Differences in assessment of macrolide interaction with human MDR1 (ABCB1, P-gp) using rhodamine-123 efflux, ATPase activity and cellular accumulation assays

Vesna Munić*, Željko Kelnerić, Lara Mikac, Vesna Eraković Haber

GlaxoSmithKline Research Centre Zagreb Ltd., Prilaz baruna Filipovića 29, HR-10000 Zagreb, Croatia

ARTICLE INFO

ABSTRACT

In this study five macrolide antibiotics (azithromycin, erythromycin, clarithromycin, roxithromycin and telithromycin) were compared based on their ability to interact with human MDR1 (ABCB1, P-glycoprotein), studied from two main aspects: by determining the influence of macrolide antibiotics on MDR1 function, as well as the influence of MDR1 on macrolide accumulation in MES-SA/Dx5 cells overexpressing human MDR1.

At higher micromolar concentrations five tested macrolides were shown to inhibit MDR1 function in terms of rhodamine-123 efflux and verapamil-activated ATPase function, whereas at lower concentrations they activated MDR1 ATPase. They were confirmed to be substrates of MDR1 and to compete with each other, as well as with verapamil for transport via this transporter. Expression of MDR1 on cells decreased macrolide accumulation in cells from 2- to 80-fold with the most pronounced change observed for azithromycin and erythromycin. Moreover, presence of active MDR1 highly affected the relative ranking of tested macrolides according to their accumulation in cells. In conclusion, out of seven applied methods and assessed parameters, four of them gave similar rough evaluation on the strength of interaction of five macrolides with MDR1, with clarithromycin, roxithromycin and telithromycin showing stronger interaction than azithromycin and erythromycin.

© 2010 Elsevier B.V. All rights reserved.

1. Introduction

Macrolides are a well-known class of antimicrobial agents, which has been in clinical use for several decades. Macrolide pharmacokinetics is characterized by a substantial affinity for tissues and accumulation within cells. In 3-h incubation with cells some macrolides can reach intracellular concentration up to 500-fold higher than extracellular (Bosnar et al., 2005; Miossec-Bartoli et al., 1999).

The fact that macrolide ranking according to their accumulation in various cell types differs (Bosnar et al., 2005; Miossec-Bartoli et al., 1999), suggests that mechanisms of macrolide transport differs among cell types and differences in expression profiles of some transporters may play a significant role in this phenomenon. To our knowledge, the effect of various transporters on macrolide accumulation and affinity for different tissues has not been studied so far.

MDR1 (ABCB1, P-glycoprotein, P-gp) is the most extensively studied human ATP-binding cassette (ABC) transporter. MDR1 is known to transport a huge spectrum of structurally very diverse substances (many drugs, but also natural compounds). As well as for other ABC transporters, the transport process occurs at cost of ATP and can act against the concentration gradient of a substrate. Due to its highly polyspecific nature and strategic localization on epithelial barriers, impairment of absorption and facilitation of elimination of xenobiotics from the organism or some of its compartments is considered to be the main physiological function of MDR1 (Borst and Elferink, 2002; Litman et al., 2001). Although much work has been done on defining substrate binding sites and 3D-structure, the exact mechanism of action of human MDR1

* Corresponding author. Tel.: +385 1 6051122; fax: +385 1 6051039.
E-mail address: vesna.y.munic@gsk.com (V. Munić).

has not yet been completely clarified. Recent theories propose the model with only one rather big binding site where various compounds bind to its different regions creating opportunities for different interactions of molecules on this protein (Ambudkar et al., 2006), as confirmed by solving the structure of the murine P-gp (mdr1a, abcb1a) (Aller et al., 2009).

Several macrolides have been reported as inhibitors of MDR1 (ABCB1, P-glycoprotein). Many results originate from rather complex cellular models which are also known to have high expression of ABC transporters other than MDR1, such as human colorectal adenocarcinoma cell line Caco-2 or brain endothelial cells. In addition, the effects on MDR1 were proven in MDR1 overexpressing cells in which erythromycin, clarithromycin and azithromycin caused an increase of accumulation of MDR1 substrates (Asakura et al., 2004; Wang et al., 2000; Yasuda et al., 2002).

Potential of macrolide inhibitory effect on MDR1 has also been detected *in vivo*. Both, clarithromycin and erythromycin, in combined therapy with vinblastine significantly improved survival of mice with transplanted tumor with MDR1 overexpression, even though macrolides alone did not exert any antitumor effect (Wang et al., 2000).

Since many compounds which inhibit the MDR1 function are also transported by this transporter, some macrolides are also reported to be MDR1 substrates. In cells with MDR1 overexpression macrolides were proven to be far less active against intracellular bacterium *Listeria monocytogenes*, which was readily overcome by cotreatment with MDR1 inhibitors: verapamil, PSC833 or cyclosporin A (Nichterlein et al., 1995). For some macrolides lower accumulation in MDR1 overexpressing cells has been reported (Nichterlein et al., 1995, 1998; Yamaguchi et al., 2006), although the effect of MDR1 on accumulation of different macrolides were not in detail compared. In transport studies through the monolayer of cells with overexpression of human MDR1 or murine Mdr1a erythromycin was proven to be a substrate for both transporters (Schuetz et al., 1998).

In addition, in studies in knock-out animals it was demonstrated that after p.o. administration erythromycin concentrations in plasma, liver, kidneys, intestine, brain, heart and lungs were 2–4-fold higher in Mdr1a$^{-/-}$ mice than in the wild type (Schuetz et al., 1998). These results clearly indicate the importance of MDR1 in pharmacokinetics of erythromycin and, possibly, other macrolides as well.

MES-SA/Dx5 is a multidrug resistant cell line established from human uterine sarcoma cell line MES-SA which were grown in the presence of increasing concentrations of doxorubicin(Harker and Sikic, 1985). In contrast to MES-SA cells MES-SA/Dx5 highly express MDR1 (ABCB1, P-glycoprotein). To our knowledge expression of other transporters in these cell lines has not been extensively studied so far, with the exception of *mrp* which was on mRNA level reported to be relatively less expressed in MES-SA/Dx5 than in MES-SA cells(Chen et al., 1994). MES-SA and MES-SA/Dx5 cell lines have been used for screening of multidrug resistance modulators(Wesolowska et al., 2005).

In present study we have compared five macrolide antibiotics (azithromycin, erythromycin, clarithromycin, roxithromycin and telithromycin) on their ability to interact with human MDR1 (ABCB1, P-glycoprotein) studied from two main aspects: by determining the influence of macrolide antibiotics on MDR1 function and ATPase activity, as well as the influence of MDR1 on macrolide accumulation in cells which express this transporter. To our knowledge this is the first systematic evaluation of these macrolides in their interaction with MDR1 using several distinct methodological approaches linking together their inhibitory and substrate properties.

2. Materials and methods

2.1. Substances

Azithromycin is a kind gift from PLIVA Ltd.; clarithromycin was internally synthesized in GlaxoSmithKline Research Centre Zagreb Ltd.; erythromycin and roxithromycin were purchased from Sigma, and telithromycin from Aventis. Verapamil, cyclosporin A and rhodamine-123 were purchased from Sigma, and MK571 from Axxora. All macrolides, verapamil and MK571 were dissolved in dimethyl-sulphoxide (Sigma), cyclosporin A in 96% ethanol (Kemika) and rhodamine-123 in deionized water.

2.2. Cell lines

MES-SA/Dx5 (ATCC, CRL-1976) human uterine sarcoma cell line with overexpression of human MDR1 (ABCB1) and its parent cell line MES-SA (ATCC, CRL-1977) were grown in McCoy's medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (Biowest) and 1% GlutaMAX (Gibco, Invitrogen). Cells were detached from the plastic surface by incubation in buffer containing: 0.3 g/L disodium-EDTA, 8 g/L NaCl, 0.56 g/L NaHCO$_3$, 1 g/L dextrose and 0.4 g/L KCl. Cells were cultured for 14 days before initiation of experiments, passaged twice weekly, and used in experiments until 20th passage. There have been to noticable change in behaviour of these cells in experiments within that period.

