universität wien

DIPLOMARBEIT / DIPLOMA THESIS

Titel der Diplomarbeit / Title of the Diploma Thesis

„Multidrug resistant protein 1 in human lung epithelial cells under the influence of anti-asthmatic drugs“

verfasst von / submitted by

Lisa Springer

angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Magistra der Pharmazie (Mag.pharm.)

Wien, 2017/ Vienna, 2017

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	A 449
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Diplomstudium Pharmazie
Betreut von / Supervisor:	ao. Univ.-Prof. Mag. Dr. Franz Gabor

ACKNOWLEDGEMENTS

First of all, I would like to thank Assoc. Prof. Dr. rer. nat. Carsten Ehrhardt for all of his support and for letting me be a part of his team for half a year. I really appreciated the great work environment and enjoyed getting to know another facet of scientific work by joining congresses and workshops.

I would also like to thank Ao. Univ.-Prof. Mag. Dr. Franz Gabor who introduced me to Prof. Ehrhardt and gave me the opportunity to do the research for my diploma thesis abroad.

Besides my advisor I would like to express my thanks to the whole team in Ireland who helped making the time there an unforgetable one and were always there for me with advise and support.

My sincere thanks goes to all my friends, fellow students and particularly Maria who I could always rely on and made the experience of moving to another country a less scary one.

Last but not least I would like to express my extreme gratitude to my mom and dad who have always been there when I needed them, encouraged me and believed in me. They have done so much more for me than can be expected and I know not to take it for granted!

TABLE OF CONTENTS

ACKNOWLEDGEMENTS I

1. ABSTRACT 5

2. LIST OF FIGURES 7

3. LIST OF TABLES 9

4. INTRODUCTION 11

4.1. Anatomy, physiology and pathophysiology of the lung 11

4.1.1. Chronic obstructive pulmonary disease 12

2.1.2. Asthma 13

4.2. Inhalation of therapeutics 14

4.3. Absorption processes 18

4.3.1. ABC-Transporter 19

4.3.2. MRP1 21

4.4. Tested Drugs 23

4.4.1. Budesonide 23

4.4.2. Dexamethasone 24

4.4.3. Formoterol 24

4.4.4. Salbutamol 25

5. MATERIALS AND METHODS 27

5.1. Materials 27

5.2. Cell culture 28

5.3. Efflux assay 28

5.4. Uptake Assay 29

5.5. Short-term incubation efflux assay 30

5.6. Short-term incubation uptake assay 30

5.7. Protein quantification 30

6. RESULTS 33

6.1. Efflux studies 33

6.1.1. Budesonide 5 µM and 10 µM 34

6.1.2. Budesonide 20 µM and 50 µM 36

6.1.3. Dexamethasone 2 µM, 10 µM and 50 µM 39

6.1.4. Formoterol fumarate 200 µM 42

6.1.5. Salbutamol 100 µM 45

6.2. Uptake studies 48

6.2.1. Budesonide 5 µM and 10 µM 48

4.2.2 Budesonide 20 µM and 50 µM 49

4.2.3. Dexamethasone 2 µM, 10 µM and 50 µM 50

4.2.4. Formoterol fumarate 200 µM 51

4.2.5. Salbutamol 100 µM 52

6.3. Short-term studies 54

6.3.1. Budesonide 20 and 50 µM 54

6.3.2. Formoterol fumarate 200 µM 58

7. DISCUSSION 63

8. ZUSAMMENFASSUNG 65

9. REFERENCES 68

10. EXPLENATION OF ABBREVIATIONS 74

1. ABSTRACT

Background:

The present study evaluates effects of certain drugs on multidrug resistance-associated protein 1 (MRP1) in NCI-H441 cells. Drugs of interest were budesonide, salbutamol, formoterol fumarate and dexamethasone, which are often prescribed in patients suffering from asthma or chronic obstructive pulmonary disease. MRP1 is an efflux pump in plasma membranes as well as subcellular compartments and plays a protective role in cellular detoxifying. Apart from exporting toxins, MRP1 also distributes to multidrug resistance by effluxing various anticancer drugs.

Methods:

To evaluate the effects of the aforementioned drugs on MRP1-mediated transport, efflux assays were performed. Both long and short-term effects were investigated.

NCI-H441 cells were incubated with 5,6-carboxyfluorescein diacetate (CFDA), which is intracellularly converted to the fluorescent CF. This MRP1 substrate is then effluxed and its activity in samples of cell supernatant measured.

In addition, uptake assays were performed to determine, how the drugs affected uptake of CFDA. Intracellular levels of CF were investigated for that purpose.

Results:

It was shown that efflux of CF was diminished when treating cells with 10 µM, 20 µM, 50 µM budesonide, 2 µM, 10 µM, 50 µM, dexamethasone, 200 µM formoterol fumarate and 100 µM salbutamol sulphate.

Reduced uptake of CFDA was observed after treating cells for 72 h with budesonide, formoterol fumarate and the highest concentration of dexamethasone tested. Contrary to this outcome, short-term incubation with 20 µM and 50 µM budesonide led to increased intracellular levels of CF.

Conclusion:

The observed reduction in CF efflux indicates an inhibiting effect of budesonide, dexamethasone, formoterol and salbutamol sulphate on MRP1. However, most results were not significant.

2. LIST OF FIGURES

Figure 1 Picture of *pMDI with spacer (32)*.....15
Figure 2 *Illustration of subtypes of transporters (40)*.....18
Figure 3 *Illustration of ion channels (44)*.....19
Figure 4 *Efflux of substrates by ABC-transporters (50)*.....21
Figure 5 *Structure of MRP1 located in plasma membrane (55)*.....22
Figure 6 *Budesonide's structure (66)*.....23
Figure 7 *Structural formula of formoterol (66)*.....24
Figure 8 *Reaction of two BCA-molecules with copper (75)*.....31
Figure 9 *CF concentration curve ranging from 0,1 to 50 µM*.....33
Figure 10 *Effect of 5 µM and 10 µM budesonide on CF efflux. No significant influence observed. Means ±SD, n=3*.....35
Figure 11 *CF concentration of lysed cells after efflux assay with 5 µM and 10 µM budesonide. No significant differences were measured, Means ±SD, n=3*.....36
Figure 12 *Effects of 20 and 50 µM budesonide on CF efflux. The drug led to an insignificant retention of CF. Means ±SD, n=3*.....37
Figure 13 *CF concentration of lysed cells after efflux assay with 20 µM and 50 µM budesonide. No significant differences were measured. Means ±SD, n=3*.....38
Figure 14 *CF efflux with 2 µM, 10 µM and 50 µM dexamethasone in NCI-H441 cells. The drug did not have a significant effect. Means ±SD, n=3* 40
Figure 15 *CF concentration determined in lysed cells after treatment with 2 µM, 10 µM and 50 µM dexamethasone. Means ±SD, n=3*.....41
Figure 16 *CF efflux study with 200 µm formoterol fumarate. Control cells showed higher efflux. Means ±SD, n=3*.....43
Figure 17 *CF concentration of lysed cells after efflux assay with 200 µM formoterol fumarate. Means ±SD, n=3*.....44
Figure 18 *Results of efflux assay after 72 hour incubation with salbutamol and three of its salts in a concentration of 100 µM. Salbutamol sulphate showed an inhibiting, the others a stimulating effect. Means ±SD, n=3*..46
Figure 19 *CF concentrations determined in lysed cells after treatment with 100 µM salbutamol, salbutamol sulphate, (R)- and (S)-salbutamol HCL. Means ±SD, n=3*.....47
Figure 20 *30 minute uptake assay with 5 µM and 10 µM budesonide. CF levels were lower after treatment with the drug. Means ±SD, n=3*.....49
Figure 21 *Uptake assay with 20 µM and 50 µM budesonide. CF concentrations were reduced after incubation with the drug. Means ±SD, n=3*.....50
Figure 22 *Uptake assay with 2 µM, 10 µM and 50 µM dexamethasone. No significant impact observed. Means ±SD, n=3*.....51
Figure 23 *Uptake assay with 200 µM formoterol fumarate. Reduced uptake was observed after treatment with the drug. Means, n=1-2*.....52
Figure 24 *30 min uptake assay with salbutamol and three of its salts. Means ±SD, n=3*.....53
Figure 25 *Effects of short-term incubation with 20 µM and 50 µM budesonide on CF efflux. The drug led to a retention of CF. Means ±SD, n=3*.....55

Figure 26 *Intracellular CF levels after termination of efflux assay with 20 and 50 µM budesonide short-term incubation*.....56
Figure 27 *Uptake assay with 20 µM and 50 µM budesonide. Increased uptake was observed after addition of the drug. Means ±SD, n=3* 57
Figure 28 *Short-term efflux study with 200 µm formoterol fumarate. Control cells showed higher efflux. Means ±SD, n=3-4*.....59
Figure 29 *CF concentration of lysed cells after short-term efflux assay with 200 µM formoterol fumarate. Means ±SD, n=3-4*.....60
Figure 30 *30 and 60 minute uptake assay with 200 µM formoterol fumarate after short-term incubation. Treatment with formoterol resulted in decreased uptake. Means ±SD, n=3-4* 61

3. LIST OF TABLES

Table 1 *Data of CF efflux studies after 72 hour incubation with 5 µM and 10 µM budesonide* 34
Table 2 *Remaining intracellular concentration of CF after efflux assay with NCI-H441 cells treated with 5 and 10 µM budesonide for 72 hours* 35
Table 3 *Data of CF efflux studies after 72 hour incubation with 20 µM and 50 µM budesonide* 37
Table 4 *Remaining intracellular concentration of CF after efflux assay with NCI-H441 cells treated with 5 and 10 µM budesonide* 38
Table 5 *Results of efflux assay after incubation of NCI-H441 cells with 2, 10 and 50 µM dexamethasone for 72 hours.* 39
Table 6 *intracellular concentrations of CF after termination of efflux assay. Statistical significant ressults (p≤ 0.05) marked with *.* 40
Table 7 *Results of efflux assay after incubation of NCI-H441 cells with 200 µM formoterol fumarate for 72 hours.* 42
Table 8 *Intracellular CF-concentration at the end of the efflux assay with 200 µM formoterol fumarate.* 43
Table 9 *Data of efflux assay after 72 hour incubation of cells with 100 µM of various salbutamol salts. Statistical significant results (p≤0.05) marked with *.* 45
Table 10 *Intracellular CF concentration after completion of the efflux study with 100 µM salbutamol, salbutamol sulphate, (R)- and (S)-salbutamol HCl..* 46
Table 11 *Data of uptake study with 5 µM and 10 µM budesonide over the course of 30 minutes* 48
Table 12 *Data of 30 and 60 minute uptake assay with 20 µM and 50 µM budesonide* 49
Table 13 *Data of 60 minute uptake assay with 2 µM, 10 µM and 50 µM dexamethasone* 50
Table 14 *Results of 30 and 60 minute uptake assays with 200 µM formoterol fumarate* 51
Table 15 *Results of 30 min uptake study with 100 µM salbutamol and three of its salts* 52
Table 16 *Data of efflux study after short-term treatment of cells with 20 µM and 50 µM budesonide* 54
Table 17 *Remaining intracellular CF concentration after short-term incubation efflux assay with NCI-H441 cells treated with 20 and 50 µM budesonide* 55
Table 18 *Results of uptake assay with 20 µM and 50 µM budesonide. Statistical significant results (p≤0.05) marked with *.* 56
Table 19 *Data of short-term efflux assay with 200 µM formoterol fumarate* 58
Table 20 *Intracellular CF-concentration at the end of a short-term efflux assay with 200 µM formoterol fumarate. Statistical significant results (p≤ 0,05) marked with ** 59
Table 21 *Data of short-term incubation uptake study with 200 µM formoterol* 60

4. INTRODUCTION

4.1. Anatomy, physiology and pathophysiology of the lung

Lungs are organs responsible for the process of respiration and thus the exchange of oxygen and carbon dioxide. (1) During ventilation oxygen is transported to the alveoli, where gas exchange between air and blood takes place, due to differences in concentration. Venous blood is deoxygenated and has high levels of carbon dioxide. While the latter is exhaled, oxygen diffuses through lung capillaries into blood and is distributed to tissues where it permeates cells. (2)

Capacity of gas diffusion depends on the size of the surface, as well as the diameter of the barrier. (3) 200 to 600 million alveoli lining the lung are responsible for its large surface, which can reach an area of up to 140 m². This enormous expand and good permeability make gas exchange during ventilation very efficient. (4,5) The process of ventilation describes the expiration and inhalation of gas out of the lung and into it, respectively. The volume of ventilation depends on body mass and varies between 4 and 8 ml/kg. However, using full lung potential a maximum capacity of 4 to 5 litre is possible. But even after forceful expiration some residual air remains in the alveoli, to prevent their collapse. (6)

From an anatomical point of view the respiratory system can be separated in an upper and lower respiratory tract. The upper airways include all organs outside of the thorax (nose, larynx, pharynx), whereas the lower airways comprise organs within the thorax. (6)

Air spaces in the respiratory tract can also be functionally classified as air-conducting or respiratory regions. Nasal cavity to bronchioles form the conducting area, which spreads over the first 16 generations of the lung. (7) These air conducting regions do not only transport air, but also serve to warm, clean and humidify it. (2) The respiratory region, which is the site of gas exchange, is comprised of alveoli and airways distal to bronchioles, which equals the last seven generations. (7)

The respiratory tract consists of 23 generations. It starts with the trachea as generation 0 and at each new generation every airway is divided into two new ones, which are consistently getting smaller and smaller. (6) The trachea is a flexible tube with a length of 10 to 12 cm and a diameter of 2 cm. It divides into the two primary bronchi, the left and right main bronchus, which further branch into two and three lobar bronchi, respectively. (2) These so-called secondary bronchi are divided into the tertiary segmental bronchi. Several more generations of bronchi are followed by bronchioles, which branch into alveolar ducts and finally into alveolar sacs, consisting of alveoli. (6)

Epithelium in these distal air spaces comprises two important cell types: type I pneumocytes and type II pneumocytes. Those alveolar epithelial cells are connected by tight junction and form the zonulae occludens. (8) Alveoli mostly consist of the smaller type I cells. They make about 96% of their surface and are extremely thin, enabling gas exchange. The remaining 4 % of the alveoli's surface are the surfactant producing type II pneumocytes. (9) Surfactant prevents alveoli from collapsing during expiration, by reducing surface tension of their lining fluid. (2)

Two characteristics play an important role in the process of ventilation: resistance and compliance. The former describes the resistance to airflow, while the latter is a term for the lung's ability to expand during ventilation. (10) Many respiratory diseases are associated, with problems evolving around these two factors. Pneumonia, lung fibrosis and lung oedema are just a few examples for pathological processes resulting in a decreased compliance. (11) These restrictive lung diseases (RLD) are contrary to obstructive lung diseases (OLD), where compliance is normal or even increased. The main feature of OLDs like asthma or emphysema is an increased resistance during expiration. (12)

4.1.1. Chronic obstructive pulmonary disease

Chronic obstructive pulmonary disease (COPD) is characterized by changes of airways, parenchyma and vascular compartments. (13) Excessive sputum production, chronic coughing and exertional dyspnoea are considered the main symptoms. (14)

COPD is a disease with a high mortality rate and one of the leading causes of death worldwide, due to the lack of effective therapies. (15) What is more, underdiagnosis as well as frequent misdiagnosis, especially as asthma, can be very problematic. Especially exacerbations, which can be triggered by inflammations, tobacco smoke or air pollution, are often mistaken for asthma attacks. (14)

The key pathological entities are chronic bronchitis, increased airspaces due to emphysematous destruction and chronic obstructive bronchiolitis. (16) The latter induces remodelling of airways, resulting in progressive, hardly reversible airflow obstructions, which take place in the small conducting airways, with diameters of less than two millimetres. (17) Fibrosis and a gain of smooth muscle mass are the main processes leading to a thickening of airway walls and thus straitening the lumen.

Chronic bronchitis is associated with excessive production and secretion of mucus, as well as an inflammatory response and enlargement of submucosal glands. (16)

Emphysema describes an increased volume of airspaces distal the bronchiole. (18) Elastic recoil is insufficient to drive out air of the lung, ultimately leading to limitation of expiratory flow. Overall it can be said, that COPD patients have a reduced airflow, because of an increased compliance, an increased resistance or a combination of both. (19)

For a majority of COPD patients the disease onset happens at an age of roughly 40 years and follows prolonged exposure to tobacco smoke. (14) Direct contact between cigarette smoke and lung leads to inflammations and damages of the tissue, thus being one of the main reasons for COPD. (16)

Anticholinergics, β_2-agonists like salbutamol or a combination of these bronchodilators, are the advised first-line therapy. Antiphlogistic drugs, such as corticosteroids, are only recommended for patients suffering from particularly severe COPD and are considered not effective enough otherwise. (14) Oxygen therapy is another option for serious cases. (15)

2.1.2. Asthma

Asthma is an inflammatory disease with reversible, episodically occurring airflow limitations. Inflammations induce enlargement of smooth muscles of large airways, mucus secretion and shedding of epithelia. (14)

Early onset during childhood is typical for this disease. Besides a familial component, other risk factors like air pollution, passive smoking and pulmonary infections have been linked to asthma. Since allergic responses can trigger asthma attacks, high levels of antibodies (immunoglobulin E) can often be observed. As a consequence eczema and rhinitis are likely symptoms of asthma. (14) Further key symptoms are chest tightness, coughing, wheezing and dyspnoea. (20)

Therapy of asthma evolves around two kinds of drugs: quick acting relievers (short acting β_2-agonists, anticholinergics) and long-term prevention (corticosteroids, long acting β_2-agonists). Corticosteroids are used because of their anti-inflammatory effect, however they have been associated with numerous systemic side effects, like osteoporosis and slowed down growth, as well as local side effects, such as candidiasis. (20) Corticosteroids are combined with short-acting β_2-adrenoreceptor agonists, whenever required. If those are not sufficient, long-acting β_2-adrenoreceptor agonists can be added to the therapy. (21) Compared to short-acting ones, they enhance lung function more and enable stronger control over symptoms. (22) To increase patient compliance anti-inflammatory drugs and long acting β_2-agonists can be mixed in a single inhaler. (21)

4.2. Inhalation of therapeutics

Inhalation is the best route of administration when it comes to respiratory diseases like asthma or COPD. Compared to oral administration far lower concentrations are required. Due to direct access to the target location, first pass effect and metabolism of the gastro intestinal tract do not interfere and systemic side effects rarely happen. All these factors lead to an optimal bioavailability, high concentrations of the drug and a quick clinical response. (5)

Not only respiratory diseases, but also systemic ones can be treated by delivering the drug through the pulmonary route. Inhalation of therapeutics for systemic diseases offers many benefits, like non-invasive administration and hardly any metabolism compared to oral administration. (23) Since alveoli are dead ends, inhaled molecules have a long residence time close to the lung's absorptive surface. It is covered by fluids with antiproteases, which prevent enzymes from destructing proteins. (4) Only soluble proteins can cross surfactant and mucus, which can reach a thickness of up to 10 µm. Positively or negatively charged molecules as well as hydrophilic ones are restricted in their permeation. (4,7) Especially peptides, proteins and small molecules can be distributed via the lung. Inhaled insulin, which is used for therapy of diabetes mellitus, has been subject of many studies. It has the advantage of a quick absorption and a response that is more physiological than that after an injection. (23) However, because of losses in airway lumen, oropharynx and the inhaler itself, depending on the device, bioavailability of insulin only lies between 10% and 46% relative to subcutaneous injection. (24)

Our lungs need to protect themselves from airborne particles. To eliminate them they command a variety of protective mechanisms, which also act as barriers for inhaled therapeutics. The lung's mucociliary clearance, its geometry and alveolar macrophages are some of these limiting factors. (5) Especially the alveolar macrophages serve as the main cause of drug elimination and make it difficult for molecules to reach the bloodstream and get into systemic circulation. (25) By releasing immunomodulatory as well as inflammatory mediators such as interleukins or leukotrienes macrophages contribute to the defence and elimination of therapeutics. (7)

Particles for inhalation should range within a size of 1 µm to 5 µm for proper distribution. With a diameter greater than 6 µm deposition in the oropharyngeal area occurs. If they are smaller than 1 µm they are simply exhaled again. (7) The size of the particles determines how the drug is deposited. Especially in the lung's first 10 generations inertial impaction of big particles happens, because of their inability to follow the airflow at bends. Gravitational sedimentation however, is more important in the last 5 to 6 generations. Since air velocity is much lower here, residence time increases and sedimentation as well as diffusion occur. Only particles smaller than 1 µm are deposited by diffusion, also known as Brownian motion, if they have not been expelled. (5,26)

Nebulizer, dry powder inhaler (DPI) and pressurized metered-dose inhaler (pMDI) are the three types of delivery systems available on the market. Each has its own benefits and is recommended under specific circumstances. And what is more, most drugs are only available for one or two of these systems.

