Thiopurines and inhibition of Rac1 in vascular disease
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APPENDICES

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SUMMARY

The studies presented in this thesis report on the pharmacological effect of immunosuppressive drug azathioprine and its downstream metabolites 6-mercaptopurine (6-MP) and thiopurine 6-T-GTP on different cell types, by modulation of the activity of small GTPase Rac1. This immunosuppressive drug is mostly used in the clinic after transplantation and in chronic autoimmune diseases such as in inflammatory bowel disease patients. Because of its anti-inflammatory properties it is reasonable to assume that azathioprine may also be beneficial in other chronic inflammatory diseases, such as during abdominal aortic aneurysm development.

In Chapter 1 we provide the general background information necessary to comprehend the studies presented in this thesis. We describe the disease settings for abdominal aortic aneurysm formation and inflammatory bowel disease, which both involve chronic inflammation, leading to destruction of the artery or bowel, respectively. Inflammatory macrophages (immune cells) are important in both pathologies. We give a brief overview of the main properties of azathioprine, and its metabolites 6-MP and 6-T-GTP, and its relation to small GTPase Rac1, which is the main target of this drug.

In Chapter 2 we review the available literature on the function of Rac1 in the three main cell types of the vessel wall, namely endothelial cells, smooth muscle cells and fibroblasts. Rac1 is involved in regulation of numerous cellular functions such as oxidative stress, cytoskeletal changes, migration and proliferation. We conclude that chronically enhanced Rac1 activity plays a role in many vascular pathologies, with partial overlapping and partial cell type specific effects of Rac1.

Chapter 3 describes a murine study, measuring the effect of azathioprine treatment on prevention and progression of aneurysm development in the angiotensin-II-induced aneurysm model. Azathioprine reduces aneurysm initiation and progression, by inhibition of the inflammatory response and thus decreasing macrophage influx into the vessel wall. This effect is at least in part mediated by inhibition of endothelial cell activation, a process which facilitates macrophage adhesion and transmigration, to exit from the circulation into the tissues upon inflammation. In endothelial cells, pro-inflammatory cytokines such as IL-12, CCL5 and CCL2, as well as adhesion molecule VCAM-1 mRNA expression is reduced upon 6-MP treatment, which suppressed Rac1 activation, consequently reducing activation of the JNK-mediated signaling pathway. This demonstrates for the first time that the immunosuppressive drug has anti-inflammatory effects on non-immune cells, namely endothelial cells.

Chapter 4 discusses the in depth mechanisms which are underlying the immunosuppressive effect of 6-MP on endothelial cells. In this chapter we demonstrate in an in vitro setup of cultured endothelial cells that 6-T-GTP, the downstream metabolite of 6-MP, is probably responsible for Rac1 inhibition. 6-MP can inhibit TNFα-induced endothelial activation by diminishing the activity of several pro-inflammatory transcription factors such as cJun, ATF2 and NFkB. In turn, this leads to reduced mRNA expression of many pro-inflammatory cytokines and reduced protein expression of VCAM-1. Expression of adhesion molecule ICAM-1 was not influenced by 6-MP or 6-T-GTP, however, the ICAM-1-dependent process of assembling leukocyte capturing structures, was abrogated due to inhibition of Rac1 activity. Rac1 activation is needed for the cytoskeletal changes to form mature capturing structures on the endothelial surface, which is hampered by 6-MP. The functional relevance of this finding is revealed in transmigration experiments where neutrophils should transmigrate...
through an endothelial cell monolayer. Incubation with 6-MP or 6-T-GTP indeed decreased neutrophil transmigration.

In Chapter 5 we anticipate that the Rac1 inhibiting capacity of 6-MP is not limited to endothelial cells (our data) and T cells (shown by others). Here, we investigate if macrophages are also sensitive to 6-MP and 6-T-GTP. Interferon-γ-induced activation of macrophages leads to JNK-mediated signal transduction and an increase in iNOS production, which could be reduced by 6-MP or 6-T-GTP in a Rac1-dependent manner. In addition, expression of a number of cytokines is suppressed by 6-MP or 6-T-GTP, yet not Rac1-dependent. This shows that these drugs influence additional GTP-dependent pathways beyond Rac1. Since azathioprine is most extensively used in inflammatory bowel disease patients, we investigate gut epithelial cells. In epithelial cells, 6-MP or 6-T-GTP treatment diminished expression of cytokines CCL2 and IL-8, of which only IL-8 production is Rac1-dependent. The drugs decrease the activity of transcription factor STAT-3 in a Rac1-dependent fashion, consequently diminishing cyclin D1 and thus the proliferative response. Summarizing the data shows that azathioprine and its metabolites inhibit activation of immune cells and non-immune cells, which is in part Rac1-dependent, and thereby effectively blocks disease progression.

