P-glycoprotein (P-gp/*MDR1*)-Mediated Efflux of Sex-Steroid Hormones and Modulation of P-gp Expression *In Vitro*

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Purpose. To determine whether female sex-steroid hormones and their metabolites can modulate P-glycoprotein (P-gp) expression and catalytic activity and to investigate P-gp mediated transport of these sex-steroids across *MDR1*-transfected Madine-Darby canine kidney (MDCK) cells.

Methods. Changes in P-gp protein and *MDR1* mRNA expression levels were examined in the presence of various estrogens and progestins after a 72-h induction period in the LS180 human colon carcinoma cell line via Western blotting and semiquantitative Reverse-transcription-polymerase chain reaction (RT-PCR), respectively. Concentration-dependent stimulation of vanadate-sensitive P-gp ATPase activity was measured in membranes of Sf9 insect cells infected with a recombinant baculovirus containing the human *MDR1* cDNA used with appropriate control membranes. MDCK and *MDR1*-transfected MDCK cell lines were then used to measure bi-directional P-gp transport of various steroids in the presence and absence of the P-gp inhibitor, GG918. Samples obtained were quantified using LC/MS.

Results. Our findings show that P-gp protein levels are inducible by estrone (4-fold over control), estriol (2-fold), and ethynyl estradiol (3-fold). *MDR1* mRNA expression levels were also inducible in a concentration-dependent manner from 25 nM to 10 μM. Bidirectional transport studies indicate that ethynyl estradiol, estrone, and estriol are all substrates for P-gp with respective efflux ratios of 10.3, 6.9, and 2.8. Norethindrone was not found to be a substrate for P-gp. Ethynyl estradiol and progesterone were able to significantly stimulate P-gp ATPase activity in a concentration-dependent manner.

Conclusions. Our studies indicate that several sex-steroid hormones are substrates for P-gp–mediated transport and are also able to induce P-gp expression at both the protein and mRNA level *in vitro*. Stimulation of P-gp ATPase catalytic activity by steroid hormones was also observed, suggesting physical interactions and identifying a need for further investigations to understand the *in vivo* effects of endogenous and synthetic steroid hormones on the expression and function of P-gp.

KEY WORDS: induction; LS180; MDCK; *MDR1*; *MDR1*-MDCK; P-glycoprotein; sex-steroid hormones; transport.

INTRODUCTION

The multidrug transporter P-glycoprotein (P-gp), the product of the *MDR1* (multi-drug resistance) gene, is a 170-kDa transmembrane efflux pump for various cytotoxic drugs, including different hydrophobic substrates (1,2). Overexpression of P-gp, a member of the ATP-binding cassette (ABC) transporter superfamily, is one of the major mechanisms by which cells confer multidrug resistance during cancer and AIDS chemotherapy. Expressed on the apical surfaces of epithelial cells in major drug eliminating organs in the body, P-gp is responsible for secreting passively diffused drug out of the cell (3). It uses energy gained from ATP hydrolysis to transport an assortment of structurally unrelated compounds out of cells.

In a study investigating the absorption of steroid hormones in rat intestine, progesterone was found to decrease intestinal absorption of vinblastine, a P-gp substrate (4). We postulate that progesterone may be inducing P-gp activity in the rat intestine and changes in hormone levels may affect the pharmacokinetics of other P-gp substrate drugs. P-glycoprotein is also highly expressed in the adrenal gland (5–7), and earlier studies have investigated its physiological function, suggesting that adrenal P-gp could play a role in the secretion of steroid hormones (8). Interestingly, several adrenal steroids such as cortisol, aldosterone, and dexamethasone were found to be actively transported by P-gp (9–11), whereas progesterone, although not a P-gp substrate, was found to be a potent P-gp inhibitor, together with several of its natural metabolites (12). In fact, numerous studies have shown progesterone to block P-gp mediated efflux of other steroids and P-gp substrate drugs, capable of reversing drug resistance in P-gp expressing cells (13,14). Although estradiol (E_2) has been discovered not to be transported by P-gp (15), estradiol and diethylstilbestrol (DES) were able to induce P-gp and decrease accumulation of intracellular adriamycin, causing drug resistance (16). In a more recent study, estradiol was found to increase cytoplasmic concentrations of P-gp in ERα-positive MCF7 breast carcinoma cells, exhibiting resistance to doxorubicin cytotoxicity, whereas T47D breast cancer cells lacking ERα were sensitive to doxorubicin (17).

Though the physiological interaction between steroids and P-gp is poorly understood, efforts have been made to probe this relationship. Altuvia *et al.* (18) evaluated the regulation of P-gp expression and function in rodent adrenal cells, where mdr1b expression was enhanced by steroid hormones via a feedback regulatory mechanism and inhibition of steroid biosynthesis markedly decreased mdr1b mRNA levels. Furthermore, disruption of the mdr1b allele resulted in greater intracellular accumulation of vinblastine and daunomycin. To further evaluate this specific interaction between female steroid hormones and P-gp, we investigated the transport of various estrogens and progestins by P-gp utilizing the *MDR1*-transfected Madine-Darby canine kidney (MDCK) cell model system.

The most commonly occurring mammalian steroids are all related to or derived from cholesterol. In women, the predominant circulating sex-steroids are estrogen and progesterone, produced primarily in the ovaries and placenta (19). The major estrogens produced by women are estradiol (estradiol-17β, E_2), estrone (E_1), and estriol (E_3). Estradiol is converted to estrone, estriol, their 2-hydroxylated derivatives and conjugated metabolites by the liver and subsequently excreted in the bile. These compounds control a myriad of biochemical reactions, acting as chemical messengers to induce or repress enzyme regulation and protein synthesis. Their mechanism of action is defined by passive intracellular diffusion, binding to intracellular receptors and as dimer complexes, initiating or repressing transcription via interaction with specific nucleo-

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tide sequences called hormone response elements (HRE) present in target genes (20). These endogenous hormones have significant developmental effects, neuroendocrine control of the menstrual cycle, and major actions on mineral, carbohydrate, protein and lipid metabolism (19).

Hormonal effects on the pharmacokinetics of P-gp substrate drugs have never been evaluated, yet a previous study demonstrated variable P-gp protein and mRNA levels in human endometrium dependent on the menstrual cycle (21). As orally administered synthetic derivatives of estrogens and progestins are among the most widely prescribed drugs for women, the studies here aim to investigate the effects of both natural and synthetic derivatives of estradiol and progesterone on P-gp expression *in vitro*. Additionally, we sought to determine whether these sex-steroid hormones and their metabolites are substrates of P-gp so as to identify potential interactions between hormones and P-gp substrate drugs.

