Thiopurines and inhibition of Rac1 in vascular disease
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CHAPTER 1

General Introduction
INTRODUCTION

Cardiovascular diseases such as atherosclerosis and aneurysm formation are the leading cause of morbidity and death worldwide. Together with inflammatory diseases of digestive tract they represent most common pathologies of the modern human society, induced by combination of various external factors such as smoking, unhealthy diet and genetic susceptibility. Introduction part of this thesis will provide a deeper insight of these two major organ systems, their pathologies as well as the possible targets for pharmaceutical approaches in their treatment.

1. Vascular system

Since the discovery of the circulatory system by William Harvey (1578-1657), an English anatomist and the godfather of modern physiology, our knowledge and understanding of it expanded tremendously. He established and described the circulatory system as a network of blood vessels in which blood is fluctuating in a “more or less” closed system, since he was unable to see the capillary network at that time\(^1\). Today, we are describing it as a closed organ system (Figure 1) in charge of transport of blood to supply our body with oxygen, nutrients, hormones and immune cells, and dispose of carbon dioxide, waste products and excess temperature. It consists of arteries (receiving blood from the heart), capillaries (the network of smallest vessels exchanging molecules and cells in tissues), and veins (bringing blood back to the heart). These three types of blood vessels each have unique architecture and functions.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** (A) Schematic overview of cardiovascular system, Obtained from: Dollar photo club; File #58768273. (B) Schematic overview of the healthy vessel wall

There are different diseases known for each type of vessel, such as venous thrombosis for veins, retinopathy in diabetic patients for capillaries and atherosclerosis or aneurysm formation for arteries. Vascular diseases, together with cancer, are the leading cause of
death in modern human society. This is predominantly related to arterial disease. But even in cancer, vascular pathology plays a key role, since tumor-induced vascularization leads to tumor growth. In 2008, 17.3 million people world-wide died from cardiovascular diseases, representing 30% of all deaths. By 2030, that number will rise to 23.3 million per year according to a predictions made by World Health Organization (WHO). During embryonic development, vessels are first formed by endothelial cells, which form a monolayer and barrier between blood and tissue. This morphological build closely resembles capillaries in later developmental stages. In arteries and veins, the endothelial cells form the inner lining of the vessel, the so called intima (tunica interna). In addition, these vessels have a smooth muscle cell (SMC)-rich medial layer (tunica media), where the SMC are embedded in a fine network of extracellular matrix. The outer layer of the vessel is called the adventitia (tunica externa), and consists predominantly of thick collagen fibers, which are maintained by fibroblasts. SMC are the most abundant cell type within the vessel wall. Arterial degradation of these layers causes excessive extension of the vessel wall, also known as an aneurysm, which ultimately may dissect or rupture and cause morbidity and mortality. There is no effective drug currently to target abdominal aortic aneurysm dilatation.

1.1 Aortic Aneurysms
The most common disease of the arterial vasculature is atherosclerosis, which is induced by chronically enhanced blood cholesterol levels, leading to cholesterol accumulation in the arteries and subsequent inflammation of the arterial wall. Aortic aneurysms are aortic dilatations caused by degradation of the arterial vessel wall. For the development of Abdominal Aortic Aneurysms (AAA) (Figure 2), atherosclerosis is a strong risk factor, together with age and smoking. In contrast to AAA, thoracic aortic aneurysms (TAA) affect individuals earlier in life and have a strong genetic background, mostly related to connective tissue disorders, such as in Marfan Syndrome. Aneurysm pathology is mostly related to the aorta, but it can also affect other arteries, such as arteries at the base of the brain, at the so called circle of Willis, resulting in intracranial aneurysms. Aneurysms are usually asymptomatic, nonetheless, this enlarged part of the vessel is prone to local rupture, associated with high mortality rates. Even though AAA incidence (4-9% >65 years) declined in recent years, presumably due to decreased smoking, the number of elderly people in western societies is still growing, which makes AAA an important vascular pathology. In addition, more frequent and more detailed imaging techniques in the clinic discover AAA patients with relatively small aneurysms. This also attributes to the urge to understand the developmental stages of aneurysm formation to be able to intervene pharmacologically, to prevent aneurysm growth and ultimately rupture. Currently, surgical intervention is required to prevent rupture of an aneurysm, because of a lack of effective medicinal treatment. However, in Marfan Syndrome patients it was shown that angiotensin-II receptor type 1 (AT1R) inhibitor Losartan significantly reduced aortic dilatation. In line with these data, it is known that two AT1R-related polymorphisms (angiotensin-1 converting enzyme (ACE) insertion/deletion polymorphism and AT1R A1166C polymorphism) are associated with enhanced risk for AAA in a meta-analysis in >16.700 individuals. In addition, one of the most prominent AAA mouse models is via chronic infusion of angiotensin-II, resulting in aortic dilatation throughout the whole aortic trajectory. Thus pathways downstream of AT1R seem detrimental in aneurysm development, although it is not known what the key pathological pathway is. Currently, a clinical trial with yet another AT1R inhibitor, namely Telmisartan, is running in AAA patients in The Netherlands and Australia (TEDY trial: EudraCT number2012-001859-39).
To develop drugs that may inhibit aortic dilatation, understanding the different pathological processes in vascular degradation is essential. Here, an overview is given of the current knowledge on AAA formation.