The most common delivery devices for the treatment of asthma and COPD are pMDIs. (27) Pressurised MDIs are used for medicines in the form of solutions or suspensions. Apart from the drug itself the container is also filled with compressed gases. Since the ban of chlorofluorocarbons (CFC), due to their ozone damaging properties, the preferred gases are hydrofluoroalkanes (HFA). When still in the container they are liquefied, however as soon as they are set free and under atmospheric pressure they expand volumetrically and are gasiform. After the actuation the gas is expelled and the liquid drug is aerosolised. (28)

pMDIs are not only rather cheap and quick to use, but due to their moderate size also portable. (29) On the downside the lack of dose counters makes it hard to tell how many uses are left. (30) Another disadvantage is the required coordination between inhalation and actuating mechanism, which is a source of errors. (29)

To avoid the problem of coordination breath-actuated inhalers, which expel the drug whenever they sense inhalation, can be used. (28) Another possibility to overcome this issue is the prescription of spacers to patients with poor technique. (31) Spacers are holding chambers, which can be attached to the original device as illustrated in figure 1. This way distance and time from aerosolisation to inhalation are increased. This leads to a decreased deposition in the oropharyngeal area. Furthermore cold Freon effect, a cold feeling at the back of the throat while inhaling, is diminished. Even though they are less convenient to carry along because of their size, spacers are especially practical for kids and infants, due to their inability to coordinate actuation and inhalation. (28)

Figure 1 Picture of *pMDI with spacer (32)*

The second class of inhalers is formed of DPIs, which can be further divided into three different types: multi-dose reservoir inhalers, multi-dose inhalers with single doses and single-unit dose inhalers.
When using single-unit dose inhalers, capsules with the drug have to be inserted into the device before every single use. Depending on the device, the gelatin capsule needs to be broken by a twisting motion or by penetrating it with two spikes. Once the capsule is damaged and the drug is set free the patient needs to take a breath. While inhaling a propeller turns and the powder is dispersed. (33)

Furthermore, there are multi-dose inhalers, which contain several doses and can be further differentiated. Firstly, there are reservoir inhalers with the powder in a bulk, for example a pellet. In this kind of inhalers a single dose is shaved off at each actuation. Secondly, multi-unit dose devices with single doses exist in two forms. The drug can either be in blisters or in capsules, each containing a single dose. (34)

DPIs contain a mixture comprised of drug and an excipient like lactose monohydrate, which serves as a carrier. The drug adheres to the carrier's particle and detaches once the patient inhales. Only the smaller drug reaches the lung, while the lactose impacts on the oropharyngeal surfaces. (35) The excipient has the advantage of increasing mass and thus dose uniformity. (33) Since DPIs are breath actuated they are easier to use than pMDIs. Due to their portability, the redundancy of spacers and the integrity of dose counters in multi-dose models, DPIs are often the preferred device. However, some patients cannot reach a sufficient airflow to disaggregate the particles. (30)

The last class of inhalers is that of nebulizers which transform a bulk liquid into droplets of an aerosol. It is differentiated between two systems of nebulizers: pneumatic nebulizers and ultrasonic nebulizers.
Pneumatic nebulizers contain compressed air. When it is released and therefore expands, negative pressure is created and liquid is carried off. Droplets are created when the film breaks and are finely atomized when they meet a baffle.

Contrary to pneumatic nebulizers, ultrasonic ones do not work with compressed gas, but instead utilize a piezoelectric transducer. It generates ultrasonic waves, that when passing through the liquid, generate fine droplets at the surface.

Nebulizers are required whenever medication is only available in soluble form. (36) Especially for kids nebulizers are the preferred delivery system for inhaling, since no cooperation and coordination are required. (37) Another positive aspect is the possibility to combine several therapeutics and inhale them together at high drug doses. However, use is long-winded and the devices are fairly mobile. (30)

Many problems in therapy derive from poor technique and wrong use of inhalers. (4) Since handling of most inhalers does not come natural to users, proper training is necessary. (30) Studies have shown, that a majority of patients is unable to use their device correctly. Only 10% to 50% managed to inhale without any errors. A study conducted by Bartolo *et al.* showed that the only predictor for no mistakes at all was a proper explanation by a respiratory physician. The most common mistakes and neglected steps were shaking of the device, breath holding and exhaling away from the inhaler. (31) While fast inhalation is essential for DPIs to reach deagglomeration, patients should inhale as slowly as possible when using a jet nebulizer. (4)

4.3. Absorption processes

All life depends on transporters both supplying the cells with nutritious compounds as well as exporting toxins.

Small nonpolar and small uncharged polar molecules can cross the cell membrane by passive diffusion. However, for charged compounds and larger uncharged polar ones, the lipid bilayer is an unsurpassable barrier and they need a transporter as a consequence. (38)

Transporters can be classified as active or passive. While passive transporters move their substrate along with the chemical gradient, active ones need a source of energy in order to transport against the concentration gradient. Primary active transporters, like ATP-transporters, generate their energy by hydrolyzing ATP. Secondary active transporters are driven by the energy of another molecule's chemical gradient. They couple the movement of two molecules, with one being transported against and the other one down its chemical gradient. (39)

There are four classes of membrane transport proteins: aquaporins, ion channels, ATP-powered pumps (e.g. ABC-transporters) and transporters. (39)

Uniporters, antiporters and symporters are the three subtypes of the latter (figure 2). Uniporters are passive transports, which move their substrate across the lipid bilayer, following the concentration gradient. Anti- and symporters on the other hand are classified as secondary active transporters, due to their coupling of an energetically unfavorable reaction with a favorable one. While antiporters transport two different substrates in opposite directions, symporters carry both in the same. (40)

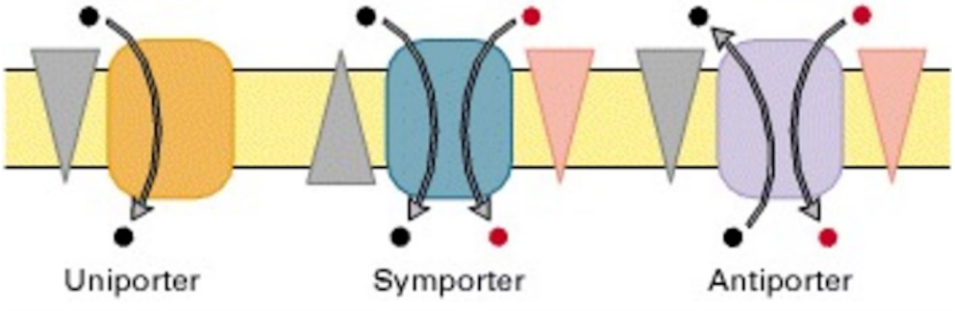

Figure 2 *Illustration of subtypes of transporters (40)*

Aquaporins are a family of integral membrane proteins. They act as channels for the transport of water across the lipid bilayer. (41) Apart from crossing the cell membrane through the pore, water can also diffuse through it. However, this process is too slow for some matters, which is why aquaporins are essential. Due to the diameter of the pore fitting exactly that of a molecule of

water, it is a very selective transporter. (42) Nevertheless aquaporins have a subfamily, aquaglyceroporins, which also move small neutral solutes, such as glycerol. (43)

The third group of transport proteins is that of ion channels. They are selective and only allow certain ions to pass. This specificity is due to a narrow opening, which only enables ions with the right charge and proportions to move through it. Contrary to aquaporins ion channels only open at certain stimuli. Depending on the impulse that opens ion channels, they are classified as voltage gated, ligand gated or mechanically gated channels. (44)

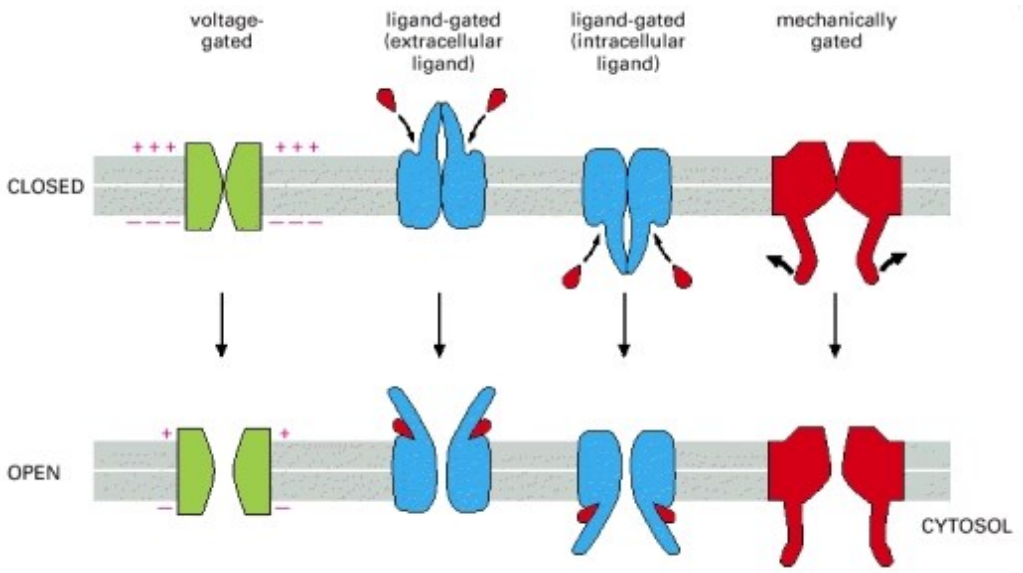

Figure 3 *Illustration of ion channels (44)*

4.3.1. ABC-Transporter

ATP-binding cassette transporters, also known as ABC-transporters, are a family of integral membrane proteins.

They can be found ubiquitous in all life forms, both in prokaryotes and eukaryotes. They import nutrients, export toxic compounds, regulate the osmolality in plants and are responsible for the resistance of bacteria to antibiotics or the resistance of insects to insecticides. (45,46)

The superfamily of ABC-transporters includes MRP1, MRP2, p-glycoprotein and BCRP. Some of these transporters are expressed in tissues, which play an important role in absorption, metabolism or elimination, like lung, gut, kidney and liver. Therefor a variety of these ABC-transporters influence these

before mentioned physiological processes among others concerning xenobiotics. (39)

There are two subtypes of ABC-transporters, depending on the direction in which they transport their substrates: ABC-importers take up substrates into the cytoplasm, whereas ABC-exporters efflux molecules out of the cell. (46)

49 genes coding for ABC-transporter have been identified so far. They form 7 subfamilies named A through G. Depending on the similarity of the amino acid sequence the proteins are grouped into these families. An identity of at least 40% results in the transporters being members of the same family. If the identity of the proteins is even higher than 55% they are assigned to the same subfamily. (39)

ABC-transporters consist of transmembrane domains (TMD) on the cytosolic side and an ATP-binding cassette, also known as nucleotide-binding domain (NBD). (47) The two NBDs (NBD1, NBD 2) have two motifs, Walker A (=P-loop) and Walker B, and the signature motif LSGGQ. The latter is a linker between the two motifs and specific for the ABC-transporter family. Walker A and B are responsible for binding ATP and are involved in hydrolyzing ATP. (48,49)

While NBDs share a high homology, TMDs are far less consistent. Each transmembrane domain consists of 6 to 10 α-helices, which makes 12 to 20 in total. However, most exporters have 6 helices per TMD. By forming a pore they provide a passageway for solutes. (49) TMDs are responsible for specificity and both substrate recognition as well as translocation. (39)

NBDs are considered the engine of the transporter. Once two ATP molecules are bound, dimerization of the two NBDs follows. The two nucleotides are sandwiched between the P-loop of one and the LSGGQ sequence of the other NBD. The association leads to a switch from inward to outward facing conformation of the TMDs. After an ATP hydrolysis not only substrate but also ADP and phosphate are released into the environment. NBDs dissociate and the original ground state with its inward facing constitution is reached again. (see figure 4) (46,49)

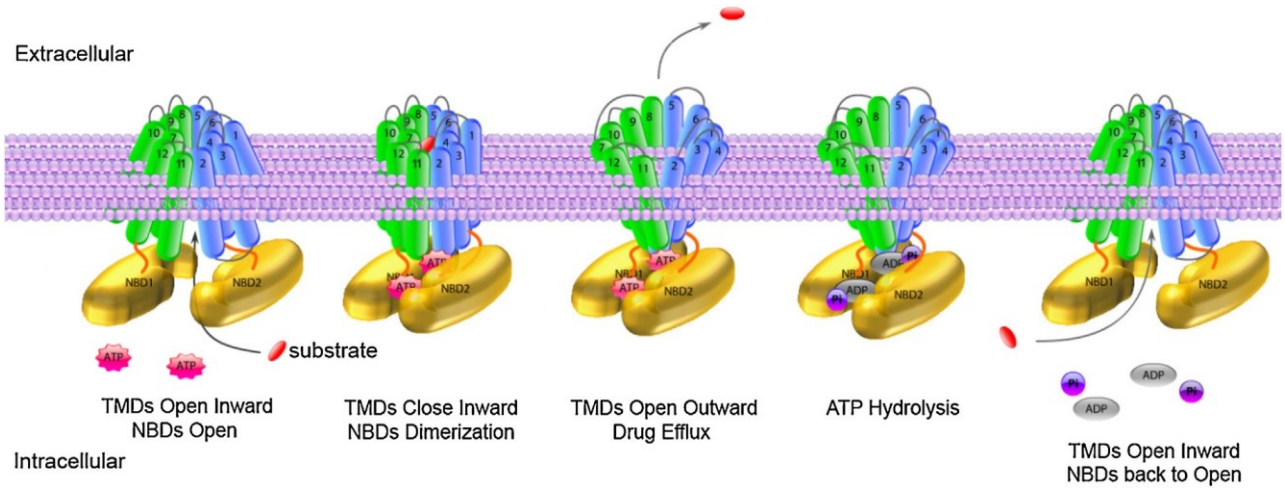

Figure 4 *Efflux of substrates by ABC-transporters (50)*

4.3.2. MRP1

Multidrug-resistance associated protein 1, also known as MRP1, is a plasma membrane efflux pump. It is a member of the ABC-transporters, more precisely their C branch. This subfamily consists of 13 proteins, with 9 of them being multidrug resistant proteins (MRP1 through MRP9). Apart from the family of MRPs, this branch also contains the cystic fibrosis transmembrane conductance regulator (CFTR) and the sulfonylurea receptors (SUR). (51,52)

As aforementioned, ABC transporters are usually composed of two NBDs and as many TMDs. However, MRP1, 2, 3, 6 and 7, comprise an additional fifth domain, which is why they are referred to as “long MRPs”. The remaining MRPs, MRP4, 5, 8 and 9, have the typical ABC-transporter structure with 4 domains and were therefore named “short MRPs”. (52)

MRP1’s additional domain is called TMD0. Contrary to the other two transmembrane domains this one comprises 5 instead of 6 transmembrane helixes as seen in figure 5. TMD0 connects to TMD1 through an intracellular linker region (L0). Studies have shown that MRP1 mutants without NBD0 still function normally and their transport activity was not affected. However, modifications of the domain led to changes in conformation of the protein, resulting in a diminished transport capacity. (53) There is also evidence, that NBD0 plays a role in the retention of MRP1 in the plasma membrane. (54)

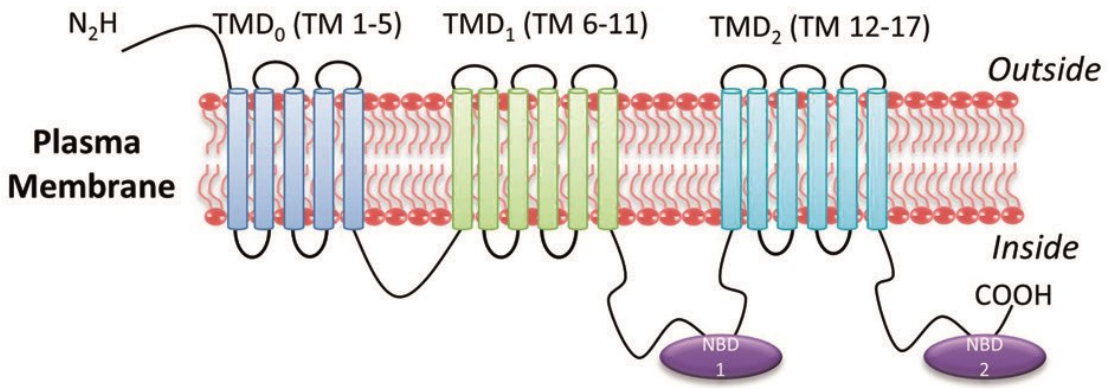

Figure 5 *Structure of MRP1 located in plasma membrane (55)*

The protein is encoded by the gen ABCC1 and has a molecular weight of 190 kDA. (56) MRP1 is located on the basolateral side of cells with the purpose to transport various substances out of the cell. That way it reduces the intracellular concentration of lipophilic and amphiphilic substances. (57) Besides being located in the plasma membrane, MRP1 can also be found in subcellular compartments like lysosomes, the Golgi apparatus and the nucleus. (58) The transporter is highly expressed in organs such as lung, kidney, testes, placenta and bladder. (59)

Substrates of MRP1 include sulphate-, glutathione- and glucuronide conjugates. (60) Some of these endobiotic conjugates are leukotriene C_4 (LTC_4), D_4 and E_4, bilirubin, prostaglandin A_2-SG and Estradiol-17-β-glucuronide. (53)

By releasing LTC_4, MRP1 is involved in inflammatory processes. This arachidonic acid derivate has an impact on allergic and asthmatic reactions. (61) Studies have shown, that Abcc1-knockout mice showed a diminished inflammatory response as a result of the decreased LTC_4 export. (52)

The transporter also plays a protective role in cells by detoxifying them. Due to the reduced concentration of endogenous metabolites and toxic compounds in lung cells, the risk of COPD is diminished. (62) During chemotherapy it transports toxins out of cardiomyocytes, thus protecting the heart from higher oxidative stress levels. (55)

However, MRP1 does not only export physiological substrates but also drugs such as methotrexate, antiandrogens, HIV-therapeutics (saquinavir, ritonavir) and therapeutics used in cancer treatment like doxorubicin, vincristine, vinblastine and paclitaxel. This can lead to multi drug resistance (MDR) and difficulties in therapy. (51)

4.4. Tested Drugs

4.4.1. Budesonide

Budesonide is an anti-inflammatory synthetic steroid of the class of glucocorticoids. (63) The drug family of corticosteroids reduces exudation of fluids, the number of inflammatory cells and mediators released by those. (64) It additionally causes apoptosis of the immune-inflammatory eosinophils, thus contributing to an anti-inflammatory effect. (65)

Budesonide features weak mineralocorticoid activity, but due to its affinity to the glucocorticoid receptor (GR), it has a high glucocorticoid effect. (64) The molecule exhibits a lipid-soluble structure, which enables diffusion across the membrane. After budesonide's binding to GR, the receptor-ligand-complex translocates into the nucleus and binds to DNA, ultimately resulting in higher transcription rates of anti-inflammatory proteins. (65)

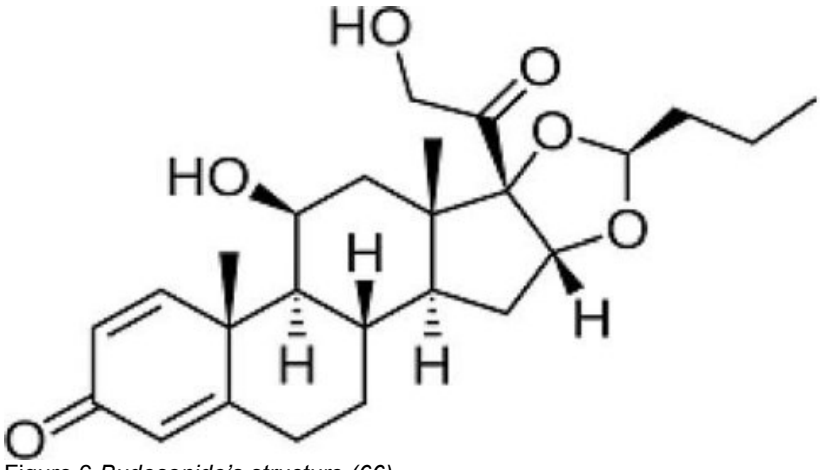

Figure 6 *Budesonide's structure (66)*

Budesonide was initially established as medication for asthma and other respiratory diseases involving inflammation. (63) It is also used as treatment of allergies, rhinitis, skin disorders and a variety of chronic gastrointestinal medical conditions like Crohn's disease. Inhalation of the drug enables quick clinical response and is applied by patients suffering from for example asthma or rhinitis. (63,64) For Crohn's disease oral application of budesonide is the administration route of choice. However, the therapeutic undergoes significant first-pass metabolism of about 90% and is poorly absorbed. (63)

4.4.2. Dexamethasone

Dexamethasone is a fluorinated synthetic glucocorticoid. Its indication ranges from asthma over arthritis to allergies. (67)

Possible ways of administration are injection, topical ophthalmic solutions and either intranasal or oral inhalation of aerosols. When it comes to treatment of asthma the latter is practiced. (68) Dexamethasone has a high bioavailability and is active for up to 72 hours. (69) Mode of action is the same as described above for budesonide, via binding of the molecule to cytoplasmic glucocorticoid receptors. (68)

4.4.3. Formoterol

Formoterol and its salt formoterol fumarate are long-acting β_2-agonists commonly used for the therapy of COPD and asthma because of their bronchodilator effect. (66)

They cause bronchodilation by stimulating adenyl cyclase, which catalyses transformation of adenosine triphosphate to cyclic-adenosine monophosphate (cAMP). A rise of cAMP levels relaxes smooth muscle cells and leads to bronchodilation. (70) This effect lasts for a minimum of 12 hours and has a rapid onset. (21)

Even though formoterol is usually inhaled, a large portion is absorbed in the gastrointestinal tract after being swallowed. (70) This and the resulting systemic bioavailability can be decreased significantly by using a spacer. (21)

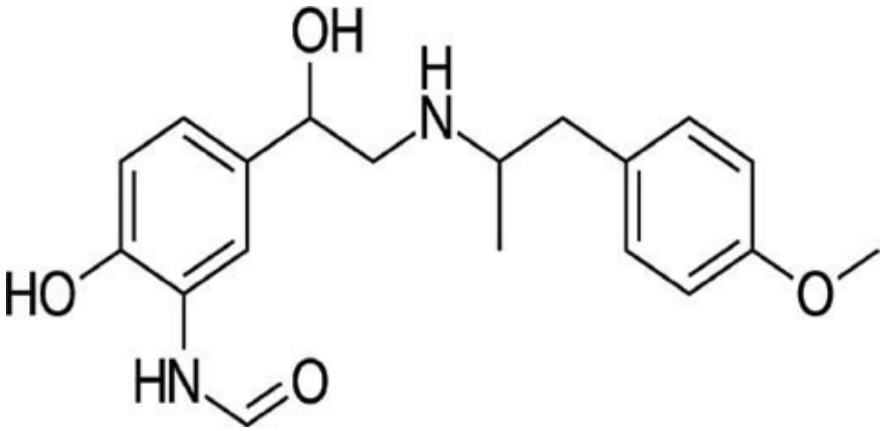

Figure 7 *Structural formula of formoterol (66)*

4.4.4. Salbutamol

Salbutamol, also known as albuterol, is another long acting agonist of the β2-adrenoreceptor. Like formoterol it is a bronchodilator and therefore generally prescribed as treatment for asthma and other airway diseases. (71)

Sympathomimetic amines often cause side effects like tachycardia, by additionally stimulating cardiac β1-adrenoreceptors. However, this is hardly the case for salbutamol, due to its selectiveness, which allows the molecule to only activate β2-receptors in the bronchial smooth muscles. (72)

Salbutamol has the same mode of action as formoterol and relaxes smooth muscles by increasing cAMP levels. (73)

5. MATERIALS AND METHODS

5.1. Materials

Cell culture

All experiments were performed on cells of the human lung adenocarcinoma cell line NCI-H441 of the passages 70 to 93.

