New insights into the pharmacology and cytotoxicity of gemcitabine and 2′,2′-difluorodeoxyuridine

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Abstract

In a clinical study with oral gemcitabine (2′,2′-difluorodeoxycytidine, dFdC), 2′,2′-difluorodeoxyuridine (dFdU) was extensively formed and accumulated after multiple oral dosing. Here, we have investigated the in vitro cytotoxicity, cellular uptake, efflux, biotransformation, and nucleic acid incorporation of dFdC and dFdU. Short-term and long-term cytotoxicity assays were used to assess the cytotoxicity of dFdC and dFdU in human hepatocellular carcinoma HepG2, human lung carcinoma A549, and Madin-Darby canine kidney cell lines transfected with the human concentrative or equilibrative nucleoside transporter 1 (hCNT1 or hENT1), or empty vector. Radiolabeled dFdC and dFdU were used to determine cellular uptake, efflux, biotransformation, and incorporation into DNA and RNA. The compounds dFdC, dFdU, and their phosphorylated metabolites were quantified by high-performance liquid chromatography with UV and radioisotope detection. dFdU monophosphate, diphosphate, and triphosphate (dFdU-TP) were formed from dFdC and dFdU. dFdU-TP was incorporated into DNA and RNA. The area under the intracellular concentration-time curve of dFdC-TP and dFdU-TP and their extent of incorporation into DNA and RNA inversely correlated with the IC50 of dFdC and dFdU, respectively. The cellular uptake and cytotoxicity of dFdU were significantly enhanced by hCNT1. dFdU inhibited cell cycle progression and its cytotoxicity significantly increased with longer duration of exposure. dFdU is taken up into cells with high affinity by hCNT1 and phosphorylated to its dFdU-TP metabolite. dFdU-TP is incorporated into DNA and RNA, which correlated with dFdU cytotoxicity. These data provide strong evidence that dFdU can significantly contribute to the cytotoxicity of dFdC.

Introduction

Gemcitabine (2′,2′-difluorodeoxycytidine, dFdC) has proven antitumor activity both in vitro and in vivo (1–6). dFdC is transported into cells by human nucleoside transporters (7) and is intracellularly phosphorylated by deoxycytidine kinase to its monophosphate (dFdC-MP) and subsequently phosphorylated to its active diphosphate (dFdC-DP) and triphosphate (dFdC-TP) metabolites (5). dFdC-TP competes with the natural substrate dCTP for incorporation into DNA (8), thereby inhibiting DNA synthesis and blocking cells in the early S phase (6). dFdC-DP inhibits ribonucleotide reductase (9), ultimately leading to depletion of dCTP pools and facilitating DNA incorporation of dFdC-TP. Moreover, dFdC can potentiate its own cytotoxic effect via other pathways (10, 11). Alternatively, dFdC is deaminated by cytidine deaminase (CDA, EC 3.5.4.5; ref. 10), which is highly expressed in human liver (12) to 2′,2′-difluorodeoxyuridine (dFdU), of which the activity is uncertain. The chemical structures of dFdC and dFdU and the biotransformation and mechanisms of action of dFdC are depicted in Fig. 1.

dFdC is a high-affinity substrate for the human concentrative nucleoside transporter type 1 (hCNT1; apparent Km = 12–36 μmol/L) and is transported at lower affinity by the human equilibrative nucleoside transporter type 1 (hENT1; apparent Km = 120–200 μmol/L; refs. 7, 13). It has been shown that the uptake of dFdC and its cytotoxicity significantly decreased following coadministration with nitrobenzylmercapturine riboside, an inhibitor of hENT-mediated transport (14). Expression of hCNT1 was shown to correlate with toxicity of dFdC in vitro in human pancreatic adenocarcinoma cell lines (15) and expression of hENT1 with overall survival in patients with pancreatic cancer (16). hENT1 is a ubiquitous transporter with significant variability in tissue abundance (17), whereas hCNT1 is considered to have more restricted distribution, with high expression in liver and kidney (18).

In a clinical study on continuous low-dose oral dFdC in patients with advanced solid tumors, dFdU was extensively formed via first-pass metabolism and accumulated, most likely in the liver (19). One patient treated with 8 mg of oral dFdC once daily for 14 days of a 21-day cycle experienced lethal hepatotoxicity. Pathologic examination revealed severe drug-induced liver necrosis.

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We hypothesized that dFdU might contribute to the toxicity and/or antitumor activity of dFdC and might become hepatotoxic after liver accumulation following chronic oral dFdC administration. We speculated that dFdU is phosphorylated to its triphosphate (dFdU-TP) and subsequently incorporated into DNA and/or RNA. Furthermore, our hypothesis was that dFdU is taken up by cells with high affinity by hCNT1.