MATERIALS AND METHODS

Materials

17 β-Estradiol, ethynyl estradiol (EE), estrone, estriol, progesterone, norethindrone, norgestrel, and 6-α methylhydroxy progesterone acetate (6α-MPA) were purchased from Sigma Chemical Company (St. Louis, MO, USA). GG918 (GF120918) was a kind donation from Glaxo-Wellcome. The *MDR1*-MDCK and MDCK cell lines were kindly provided by Dr. Ira Pastan (National Cancer Institute, Bethesda, MD, USA). The human colon carcinoma cell line, LS180, was purchased through the UCSF Cell Culture Facility (CCF; San Francisco, CA, USA) from American Type Culture Collection (ATCC; Rockville, MD, USA). Dulbecco's modified Eagle's medium (DME-H21) was obtained from UCSF CCF. RPMI 1640 (without phenol red) was custom-ordered through CCF and supplemented with the following: 10% fetal bovine serum (HyClone Laboratories), 100 U/ml penicillin, 100 μg/ml streptomycin, and 1% glutamine. Falcon polyethlylene terephthalate (PET) cell culture inserts (Becton Dickinson) and their companion Costar 6-well plates (Costar Corp.) were purchased from Fisher Scientific (Santa Clara, CA, USA). Acetonitrile (ACN) was obtained from Fisher Scientific. The P-gp antibody, C219, was obtained from Signet (Dedham, MA, USA). The 18s internal standard and *MDR1* primers were purchased from Ambion, Inc (Austin, TX, USA) and BD Gentest (Woburn, MA, USA), respectively. SuperScript OneStep RT-PCR with Platinum TAQ was purchased from UCSF CCF. Human P-gp and control membranes for ATPase activity assay were obtained from Gentest.

RT-PCR

LS180 cells were grown on either 75-cm^2 flasks or the Transwell insert-plate unit. Total RNA was isolated using TRIZOL (Gibco-BRL, Carlsbad, CA, USA), a monophasic solution of phenol and guanidine isothiocyanate. The concentration and purity of isolated RNA samples were measured using an ultraviolet spectrophotometer, confirming $A_{260/280}$ ratio values between 1.6 and 1.8. Samples were diluted into 0.5 μg/μl aliquots and frozen at –80°C. Superscript One-Step with Platinum *Taq* (Gibco-BRL) was used to detect RNA by RT-PCR, in which both cDNA synthesis and PCR was performed in a single tube. The PCR reaction encompassed 33 cycles at an annealing temperature of 57°C for 30 s.

The following gene specific, intron-spanning primer was used for *MDR1*: sense (5′- GCCTGGCAGCTGGAAGACAAATACACAAAAT -3′; bases 834-864) and anti-sense (5′- AGACAGCAGCTGACAGTCCAAGAACAGGACT -3′; bases 1088-1118) with an amplified product length of 284 bp. Human liver total RNA was used as a positive control. Integrity of RNA was confirmed by usage of an 18S ribosomal internal standard (Ambion, Austin, TX, USA) and equal volumes of each sample were run on a 2% EtBr gel. For best quantitative analysis, the linear range of PCR and optimal 18S Primer: Competimer ratio (3:7) were determined. Images were captured electronically and bands quantified using NIH Scion image analysis software.

Western Blot Analysis

Cell Lysis

LS180 colon cells were cultured on six-well inserts or 75-cm^2 flasks and harvested when confluent (~4 to 5 days). Cells were washed in ice-cold PBS without Ca^{++}/Mg^{++}, scraped and centrifuged at 4000 × *g* for 10 min (4°C). The pellet was washed again with PBS and resuspended in lysis buffer containing 1 μg/ml pepstatin, 20 μg/ml leupeptin, 20 μg/ml aprotinin, 1 mM PMSF, and a protease inhibitor cocktail in hypotonic buffer (Tris-HCl, KCl, $MgCl_2$). Samples were sonicated on ice (15 s ×2) and stored at –80°C.

Gel Electrophoresis and Probing

Protein (100 μg) was size-fractionated on a BioRad Tris-HCl gradient polyacrylamide gel (7–20%) and transferred to nitrocellulose. The membrane was blocked with 5% nonfat milk (in TBS), probed with P-gp antibody, C219 (1:100), and subsequently incubated with secondary anti-mouse (1:15,000) labeled with horseradish peroxidase, each for 1 h at RT. P-gp protein was detected with the enhanced chemiluminescence system (ECL, Amersham Biosciences, Piscataway, NJ, USA). Band density was scanned and quantified using the same method described above for RT-PCR.

Cell Culture

MDR1-MDCK and MDCK cells were grown in DME-H21 on Falcon cell culture inserts placed into 6-well plates at a seeding density of ~300,000 cells/insert. Colchicine (80 ng/ml) was added to *MDR1*-MDCK cells to select for P-gp expressing cells. LS180 cells were grown in RPMI 1640 phenol-red free media under the same conditions described for MDCK cells or in 75-cm^2 flasks at a density of 1E6. As the RPMI 1640 phenol-red free media was light sensitive, all feeding, induction, and experimental conditions were performed with minimal light exposure. Cells were grown to confluence for 3-6 days at 37°C in 5% CO_2 humidity. For induction experiments, LS180 cells were fed 24 h post-seeding and then induced with drug-containing media replacement every 24 h, 3 days before confluence. Prior to a transport experiment, transepithelial electrical resistance (TEER) was measured in

each well to ensure cellular integrity and confluence. Approximate MDCK TEER values were 150–300 Ω-cm^2, whereas *MDR1*-MDCK values ranged from 900 to 1900 Ω-cm^2.

