

MDR-ABC transporters: biomarkers in rheumatoid arthritis

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ABSTRACT

MDR-ABC transporters are widely expressed in cell types relevant to pathogenesis of rheumatoid arthritis. Many reports demonstrate the interaction of small molecule drugs with MDR-ABC transporters. Cell-based assays for disease relevant cell types can be easily gated and could reveal specific drug targets and may increase significance and utilisation of data in clinical practice. Many commonly used DMARDs (e.g. methotrexate, sulfasalazine, leflunomide/teriflunomide, hydroxychloroquine) are ABCG2 substrates. Consequently, the activity of this transporter in patients should be determined to understand the disposition and pharmacokinetics of the therapy. In addition, MDR-ABC transporters transport a variety of endobiotics that play important roles in cell proliferation, cell migration, angiogenesis and inflammation. Therefore, MDR-ABC transporters are important biomarkers in rheumatoid arthritis.

Introduction

Rheumatoid arthritis (RA) is one of the most common chronic inflammatory autoimmune diseases and affects about 0.5–1% of the world population. The disorder is characterised by pain and swelling of the symmetrical joints. As a consequence of widespread inflammation the function of other organs and tissues such as the heart, the lung and the blood vessels are impaired as well.

The trigger of pathogenesis of RA is still obscure. The pathophysiology of RA involves interactions of innate and adaptive immune systems. Cells participating in pathogenesis are the valid cellular targets for small molecule therapy. Interplay of T cells and B cells determines the autoimmune process leading to inflammation and destruction of affected joints. During this process the Th1/Th2 and Th17/Treg balance becomes shifted towards formation of

the inflammatory Th1 and autoreactive Th17 cells (1). These cell subsets then produce various inflammatory cytokines upon interaction with antigen presenting cells. Th1 cells activate the B cells to produce auto-antibodies (e.g. rheumatoid factor (RF), anti-citrullinated protein antibodies (ACPA) (2). Activated B cells differentiate into plasma cells that produce large quantities of these antibodies. Importance of B cells is substantiated by the therapeutic efficacy of rituximab, the anti CD-20 antibody that efficiently deletes B cells (3). The other cellular targets of two T cell subsets (Th1, Th17) are macrophages in the synovial tissue. Macrophages contribute to abundance of inflammatory cytokine, tumour necrosis factor (TNF) in the synovium (4). The T cell macrophage interaction is mediated via secreted cytokines interferon (IFN)-gamma and interleukin (IL)-17. IL-17 plays a major role in tissue destruction as this cytokine activates fibroblast-like synoviocytes (FLS) and osteoclasts, two effector cell types secreting matrix metalloproteases and invading cartilage (5). FLS express both IL-15 and IL-15 receptor (IL-15R), therefore they may proliferate in an autocrine manner (6). Activation of polymorphonuclear leukocytes in RA exacerbates inflammation due to production of prostaglandins and leukotrienes as well as direct tissue damage via released lysosomal enzymes and superoxide anions (7).

Small molecular anti-rheumatoid drugs and their cellular targets

Due to clinical diagnostic limitations, therapy resistance can only be defined by lack of clinical response. In order to develop drugs or improve diagnostic precision and sensitivity the resistance and response must be defined at a cellular level. Resistance often arises at the level of the molecular target. For some drugs commonly used in RA such as gold, antimalarials and sulfasalazine

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primary molecular targets are not well defined, although, toll-like receptors (TLR), particularly TLR9 have been suggested to mediate some of the effects of antimalarials (8). For drugs where primary molecular targets are known, such as glucocorticoids, methotrexate, leflunomide and cyclosporine A, the pharmacogenomics data linking genotype with response are rare. The -317AA genotype (-317G>A, rs408626) of dihydrofolate reductase (DHFR) the primary target of methotrexate was linked with less favourable response to the drug (9). A 19 bp deletion in DHFR (rs70991108) has been associated with increased methotrexate hepatotoxicity in acute lymphoblastic leukaemia (ALL) patients (10). No data have been published on the effect of polymorphism on therapeutic response to methotrexate in RA despite the observed high allelic frequency of this 19 bp deletion in the Japanese population (11). Glucocorticoid receptor (GR) polymorphism 1220A>G (rs6195) (12, 13) that leads to an amino acid change (N363S) and polymorphism BclI (rs41423247) (14) that leads to a C>G substitution in intron 2 are associated with hypersensitivity to glucocorticoids. However, the ER22/23EK (198G>A (rs6189) and 200G>A (rs6190)) (15, 16) polymorphism and the 9beta (rs6198) (17) polymorphism (an A>G change in the 3' untranslated region (UTR) leading to stabilisation of GR) are associated with resistance to glucocorticoids. Interestingly, carriers of the polymorphisms associated with glucocorticoid resistance are predisposed to develop RA (18). Conversely, carriers of polymorphism associated with glucocorticoid hypersensitivity are less susceptible to develop RA (18). Nonetheless, association of the above variants with clinical response to corticosteroid treatment in RA has not been convincingly shown. In regard to cyclosporine A and tacrolimus, calcineurin is the most important primary molecular target in T cells (19), synoviocytes (20), and osteoclast precursors (21). Calcineurin variants have been mapped (22), but association with response to cyclosporine A or tacrolimus in RA patients has not been studied. Leflunomide is thought to impair

