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Extensive metabolism and hepatic accumulation of gemcitabine following multiple oral and intravenous administration in mice

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Running title: Hepatic accumulation of gemcitabine and metabolites

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List of abbreviations:

dFdC, 2',2'-difluorodeoxycytidine (gemcitabine); dFdU, 2',2'-difluorodeoxyuridine; dFdC-MP, gemcitabine monophosphate; dFdC-DP, gemcitabine diphosphate; dFdC-TP, gemcitabine triphosphate; dFdU-MP, dFdU monophosphate; dFdU-DP, dFdU diphosphate; dFdU-TP, dFdU triphosphate; RR, ribonucleotide reductase; dCTP, deoxycytidine triphosphate; CDA, cytidine deaminase; dCK, deoxycytidine kinase; PBMCs, peripheral blood mononuclear cells; hCNT1, human concentrative nucleoside transporter type 1.

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 intravenous (i.v.) dFdC at a single dose (1qdx1d) or at multiple doses once daily for 7 days (1qdx7d) or seven times daily (7qdx1d). Blood, liver, kidneys, and lungs were collected at several time-points. Urine samples were collected after i.v. dFdC and peripheral blood mononuclear cells after 7qdx1d dosing of dFdC. The nucleosides dFdC and dFdU as well as the nucleotides gemcitabine monophosphate (dFdC-MP), diphosphate (dFdC-DP), triphosphate (dFdC-TP), and dFdU monophosphate (dFdU-MP), 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 has 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.

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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 (hNTs) 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 deoxycytidine triphosphate (dCTP), resulting in inhibition of DNA synthesis (Grunewald et al., 1992). In addition, gemcitabine diphosphate (dFdC-DP) inhibits ribonucleotide reductase (RR), 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 (Plunkett et al., 1995;Heinemann et al., 1992). 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 at continuous dosing regimens at low dose-levels in patients with advanced solid tumors (Veltkamp et al., 2008). The exposure to dFdC was low due to 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 oral dFdC once daily for 14 days (1qdx14d) 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 hepatotoxicity of dFdC. We recently found

that dFdU is 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 (submitted). The chemical structures of dFdC and dFdU as well as the proposed routes of biotransformation and pharmacological mechanisms of action of dFdC and metabolites are depicted in Figure 1.

Shipley and colleagues 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 333-500 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 following multiple oral and i.v. dosing of dFdC, which we couldn't 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 doses of 1-8 mg oral dFdC. The following dosing schedules were investigated: 1) a single dose on day 1 (1qdx1d) or 2) once daily dosing for 7 days (1qdx7d), and 3) 7 times dosing on day 1 (7qdx1d). A treatment period of maximal 7 days was chosen to reduce the discomfort for the mice. To assess the PK of dFdC in mice following multiple oral dosing, the 7qdx1d (every 1.5 h) dosing regimen was compared with 1qdx7d (every 24 h) dosing, since 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. Additionally, we determined the drug exposure levels in PBMCs, which are often used as surrogate for tumor tissue in assessment of the PK for dFdC.

Material and methods

Materials

The nucleosides dFdC and dFdU, and the nucleotides dFdC-MP, dFdC-DP, dFdC-TP, dFdU-MP, dFdU-DP, dFdU-TP were kindly provided by Eli Lilly and Company (Indianapolis, CA, USA). Tetrahydrouridine (THU) was obtained from Calbiochem (La Jolla, CA, USA). Calf intestine alkaline phosphatase (AP) (activity: 1 µmol 4-nitrophenyl phosphate/min/unit protein) was purchased from Roche Diagnostics GmbH (Penzberg, Germany). Adenosine triphosphate ($Na_2ATP.3H_2O$) was obtained from Boehringer Mannheim (Almere, The Netherlands). [^{3}H]-dFdC (21.3 Ci/mmol) was purchased from Moravek Biochemicals Inc. (Brea, CA, USA) and [γ^{32}P]-ATP was obtained from Amersham Life Science (Amersham, 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/12-h 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 x 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 3 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 sacrified by cervical dislocation followed by collection of urine (for i.v. dFdC only), and liver, kidney and lungs were removed. Whole blood was immediately transferred to a 3 mL ethylenediaminetetraacetic acid (EDTA) vacutainer on ice containing 100 µg 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 x *g* at 4 °C for 5 min. The buffycoat was carefully collected for isolation of PBMCs. The buffycoat was diluted with 10 mL red blood cell (RBC) lysis buffer (0.83% NH_4Cl, 0.1% $KHCO_3$, 1 mM EDTA), and placed on ice for 20 min. The sample was centrifuged at 2000 x *g* at 4 °C for 5 min, and washed twice with ice cold PBS. The PBMC pellet was resuspended in 100 µL 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 number 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 following 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 100% MeOH was added to 400 µL of plasma, urine, and tissue homogenate, vortex-mixed and placed at -20 °C for 2 h to precipitate proteins. Then, each mixture was centrifuged at 21,000 x *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 elution buffer (10 mM potassium phosphate pH 7.0, 10 mM tetrabutylamine (TBA)). The mixture was transferred onto an OASIS column after equilibration of the column with 2 x 1 mL Milli-Q, 2 x 1 mL 100% MeOH, 2 x 1 mL Milli-Q, and 2 x 1 mL elution buffer. The column was rinsed twice with 500 µL elution buffer and the eluate was rejected. Subsequently, nucleosides and nucleotides were eluted from the column using 500 µL 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 eluent A (80 mM potassium phosphate pH 7.0, 11 mM TBA, and 1.0% MeOH), sonicated for 5 min, centrifuged at 21,000 x *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, Inc., CA, USA) with ultraviolet (UV) and off-line radioisotope detection (Packard, Instrument Co., Inc., CT, USA). A C18 HDO column, 5 µm, 150 x 4.6 mm (Uptisphere, Interchrom, France) was used with a column temperature of 40 °C, a 1:1 mixture of Ultima-flow and 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 = 0-10 min: 17% B; t = 10-65 min: 22% B; t = 65-85 min: 100% B, and t = 85-90 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 in order to confirm their elution times. A quality control sample spiked with each

