ROLE OF EQUILIBRATIVE NUCLEOSIDE TRANSPORTERS 1 AND 2 IN THE TRANSPORT AND DISPOSITION OF GEMCITABINE AND ITS METABOLITES IN CERVICAL CARCINOMA

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Dedication

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Abstract

Gemcitabine is a nucleoside analog used as a radiosensitizer for the treatment of locally advanced cervical carcinoma. Yet, despite its efficacy when administered concomitantly with radiation, gemcitabine therapy is not without side effects. The utility of delivering gemcitabine directly to the cervix was explored through the use of a novel drug delivery device, CerviPrep™. Local administration to the cervix led to clinically relevant concentrations of gemcitabine in cervical tissue and plasma, while no gemcitabine was detected in the systemic circulation and no side effects were reported. Our data suggest that targeting gemcitabine delivery to the cervix can limit systemic exposure and toxicity while achieving cytotoxic concentrations of drug at the target site.

Despite its widespread use in cervical carcinoma, little is known about the disposition of gemcitabine in this tissue. As a nucleoside analog, gemcitabine is a substrate for the equilibrative nucleoside transporters (hENT), and patient response to gemcitabine therapy has been associated with the expression of these proteins. A characterization of hENT1 and hENT2 in both malignant and normal cervical tissue was undertaken, and while no effect of malignancy was observed on hENT1 protein expression, hENT2 protein was nearly three-fold higher in malignant cervical tissue when compared to normal tissue. Expression of hENT mRNA was highly variable and not associated with malignancy.

We also examined the effect of dFdU on gemcitabine disposition, as this relatively inactive metabolite is present at much higher concentrations in the plasma than gemcitabine following intravenous administration of the parent compound. We report a novel interaction between dFdU and gemcitabine whereby dFdU competes with

gemcitabine for transport via hENT1 and hENT2. The presence of dFdU appears to enhance the retention of gemcitabine intracellularly leading to an increase in the amount of active gemcitabine triphosphate. As more gemcitabine is phosphorylated in the presence of dFdU, a "metabolic sink" is created, further increasing gemcitabine uptake into the cell via hENT1 and hENT2. These data suggest that both transport and intracellular metabolism are equally important components of gemcitabine disposition and cytotoxic potential, and that the presence of dFdU increases intracellular exposure to this nucleoside analog.

Table of Contents

List of Tables

List of Figures

List of Terms and Abbreviations

5’-NT	5’ Nucleotidase
ABC	ATP binding cassette
CNT	Concentrative nucleoside transporter
CDA	Cytidine deaminase (also dCDA)
dCK	Deoxycytidine kinase
dFdC	2’,2’-diifluorodeoxycytidine (Gemcitabine)
dFdCDP	Gemcitabine diphosphate
dFdCMP	Gemcitabine monophosphate
dCMPD	Deoxycytidine monophosphate deaminase
dFdCTP	Gemcitabine triphosphate
dFdU	2’,2’-difluorodeoxyuridine
dFdUDP	dFdU diphosphate
dFdUMP	dFdU monophosphate
dFdUTP	dFdU triphosphate
ENT	Equilibrative nucleoside transporter
FU	5’-fluorouracil
HPV	Human papilloma virus
HU	Hydroxyurea
LACC	Locally advanced cervical carcinoma
mRNA	Messenger RNA
MRP	Multidrug resistance protein
PNGase F	Purified N-glycosidase F
SNP	Single nucleotide polymorphism
THU	Tetrahydrouridine
UCK	Uridine/cytidine kinase
UMP/CMPK	Cytidylate kinase

Chapter I.

Literature Review

1.1 Cervical Cancer Background

1.1.1 Epidemiology and Risk Factors

The prevalence of cervical cancer in the United States has decreased by more than eighty percent in the last few decades due to an increase in the use of the Pap smear as a cytological screening technique[1,2]. Yet, over 11,000 cases of cervical cancer were diagnosed in the United States in 2009 with 4,000 deaths reportedly due to the disease. Unfortunately, due to the limited availability of screening programs worldwide, cervical cancer remains one of the leading causes of death amongst women in developing countries, with nearly a half a million new cases each year and 270,000 deaths[3].

Significant evidence has accumulated implicating human papilloma virus (HPV) infection in the pathogenesis of cervical carcinoma, with nearly 99% of cervical cancer tissue samples testing positive for HPV DNA[4]. There are over forty HPV types known to infect the human genital tract, fifteen of which are known to be oncogenic. HPV16 accounts for nearly half of all cervical cancers and is strongly associated with the more common squamous cell carcinoma, while HPV18 is typically detected in adenocarcinoma of the cervix, the more severe histological subtype [5-7]. Infection with HPV is not sufficient for carcinogenesis, as demonstrated by the very low prevalence rate of cervical cancer compared with the high incidence of high-risk HPV infections[8]. However, it has been demonstrated that expression of the viral oncoproteins E6 and E7 in the cervix is necessary for neoplastic transformation of this tissue [9,10]. Additional interactions involving host immunity, genomic instability, and genetic predisposition control the effects of HPV oncogenes in the cervix and resulting tumorigenesis[11-13].

Risk factors for the development of cervical carcinoma include young age at first

sexual intercourse, multiple sexual partners, and a history of other sexually transmitted diseases, all of which are typically associated with exposure to HPV [14]. Additionally, cigarette smoking, poverty, obesity, and use of hormonal contraceptives are all risk factors for cervical cancer[15,16].

Over the last decade, vaccines for cervical cancer targeting both low- and high-risk HPV subtypes have become available. Gardasil® is a quadrivalent recombinant vaccine approved by the FDA in 2006 for vaccination against HPV 6, 11, 16, and 18 in females and males aged 9 to 26. Similarly, Cervarix® has been recently approved with efficacy against HPV 16 and 18 in the same population[17]. Studies evaluating the long-term efficacy and stability of these vaccines are currently ongoing. However, preliminary results from phase III trials in over 20,000 women have been promising. Over a three-year follow-up period, efficacy rates above 98% against HPV 16/18-related high-grade precancerous and noninvasive cervical lesions have been reported[18,19].

Based on these data, vaccination against HPV has great potential for reducing the incidence of HPV-related diseases, including cervical cancer. However, as the average age of cervical cancer diagnosis in the United States is forty-eight, the ultimate protective effects of vaccination against HPV will not be observed for many years. Additionally, as the current life-time risk for HPV infection is near 80%, with a prevalence of 33% in the United States, the need for developing and optimizing therapeutic regimens for the treatment of cervical cancer remains apparent[20].

1.1.2 Staging and Treatment

Cervical cancers are staged clinically, and the stage determined at diagnosis typically dictates the type of therapy administered and long-term prognosis. Nearly half

of all women presenting with cervical cancer are diagnosed with stage I disease, defined as a carcinoma confined to the cervix, ranging in size from microscopic to clinically visible[21]. The prognosis for early stage I disease is excellent with little risk for recurrence or metastasis. While requiring more intensive treatment, outcomes for patients with late stage I cervical cancer are still good, with over a 90% survival rate at five years[22]. Surgery is the standard of care for the treatment of stage I disease, ranging from minimally invasive outpatient procedures to radical hysterectomies followed by external beam or internal radiation.

Locally advanced cervical carcinoma (LACC), defined as stages IIB through IVA, includes all tumors extending beyond the cervix, but limited to local metastases[23]. The prognosis for advanced cervical cancer is less optimistic than with earlier stages. Five-year survival rates range from 41-51% and 8-17% for patients with local and distant disease, respectively[24]. Surgical resection continues to be the mainstay of therapy for LACC; yet, during the last decade, the inclusion of chemoradiation therapy for women with locally advanced disease has become a viable treatment option.

Previously administered in conjunction with hysterectomy, radiation therapy has been included in nearly all treatment plans for LACC since its inception in the early twentieth century[25,26]. While considered to be the gold standard, studies of women with stage II or stage III locally advanced cervical cancer treated with radiation alone exhibited a 70% and 83% likelihood, respectively, of recurrence within 24 months following cessation of therapy[27]. Similarly, treatment with single agent chemotherapy alone has exhibited limited utility in patients with cervical cancer, with overall response rates typically less than 15%. However, initial studies reported significant improvements

in the rates of overall survival, local recurrence and distal disease control when chemotherapy was combined with pelvic radiation. Specifically, five phase III trials, each enrolling several hundred women with invasive cervical cancer, were conducted to directly compare outcomes with radiation plus concomitant chemotherapy (chemoradiation) compared to treatment with radiation alone[28-32]. While the chemotherapeutic agent used varied amongst the studies, regimens including cisplatin produced the most significant effect on survival. In fact, in the studies where cisplatin was administered in combination with radiation therapy, the three-year relative survival rate increased by over thirty percent when compared to patients receiving radiation alone. These results prompted the National Cancer Institute to issue a clinical announcement recommending the use of cisplatin-based chemoradiation for the treatment of locally advanced cervical carcinoma. Over the past ten years, data from follow-up studies and meta-analyses have continued to support the use of chemoradiation for the treatment of LACC[33-35].

1.1.4 Chemoradiation in Cervical Cancer

Many chemotherapeutic agents are administered concurrently with radiation therapy for the treatment of solid tumors, including cervical cancer, either in an attempt to preserve affected organs or to act systemically and eliminate any potential metastatic lesion that may exist. Additionally, drugs like cisplatin are given in conjunction with radiation to act as radiosensitizers, increasing the elimination of radioresistant cells and synergistically enhancing the efficacy of radiation. However, direct cytotoxicity is not necessarily the primary mechanism responsible for the radiosensitization properties of a particular agent, and most radiosensitizers are believed to be effective due to their ability

to affect various cell processes simultaneously. For example, many chemotherapeutic agents, including cisplatin, are cytotoxic due to their incorporation into nascent RNA and DNA strands[36]. When this is combined with the DNA and RNA damage induced by radiation, a synergistic effect is observed. Many radiosensitizers are able to interfere with DNA repair processes, limiting the reversal of radiation-induced damage. Interference with the normal cell cycle is another radiotoxic mechanism of many radiosensitizers. These agents are effective at increasing the number of cells in the more radiosensitive G2 and M phases while limiting transition to the more radioresistant S phase[37]. Additionally, some agents are administered with the intent to block growth and proliferation processes involved in radioresistance, which also renders cells more sensitive to ionizing radiation[38].

While overall outcomes improve with chemoradiation, there is a significant increase in toxicity related to combination treatment. Several randomized trials have demonstrated that cisplatin-based chemoradiation significantly increased higher grade hematological and gastrointestinal toxicities[29,32]. In one study examining the use of cisplatin, radiation, and hysterectomy for cervical carcinoma, 35% of patients developed severe adverse effects [32]. In a separate study, toxicities incurred due to pelvic radiation alone were compared to those reported in women receiving both radiation and weekly cisplatin for LACC[39]. Over 40% of patients in the combination therapy group suffered acute grade 3-5 toxicities compared with only 4% of patients who received radiation alone. Similarly, a meta-analysis examining the utility of cisplatin-based radiation therapy reported that only 67% of patients received the entire course of cisplatin therapy due to dose limiting toxicities[40]. Additionally, nephrotoxicity, one of the most common

side effects of cisplatin therapy, is of particular concern in women with cervical cancer. Due to the invasive properties of later stage cervical carcinomas, urethral obstruction may occur as the disease spreads beyond the cervix, which often leads to renal dysfunction, making this population more susceptible to cisplatin-induced nephrotoxicity[41]. These side effects not only affect a patient's quality of life, but may also prevent a patient from receiving their prescribed dose of chemotherapy, reducing the efficacy of the treatment regimen.

In addition to the dose-limiting toxicities listed above, although cisplatin is the most effective single agent used in the treatment of cervical cancer, response rates remain low in patients with advanced disease. As such, therapy for the treatment of metastatic and recurrent disease is frequently ineffective. Numerous trials have been conducted or are currently ongoing to assess the utility of other chemotherapeutics, both with and without cisplatin, for use as radiosensitizing agents for the treatment of cervical cancer.

Another class of chemotherapeutics observed to have radiosensitizing properties includes nucleoside analogs such as 5-fluorouracil (FU) and hydroxyurea (HU). As DNA synthesis inhibitors, these compounds have the ability to inhibit the repair of DNA damaged by radiation, and consequently can trigger an apoptotic response. In fact, many of the initial trials demonstrating the superiority of chemoradiation for cervical cancer employed FU in combination with cisplatin[28]. However, it was soon identified that this combination offered no advantage over cisplatin alone, but significantly increased the number of adverse events[30]. Additionally, one study investigating the use of FU as a single agent radiosensitizer compared with cisplatin was prematurely closed when an interim analysis of the data collected suggested that the FU-only regimen was associated

with a higher failure rate[42]. Similarly, despite its inclusion in three of the five sentinel studies examining chemoradiation in cervical cancer, evidence supporting the use of HU in combination radiosensitizing regimens is lacking[43].

1.2 Gemcitabine

1.2.1 Cytotoxicity

Despite the disappointing results obtained with FU and HU, newer nucleoside analogs, including gemcitabine (2',2'-difluorodeoxycytidine, dFdC, Gemzar®), have been used in combination with radiation therapy and have achieved remarkable success. FDA-approved for the treatment of ovarian and metastatic breast cancer, gemcitabine is also used successfully against bladder, non-small cell lung, and pancreatic cancers.

Gemcitabine is a deoxycytidine analog, structurally similar to cytarabine, but with the substitution of two fluorine atoms on the 2'-position of the deoxyribose ring. As a prodrug, gemcitabine requires phosphorylation to its active di- (dFdCDP) and triphosphate (dFdCTP) moieties to become cytotoxic. The main mechanism for gemcitabine cytotoxicity is the competitive incorporation of the triphosphate moiety into growing DNA strands[44]. After this addition, DNA polymerase will incorporate one additional deoxynucleotide before synthesis is terminated. This additional deoxynucleotide 'masks' the fraudulent gemcitabine base, preventing its detection and excision by DNA repair enzymes. dFdCTP is also incorporated into nascent RNA strands as well, an activity that appears to be cell line-dependent[45]. However, the cytotoxic effect of gemcitabine-mediated inhibition of RNA synthesis remains unclear. Gemcitabine also functions as a topoisomerase I poison, by stabilizing topoisomerase I-

DNA cleavage complexes [46]. DNA-strand breaks are generated when advancing replication or transcription forks encounter these cleavage complexes, and as these complexes become stabilized in the presence of gemcitabine, DNA-strand breaks increase in number, which ultimately leads to cell death.

Gemcitabine di- and triphosphate also inhibit various cellular enzymes, resulting in self-potentiation of gemcitabine cytotoxicity. The diphosphate interferes with ribonucleotide reductase, limiting the formation of the endogenous deoxynucleotides required for DNA synthesis and repair[47]. Decreasing these natural substrates for DNA polymerase increases the incorporation of gemcitabine into DNA. Also, this same mechanism causes depletion of dCTP, a potent feedback inhibitor of dCK, leading to increased phosphorylation of gemcitabine. This particular mechanism of action seems to be a critical component of gemcitabine cytotoxicity. In cell models, overexpression of ribonucleotide reductase subunit 1 (RRM1) leads to the development of gemcitabine resistance, and both the genotype and expression level of RRM1 are significant predictors of treatment response to gemcitabine therapy[48,49]. Similarly, these metabolites (gemcitabine phosphates) are also inhibitors of cytidine triphosphate synthetase and deoxycytidylate deaminase. Cytidine triphosphate is a natural competitor of dFdCTP for incorporation into RNA, but by depleting the intracellular cytidine triphosphate pool, the level of dFdCTP utilized for RNA synthesis is significantly increased. Deoxycytidylate deaminase is involved in gemcitabine inactivation, and inhibition of this enzyme increases the retention and intracellular half-life of dFdCTP (Figure 1-2).

1.2.2 *Radiosensitization*

While gemcitabine possesses potent cytotoxic activity, the radiosensitization

induced by gemcitabine is not thought to be related to its mechanism of cellular toxicity. Neither the phosphorylation of gemcitabine to its triphosphate nor the incorporation of the triphosphate into DNA correlate with the level of radiosensitization that occurs when patients are administered gemcitabine in combination with radiation therapy[50]. In fact, in vitro incubations with non-toxic concentrations of gemcitabine still result in synergistic radiosensitization, and the concentrations of gemcitabine necessary for this synergism are nearly 1,000-fold lower than typical plasma levels measured after intravenous gemcitabine administration[50]. While many nucleoside analogs increase radiotoxicity by increasing double strand breaks or inhibiting their repair, gemcitabine has no effect on radiation-induced double strand breaks[51]. Additionally, many radiosensitizers redistribute cells to more radiosensitive phases of the cell cycle. Yet, gemcitabine causes a redistribution of cells to the less sensitive S-phase. The inhibition of ribonucleotide reductase by gemcitabine diphosphate does appear to play a role in enhancing the cytotoxic effect of ionizing radiation, as the imbalance in the deoxynucleotide pools resulting from ribonucleotide reductase inhibition, correlates with radiosensitization. High levels of dFdCDP are correlated with decreased amounts of dATP, which leads to significant increases in single-base substitutions at A:T residues during DNA replication[52]. As the level of mismatches increases with higher concentrations of gemcitabine and radiation, the mismatch repair system is overwhelmed, resulting in radiosensitization. As the proficiency of mismatch repair varies amongst different cell types, the extent of gemcitabine radiosensitization may be tissue specific. Along with gemcitabine, the deaminated metabolite, 2',2'-difluorodeoxyuridine (dFdU), also exhibits radiosensitizing properties despite a significantly reduced cytotoxic potency when

compared with the parent compound. As concentrations of dFdU are quite high following intravenous administration and its clearance is relatively low, dFdU may also play a role in determining the efficacy of gemcitabine as a radiosensitizer[53].

1.2.3 Clinical Use of Gemcitabine as a Radiosensitizer in Cervical Cancer

While thought to be largely ineffective as a single agent for the treatment of cervical cancer, early *in vitro* studies utilizing cell lines derived from pancreatic, breast, colorectal, and lung cancers, in addition to cervical cancer, have identified gemcitabine as a potent radiosensitizer[54,55]. Radiosensitization was observed to most be effective when exposure to gemcitabine occurred shortly before ionizing radiation[56]. Most preclinical *in vivo* research focused on determining the optimal schedule for gemcitabine delivery when used in conjunction with radiation. Animal studies demonstrated that the maximal radiosensitizing effect of gemcitabine was observed when administered 24 hours prior to radiation therapy[57,58].

Since its initial introduction into humans, use of gemcitabine as a radiosensitizer alone and in combination with other chemotherapeutics has been explored in many types of solid tumors including head and neck, pancreatic, non-small cell lung, glioblastoma, and esophageal cancers[59-63]. The first study examining the use of gemcitabine as a radiosensitizing agent in the treatment of cervical cancer was performed in ten women diagnosed with stages IB2 thru IIIB cervical cancer[64]. Gemcitabine doses (50-150 mg/m^2) were given two to four hours prior to the delivery of external beam radiotherapy. Toxicities were mild in nature and included diarrhea and myelosuppression. After a median follow-up time of 29 months, all but one patient was reported to be disease-free. These initial results were consistent with the preclinical data obtained from studies in

cervical cancer cell lines indicating the utility of gemcitabine as a radiosensitizer.

Two follow-up studies in a total of 38 women with locally advanced cervical carcinoma provided equally promising results. After receiving weekly doses of gemcitabine ranging from 300-600 mg/m^2 followed by ionizing pelvic radiation, the complete response rate was over 80% for both trials. Additionally, no grade 4 toxicities were reported, with only one patient developing grade 3 diarrhea and anemia[60,65].

Gemcitabine may also be successfully used as an alternative to cisplatin in patients with poor renal function due to urethral obstruction. Cetina et al. administered weekly gemcitabine (300mg/m^2) in combination with radiotherapy to eight women with advanced stage III cervical cancer and elevated serum creatinine (range 1.6-18)[66]. Not only did patients exhibit significant improvement in creatinine clearance and serum creatinine values, but at a median follow-up of eleven months, six of the eight patients remained disease free.

In the only direct, head-to-head study comparing cisplatin- and gemcitabine-based chemoradiation conducted to date, weekly gemcitabine administration resulted in similar response rates to those observed with cisplatin, including overall survival rates at follow-up of 68.8% for gemcitabine and 60% for cisplatin ($p=0.53$)[67]. Additionally, the toxicity profiles for both regimens were similar as well, with comparable numbers of patients developing grade 3 and 4 anemia, leukopenia and thrombocytopenia. Based on these data, gemcitabine appears to have a promising role in the treatment of cervical cancer, and may present a better therapeutic option than cisplatin in the treatment of women with preexisting nephrotoxicity or renal failure due to metastasis.

1.2.4 Gemcitabine-Based Combination Regimens

Cisplatin remains the standard of care for the treatment of cervical cancer, but the promising results obtained with gemcitabine as a single-agent radiosensitizer led researchers to explore the use of both drugs concomitantly. The mechanisms of cytotoxicity and radiosensitization exhibited by either cisplatin or gemcitabine are different but complementary, allowing for the targeting of multiple cellular processes. In vitro studies have demonstrated significant synergism when cisplatin and gemcitabine are used as combination therapy. For example, one mechanism of cisplatin cytotoxicity involves the formation of intrastrand adducts of DNA. These adducts are typically repaired by the nucleotide excision repair pathway. However, this pathway requires the availability of deoxynucleotides for resynthesis of the DNA following excision of the offending adduct. When cisplatin and gemcitabine are combined, the inhibition of ribonucleotide reductase by dFdCDP and the subsequent decrease in deoxynucleotide pools significantly limits repair of the intrastrand adducts[68]. Additionally, this same mechanism results in an increase in the incorporation of dFdCTP into the DNA intrastrand crosslinks resulting in irreparable damage to the DNA and leading to cell death. Gemcitabine also inhibits the repair of cisplatin interstrand crosslinks.

Unfortunately, neither gemcitabine alone or in combination with cisplatin has proven efficacious when combined with radiation for the treatment of metastatic or recurrent cervical carcinoma. However, for locally advanced disease, the combination frequently results in complete response rates ranging from 74 to 90% [41,69-71]. An additional benefit to combining cisplatin and gemcitabine is that both agents can be administered at lower doses, with doses of gemcitabine typically around 125 mg/m^2

compared with the 300-1000 mg/m^2 of gemcitabine administered as a single agent. However, the effects of this combination therapy on treatment-related adverse events have been inconsistent. While some trials have reported a decrease in higher grade toxicities, similar studies using the same doses had to be halted prematurely due to unacceptable rates of toxicity. However, there may be a scheduling component to the development of toxicity. For example, in one Gynecologic Oncology Group dose escalation study, acute dose-limiting toxicity occurred with starting doses of 100 mg/m^2 gemcitabine followed by 40 mg/m^2 cisplatin, administered weekly. Even after dosage adjustment, high rates of late grade 3 and 4 gastrointestinal and genitourinary toxicities in addition to hematologic toxicities forced suspension of the trial[72]. Yet, when cisplatin was administered prior to administration of gemcitabine and radiotherapy, the rate of grade 3 and 4 toxicities was typically less than 20% of all reported adverse events[73]. Determining the optimal schedule and dosing for cisplatin/gemcitabine combination therapy will require additional research. However, these data suggest that gemcitabine, both with and without cisplatin, may prove beneficial in the treatment of locally advanced cervical cancer when used in combination with radiation.

1.3 Gemcitabine Disposition

1.3.1 Nucleoside Transporters

Due to their hydrophilicity, nucleoside analogs, including gemcitabine, exhibit limited passive diffusion across plasma membranes and require specialized nucleoside transporters for uptake into and efflux out of cells. Physiologically, these transporters are involved in mediating the salvage of endogenous nucleosides as well as regulating the

autocrine and paracrine effects of adenosine. However, due to their structural similarity to the endogenous substrates of nucleoside transporters, multiple nucleoside analogs used as anticancer and antiviral therapies are substrates for these transporters as well.

Two major families of nucleoside transporters have been identified in humans: the equilibrative (hENT) and concentrative (hCNT) nucleoside transporters. Classified according to their mode of transport, hENTs are involved in facilitated diffusion, and move substrates down their concentration gradients both into and out of the cell. However, as their name implies, hCNTs transport nucleosides intracellularly against a concentration gradient. They are considered secondary active transporters due to their coupling of substrate transport to inwardly directed sodium gradients maintained by the Na^+/K^+-ATPase pump. Although gemcitabine is a substrate for hCNTs, namely hCNT1, the limited distribution of hCNTs in human tissues suggests that hENTs may be the more predominant transporters involved in gemcitabine disposition in humans. As this thesis work focused on the role of equilibrative nucleoside transporters in the disposition of gemcitabine in cervical cancer, this literature review will focus on hENTs as well.

There are currently four known human equilibrative nucleoside transporter subtypes (hENT) belonging to the solute carrier 29 (SLC29) family of transporters. Of these, two proteins, hENT1 and hENT2, are most commonly associated with the transport of chemotherapeutic and antiviral nucleoside analogs, whereas hENT3 and hENT4 are involved in lysosomal transport and uptake of endogenous monoamines, respectively. While members of the hENT family exhibit limited sequence homology, all family members have a similar protein primary structure comprised of eleven transmembrane segments with a cytoplasmic N-terminus and an extracellular C terminus (Figure 1-3). As

a family, hENTs are inhibited by the coronary vasodilators dilazep and dipyridamole. Prior to cloning, the individual equilibrative transporters were classified on the basis of their sensitivity (*es*) or insensitivity (*ei*) to inhibition by nitrobenzylthioinosine (NBTI, nitrobenzylmercaptopurine riboside; NBMPR). However, today, these same transporters go by the designation hENT1 and hENT2, respectively.

Unlike concentrative transporters whose tissue distribution is limited to specialized cell types, hENTs are ubiquitously expressed. hENT1, which is a 456-residue protein typically residing on the basolateral surface of cells, exhibits higher expression levels in the kidneys, erythrocytes, heart, and colon [74]. hENT2, which is 46% similar in amino acid sequence to hENT1, is especially abundant in skeletal muscle [75].

Both hENT1 and hENT2 are glycoproteins, although evidence suggests that the extent of glycosylation of these proteins may be tissue specific. Site-directed mutagenesis at three potential glycosylation sites of hENT1 failed to alter transport of nucleosides but affected inhibition by NBMPR, dilazep, and dipyridamole[76]. Specifically, the loss of glycosylation due to the conversion of the asparagine at residue 48 to glutamine, resulted in a significant decrease in affinity of hENT1 towards NBMPR, but an increase in affinity towards dilazep and dipyridamole. These data imply that this glycosylation site is very near the binding site for both nucleosides and inhibitors. hENT2 possesses two such glycosylation sites, at Asn48 and Asn57[77]. While de-glycosylation did not affect the function of the hENT2 protein, glycosylation is necessary for efficient targeting of hENT2 to the plasma membrane.

The substrate specificities of hENT1 and hENT2 overlap as both are able to transport endogenous purine and pyrimidine nucleosides and nucleoside analog therapies

including gemcitabine and cytarabine and the anti-virals ribavirin and didanosine[78]. However, the efficiency of this transport is protein-dependent. Typically, hENT1 exhibits a higher affinity for nucleoside substrates, with apparent K_m values ranging from 40 μM for adenosine to 580 μM for cytidine[79]. However, hENT2 is unique among nucleoside transporters for its ability to transport purine and pyrimidine nucleobases[80].

Inhibition of nucleoside transport by NBMPR is a property specific to hENT1. This transporter is potently inhibited by NBMPR, exhibiting a K_i value of around 5 nM in human cells[81]. While NBMPR itself is not transported by hENTs, the binding of NBMPR to the extracellular portion of the hENT1 protein is thought to 'lock' the transporter in its outward-facing conformation, hence limiting intracellular transport. The selective inhibition of hENT1 over hENT2 by NBMPR allows for determination of the individual contributions of hENT1 and hENT2 in cell systems expressing both transporters, making this compound particular useful when performing hENT transporter assays.

The study of hENT transporters is complicated by their bidirectional transport properties and the rapid equilibration of substrates across the plasma membrane. For most nucleosides, this equilibration half-life ranges from three to twelve seconds[82]. Another confounding factor requiring consideration in hENT research is the ongoing intracellular metabolism of the substrate under investigation. For substrates with no intracellular metabolism, the intracellular concentration should remain constant after equilibrium conditions have been achieved. However, based on studies with radiolabeled nucleosides, the total intracellular radioactivity continues to increase with time even after intra- and extracellular concentrations have reached equilibrium, suggesting that

intracellular metabolism is just as much an integral part of nucleoside accumulation as is transporter-mediated uptake. As such, initial work with radiolabeled fluorodeoxyuridine by Bowen et al. suggests that the uptake of this nucleoside in Ehrlich ascites tumor cells occurs in two phases[83]. The first phase is characterized by a rapid, initial, unidirectional uptake that is linear up to ~fifteen seconds. At this point transport becomes saturated, the contribution of efflux begins to impact accumulation and the increase in net radiolabel observed is no longer due to increasing amounts of parent compound, but to accumulation of the mono-, di-, and triphosphate compounds. Hence, at any point after fifteen seconds, the net accumulation of radiolabel measured intracellularly is comprised of the steady state level of parent compound plus any metabolites that are being formed at a constant rate. In fact, it is predicted that accumulation only plateaus when metabolism ceases or if gemcitabine and its metabolites are eliminated at the same rate at which they are being taken up into the cell. Based on these data, the majority of kinetic analyses performed on hENTs have been conducted over short time intervals in an attempt to limit the confounding effect of intracellular metabolism.

Transport of gemcitabine by hENT1 and hENT2 was first examined by Mackey et al., who hypothesized that low levels of either hENT expression or activity could serve as a potential mechanism of gemcitabine resistance. Studies comparing gemcitabine cytotoxicity in nucleoside transporter-competent and -incompetent cell lines observed a greater than 3200-fold increase in the IC_{50} of gemcitabine (cytotoxicity) when nucleoside transporters were deficient, suggesting that uptake of gemcitabine in the absence of nucleoside transporters was limited and insufficient to attain cytotoxic levels of gemcitabine intracellularly[84]. Interestingly, in HeLa cells expressing hENT1 and hENT2,

elimination of hENT1 activity by the addition of NBMPR had no effect on the IC_{50} of gemcitabine, suggesting that hENT2 is able to compensate, with respect to gemcitabine transport, in the absence of hENT1. The kinetics of gemcitabine transport by the individual hENTs were characterized in HeLa and human leukemic CEM cells. Similar to previous work, uptake was observed to be linear out to ten seconds. Gemcitabine uptake in hENT1-only cells was rapid, with initial rates demonstrating an apparent K_m of 329 μM and V_{max} of 17.0 pmol/s/10^6 cells. Kinetic analysis of hENT2-mediated transport revealed a K_m of 832 μM and corresponding V_{max} of 4.3 pmol/s/10^6 cells.