de novo synthesis of pyrimidine nucleotides via inhibition of dihydroorotate dehydrogenase (DHODH) (23) a mechanism shown to be effective in T cells (24). The C19 variant (rs3213422) of DHODH was associated with increased frequency of remission in RA patients (25). As the effect of this polymorphism on DHODH activity is not known, a mechanistic linkage of drug response to polymorphism is not justified. In addition, many of the latter drugs have profound effects on cytokine release and antibody production that is not necessarily linked to the primary target (26, 27). Moreover, many other effects such as inhibition of proliferation and induction of apoptosis also manifest at the cellular level. Therefore, effects of anti-rheumatic drugs can be better defined and tested at the cellular level.

Glucocorticoids induce apoptosis of activated T cells which are considered their main cellular therapeutic targets (28) in RA. T cells are also targets of antiproliferative activities of leflunomide (29) and gold (30) as well as cytokine production suppressive activities of methotrexate, cyclosporine A (19) and sulfasalazine (31). B cells are also targets of anti-rheumatic drugs. The gold compounds, gold sodium thiomalate and auranofin inhibit B-cell activation (32) whereas methotrexate (33) and sulfasalazine inhibit antibody production (34). Teriflunomide, the active metabolite of leflunomide may also have a direct effect on B cells (35), although a T cell dependent effect is likely more significant (24). Similarly, cyclosporine A, azathioprine (36) and glucocorticoids (37) exert their effect on antibody production through modulation of T cell function. The third major cell type involved in the pathogenesis of RA is the monocyte-macrophage lineage that includes osteoclasts. Multiple studies employing peripheral monocytes or cells derived from synovium have shown macrophages as targets for different small molecule anti-rheumatic drugs. Gold compounds (38, 39), hydroxychloroquine (40), leflunomide (41), cyclosporine A (42), sulfasalazine (43) and glucocorticoids (44) inhibit cytokine release by macrophages. It is generally thought that most of these

effects are mediated through inhibition of the nuclear factor kappa beta (NF- κ B) (45, 46) pathway. Furthermore, methotrexate (45), sulfasalazine, teriflunomide (41), gold (39), cyclosporine A but not hydroxychloroquine (45) inhibited osteoclast formation and/or function. Glucocorticoids, on the contrary, increase osteoclast formation (47). Finally, glucocorticoids (48, 49), gold compounds (48), sulfasalazine (50), methotrexate, cyclosporine A and hydroxychloroquine (51) all suppressed some of the activities of FLS. Data on teriflunomide are controversial as inhibition (52, 53) as well as potentiation (54) of FLS mediated cytokine secretion has been published. A recent article (55) reviewed human synovial tissue response to small molecular drugs. The number of CD68⁺ macrophages in the synovium significantly decreased upon treatment with glucocorticoids (56), gold (57), methotrexate and leflunomide (58). Decreased T cells in the synovium of patients treated with prednisolone or methotrexate was also shown (56). The only study showing decreased synovial B cell content measured CD5⁺ cells that include T cells (56). These data show that most drugs have multiple cellular targets and that cellular assays are feasible *in vitro* models to test drug response.

