Targeting DMARD resistance in Rheumatoid Arthritis

Joost van der Heijden
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The research described in this thesis was performed at the department of Rheumatology (head prof. dr. B.A.C. Dijkmans) in collaboration with the department of Pathology (head prof. dr. C.J.L.M. Meijer), VU University Medical Center, Amsterdam, the Netherlands. Joost van der Heijden was supported by the Dutch Arthritis Foundation (Grant NRF-03-I-40) and ZonMW (The Netherlands Organization for Health Research and Development; Grant 920-03-362).

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ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad Doctor aan de Vrije Universiteit Amsterdam, op gezag van de rector magnificus prof.dr. L.M. Bouter, in het openbaar te verdedigen ten overstaan van de promotiecommissie van de faculteit der Geneeskunde op vrijdag 12 december 2008 om 10.45 uur in de aula van de universiteit, De Boelelaan 1105

by

Johan Willem van der Heijden

geboren te Gouda
'I, a physician, am delighted to stand here with two distinguished chemists, Drs. Reichstein and Kendall. Perhaps the ratio of one physician to two chemists is symbolic, since medicine is so firmly linked to chemistry by a double bond. For medicine, especially during the past twenty-five years, has been receiving its finest weapons from the hands of the chemists, and the chemist finds his richest reward as the fruits of his labor rescue countless thousands from the long shadows of the sickroom'.

Philip S. Hench, rheumatologist *

*Nobel Prize Laureate (together with prof. dr. Tadeus Reichstein and prof. dr. Edward C. Kendall) in Physiology and Medicine in 1950 for their discoveries relating to the hormones of the adrenal cortex, their structure and biological effects.
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<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>AICAR</td>
<td>5-aminomimidazole-4-carboxamide ribonucleotide</td>
</tr>
<tr>
<td>AICARTF</td>
<td>5-aminomimidazole-4-carboxamide ribonucleotide formyltransferase</td>
</tr>
<tr>
<td>APRTF</td>
<td>Amidophosphoribosyltransferase</td>
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<tr>
<td>ATRA</td>
<td>All-trans retinoic acid</td>
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<tr>
<td>BCRP</td>
<td>Breast cancer resistance protein</td>
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<tr>
<td>CCP</td>
<td>Cyclic citrullinated peptide</td>
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<tr>
<td>CHQ</td>
<td>Chloroquine</td>
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<tr>
<td>DAS</td>
<td>Disease activity score</td>
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<td>DC</td>
<td>Dendritic cell</td>
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<td>DHFR</td>
<td>Dihydrofolate reductase</td>
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<td>DMARD</td>
<td>Disease modifying antirheumatic drug</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immuno Sorbent Assay</td>
</tr>
<tr>
<td>FA</td>
<td>Folic acid</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescent-activated cell sorting</td>
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<tr>
<td>FPGH</td>
<td>Folylpolyglutamate hydrolase</td>
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<td>FPGS</td>
<td>Folylpolyglutamate synthetase</td>
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<td>FR</td>
<td>Folate receptor</td>
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<tr>
<td>GARTFase</td>
<td>Glycinamide ribonucleotide transformylase</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
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<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
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<tr>
<td>IC-50</td>
<td>Drug concentration required to inhibit cell growth or cytokine production by 50% compared to non-treated cells</td>
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<tr>
<td>IκB</td>
<td>Inhibitor-IκB</td>
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<tr>
<td>IκK</td>
<td>Inhibitor-IκB kinase</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>LV</td>
<td>Leucovorin</td>
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<td>MDR</td>
<td>Multi-drug resistance</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<td>MMP</td>
<td>Matrix metalloproteinase</td>
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<tr>
<td>MRP</td>
<td>Multi-drug resistance protein</td>
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<td>MTHFR</td>
<td>Methylene tetrahydrofolate reductase</td>
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<td>MTX</td>
<td>Methotrexate</td>
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<tr>
<td>NFκB</td>
<td>Nuclear factor κB</td>
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<tr>
<td>NSAID</td>
<td>Non steroidal anti-inflammatory drug</td>
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<tr>
<td>PBL</td>
<td>Peripheral blood lymphocyte</td>
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<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>Pgp</td>
<td>P-glycoprotein</td>
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<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
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<tr>
<td>RF</td>
<td>Rheumatoid factor</td>
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<tr>
<td>RFC</td>
<td>Reduced folate carrier</td>
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<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
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<tr>
<td>SSZ</td>
<td>Sulfasalazine</td>
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<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
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<td>TS</td>
<td>Thymidylate synthase</td>
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General introduction and introduction into the chapters
General introduction

Rheumatoid Arthritis (RA) is a chronic inflammatory and joint destructive disease that affects approximately 1% of the Dutch population and commonly leads to significant disability and consequent reduction in the quality of life of RA patients. RA can start at any age, with a peak incidence between the fourth to sixth decades of life, and is three times more frequent in women than in men. RA is associated with high costs and, if not treated appropriately, with a reduction in life expectancy (1, 2).

Pathogenesis of RA

The exact pathogenesis of RA is not fully elucidated, but it is a disease where the immune and inflammatory systems are intimately linked to the destruction of cartilage and bone. Given the detection of auto-antibodies, with IgM-rheumatoid factor (RF) as the classical and prototypical serum marker, RA is classified as an auto-immune disorder. During the last decade, auto-antibodies against citrullinated peptides (anti-CCP antibodies) have been identified in two third of RA patients, which turned out to be more specific for this disease (3). These antibodies reflect an abnormal humoral response against citrullinated self-proteins (4). Interestingly, these antibodies can be detected years before the first onset of clinical symptoms and antibody levels are correlated with RA disease activity (5, 6). The question remains whether the occurrence of anti-CCP antibodies are of pathogenetic relevance in RA.

Conceptually, the onset of joint inflammation in RA is induced by the following events, depicted in figure 1. Influx of dendritic cells (DCs) into the synovial compartment occurs early in disease pathology, and it is thought that DCs play a role in the initiation and perpetuation of disease by presentation of (unknown) arthritogenic (auto) antigens. Several genetic loci are known to be associated with the susceptibility for and the severity of RA; the most well established being a specific amino-acid sequence in the HLA-DR4 allele, the so called shared epitope, which is involved in antigen presentation (7). Upon presentation of auto-antigens by appropriate HLA molecules, invading (pre-activated) T-cells become (further) activated by cell-cell contact or cytokine signalling and start to produce cytokines. These cytokines in turn stimulate on one hand B-cells to produce auto-antibodies (e.g. RF and anti-CCP) and on the other hand macrophages and synovial fibroblasts to produce pro-inflammatory cytokines (like TNFα and ILs-1β) and matrix metalloproteinase’s (MMPs) which provoke cartilage destruction. Activation of joint osteoclasts eventually leads to bone destruction. Tissue infiltration by inflammatory cells results in thickening of the synovial membrane (referred to as synovitis) and is accompanied by neovascularisation (1, 8, 9).

Therapeutic strategies

For decades therapy of RA patients consisted of non-steroidal anti-inflammatory drugs (NSAIDs), reducing the pain and inflammation but not modifying the course of the disease. The first Disease Modifying Anti-Rheumatic Drugs (DMARDs) were intramuscular gold (J. Forestier, 1928) and sulfasalazine (N. Svartz, 1938), interfering with the course and progression of the disease (1,10). The modifying effect of DMARDs is reflected in improvement of the Disease Activity Score (DAS) (a composite index of the patients opinion of disease activity, a count of tender and swollen joints by the physician and the erythrocyte sedimentation rate) (n) and parameters of joint damage as seen on X-rays of hands and feet.

Corticoids are used since the 1940’s with very good clinical response, but there are limitations to treatment duration because of the occurrence of side effects upon prolonged use, like osteoporosis, hypertension and diabetes. Methotrexate (MTX) was first introduced around 1950, but it was not given the credits it deserves at that time. Three developments can be considered as real breakthroughs in the treatment of RA patients:
(a) the rediscovery of MTX in the 1980s, (b) the introduction of combination therapies and (c) the introduction of biologic agents in the 1990s. The term biologics refers to a group of therapeutics that specifically target a particular cell or cytokine involved in the pathogenesis of RA. These highly effective anti-inflammatory agents include infliximab and adalimumab (both antibodies against TNFα), etanercept (soluble TNFα receptors), rituximab (antibodies to CD20/B-cells) and abatacept (antibodies to the co-stimulatory molecule B7) on antigen presenting cells to prevent T-cell activation) (1, 8, 10, 12-14). These drugs are usually combined with MTX. In the Netherlands physicians agreed with the government to prescribe biologics only to those patients that sequentially failed on two regular DMARDs, mainly to reduce drug related costs.

Today, many studies have proven that early and aggressive therapy with (combinations of) DMARDs and/or biologic agents is beneficial in terms of preservation of functional ability and slowing down of joint damage progression (12). After remission induction, DMARDs and/or biologic agents can then be tapered and in some cases even stopped (15).

Despite the current success of biologic therapies, DMARDs have an established place in the treatment of RA because of their efficacy, convenience, safety and low related drug costs (16). Currently, the most commonly applied DMARDs are MTX, sulphasalazine, leflunomide, prednisone and antimalarials like hydroxychloroquine. Their therapeutic efficacy can be enhanced by combining these DMARDs, however a rationale behind these combinations is often lacking (17, 18). Sulphasalazine plus MTX is a frequently used combination; however, this combination does not display additive or synergistic effects over monotherapy (19, 20), unless a third DMARD (hydroxychloroquine or prednisone) is added (21-25).

Figure 2: Reasons for discontinuation of traditional DMARDs in patients with RA.

DMARD-resistance
Observational studies and meta-analyses of treatment efficacy, average treatment duration and adverse effects for RA-patients consistently demonstrate variable responses for individual DMARDs and DMARD combinations (26, 26-30). Unfortunately, many RA patients experience loss of efficacy upon chronic treatment with DMARDs, which could point to the onset of acquired drug resistance. Galindo-Rodrigues et al showed in a retrospective practice-based study between 1985 and 1994, including 2296 DMARD therapies, that after 16 months 50% of treatments had been discontinued because of inefficacy and/or toxicity and that after 4.5 years 75% of therapies had been discontinued. MTX appeared to be the best drug in the first 5 years of disease; approximately 50% of RA patients were still receiving MTX after 3 years of treatment, compared to 33% for antimalarials and 25% for sulphasalazine (31) (figure 2). At the time this study was performed however, TNFα antagonists were not yet available. Despite the initial good response to MTX for many patients, there is room for improvement of DMARD therapy and a need for studies that unravel possible mechanisms of DMARD resistance and studies that define parameters for predicting DMARD efficacy and toxicity.

The issue of drug resistance as a cause of therapy failure and the search for new therapeutic approaches to circumvent drug-resistance has received much attention in cancer treatment (32); however it just starts to be appreciated in RA treatment (33-36). Human beings have intrinsic and inducible mechanisms against daily exposure to hundreds or even thousands of xenobiotic substances present in our environment and food (32, 37, 38). Most chemically designed therapeutic drugs, including DMARDs, are recognized by target cells as foreign substances. Given this notion, sooner or later immunologic or cellular defence mechanisms will become operative and inactivate the drugs at various molecular levels. Cellular drug resistance can be categorized under two main headings: (1) inherited or primary resistance (referring to cells that are already resistant before receiving therapy) and (2) acquired drug resistance (which indicates that cells were initially sensitive to the drug, but developed resistance during the course of treatment).

General mechanisms of cellular drug resistance are: diminished drug delivery, impaired drug uptake, energy dependent cellular drug extrusion via MDR transporters, decreased drug activation, drug sequestration or enhanced drug detoxification, alterations in the target of the drug or enhanced repair of damage/ impaired capacity for cells to go into apoptosis (33) (figure 3). These and other mechanisms of drug resistance are extensively described in chapter two.

Resistance to biologic agents
While treatment with biologic agents provides great benefit to the majority of RA patients, some patients experience persistent active disease or loss of efficacy upon prolonged treatment. Biologic agents are proteins and therefore antibodies can be

Figure 2: Reasons for discontinuation of traditional DMARDs in patients with RA.

produced against these agents, diminishing clinical efficacy. Wolbink et al showed that in serum of 43% (22/51) of consecutive RA patients treated with infliximab, anti-infliximab antibodies were detectable. A negative correlation was found for the titer of anti-infliximab antibodies and the clinical response (39). The same observation was made in a group of Ankylosis Spondylitis (AS) patients treated with infliximab (40). Adalimumab, a fully human anti-TNFα antibody, was thought to be less immunogenic than the chimeric infliximab. Nevertheless Bartelds et al showed that in serum of 17% (21/123) of consecutive RA patients anti-adalimumab antibodies were detectable and that the antibody-titer was associated with diminished adalimumab concentrations and subsequently with an impaired clinical response (41). There is evidence that MTX administration prolongs the duration of response to biologic agents, probably due to the inhibition of the production of anti-biologic antibodies (42); in several trials, the efficacy of the combination of MTX plus a biologic agent appeared to be superior over monotherapy with MTX or the biologic agent (43-48). For this reason, it is recommended to combine a biologic agent with MTX.

Aim and outline of the thesis

This thesis focuses on the mechanisms involving DMARD resistance in the treatment of RA patients and alternative ways to target RA inflammatory cells in case of resistance to currently available DMARDs.

From the field of oncology, increased drug efflux has been recognized as one important mechanism of drug resistance. Cell-membrane proteins responsible for drug efflux belong to the family of ATP-binding cassette (ABC) transporters of which 49 different proteins were identified from the human genome project (http://nutrigene.4t.com/humanabc.htm). Tissue distribution studies showed that these proteins are expressed in tissues that are heavily exposed to toxic agents, microbial and exogenous compounds, eg. liver, kidney and intestine (49). Consistently, one of the primary functions of ABC-transporters is extrusion of toxic substances. Some of these transporters are extensively characterized while the functional properties of others are still unknown (37, 38, 50). A wide range of structurally and functionally different drugs can be pumped out of cells by these ABC-transporter proteins, thereby conferring a so-called multiple-drug resistant (MDR) phenotype. Therapeutic drugs are frequently extruded in co-transport with glutathione, or after conversion to glutathione-, glucuronide- or sulphate conjugates (figure 4). Several DMARDs seem to be among the substrates of MDR proteins (33). This notion led to our hypothesis that these proteins might also provide a cellular defence mechanism against DMARDs, leading to DMARD-resistance. In the first section of this thesis we evaluated the potential role of ABC-transporters in conferring resistance to DMARDs. To this end, we first investigated whether MDR proteins become upregulated on inflammatory model cells upon chronic exposure to DMARDs in vitro. In addition, we evaluated the expression of MDR proteins on inflammatory cells (T-cells, macrophages) in synovial tissue of RA patients and assessed whether expression of these proteins correlates with clinical outcome after treatment with MTX or leflunomide.

Besides MTX efflux by MDR transporters (33, 37, 38, 50-52), cellular resistance to MTX might be conferred by diminished uptake via its cell membrane carrier, the reduced folate carrier (RFC). Also impaired polyglutamylation by the enzyme folylpolyglutamate synthetase (FPGS) or alterations in the target enzymes of MTX (a.o. dihydrofolate reductase) may contribute to a resistant phenotype (53-59). The second section of this thesis focuses on novel experimental therapeutic strategies to overcome MTX resistance. In this context, we utilized activated T-cells from RA patients to test potential anti-inflammatory effects of novel generation antifolate drugs that were rationally designed to overcome known mechanisms of MTX resistance in cancer patients (60). Furthermore, we evaluated whether the Folate Receptor (FR), expressed on synovial tissue macrophages, could be selectively targeted by novel generation antifolate drugs that display a higher affinity for this receptor than MTX (61, 62).

Finally, in the last section of this thesis we focussed on a novel class of experimental drugs that may retain therapeutic activity against resistant cells, due to the fact that they are no substrates for MDR transporters. Such class of compounds include proteasome inhibitors, which interfere in intracellular protein degradation (63). Bortezomib, a boronic acid dipeptide, is the clinically used representative of this class of drugs (64-66). Bortezomib has recently been approved for the treatment of therapy refractory multiple myeloma.
Conceptually, bortezomib may elicit potential anti-inflammatory effects by inhibiting the degradation of the natural inhibitor of the transcription factor NF\(\kappa\)B, i.e. I\(\kappa\)B\(\alpha\), thereby inhibiting nuclear translocation of NF\(\kappa\)B and transcription of several pro-inflammatory cytokines such as TNF\(\alpha\) (67, 68) (figure 5). We therefore tested the anti-inflammatory effects of this drug, using TNF\(\alpha\) release from ex-vivo activated T-cells of RA patients as a read out.

Introduction into the chapters

In chapter two, a literature review is given that outlines molecular mechanisms that could be involved in the onset of resistance to DMARDs in RA patients, including methotrexate, sulfasalazine, hydroxychloroquine, azathioprine and leflunomide. The mechanisms suggested are based on findings from experimental laboratory studies of specific drug-uptake and drug-efflux transporters, alterations in intracellular drug metabolism and genetic polymorphisms of drug transporters and metabolic enzymes. In this chapter we also discuss strategies to overcome resistance and the current clinical studies aiming to predict the response to treatments and risk of toxic effects.

In chapter three we determined whether overexpression of MDR proteins contribute to a diminished efficacy of sulfasalazine after prolonged exposure of human T-cells to this DMARD. For this purpose, a sulfasalazine resistant human T-cell line was characterized for expression of the MDR-proteins P-glycoprotein (Pgp), Multidrug resistance protein 1 (MRPs) and breast cancer resistance protein (BCRP). In addition, we assessed the impact of sulfasalazine resistance on the ability of T-cells to secrete TNF\(\alpha\). Chapter four describes the dynamics of sulfasalazine resistance in human T-cells after withdrawal of sulfasalazine and after rechallenging these cells again with this drug, by measuring the expression of MDR transporters under these conditions. Finally, we evaluated the impact of sulfasalazine resistance on responsiveness to other, non-related DMARDs.

Since we observed (chapter four) that sulfasalazine resistant T-cells in vitro were cross-resistant to MTX when co-incubated with sulfasalazine, along with the clinical notion that combination therapy of sulfasalazine and MTX does not show improvement over monotherapy with MTX or sulfasalazine, we investigated whether exposure of cells to sulfasalazine provokes intervention with the cellular pharmacology of MTX. For this purpose in chapter five we studied the effect of sulfasalazine treatment on the functional activity and expression of the cell membrane transporter responsible for the uptake of MTX, the Reduced Folate Carrier (RFC).

In chapter six we described an inventory study of expression of MDR-transporters on inflammatory cells in RA synovial tissue before and after treatment with MTX or
leflunomide. With immunohistochemical techniques we assessed the expression of Pgp, MRP1-5, MRP8, MRP9 and BCRP. Since the latter two groups of MDR transporters have MTX and leflunomide among their substrates, we examined whether expression of these drug efflux transporters correlated with the clinical response to these DMARDs.

Chapter seven reviews the leading manuscripts involving RA treatment with the antifolate drug MTX and leflunomide as monotherapy and in combination regimes with other DMARDs or biologic agents. This chapter serves as a clinical introduction to chapter 8-10, where we describe: (1) possible alternative targets in the folate pathway in inflammatory cells making use of novel, rationally designed, antifolate drugs; (2) selective targeting of synovial tissue macrophages by folate antagonists and (3) experimental drugs possessing novel mechanisms of action.

From the field of oncology, where MTX is used against childhood leukemia, novel generation of antifolate drugs are available that were designed to circumvent known mechanisms of resistance against MTX. In chapter eight we describe the potential anti-inflammatory properties of these drugs by measuring the inhibition of TNFα release from activated T-cells in whole blood of RA patients.

In chapter nine we focus on the folate receptor β (FRβ) as a potential target for RA treatment with novel antifolate drugs. Since it has been described that this receptor is selectively expressed on activated macrophages in inflamed synovial fluid of RA patients, and may be involved in MTX transport in these cells, we assessed the expression of FRβ on inflammatory cells in intact RA synovial tissue by immunohistochemistry and PCR analysis. Beyond this, we determined FRβ binding affinities for several novel antifolate drugs, in search for drugs that may be more selective than MTX in targeting activated synovial tissue macrophages via the FRβ.

Chapter ten describes a study of the anti-inflammatory properties of the proteasome inhibitor bortezomib, a drug currently used in the treatment of therapy refractory multiple myeloma. Since activation of the nuclear transcription factor NFκB, that is dependent on the proteasome-mediated breakdown of its inhibitor (IκB), is thought to play a central role in the onset and progression of inflammation in RA, selective inhibition of the activity of this transcription factor by low-dose bortezomib treatment might be a very efficient therapeutic option that warrants further investigation. In this study, we measured the inhibitory effects of bortezomib on production of TNFα by activated T-cells from RA patients, along with the induction of apoptosis in these cells.

In chapter eleven the general discussion is provided. Chapter twelve summarizes the highlights of this thesis and is followed by a summary in Dutch.
References


34. Wollheim F.A. Drug resistance in rheumatology: an area in search of investigators, Curr. References
References


References


Resistance to methotrexate and other disease-modifying antirheumatic drugs – from bench to bedside

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Departments of ¹Rheumatology and ²Pathology, VU University Medical Center, Amsterdam, The Netherlands

Nature Clinical Practice Rheumatology 2007; 3: 26-34
Summary

The chronic nature of rheumatoid arthritis (RA) means that patients require drug therapy for many years. Many RA patients, however, have to discontinue treatment due to drug-related toxic effects, loss of efficacy, or both. The underlying molecular cause for loss of efficacy of antirheumatic drugs is not fully understood, but it might be mediated, at least in part, by mechanisms shared with resistance to anticancer drugs. This review outlines molecular mechanisms that could be involved in the onset of resistance to, or the loss of efficacy of, disease-modifying antirheumatic drugs in RA patients, including methotrexate, sulfasalazine, chloroquine, hydroxychloroquine, azathioprine and leflunomide. The mechanisms suggested are based on findings from experimental laboratory studies of specific drug-uptake and drug-efflux transporters belonging to the superfamily of multidrug-resistance transporters, alterations in intracellular drug metabolism, and genetic polymorphisms of drug transporters and metabolic enzymes. We also discuss strategies to overcome resistance and the current clinical studies aiming to predict response and risk of toxic effects. More in-depth knowledge of the mechanisms behind these features could help facilitate a more efficient use of disease-modifying antirheumatic drugs.

Review criteria

We searched PubMed using the keywords “DMARDs”, “resistance”, “multidrug resistance”, “methotrexate”, “sulfasalazine” and “rheumatoid arthritis”. Searches included full text papers and abstracts from annual American College of Rheumatology (ACR) meetings and European League Against Rheumatology (EULAR) meetings from 1995–2006. We have also included experimental data from our own research aimed at identifying the molecular mechanisms of resistance to DMARDs in experimental model systems and patients.

Introduction

Despite the current success of biological therapies (inhibitors of tumor necrosis factor [TNF], interleukin-1 receptor antagonists and antibodies to CD20) (1-3), disease-modifying antirheumatic drugs (DMARDs) have an established place in the treatment of rheumatoid arthritis (RA) because of their convenience, safety and low related costs. One common phenomenon associated with chronic DMARD treatment, however, is a gradual reduction in drug efficacy, which could point to the onset of drug resistance. Increases in drug doses might partly regain therapeutic efficacy, but this approach also increases the risk of adverse effects and could ultimately lead to discontinuation of treatment. The issue of drug resistance as a cause for therapy failure has received considerable attention in the treatment of cancer and infectious diseases (4), but has just started to be appreciated in RA treatment (5, 6). Whereas endpoint evaluations for anticancer drug resistance usually refer to loss of antiproliferative effects of target cells, DMARD resistance might also be defined as loss of ability to block the release of proinflammatory cytokines, in addition to a loss of antiproliferative effects.

This review describes the current knowledge on the molecular mechanisms of cellular resistance to DMARDs (methotrexate, sulfasalazine, chloroquine, hydroxychloroquine, azathioprine, auranofin, aurothiomalate, ciclosporin, gold and leflunomide). The suggested mechanisms are mainly based on in vitro laboratory studies and theories about the emergence of DMARD resistance in RA. We also discuss potential strategies to deal with drug resistance.

General features of drug resistance

Human beings have defense mechanisms against daily exposure to hundreds or even thousands of xenobiotic substances present in our environment and food. For this reason, most chemically designed therapeutic drugs, including DMARDs, are recognized by target cells as foreign substances. Sooner or later, immunological or cellular defense mechanisms will become operative and inactivate the drugs. In a clinical setting, such a process manifests as reduced drug efficacy, for which, at a molecular level, the onset of drug resistance might be an underlying cause (7-12). Cellular drug resistance can be categorized under two main headings: inherent or primary resistance (referring to cells that are already resistant before receiving therapy) and acquired drug resistance (which indicates that cells were initially sensitive to drugs but developed resistance during the course of treatment). In RA treatment, a study by Morgan et al (13) showed that 5–10% of RA patients had inherent or primary resistance to DMARDs, which might account for variable responses to DMARDs seen among RA patients during initial therapy. Similarly, some animal strains have displayed an inherent genetic basis for treatment failure of methotrexate in collagen-induced arthritis (14). The majority of cases of DMARD treatment failure are, however, considered to be the result of various underlying mechanisms, some of which could be specific to the type of DMARD used.

Experimental studies of drug resistance mechanisms

The mechanisms of resistance to DMARDs might be similar to those of resistance to anticancer and antimicrobial drugs. Box 1 outlines several mechanisms of drug resistance for disease-specific target cells; in RA, the causes of resistance could involve cells of the immune system (T lymphocytes and macrophages) implicated in the disease pathophysiology. Possible mechanisms of drug resistance include impaired drug


Box 1 Multiple mechanisms of cellular resistance to different DMARDs.

**Methotrexate**
- Defective transport via reduced folate carrier (decreased protein levels, altered kinetics)
- Slow transport via folate receptor (lower affinity for methotrexate than folic acid)
- Increased efflux via ATP-binding cassette (ABC) subfamily transporters ABCC1–ABCC5 and ABCG2 (polymorphic variations)
- Impaired polyglutamylation (increased expression or activity of folypolyglutamate synthetase protein; increased expression or activity of folypolyglutamate hydrolase protein, polymorphic variants)
- Altered target enzymes (increased dihydrofolate reductase activity or expression, kinetically altered dihydrofolate reductase, increased 5-aminomimidazole-4-carboxamide ribonucleotide formyltransferase activity, polymorphic variants)
- Increased salvage (exogenous/endogenous folates, purines)
- Increased metabolism of methotrexate to 7-OH-methotrexate in the liver

**Sulfasalazine**
- Increased efflux via ABCG2 (polymorphic variants)
- Increased metabolism to 5-aminosalicylic acid and sulfapyridine in colon

**Chloroquine and hydroxychloroquine**
- Increased efflux via ABCB1, ABCC1 (polymorphic variants)
- Sequestration in intracellular compartments (lysosomes)

**Auranofin/aurothiomalate/gold**
- Increased expression of metallothioneins (metal-binding proteins)

**Leflunomide**
- Altered target enzyme (increased activity or expression of dihydro-orotate dehydrogenase, kinetically altered protein)

**Azathioprine**
- Increased efflux via ABCC4, ABCC5
- Increased activity of thiopurine methyltransferase linked to genetic polymorphism

**Glucocorticoids**
- Decreased levels of glucocorticoid receptor α
- Increased ratio of glucocorticoid receptor β isoforms to glucocorticoid receptor α isoform
- Diminished phosphorylation of glucocorticoid receptors, leading to shorter half-life
- Diminished crosstalk with transcription factors
- Enhanced efflux via ABCB1

delivery to target cells, defective cellular uptake, increased drug extrusion, alterations in intracellular drug activation, target inhibition, processes downstream of target inhibition, or a combination of these features. For methotrexate, an example of impaired drug delivery is its metabolism to the less active metabolite 7-hydroxymethotrexate. For sulfasalazine, the activity of intestinal drug-efflux transporters is a determining factor in variability of plasma drug concentrations (15).

Increased drug efflux has been recognized as an important mechanism of drug resistance. Cell membrane proteins responsible for drug efflux belong to the family of ATP-binding cassette (ABC) transporters (4, 16). A wide range of structurally and functionally different drugs can be pumped out of the cell by these proteins, leading to multidrug resistance. Several DMARDs seem to be among the substrates of ABC transporters.

A summary of reported mechanisms of resistance to various DMARDs is shown in Box 1. On the basis of laboratory and preclinical data from all the DMARDs, resistance mechanisms to methotrexate have been best characterized (17, 18) and are shown in Figure 1. A first limiting step in the mechanism of action of methotrexate is cellular uptake via the reduced folate carrier (RFC, also designated SLC19A1), which is constitutively expressed in almost all immune effector cells (19), or by folate receptor-β, which has a restricted expression on activated macrophages, such as in the synovium (20). Reduced expression of, or alterations in, the transport kinetics of these proteins will diminish cellular uptake of methotrexate, resulting in lower intracellular concentrations of the drug, which will thus not reach thresholds for therapeutic effects. Within the cell, methotrexate is converted to polyanionic polyglutamated forms via the action of the enzyme folypolyglutamate synthetase, which improves drug retention because the polyglutamated forms are poorer substrates for efflux transporters such as ABCC1–5 or ABCG2 (21, 22). Thus, cells with a low capacity to retain polyglutamated forms of methotrexate (because of decreased folypolyglutamate synthetase activity or increased activity of the breakdown enzymes folypolyglutamate hydrolase) will be more prone to reduced activity because of drug efflux mechanisms mediated by drug-transporters. Polyglutamated forms of methotrexate inhibit several key enzymes in folate metabolism (dihydrofolate reductase and thymidylate synthase) and purine metabolism (aminomimidazole carboxamide ribonucleotide transformylase). Elevated levels of these enzymes or kinetically altered enzymes with lower binding affinities will necessitate higher doses of methotrexate to achieve optimum inhibition.

For the DMARD sulfasalazine, increased drug efflux via ABCG2 has been reported as a potential mechanism of cellular resistance (23, 24). Molecular mechanisms of resistance for the antimalarial DMARDs chloroquine and hydroxychloroquine include drug sequestration in lysosomal compartments or drug efflux via ABC transporters ABCB1 (also called Pgp) and ABCCs (25). With respect to the DMARDs auranofin and aurothiomalate, studies by Glennas et al (26) revealed that upregulated expression of the metal-binding protein
chapter 2

Clinical laboratory studies have identified several molecular mechanisms that could contribute to a reduction in efficacy of various DMARDs. To test whether these mechanisms, alone or in combination, are actually operative in a clinical setting will require further evaluation of specific markers for DMARD resistance in prospective clinical studies. One additional point of consideration is that the onset of resistance to a specific DMARD in immune effector cells might be accompanied by other genotypic and phenotypic changes that collaboratively influence the efficacy of other DMARDs, the release of proinflammatory cytokines, or both. Findings from in vitro studies showed that acquired resistance to chloroquine in T cells is accompanied by a markedly reduced release of TNF and interleukin-8, along with a marked loss of sensitivity for glucocorticoids (25). Conversely, acquired resistance to sulfasalazine in the same T cells is accompanied by a markedly increased sensitivity for glucocorticoids (23). Hence, upon chronic exposure, DMARDs might exert both beneficial and adverse pleiotropic effects. Such effects might have been unrecognized when the anti-inflammatory effects of specific DMARDs were tested over a short time frame in a laboratory setting.

DMARD resistance or inefficacy in RA

Observational studies and meta-analyses of treatment efficacy, average treatment duration and adverse effects for RA patients consistently demonstrate variable responses for individual DMARDs and DMARD combinations (7, 8, 10-12, 32, 33). For example, in a 10-year follow-up study (1985-1994), that included 2,296 RA patients, 25% of patients had to discontinue DMARD treatment owing to inefficacy and 20% as a result of adverse effects (7, 33). The percentage of RA patients who had to discontinue DMARD monotherapy for inefficacy was highest for auranofin (37%), sulfasalazine (36%), and the antimalarials chloroquine and hydroxychloroquine (25%), and lowest for methotrexate (9%) (7). Unfortunately, except for clinical endpoints, the molecular basis for DMARD inefficacy was not further unraveled in these studies. This lack of findings was probably the result of an insufficient number of clinical samples and sample sizes of peripheral blood or synovial tissue, which made biochemical and molecular analyses of potential DMARD resistance-related parameters impossible.

Mechanisms of resistance to leflunomide in B cells revealed a marked upregulation of its target enzyme dihydro-orotate dehydrogenase (29), which therefore requires higher drug doses to be effectively inhibited. Finally, two reviews have summarized molecular mechanisms of resistance to glucocorticoids in RA. These mechanisms might involve reduced levels of the α isoform of the glucocorticoid receptor and an increased ratio of the β isoform to the α isoform, (the β isoform lacks the high affinity glucocorticoid-binding capacity and acts as a dominant-negative regulator of the α isoform), impaired crosstalk with transcription factors, or increased drug efflux via the multidrug resistance protein ABCB1 (30, 31).

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Multidrug resistance and RA

Over the past decade, attempts have been made to associate or correlate loss of DMARD efficacy with one or more of the resistance parameters that have been identified in laboratory studies. In particular, studies have assessed the potential role of multidrug resistance proteins and aimed to establish parameters that could predict the response to methotrexate. Early studies by Salmon and Dalton (34) suggested that the drug efflux transporter ABCB1 might mediate the cellular release of TNF, but this hypothesis could not be confirmed experimentally. Mailllefer et al. (35) observed ABCB1 expression on peripheral blood cells in higher percentages of prednisone-treated RA patients (10.7%) than controls (3.3%). This finding might be consistent with the notion that prednisone is a substrate for ABCB1. In a small group of eight RA patients, Llorente et al. (36) noted a higher percentage of peripheral blood lymphocytes with high ABCB1 activity among patients who did not respond to therapy than among those who did (34.4% vs. 3.3%).

