Short Communication:

The deaminated metabolite of gemcitabine, 2’,2’-difluorodeoxyuridine, modulates the rate of gemcitabine transport and intracellular phosphorylation via deoxycytidine kinase

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Nonstandard abbreviations: dFdC, gemcitabine: dFdU, 2’,2’-difluorodeoxuryridine; dCK, deoxycytidine kinase; CdA, cytidine deaminase; NBMPR, nitrobenzylmercaptopurine riboside; hENT, human equilibrative nucleoside transporter; hCNT, human concentrative nucleoside transporter
Abstract

Gemcitabine (dFdC) is a chemotherapeutic nucleoside analog that undergoes uptake via equilibrative nucleoside transporters (hENT) followed by sequential phosphorylation to the active triphosphate moiety (dFdCTP). Its deaminated metabolite, 2',2’-difluorodeoxyuridine (dFdU), competes with the parent compound for cellular entry via hENTs, but over time dFdU increases the net intracellular accumulation of dFdC by a currently unknown mechanism. In this study we investigated whether dFdU affects intracellular phosphorylation of gemcitabine by modulating the activity of deoxycytidine kinase (dCK). We report here that co-incubation of dFdU with dFdC significantly increases intracellular levels of dFdCTP. dFdCTP was not identified as a substrate for hENTs, suggesting dFdU affects the formation rather than elimination of the triphosphate. To further characterize the disposition of dFdC in the presence of dFdU, the net intracellular radioactivity of [5-3H]-dFdC and corresponding metabolic profile were evaluated in HeLa cells transfected with dCK-targeting siRNA. Intracellular radioactivity significantly decreased in cells with compromised intracellular phosphorylation, which was mainly due to a loss in dFdCTP. Although dFdU increased the net intracellular radioactivity of [5-3H]-dFdC at 24 h in control cells, this increase was abolished in the absence of dCK activity, strongly suggesting that the interaction between dFdU and dFdC occurs via modulation of both transport and metabolism. In conclusion, we have demonstrated that the intracellular distribution of dFdC is dependent on both transport and metabolic processes, and that by affecting the rate at which dFdC enters the cell, the presence of dFdU may be altering the metabolic fate of the parent compound (dFdC).
Introduction

Gemcitabine (2’,2’-difluorodeoxycytidine, dFdC) is a deoxycytidine analog effective against many solid tumors (Hansen SW, 2001; Symon Z et al., 2002; Heinermann V, 2005). Due to its hydrophilicity, cellular uptake of dFdC is facilitated by various human transporters. For example, nucleoside transporters (hNT), and both the unidirectional concentrative (hCNT) and bidirectional equilibrative nucleoside transporter (hENT) families actively transport dFdC (Mackey JR et al., 1998). Once inside the cell, dFdC is sequentially phosphorylated to the active triphosphate, which is subsequently incorporated into nascent RNA and DNA strands resulting in cytotoxicity. (Mini E et al., 2006) The first and rate-limiting step in this phosphorylation pathway is mediated by deoxycytidine kinase (dCK). dFdC also undergoes rapid deamination in plasma as well as tissue via cytidine deaminase (CdA), yielding high concentrations of the less active metabolite, 2’,2’-difluorodeoxyuridine (dFdU) (Giusti G et al., 1970).

One of the challenges in characterizing the tissue distribution of dFdC is distinguishing between nucleoside transport and intracellular metabolism. Equilibration of nucleoside analogs via hNT present on cell membranes occurs at such a rapid rate that steady-state levels are frequently achieved within thirty seconds, after which intracellular phosphorylation becomes the limiting step to determine net nucleoside accumulation (Plagemann PGW et al., 1982). Thus, kinetic parameters based on incubations longer than 1 min are merely reflective of the rate of dFdC phosphorylation as opposed to the rate of nucleoside transport into the cell.