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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 HPLC. The inter- and intra-assay accuracy and precision were measured from these samples in threefold on three consecutive days for all analytes. The radioactivity was determined with a Tri-Carb® 2800 TR Liquid Scintillation analyzer (Perkin Elmer, USA). Gemcitabine and its metabolites were identified based on 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 3 mice at 4 h after the last dose of 7qdx1d 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 liver homogenate each were mixed with 600 µL MeOH 100%, stored for 2 h at -20 °C to precipitate proteins, and centrifuged at 21,000 x *g* 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 TrisHCl pH 8.0, 100 mM NaCl, 10 mM $MgCl_2$ and 1 mM $ZnCl_2$ with 2 units 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 3 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 non-compartmental analysis, using WinNonLin™ (version 5.0.1, Pharsight Corporation, Mountain View, CA, USA). The area under the concentration-time curve (AUC) up to the last measured concentration-time point (AUC_{0-8}; 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 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 * (Cu/Cp) (Roland M and Tozer TN, 1995), in which V presents the urine volume produced over 24 h, and Cu and Cp present the mean concentration between 4 and 24 h in urine and plasma, respectively. Pharmacokinetic parameters were reported as mean ± standard deviation (SD) (n≥3). Two-sided unpaired Student 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, USA). 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 Figure 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 PBMC protein and 1.1 ± 0.7 x 10^6 PBMCs (~ 5.9 x 10^6 PBMCs/mg protein) (n = 7). The limit of detection (LOD) for each of the metabolites was 14 fmol, corresponding to 147 fmol/mL plasma and urine or g tissue and PBMCs (~ 9.7 fmol/mg 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 one month of storage at -80 °C (97 ± 3.0%, 97 ± 2.9% and 96 ± 3.2%, respectively), with comparable results plasma, urine, and kidneys.

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Accumulation of dFdU, dFdC-MP, dFdC-TP and dFdU-TP in liver and kidney after multiple dosing of dFdC

Following oral and i.v. administration, dFdC was very rapidly cleared from plasma and organs (Figure 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, Figures 3 and 4). The highest observed concentrations of dFdC and dFdU in plasma and organs were achieved at 15 min after oral administration (Figure 3). The time to the maximum concentration (T_{max}) could not be accurately determined based on the sparse data points. The nucleotides dFdC-MP, dFdC-TP, and dFdU-TP were not only detected in the organs, but also in plasma and urine. The nucleotides dFdC-DP, 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 following single i.v. administration of dFdC (Table 2), demonstrating an apparent bioavailability of about 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 about twofold higher after a single oral dose of dFdC (ratio = 17) compared to 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), while 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 to plasma following 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 to plasma.

Multiple once daily dosing of oral dFdC for seven days (1qdx7d) resulted in a significant increase in liver exposure to dFdC-MP (1.6-fold) and dFdU-TP (2-fold) compared to 1qdx1d dosing. In the kidneys, the exposures to dFdC-TP and dFdU-TP increased approximately 2.5-fold following oral dFdC 1qdx7d (Table 1, Figure 5). In contrast, no accumulation was seen in lung tissue. Multiple i.v. administration of dFdC 1qdx7d 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 7qdx1d 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 to 1qdx7d oral dFdC (Table 1, Figure 6). A similar pattern was observed following 7qdx1d compared to 1qdx7d 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 to plasma following 7qdx1d oral dFdC, suggesting high efflux of dFdC-MP. Furthermore, the exposures to dFdC-MP, dFdC-TP, and dFdU-TP were 29-fold, 2.8-fold, and 16-fold significantly higher in the liver compared to PBMCs following 7qdx1d dosing of oral dFdC. Besides dFdC and dFdU, also dFdC-TP, dFdU-TP, and particularly dFdC-MP were detected in the urine (Figure 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 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 of Cl of dFdU, dFdC-MP, dFdC-TP, and dFdU-TP after a single i.v. dose of dFdC were 4 µL/min, 368 µL/min, 833 µL/min, 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.

