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The Effect of Low pH on Breast Cancer Resistance Protein (ABCG2)-Mediated Transport of Methotrexate, 7-Hydroxymethotrexate, Methotrexate Diglutamate, Folic Acid, Mitoxantrone, Topotecan, and Resveratrol in In Vitro Drug Transport Models

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ABSTRACT

Some cellular uptake systems for (anti)folates function optimally at acidic pH. We have tested whether this also applies to efflux from cells by breast cancer resistance protein (BCRP; ABCG2), which has been reported to transport folic acid, methotrexate, and methotrexate di- and triglutamate at physiological pH. Using *Spodoptera frugiperda*-BCRP membrane vesicles, we showed that the ATP-dependent vesicular transport of 1 μM methotrexate by BCRP is 5-fold higher at pH 5.5 than at physiological pH. The transport of methotrexate was saturable at pH 5.5, with apparent K_m and V_{max} values of 1.3 $\pm$ 0.2 mM and 44 $\pm$ 2.5 nmol/mg of protein/min, respectively, but was linear with drug concentration at pH 7.3 up to 6 mM methotrexate. In contrast to recent reports, we did not detect transport of methotrexate diglutamate at physiological pH, but we did find transport at pH 5.5. We also found that 7-hydroxy-methotrexate, the major metabolite of methotrexate, is transported by BCRP both at physiological pH and (more efficiently) at low pH. The pH effect was also observed in intact BCRP-overexpressing cells: we found a 3-fold higher level of resistance to both methotrexate and the prototypical BCRP substrate mitoxantrone at pH 6.5 as at physiological pH. Furthermore, with MDCKII-BCRP monolayers, we found that resveratrol, which is a neutral compound at pH $\leq$ 7.4, is efficiently transported by BCRP at pH 6.0, whereas we did not detect active transport at pH 7.4. We conclude that BCRP transports substrate drugs more efficiently at low pH, independent of the dissociation status of the substrate.

Uptake of weak acid and weak base chemotherapeutic drugs by tumors is greatly influenced by the dissociation properties of the drug itself and by the cellular pH gradient (i.e., the difference of extracellular pH in the tumor and the intracellular pH maintained by the cells) (Tannock and Rotin, 1989; Boyer and Tannock, 1992; Kozin et al., 2001; Mahoney et al., 2003). Whereas the median pH value in normal tissues is 7.5, in many tumor tissues, the extracellular pH is more acidic and may be as low as 5.8 (Tannock and Rotin, 1989). This is a consequence of a high rate of lactic acid production in tumors even under aerobic conditions (Tannock and Rotin, 1989; Boyer and Tannock, 1992; Gatenby and

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ABBREVIATIONS: MTX, methotrexate; BCRP, breast cancer resistance protein; MRP, multidrug resistance-associated protein; 7-OH-MTX, 7-hydroxy-methotrexate; MTX-glu2, methotrexate diglutamate; Sf9, *Spodoptera frugiperda*; HPLC, high-performance liquid chromatography; LY335979, zosuquidar trihydrochloride; GF120918, *N*-(4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinolinyl)ethyl]-phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide; SN-38, 7-ethyl-10-hydroxycamptothecin; solution A, formic acid and acetonitrile 5%; solution B, formic acid and acetonitril 23%; Ko143, 3-(6-isobutyl-9-methoxy-1,4-dioxo-1,2,3,4,6,7,12,12*a*-octahydropyrazino[1′,2′:1,6]pyrido[3,4-*b*]indol-3-yl)-propionic acid *tert*-butyl ester.

Gillies, 2004). The intracellular pH of mammalian cells is normally maintained within a narrow range (i.e., from 7.1 to 7.3) and is tightly regulated by specific ion exchangers, and this also holds for tumor cells (Moolenaar et al., 1984; Boyer and Tannock, 1992). As a consequence, tumor cells are exposed to a pH gradient that can enhance the toxicity of certain weak acid drugs and impair the uptake of weak bases (Tannock and Rotin, 1989; Boyer and Tannock, 1992; Kozin et al., 2001; Mahoney et al., 2003; Greijer et al., 2005).

Folic acid and the structurally related antifolate drug methotrexate (MTX) and its metabolite 7-hydroxy-methotrexate (7-OH-MTX) and polyglutamates of MTX contain several dissociable groups, as shown in Fig. 1 (Poe, 1977). In the case of MTX, the dissociation constants of the γ-carboxyl (pK_a 4.7), α-carboxyl (pK_a 3.4), and N(1) of the pteridine-ring (pK_a 5.7) determine the overall dissociation status in the pH range from 5.8 to 7.6 (Fig. 1). Both at physiological pH and at low tumor pH, the two carboxyl groups of MTX are predominantly in their negatively charged dissociated form (i.e., ≥ 99.8% at pH 7.3 and ≥99.6% at pH 5.8 for total [COO^-]), as calculated from the Henderson-Hasselbalch equation (Rowland and Tozer, 1995). However, at pH 6, a substantially higher percentage of the N(1) of MTX is in its protonated form than at physiological pH. The N(1) dissociation constant of MTX is considerably more basic than of folates, which have a pK_a value of N(1) of 1.2 to 2.4 (Rowland and Tozer, 1995). At pH 6, 33% of MTX exists in its $MTX^{+\ -\ -}$ zwitterionic form, whereas for folic acid, this is only 0.007% (Rowland and Tozer, 1995). The ABC drug transporter BCRP (ABCG2) has been reported to transport folic acid, MTX, and MTX di- and triglutamate (Chen et al., 2003; Mitomo et al., 2003; Volk and Schneider, 2003) and is consistent with the folate exporter function to have a possible role in the maintenance of cellular folate homeostasis (Ifergan et al., 2004). How BCRP transports its substrates and whether BCRP transports substrates preferably in their undissociated or dissociated form is not known in detail.

Several studies have identified in normal intestinal cells and in tumor cells a folate transport system that operates optimally at acidic pH (Sierra et al., 1997; Assaraf et al., 1998; Said, 2004; Zhao et al., 2004). We hypothesized that low pH might also affect BCRP-mediated transport of (anti-)folates, either by affecting the dissociation status of these compounds or by affecting the protein structure of BCRP. To test this hypothesis, we first investigated whether low pH affected the BCRP-mediated transport of (anti)folates using inside-out membrane vesicles from Sf9 cells infected with baculovirus containing human *BCRP* cDNA. In addition, we studied the effect of low pH on the BCRP-mediated efflux on MTX sensitivity for intact BCRP-overexpressing MDCKII cells.

