Drug Efflux Transporter Multidrug Resistance-Associated Protein 5 Affects Sensitivity of Pancreatic Cancer Cell Lines to the Nucleoside Anticancer Drug 5-Fluorouracil


Research Center of Pharmacology and Experimental Therapeutics (P.K.N., T.H., K.K., M.G., G.J., C.R., D.R., H.K.K., C.A.R.), Institute of Pharmacy (S.M., C.A.R.), and Department of Internal Medicine A (M.S., F.U.W., J.M., M.M.L.), Ernst-Moritz-Arndt-University, Greifswald, Germany; Laboratoire de Toxicologie, Faculté de Pharmacie de Lyon, Université de Lyon, Lyon, France (L.P., J.G.; and Department of Tumor Immunology, Wielkopolska Cancer Center, Poznan, Poland (D.W.K.)

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ABSTRACT:

Pancreatic adenocarcinoma is one of the malignancies that is highly resistant to therapy and among the leading causes of cancer-related death. Several factors may influence pancreatic cancer resistance, and expression of ATP-binding cassette transport proteins is one of the major mechanisms of drug resistance. Members of this family’s C-branch, also referred to as multidrug resistance-associated proteins (MRPs), might be of particular interest because they are able to efflux nucleoside analogs used in the treatment of pancreatic cancer. Expression of MRP1, MRP3, MRP4, and MRP5 in human pancreas and pancreatic carcinoma has been reported. However, contributions of MRPs to chemoresistance of pancreatic cancer are not fully understood. MRP5 mRNA expression in pancreatic adenocarcinoma cell lines correlated significantly with cellular sensitivity to 5-fluorouracil (5-FU) (p < 0.05). Long-term treatment with 5-FU increased expression of MRP5 by 2.4-fold and was associated with significant drug resistance IC50 values for control and 5-fluorouracil (5-FU)-resistant Patu-T cell lines were 11.3 ± 5.3 and 33.2 ± 6.9 μM, respectively (p < 0.05). Consequently, overexpression of MRP5 in Colo-357 cells resulted in significantly reduced accumulation of 5-FU related radioactivity and 5-FU cytotoxicity. Knockdown of MRP5 significantly increased cellular cytotoxicity of 5-FU to Patu-02 cells and enhanced accumulation of radioactivity related to 5-FU and its metabolites. Our results suggest that MRP5 is expressed and functionally active and contributes to variable sensitivities of pancreatic adenocarcinoma cell lines to 5-FU. Further investigations using models that resemble human pancreas tumors are necessary to prove a causative relation between expression and activity of MRP5 and tumor resistance to 5-FU.

Introduction

Pancreatic cancer is one of the most resistant human cancers with a poor prognosis and survival rate and is the fourth leading cause of cancer-related death in the world. Conventional treatment approaches have little impact on the outcome of this devastating cancer, and 5-year survival is still very poor (overall less than 6%) (Jemal et al., 2006). Pancreatic cancer is usually diagnosed at an advanced stage and to date only surgical interventions offer a likelihood of cure. However, only 15 to 20% of tumors are resectable at the stage of diagnosis (Cress et al., 2006). Chemotherapy is the option of choice for the treatment of nonresectable tumors. Chemotherapeutic regimens generally include 5-fluorouracil (5-FU) alone or in combination with other cytotoxic agents (Blaszkowsky, 1998). Gemcitabine (2’,2’-difluorodeoxycytidine), a novel nucleoside analog, has...
also shown some efficacy in the treatment of pancreatic cancers (Oettle et al., 2007). However, it is only moderately effective, showing a tumor response rate of 12% (Storniolo et al., 1999).

Resistance of tumors to chemotherapy is a common clinical problem in human cancer. Pancreatic cancer is considered to be one of the malignancies most resistant to therapy, and the underlying mechanisms are very complex. They include alterations in drug metabolism, DNA repair, and antiapoptotic pathways and alterations of drug targets. In addition, decreased expression of influx transporters (i.e., hENT) and increased expression of efflux transporters (MRPs) can make the tumor cells more resistant by reducing the intracellular drug levels (Giovannetti et al., 2006).

The ATP-binding cassette (ABC) family of drug efflux transporters comprises approximately 50 members and is subdivided into seven groups (ABCA to ABCG) (Borst and Ellerink, 2002). In particular, members of the ABCG subfamily are expected to be of importance for the emergence of multidrug resistance in cancer. ABCG2 (MRP4), ABCG5 (MRP5), and ABCG11 (MRP8) are able to efflux cyclic nucleotides and nucleoside analogs (Borst et al., 2000; Jedlitschky et al., 2000; Guo et al., 2003; Reid et al., 2003), the latter of which are commonly used in cancer therapy and have shown efficacy in the treatment of pancreatic cancer (Blaszkowsky, 1998). Overexpression of MRP4 confers resistance to 6-mercaptopurine (6-MP) and 6-thioguanine (6-TG) (6-TG) (Chen et al., 2001), whereas MRP5 confers significant resistance to 5-FU (Pratt et al., 2005). Intracellularly, 5-FU is converted to 5'-fluoro-2'-deoxyuridine (5-FdUrd) and subsequently phosphorylated by thymidine kinase to 5'-fluoro-2'-deoxyuridine mono-phosphate (5-FdUMP). Studies with membrane vesicles prepared from MRP5-transfected cells demonstrate that MRP5 shows ATP-dependent transport of FdUMP but not FdUrd or 5-FU (Pratt et al., 2005). MRP5, which is closely related to MRP5, has also been shown to be capable of causing resistance to 5-FU (Oguri et al., 2007). Expression of the MRP family members MRP1, MRP3, MRP4, and MRP5 in human pancreas and pancreatic carcinoma has been reported (König et al., 2005), but the contribution of MRPs to chemoresistance of pancreatic cancer is not fully understood.

