NT5C3 and NTPDase 1 Dephosphorylate the Pharmacologically Active Metabolites of Gemcitabine and Emtricitabine

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Abbreviations: dFdC, Gemcitabine; FTC, Emtricitabine; dCMP, Deoxycytidine monophosphate; dFdCMP, Gemcitabine monophosphate; FTC-MP, Emtricitabine monophosphate; dCTP, Deoxycytidine triphosphate; dFdCTP, Gemcitabine triphosphate; FTC-TP, Emtricitabine triphosphate; 5’-NTs, 5’-Nucleotidases; NTPDases, Nucleoside triphosphate diphosphohydrolases; LC-MS, Liquid chromatography-mass spectrometry; NT5C1A, Cytosolic
5'-nucleotidase IA; NT5C2, Cytosolic 5’-nucleotidase II; NT5C3, Cytosolic 5’-nucleotidase III; NT5C, Cytosolic 5′(3′)-deoxyribonucleotidase; NT5M, Mitochondrial 5′(3′)-deoxyribonucleotidase.

Assignment: DrugDisposition, DrugMetabolism
Abstract

Gemcitabine (dFdC) and emtricitabine (FTC) are first-line drugs that are used for the treatment of pancreatic cancer and HIV, respectively. The above drugs must undergo sequential phosphorylation to become pharmacologically active. Interindividual variability associated with the responses of the above drugs has been reported. The molecular mechanisms underlying the observed variability are yet to be elucidated. Although this could be multifactorial, nucleotidases may be involved in the dephosphorylation of drug metabolites due to their structural similarity to endogenous nucleosides. With these in mind, we performed in vitro assays using recombinant nucleotidases to assess their enzymatic activities toward the metabolites of dFdC and FTC. From the above in vitro experiments, we noticed the dephosphorylation of dFdC-monophosphate in the presence of two 5’-nucleotidases (5’-NTs), NT5C1A and NT5C3, individually. Interestingly, FTC monophosphate was dephosphorylated only in the presence of NT5C3 enzyme. Additionally, nucleoside triphosphate diphosphohydrolase 1 (NTPDase 1) exhibited enzymatic activity toward both triphosphate metabolites of dFdC and FTC. Enzyme kinetic analysis further revealed Michaelis-Menten kinetics for both NT5C3-mediated dephosphorylation of monophosphate metabolites, as well as NTPDase 1-mediated dephosphorylation of triphosphate metabolites. Immunoblotting results confirmed the presence of NT5C3 and NTPDase 1 in both pancreatic and colorectal tissue that are target sites for dFdC, and FTC treatment, respectively. Furthermore, sex-specific expression patterns of NT5C3 and NTPDase 1 were determined using mass spectrometry-based proteomics approach. Based on the above results, NT5C3 and NTPDase 1 may function in the control of the levels of dFdC and FTC metabolites.
Significance Statement

FTC and dFdC are commonly used drugs for the treatment of HIV and pancreatic cancer. To become pharmacologically active, both the above drugs must be phosphorylated. The variability in the responses of the above drugs can lead to poor clinical outcomes. Although the sources of drug metabolite concentration variability are multifactorial, it is vital to understand the role of nucleotidases in the tissue disposition of the above drug metabolites due to their structural similarities to endogenous nucleosides.
Introduction

Nucleoside analog drugs are a class of medications that work by mimicking the structure of naturally occurring nucleosides (Galmarini et al., 2001). For example, gemcitabine (dFdC) is a deoxycytidine analog drug used (as monotherapy or combination therapy) for the treatment of a range of cancers such as pancreatic, ovarian, bladder, non-small cell lung, and head and neck cancers (Manegold et al., 2000; Raguse et al., 2005; Lorusso et al., 2006; Von Hoff et al., 2013; Steinberg et al., 2020). Notably, dFdC must undergo consecutive phosphorylation to monophosphate (dFdCMP), diphosphate (dFdCDP), and finally triphosphate (dFdCTP) by kinases (Mini et al., 2006; Saiki et al., 2020). The kinases responsible for dFdC have been reported (Bergman et al., 2002). Since dFdCTP is the pharmacologically active metabolite, incorporation of it into the DNA and RNA of tumor cells leads to their death (van Haperen et al., 1993). The mode of action and cellular pharmacology of gemcitabine have been studied previously (Mini et al., 2006; Ciccolini et al., 2016; Saiki et al., 2020).

Emtricitabine (FTC), an antiretroviral drug is also a deoxycytidine analog that is used to treat HIV infection (Frampton and Perry, 2005) and hepatitis B virus infection (Gish et al., 2002). It is a nucleoside reverse transcriptase inhibitor that blocks reverse transcriptase enzyme (Bang and Scott, 2003). Similar to dFdC, the pharmacologically active form of FTC is its triphosphate metabolite, FTC-TP. FTC-TP competes with endogenous substrate 2′-deoxycytidine 5′-triphosphate to be incorporated into the HIV DNA primer strand. Due to the absence of a hydroxyl group at the 3′ position of its sugar moiety, emtricitabine triphosphate leads to chain termination when it incorporates into the HIV DNA chain (Amblard et al., 2022).
High interindividual variability among patients has been observed in dFdC and dFdCTP concentrations during dFdC treatment (Khatri et al., 2014; Ciccolini et al., 2016). Specifically, one study evaluated the concentration of dFdCTP in forty-nine patients who received 2200 mg/m² of dFdC over a 30 min intravenous infusion, and the results from the above study showed the range of dFdCTP concentration (41 µM to 373 µM) among individuals (Tempero et al., 2003). Similarly, high interindividual variability has also been observed in FTC treatments and it has been reported that a FTC-TP concentration in peripheral blood mononuclear cells were 1.3 to 6.6 pmol/10⁶ cells at 2 h after single dose of 400 mg FTC among twelve patients (Fonsart et al., 2017). The above observed variability could be multifactorial. However, due to structural similarities to endogenous nucleosides, it could be hypothesized that nucleotidases play a role in controlling the metabolites of nucleoside analog drugs.

