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“The impact of MRP1 activation on tobacco smoke-induced toxicity in human respiratory epithelial cells in vitro”

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Table of Contents

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1. Abstract

Background

The multidrug resistance-associated protein 1 (MRP1) not only potentially results in chemotherapeutic failure by causing multidrug resistance in cancer treatment, but also plays a pivotal role in cellular detoxification owing to its ability to extrude noxious substances causing oxidative stress and inflammation. In the lung, MRP1 has been shown to be expressed at high levels. The present study aimed to evaluate the effects of various pharmacological MRP1 modulators, to assess whether the activation of the transporter could be a novel target in the treatment of chronic obstructive pulmonary diseases (COPD).

Methods

In order to study MRP1 modulating properties, cigarette smoke extract (CSE), sulforaphane, perphenazine and quercetin were tested on the NCI-H441 cell line and human alveolar epithelial cells in primary culture. Cells were incubated either short or long-term with the respective compound, before being loaded with 5,6-carboxyfluorescein diacetate (CFDA), which was intracellularly converted into the fluorescent MRP1 substrate, carboxyfluorescein (CF). CF was measured in the cell supernatant and intracellularly. By Western blot, the substances´ effects on MRP1 protein levels was evaluated.

Results

In the long-term incubation assays, 5% and 10% CSE reduced MRP1's ability to efflux CF efficiently. Aged CSE caused a less pronounced retention compared to freshly prepared CSE. Sulforaphane only showed stimulatory properties when tested in primary cells, whereas quercetin inhibited MRP1 activity. In the Western blots, only sulforaphane showed slightly significant results by raising the total protein abundance of MRP1.

Conclusions

MRP1 activity could be stimulated by sulforaphane in primary alveolar epithelial cells. This might be an interesting new mechanism to increase cellular detoxification in lungs of COPD patients.

1. Zusammenfassung

Hintergrund

Das Multidrug-Resistenz assoziierte Protein (MRP1) ist nicht nur für ein mögliches Versagen einer Chemotherapie in der Krebstherapie aufgrund von Multidrug-Resistenzen verantwortlich, sondern spielt auch eine Rolle bei zellulären Entgiftungsprozessen durch seine Fähigkeit schädliche Substanzen, die zu oxidativem Stress und Entzündungsprozessen führen, aus Zellen heraus zu transportieren. In der Lunge ist das Protein stark exprimiert.
Das Ziel der vorliegenden Studie war es die Effekte von diversen pharmakologischen MRP1 Modulatoren zu evaluieren und abzuwägen, ob eine Aktivierung des Transporters ein neues therapeutisches Target in der Behandlung von COPD sein könnte.

Methoden

Zigarettenrauchextrakt, Sulforaphan, Perphenazin und Quercetin wurden an der NCI-H441 Zelllinie und humanen Alveolarepithelprimärzellen in Efflux-Experimenten getestet, um etwaige MRP1 modulierende Eigenschaften zu untersuchen. Die Zellen wurden zunächst entweder kurzfristig oder über einen längeren Zeitraum mit der jeweiligen Substanz inkubiert und dann mit 5,6-Carboxyfluorescein Diacetat (CFDA) behandelt, welches intrazellulär in das fluoreszierende Substrat von MRP1 Carboxzfluorescein (CF) metabolisiert wird. CF wurde im Zellüberstand und intrazellulär gemessen.
Mittels Western Blot wurden die Effekte der Substanzen auf die Proteinexpression von MRP1 untersucht.

Ergebnisse

Im Efflux-Experiment nach Langzeitinkubation reduzierten 5 und 10% CSE die Fähigkeit des Transporters, CF effektiv aus der Zelle zu transportieren. Gelagertes CSE verursachte eine weniger offensichtliche Retention verglichen zu frisch zubereitetem CSE. Sulforaphan wiess MRP1-stimulierende Eigenschaften an den Primärzellen auf, während Quercetin die MRP1-Aktivität hemmte. Nur Sulforaphan verursachte signifikante Veränderungen der Proteinexpression, indem es die MRP1-Menge erhöhte.

Fazit

Die Aktivität von MRP1 kann durch Sulforaphan in primären Alveolarepithelzellen stimuliert werden. Dies könnte ein interessanter neuer Mechanismus sein, um die zelluläre Entgiftung in der Lunge von COPD Patienten zu unterstützen.

2. Introduction

2.1 The Anatomy and Physiology of the Lung

The lungs are located in the thoracic cavity and are the organs responsible for the gas exchange of oxygen and carbon dioxide in the organism. They are part of the respiratory system which can be divided into the upper and the lower tract. Thanks to their elasticity, the lungs are capable of expanding and contracting during inspiration and expiration, which is crucial for a sufficient gas exchange (National Heart Lung and Blood Institute. 2019)

Pulmonary ventilation and blood flow mediate the circulation of O_2 and CO_2 within the air and the blood. The lung also regulates the balance between acids and bases due to the release of CO_2. During ventilation, which comprises inhalation and expiration, total gas volumes between 4 and 8 litres are exchanged (Patwa et al. 2015) by either the uptake of O_2 and the release of CO_2, which, however, may vary considerably in accordance with muscular activity (Buehlmann et al. 1979). During the process of ventilation oxygen is transported to the alveoli which are the sites of gas exchange between air and blood according to differences in concentration. The thin cells walls of alveolar cells allow a fast and efficient gas diffusion and, as a result, the gas exchange to occur (Ochs et al. 2004).

During inhalation the concentration of oxygen entering the alveolus is above the concentration in the erythrocytes which results in the oxygen diffusion from the alveolus to the red blood cells. While exhaling the opposite occurs leading to the exhalation of CO2.

The lung boasts a large surface area reaching to 140 m^2 which is lined by 200 to 600 million alveoli (Pfister et al. 2004), which in addition to the good permeability of the alveolar cells not only leads to a rapid and efficient gas exchange during ventilation but can also enable and facilitate the systemic delivery via pulmonary administration of drugs or biomolecules.

The airway epithelium is located at the interface separating the internal and external environment and has long been in the centre of scientific attention as it plays an undeniable role in initiating immune functions in the lungs while also serving as an important physical barrier against the external environment (Yasuhiro et al. 2018). Airway epithelial cells form the apical junctional complex that comprises tight- and adherens junctions which both provide solid adhesive contact between neighbouring epithelial cells and play a central role

in establishing cell polarity. Adherens junctions initiate the contact between cells, and mediate the maintenance of mature contact, whereas tight junctions are responsible for moving ions and solutes within paracellular pathways.

Despite comprising different proteins involved in the processes, these junctions owe similarities in their ways of forming extracellular adhesive contact between cells, attaching intracellularly to the actin cytoskeleton and mediating signalling pathways including the regulation of gene transcription (Hartsock et al. 2008). As a result, harmful substances or processes that interfere with the regulatory function and immune defence mechanism exerted by the airway epithelial barrier have recently shown to be closely linked to the development and progression of inflammation and lung diseases such as chronic obstructive pulmonary disease (COPD) and asthma (Yasuhiro et al. 2018).

2.2 The Toxic Effects of Tobacco Smoke

Tobacco smoke generates oxidative stress in lung cells and is therefore a widely known risk factor in the development of lung cancer and COPD (Van der Deen et al. 2007) which is predicted to become the third leading cause of death and disabilities worldwide by 2030 (World Health Organization. 2008).
Even though the exact pathophysiology of COPD remains elusive, tobacco smoking (actively or passively) is one of the most common causes for its occurrence as it results in excessive inflammation and tissue injury (Singh et al. 2018).

While nicotine is the constituent in tobacco primarily responsible for developing an addiction to cigarettes, most of the harm associated with smoking is caused by the by-products of tobacco combustion. Tobacco smoke contains over 5000 substances such as tar, acids, phenols, aliphatic and aromatic hydrocarbons, nitrosamines and heavy metals of which nearly 90 compounds are highly carcinogenic (Smith et al. 2018).

In order to reduce the harm triggered by smoking, two different approaches have emerged: First, the addictive potential could be minimised by significantly reducing the nicotine content of cigarettes. Second, alternative nicotine delivery systems such as electronic

cigarettes could provide sufficient nicotine to act as appropriate substitutes for regular cigarettes while exposing the smoker to much lower levels of toxicants (Smith et al. 2018).

Tobacco smoke also triggers an imbalance between oxidants and antioxidants due to exogenous reactive oxygen species (ROS). Oxidative stress and endogenously released ROS can lead to inflammatory processes and cause mitochondrial dysfunction, which may contribute to the progression of COPD. ROS and reactive nitrogen species (RNS) can oxidise a vast variety of biomolecules such as proteins, lipids and even the DNA leading to epithelial cell distress and death. ROS removal could be achieved by various detoxifying enzymes and antioxidant defence systems which could potentially act as cytoprotective mechanisms and fight the disease progression (Boukhenouna et al. 2018).

The detoxification and elimination of this harmful toxins and noxious substances found in tobacco smoke would therefore contribute to both disease prevention and efficient treatment (Van der Deen et al. 2008).

2.3 Chronic Obstructive Pulmonary Disease (COPD)

Chronic obstructive pulmonary disease (COPD) is a progressive and at an advanced stage irreparable disease marked by the permanent damage of lung tissue including chronic bronchitis and emphysema leading to serious breathing problems, poor airflow, heart failure and even psychological issues such as depression. The progressive chronic inflammatory disease affects both the large and small airways and lung parenchyma and thus leads to severe symptoms such as chronic coughing accompanied by excessive sputum production, chest tightness, shortness of breath and dyspnoea especially during physical activity (National Clinical Guideline Centre, 2010). In COPD, inflammation is significant early on and increases steadily according to the disease´s severity (Molfino et al. 2007).

Even though COPD can be triggered by various causes such as environmental and genetic factors, smoking is still considered the principle risk factor (Van der Deen et al. 2008). The disease is mostly prominent among people older than 65 years and leads to disruptive limitations at work and physical exercise, an urgent need for special equipment such as

portable oxygen tanks and is often accompanied by other chronic diseases such as congestive heart failure, diabetes, coronary heart disease, arthritis, stroke or asthma (COPD-CDC Centres for Disease and Prevention, 2018).
As a result of their poor health status, COPD patients share a high mortality rate and the disease is considered one of the leading causes of death worldwide, mostly because of the lack of effective therapies.

So far, COPD is recognised as a relentlessly progressive disease and merely a complete abstinence from smoking can effectively reduce and prevent the accelerated lung function decline. There is no real cure for COPD, yet recent studies have shown that some drugs are beneficial in the disease´s management and may reduce its symptoms. The number of exacerbations can be diminished by inhaled corticosteroids and long-acting beta-agonists such as budesonide and formoterol and their combination has been considered an effective treatment (Alsaeedi et al. 2002; Sutherland et al. 2003; Calverley et al. 2003). Furthermore, anticholinergics like ipratropium bromide result in minor improvement of lung function, however, they do not influence the long-term decline in more severe forms of COPD (Anthonisen et al. 1994).

2.4 Transporters

Cells are dependent on the constant supply with nutritious compounds as well as the export of toxic substances. While nonpolar and small uncharged polar molecules are able to cross cell membranes through passive diffusion, charged compounds and large uncharged polar molecules need specified transporters as for them the lipid bilayer is an unsurpassable barrier.

Transport proteins are integral transmembrane proteins that serve the function of moving ions, small molecules, macromolecules or other proteins across biological membranes. The proteins exist permanently within and span the membrane and be categorized as either channels or carriers (Müller-Esterl et al. 2018). Unlike channels which allow molecules to diffuse without interruptions, carriers showcase binding sites designed to recognize only specific substances.

This exchange of various substances is achieved by two basic mechanisms: active and passive transport. The main difference between these mechanisms of movement is the fact that passive transport does not require any external energy input due to the tendency of a biological system to grow and mature in entropy, which leads to processes like filtration and osmosis, diffusion and facilitated diffusion (https://biologywise.com/passive-transport-active-transport). Passive transport occurs according to the concentration gradient and is mostly achieved due to the permeability of the cell membrane structure.

Whereas active transport requires some energy input for its execution as molecules and substances move across the membrane against their concentration gradient. While primary active transport depends on ATP, secondary active transport occurs due to an electrochemical gradient.

Transport proteins are usually selective to a specific type of substrate which is then recognized at the binding site. Uniporters are integral membrane proteins that transport merely one type of substrate species, whereas symporters also specified as co-transporters are involved in the transport of various types of molecules across the cell membrane. Their name already implies that molecules will travel in equal directions, which is contrary to so-called antiporters which exchange substrates in opposite directions (Müller-Esterl et al. 2018).

In general, all transporters are encoded by three major gene superfamilies: the ATP-binding cassette transporters (ABC), the solute carrier transporter family (SLC) and the organic anion transporting polypeptides (OATP) (Bosquillon et al. 2010).

The SLC-family comprises for instance the organic cation transporter family (OCT), the peptide transporter family (PEPT) and the organic anion transporters (OAT) (He et al. 2009). The organic anion transporting polypeptides (OATP) originally belonged to the SLC family but are now reclassified as a separate transporter gene family (Müller-Esterl et al. 2018).

2.5 Transporters in the Lung

As the lungs express both efflux and uptake transporters, numerous recent studies have put their emphasis on the role of transporters in pulmonary drug metabolism. In order to

examine MRP1´s ability to affect the lungs´ drug absorbing properties, the focus has been put on the expression, localisation and functionality of transporters, which either affect the bioavailability of inhaled drugs or could be hindered in their function by lung diseases or exogenous compounds (Bosquillon et al. 2010). It has been shown that active transport processes might affect the local disposition of drugs in the lung but are also involved in the uptake of drugs in the lung from the systemic circulation (Gustavsson et al. 2016).

Due to the complex anatomical structure of the lung, which contains more than 40 different cell types with different morphological and functional features, lung transporters have become an emerging research area. In the future, a broader knowledge about those transporters could potentially enable rational drug design providing huge benefit in the treatment of respiratory diseases and increase the understanding of the mechanisms of pulmonary toxicity (Gustavsson et al. 2016).

2.6 ABC Transporters

ATP-binding cassette (ABC) transporters are referred to as ATP-dependent efflux or influx pumps and are ubiquitous membrane-bound proteins basically present in all prokaryotes, plants, fungi and animals (Vasiliou et al. 2009).

Being a family of integral membrane proteins, they supply cells with nutrients, export toxins and even regulate the osmolality in plants and extrude xenobiotics including pharmaceutical compounds and drugs. As a result, they can cause insects´ resistance to insecticides or bacterial resistance to antibiotics.

ABC transporters usually consist of multiple subunits including transmembrane proteins and membrane-associated ATPases. By using the energy output of ATP binding and hydrolysis, they are able transport a vast variety of substrates across membranes including ions, lipids, peptides and even proteins (Dean et al. 2005). In humans, the ABC superfamily consists of 49 ATP-binding transporters and includes proteins like the multidrug resistance-associated protein 1 (MRP1), P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP) (Keppler et al. 2011). The proteins form 7 subfamilies named A to G (i.e. ABCA-ABCG) and are grouped according to their amino acid sequence.

ABC exporters are marked by their conserved core of exactly 12 transmembrane (TM) helices, whereas importers can feature up to 20 TM helices. Both form highly specified passages for fitting substrates. The two regulatory domains, also known as the nucleotide binding domains (NBD) located on the cytosolic side are regulated in their functionality by ATP binding and hydrolysis as well as reversible phosphorylation and are considered the "motor" domains of ABC-transporters.

During the ATP binding confirmation, two ATP molecules are located at the interface of two NBDs, which serves as the structural basis for the binding and hydrolysis of ATP. Any minor change concerning the conformation of the NBDs is converted into a signal which is transmitted immediately to the TMDs, which then triggers a flipping from an inward-facing to an outward facing conformation. Unlike the NBDs, the TMDs which are spanning the membranes boast significant variations in regard to the primary sequence, length, architecture as well as number of TM helices (Hollenstein et al. 2007).

ABC transporters also play a crucial role in the presentation of antigens to macrophages and lymphocytes: they transport lipids that have emerged from antigen fragmentation happening in the cytosol to the endoplasmic reticulum (Müller-Esterl et al. 2018).

By extruding substances out of cells, ABC transporters provide species with protection against oxidative stress, inflammatory substances or xenobiotics. However, they have also drawn attention as anti-targets in cancer research as their overexpression or increased activity could potentially cause multidrug resistance (MDR) in tumorous cells to chemotherapeutic drugs (Van der Deen et al. 2008).

2.7 The Multidrug Resistance-Associated Protein - MRP

In the human organism, the MRP family also referred to as ABCC contains 9 members, specifically defined as MRP1 (ABCC1), MRP2 (ABCC2) and so on (Zhou et al. 2008). Apart from extruding toxins, MRP also leads to multidrug resistance, by exporting therapeutically crucial drugs such as methotrexate, antiandrogens, HIV-therapeutics and chemotherapeutics like vincristine, vinblastine, doxorubicin and paclitaxel (Munoz et al. 2007).
Whereas, as they transport exogenous compounds as well as endogenous substances like hormones including leukotrienes and oestrogen conjugates (He et al. 2011) and by-

products of endogenous metabolism, which could be equally harmful, they reduce the organism´s exposure to toxins and noxious substances (Choudhuri et al. 2006).

Just like other ABC transporters, MRP proteins feature two transmembrane domains each followed by a nuclear binding domain, which is in charge of the ATP binding and hydrolysis process, and a third NH_2-terminal "TMD0". Many recent studies have outlined the structure activity relationships (SAR) concerning MRP, however, its crystal structure still remains unresolved (He et al. 2011).

2.8 MRP1

In humans, multidrug resistance-associated protein-1 (MRP1) with the molecular weight of, 190 kDa belongs to the C-subfamily of the ABC transporter superfamily (Wang et al. 2013). First, Cole detected the protein in lung cancer cells and then cloned MRP1 from a human lung cancer cell line (Cole et al. 1992).
MRP1 belongs to the phase II metabolism that comprises a complex detoxification system not only exporting endogenous metabolites but also actively secreting foreign substances and their metabolites out of tissues and, as a result, providing effective protection from xenobiotic insult (Harris et al. 2008).
As an ATP-dependent efflux pump, MRP1 protein acts as a transporter of mostly conjugates of glutathione while being a cotransporter of unconjugated compounds (Lehmann et al. 2001). Moreover, glucuronate and sulphate-conjugated organic anions are MRP1 substrates, suggesting an important role in lung physiology by protecting cells against oxidative stress and toxic xenobiotics. Additionally, many conjugated organic anions have been identified as MRP1 substrates *in vitro*, including a glutathione-conjugated arachidonic acid metabolite specified as the leukotriene LTC_4 which is heavily involved in the development of inflammatory processes (Leslie et al. 2001a).

