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Nucleoside transporters in drug response

Author
Gray, Jennifer

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Nucleoside Transporters in Drug Response

by

Jennifer Gray

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

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of the

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Jennifer Gray

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ABSTRACT

Nucleoside Transporters in Drug Response

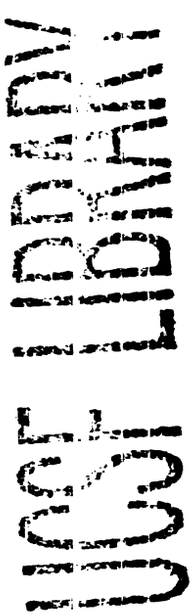
Jennifer Gray

Nucleoside analogs are widely used clinically as antimetabolites, however there is great variation in therapeutic response. Of particular interest is the nucleoside analog gemcitabine which is the first-line drug in the treatment of pancreatic cancer. As nucleoside transporters initiate gemcitabine activation, we examined the role of nucleoside transporter genetic and expression level variation in determining gemcitabine sensitivity in pancreatic cancer.

Five of the seven cloned and characterized nucleoside transporters transport clinically relevant nucleoside analogs at pharmacologic concentrations. However, gemcitabine is transported specifically by the equilibrative transporter, ENT1 (*SLC29A1*), and by the concentrative transporters, CNT1 and CNT3 (*SLC28A1* and *SLC28A3*) which are present in normal pancreas and in pancreatic cancer cell lines.

Our study of membrane transporter natural variation revealed that *SLC28A1* is a highly variable gene; 15 protein altering variants of CNT1 were studied including CNT1-Ser546Pro and CNT1-1153del (non-functional) and CNT1-Ala230Val (hyper-functional). The common variant CNT1-Val189Ile exhibited a kinetic difference in its interaction potency with gemcitabine, which may be important for clinical drug therapy as this variant is found at high allele frequencies in multiple populations. As approximately 8 to 12% of the population will be homozygous for CNT1-Val189Ile this variant may be clinically important in gemcitabine therapy.

We examined a panel of 16 pancreatic cancer cell lines to determine the relationship between gemcitabine sensitivity and mRNA expression level of ENT1, CNT1, and CNT3. In contrast to previous studies, we observed that ENT1 expression level was associated with reduced gemcitabine sensitivity. ENT1 inhibitors effected a range of gemcitabine responses, from enhanced resistance to enhanced sensitivity, depending on the relative expression level of concentrative and equilibrative transporters. Our results suggest a model in which equilibrative and concentrative nucleoside transporters work in concert to modulate the intracellular concentrations of gemcitabine. ENT1, when expressed in the absence of CNTs acts as a mediator of sensitivity, however, when expressed concomitantly with CNT1 or CNT3, ENT1 acts predominantly as an efflux transporter, mediating resistance to gemcitabine. As such, both the relative expression level and activity of these transporters will ultimately determine the intracellular concentration and therapeutic effect of gemcitabine.



Kathleen M. Giacomini

Dec. 20, 2005

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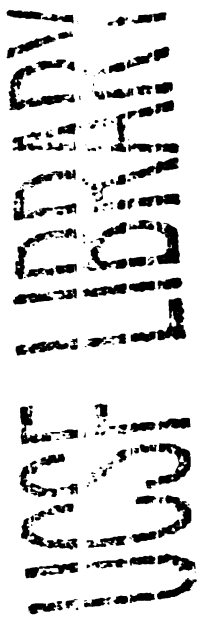

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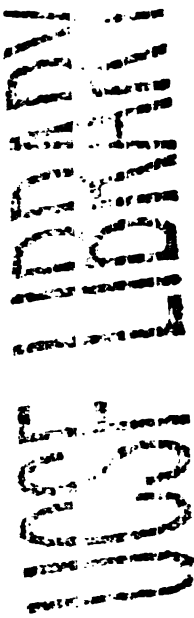

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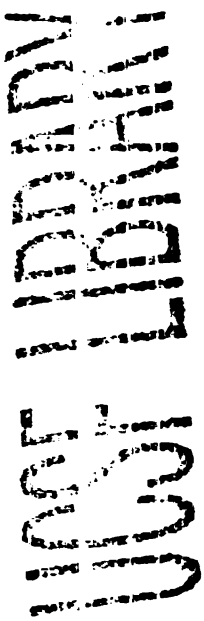


Chapter 5

CHAPTER 1

INTRODUCTION: MECHANISMS OF RESISTANCE TO ANTIMETABOLITE NUCELEOSIDE ANALOGS - THE ROLE OF NUCLEOSIDE TRANSPORTERS

1.1 Endogenous Nucleoside Metabolism

Nucleosides are small hydrophilic molecules consisting of either a ribose or deoxyribose ring conjugated to a purine or pyrimidine base (Fig. 1.1). The major physiological role of endogenous nucleosides is as a precursor to nucleotides, the essential building blocks of nucleic acids – both RNA and DNA. As such, any cells rapidly dividing or undergoing transcription will require nucleotides. There are two biological systems present for the formation of nucleotides – *de novo* synthesis and salvage of circulating nucleosides. Nucleoside salvage is often favored over *de novo* synthesis since the energy requirements of the salvage pathway are much lower. [1] Additionally, some cells, such as enterocytes, leukocytes, and erythrocytes, are not capable of *de novo* synthesis and must rely on salvaged nucleosides for nucleotide production. [2]

Through the salvage pathway nucleosides and deoxynucleosides are brought into the cell via nucleoside transporters and phosphorylated to nucleoside triphosphates (NTPs) and deoxynucleoside triphosphates (dNTPs) by a series of kinases (Fig. 1.2). Deoxycytidine kinase (dCK), deoxyguanosine kinase (dGK), thymidine kinases 1 and 2 (TK1 and TK2), and uridine-cytidine kinases 1 and 2 (UCK1 and UCK2) are responsible for the initial phosphorylation step of nucleosides and deoxynucleosides.

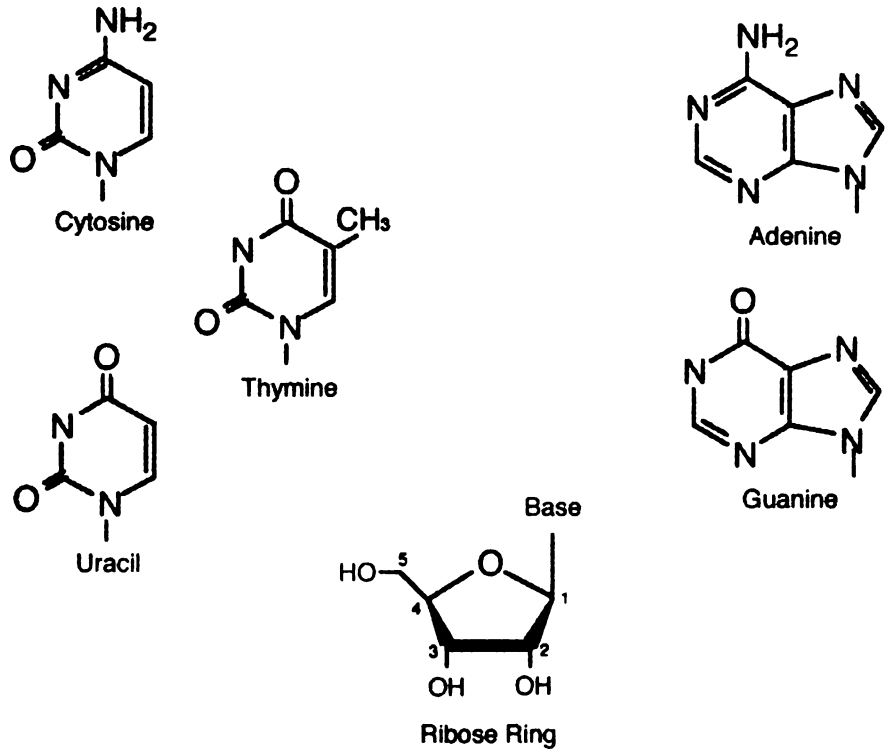


Figure 1.1 Structure of naturally occurring nucleosides. Nucleosides consist of a ribose ring conjugated to either a pyrimidine (cytosine, thymine, or uracil) or purine (adenine or guanine) base. The nomenclature for the nucleosides are: cytidine, thymidine, uridine, adenosine, and guanosine. Thymidine contains a deoxyribose ring, lacking the 2' hydroxyl group.

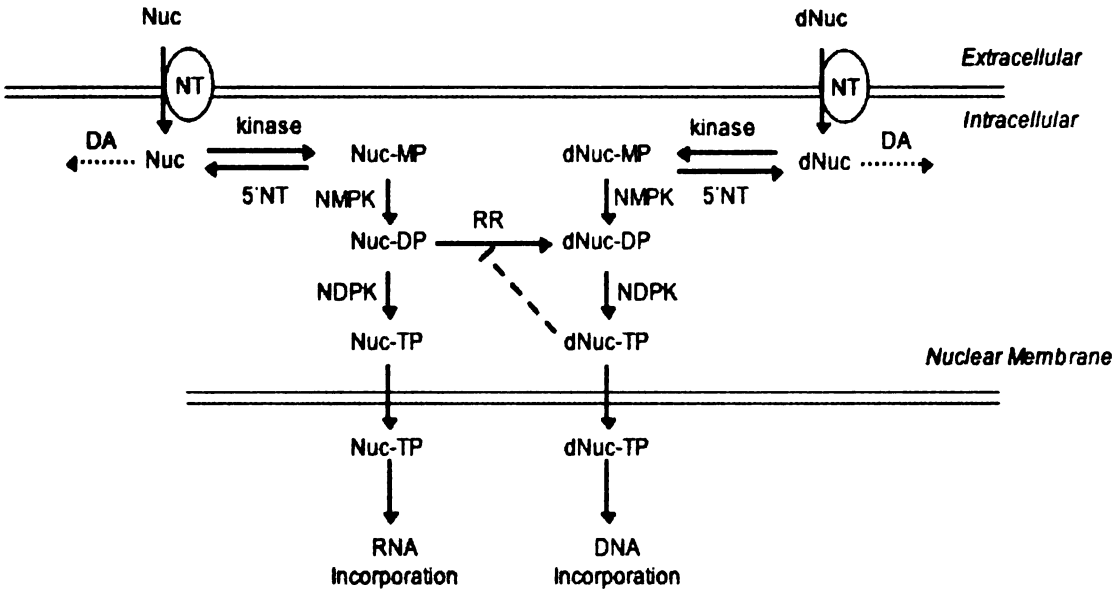


Figure 1.2 Salvage pathway of endogenous nucleosides and deoxynucleosides. Nucleoside transporters (NT) transport both nucleosides (Nuc) and deoxynucleosides (dNuc) into cells. Phosphorylation is completed stepwise through a series of enzymes; kinases, NMPKs, and NDPK which are described in the text.

Monophosphates are further converted to diphosphates by nucleoside monophosphate kinases (NMPK); UMP-CMP kinase, thymidylate kinase, guanylate kinase and adenylate kinase. The final phosphorylation step is completed by nucleoside diphosphate kinase (NDPK). Once in the nucleus, nucleoside triphosphates (NTPs) and deoxynucleoside triphosphates (dNTPs) are incorporated into RNA and DNA respectively. Nucleotide pools are kept in balance through degradation by cytoplasmic 5'-nucleotidase (5'NT), which carries out the reverse reaction of the initial phosphorylation step, and nucleoside deaminases, (DA); cytidine deaminase (CD), adenosine deaminase (AD) and deoxycytidylate deaminase (dCMP-deaminase). Nucleoside diphosphates (NDPs) can be reduced to deoxynucleoside diphosphates (dNDPs) by ribonucleotide reductase (RR). dNTPs inhibit RR and this feedback loop helps keep nucleotide and deoxynucleoside pools in balance.

1.2 Deoxynucleoside Antimetabolites

In the past twenty-five years, numerous deoxynucleoside analogs have been developed clinically as antimetabolites to treat a variety of leukemias and solid tumors (Fig. 1.3). The mechanism of action of nucleoside analogs depends on whether they act in quiescent or replicating cells. In resting cells, cytotoxicity of nucleoside analogs is due to the inhibition of DNA repair machinery. Inhibition of DNA repair leads to DNA fragmentation and an accumulation of single strand breaks that signal apoptosis. [3-5] In dividing cells, the major mechanism of cytotoxicity is the misincorporation of nucleoside analog triphosphates (NA-TP) into DNA instead of naturally occurring deoxynucleoside

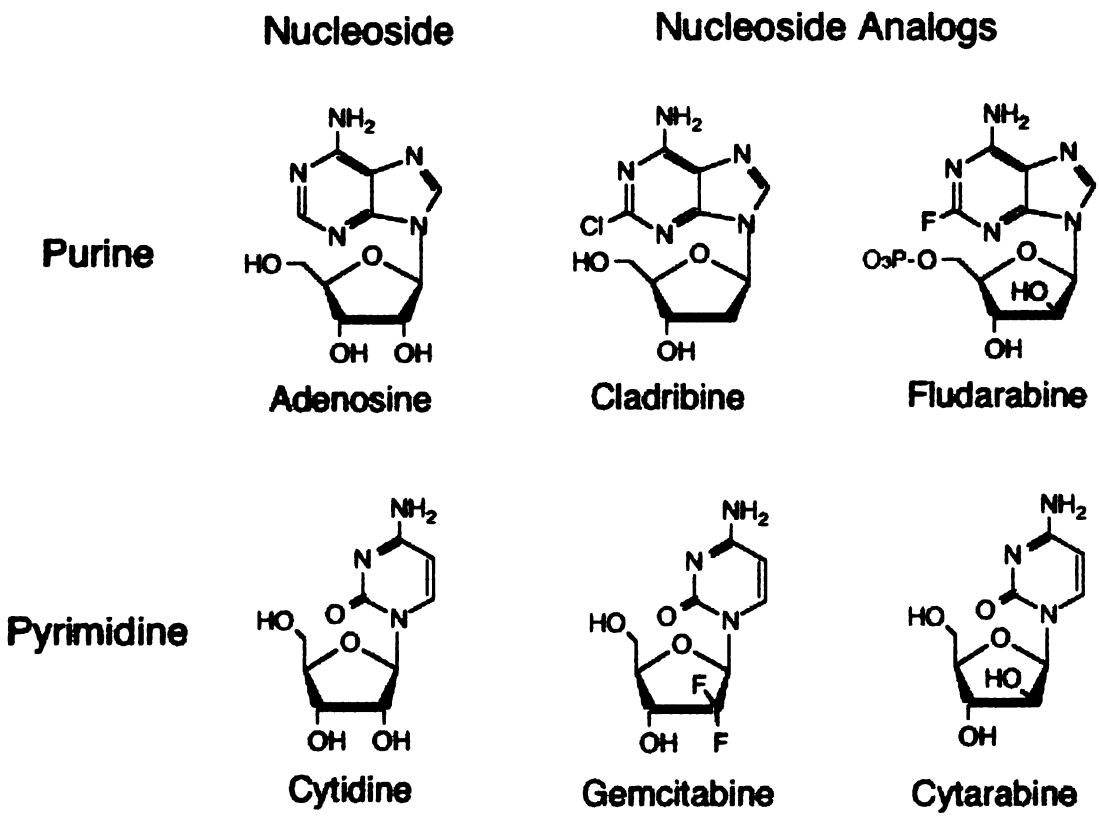


Figure 1.3 Clinically used nucleoside analogs. Both purine and pyrimidine nucleoside analogs are used clinically. Cladribine (2'-chlorodeoxyadenosine, 2'CdA) and fludarabine are adenosine analogs used in the treatment of leukemias. Cytarabine (cytosine arabinoside, araC) and gemcitabine (difluorodeoxycytidine, dFdC) are both cytidine analogs. Cytarabine is used to treat leukemias while gemcitabine is used to treat a number of solid tumors.

triphosphates (dNTP). This incorporation forces DNA into an unfavorable structure and decreases DNA stability which in turn interferes with DNA replication and repair. [6]

Drugs that are analogs of naturally occurring nucleosides rely on the nucleoside salvage pathway to exert their cytotoxic effects. Phosphorylated nucleoside analogs substitute for naturally occurring nucleotides and inhibit a variety of chemical reactions required for host-cell nucleic acid replication. Both purine (e.g. cladribine and fludarabine) and pyrimidine (e.g. cytarabine and gemcitabine) nucleoside analogs are used clinically, although the drugs differ in the types of malignancies they are used to treat. Cladribine, fludarabine, and cytarabine are used exclusively for the treatment of leukemias. Gemcitabine, however, has shown cytotoxicity against adenocarcinomas in a wide spectrum of tissue types including pancreatic, bladder, lung, ovarian and breast cancer. [7] Although the antimetabolite nucleoside analogs share common characteristics including uptake by nucleoside transporters, kinase activation, dephosphorylation by cytoplasmic 5'-nucleotidase, and formation of the active nucleoside analog triphosphate, they display varying degrees of self-potentiation. In addition to self-potentiation, the tissue specific expression of genes in the activation pathway, as well as a drug's affinity for specific transporters and kinases, act in concert to explain the various activities that the nucleoside analogs show. In this chapter, I will discuss the mechanism of action and resistance for these anticancer nucleoside analogs, paying particular attention to their relation to nucleoside transporters - the obligate first step in nucleoside analog cytotoxicity.

