The role of T cell potassium channels, $K_V1.3$ and $K_{Ca}3.1$, in the inflammatory cascade in ulcerative colitis

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INTRODUCTION

Study Background

Ulcerative Colitis (UC) and Crohn's disease (CD) are relapsing chronic inflammatory bowel diseases (IBD) that affect many people all over the world. In this study, the main focus is on UC although the results of studies in patients suffering from CD are also included. Current medical treatment consists of anti-inflammatory drugs: glucocorticoids, tumor-necrosis-factor alpha inhibitors, cyclosporine, 5-aminosalicylic acid (5ASA), azathioprine, 6-mercaptopurine, and in some cases methotrexate and tacrolimus[1]. There is a clear need for new treatment options as medical treatment often fails and patients must undergo surgery to have the affected area removed. If medical treatment fails in patients with severe UC a colectomy is necessary. However, the handling of UC has definitely improved over time. In the 1930s, the mortality rate was 75% in patients with acute severe UC[2] decreasing to about 25% in the 1950s[3], and a recent audit from the United Kingdom showed that the current mortality rate was 0.7% in 2010[4]. A concordant mortality rate about 1% was found in a systematic review from 2007[5].

The role of T cell $K_V1.3$ and $K_{Ca}3.1$ channels in UC has not yet been clearly defined. $K_V1.3$ and $K_{Ca}3.1$ are known to be involved in regulating T cell function, proliferation, and cytokine production[6] thus suggesting that they could be involved in the pro-inflammatory response in IBD further up-stream of inflammatory cytokines such as IFN-γ and TNF-α. If $K_V1.3$ and $K_{Ca}3.1$ channels play fundamental roles in inflammation in IBD, the expression of the channels may function as a novel severity score and could be a novel pharmacological target. Moreover, $K_V1.3$ and $K_{Ca}3.1$ expression may be used to guide the physician in the choice of therapy and maybe even identify risk patients in terms of treatment failure and early relapse.

Aim of the study

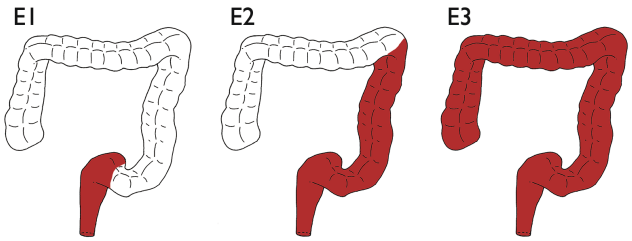
This study aimed to investigate the role of T cell potassium channels, $K_V1.3$ and $K_{Ca}3.1$, in IBD with focus on UC. The research projects were setup as a translational study where we wanted to investigate if gene expression of $K_V1.3$ and $K_{Ca}3.1$ were increased in a disease-related matter and could be used to predict responders vs. non-responders, to estimate the risk of relapse, and if $K_V1.3$ and $K_{Ca}3.1$ were involved in the inflammatory cascade. Additionally, we arranged an animal study in order to put our human results into a larger perspective; hence we wanted to evaluate $K_V1.3$ and $K_{Ca}3.1$ as potential pharmacological targets in humans and in DSS-induced colitis in rats.

ULCERATIVE COLITIS

Background and Epidemiology

UC is the most frequent of the inflammatory bowel diseases. UC is a disease located in the large intestine where it causes a mucosal inflammation starting in the distal part of the colon (proctitis) and spreads, in a continuous manner, in the proximal direction towards the caecum (pancolitis) (see figure on next page). UC primarily affects younger people between 15-30 years of age and has no sex predominance [7, 8]. The disease often may affects these young people both psychically and psychologically and leads to increased society costs due to sickness leave and expensive costs of the treatment. In recent epidemiologic studies the incidence rate is about 10-20/100,000 person years and has a prevalence rate of 156-291/100,000 persons in northern Europe and North America [8-12].

In Denmark, recent data from the Danish Drug registry show that the use of biologics such as anti-TNF-α (Infliximab, Adalimumab, Certolizomab pegol and Golimumab) has doubled from 2007-2012[13]. In 2013, Jess et al showed consistent data, in a nationwide Danish cohort study, that AZA and TNF-α treatments have increased[14].



Montreal classification for ulcerative colitis. E1) ulcerative proctitis limited to the rectum, E2) left-sided UC limited to the colon distal from the splenic flexure, E3) extensive colitis (or pancolitis) if involvement extends proximal to the splenic flexure (adapted from [15]).

Overall in Denmark, cancer risk and mortality have not increased compared to the general population but when stratifying UC patients into subgroups that have: an early onset of UC, primary sclerosing cholangitis, a predisposition to colonic cancer, and those with extensive and long-lasting relapses a slight increase in risk of developing colorectal cancer (CRC) is observed [16-20]. This is in contrast to previously reported results from historical studies, where it was found that the risk of CRC is 2% after 10 years, 7-8% after 20 years and 7-18% after 25-30 years of disease[21-26], which could suggest that current medical management of the colonic inflammation has improved and may have led to a decreased risk of CRC. These findings might not be applicable for other populations. However, in February 2014, a meta-analysis [27] was published, which showed that the risk of CRC has indeed decreased over that last six decades and similar to the Danish data, patients with extensive and long-lasting active inflammation have an increased risk of developing CRC. Overall, the meta-analysis reports that the risk is 2%, 4% and 5% for each decade after diagnosis.

Only few diseases have gains from tobacco use – UC may be one of those. There is definitely evidence that smoking alleviates the clinical course of UC[28, 29], but other studies fail to show the same connection[30].

Etiology

The cause of UC is still unclear but is believed to involve both genetically induced immunological factors and environmental factors. Different loci but also familial predispositions have been associated with increased risk of UC [31]. According to a Danish study, the most profound single risk factor for developing UC is having a relative with inflammatory bowel disease. This risk factor alone increases the risk of developing UC by 10-fold. Moreover, the risk of developing UC is 5.7-15.5% if a 1st degree relative has UC[32] and twin studies show that monozygotic twins have a pair concordance rate of 6-18% for UC (for CD it is up to 50%)[33-35] confirming the genetic influence on developing UC.

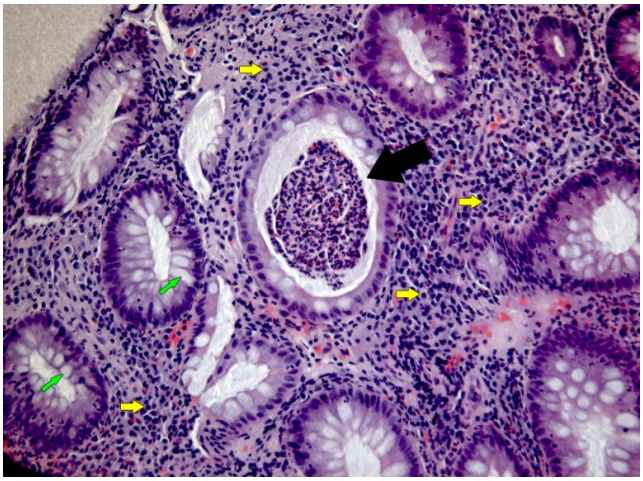
Another important feature of UC is barrier dysfunction in the intestinal wall, which causes an inadequate immune response leading to both acute and chronic inflammation [31, 33]. It has been suggested that increasingly better sanitary standards decrease exposure to mucosal pathogens during childhood, thus decreasing mucosal resistance later in life, hence increasing the risk of IBD[36]. This idea is also supported by other studies showing that previous gastrointestinal infections increase the risk of IBD[37, 38]. A German twin study, conducted using a questionnaire, reported that different environmental factors were associated with UC[38]. Interestingly, the study found that the diseased twin had a higher frequency of living abroad compared to the healthy twin, thus suggesting that the exposure to a new environment and intestinal flora somehow affects the epithelial barrier. Another interesting point was that high consumption of processed meat, soft drinks, and increased usage of antibiotics were also associated with IBD (both UC and CD) all of which supports the notion that the microbiota in the intestine is a key player in a functional barrier.

Clinical presentation and diagnosis

The main symptom of UC is bloody, mucilaginous diarrhea. Upon relapse of symptoms patients undergo endoscopic examination to evaluate the extent of disease activity and scoring of inflammation. Inflammation scoring is usually done according to the Mayo Endoscopic Subscore (none, mild, moderate and severe). Endoscopic findings of active disease are superficial edema and inflammation to deep ulcerations; hence the patients experience bloody diarrhea. During the endoscopic evaluation multiple biopsies are taken from the inflamed mucosa and a gastro-intestinal (GI) pathologist evaluates the degree of inflammation.

Histological presentation

In active UC, the colonic crypts' architectural structure is distorted with an increase of inflammatory cells[39]. Neutrophils migrate from the small capillaries into the epithelium surrounding the crypts and often form crypt abscesses (figure below). Neutrophils are a part of the innate immune system and are the most abundant white blood cell; they are easy to recognize due to the segmented nuclei. Moreover, lymphocytes, eosinophils, and basal plasmacytosis are also seen as a sign of chronic inflammatory infiltrates. Basal lymphoid aggregates may be present and in contrast to CD no granulomas are seen. The constant inflammation causes edema around the crypts and leads to depletion of mucin from goblet cells (seen as an apparent decrease in the number of goblet cells as only the mucin-loaded goblet cells are visible (figure below)). In general, the inflammation is confined in the mucosal layers and does not involve the muscularis propria and serosa. However, in severe or fulminant colitis these layers can be involved too[39].



Hematoxylin and Eosin (HE) staining from a patient with ulcerative colitis. The staining shows colonic crypts surrounded by heavy infiltration of inflammatory cells (yellow arrows), a crypt abscess (large arrow), and goblet cells with mucus (green arrows).

Surgery

In spite of current medical therapy, 5-6% of UC patients undergo surgery (colectomy) within the first year and up to 16-24% after 10 years[8, 18, 40]. In a meta-analysis from 2013 by Frolkis et al[40], the risk of surgery for IBD was generally found to have decreased over the last six decades. The lower risk of surgery seems to be consistent with the decreased incidence rate for CRC. As before, this may likely be explained by the improved control of UC thus decreasing the inflammatory impact on the colon. Historical data have shown that the 25-year cumulative colectomy rate is about 32-65% depending on the extent of inflammation, thus proctitis has a lower risk of colectomy than pancolitis[41, 42]. The risk of colectomy is still too high and there is a clear need for even better medical treatment to decrease the risk further. Colectomy may be performed as acute surgery if the patients develop toxic megacolon, massive bleeding, perforation, or fail medical treatment of an acute severe relapse [43]. Surgery may also be done as elective surgery, which is indicated if medical treatment fails or for recurrent relapses, and premalignant/malignant changes in the colon [44]. The ileal pouch-anal anastomosis is the standard reconstruction method after colectomy. The pouch functions as stool reservoir and leaves the patient with a defecation function close to normal. It is constructed as a J-pouch of the distal ileum with an anastomosis to the anal canal. Common adverse effects are pouchitis, incontinence to flatus and feces [44]. For women, a very important complication to the pouch operation is the decreased chance of becoming pregnant. This must be considered before establishing the ileal-anal pouch in an open operation, as the infertility rate increases to about 26-40% compared to about 10-12% in the background population[45, 46]. However, it seems as if newer surgical methods such as laparoscopic and robotic techniques eliminate the decrease in female fecundity[47].

IMMUNE SYSTEM, T CELLS, AND POTASSIUM CHANNELS

The innate and adaptive immune system

The human immune system consists of both the innate (IIS) and adaptive immune system (AIS). It is a collection of cells, molecules and tissues that all protect the body from pathogenic microbes. Both of the systems are vital, but work in very different ways.

The IIS is constantly ready and active in the fight against microbes. IIS consists of the body's barriers such as skin and mucosa (e.g. lungs or gastrointestinal tract), dendritic cells, natural killer cells (NK cells), the complement system and phagocytic cells (such as neutrophils and macrophages). The ISS reacts with equal force against an exposure to a pathogen and reacts immediately[48-52].

The adaptive immune system (AIS) is activated by pathogens that require an increased response. It usually takes days to ramp up the immunological response, but then the response is targeted at a specific microbe. The AIS consists of B and T lymphocytes (also called B or T cells). T cells are either $CD4^+$ or $CD8^+$ and have different functions accordingly.

$CD8^+$ T cells are called cytotoxic T cells (T_C) because they can act directly on another cell and kill it – it is the army of the AIS. The $CD4^+$ T cells are called T Helper Cells (T_H) as they help the phagocytosing cells and activate B cells for production of specific antibodies. Based on these characteristics, $CD4^+$ T helper cells are divided into T_H1 and T_H2. T_H1 are used to activate and attract macrophages and cytotoxic T cells upon presentation of antigen. Mainly, the T_H2 cells activate B-lymphocytes to produce antibodies[49, 51, 52].

The Major Histocompatibility Complexes (MHC), MHC class 1 and MHC class 2, are surface molecules that are used by the immune cells to interact with other cells in the body. MHC-1 is found on every cell in the body except red blood cells. The cells use MHC-1 to presents a small protein fragment from within the cell to the patrolling immune cells (NK cells and $CD8^+$ lymphocytes). If the presented protein fragment is foreign, T_C will destroy the cell. NK cells function as a patrolling identity checker, checking that all cells express MHC-1. If the MHC-1 is altered or missing on a cell (caused by cancer or if infected by virus) the cell will be killed by secretion of cytokines (granzymes), which causes cell lysis or apoptosis. Antigen presenting cells (APC) express MHC-2. APCs consist mainly of dendritic cells, macrophages and B-lymphocytes. MHC-2 interacts with $CD4^+$ lymphocytes, whereas MHC-1 interacts with $CD8^+$ lymphocytes[53]. The MHC/T-cell interaction is mediated through the T Cell Receptor (TCR) located on all T cells.

The mature B cells are able to secrete antibodies. This will happen when an APC presents the fragment of an engulfed protein from e.g. a bacterium. When presented to the T_H2 cells by the MHC-2, the $CD4^+$ T_H2 will interact with the specific B cells that produce the antibody against the bacteria presented[54, 55].

To sum up: Following activation of the innate immune system, APCs activate T cells that are part of the adaptive immune system. Following their activation, T cells respond by secreting soluble mediators called cytokines. These cytokines serve to suppress or activate the immune response and it has been suggested that the balance between pro-inflammatory and anti-inflammatory cytokines plays an important role in IBD.

T cells and potassium channels

Ion channels in the T cell membrane play pivotal roles in T cell function by controlling intracellular Ca^{2+}-homeostasis thereby regulating cytokine production and clonal expansion after initial T-cell receptor (TCR) activation[6]. Of particular importance are the voltage-gated potassium channel, $K_V1.3$ (shaker-related subfamily member 3, encoded by the *KCNA3* gene) and the calcium-activated potassium channel, $K_{Ca}3.1$ (also known as IKCa1, intermediate conductance Ca^{2+}-activated K^+ channel, encoded by the *KCNN4* gene). $K_V1.3$ and $K_{Ca}3.1$ are tetramers consisting of subunits that thread the membrane six times (see illustration below). Apart from their important role in T cells, $K_V1.3$ channels have also been discovered in the olfactory bulb[56], oligodendrocytes[57, 58], microglia[59, 60], macrophages[61, 62], platelets[63], osteoclasts[64], and B-lymphocytes[65].

The Ca^{2+} activated potassium channel, $K_{Ca}3.1$, has also been found in various cell types: B lymphocytes[65], fibroblasts[66], erythrocytes[67, 68], vascular endothelial and smooth muscle cells[69-73], secretory epithelial cells[74], stem cells[75], enteric neurons[76], macrophages[77], and microglia[59, 60]. Neither channel has been crystalized, so modeling the whole channel is still an issue however, physiological roles have been studied and are well described[6, 62, 78-85].

$K_V1.3$ in T cells

The human T cell has about 300-400 voltage-gated $K_V1.3$ potassium channels in the cell membrane[87]. As the name suggests, the $K_V1.3$ channel is activated when a depolarization occurs. Upon depolarization, the $K_V1.3$ channels undergo a conformational change, which opens the pore allowing K^+ ions to leave the cell.

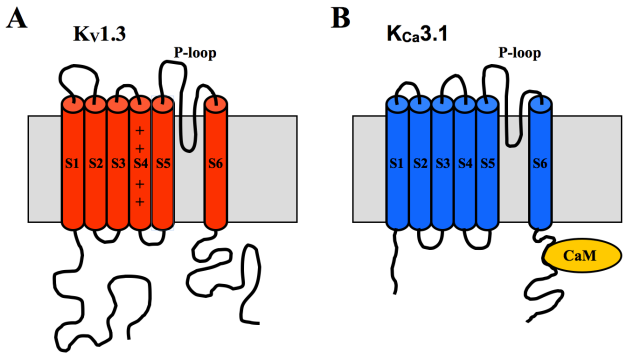


Illustration of the subunits in T cell potassium channels. The proposed membrane topology of $K_V1.3$ (A) and $K_{Ca}3.1$ (B). CaM = Calmodulin, which functions as the calcium sensing-domain for the $K_{Ca}3.1$ (Illustration adapted from Wulff et al[86]).

