Thiopurines and inhibition of Rac1 in vascular disease
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6-Mercaptopurine reduces macrophage activation and gut epithelium proliferation through inhibition of GTPase Rac1

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ABSTRACT

Background: Inflammatory bowel disease is characterized by chronic intestinal inflammation. Azathioprine and its metabolite 6-mercaptopurine (6-MP) are effective immunosuppressive drugs that are widely used in patients with inflammatory bowel disease. However, established understanding of their immunosuppressive mechanism is limited. Azathioprine and 6-MP have been shown to affect small GTPase Rac1 in T cells and endothelial cells, whereas the effect on macrophages and gut epithelial cells is unknown.

Methods: Macrophages (RAW cells) and gut epithelial cells (Caco-2 cells) were activated by cytokines and the effect on Rac1 signaling was assessed in the presence or absence of 6-MP.

Results: Rac1 is activated in macrophages and epithelial cells, and treatment with 6-MP resulted in Rac1 inhibition. In macrophages, interferon-g induced downstream signaling through c-Jun-N-terminal Kinase (JNK) resulting in inducible nitric oxide synthase (iNOS) expression. iNOS expression was reduced by 6-MP in a Rac1-dependent manner. In epithelial cells, 6-MP efficiently inhibited tumor necrosis factor-a–induced expression of the chemokines CCL2 and interleukin-8, although only interleukin-8 expression was inhibited in a Rac1-dependent manner. In addition, activation of the transcription factor STAT3 was suppressed in a Rac1-dependent fashion by 6-MP, resulting in reduced proliferation of the epithelial cells due to diminished cyclin D1 expression.

Conclusions: These data demonstrate that 6-MP affects macrophages and gut epithelial cells beneficially, in addition to T cells and endothelial cells. Furthermore, mechanistic insight is provided to support development of Rac1-specific inhibitors for clinical use in inflammatory bowel disease.

Key Words: 6-mercaptopurine, Rac1, macrophages, gut epithelium, IBD
INTRODUCTION

Crohn’s disease (CD) and ulcerative colitis (UC), the 2 major forms of inflammatory bowel disease (IBD), are characterized by chronic relapse of intestinal inflammation. In Europe, an estimated 2.5 to 3 million people are affected by IBD.\textsuperscript{1} Over the years, various pharmaceutical approaches were evaluated, but only the thiopurines, consisting of azathioprine and its metabolites, have proven effective to suppress IBD relapse.\textsuperscript{2,3} The immunosuppressive drug azathioprine has been used in clinical practice for more than 50 years.\textsuperscript{4} Since the CD clinical trial from 1980, reported by Present et al,\textsuperscript{5} described the efficacy of 6-mercaptopurine (6-MP), various randomized trials have reported remission rates for 6-MP/azathioprine in adult CD populations of 40% to 80%.\textsuperscript{6–9} In approximately 50% of patients with IBD, a low dose of azathioprine or 6-MP is prescribed and well tolerated without major complications.\textsuperscript{10} Patients with IBD (both UC and CD) have an increased risk of developing colorectal cancer\textsuperscript{11,12} through inflammation-induced genetic mutations.\textsuperscript{13,14} 6-MP has been shown to prevent advanced colorectal cancer in patients with IBD.\textsuperscript{15}

