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Involvement of C3435T and G2677T multidrug resistance gene polymorphisms in release of cytokines from peripheral blood mononuclear cells treated with methotrexate and dexamethasone

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Abstract

P-Glycoprotein is a cell membrane-associated protein that transports a variety of exogenous (including drugs) and endogenous substances. P-Glycoprotein may also be involved in transmembrane transport of some endogenous proteins; thus, it may have physiological function in cytokine transport. Previous studies suggested that P-glycoprotein expression is genetically determined.

The aim of this study was to examine involvement of multidrug resistance gene (MDR1) C3435T and G2677T polymorphisms in release of cytokines from phythemaglutynin (PHA)-stimulated peripheral blood mononuclear cells, as well as treated with methotrexate or dexamethasone. The release of cytokines: interleukin-2 (IL-2), IL-4, IL-6, IL-10, interferon-γ (INF-γ) and tumor necrosis factor-α (TNF-α) was determined in supernatants of mononuclear cell cultures from 72 healthy subjects, measured by flow cytometry. The release of INF-γ, IL-2, IL-4 and TNF-α in cultures from subjects with 2677(T–T) 3435(T–T) haplotype pair was significantly decreased as compared to subjects with other haplotypes. There were no statistically significant differences in release of IL-6 and IL-10.

The results of this study suggest an association between C3435T and G2677T MDR1 polymorphisms and transmembrane transport of some cytokines. Although the studied polymorphisms may be in linkage with polymorphisms of other transporters involved in cytokine release, it seems that the present results indirectly indicate involvement of P-glycoprotein in transport of some cytokines. Moreover, determination of C3435T and G2677T MDR1 polymorphisms might be useful in response prediction to therapy with methotrexate and dexamethasone.
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Keywords: Multidrug resistance; Polymorphism; Methotrexate; Dexamethasone

1. Introduction

The multidrug resistance (MDR1) gene product P-glycoprotein is an ATP-dependent efflux transporter. Its importance has appeared following the discovery of multidrug resistance during chemotherapy. The expression of P-glycoprotein in normal tissues plays an important role for disposition and effects of a broad variety of drugs (Fojo et al., 1987; Higgins and Gottesman, 1997). P-Glycoprotein is expressed in the apical membrane of cells with excretory functions such as: liver, kidney, small intestine, stomach, the blood–brain barrier and hematopoietic cells. The tissue distribution suggests physiological role of P-glycoprotein in secretion of metabolites and natural xenobiotics (Lum and Gosland, 1995). High levels of P-glycoprotein were also detected in the adrenal cortex, and subsequent studies provided evidence that cortizol and aldosterone are physiological substrates of P-glycoprotein (Cordon-Cardo et al., 1990). Functional P-glycoprotein was found in several types of human leukocytes and stem cells. Among hematological cells, P-glycoprotein expression is highest in natural killer cells, CD4+ and CD8+ lymphocytes, and bone marrow progenitor cells (Klimecki et al., 1994). Leukocyte expression of P-glycoprotein may play important role in therapy of some diseases. Effective therapy in several pathologies, including treatment of HIV, autoimmune disorders and suppression of the immune system after organ transplantation, requires

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drug access to leukocyte intracellular space. P-Glycoprotein expression and up-regulation was detected in several hematologic malignancies and correlates with poor outcome of treatment (Marie, 1995).

Although the function of leukocyte P-glycoprotein is unknown, the role of this transporter in cell-mediated cytotoxicity, cytokine secretion and protection from toxic substances was proposed.

Reports focusing involvement of P-glycoprotein in transmembrane transport of cytokines are controversial. Some authors suggested that P-glycoprotein might be involved in the cytokine transport from activated normal lymphocytes into surrounding medium (Raghu et al., 1996; Drach et al., 1996). However, the results of Gollapudi et al. (2000) demonstrated the lack of P-glycoprotein involvement in release of cytokines from stimulated CD4+ and CD8+ T cells.

Recently, a number of single nucleotide polymorphisms (SNPs) of the MDR1 gene have been identified and correlated with the P-glycoprotein expression (Hoffmeyer et al., 2000). Most of the detected polymorphisms are intronic or silent. However, the silent polymorphism in exon 26 C3435T was found to correlate significantly with reduced intestinal P-glycoprotein expression and increased bioavailability of digoxin. The C3435T mutation is a silent mutation that does not cause amino acid substitution and is suggested to be linked in, a majority of subjects, with functional mutation in exon 21, position 2677 (2677G > T/A), resulting in substitution of alanine in position 893 by serine (2677T) or threonine (2677A), respectively (Hoffmeyer et al., 2000; Kim et al., 2001; Tanabe et al., 2001). The effect of C3435T mutation may reflect that of G2677T, and there may be racial differences in the relation between C3435T and G2677T. Whether the C3435T mutation is related to MDR1 mRNA processing, including transcriptional activation and mRNA maturation, remains unclear. Further studies on MDR1 gene haplotype analysis are needed to understand the effect of C3435T and G2677T on P-glycoprotein function (Johne et al., 2002).

Methotrexate and dexamethasone are commonly used in the therapy of autoimmune diseases. Methotrexate is a folate antagonist first developed for treatment of malignances, and subsequently used in other diseases as an anti-inflammatory and immunosuppressive drug (Cronstein, 1996). Glucocorticoids are the inhibitors of inflammatory responses and are widely used as anti-inflammatory and immunosuppressive agents. These drugs induce lymphocyte apoptosis, and inhibit synthesis of cytokines and cell surface molecules required for immune function (Auphan et al., 1997). Methotrexate is not a P-glycoprotein substrate and the role of P-glycoprotein in dexamethasone transport is not clear either.

Nevertheless, it is possible that P-glycoprotein may modulate anti-inflammatory properties of these drugs both via involvement in release of cytokines, and influence methotrexate and dexamethasone concentration in the immune cells, as well as via not known mechanisms so far.

The aim of this study was to examine the association between MDR1 C3435T and G2677T polymorphisms and cytokine release by stimulated peripheral blood mononuclear cells, as well as treated with methotrexate and dexamethasone.

2. Materials and methods

2.1. Cell preparation

Mononuclear cells were isolated from peripheral blood of 72 healthy Caucasian subjects using Ficoll Paque. Their viability was assayed by a trypan blue exclusion test. The cells were counted using a haemocytometer and subsequently used for further experiments. Phythemaglutynin (PHA) stimulation of

Table 1
Impact of MDR1 2677 G/T polymorphism on cytokine concentrations

Genotype at locus 2677	Number		IL-2			IL-4			INF-γ		
	N	%	Mean [pg/ml]	S.D.	95% CI	Mean [pg/ml]	S.D.	95% CI	Mean [pg/ml]	S.D.	95% CI
TT	15	21	14 [a]	7	9–17	27 [a]	6	23–30	146 [a]	10	141–151
GT	32	45	36	14	30–39	48	8	45–51	189	27	179–198
GG	24	33	39	15	32–43	48	9	44–51	194	16	186–200
Rare genotype											
GA	1	1	23	–	–	45	–	–	165	–	–
Genotype at locus 2677	Number		TNF-α			IL-10			IL-6		
	N	%	Mean [pg/ml]	S.D.	95% CI	Mean [pg/ml]	S.D.	95% CI	Mean[pg/ml]	S.D.	95% CI
TT	15	21	186 [a]	13	178–193	109	52	72–138	4293	1435	3671–4963
GT	32	45	255	53	234–263	106	49	74–135	4093	1393	3711–4863
GG	24	33	268	68	236–279	107	53	71–140	4337	1487	3628–4941
Rare genotype											
GA	1	1	220	–	–	108	–	–	3872	–	–

95% CI—95% confidence interval for mean.
No significant differences were found between GT and GG genotypes.
Rare GA genotype was not introduced into ANOVA.
[a] Significantly lower ($p < 0.001$) in comparison with GT and GG genotypes (ANOVA + Tukey post-hoc test).

