

Gemcitabine Cytotoxicity: Interaction of Efflux and Deamination

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Abstract

Gemcitabine is a cytidine analogue used in the treatment of various solid tumors. Little is known about how gemcitabine and its metabolites are transported out of cells. We set out to study the efflux of gemcitabine and the possible consequences of that process in cancer cells. We observed the efflux of gemcitabine and its deaminated metabolite, 2',2'-difluorodeoxyuridine (dFdU) using high performance liquid chromatography and tandem mass spectrometry (LC-MS/MS) after gemcitabine treatment. Non-selective ABC-transport inhibition with probenecid significantly increased intracellular dFdU concentrations, with a similar trend observed with verapamil, a non-selective ABCB1 and ABCG2 transport inhibitor. Neither probenecid nor verapamil altered intracellular gemcitabine levels after the inhibition of deamination with tetrahydrouridine, suggesting that efflux of dFdU, but not gemcitabine, was mediated by ABC transporters. MTS assays showed that probenecid increased sensitivity to gemcitabine. While dFdU displayed little cytotoxicity, intracellular dFdU accumulation inhibited cytidine deaminase, resulting in increased gemcitabine levels and enhanced cytotoxicity. Knockdown of ABCC3, ABCC5 or ABCC10 individually did not significantly increase gemcitabine sensitivity, suggesting the involvement of multiple transporters. In summary, ABCC-mediated efflux may contribute to gemcitabine resistance through increased dFdU efflux that allows for the continuation of gemcitabine deamination. Reversing efflux-mediated gemcitabine resistance may require broad-based efflux inhibition.

Keywords: Gemcitabine; Cytotoxicity; Drug Efflux; Transport

Abbreviations: dFdU: Deaminated Metabolite, 2',2'-difluorodeoxyuridine; dCK: deoxycytidine kinase; CDA: Cytidine Deaminases; DCTD: Deoxycytidylate deaminases; THU: Tetrahydrouridine

Introduction

Gemcitabine (2'-deoxy-2',2'-difluorocytidine, dFdC) is a cytidine nucleoside analogue used to treat a wide variety of solid tumors including pancreatic, breast and non-small lung cancer [1-4]. It is a prodrug that requires active cellular uptake by members of the Solute Carrier SLC28 and SLC29 families, followed by intracellular activation to its monophosphorylated metabolites in a rate limiting step catalyzed by the enzyme deoxycytidine kinase (dCK), followed by subsequent phosphorylation reactions to form active di- and triphosphates [5-7]. Active phosphorylated gemcitabine metabolites induce apoptosis by incorporation into DNA and by the inhibition of ribonucleotide reductase (RR), an enzyme that catalyzes the conversion of ribonucleotides to deoxyribonucleotides [5,8]. Gemcitabine can also be metabolized to form a less active metabolite by deamination catalyzed by cytidine deaminase (CDA), while deoxycytidylate deaminase (DCTD) catalyzes the deamination of the monophosphorylated nucleotide [5,9].

There are many possible mechanisms for gemcitabine resistance. Resistance can result from a decrease in gemcitabine cellular uptake, as observed with decreased SLC29A1 (hENT1) transporter expression [10]. Levels of SLC29A1 and SLC28A3 (hCNT3) expression are correlated with prognosis after gemcitabine therapy [11,12]. In addition, changes in the expression or function of gemcitabine metabolizing enzymes could result in resistance as a result of decreased DCK or increased CDA expression or activity [10,12,13], leading to decreased gemcitabine effect [10,12,13]. Resistance could also result from alterations in cell proliferation, survival or apoptosis signaling pathways that are affected by gemcitabine treatment [10]. However, while the potential role of efflux in gemcitabine resistance has been studied in the past [14], it is not yet well understood.

ATP-binding cassette transporters make up a large family of proteins with membrane-spanning regions and cytoplasmic ATP binding

domains that are involved in the translocation of various substrates across membranes. Members of this family include ABCB1 (MDR), ABCG2 (BCRP) and ABCC (MRP) transporters that play an important role in drug resistance [14]. Previous studies of the role of drug efflux in resistance to gemcitabine have yielded confusing results. Zhou et al. [14] suggested that expression of ABCB1 and ABCG2 (BCRP) transporters might contribute to gemcitabine resistance and tumor relapse, but Bergman et al. reported that the expression of ABCB1 and ABCC1 (MRP1) enhance gemcitabine sensitivity [8]. Another study reported that non-specific induction of ABC transporters, including ABCB1, ABCG2, ABCC10 and ABCC11 reduced sensitivity to gemcitabine [15]. A study by Hopper et al. highlighted the importance of ABCC10 in gemcitabine resistance [16], but data with regard to the importance of drug efflux in gemcitabine response is limited, and the transporters involved in that process and its functional implications are even less well understood.

In the current study, we set out to explore the role of gemcitabine efflux in gemcitabine action. We also wanted to investigate the implications of efflux for gemcitabine sensitivity, to identify potential transporters involved and to examine the impact of efflux on gemcitabine metabolism. We found that the gemcitabine metabolite, dFdU, is transported out of cells by ABCC transporters and inhibition of that process increases cellular sensitivity- apparently as a result of dFdU inhibition of gemcitabine deamination.

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Materials and Methods

Cell lines and reagents

Human pancreatic cancer SU86 cells and human ovarian adenocarcinoma IGROV-1 cells were gifts from Drs. Daniel D. Billadeau and Scott H. Kaufmann, respectively (Mayo Clinic, Rochester, MN). Human breast cancer MDA-MB-231 cells, human non-small lung cancer H1437 cells, human non-small lung cancer H1792 cells, human pancreatic cancer MIAPaCa2 cells and human liver carcinoma (HepG2) cells were obtained from the American Type Culture Collection (Manassas, VA). Phosphorylated gemcitabine metabolites and dFdU were kindly provided by Dr. Matthew M. Ames (Mayo Clinic, Rochester, MN).

Cell culture

SU-86, MIAPaCa2, MDA-MB-23, H1437, H1792 and IGROV1 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% Fetal Bovine Serum (SU-86, MIAPaCa2, MDA-MB-231) or RPMI-1640 containing 15% FBS (H1437, H1792 and IGROV1). The cells were plated in 6 well plates and cultured until 80-90% confluent and were then treated with gemcitabine (Eli Lilly, 500 nmol/ml), followed by incubation for 4 h. After incubation, media was aspirated, centrifuged and used for HPLC analysis. To study the efflux of gemcitabine without confounding the findings by the presence of exogenous drug, after the initial media aspiration, cells were washed with PBS and incubated with additional fresh media for 1 h, and the media was then aspirated, centrifuged and used for HPLC analysis (see Figure 1).

HPLC analyses

To assay gemcitabine and its metabolites, high performance liquid chromatography (HPLC) was performed with a ZirChrom SAX HPLC column with Shimadzu SPD-M20A photo-diode array with detection at 275 nm. The HPLC analysis was performed using an isocratic system, with the analytical eluent consisting of 50 mmol/L KH_2PO_4 (pH 4), 1 ml/min flow rate and a 40°C column temperature. Retention times and Areas Under the Curve (AUC) were recorded. Gemcitabine (dFdC) and its phosphorylated metabolites dFdC-MP, dFdC-DP, dFdC-TP as well as its deaminated metabolite 2',2'-difluorodeoxyuridine (dFdU) were used as internal standards. The CDA inhibitor tetrahydrouridine (THU) (Calbiochem, San Diego, CA) was used to confirm the presence of extracellular dFdU. Specifically; cells were incubated with THU for 1 h prior to their incubation with gemcitabine, leading to inhibition of dFdU formation and thus disappearance of the peak corresponding to this metabolite.