To appropriately assess the kinetics of dFdC uptake, the complicating factor of its rapid intracellular metabolism needs to be controlled. Thus, short incubation periods are frequently employed in the study of dFdC transport. We have previously identified an interaction whereby the deaminated metabolite, dFdU, inhibits dFdC transport via hENTs during short incubation periods.
(Hodge LS et al., 2011). Yet, when incubation periods were increased to 24 h, a significant increase in the net accumulation of [5-\(^3\)H]-dFdC (dFdC and subsequently radiolabeled metabolites) was observed. Structurally similar to dFdC, dFdU undergoes intracellular phosphorylation via dCK in the same manner as the parent drug (Veltkamp SA et al., 1992). While dFdU exhibits a much lower affinity for dCK than dFdC, the possibility of an effect of dFdU on intracellular accumulation of dFdC must still be taken into consideration (Veltkamp SA et al., 2008). Our goal was to elucidate the role of dCK as it pertains to the interaction between dFdU and dFdC. To better separate the effects of dFdU on hENT-mediated transport and dCK-mediated phosphorylation, a novel experimental design was used in which cells were transfected with dCK-targeting siRNA to eliminate the activity of this enzyme. Our data suggest suppression of dCK-mediated phosphorylation results in the removal of an intracellular dFdC ‘sink’ that serves as the driving force for equilibrative uptake of this nucleoside. The net accumulation of [5-\(^3\)H]-dFdC and its radiolabeled metabolites is significantly decreased in the absence of phosphorylation, and this effect is independent of dFdU.

**Materials and Methods**

**Chemicals**

2’,2’-difluorodeoxycytidine (dFdC), 2’,2’-difluorodeoxyuridine (dFdU), and 2’,2’-difluorodeoxycytidine triphosphate (dFdCTP) 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 from Moravek chemicals (La Brea, CA). Nitrobenzyl-mercaptopurine riboside (NBMPR) was obtained from Sigma-Aldrich (Saint Louis, MO). 13C, 15N2-gemcitabine, 13C, 15N2-dFdU and 2’deoxycytidine were purchased from Toronto Research
Chemicals (North York, Ontario) while $^{13}$C, $^{15}$N$_2$-cytidine triphosphate (CTP) was purchased from Cambridge Isotopes (Andover, MA). All other chemicals were obtained from Fisher Scientific (Pittsburgh, PA) unless specified.

**Cell culture and transporter assays**

Hela cells, which endogenously express hENT1 and hENT2, were cultured and utilized for transport experiments as described (Hodge LS et al., 2011). Briefly, for radiolabeled experiments, tracer solutions were prepared with $[5-^3$H]-dFdC and unlabeled dFdC at a total concentration of 5 µM. Where indicated, dFdU and dFdCTP were added directly to tracer solutions. After incubations of either 1 h or 24 h in length, cells were solubilized in 1% Triton X-100, and net intracellular radioactivity (including $[5-^3$H]-dFdC and any subsequently formed radiolabeled metabolites) was determined by liquid scintillation counting. Total protein concentration in each well was determined by the BCA protein assay (Pierce, St Louis, MO). For experiments with unlabeled gemcitabine, cells were lysed in 70:30 methanol:water in a dry ice bath prior to quantification of dFdC, dFdU, and dFdCTP. As methanol interfered with the BCA protein assay, data obtained from unlabeled dFdC studies were instead normalized to the number of cells initially plated in each well. To assess hENT2-mediated transport, cells were pre-incubated for 30 minutes prior to the start of the experiment in 100 nM NBMPR (a known inhibitor of hENT1), which was also added to tracer solutions as indicated (Cass CE et al., 1992).

**Analysis of gemcitabine and its metabolites**

Quantitation of dFdU was performed as described previously (Hodge LS et al., 2011). A similar method was used for the analysis of dFdC, with $^{13}$C, $^{15}$N$_2$-dFdC serving as an internal standard. Detection of dFdC and dFdU was obtained through selected reaction monitoring (SRM)
of the following transitions: m/z 264/95 for dFdC, m/z 265/113 for dFdU, m/z 267/97 for $^{13}$C, $^{15}$N$_2$ -dFdC (IS), and m/z 268/116 for $^{13}$C, $^{15}$N$_2$ -dFdU (IS). The calibration range for this method was 2.4 – 4990 pmol for dFdC and 4.0 – 8021 pmol for dFdU. For measurement of dFdCTP, cell lysates were processed as for dFdC and dFdU analysis using $^{13}$C, $^{15}$N$_2$- cytidine triphosphate (CTP) as an internal standard. Samples were injected onto a Thermo Scientific Bio Basix AX (50 x 2.1 mm, 5 μm) column at 30º C. Chromatographic separation was achieved with a gradient (0 min-90:10, A:B; 0.51 min- 50:50, A:B; 1.76 min- 0:100, A:B; 2.5 min-0:100, A:B; 8.5 min-90:10, A:B; 9.5 min- 90:10, A:B; 12 min- 90:10, A:B) consisting of mobile phases A (30:70 ACN: 10 mM ammonium acetate in de-ionized water (DI), pH 6.0) and B (30:70 ACN: 1 mM ammonium acetate in DI, pH 10.5). The LC-MS/MS system was run in negative ionization mode with the following SRM events: m/z 502/159 for dFdCTP and m/z 494/159 for $^{13}$C9, $^{15}$N$_2$ -CTP. This method was linear within the range of 5 – 1000 pmol.