Apart from than, MRP1 is also a transporter of reduced glutathione (GSH), though merely with low affinity, and thus, many drugs are co-transported by MRP1 being associated with the reduced compound (Leslie et al. 2001b). GSH plays a central role in

maintaining and regulating the redox-status within cells and has emerged as one of the most effective antioxidants, especially in the lung (Cole et al. 2013).

MRP1 is expressed in a wide array of tissues, including gut, lung, liver, kidneys and brain which are all vital for absorption, metabolism and elimination. In healthy human lung tissue, MRP1 is mainly expressed on the basolateral side of bronchial epithelial cells. While conferring resistance to diverse xenobiotics (Leslie et al. 2001), this efflux pump can significantly modulate the bioavailability, distribution, excretion and toxicity of xenobiotics and medically used drugs (Choi et al. 2014; Leslie et al. 2005).

2.9 Pathways involved in the MRP1 expression

As the human organism suffers from daily exposure to reactive oxidants from both internal processes surrounding the metabolism and environmental pollution a complex antioxidant defence system is crucial in order to counteract any occurring oxidative or electrophilic stress. The nuclear factor erythroid 2 related factor (Nrf2) confers cellular resistance to oxidants while regulating the basal and actively induced expression of a wide range of genes involved in physiological and pathophysiological processes and also preventing genome instability (Qiang Ma 2015).

The activation of Nrf2 signalling results in the induction of an antioxidant responsive element (ARE) dependent expression of detoxifying and antioxidant defence proteins (Krajka-Kuźniak et al. 2017) including enzymes of phase II metabolism such as MRP, γ-glutamylcysteine-synthetase, heme-oxygenase and NAD(P)H quinone oxide-reductase (Kleszczyński et al. 2013). Nrf2-ARE signalling has become an attractive target or anti-target in the treatment of neurodegenerative diseases, COPD, inflammatory diseases as well as cancer. However, processes leading to constitutive over-activation of NRF2 which is often found in cancer cells have been implicated to result in cancer progression and tumour growth advantage by causing resistance to chemotherapeutics (Kansanen et al. 2012).

2.10 MRP1 and COPD

Interestingly, COPD patients usually show a lower expression of MRP1 in bronchial epithelial cells compared to healthy humans (Van der Deen et al. 2008), suggesting a link to the progressive decline of lung function (Budulac et al. 2012).

In 2007, Van der Deen et al. examined the competitive inhibition of MRP1 activity in the human bronchial epithelial cell line 16HBE140- in vitro caused by CSE as well as the increased cytotoxicity of CSE correlated to the inhibition of MRP1 by MK-571.

MRP1 could likely serve as a defence mechanism in the prevention of COPD due to its protective function against tobacco smoke in the lung. An increased activity of MRP1 could therefore reduce the toxicity of CSE on human bronchial epithelial cells thanks to the extrusion of harmful substances (Van der Deen et al 2008).

2.11 MRP1 Stimulation

As a couple of studies have suggested a reduced expression of MRP1 in the bronchial epithelium of patients suffering from COPD, a lower functional MRP1 activity could be linked to the COPD development. MRP1 owes an essential function in maintaining tissue homoeostasis and provides defence against xenobiotic and exogenous insult, and substances which upregulate the MRP1 expression and raise activity levels could positively affect respiratory symptoms caused by oxidative stress such as smoking (Wang et al. 2015).

2.12 Phenothiazine derivates

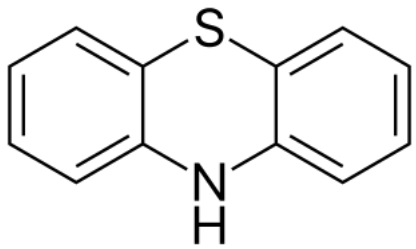
Due to their widely varying chemical structure, phenothiazines are among the most valuable molecular templates for the development of agents able to interact with a vast variety of biological systems and processes (Mosnaim et al. 2006). Their structure enables specific interactions with different membrane proteins as well as non-specific interactions with the lipid phase of membranes (Michalak et al 2007). Thus, phenothiazine derivates are able to modulate and model the physicochemical properties of cell membranes and lipid layers and have therefore become effective tools in the treatment of numerous medical conditions with a widely different aetiology as they boast antipsychotic, anticholinergic, antihistaminic and antiemetic properties (Mosnaim et al. 2006).

"Phenothiazines are known as the most potent antipsychotic drugs due to their interaction with various ion channels. They are calmodulin antagonists, inhibitors of protein kinase C and adenylate cyclase" (Wesolowska et al. 2009).

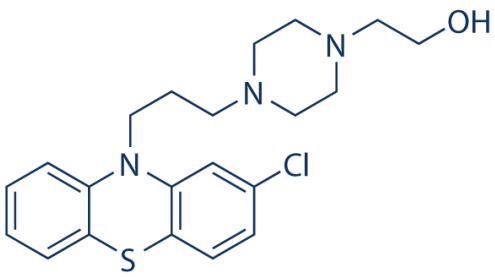
Phenothiazines are defined as a group of atypical MDR modulators, as they interact with P-gp and MRP1 in opposite ways: Phenothiazines have been the first group of known P-gp inhibitors and could therefore diminish the occurrence of primary or therapy-induced drug resistance in tumour cells, which is among the most threatening reasons for chemotherapy failure in cancer treatment. The exact mechanisms behind this MDR reversal have yet to be resolved but could be due to independent biological processes either resulting in a reduced activity of the efflux pump or in a down-regulation of the MDR gene (Wesolowska et al. 2009).

On the contrary, this group of neuroleptics has equally been suggested as potent MRP1 stimulators (Wesolowska et al. 2009). However, it should be taken into consideration that the MRP1 boosting effect exerted by phenothiazines might be strongly substrate-dependent and therefore requires further investigation. The differences observed concerning intracellular drug accumulation or a stimulated cell detoxification linked to phenothiazine derivates could be the outcome of the diversity in chemical structures, which undeniably

impacts the interaction between cell membranes and either the uptake or increased efflux of xenobiotics.



Picture 1: basic structure of phenothiazines (https://en.wikipedia.org/wiki/Phenothiazine)



Picture 2: Chemical structure of perphenazine (InvivoChem. 2018)

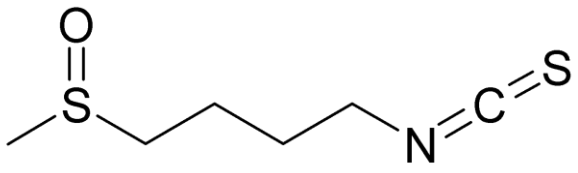
2.13 Sulforaphane

Sulforaphane (SFN) is a widely studied member of isothiocyanates (ITC), which are sulphur containing compounds that are most commonly synthesised and stored in plants and broadly distributed among cruciferous vegetables such as broccoli, cabbage, brussels sprouts, kale and radishes (Wang et al. 2014).

Many recent studies have highlighted the significant anticancer potential of ITC linked to their antioxidant and anti-inflammatory properties (Bai et al. 2015). ITC cause the cell cycle to arrest and promote apoptosis in cancer cells by inducing various mechanisms such as the release of reactive oxygen species and modulate the levels of genes and proteins that play a role in survival and proliferation of tumorous cells (Briones-Herrera et al. 2018).

Isothiocyanates like sulforaphane enhance the expression levels of phase II detoxification enzymes and thus, increase the intracellular levels of MRP1 by modulating the mRNA, the protein expression and the transporter activity (Harris et al. 2008). As a result, being a dietary component that strengthens detoxification systems, sulforaphane is considered a phytochemical at the forefront of phytomedicine.

Herbal remedies containing high concentrations of ITC could alleviate lung inflammation and improve lung function by up-regulating or boasting the function of MRP1, which could benefit COPD treatment as this illness has been linked to a reduced activity of MRP1 (Wang et al. 2014). However, further studies are required to investigate the exact mechanisms of ITC concerning the expression and function of MRP1 in lung epithelial cells in order to affirm that sulforaphane could potentially reduce the harm of tobacco smoke induced toxicity (Bai et al. 2015).



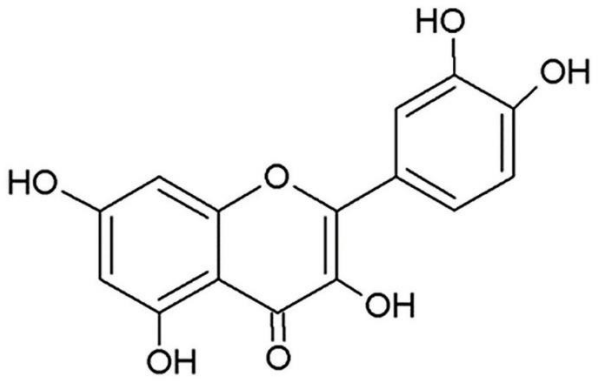
Picture 2: Chemical structure of sulforaphane (wikipedia.org/wiki/Sulforaphane#/media/File:Sulforaphane.png)

2.14 Quercetin

Quercetin is a derivate from the flavonoid group of polyphenols found in various fruits, vegetables, leaves and grains. Several studies have outlined flavonoids as potential therapeutic agents in cancer treatment, among which quercetin is considered the most effective. Quercetin may have the ability of potentiating the efficacy of anticancer drugs (Lei et al. 2017) and improving anticancer properties such as cell signalling, initiating immune response, pro-apoptotic, anti-proliferative and anti-oxidant effects and growth suppression of tumorous tissue.

In addition, potential synergistic effects when combining quercetin with established chemotherapeutic agents or radiotherapy have been reported (Brito et al. 2015). Quercetin also diminishes multidrug resistance linked to the reduction of anticancer potential of drugs, as this model flavonoid has the capacity to noticeably inhibit human MRP1 and MRP2 activity (Van Zanden et al. 2005).

This inhibitory potential is due to the outcomes of phase II metabolism, especially methylation and glucuronidation. In general, glucuronidation resulting in O-glucuronosyl quercetin derivates significantly increases the potential of the compound to inhibit both MRP1 and MRP2 mediated transport. Is has therefore been indicated that the phase II metabolism of quercetin could positively impact the potential of quercetin as an inhibitor to overcome MRP-mediated multidrug resistance (Van Zanden et al. 2007).



Picture 3: chemical structure of Quercetin (Look for Diagnosis. Quercetine)

3. Aims of the study

The Aims of this study were to assess the effect of CSE on MRP1 activity and to determine the influence of the potential MRP1 stimulators, perphenazine and sulforaphane on MRP1 activity and expression. In addition, another aim was to study the effect of the potential MRP-inhibitor, Quercetin, on MRP1 activity and expression.

4. Materials and Methods

4.1 Cell Culture

4.1.1 NCI-H441 cell line

All experiments were performed using NCI-H441 cells, a bronchiolar epithelial cell line generated from a human lung adenocarcinoma. Passage numbers 59-83 were used and the cells were cultured in T-75 flasks with filter screw caps at a temperature of 37°C in 5% CO_2 atmosphere in an incubator (Binder, Tuttlingen Deutschland). Cells were cultured in RPMI 1640 medium containing 5% Foetal Bovine Serum (FBS), 1% penicillin-streptomycin solution and 1% sodium pyruvate solution all bought from Sigma-Aldrich, until they reached 70% confluence. The medium was exchanged every other day. All reagents were preheated to 37°C before use and all steps were performed in a class 2 biosafety cabinet.

For sub-culturing, the medium was aspirated and the cells were washed twice with 5 ml Dulbecco´s phosphate-buffered saline (PBS). In order to detach the cells, 2.5 ml trypsin-EDTA solution was added, and after an incubation for 4 min, the trypsinisation was stopped by adding 7.5 ml of FBS-containing cell culture medium to resuspend the cells. PBS as well as trypsin-EDTA solution were both acquired from Sigma-Aldrich.

After a centrifugation at 900 rpm for 4 min, the supernatant was removed and replaced by 10 ml of fresh medium and the cells were counted with a haemocytometer (Hausser, Horsham, United Kingdom). Then the cells were seeded on a new 75 cm² flask and on 24-well plates at either high (i.e. 70,000 cells/cm^2) or low (i.e. 30,000 cm^2/cm) density.

4.1.2 Primary cells

The effect of CSE and the potential MRP1 activators on MRP1 activity was also determined in freshly isolated human alveolar epithelial cells in primary culture.

“Human alveolar type 2 epithelial (AT2) cells were isolated according to a protocol modified from Demling et al. from non-tumour lung tissue obtained from patients undergoing lung surgery. Briefly, purified AT2 cells were (…) seeded at a density of 200,000 cells/cm2 on collagen/fibronectin-coated surfaces using small airways growth medium (SAGM, Lonza, Verviers, Belgium) supplemented with 1% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were kept in culture for at least one week, so that transdifferentiation into an alveolar type 1-like (AT1-like) phenotype could occur. The use of human tissue specimens was approved by Saarland State Medical Board (Saarbrücken, Germany)” (Nickel et al. 2017).

4.2 The preparation of cigarette smoke extract

All experiments were carried out in bi-carbonated Krebs-Ringer buffer (KRB), containing sodium chloride 116.4 mM (Fluka, St. Gallen, Switzerland), sodium dihydrogen phosphate 0.78 mM (Riedel-de Haën, Seelze, Germany), 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), which were all dissolved in purified water. KRB was then adjusted to a pH of 7.4 using hydrochloric acid and potassium hydroxide.

Cigarette smoke extract (CSE) was prepared by bubbling the smoke of two 3R4F Kentucky research cigarettes (Kentucky Tobacco Research and Development Center, University of Kentucky, KY) through 20 ml RPMI 1640 medium using a vacuum pump. Each cigarette was combusted within two minutes after the airflow was manually interrupted in regular intervals to simulate puffs. The resulting solution was filtered resulting in a 100% CSE solution which was then diluted with KRB to prepare 5 and 10% CSE to be used in exposure studies.

In case of the experiment with aged CSE, the extract was prepared as mentioned above and stored in the freezer for at least two weeks. Prior to the experiment, the CSE was defrosted and diluted with KRB to reach the desired concentrations.

4.3 Cytotoxicity Assay

A cytotoxicity assay was conducted in order to estimate the appropriate concentration of a potentially harmful substance to be used in the efflux experiment without causing cell apoptosis or the monolayers to detach.

Once cells cultured on a 96-well plate reached confluence, they were incubated with different concentrations of the relevant test compound (e.g. sulforaphane at 5, 10, 20, 30, 40, 60, 80, 100, 150 and 200 µM) for 24 h. The medium was gently removed, and the cells were washed with KRB. Cells from four wells were used as blanks and all wells were incubated with 100 µl Alamar Blue cell viability reagent for 3 h. The AlamarBlue Cell Viability reagent was purchased from Thermo Fisher Scientific (Vienna, Austria).
Finally, the fluorescence intensity was measured as the emission at 590nm after an excitation at 530-560nm and the IC_{50} value was calculated using Excel.

4.4 Efflux Studies

The aim of the efflux studies was to evaluate the effect of various substances on the MRP1 function. As 5(6)-carboxyfluorescin (CF) is a substrate of the efflux transporter MRP1, CF retention is an indicative for an increased inhibition of MRP1. CF, sulforaphane and quercetin were dissolved in dimethyl sulfoxide (DMSO), which was purchased from Sigma.

Confluent NCI-H441 cell monolayers cultured on 24-well plates were washed with pre-warmed KRB (37°C) and then incubated with KRB containing one of several MRP1 modulators for 1 h. Cells treated with merely the respective solvent were used as the control group.

For long term studies, cells were incubated with CSE for 24 h. To initiate the efflux experiment, the KRB solution was removed and the cells were loaded with 1 ml per well of a

100 µM solution of 5(6)-carboxyfluorescein-diacetate (CFDA) bought from Santa Cruz Biotechnology (Heidelberg, Deutschland). The CFDA was prepared from a 200 mM stock solution in DMSO, diluted with KRB alone or containing the substances of interest and incubated again at 37°C for 1 h while being protected from light.

Afterwards, CFDA solution was removed and the cells were washed again with prewarmed KRB. At this stage some specimens were washed with cold KRB and lysed with 1% Triton X-100 diluted with KRB in order to determine the intracellular CF content at t_0. Fresh KRB was added to each well (with or without the respective MRP1 modulator) and samples of the supernatant were drawn from each well after 15, 30, 45, 60 and sometimes also 90 minutes. Each time the sampled volume of 200 µl was replaced with fresh KRB. At the end of the experiment, cell monolayers were washed with cold KRB and then lysed in 1% Triton X-100 to stop the experiment. The fluorescence activity of samples was assessed using an automated plate reader (FLUOstar Optima, BMG Labtech, Offenburg, Germany) at excitation and emission wavelengths of 485 nm and 520 nm, respectively. Samples were diluted with KRB solution, where appropriate.

For standardisation, the total protein concentration of the whole cell lysate was determined by Pierce BCA assay (Thermo Fisher Scientific) and the amount of CF released per microgram of protein was calculated.
All data obtained from the efflux and uptake studies as well as the protein quantification were analysed with GraphPhad Prism 5 and edited using Microsoft Excel.

4.5 Protein Quantification

The protein content of each sample was measured by BCA assays. BCA stands for bicinchoninic acid and the method is based on the reduction of Cu^{+2} to Cu^{+1}. Two BCA molecules chelate each Copper ion forming a purple-coloured complex and its intensity depends on the protein concentration. The amount of protein present is then quantified by measuring the absorbance at a wavelength of 562 nm and comparing it with the absorbance spectrum of a standard protein solution of known concentration.

After cooling the cell lysates to a temperature of 4°C and sonicating them to achieve a better homogenisation, standard solutions containing bovine serum albumin (BSA) in concentrations from 0 to 2,000 µg/ml were prepared and measured. Additionally, a working reagent consisting of the two reagents A (i.e. sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide) and B (i.e. 4% cupric sulphate) was prepared from which 200µl were added to each 10 µl sample in a 96-well plate. Duplicates of the samples and standards were first incubated for 45 min at 37°C, during which time the reduction of copper took place and the colour therefore changed from green to purple. Finally, the absorbance was measured, and the results were calculated via Excel.