Cells were cultured in flasks with a surface area of 75 cm² and filter screw caps. They were incubated at 37°C at a humidified atmosphere containing 5% CO_2 in an incubator by Binder. Cells were seeded on 24-well plates acquired from Greiner bio-one at a density of 50,000-70,000 cells/cm². Dulbecco's phosphate buffered saline was used for washing and Trypsin-EDTA solution for cell dissociation, both acquired from Sigma-Aldrich. For cell counting a haemocytometer by Hausser scientific was used.

Cells were cultivated using RPMI Medium1640 (Gibco) containing 5% FBS (foetal bovine serum), 1% Penicillin-Streptomycin (10,000 units penicillin and 10 mg streptomycin/ml), and 1% sodium pyruvate (10 mM) all bought from Sigma-Aldrich.

Bi-carbonated Krebs-Ringer buffer (KRB) was used as a medium for the experiments. It contained sodium chloride 116.4 mM (Fluka), sodium dihydrogen phosphate 0.78 mM (Riedel-de Haën), potassium chloride 5.4 mM, sodium bicarbonate 25 mM, glucose 5.55 mM, HEPES 15 mM, magnesium sulphate heptahydrate 0.81 mM and calcium chloride dihydrate 1.8 mM (Sigma-Aldrich). Everything was dissolved in purified water, which was acquired via an Elga's pure lab option. The KRB's pH was set to 7.4 with hydrochloric acid and potassium hydroxide with the help of Mettler Toledo seven easy pH meter.

Efflux and Uptake studies

The efflux of 5(6)Carboxyfluorescein (CF) and the Uptake of 5(6)Carboxyfluorescein Diacetate (CFDA) were tested under the influence of budesonide (Mundipharma), R- and S-albuterol hydrochloride (Sunovion), formoterol fumarate (Santa Cruz Biotechnology) and dexamethasone (Sigma-Aldrich).

The CF for the standard curve was purchased from Sigma-Aldrich just like dimethyl sulfoxide (DMSO), which was used as a solvent for some of the drugs and CF.

Software

The data was analysed with GraphPhad Prism 5. Furthermore Microsoft Excel was used to edit data.

5.2. Cell culture

All experiments were performed on cells of the adherent bronchiolar epithelial cell line NCI-H441.

Once the cells reached a confluence of about 70% they were subcultured from a flask to a 24-well plate on which the experiments were performed. First the cells were washed twice with 10 ml PBS before they were incubated with 3.5 ml Trypsin for 6 minutes to dissociate. The trypsinisation was stopped by adding 6.5 ml medium and the cells were separated by resuspending them. After centrifugation at a speed of 900 rpm for 5 minutes the supernatant was removed and the cell pellet was resuspended in 10 ml fresh medium. Cells were counted with a haemocytometer and 50,000-70,000 cells/cm² were seeded on a 24-well plate (1.9 cm²/well).

The reagents were preheated to a temperature of 37°C and all steps were performed under the laminar air flow.

5.3. Efflux assay

An efflux assay was performed in order to evaluate the long-term influence of drugs on MRP1. Efflux is the term for the transport of molecules out of the cell.

CFDA was added to the cells and after being taken up it is intracellularly converted to the fluorescent Carboxyfluorescein. CF is a MRP1 substrate and effluxed by it, which is why an increased retention showcases a reduced activity of MRP1. (74)

Cells were seeded on a 24-well plate and incubated (37°C, 5% CO_2) for five to seven days until they were confluent. Before starting the experiment the cells were incubated with one of several different drugs, which were to be tested, for 72 hours. Cells treated with just the solvent of each drug were used as control.

After these 72 hours the medium with the drug was removed and the cells were washed twice with KRB (1x, prepared from a 10x stock, pH 7.4). Afterwards the cells were incubated again with 0.5 ml of 50 µM CFDA solution (prepared from 20 mM CFDA in DMSO stock solution, diluted with KRB) at

37°C for one hour. Cells were protected from light during this time and the incubator's shaker was activated. At the end of the incubation the CFDA was removed again and cells were washed twice with KRB.

Fresh KRB was added to each well and samples of the supernatant were drawn after 15, 30, 45 and 60 minutes from different wells. Afterwards the cells were washed twice with ice cold KRB (4°C) to inactivate them and stop any further efflux of CF. Monolayers were lysed with 150 µl of 1% Triton X-100 in KRB. After a 15 min incubation the cell lysate of each well was transferred into a reaction tube and cooled. To remove all cell fragments samples were centrifuged at a temperature of 4°C and a speed of 13,000 g for 20 minutes. Trying not to harm the cell pellet the supernatant was transferred to new reaction tubes.

All of this was done parallel with the control cells and the cells treated with the drug.

100 µl of the supernatant of each sample were transferred into a black 96-well plate in triplicates and the fluorescence of carboxyfluorescein was determined with a fluorescence plate reader. In order to do so the samples were excited at a wavelength of 485 nm and the emission was registered at a wavelength of 520 nm. KRB was used as a blank. CF concentration was determined by comparing the fluorescence to that of a CF concentration curve.

BCA was performed to quantify the proteins for standardization of the results.

5.4. Uptake Assay

To see how the drugs affect the uptake of CFDA an uptake study was performed.

Parallel to the efflux assay the cells were seeded on a 24-well plate and cultivated until they had formed a monolayer. For the last 72 h the cells were incubated with various drugs.

After removing the medium with the drug, cells were washed twice with 1 ml KRB, which was heated to 37°C. The cells were incubated again with 0.5 ml of a 50 µM CFDA solution in the dark for 30 minutes or 1 hour, respectively. The CFDA solution was removed and cells were washed twice with 0.5 ml of ice cold KRB. After lysis with 150 µl of 1% Triton X-100 in KRB, cell fragments were centrifuged.

Plates were analysed as described above.

5.5. Short-term incubation efflux assay

Short-term incubation efflux studies were performed to investigate more imminent effects of drugs.

As described above cells were seeded on 24-well plates and incubated with RPMI medium for five to eight days until confluence was reached.

After aspirating the medium and washing the cells twice with warm KRB they were incubated with 0.5 ml of KRB for 30 min for equilibration. Next the cells were loaded with CFDA by adding 0.5 ml of a 50 µM CFDA solution for one hour. Following the aspiration of CFDA the cells were washed twice with warm KRB.

Fresh KRB with different drugs was added to the monolayers. Samples of the supernatant were drawn after 15, 30, 45, 60 min to measure the concentration of effluxed CF. Triplicates of every sample were transferred into a black 96-well plate and fluorescence was measured using a plate reader with an excitation wavelength of 485 nm and an emission wavelength of 520 nm.

At the end all the cells were lysed with 1% Triton X-100 in KRB and the intracellular CF concentration was measured as well.

To find out the protein concentration a BCA was performed. The CF concentration was then put in relation with the protein concentration.

5.6. Short-term incubation uptake assay

The short-term incubation uptake assay was carried out like the short-term incubation efflux study. However, during the incubation with 50 µM CFDA the solution additionally contained the tested drugs in the relevant concentration.

After 30 and 60 minutes, respectively, monolayers were washed, lysed and centrifuged. After determination of the CF concentration, proteins were quantified.

5.7. Protein quantification

For standardization of the results the protein concentration of the monolayer in each well was quantified. Pierce BCA Protein Assay Kit by Thermo scientific was the method of choice.

BCA is short for bicinchoninic acid. This method is based on the reduction of Cu^{+2} to Cu^{+1} in an alkaline solution through the proteins. Two BCA molecules chelate one cuprous ion (figure 8) and a purple color develops and intensifies depending on the protein concentration. With colorimetric detection the absorbance at a wavelength of 562 nm is measured.

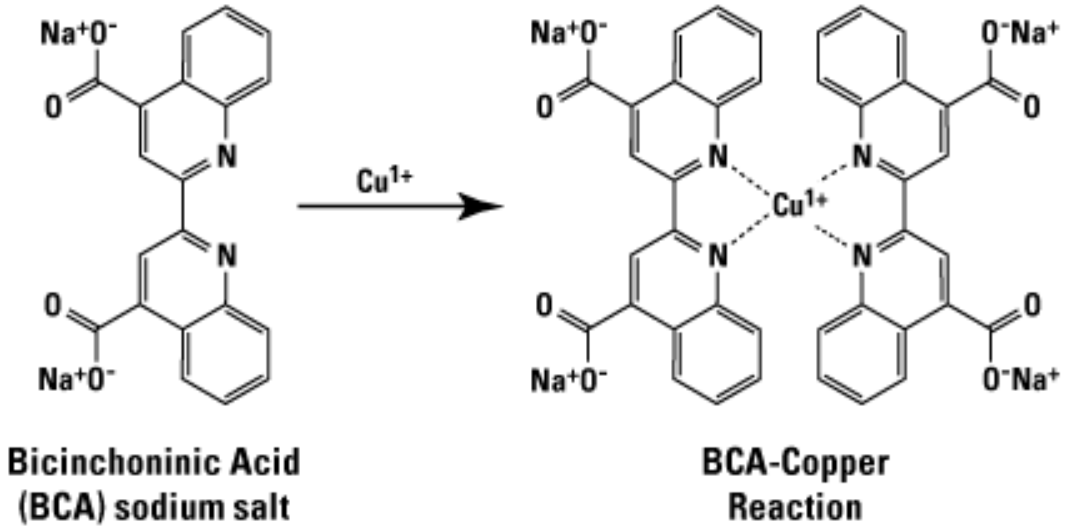

Figure 8 *Reaction of two BCA-molecules with copper (75)*

Prior to starting the protein quantification the cell lysates were cooled to a temperature of 4°C and centrifuged at a speed of 13,000 g for 20 min. The cell pellet was discarded, while the supernatant was transferred to another reaction tube and cooled on ice.

The samples were measured alongside a standard curve on the same plate to determine the exact protein quantification. Bovine serum albumin in concentrations of 0 to 2,000 µg/ml was used as a standard.

A working reagent, consisting of two reagents mixed 50:1, was prepared. Reagent A comprises sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide, while Reagent B contains 4% cupric sulfate.

25 µl of each sample or standard and 200 µl of the working reagent were pipetted into the 96-well plate. Triplicates of the samples and duplicates of the standards were measured. Protected from light the plate was incubated for 30 minutes at 37°C during which time the copper reduction took place and the color changed from green to purple. The samples were cooled down to room temperature and the absorbance was measured at 562 nm. (75)

6. RESULTS

Efflux and uptake studies were conducted to examine the influence of a variety of drugs on MRP1.

The CF concentration of each experiment was calculated using a concentration curve, ranging from 0.1 µM to 50 µM. It showed a linear correlation between fluorescence and CF concentration.

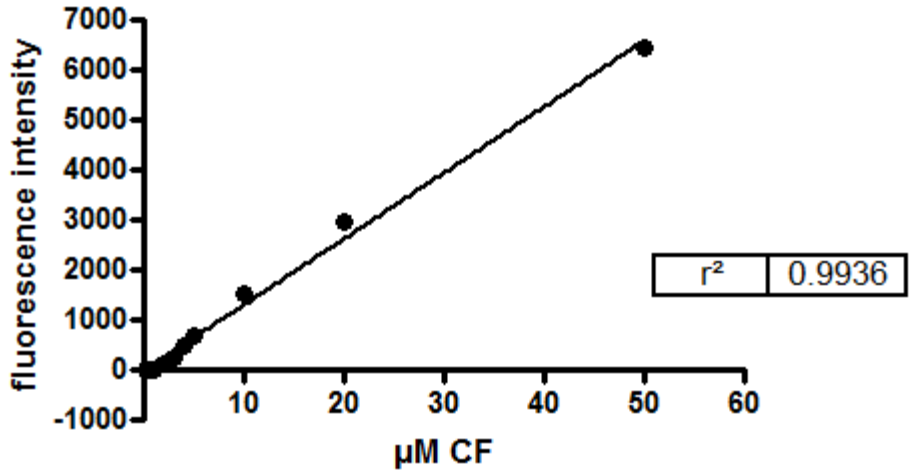

Figure 9 *CF concentration curve ranging from 0,1 to 50 µM*

A paired, two tailed t-test was used to compare the different treatment groups and determine statistical significance of the results. P-values higher than 0.05 were considered to be significant. The results were analyzed with GraphPad Prism 5.

At each experiment triplicates of the supernatant were measured.

6.1. Efflux studies

As described above the cells were cultivated to monolayers and incubated with drugs in different concentrations for 72 h before running the efflux assay. Cells were incubated with 50 µM CFDA for one hour before the solution was replaced by KRB. Samples of the supernatant were drawn in 15-minute intervals. Afterwards the cell monolayer in each well was treated with 1% Triton-X in KRB to lyse the cells. CF concentration was measured both in cells and supernatant to observe the MRP1 mediated efflux.

The efflux assay was performed with following drugs:

- 5 µM, 10 µM, 20 µM, 50 µM budesonide
- 100 µM salbutamol
- 100 µM salbutamol sulphate
- 100 µM (R)-salbutamol HCl
- 100 µM (S)-salbutamol HCl
- 200 µM formoterol fumarate
- 2 µM, 10 µM, 50 µM dexamethasone

6.1.1. Budesonide 5 µM and 10 µM

A 5 mM stock solution was prepared from which dilutions in the tested concentrations of 5 µM and 10 µM were made. The substance was dissolved in DMSO, and sterile filtered (0.45 µm) to ensure sterile conditions. As a control 2 µl of pure sterile filtered DMSO were used per monolayer. It was crucial that the concentration of DMSO wasn't higher than 1% in order not to harm the cells.

Supernatant:

time (min)	CFDA mean ± SD n=3	CFDA+ 5 µM Budesonide mean ± SD n=3	p	CFDA+ 10 µM Budesonide mean ± SD n=3	p
15	74.46 ± 10.68	82.36 ± 9.44	0.3911	63.91 ± 20.97	0.4808
30	110.12 ± 21.21	128.04 ± 22.50	0.3723	97.98 ± 27.35	0.5763
45	125.42 ± 31.59	127.36 ± 27.80	0.9400	113.35 ± 30.77	0.6602
60	153.59 ± 22.82	164.38 ± 24.78	0.6087	130.77 ± 24.99	0.3078

Table 1 *Data of CF efflux studies after 72 hour incubation with 5 µM and 10 µM budesonide*

At no time significant differences of MRP1's transport activity were observed. The cells, which were incubated with 5 µM budesonide effluxed slightly more CF, than the control cells, especially after 30 min (128.04% and 110.12% respectively). In contrast, after a treatment with 10 µM budesonide a reduction of efflux could be seen throughout the experiment. A concentration of 130.77% was measured in the supernatant, which is about 23% less than in the untreated cells'.

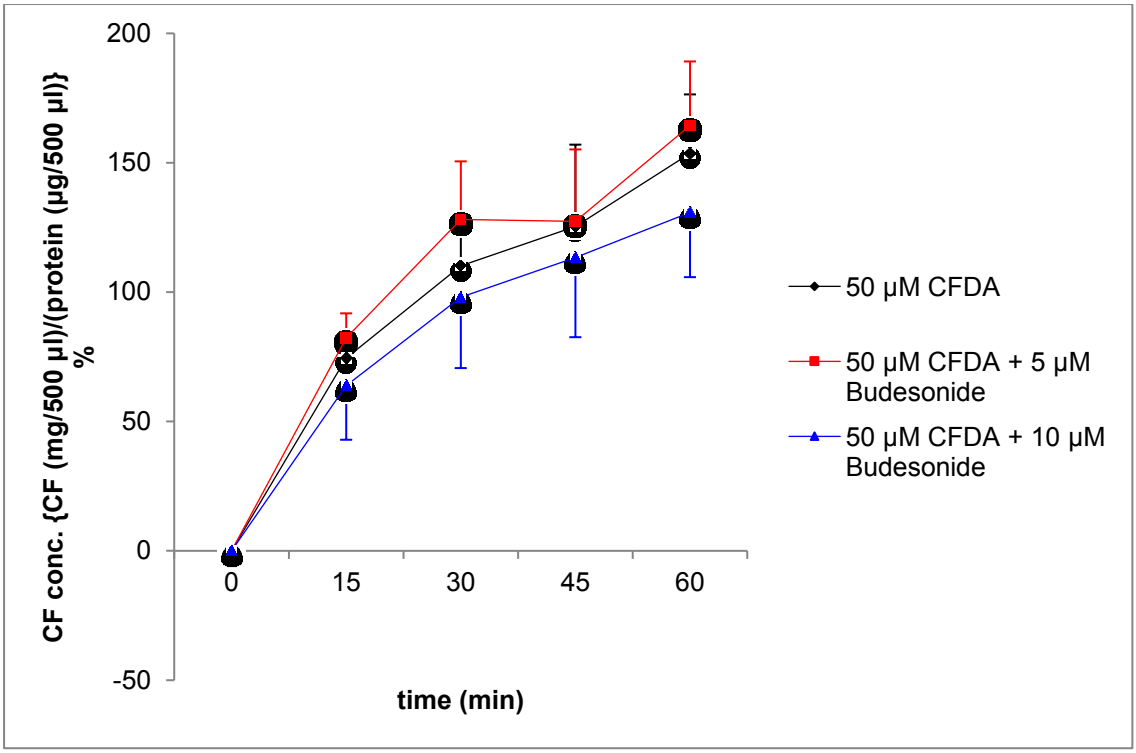

Figure 10 *Effect of 5 µM and 10 µM budesonide on CF efflux. No significant influence observed. Means ±SD, n=3*

Cell lysate:

time (min)	CFDA mean ± SD n=3	CFDA+ 5 µM Budesonide mean ± SD n=3	p	CFDA+ 10 µM Budesonide mean ± SD n=3	p
0	100 ± 34.00	100 ± 45.87	1.00	100 ± 43.35	1.00
15	82.49 ± 17.60	81.05 ± 9.50	0.9072	78.47 ± 5.23	0.7239
30	56.15 ± 9.69	63.76 ± 1.32	0.2490	58.30 ± 3.92	0.7392
45	40.95 ± 4.29	43.02 ± 2.58	0.5150	41.23 ± 2.59	0.9270
60	35.66 ± 11.34	39.18 ± 6.10	0.6603	33.95 ± 2.13	0.8100

Table 2 *Remaining intracellular concentration of CF after efflux assay with NCI-H441 cells treated with 5 and 10 µM budesonide for 72 hours*

After the efflux study was terminated all cells in the 24-well plate were lysed and the remaining intracellular concentration of CF was measured. There were no significant differences between the three treatment groups. In fact the amount of CF within the cells at each time of the efflux assay was very similar in all three groups and without any significant differences. The final concentration was ranging from 33.95% (10 µM budesonide) to 39.18% (5 µM budesonide).

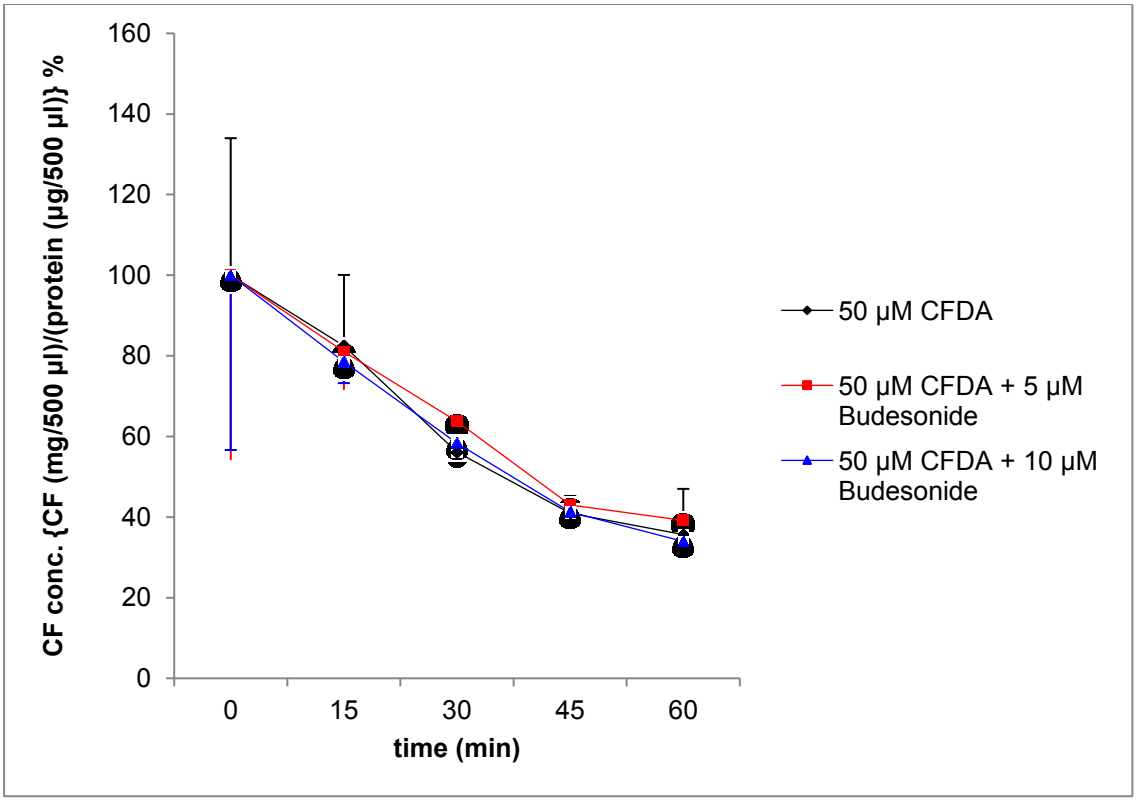

Figure 11 *CF concentration of lysed cells after efflux assay with 5 µM and 10 µM budesonide. No significant differences were measured, Means ±SD, n=3*

6.1.2. Budesonide 20 µM and 50 µM

Since no major effects were observed, when testing Budesonide at concentration 5 µM and 10 µM, higher concentrations were used this time. Budesonide was tested in concentrations 20 µM (prepared from a 5 mM stock solution in DMSO) and 50 µM (prepared from a 50 mM stock solution in DMSO). To enable a better comparison control cells were incubated with pure DMSO for the same amount of time.