2.3. Isolation of total RNA and cDNA synthesis

Total RNA was isolated using RNeasy Plus Mini kit (Qiagen) according to manufacturer's protocol. Concentration and quality of isolated total RNA was determined on Agilent Technologies 2100 Bioanalyzer. Total RNA was subjected to digestion of genomic DNA using Deoxyribonuclease I, Amplification Grade (Invitrogen). First strand cDNA was synthesized using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen).

2.4. Quantitative RT-PCR

Expression of eight human ABC transporters was analyzed by quantitative RT-PCR on ABI PRISM 7700 Sequence Detector (Applied Biosystems) using SYBR Green method and β-2-microglobulin (β2m) as a housekeeping gene. Primers were designed in Primer Express software (Table 1). Quantitative RT-PCR was performed at following conditions: 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Optimisation of primer concentrations and primer validation for use with SYBR Green PCR Master Mix (Applied Biosystems) was performed according to manufacturer's protocol. Possible generation of primer–dimers was additionally checked after RT-PCR by electrophoresis of samples in agarose gel and at selected primer concentrations there were no additional bands detected.

ABC transporter expression analysis in each cell line was performed from three independently isolated total RNA samples, and quantitative RT-PCR of each sample was done in triplicates.

To quantify the expression of the same transporter across two cell lines, treshold cycle (C_T) values obtained for ABC transporters were normalized to the values of the houskeeping gene β-2-microglobulin, and ratios of relative amount of ABC transporter and β-2-microglobulin mRNAs were multiplied by 10^6 and their log$_{10}$ values were compared. To estimate expression levels of eight ABC transporters in the same cell line, due to possible differences in efficacy of reverse transcription reaction for different genes, only

Table 1
Primers and their final concentrations used in quantitative RT-PCR.

Gene	Primer	Concentration
β-2-microglobulin	F: TCCGTGGCCTTAGCTGTGC	300 nM
	R: TTCTCTGCTGGATGACGTGAGTAA	50 nM
ABCB1 (MDR1)	F: CGTGTCCCAGGAGCCCAT	50 nM
	R: CTGCCCTCACAATCTCTTCCTGT	50 nM
ABCB4 (MDR3)	F: CAGAGCTCAACTCGGAATCGTG	50 nM
	R: CCTGTGATACAACCCGGCTG	300 nM
ABCC1 (MRP1)	F: CGCTGGCTTCCAACTATTGG	900 nM
	R: CAGGGCTCCATAGACGCTCA	50 nM
ABCC2 (MRP2)	F: GCTGGAAAGTCATCCCTCACAA	300 nM
	R: TGGAGCCCAATGGAAGCA	50 nM
ABCC3 (MRP3)	F: GAGGACATTTGGTGGGCTTTG	50 nM
	R: GAGATTCTCCCCGCCCTCT	50 nM
ABCC4 (MRP4)	F: AGAGATGTGAAGCGCCTGGAA	50 nM
	R: TCTGCTTTGTATGCCCGGAT	300 nM
ABCC5 (MRP5)	F: GCCCTCATCACCACCACG	50 nM
	R: CCCGTTAACTGGACAGCATAAGAG	50 nM
ABCG2 (BCRP)	F: TTGAAGCCAAAGGCAGATGC	300 nM
	R: CTCTGACCTGCTGCTATGGCC	50 nM

orders of magnitude of expression relative to β-2-microglobulin were compared.

2.5. Membrane isolation

Membrane fraction of cells was isolated according to Kennedy and Mangini (2002). Cells were disrupted by nitrogen cavitation (55 bar, 30 min, +4 °C) in Cell Disruption Mini-Bomb (Kontes), followed by ultracentrifugation at $100,000 \times g$, for 60 min at +4 °C. Membrane pellet was resuspended in mannitol buffer (200 mM mannitol, 50 mM Hepes, 40 mM Tris, pH = 7.4) supplemented with protease inhibitors (10 μg/mL leupeptin, 8 μg/mL aprotinin, 1 μg/mL pepstatin and 0.5 mM phenylmethylsulphonyl fluoride, all purchased from Sigma). Total protein concentration was determined by bicinchoninic acid (BCA) method (Pierce) according to manufacturer's protocol.

2.6. Electrophoresis and Western blot

Samples for electrophoresis were quickly dissolved in disaggregation buffer (Sarkadi et al., 1992). Proteins (20 μg/well) in dissolved samples were separated on NuPAGE 4–12% Bis–Tris Gel (Invitrogen) in MOPS buffer (Invitrogen) at 150 V, run for 75 min.

Proteins were transfered onto nitrocellulose membrane in Trans-Blot SD cell (BIO-RAD) at 1 mA/cm^2 for 2 h. Transfer buffer contained 25 mM Tris, 192 mM glycine and 20% methanol. Membrane was blocked overnight at +4 °C in TBS-T buffer (50 mM Tris HCl, 150 NaCl and 0.05% Tween 20) containing 5% powdered milk. Primary antibodies used for MDR1 (clone C219), MRP2 (clone M$_2$III-5, declared by the manufacturer not to crossreact with MDR1, MDR3, MRP1, MRP3 and MRP5), MDR3 (clone P3II-26, does not crossreact with MDR1), MRP1 (clone MRPr1, does not crossreact with MDR1, MDR3, MRP2, MRP3, MRP4 and MRP5) and MRP4 (clone M$_4$I-80) were purchased from Axxora Platform and incubated with the membrane for 2.5 h at room temperature at 0.2 μg/mL, 0.5 μg/mL, 5 μg/mL, 0.5 μg/mL and 2 μg/mL, respectively. Membranes were subsequently incubated with goat anti-mouse or goat anti-rat horseradish peroxidase labelled antibody (Santa Cruz Biotechnology) for 1 h and bands were visualized by ECLPlus kit (Amersham, GE Healthcare) on Storm 860 analyzer (Molecular Dynamics).

2.7. Flow cytometric measurement of rhodamine-123 efflux

To detect rhodamine-123 efflux from MES-SA/Dx5 cells method described by Laupeze et al. (2001) was used with minor modifications. Briefly, cells were loaded with 0.5 μg/mL rhodamine-123 for 30 min at 37 °C in RPMI 1640 medium w/o phenol-red (Promocell), washed twice in ice-cold phosphate buffered saline (PBS) and resuspended in medium containing test ABC transporter inhibitor or macrolide. Samples were then incubated at 37 °C for 15 min to allow rhodamine-123 to be released from cells. Rhodamine-123 fluorescence in cells was measured at 530 nm on FACScan flow cytometer (Becton Dickinson) supplied with 488 nm argon laser by measuring median fluorescence intensity (MFI). To calculate the percentage of rhodamine-123 that remained in cells, MFIs of cells after 15-min incubation for rhodamine-123 efflux were compared to values obtained for control cells immediately after loading. Final concentrations of DMSO or ethanol applied to cells during 15-min incubation with macrolides were 1 and 0.1%, respectively. In tested setup these concentrations had no adverse effect on cell viability, cell morphology (assesed by flow cytometry), nor on rhodamine-123 efflux results.

Inhibition of rhodamine-123 efflux with tested compounds was expressed relative to maximum inihibition obtained with 125 μM verapamil in the same experiment. IC$_{50}$ and % maximum inhibition were determined from minimum of three experiments with samples done in triplicates.

2.8. MDR1 ATPase activity

To measure ATPase activity, PREDEASY ATPase kit (SOLVO Biotechnologies) with Sf9 membrane vesicles with overexpression of human MDR1 were used according to manufacturer's protocol (Konya et al., 2006; Sarkadi et al., 1992). Briefly, to detect activation of ATPase activity membranes (8 mg proteins) were incubated with test macrolide and 2 mM MgATP for 10 min at 37 °C. When measuring inhibition of ATPase activity by macrolides 40 μM verapamil was added to reaction mixture to activate ATPase (Muller et al., 1996; Sarkadi et al., 1992). Final concentration of DMSO in experiment was 1% (Sarkadi et al., 1992). ATPase reaction was subsequently stopped and produced orthophosphate (Pi) was measured colorimetrically. To determine the vanadate sensitive ATPase activity, Pi values obtained in samples were reduced by the Pi values measured in the same samples prepared with addition of 1.2 mM sodium orthovanadate.