The concept of multidrug resistance

The concept of multidrug resistance (MDR) has significantly changed over the past decade. The original concept was based on the observation made in Victor Ling's lab – demonstrating pleiotropic resistance in cells selected for colchicine resistance (59). The protein responsible for the phenotype was named permeability-glycoprotein (P-gp) as it appeared to affect membrane permeability of drugs. The gene was cloned 10 years later and termed *mdr1* (60). The systemic name, ABCB1 is now used for this transporter. It was later shown that overexpression of other efflux transporters, such as ABCC1/ MRP1 (61, 62) and ABCG2/BCRP/ MXR (63) also play a role in clinical MDR. Multiple studies have linked overexpression of MDR-ABC transporters with MDR using various clini-

cal parameters. However, due to the failure of development of ABC transporter inhibitors overcoming MDR to chemotherapy in malignancies, ABC transporters are still considered unvalidated therapeutic targets for conquering MDR (64). Part of the reason for this failure may be inherent toxicity and inadequate trial design and systemic PK interactions (65).

Although the MDR phenomenon was firstly described in tumour cells, MDR-ABC transporters have been identified in many normal tissues including immune cells as part of a mechanism of the resistance to antiviral (66) and immunosuppressive (67) therapies.

ABC transporters have been linked to transport of a variety of endobiotics and implicated in various processes of cancer development such as proliferation, metastasis, inflammation and stem cell survival (68). Such endobiotics secreted by the MDR transporters play important roles in inflammatory response due to the differentiation, proliferation and maturation of immune cells as well as in their migration into the inflamed

tissues (69). ABCB1 not only transports hydrophobic and positively charged drugs (70) but also transports cholesterol, platelet-activating factor (PAF) (71) and various other membrane lipids including sphingolipids (72). Transport of PAF may facilitate angiogenesis (73) while cholesterol (74) as well as sphingolipids (75) modulate drug resistance. In addition to acidic and hydrophobic drugs ABCC1 transports a variety of arachidonic acid metabolites (69), important mediators of inflammation. ABCC1 and ABCG2 transport sphingosine-1-phosphate (S1P) (76) that facilitates cell growth, survival, invasion and angiogenesis (77). ABCG2 transports drugs with a wide substrate specificity (78). It also transports various vitamins, such as folates (riboflavin/vitamin B2) (79). Cellular efflux of folates may aggravate folate deprivation in patients on methotrexate therapy. Therefore, MDR-ABC transporters are “more than just drug efflux pumps” (68). Transporter interaction of anti-rheumatic drugs is summarised in Table I. The anti-inflammatory effect

of disease-modifying anti-rheumatic drugs (DMARDs) might be at least partially attributable to the inhibition of the pathophysiological function of the MDR-ABC transporters in immune cells (80).

According to the new concept MDR-ABC transporters are biomarkers. Their role in the immune processes and MDR can only be evaluated as part of a complex panel of biomarkers for prognostic scoring (67), for monitoring disease activity (81) or to predict the responsiveness to certain medications (*e.g.* immunosuppressive treatments or chemotherapy in malignancies) (80). However, translation of MDR-ABC transporter activity into clinical decisions and treatment regimen requires robust and reliable *in vitro* diagnostic tests for the assessment of efflux transporter function in target cells.

MDR-ABC transporters in RA

The role of transporters in RA has been studied for almost two decades. Most of the studies focused on ABCB1 as the prototype ABC transporter and were

Table I. MDR-ABC transporter interaction of anti-rheumatic drugs.

Drug	Transporter	Methods / Test system	Effect	Ref.
Methotrexate	ABCC1	a. human ovarian carcinoma cell line 2008, transfected with human MRP1 and inside-out plasma membrane vesicles b. NIH3T3/MRP1 ² vesicle	a. [3H]MTX Accumulation w/wo probenecid and ATP-dependent uptake of [3H]MTX b. vesicular uptake of MTX	a. (115) b. (116)
	ABCG2	a. HEK293-wtABCG2 vesicle b. MCF7 ³ /MX ⁴ selected, MCF7/BCRP ⁵ transfected vesicle	a. vesicular uptake, inhibition by FTC b. vesicular uptake, inhibition by FTC	a. (117) b. (118)
Leflunomide	ABCG2	BCRP-HAM-Sf9 ⁶ membrane and PLB985 ⁷ -BCRP ⁸ and HEK293-BCRP ⁸ cell lines	ATPase, VT assay Hoechst assay	(119)
	ABCG2	synovial tissue from RA patients	decreased BCRP positivity after leflunomide treatment in responder group	(96)
Prednisolon*	ABCB1	LLC-PK1/MDR1 ¹⁰ cell line	monolayer assay	(120)
Sulfasalazine	ABCG2	a. Caco2 ¹¹ cell line	a. transport	a. (121)
		b. CEM ¹² /SSZ ¹³ cell line	b. cytotoxicity (IC 50 increased)	b. (122)
Chloroquine	ABCB1	a. MDCKII-MDR1 ¹⁴ monolayer b. Sf9-MDR1 membrane c. MDCKII-MDR1 ¹⁴ monolayer	a. monolayer assay b. ATPase c. Calcein assay	(123)
	ABCC1	CEM-CEM/CQ ¹⁵ cells	cytotoxicity- reversal resistance with MK571 and probenecid	(124)