Table 2
Impact of MDR1 3435 T/C polymorphism on cytokine concentrations

Genotype at locus 3435	Number		IL-2			IL-4			INF-γ		
	N	%	Mean [pg/ml]	S.D.	95% CI	Mean [pg/ml]	S.D.	95% CI	Mean [pg/ml]	S.D.	95% CI
TT	26	36	20 [a]	11.	15–23	34 [a]	11	30–39	161 [a]	18	153–168
CT	34	47	35 [b]	13	30–38	48	8	45–51	192	26	182–202
CC	12	17	49	18	39–56	51	9	46–56	197	18	187–206

Genotype at locus 3435	Number		TNF-α			IL-10			IL-6		
	N	%	Mean [pg/ml]	S.D.	95% CI	Mean [pg/ml]	S.D.	95% CI	Mean [pg/ml]	S.D.	95% CI
TT	26	36	202 [a]	23	191–209	108	55	70–142	4251	1495	3589–4899
CT	34	47	264	51	243–272	106	49	68–138	4146	1549	3543–4738
CC	12	17	281	90	227–309	107	51	64–147	4320	1454	3821–5010

95% CI—95% confidence interval for mean.
[a] Significantly lower ($p<0.001$) in comparison with CT and CC genotypes (ANOVA + Tukey post-hoc test).
[b] Significantly lower ($p<0.05$) in comparison with CC genotype (ANOVA + Tukey post-hoc test).

cells was performed by adding PHA (concentration 5 μg/ml) and incubating mononuclear cells for 24 h at 37 °C, 5% CO_2 in RPMI 1640 medium.

The study was approved by the local ethics committee and written informed consent was obtained from all subjects.

2.2. Determination of cytokine production

Levels of IL-2, IL-4, IL-6, IL-10, INF-γ and TNF-α in the culture supernatants were quantified by flow-cytometry (FACScan, Becton Dickinson, San Jose, USA) using Th1/Th2 kit (Beckton Dickinson, San Jose USA) according to instructions of the manufacturer.

2.3. Genotyping

Genomic DNA was extracted manually (precipitation with trimethylammonium bromide salts from leukocytes contained in 450 μl of venous blood with ethylene diamine tetraacetic acid as an anticoagulant). DNA was then precipitated in 95% ethanol,

Table 3
Impact of MDR genotypes (haplotype pairs of SNPs at loci 2677 and 3435) on cytokine concentrations

	Number		IL-2			IL-4			INF-γ		
	N	%	Mean [pg/ml]	S.D.	95% CI	Mean [pg/ml]	S.D.	95% CI	Mean [pg/ml]	S.D.	95% CI
(T–T) (T–T)	15	21	13.8 [a]	7	9–17	26.5 [a]	6.3	23.3–29.7	146.1 [a]	10.2	141.0–151.3
(T–T) (G–T)	10	14	29.5 [b]	10.2	23.3–34.7	45.8	9.4	40.0–51.6	171.4 [c]	14.8	162.2–180.6
(G–C) (G–T)	13	18	32.1	9.6	26.7–36.5	45.6	7.0	41.8–49.4	189.0	14.1	181.3–196.7
(G–C) (T–T)	21	29	37.4	14.9	30.5–41.7	49.5	8.3	46.0–53.1	196.7	29.2	184.2–209.2
(G–C) (G–C)	11	15	47.4 [d]	18.0	37.1–55.3	51.1	9.9	45.2–56.9	197.3	18.9	186.1–208.4
Rare genotype											
(G–C) (T–C)	1	1.5	65	–	–	48	–	–	182	–	–
(G–T) (A–T)	1	1.5	23	–	–	45	–	–	165	–	–

	Number		TNF-α			IL-10			IL-6		
	N	%	Mean [pg/ml]	S.D.	95% CI	Mean [pg/ml]	S.D.	95% CI	Mean [pg/ml]	S.D.	95% CI
(T–T) (T–T)	15	21	186.4 [a]	13.3	177.9–192.9	109	52	72–138	4293	1435	3671–4963
(T–T) (G–T)	10	14	224.3 [e]	12.1	216.4–231.4	106	49	67–141	4297	1326	3654–4925
(G–C) (G–T)	13	18	250.7	35.6	229.0–266.4	108	50	62–153	4220	1292	3726–4936
(G–C) (T–T)	21	29	272.0	58.6	243.9–285.8	107	48	64–147	4082	1347	3675–5284
(G–C) (G–C)	11	15	287.5	91.7	229.9–319.9	106	51	63–145	4293	1425	3409–5563
Rare genotype											
(G–C) (T–C)	1	1.5	210	–	–	115	–	–	4368	–	–
(G–T) (A–T)	1	1.5	220	–	–	102	–	–	3811	–	–

Genotypes are haplotype pairs deduced from MDR1 SNPs (2677 G/T/A–3435 C/T).
Statistically significant differences in concentrations among the five groups of frequent genotypes were observed for all cytokines (ANOVA, $p<0.001$). Differences between specific genotypes were analysed using Tukey post-hoc tests.
[a] Significantly lower in comparison with any other frequent genotype group.
[b] Significantly lower in comparison with (G–C) (G–C) genotype.
[c] Significantly lower in comparison with (G–C) (T–T) and (G–C) (G–C) genotypes.
[d] Significantly higher in comparison with combined genotype groups containing one G–C haplotype (Student's *t*-test).
[e] Significantly lower in comparison with combined genotype groups containing G–C haplotype (Student's *t*-test).

