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In vitro and ex vivo evidence for modulation of P-glycoprotein activity by progestins

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Abstract

The well known gender-related differences in drug action may partly be explained by changes in activity and expression of drug metabolising enzymes, but also by modulation of active drug transport systems (e.g. P-glycoprotein, Pgp) by sexual steroids, which is yet not well investigated. Because many women are using hormones (e.g. as oral contraceptives) we investigated the influence of different synthetic progestins on Pgp activity. Pgp inhibition of progesterone, medroxyprogesterone, chlormadinone, cyproterone, levonorgestrel, norethisterone, desogestrel, and norgestimate was measured in vitro in two Pgp over-expressing cell lines (L-MDR1, P388/dx cells) and the corresponding parental cell lines by means of calcein assay, and ex vivo in human peripheral blood mononuclear cells (PBMCs) by rhodamine123 efflux. For most progestins tested, concentrations needed to double baseline fluorescence (*f*2) in L-MDR1 cells were similar to that of the potent Pgp inhibitor quinidine, whereas levonorgestrel and norethisterone did not reach *f*2. The results in P388/dx cells essentially confirmed our findings in L-MDR1 cells. Additionally, Pgp inhibitory activity of all progestins tested was also shown ex vivo in PBMCs. The potent Pgp inhibition by several synthetic progestins in vitro and ex vivo suggests that such an interaction might be clinically relevant despite generally low plasma concentrations of progestins. The results may be of particular importance for Pgp substrates, such as protease inhibitors and chemotherapeutic agents, for which intracellular concentrations are critical.
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P-glycoprotein (Pgp) is an ATP-dependent efflux pump, which plays a key role in regulating absorption, excretion, and tissue distribution of many drugs [1]. It is expressed in a variety of tissues, mainly the gut, kidneys, liver, and capillary endothelial cells of the brain, testis, and placenta [2] which implicates a role for Pgp in the excretion of drugs and endogenous metabolites into urine and bile, as well as a barrier function to certain tissues. Consequently, systemic pharmacokinetics and also tissue concentrations of Pgp substrates may be altered by compounds, which induce or inhibit Pgp.

The pharmacokinetics and pharmacodynamics of several drugs are influenced by gender and many of the differences have been attributed to hepatic drug metabolism e.g. by the cytochrome P450 (CYP) system [3]. In recent years it has been suggested that gender could also alter the expression and/or activity of drug transporters, such as Pgp [4]. However, up to now data on the effect of gender, endogenous sexual hormones, or hormone therapies on Pgp are sparse. One example for the interaction of progestins with Pgp, which might be of potential clinical and therapeutic relevance, is the modulation of multidrug resistance of malignant cells. Multidrug resistance is frequently associated with the over-expression of Pgp in the plasma membrane [5] and is reversed by medroxyprogesterone acetate and megestrol acetate [6,7] in vitro. In this context, it is also conceivable that different sexual steroid levels contribute to the observed

Abbreviations: Calcein-AM, calcein-acetoxymethylester; DMSO, dimethyl sulfoxide; *f*2, concentration needed to double baseline fluorescence; FCS, foetal calf serum; LY335979, zosuquidar; HBSS, Hank's balanced salt solution; HHBSS, with HEPES supplemented HBSS; HRT, hormone replacement therapy; MRP, multidrug resistance associated protein; OC, oral contraceptives; PBMC, peripheral blood mononuclear cells; PE, phycoerythrin; Pgp, P-glycoprotein

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lower Pgp activity in female patients with B-type chronic lymphocytic leukaemia compared to male patients [8] which may contribute to the overall better prognosis of female patients with this disease [9].

Up to now, for only few female sexual steroids modulation of Pgp activity has been assessed [10–12]. Because in Germany 34% of women aged 15–50 years use OCs [13] and 29% of postmenopausal women are HRT [14], we systematically investigated the interaction of the majority of commonly used commercially available progestins with Pgp in vitro and ex vivo.