Tandem mass spectrometry analyses

To confirm the identity of effluxed gemcitabine metabolites, we used LC-MS/MS to analyze the samples. Specifically, the HPLC peaks were analyzed by nano-flow liquid chromatography electrospray tandem mass spectrometry (nanoLC-ESI-MS/MS) using a ThermoFinnigan LTQ Orbitrap Hybrid Mass Spectrometer2 (ThermoElectron Bremen, Germany) coupled to an Eksigent nanoLC-2D HPLC system (Eksigent, Dublin, CA). The samples were loaded onto a 250nl OPTI-PAK trap (Optimize Technologies, Oregon City, OR) custom packed with Michrom Magic C8 solid phase (Michrom Bioresources, Auburn, CA) and were eluted with a 0.2 % formic acid/ acetonitrile gradient through a Michrom packed tip capillary Magic C18 column (75 μm x 150 mm).

The LTQ Orbitrap mass spectrometer experiment was set to perform a FT full scan from 150-1000 m/z with resolving power set at R= 60,000 (at 400 m/z), followed by linear ion trap CID MS/MS scans on the top three ions. The lock-mass option was enabled for the FT full scans using the common phthalate ion of m/z = 391.284286 for real time internal calibration [17].

Pharmacologic efflux pump inhibition and metabolite analyses

Pharmacologic efflux pump inhibition was performed by treating cells with verapamil HCl (Hospira, Lake Forest, IL) or probenecid (Invitrogen, Carlsbad, CA). SU-86, MIAPaCa2, MDA-MB-231, H1437, H1792 and IGROV1 cells were plated and cultured in 6 well plates until 80%-90% confluent, and were then treated with PBS, verapamil (50 nmol/ml) or probenecid (2mmol/ml for SU-86, MIAPaCa2, MDA-MB-231 cells, 1 mMol/ml for H1437, H1792, IGROV-1 cells), followed by incubation for 24 h. Following incubation with verapamil or probenecid, cells were treated with gemcitabine (500 nmol/ml) for an additional 4 h, and the media was collected for HPLC analysis of extracellular metabolites. All replicates were done on separate days. Cells were then washed with PBS and lysed using CelLytic M Cell Lysis Reagent (Sigma Aldrich, St. Louis, MO), with the lysate collected for HPLC analysis of intracellular metabolites. The direct effects of verapamil or probenecid on the parent drug gemcitabine were examined by blocking deamination by incubation with THU for 1 h prior to pharmacologic transporter inhibition and gemcitabine treatment.

Cytotoxicity assays

Cells were cultured, plated in 6 well-plates and treated with verapamil, probenecid or PBS. After 24 h, the cells were plated overnight in triplicate in 96-well plates (Corning, Corning, NY) at a density of 10^4 cells per well, and were treated with PBS or gemcitabine at increasing concentrations (0.001 micromol/ml to 10 micromol/ml). After 24 h, cytotoxicity assays were performed using the CellTiter 96 AQ_{ueous} Non-Radioactive Cell Proliferation Assay (MTS, Promega, Madison, WI). Plates were read in a Safire² microplate reader (Tecan AG, Männedorf Switzerland) following 2 h incubations. Additionally, to compare gemcitabine and dFdU cytotoxicity, we treated HepG2 cells with dFdU or gemcitabine at various concentrations (1 mcmol/ml, 1:5 dilution). After 24 h incubation, cytotoxicity assays were performed.

Interaction between cytidine deaminase and dFdU efflux

The effect of intracellular dFdU accumulation on CDA activity was assessed in two different cell lines. HepG2 were selected because of their ability to take up dFdU [9]. HepG2 cells expressing CDA were cultured and plated in 6 well plates and were incubated in DMEM + 10% FBS until 80-90% confluent. Medium was replaced and supplemented with (1.) gemcitabine (1 micromol/ml), (2.) gemcitabine (1 micromol/ml) + dFdU (1 micromol/ml) or (3.) dFdU (1 micromol/ml). After a 30 min incubation, intra- and extracellular metabolites were analyzed using HPLC as previously described. Newly formed dFdU after combined gemcitabine and dFdU treatment was calculated as follows: Newly formed dFdU = Total dFdU - Added dFdU. In addition, African green monkey kidney (COS1) cells that are inherently CDA deficient were transfected with CDA (Lipofectamin 2000, Invitrogen) [5,8]. Transfected COS1 cells were plated in 6 well plates and were incubated until 80-90% confluent. The cells were treated with PBS or increasing concentrations of dFdU (5-500 nmol/ml), followed by gemcitabine treatment (1 micromol/ml). After incubation, sample collection and HPLC analyses were conducted as described above.

Expression array

Total RNA was extracted from all studied cancer cell lines using Qiagen RNeasy Mini kits (QIAGEN, Inc., Valencia, CA). RNA quality was tested using an Agilent 2100 Bioanalyzer, followed by hybridization to Affymetrix U133 Plus 2.0 GeneChips (18).

Transporter siRNA knockdown

H1437 cells were cultured in 6 well plates until 30-50% confluent. siRNA knockdown studies with human siRNA for ABCC3, ABCC5 or ABCC10 ON-Target Plus Smart-pool (Thermo-Scientific Dharmacon, Lafayette, CO), separately or in combination, were performed using Lipofectamin RNAiMAX (Invitrogen). Non-targeting siRNA (Thermo-Scientific Dharmacon, Rockford, IL) was used for negative controls. The transfected cells were then incubated for 24 h, plated in triplicate in 96 well plates, and cultured overnight. Gemcitabine treatment and cytotoxicity assays were conducted as described above. Knockdown efficiency was assessed using quantitative reverse transcription-PCR (QRT-PCR) with the 1-step, Brilliant SYBR Green QRT-PCR kit (Stratagene, La Jolla, CA) using primers purchased from Qiagen. All QRT-PCR experiments were performed in duplicate, using β -actin as an internal control. All replicates were done on different days.

Statistical analysis

For each of the samples, 4 metabolites (extracellular dFdC and dFdU, as well as intracellular dFdC and dFdU) under 3 treatment conditions: gemcitabine, gemcitabine+probenecid or gemcitabine+verapamil, were measured in triplicate. All values within a replicate for a subject

were divided by gemcitabine concentration in that replicate, resulting in 2 measurable variables: concentration of metabolites in the gemcitabine+verapamil group relative to the gemcitabine group, and concentration of metabolites in the gemcitabine+probenecid group relative to the gemcitabine group [18]. Dose response curves for the cytotoxicity experiments were generated by fitting the data to a standard 4 parameter logistic model. AUC was then calculated based on the model. A two-tailed paired student t-test was used for analyses of differences in metabolite abundance and AUC values within each cell line.