dissolved in distilled water and stored at −20 °C until analysis. The MDR1 C3435T polymorphism was determined using the polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) assay as previously described (Cascorbi et al., 2001). A 197-bp fragment of P-glycoprotein situated in exon 26 of the MDR1 gene was amplified from genomic DNA with the primer pair P1 and P2. The primer sequences were: P1 (sense): 5′-TGTTTTCAGCTGCTTGATGG-3′ and P2 (antisense): 5′-AAGGCATGTATGTTGGCCTC-3′. PCR amplification was performed in a total volume of 100 μl that contained 200 ng genomic DNA dATP, dCTP, dGDP and dTTP (200 μmol/l each, MBI Fermantas, Vilnius, Lithuania); 250 ng of each primer, 1.5 mmol/l magnesium chloride and 2 U Tag DNA polymerase (Gibco BRL Life Technologies, Glasgow, UK). The amplification reaction was performed using the Mastercycler 5330 (Eppendorf, Hamburg, Germany). PCR amplification consisted of an initial denaturation for 2 min at 94 °C, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and, finally, extension at 72 °C for 30 s. The terminal elongation was performed at 72 °C for 7 min. In an amplified 197 base pair fragment, the C3435T polymorphism destroys a restriction enzyme cleavage site for Sau 3 AI such that, after digestion with this enzyme for 16 h at 37 °C, the 3435C allele can be detected by the presence of two fragments, which are 158 base pair and 39 base pair long. The presence of the 3435T allele in the amplified segment remaining uncut, and the presence of a heterozygous genotype, results in the presence of all three bands. DNA fragments generated after restriction enzyme digestion were separated in a 3.5% agarose gel. Restriction fragments were visualized after ethidium bromide staining of the agarose gel using an ultraviolet transilluminator. To analyze G2677T polymorphism, an allele-specific PCR method was developed. The following set of primers was used: FA: 5′-TGA AAG ATA AGA AAG AAC TAG AAG GTA-3′, FG: 5′-TGA AAG ATA AGA AAG AAC TAG AAG GTG-3′, FT: 5′-TGA AAG ATA AGA AAG AAC TAG AAG GTT-3′—forward sequence-specific primers, FK: 5′-AGC AAA TCT TGG GAC AGG AA-3′—a forward internal control primer and RR: 5′-AGT CCA AGA ACT GGC TTT GC-3′—a common reverse primer. Three separate amplification reactions were performed for each DNA sample for detection of 2677G, 2677T and 2677A alleles, using Eppendorf Mastercycler (Hamburg, Germany). Amplification mix (15 μl) contained 0.7 μM of an applicable allele-specific primer, 0.2 μM of a control primer, 0.7 μM of a reverse primer, 60–120 ng of genomic DNA, 0.5 U of RedTaq Polymerase (Sigma) in 1× enzyme-specific buffer (containing magnesium chloride at final concentration of 1.1 mmol) and deoxynucleoside triphosphates (Sigma), 200 μmol of each. The temperature

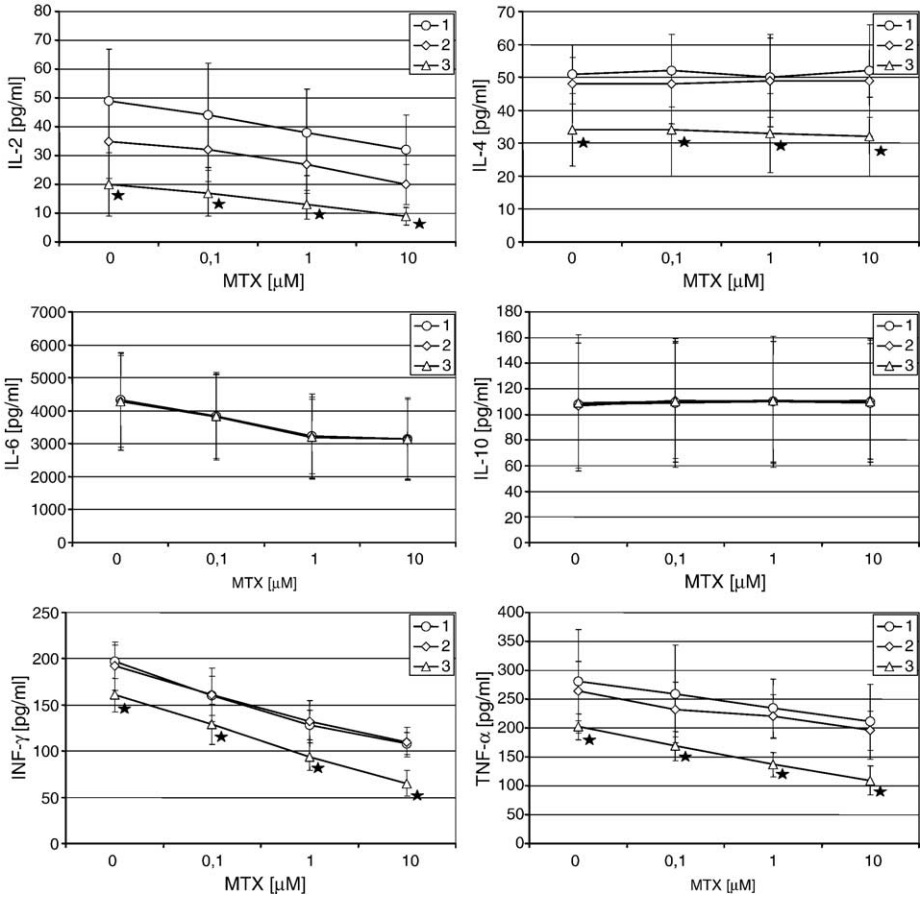

Fig. 1. The release of cytokines from PHA-activated peripheral blood mononuclear cells (MNC) treated with methotrexate (MTX) in relation to MDR1 C3435T polymorphism *$p<0.05$—comparison of the cytokine concentrations between: MDR1 3435TT genotype (3) vs. MDR1 3435CC (1) and MDR1 3435CT (2) genotypes.

profile was: initial denaturation at 94 °C for 4 min, 8 cycles at 57/72/94°C for 30 s, followed by 25 cycles at 55/72/94 °C for 30 s with a final extension step at 72 °C for 7 min. The PCR products were analyzed in 2% agarose gels, stained with ethidium bromide. Amplified control DNA fragment (353 base pair) was followed by allele-specific fragment (222 base pair) in a presence of the detected allele. Genotyping results were confirmed by sequencing of DNA fragments, obtained using FK and RR primers, for each genotype variant detected (2677GG, 2677GT, 2677TT and 2677GA, respectively).

2.4. Statistical analysis

Each genotype for single nucleotide polymorphisms G2677T and C3435T was assigned to haplotype pairs. For those individuals who were homozygous in at least one variant ($n=51$), the haplotypes could be assigned explicitly. Those belonging to the group of 21 patients heterozygous at both loci (2677 GT and 3435 CT) were assigned (2677G–3435C) (2677T–3435T) haplotype pair because of its 123-fold higher probability than (2677G–3435T) (2677T–3435C) haplotype pair, as calculated by the EH program (Terwilliger and Ott, 1994).

Chi-square test was used to examine for conformation of genotypes and haplotypes to Hardy–Weinberg equilibrium.

Analysis of variance (ANOVA) followed by Tukey post-hoc test was used to reveal differences in cytokine concentrations between genotype groups. Student's *t*-test was used for some comparisons between two groups where indicated. IL-2 and TNF-α distributions were right-skewed and significantly different from normal (Shapiro–Wilk test), so they were transformed logarithmically to obtain normal distributions before ANOVA. $P<0.05$ was considered statistically significant.

