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Extensive Metabolism and Hepatic Accumulation of Gemcitabine After Multiple Oral and Intravenous Administration in Mice

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ABSTRACT:

In a clinical study with oral gemcitabine (2′,2′-difluorodeoxycytidine, dFdC), we found that gemcitabine was hepatotoxic and extensively metabolized to 2′,2′-difluorodeoxyuridine (dFdU) after continuous oral dosing. The main metabolite dFdU had a long terminal half-life after oral administration. Our hypothesis was that dFdU and/or phosphorylated metabolites of gemcitabine accumulated in the liver after multiple oral dosing. In this study, mice were treated with oral or i.v. dFdC at a single dose (1qd×1d) or at multiple doses once daily for 7 days (1qd×7d) or seven times daily (7qd×1d). Blood, liver, kidneys, and lungs were collected at several time points. Urine samples were collected after i.v. dFdC, and peripheral blood mononuclear cells were collected 7qd×1d dosing of dFdC. The nucleosides dFdC and dFdU as well as the nucleotides gemcitabine monophosphate (dFdC-MP), diphosphate, and triphosphate (dFdC-TP) and dFdU monophosphate, diphosphate (dFdU-DP), and triphosphate (dFdU-TP) were simultaneously quantified by high-performance liquid chromatography with ultraviolet and radioisotope detection. We demonstrate that phosphorylated metabolites of both dFdC and dFdU are formed in mice, primarily consisting of dFdC-MP, dFdC-TP, and dFdU-TP. Multiple dosing of dFdC leads to substantial hepatic and renal accumulation of dFdC-TP and dFdU-TP, which have a more pronounced liver accumulation after oral than after i.v. dosing. The presence of dFdC-MP, dFdC-TP, and dFdU-TP in plasma and urine suggests efflux of these potentially toxic metabolites. Our results show that dFdU, dFdC-TP, and dFdU-TP accumulate in the liver after multiple dosing of dFdC in mice and might be associated with hepatotoxicity of oral dFdC in patients.

Gemcitabine (2′,2′-difluorodeoxycytidine, dFdC), a pyrimidine nucleoside anticancer drug, is used in the treatment of patients with a variety of solid tumors (Hertel et al., 1990; Noble and Goa, 1997). Transport by human nucleoside transporters enables dFdC to enter cells (Mackey et al., 1999), after which dFdC is phosphorylated by deoxycytidine kinase (dCK) to its monophosphate (dFdC-MP) and subsequently into its active diphosphate and triphosphate metabolites (Heinemann et al., 1988). Gemcitabine triphosphate (dFdC-TP) is incorporated into DNA (Huang et al., 1991), thereby competing with the natural substrate dCTP, resulting in inhibition of DNA synthesis (Grunewald et al., 1992). In addition, gemcitabine diphosphate (dFdC-DP) inhibits ribonucleotide reductase, which depletes dCTP pools and facilitates incorporation of dFdC-TP into DNA. Also, dFdC can potentiate its own cytotoxic effect via multiple mechanisms of action (Heinemann et al., 1992; Plunkett et al., 1995). Alternatively, dFdC is deaminated to 2′,2′-difluorodeoxyuridine (dFdU) by cytidine deaminase (CDA), which is highly expressed in human liver and mice kidney (Camiener and Smith, 1965).

In a clinical study, dFdC was orally administered in continuous dosing regimens at low dose levels in patients with advanced solid tumors (Veltkamp et al., 2008). The exposure to dFdC was low because of extensive first-pass metabolism to dFdU. Additionally, we found that the triphosphate form of dFdU (dFdU-TP) was formed at high exposure levels in peripheral blood mononuclear cells (PBMCs). One patient treated with 8 mg of oral dFdC once daily for 14 days of a 21-day cycle developed lethal hepatic toxicity during the second cycle. Pathological examination revealed severe drug-induced liver necrosis. Pharmacokinetic analysis demonstrated that dFdU has a long terminal half-life ($t_{1/2}$) (~89 h) and appeared to accumulate in the liver of patients. Based on these findings, we hypothesized that continuous daily oral dosing of dFdC results in liver accumulation of dFdU and/or phosphorylated metabolites in patients, possibly associated with the hepatotoxicity of dFdC. We recently found that dFdU is

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ABBREVIATIONS: dFdC, 2′,2′-difluorodeoxycytidine (gemcitabine); dCK, deoxycytidine kinase; dFdC-MP, gemcitabine monophosphate; dFdC-TP, gemcitabine triphosphate; dFdC-DP, gemcitabine diphosphate; dFdU, 2′,2′-difluorodeoxyuridine; CDA, cytidine deaminase; dFdU-TP, dFdU triphosphate; PBMC, peripheral blood mononuclear cell; hCNT1, human concentrative nucleoside transporter type 1; PK, pharmacokinetics; 1qd×1d, single dose on day 1; 1qd×7d, once daily dosing for 7 days; 7qd×1d, seven times daily dosing for 1 day; dFdU-MP, dFdU monophosphate; dFdU-DP, dFdU diphosphate; THU, tetrahydrouridine; AP, alkaline phosphatase; HPLC, high-performance liquid chromatography; AUC, area under the curve.

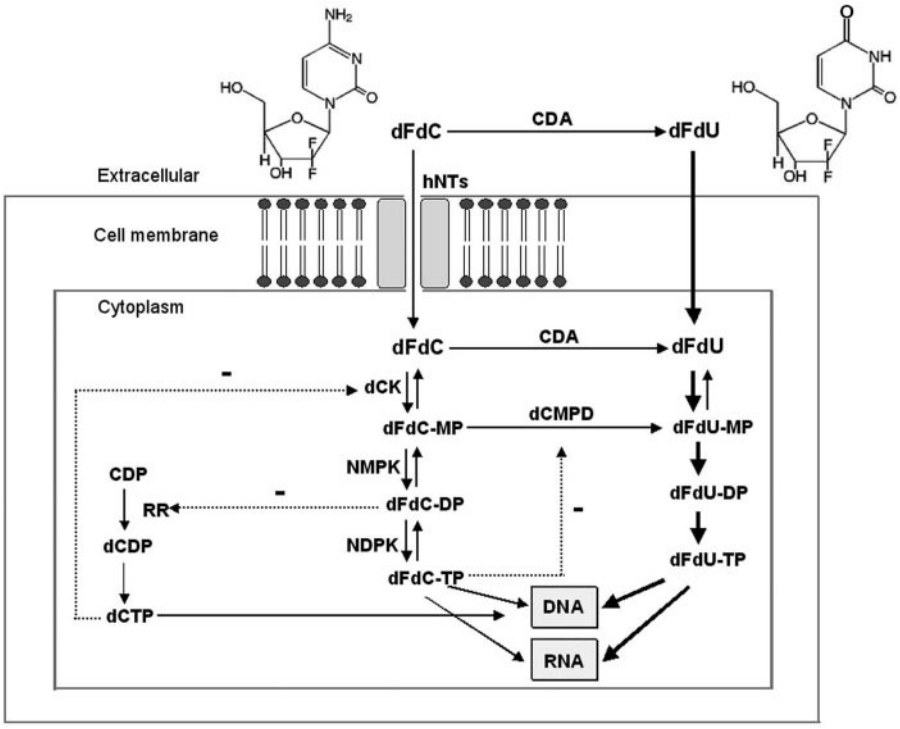

FIG. 1. Chemical structures of dFdC and dFdU and proposed routes of biotransformation and pharmacological actions of dFdC and its metabolites. dFdC is taken up by human nucleoside transporters and phosphorylated by dCK to its monophosphate (dFdC-MP), and by human nucleoside monophosphate and diphosphate kinases (NMPK and NDPK) into its diphosphate (dFdC-DP) and triphosphate (dFdC-TP) metabolites. dFdC-TP is incorporated into nucleic acids, mainly DNA, thereby competing with dCTP for incorporation. dFdC-DP inhibits ribonucleotide reductase (RR), which inhibits the conversion of CDP to dCDP and depletes dCTP pools, resulting in less inhibition of dCK and stimulation of phosphorylation of dFdC. dFdC is deaminated by CDA to dFdU. dFdC-MP is converted to dFdU-MP by deoxycytidylate deaminase (dCMPD). dFdC-TP can inhibit dCMPD, thereby decreasing deamination of dFdC-MP. dFdC, dFdC-MP, dFdC-DP, dFdC-TP, and dFdU-MP, dFdU-DP, and dFdU-TP were formed in vivo (present study). dFdU was found to be a high affinity substrate for transport by hCNT1 and was phosphorylated to dFdU-TP and incorporated into DNA and RNA in vitro in human cancer cell lines.

efficiently transported by the human concentrative nucleoside transporter type 1 (hCNT1), which is highly expressed in liver and kidney (Govindarajan et al., 2007). Additionally, we found that dFdU is phosphorylated to dFdU-TP and incorporated into nucleic acids, which correlated with the cytotoxicity of dFdU (Veltkamp et al., 2008). The chemical structures of dFdC and dFdU as well as the