Azathioprine and 6-MP are inactive prodrugs demanding enzymatic conversion to become biologically active as purine analogs. Azathioprine is enzymatically converted to 6-MP in the intestinal mucosa and liver, which is, in turn, converted to 6-thioguanosine monophosphate (6-T-IMP) and subsequently to purine analogs 6-thioguanosine-50-monophosphate (6-T-GMP) or 6-thiadenosine-5'-monophosphate (6-T-AMP).\textsuperscript{16} Azathioprine is mostly known for its mechanism of action related to decreased purine synthesis and subsequent apoptosis of fast proliferating cells such as CD4+ T cells.\textsuperscript{17} This cytotoxic effect was for a long time the only proposed mechanism for Azathioprine-induced immunosuppression.\textsuperscript{18} Relatively recently, Azathioprine, 6-MP and 6-thioguanosine-5'-triphosphate (6-T-GTP) have been shown to inhibit specific GTP-dependent proteins Rac1 and Rac2 in CD4+ T-cells, blocking T-cell activation.\textsuperscript{19} Interestingly, 6-T-GTP can also bind other small Rho-GTPases such as Cdc42 and RhoA, yet, it can only block the activity of Rac1 and Rac2.\textsuperscript{20} In addition, we have recently shown that 6-MP reduces endothelial cell (non-immune cell) activation in a Rac1-dependent manner, thereby decreasing leukocyte attachment and transmigration.\textsuperscript{21,22} Rac1 cycles between an active and inactive conformation and the GTPase is activated when guanosine-5'-diphosphate (GDP) is exchanged by guanosine-5'-triphosphate (GTP). On activation, the GTP-bound form of Rac1 can be hydrolyzed to Rac1-GDP, thereby becoming inactive again.

The potential role of 6-MP in Rac1-mediated signals has not yet been investigated in macrophages and gut epithelium, 2 key cell types involved in IBD pathology. The intestinal epithelium acts as a protective physical barrier and is actively involved in immune cell regulation. These epithelial cells are connected by intercellular junctions, and defects in this structure have been reported in patients with IBD to lead to increased permeability.\textsuperscript{23,24} Epithelial cells are also able to take up antigen, deliver it across the cell, and efficiently transfer it to antigen presenting cells, such as dendritic cells and macrophages.\textsuperscript{25} In addition, epithelial cells can express a range of inflammatory cytokines and chemokines such as tumor necrosis factor-\alpha (TNF\alpha) and interleukin (IL)-8 to regulate a proper immune response when necessary.\textsuperscript{26–28} Key immune cells that maintain intestinal immune homeostasis are T cells and macrophages.\textsuperscript{29–31} Dysregulation of macrophages leads to development of IBD in part through activation of specific T-cell populations. Thiopurines have been shown recently to cause cytotoxicity of specific subsets of T cells.
by modulation of a small number of transcription factors.\textsuperscript{32} The effect of thiopurines on macrophages has been relatively unstudied, but we already demonstrated that 6-MP enhances macrophage apoptosis and induces an anti-inflammatory macrophage phenotype.\textsuperscript{33} Because Muise et al\textsuperscript{34} revealed that single-nucleotide polymorphisms-enhancing Rac1 activity strongly affect IBD onset and progression, we hypothesized that azathioprine and its downstream metabolites are effective in treatment of IBD because of their Rac1 inhibitory capacity in cells localized in the gut. Here, we demonstrate that 6-MP and 6-T-GTP reduce pro-inflammatory signaling pathways in macrophages and have an antiproliferative and anti-inflammatory effect on epithelial cells, in part, through inhibition of Rac1.
CHAPTER 5

MATERIALS AND METHODS

RAW264.7 and CaCo-2 Cell Cultures and Transfection Procedure
RAW264.7 cells were cultured in RPMI-1640 (GIBCO; Invitrogen; Life Technologies, Bleiswijk, The Netherlands) supplemented with 100 U/mL Penicilline/Streptomycine and 10% heat-inactivated fetal calf serum (FCS; GIBCO; Invitrogen). CaCo-2 cells (p41–55) were cultured in Dulbecco’s modified eagle’s medium-high glucose (GIBCO; Invitrogen) supplemented with 100 U/mL Penicilline/Streptomycine, 20% FCS, and 4 mM L-glutamine (Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands) and were passaged when 50% confluent.

Transfection with empty plasmid or plasmid encoding constitutively active Rac1 Q61L was performed using the LTX lipofectamine kit (Invitrogen) according to the manufacturer’s instructions for these specific cell lines.

