Drug resistance is generally accepted as an important cause of treatment failure for patients with neoplastic or infectious diseases. Molecular mechanisms underlying drug resistance include the action of drug efflux pumps belonging to the super-family of ATP binding cassette (ABC) proteins, which mediate the cellular extrusion of a large variety of therapeutic drugs, a phenotype that is referred to as multidrug resistance (MDR). Unlike neoplastic and infectious diseases, chronic inflammatory diseases have received little attention. The potential role of ABC transporters in determining the efficacy of anti-rheumatic drugs, notably disease modifying anti-rheumatic drugs (DMARDs), in patients with rheumatoid arthritis is unclear. Based on knowledge from the field of oncology and immunology, this review concentrates on the pharmacological role of MDR proteins in the (clinical) efficacy of several DMARDs, as well as the physiological role of MDR proteins in transporting signalling molecules important in inflammatory processes.

Key words: rheumatoid arthritis, DMARDs, multidrug resistance, ABC transporters, P-glycoprotein, multidrug resistance associated protein, methotrexate, sulphasalazine, chloroquine, review

Rheumatoid arthritis (RA) is a common autoimmune disease that is characterized by a chronic inflammation of the synovial joints, and infiltration by blood-derived cells (T cells, macrophages), leading to progressive destruction of cartilage and bone (1). Current medical therapy of patients with RA consists of treatment with:

- Non-steroidal anti-inflammatory drugs (NSAIDs), which reduce pain and inflammation but do not alter the course of the disease
- Disease modifying anti-rheumatic drugs (DMARDs), which can influence or modify the course of RA.

Over the past decade ‘biologics’ have also been introduced in the treatment of RA (2). These biologicals are specifically aimed at antibody-guided blocking of pro-inflammatory cytokines [e.g. tumour necrosis factor α (TNFα)] that play a major role in the pathophysiology of RA (3). Novel anti-TNFα agents, such as infliximab (4) or etanercept (5), displayed marked anti-rheumatic activity, but their long-term superiority over DMARDs — with known efficacy and low costs — still needs to be established, especially in the treatment of early RA. For this reason, DMARDs or combinations of DMARDs remain a common treatment option for patients with RA (6–9). However, it is well recognized that with successive treatment with DMARDs, many RA patients demonstrate loss of drug efficacy over time. Whether this is due to the onset of acquired drug resistance has not been extensively investigated.

Meta-analyses of DMARD efficacy for treatment of RA patients showed that (hydroxy)chloroquine and sulphasalazine were among the DMARDs with the relatively shortest duration of efficacy (1–2 years), while methotrexate (MTX) had the longest-lasting efficacy (6–7 years) (10–13).

Recently, a study by Morgan (14) showed that a small population of RA patients may be primary unresponsive to DMARDs, possibly via a multidrug resistance (MDR)-related mechanism.

This review will further elaborate on the possible role of MDR proteins in the efficacy of DMARDs in RA treatment. However, it should be kept in mind that, as with anti-cancer drugs, DMARD resistance may be a multifactorial event, not only involving enhanced drug efflux via specific MDR proteins, but also other mechanisms, outlined in Figure 1A. In short, other mechanism of drug resistance can include:

- Defective drug delivery (pharmacokinetic resistance)
- Impaired drug uptake
- Impaired drug activation
- Enhanced drug detoxification/sequestration
- Altered (downstream) effects after drug–target interaction (15).
Drug resistance can be an inherent/intrinsic property of cells, indicating that cells are resistant to specific classes of drugs without ever being exposed to these drugs previously. Alternatively, drug resistance can be acquired, indicating that cells were primary sensitive, but develop resistance over successive drug treatments. Drug resistance can be restricted to one specific class of drugs, e.g. antifolates like MTX, or can extend to multiple structurally unrelated drugs, a phenotype designated as MDR (16 – 21). Molecular mechanisms of cellular drug resistance can take place at various levels, involving, for example, impaired cellular uptake, enhanced metabolism, increased levels of target enzymes, defective apoptotic pathways and/or enhanced cellular extrusion of drugs (15, 22).

This latter mechanism can be mediated by the up-regulation of specific members of the family of ATP binding cassette (ABC) transporters (17, 21, 23 –26). Drug extrusion via these transporters is an active process requiring ATP as an energy source (Figure 1B). Data from the human genome project revealed that the ABC transporter family includes 48 proteins (25, 27, 28), divided into seven distinct subfamilies (ABC A – G), some of which are extensively characterized, while the functional properties of others are still unknown.