In this study, we investigated whether MRP4, MRP5, and MRP8 are expressed, whether they are functionally active, and whether expression and activity correlate with chemoresistance in pancreatic cancer cell lines. We assessed expression and localization of MRP4 and MRP5 in pancreatic cancer cell lines and their relation to cellular sensitivity to cytotoxic drugs. We observed a significant correlation between MRP5 mRNA expression and cellular sensitivity to 5-FU. In addition, acquired resistance to 5-FU was accompanied by a significant up-regulation of MRP5 in resistant subclones of pancreatic cancer cell lines. Furthermore, stable knockdown of MRP5 resulted in a significant re-sensitization of pancreatic cancer cells to 5-FU. These results suggest that MRP5 is expressed in pancreatic carcinoma cells and that modulation of its expression modifies sensitivity to 5-FU.

**Materials and Methods**

**Cell Lines and Chemicals.** The following human pancreatic cancer cell lines were used in this study: Colo-357, T3M4, Aspc-1, Dan-G, Panc-1, Patu-8988T, and Patu-8988S (cell lines Colo-357, T3M4, Aspc-1, and Panc-1 were a kind gift from the University Clinic, Heidelberg, Germany). Cells were grown in Dulbecco’s modified Eagle’s medium or RPMI 1640 medium (PAN Biotech GmbH, Aidenbach, Germany) containing 10% fetal calf serum, 2 mM L-glutamine, 1% minimal essential medium nonessential amino acids, and penicillin/streptomycin (0.5 U/ml and 150 μg/ml). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO2, 5-FU-resistant subclones were selected from Patu-8988T cells by cultivation in the presence of increasing concentrations of 5-FU over a 6-month period. Resistant subclones were grown in drug-free medium for at least 1 week before being used for the experiments. 6-MP, 6-TG, 5-FU, and 5-FdUrd were purchased from Sigma-Aldrich (Schnelldorf, Germany). Gemcitabine was purchased from Eli Lilly (Gießen, Germany). [3H]-5-FU (52 mCi/nmol) was purchased from Moravek Biochemicals (Brea, CA).

**Stable MRP5 Expression in Mammalian Cells.** Human pancreatic cancer cell lines Colo-357 were transfected with the pcDNA3.1/hyrho-MRP5 construct or vector only using FuGENE 6.0 transfection reagent (Roche Diagnostics, Mannheim, Germany). After 48 h, the cells were split, and stable transfectants were selected using medium containing hygromycin B (Invitrogen, Karlsruhe, Germany). Resistant clones were screened by RT-PCR for MRP5 expression.

**Small Interfering RNA Knockdown of MRP5.** Transient knockdown was achieved by annealing sense and antisense RNA oligonucleotide strands of MRP5 and GFP as a negative control. In brief, sense and antisense oligonucleotide strands of each target transcript were diluted separately in annealing buffer containing 50 mM Tris, pH 7.6, and 100 mM NaCl; sense and antisense strands of the respective target transcript were then mixed and incubated for 2 min at 95°C and allowed to cool to room temperature. Sequences of oligonucleotides used were MRP5 (sense) 5'-AUACUCCUUCCUUAAGC-3' (antisense) 5'-GCAAGAAAGAAGAAAUU-3' and GFP (antisense) 5'-UAUCCUUUUCUAGCGCUU-3' (sense) 5'-GCAAGAAAAAGAAGAAUU-3'. Small interfering RNAs (siRNAs) were then transfected into cells using Nanofectin according to the manufacturer’s protocol (PAA Laboratories GmbH, Colbe, Germany). After 48 h, mRNA was detected using real-time RT-PCR. For stable knockdown, MRP5-specific siRNA expression vectors were generated by cloning the sequences 5'-GACGCGCAGACACGCGTGAAATCTCGTGTTGACGGAATATC- CACGGTCTCTGTCGGGTTT-3' (forward) and 5'-TCAAGGTGTC-TAAAAGAGACGACGAGCGTGAAATCTCGTGTTGACGGAATATC- CGCGTCTCTGTCGGGTTT-3' (reverse) encoding the hairpin siRNA targeted to MRP5 into the Xhol/Bsp1191 site of the modified pQChX vector as described previously (López De Jesús et al., 2006). Cells were transfected with vector harboring the expression cassette or the empty vector using FuGENE 6.0 transfection reagent (Roche Diagnostics). After 48 h, the cells were split, and stable transfectants were selected using medium containing hygromycin B (Invitrogen). To generate clones with a stable knockdown, one cell per well was plated in 24-well plates, selected with hygromycin B, and screened by RT-PCR for MRP5 expression.

