Interaction of progestins with the human multidrug resistance-associated protein 2 (MRP2)

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Abbreviations: CMF, 5-chloromethylfluorescein; CMFDA, 5-chloromethylfluorescein diacetate; DMEM, Dulbecco’s modified Eagle’s medium; DMSO, dimethyl sulfoxide; FCS, fetal calf serum; HBSS, Hank’s balanced salt solution; HRT, hormone replacement therapy; MF-SG, methylfluorescein-sulfoglutathione; MRP, human multidrug resistance-associated protein; OC, oral contraceptives; PBS, phosphate buffered saline; P-gp, P-glycoprotein.
Abstract

Progestins are widely used as oral contraceptives and hormone replacement therapy. Recently it has been demonstrated that many progestins are inhibitors of P-glycoprotein possibly explaining gender differences in drug actions. In vitro evidence suggested that at least norgestimate might also inhibit other transporters like the multidrug resistance-associated protein 2 (MRP2). We therefore investigated, whether norgestimate, desogestrel, medroxyprogesterone acetate, norethisterone, progesterone, cyproterone acetate, chlormadinone acetate, and levonorgestrel inhibit MRP2 in vitro using confocal laser scanning microscopy and 5-chloromethyl-fluorescein diacetate (CMFDA) as a prodrug of the fluorescent 5-chloromethylfluorescein (CMF), which is actively transported by MRP2 as glutathione conjugate. Of the progestins tested only norgestimate (50 µM) and progesterone (100 µM) significantly increased intracellular CMF fluorescence by 62% and 53%, respectively. In conclusion, the progestins norgestimate and progesterone significantly inhibit the transport activity of MRP2 in vitro.
Multidrug resistance-associated proteins (MRPs) are a subfamily of the ATP-binding cassette transport protein family involved in drug resistance (Schinkel and Jonker, 2003). MRP2 is a key member of this group and was identified in the canalicular membrane of hepatocytes as a transporter for organic anions extruding a wide range of glutathione, glucuronate, and sulphate conjugates into the bile (Ishikawa et al., 1993; Ito et al., 1997; Paulusma et al., 1997). It was also detected in renal brush-border membranes, the intestine (Schaub et al., 1997), placenta (St.-Pierre et al., 2000; Gerk and Vore, 2002), and the brain (Török et al., 2003). In human carcinoma, MRP2 may confer resistance to chemotherapy (Norris et al., 1996; Nies et al., 2001; Young et al., 2001; Schinkel and Jonker, 2003) and it appears also to play a role in drug-drug interactions (Fromm et al., 2000; Bramow et al., 2001; Bode et al., 2002; Huisman et al., 2002; Giessmann et al., 2004).

In recent years it has been suggested that gender could alter the activity of drug transporters like P-glycoprotein (P-gp) (Cummins et al., 2002). Hence in a previous study we have investigated, whether P-gp is inhibited by progestins used in oral contraceptives (OCs) and hormone replacement therapy (HRT). All progestins tested had P-gp inhibitor properties with some of them being as potent as the well known P-gp inhibitor quinidine (Fröhlich et al., 2004). Interestingly, one of the tested progestins (norgestimate) revealed an inhibitory effect on the transport of calcein-acetoxymethylester (calcein-AM) not only in the P-gp overexpressing cell line L-MDR1, but also in the parental cell line LLC-PK1, which only expresses low amounts of P-gp (Decorti et al., 2001; Weiss et al., 2003; Fröhlich et al., 2004) indicating that this effect was not selective. Because LLC-PK1 express MRP2 (Decorti et al., 2001; Evers et al., 1996) and MRP2 can transport calcein-AM (Evers, 2000) these data may suggest that norgestimate also inhibits MRP2. We thus aimed to characterize the MRP2 inhibitory potencies of norgestimate and a series of frequently used progestins in vitro in the canine kidney cell line MDCK II/Par and its analog MDCK II/MRP2 which stably overexpresses human MRP2.
The functional activity of MRP2 can be demonstrated with the cell tracker reagent 5-chloromethylfluorescein diacetate (CMFDA). The non-fluorescent CMFDA passively diffuses into the cells where cytosolic esterases cleave off its acetate residues, thereby releasing the fluorescent and membrane-impermeable product 5-chloromethylfluorescein (CMF) which can react e.g. with glutathione to form fluorescent conjugates. This methylfluorescein-glutathione complex (MF-SG) is then actively secreted by MRP2 (Bogman et al., 2003). CMF fluorescence was measured by confocal laser scanning microscopy.
Materials and Methods