3. Results

3.1. MDR1 polymorphism

From 72 healthy subjects, the 3435CC, 3435CT and 3435TT genotypes were found in 12 (16.7%), 34 (47.2%), 26 (36.1%) cases, respectively, whereas the 2677TT genotype was detected in 15 subjects (21%), 2677GT in 32 (45%) and 2677GG in 24 (33%).

Five haplotypes and seven haplotype pairs (genotypes) were observed for loci 2677 and 3435 in examined subjects. The 3435T and 3435C alleles were in linkage disequilibrium with the 2677T and 2677G alleles, respectively.

Distribution of genotypes in both loci and their haplotypes followed the Hardy–Weinberg equilibrium (each $p>0.20$). Rare haplotypes A–T and T–C were found only in

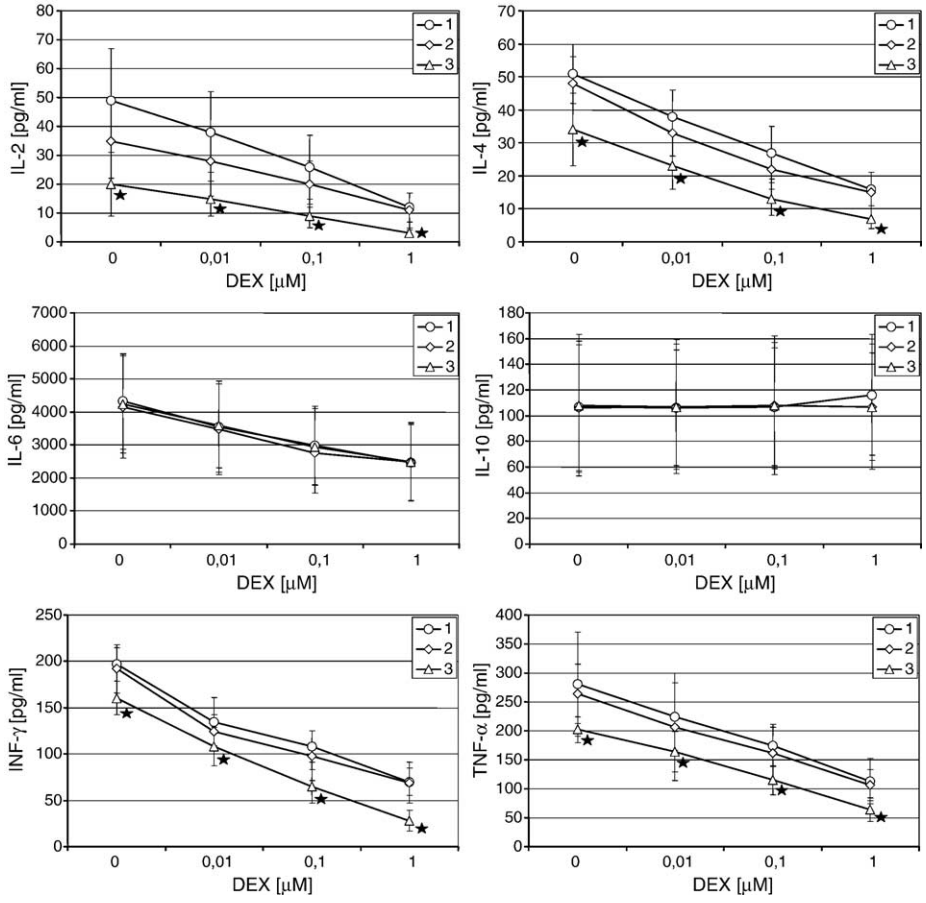

Fig. 2. The release of cytokines from PHA-activated peripheral blood mononuclear cells (MNC) treated with dexamethasone (DEX) in relation to MDR1 C3435T polymorphism *$p<0.05$—comparison of the cytokine concentrations between: MDR1 3435TT genotype (3) vs. MDR1 3435CC (1) and MDR1 3435CT (2) genotypes.

single subjects (one with genotype (G–T) (A–T) and one with (G–C) (T–C)) so they were not further analysed statistically.

3.2. The involvement of P-glycoprotein in transport of cytokines. Release of cytokines from PHA-stimulated mononuclear cells in relation to MDR1 genotypes

To study the release of cytokines from PHA-stimulated mononuclear cells in association with MDR1 genotypes, the peripheral blood mononuclear cells were stimulated with PHA (5 μg/ml) for 24 h. The cytokines (IL-2, IL-4, IL-6, IL-10, INF-γ and TNF-α) were measured in supernatants after 24 h by FACS analysis.

The release of cytokines in relation to 3435CT and 2677GT MDR1 genotypes is shown in Tables 1 and 2.

Table 3 demonstrates concentrations of cytokines for seven genotype groups (haplotype pairs). The cytokine concentrations in five groups of the most frequent genotypes were analysed with ANOVA followed by Tukey post-hoc test.

The release of INF-γ, IL-2, IL-4 and TNF-α in cultures from subjects with (T–T) (T–T) genotype was significantly decreased as compared to subjects with other genotypes. There were no statistically significant differences in release of IL-6 and IL-10 (Table 3).

3.3. The effect of methotrexate and dexamethasone on release of cytokines by PHA-stimulated mononuclear cells in relation to MDR1 genotypes

In order to establish the effect of methotrexate and dexamethasone on release of cytokines from PHA-stimulated mononuclear cells, the peripheral blood mononuclear cells were stimulated with PHA (5 μg/ml) and incubated with methotrexate (conc. 0.1–10 μM) and dexamethasone (conc. 0.01–1 μM). The level of cytokines was measured in the supernatants after 24 h by FACS analysis.

As shown in Figs. 1, 3 and 5, methotrexate significantly decreased the release of IL-2, IL-6, INF-γ and TNF-α. There was no significant effect on release of IL-4 and IL-10.

The treatment with dexamethasone resulted in decrease of IL-2, IL-4, IL-6, INF-γ and TNF-α concentrations (Figs. 2–4 and 6).