Several review papers have appeared over the past few years covering specific aspects of ABC transporters in relation to resistance to anti-cancer drugs (17 – 19, 21, 23 – 26, 29 – 34). ABC transporters that have an established or putative role in drug resistance include (Table 1):

- **ABCB1** [P-glycoprotein (Pgp), primary MDR (MDR1)] (16, 35 – 37)
- **ABCC1-7, ABCC10/11**, also denoted as MDR-associated protein 1 – 9 (MRP1 – 9) (38 – 49)
- **ABCG2**, also known as breast-cancer resistance protein (BCRP) (20, 34, 50 – 53).

Pgp and MRPs are ‘full transporters’ with 12 – 17 transmembrane domains (TMDs) assembling a pore-forming protein (25). In contrast, ABCG2 is referred to as a ‘half transporter’ (six TMDs) requiring homo- or hetero-dimerization to form a functionally active protein (21, 54).

Although there is overlap in substrate specificity, Pgp is known to prefer neutral hydrophobic substrates, while MRPs and ABCG2 transport anionic compounds, mostly as conjugates of glutathione, glucuronides or sulphates (25, 55 – 60). Tissue distribution studies showed that MDR proteins are expressed at locations and by tissues that are heavily exposed to toxic agents, microbials and exogenous.
compounds (31, 53, 61, 62). Consistently, one of the primary functions of MDR proteins is the extrusion of toxic substances.

MDR proteins Pgp and MRP1 have been detected in immunologically relevant cells [T cells, monocytes, dendritic cells (DCs) and macrophages] (61, 63–73), where they are thought to play not only a pharmacological, but also a physiological role in inflammatory processes (see later sections). Interestingly, ABCG2 has been identified in a side population of CD34^+ haematopoietic progenitor cells (74–76), although its pharmacological/physiological relevance is still unclear.

MDR proteins and DMARDs/anti-inflammatory drugs

Substrate specificity studies suggest that several DMARDs, NSAIDs and other anti-inflammatory drugs (e.g. glucocorticoids) can be transported by, or inhibit, specific MDR proteins (summarized in Table 2). The putative role of MDR proteins in loss of efficacy/resistance for these anti-rheumatic drugs is discussed in greater detail below.

Methotrexate

The folate antagonist MTX is the anchor drug in the treatment of RA patients. The precise mechanism of the anti-rheumatic action of MTX has still not been fully elucidated, but involves the inhibition of key enzymes in folate metabolism and purine biosynthesis de novo, both of these are required for DNA synthesis (77). This inhibition provokes folate depletion and enhanced adenosine release, leading to apoptosis (78) and/or interaction with adenosine receptors, resulting in an anti-inflammatory effect (79). Cell entry of MTX requires a specific transporter, the reduced folate carrier (RFC), which normally internalizes natural folates (80–82). Once taken up intracellularly, MTX is complexed to polyglutamate forms, which prevents its leakage from the cells (83, 84). In cancer patients, the most common mechanisms of acquired resistance to MTX include impaired RFC-mediated uptake of MTX, rather than Pgp-mediated efflux. Consistent with the notion that Pgp preferentially transports neutral hydrophobic compounds, rather than a divalent anion like MTX, there is currently broad consensus that Pgp is not a major player in MTX efflux (16, 25, 55, 89). Instead of Pgp, there is compelling evidence that the MDR transporters MRP1-4 and ABCG2 have the capacity to extrude non-polyglutamated MTX (90–99). Of note is that ABCG2 appears to be capable of transporting di- and tri-glutamate forms of MTX (58).

Transport kinetic data from these studies showed that MTX efflux via MRP1-4 and BCRP is a low affinity/high capacity process, in contrast to MTX influx via RFC, which is a high affinity/low capacity process (80, 82). Apparently, a dynamic balance exists between the influx and efflux routes in

