The multidrug resistance protein 5 (ABCC5) confers resistance to 5-fluorouracil and transports its monophosphorylated metabolites

Susan Pratt,[1] Robert L. Shepard,[1] Ramani A. Kandasamy,[2] Paul A. Johnston,[2] William Perry III,[1] and Anne H. Dantzig[1]

[1]Cancer Research, Lilly Research Laboratories, Lilly Corporate Center, Indianapolis, Indiana and [2]RTP Laboratories, Durham, North Carolina

Abstract

5′-Fluorouracil (5-FU), used in the treatment of colon and breast cancers, is converted intracellularly to 5′-fluoro-2′-deoxyuridine (5-FUdR) by thymidine phosphorylase and is subsequently phosphorylated by thymidine kinase to 5′-fluoro-2′-dUMP (5-FdUMP). This active metabolite, along with the reduced folate cofactor, 5,10-methylenetetrahydrofolate, forms a stable inhibitory complex with thymidylate synthase that blocks cellular growth. The present study shows that the ATP-dependent multidrug resistance protein-5 (MRP5, ABCC5) confers resistance to 5-FU by transporting the monophosphate metabolites. MRP5- and vector-transfected human embryonic kidney (HEK) cells were employed in these studies. In 3-day cytotoxicity assays, MRP5-transfected cells were ~9-fold resistant to 5-FU and 6-thioguanine. Studies with inside-out membrane vesicles prepared from transfected cells showed that MRP5 mediates ATP-dependent transport of 5 μmol/L [^{3}H]5-FdUMP, [^{3}H]5-FUMP, [^{3}H]dUMP, and not [^{3}H]5-FUdR, or [^{3}H]5-FU. The ATP-dependent transport of 5-FdUMP showed saturation with increasing concentrations and had a K_m of 1.1 mmol/L and V_{max} of 439 pmol/min/mg protein. Uptake of 250 μmol/L 5-FdUMP was inhibited by dUMP, cyclic nucleotide, cyclic guanosine 3′,5′-monophosphate, amphiphilic anions such as probenecid, MK571, the phosphodiesterase inhibitors, trequinsin, zaprinast, and sildenafil, and by the chloride channel blockers, 5-nitro-2-(3-phenylpropylamino)-benzoic acid and glybenclamide. Furthermore, the 5-FU drug sensitivity of HEK-MRP5 cells was partially modulated to that of the HEK-vector by the presence of 40 μmol/L 5-nitro-2-(3-phenylpropylamino)-benzoic acid but not by 2 mmol/L probenecid. Thus, MRP5 transports the monophosphorylated metabolite of this nucleoside and when MRP5 is overexpressed in colorectal and breast tumors, it may contribute to 5-FU drug resistance. [Mol Cancer Ther 2005;4(5):855–63]

Introduction

The fluorinated pyrimidine, 5-fluorouracil (5-FU; Fig. 1) is used in the treatment of a variety of epithelial tumors including colorectal, breast, ovarian, and head and neck cancers (1). Typical patient response rates for treatment with this single anticancer agent are between 10% and 30%. 5-FU enters the cell by a facilitated nucleobase transporter and is converted by a complex metabolic pathway shown in Fig. 1A to metabolites that interfere with both DNA and RNA synthesis (2). Of particular interest is the conversion of 5-FU to 5′-fluoro-2′-deoxyuridine (5-FUdR) by thymidine phosphorylase with the subsequent phosphorylation by thymidine kinase to the active metabolite 5′-fluoro-2′-dUMP (5-FdUMP). The primary mode of action of 5-FdUMP is believed to be thymidylate synthase inhibition resulting in the reduction in the thymidine pool and an increase in the uracil pool, leading to inhibition of DNA synthesis and UTP incorporation. 5-FdUMP competes with the natural substrate, dUMP for binding sites within thymidylate synthase and has potency in the nanomole range (3, 4). 5-FdUMP forms a relatively stable ternary complex with thymidylate synthase and the reduced folate cofactor, 5,10-methylenetetrahydrofolate (3). The ratio of cellular levels of free 5-FdUMP to dUMP determines the rate of inhibition of thymidylate synthase activity as well the duration of inhibition (5). Several mechanisms are thought to be responsible for resistance to 5-FU. Clinically, the responsiveness of colon tumors to this therapeutic agent correlates with the reduced thymidylate synthase activity (6). Although in cellular systems, the disappearance of 5-FdUMP from the cell is correlated with resumption of DNA synthesis and the rate of 5-FdUMP disappearance is higher in some resistant cell lines than in their drug-sensitive parental cell lines (7, 8). The ATP-binding cassette (ABC) transporter superfamily consists of 49 members and contains several family members that confer drug resistance to drug-sensitive cells by effluxing anticancer or antiviral agents or their metabolites from cells when expressed at high levels. P-glycoprotein (ABCB1) was the first member identified of this superfamily that conferred resistance to many structurally unrelated anticancer drugs when transfected into drug-sensitive cells (9). Several other ABC transporters within the cystic fibrosis transmembrane conductance regulator

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Requests for reprints: Anne H. Dantzig, Cancer Research, Lilly Research Laboratories, Lilly Corporate Center, Indianapolis, IN 46285. Phone: 317-276-5083; Fax: 317-276-6510. E-mail: Dantzig@lilly.com