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. It identifies and quantifies 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 (Kawai et al., 1995;Esumi et al., 1994;Shipley et al., 1992).

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 following oral compared to i.v. administration could be explained by high first-pass metabolism of dFdC (i.e. deamination of dFdC and dFdC-MP) following 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 following i.v. versus oral administration of dFdC might be associated with partial pre-systemic metabolism of dFdC. Although dFdU was likely formed in the liver and other organs, it might have been formed in part pre-systemically via deamination of dFdC in the gut wall after which dFdU could be taken up into the systemic circulation, liver, and other organs. This 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-fold 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 administered a single oral dose of 1-8 mg dFdC was more than 50-fold higher compared to the mice who received a single oral dose of 0.1 mg/kg dFdC. This is probably due to the lower Cl of dFdU and the higher extent of first-pass metabolism of dFdC in humans compared to 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 (Damaraju et al., 2007;Govindarajan et al., 2007;Gutierrez et al., 1992), 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, Jr. and Nelson, 1982; Lee et al., 1988). The estimated Cl for dFdU (~ 4 µL/min) in this study was much lower compared to the reported creatinine Cl of 255 ± 68 µL/min (Dunn et al., 2004), which reflects glomerular filtration rate (GFR), 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 protein (MRP) 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 about 1.0 h*pmol/mg protein (~ 15 h*pmol/g) after multiple oral dosing of 0.1 mg/kg dFdC 7qdx1d. In contrast, the AUC_{0-8} values of dFdC-TP and dFdU-TP in PBMCs from patients were about 40

h*pmol/mg protein and 470 h*pmol/mg protein following multiple oral dosing of 8 mg dFdC 1qdx14d. 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 to 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 following 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 following continuous oral administration of dFdC, associated with their cytotoxic potential, might lead to hepatic- and renal toxicity. Since 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 hepato- 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 evident 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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Legends for Figures

Figure 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 hNTs and phosphorylated by deoxycytidine kinase (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 deoxycytidine triphosphate (dCTP) for incorporation. dFdC-DP inhibits ribonucleotide reductase (RR), which inhibits the conversion of cytidine diphosphate (CDP) to deoxycytidine diphosphate (dCDP) and depletes dCTP pools resulting in less inhibition of dCK and stimulation of phosphorylation of dFdC. dFdC is deaminated by cytidine deaminase (CDA) to 2',2'-difluorodeoxyuridine (dFdU). dFdC-MP is converted to difluorodeoxyuridine monophosphate (dFdU-MP) by deoxycytidylate deaminase (dCMPD). dFdC-TP can inhibit dCMPD thereby decreasing deamination of dFdC-MP.

Both dFdC, dFdC-MP, dFdC-DP, dFdC-TP, and dFdU monophosphate (dFdU-MP), diphosphate (dFdU-DP), and triphosphate (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.

Figure 2

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

Figure 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 single oral dosing on day 1 (1qdx1d) of dFdC 0.1 mg/kg. Data are presented as mean ± SD (n≥3).

Figure 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 (1qdx1d) of dFdC 0.1 mg/kg. Data are presented as mean ± SD (n≥3).

Figure 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 (1qdx1d) and multiple daily dosing for 7 days (1qdx7d) of oral dFdC 0.1 mg/kg. Data are presented as mean ± SD (n≥3).

Figure 6

Comparison of the 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) following oral administration of dFdC 0.1 mg/kg at three different dosing schedules: 1qdx1d, 1qdx7d, and 7qdx1d. Data are presented as mean ± SD (n≥3).

Figure 7

Excretion of dFdC, dFdU, dFdC-MP, dFdC-TP, and dFdU-TP in urine after 1qdx1d (A) and 1qdx7d (B) administration of i.v. dFdC 0.1 mg/kg. Data are presented as mean ± SD (n≥3).

Table 1. PK of dFdC, dFdU and their nucleotides in plasma, liver, kidney, lung, and PBMCs following 1qdx1d, 1qdx7d, and 7qdx1d dosing of oral dFdC 0.1 mg/kg. Data are presented as mean ± SD (n≥3)

Oral - 1qdx1d					
Plasma	**dFdC**	**dFdU**	**dFdC-MP**	**dFdC-TP**	**dFdU-TP**
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 - 1qdx7d					
Plasma	**dFdC**	**dFdU**	**dFdC-MP**	**dFdC-TP**	**dFdU-TP**
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**]

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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 - 7qdx1d					
Plasma	**dFdC**	**dFdU**	**dFdC-MP**	**dFdC-TP**	**dFdU-TP**
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	$7033 \pm 366^{c***}/^{d***}$	$200 \pm 4^{d***}$	$45 \pm 6^{c***}/^{d***}$	$217 \pm 10^{c***}/^{d***}$
PBMCs					
AUC_{0-8} (h*pmol/g)	58 ± 11	$1202 \pm 63^{a**}$	$6.8 \pm 2^{a**}$	$16 \pm 8^{a**}$	$14 \pm 7^{a**}$

a**, $p<0.01$ for the comparison of the drug AUC between tissue (liver, kidney, lung) or PBMCs and plasma; b**, $p<0.01$ for the comparison of the drug AUC within tissues (liver, kidney) between 1qdx7d and 1qdx1d dosing; c***, $p<0.001$ for the comparison of the drug AUC within the liver between 7qdx1d and 1qdx7d dosing; d***, $p<0.001$ for the comparison of the drug AUC between the liver and PBMCs after 7qdx1d dosing.

