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Effect of its deaminated metabolite, 2′,2′-difluorodeoxyuridine, on the transport and toxicity of gemcitabine in HeLa cells

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ABSTRACT

Gemcitabine is a pyrimidine analog effective against many solid tumors. Following intravenous administration, deaminases in the plasma rapidly convert the parent compound, gemcitabine, to its deaminated metabolite, 2′,2′-difluorodeoxyuridine (dFdU), resulting in an elimination half-life for gemcitabine of 8 min. The half-life of dFdU, however, is upwards of 14 h, yielding plasma concentrations that are frequently 10–20-fold higher than that of gemcitabine. The uptake of gemcitabine into tumor cells is facilitated by both concentrative (hCNT) and equilibrative (hENT) nucleoside transporters. Recently, it was observed that dFdU is a substrate for hCNT as well. The purpose of this study was to investigate the effects of dFdU on gemcitabine uptake and efflux via hENT1 and hENT2 in HeLa cells. Our results suggest that dFdU is a substrate for both hENT1 and hENT2 as well as a competitive inhibitor of gemcitabine transport at concentrations >100-fold lower than those typically achieved in plasma (IC_{50} = 0.45 and 1.2 μM for hENT1/2 and hENT2, respectively). However, inhibition of gemcitabine uptake is time-dependent, as dFdU limits gemcitabine uptake into HeLa cells by more than 80% during short (<20 s) incubation periods but increases net gemcitabine retention as incubation length increases. While dFdU enhances the accumulation of gemcitabine by up to 1.5-fold following a 60 min incubation, dFdU did not enhance gemcitabine cytotoxicity. In conclusion, this is the first report of an interaction between dFdU and gemcitabine suggesting that the deaminated metabolite may play an important role in the disposition of gemcitabine in tumor cells.

1. Introduction

Gemcitabine (2′,2′-difluorodeoxycytidine, dFdC), is an analog of deoxycytidine with high activity against many types of solid tumors including pancreatic, cervical, ovarian, breast, bladder, and non-small cell lung cancers [1–4]. As a hydrophilic nucleoside analog, gemcitabine utilizes nucleoside transporters to cross plasma membranes. Once inside the cell, this prodrug is quickly phosphorylated to its active di- and triphosphate moieties (dFdCDP, dFdCTP) [5]. dFdCDP and dFdCTP are then incorporated into nascent DNA and RNA strands, eventually leading to inhibition of DNA polymerases, chain termination and cessation of DNA replication [6].

In addition to phosphorylation, gemcitabine may also undergo intra- and extracellular deamination to the much less active form, 2′,2′-difluorodeoxyuridine (dFdU), via cytidine deaminase (CDA), which is present at high concentrations in many types of normal and malignant tissues as well as in the plasma [7,8]. In fact, after intravenous administration, cytidine deaminases in the plasma rapidly convert gemcitabine to dFdU, resulting in a gemcitabine plasma elimination half-life of only 8 min [9]. The long half-life of dFdU (14 h), leads to plasma concentrations of the dFdU that are frequently 10–20-fold higher than those of the parent compound when measured shortly after gemcitabine administration [10–12]. Additionally, deamination of the monophosphate form of gemcitabine may also occur intracellularly via deoxycytidine monophosphate deaminase (dCMPD) to yield dFdUMP, which may then be further phosphorylated to dFdUDP and dFdUTP or dephosphorylated to dFdU [5]. As with gemcitabine, dFdU may also be directly phosphorylated intracellularly to its mono-, di-, and triphosphate metabolites by deoxycytidine kinase. However, the majority of dFdU nucleotides are thought to be formed by the breakdown of gemcitabine nucleotides as dFdU is predicted to have a low affinity for deoxycytidine kinase (dCK) [13–15].

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Gemcitabine is transported into cells by both the concentrative (hCNT) and equilibrative (hENT) nucleoside transporters. Human concentrative nucleoside transporter 1 (hCNT1) is the most efficient transporter of gemcitabine, with a K_m of around 18 μM, but the distribution of this protein throughout the body is limited when compared to the more ubiquitously expressed ENT family [16,17]. hENT1 and hENT2 also facilitate gemcitabine uptake into cells, and clinical studies have demonstrated a correlation between the expression of these transporters and response to gemcitabine therapy [18–20].

Recently, it has been reported that dFdU is transported by both hCNT1 and hENT1 into hCNT1-transfected MDCK cells [21]. The extent of dFdU uptake was nearly the same as that of gemcitabine, suggesting that dFdU is a high affinity substrate for these transporters as well. Additionally, it was determined in the same study that dFdU undergoes biphasic efflux from HepG2 and A549 cells preloaded with gemcitabine, presumably via the same nucleoside transporters.