*methyl-prednisolone- 26-fold higher affinity

¹NIH3T3: Mouse embryonic fibroblast cell line; ²NIH3T3/MRP1: MRP1 overexpressing in NIH3T3; ³MCF7: human breast adenocarcinoma cell line; ⁴MCF7/MX and ⁵MCF7/BCRP: BCRP overexpressing cell line; ⁶Sf9: membrane from *Spodoptera Frugiperda* 9; ⁷PLB985: human myelomonoblastic leukaemia cell line; ⁸PLB985-BCRP: BCRP over-expressing cell line; ⁹HEK293-BCRP: BCRP overexpressing HEK 293 cell line; ¹⁰LLC-PK1/MDR1: MDR1 overexpressing LLC-PK1 cell line; ¹¹Caco2: immortalised human epithelial colorectal adenocarcinoma cell line; ¹²CEM: human acute lymphocytic leukaemia cell line; ¹³CEM/SSZ: sulfasalazine resistant human acute lymphocytic leukaemia cell line; ¹⁴MDCKII-MDR1: MDR1 overexpressing MDCKII cell line; ¹⁵CEM/CQ: chloroquine resistant human acute lymphocytic leukaemia cell line.

trying to correlate ABCB1 expression with disease status and more importantly drug resistance. These studies with one exception (82) have found increased levels of ABCB1 expression in peripheral blood lymphocytes (PBL) or peripheral blood mononuclear cells (PBMC) that correlated with lower intracellular dexamethasone levels in these cells (83-86). Moreover, ABCB1 activity was higher in refractory than in non-refractory subgroups (83, 87). In contrast, no correlation was seen between ABCB1 expression and disease activity in synovial cells (88), though prior treatments may induce ABCB1 expression both in lymphocytes (82) and synovial cells (88) and published data were not always correlated for this important covariate. Dependence of ABCB1 activity on the genotype is controversial. Tumour cells of B cell chronic lymphocytic leukaemia patients of 3435CC genotype were shown to have greater ABCB1 activity than carriers of the T-allele (89) while no difference was observed in PBMCs from healthy volunteers (90). No difference in representation of variants between patients and controls was shown (91). However, probability of remission upon methotrexate and glucocorticoid co-administration was significantly higher in patients of 3435TT genotype than in carriers of the C allele (91, 92). Conversely, methotrexate monotherapy leads to statistically significantly more non-responders in the 3435TT cases than in the 3435CC cases (93). Cyclosporine A (94) or tacrolimus (86) treatment reduced ABCB1 levels in lymphocytes and reversed resistance.

No difference was observed in ABCC1 status of RA patients and controls (95). Unexpectedly, methotrexate and / or folate treatment lead to downregulation of ABCC1 (95). On the contrary, ABCG2 expression was 2-fold higher in synovial macrophages of RA patients than in controls and a 3-fold increase was observed in non-responders over responders to methotrexate and/or leflunomide (96). Intriguingly, combination therapies of the ABCG2 substrate methotrexate with other ABCG2 substrate and/or inhibitor DMARDS

(sulfasalazine, leflunomide, hydroxy-chloroquine, cyclosporine A) yielded better response rates than the monotherapy (97). But no difference was observed when methotrexate was co-administered with ABCG2 non-interactors such as azathioprine and gold (97). In summary, the ABCG2 data clearly show the importance of this transporter in pathogenesis as well as therapeutic response of the disease. The fact that most small molecular DMARDs are ABCG2 substrates substantiates the importance of ABCG2 in RA. The ABCB1 data are somewhat controversial. The controversy may stem from the fact that methotrexate, the drug used in most studies is not an ABCB1 substrate and/or inhibitor. Early data suggested that methotrexate showed an ABCB1 dependent cytotoxicity (98) but substrate nature of methotrexate has not been confirmed in bona fide transport experiments (99). Nonetheless, ABCB1 may play a role through a mechanism other than drug transport as treatment-induced down-regulation of ABCB1 correlated with decreased secretion of cytokines in patients (99) and administration of siMDR1 reduced synovial cytokine production *in vitro* and *in vivo* in rat (100).

