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Human Breast Cancer Resistance Protein (BCRP/ABCG2): interactions with steroid drugs, hormones, the dietary carcinogen PhIP, and transport of cimetidine

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BCRP, breast cancer resistance protein; PhIP, (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine); MRP, multidrug resistance protein; P-gp, P-glycoprotein; MDR, multidrug resistance; Ko143, 3-(6-Isobutyl-9-methoxy-1,4-dioxo-1,2,3,4,6,7,12,12a-octahydro-pyrazino[1',2':1,6]pyrido[3,4-*b*]indol-3-yl)-propionic acid *tert*-butyl ester

Abstract

The breast cancer resistance protein (BCRP/ABCG2) is an ATP binding cassette (ABC) drug efflux transporter that extrudes xenotoxins from cells, mediating drug resistance and affecting the pharmacological behaviour of many compounds. To study the interaction of human wild-type BCRP with steroid drugs, hormones and the dietary carcinogen PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine), we expressed human BCRP in the murine MEF3.8 fibroblast cell line, which lacks Mdr1a/1b P-glycoprotein and Mrp1, and in the polarized epithelial MDCKII cell line. We show that PhIP was efficiently transported by human BCRP in MDCKII-BCRP cells, as was found previously for murine Bcrp1. Furthermore, we show that six out of nine glucocorticoid drugs, corticosterone and digoxin increased the accumulation of mitoxantrone in the MEF3.8-BCRP cell line, indicating inhibition of BCRP. In contrast, aldosterone and ursodeoxycholic acid had no significant effect on BCRP. The four most efficiently reversing glucocorticoid drugs (beclomethasone, 6α-methylprednisolone, dexamethasone and triamcinolone) and 17β-estradiol showed a significantly reduced BCRP-mediated transepithelial transport of PhIP by MDCKII-BCRP cells, with the highest reduction of PhIP transport ratio for beclomethasone (from 25.0 ± 1.1 to 2.7 ± 0.0). However, none of the tested endogenous steroids or synthetic glucocorticoids nor digoxin were transported substrates of BCRP. We also identified the H_2-receptor antagonist drug cimetidine as a novel efficiently transported substrate for human BCRP and mouse Bcrp1. The generated BCRP expressing cell lines thus provide valuable tools to study pharmacological and toxicological interactions mediated by BCRP, and to identify new BCRP substrates.

Introduction

The breast cancer resistance protein (BCRP/ABCG2) belongs to the ATP Binding Cassette family of drug transporters. Human BCRP has been shown to mediate drug resistance through energy-dependent efflux of drug substrates without the need for glutathione. The range of drugs to which BCRP can confer resistance in tumor cell lines includes mitoxantrone, methotrexate, topotecan derivatives, bisantrene, etoposide, SN-38 and flavopiridol (Doyle and Ross, 2003; Litman et al., 2001; Maliepaard et al., 2001b). In several drug-selected cell lines, mutations in BCRP at arginine 482 (R482) have been described, resulting in an altered substrate specificity (e.g. increased resistance to the anthracyclin doxorubicin) (Honjo et al., 2001; Allen et al., 2002a).

BCRP is present in many normal tissues, for instance in the apical membrane of placental syncytiotrophoblasts, in the bile canalicular membrane of hepatocytes, in the luminal membranes of villous epithelial cells in the small intestine and colon and in venous and capillary endothelial cells of almost all tissues (Maliepaard et al., 2001a). The localization of BCRP in tissues with barrier or elimination functions causes the transporter to have a substantial pharmacological role in handling substrate drugs and xenobiotics.

We found earlier that mouse Bcrp1 restricts intestinal absorption of topotecan, contributes to hepatobiliary elimination of the drug and limits the entry of topotecan into fetuses by reverse pumping in the placenta (Jonker et al., 2000, 2002). Moreover, we demonstrated that dietary pheophorbide *a*, a breakdown product of chlorophyll, is a BCRP/Bcrp1 substrate that causes phototoxicity in Bcrp1$^{-/-}$ mice but not in wild-type mice (Jonker et al., 2002). Also PhIP, a carcinogen present in baked food and cigarette smoke, is a very good substrate of mouse Bcrp1 and its absorption and hepatobiliary and intestinal elimination are clearly affected by Bcrp1 in mice (van Herwaarden et al., 2003). As Bcrp1$^{-/-}$

mice are normally viable without pronounced abnormalities, protection from naturally occurring toxins appears to be a major biological function of BCRP/Bcrp1. Clinical studies demonstrated that oral administration of GF120918, a P-gp and BCRP inhibitor, can substantially increase bioavailability of topotecan after oral administration in humans (Kruijtzer et al., 2002).

Recently, estrone, 17β-estradiol, and estrogen antagonists such as diethylstilbestrol, tamoxifen and their derivatives, were shown to efficiently reverse BCRP-mediated resistance to mitoxantrone, topotecan and SN-38 (Imai et al., 2002; Sugimoto et al., 2003). Inhibitory potency of estradiol to human BCRP was also demonstrated in a Lactococcus expression model (Janvilisri et al., 2003). The latter authors also suggested that 17β-estradiol is a transported substrate of human BCRP. This conclusion, however, conflicts with the report of Imai et al. (2003), who found that BCRP transports only sulfated conjugates of estrone and 17β-estradiol, but not free estrogens.

The steroids dexamethasone and prednisone are frequently used in chemotherapeutic regimes for lymphoid malignancies, after radiotherapy, and during treatment of solid tumors for their antiemetic and antiedematous effects. Gruol and Bourgeois (1997) suggested that chemosensitizing glucocorticoids can serve simultaneously as glucocorticoid receptor agonists and modulators of multidrug resistance mediated by P-glycoprotein. A similar dual activity might apply to steroid drugs and BCRP, but relations between clinical BCRP-mediated resistance of tumor cells and potential modulation of the resistance by glucocorticoids have not been investigated yet.

In order to further characterize the interactions of human wild-type BCRP with frequently used steroid drugs, and with the carcinogen PhIP, we expressed BCRP cDNA in various cell lines. Using these lines we also identified a new BCRP/Bcrp1 substrate, the H_2-receptor antagonist cimetidine.