When administered alone, dFdU displays minimal cytotoxicity when compared with gemcitabine. Yet, with both the parent and metabolite competing for the same transporters, high plasma concentrations of dFdU may affect the efficacy of gemcitabine against solid tumors by decreasing its cellular uptake. Therefore, the objective of this study was to investigate the effects of dFdU on gemcitabine uptake and efflux via hENT1 and hENT2 in HeLa cells, a cell line which endogenously expresses these two nucleoside transporters. Based on our results, we have developed a mechanistic model whereby dFdU limits the uptake of gemcitabine into the cell, yet ultimately results in an increased intracellular sequestration of gemcitabine within cells.

2. Materials and methods

2.1. Chemicals

2′,2′-Difluorodeoxycytidine (dFdC; gemcitabine) and 2′,2′-difluorodeoxyuridine (dFdU) were synthesized by the Institute for Therapeutics, Discovery, and Development at the University of Minnesota, Minneapolis, MN. [5-^{3}H]-gemcitabine (11 Ci/mmol) was obtained from Moravek chemicals (La Brea, CA). Uridine, dilazep, and nitrobenzyl-mercaptopurine riboside (NBMPR) were obtained from Sigma–Aldrich (Saint Louis, MO). $^{13}C,^{15}N_2$-dFdU was purchased from Toronto Research Chemicals (North York, Ontario, Canada). All other chemicals were obtained from Fisher Scientific (Pittsburgh, PA) unless specified.

2.2. Cell culture

HeLa cells, derived from a cervical adenocarcinoma, were obtained from ATCC (Manassas, VA). Cells were grown as a monolayer at 37 °C under 5% CO_2 in Dulbecco's Minimum Essential Media supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 10 μg/mL streptomycin, and fortified with 10% FBS (Invitrogen; Carlsbad, CA). Cells used in transporter experiments were in the exponential growth phase and had undergone between 5 and 20 passages.

2.3. Transporter assays

HeLa cells were seeded in 12- or 24-well plates and allowed to adhere overnight. For radiolabeled experiments, tritiated gemcitabine was combined with cold gemcitabine to make a 5 μM tracer solution in transport buffer (128 mM NaCl, 4.73 mM KCl, 1.25 mM $CaCl_2$, 1.25 mM $MgSO_4$, and 5 mM HEPES, pH 7.4). For non-radiolabeled experiments, 100 μM solutions of dFdU were prepared in transport buffer. Incubations were initiated by aspirating the media from the cells, which were then loaded with tracer solution. Plates were incubated at 37° C on a rotary shaker for a time period ranging from 3 s to 24 h, after which drug was removed and cells washed three times in ice-cold transport buffer containing 50 μM dilazep, an inhibitor of both hENT1 and hENT2 [22,23]. Cells were solubilized in 1% Triton X-100, and 200 μL of each solubilized cell fraction was added to 4 mL of scintillation cocktail (ScintiSafe Econo Cocktail, Fisher Scientific, Pittsburgh, PA). Radioactivity was then determined with a Beckman Coulter LS-6500 liquid scintillation counter (Fullerton, CA). Total protein concentration in each well was determined by the BCA protein assay (Pierce, Saint Louis, MO). For cold substrates, cells were lysed with −80 °C 70:30 methanol:water, and intracellular dFdU quantified via HPLC with mass spectrometric detection. Because methanol present in the quenched incubations interfered with the absorbance readings obtained with the BCA protein assay, data from studies with non-radiolabeled substrate were normalized to the number of cells initially plated in each well.

Inhibition experiments: To determine the effects of dFdU on gemcitabine uptake, dFdU prepared in a 1:1 solution of DMSO:transport buffer was added directly to the gemcitabine tracer solutions where indicated. The amount of organic solvent in each incubation was kept below 0.5%.

hENT-mediated transport: To characterize gemcitabine and dFdU uptake in the absence of endogenous hENT, experiments were performed after cells were pre-treated with 50 μM dilazep [22,24]. Intracellular levels of both gemcitabine and dFdU in dilazep-treated cells were minimal (<5% of total uptake), allowing for the assumption that uptake of gemcitabine and dFdU in Hela cells proceeds through hENT1- and hENT2-mediated transport.

hENT2-mediated transport: To examine the effects of hENT2-mediated transport alone, cells were pre-incubated for 30 min prior to the start of the experiment in 100 nM NBMPR (a known inhibitor of hENT1), and 100 nM NBMPR was also added to tracer solutions [23,24].