4.6 CSE - Fluorescence Experiment

To examine whether CSE itself causes a fluorescence signal at the relevant wavelengths and thus could potentially lead to artefacts, CSE was prepared in concentrations ranging from 1 to 20% and the fluorescence was measured at the same wavelength used in the experiments.

4.7 Western Blot

Western blotting was used to determine the effect of various MRP1 modulators on the expression level of the transporter.

Cells were seeded on a 6-well plate and incubated with either sulforaphane (5, 10 and 20 µM) perphenazine (5 and 10 µM) or quercetin (15 and 30 µM) for 72 h. The medium was removed and the cell monolayers were washed with cold PBS and 200 µl of Invitrogen cells extraction buffer were added. Afterwards, the cell lysates were transferred to sterile microcentrifuge tubes using a cell scraper.

Equal amounts of protein (i.e. 20 - 40 µg) were mixed with loading buffer and the mixtures were heated up to 95°C for 5 min. Samples were loaded onto sodium dodecyl sulphate-

polyacrylamide gel electrophoresis (SDS-PAGE) together with 5 µl of WB protein ladder (Thermo Fisher Scientific). Electrophoresis was performed at 120 V, followed by transfer onto immunoblot polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA) at 22 V for 40 min using semi dry transfer apparatus (Bio-Rad).

The unspecific binding sites were blocked by incubating the membranes with a 3% BSA in PBST solution for at least 1 h. After being washed twice, blots were incubated overnight at 4°C with a rat monoclonal anti-MRP1 antibody (clone MRPr1, GTX13368, Gene Tex, Irvine, CA).

The next day, the membranes were washed with PBST and incubated with antirat secondary antibodies for 1 h at room temperature. The activity of the peroxidase was detected with Immobilon Western Chemiluminescent HRP substrate (Millipore, Carrigtwohill, Ireland). Signals were documented using a ChemiDoc system (Bio-Rad, Hercules, CA).

4.8 Statistical analysis

Each experiment was carried out with duplicates and repeated at least 2 times (apart from the efflux assay with the primary cells) and the results were expressed as means and the standard deviation. In order to compare two groups two-way ANOVA with Bonferroni post-tests were conducted and p-values under 0,05 were considered significant.

5. Results

Efflux and uptake studies were carried out in order to examine the effect of CSE as well as various substances on the function of MRP1. The CF concentration was calculated after each experiment by using a standard curve, ranging from 0.0003 to 10 µM, which showed a linear correlation between the fluorescence and the CF concentration.

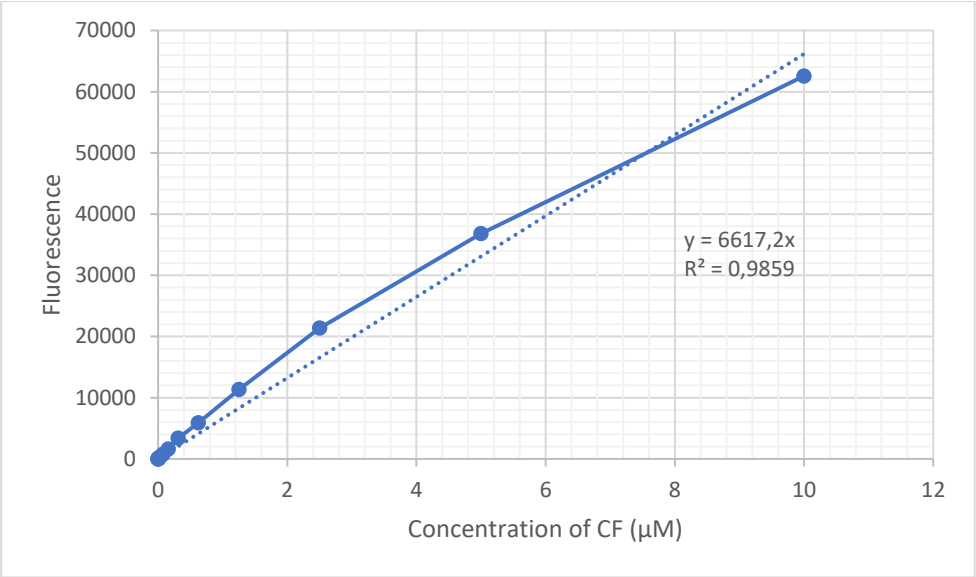


Figure 1: CF concentration curve ranging from 0 to 10 µM

The results were then calculated with Excel and analysed with GraphPad.

5.1.1 Efflux studies

Confluent monolayers of cells were incubated with either CSE or substances in different concentrations for either a short term or overnight or 72 h before running the efflux assay. In case of a short-term incubation the cells were only treated with the substance diluted with KRB for 1 h and the experiment was run directly afterwards. After the incubation with CFDA samples of the supernatant were drawn in 15-min intervals.

The CF concentration was measured both in cells and the supernatant in order to examine the MRP1 mediated efflux. In order to standardize the results, the protein concentration in each well was measured using a BCA assay.

5.1.2 Uptake studies

Uptake studies were conducted in order to observe the intracellular uptake of 5(6)-carboxyfluorescein-diacetate. The CFDA solutions were removed at different time points and the cell monolayers were lysed in order to detect the intracellular activity of CF, which was measured subsequently after 0 and 60 or 90 minutes.

5.2 MK571

Prior to experiments investigating the effects of CSE on MRP1 activity MK571 known for its inhibiting properties was tested. After a short-term incubation with 20 µM MK571, diluted in KRB the CF efflux of the supernatant as well as the cell lysates was measured.

Due to MK571 a significant reduction of MRP1 mediated CF efflux could be observed. The CF efflux was time dependent and significantly increased over time in the control group. Whereas, the cells treated with MK571 did not show any increased concentration of the fluorophore. After 1 h, the inhibition caused by MK571 led a total difference of 618.9% (p<0.001) compared to the control group but also the effects after 15 min (223.3%) and 45 mins (402.3%) were definitely significant (p<0.001).

Time (min)	Control mean ± SD, n=6	20 µM MK571 ± SD, n=6
0	120.05 ± 15.24	80.25 ± 40.73
15	321.66 ± 81.82	98.50 ± 30.92***
45	525.35 ± 84.20	123.10 ± 29.56***
60	758.71 ± 109.84	139.83 ± 26.37***

*Table 1: Data of CF efflux studies after short-term incubation with 20 µM MK571, n = 6, *** (p<0.001).*

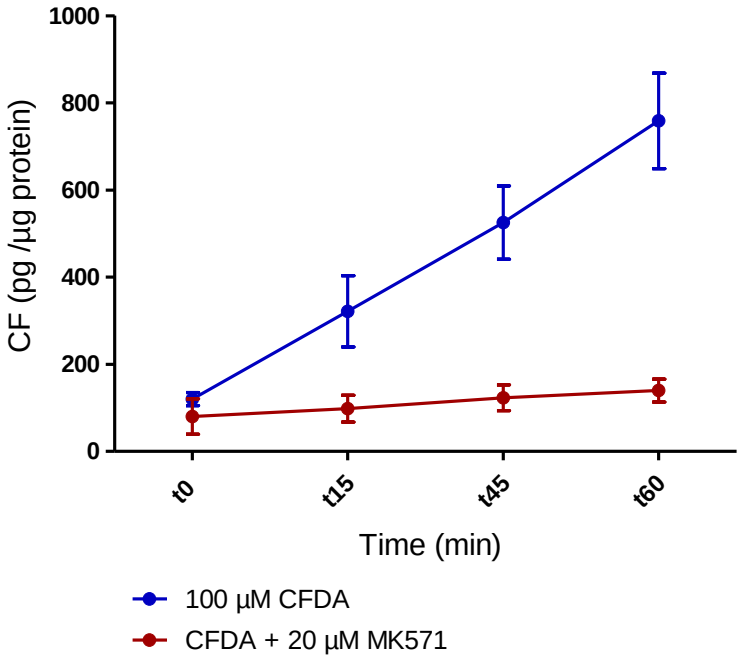


Figure 2: Effects of MK571 on CF efflux: a significant (p<0.001) inhibition of MRP1 could be measured

5.3 Effects of CSE 5%, 10% on the CF efflux

5.3.1 CSE - Fluorescence Experiment

In order to prevent the possibility of CSE itself causing a fluorescent signal that could manipulate the results from the efflux assays, CSE concentrations were prepared ranging from 1 to 20% and measured equally to the experiments.

Fortunately, CSE did not cause any change in absorbance even when tested in higher concentrations and could therefore be safely used in the following efflux experiments.

5.3.2 Short-term incubation with CSE 5%, 10%

A 100% CSE solution in KRB was prepared from which dilutions in the tested concentrations of 5 and 10% CSE were made. The 100% CSE was dissolved in KRB and sterile filtered (0.45 µm). For the short- term incubation the cells were treated with either KRB, 5 and 10% CSE for 1 h before being incubated with CFDA and measuring the CF concentration.

A significant difference of 135.2% ($p<0,01$) between the control group and 5% CSE could be observed only after 60 min. Surprisingly, overtime the cells treated with 10% CSE boasted slightly higher CF concentrations than the control cells, though there were no significant differences ($p>0,05$). In contrast, the treatment with 5% CSE led to a slightly increased retention of the fluorophore.

Time (min)	Control mean ± SD, n=5	CFDA + 5% CSE mean ± SD, n=5	CFDA + 10% CSE mean ± SD, n=5
0	128.37 ± 34.70	84.49 ± 8.47	79.04 ± 13.38
15	254.86 ± 48.13	179.72 ± 29.47	213.68 ± 42.75
30	351.81 ± 60.93	286.59 ± 59.86	371.87 ± 86.52
45	481.30 ± 78.16	397.96 ± 39.31	491.54 ± 76.88
60	612.56 ± 103.45	477.31** ± 42.46	594.66 ± 114.47

*Table 2: Data of the efflux study after short-term incubation with 5%, 10% CSE: the only significant difference ($p<0.01$) after 60 min between 5% CSE and the control group has been marked with **.*

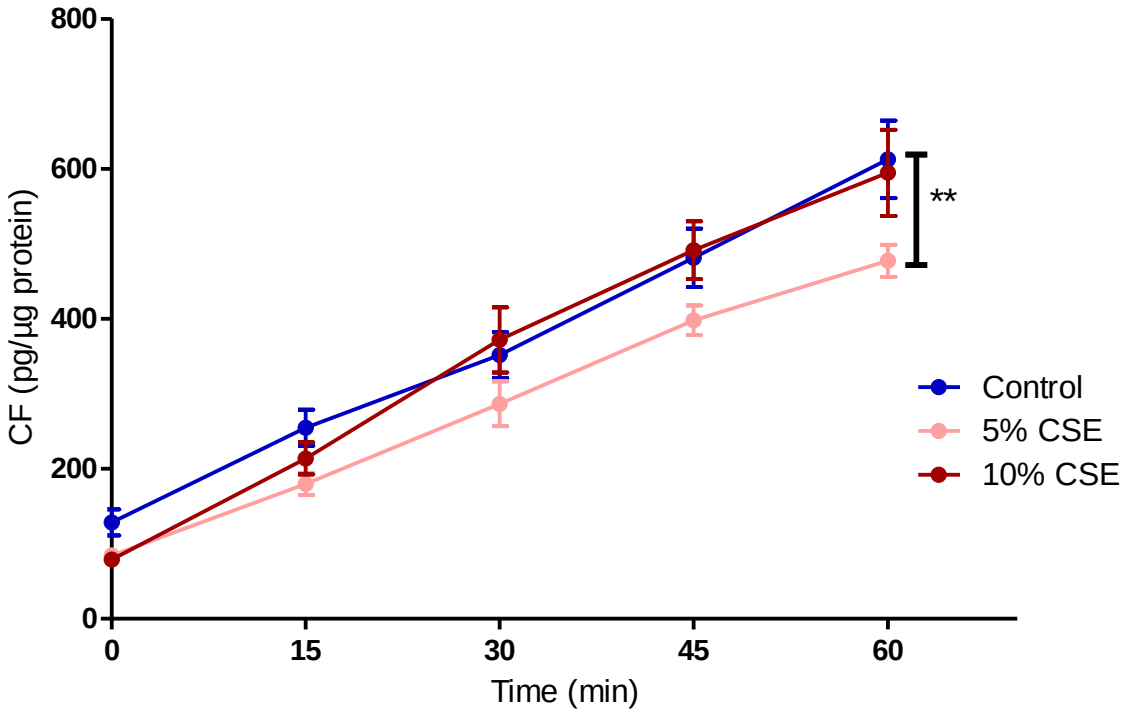


*Figure 3: Effects of 5%, 10% CSE on CF efflux after short-term incubation: a significant difference (p<0.01) after 60 min between 5% CSE and the control group has been marked with **.*

Uptake study

The uptake of the fluorescent was measured after 0 and 60 min to examine the cells´ ability to internalise CFDA, which is then converted into CF, and then excrete the substrate into the supernatant from which the samples for the efflux experiment were drawn.

In all 3 groups the intracellular CF concentrations were reduced after one hour. Initially, 5% CSE led to a very similar uptake of CFDA, whereas the cells treated with 10% of the toxic compound marked a decreased CF content. However, none of the results were significantly different (p>0.05).

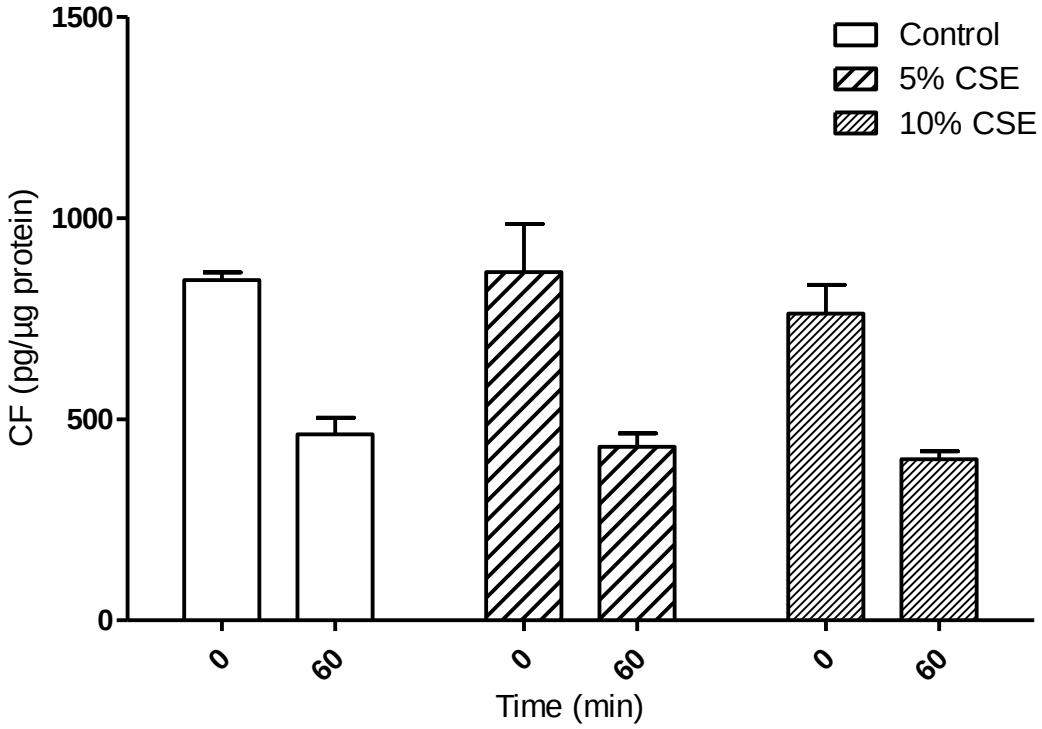


Figure 4: Uptake assay after short-term incubation with 5%,10% CSE. CF concentrations were reduced after 60 minutes in all 3 groups but no statistically significant differences could be seen (p>0.05).

5.3.3 Long-term incubation with CSE 5%, 10%

Since no major effects after the short-term treatment with CSE were observed, the cells were incubated with 5 and 10% CSE overnight to maximise the uptake of CSE. This time the 100% CSE was prepared using the feeding medium and sterile filtered (0.45 µm), which both happened under the laminar air flow to ensure sterile conditions.

After the long-term incubation a significant reduction of CF efflux in the cells treated with 5% and 10% CSE could be measured. The CF efflux by MRP1 was time dependent, and clearly reduced in the cells treated with CSE, especially after the incubation with 10% CSE.

When comparing the control group with 5% CSE minor significant differences of 37.7% ($p<0.01$) after 60 min and 32.24% after 90 min could be observed.

Whereas, 10% CSE led to a slightly increased retention even after 15 and 30 min (31.45%, 32.02% difference, p<0,05) and significant differences (p<0.01) compared to the control group could be marked after 45 min (56.3%), 60 min (74.61%) and 90 min (98.47%). 10% CSE, therefore reduced the CFDA efflux by 27.95% and could be identified as a potent inhibitor of the MRP1 transporter.

