EXTENDED REPORT

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

J van der Heijden, M C de Jong, B A C Dijkmans, W F Lems, R Oerlemans, I Kathmann, C G Schalkwijk, G L Scheffer, R J Scheper, G Jansen


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 antirheumatic drug (DMARD).

Methods: A model system of human T cells (CEM) was used to expose cells in vitro to increasing concentrations of SSZ for a period of six months. Cells were then characterised 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 cells to SSZ provoked resistance to SSZ as manifested by a 6.4-fold diminished antiproliferative effect of SSZ compared with parental CEM 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 in SSZ resistance was demonstrated by 60% reversal of SSZ resistance by the ABCG2 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 release 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 DMARDs.

Sulfasalazine (SSZ) is a widely applied disease modifying antirheumatic drug (DMARD), either as a single agent or in combination with other DMARDs. The anti-inflammatory properties of SSZ have been attributed to diminished production of proinflammatory cytokines such as tumour necrosis factor α (TNFα) through multiple mechanisms, including (a) inhibition of the activation of nuclear factor kappa B (NFκB) by inhibition of inhibitor kappa B kinase (IKK)7; (b) inhibition of the purine biosynthesis de novo enzyme 5-aminoimidazole-4-carboxamidobonucleotide transformylase8; and (c) induction of apoptosis in lymphocytes and macrophages.10

In clinical practice the median duration of use of SSZ is 1–2 years. 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.11 The underlying mechanism(s) for this lack of efficacy has not been established. Given the long term DMARD treatment patients with rheumatoid arthritis (RA) receive, we rationalised that the onset of acquired drug resistance to SSZ, analogous to resistance to anticancer drugs or anti-infectious drugs, 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 proinflammatory cytokines by inflammatory cells. Mechanistically, this can be provoked by either augmented basal levels of cytokine secretion or, indirectly, through attenuated antiproliferative/apoptotic effects of DMARDs. Diminished antiproliferative 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 superfami of ATP binding cassette (ABC) transporters. ABC transporters with an established role in drug resistance include: P-glycoprotein (Pgp/ABCB1), multidrug resistance associated proteins 1–5 (MRP1–5/ABCC1–5), and breast cancer resistance protein (BCRP/ABCG2). Currently, very little is known about SSZ as a potential substrate for one or more of the different MDR pumps or other transporters. On the basis of its chemical structure as an organic anion, SSZ may belong to a class of compounds transported by the drug efflux pump MRPI that has a preferred substrate affinity for anionic (glutathione conjugated) compounds.

To gain insight from a rheumatological perspective into possible mechanism(s) of resistance to the antiproliferative/

Abbreviations: ABC, ATP binding cassette; 5'-ASA, 5'-aminosalicylic acid; BCRP, breast cancer resistance protein; DMARD, disease modifying antirheumatic drug; DTT, dithiothreitol; ECL, enhanced chemiluminescence; ELISA, enzyme linked immunosorbent assay; FCS, fetal calf serum; IC50, drug concentration required to inhibit cell growth by 50%; IKK, inhibitor κB kinase; MDR, multidrug resistance; MRP1, multidrug resistance associated protein 1; NFκB, nuclear factor κB; Pgp, P-glycoprotein; PIC, protease inhibitor cocktail; PMSF, phenylmethylsulfonyl fluoride; RA, rheumatoid arthritis; SP, sulphapyridine; SSZ, sulfasalazine; TNFα, tumour necrosis factor α.
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 twofold 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 Chemical Co (St Louis, MO, USA). Protease inhibitor cocktail (PIC) and Triton X-100 were from Boehringer Mannheim (Ingelheim, Germany). The ABCG2 inhibitor Ko143 was kindly provided by Dr A Schinkel (Netherlands Cancer Institute, Amsterdam, The Netherlands). The MRPI blocker MK571 was provided by Merck Frosst, Quebec, Canada. RPMI-1640 tissue culture medium and fetal calf serum (FCS) were obtained from Gibco Chemical Co, Grand Island, NY, USA.