Cladribine. Cladribine (2'-chlorodeoxyadenosine, 2'CdA) is a structural analog of deoxyadenosine used to treat indolent lymphoid malignancies, particularly hairy-cell

leukemia (HCL), although it has also been approved for use in chronic lymphocytic leukemia (CLL), acute myelogenous leukemia (AML) and chronic myelogenous leukemia (CML). In 1990, Piro *et al.* determined that patients achieved a high rate of complete remission from HCL following a single course of 2'CdA treatment. [8] Subsequent studies found that relapses do occur with longer follow-up, but there is a high success rate with a second treatment of cladribine. [9]

2'CdA is cytotoxic to both dividing and resting cells. In dividing cells, 2'CdATP is incorporated into elongating DNA which leads to chain termination and apoptosis. [10] Cladribine also indirectly inhibits DNA replication by inhibition of ribonucleotide reductase (RR) an enzyme responsible for maintaining endogenous dNTP pools. [11] RR inhibition lowers cellular pools of dATP leading to the preferential incorporation of 2'CdATP into DNA. [12] Numerous mechanisms have been proposed to explain cladribine's activity in resting cells. One explanation is that incorporation of 2'CdATP during DNA repair terminates the nucleotide excision-repair process, making misincorporated DNA less likely to be repaired. This causes an accumulation of DNA single strand breaks and induces apoptosis. [13, 14] A second explanation is that TATA box consensus sequences with incorporated 2'CdATP have a reduced affinity for RNA polymerase II and a corresponding 30-35% reduction of DNA transcription. [15] Lastly, cladribine has been shown to disrupt the integrity of mitochondria, releasing cytochrome c which initiates a pro-apoptotic caspase cascade. [16]

Fludarabine. Fludarabine (F-ara-A) is a 5'-monophosphate analog of adenosine typically used in the treatment of chronic lymphocytic leukemia (CLL). CLL is characterized by the accumulation of morphologically mature but functionally inactive

neoplastic β-lymphocytes. [17] The disease has a slow rate of progression caused by the gradual buildup of β-cells with apoptotic defects. These leukemic cells eventually crowd out healthy functional leukocytes and erythrocytes. Although CLL is considered incurable, fludarabine and sometimes cladribine are used to control the disease. Unlike acute leukemias, chronic lymphocytic leukemia seems to result not from increased proliferation of cells but from the accumulation of abnormally long-lived cells. [18] Although fludarabine is active in both dividing and quiescent cells, the low proliferation rate of CLL cells suggests that apoptosis of resting cells is the major function of fludarabine therapy in this disease.

The monophosphate group found in fludarabine is present solely for improving the water solubility of the clinical formula. Prior to transport into cells, 5'-monophosphate F-ara-A is dephosphorylated to F-ara-A which is then transported by nucleoside transporters. Fludarabine follows the same activation pathway as cladribine; phosphorylation to the active compound is completed through deoxycytidine kinase, adenylate kinase, and NDPK. Fludarabine displays self-potentiation similar to 2'CdA, inhibiting RR and incorporating into elongating DNA. [19, 20] F-ara-ATP further self-potentiates by interfering with DNA primase, DNA ligase, and DNA polymerase α; enzymes responsible for DNA replication, repair, and synthesis. [21] As such, F-ara-A cytotoxicity in dividing cells is mainly cell cycle specific with S-phase incorporation leading to apoptosis. In non-dividing cells, the inhibition of DNA repair is the major mechanism of cytotoxicity. As with cladribine, fludarabine incorporation into DNA halts the nucleoside excision repair process, resulting in extensive DNA fragmentation. [13] This causes irreversible cellular damage and apoptosis.

Cytarabine. Cytarabine (cytosine arabinoside, araC) was the first nucleoside analog used in cancer therapy and is extensively used to treat acute leukemias, primarily acute myelogenous leukemia (AML), the most common form of acute leukemia in adults. For over twenty years the standard therapy of AML has been treatment with anthracyclines and the cytidine analog cytarabine. [22] Although 65-80% of patients achieve complete remission, defined as normal values for neutrophil and platelet count and no blast cells in bone marrow biopsy, of these patients 75-80% will relapse within 12 months. Relapsed patients are retreated with cytarabine, however only 40-60% of patients obtain a second remission and have a shorter duration of remission, approximately 6 months. [23] As such, cytarabine resistance is a major drawback to therapy.

AraC is transported into the cell primarily via ENT1, phosphorylated by deoxycytidine kinase, UMP-CMP kinase, and NDPK to form the active compound, araC-TP. AraC-TP is then incorporated into elongating DNA instead of the natural nucleoside triphosphate, dCTP. Misincorporation leads to DNA chain termination which subsequently stimulates apoptosis and inhibits DNA synthesis. [24] High levels of araC-TP relative to dCTP favor the incorporation of the nucleoside analog into elongating DNA. [25] Incorporation of araC-TP into blast cells *in vitro* is predictive of clinical outcome. Low araC-TP incorporation is correlated with poor outcome in AML patients on araC therapy. [26] Numerous models exist for the acquired resistance to araC including decreased expression or activity of dCK and overexpression of cytidine deaminase (CD), the enzyme responsible for catabolism of araC.

Gemcitabine. Gemcitabine (difluorodeoxycytidine, dFdC) is unique among deoxynucleoside analogs. Unlike cladribine, fludarabine, and cytarabine, which have only shown activity in leukemias, gemcitabine is a potent cytotoxic agent with a broad spectrum of activity in pancreatic, bladder, lung, ovarian and breast cancer. [7] It is thought that gemciatabine's improved activity in solid tumors over other nucleoside analogs is due to a unique method of chain termination as well as a greater degree of self-potentiation. As with the other nucleoside analogs, gemcitabine uses the nucleoside salvage pathway for activation and cytotoxicity. Gemcitabine is transported by nucleoside transporters and phosphorylated to dFdCTP by deoxycytidine kinase, UMP-CMP kinase, and nucleoside diphosphate kinase (NDPK).

Unlike other nucleoside analogs, the active metabolite of gemcitabine, dFdCTP, is the penultimate triphosphate incorporated into elongating DNA followed by the addition of a natural nucleoside triphosphate. This additional endogenous nucleoside masks the misincorporated dFdCTP from polymerase ε, making dFdCTP excision and DNA repair highly unlikely. [27] DNA polymerases are unable to proceed leading to chain termination and apoptosis. This process has been termed "masked DNA chain termination." In addition to this unique DNA incorporation, dFdCDP, inhibits RR which in turn inhibits the formation of natural nucleoside pools. [28] Low dCTP levels favor the incorporation of dFdCTP into elongating DNA. dCTP inhibits dCK activity, therefore lower dCTP levels increases the production of gemcitabine monophosphate. Further self-potentiation is exhibited through direct inhibition of CD thereby reducing the elimination of the parent compound. [29] All of these factors act in concert along with

masked chain termination to help explain the superior activity of gemcitabine in solid tumors.

1.3 Mechanism of Resistance to Deoxynucleoside Antimetabolites

As deoxynucleoside analogs act via the nucleoside salvage pathway, alterations in expression level or activity of any of the transporters and enzymes along this pathway could theoretically contribute to drug resistance. However, the proteins most commonly implicated in nucleoside analog resistance are deoxycytidine kinase, nucleoside deaminases, ribonucleotide reductase, and the nucleoside transporters.

Deoxycytidine Kinase. Deoxycytidine kinase (dCK) is a homodimer consisting of two identical 30.5 kDa subunits responsible for the initial phosphorylation of cladribine, fludarabine, cytarabine, and gemcitabine. [30-32] The enzyme is constitutively expressed however dCK activity is increased in cells entering S-phase. [33, 34] This is presumably for the phosphorylation of salvaged nucleosides for use in DNA synthesis. *In vitro* models have shown that reduced expression or activity of dCK is correlated with resistance to araC and gemcitabine. [35-37] Additional *in vitro* studies have shown an underlying reduction of dCK in lymphoid cell lines cross-resistant to cladribine, fludarabine, cytarabine, and gemcitabine. [38, 39] Finally, Hapke *et al.* determined that there was a linear relationship between dCK activity and cell sensitivity to cladribine, fludarabine, and cytarabine. [40] These findings were later validated in a xenograph model using gemcitabine as a treatment. Blackstock *et al.* found that increased dCK expression led to increased accumulation of the active metabolite dFdCTP and improved tumor response of human colon carcinoma xenographs implanted in nude

mice. [41] Clinical studies relating dCK expression and activity, however, are somewhat controversial. Numerous studies have shown that decreased expression or activity of dCK was related to resistance to fludarabine, cladribine, cytarabine, and gemcitabine. [42-46] However, several authors have found no relationship between dCK and clinical outcome. [47-50]

Cytidine Deaminase and Cytoplasmic 5'-Nucleotidase. Cytidine deaminase (CD) and cytoplasmic 5'-nucleotidase (5'NT) oppose the production of the active metabolites of nucleoside analogs. The physiologic role of cytidine deaminase is the deamination of cytidine to uridine and deoxycytidine to deoxyuridine. However, cytidine deaminase also transforms araC and gemcitabine to their inactive metabolites. Several studies have shown an in vitro relationship between increased CD and resistance to gemcitabine and araC. [51-55] However, cytidine deaminase's role *in vivo* remains unclear. Although some investigators have found a correlation between CD and resistance to araC [56, 57], other investigators have found no relationship. [48, 50] The enzyme responsible for the deamination of adenosine and deoxyadenosine, adenosine deaminase, is not a factor in cladribine or fludarabine resistance. Both cladribine and fludarabine are resistant to deamination by adenosine deaminase.

Cytoplasmic 5'-nucleotidase catalyzes the reverse reaction of deoxycytidine kinase, excising the monophosphate group from nucleoside and nucleoside analog monophosphates. Again, although the enzyme can be shown to influence resistance of nucleoside analogs *in vitro*, the *in vivo* relevance has yet to be elucidated. [45, 58, 59] This is most likely due to the complex interplay between the five cytosolic 5'-nucleotidases. [60, 61] As further work progresses to distinguish the individual

contribution of each of these enzymes, the actual influence of 5'NT to nucleoside analog resistance will become more clear.

Ribonucleotide Reductase. Ribonucleotide reductase (RR) consists of a large (M1) and a small (M2) subunit, both of which are required for the reduction of nucleoside diphosphates to deoxynucleoside diphosphates. Although the M1 subunit is present throughout the cell cycle, the M2 subunit is specific to the S-phase of proliferating cells. As such, M2 levels modulate the activity of RR. [62] Of the deoxynucleoside antimetabolites, cladribine, fludarabine, and gemcitabine inhibit RR. However, the major mechanism of action for cladribine and fludarabine is in quiescent cells. As such, the inhibition of RR may not play as vital a role in these cytotoxic agents as it does for gemcitabine, as is evident from small increases in RR in fludarabine and 2CdA resistant cell lines. [38] In contrast, RR has been shown to be a strong determinant of gemcitabine sensitivity in tumor cell lines. [63, 64] In addition, Duxbury *et al.* recently demonstrated that reduced RR expression correlated with sensitivity to gemcitabine in an orthotopic xenograph model of pancreatic cancer. [65]

Nucleoside Transporters. The nucleoside transporters (NTs) are the obligate first step in the activation pathway of nucleoside analogs. [66] There are two families of nucleoside transporters; the equilibrative (ENTs) and concentrative (CNTs) transporters. ENTs transport via facilitated diffusion, moving nucleosides into or out of the cell based solely on the substrate concentration gradient. CNTs, on the other hand, actively transport nucleosides and nucleoside analogs into cells by coupling transport to the inwardly directed sodium gradient. Five of the seven cloned and characterized nucleoside transporters are known to contribute to the transport and activation of

nucleoside analogs. ENT1, ENT2, CNT1, CNT2, and CNT3 transport clinically relevant nucleoside analogs at pharmacologic concentrations. However these transporters show different tissue localization and specificity for therapeutic nucleoside analogs (Table 1.1 and Table 1.2). Therefore, the individual contribution of each transporter will depend on the drug used and the tissue type of target cells.

Four equilibrative nucleoside transporters have been cloned and characterized, however ENT3 and ENT4 are not involved in the cellular uptake of nucleosides. ENT3 is not expressed at the plasma membrane and ENT4 (also know as the Plasma Membrane Monoamine Transporter, PMAT) transports organic cations but not nucleosides or nucleoside analogs. [67, 68] ENT1 and ENT2 are broadly selective transporters interacting with both purine and pyrimidine nucleosides. ENT1 and ENT2 differ primarily in their substrate affinities and in their sensitivity to inhibitors, primarily NBMPR. While ENT2 is inhibited by μM concentrations of NBMPR, ENT1 is sensitive to nM concentrations. [69-71] Both ENT1 and ENT2 are ubiquitously expressed although ENT2 expression is concentrated in skeletal muscle (Table 1.1) [72]

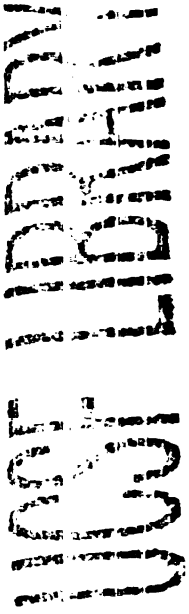
Unlike ENTs, the three cloned and characterized CNTs are substrate selective and differentially expressed. In epithelia, CNT1 is localized to the apical membrane and works in concert with equilibrative nucleoside transporters, predominately localized to the basolateral membranes of these tissues, to mediate transepithelial nucleoside flux. [73, 74] CNT1 transports naturally occurring pyrimidine nucleosides as well as the pyrimidine nucleoside analogs cytarabine and gemcitabine. [75-79] Although CNT1 appears to be expressed primarily in the liver, kidney and small intestine, it is likely that it is expressed in epithelial cells of many tissues. [72] Northern blot analysis

Table 1.1 *Tissue expression of nucleoside transporters relevant to nucleoside analog cytotoxicity*		
Transporter	**Gene Name**	**Tissue Expression**
ENT1	SLC29A1	Ubiquitously expressed. Highly expressed in erythrocytes and leukocytes. [70, 72, 80]
ENT2	SLC29A2	Ubiquitously expressed. Highly expressed in skeletal muscle. Expressed in leukocytes. [69, 72, 80]
CNT1	SLC28A1	Primarily expressed in liver, kidney and small intestine. [72, 79]
CNT2	SLC28A2	Expressed in kidney, liver, heart, brain, placenta, pancreas, skeletal muscle, colon, rectum, duodenum, jejunum, and ileum. Expressed in some leukocytes. [72, 80, 81]
CNT3	SLC28A3	Primarily expressed in pancreas, trachea, bone marrow, and mammary gland. Expressed in some leukocytes. [72, 80, 82]

Table 1.2 *Transport of endogenous nucleosides and nucleoside analogs*[1]				
	CNT1	CNT2	CNT3	
Uridine	45 ± 8 * [79] 22 ± 3 * [83]	~ 40 * [84] 80 ± 10 * [81]	1.7 ± 0.3 ⊗ [85] 1.1 ± 0.2 ‡ [86]	K_m
				K_i
				IC_{50}
2' deoxy-uridine	23 ± 4 * [83]			K_m
		~ 39 ‡ [87]		K_i
			inhibition (100 μM) ‡ [86]	IC_{50}
2' 3' dideoxy-uridine	slight transport (1 mM)* [83]			K_m
				K_i
	slight inhibition (1 mM)* [88]	slight inhibition (1 mM)* [88]	slight inhibition (2 mM) ‡ [86]	IC_{50}
5' fluoro-uridine	18 ± 3* [83]			K_m
		~ 34 ‡ [87]		K_i
			inhibition (100 μM) ‡ [86]	IC_{50}
5'fluoro-2' deoxy-uridine	15 ± 2 * [83]	no transport (500 μM) * [89]		K_m
		~ 82 ‡ [87]		K_i
		76 ± 23 ‡ [90]	inhibition (100 μM) ‡ [86]	IC_{50}
Cytidine	34 ± 7 * [77]	slight transport (500 μM) * [89]	2.2 ± 0.7 ⊗ [85] 3.4 ± 1.1 ‡ [86]	K_m
	~ 6 ‡ [91]			K_i
				IC_{50}
2' deoxy-cytidine				K_m
	~ 10 ‡ [91]			K_i
				IC_{50}
2' 3' dideoxy-cytidine				K_m
	~ 10,000 ‡ [91]			K_i
			slight inhibition (2 mM) ‡ [86]	IC_{50}
Cytarabine (araC)	no transport (1 mM) * [83]			K_m
				K_i
	no inhibition (1 mM)* [88]		slight inhibition (2 mM) ‡ [86]	IC_{50}