Investigation of transporter function in a clinical setting

There are numerous technical approaches to assay MDR function, such as (i) detection of known functional sequence variants in ABCB1, ABCC1 and ABCG2 genes, (ii) quantitation of the transporter expression or (iii) measurement of the activity of the transporters. Inter-individual variability in response to drug therapy might be, at least in part, explained by genetic factors, such as the mutations and polymorphisms identified in the genes of ABC-transporters. Some of these allelic variants have been associated with altered gene expression (101-103) or substrate specificity (104, 105) of the transporters, which might affect the response to certain drugs. The most commonly tested functional polymorphisms of the MDR transporters are the 3435C>T (rs1045642), 2677G>T/C (rs2032582), 1236C>T (rs1128503) variants in

the ABCB1 and the SNP 421C>A in ABCG2 (rs2231142). The analysis of these genetic alterations is straightforward using multiparametric assays. Efforts to identify pharmacogenetic markers in ABCB1 have led to conflicting and inconclusive results. As a consequence of a huge variety of inducers (drugs, hormones and cytokines), complexity of gene-gene interactions, nutritional factors, tissue-specific expressions and various inhibitions by co-medications as well as influence of co-morbidities the MDR phenotype cannot be predicted from the genotype of the patients (*i.e.* prediction of the treatment efficacy) (106). On the contrary, numerous studies have shown that the ABCG2 421C>A polymorphism leads to significantly decreased activity of the transporter leading to increased exposure to ABCG2 substrate drugs, DMARDs, sulfasalazine (107, 108), teriflunomide (109) among them. Translation of these data into the MDR phenotype is still missing with perhaps one notable exception. Psoriasis patients carrying the ABCG2 variant alleles responded favourably (110) to treatment with methotrexate, an ABCG2 substrate drug (111). Characteristics of assay protocols for assessment of MDR-ABC transporter genotype is shown in Table II.

Quantifying mRNA levels (106, 112, 113) is difficult due to preanalytical challenges, such as the proper selection, isolation of target cells and the instability of mRNA transcripts, but then the correlation with the activity is closer than correlation of activity with pharmacogenomic variants. Alternatively, MDR gene expression can be measured by quantifying proteins directly using antibody based or liquid chromatography tandem mass spectrometry (LC/MS/MS) based techniques. For testing clinical specimens (*e.g.* tissue or blood samples), the most commonly employed methods are immunohistochemistry and flow cytometry. Immunohistochemistry is widely used to assess the MDR phenotype in solid tumours; however, it has obvious limitations in quantification and is less amenable to characterise white blood cells in autoimmune diseases. Flow cy-

Table II. Methods to assess MDR-ABC transporters in clinical setting.