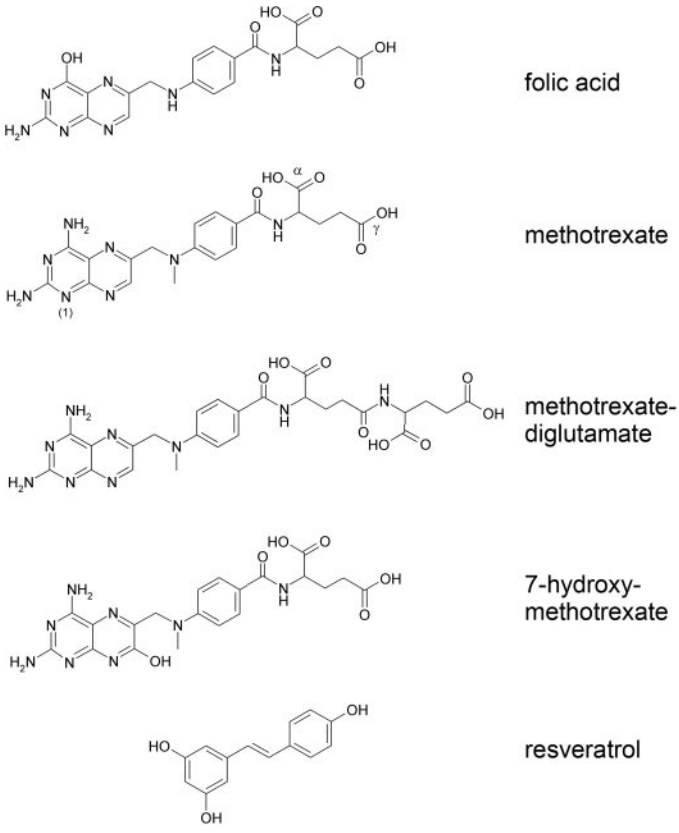

Fig. 1. Structural formulas of the studied compounds.

Materials and Methods

Materials. [^{3}H]MTX was obtained from GE Healthcare (Little Chalfont, Buckinghamshire, UK). [^{3}H]MTX-glu2, [^{3}H]folic acid, [^{3}H]resveratrol, and resveratrol were from Moravek Biochemicals (Brea, CA). MTX-glu2 and folic acid were from Schircks Laboratories (Jona, Switzerland). Topotecan (Hycamtin) and [^{14}C]topotecan were obtained from SmithKline Beecham Pharmaceuticals (King of Prussia, PA). Mitoxantrone dihydrochloride was purchased from Sigma Chemical Co. (St. Louis, MO) Pantoprazole (Pantozol 40 mg i.v.; Altana Pharma, Hoofddorp, the Netherlands) was obtained from the pharmacy of the Netherlands Cancer Institute. Elacridar (GF120918) was a generous gift from GlaxoSmithKline (Research Triangle Park, NC). Zosuquidar trihydrochloride (LY335979) was kindly provided by Eli Lilly (Indianapolis, IN). Monoclonal antibody, 5D3, detecting an external epitope BCRP, was obtained from Chemicon International Inc. (Temecula, CA). W6/32 monoclonal antibody (detecting MHC class I) and fluorescein isothiocyanate-labeled rabbit F(ab)$_2$ anti-mouse Ig were purchased from Dako Denmark A/S (Glostrup, Denmark). Ko143 (Allen et al., 2003) was kindly provided by Professor G. J. Koomen (Department of Organic Chemistry, University of Amsterdam, Amsterdam, the Netherlands).

Preparation of 7-OH-MTX and [^{3}H]7-OH-MTX. 7-OH-MTX was prepared as described by Cairnes and Evans (1983). In brief, fresh rabbit liver (80 g) was mechanically homogenized at 0°C in 500 ml of 10 mM Tris-HCl, pH 7.6, containing 20 mM $MgCl_2$. The homogenate was centrifuged at 1000*g* for 20 min at 4°C. MTX (200 mg) was added to the supernatant, which was then immediately incubated at 37°C for 2 h with constant shaking and aeration. Then, the solution was boiled for 15 min with constant shaking, filtered, and cooled down to room temperature. This resulted in precipitation of the dark yellow 7-OH-MTX. Next, the solution was filtered using a Buchner funnel. Crystals were collected from the filter and dissolved in 10 ml of distilled water, adjusted to pH 7 to 8. The purity of 7-OH-MTX was determined by HPLC analysis. The chromatographic system consisted of a Waters 616 dual piston pump, a Waters 717 Plus autosampler, and a thermostated column compartment. Elutions were performed using a Chrompack ODS-2 glass column (5 μm; 100 × 3 mm). Ultraviolet detection was performed at 308 nm using a SPECTRA system UV2000 (Thermo Electron Corp., Waltham, MA). Retention times and peak areas were analyzed with Waters Millennium integration software. Mobile phases consisted of a mixture of 10 mM formic acid and acetonitrile 5% (v/v) adjusted to pH 3.5 (solution A) and of a mixture of 10 mM formic acid and acetonitrile 23% (v/v), pH 3.5 (solution B). The column was eluted with a gradient

linearly increasing from 0 to 70% of solution B during 19 min. The flow rate was 1.0 ml/min, and all of the separations were carried out at 35°C. HPLC analysis revealed a 99.8% purity of the compound, and mass spectrometry produced a protonated molecular ion $(M + H)^+$ at *m/z* 471 Da, indicating a molecular weight of 470, compatible with 7-OH-MTX (data not shown).

The preparation of [^{3}H]7-OH-MTX was different from the preparation of 7-OH-MTX for the following steps: a smaller fraction of the supernatant was used (i.e., 250 μCi of [^{3}H]MTX in 550 μl of 10 mM Tris-HCl, pH 7.6) containing 20 mM $MgCl_2$ was added to 50 μl of liver homogenate. After the filtration step, the solution was lyophilized overnight. Then, the pellet was dissolved in 150 μl of solution A. Purity was checked by HPLC coupled to an online radioisotope detector (Radiomatic 500 TR flow scintillation analyzer; Canberra Industries, Meriden, CT). This revealed a 70.6% purity of [^{3}H]7-OH-MTX and 29.4% of [^{3}H]MTX. Further purification was achieved by loading the HPLC column with 50 μl of supernatant and collecting the 7-OH-MTX fraction from 14 to 16 min. This was repeated twice, and then fractions were pooled. After lyophilization, the pellet was dissolved in 500 μl of purified water adjusted to pH 8. Radiochemical analysis now revealed a purity of 99.0% [^{3}H]7-OH-MTX, 0.2% [^{3}H]MTX, and 0.8% unknown. The concentration of 7-OH-MTX was 46.5 μM, as determined spectrophotometrically at 308 nm.

Preparation of Membrane Vesicles and Vesicular Transport Assays. Membrane vesicles from Sf9 cells were prepared in isotonic vesicle buffer consisting of 50 mM Tris and 250 mM sucrose adjusted to pH 7.4. The experiments shown in Fig. 2C were performed with vesicles from BCRP-transfected Sf9 cells prepared with vesicle buffer adjusted to a pH of either 5.5 or 7.3. Vesicular transport assays were performed in vesicular transport assay buffer (50 mM HEPES/KOH, 100 mM KCl) as described before (Breedveld et al., 2004). The vesicular transport assay buffer and the solutions of all other reagents used in transport assays were adjusted to the appropriate pH (range, 5.5 to 8.0) by adding aliquots of HCl (37% w/w) or 2 M NaOH. The pH of the final solution was controlled and was in the range of pH 5.1 to 7.9. It is noteworthy that at pH 6.5 or lower, when 4 mM ATP (adjusted to the pH of the vesicular buffer) was added to the reaction mixture, the final pH of the solution decreased approximately 0.5 log. This is due to the formation of Mg^{2+}-ATP complexes, thereby releasing H^+, and the weak buffer capacity of the HEPES buffer at pH ≤6.5. The time- and concentration-dependent uptake of substrates into membrane vesicles was studied by following the rapid-filtration method as described previously (Breedveld et al., 2004). The ATP-dependent transport was calculated by the difference in transport in the presence or absence of ATP.