	CNT1	CNT2	CNT3	
Gemcitabine (dFdC)	17 ± 2 * [77] ~ 24 * [78]	no transport (10 μM) * [78]		K_m
				K_i
	13.8 ± 0.6 ‡ [92]	no inhibition (1 mM) ‡ [90]		IC_{50}
Thymidine	transport (10 μM) * [92]	slight transport (125 μM) * [89]	3.7 ± 1.2 ‡ [86]	K_m
		> 500 ‡ [87]		K_i
				IC_{50}
Adenosine	slight transport (1 mM)* [83]	~ 8 * [84]	3.6 ± 1.3 ⊗ [85] 4.6 ± 1.3 ‡ [86]	K_m
	50 ± 11 * [79]	6 ± 1* [81]	68.7 ± 11* [93]	K_i
	inhibition (1 mM) * [88]			IC_{50}
2' deoxy-adenosine	no transport (1 mM) * [83]	slight transport (500 μM) * [89]		K_m
	46 ± 14 * [79]	30 ± 7 * [81]		K_i
				IC_{50}
Cladribine (2CdA)	slight transport (1 mM)* [83]	slight transport (500 μM) * [89]		K_m
			274 ± 65* [93] ~ 20 ⊗ [94]	K_i
	inhibition (1 mM)* [88]	371 ± 209 ‡ [95]		IC_{50}
Fludarabine				K_m
			1066 ± 283* [93] ~ 350 ⊗ [94]	K_i
	slight inhibition (1 mM)* [88]	slight inhibition (1 mM)* [88]		IC_{50}
Guanosine	no transport (1 mM) * [83]	transport (1 μM) * [96]	5.1 ± 2.2 ‡ [86]	K_m
				K_i
				IC_{50}

	ENT1	ENT2	
Uridine	43 ± 10 ⊗ [97] 37 ± 7 ⊗ [98] 44 ± 3 ⊗ [99]	184 ± 22 ⊗ [98] 195 ± 14 ⊗ [99] 320 ± 70 ‡ [100]	K_m
			K_i
	20 ± 10 ⊗ [101] 43 ± 28 ⊗ [102]	210 ± 90 ⊗ [101]	IC_{50}
2' deoxy-uridine	171 ± 19 ⊗ [98]	155 ± 11 ⊗ [98]	K_m
			K_i
	170 ± 40 ⊗ [101]	310 ± 20 ⊗ [101]	IC_{50}
2' 3' dideoxy-uridine			K_m
			K_i
	no inhibition (2 mM) * [103]		IC_{50}
5' fluoro-uridine			K_m
			K_i
	50 ± 10 ⊗ [101]	220 ± 30 ⊗ [101]	IC_{50}
5' fluoro-2' deoxy-uridine			K_m
			K_i
	170 ± 70 ⊗ [101]	340 ± 30 ⊗ [101]	IC_{50}
Cytidine	207 ± 47 ⊗ [97] 234 ± 47 ⊗ [99] 580 ± 110 ‡ [104]	4500 ± 700 ‡ [100] 5600 ± 420 ‡ [104]	K_m
		1380 ± 170 ⊗ [99]	K_i
	170 ±140 ⊗ [101]	1980 ± 730 ⊗ [101]	IC_{50}
2' deoxy-cytidine			K_m
			K_i
	2000 ± 400 ⊗ [101]	3060 ± 2200 ⊗ [101]	IC_{50}
2' 3' dideoxy-cytidine			K_m
			K_i
	2510 ± 600 ⊗ [101]	1100 ± 460 ⊗ [101]	IC_{50}
Cytarabine (araC)			K_m
			K_i
	1430 ± 260 ⊗ [102] 1500 ± 510 ⊗ [101]	1200 ± 260 ⊗ [101]	IC_{50}



	ENT1	ENT2	
Gemcitabine (dFdC)	~ 160 * [78]	~ 740 * [78]	K_m
			K_i
	475 ± 138 ⊗ [102]		IC_{50}
Thymidine	300 ± 30 ‡ [104]	710 ± 50 ‡ [104]	K_m
	82 ± 3 ⊗ [99]	129 ± 9 ⊗ [99]	K_i
			IC_{50}
Adenosine	21 ± 4 ⊗ [105] 62 ± 18 * [74] 11 ± 1.2 ⊗ [97] 18 ± 0.8 ⊗ [99] 40 ± 4 ‡ [104]	76 ± 22 * [74] 106 ± 6 ⊗ [99] 140 ± 20 ‡ [104]	K_m
	~ 60 [106]		K_i
			IC_{50}
2' deoxy-adenosine			K_m
	~71 [106]		K_i
	inhibition (2 mM) * [103]		IC_{50}
Cladribine (2CdA)	23 ± 6 ‡ [94]		K_m
	~ 36 ⊗ [94]	~ 50 ⊗ [94]	K_i
	inhibition (2 mM) * [103]		IC_{50}
Fludarabine	~ 110 ‡ [94]		K_m
	~ 60 ⊗ [94]	~ 170 ⊗ [94]	K_i
			IC_{50}
Guanosine	48 ± 5 ⊗ [99] 140 ± 10 ‡ [104]	2700 ± 1600 ‡ [104]	K_m
	178 ± 80 ⊗ [105]	394 ± 70 ⊗ [99]	K_i
			IC_{50}

[1] Symbols and notation:

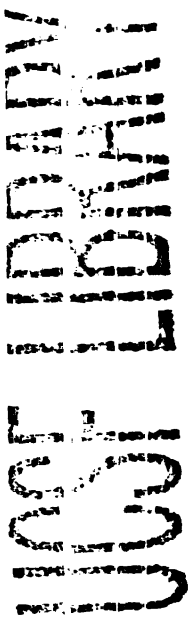
- Cell systems used are abbreviated as follows: ‡ – mammalian cells, * – oocyte, ⊗ – yeast.
- References are presented in brackets following cell system notation.
- All inhibition data was determined by inhibiting a model substrate of the transporter.
- In the absence of kinetic values the known transport or inhibition data is reported followed by the concentration tested (in parentheses).
- Cells left blank have not yet been determined.

demonstrated that CNT2 is present in the kidney, liver, heart, brain, placenta, pancreas, skeletal muscle, colon, rectum, duodenum, jejunum, and ileum. [70, 81] CNT2 transports purine nucleosides and uridine, however, of the nucleoside analogs currently used in chemotherapy, none appear to be translocated by human CNT2. [81, 89, 107] CNT3 is broadly selective and transports both purine and pyrimidine nucleosides into cells in a sodium-dependent manner. CNT3 transports a number of anticancer nucleoside analogs including cladribine, fludarabine, and gemcitabine. [82] Interestingly, the Na^+: nucleoside coupling ratio of CNT3 differs from CNT1 and CNT2. Both CNT1 and CNT2 display a 1:1 coupling ratio, however CNT3 transports nucleosides at a ratio of 2-Na^+: 1-nucleoside. This stoichiometric difference allows CNT3 to concentrate more substrate into cells against its concentration gradient. High levels of human CNT3 mRNA transcripts are found in the pancreas, trachea, bone marrow, and mammary gland; lower levels are found in the intestine, lung, placenta, prostrate, testis, and liver. [82]

Nucleoside analogs are relatively hydrophilic molecules and, at therapeutic dosages, generally will not enter cells via simple diffusion but require active transport to enter target cells. Numerous studies have found that NT deficient cells are resistant to nucleoside analogs or exhibit cross-resistance to a spectrum of nucleoside analogs due to reduced transport. [66, 108-110] Although nucleoside transporters have been implicated in nucleoside analog resistance *in vitro*, the *in vivo* data remains inconclusive. [80, 111-113] Galmarin *et al.* and Stam *et al.* found that ENT1 expression was associated with sensitivity to cytarabine clinically. [48, 50] However, in fludarabine treated CLL patients, Molina-Arcas *et al.* found no relationship between nucleoside transporter

expression and sensitivity [80], and Mackey *et al.* found that CNT3 expression was actually associated with increased resistance to fludarabine [114].

One reason for these discrepancies is the variability in tissue expression and substrate preferences of these transporters. Since fludarabine, cladribine, and cytarabine are predominantly used in these leukemic and lymphomic malignancies, much of the work regarding the relationship between nucleoside transporters and nucleoside analogs has been performed in these cells. Recent work has shown that the equilibrative transporters are predominantly expressed in these cells types. Although ENT1 and ENT2 were highly expressed in all normal leukocytes and leukemic cell lines, CNT2 and CNT3 displayed extremely variable expression. Importantly, CNT1 was not expressed in any of the normal leukocytes and leukemic cell lines tested. [80] Therefore, experiments in these cell lines may not be directly applicable to tissues that express different nucleoside transporter profiles.



Recent studies have begun to investigate the relative roles of individual nucleoside transporter homologs in antimetabolite chemotherapy. Fludarabine sensitivity in CLL cells from patients correlates to fludarabine influx but not to NT mRNA levels. [80] Further observations revealed that protein expression of ENT1 did not correlate with either fludarabine uptake or with *ex vivo* cytotoxicity. However, ENT2 protein expression correlated with both fludarabine uptake and sensitivity to fludarabine. [115] This result is unexpected since substrates typically have a greater affinity for ENT1 than ENT2 (see Table 1.2). It is possible that protein expression does not reflect function since ENT1 activity can be post-transcriptionally modified by protein kinase C. [116] In

addition, total ENT1 protein may not reflect ENT1 expressed at the membrane since ENT1 has also been shown to be expressed intracellularly in mitochondria. [117]

The determination of functional nucleoside transporter protein expression in tumor samples has previously been hampered by the lack of appropriate antibodies. Recently, Farre *et al.* compared the protein expression of ENT1, ENT2, and CNT1 in gynecologic cancers relative to normal tissue samples. [118] They found that while ENT1 and ENT2 were retained in most ovarian and uterine tumors, the loss of CNT1 occurred in 33-39% of the samples. In addition, they found that CNT1 loss was correlated with histotypes with poor prognosis. This study confirms the earlier work of Mackey *et al.*, which found that the ENT1 protein was rarely lost in breast cancer tumors [119], and Pennycooke *et al.*, who found that CNT1 and CNT2 mRNA were frequently missing in tumors form various tissue types. [72]

Clearly, nucleoside analog cytotoxicity is a complex mechanism, depending on the expression and affinity of numerous transporters and enzymes. Most studies investigating this pathway have been performed in leukocytes or leukemic cells and may not be applicable to other tissue types. As such, antimetabolites such as gemcitabine, which is active in neoplasms from a variety of tissue type, need to be studied in the appropriate cell system. Although some components of the nucleoside analog activation pathway, such as ribonucleotide reductase, are ubiquitously expressed, nucleoside transporters have specific tissue expression. Ideally, the constellation of nucleoside transporters, taken together, should be tested in a relevant cell tissue type to determine the association to a particular nucleoside analog.

Summary of Chapters

The goal of my dissertation research was to understand the role that variation in gene sequence and expression level of nucleoside transporters play in determining gemcitabine sensitivity in pancreatic cancer. Most studies investigating the role of nucleoside transporters in antimetabolite nucleoside analogs have used leukemic/lymphomic cells. The combined contribution of nucleoside transporters to gemcitabine sensitivity in pancreatic tumors has not yet been elucidated.

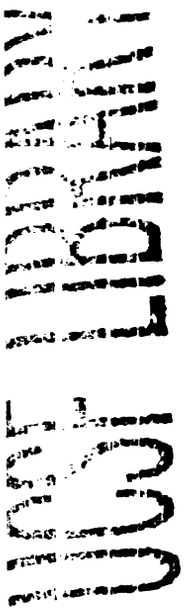
In **Chapter 2** of this dissertation, genetic variants of the concentrative nucleoside transporter CNT1 are determined in an ethnically diverse population and functionally characterized. We found two non-functional CNT1 variants occurring at low frequencies in the population. In addition, we found one common variant, CNT1-Val189Ile (allele frequency of 26%), which had a reduced affinity for gemcitabine compared to the reference CNT1.

Chapter 3 describes, in detail, our characterization of a panel of 16 pancreatic cancer cell lines and emphasizes methodologies. Using this panel, we determined the relative expression of ENT1, CNT1, and CNT3, the three nucleoside transporters responsible for gemcitabine cytotoxicity in pancreatic adenocarcinomas. We also tested this panel for gemcitabine cytotoxicity with and without the ENT1 inhibitor NBMPR.

Chapter 4 examines the complex interplay between concentrative and equilibrative nucleoside transporters that our studies revealed. In striking contrast to previous studies, we observed that ENT1 expression level was associated with reduced gemcitabine sensitivity. ENT1 inhibitors effected a range of gemcitabine responses, from enhanced resistance to enhanced sensitivity, depending on the relative expression level of

concentrative and equilibrative transporters. The observation that ENT1 can mediate both resistance and sensitivity to gemcitabine was confirmed in stably transfected cells. Our results suggest a model in which sensitivity to gemcitabine is dependent on the relative expression of equilibrative and concentrative nucleoside transporters. When expressed in the absence of concentrative nucleoside transporters, ENT1 acts as a mediator of sensitivity, however, when expressed concomitantly with CNT1 or CNT3, ENT1 acts predominantly as an efflux transporter, mediating resistance to gemcitabine.

Finally, in **Chapter 5**, we use the results of Affymetrix array data along with our gemcitabine sensitivity results to hypothesize further genes that may be involved in resistance or sensitivity to gemcitabine. As gemcitabine relies on DNA machinery and genes in the apoptotic pathway for cytotoxicity, perturbances in these pathways could also affect cytotoxicity.



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CHAPTER 2

FUNCTIONAL AND GENETIC DIVERSITY IN THE CONCENTRATIVE NUCLEOSIDE TRANSPORTER, SLC28A1, IN HUMAN POPULATIONS[1]

2.1 Introduction

Nucleoside analogs such as cladribine, fludarabine, cytarabine and gemcitabine are clinically used as anticancer drugs. These synthetic analogs are modified derivatives of naturally occurring nucleosides and exert their effects by entering nucleoside salvage pathways. Once in salvage pathways, the phosphorylated analogs substitute for naturally occurring nucleotides and inhibit a variety of chemical reactions required for nucleic acid replication.

Interindividual differences in response to anticancer nucleoside analogs represent a major obstacle in drug therapy. Such differences have been associated with variation in systemic and intracellular levels of phosphorylated nucleotide analogs. [1] For example, recent studies have shown that variation in intracellular levels of phosphorylated gemcitabine nucleotides is dependent on the rate of delivery of gemcitabine to cells. [2] An optimal rate of entry is critical in maximizing the cytotoxic response to gemcitabine.

To enter cells, naturally occurring nucleosides as well as many synthetic nucleoside analogs require plasma membrane nucleoside transporters. The concentrative

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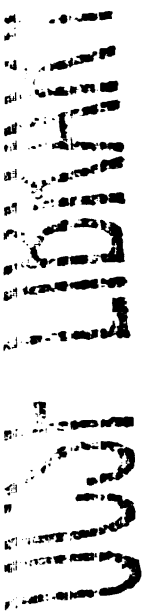
[1] This data has been published previously: Gray, J. H., *et.al.* "Functional and Genetic Diversity in the Concentrative Nucleoside Transporter, SLC28A1, In Human Populations." *Mol Pharmacol* 65:512–519, 2004. Reprinted with permission of the American Society for Pharmacology and Experimental Therapeutics.

nucleoside transporter, CNT1 (SLC28A1), is one of three members of the SLC28 family of sodium-dependent nucleoside transporters. [3] In contrast to CNT2, which is purine nucleoside preferring, and CNT3, which does not discriminate between purine and pyrimidine nucleosides, CNT1 prefers pyrimidine nucleosides and nucleoside analogs such as zidovudine (AZT), lamivudine (3TC), zalcitabine (ddC), cytarabine (AraC), and gemcitabine (dFdC). [4] CNT1 is found primarily on the apical membrane of epithelial tissues including small intestine, kidney, and liver and thus plays a role in setting systemic as well as intracellular levels of its substrates. [5]

As part of a large project investigating the natural variation in membrane transporters, variants in the coding region and flanking intronic region of CNT1 were identified in 247 DNA samples from an ethnically diverse population. [6] Of the 58 variable sites identified in this sample set, 15 were protein altering. That is, 13 resulted in amino acid substitutions, one 3-base pair insertion led to the insertion of a valine residue, and one base pair deletion led to the truncation of the protein. Four of the protein altering variants of CNT1 were found at allele frequencies greater than or equal to 20%, and four others were found at allele frequencies greater than 1%.



Due to the large number of high frequency variants of CNT1, and the critical role that the transporter plays in cellular entry of naturally occurring nucleosides and synthetic nucleoside analogs, we proposed to study the function of these variants. The aims of the present study were two-fold: to analyze the haplotype structure of CNT1, particularly with respect to protein altering variants, and to functionally characterize the protein altering variants of CNT1. Kinetic studies examining gemcitabine interaction with the most common protein altering variants of CNT1 were carried out as a first step in

determining whether functional differences in CNT1 variants may ultimately contribute to variation in systemic and intracellular levels of anticancer nucleoside analogs.