Nucleotidases involve in nucleotide metabolism by hydrolyzing nucleotides to their corresponding nucleosides and phosphates (Yegutkin, 2008). Of note, nucleoside triphosphate diphosphohydrolases (NTPDases) and 5’-nucleotidases (5’-NTs) are two major types of nucleotidases that have been characterized (Schetinger et al., 2007). Specifically, 5’-NTs catalyze the dephosphorylation of nucleoside monophosphates that leads to the regulation of endogenous nucleotide levels (Hunsucker et al., 2005). On the other hand, NTPDases catalyze the dephosphorylation of nucleoside triphosphates (Robson et al., 2006). Further, eight different NTPDases and seven 5’-NTs have been found in mammals (Zimmermann et al., 2012). Although it is known that these nucleotidases are involved in nucleotide metabolism by dephosphorylating nucleoside mono, di, and triphosphates (Yegutkin, 2008), their role in the dephosphorylation of pharmacologically active metabolites of dFdC and FTC has yet to be determined. Thus, a
comprehensive investigation on the role of nucleotidases toward the metabolites of dFdC and FTC is required to understand the variations in tissue metabolite levels.

In this study, the enzymatic activities of 5’-NTs toward dFdCMP and FTCMP, as well as NTPDases toward dFdCTP and FTC-TP were investigated. Since the pancreas is a target site for dFdC treatments and colorectal tissue is for FTC treatment, we tested the presence of nucleotidases in mouse pancreatic and colorectal tissue using an immunoblotting approach. Mass spectrometry-based proteomics approach was used to study the expression levels of the above enzymes and potential sexual dimorphisms. Since human and mouse nucleotidases share high sequence similarities, murine tissues (C57BL/6J) were used to investigate the tissue expression of the nucleotidases. To determine the expression of nucleotidases during pancreatic cancer, we used a genetically engineered mouse model of pancreatic cancer (KPC). In conclusion, the results from this work suggest that NT5C3 and NTPDase 1 may play a role in the control of pharmacologically active metabolites of dFdC and FTC.
Materials and Methods

Chemicals and reagents

The chemicals dCMP and dCTP were purchased from MilliporeSigma (Burlington, MA, USA), whereas dFdCTP was obtained from Sierra Bioresearch (Tucson, AZ, USA). FTC-MP, dFdCMP, and FTC-TP were purchased from Toronto Research Chemicals, Inc., (North York, ON, Canada). Purified human recombinant 5'-NTs (NT5C1A, NT5C2, NT5C3, NT5C, and NT5M) and NTPDases (NTPDase 1, 3, 4, 5, 6, and 7) were obtained from OriGene (Rockville, MD, USA).

Investigation of enzymatic activities of nucleotidases toward metabolites of dFdC and FTC using in vitro incubations

The activities of 5'-NTs (NT5C1A, NT5C2, NT5C3, NT5C, and NT5M) toward dCMP, dFdCMP, and FTC-MP, as well as NTPDases (NTPDase 1, 3, 4, 5, 6, and 7) toward dCTP, dFdCTP, and FTC-TP, were evaluated through in vitro incubation assays (n=3 for each protein and substrate, separately). We did not test the nucleotidases that were not available commercially. In vitro incubations were conducted according to previously described methods, with slight modifications (Seneviratne, 2023). Specifically, in a reaction volume of 100 μL, 10 nM of human recombinant protein, 1 mM monophosphate substrate (dCMP, dFdCMP, and FTC-MP) or 0.1 mM triphosphate substrate (dCTP, dFdCTP, and FTC-TP), and 5 mM MgCl₂ were incubated in 50 mM TRIS-HCl (pH 7.5) buffer at 37°C for 30 minutes. Preliminary work was performed to optimize the substrate concentration for in vitro incubation assays. Control experiments were also performed in the absence of recombinant nucleotidases. Following
incubation, the reactions were quenched by adding 20 µL of 10% trichloroacetic acid. The amount of inorganic phosphate released was determined using a malachite green phosphate assay kit (MilliporeSigma, Burlington, MA, USA) according to the manufacturer’s instructions. The absorbance at 620 nm for each assay reaction was recorded using a microplate reader (Synergy HTX, BioTek Instruments, USA). The concentration of inorganic phosphate was ascertained from the absorbance values by using the standard curve plotted in accordance with the manufacturer's instructions. In order to examine the differences in inorganic phosphate release in presence or absence of nucleotidases, a two tailed unpaired t-test was employed. The statistical significance was denoted as follows: *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001. The statistical analyses were performed by using GraphPad Prism (version 9.5.0) software (San Diego, California, USA).

**Enzyme kinetics of nucleotidase-mediated drug dephosphorylation**

Enzyme kinetics of drug metabolite dephosphorylation mediated by NT5C3 for dCMP, dFdCMP, and FTC-MP, and NTPDase 1 for dCTP, dFdCTP, and FTC-TP was conducted as previously described with some modifications (Seneviratne, 2023). Initial experiments were conducted to ascertain the linearity of enzyme-mediated dephosphorylation of the aforementioned substrates with respect to time and enzyme concentrations. Based on the results of the above preliminary analyses, subsequent kinetic experiments were carried out for a duration of 10 min, utilizing a human recombinant protein concentration of 5 nM. The nucleotidase activity was measured at 37°C in a reaction volume of 100 µl containing 5 mM MgCl₂ and 50 mM TRIS-HCl (pH 7.5) buffer. Seven different concentrations (0.01, 0.05, 0.1, 0.5, 1, 2, and 4 mM) of each monophosphate and six different concentrations (0.01, 0.05, 0.1, 0.5, 1, and 2 mM)
of each triphosphate were tested in triplicate. Following the incubations, reactions were terminated by adding 20 μL of 10% trichloroacetic acid followed by incubation on ice for 5 minutes. A malachite green phosphate assay kit (MilliporeSigma, Burlington, MA, USA) was used to measure the release of inorganic phosphate. The kinetic parameters, including $K_m$ and $V_{\text{max}}$, were calculated using GraphPad Prism (GraphPad Software V. 9.5.0, San Diego, CA, USA).