1. Methods

1.1. Materials and drugs

Culture media, FCS, medium supplements, antibiotics, and HBSS were purchased from Invitrogen, collagen-R was from Serva, DMSO and Triton X-100 were from AppliChem, doxorubicin hydrochloride from Sigma–Aldrich, calcein-AM from MoBiTec, rhodamine123 from Calbiochem, vincristine from Merck Biosciences, 96-well microtiter plates and culturing bottles were from Nunc. The PE labelled antibodies against human $CD8^+$ and mouse IgG2a as well as the vacutainer®CPT™ were purchased from BD Biosciences.

Norgestimate was a kind gift from Janssen-Cilag GmbH, desogestrel from Grünenthal GmbH, and LY335979 from Eli Lilly and Co. Progesterone, medroxyprogesterone acetate, chlormadinone acetate, cyproterone acetate, levonorgestrel, norethisterone, and verapamil were purchased from Sigma–Aldrich, and quinidine from Roth.

1.2. LLC-PK1 and L-MDR1 cells

As a test system for human Pgp we used L-MDR1 cells, a cell line generated by transfection of the porcine kidney epithelial cell line LLC-PK1 with the human MDR1 gene [15], and the parental cell line LLC-PK1 (ATCC) as a control. The L-MDR1 cell line was kindly provided by Dr. A.H. Schinkel. The cells were cultured under standard cell culture conditions as previously described [16] with medium M199 supplemented with 10% heat inactivated FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate. To maintain Pgp expression, the culture medium for L-MDR1 was supplemented with 0.64 μM vincristine. For the calcein assay, cells were seeded on collagen-coated microtiter plates in a density of 10,000 cells/cm^2 and cultured for three days. One day before the assays, both cell lines were fed with vincristine-free culture medium.

1.3. P388 and P388/dx cells

As an alternative model for Pgp, we used the murine monocytic leukaemia cell line P388 and the corresponding doxorubicin-resistant cell line P388/dx over-expressing *mdr1a/1b* [17]. Both cell lines were kindly provided by Dr. Dario Ballinari (Pharmacia and Upjohn). The cells were cultured under standard cell culture conditions (5% CO_2, 95% air at 37 °C) with RPMI 1640 medium supplemented with 10% heat inactivated FCS and 100 μM β-mercaptoethanol. To maintain Pgp expression, the culture medium for P388/dx was supplemented with 20 μM doxorubicin. One day before the assay, both cell lines were fed with doxorubicin-free culture medium.

1.4. Stock solutions

Stock solutions of test compounds were prepared strictly following the manufacturers instructions. All test compounds were dissolved in DMSO. All progestins were tested up to the highest soluble concentration. The DMSO concentration in the assays never exceeded 1% (v/v), a concentration that was found not to influence the results of the assay in pilot experiments.

1.5. Calcein uptake assay and cytotoxicity assay

To determine the Pgp inhibitor potency of the test compounds, the calcein uptake assay was carried out with LLC-PK1 and L-MDR1 cells as described previously [16] with a final calcein-AM concentration of 0.5 μM.

The calcein assay with the P388 and the P388/dx cell lines was also conducted in 96-well plates (with round bottom) in octuplet. Cells were used at a concentration of 5×10^5 cells/well. All incubation steps and cell lysis were conducted at 37 °C on a rotary shaker at 450 rpm. Prior to the uptake assay, the cells were washed with pre-warmed HHBSS and pre-incubated with HHBSS for 30 min. After centrifugation (300 × *g*, 5 min, room temperature) cells were incubated with the test compound or with the controls (HHBSS and verapamil 200 μM) for 15 min. After pre-incubation, calcein-AM was added (final concentration 1 μM) and the cells were incubated for 30 min. The uptake was stopped by centrifugation (300 × *g*, 5 min, 4 °C) and washing the cells twice with HHBSS pre-cooled to 4 °C. Subsequently, cells were lysed in 1% Triton X-100 for 15 min and calcein fluorescence generated within the cells was analysed in a Fluoroskan Ascent fluorometer (Labsystems) with 485 nm excitation and 535 nm emission filters. Each experiment was performed at least in triplicate on different days.