The concentrations of IL-2, IL-4, INF-γ and TNF-α in cultures from subjects with (T–T) (T–T) haplotype pair were

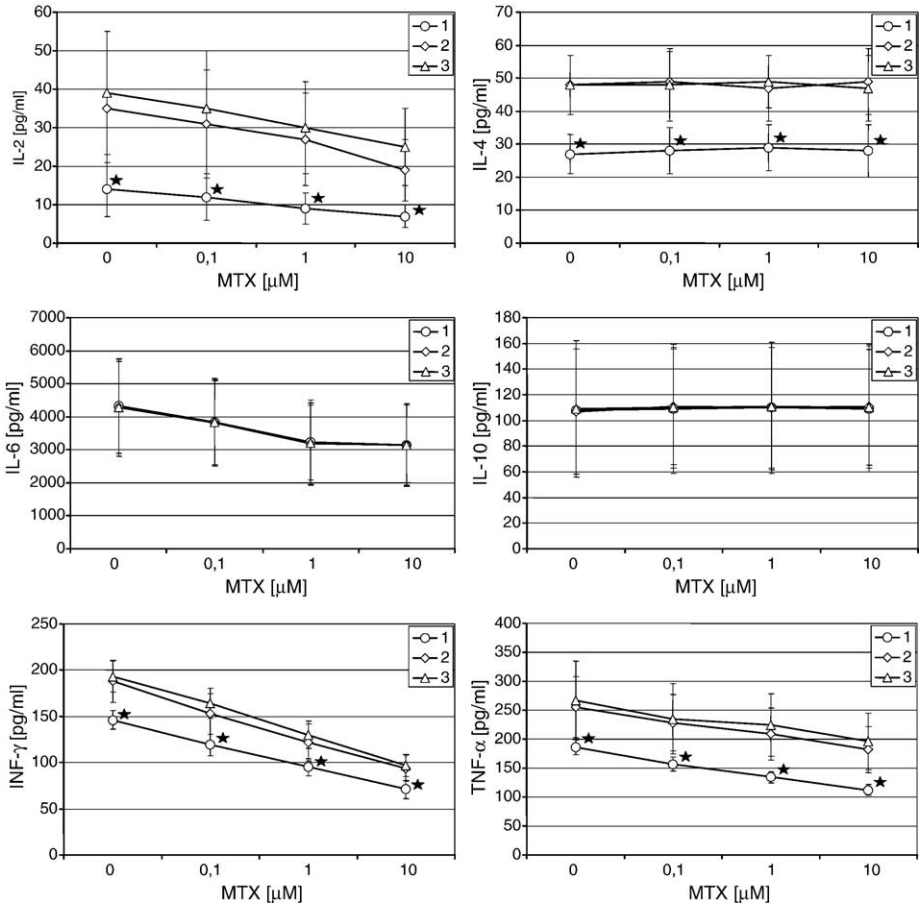

Fig. 3. The release of cytokines from PHA-activated peripheral blood mononuclear cells (MNC) treated with methotrexate (MTX) in relation to MDR1 G2677T polymorphism *$p<0.05$—comparison of the cytokine concentrations between: MDR1 2677TT genotype (1) vs. MDR1 2677GT (2) and MDR1 2677GG (3) genotypes.

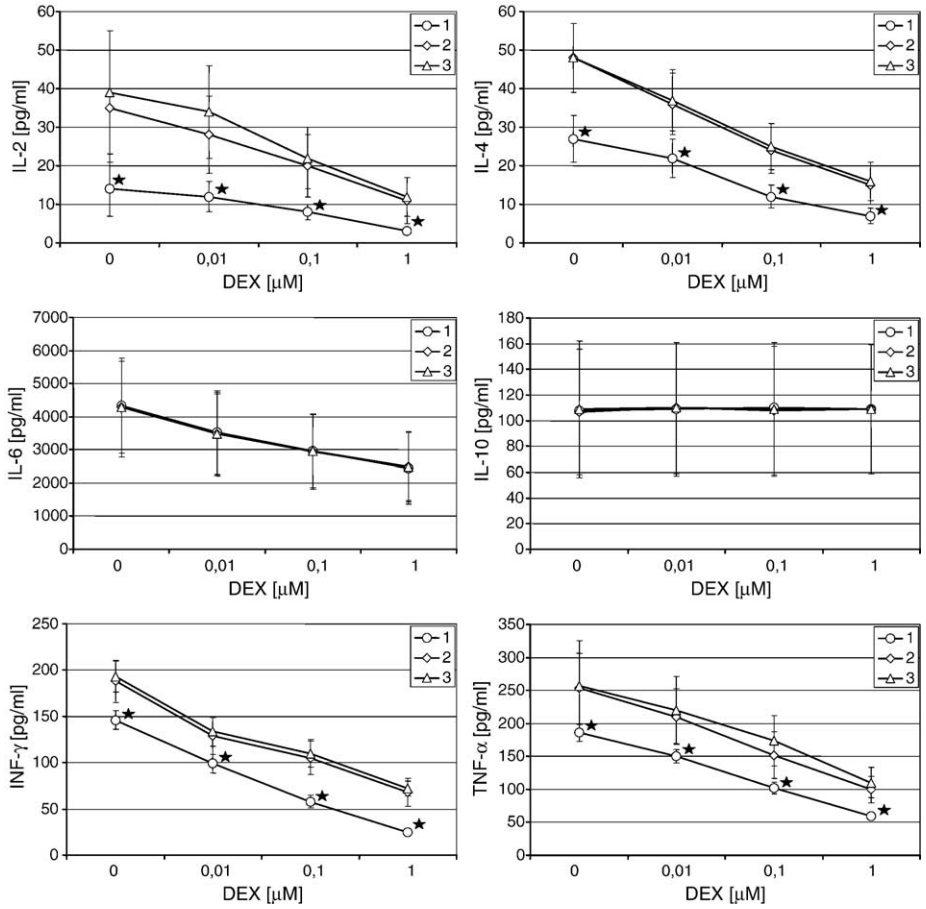

Fig. 4. The release of cytokines from PHA-activated peripheral blood mononuclear cells (MNC) treated with dexamethasone (DEX) in relation to MDR1 G2677T polymorphism *$p<0.05$—comparison of the cytokine concentrations between: MDR1 2677TT genotype (1) vs. MDR1 2677GT (2) and MDR1 2677GG (3) genotypes.

significantly decreased as compared with any other genotype group (Figs. 5 and 6).

4. Discussion

In this study, we examined the hypothesis whether the G2677T and C3435T MDR1 polymorphisms influence release of cytokines from PHA-stimulated peripheral blood mononuclear cells.

Previous studies revealed that separate analysis of C3435T or G2677T polymorphisms was not sufficient to predict P-glycoprotein activity. Therefore, in our study, the analysis of different MDR1 gene haplotypes was performed as proposed by Johne et al. (2002).

The results demonstrate that T–T haplotype is associated with low release of IL-2, IL-4, INF-γ and TNF-α from PHA-stimulated cells, whereas the G–C haplotype with extensive release of these cytokines. From analyzed haplotypes, the lowest cytokine levels for pair homozygotes (T–T) (T–T) were detected, but the highest were observed in carriers of (G–C)(G–C) genotypes. Moreover, in cultures treated with methotrexate and dexamethasone, the levels of IL-2, IL-4, INF-γ and TNF-α were the lowest in carriers of (T–T) (T–T) genotype.

Previous studies reported involvement of P-glycoprotein in release of IL-2, IL-4 and INF-γ by activated lymphocytes. Raghu et al. (1996) reported treatment of PHA-activated peripheral blood lymphocytes with anti-P-glycoprotein monoclonal antibodies resulted in a significant reduction of IL-2 levels in the culture. Drach et al. (1996) studied involvement of P-glycoprotein in the transmembrane transport of IL-2, IL-4, IL-6 and INF-γ in T lymphocytes from healthy subjects. The authors investigated the release of cytokines in PHA-stimulated lymphocyte cultures treated with P-glycoprotein inhibitors, i.e. verapamil, tamoxifen and P-glycoprotein-specific monoclonal antibodies. The release of IL-2, IL-4 and INF-γ was significantly inhibited by P-glycoprotein inhibitors, whereas secretion of IL-6 remained unaffected. The authors concluded that P-glycoprotein is involved in the transport of cytokines: IL-2, IL-4 and INF-γ in peripheral blood lymphocytes.