Rac1 and c-Jun Activity Assays
RAW264.7 and CaCo-2 cells were treated for 16 hours with 10 mM 6-MP, 10 mM 6-T-GTP, or 100 mM Rac-1 inhibitor (553502; Calbiochem, San Diego, CA) followed by a 24-hour incubation with either 50 ng/mL interferon-g (IFNγ) for RAW264.7 or 50 ng/mL TNFa for CaCo-2 cells. Cell lysates were prepared, and levels of active Rac1-GTP were measured using a colorimetric Rac1 activation G-LISA assay (BK128; Cytoskeleton Inc., Denver, CO) according to the manufacturer’s protocol. Transcriptional activity of c-Jun was measured using the TransAM Transcription Factor Assay Kits (Active Motif, La Hulpe, Belgium). Nuclear extracts were prepared, and DNA binding activity of the transcription factors was measured in an enzyme-linked immunosorbent assay setup, and Optical density was determined at 450 nm.

DNA Synthesis Assay
Caco-2 cells were seeded in 96-well plates at a density of 2 · 103 cells per well and incubated overnight in full medium. Cells were made quiescent by incubation in medium without FCS for 24 hours. Subsequently, the cells were treated with 10 mM 6-MP, 10 mM 6-T-GTP, 100 mM Rac-1 inhibitor, or 10 mM STAT3 inhibitor Stattic (Sigma) for 16 hours and stimulated with FCS (20% vol/vol) and TNFa (10 ng/mL) for 24 hours. DNA synthesis was measured by the bromodeoxyuridine (BrdU) incorporation assay (Roche, Woerden, The Netherlands) according to manufacturer’s instructions.

mRNA Isolation and Inflammatory Gene Expression
Both RAW264.7 and Caco-2 cells were treated for 16 hours with 10 mM 6-MP, 10 mM 6-T-GTP, or 100 mM Rac-1 inhibitor, additionally, RAW264.7 were treated with 50 mM JNK activation inhibitor SP600125 and CaCo-2 cells with 10 mM STAT3 inhibitor Stattic (Sigma) for 16 hours and stimulated with FCS (20% vol/vol) and TNFa (10 ng/mL) for 24 hours. Total RNA was extracted using the Aurum Total RNA Mini Kit (Bio-Rad, Veenendaal, The Netherlands), and cDNA was synthesized from 200 ng total RNA using iScript cDNA Synthesis kit (Bio-Rad). Semiquantitative real-time PCR was performed using MyIQ SYBR Green Supermix (Bio-Rad) and was measured with the MyIq system. Specific primers for inducible nitric oxide synthase (iNOS), monocyte chemo-attractant protein-1 (MCP-1/CCL2), IL-6, IL-8 (and KC as murine equivalent), and ribosomal protein P0 (and 36B4 as murine equivalent, to correct for cDNA content) were designed (Table 1). Each experiment was performed at least in duplicate.
Table I- List of primers