Transport Experiments

Bidirectional transport across *MDR1*-MDCK and MDCK cell monolayers experiments were conducted as originally described (22) with minor adjustments. After TEER measurements, cells on inserts and plates were washed with Hank's Balanced Salt Solution (HBSS-FH) containing 1% FBS and 22.5 mM HEPES (pH 7.4) and then incubated in a 37°C shaker for 30 min. To measure P-gp mediated transport of selected sex-steroid hormones (B→A), hormone-containing HBSS-FH was added to the basolateral (B) chamber, whereas HBSS-FH alone was added to the apical (A) chamber of the Transwell insert-plate unit. At the same time, (A→B) was measured via hormone dosing on the (A) side and time point sampling on the (B) side. Each measurement was evaluated in triplicate at up to 3 separate occasions. Cells were incubated in a 37°C shaker, and 150 μl aliquots were taken from the receiver side at 0.5, 1, and 2 h time points. Throughout the experiment, the final volume was maintained at 1.5 ml on the (A) side and 2.5 ml on the (B) side via addition of 150 μl replacement fresh media after aliquot samples were taken. Inhibition of P-gp transport activity was obtained by addition of 1 μM GG918 in the HBSS-FH media (+/− hormone) to both chambers. To measure intracellular concentrations, cell inserts were removed and washed by dipping twice into 3 separate solutions of PBS. Membrane inserts were then dried, cut out with a needle and placed into scintillation vials containing 1 ml of ACN:H_20 (70:30, v/v). Vials were sonicated in a water bath for 10 min. and centrifuged for 10 min at 12,000 × *g*. The supernatant was then analyzed via Liquid chromatography/mass spectrometry (LC/MS).

Transport Calculations

Apparent permeability values (P_{app}) were determined as follows:

$$P_{app} = (\Delta Q/\Delta t) \div (C_0 \times A)$$

where ΔQ is the linear accumulation of drug concentration in the receiver (basolateral) chamber over time, C_0 is the initial concentration of the test compounds, and A is the surface area of the filter (4.2 cm^2). The net efflux of the compound was calculated by taking the ratio of the P_{app} (B→A) over the P_{app} (A→B) values. A minimum net efflux ratio of 2.0 was used as the threshold value to identify P-gp mediated transport.

LC/MS Analysis

All test and hormone compounds were detected and quantified via electrospray ionization interface (ESI) using a Hewlett Packard Series 1100 LC/LC-MSD (Houston, TX, USA) equipped with an on-line six-port column switching extraction step. The first HPLC system comprises an autosampler with a quarternary pump (G1311A), whereas the second consists of a selective mass detector (G1946A) and binary pump (G1312). In brief, 50 μl of sample was injected into a MOS Hypersil extraction precolumn (Hewlett Packard). After 2 min, samples were backflushed and separation was achieved using a Prodigy 5u ODS column [50 × 2 mm] via activation of the switching valve. The mobile phase consisted of 2 mM ammonium acetate and 9:1 acetonitrile:H_20. A flow rate of 2 ml/min was used. Using selected ion monitoring (SIM), steroid estrogen ions $(M-H)^-$ were detected in negative polarity (NI), whereas progestins were measured in positive ion mode (PI).

P-gp ATPase Activity Assay

Stimulation of P-gp ATPase catalytic activity was measured in membranes of Sf9 insect cells infected with a recombinant baculovirus containing *MDR1* cDNA (Gentest). The P-gp ATPase assay method used was a modification of the original assay described by Sarkadi *et al.* (23) and as directed in the Gentest product insert. Control and P-gp membranes were separately incubated at 37°C for 20 min with positive control (20 μM verapamil) or varying concentrations of test hormone-drug, 3 mM MgATP, and buffer solution (50 mM Tris-Mes, 2mM EGTA, 50 mM KCl, 2 mM dithiothreitol, and 5 mM sodium azide). A duplicate reaction mixture also containing a P-gp ATPase inhibitor, sodium orthovanadate (100 μM) was assayed in parallel as a negative control. ATPase activity measured in the presence of sodium orthovanadate (non P-gp ATPase activity) was subtracted from the activity produced without vanadate to determine vanadate-sensitive ATPase activity. Addition of 10% SDS was used to terminate the reaction. Reaction mixtures were then incubated for 20 min in a zinc acetate (15 mM) solution containing ammonium molybdate (35 mM) to measure levels of inorganic phosphates released by its absorbance at 800 nm. A phosphate standard curve was used to compare and quantify absorbance measurements.

RESULTS

Induction of Endogenous *MDR1* mRNA by Hormones in LS180

To investigate the effect of steroid hormones on *MDR1* expression, the LS180 human colon cell line was used as an *in vitro* model. Rifampin, a known inducer of intestinal P-glycoprotein *in vivo* (24) and *in vitro* via the PXR response element, was used as a positive control (25). LS180 cells were incubated with various estrogens and progestins for 48 or 72 h prior to harvesting and isolation of total RNA. Maximal induction was observed at 72 h indicating time-dependent induction. Using RT-PCR, endogenous *MDR1* mRNA expression levels as shown in Fig. 1 were inducible 10.7-fold (over control) by 5 μM ethynyl estradiol, and 11.0-fold by 5 μM estriol. Both estrone and progesterone, at 5 μM, similarly induced *MDR1* approximately 2-fold, whereas 17 β-estradiol showed no significant induction over 18s control. However, at 50 μM, estrone and estriol demonstrated significant *MDR1* induction (7.2- and 6.3-fold, respectively) on *par* with ethynyl estradiol (9-fold). Progesterone also showed a smaller, but significant increase in *MDR1* induction at the 50 μM level (3.2-fold), whereas only a slight induction was observed with 17 β-estradiol (Fig. 1). LS180 cells were treated with increasing concentrations of four steroid hormones ranging from 25 nM to 10 μM. A concentration-dependent induction of *MDR1* mRNA was observed with D-norgestrel (Fig. 2, bot-

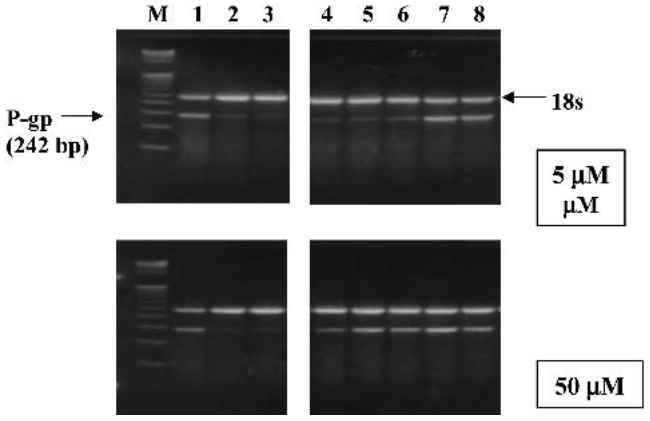

Fig. 1. Amplification of *MDR1* from untreated control or hormone-treated LS180 cells using RT-PCR. First-strand cDNA was synthesized from total RNA. LS180 cells were treated with either 5 μM of selected hormones (top panel) or 50 μM (bottom panel) as indicated: lanes 1, positive control (10 μM rifampin); lanes 2, negative control (media only); lanes 3, negative control (with 50 μM DMSO); lanes 4, β-estradiol; lanes 5, progesterone; lanes 6, estrone; lanes 7, estriol; lanes 8, ethynyl estradiol. The amplification products were resolved on 2% agarose gels stained with EtBr.