2.2 Materials and Methods

Genetic Analysis of CNT1. CNT1 variants were identified in the study of Leabman *et al.* by direct sequencing of genomic DNA from an ethnically diverse population of 247 unrelated individuals, consisting of: 100 African Americans, 100 European Americans, 30 Asian Americans, 10 Mexican Americans, and 7 Pacific Islanders. Primer sequences used for the amplification of exons and flanking intronic regions can be found at www.pharmgkb.org and www.pharmacogenetics.ucsf.edu. The neutral parameter (θ), nucleotide diversity (π), and Tajima's D statistic were calculated as described by Tajima. [7] These parameters were calculated for synonymous and non-synonymous sites for the entire population and for each racial and ethnic group. Synonymous and non-synonymous sites were defined as described by Hartl and Clark. [8] Haplotypes were reconstructed from variant sites using the Bayesian statistical method of PHASE. [9] Prior to PHASE analysis, all singleton samples were removed. Only haplotypes that were found in at least 7 out of 10 PHASE runs were reported. Results for the population parameters, variant sites, and PHASE analysis utilized version 23 of the CNT1 data.

Construction of CNT1-reference and CNT1-variant Plasmids. Human CNT1 cDNA (GenBank accession no. U62968) was subcloned into the amphibian high-expression vector, pOX. [10] The Stratagene QuikChange site-directed mutagenesis kit was then used to construct CNT1-reference, which is defined as the common allele at

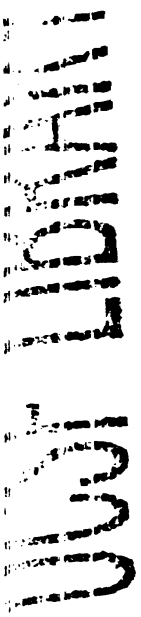
every coding region variable site. CNT1-reference corresponds to the most common haplotype in the African American population, *CNT1**1. The following changes were made to CNT1 to construct CNT1-reference: 565A>G and 1561A>G (nucleotide numbering begins at the translation start site of CNT1 cDNA). Each amino acid variant was then constructed using CNT1-reference in pOX as the template with the exception of CNT1-Ser546Pro-reversal, which used CNT1-Ser546Pro as the template. Reference and variant sequences were confirmed by sequencing the entire cDNA insert.

Functional Screening of Variants in Oocytes. Healthy stage V and VI *Xenopus laevis* oocytes were injected with 30 ng of capped cRNA transcribed *in vitro* with T3 RNA polymerase (mCAP RNA Capping kit, Stratagene) from *NotI*-linearized pOX plasmids containing either reference or variant CNT1. RNA concentrations were determined by spectrophotometry. An aliquot of cRNA was run on an agarose gel prior to injection to confirm that it was not degraded. Injected oocytes were maintained in modified Barth's solution (88 mM NaCl, 1 mM KCl, 0.82 mM $MgSO_4$, 0.41 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, 2.4 mM $NaHCO_3$, 10 mM HEPES/Tris, pH 7.4; supplemented with 20 mg/L gentamicin and 50 mg/L tetracycline) at 18 °C for 2-4 days of expression before uptake studies were performed. Groups of seven to nine oocytes were incubated in sodium buffer containing 0.1 μM ^{3}H-thymidine (61 Ci/mmol; Moravek) and 10 μM unlabeled thymidine at room temperature for 1 hour. Uptake was terminated by the rapid removal of radiolabeled buffer and the oocytes were washed five times with ice-cold choline buffer. Oocytes were then lysed individually with 100 μL of 10% SDS, and the radioactivity associated with each oocyte was determined. Uptake of thymidine in oocytes expressing each variant was determined in 7 to 10 oocytes from a single frog.

Functional studies were repeated using different batches of oocytes from at least two frogs. Variant data were normalized to the uptake of CNT1-reference for each experiment. Data are presented as mean of normalized value ± standard error. Uninjected oocytes and oocytes incubated with sodium-free buffer containing ^{3}H-thymidine serve as controls within each batch of oocytes.

Gemcitabine Interaction with Common CNT1 Variants. Four CNT1 variable sites were found at an allele frequency of ≥ 20% in the total population: CNT1+140Val, CNT1-Val189Ile, CNT1-Gln237Lys, and CNT1-Asp521Asn. CNT1 reference and these common CNT1 variants were subcloned into the pGEMT expression vector and heterologously expressed in HeLa cells using the vaccinia virus-T7 polymerase system. [11, 12] The vaccinia virus and HeLa cells were generous gifts from RH Edwards (UCSF, San Francisco, CA). Vaccinia virus stocks were prepared as described by Povlock and Amara. HeLa cells were cultured using H-21 DMEM and cell culture supplies purchased from the Cell Culture Facility (UCSF, San Francisco, CA). HeLa cells were plated on 24-well plates at a density of 80,000 cells per well and grown for 24 hours. Cells were then infected with vaccinia virus (1 x 10^{6} pfu per well) and incubated at 37°C. After thirty minutes, cells were transfected with 1 μg reference or variant CNT1 cDNA (or empty vector pGEMT as control) and 3 μg Lipofectin (Invitrogen, Carlsbad, CA). Cells were incubated 16-20 hours and analyzed for heterologous expression by ^{3}H-thymidine uptake. To do this, cells were washed one time in Na^{+} buffer (128 mM NaCl, 4.73 mM KCl, 1.25 mM, $CaCl_2$, 1.25 mM $MgSO_4$, 5 mM HEPES-Tris, pH 7.4) and exposed for four minutes to 0.1 μM ^{3}H-thymidine (60.1 Ci/mmol, Morevak) in the presence of 20 μM nitrobenzylthioinosine (NBMPR), an equilibrative nucleoside

transporter (ENT) inhibitor, and varying concentrations of gemcitabine (0-400 μM) in Na^+ buffer at 37°C. Cells were then rinsed three times in ice-cold Na^+-free buffer (128 mM Choline Chloride, 4.73 mM KCl, 1.25 mM, $CaCl_2$, 1.25 mM $MgSO_4$, 5 mM HEPES-Tris, pH 7.4). Cells were lysed in 500 μl of 1% SDS, and radioactivity was measured using a Beckman scintillation counter. All measurements were made in triplicate and were normalized to protein content as measured using the method of Lowry. Data are reported as mean ± SE. All experiments were repeated three to eight times. Data were fit to the equation $V = V_o - [V_\Delta \times I/(IC_{50}+I)]$, where V is the uptake of ^{3}H-thymidine in the presence of gemcitabine, V_o is ^{3}H-thymidine uptake in the absence of gemcitabine, V_Δ is the maximal change in ^{3}H-thymidine uptake, I is the gemcitabine concentration and IC_{50} is the concentration of gemcitabine that inhibits ^{3}H-thymidine uptake by 50%.



2.3 Results

Genetic Variation in SLC28A1. Variants of SLC28A1 were identified as part of a large-scale project whose goal was to determine natural variation in membrane transporter genes and can be found at www.pharmgkb.org. [6] Fifty-six single nucleotide polymorphisms (SNPs), one insertion mutation, and one deletion mutation were identified in the 3846 bp of exons and flanking introns in SLC28A1 resulting in an overall frequency of 1.5 variant per 100 bp. This was the highest frequency of variant/100 bp of any of the 24 membrane transporters analyzed by Leabman et al. Thirty SNPs were found in the non-coding intronic region. Of the remaining 28 coding region variants (Table 2.1), 13 were non-synonymous. Nineteen of these 28 variants were not previously reported in dbSNP. The position of each coding region

Table 2.1. *Coding region variants of SLC28A1 identified in ethnically diverse populations*								
Exon	Nucleotide Position‡	Nucleotide	Amino Acid Position	Amino Acid	Frequency (%)			
					Total	AA	EA	AS
1	9	C -> T	3	syn	4.2	10.5	0	0
1	38	C -> G	13	Ser -> Cys	0.4	1.1	0	0
2	124	T -> C	42	syn	0.8	0.5	1.5	0
2	128	G -> A	43	Ser -> Asn	1	2.5	0	0
2	*132*	*C -> G*	*44*	*syn*	*0.2*	*0.5*	*0*	*0*
4	295	C -> T	99	syn	0.4	1	0	0
4	**419**	**T -> +TTG**	**140**	**Leu -> INS**	**29.1**	**31.5**	**32**	**18.3**
5	**565**	**G -> A**	**189**	**Val -> Ile**	**26.1**	**18.9**	**28.5**	**35**
5	568	G -> T	190	Ala -> Ser	1.8	2.6	2	0
6	630	A -> T	210	syn	4	9.5	0	0
6	689	C -> T	230	Ala -> Val	0.4	0	0	3.3
6	*705*	*C -> T*	*235*	*syn*	*0.2*	*0.5*	*0*	*0*
6	**709**	**C -> A**	**237**	**Gln -> Lys**	**19.6**	**14.5**	**19.5**	**28.3**
8	*835*	*G -> A*	*279*	*Val -> Ile*	*0.2*	*0.5*	*0*	*0*
10	*969*	*A -> G*	*323*	*syn*	*0.2*	*0*	*0*	*1.7*
10	1017	C -> T	339	syn	11.8	3.5	7	53.4
11	*1098*	*G -> A*	*366*	*syn*	*0.2*	*0.5*	*0*	*0*
11	1149	G -> A	383	syn	42.9	32	34.8	93.3
11	1153	G -> -	385	Val -> DEL	1.2	3	0	0
12	*1234*	*G -> C*	*412*	*Glu -> Gln*	*0.2*	*0.5*	*0*	*0*
12	1368	G -> A	456	syn	9.2	6.5	14.3	3.3
13	1405	C -> A	469	syn	0.8	2	0	0
13	1528	C -> T	510	Arg -> Cys	7.3	4.5	0.5	43.3
13	**1561**	**G -> A**	**521**	**Asp -> Asn**	**26.2**	**10**	**51.1**	**3.4**
14	*1636*	*T -> C*	*546*	*Ser -> Pro*	*0.2*	*0.5*	*0*	*0*
16	*1821*	*C -> T*	*607*	*syn*	*0.2*	*0.5*	*0*	*0*
17	*1880*	*A -> G*	*627*	*Asn -> Ser*	*0.2*	*0.5*	*0*	*0*
17	*1903*	*C -> G*	*635*	*Leu -> Val*	*0.2*	*0*	*0*	*1.7*

Data are available in Leabman *et al.* and at www.pharmgkb.org. Frequencies of each variable site in each ethnic group are shown. Some samples contained amplicons that could not be sequenced. Therefore, allele frequencies are based on non-missing data. Abbreviations are as follows: Total = entire sample, AA = African American, EA = European American, AS = Asian American. Common variants (>20%) are shown in boldface. Singletons are shown in italics.

‡cDNA numbers are relative to the ATG start site and based on the cDNA sequence from GenBank accession number U62968.

variant in the proposed secondary structure of CNT1 is shown in Figure 2.1. [13] Both synonymous and non-synonymous variants were found in the cytosolic and extracellular loops as well as in the transmembrane domains. In addition, two insertion and deletion (indel) mutations were identified in SLC28A1. One of the two was a 3-bp insertion resulting in a valine insertion, CNT1+140Val. This variant was present at high frequencies in all populations (Table 2.1). The other indel was a guanosine bp deletion which resulted in a frameshift. This frameshift allele had a frequency of 3% in the African American population.

In addition to having a large number of variable sites, *SLC28A1* also has a number of common variants (frequency > 20%, Table 2.1). These two factors result in high values of the neutral parameter (θ) and the nucleotide diversity (π) of *SLC28A1*. Values of θ and π are given as mean $\times 10^{-4} \pm$ standard deviation. The estimate of θ over the total population was 22.5 ± 5.12 and π was 11.8 ± 6.44 for *SLC28A1*. The π for non-synonymous sites (π_{NS}) and synonymous sites (π_S) were as follows: $\pi_{NS} = 8.59 \pm 5.99$ and $\pi_S = 22.55 \pm 16.51$.

Haplotype Structure of CNT1. Haplotypes were reconstructed from population variable sites using the Bayesian statistical method of PHASE. [9] A total of 153 haplotypes were reported for CNT1, 82 of which were found on two or more chromosomes. These 153 haplotypes represent a total of 58 coding region haplotypes, that is, haplotypes with distinct coding region sequences (Fig. 2.2). Of the 58 coding region haplotypes, there were 14 with only synonymous changes, 18 with a single amino acid change, and 15 with two amino acid changes. Eleven of the 58 coding region

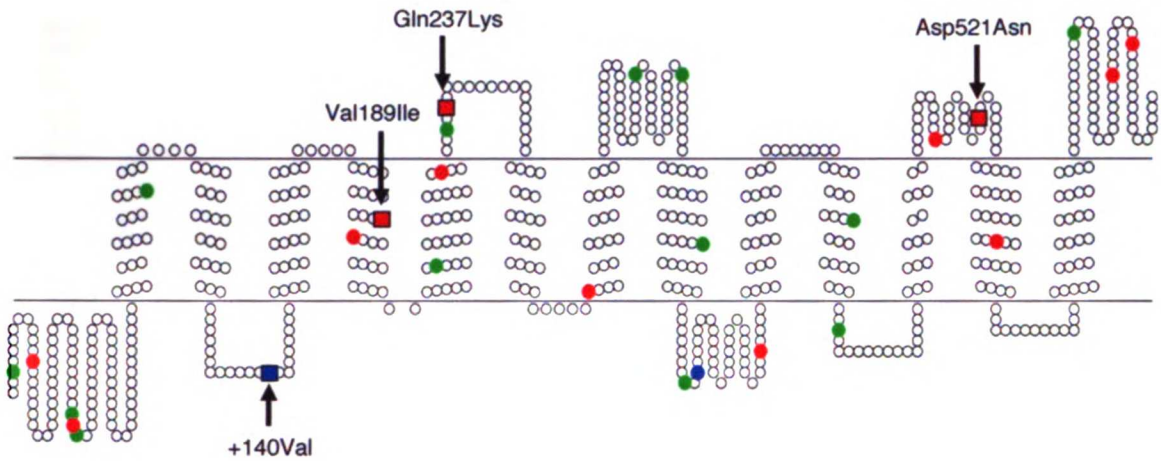
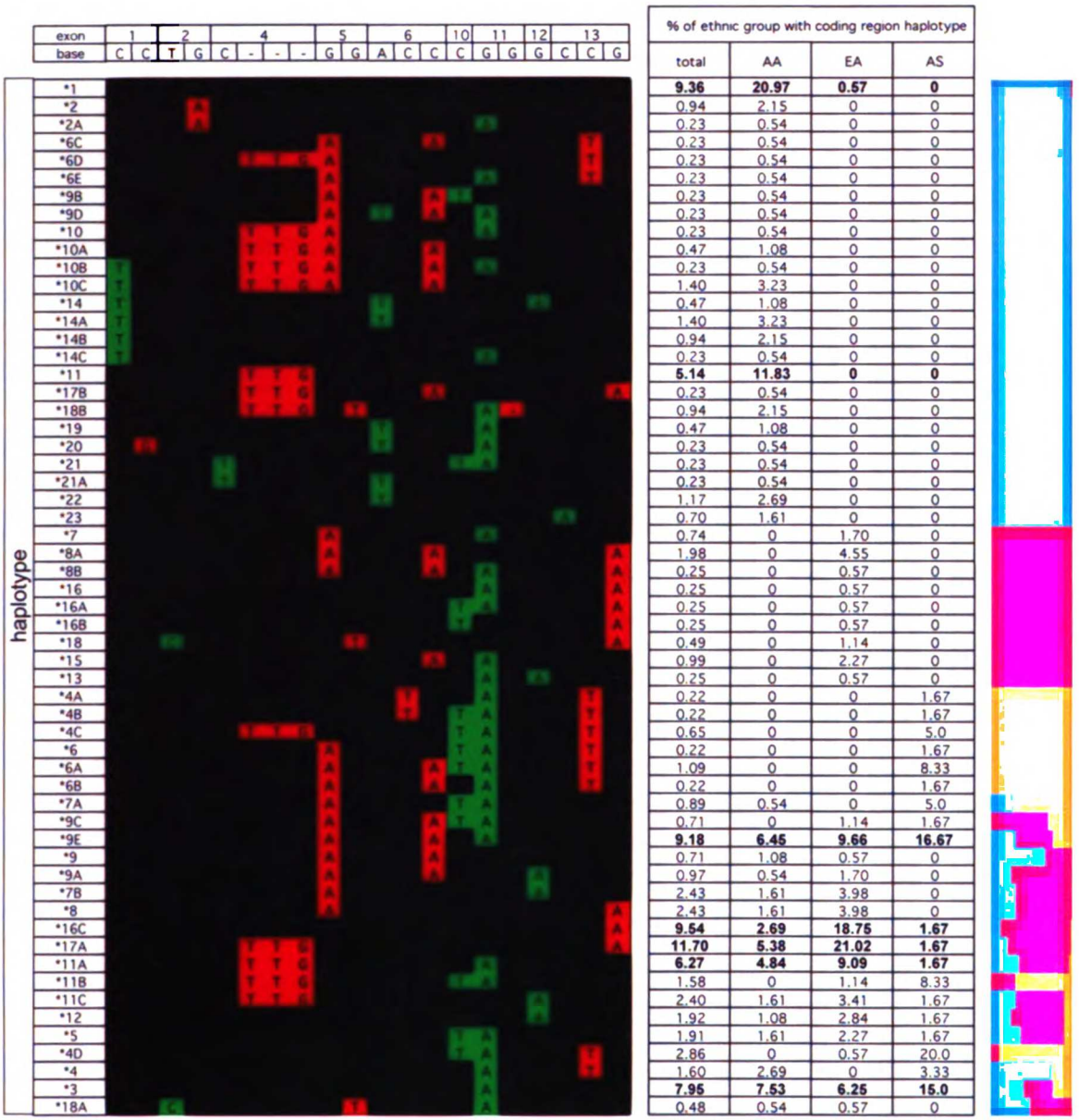