2.4. dFdU analysis

Quantitation of dFdU was performed as previously described [10]. Briefly, internal standard ($^{13}C,^{15}N_2$-dFdU) was added to the HeLa cell lysate, and the lysate evaporated to dryness followed by reconstitution in 100 μL of mobile phase. The sample was injected onto a Fusion® RP 50 mm × 3.0 mm, 3.5 μm, column at 30 °C (Phenomenex, Torrance, CA). The mobile phase (flow rate, 0.3 mL/min) consisted of 5:95 (v/v) methanol:aqueous buffer (5 mM ammonium acetate, pH 6.8). The HPLC system (Agilent 1200) was interfaced to a Thermo TSQ Quantum mass spectrometer with electrospray interface operating in positive ionization mode. Detection of dFdU was obtained through selected reaction monitoring (SRM) of the following transitions: *m/z* 265/113 for dFdU and *m/z* 268/116 for $^{13}C,^{15}N_2$-dFdU (IS). The linear calibration range for dFdU using this method was 4.0–8021 pmol, with an average accuracy of 104% and mean precision (%CV) of 4.2%. The limit of quantitation (defined as 10 times the signal-to-noise ratio) was set at 0.05 μM.

2.5. Cytotoxicity assays

MTT (3-(4,5-*Dimethylthiazol*-2-*yl*)-2,5-*diphenyltetrazolium bromide*) *assay*: Cells were plated in 96-well plates, allowed to adhere overnight, and then exposed to media containing gemcitabine alone or in combination with 100 μM dFdU for 48 h. This was followed by an additional 24-h recovery period in fresh media. MTT (10 μL of 5 mg/mL solution; Sigma–Aldrich; Saint Louis, MO) was added to each well followed by an incubation time of 2 h or until purple crystals (formazan) could be observed. The media was

then removed and 100 μL DMSO added to each well to solubilize the formazan crystals. The absorbance at 562 nm was determined using a Synergy HT Multi-Mode Microplate Reader (Biotek, Winooski, VT), and viability expressed as the percentage of A_{562} of treated cells relative to untreated controls.

2.6. Statistical analysis

IC_{50} values were determined by nonlinear regression using the following equation within SigmaPlot (Systat Software, Inc., Chicago, IL):

$$IC_{50} = \min + \frac{\max - \min}{1 + [x/EC_{50}]^{Hillslope}}$$

All experiments were performed in triplicate and values are presented as the mean ± standard deviation. Statistical comparisons were assessed using a Student's *t*-test with differences between groups considered statistically significant when the *p*-value was <0.05 (SigmaStat 3.11 software, SyStat Software, Inc., Chicago, IL).

3. Results

3.1. Uptake of gemcitabine over short time periods

Due to rapid equilibration, experiments designed to examine nucleoside uptake via equilibrative transporters were performed over a very short timeframe to minimize intracellular metabolism and limit efflux of substrate and metabolites [25]. Therefore, initial experiments to characterize the effects of dFdU on gemcitabine uptake via hENTs in HeLa cells were conducted with incubation periods of <1 min, under the assumption that all detectable intracellular radiolabel was in the form of the parent compound.

Gemcitabine uptake into HeLa cells via both hENT1 and hENT2 was linear within the first 10 s, which is consistent with previous reports [24]. The addition of 100 μM dFdU significantly inhibited gemcitabine transport out to 20 s, with nearly a five-fold decrease in gemcitabine uptake observed at 20 s (Fig. 1). When hENT1 activity was inhibited by the addition of 100 nM NBMPR, the uptake of gemcitabine decreased by about 25%, confirming that gemcitabine is a substrate for both hENT1 and hENT2 in HeLa cells. Adding 100 μM dFdU to cells pretreated with NBMPR resulted in a 9-fold decrease in overall gemcitabine uptake, indicating that along with gemcitabine, dFdU is also a substrate for both hENT1 and hENT2. The uptake of dFdU alone was studied in HeLa cells and was observed to be transported at nearly twice the velocity via hENT1 than hENT2 with initial uptake rates of 14.5 and 8 nmol/10^5 cells/min, respectively (Fig. 2).

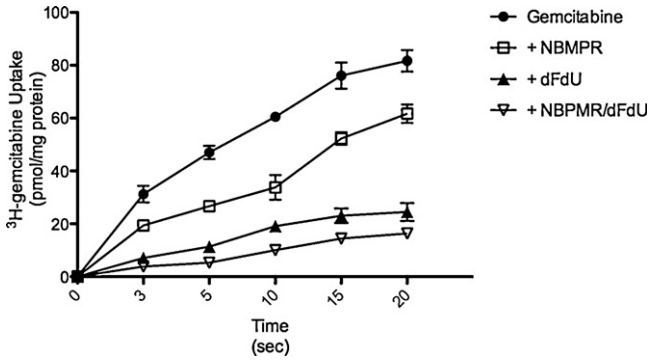

Fig. 1. Gemcitabine uptake via equilibrative nucleoside transporters in the presence of dFdU (100 μM). Intracellular accumulation of ^{3}H-gemcitabine (5 μM) in HeLa cells was determined over a 20-s interval. To examine hENT2-mediated uptake independently of hENT1, cells were pretreated with NBMPR (100 nM) for 30 min. Experiments were performed in triplicate, and data represent the mean ± S.D.