tom panel, lanes 8–12) and estrone (Fig. 2, upper panel, lanes 7–11). For norethindrone and ethynyl estradiol, a more constant level of induction was observed over the concentration range tested. Induction in some lanes seemed to look weaker at higher concentrations, however, calculated *MDR1*/18s ratio values using NIH imaging software suggest otherwise, indicating that the amount of cDNA loaded per lane may fluctuate slightly due to inherent variability in technique from lane to lane. These results indicate that *MDR1* mRNA is inducible by both natural and synthetic sex-steroid hormones suggesting regulation of the multi-drug resistance gene at the transcriptional level. The levels of these transcripts were quantitatively measured using NIH Scion imaging software and normalized to uninduced control cells. The results of the quantification are given in Table I.

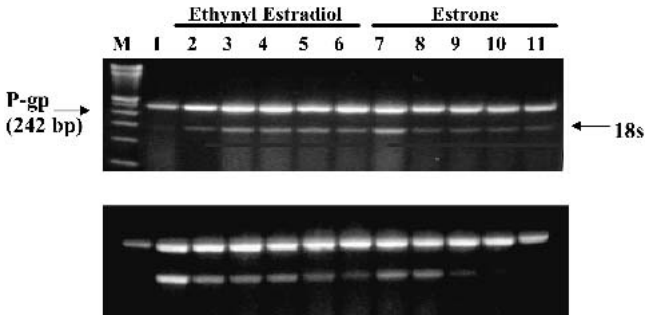

Fig. 2. Concentration-dependent induction of *MDR1* mRNA in LS180 cells using RT-PCR. *MDR1* mRNA induction was within quantitative linear range from 25 nM to 10 μM for ethynyl estradiol and estrone (top panel) as indicated: lane 1, negative control LS180 cells; lanes 2–6, 10 μM, 1 μM, 500 nM, 50 nM, 25 nM ethynyl estradiol; and lanes 7–11, same sequence of concentrations for estrone, respectively. Cells were also induced with norethindrone and D-norgestrel (bottom panel) as indicated: lane 1, negative control; lane 2, positive control (10 μM rifampin); lanes 3–7, 10 μM, 1 μM, 500 nM, 50 nM, 25 nM norethindrone; lanes 8–12, same sequence for D-norgestrel, respectively. M: Marker 100 bp DNA ladder.

Western Blotting of P-gp

The presence and expression level of P-gp was detected using Western blot analysis. LS180 cell lysate samples were run unboiled on a gradient Tris-HCl polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose and cross-reacted with the P-gp antibody, C219. The resulting P-gp protein bands ran at an apparent molecular weight of ~170 kDa. P-gp protein levels were significantly induced over control cells by 50 μM estrone (3-fold), estriol (2.2-fold), and ethynyl estradiol (3.4-fold) in LS180 cells (Fig. 3). Tamoxifen (10 μM), an estrogen receptor antagonist, was used as a positive control of P-gp induction with a 4-fold increase over control. The synthetic progestin derivative, 6α-methyl progesterone acetate (6α-MPA) was also investigated for P-gp inducibility. Interestingly, there was only a 0.1-fold induction of P-gp at the protein level, but at the same concentration (50μM), 6α-MPA induced *MDR1* mRNA 3.7-fold over control, normalized to the 18s internal standard (data not shown). Protein band intensities were scanned and pixel count measured against control cells using the same method as described above for RT-PCR.

Bidirectional Transport of Sex-Steroid Hormones Across MDCK and *MDR1*-MDCK Cell Monolayers

Natural and synthetic derivatives of female steroid hormones were tested to learn if they were substrates for P-gp in an established MDCK monolayer culture system. As an example, transport of 20 μM estriol in both [B→A] and [A→B] directions is depicted in Fig. 4, and P_{app} values were determined (Table II). Results indicate that estriol appears to be a good substrate of P-gp as the P_{app} in the [B→A] direction was found to be significantly greater than in the [A→B] direction in the *MDR1*-transfected MDCK (MM) cells (B→A/A→B = 146.9/46.5 = 3.2) compared to the control MDCK (M) cell monolayers (B→A/A→B = 111.8/97.5 = 1.15).

Other naturally occurring estrogens circulating in women and ethynyl estradiol, a synthetic derivative of 17 β-estradiol and commonly used in oral contraception, were also tested. Estrone is metabolized from and interconvertible with β-estradiol via 17-HSD (hydroxy-steroid dehydrogenase) in the liver. It is the predominant estrogen during pregnancy. In our studies, estrone (1 μM) was also discovered to be transported by P-gp. The P_{app} for estrone in the [B→A] direction was found to be greater than the [A→B] direction for the *MDR1*-MDCK cells compared to an insignificant difference in the MDCK cells. Ethynyl estradiol was found to have a markedly significant larger P_{app} in the [B→A] than [A→B] direction for *MDR1*-MDCK cells, but showed no difference in MDCK cells (Table II). 17 β-Estradiol and progesterone were also tested for transport by P-gp. Results from our studies indicate that they are most likely not P-gp substrates as the P_{app} in the [B→A] direction was not significantly different from [A→B] for both *MDR1*-transfected and control MDCK cells (results not shown).