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Table 2. PK of dFdC, dFdU and their nucleotides in plasma, liver, kidney, and PBMCs following 1qdx1d, 1qdx7d, and 7qdx1d i.v. dosing of dFdC 0.1 mg/kg. Data are presented as mean ± SD (n≥3).

IV - 1qdx1d					
Plasma	**dFdC**	**dFdU**	**dFdC-MP**	**dFdC-TP**	**dFdU-TP**
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**]
IV - 1qdx7d					
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**]
IV - 7qdx1d					

Plasma	**dFdC**	**dFdU**	**dFdC-MP**	**dFdC-TP**	**dFdU-TP**
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**}

a**, $p<0.01$ for the comparison of the drug AUC between tissue (liver, kidney, lung) or PBMCs and plasma; b**, $p<0.01$ for the comparison of the drug AUC within tissues (liver, kidney) between 1qdx7d and 1qdx1d dosing; c***, $p<0.001$ for the comparison of the drug AUC within the liver between 7qdx1d and 1qdx7d dosing; d***, $p<0.001$ for the comparison of the drug AUC between the liver and PBMCs after 7qdx1d dosing.

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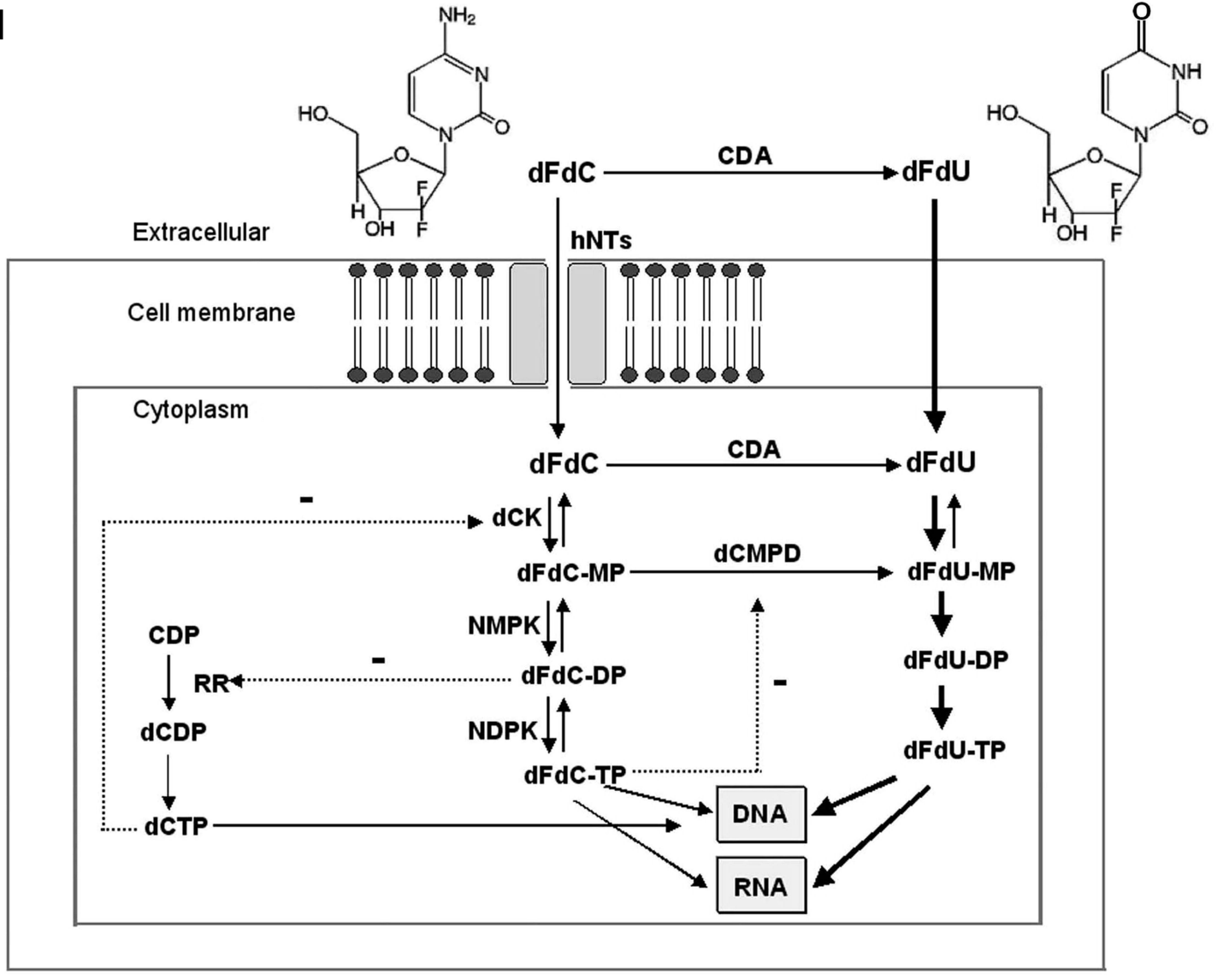
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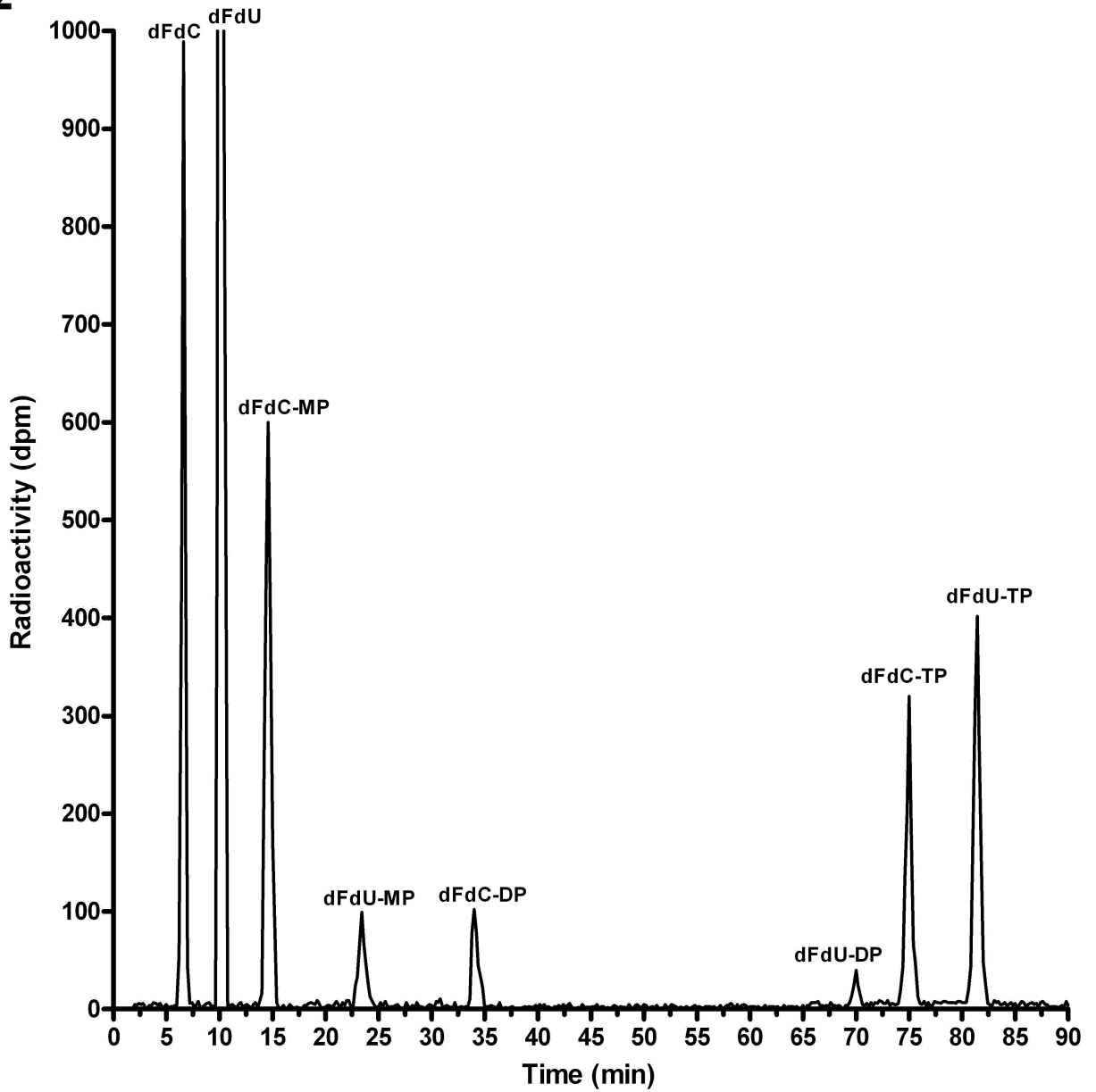
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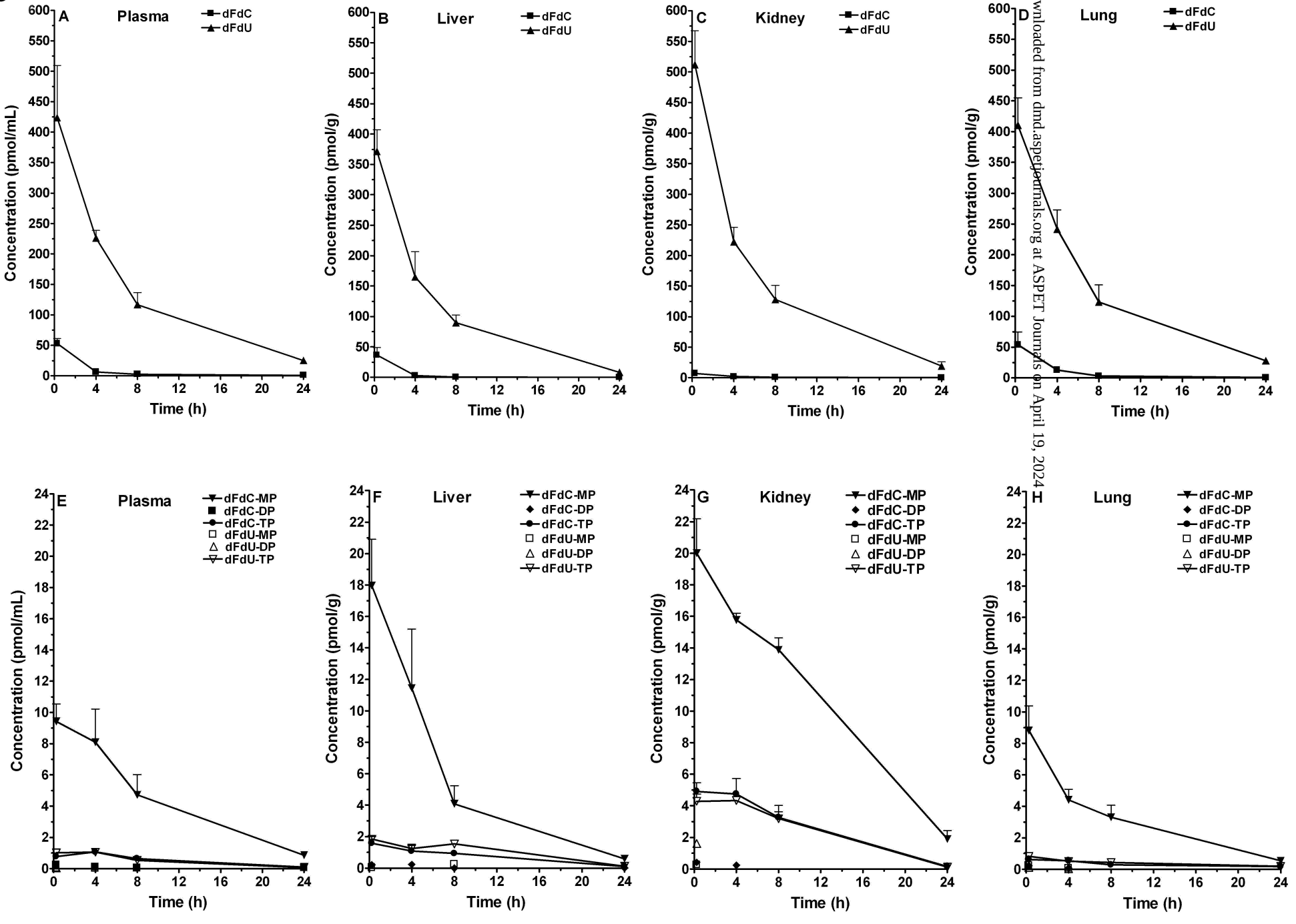