In addition to gemcitabine, recent evidence suggests that 2',2'-difluorodeoxyuridine (dFdU), the deaminated metabolite of gemcitabine, is also transported by nucleoside transporters. Examined in cells expressing hCNT1 and hENT1, dFdU exhibited uptake rates similar to dFdC via both transporters, with both compounds (dFdU and dFdC) demonstrating significantly higher intracellular levels after incubation in cells expressing hCNT1 vs. hENT1[85]. Interestingly, both dFdC and dFdU are effluxed from HepG2 and A549 cells as well, and while it is assumed that the same transporters are involved in efflux as influx, this has not yet been proven. Both dFdC and dFdU displayed biphasic efflux with a rapid elimination phase ($t_{1/2,\alpha}$ = 2.6 minutes vs. 28 minutes, respectively) followed by a slower elimination phase ($t_{1/2,\beta}$ = 28 minutes vs. 90 minutes, respectively).

This initial research supporting the important role of nucleoside transporters in gemcitabine distribution ultimately led to studies examining the relationship between hENTs and gemcitabine in vivo. Extensive evidence indicates that the in vitro findings are replicated in vivo, with hENTs being a major determinant of gemcitabine efficacy in

humans. For example, in a population of 24 patients with non-small cell lung carcinoma, responses to gemcitabine-containing chemotherapy were associated with hENT1 staining in tumor biopsies[86]. All six patients with significant hENT1 expression demonstrated therapeutic response to gemcitabine, whereas patients who exhibited no hENT1 staining did not respond to the same therapy. Similar results have been reported in patients with gemcitabine-treated pancreatic adenocarcinoma. In one study, in which twenty-one patients were enrolled and received gemcitabine therapy, those with detectable hENT1 in the adenocarcinoma cells had a significant increase in median survival time compared with those patients with low or absent levels of hENT1[87]. These findings were repeated in a separate, larger study, in which 102 patients with pancreatic cancer received gemcitabine therapy and expression levels of hENT1 significantly correlated with overall survival[88]. Patients with high levels of hENT1 expression in tumor tissue exhibited a survival time of 17.6-33.7 months compared with 7.0-9.9 months in patients with low levels of expression. Additionally, the relationship between hENT1 and response to gemcitabine has been confirmed in non-solid tumors as well, as drug uptake and sensitivity to gemcitabine was significantly correlated with hENT1 expression in mantle cell lymphoma[89].

Because hENT1 is thought to play the predominant role in gemcitabine transport and hENT2 a more secondary role, few studies have examined the relationship between the expression of this transporter (hENT2) and patient outcomes after gemcitabine-based therapy. However, initial evidence suggests that hENT2 is indeed involved in determining the efficacy of the thiopurine agents 6-mercaptopurine and 6-thioguanine in vitro, as decreased expression of this transporter protein conferred resistance against

these chemotherapeutics[90]. Additionally, expression of hENT2 has been observed to vary 11-fold amongst cells from patients with chronic lymphocytic leukemia, and reportedly, hENT2 expression is highly correlated with patient response to the nucleoside analog fludarabine[91]. Thus, the hENT2 transporter may also play a role in gemcitabine uptake and disposition, especially in tumors where hENT1 is poorly expressed.

Due to their importance in maintaining adenosine levels and providing nucleosides for the salvage pathway, ENTs are highly conserved compared to other human transporter genes. Three promoter-region SNPs have been identified in *hENT1* (*SLC29A1 -760G>C, -1050G>A, and -1345C>G),* of which the latter two are associated with increased expression of SLC29A1 leading to higher activity in vitro. However, haplotypes associated with these variants were not associated with the level of SLC29A1 (hENT1) mRNA expression in peripheral blood mononuclear cells[92]. Variants identified in the coding region are uncommon, with allele frequencies ranging from 0.5 to 2% in Caucasian, African, and Asian populations, and these variants appear to exhibit functional activity similar to that of the wild-type protein[93-95]. Similarly, the overall variation in *SLC29A2* is much lower than has been observed for other transporters of the SLC family. Owen et al. report the identification of fourteen polymorphic sites in *SLC29A2* (hENT2), eleven of which are located in the coding region, and five of which result in an alteration in protein sequence[96]. These latter five mutations were present at a very low frequency in the populations studied, each only occurring once in the sample population. Functional studies involving these five hENT2 variants demonstrated no significant difference in the transport of gemcitabine when compared with the wild-type protein, aside from *hENT* Δ845-846, which results in a frameshift mutation and a

prematurely truncated protein. *hENT* Δ845-846 was not observed to transport any of the substrates tested, including gemcitabine. Based on these results, it is not likely that genetic variation in *SLC29A1* or *SLC29A2* plays an important role in inter-individual response to nucleoside analog therapies.

1.3.2 Deoxycytidine Kinase and Cytidylate Kinase

Once a nucleoside analog is transported into the cell via hENTs or hCNTs, the majority of these compounds, including gemcitabine, require phosphorylation to become active. Considered to be the rate-limiting step in gemcitabine activation, the initial phosphorylation of gemcitabine to dFdCMP is mediated by deoxycytidine kinase (dCK). Low levels of this primarily cytoplasmic enzyme have been associated with a significant decrease in gemcitabine sensitivity, both in vitro and in vivo, and dCK deficiency is frequently implicated in acquired and intrinsic resistance to gemcitabine therapy [97-99].

Using purified human deoxycytidine kinase, the rate of phosphorylation for dFdC is similar to that of the endogenous substrate (deoxcytidine), with apparent K_m and V_{max} values of 4.6 uM and 14.9 pmol/min, respectively, compared with 1.5 uM and 5.5 pmol/min for deoxycytidine[100]. Being a saturable process, in vivo studies have identified the plasma concentration of gemcitabine necessary to achieve maximal accumulation of dFdCTP. In a phase I study of patients with solid tumors, doses of 350 mg/m^2 over thirty minutes achieved plasma concentrations of gemcitabine near 20 uM, which were associated with maximal phosphorylation[101]. Similar studies in vitro have observed the optimal concentration of gemcitabine resulting in maximal phosphorylation to be 12.5 uM. As such, clinical practice dictates the use of gemcitabine dosing schedules that achieve plasma concentrations of gemcitabine ranging from 10 to 20 uM to maximize the

formation of the active triphosphate moiety.

Interestingly, gemcitabine can increase its own phosphorylation by limiting the formation of deoxycytidine triphosphate, an inhibitor of dCK[100]. Gemcitabine diphosphate inhibits ribonucleotide reductase, which converts ribonucleosides to deoxyribonucleosides. By blocking this conversion, deoxycytidine formation is limited, and its end product, dCTP is not produced, thus eliminating the negative feedback mechanism involved in regulating dCK activity. This is one of the many ways in which gemcitabine is able to potentiate its own cytotoxicity.

As with other proteins and enzymes involved in the disposition of gemcitabine, genetic variation in deoxycytidine kinase may contribute to the high variability in patient response to gemcitabine therapy. To date, more than fifty novel SNPs have been identified in the dCK gene based on the examination of DNA from multiple ethnicities[102-104]. Compared to the wild-type protein, the activity of dCK variants examined thus far has ranged from 32 to 105% when gemcitabine is used as a substrate. Clinically, initial studies with gemcitabine have observed a significant correlation between dCK genotype and histological tumor response after chemo-radiotherapy in patients with pancreatic cancer[105].

After phosphorylation via dCK, nucleoside monophosphates are further converted to diphosphates via cytidylate kinase (UMP/CMPK). The typical substrate for UMP/CMPK is cytidine monophosphate, which is phosphorylated in the presence of ATP with a K_m of 15 uM[106]. The phosphorylation of gemcitabine monophosphate occurs at a rate that is nearly 7-fold slower than for cytidine monophosphate with an apparent K_m of 581 uM. Yet, this enzyme may play a role in gemcitabine efficacy as over-expression of

UMP/CMPK in HCT-1 cells leads to a significant increase in both dFdCDP and dFdCTP levels and cell sensitivity towards gemcitabine[107].

1.3.4 *Deaminases and 2',2'-difluorodeoxyuridine*

The half-life of gemcitabine following intravenous administration is a mere eight minutes, due to its rapid plasma conversion to 2',2'-difluorodeoxyuridine (dFdU), its deaminated metabolite. This deamination occurs via cytidine deaminase (CDA, also dCDA), which is found throughout the body with the highest activity reported in the liver and skeletal muscle[108]. The enzyme has a higher affinity towards its natural substrate, cytidine, than gemcitabine, with apparent K_m values of 46.3 uM and 95.7 uM, respectively[100]. As CDA metabolizes gemcitabine to its much less active form, the activity of this enzyme plays a large role in determining the efficacy and toxicity of gemcitabine. For example, several in vitro studies have reported an increase in gemcitabine resistance due to up-regulation of CDA[109,110]. Conversely, inhibition of CDA increases triphosphate formation and sensitivity towards cytidine analogs and gemcitabine [111,112]. In one study in human leukemic cells, the addition of tetrahydrouridine, a known inhibitor of CDA, to incubations with gemcitabine resulted in a four-fold decrease in the IC_{50} value of gemcitabine when compared with incubations in the absence of THU[113]. In a clinical study of patients with pancreatic cancer, higher expression levels of CDA in PBMCs were significantly associated with shorter time to progression and overall survival rates[114].

Similarly, CDA genotype has proven to be correlated with gemcitabine-associated toxicities. Systemic exposure to gemcitabine was five-fold higher in Japanese patients homozygous for the coding region nonsynonymous, SNP, *CDA* 208G>A, compared with

wild-type individuals, resulting in an increase in severe hematologic and non-hematologic toxicities after therapy with gemcitabine and cisplatin[115]. A separate study reported decreased gemcitabine clearance and significantly increased rates of neutropenia for haplotypes containing *CDA* 208G>A[116]. Another coding region SNP, *CDA* 79A>C, with an allele frequency of 3.5% in Africans and 36.5% in Europeans, is also associated with higher rates of toxicity and poorer outcomes with gemcitabine therapy[117].

While typically thought of as an inactive metabolite, dFdU possesses both cytotoxic and radiosensitizing properties, albeit requiring much higher concentrations than for gemcitabine. IC_{50} values for dFdU as a single agent range from 4 μM to around 3000 μM, depending on the cell line, making dFdU 500- to 113,000-fold less toxic than the parent compound[85,118]. However, as average C_{max} values for dFdU after intravenous gemcitabine dosing can reach upwards of 150 μM, it is likely that dFdU contributes somewhat to the cytotoxicity of dFdC.

In vitro work by Veltkamp et al. has demonstrated similar cytotoxic properties for dFdU as for the parent compound. The triphosphate form of dFdU is incorporated into both DNA and RNA, with the extent of incorporation being significantly associated with its cytotoxicity[85]. In fact, both dFdUTP and dFdCTP display similar incorporation into DNA in cells treated with dFdU and dFdC at their respective IC_{50} values, suggesting that both compounds may have similar intrinsic activity.

Another pathway by which gemcitabine is inactivated is through deamination of dFdCMP to dFdUMP via dCMP deaminase (dCMPD). dFdUMP then undergoes either phosphorylation to dFdUDP and dFdUTP, or can be dephosphorylated and excreted from the cell as dFdU. Interestingly, levels of dFdUTP in white blood cells from patients

administered oral gemcitabine were nearly 5.6-fold higher than the levels of dFdCTP detected in the same patients after a fourteen day dosing period[119]. Despite these high levels of dFdUTP intracellularly, dFdU is not a very good substrate for dCK, suggesting that the majority of phosphorylated dFdU detected intracellularly is most likely due to the breakdown of dFdC nucleotides and subsequent phosphorylation to dFdUTP. Incubations with both gemcitabine and the CDA inhibitor, tetrahydrouridine, exhibited similar levels of dFdUTP formation as cells solely treated with gemcitabine, providing further evidence that the majority of phosphorylated dFdU is not a consequence of the direct conversion of gemcitabine to dFdU[85]. A large intraindividual variation in dFdUMP formation has been identified, as dCMPD activity is high in normal cells undergoing rapid replication as well as in tumor cells, increasing the likelihood that the majority of dFdUMP, dFDUDP, and dFDUTP metabolites in tumors such as cervical carcinoma are derived from dFdC nucleotides[120].

Yet, evidence does exist to the contrary suggesting that in some cell lines, dFdU is directly phosphorylated by dCK. For example, after treatment of HepG2, A549 and MDCK cells with dFdU in the absence of dFdC, the nucleotides of dFdU contributed to 47%, 15%, and 8% of the total intracellular drug content, respectively[85]. Even more, comparisons of triphosphate AUCs after incubation of HepG2 cells with either dFdU or dFdC at their respective IC_{50} concentrations revealed seven-fold higher levels of dFdUTP compared to dFdCTP. Additionally, dFdCTP itself is thought to inhibit dCMPD activity leading to another self-potentiation mechanism whereby as more gemcitabine is phosphorylated, less of the gemcitabine nucleotides are broken down. These conflicting data suggest that the derivation of dFdU nucleotides and their contribution to dFdC

cytotoxicity may be tissue-specific.

1.3.5 *5'Nucleotidase (5'-NT)*

Whereas dCK and dCMPK are involved in the phosphorylation of dFdC and dFdCMP, 5'NT opposes the action of these enzymes by catalyzing the dephosphorylation of nucleoside monophosphates. 5'NT I (cN-I) is the nucleotidase most likely involved in pyrimidine dephosphorylation, and over-expression of this enzyme in Jurkat and HEK293 cells resulted in 82- and 22-fold increases in IC_{50} to gemcitabine[121]. Additionally, examination of the gemcitabine-resistant leukemia cell line, K562, revealed an increased 5'-NT I activity coupled with a decrease in dCK-mediated phosphorylation[122]. While the effects of 5'-NT I activity on gemcitabine disposition in vivo are yet to be examined, these in vitro data suggest that 5'NT I may also contribute to the therapeutic efficacy of gemcitabine.

1.3.6 *Active Efflux Transporters*

While nucleosides are known to be most efficiently transported by hENTs and hCNTs, evidence suggests that the phosphorylated derivatives of nucleoside analogs may be effluxed by members of the ABC (ATP-binding cassette) protein family. Initially, MRP4, MRP5, and MRP8 were observed to confer resistance to antiviral nucleoside analogs, such as ddC[123]. However, similar studies suggested that MRP5 and MRP7 may contribute significantly to the transport of chemotherapeutic nucleoside analogs. Specifically, expression of MRP5 mRNA is significantly associated ($r^2 = 0.63$) with gemcitabine IC_{50} values, with lower levels of mRNA expression associated with higher cytotoxicity[124]. MRP7 has demonstrated protection against gemcitabine cytotoxicity as well, with IC_{50} values increasing 2.8- to 3.0-fold in MRP7-transfected HEK293 cells[125].

Both MRP5 and MRP7 are established transporters of amphipathic anions, and it is most likely that it is the monophosphorylated metabolite of gemcitabine that is being effluxed as opposed to the uncharged parent compound. Indeed, multidrug resistance proteins 4 and 5 have been shown to transport endogenous cyclic monophosphorylated nucleosides such as cAMP and cGMP[126]. Yet, the above in vitro data notwithstanding, MRP-family transporters are not thought to make a large contribution to nucleoside analog resistance clinically, as the affinity of these transporters for nucleoside monophosphate substrates is relatively low[127]. Additionally, while MRP7 has been detected in the normal ovary and ovarian adenocarcinoma, expression levels of MRP5 and MRP7 in the cervix are unknown[128]. As such, it is not likely that the MRP family of transporters contribute significantly toward the disposition of gemcitabine in cervical carcinoma.

1.4 Research Study Objectives

The major aim of this thesis project was to characterize the disposition of gemcitabine in the cervix with the hope of improving the delivery and efficacy of this radiosensitizing chemotherapeutic in women with locally advanced cervical carcinoma. As such, the following objectives of this research are to:

1.) Examine the utility of a cervical drug delivery system for targeting the cervical tissue and circulation while limiting systemic exposure and toxicity. Using gemcitabine as the test drug for this proof-of-concept study, both local and systemic drug concentrations were measured, and adverse events associated with localized delivery

were monitored and recorded.

2.) Characterize the expression of the human equilibrative nucleoside transporters 1 and 2 in tissue from both normal and malignant cervix. Both mRNA and protein expression were quantified, and the two sets of tissue compared to assess the effect of carcinogenesis on hENT expression. hENT protein expression was also correlated to levels of gemcitabine detected in the cervix of women participating in the clinical study outlined in Objective #1.

3.) Determine the effect of the deaminated metabolite of gemcitabine, dFdU, on the disposition and efficacy of the parent compound in a representative cervical adenocarcinoma cell line, HeLa. Studies were conducted to examine the role of hENT1 and hENT2 in interactions between dFdC and dFdU. The effect of intracellular metabolism on the interplay between dFdC and dFdU was evaluated through siRNA-mediated knockdown of deoxycytidine kinase.

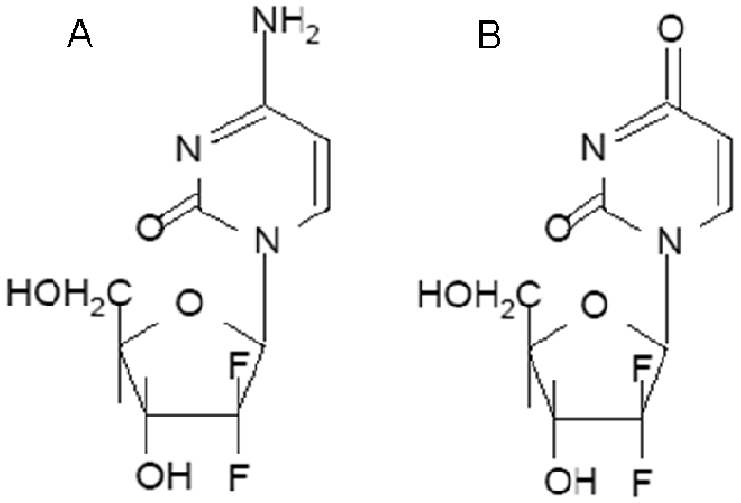

Figure 1-1: Chemical structures of A) the deoxycytidine analog, 2',2'-difluorodeoxycytidine (dFdC, gemcitabine) and B) its deaminated metabolite, 2',2'-difluorodeoxyuridine (dFdU)

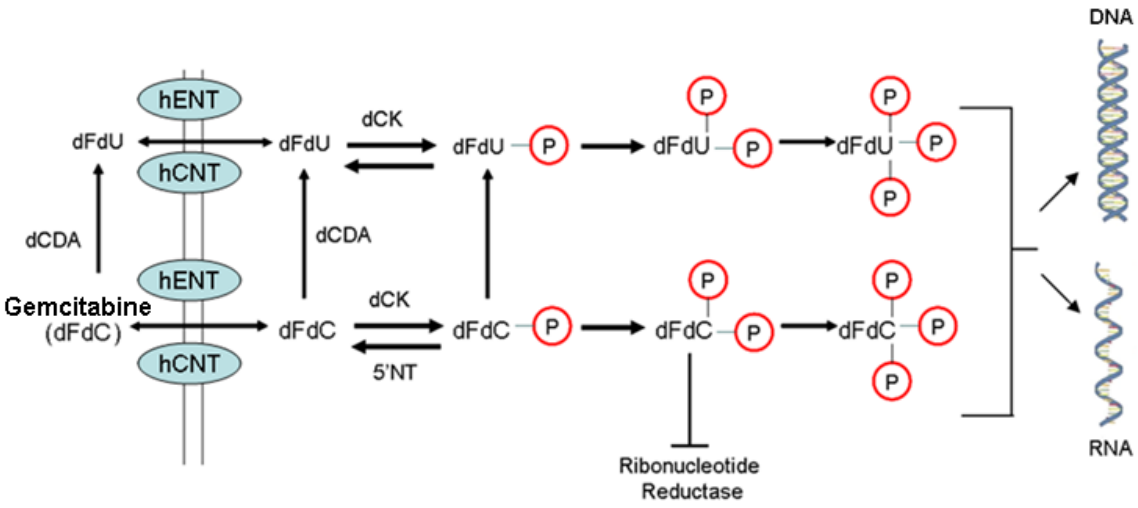

Figure 1-2: Uptake and intracellular metabolism of gemcitabine. After transport into the cell via SLC28A (CNT) and SLC29A (ENT) transporters, gemcitabine is rapidly phosphorylated to dFdCMP. A second phosphorylation step yields dFdCDP, which is an inhibitor of ribonucleotide reductase. The final product, dFdCTP, is incorporated into DNA and RNA, where it inhibits DNA polymerase and is a 'masked' chain terminator. dFdCTP also regulates dCMPDA activity by inhibiting the conversion of dFdCMP to dFdUMP. If not phosphorylated, gemcitabine may also be metabolized via dCDA (also abbreviated as CDA) to dFdU, which can then be effluxed via ENT and CNT from the cell, or it can be phosphorylated in the same fashion as the parent compound.

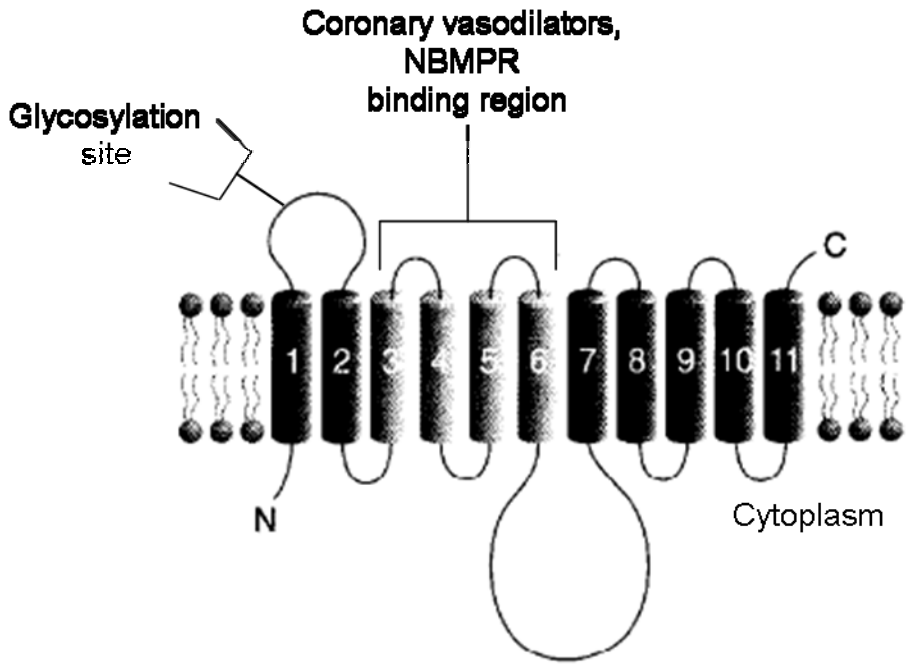

Figure 1-3: Topology model of hENT. Important sites for glycosylation have been identified on the extracellular loop extending from transmembrane regions (TM) 1 and 2. Two such sites (Asn48 and Asn57) are associated with protein trafficking and membrane localization of hENT2. A similar site has been identified on hENT1 (Asn48) and is necessary for protein activity. TMs 3-6 are necessary for interactions with nucleoside substrates and inhibitors. (Image adapted from Baldwin and Young, 1999[75])

Human gene name	*Protein name*	*Endogenous Nucleoside Substrates*	*Nucleoside Analog Substrates*	*Mechanism of Transport*
SLC28A1	CNT1	Adenosine Uridine Thymidine Cytidine	Cytarabine Gemcitabine Zidovudine Zalcitabine	Na^{2+}-coupled, secondary active transport
SLC28A2	CNT2	Guanosine Adenosine Uridine	Cytarabine Gemcitabine Cladribine Didanosine Ribavirin	Na^{2+}-coupled, secondary active transport
SLC28A3	CNT3	Adenosine Uridine Thymidine Cytidine	Gemcitabine Cladribine Fludarabine Zidovudine Zalcitabine Didanosine Ribavirin	Na^{2+}-coupled, secondary active transport
SLC29A1	ENT1	Guanosine Adenosine Uridine Thymidine Cytidine	Cytarabine Gemcitabine Cladribine Fludarabine Zalcitabine Didanosine Lamivudine Ribavirin	Facilitative
SLC29A2	ENT2	Guanosine Adenosine Uridine Thymidine Cytidine Uracil Hypoxanthine Adenine	Cytarabine Gemcitabine Zidovudine Zalcitabine Didanosine	Facilitative
SLC29A3	ENT3	Adenosine		Facilitative
SLC29A4	ENT4	Adenosine		Facilitative

Table 1-1: Representative substrates for human concentrative and equilibrative nucleoside transporters[75,78,129,130].

Chapter II

Localized Delivery of Gemcitabine to the Cervix *in vivo* for Radiosensitization

Sections of this chapter were adapted from the original manuscript "Localized delivery of gemcitabine to the cervix *in vivo* for radiosensitization" by Hodge LS, Downs Jr. LS, Chura JC, Thomas SJ, and Tracy TS.

Introduction

For the last decade, radiation with concomitant intravenous chemotherapy (chemoradiation) has been the standard of care for women with locally or regionally advanced cervical carcinoma (LACC). Clinical practice has been guided by a 1999 alert issued by the National Cancer Institute recommending the addition of chemotherapy to all radiation regimens used for the treatment of LACC. This recommendation was based on data from five clinical studies providing strong evidence for a synergistic effect between chemotherapy and radiation in treating cervical carcinoma, resulting in increased therapeutic efficacy and high patient response rates [28,29,31,32,131]. While cisplatin (CDDP) is the best studied and most widely used cytotoxic agent used as a radiosensitizer for the treatment of cervical cancer, other chemotherapeutics, including the nucleoside analog 2',2'-difluorodeoxycytidine (gemcitabine, dFdC), have been found to be effective radiosensitizers either as single agents or in addition to cisplatin therapy [60,72,132].

Gemcitabine has been used successfully as a radiosensitizing agent for many solid tumors in addition to cervical cancer, including head and neck cancer, glioblastoma, and pancreatic cancer [59,61,62,133]. As a deoxycytidine analog, gemcitabine is able to utilize equilibrative and concentrative nucleoside transporters for rapid entry into the cell. Once inside the cell, gemcitabine, a prodrug, requires intracellular activation via deoxycytidine kinase to its active diphosphate (dFdCDP) and triphosphate moieties (dFdCTP) (See Figure 2) [134,135]. However, deaminases present intracellularly and in the plasma may inactivate gemcitabine prior to cellular entry, resulting in production of the deaminated metabolite, difluorodeoxyuridine (dFdU) (Figure 3). The majority of gemcitabine's cytotoxicity is credited to the same mechanism that provides gemcitabine with strong

radiosensitizing properties, the incorporation of the active diphosphate and triphosphate compounds into nascent RNA and DNA strands [44,136]. Additionally, the ability of gemcitabine to inhibit ribonucleotide reductase, interfere with the cell cycle, and lower the threshold for cellular apoptosis have all been cited as potential mechanisms of gemcitabine toxicity, all of which ultimately increase the efficacy of ionizing radiation [50,54].

Although the use of chemoradiation is currently the most effective treatment available for LACC, there is a significant increase in toxicity related to the use of combination therapy, regardless of the radiosensitizing agent used. For example, radiation treatment with concomitant gemcitabine administration has been associated with high rates of dose-limiting hematological toxicity, severe nausea and vomiting, and dehydration, often resulting in cessation of therapy [60,137]. These side effects not only affect a patient's quality of life, but may also limit the administration frequency of the prescribed therapy, reducing the efficacy of the treatment regimen. Because of the high incidence of toxicity incurred with chemoradiation, neither intravenous gemcitabine nor cisplatin is an ideal radiation sensitizer.

Recently a novel drug device (CerviPrep™) (Figure 2-1) was developed for the application of pharmaceuticals directly to the cervix. Drug is administered through a tube-like syringe attached to a plastic cap covering the cervix, limiting exposure to surrounding vaginal tissues. In an initial proof-of-concept study, the device was used to successfully deliver diazepam to the cervix [138]. Administration of diazepam via the device resulted in high local concentrations of the drug within the uterine circulation with minimal systemic exposure.

Local delivery of a radiation sensitizing chemotherapeutic would provide direct contact with the target tissue while limiting systemic absorption and toxicity, potentially allow for more frequent dosing schedules, and improve the efficacy of chemoradiation. However, the utility of locally administered chemotherapy in cervical cancer is yet to be examined. Therefore, the aims of the current study were two-fold; first, to assess the efficacy of the CerviPrep™ device in localizing gemcitabine delivery to the cervix while limiting systemic exposure to the drug, and second, to document any adverse effects that could be attributed to the local administration of gemcitabine.

Materials and Methods

Patient Recruitment and Enrollment

Women undergoing hysterectomies for the staging or treatment of ovarian, endometrial, or cervical cancer were recruited from the Women's Health Center at the University of Minnesota Medical Center-Fairview in Minneapolis, Minnesota. Subjects were deemed eligible if all inclusion/exclusion criteria were met. Patients were eighteen years of age or older and had adequate organ function within 28 days of study entry as defined by an absolute neutrophil count $\geq$1500 cells/mm^3; platelet count $\geq$100,000 cells/ mm^3; serum creatinine $\leq$2.5 mg/dL; serum total bilirubin $\leq$ 2.0 mg/dL; and serum AST or ALT $\leq$ 3 times the upper limit of normal. Additionally, patients were required to have a Gynecologic Oncology Group (GOG) performance status less than two. Patients with prior history of whole abdomen or pelvic radiation therapy were excluded, as were women with a known hypersensitivitiy to gemcitabine. Also ineligible were women concurrently using any other anti-cancer agents or any standard/experimental chemotherapeutic within 28 days of the surgical procedure. This study was approved by the University of Minnesota Institutional review board, and voluntary, written consent was obtained from all patients.

Gemcitabine Preparation and Administration

A gemcitabine dose of 100 mg/m^2 was compounded as a gel in Surgilube® lubricating jelly prior to the scheduled hysterectomy and stored at 4˚C until use. After the induction of general anesthesia but prior to the start of surgery, the compounded gel was applied directly to the cervix via a sterile CerviPrep™ device, and the device was

subsequently removed.

Sample Collection

All blood samples were approximately 10 mL in volume and were collected in heparin-containing tubes. Samples from the uterine vein were aspirated and collected thirty minutes after gemcitabine application but prior to clamping of the uterine vessels and subsequent removal of the uterus. Three separate peripheral blood samples were collected through an intravenous line located in one of the upper extremities at thirty, sixty, and ninety minutes. After sampling, blood specimens were immediately centrifuged and plasma harvested. Plasma was stored at -20˚C prior to analysis. A section of tissue from the lower uterine segment was collected *in vivo* by the University of Minnesota Tissue Procurement Services and frozen in liquid nitrogen. Tissue samples were stored at -80˚C prior to analysis.

Safety Monitoring

Any signs or symptoms of gemcitabine toxicity were documented and graded by the National Cancer Institute's Common Terminology Criteria for Adverse Events V. 3.0. Toxicity assessments were by performed by clinicians within 28 days of study entry, on the day of surgery, and at a post-operative visit approximately two to four weeks after surgery. All toxicities were reported to the University of Minnesota Institutional Review Board and the Clinical Trials Office's Cancer Protocol Review Committee.