Supernatant:

time (min)	CFDA mean ± SD n=3	CFDA+ 20 µM Budesonide mean ± SD n=3	p	CFDA+ 50 µM Budesonide mean ± SD n=3	p
15	50.01 ± 2.12	45.00 ± 3.48	0.1005	59.24 ± 8.10	0.1286
30	81.71 ± 13.59	66.60 ± 17.33	0.3006	78.95 ± 8.59	0.7816
45	94.53 ± 4.89	90.23 ± 15.01	0.6616	98.83 ± 30.49	0.8215
60	115.21 ± 14.98	89.00 ± 19.64	0.1400	102.08 ± 8.08	0.2524

Table 3 *Data of CF efflux studies after 72 hour incubation with 20 µM and 50 µM budesonide*

The CF efflux by MRP1 was time dependent, however after one hour the two cell groups which were treated with budesonide showed a flattening curve and seemed to reach a plateau as seen in figure 12.

Both 20 µM and 50 µM budesonide led to a higher retention of CF over the course of one hour. The control cells transported 115.21% CF out of the cell, while the ones incubated with budesonide only effluxed 89.00 % (20 µM budesonide) and 102.08% (50 µM budesonide). Interestingly the effect of the lower concentration was even stronger than that of the higher one.

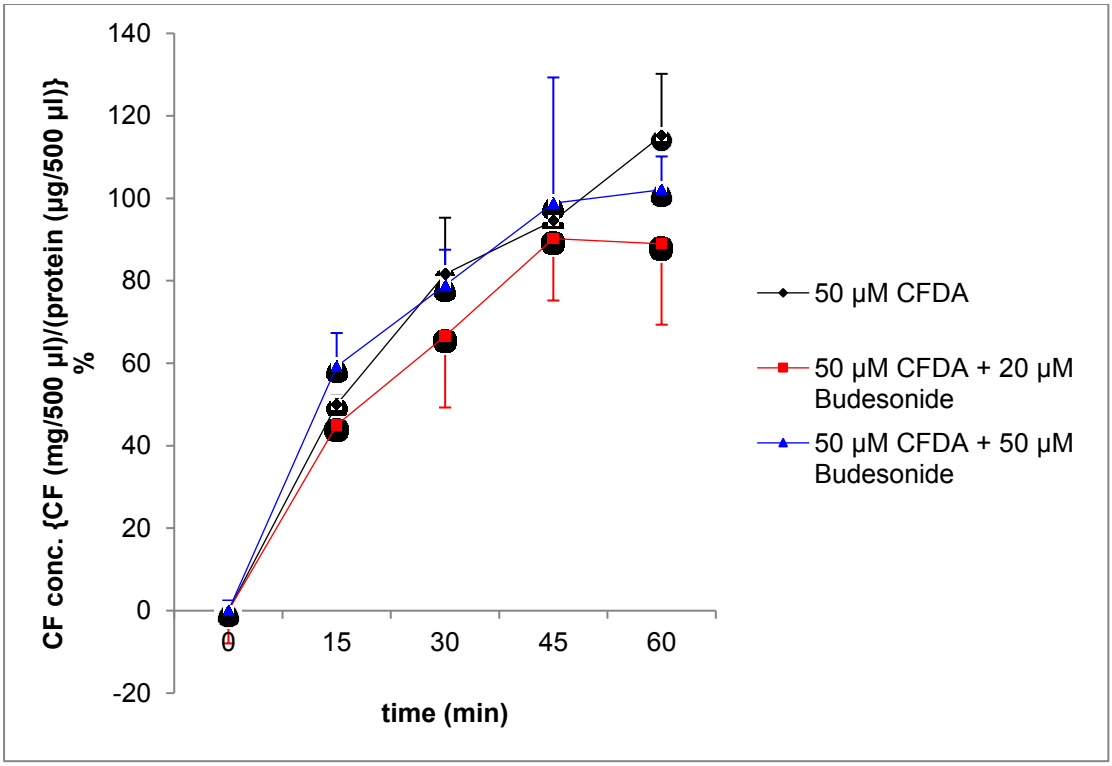

Figure 12 *Effects of 20 and 50 µM budesonide on CF efflux. The drug led to an insignificant retention of CF. Means ±SD, n=3*

Cell lysate:

time (min)	CFDA mean ± SD n=3	CFDA+ 20 µM Budesonide mean ± SD n=3	p	CFDA+ 50 µM Budesonide mean ± SD n=3	p
0	100.00 ± 15.08	100.00 ± 19.92	1.00	100.00 ± 20.80	1.00
15	77.27 ± 15.99	74.20 ± 8.56	0.7844	84.09 ± 8.38	0.5481
30	62.97 ± 14.54	57.54 ± 4.99	0.5745	67.05 ± 9.68	0.7061
45	48.67 ± 4.36	50.33 ± 8.66	0.7817	55.22 ± 16.81	0.5491
60	37.94 ± 6.44	37.21 ± 15.42	0.9431	41.18 ± 11.43	0.6904

Table 4 *Remaining intracellular concentration of CF after efflux assay with NCI-H441 cells treated with 5 and 10 µM budesonide*

After drawing samples of the supernatant, cells were lysed and the intracellular CF concentration was determined. However, the results did not significantly differ between the three treatment groups even though efflux had been reduced after the addition of budesonide. After one hour the concentration of the fluorophore was reduced by roughly 60% in all three cell types.

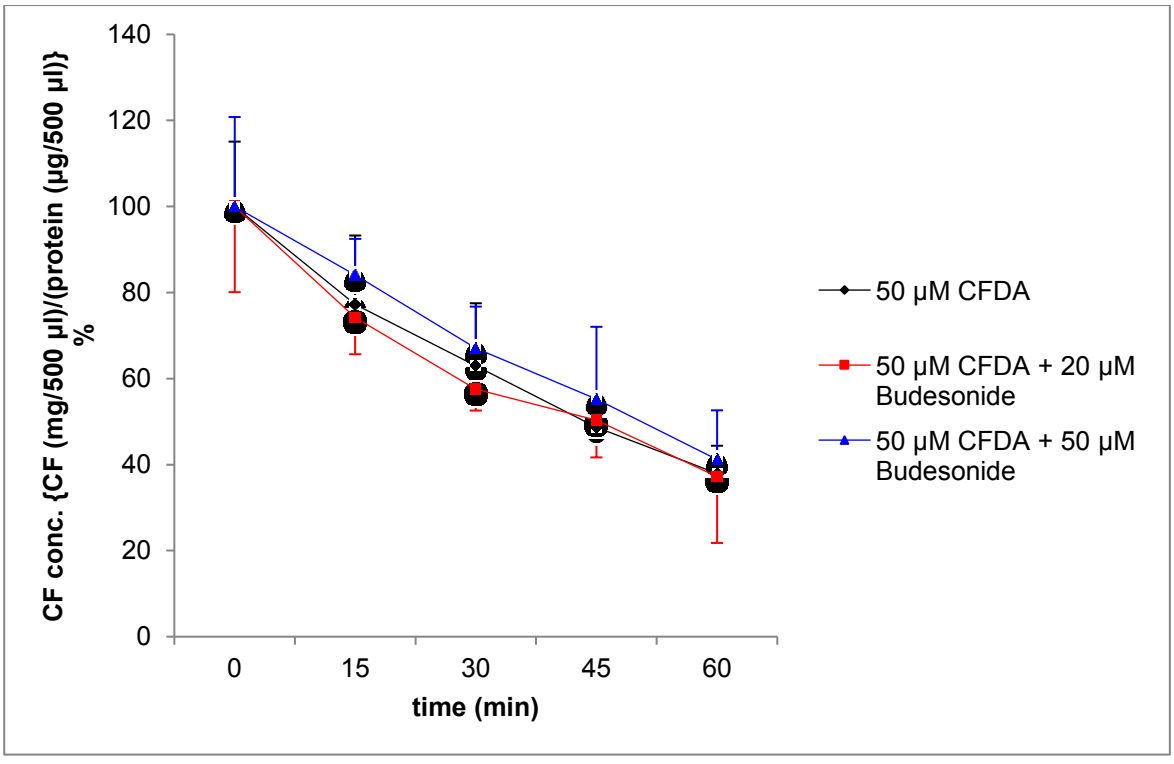

Figure 13 *CF concentration of lysed cells after efflux assay with 20 µM and 50 µM budesonide. No significant differences were measured. Means ±SD, n=3*

6.1.3. Dexamethasone 2 µM, 10 µM and 50 µM

To evaluate the effect of dexamethasone on MRP1 an efflux assay was conducted. A 20 mM stock solution of dexamethasone in DMSO was prepared and diluted to reach concentrations of 2 µM, 10 µM and 50 µM. As control cells were incubated with DMSO for 72 hours.

Supernatant:

time (min)	CFDA mean ± SD n=3	CFDA+ 2 µM Dexamethasone mean ± SD n=3	P	CFDA+ 10 µM Dexamethasone mean ± SD n=3	P	CFDA+ 50 µM Dexamethasone mean ± SD n=3	P
15	75.75 ±30.43	47.91 ±6.17	0.1953	52.18 ±14.03	0.2901	50.98 ±12.42	0.2617
30	109.91 ±27.91	72.44 ±10.41	0.0948	80.84 ±16.07	0.1929	77.18 ±21.98	0.1857
45	120.44 ±23.20	89.97 ±10.80	0.1082	107.03 ±21.02	0.4993	93.94 ±20.29	0.2107
60	148.62 ±36.48	110.57 ±15.42	0.1715	122.40 ±28.43	0.3818	111.52 ±24.80	0.2190

Table 5 *Results of efflux assay after incubation of NCI-H441 cells with 2, 10 and 50 µM dexamethasone for 72 hours.*

After one hour the concentration of CF measured in the supernatant of the control cells was 148.62%, while it ranged between 110.57% (2 µM dexamethasone) and 122.40% (10 µM dexamethasone) for cells treated with the drug. Dexamethasone led to retention of the fluorophore in all tested concentrations, however with p-values too high to consider results to be significant.

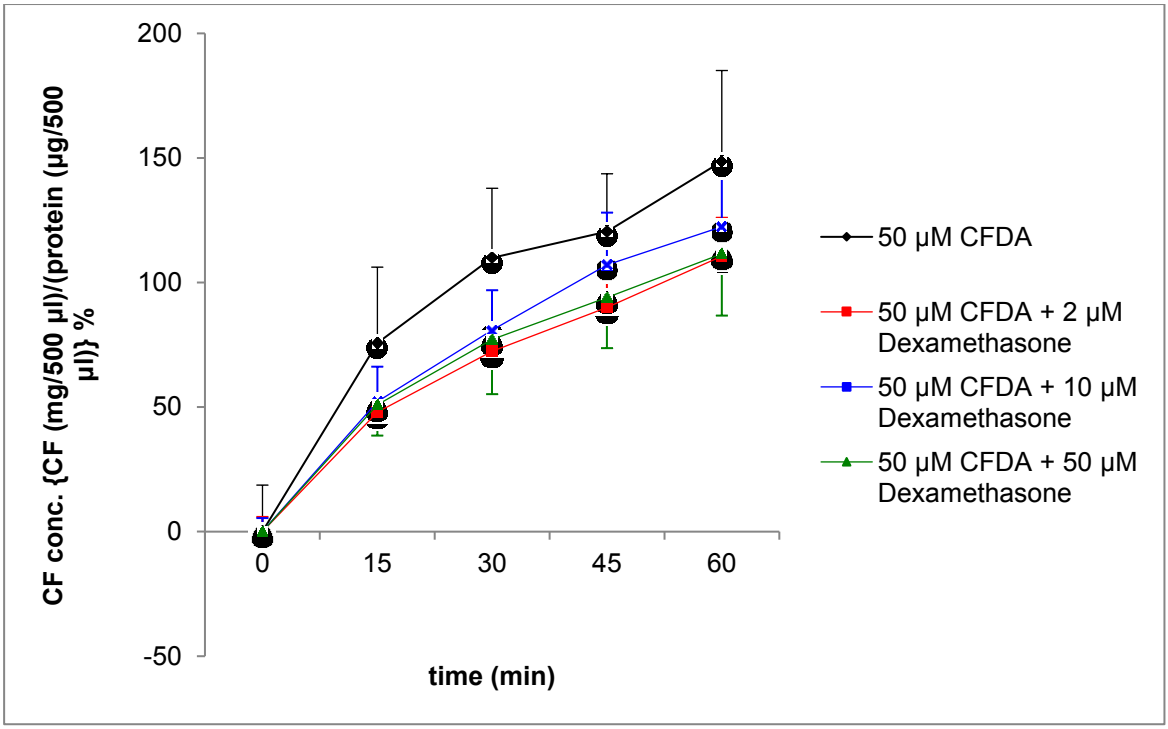

Figure 14 *CF efflux with 2 µM, 10 µM and 50 µM dexamethasone in NCI-H441 cells. The drug did not have a significant effect. Means ±SD, n=3*

Cell lysate:

time (min)	CFDA mean ± SD n=3	CFDA+ 2 µM Dexamethasone mean ± SD n=3	P	CFDA+ 10 µM Dexamethasone mean ± SD n=3	P	CFDA+ 50 µM Dexamethasone mean ± SD n=3	P
0	100.00 ± 18.51	100.00 ± 19.21	1.00	100.00 ± 23.93	1.00	100.00 ±16.82	1.00
15	98.38 ± 10.12	99.86 ± 2.45	0.8178	100.19 ± 12.27	0.8831	79.87 ± 2.82	0.0379*
30	73.60 ± 9.77	77.07 ± 3.09	0.5893	84.35 ± 9.59	0.2454	61.80 ± 5.43	0.1415
45	58.02 ± 11.75	60.45 ± 4.99	0.7574	66.65 ± 11.87	0.4212	53.05 ±12.51	0.6429
60	44.69 ± 10.51	51.31 ± 1.91	0.3434	52.23 ± 10.53	0.4240	38.01 ± 5.49	0.3848

Table 6 *intracellular concentrations of CF after termination of efflux assay. Statistical significant results (p≤ 0.05) marked with *.*

The only significant difference (p=0.0379) between control cells and one of the three treatment groups was observed with 50 µM dexamethasone after 15 minutes. A CF level of 79.87% was measured in these cells, whereas a concentration of 98,38 % was left in cells used for comparison.

After one hour 44.69% (control cells), 51.31% (2 µM dexamethasone), 52.23% (10 µM dexamethasone) and 38.01% (50 µM dexamethasone) of the initial intracellular CF concentration were determined in cells.

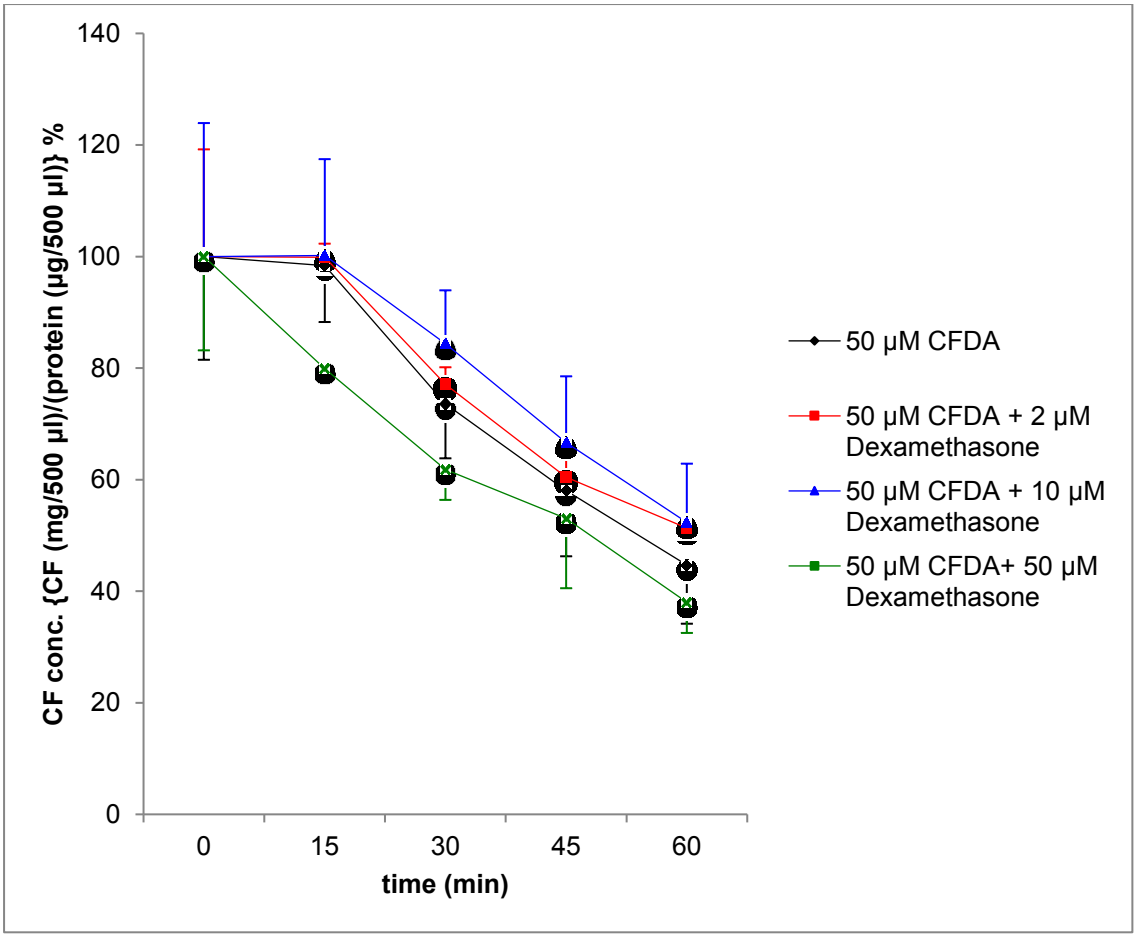

Figure 15 *CF concentration determined in lysed cells after treatment with 2 µM, 10 µM and 50 µM dexamethasone. Means ±SD, n=3*

6.1.4. Formoterol fumarate 200 µM

To investigate effects of formoterol fumarate on MRP1 a concentration of 200 µM (40 mM stock solution in DMSO) was chosen. DMSO was used to treat control cells.

Supernatant:

Time (min)	**CFDA mean ± SD n=3**	**CFDA + 200 µM Formoterol mean ± SD n=3**	p
15	50.51 ±14.16	46.49 ± 12.13	0.7274
30	71.27 ± 18.09	69.25 ± 17.70	0.8963
45	89.86 ± 28.86	80.12 ± 21.98	0.6659
60	101.56 ± 28.05	86.86 ± 26.04	0.5422

Table 7 *Results of efflux assay after incubation of NCI-H441 cells with 200 µM formoterol fumarate for 72 hours.*

As seen in figure 16 the control cells showed a marginally higher efflux of CF at all times. After one hour a final concentration of 101.56% was measured in their supernatant, whereas it was only 86.86% for the cells treated with formoterol fumarate. The differences between the two treatment groups were not significant according to a t-test.

