Chapter 12

Summary
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Rheumatoid arthritis (RA) requires chronic treatment with anti-inflammatory drugs. Unfortunately, upon prolonged treatment with potent disease modifying anti-rheumatic drugs (DMARDs), many patients sooner or later experience loss of efficacy of these drugs which could be related to the onset of acquired drug resistance in the target inflammatory cells. Obviously, a phenomenon of drug resistance is not unique to DMARDs in RA; it is generally accepted that the onset of drug resistance is a common mechanism of treatment failure for many types of drugs, for example antibiotic drugs, antimalarial drugs and anti-cancer drugs. We started our research to address this clinically relevant problem to explore mechanisms that could confer cellular resistance against methotrexate (MTX) and sulfasalazine, two widely applied DMARDs in RA. In addition we assessed whether rationally designed therapeutic drugs or drug targets may be exploited to circumvent drug resistance related mechanisms. This issue is described in the general introduction (chapter one), followed by a literature review that outlines molecular mechanisms involved in DMARD resistance in chapter two.

Mainly based on oncological research, increased drug efflux by multi-drug resistance (MDR) proteins has been recognized as an important mechanism of resistance to anti-cancer agents. MDR proteins belong to the family of ATP-binding (ABC) transporters. A wide range of structurally and functionally different drugs can be pumped out of the cells by these proteins, resulting in a multi-drug resistance phenotype. There is now accumulating evidence that several DMARDs may be among the substrates of distinct MDR proteins and thereby cause a diminished efficacy of DMARDs. Table 1 summarizes the MDR proteins that can be involved in drug efflux of selected DMARDs.
Table 1: MDR transporters and their pharmacological (DMARD) substrates

<table>
<thead>
<tr>
<th>MDR transporter</th>
<th>DMARD substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCB1 (Pgp)</td>
<td>chloroquine, glucocorticoids</td>
</tr>
<tr>
<td>ABCC1 (MRP1)</td>
<td>methotrexate, chloroquine</td>
</tr>
<tr>
<td>ABCC2 (MRP2)</td>
<td>methotrexate</td>
</tr>
<tr>
<td>ABCC3 (MRP3)</td>
<td>methotrexate</td>
</tr>
<tr>
<td>ABCC4 (MRP4)</td>
<td>methotrexate, azathioprin</td>
</tr>
<tr>
<td>ABCC5 (MRP5)</td>
<td>methotrexate, azathioprin</td>
</tr>
<tr>
<td>ABCG2 (BCRP)</td>
<td>methotrexate, sulfasalazine, leflunomide</td>
</tr>
</tbody>
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In chapter three we determined whether overexpression of MDR proteins contributed to a diminished efficacy of sulfasalazine in a human T cell line after prolonged cellular exposure to this drug. The rationale to select sulfasalazine for these studies was that this DMARD displayed a relatively rapid onset of loss of efficacy following prolonged administration. We found that after a period of 4-6 months of sulfasalazine exposure, human T-cells became 3.5-6.0 fold resistant to this drug. Characterization of the resistant cell line revealed a marked induction of the MDR protein ‘breast cancer resistance protein’ (BCRP), no involvement of P-glycoprotein (Pgp) and even a down regulated expression of multi-drug resistance protein 1 (MRP1). Sulfasalazine sensitivity could be largely restored by adding a specific BCRP blocking agent to the culture medium, proving the functional relation of BCRP expression and sulfasalazine resistance. In sulfasalazine resistant T-cells, basal levels of TNFα production after stimulation were markedly increased compared to parental T-cells, pointing to an enhanced inflammatory state of these cells. Subsequently TNFα production was less efficiently blocked by sulfasalazine, consistent with BCRP-mediated efflux of this drug.

In chapter four we determined the stability of the sulfasalazine resistant phenotype in human T-cells. We noted that BCRP expression in these cells was stable for at least 4 weeks after withdrawal of sulfasalazine but gradually declined, along with sulfasalazine resistance levels, to non-detectable levels of BCRP after 6 months. Strikingly, rechallenging with sulfasalazine led to a rapid resumption of sulfasalazine
resistance and BCRP expression within 2 weeks. In this chapter we also assessed the impact of sulfasalazine resistance on responsiveness to other, non-related, DMARDs. Sulfasalazine resistant cells displayed diminished sensitivity to leflunomide (5.1-fold), and MTX (1.8-fold), were moderately more sensitive (1.8-fold) to cyclosporine A and chloroquine, and markedly more sensitive (13-fold) to dexamethasone as compared with parental T-cells. This observation may provide a further rationale for sequential mono- or combination therapies with distinct DMARDs upon decreased efficacy of sulfasalazine.