Chemicals and Reagents

Acetonitrile, ammonium acetate, methanol, sodium phosphate, and octyl sulfonic acid were purchased from Fisher Scientific (Pittsburgh, PA). $^{13}C,^{15}N_2$-gemcitabine, $^{13}C,^{15}N_2$-dFdU and 2'deoxycytidine were purchased from Toronto Research Chemicals (North York, Ontario) while $^{13}C,^{15}N_2$-cytidine triphosphate (CTP) was purchased from Cambridge Isotopes (Andover, Massachusetts). The 2',2'-difluorodeoxycytidine (dFdC), 2',2'-difluorodeoxyuridine (dFdU), and 2',2'-difluorodeoxycytidine triphosphate (dFdCTP) standards used for mass spectrometry and HPLC analysis were synthesized by the Institute for Therapeutics, Discovery, and Development at the University of Minnesota, Minneapolis, Minnesota.

Gemcitabine and dFdU Analysis in Plasma

Plasma samples were processed and analyzed via HPLC-UV according to previously published methods [139]. Briefly, plasma samples were deproteinized with the addition of perchloric acid. After vortexing and centrifugation, 25 μL of the extract sample was injected onto a Waters Spherisorb 4.6 X 250 mm, 5 μm, C18 column at 40°C (Waters Corporation, Milford, MA, USA). The mobile phase (flow rate, 1.0 mL/min) consisted of 10:90 (v/v) acetonitrile (ACN)–aqueous buffer (50 mM sodium phosphate and 3.0 mM octyl sulfonic acid, pH 2.9). Gemcitabine, dFdU, and the internal standard, 2'-deoxycytidine (2'dC) were detected with UV wavelength set at 267 nm via an 1100 Variable Wavelength Detector with deuterium lamp (Agilent Technologies, Palo Alto, Ca, USA).

Gemcitabine and dFdU Analysis in Tissue

Pre-weighed sections of cervical tissue (~50 mg) were homogenized on ice in 600 µL of cold acetonitrile. After addition of internal standard ($^{13}C,^{15}N_2$-dFdC, and $^{13}C,^{15}N_2$-dFdU) and centrifugation, the lysate was dried and reconstituted in 100 µL of mobile phase. The sample was then injected onto a Phenomenex Fusion RP 50 X 3.0 mm, 3.5 µm, column at 30º C. The mobile phase (flow rate, 0.3mL/min) consisted of 5:95 (v/v) methanol:aqueous buffer (5 mM ammonium acetate, pH 6.8). The HPLC (Agilent 1200) was interfaced to a Thermo TSQ Quantum mass spectrometer with electrospray interface operating in positive ionization mode. Detection of dFdC and dFdU was obtained through selected reaction monitoring (SRM) of the following transitions: *m/z* 264/95 for dFdC, *m/z* 265/113 for dFdU, *m/z* 267/97 for ^{13}C, $^{15}N_2$ -dFdC (IS), and *m/z* 268/116 for ^{13}C, $^{15}N_2$ -dFdU (IS). The calibration range for this method was 2.4 – 4990 pmoles for dFdC and 4.0 – 8021 pmoles for dFdU.

dFdCTP Analysis in Tissue

The same sample preparation was performed as for dFdC and dFdU, only $^{13}C,^{15}N_2$- cytidine triphosphate (CTP) was used for an internal standard. Sample was injected onto a Thermo Scientific Bio Basix AX (50 x 2.1 mm, 5 µm) column at 30º C. Chromatographic separation was achieved with a gradient (0 min- 90:10, A:B; 0.51 min-50:50, A:B; 1.76 min- 0:100, A:B; 2.5 min-0:100, A:B; 8.5 min-90:10, A:B; 9.5 min-90:10, A:B; 12 min- 90:10, A:B) consisting of mobile phases A (30:70 ACN: 10 mM ammonium acetate in de-ionized water (DI), pH 6.0) and B (30:70 ACN: 1 mM

ammonium acetate in DI, pH 10.5). The LC-MS/MS system was run in negative ionization mode with the following SRM scan events: *m/z* 502/159 for dFdCTP and *m/z* 494/159 for $^{13}C9,^{15}N_2$-CTP. This method was linear within the range of 5 – 1000 pmol.

Results

Patient characteristics

Eighteen patients were enrolled in the study between January 2007 and February 2008. One patient was later excluded as a previously diagnosed malignancy was found to be benign. Clinical data for all remaining patients are presented in Table 2-1. The majority of women (76%) were diagnosed with either endometrial or cervical carcinomas. One woman had multi-site disease of both the endometrium and ovary. All women underwent either a total abdominal hysterectomy (65%) or a radical hysterectomy (35%). Doses of locally administered gemcitabine averaged 208 ± 87 mg in these seventeen patients (range 150-380 mg). When gemcitabine was not detected in the first two patients enrolled, IRB approval was obtained to increase the dose of gemcitabine to 200 mg/m^2. The sixth and seventh patients enrolled received the higher dose, whereas all other doses were 100 mg/m^2. No toxicities were reported by any of the subjects during the study period.

Analysis of gemcitabine and dFdU in uterine vein plasma

Quantifiable concentrations of gemcitabine were detected in the uterine vein of five patients (See Figure 2-2). Ranging from 4.4 to 26.6 μM, these levels are consistent with previously reported gemcitabine plasma concentrations observed following intravenous administration [140]. Three patients had detectable dFdU levels in the uterine vein (18-165 μM). Peripheral plasma concentrations of gemcitabine and dFdU were below the limit of quantitation in all patients at all time points assessed.

Analysis of gemcitabine and metabolites in cervical tissue

Tissue collected from the lower uterine segment yielded gemcitabine levels above the limit of quantitation in eleven of the fifteen specimens analyzed. After adjusting for the dose of gemcitabine applied, concentrations within the tissue ranged from 0.12 to 43.73 pmol/g tissue/mg gemcitabine (See Figure 2-3). Similarly, levels of dFdU ranging from 0.32 to 58.9 pmol/g tissue/mg gemcitabine were above the limit of quantitation in fourteen of the fifteen samples. Roughly half of the variability in dFdU levels could be accounted for by the level of gemcitabine (Figure 2-4). Despite a highly sensitive LC-MS/MS assay, dFdCTP levels were below the limit of quantitation in all samples analyzed.

Discussion

While chemoradiation remains the gold standard for the treatment of locally advanced cervical cancer, the risk of dose-limiting toxicities increases greatly when chemotherapeutics and radiation are combined. To overcome this limitation, most clinical studies have focused on optimizing doses of individual agents, attempting to define the lowest dose necessary to achieve the desired radiosensitizing effect while limiting systemic exposure and toxicity. However, the utilization of locally delivered gemcitabine in the treatment of other malignancies has proved promising, improving outcomes with minimal toxicity. For example, gemcitabine has been administered as an intravesical therapy for the treatment of superficial bladder cancers with good success. Results from Phase I studies suggest that intravesical administration is well tolerated, with minimal local toxicity and an absence of systemic toxicity [141,142]. Gemcitabine was undetectable in the peripheral circulation in doses up to 1500 mg, with dFdU present at concentrations less than 2 μM. At higher doses, gemcitabine levels in the plasma were still well below those observed after intravenous administration [143]. Similarly, neither gemcitabine nor dFdU were present in the systemic circulation of patients with neoplastic meningitis treated with intrathecal gemcitabine [144].

Based on these data, the objective of the current study was to assess whether or not localized delivery of gemcitabine to the cervix could be used to reduce the side effects associated with intravenous therapy while providing clinically relevant concentrations of drug to this target tissue. To our knowledge, this study is the first to demonstrate successful localization of gemcitabine to the uterine circulation via a novel drug delivery device.

Concentrations of gemcitabine detected in the uterine vein plasma after local delivery were similar to those reported previously after intravenous administration [140]. However, localized doses of gemcitabine used in this study were more than three-fold lower than those used when gemcitabine is given intravenously, suggesting that localized delivery of gemcitabine can limit overall exposure to chemotherapy both by taking advantage of the local anatomy and physiology that minimizes systemic exposure and by a reduction in dosage requirements. More importantly, uterine plasma concentrations were also within the range determined to be pharmacologically relevant. As gemcitabine is a prodrug, intracellular activation to the phosphorylated metabolite is required for cytotoxicity. The rate-limiting enzyme responsible for this conversion, deoxycytidine kinase, reportedly becomes saturated at plasma gemcitabine concentrations greater than 20 μM [101,145,146]. Hence, to achieve maximal rates of triphosphate accumulation, doses should be optimized to provide plasma concentrations in the range of 10-20 μM [140,147]. While parent gemcitabine was not detected in the uterine plasma of all patients, the average concentration in samples with measurable levels, 13.7 +/- 8.1 μM, was well within this recommended range.

A major strength and novelty of this study lies in our ability to perform *in vivo* sampling from the organ of interest, while the organ is still within the body, allowing for analysis of gemcitabine levels in both the circulation and tissue of the uterine cervix. The amount of gemcitabine detected in the tissue specimens from this study varied widely, from 10 to 6,559 pmol/g tissue (without adjusting for dose), with four samples having gemcitabine levels below the limit of quantitation. Yet, all samples had measureable amounts of dFdU (41-8835 pmol/g tissue). While gemcitabine is quickly converted to

dFdU in the plasma where it can then be transported into the tissue, there is no evidence for dFdU formation in the vaginal cavity. This suggests that all dFdU measured in the uterine tissue was formed within the tissue itself, implying that gemcitabine must have been present in all tissue samples if even at very low levels. A correlation was observed between the levels of dFdU formed in the tissue and the levels of dFdC detected in the same sample. However, the fact that this correlation was not stronger suggests that interindividual variation in intracellular metabolism of gemcitabine to its metabolites may contribute to the disposition of this compound in the cervix.

Reports of gemcitabine levels at the target site are scarce, making comparisons of gemcitabine distribution between tissues difficult. However, in one related study, gemcitabine was measured in tumor biopsies from patients with glioblastoma multiforme after intravenous administration at doses of 500 to 1000 mg/m^2 [148]. Similar to the current study, gemcitabine and dFdU levels in glioblastoma tissue were highly variable, ranging from 60 to 3580 pmol/g tissue and 29 to 60 nmol/g tissue, respectively. While tissue was collected in our study thirty minutes after the application of gemcitabine, collection occurred up to three and a half hours post-administration in the glioblastoma study, which may explain detection of substantially higher levels of dFdU in the brain. Both the glioblastoma study and a separate study examining phosphorylation of gemcitabine in patients with advanced head and neck cancers were able to detect low levels of the active dFdCTP moiety in biopsied tissue [137]. Interestingly, despite relatively high levels of gemcitabine, the levels of dFdCTP in the uterine tissue samples were too low to be quantified. This is not necessarily indicative of the absence of dFdCTP as the triphosphate metabolite is unstable, and its identification is highly dependent on sample

procurement times and processing procedures.

Extensive data also exist suggesting differential expression of the transporters and enzymes involved in the uptake and metabolism of gemcitabine between different tissues and carcinomas. These differences may be responsible for the highly variable levels of gemcitabine and dFdU detected in the cervical tissue. To enter the cell, hydrophilic nucleoside analogs, such as gemcitabine, utilize both concentrative and equilibrative transporters to cross the plasma membrane. The distribution of these concentrative (CNT) and equilibrative (ENT) nucleoside transporters is known to be tissue specific, such that certain tissues may be inherently more or less responsive to gemcitabine therapy [74]. Nucleoside transporter expression and activity may also be related to carcinogenesis. Significant relationships between low levels of ENT1 and CNT1, both highly involved in gemcitabine transport, and decreases in therapeutic efficacy of gemcitabine have been observed in patients with pancreatic adenocarcinoma and non-small cell lung cancer among other tumors [86,87,89,149]. Additionally, protein levels of CNT1, ENT1, and ENT2 were observed to vary considerably amongst gynecological tumors, with high levels of CNT1 loss noted in cervical tumors of the more severe, adenocarcinoma subtype [150].

Once inside the cell, deoxycytidine kinase (dCK) is the major enzyme involved in the phosphorylation of gemcitabine to its active form [135]. Low levels of this enzyme have been associated with a significant decrease in gemcitabine sensitivity, both in vitro and in vivo [97-99]. The expression of dCK in the cervix remains to be characterized. It is possible that dCK levels in this tissue are quite low, resulting in a slow conversion of gemcitabine to the triphosphate form. This may explain the minimal amount of dFdCTP detected in the cervical tissue from this study, especially as the samples were collected only thirty

minutes after gemcitabine application compared to the later collection times reported in other studies.

Similar to dCK, enzymes involved in the inactivation of gemcitabine may contribute to variability in patient response to therapy as well. Pharmacogenetic differences in cytidine deaminase, which converts gemcitabine to its inactive deaminated metabolite, have been reported to alter rates of gemcitabine deamination and affect the incidence of side effects associated with gemcitabine therapy [151,152]. In this study, the ratio of dFdU/dFdC was calculated to assess whether any differences in deamination of gemcitabine existed based on the disease site (data not shown). While sample sizes for each tissue group were too small to provide definitive conclusions, it appears that some differences in metabolism may be present. These data are intriguing as it suggests that levels of cytidine deaminase may differ in malignant versus healthy cervical tissue, which could explain the variability in gemcitabine and dFdU levels amongst subjects. Also, it is possible that gemcitabine may be more efficacious in the treatment of some gynecologic malignancies versus others.

In conclusion, we have successfully delivered gemcitabine to the cervix while limiting overall systemic exposure. As a result, detectable concentrations of gemcitabine and dFdU were measured in both uterine vein plasma and cervical tissue. No side effects were reported during this study, suggesting that local delivery can limit toxicity while achieving clinically relevant concentrations of drug at the target site. Additionally, our data provide evidence for variability in the uptake and/or metabolism of gemcitabine between subjects. These differences may be due to pharmacogenetics or possibly to changes in cervical tissue that may occur with carcinogenesis. Future investigations in

this laboratory are focusing on the cervical distribution of gemcitabine at a more molecular level, assessing the transport of gemcitabine across the cervical membrane in both the healthy and malignant cervix. Additional research into how differences in the expression and activity of metabolic enzymes may affect the cytotoxicity of gemcitabine in cervical cells is also ongoing. Data from such studies will be useful in optimizing the delivery of this radiosensitizing chemotherapeutic to the cervix of women with cervical carcinoma.

Average gemcitabine dose (mg)	208 (range 150-380)
Disease site	
Cervix	6 (35%)
Endometrium	7 (41%)
Ovary	2 (12%)
Uterus	1 (6%)
Endometrium/Ovary	1 (6%)
Histology	
Adeno	10 (59%)
Squamous	6 (35%)
Other	1 (1%)

Table 2-1: Characteristics of patients undergoing hysterectomy.

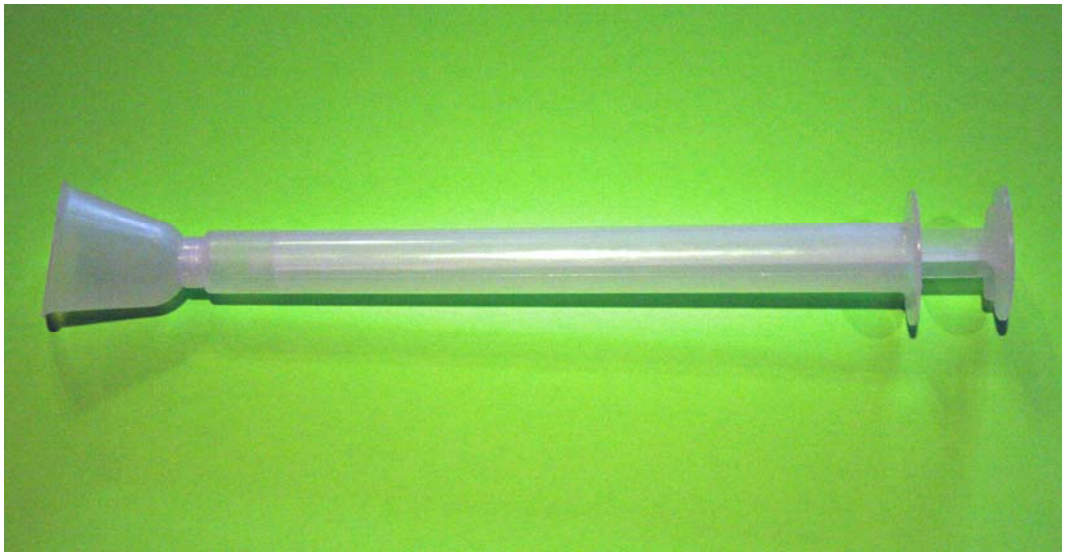

Figure 2-1: CerviPrep™ device used to deliver gemcitabine to the cervix.

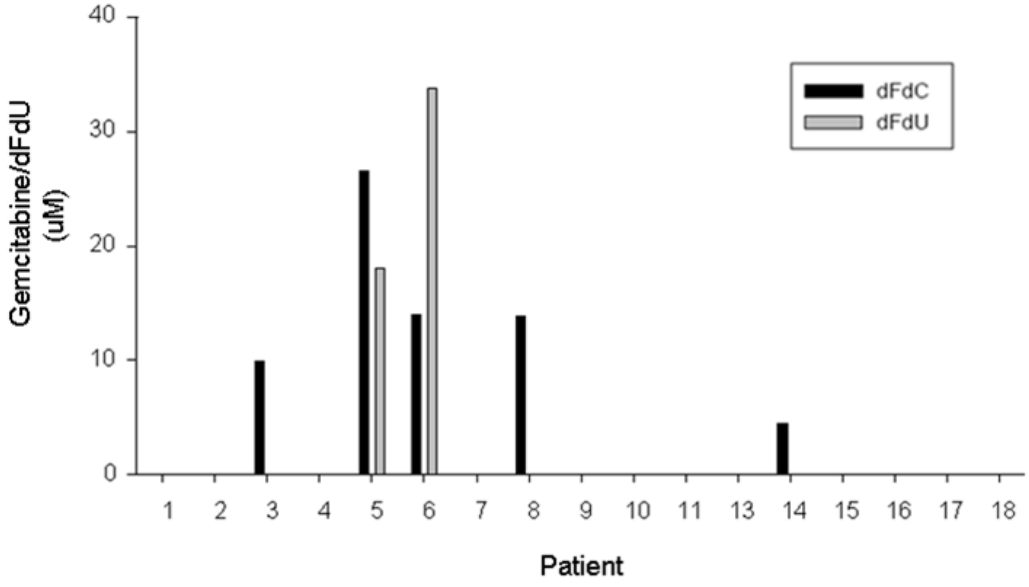

Figure 2-2: Concentration of gemcitabine (dFdC) and dFdU metabolite detected in plasma collected from the uterine vein. Levels of parent and metabolite were undetectable in all samples collected from the peripheral circulation.

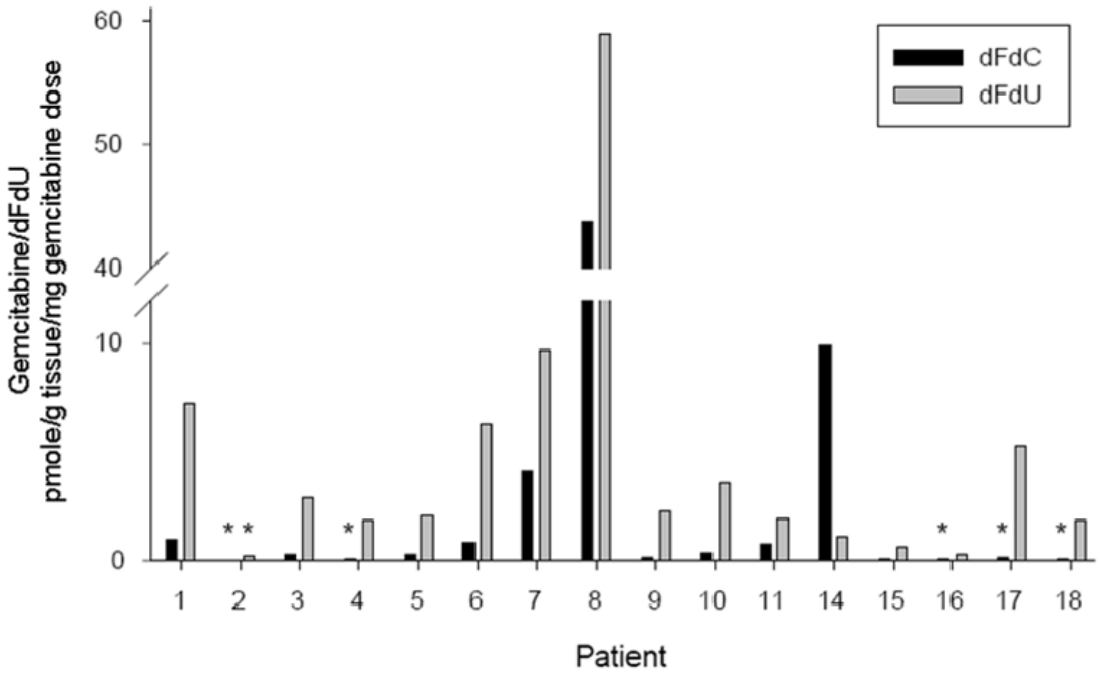

Figure 2-3: Amount of gemcitabine (dFdC) and dFdU metabolite detected in cervical tissue. All values were standardized to the dose of gemcitabine administered. *Values below the lower limit of quantitation.

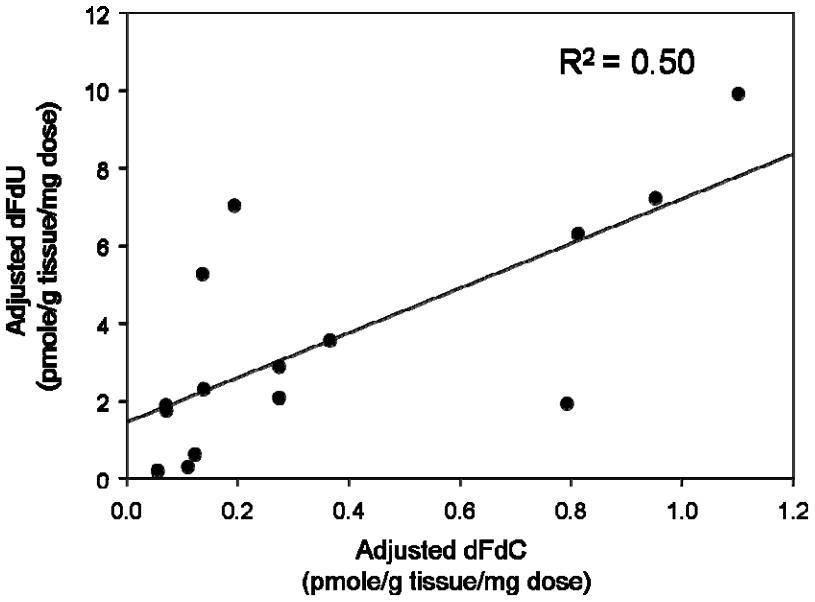

Figure 2-4: Correlation between cervical tissue levels of gemcitabine (dFdC) and dFdU in women receiving locally administered gemcitabine. Levels of both dFdC and dFdU were adjusted based on tissue weight and administered gemcitabine dose. Values for patient #8 (58.9 and 43.7 pmole/g tissue/mg dose for dFdU and gemcitabine, respectively) were excluded as they were mathematical outliers.

Chapter III

Characterization of Human Equlibrative Nucleoside Transporters in Normal and Malignant Cervical Tissue

Sections of this chapter were adapted from the original manuscript "Localized delivery of gemcitabine to the cervix *in vivo* for radiosensitization" by Hodge LS, Downs Jr. LS, Chura JC, Thomas SJ, and Tracy TS.

3.1 Introduction

Cervical cancer affects nearly a half million women worldwide each year, resulting in 270,000 deaths. While cisplatin remains the most commonly used therapy for the treatment of cervical carcinoma, use of gemcitabine, a deoxycytidine analog, is increasing due to its demonstrated efficacy as a single agent and in combination with cisplatin against locally advanced cervical carcinoma [68-70,153]. Used mainly for its radiosensitizing properties, gemcitabine requires intracellular activation prior to demonstrating any cytotoxicity. However, due to the hydrophilic nature of this compound, diffusion across lipid membranes is minimal. As such, gemcitabine and other nucleoside analogs utilize nucleoside transporters for cellular entry, upon which, this compound is further metabolized to its active triphosphate moiety [134].

Nucleoside transporters involved in mediating gemcitabine uptake belong to two gene families, SLC28 and SLC29, which encode the human concentrative (hCNT) and equilibrative (hENT) transporter proteins, respectively. Classified based on their mechanism of transport, hENT are bidirectional transporters mediating transfer across plasma membranes based solely on substrate concentration gradients. The hCNT transporters are considered secondary active transporters and move substrates against their concentration gradient into the cell, due to coupling with the intracellularly-driven sodium gradient. Specifically, hENT1 and hCNT1, and to a lesser extent, hENT2 and hCNT3, demonstrate a high affinity towards gemcitabine [154]. Yet, the ubiquitous tissue distribution of hENT compared with the more selective expression of hCNT suggests that the equilibrative transporters may have a larger contribution to gemcitabine transport[74]. High levels of both hENT1 and hENT2 protein have been strongly correlated with in vivo

efficacy of gemcitabine and other nucleoside analogs when used for the treatment of non-small cell lung cancer, mantle cell lymphoma, Hodgkin's and Non-Hodgkin's lymphoma, and pancreatic cancer [86,87,89,91]. These results suggest that the equilibrative transporters may be useful predictive markers of response to gemcitabine therapy.

While an individual's genetic makeup plays a large role in determining the level of transporter expression in a particular tissue, initial studies suggest that carcinogenesis is often associated with altered expression of these proteins, affecting tissue sentsivity to gemcitabine therapy [91,155]. Despite the increasing importance of gemcitabine in the treatment of cervical carcinoma, data regarding expression of equilibrative nucleoside transporters in this tissue is limited. An initial study in gynecological tumors reported a loss of hCNT1 staining associated with the severity of cervical carcinoma, with over 50% of tumors of the more severe adenocarcinoma subtype lacking this protein.[150] Levels of hENT1 and hENT2 staining in cervical tissue sections were reported to be highly variable as well, suggesting that some women may be inherently more likely to respond to gemcitabine therapy. To better understand the role of these transporters in determining the extent of gemcitabine distribution in the cervix, the aim of this study was to characterize the mRNA and protein expression of equilibrative nucleoside transporters 1 and 2 in human cervical tissue and to correlate these findings with cervical gemcitabine levels measured after local administration.

3.2 Materials and Methods

Tissue and Cell Lysate preparation

Malignant and normal human cervical tissue was collected by the Tissue Procurement Services at the University of Minnesota Medical Center, Minneapolis, MN and stored at -80ºC prior to processing. The tissue sections were weighed (50-150 mg) on a Petri dish and sectioned into small pieces on ice (1 mm). The tissue was transferred to a cold Dounce homogenizer containing extraction buffer (250 mM sucrose, 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$) supplemented with protease inhibitors and mechanically homogenized on ice. The lysate was then centrifuged at 4,000 rpm for ten minutes, and the supernatant collected and stored at -80ºC. Protein content was determined using the bicinchoninic acid assay (BCA) (Pierce, Rockfored, IL). Similarly, to collect lysate from cell monolayers, cells were washed once with cold PBS following media aspiration. Cold RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Triton-X 100, and 0.1% SDS) was applied directly to the cells, which were allowed to incubate on ice for ten minutes. Lysate was collected and centrifuged as described.

Immunoblotting

Tissue lysate (35 μg) was separated on a 4-20% polyacrylamide gel (Pierce, Rockford, IL) and transferred to an Immobilon-P polyvinyl difluoride membranes (Millipore, Billerica, MA) using a Mini-Trans-Blot Cell (Bio-Rad) at 230 mA for one hour. Transfer buffer consisted of 25 mM Tris and 192 mM glycine. Equal protein transfer onto the membrane was assessed via Ponceau S staining. The membrane was

then blocked in 5% non-fat milk for one hour followed by overnight incubation at 4°C with either polyclonal rabbit anti-ENT1 (Abgent, San Diego, CA) or anti-ENT2 (Abcam, Cambridge, MA) antibody. Following a wash step, the membrane was incubated in horseradish peroxidase-conjugated goat anti-rabbit (Santa Cruz Biotechnology, Santa Cruz, CA, 1:10,000 dilution in TBST) at room temperature. The membrane was treated with Pierce ECL Western Blotting Substrate (Pierce, Rockford, IL) followed by detection on high-performance chemi-luminescence film (Denville Scientific, Metuchen, NJ). If necessary, blots were then stripped in 0.1 M glycine, pH 2.5 stripping buffer, reblocked and reprobed with monoclonal mouse anti-beta actin (Abgent, San Diego, CA). The size and intensity of each band was determined using Adobe photoshop with all values normalized to β-actin. For band validation, membranes were incubated in primary antibody solution containing either hENT1 or hENT2 blocking peptides, and these blots were then compared to those prepared in the absence of blocking peptide.

In Vitro Endoglycosidase Treatment

Tissue and HeLa cell lysates were harvested as described previously. Twenty to forty micrograms of total protein were combined with 750 U of recombinant N-glycosidase F (PNGase F) (New England Biolabs, Ipswich, MA) as per the protocol supplied by the manufacturer. The reaction was allowed to proceed for sixty minutes at 37°C and then terminated by storage at -80°C. The reaction mixture was subjected to immunoblotting analysis as above.

qRT-PCR

Total RNA was isolated from cervical tissue using Trizol (Invitrogen, Carlsbad, CA) followed by purification with the PureLink RNA MiniKit (Invitrogen, Carlsbad, CA). Purity and concentration of RNA was determined using the A260/230 and A260/280 readings obtained with a NanoDrop ND-1000 spectrophotometer. A first-strand DNA template was synthesized from 1 μg of RNA using the AffinityScript QPCR cDNA Synthesis Kit (Stratagene, Santa Clara, CA). Samples were prepared in triplicate along with a control lacking reverse transcriptase to assess the presence of genomic DNA. No template controls were included. An ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) was used to perform quantitative real-time PCR using the Brilliant II SYBR® Green QPCR Master Mix (Stratagene, Santa Clara, CA) and 2 μL of cDNA template for amplification. Sequences for hENT1 and hENT2 sense and anti-sense primers were 5'-TCAGCCCACCAATGAAA-3' and 5'GGCCCAACCAGTCAAATA-3', and 5'-GCTCTTTGCCGTTTCTAATGG-3' and 5'-TCAGAGCAGCGCCTTGA-3' respectively[156]. Beta actin was used as an internal control (5'-CCCAGAGCAAGAGAGG-3' and 5'-GTCCAGACGCAGGATG-3')[157]. Reaction conditions included an initial activation step of 10 minutes at 95°C followed by forty cycles of ten seconds at 95°C and thirty seconds at 60°C and concluded with a melting curve analysis. The threshold cycle number indicating exponential amplification (C_t) was determined using the ABI 7900HT software and data analysis performed using relative quantification ($2^{-\Delta\Delta Ct}$). A validation experiment was performed to compare amplification efficiencies for hENT1, hENT2, and beta actin. Serial dilutions of the starting amount of RNA were analyzed on the ABI 7900HT as previously described, and the log DNA input

was graphed versus the ΔC_t(ENT – actin). The slope of this line was found to be less than 0.1 for both hENT1 and hENT2, allowing for the assumption of equal amplification efficiencies for all genes of interest. Reaction products from all samples were run on a 1% (w/v) agarose gel to confirm that all products were of the appropriate length for the primers used.

