universität wien

DIPLOMARBEIT / DIPLOMA THESIS

Titel der Diplomarbeit / Title of the Diploma Thesis

„The influence of NAC on the gene expression of intestinal epithelial cells (Caco-2)“

verfasst von / submitted by

Larissa Koidl

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

Magistra der Pharmazie (Mag.pharm.)

Wien, 2019 / Vienna, 2019

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	A 449
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Diplomstudium Pharmazie
Betreut von / Supervisor:	Assoz.-Prof. DDr. Eva Untersmayr-Elsenhuber

Acknowledgments

First, I want to say thank you to Prof. Franz Gabor (University of Vienna, Department of Pharmaceutical Technology and Biopharmaceutics) for introducing me to my supervisor Prof. Eva Untersmayr-Elsenhuber and thus being the enabler of this work. Without him I would have never found my way to the Institute for Pathophysiology and Allergy Research at the Medical University of Vienna and would not have been able to make the great experiences while doing the practical work for my thesis.

A very big thank you also goes to Prof. Enikö Kállay and her working group, especially to Teresa Manhardt, Taha Elajnaf, Luca Iamartino, Martin Schepelmann and to Prof. Kállay from whom I received tremendous help during the experimental part.

I also want to thank Dr. Diana Mechtcheriakova and Anastasia Meshcheryakova for giving me practical tips in handling the qPCR.

Thank you also to Miroslawa Matz, Martina Klems and Anne-Sofie Ravn Ballegaard for helping with general lab work and cell culture work.

Last but not least, I want to thank Prof. Eva Untersmayr-Elsenhuber, my incredible supervisor, for guiding me through my thesis, for the great support and for the wonderful and invaluable experience I was able to make and I greatly appreciate all the time and support I received.

Table of contents

Abbreviations

DEPC: Diethylpyrocarbonate

DMEM: Dulbecco's modified eagle medium

DMSO: Dimethylsulfoxid

FAE: follicle-associated epithelia

FCS: Fetal calf serum

GALT: Gut associated lymphatic tissue

HEPES: 4-(2-Hydroxyethyl)-1-piperazinethanesulfonic acid)

IV: intravenous

JAM: junctional adhesion molecules

NAC: N-Acetylcystein

OTC: Over the counter

ZO: Zonula occludens

TBE: Tris/Borate/EDTA

PBS: Phosphate buffered saline

qPCR: Quantitative polymerase chain reaction

Abstract

N-acetylcysteine (NAC), a very common over-the-counter available mucolytic drug, is often used to treat symptoms of frequent respiratory diseases like common cold. It lessens the viscosity of mucus by cleaving disulfide bonds between MUC proteins. But mucus does not only exist in the airways but also in the gastrointestinal system.

In a previous diploma thesis the *in vivo* effects of NAC (and Ambroxol) on mice have been investigated to determine the effect of mucolytic drugs on the intestinal mucus and the immune response. It was shown that NAC does effect the mucus composition and induces an immune response.

The aim of this diploma thesis was to *in vitro* investigate the effect of NAC on enterocytes directly, whether the stimulation of Caco-2 cells (as a model for the small intestine epithelial layer) with NAC leads to an altered expression of genes associated with intestinal barrier function and immune response. The potential up- or down-regulation of certain genes was measured by determining mRNA levels.

Abstract (deutsch)

N-acetylcysteine (NAC), ein weit verbreiteter, ohne Rezept verfügbarer mukolytischer Arzneistoff, wird oft zur Behandlung der Symptome von häufigen respiratorischen Erkrankungen, wie z.B. bei Erkältung, angewendet. NAC reduziert die Viskosität des Schleimes, in dem Disulfid-Brücken zwischen MUC-Proteinen spalten werden. Schleim kommt aber nicht nur in den Atemwegen vor, sondern auch im Gastrointestinaltrakt.

In einer vorhergehenden Diplomarbeit wurden die *in vivo* Effekte von NAC (und Ambroxol) auf Mäuse untersucht, um die Wirkung von Mukolytika auf den Darmschleim und die Immunantwort zu studieren. Es wurde gezeigt, dass NAC die Schleimzusammensetzung beeinflusst und eine Immunantwort induziert.

Ziel der vorliegenden Diplomarbeit war es, *in vitro* die Wirkung von NAC auf die Enterozyten direkt zu studieren, in dem untersucht wurde, ob die Stimulation von Caco-2 Zellen (als Modell für die Dünndarmepithelschicht) mit NAC zu einer veränderten Expression von Genen führt, die im Zusammenhang mit der intestinalen Barrierefunktion und Immunantwort stehen. Potenzielle Up- und Down-Regulierungen bestimmter Gene wurden über die exprimierte mRNA gemessen.

1 Introduction

The intestinal barrier is the biggest surface protecting our body towards the external environment. Its main function is to perform seemingly contradictory tasks: to allow the passage of important nutrients that are critical for maintaining life while disposing waste into the lumen and denying access to undesirable luminal content such as pathogens, toxins and antigens. A central hub for all these activities is the epithelial layer. The intestinal epithelial surface consists of a single layer of mostly nutrient absorbing enterocytes with mucus-producing goblet cells (and some other cells) strewn in between. [1, 2]

Every compound ingested orally will come in direct contact with the gastrointestinal barrier, interact with it and might potentially influence it. This is the case for all kinds of orally administered medications. Nearly every medication has gastrointestinal effects (i.e. diarrhea, obstipation, nausea, others) listed as side effects and thus, it is evident that medications influence the gastrointestinal tract and its function in some way, even though most times the target is not the GI tract.

Due to their over-the-counter availability, mucolytic medication can be easily bought even without a medical diagnosis or prescription. They are frequently used to treat symptoms of frequently occurring respiratory diseases like the common cold. While the effect of mucolytic drugs on the respiratory tract is well documented, their effect on the mucus layer of the intestinal barrier, and as a consequence on the local as well as systemic immune response, has yet to be determined.

N-acetylcysteine (NAC), a very common mucolytic drug, is metabolized into its active form cysteine which lowers the viscosity of bronchial mucus by cleaving the disulfide bonds. [3] To achieve the mucolytic effect of NAC in the airways, the compound has to be absorbed systemically and transported to its target via circulation. This means that NAC does not work locally through direct contact with the airway mucus but systemically. NAC affects not only the mucus in the airways, but might also affect gastrointestinal mucus. The mucus layer is a crucial part of the gastrointestinal barrier Mucus proteins for large molecules being assembled by disulfide bonds as well as Ca2+-mediated links, hydrogen bonds and non-mucin proteins. [6, 7] Thus, NAC should be able to cleave the bonds of the

gastrointestinal mucus proteins as well (and consequently lower its viscosity), however, the effect of NAC on gastrointestinal mucus in not well researched.

Of interest, side effects induced by NAC are rare but include gastrointestinal disorders, reflux disease, and allergic reactions, amongst others. [3]

In a previous diploma thesis (from our working group) the *in vivo* effects of NAC (and Ambroxol) on mice were investigated to determine the effect of mucolytic drugs on the intestinal mucus and the immune response. The NAC-treated-group showed heightened IFNγ-levels while IgA-levels were lower compared to the untreated control groups. Moreover, less free sulfhydryl groups per protein were found in the NAC-treated group, indicating a reduced viscosity of the harvested intestinal mucus due to NAC binding to the free sulfhydryl groups. [4]

The results suggested that mucolytic drugs have an influence on the mucus layer and consequently on the intesinal barrier function and the immune response.

2 Background and aim

2.1 Intestinal barrier

Several mechanisms contribute to the intestinal barrier. The most obvious is the mechanical/physical barrier, consisting of a single epithelial cell layer (*Lamina epithelialis mucosae*) and the mucus layer on top.

The majority of the intestinal epithelial cells are enterocytes. The enterocytes form microvilli on the apical side, which play an important role in intestinal surface enlargement. Adjacent cells in the epithelial layer are sealed by tight junctions. Tight junctions are protein complexes that seal – together with the adherens junctions - the paracellular pathway between adjacent epithelial cells. They are located on the apical side of the cells. The tight junction proteins include transmembrane proteins, such as the claudin family and occludin, and cytosolic proteins like zonula occludens. [2, 5]

Scattered between the enterocytes, goblet cells produce large glycoproteins called mucins. In the intestines, the gel-forming MUC2 mucin plays a particularly prominent role. The mucins assemble to form large polymer networks via inter- and intramolecular crosslinking, amongst others, through disulfide bonds between cysteines. This mucus layer acts as an additional physical/chemical barrier by protecting the intestinal epithelium from direct contact with luminal content and bacteria. Intestinal diseases like infections, inflammatory bowel diseases, Crohn's disease, and cystic fibrosis were shown to be associated with a malfunction of the intestinal barrier, in part via a changed mucus composition, showing that functional intestinal mucus is essential for the maintenance of health. [2, 6, 7]

Research in mice has shown that the lack of the special intestinal enzyme protein disulfide isomerase (PDI) results in the inability to produce intestinal mucin and is therefore associated with a higher susceptibility to colitis. The formation of a tight network by disulfide bonds is crucial for the stabilization of the mucus layer and consequently its physiological function within the intestinal barrier. [8]

In addition to enterocytes and goblet cells, several other cell types participate in the formation of the epithelial layer. These include: various endocrine cells; Paneth-cells, which multiple functions include secretion of defensins and

lysozyme (antimicrobial enzymes for chemical defense) and are located in the small intestinal crypts; and microfold cells (M-cells), which are part of the GALT (gut associated lymphatic tissue). M-cells are located in regions called the follicle-associate epithelia (FAE) and they have the ability to take up antigens. The GALT is a largely autonomous immune system of the gut and comprises Peyer's plaques and scattered immune cells, such as dendritic cells, T-cells, monocytes, and mast cells and soluble IgA antibodies that form the immune barrier. [1, 5, 40, 41, 42]

Tuft cells (or brush cells) are another type of intestinal epithelial cell. They are recognized by their characteristic microvilli and recent studies suggest that they function as intestinal sensory cells (mechano- or chemoreceptors) and play a role in the intestinal immune response, but their exact functions have yet to be determined. [9, 10]

The intestinal epithelial cells are derived from the intestinal stem cells located in the intestinal crypts. These undifferentiated and long-lived cells are able to differentiate into the many different types of intestinal epithelial cells and are the reason for the high self-renewal capacity of the intestinal epithelium. [11]

The commensal bacteria are a microcosm within themselves and research on this fascinating topic is booming. The microbiota seems to be connected to an endless amount of physiological functions and diseases. A majority of the microbiota resides in the gut and they play an essential role in many processes. They support the host in digestion, provide essential nutrients, and form the microbial barrier that defends the host against pathogenic microbes, amongst others. [12, 13]