Transport Across MDCKII Monolayer. MDCKII cells were cultured as described before (Breedveld et al., 2004). Polarized MDCKII cells stably expressing human BCRP (ABCG2) cDNA were kindly provided by Alfred Schinkel (Netherlands Cancer Institute, Amsterdam, the Netherlands) (Pavek et al., 2005). Transepithelial transport assays were performed as described previously (Breedveld et al., 2004). Media were adjusted to pH 6.0, 6.5, or 7.4 with HCl (37% w/w) and—in case of transport of topotecan—buffered with 25 mM HEPES immediately before the addition of radiolabeled substrate (i.e., resveratrol or topotecan).

Growth Inhibition Assay. Parental MDCKII cells and MDCKII cells stably overexpressing human BCRP were cultured as described

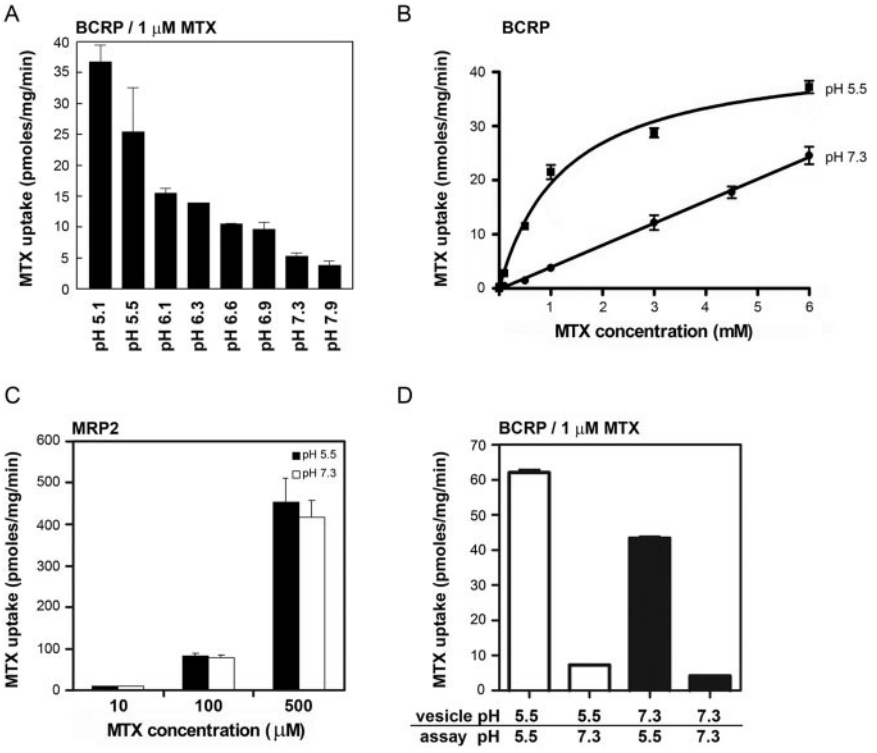

Fig. 2. Effect of pH on ATP-dependent transport of MTX by BCRP. A, effect of pH on ATP-dependent transport of 1 μM [^{3}H]MTX by BCRP. Sf9-BCRP membrane vesicles were incubated with 1 μM [^{3}H]MTX at the indicated pH at 37°C for 5 min in the presence or absence of 4 mM ATP. The ATP-dependent uptake of MTX into Sf9-BCRP membrane vesicles is plotted in picomoles per milligram of protein per minute. B, concentration-dependence of MTX transport by BCRP at pH 5.5 (■) versus pH 7.3 (●). Sf9-BCRP membrane vesicles were incubated with the concentrations of [^{3}H]MTX and at the pH indicated at 37°C for 5 min in the presence or absence of 4 mM ATP. The ATP-dependent uptake of MTX into Sf9-BCRP membrane vesicles is plotted in nanomoles per milligram of protein per minute. C, comparison of MTX transport at pH 5.5 and 7.3 by vesicles prepared at pH 5.5 (□) or pH 7.3 (■). Vesicles were prepared from Sf9-BCRP cells at physiological pH 7.3 or pH 5.5 (see *Materials and Methods*) and incubated exactly as in A at pH 5.5 or pH 7.3. The ATP-dependent uptake of MTX into Sf9-BCRP membrane vesicles is plotted in picomoles per milligram of protein per minute. D, MTX transport by MRP2 at pH 5.5 (■) versus pH 7.3 (□). Sf9-MRP2 membrane vesicles were incubated with 10, 100, or 500 μM [^{3}H]MTX at 37°C for 5 min in the presence or absence of 4 mM ATP. The ATP-dependent uptake of MTX into Sf9-BCRP membrane vesicles is plotted in picomoles per milligram of protein per minute. Values shown are means ± S.E. of triplicate (A, B, and D) or quintuplicate (C) experiments.

before (Breedveld et al., 2004; Pavek et al., 2005). The growth inhibition assay with MTX was performed essentially as described before (Hooijberg et al., 1999; Ifergan et al., 2004). In brief, cells were plated in 1 ml of medium in individual wells of a 24-well plate at an initial density of 1.25×10^4 cells/cm^2. One day after cell plating, cell culture medium was replaced by Opti-MEM medium (Invitrogen) adjusted to pH 7.4 or 6.5, and cells were allowed to habituate for 1 h. MTX was added at 12 different concentrations covering a concentration range from 1 to 2500 μM. Cells were exposed to MTX for a short term (i.e., 4 h). After short-term exposure at 37°C in a 5% CO_2 humidified incubator, the medium was aspirated, and cells were washed four times with 2 ml of drug-free medium (over 10-min time intervals and at 37°C), whereas after 68 h, cells were collected by trypsinization and counted using a hemocytometer and trypan blue exclusion, as described previously (Hooijberg et al., 1999; Ifergan et al., 2004). The growth inhibition assay with mitoxantrone was performed essentially as described for MTX, except that mitoxantrone was added at 10 different concentrations covering a concentration range from 1 nM to 1 μM.

Flow Cytometric Analyses. A monoclonal antibody 5D3, recognizing an external epitope of BCRP, was used to assess potential conformation changes in BCRP protein as a function of extracellular pH (Özvegy-Laczka et al., 2005b). Because BCRP-transfected MDCKII cells harbor a green fluorescent protein construct that interferes with flow cytometric analyses (Pavek et al., 2005), we performed these experiments with another BCRP-overexpressing cell line, MCF7/MR human breast cancer cells (Maliepaard et al., 2001). In brief, MCF7/WT and MCF7/MR cells were trypsinized and incubated at a concentration of 2×10^6 cells/ml in Opti-MEM medium/1% bovine serum albumin adjusted to pH 6.5 or 7.4. All further incubation and washing steps were performed in Opti-MEM/1% bovine serum albumin adjusted to the appropriate pH. Per sample, 2×10^5 cells/100 μl were coincubated at room temperature for 1 h with 100 μl 5D3 (1:100). As a positive staining control and as a control for the absence of a general effect of pH on antibody binding affinity, W6/32, anti MHC class I, (1:20) was used under similar incubation conditions. As a negative control, the primary antibody was omitted. After three washing steps, the cells were incubated with fluorescein isothiocyanate-labeled rabbit $F(ab)_2$ anti-mouse Ig for 1 h at room temperature (1:100) and after another round of washing was analyzed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) equipped with CellQuest analysis software.