Finally, Yudoh et al. (37) noted higher percentages of T-helper-1 cells expressing ABCB1 in a group of RA patients who did not respond to therapy with bucillamine and sulfasalazine; 9.6% at baseline and 15.4% after 3 months of therapy versus 3.3% in responding patients at both baseline and 3 months of therapy. Commenting on this study, Rahman et al. (38) suggested that increased ABCB1 expression might also contribute to resistance to methotrexate and referred to the findings of Norris et al. (39). In the study by Norris et al., however, human lymphoblastic cell lines were used that already had an extremely high level of resistance to methotrexate (four orders of magnitude higher than their methotrexate-sensitive counterparts) as measured from the defective cellular uptake via the reduced folate carrier. In addition to this finding, ABCB1 contributed only to a minor extent to the methotrexate resistant phenotype. Given the general substrate preference of ABCB1 (neutral or cationic compounds) a general role in transporting an anionic drug such as methotrexate seems unlikely. Studies of ABCB1 in reconstituted vesicles produced similar findings. The involvement of other drug transporters in methotrexate efflux has been demonstrated (Figure 1) and could contribute to resistance to this drug. Preliminary data by Oerlemans et al. (40) show that the transporters ABCCs, ABCG5 and ABCG2 could be identified on monocyte-derived macrophages of RA patients. Detailed studies on the presence of drug efflux transporters in RA synovial tissue are lacking. Preliminary data from our laboratory revealed the presence of ABCG2 on macrophages in the synovial sublining of a small group of RA patients (41). Studies are required to further elaborate on or confirm possible correlations with DMARD efficacy.

Multidrug resistance and inflammation

As well as their possible role in DMARD resistance, drug efflux transporters have other physiologic functions. The substrates of ABCB1, ABCC1–ABCc5 and ABCG2, for example, include leukotrienes, prostaglandins, cyclic AMP, cyclic GMP and steroids, through which inflammatory responses can be modulated (5). Furthermore, evidence increasingly shows that the transcriptional regulation of these transporters involves cytokine mediation (42). Hence, basal expression of individual drug efflux transporters on immune effector cells of RA patients could be a reflection of disease activity at the time therapy is started. Hider et al. (43) showed that ABCC1 expression on peripheral blood cells of RA patients declined compared with baseline after 6 months of therapy with methotrexate. Consistent with the notion of cytokine mediation, expression of this drug efflux transporter might be upregulated again when the efficacy of methotrexate treatment is reduced over time.

Methotrexate efficacy or resistance in RA

The discovery of different mechanisms of resistance to methotrexate from in vitro studies has led to the identification of common mechanims for sensitivity or resistance in RA patients. Stranzl et al. (44) observed in a cross-sectional study that messenger RNA levels of folylpolyglutamate synthetase were negatively correlated with response to methotrexate therapy. In 55% of 141 RA patients, no folylpolyglutamate synthetase mRNA was measurable; the response rate to methotrexate therapy was significantly higher than among patients with folylpolyglutamate synthetase mRNA expression (57% vs. 33%; $P = 0.009$). A positive correlation would have been expected between expression and response, as proficient folylpolyglutamate synthetase activity should allow better intracellular retention of methotrexate; however, as cellular activation or proliferation can upregulate folylpolyglutamate synthetase mRNA levels and activity, the reduced response rates might reflect augmented disease activity.

In a study of 163 RA patients, Wolf et al. (45) analyzed parameters of cellular uptake transporters (RFC mRNA) and efflux transporters (functional activity of ABCC1–4). Patients with a high RFC status and low ABCC activity might be expected to respond well to methotrexate treatment. Irrespective of RFC status, however, more patients with ABCC activity on peripheral blood cells had better response rates, according to the European League Against Rheumatism criteria, than those with a low ABCC activity (53–60% versus 29%, respectively). Whether low levels of RFC mRNA were actually translated into negligible RFC protein levels was not established, and it is unclear how defective methotrexate uptake could confer a good response. These parameters should be further evaluated in prospective studies.

Several groups have investigated the contribution of common polymorphisms of genes coding for folate or methotrexate transporters, such as RFC, and key enzymes in folate or purine metabolism (Figure 1) (46–51). They have been searching for associations with toxic effects and response to methotrexate in RA patients. Dervieux et al. (47) reported that RA patients with homozygous variant genotypes, such as 80AA in the RFC region, 347GG in the aminoimidazolecarboxamide ribonucleotide transformylase region and two 28 bp tandem repeats in the thymidylate synthase enhancer region (*2/*2), had a 3.7-
fold increased likelihood of a good response after at least 3 months’ therapy. In addition, patients with red blood cell concentrations of methotrexate long-chain polyglutamates of higher than 60 nmol/l were 14-fold more likely to have a good response. In another cross-sectional study, Dervieux et al. (48) showed that RA patients harboring the 401TT genotype in the promoter region of folylpolyglutamate hydrolase, a lysosomal folate/methotrexate polyglutamate breakdown enzyme, were 4.8-fold more likely to have long-chain methotrexate polyglutamates in their red blood cells than patients with the CC or CT genotypes. A composite of cumulative homozygous genotypes associated with toxic side effects included methylenetetrahydrofolate reductase 677TT, thymidylate synthase enhancer region *2/*2, aminimidazolecarboxamide ribonucleotide transformylase 347GG and serine hydroxymethyltransferase 1420CC (49). For methotrexate treatment in early RA, Wessels et al. (50) observed that patients harboring the methylenetetrahydrofolate reductase 1298AA and 677CC genotypes showed a greater clinical improvement than those with the other genotype combinations, and patients carrying the methylenetetrahydrofolate reductase 1298C allele were more prone to methotrexate-related toxic effects. For all these polymorphic variants, the extent to which they translate into functionally altered proteins in terms of kinetics, stability or other properties needs to be established. Whether these features are causative in relation to methotrexate inefficacy or toxic effects also requires clarification. Ultimately, these polymorphic variants should prove their value in predicting responsiveness and toxicity in prospective studies.

Strategies to overcome DMARD resistance

Since resistance to anticancer drugs has various causes, several strategies have been proposed to overcome resistance (4), some of which might also apply to DMARD resistance in RA. In a clinical setting, when loss of efficacy for a particular DMARD is observed, making controlled, incremental increases in dose is a logical option until toxic effects manifest or, if drugs are used in combination, drug interactions become apparent (52, 53). In general, alternating drugs, either in a step-up approach (therapy is started as monotherapy and drug(s) are added in cases of insufficient response) or a step-down approach (therapy is started with multiple drugs but one is stopped at a time), is thought to slow the onset of drug resistance seen with monotherapy. In cases where combination DMARD therapy has proved unsuccessful, addition of a third DMARD has proved to be highly efficacious in certain combinations (e.g. for methotrexate plus sulfasalazine plus hydroxychloroquine (54) or methotrexate plus sulfasalazine plus prednisolone (31). Whether the superiority of these triple therapy schedules is explained by minimizing the contribution of resistance-related parameters is not fully clear (25).

When a specific mechanism of resistance has been identified for a type of drug, including DMARDs, strategies to overcome resistance might include engagement with, bypassing, or taking advantage of the mode of resistance. Engagement, for example in the case of drug-efflux transporters, could involve the use of inhibitors specific to these proteins. In cancer chemotherapy, however, this approach had, for various reasons, limited success (4). The lower drug doses given to RA patients might make an engagement strategy more efficacious, but the likelihood of this outcome has not yet been established. Blocking of drug efflux transporters could raise plasma and intracellular drug concentrations. Zaher et al. (15) showed that ABCG2-knockout mice had markedly altered pharmacokinetics for sulfasalazine; plasma levels were more than 100-fold higher than in control mice. These same raised plasma levels of sulfasalazine could be achieved in normal mice by co-administering gefitinib, an ABCG2 inhibitor. This example suggests that when DMARDs are substrates for drug efflux transporters, therapy with transporter blockers could modulate plasma levels, reducing the risk of adverse effects.

Box 2 General mechanisms of cellular drug resistance.

**Impaired drug delivery to cells**
Pharmacokinetic resistance or bioavailability (impaired intestinal absorption, raised urinary secretion, extensive protein binding)

**Impaired cellular uptake**
Quantitative or qualitative defects of specific drug-uptake transporters
Increased cellular efflux

**Impaired drug activation or increased intracellular drug inactivation**
Defective activation (phosphorylation, polyglutamylation) of drugs
Raised metabolism to non-active drugs
Sequestration in intracellular compartments (e.g. lysosomes)

**Alterations in drug target levels**
Increase in drug target (enzyme) levels (e.g. by transcriptional activation or gene amplification)
Altered target (modified drug-binding properties)

**Altered events downstream of target**
Improved repair of drug-induced damage
Defective drug-induced apoptosis
Improved bypassing of drug-induced effect
Accumulated knowledge on resistance mechanisms for methotrexate has led to the rational design of second-generation folate antagonists. Use of these drugs might overcome the issue of methotrexate resistance, because they are more efficiently taken up by RFC and are better substrates for polyglutamate synthetase, which prevents efflux by drug exporters (55). Preliminary results in ex vivo experiments indicate that some of these second-generation folate antagonists are potent inhibitors of TNF production in activated T cells of RA patients. Some second-generation folate antagonists might also target folate receptors (see Figure 1). By following this route of entry, they could bypass efflux routes encountered after cell entry via the RFC route. The current status of this research is not, however, advanced enough to predict whether any of the second-generation therapies will be able to replace methotrexate.

Conclusions

Given the long duration of drug treatment that most RA patients face, maintaining the efficacy of DMARDs and biological agents for as long as possible is a challenge (3, 56). When efficacy is insufficient or lost, rheumatologists switch to alternative regimens, the long-lasting effects of which might be unpredictable. Interdisciplinary research into drug resistance has provided extensive knowledge of mechanisms associated with resistance and toxic effects. Some of the findings might apply to DMARD treatment in RA. Laboratory studies have revealed various factors that can contribute to diminished activity of specific DMARDs (Box 2), some of which are currently being evaluated in clinical practice. Of particular interest are studies aiming to predict response to methotrexate as it is used as the main drug in RA treatment. The most useful studies will assess response in relation to specific resistance-related parameters during the course of therapy that may precede clinical signs of loss of efficacy. This approach will allow the extrapolation of data into predictive criteria. Thus, pharmacogenetically guided prospective clinical studies in early RA are warranted.

The investigations of ways in which to make more efficient use of the currently available DMARDs would also be useful. For example, modulation of pharmacokinetics by blocking intestinal drug-efflux transporters and ways in which second-generation of drugs might overcome resistance deserve further attention.

Finally, beyond the level of immune effector cells in peripheral blood, more insight into factors of DMARD resistance related to inflamed synovial tissue would be helpful to assist therapeutic interventions. Any extension of DMARD efficacy over time in monotherapy or in combination with other DMARDs or biological agents will be beneficial from therapeutic and socioeconomic perspectives.
References

15. Zacher, H., Khan, A.A., Palandra, J., Brayman, T.G., Yu, L. and Ware, J.A. Breast Cancer Resistance Protein (Bcrp/Abcg2) is a major determinant of sulfasalazine absorption and elimination in mice, Molecular Pharmaceutics, 3: 55-61, 2006.


References

Carli,P.M. and Tavernier,C. Expression of the multidrug resistance glycoprotein 170 in the peripheral blood lymphocytes of rheumatoid arthritis patients. The percentage of lymphocytes expressing glycoprotein 170 is increased in patients treated with prednisolone, Br.J.Rheumatol., 35: 430-435, 1996.


Development of sulfasalazine resistance in human T cells induces expression of the multidrug resistance transporter ABCG2 (BCRP) and augmented production of TNFα

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Abstract

Objective: To determine whether overexpression of cell membrane associated drug efflux pumps belonging to the family of ATP binding cassette (ABC) proteins contributes to a diminished efficacy of sulfasalazine (SSZ) after prolonged cellular exposure to this disease modifying anti-rheumatic drug.

Methods: A T-cell model system of human CEM (T) cells was used to expose cells in vitro to increasing concentrations of SSZ for a period up to six months. Cells were then characterized for the expression of drug efflux pumps: P-glycoprotein (Pgp, ABCB1), multidrug resistance protein 1 (MRP1, ABCC1) and Breast Cancer Resistance Protein (BCRP, ABCG2).

Results: Prolonged exposure of CEM (T) cells to SSZ provoked resistance to SSZ as manifested by a 6.4-fold diminished antiproliferative effect of SSZ compared with parental CEM (T) cells. CEM cells resistant to SSZ (CEM/SSZ) showed a marked induction of ABCG2/BCRP. Pgp expression was not detectable, while MRP1 expression was even down regulated. A functional role of ABCG2/BCRP in SSZ resistance was demonstrated by a 60% reversal of SSZ resistance by the ABCG2/BCRP blocker Ko143. Release of the proinflammatory cytokine tumour necrosis factor α (TNFα) was threefold higher in CEM/SSZ cells than in CEM cells. Moreover, twofold higher concentrations of SSZ were required to inhibit TNFα production from CEM/SSZ cells compared with CEM cells.

Conclusion: Collectively, ABCG2 induction, augmented TNFα release and less efficient inhibition of TNFα production by SSZ may contribute to diminished efficacy after prolonged exposure to SSZ. These results warrant further clinical studies to verify whether drug efflux pumps, originally identified for their roles in cytostatic drug resistance, can also be induced by SSZ or other disease-modifying anti-rheumatic drugs.

Introduction

Sulfasalazine (SSZ) is a widely applied disease modifying anti-rheumatic drug (DMARD), either as a single agent or in combination with other DMARDs (1-5). The anti-inflammatory properties of SSZ have been attributed to diminished production of pro-inflammatory cytokines such as Tumor Necrosis Factor α (TNFα) through multiple mechanisms, including (a) inhibition of the activation of nuclear factor kappa B (NFkappaB) by inhibition of Inhibitor kappa B Kinase (IKK) (6, 7), (b) through inhibition of the purine biosynthesis de novo enzyme 5-aminoimidazole-4-carboxamideribonucleotide (AICAR) transformylase (8, 9) and (c) by induction of apoptosis in lymphocytes and macrophages (10, 11).

In clinical practice the median duration of use of SSZ is 1-2 years (12). A meta-analysis of DMARD treatment termination rates based on 159-studies showed that after initial activity, most withdrawals of SSZ resulted from the lack of efficacy rather than toxicity (13). The underlying mechanism(s) for this lack of efficacy has not been established. Given the long term DMARD treatment RA patients receive, we rationalized that the onset of acquired drug resistance to SSZ, analogous to resistance to anticancer drugs or anti-infectious drugs (14-16), might contribute to its lack of efficacy. From an anti-inflammatory perspective, drug resistance may refer to a diminished ability of DMARDs to inhibit secretion of pro-inflammatory cytokines by inflammatory cells. Mechanistically, this can be provoked by either augmented basal levels of cytokine secretion or, indirectly, through attenuated anti proliferative/apoptotic effects of DMARDs. Diminished anti proliferative effects mediated by overexpression of specific energy (ATP) dependent drug efflux pumps are a common and established mechanism of resistance to anticancer drugs. These drug efflux pumps belong to a superfamily of ATP-Binding Cassette (ABC) transporters (17-19). ABC transporters with an established role in drug resistance include: P-glycoprotein (Pgp(ABCB1) (20), Multidrug Resistance associated Proteins 1-5 (MRP1-5/ABCC1-5) (21, 22) and Breast Cancer Resistance Protein (BCRP/ABCG2) (15, 23). Currently, very little is known about SSZ as a potential substrate for one or more of the different MDR pumps or other transporters (24). On the basis of its chemical structure as organic anion, SSZ might belong to a class of compounds transported by the drug efflux pump MRP1 that has a preferred substrate affinity of anionic (glutathione-conjugated) compounds (21, 22).

To gain insight from a rheumatological perspective into possible mechanism(s) of resistance to the anti proliferative/anti-inflammatory effects of SSZ, we provoked acquired resistance to SSZ in an in vitro model system for human T cells (CEM) by stepwise exposure of CEM (T) cells to gradually increasing concentrations of SSZ.

In this study we observed that SSZ resistance in CEM (T) cells was conferred by overexpression of a drug efflux pump, the multidrug resistance transporter BCRP/ABCG2. Consistent with reduced drug uptake, inhibition of nuclear NFκB activity in SSZ-resistant cells, as reflected by TNFα production, required at least two-fold higher SSZ concentrations as compared with CEM (T) cells.

Materials and methods

Materials

Sulfasalazine, 5′-aminosalicylic acid, sulfapyridine, phorbol 12-myristate 13-acetate (PMA), ionomycin, verapamil and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma Chem Co. (St. Louis, MO, U.S.A.). Protease Inhibitor Cocktail (PIC) and Triton X-100 were from Boehringer Mannheim (Ingelheim, Germany). The ABCG2 inhibitor Ko143 (25) was kindly provided by Dr A. Schinkel (Netherlands Cancer Institute, Amsterdam, the Netherlands). The MRP1 blocker MK571 was provided by Merck Frosst, Quebec, Canada.
Sulfasalazine resistance in T-cells is mediated by BCRP

Western blotting
For analysis of expression of Pgp, MRP1 and BCRP/ABCG2 cells were harvested in the mid-log phase of growth and washed three times with ice cold Heps buffered saline, pH 7.4. Total cell lysates of 10^6 cells were prepared by suspending them in 500 µl of lysis buffer containing 50 mM Tris-HCl (pH 7.6), 5 mM DTT, 20 µl PIC (1 tablet/ml H2O), 20% glycerol and 0.5% NP-40. The suspension was sonicated (MSE sonicator, amplitude 6, for 3×5 seconds with 30 second time intervals at 4°C) and centrifuged in an Eppendorf microcentrifuge (10 minutes, 14000 rpm, 4°C). Protein content of the supernatant was determined by Biorad protein assay. Fifty microgram of total cell lysates was fractionated on a 7.5% polyacrylamide gel and transferred onto a nitrocellulose membrane. The nitrocellulose membranes were pre-incubated overnight at 4°C in blocking buffer (5% Biorad Blocker in TBS-T (10 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 0.1% Tween-20) to prevent aspecific antibody binding. After blocking, the membranes were incubated for 1 hour at room temperature with the primary antibodies for Pgp (JSB1, 1:500), MRP1 (MRP1, 1:500) or ABCG2/BCRP (BXP21, 1:400) as described by Scheffer et al. (32, 33). As control for loading, β-actin was used (MAB1501R, 1:3000, Chemicon International, Ca, U.S.A.). After three washing steps with TBS-T, the membrane was incubated for one hour with horseradish peroxidase labelled anti-rat/mouse (1:2000, Dako) as secondary antibody. Detection of the antibody binding was measured by enhanced chemoluminescence (ECL) according to the manufacturers’ instructions (Amersham International, Buckinghamshire, UK). Protein levels were determined by densitometric scanning (GelDoc and Molecular Analyst, Biorad Laboratories) of the X-ray films (Hyperfilm ECL, Amersham International, Buckinghamshire, UK).

For analysis of the presence of the p65 subunit of NFκB in the nucleus, 30 µg of nuclear protein was fractionated on a 7.5% polyacrylamide gel, transferred to nitrocellulose membranes, and probed with NFκBp65 antibody (Santa Cruz, sc8008, 1:2000) before ECL detection as described above.

TNFα ELISA
TNFα production was analyzed after stimulation of CEM (T) and CEM/SSZ cells (3×10^4/m) for 24 hours with PMA (10 ng/ml) (1 µM). After this period, supernatants were collected by centrifugation (5 minutes; 3000 rpm) and analyzed for TNFα by ELISA (Central Laboratory of Blood Transfusion, Amsterdam) according to the manufacturer’s instruction. The detection limit of the TNFα ELISA was 5 pg/ml.

Statistical analysis
Statistical significance of differences was analyzed by Student’s t-test. A p-value < 0.05 was considered to be statistically different.
Results

Onset of SSZ resistance in CEM (T) cells
To investigate whether CEM (T) cells developed over time less sensitivity to SSZ, CEM (T) cells were continuously exposed in vitro to stepwise increasing concentrations of SSZ. After a period of 4-6 months’ culture, CEM (T) cells could be maintained in SSZ concentrations that exceeded 3.5-6 fold the initial SSZ selection concentration of 0.4 mM SSZ (Figure 1A). Cells selected at an intermediate level of resistance (1.5 mM SSZ) and a final level of 2.5 mM SSZ were used for further characterization. Figure 1B shows the antiproliferative effect of SSZ against CEM (T) cells, CEM/SSZ1.5 and CEM/SSZ2.5. Concentrations of SSZ required for 50% growth inhibition were 4.3-fold (p<0.001) and 6.4-fold (p<0.001) higher for CEM/SSZ1.5 (IC50: 1.72 ±0.17 mM) and CEM/SSZ2.5 cells (IC50: 2.55 ±0.21 mM), respectively, as compared to CEM (T) cells (IC50: 0.40 ± 0.03 mM).

Because in vivo SSZ can be catabolized into two components, that is 5'-aminosalicylic acid (5'-ASA) and sulfapyridine (SP) (34), we analyzed whether one or both of these components might contribute to the SSZ resistant phenotype. It was noted that neither 5'-ASA nor SP, either separate or combined, displayed an antiproliferative effect at equimolar concentrations of intact SSZ (results not shown).

These results indicate that human CEM (T) cells can develop resistance to SSZ upon long term exposure to this DMARD. Resistance to SSZ does not involve its in vivo catabolites 5'-ASA and SP.

Expression of multidrug resistance transporters in CEM (T) and CEM/SSZ cells
Because SSZ is an organic anion that might be a potential substrate for ATP-driven drug efflux pumps, we investigated whether SSZ resistance in CEM (T) cells was mediated by upregulated expression of one or more of the major multidrug resistance transporters Pgp, MRP1 and ABCG2/BCRP.

Figure 1: (A) Onset of SSZ resistance in human CEM (T) cells. SSZ resistance was provoked by culturing CEM (T) cells in stepwise increasing concentrations SSZ over time. (B) SSZ sensitivity for CEM (T) cells, CEM/SSZ1.5 and CEM/SSZ2.5 cells. Antiproliferative effects were assessed after 72 hours exposure to SSZ. Results are the mean of 3-5 separate experiments (SD <20%).

Figure 2: (A) Expression of MDR transporters Pgp, MRP1 and ABCG2/BCRP in CEM (T) and CEM/SSZ cells. Western blots were performed using total cell lysates of CEM (T) and CEM/SSZ1.5 cells. Cell lysates of CEM/ VBL106 (64), 2008/MRP1 (32) and MCF7/MR (40) served as positive control for Pgp, MRP1 and ABCG2/BCRP, respectively. For CEM (T) and CEM/SSZ1.5 cells, 50 µg of cell lysate was applied on the SDS-PAGE gel and for the controls 10 µg protein. Pgp was detected by the monoclonal antibody JSB1, MRP1 was detected by the monoclonal antibody MRPr1 and ABCG2/BCRP was detected by the monoclonal antibody BXP21 (32, 33). (B) Reversal of SSZ resistance in CEM/SSZ1.5 cells by ABCG2/BCRP blocker Ko143, but not by Pgp blocker Verapamil or MRP1 blocker MK571. CEM/SSZ1.5 cells were incubated in medium containing non-toxic concentrations of the ABCG2/BCRP blocker Ko143 (0.5 µM), MRP1 blocker MK571 (20 µM) or Pgp blocker verapamil (10 µM) and evaluated for SSZ sensitivity compared to parental CEM (T) cells as described in Figure 1B. Results are presented as the mean of 3 experiments (SD <20%).
Reversal of ABCG2/BCRP mediated SSZ resistance in CEM/SSZ cells

To establish whether ABCG2/BCRP is functionally active in conferring resistance to SSZ, ABCG2/BCRP was blocked by a specific inhibitor, Ko143 (25), to verify reversal of resistance. Blocking of ABCG2/BCRP reverted SSZ resistance by 50-60%, but not completely to CEM (T) cell sensitivity (Figure 2B). Blockers of MRP1 (MK571) or Pgp (verapamil) did not reverse SSZ resistance. These results demonstrate that ABCG2/BCRP plays a functional role in conferring resistance to SSZ.

TNFα production and nuclear NFκB p65 expression in CEM (T) and CEM/SSZ cells

SSZ is a potent inhibitor of the production of the pro-inflammatory cytokine TNFα by inhibiting the nuclear activation of the transcription factor NFκB (6, 36). To assess whether SSZ resistance affected production of TNFα, secretion of this cytokine was determined for CEM (T) cells and CEM/SSZ cells after 24 hours’ PMA/ionomycin stimulation in the absence or presence of a concentration range of SSZ (Figure 3A). The basal level of TNFα production by CEM/SSZ1.5 and CEM/SSZ2.5 cells was markedly increased: 2.0-fold (p<0.001) and 2.9-fold (p<0.001), respectively compared with CEM (T) cells. Beyond this, twofold higher concentrations of SSZ were required to inhibit TNFα production by 50% in CEM/SSZ1.5 cells (IC50: 0.95 mM) and CEM/SSZ2.5 cells (IC50: 1.1 mM) compared with CEM (T) cells (IC50: 0.55 mM). Consequently, the area under the curve of TNFα production measured over the range of SSZ concentrations (Figure 3A) was 2.5-fold and 4.6-fold greater for CEM/SSZ1.5 and CEM/SSZ2.5 cells, respectively, compared with CEM (T) cells.

Because SSZ inhibits TNFα production by preventing the cytosolic to nuclear translocation of the nuclear transcription factor NFκB (6, 36), we analyzed whether increased nuclear NFκB levels themselves and/or an impaired potency of SSZ to reduce nuclear NFκB expression could explain the observed differences in absolute TNFα production and the diminished inhibitory effect of SSZ on TNFα production for CEM/SSZ2.5 cells compared to CEM (T) cells. At the time of TNFα analysis, after 24 hours’ PMA/ionomycin stimulation, expression of the nuclear NFκB p65 subunit was determined in CEM (T) cells and CEM/SSZ1.5 cells in the absence or presence of SSZ. Unstimulated CEM (T) cells and CEM/SSZ1.5 cells did not appear to differ in their basal level of nuclear NFκB p65 expression (Figure 3B, lanes A vs. E). SSZ exposure to CEM (T) cells resulted in a markedly decreased nuclear NFκB p65 expression, both in unstimulated cells (lanes A vs. B) or after PMA/ionomycin exposure (lanes C vs. D). In contrast, SSZ exposure had little effect on nuclear NFκB p65 expression in either unstimulated CEM/SSZ1.5 cells (lanes E vs. F) or PMA/ionomycin stimulated CEM/SSZ1.5 cells (lanes G vs. H). Similar results were observed for CEM/SSZ2.5 cells (results not shown). Together, these results suggest that, owing to ABCG2/BCRP mediated efflux, SSZ is less effective in inhibiting nuclear NFκB p65 activation in CEM/SSZ cells than in CEM (T) cells.

Discussion

As far as we know, this study is the first report to show that long term exposure of human T cells to the DMARD sulfasalazine can lead to development of resistance by induction of a drug efflux pump, the MDR transporter BCRP/ABCG2. Reduced intracellular drug levels not only led to impaired antiproliferative effects, but also to a less effective inhibition of nuclear NFκB activation by SSZ in the resistant cells. Thus, SSZ-resistant cells required more SSZ to inhibit TNFα secretion.

Development of drug resistance is a common cause of treatment failure for anticancer drugs or antimalarial drugs (16, 37). Drug resistance to DMARDs has received little attention in rheumatology even though loss of efficacy for several DMARDs, including SSZ, is well established upon long term treatment (12, 13). In this study we mimicked the onset of SSZ
resistance by exposing human CEM (T) cells in vitro to stepwise increasing concentrations of SSZ. Interestingly, SSZ resistance was associated with an upregulated expression of a recently discovered MDR transporter, ABCG2/BCRP, originally discovered in breast cancer cells selected for resistance (loss of antiproliferative effects) to the anticancer agents mitoxantrone and doxorubicin (38-41). Tissue distribution studies demonstrated that ABCG2/BCRP expression is not restricted to breast cancer tissues. ABCG2/BCRP is expressed in a limited number of normal tissues (liver, colon epithelium, kidney, mammary gland and blood vessels) (33). Expression of ABCG2/BCRP in synovial cells and tissues has not been explored. Very recently, ABCG2/BCRP expression was described in blood cells, notably CD34+ hematopoietic progenitor cells (42) and in acute myeloid leukemia cells (43, 44). The physiological function of BCRP/ABCG2 is as yet unknown, although recent studies with BCRP+/- knock out mice indicated a crucial role in the cellular extrusion and detoxification of dietary phototoxins (45).

In our study, evidence for a functionally active ABCG2/BCRP drug efflux pump in CEM/SSZ cells was demonstrated by the fact that Ko143 (25), a blocker of BCRP/ABCG2, could reverse resistance to SSZ. In addition, CEM/SSZ displayed cross resistance and reversal by Ko143 to mitoxantrone (39), a prototypical substrate for ABCG2. The observation that the antirheumatic drug SSZ, like distinct anticancer drugs, can induce upregulation of ABCG2/BCRP expression may imply that these drugs share a common target that triggers a response of ABCG2/BCRP upregulation. Unlike the ABC transporter Pgp (46), there is no evidence for a direct transcriptional regulation of ABCG2 via an NFκB binding site on the ABCG2/BCRP promoter (47). However, most drugs involved in ABCG2 upregulation, mitoxantrone (48), doxorubicin (49), daunorubicin (50), CPT-11/ SN38 (49, 51) and flavopiridol (52) all have been documented to target the NFκB signalling pathway by activating NFκB and stimulating IκBα degradation. Together, these results suggest that sustained (danger) signals mediated through the NFκB signalling pathway may provoke upregulation of drug efflux pumps like ABCG2/BCRP.

Along with the loss of antiproliferative effects of SSZ against CEM/SSZ cells (Figure 1B), a diminished anti-inflammatory effect was also noted as markedly higher concentrations of SSZ were required to inhibit TNFα production in CEM/SSZ cells than in CEM (T) cells (Figure 3A). Mechanistically this might be consistent with the fact that after PMA/ionomycin stimulation the activation and nuclear translocation of NFκB in CEM/SSZ cells is less effectively inhibited by SSZ (6, 7, 36) because of drug efflux via ABCG2/BCRP. Consequently, TNFα production is less inhibited than in CEM (T) cells. Besides differences in the potency of SSZ to inhibit TNFα production, it is important to recognize that after PMA/ionomycin stimulation, basal levels of TNFα production were 2-3 fold higher in CEM/SSZ cells than in CEM (T) cells. This may indicate that induction of SSZ resistance is associated with alterations in the NFκB signalling pathway that controls the transcription of anti-apoptotic and pro-inflammatory cytokine/chemokine genes (53-55).

In contrast with the up regulated expression of ABCG2/BCRP in CEM/SSZ cells, we noted a down regulated expression of another drug efflux pump MRP1 (Figure 2A). MRP1 has an established role in exporting hydrophobic and hydrophilic drugs, the latter often as glutathione conjugates (21, 22). Immunologically, MRP1 has an important function in dendritic cells by exporting the cysteinyl leukotriene LTC4, which mediates the signalling for chemokine CCL19 chemotaxis and migration of dendritic cells to the lymph nodes (56). Because SSZ has been reported to inhibit LTC synthetase (57), this may suggest that a diminished LTC production coincides with a lowered expression of the LTC4 transporter MRP1.

Collectively, this in vitro study illustrates that long term exposure of human T cells to SSZ can result in up regulated expression of the MDR pump ABCG2/BCRP and increased production of TNFα, a key component of progressive disease in RA (58, 59). In this respect, we also noted up regulation of ABCG2/BCRP expression in human monocyctic THP1 and U937 cells after in vitro exposure to SSZ (Oerlemans R et al, unpublished observation) suggesting that this phenomenon is also of relevance for other inflammatory-related cell types. Given the accumulating evidence for involvement of MDR pumps in conferring resistance to at least three DMARDs; SSZ (ABCG2/BCRP) (this study), chloroquine (MRP1) (60), and MTX (MRP1, ABCG2/BCRP) (61, 62) further research is warranted to examine whether MDR pumps also have a role in the reduced (preclinical and clinical) efficacy of other DMARDs after long term treatment of patients with RA. Thus studies evaluating inhibitors of ABCG2/BCRP (23, 25, 63) as possible chemosensitizers for SSZ activity deserve further attention.
References


32 Scheffer, G.L., Kool, M., Heijn, M., de Haas, M., Pijnenborg, A.C., Wijnholds, J., van Velthoort, A., de...
References


Acquired resistance of human T cells to sulfasalazine: stability of the resistant phenotype and sensitivity to non-related DMARDs

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Abstract

Background: A recent study from our laboratory showed that induction of the multidrug resistance related drug efflux pump ABCG2/BCRP contributed to acquired resistance of human T cells to the disease-modifying antirheumatic drug (DMARD) sulfasalazine (SSZ).

Objective: To investigate the duration of SSZ resistance and ABCG2/BCRP expression after withdrawal of SSZ and rechallenging with SSZ, and to assess the impact of SSZ resistance on responsiveness to other DMARDs.

Methods: Human CEM (T) cells with acquired resistance to SSZ (CEM/SSZ) were characterized for (a) SSZ sensitivity and ABCG2/BCRP expression during withdrawal and rechallenge of SSZ, and (b) antiproliferative efficacy of other DMARDs.

Results: ABCG2/BCRP protein expression was stable for at least four weeks when CEM/SSZ cells were grown in the absence of SSZ, but gradually declined, along with SSZ resistance levels, to non-detectable levels after withdrawal of SSZ for six months. Rechallenging with SSZ led to a rapid (<2.5 weeks) resumption of SSZ resistance and ABCG2/BCRP expression as in the original CEM/SSZ cells. CEM/SSZ cells displayed diminished sensitivity to the DMARDs leflunomide (5.1-fold) and methotrexate (1.8-fold), were moderately more sensitive (1.6-2.0 fold) to cyclosporin A and chloroquine, and markedly more sensitive (13-fold) to the glucocorticoid dexamethasone as compared to parental CEM cells.