Test (Manufacturer)	Measured parameter	Remarks	Regulatory status
<i>Genotyping assays</i>			
In-house tests	Genotyping single or multiple polymorphisms in the ABC transporter genes	Performance characteristics of the assays are variable. Applied often only in one laboratory and/or only in a few studies	LDT
MDR1 C3435T ToolSet (Genes-4U)	genotyping of the C3435T polymorphism	Qualitative real-time PCR assay optimized for the Roche LightCycler instruments	RUO
<i>mRNA expression tests</i>			
In-house tests	Quantitation of gene expression of a single or multiple ABC transporter(s)	Variable performance characteristics. Applied often only in one laboratory and/or only in a few studies	LDT
RealTime Ready ABC Transporter Panel (Roche)	gene expression of 42 ABC-transporters	Prevalidated real-time quantitative PCR assays in 96 well plate format	RUO
Human Drug Transporters RT ² Profiler (Qiagen)	PCR array of 84 human transporters including 29 ABC-transporters	96 and 384 well plate and 100 well disc formats	RUO
Taqman Array Human ABC Transporters (Applied Biosystems)	mRNA expression of 44 ABC-transporters	96 well plate	RUO
<i>Antibody-based assays</i>			
Anti-MDR1 antibodies (Different suppliers)	Protein expression (immunocytochemistry, flow cytometry, etc.)	<i>Clones:</i> Extracellular: MRK16, MC57, UIC2* Cytoplasmic: JSB-1	RUO
Anti-MRP1 antibodies (Different suppliers)	Protein expression (immunocytochemistry, flow cytometry, etc.)	<i>Clones:</i> Extracellular: – Cytoplasmic: MRPm5, QCRL-3	RUO
Anti-BCRP antibodies (Different suppliers)	Protein expression (immunocytochemistry, flow cytometry, etc.)	<i>Clones:</i> Extracellular: 2J39, 5D3* Cytoplasmic: BXP-21, BXP-34	RUO
* conformational antibodies			
<i>Functional (Dye Efflux) Assays</i>			
In-house functional assays	MDR1 and/or MRP1 and/or BCRP activity	<i>Widely applied substrates:</i> MDR1: DiOC ₂ , Calcein-AM, Rhodamine-123 MRP1: Calcein-AM, BCRP: Hoechst-33342, Mitoxantrone, Pheophorbide A. The results are not comparable between the individual laboratories applying different protocols.	LDT
MDR1 Direct Dye Efflux Assay (Millipore)	MDR1, MRP1, BCRP	<i>Substrates:</i> MDR1 and BCRP: DiOC ₂ MRP1: Rhodamine-123 The assay protocol is long and depends on the activities of the transporters.	RUO
eFluxx-ID Green/Gold kit (Enzo- Life Sciences)	MDR1, MRP1, BCRP	<i>Substrates:</i> EFluxx-ID Green kit: Fluo-8, EFluxx-ID Gold kit: Rhod-4 Interaction of these dyes with the BCRP transporter could not be confirmed on transfected cell lines.	RUO
MultiDrugQuant kit (Solvo Biotechnology)	MDR1, MRP1, BCRP	<i>Substrates:</i> MDR1 and MRP1: Calcein-AM BCRP: Mitoxantrone	RUO
Solvo MDQ kit (77 Elektronika Kft.)	MDR1, MRP1, BCRP	<i>Substrates:</i> MDR1 and MRP1: Calcein-AM BCRP: Mitoxantrone	CE-IVD

tometry is a complementary technique as it is a powerful tool to investigate protein expression on thousands of immune cells or even in a given lymphocyte subpopulation(s) of interest. The LC/MS/MS-based protein quantitation

is not routinely used in diagnostics yet. Significant drawbacks of these techniques are the indirect link between the protein expression and function and the effect of characteristics of the antibody (*e.g.* affinity, specificity and cross

reactivity) used in the study. Description of assay protocols for assessment of MDR-ABC transporter expression is shown in Table II.