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**Immunoblotting**

RAW264.7 and CaCo-2 cells were treated for 16 hours with 10 mM 6-MP, 10 mM 6-TGTP, 100 mM Rac-1 inhibitor or in the case of Caco-2 cells, with 10 mM STAT3 inhibitor Stat6, followed by a 15-minute incubation with either 50 ng/mL IFNγ or 50 ng/mL TNFα, respectively, after which the cells were washed twice with phosphate-buffered saline and lysed in ice-cold NP-40 lysis buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM NaF, 1 mM Na3PO4, 10% glycerol, and 1% Nonidet, supplemented with Sigma protease inhibitor cocktail). After 10 minute incubation on ice, the lysates were collected, sonicated for 1 minute, and boiled in sample buffer containing DTT. Samples were thereafter analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 12% gels. Proteins were transferred to either 0.2 mm nitrocellulose membranes or PVDF (Bio-Rad) using the Transblot Turbo transfer system (Bio-Rad). Nitrocellulose membranes were subsequently blocked in 5% (wt/vol) nonfat milk in Tris-buffered saline and incubated with specific primary antibodies overnight at 48C, followed by horse radish peroxidase-labeled secondary antibodies (Bio-Rad) for 1 hour. Proteins were visualized with an enhanced chemiluminescence detection system (Thermo Scientific, Leusden, The Netherlands), and quantification of signal was performed using intensity measurements in ImageJ software (by NIH, Bethesda, MD). PVDF membranes were blocked with Odyssey blocking buffer (LI-COR Biosciences, Bad Homburg, Germany)/Tris-buffered saline (1:1) and incubated with specific primary antibodies overnight at 48C, followed by IRDye-labeled secondary antibodies (LI-COR Biosciences) for 1 hour. Proteins were visualized with an Odyssey infrared imaging system (LI-COR Biosciences), and quantification was performed using Odyssey software (LI-COR Biosciences). Phosphorylated (p) STAT3 and pJNK expression were corrected for total STAT3 and JNK, respectively. The following primary antibodies were used: anti-Cyclin D1 (Cell Signaling Technology, Inc., Leiden, The Netherlands), anti-STAT3 (Millipore, Amsterdam, The Netherlands), anti-pSTAT3 (Y705), anti-JNK, anti-pJNK (all 3; Cell Signaling Technology, Inc.), and anti-a-tubulin (Cedarlane, Tebu-Bio, Heerhugowaard, The Netherlands).
IL-8 Determination
IL-8 protein levels were measured in the supernatants of Caco-2 cells treated for 16 hours with 10 mM 6-MP, 10 mM 6-T-GTP, and 100 mM Rac1 inhibitor followed by a 24-hour incubation with 50 ng/mL TNFα using the Cytometric Bead Array Human Inflammation kit (BD Biosciences, San Jose, CA) according to the manufacturer’s instructions.

Statistical analysis
Statistical analysis was performed using GraphPad Prism 5 software (La Jolla, Ca). Statistical significance was calculated using the unpaired Student’s t-test (Welch corrected when necessary). Values are represented as mean ± SEM. The significance level was set at p<0.05.
RESULTS

6-Mercaptopurine Inhibits the Inflammatory Response of Macrophages

Macrophages play a key role in the altered immune response as a consequence of disturbed normal intestinal flora, a hallmark of IBD pathogenesis. To elucidate the potential beneficial effect of 2 metabolites of azathioprine on macrophages, we investigated the in vitro response of macrophage cell line RAW264.7 to IFNγ-activation in the presence or absence of 6-MP or 6-T-GTP. Because 6-MP/6-T-GTP is known to exert part of their anti-inflammatory effect through repression of Rac1 activity, we first studied Rac-1 activation. As shown in Figure 1A, Rac1 activity was significantly induced by IFNγ and diminished by pretreatment with 6-MP or 6-T-GTP. The reduction in Rac1 activation was similar in cells treated with the synthetic Rac1 inhibitor (Fig. 1A). c-Jun N-terminal Kinase (pJNK) is activated by Rac1 and both 6-MP/6-T-GTP strongly downregulated IFNγ-induced phosphorylation of JNK (Fig. 1B) and c-Jun activity (Fig. 1C), like the Rac1 inhibitor. To determine which downstream genes were affected, the expression of a number of proinflammatory genes, such as cytokines and chemokines, was screened. 6-MP/6-T-GTP significantly decreased expression of IL-6, IL-8, CCL2, and CCL5 (Fig. 1D). Interestingly, the expression of none of these genes was reduced by the Rac1 inhibitor, indicating that 6-MP/6-T-GTP also have Rac1-independent anti-inflammatory effects in macrophages. Inducible nitric oxide synthase (iNOS) was the only gene screened, which was repressed in a Rac1-dependent manner (Fig. 1E). Of note, a specific iNOS single-nucleotide polymorphism (rs2297518) is considered detrimental in 2 early onset IBD cohorts. 36 Excessive iNOS activity may lead to enhanced NO production, causing increased tissue damage. In addition to the Rac1 inhibitor, iNOS expression could also be reduced by inhibition of JNK signaling, suggesting that 6-MP/6-T-GTP decreased iNOS expression in a Rac1/JNK-dependent fashion. To substantiate these findings, iNOS mRNA levels were measured in macrophages transfected with a construct encoding constitutively active Rac1 mutant (Q61L). Transcription of iNOS was strongly induced by IFNγ and was markedly reduced by 6-MP, 6-T-GTP, and the Rac1 inhibitor in mock transduced cells, whereas iNOS transcriptional repression was not observed in cells overexpressing the Rac1-Q61L protein (Fig. 1F). In conclusion, these data reveal that part of the beneficial effect of azathioprine in patients with IBD involves inhibition of Rac1 in macrophages and suppression of excessive iNOS expression.
CHAPTER 5