Figure 2.1 Secondary structure of CNT1 with coding-region SNPs. The transmembrane topology schematic was rendered using TOPO (S.J. Johns [UCSF, San Francisco] and R.C. Speth [Washington State University, Pullman], transmembrane protein display software, available at the UCSF Sequence Analysis Consulting Group website, www.sacs.ucsf.edu/TOPO/topo.html. Non-synonymous amino acid changes are shown in red; synonymous changes in green, insertions and deletions in blue. Arrows indicate the four most common non-synonymous variants that were kinetically characterized.



haplotype	total	AA	EA	AS
*1	**9.36**	**20.97**	**0.57**	**0**
*2	0.94	2.15	0	0
*2A	0.23	0.54	0	0
*6C	0.23	0.54	0	0
*6D	0.23	0.54	0	0
*6E	0.23	0.54	0	0
*9B	0.23	0.54	0	0
*9D	0.23	0.54	0	0
*10	0.23	0.54	0	0
*10A	0.47	1.08	0	0
*10B	0.23	0.54	0	0
*10C	1.40	3.23	0	0
*14	0.47	1.08	0	0
*14A	1.40	3.23	0	0
*14B	0.94	2.15	0	0
*14C	0.23	0.54	0	0
*11	**5.14**	**11.83**	**0**	**0**
*17B	0.23	0.54	0	0
*18B	0.94	2.15	0	0
*19	0.47	1.08	0	0
*20	0.23	0.54	0	0
*21	0.23	0.54	0	0
*21A	0.23	0.54	0	0
*22	1.17	2.69	0	0
*23	0.70	1.61	0	0
*7	0.74	0	1.70	0
*8A	1.98	0	4.55	0
*8B	0.25	0	0.57	0
*16	0.25	0	0.57	0
*16A	0.25	0	0.57	0
*16B	0.25	0	0.57	0
*18	0.49	0	1.14	0
*15	0.99	0	2.27	0
*13	0.25	0	0.57	0
*4A	0.22	0	0	1.67
*4B	0.22	0	0	1.67
*4C	0.65	0	0	5.0
*6	0.22	0	0	1.67
*6A	1.09	0	0	8.33
*6B	0.22	0	0	1.67
*7A	0.89	0.54	0	5.0
*9C	0.71	0	1.14	1.67
*9E	**9.18**	**6.45**	**9.66**	**16.67**
*9	0.71	1.08	0.57	0
*9A	0.97	0.54	1.70	0
*7B	2.43	1.61	3.98	0
*8	2.43	1.61	3.98	0
*16C	**9.54**	**2.69**	**18.75**	**1.67**
*17A	**11.70**	**5.38**	**21.02**	**1.67**
*11A	**6.27**	**4.84**	**9.09**	**1.67**
*11B	1.58	0	1.14	8.33
*11C	2.40	1.61	3.41	1.67
*12	1.92	1.08	2.84	1.67
*5	1.91	1.61	2.27	1.67
*4D	2.86	0	0.57	20.0
*4	1.60	2.69	0	3.33
*3	**7.95**	**7.53**	**6.25**	**15.0**
*18A	0.48	0.54	0.57	0

Figure 2.2 Structure and population frequency of 58 coding region haplotypes of SLC28A1.

Legend on following page.

Figure 2.2 Structure and population frequency of 58 coding region haplotypes of SLC28A1. Left panel: Sequences of the haplotypes at each of the 20 variable coding region sites. The first row denotes exons of CNT1. The second row denotes the sequence of the common (reference) allele at each site. A (-) signifies no insertion. Subsequent rows denote each of the 58 coding region haplotypes. A black filled block indicates that there is no change from the reference base pair. A red filled block indicates that there is a change in a base pair leading to a protein altering change (non-synonymous, insertion or frameshift). A green filled block indicates a change in a base pair that does not lead to an amino acid change (synonymous). Haplotype names were assigned based on evolutionary divergence and frequency in the sample. Middle Panel: This panel shows the frequency of each coding region haplotype within an ethnic population and within the total population. Common coding region haplotypes (>5% in the total population) are boldfaced. Right Panel: This panel shows the ethnicity specificity (as a proportion) of each coding region haplotype. Haplotypes in African Americans only are indicated by blue; haplotypes in European Americans are pink; and haplotypes in Asians are orange. Haplotypes that are shared among ethnic groups are indicated by multiple colors approximating the fraction of the haplotype that is possessed by a particular ethnic group. It should be noted that 200 chromosomes were analyzed for African American and European American samples, whereas 60 were analyzed for the Asian sample set. The TTG insertion in exon 4 leads to the valine insertion; the G to A change in exon 5 leads to Val189Ile; the C to A change in exon 6 leads to Gln237Lys; and the G to A change in exon 13 leads to Asp521Asn.

haplotypes had three amino acid changes. Many of the common non-synonymous variants, such as CNT1+140Val, CNT1-Val189Ile, and CNT1-Gln237Lys were found together in the context of coding region haplotypes. Of the 13 coding region haplotypes containing the 140Val insertion, ten also contained another amino acid change. Of the 22 coding region haplotypes containing Val189Ile, 19 contained at least one other amino acid change and nine had two additional amino acid changes. Finally, 15 of the 16 coding region haplotypes with Gln237Lys also contained another amino acid change.

The population specificity of coding haplotypes is shown in Figure 2.2. Most of the common coding region haplotypes (>5% frequency) were found in more than one ethnic group, with the exception of *CNT1**11 which was found only in the African American population. The African American population contained the greatest number of coding region haplotypes and a total of 24 out of 58 haplotypes were specific to this ethnic group. The most common coding region haplotype in the European American population, *CNT1**17A, had both the 140Val insertion and Asp521Asn. The second most common haplotype in this ethnic group contained the Asp521Asn variant alone. All of the Asian specific haplotypes contained two or more amino acid changes.

Functional Analysis of Variants of CNT1. Each of the single amino acid CNT1 variants were constructed by site-directed mutagenesis on a CNT1-reference template. The uptake of thymidine in oocytes expressing each of the 15 protein altering variants of CNT1 is shown in Figure 2.3. All but two of the 15 variants were able to transport thymidine. Of the two non-functional variants, one is a frameshift mutation, CNT1-1153del, that results in the truncation of the protein after transmembrane domain 8 (Fig. 2.4). This frameshift deletion occurs exclusively in the African American population at

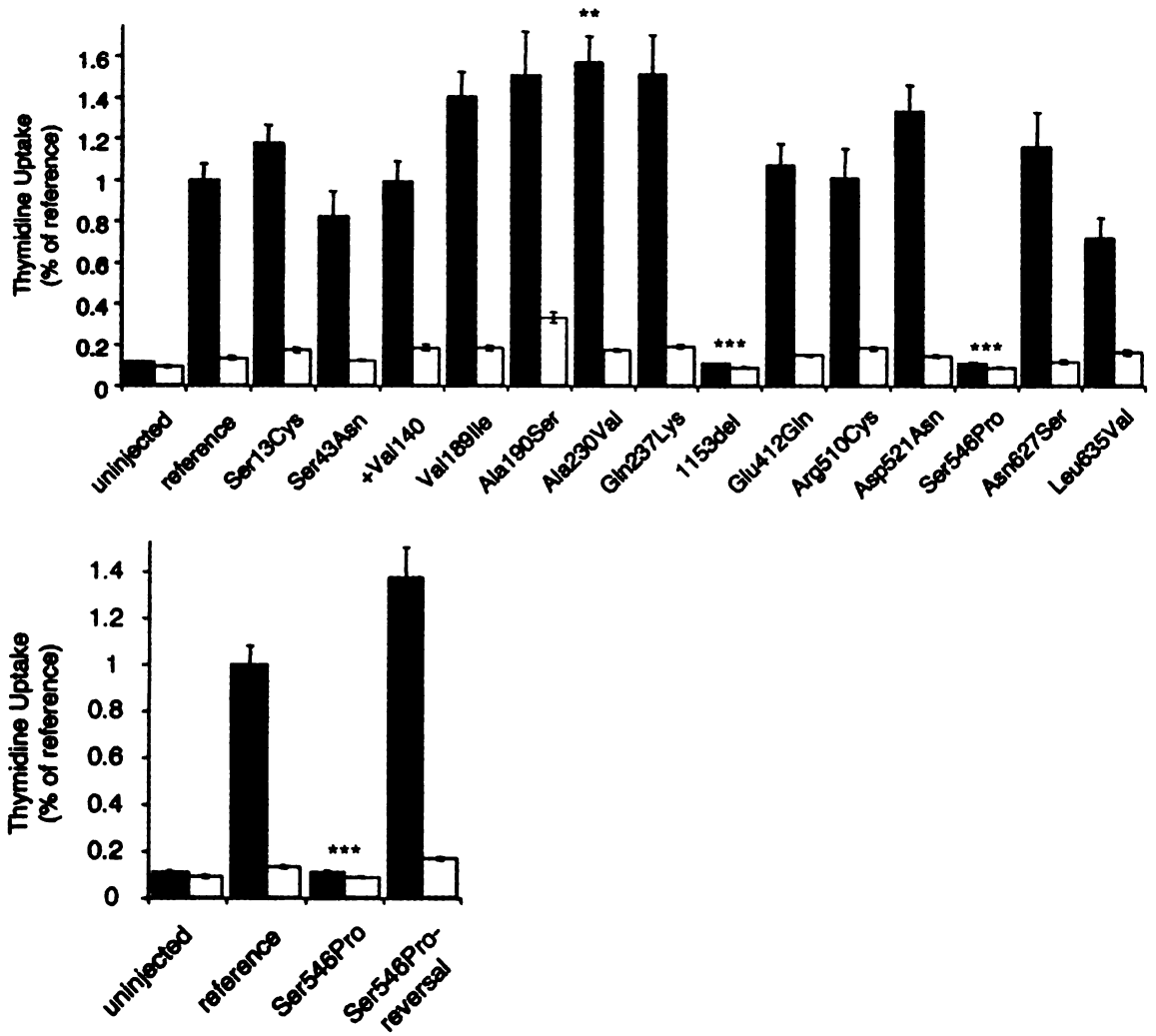


Figure 2.3 ^{3}H-thymidine uptake in *Xenopus laevis* oocytes expressing CNT1 and its protein altering variants. (top) ^{3}H-thymidine uptake in oocytes expressing the reference CNT1 (reference) and each of the 15 protein altering CNT1 variants. Closed bars represent data in the presence of Na^{+}; open bars represent data in the absence of Na^{+}. (bottom) ^{3}H-thymidine uptake in oocytes expressing the reference CNT1 and CNT1-Ser546Pro and its reversal (CNT1-Ser546Pro-reversal) in the presence (closed bars) and absence (open bars) of Na^{+}. *Xenopus laevis* oocytes were injected with approximately 30 ng of RNA. Uptake of ^{3}H-thymidine (0.1 μM ^{3}H- thymidine plus 10 μM unlabeled thymidine) was measured at room temperature after a 1 hour incubation. Data are representative of experiments carried out with two to three different batches of oocytes, normalized within each experiment. Each value represents mean ± S.E. from 7-9 oocytes. **, $P < 0.001$; ***, $P < 0.0001$ (significantly different from CNT1-reference). Using the Bonferroni correction for multiple comparisions, $P < 0.003$ is needed for an overall significance of 0.05.

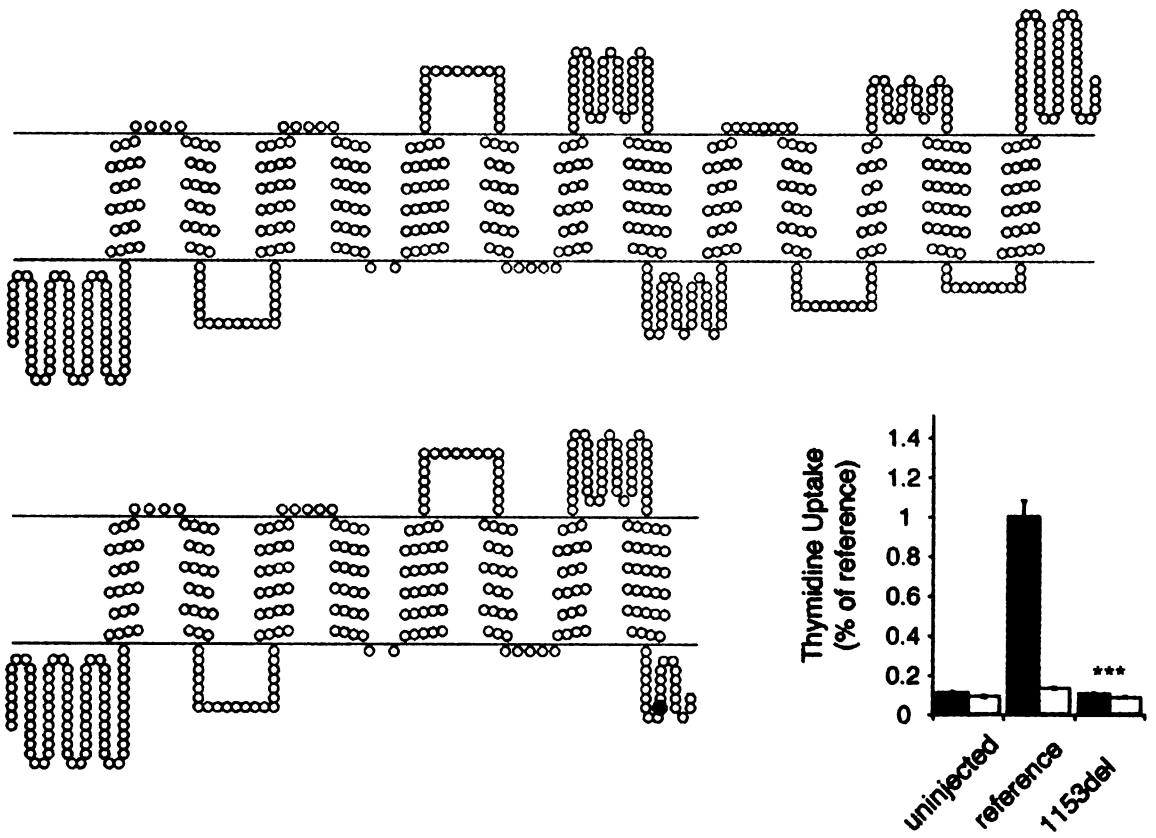


Figure 2.4 Secondary structure and function of CNT1 and CNT1-1153del. Comparison of the transmembrane topology schematic for CNT1 (top) and CNT1-1153del (bottom left) indicates that CNT1-1153del has been severely truncated. (bottom right) ^{3}H-thymidine uptake was determined in oocytes expressing the reference CNT1 and CNT1-1153del in the presence (closed bars) and absence (open bars) of Na^{+}. Each value represents mean ± S.E. from 7-9 oocytes. **, $P < 0.001$; ***, $P < 0.0001$ (significantly different from CNT1-reference). Using the Bonferroni correction for multiple comparisons, $P < 0.003$ is needed for an overall significance of 0.05. Details of the experiments are described in the Methods and in the legend of Figure 2.3.

an allele frequency of 3.0% (Table 2.1). The other non-functional CNT1 variant, Ser546Pro, was a singleton which changed an evolutionarily conserved serine to a proline. To confirm that this mutation altered function, the mutation was reversed and the function of CNT1 was recovered (Fig. 2.3). CNT1 also appears to have a statistically significant hyper-functional mutation (defined as > 150% of reference uptake), CNT1-Ala230Val (^{3}H- thymidine uptake = 157% of reference; $p<0.001$). This mutation is rare, present only in the Asian population (Table 2.1), and occurs at an evolutionarily conserved residue.