The concentration range used in the calcein assays was also screened for possible cytotoxic effects of the test compounds in each cell line with the Cytotoxicity Detection Kit (Roche Applied Science), because cytotoxic effects may diminish the increase in fluorescence in the calcein assay leading to underestimation of Pgp inhibitory potency. When cytotoxicity was observed (>30% LDH activity), the respective concentration was not included in the evaluation. Moreover, none of the test compounds

quenched the calcein fluorescence. A detailed description of the methods and the validation of the calcein assay have been published previously [16].

1.6. Isolation of human PBMCs

The study was approved by the local ethics committee and the two participants (one female, one male) provided written informed consent for blood donation and were free of any drugs for several months.

For isolation of PBMCs the vacutainer®CPT™ was centrifuged (1700 × *g*, 20 min at room temperature). After centrifugation PBMCs were separated from erythrocytes and granulocytes by the polyester gel in the vacutainer®CPT™ and were resuspended into the plasma by inverting the tube. PBMCs were then washed twice with phosphate-buffered saline (PBS) at room temperature and immediately used for the rhodamine123 efflux assay.

1.7. Rhodamine123 efflux

The rhodamine123 efflux is a widely used method to measure Pgp activity in PBMCs [18–20], although rhodamine123 is not only transported by Pgp, but also by MRPs but with significantly lower transport efficiency [21].

For rhodamine123 efflux, PBMCs were incubated with rhodamine123 at a concentration of 0.4 μM on a rotary shaker (30 min, 37 °C, 450 rpm). Subsequently, cells were washed with pre-cooled RPMI medium (4 °C) and incubated another 50 min at 37 °C in rhodamine123 free medium (with or without Pgp inhibitor) to allow rhodamine123 efflux. After washing the cells with pre-cooled RPMI (4 °C), they were stained with PE-labelled $CD8^+$ antibody or PE-labelled mouse IgG2a as a negative control (15 min on ice in darkness).

For analysis a Becton Dickinson FACS Calibur with a 488-nm argon laser was used. Rhodamine123 fluorescence was measured using a 530 bandpass filter and PE fluorescence with a 585 bandpass filter. In each sample 10,000 PBMCs were counted.

Median rhodamine123 fluorescence was determined in gated lymphocytes and $CD8^+$ cells after the efflux period. The inhibitory effect of the test compounds was then calculated as ratio of the median fluorescence of the cells incubated with and without the compound. As positive control, the specific Pgp inhibitor LY335979 [22] was used.

We did not measure the efflux in the often used $CD56^+$ cells [18–20] but in $CD8^+$ cells, because this lymphocyte subpopulation exhibits distinct advantages: while both $CD8^+$ and $CD56^+$ cells represent leukocyte subpopulations with high Pgp expression [23], the content of $CD8^+$ cells in the PBMC fraction exceeds that of the $CD56^+$ cells, and pilot experiments have demonstrated that $CD8^+$ can be gated more unequivocally in the flow cytometer than $CD56^+$ cells. Moreover, it is controversial, whether $CD56^+$ cells express full-length Pgp or a splice variant termed "mini-Pgp" with a more restricted substrate profile [24].

Each test compound was also screened for possible cytotoxic effects in PBMCs in the concentration applied in the rhodamine123 efflux assay with the Cytotoxicity Detection Kit (Roche Applied Science, Mannheim, Germany). None of the test compounds quenched the rhodamine123 fluorescence.

1.8. Statistical analysis

Due to the limited solubility, for many of the compounds tested, no plateau effects could be reached in the calcein assay. Therefore, for calculation of the inhibitor effects, the *f*2 value was determined as described previously [16]. *p*-values were determined by ANOVA with Dunnett's multiple comparison test for post hoc pairwise comparison with the control results obtained with verapamil. A *p*-value <0.05 was considered statistically significant.

2. Results

2.1. Evaluation of the Pgp inhibitory potency of progestins with the calcein assay

Due to the low solubility of the progestins and the very high expression level of Pgp in L-MDR1 cells, none of the progestins revealed plateau effects in L-MDR1 cells and levonorgestrel and norethisterone even did not reach *f*2 values (Table 1; Fig. 1A–C). We therefore also tested all compounds in P388/dx cells, a murine cell line overexpressing Pgp as well, but to a lower extent than L-MDR1 cells. In this cell line some progestins showed plateau effects and only levonorgestrel did not reach *f*2 (Table 1; Fig. 2A–C).

