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Drug efflux transporter MRP5 affects sensitivity of pancreatic cancer cell lines to the nucleoside anticancer drug 5-fluorouracil

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Running title: Role of MRP5 in pancreatic adenocarcinoma resistance

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Abbreviations: MRP, Multidrug resistance protein; ABC, ATP binding cassette; 6-MP, 6-mercaptopurine; 5-FU, 5-fluorouracil; 5-FdUrd, 5-fluoro-deoxyuridine; 5-FdUMP, 5-fluoro-deoxyuridine monophosphate; 6-TG, 6-thioguanine; GEM, gemcitabine

Abstract

Pancreatic adenocarcinoma is one of the malignancies highly resistant to therapy and among the leading causes of cancer-related death. Several aspects may influence pancreatic cancer resistance and expression of ABC 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 since they are able to efflux nucleoside analogues 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) ($r=0.738$, $p<0.05$). Long-term treatment with 5-FU increased expression of MRP5 by 2.4 fold and was associated with significant drug resistance (IC_{50} values for control and 5-FU resistant Patu-T cell lines were 11.3±5.3 µM 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. Knock down 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. Future investigations using models that resemble human pancreas tumors are necessary to proof 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 poor prognosis and survival rate and 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 the surgical interventions offer a likelihood of cure. However, only 15–20% of tumors are respectable at the stage of diagnosis (Cress et al., 2006). Chemotherapy is the option of choice for the treatment of nonresectable tumors. Until recently, generally used chemotherapeutic regimens included 5-fluorouracil (5-FU) alone or in combination with other cytotoxic agents (Blaszkowsky, 1998). Recently, gemcitabine (2',2'-difluorodeoxycytidine), a novel nucleoside analogue, showed some efficacy in the treatment of pancreatic cancers (Oettle et al., 2007). It is, however, moderately effective only, 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, 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 about 50 members and is subdivided into seven groups (ABCA to ABCG) (Borst and Elferink, 2002). In particular, members of the ABCC subfamily are expected to be of importance for the emergence of multidrug resistance in cancer. ABCC4 (MRP4), ABCC5 (MRP5), and ABCC11 (MRP8) are able to efflux cyclic nucleotides and nucleoside analogues (Borst et al.,

2000; Guo et al., 2003; Jedlitschky et al., 2000; 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) (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 (FdUrd) and subsequently phosphorylated by thymidine kinase to 5'-fluoro-2'-deoxyuridine monophosphate (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). MRP8, which is closely related to MRP5, has also been recently 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 (Konig 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, 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 upregulation of MRP5 in resistant subclones of pancreatic cancer cell lines. Furthermore, stable knock down of MRP5 resulted in a significant resensitization 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-8902, Patu-8988T and Patu-8988S (Cell lines Colo-357, T3M4, Aspc-1, and Panc-1 were a kind gift from the University Clinic, Heidelberg, Dan-G was kindly provided by Prof. Bednarski, Institute of Pharmacy, University of Greifswald, all other cell lines were purchased from the DSMZ, Braunschweig, Germany). Cells were grown in Dulbecco's modified Eagle's medium or RPMI 1640 (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% CO_2. 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 (Schnelldorf, Germany). Gemcitabine was purchased from Eli Lilly (Gießen, Germany). [^{14}C] 5-FU (52 mCi/mmol) was purchased from Moravek Biochemicals (Brea, CA).

Stable MRP5 expression in mammalian cells*:* Human pancreatic cancer cells Colo-357 were transfected with the pcDNA3.1/hygro-MRP5 construct or vector only using FuGENE 6.0 transfection reagent (Roche, 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.

siRNA knock down of MRP5: Transient knock down was achieved by annealing sense and antisense RNA oligonucleotide strands of MRP5 and GFP as negative control. Briefly, sense and antisense oligonucleotide strands of each target transcript were diluted separately in annealing buffer containing 50 mM Tris pH 7.6, 100 mM NaCl, sense and antisense strands of

the respective target transcript were then mixed and incubated for two minutes at 95°C, and allowed to cool to room temperature. Sequences of oligonucleotides used were: MRP5 (antisense) 5'-AUACUCUUUUCCUAUGUCGUU-3', (sense) 5'-CGACAUAGGAAAAGAGUAUUU-3'; GFP (antisense) 5'-AUACUCUUUUCCUAUGUCGUU-3', (sense) 5'-CGACAUAGGAAAAGAGUAUUU-3'. siRNAs were then transfected into cells using Nanofectin according to the manufacturer's protocol (PAA Laboratories GmbH, Cölbe, Germany). After 48 h mRNA was detected using real-time RT-PCR. For stable kock down, MRP5 specific siRNA expression vectors were generated by cloning the sequences 5'-CGAACCCGCACAGAGACCGTGAAGATTCTTCAAGAGAGAATCTTCACGGTCTCTG TGCTTTTTGGAAC-3' (forward) and 5'-TCGAGTTCCAAAAAGCACAGAGACCGTGAAGATTCTCTCTTGAAGAATCTTCACG GTCTCTGTGCGGGTT-3'(reverse) encoding the hairpin siRNA targeted to MRP5 into the XhoI/Bsp119I site of the modified pQCXIH vector as described (Lopez De Jesus et al., 2006). Cells were transfected with vector harboring the expression cassette or the empty vector using FuGENE 6.0 transfection reagent (Roche, Mannheim, Germany). After 48 h, the cells were split, and stable transfectants were selected using medium containing hygromycin B (Invitrogen, Karlsruhe, Germany). 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 the TaqMan reverse transcription (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. Primer and probe oligonucleotides for PCR were purchased from Invitrogen

(Karlsruhe, Germany). Real-time quantitative PCR for MRP4 was performed using forward primer 5‘-GTCTTCATTTTCCTTATTCTCCTAAACAC-3‘, reverse primer 5‘-CCATTTACAGTGACATTTAGCATACTTTGT-3‘ and the probe 5‘-(FAM)-CCAGTATGAAAGCCACCAATCTTGAAGCA-(TAMRA)-3’ and for MRP5 forward primer 5’-CACCATCCACGCCTACAATAAA-3‘, reverse primer 5‘-CACCGCATCGCACACGTA-3‘ and the probe 5‘-(FAM)-GCTTGGTTGTCATCCAGCAGCTCCTG-(TAMRA)-3’ were used. For hENT1 detection the following primers were used: 5’-GAGGGAGCTGTCAGCCAG-3’ (forward), 5’-ACAGCCCCCAGTGCCAGA-3’ (reverse) and 5’-(FAM)-CAGCACCTGGGAACGTTACTT-(TAMRA)-3’ (probe). For detection of thymidylate synthase, breast cancer resistance protein (BCRP), and 18S rRNA a pre-developed primer and probe mix was purchased from Applied Biosystems (Foster City, CA). The measurement of the gene transcriptions was performed using 20 ng cDNA.