Kinetic and Statistical Analysis. Curve fitting was performed by nonlinear regression analysis using the software package Prism version 4 (GraphPad Software, Inc., San Diego, CA). Data from concentration-dependent transport assays were analyzed according to the classic Michaelis-Menten model (Rowland and Tozer, 1995): $v = (V_{max} \times [S])/(K_m + [S])$, where v is the rate of transport, V_{max} is the maximum velocity, S is the substrate concentration, and K_m is the substrate concentration at half-maximum velocity. The two-sided unpaired Student's t test was used throughout to assess the statistical significance of the difference between two sets of data. Results are presented as means ± S.E. Differences were considered to be statistically significant when $P < 0.05$.

Results

The Effect of Low pH on Transport of MTX by BCRP (ABCG2). It has been reported that BCRP (ABCG2) is a low-affinity, high-capacity transporter of MTX at physiological pH (Chen et al., 2003; Volk and Schneider, 2003). With human embryonic kidney 293-ABCG2-R482 membrane vesicles, Chen et al. (2003) found K_m and V_{max} values of 1.3 ± 0.2 mM and 0.7 ± 0.1 nmol/mg of protein/min, respectively, whereas Volk and Schneider (2003) found K_m and V_{max} values of 0.7 ± 0.3 mM and 2.4 ± 0.4 nmol/mg of protein/min with membrane vesicles from MCF7/mitoxantrone cells. By using Sf9-BCRP membrane vesicles, we studied the effect of pH (range, 5.1–7.9) on the BCRP-mediated transport of MTX and compared this to the transport of MTX at the same chosen pH values in Sf9 wild-type vesicles. The ATP-dependent transport of 1 μM MTX by BCRP was 7-fold higher at pH 5.1 than at physiological pH, as shown in Fig. 2A, and increased gradually with increasing $[H^+]$ concentration (Fig. 2B). The pH effect seemed to be due to an effect on K_m, not on V_{max}, as shown in Fig. 2B. At pH 5.5, the transport of MTX is saturable, with apparent K_m and V_{max} values of 1.3 ± 0.2 mM and 44 ± 2.5 nmol/mg of protein/min, respectively. At pH 7.3, the transport rate increased linearly with the MTX concentration up to 6 mM, and we were unable to saturate the transport by BCRP with substrate. In Sf9 wild-type membrane vesicles, there was no ATP-dependent uptake of 500 μM MTX at pH 7.3 over the first 5 min. At pH 5.1, the uptake was minimal (approximately 0.07 nmol/mg/min) compared with the uptake in Sf9-BCRP membrane vesicles (approximately 14 nmol/mg/min).

To verify whether the observed pH effect was specific for BCRP-mediated transport of MTX, we studied the effect of low pH on the transport of MTX by MRP2 (ABCC2) in Sf9-MRP2 membrane vesicles. This transporter is known to transport MTX (Hooijberg et al., 1999) and is located in apical membranes, like BCRP. No significant difference was found between transport at pH 5.5 and 7.3 (Fig. 2D), suggesting that the increase of MTX transport at low pH is specific to BCRP. In theory, a pH gradient over the vesicular membrane might arise when vesicles are prepared at a different pH than at which the vesicular transport assay is performed. The possibility that the increase in BCRP-mediated MTX transport at low pH (Fig. 2A) was due to an artifactually introduced pH gradient over the vesicular membrane was ruled out by repeating the experiments with Sf9-BCRP vesicles that had been prepared at the same pH as at which MTX transport was subsequently determined. The results in Fig. 2C demonstrate that the preparation of vesicles at low pH had no effect on transport: BCRP-mediated MTX transport was much higher at pH 5.5 than at pH 7.3, regardless of the pH at which the vesicles were made.

In addition to the membrane vesicle studies, we investigated whether low pH also affects BCRP-mediated efflux of MTX from intact cells under conditions in which no extensive polyglutamylation of MTX takes place (≤4 h of exposure) (Hooijberg et al., 1999). For this purpose, we assessed the cell growth inhibitory effects of MTX after short-term exposure to MDCKII-BCRP cells at pH 7.4 and at pH 6.5 and compared this with the cell growth inhibitory effects of MTX after short-term exposure to control cells at the same pH values. As shown in Fig. 3 and listed in Table 1, the IC_{50} value of MTX for parental MDCKII cells was similar at pH 6.5 and at physiological pH. Resistance to MTX was 12.9-fold increased when BCRP was overexpressed in the MDCKII cells at physiological pH, which is in line with earlier findings by Ifergan et al. (2004) showing BCRP-mediated resistance to MTX in BCRP overexpressing MCF-7 cells. At pH 6.5, resistance to MTX was 3.3-fold further increased compared with resistance to MTX at pH 7.4 in the MDCKII-BCRP cells (Fig. 3, Table 1), indicating that low pH BCRP-mediated transport of MTX also confers a higher level of MTX resistance in intact cells.

The Effect of Low pH on BCRP (ABCG2)-Mediated Transport of MTX-glu2 and Folic Acid. Wild-type BCRP (ABCG2-R482) was reported to be capable of transporting MTX di- and triglutamate and folic acid at neutral pH (Chen et al., 2003; Mitomo et al., 2003; Volk and Schneider, 2003). We also found a low rate of ATP-dependent transport of MTX-glu2 by BCRP at pH 5.5 (Fig. 4A), but not at physiological pH. We observed low ATP-dependent transport of folic acid by BCRP at pH 7.3, but transport was 2- to 5-fold higher at pH 5.5, as shown in Fig. 4B. At both pH 7.3 and 5.5, the transport rate increased linearly with increasing concentration of folic acid, and we were unable to saturate the transport by BCRP with substrate. Concentrations greater than 1 mM could not be tested because of limited solubility. To verify whether the observed pH effect was specific for BCRP-mediated transport of folic acid, we studied the effect of low pH on the transport of folic acid by MRP5 (ABCC5), a transporter that was recently shown to transport folic acid (Wielinga et al., 2005). We found no significant difference in the transport of 10 μM folic acid by MRP5 at pH 5.5 and 7.3 (data not shown), suggesting that the increased folate transport at low pH is specific for BCRP.

Transport of 7-OH-MTX by BCRP (ABCG2) at Physiological and at Low pH. Given the ability of BCRP to transport several folate derivatives, we also tested transport of ^{3}H-labeled 7-OH-MTX, the major metabolite of MTX, in our vesicular transport system. As shown in Fig. 5, 9 μM 7-OH-MTX is transported by BCRP both at physiological pH and (more efficiently) at pH 5.5. The ATP-dependent uptake rate over the first 5 min at pH 7.3 was 27 (Fig. 5A) and 63 pmol/mg/min at pH 5.5 (Fig. 5B). Both at pH 7.3 and 5.5 the transport rate increased linearly with the 7-OH-MTX concentration and was approximately 3-fold higher at pH 5.5 than at 7.3, as shown in Fig. 5C. Concentrations greater than 100 μM could not be tested because of limited solubility at both pH 7.3 and 5.5. The proton-pump inhibitor pantoprazole inhibited the BCRP-mediated transport of 9 μM 7-OH-MTX in a concentration-dependent manner (Fig. 5D). A concentration of 10 μM pantoprazole inhibited the transport by 40 $\pm$ 6%, which is comparable with the inhibition of MTX transport (Breedveld et al., 2004). We also determined whether MRP2 was able to transport 7-OH-MTX. The ATP-dependent uptake of 9 μM 7-OH-MTX into the Sf9-MRP2 membrane vesicles was 10.1 $\pm$ 0.46 pmol/mg of protein/min at pH 7.3, which is 2.7-fold lower than the transport by BCRP. However, in contrast to the 3-fold higher transport of 7-OH-MTX by BCRP at pH 5.5 relative to pH 7.3, the transport by MRP2 was 1.2-fold lower at pH 5.5 than at 7.3 (data not shown).