**KPC cell culture conditions**

KPC cell line was kindly provided by Dr. Lei Zheng (Department of Oncology, Johns Hopkins University). Cells were cultured in Dulbecco's modified Eagle medium (DMEM) medium [4.5 g/L glucose, L-glutamine, and sodium pyruvate (Corning)] supplemented with 10% (v/v) fetal bovine serum (Gibco) and 1,000 U/mL penicillin-streptomycin (Gibco), and maintained at 37°C and 5% CO$_2$.

**Animal and human tissues for protein analysis**

All animal experiments were carried out in accordance with Johns Hopkins Medicine Institutional Review Board guidelines and regulations. C57BL/6J mice were obtained from Jackson Laboratories and given water and autoclaved feed (Teklad Global 2018S) ad libitum. Euthanasia was performed using isoflurane anesthesia followed by cervical dislocation.

The KPC (KrasLSL-G12D/+; Trp53LSL-R172H/+; Pdx-1-cre) is a commonly used mouse model to study pancreatic ductal adenocarcinoma (Hingorani et al., 2005). It recapitulates the
genetic mutations found in human pancreatic cancer. In order to investigate the expression patterns of the nucleotidases during pancreatic cancer, we used KPC mice in this study. C57BL/6J male (n=3 per group) and female (n=3 per group) mice aged 10 weeks old were anesthetized with isoflurane and 40 µL of PBS or cell suspension (150,000 KPC cells were mixed in 50:50 (v/v) PBS: matrigel) was injected in the tail of pancreas. On day 16 post-surgery, all mice were euthanized and pancreatic or tumor tissues were harvested. Colorectal tissues were harvested from C57BL/6J male (n=3) and female (n=3) mice aged 22 weeks old. Tissues were snap frozen and stored at -80°C.

Human pancreatic tissues (n = 3, healthy adult male, postmortem) were obtained from BioIVT (Baltimore, MD).

Immunoblotting

Immunoblotting procedures were performed as previously described (Seneviratne, 2023). Mouse pancreatic (n = 3, male and n = 3, female) and colorectal tissues (n = 3, male and n = 3, female) were lysed in 1X cell lysis buffer (Cell Signaling Technology, Beverly, MA, USA) with 1 mM Phenylmethanesulfonyl Fluoride (Cell Signaling Technology, Beverly, MA, USA) and centrifuged at 14,000 x g for 10 min at 4°C, and the supernatant was collected. A Pierce™ Bicinchoninic Acid (BCA) Protein Assay Kit was used to determine total protein concentration (Thermo Scientific). Tissue lysate containing approximately 50 µg of the protein was added to the equal amount of loading buffer (Laemmli buffer), heated at 90°C for 10 min, and then 4-15% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) (Bio-Rad, Hercules, CA, USA) electrophoresis separation was carried out. The protein samples were blotted onto a nitrocellulose membrane after electrophoresis (Invitrogen, Carlsbad, CA, USA). Subsequently,
the membranes were blocked with 5% non-fat dairy milk dissolved in tris-buffered saline with 0.1% tween 20 detergent (TBS-T) for 1 h at room temperature with shaking. Membranes were then incubated overnight at 4°C in 1:1000 primary antibody in 5% BSA in TBS-T. Primary antibodies used were anti-NT5C1A (Thermo Fisher Scientific), anti-NT5C3 (Proteintech, Rosemont, IL, USA), anti-NTPDase 1 (Abcam, Cambridge, MA, USA), anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH) (Cell signaling technology, Beverly, MA, USA), and anti-vinculin (Proteintech, Rosemont, IL, USA). Membranes were then washed three times for 5 min in TBS-T shaking at room temperature prior to incubation in 1:3000 secondary antibody in 5% BSA in TBS-T for 1 h at room temperature. Anti-rabbit IgG HRP-linked antibody (Cell signaling technology, Beverly, MA, USA) was used as a secondary antibody. Finally, the membranes were washed three times for 5 min in TBS-T, and developed with chemiluminescent substrate (Thermo Scientific), and imaged under ChemiDoc Imaging Systems (Bio-Rad, Hercules, CA, USA).

Mass spectrometry-based proteomics

Mouse pancreatic and colorectal tissues (n = 3, male and n = 3, female), human pancreatic (n = 3, adult male, postmortem), KPC tumor (n = 3, male and n = 3, female), were employed for mass spectrometry-based proteomics to investigate the expression of nucleotidases. The S-Trap method (ProtiFi) was employed for the LC-MS proteomics sample preparation. Sample preparation procedures were performed according to manufacturer’s instructions. The tissues (5 - 10 mg) were lysed in 46 μl of 5% SDS in 50 mM Triethylammonium bicarbonate (TEAB). The lysates were reduced with 5mM tris-(2-carboxyethyl) phosphine and incubated at 55°C for 15 min. After cooling samples at room temperature, and the alkylation was carried out using 20 mM
methyl methanethiosulfonate for 10 min. The alkylated proteins were then acidified with 2.5% phosphoric acid at a 1:10 acid-to-protein ratio. Next, 350 μL of S-Trap membrane binding/wash buffer (consisting of 100 mM TEAB in 90% methanol) was added to the samples and mixed thoroughly. The resulting solution was transferred to the S-Trap mini column followed by centrifugation at 10000 x g for 30 sec. The samples were washed four times with 400 μl of S-Trap binding/wash buffer before being digested with trypsin (Promega) at 47°C for 90 min. The tryptic peptides were eluted via centrifugation for 1 min at 10,000 x g, with three separate elution steps using 80 μl of 50 mM TEAB in water, 80 μl of 0.2% formic acid, and 80 μl of 50% acetonitrile. Finally, the eluted peptides were vacuum dried and reconstituted in 0.1% formic acid for mass spectrometry-based proteomics analysis.