Several progestins (progesterone, medroxyprogesterone acetate, chlormadinone acetate, cyproterone acetate, desogestrel) revealed Pgp inhibition of comparable potency to that of quinidine in L-MDR1 cells (Table 1; Fig. 1). For medroxyprogesterone acetate and chlormadinone acetate these results were confirmed in P388/dx cells (Table 1; Fig. 2). Only, for progesterone, the *f*2 value was one order of magnitude higher than that of quinidine in P388/dx cells.

Cyproterone acetate and desogestrel also significantly increased calcein accumulation in P388/dx cells (Fig. 2B and C), but due to cytotoxic effects, *f*2 value could not be calculated reliably.

Whereas, in L-MDR1 cells norethisterone did not reach *f*2, in P388/dx cells this progestin showed similar potency to quinidine (Table 1; Figs. 1 and 2). Levonorgestrel had the lowest solubility of all progestins tested and could only be tested up to 5 μM. At this concentration, it had only a minor effect on calcein accumulation in L-MDR1 and P388/dx cells.

Table 1
Inhibition of Pgp by progestins and typical Pgp inhibitors in Pgp over-expressing cells

Test compound	L-MDR1		P388/dx	
	n	*f*2 (μM)	*n*	*f*2 (μM)
Verapamil	3	4.7 ± 0.8	3	0.6 ± 0.2
Quinidine	5	13.2 ± 3.8*	4	2.4 ± 0.9
Medroxyprogesterone acetate	3	9.3 ± 2.5	3	1.6 ± 0.6
Progesterone	4	13.3 ± 3.2*	4	30.2 ± 9.8**
Cyproterone acetate	4	15.0 ± 5.1*	4	N.D.[b]
Desogestrel	3	16.8 ± 0.5**	3	N.D.[b]
Chlormadinone acetate	4	20.2 ± 6.2**	3	3.0 ± 1.24
Levonorgestrel	4	N.D.	3	N.D.
Norethisterone	6	N.D.	3	5.2 ± 2.3[b]
Norgestimate	3	N.D.[a]	3	4.2 ± 0.7**

Values represent mean ± S.D. of at least three independent assays. *p* values are determined by ANOVA with Dunnett's multiple comparison test for post hoc pairwise comparison of the results with the verapamil control. N.D., not definable; *n*, number of experiments, each performed in octuplet. The *f*2 values for verapamil and quinidine in L-MDR1 cells have already been published previously [45,46].

[a] Correct calculation of the *f*2 value for norgestimate in L-MDR1 cells was not possible due to the effect also seen in the control cell line LLC-PK1 (see also Fig. 3A).

[b] Due to cytotoxic effects *f*2 value cannot be calculated reliably and likely to underestimate the potency of the compound.

* $p < 0.05$.

** $p < 0.01$.

Progesterone, medroxyprogesterone acetate, chlormadinone acetate, cyproterone acetate, norethisterone, desogestrel, and levonorgestrel had no effect on the calcein accumulation in the control cells lines LLC-PK1 and P388.

In contrast to the other progestins, norgestimate inhibition of calcein-AM efflux was present in both, the Pgp over-expressing L-MDR1 cells and the LLC-PK1 control cells (Fig. 3A). This indicates involvement of an additional transporter protein or unspecific effects of norgestimate in both cells lines. In contrast norgestimate only increased calcein fluorescence in the Pgp over-expressing P388/dx and not in the control cell line P388 (Fig. 3B). The *f*2 value in P388/dx was similar to that of quinidine (Table 1) indicating a potent Pgp inhibition by norgestimate as well.