Figure 2. Examples of murine abdominal aortic aneurysms (AAA).
(A) Macroscopic view of an AAA after 4 weeks angiotensin-II infusion. Microscopic cross sections of such AAA reveal different type of AAA; namely atherosclerotic AAA (B) and non-atherosclerotic AAA (C). In B, a Masson Trichrome staining shows the smooth muscle cell layer in red, the fibrotic adventitia in blue and the atherosclerotic area in pink. In C, an immunohistochemical staining for smooth muscle α-actin shows the smooth muscle cell layer in red.

1.2 Cellular mechanisms of AAA development
In aneurysm tissues obtained after surgery it is clear that there is excessive inflammation and SMC death. Destruction of the arterial wall is considered the result of excessive inflammation. But since aneurysm tissue, obtained after surgery, presents a static condition of end-stage disease, animal aneurysm models have taught us the relevance of inflammation. Many animal studies indeed highlight the inflammatory processes as determined by genetic profiling. Progressive inflammation is characterized by leukocyte infiltration, which produce cytokines to activate surrounding cells, chemokines to attract cells, and proteases to facilitate infiltration and migration of cells, possibly to repair local damage. Yet, an excess of any of these proteins may also cause local cell death, causing a feed forward loop and enhancing the inflammatory response. There is a wide variety of immune cells that are identified in aneurysm tissue. Still it is very difficult to determine whether they damage the tissue or attempt to repair the tissue and thus contribute to homeostasis of the aortic tissue. Macrophages are the most common immune cell type present in murine AAA, and known to actively secrete large amounts of cytokines/chemokines and proteases. In the murine angiotensin II (AngII)-induced model of aneurysm formation, infiltration of macrophages into the aortic adventitia and media represents one of the earliest events. Most treatments in mice directed at the pro-inflammatory M1-type macrophage population have been shown to prevent AAA formation. For example, targeting chemokine (C-C motif) ligand 2 (CCL2) and its receptor CCR2 are essential for monocyte chemotaxis and the macrophage-mediated immune response. It has been demonstrated that absence/blockade of CCR2 in mice resulted in decreased aneurysms severity or prevention of aneurysm formation. Actually the most abundant inflammatory cells in human aneurysm tissue are the lymphocytes, consisting of B- and T-
cells. In human AAA, there are follicles formed inside the aneurysm wall. In mice, depletion of lymphocytes did not reduce AAA formation\textsuperscript{27}, probably because pro- and anti-inflammatory lymphocytes are both absent. This concept is illustrated by the fact that IgE, derived from B-cells, enhances AAA\textsuperscript{28}, while a subset of B-cells, namely the B2-cells, suppress AAA\textsuperscript{29}. Moreover, the presence of specific antigen-driven T-cell clonal expansion in human AAA, suggest an active role for T-cells in this disease\textsuperscript{30}. Further evaluation in mice, suggest that namely the sub-population of Th17 T-cells are detrimental in AAA\textsuperscript{31, 32}, pointing at a prominent role for the cytokine interleukin-17 (IL-17). In contrast, the regulatory T-cells are protective against AAA, with an important role for IL-10\textsuperscript{33, 34}. Interestingly, a polymorphism for the IL-10 gene (IL-10 1082 polymorphism) is associated with enhanced AAA formation in a large meta-analysis\textsuperscript{11}. Less abundant inflammatory cells in the aneurysm tissue are neutrophils, mast cells, and NK cells, however, manipulation of these cell types also influences AAA formation\textsuperscript{14, 35-39}.