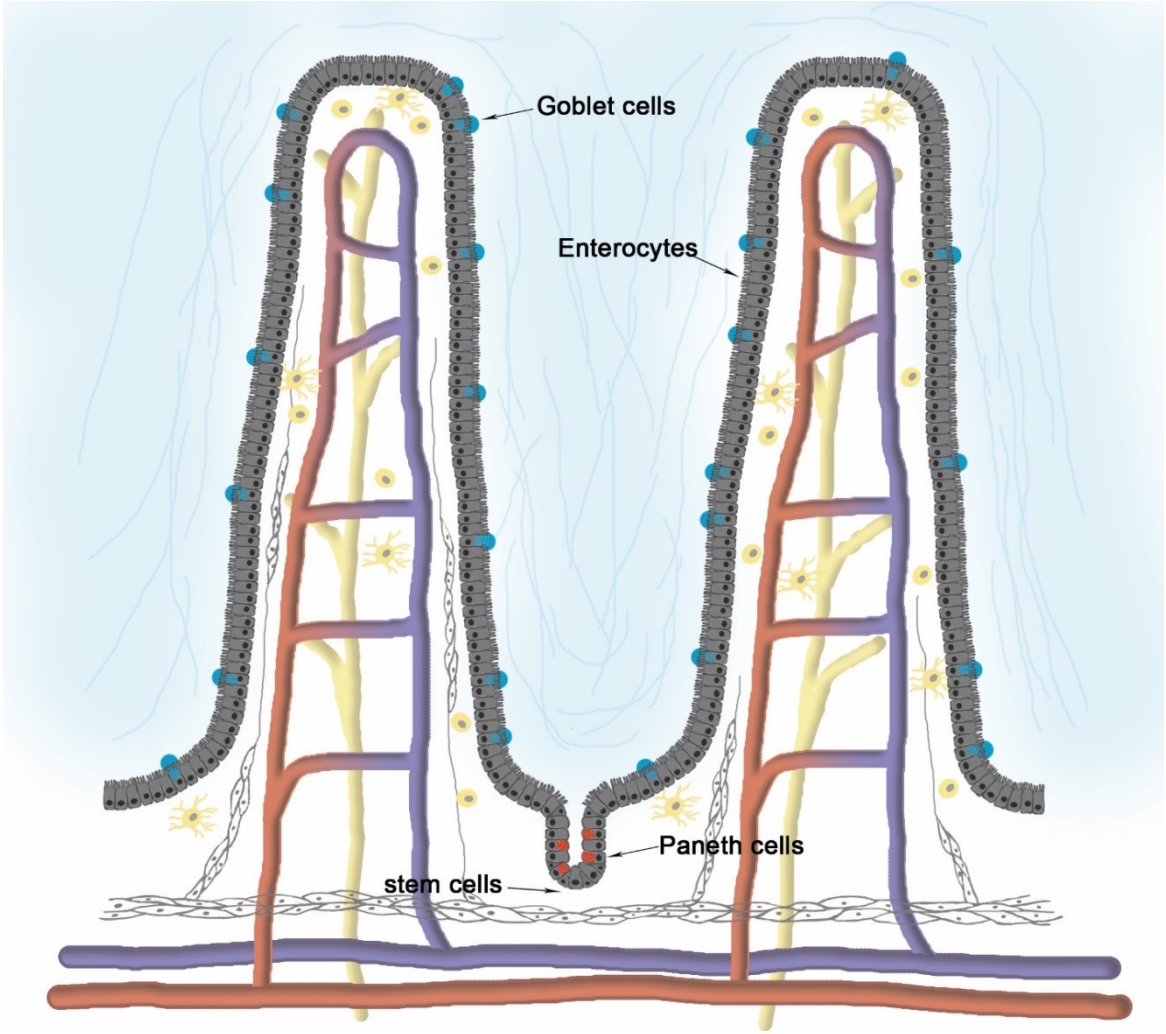


Fig. 1: Schema of small intestinal epithelial layer (Adapted after [1, 5])

2.2 Caco-2/TC7 cells

Caco-2 cells were originally isolated from a human colon adenocarcinoma. This adherent cell line has the ability to differentiate spontaneously, acquiring a small intestinal phenotype after a certain period of cultivation. Upon differentiation they reveal characteristics of small intestine enterocytes, such as formation of monolayers and tight junctions between adjacent cells, a cylindrical shape with polarization and microvilli on the apical side, small intestine enzyme activity, and directional transport of molecules, ions, and water. After reaching confluence, domes can be observed in the Caco-2 monolayers. Those domes are formed through one-directional transport of ions and water. Liquid gathers between the

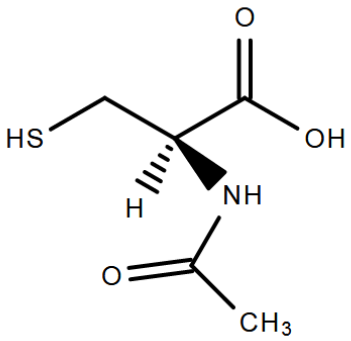
cell layer and the culture surface causing the monolayer to lift up from the surface and form the dome. [14]

Due to these characteristics, the Caco-2 line is extensively used as an *in vitro* model for the intestinal barrier. Many laboratories cultivate Caco-2 cells of different origins, making comparisons difficult. The existence of various clones adds to this difficulty.

For the experiments described in the present thesis, the Caco-2/TC7 clone was used. The Caco-2/TC7 clones were isolated from a late passage of the parental Caco-2 wild type cells. They are characterized by a high sucrose-isomaltase and UDP-GST (UDP-Glucoronosyltransferase) activity, stable CYP3A4 expression, and low glucose consumption, amongst other characteristics. SGLT1, GLUT2, and GLUT5 activity increases with time in culture, while expression of GLUT1 and GLUT3 decrease after confluence. [15]

2.3 N-Acetylcysteine

Table 1: N-Acetylcysteine basic data [16-18]

Name	N-Acetylcysteine, N-Acetyl-L-cysteine, NAC	 Adapted after Europäisches Arzneibuch 8. Ausgabe, Grundwerk 2014, band 2, S. 2225
Molecular formular	$C_5H_9NO_3S$	
Molecular weight (M_r)	163.191 g/mol	
pKa	9.52 (at 25 °C)	
CAS Nr.	616-91-1	

NAC is the precursor of the endogenous amino acid L-Cysteine. NAC is deacetylated to L-Cysteine mostly in the liver. The active metabolite L-Cysteine is able to lower the viscosity of bronchial mucus by cleaving the disulfide bonds of the mucus proteins (mucins). Other metabolites, such as cystine, diacetyl-cystine and disulfides, are inactive. Due to a high first pass effect only about 5% to 10%

(different percentages were reported) or less of the agent reaches circulation. After ingestion most of the compound can be located in the liver, the kidneys, the lung and in bronchial mucus. The mucolytic effect will start to emerge only 1 to 2 days after start of the treatment and there is no risk of mucolysis happening too fast. Most of the compound will be eliminated in form of inactive metabolites through the kidneys and a lesser portion via feces. The usual dosage is 600 mg NAC per day. No intoxication effects can be observed even when ingesting much higher doses (up to 500 mg /kg). [3, 19, 37].

The mucolytic properties are commonly used to treat symptoms of airway diseases with productive cough such as bronchitis, COPD and also cystic fibrosis, although evidence is contradictory. [19, 21-27] For example, the ECFS (European cystic fibrosis society) best practice guidelines states that the only mucolytic agent "that has proven efficacy in CF is dornase alfa" and that other mucolytic drugs, such as NAC, "have not been proven to be effective in CF patients". [26] Dornase alfa is the recombinant phosphorylated human glycoprotein desoxyribonuclease I, indicated for CF patients. It cleaves extracellular DNA, which is present in high concentrations in purulent secretions. [43]

Studies have shown that NAC has also beneficial effects on patients with heart diseases (e.g. doxorubicin-induced cardiotoxicity, stable angina pectoris) and psychiatric disorders (e.g. schizophrenia, bipolar disorder). [19]

NAC is listed on the WHO Model List of Essential Medicines, EML – 20th edition (March 2017, amended August 2017), under the antidote category. It is used as antidote for paracetamol intoxication which is caused by toxic paracetamol metabolites after overdosing with paracetamol. In the United States, paracetamol intoxication is the most common reason (40%) for acute liver failure. Acetylcysteine functions as a SH-donator (sulfhydryl group) that regenerates the depleted glutathione reserves. It intercepts the toxic metabolites and protects the liver. The intravenous application of high doses of NAC during the antidote therapy (up to 20 g) is often associated with an anaphylactoid reaction, but it is usually not necessary to stop the therapy. Most likely, histamine is released via non-IgE-mediated mechanisms. [3, 20, 46]

NAC was shown to have multiple biological activities, including regulation the expression of numerous genes, regulation of apoptosis, regulation of protein activity and immune-modulating effect, amongst others. [19, 44]

The anti-oxidative effect of NAC and its effect on oxidative stress are widely discussed in literature. Under physiological conditions NAC acts as a scavenger for the high reactive oxygen species •OH (hydroxyl radical) and for radicals $\bullet NO_2$ and $CO_3^{\bullet-}$. It is also able to bind redox-active metal ions. Studies have also shown that NAC is able to affect cell cycle regulation and to inhibit the apoptotic pathway. It was also shown that NAC exhibits immune-modulatory activity.

Although studies have shown that NAC has beneficial effects *in vitro* and *in vivo*, NAC can also act detrimentally and has the potential to act as a pro-oxidant, especially in presence of redox-active transition metals or in absence of oxidative stress. [19]

In a previous diploma thesis the *in vivo* effects of the mucolytic drugs NAC and Ambroxol on the intestinal mucus and the gut in general were researched. To evaluate their effect, BALB/c mice were gavaged either with NAC or with Ambroxol through oral feeding for 14 consecutive days. The mucus composition was examined by measuring the concentration of total protein and free sulfhydryl groups with ELISA and by characterizing MUC gene expression by quantitative real time PCR. Since mucus essentially contributes to the barrier function of the gastrointestinal barrier, the effects on cytokine and total IgA production in intestinal lavages were determined to see whether a disturbed mucus layer would change the immune response.

The NAC-treated group showed a significant reduction of the free sulfhydryl groups per protein compared to the control group.