The aims of this study were to investigate the cytotoxicity, effects on cell cycle distribution, cellular uptake, efflux, biotransformation, and nucleic acid incorporation of dFdC and dFdU in vitro using human hepatocellular carcinoma HepG2, human lung carcinoma A549, and Madin-Darby canine kidney (MDCK) cells transfected with either hCNT1, hENT1 or empty vector.

Materials and Methods

**Antibodies**

FITC-labeled goat anti-rabbit IgG antibody was purchased from Molecular Probes, Inc. Mouse anti-bromodeoxyuridine was purchased from Dakocytomation. Anti-mouse IgG-FITC was derived from Sigma-Aldrich Chemie GmbH.

**Reagents**

dFdC, dFdU, dFdC-MP, dFdC-DP, dFdC-TP, dFdU-MP, dFdU-DP, dFdU-TP, and CDA (activity: 2 μmol/min/mg for cytidine) were kindly provided by Eli Lilly and Company. Tetrahydrouridine was obtained from Calbiochem and nitrobenzylmercaptopurine riboside from Bio- mol Research Laboratories Inc. [3H]dFdC (21.3 Ci/mmol), [3H]thymidine (20 Ci/mmol), and [3H]uridine (16.2 Ci/mmol) were purchased from Moravek Biochemicals, Inc. [3H]dFdU was synthesized out of [3H]dFdC via enzymatic conversion by CDA. Nuclease P1 (endonuclease activity: 1,000 μmol/min/mg protein) was purchased from Roche Diagnostics GmbH and thymidine kinase 1 (TK1; activity: 2–12 μmol/min/mg protein for thymidine) was purchased from Biaffin GmbH & Co. Crystal violet and glutardialdehyde were obtained from Merck KgaA. All other chemicals were purchased from Sigma.

**Cell Culture**

HepG2, A549, and MDCK cells transfected with either hCNT1, hENT1, or empty vector (mock; ref. 20), were
cultured in RPMI 1640, DMEM, and modified Eagle's MEM, respectively. Medium was supplemented with 100 units/mL of penicillin, 100 μg/mL of streptomycin, and 10% fetal bovine serum. All cell lines were routinely tested to confirm that they were free of *Mycoplasma*. All experiments were done with exponentially growing cells. Cells were treated with drugs after a period of 12 h for the cells to attach.

**Sulforhodamine B Cytotoxicity Assay**

Cells in 200 μL/well were plated in 96-well plates and dFdC and dFdU were added in serial dilutions for 48, 72, and 96 h. Cells were washed and stained with sulforhodamine B (21, 22). Extinction was measured at 540 nm with a microplate reader (BioTek Instruments). The data were fitted to a sigmoidal concentration-response curve, and cytotoxicity was determined by calculation of the inhibitory concentration at 50% cell death (IC50) using GraphPad prism version 4.0 for Windows (GraphPad Software). Control wells (untreated) were set at 100% survival.

**Clonogenic Survival Assay**

Cells in 2 mL medium were plated in six-well plates and dFdC and dFdU were added at varying concentrations. Cells were allowed to form colonies over a maximal period of 14 days after the addition of drugs, which were subsequently fixed and stained with 0.2% crystal violet/2.5% glutaraldehyde. The number of colonies was counted with a Colcount (Oxford Optronix) and visually confirmed under a light microscope to contain at least 50 cells. Cell survival was corrected for plating efficiency.

**Cell Cycle Analysis**

HepG2 and A549 cells in 2 mL of medium were plated in six-well plates, dFdC and dFdU were added for 8, 24, 48, and 72 h and labeled with iododeoxuridine (23). In brief, nuclei were isolated and incubated with a mouse antimobromodeoxyuridine antibody, which binds to iododeoxuridine (1:50), followed by a 30-min incubation with FITC-conjugated anti-mouse antibody (1:50). Finally, propidium iodide was added to stain total DNA. Flow cytometry was carried out using a FACScan flow cytometer.