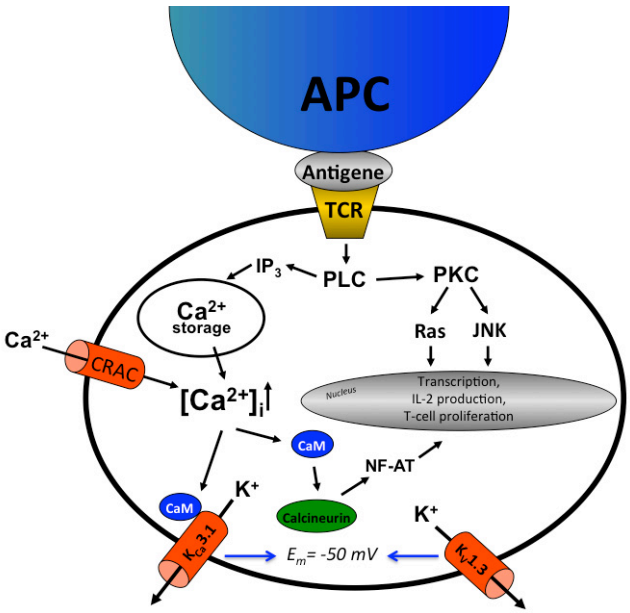
The activation threshold of the $K_V1.3$ channel is approximately -50 mV, thus almost similar to the membrane potential of a resting T cell, which has been shown to be about -60 mV [6, 88-90]. Opening of the channel increases with higher voltage until it reaches its maximum capacity at around -10 to 0 mV. $K_V1.3$ channels are vital for the function of T cells because they keep the membrane potential negative at around -50 mV in interplay with the $K_{Ca}3.1$ channels in order to maintain an adequate driving force for sustained Ca^{2+} influx via the Ca^{2+} release-activated Ca^{2+} channels (CRAC)[81]. In this way the potassium channels keep the influx of Ca^{2+} steady in order to activate nuclear factors, initiate transcription of genes leading to cytokine secretion, and T cell proliferation[87, 91-95] (See figure next page).

$K_{Ca}3.1$ in T cells

The second potassium channel in human T cells is the Ca^{2+} activated $K_{Ca}3.1$. As the name implies, Ca^{2+} activates the channel when intracellular Ca^{2+} concentrations rise above 200nM[78]. When the T cell is resting, the $K_{Ca}3.1$ channels are inactive and they only become activated upon TCR stimulation by an APC, which causes Ca^{2+} influx through CRAC channels and release from the intracellular stores (See figure below). The $K_{Ca}3.1$ channels sense the rise in intracellular calcium by calmodulin (CaM) that is bound to the c-terminus of $K_{Ca}3.1$ subunits. Overall, $K_{Ca}3.1$ channels are highly sensitive to Ca^{2+} with a Hill coefficient of four[91, 96] meaning that gating is highly co-operative and that K^+ efflux begins at very low concentrations of Ca^{2+}. Importantly, they are not affected by the change in membrane potential[96].

Chemokine-receptor-7

Chemokine-receptor-7 (CCR7) is required for T cells to circulate between the lymphoid tissues and peripheral tissues[97]. CCR7 divides T cells into two populations: CCR7 positive ($CCR7^+$) and CCR7 negative ($CCR7^-$). There is evidence that this specific molecule controls how the T cell circulates. If a T cell is $CCR7^+$ (naïve and central memory T cells (T_{CM})) it seems as if the cell is in a resting state, whereas the $CCR7^-$ T cells have constitutive effector functions. Moreover, the CCR7 molecule can be used to distinguish T cells that are "allowed" to circulate into the lymphoid tissues ($CCR7^+$) as opposed to those with immediate effector functions ($CCR7^-$) that lose their ability to enter the lymphoid tissues and stay in the blood and spleen[98, 99]. When $CCR7^+$ T cells are primed by the presentation of an antigen (via APCs and



Calcium regulation upon activation of a $CD4^+$ T cell. A $CD4^+$ T cell is presented to an antigen via the T Cell Receptor (TCR) by an antigen-presenting cell (APC). When the T cell and APC interact via the TCR, an intracellular cascade begins resulting in release of calcium from the Ca^{2+} stores of the endoplasmatic reticulum. This emptying of the intracellular Ca^{2+} stores leads to activation of Ca^{2+} release-activated Ca^{2+} Channels (CRAC). This causes a depolarization by the following Ca^{2+} influx and an increase in cytosolic Ca^{2+}. The Ca^{2+} sensor calmodulin (CaM), bound to the $K_{Ca}3.1$ channel, senses the increase and activates the $K_{Ca}3.1$ channel. Moreover, the $K_V1.3$ channel is also activated due to the change in membrane potential. Both $K_{Ca}3.1$ and $K_V1.3$ result in an efflux of K^+ thus causing a hyperpolarization. The two K^+ channels thereby counteract the depolarization caused by the constant influx of Ca^{2+} via the CRAC channels, and stabilize the membrane potential around -50 mV keeping the T cell active. The sustained intracellular rise in Ca^{2+} activates the phosphatase Calcineurin, which dephosphorylates the Nuclear Factor of Activated T cells (NF-AT). This allows NF-AT to bind to the promoter of the interleukin-2 gene in the nucleus. Together with a parallel pathway where Protein Kinase C (PKC) is activated leading to activation of the c-JUN N-terminal kinase (JNK) and Ras, the rise in intracellular calcium results in activation of various transcription factors, initiation of the transcription of various genes, and T cell proliferation. PLC = Phospholipase C; IP_3 = 1,4,5-inositol triphosphate (Adapted from Wulff et al[87]).

TCR) they become activated and differentiate into T_{CM} ($CCR7^+$). When $CCR7^+$ T_{CM} encounter the second stimulus they are now able to respond in a more efficient manner by recognizing the antigen immediately. Some of the T_{CM} develop into the effector memory T cells (T_{EM}). T_{EM} are memory cells as well as T_{CM} but CCR7 negative. This feature enables them to home directly to an infected or inflamed area with immediate effector functions such as secreting Interleukin 2 (IL-2) and other inflammatory cytokines[100, 101]. This response attracts other immune cells such as macrophages[102, 103].

Inhibition of $K_V1.3$ and $K_{Ca}3.1$ channels – other studies

$K_V1.3$

Many studies have examined the role of $K_V1.3$ and $K_{Ca}3.1$ in various diseases and disease models. $K_V1.3$ have been shown to play a role in autoimmune diseases such as type-1-diabetes, multiple sclerosis, and rheumatoid arthritis [104-107]. Positive results

were found in a rat type-1-diabetes-model, which suggested that pharmacological inhibition of $K_V1.3$ prevented the autoimmune destruction of the pancreatic islets[107]. In an experimental model of autoimmune encephalomyelitis (model of multiple sclerosis), the blockade of $K_V1.3$ also improved the course of the disease[79]. Additionally, pharmacological blockade of $K_V1.3$ was found to improve affected joints both radiologically and histopathologically in a model of rheumatoid arthritis in rats[107] IBD has also been suggested to have an autoimmune component [108] even though this remains elusive. Nevertheless, this is why the $K_V1.3$ and $K_{Ca}3.1$ channels could be important in IBD. It is known that T_{EM} ($CCR7^-$) use high numbers of $K_V1.3$ to control the membrane potential upon activation. Resting T_{EM} have approximately 300 $K_V1.3$ channels per cell, whereas activated T_{EM} have around 1,500 [106]. Additionally, T_{CM} and naïve T cells ($CCR7^+$) have 200-250 $K_V1.3$ channels in the resting state and about 300 after activation[106, 109].

Selective inhibitors of $K_V1.3$ are for instance ShK-L5 and PAP-1. ShK-L5 is the modified peptide originally from the toxin of the sea anemone Stichodactyla helianthus. This modification made the ShK-L5 peptide highly selective of the $K_V1.3$ channels[82]. PAP-1, the other $K_V1.3$ inhibitor, was synthesized in the laboratory of Heike Wulff at UC Davis with the aim to develop a small-molecule inhibitor instead of a peptide, thus entailing increased selectivity and potency. Contrary to the peptide, the small-molecule PAP-1 has the advantage that it can be taken orally [110].

Studies examining the role of $K_V1.3$ in animals are usually performed in rats rather than mice as human and rat T cells in this regard are similar. Rats use the same set of potassium channels ($K_V1.3$ and $K_{Ca}3.1$) to control calcium homeostasis after activation as humans in contrast to mice that in addition to $K_V1.3$ use several other voltage-gated potassium channels ($K_V1.1$, $K_V1.2$, $K_V3.1$), hence being unaffected by $K_V1.3$ inhibitors[80, 82, 111, 112].

Furthermore, it is not only in autoimmune diseases that $K_V1.3$ blockade is believed to play a role. Recently, pharmacological blockade of $K_V1.3$ was shown to improve the outcome in different disease models such as ovalbumin-induced asthma in rats, and oxazolone-induced contact dermatitis in rats, but also as an immunosuppressant comparable to cyclosporine in regards of preventing kidney allograft rejection in rats [113-115]. These findings suggest $K_V1.3$ as a key player in the inflammatory response in T cell mediated diseases. Interestingly, Peng et al. just reported that pharmacological blockade of $K_V1.3$ ameliorated radiation-induced brain injuries in mice[116]. The effect is believed to be due to the inhibition of constitutively expressed $K_V1.3$ channels in microglia (the macrophages of the brain), dendritic cells and lymphocytes causing inactivation and decreased production of inflammatory cytokines[59, 117]. In addition, $K_V1.3$ blockade causes proliferation of the neural progenitor cells[118]. Overall, these effects ameliorate the damages caused by radiation with no difference between the non-irradiated controls and mice subjected to $K_V1.3$ blockade[116].

$K_{Ca}3.1$

Previous studies have also examined the role of the $K_{Ca}3.1$ channel in different disease models. Upon T cell activation, the $K_{Ca}3.1$ channels are upregulated too, but in a different way than $K_V1.3$: resting T_{EM} only have about 35 $K_{Ca}3.1$ channels per cell and 50 after activation. In contrast, resting T_{CM} and naïve T cells have very low numbers of $K_{Ca}3.1$ (5-20 channels per cell) but after activation the number of $K_{Ca}3.1$ channels increases to about 500 per cell[106].

The $K_{Ca}3.1$ channels have been suggested to be involved in IBD as well as other diseases. Pharmacological blockade of $K_{Ca}3.1$ improved experimental colitis both in rats and mice[119-121], which emphasizes the importance of the $K_{Ca}3.1$ channel in experimental colitis. Moreover, in a study on $K_{Ca}3.1$ knockout mice it was shown that $K_{Ca}3.1$ channels were involved in chloride secretion thus decreasing electrolyte and water secretion causing stool dehydration[122]. Apart from IBD, $K_{Ca}3.1$ channels are suggested to be a novel target for treating various diseases such as idiopathic pulmonary fibrosis [123], airway inflammation (allergic asthma) [124] and ischemia/stroke[125] where the $K_{Ca}3.1$ channels seem to be beneficial. Senicapoc, a $K_{Ca}3.1$ blocker, was suggested as a treatment for sickle cell disease by inhibiting red blood cell dehydration thus increasing hemoglobin. Senicapoc showed positive results both in mice and humans, and there were no adverse effects compared to placebo[126, 127]. Senicapoc reached a phase 3 study. Yet, the trial was terminated before end of study, as it seemed impossible to reach the primary endpoint (reduction in sickle cell crises). Even though an increase in hemoglobin and decrease in hemolysis markers were observed, there was no difference in the number of sickle cell crises when comparing Senicapoc vs. placebo[128]. Senicapoc was the drug of choice for the $K_{Ca}3.1$ blocker in our animal study as it was already approved for a phase 3 trial.

CYTOKINE PROFILE OF ULCERATIVE COLITIS

T_H1, T_H2 and cytokine characteristics

The pattern of cytokine secretion has been traditionally divided into T_H1 and T_H2 type cytokine profiles. T_H1 and T_H2 are two polarized types of $CD4^+$ T helper cells that have very different types of cytokine profiles[129]. T_H1 cytokines include mainly IL-12 and IFN-γ that lead to release of downstream cytokines, such as TNF-α and IL-6, by activating macrophages and granulocytes; T_H2 cytokines include primarily IL-4, IL-5, IL-10 and IL-13[130-132]. It is generally believed that in UC there is a somewhat atypical T_H2 response in contrast to CD where the inflammatory response is believed to be more T_H1[31, 33]. However, also in UC some studies report an increased level of IFN-γ[133], suggestive of a T_H1 response, while others do not find evidence of a T_H1 response due to decreased levels of IFN-γ and IL-12[132, 134, 135]. The consensus on the atypical T_H2 response is based on studies showing that typical T_H2 cytokines are elevated and others are not. IL-4 and IL-5 are two of the key cytokines produced by T_H2 cells and UC patients reveal unchanged levels of IL-4 and increased levels of IL-5 compared to controls [132, 135-137]. In 2004, Fuss et al. showed that IL-13 production was indeed increased when stimulating lamina propria mononuclear cells from UC patients compared to CD and controls[135]. These findings support an "atypical" T_H2 response rather than a T_H1 response.

Recently, IL-23, a cytokine produced by antigen-stimulated dendritic cells and macrophages, was found to stimulate naïve $CD4^+$ T cells to differentiate into IL-17 secreting T helper cells called T_H17. IL-17 facilitates the secretion of more proinflammatory cytokines such as IL-6 and TNF-α from other cell types thus leading to an increased inflammatory response[138, 139].

Nevertheless, there are still a lot of unknown factors when it comes to initiating and maintaining the disease-specific inflammation seen in UC.

NOVEL MEDICAL TREATMENTS FOR ULCERATIVE COLITIS

For UC, there are some promising treatments currently being investigated in phase II and III trials. Vedolizumab, a humanized monoclonal antibody that blocks $\alpha_4\beta_7$ integrin, thus inhibiting the interaction with the mucosal addressin-cell adhesion molecule 1 (MAdCAM-1) on the intestinal vasculature, blocks the B and T lymphocyte trafficking into the inflamed tissues and has shown a positive effect on inflammation in a phase 3 trial in 2013[140-142]. Vedolizumab already demonstrated promising results for induction and maintenance therapy of UC.

Etrolizumab, another adhesion molecule blocker, is currently in phase 2 [143] after phase 1 studies were finished positively. Etrolizumab blocks the β_7 subunit of the same integrin blocked by Vedolizmab but it also targets $\alpha E\beta_7$ integrin[144, 145]. Etrolizumab was presented at the Digestive Disease Week 2013 where beneficial effects were reported compared to placebo and guaranteed to proceed to phase 3[146]. These new adhesion molecule blockers could be a novel way of targeting the mucosal inflammation in UC.

Another promising option is Tofacitinib, a Janus Kinase (JAK) inhibitor, which is currently recruiting to phase III trial. Tofacitinib primarily inhibits the T_H2 and T_H17 responses[147] and significant improvement of inflammation was demonstrated compared to controls in the phase 2 trial[148].

Overall, current medical treatment for UC is insufficient in a number of patients as documented by the need for surgery in patients with severe disease and as patients may experience relapses in spite of intensive treatment. So, there is a clear need for new treatment options and adhesion molecules may improve handling of the disease in some patients. Evidently, lymphocytes play a role in the inflammatory process of IBD, but we also need new pathomechanistic insights, which may then lead to the discovery of novel pharmacological targets and the development of novel molecular modulators.

In this PhD project, we therefore aimed to identify other lymphocyte treatment targets by investigating the role of T cell potassium channels, $K_V1.3$ and $K_{Ca}3.1$, in UC and to some extent CD.

SPECIFIC HYPOTHESES

Before commencing the studies we formulated the following hypotheses:

Study 1

Expression of T-cell $K_V1.3$ potassium channel correlates with pro-inflammatory cytokines and disease activity in ulcerative colitis

1) High expression of $K_V1.3$ and $K_{Ca}3.1$ in T cell infiltrates in the inflamed mucosa of UC patients correlates with disease activity and synthesis of the pro-inflammatory cytokines TNF-α, IFN-γ, and IL-17A.
2) In vitro blockade of $K_V1.3$ and $K_{Ca}3.1$ on human $CD3^+$ T cells decreases production of TNF-α, IFN-γ, and IL-17A.
3) Mucosal mRNA expression levels of $K_V1.3$ and $K_{Ca}3.1$ at inclusion predict the risk of relapse due to increased levels of $K_V1.3^{high}$ T cells.

Study 2

Pharmacological inhibition of $K_V1.3$ and $K_{Ca}3.1$ in Dextran Sodium Sulfate-induced colitis in rats

1) Oral administration of PAP-1, a $K_V1.3$ blocker, ameliorates DSS-induced colitis in rats assessed by endoscopy, weight, and histological inflammation.
2) Oral administration of PAP-1 and Senicapoc, blockers of $K_V1.3$ and $K_{Ca}3.1$, ameliorates DSS-induced colitis in rats significantly more than PAP-1 alone assessed by endoscopy, weight, and histological inflammation.
3) Messenger-RNA expression levels of T cell potassium channels, $K_V1.3$ and $K_{Ca}3.1$, together with pro-inflammatory cytokines, IFN-γ, TNF-α and IL-17A, are increased in DSS-induced colitis rats compared to uninflamed controls.

Study 3

Expression of T-cell $K_V1.3$ potassium channels is increased in mucosal biopsies from patients with active Crohn's disease

1) The mRNA expression levels of $K_V1.3$ and $K_{Ca}3.1$ are increased in mucosal biopsies from patients with active CD compared to controls, and correlates with disease activity and the pro-inflammatory cytokines: IFN-γ, TNF-α, and IL-17A.

MAIN METHODS

The methods are described in details in manuscript I and II. In the following, additional information about the methods used during the project is provided, but only the methods shortened in the manuscript.

Inclusion, exclusion and data storage

Inclusion criteria

- Above 18 years of age
- Previously diagnosed with UC and scheduled for acute/subacute endoscopy due to relapse or if newly referred to endoscopic evaluation because of symptoms compatible with UC (typically bloody diarrhea).
- The control group consisted of persons scheduled for endoscopy at outpatient clinics due to cancrophobia, control endoscopy after previous polyp removal, and moreover with no signs of inflammatory bowel disease when assessed during endoscopy).