Time (min)	100 µM CFDA mean ± SD, n=5	CFDA+ 5% CSE mean ± SD, n=5	CFDA+10% CSE mean ± SD, n=5
0	10.54 ± 1.01	9.56 ± 0.81	8.03 ± 0.89
15	92.45 ± 7.83	74.82 ± 4.13	60.99* ± 2.81
30	144.46 ± 14.70	135.20 ± 5.91	112.44* ± 6.63
45	205.61 ± 20.56	179.31 ± 11.12	149.31*** ± 11.98
60	251.54 ± 25.85	213.85** ± 15.48	176.93*** ± 8.00
90	352.33 ± 22.63	320.08* ± 23.05	253.86*** ± 8.35

*Table 3: Data of the efflux study after long-term incubation with 5%, 10% CSE. The statistically significant differences between either 5% or 10% CSE and the control group were marked with * (p<0.05), ** (p<0.01) and *** (p<0.001).*

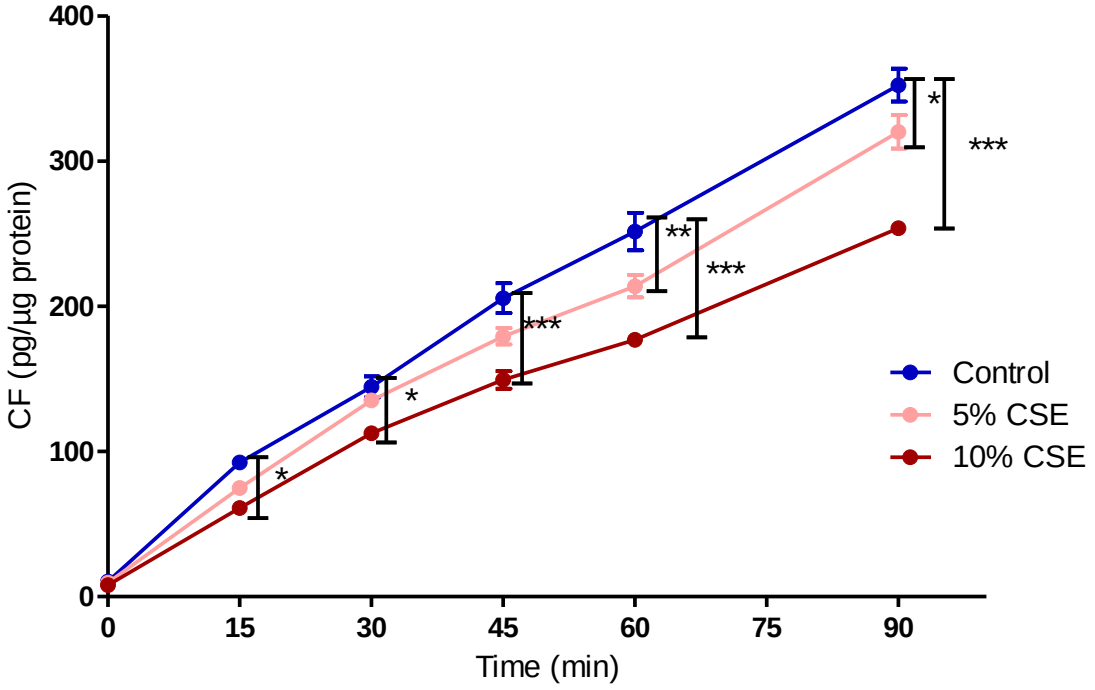


Figure 5: Efflux assay with 5%, 10% CSE after long-term incubation: a reduced CF efflux in cells treated

*with CSE could be measured. The statistically significant differences between either 5% or 10% CSE and the control group were marked with * (p<0.05), ** (p<0.01) and *** (p<0.001).*

Uptake study

After 0 and 60 min, an uptake study was performed with 5 and 10% CSE. Interestingly, a reduced uptake in the cells treated with 10% CSE was observed directly after the incubation with CFDA, suggesting that due to the treatment with CSE overnight and its toxic compounds the cells were initially unable to initiate the fluorescent initially. The difference of 259.4% with $p<0.05$ was statistically significant and after 60 min the intracellular CF concentration remained nearly stationary as MRP1 was clearly inhibited therefore could not efflux CF sufficiently.

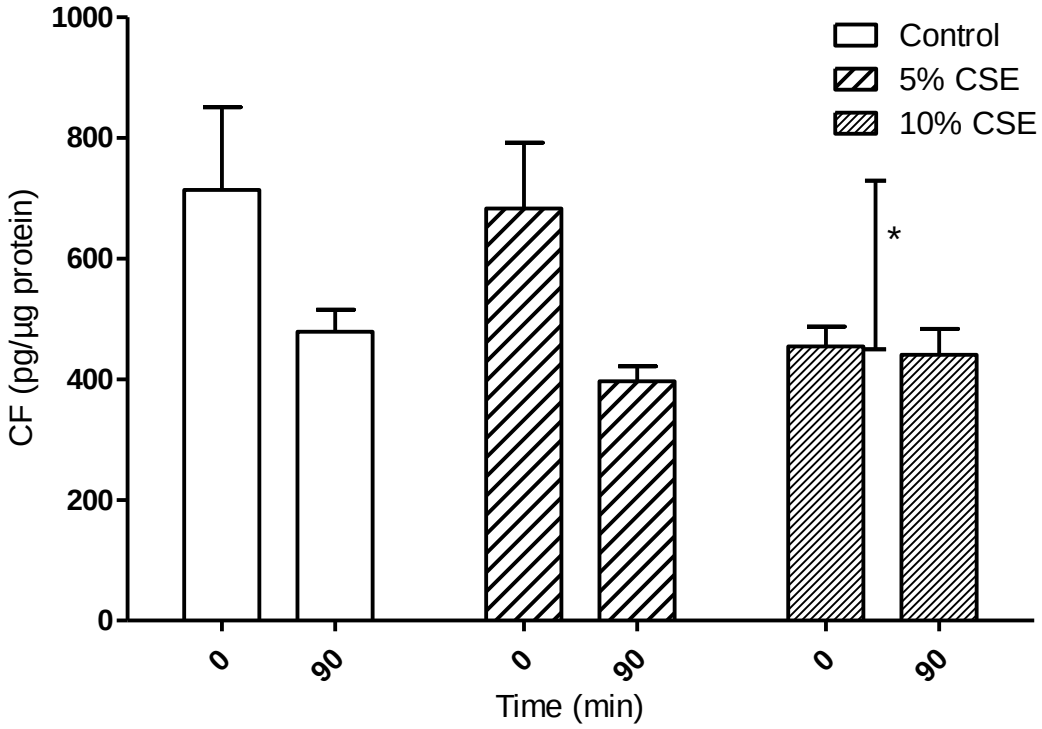


*Figure 6: Uptake study with 5%,10% CSE. CF concentrations were reduced after the exposure to 10% CSE. The statistically significant differences between either 5% or 10% CSE and the control group were marked with * (p<0.05).*

5.3.4 Fresh and aged CSE 10%

As the prior experiments have demonstrated the inhibiting effect of CSE on MRP1 the aim of the following efflux assay was to assess the inhibitory potential of aged CSE and to find out whether the storage in the freezer for a longer time period could reduce this effect. For this purpose, CSE was prepared as mentioned before, sterile filtered and stored in the freezer for more than 2 weeks before the experiment was run again

Aged CSE was compared to fresh CSE, both in concentrations of 10% diluted with KRB as well as a control group only treated with feeding medium and KRB to ensure optimal results. The cells were incubated with the solutions overnight since long-term incubation led to obvious results in the previous experiment.

As anticipated, in comparison to fresh CSE aged CSE led to a decreased retention of CF, which could be due to the reduced toxicity and oxidative stress leading to the lower transporter activity when keeping CSE frozen over a longer period of time. The sample of the group treated with 10% aged CSE taken after 60 minutes showed a significantly increased retention even after 15 min (65.47%, $p<0.05$) with the biggest difference to the control group after 90 min (150.3%, $p<0.001$).

Whereas aged CSE only triggered a slightly significant inhibition after 30 min (53.22%, $p<0.05$), 45 min (52.95%, $p<0.05$) and 90 min (52.69%, $p<0.05$), while at the other time points the effects were statistically irrelevant ($p>0.05$).

Time (min)	100 µM CFDA mean ± SD, n=5	CFDA+ 10% fresh CSE mean ± SD, n=5	CFDA+ 10% aged CSE mean ± SD, n=5
0	63.77 ± 10.51	63.12 ± 13.68	48.91 ± 3.49
15	231.91 ± 11.04	166.44** ± 15.15	196.62 ± 10.34
30	358.53 ± 17.48	264.20*** ± 17.35	305.32* ± 30.79
45	426.68 ± 21.73	322.12*** ± 17.50	373.73* ± 28.09
60	499.35 ± 19.16	384.81*** ± 27.92	463.99 ± 37.11
90	612.37 ± 36.95	462.11*** ± 26.70	559.67* ± 45.01

*Table 4: Efflux assay with 10% fresh- and 10% aged CSE, long-term incubation. The statistically significant differences between either 10% fresh or aged CSE and the control group were marked with * (p<0.05), ** (p<0.01) and *** (p<0.001).*

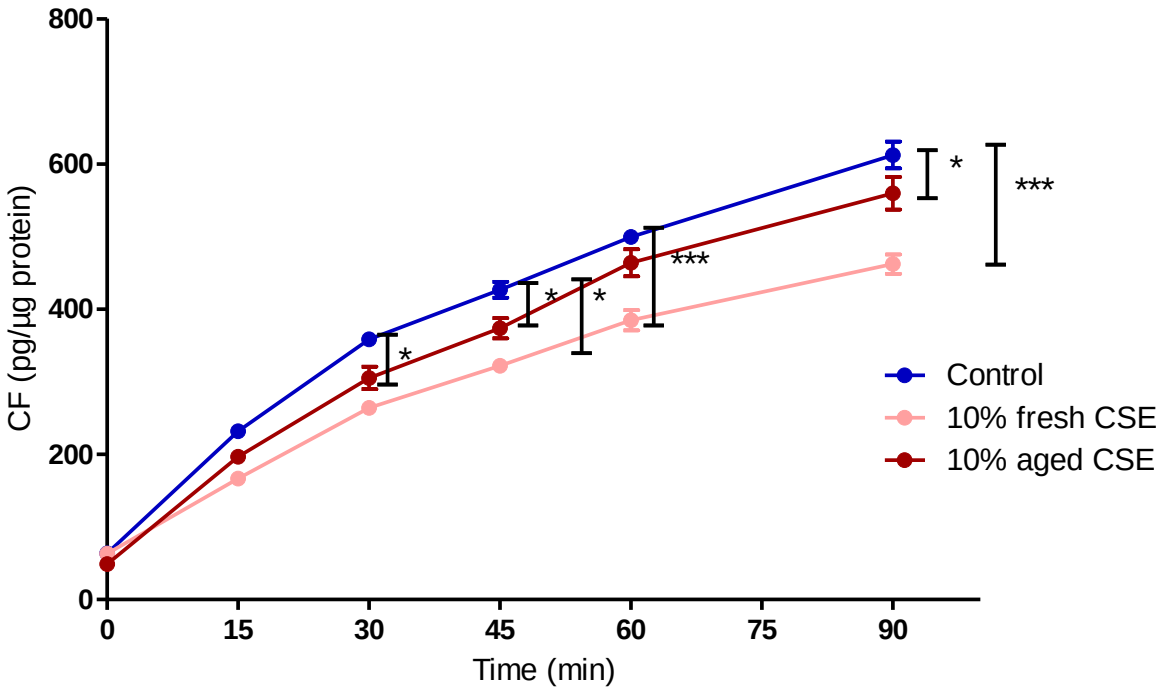


*Figure 7: efflux assay comparing 10% fresh and 2-week old CSE after 24-hours incubation. The statistically significant differences between either 10% fresh or aged CSE and the control group were marked with * (p<0.05) and *** (p<0.001).*

Uptake study

Both fresh and aged CSE marked an initially increased CF uptake but due to the inhibitory potential of the toxic compounds the cells could not efflux the fluorescent efficiently leading to higher intracellular CF concentrations when compared to the control group. Furthermore, the increased cytotoxicity of fresh CSE triggered an increased CF uptake and an insufficient efflux and therefore higher levels of intracellular CF content than older CSE. Fresh CSE therefore led to the significant difference of initially 345.5% (p<0.001) and 199.9% (p<0.01) after 90 min, while aged CSE decreased MRP1 activity by 264.9% (p<0.01, t0) and 132.6% (p<0.05, t90) compared to the control group.

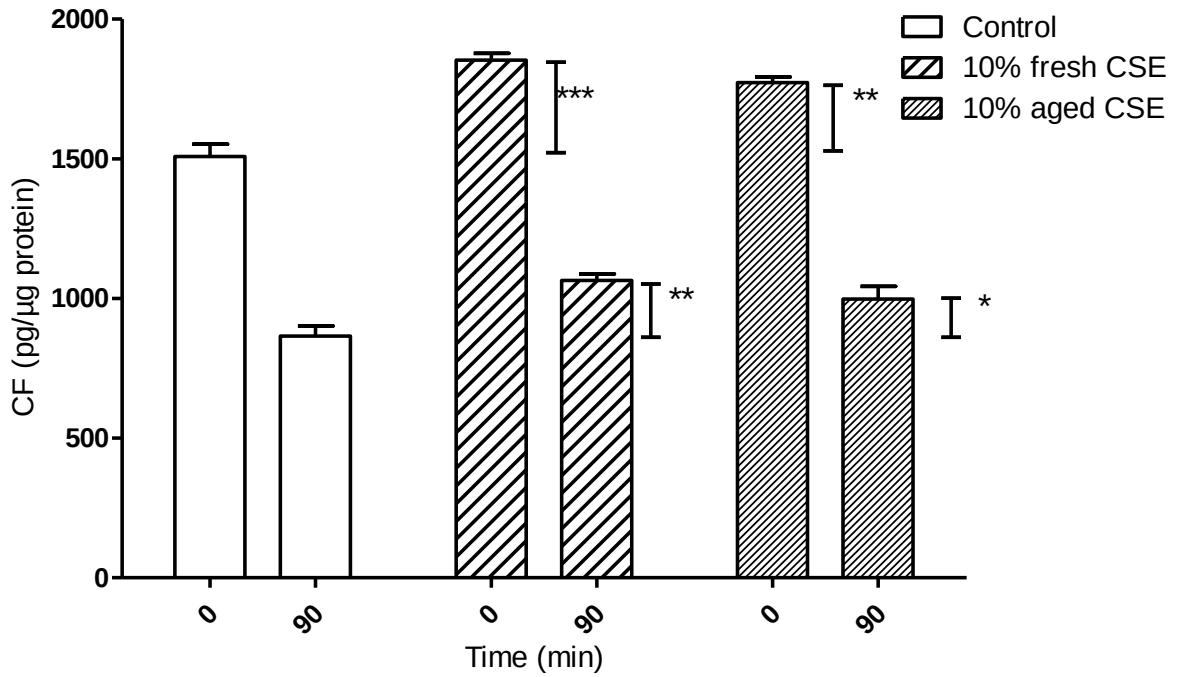


*Figure 8: uptake study with 10% fresh- and 10% old CSE after a long-term incubation. The statistically significant differences between either 10% fresh or aged CSE and the control group were marked with * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).*

5.4 Substances that could raise MRP1 activity in cells

5.4.1 Sulforaphane

Being a naturally occurring agent in chemotherapy and Nrf2 inducer sulforaphane has been tested on its cytoprotective properties by enhancing MRP1 due to an up-regulation of the Nrf2-pathway (Lubelska et al. 2016). Prior to the efflux experiment a cytotoxicity assay was carried out in order to estimate the maximum concentrations that wouldn´t harm or kill the cells.

5.4.1.1 Cytotoxicity assay with sulforaphane

Confluent cells on a 96-well plate were incubated with sulforaphane in concentrations ranging from 5 to 200 µM for 24 h. After removing the medium and washing the cells they were treated with 100 µl AlamarBlue for 3 h, while the cells from 4 wells were aspirated and used as blanks. Then, the fluorescence intensity was measured and the IC_{50} was calculated in order to estimate the cytotoxicity.

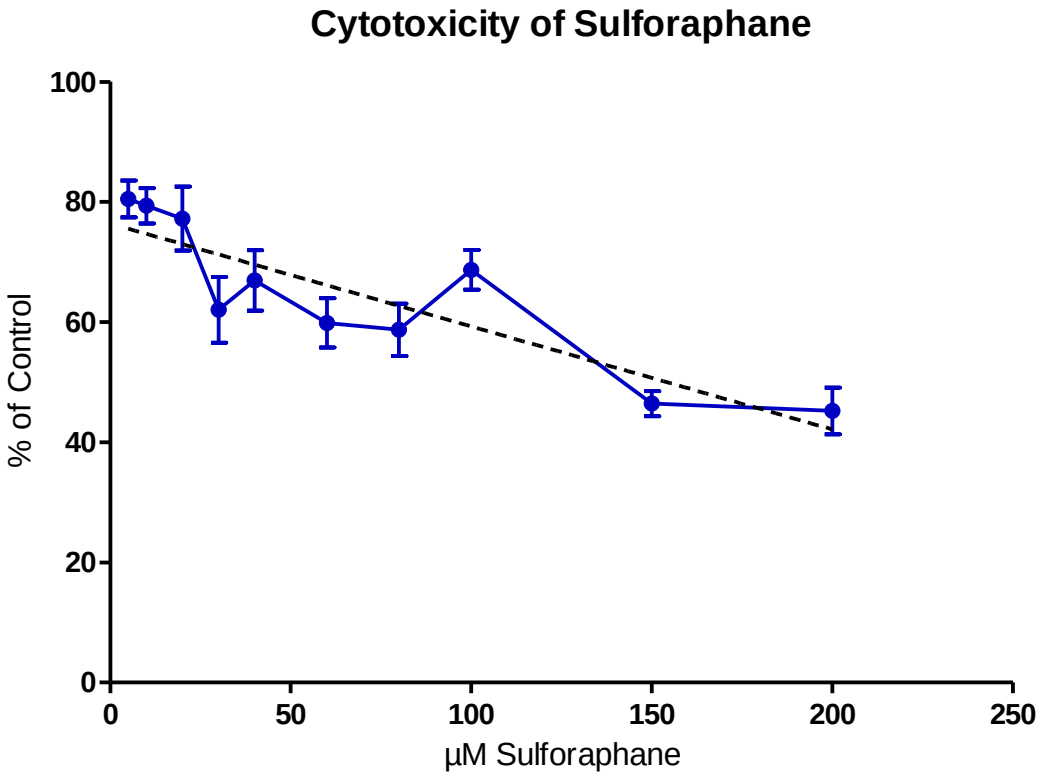


Figure 9: Cytotoxicity assay with 5, 10, 20, 30, 40, 60, 80, 100, 150 and 200 µM sulforaphane

5 µM	10 µM	20 µM	30 µM	40 µM	60 µM	80 µM	100 µM	150 µM	200 µM	Control
80.47	79.36	77.23	62.05	66.95	59.86	58.71	68.70	46.43	45.21	100.00

Table 5: Cytotoxicity assay with sulforaphane: comparing the fluorescence intensity of the cells treated with sulforaphane to a control group.

When comparing the fluorescence intensity that is linked to cell density and therefore survival of those incubated with sulforaphane to the non-treated control group a significant decrease in cell growth and density could be witnessed.

As a result, the assay suggests that sulforaphane has highly active cytotoxic properties that could kill the cells already at low concentrations. Any concentrations higher than 10 µM lead to over 20% cell death and should therefore not be used if appropriate functionality concerning the transport across the membranes is required.