**RNA Isolation and Real-Time RT-PCR.** Total RNA was prepared from cells using PeqGold RNAPure (Peqlab, Erlangen, Germany) according to the manufacturer’s instructions. The isolated RNA was reverse-transcribed using random hexamer primers and a TaqMan RT kit (Applied Biosystems, Foster City, CA). The resulting cDNA was amplified by real-time PCR with intron-spanning primers and probes for human MRP4 and MRP5. siRNA expression vectors for MRP4 and MRP5 were purchased from Invitrogen. Real-time quantitative PCR for MRP5 was performed using the forward primer 5'-GTTCTTCTTTTCCTTTTCTCAACAAAC-3', reverse primer 5'-CCATTATCACTGACATTTCATTTGTT-3', and the probe 5'-FAM-CCAGTTAAGACCCCAATCTTGAGGAAC-TAMRA-3'. For MRP5 forward primer 5'-CACCATTTGCTACGTAAACTAAAC-3', reverse primer 5'-CAGCCCTGCACTACCTGAAATC-3', and the probe 5'-FAM-GCGCCTCTGCACTACCTGAAATC-TAMRA-3'. For MRP5, hENT1 detection, the following primers were used: 5'-GAGGGAGCTGCTAGCAG-3' (forward), 5'-ACAGCCCACTGCACTACCTGAAATC-3', and the probe 5'-FAM-CAGACCATGCTAGCAGCTACCTGAAATC-TAMRA-3' (probe). For hENT1 detection of thymidylate synthase, breast cancer resistance protein (BCRP) and 18S rRNA, a predeveloped primer and probe mix was purchased from Applied Biosystems. Measurement of the gene transcripts was performed using 20 ng of cDNA.

A 2× PCR Master Mix (45 mM Tris HCl, pH 8.4, 115 mM KCl, 7 mM MgCl2, 460 μM deoxynucleoside-5’-triphosphate, 9% glycerol, 2.3% ROX reference dye, and 0.035 U/ml platinum TaqDNA polymerase; Invitrogen, Carlsbad, CA) was used for real-time PCR (ABI Prism 7700 Sequence Detector; Applied Biosystems). For quantification of MRP4 and MRP5, a PCR product of MRP4 and MRP5 cloned in the vector pGEM-Teasy (Promega GmbH, Mannheim, Germany) was used as standard.
Immunofluorescence Microscopy. Cells were cultured on cover slides and grown to confluence. After washing with PBS, cells were fixed with 4% paraformaldehyde for 15 min; next, cells were washed with PBS again before permeabilization with Triton X-100 (0.1%) for 10 min. After washing with PBS, samples were blocked with 5% fetal calf serum followed by incubation with the primary antibodies for 2 h. MRP4 was detected with the primary antibody SNG, and MRP5 was detected using the polyclonal antibody AMF. Both antibodies were kindly provided by Prof. D. Keppler, Deutsches Krebsforschungszentrum (Heidelberg, Germany). After three washes with PBS, samples were stained with the secondary antibody (Alexa Fluor 488-labeled anti-rabbit IgG, dilution 1:200; Invitrogen) for at least 1 h at room temperature and also washed five times with PBS. Nuclei counterstaining with TOTO-3-iodide dye (dilution 1:2000; Invitrogen) was performed within Fluorescent Mounting Medium (Dako North America, Inc., Carpinteria, CA). Immunostained samples were photographed using a constant exposure time with a 40× lens, confocal Laser Scanning unit QLC 100 (Visitech International, Sunderland, UK), and Nikon Eclipse TE300 Microscope (Nikon GmbH, Düsseldorf, Germany).

Crystal Violet Assay. Cells at concentrations ranging from 5000 cells/well to 10,000 cells/well were plated onto a 96-well microtiter plate in 200 μl of medium. After a 24-h incubation, medium was replaced with medium containing drug and incubated for different time points; at the end of the incubation period, medium was removed, and the cells were washed once with phosphate-buffered saline. Cells were incubated for 15 min with a 4% paraformaldehyde solution for fixation. The fixed solution was discarded, and a solution of 0.5% crystal violet (Sigma-Aldrich, Taufkirchen, Germany) in 99% methanol was added into each well and incubated for 5 min. The stain was drained, and the plate was rinsed with tap water until no further blue stain could be removed. A solution of 1% SDS was added to lyse the cells, and after 20 min of incubation on a shaker, OD at 570 nm was read by a microtiter plate reader (Wallac Victor2; PerkinElmer Life and Analytical Sciences–Wallac Oy, Turku, Finland). OD of untreated cells was set at 100%. Viability and the relative viability were calculated from the OD values of drug-treated cells.