**dCK Silencing with siRNA**

HeLa cells were transfected with dCK siRNA (Silencer Validated siRNA #70, Ambion, Cambridge, UK) or negative control siRNA (Silencer Select Negative Control #2) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) per the manufacturer’s protocol. dCK activity was assessed at 24, 48, and 72 hr by incubating transfected cells with 5 μM dFdC. After 4 h, cell lysate was analyzed for dFdCT, the end product of intracellular dFdC phosphorylation, via LCMS. Net radioactivity and intracellular profiles of dFdU, dFdC, and dFdCTP were determined in cells 48 h post-transfection using incubations of 1 and 24 h in length.
Results and Discussion

Investigations into the disposition of nucleoside analogs, including dFdC, are complicated by the multiple transport and metabolic processes involved. In an attempt to focus solely on dFdC transport, we have used incubation periods of short duration to limit the effects of intracellular metabolism. Our research revealed an interaction between dFdC and dFdU whereby the deaminated metabolite competes with the parent compound for transport via hENT1 and hENT2 (Hodge LS et al., 2011). However, when the incubation interval was lengthened to 24 h, during which time extensive metabolism of dFdC is likely occurring, an opposite effect was observed, and adding dFdU to the incubation increased the net intracellular radioactivity detected in cells treated with [5-3H]-dFdC. To more accurately characterize the disposition of dFdC in the presence of dFdU, we assessed the contribution of intracellular phosphorylation to this interaction.

In the current study, measuring intracellular dFdCTP levels following a 24 h incubation with dFdC provided substantial evidence that dFdU is affecting dFdC phosphorylation, as cells treated with dFdC and dFdU contained 6.4-fold higher (p<0.0001) levels of dFdCTP than cells treated solely with dFdC (Figure 1A). This dFdU-related increase in phosphorylation was even more pronounced in cells pre-treated with NBMPR, with greater than 10-fold higher (p<0.0001) levels of dFdCTP detected (Figure 1B). These data suggest the increase in net radioactivity measured in cells incubated with [5-3H]-dFdC and dFdU in our previous study is primarily due to an increase in the triphosphorylated moiety (Hodge LS et al., 2011).

We then investigated whether dFdCTP may also be a substrate or inhibitor of hENTs and thus, whether dFdCTP contributes to the enhanced net radioactivity observed in cells treated with both [5-3H]-dFdC and dFdU. dFdC was transported by both hENT1 and hENT2 (Figures 1C, 1D). While hENT2 is typically thought to have a lower binding affinity for dFdC than hENT1 (Km of
740 µM vs. 160 µM, respectively), hENT2 transport is characterized by a higher \( V_{\text{max}} \) value, such that dFdC transport via hENT2 often exceeds that of hENT1 (Mackey JR et al., 1999). In our studies, the addition of NBMPR as an inhibitor of hENT1 decreased dFdC uptake by about 50% at when studied at incubation times up to 20 sec (Figures 1C, 1D). Additionally, consistent with other studies demonstrating that nucleotides are not substrates for hENTs, we observed no difference in dFdC uptake via either hENT1 or hENT2 when clinically relevant concentrations of dFdCTP were added to dFdC tracer solutions (Figure 1C, 1D) (Endres CJ et al., 2009). As the kinetics of import and export via bidirectional hENTs are thought to be relatively similar, the lack of effect of dFdCTP on dFdC influx is assumed to be consistent with a lack of effect on efflux (Plagemann PGW et al., 1987). This also implies that the addition of dFdU to the incubations is not increasing dFdCTP by preventing its hENT-mediated elimination.