In addition to the functional screening study with thymidine, we carried out detailed kinetic studies of common CNT1 variants using the anticancer nucleoside analog, gemcitabine. In oocytes, we showed that radiolabeled gemcitabine was taken up by the reference CNT1 (data not shown); however, because of batch to batch variation in oocytes, we decided to carry out inhibition studies with gemcitabine in a mammalian heterologous expression system. Therefore, vaccinia infected HeLa cells were used for these studies and inhibition data were highly reproducible within and between experiments allowing us to identify small differences in kinetic parameters. The potency of gemcitabine in inhibiting ^{3}H-thymidine uptake in cells expressing CNT1+140Val, CNT1-Gln237Lys, and CNT1-Asp521Asn was not different from that of reference CNT1 (Fig. 2.5, Table 2). In contrast, gemcitabine interacted less potently with CNT1-Val189Ile (IC_{50} = 23.3 ± 1.55 μM for CNT1-Val189Ile versus 13.8 ± 0.60 μM for CNT1-reference). Similar trends were obtained in oocytes; that is, gemcitabine was a less potent inhibitor of thymidine uptake in oocytes expressing CNT1-Val189Ile in comparison to

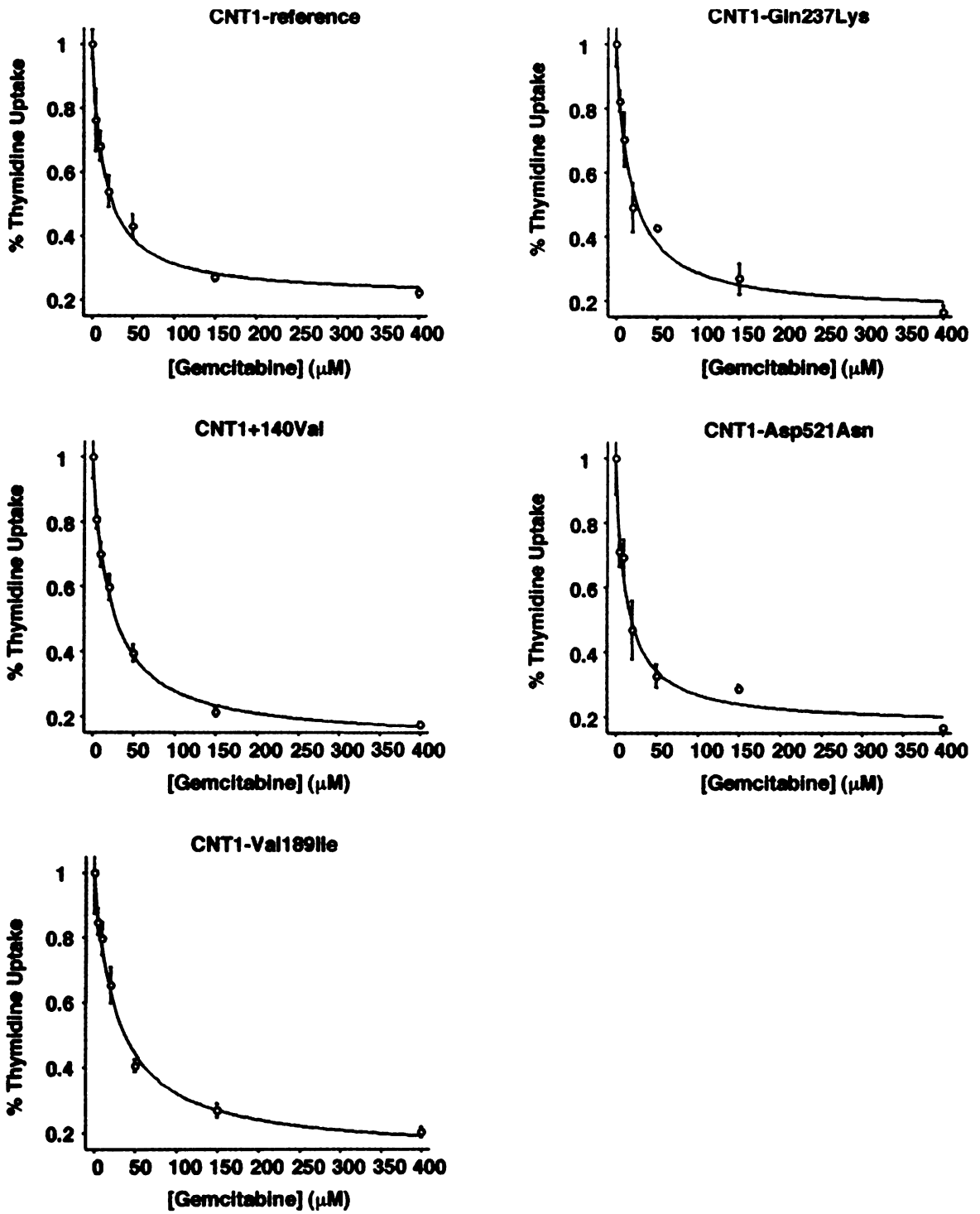


Figure 2.5 Interaction kinetics of gemcitabine with CNT1-reference and its four common protein altering variants. Legend on following page.

Figure 2.5 Interaction kinetics of gemcitabine with CNT1-reference and its four common protein altering variants. Kinetics of ^{3}H-thymidine inhibition by gemcitabine in HeLa cells expressing CNT1-reference and four common amino acid variants of CNT1, CNT1+140Val, CNT1-Gln237Lys, CNT1-Val189Ile, CNT1-Asp521Asn. Vaccinia infected HeLa cells were transfected with CNT1 and its variants as described in the Methods section. The uptake of ^{3}H-thymidine in the presence of increasing concentrations of gemcitabine was measured after a 4 minute incubation. Data are representative of experiments carried out in at least three different passages of HeLa cells. Each point represents mean ± standard error. The solid line represents the curve fit of the IC_{50}.

Table 2.2 *Kinetics of interaction of gemcitabine with CNT1-reference and its four common protein altering variants*	
variant	Gemcitabine IC_{50} (μM)
CNT1-reference	13.8 ± 0.60
CNT1+140Val	16.9 ± 1.21
CNT1-Val189Ile	23.3 ± 1.55*
CNT1-Gln237Lys	14.4 ± 0.43
CNT1-Asp521Asn	10.8 ± 1.08

Vaccinia infected HeLa cells were transfected with CNT1 and its variants as described in the Methods section. The uptake of ^{3}H-thymidine in the presence of increasing concentrations of gemcitabine was measured after a 4 minute incubation. Data represents mean ± standard error from at least 3 different experiments.

* Significantly different from value obtained for CNT1-reference, $p < 0.05$.

reference CNT1 (IC_{50} = 62.5 ± 13.8 μM for CNT1-Val189Ile versus 19.2 ± 1.83 μM for CNT1-reference).

2.4 Discussion

Recent studies have sought to elucidate the underlying diversity in the human genome. These studies have sequenced genes that are associated with a particular disease state, e.g. cardiovascular disease or blood-pressure homeostasis, or have sequenced more broadly across the genome. [14-17] Recently, Leabman *et al.* examined the diversity in a particular class of genes, membrane bound transporters. [6] From these studies, it is apparent that nucleotide diversity varies greatly among genes, with some genes having considerably more variation than others.

We observed that *SLC28A1* is a highly variable gene. That is, the average nucleotide diversity (π_T) of *SLC28A1* over the total population (11.8×10^{-4}) is higher than average values reported in the studies of Cargill *et al.* (5.05×10^{-4}) and Sachidanandam *et al.* (7.51×10^{-4}). Moreover, *SLC28A1* has more nucleotide variation than average for the 24 membrane transporter genes reported by Leabman *et al.* (5.09×10^{-4}). In addition to having high frequency variants, which is reflected in its high π_T, *SLC28A1* also has a large number of variable sites leading to a high neutral parameter (θ). The neutral parameter for *SLC28A1* (22.5×10^{-4}) was the highest of those reported by Leabman *et al.* for 24 membrane transporter genes.

For *SLC28A1*, nucleotide diversity at non-synonymous sites (8.59×10^{-4}) is lower than nucleotide diversity at synonymous sites (22.55×10^{-4}), consistent with evolutionary constraints on amino acid changes. The ratio of π_{NS}/π_S is used to assess the degree of selective pressure on a gene, where values approaching 0 indicate the greatest level of

selective pressure and values approaching 1 are consistent with no selective pressure. [18] The ratio of π_{NS}/π_S of 0.381 for *SLC28A1* was higher than the median ratio found for other membrane transporter genes (0.182) suggesting that *SLC28A1* is under less selective pressure than many other membrane transporter genes. The presence of a high frequency single bp deletion in the coding region leading to a non-functional frameshift mutation (3% in African Americans) is consistent with reduced selective pressure on CNT1. CNT1-1153del was the highest frequency frameshift mutation found in either Leabman *et al.* or in an additional 16 membrane transporter genes that have since been examined (unpublished observations, data at www.pharmacogenetics.ucsf.edu). All other frameshift mutations were either singletons, found only on one chromosome, or doubletons, found on two chromosomes in the sample population.

Consistent with its high nucleotide diversity, *SLC28A1* also has a high degree of haplotype diversity. With a total of 153 haplotypes, *SLC28A1* has the greatest number of haplotypes of the 24 genes examined by Leabman *et al.* (unpublished observations). A recent study by Stephens *et al.* analyzed both the SNP diversity and haplotype structure of the coding and flanking intronic regions of 313 genes in 82 unrelated individuals. [17] These 313 genes contained from 2-53 haplotypes and, on average, had 14 haplotypes/gene. In contrast, *SLC28A1* had a total of 153 haplotypes, 82 of which were found on two or more chromosomes. Of the 153 haplotypes, 116 were found in only one ethnic group. As expected, the African American sample had the highest number of ethnic specific haplotypes; 65 of the 116 ethnic specific haplotypes were found only in African Americans. Stephens *et al.* also found that the highest number of population specific haplotypes were in the African American sample (1335 of 2782 population-

specific haplotypes). [17] Interestingly, in *SLC28A1*, the European American and Asian sample sets also contained a large number of ethnic specific haplotypes; 38 of the 116 population specific haplotypes were found only in the European American samples and 13 of the 116 population specific haplotypes were unique to the Asian sample. The high frequency of population specific haplotypes was due to a high number of singleton haplotypes which by definition are specific to a population. In fact, approximately half of the population-specific haplotypes were singleton haplotypes.

Due to the large number of haplotypes, we focused on coding region haplotypes (Fig. 2.2). Even then there were a large number of haplotypes. Forty-four of the 58 coding region haplotypes contained one or more amino acid changes. Although most of the common coding region haplotypes were found in more than one ethnic group, there were a number of coding region haplotypes that were ethnic specific. Twenty-four of 58 coding region haplotypes were African American specific whereas 9 and 6, were specific to the European American and Asian population samples, respectively. It is interesting to note that all of the coding region haplotypes specific to the Asian population contained at least two amino acid changes.

Of the 15 protein altering variants found in CNT1, two variants, CNT1-Ser546Pro and CNT1-1153del were non-functional and one variant, CNT1-Ala230Val, was hyper-functional (Fig. 2.3). Both CNT1-Ser546Pro and CNT1-Ala230Val were rare, ethnic specific variants. CNT1-Ser546Pro was a singleton found only in the African American population and CNT1-Ala230Val was a doubleton found only in the Asian population. The non-functional variant, CNT1-1153del, was found at a frequency of 3% in the African American population. Since rat CNT1 had previously been found to function

without the first three N-terminal transmembrane domains, it was important to evaluate the function of CNT1-1153del. [13] This truncated protein does not function; however, further studies are needed to determine the molecular mechanisms responsible for its lack of function.

Both the non-functional CNT1 variant, Ser546Pro, as well as the hyper-functional variant, Ala230Val, were rare and changed evolutionarily conserved residues. It was surprising that Ala230Val was hyper-functional as changes in evolutionarily conserved amino acids are likely to cause deleterious changes in protein function. [19] For example, in previous studies of the membrane transporters OCT1, OAT1, and OAT3 all non-function variants changed an evolutionarily conserved amino acid. [20-22] Of the 155 amino acid variants uncovered in Leabman *et al.*, the only other hyper-functional variant that has thus far been characterized is OCT1-Ser14Phe. Although human OCT1 contains a serine residue at codon 14, mouse, rat, and rabbit OCT1 has a phenylalanine residue in this position. OCT1-Ser14Phe returns the human amino acid to the phenylalanine residue highly conserved among other mammalian orthologs. It has been speculated that amino acid mutations that restore evolutionary conservation are more likely to cause increased transport function, although CNT1-Ala230Val does not support this speculation.

CNT1-Ser546Pro introduces a proline into a transmembrane domain which helps explain the non-functional nature of this variant. Proline residues are generally tolerated only at the ends or in the center of α-helices due to the physical constraint introduced by the proline side chain. The presence of a proline in the center of a transmembrane domain usually indicates a bend in the α-helix. [23] Therefore, this Ser546Pro mutation

most likely results in a conformational change which renders the protein non-functional. However, the molecular mechanisms responsible for this loss of function need to be explored. It is important to note that this residue is not in regions previously identified as substrate recognition domains. [24-26]

Our data demonstrating a high degree of genetic and functional diversity in CNT1 are in contrast to data obtained by Osato *et al.* and Owen *et al.* for the equilibrative nucleoside transporters, ENT1 and ENT2. [27, 28] It appears that there is a continuum of diversity among nucleoside transporters with ENT1 at one end of the spectrum and CNT1 at the other. The equilibrative transporters, ENT1 and ENT2, display very little genetic and functional diversity. A total of seven protein altering variants were found in ENT1 and ENT2 however only one was found at an allele frequency greater than 1%, ENT1-Ile216Thr (1.2% allele frequency). [27, 28] Additionally, the only non-functional equilibrative nucleoside transporter variant was the rare frame-shift mutation ENT2-Δ845-846. [28] In contrast, of the 31 protein altering variants found in the concentrative nucleoside transporters, 13 have an allele frequency greater than 1% and six variants display altered function (Table 2.3). [29-31]

The greater diversity of the concentrative nucleoside transporters extends to haplotype diversity as well. In contrast with the 153 haplotypes found in CNT1, ENT1 and ENT2 have ten and five haplotypes respectively. CNT2 and CNT3 reside between the two extremes with 17 and 52 total haplotypes. When focusing on coding region haplotypes this disparity remains. ENT1 has only four coding region haplotypes while ENT2 has three. CNT2 and CNT3 have nine and thirteen amino acid haplotypes,

Table 2.3 *Protein altering variation of equilibrative and concentrative nucleoside transporters*					
	Protein altering variants	Protein altering variants, ≥ 1% allele frequency	Functionally different variant	Functional difference	Ref
ENT1	2	1	-	-	[27]
ENT2	5	0	ENT2-Δ845-846	non-functional	[28]
CNT1	15	8	CNT1-Val189Ile	reduced affinity for gemcitabine	[30]
			CNT1-Ala230Val	hyper-functional	
			CNT1-Δ1153	non-functional	
			CNT1-Ser546Pro	non-functional	
CNT2	6	2	CNT2-Phe355Ser	substrate specificity change	[31]
CNT3	10	3	CNT3-Gly367Arg	reduced function	[29]

Variants were identified by direct sequencing of genomic DNA from an ethnically diverse population. Primer sequences used for the amplification of exons and flanking intronic regions can be found at www.pharmgkb.org and www.pharmacogenetics.ucsf.edu.

The population sequenced for ENT1, ENT2, CNT1, and CNT2 consisted of 247 unrelated individuals and contained: 100 African Americans, 100 European Americans, 30 Asian Americans, 10 Mexican Americans, and 7 Pacific Islanders, as described in Leabman *et al.* [6]

The population sequenced for CNT3 consisted of 276 unrelated individuals and contained: 80 African Americans, 80 European Americans, 60 Asian Americans, 50 Mexican Americans, and 6 Pacific Islanders.

respectively, however, CNT1 has a total of 58 coding region haplotypes. Although 26 of the 58 CNT1 coding region haplotypes contain two or more amino acid changes, the only other nucleoside transporter to have more than one amino acid change in a single coding region haplotype was CNT2. This CNT2 double amino acid mutation (containing Pro22Leu and Ser75Arg) occurred as often as the reference coding region haplotype which had no mutations (each at a frequency of approximately 36%). Although speculative, it is possible that CNT1 and, to a lesser extent, CNT2 are able to tolerate greater functional diversity due to the presence ENT1, ENT2, and CNT3 which are tightly constrained transporters with redundant function.

Genetic variants of CNT1 have less functional diversity than those of the xenobiotic transporter, OCT1 (SLC22A1). [22] Five of the 15 naturally occurring protein-altering variants of OCT1 found in the study of Leabman *et al.* exhibited significant or complete loss of function and four of these were found at allele frequencies greater than 1% in either the African American or European American sample. [22] It is interesting to speculate why OCT1 has more deleterious alleles in comparison to CNT1. Both are epithelial transporters in the SLC superfamily and both have functionally redundant paralogs. However, OCT1, found primarily in the liver, functions in concert with drug metabolizing enzymes as part of the body's defense against xenotoxins. [32, 33] In contrast, CNT1, found on the apical membranes of epithelial tissues including the liver, serves in maintaining total body homeostasis by salvaging naturally occurring nucleosides. [34] It is possible that transporters that serve primarily in detoxification are functionally less constrained than transporters, such as CNT1, which function primarily in handling endogenous compounds.