A 2x PCR Master mix (45 mM Tris HCl, pH 8.4, 115 mM KCl, 7 mM $MgCl_2$, 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, Foster City, CA). 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-X100 (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 (AlexaFluor 488-labeled anti-rabbit IgG, dilution 1:200; Molecular Probes, Eugene, OR) for at least 1 h at room temperature and additionally washed five times with PBS. Nuclei counterstaining with TOTO-3-iodide dye (dilution 1:2000; Molecular Probes, OR) was performed within the Fluorescent Mounting Medium (DakoCytomation, Carpinteria, CA). Immunostained samples were photographed using a constant exposure time with a 40x 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 5,000 cells/well to 10,000 cells/well were plated onto a 96-well microtiter plate in 200 µl of medium. After 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 buffer solution for fixation. The fixing solution was discarded and solution of 0.5% crystal violet (Sigma, 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 the optical density (OD) at 570 nm was read by a microtiter plate reader Wallac Victor^2 (Perkin Elmer, Turku, Finland). OD of untreated cells was set 100%. Viability and the relative viability were calculated from the OD values of drug treated cells.

Cytofluorometric analysis of cell death: To determine cell death, cells were harvested after 48 h cultivation in the presence of respective drugs, followed by a 5 min incubation step in 2 µg/ml propidium iodide (PI; Sigma, Taufkirchen, Germany) in PBS at 4°C in the dark. PI uptake was assessed by flow cytometry analysis on a Becton Dickinson (Heidelberg,

Germany) FACS Calibur using CellQuest software. 10,000 cells were analyzed in each sample; 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 [^{14}C] 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 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 (type 1409; PerkinElmer Wallac, Turku, Finland) and normalized to protein content.

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-FdUrd in incubation buffer containing 140 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.5 mM $CaCl_2$, 5 mM glucose, and 12.5 mM HEPES, pH 7.3 for 20 min at 37°C, washed three times with warm PBS and 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 carried out by LC-MS as described previously (Carli et al., 2009; Honorat et al., 2008).

Data Analysis: Values are presented as means ± SD unless stated otherwise. Graphs and calculations were prepared using Excel (Microsoft Corp., Redmond, WA) or Prism 3.0 software (GraphPad Software, San Diego, CA). The IC_{50} values were calculated by nonlinear regression from a sigmoidal dose-response curve (variable slope, bottom value ≥ 0) using GraphPad. Spearman's test was used to analyze the correlation between the expression of MRP4 and MRP5 and the IC_{50} values. A p-value ≤ 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-89O2, Patu-8988T and Patu-8988S) was measured by quantitative real-time RT-PCR. Expression of MRP8 was undetectable (data not shown) while 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 in the methods section. MRP4 and MRP5 were visualized by immunofluorescence staining using the anti-MRP4 polyclonal antibody SNG and anti-MRP5 polyclonal antibody AMF, respectively. A predominant staining of the plasma membranes for MRP4 and MRP5 could be detected (Fig. 1B).

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-89O2, Patu-8988T and Patu-8988S) to the nucleotide analogues 5-FU, GEM, 6-MP and 6-TG. IC_{50} 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, IC_{50} 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 drugs tested.

IC_{50} values of all cell lines to different drugs (5-FU, GEM, 6-MP, 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 ($r=0.841$, $p<0.01$) (Fig. 2A), while the correlations between MRP5 and gemcitabine ($r=0.626$), 6-mercaptopurine ($r=-0.086$), and 6-

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thioguanine (r=0.418) were not statistically significant nor was MRP4 mRNA expression significantly correlated to any of the investigated drugs (5-FU r=0.386; GEM r=0.559; 6-MP r=0.553; 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 six 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 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). Interestingly, MRP4 also increased significantly by 2.5 fold, while thymidylate synthase and BCRP were not significantly altered in 5-FU resistant cells as compared to the controls (data not shown). Sensitivity to 5-FU was assessed by crystal violet assay as described in the methods section. 5-FU treated cells were significantly more resistant compared to untreated cells, the IC_{50} values of Patu-T (control) and 5-FU resistant (Patu-T/5-FU) cells were 11.3±5.3 µM and 33.2±6.9 µM 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 in the methods section. 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 as compared to 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 propidium iodide (PI) uptake. Vector-transfected and MRP5-transfected cells were cultured in the absence or presence of 5-FU (1–33 μM) for 48 h. We observed a concentration-dependent but significantly diminished sensitivity of MRP5-overexpressing cells to 5-FU (Fig. 4B, $p<0.05$). Percentages of dead cells were 26.4, 45.4, 54.6, and 53.0% in control cells compared to 16.4, 31.2, 39.1, and 44.9% in MRP5-overexpressing cells.

Analysis of accumulation and efflux of [^{14}C] 5-FU and 5-FdUMP in MRP5 transfected Colo-357 cells

We analyzed substrate specificity in MRP5 overexpressing Colo-357 cells and performed transport assays with radiolabeled [^{14}C] 5-FU to ensure that the increased 5-FU resistance in these cells correlates with increased efflux of 5-FU. Upon incubation for 2 h in medium containing [^{14}C] 5-FU, Colo-pcDNA3.1/hygro-MRP5 cells accumulated 59.2% less drug intracellularly compared with control Colo-pcDNA3.1/hygro cells (Fig. 4C, $p<0.01$). We also determined whether the decreased accumulation was a consequence of enhanced drug efflux (Fig. 4D). MRP5-transfected cells exhibited significantly enhanced drug efflux (2.1fold, $p<0.05$) compared to 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 as 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 as compared to 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 to control cells contributes to the variable sensitivity of these cells to 5-FU. We can, however, not exclude that active metabolites such as 5-FUMP contribute to 5-FU's toxicity as well, as we did not assess this metabolite in our system.

Sensitivity of MRP5 siRNA transfected Patu-02 cells to 5-FU

As MRP5 expression in pancreatic cancer-derived cell lines is clearly detectable and MRP5 expression correlates with cellular sensitivity to 5-FU, this may suggest a potential role of MRP5 in intrinsic drug resistance in pancreatic cancer. To investigate if 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. Both, transient and stable transfection with siRNA constructs decreased MRP5 mRNA levels to 36% and 55% of control cells, respectively (Fig. 5A, B). Cytofluorometric cell death assays revealed similar death rates of stable control and MRP5 knock down cells under normal growth conditions. However, addition of 5-FU (3-100 μM) caused a significant increase in cell death in MRP5 knock down cells (36, 53, 80, 94%) when compared to the vector transfected cells (10, 27, 63, 66%) (Fig. 5C, $p<0.01$).

Analysis of [^{14}C] 5-FU accumulation and efflux in MRP5 knock down Patu-02 cells