Results

Gemcitabine and dFdU efflux from cancer cell lines

Gemcitabine response varies widely, and multiple mechanisms are involved in determining sensitivity to this drug [18, 19]. We hypothesized that efflux of gemcitabine and its metabolites might contribute to this variation in drug sensitivity. To identify compounds transported outside of cells after gemcitabine treatment, we used HPLC and tandem mass spectrometry analysis. SU-86, MIA PaCa2, MDA-MB-231, H1437, H1792 and IGROV1 cells were treated with gemcitabine for 24 h, and the extracellular media was collected for HPLC analysis. These analyses revealed two peaks across all of the cancer cell lines tested, with retention times of 8 min and 13 min, corresponding to the internal standards gemcitabine and dFdU, respectively (Supplemental Figure 1). Since gemcitabine is deaminated to form dFdU, a reaction catalyzed by CDA, to help confirm that one of the peaks was dFdU, we used the CDA inhibitor THU to inhibit this reaction in SU86 cells. CDA

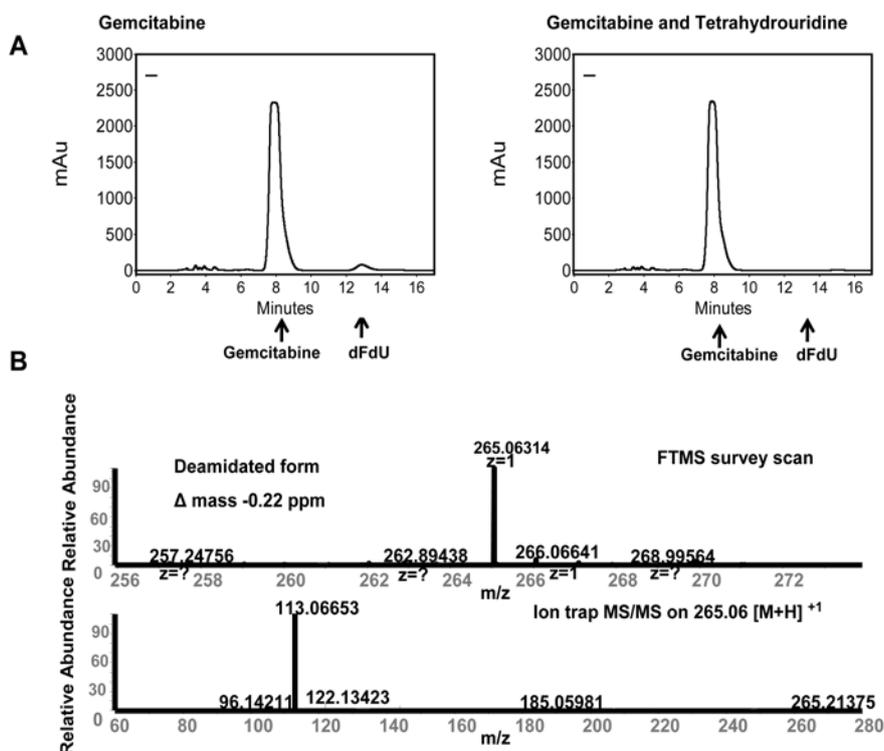


Figure 1: HPLC and LC-MS/MS efflux analyses in SU-86 cells. (A) HPLC analyses of the extracellular media after gemcitabine treatment revealed two peaks with retention times of 8 min and 13 min, corresponding to gemcitabine and dFdU standards, respectively (left). The second peak disappeared after pretreatment with the deamination inhibitor, THU (right) confirming that it was dFdU. (B) Tandem mass spectrometry showed that the second retention peak had a precursor mass of 265.06314, and MS/MS spectra showed the same dominant 113.06 Da mass which corresponds to the 4-hydroxy-pyrimidin-2-one [M+H]⁺ +1 fragment, confirming that the second peak was dFdU.

inhibition with THU resulted in disappearance of the second peak, suggesting that it corresponded to dFdU (Figure 1A). Tandem mass spectrometry showed that the second retention peak had a precursor mass of 265.06314, which is within 1 ppm of the calculated mass for the deaminated form of gemcitabine (Figure 1B). The MS/MS spectra for the second peak showed a dominant 113.06 Da mass which corresponds to the 4-hydroxy-pyrimidin-2-one [M+H]⁺ fragment, confirming that the second peak was dFdU (Figure 1B). We did not observe peaks that corresponded to the retention times of phosphorylated internal standards for gemcitabine mono-, di- and triphosphates.

Non-selective ABC-transporter inhibition with probenecid or verapamil

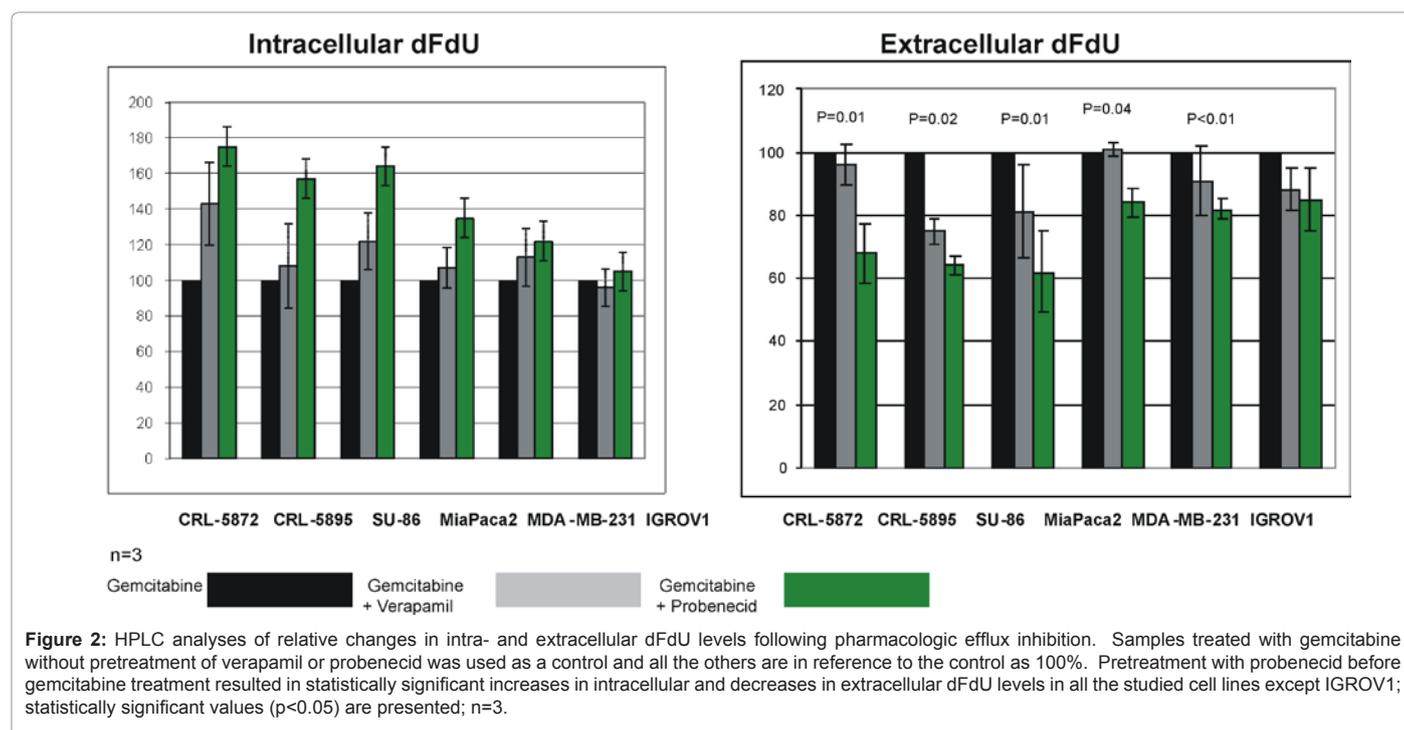
We next wanted to determine which transporters might be involved in gemcitabine and dFdU efflux. Previous studies had shown that ABC transporters were involved in drug efflux and in determining gemcitabine sensitivity [16]. We first used non-selective pharmacological inhibitors of ABC transporter family members, verapamil and probenecid, neither of which affects SLC transporter family members. Both drugs are non-selective efflux pump inhibitors, although verapamil primarily effects ABCB1 and ABCG2 [20,21], while probenecid primarily effects ABCC transporters [22,23]. We treated six different cancer cells, including two lung cancer cell lines, H1437 and H1792, two pancreatic cell lines, SU86 and MIAPaCa2, as well as one breast cancer cell line, MDA-MB-231 and one ovarian cell line, IGROV1, with probenecid and verapamil. These cells were chosen for their clinical relevance to gemcitabine treatment. Non-selective inhibition of ABCC transporters with probenecid resulted in a statistically significant increase in intracellular dFdU metabolite levels compared to controls in H1437, SU86 and MIAPaCa2 cells ($p < 0.05$) and approached significance in H1792 and MDA-MB-231 cells ($p = 0.058$ and 0.089 , respectively) but not in IGROV1 cells (Figure 2). Non-selective inhibition of ABCC transporters with probenecid also resulted in a statistically significant decrease in extracellular dFdU levels in H1792, MIAPaCa2 and MDA-MB-231