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Methods

Chemicals and cell lines. PhIP and [^{14}C]PhIP (10 Ci/mol) were from Toronto Research Chemicals, Inc. (Toronto, Ontario, Canada). [^{14}C]topotecan (56 Ci/mol) was from GlaxoSmithKline (GSK, Research Triangle Park, North Carolina, USA). [1,2,4,6,7-^{3}H]dexamethasone (70-110 Ci/mmol), [2,4,6,7-^{3}H] estradiol (70-120 Ci/mmol), [1,2-^{3}H]aldosterone (40-60 Ci/mmol), [1,2,6,7-^{3}H]corticosterone (75-105 Ci/mmol), [N-methyl-^{3}H]cimetidine (20 Ci/mmol), [^{14}C]inulin carboxylic acid (5-20 Ci/mol) and [^{3}H]inulin (0.5-3 Ci/mmol) were from Amersham Biosciences (Little Chalfont, Buckinghamshire, United Kingdom). Scintillation liquid Ultima-Gold was from Packard (Meriden, CT). Ko143 was described previously (Allen et al., 2002b). Dexamethasone, hydrocortisone succinate sodium salt and dexamethasone 21-phosphate disodium salt were from ICN Biochemicals, Inc. (Aurora, OH, USA). Estradiol, betamethasone, 6α-methylprednisolone and cimetidine were from Sigma. Prednisone and prednisolone were purchased from Kulich (Hradec Králové, Czech Republic). Triamcinolone acetonide, beclomethasone dipropionate and digoxin were purchased from Zentiva, Czech Republic. Complete high-glucose DMEM GlutaMax® and serum-free Optimem media were manufactured by Gibco. Ursodeoxycholic acid was from ProMed (Hradec Kralove, Czech Republic). GF120918 (Elacridar) was kindly provided by GSK (Greenford, England) to Dr. J.H.M. Schellens. PSC833 (Valspodar) was kindly provided to A.H.S. by Dr. D. Cohen, Novartis, Hanover, NJ.

MEF3.8, an adherent spontaneously immortalized embryo fibroblast cell line derived from triple knockout Mdr1a/b$^{-/-}$, Mrp1$^{-/-}$ mice was maintained as described (Allen et al., 1999; Allen et al., 2000). Murine Bcrp1- and human MDR1-and MRP2-transduced MDCKII sublines were previously described and characterized (Bakos et al., 1998; Evers et al., 1998; Jonker et al., 2000; Evers et al., 2000; Huisman et al., 2002; van Herwaarden et al., 2003).

Parent MDCKII cell line and its transduced sublines were cultured in DMEM complete high-glucose medium with L-glutamine (GlutaMax®, Gibco) supplemented with 10% fetal calf serum, 100 U/mL penicillin and 100 µg/mL streptomycin.

Transduction of MDCKII and MEF3.8 cell lines with wild-type human BCRP. The full-length wild-type human BCRP complementary DNA (cDNA), a kind gift of Dr Susan E. Bates (NCI, Bethesda, MD) was excised from pcDNA3 with BamHI and NotI (Roche) and inserted into the SnaBI restriction site of the LZRS-IRES-GFP expression vector (Michiels et al., 2000) by blunt end ligation. The resulting vector is a monocistronic construct containing BCRP followed by an internal ribosome entry site and the enhanced green fluorescent protein (GFP). This construct was transfected into the amphotropic Phoenix producer cell line (Kinsella and Nolan, 1996) using the calcium phosphate precipitation method. Viral supernatants from these transfected cells were used to transduce MDCKII or MEF3.8 cells by coincubation in the presence of 4 µg/mL Polybrene. After 24 h, 2% of MDCKII and 60% of MEF3.8 cells were positive for GFP fluorescence. Single GFP^+ cells were sorted into 96-well plates containing MDCKII- or MEF3.8-conditioned medium. After expansion, clones were screened for expression of functional BCRP activity on the basis of reduced mitoxantrone accumulation using a FACSCalibur cytometer (Becton-Dickinson, San Jose, CA) and topotecan transport in the case of MDCKII-BCRP. The expression of BCRP in selected clones was verified by Western blotting analysis.

Western blotting analysis. Cells were washed with PBS and homogenized in ice-cold RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 1% Sodium deoxycholate, 0.1% SDS, 5 mM EDTA, 10 µg/mL leupeptin, 10 µg/mL pepstatin and 1 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.4). Lysates were centrifuged at 10,000×g for 10

min at 4°C and protein concentrations in supernatants were determined using a BCA Protein Assay Kit (Pierce). 40 µg of protein pre-heated for 3 min at 98°C and loaded in sample buffer with 2-mercaptoethanol was resolved in an 8% polyacrylamide SDS-PAGE gel and eletrotransferred to nitrocellulose Hybond® membranes (Amersham Bioscience, Little Chalfont, UK). Membranes were incubated overnight at 4°C with BXP-21 monoclonal antibody (Monosan, the Netherlands) in a 1:500 dilution in TBST buffer (100 mM Tris-HCl, 150 mM NaCl, 0.1% w/v Tween 20). Secondary horseradish peroxidase-conjugated antibody (1:2000 dilution, 1 h incubation at room temperature) and ECL Western blotting kit (both Amersham Bioscience) were used for visualisation of BCRP on FOMA® Blue Medical X-Ray films (Foma Bohemia a.s, Hradec Kralove, Czech Republic).

Accumulation Assays. Cells were cultured in 12-well plates (30×10^3 cells/well) in complete medium for 36 hours to subconfluence. Medium was aspirated, and cells were preincubated in prewarmed Optimem medium with or without inhibitor or tested steroid for 60 min before adding fluorescent BCRP substrate. Accumulation of fluorescent substrates was allowed for 1 hour at 37°C and was arrested by prompt cooling on ice and removal of medium. Cells were kept on ice during subsequent washes with ice-cold PBS (2×1 mL) and trypsinized in ice-cold 1x Trypsin/EDTA solution (phenol red dye-free, TPP, Austria) per well. Collected cells were sedimented and resuspended in PBS with 2.5% of fetal calf serum. Relative cellular accumulation of fluorescent compounds was determined by flow cytometry using a FACSCalibur cytometer. Samples were gated on forward scatter *versus* side scatter to exclude cell debris and clumps. Excitation and emission wavelengths for mitoxantrone were 633 nm and 661 nm, respectively. Fluorescence of accumulated substrate in tested populations of at least 10,000 cells was quantified from histogram plots using the median of fluorescence (MF). BCRP inhibition increases accumulation of a fluorescent substrate in BCRP-transduced cells

and thus increases MF. Possible background fluorescence of all tested steroids and inhibitors was checked in appropriate channels, but the fluorescence was negligible in all cases. Flow cytometry data were processed and analyzed using WinMDI ver.2.8 software.