Gollapudi et al. (2000) examined secretion of IL-2 by anti-CD3-stimulated purified P-glycoprotein+ and P-glycoprotein− subsets of CD4+ and CD8+ T cells from peripheral blood lymphocytes of healthy subjects as well as the release of IL-2, IL-4, IL-10 and INF-γ in mice. The authors showed that anti-CD3-stimulated lymphocytes from wild type, mdr1a knockout and mdr1ab double knockout mice produced similar amounts

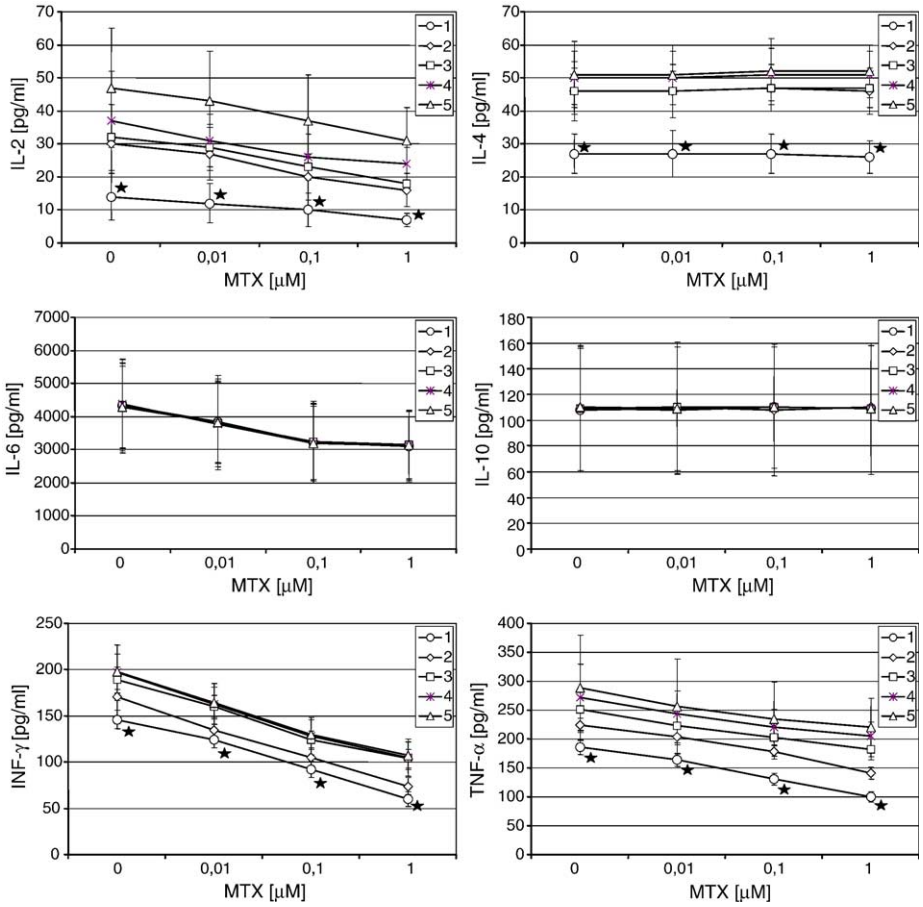

Fig. 5. The release of cytokines from PHA-activated peripheral blood mononuclear cells (MNC) treated with methotrexate (MTX) in relation to MDR1 haplotypes: *$p<0.05$—comparison of the cytokine concentrations between: subjects with (T–T) (T–T) haplotype pair (1) vs. subjects with other pairs of haplotypes; 1—(T–T) (T–T) haplotype pair; 2—(T–T) (G–T) haplotype pair; 3—(G–C) (G–T) haplotype pair; 4—(G–C) (T–T) haplotype pair; 5—(G–C) (G–C) haplotype pair.

of IL-2, IL-4, IL-10 and INF-γ. Jurkat T cells that lack P-glycoprotein and MDR1-transfected Jurkat T cells produced comparable amounts of IL-2. The authors suggested that P-glycoprotein was not required for secretion of IL-2, IL-4, IL-10 and INF-γ in mice and IL-2 in humans.

P-Glycoprotein is a known transporter that mediates efflux of chemotherapeutic agents from the intracellular milieu and thereby contributes to the drug resistance. Transported drugs vary widely in their structure, and the mechanisms by which P-glycoprotein can recognize such diverse substrates and translocate them across the membranes, are not fully understood. The only common structural feature of substrate compounds identified so far is their relatively hydrophobic amphipatic nature (Varma et al., 2003). Those transported compounds include many clinically important drugs such as anticancer agents, cardiac drugs and steroids (dexamethasone, cortisone). Previous studies reported that P-glycoprotein may be involved in resistance to dexamethasone and methotrexate. It has been shown that P-glycoprotein can confer resistance against dexamethasone in the mouse thymoma cells (Schinkel et al., 1995). Dexamethasone is known to be a substrate for P-glycoprotein. The excretion of both endogenous and exogenous steroids is mediated by P-glycoprotein. In the mdr-gene knockout mouse model, the absence of P-glycoprotein is associated with the higher concentrations of dexamethasone in a brain (Bourgeois et al., 1993). Dexamethasone has also been shown to modulate P-glycoprotein expression in the rat hepatocyte culture (Fardel et al., 1993). Verapamil was found to enhance the absorption of methylprednisolone in the jejunum of rat (Saitoh et al., 1998). Accumulation of hydrocortisone is reduced in mdr-positive pituitary tumor cells and is restored after blockade with P-glycoprotein specific monoclonal antibodies (Nelson and Hinkle, 1992). Steroid accumulation and sensitivity were reduced in variants of murine thymoma cells with high expression of P-glycoprotein. Intracellular accumulation and sensitivity to the drugs could be restored by P-glycoprotein blockade.