In general, inflammatory cells can either directly target vascular cells, or indirectly by degrading the extracellular matrix. Both processes are considered detrimental. Important proteases are the matrix metalloproteinases, cathepsins, serine proteases, tryptase/chymase and elastase\textsuperscript{40-46}. These proteases do not only degrade matrix, but some do also activate other proteases, or receptors by protein cleavage\textsuperscript{40, 47, 48}. In this way, the vascular cells also become activated and participate in the inflammatory response.

Thus besides immune cells, endothelial and SMC play an active role in AAA. To illustrate this, an endothelial cell specific overexpression mouse for NADPH oxidase NOX2, specifically in endothelial cells, shows enhanced risk of dissections after angiotensin-II infusion\textsuperscript{49}. Moreover, an endothelin-1 overexpression mouse, also specifically in endothelial cells, showed AAA in the majority of the mice, even without angiotensin-II infusion\textsuperscript{50}. In both of these studies there is enhanced reactive oxygen species generation (ROS) and inflammation. SMC are also important, since mutations in SMC proteins, such as smooth muscle α-actin (encoded by ACTA2) and smooth muscle β-myosin heavy chain (MYH11), give rise to aortic aneurysms\textsuperscript{31, 52}. In mice, it was revealed that microRNA-21 (miR-21) is a key modulator of proliferation and apoptosis of SMC during development of AAA in two murine aneurysm models\textsuperscript{53}. Preventing SMC apoptosis and inducing SMC proliferation via a miR-21-induced reduction in phosphatase and tensin homolog (PTEN) seems necessary as repair mechanism during aneurysm formation. In addition, sirtuin-1 deficiency specifically in SMC resulted in spontaneous aneurysms in pro-atherogenic mice\textsuperscript{54}. Sirtuin-1 is an NAD+-dependent deacetylase involved in metabolism, potentially influencing the SMC energetic state.

So far, in animal models many molecular targets have been identified. Yet, translation from bench to bedside does not seem straightforward, since trials with β-blockers, ACE-inhibitors, statins, or antibiotics (most of these potentially inhibiting inflammation) did not protect AAA patients until now\textsuperscript{7}. The ACE-inhibitors and doxycycline trials even showed evidence for increased AAA growth\textsuperscript{55, 56}. Therefore, development of suitable pharmacological approaches remains a clinical imperative\textsuperscript{57, 58}.

2. Inflammatory Bowel Disease

The digestive system is a complex organ system which is composed of the gastrointestinal and digestive tract (Figure 3). The gastrointestinal tract consists of the mouth, esophagus, stomach, small (jejunum and ileum) and large intestine (colon), and at the end the rectum and anus. The digestive tract on the other hand is represented by organs regulating energy metabolism, such as the liver, pancreas and gallbladder, which provide the enzymes and bile
acids necessary for food digestion. Upon digestion the food is degraded into small molecules. These smaller molecules, such as carbohydrates, proteins, fats and vitamins, are then absorbed by the wall of the small intestine, and are distributed throughout the body to use for energy supply, growth, metabolic processes, and cell renewal and repair. Undigested parts of food, together with excreted products, passage the large intestine, which absorbs any remaining nutrients and water, before exit.