Flow cytometry calculations: To compare and semi-quantify inhibitory effects of tested compounds on BCRP, a modification of the methods published by van der Kolk et al. (2002) and Wang et al. (2000) was employed. Inhibitory potencies of compounds were calculated from the shift of MF caused by the tested compound in MEF3.8-BCRP cells related to the shift of MF caused by the potent BCRP inhibitor Ko143 (1 μM) according to the following equation :

$$\textit{Inhibitory potency} = \frac{MF_{(MEF3.8\text{-}BCRP)} \text{ with tested compound} - MF_{(MEF3.8\text{-}BCRP)} \text{ without inhibitor}}{MF_{(MEF3.8\text{-}BCRP)} \text{ with Ko143} - MF_{(MEF3.8\text{-}BCRP)} \text{ without inhibitor}} \times 100\% \quad (1.)$$

Transport Assays. Transport assays were performed on microporous polycarbonate membrane filters (3.0 μm pore size, 24 mm diameter; Transwell 3414; Costar, Corning, NY) as reported, with slight modifications (van Herwaarden et al., 2003). Cells were seeded on filters at a density of 1.0×10^6 cells per well, grown for 3 days, and medium was replaced every day. One hour before the start of the experiment, medium was replaced in both compartments with Optimem medium (Gibco) containing inhibitor. At time zero the experiment was started by replacing the medium with fresh Optimem medium, with substrate and inhibitor (or tested compound) and radiolabeled inulin in the appropriate compartment. Topotecan and PhIP were used as substrates at a concentration of 2 μM in the starting compartment traced with [^{14}C]topotecan or [^{14}C]PhIP (0.02 μCi/mL). Cimetidine was tested at a concentration of 5 μM traced with [^{3}H]cimetidine (0.04 μCi/mL). Aliquots of 50 μl were

taken each hour from the opposite compartment up to 4 h, and radioactivity was measured. Inulin leakage was tolerated up to one percent per hour per well. In case of topotecan, a slowly permeable cytostatic drug, 1.5 ml of Optimem medium was used in both compartments and experiments lasted 8 hours with sampling at 2-hour intervals. Dexamethasone, corticosterone, estradiol and aldosterone were tested at a concentration of 2 μM (0.02 μCi/mL). At the end of the experiment, filters with cell layers were washed two times with ice-cold PBS, excised and measured for radioactivity. The percentage of initially applied radioactivity appearing in the opposite compartment was calculated and plotted in figures. The "transport ratio" (r) was calculated, which is defined as the ratio of apically directed translocation divided by the basolaterally directed translocation of tested substrate as measured by the end of experiment.

Statistical Analysis. Student's unpaired, two-tailed *t* test was used when appropriate to perform statistical analysis of differences between two sets of data. $P < 0.05$ was considered statistically significant. Errors are represented as standard deviations (SD).

Results

Generation and characterization of a MEF3.8-BCRP cell line. To generate a cell line with minimal background transporter activity, we expressed wild-type BCRP in the mouse embryonic fibroblast cell line MEF3.8 which was derived from Mdr1a/b$^{-/-}$, Mrp1$^{-/-}$ knockout mice (Allen et al., 1999; Allen et al., 2000). Transduction of MEF3.8 cells with retrovirus containing wild-type BCRP cDNA yielded a clone with substantial BCRP levels (Fig. 1A) and 5-10-fold reduced accumulation of mitoxantrone (Fig. 2) and pheophorbide a (not shown). The effects of Ko143 (a potent BCRP inhibitor), GF120918 (shared BCRP and P-gp inhibitor) and PSC833 (P-gp inhibitor) on accumulation of mitoxantrone (a substrate of both BCRP and P-gp) were evaluated using flow cytometry (Fig. 2). Ko143 increased the accumulation of mitoxantrone 5-10 times in the BCRP-transduced cell line whereas it had only negligible effect in the parent cells (Fig. 2A-C). GF120918 also markedly increased accumulation of mitoxantrone in the BCRP-transduced cells with little effect on the parent cells (Fig. 2D). PSC883 increased accumulation of mitoxantrone slightly in both BCRP-transduced and parent cells, suggesting little or no inhibition of BCRP and perhaps involvement of an unidentified PSC833-sensitive mitoxantrone transporter (Fig. 2E). As the MEF3.8-BCRP line was derived from a triple knockout Mdr1a/b$^{-/-}$, Mrp1$^{-/-}$ cell line which also expresses little or no endogenous murine Bcrp1, Mrp2, Mrp3, Mdr2 or Spgp (Allen et al., 1999; 2000; 2002a) it yields minimal non-specific transporter background in accumulation experiments.

Generation and characterization of a MDCKII-BCRP cell line. Transduction of the polarized epithelial MDCKII cell line yielded a clone with substantial BCRP levels as verified by Western blot (Fig. 1B). These MDCKII-BCRP cells demonstrated greatly reduced

mitoxantrone accumulation which was largely reversed by Ko143 or GF120918 treatment, indicative of BCRP overexpression and activity (Fig. 2C, D). PSC833 significantly increased mitoxantrone accumulation in both BCRP-transduced and parent cells (Fig. 2E), presumably due to inhibition of endogenous P-gp in the MDCKII lines. In transepithelial transport experiments with the BCRP/Bcrp1 substrate topotecan (Fig. 3) and the Bcrp1 substrate PhIP (Fig. 4), translocation of both compounds was considerably increased in the apical direction, and decreased in the basolateral direction in MDCKII-BCRP cells compared to the parental cells (Fig. 3A, 4A). Ko143 completely abolished the asymmetry in translocation for PhIP (Fig. 4B), but only partly for topotecan, roughly equalizing residual transport in parental and transduced cells (Fig. 3B). Because topotecan is also a P-gp substrate, we attribute the residual transport (also apparent in the parental line, Fig. 3A) to endogenous canine P-gp. Indeed, adding the P-gp inhibitor PSC833 abolished topotecan transport in the parental line, but hardly affected the (BCRP-mediated) transport in the BCRP line untouched (Fig. 3C). The data thus indicate high expression and activity of human BCRP in the MDCKII-BCRP cells, and they show that human BCRP, like murine Bcrp1, is a highly efficient transporter of PhIP.