The proteomics data acquisition was carried out using a nanoElute ultrahigh pressure liquid chromatography system (Bruker Daltonics, Bremen, Germany) coupled with the trapped ion mobility quadrupole time-of-flight mass spectrometer (timsTOF Pro, Bruker Daltonics, Bremen, Germany) with CaptiveSpray electrospray ion source (Bruker Daltonics, Bremen, Germany). A peptide aliquot (2 μL) was loaded on a ReproSil C18 column (150 mm length, 0.75 mm inner diameter with 1.9 μm particle size, and 120 Å pore size). Peptides were separated at 50°C using a binary mobile phase of water containing 0.1% formic acid (A), and 5% acetonitrile/0.1% formic acid (B) at a flow rate of 0.5 μL/min. The gradient setting was as follows: 0-18 min, 5% B; 18-18.5 min, 30% B; and 18.5-21 min, 95% B. The mass spectrometer was operated in parallel accumulation-serial fragmentation (PASEF) mode, and mass spectra were recorded in the range from m/z 100 to 1700 in positive ion polarity mode. The ion mobility (1/K_0) was scanned from 0.85 to 1.30 (V·s)/cm².
Raw files were processed and analyzed using FragPipe (V19.1) with MSFragger, an open-source software suite for proteomics data analysis (Kong et al., 2017). FragPipe was used to import the raw files, perform a database search against the UniProt mouse proteome database (downloaded on 09/16/2021), and post-process the search results. Search parameters included trypsin digestion, a maximum of two missed cleavages, and a fragment ion tolerance of 20 ppm.
Results

Enzymatic activities of nucleotidases toward the metabolites of FTC and dFdC

The enzymatic activities of nucleotidases toward the metabolites of FTC and dFdC were tested \textit{in vitro} using human recombinant 5’-nucleotidases (NT5C1A, NT5C2, NT5C3, NT5C, and NT5M) and NTPDases (NTPDase 1, 3, 4, 5, 6, and 7). Specifically, 5’-NTs were employed to assess the dephosphorylation of monophosphate metabolites, whereas the dephosphorylation of triphosphate metabolites was tested using NTPDases. To compare the activities of nucleotidases toward drug metabolites versus endogenous nucleotides, \textit{in vitro} incubation assays were performed using endogenous deoxycytidine monophosphate and triphosphate, dCMP, and dCTP. The chemical structures of deoxycytidine, FTC, dFdC, and their triphosphate metabolites are shown in Figure 1. The dephosphorylation of drug metabolites was measured using the release of inorganic phosphate groups. The release of inorganic phosphate was ascertained in the presence or absence of recombinant nucleotidases using a malachite green assay.

From the above \textit{in vitro} assays, NT5C1A and NT5C3 exhibited approximate 49- and 69-fold increases in the release of inorganic phosphate for dCMP, and 10- and 5-fold increases in the release of inorganic phosphate for dFdCMP, respectively (Figure 2). However, FTC-MP dephosphorylation was observed only in the presence of NT5C3 (Figure 2). Further, NTPDase 1
exhibited an approximate 37-, 44-, and 31-fold increases in the release of inorganic phosphate for dCTP, dFdCTP, and FTC-TP, respectively (Figure 3). Additionally, NTPDase 4 showed approximate 12-, 3-, and 2-fold increases for dCTP, dFdCTP, and FTC-TP, respectively (Figure 3). The dephosphorylation of dCTP, dFdCTP, and FTC-TP was not observed in the presence of NTPDase 3, 5, 6, and 7 (Figure 3).

**Enzyme kinetics of nucleotidase-mediated dephosphorylation of dFdCMP, FTC-MP, dFdCTP, and FTC-TP**

To determine the kinetic parameters of nucleotidase-mediated dephosphorylation, *in vitro* incubation assays were carried out using recombinant NT5C3 and NTPDase 1 proteins. The kinetics of nucleotidase-mediated dephosphorylation of monophosphate metabolites (dCMP, dFdCMP, and FTC-MP) was investigated using recombinant NT5C3 protein. Similarly, NTPDase 1 was employed for the determination of kinetic parameters of nucleotidase-mediated dephosphorylation of triphosphate metabolites dCTP, dFdCTP, and FTC-TP. As an initial step, the linearity of NT5C3-mediated dephosphorylation of monophosphate metabolites and NTPDase 1-mediated dephosphorylation of triphosphate metabolites with respect to different enzyme concentrations and time was determined. Based on the above initial experiments, an enzyme concentration of 5 nM and 10 min incubation time were used for the subsequent kinetics experiments.

Enzyme activity was measured at varying substrate concentrations by determining the release of inorganic phosphate. The initial velocity of nucleotidase-mediated dephosphorylation was
calculated from the concentration of inorganic phosphate release, enzyme concentration, and incubation time, which was then plotted against substrate concentrations. Interestingly, both NT5C3- and NTPDase 1-mediated dephosphorylation reactions exhibited Michaelis-Menten kinetics as shown in Figure 4. The kinetic parameters of NT5C3-mediated dephosphorylation of dCMP, dFdCMP, and FTC-MP are listed in Table 1. In addition, NTPDase 1-mediated dephosphorylation of dCTP, dFdCTP, and FTC-TP are shown in Table 2.