Figure 3. (A) Schematic overview of digestive system. Obtained from: Dollar photo club; File #35578366. (B) Schematic overview of the healthy intestine. (C) Inflammation and epithelial crypt loss (arrow) upon induction of colitis in a pathological murine colon cross section (photograph kindly provided by dr. AAJ Hamers)

The main disease of the gastrointestinal tract is Inflammatory Bowel Disease (IBD), which affects in Europe alone already 3 million people\textsuperscript{59}. IBD includes actually two intestinal pathologies, namely ulcerative colitis (UC) and Crohn’s disease (CD). UC is predominantly localized within the colon and it affects the epithelial lining, causing constant inflammation. On the other hand, CD is a pathology which can virtually affect any part of gastrointestinal tract, although it is mainly localized within the ileum (last part of the small intestine). In CD, inflammation can affect all layers of the intestinal tissue. For both of these intestinal disorders, a common feature is that they manifest in adulthood, with periods of remission and relapse\textsuperscript{60}. Because both pathologies are lifelong disorders, they are affecting the healthcare system with an immense burden, which costs approximately 4.6-5.6 billion euro in Europe alone\textsuperscript{59}. Thus to identify safe, effective and cheap treatment strategies is an unmet medical and financial need. This can only be achieved by an in depth understanding of the disease.
Pharmaceutical treatment, in combination with surgical intervention for the patients who do not respond to therapy, is the widely accepted manner of IBD treatment. Pharmacological approaches are classified into several different categories: anti-inflammatory drugs/immunosuppressive drugs, antibiotics, and biological agents. Within these categories, the immunosuppressive thiopurines, consisting of Azathioprine (Aza) and its metabolites, have proven effectiveness to suppress IBD and relapse. Besides decreased inflammation and relapse, Aza also prevents development of colorectal cancers in IBD patients. Interestingly, Aza is used in clinical practice for more than half a century, yet, its working mechanism in treatment of chronic disease, such as in IBD, is still unclear. The effect of Aza can not be attributed to decreased DNA synthesis alone, when patients are treated for decades with Aza. Thus a more subtle mechanism is thought to be involved, which is investigated in this thesis.

2.1 Immune response in IBD

In IBD an impaired mucosal immune response is caused by environmental factors and/or genetic susceptibility. In the past decade, a considerable amount of evidence appeared, identifying different immune cell subsets involved in chronic inflammation, which is characteristic for IBD. IL-17 is one of the cytokines strongly increased in individuals with IBD, both in UC or CD. The source of increased IL-17 production is the Th17 cells, which are increased in patients with active IBD in comparison to patients in remission/silent. Intestinal fibroblasts were shown to be highly responsive to IL-17. Fibroblast-mediated fibrosis manifests itself by a shortened and stiff colon as well as thickening of the intestinal wall. IL-17 has been proposed as a cytokine candidate to promote fibrosis. Next to the Th17 cells, macrophages and dendritic cells play a key role in IBD pathogenesis. Intestinal macrophages are short living cells, with a constant need for repopulation from bone marrow derived monocytes. They produce the anti-inflammatory cytokine IL-10, which is essential for maintaining T-regulatory cell-mediated gut homeostasis. If the gut homeostasis is disturbed, Th17 cell, CD4+ T helper 1 (Th1) cell (highly correlated with CD) and CD4+ T helper 2 (Th2) cell activation (dominant in patients with UC) is causing IBD. Thus taken together, anti-inflammatory drugs seem the logical treatment strategy in IBD patients, with the focus on T cells and macrophages/dendritic cells. Indeed, the immunosuppressive drug Aza (and its derivatives), is still the most commonly used drug against IBD.