Sulfasalazine is often combined with MTX and other DMARDs in RA combination-therapy schedules. However, the combination of sulfasalazine and MTX does not display clinical additive effects compared to monotherapy with either sulfasalazine or MTX. Moreover, we observed that sulfasalazine-resistant human T cells were cross-resistant to MTX (described in chapter four) by a mechanism that does not involve enhanced cellular efflux of MTX by BCRP. To further unravel interaction(s) of sulfasalazine and MTX we investigated in chapter five whether exposure of cells to sulfasalazine interferes with the cellular pharmacology of MTX. For this purpose human T-cells were used to analyse the effect of sulfasalazine on the cellular uptake of radiolabelled MTX and the natural folate leucovorin by the reduced folate carrier (RFC). Moreover T-cells with and without acquired resistance to sulfasalazine were used to assess the anti-proliferative effects of MTX in combination with sulfasalazine. Transport kinetic analysis revealed that sulfasalazine is a potent, non-competitive inhibitor of RFC mediated cellular uptake of MTX and the natural reduced folate analogue leucovorin. Consistent with this proven interaction, a marked loss of MTX efficacy was observed when MTX was co-administered with sulfasalazine: up to 3.5 fold for parental T-cells in the presence of 0.25 mM sulfasalazine and >400-fold for sulfasalazine-resistant cells in the presence of 2.5 mM sulfasalazine. Along with the diminished efficacy of MTX, a sulfasalazine dose-dependent decrease in leucovorin accumulation was observed, suggesting the onset of cellular folate depletion. Thus, when considering use of these drugs in combination therapies, these results provide a rationale for both the use of folate supplementation and for spacing administration of sulfasalazine and MTX over time, because the inhibitory effects of sulfasalazine on RFC dependent uptake of MTX are only transient.
Chapter six described an inventory study of expression of MDR transporters on inflammatory cells in synovial tissue of RA patients. In this study we determined the expression of Pgp, MRP1-5, MRP8, MRP9 and BCRP by immunohistochemical techniques in synovial tissue of RA patients with active disease before and after treatment with MTX (7.5-15 mg/week) or leflunomide (20mg/day) and non-inflammatory synovial tissue from orthopaedic patients suffering from mechanical joint injury. We found abundant expression of BCRP in all RA synovial biopsies, both prior to treatment and after 4 months of MTX treatment, 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 (defined by the disease activity score of 28 joints/DAS28). Furthermore, median BCRP expression was 4-8 fold higher for MTX-non-responders 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’ compared to biopsies from patients with a good response on leflunomide. For other MDR proteins, moderate expression of MRP1 was observed in T-cell areas of some synovial biopsies, while expression of Pgp, MRP2-5, MRP8 and MRP9 were below the immunohistochemistry detection levels. In control synovial 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 inflammation dependent rather than drug induced. Because MTX, sulfasalazine and leflunomide are identified as substrates for BCRP, this transporter may contribute to a reduced therapeutic effect of these drugs.

In chapter seven a clinically-oriented review on the status of MTX and leflunomide (monotherapy and in combination schedules) in RA treatment served as an introduction to our laboratory-directed studies on novel experimental antifolate drugs, described in chapter eight and nine. Publications on efficacy of MTX and leflunomide show that both drugs are very potent DMARDs in RA treatment. Long-term observational studies of MTX, with follow-up periods of more than 10 years, showed long-lasting effectiveness with low discontinuation rates and drug survival rates of more than 5 years in approximately 50% of patients, which compares favourably with
25% reported for other DMARDs. The efficacy of low dose MTX also includes a reduction in joint damage progression in RA patients, as seen on X-rays of hands and feet. Moreover, it has been proven that the effective TNFα blocking biologic agents are even more powerful if concomitantly MTX is prescribed. This might be partly due to suppression of the production of antibodies against these agents by plasma cells.

Despite an initially good response to MTX and relatively long-lasting effectiveness in approximately 50% of RA patients, many patients ultimately experience loss of efficacy upon prolonged treatment. Beyond the mechanism of MTX efflux by specific MDR transporters (e.g. BCRP and MRP1), several other causes for impaired responsiveness to MTX have been revealed in cancer chemotherapy. Some of these mechanisms may also be of relevance for RA treatment, e.g. (a) impaired cellular uptake of MTX via the Reduced Folate Carrier (RFC), (b) reduced conversion to polyglutamate forms of MTX due to decreased activity of folylpolyglutamate synthetase (FPGS), (c) increased expression of the target enzyme of MTX, dihydrofolate reductase (DHFR) or (d) polymorphism in genes coding for folate-depened enzymes.