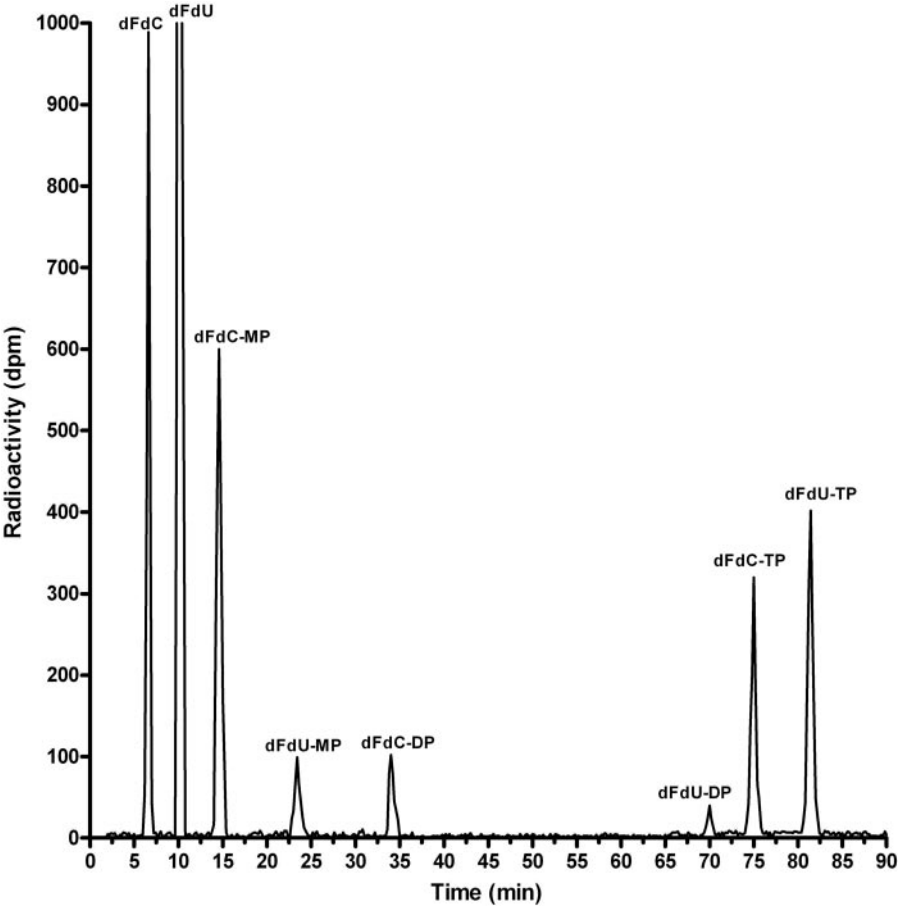

FIG. 2. Representative mouse liver chromatogram at $t = 4$ h after oral administration of 0.1 mg/kg dFdC.

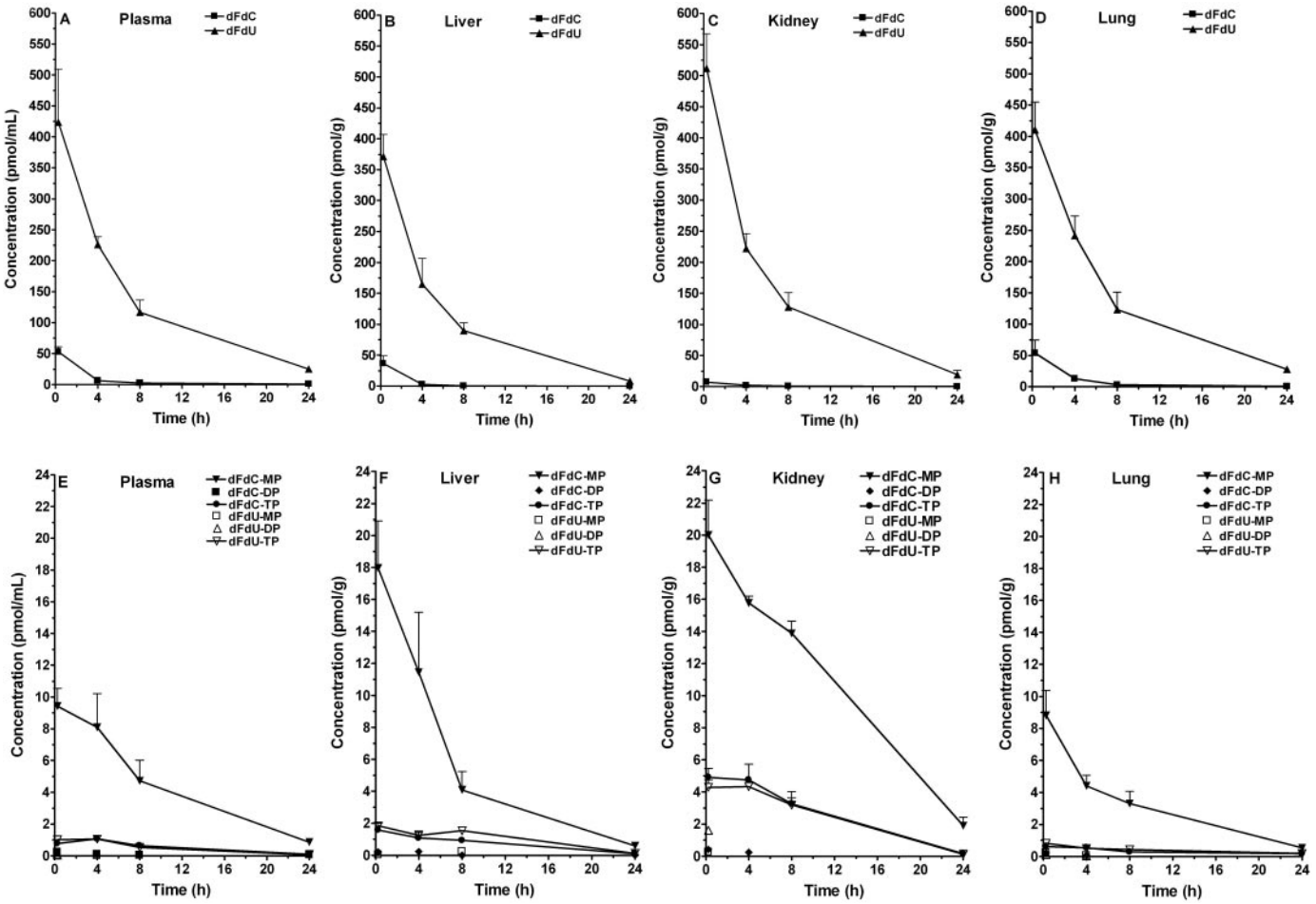

FIG. 3. Concentration versus time profiles of dFdC, dFdU, dFdC-MP, dFdC-TP, and dFdU-TP in plasma (A and E), liver (B and F), kidney (C and G), and lung (D and H) after a single oral dosing on day 1 of 1 mg/kg dFdC. Data are presented as mean ± S.D.; $n \geq 3$.

proposed routes of biotransformation and pharmacological mechanisms of action of dFdC and metabolites are depicted in Fig. 1.

Shipley et al. (1992) investigated the pharmacokinetics (PK) of dFdC in plasma after single i.v. administration at a dose of 20 mg/kg (Shipley et al., 1992). However, no phosphorylated metabolites were quantified and no drug exposures in tissues were determined. Dose-responsive intestinal lesions and hepatotoxicity were found in mice after single oral administration of dFdC at high doses of 333 to 500 mg/kg (Horton et al., 2004).

The objectives of this study were to assess the PK and metabolism of dFdC in mice and to investigate whether the drug accumulates in the liver after multiple oral and i.v. dosing of dFdC, which we could not measure in patients. Gemcitabine was administered to mice at a low dose level of 0.1 mg/kg to compare the PK to that in patients treated with low oral doses of 1 to 8 mg of dFdC. The following dosing schedules were investigated: 1) a single dose on day 1 (1qd×1d) or 2) once daily dosing for 7 days (1qd×7d), and 3) 7 times dosing on day 1 (7qd×1d). A maximal treatment period of 7 days was chosen to reduce the discomfort for the mice. To assess the PK of dFdC in mice after multiple oral dosing, the 7qd×1d (every 1.5 h) dosing regimen was compared with 1qd×7d (every 24 h) dosing, because dFdU had an approximately 15-fold higher clearance (Cl) in mice than in humans. Furthermore, excretion of dFdC and its metabolites was measured in the urine. In addition, we determined the drug exposure levels in PBMCs, which are often used as a surrogate for tumor tissue in assessment of the PK for dFdC.