2.2. Inhibition of Pgp in human PBMCs ex vivo

The results obtained with the calcein assay were confirmed with an independent ex vivo assay for Pgp activity. Inhibition of rhodamine123 efflux was measured in lymphocytes and the subpopulation of $CD8^+$ cells of two individuals (one male and one female) at a concentration of 40 μM (except levonorgestrel, which is not soluble above 10 μM). The specific Pgp inhibitor LY335979 (1 μM) was used as a positive control for Pgp inhibition. One representative FACS histogram overlay is shown in Fig. 4. All progestins inhibited rhodamine123 efflux similarly in lymphocytes and $CD8^+$ cells (Fig. 5) confirming Pgp inhibitory activity in native human cells. The results

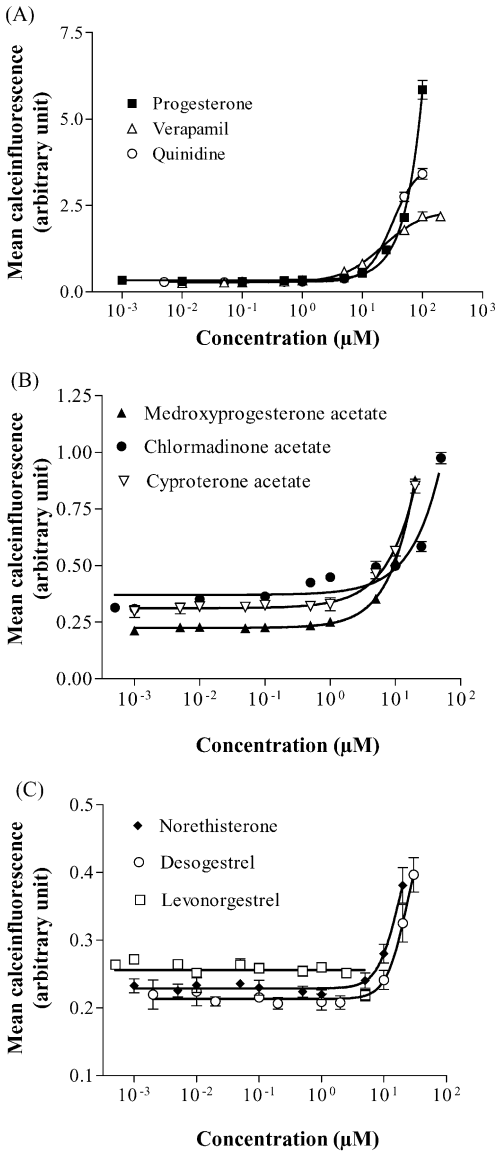

Fig. 1. Calcein assay. Concentration-dependent effect of progestins (A–C) and control compounds (A) on the calcein accumulation in L-MDR1 cells over-expressing human Pgp. Each curve depicts one representative experiment of a series of three to five. Data are expressed as mean ± S.D. for $n = 8$ wells.

(% Pgp inhibition compared to control) were similar in the cells of the female and the male individual tested. In this assay, norethisterone and progesterone revealed the weakest Pgp inhibitory activity. Only desogestrel revealed slight cytotoxic effects (31% LDH activity) in PBMCs presumably leading to underestimation of the Pgp inhibitory effect.

3. Discussion

The results of the present in vitro/ex vivo study revealed a potent Pgp inhibitor effect of several progestins, which are widely used for OC and HRT. The extent of Pgp inhibition in L-MDR1 cells by most progestins was com-