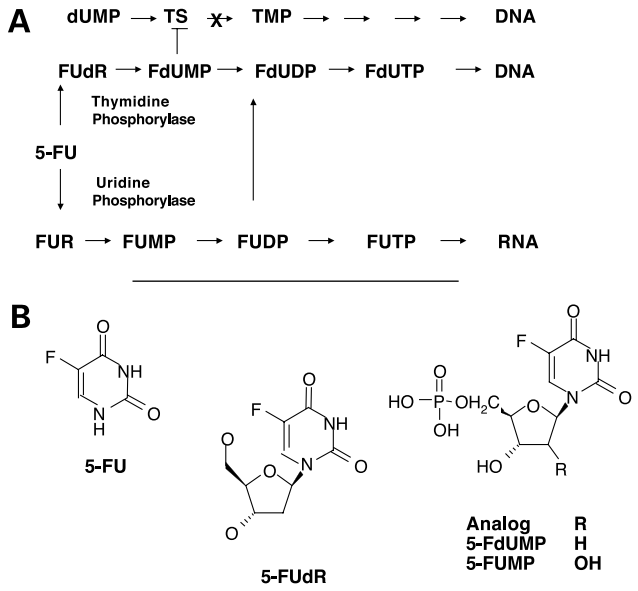

Figure 1. Pathway of 5-FU metabolism with structures of key metabolites. **A,** the diagram of the pathway for the metabolism of 5-FU to metabolites that interfere in both DNA and RNA synthesis. 5-FU catalyzed by thymidine phosphorylase followed by thymidine kinase results in metabolites that ultimately block DNA synthesis, whereas uridine phosphorylase along with uridine kinase are important in generating metabolites that block RNA synthesis. DNA synthesis is also blocked when 5-FdUMP, along with 5,10-methylenetetrahydrofolate, forms a stable ternary complex with thymidylate synthase thereby inhibiting the enzyme and resulting in depleted cellular thymidine pools. **B,** structures of 5-FU and its metabolites used in this study.

(CFTR)/MRP (ABCC) family also transport a wide array of drugs and confer multidrug resistance when ectopically expressed in drug-sensitive cell lines (10–12). Recently, one family member, MRP8 (ABCC11) was shown to confer resistance to 5-FU and certain fluoropyrimidines when ectopically expressed into LLC-PK1 cells (13). Moreover, MRP8 mediated the ATP-dependent transport of the monophosphorylated metabolite of the deoxyribose form of 5-FU, 5-FdUMP, into inside-out membrane vesicles prepared from these cells. MRP8 shares the greatest structural similarity with MRP4 (ABCC4) and MRP5 (ABCC5) that lack a transmembrane domain present in other family members MRP1-3 (ABCC1-3). When transfected into drug-sensitive cells, MRP4 and MRP5 confer resistance to several anticancer and antiviral nucleosides (14–16). The MRP4 and MRP5 transporters confer resistance to 6-thioguanine (6-TG), 6-mercaptopurine (6-MP), as well as 9-(2-phosphonylmethoxynyl)-adenine (PMEA), whereas MRP8 confers resistance to PMEA but not 6-TG. Neither MRP4 nor MRP5 has been shown to confer resistance to 5-FU; in fact, initial studies by Wijnholds and coworkers did not observe MRP5-mediated resistance to 5-FU (17).

In the present study, we reexamine the role of MRP5 in 5-FU resistance. Human embryonic kidney (HEK) 293 cells were transfected with human MRP5 and were resistant to 6-TG and several other anticancer agents including 5-FU and the platinum-containing drugs, cisplatin and oxaliplatin used in the treatment of colon cancer. Moreover, MRP5-mediated ATP-dependent uptake of the 5-FU monophosphate metabolites, 5-FdUMP and 5-FUMP, into inside-out membrane vesicles prepared from these HEK-MRP5 transfectants. In addition, competition studies of 5-FdUMP uptake indicated that the chloride channel blocker, 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB), which inhibits the CFTR (15, 18), is also a potent inhibitor of MRP5 and modulates 6-TG and 5-FU drug resistance *in vitro*.

Materials and Methods

Materials

Moravek Biochemicals Inc. (Brea, CA) was the supplier of the radiolabeled compounds: [^{3}H]5-FdUMP (14-20 Ci/mmol, 99.9% radiochemical purity), [^{3}H]5-FUMP (19.5 Ci/mmol, 97.7% radiochemical purity), [^{3}H]5-FUdR (13.9 Ci/mmol, 99.3% radiochemical purity), and [^{3}H]5-FU (5-20 Ci/mmol, 97% purity). Sildenafil was purified from Viagra (Pfizer, Groton, CT). MK571 was purchased from Alexis Biochemicals (Carlsbad, CA). Pemetrexed (Alimta) was obtained from Eli Lilly and Co. (Indianapolis, IN). All other reagents were purchased from Sigma (St. Louis, MO). Other than those noted, cell culture materials were purchased from Invitrogen (Life Technologies, Grand Island, NY).

Transfection of Mammalian Cells

HEK 293 cells cultured in DMEM supplemented with 2 mmol/L L-glutamine, 10% fetal bovine serum (Hyclone, Logan, UT), and 0.5 mg/mL G418 were transfected with a cDNA encoding human MRP5 cloned into pIRESNeo vector (BD Biosciences-Clontech, Palo Alto, CA), or the empty vector as a control. The encoded MRP5 protein is identical to that previously reported by Wijnholds et al. (ref. 17; Genbank accession number aab71758). After 24 hours of recovery, cells were selected with 1.5 mg/mL G418. MRP5-HEK clone 36 was selected after screening >200 colonies for MRP5 expression and drug sensitivity to 6-TG and retained these properties after 1 month of subculturing cells in the presence of 0.5 mg/mL G418. HEK-MRP5 (clone 36) and HEK-vector transfectants used in this study were determined to be *Mycoplasma*-free (Bionique, Saranac Lake, NY). For the transient transfection studies, PEAK^{STABLE} cells (transformed HEK cells that stably express the *EBNA-1* gene; Edge Biosystems, Gaithersburg, MD) were transfected with MRP5 in EW1969 using Fugene (Roche Diagnostics, Indianapolis, IN) as previously described (19). Cells were used for drug uptake studies or membrane vesicle preparation 3 days after transfection.