Figure 1. 6-MP and 6-T-GTP inhibit inflammation in macrophages.
(A) Rac1 activity is increased after IFNγ stimulation in RAW264.7 macrophages, and decreased by 6-MP and 6-T-GTP to a similar extent as with Rac1 inhibitor (*P<0.03). (B) Increased phosphorylated (p)-JNK is observed in response to IFNγ stimulation, which is lowered after 6-MP, 6-T-GTP and Rac1 inhibitor pre-treatment. Quantification of p-JNK is corrected for total JNK (*P<0.02). (C) In line with these findings, the activity of the transcription factor c-Jun was significantly increased in IFNγ treated macrophages and reduced by 6-MP, 6-T-GTP and Rac1 inhibitor (*P<0.01). (D) Increased gene expression of cytokine IL-6, and chemokines IL-8, CCL2 and CCL5 was observed in response to IFNγ, which is reduced by 6-MP/6-T-GTP, but not by the Rac1 inhibitor. (*P<0.05). (E) iNOS mRNA expression in IFNγ-activated macrophages is reduced by 6-MP/6-T-GTP, as well as by the Rac1- and JNK- inhibitors (*P<0.04). (F) RAW264.7 cells were transfected with control plasmid or plasmid encoding the constitutively active Rac1-mutant (Q61L). iNOS mRNA expression was no longer inhibited by 6-MP/6-T-GTP and Rac1 inhibitor, when Rac1- Q61L was overexpressed (*P<0.03), indicating that iNOS production is Rac1-mediated.
Reduced Inflammatory Response of Intestinal Epithelial Cells by 6-MP

Because 6-MP and downstream metabolite 6-T-GTP reduce the inflammatory gene expression profile in T cells, endothelial cells, and now in macrophages, we analyzed gene expression of 2 macrophage-attracting chemokines in an intestinal epithelial cell line (Caco-2). Caco-2 cells were pretreated overnight with 6-MP, 6-T-GTP, or the Rac1 inhibitor and subsequently incubated with TNFα for 24 hours. TNFα significantly induced mRNA expression of CCL2 and IL-8, whereas 6-MP/6-T-GTP strongly diminished the expression of both chemokines, similarly as observed in endothelial cells and macrophages (Fig. 2A). Comparable with the macrophages, CCL2 expression was not diminished by the Rac1 inhibitor; however, in contrast, IL-8 expression was sensitive to Rac1 inhibition. To confirm that reduced levels of IL-8 mRNA result in reduced IL-8 protein expression, we measured this chemokine in the supernatant of the epithelial cells. As expected, IL-8 protein expression was increased upon TNFα stimulation and reduced by 6-MP/6-T-GTP or the Rac1 inhibitor (Fig. 2B), suggesting a possible Rac1-dependent decrease in IL-8 by the thiopurines, which may reduce macrophage and neutrophil attraction to the gut.