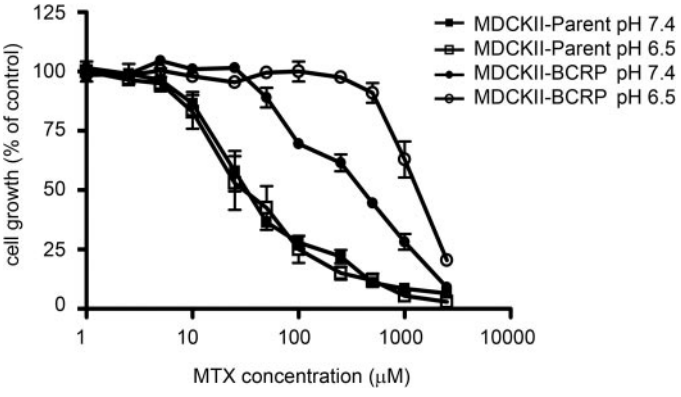

Fig. 3. Effect of pH on growth inhibition of BCRP overexpressing cells by MTX parental MDCKII cells and MDCKII cells stably overexpressing human BCRP were incubated with MTX at 12 different concentrations (in 1 ml of Opti-MEM per compartment using a 24-well plate), covering a concentration range from 1 to 2500 μM, at the indicated pH at 37°C for 4 h. Cellular growth inhibition with MTX was determined as described under *Materials and Methods*. Cell growth is plotted as a percentage of control. Values shown are means $\pm$ S.D. of four independent experiments.

TABLE 1
Effect of pH on growth inhibition of BCRP overexpressing cells by MTX during 4-h exposure

pH	IC_{50}[a]		RF[b]
	MDCKII Cells	MDCKII-BCRP Cells	
	μM		
7.4	33 $\pm$ 7	420 $\pm$ 5[c]	13
6.5	33 $\pm$ 11	1419 $\pm$ 209[c]	43

[a] IC_{50} values are presented as means $\pm$ S.D. of four experiments.
[b] RF, resistance factor, relative to parental MDCKII cells.
[c] Significantly different from parental, $P < 0.05$.

The Effect of Low pH on Transport of the BCRP Substrate Drugs Mitoxantrone and Topotecan. To verify whether the pH effect is also observed for established BCRP substrates other than folates and antifolates, we studied the effect of low pH on the BCRP-mediated transport of the weak base mitoxantrone, which has a pK_a value of 8.3 (Doyle et al., 1998; Mahoney et al., 2003) and the weak acid topotecan (Gabr et al., 1997; Maliepaard et al., 1999), which is known to undergo a pH-dependent, reversible hydrolytic dissociation of its lactone function (Underberg et al., 1990). In the case of mitoxantrone, we assessed the cell growth inhibitory effects of mitoxantrone after short-term exposure to MDCKII-BCRP cells at pH 7.4 and 6.5 and compared this with the cell growth inhibitory effects of mitoxantrone after short-term exposure to control cells at the same pH values. As shown in Fig. 6A, the IC_{50} value of mitoxantrone for parental MDCKII cells was similar at pH 6.5 and at physiological pH. Resistance to mitoxantrone was 14-fold increased when BCRP was overexpressed in the MDCKII cells at physiological pH. After incubations at pH 6.5, resistance levels for mitoxantrone were increased by another 2.8-fold compared with resistance levels for mitoxantrone at pH 7.4. At both pH values, resistance to mitoxantrone was reversible by the BCRP blocker Ko143 (data not shown).

Furthermore, we studied the human BCRP- and mouse Bcrp1-mediated transport of another prototypical BCRP substrate (i.e., topotecan) across MDCKII monolayers of cells at pH 6.5 and at physiological pH. At $t = 4$ h, we found that the BCRP- and Bcrp1-mediated transport of topotecan was increased by 1.4- and 1.6-fold, respectively, at low pH compared with physiological pH; Fig. 6B). Together, these results show that beyond (anti)folates, also the BCRP/Bcrp1-mediated transport of the established BCRP substrates mitoxantrone and topotecan is increased at low pH compared with their transport at physiological pH, independent of whether the drug is a weak base or a weak acid.

The Effect of Low pH on Transport of Resveratrol by BCRP (ABCG2). To further exclude that only the pH-dependent dissociation status of the substrate drug is responsible for the increased BCRP-mediated transport, we studied the effect of low pH on the BCRP-mediated transport of a compound that is neutral at physiological and at low pH. We used

resveratrol, a plant-derived polyphenol, which was shown recently to increase the accumulation of the BCRP substrates mitoxantrone and BODIPY-FL-prazosin in two separate BCRP-overexpressing cell lines (Cooray et al., 2004). The pK_a value of each phenol group of resveratrol is ~10, as has been determined by Blanco et al. for the polyphenolic natural

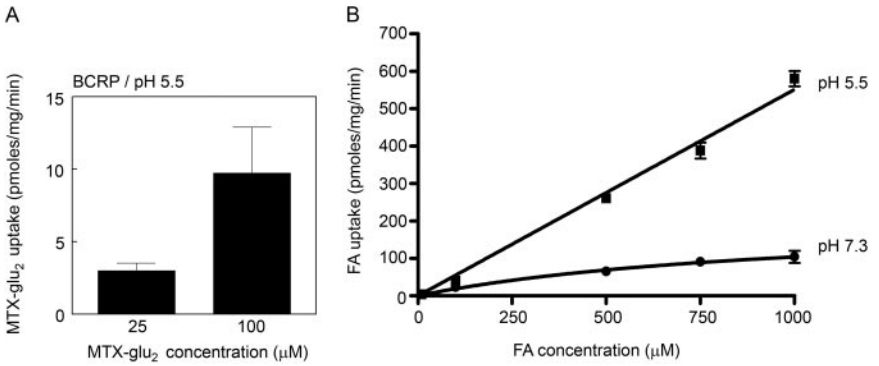

Fig. 4. Effect of low pH on ATP-dependent transport of MTX-glu2 and folic acid by BCRP. A, concentration-dependence of MTX-glu2 uptake by BCRP. Sf9-BCRP membrane vesicles were incubated with the indicated concentration of [^{3}H]MTX-glu2 at pH 5.5 at 37°C for 10 min in the presence or absence of 4 mM ATP. The ATP-dependent uptake of MTX-glu2 is plotted in picomoles per milligram of protein per minute. Values shown are means ± S.E. of each experiment in triplicate. B, concentration-dependence of folic acid transport by BCRP at pH 5.5 versus pH 7.3. Sf9-BCRP membrane vesicles were incubated with the concentrations of [^{3}H]folic acid and the pH indicated at 37°C for 10 min in the presence or absence of 4 mM ATP. The ATP-dependent uptake of folic acid is plotted in picomoles per milligram of protein per minute. Values shown are means ± S.E. of each experiment in triplicate.