To further evaluate the role of phosphorylation in the interaction between dFdU and dFdC, studies measuring net radioactivity and intracellular metabolite composition were repeated in cells with minimal capacity for intracellular phosphorylation, achieved though the siRNA-mediated knockdown of dCK (Figure 1E). A significant decrease in net radioactivity was noted at 1 and 24 h in [5-\(^{3}\)H]-dFdC-treated cells lacking dCK activity (Figures 2A, 2C). The metabolite composition in the same cells suggested that the decrease in net radioactivity was due in part to a loss of dFdCTP (Figures 2B, 2D). However, levels of dFdU were not affected. Interestingly, the collective concentration of dFdC, dFdCTP, and dFdU measured at 1 and 24 h in dFdC-treated cells (Figure 2B, 2D) remained relatively constant although the net radioactivity associated with these time points was significantly greater at 1 h. We suspect the net radioactivity at 1 h also includes metabolites not accounted for in our metabolite profiling. Specifically, Veltkamp et al. have
reported high intracellular concentrations of both dFdCMP and dFdCDP in cells treated with dFdC (Veltkamp SA et al., 2008).

Consistent with our finding that the addition of dFdU to cells treated with dFdC increases intracellular levels of dFdCTP, the interaction between dFdU and dFdC appears to be at least partially dependent on dCK activity. Specifically, in cells with active dCK, the addition of dFdU yielded an increase in net intracellular radioactivity after treatment with [5-3H]-dFdC for 1 h whereas a profound decrease was observed in cells with limited phosphorylative capacity (Figure 2A). At 24 h, the presence of dFdU again increased net intracellular radioactivity in control cells, however in dCK knockdown cells, accumulation remained constant regardless of dFdU treatment (Figures 2C). While the increase in net radioactivity at 1 h in cells treated with dFdC and dFdU appears to be due to unquantitated metabolites, at 24 h, the increase in net radioactivity is associated with a large increase in dFdCTP, an effect that is abolished upon inhibition of dCK. Even more, the addition of NBMPR, and the subsequent inhibition of hENT1, had little effect on net radioactivity or the metabolite profile at 1 h. However at 24 h, the addition of NBMPR led to a significant increase in net radioactivity over cells treated solely with [5-3H]-dFdC or the combination of [5-3H]-dFdC and dFdU. Again, the increase in net radioactivity associated with the addition of NBMPR appears to be secondary to an increase in dFdCTP (Figure 2D).

Our previous work suggests that by competing with dFdC for hENT-mediated uptake, dFdU affects the rate at which dFdC enters the cell (Hodge LS et al., 2011). The current studies elaborate on these findings and imply that once an equilibrium between intracellular and extracellular dFdC concentrations is reached in the absence of intracellular metabolism, dFdU has no further effect on the kinetics of dFdC uptake. Yet in the presence of dCK, dFdU significantly increases net radioactivity in [5-3H]-dFdC –treated cells and increases intracellular levels of
dFdCTP. Therefore, dCK appears to be a necessary component of the interaction between dFdC and dFdU.

A report detailing a simulation of intracellular ribavirin distribution, the results of which were subsequently validated following the conduct of in vitro studies, has provided significant insight into the interplay between nucleoside transporter-mediated uptake and intracellular phosphorylation (Endres CJ et al., 2009). Similar to the disposition of dFdC, ribavirin is a nucleoside analog that is a substrate for hENTs and adenosine kinase. In models of hENT1-expressing erythrocytes, which are not metabolically competent, the simulated data predicted no change in net intracellular ribavirin after equilibrium was reached. More complex modeling, incorporating ribavirin transport by hENTs and intracellular metabolism via a nonphosphorylative and a phosphorylative pathway, suggested that ribavirin concentrations still reach equilibrium within 60 sec. Yet, the net radioactivity (ribavirin plus its metabolites) was predicted to increase over the course of a 6 h incubation due to a “metabolic sink effect” incurred as ribavirin was intracellularly phosphorylated, leading to the continual uptake of parent compound from the extracellular compartment. Analogously, our data demonstrate that gemcitabine phosphorylation via dCK creates a “metabolic sink” in HeLa cells, as observed by comparing the net dFdC radioactivity in cells with and without active dCK. Therefore, it appears that both nucleoside transport and intracellular metabolism are key determinants of intracellular dFdC concentrations. The complementary interplay of hENTs and dCK as it pertains to dFdC and dFdU was confirmed in that knockdown of dCK activity abrogated the significant increase in dFdC accumulation associated with concomitant dFdU treatment (Figures 2C, 2D).