<table>
<thead>
<tr>
<th>ABC code</th>
<th>Common name</th>
<th>Chromosome localization</th>
<th>Size (AA)</th>
<th>Normal tissue distribution</th>
<th>Immunochemical cells</th>
<th>Preferred substrates</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCB1</td>
<td>P-gp, MDR1</td>
<td>7q21</td>
<td>1279</td>
<td>Many tissues</td>
<td>T cells (CD4, CD8) DCs, monocytes T cells (CD56, CD8, CD4, CD34^+), DC, Mø</td>
<td>Neutral hydrophobic compounds Anionic conjugates (glutathione, glucoronide)</td>
<td>(18, 25, 68, 71, 125, 65, 69, 73)</td>
</tr>
<tr>
<td>ABCB2</td>
<td>MRP1</td>
<td>16p13.1</td>
<td>1531</td>
<td>Ubiquitous</td>
<td>T cells (CD56, CD8, DC, Mø)</td>
<td>Anionic conjugates, glutathione</td>
<td>(19, 39)</td>
</tr>
<tr>
<td>ABCB3</td>
<td>MRP2</td>
<td>10q24</td>
<td>1545</td>
<td>Liver, intestine, kidney</td>
<td>T cells (CD56, CD8, DC, Mø)</td>
<td>Anionic conjugates, glutathione</td>
<td>(25, 91)</td>
</tr>
<tr>
<td>ABCB4</td>
<td>MRP3</td>
<td>17q21.3</td>
<td>1527</td>
<td>Liver</td>
<td>T cells (CD56, CD8, DC, Mø)</td>
<td>Anionic conjugates, glutathione</td>
<td>(39, 41, 93, 113)</td>
</tr>
<tr>
<td>ABCB5</td>
<td>MRP4</td>
<td>13q32</td>
<td>1325</td>
<td>Many tissues (kidney)</td>
<td>T cells (CD56, CD8, DC, Mø)</td>
<td>Anionic conjugates, glutathione</td>
<td>(39, 43, 113)</td>
</tr>
<tr>
<td>ABCB6</td>
<td>MRP5</td>
<td>16p13.1</td>
<td>1503</td>
<td>Liver, kidney</td>
<td>T cells (CD56, CD8, DC, Mø)</td>
<td>Anionic conjugates, glutathione</td>
<td>(45, 206)</td>
</tr>
<tr>
<td>ABCB7</td>
<td>MRP6</td>
<td>6q21</td>
<td>1513</td>
<td>Low in all tissues</td>
<td>T cells (CD56, CD8, DC, Mø)</td>
<td>Anionic conjugates, glutathione</td>
<td>(46, 207)</td>
</tr>
<tr>
<td>ABCB8</td>
<td>MRP7</td>
<td>16q12.1</td>
<td>1382</td>
<td>Low in all tissues</td>
<td>T cells (CD56, CD8, DC, Mø)</td>
<td>Anionic conjugates, glutathione</td>
<td>(47, 48)</td>
</tr>
<tr>
<td>ABCB9</td>
<td>MRP8</td>
<td>1q12.1</td>
<td>655</td>
<td>Liver, breast, placenta</td>
<td>CD34^+ (side population)</td>
<td>Amphipatic drugs</td>
<td>(21, 51, 53, 74)</td>
</tr>
</tbody>
</table>

AA = Number of amino acids, Mø = macrophages. For timely updates on properties of ABC transporters, see http://www.nutrigene.4t.com/humanabc.htm
controlling intracellular levels of MTX. In this respect, MTX exposure time, which allows conversion to non-effluxable polyglutamated forms, can also be a determining factor in the efficacy of MTX. This is illustrated in Table 3, where the efficacy (anti-proliferative effect) of MTX was evaluated against Pgp-, MRP1- and BCRP-over-expressing cells compared with controls after either a 72 h continuous exposure to MTX (permissive for polyglutamylation), or a short-term (4 h) exposure to MTX (incomplete polyglutamylation) followed by a 68 h drug-free period. Following a 4 h exposure to MTX, only cells over-expressing MRP1 and BCRP displayed a high level of resistance to MTX, indicating that MRP1 and BCRP, but not Pgp, were able to extrude unpolyglutamated MTX, resulting in lower intracellular levels and diminished efficacy. Following a 72 h exposure to MTX, low levels of resistance were still noted for the MRP1 and BCRP1, but not for the Pgp over-expressing cells.

Finally, it is of importance to note that MRP1-3 can also mediate the efflux of natural folates (100). In this respect, diminished expression of MRP1 may lead to increased intracellular folate pools, which can antagonize MTX activity (101).

Sulphasalazine

Sulphasalazine (SSZ) is a potent DMARD used as a single agent and in combination regimens with MTX, hydroxychloroquine and prednisone (8, 9). SSZ is an organic anion that, based on structure–activity relationships, could be a potential substrate for members of the ABCC family of MDR transporters. In fact, a role for MRP1 in SSZ transport was indicated from studies by Lightfoot (102): however, studies from our laboratory (unpublished) did not demonstrate resistance to SSZ in MRP1-overexpressing cells. This may be caused by the fact that SSZ is also an inhibitor of glutathione-S-transferase (103), the enzyme responsible for glutathione conjugation prior to MRP1 transport. Recent studies by our laboratory have shown that acquired resistance to SSZ was accompanied by induction of ABCG2 (see below).

Table 3. Role of MDR proteins in MTX resistance.