P-Glycoprotein expression can also affect the resistance to methotrexate. Although methotrexate is not known to be a substrate for P-glycoprotein, several studies suggested P-glycoprotein involvement in methotrexate resistance. Norris et al. (1996) examined the expression of P-glycoprotein in a series of leukemia sublines resistant to methotrexate. The authors demonstrated increased expression of MDR1 messenger RNA and increased P-glycoprotein expression in those sublines. The resistance to methotrexate was reversed by P-glycoprotein specific monoclonal antibodies as well as by verapamil or cyclosporine. In a second study, Graaf et al. (1996) hypothesized

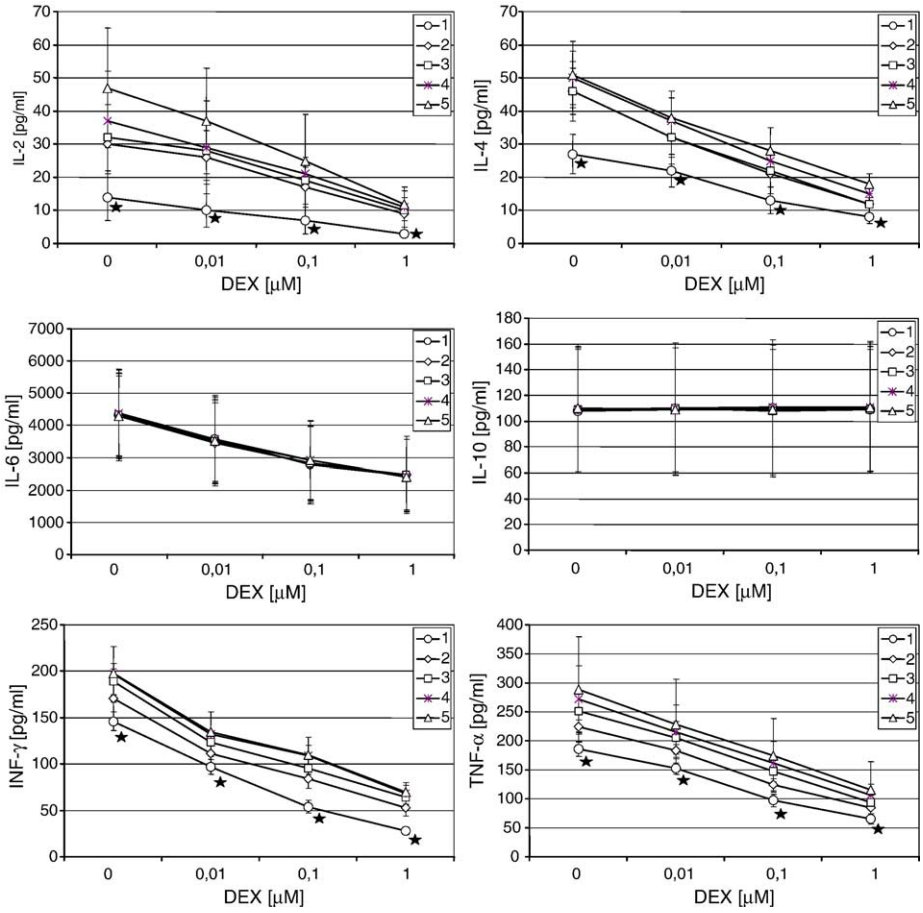

Fig. 6. The release of cytokines from PHA-activated peripheral blood mononuclear cells (MNC) treated with dexamethasone (DEX) in relation to MDR1 haplotypes: *$p<0.05$—comparison of the cytokine concentrations between: subjects with (T–T) (T–T) haplotype pair (1) vs. subjects with other pairs of haplotypes: 1—(T–T) (T–T) haplotype pair; 2—(T–T) (G–T) haplotype pair; 3—(G–C) (G–T) haplotype pair; 4—(G–C) (T–T) haplotype pair; 5—(G–C) (G–C) haplotype pair.

that P-glycoprotein might be involved in methotrexate resistance in cells with deficient carrier-mediated methotrexate uptake. The authors reported that insertion of recombinant retrovirus expressing the human MDR1 gene resulted in increased survival of resistant cells. These studies demonstrated the potential of P-glycoprotein overexpression in conferring resistance to methotrexate.

Llorente et al. observed an increased number of lymphocytes with high P-glycoprotein activity in patients with rheumatoid arthritis and suggested that P-glycoprotein expression might be related to the active course of rheumatoid arthritis and poor response to treatment. Moreover, P-glycoprotein expression was higher in refractory patients than in treatment responding patients (Llorente et al., 2000).

In our previous study, we demonstrated association between genetically determined P-glycoprotein expression and resistance to methotrexate and glucocorticosteroids in patients with rheumatoid arthritis (Pawlik et al., 2004). The probability of remission of rheumatoid arthritis symptoms after therapy with these drugs was significantly higher in patients with MDR1 3435TT genotype than in subjects with MDR1 3435CT and CC genotypes. Previous reports suggested also that C3435T MDR1 polymorphism might influence therapy outcome with some drugs. Fellay et al. (2002) studied association between C3435T MDR1 polymorphism and response to antiretroviral treatment. Patients with MDR1 TT genotype were characterized by greater rise in CD4-cell count than patients with the CT and CC genotypes, and the best recovery of naive CD4-cells 6 months after starting treatment. The C3435T and G2677T MDR1 polymorphisms may also be involved in steroid weaning in children after heart transplantation. The children homozygous for the 3435C and 2677G alleles more frequently received prednisone 1 year after transplantation. The authors also found linkage disequilibrium between C3435T and G2677T alleles (Zheng et al., 2002).

The present ex vivo study demonstrated that the concentrations of IL-2, IL-4, INF-γ and TNF-α in cultures treated with methotrexate and dexamethasone are significantly lower in subjects with (T–T) (T–T) haplotype pair. This is the result of decreased baseline release of cytokines in these subjects. Therefore, in subjects with (T–T) (T–T) genotype, there might be reduced incidence and activity of rheumatoid arthritis and higher probability of remission of the disease symptoms after therapy with methotrexate and dexamethasone.

The results of this study suggest the association between C3435T and G2677T MDR1 polymorphisms and transmembrane transport of some cytokines. Although the studied polymorphisms may be in linkage with polymorphisms of

other transporters involved in release of cytokines, it seems that the present results indirectly indicate involvement of P-glycoprotein in transport of some cytokines. Moreover, the determination of C3435T and G2677T MDR1 polymorphisms might be useful in response prediction to therapy with methotrexate and dexamethasone.

References

Auphan, N., Didonato, J.A., Helmberg, A., Rosette, C., Karin, M., 1997. Immunoregulatory genes and immunosuppression by glucocorticoids. Arch. Toxicol., Suppl. 19, 87–95.

Bourgeois, S.D., Gruol, J., Newby, R.F., Rajah, F.M., 1993. Expression of an mdr gene is associated with a new form of resistance to dexamethasone-induced apoptosis. Mol. Endocrinol. 7, 840–851.

Cascorbi, I., Gerloff, T., Johne, A., Meisel, C., Hoffmeyer, S., Schwab, M., Schaeffeler, E., Eichelbaum, M., Brinkmann, U., Roots, I., 2001. Frequency of single nucleotide polymorphisms in the P-glycoprotein drug transporter MDR1 gene in white subjects. Clin. Pharmacol. Ther. 69, 169–174.

Cordon-Cardo, C., O`Brien, J.P., Boccia, J., Casals, D., Bertino, J.R., 1990. Expression of the multidrug resistance gene product (P-glycoprotein) in human normal and tumor tissues. J. Histochem. Cytochem. 38, 1277–1287.

Cronstein, B.N., 1996. Molecular therapeutics. Methotrexate and its mechanism of action. Arthritis Rheum. 39, 1951–1960.