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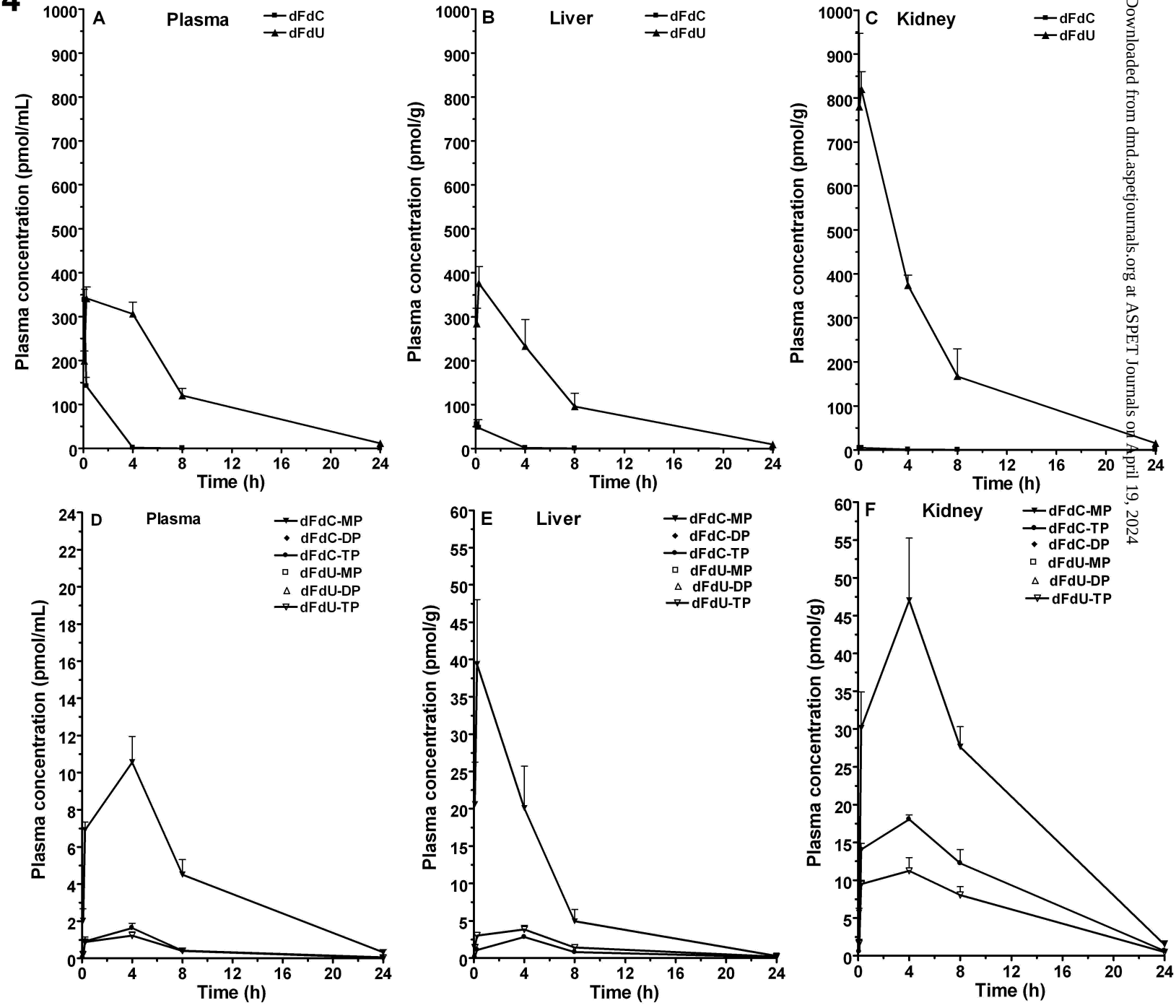