2.9. Macrolide accumulation

Macrolide accumulation in cells was measured as described previously (Bosnar et al., 2005) with slight modifications. Since MES-SA/Dx5 cells were found to contain a small population of MDR1-negative cells, to select only MDR1 positive cells, MES-SA/Dx5 were for four days before experiment grown in medium containing 0.2 μg/mL doxorubicin (Sigma). A day before experiment cells were seeded in 6-well plates at density of 2×10^6 cells/well in culture medium. On the next day cells were washed and incubated for 180 min at 37 °C, 5% CO$_2$ and 95% relative humidity, in 3 mL RPMI-1640 medium without phenol-red containing 10 or 50 μM macrolides with or without 50 μM verapamil. After incubation samples were washed 4 times with ice-cold PBS and scraped in 300 μL of lysis solution (0.5% Triton X-100 (Sigma) in deionized water). Samples were frozen, thawed, sonicated for 15 s on ice, centrifuged at $18,000 \times g$ for 10 min, at 4 °C and obtained supernatants were collected for measurement of macrolide concentrations. Final concentration of DMSO during incubation with macrolides was 0.1%.

Macrolide standards for calibration curve were prepared by spiking test macrolide to cell lysates. Briefly, drug naive cells were lysed in lysis solution containing 0.05–10 μM test macrolide, and were further processed in the same way as samples.

2.10. Macrolide quantitation

Intracellular concentration of macrolides was detemined by microbiological agar diffusion method using *Micrococcus luteus* (ATCC 9341) as test microorganism (Foulds et al., 1990). Macrolide concentration was determined using a calibration curve obtained with series of standards prepared as described above. Intracellular concentrations were normalized on total protein content in cells determined by BCA method (Pierce) according to manufacturer's protocol. Lowest limit of detection corresponded to <0.005 μmol g^{-1} of total protein content in samples for all measured macrolides.

When more than one macrolide was present in samples (e.g. when measuring competition of macrolides for accumulation in cells), intracellular concentrations of macrolides were determined by liquid chromatography with tandem mass spectrometry (LC–MS/MS) method. Samples were precipitated by addition of three volumes of acetonitrile:methanol mixture (2:1), followed by centrifugation at $18,000 \times g$ for 10 min at +4 °C, and obtained supernatants were analyzed on API2000 (Applied Biosystems) triple quadrupole mass spectrometer, with ionization at atmospheric pressure, and using binary pump for liquid chromatography Agilent 1100. Quantification was carried out using the ratio of area under the curve for analyte and internal standard (6-O-methyl-9a-aza-9a-homoerythromycin A), and standard calibration curve of the analyte prepared as described above. Response was linear for tested macrolide concentrations of 0.2–10 μM with $R^2 > 0.99$, and all measured samples were within the same range. LLOQ was 0.2 μM with less than 15% error. LLOD for all tested macrolides was approximately 0.02 μM. To check for the specificity for methods of detection of each macrolide, all five macrolides were measured in all samples, standards and blanks and no false detection of any of macrolides was detected in samples that did not contain that particular macrolide.

2.11. Statistic analysis

To compare accumulation of individual macrolide with and without verapamil unpaired two-tailed Student's *t*-test was performed, and for comparison of accumulation of all five macrolides together one way analysis of variance (ANOVA) with Bonferroni multiple comparison test as a post-test were used.

2.12. Cell viability

Viability of cells in treatments with macrolides and ABC transporter inhibitors was assesed by two methods. Possible induction of necrosis was checked by luminescent measurement of the release of cytosolic enzyme adenylate kinase (ToxiLight, Lonza) (Benachour and Seralini, 2009; Heinrich et al., 2009) in supernatants of treated cells using 0.5% Triton X-100 as a positive control which induced 10-fold increase in adenylate kinase release. Induction of apoptosis was determined by luminescent measurement of caspase 3 and 7 activities in cell lysates (Caspase-Glo 3/7, Promega) with 10 μM staurosporine (Sigma) used as positive control which induced 5-fold increase in caspase activity in this setup.
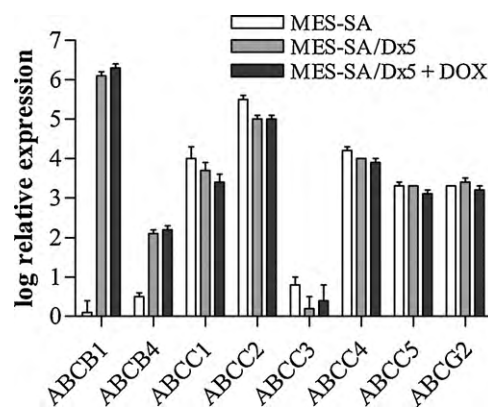
Fig. 1. mRNA expression of eight ABC transporters measured by quantitative RT-PCR. Results are expressed as log$_{10}$ of relative amount of ABC transporter mRNA normalized on β-2-microglobulin expression ± S.D. Results are mean values of three independent experiments.

3. Results

3.1. ABC transporter expression analysis in model cell lines

To study interaction of macrolides with MDR1 on cellular level we have used MES-SA/Dx5 cell line originally obtained by cultivation of parent cell line MES-SA in the presence of doxorubicin. In order to confirm overexpression of MDR1 in MES-SA/Dx5 cell line as well as to characterize all selected cell models regarding expression of other ABC transporters that could potentially influence our results, endogenous expression of eight ABC transporters, reported in literature to transport various drugs (Borst and Elferink, 2002; Litman et al., 2001), was studied by quantitative RT-PCR. Since for macrolide accumulation experiments MES-SA/Dx5 needed to be preselected with 0.2 μg/mL doxorubicin for four days, expression was analyzed in these samples as well.

Quantitative RT-PCR results in Fig. 1 show relative intensity of mRNA expression for eight ABC transporters in three tested cell models. MDR1 (ABCB1) is clearly overexpressed in MES-SA/Dx5 cells showing relative amounts of mRNA in the range of housekeeping gene β-2-microglobulin, whereas MES-SA do not express this transporter. Among other ABC transporters studied, both cell lines have significant amounts of MRP2 (ABCC2) mRNA, approximately one order of magnitude less than that of MDR1 in overexpressing cells. Expression of MRP2 is about 2-fold higher in MES-SA than in MES-SA/Dx5 cells. Expression of MRP1 (ABCC1), MRP4 (ABCC4), MRP5 (ABCC5) and BCRP (ABCG2) is about 100–1000-fold lower than the expression of the β-2-microglobulin, and their expression intensities are about the same in all three cell models studied. Even though it appears that ABCB4 is much more expressed in MES-SA/Dx5 cells than in the parent cell line, its overall expression is extremely low, so that it was assumed that it could not significantly influence results of further experiments. MES-SA/Dx5 cells grown with doxorubicin show ABC transporter expression profile equal to non-selected cells.

Expression of transporters highly expressed on mRNA level was also analyzed in membrane fractions of model cells by Western blot. As shown in Fig. 2, the results obtained on protein level are largely in agreement with those observed on mRNA level. The only transporter present in MES-SA/Dx5 (with or without doxorubicin) and not in MES-SA cells was, as expected, MDR1. MDR3 could not be detected in tested conditions. MRP1 is equally expressed in all three cell models. Interestingly, apart from MRP2, for which higher mRNA expression was observed in MES-SA cells, MRP4 showed the same pattern.
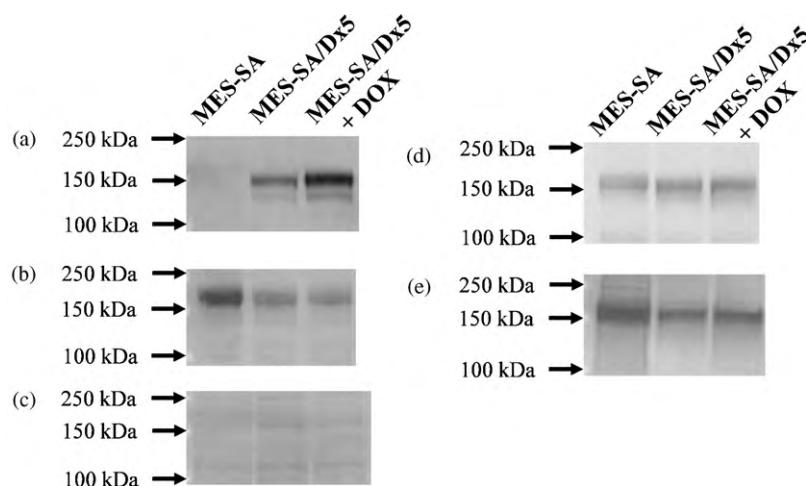
Fig. 2. Western blot analysis of membrane fractions of MES-SA, MES-SA/Dx5 and MES-SA/Dx5 pretreated with doxorubicin for (a) MDR1 (ABCB1), (b) MRP2 (ABCC2), (c) MDR3 (ABCB4), (d) MRP1 (ABCC1), and (e) MRP4 (ABCC4). 20 µg total proteins per lane. DOX: doxorubicin.