Conclusion: The drug efflux pump ABCG2/BCRP has a major role in conferring resistance to SSZ. The collateral sensitivity of SSZ resistant cells for some other (non-related) DMARDs may provide a further rationale for sequential mono- or combination therapies with distinct DMARDs upon decreased efficacy of SSZ.

Introduction

Acquired drug resistance is recognized as a common cause of treatment failure for patients with cancer after sustained treatment with cytostatic drugs (1, 2). An important mechanism of acquired cellular drug resistance to anticancer drugs is the overexpression of drug efflux pumps belonging to the superfamily of adenosine triphosphate (ATP)-dependent binding cassette (ABC) transporters (2-6). ABC transporters with an established role in drug efflux and drug resistance include: (a) P-glycoprotein (Pgp, ABCB1), which confers resistance preferentially to lipophilic drugs (7); (b) members of the multidrug resistance associated protein subfamily (MRP1-6, ABCC1-6), which confer resistance to various anionic charged/glutathione conjugated drugs such as methotrexate (MTX) (8), thiopurines (9, 10) and steroids (5, 11); and (c) ABCG2, also known as Breast Cancer Resistance Protein (BCRP), a recently identified ABC transporter that confers resistance to amphiphilic drugs, including mitoxantrone and MTX (12, 13).

In clinical rheumatology, loss of drug efficacy is also noted for patients with rheumatoid arthritis (RA) receiving long term treatment with disease modifying anti-rheumatic drugs (DMARDs) (14-17). Whether this loss of DMARD efficacy is related to the onset of acquired resistance to DMARDs, with mechanisms similar to anticancer drug resistance, is not known (18). Meta-analyses of DMARD treatment termination rates showed that the DMARD sulfasalazine (SSZ) (19, 20) had a relatively shorter lasting efficacy than other DMARDs, in particular the ‘golden standard’ MTX (14, 16-18). In general, lack of efficacy may be related to diminished anti-inflammatory and/or antiproliferative effects of DMARDs against pro-inflammatory cytokine releasing cells at inflammatory sites (21).

To gain insight into the possible mechanism(s) of resistance to the antiproliferative/anti-inflammatory effects of the DMARD SSZ, we previously provoked acquired resistance to SSZ in an in vitro model system of human T-lymphocytes by stepwise exposure of CEM (T) cells to gradually increasing concentrations of SSZ (22). This study showed that SSZ resistance in CEM (T) cells was conferred by overexpression of a drug efflux pump - notably the multidrug resistance transporter ABCG2/BCRP. Owing to enhanced drug efflux in SSZ resistant cells (CEM/SSZ), higher concentrations of SSZ were required to inhibit the secretion of the proinflammatory cytokine tumour necrosis factor α (TNFα) (22).

In the present study, we investigated the stability of the resistant phenotype of CEM/SSZ cells after withdrawal of SSZ in order to establish whether SSZ resistance is slowly/rapidly lost over time and reversible after SSZ rechallenge. Moreover, we analyzed to what extent SSZ resistance affected antiproliferative effects of other DMARDs and anti-inflammatory drugs. The results demonstrate that SSZ resistance, along with ABCG2/BCRP expression, is gradually lost over a period of six months after withdrawal of SSZ, but rapidly re-emerged, within 2 weeks upon rechallenge with SSZ. Drug sensitivity was as follows: CEM/SSZ cells displayed lower sensitivity (fivefold) to the DMARD leflunomide, a slightly diminished sensitivity to MTX (twofold), moderately increased sensitivities for cyclosporin A and chloroquine (up to twofold), but a markedly enhanced sensitivity for dexamethasone (13-fold).

Materials and methods

Materials: Sulfasalazine, leflunomide, chloroquine, phenylmethylsulfonyl fluoride (PMSF) and dexamethasone were purchased from Sigma Chem Co. (St. Louis, MO, U.S.A.). Protease Inhibitor Cocktail (PIC) and Triton X-100 were from Boehringer Mannheim (Ingelheim, Germany). MG132 was from Calbiochem (Germany). Methotrexate (MTX) was a generous gift from Pharmachemie Haarlem, the Netherlands. Cyclosporin A was a gift from Novartis (Arnhem, the Netherlands). The ABCG2/BCRP inhibitor Ko143 (23) was kindly
provided by Dr A. Schinkel (Netherlands Cancer Institute, Amsterdam, the Netherlands). RPMI-1640 tissue culture medium and fetal calf serum (FCS) were obtained from Gibco Chemical Co, Grand Island, NY, USA.

Cell culture and selection of SSZ-resistant CEM (T) cells
SSZ resistant human CEM (T) cells were isolated as described previously (22). In short, human CEM (T) cells were cultured at an initial density of 3x10^6 cells/ml in RPMI-1640 medium supplemented with 10% FCS, 2 mM L-glutamine, and 100 µg/ml penicillin and streptomycin in a 5% CO\textsubscript{2} incubator at 37°C (24, 25). Cell cultures were refreshed twice weekly and SSZ was added to the cell culture medium at an initial concentration of 0.4 mM. This concentration of SSZ could be stepwise increased to 1.5 mM SSZ (after 4 months) and 2.5 mM SSZ after a period of 6 months.

Extensive binding of SSZ to plasma proteins (serum albumin) is known to limit its therapeutic effect (26, 27). Based on the presence of at least three classes of SSZ binding sites on serum albumin and their association rate constants (28) it can be calculated that a protein concentration of about 100 µmol/l in 10% fetal calf serum can bind 320 µM SSZ. For the two selected SSZ-resistant CEM cells grown the presence of 1500 and 2500 µM SSZ, this would imply that 21% and 13% of SSZ, respectively, is protein bound.

It should be recognized that CEM cells are of T cell leukaemic origin, proliferating with a doubling time of 26-30 hours. Thus CEM cells may not fully representative for normal T cells or T cells applicable to RA. However, CEM cells may serve as a valuable in vitro model system for RA research as they respond to T cell stimuli, producing pro-inflammatory cytokines like TNF\textsubscript{α} (24). Furthermore, CEM cells have been exploited to disclose resistance mechanisms to MTX that are clinically encountered (24, 29-31).

Antiproliferative effects of DMARDs and other drugs were analyzed by plating 1.25x10\textsuperscript{4} cells/ml of parental CEM (T) cells, CEM/SSZ1.5 and CEM/SSZ2.5 cells (in the absence of SSZ) in individual wells of a 24 well plate containing up to 50 µl of drug solution. Inhibition of cell growth was determined after 72 hours incubation by counting cells with a haemocytometer and cell viability by trypan blue exclusion. The drug concentration required to inhibit cell growth by 50% compared with control growth is defined as IC\textsubscript{50} concentration.

Western blotting
For analysis of expression of ABCG2/BCRP and ABC1/MRP1 and 1:3000 for β-actin (Chemicon International, CA, USA., 1:3000) was used. After three washing steps with TBS-T, membranes were incubated for 1 hour with horseradish peroxidise labelled anti-rat/mouse (Dako: 1:2000 for ABCG2/BCRP and ABC1/MRP1 and 1:3000 for β-actin) as secondary antibody. Detection of the antibody binding was measured by enhanced chemoluminescence (ECL) according to the manufacturers instructions (Amersham International, Buckinghamshire, UK). Protein levels were determined by densitometric scanning (GelDoc and Molecular Analyst, Biorad Laboratories) of the X-ray films (Hyperfilm ECL, Amersham International, Buckinghamshire, UK).

Statistical analysis
Statistical significance of differences was analyzed by analysis of variance, using SPSS 9.0 computer software. A p-value <0.05 was considered to be statistically different.

Results
Onset and stability of SSZ-resistance in human CEM (T) cells
Acquired resistance to SSZ in human CEM (T) cells could be provoked by culturing cells in stepwise increasing concentrations of SSZ (22). Figure 1A (point A) shows the antiproliferative effect of SSZ (IC-50: concentration for 50% growth inhibition) for CEM (T) cells that were grown in the presence of stepwise increasing concentrations of SSZ. After a period of 4 months, CEM (T) could be maintained at an IC-50 concentration of 1.5 mM SSZ (CEM/SSZ1.5) which was about fourfold higher than parental CEM (T) cells. When CEM/SSZ1.5 cells were grown in the presence of 1.5 mM SSZ for another 2 months, the IC-50 value slightly increased to 1.7 mM (Figure 1A, point B). When, in parallel, CEM/SSZ1.5 cells obtained after 4 months were exposed to further increasing concentrations of SSZ for 2 months, eventually CEM/SSZ2.5 cells were obtained that could grow in the presence of 2.5 mM SSZ (not shown).

To establish whether the SSZ-resistance in CEM/SSZ1.5 cells is a transient or stable phenotype, CEM/SSZ1.5 cells (at point B) were further grown in the absence of SSZ and IC-
resistance would be reinduced upon exposure to SSZ, revertant CEM/SSZ1.5 cells were re-
original level of SSZ sensitivity of parental CEM (T) cells (point C). To assess whether SSZ

From this point on CEM/SSZ1.5 were grown in the absence of SSZ to finally yield revertant CEM/SSZ1.5 cells

0.4 mM SSZ that was gradually increased to 1.5 mM over a period of 6 months to yield SSZ resistant CEM/SSZ1.5 cells (point B). From this point on CEM/SSZ1.5 cells were grown in the absence of SSZ to finally yield revertant CEM/SSZ1.5 cells that displayed again parental CEM (T) cell sensitivity to SSZ (point C). When revertant cells were re-exposed to 1.5 mM SSZ for 2.5 weeks (point D), IC-50 values for SSZ only slowly declined. At 70 days after withdrawal of SSZ from the cell culture of CEM/SSZ1.5 for a period of six months (Figure 1A, point C), ABCG2/BCRP and ABCC1/MRP1 expression reverted to the original levels of parental CEM (T) cells. Notably, when revertant CEM/SSZ1.5 cells where re-exposed to 1.5 mM SSZ for 2.5 weeks (Figure 1A, point D), ABCG2/BCRP expression was rapidly up regulated, while ABCG1/MRP1s was again down regulated.

As a control, fluctuations in the expression of ABCG2/BCRP paralleled the growth inhibitory effects (IC-50 values) for the cytostatic drug mitoxantrone (MXR), which is a known substrate for efflux by ABCG2/BCRP (6, 34) (data not shown).

Because Figure 1A showed that changes in IC-50 values for CEM/SSZ1.5 cells were particularly noted within the first 10 weeks after withdrawal of SSZ (Figure 1A, point B→ C), we fine-tuned the analysis of IC-50 values for SSZ and changes in expression of ABCG2/BCRP and ABCC1/MRP1 during this period of 10 weeks. IC-50 values for SSZ dropped by approximately 40-50% after growing of CEM/SSZ1.5 cells in the absence of SSZ for 1 week (Figure 2A). From this time point on, IC-50 values for SSZ only slowly declined. At 70 days after withdrawal of SSZ from CEM/SSZ1.5 cells, resistance to SSZ could still be noted compared with parental CEM (T) cells. Figure 2B shows the expression of ABCG2/BCRP and ABCC1/MRP1 in parental CEM (T) cells (lane A) compared with CEM/SSZ1.5 cells before (lane B) and after withdrawal of SSZ for 3 days, 7 days, 4 weeks and 10 weeks (lanes C-F, respectively). ABCG2/BCRP protein expression in CEM/SSZ1.5 cells appeared to be stable for at least 4 weeks upon withdrawal of SSZ, after which a diminished, but still detectable level of expression could be seen after 10 weeks. ABCG1/MRP1 expression, which was down regulated in CEM/SSZ1.5 cells in the presence of SSZ, was rapidly reinduced within 3-7 days after withdrawal of SSZ, even overshooting the level of ABCG1/MRP1 expression in parental CEM (T) cells after 70 days of SSZ withdrawal from CEM/SSZ1.5 cells. These results indicate that although SSZ resistance in CEM/SSZ is transiently lost over a period of 6 months, rechallenge of revertant cells to SSZ rapidly reinduces resistance to SSZ.

Expression of multidrug resistance (MDR) transporters ABCG2/BCRP and ABCC1/ MRP1 in CEM (T) and CEM/SSZ cells

Initial studies from our laboratory (22) reported differential expression of multidrug resistance proteins upon development of SSZ resistance in CEM/SSZ cells: induction of ABCG2/BCRP and down regulation of ABCC1/MRP1 expression. Figure 2B illustrates the expression of ABCG2/BCRP and ABCC1/MRP1 at the various times A-D in Figure 1A. Parental CEM (T) cells (Figure 1A, point A) exhibited undetectable levels of expression of ABCG2/BCRP protein, but had an appreciable level of ABCC1/MRP1. In contrast, SSZ resistant CEM cells isolated at 1.5 mM SSZ (CEM/SSZ1.5 cells) (Figure 1A, point B) showed a marked induction of ABCG2/BCRP expression and down regulation of ABCC1/MRP1 expression. After withdrawal of SSZ from the cell culture of CEM/SSZ1.5 for a period of six months (Figure 1A, point C), ABCG2/BCRP and ABCC1/MRP1 expression reverted to the original levels of parental CEM (T) cells. Notably, when revertant CEM/SSZ1.5 cells where re-exposed to 1.5 mM SSZ for 2.5 weeks (Figure 1A, point D), ABCG2/BCRP expression was rapidly up regulated, while ABCC1/MRP1s was again down regulated. As a control, fluctuations in the expression of ABCG2/BCRP paralleled the growth inhibitory effects (IC-50 values) for the cytostatic drug mitoxantrone (MXR), which is a known substrate for efflux by ABCG2/BCRP (6, 34) (data not shown).

Figure 1 A

Figure 1 B

ABC2/BCRP
ABCG1/MRP1
β-actin

Figure 1: (A) Time course of sensitivity to sulfasalazine (SSZ) of CEM (T) during development of SSZ resistance, after withdrawal of SSZ from CEM/SSZ1.5 cells and rechallenge of revertant CEM/SSZ1.5 cells with SSZ. Parental CEM (T) cells (point A) were exposed to a starting concentration of 0.4 mM SSZ that was increased gradually to 1.5 mM over a period of 6 months to yield SSZ resistant CEM/SSZ1.5 cells (point B). From this point on CEM/SSZ1.5 cells were grown in the absence of SSZ to finally yield revertant CEM/SSZ1.5 cells that displayed again parental CEM (T) cell sensitivity to SSZ (point C). When revertant cells were re-exposed to 1.5 mM SSZ for 2.5 weeks (point D), CEM/SSZ1.5 resumed their original resistance level as observed at point B. IC-50 values were determined after 72 hours exposure of cells to SSZ. (B) ABCG2/BCRP and ABCC1/MRP1 protein expression is shown for CEM (T) cells and (revertant) CEM/SSZ1.5 cells isolated at the various time points A-D. Cell lysates of 2008/MRP1 (32) and MCF7/MR (43) served as positive controls for ABCC1/MRP1 and ABCG2/BCRP, respectively (E). For CEM (T) and CEM/SSZ1.5 cells, 50 µg of total cell lysate was applied on the SDS-PAGE gel, for the controls 10 µg protein. ABCC1/MRP1 was detected by the monoclonal antibody MRP1 and ABCG2/BCRP was detected by the monoclonal antibody BN21 (32,33). β-Actin was used as a control for equal protein loading.

50 values for the CEM/SSZ1.5 cells were monitored over time. After 1 week in the absence of SSZ, IC-50 values for SSZ dropped by 40-50% to stay at this level for up to 4 weeks. From this time point on, IC-50 values for SSZ gradually declined to reach ultimately the original levels of SSZ sensitivity of parental CEM (T) cells (point C). To assess whether SSZ resistance would be reinduced upon exposure to SSZ, revertant CEM/SSZ1.5 cells were re-exposed to 1.5 mM SSZ. Within only 2.5 weeks' exposure to SSZ (D), revertant CEM/SSZ1.5 cells resumed their original level of SSZ resistance as seen at point B. These results indicate that although SSZ resistance in CEM/SSZ is transiently lost over a period of 6 months, rechallenge of revertant cells to SSZ rapidly reinduces resistance to SSZ.
indicate that ABCG2/BCRP and ABCC1/MRP1 follow an inverse pattern of expression after SSZ exposure and withdrawal. Given the apparent discrepancy of the rapid partial loss of SSZ resistance within 1 week of withdrawal of SSZ from CEM/SSZ1.5 cells (Figure 2A) and the stable expression of ABCG2/BCRP during this period (Figure 2B, lanes B-D), we further investigated the functional contribution of ABCG2/BCRP in SSZ resistance by blocking ABCG2/BCRP with a specific inhibitor, Ko143 (23). Blocking of ABCG2/BCRP reversed SSZ resistance in CEM/SSZ1.5 cells by 50-60%, but not completely to parental CEM (T) cell sensitivity (Figure 2C). After growth of CEM/SSZ1.5 cells in the absence of SSZ for 4 weeks, when IC-50 values for SSZ had stabilized at about 50% of the original CEM/SSZ1.5 cells, Ko143 fully reversed the residual SSZ resistance of CEM/SSZ1.5 cells to the SSZ sensitivity of CEM (T) cells (Figure 2C). These results indicate that SSZ resistance in CEM/SSZ1.5 is mediated by at least two components, each contributing for approximately 50%. One component is rapidly lost within one week after withdrawal of SSZ, the other component, which stabilised after 1-4 weeks' growth of CEM/SSZ1.5 cells in the absence of SSZ, can be fully accounted for by ABCG2/BCRP. The latter component is then gradually lost over a period of 5 months' culture in the absence of SSZ.

Sensitivity profile of CEM/SSZ cells for DMARDs and other anti-inflammatory drugs

In clinical rheumatology, SSZ is applied sequentially in mono- or combination therapies with other DMARDs (19, 20, 35). For this reason, we investigated whether the onset of SSZ resistance affects the sensitivity for other clinically active DMARDs, as well as other drugs with potential anti-inflammatory properties. Drug sensitivities were evaluated by their antiproliferative effects in CEM/SSZ1.5 and CEM/SSZ2.5 cells as compared with parental CEM (T) cells (Table 1).

Cross-resistance to mitoxantrone, a topoisomerase II inhibitor that is a prototypical substrate for ABCG2/BCRP (34, 36), for both CEM/SSZ1.5 (4.3-fold, p<0.002 vs. CEM) and CEM/SSZ2.5 cells (6.4-fold, p<0.001 vs. CEM) was observed. As expected, mitoxantrone cross resistance was fully reversed by the ABCG2/BCRP blocker Ko143. Furthermore,
cross resistance of CEM/SSZ cells was noted for the DMARDs leflunomide (up to 5.1-fold for CEM/SSZ2.5, p=0.005 vs. CEM) and MTX (up to 1.8-fold for CEM/SSZ2.5, p=0.027 vs. CEM). Because SSZ mediates part of its anti-inflammatory effects by targeting of the NFκB signalling pathway (37, 38) we also determined the drug sensitivity for MGI32, an inhibitor of NFκB activation via inhibition of 26S-proteasome (39). A low level of cross-resistance for CEM/SSZ cells was noted for MGI32 (1.6-fold, p=0.004 for CEM/SSZ2.5 and p=0.001 for CEM/SSZ1.5 vs. CEM). Interestingly, enhanced sensitivity of CEM/SSZ cells was noted for the DMARDs chloroquine (up to 1.9-fold CEM/SSZ2.5, p=0.043 vs. CEM) and cyclosporin A (up to 1.6-fold, p=0.001 for CEM/SSZ2.5 and p=0.028 for CEM/SSZ2.5 vs. CEM). CEM/SSZ cells displayed even more hypersensitivity (up to 13-fold, p=0.002 for CEM/SSZ1.5 and p=0.003 for CEM/SSZ2.5 vs. CEM) to the glucocorticoid dexamethasone. These results indicate that induction of SSZ resistance can coincide with a differentially altered MDR sensitivity pattern, including on the one hand cross resistance to leflunomide and MTX, but on the other enhanced sensitivity to cyclosporin A and chloroquine, and, most notably, to dexamethasone.

Discussion

Characterization of human CEM (T) cells with acquired resistance to the DMARD sulfasalazine showed differential expression of two multidrug resistance (MDR) efflux pumps: up regulation of ABCG2/BCRP and down regulation of ABCC1/MRP1. This phenotype could be reverted (slowly) and reinduced (rapidly) after SSZ withdrawal and SSZ re-exposure, respectively. Beyond this, CEM/SSZ cells were characterised by enhanced sensitivity to the DMARDs cyclosporin A and chloroquine, and most notably for dexamethasone, which may provide possible strategies to circumvent resistance to SSZ.

SSZ is commonly used in various DMARD regimens for RA treatment, including monotherapy or combination therapy with other DMARDs such as MTX, hydroxychloroquine or in the COBRA regimen (19, 20, 35, 40). SSZ can induce antiproliferative/apoptotic effects (41) as well as anti-inflammatory effects by inhibiting activation of NFκB (37), which leads to decreased production of pro-inflammatory cytokines such as TNFα (38). Although SSZ is a potent DMARD, its efficacy upon long term treatment seems to be more compromised as compared to other DMARDs, such as MTX (14, 16, 18). Because lack of efficacy may be related to either persistent/renewed disease activity and/or side effects of DMARDs, we suggested that diminished SSZ efficacy could also be associated with the development of resistance. Indeed, as previously reported (22), we found that the onset of SSZ resistance in human CEM (T) cells coincided with the induction of the MDR pump ABCG2/BCRP. Thus far, ABCG2/BCRP induction had only been noted upon development of a selected group of topoisomerase inhibitors such as mitoxantrone, doxorubicin and topotecan (12, 34, 42–44). ABCG2/BCRP expression in CEM/SSZ cells did not fully account for the resistant phenotype as about 50% of the SSZ resistance was lost within one week after withdrawal of SSZ, while ABCG2/BCRP expression was unchanged. The nature of this latter component has not been identified, but could be related to SSZ-induced transient alterations in the NFκB signalling pathway (38) that controls the transcription of anti-apoptotic and pro-inflammatory cytokine/chemokine genes (45–47). Consistent with this hypothesis is the observation of a two- to threefold enhancement of TNFα production by CEM/SSZ cells compared to CEM (T) cells (22). It remains to established whether these type of SSZ resistance-induced effects may also be responsible for the altered anti-proliferative effects (Table 1) of NFκB signalling pathway drugs, including leflunomide (48), MGI32 (39) and dexamethasone (45, 49, 50).

The gradual loss of ABCG2/BCRP expression from cell cultures of CEM/SSZ1.5 cells after 6 months (approximately 50 passages) withdrawal of SSZ is consistent with a study by Malepaard et al (51) who noted loss of ABCG2/BCRP expression from a topotecan-resistant ovarian carcinoma cell line over 30 passages in drug-free medium. These results indicate that ABCG2/BCRP expression gradually declines when the selective pressure is absent, but can be rapidly resumed upon renewed exposure to the selective drug. Besides the effects on ABCG2/BCRP expression, it is of interest to note that SSZ exposure/resistance had down regulatory effects on the expression of ABCC1/MRP1. Although this MDR transporter has an established role in extrusion of various toxic drugs (2, 5, 11), it also has important immunological functions in for instance dendritic cells, where, by exporting the cytokine leucotriene LTC4, it mediates the signalling for chemokine CCL19 chemotaxis and migration of dendritic cells to the lymph nodes (52). Because SSZ is an inhibitor of LTC synthetase (53), impaired synthesis of LTC may parallel reduced expression of its transporter ABCC1/MRP1. Another example of ABCC1/MRP1 down/up-regulation was recently identified by our laboratory after depletion/repletion of cellular folate status (54) in CEM (T) cells. Consistent with the notion that SSZ can also exert antifolate effects (55), we obtained preliminary evidence that the differential expression of ABCC1/MRP1 is indeed correlated with the cellular folate status in CEM/SSZ cells (Jansen G, Scheper RJ, Dijkmans BAC, unpublished data).

The present study also provides insight into strategies that may or may not be successful to circumvent/reverse SSZ resistance. Firstly, temporary discontinuation of DMARD treatment after an initial response and resumption of DMARD treatment at the time of progressive disease, proved to be effective (56). Whether a similar strategy is also effective for patients with RA for whom DMARD treatment has failed, has not been investigated. When such a clinical strategy was mimicked in vitro – discontinuation of SSZ treatment after development of SSZ resistance and rechallenging
Characterization of sulfasalazine resistant T-cells

with SSZ at the time SSZ resistance had apparently disappeared—it did not seem to be effective. SSZ resistance, along with ABCG2/BCRP overexpression, was rapidly reinduced (<2.5 weeks). Secondly, development of resistance to SSZ may be accompanied by adverse effects of collateral resistance to other DMARDs (e.g. leflunomide). Potentially positive strategies to target SSZ resistance may include the use of (a) blockers of ABCG2/BCRP that serve as chemosensitizers to reverse SSZ resistance (e.g. Ko143); (b) DMARDs that were not impaired in their anti-proliferative activity during development of SSZ resistance, such as cyclosporin A and chloroquine. Since chloroquine is a substrate for efflux by ABCC1/MRP1, a reduced expression of ABCC1/MRP1 as observed in CEM/SSZ cells may confer an enhanced sensitivity to chloroquine (57) which could increase the efficacy of hydroxychloroquine containing therapies (20). Finally, most interesting was the observation that CEM/SSZ cells displayed hypersensitivity (13-fold) to the glucocorticoid dexamethasone. One previous report has described collateral sensitivity to dexamethasone (10-fold) in human myeloma cells with acquired resistance to doxorubicin (58). The mechanistic basis for the markedly enhanced dexamethasone sensitivity is not clear, but is not associated with an increase in glucocorticoid receptor levels in CEM/SSZ cells compared to CEM (T) cells (not shown).

Altogether, this study shows that prolonged exposure of CEM (T) cells to SSZ can have differential effects on expression levels of at least two important MDR pumps: ABCG2/BCRP and ABCC1/MRP1. As these MDR pumps are normally expressed on various peripheral blood cell types (59-61), further research is warranted to identify the expression levels of these MDR pumps during treatment of patients with RA with DMARDs which include SSZ. Thus far, only a few studies have investigated blood samples of patients with RA for differential expression of the MDR transporter Pgp (62-65). Although these studies included limited numbers of patients, data were supportive for a role of Pgp in DMARD resistance. Our present study, though based on in vitro data using a T cell model system, demonstrates also that MDR pumps other than Pgp may be relevant for DMARD resistance. Once specific MDR proteins are identified for their contributing role in DMARD (in)efficacy, this information may be exploited to use specific blockers for the various MDR pumps (2) as chemosensitizers for DMARD activity.
References


References
References


Sulfasalazine is a potent inhibitor of the Reduced Folate Carrier: implications for combination therapies with methotrexate in rheumatoid arthritis

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Abstract

Objective: To investigate whether interactions of sulfasalazine (SSZ) with the reduced folate carrier (RFC), the dominant cell membrane transporter for natural folates and methotrexate (MTX), may limit the efficacy of combination therapy with MTX and SSZ in patients with rheumatoid arthritis.

Methods: Human RFC-overexpressing CEM cells of T-cell origin were used to analyze the effect of SSZ on the RFC-mediated cellular uptake of radiolabeled MTX and the natural folate leucovorin. Moreover, both cells with and those without acquired resistance to SSZ were used to assess the antiproliferative effects of MTX in combinations with SSZ.

Results: Transport kinetic analyses revealed that SSZ was a potent noncompetitive inhibitor of RFC-mediated cellular uptake of MTX and leucovorin with mean Kᵢ (50% inhibitory concentration) values of 36 ± 6 µM and 74 ± 7 µM SSZ, respectively. Consistent with the inhibitory interaction of SSZ with RFC, a marked loss of MTX efficacy was observed when the latter was coadministered with SSZ: up to 3.5-fold for CEM cells in the presence of 0.25 mM SSZ, and >400-fold for SSZ-resistant cells in the presence of 2.5 mM of SSZ. Importantly, along with a diminished efficacy of MTX, evidence for cellular folate depletion was obtained by the demonstration of a SSZ dose-dependent decrease in leucovorin accumulation.

Conclusion: At clinically relevant plasma concentrations, interactions of SSZ with the RFC provide a biochemical rationale for two important clinical observations: (1) the onset of a (sub)clinical folate deficiency during SSZ treatment, and (2) the lack of additivity/synergism of the combination of SSZ and MTX when these disease-modifying antirheumatic drugs are administered simultaneously. Thus, when considering use of these drugs in combination therapies, the present results provide both a rationale for folate supplementation and for spacing administration of these drugs over time.

Introduction

Although novel biologic agents (e.g. anti-tumour necrosis factor α or anti-interleukin-1) have become available for the treatment of patients with rheumatoid arthritis (RA) (1-3), traditional disease modifying anti-rheumatic drugs (DMARDs) or combinations of DMARDs, still find widespread application because of their proven efficacy and low cost (1, 3, 4). Moreover, long term superiority of expensive biologic agents over current (combinations of) DMARDs has not yet been proven. Therefore, further research on combination therapies remains warranted, at the levels of both clinical trials and fundamental research, in particular regarding mechanisms of action and possible drug interactions (5).

Sulfasalazine (SSZ) is commonly prescribed for the treatment of patients with RA (6-8) and those with inflammatory bowel disease (9). For the treatment of RA, SSZ (2-3 g/day) is often combined with the anchor drug MTX (7.5-15 mg/week), although this combination therapy does not display additive/synergic effects compared with monotherapy with either SSZ or MTX (10, 11). In fact, addition of a third component such as hydroxychloroquine (12, 13) or prednisone (14) is usually required to significantly improve clinical efficacy. Furthermore, one notable adverse effect was a persistent increase in plasma homocysteine levels in patients with RA who were receiving the combination of MTX and SSZ, compared with those receiving single-drug therapy (15, 16). Elevated plasma homocysteine levels are associated with cellular folate depletion (17-22), which has been ascribed to the inhibitory effects of SSZ on several intracellular enzymes in folate metabolism (23, 24) and/or inhibition of intestinal folate uptake (25-27).

In this study we present various lines of evidence for a novel site of interaction of SSZ with the reduced folate carrier (RFC) (28-32). RFC is the dominant cell membrane transporter for natural folates such as the main circulating folate 5-methyltetrahydrofolate, 5-formyltetrahydrofolate/leucovorin and folic acid (29, 32). Importantly, MTX also utilizes RFC for cellular uptake with an affinity (Kᵢ: 1-5 µM) close to 5-methyltetrahydrofolate (29, 32). Given their anionic nature, natural folates and MTX can not traverse the plasma membrane; therefore RFC-mediated cellular uptake should be regarded as the first critical step in any anti-inflammatory/antiproliferative mode of action of MTX (33-36). Consequently, diminished transport activity of RFC has been associated with loss of MTX efficacy and with folate deficiency (31, 37-40).

Herein we report that SSZ is a potent, non-competitive inhibitor of the RFC. Consistently, inhibition of RFC transport activity by SSZ resulted in a diminished efficacy of MTX when coadministered with SSZ and, as a side-effect, a cellular folate depletion. The possible clinical implications of SSZ-RFC interactions for SSZ/MTX-based therapies for RA are discussed.

Materials and methods

Materials

SSZ, 5'-amino salicylic acid (5'-ASA), sulfapyridine (SP) and folic acid were purchased from Sigma (St. Louis, MO, USA). Leucovorin was obtained from Eprova Co (Schaffhausen, Switzerland). Methotrexate was a generous gift from Pharmacien (Haarlem, The Netherlands). Trimetrexate was a gift from Dr. D. Fry, (Park-Davis, Ann Arbor, MI, USA). Stock solutions of 25 mM SSZ were prepared by dissolving SSZ in 50 mM NaHCO₃, at 37°C. Following filter sterilization (through a 0.22 µm filter), stock solutions were stored at -20°C. Stock solutions (0.5 M) of 5'-ASA and SP were prepared in DMSO. 3', 5',7'-[H]-MTX (specific activity 20-25 Ci/mmol) and 3',5', 7,9'-[H]-Leucovorin (specific activity 15-20 Ci/
mmol) were obtained from Moravek Biochemicals (Brea, CA) and purified prior to use by thin layer chromatography as described previously (41). RPMI 1640 tissue culture medium (with or without 2.2 µmol/l folic acid) and whole and dialyzed fetal calf serum (FCS) were purchased from Gibco BRL (Grand Island, NY, USA).

Cell lines
Cell cultures of human CEM cells (T-cell origin) were maintained in RPMI 1640 medium (with 2.2 µmol/l folic acid, unless otherwise indicated) supplemented with 10% FCS, 2 mM L-glutamine and 100 U/ml penicillin/streptomycin. Cells were cultured at an initial density of 3x10^6 cells/ml in a 5% CO_2 incubator as described previously (42). A subline of CEM cells (CEM-7A) with RFC overexpression was grown in folic acid-free RPMI medium, 10% dialyzed FCS, 2 mM L-glutamine, 100 U/ml penicillin/streptomycin and 0.15 nM L-leucovorin as the sole folate source (43-45). CEM cells with acquired resistance to SSZ were obtained by growing CEM cells in stepwise increasing concentrations of SSZ over a period of 6 months (46, 47). CEM cells selected to grow in the presence of 1.5 mM SSZ (CEM/SSZ1.5) and 2.5 mM SSZ (CEM/SSZ2.5) were used in this study.

Cell growth inhibition/stimulation studies
The antiproliferative effects of DMARDs were assessed by seeding cells into individual wells of 24 well plates (1.25x10^5 cells/ml; 1 ml/well). For each drug, 8 concentrations covering 2-3 logs were added to the individual wells. Thereafter, cells were exposed continuously to the drugs for 72 hours after which viable cell numbers were determined by a haemocytometer and trypan blue exclusion. The drug concentration required to inhibit cell proliferation by 50% (IC-50 value) is determined to assess antiproliferative effects. For folic acid and leucovorin growth stimulation analysis, cells were suspended in folic acid-free modified RPMI medium supplemented with 10% dialyzed FCS and transferred to individual wells of a 24 well plate containing increasing concentrations of either leucovorin (range: 0-250 nM) or folic acid (0-250 µM). Stimulation of cell growth was analyzed after 72 hours of incubation by determining the cell number with a hemocytometer.