In Chapter 6 we perform in silico docking studies to determine if and how 6-T-GTP can inhibit Rac1, if this would be via a direct interaction with Rac1. We examine the relative binding affinities of 6-T-GTP and GTP for the Rac1 GTP-binding pocket. 6-T-GTP can bind into the GTP-binding pocket of Rac1, although with low specificity and lower affinity than its natural substrate GTP. In addition, 6-T-GTP shows a high specificity and affinity for a groove on the surface of Rac1, when Rac1 is in the conformation to bind its guanine nucleotide exchange factor (GEF). This groove is at the Rac1-GEF interface and necessary for binding of the GEF to Rac1. Binding of 6-T-GTP at this position suggests that it can block GEF binding and thus Rac1 activation, which is what we observe in our previous studies. Interestingly, two known Rac1 inhibitors bind in the same groove, which is considered their mode of function.

This thesis ends with Chapter 7 where the general outcomes of the different studies are reviewed in the context of other relevant literature, extrapolated to human data, and future research is discussed.
SAMENVATTING

De studies in dit proefschrift beschrijven het farmacologische effect van het immuunsuppressivum azathioprine en zijn metabolieten 6-mercaptopurine (6-MP) en thiopurine 6-T-GTP op verschillende cel types, door de activiteit van small GTPase Rac1 te moduleren. Dit immuunsuppressivum wordt het meest gebruikt na transplantatie of bij autoimmuun ziekten, zoals bij patiënten met chronische darmontstkingen. Door de anti-inflammatoire eigenschappen van dit medicijn is het denkbaar dat azathioprine ook een gunstig effect zou kunnen hebben op andere chronische ontstekingsziekten, zoals bij het ontstaan van een buik-aneryysma van de aorta.

In Hoofdstuk 1 wordt een algemene introductie gegeven om de verdere studies in dit proefschrift te begrijpen. Het ontstaan van buik-aneryysmata en darmontstkingen wordt beschreven, waarbij chronische ontsteking leidt tot afbraak van respectievelijk de vaatwand en de darmwand. Macrofagen zijn immuun cellen die belangrijk zijn bij beide ziektebeelden. Er wordt ook een overzicht gegeven van de omzetting van azathioprine in zijn metabolieten 6-MP en 6-T-GTP, en de relatie met GTPase Rac1, wat een belangrijk aangrijpingspunt is van deze metabolieten.

In Hoofdstuk 2 wordt een overzicht gegeven van de huidige literatuur over de functie van Rac1 in de drie voornaamste cel typen van de vaatwand; namelijk de endotheelcellen, gladde spiercellen en fibroblasten. Rac1 is betrokken bij de regulatie van verschillende cellulaire functies, zoals oxidatieve stress, cytoskelet veranderingen, migratie en proliferatie. We kunnen concluderen dat chronisch verhoogde Rac1 activiteit een rol speelt in veel vasculaire ziekten, met overeenkomstige en cel type specifieke effecten van Rac1.

In Hoofdstuk 3 wordt een muis studie beschreven in het angiotensine-II geïnduceerde aneurysma model, waarbij het effect van azathioprine op de preventie en progressie van aneurysma ontwikkeling wordt gemeten. Azathioprine vermindert de initiatie en progressie van aneurysma ontwikkeling, door de inflammatoire respons te remmen, waardoor er minder macrofaag influx plaats vindt in de vaatwand. Dit effect is gedeeltelijk afhankelijk van de remming van endotheelcel activatie, een proces dat normaliter macrofaag adhesie en migratie faciliteert, waardoor macrofagen vanuit de circulatie het ontstoken weefsel in trekken. In endotheelcellen is de expressie van cytokines zoals IL-12, CCL5, CCL2 en adhesie molecuul VCAM-1 verlaagd na behandeling met 6-MP, door middel van Rac1 remming, wat activatie van de JNK signaal transductie route vermindert. Het laat voor het eerst zien dat dit immuunsuppressivum ook een anti-inflammatoire werking heeft in niet-immuun cellen zoals endotheelcellen.

Hoofdstuk 4 bestudeert de verschillende mechanismen die ten grondslag liggen aan het immuunsuppressieve effect van 6-MP op endotheelcellen. In dit hoofdstuk wordt aangetoond in gekweekte endotheelcellen dat 6-T-GTP, gevormd uit 6-MP, waarschijnlijk verantwoordelijk is voor de Rac1 remmende werking. 6-MP blokkeert TNFα-geïnduceerde endotheelcel activatie door de activiteit van meerdere pro-inflammatoire transcriptie factoren, zoals cJun, ATF2 en NFkB, te verlagen. Dit leidt tot verminderde mRNA expressie van veel pro-inflammatoire cytokines en verminderde eiwit expressie van VCAM-1. Expressie van adhesie molecuul ICAM-1 wordt niet beïnvloed door 6-MP of 6-T-GTP, maar de vorming van membraan uitstulpingen voor het omkapselen van migrerende leukocyten, een ICAM-1-afhankelijk proces, is wel verminderd door Rac1 remming. Rac1 activatie is nodig om de cytoskelet veranderingen mogelijk te maken om de membraan uitstulpingen te vormen, wat verstoord wordt door 6-MP. De functionele relevantie wordt aangetoond door middel van
transmigratie experimenten, waarbij neutrofielen migreren over een endotheelcel monolaag. Incubatie met 6-MP of 6-T-GTP vermindert inderdaad de neutrofiel transmigratie.