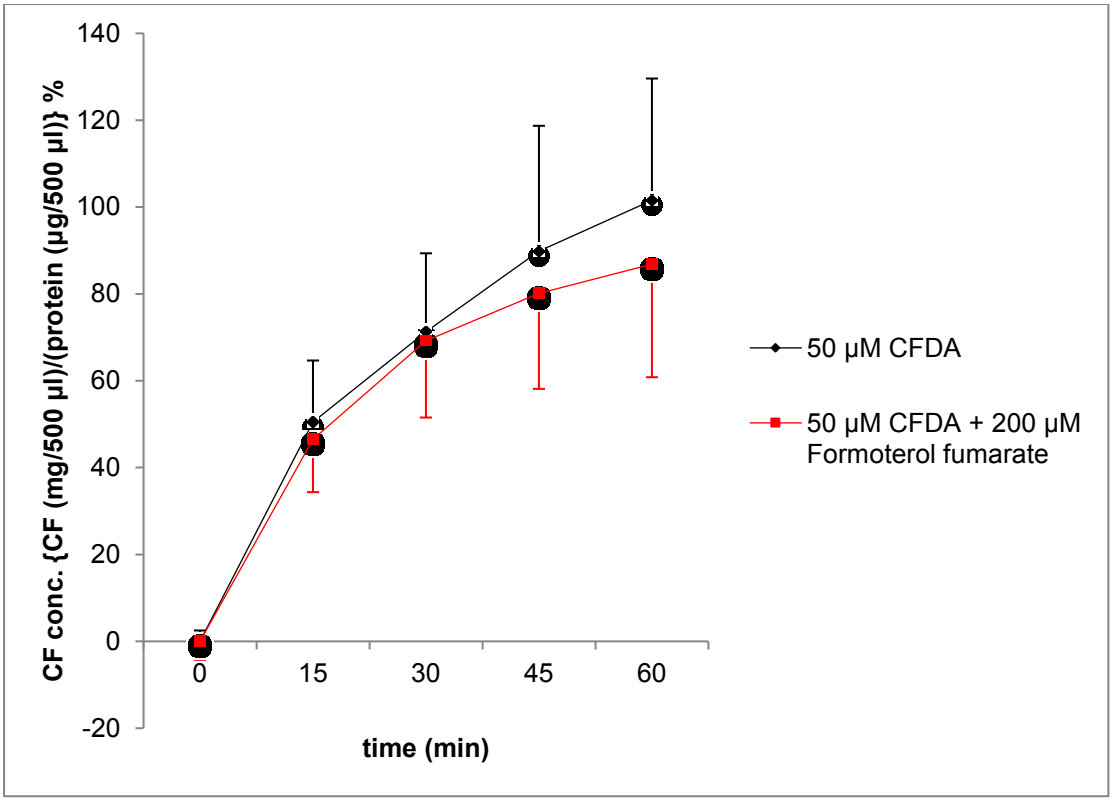

Figure 16 *CF efflux study with 200 µm formoterol fumarate. Control cells showed higher efflux. Means ±SD, n=3*

Cell lysate:

Time (min)	**CFDA mean ± SD n=3**	**CFDA + 200 µM Formoterol mean ± SD n=3**	**p**
0	100.00 ± 20.27	100.00 ± 22.78	1.00
15	77.87 ± 5.51	87.07 ± 6.82	0.1432
30	59.09 ± 3.11	65.04 ± 6.69	0.2351
45	45.60 ± 9.23	48.07 ± 3.02	0.6831
60	35.22 ± 8.23	34.36 ± 3.44	0.8743

Table 8 *Intracellular CF-concentration at the end of the efflux assay with 200 µM formoterol fumarate.*

The intracellular concentration of CF was determined after termination of the efflux assay. Very similar results were achieved for both groups, despite the fact that formoterol had led to decreased transport of the fluorophore. After one hour 35.22% of the initial concentration were left inside of the control cells and 34.36% in the ones treated with formoterol fumarate.

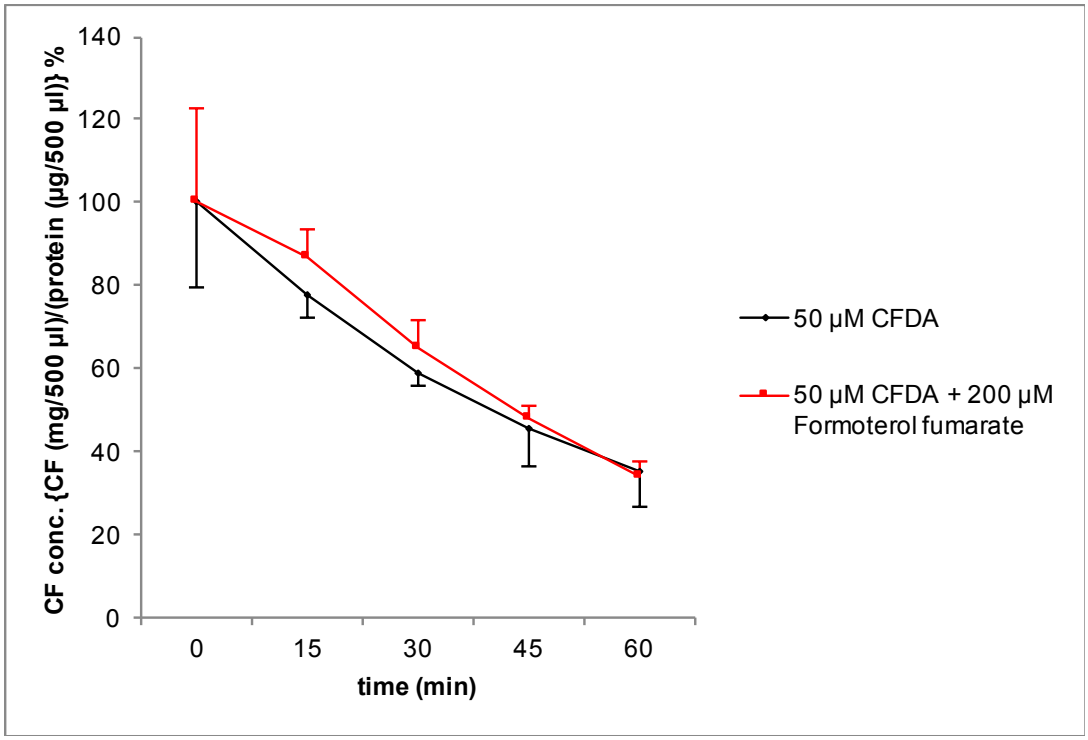

Figure 17 *CF concentration of lysed cells after efflux assay with 200 µM formoterol fumarate. Means ±SD, n=3*

6.1.5. Salbutamol 100 µM

Salbutamol and three of its salts, salbutamol sulphate (20 mM stock solution in water), (R)-salbutamol HCl and (S)-salbutamol HCl (both prepared from a 10 mM stock solution in water) were compared in the concentration of 100 µM each.

Supernatant:

time (min)	CFDA mean ± SD n=3	CFDA+ 100 µM Salbutamol mean ± SD n=3	p	CFDA+ 100 µM Salbutamol sulphate mean ± SD n=3	p	CFDA+ 100 µM (R)-Salbutamol HCl mean ± SD n=3	p	CFDA+ 100 µM (S)-Salbutamol HCl mean ± SD n=3	p
15	59.59 ±5.54	61.13 ±9.92	0.8253	49.27 ±11.41	0.2316	78.89 ±0.84	0.0415 *	71.24 ±11.42	0.1870
30	86.50 ±10.72	97.03 ±16.23	0.4016	75.91 ±16.07	0.3958	99.34 ±11.80	0.2356	105.29 ±24.27	0.2873
45	114.65 ±0.48	124.77 ±11.25	0.1942	96.49 ±14.83	0.1015	127.80 ±7.05	0.0321 *	149.77 ±20.69	0.0424 *
60	136.59 ±3.80	147.30 ±5.59	0.0517	110.36 ±10.20	0.0140 *	149.05 ±4.44	0.0209 *	148.93 ±9.48	0.1045

Table 9 *Data of efflux assay after 72 hour incubation of cells with 100 µM of various salbutamol salts. Statistical significant results (p≤0.05) marked with *.*

Salbutamol sulphate was the only one of these drugs that led to a significant (p= 0.014) retention of CF over the course of one hour. After 60 minutes 110.36% of the fluorescent were measured in the supernatant of this treatment group, which is about 26% less than in the supernatant of the control cells.

Contrary to this result, salbutamol as well as (R)- and (S)-salbutamol HCl led to increased levels of CF in the supernatant. Significant differences were determined between control cells and (R)-salbutamol HCl after 15 min (59.59% and 78.89%, respectively), 45 min (114.65% and 127.80%) and 60 minutes (136.59% and 149.05%). (S)-salbutamol HCl showed significantly higher efflux over the course of 45 minutes. Here a discrepancy of about 35% was measured.

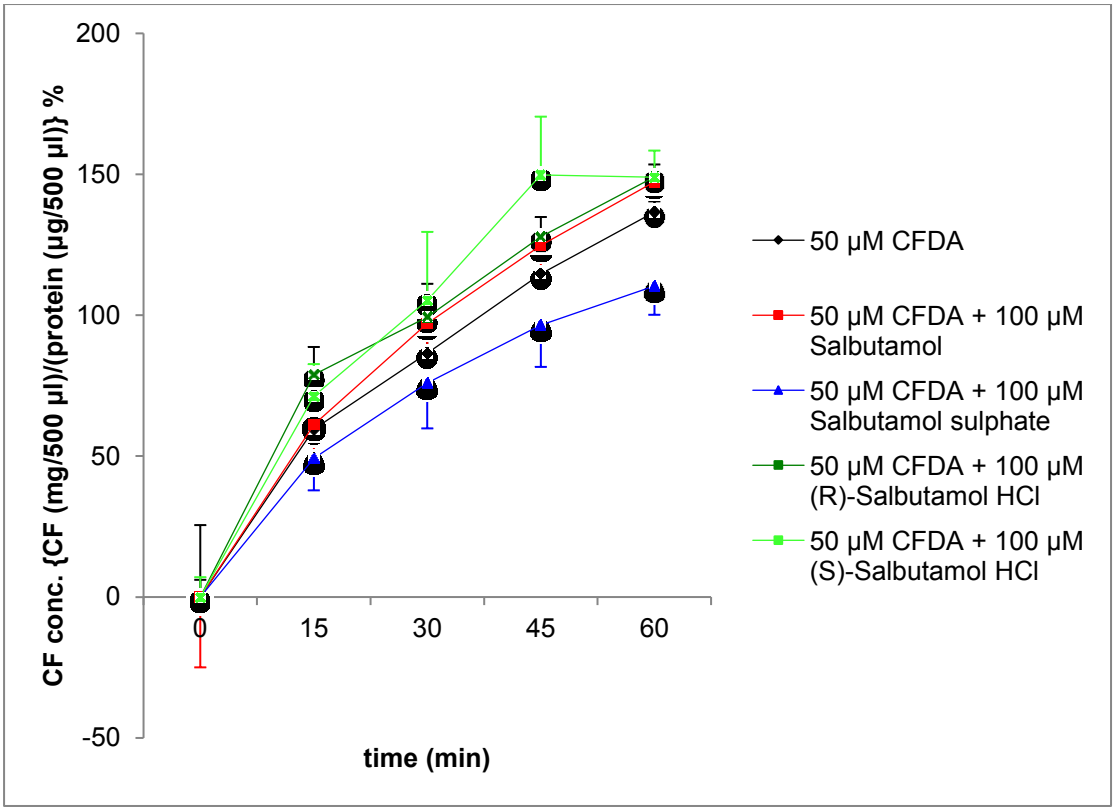

Figure 18 *Results of efflux assay after 72 hour incubation with salbutamol and three of its salts in a concentration of 100 µM. Salbutamol sulphate showed an inhibiting, the others a stimulating effect. Means ±SD, n=3*

Cell lysate:

time (min)	CFDA mean ± SD n=3	CFDA+ 100 µM Salbutamol mean ± SD n=3	p	CFDA+ 100 µM Salbutamol sulphate mean ± SD n=3	p	CFDA+ 100 µM (R)-Salbutamol HCl mean ± SD n=3	p	CFDA+ 100 µM (S)-Salbutamol HCl mean ± SD n=3	p
0	100.00 ±85.85	100.00 ±79.37	1.00	100.00 ±84.75	1.00	100.00 ±78.21	1.00	100.00 ± 87.17	1.00
15	74.10 ±8,97	81.44 ± 3.55	0.2576	75.73 ±3.07	0.7804	72.95 ± 6.08	0.8632	73.13 ± 6.90	0.8889
30	53.15 ±7.58	64.78 ± 4.22	0.0809	58.47 ±11.07	0.5296	55.03 ± 3.88	0.7205	57.20 ± 4.85	0.4788
45	42.80 ±6.68	50.63 ± 9.40	0.3048	44.33 ±6.20	0.7858	41.76 ± 2.47	0.8114	47.52 ± 4.34	0.3633
60	33.81 ±3.53	39.07 ± 6.14	0.2671	35.41 ±6.79	0.7341	32.38 ± 5.41	0.7219	33.46 ± 6.27	0.9382

Table 10 *Intracellular CF concentration after completion of the efflux study with 100 µM salbutamol, salbutamol sulphate, (R)- and (S)-salbutamol HCl..*

The high standard deviation of the initial CF concentration in all five treatment groups might be a result of a drastic change of cell passage number (P90, P91, P70). However, apart from a varying uptake of CFDA no differences were noticeable when it comes to the intracellular or effluxed CF concentration and SDs were far more moderate at the remaining time points.

Intracellular concentrations of each treatment group were comparable and final CF levels ranging between 32% and 39% were measured after one hour.

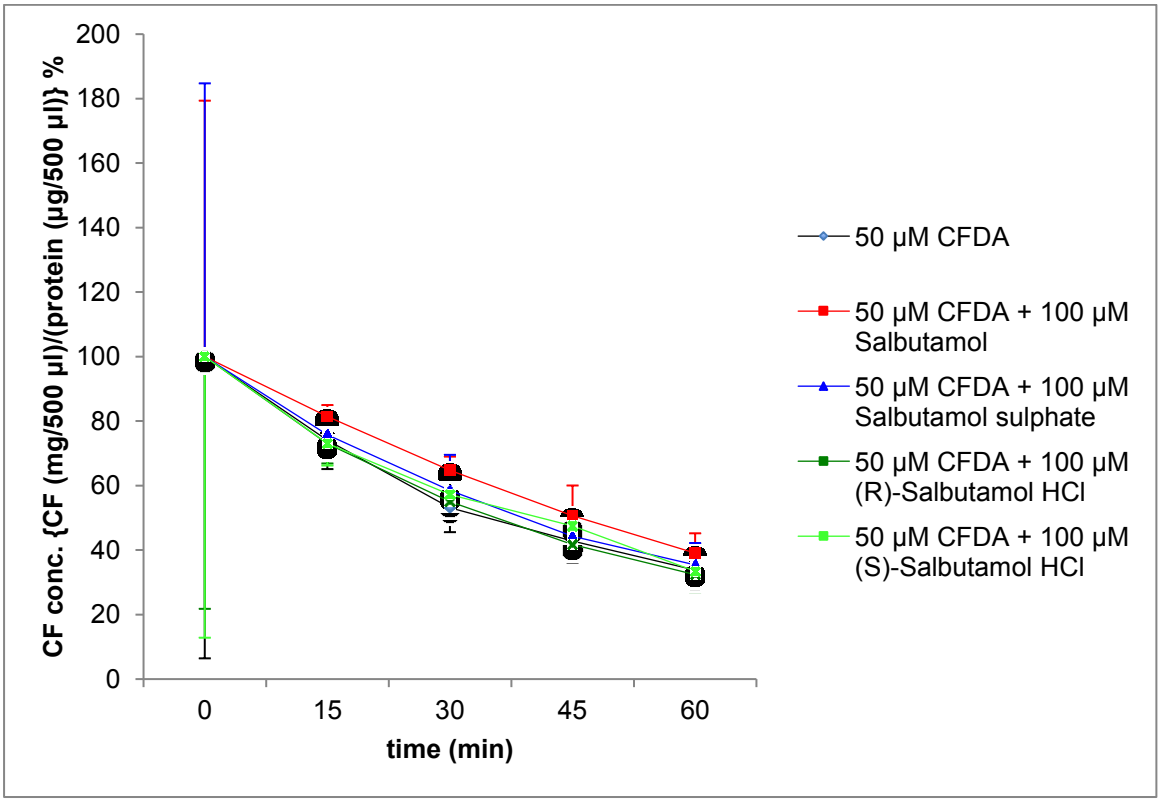

Figure 19 *CF concentrations determined in lysed cells after treatment with 100 µM salbutamol, salbutamol sulphate, (R)- and (S)-salbutamol HCl. Means ±SD, n=3*

6.2. Uptake studies

Cells in a 24-well plate were incubated with drugs for 72 h once monolayers had reached confluence. As described above the cells were loaded with 50 µM CFDA for 30 min or one hour, respectively, before being lysed with 1% Triton X-100 in KRB. The intracellular CF concentration was measured subsequently.

For better readability the unit "{(mg/500 µl)/(protein (µg/500 µl)}" will be left out in the following paragraphs.

The uptake assay was performed with following drugs:

- 5 µM, 10 µM, 20 µM, 50 µM budesonide
- 100 µM salbutamol
- 100 µM salbutamol sulphate
- 100 µM (R)-salbutamol HCl
- 100 µM (S)-salbutamol HCl
- 200 µM formoterol fumarate
- 2 µM, 10 µM, 50 µM dexamethasone

6.2.1. Budesonide 5 µM and 10 µM

CFDA mean ± SD n=3	CFDA+ 5 µM Budesonide mean ± SD n=3	P	CFDA+ 10 µM Budesonide mean ± SD n=3	P
1.21×10^{-6} ± 3.32×10^{-7}	9.74352×10^{-7} ± 3.18127×10^{-7}	0.4161	9.48×10^{-7} ± 3.54×10^{-7}	0.3948

Table 11 *Data of uptake study with 5 µM and 10 µM budesonide over the course of 30 minutes*

To investigate budesonide's effects on CFDA uptake, cells were treated with concentrations of 5 µM and 10 µM of the substance and a 30 min uptake study was conducted 72 h later. Although the addition of 5 µM budesonide (9.74352×10^{-7}) and 10 µM budesonide (9.48×10^{-7}) led to a reduced uptake compared to the control cells (1.21×10^{-6}), no statistical differences were observed.

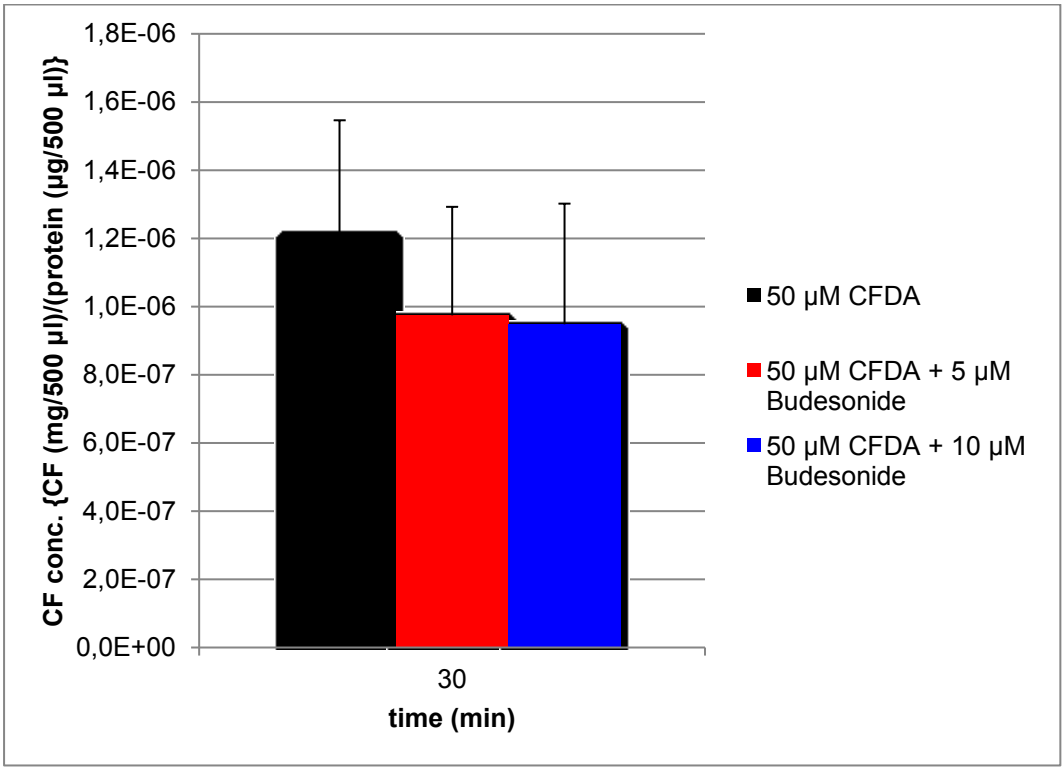

Figure 20 *30 minute uptake assay with 5 µM and 10 µM budesonide. CF levels were lower after treatment with the drug. Means ±SD, n=3*

4.2.2 Budesonide 20 µM and 50 µM

time (min)	CFDA mean ± SD n=1-2	CFDA+ 20 µM Budesonide mean n=1-2	CFDA+ 50 µM Budesonide mean n=1-2
30	8.50×10^{-7}	8.50×10^{-7}	8.72×10^{-7}
60	8.98×10^{-7}	6.36×10^{-7}	6.15×10^{-7}

Table 12 *Data of 30 and 60 minute uptake assay with 20 µM and 50 µM budesonide*

An uptake study with 20 µM and 50 µM budesonide was performed both over 30 and 60 min. Reduced uptake was observed in cells exposed to budesonide, with barely a difference between the tested concentrations. The experiments revealed lower intracellular CF levels in all three treatment groups after one hour than after half the amount of time. The biggest discrepancy was observed after treatment with 50 µM budesonide with

measured concentrations of $8.72\times10^{-7}$and 6.15×10^{-7}over 30 and 60 min, respectively.

No t-Test was performed, since each assay was only run one or two times.