Drach, J., Gsur, A., Hamilton, G., Zhao, S., Angerler, J., Fiegl, M., Zojer, N., Raderer, M., Haberl, I., Andreeff, M., Huber, H., 1996. Involvement of P-glycoprotein in the transmembrane transport of interleukin-2 (IL-2), IL-4, and interferon-gamma in normal human T lymphocytes. Blood 88, 1747–1754.

Fardel, O., Lecureur, V., Guillouzo, A., 1993. Regulation by dexamethasone of P-glycoprotein expression in cultured rat hepatocytes. FEBS Lett. 327, 189–193.

Fellay, J., Marzolini, C., Meaden, E.R., Back, D.J., Buclin, T., Chave, J.P., Decosterd, L.A., Furrer, H., Opravil, M., Pantaleo, G., Retelska, D., Ruiz, L., Schinkel, A.H., Vernazza, P., Eap, C.B., Telenti, A., 2002. Response to antiretroviral treatment in HIV-1-infected individuals with allelic variants of the multidrug resistance transporter 1: a pharmacogenetic study. Lancet 359, 30–36.

Fojo, A.T., Ueda, K., Slamon, D.J., Poplack, D.G., Gottesmann, M.M., Pastan, I., 1987. Expression of a multidrug-resistance gene in human tumors and tissues. Proc. Natl. Acad. Sci. U. S. A. 84, 265–269.

Gollapudi, S., Kim, C., Gupta, S., 2000. P-glycoprotein (encoded by multidrug resistance genes) is not required for interleukin-2 secretion in mice and humans. Genes Immun. 1, 371–379.

Graaf, D., Sharma, R.C., Mechetner, E.B., Schimke, R.T., Roninson, I.B., 1996. P-glycoprotein confers methotrexate resistance in 3T6 cells with deficient carrier-mediated methotrexate uptake. Proc. Natl. Acad. Sci. U. S. A. 93, 1238–1242.

Higgins, C.F., Gottesman, M.M., 1997. Is the multidrug transporter a flippase? Trends Biochem. Sci. 17, 18–21.

Hoffmeyer, S., Burk, O., von Richter, O., Arnold, H.P., Brockmoller, J., Johne, A., Cascorbi, I., Gerloff, T., Roots, I., Eichelbaum, M., Brinkmann, U., 2000. Functional polymorphisms of the human multidrug-resistance gene: multiple sequence variations and correlation of one allele with P-glycoprotein expression and activity in vivo. Proc. Natl. Acad. Sci. U. S. A. 97, 3473–3478.

Johne, A., Kopke, K., Gerloff, T., Mai, I., Rietbrock, S., Meisel, C., Hoffmeyer, S., Kerb, R., Fromm, M.F., Brinkmann, U., Eichelbaum, M., Brockmoller, J., Cascorbi, I., Roots, I., 2002. Modulation of steady-state kinetics of digoxin by haplotypes of the P-glycoprotein MDR1 gene. Clin. Pharmacol. Ther. 72, 584–594.

Kim, R.B., Leake, B.F., Choo, E.F., Dresser, G.K., Kubba, S.V., Schwarz, U.I., Taylor, A., Xie, H.G., McKinsey, J., Zhou, S., Lan, L.B., Schuetz, J.D., Schuetz, E.G., Wilkinson, G.R., 2001. Identification of functionally variant MDR1 alleles among European Americans and African Americans. Clin. Pharmacol. Ther. 70, 189–199.

Klimecki, W.T., Futscher, B.W., Grogan, T.M., Dalton, W.S., 1994. P-glycoprotein expression and function in circulating blood cells from normal volunteers. Blood 83, 2451–2458.

Llorente, L., Richaud-Patin, Y., Diaz-Borjon, A., Alvarado de la Barrera, C., Jakez-Ocampo, J., de la Fuente, H., Gonzalez-Amaro, R., Diaz-Jouanen, E., 2000. Multidrug resistance-1 (MDR-1) in rheumatic autoimmune disorders: Part I. Increased P-glycoprotein activity in lymphocytes from rheumatoid arthritis patients might influence disease outcome. Jt. Bone Spine 67, 30–39.

Lum, B.L., Gosland, M.P., 1995. MDR expression in normal tissues: pharmacological implications for the clinical use of the P-glycoprotein inhibitors. Hematol. Oncol. Clin. North. Am. 9, 319–336.

Marie, J.P., 1995. P-glycoprotein in adult hematologic malignances. Hematol. Oncol. Clin. North. Am. 9, 239–249.

Nelson, E.J., Hinkle, PM., 1992. Characterization of multidrug-resistant pituitary tumor cells. Endocrinology 130, 3246–3256.

Norris, M.D., De Graaf, D., Haber, M., Kavallaris, M., Madafiglio, J., Gilbert, J., 1996. Involvement of MDR1 P-glycoprotein in multifactorial resistance to methotrexate. Int. J. Cancer 65, 613–619.

Pawlik, A., Wrzesniewska, J., Fiedorowicz-Fabrycy, I., Gawronska-Szklarz, B., 2004. The MDR1 3435 polymorphism in patients with rheumatoid arthritis. Int. J. Clin. Pharmacol. Ther. 42, 496–503.

Raghu, G., Park, S.W., Roninson, I.B., Mechetner, E.B., 1996. Monoclonal antibodies against P-glycoprotein an MDR1 gene product, inhibit interleukin-2 release from PHA-activated lymphocytes. Exp. Hematol. 24, 1258–1264.

Saitoh, H., Hatakeyama, M., Eguchi, O., Oda, M., Takada, M., 1998. Involvement of intestinal P-glycoprotein in the restricted absorption of methylprednisolone from rat small intestine. J. Pharm. Sci. 87, 73–75.

Schinkel, A.H., Wagenaar, E., van Deemter, L., Mol, C.A., Borst, P., 1995. Absence of the mdr1a P-glycoprotein in mice affects tissue distribution and pharmacokinetics of dexamethasone, digoxin, and cyclosporin A. J. Clin. Invest. 96, 1698–1705.

Tanabe, M., Ieiri, I., Nagata, N., Inoue, K., Ito, S., Kanamori, Y., Takahashi, M., Kurata, Y., Kigawa, J., Higuchi, S., Terakawa, N., Otsubo, K., 2001. Expression of P-glycoprotein in human placenta: relation to genetic polymorphism of the multidrug resistance (MDR)-1 gene. J. Pharmacol. Exp. Ther. 297, 1137–1143.

Terwilliger, J., Ott, J., 1994. Handbook of Human Genetic Linkage. Johns Hopkins University Press, Baltimore.

Varma, M.V., Ashokraj, Y., Dey, C.S., Panchagnula, R., 2003. P-glycoprotein inhibitors and their screening: a perspective from bioavailability enhancement. Pharmacol. Res. 48, 347–359.

Zheng, H., Webber, S., Zeevi, A., Schuetz, E., Zhang, J., Lamba, J., Bowman, P., Burckart, G.J., 2002. The MDR1 polymorphisms at exons 21 and 26 predict steroid weaning in pediatric heart transplant patients. Hum. Immunol. 63, 765–770.