Statistical Analysis

Correlations between nucleoside transporter RNA, protein, and gemcitabine levels were assessed using SigmaStat 3.11 software (SyStat Software, Inc., Chicago, IL). Significance of the correlations was determined using Pearson's test. Comparisons in expression levels between normal and malignant tissue were performed using the Student t-test. Significance was set at a p-value of 0.05.

3.3 Results

Equilibrative nucleoside transporter mRNA expression in cervical tissue

To examine the relationship between the human equilibrative nucleoside transporters and gemcitabine disposition in the cervix, an investigation into the expression of hENT mRNA and protein in twenty normal and sixteen malignant cervical tissue samples was conducted. Expression levels of hENT mRNA were highly variable, with a 28-fold and nearly 60-fold range of variability for hENT1 and hENT2, respectively (Figure 3-1). This extensive variation was present in both malignant and normal tissue, and as a result, no significant difference in mRNA expression due to carcinogenesis was observed for either hENT1 or hENT2.

Equilibrative nucleoside transporter protein expression in cervical tissue

ENT protein levels were assessed via western blotting. Consistent with previous reports, hENT1 protein migrated as a band around 55 kDa. After subjecting lysates to deglycosylation treatment, this band shifted to 50 kDa, suggesting that hENT1 is glycosylated in cervical tissue (Figure 3-2). No differences in hENT1 glycosylation were observed between normal and malignant tissue.

Despite having a predicted molecular weight of 50 kDa, hENT2 protein is known to migrate slowly during SDS-PAGE, resulting in a band around 60 kDa. In our study, we observed that the banding pattern for hENT2 differed significantly depending upon how the protein was prepared prior to electrophoresis. As many membrane proteins aggregate upon heating at high temperatures, our methodology avoided a prolonged denaturation step at 100°C [158]. Instead, SDS and dithiothreitol were added to each sample

at final concentrations of 0.5% and 40 mM, respectively, followed by a twenty-minute incubation at 37°C. This procedure led to the isolation of one band at 100 kDa .This band was not only present in an hENT2-overexpressed lysate used as a positive control, but also disappeared upon addition of a synthetic hENT2 peptide to the primary antibody solution (Figure 3-3). As treatment of the lysate with PNGase F, an enzyme specific for N-deglycosylation, did not alter the migration of the 100 kDa band, this higher molecular weight was not likely due to extensive glycosylation (Figure 3-2). Based on these findings, it was determined that the band at 100 kDa was most likely a dimer of hENT2. Whether or not this dimer is present physiologically or is merely due to our preparation methods is not yet known. However, all quantification was based on the 100 kDa band.

Densitometry analysis produced ratios of hENT protein vs. β-Actin, which were then compared amongst normal and malignant cervical tissue samples (Figures 3-4 and 3-5). These ratios of hENT to beta-Actin were highly variable in cervical tissue, regardless of pathological status. Protein levels of hENT1 were found to be very similar between normal and malignant samples (1.64 +/- 1.41 vs. 1.50 +/- 0.72, p=0.728) (Table 3-1). However, after the removal of normal sample number two, a mathematical outlier, the average amount of hENT2 protein in malignant tissue was over twice that observed in the normal cervix (0.884 +/- 0.739 vs. 0.356 +/- 0.264, p=0.01).

Relationship between hENT Expression and Tissue Levels of Gemcitabine

Due to the wide variation in hENT protein levels observed in cervical tissue, the uptake of gemcitabine into tumor tissue may differ from patient to patient as well. As such, we were interested in determining whether hENT protein expression correlates with

gemcitabine distribution in the cervix, contributing to the wide variability in cervical levels of gemcitabine detected after locally administered therapy. To this end, expression levels of hENT1 and hENT2 protein were quantified in the cervical tissue of women participating in our previous clinical study and were correlated to the corresponding level of gemcitabine and dFdU detected in the same tissue samples. The amount of hENT1 and hENT2 mRNA detected in cervical samples from women participating in the CerviPrep™ study did not correlate with either the level of gemcitabine or dFdU present in the tissue (Figure 3-6). Protein levels were not correlated with cervical gemcitabine or dFdU either (Figure 3-7). Additionally, hENT protein not was related to the amount of hENT mRNA quantified in the same tissue samples (Figure 3-8). The level of hENT mRNA and protein expression was also independent of tumor grade and stage.

Discussion

As nucleoside transporters are required for the intracellular uptake of nucleoside analogs, including gemcitabine, their presence appears to be a prerequisite for adequate tissue distribution and therapeutic efficacy of these compounds. In vitro, a lack of hENT results in a diminished cytotoxic effect of gemcitabine, suggesting that limited uptake may be a mechanism of resistance to this nucleoside analog[84]. In vivo, mRNA and protein expression of hENT have been observed to correlate with patient outcome after gemcitabine therapy, supporting the clinical use of these transporters as prognostic and predictive markers of therapeutic efficacy.

Despite the increased use of gemcitabine as a radiosensitizer for the treatment of cervical carcinoma, knowledge of hENT expression in the cervix is limited. Initial immunostaining of hENT in cervical tumors indicated a wide variability in levels of nucleoside transporter protein, suggesting that some cervical tumors may be more sensitive to gemcitabine therapy than others[150]. For these reasons, the goal of the current study was to examine the pattern of hENT1 and hENT2 mRNA and protein expression in the normal and malignant cervix and to explore the relationship between hENT expression and cervical gemcitabine disposition in vivo.

In this study, tissue samples from twenty normal and sixteen malignant cervixes were analyzed for hENT mRNA and protein content. When considering all samples together, a 28-fold and 60-fold variability in the mRNA expression of hENT1 and hENT2, respectively, was observed. Even when the samples were separated according to pathological status, the high interindividual variability in transcript levels prevented a statistically significant difference in expression from being detected between the normal

and malignant groups. Additionally, mRNA levels of neither hENT1 nor hENT2 were associated with gemcitabine or dFdU levels in the cervix and were also not related to the level of hENT protein detected in the same samples.

Protein levels of hENT1 and hENT2 were highly variable as well, with hENT2 displaying more variability than hENT1. This heterogeneity is consistent with reports of hENT expression in tumor tissue from patients with lymphoma, breast, and pancreatic cancers[87,155,159]. The effect of this variation in hENT expression on patient response to gemcitabine therapy is unclear. Initial studies observed improved survival outcomes in patients with high hENT expression, implicating the necessity for hENT in the uptake of gemcitabine into tumor cells. For example, in a study of 105 patients with pancreatic cancer, patients in the lowest tertile of hENT1 gene expression had significantly poorer prognoses than those with the highest level of expression. Additioanlly, the presence of high levels of hENT1 was significantly associated with longer median overall survival times when compared with patients with lower expression of hENT1 (25.69 vs. 15.74 and 8.48 months)[113]. In a separate study of 45 patients with resected pancreatic adenocarcinoma treated with gemcitabine, the three-year survival rate for patients with high hENT1 staining of tumor tissue was over three-fold higher than the survival rate for patients with low or no staining[160].

Yet, more recent studies describe an inverse correlation between hENT expression and patient outcome. Santini et al. observed a decrease in overall survival and disease-free survival associated with overexpression of hENT1 immunostaining in patients with resected gastric cancer[161]. Similarly, a study of patients with refractory Hodgkin's lymphoma observed hENT1 positive staining of Reed-Sternberg cells to be an

independent predictor of gemcitabine-based treatment failure[162].

As this was not a treatment study, the relationship between hENT protein expression and overall patient outcome in patients treated with gemcitabine for cervical carcinoma remains unknown. Yet, this is the first study to directly examine the relationship between the expression of equilibrative nucleoside transporters and tissue disposition of gemcitabine in vivo. While our sample size was small, no association was observed between hENT expression and either gemcitabine or dFdU levels in the cervix. Assuming intratumoral gemcitabine concentration to be a good marker of cytotoxicity, these results suggest that since hENT had no effect on cervical gemcitabine concentrations, it is unlikely that hENT would be associated with toxicity either.

In fact, despite the previously mentioned studies suggesting a relationship between hENT and gemcitabine efficacy, there are other reports which are consistent with our data, finding no association between transporter expression and patient outcome. Ferrandina et al. reported a relationship between neither hENT1 nor hCNT1 mRNA expression in ovarian carcinoma and any survival outcome despite observing a strong relationship between the expression of ribonucleotide reductase and overall survival[163]. A lack of correlation was obserbed between hENT1 protein in breast cancer patients and patient response to gemcitabine as well[155]. This lack of a significant relationship may be partly accounted for by the common misconception that hENT protein levels are an adequate marker of hENT functional activity, which is highly associated with nucleoside analog cytotoxicity but much more difficult to measure in vivo. As hENT1 expression is not limited to the plasma membrane but is also detected on the mitochondrial membrane, and both hENT1 and hENT2 undergo post-translational modification to aid with

membrane trafficking and function, and as such it is not surprising that a correlation between hENT protein and activity is often lacking[76,77,164]. Thus, these conflicting clinical results suggest that the strong relationship between expression of hENTs and the clinical efficacy of gemcitabine observed in pancreatic adenocarcinoma may not be applicable towards all tumor types, and that expression levels of hENTs may not be as useful as a general predictor of patient outcome after gemcitabine therapy as was previously thought.

While protein levels of hENT1 and hENT2 have been reported to vary considerably amongst gynecological tumors, this is the first study comparing hENT expression in malignant versus healthy cervical tissue[150]. hENT2 expression was nearly three-fold higher in malignant cervical tissue, suggesting potential up-regulation of this transporter during carcinogenesis. This upregulation may reflect an increased need for nucleosides in malignant cells due to heightened proliferation rates when compared with normal tissue. While mechanisms of hENT2 regulation remain poorly understood, expression of hENT1 protein is known to correlate with cellular proliferation, with higher rates of replication associated with an increase in hENT1 abundance[165]. In HeLa cells, the number of NBMPR-binding sites (a measure of hENT1 protein) doubled between late G_1 and S phases, resulting in a corresponding doubling of V_{max} values for cytidine and adenosine transport[166]. Additionally, hENT1 expression in immortalized cell lines is significantly higher than in their respective primary counterparts[167]. Additionally, while sample sizes were small, an analysis of tissue from 25 ovarian carcinomas observed that those of the more aggressive clear cell subtype had significantly more hENT1 protein than the more common serous carcinoma, possibly corresponding to an increased rate of proliferation[163]. Since hENTs are bidirectional transporters, an increase in hENT protein

due to increased proliferation could also be associated with an increase in efflux of the nucleoside analogs used to treat these tumors, providing a mechanism of inherent resistance. Yet, despite the increase in hENT2 protein observed in malignant cervical tissue, the level of hENT1 transporter protein was relatively similar between normal and malignant samples, suggesting not only that carcinogenesis has no effect on the expression of cervical hENT1, but also the possibility of differential regulation of the equilibrative transporters in the cervix.

In conclusion, this is the first study to quantify the expression of hENT1 and hENT2 mRNA and protein in cervical tissue. While levels of these transporters were highly variable in the cervix, no relationship between hENT expression and in vivo gemcitabine disposition in this tissue was observed, suggesting that hENT expression may not be an optimal predictor of clinical outcome in patients receiving gemcitabine for cervical carcinoma. However, further confirmatory studies examining the association between hENT expression and activity in the cervix and therapeutic efficacy are warranted.

Protein	Mean	SD	Significance
hENT1			
Normal (n=20)	1.64	1.41	p=0.73
Malignant (n=15)	1.50	0.72	
hENT2			
Normal (n=19)	0.36	0.26	p=0.01
Malignant (n=14)	0.88	0.74	

Table 3-1: Protein expression of hENT1 and hENT2 in cervical tissue as determined by western blotting. Protein amounts were calculated as the ratio of hENT to β-actin protein as determined by densitometry. A p-value of less than 0.05 was considered statistically significant.

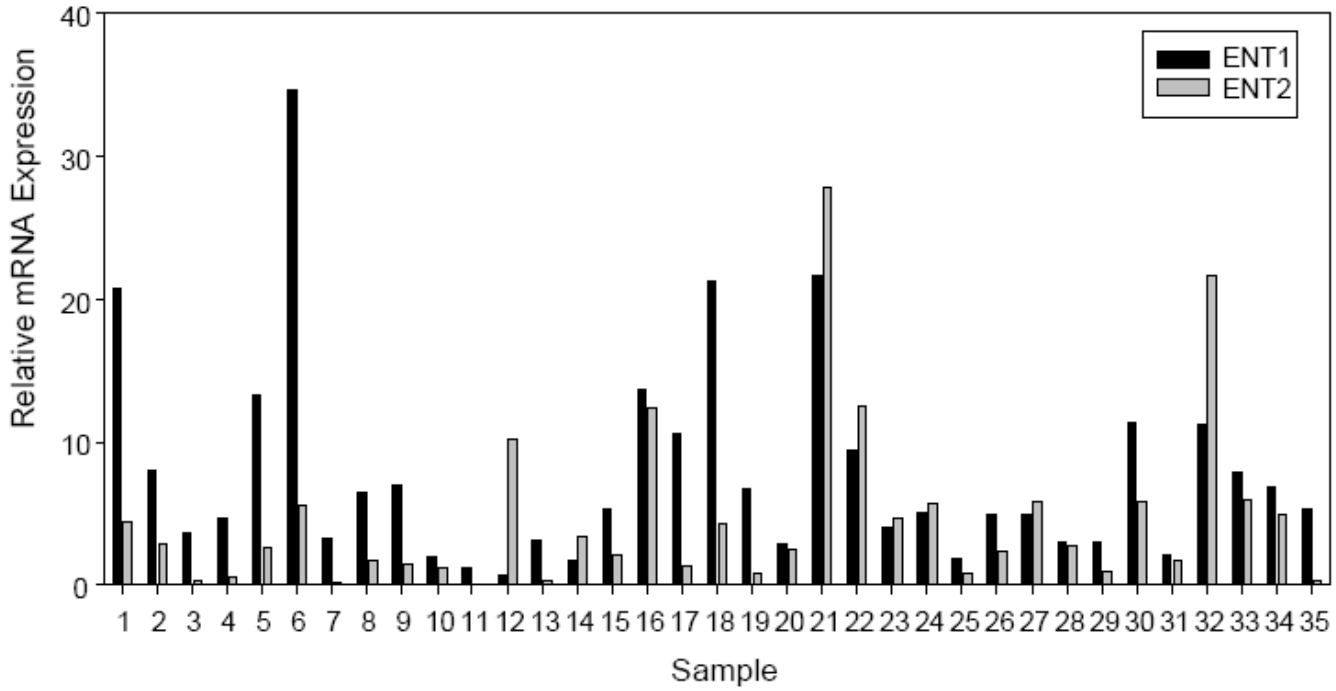

Figure 3-1: hENT mRNA expression determined by real-time PCR quantitation (SYBR-Green) in RNA from normal and malignant cervical tissue. Values are the ratio of hENT expression relative to that of the internal control, β-Actin. Samples 1-20 were extracted from normal cervical tissue with samples 21-35 extracted from malignant cervical tissue.

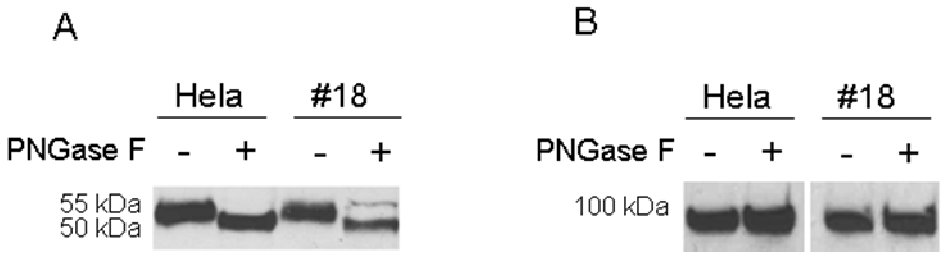

Figure 3-2: Representative western blots of A) hENT1 and B) hENT2 from HeLa and tissue (#18) lysates subjected to N-linked deglycosylation via incubation with PNGase F for one hour. Analysis for hENT2 was performed based on the 100 kDa band.

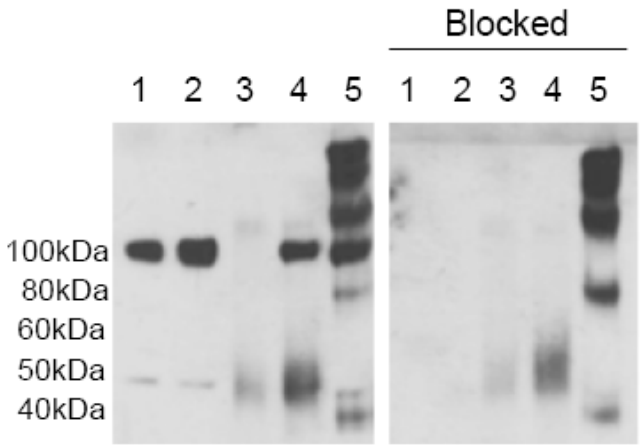

Figure 3-3: Immunoblotting of hENT2 protein in the presence and absence of synthetic hENT2 peptide demonstrating specificity of anti-hENT2 antibody to 100 kDa band. HeLa cell lysate (Lanes 1), purified hENT2 protein (Lanes 2), negative control (bovine serum albumin, Lanes 3), tissue lysate loaded after the addition of 0.5% SDS and 40 mM DTT and heating at 37ºC for twenty minutes (Lanes 4), and tissue lysate loaded after a ten minute incubation at 100 ºC (Lanes 5). Amount of hENT2 blocking peptide added to the primary antibody solution was fifteen-fold greater than the amount of hENT2 antibody.

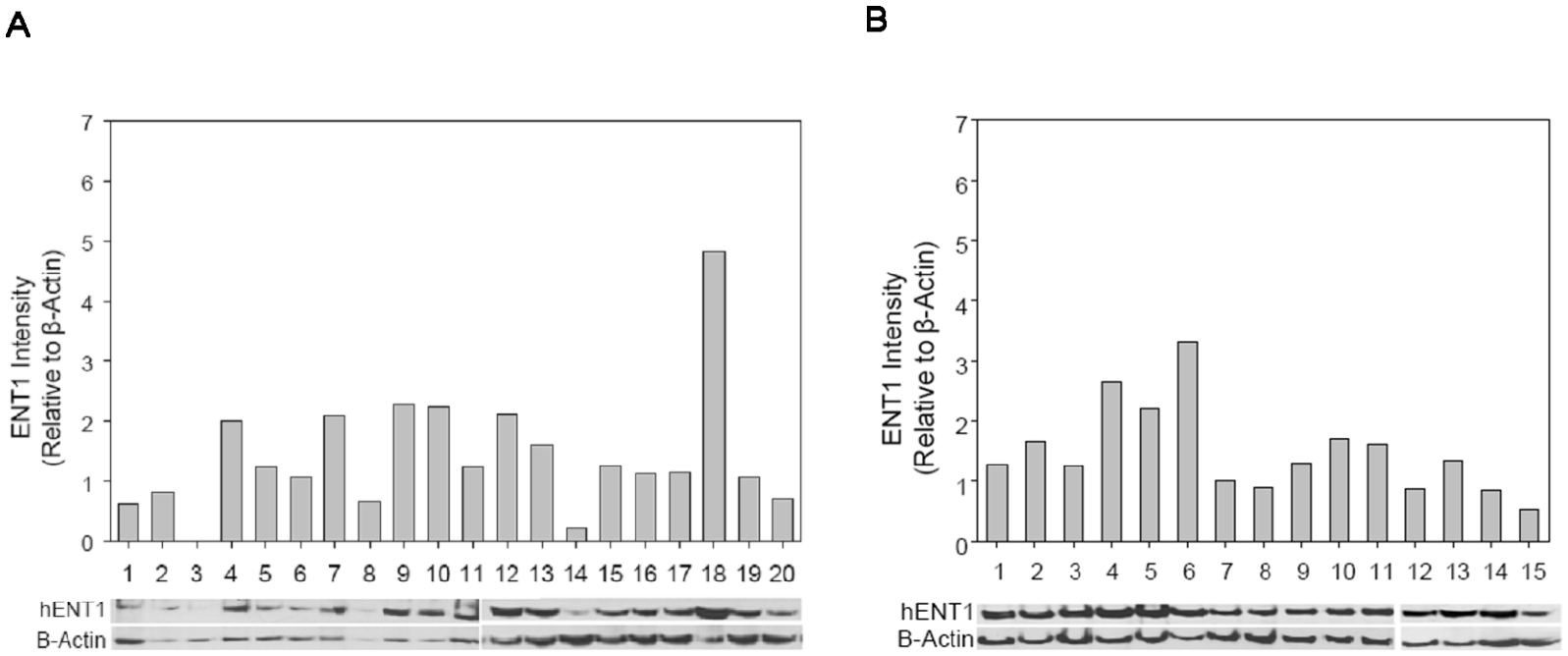

Figure 3-4: hENT1 protein expression in malignant versus normal cervical tissue. Normalized hENT1 protein levels in A) twenty normal and B) fifteen malignant cervical tissue samples. Protein amounts were calculated as the ratio of hENT1 to β-Actin protein as determined by densitometry. The respective western blots for hENT1 and corresponding β-Actin controls are shown below for all samples.

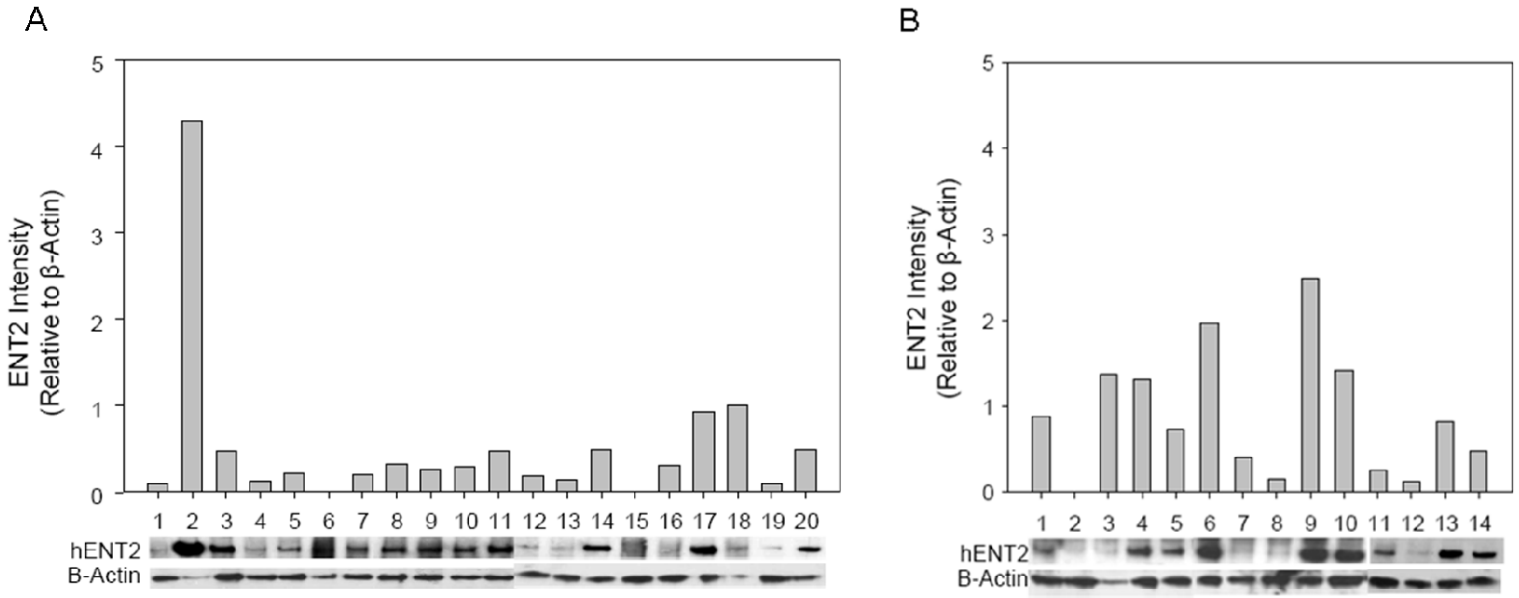

Figure 3-5: hENT2 protein expression in malignant versus normal cervical tissue. Normalized hENT2 protein levels in A) twenty normal and B) fourteen malignant cervical tissue samples. Protein amounts were calculated as the ratio of hENT2 to β-Actin protein as determined by densitometry. The respective western blots for hENT2 and corresponding β-Actin controls are shown below for all samples. The band at 100 kDa was used for quantification of hENT2 protein.

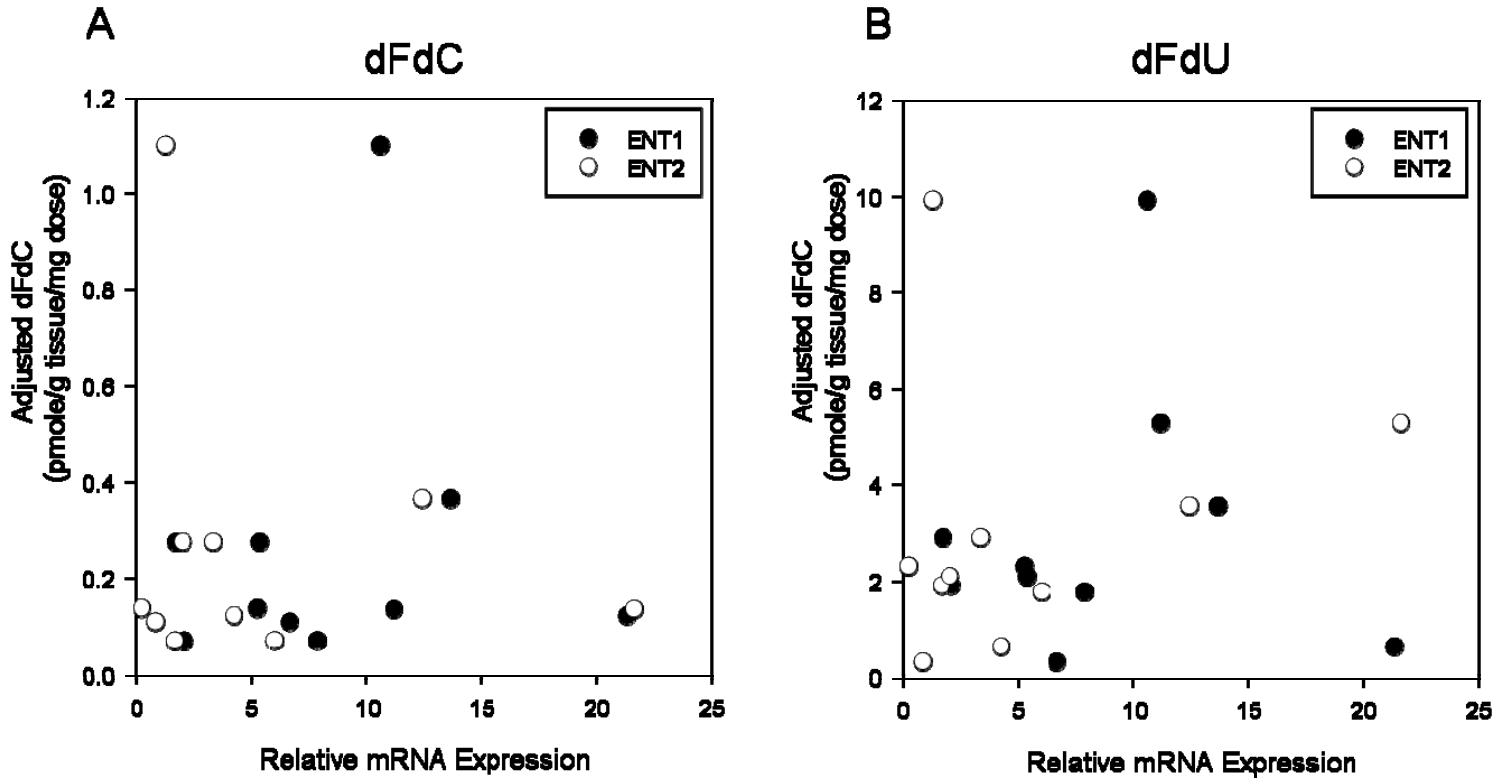

Figure 3-6: Correlation between hENT1 and hENT2 mRNA levels with A) dFdC levels or B) dFdU levels in cervical tissue harvested after localized delivery of gemcitabine via the CerviPrep ™ device. The mRNA levels are normalized relative to beta actin. dFdC and dFdU levels are adjusted per gram of tissue analyzed and dose of gemcitabine administered.

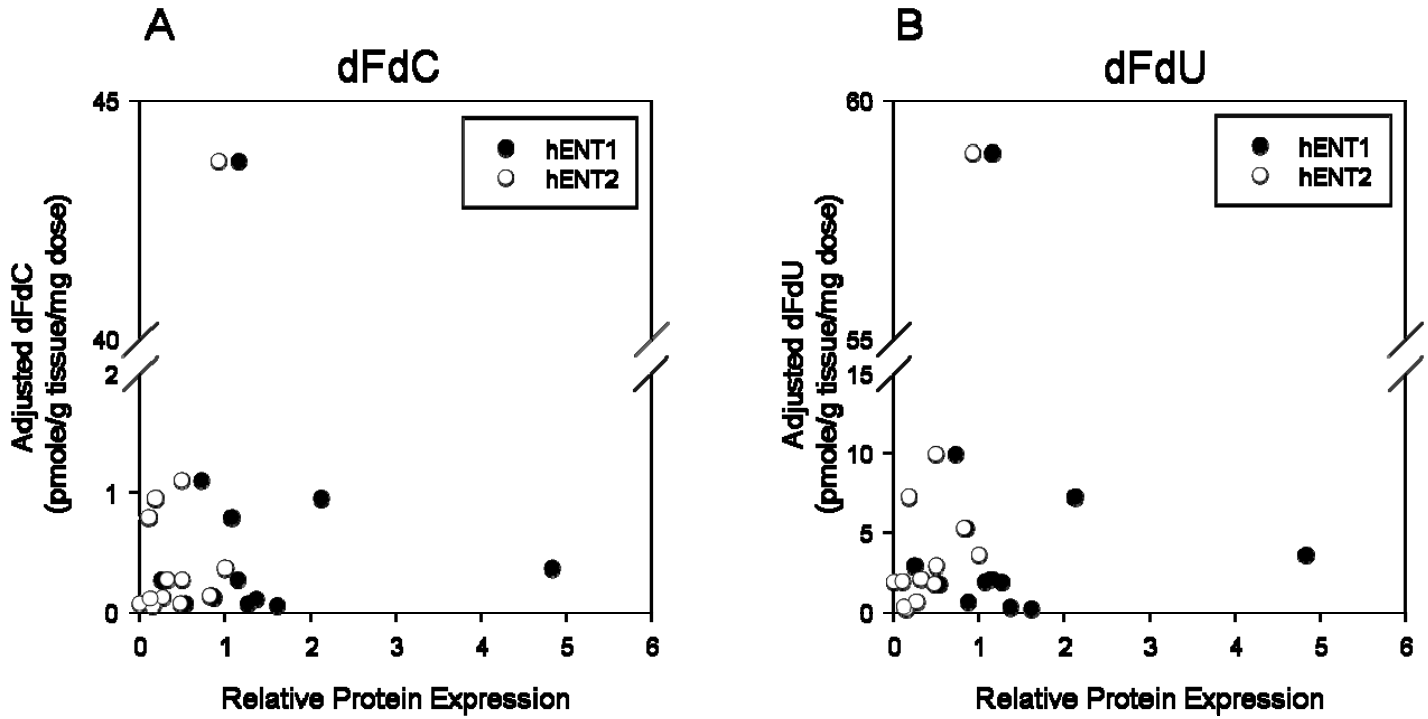

Figure 3-7: Correlation between hENT1 and hENT2 protein levels with A) dFdC or B) dFdU in twelve cervical tissue samples harvested after localized in vivo delivery of gemcitabine via the CerviPrep ™ device. Protein levels are normalized relative to β-Actin. dFdC and dFdU levels are adjusted per gram of tissue analyzed and gemcitabine dose administered.