<table>
<thead>
<tr>
<th>Exposure time MTX</th>
<th>ABCB1&lt;sup&gt;(a)&lt;/sup&gt; (Pgp)</th>
<th>ABCC1&lt;sup&gt;(###)&lt;/sup&gt; (MRP1)</th>
<th>ABCG2&lt;sup&gt;(####)&lt;/sup&gt; (BCRP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>72 h</td>
<td>0.8</td>
<td>1.9</td>
<td>1.7</td>
</tr>
<tr>
<td>4 h (+68 h drug free)</td>
<td>1.1</td>
<td>&gt;80</td>
<td>28</td>
</tr>
</tbody>
</table>

<sup>(a)</sup>Resistance factor is defined as the ratio of IC<sub>50</sub> concentration (MTX concentration resulting in 50% inhibition of cell proliferation) of cells with over-expression of the indicated ABC transporter versus control cells without over-expression of the ABC transporter.

<sup>(##)</sup>ABCB1/Pgp-overexpressing cells were vinblastin-resistant CEM cells (106).

<sup>(###)</sup>ABCC1/MRP1-overexpressing cells were MRP1 transfected 2008 cells (90).

<sup>(####)</sup>ABCG2/BCRP-overexpressing cells were mitoxantrone-resistant MCF-7/MR cells (50).
(Hydroxy)chloroquine

The antimalarial DMARD (hydroxy)chloroquine has relatively little activity as a single agent, but is used in combinations with other DMARDs (6, 104, 105). Chloroquine has been identified as a substrate, as well as an inhibitor of Pgp and MRP1 (106–108). Its inhibitory activity suggests its use as a chemosensitizer, as it elevates the intracellular concentration and efficacy of therapeutic drugs that are substrates for these MDR proteins (see below).

Leflunomide

Leflunomide is a DMARD with immunosuppressive activity due to inhibition of dihydroorotate dehydrogenase (DHODH), a key enzyme in pyrimidine biosynthesis de novo (109, 110). To date, it is unknown whether leflunomide, or its active metabolite A771726, is a substrate for MDR proteins, or whether over-expression of specific MDR proteins confers resistance to leflunomide. Acquired resistance to leflunomide does, however, seem to be associated with increased activity and/or structural alterations of the target enzyme DHODH (111).

Azathioprine

Azathioprine is a prodrug of 6-mercaptopurine (6-MP) and has an established place in RA treatment (112). 6-MP, along with 6-thioguanine and other nucleoside analogues, proved to be a potential substrate for MRP4/ABCC4 and MRP5/ABCC5 (41, 43, 113–115). Over-expression of MRP4 or MRP5 is associated with resistance to this class of compounds. As for MTX, where polyglutamylation abolished efflux, metabolism of 6-MP to its diphosphate and triphosphate metabolites diminished substrate affinity for MRP4 and MRP5.

Auranofin/aurothiomalate

Although gold preparations belong to the first generation of DMARDS, they are still prescribed in some cases of RA. Glennas and colleagues (116, 117) are the only investigators who have studied mechanisms of acquired resistance to aurothiomalate. They observed the onset of resistance, but an underlying mechanism was not revealed. No evidence for an increase in methallothionein was observed (118). Evidence has been presented that MRP1 and MRP2 can transport glutathione conjugates of heavy metal ions (arsenite, cis-platin) (19) but whether this also includes gold preparations remains to be established.

Cyclosporin A

In RA treatment, cyclosporin A (CSA) displayed activity in combination with MTX (119). CSA is well recognized for its inhibitory interaction with P-gp (35, 120) (see also below). Data on mechanisms of acquired resistance to CSA are limited, but surprisingly do not seem to involve up-regulation of Pgp expression (121, 122).

Glucocorticoids

Glucocorticoids (GC) display DMARD activity. Given their hydrophobic nature, GCs like prednisone and dexamethasone were identified as substrates for Pgp (18, 123). Over-expression of Pgp reduces the cellular uptake of dexamethasone, which can be normalized by blocking of Pgp (124).

Altogether, there is ample evidence that several DMARDs can be substrates for MDR proteins. To what extent specific MDR proteins actually contribute to clinical DMARD resistance requires further investigations.

Physiological role of MDR proteins

MDR proteins have received most attention for their capacity to extrude therapeutic drugs from cells. Less well understood is the putative physiological role of MDR proteins in transporting signalling molecules, peptides or small proteins that are involved in immunological/inflammatory processes (Table 2). Pgp is expressed on CD4 T cells and CD14 DCs, where it may mediate interferon-γ (IFN-γ) and interleukin-12 (IL-12) release (125), and DC migration to draining lymph nodes (68). Whether these processes involve the Pgp-mediated transport of lipid molecules, such as platelet-activating factor (PAF), is the subject of ongoing studies (126, 127).