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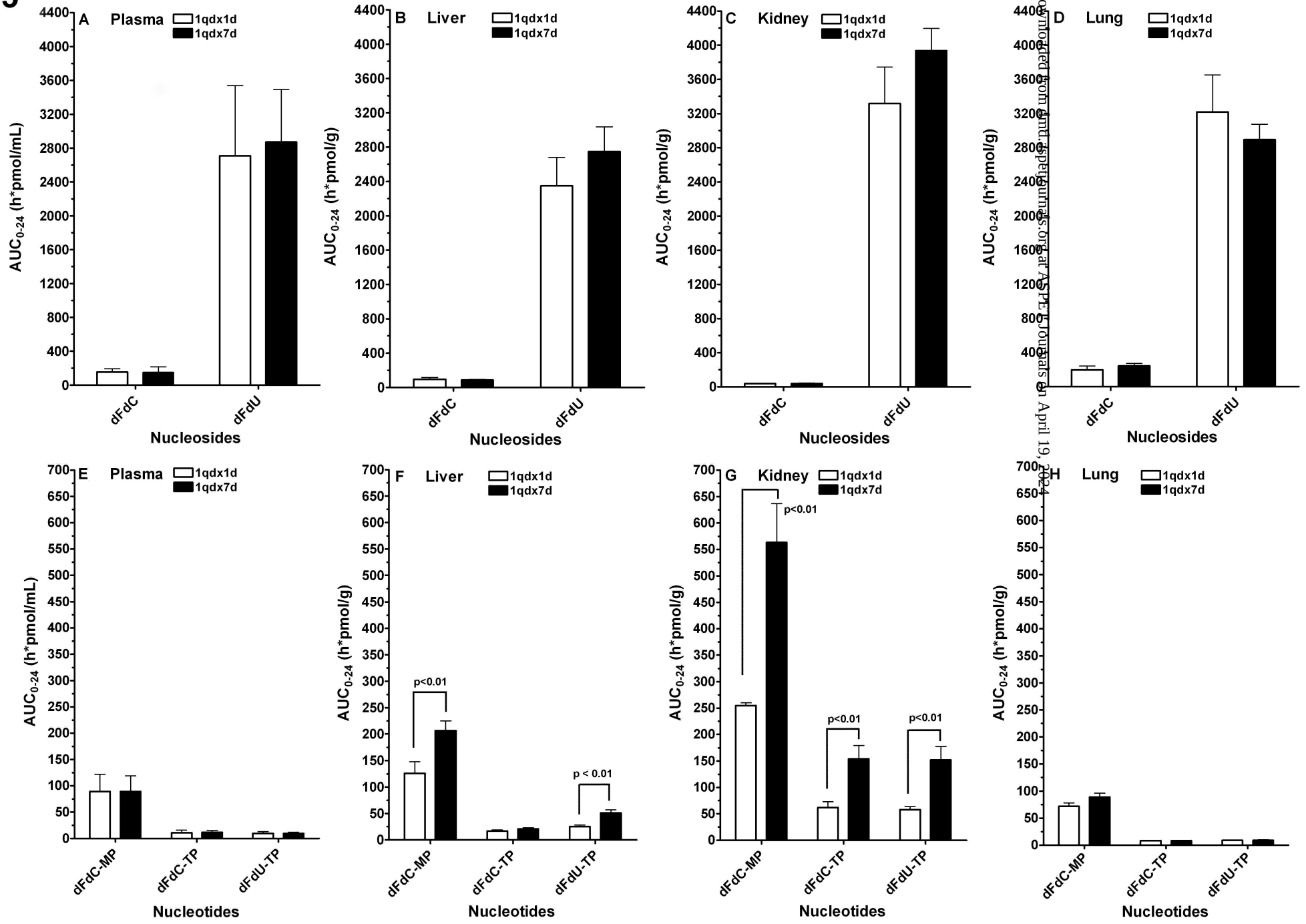