**Nucleoside and Nucleotide Extraction and Analysis**

Separation, identification, and quantification of dFdC, dFdU, dFdC-MP, dFdC-DP, dFdC-TP, dFdU-MP, dFdU-DP, and dFdU-TP were done using ion-pairing reversed phase high-performance liquid chromatography (Packard, Instrument Co., Inc.) with UV and off-line radioisotope detection. The reference compounds dFdC and dFdU were used prior to each sequence to assess system suitability. The nucleosides and nucleotides eluted at the following retention times: *t* = 6.7 min (dFdC), 10.3 min (dFdU), 14.5 min (dFdC-MP), 23 min (dFdU-MP), 34 min (dFdC-DP), 70 min (dFdU-DP), 75 min (dFdC-TP), and 81 min (dFdU-TP). In HepG2 cells, 1 mg of cellular protein corresponded to 12 × 10^6 cells with a volume of 17 fL per cell (*n* = 36) and to 13 × 10^6 cells with a volume of 14 fL per cell in A549 cells (*n* = 36). The limit of detection for each of the detected metabolites in whole cell lysate was 1.5 fmol, corresponding to 2.5 fmol/mg protein in HepG2 cells, 3.8 fmol/mg protein in A549 cells, and 15 fmol/mg protein in MDCK-hCINT1 cells. The limit of detection for each metabolite in DNA and RNA was 0.6 fmol, corresponding to 8.0 fmol/mg DNA and 2.0 fmol/mg RNA (HepG2 cells), 8.0 fmol/mg DNA and 2.7 fmol/mg RNA (A549 cells), and 24 fmol/mg DNA and 4.0 fmol/mg RNA (MDCK-hCINT1 cells).

**Synthesis of [3H]dFdU and Determination of Phosphorylation by TK1**

[3H]dFdU was synthesized from [3H]dFdC via conversion by CDA. A mixture of 1.0 mL of [3H]dFdC (1.0 mCi/mL), 80 μL of CDA (134 μg/mL), and 8.9 mL of 10 mmol/L Tris-HCl (pH 7.5) was incubated for 60 min at 37°C. The solution was snap-frozen on a dry ice/ethanol bath, freeze-dried, and the pellet was dissolved in 500 μL of eluent [10 mmol/L NaH2PO4, 2.0% methanol (pH 6.0)] and injected onto the high-performance liquid chromatograph. [3H]dFdU was identified with UV and radioisotope detection as described above. The reference compounds dFdC and dFdU were used prior to each sequence to assess system suitability. The elution times for dFdC and dFdU were 15 and 26 min, respectively. The [3H]dFdU fraction was collected, freeze-dried, and the pellet was dissolved in 1.0 mL of Milli-Q. The recovery was calculated based on the radioactivity of [3H]dFdU relative to the radioactivity of the initial [3H]dFdC solution, and purity was checked by high-performance liquid chromatography. Recovery and purity were >97% and >99%, respectively.

**Phosphorylation of [3H]dFdC, [3H]dFdU and [3H]thymidine** (positive control) was investigated by incubation with TK1 [in 50 mmol/L Tris-HCl (pH 7.6), 0.5 mmol/L MgCl2, 0.1% Tween 20, and 2.5 mmol/L ATP in 0.9% NaCl] at 37°C for 30 min, after which the mixture was placed at 100°C for 2 min, following centrifugation at 21,000 × g for 5 min and injection of the supernatant onto the high-performance liquid chromatograph.
Cellular Uptake and Biotransformation of dFdC and dFdU

Cells in 10 mL medium/well were plated in 8-cm dishes and incubated for 4, 12, and 24 h with [3H]dFdC or [3H]dFdU (40 × 10^6 dpm/well) complemented with cold dFdC and dFdU, respectively, to total concentrations of 0.5 and 5.0 μmol/L. In HepG2 cells, it was able to measure intracellular drug at the lower IC_{50} of 5 nmol/L dFdC, which was compared with the IC_{50} of 500 μmol/L dFdU. dFdC was also co-inoculated with 100 μmol/L of tetrahydrodoridine, a competitive inhibitor of CDA (25–27), to reduce deamination of dFdC to dFdU to better compare the relative toxicity of dFdC with dFdU. Experiments were stopped, medium was collected, and cells were washed thrice with 10 mL of ice-cold PBS. Subsequently, cells were scraped in 10 mL ice-cold PBS, transferred into a 15 mL tube, and centrifuged at 1,100 × g for 5 min. The supernatant was removed and cells were resuspended into 200 μL ice-cold PBS. Twenty microliters was used to determine the protein concentration. The remaining solution was mixed with methanol to a final concentration of 60% and placed at −20°C for 2 h to precipitate proteins and to extract dFdC, dFdU, and their phosphorylated metabolites. After centrifugation at 21,000 × g for 5 min, the supernatant was freeze-dried, resuspended in 240 μL of eluent A, sonificated for 5 min, and centrifuged at 21,000 × g for 5 min. The supernatant was collected for analysis of dFdC, dFdU, and phosphorylated metabolites by high-performance liquid chromatography. A comparable procedure was done for the medium.