An established substrate for MRP1 and other members of the MRP family is leucotriene C4 (LTC4) (25, 128). Leucotrienes are biologically active metabolites derived from arachidonic acid that can activate inflammatory processes by interaction with specific cysteinyl leucotriene receptors, which are coupled to G proteins and downstream signalling pathways (129, 130). LTC4 is a glutathione conjugate of LTA4, which explains its substrate affinity for MRP1 as this protein preferentially transports glutathion conjugates. As for Pgp, Robbiani (72) has demonstrated an important role for MRP1 and LTC4 in antigen-presenting DC migration to lymph nodes. MRP1-mediated transport of LTC4 initiated binding to cysteinyl leucotriene receptors, which promoted CCL19-dependent trafficking of DCs to the lymph nodes. MRP1−/− knock-out mice consistently demonstrated a markedly impaired DC mobilization capacity, which could be restored by
exogenous LTC4. Of note, studies by Wijnholds (131) had shown that MRPI−/− mice had a decreased inflammatory response (ear oedema) after arachidonic acid, LTC4 or phorbol ester treatment.

Steroids are another class of biologically active compounds that are potential physiological substrates for Pgp, MRPs and BCRP (25, 57, 59, 132, 133). A well-known high-affinity substrate for these MDR transporters is 17β-oestradiol 17β-o-glucuronide (E217βG). Recently, cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) were found to be high-affinity substrates for MRP4 and MRP5 (93, 114, 134, 135). Since cyclic nucleotides play essential roles as activators of protein kinases and signalling pathways, one may speculate that MRP4 and MRP5 may (indirectly) contribute to control these processes by extrusion of cAMP and cGMP.

Finally, Reid (136) reported that MRPI is a major efflux transporter for prostaglandins (PG) such as PGE2, PGE3 and PGA1. Interestingly, several non-steroidal anti-inflammatory drugs (NSAIDs), notably indomethacin and indoprofen, proved to be potent inhibitors of PG efflux via MRPI. This suggests that, beyond inhibition of PG synthesis, part of the anti-inflammatory effects of these NSAIDs may be mediated via inhibition of MRPI.

In an early report, Salmon and Dalton (137) hypothesized that MDR proteins, such as P-gp, could function as efflux transporters for pro-inflammatory cytokines, such as TNFα. The anti-inflammatory effect of a DMARD like CSA could then be compatible with it being an inhibitor of Pgp. In line with this hypothesis were studies by Drach (138) and Raghu (139), demonstrating that inhibition of Pgp blocked the release of IL-2, IL4 and IFNγ. In contrast, however, Eisenbraum and Miller (140) showed that T cells from Pgp−/− mice had unaltered release of IL2, IL4 and IFNγ compared with T cells from wild-type mice. Thus, a direct role for Pgp in transport of cytokines with a molecular weight of 10–25 kD is still controversial.

An alternative explanation for these observations may be that Pgp effluxes compounds that control the release of cytokines via other routes. A few reports also suggested a role for MRPI in the transmembrane transport of distinct cytokines, e.g. basic fibroblast growth factor and IL-4 (141, 142). Vellenga (143) reported a marked increase in the release of IL-6 from activated monocytes following inhibition of MRPI by MK571. Again, as for Pgp, conclusive evidence for MRPI-mediated efflux of cytokines will require additional studies, including competition studies of putative protein substrates with established MRPI substrates, such as LTC4. Rather than for proteins, there is substantial evidence that Pgp and MRPI can efflux small (cyclic) peptides (up to 10 mer) (144, 145).

ANLL (N-acetyl-leu-leu-norleucinal) is a tripeptide with Pgp and MRPI substrate affinity (144, 145). It is an inhibitor of 26S-proteasome activity, which plays a key role in the activation of nuclear factor κB (NFκB), the nuclear transcription factor that mediates the transcription of TNFα (146, 147). Hence, cellular Pgp and MRPI expression should be considered as factors determining the efficacy of targeting NFκB signaling by peptide-based 26S-proteasome inhibitors.

In summary, beyond transport of therapeutic drugs, some MDR proteins have the capacity to transport small signalling molecules/peptides. A direct role in transporting higher molecular weight cytokines is still under debate, although (in)direct effects on cytokine release seem evident.