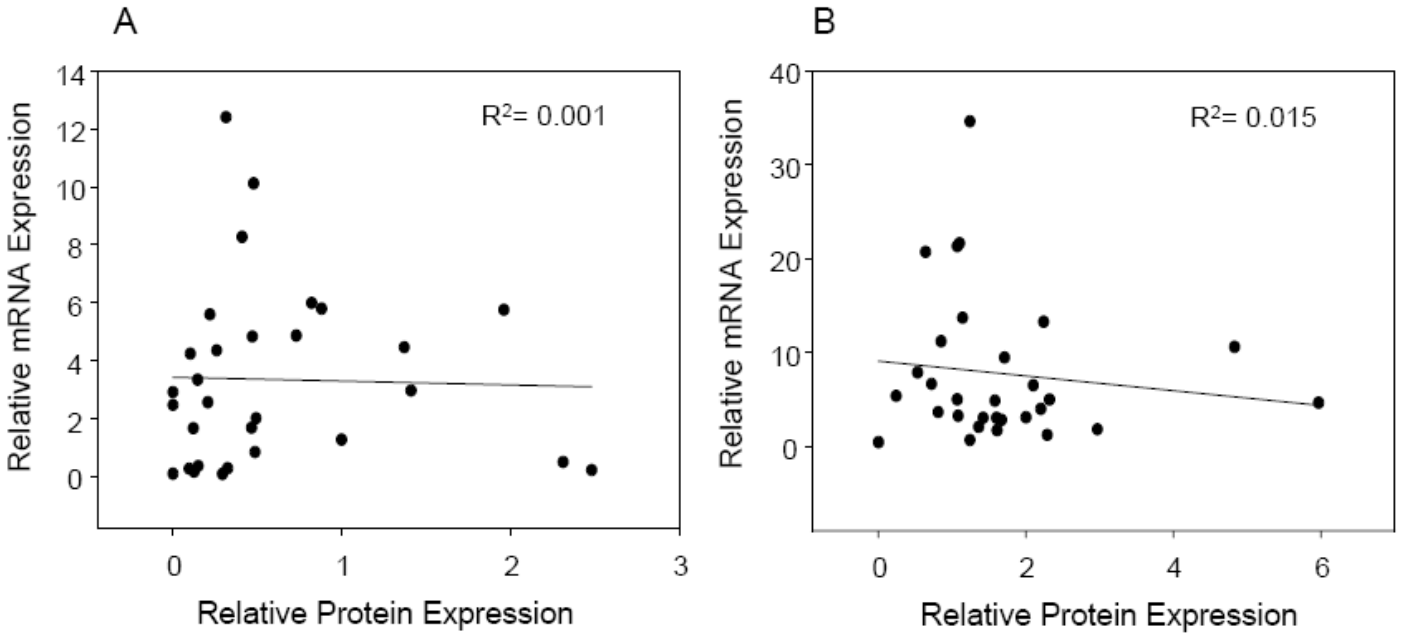

Figure 3-8: Correlation between A) hENT1 and B) hENT2 protein expression levels with the corresponding level of gene expression in cervical tissue. Data sets include both normal and malignant cervical tissue samples collectively. R^2 values are included.

Chapter IV.

Initial System Validation with Uridine as a Substrate

Introduction

Uridine is a pyrimidine nucleoside, formed by the conjugation of the nucleobase, uracil, to a ribofuranose ring (Figure 4-1). One of the four main nucleosides involved in RNA synthesis, uridine is also able to serve as a substrate for the salvage pathway of pyrimidine nucleotide synthesis and as a precursor for uridine diphosphate and triphosphate, which are required for proper functioning of the glycolysis pathway[168].

Uridine is the predominant circulating pyrimidine in humans, most likely the result of conversion of cytidine to uridine by cytidine deaminase, which is present at high circulating plasma levels[169]. As such, plasma concentrations of uridine in humans range from 2.5 μM in children to 5.3 μM in adults, with intracellular concentrations reaching 15.4 μM[170].

Uridine is one of the most extensively studied substrates of the nucleoside transporters, and to date, it has been reported to be transported by hENT1-3 and hCNT1-3. hENTs are involved in low-affinity transport of uridine, with K_m values of 0.24 mM, 0.2 mM, and 2 mM for hENT1, hENT2, and hENT3, respectively[84]. As with other nucleosides, the rapid intracellular conversion of uridine to its phosphorylated metabolites, with initial monophosphorylation occurring via uridine/cytidine kinase (UCK), makes examination of nucleoside transporter-mediated uptake of uridine more challenging in cell-based systems. As such, typical methodologies utilize short incubation periods, frequently less than twenty seconds in length, to limit formation of the uridine phosphates.

While the focus of this thesis research is on gemcitabine and its metabolites, in order to validate both the cell culture system and experimental techniques, transporter

assays were first performed with radiolabeled uridine. Results from these experiments were then compared with previous characterizations of uridine transport via equilibrative nucleoside transporters and were observed to be consistent with these previous studies. Based on this information, studies were then conducted with gemcitabine and its metabolites, working under the assumption that any novel findings concerning gemcitabine transport were due to the unique characteristics of its transport and not due to differences in experimental design. This chapter presents the findings of these initial validation experiments with uridine.

Materials and Methods

Chemicals

Uridine, dilazep hydrochloride, and nitrobenzyl-mercaptopurine riboside (NBMPR) were obtained from Sigma-Aldrich (Saint Louis, MO). ^{3}H-uridine was obtained from Moravek (La Brea, CA). Gemcitabine (2',2'-difluorodeoxycytidine, dFdC) and 2'2'-difluorodeoxyuridine (dFdU) were synthesized by the Institute for Therapeutics, Discovery, and Development at the University of Minnesota. All other chemicals were obtained from Fisher Scientific (Pittsburgh, PA), unless specified.

Cell Culture

HeLa cells, derived from a cervical adenocarcinoma, were obtained from ATCC (Manassas, VA). Cells were grown as a monolayer at 37ºC under 5% CO_2 in Dulbecco's Minimum Essential Media (DMEM) supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 ug/mL streptomycin, and fortified with 10% FBS. Cells used in transporter experiments were in the exponential growth phase and had undergone between five and twenty passages.

Transporter Assays

HeLa cells were seeded in 12- or 24-well plates and allowed to adhere overnight. Cells had achieved approximately 80% confluence by the start of the experiment. For radiolabeled experiments, tritiated uridine was combined with cold uridine to make a 5 μM tracer solution in transport buffer (128 mM NaCl, 4.73 mM KCl, 1.25 mM $CaCl_2$, 1.25 mM $MgSO_4$, and 5 mM HEPES, pH 7.4). The 5 μM concentration of uridine was

used to mimic physiological levels of uridine detected in the extracellular compartment. Incubations were initiated by aspirating the media from the cells, which were then loaded with tracer solution. Plates were incubated at 37º C on a rotary shaker for a time period ranging from three seconds to twenty-four hours, after which drug was removed, and cells washed three times in ice-cold transport buffer containing 50 μM diazep. Cells were solubilized in 1% Triton X-100, and 200 μL of each solubilized cell fraction was added to 4 mL of scinitillation cocktail (ScintiSafe Econo Cocktail, Fisher Scientific, Pittsburgh, PA) Radioactivity was then determined with a Beckman Coulter LS-6500 liquid scintillation counter (Fullerton, CA). Total protein concentration in each well was determined by the BCA protein assay (Pierce, St Louis, MO).

Inhibition experiments

To determine the effects of unlabeled dFdU and dFdC on uridine uptake, concentrated stock solutions of dFdU or dFdC were prepared in either a 1:1 solution of DMSO:transport buffer or 100% transport buffer, respectively, and added directly to the uridine tracer solutions where indicated. The organic concentration of each solution was kept below 0.5%.

hCNT and hENT-mediated transport

To assess the transport of uridine via hCNT and hENT, initial experiments were performed in HeLa cells to assess the presence of endogenous nucleoside transporters in this cell line. To determine if hCNTs were expressed in these cells, uptake studies using radiolabeled uridine were performed in transport buffer containing 128 mM choline

chloride in place of sodium chloride, as hCNTs require sodium ions to move nucleoside substrates against their concentration gradient. To examine uridine uptake by hCNTs (i.e., in the absence of hENT), cells were pre-incubated for thirty minutes prior to the start of the incubation in 50 μM dilazep (a known inhibitor of hENT), and 50 μM dilazep was also added to individual tracer solutions to assure consistent dilazep concentrations in the incubation media. The temperature dependence of hENT-mediated uridine transport was also assessed by performing experiments at 4°C.

hENT2-mediated transport

To examine the effects of hENT2-mediated transport alone, cells were pre-incubated for thirty minutes prior to the start of the experiment in 100 nM NBMPR (a known inhibitor of hENT1), and 100 nM NBMPR was also added to individual tracer solutions, again to assure adequate concentrations of NBMPR in the incubation media.

Statistical Analysis

All experiments were performed in triplicate and values are presented as the mean ± standard deviation. Statistical comparisons were performed using the Student's t-test via SigmaStat 3.11 software (SyStat Software, Inc., Chicago IL). Differences between groups were considered statistically significant when the p-value was <0.05.

Results

Nucleoside Transporter Activity in HeLa Cells

To better characterize the endogenous activity of the nucleoside transporters in HeLa cells, initial experiments were performed to assess the contribution of hCNTs and hENTs to uridine transport in this cell line. As hCNTs require sodium ions for activity, any loss of uridine uptake occurring with the use of choline chloride-based transport buffer, in place of sodium chloride, is attributable to hCNT transport. Consistent with previously published reports, no significant difference in uridine uptake occurred whether or not sodium was present in the transport buffer, suggesting that hCNTs are not endogenously present in HeLa cells (data not shown).

Pre-treatment of cells with 50 μM dilazep (inhibition of hENT1 and hENT2) prior to performing incubations resulted in minimal accumulation of radiolabeled uridine, suggesting limited passive diffusion of this hydrophilic nucleoside and lack of transport via other endogenous proteins expressed in HeLa cells (Figure 4-2). Additional incubations with NBMPR, which selectively inhibits hENT1 at low concentrations, also demonstrated a decrease in intracellular uridine levels, although not to the same extent as that noted in the presence of dilazep. This indicates that hENT1 and hENT2 are endogenously active in HeLa cells, and that uridine uptake can occur via both of these equilibrative transporters.

Finally, incubations were performed at 4°C, a temperature at which the function of most active transporters is significantly inhibited. While a decrease in uridine uptake was observed at 4°C, the uptake was still much higher than that measured in the presence of dilazep and NBMPR (Figure 4-2). This is consistent with facilitative diffusion via

hENTs as opposed to active transport processes involving ATP.

Uptake of Uridine over Short Incubation Periods (less than one minute)

Initial incubations with uridine were performed for less than twenty seconds, consistent with previously published experimental methods for examining transport via hENT (Figure 4-3). Uptake of uridine achieved saturation after approximately ten seconds, indicative of a transporter-mediated process. Also consistent with a transporter-mediated mechanism of uptake is the decrease in total uridine accumulation observed after addition of 15 μM unlabeled uridine to the radiolabeled tracer solutions. Both 100 μM dFdU and 5 μM dFdC also significantly decreased uptake of uridine at twenty seconds by nearly forty percent.

Accumulation of Uridine over Long Incubation Periods (up to one hour)

To examine long-term uridine accumulation, incubations were performed over intervals varying from one minute to one hour. When both hENT1 and hENT2 were active, uridine accumulation approached its maximum at around ten minutes (Figure 4-4A). The addition of 40 μM unlabeled gemcitabine to uridine tracer solutions resulted in a significantly higher net accumulation of radiolabeled uridine, such that by sixty minutes, the intracellular levels achieved were nearly twice those observed in the absence of dFdC. An even more significant effect was noted in the presence of 100 μM dFdU, increasing the net accumulation of tritiated radiolabel over three-fold at thirty minutes.

To allow for explicit examination of the role of hENT2 in uridine disposition, cells were pretreated with 100 nM NBMPR (inhibitor of hENT1). While net

accumulation was significantly decreased when compared to cells with both active hENT1 and hENT2, intracellular radiolabel was still detectable, suggesting that uridine is transported via both of these equilibrative transporters (Figure 4-4B). However, net accumulation of uridine at one minute decreased by 2-fold, and at sixty minutes decreased by >9-fold compared to incubations conducted under conditions when both transporters are active, suggesting that the majority of uridine transport into HeLa cells occurs via hENT1. When unlabeled dFdC and dFdU were added to NBMPR-treated cells (i.e., potential competition for hENT transport), a decrease in total accumulation of uridine was observed at nearly all time points suggesting that all three agents (uridine, dFdC and dFdU) are transported by hENTs.

Discussion

Uridine is an endogenous pyrimidine base formed by both de novo synthesis and the salvage of nucleosides in humans. Plasma concentrations of uridine range from 3-5 μM and remain tightly regulated across many species, with uridine degradation and formation under homeostatic control of the liver[171]. In addition to its widely known role in the synthesis of RNA, evidence suggests that uridine is an integral component in the regulation of many physiological functions. For example, high concentrations of uridine detected in seminal fluid support a function for this pyridimine in spermatogenesis[172]. Similarly, low levels of uridine have been measured in patients with prostatitis, suggesting that uridine may play a role in the etiology of this disease. Uridine also has been observed to be involved in modulating the peripheral nervous sytem, as low levels of pyrimidines, including uridine, have been associated with autism, convulsions, mental retardation, and decreased motor coordination[173]. Recently, evidence suggests that uridine may be beneficial in recovery from ischemic injury to the heart as well as with neuronal degeneration associated with diabetic neuropathy in the brain[174,175].

As an endogenous substrate for nucleoside transporters including hENTs, uridine is a useful experimental probe for hENTs in HeLa cells. These initial experiments with uridine confirmed that hENT1 and hENT2 are endogenously expressed in HeLa cells, whereas hCNTs are not[84,176]. As such, HeLa cells are an ideal cellular model for characterizing nucleoside transport via hENTs in an endogenous system.

Despite having a 50% amino acid sequence homology, significant differences exist in the capacity of hENT1 and hENT2 to bind both substrates and inhibitors. These differences can be exploited to evaluate the individual contributions of hENT1- or

hENT2-mediated nucleoside transport. In fact, the early classification system for equilibrative nucleoside transporters was based on their relative sensitivity (*es*) or insensitivity (*ei*) to inhibition by NBMPR. For example, hENT1, considered an *es*-type transporter, is competitively inhibited by very low concentrations of NBMPR, with a reported K_i value of 5 nM[81]. However, hENT2, an *ei*-type transporter, displays a loss of activity of less than 15% in the presence of NBMPR in concentrations upwards of 1 μM. These initial studies with uridine in HeLa cells confirmed the utility of NBMPR as a potent inhibitor of hENT1, allowing for a direct comparison of hENT1 and hENT2 activity.

hENT1 is typically thought to have a higher affinity for uridine than hENT2[84]. The current studies are congruent with this argument which was confirmed when uridine was incubated for both short (< 1 minute) and longer incubation periods (1 minute to one hour). When cells were pretreated with the hENT1 inhibitor NBMPR, the intracellular uridine concentration was less than half of what was observed initially, suggesting that hENT1 is involved to a greater extent in uridine transport than hENT2, at least over the time periods measured in these experiments.

In addition to differences in NBMPR binding and inhibition, hENT1 and hENT2 also differ in their sensitivities to inhibition by coronary vasodilators. For example, hENT2 is two and three orders of magnitude less sensitive to dipyridamole and dilazep, respectively, than hENT1[177]. Chimeric proteins derived from rat and human hENT1 have identified the N-terminal half of this protein, containing transmembrane domains 3-6, to be critical for the interaction between dilazep and hENT1[178]. Similar areas of amino acid sequence deemed to be critical for substrate/inhibitor binding have been identified for

hENT2 as well, with an Ile33Met mutation in this same transmembrane region significantly altering the sensitivity of this protein to inhibition via dilazep[179].

Despite the reduced sensitivity of hENT2 to inhibition by dilazep, the 50 μM concentration of dilazep used in the present validation studies was significantly higher than the reported IC_{50} values for inhibition of both hENT1 and hENT2 of 5 nM and 0.356 μM, respectively[79]. Thus, with the transport activity of both hENT1 and hENT2 sufficiently inhibited by dilazep, any remaining uptake was attributed to passive diffusion. The addition of dilazep inhibited nearly all accumulation of intracellular uridine demonstrating that as hydrophilic nucleosides, compounds such as uridine and gemcitabine require specific transport systems for cellular influx and efflux and generally are not capable of crossing cell membranes extensively via passive diffusion (similar studies were repeated with gemcitabine in Chapter V). This also suggests that other transport proteins present in HeLa cells are not likely involved in the transport of nucleosides, allowing for the assumption that transport of uridine and gemcitabine in HeLa cells is occurring solely through hENTs.

The addition of dFdU and dFdC to the incubations resulted in opposing effects on uridine accumulation depending on whether or not the cells were first pre-treated with 100 nM NBMPR. For example, when both hENT1 and hENT2 were active, both compounds significantly increased the net accumulation of uridine, such that by thirty minutes, uptake of uridine in the presence of dFdU was nearly four-fold higher than in the absence of dFdU. Conversely, when only hENT2 was active, both dFdU and dFdC were observed to inhibit uridine accumulation, consistent with their inhibitory effect observed during the short incubation periods. While it is not the goal of this thesis work

to define the mechanism by which dFdU and dFdC affect uridine accumulation, it is most likely quite similar to the mechanism by which dFdU affects gemcitabine disposition, which will be proposed in Chapters V and VI. Namely, it is possible that dFdU is competitively inhibiting uridine transport via hENT1 and hENT2 during short incubations, but as the length of the incubation period increases, dFdU starts to inhibit efflux of uridine as well, hence the heightened intracellular detection of tritium in the presence of dFdU.

In addition to exploring the effect of different inhibitors on uridine uptake in HeLa cells, experiments were also performed to analyze the effect of temperature. Active transport processes are known to be temperature-dependent, such that lower temperatures significantly decrease activity[180]. As hENTs are facilitative transporters, changes in temperature should have much less of an effect on their function. However, as cold temperatures slow down the equilibration of substrate extracellularly and affect the fluidity of the plasma membrane, an effect of temperature may be observed with facilitative transport processes as well. As such, when incubations were performed at 4°C, intracellular levels of radiolabeled uridine were lower than what was observed in experiments performed at room temperature. However, uptake was still significantly higher than accumulation in the presence of dilazep, consistent with the hypothesis that temperature is not likely affecting the function of hENTs directly, but is more likely a secondary effect of alterations to other temperature-sensitive cellular processes involved in uridine disposition.

In conclusion, these initial experiments using the well-studied nucleoside transporter substrate uridine provide a sound rationale for the use of HeLa cells in studies

examining the role of hENTs in the disposition of other nucleosides such as gemcitabine. The expression of nucleoside transporters in HeLa cells includes both hENT1 and hENT2, and while other endogenous transport systems may be present in this adenocarcinoma cell line, these other proteins are unlikely to contribute significantly to the active uptake of nucleosides that will be used as probe substrates for experiments involving hENT characterization. Additionally, the use of dilazep and NBMPR as tools for further characterization of nucleoside transport has been validated.

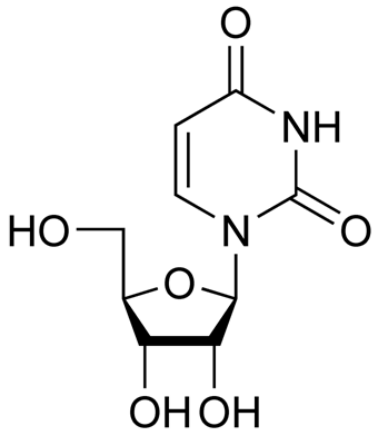

Figure 4-1: Structure of uridine.

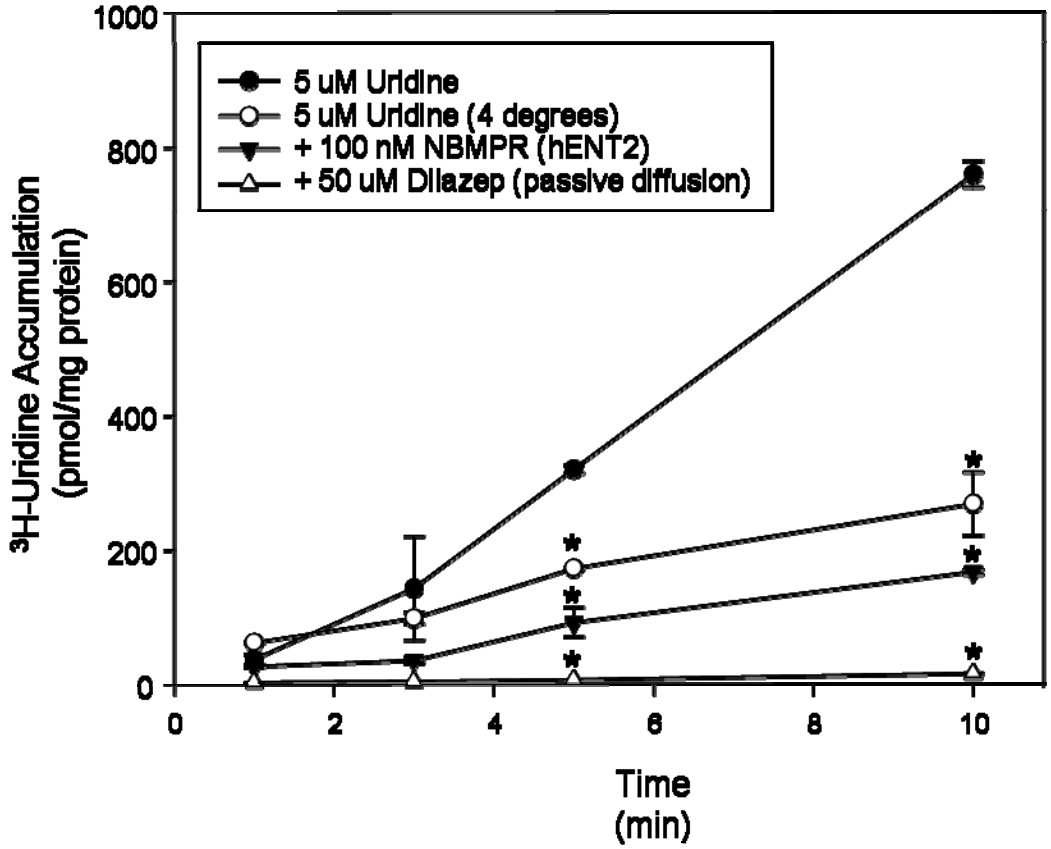

Figure 4-2: Effect of temperature and chemical inhibition on hENT-mediated transport of tritiated uridine in HeLa cells. Data represent total tritium detected in the cell at each incubation time point including uridine and its metabolites. *Significantly different from accumulation of uridine alone ($p<0.05$). Data points for + 100 nM NBMPR (hENT2) and + 50 μM dilazep (passive diffusion) at three seconds are both significantly different than 5 μM uridine alone.

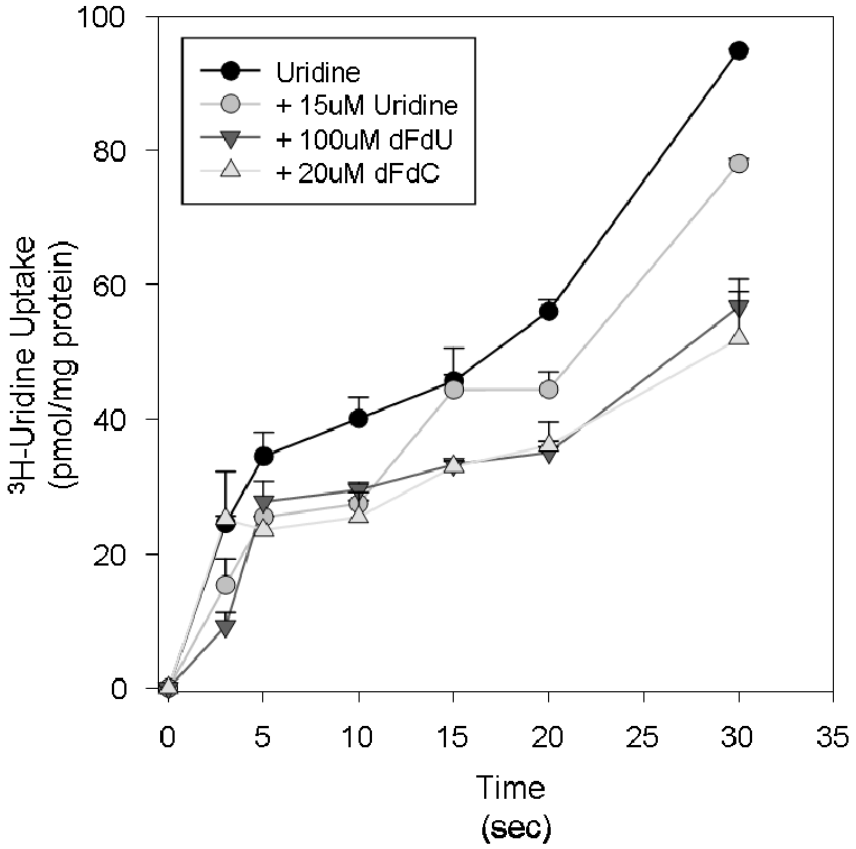

Figure 4-3: Effect of uridine, dFdU, and dFdC on uridine uptake over short incubation periods. The concentration of uridine used in all experiments was 5 μM. Data represent total tritium detected in the cell at each incubation time point including uridine and its metabolites.

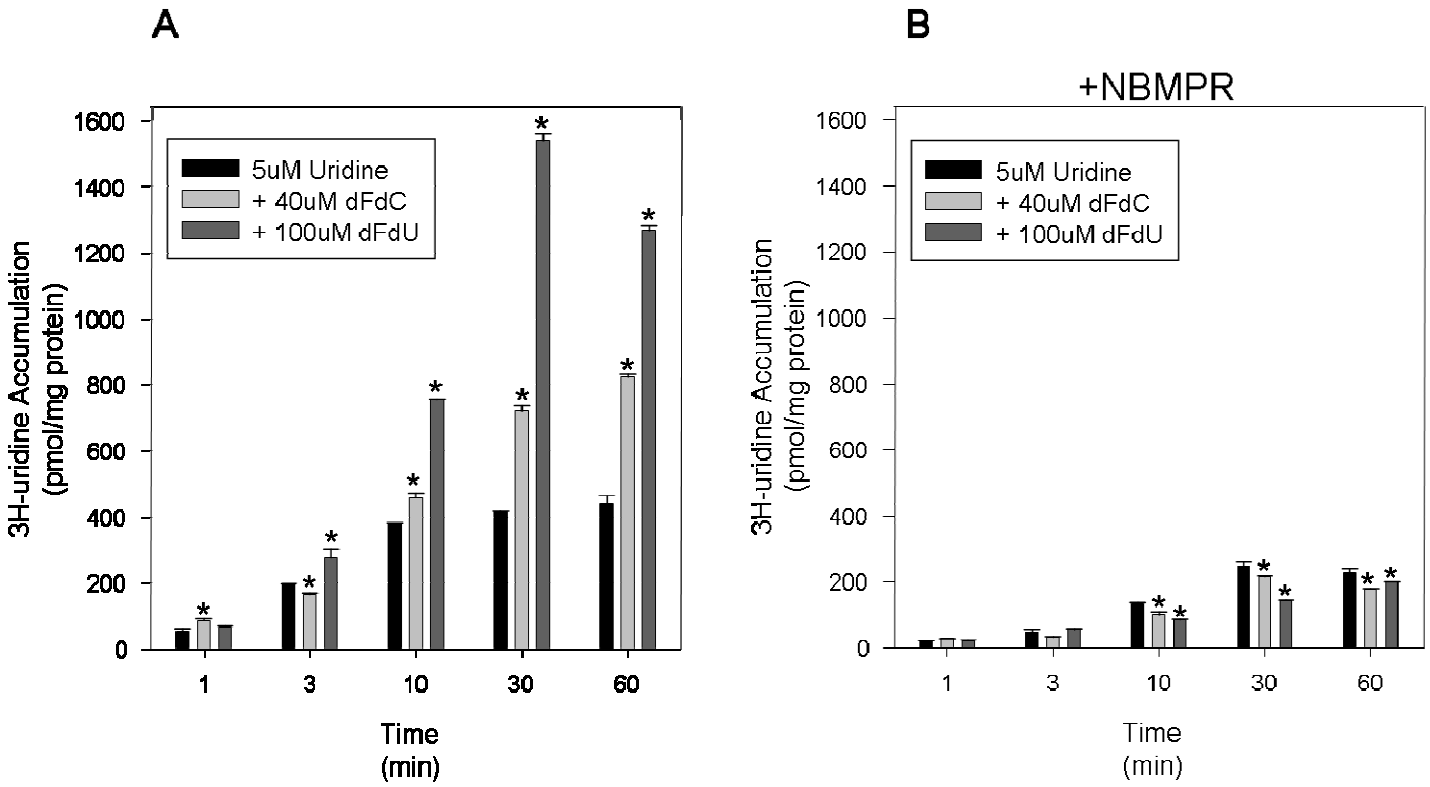

Figure 4-4: Effect of gemcitabine (dFdC) and dFdU on the net accumulation of uridine over a one-hour period via A) hENT1 and hENT2, and B) hENT2. Addition of 100 nM NBMPR allowed for the determination of hENT2-mediated accumulation alone. Data represent total tritium detected in the cell at each incubation time point including uridine and its metabolites. *Significantly different from accumulation of uridine alone ($p<0.05$).

Chapter V.

