Chapter 13

Summary

Rheumatoid arthritis (RA) is a chronic inflammatory disease, which, in the Netherlands, affects approximately 1% of the population. The disease is mainly characterized by inflammation of the joints which become swollen and painful. The chronic inflammation will eventually be destructive to the cartilage and bone of the joints, leading to serious disability, immobility, reduction of quality of life and reduction of life expectancy. Furthermore, due to the high prevalence of RA and the chronic burden of the disease, RA also has a major socio-economic impact due to substantial health care costs and because of its impact on work ability (or capacity). Therefore, current strategies for RA treatment include rapid and aggressive interventions with classical and biological disease modifying anti-rheumatic drugs aiming for prolonged suppression of the disease and ideally achieving complete remission. In this context, worldwide efforts are now going on to explore, design and evaluate novel treatment options for RA, taking advantage of the cumulative knowledge of the pathophysiology of RA.

Although the exact cause of RA remains unknown, several risk factors have been clearly linked to the onset of RA (Chapter 1). It is assumed that a combination of a certain genetic background and external events can initiate an autoimmune response. In an autoimmune disease setting, the immune system is deregulated and cannot make an adequate distinction between self and non-self. For rheumatoid arthritis it is believed that the immune system recognizes a certain component in the joint, possibly cartilage, as a foreign and dangerous substance that needs to be eliminated. The process of elimination is regulated by the inflammatory response executed by the immune system. For this process, the immune system can recruit several types of immune-effector cells, including: (a) dendritic cells (involved in antigen processing and presentation), T cells (involved in cellular defense by specific killing of infected cells), B cells (can differentiate to antibody producing plasma cells) and monocytes/macrophages (involved in phagocytosis and digestion of cellular debris and pathogens). In a complex manner all cells of the immune system communicate with each other by the secretion of specific hormonal-like signals, which are called chemokines and cytokines. Within this advanced and complex communication network, the cytokine Tumor Necrosis Factor α (TNFα) is considered to be a dominant mediator of the inflammatory response. In RA it is now generally accepted that immune-effector cells (macrophages, dendritic cells, T-lymphocytes and B-lymphocytes) together with an unbalanced secretion of pro-inflammatory cytokines such as TNFα are essential for the onset as well as the maintenance of the disease. Therefore most treatment options for RA are currently focused on the control of the inflammatory response and
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prevention of bone lesions by either eliminating inflammatory cells or by interfering in the cytokine/chemokine communication network.

Classical and biological disease modifying anti-rheumatic drugs (DMARDs) are commonly prescribed to patients with RA. These molecules/antibodies are able to dampen the inflammation and they are even capable of inducing temporary remissions of the disease in a number of patients. However, since these drugs are unable to cure the disease, they require chronic administration to sustain clinical benefit. Unfortunately, for many RA patients therapeutic benefits of DMARDs are temporary, as DMARDs will often gradually lose their efficacy after several months or years of chronic use. This process is also known as acquired resistance to therapeutic drugs. In a small number of patients, lack of efficacy may already be observed at treatment initiation, which is referred to as primary or inherent resistance to DMARDs. However, usually treatment failure to DMARDs occurs after a period of adequate treatment response, referred to as secondary failure. Additional distinctions can be made when resistance to DMARDs includes only one class of DMARDs (e.g. folic acid analogues) or when it involves multiple DMARDs that are chemically and functionally unrelated. In the latter case, this is referred to as a multidrug-resistant phenotype. Important questions are how patients acquire resistance to DMARDs and what is/are the underlying mechanism(s) of resistance. In this thesis we focused on one important mechanism which concerns the involvement of multi-drug resistance (MDR) drug efflux transporters. MDR proteins belong to the family of energy (Adenosine Triphosphate, ATP)-driven ATP-binding cassette (ABC) transporters, which may pump a wide range of different compounds out of cells. There is now accumulating evidence that several DMARDs are substrates of distinct MDR proteins and thereby cause a diminished efficacy of DMARDs. Specifically, we set out to address two main goals in this thesis:

1. To define the role of drug efflux transporters in conferring resistance/loss of efficacy to DMARDs in immune-competent cells implicated in the pathophysiology of RA.