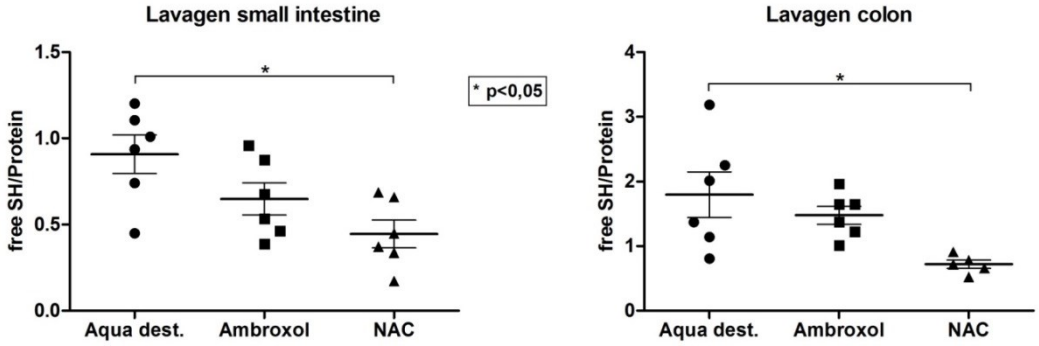


Fig. 2: Measurement of free sulfhydryl groups per protein by Ellman's test [4]

The total IgA level in intestinal lavages was measured to evaluate the local immune response. The IgA levels were lower in the mucolytic-treated groups compared to the untreated control group.

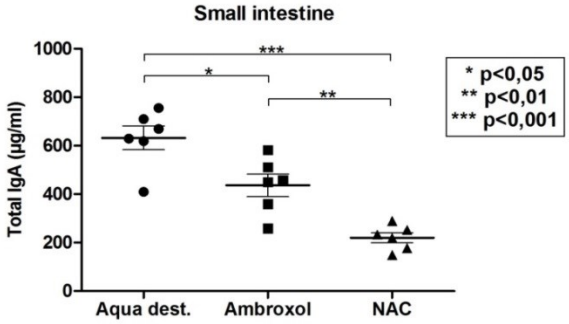


Fig. 3: Measurement of total IgA-concentration by ELISA [4]

Regarding the systemic immune response, IFN-γ levels of both the NAC-treated and the Ambroxol- treated groups were higher compared to the control group.

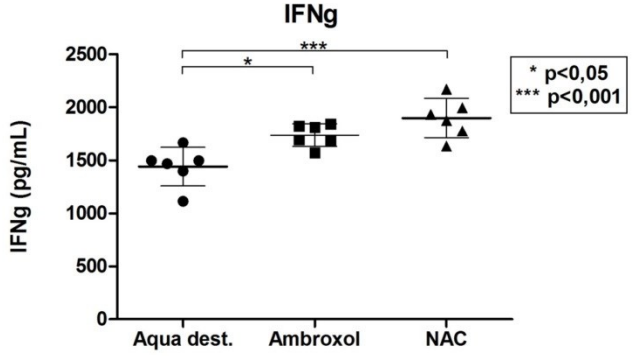


Fig. 4: Measurement of IFN-γ levels by ELISA [4]

This study suggested that treatment with mucolytic drugs influences intestinal and systemic immune homeostasis by changing the intestinal mucus layer. [4]

2.4 Aim and investigated parameters

2.4.1 Aim

The aim of this diploma thesis was to investigate the effect of NAC on expression of barrier and immune function associated genes in enterocytes and whether the stimulation of Caco-2 cells as a model for the small intestine epithelial layer leads to an altered gene expression. The potential up- or down-regulation of certain genes was measured by determining the mRNA levels with qPCR.

The expression of the mRNA levels of tight junction proteins occludin, claudin-1, claudin-4, zonula occludens 1 and 2, was determined in order to investigate whether NAC has an effect on the barrier function of the intestinal epithelial layer.

Moreover, the mRNA expression of cytokines IL-8 and CCL20 was assessed to evaluate whether NAC induces an immune response via enterocyte stimulation.

2.4.2 Cytokines

Interleukin 8 (IL-8)

IL-8 (synonym: CXCL8) is a chemokine of the CXC chemokines family. It is the ligand to the CXCR1 and CXCR2 receptors. Similarly to many other chemokines of the CXC family, its main function lies in neutrophil trafficking, but it also acts as a chemotactic factor for basophiles and T-cells. High IL-8 levels are associated with certain diseases such as pulmonary diseases and certain chronic inflammatory diseases (psoriasis, rheumatoid arthritis). IL-8 is also overexpressed after septic shock and systemic distribution of endotoxins. [28, 29] IL-8 is also associated with tumor growth by promoting neovascularization and angiogenesis. [30] A previous study has shown that IL-1α-induced IL-8 secretion was reduced by NAC in a concentration-dependent manner in bronchial epithelial cells. [31]

The same study suggests that underlying antioxidant-sensitive mechanisms might play a role.

CCL20

CCL20 (synonym: MIP-3, LARC, Exodus) is the name of the gene that codes for the C-C motif chemokine 20. It is a cytokine of the CC chemokines family. CCL20 is expressed in different types of epithelial cells, and amongst others, by the cells of the follicle-associated epithelial (FAE), which overlay the Peyer's Patches in the gut. CCL20 in particular binds to the CCR6, a receptor expressed in immature dendritic cells, B-cells and T-cells, and has both inflammatory and homeostatic function. The CCL20-CCR6 ligand-receptor pair functions as a chemoattractant that recruits especially dendritic cells, B- and T-cells, and plays an important role in the gut associated lymphatic tissue (GALT) by recruiting B-cells. [29, 30, 32] CCL20 is expressed at a low basal level, but expression can be increased strongly by cytokines, such as TNF-α and IL-34. The inflammatory bowel diseases Morbus Crohn und Colitis ulcerosa are associated with a higher CCL20 expression. [33]

2.4.3 Tight junction proteins

Tight junctions (zonula occludens) are dynamic structures in epithelial cells responsible for preventing uncontrolled paracellular transport and for regulating this pathway. Tight junctions are formed between adjacent cells and consist of several proteins, such as the transmembrane proteins occludin, the claudin family (>20 members), tricellulin, and junction adhesion molecules (JAM), and the cytosolic proteins zonula occludens (ZO) family, and cingulin, amongst others. These proteins interact not only with each other but also with the cytoskeleton. Functional tight junctions are crucial for the integrity of the epithelium. The tightness of epithelial barrier seems to depend on the number of tight junction strands, with leakier epithelia having fewer and tighter epithelia having more.

Further down (in the basolateral direction), adherens junctions (zonula adherens) and desmosomes also contribute to the sealing of the epithelial cell layers but they are also responsible for intercellular adhesion and communication. [2, 12, 34, 38, 39]

Gap junctions are responsible for intracellular transport between adjacent cells. [5]

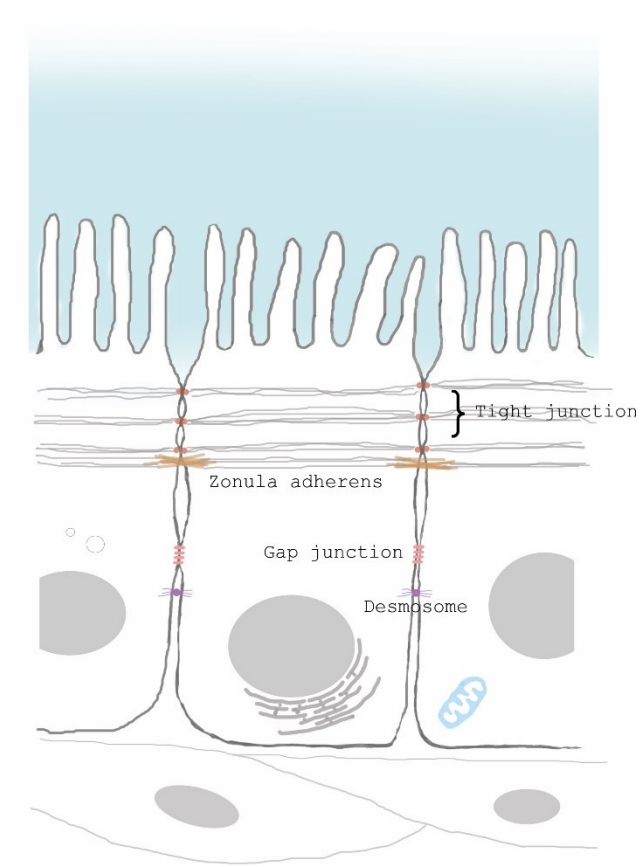


Fig. 5: Schema of intestinal epithelial cells - Sealing of the paracellular pathway. (Adapted after [5])

Occludin, Claudin-1 and Claudin-4

Occludin, claudin-1, and claudin-4 are transmembrane tight junction proteins. They form strands that regulate transport through the tight junctions, thus contributing to the tightness/permeability of the epithelial layer. Compounds seem to be able to pass through tight junctions in two ways: the leak pathway and the pore pathway. While occludin appears to form the lower capacity leak pathways,

which allow for the passage of macromolecules (such as antigens, etc.), claudins seem to be able to build ion/charge selective pores.

Occludin can be phosphorylated and the phosphorylation status of the protein seems to play an important role in the regulation and function of occludin. However, the precise role of occludin is even to date not completely understood and studies report conflicting results about the significance of occludin for tight junction functionality.

Claudins are a family of proteins with more than 20 identified members. They build charge-selective pores which can be passed by ions with a radius of up to 4 Å. Different claudin proteins show different selectivity, which seems to depend on the charged amino acid side chains of the first extracellular domain of claudins. Also, different strands seem to be able to interact with each other building a variety of pores with various properties that are specific to the epithelia. These pores allow passage of water molecules and salts and are therefore important for the maintenance of physiological functions.

TEER (transepithelial electrical resistance) is often used to assess the tightness of an epithelial barrier and it seems to depend on the ion transport through the pore pathway. [12, 34, 36, 38, 39]

Zonula occludens proteins: ZO-1 and ZO-2

The tight junction proteins zonula occludens 1 (ZO-1) and zonula occludens 2 (ZO-2) are intracellular proteins. Their function is to assemble and stabilize the tight junction. ZO-1 binds directly to occludin and ZO-1 and ZO-2 bind to each other. ZO-1 also binds to the cytoskeleton proteins actin and F-myosin and links occludin to actin and myosin. ZO-1 also has binding sites for claudins and a number of other tight junction proteins, such as tricellulin and JAM. ZO-1 also seems to play a role in the regulation of leak pathways, as it was demonstrated that ZO-1 depletion in MDCK cells lead to an increased permeability of larger molecules, but this did not occur when ZO-2 was depleted. [2, 12, 35, 36]

3 Material and Methods

3.1 Culturing and stimulation of cells

Cell culturing and seeding the cells for stimulation experiments

The Caco-2/TC7 cell line was used for the experiments. The frozen cells (in DMSO) were thawed and cultured in Dulbecco's modified eagle medium (DMEM, Glibco, Ref 41965-039; for components see appendix). 10% of FCS and 1% of PenStrep, HEPES buffer, and Glutamin each were added to the medium. The medium in the cell culture flask was changed three times per week. The cells were passaged before reaching confluence (see *Protocol for splitting*).