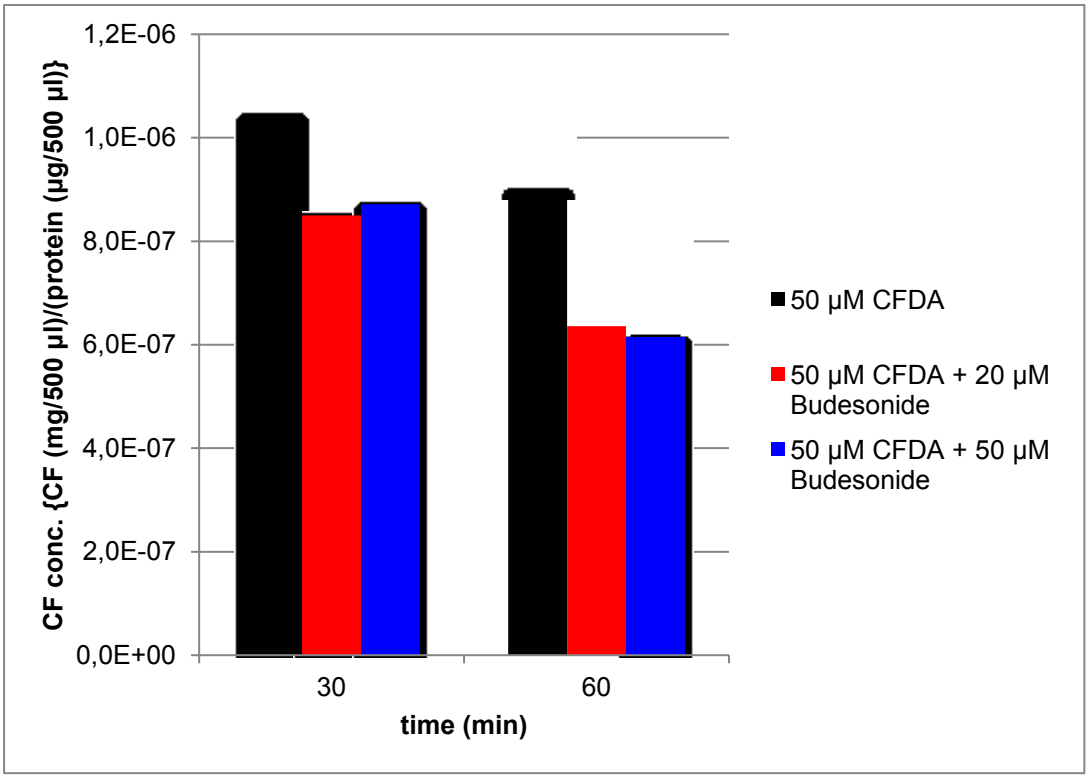

Figure 21 *Uptake assay with 20 µM and 50 µM budesonide. CF concentrations were reduced after incubation with the drug. Means ±SD, n=3*

4.2.3. Dexamethasone 2 µM, 10 µM and 50 µM

CFDA mean ± SD n=3	CFDA+ 2 µM Dexamethasone mean ± SD n=3	P	CFDA+ 10 µM Dexamethasone mean ± SD n=3	P	CFDA+ 50 µM Dexamethasone mean ± SD n=3	P
7.68×10^{-7} ± 1.73×10^{-7}	8.50×10^{-7} ± 1.71×10^{-7}	0.5900	8.63×10^{-7} ± 1.51×10^{-7}	0.5129	6.49×10^{-7} ± 1.10×10^{-7}	0.3753

Table 13 *Data of 60 minute uptake assay with 2 µM, 10 µM and 50 µM dexamethasone*

Depending on the tested concentration of dexamethasone, the uptake was slightly increased or decreased in relation to the control cells (7.68×10^{-7}). The lowest concentration was measured in cells treated with 50 µM

dexamethasone (6.49×10^{-7}), the highest in those incubated with 10 µM dexamethasone (8.63×10^{-7}).

Having said this, results are not significant according to t-tests, which showed high p-values.

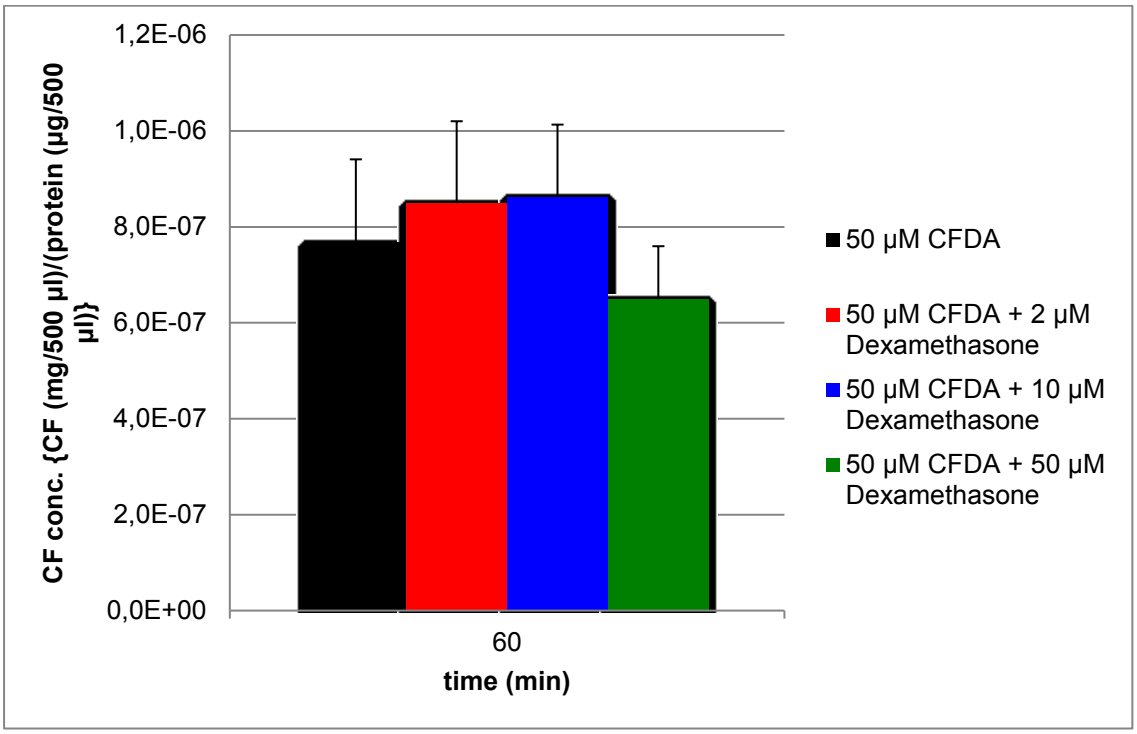

Figure 22 *Uptake assay with 2 µM, 10 µM and 50 µM dexamethasone. No significant impact observed. Means ± SD, n=3*

4.2.4. Formoterol fumarate 200 µM

time (min)	CFDA mean n=1-2	CFDA + 200 µM Formoterol mean n=1-2
30	8.38×10^{-7}	7.38×10^{-7}
60	9.47×10^{-7}	8.61×10^{-7}

Table 14 *Results of 30 and 60 minute uptake assays with 200 µM formoterol fumarate.*

Uptake studies indicate a reduced uptake of CFDA following a 72 h incubation with 200 µM formoterol fumarate.

After 30 min of incubation with 50 µM CFDA a CF concentration of 8.38×10^{-7} was measured in control cells. The level went up to 9.47×10^{-7} after twice the

time. Formoterol treated cells showed lower concentrations, 7.38×10^{-7} and 8.61×10^{-7}, respectively, to be precise.

Due to running the assays a mere one time over 30 mins and twice over 60 min, no t-tests were performed and no standard deviations were evaluated.

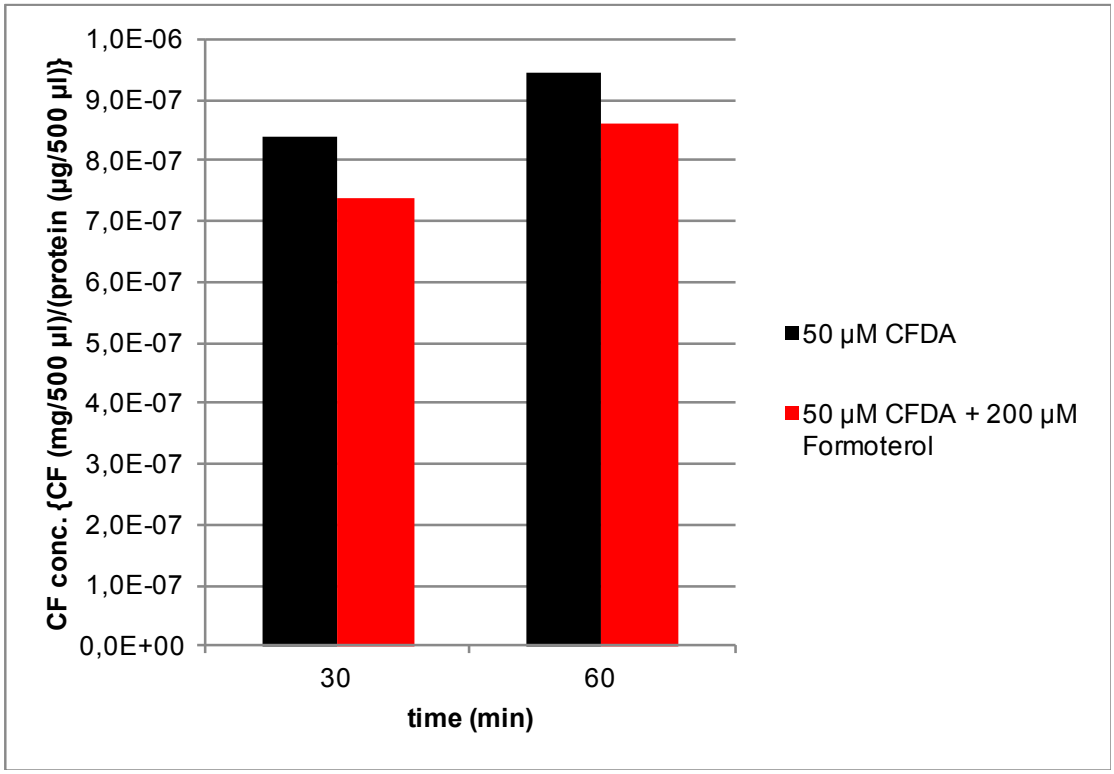

Figure 23 *Uptake assay with 200 µM formoterol fumarate. Reduced uptake was observed after treatment with the drug. Means, n=1-2*

4.2.5. Salbutamol 100 µM

CFDA mean ± SD n=3	CFDA+ 100 µM Salbutamol mean ± SD n=3	p	CFDA+ 100 µM Salbutamol sulphate mean ± SD n=3	p	CFDA+ 100 µM (R)-Salbutamol HCl mean ± SD n=3	p	CFDA+ 100 µM (S)-Salbutamol HCl mean ± SD n=3	p
8.86×10^{-7} ± 3.04×10^{-7}	8.93846×10^{-7} ± 3.49×10^{-7}	0.9790	8.78×10^{-7} ± 3.52×10^{-7}	0.9780	8.67×10^{-7} ± 4.91×10^{-7}	0.9562	8.53×10^{-7} ± 4.48×10^{-7}	0.9212

Table 15 *Results of 30 min uptake study with 100 µM salbutamol and three of its salts*

According to uptake assays conducted after treating NCI-H441 cells with 100 µM salbutamol, salbutamol sulphate, (R)-salbutamol HCl and (S)-salbutamol HCl neither drug showed a significant effect on CFDA uptake.

After a period of 30 min CF levels ranged between 8.53×10^{-7} (100 µM (S)-salbutamol HCl) and 8.93846×10^{-7} (100 µM salbutamol).

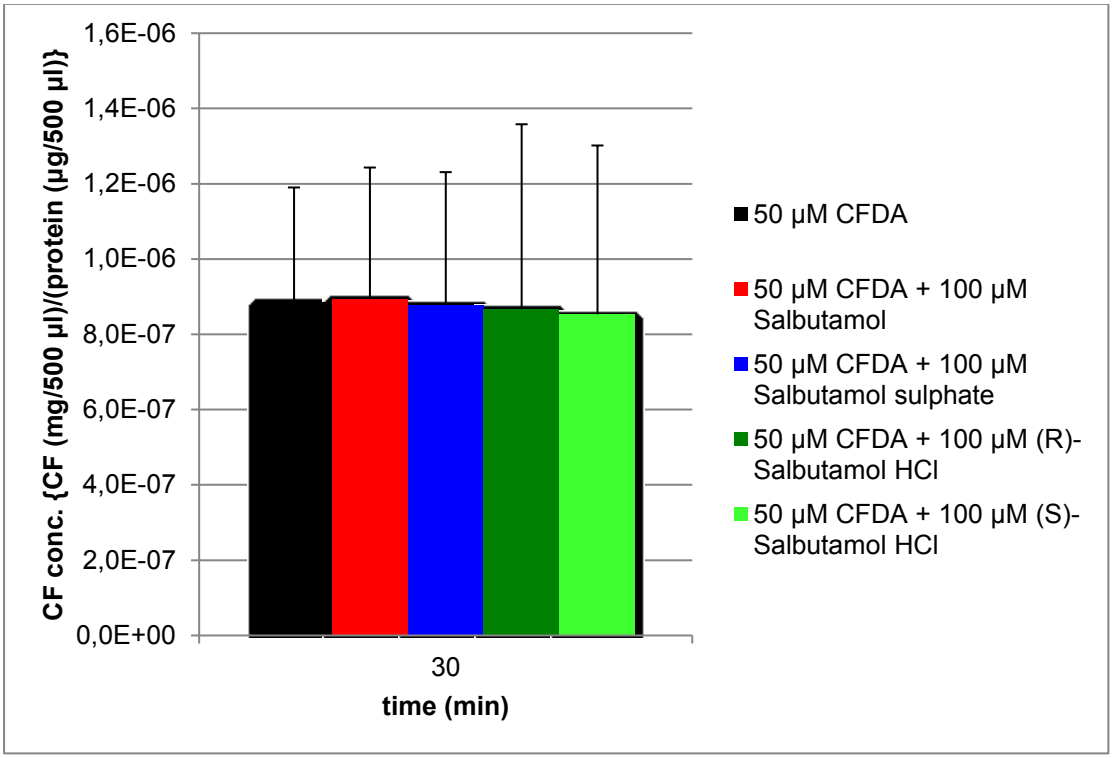

Figure 24 *30 min uptake assay with salbutamol and three of its salts. Means ±SD, n=3*

6.3. Short-term studies

Short-term uptake assays and efflux assays were run to investigate more immanent effects of drugs. For that purpose cells were incubated with the substances in question only for the duration of the experiment and not for 72 hours prior to starting them.

6.3.1. Budesonide 20 and 50 µM

4.3.1.1. Efflux assay

Supernatant:

time (min)	CFDA mean ± SD n=3	CFDA+ 20 µM Budesonide mean ± SD n=3	p	CFDA+ 50 µM Budesonide mean ± SD n=3	p
15	63.96 ± 17.75	61.18 ± 17.32	0.8558	73.87 ± 16.78	0.5209
30	103.85 ± 16.99	85.18 ± 24.46	0.3402	96.45 ± 17.95	0.6317
45	129.51 ± 25.59	105.16 ± 19.99	0.2638	101.16 ± 18.72	0.1963
60	148.34 ± 37.57	116.20 ± 24.35	0.2816	122.01 ± 21.58	0.3519

Table 16 *Data of efflux study after short-term treatment of cells with 20 µM and 50 µM budesonide*

Results were similar to those of the same experiment after an incubation time of 72 hours with the drug. Again budesonide led to a diminished activity of MRP1, with 20 µM of the drug having a slightly higher effect. After one hour the CF concentration in the supernatant of cells treated with budesonide was about 30% lower than that of the cells used for comparison. The analysis did not reveal any significant differences.

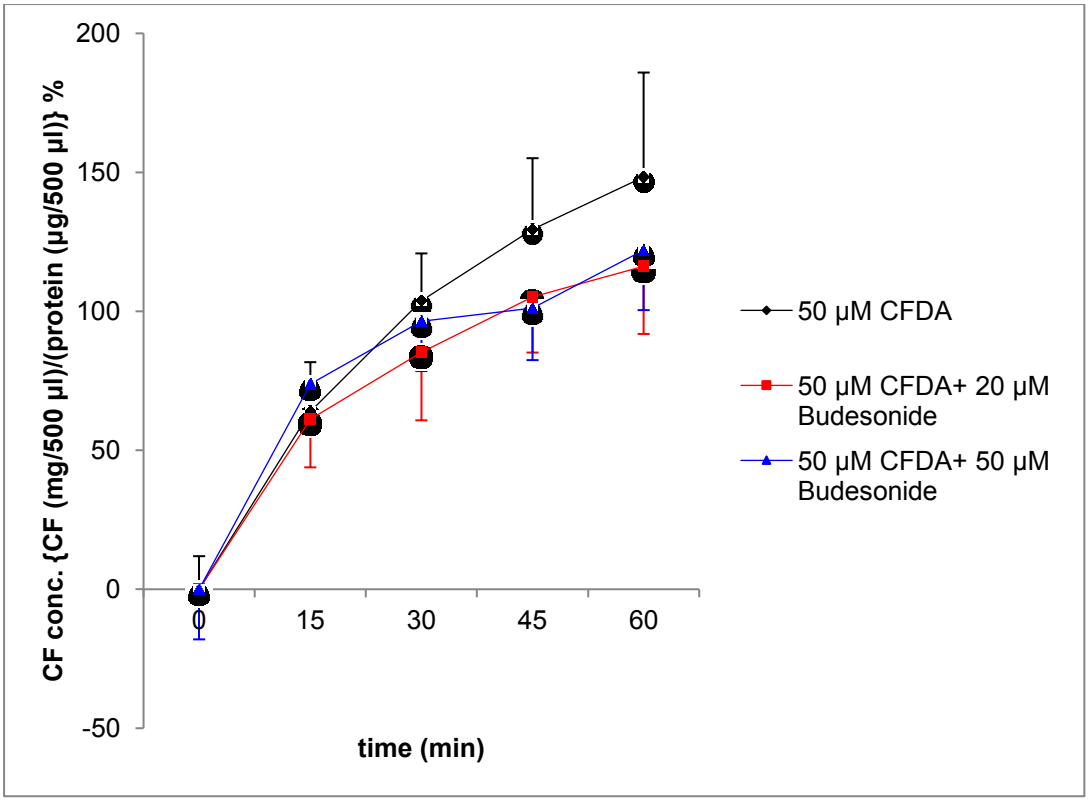

Figure 25 *Effects of short-term incubation with 20 µM and 50 µM budesonide on CF efflux. The drug led to a retention of CF. Means ±SD, n=3*

Cell lysate:

time (min)	CFDA mean ± SD n=3	CFDA+ 20 µM Budesonide mean ± SD n=3	p	CFDA+ 50 µM Budesonide mean ± SD n=3	p
0	100.00 ± 7.97	100.00 ± 5.33	1.00	100.00 ± 6.09	1.00
15	92.35 ± 16.48	91.34 ± 12.55	0.9369	90.69 ± 10.58	0.8902
30	68.62 ± 3.06	77.74 ± 8.04	0.1405	80.03 ± 6.48	0.0512
45	54.81 ± 9.48	61.71 ± 10.22	0.4398	60.73 ± 8.29	0.4617
60	42.99 ± 9.89	46.34 ± 9.80	0.6986	47.20 ± 8.41	0.6048

Table 17 *Remaining intracellular CF concentration after short-term incubation efflux assay with NCI-H441 cells treated with 20 and 50 µM budesonide*

Determined intracellular residues of CF were without significant differences between the three treatment types. The lowest concentration was measured in control cells (42.99%) after one hour. After treatment with 50 µM budesonide a CF level of 47.20% was observed, which was about 1% more than after the addition of 20 µM of the drug.

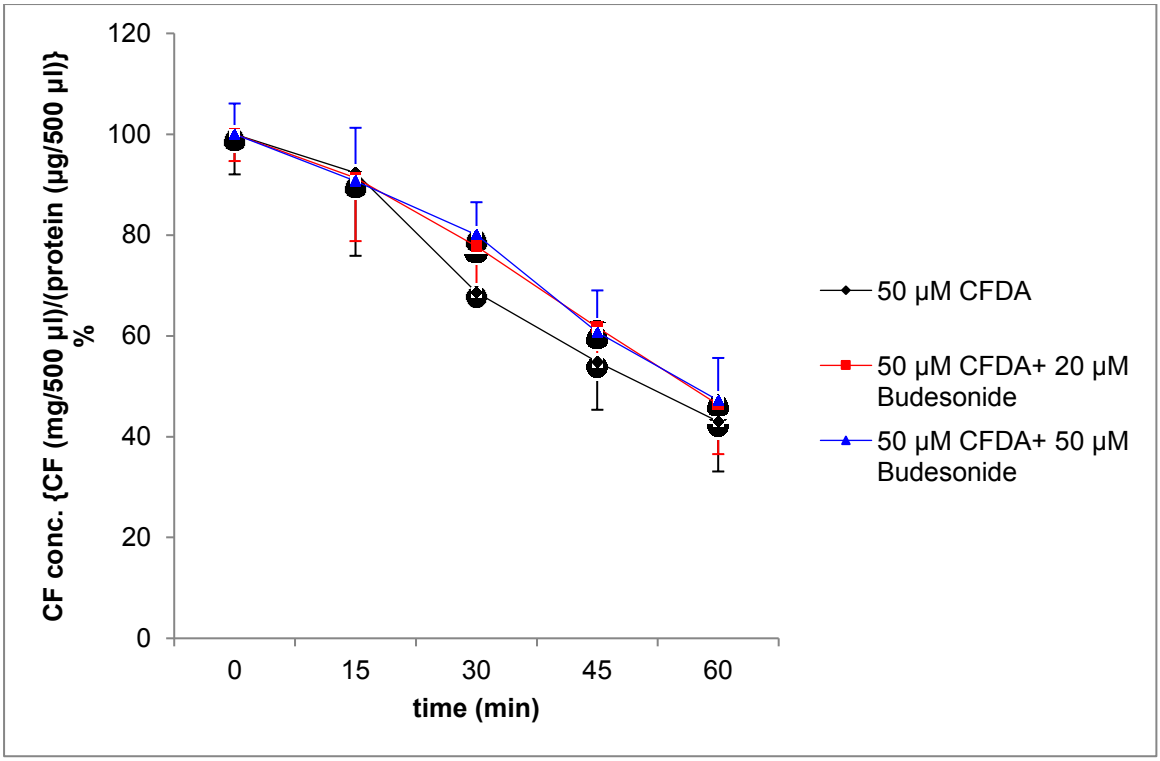

Figure 26 *Intracellular CF levels after termination of efflux assay with 20 and 50 µM budesonide short-term incubation*

4.3.1.2. Uptake assay

time (min)	CFDA mean ± SD n=3	CFDA+ 20 µM Budesonide mean ± SD n=3	p	CFDA+ 50 µM Budesonide mean ± SD n=3	p
30	6.64×10^{-7} ± 8.32×10^{-8}	7.35×10^{-7} ± 3.79×10^{-8}	0.2498	8.52×10^{-7} ± 1.07×10^{-7}	0.0747
60	7.22×10^{-7} ± 2.72×10^{-8}	9.05×10^{-7} ± 1.41×10^{-7}	0.0911	9.55×10^{-7} ± 1.03×10^{-7}	0.0190 *

Table 18 *Results of uptake assay with 20 µM and 50 µM budesonide. Statistical significant results (p≤0.05) marked with *.*

As illustrated in figure 25 uptake studies revealed that intracellular CF levels were lower in control cells than when exposing cells to budesonide over a short period of time. This finding is contrary to results of assays run after incubating cells with the same drug for 72 h before performing the experiment.