2. To explore the efficacy of some novel experimental targeted drugs with the potency to overcome DMARD resistance, taking into account that they may themselves also be subject to the development of resistance.

Chapter 1 provides an overview of the pathophysiological aspects of RA and summarizes current treatment modalities for this disease. Furthermore, the potential role of drug efflux transporters in conferring resistance to DMARDs is described. Finally, the introduction discusses new experimental therapeutic options for RA, such as proteasome inhibitors, a new class of small molecule drugs with potential anti-inflammatory properties.
In Chapter 2 we reviewed the current state of the literature concerning MDR transporters and their relation to the immune system. It is recognized that MDR transporters are present on most immune-effector cells (T-cells, B-cells, monocytes/macrophages, dendritic cells), including those that play a role in RA pathophysiology. Apart from a possible pharmacological function in conferring drug resistance by extruding therapeutic drugs including DMARDs, several MDR transporters also fulfil important immuno-regulatory functions by facilitating the transport of compounds that serve as mediators of inflammation (e.g., leukotrienes) and/or are involved in cell differentiation and maturation. This dual function of MDR transporters and the implications for autoimmune disorders as well as for cancer is extensively discussed. Conceivably, interference of physiological MDR function in autoimmune diseases by specific MDR blockers may attenuate the aberrant immune response in RA patients however, in immune-compromised cancer patients, MDR blocking may elicit an adverse effect.

Though not being the most potent DMARD, the antimalarial (hydroxy) chloroquine (CHQ) has a modest but established place in RA treatment, also because it is easy to combine it with other DMARDs. In RA clinical practice, however, CHQ is among the DMARDs of which a relatively rapid loss of efficacy has been reported upon prolonged administration. The mechanistic basis for this is largely unknown. In Chapter 3 we mimicked the development of resistance mechanism to CHQ by in vitro exposure of a human T-cell line to a stepwise increasing concentration of CHQ. Following this procedure, cells acquired a 3-4 fold level of resistance to CHQ over a period of 5 months. Examination of the mechanism of resistance revealed the overexpression of multidrug resistance-associated protein 1 (MRP1). The role of this drug efflux transporter was further confirmed by the fact that blocking of MRP1 reversed CHQ sensitivity. CHQ-resistant T-cells remained fully sensitive to other DMARDs, including methotrexate (MTX), leflunomide, cyclosporine A and sulphasalazine. Rather strikingly, CHQ-resistant cells were highly resistant (> 1,000-fold) to the glucocorticoids (GCs) dexamethasone and prednisolone due to a disturbed cyclic-AMP dependent protein kinase A signaling pathway. Consistently, transient activation of cAMP-dependent protein kinase A sensitized cells for GCs. Finally, CHQ-resistant T cells demonstrated a markedly impaired capacity to release the pro-inflammatory cytokine TNF-α and the chemokine IL-8, suggesting that CHQ-resistance in this model T cell line does not necessarily compromise its anti-inflammatory effects.

Inherent or acquired resistance to glucocorticoids (GCs) is a well recognized problem in the treatment of inflammatory diseases and it limits the optimal efficacy of GCs. In addition, increase of dosage could be necessary but will lead to an increase incidence of substantial adverse events. As shown in chapter 3, resistance to CHQ was associated with a marked cross-resistance to GCs. However, in Chapter 4 we reported an
opposite phenomenon, namely that prolonged exposure of two primary GC-resistant human monocyctic/macrophage cell lines could be sensitized for GCs after prolonged exposure to another DMARD; sulphasalazine (SSZ). Recovery of GC-sensitivity in SSZ-exposed cells was conveyed via GC-induced apoptotic cell death, together with inhibition of NFkB activation. GC-sensitivity was also greatly improved by the fact that in SSZ-exposed cells, the expression of the GC receptor $\alpha$ protein was markedly increased, probably by increased stabilization of GR$\alpha$ protein. These results deserve further experimental elaboration to disclose potential mechanisms of clinical activity of the triple DMARD combination therapy of methotrexate, sulphasalazine and prednisolone as part of the COBRA treatment protocol for RA patients.