**Selection of SSZ resistance in CEM (T) cells**

In this study human CEM T lineage lymphocytic cells were used as a model system for human T cells. CEM (T) cells were originally isolated by Foley et al. and characterised by several T cell marker molecules, including CD3, CD4, CD8, and CD25. CEM (T) cells have been employed as a representative model to study resistance mechanisms for DMARDs like methotrexate. CEM (T) cells were cultured at an initial density of 3 x 10^5 cells/ml in RPMI-1640 medium supplemented with 10% FCS, 2 mM l-glutamine, and 100 µg/ml penicillin and streptomycin. After a period of 4–6 months' culture, CEM (T) cells developed over time less sensitivity to SSZ, CEM (T) cells were continuously exposed in vitro to stepwise increasing concentrations of SSZ. 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 were harvested in the mid-log phase of growth and washed three times with ice cold Hepes buffered saline, pH 7.4. Total cell lysates of 10^7 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 (one tablet/ml H2O), 20% glycerol, and 0.5% NP-40. The suspension was sonicated (MSE sonicator, amplitude 6, for 3×5 seconds with 30 sec between each pulse) to release CEM (T) cell nuclei. The cell lysates were incubated for one hour at room temperature with the primary antibodies for Pgp (JSB1, 1:500), MRPI (MRPI, 1:500) or ABCG2/BCRP (BXP21, 1:400) as described by Scheffer et al. As control for loading β-actin was used (MAB1501R, 1:3000, Chemicon International, Ca, USA). After three washing steps with TBS-T, the membranes were incubated for one hour with horseradish peroxidase labelled antirat/mouse (1:2000, Dako) as secondary antibody. Detection of the antibody binding was measured by enhanced chemiluminescence (ECL) according to the manufacturer's 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).

**Western blotting**

For analysis of expression of Pgp, MRPI, and BCRP/ABCG2 cells were harvested in the mid-log phase of growth and washed three times with ice cold Hepes buffered saline, pH 7.4. Total cell lysates of 10^7 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 (one tablet/ml H2O), 20% glycerol, and 0.5% NP-40. The suspension was sonicated (MSE sonicator, amplitude 6, for 3×5 seconds with 30 sec between each pulse) to release CEM (T) cell nuclei. The cell lysates were incubated for one hour at room temperature with the primary antibodies for Pgp (JSB1, 1:500), MRPI (MRPI, 1:500) or ABCG2/BCRP (BXP21, 1:400) as described by Scheffer et al.

**Statistical analysis**

Statistical significance of differences was analysed by Student's t test. A p value <0.05 was considered to be significantly different.

**RESULTS**

**Onset of SSZ resistance in CEM (T) cells**

Development of sulfasalazine resistance

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 twofold higher SSZ concentrations as compared with CEM (T) cells.

**Selection of SSZ resistance in CEM (T) cells**

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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 and transferred onto a nitrocellulose membrane. The nitrocellulose membranes were preincubated overnight at 4°C in blocking buffer (5% Biorad blocking, the membranes were incubated for one hour with horse-radish peroxidase labelled antirat/mouse (1:2000, Dako) as secondary antibody. Detection of the antibody binding was measured by enhanced chemiluminescence (ECL) according to the manufacturer's 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 NFκB p65 antibody (Santa Cruz, sc8008, 1:2000) before ECL detection as described above.

**TNFα ELISA**

TNFα production was analysed after stimulation of CEM (T) and CEM/SSZ cells (3 x 10^5/ml) for 24 hours with PMA (10 ng/ml)/ionomycin (1 µM). After this period supernatants were collected by centrifugation (five minutes, 3000 rpm) and analysed for TNFα by enzyme linked immunosorbent assay (ELISA; Central Laboratory of Blood Transfusion, Amsterdam) according to the manufacturer's instruction. The detection limit of the TNFα ELISA was 3 pg/ml.
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%).

could be maintained in SSZ concentrations that exceeded 3.5–6-fold the initially selected SSZ concentration of 0.4 mM SSZ (fig 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 characterisation. Figure 1B shows the antiproliferative effect of SSZ against CEM (T), CEM/SSZ1.5, and CEM/SSZ2.5 cells. 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 with CEM (T) cells (IC50 0.40 (0.03) mM).

Because in vivo SSZ can be catabolised into two components, that is 5’-aminosalicylic acid (5’-ASA) and sulfapyridine (SP), we analysed whether one or both of these components might contribute to the SSZ resistant phenotype. It was noted that neither 5’-ASA nor SP, either separately 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 over 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 up regulated expression of one or more of the major MDR transporters Pgp, MRP1, or ABCG2 (BCRP). CEM/SSZ1.5 cells and CEM/SSZ2.5 cells displayed a marked up regulation of ABCG2 compared with CEM (T) cells, in which ABCG2 expression was negligible (fig 2A). In contrast, control levels of MRP1 expression in CEM (T) cells were down regulated in CEM/SSZ1.5 and CEM/SSZ2.5 cells compared with CEM (T) cells. Along with Pgp, other MDR related molecules pumps (MRP2, MRP3, and LRP) also were undetectable in CEM (T), CEM/SSZ1.5, and CEM/SSZ2.5 cells (not shown). These results demonstrate that the drug resistance efflux pump ABCG2 is markedly up regulated during acquired resistance of CEM (T) cells to SSZ.