cells ($p < 0.05$) and approached significance in H1437 and SU86 cells ($p = 0.08$ and 0.067 , respectively) but not in IGROV1 cells (Figure 2). A similar although smaller effect that did not reach statistical significance was observed with non-selective inhibition with verapamil (Figure 2). Treatment with probenecid also resulted in increases in intracellular gemcitabine levels in all cancer cells except IGROV1, that reached statistical significance in H1437 and H1792 cells ($p < 0.05$) (Figure 3). No statistically significant changes in intracellular gemcitabine levels were observed with verapamil treatment. In contrast, extracellular gemcitabine levels did not decrease as expected (Figure 3), but rather remained unchanged or increased ($p < 0.05$) following treatment, suggesting that the increase in intracellular gemcitabine levels might result from a decrease in the breakdown of the parent drug rather than from efflux inhibition. Following THU treatment, no appreciable effect on intra- or extra-cellular gemcitabine levels after probenecid or verapamil treatment was observed, compatible with the conclusion that ABC transporters mainly mediate dFdU and not gemcitabine efflux.

Cytotoxicity after non-selective ABC-transporters inhibition

We next set out to determine the influence of the efflux of gemcitabine and especially its deaminated metabolite on gemcitabine sensitivity. We hypothesized that ABC transporter-mediated efflux might play a role in gemcitabine sensitivity, given the changes that we had observed in intracellular gemcitabine levels and intra- and extracellular dFdU levels following probenecid treatment (Figure 2 and Figure 3). Therefore, we performed gemcitabine cytotoxicity studies with these cancer cell lines after blocking the transporters with probenecid or verapamil to determine the influence of efflux on gemcitabine sensitivity.

Non-selective inhibition of ABCC-transporters with probenecid increased gemcitabine sensitivity in all of the studied cell lines, reaching a significant decrease in AUC values in H1437, H1792, SU86 and MDA-MB-231 cells ($p = 0.016$, 0.019 , $p < 0.01$ and $p = 0.01$, respectively) (Figure 4), but no significant change was observed for the MIAPaCa2 and



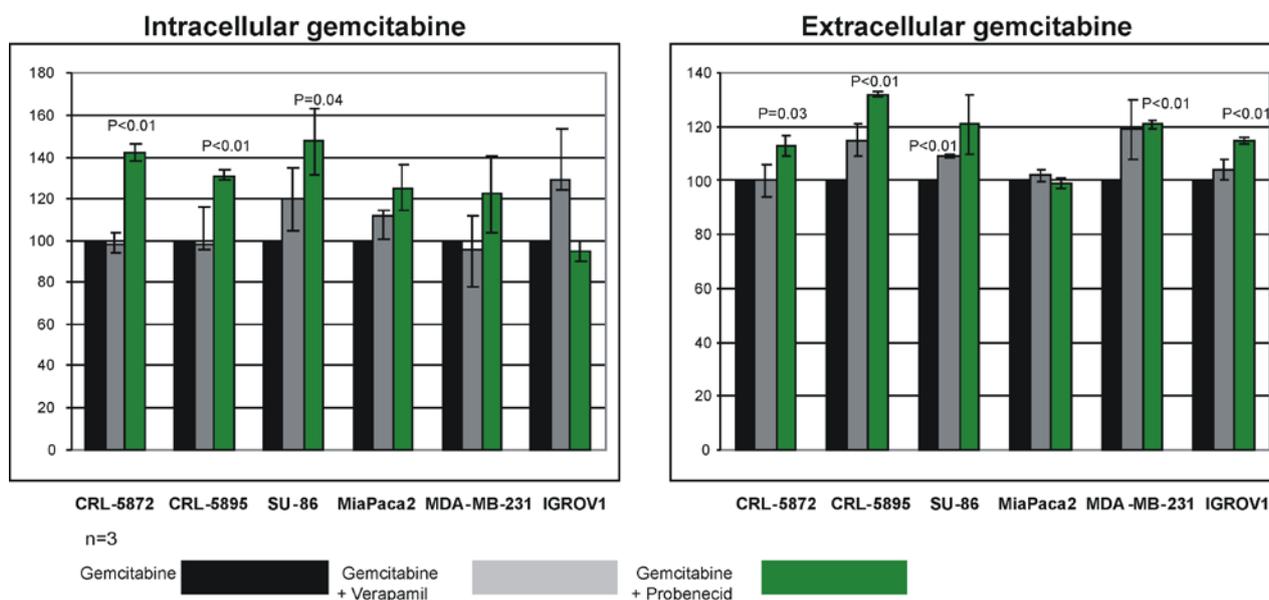


Figure 3: HPLC analyses of relative changes in intra- and extracellular gemcitabine levels following pharmacologic efflux inhibition. Pretreatment with probenecid before gemcitabine treatment resulted in significant increases in intracellular gemcitabine levels in H1437, H1792, SU86 and MDA-MB-231 cells ($p < 0.05$) while extracellular gemcitabine levels increased ($p < 0.05$) or remained unchanged; statistically significant values are presented ($n = 3$).