For the three experiments, cells from passage numbers 26, 26, and 28 were used. After splitting and counting the cells, they were seeded into two 12-well plates with 228,000 cells per well (60,000 cells per cm², area per well: 3.8 cm²) in 1.5 mL. Since a confluent monolayer and differentiated cells was needed for the experiments, cells were cultivated for 21 days before stimulation.

Cells from passage 34 were used to create a reference value for calculation of ΔCq-values with results of the qPCR experiment.

Protocol for splitting

After thawing, DMEM was removed and cells were washed with PBS. After PBS removal, a corresponding amount of Trypsin (1 mL for T25 culture flask, 2 mL for T75, 4 mL for T175) added. The cells were incubated in Trypsin at 37 °C for 10 minutes or until the cells were detached from the culture flask. The detached cells were checked under the microscope. The necessary amount of medium (with FCS) was added to quench Trypsin activity (5 mL for T25, 10 mL for T75, 20 mL for T175). The cell suspension was transferred into a centrifuge tube and centrifuged at 150G for 8 minutes at room temperature.

After centrifugation the medium was carefully removed without disturbing the cell pellet, the cells were re-suspended in 1 to 2 mL medium, depending on the size of the cell pellet, and then counted in the in TC20 Automated Cell Counter (Bio-Rad).

A corresponding amount of medium was added to a new cell culture flasks (5 mL for T25, 20mL for T75, 40 mL for T175) and, depending on the flask size, a corresponding cell number was transferred into the new flask (50,000 for cells for T25, 300,000 cells for T75, 1 Mio. cells for T175).

Preparation of NAC solutions and stimulation of cells

The NAC solution was prepared by using the OTC medication Aeromuc (Aeromuc 600 mg lösliche Tabletten, Astellas Pharma Ges.m.b.H), which contains 600 mg of NAC per dissolvable tablet. The filling material is sodium hydrogen carbonate, citric acid, aspartame, and lemon flavoring. [37]

The tablet was dissolved in 5 mL PBS and then sterilized by filtration. Using the sterile stock solution, three solutions were prepared using DMEM medium. The final concentrations of the solutions were: 10 mM, 1 mM, 0.1 mM. The 10 mM NAC solution was slightly acidic with a pH of 6.32. The other solutions had a neutral pH.

First the medium in the two 12-well plates was removed and the 1.5 mL of the NAC solution was pipetted into each well in triplicates. Cells were incubated with the solution for 30 minutes or 60 minutes respectively.

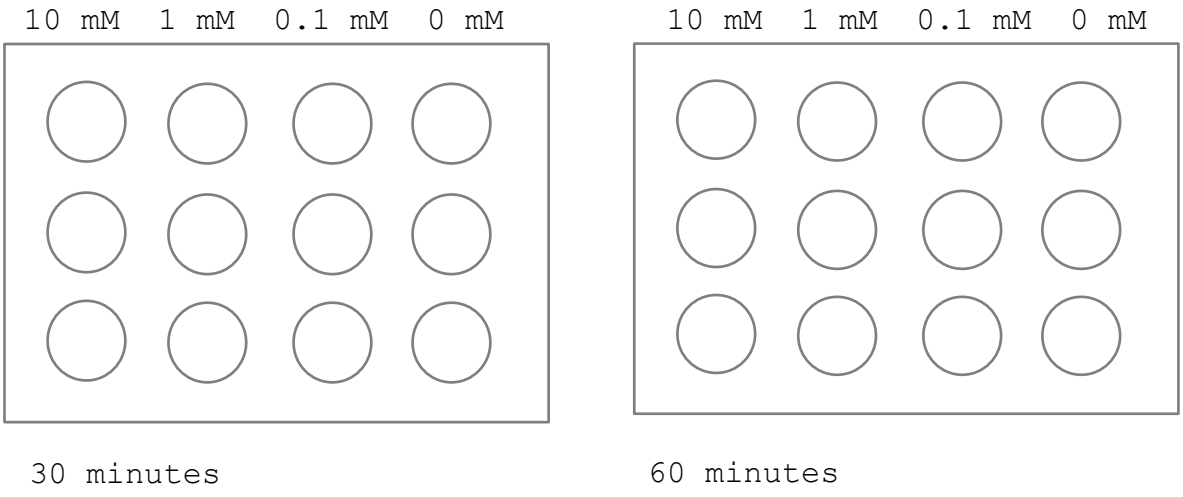


Fig. 6: Schema for the stimulation with NAC

After incubation, the stimulation was stopped by removing the NAC solution. The cells were immediately washed with PBS, put on ice, and 380 µL of TRIzol® Reagent was added to each well.

After 3 minutes of incubation time with TRIzol® Reagent, the lysed cells were homogenized with a Terumo syringe and transferred into a DNAse and RNAse free Eppi. Ssamples were frozen at -80 °C until further use.

3.2 RNA extraction

After thawing the samples at room temperature, 76 µL of chloroform was added to the tube containing the cell lysates. After shaking and incubating for 3 minutes at room temperature, the samples were centrifuged at 12,000 G and 4 °C for 10 minutes. Afterwards, the transparent upper phase was transferred into a fresh tube containing 190 µL isopropanol. After shaking and incubating for 10 minutes at room temperature the samples were again centrifuged at 12,000 G and 4 °C for 10 minutes. After the second centrifugation, a small pellet (RNA) could be observed at the bottom of the tube. The supernatant was removed completely by inverting the tube. The pellet was washed with 1 mL of 75% ethanol and after a further centrifugation (7,500 G at 4 °C for 5 minutes) the ethanol was removed by inverting the tube. The washing process was repeated once. The pellet in the inverted tube was dried for 5 to 10 minutes at room temperature. The dried pellet was dissolved into 40 µL RNase-free water and put on ice. The samples were heat treated in the Thermomixer for 10 minutes at 65 °C while shaking. Afterwards, the samples were immediately transferred back on ice.

The RNA concentration was measured with a NanoDrop spectrophotometer (ND-1000, PEQLAB Biotechnologie GmbH).

In case of low 260/230 values the samples were purified by re-precipitation (see re-precipitation protocol). The integrity of the RNA was tested by performing gel electrophoresis on an agarose gel (see *RNA integrity test protocol*).

The RNA samples were frozen at -80 °C until further use.

Re-precipitation protocol

9 parts of ice cold 75% ethanol was mixed with 1 part sodium acetate and homogenized. 120 µL of the mixture was added to the samples (which contained 40 µL of fluid) and shaken by hand. The samples were store over night at -20 °C and centrifuged the following day at 16,000 G and 4 °C for 20 minutes. After centrifugation a small pellet was observed on the bottom of the tube. The supernatant was removed by inverting the tube and the pellet was washed with 75% ethanol. After a second centrifugation (16,000 G at 4 °C for 10 minutes) the ethanol was removed by inverting the tube. The washing step was repeated once. Afterwards, the pellet was dried at room temperature in the inverted tube. The dry pellet was dissolved in 20 µL RNase free water and boiled in a shaking thermoblock at 55 °C for 10 minutes.

RNA integrity test protocol

1 g of agarose was dissolved into TBE (Tris/Borate/EDTA) buffer (in DEPC-treated water) using a microwave (750 W). After cooling down the solution 3 µL peqGreen was added. The gel was poured into the electrophoresis apparatus and the samples were prepared as following: 4 µL RNase free water + 1.5 µL loading dye + 1 µL sample.

The samples were pipetted into the pockets and the gel was run at 150 V for 25 minutes.

The gel was analyzed using the ChemiDoc Imaging System (Bio-Rad). The 28S and 18S bands were visible.

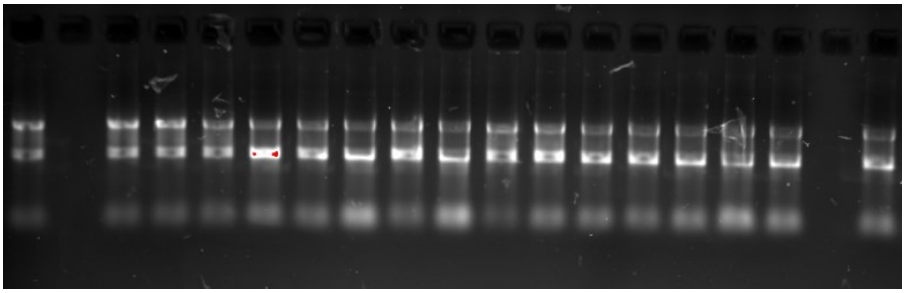


Fig. 7: RNA integrity test gel with visible 28S and 18S bands.

3.3 PCR

Reverse transcription PCR

The RNA samples were thawed slowly on ice. The reverse transcription from RNA to cDNA was achieved by using a reverse transcription kit (High-Capacity cDNA Reverse Transcription Kit, Applied Biosystems™). The master mix was prepared according to the manufacturer's protocol and the reactions were run in a thermal cycler (MyCycler™ Thermal Cycler, Bio-Rad) with following conditions: 10 minutes at 25 °C, then 120 minutes at 37 °C, 5 minutes at 85°C and finally 4°C at the end.

The resulting cDNA samples were diluted 1:4 with RNase free water and frozen at -20 °C until further use.

Table 2: Reagent measurements for 1 PCR reaction

RNA template	10 µL (1 µg)
10x RT Puffer	2 µL
25x dNTP Mix	0.8 µL
10x RT Random Hexamers	2 µL
Reverse transcriptase	1 µL
RNase free water	4.2 µL
TOTAL	20 µL

qPCR

The cDNA samples were analyzed by using a qPCR kit (GoTaq® qPCR Master Mix, Promega) and a real-time PCR system (7900HT Fast Real-Time PCR System, Applied Biosystems™) in 384-well plates. The master mix was prepared according to the manufacturer's protocol. The predesigned primers were purchased from Sigma-Aldrich. RPLP0 was used as housekeeping gene.