Materials and Methods

Materials. The nucleosides dFdC and dFdU and the nucleotides dFdC-MP, dFdC-DP, dFdC-TP, dFdU-MP, dFdU-DP, and dFdU-TP were kindly provided by Eli Lilly and Company (Indianapolis, CA). Tetrahydrouridine (THU) was obtained from Calbiochem (La Jolla, CA). Calf intestine alkaline phosphatase (AP) (activity: 1 μmol of 4-nitrophenyl phosphate/min/unit of protein) was purchased from Roche Diagnostics GmbH (Penzberg, Germany). Adenosine triphosphate ($Na_2ATP \cdot 3H_2O$) was obtained from Boehringer Mannheim (Almere, The Netherlands). [^{3}H]dFdC (21.3 Ci/mmol) was purchased from Moravek Biochemicals Inc. (Brea, CA) and [γ^{32}P]ATP was obtained from GE Healthcare (Chalfont St. Giles, UK).

Animals. The study protocol was approved by the institutional committee for animal experiments. Mice were housed and handled according to institutional guidelines complying with Dutch legislation. Animals used in this study were female wild-type mice (FVB) between 9 and 14 weeks of age. Animals were kept in a temperature-controlled environment with a 12-h light/dark cycle, and received a standard diet (AM-II; Hope Farms, Woerden, The Netherlands) and acidified water ad libitum.

Drug Preparation, Administration, and Collection of Samples. The dFdC stock solution was diluted with 0.9% NaCl to a final concentration of 10 μg/ml. A tracer quantity of 5 μCi (~12×10^6 dpm) [^{3}H]dFdC was added to the dFdC solution. Mice received dFdC at a low dose of 0.1 mg/kg either orally by gavage into the stomach or by i.v. injection into the tail vein. Each test group consisted of at least three mice per time point. Blood samples (1 ml) were taken at $t = 0.08$, 0.25, 4, 8, and 24 h after i.v. administration and at $t = 0.25$, 4, 8, and 24 h after oral administration. Mice were anesthetized with methoxyflurane, their blood was collected by cardiac puncture, and they were sacrificed by cervical dislocation followed by collection of urine (for i.v. dFdC

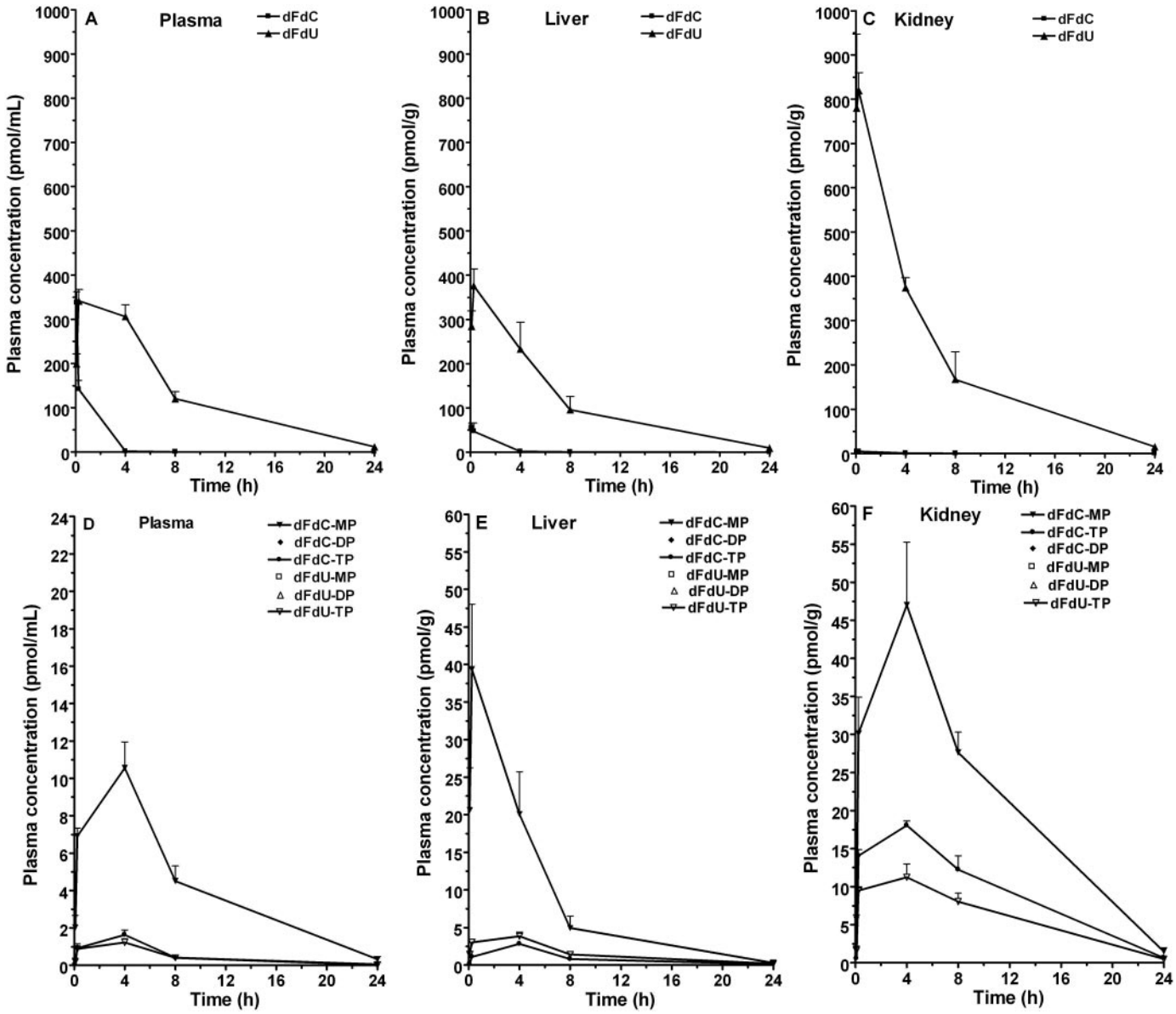

FIG. 4. Concentration versus time profiles of dFdC, dFdU, dFdC-MP, dFdC-TP, and dFdU-TP in plasma (A and D), liver (B and E), and kidney (C and F) after a single i.v. administration on day 1 of 0.1 mg/kg dFdC.1 mg/kg. Data are presented as mean ± S.D.; $n \geq 3$.

only), and liver, kidney and lungs were removed. Whole blood was immediately transferred to a 3-ml EDTA Vacutainer on ice containing 100 μg of THU (10 μl of a 10 mg/ml THU solution) to prevent any ex vivo deamination of dFdC. The plasma fraction of the blood samples was collected after centrifugation at 2000*g* at 4°C for 5 min. The buffy coat was carefully collected for isolation of PBMCs. The buffy coat was diluted with 10 ml of red blood cell lysis buffer (0.83% NH_4Cl, 0.1% $KHCO_3$, and 1 mM EDTA), and placed on ice for 20 min. The sample was centrifuged at 2000*g* at 4°C for 5 min, and washed twice with ice-cold PBS. The PBMC pellet was resuspended in 100 μl of ice-cold PBS. The cellular protein content was determined by the Bio-Rad protein assay (Bradford, 1976) and was used to correct for differences in numbers of isolated cells between samples. Cell number was determined using a Coulter counter (Beckman, Mijdrecht, The Netherlands). Plasma, PBMCs, urine, and organ samples were immediately weighted and snap-frozen in liquid nitrogen after storage at −80°C until analysis.

Nucleoside and Nucleotide Extraction, Recovery, and Analysis. Samples were thawed on ice and tissues were homogenized in 4% (m/v) bovine serum albumin (5 ml for liver and 3 ml for kidneys and lungs). Then, 600 μl of 100% MeOH was added to 400 μl of plasma, urine, and tissue homogenate, vortex-mixed, and stored at −20°C for 2 h to precipitate proteins. Then, each mixture was centrifuged at 21,000*g* for 5 min, and the supernatant was dried overnight in a SpeedVac at room temperature. The pellet was dissolved by sonication for 15 min in 500 μl of elution buffer (10 mM potassium phosphate, pH 7.0, and 10 mM tetrabutylamine). The mixture was transferred onto an OASIS column after equilibration of the column two times with 1 ml of Milli-Q, two times with 1 ml of 100% MeOH, two times with 1 ml of Milli-Q, and two times with 1 ml of elution buffer. The column was rinsed twice with 500 μl of elution buffer, and the eluate was rejected. Subsequently, nucleosides and nucleotides were eluted from the column using 500 μl of 60% MeOH, and the eluate was dried in a SpeedVac for approximately 3 h at room temperature. The dry pellet was resuspended in 100 μl of eluent A (80 mM potassium phosphate, pH 7.0, 11 mM tetrabutylamine, and 1.0% MeOH), sonicated for 5 min, and centrifuged at 21,000*g* for 5 min and the supernatant was collected for analysis.