Electrophoresis and Western Analysis

Proteins were separated from cell lysates on a 4% to 20% SDS-polyacrylamide gel and transferred to a nitrocellulose filter by electroblotting as previously described (20). The blot was incubated with the anti-MRP5 monoclonal antibody M_5I-1 (Kamiya Biomedical Corp., Seattle, WA)

at a 1:200 dilution and subsequently incubated with an enhanced chemiluminescence Western blotting analysis system (Amersham Biosciences, Buckinghamshire, United Kingdom) employing anti-rat IgG conjugated to horseradish peroxidase (Santa Cruz, Santa Cruz, CA) at a 1:10,000 dilution.

Cytotoxicity Studies

HEK transfectants were grown in 96-well tissue culture plates (Costar, Corning, NY) at a density of 5.0×10^3 cells per well in DMEM supplemented with 2 mmol/L L-glutamine, 10% fetal bovine serum, and 0.5 mg/mL G418. For studies with methotrexate and pemetrexed, the growth medium was folic acid-deficient RPMI with 10% dialyzed fetal bovine serum (Life Technologies) and 0.5 mg/mL G418. After 24 hours of attachment, the cytotoxic drug was added to the growth medium. Cell viability was determined after 3 days of continued drug treatment using CellTiter 96 Aqueous One (Promega, Madison, WI). Maximal percentage of inhibition denotes the greatest growth inhibition observed over an extended range of drug concentrations; EC_{50} values represent the effective concentration of drug which gives 50% inhibition of the maximum inhibition achieved and were generated with a curve-fitting program, BRAVO/SAS. Resistance factor is the ratio of EC_{50} value of the HEK-MRP5 transfectant to that of the HEK-vector cells. For studies with 5-FU, EC_{50} values were fitted using the maximum inhibition determined for the vector cells as the maximum for both transfectants. For the modulator studies, the EC_{50} value for each anticancer agent was measured in the absence or presence of a noncytotoxic concentration of the indicated modulator.

Membrane Vesicle Preparation

Cells were scraped into Dulbecco's PBS (pH 7.5), pelleted, and hypotonically lysed at room temperature in 1 mmol/L sodium bicarbonate buffer (pH 8) prepared with Complete protease inhibitors without EDTA (Roche Diagnostics). At 4°C, the lysate was centrifuged for 5 minutes at $800 \times g$ and the resulting supernatant was applied to a one-step 38% sucrose gradient centrifuged for 30 minutes at $113{,}000 \times g$ in a swinging bucket rotor, Beckman ultracentrifuge. The band at the sucrose interface was diluted with 250 mmol/L sucrose and 50 mmol/L Tris-HCl buffer (pH 7.5) and centrifuged at $133{,}000 \times g$ for 40 minutes. The pellet of membrane vesicles was resuspended in a small volume of 250 mmol/L sucrose and 50 mmol/L Tris-HCl buffer (pH 7.5) and passed 20 times through a 27-gauge syringe needle and frozen in liquid nitrogen and maintained under argon at −70°C.

Membrane Vesicle Transport Studies

Assays were determined in a 96-well format as previously described (21). Unless noted otherwise, uptake of the radiolabeled compound was measured at 37°C for 20 minutes and was initiated with the addition of 10 μg of membrane protein of inside-out membrane vesicles to buffer containing the indicated [^{3}H]-radiolabeled substrate, 20 mmol/L $MgCl_2$, 4 mmol/L ATP, or 4 mmol/L AMP-PNP prepared in 250 mmol/L sucrose and 50 mmol/L Tris-HCl sucrose buffer (pH 7.5) with an ATP-regenerating system consisting of phosphocreatine and creatine kinase (Roche Diagnostics). Uptake was terminated by filtration; filters were dried overnight and counted as previously described (21). ATP-dependent uptake was calculated by subtracting uptake measured in the presence of AMP-PNP from uptake measured in the presence of ATP. ATP-dependent MRP5-mediated transport was calculated by subtracting ATP-dependent uptake by the membrane vesicles prepared from HEK-vector control cells from the ATP-dependent uptake measured with vesicles prepared from HEK-MRP5 cells. For inhibition studies, compounds were added from a stock solution prepared in DMSO with a final concentration not exceeding 2% in the assay buffer and were compared with control samples with the same solvent concentration. For the concentration-dependence studies, uptake was measured at several time points that were determined to be within the linear range for uptake at a given concentration. Kinetic variables were determined by fitting the data to a single transporter with a Michaelis-Menten curve-fitting program by SigmaPlot 8.0. Protein was measured using the bicinchoninic acid protein assay reagent (Pierce, Rockford, IL) using bovine serum albumin as the standard.

Analysis of 5-FdUMP

The integrity of 5-FdUMP was determined at 1 mmol/L after incubation of MRP5 inside-out membrane vesicles under the same conditions employed for the uptake studies: 0.5 mg/mL membrane vesicle protein with 20 mmol/L $MgCl_2$ in 50 mmol/L Tris/250 mmol/L sucrose buffer (pH 7.5), but in the absence of ATP or AMP-PNP and the creatine kinase regenerating system. The incubation time was for 0, 20, and 60 minutes at 37°C and was halted by the addition of 2 volumes of high-pressure liquid chromatography grade acetonitrile. After centrifugation to precipitate insoluble material, supernatants were concentrated by vacuum centrifugation and resuspended with 2% acetonitrile/distilled H_2O to a concentration of 0.188 mmol/L 5-FdUMP with 0.25 mmol/L uridine added as an internal standard. High-pressure liquid chromatography was used to resolve 50 μL samples on a Zorbax SB-C18 reversed-phase column (4.6 × 250 mm, Agilent Technologies, Palo Alto, CA) and isocratic elution with 2% acetonitrile/distilled H_2O at 1.5 mL/minute. Under these conditions, the retention time for 5-FdUMP was 2.7 minutes and a standard curve of 5-FdUMP from 0.094 to 0.188 mmol/L indicated that ≥10% decrease of 5-FdUMP concentration was measurable.