Incorporation of dFdC-TP and dFdU-TP into DNA and RNA

Cells in 10 mL of medium were plated in 8-cm dishes and incubated for 24 h with [3H]dFdC or [3H]dFdU (100 × 10^6 dpm/dish), both complemented with cold drugs to total concentrations of 0.5 μmol/L (as well as the IC_{50} concentrations of 5 nmol/L dFdC and 500 μmol/L dFdU in HepG2 cells). After termination of the experiments, medium was discarded, and cells were washed thrice with 10 mL of ice-cold PBS. Subsequently, cells were scraped in 10 mL of ice-cold PBS, transferred into a 15 mL tube, centrifuged at 1,100 × g at 0°C for 5 min and the supernatant was discarded. From the remaining cell pellet, DNA and RNA were purified using a QIAamp DNA Mini Kit and RNAeasy Mini Kit, respectively (Qiagen, Inc.). Purity was determined by a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Inc.). After purification, 80 μL of the DNA/RNA solution (maximal 100 μg DNA/RNA) was diluted with 420 μL of 50 mmol/L ammonium acetate (pH 5.0) and digested by the addition of 6 μL of nuclease P1 (0.5 units/μL) at 60°C for 3 h. Final solutions were freeze-dried, resuspended in eluent A, centrifuged at 21,000 × g for 5 min, and the supernatant was collected for quantification of dFdC-TP and dFdU-TP and determination of the amount of incorporated dFdC-TP and dFdU-TP. The amount of DNA and RNA that was synthesized during treatment with dFdC or dFdU was assessed by coincubation of the cells with [3H]thymidine and [3H]uridine (10 × 10^6 dpm each). Two wells were treated with nonradioactive dFdC or dFdU only following incubation with [3H]thymidine or [3H]uridine for 10 min to exclude any nonspecific binding of [3H]thymidine and [3H]uridine to DNA/RNA. Total amounts of incorporated dFdC-TP and dFdU-TP were expressed as pmol/mg of DNA or RNA to compare within cell lines and in pmol drug/μmol synthesized DNA or RNA to be able to compare between cell lines, which had different DNA and RNA content.

Transport of dFdC and dFdU by hCNT1

MDCK-hCNT1 and MDCK-mock cells in 1.0 mL medium/well were plated in 24-well plates. Transport experiments were carried out in sodium-containing transport buffer [20 mmol/L Tris-HCl, 3 mmol/L K2HPO4, 1 mmol/L MgCl2, 6 mmol/L NaCl, 5 mmol/L glucose, 130 mmol/L NaCl (pH 7.4), or sodium-free transport buffer in which NaCl was replaced by 130 mmol/L of N-methyl-d-glucamine (pH 7.4)]. Cells were preincubated for 15 min at 37°C with 250 μL transport buffer in sodium-containing or sodium-free transport buffer containing 10 μmol/L of nitrobenzylmercaptopurine riboside (28). Subsequently, 250 μL of the corresponding transport buffer containing [3H]dFdC or [3H]dFdU (150,000 dpm/well) both complemented with cold dFdC and dFdU, respectively, at varying concentrations were added for 15 min at 37°C. Experiments were stopped, medium was discarded, and cells were washed thrice with 1.0 mL of ice-cold PBS. Cells were lysed in 500 μL of 1N NaOH for 30 min at 37°C, resuspended, radioactivity was counted and protein concentration was determined.

Efflux of dFdC and dFdU

HepG2 and A549 cells in 1.0 mL medium/well were plated in 24-well plates. Cells were loaded with [3H]dFdC (40 × 10^6 dpm) for 15 min at a total concentration of 15 μmol/L or with [3H]dFdU for 4 h complemented with cold drugs to 5 μmol/L. The short period of time and relatively high dFdC concentration were chosen to prevent the metabolism of dFdC, and to obtain measurable amounts of dFdC. After loading the cells, medium was replaced by fresh medium and samples were taken at t = 0, 5, 10, 30, and 60 min. Cells were washed thrice, scraped and resuspended in ice-cold PBS, centrifuged at 21,000 × g for 5 min, the supernatant was discarded, and cells were resuspended in 200 μL of ice-cold PBS. The protein concentration was determined and nucleosides and nucleotides were quantified.

In vitro Pharmacokinetic and Statistical Analysis

The cellular pharmacokinetic variables of dFdC, dFdU, dFdC-MP, dFdC-TP, dFdC-MP, dFdU-TP, dFdU-TP, and dFdU-TP were determined by noncompartmental analysis using WinNonLin (version 5.0.1, Pharsight Corporation). The area under the intracellular concentration-time curve (AUC) up to the last measured concentration-time point (AUC_{0-24}) was determined using the trapezoidal method. Furthermore, the maximal observed drug concentration (C_{max}) and the terminal half-life (t_{1/2}) were determined. Pharmacokinetic variables and IC_{50} values were reported as mean ± SD (n ≥ 3). Two-sided unpaired Student’s t tests were performed.
were applied on the log-transformed values of the IC\textsubscript{50}. Relationships between (a) the dFdC or dFdU concentration that was added to HepG2 cells and the intracellular levels of metabolites, (b) the AUC of dFdC-TP/dFdU-TP and the IC\textsubscript{50} of dFdC/dFdU, (c) the extent of incorporation into DNA/RNA of dFdC-TP/dFdU-TP and the IC\textsubscript{50} of dFdC/dFdU, and (d) the AUC of dFdC-TP/dFdU-TP and the extent of incorporation into DNA/RNA were assessed by scatter plots and Spearman correlation coefficients. Statistical analysis was done using SPSS 15.0 (SPSS, Inc.). Differences were considered to be statistically significant at \( P < 0.05 \).