3.2. Inhibition of rhodamine-123 efflux via MDR1 by macrolides

In order to evaluate macrolide effect on MDR1 function, a rhodamine-123 efflux assay was performed. At maximum inhibition of rhodamine-123 efflux (125 µM verapamil) from MES-SA/Dx5 cells 35–44% of initially accumulated rhodamine-123 remained in cells (result not shown). Results for test compounds are expressed relative to maximum inhibition which was obtained with 125 µM verapamil in the same experiment. IC_{50} values ± S.D., as well as maximums of inhibition obtained with test compounds (max) were calculated from three to four independent experiments.

From Fig. 3, it is clear that all tested macrolides and standard ABC transporter inhibitors display inhibitory effect on rhodamine-123 efflux, but in very different concentration ranges. The most potent one is cyclosporin A (IC_{50} = 2.1 ± 0.5 µM, max = 101 ± 11%) followed by verapamil (IC_{50} = 9.2 ± 3.8 µM, max = 100 ± 0%). MK571 (IC_{50} > 65 µM, max > 50%), a standard inhibitor more specific for ABCC class of ABC transporters (Gekeler et al., 1995) which inhibits ABCC1 (MRP1) in lower micromolar range, also inhibits MDR1, but only at rather high concentrations. Concentrations of MK571 higher than 100 µM could not have been tested since they were cytotoxic. Azithromycin (IC_{50} > 1174 µM, max > 18%) and erythromycin (IC_{50} > 1714 µM, max > 64%) were clearly the least potent inhibitors in this assay, which in their tested concentrations did not reach their maximal effect. Higher concentrations could not have been tested due to problems with solubility and
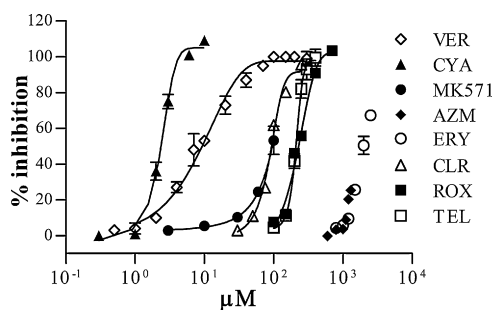

Fig. 3. Inhibition of rhodamine-123 efflux from MES-SA/Dx5 cells. Results are expressed as percentage of maximal inhibition obtained with 125 µM verapamil ± S.D. Shown results are from one respresentative experiment done in triplicates. VER: verapamil, CYA: cyclosporin A, AZM: azithromycin, ERY: erythromycin, CLR: clarithromycin, ROX: roxithromycin, and TEL: telithromycin.

cytotoxicity. Roxithromycin (IC_{50} = 213 ± 17 µM, max = 109 ± 7%) and telithromycin (IC_{50} = 214 ± 15 µM, max = 103 ± 3%) were at least 7 times more potent inhibitors of rhodamine-123 efflux than azithromycin and erythromycin, whereas clarithromycin (IC_{50} = 83 ± 7 µM, max = 94 ± 3%) was about 2.5-fold more potent than roxithromycin and telithromycin. These last three macrolides were also tested in concentrations of up to 1000 µM and maximum values obtained were never higher than 115% of verapamil's. Therefore, it can also be concluded that all tested macrolides that could be tested at high enough concentrations reach the same maximum of rhodamine-123 efflux inhibition as verapamil and based on their inhibition of rhodamine-123 efflux via MDR1 they can be ranked as follows: clarithromycin > roxithromycin ≈ telithromycin ≫ azithromycin ≈ erythromycin.

3.3. Effects of macrolides on MDR1 ATPase function

To further evaluate the effect of macrolides on MDR1 function, MDR1 ATPase activity was assessed using Sf9 membaranes with MDR1 overexpression. Firstly, effect of macrolides on basal ATPase activity was assessed. Control activator of MDR1 ATPase activity verapamil (40 µM) caused a 3.3-fold increase in ATPase activity over basal. For ATPase activation and inhibition EC_{50} and IC_{50} values, respectively, were calculated. Maximal activation (max_a) obtained with a test compound was expressed as % of maximum activation achieved with 40 µM verapamil, whereas maximal inhibition (max_i) is expressed as % of difference between basal and maximal activity (reached with verapamil) for which a compound decreases ATPase activity.

All five tested macrolides activated ATPase activity, but with different intensities and concentration range (Fig. 4). Only clarithromycin (EC_{50} = 11 µM, max_a = 100%) reached the same level of activation as verapamil. Even though it activated ATPase in the lowest concentration range, telithromycin (EC_{50} = 0.55 µM, max_a = 43%) also exhibited the lowest maximum activation of all tested macrolides. On the other hand, azithromycin (EC_{50} = 120 µM, max_a = 85%) activates MDR1 ATPase at highest concentrations, but reaches approximately the same maximum as verapamil. The last two macrolides, erythromycin (EC_{50} = 25 µM, max_a = 70%) and roxithromycin (EC_{50} = 2.5 µM, max_a = 66%), reach about the same maximum but in clearly distinct concentration ranges. To compare molecules with such different activation curves two parameters were compared:
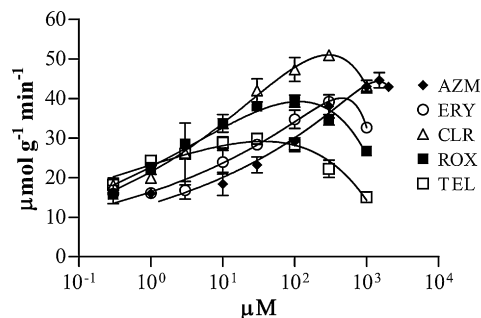
Fig. 4. Activation of human MDR1 ATPase activity by macrolides. Results are expressed as the amount of produced Pi per gram total membrane proteins per min ± S.D. Basal activity of the membranes and maximal activity obtained with 40 μM verapamil were 13–17 and 49–51 μmol g^{-1} min^{-1}, respectively. Shown results are from one representative experiment. AZM: azithromycin, ERY: erythromycin, CLR: clarithromycin, ROX: roxithromycin, and TEL: telithromycin.
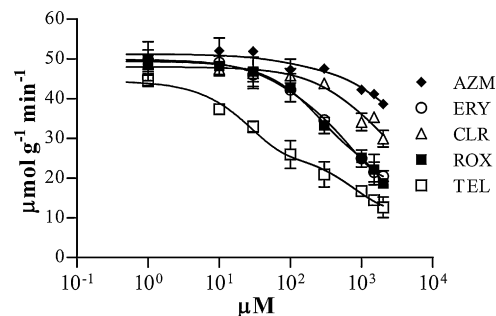
Fig. 5. Inhibition of human MDR1 ATPase activity by macrolides. Membranes were activated with 40 μM verapamil. Results are expressed as the amount of produced Pi per gram total membrane proteins per min ± S.D. Basal activity of the membranes and maximal activity obtained with 40 μM verapamil were 13–17 and 49–51 μmol g^{-1} min^{-1}, respectively. Shown results are from one representative experiment. AZM: azithromycin, ERY: erythromycin, CLR: clarithromycin, ROX: roxithromycin, and TEL: telithromycin.

according to the concentration range of the beginning of their activation curves, tested macrolides were ranked as follows: clarithromycin ≈ roxithromycin ≈ telithromycin ≪ erythromycin < azithromycin, whereas according to the reached maximum of activation the ranking was: clarithromycin > azithromycin > erythromycin ≈ roxithromycin > telithromycin.