Results

Drug Resistance

MRP5 has previously been shown to confer drug resistance to 6-TG, 6-MP, and PMEA (17). We compared the drug sensitivity of MRP5- and vector-HEK transfectants to several anticancer agents. Analysis of lysates from these transfectants by Western blotting showed that MRP5 was

highly expressed in the MRP5 transfectant relative to the vector transfectant (data not shown). Drug sensitivity was evaluated with anticancer agents, 5-FU, 5′-deoxy-5′-fluorouridine, a metabolite of the orally administered prodrug of 5-FU, capecitabine, the anti-folate drugs methotrexate and pemetrexed, the platinum-containing drugs cisplatin and oxaliplatin, and natural products doxorubicin and vincristine. The effective concentration necessary for 50% growth inhibition (EC_{50}) of each transfectant in a 3-day cytotoxicity assay was determined and is presented in Table 1. Comparison of the EC_{50} values for HEK-vector and HEK-MRP5 indicated that HEK-MRP5 is ~9-fold resistant to both 6-TG and 5-FU and ~2-fold to 5′-deoxy-5′-fluorouridine, which is subsequently metabolized to 5-FU in tumor cells (22). These MRP5-transfected HEK cells were also 4- to 8-fold resistant to methotrexate and pemetrexed, approximately 3-fold resistant to the platinum-containing anticancer agents cisplatin and oxaliplatin, and 2-fold resistant to doxorubicin. These HEK-MRP5 cells were as sensitive as HEK-vector cells to the natural product anticancer agent, vincristine.

Cellular Drug Accumulation

5-FU is such an important anticancer agent used in the treatment of colorectal and breast cancer. We therefore examined whether 5-FU resistance is the result of a reduction in drug accumulation. The uptake of 1 μmol/L [^{3}H]5-FU was examined over a 40-minute time course in the vector- and MRP5-transfected HEK cells (Fig. 2); the cellular accumulation of 5-FU was significantly reduced by 2-fold within 10 minutes and throughout the 40-minute incubation (Fig. 2A). Reduced 5-FU accumulation was also apparent in MRP5 transiently transfected in PEAK^{STABLE} HEK cells compared with the vector (Fig. 2B). This verifies that the observation that MRP5 reduces 5-FU drug accumulation is not due to clonal variation because similar results were obtained by transient transfection of MRP5.

MRP5-Mediated Uptake into Inside-Out Membrane Vesicles

Because 5-FU is rapidly metabolized by thymidine phosphorylase and uridine phosphorylase to several metabolites that ultimately result in termination of DNA and RNA synthesis (Fig. 1), we examined the uptake of several metabolites for uptake into inside-out membrane vesicles prepared from HEK-vector, HEK-MRP5 clone 36, and MRP5-transiently transfected HEK cells (Fig. 3). Uptake into vesicles was measured in the absence and presence of ATP with the nucleobase 5-FU, the metabolite 5-FUdR, and the monophosphorylated metabolites of 5-FU, 5-FdUMP, and 5-FUMP. Uptake of the natural nucleotide dUMP was also examined. ATP-dependent uptake of 5 μmol/L [^{3}H]5-FU and [^{3}H]5-FUdR was minimal in vesicles prepared from both the vector and MRP5 transfectants, whereas the uptake of [^{3}H]5-FdUMP was enhanced 4- to 8-fold in MRP5-expressing membrane vesicles when compared with vector membrane vesicles (Fig. 3A and D). Uptake of 5-FdUMP was osmotically sensitive (data not shown). To confirm that the uptake of 5-FdUMP was mediated by MRP5, vesicles were prepared from MRP5- and vector-transiently transfected HEK cells (Fig. 3D); Fig. 3B shows greater uptake into the MRP5 vesicles. When the ATP-dependent uptake of 5 μmol/L [^{3}H]5-FdUMP, [^{3}H]5-FUMP, and [^{3}H]dUMP was compared, MRP5-mediated uptake of the deoxyribose monophosphate metabolite and the ribose metabolite was similar to that of the natural substrate, dUMP (Fig. 3C). Subsequently, the rate of [^{3}H]5-FdUMP uptake into HEK-MRP5 vesicles was examined over a wide concentration range (0.15-10 mmol/L) and was found to saturate with increasing concentrations (Fig. 4). The Michaelis-Menten kinetic variables were determined to be a V_{max} of 439 ± 113 pmol/minute/mg protein and a K_m of 1.10 ± 0.13 mmol/L (n = 4). As a control, the integrity of 5-FdUMP was evaluated by high-pressure liquid chromatography after incubation with

Table 1. Drug sensitivity of HEK-MRP5 and HEK-vector transfectants to various anticancer agents

Drug	HEK-vector	HEK-MRP5	Resistance factor (n)
	EC_{50} (μmol/L)		
6-TG	2.7 ± 0.10	21.76 ± 1.39*	8.0 (34)
5-FU	12.85 ± 1.38	134.37 ± 43.30*	10.5 (8)
5′-Deoxy-5′-fluorouridine	38.78 ± 9.71	84.93 ± 26.16 †	2.2 (3)
Methotrexate	0.014 ± 0.003	0.07 ± 0.01*	4.7 (3)
Pemetrexed	0.048 ± 0.004	0.36 ± 0.02*	7.5 (15)
Cisplatin	0.76 ± 0.18	1.78 ± 0.11*	2.6 (3)
Oxaliplatin	1.11 ± 0.16	3.40 ± 0.77*	3.1 (4)
Doxorubicin	0.03 ± 0.01	0.05 ± 0.01*	2.0 (2)
Vincristine	0.02 ± 0.003	0.019 ± 0.002 ‡	1.0 (2)