Materials

Dulbecco’s modified Eagle’s medium (DMEM) was purchased from PAA Laboratories GmbH (Parching, Austria), fetal calf serum (FCS), medium supplements, antibiotics, glutamine, buffers and cell culture medium M 199 from Invitrogen (Karlsruhe, Germany), NaCl, MgSO\(_4\) and dimethyl sulfoxide (DMSO) were from AppliChem (Darmstadt, Germany). Collagen-R was obtained from Serva (Heidelberg, Germany). Cytotoxicity Detection Kit was obtained from Roche Applied Science (Mannheim, Germany). CMFDA was purchased from MobiTec (Göttingen, Germany), norgestimate was a kind gift from Janssen-Cilag GmbH (Neuss, Germany), desogestrel from Grüenthal GmbH (Santiago, Chile), medroxyprogesterone acetate, norethisterone, progesterone, cyproterone acetate, chlormadinone acetate, levonorgestrel, and pyruvate were from Sigma-Aldrich (Taufkirchen, Germany), MK 571 was from Biomol (Plymouth Meeting, PA, USA), 6-well-plates from Biochrom AG (Berlin, Germany), 96-well microtiter plates and culturing bottles were from Nunc (Wiesbaden, Germany), the coverslips for confocal laser scanning microscopy from H. Saur (Reutlingen, Germany), and the QuantiChrom™ Glutathione Assay Kit was from BioAssay Systems (Hayward, CA, USA).

MDCK II/Par and MDCK II/MRP2 Cells

As an in vitro model for human MRP2 we used MDCK II/MRP2 cells, a cell line generated by stable transfection of MRP2 cDNA into MDCK II cells (Evers et al., 1998). The cell line was kindly provided by Dr. Piet Borst (The Netherlands Cancer Institute, Amsterdam). MDCK II/Par cells served as a control. Both cell lines were cultured in DMEM supplemented with 10% heat inactivated FCS, 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin sulphate.

L-MDRI and LLC-PK1 Cells
The porcine kidney epithelial cell line LLC-PK1 (available at the American Type culture Collection, Manassas, VA, USA) and the L-MDR1 cell line with overexpression of human P-gp (kindly provided by Dr. Alfred H. Schinkel, The Netherlands Cancer Institute, Amsterdam) were also tested for MRP2-activity. The cells were cultured under standard cell culture as described previously (Weiss et al., 2003).

**Confocal Laser Scanning Microscopy: CMFDA Accumulation Assay**

Intracellular accumulation of the MRP2 substrate MF-SG in cells was analyzed with a DM IRE 2 TCS SP II confocal laser scanning microscope from Leica (Bensheim, Germany) using an adapted protocol published by Bogman and coworkers (Bogman et al., 2003). Pilot experiments confirmed, that 15 min incubation with CMFDA are sufficient to achieve maximal loading with this compound. For excitation a 488 nm argon laser line was used and a 500 to 550 nm band-pass filter to detect emission. The objective used was a Leica HCX PL APO CS 63x with a numerical aperture of 1.2. Living cells (6 x 10^5) were seeded on poly-D-lysine and collagen coated coverslips in a closed miniperfusion chamber (H. Saur, Reutlingen, Germany) directly before the experiment and preincubated for 10 min with or without the test compound in darkness at 37 °C in 1 ml transport buffer consisting of HBSS and 1 mM pyruvate for energy supply. Subsequently, CMFDA in a final concentration of 50 nM was added. After incubation for 15 min three series of 20 sections in z-plane through the cells were acquired for each coverslip. The thickness of each optical section was about 0.15 to 0.2 µm. The mean amplitude of fluorescence intensities between cells without (control) and those treated with the specific compound were compared. In each series 30 regions of interest with an area of 350 µm² each were automatically drawn in the image. Fluorescence was quantified with the stack profile function in the quantify modus (Leica software) which calculates the statistical mean average. The experiments were performed at least in triplicate on different days. All progestins were tested in the highest soluble concentration (Fröhlich et al., 2004): norgestimate (50 µM), desogestrel (30 µM), medroxyprogesterone (20 µM), norethisterone...
(20 µM), progesterone (100 µM), cyproterone acetate (20 µM), chlormadinone acetate (50 µM), and levonorgestrel (5 µM).