All tested compounds display the same profile of the ATPase activation curve: they activate ATPase at lower concentrations, and inhibit it at higher. For azithromycin the inhibitory part of the ATPase activity curve seems to be just at its beginning, but higher concentrations could not have been tested due to compounds' insolubility.

In further experiments effect of macrolides was measured on membranes activated with 40 μM verapamil. At concentrations higher than 100 μM all five tested macrolides inhibited ATPase activity of MDR1 (Fig. 5). The most potent inhibitor was telithromycin (IC$_{50}$ > 37 μM, max$_i$ > 100%), and the least potent clarithromycin (IC$_{50}$ > 600 μM, max$_i$ > 61%) and azithromycin (IC$_{50}$ > 2000 μM, max$_i$ > 32%). Erythromycin (IC$_{50}$ > 290 μM, max$_i$ > 81%) and roxithromycin (IC$_{50}$ > 270 μM, max$_i$ > 94%) exhibited medium inhibitory activity. Relative position of ATPase inhibition curves for the five macrolides correlates with those obtained on membranes activated with verapamil (Fig. 5), although in the presence of verapamil inhibitory action of macrolides is detected at lower concentrations. Depending on their potency in inhibiting ATPase activity macrolides can be ranked in this way:

telithromycin > roxithromycin ≈ erythromycin > clarithromycin > azithromycin.

Among tested compounds only telithromycin showed inhibitory effect on ATPase activity insensitive to vanadate, meaning that this macrolide might also inhibit some other ATPases outside ABC transporter superfamily.

3.4. Effect of MDR1 on macrolide accumulation in cells

To determine the influence of MDR1 on macrolide accumulation in cells, MES-SA/Dx5 (Fig. 6a) or MES-SA cells (Fig. 6b) were incubated with a test macrolide with or without verapamil as an inhibitor of MDR1. Afterwards, the amount of cell-associated macrolide was measured.

Since all five tested macrolides accumulate far better in cells that either do not have MDR1 expressed (MES-SA), or have inhibited MDR1 function (MES-SA/Dx5 with verapamil), than in cells with functional MDR1 (MES-SA/Dx5 with vehicle, dimethylsulphoxide), as already reported in literature, these macrolides can be considered MDR1 substrates. In MES-SA/Dx5 cells at both concentrations tested, intracellular concentrations of all five macrolides were significantly higher in cotreatment with verapamil compared to treatment with macrolides alone ($p < 0.01$, Student's t-test). Macrolide concentrations in MES-SA/Dx5 cells cotreated with verapamil were still a bit lower than those in MES-SA cells cotreated only with vehicle, possibly due to to the
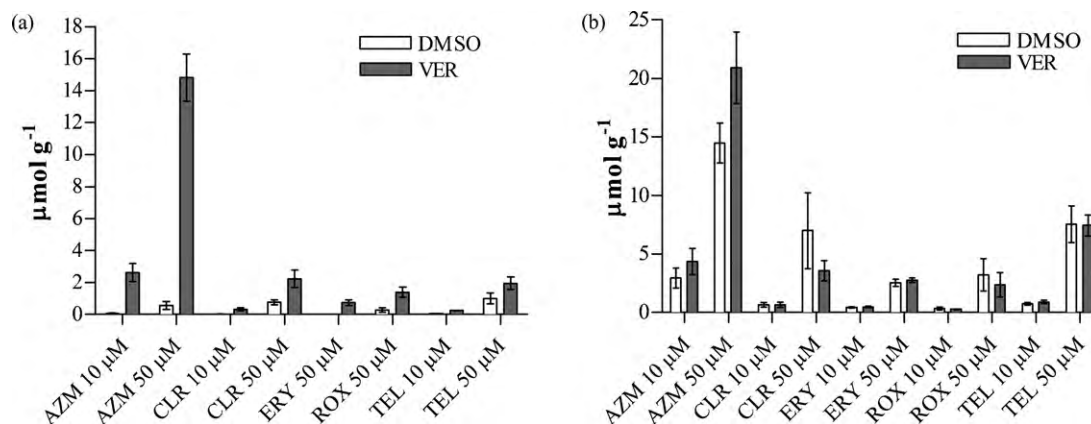
Fig. 6. Macrolide accumulation in (a) MES-SA/Dx5 and (b) MES-SA cells after 3 h incubation in cotreatment with 50 μM verapamil. Results are expressed as amount of macrolide per gram total cellular proteins ± S.D. Results are mean values from three to five independent experiments. AZM: azithromycin, ERY: erythromycin, CLR: clarithromycin, ROX: roxithromycin, and TEL: telithromycin.

fact that 50 μM verapamil does not cause complete inhibition of MDR1.

Furthermore, inhibition of MDR1 on MES-SA/Dx5 cells caused an increase in intracellular concentration of azithromycin and erythromycin (limit of quantitation for erythromycin was $0.005\,\mu\text{mol g}^{-1}$ and measured concentration in cells with verapamil was $0.745 \pm 0.166\,\mu\text{mol g}^{-1}$) for an order of magnitude higher than the increase of clarithromycin, roxithromycin and telithromycin (>35-fold for azithromycin and erythromycin vs. 2–8-fold for clarithromycin, roxithromycin and telithromycin). This means that MDR1 has much higher impact on accumulation of azithromycin and erythromycin than on the accumulation of the remaining three macrolides.

Consequently, cell affinity for selected five macrolides differs depending on the presence of the active MDR1 on their surface. Indeed, comparing all macrolides dosed at 50 μM, in MES-SA cells with and without cotreatment with verapamil, azithromycin accumulates significantly better than all other macrolides tested ($p < 0.001$, ANOVA, Bonferroni post-test). The same result was observed in MES-SA/Dx5 cells cotreated with verapamil ($p < 0.001$, ANOVA, Bonferroni post-test), whereas in the same cell line without verapamil cotreatment azithromycin accumulates with the same intensity to three other macrolides and significantly more only compared to erythroymcin ($p < 0.01$, ANOVA, Bonferroni post-test). It can therefore be concluded that azithromycin accumulates much more than other tested macrolides in cells that do not express MDR1, or its function was inhibited, whereas in cells with active MDR1 accumulation of azithromycin is in the range of clarithromycin, telithromycin and roxithromycin.

In most cases in MES-SA cells, as expected, verapamil did not change accumulation of tested macrolides. However, it induced a slight but significant increase (1.5-fold) in intracellular concentration of azithromycin ($p < 0.05$, Student's t-test). Apart from that, in the same cell line a statistically significant decrease in clarithromycin accumulation was observed when macrolide and verapamil were applied at 50 μM ($p < 0.05$, Student's t-test).

Therefore, comparing five macrolides based on the relative increase in cellular accumulation upon MDR1 inhibition, following ranking was observed: erythromycin ≥ azithromycin ≫ roxithromycin ≈ clarithromycin ≈ telithromycin.

On the other hand, comparing the absolute intensities of macrolide accumulation for cells with MDR1 the ranking is: telithromycin ≈ clarithromycin ≈ azithromycin ≈ roxithromycin > erythromycin, whereas for cells without active MDR1 it is: azithromycin ≫ telithromycin ≈ clarithromycin ≥ roxithromycin ≥ erythromycin.

3.5. Macrolide competition for cellular transport and accumulation mechanisms

With the aim to explore how do macrolides compete for MDR1 transport and to compare the affinities of MDR1 for these compounds, accumulation of two macrolides added simultaneously to MES-SA/Dx5 cells was determined by LC–MS/MS method. Results in Table 2 are expressed relative to control accumulation where each macrolide was added to the cells alone, only with vehicle dimethyl-sulphoxide.