The third option to quantify MDR function in target cells is measurement of

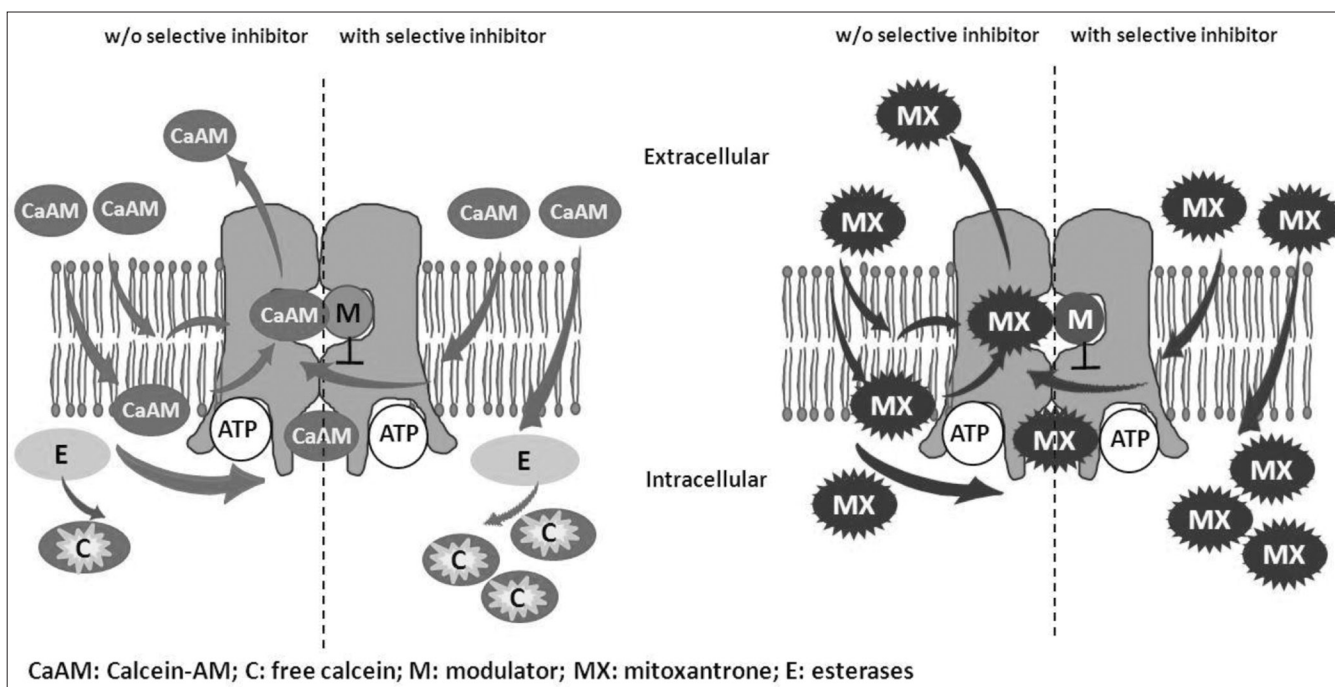


Fig. 1. Dye efflux assays applied in the Solvo MDQ-kit. The Calcein-assay (left panel) is based on determining fluorescence intensity using a flow cytometer. After short *in vitro* incubation of the cell suspension with the fluorogenic dye calcein-acetoxymethyl ester (calcein AM), activity ABCB1 and ABCC1 is determined using selective inhibitors of the transporters. Intracellularly calcein-AM is rapidly hydrolysed by esterases to yield the highly fluorescent free acid, calcein, which due to its hydrophilic character becomes trapped in the cytoplasm. The Mitoxantrone-assay (right panel) measures the activity of ABCG2 transporter applying a similar principle. The assay utilises mitoxantrone as a fluorescent dye and Ko134 as an ABCG2-specific inhibitor. Pls note that mitoxantrone is fluorescent and, therefore, does not require further intracellular processing.

the translocation of a probe substrate. Such a measurement is only possible if the substrate can be easily detected and visualised/quantified within the cell/tissue of interest. Except for the isotope labeled probes used for imaging barrier penetrations and mapping solid tumours (114), the compounds applied in functional assays are fluorescent dyes. Therefore, these tests are often referred as fluorescent dye uptake assays. Cells expressing more MDR transporters accumulate the fluorescent substrate at a slower rate, thus, the difference in the fluorescent signal intensities measured with/without the specific inhibitor is proportional with the activity of the transporter in the target cells. These functional assays measure the MDR function directly and cell subpopulation specific values can be obtained when employing a FACS-based method. Some fluorescent dye-uptake tests require long incubation times, extensive washing or have serious shortcomings in their kinetics (*e.g.* intracellular sequestration, poor cellular retention, etc.) and/or fluorescent characteristics (spectral and intensity shifts).

A multitude of assay protocols for assessment of MDR-ABC transporter activity has been developed (Table II). Nevertheless, most of these tests failed to conform to the robustness and reproducibility required from routine diagnostic methods. Furthermore, transporter activities measured in the same patient sample can vary depending on the fluorescent substrates and testing procedure applied.

Each of these approaches are characterised by a huge variety of MDR testing methods usually applying individual reagents (*e.g.* primers, probes, antibodies, fluorescent substrates and inhibitors) according to an individual procedure adapted from the literature or developed in-house by the individual laboratory (Table II). The performance (measured in terms of specificity, reproducibility and robustness) of such laboratory developed tests (LDTs) varies from laboratory to laboratory because they are not subjected to the same quality standards as commercial kits.

At present, there is only one commercial kit which has been registered (CE-marked) for diagnostic purposes in

the EU and some other countries with similar IVD regulatory requirements (Table II). This kit applies the calcein-assay technology (Fig. 1, left) for quantitative measurement of ABCB1 and ABCC1 activities. ABCG2 activity is measured using a similar principle: intracellular accumulation of mitoxantrone, a fluorescent drug is measured in the presence and absence of the selective ABCG2-inhibitor, Ko134 (Fig. 1, right).

Conclusions

MDR-ABC transporters influence susceptibility to develop RA and also may define prognosis and therapeutic response. Diagnostic tools that allow for assessment of MDR-ABC transporter activity in the cell type most relevant to the disease and/or the therapeutic drug are clearly favoured. Clinical trials correlating MDR-ABC transporter activity and prognosis are warranted.

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