Transport studies
RFC-mediated uptake of [3H]-MTX or [3H]-LV was analyzed for CEM cells, CEM-7A cells and CEM/SSZ cells in the mid-log phase of growth. Cells were harvested and washed with transport buffer (140 mM NaCl, 20 mM HEPES, 6 mM KCl, 2 mM MgCl_2, and 6.0 mM D-glucose, pH 7.4). Cells were suspended to a final density of 5x10^5/ml for RFC-overproducing CEM-7A cells, and 1.5x10^5/ml for CEM or CEM/SSZ cells. Incubations with 0.05-5 µM [3H]-MTX or [3H]-LV (final specific activity: 1000 dpm/pmol), in the absence or presence of inhibitors, were made for 1.5 minutes (for CEM-7A cells) and 3 minutes (for CEM and CEM/SSZ cells) at 37°C in a shaking water bath. Transport was terminated by the addition of 10 ml ice-cold transport buffer, after which the cell suspension was centrifuged (500g for 5 minutes at 4°C) and the cell pellet was washed once more with 10 ml ice-cold transport buffer. The final pellet was suspended in water and processed for radioactivity counting.

Quantification of RFC and multidrug resistance-associated protein 1 (MRPs) expression by Western blot analysis
Triton X-100-soluble microosomal proteins (50 µg) were resolved by electrophoresis on 10% polyacrylamide gels containing SDS and electroblotted onto nitrocellulose nylon membranes. The blots were then blocked for 1 hour at room temperature in Tris buffered saline (TBS) buffer (150 mM NaCl and 0.5% Tween 20 and 10 mM Tris/HCl at pH 8.0) containing 1% skim milk. The blots were reacted with a polyclonal antiserum (1:700) prepared in mice against a C-terminal hRFC peptide. Blots were then rinsed in the same buffer for 10 minutes at room temperature and reacted with horseradish peroxidase-conjugated goat anti-mouse IgG (1:40,000 dilution, Jackson Immunoresearch, West Grove, PA) for 1 hr at room temperature (38). Following three 10 minutes washes in TBS at room temperature, enhanced chemoluminescence detection was performed according to the manufacturer’s instructions (Biological Industries, Kibbutz, Beth Haemek, Israel). Protein expression of MRPs was analyzed by Western blotting according to previously described procedures, using MRPs (1:500) monoclonal antibody to MRPs (46, 48). Protein was determined by the colorimetric method (Biorad, Hercules, CA) described by Bradford.

Statistical analysis
Results were analyzed by Student’s t-test. P values less than 0.05 were considered significant.

RESULTS
Effects of SSZ on MTX efficacy
To test the effect of SSZ on the efficacy of MTX, human T cells (CEM cells) were exposed to MTX in the presence of non-toxic concentrations of SSZ (up to 0.25 mM) after which the degree of abrogation of the antiproliferative capacity of MTX was determined (Figure 1A). In the presence of 250 µM SSZ, the antiproliferative efficacy of MTX was markedly decreased (3.5-fold) when compared with that in absence of SSZ (p<0.001); the mean ±SD IC-50 values were 34.1 ±2.1 and 9.7 ±1.5 nM MTX, respectively. In contrast, equimolar concentrations (0.25 mM) of the SSZ catabolic products 5'-ASA and sulfapyridine (49), did not alter MTX efficacy against CEM cells (Figure 1A).

We next determined the efficacy of MTX against SSZ-resistant CEM cells (CEM/SSZ),
Effects of SSZ on RFC-mediated transport of MTX and natural folates

To test the hypothesis that SSZ exerts an inhibitory action on RFC transport activity, we determined whether SSZ is capable of inhibiting RFC-mediated transport of radiolabeled MTX in human CEM-7A cells that overexpress RFC (43). Indeed, experiments shown in Figure 2A demonstrate that RFC-mediated cellular uptake of radiolabeled MTX was inhibited by SSZ in a concentration-dependent manner, whereas the catabolic products of SSZ, 5’-ASA and sulfapyridine had no effect on RFC-mediated transport of MTX. Similar results were obtained for CEM cells and CEM/SSZ cells (data not shown). Because the physiologic function of RFC is to transport natural folates, we determined whether SSZ could also inhibit RFC-mediated transport of leucovorin as a representative natural folate. Indeed, SSZ also inhibited RFC mediated transport of leucovorin, although, when compared with MTX, a 2-fold higher concentration of SSZ was required to elicit a 50% inhibition of leucovorin transport (Figure 2B). Interestingly, the potential of SSZ to inhibit RFC-mediated transport of MTX was not affected by extracellular concentrations of MTX; at an extracellular concentration of either 0.05 µM or 2 µM MTX, the same level of inhibition was observed (Figure 2B).

To identify the mode of inhibition of RFC-dependent transport by SSZ, the inhibitory effects of SSZ on RFC transport were measured at extracellular concentrations of MTX or leucovorin (1-5 µM) in the relevant range of substrate affinities (Kᵣ) for RFC. Dixon plots, representing reciprocal transport rates of MTX uptake (Figure 2C) or leucovorin uptake (Figure 2D) at various concentrations of SSZ (0-100 µM), revealed a noncompetitive mode of inhibition, with mean ± SD Kᵣ (50% inhibitory concentration) values of 36 ± 6 µM SSZ for RFC-mediated MTX transport and 74 ± 17 µM SSZ for leucovorin transport.

Effect of SSZ on cellular folate accumulation and folate growth dependency

Consistent with the inhibitory interaction of SSZ with RFC, SSZ induced a dose-dependent inhibition of [3H]-LV accumulation in CEM and CEM/SSZ cells incubated for 24 hours which can tolerate higher concentrations of SSZ than parental CEM cells due to the overexpression of the multidrug resistance transporter ABCG2 (46, 47). In the absence of SSZ, CEM/SSZ cells and parental CEM cells were equally sensitive to MTX, however, in the presence of SSZ, a significant (p<0.001), concentration-dependent loss of MTX efficacy (i.e. decrease in IC-50 value) was observed: 3.4-fold at 0.25 mM SSZ, 41-fold at 1.0 mM SSZ and 345-fold at 2.5 mM SSZ (Figure 1B).

The changes in the IC-50 values for MTX in the presence of increasing concentrations of SSZ are illustrated in Figure 1C. In order to explore whether coadministration of SSZ also affects the efficacy of folate antagonists other than MTX, we performed similar experiments with the folate antagonist trimetrexate. Like MTX, trimetrexate has the same target in folate metabolism, i.e. dihydrofolate reductase (DHFR), but unlike MTX, trimetrexate does not require cellular entry through the RFC, because it enters cells by passive diffusion (32, 33, 50, 51). Strikingly, in combination with increasing concentrations of SSZ, markedly increased efficacy (i.e., a decrease in the IC-50 values) of trimetrexate (up to 8-fold; p<0.001) was noted against CEM/SSZ cells as compared with CEM cells (Figure 1C). These results demonstrate that the differential effects of SSZ on MTX and trimetrexate efficacy cannot be explained at the level of the target enzyme DHFR. Rather, these results suggest that SSZ interferes with the cell membrane transport of MTX via RFC, resulting in a dose-dependent abrogation of MTX efficacy.
with a physiologically representative concentration of 50 nM leucovorin (Figure 3A). Consequently, this implies that in the presence of SSZ, higher concentrations of folates will be required to support cell growth. This is illustrated for CEM/SSZ cells which required 80-fold higher concentrations of leucovorin (EC₅₀ = 16 nM vs. 0.21 nM; p<0.001) (Figure 3B) or 27-fold higher concentrations of folic acid (EC₅₀ = 1960 nM vs. 27 nM; p<0.001) (Figure 3C) in order to support half-optimal growth in the presence of 1.5 mM of SSZ when compared

**RFC and MRP1 expression in CEM and CEM/SSZ cells**

At the level of the cell membrane, cellular folate homeostasis is regulated by at least two groups of transporters. The RFC mediates folate transport inward (29, 32), while members of the MRP family (52), in particular MRP1, control cellular export of folates (53-55). In order to assess effects of long term exposure to SSZ, we analyzed RFC protein expression and transport activity and MRP1 protein expression in CEM/SSZ cells. RFC protein expression appeared to be down-regulated 4-fold and 8-fold in CEM/SSZ1.5 cells and CEM/SSZ2.5 cells, respectively (Figure 4). RFC transport activity, represented by relative rates of [³H]-MTX uptake, closely corresponded to RFC protein levels in RFC-overproducing
CEM-7A, CEM cells and CEM/SSZ cells (Figure 4). Interestingly, MRPI levels were markedly decreased in CEM/SSZ cells compared with CEM cells (Figure 4). It is conceivable that this down-regulation of MRPI is a response to minimize export of folates after inhibition of RFC-mediated uptake of natural folates by SSZ (53, 55).

Figure 4: RFC and MRPI protein expression of CEM/SSZ cells versus CEM and CEM-7A cells. Western blot analysis of RFC and MRPI expression in CEM-7A, CEM, CEM/SSZ1.5 and CEM/2.5 cells. A CEM line with a mutated RFC gene, CEM/R0.6MTX (38), served as negative control for RFC expression. Na+/K+-ATPase expression served as control for equal loading.

Discussion

Notwithstanding current successes with biologic agents, DMARDs continue to have an established place in the treatment of patients with RA. MTX, the anchor drug in RA treatment, is effective as a single agent but also serves as the basis for combination therapies with other DMARDs such as SSZ. Initial clinical studies with the combination of MTX plus SSZ were empirically driven by the proven efficacy of both DMARDs as single agents (10, 11), although a biochemical basis for the putative additive or synergistic effects of the combination was still lacking. In fact, extensive clinical evaluation of the combination of MTX plus SSZ revealed no superior activity over mono-therapy with either DMARDs (9). Moreover, side effects of the combination of MTX plus SSZ included elevated plasma homocysteine levels, revealing folate deficiency. Manifestations of (sub)clinical folate deficiency requiring folate supplementation had been reported previously during SSZ single-drug therapy in patients with RA (17-22). Thus far, the mechanisms proposed to underlie these effects were inhibitory effects of SSZ on intestinal folate absorption (25-27) and several enzymes in intracellular folate metabolism (23-24).

In this study, data are presented revealing an as yet unknown mechanism explaining not only the lack of additivity/synergism of SSZ when combined with MTX, but also the side effect of folate deficiency. This mechanism is based on interactions of SSZ with the RFC, the dominant cell membrane transport protein for natural folates as well as structurally related drugs like MTX. RFC is constitutively expressed in many cell types, particularly those relying on folate uptake for DNA synthesis and proliferation (29, 32).

In the current study, several lines of evidence support RFC inhibition by SSZ. First, the efficacy of MTX was progressively reduced when it was combined with increasing concentrations of SSZ. In fact, the levels of loss of MTX efficacy at high concentrations of SSZ (>400-fold in the presence of 2.5 mM SSZ) were similar to those obtained in cells displaying loss of MTX efficacy due to decreased expression of the RFC protein (28, 38, 39, 56). In this respect, the decreased expression of RFC (up to 8-fold) in CEM/SSZ cells (Figure 4) could have contributed to a diminished efficacy of MTX, but data shown in Figures 1a and 1b did not reveal a difference between MTX efficacy in CEM/SSZ cells and that in CEM cells. This may be explained by the fact that MTX efficacy, as shown in Figures 1a and 1b, is determined mainly by the duration of inhibition of the intracellular target enzyme DHFR by MTX. When sufficient MTX is internalized via RFC to bind DHFR stoichiometrically, MTX will start to be efficacious. Experiments described in Figures 1a and 1b included a 72-hour exposure to MTX, a time frame that is permissive to fully saturate DHFR over time, even when the internalization rate of MTX is 8-fold lower in CEM/SSZ cells compared with CEM cells. It can also be argued that the decrease in RFC expression in CEM/SSZ cells provokes lower intracellular pools of natural folates, which are also transported by the RFC (see below). Consequently, less MTX is required to deplete intracellular folate pools as part of the secondary effect of DHFR inhibition. Thus, a lower intracellular folate pool may compensate for any decrease in MTX uptake to confer a similar efficacy of MTX between CEM/SSZ and CEM cells when analyzed in the absence of SSZ.

Second, transport kinetic studies revealed a concentration-dependent inhibition of (RFC-mediated) uptake of MTX or leucovorin by SSZ in CEM cells and RFC-overproducing CEM-7A cells. These observations are not specific for cells of T cell origin, because we observed similar effects of SSZ-RFC interaction in monocytic/macrophage cells (Oerlemans R, Dijkmans BAC, Jansen G: unpublished observation).

Third, SSZ-mediated inhibition of RFC transport resulted in a diminished uptake of natural folates required for cell proliferation. To counteract this effect, folate supplementation may be required whereby leucovorin, concentration-wise, may be more effective than folic acid, because leucovorin is more efficiently transported via RFC than folic acid (for leucovorin, K_M = 1-5 µM; for folic acid, K_M = 200-400 µM) (29, 32). SSZ-induced cellular folate deficiency is also reflected by hypersensitivity to the folate antagonist trimetrexate. Hypersensitivity to trimetrexate has been described in cells with low intracellular folate pools, which are also transported by the RFC (see below). Consequently, less MTX is required to deplete intracellular folate pools as part of the secondary effect of DHFR inhibition. Thus, a lower intracellular folate pool may compensate for any decrease in MTX uptake to confer a similar efficacy of MTX between CEM/SSZ and CEM cells when analyzed in the absence of SSZ.
Recent studies by Chen et al (59) and Volk et al (60) showed that the multidrug resistance transporter ABCG2, which is upregulated in CEM/SSZ cells (46, 47), could mediate cellular export of MTX and contribute to diminished efficacy of MTX. However, recent studies from our laboratory (61) showed that ABCG2 did not play a major role in MTX resistance upon prolonged (>72 hrs) exposure to MTX, the conditions that were used in the experiments illustrated in Figures 1A and 1B. Of note, ABCG2-mediated MTX resistance was observed when MTX exposure was limited to a short time period (<4 hours), during which no long-chain polyglutamate forms of MTX were formed.

Although the effects observed in this in vitro study may be more pronounced because cells, in particular SSZ-resistant CEM cells, could be exposed to high concentrations of SSZ, the present results are most likely also of clinical relevance. The ability of SSZ to block RFC-mediated transport of MTX and leucovorin by 50% was noted at SSZ concentrations of 36 and 74 µM, respectively. Of note, daily administration of SSZ (2-3 g/day) to patients with RA can result in mean plasma levels of up to 100 µM SSZ (24, 49), which is thus clearly sufficient to exert an inhibitory effect on RFC-mediated uptake of MTX and natural folates. Consequently, this may trigger a (sub)clinical folate deficiency, especially when dietary intake, absorption or cellular retention of folates is poor. With respect to folate absorption, it is important to note that the intestinal folate transporter is structurally and functionally identical to the RFC expressed in CEM cells (62-64). Thus, original reports on impaired intestinal folate absorption following SSZ treatment (25-27) can be attributed to direct inhibition of intestinal RFC by SSZ. Complementary inhibitory effects of SSZ on enzymes in the folate metabolism (23, 24) may then further aggravate the folate-deficient status.

It has been well established that a variety of anorganic and organic anions can influence functional RFC transport activity (29, 30). For example, recently we have shown that prostaglandin A1 (PGA1), which is a charged organic anion at physiological pH, is a potent noncompetitive inhibitor of RFC-mediated transport of MTX with a K1 of 21 µM (65). Our current study revealed that the monovalent anion SSZ also displays an interaction with the RFC. Of particular interest is that the mode of inhibition of RFC activity by both PGA, and SSZ is noncompetitive, indicating that both interact at an RFC domain that is distinct from the folate/MTX binding site. Furthermore, SSZ may interact in an inhibitory mode with the RFC, regardless of whether the latter is loaded with MTX or natural folate substrates. Consequently, this implies that the inhibition of RFC-mediated uptake of MTX by SSZ cannot be overcome by increasing doses of MTX. Rather, one could speculate that a possible strategy to improve the efficacy of the combination of MTX plus SSZ may be to alleviate the block on RFC by discontinuing the daily administrations of SSZ for the one day at which the weekly dose of MTX is administered.

Another strategy of the rheumatologist for enhancing the efficacy of the combination of MTX plus SSZ is the addition of a third drug (e.g. hydroxychloroquine (13) or prednisone (14)) to the MTX-SSZ combination. Although the objective of this study was not to elucidate a mechanistic basis for the efficacy of the triple DMARD therapy, recent studies by our group (47) did indicate that CEM/SSZ cells displayed enhanced sensitivity to both chloroquine and dexamethasone. In the case of chloroquine this may be related to down-regulation in the expression of MRPs (see Figure 4), which, apart from a physiological role of MRPs in folate homeostasis (53-55), is best known as a drug efflux pump that can export various drugs (52, 66), including chloroquine (67, 68). Down-regulation of MRPs may therefore lead to increased intracellular retention of chloroquine, resulting in enhanced efficacy.

In conclusion, this is the first study to demonstrate that SSZ is a potent noncompetitive inhibitor of RFC-mediated cellular uptake of MTX and leucovorin. Consistently, the inhibitory interaction of SSZ with RFC resulted in a marked loss of MTX efficacy when MTX was coadministered with SSZ. Furthermore, along with diminishing efficacy of MTX, the inhibition of natural folate uptake by SSZ induced cellular folate depletion. Hence, results of this study points to the RFC as an important site of interaction with SSZ and downstream effects on folate and MTX metabolism (see Figure 5). RFC-SSZ interactions warrant further consideration for optimal scheduling of MTX/SSZ-based DMARD combination therapies and folate supplementation in the treatment of patients with RA.

![Figure 5](image_url)
References


References


60 Volk,E.L. and Schneider,E. Wild-type breast cancer resistance protein (BCRP/ABCG2) is a methotrexate polyglutamate transportator, Cancer Res., 63: 5538-5543, 2003.


Involvement of Breast Cancer Resistance Protein expression on RA synovial tissue macrophages in resistance to methotrexate and leflunomide

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Abstract

Objective: To determine whether multidrug-resistance efflux transporters are expressed on immune effector cells in synovial tissue from rheumatoid arthritis (RA) patients and may compromise the efficacy of methotrexate (MTX) and leflunomide.

Methods: Synovial tissue biopsies obtained from RA patients before treatment and 4 months after starting treatment with MTX (n=17) and leflunomide (n=13) were examined by immunohistochemical staining and digital image analysis for the expression of the drug efflux transporters: P-glycoprotein (Pgp), Multidrug Resistance-associated Proteins MRP1-5, MRP8, MRP9 and Breast Cancer Resistance Protein (BCRP).

Results: BCRP expression was observed in all RA synovial biopsies, both pre- and post-treatment, but not in control non-inflammatory synovial tissue of orthopedic patients. BCRP expression was found both in the intimal lining layer as well as on macrophages and endothelial cells in the synovial sublining. Correlation analysis showed a trend towards more abundant BCRP expression at higher disease activity. Furthermore, median BCRP expression was 4-fold higher for MTX-non responders than for MTX-responders (p=0.08), and 2.5-fold higher in leflunomide failures compared to leflunomide-responders (p=0.12).

Conclusion: The drug resistance related proteins BCRP and MRP1 are expressed on inflammatory cells in RA synovial tissue. Since MTX is a substrate for both BCRP and MRP1 and leflunomide is a high affinity substrate for BCRP, these transporters may contribute to a reduced therapeutic efficacy of these DMARDs.

Introduction

The disease modifying anti-rheumatic drugs (DMARDs) methotrexate (MTX), leflunomide, sulfasalazine and (hydroxy)chloroquine have an established place in the treatment of patients with Rheumatoid Arthritis (RA), either as single agents or in combination with other DMARDs or biological agents (1, 2). Despite the potent disease modifying effects of these DMARDs, it is well recognized that chronic treatment is often accompanied by a gradual loss of efficacy, requiring dose escalation, up to a stage where treatment has to be discontinued for reasons of inefficacy and/or toxicity (3-5). From oncology research, it has been established that specific cell membrane-associated drug efflux transporters belonging to the family of ATP Binding Cassette (ABC) proteins, notably P-glycoprotein (Pgp; ABCB1), Multidrug Resistance Proteins 1-5 (MRP1-5; ABCG2+5) and Breast Cancer Resistance Protein (BCRP; ABCG2), can confer resistance to various types of drugs including MTX (6-9). Studies from our laboratory showed that acquired resistance to the DMARDs sulfasalazine and chloroquine could be mediated by the increased expression of the ABC transporters BCRP and MRP1, respectively (10-12). Furthermore, very recently, it was demonstrated that leflunomide is a high affinity substrate for BCRP (13).

A number of studies have reported on the expression of Pgp and MRPs (mRNA levels, protein levels, functional activity) on peripheral blood cells of RA patients. Maillefert et al (14) observed that Pgp levels were increased on peripheral blood cells of prednisolone-treated RA patients compared to untreated healthy individuals, which may be compatible with the notion that steroids are known substrates for Pgp. Likewise, Llorente et al (15) showed that RA patients had functionally active Pgp on peripheral blood lymphocytes which was markedly higher in treatment-refractory RA patients than in RA patients with a good clinical response to DMARD therapy. More recently, two studies evaluated the expression of MRP1 (by functional activity and immunohistochemical staining) as a potential MTX drug efflux pump in peripheral blood cells of RA patients. In a cross-sectional study, Wolf et al (16) counter-intuitively observed that patients with functional MRP1 expression who were treated with MTX had better EULAR response rates than patients with a low MRP1 activity. Hider et al (17) showed that MRP1 expression on peripheral blood cells declined from baseline to 6 months therapy with MTX. These results suggest that beyond drug-induced changes in MRP1 expression during dose escalations, basal levels of MRP1 expression in immune effector cells may also be induced by disease activity/inflammation status (9). In contrast to studies on peripheral blood cells from RA patients, little is known about expression levels of ABC transporters on inflammatory cells in RA synovial tissue.

In the present study we assessed the expression of a panel of ABC transporters with an established role in drug resistance in RA synovial tissue biopsies that were taken prior to and after 4 months of therapy with MTX and leflunomide. Beyond Pgp, we focused on more recently identified ABC transporters: MRP1-5, MRP8 and MRP9, which have MTX among their substrates (8) and BCRP for which both MTX and leflunomide are known substrates (13), thereby allowing correlation analysis with response to these drugs.

Materials and Methods

Patients

In this study we analyzed synovial tissue biopsies derived from the same knee joints of 17 RA patients with active disease, both before treatment and after 4 months of treatment with MTX (starting dose of MTX: 7.5 mg/week; increasing stepwise to 15 mg/week over 12 weeks) and 13 RA-patients before and after 4 months of treatment with leflunomide (20 mg/day; loading dose 100 mg/day for the first 3 days). Active disease was defined as ≥6 swollen or
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Expression of multi-drug resistance proteins in synovial tissue of Rheumatoid Arthritis patients

tender joints and levels of moderate or worse on the physician’s and patient’s assessments of disease activity. All patients had at least 1 clinically involved knee joint. Concomitant treatment with low dose prednisone (<10 mg/day) and stable doses of nonsteroidal anti-inflammatory drugs (NSAIDs) was allowed. None of the patients ever used methotrexate or leflunomide before enrolling the study. In patients taking other DMARDs, these drugs were terminated following a washout phase of 28 days. Before treatment and after 4 months of treatment, the DAS28 score was determined (18). Arthroscopies were performed as described previously (19) as part of a joined study that was approved by the Medical Ethics committees of the Leiden University Medical Center and the Leeds University Medical Center (20). As non-inflammatory control synovial tissue we included 7 samples from patients with mechanical joint injury, kindly provided by dr. B.J. van Royen, department of Orthopedic Surgery, VU University Medical Center, Amsterdam, The Netherlands.

For statistical analysis, RA patients were divided in a group of DMARD-responders and a group of DMARD-non-responders, according to the DAS28 response criteria (18). Responders were patients with a significant decrease in DAS28 score (>1.2) upon treatment and patients with a moderate change in DAS28 score (>1.2 and <0.6) and low/moderate disease activity (<5.1). The remaining patients were classified as non-responders. An additional analysis was performed, categorizing patients with a normalized CRP level (<10 mg/l) after 4 months of treatment as responders.

Immunohistochemical analysis

Immunohistochemical staining of cryostat sections (5 µm) of synovial tissue biopsies from RA patients and controls was performed using a 3-step immunoperoxidase method as described previously (21). Sections were stained with the following monoclonal antibodies (5-10 µg/ml): C219 (anti-Pgp) (Alexis, Lausen, Switzerland), MRPl (anti-MRP1), M2II6 (anti-MRP2), M3II9 (anti-MRP3), M4II10 (anti-MRP4), M5II1 (anti-MRP5), M8II16 (anti-MRP8), M9II-27 (anti-MRP9), BXP-21 (anti-BCRP), as described by Scheffer et al (21, 22). T-cells and macrophages were stained with anti-CD3-PE (Dako, Glostrup, Danmark) and anti-mouse biotin-labeled secondary antibodies (Dako), Streptavidin thyramine (red fluorescence) according to the instructions of the manufacturer. T-cells and macrophages (primary antibodies anti-CD3 and anti-CD68, respectively) were detected by secondary reagents (Dako), and development was with rhodamine/ABC transporter protein expression was detected by species specific HRP-labeled secondary reagents (Dako, Glostrup, Danmark), and development was with rhodamine/thyramine (red fluorescence) according to the instructions of the manufacturer. T-cells and macrophages (primary antibodies anti-CD3 and anti-CD68, respectively) were detected by anti-mouse biotin-labeled secondary antibodies (Dako, Glostrup, Danmark), Streptavidin Alexa-488 serving as substrate (green fluorescence; Molecular Probes, Eugene, OR). Slides were mounted with Vectashield, containing 1 µg/ml DAPI (for staining of nuclei) (Vector laboratories Inc., Burlingame, CA). Cells were examined using a fluorescence microscope (Leica DMRB, Rijswijk, the Netherlands).

Statistical analysis

Statistical analysis was performed using non-parametric tests. Correlation of BCRP-protein expression with DAS28 score and BCRP-protein expression for individual patients before treatment and after 4 months of treatment with MTX was calculated by Spearman Rho analysis. For determining significant differences in macrophage counts before and after treatment with MTX and BCRP-protein expression between DMARD-responding and DMARD-non-responding RA patients, the Mann-Whitney U test was used. P-values <0.05 were considered to represent significant differences.

Results

Expression of drug resistance related transporters on macrophages, T-cells and endothelial cells in RA synovial tissue

Immunohistochemical staining for the drug-resistance related transporters Pgp, MRPl-5, MRPl8, MRPl9 and BCRP was performed on synovial biopsies obtained from RA patients prior to and after 4 months of treatment with MTX (n=17) and leflunomide (n=13). Negative staining was observed for all isotype controls (Figure 1A). 3A5 staining showed that synovial tissue samples were highly enriched for infiltrated macrophages in the intimal lining layer and synovial sublining (Figure 1B). The most abundantly expressed drug efflux transporter in RA synovial tissue, both in the intimal lining layer and synovial sublining, appeared to be BCRP (Figure 1C). Only a few BCRP-positive cells could be identified in ‘non-inflammatory’ synovial tissues of orthopedic patients (Figure 1F), which also displayed low levels of infiltrated macrophages (3A5 staining, Figure 1E) as compared to RA synovium (Figure 1B). More detailed analysis by fluorescence microscopy, demonstrated the co-localization of BCRP and CD68 in the intimal lining layer and synovial sublining, proving that BCRP is expressed on RA macrophages. BCRP was also found to be expressed on endothelial cells (Figure 2A-H), and double staining techniques showed that BCRP is weakly expressed on

Double-labeling immunofluorescence

ABC transporter protein expression was detected by species specific HRP-labeled secondary reagents (Dako, Glostrup, Danmark), and development was with rhodamine/thyramine (red fluorescence) according to the instructions of the manufacturer. T-cells and macrophages (primary antibodies anti-CD3 and anti-CD68, respectively) were detected by anti-mouse biotin-labeled secondary antibodies (Dako, Glostrup, Danmark), Streptavidin Alexa-488 serving as substrate (green fluorescence; Molecular Probes, Eugene, OR). Slides were mounted with Vectashield, containing 1 µg/ml DAPI (for staining of nuclei) (Vector laboratories Inc., Burlingame, CA). Cells were examined using a fluorescence microscope (Leica DMRB, Rijswijk, the Netherlands).

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Expression of multi-drug resistance proteins in synovial tissue of Rheumatoid Arthritis patients

With light microscopy, weak MRP1 staining was observed in synovial tissue biopsies of approximately one-third of RA patients. With more sensitive detection methods (immunofluorescence; rhodamine/thyramine development), clear MRP1 expression was observed on CD3-positive lymphocytes, being most abundant in lymphocytic aggregates and less pronounced on neighboring T-cells (Figure 3A-D). With this detection method we also observed moderate MRP1 expression on CD68 positive macrophages in biopsies from the same patients (Figure 3E-F). No co-localization of MRP1 was found with CD90, a marker for synovial tissue fibroblasts (not shown). Expression levels of Pgp, MRP2-5, MRP8 and MRP9 in synovial tissue of RA patients were below the immunohistochemical detection limit.

BCRP expression and response to MTX and leflunomide therapy

Since MTX and lefunomide are BCRP substrates (8, 13) and thus can be extruded from synovial tissue macrophages via this transporter, expression levels of BCRP in the synovial sublining were correlated with the response to MTX and leflunomide therapy. Correlation analysis showed a trend towards a more abundant BCRP expression at higher disease activity (r=0.30, p=0.12) (Figure 4A). BCRP expression for individual patients appeared to be rather constant over time, as a significant correlation was found between BCRP expression before treatment and after 4 months of treatment with MTX (r=0.54, p=0.025) (Figure 4B). The median number of 3A5 macrophages decreased upon MTX treatment: 219/mm² (range 11-622) to 119/mm² (range 12-844) (Figure 4C, p=0.14). The

peri-vascular CD3 positive T-cells (not shown).
Expression of multi-drug resistance proteins in synovial tissue of Rheumatoid Arthritis patients

Figure 4: (A) Correlation between DAS28 score and BCRP expression (positive cells/mm²; sublining layer) before treatment. Spearman rho: r=0.30; p=0.12. (B) Correlation between BCRP expression (positive cells/mm²; sublining layer) before treatment and after 4 months of treatment with MTX for individual patients. Spearman rho: r=0.54; p=0.025. (C) Median number of 3A5 positive macrophages (positive cells/mm²; sublining layer) before treatment: 219, range 11-622 and after 4 months of treatment with MTX: 119, range 12-844) (p=0.14). The median expression of BCRP (positive cells/mm²; sublining layer) after 4 months of therapy is depicted for patients with high macrophage counts (upper right square) and low macrophage counts (lower right square).

Figure 5: Expression of BCRP (median number of positive cells/mm²: sublining layer) for controls and RA patients after 4 months of treatment with MTX. (A) MTX-responders: 75 (range 0-484) and MTX-non-responders: 300 (range 2-1159). (B) Number of BCRP positive cells for RA-patients with normalized CRP levels (mean CRP: 5 mg/l): 29 (range 0-484) and patients with persistent high CRP levels (mean CRP: 54 mg/l): 224 (range 2-1159). (C) Expression of BCRP (median number of positive cells/mm²: sublining layer) for controls and RA patients after 4 months of treatment with leflunomide. Median BCRP expression in leflunomide-responders was 127 (range 1-223) and 297 (range 11-1007) for leflunomide-non-responders. Statistical analysis: Mann-Whitney U test.

A group of patients with a persistent high macrophage count in their synovial biopsies after 4 months of treatment with MTX (Figure 4C, upper right square) had a 3.9-fold higher count of BCRP positive cells (293/mm²) compared to patients with a low macrophage count (75/mm²) (p=0.12) (Figure 4C, lower right square).

For MTX non-responders (n=8), the median number of BCRP positive cells appeared to be 4-fold higher after 4 months of treatment with MTX than for MTX-responders (n=9): 300 (range 2-1159) and 75 (range 0-484) cells/mm², respectively (p=0.08) (Figure 5A). Additional analysis (Figure 5B) revealed a statistically significant (p=0.016) 8-fold higher BCRP expression for RA-patients with persistent high CRP levels (median 224 BCRP positive cells/mm²; sublining layer) before treatment and after 4 months of treatment with MTX for individual patients. Spearman rho: r=0.30; p=0.12.
Discussion

This is the first detailed study in RA synovial tissue investigating the expression of a panel of multidrug transporter proteins from the family of ATP-binding cassette (ABC) transporters, known to confer resistance to multiple anticancer drugs as well as DMARDs (9, 25). Immunohistochemical detection of Pgp and MRP2-5, MRPs and BCRP in RA synovial tissue was not observed, in contrast to the drug transporters MRPs and BCRP, the latter being markedly expressed on RA synovial tissue macrophages in the intimal lining layer and the synovial sublining and on endothelial cells. Importantly, BCRP expression could already be observed in the synovial tissue of RA patients prior to MTX therapy. This finding suggests that BCRP expression may be intrinsically associated with the inflammatory status of RA synovial tissue, rather than being a therapy-induced phenomenon. It should be noted that some patients had received sulfasalazine as initial treatment prior to a wash-out period and MTX or leflunomide treatment in the current study. Previous studies from our laboratory have shown that prolonged in vitro exposure of T cells to sulfasalazine may provoke induction of BCRP (10), but this induction was transient and reversed upon discontinuation of sulfasalazine exposure (11). Thus, it seems unlikely that the marked expression levels of BCRP on synovial macrophages at the onset of MTX/leflunomide treatment may be reminiscent to any previous sulfasalazine treatment. Consistent with our hypothesis that BCRP expression is inflammation-driven, is the correlation between BCRP expression and DAS28 scores (Figure 4A), although statistical significance should be further corroborated in a larger group of RA patients.

One pathophysiological condition in RA synovial tissue that could be implicated in BCRP expression is its hypoxic environment. It is well recognized that synovial infiltrating cells suffer from hypoxic stress (26). Given the notion that BCRP transcription is induced by hypoxia via the hypoxia-inducible transcription factor complex HIF-1α (27), and that HIF-1α is particularly expressed in synovial tissue macrophages (28, 29), this may underlie the expression of BCRP on these cells.