From these analyses, NT5C3 exhibited different substrate affinities (K\textsubscript{m}) for dCMP (0.057 ± 0.005 mM), dFdCMP (0.095 ± 0.017 mM), and FTC-MP (1.284 ± 0.320 mM) (Table 1). Furthermore, the calculated V\textsubscript{max} values (maximal velocity) of NT5C3-mediated dephosphorylation of dFdCMP and FTC-MP were 5494 ± 196 and 5717 ± 606 pmol min\textsuperscript{-1} \mu g\textsuperscript{-1}, respectively (Table 1). The turnover numbers (k\textsubscript{cat}) of dCMP, dFdCMP, and FTC-MP were 33.62 ± 0.59, 20.32 ± 0.73, and 21.14 ± 2.24 min\textsuperscript{-1}, respectively (Table 1). In order to measure the substrate specificity, catalytic efficiency (k\textsubscript{cat}/K\textsubscript{m}) values were calculated. The calculated k\textsubscript{cat}/K\textsubscript{m} values of dCMP, dFdCMP, and FTC-MP were 589.78 ± 34.51, 213 ± 20.78, and 17.76 ± 4.41 mM\textsuperscript{-1} min\textsuperscript{-1}, respectively (Table 1).

The calculated K\textsubscript{m} values of NTPDase 1 for dCTP, dFdCTP, and FTC-TP were 0.276 ± 0.088, 0.048 ± 0.007, and 0.306 ± 0.072 mM, respectively (Table 2). The V\textsubscript{max} values of NTPDase 1-mediated dephosphorylation of dCTP, dFdCTP, and FTC-TP were 44201 ± 3935, 35464 ± 1134, and 34999 ± 2382 pmol min\textsuperscript{-1} \mu g\textsuperscript{-1}, respectively (Table 2). The substrates, dCTP, dFdCTP, and FTC-TP exhibited k\textsubscript{cat} values of 256.4 ± 22.82, 205.7 ± 6.58, and 203.00 ± 13.82 min\textsuperscript{-1}, respectively (Table 2). NTPDase 1-mediated dephosphorylation of dFdCTP exhibited relatively
high $k_{cat}/K_m$ ($4381.03 \pm 481.32 \text{ mM}^{-1} \text{ min}^{-1}$) as compared to dCTP ($953.70 \pm 395.66 \text{ mM}^{-1} \text{ min}^{-1}$) and FTC-TP ($666.02 \pm 76.07 \text{ mM}^{-1} \text{ min}^{-1}$) (Table 2).

**Protein expression in mouse tissues**

Since NT5C3, NT5C1A, and NTPDase 1 exhibited enzymatic activities toward the metabolites of dFdC and FTC, it was next instructive to determine the presence of the above proteins in relevant tissues. In order to confirm the presence of NT5C3, NT5C1A, and NTPDase 1 in the pancreatic (target tissue for dFdC treatments) and colorectal (target tissue for FTC treatments) tissues, immunoblotting was performed. For immunoblotting analysis, pancreatic and colorectal tissues were obtained from mice (C57BL/6J). From these analyses, expression of NT5C3 and NTPDase 1 was confirmed in both pancreatic and colorectal tissue lysates (Figures 5 and 6). However, we did not detect NT5C1A in pancreatic tissue (Figures 5 and 6). In these immunoblot analyses, GAPDH and vinculin were utilized as loading controls.

**Mass spectrometry-based proteomics analysis**

To determine the expression levels (relative abundance) of nucleotidases, including 5’NTs and NTPDases in healthy mouse pancreatic and colorectal tissues, human pancreatic, and KPC tumor tissues, as well as to investigate the potential sexual dimorphisms of the expression patterns of the above proteins, an unbiased mass spectrometry-based proteomics approach was utilized. In this work, mass spectrometry analyses were coupled to ion mobility mass spectrometry to increase the coverage of the detected proteins. From the proteomics analysis, approximately a total of 5600 and 5000 proteins were detected in healthy mouse pancreatic and colorectal tissue.
lysates, respectively. A total of four 5’NTs and three NTPDases were detected in pancreatic tissue. Similarly, a total of four 5’-NTs and four NTPDases were detected in colorectal tissue. Of the detected nucleotidases, NT5C3 and NTPDase 1 exhibited high abundance across male and female mice pancreatic and colorectal tissue (Tables 3 and 4). We did not detect NT5C1A, NT5M, NTPDase 3, 4, 6, and 7 in mouse pancreatic tissue. Additionally, NT5C1A, NTPDase 3, 6, and 7 were not detected in mouse colorectal tissue. In healthy human pancreatic tissue lysate, an approximate 3900 proteins were detected, among which we observed the expression of NT5C2, NT5C, and NTPDase 1 (data not shown). However, NT5C3 was not detected in any of three tissue samples analyzed. Our laboratory reported the NTPDase 1 expression in human colorectal tissue previously (Seneviratne, 2023).

In order to investigate the expression patterns of nucleotidases during disease states such as pancreatic cancer, we analyzed KPC cell-derived orthotopic tumors. The KPC mouse pancreatic cell line was isolated from a tumor of KPC mouse model of pancreatic ductal adenocarcinoma. Importantly, KPC mouse model recapitulates the genetic mutations found in human pancreatic cancer (Hingorani et al., 2005). For these experiments, cultured KPC cells were implanted into the pancreas of a wild-type mouse, and tumors were allowed to form over 16 days. In these KPC tumors, we detected relatively high expression of NT5C3 and NTPDase 1 in KPC tumor tissues across all the mice (Table 5). However, we did not observe any statistically significant differences in the expression of the above nucleotidases in healthy versus KPC tumor tissues.
Discussion

In the present study, we investigated the nucleotidases responsible for the dephosphorylation of pharmacologically active metabolites of two first-line nucleoside analog drugs: dFdC and FTC. Based on the structural similarities of the above drugs to the endogenous nucleoside, deoxycytidine, we hypothesized that the nucleotidases that contribute to the dephosphorylation of deoxycytidine metabolites may also be involved in controlling metabolism of the above drugs. The kinase activities toward dFdC and FTC have already been studied well (Bergman et al., 2002; Alvarellos et al., 2014; Figueroa et al., 2018). Specifically, deoxycytidine kinase catalyzes the first phosphorylation step of dFdC whereas cytidine monophosphate kinase 1 and nucleoside diphosphate kinase involve in subsequent phosphorylations of dFdC monophosphate (Alvarellos et al., 2014). Although the specific kinases that activate FTC in peripheral blood mononuclear cells are known (Figueroa et al., 2018), the kinases that are responsible for the phosphorylation of FTC in colorectal and vaginal tissues are yet to be elucidated. Of note, tissue drug metabolite levels play a key role in yielding the efficacy of drugs. Therefore, it is important to understand the sources of variability that affect the levels of pharmacologically active metabolites of drugs.
Our findings from this study provide novel insights to increase our understanding of the enzyme-mediated drug metabolite dephosphorylation that may lead to drug inactivation.