Figure 2. 6-MP/6-T-GTP treatment decreases chemokine expression in gut epithelial cells.

(A) mRNA expression of chemokines CCL2 and IL-8 is increased upon TNFα stimulation of gut epithelial Caco-2 cells. 6-MP/6-T-GTP modulate the pro-inflammatory gene expression response by suppressing mRNA production of both chemokines. IL-8 mRNA production is also reduced by the Rac1 inhibitor (*P<0.03). (B) IL-8 protein expression in conditioned culture medium is also suppressed by 6-MP, 6-T-GTP and the Rac1 inhibitor (*P<0.04).
6-MP Inhibits Rac1-mediated STAT3 signaling and proliferation of epithelial cells

Patients with IBD exhibit a proliferative epithelial cell phenotype that may result in colon cancer. To determine the involvement of Rac1 in proliferation of intestinal epithelial cells, we investigated their response to TNFα stimulation in the presence or absence of 6-MP/6-T-GTP or the Rac1 inhibitor. In line with our previous results in non-immune cells, such as endothelial cells, the amount of active Rac1 was increased on TNFα stimulation and decreased in the presence of 6-MP/6-T-GTP or the Rac1 inhibitor (Fig. 3A). To study the effect of 6-MP/6-T-GTP on proliferation of epithelial cells, serum-induced BrdU incorporation was measured in Caco-2 cells. The 10 mM dosage of 6-MP/6-T-GTP is low and therefore considered not to interfere directly with DNA synthesis. Yet, pretreatment of epithelial cells with 6-MP/6-T-GTP resulted in decreased DNA synthesis, similar to the use of the Rac1 inhibitor (Fig. 3B). In contrast, overexpression of constitutively active Rac1-Q61L resulted in increased proliferation which could not be reduced by 6-MP/6-T-GTP or the Rac1 inhibitor. Signal transducer and activator of transcription-3 (STAT3) is an important transcription factor in activated epithelial cells in IBD, known for its pro-proliferative effect and Rac1-dependency. Here, we show that STAT3 inhibitor Stattic reduced proliferation of the Caco-2 cells, suggesting that 6-MP/6-T-GTP may inhibit proliferation in a Rac1/STAT3-dependent fashion. To further substantiate these findings, we revealed that STAT3 phosphorylation (pSTAT3) is induced by TNFα in epithelial cells, which was reduced by 6-MP/6-T-GTP or the Rac1 inhibitor (Fig. 3C). In hepatocellular carcinoma cells, it is known that 6-MP has a strong anti-proliferative effect, which involves 6-MP-mediated reduction of Cyclin D1. Here, we demonstrated that indeed, levels of Cyclin D1 were decreased on 6-MP/6-T-GTP and Rac1 inhibitor treatment, as well as with the STAT3 inhibitor (Fig. 3D), suggesting that the thiopurines block epithelial proliferation in a Rac1/STAT3/Cyclin D1-dependent manner.
Figure 3. Inhibition of epithelial cell proliferation by 6-MP/6-T-GTP.
(A) Rac1 activity is increased in gut epithelial Caco-2 cells upon TNFα stimulation, and is inhibited by 6-MP/6-T-GTP pre-treatment to a similar extent as the Rac1 inhibitor (*P<0.03). (B) TNFα activation increases epithelial cell proliferation as measured by enhanced BrdU incorporation. 6-MP, 6-T-GTP or Rac1 inhibitor treatment results in reduced DNA synthesis in cells transfected with the control empty vector (*P<0.003). Upon overexpression of constitutively active Rac1-mutant Q61L, proliferation is increased, already at baseline, and is not influenced by TNFα stimulation, 6-MP, 6-T-GTP or the Rac1 inhibitor. (C) Phosphorylation of transcription factor STAT-3 is significantly increased by TNFα and repressed by 6-MP, 6-T-GTP and the Rac1-inhibitor, similarly as by the STAT-3 inhibitor (*P≤0.05). Quantification of p-STAT-3 is corrected for total STAT-3. (D) CyclinD1 is significantly increased by TNFα and repressed by 6-MP, 6-T-GTP, the Rac1-inhibitor, and the STAT-3 inhibitor (*P<0.05).
In conclusion, the azathioprine metabolites 6-MP and 6-T-GTP display Rac1-independent and Rac1-dependent anti-inflammatory properties in immune and non-immune cells and have an anti-proliferative effect on gut epithelial cells, possibly decreasing susceptibility to cancer development in IBD. The Rac1-dependent functions of 6-MP/6-T-GTP in different cell types in the gut are summarized in a scheme (Fig. 4).