Interaction of glucocorticoid drugs, endogenous glucocorticoids, aldosterone and steroid drugs with BCRP. Interactions of 9 glucocorticoid drugs, 2 steroid hormones (corticosterone and aldosterone), ursodeoxycholic acid (a representative of steroid choleretic drugs) and digoxin (a cardiac glycoside with a steroid core structure) with BCRP were evaluated by measuring their ability to reverse the reduced mitoxantrone accumulation in MEF3.8-BCRP cells in flow cytometry experiments. Digoxin and beclomethasone had the strongest inhibitory potency, followed by 6α-methylprednisolone, corticosterone, triamcinolone, dexamethasone, betamethasone, and prednisone (Table 1). Prednisolone, hydrocortisone, aldosterone and hydrophilic steroid drugs such as dexamethasone 21-phosphate and ursodeoxycholic acid had

little or no significant effect on BCRP (Table 1). None of the tested compounds had significant effects on mitoxantrone accumulation in the parental MEF3.8 cell line (not shown). Our data show that several endogenous and synthetic steroids are capable of inhibiting wild-type BCRP, albeit at relatively high micromolar concentrations.

Influence of glucocorticoids on transepithelial transport of PhIP. Beclomethasone, 6α-methylprednisolone, dexamethasone and triamcinolone, the four most efficiently reversing glucocorticoid drugs, were tested for their inhibitory potential on transepithelial transport of PhIP. Beclomethasone (at 20 μM, its solubility limit) markedly reduced transport of PhIP across MDCKII-BCRP cells (reducing the transport ratio from 25.0 ± 1.1 to 2.7 ± 0.0), without a significant effect on the parent cells (Fig. 4C). The radioactivity associated with the MDCKII-BCRP cell layer was also 4-fold increased by beclomethasone treatment, in accordance with inhibition of BCRP-mediated efflux (Fig. 4C). 6α-methylprednisolone, dexamethasone and triamcinolone at a concentration of 50 μM also significantly impaired the BCRP-mediated transport of PhIP, reducing transport ratios to 5.3 ± 0.9, 12.5 ± 0.2, and 18.7 ± 0.3, respectively, compared to 25.0 ± 1.1 in controls (Fig. 4D; for dexamethasone and triamcinolone data not shown).

Transport experiments with steroids. Dexamethasone, corticosterone, aldosterone and digoxin were not themselves transported by BCRP, as indicated by similar apically and basolaterally directed translocations of these compounds in MDCKII-BCRP cells (Fig. 5A-D), as was also seen in the parental cells (not shown). With the estrogen 17β-estradiol, somewhat more translocation in the basolateral compared to the apical direction was observed, and this difference was slightly decreased in MDCKII-BCRP cells (Fig. 5E-F). However, differences were minor, also after treatment with Ko143 (Fig. 5G-H), suggesting

that 17β-estradiol itself in its unconjugated form is hardly (if at all) transported by BCRP. On the other hand, 17β-estradiol did boost accumulation of mitoxantrone in MEF3.8-BCRP cells (not shown). Similarly, transepithelial BCRP-mediated transport of both topotecan and PhIP was virtually completely abolished by 100 μM of 17β-estradiol (Fig. 3D, 4E). The results are in line with data from Imai et al. (2002) and Janvilisri et al. (2003), indicating that 17β-estradiol is a fairly good inhibitor of human BCRP.

***In vitro transport of cimetidine*.** We next determined whether human BCRP and murine Bcrp1 could transport the H_2-receptor antagonist cimetidine. The possible involvement of two other pharmacologically important ABC transporters, MDR1 P-gp and MRP2 (ABCC2), was also tested. In the BCRP- and Bcrp1-transduced MDCK-II cell lines, apically directed translocation of cimetidine was highly increased and basolaterally directed translocation drastically decreased when compared to the parental cell line (Fig. 6A, B, D). Ko143 completely abolished this BCRP/Bcrp1 mediated transport (Fig. 6C, E). In contrast, in the MDR1- and MRP2-transduced MDCK-II cell lines, the vectorial translocation was similar to the MDCK-II parental cell line (Fig. 6F, G). These results indicate highly efficient transport of cimetidine by BCRP and Bcrp1, but not by MDR1 P-gp or MRP2.

Discussion

In this study we describe the generation and validation of non-polarized MEF3.8-BCRP cells, and of polarized MDCKII-BCRP cells, expressing wild-type human BCRP cDNA. We applied these cell lines to analyze the interaction of BCRP with a range of steroid compounds, the dietary carcinogen PhIP, and the H_2-receptor antagonist cimetidine. Next to many other applications, comparisons between these cells and the previously obtained MEF3.8-Bcrp1 and MDCKII-Bcrp1 cells expressing wild-type murine Bcrp1 (Allen et al., 2000; van Herwaarden et al., 2003), will support extrapolation of data obtained in Bcrp1 knockout mice (Jonker et al., 2002) to their possible relevance in humans. For instance, this study indicates that the dietary carcinogen PhIP is transported very efficiently by human BCRP as was shown earlier for mouse Bcrp1. Hence, the pronounced pharmacokinetic and toxicologically protective role of Bcrp1 seen for PhIP in mice (van Herwaarden et al., 2003) may also apply to BCRP in humans.

Utilizing two independent cell lines, our data clearly show that cimetidine is an efficiently transported substrate of human BCRP and murine Bcrp1, which was further confirmed by inhibition with the BCRP/Bcrp1 inhibitor Ko143. Cimetidine is a widely used drug, and its being a BCRP substrate might affect its clinical applications, for instance by causing reduced brain penetration (Cisternino et al., 2004) and thus reduced CNS side effects.

In contrast to previous studies, we did not detect significant transport of cimetidine by human MDR1 P-gp (Pan et al., 1994; Collett et al., 1999; Karyekar et al., 2003). Also in the well-characterized pig-kidney cell line LLC-PK1 transfected with human MDR1, which is routinely used in MDR1 transport experiments (Schinkel et al., 1995; Jonker et al., 1999; Wandel et al., 2000; Lecureur et al., 2000; Karssen et al., 2002), we found no indications for MDR1-mediated cimetidine transport (data not shown). Possibly some MDR1-transfected or –transduced cell lines used in these earlier cimetidine studies display clonal variation in the

expression of endogenous BCRP, especially when they are maintained under continuous drug selection (e.g., Pan et al., 1994, Karyekar et al., 2003). We observed earlier that there can be significant clonal variation in expression of endogenous drug transporters in epithelial cell lines (e.g., Huisman et al., 2002), so utilization of several independent cell lines and/or inhibitors is advisable.