**Results**

**Cytotoxicity of dFdU Is Highly Dependent on Time Period of Drug Exposure and Influenced by Expression of hCNT1**

The IC\textsubscript{50} of dFdC and dFdU decreased 13-fold and 196-fold, respectively, in HepG2 cells and 3.5-fold and 48-fold, respectively, in A549 cells when the exposure time was increased from 72 hours to 14 days (Table 1). The IC\textsubscript{50} for dFdC at 72 h was \( \sim 94,000 \)-fold, 113,000-fold, and 2,500-fold lower than for dFdU in HepG2, A549, and MDCK-hCNT1 cells, respectively. Tetrahydrouridine lowered the IC\textsubscript{50} of dFdC \( \sim 2 \)-fold in HepG2 and A549 cells (\( P = 0.005 \) for both) and did not influence dFdU cytotoxicity. The cytotoxicity at 72 hours for dFdC and dFdU was \( \sim 3 \)-fold to 4-fold higher in HepG2 compared with A549 cells (\( P = 0.005 \) for dFdC and \( P < 0.001 \) for dFdU). hENT1- and hCNT1-MDCK cells had a 3-fold higher sensitivity to dFdC compared with mock cells (\( P = 0.032 \)) without significant differences for dFdU (Table 1). IC\textsubscript{50} values at 72 hours were significantly lower for dFdC (8-fold, \( P = 0.015 \)) and dFdU (26-fold, \( P < 0.001 \)) in hCNT1-overexpressing cells compared with mock cells (Table 1). These data show that dFdC cytotoxicity is significantly affected by CDA activity and expression of hENT1 and dFdU cytotoxicity is significantly influenced by expression of hCNT1.

**dFdU-TP Is Intracellularly Formed and its Accumulation Correlates with dFdU Cytotoxicity**

As depicted in Fig. 2A and C and Table 2, dFdU, dFdC-TP, and dFdU-TP had a long \( t_{1/2} \), demonstrating the long retention of these metabolites in HepG2 cells. Coadministration of dFdC with tetrahydrouridine in HepG2 cells resulted in a 1.5-fold to 2-fold increase in intracellular dFdC, dFdC-MP, dFdC-DP, and dFdC-TP, consistent with the somewhat higher cytotoxicity of dFdC when coadministered with tetrahydrouridine. The metabolites dFdU-MP, dFdU-DP, and dFdU-TP were the only detected compounds following incubation with dFdU (Fig. 2D; Table 2). This shows that dFdU itself enters the cell and is further phosphorylated to its metabolites (Fig. 1). No dFdC-MP or dFdU-MP were detected after incubation of \( ^{[1]} \text{H} \text{dFdC} \) or \( ^{[1]} \text{H} \text{dFdU} \) with TK1, whereas \( ^{[1]} \text{H} \text{thymidine} \) was completely phosphorylated to its monophosphate form.

The nucleotides of dFdU contributed to 47%, 15%, and 8% of the total intracellular drug content following dFdU treatment in HepG2, A549, and MDCK cells, respectively, demonstrating relatively high phosphorylation of dFdU in HepG2 cells. In HepG2 cells, the AUC of dFdC-TP increased with the concentration of dFdC in the range from 5 nmol/L to 0.5 \( \mu \)mol/L to 5 \( \mu \)mol/L (\( r = 0.949, P = 0.001 \)). Intracellular drug accumulation increased up to 20 \( \mu \)mol/L of dFdC reaching a plateau at higher concentrations (data not shown). The AUC of dFdU-TP, after incubation with dFdU, increased linearly in the range from 0.5 \( \mu \)mol/L to 5 \( \mu \)mol/L to 500 \( \mu \)mol/L (\( r = 0.949, P = 0.001 \)).