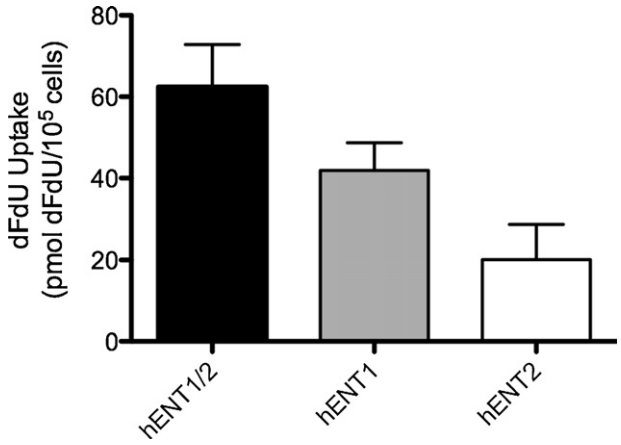

Fig. 2. dFdU uptake via endogenously expressed hENT1 and hENT2 in HeLa cells. 100 μM dFdU was added to the extracellular compartment, and intracellular levels of dFdU were determined via mass spectrometry after a 1-min incubation period. To calculate hENT1-mediated transport of dFdU, uptake of dFdU in cells pre-treated with 100 nM NBMPR (hENT2-mediated) was subtracted from the total uptake (hENT1/2). Experiments were performed in triplicate, and data represent the mean ± S.D. from replicate experiments.

3.2. dFdU-Mediated inhibition of gemcitabine uptake via hENT1 and hENT2

Experiments described in Section 3.1 demonstrated that dFdU is a substrate for hENT1 and hENT2 and is capable of limiting gemcitabine transport via these same transporters, most likely through competitive inhibition. To further characterize this interaction, the relative potency of dFdU as an inhibitor of gemcitabine uptake via hENTs was determined. IC_{50} values for the dFdU-mediated inhibition of hENT1 and hENT2 with gemcitabine as a substrate were 1.2 μM (hENT1/2) and 0.45 μM (hENT2), respectively (Fig. 3).

3.3. Uptake of gemcitabine over longer incubation periods

As efflux and intracellular metabolism of gemcitabine occurs with increasing incubation length, studies with radiolabeled gemcitabine and unlabeled dFdU were repeated over longer incubation periods to define interactions that may occur in a clinical setting after gemcitabine administration. To account for the effects of metabolism occurring with longer incubations, the accumulated intracellular radioactivity is assumed to represent the parent compound and all subsequently radiolabeled intracellular metabolites. This total gemcitabine accumulation, was determined at time points ranging from 3 min to 24 h. Experiments were also performed with and without NBMPR pre-treatment, allowing for determination of hENT1- and hENT2-mediated transport.

In HeLa cells with active hENT1 and hENT2, the addition of 100 μM dFdU to gemcitabine tracer solutions resulted in an initial decrease in the total accumulation of gemcitabine at 3 and 10 min. However, accumulation of gemcitabine significantly increased over the remaining incubation periods (Fig. 4A). By 24 h, gemcitabine accumulation in the presence of 100 μM dFdU was nearly twice that measured in the absence of dFdU.

When hENT1-mediated transport was inhibited by NBMPR, net gemcitabine accumulation in HeLa cells was nearly halved (Fig. 4B). Again, this confirms that both hENT1 and hENT2 are significant contributors to gemcitabine transport into HeLa cells. When 100 μM dFdU was added to HeLa cells pre-treated with NBMPR, net gemcitabine accumulation was significantly inhibited out to 60 min, at which time dFdU significantly increased total intracellular gemcitabine concentrations. By 24 h, hENT2-mediat-