We found that some glucocorticoid drugs can inhibit BCRP, but that none of the tested steroids was substantially transported by BCRP. Applied at micromolar concentrations, glucocorticoids such as beclomethasone, 6α-methylprednisolone, dexamethasone and triamcinolone could reverse BCRP-mediated transport of several substrates in both accumulation and transport experiments. Similarly, digoxin, a drug with a steroid-like core structure, was not transported itself, but it did inhibit BCRP-mediated transport of mitoxantrone. Thus, in vivo pharmacokinetic interactions of some of the tested glucocorticoids and drugs with transported BCRP substrates might occur at physiological barriers in the intestine and in the liver, where BCRP affects absorption and elimination of its substrates. Especially after oral administration relatively high levels of these BCRP-modulating compounds might be reached in the intestine and liver.

Many of the BCRP substrates identified so far are anticancer drugs (for review see Schinkel and Jonker, 2003; Abbott, 2003). Some glucocorticoids, especially dexamethasone and prednisone, are frequently used in chemotherapeutic regimens together with such anticancer drugs in the treatment of both lymphoid leukemias and solid tumors, either for their intrinsic anticancer activity, or for their ability to reduce adverse side effects of chemotherapy. When BCRP present in the malignant cells would play a significant role in their chemotherapy resistance - which is still an open question (Steinbach et al., 2002; van den Heuvel-Eibrink et al., 2002; Abbott, 2003) - one could consider the possibility that high systemic levels of the glucorticoids might reverse this BCRP-mediated resistance.

We found that the steroid drugs dexamethasone and digoxin, and endogenous steroid hormones such as corticosterone, estradiol and aldosterone are not substantially transported by BCRP in polarized monolayers. This is in line with the results of Imai et al. (2003) who reported that estradiol, estrone, cortisol and progesterone are not transported substrates of wild-type BCRP in their native form; however, sulfate conjugates of estradiol and estrone are transported by BCRP. In contrast to the data of Imai et al. (2003) and to our observations, both obtained in mammalian cell systems, estradiol appears to be a transported BCRP substrate in Lactococcus lactis bacteria expressing human BCRP (Janvilisri et al., 2003). However, as pointed out by the latter authors, there are marked differences in membrane composition between mammalian cells and these bacteria, which lack mammalian sterols. Such differences in membrane environment might affect the apparent substrate specificity of BCRP (and possibly other ABC transporters as well), for instance due to altered competitive interactions between endogenous membrane compounds and exogenous substrates of BCRP. Overall, we therefore consider it unlikely that BCRP would have a significant physiological impact by transport of the tested steroid hormones out of mammalian cells.

Several endogenous steroids, however, do inhibit BCRP quite efficiently. Estrogens such as estrone and 17β-estradiol, as well as the bile salt taurolithocholate efficiently inhibit BCRP-mediated transport of topotecan, mitoxantrone, SN-38, PhIP and Hoechst 33342 in BCRP-expressing cells and membrane vesicles (Imai et al., 2002; 2003; Janvilisri et al., 2003; this study). Moreover, their sulfated conjugates have the same or even stronger inhibitory potency to BCRP-mediated transport of [^{3}H]estrone 3-sulfate in membrane vesicles (Imai et al., 2003). Therefore, the transport functions of BCRP under physiological conditions might perhaps be affected by endogenous steroids or their conjugates, especially in tissues with high levels of steroids such as the liver.

In summary, this study illustrates the usefulness of the generated BCRP-expressing cell lines in better understanding the impact of human BCRP on the pharmacology and toxicology of a range of steroid and other drugs, steroid hormones and of dietary carcinogens.

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Legends for Figures:

Figure 1. Expression of BCRP cDNA in transduced MEF3.8 (A) and MDCKII (B) cell lines. Western blot analysis was performed with 40 μg of cellular protein using monoclonal antibody BXP-21 (dilution 1:500). BCRP is detected as a band of about 70 kDa. Position of molecular weight markers is indicated.

Figure 2. Effect of inhibitors on accumulation of mitoxantrone in parent MEF3.8 and MDCKII cells and in their BCRP-transduced derivatives. Representative histograms documenting mitoxantrone accumulation (shown in terms of relative arbitrary units of mitoxantrone fluorescence) in both MEF3.8-parent (A) and MEF3.8-BCRP (B) cell lines, 1 hour after adding 10 μM of mitoxantrone. *Narrow line*, fluorescence in a control experiment without inhibitor; *bold line*, fluorescence, where BCRP was inhibited by 1 hour of preincubation with 1 μM Ko143; *shaded histogram*, endogenous cellular fluorescence without mitoxantrone treatment. Mitoxantrone accumulation in cells preincubated with or without Ko143 (C), GF120918 (D) or PSC833 (E) at the indicated concentrations. Results are expressed as means; the error bars indicate the standard deviations from at least three experiments $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

Figure 3. Transepithelial transport of [^{14}C]topotecan (2 μM) across MDCKII-parent and MDCKII-BCRP monolayers. The experiment was started with the application of [^{14}C]topotecan to one compartment (basal or apical). The percentage of radioactivity appearing in the opposite compartment was measured and plotted. The BCRP inhibitor Ko143 (B), P-gp inhibitor PSC833 (C) or estradiol (D) were added in both compartments. Results are presented as the means; the error bars indicate the standard deviations from at least three experiments. Translocation from the basolateral compartment to apical compartment (■); translocation from the apical compartment to the basolateral compartment (○). *r* – transport ratio; *Ap, Bl* - percentage of total radioactivity applied to the apical (*Ap*) or basolateral (*Bl*) compartment that was retrieved from the cell layer.

Figure 4. Transepithelial transport of [^{14}C]PhIP (2 μM) across MDCKII-parent and MDCKII-BCRP monolayers. The experiment was started with the application of [^{14}C]PhIP to one compartment (basal or apical). The percentage of radioactivity appearing in the opposite compartment was measured and plotted. The BCRP inhibitor Ko143 (B), beclomethasone (C), 6α-methylprednisolone (D) or estradiol (E) were added in both compartments. Results are presented as the means; the error bars indicate the standard deviations from at least three experiments. Translocation from the basolateral compartment to apical compartment (■); translocation from the apical compartment to the basolateral compartment (○). *r* – transport ratio; *Ap, Bl* - percentage of total radioactivity applied to the apical (*Ap*) or basolateral (*Bl*) compartment that was retrieved from the cell layer.

Figure 5. Transepithelial transport of [^{3}H]dexamethasone (A), [^{3}H]corticosterone (B), [^{3}H]aldosterone (C), [^{3}H]digoxin (D), and [^{3}H]estradiol (E-H) across MDCKII-parent and MDCKII-BCRP monolayers. The experiment was started with the application of tested compound to one compartment (basal or apical) at a concentration of 2 μM. The percentage of radioactivity appearing in the opposite compartment was measured and plotted. The BCRP inhibitor Ko143 (1 μM) was present as indicated (G and H). Results are the means; the error bars indicate the standard deviations from at least three experiments. Translocation from the basolateral compartment to apical compartment (■); translocation from the apical compartment to the basolateral compartment (○). *r* indicates the transport ratio.