CF levels in control cells rose from 6.64×10^{-7} to 7.22×10^{-7}, when doubling the time of the uptake study. The addition of budesonide resulted in higher uptake both over half an hour and 60 min. When treating cells with 20 µM of the substance concentrations of 7.35×10^{-7} and 9.05×10^{-7} were observed. 50 µM had an even stronger effect on the fluorescent's intake, leading to intracellular CF levels of 8.52×10^{-7} and 9.55×10^{-7}, respectively.

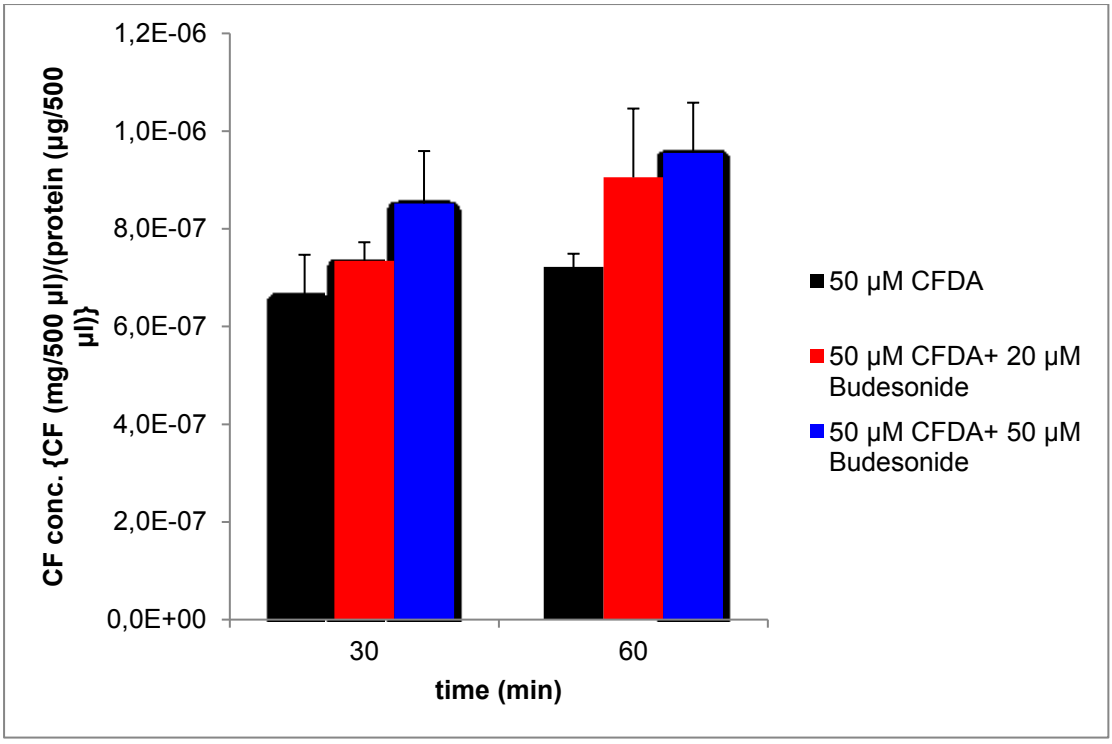

Figure 27 *Uptake assay with 20 µM and 50 µM budesonide. Increased uptake was observed after addition of the drug. Means ± SD, n=3*

6.3.2. Formoterol fumarate 200 µM

4.3.2.1. Efflux assay

Supernatant:

Time (min)	**CFDA mean ± SD n=3-4**	**CFDA + 200 µM Formoterol mean ± SD n=4**	p
15	72.86 ± 13.44	57.01 ± 16.05	0.1810
30	100.18 ± 31.49	90.18 ± 28.16	0.6760
45	126.80 ± 24.68	110.90 ± 30.30	0.4467
60	145.59 ± 32.93	123.59 ± 25.06	0.3287

Table 19 *Data of short-term efflux assay with 200 µM formoterol fumarate*

At all times CF concentrations in the supernatant were lower after the addition of 200 µM formoterol fumarate. After one hour a discrepancy of 22% was measured. This is consistent with the results of the same experiment after 72 hours of treatment with this drug.

Although formoterol led to decreased efflux of CF, no statistical significant differences were observed.

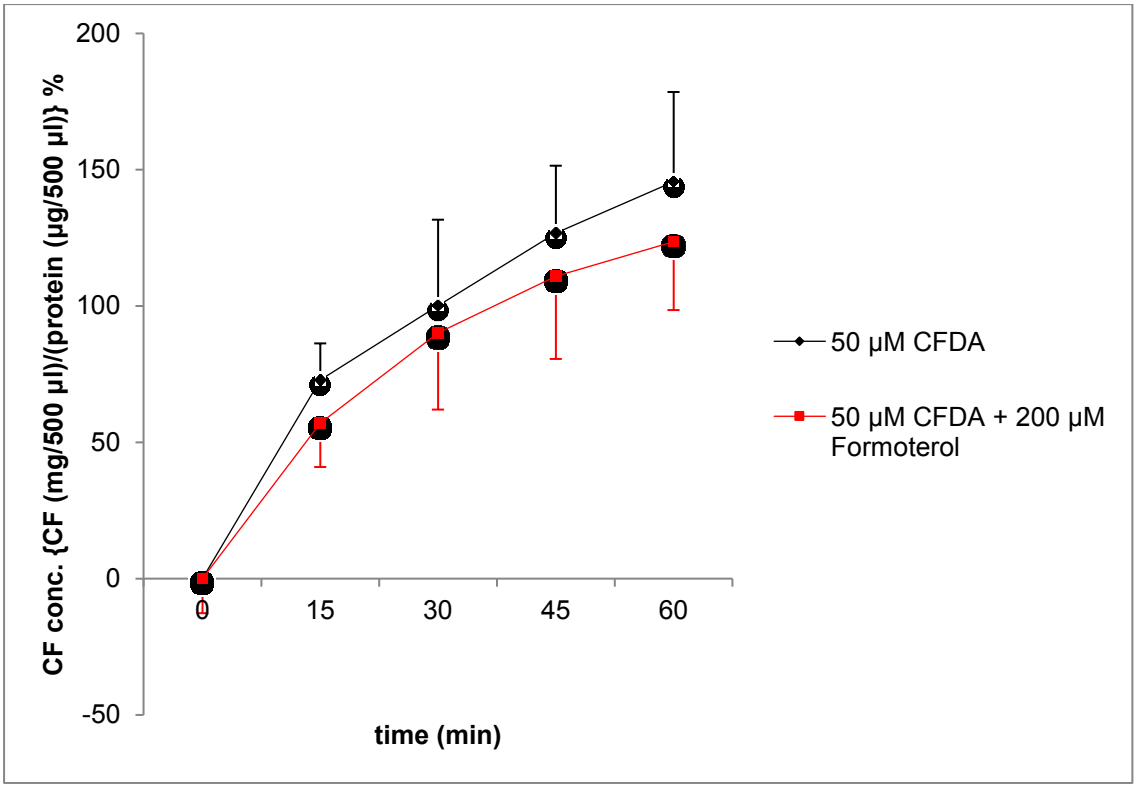

Figure 28 *Short-term efflux study with 200 µm formoterol fumarate. Control cells showed higher efflux. Means ±SD, n=3-4*

Cell lysate:

Time (min)	**CFDA mean ± SD n=3-4**	**CFDA + 200 µM Formoterol mean ± SD n=4**	p
0	100.00 ± 31.02	100.00 ± 22.87	1.00
15	82.83 ± 3.27	78.39 ± 10.02	0.4311
30	66.68 ± 8.81	53.22 ± 9.07	0.1065
45	49.10 ± 3.82	34.94 ± 7.12	0.0127*
60	38.27 ± 4.53	26.43 ± 6.47	0.0240*

Table 20 *Intracellular CF-concentration at the end of a short-term efflux assay with 200 µM formoterol fumarate. Statistical significant results (p≤ 0,05) marked with **

Intracellular CF levels determined after completion of efflux studies were decreased with 200 µM formoterol fumarate compared to control cells, as seen in figure 29. Statistical significant differences were observed after 45 and 60 min. The final concentration was significantly (p=0.0240) reduced from 38.27% to 26.43% while 15 minutes earlier CF levels of 49.10% (control cells) and 34.94% (200 µM formoterol fumarate) were observed (p=0.0127).

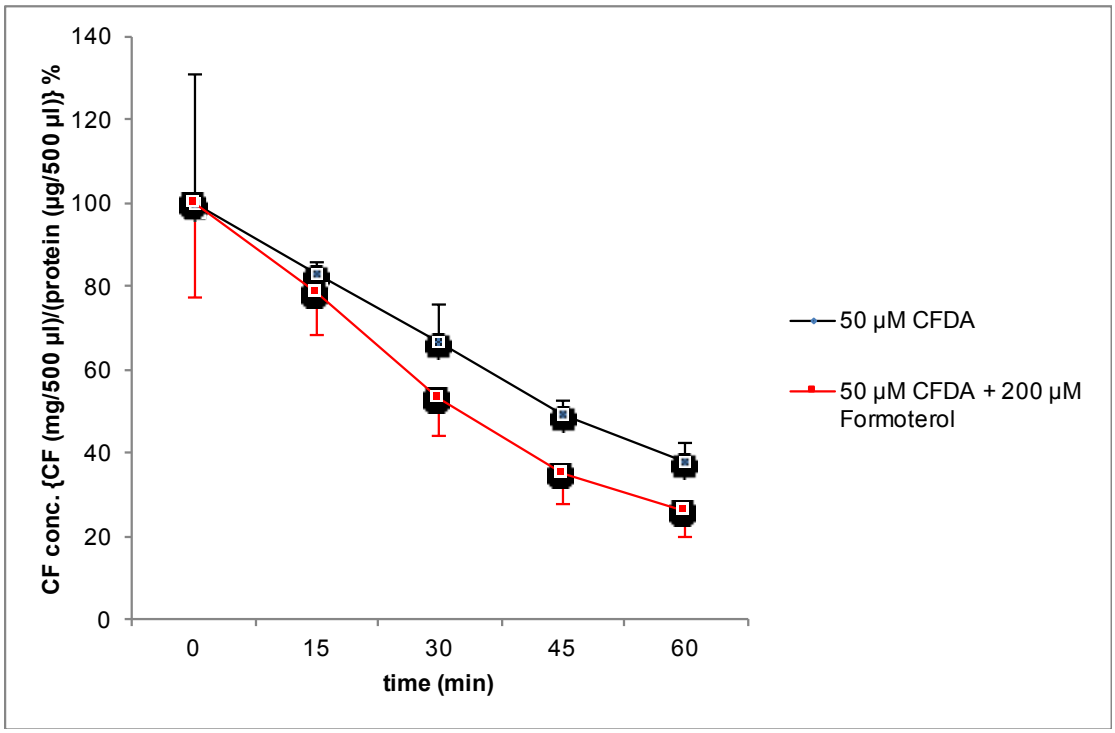

Figure 29 *CF concentration of lysed cells after short-term efflux assay with 200 µM formoterol fumarate. Means ±SD, n=3-4*

4.3.2.2. Uptake assay

time (min)	CFDA mean ± SD n=3-4	CFDA + 200 µM Formoterol mean ± SD n=3-4	p
30	$6.12\times10^{-7} \pm 2.39\times10^{-7}$	$4.98\times10^{-7} \pm 1.57\times10^{-7}$	0.4537
60	$5.91\times10^{-7} \pm 2.64\times10^{-7}$	$4.30\times10^{-7} \pm 1.63\times10^{-7}$	0.4196

Table 21 *Data of short-term incubation uptake study with 200 µM formoterol*

The short-term incubation with 200 µM formoterol fumarate resulted in a reduced uptake of CFDA, just like the 72 h incubation with the drug did. After one hour slightly lower concentrations than after 30 min were observed, in both the control cells and the ones treated with formoterol.

The highest amount of CF (6.12×10^{-7}) was measured in control cells after half an hour. The concentration determined after 60 min was just marginally lower (5.91×10^{-7}). After the addition of 200 µM formoterol intracellular CF levels were insignificantly reduced to 4.98×10^{-7} and 4.30×10^{-7}, respectively.

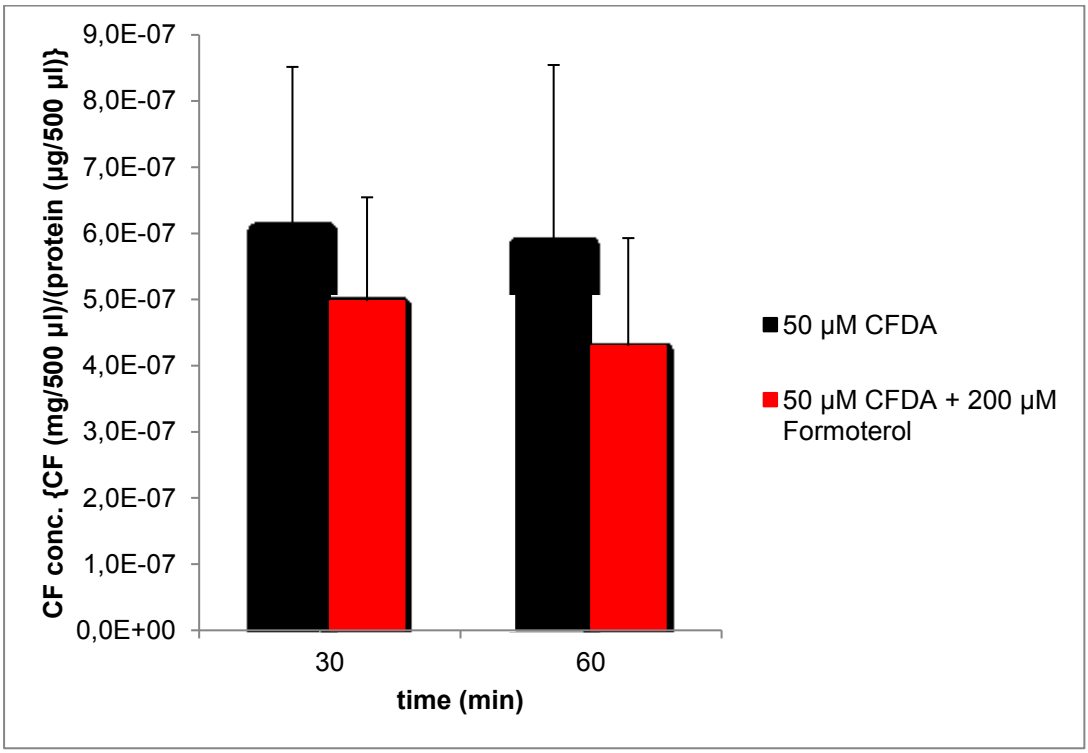

Figure 30 *30 and 60 minute uptake assay with 200 µM formoterol fumarate after short-term incubation. Treatment with formoterol resulted in decreased uptake. Means ±SD, n=3-4*

7. DISCUSSION

The present study evaluates effects of drugs in COPD and asthma therapy on MRP1 in NCI-H441 cells. MRP1 is an efflux pump in plasma membrane and subcellular compartments like lysosomes, Golgi apparatus and nucleus, with particularly high expression in lung, testes, kidney, placenta and bladder. (58,59)

It plays a protective role in cells by detoxifying them and as a consequence diminishes the risk of developing chronic obstructive pulmonary disease. (62) Apart from exporting toxins, MRP1 also contributes to multidrug resistance, by effluxing drugs such as methotrexate, antiandrogens, HIV-therapeutics (saquinavir, ritonavir) and therapeutics used in cancer treatment like doxorubicin, vincristine, vinblastine and paclitaxel. (51)

Uptake and efflux assays, both after short-term incubation and long-term incubation over 72 h, were performed with budesonide, dexamethasone, salbutamol and formoterol to find out if these drugs somehow affect the transporter.

It was shown that budesonide had an effect on MRP1 in concentrations higher than 5 µM, however, while efflux of CF was indeed reduced, it was in a non-significant manner. The results of efflux studies performed after long and short-term incubation with the substance in question were conformable. This confirms previous findings by van der Deen who showed that 10 µM and 100 µM budesonide led to significant accumulation of CF in the bronchial epithelial cell line 16HBE14o$^-$. (74)

Reduced uptake of CFDA was investigated in cells treated with 20 µM and 50 µM budesonide for 72 hours. However, this finding is contrary to the results of an uptake study where cells were only in contact with the drug for the duration of the experiment. Here the quantity of CF was higher in cells treated with budesonide than in those that were not.

Uptake assays performed after treating cells with 20 µM and 50 µM budesonide for 72 h yielded another surprising result. Intracellular CF concentrations were lower after incubating cells with 50 µM CFDA for one hour than after half the amount of time.

Just like budesonide 2 µM, 10 µM and 50 µM dexamethasone had a statistical insignificant inhibiting effect on MRP1 when running an efflux study after long-term incubation. The highest effect was achieved when treating cells with 2 µM of the substance. After 60 minutes the investigated concentration of CF in the supernatant of these cells was roughly 38% below that of control cells. An

also performed uptake study revealed that depending on the tested concentration of dexamethasone, the intake was slightly increased (2 µM and 10 µM dexamethasone) or decreased (50 µM dexamethasone) in relation to the control cells.

Efflux studies, both after long- and short-term incubation with formoterol fumarate, revealed a decreased efflux of CF after adding 200 µM of the drug to cells. After termination of the experiment following a 72 hour incubation with formoterol, a difference of 15% in CF concentration was determined between the supernatants of treated and untreated cells. Performing the experiment after a shorter treatment with the substance, the discrepancy increased to 22%. However, it must be said that results were not statistical significant according to t-tests.

By running uptake assays it was shown that formoterol caused cells to contain less CF than untreated control cells after incubation with CFDA for 30 or 60 minutes. When prolonging incubation time with CFDA from half an hour to one hour, the concentration increased following a long-term treatment with formoterol. As opposed to that, the quantity of CF was slightly reduced when doubling the time after a short-term incubation. Since long-term uptake studies with 200 µM formoterol fumarate over 30 and 60 min were only performed once and twice, respectively, no p-values were evaluated and it might be worth looking further into it.

100 µM salbutamol, salbutamol sulphate as well as (R)-and (S)-salbutamol HCl were examined regarding their effects on efflux of CF. The only drug that led to a significantly reduced transport of the fluorophore was salbutamol sulphate. When it comes to salbutamol, (R)- and (S)- salbutamol HCl a slight increase in CF concentration in the supernatant was determined. Uptake assays conducted with the same drugs revealed no differences compared to the control cells after 30 minutes.

Point of criticism regarding this study is that several experiments were not run a sufficient number of times. In addition standard deviations and p-values were quite high in most experiments, making results insignificant. To verify this data further testing is required. Yet another problem was a drastic change of cell passage number in between efflux studies with salbutamol and its salts. This led to extreme differences in uptake of CFDA and as a result to high standard deviations when it comes to the intracellular level of CF.

In summary it can be stated that budesonide, dexamethasone, formoterol fumarate and salbutamol sulphate led to reduced efflux of CF and thus seem to have an inhibiting effect on MRP1, however further studies should be conducted to reinforce these results.

8. ZUSAMMENFASSUNG

Die vorliegende Studie beschäftigt sich mit den Einflüssen verschiedener Medikamente, die bei der Therapie von Asthma und COPD zum Einsatz kommen, auf den Transporter MRP1 in Zellen der Zelllinie NCI-H441. Bei MRP1 handelt es sich um eine Effluxpumpe in Plasmamembranen und subzellulären Kompartiments wie Lysosomen, Golgi-Apparat und Nukleus. Besonders hohe Expressionsraten finden sich in Lunge, Hoden, Niere, Plazenta und Harnblase. (58,59)

MRP1 hat eine Schutzfunktion in Zellen inne, indem es sie entgiftet und so das Risiko, an COPD zu erkranken reduziert. (62) Neben dem Export von Toxinen trägt der Transporter allerdings durch den Abtransport von beispielsweise Methotrexat, Antiandrogenen, HIV-Medikamenten (Saquinavir, Ritonavir) und Therapeutika, die bei der Behandlung von Krebs zum Einsatz kommen (Doxorubicin, Vincristin, Vinblastin, Paclitaxel) auch zu der Entwicklung von Multidrug-Resistenzen bei. (51)

Nach kurzzeitiger oder 72-stündiger Behandlung der Zellen mit Budesonid, Dexamethason, Salbutamol und Formoterol wurden Studien bezüglich der Aufnahme von CFDA und dem Efflux von CF durchgeführt um festzustellen, ob die zuvor erwähnten Arzneistoffe die Funktion von MRP1 beeinträchtigen.