Separation, identification, and quantification of dFdC, dFdU, dFdC-MP, dFdC-DP, dFdC-TP, dFdU-MP, dFdU-DP, and dFdU-TP were performed using ion-pairing reversed-phase high-performance liquid chromatography (HPLC) (Beckman Coulter, Fullerton, CA) with UV and off-line radioisotope detection (PerkinElmer Life and Analytical Sciences, Meriden, CT). A C18 HDO column (5 μm, 150 × 4.6 mm; Uptisphere, Interchrom, France) was used with a column temperature of 40°C, a 1:1 mixture of Ultima Flow and

TABLE 1

PK of dFdC, dFdU, and their nucleotides in plasma, liver, kidney, lung, and PBMCs after 1qd×1d, 1qd×7d, and 7qd×1d dosing of oral 0.1 mg/kg dFdC

Data are presented as means ± S.D.; $n \geq 3$.

	dFdC	dFdU	dFdC-MP	dFdC-TP	dFdU-TP
Oral 1qd×1d					
Plasma					
AUC_{0-8} (h · pmol/ml)	136 ± 20	1957 ± 255	60 ± 7	7 ± 1	7 ± 1
AUC_{0-24} (h · pmol/ml)	157 ± 36	2708 ± 827	89 ± 33	11 ± 3	10 ± 3
$t_{1/2}$ (h)	1.7 ± 0.5	5.7 ± 1.1	6.7 ± 1.2	5.7 ± 0.2	6.9 ± 1.2
Liver					
AUC_{0-8} (h · pmol/g)	63 ± 47	1562 ± 254	88 ± 12	9 ± 2	12 ± 1
AUC_{0-24} (h · pmol/g)	68 ± 46[a]**	2347 ± 333	126 ± 22	17 ± 2[a]**	25 ± 3[a]**
Kidney					
AUC_{0-8} (h · pmol/g)	25 ± 0.1	2142 ± 185	129 ± 3	35 ± 6	32 ± 2
AUC_{0-24} (h · pmol/g)	36 ± 0.1[a]**	3319 ± 426	255 ± 5[a]**	62 ± 11[a]**	58 ± 6[a]**
Lung					
AUC_{0-8} (h · pmol/g)	165 ± 51	2005 ± 247	41 ± 2	4 ± 0.1	4 ± 0.1
AUC_{0-24} (h · pmol/g)	195 ± 49[a]**	3217 ± 434	72 ± 6	8 ± 1	9 ± 0.1
Oral 1qd×7d					
Plasma					
AUC_{0-8} (h · pmol/ml)	113 ± 72	1794 ± 559	89 ± 22	6 ± 2	6 ± 2
AUC_{0-24} (h · pmol/ml)	149 ± 68	2870 ± 622	89 ± 30	12 ± 3	10 ± 2
$t_{1/2}$ (h)	1.9 ± 0.5	6.1 ± 0.7	5.9 ± 0.5	9.5 ± 2.3	7.7 ± 3.0
Liver					
AUC_{0-8} (h · pmol/g)	16 ± 3.1	1947 ± 226	145 ± 20	11 ± 2	29 ± 3
AUC_{0-24} (h · pmol/g)	22 ± 3.9	2748 ± 289	207 ± 18[b]**	21 ± 2	51 ± 6[b]**
Kidney					
AUC_{0-8} (h · pmol/g)	27 ± 3.8	2642 ± 162	282 ± 42	88 ± 14	89 ± 11
AUC_{0-24} (h · pmol/g)	38 ± 4.0	3935 ± 260	563 ± 74[b]**	154 ± 25[b]**	152 ± 25[b]**
Lung					
AUC_{0-8} (h · pmol/g)	210 ± 27	1908 ± 138	51 ± 5	4 ± 0.1	5 ± 1
AUC_{0-24} (h · pmol/g)	245 ± 27	2893 ± 180	89 ± 7	8 ± 0.1	9 ± 1
Oral 7qd×1d					
Plasma					
AUC_{0-8} (h · pmol/ml)	79 ± 22	9706 ± 410	271 ± 44	36 ± 4	36 ± 7
Liver					
AUC_{0-8} (h · pmol/g)	30 ± 4	7033 ± 366[c]***[,d]***	200 ± 4[d]***	45 ± 6[c]***[,d]***	217 ± 10[c]***[,d]***
PBMCs					
AUC_{0-8} (h · pmol/g)	58 ± 11	1202 ± 63[a]**	6.8 ± 2[a]**	16 ± 8[a]**	14 ± 7[a]**

[a]** $P < 0.01$ for the comparison of the drug AUC between tissue (liver, kidney, and lung) or PBMCs and plasma.
[b]** $P < 0.01$ for the comparison of the drug AUC within tissues (liver and kidney) between 1qd×7d and 1qd×1d dosing.
[c]*** $P < 0.001$ for the comparison of the drug AUC within the liver between 7qd×1d and 1qd×7d dosing.
[d]*** $P < 0.001$ for the comparison of the drug AUC between the liver and PBMCs after 7qd×1d dosing.

eluent, and a flow rate of 1.0 ml/min. The injection volume was 95 μl. The mobile phase consisted of a mixture of eluent A mixed with eluent B (80 mM potassium phosphate, pH 7.0, and 10% MeOH) in the following gradient: t = 0 to 10 min: 17% B; t = 10- to 65 min: 22% B; t = 65 to 85 min: 100% B, and t = 85 to 90 min; 17% B.

Three individual blank samples of liver, kidney, lung, and plasma from mice were used to determine the radioactivity for evaluation of specificity. Each sequence was preceded and ended by a system suitability test, consisting of a blank sample to confirm absence of radioactive peaks followed by a sample containing a mixture of all detected reference compounds to confirm their elution times. A quality control sample spiked with each analyte (963 ng/ml) was measured before and after each sequence and processed together with each sample batch. The linearity and recovery were determined over the complete range of analyte concentrations found in the mice samples. Samples were spiked with a mixture of [^{3}H]dFdC, [^{3}H]dFdU, and [^{32}P]ATP (as a surrogate for the phosphorylated nucleosides) at concentrations of 39, 193, and 963 ng/ml and were processed and measured on the high-performance liquid chromatograph. The inter- and intra-assay accuracy and precision were measured from these samples in 3-fold on 3 consecutive days for all analytes. The radioactivity was determined with a Tri-Carb 2800 TR liquid scintillation analyzer (PerkinElmer Life and Analytical Sciences, Boston, MA). Gemcitabine and its metabolites were identified on the basis of their retention time and quantified by their radioactivity relative to the total administered radioactivity of [^{3}H]dFdC multiplied by the total amount of drug.

Dephosphorylation of Metabolites in Liver Homogenates. Liver homogenates obtained and prepared from three mice at 4 h after the last dose of 7qd×1d oral dFdC were pooled. Previous analysis revealed the presence of dFdC, dFdU, dFdC-MP, dFdC-DP, dFdC-TP, dFdU-MP, dFdU-DP, and dFdU-TP in these liver homogenates. Four fractions of 400 μl of liver homogenate each were mixed with 600 μl of MeOH 100%, stored for 2 h at −20°C to precipitate proteins, and centrifuged at 21,000g for 5 min. The supernatants of the four fractions were transferred into a new vial and dried in a SpeedVac. The dried pellets were dissolved in eluting buffer followed by OASIS extraction and purification as described above. The pellets were again pooled after dissolving in 500 μl of a mixture containing 10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10 mM $MgCl_2$, and 1 mM $ZnCl_2$ with 2 units of AP or without AP (negative control) and incubated at 37°C for 4 h. Samples were lyophilized overnight and solubilized in 100 μl eluent A. The nucleosides and nucleotides were analyzed by HPLC with UV and radioisotope detection to confirm the identification of the phosphorylated metabolites.

Stability of Nucleosides and Nucleotides. Stability was assessed by spiking freshly obtained plasma, liver, and kidney homogenates from three blank (untreated) mice with [^{3}H]dFdC, [^{3}H]dFdU, and [^{32}P]ATP supplemented with cold drugs to a total concentration of 963 ng/ml. Extraction and analysis of dFdC, dFdU, and ATP was performed as described above. Stability was determined at t = 0, t = 1 week, and t = 1 month after storage at −80°C followed directly by OASIS solid-phase extraction and HPLC analysis as described previously.