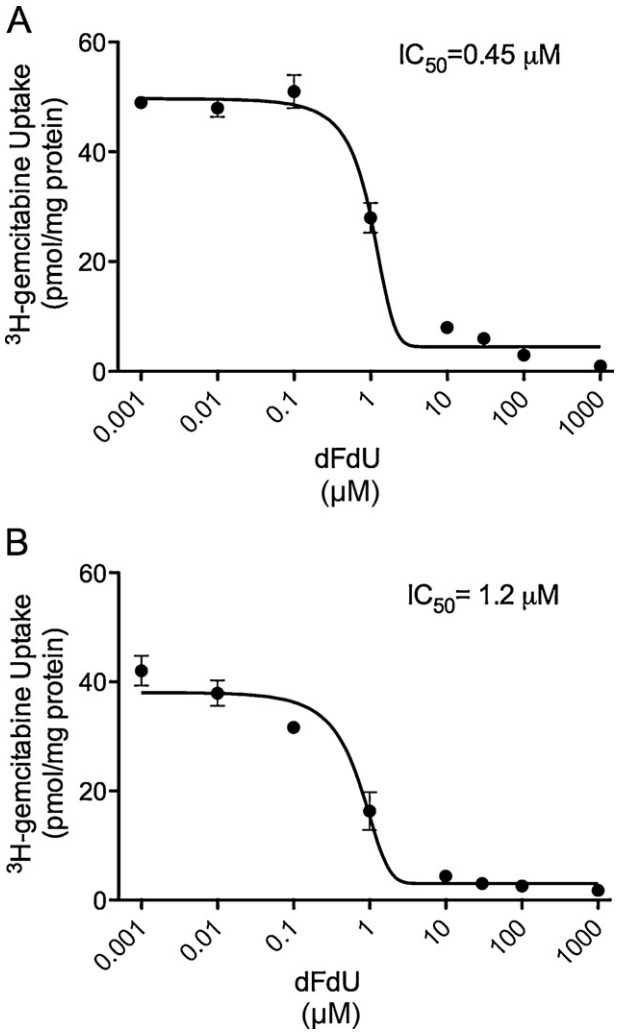

Fig. 3. Dose-dependent inhibition of 5 μM gemcitabine transport across the Hela cell monolayer by dFdU. (A) hENT1- and hENT2-mediated transport, (B) hENT2-mediated transport. Intracellular accumulation of ^{3}H-gemcitabine (5 μM) was measured in the presence of increasing concentrations of dFdU over a 10 s interval. IC_{50} values were calculated by nonlinear regression as specified in Section 2.6. Data points represent the mean ± S.D.

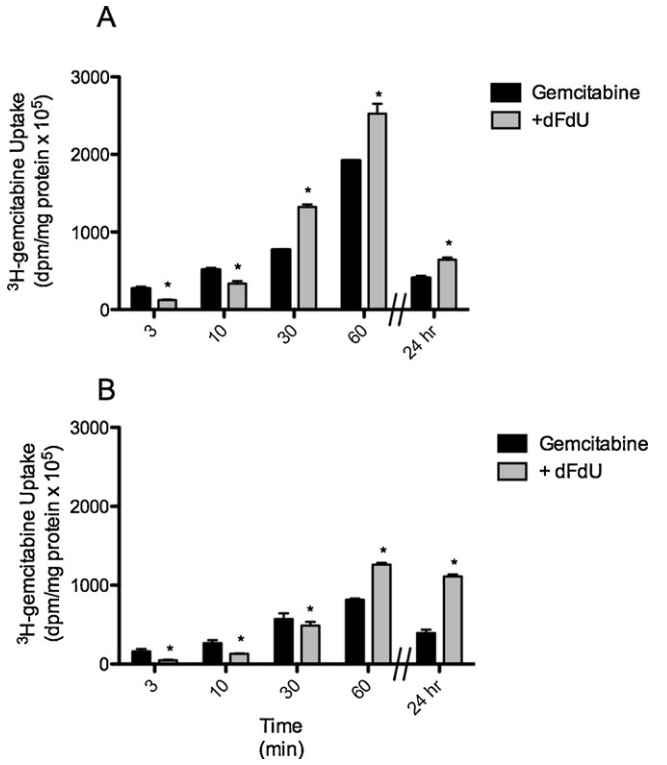

Fig. 4. Time-dependent inhibition of 5 μM gemcitabine transport in the presence of dFdU. Hela cells were treated with ^{3}H-gemcitabine (5 μM) and dFdU (100 μM) at intervals up to 24 h, at which time cells were lysed, and total intracellular radioactivity was measured. (A) hENT1- and hENT2-mediated uptake, (B) hENT2-mediated uptake. Pretreatment of cells with NBMPR (100 nM) allowed for the determination of hENT2-mediated transport independently of hENT1. Data are represented as the mean ± S.D. of replicate experiments. Asterisk indicates (*) significantly different compared to the total accumulation of gemcitabine in the absence of dFdU ($p < 0.05$).

ed net accumulation of gemcitabine in the presence of 100 μM dFdU was nearly 3-fold higher than in the absence of dFdU.

3.4. Effect of dFdU on gemcitabine cytotoxicity

As the addition of physiologically relevant concentrations of dFdU increased the net intracellular level of gemcitabine at 24 h, it is possible that dFdU may have an effect on gemcitabine toxicity as well. As a single agent, dFdU is cytotoxic to HeLa cells, with an apparent IC_{50} value of 260 μM compared with an IC_{50} value of 0.03 μM for gemcitabine alone (Fig. 5A and B). However, despite the significantly higher level of gemcitabine accumulation in the presence of dFdU, the effect of dFdU on gemcitabine cytotoxicity was merely additive (Fig. 5B). In Fig. 5B the percentage of viable cells remaining after treatment with dFdU and dFdC was not corrected for the cytotoxic effect of dFdU itself, to better allow for visualization of the additive effect of these compounds on cell viability. When the viability is corrected for dFdU-mediated toxicity, no significant effect on the anti-proliferative activity of gemcitabine is observed.