Biodistribution and functional studies indicated that BCRP has important physiological and pharmacological roles, particularly in the clearance of toxic agents from the gastrointestinal tract, blood-brain barrier, liver, kidney, placenta and mammary glands (21, 30, 31). With respect to hematological cells, BCRP was identified in hematopoietic stem cells (32) and dendritic cells (33). From a therapeutic perspective, BCRP has been recognized to mediate the cellular export of several anticancer drugs (8, 34-36), and has been associated with a poor clinical outcome in the treatment of AML (37). Beyond this, DMARDs like MTX, sulfasalazine and leflunomide can also be extruded from cells via BCRP (8, 10, 13, 38). Of note, BCRP can export MTX as well as its di- and triglutamate metabolites, which could thus lead to a diminished efficacy of MTX (38).

The present study revealed BCRP expression on synovial tissue macrophages of all tested RA patients. High BCRP expression was associated with the persistence of infiltrated 3A5 positive synovial tissue macrophages after treatment with MTX (Figure 4C), which might be related with BCRP-mediated resistance to MTX in these macrophages. Accordingly, a trend but no significant correlation was found between BCRP expression in RA synovial tissue and a diminished clinical response to MTX and leflunomide therapy, assessed by an attenuated improvement of DAS28 scores after 4 months of treatment. In secondary analyses, a 8-fold significant increase in BCRP expression was observed in patients with a persistent high CRP level after treatment compared to patients with normalized CRP levels, providing additional support for a role of BCRP in conferring MTX resistance.

In addition to BCRP expression on synovial tissue macrophages, moderate expression of MRPs was found on macrophages in synovial tissue of one-third of RA patients, while more prominent expression of MRPs could be noted on CD3 positive T-cells in lymphocytic aggregates of these patients by more sensitive staining methods. MTX and the anti-malarial chloroquine are among the DMARD substrates of MRPs, and expression of this transporter may thus confer resistance to these drugs (6, 12, 39). However, since MRPs levels in synovial T-cells are rather moderate, a major contribution to a reduced response to MTX or anti-malarial drugs remains elusive. Apart from its putative pharmacological role in MTX extrusion, MRPs also has an important physiological function as it exports inflammatory mediators such as leukotriene C4 (LTC4), which triggers antigen-presenting dendritic cell migration to lymph nodes via chemo-attraction by CCL19 (40, 41). Since high levels of CCL19 have been identified in RA-synovial tissues (42, 43), MRPs may be instrumental in the homing of inflammatory cells in RA synovial tissues.

Current experimental strategies to reverse drug resistance associated with efflux transporters include the utilization of specific inhibitors of ABC transporters. Small molecule inhibitors of BCRP have been tested in a laboratory setting (34, 36). Whether such an approach may also be of therapeutic benefit for RA treatment remains to be established. Recently, Zaher et al. (44) reported that Iressa, an EGFR receptor antagonist and BCRP transport inhibitor, could markedly enhance the bioavailability of the DMARD sulfasalazine (a BCRP substrate (10, 11)) by blocking intestinal extrusion of sulfasalazine via BCRP. Based on this proof of principle, BCRP expression in RA synovial tissue may also be an interesting target for intervention aimed at increasing MTX, leflunomide or sulfasalazine levels in RA macrophages.

In conclusion, along with an established role for the efficacy of cancer therapy, this study revealed that multidrug resistance related transporters, notably BCRP and to a lesser extent MRPs, may play a role in the efficacy of RA treatment with specific DMARDs. Delineation of their precise pharmacological and physiological role warrants further evaluation and should be helpful in improving the efficacy of DMARDs such as MTX, leflunomide, sulfasalazine and (hydroxy)chloroquine, being substrates for these drug efflux transporters.
References


44 Zaher,H., Khan,A.A., Palandra,J., Brayman,T.G., Yu,L. and Ware,J.A. Breast Cancer Resistance Protein (Bcrp/abcg2) is a major determinant of sulfasalazine absorption and elimination in mouse, Molecular Pharmaceutics, 3: 55-61, 2006.
Anti-folate Drug Combinations for Inflammatory Diseases

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Abstract

Drugs used in daily medical practice to treat inflammatory diseases include the anti-folate methotrexate (MTX) and the anti-pyrimidine leflunomide. Treatment with MTX is widespread for various inflammatory diseases and this drug has become the anchor drug in the treatment of rheumatoid arthritis (RA) patients. In RA-patients MTX is administered as monotherapy, but nowadays it is often combined with other disease modifying antirheumatic drugs (DMARDs) like sulfasalazine, hydroxychloroquine and prednisone, and biologic agents like infliximab, etanercept and adalimumab. The beneficial effects of biologic agents, like anti-tumour necrosis factor α (TNFα) antibodies, have been assessed after partial failure on MTX, as monotherapy or in combination with MTX. Thus, MTX has gained a very important place in the treatment of RA patients; the definite place for various combination strategies is still under investigation. Leflunomide has also proven to be an effective drug in the treatment of RA-patients, but has a less established place than MTX.

Introduction

Rheumatoid arthritis (RA) is a disease with synovitis as hallmark, leading to cartilage and bone destruction (1). Moreover, RA is accompanied by general effects of inflammation, such as malaise, fatigue and an increased sedimentation rate. It affects on average 1% of the Dutch population, 70% of patients being women. For decades, therapy of RA-patients consisted of non-steroidal anti-inflammatory drugs (NSAIDs), reducing pain and inflammation but not modifying the course of the disease. In the 1930’s, the first Disease Modifying Anti-rheumatic Drugs (DMARDs) -gold and sulfasalazine- were introduced, interfering with the course and progression of the disease (2, 3). The modifying effect of DMARDs is reflected in improvement of the Disease Activity Score (DAS), a composite index of the patients opinion of disease activity, a count of tender and swollen joints by the physician and the erythrocyte sedimentation rate. The most important DMARDs are listed in an historical perspective in figure 1. This figure illustrates that intramuscular gold is the eldest DMARD, followed by sulfasalazine. Corticoids are used since the 1940’s for RA, with good clinical response, but it induces many side effects in patients, for example osteoporosis, hypertension and diabetes mellitus upon prolonged use.

Two developments can be considered as real breakthroughs in the treatment of RA-patients: (a) the rediscovery of methotrexate (MTX) in the 1980’s and (b) the introduction of biologic agents in the 1990’s. Biologic agents are monoclonal antibodies directed against pro-inflammatory cytokines (e.g. TNFα) that play a major role in the pathophysiology of RA (4). These agents include infliximab and adalimumab (both antibodies against TNFα) and etanercept (soluble TNFα receptors). At present, biologic agents have an established place in the treatment of patients with RA, in principle in combination with MTX. This manuscript provides an overview of the current status of MTX and leflunomide in the treatment of RA-patients, as monotherapy or in combination with other DMARDs or biologic agents. It is a review of the leading manuscripts involving RA treatment. The efficacy of drugs in the various trials is defined by the ACR response criteria (developed by the American College of Rheumatology), that is the 20%, 50% and 70% improvement in RA disease activity. In Europe, rheumatologists also commonly use the Disease Activity Score 28 (DAS28), evaluating swelling and tenderness of 28 joints and counting BSE scores and patient-reported disease activity, to scale disease severity of RA patients (5).

Methotrexate in rheumatoid arthritis

MTX is a folate analogue originally developed in the 1940, as a highly selective inhibitor of dihydrofolate reductase (DHFR) (6). Its beneficial effect in reduction of RA synovitis was first reported in 1951. Subsequently, its efficacy in RA was proven in a series of papers in the mid 1980’s and nowadays it is the anchor drug in the treatment of RA-patients, either as monotherapy or in combination therapy as recommended by the American College of Rheumatology (ACR).

The following features of MTX are supposed to explain its efficacy in RA:

1. Induction of apoptosis in T cells or monocytic/macrophage cells,
2. Inhibition of the production of proinflammatory cytokines: IL1 and IL6,
3. Stimulation of the production of anti-inflammatory cytokines: IL4 and IL10 and
4. Inhibition of metalloproteinase production, thereby reducing cartilage and bone destruction (7)
MTX is metabolised in the liver to active polyglutamylated forms and inactive 7-hydroxy-methotrexate forms. Approximately 60% is bound to albumin. The elimination half-life increases with dose and ranges form 3 to 15 hours. MTX and its metabolites are predominantly renally excreted. Hence, drugs that might influence renal function should be co-administered with caution (8).

MTX can be administered orally, parenterally (subcutaneously or intramuscularly). The bioavailability of low oral doses MTX (up to 10 mg) is relatively high but decreases with higher doses. Hence, for a patient not responding to higher doses of MTX (25 mg/week) parenteral administration should be considered. MTX is administered once weekly, with a starting dose of 7.5 mg, up to 25-30 mg/week. In the Netherlands, it is recommended to prescribe folic acid or folinic acid along with MTX-therapy to reduce systemic toxicity (9).

**MTX monotherapy**

Open studies in the early 1980’s indicated the efficacy of MTX, up to 15 mg per week, in patients with RA refractory to other DMARDs (10, 11). The efficacy of MTX was confirmed in placebo-controlled studies assessing dosages up to 25 mg per week (12, 13). A meta-analysis of these studies showed that patients treated with MTX had a 39% reduction in painful joints and 29% reduction in swollen joints compared with placebo (14).

Long-term observational studies 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. Moreover, from these studies it has been proven that MTX prevents joint damage in RA patients (15).

**MTX in combination with other DMARDs**

So far, five comparative trials have been published investigating combination therapy of sulfasalazine and MTX (16-20) (see also Table 1). One trial indicated that triple therapy consisting of sulfasalazine, MTX and hydroxychloroquine was more effective than MTX alone or the sulfasalazine/hydroxychloroquine combination (21). Another comparative trial showed no efficacy differences between sulfasalazine alone, MTX alone, and their combination (17). Two trials had a step-down approach (16, 19). In one study the combination sulfasalazine, MTX, hydroxychloroquine and low-dose prednisolone was compared with sulfasalazine alone, which could be replaced by MTX alone (19). In the combination therapy group, prednisolone and MTX could be discontinued provided remission was achieved during the first year. The remission rate in this group after two years was doubled compared with the sulfasalazine group.

In the COBRA-trial (16) 56 patients with early active RA were randomised to either treatment with a combination of sulfasalazine, MTX and prednisolone (COBRA-schedule) or sulfasalazine alone. Prednisolone and methotrexate were tapered and stopped after 28 and 40 weeks, respectively. The primary efficacy outcome measure was a composite index comprising improvement in erythrocyte sedimentation rate, grip strength, tender joint count, global assessment by the observer, and improvement in functional ability. The improvement of this pooled index at week 28 was strongly in favour of the combination-therapy group, but at week 56 the improvement in both groups was equal. The efficacy of the COBRA schedule was further demonstrated in a larger cohort of RA patients (22).

At present, there is no evidence that the combination of MTX and sulfasalazine is superior to monotherapy with these agents. The initial addition of prednisolone improved the clinical efficacy and might have long-term benefits in the treatment of RA-patients. Recently in-vitro studies showed that sulfasalazine is a potent inhibitor of the Reduced Folate Carrier (RFC; carrier of MTX), which may explain the lack of potentiation of the combination therapy of MTX and sulfasalazine compared to monotherapy with these drugs (23).

**MTX in combination with biological agents**

If, because of loss of DMARD efficacy, RA-patients switch to ‘third-line’ treatment with biological agents, MTX usually remains included in therapeutic protocols. There is evidence

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**Table 1: Combinations of MTX with other DMARDs and biological agents in current clinical treatment of Rheumatoid Arthritis.**

<table>
<thead>
<tr>
<th>DMARDs</th>
<th>Remark</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTX + SSZ</td>
<td>COBRA schedule</td>
<td>(17)</td>
</tr>
<tr>
<td>MTX + SSZ + prednisolone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTX + HCHQ</td>
<td>O’Dell schedule</td>
<td>(20)</td>
</tr>
<tr>
<td>MTX + HCHQ + SSZ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTX + leflunomide</td>
<td></td>
<td>(34)</td>
</tr>
</tbody>
</table>

**Biological agents**

<table>
<thead>
<tr>
<th>Biological agents</th>
<th>Remark</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTX + Infliximab (anti-TNFα)</td>
<td>Chimeric Moab to TNFα</td>
<td>(25)</td>
</tr>
<tr>
<td>MTX + Etanercept (anti-TNFα)</td>
<td>Soluble TNFα receptor II</td>
<td>(27)</td>
</tr>
<tr>
<td>MTX + Adalimumab (anti-TNFα)</td>
<td>Human Moab to TNFα</td>
<td>(30)</td>
</tr>
<tr>
<td>MTX + Anakinra (anti-IL1β)</td>
<td>IL-1β antagonist</td>
<td>(35)</td>
</tr>
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**Abbreviations:** SSZ, sulfasalazine; HCHQ, hydroxychloroquine; MTX, methotrexate; TNFα, Tumor Necrosis Factor α; IL-1β, Interleukin-1β.
that MTX administration prolongs the duration of response to biological agents, probably due to the inhibition of the formation of antibodies against these agents. In several trials the efficacy of the combination therapy of MTX plus a biological agent was compared with monotherapy with MTX or the biological agent (Table 1).

Infliximab (anti-TNFα antibody) was administered in doses of 1, 3 and 10 mg/kg, with or without weekly MTX (24). Significant clinical benefit was seen in the combination therapy group, with responses up to 55% at week 26 compared with 35% in patients who were treated with MTX alone. This trial indeed indicated that MTX administration prolonged the duration of response to infliximab.

In a later trial, 428 patients with established RA who were treated with MTX were randomised to either infliximab 3 or 10 mg/kg given monthly or bimonthly, or placebo (25). At week 30, the response criteria were met by 53% and 50% of patients receiving the 3 mg/kg every 4 or 8 weeks, respectively, and 58% and 52% for those receiving 10 mg/kg every 4 or 8 weeks. In the placebo group, 20% of the patients met the response criteria at week 30.

The BeSt trial is a multicenter, randomized, single-blind trial conducted in the Netherlands, including 598 patients with newly diagnosed RA who were not previously treated with DMARDs (26). In this trial 4 different treatment strategies in early rheumatoid arthritis were compared. The one-year follow-up results indicated that patients on a step-down therapy from either MTX + sulfasalazine + prednisone 60 mg tapered to 7.5 mg or a treatment with MTX + infliximab showed a faster clinical response (reflected by a reduction in DAS) and less radiological damage than patients on either a sequential monotherapy starting with MTX → sulfasalazine → leflunomide or a step-up therapy including MTX + sulfasalazine + hydroxychloroquine. Interestingly, follow-up analysis beyond one year showed that all four therapy arms were equally efficacious in DAS reduction.

The results of the ASPIRE trial in 1049 patients with early RA indicated that infliximab plus MTX was more effective than MTX alone in preventing joint destruction and disability (2). The response criteria were met by 66%, 62% and 54% of patients treated with 3 mg/kg plus MTX, 6 mg/kg plus MTX, and placebo plus MTX, respectively. Progression of joint destruction in the infliximab plus MTX arms were significantly decreased compared to the placebo plus MTX arm.

Studies investigating the combination of Etanercept, (soluble TNFα receptor) and MTX showed that in patients with advanced RA, who were not responding to MTX, the addition of etanercept 25 mg twice weekly resulted in a marked clinical improvement (27). At 6 months, 71% of the patients treated with etanercept plus MTX achieved the response criteria versus 27% of the patients treated with placebo plus MTX.

Etanercept was also compared with MTX in another trial involving 632 patients with early RA (28). Patients were either treated with etanercept twice-weekly 10 mg or once weekly 25 mg or MTX monotherapy for one year. During the first 6 months, the proportion of patients achieving the response criteria was significantly higher in the etanercept 25 mg group than in the MTX monotherapy group.

In 2004 the results of the TEMPO study have been published (29). This study is a comparative double-blind trial evaluating etanercept with or without MTX versus MTX alone. After 1 year, the 20% response criteria were met by 85% of patients treated with etanercept plus MTX, 76% of patients treated with etanercept alone and 75% of patients treated with MTX. 43%, 24% and 19% of the patients receiving the combination, etanercept alone or MTX alone, respectively, achieved the 70% response criteria. Of the patients with combination therapy, 80% experienced no radiographic progression throughout one year, compared with 68% of patients treated with etanercept alone and 57% of patients treated with MTX alone.

FDA-approval of adalimumab (a complete human antibody against TNFα) was based on several randomised double-blind trials. In the ARMADA-trial 271 RA-patients, who had an inadequate response to MTX and had no response to other DMARDs, were treated with adalimumab (either 20, 40 or 80 mg) or placebo every 2 weeks (30). At week 24, patients receiving treatment with adalimumab 40 mg every other week achieved the 20% response criteria in 65% of cases and the 70% response criteria in 24% of cases, compared with response rates of 15% and 3% for placebo, respectively.

In summary, there is a strong indication that the combination of MTX plus one of the mentioned biologic agents is superior above monotherapy with MTX or the biologic agent, not only in reducing disease activity but also in reducing joint damage as seen on X-rays of hands and feet.

**Leflunomide in rheumatoid arthritis**

Following oral administration, leflunomide is rapidly converted in the gut wall and liver to the active metabolite A 77 1726. Binding of A77 1726 to the enzyme dihydro-orotate dehydrogenase results in inhibition of pyrimidine biosynthesis (31, 32). A consequence of this inhibition is a reversible cell cycle arrest in rapidly dividing cell populations such as activated lymphocytes; the immune cells that mediate joint inflammation. Elimination of leflunomide from the body is by both renal and gastro-intestinal routes. The mean half-life of leflunomide is 14-16 days. Administration of cholestyramine in a dosage of 4 gram 3 times daily reduces the half-life of leflunomide to approximately one day, indicating that significant levels of the drug undergo enterohepatic circulation. Due to the long half-life of the active metabolite, a starting dose of 100 mg, taken daily for 3 days, has been suggested to reach effective steady-state blood levels within 4-6 weeks.

**Leflunomide monotherapy**

Recently, a systemic review and meta-analysis of six double-blind comparative trials
with leflunomide was published (33). In these trials, 1144 patients were treated with leflunomide, 680 were on MTX, 132 on sulfasalazine and 312 patients got placebo. Overall, the trials indicated that leflunomide is significantly superior to placebo at 6 and 12 months treatment and has a similar efficacy compared to sulphasalazine and MTX, however leflunomide efficacy has a more rapid onset than MTX and sulfasalazine. After 24 months leflunomide was more effective than sulfasalazine and had equal efficacy compared with MTX.

**Combination of leflunomide and MTX**

The rationale for combination therapy of leflunomide with MTX is the simultaneous inhibition of the purine metabolism by MTX and inhibition of the pyrimidine metabolism by leflunomide, resulting in a subsequently more efficient inhibition of proliferation of inflammatory cells.

An open label study, in which leflunomide was added to MTX, showed that the combination was well tolerated and efficacious in 53% of patients. However, liver enzyme elevations were observed in 63% of patients. Subsequently, a 24-week double blind, placebo-controlled trial in RA patients with a partial response to MTX was conducted. A total of 130 patients were treated with leflunomide 100 mg/day for two days followed by 10 mg/day. This dosage could be increased to 29 mg/day in case of persistent active disease. Elevated liver enzymes were seen in 28% of patients and the authors concluded that this combination could be administered safely, provided appropriate laboratory monitoring. The 20% response criteria at week 24 were met by 46% of the leflunomide-treated patients compared to 20% of the placebo treated patients. The 70% response rates were 10% and 2% in patients receiving leflunomide and placebo, respectively (34)

**Conclusion**

The anti-folate drug MTX and, to a lesser extend, the anti-pyrimidine drug leflunomide have an established place in the treatment of patients with rheumatoid arthritis. At present these DMARDs are often prescribed in combination schedules with other DMARDs or in combination with biological agents. There is accumulating clinical evidence that MTX in the COBRA schedule or MTX in combination with a biological agents is superior above monotherapy, not only in reducing disease activity but also in reducing joint damage. Finally, co-administration of MTX is supposed to prolong drug-survival of the biological agents, probably by preventing antibody formation against these agents.
References


Enhanced capacity of selected methotrexate-analogues to inhibit TNFα production in whole blood from RA patients

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Submitted for publication
Abstract

Objective: Although methotrexate (MTX) is the anchor drug in the treatment of patients with rheumatoid arthritis (RA), many patients experience clinical resistance to MTX upon prolonged treatment. Here we explored whether novel generation antifolate drugs elicit improved anti-inflammatory properties compared to MTX, based on their capacity to inhibit TNFα production by activated T-cells from RA patients.

Methods: T-cells in whole-blood from 18 RA patients (including MTX-naïve, MTX responsive and MTX non-responsive patients) and 7 healthy volunteers were stimulated with αCD3/αCD28 antibodies and incubated ex-vivo with MTX and eight novel antifolate drugs with potential favourable pharmacological properties. Inhibition of TNFα production was measured after 72 hours by ELISA and drug concentrations exerting 50% inhibition of TNFα production (IC-50) were monitored as an estimate for their anti-inflammatory capacity. In parallel, induction of T-cell apoptosis was studied by FACS analysis.

Results: The novel generation antifolates PT523, PT644, raltitrexed and GW1843 proved to be potent inhibitors of TNFα production in activated T-cells from all three groups of RA patients as well as healthy volunteers. Based on IC-50 values, these antifolates were 1.3-10.3 fold more potent than MTX. The anti-inflammatory effects were observed at drug-concentrations that provoked apoptosis in 20-40% of activated T-cells. Beyond this, also suppression of T-cell activation was observed, being partially independent from apoptosis induction.

Conclusion: In an ex-vivo setting, novel generation antifolate drugs elicited effective inhibition of TNFα production by induction of apoptosis in activated T-cells from RA patients. Conceivably, further clinical evaluation is warranted to investigate whether low dosages of one of these novel antifolate drugs can induce immuno-suppressive effects equivalent to MTX, or be superior to MTX in patients who do not respond to MTX.

Introduction

For many decades the folate antagonist methotrexate (MTX) is considered the anchor drug in the treatment of neoplastic diseases (1) as well as chronic inflammatory diseases such as rheumatoid arthritis (RA) (2). From an anti-cancer perspective, the molecular basis for the anti-proliferative activity of MTX relies on inhibition of key enzymes in folate metabolism, e.g. dihydrofolate reductase (DHFR) or thymidylate synthase (TS) and folate dependent enzymes in purine biosynthesis de novo (3). These features lead to intracellular folate depletion and diminished pools of purines and pyrimidines required for DNA synthesis. Anti-proliferative properties of MTX may also be of relevance in RA treatment, but the anti-inflammatory properties of MTX have most been related to its ability to inhibit the release of pro-inflammatory cytokines such as tumor necrosis factor α (TNFα) or interleukin 1β (IL-1β) (4). Multiple mechanisms for these anti-inflammatory effects have been postulated (2, 4-6): (a) MTX-induced extracellular release of adenosine by inhibition of the enzyme AICARFase (involved in purine biosynthesis), causing anti-inflammatory signalling via the adenosine receptor (7-9), (b) MTX-induced inhibition of the first enzyme in purine biosynthesis de novo: amidophosphoribosyltransferase (10), (c) apoptosis of activated T-cells (11) possibly facilitated by MTX-induced generation of reactive oxygen species (12, 13), (d) MTX-induced inhibition of methyltransferases leading to impairment of Ras signalling (14) and/or (e) direct inhibition of the activation of the transcription factor NFκB that controls the transcription of TNFα and IL1β (15).

Notwithstanding the potent anti-rheumatic capacity of MTX, up to 50% of RA patients ultimately experience clinical resistance to MTX during chronic therapy (16). In this context, still little is known about the mechanism(s) of resistance to MTX in RA patients (17). For MTX as an anti-cancer agent, several mechanisms of resistance have been identified, including (a) down regulation of the reduced folate carrier (RFC) leading to impaired cellular uptake of MTX, (b) decreased cellular retention of MTX due to impaired polyglutamylation by folylpolyglutamate synthetase (FPGS), (c) elevated levels of the target enzymes of MTX: dihydrofolate reductase (DHFR) and thymidylate synthase (TS), (d) enhanced cellular efflux of MTX by specific members of the ATP-Binding Cassette family of drug efflux transporters, and/or (e) increased folate levels that compete with MTX for cellular uptake and polyglutamylation (18-24).

Extended knowledge on the molecular basis of resistance mechanisms to MTX has facilitated the rational design of new generation antifolate drugs with the potential ability to circumvent MTX-resistance due to any of the above-mentioned mechanisms. In this context, second and third generation of folate-antagonists exhibit one or more of the following characteristics: (a) are transported more efficiently via the RFC, (b) have higher affinity for FPGS or being independent of polyglutamylation for their activity, (c) have the ability to target other enzymes in the folate metabolic pathway besides DHFR like TS and glycaminide ribonucleotide transformylase (GARTFase), (d) exhibiting poor affinity for drug efflux transporters or (e) show retention of activity regardless of extra/intracellular folate status (reviewed in (18, 25)). The present study was undertaken to assess the anti-inflammatory effects of a selected set of these novel generation antifolates. To this end, we investigated the potency to suppress the release of the pro-inflammatory cytokine TNFα from activated T cells in whole blood cell cultures of RA patients, including those who were clinically unresponsive to MTX.
Materials and methods

Patient characteristics
All patients included in this study signed an informed consent form and the study on ‘DMARD-resistance’ was approved by the Medical Ethics committee of the VU University Medical Center, Amsterdam, The Netherlands. All patients fulfilled the American College of Rheumatology (formerly, the American College of Rheumatism Association) 1987 revised criteria for RA and were on stable doses of MTX (15-30 mg/week) for at least three months. Newly diagnosed RA patients, who were not yet receiving Disease Modifying Anti-Rheumatic Drugs (DMARDs), were included as MTX-naïve patients. Characteristics of RA patients included in this study were: 18 RA patients (male/female: 5/13; mean age: 52 years; mean DAS28 score: 4.3, range 1.3-7.2). Five patients were MTX-naive and 13 patients received MTX; RA-patients were defined as MTX-non-responders if they had a DAS28 score of >3.2 at the time of enrolment (n=7), patients with a DAS28 score of ≤3.2 were defined as MTX-responders (n=6). In addition, seven healthy volunteers were included (male/female: 4/3; mean age: 38 years).

Drugs
Methotrexate was a kind gift of Pharmachemie, Haarlem, The Netherlands. Pemetrexed/ALIMTA® (Eli Lilly) was obtained via the VUmc pharmacy department. The following folate antagonist drugs were obtained from the indicated companies/institutions; Raltitrexed/Tomudex®/ZD1694 (26) (Astra-Zeneca, UK), PT523 and PT644 (27, 28) (Dr. A. Rosowsky, Harvard Medical School, Boston, U.S.A), TMQ (Trimetrexate/Neutrexin®; Warner Lambert Park Davis, now MedImmune Pharma Netherlands) (1, 25), Plevitrexed/BGC9331 (30) (BTG International Ltd) and AG2037 (31) (Agouron/Pfizer, San Diego, U.S.A). Chemical structures of these folate antagonists are provided in the supplementary file. A schematic overview of their targets in the folate metabolism is depicted in Figure 1. Properties regarding affinities of these drugs for RFC and FPGS are shown in Table 1.

T-cell activation and TNFα ELISA procedures
Whole blood of 18 RA patients and 7 healthy volunteers was collected in heparin containing tubes that were tested to be endotoxin-free (Becton Dickinson; San Jose, CA, U.S.A.). Blood was diluted 1:10 with DMEM medium supplemented with heparin (15 U/ml), 100 µg/ml penicillin/streptomycin and incubated ex-vivo with αCD3/αCD28 antibodies (0.1 µg/ml and 1 µg/ml, respectively) as T-cell activating agents as described previously (32). To these incubates 7 concentrations (range: 0-300 nM) of antifolates were added, followed by an incubation period of 72 hours. Inhibition of TNFα production was measured after 72 hours drug exposure by ELISA as described before (32).

The concentration of each drug required to inhibit TNFα production by 50% is defined as the anti-inflammatory IC50 concentration.

FACS analysis
Peripheral blood mononuclear cells (PBMCs) of RA patients were isolated by Ficoll-Paque Plus density centrifugation and suspended in DMEM-medium supplemented with 10% autologous donor-serum. Apoptosis in PBMCs was analyzed by flow cytometry (FACS) after 72 hours exposure to MTX and novel anti-folate drugs, using Annexin-V-FITC/7-aminoactinomycin D (7-AAD) staining (APOPTEST™-FITC A700, VPS Diagnostics, Hoeven, the Netherlands) according to the manufacturer’s protocol. At the highest drug-concentrations, 2 µM of the natural reduced folate 5-formyltetrahydrofolate/leucovorin (Merck Eprova, Switzerland) was added to test folate competition-induced prevention of apoptosis. Phycoerythrin labeled anti-CD25 (Becton Dickinson; San Jose, CA, U.S.A.) was used for monitoring of T-cell activation.
Novel antifolate drugs are potent inhibitors of TNFα production by activated T-cells from healthy volunteers and RA patients

To explore whether novel folate antagonists could elicit potential anti-inflammatory effects, the efficacy of these drugs was tested to inhibit TNFα production by activated T-cells in whole blood cultures of healthy controls and RA patients, including MTX-naïve, MTX-responsive and MTX non-responsive patients. TNFα levels were determined after 72 hours of continuous drug exposure, as at this time T-cell activation appeared to be optimal according to CD25 expression (Figure 2). Basal levels of TNFα production after T-cell stimulation were not significantly different between healthy controls ($3208 \pm 2554$ pg/ml) and (subgroups of) RA patients ($3400 \pm 2818$ pg/ml). For each individual patient, the IC-50 values for TNFα inhibition by all nine folate antagonists are shown in Figure 3. The drugs are grouped according to their target in the folate metabolic pathway (see figure 1): (1) the group of primary DHFR inhibitors: MTX, TMQ, PT523 and PT644 (Figure 3A-D); (2) the group of primary TS inhibitors: Raltitrexed, Pemetrexed, GW843 and BGC9331 (Figure 3E-H) and the CARGFase inhibitor AG2037 (Figure 3I). From these figures, an overview of all median IC-50 values is presented in Table 1.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Target</th>
<th>RFC affinity</th>
<th>FPGS affinity</th>
<th>Controls RA-total DMARD naive Responders Non-responders</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTX</td>
<td>DHFR</td>
<td>++</td>
<td>-</td>
<td>53 (48-65) 60 (16-325) 55 (39-102) 47 (18-260) 60 (16-325)</td>
</tr>
<tr>
<td>TMQ</td>
<td>DHFR</td>
<td>-</td>
<td>-</td>
<td>254 (21-300) 232 (44-461) 259 (51-461) 185 (34-278) 157 (24-355)</td>
</tr>
<tr>
<td>PT523</td>
<td>DHFR</td>
<td>+++</td>
<td>-</td>
<td>3.0 (1.9-23) 7.4 (1.9-10) 20 (4.6-10) 7.0 (1.9-10) 6.2 (1.9-33)</td>
</tr>
<tr>
<td>PT644</td>
<td>DHFR</td>
<td>+++</td>
<td>-</td>
<td>2.0 (1.0-25) 5.8 (2.3-11) 29 (6.3-10) 8.4 (3.9-35) 2.8 (2.3-11)</td>
</tr>
<tr>
<td>Raltitrexed</td>
<td>TS</td>
<td>+++</td>
<td>+++</td>
<td>4.0 (2.6-10) 8.1 (3.3-15) 46 (3.7-90) 9.0 (3.3-93) 8.3 (6.7-152)</td>
</tr>
<tr>
<td>Pemetrexed</td>
<td>TS</td>
<td>+++</td>
<td>+++</td>
<td>41 (22-88) 46 (7.2-366) 75 (17-427) 36 (19-79) 30 (2.2-346)</td>
</tr>
<tr>
<td>GW843</td>
<td>TS</td>
<td>+++</td>
<td>+++</td>
<td>3.0 (2.2-17) 7.6 (2.5-32) 37 (5.5-122) 6.4 (3.5-42) 7.0 (6.0-11)</td>
</tr>
<tr>
<td>BGC9331</td>
<td>TS</td>
<td>+++</td>
<td>-</td>
<td>27 (19-59) 47 (8.7-405) 68 (25-495) 58 (16-195) 29 (8.7-405)</td>
</tr>
<tr>
<td>AG2037</td>
<td>CARGFase</td>
<td>+++</td>
<td>+++</td>
<td>104 (56-208) 143 (79-250) 313 (184-455) 152 (94-625) 119 (79-1250)</td>
</tr>
</tbody>
</table>

Table 1: Properties of MTX and novel generation folate antagonists.

Properties of MTX and novel folate antagonists regarding affinity for the reduced folate carrier (RFC) and folylpolyglutamate synthetase (FPGS). Kinetic properties (affinity; in µM) of RFC and FPGS for folate antagonist drugs are categorized as follows: RFC affinity Km: <5 µM (+++), Km: 5-25 µM (++), Km: 25-100 µM (+), no substrate (-). FPGS affinity: Km: <5 µM (+++), Km: 5-25 µM (++), Km: 25-100 µM (+), no substrate (-). Anti-inflammatory properties of MTX and novel folate antagonists in ex vivo activated T-cells from healthy controls and RA patients are depicted as median drug concentrations (nM) required to inhibit TNFα production by 50% (IC-50). The RA group is sub-categorized in MTX-naïve patients, MTX-responders and MTX non-responders. *Asterisk denotes significant differences between RA-patients and healthy volunteers (p< 0.05).

Statistical analysis

Statistical analysis was performed using non-parametric tests. For determining significant differences in anti-inflammatory IC-50 values between healthy controls and RA patients the two tailed Mann-Whitney test was used. A p-value <0.05 was considered statistically significant.