Transport of norethindrone, a synthetic progestin, was tested under the same conditions. The net efflux ratio in the *MDR1*-transfected cell monolayers was approximately 1.3, which did not reach the minimum threshold associated with

Table I. Semiquantitative Measurements for P-gp and *MDR1* Induction by Steroid Hormones

		*MDR1**	18s*	Ratio (*MDR1*/18s)	Fold induction (Over control)
Figure 1					
	10 μM Rifampin	17.7	113.3	0.16	1.9
	Control	10.0	119.0	0.08	—
	Control (+50 μM DMSO)	13.7	120.7	0.11	1.3
5 μM	β-Estradiol	10.4	114.2	0.09	1.1
5 μM	Progesterone	22.5	114.9	0.20	2.3
5 μM	Estrone	19.9	108.1	0.18	2.2
5 μM	Estriol	86.5	96.3	0.90	11.0
5 μM	Ethynyl estradiol	70.8	90.1	0.79	10.7
	Control	10.9	119.9	0.09	—
50 μM	β-Estradiol	14.5	115.1	0.13	1.4
50 μM	Progesterone	35.0	121.7	0.29	3.2
50 μM	Estrone	79.4	121.9	0.65	7.2
50 μM	Estriol	68.9	121.0	0.57	6.3
50 μM	Ethynyl estradiol	99.2	121.7	0.82	9.0
Figure 2					
	Control	50.9	79.6	0.08	—
	10 μM Ethynyl estradiol	61.0	94.6	0.27	3.4
	1 μM Ethynyl estradiol	22.8	62.3	0.37	4.6
	500 nM Ethynyl estradiol	23.4	64.3	0.36	4.5
	50 nM Ethynyl estradiol	25.1	62.0	0.39	4.9
	25 nM Ethynyl estradiol	19.8	62.3	0.31	3.9
	10 μM Estrone	29.2	60.5	0.48	6.0
	1 μM Estrone	12.3	52.7	0.23	2.9
	500 nM Estrone	10.8	49.3	0.22	2.8
	50 nM Estrone	7.8	43.6	0.18	2.3
	25 nM Estrone	5.0	34.4	0.14	1.8
	Control	13.8	68.4	0.20	—
	10 μM Norethindrone	57.9	92.3	0.63	3.1
	1 μM Norethindrone	47.4	91.0	0.52	2.6
	500 nM Norethindrone	48.5	87.7	0.55	2.7
	50 nM Norethindrone	44.5	87.5	0.51	2.5
	25 nM Norethindrone	32.9	86.4	0.38	1.9
	10 μM Norgestrel	42.4	80.4	0.53	2.6
	1 μM Norgestrel	38.2	72.7	0.53	2.6
	500 nM Norgestrel	18.8	72.0	0.26	1.3
	50 nM Norgestrel	13.4	65.4	0.21	1.0
	25 nM Norgestrel	0.4	40.3	0.01	0.0
Figure 3		MDR1**	SD	Fold Induction	
	Control	24.7	0.42	—	
	10 mM Tamoxifen	95.8	—	3.9	
	50 mM Estrone	71.1	2.12	2.9	
	50 mM Estriol	54.7	0.04	2.2	
	50 mM Ethynyl Estradiol	84.1	1.06	3.4	
	50 mM MPA	26.7	—	1.1	

* Values for *MDR1* and 18s were subtracted from background pixel count.
** Values reflect average of duplicates when applicable.

P-gp transport. The apparent permeability for both apical and basolateral directions in both cell types did not show a noticeable difference, which was validated by the efflux ratios. This suggests norethindrone may not be a P-gp substrate.

Inhibition of P-gp–Mediated Steroid Hormone Transport by GG918

To validate the active [B→A] transport of these estrogens mediated by P-gp, a relatively specific P-gp inhibitor, GG918, was used. P_{app} was determined in both directions in the presence and absence of GG918 (1μM). GG918 was placed in both apical and basolateral compartments and at a concentration of 1μM completely inhibited polarized efflux of ethynyl estradiol, estrone and estriol, as shown in the efflux ratios (Table III). [B→A] flux was significantly decreased, whereas [A→B] flux was slightly increased. This decreased the efflux ratio [*MDR1*-MDCK/MDCK] for all three estrogens to ~1.0, indicating complete inhibition of P-gp–mediated transport and confirming P-gp substrate specificity. Norethindrone was not tested because it was not found to be a likely

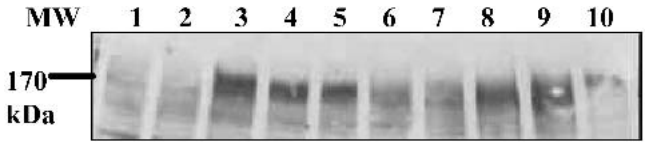

Fig. 3. Western blot of efflux transporter, P-gp, in the LS180 human colon carcinoma cell line. LS180 cells were incubated in media containing various steroid hormones 72 h prior to harvesting. Samples are shown as indicated: lanes 1,2, negative control (cells grown in media only); lane 3, positive control (10 μM tamoxifen); lanes 4,5, 50 μM estrone; lanes 6,7, 50 μM estriol; lanes 8, 9, 50 μM ethynyl estradiol; lane 10, 50 μM 6α-methyl hydroxy progesterone acetate. 50 μg samples were run on a 4–20% gradient gel, transferred to nitrocellulose and hybridized with C219 monoclonal Ab.

P-gp substrate. Intracellular concentrations were also determined and as expected, amount of intracellular drug in *MDR1*-MDCK cells was considerably decreased in contrast to MDCK cells. Furthermore, in the presence of GG918, an 81% increase of intracellular estriol accumulation was observed after an apical dose (A to C), and a 40% increase after a basolateral dose (B to C) for *MDR1*-transfected MDCK cells compared to unchanged levels in MDCK cells with or without GG918 (Fig. 5). This suggests that the expected decrease in intracellular estriol observed in *MDR1*-MDCK due to P-gp mediated efflux is inhibited by GG918, thereby accruing intracellular levels similar to that observed in MDCK cells. The results of these studies additionally confirm the P-gp mediated transport of both natural and synthetic steroid hormones.

DISCUSSION

Studying the integral elements that dictate the role of P-gp in major drug-eliminating organs (i.e., intestine, liver) is essential for better understanding the absorption and elimination of many administered drugs and hormones prevalent in women's health. We hypothesize that steroid hormones and both their natural and synthetic metabolites can modulate the expression and function of P-gp. This in turn can affect the pharmacokinetics and bioavailability of drugs that are substrates for P-gp in women. Upon further investigation of this hormone-transporter relationship, several estrogens and their synthetic metabolite, ethynyl estradiol, were found to be transported by P-gp. Furthermore, ethynyl estradiol and progesterone significantly stimulated P-gp catalytic ATPase activity in a concentration-dependent manner, suggesting a physical interaction with mechanisms that have yet to be characterized. The results of our studies have significant clinical implications in identifying potential interactions and/or complications between exogenously administered hormone therapies and co-administered P-gp substrate drugs. The discovery of P-gp induction by several endogenous estrogens has also exposed a critical need for more women's health research investigating menstrual cycle effects on the therapeutic efficacy of many drugs transported by P-gp.