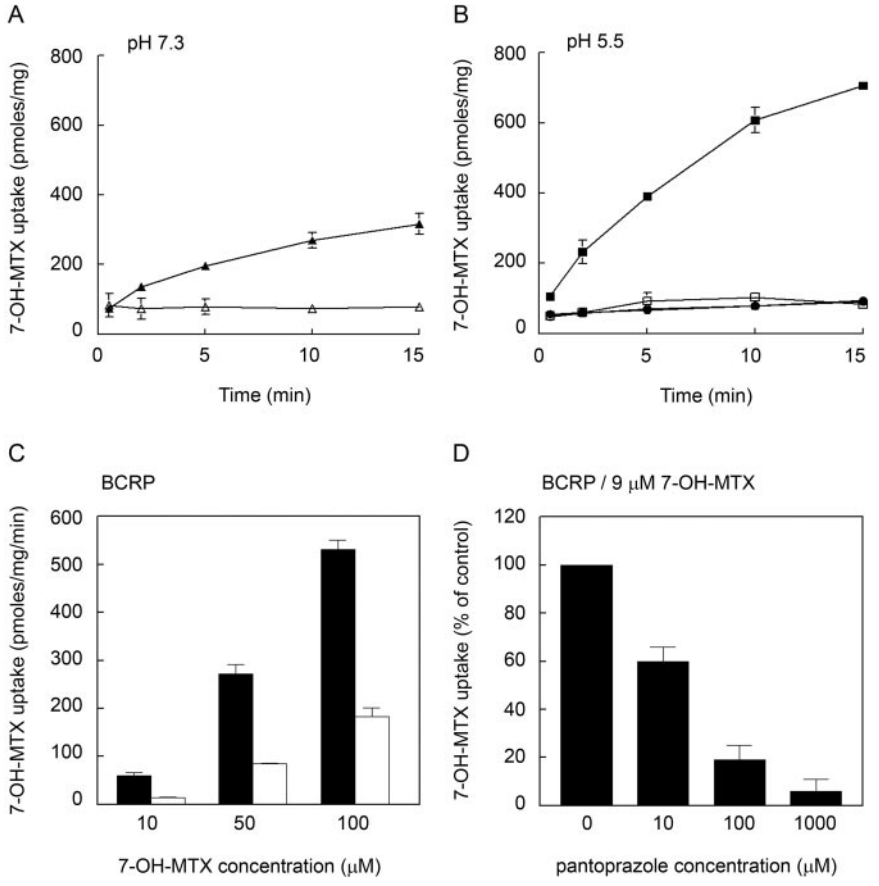

Fig. 5. ATP-dependent transport of 7-OH-MTX by BCRP. A, time course of ATP-dependent uptake of 9 μM [^{3}H]7-OH-MTX at pH 7.3 and 5.5 into membrane vesicles containing BCRP. Sf9-BCRP membrane vesicles were incubated at 37°C with 9 μM [^{3}H]7-OH-MTX at pH 7.3 (△, ▲) for the indicated time-periods in the presence (closed symbols) or absence (open symbols) of 4 mM ATP. B, membrane vesicles from Sf9 insect cells infected with a BCRP baculovirus (□, ■) or infected with a wild-type baculovirus (○, ●) were incubated at 37°C with 9 μM [^{3}H]7-OH-MTX at pH 5.5 for the indicated time periods in the presence (closed symbols) or absence (open symbols) of 4 mM ATP. Values shown are means ± S.E. of experiments in triplicate. C, concentration-dependence of 7-OH-MTX transport by BCRP at pH 5.5 versus 7.3. Sf9-BCRP membrane vesicles were incubated with the concentrations of [^{3}H]7-OH-MTX indicated at pH 5.5 (■) and pH 7.3 (□) at 37°C for 5 min in the presence or absence of 4 mM ATP. The ATP-dependent uptake of 7-OH-MTX is plotted in picomoles per milligram of protein per minute. Values shown are means ± S.E. of each experiment in triplicate. D, effect of the BCRP-inhibitor pantoprazole on ATP-dependent transport of 7-OH-MTX by BCRP. Sf9-BCRP membrane vesicles were incubated with 9 μM [^{3}H]7-OH-MTX for 5 min at 37°C at pH 7.3 in the presence or absence of the concentrations of pantoprazole indicated. The ATP-dependent transport is plotted as percentage of the control value. Values shown are means ± S.E. of each experiment in triplicate.