**Regulation of MDR protein expression**

Drug-induced up-regulation of MDR protein expression is well documented, however, mechanisms in control of the transcriptional regulation of MDR genes are still relatively poorly understood. There is accumulating evidence that endogenous or exogenous stress signals (e.g. oxidative stress, toxic drugs, inflammation, cytokines and growth factors) play a central role in the transcriptional regulation of Pgp and MRPI (148): for most other MDR proteins this needs to be established. In this respect, several downstream signal transduction pathways/proteins (MAPK, JNK) and transcription factors (NFκB, and notably cAMP-responsive transcription factors AP1 and SP1) have been implicated in the expression of Pgp and MRPI (149–151).

NFκB binding sites were identified in the promoter region of the Pgp gene (152, 153), and actually appeared to control the regulation of Pgp gene expression (154). The status/activity of p53, a key regulatory gene in tumourogenesis, as well as chronic inflammation (155), also appeared to be of importance in the regulation of MRPI expression: wild type p53 suppresses (156), while mutated p53 enhances (157) MRPI gene transcription. Finally, a recent study by Assaraf (158) showed that cellular folate depletion resulted in down-regulation of MRPI expression, tentatively as a physiological response to prevent loss of intracellular folates.

A number of studies showed that cytokines can have differential effects on MDR protein expression. In fact, TNFα (159, 160) and IL-1β and IL-6 (148) down-regulated expression of Pgp, whereas IFN-γ can up-regulate Pgp expression (161). TNFα was found to up-regulate expression of MRPI, but not of LRP (162). Effects of cytokines/chemokines on expression levels of MDR proteins other than Pgp and MRPI are currently unknown.

Cell differentiation and maturation are processes that are associated with changes in MDR protein
expression. For example, an increase in Pgp and MRP1 expression was noted during monocyte–DC differentiation, and during DC maturation (71, 73). Of note are also observations that MDR expression may alter as a function of ageing (163). In particular, Pgp expression in CD4+ and CD8+ T cells increased during ageing (164). Hence, clinical studies with RA patients for the evaluation of MDR protein expression should include age-matched controls. Taken together, these studies demonstrate that up/down-regulated expression of MDR proteins can be either drug-induced or a response to stress signals, cytokines and other inflammatory effects.

**MDR protein induction by DMARDs**

Induction of MDR proteins during development of resistance to anti-cancer drugs is well established. With the exception of MTX, it is unknown whether acquired resistance to DMARDs can also be associated with the induction of specific MDR proteins. In recent studies from our laboratory (165–168), resistance was provoked in vitro in human T cells (CEM cell line) to the DMARDs chloroquine (CHQ) and sulphasalazine (SSZ). These two DMARDs were selected as, in clinical practice, they present a relatively more rapid onset of diminished efficacy than other DMARDs (10, 12, 13). Following exposure of CEM to stepwise increasing concentrations of CHQ or SSZ for 6 months, appreciable levels of resistance to CHQ (3.5-fold) and SSZ (6.5-fold) were noted. Analysis of MDR protein expression revealed a marked induction of MRP1 in CHQ-resistant cells and BCRP in SSZ-resistant cells. CHQ-resistant cells retained sensitivity to most other DMARDs (MTX, CSA, leflunomide and SSZ), but were highly cross-resistant (>1000-fold) to the glucocorticoid dexamethasone. In contrast, SSZ-resistant T cells displayed markedly increased (13-fold) sensitivity to dexamethasone compared to parental cells. This may actually reflect the improved efficacy of glucocorticoids (prednisone) in combination with MTX+SSZ, compared to MTX+SSZ alone for the treatment of RA patients (9). SSZ-resistant cells showed unaltered sensitivity to CHQ and CsA.

Interestingly, SSZ-resistant cells displayed a low level of cross-resistance to MTX (<2-fold), which markedly increased (>300-fold) when MTX sensitivity was tested in the presence of the selective concentrations of SSZ. This loss of MTX efficacy appeared to be due an unexpected and hitherto unknown inhibition by SSZ of the reduced folate carrier, the cell membrane transporter for MTX, which abolished cell entry of MTX to confer resistance (169). Finally, an altered release of cytokines was observed in SSZ-resistant cells, as indicated by a 3-fold higher secretion of TNFα compared with control CEM cells.

Collectively, these in vitro results demonstrate that the onset of DMARD resistance can be accompanied by induction of specific MDR proteins, altered drug sensitivity and increased TNFα release.

**MDR proteins in RA patients under DMARD therapy**

A large number of cancer therapy studies have included an evaluation of MDR protein expression, notably Pgp, MRP1-5 and BCRP, as part of an assessment of the efficacy of chemotherapeutic drugs (36, 170–173). Other than in a pilot setting, similar studies have not been carried out for RA patients to assess the (long term) efficacy of various DMARDs.