A dramatic increase in intracellular accumulation (2.3–5.3-fold) was observed for azithromycin and erythromycin in cotreatment with clarithromycin, telithromycin or roxithromycin, and for telithromycin in cotreatment with clarithromycin. From the magnitude of the effects on accumulation of other macrolides, as well as from the magnitude of changes of their own accumulation under the influence of other macrolides, macrolides can, based on their affinity for MDR1, be ranked as follows:

Table 2
Macrolide uptake[a] in MES-SA/Dx5 cells in cotreatment with other macrolides.[b]

Effector[b]	% control uptake[a] ± S.D.				
	AZM	ERY	CLR	ROX	TEL
AZM		101 ± 6	50 ± 4	68 ± 9	86 ± 6
ERY	123 ± 6		89 ± 3	109 ± 6	116 ± 8
CLR	525 ± 99	491 ± 83		155 ± 17	249 ± 28
ROX	231 ± 39	299 ± 44	53 ± 16		124 ± 6
TEL	332 ± 65	499 ± 98	107 ± 41	144 ± 20	

Effector and measured macrolides were both applied to cells at 50 μM concentration and incubated for 3 h. Macrolide concentration was determined by LC–MS/MS method and expressed as percentage of macrolide accumulated without presence of effector. Results are mean values ± S.D. from three independent experiments. AZM: azithromycin, ERY: erythromycin, CLR: clarithromycin, ROX: roxithromycin, and TEL: telithromycin.

clarithromycin > roxithromycin ≈ telithromycin ≫ azithromycin ≈ erythromycin.

Even though it would seem most likely that two substrates for MDR1, which compete for the transporter and possibly, at least partially, bind to the same binding site, would either increase each other's accumulation, or at least not change it; in a few cases an opposite effect was detected. Thus, azithromycin significantly lowered the accumulation of clarithromycin and roxithromycin for 50 and 32%, respectively; and cotreatment with roxithromycin decreased the accumulation of clarithromycin for 47%.

3.6. Cell viability

As measured by the release of adenylate kinase or caspase 3/7 activation, applied macrolide treatments had no adverse effect on cell viability within the tested concentration range and monitored incubation time (maximum fold change for both parameters was ≤1.1; results not shown).

4. Discussion

In this study five macrolide antibiotics (azithromycin, erythromycin, clarithromycin, roxithromycin and telithromycin) were compared based on their ability to interact with human MDR1 (ABCB1, P-glycoprotein). The interactions were addressed in terms of two major questions: how do macrolides influence MDR1 function, and how does MDR1 affect macrolide accumulation in cells? Presented results show that estimation of the relative interaction of the five macrolides with MDR1 differs considerably depending on the method applied.

Apart from inhibiting rhodamine-123 export via MDR1, all tested macrolides display the ATPase activation curve characteristic for quickly transported substrates with the possibility to bind to at least two binding sites in MDR1: they activate ATPase at lower concentrations, and inhibit it at higher. The same was observed with other MDR1 substrates like verapamil, vincristin, vinblastin, etoposide and colchicine (Muller et al., 1996). Thus, it is most likely that tested macrolides inhibit rhodamine-123 export by competing for the transport via the same transporter.

By measuring macrolide accumulation in MES-SA and MES-SA/Dx5 cells with and without MDR1 inhibitor verapamil, it was found that MDR1 has a marked impact on macrolide accumulation in cells. As expected from previous reports (Nichterlein et al., 1995, 1998; Yamaguchi et al., 2006) high expression of functional MDR1 on cell surface decreases intracellular concentrations of these five macrolides. Furthermore, in this study it was shown that inhibition of MDR1 had the greatest influence on the accumulation of azithromycin and erythromycin, which is very similar to the result reported on murine cell line J774A.1 (Seral

et al., 2003), which possibly express murine homologue of this gene.

Consequently, relative ranking of macrolides according to their accumulation in cells is dramatically different depending on the presence of the active MDR1 on cell surface. Therefore, it is likely that even *in vivo* in cells with high MDR1 expression macrolides would, firstly, reach lower intracellular concentrations, and secondly, display different relative ranking order according to their accumulation than in other cell types. These results indicate how expression of a single transporter can dramatically change cell affinity for structurally related compounds which could at least partially explain some differences in their pharmacokinetic behaviour and even in the intensity of their pharmacodynamic effects.

In our previous studies two tested cell lines showed similar profile of macrolide ranking as cells used in this study (Bosnar et al., 2005). Canine kidney epithelial cell line MDCK resembles MES-SA/Dx5 cells with active MDR1 in their affinity for macrolides, whereas human monocytic leukemia cells THP1 are more like MES-SA cell line. Similarity of MDCK to MES-SA/Dx5 cells in this system may well be due to the endogenous expression of Mdr1, which has already been proven in these cells (Raggers et al., 2002).

Moreover, since the measurement of ATPase activity revealed that both verapamil and tested macrolides activate MDR1 ATPase activity (which is characteristic for quickly transported substrates), macrolides inhibit verapamil-activated ATPase activity of MDR1, and verapamil increases macrolide accumulation in MDR1-expressing cells; it can be concluded that tested macrolides clearly compete with verapamil for transport via MDR1, which is most likely due to the binding to the same part of the MDR1 substrate binding site.

In order to further evaluate macrolide interactions with MDR1 we have measured the accumulation of two macrolides added simultaneously to MES-SA/Dx5 cells. Since in all tested combinations of macrolides intracellular concentration of one of them was higher due to the presence of the other, we conclude that macrolides compete with each other for transport via MDR1, which could be due to the binding to the same part of the MDR1 substrate binding site. However, even though it would seem most likely that two substrates for MDR1, which compete for MDR1, would either increase each other's accumulation, or at least leave it unchanged, azithromycin caused a significant decrease in accumulation of clarithromycin, as well as that of roxithromycin.

Possible explanation for the mentioned effect of azithromycin could be that in incubation of a compound with a high affinity for MDR1 (e.g. clarithromycin or roxithromycin) a compound with a significantly lower affinity for MDR1 (e.g. azithromycin) does not considerably affect the transport of the high-affinity compound via this transporter, but the interaction rather occurs on some other mechanism of transport or accumulation in the cell. Since azithromycin is known to highly accumulate in cells (Bosnar et al., 2005; Miossec-Bartoli et al., 1999) interaction could happen inside the cell, for instance in lysosomes which are proven to be the sites of macrolide intensive accumulation (McDonald and Pruul, 1991; Miossec-Bartoli et al., 1999). In addition, even though it showed a rather low affinity for MDR1, erythromycin does not display the same effect as azithromycin on clarithromycin accumulation, which could be due to the fact that the overall concentration of erythromycin in cells is quite low.

Comparing all presented data it is apparent that four methods offer the same rough estimation of five selected macrolides according to the strength of their interaction with MDR1. By measuring the inhibition of rhodamine-123 efflux, by determining the concentration range of the beginning of ATPase activation curve, by estimation of the affinity for MDR1 by measurement of cellular accumulation of two macrolides simultaneously, as well as

by determining the magnitude of an increase in cellular accumulation upon MDR1 inhibition, five macrolides were divided into two groups: azithromycin and erythromycin in one, and clarithromycin, roxithromycin and telithromycin in the other. By these methods it turned out that azithromycin and erythromycin have the lowest potency to inhibit rhodamine-123 efflux via MDR1 and to activate its ATPase activity, they show the lowest affinity for MDR1 when competing with other macrolides, but the increase of their accumulation in cells upon MDR1 inhibition appears the highest. Even though at first glance the last fact looks a bit contradictory to previous three, obviously these two macrolides have even lower affinity for MDR1 than verapamil. Therefore, a cotreatment with verapamil possibly causes a complete abrogation of azithromycin and erythromycin transport via MDR1, which consequently causes a dramatically higher increase in their intracellular concentrations. On the other hand, clarithromycin, roxithromycin and telithromycin have a much higher affinity for MDR1, so that verapamil in tested dose only partially impairs the export of these macrolides by MDR1, and the change in the intracellular accumulation upon cotreatment with verapamil is not so pronounced as in the case of azithromycin and erythromycin.

Moreover, it is possible that the differences in permeability of these compounds through the cell membrane at least partially contribute to the mentioned phenomena. Such an effect was already observed with verapamil (Litman et al., 2001), which, even though it is intensively transported via MDR1, by its passive diffusion through the membrane abolishes the effect of the unidirectional transport via this ABC transporter.