Results

Novel antifolate drugs are potent inhibitors of TNFα production by activated T-cells from healthy volunteers and RA patients

Four folate antagonists (PT523, PT644, raltitrexed and GW843) could be identified with a greater potency (up to 10-fold) than MTX in RA T-cells, two had comparable activity (pemetrexed and BGC9331), while two (TMQ and AG2037) were less potent (up to 4-fold) than MTX. Data from Figure 3 further revealed a marked inter-patient variability in sensitivity for the drugs. In general, IC-50 values for TNFα release were lower for healthy volunteers compared to RA patients, but these differences did only reach statistical significance for the TS inhibitors Raltitrexed and GW843 (p<0.05). Of note, compared to healthy controls, 5 patients within the total group of 18 RA patients (equally distributed among RA subgroups) were less sensitive to MTX and most of the antifolate drugs. Interestingly, a slightly greater potency in suppressing TNFα production was observed in the group of MTX treated RA patients compared to MTX naïve patients (Table 1; not statistically significant). Clinical unresponsiveness to MTX was not reflected by higher IC-50 values in the whole blood assay; T-cells from clinically MTX non-responsive RA patients were equally
sensitive to MTX compared to T-cells from MTX responsive RA patients. Similarly, T-cells from MTX non-responsive RA patients were equal sensitive for each of the eight novel antifolate drugs as T-cells from MTX-responsive RA patients.

MTX and novel antifolates induce apoptosis in activated (CD25+) but not resting (CD25-) T-cells and attenuate T-cell activation

Since one of the proposed mechanism of action of low dose MTX is induction of apoptosis in auto-reactive T-cells (11), we determined the onset of apoptosis in PBMC cultures of three drug-naïve RA patients after exposure to MTX and novel generation antifolate drugs. Upon stimulation with αCD3/αCD28, polyclonal expansion of T-cells was observed microscopically between 48 and 72 hours along with a 1.5-2 fold increase in cell counts. For all antifolates tested, we observed a dose dependent decrease in CD25+ cells, which was partially independent of apoptosis (Figure 4; white bars). Furthermore, we observed apoptosis in up to 70% of activated (CD25+) T-cells (Figure 4; dark grey bars), but not in resting (CD25-) T-cells (Figure 4; light grey bars). To verify the role of apoptosis in inhibition of TNFα production by activated T-cells, we calculated the level of apoptosis at the IC-50 concentration for TNFα production in RA whole-blood for all nine antifolate drugs. As shown in Figure 5, at the IC-50 value for inhibition of TNFα production, 20-40% apoptosis was observed in activated (CD25+) T-cells, except for PT523, exhibiting a more apoptosis-unrelated anti-inflammatory effect. Addition of the natural reduced folate leucovorin (2 µM) (partially) prevented apoptosis induction and inhibition of T-cell activation by MTX, PT523, TMQ, Raltitrexed and Pemetrexed, suggestive for competitive effects with these antifolates. No rescue effect of leucovorin was observed for PT644, GW1843, BGC9331 and AG2037, suggesting that the activity of these anti-folates are not influenced at this concentration of leucovorin.
Figure 3: Inhibition of TNFα release from activated T-cells in whole blood cell cultures by antifolate drugs.

Drug concentrations (nM) establishing 50% inhibition of TNFα release are depicted as IC_{50} values for each patient/control. Horizontal lines represent median IC_{50} values. Tested drugs include DHFR inhibitors: (A) MTX, (B) PT523, (C) PT644, (D) TMQ; TS inhibitors: (E) Raltrexed, (F) Pemetrexed, (G) GW1843, (H) BGC9331 and the GARTFase inhibitor: (I) AG2037.

Figure 4: Inhibition of T-cell activation and apoptosis induction in PBMCs of RA patients by antifolate drugs.

PBMCs of RA patients were stimulated with αCD3/αCD28 for 72 hours in the absence or presence of MTX and novel generation antifolate drugs. FACS analysis was used to monitor T-cell activation (CD25 expression) and apoptosis induction (Annexin-V/7AAD staining). Tested drugs include; DHFR inhibitors: (A) MTX, (B) PT523, (C) PT644, (D) TMQ; TS inhibitors: (E) Raltrexed, (F) Pemetrexed, (G) GW1843, (H) BGC9331 and the GARTFase inhibitor: (I) AG2037.

White bars represent the percentage of activated (CD25+) T-cells; Light grey bars represent the percentage of apoptotic (Annexin-V+/7AAD+) cells in the CD25- cell population; Dark grey bars show the percentage of apoptotic (Annexin-V+/7AAD+) cells in the CD25+ cell population. At the highest drug-concentrations, reversibility of apoptosis was tested by addition of 2 µM of the natural folate leucovorin. Results depicted represent the mean ± S.D. of 3 separate experiments.
Discussion

The current study demonstrates that selected novel generation folate antagonists (25) share with MTX the ability to exert potential anti-inflammatory effects by suppressing the release of TNFα by activated T-cells in whole blood from RA patients. Notably, this property is observed at low drug dosages and continuous exposure (72 hours) for: (a) non-polyglutamatable DHFR inhibitors (TMQ, PT523 and PT644), (b) folate-based TS inhibitors; both polyglutamatable (Raltitrexed, Pemetrexed, GW1843) and non-polyglutamatable (BGC9331), and (c) a folate-based GARTFase inhibitor (AG2037). Moreover, the current study indicates that induction of apoptosis in activated T-cells by antifolate drugs explains at least part of the the anti-inflammatory properties, as described previously for MTX (11). Finally, we observed attenuation of T-cell activation at low concentrations of anti-folate drugs, an effect that was partially unrelated to apoptosis induction. This latter observation might be supportive for additional mechanisms of action conveyed by MTX and other anti-folate drugs (7, 9, 15).

We observed a marked inter-patient variability in anti-inflammatory activity of novel antifolates, which could not be related to differences in basal levels of TNFα production or clinical response to MTX treatment. However, the whole-blood assay (32) proved to be an useful clinical mimick to assess differential potencies of drugs as described here for a series of folate antagonists. Nonetheless, it should be anticipated that besides T-cells, monocytes/macrophages are contributors to TNFα production in this assay, as they get stimulated by cytokines (like IL-17) produced by activated T-cells (33).

Four folate antagonists (PT523, PT644, raltitrexed and GW1843) could be identified with a greater potency (up to 10-fold) than MTX to inhibit TNFα production. Since the RFC is the dominant folate antagonist transport route in T-cells, at least part of the enhanced activity by these novel folate antagonists over MTX can be attributed to their more efficient cellular uptake via this transporter (18, 19, 34, 35).

One important parameter for the efficacy of folate antagonists ex-vivo and in-vivo is the extracellular and intracellular folate status, as natural folates may compete for cellular uptake and polyglutamylation, respectively (36). The wide-spread application of folate supplementation to RA patients (37) along with folate food fortification programmes of the past decade resulted in markedly higher plasma folate levels in RA patient populations (38), which could confer diminished efficacy of MTX. Beyond MTX, the activity of the TS inhibitor pemetrexed and the lipophilic DHFR inhibitor TMQ was also found to be influenced by changes in folate status, while other drugs (e.g. BGC9331 and GW1843) retain potent activity regardless of folate status (22, 36, 39). Consistently, such a profile was also observed in Figure 4, revealing that addition of leucovorin rescued activated T-cells from apoptosis induced by MTX, TMQ, Raltitrexed, Pemetrexed and to some extent PT523, whereas the efficacy of PT644, BGC9331 and GW1843 was not influenced by this natural folate. On top of high folate levels, exogenous purines may bypass inhibitory effects of AICARTFase and GARTFase inhibitors (40). As a result, the presence of exogenous purines/pyrimidines in whole blood samples of RA patients could have attenuated the efficacy of the GARTFase inhibitor AG2037 in this study, as shown in Figure 4.

From the above considerations, it is anticipated that an enhanced therapeutic effect may be elicited from novel antifolates that have the following properties: (a) high RFC affinity and (b) being less influenced in their activity by fluctuations in the patient’s folate status. The drugs identified from this study that harbor these features are PT644, GW1843 and BGC9331. With respect to the non-polyglutamatable folate antagonists (PT523, PT644 and BGC9331), it should be taken into account that continuous drug exposure will be required for optimal activity of these drugs, as these drugs may otherwise be rapidly effluxed from cells when a short term exposure is followed by a drug-free period (17, 25, 41). Where T-cells almost exclusively depend on RFC for cellular uptake of novel antifolate drugs, it should be mentioned that alternative folate transport routes are operative in activated macrophages in RA synovial tissue. These activated macrophages utilize a folate-receptor (FR) mediated uptake mechanism (42). Given the marked differences in affinities for RFC and FR, novel antifolates with a good affinity for RFC are not necessarily effective against FR expressing activated macrophages. From this perspective GW1843, having a high affinity for both RFC and FR, may be an effective drug for targeting both T-cells and activated synovial tissue macrophages (J.W. van der Heijden, manuscript accepted for publication in Arthritis & Rheumatism 2008).

In light of one of the proposed mechanisms of action of MTX in RA, i.e. via AICARTFase inhibition, adenosine release and adenosine receptor stimulation (5, 43), it is interesting to note that also non-polyglutamatable folate antagonists displayed potential anti-inflammatory effects. By definition, non-polyglutamatable DHFR inhibitors such as TMQ, PT523 and PT644 can not be converted to polyglutamate forms and consequently do not directly target AICARTFase. This suggests that their mechanism of action is probably indirect via dihydrofolate that is built up due to DHFR inhibition (Figure 2), whereby polyglutamate forms of dihydrofolate are potent inhibitors of AICARTFase. Consistent with this notion may be the potential anti-inflammatory properties of non-polyglutamatable DHFR inhibitors such as MX-68 in animal models of arthritis (44) and CH-1054 in a controlled clinical trial for RA treatment (45). Interestingly, the non-polyglutamatable and specific TS inhibitor ZD9331 (46) also exerted, at low dosages, potent TNFα inhibitory effects. While this drug does not convey any inhibitory effects on AICARTFase, this finding suggests that
Beyond inhibition of AICARTFase, inhibition of other key-enzymes in the folate metabolic pathway such as TS also facilitates a potential anti-inflammatory response.

Conclusion

The present study indicates that novel generation antifolates, in particular PT523, PT644, Raltitrexed and GW1843, elicit potential anti-inflammatory effects at low dosages. Even though the clinically accepted good window for MTX efficacy could hold back a timely introduction of these novel generation folate antagonists, some of their superior pharmacological properties over MTX may be exploited in future RA treatment. This option may particularly hold for clinical conditions of MTX resistance (17) or in strategies to improve RA remission. In this context, some recently described prediction models for MTX efficacy and toxicity in RA treatment (47-50) may also be helpful to assess the potential clinical value of second/third generation of folate-based therapeutic drugs. Furthermore, many of the drugs presented in this study have been registered for application as anticancer drugs, with known efficacy and toxicity profiles (25).

Chemical structures of folic acid, leucovorin, methotrexate and novel generation anti-folate drugs.
References

References


Folate receptor-β as potential delivery route for novel folate antagonists to macrophages in synovial tissue of rheumatoid arthritis patients

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Abstract

Purpose: To determine (a) the expression of folate receptor-β (FR-β) in human synovial tissue biopsies and peripheral blood lymphocytes from rheumatoid arthritis (RA) patients and (b) to identify novel folate antagonists being more selective in FR-β targeting and internalization than methotrexate (MTX).

Methods: Immunohistochemistry and computer-assisted digital imaging was used for the detection of FR-β protein expression on immune-competent cells in synovial biopsies of RA patients with active disease and in non-inflammatory control synovial tissue. FR-β mRNA levels were determined by RT-PCR. Binding affinities of FR-β for folate antagonists were assessed by competition experiments for [3H]-folinic acid binding on FR-β-transfected cells. Efficacy of FR-β-mediated internalization of folate antagonists was evaluated by assessment of anti-proliferative effects against FR-β-transfected cells.

Results: Immunohistochemical staining of RA synovial tissue showed high expression of FR-β on macrophages in the intimal lining layer and synovial sublining, 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 RA monocyte-derived macrophages, but low in peripheral blood T-cells and monocytes. Screening of 10 novel generation folate antagonists revealed 4 compounds for which FR-β had a high binding affinity (20-77 fold higher than for MTX). One of these, the thymidylate synthase inhibitor BGC945, displayed selective targeting against FR-β-transfected cells.

Conclusion: Abundant FR-β expression on activated macrophages in synovial tissue from RA patients deserves further exploration for selective therapeutic interventions with high affinity binding folate antagonists, of which BGC945 may be a prototypical representative.

Introduction

The folate antagonist methotrexate (MTX) is the most widely applied disease modifying anti-rheumatic drug (DMARD) in the treatment of patients with rheumatoid arthritis (RA) (5). It is used either as a single agent or in combination with other DMARDs (e.g. sulfasalazine and hydroxychloroquine) and MTX use is obligate in most treatment strategies involving biological agents (anti-TNFα or anti-CD20 monoclonal antibodies) (2-5).

The first pivotal step in the cellular pharmacology of MTX is its cell entry, which can be mediated by at least 3 different routes; the reduced folate carrier (RFC) (6, 7), membrane-associated folate receptors (MFR) (8, 9) or a proton-coupled (low pH) folate transporter (PCFT) (10). The latter transporter is mainly involved in intestinal folate uptake, the other transport routes harbour physiological and pharmacological relevance for immune-competent cells by facilitating the uptake of natural reduced folate cofactors and folate antagonists such as MTX (6). The RFC and MFR differ considerably in the mechanism of (anti)folate uptake (transmembrane carrier vs. endocytosis), substrate specificity (low affinity folic acid/high affinity MTX vs. high affinity folic acid/low affinity MTX) and tissue specificity (constitutive vs. restricted expression). For MFR 3 isoforms (α, β and γ) have been identified. The α-isof orm of MFR is overexpressed in specific types of cancer (e.g. ovarian cancer) while the γ-isof orm is a secreted protein from haematopoietic cells (8).

Interestingly, selective expression of the FR-β isoform has been described on activated macrophages in inflamed synovial fluid of RA patients (11) and animal models of arthritis (12, 13). Subsequently, FR-β was recognized as an attractive target for imaging of arthritis and therapeutically for selective antibody-guided or folate-conjugate guided delivery of toxins and other small/macro-molecules (14-15). Thus far, targeting of FR with folate antagonists has only been explored on cancer cells/tissues overexpressing FR-α (16, 17).

Over the past two decades, a second generation of folate antagonists has been designed and clinically evaluated from a perspective of circumventing common mechanisms of resistance to MTX, including impaired transport via the RFC, defective polyglutamylation, increased activity of the target enzyme DHFR and/or enhanced drug efflux (2). Based on this background, second generation antifolates include compounds that are more efficiently transported via the RFC, are more efficiently polyglutamylated or being independent of polyglutamylation, or target other key enzymes in folate metabolism than DHFR, e.g. thymidylate synthase (TS) or glycaminde ribonucleotide transformylase (6, 18, 19). In the present study we set out to investigate whether distinct second generation antifolates may serve as selective targeting drugs for FR-β expressing cells in synovial tissue and/or immune-competent cells of RA patients. We identified the TS inhibitor BGC945 as a prototypical antifolate drug that fulfilled the criteria of a high FR-β binding affinity and a low RFC affinity, thereby enabling selective uptake by FR-β expressing cells.

Materials and methods

Drugs
Folic acid was obtained from Sigma Chem Co, St. Louis, Mo. L-Leucovorin was purchased from Merck Eprova, Schaffhausen Switzerland. Methotrexate was a kind gift of Pharmachemie, Haarlem, The Netherlands. Pemetrexed/ALIMTA® (Eli Lilly) (20) was obtained via the VUmc pharmacy department. The following folate antagonist drugs were obtained from the indicated companies/ institutions; raltitrexed/Tomudex®/ZD1694 (21) (AstraZeneca, UK), PT523 and PT644 (22, 23) (Dr. A. Rosowsky, Harvard Medical School, Boston, MA),
GIW843 (24) (Glaxo-Wellcome, USA), CB300635 (29) (Institute of Cancer Research, Sutton, UK), Plevitrexx/BGC 9371 (26) and BGC 945 (6R,S and 6S) (27) (BTG International Ltd), 5,10-dideazatetrahydrofolate (DDATHF) (28) (Eli Lilly, Indianapolis, IN), and AG2034 (29) (Agouron Pfizer Pharmaceuticals, San Diego, Ca). The chemical structures of these folate antagonists are provided in Supplementary Figure. Preclinical and clinical background information on these antifolates is published in (6, 18). [3',5',7,9-3H]-folic acid (20-40 Ci/mmol, MT783) was purchased from Moravek, Brea, Ca.

Cell lines

Wild type Chinese Hamster Ovary (CHO/WT) cells, CHO cells transfected with FR-β (CHO/FR-β) (30) and human nasopharyngeal epidermoid KB cells, expressing FR-α (31) (American Type Culture Collection, Manassas, VA) were grown in folic-acid free RPMI 1640 medium (Gibco, Grand Island, NY), supplemented with 10% fetal calf serum, 2 mM L-glutamine, 0.15 mg/ml proline and 100 units/ml penicillin and streptomycin. [3H]-folic acid binding capacities of CHO/FR-β and KB cells were 0.5-1 pmol/10^6 cells and 20-40 pmol/10^6 cells, respectively. Human monocytic-macrophage THP-1 cells (American Type Culture Collection, Manassas, VA) were grown in RPMI 1640 medium (Gibco, Grand Island, NY) containing 2.2 µM folic acid, supplemented with 10% fetal calf serum, 2 mM L-glutamine, 0.15 mg/ml proline and 100 units/ml penicillin and streptomycin. All cell lines were kept at 37°C in a humidified atmosphere containing 5% CO₂.

Synovial tissue samples

In this study we analyzed synovial tissue biopsies derived from the knee joints of 15 RA-patients with active disease before treatment and after 4 months of treatment with MTX (starting dose of MTX: 7.5 mg/week; increasing stepwise to 15 mg/week over 12 weeks). Active disease was defined as ≥ 6 swollen or tender joints and levels of moderate or worse on the physician's and patient's assessments of disease activity (Disease Activity Score-28; DAS-28). All patients had at least 1 clinically involved knee joint. Concomitant use of low dose prednisone (< 10 mg/day) and stable doses of nonsteroidal anti-inflammatory drugs (NSAID) was allowed. None of the patients ever used MTX before enrolling the study. In patients taking other DMARDs, the treatment was terminated following a washout phase of 28 days. The arthroscopy procedure was performed as described previously (31) as part of a joined study that was approved by the Medical Ethics committees of the Leiden University Medical Center (The Netherlands) and the Leeds University Medical Center (United Kingdom) (32). As non-inflammatory control synovial tissue we included 7 samples from patients with mechanical joint injury, kindly provided by dr. B.J. van Royen, department of Orthopedic Surgery, VU University Medical Center, Amsterdam, The Netherlands. For peripheral blood sample collection, all patients signed an informed consent form and the study on ‘DMARD resistance’ was approved by the Medical Ethics committee of the VU University Medical Center, Amsterdam, The Netherlands.

FR-β immunohistochemistry

Immunohistochemical staining of cryostat sections (5 μm) of synovial tissue biopsies from RA patients and controls was performed using a 3-step immunoperoxidase method as described previously (33, 34). Sections were stained with a specific antibody for FR-β (30) (dilution 1: 3000) (isotype control: normal rabbit-serum). Macrophages and T-cells were stained with 3A5 (dilution 1: 100) (35) and anti-CD3-PE (dilution 1: 25; Dako, Glostrup, Denmark) monoclonal antibodies (isotype control: mouse immunoglobulin), respectively. Biotinylated swine anti-rabbit IgG (Dako; dilution 1:200) and rabbit anti-mouse IgG (Dako; dilution 1:300) were used as secondary antibodies. Color development was performed using 0.4 mg/ml AEC (aminooethyl carbazole). After counterstaining with hematoxylin, slides were mounted. Stained sections were analysed for FR-β, 3A5 and CD3 expression by digital image analysis, as described previously (36). In short, for each marker representative regions were used for image acquisition, using 40× magnification. These regions were divided into 6 high-power fields (hpf) with a 3-pixel overlap. Positive cells were evaluated by analysing ≥8 consecutive hpf, scoring numbers of positive cells in the intimal lining layer and the synovial sublining per mm².

Double-labeling immunofluorescence

FR-β was detected by swine-anti-rabbit HRP-labelled antibodies (dilution 1:200; Dako, Glostrup, Denmark) and development was with rhodamine/thyramine (red fluorescence) according to the instructions of the manufacturer (dilution 1:1000) (37). CD68 was detected by goat-anti-mouse biotinylated antibodies (dilution 1:100; Dako, Glostrup, Denmark) utilizing streptavidin Alexa-488 as a substrate (dilution 1:750; green fluorescence; Molecular Probes, Eugene, OR). Slides were mounted with Vectashield, containing 1 μg/ml DAPI (for staining of nuclei) (Vector laboratories inc., Burlingame, CA). Cells were examined using a fluorescence microscope (Leica DMRB, Rijswijk, the Netherlands).

Isolation of peripheral blood cells of RA-patients and culture conditions

Peripheral blood mononuclear cells were isolated from freshly obtained blood samples by gradient centrifugation (35° at 400xg) on Ficoll-Paque Plus (Amersham Pharmacia Biotechnologies, UK). After centrifugation the interphase was carefully collected and washed 3 times using Phosphate Buffered Salt solution (PBS) supplemented with 1% BSA. The lymphocyte fraction was counted and resuspended in IMDM culture medium (Invitrogen, Breda, The Netherlands) which contained 10% FCS, 2 mM L-glutamine and 100 μg/ml penicillin and streptomycin. Monocytes were isolated by adherence after 2 hours incubation at 37°C in culture flasks followed by RNA extraction or macropagedifferentiation by culturing the monocytes for 7 days in the presence of 10 ng/ml Macrophage Colony
Stimulating Factor (M-CSF) (Strathmann Biotech, Hamburg, Germany). Peripheral blood lymphocytes (PBLs) remaining in suspension after monocyte adherence were collected for RNA isolation or used for T-cell activation by incubating them at a density of 1 × 10^6 cells/ml with monoclonal anti-CD28 (5 µg/ml, CLB-CD28/1, Sanquin, Amsterdam, The Netherlands) and anti-CD3 (1 µg/ml, CLB-T3/4.E, Sanquin, The Netherlands) in goat-anti-mouse (Dako, Glostrup, Denmark) coated 24 well plates. After 48 hours stimulation, activated T-cells were harvested for RNA isolation and the activation status was determined by measuring CD25 expression using flow-cytometry (FACScalibur, Beckton & Dickinson).

**FR-β mRNA expression in synovial tissue and peripheral blood cells of RA-patients**

RNA from synovial tissue (n=7), PBLs (n=9), monocytes (n=9), macrophages (n=25), and activated T-cells (n=22) from RA patients was isolated using the Qiagen RNeasy Plus isolation kit (Qiagen, Venlo, The Netherlands) following the instructions provided by the manufacturer. Prior to RNA isolation, the frozen synovial tissue samples were powdered by grinding them in a liquid nitrogen pre-chilled mortar where after RPE buffer was added. Total RNA concentrations were determined using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, USA). Real-time reverse transcription-PCR (RT-PCR) methodology described previously by Qi et al (38) was used to measure simultaneously mRNA levels for FR-β and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; reference gene). The reverse transcription step was carried out using Taqman Reverse Transcript Reagents from Applied Biosystems (Foster City, CA), following the protocol of the manufacturer. Briefly, 400 ng of total RNA was mixed with random hexamer primers (50 µmol/L), RNase inhibitor (1 unit/µl), MultiScribe reverse transcriptase (5 units/µL), and deoxynucleoside triphosphate mix (2.5 mmol/L each) in reverse transcriptase buffer. The 10-µL reaction mixture was first incubated at 25°C for 10 minutes, then at 48°C for 30 minutes and finally at 95°C for 5 minutes. The subsequent real-time PCR step for FR-β was carried out in the presence of 12.5 µL of PCR Mastermix (Applied Biosystems), 0.5 µL each of forward primer (CTGGCTCCTTGGCTG-AGTTC) and reverse primer (GCCCAGCCTGGTTATCCA), and 0.5 µL of Taqman probe (6FAM-TCTTCCAGACTACCTGACCTACCAG-TAMERA). The primers and the Taqman probe for control GAPDH gene were purchased from Applied Biosystems. The PCR conditions were 2 minutes at 50°C, 10 minutes at 95°C, followed by 40 cycles of 15 seconds each at 95°C, and finally 1 minute at 60°C. Fluorescence data generated were monitored and recorded by the Gene Amp 5700 sequence detection system (Applied Biosystems). All samples were set up in triplicate and normalized to GAPDH values.

**Results**

**FR-β synovial tissue immunohistochemistry**

Immunohistochemical staining of all RA synovial tissue showed high expression of FR-β in the intimal lining layer as well as in the synovial sublining. The staining pattern for FR-β was consistent with 3A5 (macrophage) staining, whereas T-cell areas showed no staining (figure 1 A-C). In fact, more detailed fluorescence microscopic analysis demonstrated co-localization of FR-β and CD68 (macrophage marker) on the cellular membranes of synovial tissue infiltrating macrophages and intimal macrophages (figure 1 D-F). In non-
inflammatory synovial tissue of orthopedic controls, no staining for FR-β was observed, consistent with low numbers of macrophages (not shown).

Staining results were analyzed by computer-assisted digital image analysis. A significant correlation was found between 3A5 and FR-β expression in the synovial sublining layer (cell counts/mm²) (p=0.04) (Figure 2A). Median positive cell counts in the synovial sublining layer/mm² were 126 (range 9-630) for FR-β and 219 (range 11-622) for 3A5. Median numbers of macrophages decreased upon 4 months of treatment with MTX (from 219 to 119 positive cell counts/mm², p=0.14). FR-β expression after 4 months of treatment with MTX was positively correlated (R=0.32) with DAS28 improvement (∆DAS28), but did not reach statistical significance (p=0.12) (Figure 2B).

Figure 2: (A) Correlation between 3A5 (macrophages) and FR-β positive cell/mm² in synovial tissue of RA patients (n=15) before treatment with MTX. Median positive cell counts in the synovial sublining layer/mm² were 126 (range: 9-630) for FR-β and 219 (range: 11-622) for 3A5. Linear regression: R=0.64, p=0.004. (B) Correlation between DAS28 improvement (∆DAS28) and FR-β expression on macrophages (positive cells/mm²) after 4 months treatment with MTX (R=0.32; p=0.12).

CHO-β cells > peripheral blood lymphocytes (PBLs) (0.7%) >> monocytes (0.02%) and ex-vivo activated T-cells (< 0.001%) (Figure 3).

Binding affinities of FR-β vs. FR-α for folate antagonists

Binding affinities of FR-α for selected folate antagonists were previously reported (16) but to what extent they overlap or differ for the FR-β isoform has not been established. To this end, binding affinities (relative to folic acid) to FR-β versus FR-α for a series of folate-based inhibitors of DHFR, TS and GARTFase were determined and shown in Figure 4. Compared to folic acid, both FR-β and FR-α displayed a rather low affinity for the group of DHFR inhibitors. Binding affinity of FR-β for MTX is approximately 50-fold lower than for folic acid. The binding affinity for PT523 is markedly lower than for MTX (0.3% of folic acid) while PT644, the 5-methyl analogue of PT523, showed an affinity comparable to MTX. Of note, FR-α exhibits a good binding affinity for all tested folate-based TS inhibitors, but binding affinities of FR-β for pemetrexed, raltitrexed and BGC9331 were markedly lower (16-30 fold) than for FR-α. High binding affinity of FR-β was observed for the TS inhibitors CB306035 (16% of folic acid) and (6R, S)-BGC945 (83% of folic acid) and (6S)-BGC945 (46% of folic acid). FR-β also exhibited a proficient binding affinity for the GARTFase inhibitors DDATHF (27% of folic acid) and AG2034 (54% of folic acid) even though FR-α binding affinities for these compounds were 2.5-fold higher. Together, these results demonstrate a broad differential in binding affinities of FR-β for folate antagonists, among which several folate antagonists revealed a markedly higher binding affinity compared to MTX.

Folate antagonist induced growth inhibition of FR-β expressing cells

To investigate whether the folate antagonists used in the current study would convey a
Targeting RA synovial macrophages via the FR-β

Figure 3: FR-β mRNA expression in synovial tissue and ex-vivo cultured RA peripheral mononuclear cells (PBMCs).

Median FR-β mRNA expression was determined in peripheral blood lymphocytes (PBLs; n=9), monocytes (n=9), ex-vivo monocyte-derived macrophages (n=25), ex-vivo activated T-cells (n=22) and synovial tissue of RA patients (n=7). FR-β expression in these samples is shown as a percentage of the expression in CHO/FR-β cells (Chinese Hamster Ovarian cells transfected with FR-β; expression in these cells was set to 100%).

Table 1: Growth inhibitory effects of folate antagonists against human monocytic-macrophage THP1 cells.

<table>
<thead>
<tr>
<th>Folate antagonist</th>
<th>IC₅₀ (nM)</th>
</tr>
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<tbody>
<tr>
<td>MTX</td>
<td>71 ± 0.5</td>
</tr>
<tr>
<td>PT523</td>
<td>2.2 ± 0.3</td>
</tr>
<tr>
<td>PT644</td>
<td>1.0 ± 0.4</td>
</tr>
<tr>
<td>RAhitred</td>
<td>2.4 ± 0.5</td>
</tr>
<tr>
<td>Pemetrexed</td>
<td>10.7 ± 2.2</td>
</tr>
<tr>
<td>GW1443</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>BGC9331</td>
<td>8.7 ± 0.8</td>
</tr>
<tr>
<td>CB300635</td>
<td>4850 ± 285</td>
</tr>
<tr>
<td>BGC945</td>
<td>3830 ± 350</td>
</tr>
<tr>
<td>DDATHF</td>
<td>9.8 ± 0.8</td>
</tr>
<tr>
<td>AG2034</td>
<td>3.2 ± 0.2</td>
</tr>
</tbody>
</table>

THP1 cells were grown in RPMI-1640 medium containing 2.2 µM folic acid (which would block any FR activity). Drug exposure time: 72 hours. Results are the mean ± SD of 3 separate experiments.

Figure 4: Relative binding affinities of FR-α (white bars) and FR-β (grey bars) for novel generation folate antagonists.

Potential growth inhibitory effects against macrophage-like type of cells, this parameter was investigated for human monocytic-macrophage THP1 cells (Table 1). Consistent with RFC as the dominant transport route in THP1 cells, potent growth inhibitory effects were observed for all folate antagonists, except CB300635 and BGC945, two compounds that have a poor affinity for RFC (25, 27).

Three folate antagonists for which FR-β displayed the highest binding affinity (CB300635, AG2034 and BGC945) were evaluated for their potency to target FR-β by provoking cell growth inhibition of CHO/FR-β cells. Against CHO/WT cells, BGC945 only induced growth inhibition at extracellular concentrations of > 1000 nM (Figure 5A). Remarkably, growth inhibition of CHO/FR-β cells was induced at markedly lower concentrations (10-50 nM) of BGC945. The addition of folic acid to these cell cultures completely abrogated the activity of BGC945, consistent with a blockade of FR. Despite displaying the highest binding affinity for FR-β, CB300635 was not markedly potent in inducing growth inhibition in CHO/FR-β cells (Figure 5B). Finally, AG2034 may utilize both the constitutively expressed RFC and FR as route for cell entry (Figure 4). As such, AG2034 displayed a growth inhibitory potential...
against CHO/WT cells and to a greater extent to CHO/FR-β cells. Consistently, abrogation of AG2034 growth inhibitory effects by FR-β blocking (with folic acid) and RFC blocking (with leucovorin) are only partial (Figure 5C/D).

**Discussion**

Since MTX is the anchor-drug in many therapeutic regimens for RA treatment, delineation of genetic, biochemical and metabolic parameters that could assist in predicting and/or improving the therapeutic response to MTX have received considerable recent interest (1, 19, 40, 41). This study focussed specifically on the role of cell membrane transport of MTX, which, in activated synovial macrophages, is mediated predominantly by the folate receptor β isoform (11). Given the notion that the molecular and functional properties of FRs and the constitutively expressed RFC differ considerably, a better understanding of the properties of FR-β may facilitate a better therapeutic window by selective targeting of FR-β over RFC.

Here we showed that FR-β expression primarily co-localized with macrophages in the intimal lining layer and the sublining of RA patients and may therefore be an attractive target for folate antagonists. It has been previously reported that both FR-α and FR-β isoforms exhibit a rather low affinity for MTX as compared to folic acid (16, 42). In this context, it would be worthwhile to investigate whether folate antagonists with a higher binding affinity to FR would provide a greater degree of selectivity and thereby elicit a potentially improved therapeutic response. Screening for binding affinities of a series of second generation folate antagonists, some of which with proven anticancer activity (18), revealed that the group of DHFR inhibitors all had a rather low FR-β affinity. This is consistent with previously reported structure/activity relationship, demonstrating that the α isomer of FR had low affinities for folate antagonists with a 2,4-diamino-based structure (see Supplementary File). Interestingly, while FR-α demonstrated a relatively high binding affinity for all tested folate-based inhibitors of thymidylate synthase, for FR-β this was only retained for 3 compounds (CB300635, GW1843 and BGC945) that share a common chemical property of 3-ring structures and/or glutamate side chain modifications. The latter modification also markedly suppresses its ability to be transported via the RFC and thus contributes to a greater FR-selectivity. In fact, selective targeting by BGC945 for FR-β and not RFC was demonstrated in FR-α over expressing tumour cell lines. (17, 27).