In one of four studies published on the putative role of DMARD resistance and MDR expression, Maillefert (174) observed a significant increase in the percentage of Pgp-positive peripheral blood lymphocytes in a group of RA patients (n = 68, mean disease duration 10.7 years) treated with prednisone (n = 40, 10.7% Pgp+) compared with RA patients treated with various DMARDs (n = 25) and NSAIDs (n = 34), or controls (n = 44) (3.3% Pgp+). Pgp expression did not correlate with gender, disease duration, past resistances to DMARDs, RF, or C-reactive protein (CRP). Consistent with prednisone being a potential substrate for Pgp, these results may suggest a role for prednisone in Pgp induction.

Yudoh (175) studied percentages of Pgp positive cells in Th1 and Th2 subpopulations of CD4+ T cells of 14 RA patients and eight RA patients non-responsive to DMARD therapy (sulphasalazine/bucillamine). The percentage of Pgp+ cells was significantly higher in Th1 cells of non-responders versus responders, both at baseline (9.6% versus 3.3%) and two months after therapy (15.4% versus 3.3%). No differences in Pgp expression (2.2–2.6%) were observed in Th2 cells of responders and non-responders.

Llorente (176) analysed the percentage of peripheral blood lymphocytes for Pgp activity (based on daunorubicin efflux) in 24 controls, eight therapy-responsive RA patients and eight therapy non-responsive RA patients. Therapy non-responsive patients revealed a markedly higher percentage of Pgp-active cells (34.4%) compared with therapy responsive patients (3.9%) or controls (<1.5%).

Finally, in a prospective study, Maillefert (177) analysed Pgp expression (by indirect immunofluorescence) on CD4+ and CD8+ T cells, B cells and monocytes in a group of RA patients (n = 24, mean age 56 years, disease duration 5.9 years). Pgp expression was analysed at baseline and 6 months after therapy with one of the following DMARDs: MTX, sulphasalazine, leflunomide or gold salts. At this stage, 17 out of 19 evaluable patients displayed a clinical response. Most significant differences at baseline and after 6 months therapy were a decrease in
the percentage of Pgp+ monocytes in responders: 16% versus 0%, respectively, suggesting that Pgp expression on monocytes may be a marker of response to DMARDs.

To date, only one study, by Jorgensen (178), has reported on the expression of Pgp in synovial tissue of RA patients. Immunohistochemical staining of Pgp was observed in synovial membranes of five out of 16 RA patients treated with various DMARDs. Surprisingly, however, Pgp mRNA was identified in only one out of these five patients, from which the investigators suggested they have identified an atypical MDR phenotype, distinct from classical Pgp, that was previously described in a T-cell line selected for extremely high concentrations of MTX (179).

In summary, studies on MDR expression on clinical specimens of RA patients are currently limited to the analysis of Pgp expression on peripheral blood lymphocytes. Data on expression of other MDR proteins and in synovial cells are not readily available.

**MDR reversal agents/inhibitors**

In cancer chemotherapy, blocking or modulating of MDR activity has received considerable attention as a possible strategy to overcome diminished drug efficacy due to MDR-mediated drug efflux. Inhibitors/modulators for various MDR proteins are now available (Table 2), which confer in vitro reversal of resistance to chemotherapeutic drugs transported by the MDR proteins. Verapamil and CSA are first generation inhibitors of Pgp activity (37, 180, 181). PSC833 (120), a structural analogue of CSA devoid of immunosuppressive effects, is a second generation Pgp inhibitor currently undergoing clinical trials. Inhibitors of MRP1 include probenecid, which also inhibits MRP2-4 (25, 90, 93), and MK571, a leuco triene D4 analogue (182). Interestingly, several NSAIDs also appeared to be potent inhibitors of MRP1 (e.g. sulindac and indomethacin) (183) and MRP4 (e.g. indoprofen, indomethacin) (136). Recently identified inhibitors of BCRP include GF120918 (184, 185) and a fumitremorgine analogue Ko143 (186). Based on in vitro potency to reverse drug resistance, several MDR inhibitors are currently being evaluated in a (pre)clinical setting (29, 187, 188) for their ability to modulate clinical drug resistance.

Evaluating the outcome of the various MDR reversal trials, van Zuylen (189) concluded that in the cancer chemotherapeutic setting the clinical benefit of MDR modulators is rather modest. This may be due to several reasons:

- Plasma levels of MDR modulators are insufficient to achieve optimal reversal
- Pharmacokinetic drug interact with some MDR modulators, requiring dose reductions
- A multifactorial basis for drug resistance renders reversal of the resistant phenotype incomplete.