In this work, we observed that two 5’-NTs, NT5C1A and NT5C3 exhibited enzymatic activities toward dCMP and dFdCMP. Based on sequence analysis data, the amino acid sequence homology between NT5C1A and NT5C3 was low. Previously it has been reported that NT5C1A and NT5C3 are associated with dFdCMP inactivation by dephosphorylating it to dFdC (Li et al., 2008; Patzak et al., 2019). Our results are in concordance with the above findings. In our study, we performed a comprehensive comparison of enzymatic activities of a range of human recombinant 5’-NTs toward dCMP, dFdCMP, and FTC-MP. In addition, we determined the enzyme kinetic parameters of NT5C3-mediated dephosphorylation of dCMP, dFdCMP and FTC-MP. Based on our data, we did not observe enzymatic activity of NT5C2, NT5C, and NT5M toward any of the substrates (dCMP, dFdCMP, and FTC-MP). However, NT5C exhibited enzymatic activity toward a metabolite of another nucleoside analog drug, acyclovir monophosphate (supplementary figure). These observations may be due to the substrate preference of the above enzymes. NT5C2, NT5C, and NT5M contain approximately 561, 201, and 228 amino acids, respectively. It has been reported that NT5C and NT5M exhibit 52% sequence identity and both proteins contain two domains (Walldén et al., 2007a). Moreover, NT5C2 is a tetrameric enzyme structurally similar to NT5M and NT5C3; however, it contains additional allosteric sites (Walldén et al., 2007b).

Unlike NT5C1A, NT5C3 exhibits six different isoforms, with amino acid lengths of 331, 297, 285, 298, 264, and 264 in isoforms 1 to 6, respectively, as reported in the National Center for...
Biotechnology Information (NCBI) database (Aksoy et al., 2009). In this study, we used NT5C3 isoform 2, which shares sequence similarities of 88.3%, 96.0%, 83.6%, 68.1%, and 88.9% with isoforms 1, 3, 4, 5, and 6, respectively. NT5C1A and NT5C3 are cytosolic divalent cation-dependent enzymes that catalyze the dephosphorylation of pyrimidine analogs and they exhibit optimum activity at 5 mM Mg\(^{2+}\) and 7.5 pH (Hunsucker et al., 2001; Amici and Magni, 2002). The 3D structure of *Escherichia coli* periplasmic 5’-NT provides a structural basis to understand the mode of action of mammalian 5’-NTs (Knöfel and Sträter, 1999). The above enzyme consists of two domains: N-terminal and C-terminal, and between these two domains lies a catalytic cleft containing a divalent metal center (Knöfel and Sträter, 2001). Substrate binding occurs at the interface between two domains (Knöfel and Sträter, 1999; Knöfel and Sträter, 2001; Sträter, 2006). The presence of common domains suggests a common catalytic mechanism for all 5’-NTs (Bianchi and Spychala, 2003). On the basis of our data, we did not observe the enzymatic activity of NT5C1A toward FTC-MP. This observation suggests that NT5C1A may have specific substrate preference.

One of the notable findings of this work is that NTPDase 1 showed relatively high enzymatic activity toward all three triphosphate substrates, dCTP, dFdCTP, and FTC-TP. Specifically, NTPDase 1 exhibited roughly 37-, 44-, and 31-fold increases in the release of inorganic phosphate for dCTP, dFdCTP, and FTC-TP, respectively (compared with no-protein control). Through this work, for the first time, we demonstrate the enzymatic activity of NTPDase 1 toward dFdCTP, and FTC-TP. Of note, seven different isoforms of NTPDase 1, with amino acid lengths of 510, 517, 522, 469, 402, 372, and 482 in isoforms 1 to 7, respectively, are reported in the NCBI database. We used NTPDase 1 isoform 1, which shows sequence similarities of 86.3%,
96.9%, 92.0%, 78.8%, 72.9%, and 84.2% with isoforms 2, 3, 4, 5, 6, and 7, respectively.

NTPDase 1 is a divalent cation-dependent enzyme that catalyzes the dephosphorylation of nucleoside tri- and/or diphosphates at millimolar concentration of Mg$^{2+}$ at pH between 7 and 8 (Zimmermann, 2001). Previously, it has been reported that several nucleotidases, including NTPDase 1, 3, 4, and 8 are known to dephosphorylate ATP and UTP (Kukulski et al., 2005; Corriden et al., 2008; Zimmermann et al., 2012). More recently, our laboratory found that NTPDase 1 exhibit enzymatic activity toward the pharmacologically active metabolite of a nucleoside analog drug, tenofovir (Seneviratne, 2023). It has been reported that NTPDase 1, 2, 3, and 8 have broad substrate specificity toward pyrimidine nucleotides (Zimmermann et al., 2012). Based on our data, only NTPDase 1 exhibited relatively high enzymatic activity toward dFdCTP and FTC-TP. In addition, we also observed the enzymatic activity of NTPDase 4 toward dFdCTP and FTC-TP but to a low extent.