Exclusion criteria

- In need of an interpreter
- Active treatment with anticoagulants, non-steroidal anti-inflammatory drugs(NSAID) or acetylsalicylic acid
- Pregnancy or lactation
- Positive pathogenic stool cultures
- Endoscopy contraindicated
- For controls, diarrhea was an exclusion criterion as we did not want any hidden or undiagnosed IBD

Other variables that were registered at inclusion
We registered the following variables at inclusion: age, sex, mayoscore, medical treatment, weight, height, smoker, and autoimmune diseases.

Place of inclusion and data storage
Participants were included at the outpatient clinics at Odense University Hospital and at the Hospital of South-West Jutland in Esbjerg, both hospital are located in Denmark. The participants were included after verbal and written informed consent, which were retained at the Dept. of Gastroenterology at the Hospital of South-West Jutland, Esbjerg, and Odense University Hospital throughout the study and are available on demand by the Danish authorities. Patient data were handled anonymously, inaccessible to the public and social security numbers were encrypted with Truecrypt 7.1a (256-bit)[149].

Ethics statement
The study was approved by the Danish Ethics Committee (permit no. S-20110007) and data collection was approved by the Danish Data Protection Agency (permit no. 2008-58-0035).

Collection of biopsies
Blood samples (C-reactive protein (CRP), leucocyte count, fecal calprotectin, stool cultures, and endoscopy were performed the same day. Fecal calprotectin samples were collected prior to performing the endoscopy as calprotectin levels increase due to leaking of neutrophils from the mucosal biopsy site into the feces[150, 151]. During endoscopic examination of UC patients with relapse, biopsies were obtained from the inflamed rectal mucosa and from a "healthy looking" part of the colon if possible. The "healthy looking" part of the colon must be in the next segment (sigmoid, transverse, or the ascending colon) and have a Mayo Endoscopic Subscore of 0. The control population only had biopsies taken from the rectal mucosa.
Biopsies were immediately stored at 4 °C in sterile 2.0 mL tubes containing RNAlater® (Ambion, Austin, TX, USA). According to manufacturer's instructions biopsies were stored overnight at 4°C. The next day we proceeded with either RNA isolation or they were put in a -80 °C freezer for later processing.

mRNA isolation, DNase digestion and cDNA synthesis

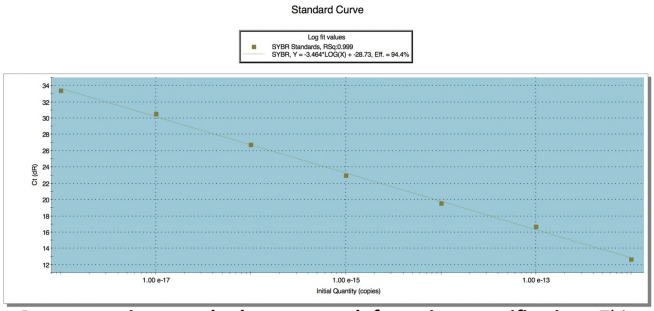
Isolation of mRNA
The RNA-stabilized biopsies were cut into pieces and homogenized thoroughly with a power homogenizer (Kinematica Polytron PT3100, Switzerland). Total RNA was isolated from the biopsies using TRIZOL reagent® (Invitrogen, United Kingdom) following the manufactures instructions. For each biopsy 800 µL TRIZOL reagent® was used. Depending on the pellet size at the end of the procedure RNA was dissolved in 30-50 µL RNase free water. The total RNA concentration was measured as duplicates on a nanophotometer (Implen, Germany). Absorbance rate (A260/280) of purified RNA was measured to assess the purity of the RNA and were accepted if A260/280 absorbance rate was >2.0. Afterwards we proceeded with the cDNA synthesis or stored the RNA at -80 °C for later processing.

DNase digestion
The RNase-Free DNase Set (Qiagen, Germany) for DNase digestion. 2 µg of RNA from the human biopsies were mixed with 3 µL of the RNase-free buffer and 1 µL RNase-free DNase. RNase-free water was added to a total volume of 30 µL. The mixture was incubated on Eppendorf Mastercycler Personal: 25 °C for 10 minutes, 75 °C for 5 minutes, and hold at 4 °C.

Synthesis of cDNA
The iScript cDNA Synthesis Kit (BioRad, CA, USA) was used to create complementary DNA (cDNA). Two reactions were made: one with reverse transcriptase (+RT) and one without (-RT). Each reaction consisted of 15 µL of the DNase reaction and 8 µL 5x iScript Reaction Mix. 2 µL of iScript Reverse Transcriptase were added to the +RT reaction. Nuclease free water was added to both reactions, to a total volume of 40 µL.
The reverse transcription was completed with the following steps of incubation on the Eppendorf Mastercycler Personal: 25 °C for 5 minutes, 42 °C for 30 minutes, 85 °C for 5 minutes, Hold at 4 °C. RNA input in the cDNA synthesis reaction is 500ng/20µL reaction (25ng/µL). The synthesized cDNA was used immediately to perform quantitative real time polymerase chain reactions (qPCR) or stored at -20 °C for processing within two weeks to avoid degrading of the DNA.

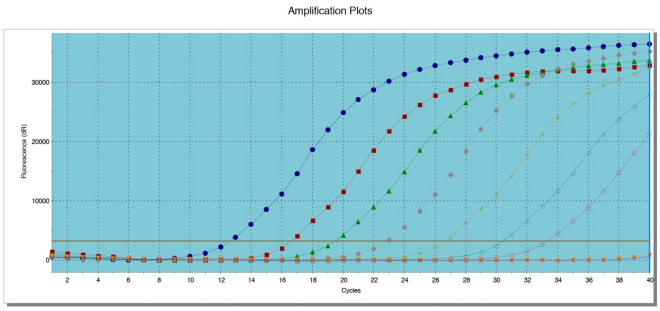
Primer design, PCR efficiency, and qPCR setup

Quantitative Real Time Polymerase Chain Reaction
Quantitative PCR was performed on Stratagene MX3000P qPCR instrument (Agilent Technologies, Santa Clara, CA, USA). The following primers were used: GAPDH as reference gene; $K_{Ca}3.1$ and $K_V1.3$ as our target genes; IFN-γ, TNF-α, IL-10 and IL-17A as cytokines involved in acute and chronic inflammation; CD4 and CD8 as T-cell markers, and CD14 and CD16 as a marker of resting or activated monocytes/macrophages respectively (Table 2 in Manuscript 1). Quantitative real-time PCR (qPCR) was performed using SYBR Green Supermix (Bio-Rad, CA, USA) according to the MIQE guidelines[152] (except from using only one reference gene). All primers were obtained from Sigma-Aldrich (St. Louis, MO, USA) and created using the PubMed Gene Bank[153], Primer3 Web[154], and the Basic Local Alignment Search Tool (BLAST)[155] to be sure that the designed primers targeted only the gene of interest. Moreover, primer efficiencies (PCR efficiencies) were determined by serial dilutions to make sure that the amplification at each cycle was close to 100% (See figure below). All primers were found to have efficiency between 90-105%. The melting curves of the PCR products were also evaluated after each PCR run to make sure that the correct product was amplified and that only one product was present (See figure on next page).



Representative standard curve used for primer verification. This sample curve was done as a part of the primer efficiency test for the $K_V1.3$ primer. Serial dilutions from 1 x 10^{-12} to 1x 10^{-18} were performed. Afterwards the efficiency of the PCR reactions was calculated. In this example the PCR efficiency for $K_V1.3$ is 94.4%.

The PCR product size was also verified by agarose gel electrophoresis. Each qPCR reaction was made using 2µL of cDNA template (+RT), 1 µL of each primer (forward and reverse), 12 µL iQ™ SYBR Green Supermix (Bio-Rad) and adding RNase free water to a total volume of 24 µL. The final primer concentration was 400 nM. All genes were tested in duplicates and moreover both non-template-controls and –RT reactions for each gene were used. The mix was loaded on a 96 well plate (Mx3000P 96-well plates, Agilent Technologies) and sealed with microseal film (Bio-Rad). Evaluation of fluorescence signals the threshold was set to 4000, matching the exponential phase of the amplification. The qPCR cycles were run as shown here:



Representative Melting curves and amplification plot used for primer verification. Amplification plots of the $K_V1.3$ primer pair tested in serial dilutions in concentrations from 1 x 10^{-12} to 1x 10^{-18} (top). The second picture shows the melting curve of $K_V1.3$ product amplified at the different dilutions. The separate dilutions (depicted by an individual color and symbol) show only one product, the $K_V1.3$ gene. The two flat lines in the bottom (orange triangle and blue X) are the non-template controls, which should not show any amplified product.

Table showing the qPCR setup

	Temperature	Duration	Cycles
Denaturation	95 °C	3:00 min.	1 Cycle
Denaturation	95 °C	0:20 min.	
Annealing	60 °C	0:20 min.	40 Cycles
Extension	72 °C	0:20 min.	
Denaturation	95 °C	1:00 min.	1 Cycle
Melting point curves	55-95 °C	**≈ 15:00 min.**	

In polymerase-chain-reactions, the denaturation at 95°C is performed to separate the DNA strand by disrupting the hydrogen bonds that holds them together. The separation of DNA strands and lowering of temperature to 60°C (Annealing phase) allows the primers to attach to their specific sequence on the single-stranded DNA. The hydrogen bonds between the primer and DNA are only formed when the base-sequence matches closely. The last step is the extension phase. Here, the temperature is increased to 72°C to activate the Taq-polymerase, which starts synthesizing the complementary DNA strand. These steps (Denaturation, Annealing, Extension) are repeated for 40 cycles and each cycles doubles (according to the primer efficiency) the number of amplicons causing an exponential amplification (figure below).

The mRNA expressions were presented as "percentage of

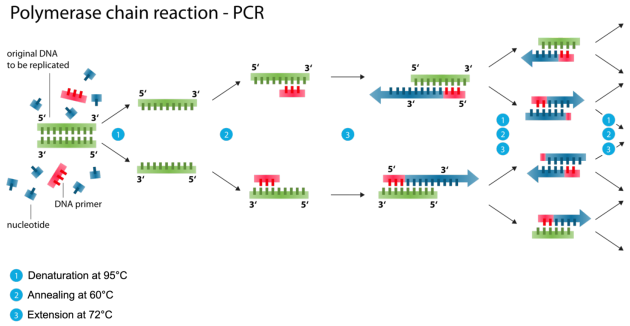


Illustration of the amplification process during the polymerase chain reaction (adapted from [156])

GAPDH" (%GAPDH). No significant difference was found in GAPDH expression between UC and controls (please refer to additional results section). The ΔCq-value and the percentage of GAPDH (% GAPDH) were calculated as follow:

$$\Delta\mathrm{Cq} = \frac{\left(Cq_{product(1)} + Cq_{product(2)}\right)}{2} + \frac{\left(Cq_{GAPDH(1)} + Cq_{GAPDH(2)}\right)}{2}$$

$$\%\ \boldsymbol{of\ GAPDH} = \frac{1}{2^{\Delta\mathrm{Cq}}} \times 100$$

Quantitative real-time polymerase chain reaction data exclusion criteria

- Cq-value (formerly known as Ct-value) of GAPDH above 25, indicating low amounts or bad quality of cDNA/mRNA)
- Cq-values in a double determination differed by more than 2 Cq-values (inconclusive data)
- Non-exponential amplification slopes (false-positive, incomplete reaction)
- Wrong product size or if more than one product assessed by the respective melting curves.

Immunohistochemical staining

Immunohistochemical (IHC) stainings were done on the same paraffin-embedded biopsies cut in 5 µm slices as we used for the pathological assessment. Tissue-Tek® Tissue-Clear® was used to deparaffinize the slides; three baths of 3.5 minutes each. After deparaffinisation slides were hydrated through an ethanol gradient from 99.9% ethanol to mQ water; two baths of 99.9% ethanol, two baths of 95% ethanol, two baths of 70% ethanol and then 5 minutes in mQ water. After hydration the slides were put in TBS (Tris-Buffered Saline) with 1.5% H_2O_2 for 10 minutes. Afterwards, Heat-Induced Epitope Retrieval (HIER) was used to enhance the antibody/antigen binding capability. All antibodies were tested using three different buffers (Citrate (Dako #S2031), Tris-EGTA (TEG), and TRS (Dako #S1699)) and in serial dilutions to

find the best HIER buffer and the optimum concentration of the antibodies.
The primary antibodies were identified by Horseradish Peroxidase-labeled secondary antibodies (DAKO Envision ™+ Kit, Glostrup, Denmark). DAB+ (DAKO) was used as substrate-chromogen system. IHC slides were counterstained with Hematoxyline. We used the following antibodies: CD3 (AB Serotec, #MCA1477), CD4 (Thermo Scientific, #MA5-12259), CD8 (AB Serotec, #MCA1817), Macrophage/L1 molecule (MAC) (AB Serotec, #MCA874G), K_V1.3 (Novus Biologicals, #NBP1-19415) and K_{Ca}3.1 (Sigma-Aldrich, #AV35098).

Immuno-fluorescent staining
Immunofluorescent stainings (IF) were run in the same way as standard IHC. We followed the same protocol until the primary antibody was washed off and then the secondary antibody was applied (combined with fluorochromes: Alexa Fluor 488 and 568). Nuclei staining were done with 4'-6-Diamidino-2-phenylindole dihydrochloride (DAPI). DAPI binds to the A-T region of DNA and can stain the nuclei of both fixed and live cells. Afterwards, coverslips were mounted. We used the same antibodies as we used for IHC, and they were all tested in IHC before proceeding to IF.

Follow-up data
Two and a half years after the first patient was included in the study we reviewed patient files to verify if the patients had had a clinical relapse. A relapse was defined by symptoms compatible with active UC combined with alterations in treatment by the patient's gastroenterologist. Not all relapses were verified by endoscopic examination. If the relapse was only identified by phone or in the outpatient clinic, we used the date of contact. We then identified the "days to relapse from the inclusion date" and "days to relapse after initial remission was achieved". Furthermore, we registered the "days to achieve remission" to investigate if it was possible to identify patients that responded to initiated treatment.
In addition to the manual review of patient files, UC patients were asked to participate in additional follow-ups after 4 weeks, 8 weeks, and after 12 months (or earlier if they got relapses). At the follow-up we repeated the biopsies from the inflamed (if present) and un-inflamed mucosa. These biopsies were collected to investigate whether the expression levels of K_V1.3 and K_{Ca}3.1 channels decrease in parallel with the individual patient's mucosal inflammation over time.

RESULTS

MANUSCRIPT 1: EXPRESSION OF T-CELL K_V1.3 POTASSIUM CHANNEL CORRELATES WITH PRO-INFLAMMATORY CYTOKINES AND DISEASE ACTIVITY IN ULCERATIVE COLITIS

Authors:
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Abstract

Background:
Potassium channels, K_V1.3 and K_{Ca}3.1, have been suggested to control T-cell activation, proliferation, and cytokine production and may thus constitute targets for anti-inflammatory therapy. Ulcerative colitis (UC) is a chronic inflammatory bowel disease characterized by excessive T-cell infiltration and cytokine production. It is unknown if K_V1.3 and K_{Ca}3.1 in the inflamed mucosa are markers of active UC. We hypothesized that K_V1.3 and K_{Ca}3.1 correlate with disease activity and cytokine production in patients with UC.

Methods:
Mucosal biopsies were collected from patients with active UC (n=33) and controls (n=15). Protein and mRNA expression of K_V1.3 and K_{Ca}3.1, immune cell markers, and pro-inflammatory cytokines were determined by quantitative-real-time-polymerase-chain-reaction (qPCR) and immunofluorescence, and correlated with clinical parameters of inflammation. In-vitro cytokine production was measured in human $CD3^+$ T-cells after pharmacological blockade of K_V1.3 and K_{Ca}3.1.

Results:
Active UC K_V1.3 mRNA expression was increased 5-fold compared to controls. Immunofluorescence analyses revealed that K_V1.3 protein was present in inflamed mucosa in 57% of $CD4^+$ and 23% of $CD8^+$ T-cells. K_V1.3 was virtually absent on infiltrating macrophages. K_V1.3 mRNA expression correlated significantly with mRNA expression of pro-inflammatory cytokines TNF-α (R^2=0.61) and IL-17A (R^2=0.51), the mayo endoscopic subscore (R^2=0.13), and histological inflammation (R^2=0.23). In-vitro blockade of T-cell K_V1.3 and K_{Ca}3.1 decreased production of IFN-γ, TNF-α, and IL-17A.

Conclusion:
High levels of K_V1.3 in CD4 and CD8 positive T-cells infiltrates are associated with production of pro-inflammatory IL-17A and TNF-α in active UC. K_V1.3 may serve as a marker of disease activity and pharmacological blockade might constitute a novel immunosuppressive strategy.

MANUSCRIPT 2: PHARMACOLOGICAL INHIBITION OF K_V1.3 AND K_{CA}3.1 IN DSS-INDUCED COLITIS IN RATS

Authors
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[Unpublished]

Abstract

Background

Ulcerative colitis (UC) is a chronic inflammatory bowel disease that involves patients from the entire world. The key symptom is bloody diarrhea. Treatments have been improved over the last decades especially because of usage of monoclonal antibodies against TNF-α, but there is still a need for improved treatment options, especially to avoid side effect by systemic immunosuppression. T cell potassium channels, $K_V1.3$ and $K_{Ca}3.1$, are increased in the inflamed mucosa in UC patients and PAP-1 and Senicapoc are known to be blockers of $K_V1.3$ and $K_{Ca}3.1$ respectively. Dextran sodium sulfate (DSS)-induced colitis is a well-established experimental model of UC. Human and rat T cells share the same set of potassium channels to control their activity, thus the DSS-induced colitis in rats is a relevant model of UC in humans. In this study, we hypothesized that pharmacological blockade of $K_V1.3$ and $K_{Ca}3.1$ ameliorate DSS-induced colitis in rats.