Table 3: Primer sequences

Gene name	Forward sequence (5'-3')
	Reverse sequence (5'-3')
RPLP0	CCTCATATCCGGGGGAATGTG
	GCAGCAGCTGGCACCTTATTG
IL-8	AGCTCTGTGTGAAGGTGCAGT
	GTCCACTCTCAATCACTCTCA
CCL20	GCGAATCAGAAGCAGCAAGCA
	TGTGTGAAAGATGATAGCATT
Occludin	GATGAGCAGCCCCCCAAT
	GGTGAAGGCACGTCCTGTGT
Claudin 1	CAGTCAATGCCAGGTACGAATTT
	AAGTAGGGCACCTCCCAGAAG
Claudin 4	TGTACCAACTGCCTGGAGGAT
	GACACCGGCACTATCACCATAA
ZO-1	ACAGTGCCTAAAGCTATTCCTGT
	TCGGGAATGGCTCCTTGAG
ZO-2	GGGAACCACTGGGTGTAATTCA
	CGGTTAAATACCGTGAGGCAAA

Table 4: Reagents for Primer mix

RNase free water	90 µL
Forward primer	5 µL
Reverse primer	5 µL
TOTAL	100 µL

Table 5: Reagents for 1 PCR reaction

Template (cDNA)	2 µL
2x GoTaq Master Mix	5 µL
RNase free water	2.4 µL
Primer mix	0.5 µL
CXR	0.1 µL
TOTAL	10 µL

The preparations of the reagents and the pipetting were done under a RNA-hood. After covering, the 384-well plates were centrifuged with 258 G at 4°C for 5 minutes. The thermal profile for the PCR reactions were set-up as follows:

Initiation stage: 95 °C for 2 minutes

Denaturation, hybridization, elongation: 95 °C for 15 seconds, then 60 °C for 1 minute, in total 40 cycles

Disassociations stage: 95 °C for 15 seconds, 60°C for 15 seconds, the 95 °C for 15 seconds

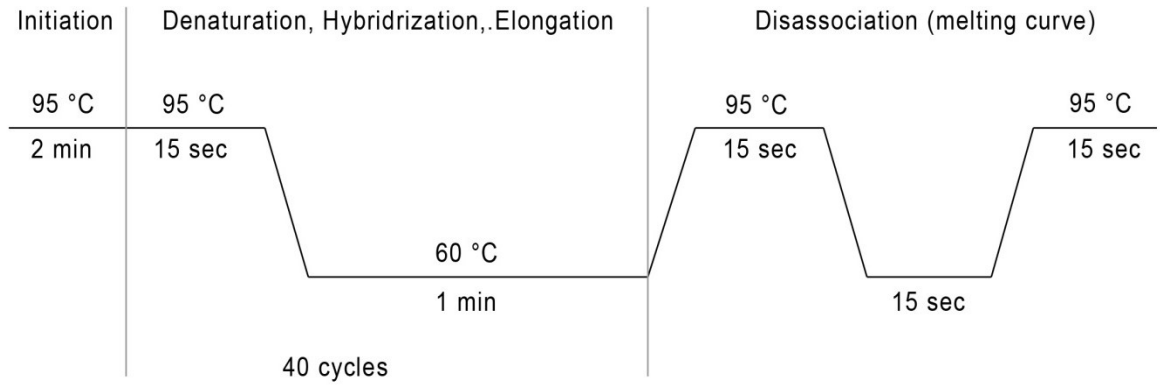


Fig. 8: Thermal profile of the qPCR reaction

3.4 Statistics

In the qPCR experiment, triplicates of each sample were made. After adjusting the threshold accordingly, the resulting mean Ct of the triplicates was used for further calculations. The ΔCq-, ΔΔCq-values and the fold induction were calculated.

The results were analyzed with the statistic software GraphPad Prism version 5.00 for Windows. The Kolmogorov-Smirnov test was used to check for normal distribution. Afterwards, the data was statistically compared using the one-way ANOVA test with a p value < 0.05 being considered significant. (* 0.01 to 0.05, ** 0.001 to 0.01, *** < 0,001)

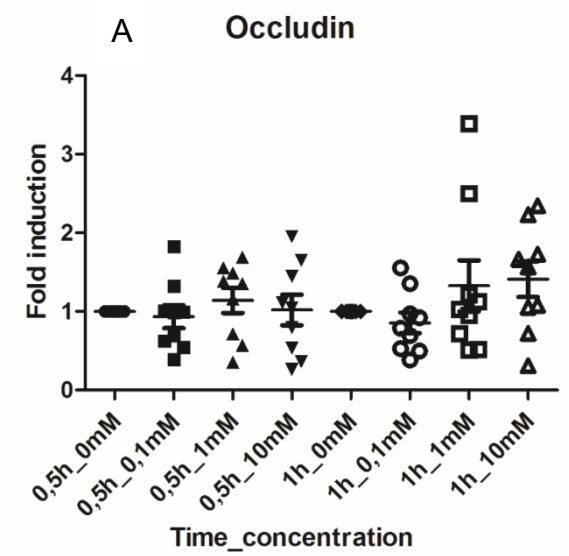
4 Results

4.1. mRNA levels of tight junction proteins

Incubation of confluent Caco-2 cells with three different concentrations of NAC (0.1 mM, 1 mM, 10 mM) for 30min or 1h induced no significant change on the mRNA levels of tight junction proteins occludin, claudin-1, claudin-4, ZO-1 and ZO-2, which were measured by qPCR.

Still, a slight dose-dependent trend could be seen in the mRNA levels of claudin-1. In cells stimulated for 1 h with higher concentrations of NAC the gene expression of claudin-1 was upregulated, however without reaching statistical significance. This effect could not be observed after 30 minutes of stimulation. This might be, as a longer incubation might lead to a more pronounced effect.

Neither a time-dependent nor a dose-dependent trend could be seen for the mRNA levels of the other tight junction proteins.



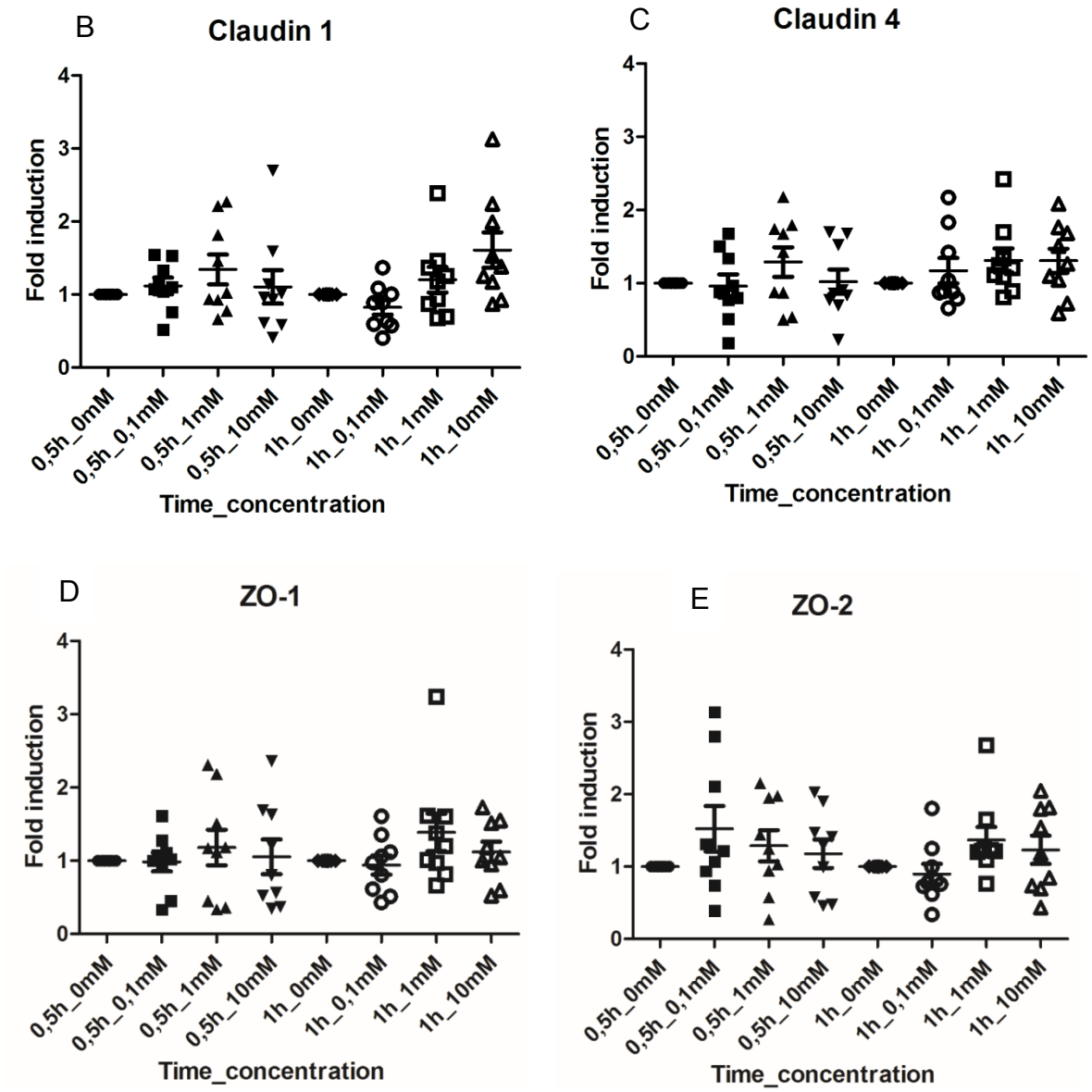


Fig 9: mRNA levels of tight junction proteins occludin, claudin-1, claudin-4, ZO-1 and ZO-2 after 30 minutes or 1 h of stimulation with 0 mM, 0.1 mM, 1mM and 10 mM NAC solution of confluent Caco-2 cells. The fold induction of mRNA was calculated with the Ct values from the qPCR experiment. Data are presented as the mean ± SEM. No significant change on the expression of tight junction proteins was observed. A slight dose-dependent upregulation of claudin-1 was seen after 1 h stimulation, but this effect is not significant. Time- and dose-dependent fold induction of (A) occludin (B) claudin-1 (C) claudin-4 (D) zonula occludens protein 1 (E) zonula occludens protein 2

4.2. mRNA levels of cytokines

When the effect of NAC incubation on cytokine levels was evaluated, we observed a significant dose-dependent effect on the mRNA levels of the cytokines IL-8 and CCL20, thus, suggesting an enhanced expression of cytokine genes indicating a potential induction of an immune response via epithelial Caco-2 cells. While no discernable effect could be observed after 30 minutes of incubation, a concentration-dependent effect on the immune activating response was observed after 60 minutes of incubation. When stimulated with higher NAC concentrations (10 mM and 1 mM), the mRNA levels of the inflammatory proteins IL-8 and CCL20 showed a higher fold induction compared to the control group. When stimulated with 0.1 mM the fold induction of IL-8 and CCL20 remained equal to or was even lower than in the unstimulated cells.

