Chapter 1

Introduction and scope of the thesis

Protein-protein interactions (PPIs) underlie a great number of biological processes found in signal transduction cascades, and play crucial roles in disease progression [1]. PPIs have the potential to provide a vast number of both intracellular and extracellular therapeutic targets. The potential of modulation of PPIs for drug discovery triggered great interest in development of inhibitors over the past decade [2]. However, there is a number of challenges inherent in developing PPI inhibitors that prevented this effort from reaching its full fruits. Expression of the interacting proteins for *in vitro* experiments proves often to be difficult, in particular when the interaction partners include membrane bound receptors. Another challenge is the development of convenient assay formats to screen for novel hit and lead compounds. Finally, it is challenging to translate PPI inhibition to cell-based studies, animal models and ultimately clinical applications. In this thesis, we focus on the PPI interactions of macrophage migration inhibitory factor (MIF) and its binding partners such as the Cluster of Differentiation 74 (CD74) receptor. We aim to improve the production of the purified CD74 protein in bacteria in order to provide a suitable MIF-CD74 binding assay. Furthermore, we aim to identify novel MIF binders. Ultimately, this will contribute to drug discovery that employs MIF as a molecular target.

Initially, MIF was described as a T cell-derived mediator that prevents random movement of macrophages. Its activity was associated with delayed-type hypersensitivity reactions, which is a feature of some human chronic diseases [3]. Moreover, it is released at infection sites, causing macrophages to localize and perform antigen processing and phagocytosis [4]. Currently, MIF is identified as a cytokine with a key role in innate and adaptive immune responses that is associated with the progression of multiple diseases [5][6]. Consequently, an increasing number of roles in the pathogenesis of various inflammatory diseases and cancer have been described for MIF [7].

MIF is produced in many organs and tissues by various cells [8]. *In vivo*, MIF exerts its action by binding to membrane receptors, such as the CD74 receptor. Although MIF-CD74 binding is the best-characterized interaction, also binding to other receptors has been reported. It has been elucidated that MIF binding to the chemokine receptors CXCR2, CXCR4 and CXCR7 also plays an important role in MIF actions [9]. Apart from its cytokine activity, MIF possesses

tautomerase enzyme activity and is a member of the tautomerase superfamily [10]. The tautomerase enzyme activity is a property that is extensively employed in the screening for small molecule MIF binders (see Chapter 2). MIF has also a close relative as identified from the human genome. D-dopachrome tautomerase (D-DT) has been identified as a gene with marked homology to MIF. Because of its similarity, D-DT is also called as MIF2. An overlapping functional spectrum of MIF and D-DT has been suggested [6][11][12]. Therefore, the close homology of MIF with D-DT should be taken into account in the evaluation of MIF cytokine activities and the development of small molecule MIF modulators.

The CD74 receptor is also referred to as HLA class II histocompatibility antigen gamma chain or HLA-DR antigen-associated invariant chain Ii. This is a non-polymorphic type II transmembrane glycoprotein that has been described to be involved in many biological processes in the cell, such as antigen loading and transport of MHC class II molecules from the endoplasmic reticulum to the Golgi complex [13]. This receptor is also recognized as part of a complex to which MIF binds and that enables initiation of MIF induced signaling in inflammation [9]. Thus this receptor complex participates in the progression of MIF cytokine-related diseases. The extracellular domain of CD74 is involved in direct interaction with MIF. It has been reported that CD74 undergoes progressive proteolytic degradation in the endosomal/lysosomal system, eventually leaving the small class-II-associated invariant chain peptide (CLIP) as a fragment that binds MIF [14].

Production of relatively large amounts of the functional extracellular moiety of CD74 is indispensable for further exploration of MIF-CD74 interaction and discovery of novel inhibitors to disrupt this interaction. Our previous findings showed that the production of extracellular moiety of CD74 in bacterial cells gave very low yields. To overcome this problem, we aim for the production of the extracellular domain of CD74 as fusion protein for enhancing the solubility and stability (see Chapter 3). Towards this aim we applied two different fusion partners: MBP (maltose-binding protein) and Fh8 (a small protein secreted by the parasite *Fasciola hepatica*). Both fusion partners are also intended to facilitate purification of the fusion proteins: MBP fusion proteins bind to immobilized amylose resins and can be eluted using maltose, and Fh8 fusion proteins make calcium-dependent interaction with hydrophobic resins and can be eluted using a calcium chelating agent, such as EDTA [15]. We put factor Xa and 3C cleavage sites on MBP-CD74 and Fh8-CD74 proteins, respectively. Following the production and purification of the fusion proteins, the MBP and Fh8 can be removed by cleaving them with factor Xa and 3C protease, respectively. All the fusion proteins and the cleaved products are characterized by SDS-PAGE and mass spectrometry, and their functionality in PPI are evaluated by ELISA and ITC.