Cytotoxic response to increasing concentrations of dFdU combined with 0.5 or 5 μM gemcitabine was also examined. At the higher concentration of gemcitabine, the addition of dFdU produced no additional loss of cell viability (Fig. 6). At 0.5 μM of gemcitabine, the effect of dFdU was merely additive and was independent of dFdU concentration.

4. Discussion

Following intravenous administration of gemcitabine, dFdU reaches peak plasma levels within 5–15 min post-infusion. Unlike gemcitabine, concentrations of dFdU remain elevated for a prolonged period of time with an elimination half-life of up to 24 h [9]. Yet, despite the difference in their respective pharmacokinetic profiles, the contribution of dFdU to gemcitabine disposition is unknown. A nucleoside analog itself, dFdU has been observed to undergo influx via both hCNT1 and hENT1 in MDCK-transfected cells [21]. Presumably, due to its bidirectional transport properties, hENT1 also facilitates the efflux of dFdU in transfected MDCK cells as well. In the current study, dFdU has been identified as a substrate for both hENT1 and hENT2 in HeLa cells, as well as a competitive inhibitor of gemcitabine influx via these same transporters.

When measuring net intracellular accumulation of a nucleoside that is also a substrate for metabolizing enzymes, distinguishing between the effects of transport and intracellular metabolism is often difficult, since the concurrent occurrence of both processes may confound interpretation of the results [25]. To effectively examine interactions between dFdU and gemcitabine while minimizing the contribution of metabolism and efflux, incubation periods were initially limited to 20 s. Evidence suggests that longer

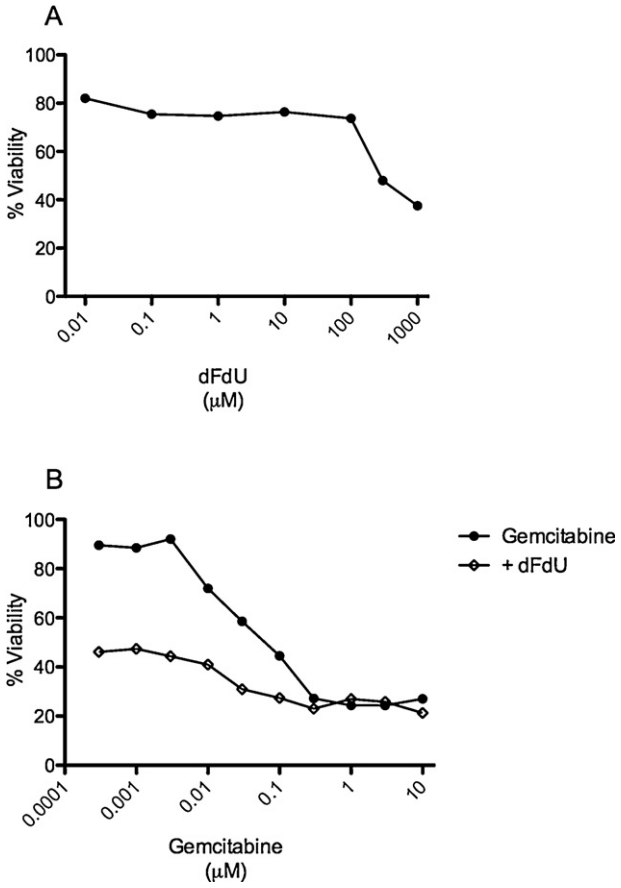

Fig. 5. Dose-dependent antiproliferative effects of dFdU and gemcitabine in HeLa cells. (A) dFdU, (B) gemcitabine in combination with dFdU (100 μM). Percent viability calculated by taking the ratio of absorbance in treated wells to the absorbance in untreated control wells. Data points represent the mean results of a single representative experiment ($n = 6$ replicates at each concentration).