NOTE: Transfectants were grown for 3 days in the presence of the indicated drug; EC_{50} values were determined as described in Materials and Methods. Values are the mean ± SE of 2 to 34 independent experiments (n) determined in duplicate or triplicate. The resistance factor was calculated as the ratio of the EC_{50} value of the indicated HEK-MRP5 transfectant to the HEK-vector transfectant.
*Significantly different from HEK-vector cells by Student's paired t test ($P \leq 0.05$).
† Student's paired t test ($P = 0.06$).
‡ Student's paired t test ($P = 0.24$).

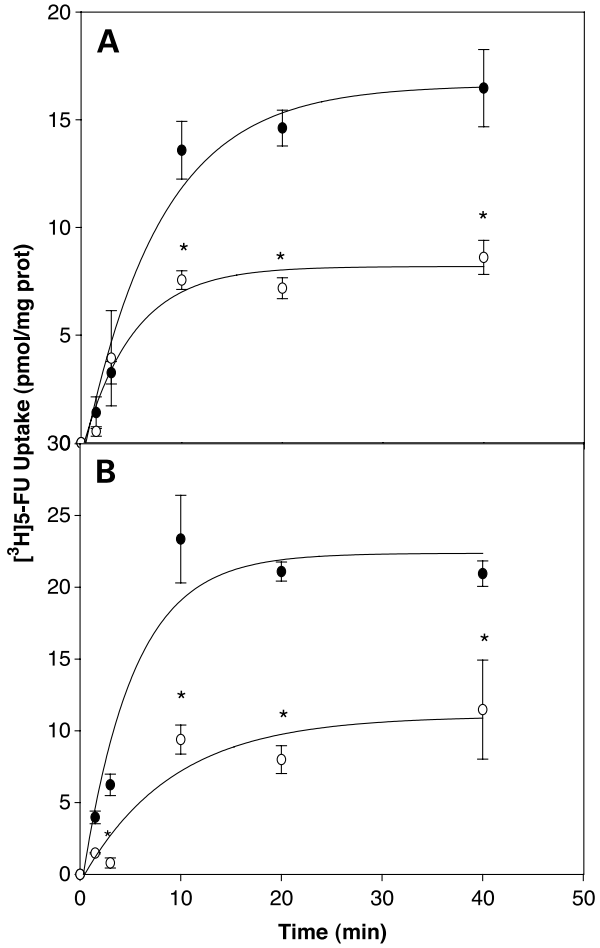

Figure 2. Cellular accumulation of 5-FU. The uptake of 5 μmol/L [^{3}H]5-FU was measured at 37°C over 40 min in (**A**) MRP5- and vector-HEK stable transfectants (**B**) MRP5- and vector-transiently transfected HEK (PEAK^{stable}) cells. ○, MRP5-transfected cells; ●, vector-transfected cells. *Points*, mean; *bars*, ±SE of three independent experiments measured in triplicate; *, significant reduction (~2-fold) in drug accumulation was observed at the data points as determined by Student's *t* test ($P \leq 0.05$).

membrane vesicles for up to 60 minutes. The 5-FdUMP concentration did not change, indicating that metabolism was <10% over the duration of the assay (data not shown). These findings further support that MRP5 mediates 5-FdUMP uptake with low affinity.

Inhibition of MRP5-Mediated 5-FdUMP Uptake

To further explore the specificity of this transporter, the effect of the presence of several amphiphilic anions known to be substrates or inhibitors of members of the CFTR/MRP family (ABCC) were examined on the uptake of 250 μmol/L 5-FdUMP. These compounds were tested over a wide concentration range and EC_{50} values are listed in Table 2. 5-FdUMP inhibited its own uptake with an EC_{50} of ~1.7 mmol/L, similar to the determined K_m value of 1.1 mmol/L, whereas its natural nonfluorinated dUMP had an EC_{50} value of 9.5 mmol/L. Even though the nonphosphorylated intermediate 5-FUdR is not a substrate, this metabolite had a ~2-fold lower EC_{50} value of 0.48 mmol/L than 5-FdUMP. Two anions, methotrexate and pemetrexed, and the cyclic nucleotide, cyclic guanosine 3′,5′-monophosphate, had EC_{50} values in the ~0.8 to 2.2 mmol/L range. The cyclic guanosine 3′,5′-monophosphate-specific phosphodiesterase (PDE5) inhibitors, trequinsin, sildenafil, and zaprinast, also blocked 5-FdUMP uptake; zaprinast was the most potent inhibitor with an EC_{50} value of 0.02 versus ~0.6 mmol/L for the other two PDE5 inhibitors. The organic anion transporter inhibitors that are nonspecific MRP inhibitors, probenecid and MK571 inhibited with EC_{50} values of ~0.07 and 0.02 mmol/L, respectively. Two inhibitors of CFTR, glybenclamide and NPPB, gave strong inhibition with EC_{50} values in the 2 to 15 μmol/L range.