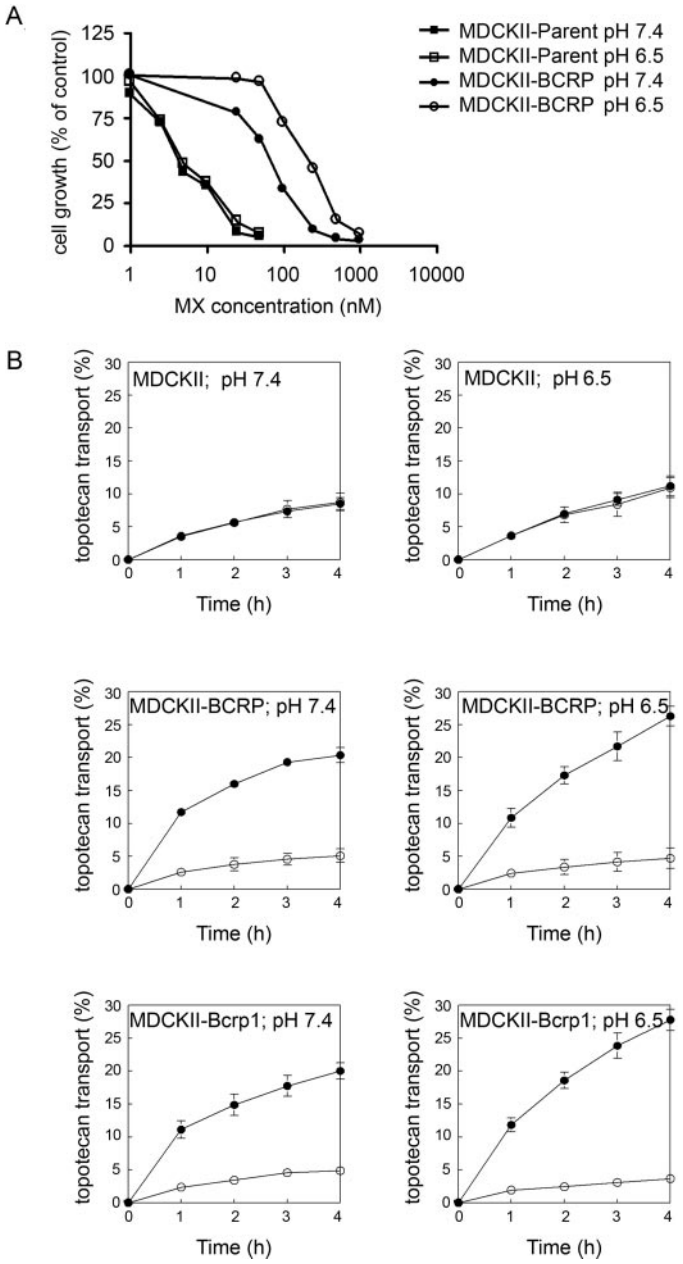

Fig. 6. Effect of pH on the transport of the established BCRP substrate drugs mitoxantrone and topotecan. A, effect of pH on growth inhibition of BCRP (ABCG2)-overexpressing cells by mitoxantrone (MX). Parental MDCKII cells and MDCKII cells stably overexpressing human BCRP were incubated with mitoxantrone at seven different concentrations (in 1 ml of Opti-MEM per compartment using a 24-well plate), covering a concentration range from 1 to 1000 nM, at the indicated pH at 37°C for 4 h. Cellular growth inhibition with mitoxantrone was determined as described under *Materials and Methods*. Cell growth is plotted as the percentage of control. Data shown are mean values of two independent experiments. B, transport of topotecan by human BCRP (ABCG2) and mouse Bcrp1 (Abcg2) at low and at physiological pH. Parental MDCKII cells and MDCKII cells stably overexpressing human BCRP or mouse Bcrp1 were preincubated for 2 h with 5 μM zosuquidar trihydrochloride (LY335979), a P-glycoprotein inhibitor (in 2 ml of Opti-MEM per compartment). The pH of the media was adjusted to pH 6.5 or 7.4 and buffered with 25 mM HEPES after the preincubation and before the start of the experiment. At t = 0 h, 5 μM [^{14}C]topotecan was applied to either the apical or basolateral side, and the amount of [^{14}C]topotecan appearing in the opposite basolateral compartment (open symbols) or apical compartment (closed symbols) was determined. Samples were taken at indicated time points. Values shown are means ± S.D. of each experiment (n = 3).

flavonoid resorcinol (pK_{a1} = 9.20 and pK_{a2} = 10.9) (Blanco et al., 2005; see Fig. 1 for structural formula of resveratrol). Thus, under physiological pH conditions, ~0.4% of resveratrol is in its dissociated form (O^-), whereas at pH 6, this is only ~0.016%. Figure 7 shows that resveratrol is efficiently transported at low pH in MDCKII cells stably overexpressing human BCRP, but transport was not detectable at physiological pH. The P-glycoprotein and BCRP inhibitor elacridar effectively inhibited the transport of resveratrol at low pH (Fig. 7). These results demonstrate that also a neutral compound is more efficiently transported by BCRP at low pH than at physiological pH, indicating that the increased transport of BCRP substrates at low pH is not dependent on the protonation status and net charge of the substrate.

Discussion

In this study, we showed that BCRP transports both (anti)folates and the established BCRP substrate mitoxantrone at a much higher rate at low pH than at physiological pH. At pH 5.5, we found that transport of MTX was saturable, with apparent K_m and V_{max} values of 1.3 ± 0.2 mM and 44 ± 2.5 nmol/mg of protein/min, respectively. At physiological pH, the transport rate of MTX increased linearly with increasing concentrations of substrate, and we were unable to saturate the transport by BCRP with MTX. For 7-OH-MTX and folic acid, the affinity of BCRP at physiological pH is so low that we could not determine a K_m value, given the solubility limits of these substrates, which are 1 mM and 100 μM, respectively. For MTX-glu2 we were not able to detect any transport at physiological pH. These results are markedly different from those of Chen et al. (2003) and Volk and Schneider (2003), who found saturable transport of MTX and significant transport of MTX-glu2 at physiological pH. A possible explanation for this discrepancy could lie in the actual pH at which the experiments in these studies were done. These authors used 10 mM Tris-HCl/250 mM sucrose buffer at pH 7.3. We found that the addition of 4 mM disodium ATP to this assay buffer results in a pH of 7.2 because of the formation of Mg^{2+}-ATP complexes, and results in a pH value of ~5, when the ATP is not neutralized before addition. This explanation is in line with the recent finding of Rhee and Schneider (2005) that BCRP was unable to transport MTX polyglutamates from intact cells at neutral pH (Rhee and Schneider, 2005).

In vesicle studies, we found no significant difference in the transport of MTX and folic acid by MRP2 and MRP5, respectively, at pH 5.5 and 7.3, suggesting that the increased folate transport at low pH is specific for BCRP. In addition, changes in intra- or extracellular pH have been reported not to affect drug extrusion by P-glycoprotein (Altenberg et al., 1993; Goda et al., 1996). The results with the MRP vesicles also exclude that uptake of (anti)folates into cells via a low pH transport route (Sierra et al., 1997; Assaraf et al., 1998; Said, 2004; Zhao et al., 2004) could have contributed to the observed transport differences at low pH compared with physiological pH. Any contribution of the reduced folate carrier, a bidirectionally operating folate transporter, can be ruled out as well, because this transporter does not function below pH 6.0 (Jansen, 1999).

The pH effect was observed not only for BCRP-mediated transport in BCRP-overexpressing membrane vesicles but

also for efflux of MTX from intact BCRP-overexpressing cells, which was significantly increased (3- to 4-fold) at low pH compared with physiological pH. Low pH did not affect MTX efflux from control cells, indicating that the decreased accumulation at low pH was related to BCRP and was not a result of an imbalance of a decreased uptake of MTX in the MDCKII-BCRP cells via the reduced folate carrier at low pH and an increased activity of the low pH (anti)folate transporter (Sierra et al., 1997; Assaraf et al., 1998; Said, 2004; Zhao et al., 2004).

The increased BCRP-mediated transport at low pH cannot be explained solely by a decreased dissociation of (anti)folates, because we observed that mitoxantrone and resveratrol, a weak base and a neutral compound, respectively, are more efficiently transported by BCRP at low pH than at physiological pH. For the weak acid topotecan, we also found an increased efflux (~1.4-fold) by BCRP from intact MDCKII-BCRP cells at low pH, but this increase was not as pronounced as for the weak acid MTX (3- to 4-fold). Furthermore, it seems unlikely that the 1.4-fold increase in topotecan transport is explained by an increased uptake of topotecan in the MDCKII cells as a result of the increased formation of the lactone form of topotecan at pH 6.5 compared with physiological pH (Underberg et al., 1990; Gabr et