Numerous studies describe the involvement of the MIF-CD74 interaction in the progression of inflammatory diseases and cancer. In this perspective, it is not surprising that over the past few years many efforts have been taken to develop small-molecule inhibitors targeting the MIF-CD74 interaction as potential therapeutics. Small-molecule inhibitors offer certain advantages compared to biologicals, such as antibodies. Advantages of small-molecule inhibitors are their low manufacturing cost, their low immunogenicity on repeated administration and their flexible delivery options, including oral administration. Development of MIF binding inhibitors often starts from screening for inhibition of the MIF tautomerase activity, which does not necessarily imply interference with the MIF-CD74 interaction. Nevertheless, MIF tautomerase inhibitors have potential to interfere with the MIF-CD74 interaction. For instance, allosteric inhibitors of MIF tautomerase activity may be capable to induce conformational changes that result in disruption of the MIF-CD74 interaction. While purposeful design of this type of compounds could be tricky, due to not-easy-to predict induced conformational modifications, targeting MIF tautomerase activity remains a convenient and efficient approach to develop MIF inhibitors [16]. Moreover, recent findings indicating that the interaction of MIF-CD74 take places in the area surrounding MIF tautomerase enzyme active site [17], support the idea that structure-based designed MIF tautomerase inhibitors holds promises to interfere with the MIF-CD74 interaction.

Several methods can be applied to develop small inhibitors in drug discovery. These methods range from random screening of large libraries of compounds to screening of focused compound collections and structure-based design methods. Random screening of large libraries usually covers a broad chemical space and enables the identification of unique hit and lead compounds. [1]. MIF inhibitors identified by Orita [19] and Cournia [20] are examples of this. Screening of focused compound collections enables a more comprehensive exploration of a predefined chemical space in which MIF binding has been identified or is to be expected. Several selection methods for screening of focused compound collections have been described. A method defined in our groups is the substitution-oriented screening (SOS) [1][18] in which scaffolds of inhibitors with known interactions with the target are employed for library design. This enables the screening of a large variety in substitution around scaffolds with known activity. Thus, SOS covers a smaller chemical space but enables a more profound exploration of the chemical space around a known scaffold [18]. The disadvantage of this method is it does not enable the identification of inhibitors with unique scaffolds. MIF inhibitors identified by Alam *et al*. with the isoxazoline scaffold [21], Jorgensen *et al*. with the triazole scaffold [22] are examples of MIF inhibitors

developed using this method. We apply this method to identify chromene-based MIF inhibitors in Chapter 4.

In contrast to screening methods, structure-based design provides the possibility for a directed exploration of the structure-activity relationship. Structure-based design requires a known inhibitor scaffold that has been crystallized with the target to provide an experimental structural basis for inhibitor design. Using systematic variations at specific positions in the inhibitor, the binding space in relation to the structure of the target can be explored systematically. The aim is to optimize the inhibitor potency and selectivity [18]. This method is often applied in combination with screening methods for generation of hit compounds that are subsequently optimized using structure-based design. This methods has been used to explore and optimize the binding properties of MIF inhibitor with the isoxazoline scaffold [21][23], the triazole scaffold [22], or the biaryltriazole scaffold [24]. In this thesis we employ the structure-based design to explore structure-activity relationship for inhibitors of the triazole-phenol type in Chapter 5.

In **Chapter 2**, we provide a review of the role of MIF in the pathogenesis of inflammatory diseases and cancer. In addition, we provide an overview of small-molecule inhibitors of MIF tautomerase activity and we give future perspective on the development of such inhibitors [7].