Methods

Forty-one Sprague Dawley rats were randomized into 4 groups. One group with untreated animals (n=5) and 3 DSS groups (n=12) where colitis was induced using 5% DSS in drinking water for 5 days and 1% DSS to maintain a chronic inflammatory response until sacrifice at day 21. One group received vehicle (Miglyol), one group PAP-1 (50 mg/kg), and one group PAP-1 + Senicapoc (50 mg/kg). Video endoscopy was done at day 0, 7, 10 and 14 to score inflammation. Moreover, histological evaluation of inflammation, edema, and mucosal necrosis was performed. Plasma concentrations of PAP-1 and Senicapoc were determined by Ultra Performance Liquid Chromatography – tandem Mass Spectrometry. Gene expression in colonic samples was assessed by qPCR.

Results

Measurement of plasma concentration revealed low levels of PAP-1 and Senicapoc. A trend towards amelioration of endoscopic inflammation was found on day 7 and 10 ($p=0.07$ and $p=0.08$). Overall, no significant differences in endoscopic inflammation, histological inflammation, edema, or necrosis were observed between groups receiving DSS. Gene expressions of $K_V1.3$, TNF-α, and IL-17A were increased in DSS vehicle group compared to controls. No differences in gene expression were observed between the expression of the three DSS groups: vehicle, PAP-1, and PAP-1 + Senicapoc.

Conclusion

Pharmacological blockade of $K_V1.3$ (PAP-1) and $K_{Ca}3.1$ (Senicapoc) did not ameliorate DSS-induced colitis in rats when administered by oral gavage in Miglyol at 50 mg/kg assessed by endoscopic and histological inflammation, however a trend was found towards amelioration of endoscopic inflammation on day 7 and 10 but the effect faded subsequently. The absence of an effect on inflammation may be due to 1) insufficient enteral uptake of PAP-1 and Senicapoc, 2) T cell potassium channels not being important for the DSS-induction of colitis in rats. Further studies should apply higher doses or parenteral administration of the drugs.

Introduction

Ulcerative colitis (UC) is a chronic inflammatory bowel disease affecting an increasing number of adults as well as children worldwide [1]. The classical symptoms of UC are mucilaginous and bloody diarrhea. Anti-inflammatory drugs such as 5-aminosalicylates (mesalazine) and corticosteroids are first line medications for the treatment of UC to induce remission [2]. For maintaining remission 5-aminosalicylates is the mainstay together with drugs affecting the immune system such as azathioprine, 6-mercaptopurine, and anti-tumor necrosis factor-α (anti-TNF-α). Severe relapses in patients with UC require admission and intravenous corticosteroids. If corticosteroids fail to induce remission, cyclosporine (a calcineurin inhibitor) or anti-TNF-α may be used as rescue therapy [2]. In a number of patients treatment is not satisfactory documented by chronic disease activity and frequent relapses, thus new treatment modalities are warranted. These new and promising treatments all address lymphocyte response. One target is the blocking of integrins or the integrin subunits, which prevent the lymphocytes from interacting with the mucosal addressin cell adhesion molecule-1 (MAdCAM-1) thus blocking the trafficking into the inflamed tissues from the vascular bed [3, 4]. The second target is to inhibit the Janus Kinase (JAK), which inhibits the T_H2 and T_H17 [5] response thus decreasing the production of inflammatory cytokines. These promising treatments are already in phase 2 and 3 trials [4, 6-8].

The development of animal models that reflect aspects of UC biology have contributed to the evaluation of therapies for IBD[9]. The dextran sodium sulfate (DSS) induced colitis in rodents is a well-established model of UC and has been used for testing different treatments over time, e.g. mesalazine, cyclosporine, and anti-TNF-α [10-12]. The DSS-induced colitis has an acute and a chronic inflammatory response that includes T helper cell (T_H) response [13, 14]. However, while some studies report an increased interferon-γ production in UC when compared to controls, other studies do not find any difference in IFN-γ production and rather report that UC is characterized by an atypical T_H2 response (the typical T_H2 response's key cytokine is IL-4), whereas Crohn's disease (CD) is believed to be T_H1 driven (key cytokine IFN-γ) [1, 15]. In the DSS-induced colitis model, oral administration of DSS damages the colonic mucosa and the resulting tissue damage and inflammation leads to the development of diarrhea and rectal bleeding. The tissue damage and inflammation observed in this model is restricted to the mucosa and generally affects the entire colon and rectum with a more severe disease in the distal colon mimicking UC in humans. We chose this model because, unlike mice, T cells from rats and humans use the same set of potassium channels to regulate the membrane potential [16]. When the T cell is stimulated via the T cell receptor causing a depolarization, the T cell potassium channels, $K_V1.3$ and $K_{Ca}3.1$, counteract the depolarization by an efflux of K^+ resulting in a hyperpolarization thus keeping the Ca^{2+} influx steady through the Ca^{2+} release-activated Ca^{2+} channels (CRAC) channels. The resulting sustained rise in intracellular Ca^{2+} is crucial for T cell cytokine production and proliferation [17]. In line with the $K_{Ca}3.1$ channels in T cells, pharmacological blockade

of $K_{Ca}3.1$ has been shown to ameliorate T cell-mediated colitis in rats [18] and in mice [19, 20], but only very little is known about $K_V1.3$ and inflammatory bowel diseases.
Previously, our group found that T cell $K_V1.3$ protein and mRNA expression were significantly increased in colonic biopsies from patients with active UC compared to controls [21]. $K_{Ca}3.1$ mRNA expression were unchanged but $K_{Ca}3.1$ was abundant in T cell infiltrates.

In the present study, we hypothesized that pharmacological inhibition of $K_V1.3$ and $K_{Ca}3.1$ with PAP-1 and Senicapoc alleviates mucosal inflammation in DSS-induced colitis in rats.

Materials and methods

Compounds
To block the T cell potassium channels we used PAP-1 (Patent # US20060079535) as a specific blocker of $K_V1.3$ (IC_{50} = 2 nM) [22] and Senicapoc (ICA-17043) as a blocker of $K_{Ca}3.1$ (IC_{50} = 11 nM) [23]. PAP-1 and Senicapoc were synthesized at the Wulff Laboratory, Dept. of Pharmacology, University of California, Davis, CA, USA. PAP-1 and Senicapoc were given at a concentration of 50 mg/kg by oral. The vehicle consisted of MIGLYOL 812 at 2 µL/g bodyweight instead of oils containing longer carbon chains and polyunsaturated fatty acids in order to avoid potential anti-inflammatory effects.

Experimental design
Forty-one male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA, USA) weighing 200 to 250 g were acclimatized for 3 days and randomized into 4 groups: a group of 5 untreated controls (no DSS) and 3 groups of 12 rats that were treated with DSS + vehicle, DSS + PAP-1, and DSS + PAP-1 + Senicapoc, respectively (Table 1).

Animals were kept in standard laboratory conditions (room temperature of 21°C/70°F with a controlled 12 hours light/dark cycle and free access to animal chow and water). All animals were fed with sterile Purina LabDiet® 5053 (LabDiet, St. Louis, MO, USA). Colitis was induced by exposure 5% DSS (Cat. #160110, MP Biomedicals, Solon, OH, USA) in drinking water from Days 0-5 and afterwards continued on a maintenance dose of 1% DSS in the drinking water thus mimicking both acute and semi-chronic colitis, respectively. The volume administered was 0.2 mL/100g for each animal. Study endpoints were endoscopy colitis score, body weight change, survival, colon length, colon weight, and colon histology. All animals were sacrificed on day 21. Blood was drawn (EDTA) for determination of plasma levels of PAP-1 and Senicapoc by Ultra Performance Liquid Chromatography – tandem Mass Spectrometry (UPLC/MS). The colon was excised and measured (length) after removal of the faeces .

The colon was trimmed to a length of 8 cm: the distal 3 cm and the proximal 3 cm were stored in formalin and embedded in paraffin. The remaining 2 cm was put in RNAlater and used for quantitative real-time PCR analyses (qPCR).

Evaluation of endoscopic inflammation
Each rat underwent video endoscopy (Karl Storz Endoskope, Tuttlingen, Germany) under isoflurane anesthesia on days 7, 10, 14, and 21. During each endoscopic procedure still images as well as video were recorded to evaluate the extent of colitis and the response to treatment. A blinded observer scored the endoscopic inflammation using a 0-4 scale (0= normal mucosa; 1= loss of vascularity; 2= loss of vascularity and friability; 3= friability and erosions; 4= ulcerations and bleeding).

Evaluation of histological inflammation
The colon samples (distal and proximal part as describe in the above) were trimmed into 7-10 equally spaced transverse sections. Tissues were embedded in paraffin and sectioned at approximately 5 microns. One slide for each colon (with all transverse sections per slide) was stained with hematoxyline and eosin (HE) and examined by a board-certified veterinary pathologist who was blinded to the treatment each group received at the time of assessment. Tissues were scored for inflammation, edema, and mucosal necrosis according to the scoring criteria listed below in Table 2. Each of the transverse sections was scored for these 3 parameters and the mean was reported for each animal for each parameter along with the mean sum score, which is simply the sum of the three individual parameter scores.

RNA preparation, DNase digestion, cDNA synthesis and quantitative Real Time PCR
RNA was initially isolated from biopsies using TRIZOL reagent® (Invitrogen, United Kingdom) and RNA concentrations were measured as duplicates on a nanophotometer (Implen, Germany). Absorbance rate (A260/280) of purified RNA was measured to assess the purity of the RNA and were accepted if the A260/280 absorbance rate was >2.0. Dextran Sodium Sulfate is known to inhibit PCR reactions[18] and therefore, we purified the RNA using the Dynabeads® mRNA Direct™ Purification Kit (life-technologies, Carlsbad, CA, USA) according to manufacturers instruction. Afterwards, we proceeded with DNase digestion. Thermo Scientific DNase I, RNase-free kit was used for DNase digestion according to the manufacturers instructions. Complementary DNA (cDNA) was synthesized using iScript cDNA Synthesis Kit (Bio-Rad, CA, USA) and quantitative real-time PCR (qPCR) was performed according to the MIQE guidelines[24] (except from using only one reference gene) using SYBR Green Supermix (Bio-Rad, CA, USA) on a Strata-

Group #	Number of rats	Treatment (p.o.) 0.2mL/100g	Dosing Schedule	DSS	Video Endoscopy
1	5 males	Untreated Control	---	---	Days 7, 10, 14, and 21
2	12 males	Vehicle (Miglyol only)	Days 0-21	5% Day 0-5 1% Day 6-21	Days 7, 10, 14, and 21
3	12 males	PAP-1 (50 mg/kg)	Days 0-21	5% Day 0-5 1% Day 6-21	Days 7, 10, 14, and 21
4	12 males	PAP-1 (50mg/kg) + Senicapoc (50mg/kg)	Days 0-21	5% Day 0-5 1% Day 6-21	Days 7, 10, 14, and 21

Table 1: Study overview

gene MX3000P qPCR instrument (Agilent Technologies, Santa Clara, CA, USA). The efficiencies of all primers where calculated using serial dilutions and found to be between 90-105%. The qPCR plates were run in duplicates with all samples (n=41) on the same plate to decrease plate-to-plate variation. Data were excluded if: Cq-values of GAPDH were above 25, Cq-values differed more than 2, non-exponential amplification curves, melting curves indicating more than 1 product. Primer sequences are shown in table 3.

Table 2: Histopathologic Scoring Criteria

Inflammation

Score	Description
0	None present
1	Rare foci; minimal
2	Scattered aggregates or mild diffuse inflammation
3	Numerous aggregates or moderate diffuse inflammation
4	Marked diffuse inflammation

Edema

Score	Description
0	None present
1	Rare foci; minimal
2	Scattered regions or mild diffuse edema
3	Numerous regions or moderate diffuse edema
4	Marked diffuse edema

Mucosal Necrosis

Score	Description
0	None present
1	<25% of the mucosa affected
2	26-50% of the mucosa affected
3	51-75% of the mucosa affected
4	>76% of the mucosa affected

Ethics statement

The animal study was approved by the Institutional Animal Care and Use Committee (approval number 12-1231-02) and followed good work practices for all laboratory procedures. The experiments took place at the animal facility at Biomodels LLC (Watertown, MA, USA), which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (Frederick, MD, USA).

Statistics

Statistical differences between treatment groups and the vehicle group were determined using 1 and 2-way ANOVA with Tukey's or Holm-Sidak's Multiple Comparison post-hoc test (and Kruskal Wallis with Dunn's multiple comparison test if non-Gaussian data). Results were considered significant if $p < 0.05$. Analyses were performed using GraphPad Prism 6.0d (GraphPad Software Inc., La Jolla, CA, USA).

Table 3: Primers specifications

Primer	Size (bp)	NCBI reference sequence	Sense	Anti-sense
GAPDH	207	NM017008.4	AGACAGCCGCATCTTCTTGT	CTTGCCGTGGGTAGAGTCAT
K_V1.3	232	NM019270.3	CTCTGCCAGTTCCCTGAGAC	CACGGAACTTCTCCATAGCC
K_{Ca}3.1	187	NM023021.2	TTATGCCAAAGAGATGAAGG	CATGGAATTCACTTGTTCCC
IFN-γ	119	NM138880.2	GAAAGCCTAGAAAGTCTGAAG	AGTATTTTCGTGTTACCGTC
TNF-α	195	NM012675.3	CTCACACTCAGATCATCTTC	GAGAACCTGGGAGTAGATAAG
IL-17A	118	NM1106897.1	AAAGTCCTCAACTCCCTTAG	CAGAAGGATATCTATCAGGGTC

Results

Colitis induction and endoscopic inflammation score

To assess that the inflammation was induced correctly and to follow treatment response over time, animal underwent endoscopic examination at day 7, 10, 14 and 21. In regards to the induction of colitis, we found that all DSS-treated rats (Vehicle, PAP-1, PAP-1 + Senicapoc) had developed colitis at day 7 compared to the non-DSS control group, which implies that the induction of colitis was successful (figure 1 and 2).

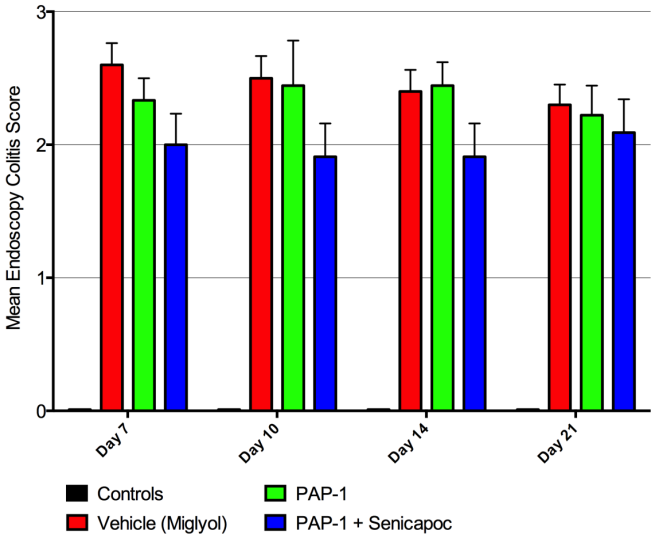

Figure 1. Mean Endoscopy Colitis Scores. Animals underwent video endoscopy on Day 7, 10, 14 and 21. Colitis severity was scored on a scale of 0-4. Data represent group means and standard error of the mean (SEM). No significant difference was observed at any time point when comparing PAP-1 and PAP-1 + Senicapoc to the Vehicle group, however an encouraging trend towards a decrease in endoscopic inflammation score was found on day 7 and 10.

By the pharmacological inhibition of T cell potassium channels, K_V1.3 and K_{Ca}3.1, we observed a promising trend towards amelioration of the endoscopic inflammation although it failed to become statistically significant (Figure 1). The concomitant administration of PAP-1 and Senicapoc consistently decreased endoscopy scores, particularly in the acute induction phase on Day 7 and 10 compared to vehicle group (p=0.07 and p=0.08). No significant differences were found in endoscopic inflammation between the vehicle group and PAP-1 group at any day.

Adverse events and body weight

No unanticipated deaths were observed in either group during the animal trial. Moreover, all groups showed a consistent weight gain over the course of the study. No other adverse effects were observed. For the 3 DSS treated groups (Vehicle, PAP-1, PAP-1 + Senicapoc) the mean weight at day 21 were 365.7 grams, 362.8 grams, and 356.3 grams respectively. There were no statistically

Figure 2. Representative Endoscopy Images. Animals underwent video endoscopy on Day 7, 10, 14 and 21. Images were captured from each animal during the procedure and representative images from each treatment group are presented.

significant differences in mean body weight or in mean percent body weight change among any of the groups in comparison with the Vehicle Control Group at any day (all p values above 0.76) (Figure 3).

Colon length and weight

Upon sacrifice the rat colons were removed and measured. None of the treatments had any significant effect on colon length or colon weight when compared to the vehicle group (Figure 4).