Cytotoxicity Analysis of Cell Death. To determine cell death, cells were harvested after a 48-h cultivation in the presence of the respective drugs, followed by a 5-min incubation step in 2 μg/ml propidium iodide (PI) (Sigma-Aldrich) in PBS at 4°C in the dark. PI uptake was assessed by flow cytometry analysis on a Becton Dickinson (Heidelberg, Germany) FACSCalibur using CellQuest software. In each sample, 10,000 cells were analyzed; data were gated to exclude debris.

Drug Accumulation and Efflux. For accumulation experiments, subconfluent cells were seeded in triplicate in 24-well plates. After overnight growth in standard growth medium, the cells were incubated at 37°C with medium containing 7.6 μM 14C-radiolabeled 5-FU corresponding to 0.4 μCi/ml at a specific activity of 52 mCi/mmol for different time intervals. Cells were washed twice with ice-cold PBS and lysed with 0.2% SDS. An aliquot was mixed with 1 ml of scintillation cocktail (Rotiszint; Roth, Karlsruhe, Germany) and measured in a scintillation beta counter. Specific activity was determined for different time points. Cells were incubated with 140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM KH₂PO₄, 1.2 mM MgSO₄, 1.5 mM CaCl₂, 5 mM glucose, and 12.5 mM HEPES, pH 7.3, for 20 min at 37°C and washed three times with warm PBS. Efflux was then determined at several time points in the supernatant. In addition, accumulation was detected in cell lysates after stopping the efflux and three washing steps with ice-cold PBS. In a subset of samples, the MRP inhibitor MK571 was added during the incubation phase at a concentration of 10 μM. Supernatants and cell lysates were stored at −20°C until determination. Assays of 5-FdUMP were performed by liquid chromatography-mass spectrometry as described previously (Honorat et al., 2008; Carli et al., 2009).

Data Analysis. Values are presented as means ± S.D. unless stated otherwise. Graphs and calculations were prepared using Excel (Microsoft Corp., Redmond, WA) or Prism 3.0 software (GraphPad Software Inc., San Diego, CA). The IC₅₀ values were calculated by nonlinear regression from a sigmoidal dose-response curve (variable slope, bottom value ≈0) using Prism. Spearman’s test was used to analyze the correlation between the expression of MRP4 and MRP5 and the IC₅₀ values. p ≤ 0.05 was considered statistically significant using an unpaired t test analysis unless stated otherwise.

Results

Expression of MRP4, MRP5, and MRP8 mRNA and Relation to Drug Sensitivity in Pancreatic Cancer Cell Lines. Transcript expression of MRP4, MRP5, and MRP8 in pancreatic adenocarcinoma cell lines (Colo-357, T3M4, Aspc-1, Dan-G, Panc-1, Patu-8902, Patu-8988T, and Patu-8988S) was measured by quantitative real-time RT-PCR. Expression of MRP8 was undetectable (data not shown), whereas both MRP4 and MRP5 were expressed at different expression levels in all cell lines (Fig. 1A).

Furthermore, immunofluorescence staining was performed in two pancreatic carcinoma cell lines (Patu-02 and Colo-357) as described under Materials and Methods. MRP4 and MRP5 were visualized by immunofluorescence staining using the anti-MRP4 polyclonal antibody SNG and anti-MRP5 polyclonal antibody AMF, respectively. Predominant staining of the plasma membranes for MRP4 and MRP5 could be detected (Fig. 1B).

For efflux experiments, subconfluent cells seeded in triplicate in 100-mm plastic dishes were incubated overnight in growth medium. The next day the cells were washed and incubated for 2 h in energy depletion medium (glucose-free, pyruvate-free sodium azide) containing radiolabeled drug as described for accumulation assays. Cells were then washed twice with PBS, and the medium was replaced with ordinary growth medium without radiolabeled drug. At various time points medium was collected for measuring radioactivity. Data are presented as the ratio of radioactivity released from the cells to the amount of radioactivity accumulated during the energy-depleting incubation period.

5-FdUMP Transport Assay. For assessing transport of the 5-FU metabolite 5-FdUMP, subconfluent Colo-357 cells were incubated with 100 μM 5-FdUr in incubation buffer containing 140 mM NaCl, 5 mM KCl, 1 mM KH₂PO₄, 1.2 mM MgSO₄, 1.5 mM CaCl₂, 5 mM glucose, and 12.5 mM HEPES, pH 7.3, for 20 min at 37°C and washed three times with warm PBS. Efflux was then determined at several time points in the supernatant. In addition, accumulation was detected in cell lysates after stopping the efflux and three washing steps with ice-cold PBS. In a subset of samples, the MRP inhibitor MK571 was added during the incubation phase at a concentration of 10 μM. Supernatants and cell lysates were stored at −20°C until determination. Assessment of 5-FdUMP was performed by liquid chromatography-mass spectrometry as described previously (Honorat et al., 2008; Carli et al., 2009).