In addition to folate-based TS inhibitors, FR-β also exhibited moderate to high binding affinities for the folate-based GARTFase inhibitors AG2034 and DDATHF, which classifies them as folate antagonist drugs that can be transported both via RFC (16) and FR. FR-β-transfected CHO cells were used as a model system to evaluate the efficiency of FR-β-mediated cellular uptake of folate antagonists by conveying anti-proliferative effects (43). This cell line model may be clinically representative, based on [3H]-folic acid binding levels and FR-β mRNA levels that are compatible with FR-β mRNA levels in synovial tissue of RA patients (Figure 3). The largest differential in activity between control (RFC-expressing) CHO cells and FR-β transfected cells was observed for BGC945, consistent with a poor affinity for transport via RFC and a high FR-β binding affinity. FR-β mediated uptake of BGC945 could be inhibited by blocking of the receptor with excess folic acid, implying that circulating natural folates in synovial tissue/plasma could attenuate the potential activity of BGC945 in vivo either by receptor occupancy/competition or by receptor down-regulation. In this context, it may be anticipated that e.g. folate food fortification may reduce the activity of this folate antagonist whereas restrictions in dietary folate intake could further enhance the therapeutic efficacy of these type of drugs (44).

An intriguing observation was that the compound CB300635 for which FR-β had the highest affinity (even 1.5-fold higher than folic acid) was not differentially active against FR-β transfected cells. This may suggest that the high affinity binding may have a downside, which is the poor release of the compound from the receptor in an acidic compartment during endocytic cycling of the receptor (45). Hence, to allow optimal targeting of FR-β by folate
antagonists, both conditions for efficient binding to the receptor and efficient intracellular release from the receptor for target delivery should be fulfilled. The GARTFase inhibitors DDATHF and AG2034 harboured dual specificity for RFC and FRα/β as folate transport systems. Whether this property renders a different type of activity profile as compared to FR-selective compounds awaits further investigations. In this respect, two recent studies (46, 47) showed that two folate-based GARTFase inhibitors could confer suppression of rat adjuvant arthritis and collagen-induced arthritis in mice. Moreover, in a preliminary account, we showed that novel generation of folate antagonists were potent suppressors of TNFα production from activated T cells of RA patients (48).

Although MTX is the drug of first choice in RA treatment, there is room for improving the efficacy of MTX, for instance in clinical cases of primary and acquired MTX failures (19). Further evaluation of folate-antagonists with properties of high FR-β binding affinity and a low affinity for the RFC, prototypically harbored by BGC945 (27), may pave the road for a more selective targeted therapy of activated synovial macrophages. Even in cases where FR-β expression would be low, there may be strategies to increase FR-β expression by pretreatment with retinoids and/or HDAC inhibitors (38, 49, 50). Thus, beyond exploiting FR-β for delivery of therapeutic agents or for imaging purposes (12, 14), the present study also supports therapeutic strategies that include FR-β mediated uptake of clinically active second generation folate antagonists.

Supplementary figure

| Chemical structures of folic acid, leucovorin (5-formyltetrahydrofolate) and folate-based inhibitors of dihydrofolate reductase (DHFR), thymidylate synthase (TS) and glycinamide ribonucleotide transformylase (GARTFase). |
References


The proteasome inhibitor bortezomib inhibits the release of NFκB-inducible cytokines and induces apoptosis of activated T cells from rheumatoid arthritis patients

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Abstract

Objective: The proteasome is a multicatalytic protease complex regulating the intracellular breakdown of many proteins, including those mediating the activation of pro-inflammatory signaling pathways (e.g. NFκB), cell proliferation and survival. Conceptually, proteasome inhibitors may therefore elicit potential anti-inflammatory properties by inhibiting these processes and thereby impair the cellular release of pro-inflammatory cytokines such as Tumor Necrosis Factor α (TNFα) in RA patients.

Methods: Whole-blood from 19 RA patients (including methotrexate-responsive and non-responsive patients) and 7 healthy volunteers was incubated ex-vivo with the proteasome inhibitor bortezomib after T-cell stimulation with αCD3/CD28. Inhibition of cytokine production by bortezomib was measured after 24 and 72 hours by ELISA. Effects of bortezomib on apoptosis and T-cell activation (CD25 expression) were measured by FACS-analysis.

Results: Bortezomib proved to be a rapid (<24 hour) and potent inhibitor of the release of several NFκB-inducible cytokines (including TNFα, IL-1β, IL-6 and IL-10) by activated T-cells from healthy volunteers and RA patients, regardless of their clinical responsiveness to methotrexate. Median concentrations of bortezomib required to inhibit TNFα production by 50% (mIC-50) were 12 nM (range: 8-50 nM) for healthy volunteers and 46 nM (range: 18-60 nM) for RA patients. A reduction of T cell activation and a marked induction of T-cell apoptosis were revealed as late effects after bortezomib incubations beyond 24 hours.

Conclusion: Proteasome inhibitors, represented by bortezomib, may elicit potential anti-inflammatory properties that deserve further exploration in experimental therapies for RA.

Introduction

The proteasome is a multimeric proteinase complex that facilitates the degradation of approximately 80% of intracellular proteins. The catalytic activities of the proteasome not only ensure protein homeostasis; they also provide a fine-tuned mechanism of controlled (in)activation of proteins involved in cell proliferation, signaling processes and the generation of antigenic peptides to be presented on MHC class I molecules (1-3). In this context, for example, activation and nuclear translocation of the transcription factor NFκB intimately depends on proteasome-mediated breakdown of its natural inhibitor protein IκBα and thereby mediates the transcriptional regulation of several pro-inflammatory cytokines such as TNFα and IL1β (4, 5) as well as anti-apoptotic genes (6). From this perspective, it has been anticipated that inhibitors of the proteasome may counteract the NFκB activation process and eliciting potential anti-inflammatory response (7-11).

Bortezomib (Velcade®, PS341), a boronic acid dipeptide, is the first specific proteasome inhibitor that is registered in cancer chemotherapy for therapy-refractory multiple myeloma patients (12, 13). Bortezomib is used as single agent and in drug combination regimens where it has displayed additive/synergistic anti-tumor effects when combined with glucocorticoids (dexamethasone) or TNF-related apoptosis-inducing ligand (TRAIL) (13, 14). Interestingly, patients with relapsed or refractory lymphoma who responded to bortezomib had a marked reduction in plasma TNFα levels as compared to bortezomib non-responders (15). Of additional interest is a potential anti-inflammatory perspective were preliminary data from animal models for arthritis (16, 17) which revealed that bortezomib treatments conveyed clear therapeutic effects. In fact, a recent report by Neubert et al showed that bortezomib targets both short and long-lived plasma cells in mice with lupus-like disease, thereby abrogating the production of antibodies to double-stranded DNA and preventing the onset of nephritis (18). The present study was undertaken to assess the potential anti-inflammatory effects of bortezomib on the basis of inhibiting the production of TNFα and other pro-inflammatory cytokines by activated T cells from RA patients.

Materials & methods

Patient characteristics

All patients signed an informed consent form, and the study on ‘DMARD-resistance’ was approved by the Medical Ethics committee of the VU University Medical Center, Amsterdam, The Netherlands. Characteristics of 19 RA patients included in this study were: male/female: 5/14; mean age: 52.5 years; mean DAS28 score (Disease Activity Score for 28 joints): 4.3 (range 1.3-7.2). Five patients had no prior treatment with Disease Modifying Anti-Rheumatic Drugs (DMARD-naive), 13 patients used methotrexate (MTX) 15-30 mg/week, and 1 patient received the combination of sulfasalazine and hydroxychloroquine. Patients were on DMARDs for at least 3 months (range: 12 weeks to 9 years). Patients who were on DMARD therapy were defined as DMARD responders if their DAS28 was ≤3.2 at the time of enrollment (n=7); RA patients with a DAS28 score of >3.2 were defined as DMARD non-responders (n=7). Finally, 7 healthy volunteers were included (male/female: 4/3; mean age: 38 years). Additional patient characteristics are described in Table 1.

Materials

Bortezomib was kindly provided by Millennium Pharmaceuticals; Cambridge, MA, U.S.A. Stock solutions of 1 mM were prepared in dimethylsulfoxide (DMSO) and stored at -20°C. αCD3/ιCD28 antibodies were a generous gift from Prof. L. Aarden (Sanquin, Amsterdam, the Netherlands). Anti-CD3-PE was obtained from Becton Dickinson; San Jose, CA, U.S.A. Cytokine ELISA assays (PeliKine) for TNFα, IL-1β, IL-6 and IL-10 were obtained from Sanquin, Amsterdam, The Netherlands, and utilized according to the manufacturers’ instructions and as described previously (19).
Table 1: Baseline characteristics of (subgroups of) RA patients and controls and outcome of TNF inhibition from activated T-cells by bortezomib and methotrexate.

<table>
<thead>
<tr>
<th></th>
<th>Controls (n=7)</th>
<th>RA pts (n=19)</th>
<th>DMARD-naive (n=5)</th>
<th>DMARD-responders (n=7)</th>
<th>DMARD-non-responders (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (yrs)</td>
<td>38 (13)</td>
<td>53 (7)a</td>
<td>53 (6.9)</td>
<td>55 (3.9-7.2)</td>
<td>55 (3.9-7.2)</td>
</tr>
<tr>
<td>Mean ESR (mm/hr)</td>
<td>27 (7)i</td>
<td>47 (32-59)i</td>
<td>48 (44-60)i</td>
<td>27 (18-59)</td>
<td></td>
</tr>
<tr>
<td>Median DAS28 score (range)</td>
<td>12 (8-50)</td>
<td>48 (18-60)i</td>
<td>47 (32-59)i</td>
<td>48 (44-60)i</td>
<td>27 (18-59)</td>
</tr>
<tr>
<td>Median IC50 bortezomib (nM) (range)</td>
<td>53 (8-85)</td>
<td>59 (16-325)</td>
<td>53 (12-102)</td>
<td>44 (28-260)</td>
<td>60 (16-325)</td>
</tr>
</tbody>
</table>
| Median IC50 MTX (nM) (range) | IC50: drug concentration required to inhibit TNF production by 50%. *p<0.05. NS Not Significant. DAS28: Disease Activity Score (28 joints). ESR: Erythrocyte Sedimentation Rate.

Procedures & T-cell activation
Whole blood (1:10 diluted with heparin-containing DMEM medium, supplemented with 0.1% fetal calf serum) of 19 RA patients and 7 healthy volunteers were incubated ex vivo with oCD3/αCD28 antibodies (0.1µg/ml and 1µg/ml respectively) as T-cell activating agents as described previously (19). T-cell activation was monitored by CD25-PE staining by flow cytometry. The whole blood incubates were then supplemented with a concentration range (0-100 nM) of bortezomib, and, as a reference, 33-300 nM MTX, followed by an incubation period of 24-72 hours. Inhibition of TNFα production was measured after 72 hours drug exposure by ELISA as described before (19). Beyond TNFα, production of other NFκB-inducible cytokines (i.e. IL-1β, IL-6 and IL-10) was measured in whole blood from 3 separate healthy controls and 3 RA patients after T-cell stimulation and 24 hour exposure to bortezomib.

Apoptosis assay
Peripheral blood lymphocytes (PBLs) of RA patients and healthy volunteers were isolated by Ficoll-Paque Plus density centrifugation and suspended in DMEM-medium supplemented with 10% fetal calf serum. Apoptosis in PBLs was analyzed by flow cytometry (FACS) after 24 and 48 hours exposure to bortezomib using Annexin-V-FITC/7-aminoactinomycin D (7-AAD) staining (APOPTEST™-FITC A700, VPS Diagnostics, Hoeven, the Netherlands) according to the manufacturer’s protocol. Phycoerythrin labeled T-cell markers (CD4/CD8/CD25) were obtained from Becton Dickinson; San Jose, CA, U.S.A.

Statistical analysis
Statistical analysis was performed with the Mann-Whitney test to analyze differences between RA patients and healthy volunteers.

Results
Bortezomib-induced inhibition of cytokine release from activated T-cells after 24 hours
In a pilot setting, the ability of bortezomib was assessed to inhibit the release of cytokines from oCD3/αCD28 activated T-cells from controls (Figure 1A) and RA patients (Figure 1B). Production of TNFα, IL-1β, IL-6 and IL-10 production in controls was half maximally inhibited at bortezomib concentrations between 5-25 nM (Fig 1A). In activated T-cells of RA patients (Figure 1B), bortezomib also inhibited the production of IL-6, IL-1β and TNFα, although for the latter cytokine, slightly higher concentrations of bortezomib were required. Production of IL-10 in activated T-cells of RA patients was less efficiently inhibited as compared to controls.

Inhibition of TNFα release from activated T-cells by bortezomib and methotrexate after 72 hours
The potency of bortezomib to inhibit the production of TNFα was further investigated in a larger group of RA patients (n=19) which could be subcategorized in DMARD-naïve patients (n=5), DMARD-responsive patients (DAS28 <3.2, n=7) and DMARD-non-responsive RA patients (DAS28 ≥3.2, n=7). For comparison, inhibition of TNFα production was also analyzed for methotrexate (MTX) as a reference drug. Following oCD3/αCD28 stimulation of T-cells, absolute levels of baseline TNFα production were not significantly different in whole blood.

![Figure 1 A](H10_1A.pzf:Data 1 graph - Thu Jul 31 16:19:13 2008)

![Figure 1 B](H10_1B.pzf:Data 1 graph - Thu Jul 31 16:17:06 2008)

Figure 1: Bortezomib-induced inhibition of TNFα, IL-1β, IL-6 and IL-10 production in activated T-cells from controls and RA patients.
Bortezomib-induced inhibition of TNFα, IL-1β, IL-6, and IL-10 production from activated T-cells of (A) healthy volunteers (n=5) and (B) DMARD-naïve RA patients (n=7) was analyzed after 24 hour stimulation of T-cells in whole blood by oCD3/αCD28 in the presence of the indicated concentrations of bortezomib. Results are depicted as the mean percentage of cytokine production relative to controls incubated without bortezomib.
incubates from 7 healthy volunteers (baseline TNFα production: 3208± 2554 pg/ml; range: 623-7361 pg/ml) and 3 groups of RA patients: DMARD-naive (n=5), DMARD-responders (n=7) and DMARD non-responders (n=7) (Figure 2). Upon co-incubation with bortezomib, median concentrations of this drug required to inhibit TNFα production by 50% (IC50) were 3.8-fold lower (p<0.01) for healthy controls (IC50: 12 nM, range: 8-50 nM) as compared to the total group of RA patients (IC50: 46 nM, range: 18-60 nM) (Table 1, Figure 3).

Sub-analysis for the DMARD non-responsive RA patients showed a 1.7-fold greater potency of bortezomib to inhibit TNFα production compared to DMARD-naive and DMARD-responsive RA patients, but this difference was not significant (p=0.32) (Figure 4). Median concentrations of MTX to inhibit TNFα production by 50% were not significantly different (p=0.64) between healthy controls (IC50: 53 nM, range 18-85 nM) and RA patients (IC50: 59 nM, range: 16-325 nM) (Table 1).

Bortezomib-induced induction of apoptosis and inhibition of T-cell activation

Along with a rapid reduction in TNFα release, a marked induction of apoptosis was observed in peripheral blood lymphocytes (PBLs) of RA patients at a later stage of bortezomib exposure: 10-21% Annexin-V positive cells in control conditions versus 62-77% Annexin-V/7AAD-positive cells after exposure to 10-100 nM bortezomib for 48 hours, respectively (Figure 5A). A representative FACS analysis of bortezomib-induced T cell apoptosis in PBLs from a RA patient is shown in Figure 5B. Without bortezomib (control), only 7% of CD4/CD8 V/7AAD-positive cells after exposure to 10-100 nM bortezomib for 48 hours, respectively (representing IC50 value for inhibition of TNFα production in RA whole-blood), 60% of CD4/CD8 positive T-cells were apoptotic based on Annexin-V staining. Apoptosis induction by bortezomib could be partially prevented, in a concentration dependent manner, by pre-incubation with the broad-spectrum-caspase inhibitor ZVAD-fmk (10-50 µM), suggesting that cell death was mediated via apoptosis rather than by necrosis (data not shown). For comparison, no induction of apoptosis was observed after 48 hours exposure to 33 nM bortezomib for 48 hours (IC50: 12 nM, range 8-50 nM) and RA patients (IC50: 59 nM, range 16-325 nM) (Table 1).

Discussion

Nowadays, the ubiquitin-proteasome system is recognized as the major pathway for degradation of intracellular proteins, many of which play a key role in regulation of pro-inflammatory cytokines (7, 11). Data from the present study further support the proof of principle that bortezomib can confer inhibition of TNFα production in whole blood from RA patients after T-cell stimulation. Mechanistically, this effect can be explained by inhibition of the NFκB signalling pathway as a downstream effect of proteasome inhibition in conjunction with the NFκB principle that bortezomib can confer inhibition of TNFα production.

Anti-inflammatory effects of bortezomib

Dose-response curve (72 hours incubation) for bortezomib-induced inhibition of TNFα production by activated T-cells from healthy controls (squares, n=7), total group of RA patients (n=19), and after sub-classification in DMARD-naive (n=5), DMARD-responders (n=7) and DMARD non-responding RA patients (n=7). Data are presented as mean values (pg/ml) ± SD. Differences in baseline TNFα production in the tested groups were not statistically different. Note: no TNFα production was observed in unstimulated whole blood cell cultures.
Figure 5: Effects of bortezomib on T-cell activation and induction of apoptosis.
(A) Induction of apoptosis by bortezomib in CD3/CD28-stimulated peripheral blood lymphocytes of RA patients after 24 hours (white bars) and 48 hours (black bars) drug exposure. (B) Representative FACS analysis depicting induction of apoptosis (Annexin-V positivity in the CD4/CD8 positive population of activated) T-cells in control conditions (without bortezomib) and after 48 hours exposure to 33 nM bortezomib. (C) Induction of apoptosis by MTX in CD3/CD28-stimulated peripheral blood lymphocytes of RA patients after 24 hours (white bars) and 48 hours (black bars) drug exposure. (D) Percentage of activated (CD25 positive) T-cells in CD3/CD28-stimulated peripheral blood lymphocytes after incubation with bortezomib. Results presented are the mean ±SD of 3 individual RA patients.

with induction of apoptosis in activated T-cells (12-14, 17). In the whole blood assay employed in this study, it is anticipated that beside T-cells, monocytes/macrophages will be the main producers of IL-1, IL-6 and TNFα, as they get stimulated by cytokines (like IL-17) produced by activated T-cells (20). Since we observed that bortezomib abrogated T-cell activation at low concentrations, it can be speculated that the production of these cytokines by monocytes/macrophages is inhibited simultaneously.

Consistent with inhibition of NFκB were observations (Figures 1, 3, 4) that besides TNFα, bortezomib also inhibited other NFκB-inducible cytokines (IL-1, IL-6, IL-10) (Figure 1) in a concentration range (10-50 nM) that was previously shown to abrogate NFκB activity (23). Given the central role of the ubiquitin-proteasome system in regulating signalling pathways (11), it is anticipated that signalling pathways other than NFκB may be affected at higher concentrations of bortezomib and/or longer exposure times. In fact, concentrations of bortezomib that provoke apoptosis induce p38 MAPK activity along with a release of anti-inflammatory cytokines, such as IL-10 (22). The incomplete inhibition of IL-10 production at higher concentrations of bortezomib (Figure 1) may be consistent with this notion.

While bortezomib-induced inhibitory effects on NFκB activity could be observed rather rapidly (within 24 hour, Figure 1), bortezomib-induced apoptosis of activated T-cells was recognized as a later effect, emerging between 24 and 48 hours of bortezomib exposure. In this respect, our data are consistent with Blanco et al showing that bortezomib was particularly active against alloreactive (CD25+) T-cells and not resting T-cells (23). Based on the profile of inhibition of TNFα production, bortezomib exhibited potent ex vivo activity for both DMARD-naive and clinically DMARD-responsive RA patients, and an even slightly greater activity for DMARD non-responsive patients. This result may point to the fact that proteasome targeting may bypass or circumvent common mechanisms of loss of efficacy to DMARDs after chronic administration (24).

Apart from the inter-patient variability in ex vivo response to bortezomib, one other intriguing observation was that bortezomib displayed a greater potency in blocking of TNFα production by activated T-cells from healthy controls than from RA patients. An explanation for this is not readily available but could relate to possible differences between healthy controls and RA patients with respect to (i) quantitative and qualitative differences in proteasomal catalytic activity in T-cells from healthy controls versus patients with RA or other autoimmune diseases (25-27), or (ii) higher constitutive NFκB activity in activated T-cells from RA patients (4), which would require higher bortezomib dosages for inhibition. In addition, a recent study (28) indicated that plasma pharmacokinetics and activity of bortezomib can be influenced by uptake of bortezomib in red blood cells. In our study, we made use of (1:10) diluted whole blood cell samples (19) within which variability in erythrocyte concentrations could have influenced residual concentrations of bortezomib to some degree. Notably, pharmacokinetics of bortezomib in phase I clinical trials showed peak plasma levels of 50-1000 nM and steady state plasma levels of 10-20 nM (29, 30), which are within the concentration range where bortezomib showed inhibition of TNFα production by activated T-cells from RA patients (Table 1, Figure 1) and induction of T-cell apoptosis. It remains to be established whether this induction of apoptosis is based solely on the apoptotic effects of bortezomib itself or whether it also involves previously reported bortezomib-induced sensitization of apoptotic effects of TRAIL or TNFα (14). Obviously, any design of a trial for bortezomib in RA treatment should address a number of issues: (a) assessment of optimal dosing/therapeutic window for RA treatment, (b) it is anticipated that commonly long term drug administration to RA patients will require special precautions with respect to drug safety (31), (c) it should be considered that proteasome inhibition may partially abrogate intracellular degradation of (citrullinated) proteins in RA patients (32). Finally, (d)
it remains to be revealed to which extent other pro-inflammatory cytokine producing cells (monocytes, macrophages) in peripheral blood or in synovial tissue are subject to targeting by bortezomib as demonstrated for activated T cells in this study.

Despite the current success of DMARDs and biologic agents, there is still room for improvement of RA therapy (33). Given their unique profile of action, proteasome inhibitors (34, 35) certainly deserve further evaluation for future clinical application in the treatment of chronic inflammatory diseases. As a proof of concept, this pilot study demonstrated that proteasome targeting by specific inhibitors such as bortezomib, can suppress production of pro-inflammatory cytokines from activated T cells of RA patients. Thus, beyond demonstrations of good clinical activity against certain types of cancer, proteasome inhibitors may represent a new generation of targeted small molecule drugs in the therapeutic armour, in particular for patients with DMARD-refractory RA.
References


General discussion and future perspectives
It is generally accepted that the onset of drug resistance is a major limiting factor in sustaining a long term therapeutic effect by drugs, regardless whether it concerns treatment of patients with infectious diseases, neoplastic diseases or chronic inflammatory diseases. Also, multiple mechanisms can underlie the resistant phenotype of various types of therapeutic drugs, including disease modifying anti-rheumatic drugs (1, 2). In this thesis, we focussed primarily on a major mechanism of drug resistance associated with drug efflux transporters from the ABC transporter family, being aware that other possible mechanisms of resistance to DMARDs should not be excluded when a MDR phenotype can not fully explain the molecular basis of resistance.

MDR proteins & DMARD resistance

Cumulative data from the current project and studies by others have now established a clear picture of which DMARDs can be substrates for specific drug efflux transporters, notably P-glycoprotein (Pgp), Multidrug Resistance associated Protein 1-5 (MRP1-5, ABCC1-5) and Breast Cancer Resistance Protein (BCRP, ABCG2) and how upregulation of drug transporters may contribute to DMARD resistance (see chapter 2 and Table 1; summary section).

BCRP expression on synovial tissue macrophages

Induction of the drug efflux transporter BCRP appeared to play a key role in the onset of acquired resistance of T-cells to the DMARD sulfasalazine in vitro. Interestingly, rather than a DMARD-provoked induction of BCRP expression, we also observed abundant expression of BCRP on macrophages in the intimal lining layer and the synovial sublining of synovial biopsies from RA patients even prior to receiving DMARD therapy. These observations may comply with at least two mechanisms implicated in the induction of BCRP expression. First, evidence has been presented for an epigenetic regulation of BCRP expression by diminished BCRP promoter methylation (3, 4). Consistent with this possibility we showed (see chapter 4) that sulfasalazine is a potent inhibitor of the cell membrane carrier for reduced folate analogues, utilized intracellularly for DNA-methylation processes. Thus, (partial) blocking of cellular folate uptake by increasing concentrations of sulfasalazine during development of resistance may have resulted in enhanced BCRP expression due to diminished methylation of a potential CpG island in the BCRP promoter. Second, BCRP expression is also induced under hypoxic conditions, which may apply to RA synovium, by the action of the transcription factor HIF-1α (5). In this context, it has also been reported that specific RA pathophysiological conditions of aberrant production of pro-inflammatory cytokines can upregulate the expression of other MDR transporters, notably Pgp and MRP1 (6, 7). Hence, alterations in expression levels of MDR transporters can be controlled not only by chronic drug exposure, but also by environmental factors. Another example of DMARD-induced MDR protein expression is MRP1, that appeared to be dominantly involved in conferring resistance to chloroquine (8). In contrast, in sulfasalazine resistant T-cells a decreased MRP1 expression was observed and subsequently enhanced sensitivity to chloroquine. Chloroquine resistant T-cells were highly cross-resistant to glucocorticoids, in contrast to sulfasalazine-resistant cells that gained sensitivity to glucocorticoids due to increased stability of the glucocorticoid receptor α (9). In sulfasalazine resistant T-cells, cross-resistance to MTX was observed due to inhibition of cellular entry of MTX by sulfasalazine. These observations underscore that a detailed characterization of resistant cells may disclose specific drug interactions, either adverse or favourable, which may be exploited clinically (10). Specifically, the above-described effects for the drugs used clinically in the COBRA and O’Dell treatment protocols for RA (11, 12), may warrant further in depth investigations to provide a better mechanistic basis for its efficacy. Table 1 summarizes the

<table>
<thead>
<tr>
<th>Drug combinations</th>
<th>Type of Interaction</th>
<th>Mechanism of Interaction</th>
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<tbody>
<tr>
<td>SSZ + glucocorticoids</td>
<td>Synergistic</td>
<td>SSZ stabilizes the GRα, thereby increasing the expression of GRα and enhancing efficacy of glucocorticoids</td>
</tr>
<tr>
<td>SSZ + CHQ</td>
<td>Synergistic</td>
<td>SSZ downregulates MRPα for which CHQ is a substrate, thereby enhancing CHQ efficacy</td>
</tr>
<tr>
<td>SSZ + MTX</td>
<td>Negative interaction</td>
<td>(1) SSZ is a potent inhibitor of the RFC, the dominant transporter for cellular uptake of MTX, thereby inducing MTX resistance (2) SSZ induces BCRP expression upon prolonged exposure, thereby inducing MTX resistance since MTX is a BCRP substrate</td>
</tr>
<tr>
<td>CHQ + glucocorticoids</td>
<td>Negative interaction</td>
<td>CHQ induces aberrant signaling via the cAMP/PKA pathway downstream of the GRα, thereby inducing glucocorticoid resistance</td>
</tr>
<tr>
<td>MTX + CHQ</td>
<td>Negative interaction</td>
<td>CHQ induces MRPα expression for which MTX is a substrate, thereby inducing MTX resistance</td>
</tr>
</tbody>
</table>

Abbreviations: SSZ: sulfasalazine; MTX: methotrexate; CHQ: chloroquine; MRPα: multidrug resistance protein α; GR: glucocorticoid receptor; cAMP: cyclic adenine monophosphate; PKA: protein kinase A; RFC: reduced folate carrier.
possible DMARD interactions in combination therapies for RA treatment according to our in vivo studies on T-cells.

**MDR-protein expression on RA peripheral blood lymphocytes**

Beyond studies with in vitro experimental model systems and synovial tissue (chapter 3, 4 and 5), screening of peripheral blood-derived cells showed protein and/or functional expression of several MDR transporters in T cells (Pgp, MRPs), monocytes (Pgp, MRPs), dendritic cells (Pgp, MRPs, BCRP) and macrophages (MRPs, BCRP), some of which at higher expression levels in RA patients as compared to healthy controls (R. Oerlemans, Ph.D. thesis in preparation) (13). However, it should be taken into account that these indicated transporters still comprise a fraction (~20%) of the 49 members of the ABC family of drug transporters for which a function has been revealed. Interestingly, some of the other members of the ABC transporter family have recently been identified to be involved in processes that can be of direct and indirect relevance for pathophysiological processes in RA; e.g. involvement of ABCG5 in cholesterol transport (possible implications for cardiovascular disease) (14) or involvement of ABCA1 in cellular extrusion of interleukin-1β (15). From this perspective, it will be worthwhile to further pursue unravelling the role of specific ABC transporters in RA. For this purpose, customized micro-arrays have become available that may allow detection of mRNA levels of most of the known ABC transporters in one assay (16). In this manner, MDR expression in patient samples (synovial tissue biopsies; immune-effector cells from peripheral blood) can be assessed before and during therapy.

MDR-proteins that are differentially expressed can then be analyzed for functionality in terms of efflux properties and correlated to the clinical response to specific DMARDs.

**Pharmacological & physiological roles of MDR proteins: balanced dual functions**

Expression of several specific MDR transporters has been reported in almost all cell types of the haematopoietic lineage, from stems cells to fully differentiated cells (17). Although these MDR proteins constitute an efficient mode of cellular defence to therapeutic drugs and xenobiotic compounds, this is unlikely to represent their sole function. MDR transporters such as Pgp, several MRPs and BCRP can transport compounds such as bioactive lipids (prostaglandins, leucotrienes) and steroid hormones (18-21) through which they may regulate functional properties of immune-competent cells. Prototypically, this is illustrated for antigen presenting dendritic cells whose migration depends on functional Pgp and MRPs activity (22, 23). Consequently, interference with Pgp or MRPs activity may potentially attenuate an appropriate immune response; for (RA) patients with an augmented immune-response this may be therapeutically beneficial, but in immune-compromised patients, it may evoke adverse effects. Further assessment of the physiological roles of MDR transporters in patients with chronic inflammatory diseases therefore deserves further attention in future studies.

**Reversal of multiple drug resistance**

Conceptually, specific blockers of MDR proteins could be exploited to reverse a MDR phenotype. Indeed, in cancer chemotherapeutic model systems, specific blockers of MDR transporters could reverse resistance to MDR-related drugs. Unfortunately, in a clinical setting this approach proved to be far less successful (24) (25) for reasons discussed in chapter 2. Limitations in effective reversal of Pgp-mediated drug resistance by blocking agents included for example; (a) effective plasma levels of blockers could not be reached, (b) enhanced metabolism of the blocking agent, (c) toxic side effects of the blockers, (d) drug interaction phenomena, (e) presence of multiple MDR transporters, and (f) interference with physiological functions of Pgp. However, it still remains to be established whether blockers of MRPs or BCRP face similar limitations as Pgp blockers.

One interesting aspect of blocking BCRP activity in relation to DMARD bioavailability was indicated by a study by Zaher et al (26) who showed that blocking of intestinal BCRP markedly elevated plasma levels of sulfasalazine, which is a substrate for BCRP (see chapter 3). Besides enhancing sulfasalazine uptake by blocking intestinal BCRP activity, co-administration of selected BCRP blockers could also increase intracellular sulfasalazine levels in BCRP expressing synovial tissue macrophages and subsequently improve therapeutic efficacy.

**Methotrexate resistance**

There is consensus that drug efflux via MDR proteins MRPs-5 and BCRP can provide a mechanistic basis for conferring resistance to the anchor drug in RA treatment, the antifolate MTX (see also chapter 2) (27). The relative importance of MDR proteins for MTX resistance in RA however is not readily clear, as other modes of resistance to MTX (discussed in chapter 2) (28-31) may be operative separately or alongside a MDR mechanism. The contribution by MDR proteins to MTX resistance may depend on several factors: type of MDR transporter(s) being expressed in various types of immune cells, dosing of MTX, efficiency of intracellular metabolism to polyglutamate forms and cellular folate status. These latter two conditions determine whether MTX is converted to long-chain polyglutamate moieties that are no longer substrates for MDR proteins. It may be anticipated that in immune cells with either low levels of polyglutamylation capacity and/or high intracellular folate levels that compete with MTX polyglutamylation, MTX will become poorly polyglutamylated and thus becomes available for cellular extrusion by either one of the MRPs or BCRP. Regarding intracellular folate status, this may be determined by several factors, of which dietary intake and/or vitamin supplementations are most relevant, especially since food and drug administrations in the US and most recently also in the Netherlands recommended folate food fortification to reduce the incidence of neural tube defects in neonates (32).
Novel folate antagonists

MTX is the anchor drug in RA treatment, exhibiting a relatively long-lasting activity as single agent or in combination schedules. A second generation of folate antagonists was rationally developed to overcome various modes of cellular resistance to MTX by harboring properties of either: more efficient drug uptake via the Reduced Folate Carrier than MTX (e.g. pemetrexed, raltitrexed); being polyglutamylation-independent (e.g. BGC9331), targeting other key enzymes in folate metabolism (Pemetrexed, Raltitrexed); or retaining activity regardless of intracellular folate status along with folate compartmentalization (2, 41, 42). In conjunction with this knowledge, predictive models for MTX responsiveness and toxicity may be even more reliable. Thus, before clinicians can predict the response on MTX treatment, these models have to be fine-tuned and further tested in appropriate study populations.

Figure 1: Routes of cellular entry for MTX and folic acid.

Targeting of synovial macrophages via the folate receptor-β

Activated macrophages in the synovial membrane play a central role in the chronic course of RA, by facilitating a persistent pro-inflammatory state of other inflammatory cells and synovial fibroblasts and the bone-destructive activity of osteoclasts. Numbers of synovial tissue macrophages are associated with disease activity and recently Haringman et al. showed that change in the number of synovial tissue macrophages is a highly sensitive biomarker for response to treatment in RA patients (44). Therefore, selective targeting of synovial tissue macrophages via the FR-β might be an attractive strategy (figure 1). We demonstrated a high expression of FR-β on macrophages in RA-synovial tissue. We also identified an antifolate drug candidate for selective targeting of this receptor, notably the TS inhibitor BGC945. However, an important issue in FR-β targeting to take into account is again a current nutritional policy of folate food fortification (32), which may reduce the activity of folate antagonists by receptor occupancy/competition (45). For example, Arabelovic et al. recently showed that folic acid fortification is associated with higher histone deacetylase (HDAC) inhibitors (e.g. valproic acid and trichostatin A) may be capable of inducing FR-β expression on myeloid cells, consistent with the notion that
histone deacetylation regulates the transcription of the FR-β gene (51). For this reason, ATRA and HDAC inhibitors warrant further evaluation as potential positive modulators of FR-β expression on macrophages. This approach may facilitate a selective macrophage-targeted therapy in RA, with greater efficacy and low systemic toxicity (because FR-β is dominantly expressed on activated macrophages).