For compounds, which revealed inhibitory activity on CMF transport by MRP2 (MK 571, progesterone, and norgestimate), the increase in intracellular fluorescence was also recorded over the time of 30 min. For the time kinetics 5 x 10^6 cells/ml transport buffer were preincubated on a coverslip in a closed miniperfusion chamber with 50 nM CMFDA for 15 min to reach a steady-state within the cells. Afterwards MK 571 (20 µM), progesterone (100 µM), or norgestimate (50 µM) was added and time series in xytz-modus over a period of 30 min were recorded with one picture per minute. Time series were also recorded in LLC-PK1 and L-MDR1 cells with and without the MRP2 specific inhibitor MK 571.

**Quenching Test**

The absence of potential errors in the quantification of CMF because of changes in its spectral behaviour in the presence of other compounds was tested in a quenching assay by adding increasing concentrations of individual progestins to aliquots of the cell lysate after incubation with 50 nM CMFDA. Comparison of the fluorescence with control cell lysates without the respective progestin confirmed that none of the progestins showed any quenching effect on the fluorescence of CMF.

**Cytotoxicity Assay**

None of the progestins exerted cytotoxic effects as evaluated with the Cytotoxicity Detection Kit (Roche Applied Science, Mannheim, Germany).

**Glutathione Assay**

CMFDA requires the conjugation with glutathione to generate the fluorescent MRP2 substrate MF-SG. To exclude that differences observed between MDCK II/Par and MDCK II/MRP2 cells can be attributed to differences in the glutathione level in the two cell lines and to ensure that norgestimate and progesterone do not alter glutathione levels, glutathione concentrations were measured in both cell lines before and after incubation with these progestins.
Quantification of glutathione was measured with the QuantiChrom Glutathione Assay Kit (BioAssay Systems, Hayward, CA, USA). In this Assay DTNB (5,5’-dithiobis(2-nitrobenzoic acid)) reacts with reduced glutathione to form a yellow product. The optical density, measured at 412 nm, is directly proportional to the glutathione concentration in the sample. The assay was conducted according to the manufacturer’s instruction with $5 \times 10^4$ cells/100 µl.

Statistical Analysis

P values were calculated by analysis of variance (ANOVA) with Dunnett’s multiple comparison test for post hoc pair wise comparison of the results with the corresponding control (without inhibitor). All statistical analyses were performed with GraphPad InStat, version 3.05, GraphPad Software (San Diego, CA, USA). A p value of $\leq 0.05$ was considered significant.
Results

Confirmation of Active MRP2 in LLC-PK1 Cells

In the CMFDA accumulation assay the specific MRP2 inhibitor MK571 increased intracellular CMF fluorescence in LLC-PK1 (Fig. 1), but not in the P-gp overexpressing cell line L-MDR1 (data not shown).

Inhibition of MRP2 by Progestins

In the MRP2 overexpressing cell line MDCK II/MRP2 of all investigated progestins only norgestimate (50 µM) and progesterone (100 µM) significantly inhibited MRP2 (Fig. 2A). In contrast, neither the positive control MK 571 nor any of the tested progestins increased intracellular CMF fluorescence in the control cell line MDCK II/Par (Fig. 2B).

Glutathione Concentration in MDCK II/Par and MDCK II/MRP2 Cells

Intracellular glutathione concentration did not vary between MDCK II/MRP2 cells and the corresponding parental cell line MDCK II/Par (Fig. 3). Moreover, norgestimate and progesterone had no influence on intracellular glutathione levels.

Concentration Dependent Inhibition of MRP2 by Norgestimate and Progesterone

Inhibition of MRP2 by norgestimate and progesterone was concentration-dependent and reached significance for norgestimate ≥ 25 µM and for progesterone = 100 µM (Fig. 4).