DMARD combinations are quite common in RA treatment protocols. For one type of DMARD combination, i.e. MTX + SSZ, conflicting results have been reported: either this combination has an additive/synergistic or an antagonistic effect over either DMARD alone. In Chapter 5 we examined whether possible drug interactions influence the efficacy of the MTX + SSZ combination against an in vitro model of human monocytic/macrophage cells. Indeed, two types of negative interactions of SSZ with MTX were observed. First, SSZ was shown to be a potent and non-competitive inhibitor of the reduced folate carrier (RFC), the main cellular uptake route for MTX. Secondly, long term SSZ exposure provoked a marked upregulation of the multidrug resistance transporter ABCG2 (BCRP). Since this drug efflux transporter has MTX among its extrudable substrates, this may contribute to a diminished efficacy of MTX. Altogether, these observations have relevant implications for the optimal use and adverse effects of MTX+SSZ combinations in RA treatment. Considering the latter, SSZ-RFC interactions may not only hamper cellular MTX uptake, it may also impair cellular uptake of natural folate cofactors, possibly leading to a subclinical causing folate deficiency. These results also plead for giving folate supplementation and for spacing administration of sulfasalazine and MTX over time, anticipating that the inhibitory effects of SSZ on RFC-dependent MTX uptake are only transient.

In vitro studies have clearly established that MDR proteins can facilitate resistance to several DMARDs. Data demonstrating a role of MDR transporters in clinical DMARD resistance are scarce. Most clinical studies regarding this subject were focused on the P-glycoprotein, the first identified MDR transporter, but the possible contribution of other MDR transporters received little attention. Therefore, in Chapter 6, we evaluated the expression profile and functional activities of selected MDR transporters (P-gp, BCRP and MRP1 to 9) on immune-effector cells of RA patients in relation to clinical DMARD responsiveness and DMARD refractoriness. For this study peripheral blood lymphocytes (PBLs) and monocyte-derived macrophages (MDM) were obtained from RA patients (including DMARD-naive and DMARD-(non) respon-
sive patients) and healthy controls. By using different techniques, such as real-time PCR, immunohistochemistry and flow cytometry we were able to examine the presence and functional activity of MDR transporters. We demonstrated that many MDR transporters are expressed on peripheral blood lymphocytes and monocyte-derived macrophages from both RA patients and healthy controls. However, increased mRNA levels of MRP1 (2.5-fold), MRP4 (1.6-fold) and MRP7 (1.9-fold) were observed only in peripheral blood lymphocytes of RA patients. In monocyte-derived macrophages we detected significantly increased levels of BCRP protein (2.4-fold) and BCRP mRNA (2.8-fold), whereas P-gp protein expression correlated with disease activity. In T-lymphocytes, the activity of P-gp was found to be significantly increased in RA patients treated with DMARDs when compared to DMARD therapy-naive patients. To assess whether any of the MDR transporters was associated with DMARD therapy failure, only MRP2 and MRP4 mRNA levels were found to be increased in DMARD non-responders when compared to DMARD-responders. Collectively, this study suggests that upregulation of specific MDR drug efflux transporters on immune-competent cells of RA patients is more a reflection of disease activity rather than a DMARD-induced phenomenon. However, in both scenarios, elevated MDR expression can contribute to an attenuated DMARD response in RA patients. As follow up of this cross-sectional study, it will be of interest to further explore expression profiling of MDR transporters on immune-effector cells of RA patients in a longitudinal study of DMARD use and emergence of loss of efficacy.