Modulation of 5-FU Resistance

To ascertain if NPPB is an effective modulator of MRP5-mediated resistance, cytotoxicity studies were conducted with 6-TG and 5-FU as the anticancer agents in the presence of a noncytotoxic concentration of either probenecid or NPPB. Probenecid has been previously shown to inhibit the MRP5-mediated efflux of fluorescein diacetate from loaded cells and to inhibit the vectorial MRP5-mediated transport of PMEA across Madin-Darby canine kidney cells (17, 23). As shown in Fig. 5, the drug sensitivity of MRP5-HEK cells which were 6-fold resistant to 6-TG, was enhanced by 3.3- and 2.7-fold in the presence of 2 mmol/L probenecid and 40 μmol/L NPPB, respectively. In contrast, the drug sensitivity of MRP5-HEK cells to 5-FU was enhanced slightly (1.3-fold) by 2 mmol/L probenecid and significantly by 2.7-fold by 40 μmol/L NPPB. NPPB also had a small but significant effect on HEK-vector cells. Thus, NPPB is an effective modulator of MRP5-mediated drug resistance to 6-TG and 5-FU.

Discussion

Several groups have prepared MRP5 stably-transfected HEK 293 cells to examine the role of this ABC transporter in drug resistance to anticancer and antiviral agents. McAleer et al. (23) originally found that MRP5-transfected HEK 293 cells were resistant to certain metallic salts, cadmium chloride, and potassium antimonyl tartrate, but not to anticancer agents, such as cisplatin or daunomycin. Wijnholds et al. (17) then reported MRP5 transfectants of HEK 293 cells to be cross-resistant to PMEA, 6-TG, and 6-MP, but not to a number of other agents including 5-FU; Davidson et al. (24) reported MRP5-HEK cells to be resistant to several fluoropyrimidines. The present study shows for the first time that MRP5 mediates resistance to the anticancer agent 5-FU as well as several clinically used anticancer agents. These agents include the natural product, doxorubicin, the platinum-containing anticancer agents, cisplatin and oxaliplatin, the anti-folate drugs, methotrexate and pemetrexed, but not vincristine. The finding that MRP5 confers resistance to cisplatin and doxorubicin are consistent with published reports of drug-selected cells or drug-treated cells displaying elevated expression of MRP5 or its splice variant SMRP.

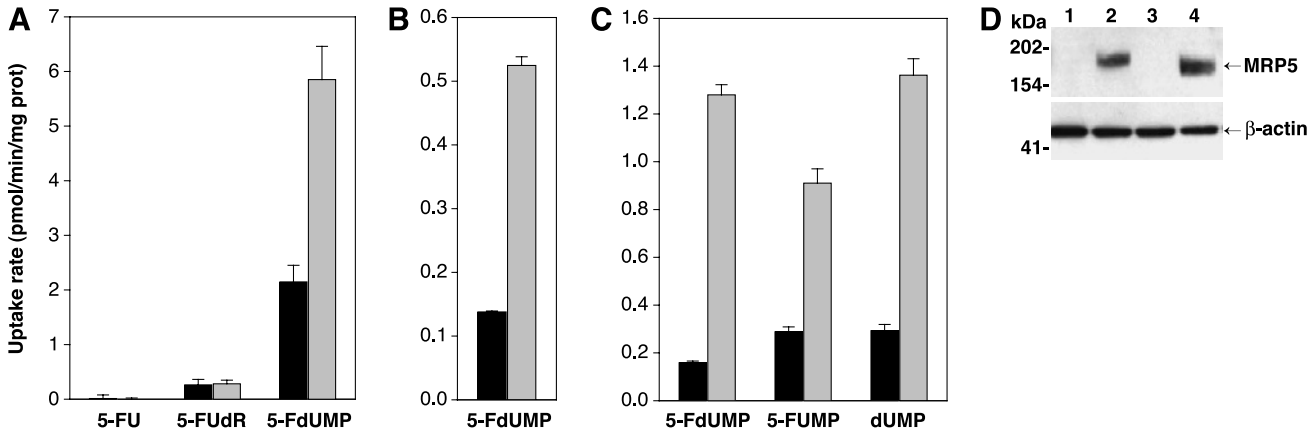

Figure 3. Comparison of the ATP-dependent uptake of dUMP and 5-FU metabolites by inside-out membrane vesicles. **A,** the uptake rate of 5 μmol/L [^{3}H]5-FU, [^{3}H]5-FUdR, and [^{3}H]5-FdUMP was measured using inside-out membrane vesicles prepared from stably transfected HEK-MRP5 cells (*gray columns*) and HEK-vector cells (*black columns*) as described in Materials and Methods. *Columns*, mean; *bars*, ±SE of two independent experiments measured in duplicate. **B,** the ATP-dependent uptake of 5 μmol/L [^{3}H]5-FdUMP by inside-out membranes vesicles prepared from HEK (PEAK^{stable}) cells transiently transfected with MRP5 (*gray columns*) or vector (*black columns*). Values are the mean ± SE of two independent experiments measured in duplicate. **C,** ATP-dependent MRP5-mediated uptake of 5 μmol/L [^{3}H]5-FdUMP, [^{3}H]5-FUMP, [^{3}H]5-dUMP. Uptake was measured into inside-out vesicles prepared from stably transfected HEK-MRP5 cells (*gray columns*) and HEK-vector cells (*black columns*). *Columns*, mean; *bars*, ±SE of two independent experiments measured in triplicate. **D,** Western analysis of MRP5 expression in membrane vesicles prepared from stable and transient HEK transfectants. Proteins (10 μg/lane) from vector- and MRP5-transfected HEK membrane vesicles were separated by SDS-PAGE on 4% to 20% gels, electroblotted to nitrocellulose membranes, and incubated with an anti-MRP5 monoclonal antibody as described in Materials and Methods. *Lane 1,* HEK-vector; *lane 2,* HEK-MRP5; *lane 3,* vector transiently transfected HEK (PEAK^{stable}), and *lane 4,* MRP5 transiently transfected HEK (PEAK^{stable}). The sizes of the molecular weight standards are indicated in kilodaltons. *Arrow,* the location of MRP5 corresponding to a molecular weight of approximately 190 kDa.