Pharmacokinetic and Statistical Analysis. The pharmacokinetic parameters of dFdC, dFdU, dFdC-MP, dFdC-DP, dFdC-TP, dFdU-MP, dFdU-DP, and dFdU-TP were determined by noncompartmental analysis, using WinNonLin (version 5.0.1; Pharsight Corporation, Mountain View, CA). The area under the concentration-time curve (AUC) up to the last measured concentration-time point (AUC_{0-8} or AUC_{0-24}) was calculated using the trapezoidal method. Furthermore, the overall terminal half-life ($t_{1/2}$) was determined. The apparent Cl and volume of distribution (V_d) were calculated for dFdC. The apparent oral

TABLE 2

PK of dFdC, dFdU, and their nucleotides in plasma, liver, kidney, and PBMCs after 1qd×1d, 1qd×7d, and 7qd×1d i.v. dosing of dFdC 0.1 mg/kg

Data are presented as mean ± S.D. ($n \geq 3$).

	dFdC	dFdU	dFdC-MP	dFdC-TP	dFdU-TP
Intravenous 1qd×1d					
Plasma					
AUC_{0-8} (h · pmol/ml)	327 ± 36	2129 ± 48	64 ± 4	9 ± 1	7 ± 1
AUC_{0-24} (h · pmol/ml)	350 ± 39	3185 ± 85	102 ± 9	13 ± 1	11 ± 1
$t_{1/2}$ (h)	1.8 ± 0.3	4.4 ± 0.6	3.9 ± 0.6	4.5 ± 1.3	4.0 ± 0.7
Cl (l/kg/h)	1.0 ± 0.1	n.a.	n.a.	n.a.	n.a.
V_d (l/kg)	12 ± 1.6	n.a.	n.a.	n.a.	n.a.
Liver					
AUC_{0-8} (h · pmol/g)	106 ± 35	1878 ± 227	168 ± 36	15 ± 1	24 ± 2
AUC_{0-24} (h · pmol/g)	110 ± 35[a]**	2716 ± 155[a]**	210 ± 41[a]**	22 ± 1[a]**	37 ± 1[a]**
Kidney					
AUC_{0-8} (h · pmol/g)	15 ± 1.0	3520 ± 219	297 ± 28	122 ± 4	78 ± 9
AUC_{0-24} (h · pmol/g)	21 ± 1.5[a]**	4976 ± 668[a]**	530 ± 53[a]**	225 ± 18[a]**	147 ± 19[a]**
Intravenous 1qd×7d					
Plasma	dFdC	dFdU	dFdC-MP	dFdC-TP	dFdU-TP
AUC_{0-8} (h · pmol/ml)	348 ± 14	2495 ± 75	58 ± 4	9 ± 1	9 ± 1
AUC_{0-24} (h · pmol/ml)	377 ± 40	4673 ± 131	114 ± 9	16 ± 1	16 ± 1
$t_{1/2}$ (h)	2.0 ± 0.3	5.9 ± 0.5	6.1 ± 1.0	8.1 ± 1.0	6.9 ± 0.8
Liver					
AUC_{0-8} (h · pmol/g)	124 ± 32	2185 ± 32	199 ± 16	16 ± 2	29 ± 3
AUC_{0-24} (h · pmol/g)	131 ± 32	3578 ± 158[b]**	284 ± 20[b]**	30 ± 5[b]**	55 ± 2[b]**
Kidney					
AUC_{0-8} (h · pmol/g)	15 ± 2.0	3555 ± 289	336 ± 33	150 ± 16	115 ± 13
AUC_{0-24} (h · pmol/g)	27 ± 4.0	5570 ± 377	657 ± 80	284 ± 37[b]**	208 ± 21[b]**
Intravenous 7qd×1d					
Plasma					
AUC_{0-8} (h · pmol/ml)	182 ± 148	9284 ± 1018	271 ± 34	37 ± 7	34 ± 6
Liver					
AUC_{0-8} (h · pmol/g)	89 ± 53	6985 ± 143[c]***,[d]***	353 ± 279[c]***,[d]***	61 ± 11[c]***,[d]***	101 ± 15[c]***,[d]***
PBMCs					
AUC_{0-8} (h · pmol/g)	125 ± 42	2242 ± 380[a]**	5.6 ± 2.9[a]**	15 ± 2[a]**	16 ± 2[a]**

n.a., not available.
[a]** $P < 0.01$ for the comparison of the drug AUC between tissue (liver, kidney, and lung) or PBMCs and plasma.
[b]** $P < 0.01$ for the comparison of the drug AUC within tissues (liver and kidney) between 1qd×7d and 1qd×1d dosing.
[c]*** $P < 0.001$ for the comparison of the drug AUC within the liver between 7qd×1d and 1qd×7d dosing.
[d]*** $P < 0.001$ for the comparison of the drug AUC between the liver and PBMCs after 7qd×1d dosing.

bioavailability (F) of dFdC was calculated by the formula: $F = AUC_{0-24}$ p.o./AUC_{0-24} i.v. · 100%. The plasma Cl of dFdU, dFdC-MP, dFdC-TP, and dFdU-TP was estimated by the formula: $Cl = V \cdot (C_u/C_p)$ (Roland and Tozer, 1995), in which V presents the urine volume produced over 24 h, and C_u and C_p represent the mean concentration between 4 and 24 h in urine and plasma, respectively. Pharmacokinetic parameters are reported as mean ± S.D. ($n \geq 3$). Two-sided unpaired Student's t tests were applied on the log-transformed values of the PK parameters to compare the groups. Statistical analysis was performed using SPSS 15.0 (SPSS Inc., Chicago, IL). Differences were considered to be statistically significant when $p < 0.05$.

Results

Nucleosides and Nucleotides Were Simultaneously Quantified Using a Sensitive HPLC Assay with Radioisotope Detection. A representative chromatogram of dFdC, dFdU, and their phosphorylated nucleosides in the liver of mice obtained at $t = 4$ h after a single oral dose of dFdC 0.1 mg/kg is presented in Fig. 2. The detected compounds eluted at the following retention times: dFdC, $t = 6.7$ min; dFdU, $t = 10.3$ min; dFdC-MP, $t = 14.5$ min; dFdU-MP, $t = 23$ min; dFdC-DP, $t = 34$ min; dFdU-DP, $t = 70$ min; dFdC-TP, $t = 75$ min; and dFdU-TP, $t = 81$ min. The mean weight of the PBMC dry pellet was 12.3 ± 4.9 mg, corresponding to 186 ± 84 μg of PBMC protein and 1.1 ± 0.7 × 10^6 PBMCs (~5.9 × 10^6 PBMCs/mg of protein) ($n = 7$). The limit of detection for each of the metabolites was 14 fmol, corresponding to 147 fmol/ml of plasma and urine or g of tissue and PBMCs (~9.7 fmol/mg of PBMC protein or 1.6 fmol/10^6 PBMCs).

Specificity was guaranteed by the absence of radioactive peaks in the control blank liver, kidney, lung, and plasma mice samples. The inter- and intra-assay accuracy and precision for all analytes (39, 193, and 963 ng/ml) were less than 10%. Quality control samples were within 15% of the expected concentration. Reproducible recovery was demonstrated for dFdC, dFdU, and ATP at all tested concentrations in liver (83 ± 5.7, 82 ± 4.9, and 79 ± 3.4%, respectively; 193 ng/ml) with comparable results in the other tissues. Treatment of liver homogenates with AP resulted in a decrease in the concentrations of all nucleotides (e.g., 33-fold for dFdC-MP, 23-fold for dFdC-TP, and 38-fold for dFdU-TP) and led to an expected increase in concentrations of dFdC and dFdU. Good stability was demonstrated for dFdC, dFdU, and ATP in liver after 1 month of storage at −80°C (97 ± 3.0, 97 ± 2.9, and 96 ± 3.2%, respectively), with comparable results for plasma, urine, and kidneys.

Accumulation of dFdU, dFdC-MP, dFdC-TP, and dFdU-TP in Liver and Kidney after Multiple Dosing of dFdC. After oral and i.v. administration, dFdC was very rapidly cleared from plasma and organs (Figs. 3 and 4) with a rapid decline in concentration between 5 and 15 min ($t_{1/2,\ \alpha}$ = ~0.17 h) followed by a slower decline up to 4 h ($t_{1/2,\ \beta}$ = ~2 h). The concentration of dFdU in plasma and organs decreased from 0.25 to 8 h ($t_{1/2,\ \alpha}$ ~ 4 h) followed by a slower decrease up to 24 h ($t_{1/2,\ \beta}$ ~6 h) (Tables 1 and 2; Figs. 3 and 4). The highest observed concentrations of dFdC and dFdU in plasma and organs were achieved at 15 min after oral administration (Fig. 3). The time to the maximum concentration (T_{max}) could not be accurately determined on the basis of the sparse data points. The nucleotides dFdC-MP, dFdC-TP, and dFdU-TP were detected not only in the organs but also in plasma and urine. The nucleotides dFdC-DP,