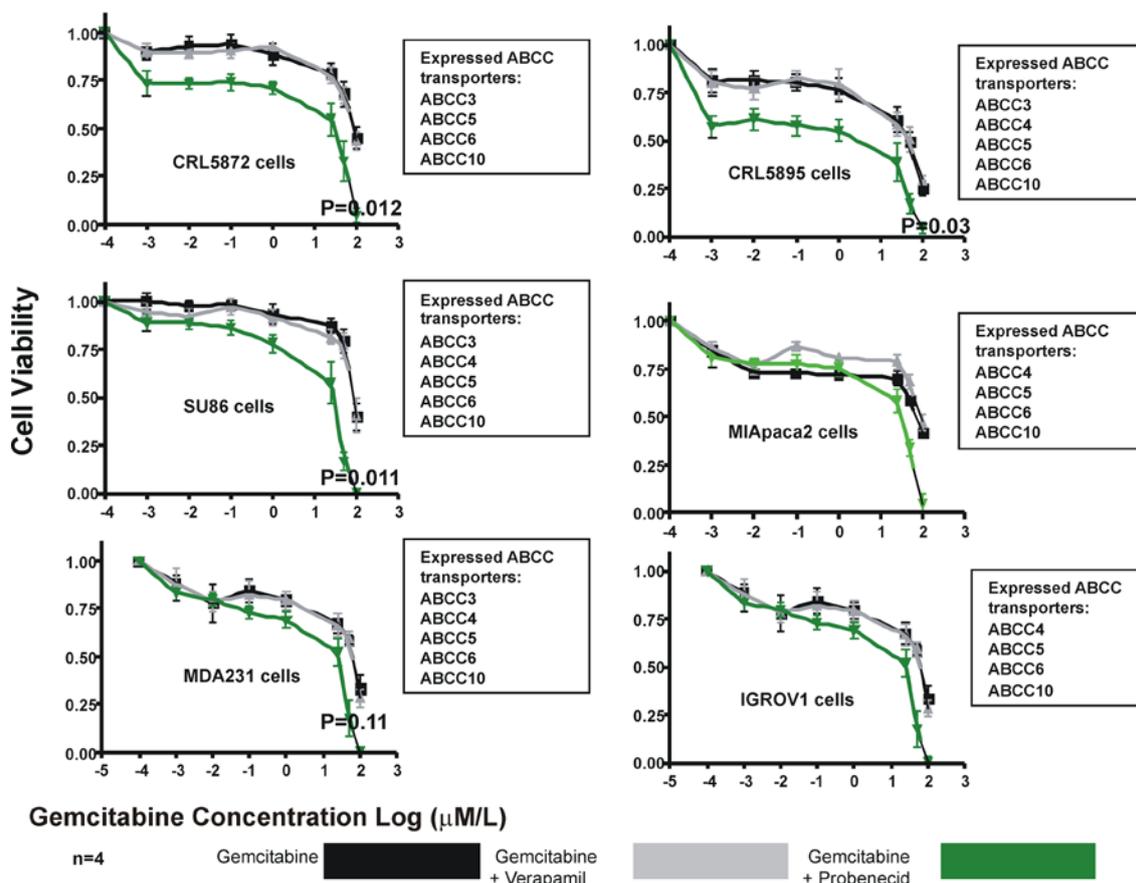


Figure 4: Gemcitabine cytotoxicity assays after pretreatment with probenecid, verapamil or PBS. Pretreatment with probenecid increased gemcitabine cytotoxicity, reaching a significant decrease in AUC values in H1437, H1792 and SU86 cells ($p = 0.01$, 0.03 and 0.01 , respectively) and approaching significance in MDA-MB-231 cells ($p = 0.11$). No significant effect was observed with verapamil ($p > 0.05$); statistically significant values are presented ($n = 4$).

IGROV1 cell lines. No significant effect on cytotoxicity was observed with the non-selective inhibitor of ABCB1 and ABCG2 transporters, verapamil ($p > 0.05$ for all) (Figure 4). These findings suggest a potential important role of ABCC-transporter mediated efflux in resistance to gemcitabine.

ABCC3, ABCC5 and ABCC10 as candidate transporters

Since probenecid showed a more significant effect than did verapamil on intra and extracellular metabolite levels (Figure 2 and Figure 3), and on sensitivity to gemcitabine (Figure 4), we hypothesized that ABCC family members might play an important role in the efflux of gemcitabine and dFdU. Therefore, we used expression arrays to study expression levels of ABCC transporters in all six cancer cell lines studied and found that ABCC5, ABCC6 and ABCC10 were expressed in all six cell lines, while ABCC3 was expressed in all but MIAPaCa2 and IGROV1 cells while ABCC4 was expressed in all but the H1437 cell line (Figure 4).

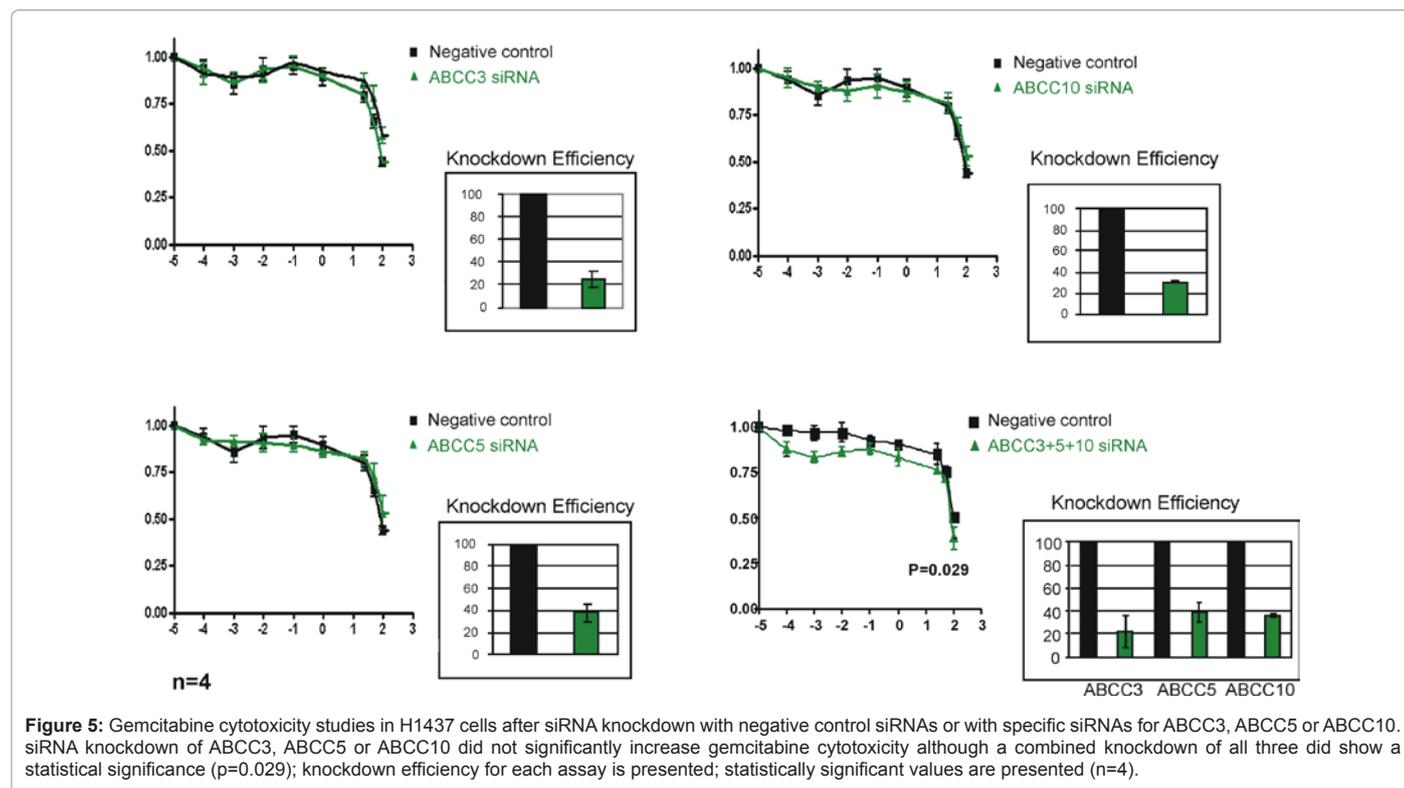
In order to determine potential ABCC family members involved in this process, based on the expression and cytotoxicity data shown in (Figure 4), we chose the transporters that were most likely to be involved in gemcitabine efflux. We selected ABCC3, ABCC5 and ABCC10 to investigate further because ABCC3 was highly expressed in cell lines that displayed a large change in cytotoxic response after probenecid (H1437, H1792, SU86) and was poorly expressed in cells that did not (MIAPaCa2, IGROV1), while ABCC5 and ABCC10 were highly expressed in all cell lines studied. ABCC6 was expressed at a relatively low level, while ABCC4 was not expressed in the H1437 cells that had a large effect, but was expressed in IGROV1 cells that did not show a significant effect, so these two transporters were not selected for further study.