Non–small cell lung cancer PC-14, selected for resistance to cisplatin, were reported to express a MRP5 splice variant called SMRP (25). Treatment of PC-14 and PC-14/cisplatin cells with Adriamycin resulted in an induction of MRP5 and the splice variant. An increased expression of both MRP5 and SMRP transcripts were also observed in three Adriamycin-selected resistant cell lines (25). Clinical studies of patients after long-term treatment with cisplatin indicated that expression of MRP5 and SMRP mRNAs was elevated in both normal lung tissue and lung tumors even though 24-hours exposure of cells in culture did not increase MRP5 transcripts (26). The present study is the first demonstration in a cellular *in vitro* system that ectopically expressed MRP5 confers resistance to cisplatin and doxorubicin. The ability to show this is most likely due to higher expression of

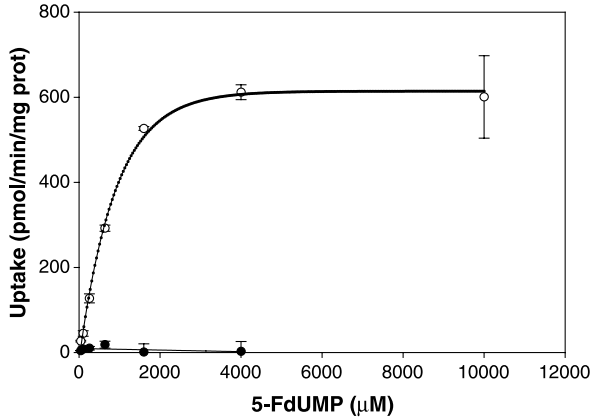

Figure 4. Kinetics of ATP-dependent MRP5 mediated 5-FdUMP uptake. Uptake by inside-out membrane vesicles (25 μg per data point) from HEK-vector (●) and HEK-MRP5 clone 36 (○) cells were determined over a broad concentration range of 5-FdUMP (0.15-10 mmol/L). Uptake was measured for 20 min at 37°C in the absence and presence of ATP and curves shown are the ATP-dependent uptake. *Points*, mean; *bars*, ±SE. Kinetic variables based upon four independent experiments were found to be: K_m of 1.10 ± 0.13 mmol/L and V_{max} of 439 ± 113 pmol/min/mg protein.

Table 2. Inhibition of ATP-dependent MRP5-mediated uptake of 5-FdUMP

Compound	EC_{50} (mmol/L)
5-FdUMP	1.69 ± 0.30
dUMP	9.45 ± 0.18
5-FUdR	0.46 ± 0.01
Methotrexate	0.78 ± 0.23
Pemetrexed	2.11 ± 0.38
Cyclic guanosine 3′,5′-monophosphate	0.96 ± 0.07
Trequinsin	0.58 ± 0.26
Sildenafil	0.58 ± 0.09
Zaprinast	0.020 ± 0.006
Probenecid	0.07 ± 0.02
MK571	0.017 ± 0.003
Glybenclamide	0.015 ± 0.009
NPPB	0.002 ± 0.001

NOTE: The uptake of 250 μmol/L [^{3}H]5-FdUMP was measured at 37°C for 40 minutes in the presence of increasing concentrations up to 10 mmol/L of the indicated test compound. The effective concentration that gave 50% inhibition over the dose range (EC_{50}) is listed below. Values are the mean ± SE of two to three independent experiments measured in triplicate.

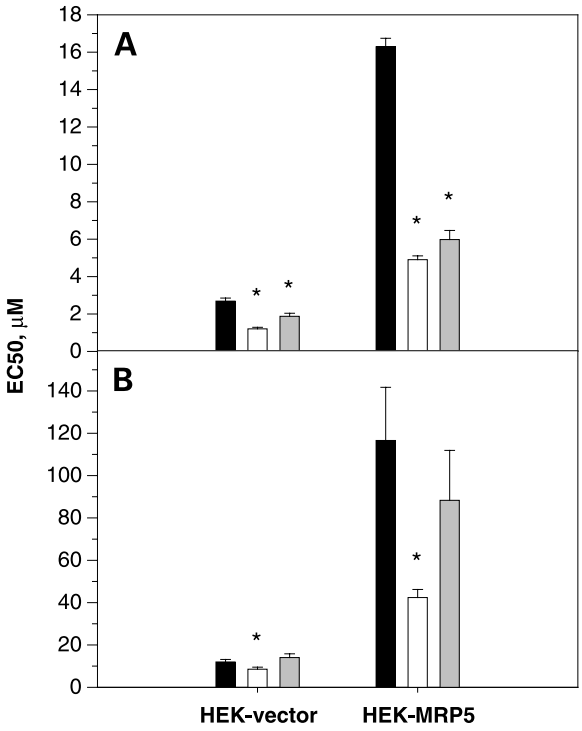

Figure 5. Effect of modulators on the cytotoxicity of 6-TG and 5-FU to MRP5- and vector-transfected HEK 293 cells. Cells were grown for 3 d in the absence (*black columns*) or presence of either NPPB (*white columns*) or 2 mmol/L probenecid (*gray columns*) in the presence of increasing concentrations of 6-TG (**A**) or 5-FU (**B**) as described in Materials and Methods. Each modulator was tested at a concentration that was nontoxic when evaluated alone. The effect of the modulator was compared with the corresponding control transfectant measured in the absence of added modulator was determined to be statistically significant by Student's *t* test ($P \leq 0.05$). Curves are representative of three independent experiments measured in triplicate.

MRP5 resulting in much higher level of resistance to 6-TG (8- to 10-fold) than was previously reported (1- to ~3-fold; refs. 17, 23, 27).