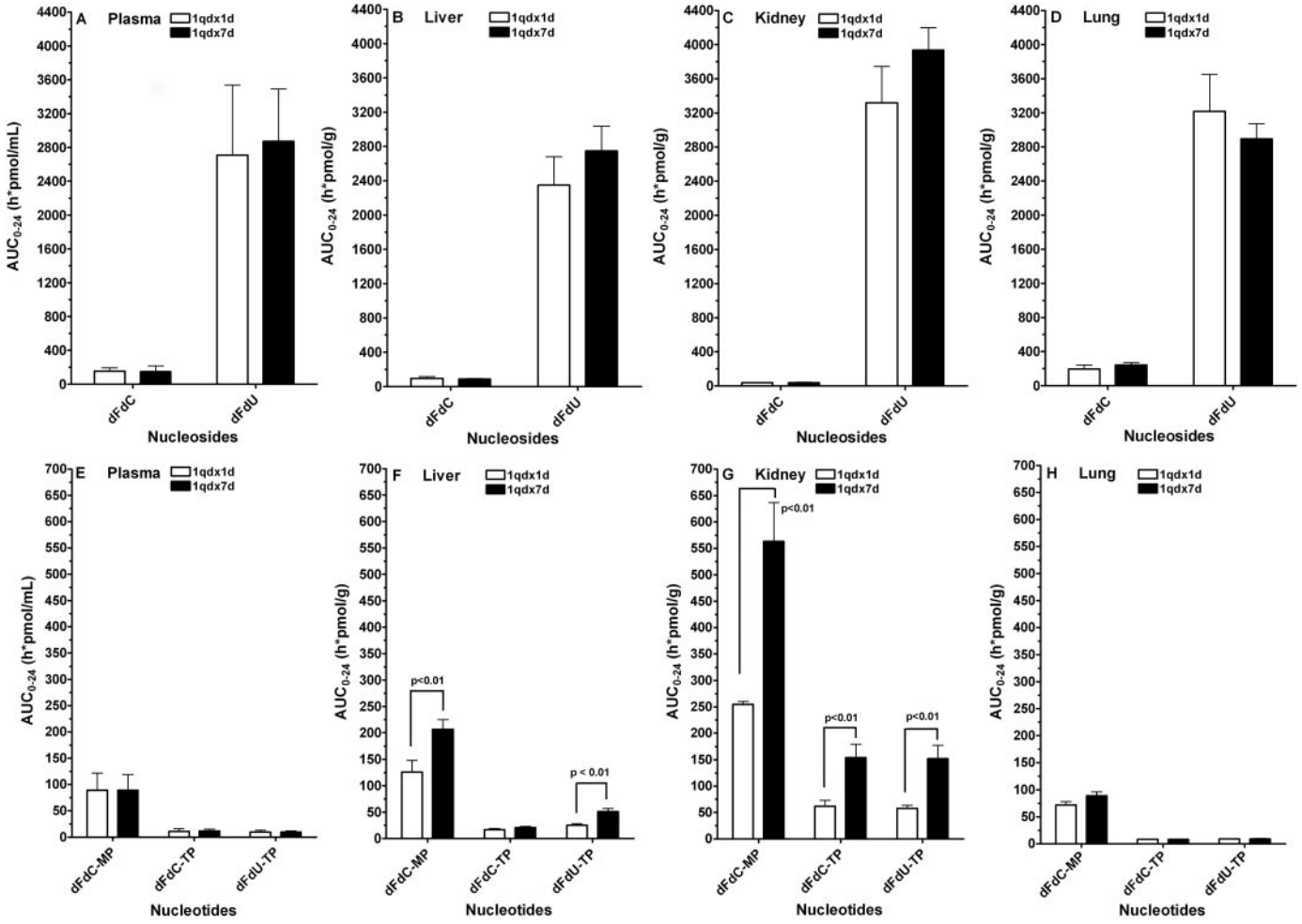

FIG. 5. Systemic exposure (AUC_{0-24}) to dFdC, dFdU, and the main phosphorylated metabolites in plasma (A and E), liver (B and F), kidney (C and G), and lung (D and H) after a single dose on day 1 and multiple daily dosing for 7 days of oral 0.1 mg/kg dFdC.1 mg/kg. Data are presented as mean ± S.D.; $n \geq 3$.

dFdU-MP, and dFdU-DP were detectable at very low concentrations at a few time points only in plasma, liver, kidney, and lung.

The mean plasma AUC_{0-24} of dFdC was 157 h · pmol/ml after a single oral dose of dFdC (Table 1) and 350 h · pmol/ml after a single i.v. administration of dFdC (Table 2), demonstrating an apparent bioavailability of approximately 45% for oral dFdC. In all organs, dFdU was the most prominent metabolite after oral and i.v. administration of dFdC (Tables 1 and 2). The ratio of dFdU AUC_{0-24}/dFdC AUC_{0-24} was approximately 2-fold higher after a single oral dose of dFdC (ratio = 17) compared with a single i.v. dose of dFdC (ratio = 9), indicating first-pass metabolism of dFdC to dFdU. The exposure values to dFdC were significantly lower in liver (2.3-fold) and kidneys (4.4-fold), whereas the exposures to the nucleoside triphosphates were significantly higher in liver (dFdC-TP, 1.5-fold; dFdU-TP, 2.5-fold) and kidneys (dFdC-TP, 5.6-fold; dFdU-TP, 5.8-fold) compared with plasma after a single oral dose of dFdC (Table 1). In lung tissue, the AUC_{0-24} of dFdC was relatively high without major differences in exposure to dFdU, dFdC-TP, and dFdU-TP compared with plasma.

Oral dFdC 1qd×7d resulted in a significant increase in liver exposure to dFdC-MP (1.6-fold) and dFdU-TP (2-fold) compared with 1qd×1d dosing. In the kidneys, the exposures to dFdC-TP and dFdU-TP increased approximately 2.5-fold after oral dFdC 1qd×7d (Table 1; Fig. 5). In contrast, no accumulation was seen in lung tissue. Multiple i.v. administration of dFdC 1qd×7d led to a lower 1.2- to 1.5-fold increase in exposure to dFdC-MP, dFdC-TP, and dFdU-TP in liver and kidneys (Table 2). The 7qd×1d dosing schedule for oral dFdC led to a significant increase in liver exposure to dFdU (3.6-fold), dFdC-TP (4.1-fold), and dFdU-TP (7.5-fold) compared with 1qd×7d oral dFdC (Table 1; Fig. 6). A similar pattern was observed after 7qd×1d compared with 1qd×7d i.v. administration of dFdC; however, the 3.5-fold increase in liver exposure to dFdU-TP (Table 2) was less pronounced than after oral dosing. The exposure to dFdC-MP was 40-fold lower in PBMCs compared with plasma after 7qd×1d oral dFdC, suggesting high efflux of dFdC-MP. Furthermore, the exposures to dFdC-MP, dFdC-TP, and dFdU-TP were 29-, 2.8-, and 16-fold significantly higher in the liver compared with PBMCs after 7qd×1d dosing of oral dFdC. Besides dFdC and dFdU, dFdC-TP, dFdU-TP, and particularly dFdC-MP also were detected in the urine (Fig. 7). Although we did not collect total urine within the first 24 h, we estimated the Cl of the main excreted metabolites after i.v. dFdC 0.1 mg/kg (see *Materials and Methods*). From previous metabolic cage experiments performed at our institute, it is known that female FVB wild-type mice between 9 and 14 weeks of age ($n = 16$) produce approximately 1.3 ± 0.5 ml of urine in 24 h (~0.903 μl/min). Estimated values for Cl of dFdU, dFdC-MP, dFdC-TP, and dFdU-TP after a single i.v. dose of dFdC were 4, 368, 833, and 521 μl/min, respectively. These values have to be interpreted with some caution as dFdC might theoretically affect the urinary output, although this was not expected after the single low dose of dFdC that was administered.

