INTERACTION OF PROGESTINS WITH THE HUMAN MULTIDRUG RESISTANCE-ASSOCIATED PROTEIN 2 (MRP2)

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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-chloromethylfluorescein diacetate as a produg 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 sulfate conjugates into the bile (Ishikawa 1993; Ito et al., 1997; Paulusma and Oude Elferink, 1997). It was also detected in renal brush-border membranes, the intestine (Schaub et al., 1993; Ito et al., 1997; Paulusma and Oude Elferink, 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 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 and hormone replacement therapy. 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 calcine-acetoxyethylster (calcine-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 expresses MRP2 (Evers et al., 1996; Decorti et al., 2001) and MRP2 can transport calcine-AM (Evers et al., 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 cystosolic 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 methyfluorescein-sulfoglutathione complex (MF-SG) is then actually 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 was purchased from PAA Laboratories GmbH (Colbe, Germany); fetal calf serum, medium supplements, antibiotics, glutamine, buffers, and cell culture medium M 199 were obtained from Invitrogen (Karlsruhe, Germany); and NaCl, MgSO4 and dimethyl sulfoxide were obtained from AppliChem (Darmstadt, Germany). Collagen-R was obtained from Serva (Heidelberg, Germany). A Cytotoxicity Detection Kit was obtained from Roche Applied Science (Mannheim, Germany). CMFDA was purchased from MoBiTec (Göttingen, Germany), norgestimate was a kind gift from Gynemed (Marburg, Germany), and desogestrel was obtained fromGrünenthal GmbH (Santiago, Chile), and medroxyprogesterone acetate, norethisterone, progesterone, cyproterone acetate, chlormadinone acetate, levonorgestrel, and pyrurate were obtained from Sigma-Aldrich (Taufkirchen, Germany), MK 571 was obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA). Six-well plates were obtained from Biochrom AG (Berlin, Germany), 96-well microtiter plates and culturing bottles were obtained from NUNC GmbH & Co. KG (Wiesbaden, Germany), the coverslips for confocal laser scanning microscopy were obtained from H. Saur (Reutlingen, Germany), and the QuantiChrom Glutathione Assay Kit was obtained from BioAssay Systems (Hayward, CA).

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 Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate.

L-MDR1 and LLC-PK1 Cells. The porcine kidney epithelial cell line LLC-PK1 (available at the American Type Culture Collection, Manassas, VA) 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 MG-S in cells was analyzed with a DMIRE 2 TCS SP II confocal laser scanning microscope from Leica (Wetzlar, Germany) using an adapted protocol published by Bogman et al. (2003). Pilot experiments confirmed that a 15-min incubation with CMFDA is 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 was used to detect emission. The objective used was a Leica HCX PL APO CS 63x with a numerical aperture of 1.2. Living cells (6 × 10^5) were seeded on poly-l-lysine and collagen-coated coverslips in a closed perfusion chamber (B. Sau). Directly before the experiment and preincubated for 10 min with or without the test compound in darkness at 37°C in 1 ml of transport buffer consisting of Hanks’ balanced salt solution and 1 mM pyruvate for energy supply. Subsequently, CMFDA in a final concentration of 50 nM was added. After incubation for 15 min, series of 20 sections in z-plane over the period 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 μm^2 each were automatically drawn in the image. Fluorescence was quantified with the stack profile function in the quantification module (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), chloromadinone acetate (50 μM), and levonorgestrel (5 μM).

For compounds that revealed inhibitory activity on CMF transport by MRP2 (MK 571, progesterone, and norgestimate), the increase in intracellular fluorescence was also recorded over a period of 30 min. For the time kinetics, 5 × 10^5 cells/ml transport buffer were preincubated on a coverslip in a closed perfusion chamber with 50 nM CMFDA for 15 min to reach a steady state within the cells. Afterward, MK 571 (20 μM), progesterone (100 μM), or norgestimate (50 μM) was added, and time series in z-stack mode over the 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 behavior 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 cells 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).

Glutathione Assay. CMFDA requires conjugation with glutathione to generate the fluorescent MRP2 substrate MG-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). In this assay, 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 × 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 Inc., San Diego, CA). A p value of ≤0.05 was considered significant.

Results

Confirmation of Active MRP2 in LLC-PK1 Cells. In the CMFDA accumulation assay, the specific MRP2 inhibitor MK 571 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, 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 MDCK 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 to 50 as oral contraceptives (Heinemann et al., 2002) and by 29% of postmenopausal women for hormone replacement therapy (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 (Frohlich 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 (Frohlich 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. Figure 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 IC50 values. Because effective concentrations for P-gp inhibition are lower (Frohlich 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 et al. (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 calcine fluorescence in LLC-PK1 cells provoked by norgestimate (Frohlich 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 to 50 nM (Kuhl, 1996) raises the question of whether...