To study the difference in transport of 5-FU in knock down cells, we performed transport assays with radiolabeled [^{14}C] 5-FU as described in the methods section. Intracellular drug

accumulation was significantly enhanced in Patu-02 cells both transiently and stably transfected with MRP5 siRNA when compared to the respective control transfected Patu-02 cells at 30 and 60 min of drug drug incubation (Fig. 5D, E). In addition, efflux assays demonstrated that Patu-02 cells stably transfected with MRP5 siRNA exported 37% less drug compared to the Patu-02-Mock cells over an efflux period of two hours (Fig. 5F, $p<0.05$). 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 drug resistance proteins is one mechanism by which drug resistance is achieved (Giovannetti et al., 2006). In a recent study 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 to normal pancreatic tissue (Konig et al., 2005). In our study, we found MRP4 and MRP5 expression in all investigated pancreatic adenocarcinoma cell lines 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, knock down 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), in cardiomyocytes (Dazert et al., 2003), brain (Nies et al., 2004; Vogelgesang et al., 2004) as well as in human placenta (Meyer zu Schwabedissen et al., 2005) on mRNA and protein levels. In these tissues, MRP5 is thought to regulate intracellular levels of cyclic guanosine monophosphate (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 cyclic adenosine monophosphate (cAMP), MRP5 has been characterized 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). Recent reports found that 5-FU metabolites are transported by MRP5 as well (Pratt

et al., 2005). The transport of other nucleosidic anticancer drugs particularly gemcitabine, however, is controversial (Oguri et al., 2006) (Reid et al., 2003). Typically, the export of nucleoside analogues by MRP5 expression increases drug resistance in the range of 2- to 10-fold to these drugs *in vitro* (Pratt et al., 2005; Borst et al., 2004), 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 knock-down 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 breast cancer patients and MRP5 was found to be significantly higher expressed in the group of non-responders after neoadjuvant chemotherapy compared to the responders group (Park et al., 2006). Notably, all patients were treated with a chemotherapy regimen containing 5-FU. Expression of most MRP drug transporters was demonstrated in pancreatic tissue (Konig et al., 2005), however only MRP3 and MRP5 were differentially expressed when comparing mRNA levels in normal pancreatic tissue and pancreatic carcinoma. These transporters were also upregulated in a pancreatic adenocarcinoma cell line that acquired resistance to 5-FU and regained sensitivity when MRP5 expression was reduced by siRNA knock-down (Hagmann et al., 2009). In our study we confirm a significant upregulation of MRP4 and MRP5 during adaptation to 5-FU and a gain of sensitivity after downregulation of MRP5 expression. Although MRP4 transcription did not significantly correlate with sensitivities to the drugs tested in our cell panel the upregulation of MRP4 mRNA after long-term incubation with 5-FU might be of relevance. Novel strategies that utilize combinations of inhibitor substances may include yet unknown substrates for this transporter. Additionally, we cannot rule out that these transporters are regulated as part of a multifactorial cellular adaptation mechanism as transporter upregulation has also been observed when cells adapt to

drugs that are no specific substrates for these transporter proteins (Yoshida et al., 2001 and P.K.N, C.A.R unpublished data). Similarly, in a recent report MRP7 was capable to transport and confer resistance to nucleoside analog drugs such as gemcitabine, cytarabine, dideoxycytidin, and PMEA (Hopper-Borge et al., 2009). Although mRNA expression of MRP7 has been detected in pancreatic tissue (Hopper et al., 2001) its impact on drug resistance in pancreatic cancer requires future investigations.

Our work further 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 a variable sensitivity to 5-FU and an altered accumulation of 5-FU or its active metabolites. We also find MRP5-dependent efflux activity for 5-FdUMP that was converted from exogenous 5-FdUrd. As 5-FU is metabolized via 5-FdUrd to the cytotoxically active 5-FdUMP intracellularly, we assume that the modified efflux capacity for 5-FdUMP by MRP5 in our model system at least in part contributes to the modified sensitivity for 5-FU. However, possible contributions of other active metabolites, such as 5-FUMP, are not considered. Finally, we not only investigated ABCC 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 and colleagues found a significant correlation between high-level thymidylate synthase and poor prognostic outcome, however, these patients were more susceptible to adjuvant treatment with 5-FU (Hu et al., 2003). In contrary, median survival was unaffected by thymidylate synthase immunoreactivity in a cohort of pancreatic ductal adenocarcinoma patients who received adjuvant 5-FU chemotherapy (Formentini et al., 2007). In our carcinoma cell line panel we found a trend towards an inverse correlation between 5-FU sensitivity and expression of thymidylate synthase which might support the findings by Hu and colleagues. 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 efflux transporter, the expression of the uptake transporter hENT1 has been related to 5-FU sensitivity in that its expression correlated with growth inhibiting activity of 5-FU in a panel of pancreatic cancer cell lines (Tsujie et al., 2007). Similarly, 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 significant correlation of hENT1 or BCRP expression with 5-FU sensitivity nor could we detect a transcriptional regulation in the process of resistance acquisition to 5-FU.

Certainly, the situation in a patient's pancreas 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 have emerged as a relevant factor influencing tumor growth and chemotherapy response. These include normal host epithelial cells, stromal fibroblasts, inflammatory cells, altered extracellular matrix as well as proliferating endothelial cells (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). In order to investigate these processes appropriately, genetic mouse models of pancreatic ductal adenocarcinoma were developed that most closely resemble human pancreatic tumors. Olive and coworkers observed that in a mouse tumor model expressing endogenous mutant Kras and p53 alleles in pancreatic cells that tumors were poorly perfused and vascularized compared to xenograft transplantation models. Depletion of tumor-associated stromal tissue, however, by an inhibitor of the hedgehog cellular signaling pathway significantly increased intratumoral vascular density and stabilization of disease when

coadministered with chemotherapy (Olive et al., 2009). Since delivery of drugs to pancreatic tumor cells seems to depend on vascular density and the drug transporter MRP5 has been localized to vascular endothelium, future research should 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 upregulation of MRP5 in resistant subclones of pancreatic cancer cell lines. Furthermore, stable knock down 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, Sendler, and Rimmbach
Contributed new reagents or analytic tools: Payen, Jedlitschky, Rosskopf, Kowalcyk, 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

References

Blaszkowsky L (1998) Treatment of advanced and metastatic pancreatic cancer. *Front Biosci* **3**: E214-E225.

Borst P, Balzarini J, Ono N, Reid G, de Vries H, Wielinga P, Wijnholds J, and Zelcer N (2004) The potential impact of drug transporters on nucleoside-analog-based antiviral chemotherapy. *Antiviral Res* **62**: 1-7.

Borst P and Elferink RO (2002) Mammalian ABC transporters in health and disease. *Annu Rev Biochem* **71**: 537-592.

Borst P, Evers R, Kool M, and Wijnholds J (2000) A family of drug transporters: the multidrug resistance-associated proteins. *J Natl Cancer Inst* **92**: 1295-1302.

Carli D, Honorat M, Cohen S, Megherbi M, Vignal B, Dumontet C, Payen L, and Guitton J (2009) Simultaneous quantification of 5-FU, 5-FUrd, 5-FdUrd, 5-FdUMP, dUMP and TMP in cultured cell models by LC-MS/MS. *J Chromatogr B Analyt Technol Biomed Life Sci* **877**: 2937-2944.

Chen ZS, Lee K, and Kruh GD (2001) Transport of cyclic nucleotides and estradiol 17-beta-D-glucuronide by multidrug resistance protein 4. Resistance to 6-mercaptopurine and 6-thioguanine. *J Biol Chem* **276**: 33747-33754.

Cress RD, Yin D, Clarke L, Bold R, and Holly EA (2006) Survival among patients with adenocarcinoma of the pancreas: a population-based study (United States). *Cancer Causes Control* **17**: 403-409.

Dazert P, Meissner K, Vogelgesang S, Heydrich B, Eckel L, Bohm M, Warzok R, Kerb R, Brinkmann U, Schaeffeler E, Schwab M, Cascorbi I, Jedlitschky G, and Kroemer HK (2003)

Expression and localization of the multidrug resistance protein 5 (MRP5/ABCC5), a cellular export pump for cyclic nucleotides, in human heart. *Am J Pathol* **163**: 1567-1577.