In addition to its role in the salvage of naturally occurring nucleosides, CNT1 is also involved in the disposition and targeting of synthetic nucleoside analogs. [35] In particular, the transporter interacts with and translocates structural analogs of pyrimidine nucleosides such as the anticancer drug, gemcitabine. Gemcitabine is the first-line drug in the treatment of pancreatic cancer. [36] Studies have shown that nucleoside transporters are essential for the cytotoxicity of gemcitabine. [37] In this study, we evaluated the potency of interaction of gemcitabine with CNT1 and four of its most common protein altering variants (Fig. 2.5). Our data demonstrated that gemcitabine exhibited about half of the potency in interacting with CNT1-Val189Ile in comparison to the reference CNT1. Clinical studies have suggested that the delivery rate of gemcitabine to tumor cells is important for therapeutic outcome and that the optimal plasma concentration of gemcitabine in cancer chemotherapy is approximately 20 μM. [38] Thus, the differences in interaction potencies of gemcitabine with CNT1 (13.8 μM) and CNT1-Val189Ile (23 μM) may be clinically important.

In summary, we have determined that CNT1 has a high degree of genetic and functional variation. The large number of common haplotypes, particularly in the coding region, suggest that this gene mutates frequently and that many mutations including amino acid mutations are tolerated. Although speculative, it is possible that the presence of functionally redundant nucleoside transporters and other proteins in nucleoside salvage pathways allow even deleterious mutations in CNT1 (e.g., CNT1-1153del) to achieve a certain degree of fixation in the population. The finding that a common variant of CNT1, CNT1-Val189Ile exhibits a kinetic difference in its interaction potency with the anticancer drug, gemcitabine, may be important for clinical drug therapy. In particular,

this variant is found at high allele frequencies in multiple populations and, therefore, individuals who are homozygous for CNT1-Val189Ile (approximately 8 to 12%) will be found in patient populations. Our data suggest that future studies should be designed to assess the role of genetic variation in CNT1 in clinical response to gemcitabine.

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CHAPTER 3

CHARACTERIZATION OF A PANEL OF PANCREATIC CANCER CELL LINES

3.1 Introduction

The pancreas is a complex, multi-functional organ located near the beginning of the small intestine. It consists of an exocrine system, which produces enzymes used in digestion, and an endocrine system, responsible for insulin and glucagon production and secretion. Of the three main cell types found in the pancreas, acinar cells make up the bulk of the tissue (Fig. 3.1). [1] Acinar cells produce digestive enzymes and account for approximately 80% of pancreatic cells. Embedded within the acinar tissue are the Islets of Langerhans that produce hormones, primarily insulin and glucagon, which are secreted into the bloodstream (Fig. 3.1). Islet cells comprise roughly 2 % of the total pancreas. The remainder of the pancreatic tissue forms a ductal system, a network of blood vessels, lymphatics, nerves, and support stroma, that runs throughout the pancreas and empties into the duodenum. Lining the ducts are epithelial cells which add mucous and bicarbonate to the digestive juices. Although ductal epithelial cells comprise only 5% of the pancreas, approximately 90% of pancreatic cancers arise from these cells. [2, 3]

Pancreatic cancer has been well characterized at the molecular and genetic level, however it remains remarkably difficult to treat clinically. This is probably because symptoms do not usually present until the disease has metastasized and the pancreatic tumors are no longer resectable. Patients with resectable nonmetastatic disease have a long-term survival rate of ~20% post resection. [1] However, in most cases there are already micrometastases circulating in the bloodstream at the time of surgery.

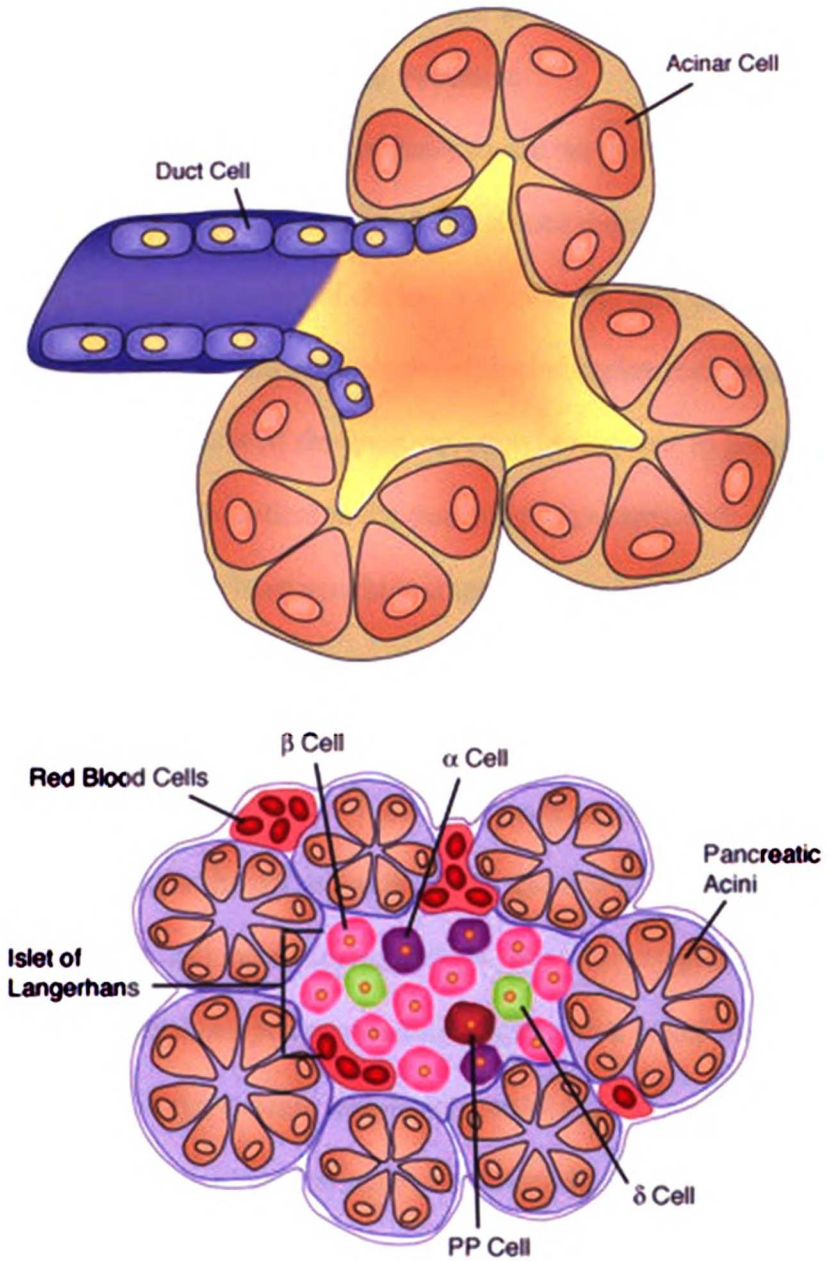

Figure 3.1 Cellular anatomy of the pancreas. The pancreas is formed from a system of acini, clusters of cells comprising the bulk of the tissue, and ducts, which run throughout the pancreas and empty into the duodenum. (top) Acinar cells produce an enzyme mixture used in digestion. Epithelial cells line the ducts and add mucous and bicarbonate to the digestive juices. (bottom) Embedded within the acinar tissue are the Islets of Langerhans consisting of α-, β-, δ-, and PP cells. α- and β- cells produce glucagon and insulin used in glucose regulation. δ- and PP cells produce somatostatin and pancreatic polypeptide which regulate the secretions of other pancreatic cell types. (Figure redrawn form [4])

Therefore, chemotherapy is almost universally given to all patients with ductal pancreatic adenocarcinoma regardless of surgical treatment.

In the last decade, gemcitabine has become the first line of therapy in pancreatic cancer. In 1997, Burris *et al.* determined that gemcitabine significantly improved outcome over the then treatment of choice, 5'-flurouracil. [5] Despite this improvement, wide variation in response has been observed, including intrinsic resistance to gemcitabine. [6-8] Because of the great variation in response to gemcitabine, optimization of therapy is critical in the treatment of patients with pancreatic cancer. Numerous clinical studies have sought to improve gemcitabine efficacy by adding concomitant drug or radiation therapy. [9, 10] In addition, recent studies have shown that intracellular levels of gemcitabine's active metabolites are dependent on the rate of delivery of gemcitabine to cells. [11] As such, there are numerous ongoing studies attempting to determine the optimal scheduling and rate of delivery of the drug.

Unlike many nucleoside analogs which show activity predominantly in various leukemias, gemcitabine is a potent cytotoxic agent with a broad spectrum of activity in pancreatic, bladder, lung, ovarian and breast cancer. [12] Gemcitabine is a prodrug that must be intracellularly activated to achieve cytotoxicity (Fig. 3.2). The drug is initially phosphorylated by deoxycytidine kinase to gemcitabine monophosphate and subsequent phosphorylation steps yield gemcitabine diphosphate and gemcitabine triphosphate. Both the di- and tri-phosphates are considered active compounds. Gemcitabine diphosphate inhibits ribonucleotide reductase which is responsible for maintaining pools of endogenous nucleotides. Ribonucleotide reductase inhibition decreases the cellular pool of deoxycytidine triphosphate (dCTP) that competes with gemcitabine triphosphate for

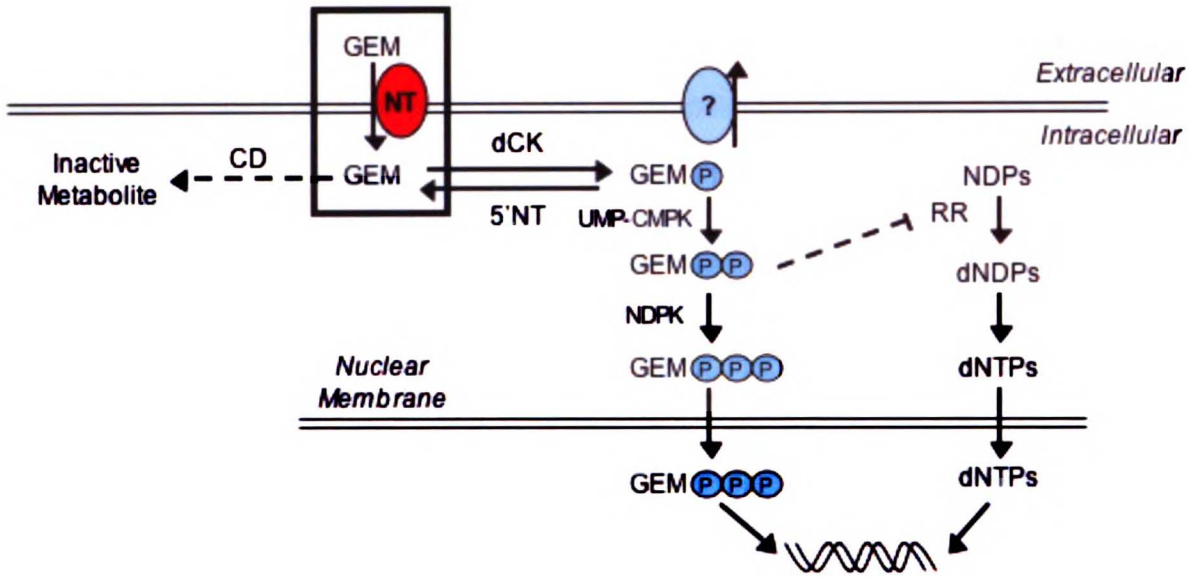

Figure 3.2 Gemcitabine activation pathway. Gemcitabine (GEM) is transported into the cell via nucleoside transporters (NT). Gemcitabine can be deaminated by cytidine deaminase (CD) or phosphorylated by deoxycytidine kinase (dCK). The reverse reaction is carried out by cytoplasmic 5'-nucleotidase (5'NT). Uridine monophosphate/cytidine monophosphate kinase (UMP-CMPK) catalyzes the reaction to GEM-DP while nucleoside diphosphate kinase (NDPK) further catalyzes the reaction to the triphosphate. Ribonucleotide diphosphates (NDP) are reduced to deoxyribonucleotide diphosphates (dNDP) by ribonucleotide reductase (RR). Further phosphorylation of dNDPs form nucleotides (dNTPs) which compete with GEM-TP for incorporation into elongating DNA.

incorporation into DNA. Incorporation of gemcitabine triphosphate into DNA inhibits replication with subsequent induction of apoptosis.

Gemcitabine is deaminated by cytidine deaminase and the velocity of this reaction is stimulated by deoxycytidine triphosphate. In addition, dCK activity is reduced by high levels of dCTP. Therefore, reduction of dCTP pools favors the formation of GEM-MP. Thus, reduction of deoxycytidine triphosphate pools by gemcitabine prolongs cellular half-life of gemcitabine triphosphate. [13] This self-potentiating action of gemcitabine is one reason for the improved spectrum of activity over other nucleoside analogs.

Gemcitabine is a hydrophilic prodrug that relies on nucleoside transporters to gain entry into the cell. [14] Studies in cell lines from various tumors have demonstrated that by mediating influx, nucleoside transporters are necessary in the efficacy of gemcitabine. These transporters include the equilibrative transporter, ENT1, *SLC29A1*; and the concentrative transporters, CNT1, *SLC28A1* and CNT3, *SLC28A3*. Gemcitabine is not taken up by CNT2, which is a purine-selective transporter, and only very weakly interacts with ENT2 (Km = 750 μM). [15]

We have used a quantitative colorimetric assay (MTT assay) to determine the sensitivity of a panel of 16 pancreatic cancer cell lines to gemcitabine. We used this characterization in a number of ways. First, we examined the relationship between gemcitabine sensitivity and mRNA expression levels, as determined by quantitative reverse transcription polymerase chain reaction (quantitative RT-PCR), of the three nucleoside transporters relevant to gemcitabine cytotoxicity: ENT1, CNT1 and CNT3. Second, we used this sensitivity data along with Affymetrix array data to discover genes that were differentially expressed in cell lines that were sensitive to gemcitabine vs. those

that were resistant. We were especially interested in genes involved in the gemcitabine activation pathway or apoptosis pathway. We used the results of this analysis as a hypothesis generator and as a method of directing future studies. Chapter 3 describes, in detail, our characterization of the panel of pancreatic cancer cell lines and emphasizes methodologies. Chapter 4 examines the complex interplay between concentrative and equilibrative nucleoside transporters our studies revealed. Finally, in Chapter 5, the results of the Affymetrix array data are presented.

3.2 Materials and Methods

Cell Culture. Cell lines were provided by our collaborator, Martin McMahon. All cell lines are available for purchase from ATCC with the exception of L.3.3, L.3.6 pL, L.3.6 sL, and MPanc96. Table 3.1 gives the known characteristics (tumor type, subcultivation ratio, etc.) of the cell lines studied. The following cell lines - CaPan2, HPAFII, L.3.3, L.3.6 pL, L.3.6 sL, MiaPaCa2, Panc1, and SW1990, were maintained in Dulbecco's Modified Eagles's Medium with 4 mM L-glutamine, 1.5 g/L sodium bicarbonate, and 4 g/L glucose (DME H-21), supplemented with 10 % fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin in 5% CO_2, 95% air. All other cell lines used RPMI 1640 medium with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, and 4.5 g/L glucose, 10 mM HEPES, 1 mM sodium pyruvate, and 10 units insulin/selenium/transferrin, supplemented with 15 % fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were passaged at 60-90% confluence using 0.25% trypsin.

TaqMan Control Plate. The TaqMan Endogenous Control Plate (Applied Biosystems, Foster City, CA) was used to determine the appropriate housekeeping gene

Table 3.1. *Known characteristics of adherent epithelial pancreatic cancer cell lines.*[1]				
Cell line[2]	Tumor Type[3]	Metastatic Site	Subcultivation Ratio	Doubling Time (hrs)
AsPc1	M	ascites	1:3 to 1:6	
BxPc3	P		1:3 to 1:6	
Capan1	M	liver	1:2 to 1:4	
CFPAC1	M	liver	1:3 to 1:10	32
HPAC[4]	P		1:3 to 1:6	41
HPAF II	M	ascitic fluid	1:2 to 1:3	
HS766T	M	lymph node	1:2 to 1:8	
Hs 700T	M	pelvis	1:2 to 1:4	
MiaPaCa2	P		1:3 to 1:8	40
MPanc96	P			27
Panc1	P		1:2 to 1:4	52
Panc 02.03	P		1:2 to 1:4	28
Panc 02.13[4]	P		1:2 to 1:4	34
Panc 03.27	P		1:3 to 1:4	37
Panc 04.03	P		1:2 to 1:3	51
Panc 05.04	P		1:3 to 1:5	46
Panc 08.13	P		1:2 to 1:4	20
Panc 10.05[5]	P		1:2 to 1:4	19
PL45[5]	P		1:2 to 1:6	
SU 86.86	M	liver	1:2 to 1:3	
SW 1990	M	spleen	1:2 to 1:4	64

[1] Characteristics as reported by ATCC. Cell lines used in gemcitabine sensitivity studies are listed in bold.

[2] Only characterized cell lines are listed here. Values have not been reported for the following cell lines: COLO357, L3.6pl, L3.6sl, NOR-P1, Panc 01.28, Panc 04.21, Panc 06.03, Panc 09.06.