The IC_{50} was calculated after creating the linear regression and turned out to be 154.08 µM, which was actually higher than anticipated. However, in order to carry out reasonable efflux assay a higher abundance of attached cells is required and therefore sulforaphane should only be tested in concentrations way below the IC_{50} value.

5.4.1.2. Sulforaphane 5 µM and 10 µM

The first experiment was carried out using 5 µM and 10 µM sulforaphane (SFN) as higher concentrations led to a survival of less than 80% of cells. A 10 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 KRB and sterile filtered to ensure sterile conditions.

24-wells of confluent cells were treated with the dilutions (except the control group which was only treated with KRB) and incubated overnight. After removing the solutions and

washing the cells were incubated again with the same concentrations of substances for one hour, which were then aspirated again and replaced with CFDA solutions containing the same sulforaphane solutions. After the incubation samples from the supernatant were drawn the absorption was measured.

The data obtained from the efflux experiment suggests only a very slight increase in MRP1 activity in the cells treated with 5 µM sulforaphane. Whereas, the incubation with 10 µM led to a decreased expression of the transporter, which could be due to the cytotoxic properties of sulforaphane that could have killed a significant number of cells, leading to a decreased CF efflux. While the control cells excreted 539.35% of the initial concentration into the supernatant, the cells treated with sulforaphane led to 543.86% (5 µM sulforaphane) and 531.85% (10 µM sulforaphane).

However, looking at the graph no major difference in transporter activity is noticeable leading to the conclusion that maybe higher concentrations of sulforaphane could cause a bigger effect, though could be harmful for the cells.

Time (min)	100 µM CFDA mean ± SD, n=5	CFDA+ 5 µM SFN mean ± SD, n=5	CFDA+ 10 µM SFN mean ± SD, n=5
0	60.32 ± 14.33	61.82 ± 8.82	41.47 ± 4.90
15	134.32 ± 16.70	141.04 ± 19.07	129.49 ± 17.16
30	225.84 ± 26.46	237.56 ± 8.78	216.10 ± 26.84
45	318.13 ± 36.80	322.82 ± 11.53	285.63 ± 25.16
60	383.53 ± 28.21	419.89 ± 16.65	369.96 ± 37.14
90	539.35 ± 40.36	543.86 ± 27.77	531.85 ± 56.41

Table 6: Data of the efflux study after 24 hours of incubation with 5 and 10 µm sulforaphane. At no time point any significant difference could be observed (p>0.05).

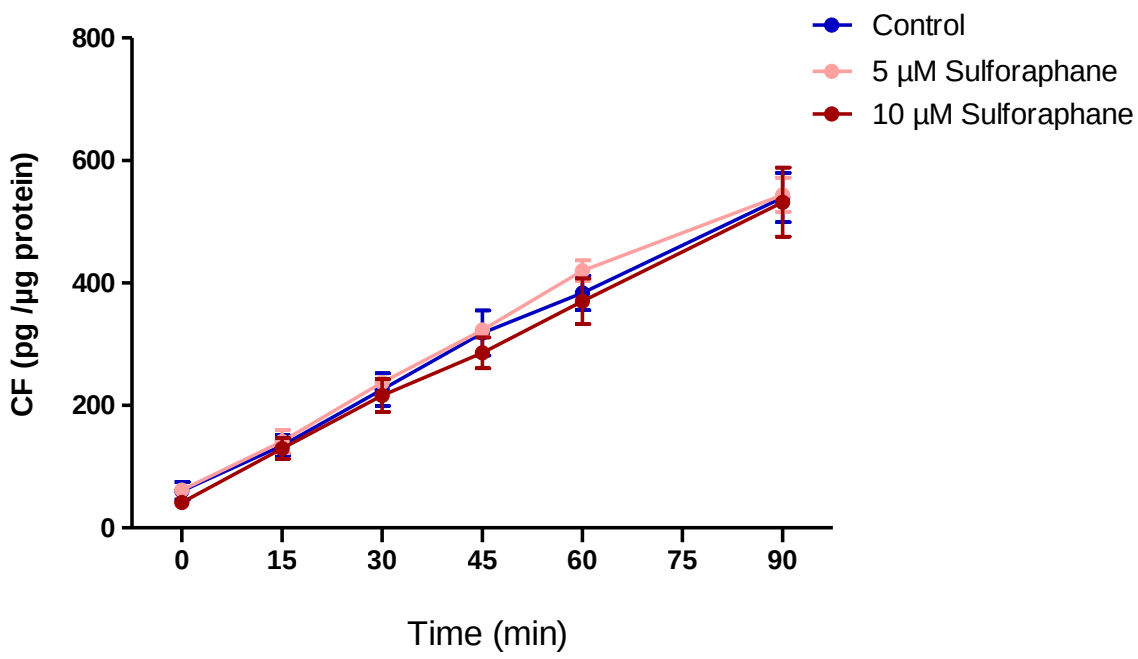


Figure 10: graph of the efflux assay with 5 µM, 10 µM sulforaphane. Neither 5 µM or 10µM sulforaphane triggered a significant effect (p>0.05).

Uptake study

After 0 and 90 min and lysing the cells with Triton X-100 an uptake study was performed, which suggested a slightly decreased CF uptake in the cells treated with sulforaphane leading to the assumption that due to the cytotoxicity of the substance the cells could not sufficiently absorb the CFDA and therefore efflux the CF.

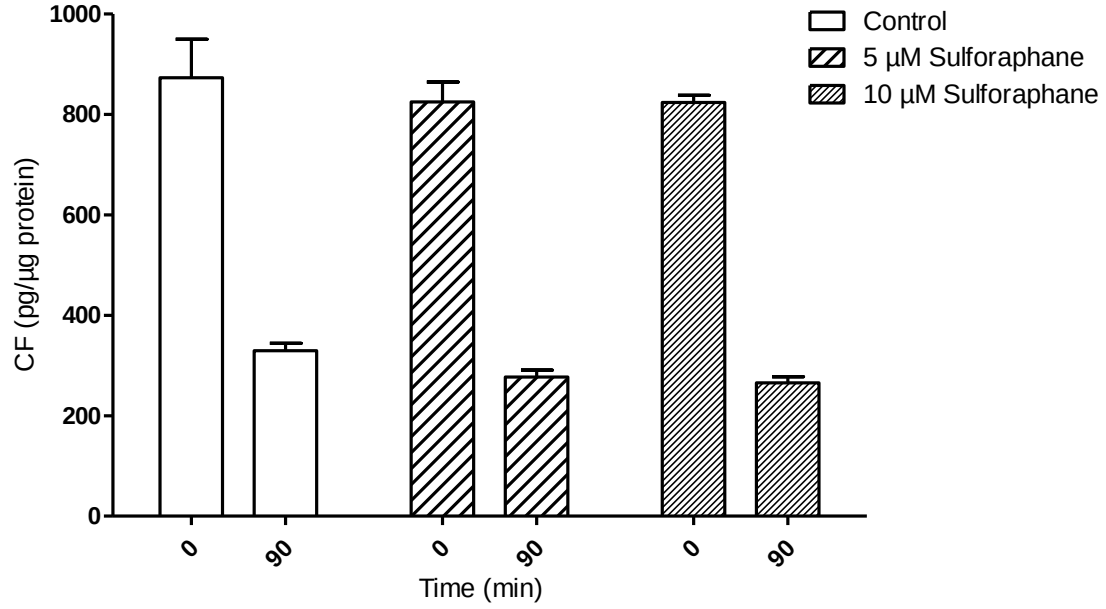


Figure 11: Uptake study with 5 µM, 10 µM sulforaphane. No significant differences could be observed (p>0.05).

5.4.1.3 Sulforaphane 50 µM, 100 µM

Since the prior experiments using low concentrations of sulforaphane showed no significant increase in transporter activity the next step was to test 50 µM and 100 µM. Despite the potential cytotoxic effect and cell death an increased CF efflux due to improved MRP1 activity was anticipated.

The solutions were prepared as mentioned before in KRB, though leading to dilutions of 50 µM and 100 µM sulforaphane. Then, the efflux experiment was run again, however this time after a short-term incubation, and the protein was quantified for standardisation purposes.

Unfortunately, despite the short incubation time the high concentrations of sulforaphane caused major cell death leading to decreased CF efflux. The surviving cells were harmed and the monolayer detached, which made them incapable of a sufficient efflux.

The short incubation with sulforaphane decreased the transporter´s activity by 61.11% (50 µM sulforaphane) and 68.86% (100 µM sulforaphane).

Time (min)	100µM CFDA mean ± SD, n=5	CFDA+ 50µM SFN mean ± SD, n=5	CFDA+ 100µM SFN mean ± SD, n=5
0	12.49 ± 2.38	6.94 ± 0.91	6.19*** ± 0.57
15	149.35 ± 12.91	54.33*** ± 3.89	49.40*** ± 3.47
30	228.69 ± 17.61	85.46*** ± 4.64	73.88*** ± 5.23
45	314.47 ± 23.75	115.16*** ± 2.56	101.32*** ± 7.58
60	374.09 ± 31.65	138.45*** ± 4.73	121.56*** ± 7.49
90	535.72 ± 57.02	208.35*** ± 12.31	166.79*** ± 13.97

Table 7: Data of the efflux study after 24 hours of incubation with 50 and 100 µM sulforaphane

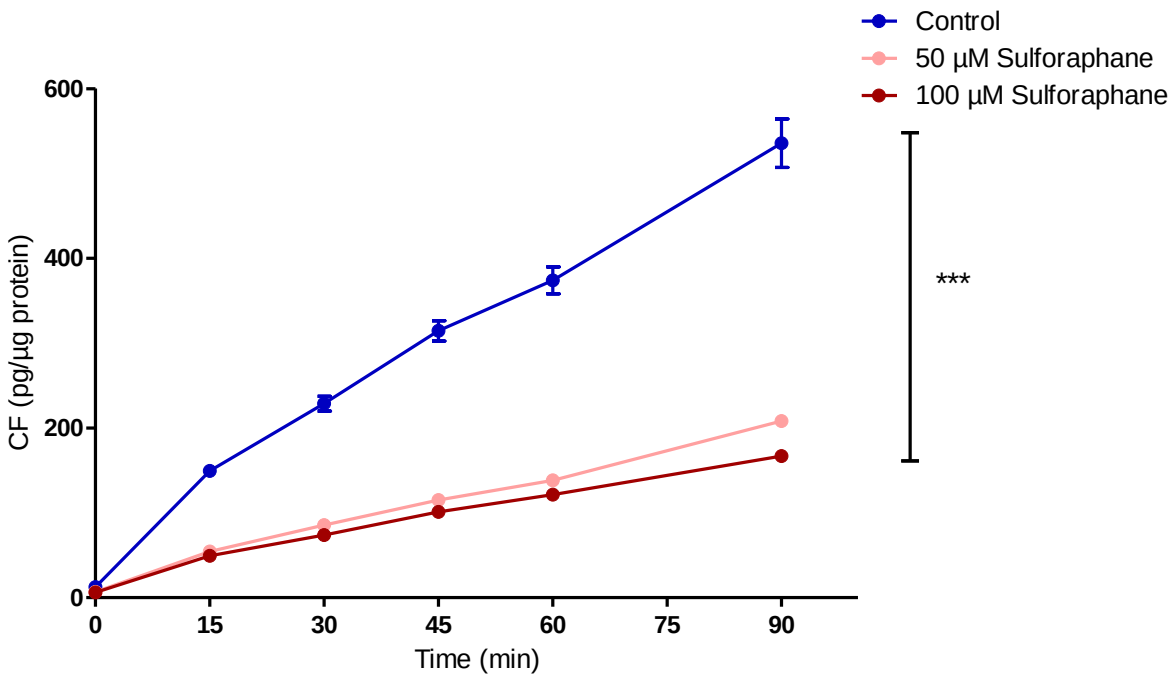


Figure 12: graph of the efflux assay with 50 µM, 100 µM sulforaphane. At every time point both 50 µM and 100 µM sulforaphane triggered a significantly decreased function of MRP1 (p<0.001).

Uptake study

The uptake study carried out after 0 and 90 minutes also highlighted the insufficient uptake of CFDA of the cells due to the toxic effects of sulforaphane when used in high concentrations. As a result, sulforaphane couldn´t prove its potential to induce the Nrf2 pathway and therefore raise the MRP1 activity as low concentrations do not show any

significant effect, while dilutions containing more than 5 µM led to more than 80% cell death and therefore unconvincing experimental results.

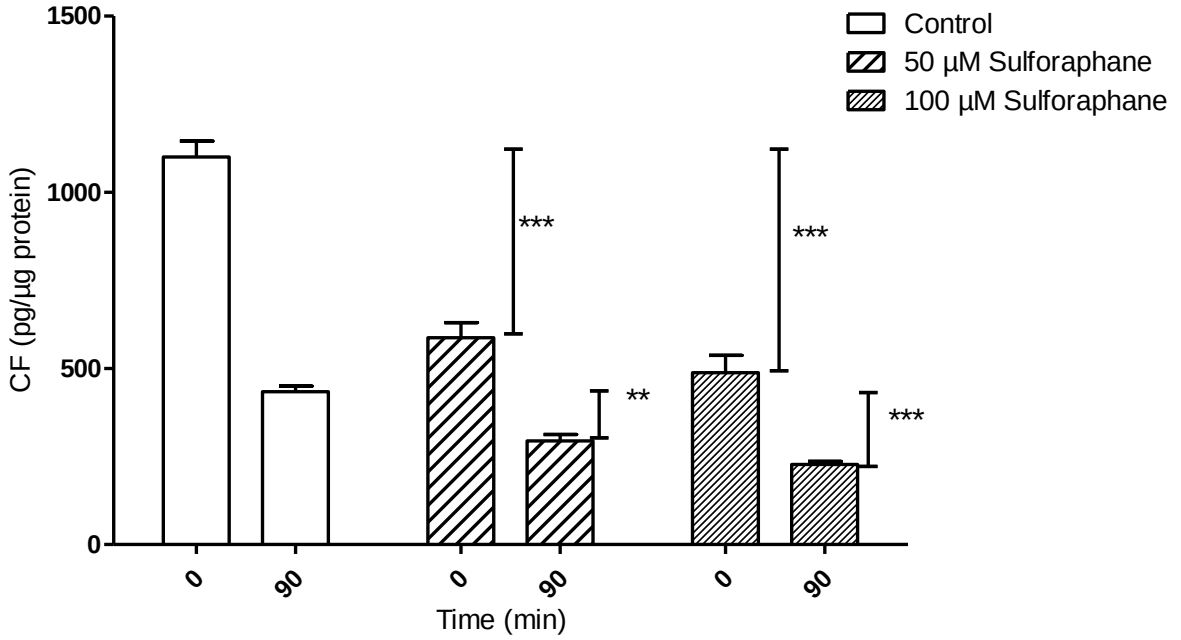


*Figure 13: Uptake study with 50 µM, 100 µM sulforaphane. The statistically significant differences between either 50 µM or 100 µM sulforaphane and the control group were marked with ** (p<0.01) and *** (p<0.001).*

4.4.1.4 Sulforaphane 5 µM and 7.5 µM, 72 hours incubation

Since 5µm showed a slight increase in CFDA efflux, whereas concentrations higher than 10 µM led to major cell death, the experiment was repeated using 5 µM and 7.5 µM. However, this time the cells were incubated with the dilutions, which were prepared from a 10 mM stock solution of sulforaphane in KRB as mentioned in previous experiments, for 72 h. To enable a better comparison the cells of the control group were incubated with pure feeding medium for the same amount of time.

Interestingly, this time even 5 µM of the substance tested led to a retention of CF efflux and 7.5 µM showed an even more significant effect. This experiment therefore suggests an inhibition of MRP1 performed by sulforaphane, which, however, could be due to the fact that this substance triggers cell apoptosis when used in higher concentrations or after long-

term incubations. While 5 µM led to the biggest difference compared to the control group after 90 min (60.15% difference, $p<0.001$), 7.5 µM sulforaphane decreased the CF efflux significantly after 45 min (44.33%, $p<0.001$), 60 min (44.83%, $p<0.001$) and 90 min (83.39%, $p<0.001$).

Time (min)	100 µM CFDA mean ± SD, n=5	CFDA+ 5 µM SFN mean ± SD, n=5	CFDA+ 7.5 µM SFN mean ± SD, n=5
0	45.34 ± 11.61	51.99 ± 9.42	58.42 ± 10.68
15	135.63 ± 6.52	123.27 ± 7.69	133.21 ± 14.03
30	214.38 ± 8.25	206.81 ± 7.00	193.68 ± 13.76
45	280.34 ± 16.96	254.96* ± 16.77	236.01*** ± 16.61
60	324.01 ± 7.43	295.32** ± 13.17	279.18*** ± 16.64
90	400.80 ± 14.61	340.65*** ± 13.04	317.41*** ± 18.72

*Table 8: Data of the efflux study after 72 hours of incubation with 5 and 7.5 µM sulforaphane. The statistically significant differences between either 5 or 7.5 µM sulforaphane and the control group were marked with * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).*

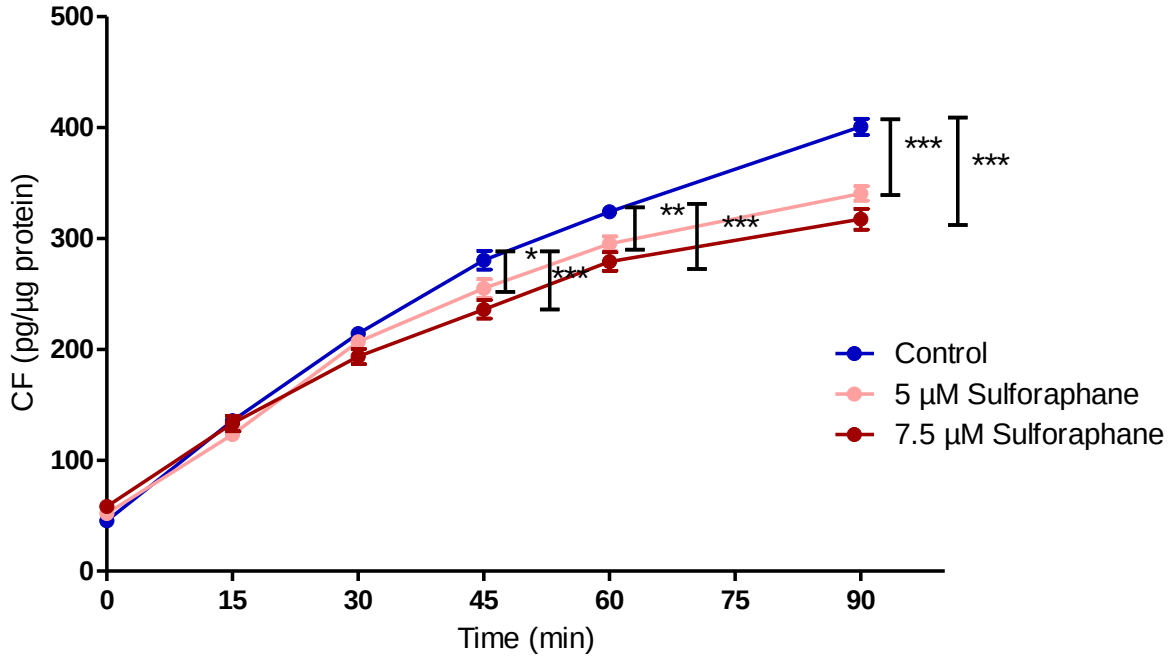


*Figure 14: graph of the efflux assay with 5 µM, 7.5 µM sulforaphane after 72 hours incubation. The statistically significant differences between either 5 µM or 7.5 µM sulforaphane and the control group were marked with * (p<0.05), ** (p<0.01) and *** (p<0.001).*

Uptake study

After drawing samples of the supernatant, cells were lysed and the intracellular CF concentration was determined. Sulforaphane led to a significant decrease in the initial CFDA uptake, which was directly proportional to the increased concentrations: 5 µM inhibited the uptake by 170.8% (p<0.001) and 7.5 µM by 235.3% (p<0.001) compared to the control group. This time the cytotoxic effect leading to a reduced intracellular CF content was even

more prominent as the cells were treated for a longer time period.