2.2 Role of Gut Epithelium and Endothelium in IBD

The intestinal epithelium represents a physical barrier between the microbe-rich luminal content and the circulation. Therefore, intestinal epithelial cells have a need to distinguish between pathogenic and commensal bacteria. To achieve this, epithelial cells express Pattern Recognition Receptors (PRRs) such as Toll-like receptors (TLRs) and NOD-like receptors (NLRs), which recognize microbial motives and pathogen-associated virulence motives. It is important for epithelial cells to stay hypo-responsive to commensal microflora in order to prevent triggering of an unprovoked immune response, and remain responsive to invading pathogens. To stay in a quiescent state, epithelial cells express negative regulators of TLR- and PRR-induced signaling. Interference with these negative regulatory pathways or activation of pro-inflammatory transcription factors, such as NFκB-light-chain-enhancer of activated B cells (NFκB), lead to activation of the epithelium. Chronic activation of TLR signaling via MyD88, leading to NFκB activation, has been shown to induce colorectal cancer in various mouse models, and is often observed in IBD patients. Thus inhibition of inflammation in IBD also reduces the risk of colon cancer.
An important non-immune contributor of inflammation is the endothelium. Endothelial cells are abundant in the highly vascularized intestine. Upon activation, the endothelium facilitates inflammation, due to its ability to selectively guide leukocyte trafficking from the blood to the intestinal mucosa, and thereby attribute to IBD initiation and progression.

Chronic inflammation in the gut generates an abundance of cytokines. Plasma levels of cytokines/growth factors reveal highly elevated pro-inflammatory IL-6, IL-23, IL-12 and TNFα in both human IBD patients and animal IBD models. Many of these cytokines are produced by infiltrated leukocytes, whereas activated gut cells, such as epithelial cells, smooth muscle cells, fibroblasts, and endothelial cells will also contribute substantially. TNFα is a cytokine with a potent pro-inflammatory effect on endothelium, inducing expression of adhesion molecules such as vascular cell adhesion molecule (VCAM)-1 and mucosal addressin cell adhesion molecule (MAdCAM)-1, and inducing expression of pro-angiogenic factors such as VEGF-A. Thus apart from promoting leukocyte influx via the adhesion molecules, there will also be enhanced angiogenesis via VEGF. Angiogenic endothelium differs from mature endothelium in many ways, among which its enhanced permeability, which impedes with protection against pathogens that are so abundant in the gut. In addition, enhanced surface area of endothelium and decreased barrier function by induction of angiogenesis, will also promote leukocyte influx. The previously discussed anti-inflammatory cytokine IL-10 was shown to protect against inflammation by preventing IFNγ (produced mostly by Th1 cells)-mediated endothelial disruption. In addition, IL-10 can suppress endothelial expression of adhesion molecules ICAM-1 and VCAM-1, thus reducing leukocyte infiltration. Mice lacking IL-10 (IL-10/-) have enhanced recruitment of leukocytes and thus more severe colitis. In conclusion, preventing activation of non-immune gut cells will probably also decrease IBD severity.

3. Immunosuppressive drug Azathioprine (Aza)

Aza is an immunosuppressive drug, which has been present for more than 60 years in clinical practice. It is used for treatment of various chronic inflammatory diseases, as well as after transplantation surgery to prevent transplant rejection. Its immunosuppressive effect has been well documented in treatment of patients with IBD, rheumatoid arthritis, myasthenia gravis, idiopathic thrombocytopenic purpura, systemic lupus erythematosus or autoimmune hepatitis. Aza is an inactive pro-drug, which in the liver undergoes enzymatic conversion to 6-Mercaptopurine (6-MP) (Figure 4). 6-MP is then distributed throughout the body and enters the cells where it is subsequently converted to 6-thioguanines (6-TGN); namely 6-Thioguanosine-monophosphate (6-T-GMP) and 6-Thio-adenosine-monophosphate (6-T-AMP), and subsequent di- and triphosphates. The alternative purines can interfere with DNA/RNA synthesis and thus inhibit active and fast proliferating cells such as lymphocytes. It is indeed known that 6-MP efficiently suppresses proliferation of T and B lymphocytes. For a long time this was the only explanation of its immunosuppressive effect. However, relatively recently, Tiede and Poppe and colleagues discovered that 6-T-GTP inhibited small GTPase Rac1 (and Rac2) in T cells, and thereby dampened its immune response. Interestingly, they demonstrated that besides structural and functional similarities between the Rho GTPase family members, RhoA and Cdc42 did bind the thiopurines, but were not inhibited by them, and thus functioned normally.
Figure 4. Chemical structures of Azathioprine (Aza), 6-Mercaptopurine (6-MP) and 6-Thioguanosine-5'-O-triphosphate (6-T-GTP) respectively.