In Hoofdstuk 5 veronderstellen we dat de Rac1 remmende werking van 6-MP niet is voorbeholden aan de endotheelcellen (onze data) of T cellen (zoals aangetoond door anderen). Hier wordt onderzocht of ook macrofagen gevoelig zijn voor 6-MP en 6-T-GTP. Interferon-γ-geïnduceerde activatie van macrofagen leidt tot JNK-gemediëerde signaal transductie en een verhoging van iNOS expressie, wat verlaagd kan worden door 6-MP en 6-T-GTP op een Rac1-afhankelijke manier. Expressie van een aantal cytokines is ook verlaagd door 6-MP en 6-T-GTP, maar blijkt niet Rac1-afhankelijk te zijn. Dit laat zien dat er meer GTP-afhankelijke routes geremd worden, naast GTPase Rac1.

Aangezien azathioprine het meest voorgeschreven wordt in patiënten met chronische darmontstekingen, zijn ook darm epitheelcellen bestudeerd. TNFα-geïnduceerde epitheelcel activatie wordt sterk geremd door 6-MP en 6-T-GTP. CCL2 en IL-8 expressie is verlaagd, maar alleen de IL-8 productie blijkt Rac1-afhankelijk te zijn. Het medicijn vermindert de activatie van transcriptie factor STAT3, welke ook Rac1-afhankelijk is, met als gevolg dat er minder cyclin D1 wordt geproduceerd, waardoor de proliferatieve respons uit blijft. Samenvattend laten de data zien dat azathioprine, en zijn metabolieten, activatie van immuun cellen en niet-immuun cellen remt, voor een deel via Rac1-afhankelijke processen, en op deze manier effectief ziekte progressie kan blokkeren.

In Hoofdstuk 6 worden in silico docking studies uitgevoerd om te bepalen of en hoe 6-T-GTP de GTPase Rac1 kan remmen, en of er een directe interactie mogelijk is met Rac1. De relatieve binding-affiniteiten van 6-T-GTP en GTP voor de GTP-bindingsplaats op Rac1 laten zien dat 6-T-GTP kan binden aan Rac1 op die locatie, maar met een minder hoge affiniteit dan zijn natuurlijke substraat GTP. Daarnaast laat 6-T-GTP een hoge specificiteit en affiniteit zien voor een groeve aan het oppervlak van Rac1, waar normaliter de guanine nucleotide exchange factor (GEF) bindt. Binding van 6-T-GTP op deze Rac1-GEF interactie locatie suggereert dat het de binding van de GEF kan voorkomen, waardoor Rac1 activatie geremd wordt, wat we ook zien in de voorgaande studies. Het is opmerkelijk dat twee andere bekende Rac1 remmers in dezelfde groeve binden, wat het werkingsmechanisme is van die Rac1 remmers.

Dit proefschrift eindigt met Hoofdstuk 7, waar de uitkomsten van de verschillende studies worden belicht in de context van relevante literatuur, geëxtrapoleerd naar de humane situatie, en vervolg onderzoek wordt besproken.
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PERSONAL DATA
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PROFILE
Five years of academic research in the field of vascular biology and vascular diseases with a main focus on atherosclerosis and aneurysm formation. As a Postdoc, spotlight of the research is to explore the role of immune response in pathology of vascular diseases with a main focus on monocyte characterization.

PROFESSIONAL EXPERIENCE
Postdoc. scientist At the Dept. of Immunology, Weizmann Institute of Science

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Researcher at the Dept. of Human and Medical Genetics, Institute of Mental Health
Prenatal screening and diagnostics of genetic aberrations (funded by Serbian Ministry of Health care)

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Ph.D. study at the Academic Medical Center
Thesis defense expected: beginning of 2014

Bachelor/Master of Science at the Faculty of Biology
Average grade: 8.8/10, Diploma thesis and exam grade: 10/10

PUBLICATIONS & AWARDS

- Marinković G, Heemskerk N, van Buul JD, de Waard V. The ins and outs of small GTPase Rac1 in vasculature. Manuscript in submission
- Marinković G, de Vries CJ, Delgado Oabarriaga S, de Waard V, Bleijlevens B. Docking of the GTP-metabolites of immunosuppressive drug azathioprine reveals an inhibitory Rac1 binding site. Manuscript in submission
- Ruiter MS, van Tiel CM, Doornbos A, Marinković G, Strang A, Attevelt NJM, de Waard V, de Winter RJ, Steendam R, de Vries CJ. Stents eluting 6-mercaptopurine reduce neointima formation and inflammation while enhancing strut coverage in rabbits. Manuscript in submission
- “Azathioprine Is Protective in Aneurysm Formation and Progression”; Won “ATVB Travel Awards for Young Investigators” at Atherosclerosis, Thrombosis and Vascular Biology conference in Chicago, USA, 2012
REFERENCES

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