Because 5-FU is such an important drug used in the treatment of colon and breast tumors and other malignancies, we conducted more extensive studies on the uptake of 5-FU. The accumulation of 5-FU was significantly reduced by 2-fold in both stably and transiently transfected HEK-MRP5 cells compared with their respective HEK-vector cells, indicating a role for MRP5 in drug efflux. Studies with inside-out oriented vesicles prepared from MRP5 and vector HEK 293 cells either stably- or transiently-transfected conclusively showed ATP-dependent MRP5-mediated uptake of both 5-FdUMP and 5-FUMP but not 5-FUdR or 5-FU. Interestingly, 5-FUdR inhibits 5-FdUMP uptake with an EC_{50} that is in the same range as 5-FdUMP (0.46 versus 1.69 mmol/L, respectively). Thus, the nonphosphorylated form inhibits but is not transported indicating that the monophosphate moiety is essential for being a substrate even though the transporter has a low affinity for 5-FdUMP with a K_m of 1.1 mmol/L with a V_{max} of 439 pmol/minute/mg protein. The finding that key intermediates of 5-FU, 5-FdUMP a potent thymidylate synthase inhibitor and 5-FUMP, are substrates support the notion that ATP-dependent efflux by MRP5 can interfere with the ability of 5-FU to inhibit both DNA and RNA synthesis (Fig. 1A) and therefore confer drug resistance. Furthermore, the finding that the monophosphate metabolites of the nucleosides are MRP5 substrates is consistent with earlier studies. For example, MRP5 also mediates the ATP-dependent influx of cyclic guanosine 3′,5′-monophosphate and cyclic AMP into inside-out membrane vesicles prepared from human MRP5-transfected Chinese hamster V79 lung fibroblasts (28). When HEK 293/MRP5 transfectants are incubated with PMEA, 6-MP, or 6-TG, MRP5 mediates the cellular efflux of PMEA (29, 30) and the metabolites of 6-TG and 6-MP, thioinosine monophosphate and thioxanthosine monophosphate and 6-methylthioinosine monophosphate, respectively (17, 31, 32). Thus, MRP5 transports amphiphatic organic anions as previously seen with other CFTR/MRP family members.

Interestingly another ABC transporter, MRP8 was recently shown to also confer resistance to PMEA and 5-FU, but not 6-TG. At the sequence level, MRP5 and MRP8 are the most structurally similar of the MRPs, and like MRP5, MRP8 mediated ATP-dependent transport of 5-FdUMP into inside-out membrane vesicles prepared from MRP8-transfected LLC-PK1 cells was observed, but transport of 5-FU or 5-FUdR was not (13). The affinity of this transporter for 5-FdUMP has not yet been reported. MRP8 is expressed in normal breast, prostrate, testis, kidney, liver, placenta, brain, and to high levels in breast tumors (33, 34), whereas MRP5 seems to be ubiquitously expressed in most tissues and is expressed to high levels in heart and brain (35, 36). MRP5 is expressed at lower levels in cystic fibrosis patients with the F508 mutation and is expressed to 2-fold higher levels in patients' hearts after ischemia (36, 37). Whether MRP5 and MRP8 work in concert in transporting substrates out of tissues or are localized to different cellular surfaces in polarized cells is yet to be determined.

Competition studies were conducted to examine the specificity of the transporter for various known amphiphatic anions that are MRP inhibitors; EC_{50} values were determined from the dose-response curves. MK571 and probenecid modulate resistance conferred by several members of the CFTR/MRP family of ABC transporter superfamily and also inhibit their transport activity in cellular and/or membrane vesicle assays (17, 28, 38–42). Previously, the PDE5 inhibitors gave potent inhibition of MRP5-mediated uptake of cyclic guanosine 3′,5′-monophosphate into membrane vesicles; trequinsin was a competitive inhibitor with a K_i of 240 nmol/L (28, 43). In the present study, these PDE5 compounds inhibited MRP5-mediated uptake of 5-FdUMP in a dose-dependent manner, however, zaprinast was the most potent with an EC_{50} value of 20 μmol/L compared with a value of 580 μmol/L for both trequinsin and sildenafil. We also examined two cyclic AMP–dependent chloride channels inhibitors, NPPB and glybenclamide. Both were found to be inhibitors of MRP5-mediated uptake, NPPB being the most potent inhibitor

of MRP5 with an EC_{50} value of ~2 μmol/L. Interestingly, CFTR, another member of the CFTR/MRP family is a cyclic AMP–dependent chloride channel that has some overlap in substrate specificity with the MRPs (44, 45) and whose activity is also blocked by NPPB and glybenclamide (46).

Previously, inhibitors of ABC drug transporters have been shown to enhance the drug sensitivity of cells that overexpress a drug transporter such as P-glycoprotein and MRP1 (47–50). Because NPPB was the most potent inhibitor of ATP-dependent MRP5-mediated uptake of 5-FdUMP (Table 2), we examined its ability to sensitize MRP5-transfectant to 5-FU and 6-TG cytotoxicity. NPPB was a more potent modulator than probenecid of drug resistance for both anticancer agents (Fig. 4), consistent with its greater potency than probenecid (~35-fold) as a MRP5 transport inhibitor. As the present study shows for the first time that MRP5 confers cross-resistance to a number of anticancer agents including 5-FU, methotrexate, pemetrexed and doxorubicin, and the platinum-containing drugs, cisplatin and oxaliplatin. These studies indicate that potent MRP5 inhibitors may have use in reversing MRP5-mediated drug resistance when used in combination with anticancer agents that are effluxed by this ABC transporter and may be useful in the treatment of MRP5-expressing tumors such as breast and colon tumors (51).

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