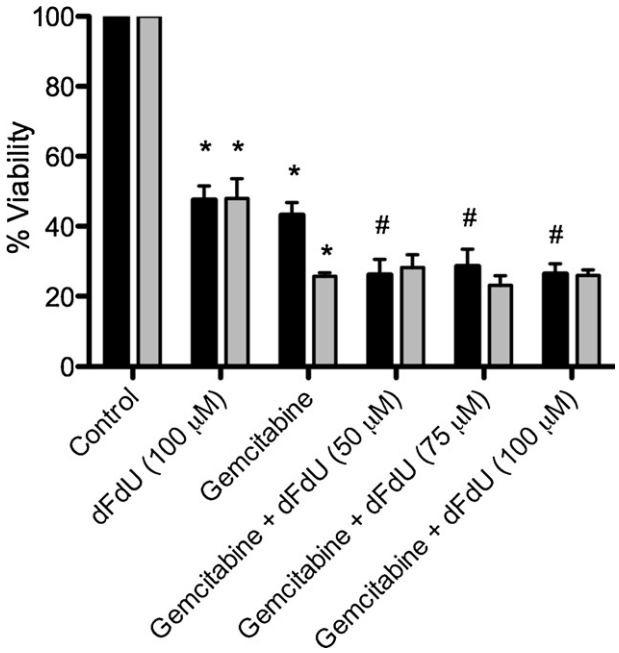

Fig. 6. Antiproliferative effects of gemcitabine in combination with increasing concentrations of dFdU (50, 75, and 100 μM). Gemcitabine concentrations of 0.5 μM (black bars) and 5 μM (gray bars) were tested. Percent viability was calculated by taking the ratio of absorbance in treated wells to the absorbance in untreated control wells. Data represent the mean ± S.D. ($n = 6$ replicates for each treatment). A single representative experiment is shown. Asterisk indicates (*) significantly different compared to cells treated with media only. Hash indicates (#) significantly different than 0.5 μM gemcitabine alone.

incubation periods result in the saturation of uptake transport, and accumulating intracellular metabolites account for the majority of net radioactivity detected within the cell [25]. In Fig. 1, clinically relevant concentrations of dFdU significantly inhibited gemcitabine accumulation at incubation periods up to 20 s. dFdU had an even greater effect on the intracellular accumulation of gemcitabine when cells were pre-treated with NBMPR. Since interactions occurring at early time points principally affect transporter-mediated uptake, these results suggest that dFdU limits gemcitabine transport via both hENT1 and hENT2. Further analyses using unlabeled dFdU provided evidence that dFdU is indeed a substrate of both of these equilibrative nucleoside transporters (Fig. 2). Also, IC_{50} values for the dFdU-mediated inhibition of gemcitabine uptake by hENTs are nearly 100-fold lower than the average plasma concentration for dFdU in humans following gemcitabine administration (Fig. 3).

To further characterize the effect of high plasma concentrations of dFdU on gemcitabine disposition, longer incubation periods were evaluated (up to 24 h) as well. During longer incubations, radiolabeled gemcitabine undergoes sequential phosphorylation, resulting in the formation of active phosphorylated metabolites, in addition to deamination to dFdU [5]. Contrary to what was observed at incubation periods <1 min, the addition of dFdU to incubations of 24 h resulted in significantly higher levels of net intracellular radioactivity (including gemcitabine and metabolites), implying that the effects of dFdU on gemcitabine transport and accumulation are time-dependent. Together, these results suggest that dFdU is simply not only a less cytotoxic metabolite but also a modulator of gemcitabine accumulation in cells.

Interestingly, this is not the first study to suggest that a temporal component governs the effects of hENT inhibition on nucleoside disposition. Plagemann and Wohlhueter examined the transport of the nucleoside analog and hENT substrate 2′deoxyadenosine (2′-dAdo) and identified a time-dependent effect of the known hENT inhibitors NBMPR and dipyridamole on the accumulation of this nucleoside analog in L1210 leukemia cells [26]. Specifically, the influx of 2′-dAdo was inhibited in the presence of NBMPR and dipyridamole over incubation periods of <1 min, but when incubation length increased to 2 h, intracellular accumulation of 2′-dAdo became significantly 'stimulated' in the presence of these inhibitors.

The mechanism underlying the interaction between gemcitabine and dFdU is most likely multi-faceted, and includes the direct competition between the parent compound and the metabolite for hENT-mediated transport. Our data demonstrate that dFdU initially limits gemcitabine uptake via hENTs. However, equilibration of gemcitabine and its metabolites is achieved with increasing incubation length, and intracellular dFdU may also begin to block gemcitabine efflux via these same transporters. If this were true, longer incubations in the presence of dFdU would conversely result in the intracellular retention of gemcitabine, which is what we have observed here (Fig. 4). This mechanism becomes even more plausible when one considers that the intracellular concentration of dFdU in our *in vitro* system (100 μM) is theoretically 20-fold higher than our starting concentration of gemcitabine (5 μM), and this does not include the additional intracellular accumulation of dFdU formed as gemcitabine undergoes deamination. While bidirectional equilibrative nucleoside transporters are thought to be symmetrical with regard to substrate affinity and rate of transport, data exist suggesting that inhibition of hENTs with dipyridamole may affect substrate efflux more significantly than influx, potentially increasing the intracellular accumulation of

nucleosides despite a significant decrease in the rate of uptake [27,28]. If dFdU differentially inhibits the efflux versus the influx of gemcitabine, this provides a mechanism by which the deaminated metabolite enhances the retention of the parent compound.

This hypothesis is consistent with earlier work by Wright et al. who observed a significant increase in the intracellular half-life of 2-chlorodeoxyadenosine (2-CdA) in human leukemic lymphoblasts when efflux via hENT1 was limited by the addition of NBMPR and dilazep [29]. Additionally, Mackey et al. reported that despite a significant decrease in the initial rate of gemcitabine uptake in Caco-2 cells pre-treated with dipyridamole, intracellular accumulation of radiolabeled gemcitabine exceeded the steady-state concentration achieved in the absence of dipyridamole, possibly due to the inhibition of gemcitabine efflux via the bidirectional hENTs [24].