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To investigate the correlation between MRP expression and cellular sensitivity to cytotoxic drugs, we measured sensitivity of eight pancreatic cancer-derived cell lines (Colo-357, T3M4, Aspc-1, Dan-G, Panc-1, Patu-8902, Patu-8988T, and Patu-8988S) to the nucleotide analogs 5-FU, GEM, 6-MP, and 6-TG. IC50 values are given in Table 1. It was observed that gemcitabine was very effective against pancreatic cancer cell lines followed by 5-FU, 6-TG, and 6-MP. However, IC50 values varied among the different pancreatic cancer cell lines, indicating differences in drug sensitivity. T3M4 and Colo-357 were among the most sensitive cell lines, whereas PANc-1, Patu-02, and Dan-G were less sensitive to the drugs tested.

IC50 values of all cell lines to different drugs (5-FU, GEM, 6-MP, and 6-TG) were correlated with their relative MRP4 and MRP5 expressions. We found a significant correlation between the MRP5 transcript expression and sensitivity to 5-FU \((r = 0.738, p < 0.05)\) (Fig. 2A), whereas the correlations between MRP5 and gemcitabine \((r = 0.626)\), 6-mercaptopurine \((r = -0.086)\), and 6-thioguanine \((r = 0.418)\) were not statistically significant nor was MRP4 mRNA expression significantly correlated with any of the drugs investigated \((5-FU r = 0.386, GEM r = 0.559, 6-MP r = 0.553, and 6-TG r = 0.031)\). In addition, we did not observe any significant correlation between sensitivity to 5-fluorouracil and mRNA expression of thymidylate synthase (Fig. 2B), BCRP (Fig. 2C), and hENT1 (Fig. 2D).

**Expression of MRP5 in Acquired Resistance of the Pancreatic Adenocarcinoma Cell Line Patu-T.** To study whether MRP5 plays any role in acquired resistance in pancreatic cancer, we incubated Patu-T cells over 6 months with increasing concentrations of 5-FU. Expression of MRP5 in Patu-T/5-FU was measured by quantitative real-time RT-PCR and compared with that in untreated Patu-T cells. We observed a significant 2.4-fold increase in MRP5 expression in 5-FU-resistant subclones (Patu-T/5-FU) (Fig. 3A). Of interest, MRP4 also increased significantly by 2.5-fold, whereas thymidylate synthase and BCRP were not significantly altered in 5-FU-resistant cells compared with the controls (data not shown). Sensitivity to 5-FU was assessed by crystal violet assay as described under Materials and Methods. 5-FU-treated cells were significantly more resistant than were untreated cells; the IC50 values of Patu-T (control) and 5-FU-resistant (Patu-T/5-FU) cells were 11.3 ± 5.3 and 33.2 ± 6.9 \(\mu M\), respectively \((p < 0.05)\) (Fig. 3B).

**Effect of MRP5 Overexpression on Cellular Sensitivity to 5-FU of Colo-357 Pancreatic Adenocarcinoma Cells.** To confirm the role of MRP5 expression in pancreatic cancer resistance to 5-FU, we stably overexpressed MRP5 in the pancreatic cancer cell line Colo-357, which itself has low MRP5 expression. The MRP5 expression vector was transfected and stable clones were selected as described under Materials and Methods. Clones were screened by RT-PCR for MRP5 expression. Transfection of Colo-357 cells with the pcDNA3.1/hygro vector containing the MRP5 cDNA cassette (Colo-pcDNA3.1/hygro-MRP5) increased MRP5 levels by 4.3-fold compared with cells transfected with the empty vector (Colo-pcDNA3.1/hygro) (Fig. 4A). To assess the sensitivity of MRP5-overexpressing cells to 5-FU we monitored cell killing by cytofluorometric analysis of PI uptake. Vector-transfected and MRP5-transfected cells were cultured in the absence or presence of 5-FU \((1–33 \mu M)\) for 48 h. We observed a concentration-dependent but significantly diminished sensitivity of MRP5-overexpressing cells to 5-FU \((p < 0.05)\) (Fig. 4B). Percentages

### TABLE 1

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC50 Value</th>
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<tbody>
<tr>
<td></td>
<td>5-FU</td>
</tr>
<tr>
<td>T3M4</td>
<td>0.33 ± 0.06</td>
</tr>
<tr>
<td>Colo-357</td>
<td>2.05 ± 0.03</td>
</tr>
<tr>
<td>Patu-T</td>
<td>2.08 ± 0.07</td>
</tr>
<tr>
<td>Patu-S</td>
<td>2.93 ± 0.04</td>
</tr>
<tr>
<td>Aspc-1</td>
<td>2.97 ± 0.03</td>
</tr>
<tr>
<td>Panc-1</td>
<td>3.94 ± 0.03</td>
</tr>
<tr>
<td>Patu-02</td>
<td>3.95 ± 0.01</td>
</tr>
<tr>
<td>Dan-G</td>
<td>4.38 ± 0.03</td>
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of dead cells were 26.4, 45.4, 54.6, and 53.0% in control cells compared with 16.4, 31.2, 39.1, and 44.9% in MRP5-overexpressing cells.