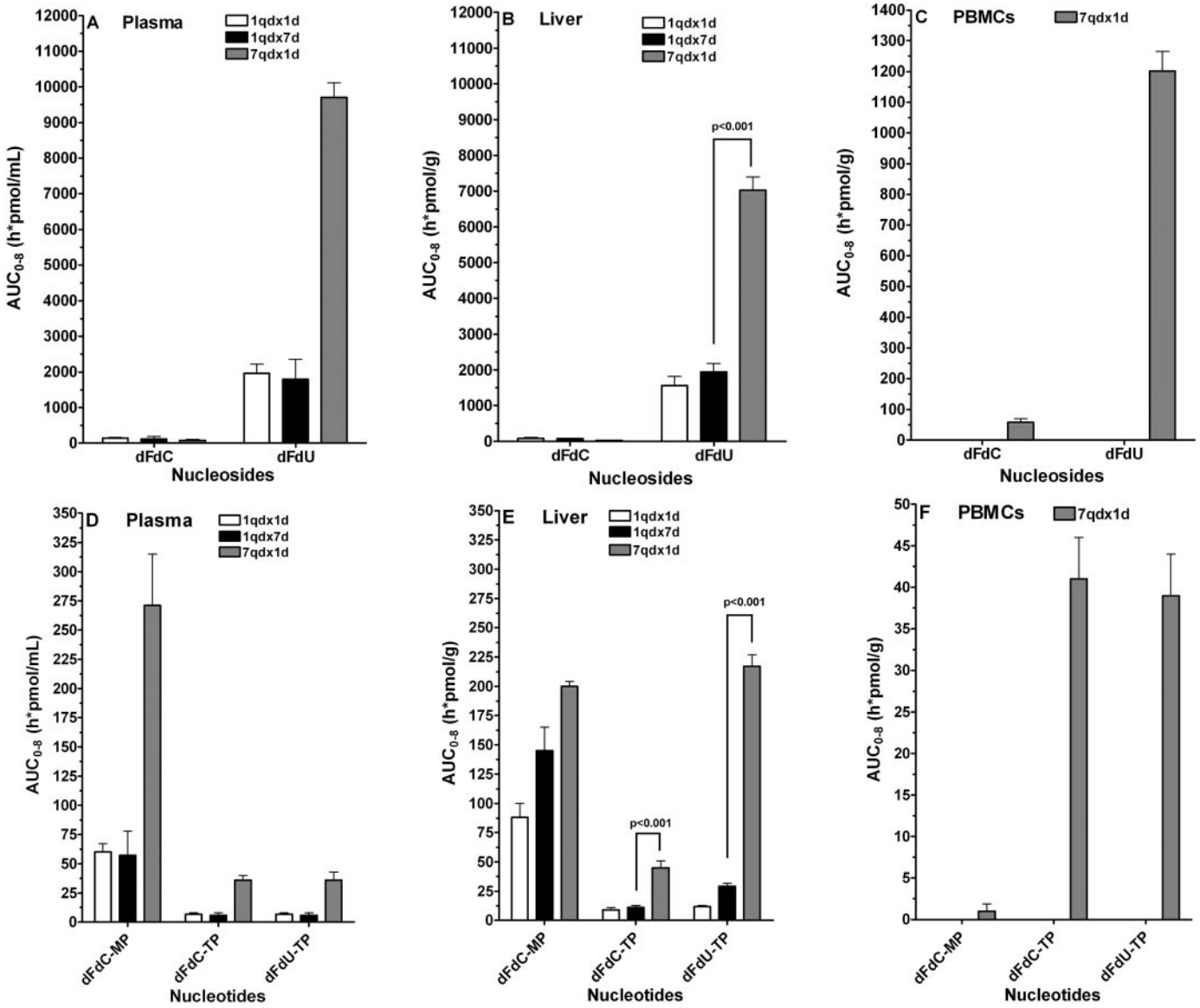

FIG. 6. Comparison of systemic exposures (AUC_{0-8}) to dFdC, dFdU, and the main phosphorylated metabolites in plasma (A and D), liver (B and E), and PBMCs (C and F) after oral administration of 0.1 mg/kg dFdC at three different dosing schedules: 1qd×1d, 1qd×7d, and 7qd×1d. Data are presented as mean ± S.D.; $n \geq 3$.

Discussion

This study provides new insights into the in vivo PK and metabolism of dFdC in mice and was initiated to better understand the biotransformation, disposition, and safety of oral dFdC in patients. We identify and quantify for the first time dFdC and its metabolites dFdU, dFdC-MP, dFdC-DP, dFdC-TP, dFdU-MP, dFdU-DP, and dFdU-TP in liver, kidney, lung, plasma, and urine. Previous studies in animal models only investigated the PK of dFdC after i.v. administration at relatively high dose levels (Shipley et al., 1992; Esumi et al., 1994; Kawai et al., 1995).

Peak concentrations of dFdC and dFdU were achieved shortly after oral administration in plasma and organs, indicating rapid absorption of dFdC from the gastrointestinal tract into the systemic circulation, rapid distribution of dFdC into tissues, and extensive deamination of dFdC to dFdU. Multiple dosing of dFdC in mice resulted in significant accumulation of dFdU, dFdC-TP, and dFdU-TP in liver and kidney. The higher accumulation of dFdU-TP in the liver after oral compared with i.v. administration could be explained by high first-pass metabolism of dFdC (i.e., deamination of dFdC and dFdC-MP) after oral administration. Furthermore, the relatively high levels of dFdU in the kidneys could be associated with the high expression levels of CDA in mice kidney (Camiener and Smith, 1965). In addition, the relatively high observed levels of dFdU in the kidneys after i.v. versus oral administration of dFdC might be associated with partial presystemic metabolism of dFdC. Although dFdU was probably formed in the liver and other organs, it might have been formed in part presystemically via deamination of dFdC in the gut wall after which dFdU could be taken up into the systemic circulation, liver, and other organs. This possibility should be investigated in more detail in future studies.

The ratio of the plasma AUC_{0-24} of dFdU over dFdC was 0.7 after a single i.v. dose of 20 mg/kg dFdC (Shipley et al., 1992). This value was 13- and 24-fold higher after i.v. and oral administration of a low dose of 0.1 mg/kg dFdC in this study, suggesting that deamination of dFdC becomes a more prominent route of metabolism at lower doses of dFdC.

The dFdU/dFdC plasma AUC ratio of 1000 in the patients who were given a single oral dose of 1 to 8 mg of dFdC was more than 50-fold higher compared with that in the mice who received a single

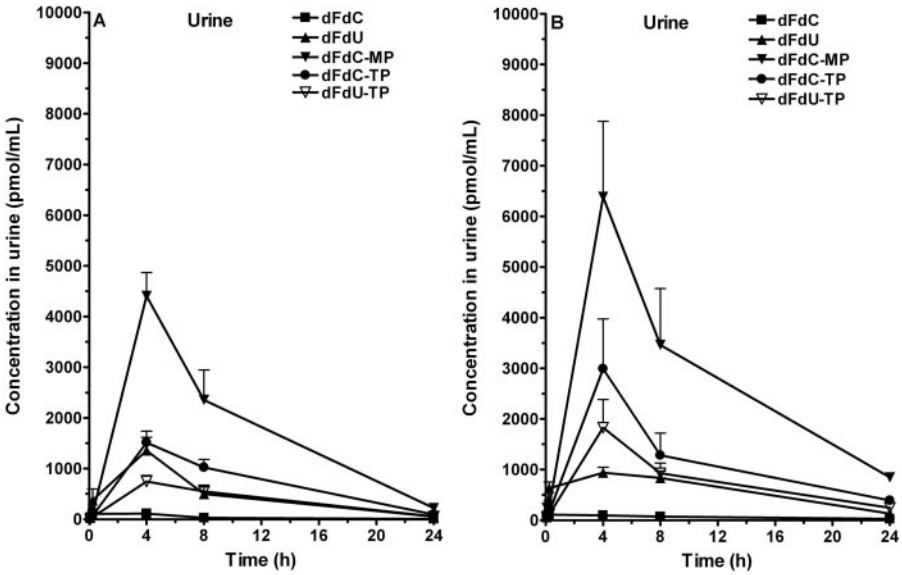

FIG. 7. Excretion of dFdC, dFdU, dFdC-MP, dFdC-TP, and dFdU-TP in urine after 1qd×1d (A) and 1qd×7d (B) administration of 0.1 mg/kg dFdC i.v. Data are presented as mean ± S.D.; $n \geq 3$.

oral dose of 0.1 mg/kg of dFdC. This is probably due to the lower Cl of dFdU and the higher extent of first-pass metabolism of dFdC in humans than in mice, consistent with the high expression of CDA in human liver and in mice kidney (Camiener and Smith, 1965).

Moreover, dFdU might be (re)absorbed by human equilibrative and concentrative nucleoside transporters, which are expressed in human liver and kidney (Gutierrez et al., 1992; Damaraju et al., 2007; Govindarajan et al., 2007), consistent with our in vitro data showing high affinity of dFdU for hCNT1. It is known that the proximal tubule of the kidney is capable of nucleoside reabsorption (Kuttesch and Nelson, 1982; Lee et al., 1988). The estimated Cl for dFdU (~4 μl/min) in this study was much lower compared with the reported creatinine Cl of 255 ± 68 μl/min (Dunn et al., 2004), which reflects glomerular filtration rate, suggesting that dFdU is actively reabsorbed from the kidneys.

The low levels of dFdC-DP, dFdU-MP, and dFdU-DP in plasma and organs suggest relatively high instability of these metabolites in vivo in mice. The presence of dFdC-MP, dFdC-TP, and dFdU-TP in the urine indicates that cells are able to eliminate these potentially toxic compounds. Thus far, it is unknown whether certain efflux transporters contribute to this phenomenon. Multidrug resistance proteins 4 and 5 were shown to transport nucleoside monophosphates, however, with low affinity and without having a significant effect on resistance to dFdC (Reid et al., 2003; Pratt et al., 2005).