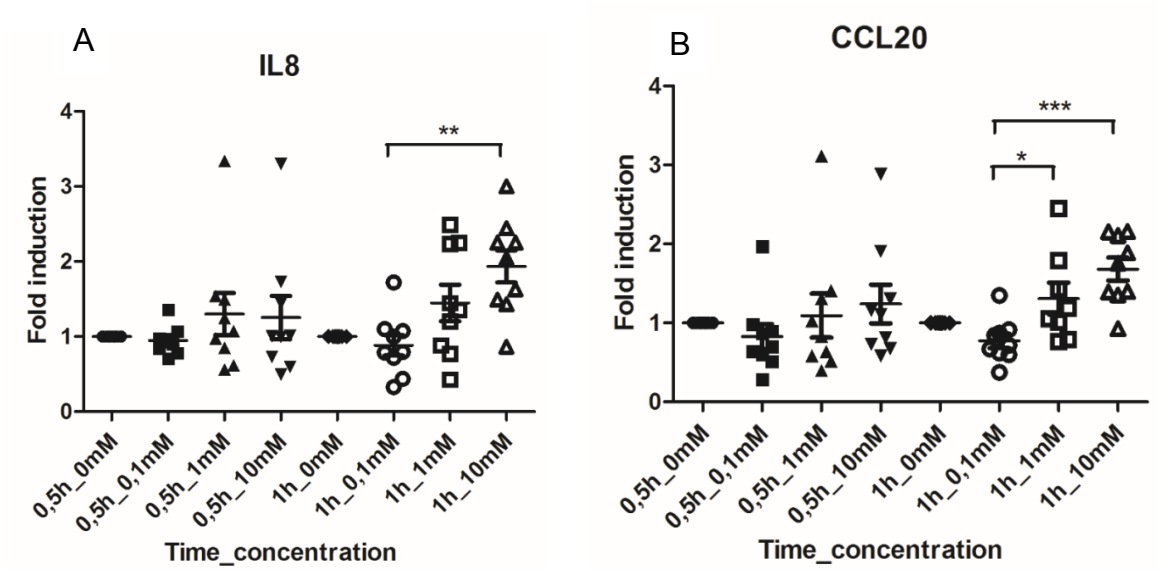


Fig 10: mRNA levels of the cytokines IL-8 and CCL20 after 30 minutes or 1 h of stimulation with 0 mM, 0.1 mM, 1mM and 10 mM NAC solution of confluent Caco-2 cells. The fold induction of mRNA was calculated with the Ct values form the qPCR experiment. Data are presented as the mean ± SEM. A significant dose-dependent effect can be seen between cells stimulated with 0.1 mM and 10 mM NAC in both IL-8 and CCL20 mRNA expression after 1 h. No discernable effect can be observed after 30 minutes of incubation. (* p-value 0.01 to 0.05, ** p-value 0.001 to 0.01, *** p-value < 0,001) (A) Fold induction of IL-8 (B) Fold induction of CCL20.

5 Discussion

NAC had no significant effect on the mRNA levels of tight junction proteins, which leads to the conclusion that the compound does not influence the barrier function of Caco-2 cells directly by influencing tight junction proteins. This might suggest that tight junctions of enterocytes in living organisms might also not be influenced by NAC. However, tight junctions are not the only mechanism contributing to the intestinal barrier. The most obvious barrier mechanism that might be disturbed by NAC is the mucus layer overlaying the enterocytes. As shown in previous work by our group (Klems M. diploma thesis), NAC has a significant effect on the concentration of free sulfhydryl groups in intestinal lavages (unattached mucus) and collected mucus samples of mice. A slight, but not significant, change could also be observed in mRNA levels of MUC2 and MUC3. These results suggest that NAC can weaken the epithelial barrier by influencing the mucus composition, however, might not directly influence tight junctions of enterocytes.

Functional mucus composed of essential mucus proteins is required for maintenance of gastrointestinal health. Intestinal mucus prevents direct contact of luminal bacteria with the epithelial cells underlying the mucus. A human study that compared the mucus of normal and inflamed colon showed that an increased severity of inflammation correlated to a decrease in the thickness and spread of the mucus. Thinner or even absent mucus seemed to allow bacteria to adhere to the epithelial cells, causing inflammation. [47] Another clinical study reported that patients with Crohn's Disease and Ulcerative Colitis have an altered expression of mucins with less MUC2 and MUC 3 and more MUC4 and TFF3 (trefoil factor). [48]

Studies in mice showed that MUC2 seemed to play an especially important role for gut homeostasis, as mice with deficient MUC2 spontaneously developed colitis or colorectal cancer and were more prone to parasitic infection. [49, 50, 51]

Further research revealed that the parasitic amoeba *Entamoeba histolytica* secreted cysteine proteases that were able to cleave MUC2 resulting in degradation of the polymeric network of the mucus. While intact mucus hindered the attachment of *E. histolytica* to the epithelial cells, the degraded mucus seemed to be less effective in preventing the invasion of the parasite. [52, 53]

The results of the present thesis showed that NAC induced a concentration dependent elevation of cytokine gene expression. Elevation of cytokine levels is observed in association with an inflammatory reaction in enterocytes. The inflammation inducing effect was, however, only observed for higher NAC concentrations (1 mM, 10 mM). The lowest NAC concentration (0.1 mM) seems to have an opposite effect, by even slightly reducing the mRNA levels of cytokines, which did not reach statistical significance.

The main function of the pro-inflammatory cytokine IL-8 lies in neutrophil trafficking. Active ulcerative colitis or Crohn's disease are associated with heightened tissue IL-8 levels, resulting in infiltration of tissue by neutrophils, which is characteristic for the aforementioned diseases. [55, 56]

CCL20 functions as a lymphocyte chemoattractant and activator. Elevated levels of CCL20 in enterocytes are found to be associated with Crohn's disease as well. [57]

The symptoms of Crohn's disease are caused by a defective immune response to commensal intestinal microbiota, resulting in impaired recruitment of neutrophils and inflammation. While it is known that genetic risk factors contribute, the exact trigger of this disease is still unknown. It was suggested that a breach of the intestinal barrier triggers Crohn's disease and by allowing contact of luminal content with epithelium. [58]

Since inflammatory bowel diseases are associated both with a higher IL-8 levels (and also higher CCL20 levels in the case of Crohn's disease), as well as decreased or degraded mucus, the results of the present thesis (NAC can induce higher IL-8 and CCL20 expression in Caco-2 cells which would lead to recruitment of neutrophils) and the previous thesis by M. Klems (NAC can cause mucus degradation and an altered MUC expression which would result in a compromised mucus layer) [4] might lead to the conclusion that ingestion of NAC is detrimental for intestinal health and homeostasis and could even be a factor that promotes inflammatory bowel diseases in healthy organisms.

However, many studies indicate that NAC (alone or in combination with other compounds) has a positive effect on parameters associated with inflammatory

bowel diseases in rats, mice and porcine, mostly due to its antioxidative effect. [59-64] It has to be mentioned, that all the mentioned studies examined the effects of NAC on sick organisms, where an inflammatory bowel disease has been induced through another compound. Without any doubt, further studies are needed to determine the effect of NAC on organisms with healthy guts.

In human, evidence shows that NAC can act both beneficially (anti-oxidative) and detrimentally (pro-oxidative). [19] High concentrations of NAC are used to treat paracetamol intoxications with 150 mg/kg IV as an initial dose and 50 mg/kg IV within 4 hours [3] or 15 g within 20 or 21 hours [20, 46]. Anaphylactoid reactions are a common adverse effect of this treatment [20, 46], suggesting an immune response or an unspecific cellular activation by NAC in high doses. This hypothesis is supported by the results of the present experiment. Still, in cases like paracetamol intoxication the benefits highly outstrip the possibly detrimental effects.

However, in another study NAC was suggested to improve immune function in post-menopausal woman. The authors stated that “aging is a chronic oxidation process in which the immune system is involved”. In this study post-menopausal women received 600 mg NAC per day for 4 months in total and TNF-α and IL-8 levels were significantly reduced during NAC supplementation. [45]

Based to these results, it was suggested that NAC might be harmful in the absence of oxidative stress or in the presence of redox active transition metals [19] (which is present in the DMEM in form of ferric nitrate, although in a very low concentration of 0.1 mg/L). The results of the present experiment fit both aspects of NAC, since higher NAC concentrations seemed to increase pro-inflammatory cytokine expression while stimulation with lower concentrations down-regulated cytokine expression, or at least, showed no significant changes in expression of the inflammatory parameters. It was suggested that NAC shows characteristics of hormesis, as it can be harmful in large doses (to healthy organisms), while beneficial in low doses. [19] Our experiment supports this hypothesis.

There are certain limitations of the here presented results. The 10 mM solution used in this experiment was slightly acidic. The cell culture medium turned yellow and a pH of 6.3 was measured for this concentration, compared to the more neutral concentration of the medium and the other concentrations (pH 7.4). There is a possibility that this is the reason for the inflammatory response of the Caco-2 cells, but there is also evidence to refute this hypothesis. The lower pH does not seem to have a significant effect in the 30 minutes groups (only in the 60 minutes group) and the 1 mM solution was neutral but still shows the same tendency as the 10 mM group. Moreover, at the end of the 60 minutes stimulation time, the yellow 10 mM solution changed back to a pinkish color, which indicates that the pH of the solution had risen again.

To summarize the results of these experiments, NAC does not affect the expression of tight junction proteins in Caco-2 cells, while in high concentrations induction of cytokine expression was observed in Caco-2 cells.

Outlook and further research

NAC is a versatile compound with a wide spectrum of biological functions.

While the effect of NAC on the respiratory tract is well investigated, its effect on the mucus layer of the intestinal barrier and in consequence on the local and systemic immune response has yet to be determined in detail. Human clinical trials regarding the effect of NAC on the gastrointestinal mucus and barrier, as well as the consequences for the immune system have yet to be undertaken.

To date, no information is available on this topic and there is a need for a better understanding of potential effects of mucolytic drugs on the intestinal mucus with consequences on the intestinal barrier.