Analysis of Accumulation and Efflux of \([^{14}\text{C}]5\text{-FU}\) and 5-FdUMP in MRP5-Transfected Colo-357 Cells. To study the difference in transport of 5-FU in knockdown cells, we performed transport assays with \([^{14}\text{C}]\text{-radiolabeled 5-FU}\) as described under Materials and Methods. Intracellular drug accumulation was significantly enhanced in Patu-02 cells, both those that were transiently transfected and those that were stably transfected with MRP5 siRNA compared with the respective control transfected Patu-02 cells at 30 and 60 min of drug incubation (Fig. 5, D and E). In addition, efflux assays demonstrated that Patu-02 cells stably transfected with MRP5 siRNA exported 37% less drug compared with the Patu-02-Mock cells over an efflux period of 2 h (p < 0.05) (Fig. 5F). These results suggest that the increase in sensitivity to 5-FU of the pancreatic adenocarcinoma cell line Patu-02 was associated with enhanced drug accumulation due to reduced drug efflux.

Discussion

Resistance to chemotherapy is a key clinical problem in pancreatic cancer and can be influenced by several factors. The expression of efflux of 5-FU. Upon incubation for 2 h in medium containing \([^{14}\text{C}]5\text{-FU}\), Colo-pcDNA3.1/hygro-MRP5 cells accumulated 59.2% less drug intracellularly than control Colo-pcDNA3.1/hygro cells (p < 0.01) (Fig. 4C). We also determined whether the decreased accumulation was a consequence of enhanced drug efflux (Fig. 4D). MRP5-transfected cells exhibited significantly enhanced drug efflux (2.1-fold, p < 0.05) compared with that in control cells over the 60-min incubation period. It has been shown that, rather than 5-FU itself, active metabolites such as 5-FUMP and 5-FdUMP, the latter after conversion of 5-FU to 5-FdUrd, are transported by MRP5 (Pratt et al., 2005). We measured 5-FdUMP intracellularly and in supernatants of cells incubated with the intermediate 5-FU metabolite 5-FdUrd. This was necessary because we experienced a low intrinsic capacity of the cells to convert 5-FU to 5-FdUrd, which is mandatory for the production of sufficient amounts of the active metabolite 5-FdUMP to be detected by the method used. After the efflux period, intracellular levels of 5-FdUMP were significantly higher in control transfected cells as well as in cells treated with the MRP inhibitor MK571 compared with those in cells overexpressing MRP5 (Fig. 4E). Moreover, 5-FdUMP that was effluxed into the supernatant was only detectable from cells that overexpressed MRP5 (Fig. 4F). From these experiments we concluded that the differential capacity of MRP5-overexpressing cells to efflux 5-FdUMP compared with control cells contributes to the variable sensitivity of these cells to 5-FU. However, we cannot exclude the possibility that active metabolites such as 5-FUMP contribute to the toxicity of 5-FU as well, because we did not assess this metabolite in our system.

Sensitivity of MRP5 siRNA Transfected Patu-02 Cells to 5-FU. MRP5 expression in pancreatic cancer-derived cell lines is clearly detectable, and MRP5 expression correlates with cellular sensitivity to 5-FU, suggesting a potential role for MRP5 in intrinsic drug resistance in pancreatic cancer. To investigate whether MRP5 expression is a major factor that controls 5-FU sensitivity in pancreatic adenocarcinoma cells, we knocked down MRP5 expression in the pancreatic adenocarcinoma cell line Patu-02. Transient and stable transfection with siRNA constructs decreased MRP5 mRNA levels to 36 and 55% of control cells, respectively (Fig. 5, A and B). Cytofluorometric cell death assays revealed that death rates of stable control and MRP5 knockdown cells were similar under normal growth conditions. However, addition of 5-FU (3–100 \(\mu\text{M}\)) for the production of sufficient amounts of the active metabolite 5-FdUMP to be detected by the method used. After the efflux period, intracellular levels of 5-FdUMP were significantly higher in control transfected cells as well as in cells treated with the MRP inhibitor MK571 compared with those in cells overexpressing MRP5 (Fig. 4E). Moreover, 5-FdUMP that was effluxed into the supernatant was only detectable from cells that overexpressed MRP5 (Fig. 4F). From these experiments we concluded that the differential capacity of MRP5-overexpressing cells to efflux 5-FdUMP compared with control cells contributes to the variable sensitivity of these cells to 5-FU. However, we cannot exclude the possibility that active metabolites such as 5-FUMP contribute to the toxicity of 5-FU as well, because we did not assess this metabolite in our system.