4. Small GTPase Rac1

GTPases are enzymes that hydrolase guanosine-5'-triphosphate (GTP), which is characteristic for their activity to rapidly transduce signals. Binding and subsequent conversion of GTP to GDP (releasing the orthophosphate) occurs at a highly conserved domain, called G domain, which is present in all GTPases. GTPases are composed of more than 100 members and are present in virtually all types of eukaryotic cells, with a key function in signal transduction. Their preserved homology in structure in various species dispersed throughout the evolutionary tree, and their diversity in functions, point to their importance. Small GTPases, also known as the Ras super-family, is composed of monomeric proteins, ranging in size from 20 to 40 kDa. According to the amino acid sequence and cellular function they are subdivided into five families: Ras, Rho, Rab, Sar1/Arf, and Ran families. Rac1, which is the main focus of this thesis, together with RhoA and Cdc42 form the Rho family. They are involved in mechanosensing, protein biosynthesis, cell cycle control, protein translocation, vesicular transport within the cell, and cell motility. Upon hydrolysis of GTP, GDP remains bound within the active pocket of the GTPase. Several classes of proteins are involved in the cycling between the active GTP-bound and inactive GDP-bound state, and back. The exchange of a GDP for a new GTP molecule within the binding pocket is facilitated by Guanine nucleotide Exchange Factors (GEFs). GTPase Activating Proteins (GAPs), enable activation of GTPases by facilitating GTP hydrolysis. Guanine nucleotide Dissociation Inhibitors (GDIs) inhibit exchange of GDP for GTP, as well as hydrolysis of bound GTP. The main function of this switch is to provide rapid and specific response of the cell to explicit stimuli. Therefore, particular GTPases, GEFS, GAPs or GDIs may be suitable targets for drug development in certain diseases. Immunosuppressive drugs Aza and its metabolite 6-MP have been shown to block Rac1 in T cells. The question remains if Aza is a T cell specific Rac1 inhibitor. In Chapter 2 of this thesis, the role of small GTPase Rac1 will be reviewed with the focus on its function in healthy and diseased vascular cells.
5. Aim and outline of this thesis
This thesis aims at unraveling the working mechanism of Aza beyond interference in DNA/RNA synthesis. If the latter would be the only mechanism of action of Aza, the drug would not be suitable for chronic use, thus there must be a more subtle mechanism. Indeed, upon the discovery of the effect of Aza on Rac1, the relevance of this pathway is explored in this thesis in two inflammatory diseases, namely AAA and IBD.

Chapter 1 provides a general overview of aortic aneurysm development, inflammatory bowel disease, GTPase Rac1 and immunosuppressive drug Aza. Chapter 2 reviews the role of Rac1 in the healthy and diseased vessel wall, with an emphasis on the different cell types in it; namely endothelial cells, SMC and fibroblasts. Chapter 3 demonstrates the ability of Aza and its metabolite 6-MP to suppress endothelial cell activation. The underlying mechanism involves suppression of Rac1 activation, resulting in reduced phosphorylation of JNK and c-Jun. Upon use of Aza in a murine model for AAA, it reveals that Aza can decrease AAA, in part by reduced leukocyte influx into the aortic wall. In Chapter 4 we demonstrate that 6-MP and downstream metabolite 6-T-GTP indeed decrease Rac1 activation in endothelial cells, resulting in reduced activation of transcription factors c-Jun, activating transcription factor-2 (ATF-2) and NFκB. Moreover, Aza inhibits transmigratory cup formation, which is essential to capture leukocytes. Chapter 5 demonstrates that reduction of Rac1 activity by 6-MP and 6-T-GTP is responsible for decreased STAT-3-mediated proliferation of epithelial cells, and decreased iNOS production in macrophages. In combination with the effect of Aza on endothelial cells and T cells, it provides insight into the effectiveness of Aza in treatment of IBD. Chapter 6 sheds light on the mechanism how 6-T-GTP inhibits Rac1, since it docks on the interface of Rac1, and its binding GEF. Chapter 7 provides a discussion of the most relevant findings of this thesis and gives an overview of future perspectives.
Reference List


CHAPTER 1


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