References

1. Mutschler E, Schaible HG, Vaupel P. Anatomie Physiologie Pathophysiologie des Menschen. 6. Auflage, 2007, Wissenschaftliche Verlagsgesellschaft mbH Stuttgart
2. Turner JR. Intestinal mucosal barrier function in health and disease. Nat Rev Immunol. 2009 Nov;9(11):799-809. doi: 10.1038/nri2653.
3. Mutschler E, Geisslinger G, Kroemer HK, Menzel S, Ruth P. Mutschler Arzneimittelwirkungen: Pharmakologie – Klinische Pharmakologie – Toxikologie. 10. Auflage, 2012, Wissenschaftliche Verlagsgesellschaft mbH Stuttgart. S581-582
4. Klems M. The impact of mucolytic drugs on the intestinal mucus and the local immune response. 2017. Diploma Thesis
5. Rainer K, Pape HC, Kurtz A, Silbernagl S. Physiologie. 6. Auflage, 2009, Thieme Verlag. Page 17, 46-47 and 417-418
6. Johansson M, Sjövall H, Hansson GC. The gastrointestinal mucus system in health and disease. Nat Rev Gastroenterol Hepatol. 2013 Jun;10(6):352-61. doi: 10.1038/nrgastro.
7. Meldrum OW, Yakubov GE, Bonilla MR, Deshmukh O, McGuckin MA, Gidley MJ. Mucin gel assembly is controlled by a collective action of non-mucin proteins, disulfide bridges, Ca2+-mediated links, and hydrogen bonding. Sci Rep. 2018 Apr 11;8(1):5802. doi: 10.1038/s41598-018-24223-3.
8. Park SW, Zhen G, Verhaeghe C, Nakagami Y, Nguyenvu LT, Barczak AJ, Killeen N, Erle DJ. The protein disulfide isomerase AGR2 is essential for production of intestinal mucus. Proc Natl Acad Sci U S A. 2009 Apr 28;106(17):6950-5. doi: 10.1073/pnas.0808722106.
9. Sato A. Tuft cells. Anat Sci Int. 2007 Dec;82(4):187-99. doi: 10.1111/j.1447-073X.2007.00188.x
10. Cheng X, Voss U, Ekblad E. Tuft cells: Distribution and connections with nerves and endocrine cells in mouse intestine. Exp Cell Res. 2018 Aug 1;369(1):105-111. doi: 10.1016/j.yexcr.2018.05.011.
11. Shahid U. Intestinal Stem Cells. Curr Gastroenterol Rep. 2010: 12(5): 340–348. doi:10.1007/s11894-010-0130-3.
12. Chelakkot C, Ghim J, Ryu SH. Mechanisms regulating intestinal barrier integrity and its pathological implications. Exp Mol Med. 2018 Aug 16;50(8):103. doi: 10.1038/s12276-018-0126-x.
13. Flint HJ, Scott KP, Louis P, Duncan SH. The role of the gut microbiota in nutrition and health. Nat Rev Gastroenterol Hepatol. 2012 Sep 4;9(10):577-89. doi: 10.1038/nrgastro.2012.156.
14. Tanner C, Frambach DA, Misfeldt DS.. Transepithelial transport in cell culture. A theoretical and experimental analysis of the biophysical properties of domes. Biophys J. 1983 Aug; 43(2): 183–190. doi: 10.1016/S0006-3495(83)84339-2
15. Sambuy Y, De Angelis I, Ranaldi G, Scarino ML, Stammati A, Zucco F.. The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics. Cell Biol Toxicol. 2005 Jan;21(1):1-26.
16. Europäisches Arzneibuch 8. Ausgabe, Grundwerk 2014. Deutscher Apotheker Verlag. Band 2, S. 2225
17. https://www.sigmaaldrich.com/catalog/search?term=616-91-1&interface=CAS%20No.&N=0&mode=partialmax&lang=de&region=AT&focus=product (27.3.19)
18. https://pubchem.ncbi.nlm.nih.gov/compound/N-Acetyl-L-cysteine#section=Top (27.3.19)

19. Samuni Y, Goldstein S, Dean OM, Berk M. The chemistry and biological activities of N-acetylcysteine. Biochimica et Biophysica Acta. 2013: 4117–4129
20. Aktories, Förstermann et al. Allgemeine und spezielle Pharmakologie und Toxikologie, 11 Auflage. 2013, Munich. Elsevier GmbH, Urban & Fischer Verlag. S 974-975
21. https://pubchem.ncbi.nlm.nih.gov/compound/12035#section=LiverTox-Summary (28.3.19)
22. Cazzola M, Calzetta L, Page C, Jardim J, Chuchalin AG, Rogliani P, Matera MG. Influence of N-acetylcysteine on chronic bronchitis or COPD exacerbations: a meta-analysis. Eur Respir Rev. 2015 Sep;24(137):451-61. doi: 10.1183/16000617.00002215.
23. Stey C, Steurer J, Bachmann S, Medici TC, Tramèr MR. The effect of oral N-acetylcysteine in chronic bronchitis: a quantitative systematic review. Eur Respir J. 2000 Aug;16(2):253-62.
24. Ayfer Aytemur Z, Baysak A, Ozdemir O, Köse T, Sayiner A. N-Acetylcysteine in patients with COPD exacerbations associated with increased sputum. Wien Klin Wochenschr. 2015 Apr;127(7-8):256-61. doi: 10.1007/s00508-014-0692-4.
25. Sadowska AM. N-Acetylcysteine mucolysis in the management of chronic obstructive pulmonary disease. Ther Adv Respir Dis. 2012 Jun;6(3):127-35. doi: 10.1177/1753465812437563.
26. Castellani C, Duff AJA, Bell SC, Heijerman HGM, Munck A, Ratjen F, Sermet-Gaudelus I, Southern KW, Barben J, Flume PA, Hodková P, Kashirskaya N, Kirszenbaum MN, Madge S, Oxley H, Plant B, Schwarzenberg SJ, Smyth AR, Taccetti G, Wagner TOF, Wolfe SP, Drevinek P. ECFS best practice guidelines: the 2018 revision. J Cyst Fibros. 2018 Mar;17(2):153-178. doi: 10.1016/j.jcf.2018.02.006.
27. Conrad C, Lymp J, Thompson V, Dunn C, Davies Z, Chatfield B, Nichols D, Clancy J, Vender R, Egan ME, Quittell L, Michelson P, Antony V, Spahr J, Rubenstein RC, Moss RB, Herzenberg LA, Goss CH, Tirouvanziam R.. Long-term treatment with oral N-acetylcysteine: Affects lung function but not sputum inflammation in cystic fibrosis subjects. A phase II randomized placebo-controlled trial. J Cyst Fibros. 2015 Mar;14(2):219-27. doi: 10.1016/j.jcf.2014.08.008.
28. https://www.uniprot.org/uniprot/P10145 (31.3.2019)
29. Palomino DC, Marti LC. Chemokines and immunity. Einstein (Sao Paulo). 2015;13(3):469–473. doi:10.1590/S1679-45082015RB3438
30. Le Y, Zhou Y, Iribarren P, Wang J. Chemokines and Chemokine Receptors: Their Manifold Roles in Homeostasis and Disease. Cell Mol Immunol. 2004;1(2):95-104.
31. Matsumoto K, Hashimoto S, Gon Y, Nakayama T, Takizawa H, Horie T.. N-Acetylcysteine inhibits IL-1α-induced IL-8 secretion by bronchial epithelial cells. Respir Med. 1998 Mar;92(3):512-5.
32. https://www.uniprot.org/uniprot/P78556 (29.3.2019)
33. Williams IR. CCR6 and CCL20 – Partners in Intestinal Immunity and Lymphoraganogenesis. Ann N Y Acad Sci. 2006 Aug;1072:52-61.
34. Weber CR. Dynamic properties of the tight junction barrier. Ann N Y Acad Sci. 2012 Jun;1257:77-84. doi: 10.1111/j.1749-6632.2012.06528.x.
35. Anderson JM, Van Itallie CM. Tight junctions and the molecular basis for regulation of paracellular permeability. Am J Physiol. 1995 Oct;269(4 Pt 1):G467-75.
36. Van Itallie CM, Fanning AS, Bridges A, Anderson JM.. ZO-1 Stabilizes the Tight Junction Solute Barrier through Coupling to the Perijunctional Cytoskeleton. Mol Biol Cell. 2009 Sep 1; 20(17): 3930–3940. doi: 10.1091/mbc.E09-04-0320

37. Austria Codex Fachinformation – Aeromuc 600 mg lösliche Tabletten
38. Colegio OR, Van Itallie C, Rahner C, Anderson JM. Claudin extracellular domains determine paracellular charge selectivity and resistance but not tight junction fibril architecture. Am J Physiol Cell Physiol. 2003 Jun;284(6):C1346-54.
39. Feldman GJ, Mullin JM, Ryan MP. Occludin: structure, function and regulation. Adv Drug Deliv Rev. 2005 Apr 25;57(6):883-917. doi:10.1016/j.addr.2005.01.009
40. Mabbott NA, Donaldson DS, Ohno H, Williams IR, Mahajan A. Microfold (M) cells: important immunosurveillance posts in the intestinal epithelium. Mucosal Immunol. 2013 Jul;6(4):666-77. doi: 10.1038/mi.2013.30.
41. Bevins CL, Salzman NH. Paneth cells, antimicrobial peptides and maintenance of intestinal homeostasis. Nat Rev Microbiol. 2011 May;9(5):356-68. doi: 10.1038/nrmicro2546.
42. Clevers HC1, Bevins CL. Paneth cells: maestros of the small intestinal crypts. Annu Rev Physiol. 2013;75:289-311. doi: 10.1146/annurev-physiol-030212-183744.
43. Austria Codex Fachinformation – Pulmozyme 2,500 E./2,5 mL
44. Zafarullah M, Li WQ, Sylvester J, Ahmad M. Molecular mechanisms of N-acetylcysteine actions. Cell Mol Life Sci. 2003 Jan;60(1):6-20
45. Arranz L, Fernandez C, Rodriguez A, Ribera JM, De la Fuente M. The gluthathion precursor N-acetylcysteine improves immune function in postmenopausal women. Free Radic Biol Med. 2008 Nov 1;45(9):1252-62. doi: 10.1016/j.freeradbiomed.2008.07.014.
46. Yarema M, Chopra P, Sivilotti MLA, Johnson D, Nettel-Aguirre A, Bailey B, Victorino C, Gosselin S, Prussel R, Thompson M, Spyker D, Rumack B. Anaphylactoid Reactions to Intravenous N-Acetylcysteine during Treatment for Acetaminophen Poisoning. J Med Toxicol. 2018 Jun;14(2):120-127. doi: 10.1007/s13181-018-0653-9.
47. Swidsinski A, Loening-Baucke V, Theissig F, Engelhardt H, Bengmark S, Koch S, Lochs H, Dörffel Y. Comparative study of the intestinal mucus barrier in normal and inflamed colon. Gut. 2007 Mar;56(3):343-50
48. Dorofeyev AE, Vasilenko IV, Rassokhina OA, Kondratiuk RB. Mucosal barrier in ulcerative colitis and Crohn's disease. Gastroenterol Res Pract. 2013;2013:431231. doi: 10.1155/2013/431231
49. Velcich A, Yang W, Heyer J, Fragale A, Nicholas C, Viani S, Kucherlapati R, Lipkin M, Yang K, Augenlicht L. Colorectal cancer in mice genetically deficient in the mucin Muc2. Science. 2002 Mar 1;295(5560):1726-9.
50. Van der Sluis M, De Koning BA, De Bruijn AC, Velcich A, Meijerink JP, Van Goudoever JB, Büller HA, Dekker J, Van Seuningen I, Renes IB, Einerhand AW. Muc2-deficient mice spontaneously develop colitis, indicating that MUC2 is critical for colonic protection. Gastroenterology. 2006 Jul;131(1):117-29.
51. Heazlewood CK, Cook MC, Eri R, Price GR, Tauro SB, Taupin D, Thornton DJ, Png CW, Crockford TL, Cornall RJ, Adams R, Kato M, Nelms KA, Hong NA, Florin TH, Goodnow CC, McGuckin MA. Aberrant mucin assembly in mice causes endoplasmic reticulum stress and spontaneous inflammation resembling ulcerative colitis. PLoS Med. 2008 Mar 4;5(3):e54. doi: 10.1371/journal.pmed.0050054.
52. Moncada D, Keller K, Chadee K. Entamoeba histolytica cysteine proteinases disrupt the polymeric structure of colonic mucin and alter its protective function. Infect Immun. 2003 Feb;71(2):838-44.
53. Lidell ME, Moncada DM, Chadee K, Hansson GC. Entamoeba histolytica cysteine proteases cleave the MUC2 mucin in its C-terminal domain and dissolve the protective colonic mucus gel. Proc Natl Acad Sci U S A. 2006 Jun 13;103(24):9298-303.