**Figure 4. Schematic overview of inflamed intestine with various cell types involved in IBD and the Rac1-dependent processes that are inhibited by 6-MP.**

6-MP affects immune cells, specifically, T-cells and macrophages, and non-immune cells, such as endothelial cells and epithelial cells, in a Rac1-dependent fashion, contributing to the resolution of IBD. Poppe et al. (45) reported that 6-MP efficiently blocks Rac1 activity in T cells. This blockade leads to a suppressed ability of T-cells to interact with antigen presenting cells (APC), reduced IFNγ, and thus a reduced immune response. Here, we showed that in macrophages (MΦ), 6-MP reduces activation of pro-inflammatory pJNK-mediated signaling and transcription of iNOS. The production of inflammatory cytokines and chemokines was also diminished by 6-MP, although not in Rac1-dependent manner. The vasculature in the gut attributes to inflammation by promoting leukocyte influx into the activated/inflamed area. We previously reported (37) that, 6-MP reduces the activity of pro-inflammatory transcription factors AP-1, ATF-2 and NFκB in a Rac1-dependent manner in endothelial cells (EC). It reduces the expression of various chemotactic proteins and VCAM-1/ICAM1-mediated transmigration of leukocytes. In the current study, we revealed that 6-MP additionally effectively decreases chemokine IL-8 expression in intestinal epithelial cells (IEC) and blocks activation of STAT-3 and cyclinD1, resulting in a reduced proliferative capacity, which is beneficial considering the increased colon cancer risk in IBD.
Azathioprine is effective to combat IBD; nonetheless, the mechanism of action of this immunosuppressive drug on the intestinal wall remained elusive. Here, we demonstrate that the Azathioprine metabolites 6-MP and 6-T-GTP inhibit inflammatory and proliferative processes in macrophages and gut epithelial cells, at least partly through Rac1 inhibition. In macrophages, these thiopurines reduce the inflammatory response, which is largely Rac1-independent, except for iNOS expression which is reduced through Rac1-inhibition. Nitric oxide produced by iNOS is known to cause tissue damage in IBD and therefore inhibition of iNOS expression by 6-MP and 6-T-GTP may be considered beneficial in IBD. In epithelial cells, the thiopurines decrease expression of the chemokines CCL2 and IL-8; and in these cells, IL-8 production was shown to be Rac1-dependent. On secretion from activated epithelial cells, CCL2 strongly attracts macrophages, whereas IL-8 is a potent chemokine for both macrophages and neutrophils, together resulting in local inflammation in the bowel. Here, we provide evidence that this process is favorably influenced by 6-MP and 6-T-GTP.

IBD coincides with an increased risk of colon cancer due to chronic inflammation and subsequent STAT3-mediated epithelial proliferation. 6-MP/6-T-GTP inhibits proliferation in a Rac1/STAT3/Cyclin D1-dependent manner and may therefore contribute to a decreased risk to develop colon cancer. In line with our results, Triptolide, a diterpenoid triepoxide isolated from a traditional Chinese medicinal herb, is a potent Rac1 inhibitor decreasing proliferation of colon cancer cells (SW480 cell line) and Caco-2 cells by inhibition of the STAT3-Cyclin D1 pathway. Moreover, Triptolide prohibits growth of primary tumors in nude mice.