Combined but not separate ABCC3, ABCC5 and ABCC10 siRNA knockdown significantly enhanced gemcitabine sensitivity

We next examined the effect of knockdown of individual ABCC transporters on gemcitabine cytotoxicity. The H1437 cells that displayed a large effect after probenecid treatment were used in these experiments. Knockdown with individual or combined siRNAs for ABCC3, ABCC5 and ABCC10 were performed in H1437 cells, followed by gemcitabine cytotoxicity assays. siRNA knockdown of ABCC3, ABCC5 or ABCC10 did not result in an increased gemcitabine sensitivity as compared to negative controls (Figure 5). However, combined knockdown of all three candidate transporters led to a statistically significant sensitization of H1437 cells to gemcitabine ($p = 0.029$) (Figure 5).

dFdU inhibition of CDA

The fact that blockage of dFdU efflux after gemcitabine treatment was associated with gemcitabine sensitization raised the question of whether accumulation of dFdU might inhibit CDA by “product inhibition”, leading to decreased gemcitabine deamination and elevated gemcitabine cellular concentration since the cytotoxicity of dFdU itself is minimal, with IC₅₀ values 693-fold lower than that of gemcitabine in HepG2 cells ($p = 0.036$, $n = 3$). To test that hypothesis, we incubated HepG2 cells with dFdU before gemcitabine treatment, and then added gemcitabine, followed by HPLC analyses to quantify dFdU concentration. Final results for the newly formed dFdU were calculated by subtracting the quantity of added dFdU from the amount of total dFdU measured by HPLC. With increasing concentrations of extracellular dFdU, less newly formed intracellular dFdU was observed as compared to controls (51% decrease, $n = 2$). We also overexpressed CDA in COS-1 cells, a cell lines that does not express endogenous CDA, and treated cells with dFdU, followed by gemcitabine. CDA-transfected



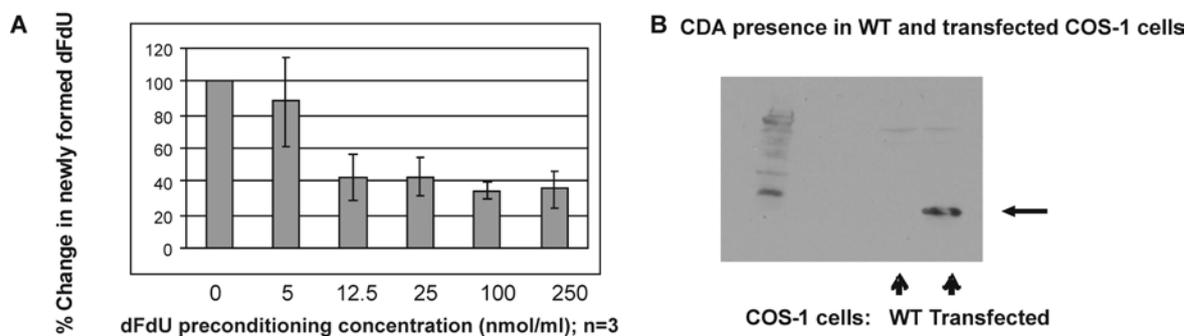


Figure 6: Changes in deamination activity after dFdU pretreatment in CDA-transfected COS-1 cells. (A) Pretreatment with dFdU before gemcitabine treatment resulted in decreased dFdU formation (n=3). (B) CDA transfection in COS-1 cells was confirmed by Western blot. Values that statistically significant differ from controls are presented.

COS-1 cells incubated with dFdU before gemcitabine treatment formed less newly intracellular dFdU than did controls (Figure 6A). Although we were unable to perform an enzyme assay because of the limited quantity of dFdU available to us, and because our assay was not sufficiently sensitive to quantify concentrations of phosphorylated metabolites, data from both experiments supported an inhibitory effect of dFdU on CDA activity. Transfection efficiency for CDA in COS-1 cells was confirmed by Western blots (Figure 6B).

Discussion

ATP-Binding Cassette (ABC) transporters are membrane proteins that translocate a wide variety of substrates across cellular membranes, facilitating many biologic processes including cellular homeostasis, nutrient uptake and cell proliferation [24]. These proteins are present in many cancer and non-cancer cells and are expressed constitutively or following induction by toxins or drugs. ABC-transporters include the well-studied ABCB1, ABCG2 and ABCC1 transporters as well as ABCC transporters. The importance of ABCC transporters in mediating drug efflux is well established [25,26]. Therefore, these transporters have therapeutic importance since they mediate resistance to many drugs, including antineoplastics and anti-infectives. Although this mechanism of resistance has been the focus of many therapeutic efforts through pharmacological inhibition and genetic silencing, attempts to target efflux-mediated resistance have generally been unsuccessful [26,27].

In addition to the role of ABC-transporters in lowering cellular sensitivity to chemotherapy, their action allows cancer stem-like side populations of cells to escape treatment, thus resulting in treatment failure and tumor recurrence [28]. This phenomenon was reported by Fokuda et al. [29], who found stem-like gastric cancer side populations of cells to have high levels of ABCB1 and ABCG2 genes expression. Those observations are in agreement with reports by Loebinger et al. [29] who found squamous carcinoma cells to contain a sub-population of stem-like cancer cells possessing high efflux capabilities, with restoration of their chemosensitivity upon ATP-dependent transport inhibition. Similar findings have been observed with pancreatic [30], esophageal [31], and nervous system cancer stem-like cells [32].