In HepG2 cells, the AUC of dFdU-TP after treatment at the IC\textsubscript{50} of 500 \( \mu \)mol/L dFdU, was approximately 7-fold higher than the AUC of dFdC-TP after treatment at the IC\textsubscript{50} of 5 nmol/L dFdC (Fig. 3A and B; Table 2). The pattern of accumulation of dFdC and its metabolites was dependent on the incubation concentration of dFdC both in HepG2 and MDCK-hCNT1 cells. In HepG2 cells, for example, dFdC, dFdU, dFdC nucleotides, and dFdU nucleotides contributed 5%, 1%, 82%, and 12%, respectively, to the total recovered intracellular drug content after treatment with 5 nmol/L of dFdC, whereas these values were 1%, 0.06%, 98% and 0.94%, respectively, following incubation at 0.5 \( \mu \)mol/L dFdC. Additionally, \( C_{\text{max}} \) of

<table>
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<th>IC\textsubscript{50} (nmol/L)</th>
<th>HepG2</th>
<th>A549</th>
<th>MDCK-&lt;br&gt;hCNT1</th>
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<tr>
<td>dFdC ( t = 48 ) h</td>
<td>16 ± 0.9</td>
<td>47 ± 11</td>
<td>549</td>
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<td>dFdC ( t = 72 ) h</td>
<td>5.2 ± 0.9</td>
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<td>+ 100 ( \mu )mol/L THU</td>
<td>2.5 ± 0.6</td>
<td>9.2 ± 1.1</td>
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<tr>
<td>dFdC ( t = 96 ) h</td>
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<td>9.6 ± 0.1</td>
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<tr>
<td>dFdC ( t = 14 ) d</td>
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<td>4.5 ± 0.4</td>
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<tr>
<td>dFdU* ( t = 48 ) h</td>
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<td>3,924 ± 390</td>
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<tr>
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</tr>
<tr>
<td>dFdU* ( t = 96 ) h</td>
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<td>1,161 ± 180</td>
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<tr>
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<td>2.5 ± 0.3</td>
<td>38 ± 0.6</td>
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NOTE: Data are presented as mean ± SD (\( n \geq 3 \)). Abbreviation: THU, tetrahydrouridine. IC\textsubscript{50} values for dFdU have to be multiplied by a factor of 1,000.
Figure 2. Intracellular concentration versus time profiles of dFdC, dFdU, and phosphorylated metabolites and corresponding AUC_{0–24} values in HepG2 cells after incubation with 0.5 μmol/L of dFdC + 100 μmol/L of tetrahydrouridine (A and B) and 0.5 μmol/L of dFdU (C and D). Insert, the profiles for dFdU and its phosphorylated metabolites only (A). Effects on cell cycle distribution after 48 h are depicted for control (untreated) cells (E and I) and after treatment with dFdU at 500 μmol/L (F), 1,000 μmol/L (G), and 6,000 μmol/L (H), and dFdC at 10 nmol/L (J), 20 nmol/L (K), and 100 nmol/L (L). Cells were labeled with iododeoxuridine for determination of S phase fraction (top, positive for iododeoxuridine) and stained with propidium iodide for distinguishing G1 (bottom left) from G2-M cells (bottom right).
inverse correlations were found for the IC$_{50}$ of dFdC with protein (Mol Cancer Ther 2008;7(8). August 2008). dFdC-TP was 2.1 pmol/mg protein (Table 2). Pharmacokinetics of dFdC, dFdU, and their nucleotides and DNA and RNA incorporation in cell lines

<table>
<thead>
<tr>
<th>Abbreviations: n.a., not applicable; n.d., not detectable; THU, tetrahydrouridine.</th>
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<tr>
<td>HepG2</td>
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<td>5 nmol/L dFdC + THU</td>
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<td>C$_{max}$ (pmol/mg protein)</td>
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<td>AUC (h pmol/mg protein)</td>
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<td>DNA (pmol/µmol)</td>
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<td>0.5 µmol/L dFdU</td>
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<td>C$_{max}$ (pmol/mg protein)</td>
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<td>AUC$_{0-24}$ (h pmol/mg protein)</td>
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<td>A549</td>
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NOTE: Data are presented as mean ± SD (n ≥ 3). Abbreviations: n.a., not applicable; n.d., not detectable; THU, tetrahydrouridine.

dFdC-TP was 2.1 pmol/mg protein (~10 µmol/L) at 5 nmol/L of dFdC, which increased to 121 pmol/mg protein (~556 µmol/L) at 0.5 µmol/L of dFdC. Significant inverse correlations were found for the IC$_{50}$ of dFdC with the AUC of dFdC-TP ($r = -0.917$, $P = 0.001$) and for the IC$_{50}$ of dFdU after dFdU treatment with the AUC of dFdU-TP ($r = -0.950$, $P = 0.001$; Fig. 3D and E).

**dFdU Induces Cell Cycle Arrest**

Figure 2 depicts representative dot plots of HepG2 cells after 48 hours of treatment with dFdU of 0 to 6,000 µmol/L.
(E–H) and dFdC 0 to 100 nmol/L (I–L). Similar to dFdC, dFdU caused a concentration-dependent arrest in the early S phase. These effects were also time-dependent and similar results were found in A549 cells (data not shown).