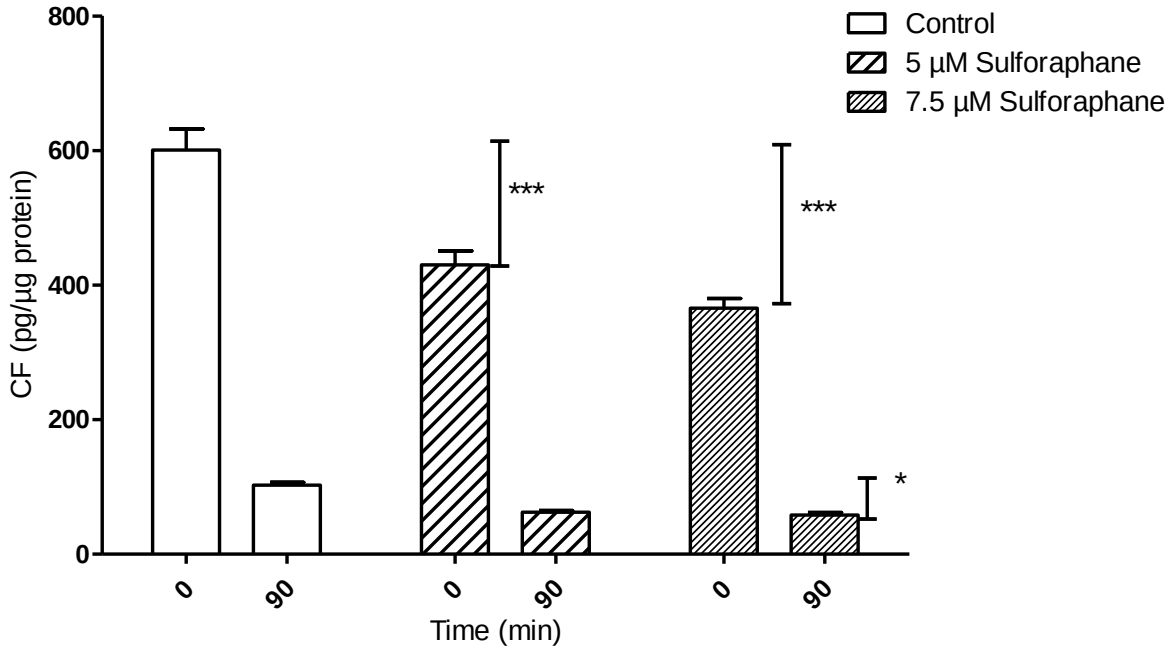


*Figure 15: Uptake study with 5 and 7.5 µM sulforaphane after 72 hours of incubation. The statistically significant differences between either 5 µM or 7.5 µM sulforaphane and the control group were marked with * ($p<0.05$) and *** ($p<0.001$).*

4.4.2 Efflux study with Perphenazine 25 µM and Quercetin 25 µM

As perphenazine is a prominent member of the phenothiazine group, which are broadly used as neuroleptics and are known to enhance levels of MRP-proteins the following experiment aimed at proofing this thesis. On the contrary, quercetin a naturally occurring flavonoid serves in chemotherapy by reducing the resistance of cancer cells to chemotherapeutic agents as inhibitor of MRP efflux transporters, thus improving their potency.

The experiment was conducted using 25 µM of both drugs which were prepared from 25 mM stock solutions diluted with DMSO and then added to the feeding medium. After an incubation time of 72 h the efflux experiment was carried out.

The experiment proved a significant inhibition of MRP1 by quercetin, which led to a significantly decreased CF efflux compared to the control group measurable at every time point: The differences of 42,87% (t15), 76,36% (t30), 109,3% (t45), 140,3% (t60) and 192,1% (t90) were all clearly significant (p<0,001) and therefore highlighted quercetin´s inhibiting properties of MRP1.

Whereas, contrary to the predicted activation of MRP1, perphenazine also decreased the efflux by 37,43% (p<0,01) after 60 minutes and 47,51% (p<0,001) after 90 minutes when compared to the control group. Perphenazine´s ability to stimulate the MRP mediated efflux could therefore be highly substrate dependent and rely on the use of specific types of cells like erythrocytes or primary cells and do not modulate the CF efflux in bronchial cell lines.

time (min)	**100 µM CFDA mean ± SD, n=5**	**CFDA+ 25 µM P. mean ± SD, n=5**	**CFDA+ 25 µM Q. mean ± SD, n=5**
0	48.28 ± 4.25	53.21 ± 3.01	39.07 ± 3.59
15	126.53 ± 5.60	123.28 ± 11.64	83.65** ± 11.43
30	199.12 ± 11.51	185.08 ± 12.85	122.76*** ± 10.09
45	261.20 ± 9.27	241.71 ± 11.65	151.92*** ± 12.59
60	323.31 ± 16.08	285.88** ± 15.34	182.98*** ± 14.92
90	438.56 ± 24.80	391.06*** ± 33.02	246.49*** ± 24.70

*Table 9: Results from the efflux experiment conducted with 25 µM perphenazine (P.) and 25 µM quercetin after a 72-hours incubation. The statistically significant differences between either 25 µM perphenazine, 25 µM quercetin and the control group were marked with ** (p<0,01) and *** (p<0,001).*

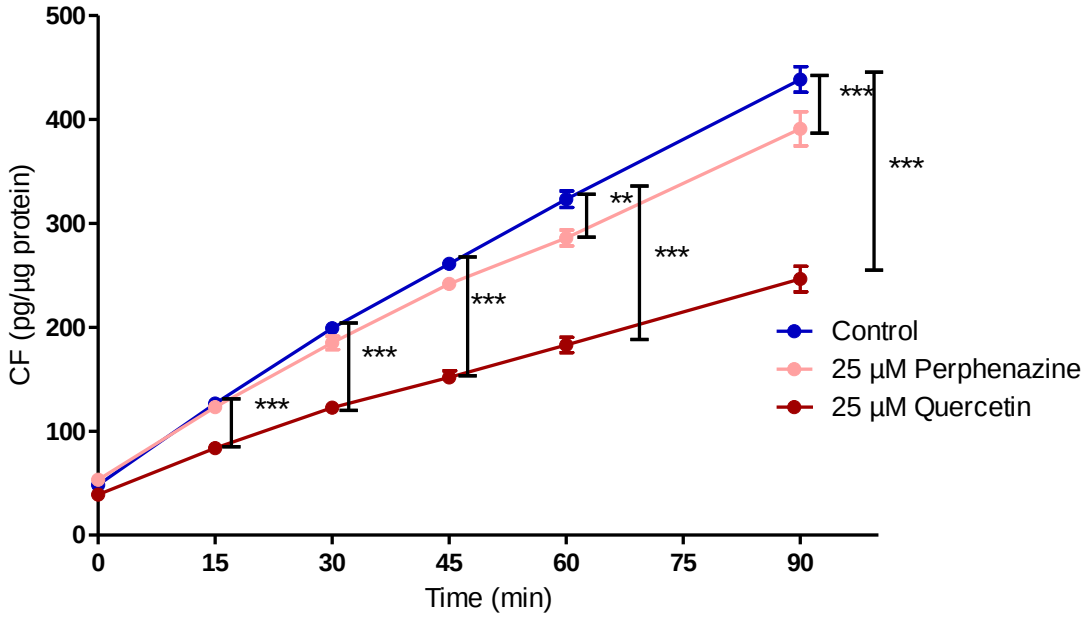


*Figure 16: graph of the efflux assay with 25 µM perphenazine and 25 µM quercetin after 72 hours incubation. The statistically significant differences between either 25 µM perphenazine, 25 µM quercetin and the control group were marked with ** (p<0.01) and *** (p<0.001).*

Uptake study

The uptake study performed with samples drawn after 0 and 90 min showed a significantly increased CFDA uptake in the cells treated with perphenazine, which then could not efflux the substrate efficiently. The differences to the control group with 345.1% (t0, $p<0.001$) and 210.2% (t90, $p<0.001$) were both obvious.

Quercetin showed the same effect, though led to a lower initial intracellular CF concentration which was only increased by 153.7% (t0, $p<0.05$) in comparison to the control group followed by a significantly increased retention after 90 min (339.3%, $p<0.001$).

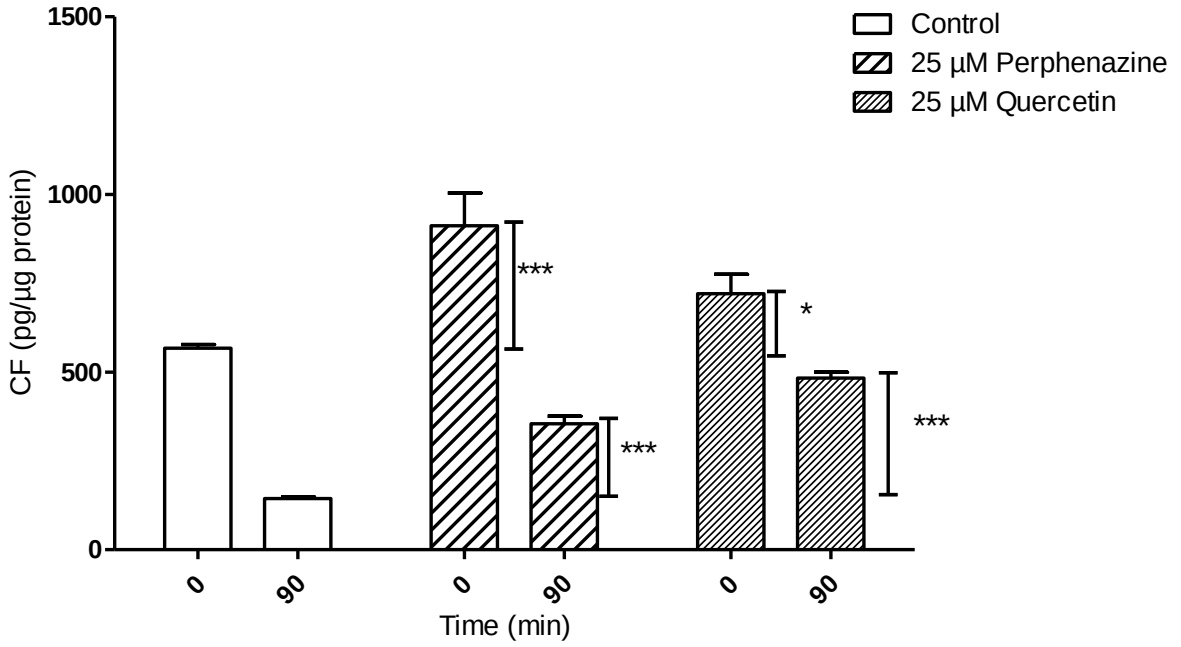


*Figure 17: Uptake study with 25 µM perphenazine and 25 µM quercetin after 72 hours of incubation. The statistically significant differences between either 25 µM perphenazine, 25 µM quercetin and the control group were marked with * (p<0.05) and *** (p<0.001).*

5.4.3 Efflux assay with primary cells: Sulforaphane 10 µM and CSE 5%

Since the experiments conducted with cells derived from cancer tissue led to unsatisfying results this time the efflux assay was carried out using primary cells. In anticipation of a significant activation and inhibition of the MRP1 transporter 10 µM sulforaphane and 5% CSE were tested after short-term incubation.

Surprisingly, the use of primary cells led to very significant results: After 90 minutes 10 µM sulforaphane increased the efflux over 20%, though the most noticeable difference when compared to the control group happened after 45 min, when sulforaphane maximized the transporter activity by nearly 28%. On the contrary, 5% CSE showed similar results as in previous experiments carried out after a 24-hours incubation and decreased the efflux of the substrate by 12.4% after 90 min.

Since the primary cells turned out to be very delicate and the monolayer detached very easily this time each group consisted of only 3 wells instead of 8 and therefore no cells were lysed in order to examine the intracellular CF content to ensure reliable efflux results by using duplicates of wells serving as the control and triplicates of those treated with 5% CSE and 10 µM sulforaphane.

Time (min)	100 µM CFDA mean ± SD, n=2	CFDA+ 10 µM SFN mean ± SD, n=3	CFDA+ 5% CSE mean ± SD, n=3
0	61.65 ± 14.89	80.83 ± 34.18	54.73 ± 3.76
15	108.17 ± 7.09	145.12 ± 31.79	107.89 ± 9.64
30	160.79 ± 2.41	211.93 ± 26.56	144.51 ± 22.60
45	196.69 ± 4.33	270.02 ± 35.14	184.21 ± 18.99
60	237.78 ± 10.94	307.46 ± 27.84	213.26 ± 27.90
90	290.61 ± 20.54	364.15 ± 27.53	253.95 ± 26.27

Table 10: efflux assay with primary cells. 10 µM sulforaphane and 5% CSE after long-term incubation

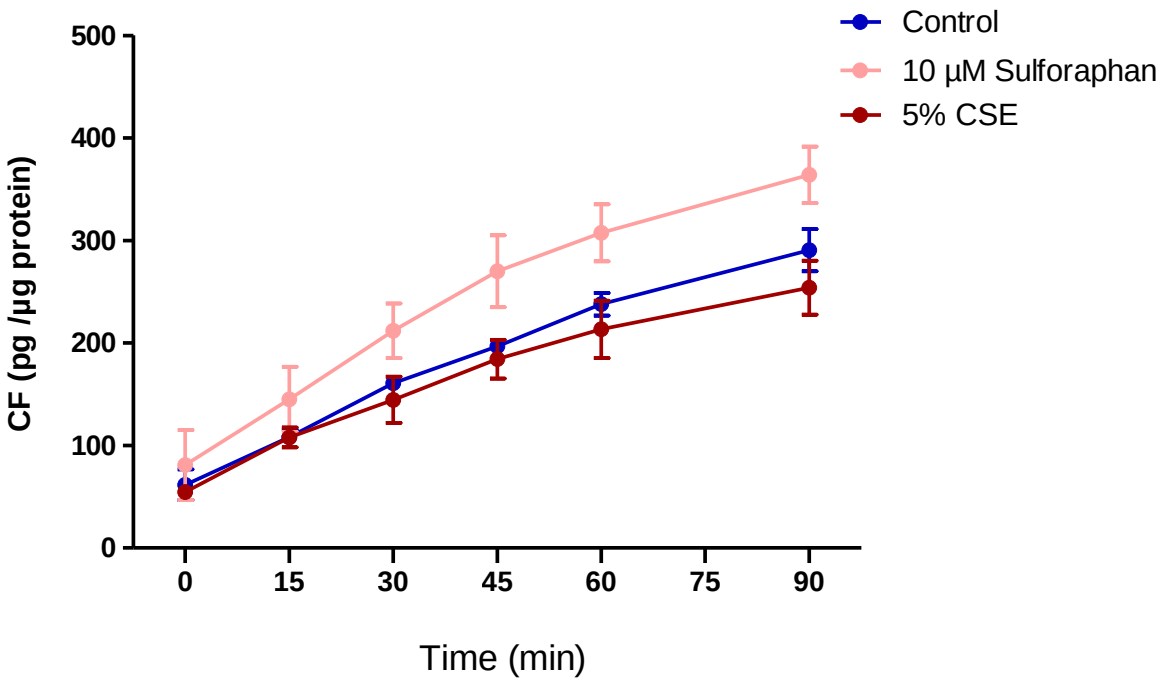


Figure 18: graph showing the results of the efflux experiment using primary cells treated with 10 µM sulforaphane and 5% CSE for 24 h.

5.5 Western Blot

In order to evaluate the influence of sulforaphane, quercetin and perphenazine on the expression of MRP1 in the lung epithelial cell line NCI-H441 Western Blots were carried out by Dr. Mohammed Ali Selo (Trinity College, Dublin).