The AUC_{0-8} values of dFdC-TP and dFdU-TP in PBMCs from mice were approximately 1.0 h · pmol/mg of protein (~15 h · pmol/g) after multiple oral dosing of 0.1 mg/kg dFdC 7qd×1d. In contrast, the AUC_{0-8} values of dFdC-TP and dFdU-TP in PBMCs from patients were approximately 40 h · pmol/mg protein and 470 h · pmol/mg protein after multiple oral dosing of 8 mg of dFdC once daily for 14 days. The higher exposure levels to dFdC-TP and dFdU-TP in humans might indicate a higher extent of nucleoside phosphorylation (e.g., due to higher expression of phosphorylating kinases) in human cells compared with mouse cells, as was also suggested for fludarabine (Plunkett and Gandhi, 1997).

In conclusion, this study demonstrates that dFdU-TP and dFdC-TP are extensively formed in vivo in mice after oral and i.v. administration of dFdC. Multiple administrations of dFdC resulted in a significant increase in dFdU, dFdC-TP, and dFdU-TP in liver and kidney of mice. Accumulation of dFdU-TP and dFdC-TP in liver and kidney of mice after continuous oral administration of dFdC, associated with their cytotoxic potential, might lead to hepatic and renal toxicity. Because dFdU was extensively formed after first-pass metabolism of dFdC in patients and dFdU was predicted to accumulate in the liver, multiple oral administration of dFdC might explain the more pronounced hepatotoxicity as observed in patients. Future studies should address the potential hepatotoxicity and renal toxicity and antitumor activity of dFdU-TP relative to dFdC-TP in vivo.

The differences in PK and metabolism of oral dFdC between humans and mice suggest that mice models are not optimal for assessment of the PK, safety, and efficacy of oral gemcitabine. Other animal models, such as rhesus monkeys, which have a high expression of CDA in the liver, appear to be more appropriate. Recently, different approaches have been attempted to decrease deamination of dFdC to dFdU, such as coupling a long-chain fatty acid or an isoprenoid chain of squalene to the terminal amino group of dFdC (Castelli et al., 2006; Couvreur et al., 2006), thereby protecting it from deamination by CDA. Future studies should determine whether these strategies lead to less first-pass metabolism of dFdC to dFdU in the liver and higher intracellular levels of dFdC and its active nucleotides in tumor tissue. The findings in this study are evidence for the application of continuous dosing regimens of dFdC and for the development of oral formulations of dFdC and might contribute to an improved efficacy/toxicity balance of dFdC in patients in the future.

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References

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:**248–254.

Camiener GW and Smith CG (1965) Studies of the enzymatic deamination of cytosine arabinoside. I. Enzyme distribution and species specificity. *Biochem Pharmacol* **14:**1405–1416.

Castelli F, Sarpietro MG, Ceruti M, Rocco F, and Cattel L (2006) Characterization of lipophilic gemcitabine prodrug-liposomal membrane interaction by differential scanning calorimetry. *Mol Pharm* **3:**737–744.

Couvreur P, Stella B, Reddy LH, Hillaireau H, Dubernet C, Desmaele D, Lepetre-Mouelhi S, Rocco F, Reuddre-Bosquet N, Clayette P, et al. (2006) Squalenoyl nanomedicines as potential therapeutics. *Nano Lett* **6:**2544–2548.

Damaraju VL, Elwi AN, Hunter C, Carpenter P, Santos C, Barron GM, Sun X, Baldwin SA, Young JD, Mackey JR, et al. (2007) Localization of broadly selective equilibrative and concentrative nucleoside transporters, HENT1 and HCNT3, in human kidney. *Am J Physiol Renal Physiol* **293:**F200–F211.

Dunn SR, Qi Z, Bottinger EP, Breyer MD, and Sharma K (2004) Utility of endogenous creatinine clearance as a measure of renal function in mice. *Kidney Int* **65:**1959–1967.

Esumi Y, Mitsugi K, Takao A, Seki H, and Kawai M (1994) Disposition of gemcitabine in rat and dog after single and multiple dosings. *Xenobiotica* **24:**805–817.

Govindarajan R, Bakken AH, Hudkins KL, Lai Y, Casado FJ, Pastor-Anglada M, Tse CM, Hayashi J, and Unadkat JD (2007) In situ hybridization and immunolocalization of concentrative and equilibrative nucleoside transporters in the human intestine, liver, kidneys, and placenta. *Am J Physiol Regul Integr Comp Physiol* **293:**R1809–R1822.

Grunewald R, Kantarjian H, Du M, Faucher K, Tarassoff P, and Plunkett W (1992) Gemcitabine in leukemia: a phase I clinical, plasma, and cellular pharmacology study. *J Clin Oncol* **10:**406–413.

Gutierrez MM, Brett CM, Ott RJ, Hui AC, and Giacomini KM (1992) Nucleoside transport in brush border membrane vesicles from human kidney. *Biochim Biophys Acta* **1105:**1–9.

Heinemann V, Hertel LW, Grindey GB, and Plunkett W (1988) Comparison of the cellular pharmacokinetics and toxicity of 2′,2′-difluorodeoxycytidine and 1-β-D-arabinofuranosylcytosine. *Cancer Res* **48:**4024–4031.

Heinemann V, Xu YZ, Chubb S, Sen A, Hertel LW, Grindey GB, and Plunkett W (1992) Cellular elimination of 2′,2′-difluorodeoxycytidine 5′-triphosphate: a mechanism of self-potentiation. *Cancer Res* **52:**533–539.

Hertel LW, Boder GB, Kroin JS, Rinzel SM, Poore GA, Todd GC, and Grindey GB (1990) Evaluation of the antitumor activity of gemcitabine (2′,2′-difluoro-2′-deoxycytidine). *Cancer Res* **50:**4417–4422.

Horton ND, Young JK, Perkins EJ, and Truex LL (2004) Toxicity of single-dose oral gemcitabine in mice (Abstract). *Am Assoc Cancer Res* **45:**486.

Huang P, Chubb S, Hertel LW, Grindey GB, and Plunkett W (1991) Action of 2′,2′-difluorodeoxycytidine on DNA synthesis. *Cancer Res* **51:**6110–6117.

Kawai M, Esumi Y, Ishizaki M, Gunji S, and Seki H (1995) Metabolism of gemcitabine in rat and dog. *Xenobiotica* **25:**405–416.

Kuttesch JF Jr and Nelson JA (1982) Renal handling of 2′-deoxyadenosine and adenosine in humans and mice. *Cancer Chemother Pharmacol* **8:**221–229.

Lee CW, Cheeseman CI, and Jarvis SM (1988) Na^+- and K^+-dependent uridine transport in rat renal brush-border membrane vesicles. *Biochim Biophys Acta* **942:**139–149.

Mackey JR, Yao SY, Smith KM, Karpinski E, Baldwin SA, Cass CE, and Young JD (1999) Gemcitabine transport in *Xenopus* oocytes expressing recombinant plasma membrane mammalian nucleoside transporters. *J Natl Cancer Inst* **91:**1876–1881.

Noble S and Goa KL (1997) Gemcitabine: a review of its pharmacology and clinical potential in non-small cell lung cancer and pancreatic cancer. *Drugs* **54:**447–472.

Plunkett W and GandhiV (1997) Nucleoside analogs: cellular pharmacology, mechanisms of action, and strategies for combination therapy, in *Nucleoside Analogs in Cancer Therapy* (Cheson BD, Keating MJ and Plunkett W eds) pp 1–36, Marcel Dekker, Inc., New York.

Plunkett W, Huang P, Xu YZ, Heinemann V, Grunewald R, and Gandhi V (1995) Gemcitabine: metabolism, mechanisms of action, and self-potentiation. *Semin Oncol* **22:**3–10.

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

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

Roland M and Tozer TN (1995) *Clinical Pharmacokinetics: Concepts and Applications.* Williams & Wilkins, Philadelphia.

Shipley LA, Brown TJ, Cornpropst JD, Hamilton M, Daniels WD, and Culp HW (1992) Metabolism and disposition of gemcitabine, and oncolytic deoxycytidine analog, in mice, rats, and dogs. *Drug Metab Dispos* **20:**849–855.

Veltkamp SA, Jansen RS, Callies S, Pluim D, Visseren-Grul CM, Rosing H, Kloeker-Rhoades S, Andre VAM, Beijnen JH, Slapak CH, et al. (2008). Oral administration of gemcitabine in patients with refractory tumors: a clinical and pharmacological study. *Clin Cancer Res* **14:**3477–3486.

Veltkamp SA, Pluim D, von Eijndhoven MAJ, Bolÿn M, Ong FHG, Govindarajan R, Unadkat JD, Beijnen JH, Schellens JHM (2008) New insights into the pharmacology and cytotoxicity of gemcitabine and 2′,2′-difluorodeoxyuridine. *Mol Cancer Ther*, in press.

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