**dFdU-TP Is Incorporated into DNA and RNA and its Incorporation Correlates with the Cytotoxicity of dFdU**

Both dFdC-TP and dFdU-TP were incorporated into nucleic acids favoring DNA over RNA (Table 2). Strong inverse correlations were found for the IC$_{50}$ of dFdC with the amount of incorporated dFdC-TP into DNA ($r = -0.856, P = 0.003$) and RNA ($r = -0.933, P = 0.001$) and for the IC$_{50}$ of dFdU following dFdU treatment with the amount of incorporated dFdU-TP into DNA ($r = -0.850, P = 0.004$) and RNA ($r = -0.910, P = 0.001$; Fig. 3F and G). As expected, significant positive correlations were found for the AUC of dFdC-TP and the extent of incorporation of dFdC-TP into DNA ($r = 0.867, P = 0.002$) and RNA ($r = 0.933, P = 0.001$) and for the AUC of dFdU-TP after treatment with dFdU with the extent of incorporation of dFdU-TP into DNA ($r = 0.942, P = 0.001$) and RNA ($r = 0.900, P = 0.001$).

In HepG2 cells treated at IC$_{50}$ concentrations of dFdC or dFdU, only a 1.3-fold difference ($P = 0.051$) was observed in DNA incorporation of dFdC-TP after dFdC treatment and DNA incorporation of dFdU-TP after dFdU treatment (Fig. 3C), suggesting equal intrinsic toxicity of dFdC-TP and dFdU-TP.

**Transport of dFdU and dFdC Is Mediated by hCNT1**

The uptake of dFdC and dFdU significantly increased with the concentration of the drugs (Fig. 4). Transport of
both dFdC and dFdU was significantly enhanced in the presence of sodium, required as cotransporter for hCNT1-mediated transport (Fig. 4A). As expected, uptake of dFdC and dFdU in MDCK-hCNT1 cells in the absence of sodium decreased to levels comparable with those in the MDCK-mock cells (Fig. 4A and B).

**dFdU and dFdC Are Effluxed**

After loading the HepG2 and A549 cells and release in drug-free medium, intracellular concentrations of dFdC rapidly decreased to 10% of the initial concentration within 10 minutes without significant formation of dFdU and phosphorylated metabolites. In HepG2 cells, the elimination pattern of dFdC was biphasic with a rapid elimination during the first 10 minutes ($t_{1/2,a} = 2.6$ minutes) followed by a slower elimination from 10 to 60 minutes ($t_{1/2,b} = 28$ minutes). Elimination of dFdU was also biphasic with an initial rapid elimination ($t_{1/2,a} = 9.2$ minutes) followed by a slower elimination ($t_{1/2,b} = 90$ minutes). Thus, dFdC and dFdU were rapidly effluxed with a slower elimination rate of dFdU compared with dFdC.

**Discussion**

This is the first study that shows the formation of dFdU-MP, dFdU-DP, and dFdU-TP from dFdC and dFdU. It shows that dFdU is a good substrate for hCNT1 and is intracellularly phosphorylated and incorporated into nucleic acids. The AUC and extent of incorporation of dFdU-TP and dFdC-TP into DNA and RNA positively correlate with the cytotoxicity of dFdU and dFdC, respectively. The $IC_{50}$ for dFdU of 2.5 μmol/L (≈ 660 ng/mL) following 14 days of incubation was of the same order of magnitude as the plasma concentrations of dFdU (≈ 700 ng/mL) in patients treated with multiple low doses of oral dFdC in our phase I study.

The $IC_{50}$ values for dFdC and dFdU in this study were somewhat higher compared with the $IC_{50}$ values at 48 hours for dFdC of 0.6 to 11 nmol/L in A2780, C26-10, and WiDr cell lines (29), and the $IC_{50}$ value at 24 hours for dFdU of 252 μmol/L in H292 cells (30). This could be due to differences in expression of uptake and efflux transporters and in metabolic enzymes. Treatment with dFdU clearly caused an arrest of cells in the early S phase, consistent with findings in ECV304 and H292 cells (31).