Obviously, these issues should be resolved in order to reveal a greater potential of the MDR inhibitors/modulators, but recent studies with third generation MDR reversal agents look promising in this respect (190). Another novel clinical application of MDR inhibitors may be in improving oral drug treatment by temporary inhibition of drug transporters in the gastrointestinal tract (191).

From a rheumatology perspective, lessons can to be learned for the exploitation of MDR reversal agents as possible modulators of DMARD resistance. First, in order to utilize the appropriate MDR modulator, the MDR protein(s) contributing to efflux of various DMARDs should be identified. Second, different endpoints may reflect the efficacy of MDR modulators in cancer treatment (restoration of antiproliferative effects) and RA treatment (restoration of anti-inflammatory effects of DMARDs). These differential effects should not necessarily be achieved at identical concentrations of MDR modulators. Ideally, if modulation of DMARD resistance is effective at lower concentrations of MDR modulators than for anticancer drugs, this would improve the therapeutic window of the modulators. Third, it is apparent that MDR proteins have physiological functions in transporting inflammatory mediators, e.g. LTC4 via MRP1. Hence, blocking of MDR proteins may turn out to be a double-edged sword, as it may reduce the anti-inflammatory response capacity — as in MRP1−/− knock out mice (131).

In conclusion, although MDR modulators are available, their full (in vitro) potential still needs to be translated into a markedly enhanced clinical benefit of cancer chemotherapeutic drugs. Exploration of MDR modulators to improve the efficacy of DMARDs may warrant further attention.

**Perspectives**

Drug resistance, inherent or acquired, is generally accepted as a common cause of treatment failure in the field of infectious diseases and cancer, but has received relatively little attention in clinical rheumatology in relation to DMARD resistance. Expanding knowledge on drug resistance has revealed that there is considerable overlap in molecular mechanisms of drugs, regardless of whether these drugs have anti-infectious, anti-cancer or anti-rheumatic effects.

Although drug resistance can be a multifactorial event, one common mode of cellular drug resistance is enhanced efflux by several members of the ABC transporter family, giving rise to the MDR phenotype. There is a growing body of evidence that
various ABC transporters are expressed in cell types that play a role in inflammatory processes, and which are targets for DMARD therapy. In this respect it will be important that future studies categorize in clinical specimens from RA patients (peripheral blood cells, synovial cells and tissue) the expression of those ABC transporters that have an established role in drug resistance. These studies are now achievable, as the technology required to analyse mRNA levels of all 48 ABC transporters is available (192), just as flow cytometric assays are available to analyse the functional activity of Pgp, MRPI and BCRP (193). However, for an assessment of the various MDR transporters in clinical specimens containing many different cell types, immunohistochemistry remains the mainstay of the analytical platform (40, 53).

Other important issues requiring further attention are:

- The assessing of whether up-regulation of MDR proteins in RA patients is: a DMARD-induced phenomenon, with possible variability dependent on the DMARD used, and/or a disease-related phenomenon. In other words, is MDR expression/functional activity a reflection of disease activity related to the physiological function of some MDR proteins in transporting inflammatory mediators.
- Patient variability in response to DMARDs may be envisioned given the identification of a large variety of Pgp, MRPI and BCRP polymorphisms (194–200). The functional implications of all these polymorphisms need to be established. Furthermore, several distinct mutations in the Pgp, MRPI and BCRP gene have been reported that may affect transport of MTX (58, 99, 201, 202).
- Studies on drug resistance will have broader implications in inflammatory diseases rather than in RA alone, as expression of Pgp was also identified in blood cells of other autoimmune diseases, e.g. systemic lupus erythematosus (SLE) (203).
- Along with clinical studies, the use of in vitro models with acquired resistance to DMARDs resistance can provide further insight into the molecular mechanisms of resistance to various DMARDs, the involvement of MDR proteins and/or other mechanisms of resistance, and the design of strategies to overcome DMARD resistance.

Answering these questions will undoubtedly contribute to better treatment options with DMARDs for patients with RA or other chronic inflammatory diseases.

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Abbreviations
MDR1; Multi Drug Resistance, Pgp; P-glycoprotein, MRP; Multidrug Resistance associated Protein, MTX; methotrexate, CsA; cyclosporin A, BCRP; Breast Cancer Resistance Proteins, ABC; ATP Binding Cassette, cAMP; Cyclic Adenosine Mono-Phosphate, NFkB; Nuclear Factor xB, DHFR; Dihydrofolate reductase, FPGS; Folyhopolyglutamate synthetase, RFC; Reduced Folate Carrier, RA; Rheumatoid Arthritis.

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