Formentini A, Sander S, Denzer S, Straeter J, Henne-Bruns D, and Kornmann M (2007) Thymidylate synthase expression in resectable and unresectable pancreatic cancer: role as predictive or prognostic marker? *Int J Colorectal Dis* **22**: 49-55.

Giovannetti E, Mey V, Nannizzi S, Pasqualetti G, Del TM, and Danesi R (2006) Pharmacogenetics of anticancer drug sensitivity in pancreatic cancer. *Mol Cancer Ther* **5**: 1387-1395.

Guo Y, Kotova E, Chen ZS, Lee K, Hopper-Borge E, Belinsky MG, and Kruh GD (2003) MRP8, ATP-binding cassette C11 (ABCC11), is a cyclic nucleotide efflux pump and a resistance factor for fluoropyrimidines 2',3'-dideoxycytidine and 9'-(2'-phosphonylmethoxyethyl)adenine. *J Biol Chem* **278**: 29509-29514.

Hagmann W, Jesnowski R, Faissner R, Guo C, and Lohr JM (2009) ATP-binding cassette C transporters in human pancreatic carcinoma cell lines. Upregulation in 5-fluorouracil-resistant cells. *Pancreatology* **9**: 136-144.

Honorat M, Mesnier A, Vendrell J, Guitton J, Bieche I, Lidereau R, Kruh GD, Dumontet C, Cohen P, and Payen L (2008) ABCC11 expression is regulated by estrogen in MCF7 cells, correlated with estrogen receptor alpha expression in postmenopausal breast tumors and overexpressed in tamoxifen-resistant breast cancer cells. *Endocr Relat Cancer* **15**: 125-138.

Hopper E, Belinsky MG, Zeng H, Tosolini A, Testa JR, and Kruh GD (2001) Analysis of the structure and expression pattern of MRP7 (ABCC10), a new member of the MRP subfamily. *Cancer Lett* **162**: 181-191.

Hopper-Borge E, Xu X, Shen T, Shi Z, Chen ZS, and Kruh GD (2009) Human multidrug resistance protein 7 (ABCC10) is a resistance factor for nucleoside analogues and epothilone B. *Cancer Res* **69**: 178-184.

Hu YC, Komorowski RA, Graewin S, Hostetter G, Kallioniemi OP, Pitt HA, and Ahrendt SA (2003) Thymidylate synthase expression predicts the response to 5-fluorouracil-based adjuvant therapy in pancreatic cancer. *Clin Cancer Res* **9**: 4165-4171.

Huanwen W, Zhiyong L, Xiaohua S, Xinyu R, Kai W, and Tonghua L (2009) Intrinsic chemoresistance to gemcitabine is associated with constitutive and laminin-induced phosphorylation of FAK in pancreatic cancer cell lines. *Mol Cancer* **8**: 125.

Jedlitschky G, Burchell B, and Keppler D (2000) The multidrug resistance protein 5 functions as an ATP-dependent export pump for cyclic nucleotides. *J Biol Chem* **275**: 30069-30074.

Jemal A, Siegel R, Ward E, Murray T, Xu J, Smigal C, and Thun MJ (2006) Cancer statistics, 2006. *CA Cancer J Clin* **56**: 106-130.

Konig J, Hartel M, Nies AT, Martignoni ME, Guo J, Buchler MW, Friess H, and Keppler D (2005) Expression and localization of human multidrug resistance protein (ABCC) family members in pancreatic carcinoma. *Int J Cancer* **115**: 359-367.

Lopez De Jesus M, Stope MB, Oude Weernink PA, Mahlke Y, Borgermann C, Ananaba VN, Rimmbach C, Rosskopf D, Michel MC, Jakobs KH, and Schmidt M (2006) Cyclic AMP-dependent and Epac-mediated activation of R-Ras by G protein-coupled receptors leads to phospholipase D stimulation. *J Biol Chem* **281**: 21837-21847.

Mahadevan D and Von Hoff DD (2007) Tumor-stroma interactions in pancreatic ductal adenocarcinoma. *Mol Cancer Ther* **6**: 1186-1197.

Meyer zu Schwabedissen HE, Grube M, Heydrich B, Linnemann K, Fusch C, Kroemer HK, and Jedlitschky G (2005) Expression, localization, and function of MRP5 (ABCC5), a transporter for cyclic nucleotides, in human placenta and cultured human trophoblasts: effects of gestational age and cellular differentiation. *Am J Pathol* **166**: 39-48.

Miyamoto H, Murakami T, Tsuchida K, Sugino H, Miyake H, and Tashiro S (2004) Tumor-stroma interaction of human pancreatic cancer: acquired resistance to anticancer drugs and proliferation regulation is dependent on extracellular matrix proteins. *Pancreas* **28**: 38-44.

Nies AT, Jedlitschky G, Konig J, Herold-Mende C, Steiner HH, Schmitt HP, and Keppler D (2004) Expression and immunolocalization of the multidrug resistance proteins, MRP1-MRP6 (ABCC1-ABCC6), in human brain. *Neuroscience* **129**: 349-360.

Nies AT, Spring H, Thon WF, Keppler D, and Jedlitschky G (2002) Immunolocalization of multidrug resistance protein 5 in the human genitourinary system. *J Urol* **167**: 2271-2275.

Oettle H, Post S, Neuhaus P, Gellert K, Langrehr J, Ridwelski K, Schramm H, Fahlke J, Zuelke C, Burkart C, Gutberlet K, Kettner E, Schmalenberg H, Weigang-Koehler K, Bechstein WO, Niedergethmann M, Schmidt-Wolf I, Roll L, Doerken B, and Riess H (2007) Adjuvant chemotherapy with gemcitabine vs observation in patients undergoing curative-intent resection of pancreatic cancer: a randomized controlled trial. *JAMA* **297**: 267-277.

Oguri T, Achiwa H, Sato S, Bessho Y, Takano Y, Miyazaki M, Muramatsu H, Maeda H, Niimi T, and Ueda R (2006) The determinants of sensitivity and acquired resistance to gemcitabine differ in non-small cell lung cancer: a role of ABCC5 in gemcitabine sensitivity. *Mol Cancer Ther* **5**: 1800-1806.

Oguri T, Bessho Y, Achiwa H, Ozasa H, Maeno K, Maeda H, Sato S, and Ueda R (2007) MRP8/ABCC11 directly confers resistance to 5-fluorouracil. *Mol Cancer Ther* **6**: 122-127.

Olive KP, Jacobetz MA, Davidson CJ, Gopinathan A, McIntyre D, Honess D, Madhu B, Goldgraben MA, Caldwell ME, Allard D, Frese KK, Denicola G, Feig C, Combs C, Winter SP, Ireland-Zecchini H, Reichelt S, Howat WJ, Chang A, Dhara M, Wang L, Ruckert F, Grutzmann R, Pilarsky C, Izeradjene K, Hingorani SR, Huang P, Davies SE, Plunkett W, Egorin M, Hruban RH, Whitebread N, McGovern K, Adams J, Iacobuzio-Donahue C, Griffiths J, and Tuveson DA (2009) Inhibition of Hedgehog signaling enhances delivery of chemotherapy in a mouse model of pancreatic cancer. *Science* **324**: 1457-1461.