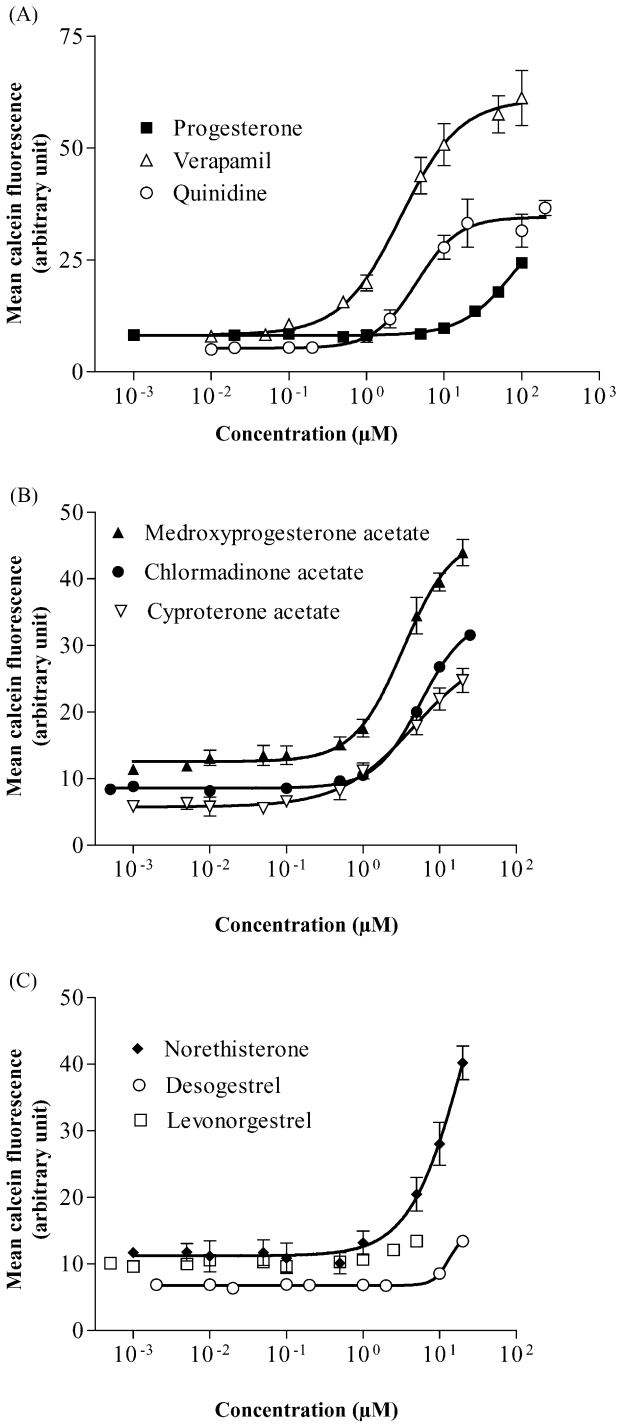

Fig. 2. Calcein assay. Concentration-dependent effect of progestins (A–C) and control compounds (A) on the calcein accumulation in P388/dx cells expressing murine *mdr1a/mdr1b*. Each curve depicts one representative experiment of a series of three to five. Data are expressed as mean ± S.D. for $n = 8$ wells.

parable to that of the potent Pgp inhibitor quinidine and slightly weaker than that of verapamil. The rank order of inhibition was essentially confirmed in P388/dx cells with one notable exception. In contrast to other progestins whose *f*2 values were roughly one order of magnitude lower than in L-MDR1 cells progesterone exhibited

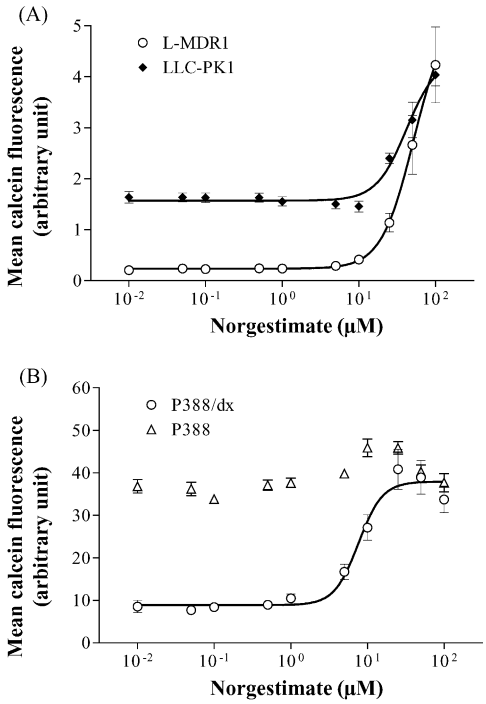

Fig. 3. Calcein assay. Concentration-dependent effect of norgestimate on the calcein accumulation in L-MDR1 and LLC-PK1 cells (A) and P388 and P388/dx cells (B). Each curve depicts one representative experiment of a series. Data are expressed as mean ± S.D. for $n = 8$ wells.

weaker inhibitory activity in P388/dx cells than in L-MDR1 cells.

The finding of lower *f*2 values in P388/dx cells, compared to L-MDR1 cells is expected and concurs with the lower Pgp expression in the former cell line and might also be attributed to species differences [25].