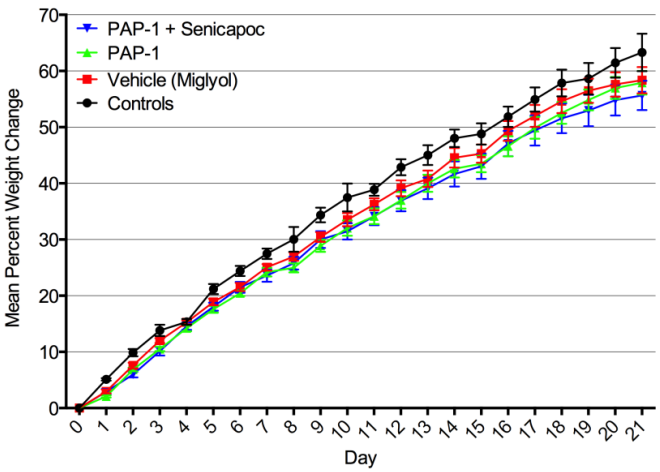

Figure 3. Mean Percent Body Weight Change. Animals were weighed daily and the percent weight change from Day 0 was calculated. Data represent group means and standard error of the mean (SEM). Group difference compared to the Vehicle Control group was analyzed using a one-way ANOVA followed by Holm-Sidak's multiple comparison test. No significant weight change was observed between the DSS groups (Vehicle, PAP-1 and PAP-1 + Senicapoc).

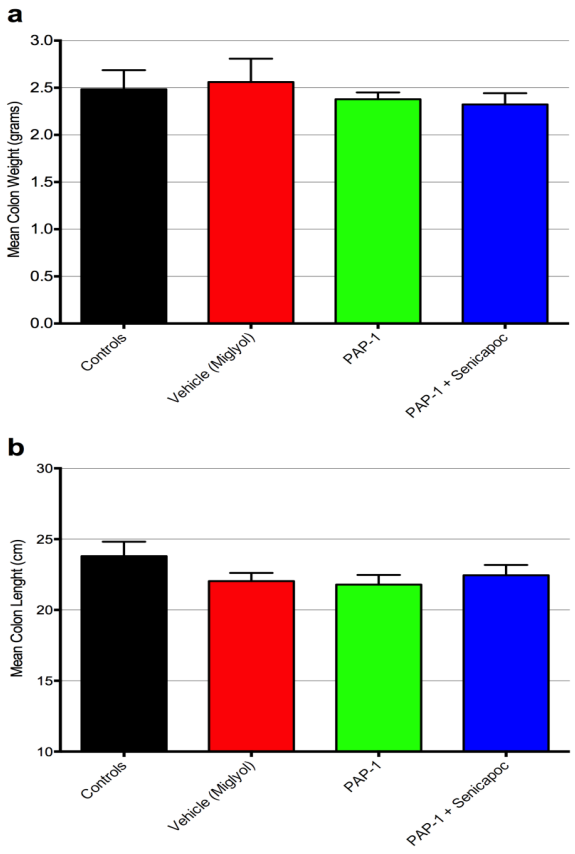

Figure 4. Mean Colon Weight and Mean Colon Length on Day 21. Rats were euthanized on Day 21 and colons were removed, weighed and measured. a) Colon weight in grams and b) Colon length in cm. Data represent group means and standard error of the mean (SEM). No significant differences were observed between the groups.

Histopathologic scoring

A histopathologic evaluation of the mucosal inflammation, edema, mucosal necrosis, and a summarizing score was performed (Sum Score) (Figure 5 and 6). Inflammation was significantly affected by DSS administration (one-way ANOVA including all groups, p<0.01; all groups increased significantly compared to untreated controls). However, when untreated control animals were removed from the analysis, treatment did not significantly affect inflammation (p=0.86). There were similar levels of inflammation in animals treated with vehicle, PAP-1, and PAP-1 + Senicapoc (Figure 5a). Edema was also significantly affected by DSS administration (one-way ANOVA including all groups, p<0.01; all groups increased significantly compared to untreated controls). However, when untreated control animals were removed from the analysis, treatment did not significantly affect edema (p=0.33). There were similar levels of edema in animals treated with vehicle, PAP-1, and PAP-1 + Senicapoc (Figure 5b). Mucosal necrosis/loss was significantly affected by DSS administration (one-way ANOVA including all groups, p<0.01; all groups increased significantly compared to untreated controls). However, when untreated control animals were removed from the analysis, treatment did not significantly affect necrosis (p=0.63). There were similar levels of necrosis in animals treated with vehicle, PAP-1, and PAP-1 + Senicapoc (Figure 5c). The colitis sum score was significantly affected by DSS administration (one-way ANOVA including all groups, p<0.01; all groups increased significantly compared to untreated controls). However, when untreated control animals were removed from the analysis, treatment did

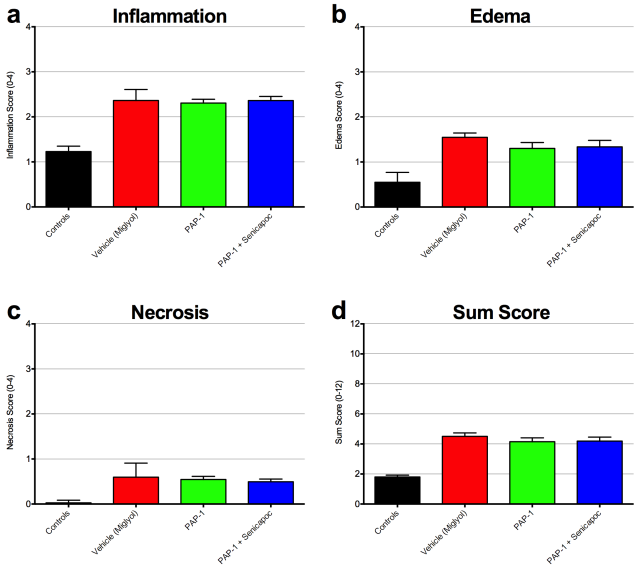

Figure 5. Histopathologic scores of all groups. Group means with standard error of the mean (SEM) bars are shown. A) Inflammation were significantly increased in all DSS groups compared to the untreated controls; no significant difference in inflammation were observed between vehicle and the two treatment groups. b) Edema was also significantly increased in the DSS treated rats compared to untreated controls; no significant difference in degree of edema was found between vehicle and the two treatment groups. C) Mucosal necrosis was increased significantly in the DSS groups compared to untreated controls; the degree of necrosis did not differ significantly between vehicle and the two treatment groups. d) The sum score (sum of the 3 other scores) was significantly lower in the untreated controls compared to DSS treated groups. We did not observe significant differences between the DSS treated groups.

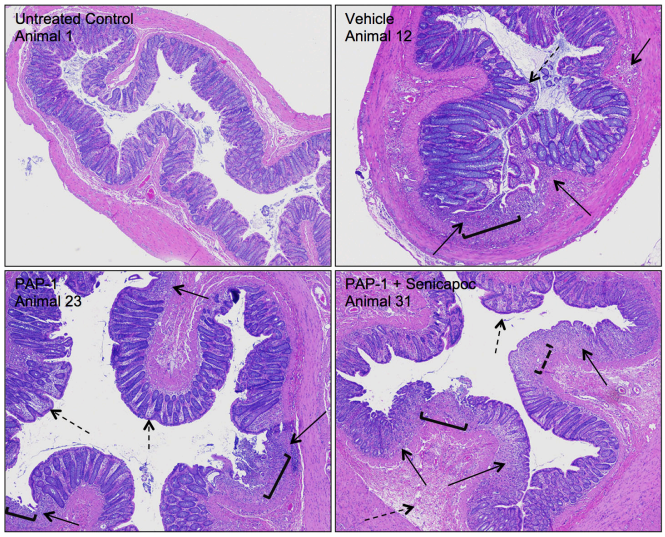

Figure 6. Representative photomicrographs for the histological changes – HE stainings at 40x.
Animals administered DSS and treated with vehicle had multifocal areas of moderate to marked inflammation (arrows) with multifocal areas of mucosal erosion and necrosis (brackets), and edema (dashed arrows). Treatment with PAP-1 and PAP-1 + Senicapoc did not significantly reduce inflammation, necrosis, or edema. Animals still had multifocal colitis with areas of inflammation, mucosal erosion, and edema. The mucosa was also attenuated multifocally (dashed bracket).

Table 4: Plasma concentrations of PAP-1 and Senicapoc (nM).

Rat no.	Group 3 (PAP-1 only) PAP-1 (nM)	Group 4 (PAP-1+Senicapoc) PAP-1 (nM)	Group 4 (PAP-1+Senicapoc) Senicapoc (nM)
1	33.2	1.2	75.6
2	20.9	1.8	< 8
3	5.3	0.6	< 8
4	2.7	1.2	< 8
5	3.3	0.4	< 8
6	4.5	0.3	< 8
7	16.4	0.5	< 8
8	2.4	1.0	< 8
9	2.5	0.7	< 8
10	7.1	0.7	< 8
11	4.1	9.9	112.7
12	18.7	2.1	< 8

not significantly affect the sum score (p=0.56). There were similar levels of colitis in animals treated with vehicle, PAP-1, and PAP-1 + Senicapoc (Figure 5d).

Plasma levels of compounds

Plasma levels of PAP-1 and Senicapoc were measured by UPLC/MS and revealed relatively low levels of PAP-1 in the group receiving PAP-1 (mean 10.09 nM; 95% CI: 3.79-16.39 nM). In the group receiving both PAP-1 and Senicapoc, the PAP-1 levels were even lower (mean 1.70 nM; 95% CI: 0.02-3.38). The plasma levels of Senicapoc were also low (< 8 nM) in 10 out of 12 rats. Data are shown in table 4. Plasma concentrations did not correlate with the endoscopic or histological subscore (data not shown).

Messenger-RNA expression levels

No significant differences in mRNA expression levels of $K_V1.3$, $K_{Ca}3.1$, IFN-γ, TNF-α , and IL-17A were found between the Vehicle, PAP-1, and PAP-1 + Senicapoc groups (data not shown). In addition, we wanted to test if there were evidence supporting that inflammation was actually present in the DSS-induced colitis model. Therefore, we tested the expression of different inflammatory cytokines and of the T cell potassium channels. Here, we found that mRNA expression of $K_V1.3$, TNF-α, and IL-17A were significantly increased in the DSS vehicle group ($p < 0.05$, $p < 0.01$, $p < 0.01$, respectively) (figure 7). No differences in $K_{Ca}3.1$ or IFN-γ were found when comparing controls and the vehicle group.

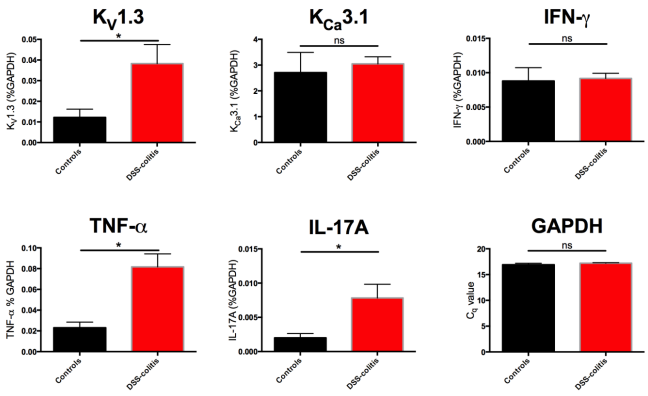

Figure 7: Mean mRNA expression levels in non-DSS animals (controls) vs. DSS-induced colitis (DSS-colitis). $K_V1.3$, TNF-α and IL-17A expression were significantly increased in DSS animals. Expression levels are stated in percentage of GAPDH. The graph to the lower right states the GAPDH levels between controls and vehicle group. Error bars are SEM.

Discussion

The main finding of our study was that pharmacological treatment with the $K_V1.3$ and $K_{Ca}3.1$ blockers, PAP-1 and Senicapoc, did not ameliorate DSS-induced colitis when administered by oral gavage at 50 mg/kg.

Blockade of potassium channels $K_V1.3$ and $K_{Ca}3.1$ showed a trend towards amelioration of DSS-induced colitis when assessed by endoscopic evaluation on day 7 and 10 of the study; the concomitant administration of PAP-1 and Senicapoc consistently decreased the endoscopic inflammation but failed to reach statistical significance. This finding could suggest that the combined therapy might be relevant in the semi-chronic colitis model used here. Overall, this reduction in endoscopic inflammation score seems potentially clinically meaningful, as this trend was evident during the beginning of the trial where it seems to have a protective efficacy. However, a sustained effect was absent. This could be due to the decrease in the drinking water DSS concentration (from 5% to 1% after the initial 5 days) as this concentration might be too low to maintain the chronic inflammation adequately. This is supported by the fact that there was no difference in body weight between animal groups. We also correlated plasma concentrations with endoscopic scores of the subgroups, but did not find a significant correlation.

There are several experimental animal models mimicking UC. In our study, we chose the semi-chronic DSS-induced colitis, as this model is known to include an acute and chronic T cell response, and the histological features of inflammation [13, 14, 25, 26]. Thus it was assumed that this model could be potentially relevant to assess the effect of pharmacological inhibition of $K_V1.3$ and KCa3.1. Several other types of experimental colitis could have been used such as the oxazolone, 2,4,6-Trinitrobenzene sulfonic acid (TNBS), IL-7 Transgenic, IL-2 knockout, T cell receptor alpha, and the dnKO (IL10R2 knockouts) colitis. Moreover, we chose the DSS-model as it is the widely used for pharmacological testing of compounds [10, 12, 27]. In respect to the type of T cell response, the DSS model has previously been shown to induce both T_H1 and T_H2 responses, which makes this model useful to study disease processes potentially relevant for UC but also for Crohn's disease (CD) [26]. According to our qPCR results, this DSS model includes T_H1 as well as TH17 response as concluded from the upregulation of TNF-α and IL-17A. Moreover with respect to the potassium channels we found that $K_V1.3$ expression was also increased in the DSS-treated rats compared to the non-DSS controls. $K_{Ca}3.1$ was not changed in DSS, thus these results indicate similar alterations as found in UC patients. This fosters the view that this model is suitable to study whether or not blockade of $K_V1.3$ and $K_{Ca}3.1$ would be relevant.

Pharmacological blockade of $K_{Ca}3.1$ has previously been shown to ameliorate T cell-mediated colitis in DNBS (dinitrobenzene sulfonic acid)-induced colitis in rats [18], in DSS-induced colitis in mice[20], and TNBS (trinitrobenzene sulfonic acid)-induced colitis mice [19]. In our study, we did not find the same anti-inflammatory effect of the $K_{Ca}3.1$ blocker as found in previous reports. On the other hand, we used a combination with a $K_V1.3$ blocker. Another difference between the two studies was that they used TRAM-34 and NS6180, and we used Senicapoc, as Senicapoc also is a selective blocker of $K_{Ca}3.1$ [28]. Pharmacological blockade of $K_V1.3$ in DSS-induced colitis has not been tested before. During the setup of the study, the concentrations of PAP-1 and Senicapoc (50mg/kg) were based on a previous study investigating $K_V1.3$ inhibition by PAP-1 in type-1 diabetic rats where it worked well [29]. However, our plasma analyses of PAP-1 and Senicapoc levels revealed that concentrations were lower than expected and were unlikely to be pharmacological active based on the IC_{50} of the compounds. The animals definitely received the compounds as we were able to measure concentrations in all plasma samples but they were just too low to be pharmacologically active. The concentration of PAP-1 was substantially higher in the monotherapy group compared to group of animals treated with combination of PAP-1 and Senicapoc. An explanation could be that Senicapoc is a P450-inducer thus increasing the turnover of the compounds. However, the difference from 10 nM and 1.7 nM is very low for both groups and probably is not sufficiently pharmacologically active, especially considering the IC50 values being in this nanomolar range. Another reason might be that the animals

were put down consequently, which gives group 4 animals more time to break down the compounds before plasma was harvested.

An explanation for the low plasma levels was that the oral administration dose of PAP-1 and Senicapoc was simply too low. Another reason might be that the diarrhea in some of the animals might have impaired the intestinal absorption of the compounds due to faster transit time. Therefore, in future studies we need to change the route of administration for this specific model to intraperitoneal injections in order to reach the levels of pharmacologically active concentrations of PAP-1 and Senicapoc. Another way could be to continue the oral administration and give the animals a higher concentration of the two blockers (e.g. 100-200 mg/kg).

In our study, we did not observe any adverse effects or deaths. Senicapoc has already been tested in a phase-3 randomized trial as treatment for sickle cell anemia. No increased risk of adverse events was observed [28]. PAP-1 has only been tested in animal studies but is well-tolerated [30, 31]. This may suggest that PAP-1 and Senicapoc are safe. Yet, we need to achieve plasma concentrations that reach pharmacologically active levels to allow definite conclusion.

In the paper by Christophersen et al. [121] pharmacological blockade of $K_{Ca}3.1$ with oral administration of NS6180 had a positive effect on the inflammation in the DNBS-induced colitis model in rats. The plasma concentrations from their study also showed very low levels of the $K_{Ca}3.1$ blocker, thus we found it relevant also to perform histopathologic evaluation in our study. Here, we did not find significant differences between any of the DSS-induced colitis groups suggesting that the mucosal concentrations of the channels blockers (via blood or through the intestine) were too low or that pharmacological blockade of the channels simply does not improve the DSS-induced inflammation in rats. Most likely, the reason for insufficient effect of PAP-1 and Senicapoc is that we did not reach therapeutic levels, which is supported by the previous reported effects of $K_{Ca}3.1$ blockade on DSS-induced colitis [18-20]. Theoretically, the additional $K_V1.3$ blockade should dampen the T cell response resulting in an even more pronounced amelioration of the inflammation as other studies have shown that $K_V1.3$ blockade alone and in combination with $K_{Ca}3.1$ lead to decrease in T cell proliferation and cytokine production [21, 32].