Effect of the Deaminated Metabolite, 2',2'-difluorodeoxyuridine (dFdU), on the Transport and Toxicity of Gemcitabine in HeLa Cells

Introduction

Gemcitabine (2',2'-difluorodeoxycytidine, dFdC), is an analog of deoxycytidine with high activity against many types of solid tumors including pancreatic, ovarian, breast, bladder, and non-small cell lung cancers. As a hydrophilic nucleoside analog, gemcitabine utilizes nucleoside transporters to cross plasma membranes. Once inside the cell, this prodrug is quickly phosphorylated to its active di- and triphosphate moieties (dFdCDP, dFdCTP) [135]. dFdCDP and dFdCTP are then incorporated into nascent DNA and RNA strands, eventually leading to inhibition of DNA polymerases, chain termination and cessation of DNA replication[44].

In addition to phosphorylation, gemcitabine may also undergo intra- and extracellular deamination to the much less active form, 2',2'-difluorodeoxyuridine (dFdU), via cytidine deaminase (CDA), which is present at high concentrations in many types of normal and malignant tissues as well as in the plasma[108,181]. In fact, after intravenous administration, cytidine deaminases in the plasma rapidly convert gemcitabine to dFdU, resulting in a gemcitabine plasma elimination half-life of only eight minutes for the parent compound. The long half-life of dFdU (fourteen hours), leads to plasma concentrations of the deaminated metabolite that are frequently ten- to twenty-fold higher than those of gemcitabine when measured shortly after gemcitabine administration[145]. Additionally, deamination may also occur intracellularly with the monophosphate form of dFdC, as dFdCMP can be converted by deoxycytidine monophosphate deaminase (dCMPD) to dFdUMP, which may then be further phosphorylated to dFdUDP and dFdUTP or dephosphorylated to yield dFdU. Similar to dFdC, dFdU may also be directly phosphorylated intracellularly to its mono-, di-, and

triphosphate metabolites by deoxycytidine kinase. However, the majority of dFdU nucleotides are thought to be formed by the breakdown of dFdC nucleotides as dFdU is predicted to have a low affinity for dCK [85,182], [118].

While typically thought to be relatively inactive if administered alone, the di- and triphosphate metabolites of dFdU contribute not only to gemcitabine's cytotoxicity, but also its efficacy as a radiation sensitizer[53]. In fact, recent in vitro evidence suggests that the intrinsic toxicities of dFdU and dFdC are nearly identical, as there was only a 1.3-fold difference in DNA incorporation between dFdCTP and dFdUTP when HepG2 cells were treated with the respective IC_{50} concentrations of dFdC and dFdU[85].

Gemcitabine is known to be transported into the cell by both the concentrative (CNT) and equilibrative (ENT) nucleoside transporters. Human concentrative nucleoside transporter 1 (hCNT1) is the most highly efficient transporter of gemcitabine, with a K_m of around 18 μM, but the distribution of this protein throughout the body is thought to be more limited than the more ubiquitously-expressed ENT family[74,183]. Both hENT1 and hENT2 are also involved in gemcitabine transport, and in vivo studies have observed a high correlation between the expression of these transporters and response to gemcitabine therapy [89,159,184].

Recently, it has been reported that dFdU is transported by both hCNT1 and hENT1 in hCNT1-transfected MDCK cells[85]. The extent of dFdU uptake was nearly the same as that of gemcitabine, suggesting that dFdU may be a high affinity substrate for these transporters as well. Additionally, it was determined in the same study that dFdU undergoes biphasic efflux from HepG2 and A549 cells preloaded with gemcitabine.

With both the parent and metabolite competing for the same transporters, high

plasma concentrations of dFdU may affect the efficacy of gemcitabine against solid tumors, by decreasing its uptake into the cell. Therefore, the objective of this study was to investigate the effects of dFdU on gemcitabine uptake and efflux via hENT1 and hENT2 in HeLa cells, which endogenously express these two nucleoside transporters. Based on our results, we have developed a mechanistic model whereby dFdU limits the uptake of gemcitabine into the cell, yet ultimately allows for increased sequestration of intracellular gemcitabine.

Materials and Methods

Chemicals

2',2'-difluorodeoxycytidine (dFdC) and 2',2'-difluorodeoxyuridine (dFdU) were synthesized by the Institute for Therapeutics, Discovery, and Development at the University of Minnesota, Minneapolis, MN. [5-^{3}H]-gemcitabine (11 Ci/mmol) were obtained from Moravek chemicals (La Brea, CA). Uridine and nitrobenzyl-mercaptopurine riboside (NBMPR) were obtained from Sigma-Aldrich (Saint Louis, MO). $^{13}C,^{15}N_2$-dFdU was purchased from Toronto Research Chemicals (North York, Ontario). All other chemicals were obtained from Fisher Scientific (Pittsburgh, PA) unless specified.

Cell Culture

HeLa cells, derived from a cervical adenocarcinoma, were obtained from ATCC (Manassas, VA). Cells were grown as a monolayer at 37ºC under 5% CO_2 in Dulbeccos Minimum Essential Media supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 ug/mL streptomycin, and fortified with 10% FBS. Cells used in transporter experiments were in the exponential growth phase and had undergone between five and twenty passages.

Transporter Assays

HeLa cells were seeded in 12- or 24-well plates and allowed to adhere overnight. For radiolabeled experiments, tritiated gemcitabine was combined with cold gemcitabine to make a 5 μM tracer solution in transport buffer (128 mM NaCl, 4.73 mM KCl, 1.25

mM $CaCl_2$, 1.25 mM $MgSO_4$, and 5 mM HEPES, pH 7.4). For non-radiolabeled experiments, 100 μM solutions of dFdU were prepared in transport buffer. Incubations were initiated by aspirating the media from the cells, which were then loaded with tracer solution. Plates were incubated at 37º C on a rotary shaker for a time period ranging from three seconds to twenty-four hours, after which drug was removed, and cells washed three times in ice-cold transport buffer containing 50 μM dilazep. Cells were solubilized in 1% Triton X-100, and 200 μL of each solubilized cell fraction was added to 4 mL of scinitillation cocktail (ScintiSafe Econo Cocktail, Fisher Scientific, Pittsburgh, PA) Radioactivity was then determined with a Beckman Coulter LS-6500 liquid scintillation counter (Fullerton, CA). Total protein concentration in each well was determined by the BCA protein assay (Pierce, St. Louis, MO). For cold substrates, cells were lysed with -80º C 70:30 methanol:water and dFdU quantified via HPLC with mass spectrometric detection. Because methanol present in the quenched incubations interfered with the absorbance readings obtained with the BCA protein assay, data from studies with cold substrate were normalized to the number of cells initially plated in each well.

Inhibition experiments: To determine the effects of dFdU on gemcitabine uptake, dFdU prepared in a 1:1 solution of DMSO:transport buffer was added directly to the gemcitabine tracer solutions where indicated. The amount of organic solvent in each incubation was kept below 0.5%.

ENT2-mediated transport: To examine the effects of ENT2-mediated transport alone, cells were pre-incubated for thirty minutes prior to the start of the experiment in 100 nM NBMPR (a known inhibitor of hENT1), and 100 nM NBMPR was also added to tracer

solutions.

dFdU Analysis

Internal standard ($^{13}C,^{15}N_2$-dFdU) was added to the HeLa cell lysate, and the lysate evaporated to dryness followed by reconstitution in 100 μL of mobile phase. The sample was injected onto a Fusion® RP 50 X 3.0 mm, 3.5 μm, column at 30º C (Phenomenex, Torrance, CA). The mobile phase (flow rate, 0.3mL/min) consisted of 5:95 (v/v) methanol:aqueous buffer (5 mM ammonium acetate, pH 6.8). The HPLC (Agilent 1200) was interfaced to a Thermo TSQ Quantum mass spectrometer with electrospray interface operating in positive ionization mode. Detection of dFdU was obtained through selected reaction monitoring (SRM) of the following transitions: *m/z* 265/113 for dFdU and *m/z* 268/116 for ^{13}C, $^{15}N_2$ -dFdU (IS). The calibration range for this method was 4.0 – 8021 pmoles for dFdU.

Cytotoxicity Assays

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) Assay

Cells were plated in 96-well plates, allowed to adhere overnight, and then exposed to media containing gemcitabine alone or in combination with 100 μM dFdU for 48 hours. This was followed by an additional 24-hour recovery period in fresh media. MTT (10 μL of 5 mg/mL solution) was added to each well followed by an incubation time of 2 hours or until purple crystals (formazan) could be observed. The media was then removed and 100 μL DMSO added to each well to solubilize the formazan crystals. The absorbance at 562 nm was determined using a Synergy HT Multi-Mode Microplate Reader (Biotek, Winooski, VT), and viability expressed as the percentage of A_{562} of

treated cells relative to untreated controls.

Statistical Analysis

IC_{50} values were determined by nonlinear regression using the following equation within SigmaPlot (Systat Software, Inc., Chicago, IL):

$$IC_{50} = \min + \frac{(\max - \min)}{(1 + [(x / EC_{50})\text{^ } Hillslope])}$$

All experiments were performed in triplicate and values are presented as the mean ± standard deviation. Statistical comparisons were assessed using a Student's t-test with differences between groups considered statistically significant when the p-value was <0.05 (SigmaStat 3.11 software, SyStat Software, Inc., Chicago, IL).

Results

Uptake of Gemcitabine over Short Time Periods

Due to rapid equilibration, experiments designed to examine nucleoside uptake via equilibrative transporters were performed over a very short timeframe to minimize intracellular metabolism and limit efflux of substrate and metabolites[83]. As such, initial experiments to characterize the effects of dFdU on gemcitabine uptake via hENTs in HeLa cells were conducted with incubation periods less than one minute in length.

Gemcitabine uptake into HeLa cells via both hENT1 and hENT2 was relatively linear within the first ten seconds, which is consistent with previous reports [84]. The addition of 100 μM dFdU significantly inhibited gemcitabine transport at all incubation times, with nearly a five-fold decrease in gemcitabine uptake observed at fifteen seconds (Figure 5-1). When hENT1 activity was inhibited by the addition of 100 nM NBMPR, the uptake of gemcitabine decreased by about 25%, suggesting that gemcitabine is a substrate for both hENT1 and hENT2 in HeLa cells. Adding 100 uM dFdU to cells pretreated with NBMPR (hENT2-only) resulted in a nine-fold decrease in overall gemcitabine uptake, indicating that along with gemcitabine, dFdU is also a substrate for both hENT1 and hENT2. As such, the uptake of dFdU alone was studied in HeLa cells and was observed to be transported at nearly twice the velocity via hENT1 than hENT2 with initial uptake rates of 14.5 and 8 nmol/10^5 cells/minute, respectively (Figure 5-2).

dFdU-Mediated Inhibition of Gemcitabine Uptake via hENT1 and hENT2

The abovementioned set of experiments demonstrated that dFdU is a substrate for hENT1 and hENT2 and is capable of limiting gemcitabine transport via these same

transporters, most likely through competitive inhibition. As human plasma concentrations of dFdU are typically much higher than gemcitabine shortly after intravenous administration, the effects of dFdU inhibition on gemcitabine influx and efflux may be quite significant in vivo. However, the relative potency of dFdU as an inhibitor of gemcitabine transport via hENTs is not known. Therefore, IC_{50} values were determined for the dFdU-mediated inhibition of hENT1 and hENT2 with gemcitabine as a substrate. Transport of gemcitabine via both hENT1/2 and via hENT2-only is inhibited by concentrations of dFdU that are at least 100-fold lower than those typically measured in human plasma after gemcitabine dosing with IC_{50} values of 1.2 μM (hENT1/2) and 0.45 μM (hENT2), respectively (Figure 5-3).

Uptake of Gemcitabine over Longer Incubation Periods

While acknowledging the potentially confounding factors of intracellular metabolism and efflux present during long-term incubations, studies with radiolabeled gemcitabine and cold dFdU were repeated for longer incubation periods to further characterize any long-term interactions that may occur over the twenty-four hour period after gemcitabine administration. Total gemcitabine accumulation was determined at time points ranging from three minutes to twenty-four hours. Experiments were also performed with and without 100 nM NBMPR pre-treatment to selectively inhibit hENT1, and thus separate interactions due to hENT1-mediated and hENT2-mediated transport, respectively.

The addition of 100 μM dFdU to gemcitabine tracer solutions resulted in an initial decrease in the accumulation of gemcitabine in HeLa cells at three and ten minutes, at

which point accumulation significantly increased for the remaining incubation periods (Figure 5-4A). At twenty-four hours, gemcitabine accumulation in the presence of 100 μM dFdU was nearly twice as high as in the absence of dFdU.

When hENT1-mediated transport was inhibited, gemcitabine accumulation was nearly halved as compared to when both hENT1 and hENT2 are active (Figure 5-4B). Again, this suggests that both hENT1 and hENT2 are significant contributors to gemcitabine transport in HeLa cells. When 100 μM dFdU was added to HeLa cells pre-treated with NBMPR, gemcitabine accumulation was significantly inhibited out to sixty minutes, at which time an increase in gemcitabine concentration was measured intracellularly. At twenty-four hours, hENT2-mediated accumulation of gemcitabine in the presence of 100 μM dFdU is nearly three times higher than in the absence of dFdU.

Effect of dFdU on dFdC Cytotoxicity

At physiologically relevant concentrations, dFdU appears to play a significant role in determining the extent of gemcitabine distribution. As the addition of dFdU significantly increased the net intracellular level of gemcitabine with increasing incubation length, it is possible that dFdU may have an effect on gemcitabine toxicity as well. dFdU itself is cytotoxic to HeLa cells, with an apparent IC_{50} value of 260 μM compared with 0.03 μM for gemcitabine (Figure 5-5). Yet, the effect of concomitant dFdU and dFdC on cytotoxicity is not known. An MTT assay was performed with increasing concentrations of gemcitabine in addition to 100 μM dFdU. Despite the significantly higher level of dFdC accumulation in the presence of dFdU, the effect of dFdU on gemcitabine cytotoxicity was merely additive (Figure 5-5B). In this graph, the

percentage of viable cells remaining after treatment with dFdU and dFdC was not corrected for the cytotoxic effect of dFdU itself, to better allow for visualization of the additive effect of these compounds on cell toxicity. When the viability is corrected for dFdU-mediated toxicity, no significant effect on the antiproliferative activity of gemcitabine is observed.

As the IC_{50} value for gemcitabine was observed to be more than ten-fold lower than the concentrations of gemcitabine used in the transporter studies, it is possible that at 5 μM gemcitabine, maximal cytotoxicity is already achieved, such that simultaneous exposure to dFdU has no additional effect. The effect of increasing concentrations of dFdU on a lower concentration of gemcitabine was examined as well; however, the effect of dFdU on 0.5 μM gemcitabine was merely additive and was not affected by increasing the concentration of dFdU (Figure 5-6).

Discussion

Typically referred to as an inactive metabolite of gemcitabine, dFdU is formed by the deamination of either gemcitabine or dFdCMP via cytidine deaminase and deoxycytidylate deaminase, respectively. While the relative cytotoxicity of dFdU is at least 1,000-fold lower than that of gemcitabine, their intrinsic cytotoxicities are quite similar [85,181]. Additionally, dFdU is also a potent radiosensitizer and can elicit effects on the cell cycle and apoptosis at concentrations well below those needed to achieve toxicity.

After intravenous administration of gemcitabine, dFdU reaches peak levels within five to fifteen minutes post-infusion, and, unlike gemcitabine, remains elevated for a prolonged period of time with an elimination half-life of up to 24 hours [145]. Yet, despite the difference in their respective pharmacokinetic profiles, little is known regarding the role of the deaminated metabolite of gemcitabine in determining the disposition of the parent compound. A nucleoside analog itself, dFdU has been observed in a previous study to undergo influx via both hCNT1 and hENT1 in MDCK-transfected cells[83,85]. Presumably, due to its bidirectional transport properties, hENT1 is involved in the observed efflux of dFdU from MDCK cells as well. In this study, dFdU is identified as a substrate for both hENT1 and hENT2 in HeLa cells, as well as a competitive inhibitor of gemcitabine influx and efflux via these same transporters.

When measuring net intracellular accumulation of a nucleoside that is also a substrate for membrane-bound transporters, distinguishing between the effects of transport and intracellular metabolism is often difficult, especially because uptake may be influenced by metabolism. In order to examine the effect of dFdU on dFdC uptake, while

minimizing the contribution of metabolism and efflux, incubation periods were limited to twenty seconds in length. Extensive evidence suggests that after twenty seconds, transport of nucleosides becomes saturated, and accumulation of intracellular metabolites accounts for the majority of net radiolabeled compound detected within the cell[83]. In Figure 5-1, clinically relevant concentrations of dFdU were observed to significantly inhibit gemcitabine accumulation at incubation periods up to twenty seconds in length. With both hENT1 and hENT2 active within the cell, the addition of 100 μM dFdU resulted in a four-fold decrease in the intracellular accumulation of gemcitabine as compared to when dFdU was absent. Intracellular accumulation of gemcitabine decreased even further when cells were pre-treated with NBMPR. Working under the assumption that interactions occurring at early time points principally affect transporter-mediated uptake, these results suggest that dFdU inhibits gemcitabine transport via both hENT1 and hENT2. In fact, further analysis with unlabeled dFdU provided evidence that the deaminated metabolite is indeed a substrate of both of these equilibrative nucleoside transporters, and that the IC_{50} values associated with dFdU-mediated inhibition of gemcitabine uptake by hENTs are nearly one hundred-fold lower than the average plasma concentration for dFdU in humans after gemcitabine dosing (Figures 5-2 and 5-3).

During longer incubation periods, radiolabeled gemcitabine undergoes sequential phosphorylation, resulting in the formation of active metabolites in addition to deamination, which yields dFdU. Contrary to what was observed when incubation length was kept to a minimum (ie. less than one minute), the addition of dFdU to incubations lasting up to 24 hours in length resulted in significantly higher levels of net radiolabeled gemcitabine (including parent and metabolites) that accumulated intracellularly. While it

is possible that dFdU is altering the phosphorylation of gemcitabine, it is more likely that dFdU is affecting gemcitabine transport, as this study has identified dFdU as a substrate of hENT1 and hENT2. As the extracellular concentration of dFdU in our *in vitro* system is twenty-fold higher than that of gemcitabine, after equilibration is allowed to occur, the intracellular concentration of dFdU is theoretically twenty-fold higher as well. Because dFdU coincubation limits gemcitabine uptake, once both compounds are inside the cell, dFdU may also alter efflux of gemcitabine and its metabolites as well, resulting in the intracellular retention of radiolabeled gemcitabine and its subsequent radiolabeled metabolites. This hypothesis is consistent with earlier work by Wright et al. who observed an increase in the retention of 2-chlorodeoxyadenosine (2-CdA) in human leukemic lymphoblasts when efflux via hENT1 was limited by the addition of NBMPR and dilazep after preloading cells with 2-CdA[185]. In the absence of hENT1 inhibition, intracellular concentrations of 2-CdA underwent a biexponential decline when suspensions of drug-containing cells were diluted in fresh media. However, when cell suspensions were diluted in media containing dilazep or NBMPR, loss of 2-CdA became monoexponential, and its intracellular half-life significantly increased. Additionally, studies by Mackey et al. were conducted using Caco-2 cells, which endogenously express hCNT, hENT1, and hENT2. They demonstrated that, despite a significant decrease in the initial rate of gemcitabine uptake in Caco-2 cells pre-treated with dipyridamole (an inhibitor of hENT), intracellular accumulation of radiolabeled gemcitabine increased above the steady-state concentration achieved without dipyridamole pre-treatment, most likely due to the inhibition of gemcitabine efflux via the bidirectional hENTs[84].

However, the increased intracellular retention of these cytotoxic nucleosides

occurring in response to interactions with hENTs does not consistently translate into an increase in cell sensitivity. In the study of 2-CdA, both leukemic lymphoblast cell lines utilized became more sensitive to this nucleoside in the presence of NBMPR and dilazep. In this study, despite a significant increase in gemcitabine retention at twenty-four hours, no synergistic effect on cytotoxicity was observed when dFdU was added to HeLa cells. The effect was merely additive, due to the known cytotoxic effects of dFdU at higher concentrations. As the high concentration of gemcitabine initially used in the these studies may have yielded maximal cytotoxic effects even in the absence of dFdU, the study was repeated with a concentration of gemcitabine closer to the IC_{50} value observed for this nucleoside analog in HeLa cells (Figure 5-6). However, the effect of dFdU on the cytotoxicity of a ten-fold lower concentration of gemcitabine was observed to be additve as well. Likewise, Mackey et al. did not observe any increase in cell sensitivity of gemcitabine towards Caco-2 cells associated with the inhibition of hENT-mediated efflux of this nucleoside and hypothesized that the elimination of gemcitabine back into the media via the bidirectional hENT transporters is not a major component of net gemcitabine retention[84]. It is also possible that the intracellular concentration of gemcitabine in the absence of dFdU was already sufficient to produce a maximal cytotoxic effect, such that increasing the concentration further by the addition of dFdU yielded no additional toxicity.

While dFdU is known to possess cytotoxic and radiosensitizing properties, the extensive conversion of gemcitabine to dFdU is frequently seen as an impediment to optimizing gemcitabine therapy. In fact, significant in vitro work exists suggesting that inhibition of intracellular cytidine deaminase actually increases the cytotoxicity of

gemcitabine by decreasing the anabolism of the more potent parent compound to the less active deaminated metabolite[109,110,186]. Additionally, in vivo studies conducted in mice have demonstrated that oral administration of 3,4,5,6-tetrahydrouridine (THU), a known inhibitor of cytidine deaminase, significantly increased oral bioavailability of gemcitabine from 10 to 40%[187]. Unfortunately, the effects of THU coadministration on in vivo gemcitabine uptake and cytotoxicity were not investigated. However, based on our data and that of others, it seems probable that while limiting intracellular breakdown of gemcitabine to dFdU may increase cell sensitivity to gemcitabine, limiting plasma formation of dFdU after intravenous administration of gemcitabine may actually have negative effects on cytotoxicity. While initially more gemcitabine would be taken up into the cell in the absence of high plasma dFdU, the intracellular retention of the parent compound may be significantly limited, ultimately affecting cytotoxicity. Future in vivo studies focusing on the effect of plasma dFdU on intracellular gemcitabine disposition and toxicity may provide important insights into the optimal role of deaminase inhibitors in gemcitabine therapy.

In conclusion, evidence has been provided suggesting for the first time that the extensive deamination of gemcitabine in the plasma, typically thought to limit the efficacy of the parent compound, may actually be an integral part of the in vivo disposition of this nucleoside analog. This work supports a mechanism by which the deaminated metabolite, dFdU, plays a potentially significant role in enhancing the intracellular retention of gemcitabine, possibly through its interactions with the bidirectional equilibrative nucleoside transporters.

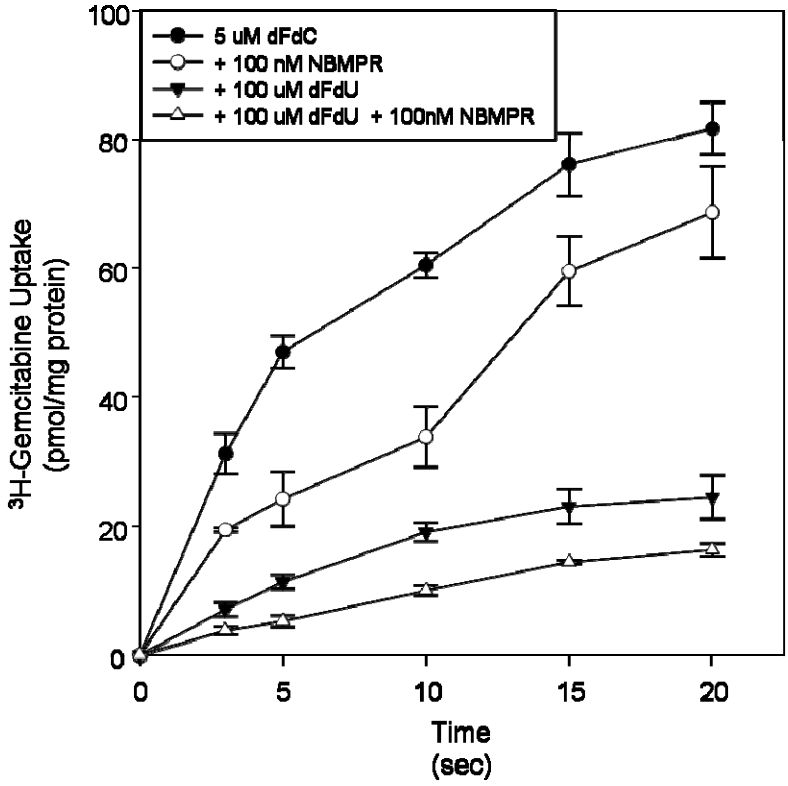

Figure 5-1: Effect of dFdU and NBMPR on gemcitabine (dFdC) uptake over short time points. Incubation periods were limited to twenty seconds in length to avoid the effects of efflux and intracellular metabolism of dFdC. To analyze hENT2-mediated uptake independently of hENT1, cells were pre-treated with 100 nM NBMPR. *Significantly different than accumulation of gemcitabine in absence of dFdU or NBMPR ($p<0.05$).

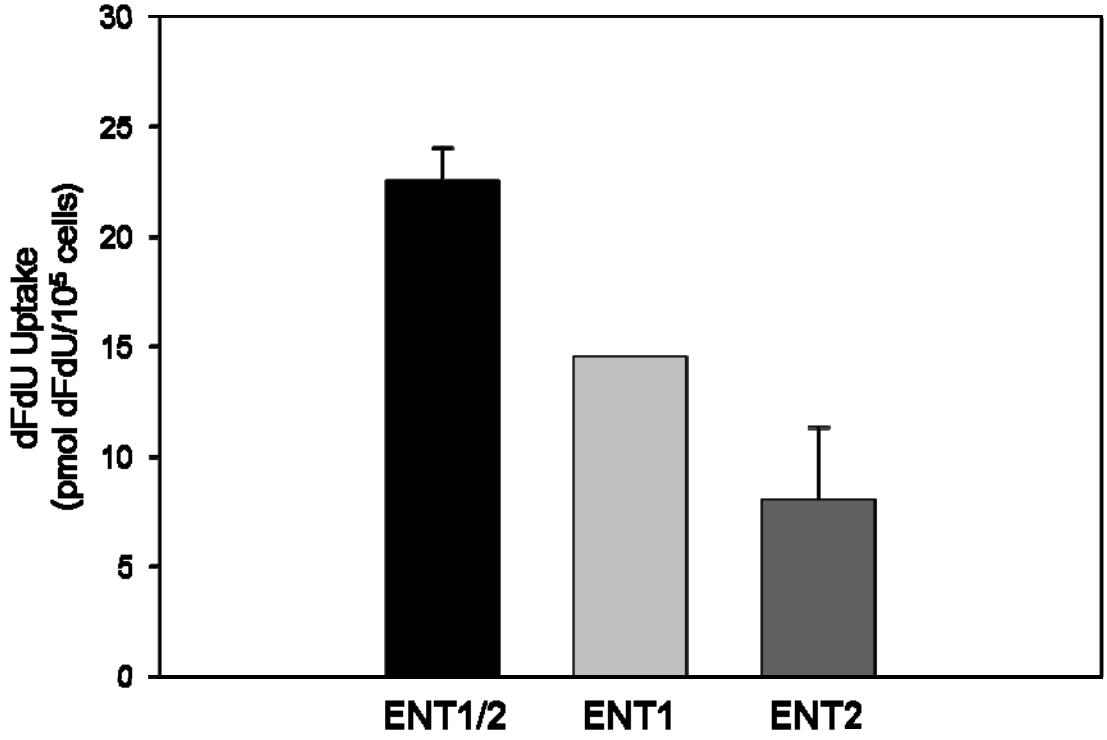

Figure 5-2: Uptake of dFdU by hENT1 and hENT 2 in HeLa cells. To calculate hENT1-mediated transport of dFdU, uptake of dFdU in cells pre-treated with 100 nM NBMPR (hENT2-mediated) was subtracted from the uptake measured via both hENT1 and hENT2. Incubations were one minute in length.

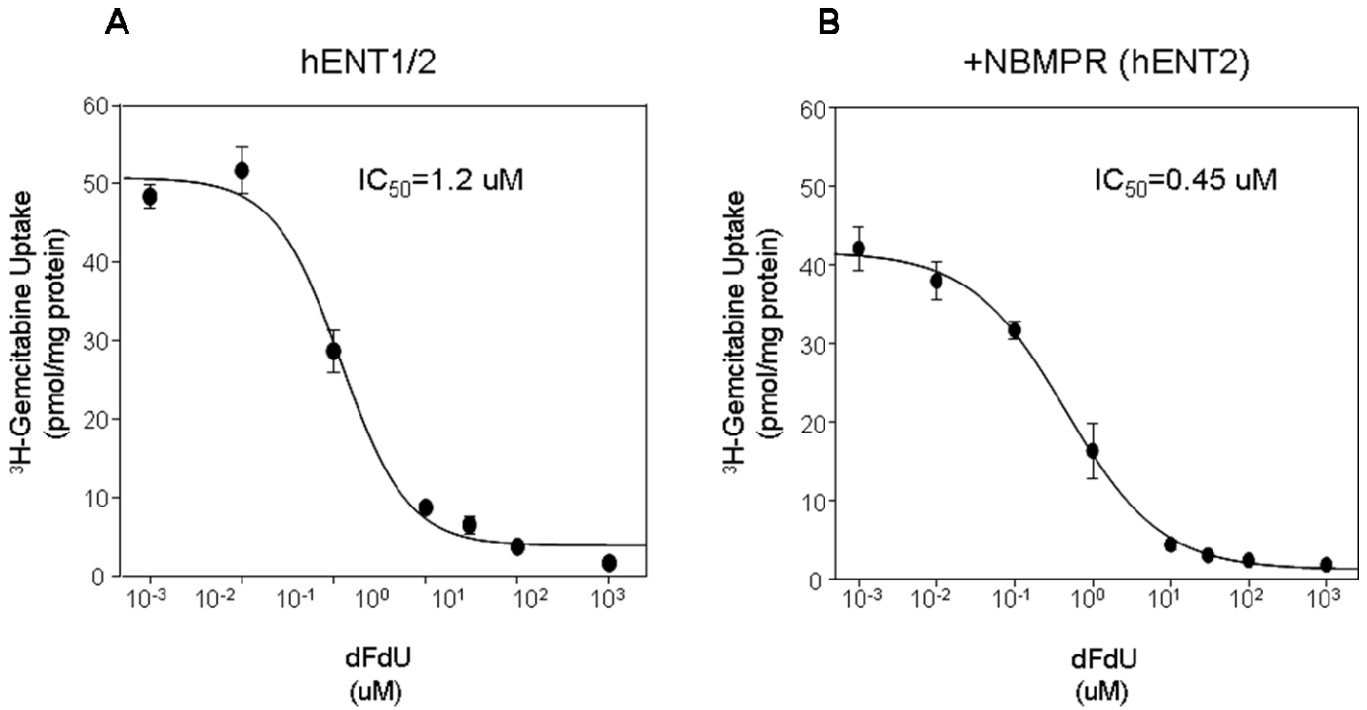

Figure 5-3: Determination of IC_{50} values for dFdU-mediated inhibition of A) hENT1/2 and B) hENT2 with gemcitabine as a substrate. All incubations were ten seconds in length. Addition of 100 nM NBMPR allowed for the determination of hENT2-mediated accumulation alone.