Curiously, the increased intracellular retention of cytotoxic nucleosides occurring in response to interactions with hENTs does not consistently translate into an increase in cell sensitivity. In a study with 2′-deoxyadenosine, the addition of dipyridamole significantly potentiated the growth inhibition of L1210 leukemia cells yet had no effect on P388 cells [26]. In the current study, despite a significant increase in gemcitabine retention at 24 h, no synergistic effect on the inhibition of cell growth was observed when dFdU was added to gemcitabine. The effect was merely additive, due to the known cytotoxic effects of dFdU at higher concentrations (Fig. 5A). As the high concentration of gemcitabine (5 μM) initially used in the these studies may have yielded maximal cytotoxic effects even in the absence of dFdU, the study was repeated with a concentration of gemcitabine closer to the IC_{50} value observed for this nucleoside analog in HeLa cells (Fig. 6). However, the effect of dFdU on the cytotoxicity of a 10-fold lower concentration of gemcitabine was also observed to be additive (Fig. 6). Likewise, Mackey et al. did not observe any increase in cell sensitivity of gemcitabine towards Caco-2 cells associated with the inhibition of hENT-mediated efflux of this nucleoside by dipyridamole [24].

As the activation pathway for gemcitabine is complex, the interaction between dFdU and gemcitabine may not be solely attributable to inhibition of hENTs. By limiting the rate at which gemcitabine enters the cell, dFdU may be affecting the rate or extent of intracellular phosphorylation or deamination, as well as inhibiting efflux. Investigations with 2′-deoxyadenosine, a substrate that also undergoes extensive intracellular metabolism, revealed significantly higher levels of 2′-deoxyadenosine triphosphate in cells treated with dipyridamole, suggesting that inhibition of hENTs with dipyridamole is also affecting phosphorylation [26]. Conversely, it is also possible that dFdU is limiting the degradation of gemcitabine metabolites, possibly through the inhibition of 5′-nucleotidases, which oppose the effects of deoxycytidine kinase through dephosphorylation of gemcitabine nucleotides [30]. Lastly, gemcitabine is also transported intracellularly by the unidirectional hCNTs. Being more ubiquitously expressed, hENTs are thought to have a larger overall impact on the disposition of gemcitabine [16]. However, the more tissue-specific expression of hCNTs has been observed to impact the efficacy and side effect profile of gemcitabine in many malignancies as well [31–33]. With initial data suggesting that dFdU is also a substrate for hCNT1, it is possible that dFdU may affect the disposition of gemcitabine through interactions with hCNTs as well [21]. Thus, further studies are required to fully characterize the molecular mechanisms by which dFdU is enhancing gemcitabine retention.

While dFdU is known to possess cytotoxic and radiosensitizing properties, the extensive conversion of gemcitabine to dFdU is frequently seen as an impediment to optimizing gemcitabine therapy, with ours being the first study to suggest that dFdU may actually improve the disposition of gemcitabine in tumor cells. In fact, significant *in vitro* work exists suggesting that inhibition of intracellular cytidine deaminase actually increases the cytotoxicity of gemcitabine by decreasing the anabolism of the more potent parent compound to the less active deaminated metabolite (dFdU.) [34]. Additionally, studies conducted in mice have demonstrated that oral administration of 3,4,5,6-tetrahydrouridine (THU), a known inhibitor of cytidine deaminase, significantly increased the oral bioavailability of gemcitabine from 10 to 40% [35]. Unfortunately, the effects of THU coadministration on *in vivo* gemcitabine uptake and cytotoxicity were not investigated. However, based on our data and that of others, it seems probable that while limiting intracellular breakdown of gemcitabine to dFdU may increase cell sensitivity to gemcitabine, limiting plasma formation of dFdU after intravenous administration of gemcitabine may actually have negative effects on cytotoxicity. While initially more gemcitabine would be taken up into the cell in the absence of high plasma dFdU, the intracellular retention of the parent compound may be significantly limited, potentially affecting cytotoxicity. Future *in vivo* studies focusing on the effect of plasma dFdU on intracellular gemcitabine disposition and toxicity may provide important insights into the optimal role of deaminase inhibitors in gemcitabine therapy.

In conclusion, our studies demonstrate that the extensive deamination of gemcitabine in the plasma, typically thought to limit the efficacy of the parent compound, may actually be an integral part of the *in vivo* disposition of this nucleoside analog. This work supports a mechanism by which the deaminated metabolite, dFdU, plays a potentially significant role in enhancing the intracellular retention of gemcitabine, possibly through its interactions with the bidirectional equilibrative nucleoside transporters.

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