Park S, Shimizu C, Shimoyama T, Takeda M, Ando M, Kohno T, Katsumata N, Kang YK, Nishio K, and Fujiwara Y (2006) Gene expression profiling of ATP-binding cassette (ABC) transporters as a predictor of the pathologic response to neoadjuvant chemotherapy in breast cancer patients. *Breast Cancer Res Treat* **99**: 9-17.

Pratt S, Shepard RL, Kandasamy RA, Johnston PA, Perry W, III, and Dantzig AH (2005) The multidrug resistance protein 5 (ABCC5) confers resistance to 5-fluorouracil and transports its monophosphorylated metabolites. *Mol Cancer Ther* **4**: 855-863.

Reid G, Wielinga P, Zelcer N, de Haas M, Van Deemter L, Wijnholds J, Balzarini J, and Borst P (2003) Characterization of the transport of nucleoside analog drugs by the human multidrug resistance proteins MRP4 and MRP5. *Mol Pharmacol* **63**: 1094-1103.

Ritter CA, Jedlitschky G, Meyer Zu SH, Grube M, Kock K, and Kroemer HK (2005) Cellular export of drugs and signaling molecules by the ATP-binding cassette transporters MRP4 (ABCC4) and MRP5 (ABCC5). *Drug Metab Rev* **37**: 253-278.

Storniolo AM, Enas NH, Brown CA, Voi M, Rothenberg ML, and Schilsky R (1999) An investigational new drug treatment program for patients with gemcitabine: results for over 3000 patients with pancreatic carcinoma. *Cancer* **85**: 1261-1268.

Tsujie M, Nakamori S, Nakahira S, Takahashi Y, Hayashi N, Okami J, Nagano H, Dono K, Umeshita K, Sakon M, and Monden M (2007) Human equilibrative nucleoside transporter 1, as a predictor of 5-fluorouracil resistance in human pancreatic cancer. *Anticancer Res* **27**: 2241-2249.

Vogelgesang S, Kunert-Keil C, Cascorbi I, Mosyagin I, Schroder E, Runge U, Jedlitschky G, Kroemer HK, Oertel J, Gaab MR, Pahnke J, Walker LC, and Warzok RW (2004) Expression of multidrug transporters in dysembryoplastic neuroepithelial tumors causing intractable epilepsy. *Clin Neuropathol* **23**: 223-231.

Wielinga PR, Reid G, Challa EE, van der Heijden I, Van Deemter L, de Haas M, Mol C, Kuil AJ, Groeneveld E, Schuetz JD, Brouwer C, De Abreu RA, Wijnholds J, Beijnen JH, and Borst P (2002) Thiopurine metabolism and identification of the thiopurine metabolites transported by MRP4 and MRP5 overexpressed in human embryonic kidney cells. *Mol Pharmacol* **62**: 1321-1331.

Yoshida M, Suzuki T, Komiya T, Hatashita E, Nishio K, Kazuhiko N, and Fukuoka M (2001) Induction of MRP5 and SMRP mRNA by adriamycin exposure and its overexpression in human lung cancer cells resistant to adriamycin. *Int J Cancer* **94**: 432-437.

Yuan J, Lv H, Peng B, Wang C, Yu Y, and He Z (2009) Role of BCRP as a biomarker for predicting resistance to 5-fluorouracil in breast cancer. *Cancer Chemother Pharmacol* **63**: 1103-1110.

Footnotes

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Figure legends

Figure 1: Expression of MRP4 and MRP5 in pancreatic cancer derived cell lines. (A) Total RNA was extracted from eight human pancreatic cancer cell lines (Patu-8988T, Patu-8988S, Patu-89O2, Panc-1, Colo-357, Aspc-1, Dan-G, and T3M4) and MRP4 (white bars) and MRP5 (black bars) mRNA expression was measured by quantitative RT-PCR. 18S ribosomal RNA was used for normalization of cDNA samples. Data are expressed as copy number x 100,000 (mean ± SD, n=3); results are representative for replicate experiments. (B) Immunolocalization of MRP4 *(top)* and MRP5 *(bottom)* in pancreatic cancer cell lines Patu-O2 and Colo-357. Drug transporter expression was detected using the polyclonal rabbit antisera SNG against MRP4 and AMF against MRP5. Fluorescence was developed by staining with a gaot anti-rabbit secondary antibody labeled with Alexa Fluor 488 (green fluorescence). The blue staining represents the nuclear staining with TOTO-3 Iodide dye (magnification 400x).

Figure 2: Correlation of IC_{50} values of 5-fluorouracil (5-FU) and expression of target mRNA of genes that are related to 5-FU resistance in a panel of pancreatic adenocarcinoma cell lines: (A) MRP5, (B) thymidylate synthase (TS), (C) BCRP, and (D) hENT1. Symbols represent means of three cell viability assays performed in triplicates. Statistical significance of the correlations was determined by Spearman's correlation test and the level of significance was set at 5% using a two-sided analysis.

Figure 3: Regulation of MRP5 expression in drug resistance acquired to 5-FU. (A) Relative expression of MRP5 mRNA in the 5-FU resistant cell line Patu-T/5-FU after cultivation in the presence of increasing concentrations of 5-FU for 6 months. (B) Dose response curves for 5-FU resistant subclone: at least five drug concentrations were used to

determine IC50 values. Experiments at each concentration were carried out in triplicates. IC_{50} values of Patu-T (open circles) and Patu-T/5-FU cells (closed circles) were 11.3±5.3 µM and 33.2±6.9 µM respectively. Data represent mean ± SD, $p<0.05$.

Figure 4: Overexpression of MRP5 in the pancreatic cancer cell line Colo-357. (A) Expression of MRP5 mRNA in Colo-pcDNA3.1/hygro and Colo-pcDNA3.1/hygro-MRP5 was measured by quantitative RT-PCR. (B) Colo-pcDNA3.1/hygro (white bars) and Colo-pcDNA3.1/hygro-MRP5 cells (black bars) were incubated with 5-FU (1-33µM) for 48 h and cell death was determined by cytofluorometric analysis of PI uptake. Data represent mean±SD of three cell death assays done in triplicate; *$p<0.05$; **$p<0.01$. (C) Time dependent accumulation of [^{14}C] 5-FU in Colo-pcDNA3.1/hygro (white bars) and Colo-pcDNA3.1/hygro-MRP5 (black bars) was measured as described in materials & methods at different time points; **$p<0.01$; ***$p<0.005$. (D) Efflux of 5-FU from Colo-pcDNA3.1/hygro (squares) and Colo-pcDNA3.1/hygro-MRP5 (triangles) into medium after various efflux times. Cells were first incubated for 2 h in the presence of [^{14}C] 5-FU under energy-depletion conditions, then the medium was changed to 5-FU free complete medium and efflux of 5-FU into the medium was measured at various time points. Data represent mean ± SD; *$p<0.05$; **$p<0.01$. (E) Accumulation of 5-FdUMP was assessed after pre-incubating Colo-pcDNA3.1/hygro (white bars) and Colo-pcDNA3.1/hygro-MRP5 (black bars) with 100 µM 5-FdUrd for 20 min in the absence or presence of 10 µM MK571. Intracellular 5-FdUMP was determined after 15 minutes of efflux as described in the Methods section. Values represent mean ± SD (n=3, *$p<0.05$; **$p<0.01$, one-way ANOVA). (F) 5-FdUMP effluxed 15 minutes after incubation period described above, n.d. not detected. Values represent mean±SD (n=3).