Reversal of ABCG2/BCRP mediated SSZ resistance in CEM/SSZ cells

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

TNFα production and nuclear NFkB 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 NFkB. To assess whether SSZ resistance affected production of TNFα, secretion of this cytokine was determined for CEM (T) and CEM/SSZ cells after 24 hours’ PMA/ionomycin stimulation in the absence or presence of a concentration range of SSZ (fig 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 (fig 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 the mechanistic basis of SSZ to inhibit TNFα production is by preventing the cytosolic to nuclear translocation of the nuclear transcription factor NFkB, we analysed whether increased nuclear NFkB levels themselves and/or an impaired potency of SSZ to reduce nuclear NFkB 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 with CEM (T) cells. At the time of TNFα analysis, after 24 hours’ PMA/ionomycin stimulation, expression of the nuclear NFkB 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 (fig 3B, lanes A v E). SSZ exposure to CEM (T) cells resulted in a markedly decreased nuclear NFκB p65 expression, both in unstimulated cells (lanes A v B) or after PMA/ionomycin exposure (lanes C v D). In contrast, SSZ exposure had little effect on nuclear NFκB p65 expression in either unstimulated CEM/SSZ1.5 cells (lanes E v F) or PMA/ionomycin stimulated CEM/SSZ1.5 cells (lanes G v H). Similar results were observed for CEM/SSZ2.5 cells (results not shown). Together, these results suggest that owing to ABCG2 mediated efflux, SSZ is less effective in inhibiting nuclear NFκB 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 up regulated expression of a recently discovered MDR transporter, BCRP, originally discovered in breast cancer cells selected for resistance (loss
of antiproliferative effects) to the anticancer agents mitoxantrone and doxorubicin. Tissue distribution studies demonstrated that ABCG2/BCRP expression is not restricted to breast cancer tissues. ABCG2 is expressed in a limited number of normal tissues (liver, colon epithelium, mammary gland, and blood vessels). Expression of ABCG2 in synovial cells and tissues has not been explored. Very recently, ABCG2 expression was described in blood cells, notably CD34+ haematopoietic progenitor cells and in acute myeloid leukemia cells. The physiological function of BCRP/ABCG2 is as yet unknown, although recent studies with BCRP−/− knockout mice indicated a crucial role in the cellular extrusion and detoxification of dietary phototoxins.

In our study, evidence for a functionally active ABCG2 drug efflux pump in CEM/SSZ cells was demonstrated by the fact that K143, a blocker of BCRP/ABCG2, could reverse resistance to SSZ. In addition, CEM/SSZ displayed cross resistance and reversal by K143 to mitoxantrone, a prototypical substrate for ABCG2.

The observation that the antineoplastic drug SSZ, like distinct anticancer drugs, can induce up regulation of ABCG2 expression may imply that these drugs share a common target that triggers a response of ABCG2 up regulation. Unlike the ABC transporter Pgp, there is no evidence for a direct transcriptional regulation of ABCG2 via an NFκB binding site on the ABCG2 promoter. However, most drugs involved in ABCG2 up regulation, mitoxantrone, doxorubicin, daunorubicin, CPT-11/SN38, and 17β-estradiol, have all been documented to target the NFκB signalling pathway by activating NFκB and stimulating IkBα degradaton. Together, these results suggest that sustained (danger) signals mediated through the NFκB signalling pathway may provoke up regulation of drug efflux pumps like ABCG2.

Along with the loss of antiproliferative effects of SSZ against CEM/SSZ cells (fig 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 (fig 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 because of drug efflux via ABCG2. 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 two- to threefold 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 proinflammatory cytokine/chemokine genes.

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 (fig 2A). MRPI has an established role in exporting hydrophobic and hydrophilic drugs, the latter often as glutathione conjugates. Immunologically, MRPI 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. Because SSZ has been reported to inhibit LTC synthesis, this may suggest that a diminished LTC production coincides with a lowered expression of the LTC4 transporter MRPI.

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 and increased production of TNFα, a key component of progressive disease in RA. In this respect, we also noted up regulation of ABCG2 expression in human mononcytic 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) (this study), chloroquine (MRP1), and methotrexate (MRP1, ABCG2)—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 as possible chemosensitizers for SSZ activity deserve further attention.

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Authors’ affiliations
J van der Heijden, B A C Dijkmans, W F Lems, R Oerlemans, G Jansen, Department of Rheumatology, Vrije Universiteit Medical Centre, Amsterdam, The Netherlands
M C de Jong, G L Scheffer, R J Schepers, Department of Pathology, Vrije Universiteit Medical Centre, Amsterdam, The Netherlands
I Kathmann, Department of Medical Oncology, Vrije Universiteit Medical Centre, Amsterdam, The Netherlands
C G Schalkwijk, Department of Clinical Chemistry, Vrije Universiteit Medical Centre, Amsterdam, The Netherlands

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