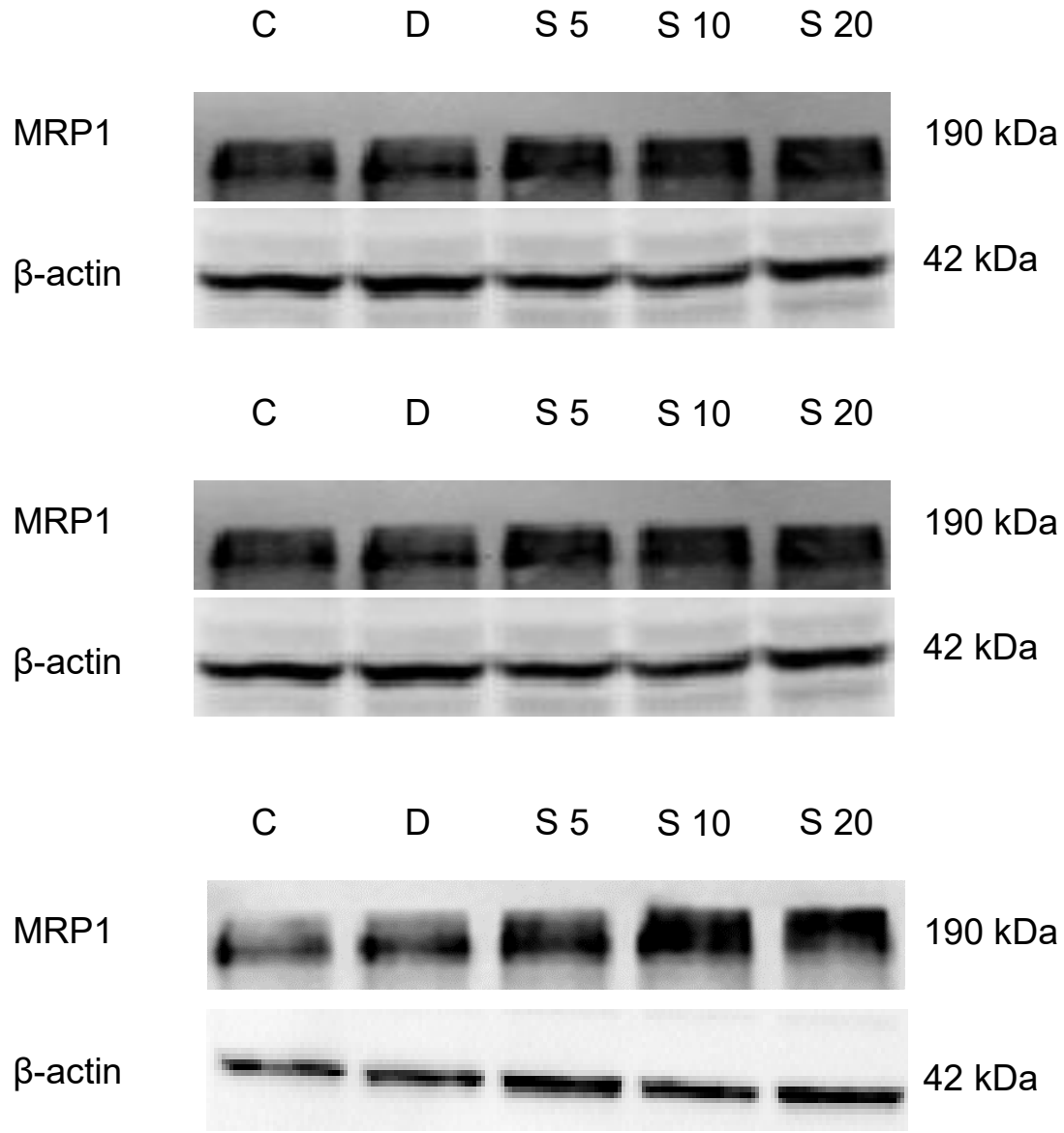
When incubated with the MRP1 modulators, the NCI-H441 immunoblots revealed a prominent band corresponding to the MRP1 protein with a molar mass of 190 kDa. The analysis was performed using protein isolations from cells incubated overnight with different concentrations of substances before lysing and extracting the cells. All passages showed a distinct clear staining and no other band was detected, which confirmed the selectivity of the MRP1 antibody and the absence of any possible contamination. The results were compared to a control group and beta-actin was used as a check value to normalize the results. The molecular weight markers allowed the clear assignment of the bands referring to beta-actin and MRP1 at 42 kDa and 190 kDa.

5.5.1 Sulforaphane 5 µM, 10 µM, 20 µM

The cells were incubated with the tested concentrations of substances before being extracted and running the western blots. As the dilutions of sulforaphane were prepared using DMSO, the dissolvent was also tested by itself to ensure no influence on the results. The samples were separated by a 12% polyacrylamide gel and transferred onto an immunoblot polyvinylidene fluoride membrane. The presence of a beta-actin-band proved the reliability of the results.

The cells treated with DMSO showed similar expression levels of MRP1 as the control group suggesting that preparing the desired concentrations of sulforaphane with DMSO has no effect on the transporter´s expression due to the dissolvent.

Whereas, the immunoblots confirmed a raised MRP1 abundance when incubating the cells with sulforaphane. Interestingly, higher concentrations did not lead to significantly increased expression levels even between 5 µM and 20 µM only a slight difference is noticeable, while the highest protein expression was observed with 10 µM, letting suggest that the effect is not concentration dependent.

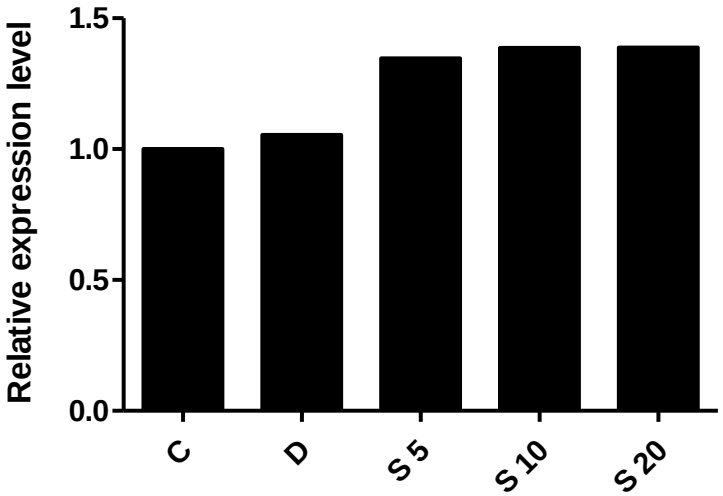


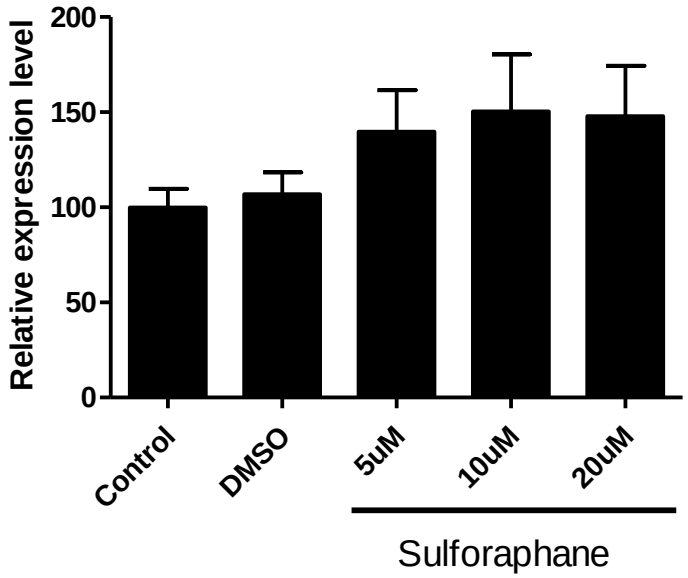
C, Control

D, DMSO

S, Sulforaphane (concentrations in µM)

Figures 19, 20, 21: Immunoblots showing the bands referring to MRP1 at 190 kDa and beta-actin at 42 kDa testing DMSO, 5 µM, 10 µM and 20 µM sulforaphane compared to a control group.



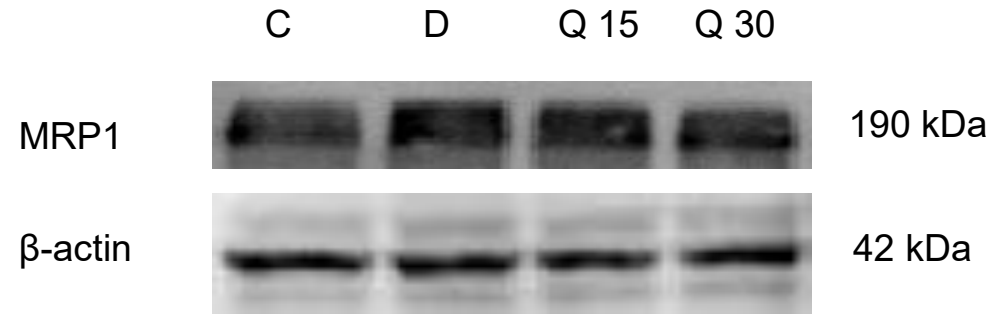


Figures 22, 23: the results from the western blots presented as bar graphs showed slight increases in the relative expression levels of MRP1 caused by sulforaphane when compared to the bars from DMSO and the control group

5.5.2 Quercetin 15 µM, 30 µM

To assess whether quercetin can lower the MRP1 mediated efflux due to decreased MRP1 expression levels cells were cultured in the presence or absence of quercetin and studied by Western blotting. As the required concentrations were prepared using DMSO, in the following experiment the dissolvent was also added to a separate group of cells when incubating them with 15 µm and 30 µM quercetin. The control group was only treated with feeding medium and the western blot was run after lysing and extracting the cells.

Again DMSO led to an insignificant increase in MRP1 levels as seen in the previous experiment. Surprisingly, quercetin did not result in any change in MRP1 abundance and caused even lower results as examined in the bar graph than DMSO.



C, CONTROL

D, DMSO

Q, QUERCETIN (concentrations in µM)

Figure 24: Immunoblot showing the bands referring to MRP1 at 190 kDa and beta-actin at 42 kDa testing DMSO, 15 µM and 30 µM quercetin compared to a control group.

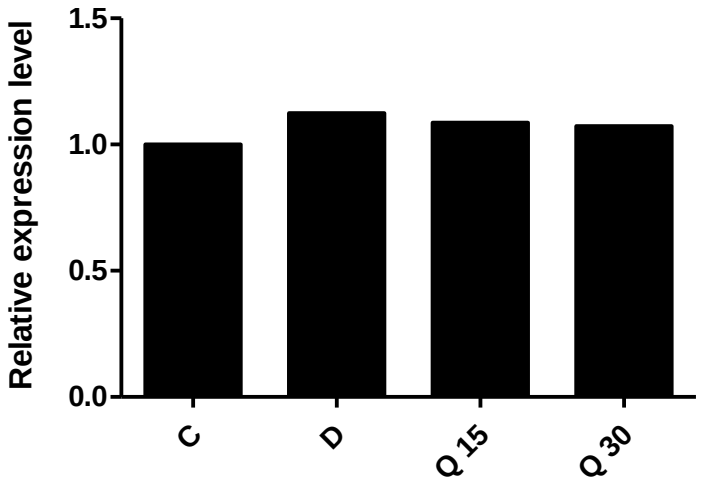
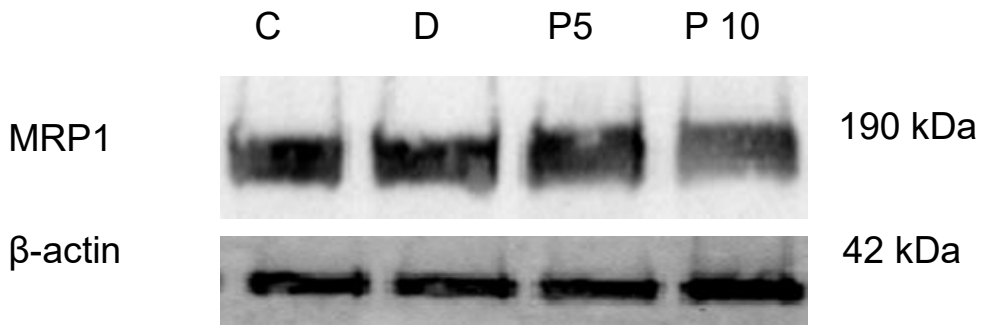


Figure 25: bar graph presenting the results from the western blot showing a slight increase in the MRP1 expression levels due to DMSO. Whereas, the bars representing quercetin did not show higher results compared to the control group.

5.5.3 Perphenazine 5 µM, 10 µM

An additional Western blot was run testing 5 µM and 10 µM perphenazine in comparison to DMSO and only feeding medium. While 5 µM of the substance slightly increased the expression levels of MRP1, 10 µM led to basically the same results as seen in case of using DMSO. The raise in protein abundance caused by perphenazine was not significant enough to confirm an effect of the phenothiazine on the transporter.



C, CONTROL

D, DMSO

P, Perphenazine

Figure 26: Immunoblot showing the bands referring to MRP1 at 190 kDa and beta-actin at 42 kDa testing DMSO, 5 µM and 10 µM perphenazine compared to a control group.

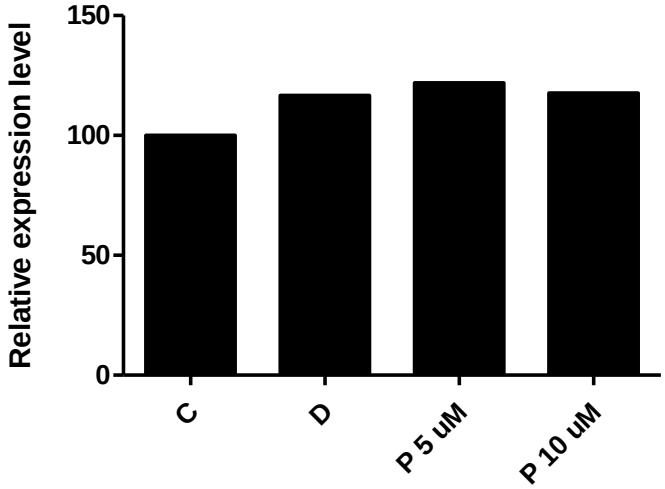


Figure 27: Bar graph comparing the results from the Western blot with 5 µM, 10 µM perphenazine, DMSO and the control group showing insignificant increases of expression levels due to perphenazine and the dissolvent.

6. Discussion

The present study evaluates the effect of CSE and other substances on MRP1, an efflux pump in plasma membranes and subcellular compartments like the Golgi apparatus, lysosomes and the nucleus primarily expressed in lung, kidney, placenta, testes and bladder (Jiye et al. 2011).

MRP1 contributes to multidrug resistance due to its ability to efflux drugs such as chemotherapeutics, antiandrogens or HIV-therapeutics. As a result, in clinical oncology MRP1 has owed a firmly established role as it effluxes xenobiotics and thus confers resistance to a wide array of chemotherapeutic agents (Cole et al. 2014).

Furthermore, the transporter serves a protective role in cells by extruding toxins and harmful substances outside the cells and could therefore contribute to a reduced risk of developing COPD and other diseases.

In 2007, Van der Deen et al. published a study evaluating the cytotoxicity of CSE in the human bronchial epithelial cell line 16HBE14o− and the analysis of the cellular retention of carboxyfluorescein combined with an MRP1 inhibitor was used to study the effect of CSE on the MRP1 function (Van der Deen et al. 2007).
This hypothesis was proven in this present study by performing efflux and uptake assays both after short- and long-term incubation over 24 hours with 5 and 10% CSE. While the short-term incubation did not lead to any noticeable changes in MRP1 activity patterns, CSE exposure over 24 hours has been shown to result in a significant reduction of CF efflux in a concentration dependant manner.

Next, the effect of storing CSE in the freezer for more than 2 weeks on the cytotoxic properties on the NCI-H441 cells was studied by carrying out efflux assays with 10% aged compared to 10% fresh CSE after 24 hours of incubation. An increase in CF retention was revealed in the cells treated with fresh CSE, whereas the incubation with 10% aged CSE led to higher levels of MRP1 function, which could be due to the decreased cytotoxic effects of the noxious compounds in CSE that affect the transporter´s ability to efficiently eliminate harmful substances.

Efflux and uptake assays were conducted with substances that have previously shown to influence MRP1 function either leading to increased or decreased activity. The first substance to be tested was sulforaphane, an isothiocyanate that has recently owed a role in cancer treatment due to pleiotropic effects such as the modulation of cellular homeostasis through the activation of the transcription factor Nrf2 (Russo et al. 2018).

Prior to the efflux study with sulforaphane a cytotoxicity assay was carried out in order to examine the highest concentration possible of this potentially cytotoxic substance that could be tested without harming the cells.
First, 5 µM and 10 µM were tested after 24-hours incubation, which, unfortunately did not lead to the desired results: While 5 µM sulforaphane only caused a marginal rise in MRP1 activity after 60 minutes, 10 µM led to decreased CF efflux when compared to the control group.
These insignificant results could be due the insufficient concentrations of sulforaphane and therefore higher concentrations were tested in the following experiment.
50 µM and 100 µM sulforaphane led to serious cell death and therefore significantly lower CF efflux, which confirmed the findings of the cytotoxicity assay suggesting that high concentrations of this potent cytotoxic substance would kill the majority of cells.

As 5 µM led to slightly increased CF efflux after 24-hours incubation the experiment was repeated using 5 µM and 7.5 µM sulforaphane but this time after a 72-hours incubation time. The long incubation time revealed an increased retention of CF even with 5 µM, which could be due to major cell apoptosis after the exposition to sulforaphane for a longer period of time. In this case, the incubation time of 72 h could have enhanced the cytotoxic effects and led to an incapability of the surviving cells to provide sufficient CF efflux by MRP1.
Apart from that, sulforaphane enhances MRP1 activity due to the Nrf2 pathway and as cancer cells have shown to often feature mutations and therefore a hyperactivation of this pathway sulforaphane could not additionally induce MRP1 as Nrf2 was already highly active in the cancer cells before.

In the following experiment, perphenazine a widely used member of the phenothiazine group and quercetin a prominent flavonoid, were both tested in concentrations of 25 µM, which yielded both surprising and already predicted results: Quercetin which owes a precious role in chemotherapy due to its ability to lower multidrug resistance, proofed its MRP1 inhibiting capacity represented by increased CF retention.

Whereas, phenothiazines have been presented as potential MRP1 inducers, however perphenazine also inhibited the CF efflux leading to lower CF levels than the control group. Any MRP1 activating properties performed by phenothiazines could therefore be closely linked to their chemical structure which strongly varies within this group playing a crucial role in substrate specificity (Wesolowska et al. 2009) Phenothiazines were among the first recognized modifiers of multidrug resistance proteins and Wesolowska et al. suggested in 2009 a stimulatory effect on MRP1 exerted by different compounds such as perphenazine, triethylperazine and chlorpromazine. However, as the drugs caused haemolysis at different concentrations the highest concentration tested in the mentioned experiment was 15 µM and the efflux assay was run using the carboxyfluorescein derivative BCPCF or BCECF as the substrate to monitor MRP1´s transport activity out of human erythrocytes.
The inhibitory effect exerted by perphenazine shown in this experiment could therefore be due to increased cell apoptosis caused by the high concentration of 25 µM. Furthermore, Wesolowska´s study used a different cell type as well as another fluorescent probe and as the MRP1 stimulatory effect of phenothiazines is highly substrate dependent, perphenazine could have inhibitory properties when used in lung epithelial cells derived from a tumour leading to lower rates of CF efflux.