Figure 5: Knock-down of MRP5 expression in the pancreatic cancer cell line Patu-02. (A, B) Expression of MRP5 mRNA in Patu-02 cell (A) transiently transfected with GFP

siRNA (white bars) and MRP5 siRNA (black bars) or (B) stably transfected Patu-02/V4 (white bar) and Patu-02/V4siMRP5 cells (black bar) was measured by quantitative RT-PCR. (C) Patu-02/V4 (white bars) and Patu-02/V4-MRP5 siRNA cells (black bars) were incubated with 5-FU (3.3-100µM) for 48 h and cell death was determined by cytofluorometric analysis of PI uptake. Data represent mean ± SD of three cell death assays done in triplicate; **p<0.01; ***p<0.005. (C,D) Time-dependent accumulation of [^{14}C] 5-FU in Patu-02 cells (D) transiently transfected with GFP siRNA (white bars) and MRP5 siRNA (black bars) or (E) stably transfected Patu-02/V4 (white bars) and Patu-02/V4-MRP5 cells (black bars) was measured as described in the Methods section; *p<0.05, **p<0.01. (F) Efflux of 5-FU from Patu-02/V4 (squares) and Patu-02/V4-MRP5 siRNA (triangles) into medium. Cells were first incubated for 2 h in the presence of [^{14}C] 5-FU under energy-depletion conditions, then the medium was changed to 5-FU free complete medium and efflux of 5-FU into the medium was measured at various time points. Data represent mean ± SD; *p<0.05; **p<0.01.

Tables

Table 1: IC_{50} values of pancreatic cancer cell lines to 5-FU, GEM, 6-TG, and 6-MP. Values were determined by crystal violet assays as described in the methods section. At least five drug concentrations were used to determine IC_{50} values. Experiments at each concentration were carried out in triplicates and the IC_{50} values were calculated by nonlinear regression from a sigmoidal concentration-response curve (Prism software 3.0). Data represent mean ± SE of cumulated data of three independent experiments performed in triplicate (n=9).

	IC_{50} value			
Cell line	5-FU (μM)	GEM (nM)	6-TG (μM)	6-MP (μM)
T3M4	0.33 ± 0.06	1.50 ± 0.01	1.28 ± 0.03	2.63 ±0.02
Colo-357	2.05 ± 0.03	0.36 ± 0.01	2.09 ± 0.33	6.12 ±0.07
Patu-T	2.08 ± 0.07	2.80 ± 0.03	3.43 ± 0.03	4.09 ± 0.04
Patu-S	2.93 ± 0.04	3.20 ± 0.03	2.21± 0.03	11.07±0.07
Aspc-1	2.97 ± 0.03	4.00 ± 0.04	2.80 ± 0.02	2.45 ±0.01
Panc-1	3.94 ± 0.03	1.00 ± 0.02	8.31± 0.27	6.39 ±0.02
Patu-02	3.95 ± 0.01	3.40 ± 0.03	2.26± 0.02	9.69 ±0.04
Dan-G	4.38 ± 0.03	3.40 ± 0.02	4.32 ± 0.09	4.06 ±0.01

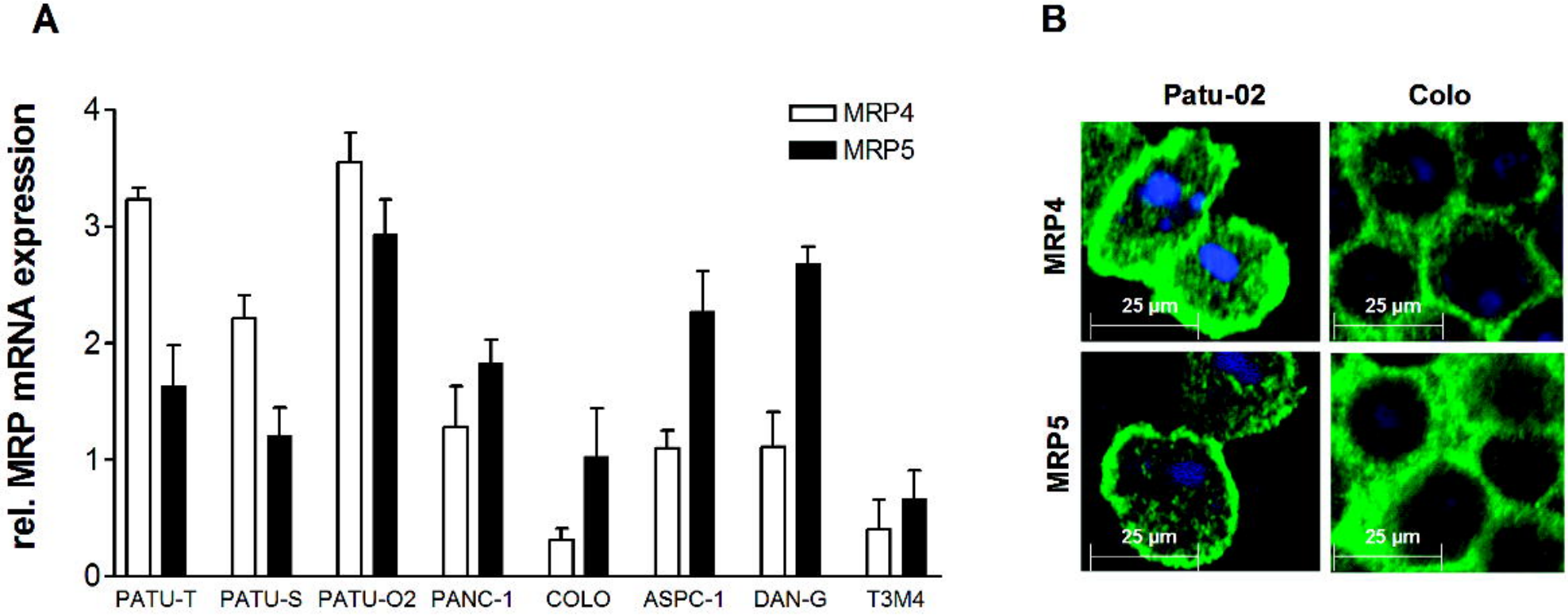
A
rel. MRP mRNA expression
0
1
2
3
4
MRP4
MRP5
PATU-T
PATU-S
PATU-O2
PANC-1
COLO
ASPC-1
DAN-G
T3M4
B
Patu-02
Colo
MRP4
MRP5
25 µm
25 µm
25 µm
25 µm