The comparison of $K_m$ values of enzymatic reactions is important as it measures the enzyme’s affinity for its substrate. We observed a $K_m$ value of 0.095 ± 0.017 mM for NT5C3-mediated dephosphorylation of dFdCMP, which is similar to the previously reported $K_m$ value of 0.088 ± 0.006 mM (Aksoy et al., 2009). The affinity of NT5C3 for both dFdCMP and FTC-MP is lower than that for endogenous substrate dCMP ($K_m = 0.057 ± 0.005$ mM). The kinetic parameters of NT5C1A-mediated dephosphorylation of dFdCMP were not determined due to its absence in mouse pancreatic tissue, as revealed from mass spectrometry based-proteomics data and further confirmed by immunoblotting. However, the substrate affinity of NT5C1A for endogenous substrate dCMP have previously been reported as 0.061 mM (Garvey et al., 1998). The $K_m$ value of NTPDase 1-mediated dephosphorylation of dFdCTP was approximately 6 times lower.
than that for dCTP, but $K_m$ values for dCTP and FTC-TP were comparable. This result implies that affinity of NTPDase 1 to dephosphorylate dFdCTP is high. Moreover, $k_{cat}/K_m$ of NTPDase 1-mediated dephosphorylation of dFdCTP is 5 times higher than that of dCTP. We did not carry out enzymatic kinetic analysis on NTPDase 4-mediated dephosphorylation of triphosphate metabolites since it showed relatively low activity for both dFdCTP and FTC-TP substrates.

Since dFdC is used for the treatment of pancreatic cancer and colorectal tissue is a putative site for HIV infection, we investigated the expression of 5'-NTs and NTPDases in mouse pancreatic, colorectal, and KPC tumor tissues. Based on immunoblotting data, NT5C3 and NTPDase 1 are expressed in relatively high abundance in both pancreatic and colorectal tissues in male and female mice. However, based on our data, we did not observe the presence of NT5C1A in mouse healthy pancreatic tissue and tumor tissues. On the contrary, a previous report indicated high expression of NT5C1A in human pancreatic ductal adenocarcinoma based on immunohistochemical analysis of tissue microarrays (Patzak et al., 2019). Further, we investigated the overall expression of nucleotidases using mass spectrometry-based proteomics in both pancreatic and colorectal tissues of male and female mice. Among the 5'-NTs, only NT5C2, NT5C3, and NT5C were found to be expressed in both pancreatic and colorectal tissues of male and female mice. NTPDases 1, 2, and 5 were detected in pancreatic tissues, while NTPDases 1, 2, 4, and 5 were detected in colorectal tissues. Interestingly, NTPDase 1 was the only NTPDase that was expressed in high abundance in both pancreatic and colorectal tissues of male and female mice. Since KPC mouse model recapitulates the genetic mutations found in human pancreatic cancer, we investigated the expression of NT5C3 and NTPDase 1 in KPC tumors. From these, we detected the expression of both NT5C3 and NTPDase 1 in KPC tumor
tissues. It would be interesting to investigate the expression patterns of NT5C3 and NTPDase 1 in other tissues that are susceptible to HIV infection.

In order to take the current findings further, it will be important to analyze tissues obtained from humans to investigate the impact of both kinases and nucleotidases toward dFdC and FTC responses in patients with pancreatic cancer and HIV/hepatitis B. Since both NT5C3 and NTPDase 1 exhibit polymorphisms, it would be interesting to study the impact of genetic variants of the above proteins on drug metabolite dephosphorylation. Moreover, siRNA knockdown of above nucleotidases in tissue samples will provide a mechanistic understanding of their role on the disposition of dFdC and FTC metabolites in vivo.

In conclusion, our results suggest that NT5C3 and NTPDase 1 may function in the regulation of pharmacologically active metabolites of dFdC and FTC. NTPDase 1 exhibited relatively high affinity for both dFdCTP and FTC-TP. Mass spectrometry based-proteomics data revealed the high abundances of NT5C3 and NTPDase 1 in mouse pancreatic, KPC tumor and colorectal tissues. Taken together, the above results provide important insights into the nucleotidase-mediated dephosphorylation of the pharmacologically active metabolites of dFdC and FTC.

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Data Availability statement

The authors declare that all the data supporting the findings of this study are contained within the paper and its supplementary material.

Authorship Contributions

N.R.P. conducted experiments, C.I. and P.J.E. provided animal tissue, while all authors participated in research design and data analysis. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Conflict-of-interest statement

The authors declare no competing financial interest.

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Seneviratne HK (2023) Nucleoside Triphosphate Diphosphohydrolase 1 Exhibits Enzymatic Activity Toward Tenofovir Diphosphate. *Drug Metabolism and Disposition* **51**:385-391.


**Figures Legends**

**Figure 1.** Chemical structures of deoxycytidine (dC), dFdC, FTC, and their triphosphate metabolites.

**Figure 2.** Enzymatic activities of 5’-NTs toward (A) dCMP, (B) dFdCMP, and (C) FTC-MP. Each substrate (1 mM) was incubated in the presence of 5 mM MgCl$_2$ for 30 minutes at 37 ºC. Release of inorganic phosphate groups from substrates in the absence or presence of nucleotidases were measured using malachite green phosphate assay. Data are representative of three independent experiments. Bars indicate mean ± standard deviation. The statistical significance was denoted as follows: ***, p ≤ 0.001.

**Figure 3.** Enzymatic activities of NTPDases toward (A) dCTP, (B) dFdCTP, and (C) FTC-TP. Each substrate (100 µM) was incubated in the presence of 5 mM MgCl$_2$ for 30 minutes at 37 ºC.
Release of inorganic phosphate groups from substrates in the absence or presence of nucleotidases were measured using malachite green phosphate assay. Data are representative of three independent experiments. Bars indicate mean ± standard deviation. The statistical significance was denoted as follows: ***, p ≤ 0.001.