In **Chapter 3**, we report the production and purification of the extracellular part of the CD74 receptor. This protein was expressed as fusion protein to solubility enhancing domains such as MBP and Fh8. We characterized the purified MBP-CD74 and Fh8-CD74 fusion proteins, as well as the CD74 cleavage products. Binding of the CD74 domain to MIF was identified using an ELISA assay. The successful production of functional CD74 in high quantities is the first important step for further characterization of its structural features and for identification of its binding characteristics to MIF. Hence, it will foster further development of the relevant small-molecule inhibitors for MIF-CD74 interaction [15].

In **Chapter 4**, we describe the development of chromenes as MIF inhibitors. Inspired by the known MIF inhibitor Orita-13, a SOS for MIF inhibitors was done with a diversely-substituted collection of compounds with a chromene scaffold. The chromene compounds were synthesized using versatile cyanoacetamide chemistry. The SOS provided several hit compounds for which the IC_{50}'s were determined. In addition, we evaluated the reversibility of binding and also analysed the enzyme kinetic of the most potent inhibitor. The newly identified

inhibitors will support further development of novel inhibitors as potential therapeutic agents against immune diseases in which MIF is involved [25].

In **Chapter 5**, we report structure-based design of compounds with isoxazole, benzoxazole and triazole-phenol scaffolds. Compounds with various substitutions were synthesized and their inhibition of MIF tautomerase activity was evaluated to explore the structure-activity relationship. This provided several substituted triazole-phenol compounds as MIF tautomerase inhibitors. It is expected that by making use of MIF enzymatic pocket to anchor small-molecule inhibitors, we can assemble substituents on the triazole ring that protrude the solvent interface of the pocket ("caps") to target the inhibition on MIF-CD74 interaction.

In **Chapter 6**, we provide a summary of our work and we describe the challenges. Finally we give suggestions and perspectives for future work.

References

[1] Laraia L, McKenzie G, Spring DR, Venkitaraman AR, Huggins DJ. Overcoming Chemical, Biological, and Computational Challenges in the Development of Inhibitors Targeting Protein-Protein Interactions. Chem Biol 2015;22:689–703. doi:10.1016/J.CHEMBIOL.2015.04.019.

[2] Arkin MR, Tang Y, Wells JA. Small-molecule inhibitors of protein-protein interactions: progressing toward the reality. Chem Biol 2014;21:1102–14. doi:10.1016/j.chembiol.2014.09.001.

[3] Bloom BR, Bennett B. Mechanism of a reaction in vitro associated with delayed-type hypersensitivity. Science 1966;153:80–2.

[4] Nathan CF, Karnovsky ML, David JR. Alterations of macrophage functions by mediators from lymphocytes. J Exp Med 1971;133:1356–76. doi:10.1084/JEM.133.6.1356.

[5] Bloom J, Sun S, Al-Abed Y. MIF, a controversial cytokine: a review of structural features, challenges, and opportunities for drug development. Expert Opin Ther Targets 2016;20:1463–75. doi:10.1080/14728222.2016.1251582.

[6] O'Reilly C, Doroudian M, Mawhinney L, Donnelly SC. Targeting MIF in Cancer: Therapeutic Strategies, Current Developments, and Future Opportunities. Inc Med Res Rev 2016;36:440–60. doi:10.1002/med.21385.

[7] Kok T, Wasiel AA, Cool RH, Melgert BN, Poelarends GJ, Dekker FJ. Small-molecule inhibitors of macrophage migration inhibitory factor (MIF) as an emerging class of therapeutics for immune disorders. Drug Discov Today 2018. doi:10.1016/j.drudis.2018.06.017.

[8] Calandra T, Roger T. Macrophage migration inhibitory factor: a regulator of innate immunity. Nat Rev Immunol 2003;3:791–800. doi:10.1038/nri1200.

[9] Leng L, Metz CN, Fang Y, Xu J, Donnelly S, Baugh J, et al. MIF Signal Transduction Initiated by Binding to CD74. J Exp Med 2003;197:1467–76. doi:10.1084/jem.20030286.

[10] Poelarends GJ, Veetil VP, Whitman CP. The chemical versatility of the beta-alpha-beta fold: catalytic promiscuity and divergent evolution in the tautomerase superfamily. Cell Mol Life Sci 2008;65:3606–18. doi:10.1007/s00018-008-8285-x.

[11] Merk M, Mitchell RA, Endres S, Bucala R. D-dopachrome tautomerase (D-DT or MIF-2): doubling the MIF cytokine family. Cytokine 2012;59:10–7. doi:10.1016/j.cyto.2012.03.014.