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Drug resistance proteins is one mechanism by which drug resistance is achieved (Giovannetti et al., 2006). Expression of MRP4 and MRP5 mRNA has been demonstrated in pancreatic cancer, and MRP5 mRNA levels were found to be significantly higher in carcinoma compared with normal pancreatic tissue (König et al., 2005). In our study, we found MRP4 and MRP5 expression in all pancreatic adenocarcinoma cell lines investigated, and the expression of MRP5 significantly correlated with cellular sensitivity to 5-FU. Long-term incubation with 5-FU was able to increase MRP5 expression, which correlated with enhanced resistance to the drug. This was confirmed by overexpression of MRP5 in a pancreatic cancer cell line. These cells showed reduced sensitivity to 5-FU, decreased accumulation of 5-FU related radioactivity, and enhanced efflux of 5-FdUMP converted from 5-FdUrd. On the other hand, knockdown of MRP5 sensitized pancreatic adenocarcinoma cells to 5-FU.

The drug transporter MRP5 has been identified in several tissues such as blood vessels and smooth muscle cells of the genitourinary tract (Nies et al., 2002), cardiomyocytes (Dazert et al., 2003), brain (Nies et al., 2004; Vogelgesang et al., 2004), and human placenta (Meyer zu Schwabedissen et al., 2005) on mRNA and protein levels. In these tissues, MRP5 is thought to regulate intracellular levels of cGMP, which has been shown to be a high-affinity substrate of MRP5 (Jedlitschky et al., 2000). Besides effluxing cGMP and to a lesser extent also cAMP, MRP5 has been noted to confer resistance to a wide range of antiviral and anticancer drugs, preferentially of nucleosidic structure in vitro (Ritter et al., 2005). Among anticancer drugs, export of the thiopurine derivatives 6-MP and 6-TG has been best characterized (Wielinga et al., 2002). Other reports showed that 5-FU metabolites are also transported by MRP5 (Pratt et al., 2005). However, the transport of other nucleosidic anticancer drugs, in particular gemcitabine, is controversial (Reid et al., 2003; Oguri et al., 2006).

In general, the export of nucleoside analogs by MRP5 expression increases drug resistance in the range of 2- to 10-fold to these drugs in vitro (Borst et al., 2004; Pratt et al., 2005), which is in good agreement with our own findings.

Clinically, the role of MRP5 expression in intrinsic or acquired resistance is unresolved. MRP5 expression significantly correlated with the sensitivity of a panel of non–small-cell lung cancer cell lines to gemcitabine. Moreover, inhibition of transport activity by small molecule inhibitors or siRNA knockdown markedly sensitized cells to the cytotoxic effect of gemcitabine (Oguri et al., 2006). The expression of drug transporters was also analyzed in a population of patients with breast cancer, and MRP5 was found to be expressed significantly more highly in the group of nonresponders after neoadjuvant chemotherapy than in the responder group (Park et al., 2006). Of note, all patients were treated with a chemotherapy regimen containing 5-FU. Expression of most MRP drug transporters was demonstrated in pancreatic tissue (König et al., 2005); however, only MRP3 and MRP5 were differentially expressed when mRNA levels in normal pancreatic tissue and pancreatic carcinoma were compared. These transporters were also up-regulated in a pancreatic adenocarcinoma cell line that acquired resistance to 5-FU and regained sensitivity when MRP5 expression was reduced by siRNA knockdown (Hagmann et al., 2009). In our study, we confirm a significant up-regulation of MRP4 and MRP5 during adaptation to 5-FU and a gain of sensitivity after down-regulation of MRP5 expression. Although MRP4 transcription did not significantly correlate with sensitivities to the drugs tested in our cell panel, the up-regulation of MRP4 mRNA after long-term incubation with 5-FU might be of relevance. Novel strategies that use combinations of inhibitor substances may include yet unknown substrates for this transporter. In addition, we cannot rule out the possibility that these transporters are regulated as part of a multifactorial cellular adaptation mechanism because transporter up-regulation has also been observed when cells adapt to drugs that are not specific substrates for these transporter proteins (Yoshida et al., 2001; P.K.N. and C.A.R., unpublished data). Likewise, in a recent report MP7 was capable of transport and conferred resistance to nucleoside analog drugs such as gemcitabine, cytarabine, dideoxyctidine, and 9-(2-phosphonomethoxethyl) adenine (Hopper-Borge et al., 2009). Although mRNA expression of MP7 has been detected in pancreatic tissue (Hopper et al., 2001), its impact on drug resistance in pancreatic cancer requires further investigations.