**Figure 4.** Kinetic analysis of (A) NT5C3 toward dCMP, dFdCMP, and FTC-MP (B) NTPDase 1 toward dCTP, dFdCTP, and FTC-TP. Picomoles of released inorganic phosphate per minute per microgram of NT5C3 or NTPDase1 enzyme (pmolPi/min/µg) were utilized to measure reaction velocity. GraphPad Prism software was used to perform curve fitting (using nonlinear regression). Data are representative of three independent experiments. Bars indicate mean ± standard deviation.

**Figure 5.** The expression of NT5C3 and NTPDase 1 in mouse pancreatic tissue. Immunoblotting was performed to investigate the presence of NT5C3 and NTPDase1 in pancreatic tissue obtained from healthy male and female mice (n = 3). M, male; F, female.

**Figure 6.** The expression of NT5C3 and NTPDase 1 in mouse colorectal tissue. Immunoblotting was performed to investigate the presence of NT5C3 and NTPDase1 in pancreatic tissue obtained from healthy male and female mice (n = 3). M, male; F, female.
**Table 1.** Kinetic parameters of NT5C3-mediated dephosphorylation of dCMP, dFdCMP, and FTC-MP determined by varying the concentration of substrates. The $V_{\text{max}}$, $K_m$, and $k_{\text{cat}}$ were calculated using non-linear regression in GraphPad Prism software. Experimental values are shown as mean ± S.E. for three independent experiments.

<table>
<thead>
<tr>
<th></th>
<th>dCMP</th>
<th>dFdCMP</th>
<th>FTC-MP</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_m$ (mM)</td>
<td>0.057 ± 0.005</td>
<td>0.095 ± 0.017</td>
<td>1.284 ± 0.320</td>
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<tr>
<td>$V_{\text{max}}$ (pmol min$^{-1}$ μg$^{-1}$)</td>
<td>9092 ± 160</td>
<td>5494 ± 196</td>
<td>5717 ± 606</td>
</tr>
<tr>
<td>$k_{\text{cat}}$ (min$^{-1}$)</td>
<td>33.62 ± 0.59</td>
<td>20.32 ± 0.73</td>
<td>21.14 ± 2.24</td>
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<tr>
<td>$k_{\text{cat}}/K_m$ (mM$^{-1}$ min$^{-1}$)</td>
<td>589.78 ± 34.51</td>
<td>213 ± 20.78</td>
<td>17.76 ± 4.41</td>
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</table>

**Table 2.** Kinetic parameters of NTPDase 1-mediated dephosphorylation of dCTP, dFdCTP, and FTC-TP determined by varying the concentration of substrates. The $V_{\text{max}}$, $K_m$, and $k_{\text{cat}}$ were calculated using non-linear regression in GraphPad Prism software. Experimental values are shown as mean ± S.E. for three independent experiments.
<table>
<thead>
<tr>
<th></th>
<th>dCTP</th>
<th>dFdCTP</th>
<th>FTC-TP</th>
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<tr>
<td>$K_m$ (mM)</td>
<td>0.276 ± 0.088</td>
<td>0.048 ± 0.007</td>
<td>0.306 ± 0.072</td>
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<tr>
<td>$V_{\text{max}}$ (pmol min$^{-1}$ μg$^{-1}$)</td>
<td>44201 ± 3935</td>
<td>35464 ± 1134</td>
<td>34999 ± 2382</td>
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<tr>
<td>$k_{\text{cat}}$ (min$^{-1}$)</td>
<td>256.4 ± 22.82</td>
<td>205.7 ± 6.58</td>
<td>203.0 ± 13.82</td>
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<tr>
<td>$k_{\text{cat}}/K_m$ (mM$^{-1}$ min$^{-1}$)</td>
<td>953.70 ± 395.66</td>
<td>4381.03 ± 481.32</td>
<td>666.02 ± 76.07</td>
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</table>

**Table 3.** Relative abundance of nucleotidases in mouse pancreatic tissues

<table>
<thead>
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<th>Protein</th>
<th>Relative abundance (relative MS intensity)</th>
</tr>
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<tr>
<td></td>
<td>Male</td>
</tr>
<tr>
<td></td>
<td>Female</td>
</tr>
<tr>
<td>NT5C3</td>
<td>119463.1 ± 21036.7</td>
</tr>
<tr>
<td></td>
<td>99302.4 ± 79505.9</td>
</tr>
<tr>
<td>NTPDase 1</td>
<td>90876.4 ± 56906.0</td>
</tr>
<tr>
<td></td>
<td>96025.6 ± 8808.5</td>
</tr>
</tbody>
</table>

**Table 4.** Relative abundance of nucleotidases in mouse colorectal tissues

<table>
<thead>
<tr>
<th>Protein</th>
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<tr>
<td></td>
<td>Male</td>
</tr>
<tr>
<td></td>
<td>Female</td>
</tr>
<tr>
<td>NT5C3</td>
<td>85847.9 ± 42643.3</td>
</tr>
<tr>
<td></td>
<td>83950.8 ± 29843.0</td>
</tr>
<tr>
<td>NTPDase 1</td>
<td>58757.0 ± 23928.3</td>
</tr>
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<td></td>
<td>73116.8 ± 39117.7</td>
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### Table 5. Relative abundance of nucleotidases in KPC tumor tissues

<table>
<thead>
<tr>
<th>Protein</th>
<th>Relative abundance (relative MS intensity)</th>
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<tbody>
<tr>
<td></td>
<td>Male</td>
</tr>
<tr>
<td>NT5C3</td>
<td>101635.1 ± 47328.1</td>
</tr>
<tr>
<td>NTPDase 1</td>
<td>61370.3 ± 23587.0</td>
</tr>
</tbody>
</table>
Fig. 1
**Fig. 2**

(A) dCMP

(B) dFdCMP

(C) FTC-MP

Each bar graph represents the pmoles of inorganic phosphate for different 5'-NTs (NT5C1A, NT5C2, NT5C3, NT5C, NT5M) in the presence or absence of protein. The bars indicate statistically significant differences (*** p < 0.001).
Fig. 3
Fig. 4
Fig. 5
Fig. 6