In a last attempt trying to showcase MRP1 stimulation by sulforaphane, 10 µM of the isothiocyanate was tested in comparison to 5% CSE but this time using primary cells directly derived from human non-tumorous tissue after a 24 hours incubation, which surprisingly led to significant results: While 5% CSE lowered the transporter´s activity, 10 µM sulforaphane noticeably rose the CF efflux compared to the control group.
The stimulatory effect on the efflux pump of sulforaphane in primary cells confirmed the initial thesis that the (Keap1)-nuclear factor E2-related factor 2 (Nrf2) signalling cascade is often mutated and dysregulated in cancer cells resulting in constitutively active Nrf2 and an

increased expression of cytoprotective Nrf2 target genes. As in primary cells the pathway is active at normal rates the incubation with sulforaphane could efficiently activate Nrf2 and MRP1 leading to increased CF efflux compared to the control cells as those did not already boast an enhanced signalling cascade.
These results confirmed the ability of sulforaphane to activate the MRP1 transporter through the Nrf2 pathway.

As efflux assays only allow the investigation of the function of MRP1, Western blotting was additionally used again on NCI-H441 cells to examine the expression levels through the immunodetection of proteins followed by gel electrophoresis (Kurien et al. 2017).
The first Western blot was carried out using 5 µM, 10 µM and 20 µM sulforaphane and revealed an increase in MRP1 abundance in the cells treated with the isothiocyanate compared to those incubated with only feeding medium or DMSO. Even though the experiment was conducted using cancer cells which likely boast a hyperactivation of Nrf2, sulforaphane could raise the expression levels of the transport protein. This result suggests that the Nrf2 pathway merely affects the transporters ability to efficiently efflux substances but not its expression levels. Sulforaphane could therefore boost MRP1 expression levels without leading to increased CF efflux as shown in the efflux assays.

In addition, a Western blot was run with 15 µM and 30 µM quercetin, which has shown an inhibitory effect on the MRP1 mediated CF efflux. Interestingly, in the Western blot analysis of the flavonoid has not showcased any impact on the transporter´s expression, which suggests that quercetin only inhibits the function but not the total intracellular abundance of the transporter.
Finally, a Western blot was conducted with 5 µM and 10 µM perphenazine, which again did not result in any significant increase of MRP1 abundance, affirming that perphenazine´s stimulatory effects might only refer to specific substrates and cells and therefore requires further investigation.

7. Conclusions

First, the outcome of a study conducted by Van der Deen *et al.* in 2007 suggesting a dose dependent cellular CF retention performed by CSE had to be proven: The CSE-triggered decreased function of MRP1 could potentiate the negative effects caused by cigarette smoking due to the reduced efficiency in cell detoxification and therefore substances including sulforaphane, perphenazine and quercetin were tested on the NCI-H441 cell line and on primary cells to showcase any effects on the transporter´s activity.

In addition to efflux studies, Western blotting was used to determine whether the suggested MRP1 modulators do not only affect the transporter´s activity and detoxifying potential but also its expression in cells. An increased CF efflux could either happen due to an increased activity or a higher expression of the transport protein in the cell membrane, which could only be evaluated by conducting a western blot analysis.

Sulforaphane only stimulated MRP1´s function in the primary cells as there the involved pathway could be activated efficiently and slightly increased the expression levels of the protein. Whereas, perphenazine did neither raise the transporter´s activity or expression levels. Furthermore, quercetin proved its ability to inhibit the transporter´s function but didn´t impact the total protein abundance.

In the future, MRP1 activators could play a crucial role in fighting illnesses and inflammation provoked by toxic compounds leading to oxidative stress and should therefore be the subject of studies trying to optimise the treatment of diseases like COPD.

8. References

Alsaeedi A, Sin DD, McAlister FA (2002) The effects of inhaled corticosteroids in chronic obstructive pulmonary disease: a systematic review of randomized placebo-controlled trials. Am J Med, 113:59–65.

Anthonisen NR, Connett JE, Kiley JP (1994) Effects of smoking intervention and the use of an inhaled anticholinergic bronchodilator on the rate of decline of FEV1. The Lung Health Study. JAMA, 272:1497–505.

Bai Y, Wang X, Zhao S, Ma C, Cui J, Zheng Y (2015) Sulforaphane Protects against Cardiovascular Disease via Nrf2 Activation. Oxid Med Cell Longev. doi: 10.1155/2015/407580. Epub 2015 Oct 25.

Bosquillon C (2010) Drug transporters in the lung- do they play a role in the biopharmaceutics of inhaled drugs? J Pharm Sci, v. 99, p. 2240-55.

Boukhenouna S, Wilson MA, Bahmed K, Kosmider B (2018) Reactive oxygen species in chronic obstructive pulmonary disease. doi: 10.1155/2018/5730395.

Briones-Herrera A, Eugenio-Pérez D, Reyes-Ocampo JG, Rivera-Mancía S, Pedraza-Chaverri J (2018) New highlights on the health-improving effects of sulforaphane. Food Funct. doi: 10.1039/c8fo00018b.

Brito AF, Ribeiro M, Abrantes AM1, Pires AS, Teixo RJ, Tralhão JG, Botelho MF (2015) Quercetin in Cancer Treatment, Alone or in Combination with Conventional Therapeutics? Curr Med Chem.;22(26):3025-39.

Budulac SE, Postma DS, Hiemstra PS, Lapperre PS, Kunz IZ, Vonk M, Timens W (2012) Multidrug resistance-associated protein 1 and lung function decline with or without long-term corticosteroids treatment in COPD. Eur J Pharmacol, v. 696, p. 136-42.

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

Calverley PM, Boonsawat W, Cseke Z, Zhong N, Peterson S, Olsson H (2003) Maintenance therapy with budesonide and formoterol in chronic obstructive pulmonary disease. Eur Respir J, 22:912–9.

Choi YH, Yu AM (2014) ABC Transporters in multidrug resistance and pharmacokinetics, and strategies for drug development. Curr Pharm Des.;20(5):793-807.

Choudhuri S, Klaassen CD (2006) Structure, function, expression, genomic organization, and single nucleotide polymorphisms of human ABCB1(MDR1), ABCC (MRP), and ABCG2 (BCRP) efflux transporters. Int J Toxicol. ;25(4):231-59.

Cole SP, Bhardwaj G, Gerlach JH, Mackie JE, Grant CE, Almquist KC, Stewart AJ, Kurz EU, Duncan AM, Deeley RG (1992) Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. Science, v. 258, p. 1650-4.

Cole SP (2014) Multidrug resistance protein 1 (MRP1, ABCC1), a "multitasking" ATP-binding cassette (ABC) transporter. The Journal of Biological Chemistry.;289(45):30880-8.

Cole SP (2013) Targeting Multidrug Resistance Protein 1 (MRP1, ABCC1): Past, Present, and Future. Annu Rev Pharmacol Toxicol. pp 95-117

Dean M (2005) The genetics of ATP-binding cassette transporters. Methods Enzymol. 2005;400:409-29.

Gon Y, Hashimoto S (2018) Role of airway epithelial barrier dysfunction in pathogenesis of asthma. Allergology International Volume 67, Issue 1, Pages 12-17

Gustavsson L, Bosquillon C, Gumbleton M (2016) Drug transporters in the lung: Expression and potential impact on pulmonary drug disposition; Available from: researchgate.net/publication/308372073;

Harris KE, Jeffery EH (2008) Sulforaphane and Erucin increase MRP1 and MRP2 in human carcinoma cell lines. Volume 19, Issue 4, Pages 246-254

Hartsock A, Nelson WJ (2009) Adherens and Tight Junctions: Structure, Function and Connections to the Actin Cytoskeleton. Biochim Biophys Acta. Author manuscript; available in PMC Biochim Biophys Acta. 2008 Mar; 1778(3): 660–669.

He L, Vasiliou K, Nebert DW (2009) Analysis and update of the human solute carrier (SLC) gene superfamily. Hum Genomics, v. 3, p. 195-206.

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

InvivoChem (2018) Perphenazine. Available from: https://www.invivochem.com/perphenazine/

Jaramillo MC, Zhang DD (2013) The emerging role of the Nrf2-Keap1 signalling pathway in cancer. Genes Dev. 2013 Oct 15;27

Kansanen E, Kuosmanen SM, Leinonen H, Levonen AL (2012) The Keap1-Nrf2 pathway: Mechanisms of activation and dysregulation in cancer. Department of Biotechnology and Molecular Medicine, A. I. Virtanen Institute for Molecular Sciences, University of Eastern Finland; doi: 10.1016/j.redox.2012.10.001

Keppler D (2011) Multidrug Resistance Proteins (MRPs, ABCCs): Importance for pathophysiology and drug therapy, in M. F. Fromm, and R. B. Kim, eds., Drug Transporters, v. Handbook of Experimental Pharmacology 201 2011, Springer.

Khan. Passive Transport and Active Transport. Available from: https://biologywise.com/passive-transport-active-transport

Kleszczyński K, Ernst IM, Wagner AE, Kruse N, Zillikens D, Rimbach G, Fischer TW (2013) Sulforaphane and phenylethyl isothiocyanate protect human skin against UVR-induced oxidative stress and apoptosis: role of Nrf2-dependent gene expression and antioxidant enzymes. Pharmacol Res. 2013 Dec;78:28-40.

Krajka-Kuźniak V, Paluszczak J, Baer-Dubowska W (2017) The Nrf2-ARE signalling pathway: An update on its regulation and possible role in cancer prevention and treatment. Pharmacol Rep. 2017 Jun;69(3):393-402.

Kurien BT, Dorri Y, Dillon S, Dsouza A, Scofield RH (2011) An overview of Western blotting for determining antibody specificities for immunohistochemistry. Methods Mol Biol. 2011; 717:55-67

Lehmann T, Köhler C, Taege C, Weidauer E (2001) Expression of MRP1 and related transporters in human lung cells in culture; Toxicology 167; 59–72

Lei CS, Hou YC, Pai MH, Lin MT, Yeh SL (2018) Effects of quercetin combined with anticancer drugs on metastasis-associated factors of gastric cancer cells: in vitro and in vivo studies. J Nutr Biochem. 51:105-113

Leinonen HM, Kansanen E, Pölönen P, Heinäniemi M, Levonen AL (2015) Dysregulation of the Keap1-Nrf2 pathway in cancer. Biochem Soc Trans. 2015 Aug;43(4):645-9.

Leslie EM, Deeley RG, Cole SP (2001 a) Toxicological relevance of the multidrug resistance protein 1, MRP1 (ABCC1) and related transporters. Toxicology, 167:3–23.

Leslie EM, Deeley RG, Cole SP, Hechti SS (2001b) Transport of the β-O-glucuronide conjugate of the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) by the multidrug resistance protein 1 (MRP1): requirement for glutathione or a non-sulfur-containing analog. J Biol Chem 276: pp 27846–27854.

Leslie EM, Deeley RG, Cole SP (2005) Multidrug resistance proteins: role of p-glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defence. Toxicol Appl Pharmacol. 1;204(3):216-37.

Look for Diagnosis. Quercetine. Available from: https://www.lookfordiagnosis.com/mesh_info.php?term=Quercetin&lang=6

Lubelska K, Wiktorska K, Mielczarek L, Milczarek M, Zbroińska-Bregisz I, Chilmonczyk Z (2016) Sulforaphane Regulates NFE2L2/Nrf2-Dependent Xenobiotic Metabolism Phase II and Phase III Enzymes Differently in Human Colorectal Cancer and Untransformed Epithelial Colon Cells. Nutr Cancer. 2016 Nov-Dec;68(8):1338-1348

Ma Q (2015) Role of Nrf2 in Oxidative Stress and Toxicity. Annu Rev Pharmacol Toxicol. 2013; 53: 401–426. doi: 10.1146/annurev-pharmtox-011112-140320

Michalak K, Wesolowska O, Motohashi N and Hendrich AB (2007) The role of the membrane actions of phenothiazines and flavonoids as functional modulators. Top Heterocyclic Chem (Bioactive Heterocycles II) 8: 223-302

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

Müller-Esterl W, Brandt U, Anderka O, Kerscher S, Kieß S, Ridinger K (2018) Biochemie- Eine Einführung für Mediziner und Naturwissenschaftler

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

National Center for Chronic Disease Prevention and Health Promotion, Division of Population Health (Page last reviewed: 2018). Chronic Obstructive Pulmonary Disease (COPD) Available from: https://www.cdc.gov/copd/index.html

National Clinical Guideline Centre (UK) (2010) Chronic Obstructive Pulmonary Disease: Management of Chronic Obstructive Pulmonary Disease in Adults in Primary and Secondary Care. London: Royal College of Physicians (UK); (NICE Clinical Guidelines, No. 101.) 1, Introduction. Available from: https://www.ncbi.nlm.nih.gov/books/NBK65043/

National Heart, Lung and Blood Institute (last reviewed 2019) The respiratory system. Available from: https://www.nhlbi.nih.gov/health-topics/how-lungs-work

Nickel S, Selo MA, Fallack J, Clerkin CG, Huwer H, Schneider-Daum N, Lehr CM, Ehrhardt C (2017) Expression and Activity of Breast Cancer Resistance Protein (BCRP/ABCG2) in Human Distal Lung Epithelial Cells In Vitro. Published online: 3 May 2017 Springer Science+Business Media New York 2017

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

Pajak B, Molnar J, Engi H, Orzechowski A (2005) Preliminary Studies on Phenothiazine-mediated Reversal of Multidrug Resistance in Mouse Lymphoma and COLO 320 Cells. Available from: https://www.researchgate.net/publication/7491329

Patton JS, Byron PR (2007) Inhaling medicines: delivering drugs to the body through the lungs. Nat Rev Drug Discov, v. 6, p. 67-74.

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

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

Russo M, Spagnuolo C, Russo GL, Skalicka-Woźniak K, Daglia M, Sobarzo-Sánchez E, Nabavi SF, Nabavi SM (2018) Nrf2 targeting by sulforaphane: A potential therapy for cancer treatment. Crit Rev Food Sci Nutr.: 1391-1405.

Singh DP, Kaur G, Bagam P, Pinkston R, Batra S (2018) Membrane microdomains regulate NLRP10- and NLRP12- dependent signalling in A549 cells challenged with cigratte smoke extract. Arch Toxicol. May 2018, Volume 92, Issue 5, pp 1767–1783

Smith TT, Hatsukami DK, Benowitz NL (2008) Whether push or pull? Nicotine reduction and non-combusted alternatives- two strategies for reducing cigarette smoking and improving public health. S0091-7435(18)30110-5.

Sutherland ER, Allmers H, Ayas NT (2003) Inhaled corticosteroids reduce the progression of airflow limitation in chronic obstructive pulmonary disease: a meta-analysis. Thorax, 58:937–41.

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

Van der Deen M, Homan S, Timmer-Bossau H, Scheper RJ, Timens W, Postma DS, De Vries EG (2008) Effect of COPD treatments on MRP1-mediated transport in bronchial epithelial cells; International Journal of COPD 2008:3(3) 469–475

Van der Deen M, Marks H, Willemse BW, Postma DS, Müller M, Smit EF, Scheper RJ, De Vries EG, Timens W (2006) Diminished expression of multidrug resistance- associated protein 1 (MRP1) in bronchial epithelium of COPD patients. Virchows Arch, v. 449

Van Zanden JJ, Van der Woude H, Vaessen J, Usta M, Wortelboer HM, Cnubben NH, Rietjens IM (2007) The effect of quercetin phase II metabolism on its MRP1 and MRP2 inhibiting potential. Biochem Pharmacol. 2007 Jul 15;74(2):345-51

Van Zanden JJ, Wortelboer HM, Bijlsma S, Punt A, Usta M, Bladeren PJ, Rietjens IM, Cnubben NH (2005) Quantitative structure activity relationship studies on the flavonoid mediated inhibition of multidrug resistance proteins 1 and 2. Biochem Pharmacol. 2005 Feb 15;69(4): p 699-708.

Vasiliou V, Vasiliou K, Nebert DW (2009) Human ATP-binding cassette (ABC) Transporter family. Hum Genomics. 2009 Apr;3(3):281-90.

Wang D, Wang C, Cao Y, Zhang X, Tao X, Yang L, Chen J, Wang S, Li Z (2013) Allyl Isothiocyanate Increases MRP1 Function and Expression in a Human Bronchial Epithelial Cell Line. Oxid Med Cell Longev. Published online 2014 Jan 14. doi: 10.1155/2014/547379

Wang S, Wang S, Wang C, Chen Y (2015) Upregulation of Multidrug Resistance-Associated Protein 1 by Allyl Isothiocyanate in Human Bronchial Epithelial Cell: Involvement of c-Jun N-Terminal Kinase Signalling Pathway; Hindawi Publishing Corporation Oxidative Medicine and Cellular Longevity Volume 2015, Article ID 903782, 8 pages

Wang Q, Beck WT (1998) Transcriptional suppression of multidrug resistance-associated protein (MRP) gene expression by wild-type p53: Cancer Res, v. 58, p. 5762-9.

Wesołowska O (2011) Interaction of phenothiazines, stilbenes and flavonoids with multidrug resistance-associated transporters, P-glycoprotein and MRP1. Acta Biochim Pol. 2011;58(4):433-48.

Wesolowska O, Ocsovszki I, Molnar J, Michalak K (2009) Differential Effect of Phenothiazines on MRP1 and P-Glycoprotein Activity. in vivo 23: 943-948

Wikipedia. Passive Transport. Available from: https://en.wikipedia.org/wiki/Passive_transport

Wikipedia Phenothiazine. Available from: https://en.wikipedia.org/wiki/Phenothiazine

Wikipedia. Sulforaphane. Available from: https://en.wikipedia.org/wiki/Sulforaphane#/media/File:Sulforaphane.png

World Health Organisation (2008) COPD predicted to be third leading cause of death in 2030. Available from: www.who.int/respiratory/copd/World_Health_Statistics_2008/en/

Zhou SF, Wang LL, Di YM, Xue CC (2008) Substrates and inhibitors of human multidrug resistance associated proteins and the implications in drug development. Curr Med Chem. 2008;15(20):1981-2039.