Figure 1

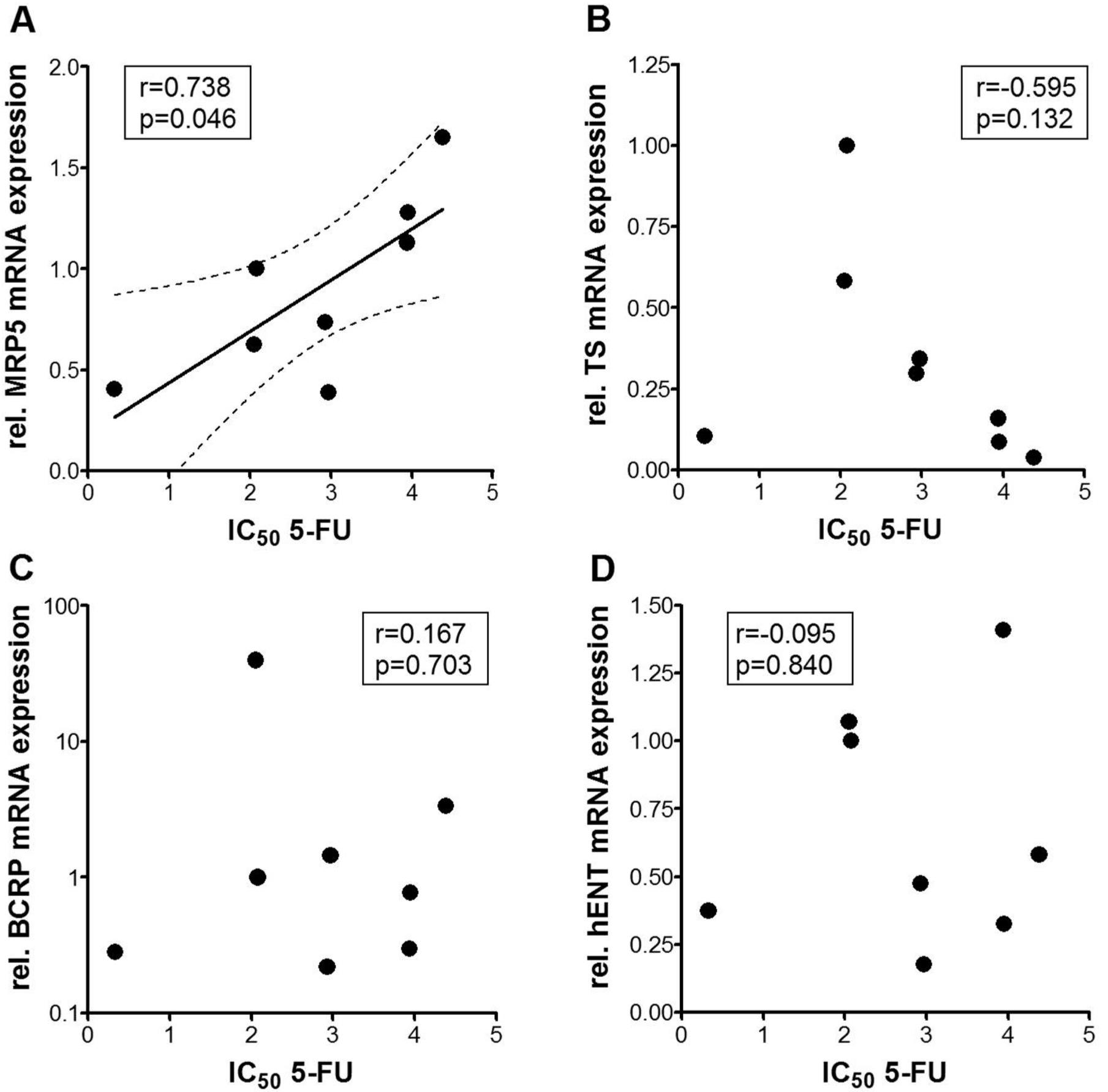
A
r=0.738
p=0.046
rel. MRP5 mRNA expression
IC50 5-FU
B
r=-0.595
p=0.132
rel. TS mRNA expression
IC50 5-FU
C
r=0.167
p=0.703
rel. BCRP mRNA expression
IC50 5-FU
D
r=-0.095
p=0.840
rel. hENT mRNA expression
IC50 5-FU

Figure 2

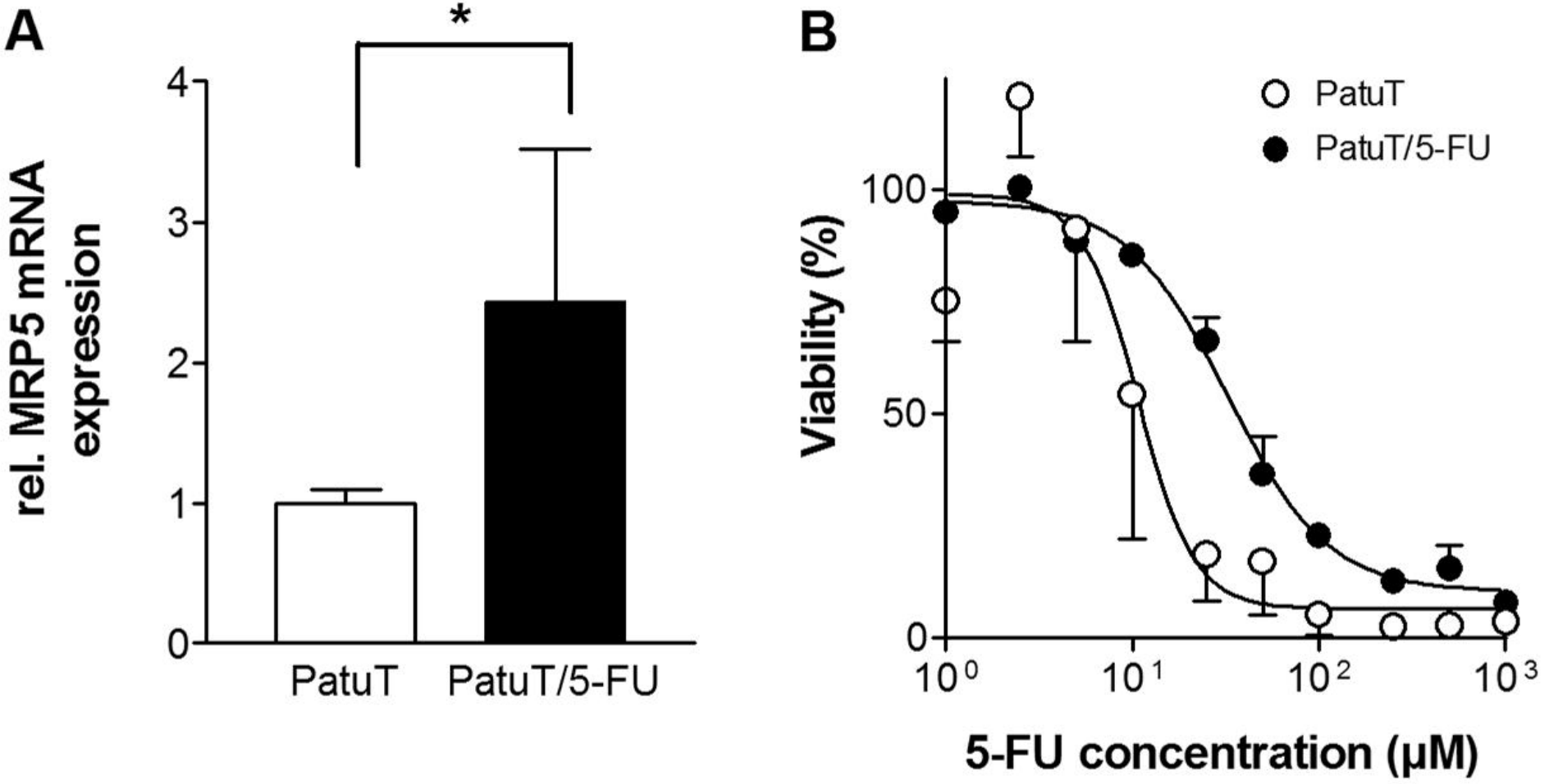
A
*
rel. MRP5 mRNA expression
4
3
2
1
0
PatuT
PatuT/5-FU
B
PatuT
PatuT/5-FU
Viability (%)
100
50
0
10 0
10 1
10 2
10 3
5-FU concentration (µM)