Of importance to our hypothesis, P-gp expression and function has been found to vary with ovulatory function and phase. The highest levels were found during the midluteal phase of the menstrual cycle, when estrogen and progestin levels peak (21). Studies in rats demonstrate highly increased levels of mdr1-type P-gp RNA in the luminal and glandular

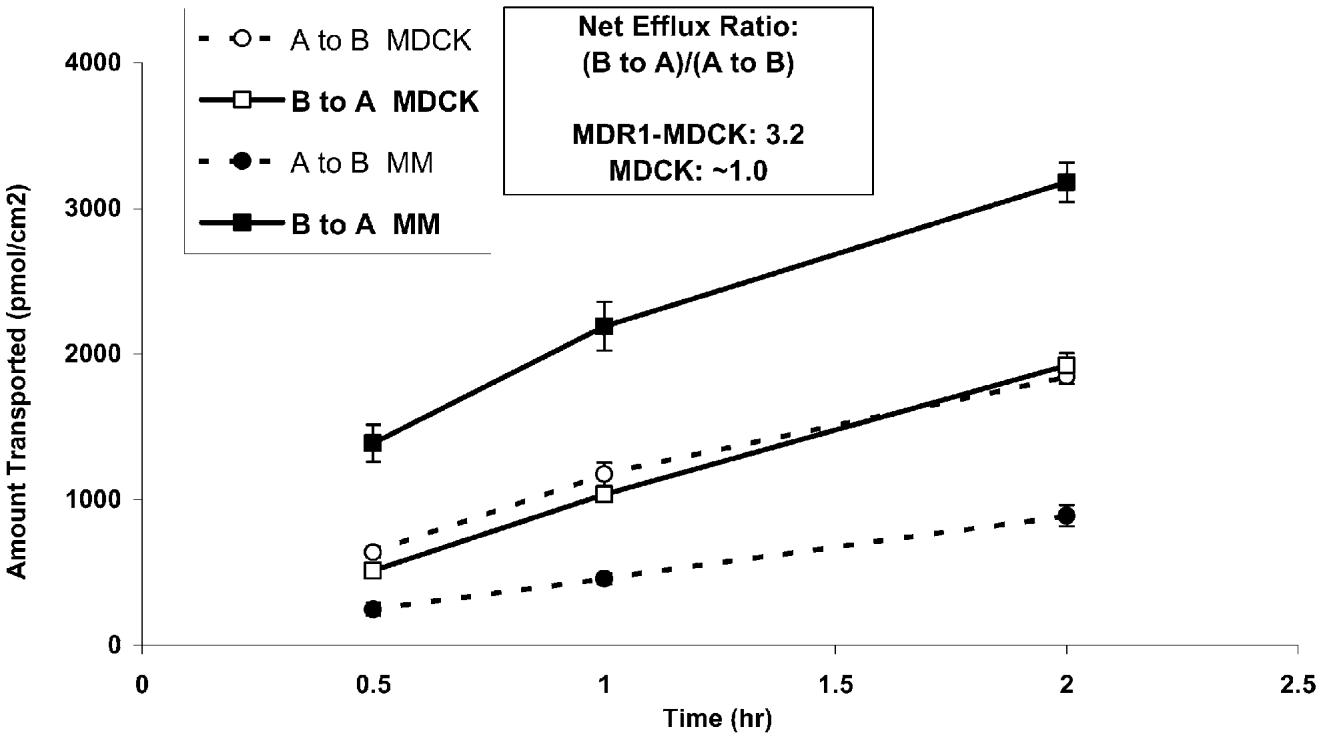

Fig. 4. Representative depiction of the results presented in Tables II and III. for transepithelial transport of 20 μM estriol across MDCK and *MDR1*-MDCK (MM) cell monolayers. Drug was added to apical (A→B) or basolateral (B→A) compartments and sampled on the opposite side at 0.5, 1, 2 h timpoints in 37°C. Each point represents the mean of ~3 monolayers from a typical experiment. *MDR1*-MDCK (B→A): —■—, MDCK (B→A): —□—, *MDR1*-MDCK (A→B):—●—, MDCK (A→B):—○—.

Table II. Apparent Permeability (P_{app}) Values of Steroid Hormones Across MDCK and *MDR1*-MDCK Cell Monolayers

Cell type	Hormone	$P_{app} \times 10^{-7}$ cm/s (avg. ± SE, n = 3)		Efflux ratio
		B → A	A → B	A → B
MDCK [M]	Ethynyl estradiol (5 μM)	50.1 (0.9)	63.3 (1.3)	0.80
***MDR1*-MDCK [MM]**		467 (9)	41.6 (2.2)	**11**
MDCK	Estrone (1 μM)	5.90 (0.24)	6.10 (0.38)	0.97
***MDR1*-MDCK**		38.7 (6.4)	5.90 (1.39)	**6.6**
MDCK	Estriol (20 μM)	112 (3)	97.5 (3.9)	1.2
***MDR1*-MDCK**		147 (10)	46.5 (19.4)	**3.2**
MDCK	Norethindrone (10 μM)	34.8 (6.8)	57.3 (7.1)	0.6
***MDR1*-MDCK**		43.8 (5.9)	33.1 (5.2)	**1.3**

epithelium of uterus and placenta during pregnancy (26). In mice, the levels of P-gp expression parallel that of progesterone in the serum, peaking during days 15–17 of gestation, then declining (27). Furthermore, *mdr1* expression was induced by the combination of β-estradiol and progesterone in the secretory epithelium of the uterus (28). As P-gp is also found in human placental trophoblasts and in human secretory and gestational endometrium (21), these observations suggest possible interactions between P-gp and steroids in humans. Therefore, an *in vitro* investigation of the physiological interaction between the ingested hormone-drug and simulated model cell system was carried out.