Es zeigte sich, dass Budesonid ab einer Konzentrationen von 5 µM eine Wirkung auf MRP1 hat. Zwar wurde CF vermindert aus den Zellen transportiert, allerdings geschah dies in insignifikantem Maße. Versuche nach einer kurzfristigen Inkubation, sowie einer 72 Stunden andauernden, erzielten hier dasselbe Resultat. Diese Ergebnisse bestätigen auch frühere Erkenntnisse von van der Deen, laut denen 10 µM und 100 µM Budesonid zu einer signifikanten Akkumulation von CF in der bronchialen Epithelzelllinie 16HBE14o$^-$ führten. (74)

Nach 72-stündiger Behandlung der Zellen mit 20 µM und 50 µM Budesonid wurde nach Beendigung der Uptake-Studie eine verminderte intrazelluläre Konzentration an CF festgestellt. Dieses Ergebnis ist gegensätzlich zu den Resultaten einer Uptakestudie, bei der die Zellen nur für die Dauer des Experiments mit besagtem Arzneistoff inkubiert wurden. In diesen wurden höhere Mengen des Fluorophors festgestellt als in den Kontrollzellen.

Uptakestudien die durchgeführt wurden nachdem die Zellen für 72 Stunden mit 20 und 50 µM des Arzneistoffs inkubiert worden waren, lieferten ein weiteres unerwartetes Ergebnis. Nach einstündiger Behandlung mit 50 µM

CFDA war die intrazelluläre Konzentrationen an CF geringer als nach nur 30 Minuten.

So wie Budesonid hatten auch 2 µM, 10 µM und 50 µM Dexamethason eine insignifikant inhibierende Wirkung auf MRP1, wie eine Efflux-Studie nach längerer Inkubation mit der Substanz zeigte. Der stärkste Effekt wurde mit 2 µM des Arzneistoffs erzielt. Nach 60 Minuten lag die Konzentration an CF im Überstand der Zellen etwa 38% unter jener der Kontrollzellen.

Eine ebenfalls vorgenommene Uptakestudie ergab, dass die Aufnahme von CFDA, abhängig von der untersuchten Konzentration, im Vergleich zu den Kontrollzellen leicht reduziert (2 µM und 10 µM Dexamethason) oder gesteigert (50 µM Dexamethason) war.

Efflux-Experimente, sowohl nach lang- als auch kurzfristiger Inkubation mit Formoterol fumarat, zeigten nach Behandlung mit 200 µM der Substanz eine Reduktion des Transports von CF. Bei Abschluss des Experiments, das nach einer 72-stündigen Inkubation mit Formoterol durchgeführt wurde, lag der Unterschied der CF-Konzentration im Überstand von behandelten und unbehandelten Zellen bei 15%. Nach einer kürzeren Inkubation mit der Substanz stieg diese Differenz auf 22%. Allerdings waren die Ergebnisse laut eines t-Tests insignifikant.

Formoterol führte außerdem dazu, dass nach 30- und 60-minütiger Inkubation mit CFDA geringere Konzentrationen an CF gemessen wurden als in Kontrollzellen. Wurden die zuvor für 72 Stunden mit Formoterol behandelten Zellen statt einer halben Stunde eine volle Stunde mit CFDA inkubiert, stieg die Menge an CF an. Im Gegensatz dazu nahm die intrazellulär festgestellte Menge nach einer kurzzeitigen Behandlung mit dem Arzneistoff bei Verdopplung der Zeit mit CFDA ab. Da die Uptakestudien über 30 und 60 Minuten nach längerem Zusatz von 200 µM Formoterol fumarat nur einmal beziehungsweise zweimal durchgeführt wurden, erfolgte hier keine Berechnung von p-Werten und weitere Tests sind erforderlich.

100 µM Salbutamol, Salbutamol sulphat sowie (R)-und (S)-Salbutamol HCl wurden ebenso bezüglich ihres Einflusses auf den auswärts gerichteten Transport von CF untersucht. Der einzige Arzneistoff der den Efflux des Fluorophors signifikant reduzierte war Salbutamol sulphat. Salbutamol (R)- und (S)- Salbutamol HCl hingegen führten zu einem leichten Anstieg der Konzentration von CF im Überstand.

Uptakestudien die über einen Zeitraum von 30 Minuten durchgeführt wurden, zeigten keine Unterschiede zwischen den zuvor genannten Substanzen.

Ein Kritikpunkt an der vorliegenden Studie ist, dass mehrere Experimente nicht genügend oft durchgeführt wurden. Zudem sind die Standardabweichungen und p-Werte oftmals sehr hoch, wodurch die erzielten Resultate insignifikant sind. Um diese Daten zu bestätigen sind weitere Tests erforderlich. Ein weiteres Problem war eine drastische Veränderung der Zellpassagennummer zwischen den Experimenten mit Salbutamol und seinen Salzen. Dies führte zu extremen Unterschieden bei der Aufnahme von CFDA und dadurch kam es zu hohen Standardabweichungen bezüglich der intrazellulären CF-Konzentration.

Zusammenfassend lässt sich sagen, dass Budesonid, Dexamethason, Formoterol fumarat und Salbutamol sulphat zu einem reduzierten Efflux von CF führten und somit einen inhibierenden Effekt auf MRP1 zu haben scheinen. Jedoch sollten weitere Experimente durchgeführt werden, um diese Ergebnisse zu stärken.

9. REFERENCES

1. Buehlmann AA. The Lungs and Respiration. In: Buehlmann PD med AA, Froesch PD med ER, editors. Pathophysiology [Internet]. Springer New York; 1979 [cited 2017 Apr 1]. p. 1–31. Available from: http://link.springer.com/chapter/10.1007/978-1-4612-9954-7_1

2. Faller A, Schünke M, Schünke G. Der Körper des Menschen. 16th ed. Stuttgart: Thieme; 2012.

3. Ochs M, Nyengaard JR, Jung A, Knudsen L, Voigt M, Wahlers T, et al. The Number of Alveoli in the Human Lung. Am J Respir Crit Care Med. 2004 Jan 1;169(1):120–4.

4. Pfister T, Dolan D, Bercu J, Gould J, Wang B, Bechter R, et al. Bioavailability of Therapeutic Proteins by Inhalation—Worker Safety Aspects. Ann Occup Hyg. 2014 Aug 1;58(7):899–911.

5. Labiris NR, Dolovich MB. Pulmonary drug delivery. Part I: Physiological factors affecting therapeutic effectiveness of aerosolized medications. Br J Clin Pharmacol. 2003 Dec;56(6):588–99.

6. Patwa A, Shah A. Anatomy and physiology of respiratory system relevant to anaesthesia. Indian J Anaesth. 2015 Sep;59(9):533–41.

7. Uchenna Agu R, Ikechukwu Ugwoke M, Armand M, Kinget R, Verbeke N. The lung as a route for systemic delivery of therapeutic proteins and peptides. Respir Res. 2001;2(4):198–209.

8. Kim K-J, Malik AB. Protein transport across the lung epithelial barrier. Am J Physiol - Lung Cell Mol Physiol. 2003 Feb 1;284(2):L247–59.

9. Castranova V, Rabovsky J, Tucker JH, Miles PR. The alveolar type II epithelial cell: a multifunctional pneumocyte. Toxicol Appl Pharmacol. 1988 May;93(3):472–83.

10. Konietzko N, Wendel H, Wiesner B. Erkrankungen der Lunge. Walter de Gruyter; 1994. 1002 p.

11. Laux DG. Lungenphysiologie und Beatmung in Narkose. In: Rossaint PDR, Werner PDC, Zwißler PDB, editors. Die Anästhesiologie [Internet]. Springer Berlin Heidelberg; 2012 [cited 2017 Apr 2]. p. 574–88. Available from: http://link.springer.com/chapter/10.1007/978-3-642-21125-6_33

12. Lutfi MF. The physiological basis and clinical significance of lung volume measurements. Multidiscip Respir Med. 2017;12:3.

13. Wright JL, Churg A. Advances in the pathology of COPD. Histopathology. 2006 Jul 1;49(1):1–9.

14. Doherty DE. The pathophysiology of airway dysfunction. Am J Med Suppl. 2004 Dec 20;117(12, Supplement 1):11–23.

15. Fellner RC, Terryah ST, Tarran R. Inhaled protein/peptide-based therapies for respiratory disease. Mol Cell Pediatr. 2016 Apr 20;3(1):16.

16. Molfino NA, Jeffery PK. Chronic obstructive pulmonary disease: Histopathology, inflammation and potential therapies. Pulm Pharmacol Ther. 2007 Oct;20(5):462–72.

17. Martin C, Frija J, Burgel P-R. Dysfunctional lung anatomy and small airways degeneration in COPD. Int J Chron Obstruct Pulmon Dis. 2013;8:7–13.

18. Silbernagel S, Lang F. Taschenatlas Pathophysiologie. 3rd ed. Thieme; 2009.

19. Hogg JC. Pathophysiology of airflow limitation in chronic obstructive pulmonary disease. The Lancet. 2004 Aug 27;364(9435):709–21.

20. Dahl R. Systemic side effects of inhaled corticosteroids in patients with asthma. Respir Med. 2006 Aug 1;100(8):1307–17.

21. McKeage K. Fluticasone Propionate/Formoterol Fumarate: A Review of Its Use in Persistent Asthma. Drugs. 2013 Feb 1;73(2):195–206.

22. Pauwels RA, Löfdahl C-G, Postma DS, Tattersfield AE, O'Byrne P, Barnes PJ, et al. Effect of Inhaled Formoterol and Budesonide on Exacerbations of Asthma. N Engl J Med. 1997 Nov 13;337(20):1405–11.

23. Patton JS, Fishburn CS, Weers JG. The lungs as a portal of entry for systemic drug delivery. Proc Am Thorac Soc. 2004;1(4):338–44.

24. Mastrandrea LD. Inhaled insulin: overview of a novel route of insulin administration. Vasc Health Risk Manag. 2010;6:47–58.

25. Lombry C, Edwards DA, Préat V, Vanbever R. Alveolar macrophages are a primary barrier to pulmonary absorption of macromolecules. Am J Physiol - Lung Cell Mol Physiol. 2004 May 1;286(5):L1002–8.

26. Finlay WH. The Mechanics of Inhaled Pharmaceutical Aerosols: An Introduction. Academic Press; 2001. 328 p.

27. Myrdal PB, Sheth P, Stein SW. Advances in Metered Dose Inhaler Technology: Formulation Development. AAPS PharmSciTech. 2014 Jan 23;15(2):434–55.

28. Newman SP. Principles of metered-dose inhaler design. Respir Care. 2005 Sep;50(9):1177–90.

29. Terzano C. Metered dose inhalers and spacer devices. Eur Rev Med Pharmacol Sci. 1999 Aug;3(4):159–69.

30. Geller DE. Comparing clinical features of the nebulizer, metered-dose inhaler, and dry powder inhaler. Respir Care. 2005 Oct;50(10):1313-1321-1322.

31. Bartolo K, Balzan M, Schembri EL, Asciak R, Mercieca Balbi D, Pace Bardon M, et al. Predictors of correct technique in patients using pressurized metered dose inhalers. BMC Pulm Med [Internet]. 2017 Feb 28 [cited 2017 Mar 10];17. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC5331728/

32. Breathe New Hampshire [Internet]. [cited 2017 Sep 11]. Available from: https://www.breathenh.org/home

33. Chrystyn H. The Diskus™: a review of its position among dry powder inhaler devices. Int J Clin Pract. 2007 Jun;61(6):1022–36.

34. Atkins PJ. Dry Powder Inhalers: An Overview. Respir Care. 2012 Nov 22;50(10):1304.

35. Mehta P. Dry Powder Inhalers: A Focus on Advancements in Novel Drug Delivery Systems. J Drug Deliv. 2016 Oct 27;2016:e8290963.

36. Hess DR. Nebulizers: principles and performance. Respir Care. 2000 Jun;45(6):609–22.

37. Smith C, Goldman RD. Nebulizers versus pressurized metered-dose inhalers in preschool children with wheezing. Can Fam Physician. 2012 May 1;58(5):528–30.

38. Cooper GM. Cell Membranes. 2000 [cited 2016 Oct 28]; Available from: https://www.ncbi.nlm.nih.gov/books/NBK9928/

39. Vasiliou V, Vasiliou K, Nebert DW. Human ATP-binding cassette (ABC) transporter family. Hum Genomics. 2009;3:281.

40. Lodish H, Berk A, Zipursky SL, Matsudaira P, Baltimore D, Darnell J. Overview of Membrane Transport Proteins. 2000 [cited 2017 Mar 7]; Available from: https://www.ncbi.nlm.nih.gov/books/NBK21592/

41. Takata K, Matsuzaki T, Tajika Y. Aquaporins: water channel proteins of the cell membrane. Prog Histochem Cytochem. 2004;39(1):1–83.

42. Kozono D, Yasui M, King LS, Agre P. Aquaporin water channels: atomic structure molecular dynamics meet clinical medicine. J Clin Invest. 2002 Jun 1;109(11):1395–9.

43. Zelenina M, Zelenin S, Aperia A. Water Channels (Aquaporins) and Their Role for Postnatal Adaptation. Pediatr Res. 2005 May;57(5 Part 2):47R–53R.

44. Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P. Ion Channels and the Electrical Properties of Membranes. 2002 [cited 2017 Mar 8]; Available from: https://www.ncbi.nlm.nih.gov/books/NBK26910/

45. Aurade RM, Jayalakshmi SK, Sreeramulu K. P-glycoprotein ATPase from the resistant pest, Helicoverpa armigera: Purification, characterization and effect of various insecticides on its transport function. Biochim Biophys Acta BBA - Biomembr. 2010 Jun;1798(6):1135–43.

46. Hollenstein K, Dawson RJP, Locher KP. Structure and mechanism of ABC transporter proteins. Curr Opin Struct Biol. 2007 Aug;17(4):412–8.

47. Bretschneider A, Heckel DG, Vogel H. Know your ABCs: Characterization and gene expression dynamics of ABC transporters in the polyphagous herbivore Helicoverpa armigera. Insect Biochem Mol Biol. 2016 May;72:1–9.

48. Frelet A, Klein M. Insight in eukaryotic ABC transporter function by mutation analysis. FEBS Lett. 2006 Feb 13;580(4):1064–84.

49. Wilkens S. Structure and mechanism of ABC transporters. F1000Prime Rep [Internet]. 2015 Feb 3 [cited 2016 Nov 1];7. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4338842/

50. Li W, Zhang H, Assaraf YG, Zhao K, Xu X, Xie J, et al. Overcoming ABC transporter-mediated multidrug resistance: Molecular mechanisms and novel therapeutic drug strategies. Drug Resist Updat. 2016 Jul;27:14–29.

51. Munoz M, Henderson M, Haber M, Norris M. Role of the MRP1/ABCC1 Multidrug Transporter Protein in Cancer. IUBMB Life. 2007 Jan 1;59(12):752–7.

52. Chen Z-S, Tiwari AK. Multidrug Resistance Proteins (MRPs/ABCCs) in Cancer Chemotherapy and Genetic Diseases. FEBS J. 2011 Sep;278(18):3226–45.

53. Bakos É, Homolya L. Portrait of multifaceted transporter, the multidrug resistance-associated protein 1 (MRP1/ABCC1). Pflüg Arch - Eur J Physiol. 2006 Dec 23;453(5):621–41.

54. Westlake CJ, Cole SPC, Deeley RG. Role of the NH2-terminal Membrane Spanning Domain of Multidrug Resistance Protein 1/ABCC1 in Protein Processing and Trafficking. Mol Biol Cell. 2005 May;16(5):2483–92.

55. Lu JF, Pokharel D, Bebawy M. MRP1 and its role in anticancer drug resistance. Drug Metab Rev. 2015 Oct 2;47(4):406–19.

56. Rosenberg MF, Mao Q, Holzenburg A, Ford RC, Deeley RG, Cole SPC. The Structure of the Multidrug Resistance Protein 1 (MRP1/ABCC1) CRYSTALLIZATION AND SINGLE-PARTICLE ANALYSIS. J Biol Chem. 2001 May 11;276(19):16076–82.

57. Daood MJ, Tsai C, Ahdab-Barmada M, Watchko JF. ABC Transporter (P-gp/ABCB1, MRP1/ABCC1, BCRP/ABCG2) Expression in the Developing Human CNS. Neuropediatrics. 2008 Aug;39(4):211.

58. Roundhill EA, Burchill SA. Detection and characterisation of multi-drug resistance protein 1 (MRP-1) in human mitochondria. Br J Cancer. 2012 Mar 13;106(6):1224–33.

59. Jiye Y, Jianting Z. Multidrug resistance-associated protein 1 (MRP1/ABCC1) polymorphism: from discovery to clinical application. Zhong Nan Da Xue Xue Bao Yi Xue Ban. 2011 Oct;36(10):927–38.

60. Jedlitschky G, Leier I, Buchholz U, Barnouin K, Kurz G, Keppler D. Transport of Glutathione, Glucuronate, and Sulfate Conjugates by the MRP Gene-encoded Conjugate Export Pump. Cancer Res. 1996 Mar 1;56(5):988–94.

61. Slot AJ, Molinski SV, Cole SPC. Mammalian multidrug-resistance proteins (MRPs). Essays Biochem. 2011 Sep 7;50:179–207.

62. Budulac SE, Postma DS, Hiemstra PS, Kunz LI, Siedlinski M, Smit HA, et al. Multidrug resistance-associated protein-1 (MRP1) genetic variants, MRP1 protein levels and severity of COPD. Respir Res. 2010;11(1):60.

63. O'Donnell S, O'Morain CA. Therapeutic benefits of budesonide in gastroenterology. Ther Adv Chronic Dis. 2010 Jul;1(4):177–86.

64. DrugBank, editor. Budesonide. In: DrugBank [Internet]. 2017 [cited 2017 Apr 12]. Available from: https://www.drugbank.ca/drugs/DB01222

65. Pelaia G, Vatrella A, Busceti MT, Fabiano F, Terracciano R, Matera MG, et al. Molecular and cellular mechanisms underlying the therapeutic effects of budesonide in asthma. Pulm Pharmacol Ther. 2016 Oct;40:15–21.

66. Wolthers OD. Budesonide + formoterol fumarate dihydrate for the treatment of asthma. Expert Opin Pharmacother. 2016 May 2;17(7):1023–30.

67. Ammon HPT, Schubert-Zsilavecz M. Hunnis Pharmazeutisches Wörterbuch. 11th ed. De Gruyter; 2014.

68. DrugBank, editor. Dexamethasone. In: DrugBank [Internet]. 2017 [cited 2017 Apr 15]. Available from: https://www.drugbank.ca/drugs/DB01234

69. Emami Ardestani M, Kalantary E, Samaiy V, Taherian K. Methyl prednisolone vs Dexamethasone in Management of COPD Exacerbation; a Randomized Clinical Trial. Emergency [Internet]. 2017 [cited 2017 Apr 4];5(1). Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC5325905/

70. DrugBank, editor. Formoterol. In: DrugBank [Internet]. 2017 [cited 2017 Apr 13]. Available from: https://www.drugbank.ca/drugs/DB00983

71. Ehrhardt C, Kneuer C, Bies C, Lehr C-M, Kim K-J, Bakowsky U. Salbutamol is actively absorbed across human bronchial epithelial cell layers. Pulm Pharmacol Ther. 2005 Jun;18(3):165–70.

72. Walker SR, Evans ME, Richards AJ, Paterson JW. The clinical pharmacology of oral and inhaled salbutamol. Clin Pharmacol Ther. 1972 Nov 1;13(6):861–7.

73. DrugBank, editor. Salbutamol. In: DrugBank [Internet]. 2017 [cited 2017 Apr 15]. Available from: https://www.drugbank.ca/drugs/DB01001

74. van der Deen M, Homan S, Timmer-Bosscha H, Scheper RJ, Timens W, Postma DS, et al. Effect of COPD treatments on MRP1-mediated transport in bronchial epithelial cells. Int J Chron Obstruct Pulmon Dis. 2008 Sep;3(3):469–75.

75. Pierce BCA Protein Assay Kit - Thermo Fisher Scientific [Internet]. [cited 2017 Oct 17]. Available from: https://www.thermofisher.com/order/catalog/product/23225

Ich habe mich bemüht, sämtliche Inhaber der Bildrechte ausfindig zu machen und ihre Zustimmung zur Verwendung der Bilder in dieser Arbeit eingeholt. Sollte dennoch eine Urheberrechtsverletzung bekannt werden, ersuche ich um Meldung bei mir.

10. EXPLENATION OF ABBREVIATIONS

ABC-transporter	ATP binding cassette transporter
ADP	adenosine diphosphate
ATP	adenosine triphosphate
BCRP	breast cancer resistance protein
cAMP	cyclic-adenosine monophosphate
CF	5(6)carboxyfluorescein
CFDA	5(6)carboxyfluorescein diacetate
CFTR	cystic fibrosis transmembrane conductance regulator
COPD	chronic obstructive pulmonary disease
DPI	dry powder inhaler
FBS	fetal bovine serum
GR	glucocorticoid receptor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
KRB	bicarbonated Krebs-Ringer buffer
MRP1	multidrug-resistance associated protein 1
NBD	nucleotide-binding domain
PBS	phosphate-buffered saline
pMDI	pressurized metered-dose inhaler
SD	standard deviation
TMD	transmembrane domain