The ex vivo assay with human PBMCs confirmed Pgp inhibitory activity of all progestins tested, even for the poorly soluble levonorgestrel. The results in lymphocytes and in the subpopulation of $CD8^+$ cells alone were similar, indicating the suitability of both PBMCs subpopulations for investigating Pgp inhibition.

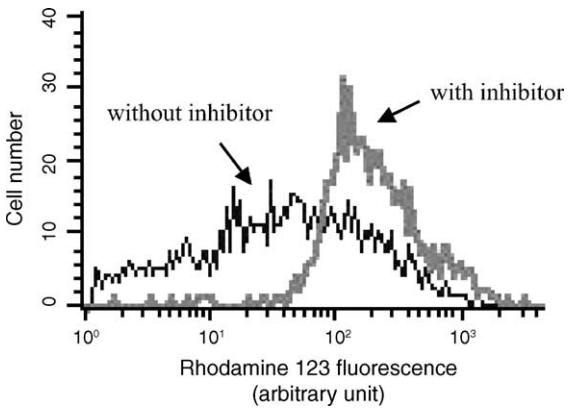

Fig. 4. Representative example for rhodamine123 efflux in $CD8^+$ cells. Overlay of the histograms of the gated $CD8^+$ cells with and without the specific Pgp inhibitor LY335979.

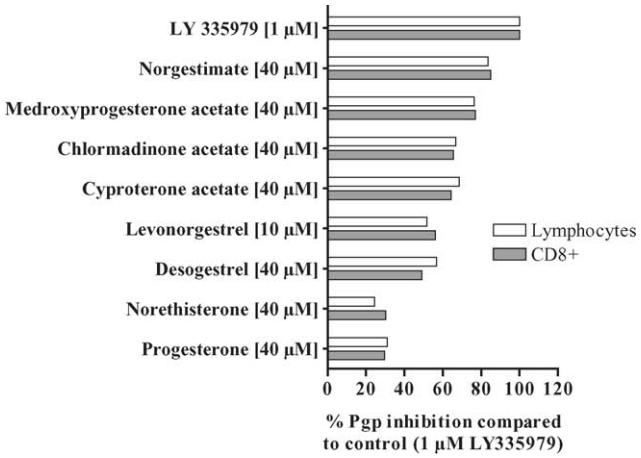

Fig. 5. Pgp inhibition of progestins in human lymphocytes and $CD8^+$ cells of one (female) individual measured as percent inhibition compared to control (1 μM of the specific Pgp inhibitor LY335979). Pgp inhibition was evaluated be measuring the increase in rhodamine123 fluorescence compared to cells without inhibitor during the efflux period of 50 min. The results obtained with the other (male) participant were similar.

Pgp inhibition of progestins seems to be a class effect, because all investigated compounds inhibited Pgp at comparable concentrations. Norethisterone and levonorgestrel could only be tested in concentrations up to 20 and 5 μM, respectively. Thus, no *f*2 values could be calculated for these two compounds in L-MDR1 cells and only for norethisterone in P388/dx cells. In general, the calcein assay with progestins was methodologically limited by the poor water solubility of the test substances. Thus, high concentrations of progestins, which are necessary to reach plateau effects, could not be tested, at least not in L-MDR1 cells with very high Pgp expression. However, our results are in agreement with the previous finding that Pgp inhibition by steroid hormones strongly depends on a relative hydrophobicity of the substances [11,12].

Whereas the calcein accumulation only in P388/dx and not in P388 cells demonstrates specific Pgp inhibitory potency of norgestimate, its effect seen in the parental cell line LLC-PK1 (without human Pgp) indicates that the increase in calcein fluorescence in L-MDR1 cells can not fully be explained by the exclusive interaction with human Pgp. In contrast to LLC-PK1 and L-MDR1 cells, there is no evidence for expression of MRPs in P388 and P388/dx cells [26], suggesting that MRPs might be responsible for the effect seen in LLC-PK1 cells. But it is also conceivable that another transporter or unspecific effects are involved. However, the breast cancer resistance protein (BCRP, *ABCG2*) cannot be responsible for this phenomenon, because it does not transport calcein-AM [27].