Figure 6. Transepithelial transport of [^{3}H]cimetidine across MDCKII-parent (A), MDCKII-BCRP (B), MDCKII-Bcrp1 (D), MDCKII-MDR1 (F) and MDCKII-MRP2 (G) monolayers. The experiment was started with the application of tested compound to one compartment (basal or apical) at a concentration of 5 □M. The percentage of activity appearing in the opposite compartment was measured and plotted. The BCRP inhibitor Ko143 (5 □M) was present as indicated (C, E). Results are the means; the error bars indicate the standard deviations from at least three experiments. Translocation from the basolateral compartment to apical compartment (■); translocation from the apical compartment to the basolateral compartment (○). *r* indicates the transport ratio.

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Table 1. **Interactions of steroid drugs and some endogenous steroid hormones with BCRP-mediated transport of mitoxantrone.** MEF 3.8 BCRP and its parental subline were pre-incubated with a test compound for 1 hour, then 10 μM mitoxantrone was applied to the medium, accumulation allowed for 1 hour and cells were subsequently evaluated by flow cytometry.

test compound	concentration	Inhibitory potency (%)[#]
Beclomethasone[##]	20 μM	40.4 ± 3.2**
6α-methylprednisolone	50 μM	21.5 ± 2.2**
Corticosterone	50 μM	20.7 ± 4.0*
Triamcinolone	50 μM	15.3 ± 4.3*
Dexamethasone	50 μM	13.2 ± 2.4**
Betamethasone	50 μM	10.9 ± 3.5*
Prednisone	50 μM	7.4 ± 1.6*
Dexamethasone 21-phosphate	50 μM	0.5 ± 1.5
Hydrocortisone	50 μM	-0.4 ± 1.8
Prednisolone	50 μM	-2.9 ± 1.6
Aldosterone	50 μM	-3.2 ± 5.0
Digoxin	50 μM	45.1 ± 1.7**
Ursodeoxycholic acid	50 μM	2.8 ± 4.3
Ko143	1 μM	100

[#] Inhibitory potency of tested compounds was related to the effect of reference inhibitor Ko143 at a concentration of 1 μM (set at 100% inhibition of BCRP).

[##] The solubility limit of beclomethasone in medium is 20 μM.

Data are expressed as a mean ± SD from at least three experiments.

* $p < 0.05$; ** $p < 0.01$

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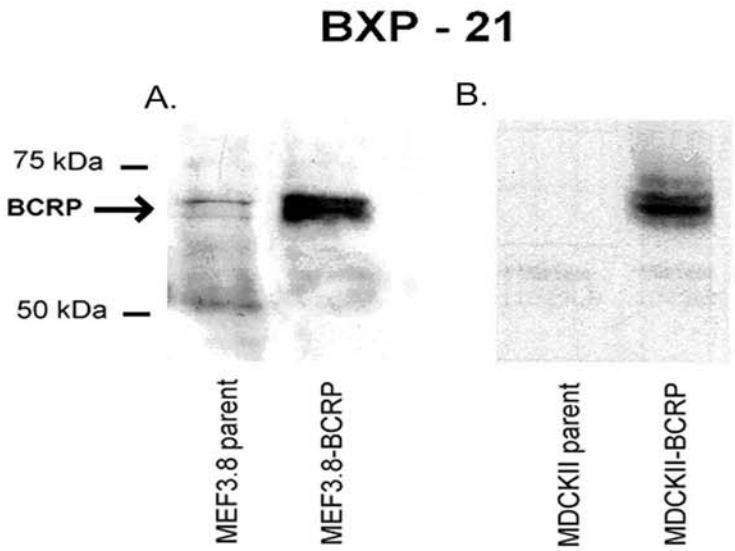
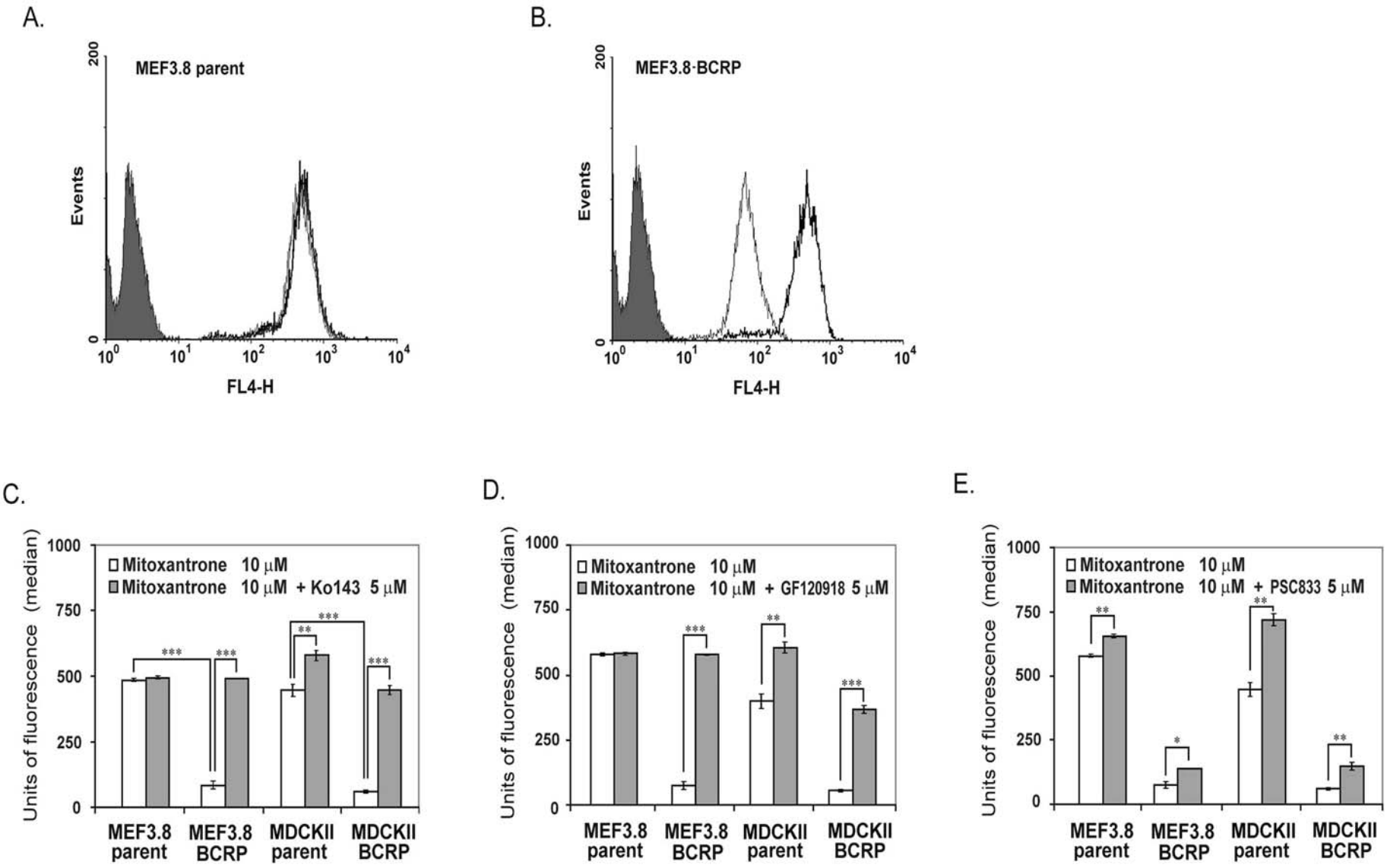
Figure 1. JPET No. 73916