[12] Benedek G, Meza-Romero R, Jordan K, Zhang Y, Nguyen H, Kent G, et al. MIF and D-DT are potential disease severity modifiers in male MS subjects. Proc Natl Acad Sci U S A 2017;114:E8421–9. doi:10.1073/pnas.1712288114.

[13] Borghese F, Clanchy F IL. CD74: an emerging opportunity as a therapeutic target in cancer and autoimmune disease. Expert Opin Ther Targets 2011;15:237–51. doi:10.1517/14728222.2011.550879.

[14] Strubin M, Berte C, Mach B. Alternative splicing and alternative initiation of translation explain the four forms of the Ia antigen-associated invariant chain. EMBO J 1986;5:3483–8.

[15] Kok T, Wasiel AA, Dekker FJ, Poelarends GJ, Cool RH. High yield production of human invariant chain CD74 constructs fused to solubility-enhancing peptides and characterization of their MIF-binding capacities. Protein Expr Purif 2018;148:46–53. doi:10.1016/j.pep.2018.03.008.

[16] Trivedi-Parmar V, Jorgensen WL. Advances and Insights for Small Molecule Inhibition of Macrophage Migration Inhibitory Factor. J Med Chem 2018: acs.jmedchem.8b00589. doi:10.1021/acs.jmedchem.8b00589.

[17] Pantouris G, Syed MA, Fan C, Rajasekaran D, Cho TY, Rosenberg EM, et al. An Analysis of MIF Structural Features that Control Functional Activation of CD74. Chem Biol 2015;22:1197–205. doi:10.1016/j.chembiol.2015.08.006.

[18] Eleftheriadis N, Neochoritis CG, Leus NGJ, van der Wouden PE, Dömling A, Dekker FJ. Rational Development of a Potent 15-Lipoxygenase-1 Inhibitor with *in Vitro* and *ex Vivo* Anti-inflammatory Properties. J Med Chem 2015;58:7850–62. doi:10.1021/acs.jmedchem.5b01121.

[19] Orita M, Yamamoto S, Katayama N, Aoki M, Takayama K, Yamagiwa Y, et al. Coumarin and chromen-4-one analogues as tautomerase inhibitors of macrophage migration inhibitory factor: discovery and X-ray crystallography. J Med Chem 2001;44:540–7. doi:10.1021/JM000386O.

[20] Cournia Z, Leng L, Gandavadi S, Du X, Bucala R, Jorgensen WL. Discovery of Human Macrophage Migration Inhibitory Factor (MIF)-CD74 Antagonists via Virtual Screening. J Med Chem 2009;52:416–24. doi:10.1021/jm801100v.

[21] Alam A, Pal C, Goyal M, Kundu MK, Kumar R, Iqbal MS, et al. Synthesis and bio-evaluation of human macrophage migration inhibitory factor inhibitor to develop anti-inflammatory agent. Bioorg Med Chem 2011;19:7365–73. doi:10.1016/j.bmc.2011.10.056.

[22] Jorgensen WL, Gandavadi S, Du X, Hare AA, Trofimov A, Leng L, et al. Receptor agonists of macrophage migration inhibitory factor. Bioorg Med Chem Lett 2010;20:7033–6. doi:10.1016/j.bmcl.2010.09.118.

[23] Ioannou K, Cheng KF, Crichlow G V, Birmpilis AI, Lolis EJ, Tsitsilonis OE, et al. ISO-66, a novel inhibitor of macrophage migration, shows efficacy in melanoma and colon cancer models. Int J Oncol 2014;45:1457–68. doi:10.3892/ijo.2014.2551.

[24] Dziedzic P, Cisneros JA, Robertson MJ, Hare AA, Danford NE, Baxter RHG, et al. Design, Synthesis, and Protein Crystallography of Biaryltriazoles as Potent Tautomerase Inhibitors of Macrophage Migration Inhibitory Factor. J Am Chem Soc 2015;137:2996–3003. doi:10.1021/ja512112j.

[25] Kok T, Wapenaar H, Wang K, Neochoritis CG, Zarganes-Tzitzikas T, Proietti G, et al. Discovery of chromenes as inhibitors of macrophage migration inhibitory factor. Bioorg Med Chem 2018;26:999–1005. doi:10.1016/J.BMC.2017.12.032.