Besides analysis of MDR transporter expression on peripheral blood cells (chapter 6), we also extended our studies to inflamed synovial tissue from RA patients. Chapter 7 reports on the immunohistochemical analysis of expression levels of the MDR transporters P-gp, MRP1-5, MRP8, MRP9 and BCRP on inflammatory cells in synovial tissue of RA patients with active disease before and after 4 months of treatment with MTX (7.5-15 mg/week) or leflunomide (20mg/day). Results were compared with non-inflamed synovial tissue from orthopedic patients. In all RA synovial biopsies, both prior to treatment and after 4 months of MTX treatment, abundant expression of BCRP was observed on macrophages in the intimal lining layer as well as on macrophages and endothelial cells in the synovial sublining. Statistical analysis showed that there was a trend towards more abundant BCRP expression at higher disease activity. Furthermore, median BCRP expression was 4-8 fold higher for MTX-non-responders when compared to MTX-responders. The same trend was observed for RA patients treated with leflunomide: a 2.5-fold higher BCRP expression was observed in synovial biopsies from ‘leflunomide-failures’ when compared to biopsies from patients with a good response on leflunomide. Moderate expression of MRP1 was observed in T-cell areas of some synovial biopsies, whereas expression of P-gp, MRP2-5, MRP8 and MRP9 were below the immunohistochemical detection levels. In control synovial
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tissue, along with very low levels of infiltrated macrophages, only a few BCRP positive cells were observed, while staining for the other MDR-proteins was negative. Since we found positive staining on macrophages in all RA synovial biopsies prior to therapeutic interventions, expression of BCRP seems to be an inflammation-dependent phenomenon rather than a drug-induced effect associated with the onset of resistance. Notwithstanding this fact, since MTX, leflunomide and sulphasalazine are established substrates for BCRP, this transporter may contribute to a reduced therapeutic effect of these DMARDs.

A controversial issue that remains is that MDR transporters could be directly or indirectly be involved in the cellular secretion of inflammation-associated chemokines and cytokines. There is ample evidence that MDR transporters can extrude small molecule compounds and peptides with molecular weight < 1kD, but whether this also holds for higher molecular weight chemokines (up to 8 kD) or cytokines (up to 20 kD) is not completely clear. In Chapter 8 we investigated whether MDR transporters MRP1-5, P-gp or BCRP are involved in the secretion of chemokines CCL20, MCP1 and IL-8 and cytokine TNFα. Cell lines with or without overexpression of one of the MDR transporters were activated with the phorbol ester PMA and the calcium ionophore ionomycin to stimulate production of chemokines and cytokines. By using specific blockers of MDR transporters we examined whether this had an effect on chemokine and cytokine secretion when compared to the condition without MDR blockade. The results indicated no robust differences in CCL20, MCP1, IL-8 and TNFα secretion between activated parent cell lines without MDR expression and counterparts with overexpression of MRP2, 3, 4, 5 and BCRP or after blocking of these transporters. Interestingly, CCL20 secretion was reduced after blocking of MRP1 or P-gp, however, this showed variability among different cell lines. One other interesting point was that P-gp showed the capability to extrude the activator of chemokine/cytokine production, PMA, which may support the conclusion that MDR transporters do not directly extrude higher molecular weight chemokines and cytokines, but that they can have an indirect effect on suppression of chemokine/cytokine secretion by extruding small molecule activators of this process.

New experimental drugs with novel mechanisms of action could be utilized to bypass MDR-associated DMARD resistance. One such class of novel experimental drugs is proteasome inhibitors which interfere in protein degradation processes. The proteasome has a crucial role in the activation of the transcription factor NFκB, which drives the transcription of several pro-inflammatory cytokines such as TNFα and IL-1β. It does so by facilitating the breakdown of IκBα protein, the natural inhibitor of NFκB. Thus, proteasome inhibitors may harbor the capacity to block IκBα protein breakdown, block NFκB activation and elicit an anti-inflammatory effect by suppress-
ing pro-inflammatory cytokine production. In Chapter 9 we evaluated the potential anti-inflammatory properties of bortezomib, a boron-containing dipeptide-based proteasome inhibitor, which is currently registered for the treatment of hematological malignancies. Indeed, we observed that bortezomib conveyed a rapid and potent inhibition of TNFα production by activated T-cells from RA patients. This inhibitory potency was also demonstrated in T cells from DMARD non-responsive RA patients. Along with a reduction in TNFα release, bortezomib had additional delayed effects of inhibiting T-cell activation by CD3/CD28 and, after > 48 hours exposure, a marked induction of apoptosis of peripheral blood lymphocytes of RA patients.