Beyond exploitation of the FR-β on synovial macrophages for targeting with small molecule folate antagonists, the high affinity binding of the receptor for any folate conjugated (toxic) macromolecule/protein or tracer allows additional therapeutic targeting options as well as opportunities for imaging of RA disease activity, e.g. by folate-conjugated Positron Emission Tomography (PET) tracers. (52-55). In fact, these latter types of studies are currently being initiated in our department.

Novel experimental drugs: proteasome inhibitors

Proteasome inhibitors represent a novel generation of therapeutic drugs that may be of interest to bypass MDR-associated drug resistance; the proteasome inhibitor bortezomib, which is registered for therapy-refractory multiple myeloma, has been identified to be a poor substrate for most MDR transporters. We demonstrated that bortezomib exhibited potent anti-inflammatory properties by blocking TNFα production from ex-vivo activated T-cells of RA patients, tentatively through inhibition of the activation of the transcription factor NFκB (56). In addition, other studies have reported that bortezomib effectively reduced inflammation in animal models of streptococcal cell wall-induced polyarthritis (57) and systemic lupus erythematosus (58).

The proteasome and auto-immunity

Apart from blocking of NFκB activation in RA inflammatory cells, bortezomib could have more potentially benefits in RA treatment. It is well known that the proteasome is engaged in the process of antigen presentation via MHC class I molecules and therefore plays an important role in the initiation and prolongation of the immune response by determination of the difference between self and non-self antigens (59). In fact, alterations in the functionality or expression of the proteasome may be involved in the onset of auto-immune diseases. In this context, Hayashi et al showed that downregulation of one of the proteasome subunits, and thereby impaired antigen presentation and T-cell education towards self, has been identified as the cause for the onset of insulinitis in type-1 diabetes in the Non Obese Diabetes (NOD) mouse model (60). On the other hand, Egerer et al observed that the catalytic subunit LMP7 of the immunoproteasome (differing from constitutive proteasomes by 3 IFNγ-inducible β-subunits) was specifically up regulated in salivary glands of patients with Sjögren syndrome (61). Whether this implicates a role for proteasomes and altered protein/peptide degradation in the pathophysiology of auto-immune diseases or just reflects disease activity, remains to be established. Finally, Yoshimura et al showed that activated NFκB is required for antigen presentation by Dendritic Cells (DCs), and that NFκB inhibition with the proteasome inhibitor bortezomib resulted in down regulation of MHC class II molecules and CD86 on DCs along with inhibition of the production of immunostimulatory cytokines like IL-12 and TNFα (62).

Based on these studies, it may be speculated that inhibition of the proteasome could evoke dampening of the over-active immune response in RA.

The proteasome and ageing

The proteasome is also thought to play a central role in the process of ageing. Several studies showed a decrease in the constitutive proteasome content in different tissues and a concomitant accumulation of oxidized and ubiquinated proteins upon ageing (63-65). Conversely, the cellular content of immunoproteasomes remains constant, even though qualitative or quantitative changes in immunoproteasome activity in elderly might lead to alterations of presented epitopes (66). This impaired processing of proteins/peptides and altered antigen presentation, may render susceptibility for an auto-immune response in ageing people.

Expectations for future research

Given the above considerations, it would be of interest to investigate whether either short or chronic-exposure of immune effector cells to bortezomib or other experimental proteasome inhibitors may elicit beneficial therapeutic effects. Obviously, any design of a trial for these group of drugs in RA treatment should address a number of issues including (a) assessment of optimal dosing/therapeutic window for RA treatment, and (b) special precautions with respect to long term drug safety given the anticipated chronic administration (67). When these issues can not be met for bortezomib, second generation of proteasome inhibitors (68-70) may meet this profile as, unlike bortezomib, these are irreversible inhibitors which may provoke less off-target (toxic) effects than bortezomib. Finally, proteasome inhibitors may also serve, at low doses, as potentiators of glucocorticoid activity (71) which may also be of interest from an RA therapeutic perspective.
References


26. Zaher, H., Khan, A.A., Palandra, J.R., Brayman, T.G., Yiu, L. and Ware, J.A. Breast Cancer Resistance Protein (Bcrp1abega) is a major determinant of sulfasalazine absorption and elimination in mouse, Molecular Pharmacology, 3: 55-51, 2005.


Summary
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.

In 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>
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<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, azathioprine</td>
</tr>
<tr>
<td>ABCC5 (MRP5)</td>
<td>methotrexate, azathioprine</td>
</tr>
<tr>
<td>BCG2 (BCRP)</td>
<td>methotrexate, sulfasalazine, leflunomide</td>
</tr>
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</table>

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 104-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, MRPl-5, MRPl8, MRPl9 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 (20 mg/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 to leflunomide. For other MDR proteins, moderate expression of MRPl1 was observed in T-cell areas of some synovial biopsies, while expression of Pgp, MRPl2-5, MRPl8 and MRPl9 were below the immunohistochemical 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 MRPl), 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-dependent 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 (GARTase). 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. Consistently, 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 NFκB 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 NFκB binding to its promoter site and (c) inhibitors of IκB kinase. Another approach to interfere with NFκB activation, which is relatively unexplored, is by blocking the 26S proteasome-mediated breakdown of the IκBα protein, the natural inhibitor of NFκB. Cell activation initiates both phosphorylation and ubiquitination of IκBα. The latter process triggers breakdown of IκBα protein by the 26S proteasome system. Upon loss of binding of its natural inhibitor protein, NFκB 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 highly effective in abrogating cytokine production in activated RA T-cells
Nederlandse samenvatting
Dankwoord/Acknowledgements
Curriculum vitae
Curriculum vitae (english)
List of publications
Samenvatting

Patiënten met reumatoïde artritis (RA) worden langdurig behandeld met ontstekingsremmende medicijnen, waaronder de zogenaamde Disease Modifying Anti-rheumatic Drugs (DMARDs). Helaas worden veel patiënten in de loop van de behandeling resistent tegen deze DMARDs. Resistentie tegen antibiotica bij infectieziekten en resistentie tegen cytotactica bij kanker is een bekend fenomeen en daar is veel onderzoek naar gedaan; naar resistentie tegen DMARDs is nog relatief weinig onderzoek verricht.

In dit proefschrift beschrijven wij mogelijke mechanismen van resistentie tegen methotrexaat (MTX) en sulfasalazine, twee veel gebruikte DMARDs bij de behandeling van patiënten met reumatoïde artritis. Daarnaast hebben we onderzoek gedaan naar nieuwe generatie geneesmiddelen die deze mechanismen van resistentie mogelijk kunnen omzeilen.

In hoofdstuk 1 en 2 wordt een overzicht gegeven van de tot nu toe bekende moleculaire mechanismen van resistentie tegen DMARDs. Vanuit de oncologie is bekend dat een verhoogde expressie van multi-drug resistentie (MDR) eiwitten op de cellomembaan van kankercellen kan leiden tot resistentie tegen cytotactica. MDR eiwitten behoren tot de familie van de ATP afhankelijke transporteiwitten, waarvan er 49 bekend zijn. Deze eiwitten zijn in staat chemisch- en functioneel verschillende medicijnen de kankercellen uit te pompen, wat aanleiding geeft tot zogenaamde multi-pele-drug resistentie. Het is bekend dat verschillende DMARDs (onder andere MTX) substraat zijn voor een of meerdere van deze MDR eiwitten (zie tabel 1). Het is echter onbekend welke MDR-eiwitten voorkomen op perifere bloedcellen of ontstekingscellen in synoviaal weefsel van RA-patiënten, en of dit aanleiding geeft tot DMARD resistentie.

In hoofdstuk 3 hebben we onderzocht of MDR-eiwitten betrokken zijn bij resistentie tegen sulfasalazine in een humane T-cellijn, wanneer deze cellen langdurig blootgesteld werden aan deze DMARD. Na 4-6 maanden blootstelling aan sulfasalazine waren deze cellen een factor 3,5-6,0 resistent geworden. In de resistent cellen bleek een duidelijke opregulatie te zijn van het MDR-eiwit BCRP en een verminderde expressie van MRP1. Door het blokkeren van BCRP, werden de resistent cellen weer gevoelig voor sulfasalazine, waarmee een directe relatie tussen BCRP expressie en sulfasalazine-resistentie werd aangetoond. Verder bleek dat sulfasalazine-resistente cellen na stimulatie meer TNFα productie te produceren dan controle cellen, wat duidt op een verhoogde ontstekingsactiviteit van deze cellen. De TNFα productie in de resistente cellen kon ook moeilijker worden geremd door sulfasalazine, samenhangend met de verhoogde expressie van BCRP.

In hoofdstuk 4 werd onderzocht of sulfasalazine-resistentie in de humane T-cellijn reversibel was als de cellen werden gekweekt in afwezigheid van sulfasalazine. We zagen dat de BCRP expressie een maand stabiel verhoogd bleef, daarna geleidelijk afnam (evenals de resistentie-factor) en na 6 maanden niet meer detecteerbaar was. Echter, wanneer sulfasalazine opnieuw aan het kweekmedium werd toegevoegd, was er weer een snelle inductie van BCRP-expressie waar te nemen en werden de cellen dientengevolge binnen 2 weken opnieuw resistent tegen sulfasalazine. In dit hoofdstuk werd ook onderzocht of er in de sulfasalazine-resistente cellen kruisresistentie bestaat tegen andere DMARDs. Er bleek sprake van kruisresistentie voor leflunomide (factor 5,1) en MTX (factor 1,8), maar juist een verhoogde gevoeligheid voor cyclosporine A, chloroquine (factor 1,8) en dexamethason (factor 13), vergeleken met controle cellen. Vanuit dit oogpunt zou het een goede keuze kunnen zijn om deze laatste middelen voor te schrijven aan patiënten die geweigerd hebben op sulfasalazine.

RA-patiënten worden vaak behandeld met een combinatie van DMARDs. Een veel gebruikte combinatie is sulfasalazine met MTX. Uit literatuurgegevens blijkt echter dat de combinatie van sulfasalazine en MTX niet effectiever is dan mono-therapie met deze middelen, tenzij een derde DMARD zoals chloroquine of prednison wordt toegevoegd. Ook in onze laboratoriumstudies zagen we dat sulfasalazine resistent T-cellijns minder gevoelig waren voor MTX (hoofdstuk 4), wat niet werd verklaard door verhoogde expressie van het MDR-eiwit BCRP. In hoofdstuk 5 onderzochten we daarom de farmacologische interactie tussen sulfasalazine en MTX. Dezelfde humane T-cellijn die beschreven werd in hoofdstuk 3 en 4, werd nu gebruikt om het effect van sulfasalazine op de cellulaire opname van MTX en natuurlijke folaten (leucovorin) door de Reduced Folate Carrier (RFC) (het eiwit dat betrokken is bij de opname van MTX en natuurlijke folaten in de cel) te bestuderen. Analyse van de transportkinetiek van de RFC toonde aan dat sulfasalazine een potente (reversible), niet-competitieve remmer is van de cellulairere opnamecapaciteit voor MTX en leucovorin. In overeenstemming met deze bevinding was dat er MTX resistentie optrad in sulfasalazine-

<table>
<thead>
<tr>
<th>MDR eiwit</th>
<th>DMARD substantie</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCB1 (Pgp)</td>
<td>chloroquine, glucocorticoiden</td>
</tr>
<tr>
<td>ABCB1 (MRP1)</td>
<td>methotrexaat, chloroquine</td>
</tr>
<tr>
<td>ABCB2 (MRP2)</td>
<td>methotrexaat</td>
</tr>
<tr>
<td>ABCB3 (MRP3)</td>
<td>methotrexaat</td>
</tr>
<tr>
<td>ABCB4 (MRP4)</td>
<td>methotrexaat, azathioprime</td>
</tr>
<tr>
<td>ABCB5 (MRP5)</td>
<td>methotrexaat, azathioprime</td>
</tr>
<tr>
<td>ABCG2 (BCRP)</td>
<td>methotrexaat, sulfasalazine, leflunomide</td>
</tr>
</tbody>
</table>

Table 1: Overzicht van de MDR eiwitten die DMARDs kunnen verpompen.
resistente T-cellen in aanwezigheid van 0.25 mM sulfasalazine (resistentiefactor: 3.5) en 2.5 mM sulfasalazine (resistentiefactor 4.0). Naast MTX-resistente werd een dosis-afhankelijk remmend effect gezien van sulfasalazine op de opname van leucovorin, wat aanleiding kan geven tot intracellulaire folaat-depletie. Dit onderzoek pleit ervoor dat sulfasalazine en MTX niet gelijktijdig (op dezelfde dag) moeten worden ingenomen maar juist gespreid, om een maximaal effect te kunnen bereiken. Daarnaast moet er bij deze combinatie in ieder geval folumzuur worden voorgeschreven om ‘gezonde cellen’ te beschermen tegen mogelijke folaat-depletie.

**In hoofdstuk 6** werd de expressie van MDR-eiwitten onderzocht op ontstekingscellen in synoviaal weefsel van RA-patiënten met actieve ziekte, voor en na behandeling met MTX (7.5-15 mg/week) of leflunomide (20mg/dag) en ‘gezond’ synoviaal weefsel van patiënten die een orthopedische operatie ondergingen. Met behulp van immunohistochemische technieken werd de expressie bepaald van Pgp, MRP1-5, MRp8, MRp9 en BCRP. Er werd een hoge expressie gevonden van BCRP in de synoviale lining en op macrofagen en endotheelcellen in de synoviale subliming bij alle patiënten. Statistische analyse liet een trend zien van hogere BCRP expressie in biorp van patiënten met een hoge ziekte-activiteit (gedefinieerd door een hoge DAS28 score). Daarnaast bleek dat de mediane BCRP expressie 4-8 keer zo hoog was in biorp van ‘MTX-falers’ vergeleken met biorp van patiënten met een goede respons op MTX na 4 maanden. Dezelfde trend werd gezien voor RA-patiënten die werden behandeld met leflunomide: een 2.5 keer hogere BCRP expressie werd waargenomen in synoviale biorp van ‘leflunomide-falers’ in vergelijking met patiënten met een goede respons op leflunomide. Naast BCRP werd een duidelijke expressie gevonden van MRP1 op T-cellen (gelegen in aggregaten) en een lage expressie op macrofagen. De expressie van Pgp, MRP2-5, MRp8 en MRp9 was te laag om met immunohistochemische technieken aan te tonen. In ‘gezond’ synoviaal weefsel, met zeer weinig ontstekingscellen, werden slechts enkele BCRP positieve cellen aangetoond. In dit weefsel werd ook geen expressie gevonden van de andere MDR-eiwitten. Omdat hoge expressie van BCRP werd aangetoond in synoviale biorp van alle patiënten reeds vóór aanvang van de therapie met MTX, is het waarschijnlijker dat de expressie wordt geïnduceerd door het ontstekingsproces dan door blootstelling aan DMARDs. Ondanks het feit dat de expressie van BCRP positieve cellen aangetoond is in ‘gezond’ synoviaal weefsel, de expressie van MRP1-5, MRp8 en MRp9 is te laag om met immunohistochemische technieken aan te tonen.

**Hoofdstuk 7** is een klinisch georiënteerd review-artikel over de status van MTX en leflunomide (als monotherapie en in combinatieschema’s) bij de behandeling van patiënten met RA. Dit hoofdstuk dient als inleiding van hoofdstuk 8, waar ex vivo analyses naar de ontstekingsremmende capaciteit van nieuwe generatie anti-folaten (MTX-analoga) worden beschreven. Effectiviteitsstudies laten zien dat MTX en leflunomide zeer potente geneesmiddelen zijn bij de behandeling van RA-patiënten. Lange termijn observationele studies tonen effectiviteit aan van MTX (op vermindering van ziekte-activiteit en gewrichtsschade) gedurende meer dan 5 jaar bij ongeveer 50% van de RA-patiënten. Daarnaast is bewezen dat MTX de effectiviteit van TNFα blokkers (antilichamen tegen TNFα) versterkt, waarschijnlijk via remming van de productie van antistoffen tegen deze middelen door plasmacellen.

Ondanks een aanvankelijk goede respons op behandeling met MTX en een relatief lange gebruiksduur bij ongeveer 50% van de RA patiënten, worden veel patiënten geconfronteerd met verlies van MTX-effectiviteit bij langdurig gebruik (klinische resistentie). Naast het mechanisme van efflux van MTX via MDR-eiwitten (zoals BCRP en MRP1) kunnen er ook andere oorzaken zijn voor de verminderde effectiviteit van MTX, zoals eerder is aangetoond voor resistentie van kankercellen tegen MTX. Sommige van deze mechanismen zouden ook een rol kunnen spelen bij de behandeling van RA-patiënten, zoals: (a) verminderde cellulaire opname van MTX via de Reduced Folate Carrier (RFC), (b) verminderde conversie naar polyglutamaat metabolieten van MTX door een verminderde activiteit van het enzym folypolyglutamaat synthetase (FPGS), (c) verhoogde expressie van het target-enzym van MTX, dihydrofolate reductase (DHFR) of (d) polymorfismen in genen die coderen voor enzymen in het foliumzuurmetabolisme. Om dit te onderzoeken werd in **hoofdstuk 8** de ontstekingsremmende capaciteit van 8 nieuwe generatie anti-folaten bepaald, die zijn ontworpen om MTX resistentie bij leukemie-patiënten te omzeilen. Deze geneesmiddelen hebben bijvoorbeeld een hogere affiniteit voor de RFC en/of FPGS in vergelijking met MTX of hebben een ander target in het foliumzuurmetabolisme dan DHFR, zoals thymidylate synthase (TS) en glycaminide ribonucleotide transformylase (GARTFase). De effectiviteit van deze geneesmiddelen om TNFα productie door geactiveerde T-cellen (na CD3/CD28 stimulatie) te remmen werd onderzocht in volbloed van RA-patiënten. Twee nieuwe generatie DHFR remmers (P523 en P564) en twee nieuwe generatie TS remmers (Raltitrexed en GW1843), welke een hogere affiniteit hebben voor zowel RFC en FPGS bleken bij lage concentraties effectief in het remmen van TNFα productie door geactiveerde T-cellen. De mediane concentraties van deze medicijnen om 50% van de TNFα productie te remmen (IC50 waarden) waren 6.9-10.5 keer lager in vergelijking met MTX, ook in volbloed van RA-patiënten die klinisch hadden gejaagd op MTX. Deze data tonen aan dat behandeling met deze nieuwe generatie anti-folaten te overwogen is bij RA-patiënten die falen op MTX.

**In hoofdstuk 9** hebben we de folaatreceptor-β (FR-β) onderzocht als potentiële nieuw target voor de behandeling van RA-patiënten. Folaatreceptoren zijn membraangebonden receptoren waarvan drie isotypen bestaan (α,β,δ). Deze receptoren hebben een hoge affiniteit voor foliumzuur en een relatief lage affiniteit voor MTX, waarna opname van deze middelen geschiedt via endocytose van het receptor-ligand complex. Het is bekend dat de FR-β selectief tot expressie komt op geactiveerde synoviale macrofagen en daarom een target voor therapie kan zijn bij patiënten met reumatoïde artritis. Om een voorspelling te kunnen
doen over de potentiële effectiviteit van deze benadering hebben we in eerste instantie de expressie bepaald van de FR-β op ontstekingscellen in intact synoviaal weefsel van RA-patiënten en op perifere bloedlymfocyten. Daarnaast werd de bindingsaffiniteit voor de FR-β onderzocht van 10 nieuwe generatie anti-folaten op basis van competitiestudies met [³H]-foliumzuur op een cellijn die getransfecteerd is met de FR-β. Zoals verwacht kon met behulp van immunohistochemische technieken hoge expressie van de FR-β worden aangetoond op synoviale lining cellen en macrofagen in de synoviale sublining van RA-patiënten, terwijl geen expressie werd gevonden in T-cel gebieden en in ‘gezond’ synoviaal weefsel. In overeenstemming hiermee werd een hoge mRNA expressie gevonden in synoviaal weefsel van RA-patiënten, een duidelijke expressie werd gezien in ex-vivo gekweekte macrofagen uit perifeer bloed van RA-patiënten, terwijl geen expressie werd waargenomen in monocyten en ex-vivo geactiveerde T-cellen. Screening van 10 nieuwe generatie anti-folaten voor affiniteit voor de FR-β leverde 4 geneesmiddelen op met een hoge affiniteit voor deze receptor (20-77 keer hogere affiniteit dan MTX). Een van deze geneesmiddelen, de TS-remmer BCG945, bleek een heel hoge affiniteit te hebben voor de FR-β, terwijl bekend is dat dit middel een heel lage affiniteit heeft voor de RFC. BGC945 verdient daarom nadere evaluatie als potentiële selectieve macrofaag-gerichte therapie bij RA.

Een ander potentieel nieuw target voor de behandeling van patiënten met reumatoïde artritis is NFkB. Aangenomen wordt dat activatie van deze transcriptie factor een belangrijke rol speelt bij het ontstekingsproces in RA. Om deze reden zijn er diverse therapeutische strategieën ontwikkeld om de activiteit van NFkB te remmen, bijvoorbeeld door gebruik te maken van directe NFkB-remmers, NFkB oligonucleotides om binding van NFkB aan de promotor tegen te gaan, of remmers van IkB-kinase. Een andere benadering is blokkade van de proteasoom-afhankelijke afbraak van IkBα, de natuurlijke remmer van NFkB. Cel-activatie resulteert normaliter in fosforlyering en ubiquitinering van IkBα. Deze processen zorgen ervoor dat IkBα kan worden afgebroken door het 26S proteasoom. Nadat IkBα is afgebroken, kan NFkB naar de kern worden getransporteerd waar het de transcriptie van pro-inflammatoire cytokines en anti-apoptotische genen kan initiëren. Vanuit dit concept is dus te verwachten dat remmers van het proteasoom deze processen kunnen remmen en daarmee een anti-inflammatoire respons bewerkstelligen. In hoofdstuk 10 werd daarom het ontstekingsremmende effect onderzocht van de proteasoomremmer bortezomib, een boronzuur bevattende dipetide, dat geregistreerd is voor de behandeling van (therapie-resistente) multipiele myeloom patiënten. We konden aantonen dat bortezomib heel effectief is in remming van de TNFα productie door ex-vivo geactiveerde T-cellen van RA-patiënten, onafhankelijk van hun klinische respons op MTX. Naast remming van TNFα productie werd ook een opvallende inductie van apoptose gezien in geactiveerde T-cellen, 48 uur na blootstelling aan dit geneesmiddel. Ook vonden we een concentratie-afhankelijke, maar apoptose-onafhankelijke, remming van T-cel activatie door αCD3/CD28. Gezien deze resultaten, het unieke werkingsmechanisme van bortezomib, en het feit dat bortezomib waarschijnlijk ook effectief is tegen DMARD resistente ontstekingscellen, verdient het aanbeveling om dit geneesmiddel en tweede-generatie proteasoomremmers verder te onderzoeken als potentiële anti-reumatica.

De belangrijkste bevindingen uit dit proefschrift kunnen als volgt worden samengevat:

**Belangrijkste bevindingen:**

- **Chronische blootstelling van ontstekingscellen aan sulfasalazine induceert expressie van het multi-drug resistentie eiwit BCRP op de celmembrana, wat resulteert in sulfasalazine resistentie**
- **Hoge expressie niveaus van BCRP werden aangetoond op de celmembrana van synoviale macrofagen, wat voor verminderde werkzaamheid van sulfasalazine, methotrexaat en leflunomide (allen BCRP substraten) kan zorgen**
- **Sulfasalazine is een potente remmer van de opname van methotrexaat via de Reduced Folate Carrier; deze bevinding zou kunnen verklaren waarom er geen synergistisch effect wordt gevonden bij de combinatie van sulfasalazine en methotrexaat in combinatietherapieën**
- **Nieuwe generatie anti-folaten, ontwikkeld om methotrexaat resistentie bij leukemiepatiënten te omzeilen, blijken zeer effectieve ontstekingsremmers van ex-vivo geactiveerde T-cellen van RA-patiënten. Deze geneesmiddelen zouden kunnen worden gebruikt bij de behandeling van RA-patiënten die falen op behandeling met methotrexaat**
- **De folat receptor-β, die hoog tot expressie komt op synoviale macrofagen, kan worden gebruikt als een selectieve target bij de behandeling van RA-patiënten met nieuwe generatie anti-folaten en voor beeldvorming van (subklinische) ziekte-activiteit**
- **Het remmen van de cellulair proteasoomactiviteit met bortezomib veroorzaakt een effectieve remming van de cytokine-productie door geactiveerde T-cellen van RA-patiënten**
Dankwoord/acknowledgements

De gezagvoerder meldt vanuit de cockpit de te vliegen route. Hij zegt dat het mooi weer is op de plaats van bestemming, maar dat weet je nooit zeker....

Promoveren is als een vliegreis, een onderneming waar veel mensen bij betrokken zijn om het mogelijk te maken. Vanaf deze plaats wil ik graag iedereen bedanken die mij op enige wijze heeft geholpen bij mijn onderzoek.

Allereerst gezagvoerder dr. Gerrit Jansen, co-promotor. Beste Gerrit, in 2001 ben ik als co-piloot begonnen in jouw laboratorium. Je enthousiaste en deskundige begeleiding heeft ervoor gezorgd dat deze stage uiteindelijk is uitgemond in dit promotieonderzoek. Je literatuurkennis is ongeëvenaard; als er iemand over een fotografisch geheugen beschikt dan ben jij het. Ik heb zeer prettig met je samengewerkt en heb veel van je geleerd. Dank voor de leuke jaren!

De overige gezagvoerders: professor Ben Dijkmans en professor Rik Scheper (promotoren) en professor Willem Lems (co-promotor).

Beste Ben, ik wil je hartelijk bedanken voor het vertrouwen en de goede begeleiding. Ik heb veel van je geleerd over de klinische reumatologie. Je vestigde steeds de aandacht op de klinische implicaties van ons onderzoek, en daar gaat het natuurlijk ook om. Je morele steun, bij zowel het onderzoek, mijn klinische carrière als ook op persoonlijk vlak waarder ik zeer.

Beste Rik, jij bracht me in contact met de afdeling reumatologie waar ik ben gaan werken op het RA/MDR-project, een samenwerking tussen de afdelingen reumatologie en pathologie. De wekelijkse MDR-besprekingen hebben mij wetenschappelijk gevormd, en je scherpe commentaar bij zowel het onderzoek als de manuscripten waren voor mij van grote waarde. Veel dank voor de prettige samenwerking!

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Ik wil al het ‘vliegend personeel’ (reumatologen, arts-assistenten en arts-onderzoekers van het VU-Medisch Centrum, het Slotervaart ziekenhuis en het Jan van Breemeninstituut) bedanken voor de hulp bij het selecteren van patiënten voor ons onderzoek: Conny van der Laken, Irene van der Horst, Irene Bultink, Alexandre Voskuyl, Dirk-Jan van Schaardenburg, Kirsti Steen, Franktien Turkstra, Natalja Basoski en Andreas Gerards (Vlietland ziekenhuis).


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Special thanks to professor Manohar Ratnam from the laboratory for Cancer Biology at the Medical University of Toledo, Ohio, USA, for the fruitful collaboration. I really enjoyed visiting your laboratory twice! Thank you very much for your hospitality and for training me in molecular biology. Marcela D’Alincourt Salazar: thank you for your help (and troubleshooting) in designing a model-system of rheumatoid arthritis macrophages and for the nice social program along the side. Mariana and Stoytcho, my Bulgarian friends: Thank you for your help in the lab and making me feel at home in Toledo. I will never forget Christmas Eve ‘Bulgarian style’! The other people in the ‘Ratnam lab’: Hala, Juan, Huiling, Mesfin and Aymen, thanks for all your help and hospitality during my stay in Toledo.

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Vertrouwend op een zachte landing!

Joost.
Curriculum vitae


Het onderzoek van Joost van der Heijden werd gewaardeerd met een tweetal prijzen. In april 2002 won hij de ‘Arthron Young Investigator Award’ voor innovatief wetenschappelijk onderzoek op het gebied van de reumatologie. Voor zijn onderzoeksvoorstel getiteld: ‘Specifieke macrofaag gerichte therapie voor de behandeling van RA-patiënten met nieuwe generatie foliumzuur antagonisten’, ontving hij in januari 2006 de ‘Rheumatology Grant 2006’ van de Nederlandse Vereniging voor Reumatologie (NVR). In het kader van dit onderzoek werkte hij gedurende 3 maanden in het laboratorium van professor M. Ratnam (een autoriteit op het gebied van de folaatreceptor) aan de Medical University of Toledo, Toledo (OH), Verenigde Staten. Vanaf november 2008 zal Joost van der Heijden de opleiding tot internist vervolgen.

Curriculum vitae

Joost van der Heijden was born in Gouda, on the 6th of april 1976. In 1995 he obtained his VWO diploma at the Gouwe College in Gouda. From 1995-1998 he studied Medical Biology at the Vrije Universiteit in Amsterdam. In 1998 he started his studies in Medicine, obtaining his MSc degree in 2001 and his medical license in January 2004. During his study, he performed a traineeship in the laboratory for Experimental Rheumatology (head: dr. G. Jansen) at the department of Rheumatology from the VU University Medical Center, Amsterdam, the Netherlands (head: prof. dr. B.A.C. Dijkmans). Next, a research project on ‘multiple drug-resistance in the treatment of rheumatoid arthritis patients’ was performed in collaboration with the department of Experimental Pathology (head: prof. dr. R.J. Scheper) and with financial support of the Dutch Arthritis Association. After obtaining his medical licence he continued his research at the department of Rheumatology. One of his research proposals was rewarded with a Netherlands Organization of Scientific Research ZonMw/NWO-AGIKO stipend. In July 2005 he started his specialization in Internal Medicine at the department of Internal Medicine from the VU University Medical Center (head prof. dr. S.A. Danner, at present prof.dr. M.H.H. Kramer). In this context, in 2006 and 2007 he worked at the department of Internal Medicine from the Kennemer Gasthuis hospital, Haarlem, the Netherlands (head: prof. dr. R.W. ten Kate).

His research was awarded twice: In April 2002 he won the ‘Arthron Young Investigator Award’ for his innovative scientific research in the field of drug resistance in rheumatology. For his research proposal entitled: ‘Specific macrophage-directed therapy for the treatment of rheumatoid arthritis patients with novel antifolate drugs’, he received the ‘Rheumatology Grant 2006’ from the Dutch Rheumatology Association. As part of this grant, he performed a traineeship of three months in the laboratory of prof. dr. M. Ratnam (a world leading investigator in the field of folate receptors) at the Medical University of Toledo, Toledo (OH), USA.

Joost van der Heijden will continue his training in Internal Medicine in November 2008.
List of publications

International journals


2. Van der Heijden JW, Oerlemans R, Lems WF, Scheper RJ, Dijkmans BAC, Jansen G. The proteasome inhibitor bortezomib inhibits the release of NFκB-inducible cytokines and induces apoptosis of activated T cells from rheumatoid arthritis patients. Accepted for publication in Clinical & Experimental Rheumatology 2008


Submitted

2. Van der Heijden JW, Gerards AH, Oerlemans R, Lems WF, Scheper RJ, Aarden LA, Dijkmans BAC, Jansen G.
Enhanced capacity of selected methotrexate-analogues to inhibit TNF-α production in whole blood from RA patients.
Submitted for publication.

National journals

1. Van der Heijden JW.
Toename van een pompeiwit in de celmembraan van CEM (T) cellen geeft aanleiding tot resistentie/ongevoeligheid voor de DMARD sulfasalazine.

2. Van der Heijden JW.
Macrofaag gerichte therapie voor de behandeling van RA-patiënten met nieuwe generatie foliumzuur antagonisten.

3. Van der Heijden JW.
Waarom worden mensen met RA resistent tegen anti-reumatica?

Abstracts (first author)

Expression of lung resistance protein on CD68 positive synovial tissue macrophages is associated with disease activity in rheumatoid arthritis patients.


3. Van der Heijden JW, Oerlemans R, Lems WF, Scheper RJ, Dijkmans BAC, Jansen G.
Potential anti-inflammatory properties elicited by the 26-S proteasome inhibitor bortezomib.

4. Van der Heijden JW, Oerlemans R, Lems WF, Scheper RJ, Dijkmans BAC, Jansen G.
The proteasome inhibitor bortezomib induces apoptosis and inhibits TNF-α release by activated T-cells from rheumatoid arthritis (RA) patients.

Expression of the multi-drug resistance protein BCRP in synovial tissue of RA-patients. A marker for inflammation or resistance to MTX?

Inhibition of TNF-α production by activated T-cells of rheumatoid arthritis patients by novel anti-folate drugs: an ex vivo pilot study.

7. Van der Heijden JW, Gerards AH, Oerlemans R, Lems WF, Scheper RJ, Aarden LA, Dijkmans BAC, Jansen G.
Inhibition of tumor necrosis factor alpha production by activated T-cells of rheumatoid arthritis patients by novel anti-folate drugs: an ex vivo pilot study.

Conference proceedings

1. Van der Heijden JW, Jansen G, Dijkmans BAC.
Antifolate drug combinations for inflammatory diseases.

2. Oerlemans R, Van der Heijden JW, Dijkmans BAC, Lems WF, Scheper RJ, Assaraf YG, Jansen, G.
Sulfasalazine exposure confers MTX resistance in human monocytic/macrophage cells by differential effects on folate influx and folate efflux systems.
List of publications