Time-Series for MRP2 Inhibition by MK571, Norgestimate, and Progesterone

In MCDK II/MRP2 cells MK 571, norgestimate, and progesterone induced a time-dependent increase of CMF-fluorescence with plateau effects in the CMFDA accumulation assay (Fig. 5). In contrast, in MDCK II/Par cells there was no difference in the CMF fluorescence between control (without inhibitor) and the compounds tested.
Discussion

Synthetic progestins are used in Germany by 34% of women aged 15-50 as oral contraceptives (OC) (Heinemann et al., 2002) and by 29% of postmenopausal women for hormone replacement therapy (HRT) (Thiel et al., 2001). Gender related differences in drug action are well known and might be caused by modulation of the activity of ABC-transporter systems by sexual steroids as recently shown for P-gp (Fröhlich et al., 2004).

Using the selective and efficient MRP2 inhibitor MK 571 in the CMF accumulation assay we first verified, that MRP2 is indeed active in LLC-PK1 cells (Fig. 1) explaining the effects observed with norgestimate in LLC-PK1 cells in a previous study (Fröhlich et al., 2004).

Interestingly, in the P-gp overexpressing cell line (L-MDR1) MK 571 had no effect suggesting that in this cell line MRP2 is down-regulated as a consequence of P-gp overexpression.

The CMFDA accumulation assay with the MRP2 overexpressing cell line MDCK II/MRP2 and the corresponding parental cell line demonstrated, that of all progestins tested only norgestimate (50 µM) and progesterone (100 µM) significantly inhibited MRP2 (Fig. 2A). Because glutathione levels in the overexpressing and in the control cell line did not differ and because norgestimate and progesterone had no influence on intracellular glutathione levels (Fig. 3), the effects observed in MDCK II/MRP2 cells clearly indicate inhibition of MRP2.

For further characterization of MRP2 inhibition by norgestimate and progesterone, concentration- and time-series were conducted. Fig. 4 demonstrates a concentration-dependent inhibition of MRP2 by both compounds. Due to the limited solubility plateau effects were not reached excluding the calculation of true IC_{50} values. Because effective concentrations for P-gp inhibition are lower (Fröhlich et al., 2004), it can be concluded that the MRP2 inhibitory effects of norgestimate and progesterone are presumably less important than their modulation of P-gp.
For progesterone, norgestimate, and MK 571 we also analyzed the increase of CMF fluorescence in MDCK II/MRP2 cells over a period of 30 min (Fig. 5). MK 571, norgestimate, and progesterone induced a time-dependent increase in intracellular CMF fluorescence confirming MRP2 inhibition. Moreover the data suggest, that 25 min of incubation with an inhibitor is sufficient for maximal inhibition, confirming the data published by Bogman and co-workers (2003), who also measured fluorescence after a total incubation time of 25 min with the inhibitor. The lack of effect in MDCK II/Par cells indicates that the fluorescence increase in MDCK II/MRP2 cells is specific for inhibition of MRP2.

Taken together our data demonstrate significant MRP2 inhibition by norgestimate and progesterone. MRP2 inhibition might also be responsible for the increase in calcein fluorescence in LLC-PK1 cells provoked by norgestimate (Fröhlich et al., 2004). Due to the poor water solubility of the test compounds higher concentrations of the progestins could not be tested. Thus, it cannot be excluded that other progestins besides norgestimate and progesterone might also inhibit MRP2 at higher concentrations.

The low therapeutic plasma concentration of progestins with a range of 5-50 nM (Kuhl, 1996) raises the question of whether circulating concentrations will ever be high enough for a clinically relevant MRP2 inhibition. Obviously, this question can only be answered in an appropriate clinical study. However, after oral administration of progestins the highest concentrations will be reached at the gut wall which expresses both P-gp and MRP2 (Schaub et al., 1997). Nakayama and co-workers have recently shown that progesterone concentrations are indeed sufficient to promote vinblastine absorption most likely through inhibition of intestinal P-gp in duodenum and jejunum (Nakayama et al., 1999). Moreover, as highly lipophilic compounds progestins have a large volume of distribution indicating that their tissue concentration is considerably higher than the corresponding plasma concentrations. For instance, cyproterone acetate studies in rats and humans demonstrated up to 16-fold higher
concentrations of this progestin in all organs investigated when compared to the plasma with the highest concentrations in the liver (Schleicher et al., 1998; Speck et al., 1976). Thus it seems conceivable, that tissue concentrations are high enough for MRP2 modulation. However, whether MRP2 inhibition by progesterone and norgestimate detected in vitro also occurs in vivo and whether this is of relevance for drug interactions can only be addressed in a clinical study.
Acknowledgments