Our studies have shown that various sex-steroid hormones can induce *MDR1* mRNA expression in a time and concentration-dependent manner in the colon carcinoma cell line LS180, establishing it to be a suitable model for intestinal *MDR1* induction. These hormones include both endogenous (β-estradiol, estrone, estriol, progesterone) and synthetic hormone derivatives (ethynyl estradiol, norethindrone, norgestrel, 6α-HPA) commonly used in oral contraception and hormone replacement therapies. Induction at the mRNA level was also observed at the protein level for nearly all of the steroid hormones, except for the synthetic progestin, 6α-HPA, albeit at higher concentrations than observed *in vivo*. Endogenous peak serum estrogen and progestin concentrations in normal, premenopausal women have been observed for estradiol at 1.5 nM, estriol at 10 nM, estrone at 1 nM, and progesterone at 40 nM. Because these estrogens are highly bound to plasma proteins such as albumin and SHBG (sex-hormone binding globulin) at a range from 50% to 99%, serum concentrations of unbound or free estrogens can be determined from serum hormone and plasma protein concentrations. Estradiol is protein bound to SHBG at 37% and albumin at 61% for a total bound fraction of ~98%. Hence, the unbound fraction for circulating estradiol is determined to be approximately 30 pM. Unbound concentrations of estriol range from 1.3 to 2 nM, whereas estrone is ~20 pM. Maximum serum concentrations of synthetic steroids from low-dose oral hormone therapies were measured at 15 nM for norgestrel (100 μg dose), 47 nM for norethindrone (1 mg) and 0.3 nM for ethynyl estradiol (10 μg) (29–31). Unbound plasma concentrations of these various synthetic steroids typically range from 0.01 to 20 nM. Despite low unbound circulating concentrations, these hormones are significantly potent. Our studies show *in vitro* induction of *MDR1* mRNA by various steroids at concentration ranges that are albeit higher, but significantly low enough to suggest *MDR1* mRNA induction may very well be observed *in vivo*.

Rifampin, used as a positive control, has been shown to transcriptionally induce *MDR1* by binding a PXR response element (DR4 motif) in the upstream enhancer region of the *MDR1* gene (25). One can speculate that the mechanism of *MDR1* induction by these steroid hormones may be similar to that of rifampin (via PXR) or possibly by other hormone response elements (i.e. ERE) present in the upstream promoter region of the *MDR1* gene. Preliminary data in hormone-induced LS180 cells transfected with a pGL3 plasmid vector containing a 4-kb section of the upstream promoter region of *MDR1*, demonstrated 2-fold (over no drug control) transcriptional activation of the *MDR1* promoter by 10 μM rifampin and 4-fold by 10 μM ethynyl estradiol (data not shown). Additionally, Piekarz *et al.* (32) demonstrated that

Table III. Apparent Permeability (P_{app}) Values of Steroid Hormones in the Presence of GG918

Cell type	Hormone	$P_{app} \times 10^{-7}$ cm/s (avg. ± SE, n = 3)		B → A	Net efflux ratio
		B → A	A → B	A → B	[MM/M] + GG918
MDCK [M]	Ethynyl estradiol (5 μM)	28.5 (6.7)	27.7 (8.9)	1.0	0.8
***MDR1*-MDCK [MM]**		27.6 (1.4)	22.6 (2.5)	**1.2**	
MDCK	Estrone (1 μM)	6.2 (1.6)	6.9 (1.3)	0.9	1.3
***MDR1*-MDCK**		7.5 (3.3)	6.3 (3.6)	**1.2**	
MDCK	Estriol (20 μM)	20.0 (4.4)	13.2 (2.1)	1.5	0.9
***MDR1*-MDCK**		16.3 (7.2)	12.3 (9.0)	**1.3**	
MDCK	Norethindrone (10 μM)	n/a	n/a	n/a	n/a
***MDR1*-MDCK**		—	—	—	

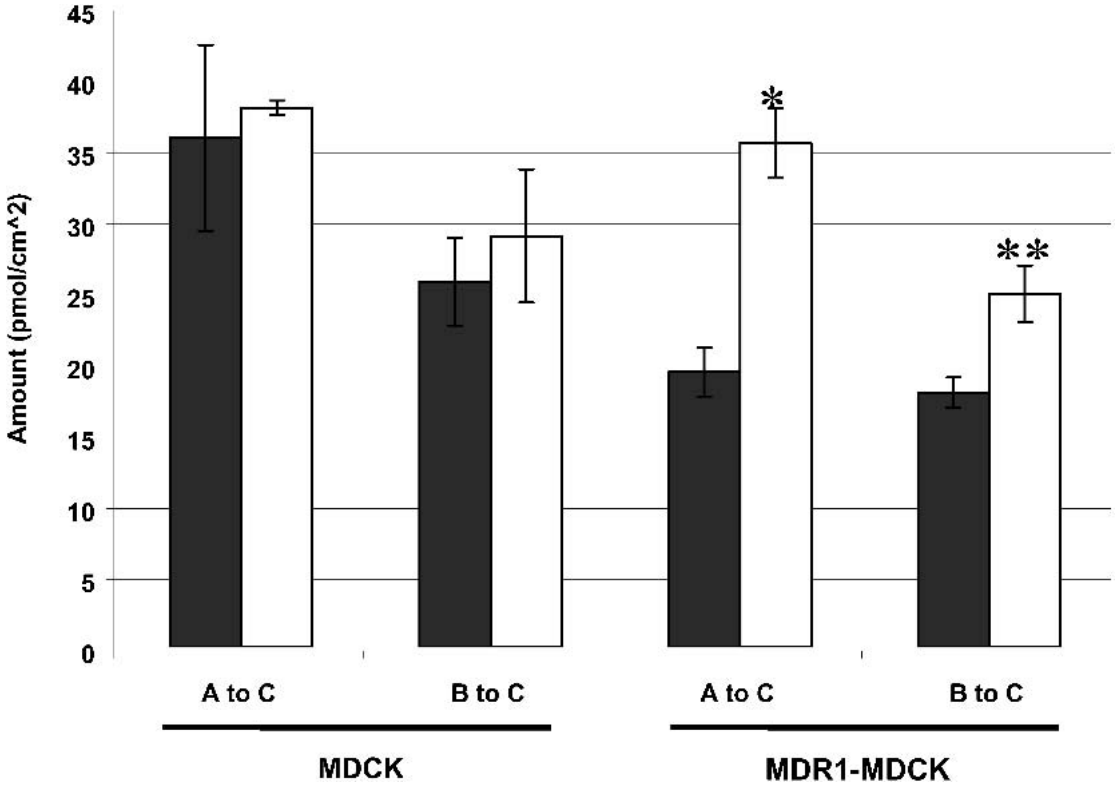

Fig. 5. Intracellular concentrations of 20 μM estriol in MDCK vs. *MDR1*-MDCK cells after bidirectional transport indicate active efflux. *A*pical to *C*ell describes amount of intracellular estriol after an apical dose of estriol, whereas *B*asolateral to *C*ell indicates the same after a basolateral dose. Solid bars (■) and open bars (□) reflect intracellular measurements in absence and presence of the P-gp inhibitor, GG918, respectively. *$p < 0.001$, statistical difference observed in *MDR1*-MDCK cells (A to C) between cells treated with and those without GG918, using paired, two-tailed *t* test. **$p < 0.01$, statistical difference observed in *MDR1*-MDCK cells (B to C), as defined above.