Our work also reveals that expression of MRP5 mRNA is significantly correlated with antiproliferative activity of 5-FU in a panel of eight pancreatic adenocarcinoma cell lines. Moreover, we show that differential expression of MRP5 in cell systems is related to variable.
sensitivity to 5-FU and altered accumulation of 5-FU or its active metabolites. We also found MRP5-dependent efflux activity for 5-FdUMP that was converted from exogenous 5-FdUrd. Because 5-FU is metabolized via 5-FdUrd to the cytotoxically active 5-FdUMP intracellularly, we assumed that the modified efflux capacity for 5-FdUMP by MRP5 in our model system contributes, at least in part, to the modified sensitivity for 5-FU. However, possible contributions of other active metabolites, such as 5-FUMP, were not considered. Finally, we investigated not only ABC drug transporters but also other factors that could confer resistance for 5-FU in our cell model. Thymidylate synthase is the main target of 5-FU, and its expression might therefore influence 5-FU efficacy. Using tissue microarrays of pancreatic cancer, Hu et al. (2003) found a significant correlation between high-level thymidylate synthase and poor prognostic outcome; however, their patients were more susceptible to adjuvant treatment with 5-FU. In contrary, median survival was unaffected by thymidylate synthase immunoreactivity in a cohort of patients with pancreatic ductal adenocarcinoma who received adjuvant 5-FU chemotherapy (Formentini et al., 2007). In our carcinoma cell line panel, we found a trend toward an inverse correlation between 5-FU sensitivity and expression of thymidylate synthase that might support the findings by Hu et al. (2003). However, expression of thymidylate synthase was not significantly changed in cells that acquired resistance to 5-FU, which suggests that the expression of this protein contributes to 5-FU resistance in a subordinate manner in our cell system. Apart from MRP5 as an efflux transporter, the expression of the uptake transporter hENT1 has been related to 5-FU sensitivity in that its expression correlated with the growth-inhibiting activity of 5-FU in a panel of pancreatic cancer cell lines (Tsujie et al., 2007). Likewise, BCRP, a half-transporter of the ABC family, reportedly mediates transport of and sensitivity to 5-FU in breast cancer cells (Yuan et al., 2009). In contrast, our cell panel did not reveal a significant correlation of hENT1 or BCRP expression with 5-FU sensitivity nor could we detect transcriptional regulation in the process of acquisition of resistance to 5-FU.

The situation in a patient’s pancreatic tumor is by far more complex than can be investigated with cell lines. In recent years, interactions of pancreatic tumor cells with the tissue environment, including normal host epithelial cells, stromal fibroblasts, inflammatory cells, altered extracellular matrix, and proliferating endothelial cells, have emerged as a relevant factor influencing tumor growth and chemotherapy response (Mahadevan and Von Hoff, 2007). For instance, when pancreatic cancer cell lines were cultivated in the presence of extracellular matrix proteins such as collagen, fibronectin, or laminin, chemotherapy resistance developed according to the differentiation grade of the cell lines (Miyamoto et al., 2004). Increased activation of focal adhesion kinase, a central mediator in extracellular matrix/integrin signaling, may trigger this response (Huanwen et al., 2009). To investigate these processes appropriately, genetic mouse models of pancreatic ductal adenocarcinoma that closely resemble human pancreatic tumors were developed. Olive et al. (2009) observed in a mouse tumor model expressing endogenous mutant Kras and p53 alleles in pancreatic cells that tumors were poorly perfused and vascularized compared with xenograft transplantation models. However, depletion of tumor-associated stromal tissue by an inhibitor of the hedgehog cellular signaling pathway when coadministered with chemotherapy significantly increased intratumoral vascular density and stabilization of disease (Olive et al., 2009). Because delivery of drugs to pancreatic tumor cells seems to depend on vascular density and the drug transporter MRP5 has been localized to vascular endothelium, further research should be conducted to assess this protein in animal models of genetically developed pancreatic ductal adenocarcinoma.

In conclusion, our data show that MRP5 is expressed, functionally active, and correlates with chemoresistance in pancreatic cancer cell lines. We observed a significant correlation between MRP5 mRNA expression and cellular sensitivity to 5-FU. In addition, acquired resistance to 5-FU was accompanied by a significant up-regulation of MRP5 in resistant subclones of pancreatic cancer cell lines. Furthermore, stable knockdown of MRP5 resulted in a significant resensitization of pancreatic cancer cells to 5-FU. Our results suggest that MRP5 modulates cellular sensitivity of pancreatic adenocarcinoma cell lines to the cytotoxic agent 5-FU. More work is needed to translate these data to the clinical situation of pancreatic cancer and to clarify whether MRP5 modulation could influence tumor resistance to 5-FU.

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Authorship Contributions

Participated in research design: Köck, Grube, Jedlitschky, Rimmbach, Rosskopf, Mayerle, and Ritter.

Conducted experiments: Nambaru, Hübner, Mews, Grube, Guitton, Senderl, and Rimmbach.

Contributed new reagents or analytic tools: Payen, Jedlitschky, Rosskopf, Kowalczyk, and Weiss.

Performed data analysis: Nambaru, Köck, Grube, Guitton, Rimmbach, Rosskopf, Weiss, and Ritter.

Wrote or contributed to the writing of the manuscript: Nambaru, Weiss, and Ritter.

Other: Rosskopf, Kroemer, Lerch, and Ritter acquired funding for the research.

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ROLE OF MRP5 IN PANCREATIC ADENOCARCINOMA RESISTANCE


Address correspondence to: Dr. Christoph A. Ritter, Institute of Pharmacy, Friedrich-Ludwig-Jahn-Str. 17, 17487 Greifswald, Germany. E-mail: ritter@uni-greifswald.de.