Figure 3

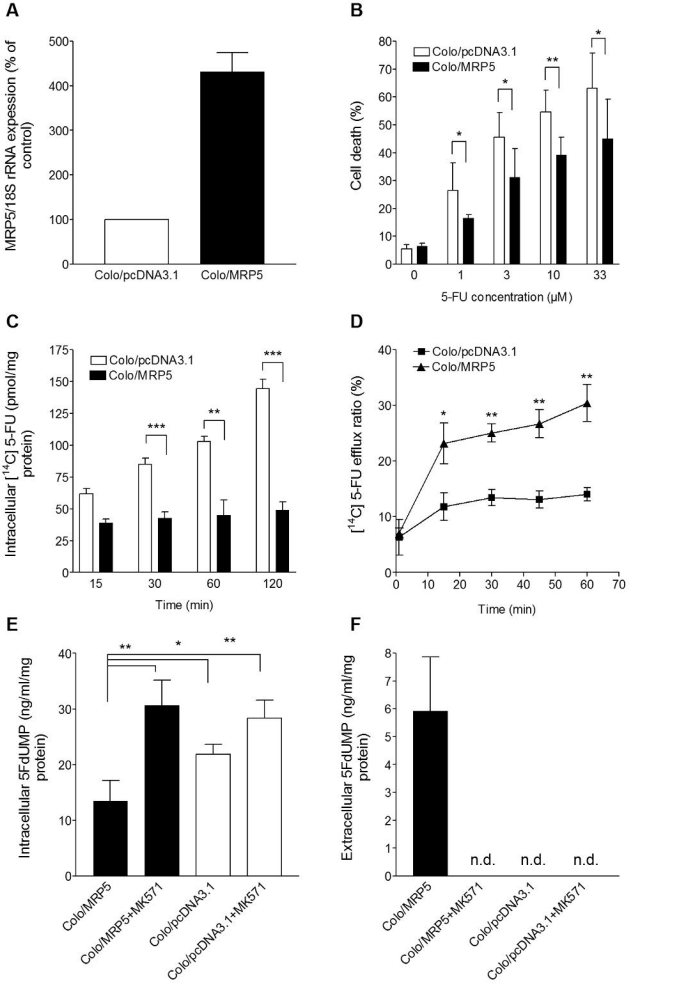
A
MRP5/18S rRNA expression (% of control)
500
400
300
200
100
0
Colo/pcDNA3.1
Colo/MRP5
B
Cell death (%)
Colo/pcDNA3.1
Colo/MRP5
80
70
60
50
40
30
20
10
0
*
*
**
*
0
1
3
10
33
5-FU concentration (μM)
C
Intracellular [14C] 5-FU (pmol/mg protein)
Colo/pcDNA3.1
Colo/MRP5
175
150
125
100
75
50
25
0
***
**
***
15
30
60
120
Time (min)
D
[14C] 5-FU efflux ratio (%)
Colo/pcDNA3.1
Colo/MRP5
40
30
20
10
0
*
**
**
**
0
10
20
30
40
50
60
70
Time (min)
E
Intracellular 5FdUMP (ng/ml/mg protein)
40
30
20
10
0
**
*
**
Colo/MRP5
Colo/MRP5+MK571
Colo/pcDNA3.1
Colo/pcDNA3.1+MK571
F
Extracellular 5FdUMP (ng/ml/mg protein)
8
7
6
5
4
3
2
1
0
n.d.
n.d.
n.d.
Colo/MRP5
Colo/MRP5+MK571
Colo/pcDNA3.1
Colo/pcDNA3.1+MK571

Figure 4

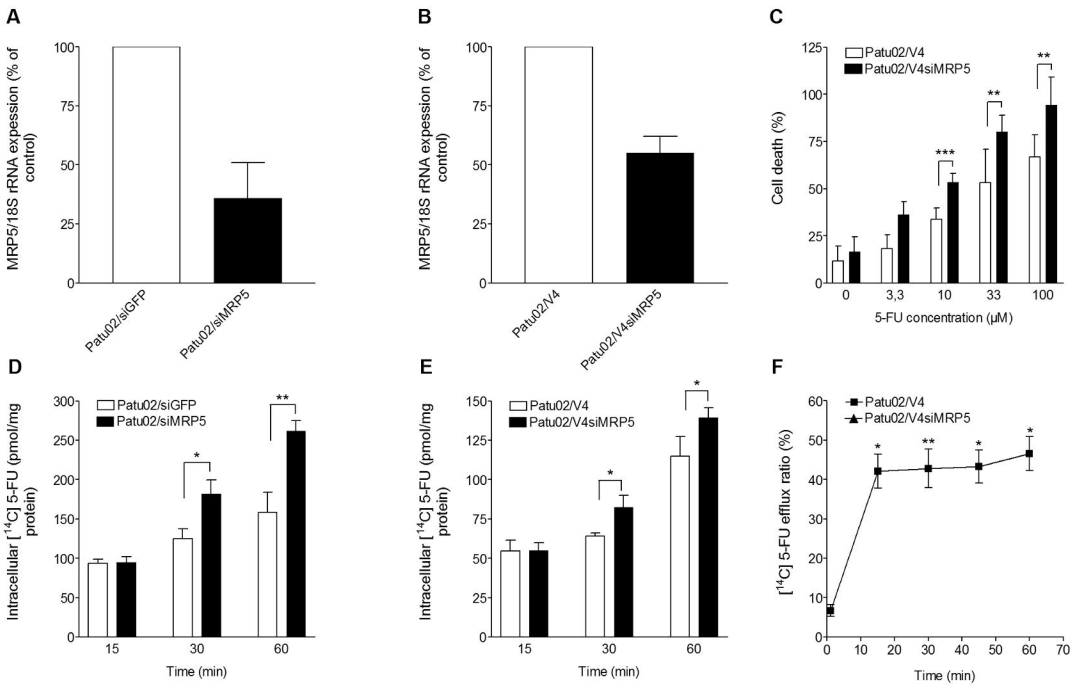
A
MRP5/18S rRNA expession (% of control)
100
75
50
25
0
Patu02/siGFP
Patu02/siMRP5
B
MRP5/18S rRNA expession (% of control)
100
75
50
25
0
Patu02/V4
Patu02/V4siMRP5
C
Patu02/V4
Patu02/V4siMRP5
Cell death (%)
125
100
75
50
25
0
***
**
**
0
3,3
10
33
100
5-FU concentration (µM)
D
Patu02/siGFP
Patu02/siMRP5
Intracellular [14C] 5-FU (pmol/mg protein)
300
250
200
150
100
50
0
*
**
15
30
60
Time (min)
E
Patu02/V4
Patu02/V4siMRP5
Intracellular [14C] 5-FU (pmol/mg protein)
150
125
100
75
50
25
0
*
*
15
30
60
Time (min)
F
Patu02/V4
Patu02/V4siMRP5
[14C] 5-FU efflux ratio (%)
60
50
40
30
20
10
0
*
**
*
*
0
10
20
30
40
50
60
70
Time (min)

Figure 5