Pgp inhibition of progesterone has previously been demonstrated in different Pgp over-expressing cells, using [H^3]vinblastine accumulation [10,11], rhodamine123 accumulation [12], and flow cytometry with daunorubicin as a fluorescent marker [28]. In one study Pgp inhibition by medroxyprogesterone acetate and megestrol acetate was reported [11]. None of these studies used the calcein-AM uptake assay or the rhodamine123 ex vivo assay to quantify Pgp activity and modulation of Pgp activity by other synthetic progestins has not been reported thus far. The present study revealed a significant Pgp inhibition (*f*2) by all progestins tested except levonorgestrel, which could only be tested up to low micromolar concentrations and thus may have been underdosed.

Pharmacokinetic drug–drug interactions with a suspected involvement of Pgp are well known. Established examples are the interactions of verapamil or quinidine with digoxin [29,30]. Regular doses of verapamil increase plasma concentrations of digoxin by 60–90% [31], and digoxin plasma concentrations are roughly doubled by 600–1200 mg/d quinidine [32]. Compared to the plasma concentrations of verapamil (440–880 nM) and quinidine (3–18 μM), therapeutic plasma concentrations of progestins are much lower (5–50 nM) [33], which raises the question of whether progestin concentrations obtained in vivo are sufficient for a clinically relevant Pgp inhibition. However, due to their highly lipophilic character, tissue concentration might be substantially higher thus explaining the large volume of distribution e.g. of cyproterone, which accumulates in tissues, especially in fat [34]. On the other hand, following oral application, one may presume that progestin concentrations at the gut wall are considerably higher than subsequent plasma concentrations. Thus, it is possible that Pgp inhibition by progestins also takes place at the site of absorption. This is supported by recent evidence from a study in rats, in which progesterone at concentrations of 20 μM enhanced vinblastine absorption from the duodenum and jejunum most likely by suppression of Pgp function [35]. If this concept holds true, then the interaction profile of exogenous progestins might differ from endogenous sexual steroids.

In addition to the potential clinical and therapeutic implications of the Pgp inhibitory activity of progestins in anticancer therapy [6,7] Pgp modulation by progestins could also reach clinical relevance in other drug therapies. For instance, failure of antiretroviral therapy in HIV infection may be due to increased Pgp expression [36], which limits the intracellular availability of protease inhibitors [37,38] as substrates of Pgp. Indeed, in vitro drug resistance to protease inhibitors was reversed by Pgp inhibition in leukocytes [39] in the same manner as in anticancer therapy. This concept is further supported by the finding that in HIV infected postmenopausal women HRT is correlated with a better prognosis [40]. However, a recent study in healthy women revealed no influence of an oral contraceptive (0.03 mg ethinylestradiol/0.075 mg gestodene) on the single dose pharmacokinetics of oral saquinavir [41], suggesting that drug absorption and metabolism were unchanged. It was, however, not suited to assess drug distribution which may be the pharmacokinetic process primarily influenced by alterations of Pgp activity. Another example for the potential influence of sexual

steroids on drug disposition is the recently published post hoc subgroup analysis of the Digitalis Investigation Group trial [42], in which digoxin therapy resulted in a statistically significant decrease in death from heart failure and hospitalisation for worsening heart failure in men, but not in women. Moreover, women randomly assigned to digoxin had a 4.2% higher rate of death from any cause than women assigned to placebo. In this context, it was speculated that the observed gender-related differences might result from pharmacokinetic interactions of progestins with the Pgp substrate digoxin, ultimately leading to higher plasma and tissue digoxin concentrations in women.

Although, gender disparity in plasma pharmacokinetics has been identified for numerous drugs, differences are generally only subtle [43] and the majority of pharmacokinetic drug–drug interactions with OC or HRT usually result in increases in plasma AUC between 20–100% [44], which is regarded to be of clinical significance only in particular cases. One possible explanation for this discrepancy may be different distribution kinetics in women resulting in altered drug tissue concentrations. This process, in which drug transporters are causally involved, has not been studied thoroughly in vivo thus far.

In conclusion, this study revealed that a majority of clinically used progestins inhibit Pgp activity in vitro and ex vivo. It is now to be evaluated whether progestins at therapeutic doses or in physiological plasma concentrations may act as modulators of Pgp activity and thus, drug distribution to intracellular target structures.

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