The relatively high amount of dFdU nucleotides in HepG2 cells treated at 5 nmol/L dFdC (corresponding with 10 μmol/L dFdC-TP) compared with the higher 0.5 μmol/L dFdC concentration (corresponding with 556 μmol/L dFdC-TP) was possibly due to less inhibition of dCDMP by dFdC-TP, leading to increased deamination of dFdC-MP. Of note, dFdC-TP concentrations of 460 μmol/L were shown to cause 50% inhibition of dCDP deaminase activity (11). The intracellular concentrations of dFdC-DP of

![Figure 4](https://mct.aacrjournals.org/)
271 µmol/L in HepG2 cells and 132 µmol/L in A549 cells likely caused maximal inhibition of ribonucleotide reductase because the IC_{50} for inhibition of ribonucleotide reductase was shown to be 0.3 µmol/L following dFdC treatment (9). The dFdC-TP concentrations of 10 pmol/10^6 cells in HepG2 cells and 5 pmol/10^6 cells in A549 cells at 4 hours of treatment with 0.5 µmol/L of dFdC were somewhat lower compared with the dFdC-TP values of 40 pmol/10^6 A2780 cells, 20 pmol/10^6 WiDr cells, and 20 pmol/10^6 C26-10 cells following 4 hours of incubation with 1.0 µmol/L of dFdC (29), consistent with the higher IC_{50} values found in our investigated solid tumor cell lines. The higher uptake of dFdC and dFdU in HepG2 compared with A549 cells might have been associated with the higher gene expression of hCNT1 in HepG2 than in A549 cells measured by quantitative-PCR (data not shown).

In patients who were treated with 8 mg of oral dFdC in our phase I study, plasma concentrations of dFdC were close to 1 ng/mL (− 4 nmol/L) and the AUC_{0–24} of dFdC-TP was 78 h pmol/mg protein in peripheral blood mononuclear cells, which was comparable to the AUC_{0–24} in HepG2 cells of 45 h pmol/mg protein after 5 nmol/L of dFdC. The AUC_{0–24} of dFdU-TP in peripheral blood mononuclear cells was 129 h pmol/mg protein and increased to 927 h pmol/mg protein following daily oral dosing for 14 days, which was 182-fold higher compared with the dFdU-TP AUC_{0–24} of 5.1 h pmol/mg protein after 5 nmol/L of dFdC and 3-fold higher than the dFdU-TP AUC_{0–24} of 309 h pmol/mg protein after 500 µmol/L of dFdC in HepG2 cells. Thus, in patients, there is a relatively high exposure to dFdU-TP in peripheral blood mononuclear cells, possibly due to differences in uptake, efflux, and biotransformation of dFdC and/or dFdU between human peripheral blood mononuclear cells and solid tumor cell lines.

Both dFdC and dFdU were effluxed from HepG2 and A549 cells, possibly by hENT1, which was found to be expressed in both cell lines in the quantitative-PCR experiments (data not shown). The presence of dFdC-MP, dFdU-MP, and dFdU-TP in the medium suggests that cells are able to efflux nucleoside phosphates. To our knowledge, only multidrug resistance proteins (MRP4 and MRP5) were shown to transport nucleoside monophosphates but with low affinity and without having significant effects on resistance to dFdC (32–34). Further studies should elucidate possible efflux transporters for these compounds. Theoretically, tetrahydrouridine might have inhibited the uptake of dFdC and dFdU, in part, by inhibition of human nucleoside transporters following simultaneous administration. However, this is unlikely based on the increase in uptake and cytotoxicity of dFdC after coinubcation with tetrahydrouridine in this study, consistent with previous findings for cytarabine plus tetrahydrouridine in hepatocytes (35).

This study showed high transport of dFdU by hCNT1, which is highly expressed in liver (18, 36), kidney (37, 38), and intestine (39, 40). This might cause the (re)uptake of dFdU into the liver of patients, leading to high exposures to dFdU and its nucleotides in the liver for prolonged periods of time. Cytotoxicity of dFdU in MDCK-hCNT1 cells largely exceeded cytotoxicity in hENT1 cells, consistent with the high transport of dFdU by hCNT1 (7, 15).

This study clearly showed the phosphorylation of dFdU to its nucleotides in all tested cell lines in contrast with previous statements (10). No phosphorylation of dFdU to dFdU-MP by cytosolic TK1 was observed, consistent with the low affinity of dFdU for TK1 (41). Possibly, dFdU is phosphorylated in part by other cytosolic enzymes and/or by TK2 in mitochondria, which warrants further investigation.

In conclusion, the unique new findings of this study are (a) the high uptake of dFdU by hCNT1, (b) the formation of dFdU-TP and dFdU-TP from dFdC and dFdU, (c) the incorporation of dFdU-TP into DNA and RNA, and (d) the significant correlations between the AUC and DNA/RNA incorporation of dFdC-TP/dFdU-TP and the cytotoxicity of dFdC and dFdU. High exposure levels to dFdU combined with significant phosphorylation to dFdU-TP and high levels of incorporation of dFdU-TP into DNA/RNA could contribute to the toxicity and/or antitumor activity of dFdC in vivo, which warrants further investigation.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


New insights into the pharmacology and cytotoxicity of gemcitabine and 2′,2′-difluorodeoxyuridine

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