The importance of ABC-transporters in gemcitabine resistance has only been examined recently. Veltkamp et al. [9] documented the presence of gemcitabine cellular efflux, reporting that both dFdC and dFdU were effluxed. Their study also hinted that efflux of phosphorylated metabolites may be possible as well, and suggested the need for further study. We did not find evidence to confirm that, which could be due to

the low level of such transport, as suggested previously [9], the limit of detection in our study, or a combination of the two. Veltkamp et al. [9] also reported that dFdU possessed some, although limited, cytotoxicity. The potential importance of this efflux was illustrated by Hopper et al. [16], who reported that gemcitabine resistance increased 3-fold following transfection of HEK293 cells with ABCC10. Furthermore, while the role of the critical gemcitabine metabolizing enzyme CDA in gemcitabine metabolism is well characterized [5], the inhibitory and stimulatory triggers for CDA activity have not been explored previously.

In our study, we found that gemcitabine and dFdU are transported out of six clinically relevant cancer cell lines and we joined those observations to studies of gemcitabine-dependent cytotoxicity. Taken together, our results suggest a novel pathway linking dFdU and gemcitabine efflux with cytotoxicity (Figure 7). We propose that dFdU efflux is mediated by several ABCC transporters. This efflux allows for continued gemcitabine deamination, while inhibition of efflux could result in intracellular accumulation of dFdU leading to inhibition of deamination and elevated intracellular gemcitabine concentrations.

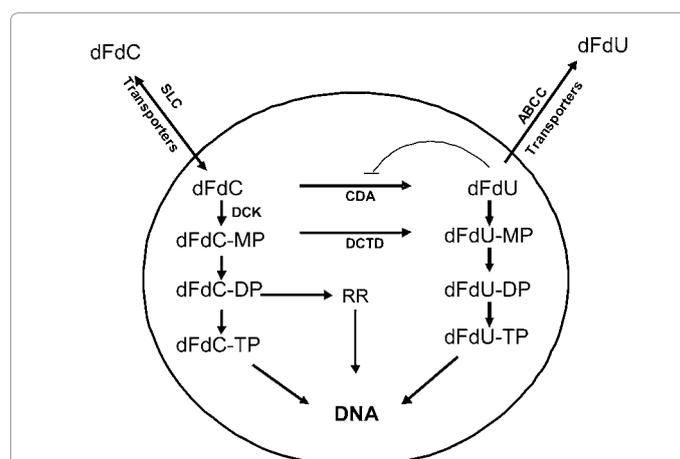


Figure 7: Interaction between gemcitabine metabolism and efflux. dFdU is effluxed by ABCC transporters allowing for continued gemcitabine deamination, while inhibition of efflux could result in intracellular accumulation of dFdU inhibiting deamination and resulting in increased intracellular gemcitabine concentrations and cytotoxicity; dFdU=2,2-difluorodeoxycytidine (gemcitabine); dFdU=2',2'-difluorodeoxyuridine (deaminated gemcitabine metabolite); MP=monophosphate; DP=diphosphate; TR=triphosphate; CDA=cytidine deaminases; DCTD=deoxycytidylate deaminases; RR=ribonucleotide reductase.

While increased cytotoxicity could be due, in part, to an increase in intracellular dFdU levels, given the minimal cytotoxicity of dFdU as compared with gemcitabine, a second mechanism is likely to be involved. That mechanism appears to be CDA inhibition as a result of dFdU accumulation, leading to higher gemcitabine concentrations, thus increasing cytotoxicity. Also, although influx inhibition by increased intracellular dFdU levels could have potentially contributed to our observations as presented in Figure 6, our findings that non-specific pharmacologic efflux inhibition increased dFdU levels intracellularly and decreased them extracellularly, while gemcitabine levels increased intra- and extracellularly, suggested that a decrease in gemcitabine deamination despite an increase in substrate availability is the favored explanation rather than inhibition of gemcitabine influx. Furthermore, since we only achieved partial efflux inhibition with non-selective inhibitors, the cytotoxic effect attributable to efflux inhibition might be even greater with complete blockade. These findings have potential clinical and therapeutic implications. Since dFdU was previously shown to sensitize cells to radiation therapy [33,34], dFdU might also sensitize cells to gemcitabine treatment and enhance its specificity for cancer cells since they often express ABC-transporters. However, pursuit of those hypotheses will have to occur during future studies.

Efflux inhibition with probenecid resulted in a considerably larger change in gemcitabine cytotoxicity than did efflux inhibition with verapamil (Figure 4). This difference favors the involvement of ABCC-transporters, although we can not rule out the possible involvement of other ABC transporter family members. Also, our data suggest that ABCC-transporters facilitate dFdU efflux, while gemcitabine efflux was present, yet was not influenced by ABC transporter inhibition by probenecid or verapamil, suggesting that its efflux was mediated by SLC transporters. Interestingly, while each of the candidate transporters examined in detail: ABCC3, ABCC5 and ABCC10, likely played a role in gemcitabine efflux; their role was not exclusive, since there were not significant increases in gemcitabine sensitivity following their individual knockdown. In contrast, knockdown of all three resulted in some sensitization to gemcitabine, suggesting that multiple ABCC transporters might transport dFdU across the cell membrane. While it is possible that suboptimal siRNA knockdown resulted in limited impact on cytotoxicity, we did see a slightly enhanced effect after simultaneous knockdown of all three. Consequently, inactivation of one or even several ABCC transporters may not be sufficient for complete efflux inhibition. In addition, such a narrow approach might lead to induction of other ABCC transporters, and thus, escape from therapy. This concept could explain the suboptimal outcome of targeted efflux pump inhibition treatments in clinical trials and the discordance across studies examining this topic, implicating multiple transporters in the process [8,16,35].

Obviously, our study focused only on constitutively expressed transporters rather than those induced by gemcitabine, which could also play an important role in gemcitabine resistance. However, the main goal of this study was to identify potential novel mechanisms involved in gemcitabine efflux and their possible contribution to drug resistance. Our findings may also be extended to other nucleoside analogues used to treat cancer or infectious diseases [34,36].

In summary, dFdU is effluxed from cancer cells through ABC transporters, decreasing its accumulation, and as a result, product inhibition of CDA. Consequently, a greater proportion of gemcitabine is converted to dFdU, decreasing gemcitabine cytotoxicity. This efflux of dFdU is mediated by several transporters, with likely overlap among them. Therefore, this study has expanded our understanding of ABC

transporters and the role of ABC transporters in gemcitabine resistance and sensitivity, and might have application to future translational studies.