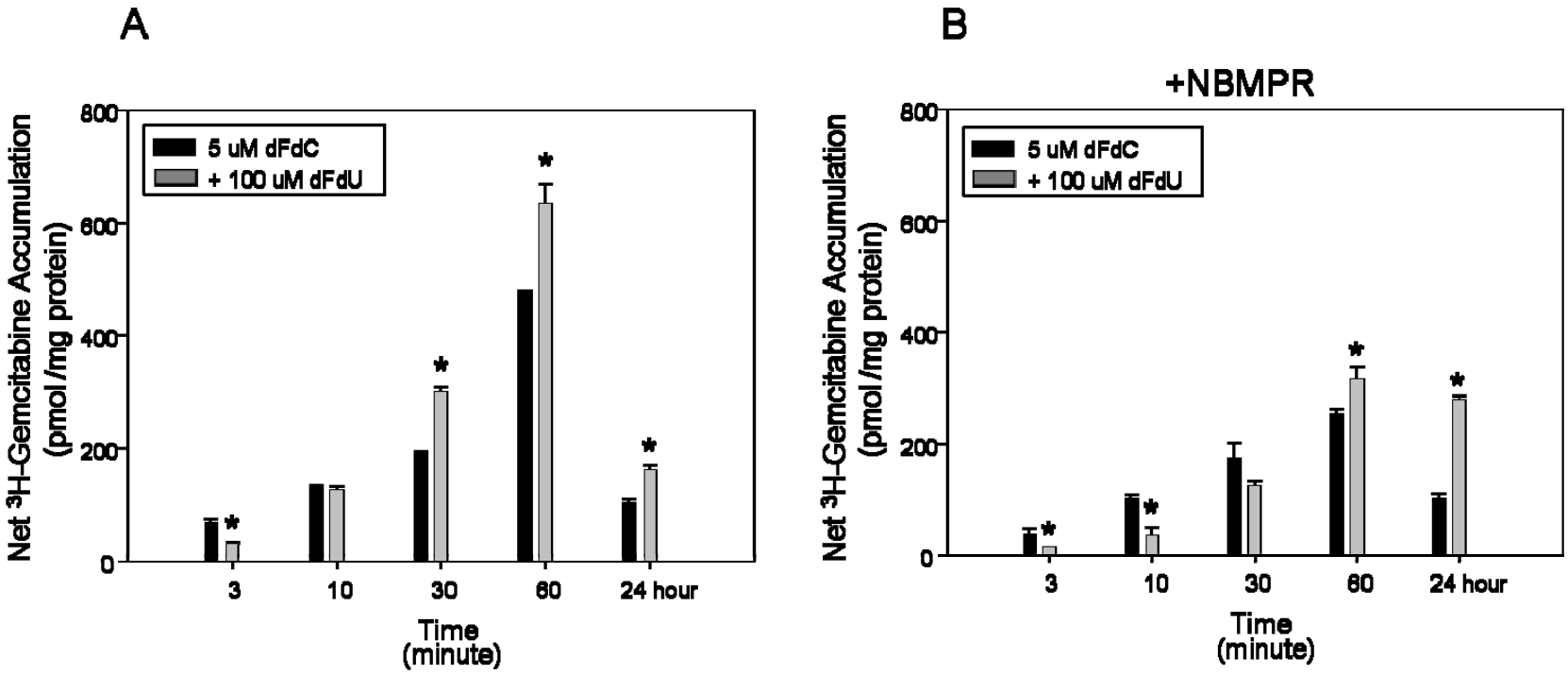

Figure 5-4: Effect of dFdU on the accumulation of gemcitabine (dFdC) over a twenty-four hour period via A) hENT1 and hENT2 and B) hENT2. Addition of 100 nM NBMPR allowed for the determination of hENT2-mediated accumulation alone. Data represent total tritium detected in the cell at each time point including gemcitabine and its metabolites. *Significantly different from accumulation of gemcitabine in the absence of dFdU ($p<0.05$).

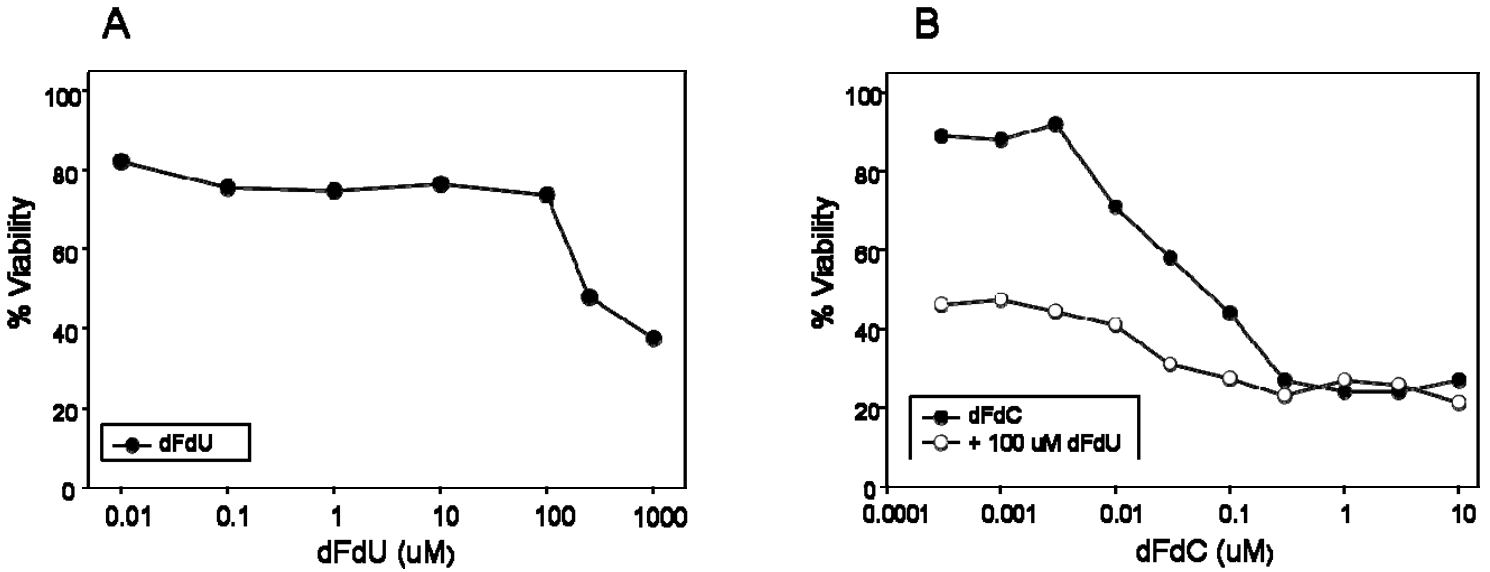

Figure 5-5: Antiproliferative effect of dFdU A) alone, and B) in combination with gemcitabine (dFdC) against HeLa cells. Percent viability calculated by taking the ratio of absorbance in the treated wells to the absorbance in untreated wells. Each point represents the mean of at least six wells. A single representative experiment is shown.

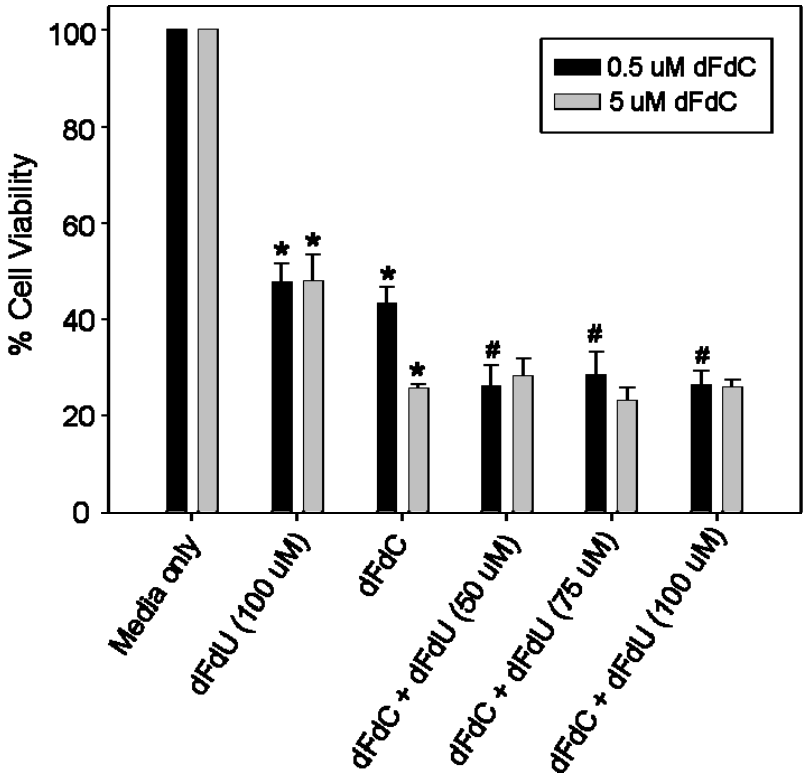

Figure 5-6: Additive effect of increasing concentrations of dFdU on antiproliferative activity of 0.5 and 5 μM gemcitabine against HeLa cells. Percent viability calculated by taking the ratio of absorbance in treated wells to the absorbance in untreated wells. In addition to the 5 μM gemcitabine concentration used in the transporter assays, the cytotoxicity of 0.5 μM gemcitabine was also examined as this concentration is close to the IC_{50} value for gemcitabine in HeLa cells. Each point represents the mean of at least six wells +/- SD. A single representative experiment is shown. *Significantly different than cells treated with media-only. #Significantly different than 0.5 μM gemcitabine alone.

Chapter VI.

Role of Deoxycytidine Kinase in the Accumulation of Gemcitabine in HeLa Cells via Human Equilibrative Nucleoside Transporters 1 and 2

Introduction

With efficacy as both a cytotoxic agent and as a radiosensitizer, gemcitabine (dFdC) is a deoxycytidine analog frequently administered in combination with cisplatin and radiation for the treatment of locally advanced cervical carcinoma[60,69,72]. Due to the hydrophilicity of this antimetabolite, gemcitabine requires specialized nucleoside transporters for uptake into cervical cells, and members of both the concentrative (hCNT) and equilibrative nucleoside transporter (hENT) families have high affinity for this compound[84]. Once inside the cell, this prodrug is quickly metabolized to its triphosphorylated moiety, which is incorporated into nascent RNA and DNA strands resulting in cytotoxicity. This phosphorylation is mediated by deoxycytidine kinase (dCK), the rate-limiting enzyme in the formation of gemcitabine triphosphate. In addition to phosphorylation, gemcitabine can also undergo deamination in tissue as well as in the plasma via cytidine deaminase, yielding 2',2'-difluorodeoxyuridine (dFdU), a metabolite possessing both cytotoxic and radiosensitizing properties, albeit at much lower potencies than observed for gemcitabine.

To gain more insight into the use of gemcitabine as a radiosensitizer in cervical carcinoma, our laboratory has undertaken investigations into the disposition of this compound in the cervix. Yet, one of the major difficulties in the study of gemcitabine's tissue distribution is distinguishing between the effects of nucleoside transport and intracellular metabolism. Specifically, equilibration of nucleoside analogs via the nucleoside transporters occurs at such a rate that steady-state levels are frequently achieved within thirty seconds after the start of the incubation, after which intracellular phosphorylation becomes the limiting step for net nucleoside accumulation (including the

parent compound plus its subsequent phosphorylated metabolites). As such, kinetic parameters based on incubations greater than one minute in length are merely reflective of the rate of gemcitabine phosphorylation versus the rate of nucleoside transport.

In an attempt to focus on uptake via nucleoside transporters, short incubation periods are frequently employed in the study of the transport of nucleoside analogs such as gemcitabine, thereby limiting the effects of intracellular metabolism. Additionally, experimental methods have included pre-incubation of cells with ATP-depleting regimens. As ATP is the primary intracellular phosphate donor required by deoxycytidine kinase for the phosphorylation of nucleosides, elimination of ATP essentially minimizes the activity of dCK. Treatment with potassium cyanide and iodoacetate or sodium fluorine followed by deoxyglucose yields intracellular concentrations of ATP that are less than 1% of those measured in untreated cells [188,189]. However, chemical depletion of ATP often requires several hours of prior incubation, and the indirect effect of the chemical perturbation on the cellular membrane as well as transporter protein function is not known. Thus the utility of this method of indirectly reducing the activity of deoxycytidine kinase is of questionable value.

Other studies examining nucleoside analog disposition have also been performed with universal nucleoside transporter substrates including formycin B, which is an inosine derivative transported via the equilibrative nucleoside transporters but is unique in its lack of intracellular phosphorylation[190]. However, while compounds such as formycin B may be useful for gaining a better understanding of the transport of non-metabolizable substrates, these results may not be generalizable towards other nucleoside analogs that require phosphorylation for cytotoxicity.

Previous experiments with gemcitabine in this laboratory have avoided the effects of intracellular phosphorylation by keeping incubation times to a minimum. Such experiments have identified an interaction between gemcitabine and its deaminated metabolite, dFdU, which significantly alters gemcitabine transport via hENTs in HeLa cells. Yet, when incubation periods were increased out to twenty-four hours to further characterize the interaction, a significant increase in net gemcitabine accumulation was observed, resulting in an enhanced retention of the parent compound. Similar in structure to gemcitabine, it is known that dFdU undergoes intracellular phosphorylation via deoxycytidine kinase in the same manner as the parent drug[85]. While dFdU is thought to exhibit a much lower affinity for dCK than gemcitabine, the possibility of an effect of dFdU on the intracellular metabolism of gemcitabine over a twenty-four hour incubation period must still be taken into consideration[191]. Therefore, the objective of these experiments was two-fold. The first was to investigate the likelihood of an interaction between dFdU, gemcitabine, and deoxycytidine kinase over a twenty-four hour period. The second objective was to examine the relationship between deoxycytidine kinase and hENTs as it pertains to gemcitabine disposition. To allow for a better separation of the effects of dFdU on hENT-mediated transport and dCK-mediated phosphorylation, a novel experimental design was used in which cells were transfected with siRNA targeting deoxycytidine kinase to eliminate the activity of this enzyme without having to limit incubation periods or add unnecessary chemicals to the cellular environment. Our data suggest that the elimination of dCK-mediated phosphorylation results in the removal of an intracellular gemcitabine 'sink' that serves as the driving force for equilibrative uptake of this nucleoside. As a result, the overall net accumulation of gemcitabine is significantly

decreased in the absence of phosphorylation, and this effect is independent of dFdU concentration.

Materials and Methods

Chemicals

2',2'-difluorodeoxycytidine (dFdC), 2',2'-difluorodeoxyuridine (dFdU), and 2',2'-difluorodeoxycytidine triphosphate (dFdCTP) were synthesized by the Institute for Therapeutics, Discovery, and Development at the University of Minnesota, Minneapolis, MN. [5-^{3}H]-gemcitabine (11 Ci/mmol) was from Moravek chemicals (La Brea, CA). Nitrobenzyl-mercaptopurine riboside (NBMPR) was obtained from Sigma-Aldrich (Saint Louis, MO). ^{13}C,^{15}N$_2$-gemcitabine, ^{13}C,^{15}N$_2$-dFdU and 2'deoxycytidine were purchased from Toronto Research Chemicals (North York, Ontario) while ^{13}C,^{15}N$_2$-cytidine triphosphate (CTP) was purchased from Cambridge Isotopes (Andover, MA). All other chemicals were obtained from Fisher Scientific (Pittsburgh, PA) unless specified.

Cell Culture

HeLa cells, derived from a cervical adenocarcinoma, were obtained from ATCC (Manassas, VA). Cells were grown as a monolayer at 37ºC under 5% CO_2 in Dulbeccos Minimum Essential Media supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 ug/mL streptomycin, and fortified with 10% FBS. Cells used in transporter experiments were in the exponential growth phase and had undergone between five and twenty passages.

Transporter Assays

HeLa cells were seeded in 12- or 24-well plates and allowed to adhere overnight. For radiolabeled experiments, tritiated gemcitabine was combined with cold gemcitabine to

make a 5 μM tracer solution in transport buffer (128 mM NaCl, 4.73 mM KCl, 1.25 mM $CaCl_2$, 1.25 mM $MgSO_4$, and 5 mM HEPES, pH 7.4). For non-radiolabeled experiments, substrates were also prepared at a concentration of 5 μM with cold gemcitabine. Incubations were initiated by aspirating the media from the cells, which were then loaded with tracer solution. Plates were incubated at 37º C on a rotary shaker for up to twenty-four hours, after which drug was removed, and cells washed three times in ice-cold transport buffer containing 50 μM dilazep. For radiolabeled experiments, cells were solubilized in 1% Triton X-100, and 200 μL of each solubilized cell fraction was added to 4 mL of scinitillation cocktail (ScintiSafe Econo Cocktail, Fisher Scientific, Pittsburgh, PA) Radioactivity was then determined with a Beckman Coulter LS-6500 liquid scintillation counter (Fullerton, CA). Total protein concentration in each well was determined by the BCA protein assay (Pierce, St Louis, MO). For cold substrates, cells were lysed with 70:30 methanol:water incubated in a dry ice bath, and gemcitabine, dFdU, and dFdCTP quantified by HPLC-UV or LCMS. Because methanol interfered with the absorbance readings obtained with the BCA protein assay, data from studies with cold substrate were normalized to the number of cells initially plated in each well.

Inhibition experiments: To determine the effects of dFdU and dFdCTP on gemcitabine uptake, either dFdU or dFdCTP prepared in a 1:1 solution of DMSO:transport buffer was added directly to the gemcitabine tracer solutions where indicated. Organic concentrations were limited to less than 0.5%.

ENT2-mediated transport: To examine the effects of hENT2-mediated transport alone, cells were pre-incubated for thirty minutes prior to the start of the experiment in 100 nM NBMPR (a known inhibitor of hENT1), and 100 nM NBMPR was also added to tracer

solutions.

Gemcitabine and dFdU Analysis

Internal standards ($^{13}C,^{15}N_2$-dFdC, and $^{13}C,^{15}N_2$-dFdU) were added to the HeLa cell lysate, and the lysate evaporated to dryness followed by reconstitution in 100 µL of mobile phase. The sample was injected onto a Fusion® RP 50 X 3.0 mm, 3.5 µm, column at 30º C (Phenomenex, Torrance, CA). The mobile phase (flow rate, 0.3mL/min) consisted of 5:95 (v/v) methanol:aqueous buffer (5 mM ammonium acetate, pH 6.8). The HPLC (Agilent 1200) was interfaced to a Thermo TSQ Quantum mass spectrometer with electrospray interface operating in positive ionization mode. Detection of dFdC and dFdU was obtained through selected reaction monitoring (SRM) of the following transitions: *m/z* 264/95 for dFdC, *m/z* 265/113 for dFdU, *m/z* 267/97 for ^{13}C, $^{15}N_2$ -dFdC (IS), and *m/z* 268/116 for ^{13}C, $^{15}N_2$ -dFdU (IS). The calibration range for this method was 2.4 – 4990 pmoles for dFdC and 4.0 – 8021 pmoles for dFdU.

Gemcitabine Triphosphate Analysis

For measurement of dFdCTP, HeLa cell lysates were processed as for dFdC and dFdU analysis using $^{13}C,^{15}N_2$- cytidine triphosphate (CTP) for an internal standard. Sample was injected onto a Thermo Scientific Bio Basix AX (50 x 2.1 mm, 5 µm) column at 30º C. Chromatographic separation was achieved with a gradient (0 min- 90:10, A:B; 0.51 min-50:50, A:B; 1.76 min- 0:100, A:B; 2.5 min-0:100, A:B; 8.5 min-90:10, A:B; 9.5 min-90:10, A:B; 12 min- 90:10, A:B) consisting of mobile phases A (30:70 ACN: 10 mM ammonium acetate in de-ionized water (DI), pH 6.0) and B (30:70 ACN: 1 mM ammonium acetate in DI, pH 10.5). The LC-MS/MS system was run in negative ionization mode with

the following SRM scan events: *m/z* 502/159 for dFdCTP and *m/z* 494/159 for $^{13}C9,^{15}N_2$-CTP. This method was linear within the range of 5 – 1000 pmoles.

dCK Silencing with siRNA

HeLa cells were washed twice in PBS and resuspended at a concentration of 1.25 x 10^6 cells/well in Dulbecco's Minimum Essential Media containing 10% FBS and 2 mM L-glutamine. Cells were transfected with either 10 nM dCK siRNA (*Silencer* Validated siRNA #70, Ambion, Cambridge, UK) or negative control siRNA (*Silencer Select* Negative Control #2, Ambion, Cambridge, UK). Transfection was conducted with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in Optimem (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Transfection media was removed after 24h and cells were grown in DMEM medium containing antibiotics for an additional 48 hours. Activity assays were performed by incubating transfected cells with 5 μM cold gemcitabine in DMEM. After four hours, media was removed and cells were washed with ice-cold transport buffer. Cells were then lysed with -80ºC 70:30 methanol:water, and lysate was analyzed for dFdCTP. The triphosphate moiety was used as a marker of dCK activity not only because it is the active end product of the sequential phosphorylation pathway of gemcitabine, but also because the product of the monophosphorylation step mediated by dCK, dFdCMP is rapidly phosphorylated to dFdCDP and dFdCTP[134].

To compare the intracellular metabolite ratio in cells transfected with dCK siRNA and negative control siRNA, 5 μM gemcitabine in DMEM was added to each well and incubations stopped after one and twenty-four hours with the addition of ice-cold transport buffer. Cells were lysed and prepared for analysis as specified previously.

Results

Effect of dFdU on gemcitabine metabolite formation

We have previously identified an interaction between gemcitabine and its deaminated metabolite whereby dFdU inhibits the bidirectional transport of gemcitabine via hENT1 and hENT2. During long incubation periods, it was noted that cells treated with both radiolabeled gemcitabine and 100 μM unlabeled dFdU contained significantly higher net levels of radioactivity (including gemcitabine and its metabolites). To assess whether dFdU was affecting the intracellular metabolism of gemcitabine, HeLa cells were incubated with unlabeled gemcitabine and dFdU, and the amount of intracellular gemcitabine and gemcitabine triphosphate (dFdCTP) were measured at one and twenty-four hours. Additionally, experiments were repeated in cells pretreated with 100 nM NBMPR to examine the interaction when only hENT2-mediated transport was active.

Gemcitabine levels were below the lower limit of quantitation at both one and twenty-four hours regardless of pretreatment with NBMPR. In cells with both hENT1 and hENT2 active (Figure 6-1A), gemcitabine triphosphate levels were measurable at both one and 24-hours, yet dFdU had no significant effect on gemcitabine triphosphate levels at the one hour time point. However, by 24 hours, the addition of dFdU resulted in dFdCTP levels that were nearly five-fold higher than with gemcitabine alone. In cells pretreated with NBMPR (hENT2-only, Figure 6-1B), again, triphosphate levels were similar at the one hour time point regardless of whether or not dFdU was present. Yet, by 24 hours, cells treated with both gemcitabine and dFdU had nearly ten-fold higher levels of gemcitabine triphosphate compared with cells incubated solely with gemcitabine. Twenty-four hour dFdCTP levels in NBMPR-treated cells were also significantly higher than the dFdCTP

levels at the same time point in cells with both hENT1 and hENT2 active.

Gemcitabine triphosphate transport via hENT

Previously we have proposed a mechanism by which dFdU enhances intracellular gemcitabine retention by inhibiting efflux of the parent compound via hENTs. Figure 6-1 also suggests that this heightened retention of gemcitabine results in increased phosphorylation to yield gemcitabine triphosphate. However, it is also possible that gemcitabine triphosphate inhibits transporter-mediated gemcitabine efflux as well, thereby potentiating its own formation by increasing gemcitabine retention.

To determine whether or not gemcitabine triphosphate is inhibiting gemcitabine efflux via hENTs, incubations less than twenty seconds in length were performed with gemcitabine co-incubated with 100 μM dFdCTP, and the amount of intracellular gemcitabine measured. As these are bidirectional transporters, if the triphosphate is inhibiting efflux, it should also inhibit influx.

Gemcitabine triphosphate was observed to have no effect on the level of gemcitabine uptake in cells regardless of whether both hENT1 and hENT2 (Figure 6-2A) or hENT2 alone (Figure 6-2B, NBMPR-treated) was active.

Interactions between gemcitabine, dFdU, hENT, and dCK

As incubations with gemcitabine and dFdU result in significantly higher levels of gemcitabine triphosphate formation, I was interested in determining whether or not dFdU was affecting gemcitabine phosphorylation directly in addition to inhibiting hENTs. To examine the effect of dFdU on dCK-mediated phosphorylation of gemcitabine,

accumulation studies with radiolabeled gemcitabine and metabolite composition experiments with unlabeled gemcitabine were repeated after siRNA-mediated knockdown of deoxycytidine kinase. Off-target effects on intracellular phosphorylation were observed when cells were transfected with negative control siRNA[195]. As such, cells transfected solely with Lipofectamine 2000 were used as a negative control.

Transfection of HeLa cells with dCK siRNA resulted in a significant decrease in deoxycytidine kinase activity by 24-hours post-transfection, which was determined by measuring gemcitabine triphosphate formation after incubation with gemcitabine, assuming that dCK is the rate-limiting enzyme in gemcitabine triphosphate formation (Figure 6-3). By 72-hours post-transfection, the level of dFdCTP formation was nearly six-fold lower when compared with cells transfected only with Lipofectamine 2000. Therefore, dCK knockdown studies were performed 72-hours post-siRNA transfection.

At one hour, net accumulation of tritiated gemcitabine was significantly decreased in cells deficient in deoxycytidine kinase activity, and this was associated with a lack of gemcitabine triphosphate formation (Figure 6-4A and B). The addition of 100 μM dFdU alone and in combination with 100 nM NBMPR produced a thirteen- and eighteen-fold decrease in total intracellular radioalabel, respectively, compared to control cells receiving the same treatment (Figure 6-5A). Additionally, a three-fold decrease in gemcitabine accumulation was observed when 100 μM dFdU was added to dCK-knockdown cells treated with gemcitabine. Similar decreases in gemcitabine accumulation in the presence of dFdU were not noted in non-transfected control cells at one hour, suggesting that at one hour dFdU produces a greater inhibitory effect on gemcitabine accumulation in the absence of dCK than when intracellular phosphorylation is occurring.

Interestingly, by 24 hours, net gemcitabine accumulation was still significantly decreased in the absence of dCK, but not nearly to the same extent as at the earlier time point (Figure 6-4C). Overall levels of dFdU metabolite were higher and dFdCTP lower at twenty-four hours, suggesting that the triphosphate may also be undergoing elimination via a deamination pathway as well over the longer incubation timeframes (Figure 6-4D). Additionally, the levels of intracellular radiolabel present in dCK-knockdown cells at 24 hours were nearly identical regardless of whether or not dFdU or NBMPR was added (Figures 6-4D and 6-5D). This implies that as intracellular and extracellular concentrations of gemcitabine equilibrate over longer incubation times, the lack of intracellular phosphorylation in dCK knockdown cells eliminates the additional accumulation of intracellular gemcitabine phosphates, and all cells ultimately yield the same amount of radiolabel independent of dFdU or NBMPR treatment. As the addition of dFdU has no effect on gemcitabine accumulation in dCK-knockdown cells, this suggests that the interaction between dFdU and gemcitabine is not solely due to inhibition of gemcitabine efflux, but also due to a subsequent increase in gemcitabine phosphorylation mediated by dCK. Correspondingly, the major differences in net gemcitabine accumulation at 24 hours between dCK knockdown and negative control cells are due to the formation of gemcitabine triphosphate. (Figures 6-4C and 6-5C). Consistent with the results of our previous study, the addition of dFdU again yielded the highest levels of dFdCTP formation in control cells both with and without preincubation in 100 nM NBMPR solution.

Discussion

After intravenous administration of gemcitabine, this nucleoside analog is extensively metabolized in humans via two different metabolic pathways. High levels of cytidine deaminase in human plasma lead to rapid deamination of gemcitabine to yield the the less active 2',2'-difluorodeoxyuridine (dFdU) metabolite[120]. Deamination also occurs intracellularly as well, once gemcitabine has been transported into the cell via nucleoside transporters. However, the intracellular expression of cytidine deaminase is highly variable, and the intracellular formation of dFdU is dependent upon the tissue in question[120].

The second metabolic fate of gemcitabine involves a series of sequential phosphorylation steps to form the active diphosphate (dFdCDP) and triphosphate (dFdCTP) moieties. The first and rate-limiting step in this phosphorylation pathway is a saturable process leading to the formation of gemcitabine monophosphate. Mediated by deoxycytidine kinase, gemcitabine is phosphorylated with a similar efficiency as the endogenous substrate for this enzyme, deoxycytidine, with apparent K_m and V_{max} values for gemcitabine of 4.6 μM and 14.9 pmol/min, respectively[100]. Due to its role in gemcitabine activation, it is not surprising that levels of deoxycytidine kinase have been observed to correlate with in vitro sensitivity towards gemcitabine in many cell types[97,99,192] Additionally, in vivo administration of a dCK fusion gene concomitantly with gemcitabine resulted in a significant reduction in tumor size in a hamster-model of pancreatic cancer further underscoring the importance of this enzyme in gemcitabine cytotoxicity[193].

Study of the disposition of nucleoside analogs such as gemcitabine is complicated by the multiple transport and metabolic processes involved. Earlier attempts to focus solely on transport have employed various methodologies in attempts to limit the effects of

intracellular metabolism, which begins within seconds after the appearance of the nucleoside in the cell. In our laboratory, we have utilized incubation periods of very short duration to limit the effects of intracellular deamination and phosphorylation. This research has revealed an interaction between gemcitabine and dFdU whereby the deaminated metabolite inhibits both the influx and efflux of the parent compound via the equilibrative nucleoside transporters 1 (hENT1) and 2 (hENT2). However, when the incubation interval was increased, an opposite effect was observed, and the presence of dFdU actually increased the retention of radiolabeled gemcitabine over periods up to twenty-four hours, during which time extensive metabolism of gemcitabine was undoubtedly occurring. In order to more accurately characterize the disposition of gemcitabine in the presence of dFdU, we became interested in studying the contribution of intracellular phosphorylation to this interaction.