54. Hasnain SZ, Wang H, Ghia JE, Haq N, Deng Y, Velcich A, Grencis RK, Thornton DJ, Khan WI. Mucin gene deficiency in mice impairs host resistance to an enteric parasitic infection. Gastroenterology. 2010 May;138(5):1763-71. doi: 10.1053/j.gastro.2010.01.045.
55. Mitsuyama K, Toyonaga A, Sasaki E, Watanabe K, Tateishi H, Nishiyama T, Saiki T, Ikeda H, Tsuruta O, Tanikawa K. IL-8 as an important chemoattractant for neutrophils in ulcerative colitis and Crohn's disease. Clin Exp Immunol. 1994 Jun;96(3):432-6.
56. Wera O, Lancellotti P, Oury C. The Dual Role of Neutrophils in Inflammatory Bowel Diseases. J Clin Med. 2016 Dec; 5(12): 118. doi: 10.3390/jcm5120118.
57. Kwon JH, Keates S, Bassani L, Mayer LF, Keates AC. Colonic epithelial cells are a major site of macrophage inflammatory protein 3alpha (MIP-3alpha) production in normal colon and inflammatory bowel disease. Gut. 2002 Dec;51(6):818-26.
58. Segal AW. Making sense of the cause of Crohn's – a new look at an old disease. F1000Res. 2016 Oct 12 [revised 2016 Nov 16];5:2510. doi: 10.12688/f1000research.9699.2.
59. Ancha HR1, Kurella RR, McKimmey CC, Lightfoot S, Harty RF. Effects of N-acetylcysteine plus mesalamine on prostaglandin synthesis and nitric oxide generation in TNBS-induced colitis in rats. Dig Dis Sci. 2009 Apr;54(4):758-66. doi: 10.1007/s10620-008-0438-0.
60. Wang Q, Hou Y, Yi D, Wang L, Ding B, Chen X, Long M, Liu Y, Wu G. Protective effects of N-acetylcysteine on acetic acid-induced colitis in a porcine model. BMC Gastroenterol. 2013 Aug 30;13:133. doi: 10.1186/1471-230X-13-133.
61. Uraz S, Tahan G, Aytekin H, Tahan V. N-acetylcysteine expresses powerful anti-inflammatory and antioxidant activities resulting in complete improvement of acetic acid-induced colitis in rats. Scand J Clin Lab Invest. 2013 Feb;73(1):61-6. doi: 10.3109/00365513.2012.734859.
62. Romagnoli C, Marcucci T, Picariello L, Tonelli F, Vincenzini MT, Iantomasi T. Role of N-acetylcysteine and GSH redox system on total and active MMP-2 in intestinal myofibroblasts of Crohn's disease patients. Int J Colorectal Dis. 2013 Jul;28(7):915-24. doi: 10.1007/s00384-012-1632-2.
63. You Y, Fu JJ, Meng J, Huang GD, Liu YH. Effect of N-acetylcysteine on the murine model of colitis induced by dextran sodium sulfate through up-regulating PON1 activity. Dig Dis Sci. 2009 Aug;54(8):1643-50. doi: 10.1007/s10620-008-0563-9.
64. Amrouche-Mekkioui I, Djerdjouri B. N-acetylcysteine improves redox status, mitochondrial dysfunction, mucin-depleted crypts and epithelial hyperplasia in dextran sulfate sodium-induced oxidative colitis in mice. Eur J Pharmacol. 2012 Sep 15;691(1-3):209-17. doi: 10.1016/j.ejphar.2012.06.014.

Appendix

I. DMEM Medium components

11965 - DMEM, high glucose

Components	Molecular Weight	Concentration (mg/L)	mM
Amino Acids			
Glycine	75.0	30.0	0.4
L-Arginine hydrochloride	211.0	84.0	0.39810428
L-Cystine 2HCl	313.0	63.0	0.20127796
L-Glutamine	146.0	584.0	4.0
L-Histidine hydrochloride-H2O	210.0	42.0	0.2
L-Isoleucine	131.0	105.0	0.8015267
L-Leucine	131.0	105.0	0.8015267
L-Lysine hydrochloride	183.0	146.0	0.7978142
L-Methionine	149.0	30.0	0.20134228
L-Phenylalanine	165.0	66.0	0.4
L-Serine	105.0	42.0	0.4
L-Threonine	119.0	95.0	0.79831934
L-Tryptophan	204.0	16.0	0.078431375
L-Tyrosine disodium salt dihydrate	261.0	104.0	0.39846742
L-Valine	117.0	94.0	0.8034188
Vitamins			
Choline chloride	140.0	4.0	0.028571429
D-Calcium pantothenate	477.0	4.0	0.008385744
Folic Acid	441.0	4.0	0.009070295
Niacinamide	122.0	4.0	0.032786883
Pyridoxine hydrochloride	206.0	4.0	0.019417476
Riboflavin	376.0	0.4	0.0010638298
Thiamine hydrochloride	337.0	4.0	0.011869436
i-Inositol	180.0	7.2	0.04
Inorganic Salts			
Calcium Chloride (CaCl2) (anhyd.)	111.0	200.0	1.8018018
Ferric Nitrate (Fe(NO3)3"9H2O)	404.0	0.1	2.4752476E-4
Magnesium Sulfate (MgSO4) (anhyd.)	120.0	97.67	0.8139166
Potassium Chloride (KCl)	75.0	400.0	5.3333335
Sodium Bicarbonate (NaHCO3)	84.0	3700.0	44.04762
Sodium Chloride (NaCl)	58.0	6400.0	110.344826
Sodium Phosphate monobasic (NaH2PO4-H2O)	138.0	125.0	0.9057971
Other Components			
D-Glucose (Dextrose)	180.0	4500.0	25.0
Phenol Red	376.4	15.0	0.039851222

Reference:
Dulbecco, R. and Freeman, G. (1959) Virology 8:396.
https://www.thermofisher.com/at/en/home/technical-resources/media-formulation.8.html (17.09.2019)

II. Zusammenfassung

Die gastrointestinale Barriere ist die größte Außenfläche unseres Körpers und stellt eine wichtige Barriere zwischen der Umwelt und unserem Körper dar. Sie hat nicht nur die Funktion lebenswichtige Nährstoffe aufzunehmen, sondern soll uns auch vor Krankheitserregern oder schädlichen Stoffen schützen.

Mehrere Komponenten tragen zur Aufrechterhaltung der gastrointestinalen Barriere bei: die Epithelschicht, eine einzellige Schicht aus Nährstoff-resorbierenden Enterozyten, die dicht miteinander über Tight Junctions verbunden sind, sind ein Teil dieser Barriere und Gegenstand der vorliegenden Diplomarbeit.

Die Schleimschicht liegt der Epithelschicht auf und wird durch Mukus-Proteine produzierende Becherzellen aufrechterhalten. Ebenso existiert eine chemische Barriere, die durch Defensine und Lysozyme aus endokrinen Zellen und Paneth-Zellen entsteht und eine Immunbarriere, die durch Immunzellen und Antikörper gebildet wird.

N-acetylcysteine (NAC) ist ein weit verbreiteter, ohne Rezept verfügbarer mukolytischer Arzneistoff, der oft zur Behandlung der Symptome von häufigen respiratorischen Erkrankungen, wie z.B. bei Erkältung, angewendet wird. NAC reduziert die Viskosität des Schleimes, in dem Disulfid-Brücken zwischen MUC-Proteinen gespalten werden. Schleim kommt aber nicht nur in den Atemwegen, sondern auch im Gastrointestinaltrakt vor.

Ziel der vorliegenden Diplomarbeit war dabei, *in vitro* die direkte Wirkung von NAC auf die Enterozyten zu studieren. Es sollte untersucht werden, ob die Stimulation von Caco-2 Zellen (als Modell für die Dünndarmepithelschicht) mit NAC zu einer veränderten Expression von Genen führt, die im Zusammenhang mit der intestinalen Barrierefunktion und Immunantwort stehen. Potenziell veränderte Genexpression wurde über die exprimierte mRNA mittels qPCR gemessen.

Um die Wirkung auf die Barriere zu untersuchen, wurden die Tight Junction-Proteine Occludin, Claudin-1, Claudin-4, ZO-1 und ZO-2 evaluiert. Um den

Einfluss auf die Immunantwort zu analysieren wurden die Zytokine Interleukin-8 und CCL20 analysiert.

Die Caco-2 Zellen wurden mit unterschiedlichen Konzentration von NAC (0 mM, 0.1 mM, 1 mM und 10 mM) für 30 Minuten bzw. 1 Stunde stimuliert. Anschließend wurden die Zellen mittels Trizol®-Reagens lysiert, die RNA extrahiert, mittels Reverse Transkription PCR in cDNA umgeschrieben und mittels qPCR analysiert. Mit dem Mittelwert der resultierenden Ct-Werte wurde die Fold induction ausgerechnet und die Ergebnisse mit einem Statistikprogramm analysiert.

Die Stimulation der Caco-2 Zellen führte zu keiner signifikanten Veränderung hinsichtlich Genexpression von Tight Junction Proteinen. Wir konnten allerdings einen signifikanten Effekt hinsichtlich Expression von Zytokinen beobachten. Eine 1-stündige Stimulation mit 1 mM und 10 mM NAC führte zu einer signifikanten Erhöhung der Fold induction bei IL-8 und CCL20. Dieser Effekt konnte nicht nach einer 30-minütigen Stimulation beobachtet werden. Die Stimulation der Zellen mit 0.1 mM NAC führte interessanterweise zu einem leichten Rückgang der Genepxression der Zytokine, wobei dieser Effekt nicht signifikant war.

Zusammenfassend kann gesagt werden, dass NAC keine direkte Auswirkung auf die Barrierefunktion von Caco-2-Zellen hatte, aber eine Immunantwort über die Enterozyten in Gang setzen könnte.