While the exact mechanism by which dFdU increases dFdCTP remains to be elucidated, our data suggest it is not likely due to a decrease in the elimination of dFdCTP by hENTs. Instead,
dFdU may trigger an increase in the formation of dFdCTP, possibly by slowing the rate at which dFdC enters the cell, thus altering its metabolic fate. Specifically, intracellular dFdC is more likely to undergo phosphorylation via dCK (k_m= 4.6 µM) before CdA-mediated deamination (k_m=95.7 µM) (Bouffard DY et al., 1993). However, at dFdC concentrations greater than 20 µM, dCK becomes saturated, and excess dFdC may undergo conversion to dFdU (Grunewald R et al., 1991). We could speculate that by slowing the entry of dFdC into the cell, intracellular concentrations remain low enough so the majority of dFdC is phosphorylated, leaving less to be inactivated through deamination. During long incubations, higher concentrations of dFdCTP would be detected in the presence of extracellular dFdU, despite having limited effects on dFdC accumulation at 1 hr. This hypothesis is supported by the higher levels of dFdU measured in cells lacking dCK activity, as more dFdC undergoes deamination in the absence of phosphorylation (Figure 2D). However, one cannot exclude the possibility that dFdU is also interfering with one of the many feedback mechanisms regulating nucleotide synthesis and degradation.

In conclusion, we have further characterized the interaction between dFdU and dFdC and have provided new data demonstrating that intracellular metabolism enhances dFdC uptake via hENTs. In fact, we predict that studies of nucleoside analog transport in the absence of intracellular metabolism may not adequately describe the distribution of these compounds, which appears to be dependent on both transport and metabolic processes.
Authorship Contributions:

Participated in research design: Hodge, Taub, Tracy

Conducted experiments: Hodge

Performed data analysis: Hodge, Taub, Tracy

Wrote or contributed to the writing of the manuscript: Hodge, Taub, Tracy
References


Figure Headings:

**Figure 1. dFdU increases intracellular levels of dFdCTP.** (A) dFdCTP content was measured in HeLa cells cultured for 1 h and 24 h with 5 µM dFdC in the presence and absence of 100 µM dFdU. (B) Addition of 100 nM NBMPR to the incubations allowed for the measurement of hENT2-mediated transport in the absence of hENT1 activity. (C) Uptake of [5-³H]-gemcitabine in the presence and absence of 100 µM dFdCTP. (D) Addition of 100 nM NBMPR to incubations of [5-³H]-dFdC and dFdCTP allowed for the measurement of hENT2-mediated transport. (E) HeLa cells transfected with either dCK-targeting or non-silencing siRNA were assessed for dCK activity at 24, 48, and 72 hr post-transfection by measuring intracellular dFdCTP after a 4 h incubation with 5 µM dFdC. All experiments were performed at least three times. Data represent averages +/- SD. *denotes p-value <0.05 as determined by Student’s t-test.

**Figure 2. Interaction between dFdU and dFdC is also dependent upon intracellular phosphorylation.** Net radioactivity ([5-³H]-dFdC and radiolabeled metabolites) was measured in HeLa cells transfected with either a non-silencing siRNA or dCK-targeting siRNA and incubated with 5 µM [5-³H]-dFdC in the presence and absence of 100 µM dFdU and/or 100 nM NBMPR for A) 1 h or C) 24 h. Experiments were performed at least three times, and data are presented as averages +/- SD. *Statistically different from accumulation in control cells as determined by a Student’s t-test (p<0.05). #Statistically different from accumulation in cells treated only with [5-³H]-dFdC (p<0.05). (B,D) The corresponding metabolite profiles including dFdC, dFdCTP, and dFdU at B) 1 h or D) 24 h in cells treated with 5 µM dFdC in the presence and absence of 100 µM dFdU and/or 100 nM NBMPR. Data represent results from two separate experiments, each performed in triplicate.
Figure 1
Figure 2