We therefore tested in chapter eight the anti-inflammatory effects of eight novel antifolate drugs, rationally designed to overcome one or more of these mechanisms of MTX resistance. These drugs, for example, exhibit higher affinity for the RFC and FPGS compared to MTX, and/or target other enzymes in the folate metabolism than DHFR; such as thymidylate synthase (TS) and glycaminide ribonucleotide transformylase (GARTFase). The ability of these antifolate drugs to inhibit TNFα production was analyzed for ex vivo stimulated T-cells in whole-blood from RA patients. Two novel DHFR inhibitors (PT523 and PT644) and two novel TS inhibitors (Raltitrexed and GW1843), all harbouring high substrate affinities for RFC and FPGS, turned out to be very effective in abrogating TNFα release by T-cells of RA patients (at nanomolar concentrations). Median concentrations of these drugs to inhibit TNFα production by 50% (IC-50 values) were 6.9-10.5 lower compared to MTX, also in RA patients who were clinically unresponsive to MTX. Conceivably, experimental therapies with one of these novel antifolate drugs deserve further consideration for patients with MTX-refractory RA.
In chapter nine we explored the folate receptor-β (FR-β) as a novel target for selective macrophage directed RA treatment with folate antagonist drugs. Folate receptors (FR) are membrane-associated proteins consisting of at least three isoforms (α,β,γ). These receptors have a high affinity for folic acid and a relatively low affinity for MTX, and cell entrance of these agents occurs via endocytosis. Given the notion that the β isoform is specifically expressed on synovial macrophages, this receptor may be exploited as a selective target in RA treatment. For this reason we determined the expression of FR-β on macrophages in cryopreserved synovial tissue from RA patients with active disease, along with expression on peripheral blood cells of RA patients (lymphocytes, monocytes, ex-vivo cultured macrophages and ex-vivo activated T-cells). We also determined FR-β binding affinities for 10 novel antifolate drugs by competition experiments with [³H]-folic acid on FR-β transfected cells. As hypothesized, immunohistochemical staining of RA synovial tissue showed high expression of FR-β on macrophages in the synovial (sub)lining, while no staining was observed in T-cell areas or control synovial tissue. Consistently, FR-β mRNA levels were highest in synovial tissue extracts and monocyte-derived macrophages, but low in peripheral blood T-cells and monocytes. Screening of 10 novel antifolate drugs revealed 4 compounds for which FR-β had a high binding affinity (20-77 folds higher than for MTX). One of these compounds, the thymidylate synthase inhibitor BCG945, displayed selective targeting against FR-β transfected cells because of a concomitant low affinity for the RFC. This drug therefore deserves further exploration for selective macrophage directed therapy in RA.

A potential novel target in the treatment of RA is NFκB, because activation of this transcription factor is thought to play a central role in the onset and progression of inflammation in RA. From this perspective, experimental and therapeutic strategies aiming at inhibition of the activation of NFkB have been developed and tested in preclinical and clinical stages of RA treatment. These strategies include the use of (a) direct NFκB inhibitors; (b) NFκB decoy oligonucleotides to prevent NFkB binding to its promotor site and (c) inhibitors of IkB kinase. Another approach to interfere with NFkB activation, which is relatively unexplored, is by blocking the 26S proteasome-mediated breakdown of the IkBα protein, the natural inhibitor of NFkB. Cell activation initiates both phosphorylation and ubiquitination of IkBα. The latter process triggers
breakdown of IkBα protein by the 26S proteasome system. Upon loss of binding of its natural inhibitor protein, NFkB can translocate to the nucleus to drive the transcription of pro-inflammatory cytokines (like TNFα and IL1β) and anti-apoptotic genes. Thus, it is anticipated that inhibitors of the proteasome may counteract this process and elicit a potential anti-inflammatory response. We therefore tested in chapter ten the anti-inflammatory properties of the proteasome inhibitor bortezomib, a boronic acid dipeptide, currently used in the treatment of advanced multiple myeloma. We found that bortezomib conveys potent inhibition of TNFα production upon activation of T-cells from RA patients, regardless of their clinical response to MTX. Along with a reduction in TNFα release, a marked induction of apoptosis was observed in peripheral blood lymphocytes of RA patients following 48 hours of bortezomib exposure. Furthermore we found that bortezomib inhibits T-cell activation by CD3/CD28 as defined by a (apoptosis unrelated) concentration dependent decrease in CD25 expression. Along with these effects, it is also appreciated that proteasome inhibitors may retain therapeutic activity against drug resistant cells. Therefore, this class of drugs with a novel mode of action would be worthy for further (pre) clinical evaluation in a RA setting.
KEY POINTS OF THIS THESIS:

- Chronic exposure of inflammatory cells to sulfasalazine induces expression of the multi-drug resistance protein BCRP on the cell membrane, causing sulfasalazine resistance.
- BCRP is highly expressed on the cell membrane of synovial tissue macrophages and may therefore cause diminished efficacy of the DMARDs sulfasalazine, methotrexate and leflunomide (BCRP substrates).
- Sulfasalazine is a potent inhibitor of methotrexate uptake via the Reduced Folate Carrier; this finding may explain the lack of additive clinical effects of the combination of methotrexate and sulfasalazine in therapeutic regimens for RA.
- Novel generation antifolate drugs, designed to overcome methotrexate resistance in oncology, are very potent anti-inflammatory drugs in ex-vivo activated T-cells of RA patients. These drugs might therefore be beneficial for RA patients who do not respond to treatment with methotrexate.
- The high folate receptor-β expression on synovial tissue macrophages may serve as a distinctive target for therapy with novel generation antifolate drugs and imaging of (sub-clinical) RA disease activity.
- Proteasome inhibition by bortezomib is very effective in abrogating cytokine production in activated RA T-cells.