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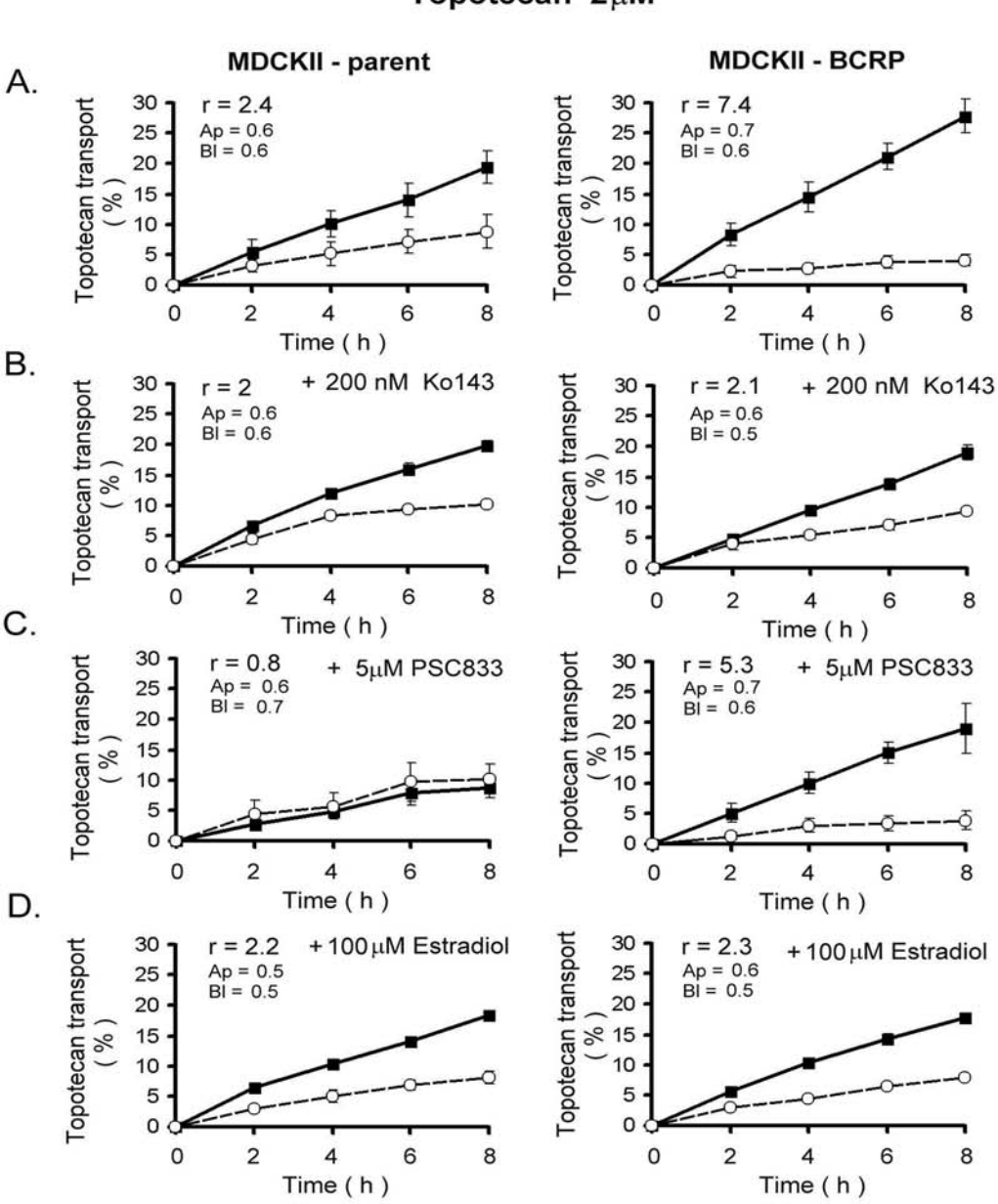