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References


Figure legends:

Figure 1: Time-dependent change of CMF fluorescence in LLC-PK1 cells incubated with 20 µM MK 571 (filled triangles), and without MK 571 (open diamonds) for 30 min. Data are expressed as mean ± SEM of n = 3 experiments. P-values were determined by ANOVA with Dunnett’s multiple comparison test for post hoc comparison of the results with control values at baseline set to 1. For MK 571 all values for t ≥ 3 min were significant, whereas in the absence of inhibitor no change in the relative fluorescence was observed.

Figure 2: Effect of MK 571 (black bar) and the progestins in the highest soluble concentrations (grey bars) on the increase of the intracellular CMF concentration in MDCK II/MRP2 cells (A) and MDCK II/Par cells (B). Data are expressed as mean ± SEM with n = 3-7 experiments. P-values (*, p < 0.05; **, p < 0.01) were determined by ANOVA with Dunnett’s multiple comparison test for post hoc comparison of the results with the known potent inhibitor MK 571.

Figure 3: Determination of glutathione concentrations in MDCK II/Par and MDCK II/MRP2 cells and effect of norgestimate (50 µM) and progesterone (100 µM) on the glutathione level. Data are expressed as mean ± SEM normalized to control without inhibitor, n = 3 experiments with sextuplets. P-values were determined by ANOVA with Dunnett’s multiple comparison test for post hoc comparison of the results with buffer control.

Figure 4: Concentration-dependent effect of norgestimate and progesterone on the intracellular CMF fluorescence in MDCK II/MRP2 cells. Data are expressed as mean ± SEM, n = 3 experiments. P-values (*, p < 0.05; **, p < 0.01) were determined by ANOVA with
Dunnett’s multiple comparison test for post hoc comparison of the results with the control without inhibitor.

**Figure 5:** Time-dependent change of CMF fluorescence in MDCK II/MRP2 cells incubated with 20 µM MK 571 (filled triangles), 50 µM norgestimate (open squares), and with 100 µM progesterone (inverted open triangles) for 30 min. Data are expressed as mean ± SEM normalized to control without inhibitor, n = 3 experiments. P-values were determined by ANOVA with Dunnett’s multiple comparison test for post hoc comparison of the results with control values at baseline set to 1. For MK 571 all values for t ≥ 17 min, for progesterone all values for t ≥ 7 min, and for norgestimate all values for t ≥ 6 min were significant.
Figure 1

Relative fluorescence compared to control at $t=0$

- ◇ Without inhibitor
- ▲ + MK 571

Time after addition of inhibitor [min]
Figure 2

A

- MK 571 [20 μM]
- Norgestimate [50 μM]
- Progesterone [100 μM]
- Desogestrel [30 μM]
- Norethisterone [20 μM]
- Medroxyprogesterone [20 μM]
- Levonorgestrel [5 μM]
- Cyproterone [20 μM]
- Chlormadinone [50 μM]

Increase in intracellular CMF fluorescence (control = 1)

B

- MK 571 [20 μM]
- Norgestimate [50 μM]
- Progesterone [100 μM]
- Desogestrel [30 μM]
- Norethisterone [20 μM]
- Medroxyprogesterone [20 μM]
- Levonorgestrel [5 μM]
- Cyproterone [20 μM]
- Chlormadinone [50 μM]

Increase in intracellular CMF fluorescence (control = 1)
Figure 3

The bar chart shows the glutathione levels in MDCK II/Par and MDCK II/MRP2 cells under various conditions. The x-axis represents different treatments: Control, Progesterone [100 μM], and Norgestimate [50 μM]. The y-axis represents glutathione levels in μM, ranging from 0 to 20 μM. The data indicates a significant increase in glutathione levels in both cell lines after treatment with Progesterone and Norgestimate compared to the Control.
Figure 5

Relative fluorescence compared to control at t=0 and normalized to treatment without inhibitor.

- ▲ + MK 571
- ▼ + Progesterone
- □ + Norgestimate

Time after addition of inhibitor [min]

0  10  20  30