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References

1. Freitas D, Fernandes Gdos S, Hoff PM, Cunha JE (2009) Medical management of pancreatic adenocarcinoma. *Pancreatology* 9: 223-232.
2. Markman M (2007) New, expanded, and modified use of approved antineoplastic agents in ovarian cancer. *Oncologist* 12: 186-190.
3. Mauri D, Polyzos NP, Salanti G, Pavlidis N, Ioannidis JP (2008) Multiple-treatments meta-analysis of chemotherapy and targeted therapies in advanced breast cancer. *J Natl Cancer Inst* 100: 1780-1791.
4. Rosell R, Cobo M, Isla D, Camps C, Massuti B (2006) Pharmacogenomics and gemcitabine. *Ann Oncol* 17 Suppl 5: 13-16.
5. Gilbert JA, Salavaggione OE, Ji Y, Pellemounter LL, Eckloff BW, et al. (2006) Gemcitabine pharmacogenomics: cytidine deaminase and deoxycytidylate deaminase gene resequencing and functional genomics. *Clin Cancer Res* 12: 1794-1803.
6. Kocabas NA, Aksoy P, Pellemounter LL, Moon I, Ryu JS, et al. (2008) Gemcitabine pharmacogenomics: deoxycytidine kinase and cytidylate kinase gene resequencing and functional genomics. *Drug Metab Dispos* 36: 1951-1959.
7. Mini E, Nobili S, Caciagli B, Landini I, Mazzei T (2006) Cellular pharmacology of gemcitabine. *Ann Oncol* 17 Suppl 5: 7-12.
8. Bergman AM, Pinedo HM, Talianidis I, Veerman G, Loves WJ, et al. (2003) Increased sensitivity to gemcitabine of P-glycoprotein and multidrug resistance-associated protein-overexpressing human cancer cell lines. *Br J Cancer* 88: 1963-1970.
9. Veltkamp SA, Pluim D, van Eijndhoven MA, Bolijn MJ, Ong FH, et al. (2008) New insights into the pharmacology and cytotoxicity of gemcitabine and 2',2'-difluoro deoxyuridine. *Mol Cancer Ther* 7: 2415-2425.
10. El Maalouf G, Le Tourneau C, Batty GN, Faivre S, Raymond E (2009) Markers involved in resistance to cytotoxic and targeted therapeutics in pancreatic cancer. *Cancer Treat Rev* 35: 167-174.
11. Maréchal R, Mackey JR, Lai R, Demetter P, Peeters M, et al. (2009) Human equilibrative nucleoside transporter 1 and human concentrative nucleoside transporter 3 predict survival after adjuvant gemcitabine therapy in resected pancreatic adenocarcinoma. *Clin Cancer Res* 15: 2913-2919.
12. Ueno H, Kiyosawa K, Kaniwa N (2007) Pharmacogenomics of gemcitabine: can genetic studies lead to tailor-made therapy? *Br J Cancer* 97: 145-151.
13. Giovannetti E, Mey V, Nannizzi S, Pasqualetti G, Del Tacca M, et al. (2006) Pharmacogenetics of anticancer drug sensitivity in pancreatic cancer. *Mol Cancer Ther* 5: 1387-1395.
14. Zhou J, Wang CY, Liu T, Wu B, Zhou F, et al. (2008) Persistence of side population cells with high drug efflux capacity in pancreatic cancer. *World J Gastroenterol* 14: 925-930.
15. Hauswald S, Duque-Afonso J, Wagner MM, Schertl FM, Lübbert M, et al. (2009) Histone deacetylase inhibitors induce a very broad, pleiotropic anticancer drug resistance phenotype in acute myeloid leukemia cells by modulation of multiple ABC transporter genes. *Clin Cancer Res* 15: 3705-3715.
16. Hopper-Borge E, Xu X, Shen T, Shi Z, Chen ZS, et al. (2009) Human multidrug resistance protein 7 (ABCC10) is a resistance factor for nucleoside analogues and epothilone B. *Cancer Res* 69: 178-184.
17. Olsen JV, de Godoy LM, Li G, Macek B, Mortensen P, et al. (2005) Parts per million mass accuracy on an Orbitrap mass spectrometer via lock mass injection into a C-trap. *Mol Cell Proteomics* 4: 2010-2021.
18. Li L, Fridley B, Kalari K, Jenkins G, Batzler A, et al. (2008) Gemcitabine and cytosine arabinoside cytotoxicity: association with lymphoblastoid cell expression. *Cancer Res* 68: 7050-7058.

19. Li L, Fridley BL, Kalari K, Jenkins G, Batzler A, et al. (2009) Gemcitabine and arabinosylcytosin pharmacogenomics: genome-wide association and drug response biomarkers. *PLoS One* 4: 7765.
20. Nobili S, Landini I, Gigliani B, Mini E (2006) Pharmacological strategies for overcoming multidrug resistance. *Curr Drug Targets* 7: 861-879.
21. Tan B, Piwnicka-Worms D, Ratner L (2000) Multidrug resistance transporters and modulation. *Curr Opin Oncol* 12: 450-458.
22. Barrand MA, Bagrij T, Neo SY (1997) Multidrug resistance-associated protein: a protein distinct from P-glycoprotein involved in cytotoxic drug expulsion. *Gen Pharmacol* 28: 639-645.
23. Wein S, Fauroux M, Laffitte J, de Nadaï P, Guaini C, et al. (2004) Mediation of annexin 1 secretion by a probenecid-sensitive ABC-transporter in rat inflamed mucosa. *Biochem Pharmacol* 67: 1195-1202.
24. Jones P M, O'Mara ML, George AM (2009) ABC transporters: a riddle wrapped in a mystery inside an enigma. *Trends Biochem Sci* 34: 520-531.
25. Eckford PD, Sharom FJ (2009) ABC efflux pump-based resistance to chemotherapy drugs. *Chem Rev* 109: 2989-3011.
26. Modok S, Mellor HR, Callaghan R (2006) Modulation of multidrug resistance efflux pump activity to overcome chemoresistance in cancer. *Curr Opin Pharmacol* 6: 350-354.
27. Pérez-Tomás R (2006) Multidrug resistance: retrospect and prospects in anti-cancer drug treatment. *Curr Med Chem* 13: 1859-1876.
28. Dean M (2009) ABC transporters, drug resistance, and cancer stem cells. *J Mammary Gland Biol Neoplasia* 14: 3-9.
29. Fukuda K, Saikawa Y, Ohashi M, Kumagai K, Kitajima M, et al. (2009) Tumor initiating potential of side population cells in human gastric cancer. *Int J Oncol* 34: 1201-1207.
30. Wang YH, Li F, Luo B, Wang XH, Sun HC, et al. (2009) A side population of cells from a human pancreatic carcinoma cell line harbors cancer stem cell characteristics. *Neoplasia* 56: 371-378.
31. Huang D, Gao Q, Guo L, Zhang C, Jiang W, et al. (2009) Isolation and identification of cancer stem-like cells in esophageal carcinoma cell lines. *Stem Cells Dev* 18: 465-473.
32. Chua C, Zaiden N, Chong KH, See SJ, Wong MC, et al. (2008) Characterization of a side population of astrocytoma cells in response to temozolomide. *J Neurosurg* 109: 856-866.
33. Pauwels B, Korst AE, Lambrechts HA, Pattyn GG, de Pooter CM, et al. (2006) The radiosensitising effect of difluorodeoxyuridine, a metabolite of gemcitabine, in vitro. *Cancer Chemother Pharmacol*: 219-228.
34. Sigmond J, Honeywell R J, Postma T J, Dirven C M, de Lange S M, et al. (2009) Gemcitabine uptake in glioblastoma multiforme: potential as a radiosensitizer. *Ann Oncol* 20: 182-187.
35. Noma B, Sasaki T, Fujimoto Y, Serikawa M, Kobayashi K, et al. (2008) Expression of multidrug resistance-associated protein 2 is involved in chemotherapy resistance in human pancreatic cancer. *Int J Oncol* 33: 1187-1194.
36. Guo Y, Köck K, Ritter C A, Chen Z S, Grube M, et al. (2009) Expression of ABC-type nucleotide exporters in blasts of adult acute myeloid leukemia: relation to long-term survival. *Clin Cancer Res* 15: 1762-1769.