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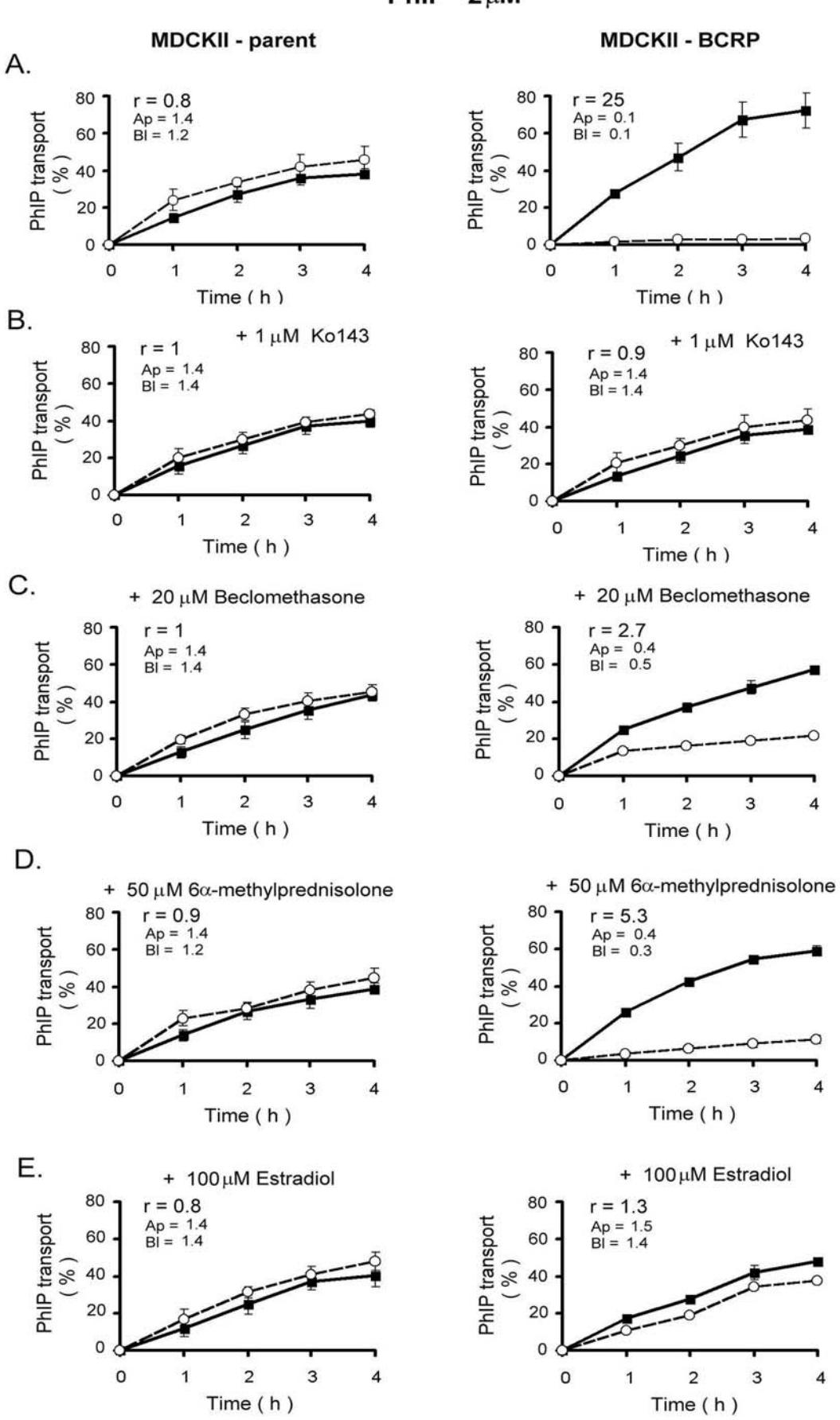

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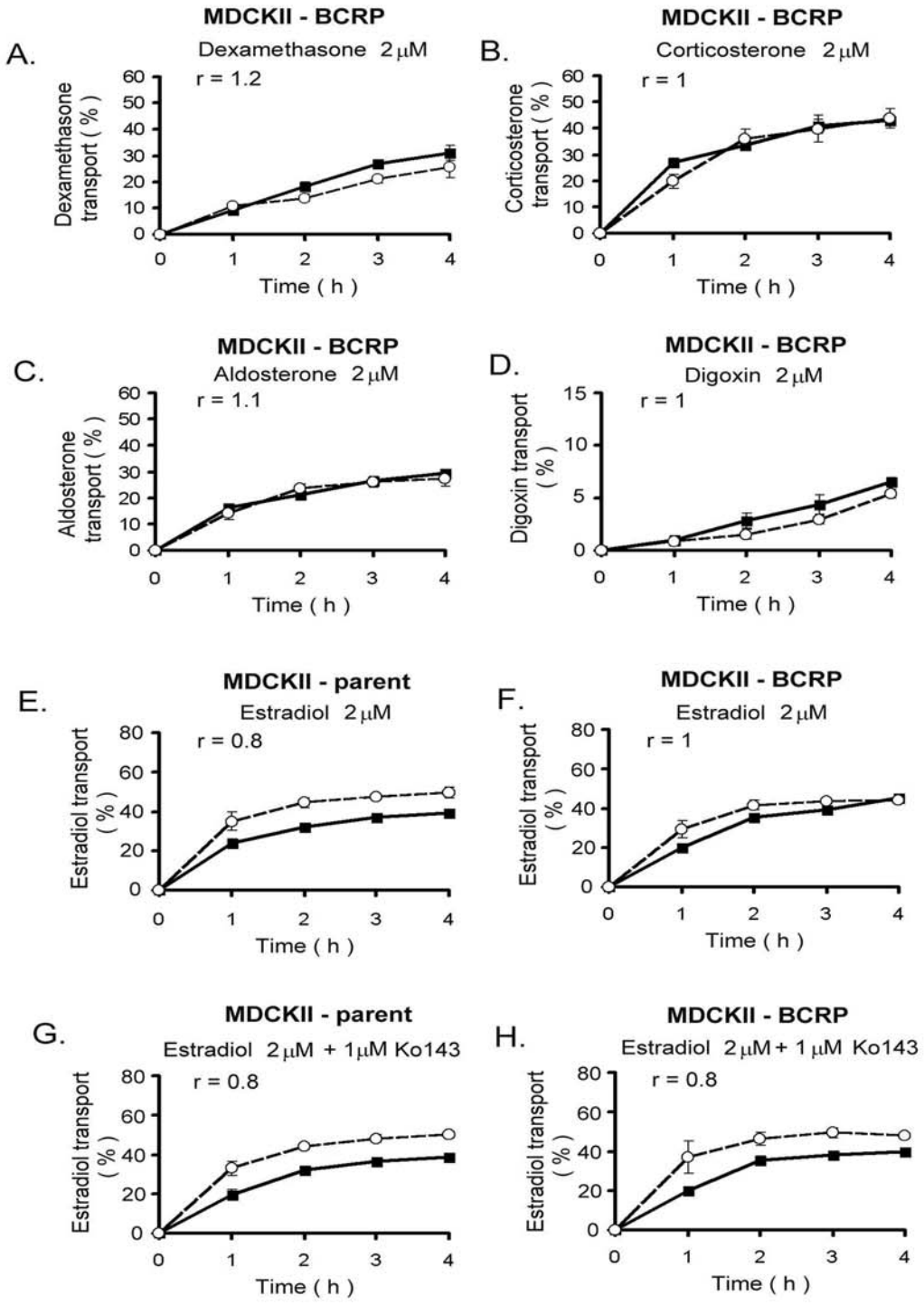

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Figure 6. JPET No. 73916