The anti-proliferative effect of 6-MP in epithelial cells has also been observed in vascular smooth muscle cells, where 6-MP induced the cell cycle inhibitor p27kip1. In gut, smooth muscle cells are abundant, and our previous data suggest that the thiopurines may also affect this cell type beneficially. This requires further investigations to assess the effect of 6-MP/6-T-GTP on both proliferation and inflammatory processes in smooth muscle cells and the impact on development and resolution of IBD.

The potent inhibitory effect of 6-MP/6-T-GTP on STAT3 activation seems highly relevant because single nucleotide polymorphisms for STAT3 were shown to associate with excessive STAT3 signaling and increased sensitivity to IBD. In addition, increased phosphorylation of STAT3 was observed in epithelium of patients with active IBD as compared with patients with inactive IBD. Of note, a dual role has been described for STAT3 signaling in the gut; STAT3 activation is required to establish quiescence after acute infection, whereas chronic STAT3 activation induces susceptibility to bacterial infection causing ulceration. Prolonged STAT3 activation increases the production of cytokines IL-6 and IL-23, which promote the maturation of pro-inflammatory Th17 cells that have been implicated in IBD. Along this line, Rac1 is essential for CD4+ T-cell differentiation, and 6-MP/6-T-GTP efficiently block Rac1 activity in these cells, thus inhibiting their activation and interaction with antigen presenting cells, such as macrophages, resulting in reduced inflammation.

The presence of activated macrophages in the intestinal wall is a key feature of IBD pathology. We have previously shown that 6-MP has a pro-apoptotic and an anti-inflammatory effect on macrophages. Pro-inflammatory stimuli, such as TNFα or IFNγ, lead to strong activation of the JNK signaling cascade, which is a consequence of Rac1 activation. Blocking the JNK pathway with inhibitors in animal IBD models led to the resolution of intestinal inflammation. In patients with IBD, JNK phosphorylation is increased.
in colon tissue with active disease. JNK phosphorylation is present in the intestinal cells, macrophages, and lymphocytes and localized predominantly in the nucleus. We have shown here that, like in endothelial cells, 6-MP/6-T-GTP can efficiently attenuate activation of JNK-mediated signaling in macrophages. In this investigation, we propose Rac1-JNK-cJun-dependent iNOS expression in macrophages. Ultimately, enhanced iNOS expression will result in increased production of nitric oxide, a reactive oxygen species, that is highly associated with tissue damage in IBD, which is prevented by 6-MP/6-T-GTP.

In conclusion, we demonstrate that the metabolites of azathioprine, namely 6-MP and 6-T-GTP, suppress the inflammatory response in macrophages and gut epithelial cells, important cell types involved in IBD pathology. The effect is in part mediated by Rac1-specific inhibition and in part through another GTP-dependent mechanism because the Rac1-independent features of 6-MP were inhibited by 6-T-GTP. In addition, epithelial cell proliferation is suppressed by 6-MP/6-T-GTP through targeting of Rac1-STAT3-Cyclin D1. Azathioprine is essential in treatment of IBD, but it has serious side effects signifying the clear need for more specific drugs. Our data provide clues why thiopurines are so effective in treatment of IBD by targeting multiple cell types. This knowledge is instrumental to eventually define the necessary characteristics of the ideal drug to control this disease. Specific inhibition of Rac1 is a realistic option, for which therapeutic strategies are being developed in the cancer field. However, blocking Rac1 with inhibitor NSC23766 delayed UC healing in a rat model for UC, revealing a beneficial role for Rac1 that should be kept in mind.

Finally, azathioprine and 6-MP are not just immnosuppressive drugs affecting T-cells and macrophages, but also favorably influence endothelial cells and as shown in this study, epithelial cells.
Reference List