Another important factor in observed phenomena may be the drug concentration in the membrane. Since MDR1 takes its substrates from the inner leaflet of the membrane (Aller et al., 2009), it is often refered to as a 'hydrophobic vacuum cleaner' (Raviv et al., 1990). Therefore, there are at least two factors important for the interaction of the drug with MDR1: drug concentration within the membrane (presumably dependent on its lipophilicity) and drug affinity for the transporter. However, comparing $\log P$ values of tested macrolides with results of each of the applied tests for interaction with MDR1 in this study no direct link with drug lipophilicity could be found. The ranking of tested macrolides according to $\log P$ is as follows: azithromycin ($\log P = 4.1$) $\gg$ roxithomycin (3.7) > telithromycin (3.3) $\approx$ clarithromycin (3.16–3.2) > erythromycin (2.8–3.06) (Evrard-Todeschi et al., 2000; McFarland et al., 1997; Zhu et al., 2008).

From presented results it is clear that tested macrolides interact with MDR1, which could, therefore, potentially lead to interactions with other drugs on this transporter. In this study tested five macrolides have mainly shown inhibitory effects (inhibition of rhodamine-123 efflux and inhibition of ATPase activity) at concentrations higher than 100 μM whereas their MDR1 substrate properties (activation of ATPase activity and accumulation in MDR1-expressing cells) were detected at concentrations as low as 1 or 10 μM. This is similar to the findings of Polli et al. (2001) who have evaluated interactions of erythromycin and clarithromycin with MDR1 and found that at 20 μM both compounds activate MDR1 ATPase activity, but in calcein-AM accumulation assay they did not show inhibitory effect in concentrations of up to 200 μM. Yasuda et al. (2002) reported K_i values for erythromycin in calcein-AM accumulation assay to be >1000 μM, whereas in vinblastine accumulation assay it was 38 μM. Similar results for other compound classes, showing that some consequences of the interaction of a compound with MDR1 occur in very distant concentration ranges, have already been discussed in literature (Polli et al., 2001; Scala et al., 1997). Similarly, Lespine et al. (2007) report differences in concentra-

tion range, as well as difference in relative potency determined for six distinct macrocyclic lactone compounds comparing them in P-gp ATPase activity assay and rhodamine-123 accumulation assay.

From all these data it is clear that for estimating the interaction of compounds with MDR1, as well as the consequences of this interaction, it is necessary to use several methods which study substrate as well as inhibitory properties of tested compounds. As shown in this and some previous reports these two aspects of interaction with MDR1 may well give different estimations of relative potency of tested drugs for interaction with this transporter.

Regarding the relevance of observed macrolide inibitory effects on MDR1, it should be noted that in standard peroral therapy concentrations that macrolides reach in extracellular compartments in the organism are much lower than 100 μM (Ciervo and Shi, 2005; Jain and Danziger, 2004; Rodvold et al., 1997), with exception of intestinal lumen where, due to their presence in higher concentrations (Masaoka et al., 2006; Saito et al., 2000; Yee, 1997), interactions of macrolides with MDR1 may have a much higher impact on absorption and elimination of other MDR1 substrates.

Apart from all these effects on MDR1, in this study it was also noted that accumulation of azithromycin in MDR1-negative cells MES-SA is slightly increased in the presence of verapamil. It could possibly be due to competition of these two compounds on some other endogenously expressed transporter for which azithromycin is also a substrate. According to our quantitative RT-PCR results this could be MRP2 (ABCC2), which could, at least according to some *in vivo* results, also be capable of azithromycin transport (Sugie et al., 2004). According to Western blot results another possible candidate might be MRP4 (ABCC4).

Another unexpected result of this study was a significant decrease in clarithromycin accumulation in MES-SA cells in cotreatment with verapamil. Possible explanations of this effect could be that verapamil and clarithromycin are co-transported via some ABC transporter out of the cell, or they could also compete for some mechanism of entry into the cell, e.g. for some importer. It should be born in mind that this effect most probably also takes place in MES-SA/Dx5 cells but is masked by MDR1 activity.

In conclusion, all five tested macrolides interact with MDR1. Depending on the method applied, the concentration range at which the interaction is detected, but also the estimation of stronger and weaker interactors, differ considerably. Four methods, however, provided similar rough evaluation of five macrolides, with clarithromycin, roxithromycin and telithromycin being capable of a stronger interaction with MDR1 than azithromycin and erythromycin. Tested macrolides affect MDR1 transport function as well as its ATPase activity, which opens questions on the possible interactions with other drugs on this transporter. The five macrolides are also transported by this transporter, which highly affects their intracellular accumulation. Since MDR1 does not have the same impact on accumulation of all five macrolides, their relative ranking order based on cellular accumulation differs depending on the presence of active MDR1 on cell surface. These data reveal the importance of studying the effect of various transporters, exporters as well as importers, on macrolide cellular accumulation, in order to better understand macrolide pharmacokinetic properties, which may in turn shed additional light on the differences in the extent of their pharmacodynamic effects.

References

Aller, S.G., Yu, J., Ward, A., Weng, Y., Chittaboina, S., Zhuo, R., Harrell, P.M., Trinh, Y.T., Zhang, Q., Urbatsch, I.L., Chang, G., 2009. Structure of P-glycoprotein reveals a molecular basis for poly-specific drug binding. Science 323, 1718–1722.

Ambudkar, S.V., Kim, I.W., Sauna, Z.E., 2006. The power of the pump: mechanisms of action of P-glycoprotein (ABCB1). Eur. J. Pharm. Sci. 27, 392–400.

Asakura, E., Nakayama, H., Sugie, M., Zhao, Y.L., Nadai, M., Kitaichi, K., Shimizu, A., Miyoshi, M., Takagi, K., Takagi, K., Hasegawa, T., 2004. Azithromycin reverses anticancer drug resistance and modifies hepatobiliary excretion of doxorubicin in rats. Eur. J. Pharmacol. 484, 333–339.

Benachour, N., Seralini, G.E., 2009. Glyphosate formulations induce apoptosis and necrosis in human umbilical, embryonic, and placental cells. Chem. Res. Toxicol. 22, 97–105.

Borst, P., Elferink, R.O., 2002. Mammalian ABC transporters in health and disease. Annu. Rev. Biochem. 71, 537–592.

Bosnar, M., Kelneric, Z., Munic, V., Erakovic, V., Parnham, M.J., 2005. Cellular uptake and efflux of azithromycin, erythromycin, clarithromycin, telithromycin, and cethromycin. Antimicrob. Agents Chemother. 49, 2372–2377.

Chen, G., Jaffrézou, J.P., Fleming, W.H., Durán, G.E., Sikic, B.I., 1994. Prevalence of multidrug resistance related to activation of the mdr1 gene in human sarcoma mutants derived by single-step doxorubicin selection. Cancer Res. 54, 4980–4987.

Ciervo, C.A., Shi, J., 2005. Pharmacokinetics of telithromycin: application to dosing in the treatment of community-acquired respiratory tract infections. Curr. Med. Res. Opin. 21, 1641–1650.

Evrard-Todeschi, N., Gharbi-Benarous, J., Gaillet, C., Verdier, L., Bertho, G., Lang, C., Parent, A., Girault, J.P., 2000. Conformations in solution and bound to bacterial ribosomes of ketolides, HMR 3647 (telithromycin) and RU 72366: a new class of highly potent antibacterials. Bioorg. Med. Chem. 8, 1579–1597.

Foulds, G., Shepard, R.M., Johnson, R.B., 1990. The pharmacokinetics of azithromycin in human serum and tissues. J. Antimicrob. Chemother. 25 (Suppl. A), 73–82.

Gekeler, V., Ise, W., Sanders, K.H., Ulrich, W.R., Beck, J., 1995. The leukotriene LTD4 receptor antagonist MK571 specifically modulates MRP associated multidrug resistance. Biochem. Biophys. Res. Commun. 208, 345–352.

Harker, W.G., Sikic, B.I., 1985. Multidrug (pleiotropic) resistance in doxorubicin-selected variants of the human sarcoma cell line MES-SA. Cancer Res. 45, 4091–4096.

Heinrich, A., Balszuweit, F., Thiermann, H., Kehe, K., 2009. Rapid simultaneous determination of apoptosis, necrosis, and viability in sulfur mustard exposed HaCaT cell cultures. Toxicol. Lett. 191, 260–267.

Jain, R., Danziger, L.H., 2004. The macrolide antibiotics: a pharmacokinetic and pharmacodynamic overview. Curr. Pharm. Des. 10, 3045–3053.