In conclusion, pharmacological blockade of $K_V1.3$ (PAP-1) alone or in combination with $K_{Ca}3.1$ (Senicapoc) did not significantly ameliorate DSS-induced colitis in rats when administered by oral gavage in Miglyol at 50 mg/kg due to insufficient uptake or insufficient drug concentrations. Nonetheless, there was a promising trend towards amelioration of endoscopic inflammation after the acute induction of colitis at day 7 and 10, thus we argue that pharmacological blockade of $K_V1.3$ and $K_{Ca}3.1$ could have immunosuppressive effect even though the difference did not reach statistical significance.

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STUDY 1: ADDITIONAL RESULTS

GAPDH variability in qPCR analyses

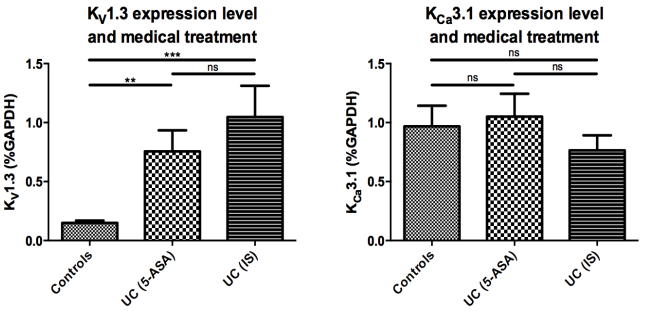
In the human UC study we stated that there was no significant difference between UC patients and controls when assessing the reference gene GAPDH. As data had a non-Gaussian distribution, we performed the analysis using the non-parametric Mann-Whitney test and found no significant differences between UC and controls (mean Cq value 22.47 vs. 22.32, p = 0.43).

K_V1.3 and K_{Ca}3.1 mRNA expression and age, weight, and height

To examine if K_V1.3 and K_{Ca}3.1 expression were influenced by demographic variables such as age, weight, and height we tested for significant correlations. We did not find any significant correlations between any of these variables (data not shown).

K_V1.3 and K_{Ca}3.1 mRNA expression and medical treatment

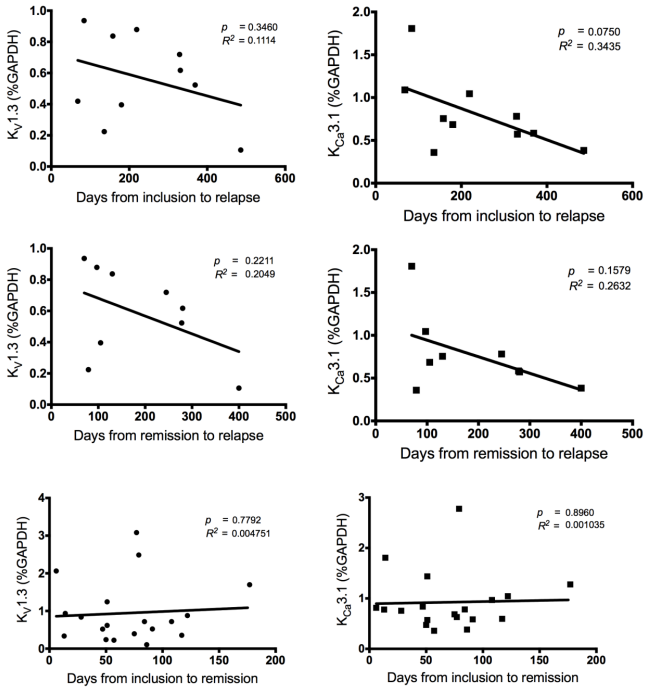
We also investigated if ongoing treatment of UC influenced the expression levels of K_V1.3 and K_{Ca}3.1. We documented that K_V1.3 was significantly increased both in the 5ASA and IS group, but there was no difference between 5ASA and IS treated patients. No differences in K_{Ca}3.1 were observed between controls and the two groups of UC patients.



Medical treatment and K_V1.3 expression. UC patients were divided into groups receiving 5ASA as monotherapy or 5ASA in addition with another immunosuppressant (IS) (glucocorticoids, azathioprine, infliximab). For K_V1.3 expression no significant difference between 5ASA and IS group was found. As expected from previous results, the controls had significantly lower expression of K_V1.3 compared to both treatment groups. For K_{Ca}3.1 no significant differences were found between any of the groups.

Prognostic value of $K_V1.3$ and $K_{Ca}3.1$ mRNA expression

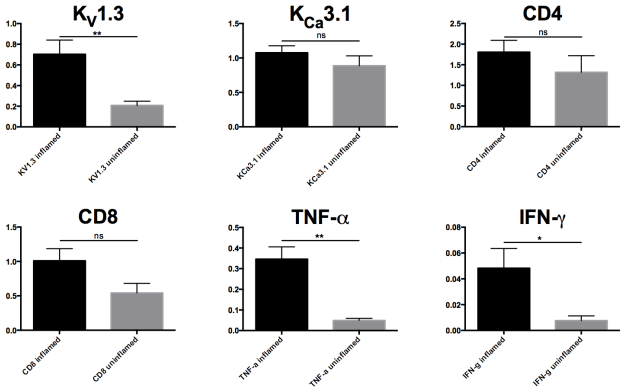
In addition, we also investigated if $K_V1.3$ and $K_{Ca}3.1$ could be used a prognostic marker. Here, we reported that there was no correlation between the mRNA expression of $K_V1.3$ or $K_{Ca}3.1$ from human mucosal biopsies obtained at inclusion and the "time to relapse". Still, we observed a trend towards a negative correlation in $K_{Ca}3.1$ expression (R2=0.03, p = 0.08). The number of days was evaluated from the inclusion date and from the date of obtained remission (n=10) until a relapse was observed. Here, we found no correlations of the expression of the channels and the number of days to relapse. By further analyzing the days from inclusion until achieving remission, we wanted to test whether mRNA expression of $K_V1.3$ and $K_{Ca}3.1$ could be used to predict patients who would achieve remission quickly and those who were not. Again, we found no significant correlations between the expression of the $K_V1.3$ and $K_{Ca}3.1$ (Please see prognostic figure below).



$K_V1.3$ and $K_{Ca}3.1$ expression and the correlations with prognostics. No significant correlations were found between the mRNA expression of $K_V1.3$ (left) and $K_{Ca}3.1$ (right) channels and the time to either remission or the next relapse. $K_V1.3$ and $K_{Ca}3.1$ are depicted on the Y-axis in percentage of the reference gene GAPDH (%GAPDH) and time is on the X-axis. R^2=Coefficient of determination.

Messenger-RNA in inflamed vs. un-inflamed mucosa in the same patient.

We also compared the mRNA expression levels of $K_V1.3$, $K_{Ca}3.1$, CD4, CD8, IFN-γ, and TNF-α in the simultaneously obtained biopsies obtained at inclusion from the inflamed rectal mucosa and the more proximal healthy mucosa with no endoscopic inflammation. Here, we found increased levels of $K_V1.3$, IFN-γ, and TNF-α when each individual was used as his/her own control. No significant difference in the other mRNA expressions.

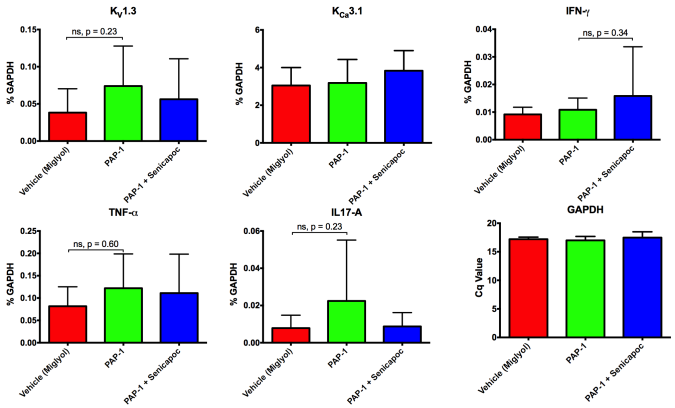


Gene expression levels in biopsies taken from the inflamed vs. uninflamed colon mucosa in patients with ulcerative colitis. Biopsies were taken from the same patients at the inflamed level and at a more proximal location with no endoscopic inflammation. $K_V1.3$, TNF-α and IFN-γ are all increased in the inflamed mucosa compare to the uninflamed. $K_{Ca}3.1$, CD4, and CD8 do not differ significantly but there was a small trend towards an increase of CD8 in the inflamed mucosa (p = 0.19)

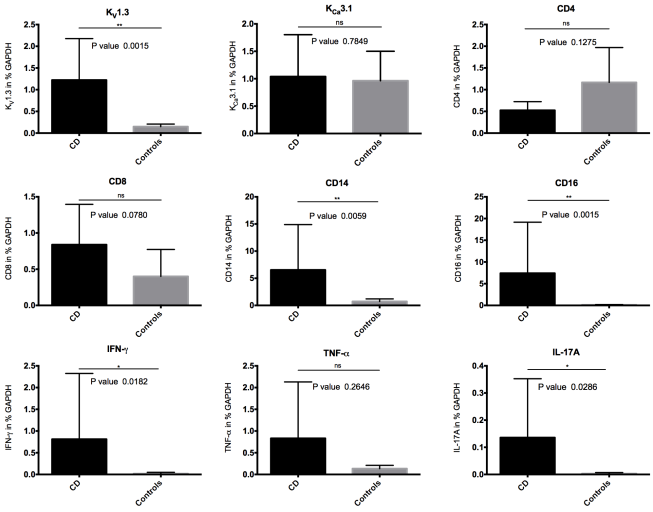
Additionally, we analyzed the $K_V1.3$ and $K_{Ca}3.1$ expression at the time of follow-up visits after 4 weeks, 8 weeks, and 1 year We wanted to test if expression levels of $K_V1.3$ and $K_{Ca}3.1$ decreased after initiation of anti-inflammatory treatment. Only 4 patients completed all three follow-up visits. The small number completing all follow-up visits does not allow us to draw definite conclusions. However, comparing the expression levels at inclusion (before treatment was initiated) with the expression levels after one month of treatment, $K_V1.3$ still correlates with the endoscopic inflammation subscore as we found it in study 1. In conclusion, the expression of $K_V1.3$ seems to correlate with the actual mucosal inflammation, thus supporting $K_V1.3$ as a pharmacological target.

STUDY 2: ADDITIONAL RESULTS

In the DSS rat study, we reported significant differences in mRNA expression between controls and rats with DSS-induced colitis (vehicle group). Moreover, we also examined if there were significant differences between the three groups with DSS-induced colitis (vehicle, PAP-1, PAP-1 + Senicapoc) but no significant differences were found between any of the groups. The figure below depicts these findings. For $K_V1.3$ and IL-17A, it seems that the addition of PAP-1 increased the mRNA expression levels. However, this is at best by trend (p=0.23) and SDs were large. Data are presented as is and outliers did not meet the requirements for exclusions as describe in the methods paragraph.



The mRNA expression levels of $K_V1.3$ and $K_{Ca}3.1$, and inflammatory cytokines. Expression is depicted as a percentage of GAPDH. No significant differences were found comparing the different groups to each other. In the figure p values have been stated where graphs suggest differences. No differences in GAPDH Cq-values were found between groups. Data had a non-Gaussian distribution and the statistical analyses were performed using the non-parametric Kruskal-Wallis test with Dunn's multiple comparisons test as post-hoc analysis. Error bars are SD.



Gene expression of $K_V1.3$, $K_{Ca}3.1$, CD4, CD8, CD14, CD16, IFN-γ, TNF-α, and IL-17A in CD patients compared to controls. Expression levels of $K_V1.3$, CD14, CD16, IFN-γ and IL-17A are significantly increased, whereas $K_{Ca}3.1$, CD4, CD8 and TNF-α are not. $K_V1.3$, $K_{Ca}3.1$, and CD4 had normally distributed data and were analysed with the parametric "unpaired t-test". The rest had non-Gaussian distributed data and were analysed using the non-parametric Mann-Whitney test. Error bars are SD.

STUDY 3: PRELIMINARY RESULTS FROM PATIENTS WITH CROHN'S DISEASE

Expression of the potassium channels, $K_V1.3$ and $K_{Ca}3.1$

In study 1 we have investigated the potassium channels in UC patients. In an ongoing study, we are investigating the expression of potassium channels in patients with CD. Preliminary data of qPCR analyses on mucosal biopsies from patients with active CD (n=13) in colon and terminal ileum reveal results similar to those seen in UC. The mRNA expression of $K_V1.3$ is increased by 7-fold in CD biopsies compared to controls ($p<0.01$). For $K_{Ca}3.1$, no significant difference was found when comparing CD vs. controls. T cell markers, CD4 and CD8, did not differ between CD and controls but CD8 turned out borderline significant ($p = 0.08$). CD14 and CD16, markers of resting or activated monocytes/macrophages, respectively, were significantly increased compared to controls. The expression levels of pro-inflammatory cytokines, IFN-γ and IL-17A were also significantly increased. The difference in TNF-α did not reach statistical significance (figure below).

Additionally, we compared the expression levels in the inflamed mucosal biopsies of CD patients with those of UC patients (and Controls) using the Kruskal-Wallis test with Dunn's multiple comparisons test. Again, $K_V1.3$ expression was increased in UC ($p<0.01$) and CD ($p<0.01$) compared to controls, but no significant difference was found in $K_V1.3$ expression between UC and CD ($p=0.87$). No difference in $K_{Ca}3.1$ expression was observed in any group.

Correlation analyses between mRNA expression and clinical scores

When biopsies from CD patients were obtained, the patient was scored according to the Harvey Bradshaw Score[157] and the Simplified Endoscopic activity Score for Crohn's Disease (SES-CD) score (maximal local segment score (0-12) and Total Score (0-48))[158]. In this pilot study, only seven CD patients were scored accordingly (n=7). Considering the small group size, correlation analyses are preliminary and should be weighed as such. Linear correlation was used to correlate the Harvey Bradshaw score to $K_V1.3$ and $K_{Ca}3.1$ expression as data had a Gaussian distribution. No significant correlations were found. In addition, we used Spearman's Rank Correlation to correlate SES-CD with $K_V1.3$ and $K_{Ca}3.1$, as data were non-Gaussian. Again, no correlations were found. However, these analyses lack power, and more patients are needed in order to conclude on this.

OVERALL DISCUSSION

In this thesis the importance of the potassium channels $K_V1.3$ and $K_{Ca}3.1$ in relation to the inflammatory cascade was addressed in patients with UC, patients with CD, and healthy controls. Furthermore, the effects of pharmacological blockade of the potassium channels were addressed in a rat DSS colitis model.

We found as a main outcome that T cell $K_V1.3$ channels were indeed present and increased in human mucosal biopsies from patients with active UC as well as in CD. $K_V1.3$ expression correlated with the inflammation scored by endoscopy and histology, and also correlated with pro-inflammatory cytokines. The mRNA expression level of $K_{Ca}3.1$ was not increased in UC compared to controls and $K_{Ca}3.1$ expression only showed a positive correlation with TNF-α. The expression of $K_V1.3$ and $K_{Ca}3.1$ could not be used to predict the risk of relapse, even though a trend towards a negative correlation was found for $K_{Ca}3.1$ expression and the days to relapse. We also demonstrated that *in vitro* inhibition of $K_V1.3$ and $K_{Ca}3.1$ leads to decreased proliferation rates and cytokine production in stimulated human $CD3^+$ T cells. Overall, the studies in patients with UC and patients with CD may serve as target identification studies for new potential pharmacological treatment of IBD.

In addition, we conducted a pilot study investigating whether pharmacological blockade of $K_V1.3$ and $K_{Ca}3.1$ with PAP-1 and Senicapoc could ameliorate inflammation in the DSS-induced colitis model in rats. Here, we found a trend towards amelioration of the acute inflammation (on day 7 and 10), although the difference failed to reach statistical significance. We did not observe a difference in histological changes between vehicle and either treatment group. One reason could be that we found low plasma concentrations of PAP-1 and Senicapoc, suggesting decreased intestinal uptake or underdosing of PAP-1 and Senicapoc.

METHODOLOGICAL CONSIDERATIONS

Statistical correlation analyses

Correlation analyses in manuscript 1 were performed using linear correlation assuming that the data were normally distributed. As some of the data did not have a Gaussian distribution it would have been more correct to use the Spearman's Rank Correlation. We set out to investigate the correlations between $K_V1.3$ and $K_{Ca}3.1$ expression and the clinical scores. We also studied if there were correlations between $K_V1.3$ and $K_{Ca}3.1$ expression levels and other genes (IFN-γ, IL17A etc.). These sub-analyses were only exploratory.

Therefore, we recalculated the statistics. The additional correlation analyses using the Spearman's Rank Correlation on the non-Gaussian distributed data reveal that apart from the previously reported positive correlation between $K_V1.3$ and CD14, CD16, TNF-α and IL17A (using linear regression) also expression levels of $K_{Ca}3.1$, IFN-γ, and CD8 have a significant positive correlation with the expression of $K_V1.3$. $K_{Ca}3.1$ correlations now showed a positive correlation with IFN-γinstead of TNF-α , CD14 and CD16.

These findings support the view that $K_V1.3$ channels, and to a lesser extent $K_{Ca}3.1$, are important in the inflammatory process in active UC.

Immunofluorescent staining

Before starting to perform immunofluorescent staining, we tested all antibodies in standard immunohistochemistry, so we were certain that CD3 and CD4/8 did stain T cells. For $K_V1.3$ and $K_{Ca}3.1$ we performed similar staining on tissue/cells where we knew that the channels were present. In order to achieve this, we tested all antibodies in different concentrations, using different boiling buffers to retrieve the antigens, and using different blocking solutions in order to eliminate background staining. To obtain specific and clear staining several antibodies were tested, as many failed to display the antigen correctly.