Measuring the intracellular metabolite profile of gemcitabine over longer incubations (up to twenty-four hours) provided substantial evidence that dFdU is affecting phosphorylation, albeit most likely by an indirect mechanism. Specifically, the increase in net gemcitabine radiolabel previously observed in the presence of 100 μM dFdU appears to be associated with an increase in the formation of gemcitabine phosphates, resulting in a significantly higher level of dFdCTP at twenty-four hours when compared with cells treated solely with gemcitabine. We hypothesize that this increase in gemcitabine triphosphate formation is due to an enhanced retention of parent gemcitabine within the cell due to the dFdU-mediated inhibition of gemcitabine efflux that occurs via the hENTs. As gemcitabine becomes 'trapped' within the cell, more is available to undergo phospohorylation via deoxycytidine kinase. As more gemcitabine is phosphorylated, an

intracellular gemcitabine 'sink' is essentially created, leading to an increase in gemcitabine uptake from the extracellular compartment. As more time passes, a significant increase in net gemcitabine radiolabel, made up of parent gemcitabine and its metabolites, is observed in cells treated simultaneously with dFdU and gemcitabine due to these combined direct and indirect effects of dFdU. Additionally, dFdCTP is known to inhibit deoxycytidine monophosphate deaminase, which converts dFdCMP to dFdUMP, and ultimately potentiates the accumulation of the active parent triphosphate[182]. This may explain why lower levels of intracellular dFdU are observed in cells with the highest levels of dFdCTP.

We then investigated whether the high concentration of dFdCTP itself may also be an inhibitor/substrate for the hENTs and thus, also contribute to the enhanced net retention of gemcitabine observed in cells treated with both gemcitabine and dFdU. However, consistent with other studies demonstrating that nucleotides are not substrates for hENT1, we observed no difference in gemcitabine uptake via either hENT1 or hENT2 when clinically relevant concentrations of dFdCTP were added to gemcitabine tracer solutions[194]. As these transporters are bidirectional, a lack of effect of dFdCTP on gemcitabine influx is assumed to be consistent with a lack of effect on efflux.

To further evaluate the role of gemcitabine phosphorylation in the interaction between dFdU and gemcitabine, studies measuring net radiolabel accumulation and intracellular metabolite composition were repeated in cells possessing minimal capacity for intracellular phosphorylation, achieved through the siRNA-mediated knockdown of deoxycytidine kinase. A significant decrease in net gemcitabine accumulation in cells lacking deoxycytidine kinase activity was observed over a twenty-four hour evaluation period. When compared with the metabolite composition in corresponding cells, the

decrease in net accumulation was associated with a loss of gemcitabine triphosphate formation. However, levels of dFdU formation were not affected. The effects of dFdU and NBMPR on gemcitabine retention were dependent on the length of the incubation. Specifically, when dFdU and NBMPR were added to the gemcitabine tracer solutions for one hour incubations, an even more profound decrease in gemcitabine accumulation was noted. However, by twenty-four hours, levels of accumulation in dCK knockdown cells were nearly identical regardless of dFdU or NBMPR treatment. These data suggest that by competing with gemcitabine for hENT-mediated transport, dFdU may affect the rate at which gemcitabine distributes into the cell, but once an equilibrium between intracellular and extracellular gemcitabine concentrations have been reached, in the absence of intracellular metabolism, dFdU has no further effect on gemcitabine disposition. Similarly, inhibition of hENT1 by NBMPR affects the rate of gemcitabine uptake, but given a long enough incubation period, one could hypothesize that the ultimate accumulation of gemcitabine would be the same as in cells with both hENT1 and hENT2 activity.

A recent simulation of the accumulation of ribavirin, a nucleoside analog that is a substrate for the equilibrative nucleoside transporters and adenosine kinase, has also provided significant insight into the interplay between nucleoside transporter-mediated uptake and intracellular phosphorylation. In models of hENT1-expressing erythrocytes lacking an intracellular metabolism component, no change in intracellular total ribavirin radioactivity was predicted after equilibrium was reached[194]. When a more complex model was used in which it was assumed that ribavirin was transported via nucleoside transporters and metabolized intracellularly via two separate pathways (a nonphosphorylative and a phosphorylative pathway), ribavirin concentrations again reached equilibrium by sixty

seconds. Yet, in this model, the total intracellular radioactivity continued to increase up to six hours due to a "metabolic sink effect" whereby ribavirin is intracellularly phosphorylated leading to a continual uptake of parent compound from the extracellular compartment. These predictions were then confirmed following the conduct of *in vitro* studies in both mouse and human erythrocytes.

We report here on the interplay between hENT-mediated uptake of gemcitabine and its subsequent intracellular phosphorylation via deoxycytidine kinase. However, another critical pathway involved in gemcitabine disposition is deamination via cytidine deaminase. In HepG2 cells, dFdU and its nucleotides made up only 0.06% and 0.94% of the total recovered intracellular drug content at 24 hours, respectively, after incubation with 0.5 uM dFdC[85]. As such, cytidine deaminse inhibitors, such as tetrahydrouridine, were not used in these experiments, as the level of deamination occurring intracellularly was so low that it did not appear to warrant inhibition. Yet in our experiments with HeLa cells, a higher, more clinically relevant concentration of 5 μM gemcitabine was used, and dFdU frequently made up more than 50% of the total intracellular drug content, suggesting that at higher concentrations of gemcitabine, deamination may also lead to the formation of the intracellular "metabolic sink" in the same manner as phosphorylation. However, despite intact deamination processes, twenty-four hour gemcitabine accumulation was two- to four-fold lower in dCK-knockdown cells than in control cells. Additionally, the increased accumulation in total drug content noted in control cells was associated with significant increases in gemcitabine triphosphate, whereas dFdU levels remained constant despite dCK knockdown, implying that phosphorylation and not deamination has a larger effect on overall gemcitabine accumulation.

In conclusion, we have further characterized the interaction between dFdU and gemcitabine and propose a novel mechanism whereby dFdU-mediated inhibition of gemcitabine efflux increases gemcitabine phosphorylation intracellularly due to higher levels of substrate available for conversion by deoxycytidine kinase. This increase in phosphorylation essentially creates a "metabolic sink", drawing more gemcitabine into the cell from the extracellular space. We also predict that studies of nucleoside analog transport in the absence of intracellular metabolism may not adequately describe the distribution of these compounds, which appears to be equally dependent on both transport and metabolic processes.

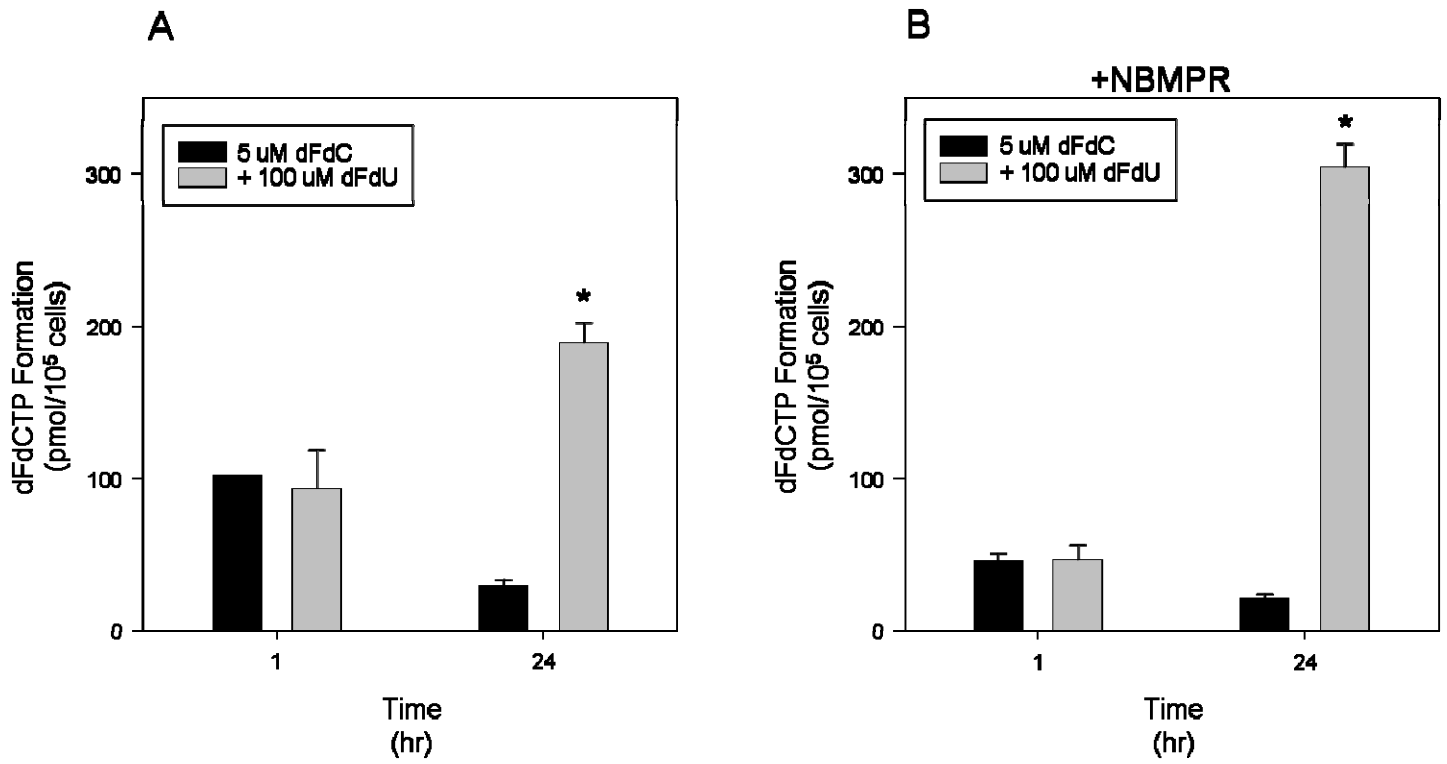

Figure 6-1. Effect of dFdU on the formation of gemcitabine triphosphate (dFdCTP) in HeLa cells with A.) hENT1- and hENT2-, and B) hENT2-mediated gemcitabine transport. Addition of 100 nM NBMPR allowed for the measurement of dFdCTP formation in cells with only hENT2 active. No gemcitabine was detected at either one or 24-hours. *Significantly different than formation in cells treated solely with gemcitabine ($p = 0.05$).

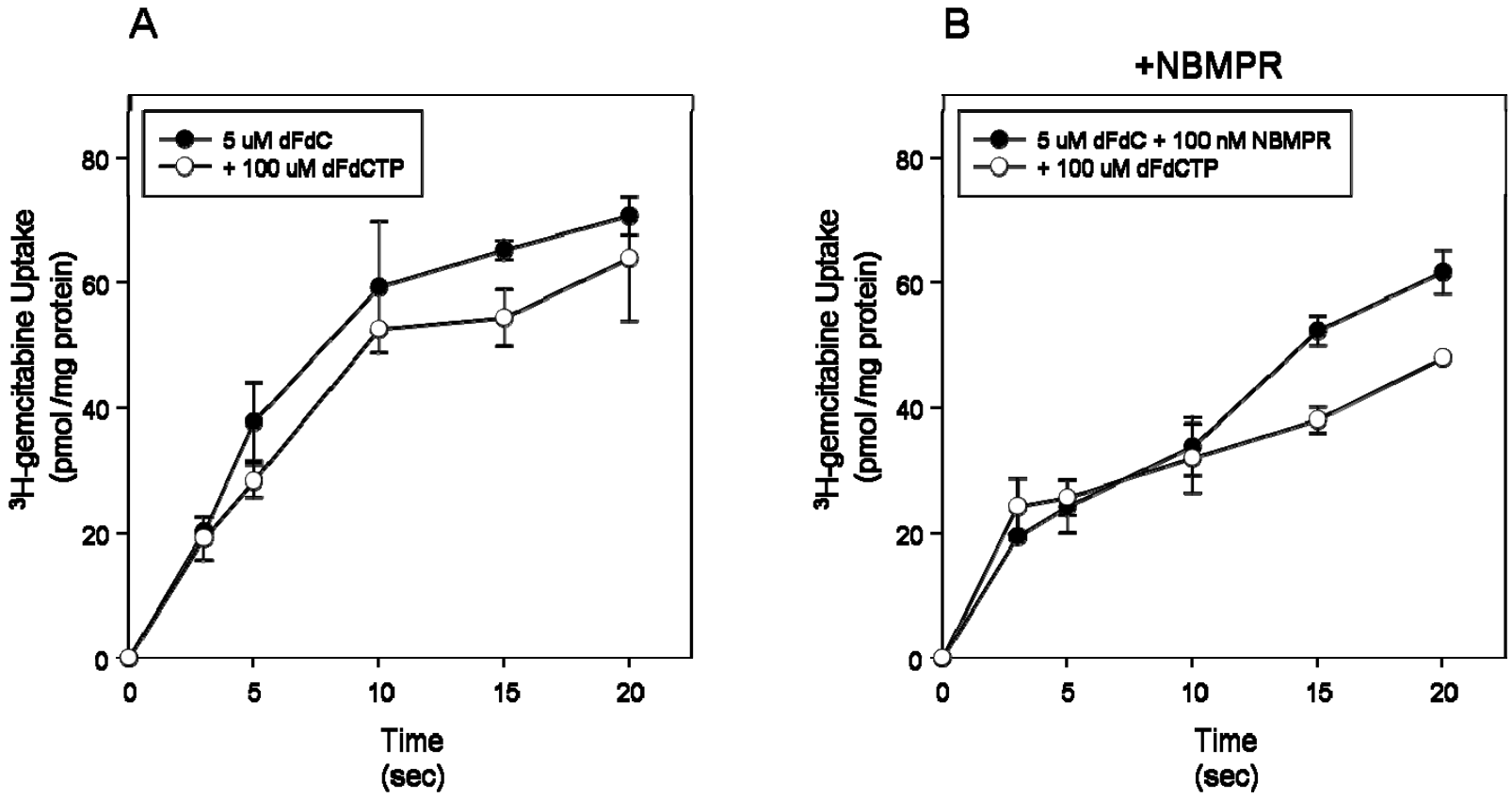

Figure 6-2. Effect of gemcitabine triphsophate (dFdCTP) on the uptake of tritiated gemcitabine via A) hENT1 and hENT2, and B) hENT2 during short incubation periods. Addition of 100 nM NBMPR allowed for analysis of gemcitabine uptake in cells with only hENT2 active.

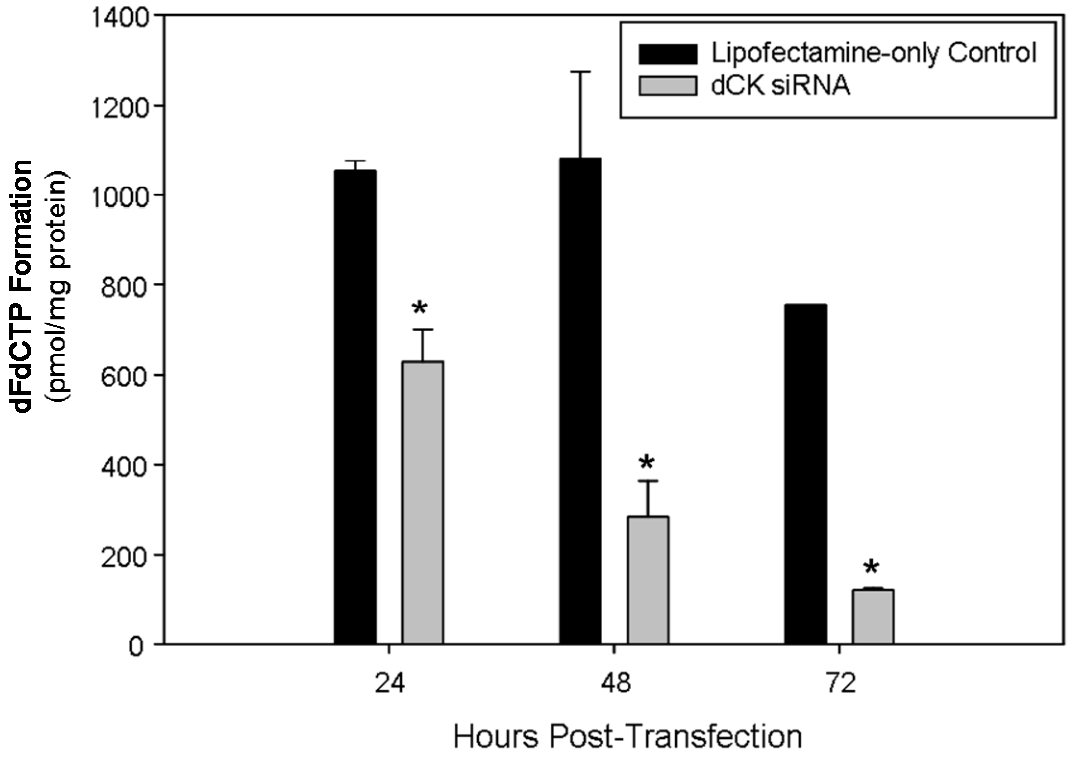

Figure 6-3. Effect of deoxycytidine kinase (dCK)-targeting siRNA on the formation of gemcitabine triphosphate in HeLa. Cells transfected with only Lipofectamine 2000 and water were used as a negative control. *Significantly different than gemcitabine triphosphate formation in the negative control-trasnfected cells ($p = 0.05$)

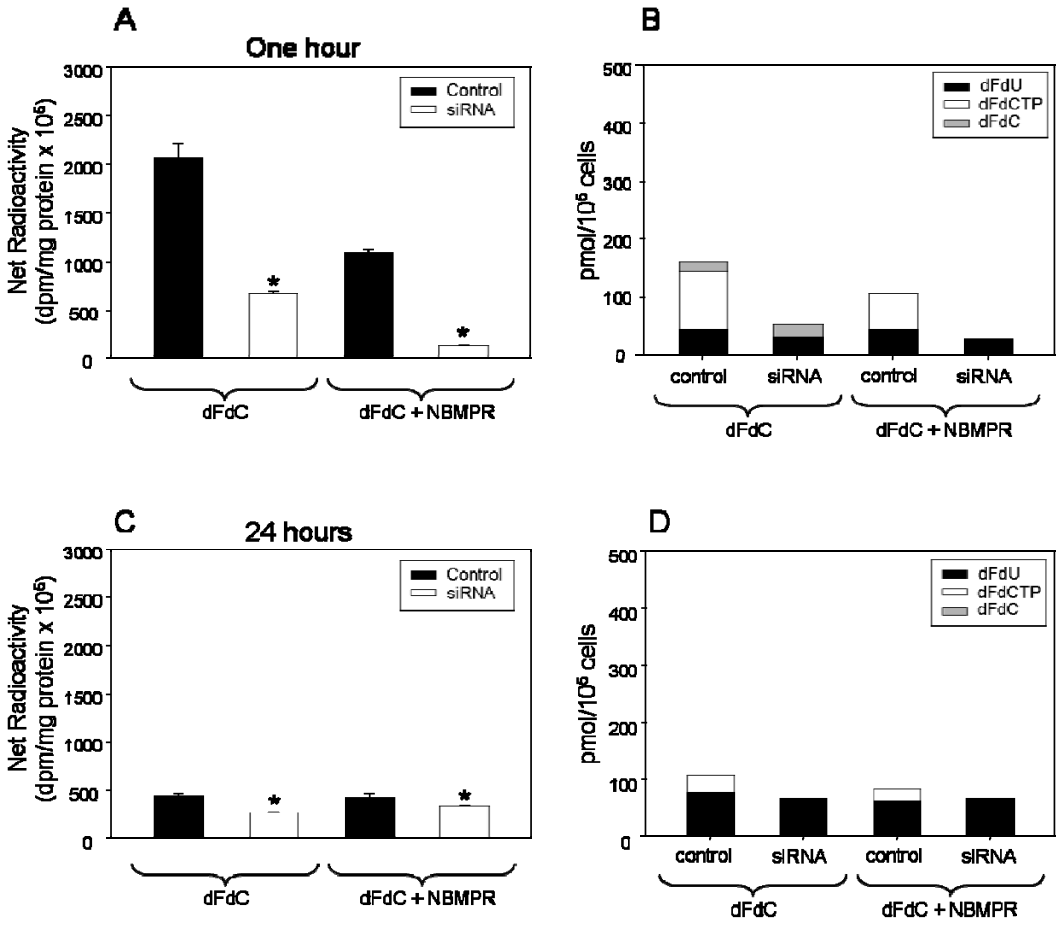

Figure 6-4. Effect of deoxycytidine kinase knockdown on accumulation and metabolite profile of gemcitabine in HeLa cells. Net accumulation of 5 μM tritiated gemcitabine measured after A) one hour or C) 24 hours in either negative control HeLa cells or HeLa cells transfected with dCK-siRNA. Corresponding formation of gemcitabine, dFdCTP, and dFdU was measured in cells treated with 5 μM gemcitabine for B) one hour or D) 24 hours in either control or dCK-knockdown (siRNA) HeLa cells. Addition of 100 nM NBMPR to tracer solutions yielded HeLa cells with only hENT2 active as compared to cells with both hENT1 and hENT2 activity. *Statistically different from accumulation in control cells, ($p < 0.05$).

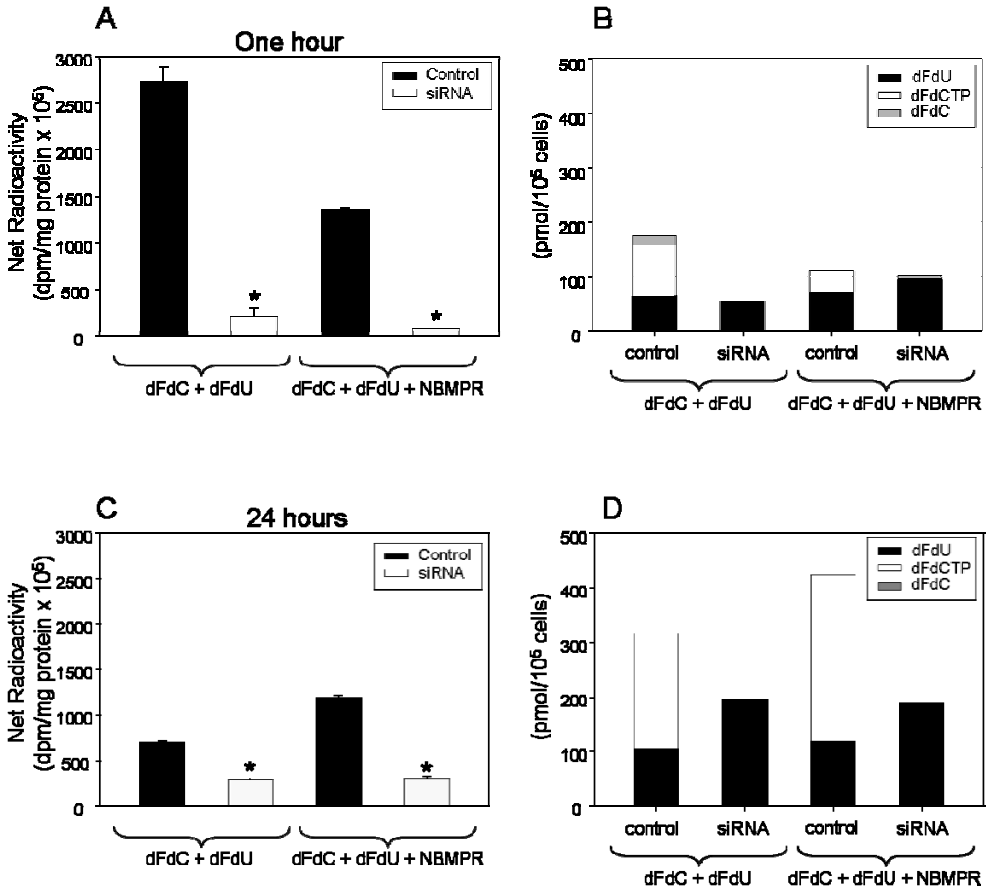

Figure 6-5. Effect of dFdU on accumulation and metabolite profile of gemcitabine in HeLa cells transfected with dCK-siRNA. Net accumulation of 5 μM tritiated gemcitabine measured after A) one hour or C) 24 hours in either negative control HeLa cells or HeLa cells transfected with dCK-siRNA. Corresponding formation of gemcitabine, dFdCTP, and dFdU was measured in cells treated with 5 μM gemcitabine for B) one hour or D) 24 hours in either control or dCK-knockdown (siRNA) HeLa cells. Addition of 100 nM NBMPR to tracer solutions yielded HeLa cells with only hENT2 active as compared with cells with both hENT1 and hENT2 activity. *Statistically different from accumulation in control cells, ($p < 0.05$).

Chapter VII.

Summary and Conclusions

While the incidence is declining in the United States, cervical carcinoma remains a major public health concern amongst women in developing countries, with nearly 500,000 cases diagnosed in 2009. Cisplatin in combination with radiation is the most commonly used therapeutic regimen for the treatment of cervical carcinoma and, in addition to surgery, has been the standard of care for more than ten years. However, despite the success of cisplatin-based chemoradiation in treating earlier stages of cervical carcinoma, once the tumor has spread beyond the border of the cervix, patient response to current therapies is significantly decreased. Additionally, side effects associated with intravenous cisplatin administration are often dose-limiting, resulting in the use of suboptimal dosing regimens. As such, researchers continue to explore alternative therapeutic options for the treatment of cervical carcinoma.

Like cisplatin, the pyrimidine analog gemcitabine has been observed to be a potent radiosensitizer as well and has demonstrated efficacy against many types of solid tumors including cervical cancer. Gemcitabine-based chemoradiation regimens yield response rates similar to those obtained with cisplatin, and the differences in the toxicity profiles of these two agents may make gemcitabine more suitable for use in women with poor renal function, often a consequence of locally invading cervical tumors. Yet, despite its promise as a radiosensitizing agent in cervical carcinoma, our understanding of the disposition of gemcitabine in the cervix is very limited, and a more thorough characterization of gemcitabine distribution in cervical tissue would be beneficial for further optimization of gemcitabine-based chemoradiation.

Typically administered intravenously, nonspecific toxicities associated with gemcitabine therapy may be alleviated if the delivery of this nucleoside analog could be

localized to the tissue of interest. We report here the utility of a novel cervical drug delivery device in targeting the administration of gemcitabine to the cervix while limiting systemic side effects. Use of this device in eighteen women demonstrated that localized delivery of gemcitabine to the cervix produced clinically relevant concentrations of drug in the cervical tissue and vasculature while limiting systemic exposure and related toxicities. However, substantial inter-individual differences in the levels of gemcitabine and dFdU measured in the cervix suggested that some women may be inherently more sensitive to gemcitabine therapy than others.

Due to the variation in cervical levels of gemcitabine noted in the clinical study, we then focused our research on the transport and metabolism of gemcitabine, as a potential source for the inter-individual differences in gemcitabine disposition in the cervix. As a hydrophilic nucleoside analog, gemcitabine undergoes limited passive diffusion and instead requires nucleoside transporter proteins to facilitate uptake into cells. Investigations in other tumor types suggest that therapeutic response to gemcitabine is highly correlated with the level of expression of these nucleoside transporter proteins in the target tissue. As such, a characterization of the expression of hENT1 and hENT2 was performed in both healthy and malignant cervical tissue. These levels were then correlated with the amount of gemcitabine and dFdU detected in the tissue samples collected during the clinical study. mRNA levels of hENT1 and hENT2 were highly variable in both the malignant and the normal cervical tissue, and carcinogenesis was not observed to have an effect on mRNA expression in our set of thirty-six tissue samples. Due to differences in cellular localization and extensive post-translation modification, hENT protein levels typically do not correlate well with mRNA expression, which we

observed to be true of hENT expression in cervical tissue. While hENT1 protein levels were similar regardless of pathological status, protein expression of hENT2 was nearly three-fold higher in the malignant cervical tissue samples, suggesting that carcinogenesis affects the regulation of hENT2 protein expression. As these are bidirectional transporters, an increase in protein expression may be associated with an increased need for nucleosides due to rapidly dividing cells, but may also be a mechanism of inherent resistance to nucleoside therapies resulting in an increased efflux of gemcitabine from the malignant cervix.

The pharmacokinetic profile of gemcitabine after intravenous administration reveals a rapid deamination of the parent compound yielding dFdU. With a plasma half-life of only eight minutes, concentrations of gemcitabine are at least five- to ten-fold lower than those observed for dFdU, which possess a half-life measured in hours. Due to their structural similarities it is not surprising that recent studies have observed dFdU to also be a substrate for hENT1 and hCNT1. Because the ratio of dFdU to dFdC in the plasma is typically quite large, we investigated what effect dFdU has on the transport of gemcitabine and explored the possibility of an in vivo interaction between the deaminated metabolite and the parent compound.

These studies are the first to report that in addition to hENT1, dFdU is also a substrate for hENT2 in HeLa cells. Not surprisingly, clinically relevant concentrations of dFdU were observed to significantly inhibit gemcitabine uptake via both hENT1 and hENT2. IC_{50} values associated with dFdU-mediated inhibition of gemcitabine uptake via hENT1 and hENT2 were quite low relative to typical plasma concentrations of the deaminated metabolite, suggesting that the likelihood for an in vivo interaction between

gemcitabine and dFdU is quite high. Interestingly, when incubations with gemcitabine and dFdU were performed for up to twenty-four hours, the presence of dFdU significantly enhanced the retention of radiolabeled gemcitabine, suggesting that dFdU limits gemcitabine uptake into the cell but also inhibits efflux as well, resulting in significantly higher levels of gemcitabine accumulation. Yet, despite the notable increases in intracellular gemcitabine levels in the presence of dFdU, this did not translate into an increase in gemcitabine cytotoxicity when dFdU was administered simultaneously.

Additional studies focused on the effect of intracellular phosphorylation on the interaction between gemcitabine and dFdU. In HeLa cells, the addition of dFdU to gemcitabine incubations resulted in nearly ten-fold higher levels of dFdCTP formation over a twenty-four hour period when compared to similar incubations in the absence of gemcitabine. Yet dFdCTP itself did not interfere with gemcitabine uptake, suggesting that the triphosphate is not a substrate or inhibitor for hENT1 and hENT2. This strengthened the hypothesis that dFdU was indeed inhibiting gemcitabine efflux increasing the availability of gemcitabine substrate for phosphorylation via deoxycytidine kinase, the enzyme responsible for the first, rate-limiting step of gemcitabine activation.

Using HeLa cells transfected with dCK-siRNA, we next examined the interplay between intracellular phosphorylation and nucleoside transporters as it pertains to the disposition of gemcitabine in the presence of dFdU. In the absence of dCK-mediated phosphorylation, overall accumulation of radiolabeled gemcitabine was significantly decreased. The corresponding metabolite profile revealed a lack of gemcitabine triphosphate formation, without any effect on dFdU levels, suggesting that in the absence

of intracellular phosphorylation, the overall uptake of gemcitabine is significantly decreased. While the increase in net gemcitabine radiolabel detected in control cells in the presence of dFdU was associated with significantly higher levels of triphosphate formation, in the dCK knockdown cells, dFdU no longer had any effect. These data led to the proposition of a novel mechanism whereby dFdU inhibition of gemcitabine efflux increases the intracellular phosphorylation of gemcitabine due to increased availability of substrate for deoxycytidine kinase. As more gemcitabine is phosphorylated, a "metabolic sink" is formed, driving extracellular gemcitabine inward via the equilibrative nucleoside transporters to maintain equilibrium conditions.

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