Kennedy, B.G., Mangini, N.J., 2002. P-glycoprotein expression in human retinal pigment epithelium. Mol. Vis. 8, 422–430.

Konya, A., Andor, A., Satorhelyi, P., Nemeth, K., Kurucz, I., 2006. Inhibition of the MDR1 transporter by new phenothiazine derivatives. Biochem. Biophys. Res. Commun. 346, 45–50.

Laupeze, B., Amiot, L., Bertho, N., Grosset, J.M., Lehne, G., Fauchet, R., Fardel, O., 2001. Differential expression of the efflux pumps P-glycoprotein and multidrug resistance-associated protein in human monocyte-derived dendritic cells. Hum. Immunol. 62, 1073–1080.

Lespine, A., Martin, S., Dupuy, J., Roulet, A., Pineau, T., Orlowski, S., Alvinerie, M., 2007. Interaction of macrocyclic lactones with P-glycoprotein: structure–affinity relationship. Eur. J. Pharm. Sci. 30, 84–94.

Litman, T., Druley, T.E., Stein, W.D., Bates, S.E., 2001. From MDR to MXR: new understanding of multidrug resistance systems, their properties and clinical significance. Cell. Mol. Life Sci. 58, 931–959.

Masaoka, Y., Tanaka, Y., Kataoka, M., Sakuma, S., Yamashita, S., 2006. Site of drug absorption after oral administration: assessment of membrane permeability and luminal concentration of drugs in each segment of gastrointestinal tract. Eur. J. Pharm. Sci. 29, 240–250.

McDonald, P.J., Pruul, H., 1991. Phagocyte uptake and transport of azithromycin. Eur. J. Clin. Microbiol. Infect. Dis. 10, 828–833.

McFarland, J.W., Berger, C.M., Froshauer, S.A., Hayashi, S.F., Hecker, S.J., Jaynes, B.H., Jefson, M.R., Kamicker, B.J., Lipinski, C.A., Lundy, K.M., Reese, C.P., Vu, C.B., 1997. Quantitative structure–activity relationships among macrolide antibacterial agents: in vitro and in vivo potency against *Pasteurella multocida*. J. Med. Chem. 40, 1340–1346.

Miossec-Bartoli, C., Pilatre, L., Peyron, P., N'Diaye, E.N., Collart-Dutilleul, V., Maridonneau-Parini, I., Diu-Hercend, A., 1999. The new ketolide HMR3647 accumulates in the azurophil granules of human polymorphonuclear cells. Antimicrob. Agents Chemother. 43, 2457–2462.

Muller, M., Bakos, E., Welker, E., Varadi, A., Germann, U.A., Gottesman, M.M., Morse, B.S., Roninson, I.B., Sarkadi, B., 1996. Altered drug-stimulated ATPase activity in mutants of the human multidrug resistance protein. J. Biol. Chem. 271, 1877–1883.

Nichterlein, T., Kretschmar, M., Schadt, A., Meyer, A., Wildfeuer, A., Laufen, H., Hof, H., 1998. Reduced intracellular activity of antibiotics against *Listeria monocytogenes* in multidrug resistant cells. Int. J. Antimicrob. Agents 10, 119–125.

Nichterlein, T., Kretschmar, M., Siegsmund, M., Hof, H., 1995. Erythromycin is ineffective against *Listeria monocytogenes* in multidrug resistant cells. J. Chemother. 7, 184–188.

Polli, J.W., Wring, S.A., Humphreys, J.E., Huang, L., Morgan, J.B., Webster, L.O., Serabjit-Singh, C.S., 2001. Rational use of in vitro P-glycoprotein assays in drug discovery. J. Pharmacol. Exp. Ther. 299, 620–628.

Raggers, R.J., Vogels, I., van Meer, G., 2002. Upregulation of the expression of endogenous Mdr1 P-glycoprotein enhances lipid translocation in MDCK cells transfected with human MRP2. Histochem. Cell Biol. 117, 181–185.

Raviv, Y., Pollard, H.B., Bruggemann, E.P., Pastan, I., Gottesman, M.M., 1990. Photo-sensitized labeling of a functional multidrug transporter in living drug-resistant tumor cells. J. Biol. Chem. 265, 3975–3980.

Rodvold, K.A., Gotfried, M.H., Danziger, L.H., Servi, R.J., 1997. Intrapulmonary steady-state concentrations of clarithromycin and azithromycin in healthy adult volunteers. Antimicrob. Agents Chemother. 41, 1399–1402.

Saito, H., Fukasawa, Y., Otsubo, Y., Yamada, K., Sezaki, H., Yamashita, S., 2000. Carrier-mediated transport of macrolide antimicrobial agents across Caco-2 cell monolayers. Pharm. Res. 17, 761–765.

Sarkadi, B., Price, E.M., Boucher, R.C., Germann, U.A., Scarborough, G.A., 1992. Expression of the human multidrug resistance cDNA in insect cells generates a high activity drug-stimulated membrane ATPase. J. Biol. Chem. 267, 4854–4858.

Scala, S., Akhmed, N., Rao, U.S., Paull, K., Lan, L.B., Dickstein, B., Lee, J.S., Elgemeie, G.H., Stein, W.D., Bates, S.E., 1997. P-glycoprotein substrates and antagonists cluster into two distinct groups. Mol. Pharmacol. 51, 1024–1033.

Schuetz, E.G., Yasuda, K., Arimori, K., Schuetz, J.D., 1998. Human MDR1 and mouse mdr1a P-glycoprotein alter the cellular retention and disposition of erythromycin, but not of retinoic acid or benzo(a)pyrene. Arch. Biochem. Biophys. 350, 340–347.

Seral, C., Michot, J.M., Chanteux, H., Mingeot-Leclercq, M.P., Tulkens, P.M., Van Bambeke, F., 2003. Influence of P-glycoprotein inhibitors on accumulation of macrolides in J774 murine macrophages. Antimicrob. Agents Chemother. 47, 1047–1051.

Sugie, M., Asakura, E., Zhao, Y.L., Torita, S., Nadai, M., Baba, K., Kitaichi, K., Takagi, K., Takagi, K., Hasegawa, T., 2004. Possible involvement of the drug transporters P glycoprotein and multidrug resistance-associated protein Mrp2 in disposition of azithromycin. Antimicrob. Agents Chemother. 48, 809–814.

Wang, L., Kitaichi, K., Hui, C.S., Takagi, K., Takagi, K., Sakai, M., Yokogawa, K., Miyamoto, K.I., Hasegawa, T., 2000. Reversal of anticancer drug resistance by macrolide antibiotics in vitro and in vivo. Clin. Exp. Pharmacol. Physiol. 27, 587–593.

Wesolowska, O., Paprocka, M., Kozlak, J., Motohashi, N., Dus, D., Michalak, K., 2005. Human sarcoma cell lines MES-SA and MES-SA/Dx5 as a model for multidrug resistance modulators screening. Anticancer Res. 25, 383–389.

Yamaguchi, S., Zhao, Y.L., Nadai, M., Yoshizumi, H., Cen, X., Torita, S., Takagi, K., Takagi, K., Hasegawa, T., 2006. Involvement of the drug transporters P glycoprotein and multidrug resistance-associated protein Mrp2 in telithromycin transport. Antimicrob. Agents Chemother. 50, 80–87.

Yasuda, K., Lan, L.B., Sanglard, D., Furuya, K., Schuetz, J.D., Schuetz, E.G., 2002. Interaction of cytochrome P450 3A inhibitors with P-glycoprotein. J. Pharmacol. Exp. Ther. 303, 323–332.

Yee, S., 1997. In vitro permeability across Caco-2 cells (colonic) can predict in vivo (small intestinal) absorption in man—fact or myth. Pharm. Res. 14, 763–766.

Zhu, Z.J., Krasnykh, O., Pan, D., Petukhova, V., Yu, G., Liu, Y., Liu, H., Hong, S., Wang, Y., Wan, B., Liang, W., Franzblau, S.G., 2008. Structure–activity relationships of macrolides against *Mycobacterium tuberculosis*. Tuberculosis (Edinb) 88 (Suppl. 1), S49–S63.