In immunofluorescent staining a high amount of $CD4^+/K_V1.3^+$ and $CD8^+/K_V1.3^+$ was found, which could indicate $K_V1.3$ as a novel target of immunosuppression. It would indeed be interesting if it were possible to divide the $K_V1.3$ expressing T cells into either high or low thus indicating a T_{EM} response or not. However, the immunofluorescent data did not allow us to distinguish between $K_V1.3^{high/low}$ and $K_{Ca}3.1^{high/low}$ populations of T cells though in some pictures it was possible to identify two peaks of fluorescence intensity; choosing the arbitrary border of high/low would be highly speculative as this border was not consistent throughout the images.

Immunofluorescent staining also showed a high degree of co-localization of CD4 and $K_{Ca}3.1$, which is in line with the findings of other studies showing that when T-cells are activated they use high numbers of $K_{Ca}3.1$ to drive this activation [106]. Interestingly, $CD8^+$ cells only co-localized in a small percentage of the $K_{Ca}3.1^+$ cells and we did not find evidence that $K_V1.3$ or $K_{Ca}3.1$ were highly expressed in macrophages either.

Antibodies

Immunohistochemistry and immunofluorescent staining are very illustrative but require that the antibodies used are "stable" and do not have batch-to-batch variation in specificity. Unfortunately, we experienced this variation in the $K_V1.3$ polyclonal antibody (Novus Biologicals, #NBP1-19415). After we had been using the $K_V1.3$ antibody for 2 years (including the staining used in quantitative analyses in Manuscript 1), we had to reorder from Novus Biologicals, as we wanted to do more $K_V1.3$ stainings for some recently included CD patients. Unexpectedly, this new batch of $K_V1.3$ antibody did not work (or had lost its specificity) even though we followed the exact same protocol using the same washing buffers, heating buffers etc. Frustratingly, the new antibody now bound predominantly to the mucin in the goblet cells made any further quantitative analyses irrelevant. As a rule of thumb, monoclonal antibodies have the least batch-to-batch variation[159] and we actually started immunostaining using a monoclonal antibody for $K_V1.3$ (AB Serotec, #MCA3212Z). In the beginning, this antibody was used with success for immunohistochemistry. When we wanted to proceed to immunofluorescent co-staining we needed two antibodies (e.g. anti-CD4 and anti-$K_V1.3$) produced in different hosts. This is a requirement if one wishes to use two different secondary fluorescent antibodies. Therefore, despite the fact that polyclonal antibodies have an increased risk of batch-to-batch variation[159, 160], we continued using both the $K_V1.3$ and $K_{Ca}3.1$ as polyclonal antibodies as they worked very well. This turned out to be a bad decision, as the $K_V1.3$ antibody (Novus Biologicals, #NBP1-19415) suddenly did not work in the newly arrived batch. For future research in patients with CD, I will certainly continue to troubleshoot and test the current antibodies, as this is a vital step in illustrating the presence of actual channels instead of levels of mRNA that may have to undergo some kind of activation before the protein is functional and expressed. A way to do this would be to focus on the monoclonal antibodies. Currently, however, most monoclonal antibodies against the potassium channels are polyclonal and the monoclonal antibodies that are available are all from mice, which then hinders double staining of $K_V1.3$ and $K_{Ca}3.1$. However, we could use a monoclonal antibody for $K_V1.3$ and find new antibodies not from mice for CD4/CD8 and macrophages.

Quantitative Polymerase Chain Reaction

Quantitative Real-Time Polymerase Chain Reaction = qRT-PCR = qPCR is a method to measure the amplification of a specific base sequence in DNA. Normally, it is used to determine absolute copies or copies relative to reference genes (also known as housekeeping genes) of specific mRNA transcripts in biological samples where the RNA has been isolated. To minimize the variations in housekeeping genes the MIQE guidelines now recommend the use of two genes [152]. Before using a reverse transcriptase to transcribe mRNA into complementary DNA (cDNA synthesis) the RNA should have been treated with DNase to avoid DNA contamination, which could result in inaccurate quantification especially if the gene of interest is not intron-spanning[161]. When cDNA is synthesized, the gene-specific primers are added and you are now able to perform a polymerase chain reaction amplifying the gene of interest. To measure how many copies of a

gene are produced during these cycles of PCR, a fluorochrome must be added. This is done either by using SYBR green fluorescence or a gene-specific probe labeled with a fluorochrome. In short, a fluorescent signal is released when the transcription of the gene has occurred and the qPCR instrument reads this signal. In our research group we used the SYBR Green assay.

SYBR green

The SYBR® Green I Dye, which is detected by the qPCR instrument, binds to double-stranded DNA (dsDNA). By using gene-specific primers the level of fluorescent signal is measured after each cycle and is dependent on how much dsDNA is in the well. This is typically repeated for 40 cycles after which billions of copies have been made. When using SYBR® Green one does not get an absolute number of amplicons but a measurement of the fluorescent signal. By using a standard curve in which you have determined the exact concentration of a given transcript and diluted this into several wells on the qPCR plate, you are able to determine the approximate number of copies in your sample. When you report your data, you compare the level of fluorescence of your gene of interest with the signal from your reference gene(s), and afterwards compare these relative numbers between groups e.g. patient vs. control. As the SYBR Green I dye binds unspecifically to dsDNA, a melting curve analysis must be performed to make sure that only one product is amplified depicted as one sharp peak (figure in methods). SYBR Green is marginally cheaper than Taqman probes, but may require additional optimization of primers.

Taqman probes

Taqman® Probes are another way of determining gene copies during the PCR reaction. A gene-specific probe binds to the sequence of the gene (on the cDNA). The primers of the same gene are created so that it anneals upstream of the probe. When the DNA polymerase starts to amplify the gene of interest, it will soon reach the area where the probe has annealed. When the polymerase encounters the probe, it cleaves a reporter dye from the probe emitting one signal per transcript. In this way Taqman® chemistry allows determination of the absolute number of gene amplifications. In Taqman assays one reports the results relative to reference genes in the same way as described under the SYBR® Green assay.

PCR inhibitors

In this project we performed PCR on both human and rat tissue. As described under methods, it is important that the primers are well-designed otherwise one will not get the amplification and product that is needed. Moreover, different compounds are known to inhibit PCR reactions e.g. heparin[162]. Another example, which I encountered when I was synthesizing cDNA, was that DSS is a PCR inhibitor. Going through studies that have used the DSS-colitis model, they did not seem to report any problems with their PCR analyses[120]. However, Kerr et al. [163] discovered the same issue and decided to investigate this further. They found that DSS indeed inhibited the PCR reactions to different degrees dependent on which RNA extraction method was used. Trizol was shown to include most of the inhibitory factor from the DSS. Therefore, we had to purify the RNA afterwards to allow the PCR to run trouble-free.

Pitfalls of qPCR when comparing results between groups

In qPCR analyses there are some pitfalls that must be considered when designing the study. Recently, the MIQE guideline was published in order to make qPCR findings reproducible, as there was too high variability between studies [152]. The two most important things that must be tested before it allows you to base conclusions on the results are:

1) Reference genes must be tested for stable expression in the diseased tissue and in the control tissue as features such as inflammation may affect expression levels of reference genes[152, 161]. Reference genes are constitutive genes involved in basic cellular mechanisms, which are used for normalization of mRNA data when reporting relative expressions from qPCR analyses.

2) PCR efficiency is a measure of how well the primers are functioning under the circumstances that have been set in the PCR instrument (temperature and length of denaturation, annealing, and elongation phases). The design of the primer is of vital importance as this makes sure that the amplicon is actually the gene of interest and diminishes the risk of primer dimers (forward and reverse primers that anneal creating false positive signals in SYBR green assays). The PCR efficiencies of primer pairs should be between 95-105%[152, 161, 164].

3) Optimally, when comparing groups, one should synthesize cDNA from all samples in the same run or in parallel to minimize differences in the setup. Afterwards, one should perform the qPCR analysis for a specific gene on a single plate to minimize the risk of plate-to-plate variation, which could be due to primer efficiency decreasing over time, RNA/cDNA degradation etc. If this is not possible due to continuous collection of specimens, one should have an internal control on each plate. The internal control could be a sample that you know should have a previously determined Cq-value. If this value is different on the plate, one should then use the internal control to normalize the rest of the sample-data.

Designing the animal trial

The design of the animal trial was based on the results obtained from the patient study. From the patient study, our results support the view that $K_V1.3$ plays a pro-inflammatory role in UC, which is why we focused primarily on $K_V1.3$ blockade as treatment strategy. As other animal studies have shown satisfactory absorption rates in bowel-healthy animals, we did not perform a pilot study in advance. Moreover, we chose the usually preferred route of compound administration, which is oral for patients with ulcerative colitis in order to achieve a luminal effect on the inflammatory process in the gut.

The lipophilic nature of the two compounds required the use of an non-polar solvent. Indeed, PAP-1 is hardly water-soluble with a maximal solubility round 2.5 microM/L and a log P about 4 [110], thus demanding a non-polar carrier. In our case we used Miglyol. A formulation with peanut oil has been previously used orally (1x daily at 50 mg/kg in peanut oil) by Beeton et al [107]. This worked out very well to prevent type-1 diabetes in the model. Miglyol was found to achieve comparable plasma levels compared to peanut oil (unpublished data from Heike Wulff), which is why we chose to use the relatively inert Miglyol over oils containing long-

er carbon chains and polyunsaturated fatty acids, such as peanut oil, to avoid potential effects. Despite our considerations we have to admit that the fast bowel transit due to the induction of colitis might have influenced absorption although we still measured compound in plasma.

We based our treatment groups on the findings from the human UC study where we found most evidence that $K_V1.3$ was involved in the inflammatory process. Therefore, we decided to include a treatment arm with vehicle, $K_V1.3$ blocker and $K_V1.3$ and $K_{Ca}3.1$ blocker in combination. Retrospectively, we might have done a pilot study to test the animal model more thoroughly to address potential absorption issues. Moreover, we might have included a treatment arm with a $K_{Ca}3.1$ blocker as monotherapy in order to reproduce the previously reported anti-inflammatory effect using another water-insoluble $K_{Ca}3.1$ blocker[119, 121].

Still, the main focus of the present study was on $K_V1.3$ channels to underscore the novel aspects of our study.

In conclusion, troubleshooting, design- and methodological considerations are especially important in basic research. The period spent on optimizing the assays is indeed time-consuming but nonetheless vital for reliable results.

REFLECTIONS ABOUT THE RESULTS

Autoimmunity

T effector memory cells (T_{EM}) have been suggested to be of importance in various diseases (including autoimmune diseases) such as type-1-diabetes, rheumatoid arthritis, multiple sclerosis, and glomerulonephritis[104, 106, 107, 165, 166]. Activated T_{EM} express increased numbers of $K_V1.3$ to maintain the driving force for continuous Ca^{2+} influx after TCR stimulation in contrast to T_{CM} and naïve T cells that use high numbers of $K_{Ca}3.1$ instead[6]. Pharmacological blockade of $K_V1.3$, thus inhibiting T_{EM}, seems to be a way of targeting the autoimmune response. Autoimmunity may also be important in UC and the lack of pathophysiologic insight is sparse and insufficient. Therefore, we wanted to study if T cell $K_V1.3$ channels are involved in active UC, and if we could show the presence of $K_V1.3^{high}$ T_{EM}, thus suggesting an autoimmune component as suggested in the studies mentioned above.

First, we wanted to address the mRNA expression of T-cell potassium channels in UC vs. controls. We documented a 5-fold increase in $K_V1.3$ mRNA expression in UC patients compared to controls. To some extent all T cells use $K_V1.3$ channels to keep a negative membrane potential; therefore we wanted to examine if the increased $K_V1.3$ expression was due to mucosal infiltration of T_{EM} ($CCR7^-/K_V1.3^{high}$) or just increased numbers of regular T cells ($CCR7^+$ / $KV1.3^{low}$). We proceeded with the immunohistochemical stainings for T cell markers (CD4 and CD8) and CCR7. Here, we did not find evidence of heavy T_{EM} infiltration, as 80% of the T cells were $CCR7^+$. Yet, we found that $K_V1.3$ was expressed in both $CD4^+$ and $CD8^+$ cells.

Correlations and inflammation scores

After the initial finding that $K_V1.3$ was increased in UC patients, we continued with the correlation analyses. Here, we found that $K_V1.3$ expression correlated with the Mayo endoscopic subscore, the histological inflammation score, the pro-inflammatory cytokines IFN-γ and TNF-α, and monocyte markers CD14 and CD16. This suggests that $K_V1.3^+$ cells are involved in the inflammatory process during active UC. Of course, this is not a proof that the $K_V1.3^+$ cells are causing the inflammation but more that they have an important role to play. Additionally, the results of the study suggested that $K_V1.3$ expression levels decreased along with the mucosal inflammation after medical treatment was initiated. This supports that the $K_V1.3$ channel may play a role in the inflammatory process during active UC.

Cytokine assay

The importance of the correlations of $K_V1.3$ with inflammation scores was further supported by the results from our in vitro study. To address the question whether $K_V1.3$ and $K_{Ca}3.1$ play a role in T cell proliferation and cytokine production, we performed an ELISA analysis on PMA + ionomycin-stimulated $CD3^+$ T cells. We used PMA + ionomycin for this reasons: It is a strong Ca^{2+} dependent stimulus, which is to be preferred when testing compounds or constituent pathways that require Ca^{2+} signaling, like $K_{Ca}3.1$. In line with previous studies[114, 165], we found that T cell proliferation was decreased together with the production of inflammatory cytokines - especially if $K_V1.3$ and $K_{Ca}3.1$ were blocked simultaneously. This supported our hypothesis that T cell $K_V1.3$ and $K_{Ca}3.1$ are involved in cytokine production.

Predictive and monitoring markers of ulcerative colitis

Finally, we looked into the clinical outcome of UC patients according to the potassium channels. In inflammatory bowel disease, a lot of studies have been performed to find biological markers that may predict response to treatment or risk of relapse or could serve as a surrogate for disease activity. If a marker could be used to predict the risk of relapse, this could be used in outpatient clinics to identify the patients at risk and schedule them for early follow-up after a disease flare. Additionally, such marker could be used to avoid unimportant follow-ups for low-risk patients thus reducing the load in the outpatient clinics. With regards to markers of disease activity, this might allow gastroenterologists to distinguish between patients that could benefit from top-down treatment instead of step-up[167].

An important feature of a biological marker is that it must be easy to collect (e.g. blood and feces). If the biological marker increases the risk of complications or requires a larger setup (e.g. mucosal biopsies), the marker will not be implemented in daily routines unless the impact of the results it is significant. A number of biological markers have been suggested as markers of disease activity and predictors of disease course in UC. CRP is a valuable marker in CD as it correlates with disease activity and other inflammation markers such as fecal calprotectin and Crohn's disease activity index[168], but when it comes to UC this association fades[169]. Lasson et al [170] showed that high levels of fecal calprotectin in UC were associated with a higher risk of relapse. Bessissow et al [171] showed that basal plasmacytosis in the histological assessment was also associated with higher risk of relapse in patients with UC and mucosal healing, which independently also is a predictive marker of a more quiet course of UC[172].

As T_{EM} ($CCR7^-/K_V1.3^{high}$) are T cells that remain chronically active while circulating until there is a need for another specific immune response, we thought that the mRNA expression of $K_V1.3$ and $K_{Ca}3.1$ at relapse might serve as a novel predictor of relapse. If $K_V1.3$ expression was high it could be indicative of increased numbers of T_{EM} cells, which might suggest that on top of the acute inflammation there is also a more severe chronic inflammatory activity from these cells, which empirically would mean a higher risk of relapse. Therefore, we performed prognostic anal-

yses with regard to "days to relapse" (study 1) and "days to achieve remission", which in the first case was based on a low numbers of individuals (n=10) whereas the number of days to remission was based on a more solid number of individuals (n=19). There seemed to be a trend towards a negative correlation between $K_{Ca}3.1$ mRNA expression levels at inclusion and the time to relapse. This suggests that high levels of $K_{Ca}3.1$ might be used as a predictive marker to identify patients that have increased risk of a rapid relapse. Clearly, more patients must be included to increase the power of the statistical analyses in order to reach such a conclusion. However, it is in line with the other findings from study 1 suggesting that expression levels of $K_V1.3$ and $K_{Ca}3.1$ are characteristic markers of UC in a disease-related fashion. But when it comes to the correlation between mRNA expression levels and "time to achieve" remission, no data supported the view that this may be used to stratify patients into fast or slow-responders.

Overall, $K_V1.3$ and $K_{Ca}3.1$ channel expression levels are not applicable as a marker for monitoring inflammation in the clinical setting at present because of understandable methodological aspects. Nonetheless, they do correlate with the mucosal inflammation. Based on the results obtained in the current study, we may still speculate that the expression levels in biopsies would provide more information than current endoscopic scores of inflammation or fecal calprotectin, beyond new mechanistic insights into disease. Accordingly, whether expression levels of the channels are better tools than established markers, remains unanswered by this study.