progesterone, acting through the A form of the progesterone receptor (PR_A) was able to regulate the expression of the *mdr1b* gene. Using ATPase activity assays, ethynyl estradiol and progesterone were both also able to significantly stimulate P-gp ATPase activity in a concentration-dependent manner (Fig. 6). These results suggest direct interactions of steroid hormones with P-gp and perhaps functional regulation to some degree.

A deeper exploration into the interactions between P-gp and these steroid hormones led us to question whether these steroid hormones may potentially be substrates for P-gp. A bidirectional transport system in *MDR1*-MDCK and control MDCK cells was used to measure P-gp mediated active transport of various steroid hormones. Results indicate that ethynyl estradiol (10 μM) is a strong P-gp substrate with a net efflux ratio (B→A/A→B) of ~11. Estrone and estriol were also found to be P-gp substrates with ratios of ~7 and 3, respectively. The addition of the specific P-gp inhibitor, GG918, collapsed this B→A net flux ratio by these estrogens to ~1. Norethindrone was not found to be a transported by P-gp. Accompanying data also determined that β-estradiol and progesterone were not P-gp substrates (data not shown). It is interesting to note that although β-estradiol is not a P-gp substrate, its successor metabolites, which are equally important in mediating estrogenic effects, are good substrates. Possible reasons can only be attributed to the promiscuous, yet specific nature of this enigmatic transporter. This is the first report to demonstrate P-gp mediated transport of these synthetic and natural steroid hormones, many of which are known to be widely metabolized by CYP3A enzymes and eliminated thereafter (33–35), but never investigated as potential P-gp substrates. These natural estrogens, as well as ethynyl estradiol, can be added to the growing list of compounds that are both P-gp and CYP3A substrates, allowing a better understanding of the elimination and overall efficacy of hormone therapies in women. Additional studies are necessary to elucidate the complex interaction between transport, induction and the effect they have on each other, not to mention its clinical relevance.

The endeavor to understand the effects of natural and synthetic steroid hormones on the expression and function of P-gp requires further *in vitro* studies investigating the regulation of P-gp at the molecular level. In humans, variations in hormone levels during the menstrual cycle have been correlated with changes in P-gp expression. Hence, the relevance of exploring hormonal effects on the pharmacokinetics of P-gp substrate drugs becomes more significant. The involvement of steroid hormones as P-gp substrates and in the modulation of P-gp expression points to an association between the effects of reproductive hormones on P-gp and potential consequential risks for drug efficacy in women. Thus, further investigations in the interactions between sex-steroids and P-gp substrate drugs are necessary to improve predictions of complications that may compromise therapeutic drug effica-

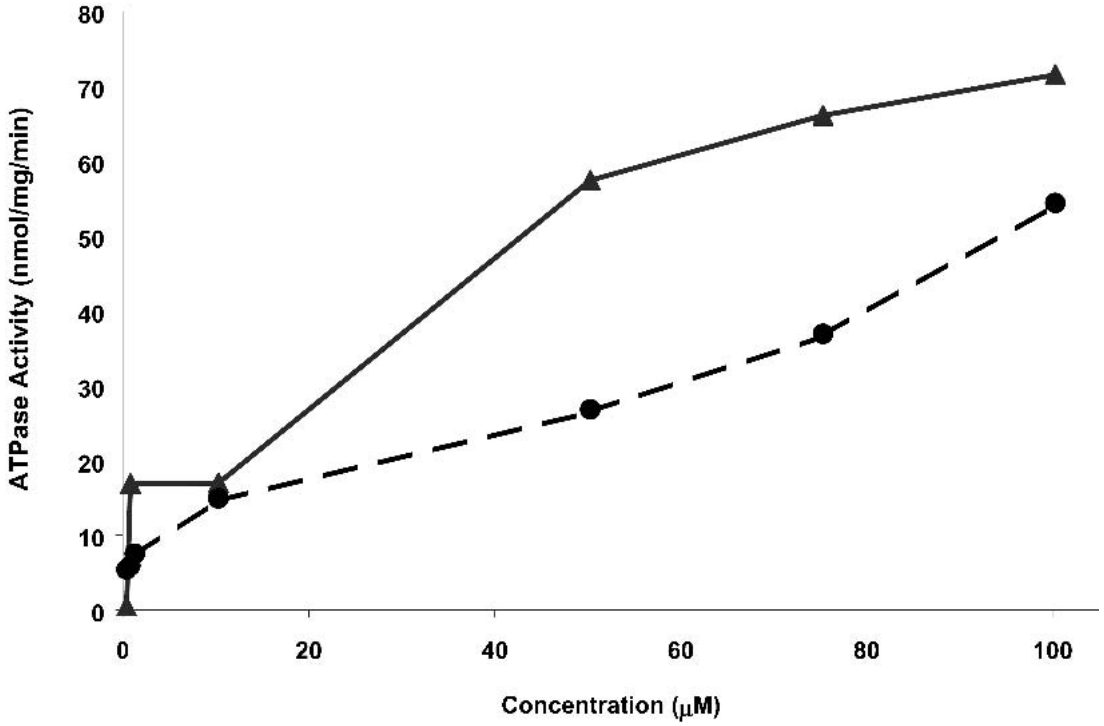

Fig. 6. Concentration-dependent stimulation of vanadate-sensitive ATPase activity of P-gp by progesterone (—▲—) and ethynyl estradiol (—●—) over a range of 0.1 μM to100 μM. The P-gp ATPase activity was measured in membranes of Sf9 insect cells infected with a recombinant baculovirus containing *MDR1* cDNA used with appropriate control membranes. Each data point is the average of duplicate determinations.

cies and better understand the effects of both endogenous and exogenously administered hormones on future drug therapy in women.

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