Despite the promising anti-inflammatory effect of bortezomib, it is not clear what the long term efficacy and toxicity of this drug will be during chronic administration as anticipated for RA patients. Furthermore, it is also not clear whether long term bortezomib exposure will provoke the onset of resistance to this drug. To obtain more insight into the possible development of acquired resistance to bortezomib and the identification of the molecular basis of resistance, we set out to expose in vitro human monocytic/macrophage THP1 cells to stepwise increasing extracellular concentrations of bortezomib from 2.5 nM to 200 nM (Chapter 10). Indeed, by this protocol, high levels of bortezomib resistance (45-129 fold compared to parental cells) could be provoked. Examination of the molecular mechanism of bortezomib-resistance in these cells revealed two major findings; (1) a mutation in the PSMB5 gene encoding for the proteasome β5 subunit protein of the 26S proteasome, being the primary target of bortezomib. This mutation resulted in an amino acid substitution at position 49 of the β5 subunit protein from alanine to threonine (Ala49Thr). This amino acid position is known to be critical for bortezomib docking in the active site of the β5 subunit. (2) A marked and selective overexpression (up to 60-fold) was observed of PSMB5 protein but not of the other two catalytically active proteasome subunits PSMB6 and PSMB7, or of one of the non-catalytic α subunits of the proteasome, PSMA7. In addition, the bortezomib-resistant cells also displayed high levels of cross-resistance to other β5 subunit-targeted cytotoxic peptides, including 4A6, MG132, MG262 and ALLN, but they retained full sensitivity to a broad spectrum of chemotherapeutic drugs as well as to all DMARDs. Bortezomib sensitivity in bortezomib-resistant cells could be restored by siRNA-mediated silencing of PSMB5 gene expression preventing the upregulation of β5 subunit protein. Altogether, these studies demonstrate that drug resistance phenomena should also be considered for proteasome inhibitors as novel experimental drugs.

Bortezomib is the first prototypical proteasome inhibitor that entered into the clinic. Continuous research being done to design, identify and evaluate second generation of proteasome inhibitors that may have superior properties over bortezomib with
regard to irreversible binding vs reversible binding, the targeting of other catalytically active proteasome subunits, or the capability to bypass bortezomib resistance. In Chapter 11 we reported on a cytotoxic peptide, 4A6, with an unknown mechanism of action. Based on cross-resistance profiling for bortezomib resistant cells it was suggested that 4A6 could be a proteasome inhibitor. More detailed characterization revealed that 4A6 is a potent and reversible inhibitor of the proteasome β5 subunit. 4A6 displayed differential activity against various leukemia and breast cancer cell lines, but its activity may be limited by the fact that it is a substrate for cellular extrusion by the MDR transporters P-gp and MRP1. Still, 4A6 may be considered as a lead compound for further drug development of novel proteasome inhibitors that lack MDR substrate affinity and elicit anti-inflammatory properties in an RA clinical treatment setting.
Key points of the thesis

Preclinical/in vitro laboratory studies:

- Acquired resistance to the DMARD chloroquine is mediated by drug efflux via upregulated expression of multidrug resistance-associated protein 1 (MRP1).
- Chloroquine resistance is accompanied by a marked cross-resistance to glucocorticoids related to suppression of the cyclic-AMP protein kinase A signalling pathway.
- Sulphasalazine and methotrexate are prone to drug interactions based on the fact that sulphasalazine is a potent inhibitor of cellular uptake of methotrexate via the reduced folate carrier. Consequently, this interaction may limit additive/synergistic effects of this DMARD combination.
- Chronic exposure to sulphasalazine sensitizes immune-effector cell lines for glucocorticoids.
- Multidrug resistance (MDR) drug efflux transporters may be indirectly involved in the secretion of chemokines and cytokines by mediating the extrusion of (small molecule) activators of this process.
- Acquired resistance to the proteasome inhibitor bortezomib is conferred by an Ala49Thr mutation in the highly conserved bortezomib-binding pocket of the proteasome β5-subunit (PSMB5) protein.

Clinically-directed laboratory studies:

- Multidrug resistance transporters are well known for their pharmacological role in conferring drug resistance; however, their physiological role in immune-effector cells may be equally important for optimal immune-function.
- Upregulated expression of specific MDR drug efflux transporters in peripheral blood cells seems primarily a disease-activity associated phenomenon rather than a DMARD-induced effect.
- The MDR transporter BCRP is highly expressed on synovial tissue macrophages and may be responsible for an attenuated response to the DMARDs methotrexate, leflunomide and sulphasalazine, all three being substrates for BCRP.
- The proteasome inhibitor bortezomib is highly effective in suppressing pro-inflammatory cytokine release by activated T cells from RA patients.