Pharmacological targeting of $K_V1.3$ and $K_{Ca}3.1$ in inflammatory bowel disease

Our study on human mucosal biopsies clearly showed that $K_V1.3$ and $K_{Ca}3.1$ are located in the inflamed mucosa, thus suggesting that the channels could be novel pharmacological targets. Moreover, we did an in vitro study showing that pharmacological blockade of $K_V1.3$ and $K_{Ca}3.1$ caused decreased proliferation rate and cytokine production in $CD3^+$ T cells. As a result, we found it relevant to proceed with an animal trial to study the effect of single and dual inhibitors of T cell potassium channels in a DSS-induced colitis model in rats. This did not turn out as we had hoped, as it seemed that we were using low doses of blockers or used an inferior way of administering the compounds as discussed in the report from the animal study above. Nevertheless, we found a promising trend towards amelioration of inflammation in the acute phase. If one assumes that pharmacological blockade of $K_V1.3$ and $K_{Ca}3.1$ actually does ameliorate DSS-induced colitis, could a similar effect be shown in humans? How do the compounds actually work in the long run? Do the proinflammatory T cells die or are they kept in standby until the treatment is discontinued whereupon the T cells again secrete inflammatory cytokines? Pharmacological inhibition of $K_V1.3$ and $K_{Ca}3.1$[114, 119, 165] in T cells does not kill the cells; instead they are inhibited of their effector functions ultimately decreasing cytokine production and proliferation. In the light of this, $K_V1.3$ and $K_{Ca}3.1$ blockers might be used to treat inflammation in combination with other anti-inflammatory drugs to gain control of a relapse by eliminating the stimuli for continuous inflammation. In this way, when the inflammation is gone, the epithelial barrier might be regenerated and the blockers could be tapered or simply discontinued. However, if the secretion of proinflammatory cytokines is inhibited then the $K_V1.3$ and $K_{Ca}3.1$ blockers might be used as mono therapy, as inflammation is dampened. Again, when the treatment is discontinued, the APCs that initially activated the immunological response via the TCR might be gone because of the reconstruction of the epithelial barrier. However, if the TCR stimulation is still present, the inflammation would reoccur and the treatment would need to be reinitiated in combination with standard medications, such as 5ASA or prednisolone.

Expression levels in the inflamed vs. un-inflamed mucosa

As we have documented that $K_V1.3$ and $K_{Ca}3.1$ channels were present in the inflamed mucosa of UC patients, it was of interest to investigate whether the increased levels of $K_V1.3$, $K_{Ca}3.1$, CD4, CD8, IFN-γ, TNF-α, and IL-17A were just characteristic features of the inflamed mucosa or if there might be widespread changes in "healthy looking" mucosa as well suggesting an underlying inflammation bound to break out. When UC patients were enrolled in the study, we collected mucosal biopsies from the inflamed area of the bowel, but we also took biopsies from the healthy-looking mucosa in the further proximal part of the colon (Mayo endoscopic subscore of 0) if reachable by the endoscope. Comparing the inflamed with the un-inflamed mucosa we found that the mRNA expression levels of $K_V1.3$ was indeed increased in the inflamed mucosa but not in the un-inflamed mucosa. As expected, IFN-γ and TNF-α were also increased in inflamed biopsies. This supports the view that $K_V1.3$ may play a role upstream of the pro-inflammatory cytokine production of IFN-γ and TNF-α. We did not find evidence that $K_V1.3/K_{Ca}3.1$ may be involved in an underlying hidden inflammation in the healthy-looking mucosa.

$K_V1.3$ and $K_{Ca}3.1$ expression and medical treatment

To explore if $K_V1.3$ and $K_{Ca}3.1$ expression might be used as a measure of determining the best medical treatment for the UC patients, we tested if the increased expression could be used to stratify patients according to their medical treatment. If there was a higher expression of $K_V1.3/K_{Ca}3.1$ in the IS group compared to 5ASA group this could be indicative of a specific characteristic of UC patients that get relapses even though they are receiving a more powerful immunosuppressant (IS) treatment. However, we did not find significant differences between the 5ASA and the IS group. As we would expect, we found that $K_V1.3$ expression was increased in both 5ASA and IS groups compared to controls. This fits the findings from study 1, and that the general $K_V1.3$ expression correlates with inflammation score.

$K_V1.3$ and $K_{Ca}3.1$ in Crohn's disease

The results from UC patients made it relevant to investigate the expression of $K_V1.3$ and $K_{Ca}3.1$ in CD patients. Therefore, we began including patients with active CD. The preliminary results suggest that the expression levels are similar to the expression levels found in the study of patients with UC. $K_V1.3$ expression was increased 7-fold in CD mucosal biopsies, whereas $K_{Ca}3.1$ did not differ between groups. Interestingly, CD4 and CD8 expression did not differ between groups; still a trend towards increased expression of CD8 was observed. The monocyte/macrophage markers, CD14 and CD16, were significantly increased in CD biopsies together with IFN-γ and IL-17A. TNF-α expression did not reach statistical significance but a small trend was observed. The qPCR analyses were performed on biopsies obtained from patients with active CD. As the inflammation in CD patients is often more scattered over the colonic surface, this might cause a higher degree of variability in the inflammatory cells. As CD is believed to be T_H1 driven with IFN-γ as the key cytokine of T_H1, the results are in line with the finding from Reese et al. who found that T cells

from CD patients secrete more IFN compared to UC and controls [173]. Our results suggest that $K_V1.3$ may indeed be involved in the inflammatory cascade of both CD and UC. Our data from the cytokine assays further support the view that $K_V1.3$ and $K_{Ca}3.1$ blockers could have a positive effect on inflammation in CD, as dual blockade of the channels led to a decrease in proinflammatory cytokines IFN-γ, TNF-α and IL-17A. Generally seen, T cells are activated by APCs that engulf and present the bacteria that penetrate the damaged epithelial. The T cell activation leads to secretion of pro-inflammatory cytokines that attract more T cells and macrophages. The macrophages secrete more TNF-α and then the "circle of inflammation" is kept alive until the stimulation is discontinued due to cessation of the APC-stimulation via the TCR or if regulatory T cells (T_{reg}) inhibit the inflammatory cascade by secreting IL-10. Overall, our data, together with previous knowledge, suggest that $K_V1.3$ and $K_{Ca}3.1$ could be of importance in UC and CD.

$K_V1.3$ and $K_{Ca}3.1$ in DSS-induced colitis

Overall, administration of PAP-1 and Senicapoc (50 mg/kg) by oral gavage did not ameliorate endoscopic and histological inflammation in the DSS-induced colitis model in rats. However, we found a trend towards improvement of the mucosal inflammation evaluated by endoscopy during the acute phase (day 7 and 10). The histopathologic evaluation was performed after study termination at day 21 and did not show differences between the DSS-groups. Maybe the pharmacological inhibition of $K_V1.3$ and $K_{Ca}3.1$ has the majority of its impact during the acute phase where 5% DSS was administered (days 0-5) compared to maintenance dose of 1% DSS, which sustains the subsequent chronic-like inflammation. Other studies found that $K_{Ca}3.1$ blockade improved the inflammation in multiple models of colitis (DNBS, TNBS, and DSS)[119-121], but we did not find the same effect after the 21-days "semi-chronic" model of colitis, supporting the view that the pharmacological blockade of $K_V1.3$ and $K_{Ca}3.1$ may be more potent in the acute phase of inflammation as the above studies all were terminated after acute induction of colitis. Nevertheless, the main problem of our pilot trial probably was that plasma concentrations were too low and did not reach pharmacologically active levels.

THE DEVELOPMENT OF NEW DRUGS

The development of drugs for treatment of inflammatory bowel disease has changed. Initially the insight in the pathological mechanisms was more or less absent. The introduction of sulfasalazine for the treatment of ulcerative colitis was a result of a clinical observation. Patients with co-existence of ulcerative colitis and rheumatoid arthritis experienced relief in the gut symptoms when treated with sulfasalazine for their joint complaints[174]. The increased knowledge of pathogenic changes in patients with Crohn's disease, showing substantially elevated TNF-α in active disease, lead to a more focused development of treatment of anti-TNF blocking agents[175]. Animal model have a number of limitations and colitis spontaneous developing in animals or chemical induced colitis does only to some degree reflect the complex human inflammatory bowel disease where genetic and environmental factors are of importance. Nonetheless, animal models are of importance in the development of new drugs. In particular it may allow a preclinical evaluation if a specific pathogenetic mechanism is suspected as in the current study. Of course, the lack of effect or the observation of an effect in an animal does not allow extrapolation to a human clinical disease[176, 177]. TNF-α is increased not only in human inflammatory bowel disease but also in animal models of IBD[178] making animal models interesting in evaluating the effect of TNF-α in the treatment. On the other hand, sulfasalazine in some models do not have effect on the inflammation. Thus caution is needed when evaluating therapeutic trials from animal model studies.

No animal model is perfect, and each model has its advantages. Therefore, it is crucial to define a specific hypothesis including a specific pathological mechanism, and afterwards choose the most appropriate model. Otherwise, one could likely reject a "true" hypothesis.

PERSPECTIVES AND FUTURE RESEARCH

We have shown the presence of $K_V1.3$ in infiltrating T cells in human biopsies from patients with active UC. In the animal trial we also found, although only by trend, that pharmacological blockade of T cell $K_V1.3$ and $K_{Ca}3.1$ seemed to ameliorate DSS-induced colitis when assessed by endoscopic evaluation in the acute phase of the induced colitis (days 7 and 10). In contrast, this was not found when evaluating the histological changes. In our animal study, we might have used an ineffective way of administering the $K_V1.3$ and $K_{Ca}3.1$ blockers, as the drugs did not reach pharmacologically active levels in the plasma. Finding the optimal way of administering $K_V1.3$ and $K_{Ca}3.1$ blockers may lead to amelioration of experimental colitis and should be the subject of future studies. If animal trials reveal positive effects of pharmacological blockade of $K_V1.3$ and $K_{Ca}3.1$ on inflammation, future larger studies could be set up to investigate safety and effect in patients with UC or CD.

Additionally, we would like to elucidate the role of T cell potassium channels, $K_V1.3$ and $K_{Ca}3.1$, in CD. Preliminary data of CD patients reveal that the mRNA expression of $K_V1.3$ and inflammatory cytokines such as IFN-γ and IL-17A are significantly increased in biopsies from inflamed areas. In addition, it would be relevant to continue with immunostaining to visualize the infiltrating cells and channel expression in the mucosal biopsies, thus supporting the view that $K_V1.3$ and $K_{Ca}3.1$ blockers could be potential targets for medical treatment of CD.

Another very interesting approach would be to use flowcytometry to investigate the infiltrating T cells. In this way one could investigate specific T cells in peripheral blood but even more exciting in the inflamed mucosa. Using flowcytometry, it would be possible to isolate infiltrating T cells from within the lamina propria thus making it possible, based on immunofluorescence, to investigate the expression of $K_V1.3/K_{Ca}3.1$ channels in the individual subtypes of lymphocytes and also the cytokine profile of these. Additionally, it would allow us to compare these characteristics among IBD patients and a control population. One could test if the cells with high levels of potassium channels also are the ones with increased levels of intracellular cytokines such as IL-17A and IFN-γ. Another interesting objective would be to test if a population of $K_V1.3/K_{Ca}3.1$-high-expressing cells exists in the inflamed mucosa. This allow us to further investigate if high levels of potassium channels are characteristic features of infiltrating T cells in IBD thus supporting $K_V1.3$ and $K_{Ca}3.1$ as potential pharmacological targets. Flowcytometry is not only limited to T cells, but may be expanded to examine macrophages, NK-cells etc.

PERSONAL GAINS AS A RESEARCH FELLOW

During my years as a research fellow I have acquired important knowledge within the area of translational research. We have been working closely together with hospitals, outpatient clinics, nurses, doctors, technicians etc. This wide collaboration has taught me how to make things work smoothly between different staff groups and the importance of clear communication to avoid misunderstandings.

I have learned various methods used in basic and translational science e.g. the importance of troubleshooting in a chronological order to make sure that most aspects are covered. I have performed numerous qPCR analyses, which have led to several frustrating hours trying to solve why a reaction suddenly did not work as it did before.

Last year, when we decided that we wanted to initiate an animal trial to investigate the effect of pharmacological inhibition of $K_V1.3$ and $K_{Ca}3.1$ on mucosal inflammation, I also learned about project management, as I was the middleman who had to design a protocol that it was possible to use in the animal facility, and that met our expectations as well. Evidently, I did not do all this by myself, but got great guidance from Heike and Ralf when setting up the study.

During the years as a research fellow, I have expanded my knowledge with regard to academic writing. This was improved through the writing of the manuscripts included in this thesis, the useful critique of my supervisors, but also by co-authoring other manuscripts. I learned a lot about writing in a scientific language; nevertheless, there is room for improvement. My scientific writing skills received a huge upgrade, similar to upgrading from DOS to Windows 8.

OVERALL SUMMARY OF THE PROJECT

We demonstrated that T cell $K_V1.3$ channels are indeed present and increased in human mucosal biopsies from patients with active UC and possibly CD. This suggests that the T cell potassium channel, $K_V1.3$, could be a new pharmacological target. Moreover, the *in vitro* inhibition of $K_V1.3$ and $K_{Ca}3.1$ led to decreased proliferation rate and cytokine production, which suggests that both $K_V1.3$ and $K_{Ca}3.1$ may be involved in the inflammatory cascade upstream of classical pro-inflammatory cytokines. Finally, pharmacological inhibition of $K_V1.3$ and $K_{Ca}3.1$ showed an encouraging trend towards a beneficial effect on the endoscopic inflammation at day 7 and 10. However, these differences failed to become statistically significant likely due to insufficient absorption of compound or underdosing. In conclusion, $K_V1.3$ and $K_{Ca}3.1$ channels seem to be involved in a disease-related fashion in UC and possibly CD.

LIST OF ABBREVIATIONS

5ASA	5-aminosalicylic acid = mesalazine
AIS	Adaptive immune system
CCR7	Chemokine-Receptor
CD	Crohn's disease
CD#	Cluster of differentiation (#=number, e.g. CD4)
cDNA	Complementary DNA
DNA	Deoxyribonucleic acid
DNBS	Dinitrobenzene sulfonic acid
DSS	Dextran Sodium Sulfate
GC	Glucocorticoids
IFN-γ	Interferon gamma
IL-#	Interleukin (#=number, e.g. IL-17A)
ISS	Innate immune system
MHC	Major Histocompatibility Complexes
mRNA	Messenger RNA
NSAIDs	Non-steroidal anti-inflammatory drugs
PCR	Polymerase chain reaction
qPCR	Quantitative real-time polymerase chain reaction
RNA	Ribonucleic acid
T_C	Cytotoxic T cells
T_{CM}	Central memory T cells
TCR	T Cell Receptor
T_{EM}	Effector memory T cells
T_H	T helper cells
TNBS	Trinitrobenzene sulfonic acid
TNF-α	Tumor necrosis factor alpha
UC	Ulcerative colitis

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From the bottom of my heart. Thank you all!

Lars Koch Hansen

Odense, August 2014

SUMMARY IN ENGLISH

Ulcerative Colitis (UC) is a chronic inflammatory bowel disease located in the mucosa of the large bowel. UC often affects young adults between 15-40 years of age with no pre-dominant sex. Over time, incidence rates are steadily increasing and the cause of the disease remains unknown. Symptoms are general discomfort and bloody diarrhea. UC is diagnosed by endoscopic examination of the large bowel, where different hallmarks are found. It is of great importance that attacks/relapses are treated medically, as flares may cause death due to inflammatory destruction of the mucosa and perforation of the colon leading to extreme infection of the abdominal cavity. UC often affects the social life of the patients, as they feel that they must be in the immediate vicinity of toilets. Therefore, many patients prefer to stay at home during

active disease. For society, UC is a costly disease due to patients reporting in sick and expensive medications. When medical treatment fails, UC patients must undergo surgery and have their colon removed (colectomy).

This PhD project focused on the immune system of the body. Specifically, we looked into T cells (the chairmen of the immune system) that we believe play an important role in disease activity. When T cells are activated in inflammatory diseases, they produce several signaling substances (cytokines) that attract and activate the other parts of the immune system. T cells regulate their effector functions through calcium regulation. Upon activation, calcium is released from intracellular stores, which causes calcium channels to be embedded in the cell membrane (CRAC channels). As long as the T cells are stimulated, the two potassium channels, $K_V1.3$ and $K_{Ca}3.1$ maintain the driving force for calcium influx, thus keeping the T cells activated.

Our aims were to investigate whether the two potassium channels $K_V1.3$ and $K_{Ca}3.1$ were upregulated in mucosal biopsies from patients with active UC and whether there were correlations between the expression of the channels and the disease severity assessed by endoscopic and histological evaluation. Moreover, we used a rat colitis model (DSS-induced) to examine the effect of pharmacological inhibition of $K_V1.3$ and $K_{Ca}3.1$ on inflammation.

We found that the expression of T cell potassium channel, $K_V1.3$, was increased in active UC and a higher expression correlated well with both the endoscopic and the histological degree of inflammation. This suggests $K_V1.3$ to be involved in the inflammatory process of UC. We did not find an increase of the other potassium channel, $K_{Ca}3.1$, at the gene expression level, but the channels were definitely present in the infiltrating T cells as examined by immunostaining. Preliminary gene expression data showed similar changes of gene expression in biopsies from CD patients. In addition, we conducted first pilot studies investigating whether pharmacological blockade of the channels ameliorates colitis in the rat DSS-model. We found a tendency towards less endoscopic inflammation in the acute phase (at day 7 and 10). However, at study termination, the improvement of inflammation failed to reach a significant level, presumably because of insufficient compound absorption from the intestine (based on low plasma concentration and previously reported amelioration of colitis by inhibiting $K_{Ca}3.1$).

Based on these findings in our target identification study, it is suggested that both $K_V1.3$ and $K_{Ca}3.1$ play a role in the inflammation of UC and possibly of CD and represent new pharmacological targets.

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