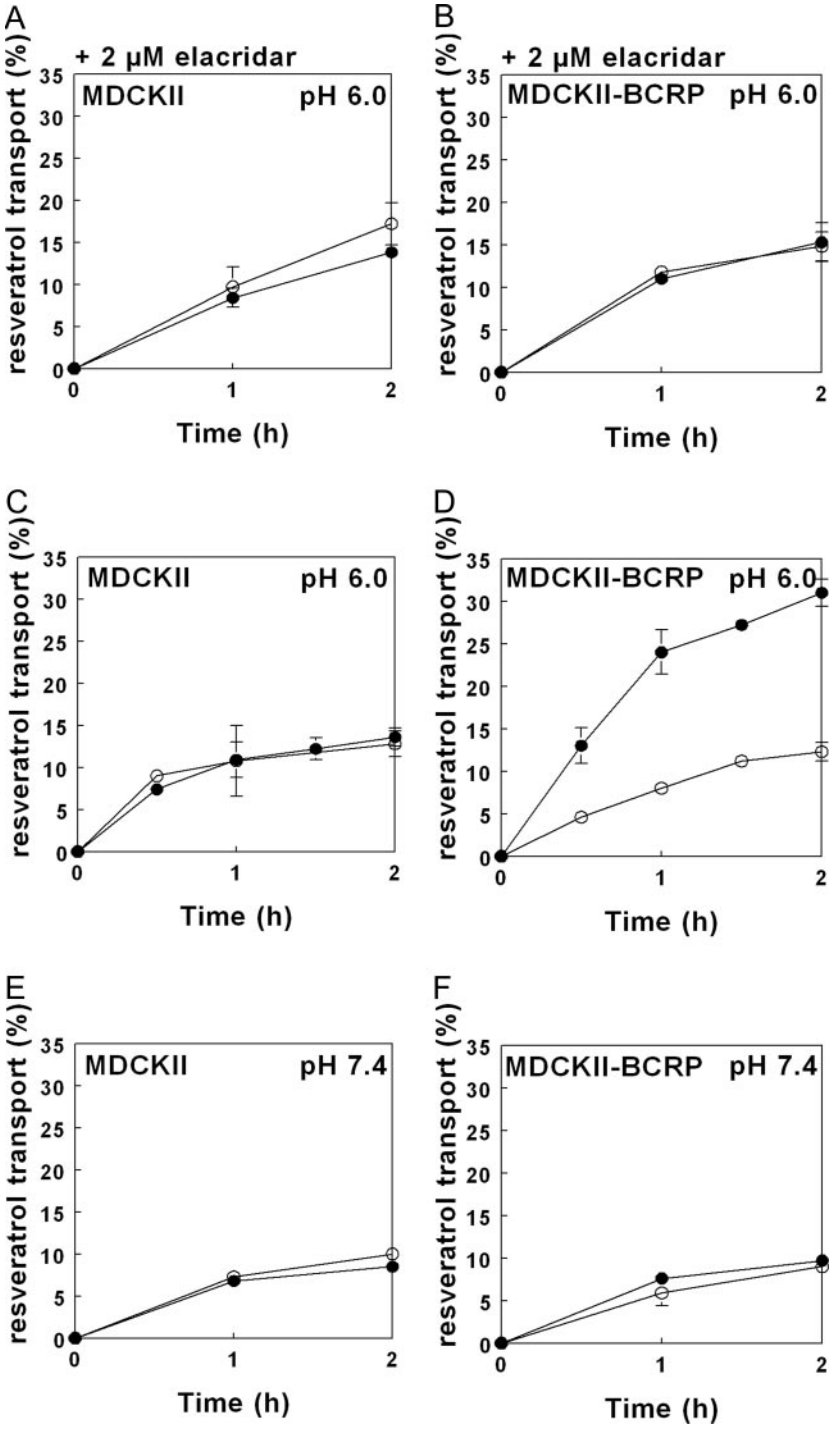

Fig. 7. Transport of resveratrol by BCRP at low and at physiological pH in the absence or presence of the P-glycoprotein and BCRP inhibitor elacridar (GF120918). Parental MDCKII cells and MDCKII cells stably overexpressing human BCRP were preincubated for 2 h with 5 μM zosuquidar trihydrochloride (LY335979), a P-glycoprotein inhibitor, with or without elacridar (2 μM) (in 2 ml of Opti-MEM per compartment). The pH of the media was adjusted to pH 6.0 or 7.4 after the preincubation and before the start of the experiment. At $t = 0$ h, 10 μM [^{3}H]resveratrol (in final concentration of 0.5% dimethyl sulfoxide) was applied to either the apical or basolateral side and the amount of [^{3}H]resveratrol appearing in the opposite basolateral compartment (open symbols) or apical compartment (closed symbols) was determined. Samples were taken at indicated time points. Values shown are means ± S.D. of each experiment ($n \geq 3$).

al., 1997), because low pH did not affect efflux of topotecan from control MDCKII cells.

In experiments using Sf9 membrane vesicles, the pH is changed at the *cis-* side (i.e., the side from which the substrate is transported, reflecting the intracellular pH). The possibility that a transmembrane pH gradient contributed to the observed increase in transport activity of BCRP at low pH was ruled out, because we found that the increase in BCRP-mediated MTX transport at low pH compared with physiological pH was similar in vesicles made at pH 5.5 and 7.4. It is interesting that we found a strong pH effect on MTX, mitoxantrone, and resveratrol export from intact MDCKII-BCRP cells when the extracellular pH (*trans-* side) was lowered to 6 or 6.5. However, we cannot exclude that a lower intracellular pH was responsible. Because the Na^+/H^+ exchanger cannot cope with increased intracellular proton accumulation at an acidic extracellular pH value of 6, the intracellular pH might temporarily decrease (Moolenaar et al., 1984; Boyer and Tannock, 1992).

Further work is required to establish the effect of low pH on 1) the transport activity of polymorphic BCRP variants (Honjo et al., 2001; Ishikawa et al., 2003; Mizuarai et al., 2004; Morisaki et al., 2005; Özvegy-Laczka et al., 2005a), on 2) substrate-induced ATPase activity (Özvegy et al., 2002), on 3) homodimerization of BCRP (Bhatia et al., 2005), and on 4) the three-dimensional configuration/folding of BCRP (Özvegy-Laczka et al., 2005b). It is well-established that nucleotide position 482 is a polymorphic hot spot for BCRP that can be associated with altered substrate specificities and transport activities (Özvegy-Laczka et al., 2005a). For instance, Honjo et al. (2001) showed that cells with R482T and R482G BCRP displayed altered levels of resistance to anthracyclines, SN-38, and topotecan. For (anti)folates, vesicular studies have shown that folic acid, MTX, and MTX-glu2 are transported by wild-type BCRP (ABCG2-R482), used in our studies, but not by mutant BCRP (ABCG2-R482T and ABCG2-R482G) (Chen et al., 2003; Mitomo et al., 2003; Volk and Schneider, 2003), suggesting that changes in the protein structure indeed have consequences for the (anti)folate substrate specificity of BCRP. Remarkable in this respect were observations by Rhee and Schneider (2005) that in intact cells wild-type BCRP did not contribute to MTX export and resistance. However, recent observations by Shafran et al. (2005) demonstrated that intact cells harboring the R482G and R482T amino acid replacements were markedly resistant to short-term exposures to antifolates, including MTX, in association with a poor accumulation of MTX-diglutamates. Whether these apparent discrepancies in transport of (anti)folates in vesicular and intact cell systems are associated with inadequate control of pH under experimental conditions remains to be established.

The 5D3 antibody can be used as a sensitive tool to reveal intramolecular changes, reflecting ATP binding, the formation of a catalytic intermediate, or substrate inhibition within the transport cycle of the BCRP protein (Özvegy-Laczka et al., 2005b). We analyzed by flow cytometry 5D3 monoclonal antibody binding to BCRP at pH 6.5 versus physiological pH for BCRP-overexpressing MCF-7/MR cells (Maliepaard et al., 2001). The latter cells were used because MDCKII-hBCRP cells harbor a green fluorescent protein construct that interferes with flow cytometric analysis. No apparent changes in 5D3 binding were observed at both pH values, neither for binding of W6/32 monoclonal (MHC class I) antibody that was used as control to detect possible general pH induced alterations in antibody binding (results not shown). These results suggest that, at least in a ground state form and within a narrow range of pH 6.5 to 7.4, no marked changes in 3D-configuration for BCRP are revealed. Whether this also is the case under conditions of substrate and/or inhibitor binding at various pH values should be subject of further studies.

Our results may have clinical implications, because BCRP could contribute to cellular export of MTX, MTX diglutamate, 7-OH-MTX, and mitoxantrone in the acidic extracellular environment of solid tumors or renal tubule lumen given the high rate of folate analog and mitoxantrone transport by BCRP. However, there is no experimental evidence to support this conjecture, and it remains to be verified in animal models.

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