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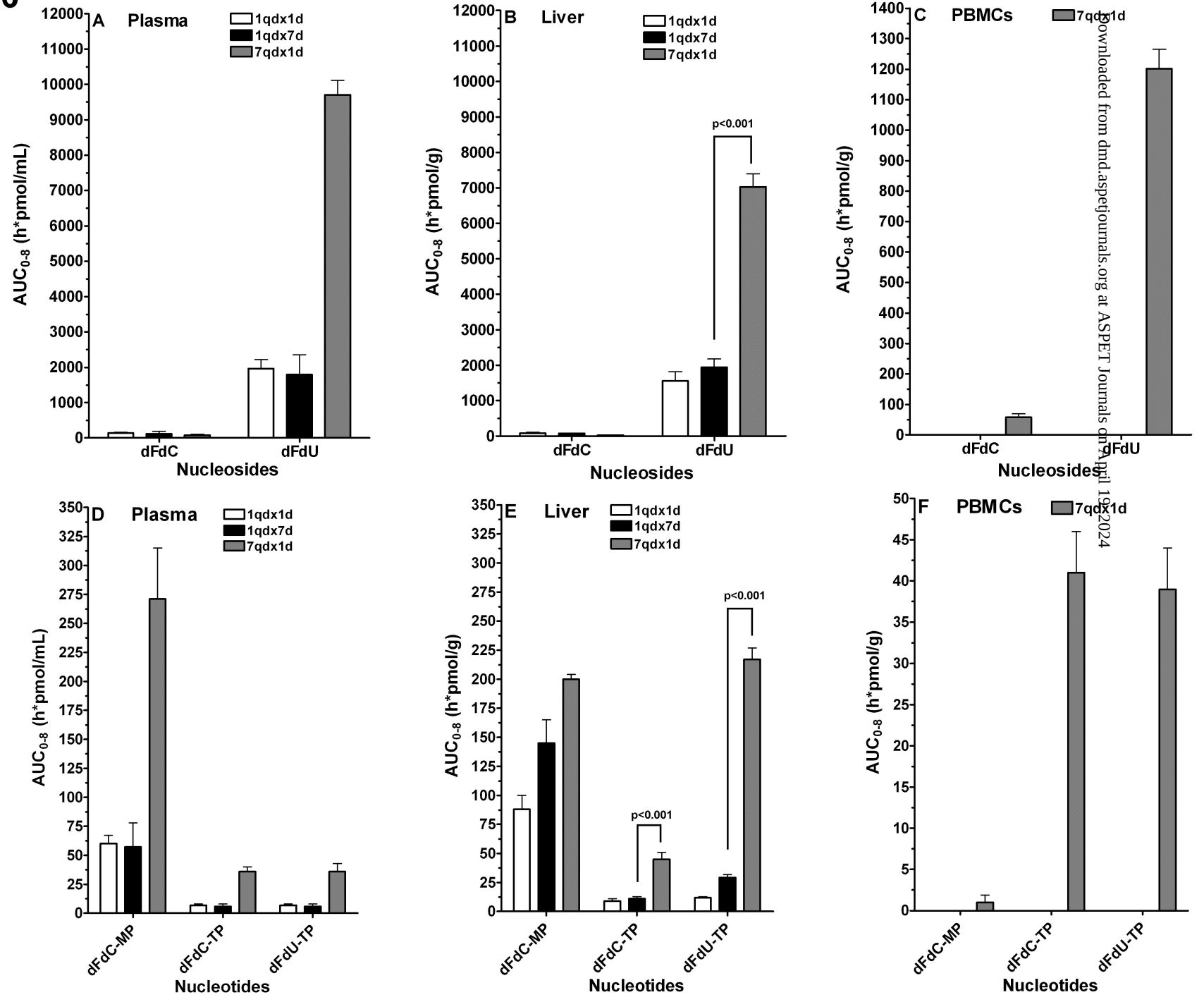

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