[3] Tumor type: P – primary tumor, M – metastatic tumor.

[4] In culture, cells form monolayers of morphologically heterogeneous polar epithelial cells

to use as the control gene in subsequent Real-Time Quantitative RT-PCR experiments. Eight cell lines were randomly picked and used to test the variation in expression of typical housekeeping genes among the pancreatic cancer cell lines. The housekeeping gene with the smallest variation in expression was used as the control gene in TaqMan assays. Panc 4.21, Panc 02.03, SU 86.86, MiaPaCa2, Panc1, MPanc96, L.3.3, and L.3.6 sL were grown on 100 mm polystyrene plates until approximately 80% confluent. Cells were then trypsinized, washed, and collected in 15 mL conical tubes. Total RNA was then isolated using the RNeasy kit (QIAGEN, Valencia, CA) (see Total RNA Isolation below). cDNA was prepared using the SuperScript III First Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) (see cDNA synthesis below). Each control plate consists of a MicroAmp® Optical 96-Well Reaction Plate divided into 12 columns. Each column consists of eight identical wells containing TaqMan primers and probes as follows - internal positive control (IPC), 18S rRNA (18S), acidic ribosomal protein (PO), beta-actin (BA), cyclophilin (CYC), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phosphoglycerokinase (PGK), beta2-microglobulin (B2M), b-glucronidase (GUS). hypoxanthine ribosyl transferase (HPRT), TATA binding protein (TBP), and transferrin receptor (TFR). For each plate, the first two rows are used for the analysis of the calibrator sample, a sample used as a basis for comparison with the other samples on the control plate, and the following six rows are used for three test samples (each sample is tested in duplicate). 2.5 μL of cDNA, 22.5 μL of RNase-free water, and 25 μL of 2X TaqMan Universal PCR Master Mix were added to each well. The control plate was sealed with a MicroAmp Optical Adhesive Cover and centrifuged to spin down the contents and eliminate any air bubbles. Reactions were run on an ABI Prism 7700 and

cycling conditions were: 50°C for 2 min, 95°C for 10 min, followed by 45 cycles of 95°C for 15s and 60°C for 1 min. The resulting output curves and C_T determination were analyzed using the Sequence Detection Systems (SDS) software.

Total RNA Isolation. Approximately 1 X 10^6 to 1 X 10^7 cells were used for total RNA extraction depending on the expected RNA content. In all cases, cells were trypsinized, washed and pelleted prior to RNA extraction. In many cases, cell pellets were stored at -80 °C until RNA preparation. Frozen pellets were thawed briefly prior to isolation to aid in lysis. Total RNA was isolated using the RNeasy kit (QIAGEN, Valencia, CA) according to the protocol. RNA concentrations were determined using a NanoDrop spectrophotometer.

cDNA synthesis. cDNA was prepared using the SuperScript III First Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) following the manufacture's protocol. A total of 1.5 μg of total RNA was used for cDNA synthesis. This was to ensure 100 ng of total RNA (converted to cDNA) per well for 12 wells per cell type. RNA, random hexamers, dNTPs, and DEPC-treated water were combined in a PCR tube according to the protocol. This solution was mixed gently and incubated at 65°C for 5 minutes, then placed on ice for at least 1 minute. cDNA Synthesis Mix (10 μl) was added to each 10 μl of RNA/primer mixture, mixed gently, and collected by brief centrifugation. The samples were incubated 10 min at 25°C, followed by 50 min at 50°C. The reaction was terminated at 85°C for 5 min and the samples were chilled on ice. Reactions were collected by brief centrifugation and RNA template was removed by adding 1 μl of RNase H to each tube and incubating for 20 min at 37°C.

Cytotoxicity Assay. Gemcitabine was obtained as a lyophilized powder from the UCSF pharmacy. NBMPR, dipyridamole, and MTT were purchased from Sigma Chemical Co.. Gemcitabine cytotoxicity, with and without ENT1 inhibitors, was assessed using the MTT assay. [16] Cultured cells were trypsinized, counted in a hemocytometer, and seeded into flat-bottomed 96-well plate (Costar) and cultured in a CO_2 incubator at 37°C for 24 hours. A portion of these cells were reserved for RNA extraction and quantitative RT-PCR. For each cell line, the optimal cell number was determined by previous cell density studies (between 8-15 X 10^3 cells/well). Either gemcitabine alone (final volume 100 μL/well) or gemcitabine plus ENT1 inhibitor (final volume 150 μL/well) was added to each well, and the cells were cultured for 72 hours at 37°C. For each 96-well plate, one row of cells was left untreated and used as a control. Cytotoxicity determination was completed by adding 20% by volume (either 20 or 30 μL) of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide, 5 mg/mL in PBS) labeling reagent to each well. After incubating for 5 hours, the media was removed from each well, and 100 μL isopropanol (made to 0.1 N with HCl) was added to each well. The plates were shaken gently at room temperature for 20 minutes to allow complete dissolution of crystals. Absorbance was measured with a VersaMax microplate reader at 580 nm with a reference wavelength of 670 nm. In each experiment, cells were plated in quadruplicate for a given concentration. All experiments were repeated at least 3 times.

The actual absorbance for each well was calculated by subtracting the background absorbance from the reading ($A=A_{580}-A_{670}$). These values were then expressed as percent of control (absorbance of untreated cell growth=100%). The log of absorbance at each

gemcitabine concentration was plotted versus % control and the curve was fitted using PRISM 4 to the equation:

$$\% \text{ control} = \% \text{ control}_{min} + \frac{\% \text{ control}_{max} - \% \text{ control}_{min}}{1+10^{((\text{LogEC}_{50} - \text{Log[gem]})*\text{HillSlope})}}$$

Where $\% \text{ control}_{max}$ is the maximum value of the % control, $\% \text{ control}_{min}$ is the minimum value of the % control, HillSlope is the value of the Hill slope coefficient, and EC_{50} is the gemcitabine concentration that results in half maximal cytotoxicity.

Real-Time Quantitative Reverse Transcriptase-PCR (RT-PCR). Cells were reserved from each MTT assay for RNA isolation. RNA from each cell line was isolated using a RNeasy kit (QIAGEN, Valencia, CA) according to the manufacture's protocol. Reverse transcription was carried out using TaqMan Universal Master Mix (Applied Biosystems, Foster City, CA). Primer and probe sets for each gene were Assays-on-Demand purchased from Applied Biosystems (Foster City, CA) (Table 3.2). Reactions were run on an ABI Prism 7700 and cycling conditions were: 50°C for 2 min, 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. The relative expression of specific transcripts was calculated by the following formula: Relative expression ($X\ 10^{-6}$) = $2^{-\Delta Ct}$ where $\Delta Ct = (Ct_{target} - Ct_{18S})$.

Table 3.2 *Assay-on-Demand primers/probes used in TaqMan experiments*

Transporter	Gene	Assay-on-Demand ID	Exon Boundary
ENT1	SLC29A1	Hs00191940_m1	Exon 1 | Exon 2
CNT1	SLC28A1	Hs00188418_m1	Exon 15 | Exon 16
CNT3	SLC28A3	Hs00223220_m1	Exon 5 | Exon 6

Assay-on-Demand primers/probes used in TaqMan experiments were purchased from Applied Biosystems (Foster City, CA) and are proprietary.

3.2 Results

Optimization of Cytotoxicity Assay. The optimal number of cells plated was determined for each cell line prior to performing a cytotoxicity assay. Cells were seeded into a flat-bottomed 96-well plate, in quadruplicate, at densities from ~200 – 100,000 cells/well. Cells were cultured for 72 hours at 37°C without drug and then analyzed using the MTT assay. The optimal number of cells is in the linear range of the absorbance vs. cell number curve (Fig. 3.3) and results in an absorbance of between 0.5 and 1.5. It is important that the optimal number of cells be determined for each cell line. If cells are seeded too densely, that is if the absorbance values have plateaued, changes in cell density due to drug cytotoxicity will not be visible. Optimal cell densities are given in Table 3.3.

In addition to cell density optimization, the % by volume of MTT was also optimized. For this optimization, cells were plated at an optimal density and incubated for 72 hours at 37°C without drug. Cells were then incubated with varying concentrations of MTT for 5 hours and analyzed as usual. Absorbance vs. % volume MTT is shown in Figure 3.4. In this case, the optimal value of MTT used will be in the plateau of the curve. There are likely to be small variations in the volume of MTT delivered to each well by the multi-channel pipetter. If the volume of MTT used was in the linear range of the absorbance vs. % volume MTT curve, variation in MTT added would result in an inaccurate absorbance reading. However, if the volume of MTT used is in the plateaued region of the curve, the amount of MTT can shift slightly to the right or left without significantly altering the absorbance reading. Absorbances began to

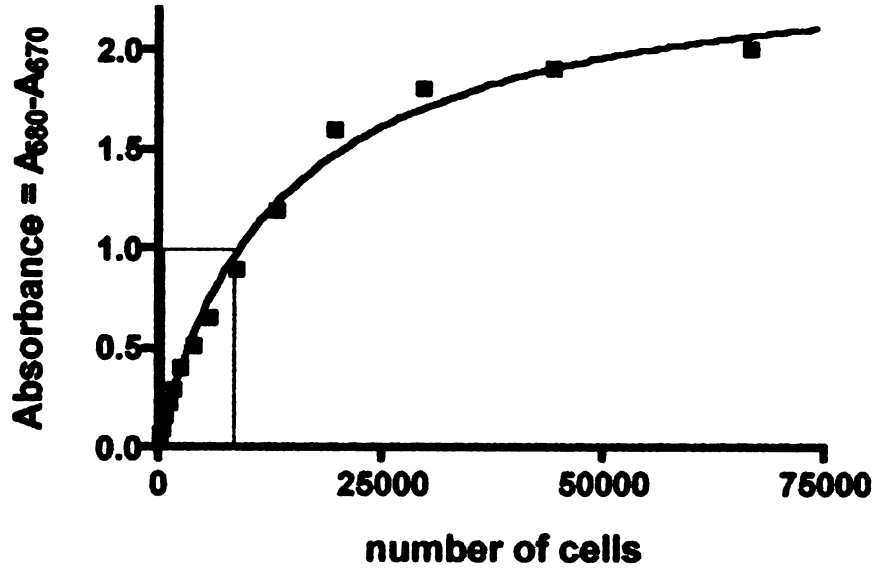

Figure 3.3 Optimal density determination. Cells were seeded at densities from ~200 – 100,000 cells/well. Cells were cultured for 72 hours at 37°C without drug and then analyzed using the MTT assay. Cell densities within the linear range (within box) are optimal for MTT assays. Optimal density determination of cell line L.3.3 shown as an example.

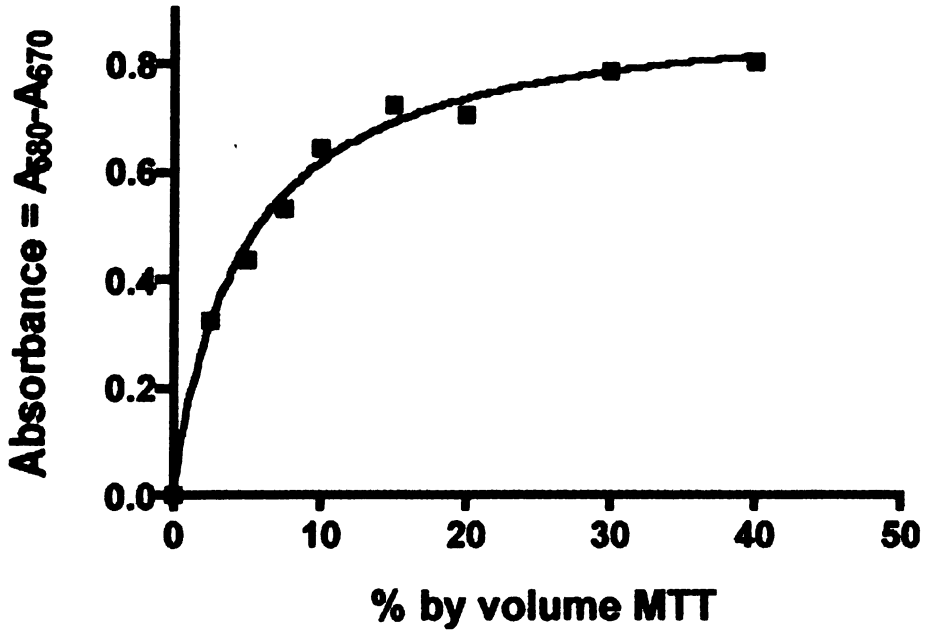

Figure 3.4 Optimal % MTT by volume determination. Cells were seeded at an optimal density and cultured for 72 hours at 37°C without drug The plates were then analyzed using the MTT assay. Absorbances began to plateau around 15% by volume MTT. A value of 20% by volume was used in cytotoxicity assays. % MTT is optimal in the plateau region.

Table 3.3. *Optimal seeding cell densities of pancreatic cancer cell lines.*[1]	
Cell line	Optimal Cell Density (cells/well) [2]
Panc 2.03	10,000
Panc 2.13	10,000
Panc 3.27	15,000
Panc 5.04	15,000
Panc 8.13	15,000
Panc 10.05	15,000
CaPan2	10,000
HPAF II	10,000
L.3.3	10,000
L.3.6 pL	10,000
L.3.6 sL	10,000
MiaPaCa2	8,000
MPanc96	15,000
Panc1	8,000
SW1990	8,000
SU 86.86	6,000

[1] Optimal cell densities were determined as described in the text for each cell line.

[2] Cell density is reported as the optimal number of cells initially seeded into one well on a 96-well plate.

plateau around 15% by volume MTT. A value of 20 % by volume MTT was used in subsequent gemcitabine cytotoxicity assays.

Sensitivity of Pancreatic Cancer Cell Lines to Gemcitabine. We examined the gemcitabine sensitivity in a panel of 16 pancreatic cancer cell lines. MTT cytotoxicity assays were performed on these cell lines to determine the concentrations of gemcitabine that inhibited cell growth by 50% (EC_{50}). Cells were exposed to a range of gemcitabine concentrations for 72 hours and growth was then quantified via the MTT assay (see Materials and Methods). Since preliminary studies suggested that EC_{50} values may vary with cell passage each cell line was tested multiple times. Of the 16 pancreatic cancer cell lines, approximately a 1400 fold difference was observed between the median EC_{50} values of the most sensitive cell line (L.3.6 sL) and the most resistant cell line (MPanc96) (Table 3.4). When each individual experiment is considered this range is even more dramatic. We observed a 10,000 fold difference in EC_{50} values between the most sensitive and the most resistant cell passage.

Cells exposed to gemcitabine and the ENT1 inhibitor NBMPR displayed similar EC_{50} variation. In the presence of NBMPR, approximately a 7800 fold difference was observed between the median EC_{50} values of the most sensitive cell line (L.3.6 sL) and the most resistant cell line (Panc1) (Table 3.4). A 15,000 fold difference in EC_{50} values was observed between the most sensitive and the most resistant cell passage when treated with gemcitabine and NBMPR. We found that there were three classes of response to ENT1 inhibition with NBMPR – cells became more resistant, more sensitive, or had no change in sensitivity to gemcitabine when treated with NBMPR. The implications of these responses will be discussed further in Chapter 4.

Table 3.4 *Gemcitabine cytotoxicity in a panel of pancreatic cancer cell lines**									
Cell line		Gemcitabine EC_{50} (μM) with and without 20 nM NBMPR for each cell passage†							
Panc 2.03	passage	6	10	16	12	13††			
	gem	52.3	51.9	31.7	20.7	0.7			
	NBMPR	755	392	186	79.6	1.6			
Panc 2.13	passage	31	1	4	8	12			
	gem	3.1	7.1	11.0	10.5	10.5			
	NBMPR		13.2	52.1	46.2	45.8			
Panc 3.27	passage	37	43	81	83	87	54	57	69
	gem	20.7	22.7	6.0	21.4	11.0	11.0	13.6	1.9
	NBMPR			9.4	32.0	17.5	6.3	18.4	4.1
Panc 5.04	passage	47	50	28	31	37††			
	gem	63.0	12.9	27.5	4.1	0.11			
	NBMPR	338	11.1	2803	244	0.03			
Panc 8.13	passage	13	16	18	21	14	22	7	12
	gem	3236	142	7340	76.2	767	3296	78.0	7777
	NBMPR	4598	10416	1714	2269	2084	2962	6505	9830
Panc 10.05	passage	6	19	26	30				
	gem	1365	1736	381	1011				
	NBMPR		1661	2299	2003				
CaPan2	passage	29	43	46	48	8	28	19	24
	gem	2906	970	807	956	561	802	817	477
	NBMPR	3014	2397	1069	1711	11120	2546	1617	1490
HPAF II	passage	14	32	36	5	8	12	18	22
	gem	6.1	3.7	4.2	8.8	3.4	7.4	8.9	13.8
	NBMPR	3.5	3.4	3.6	13.3	1.4	7.5	9.2	4.6

Cell line		Gemcitabine EC_{50} (μM) with and without 20 nM NBMPR for each cell passage†							
L.3.3	passage	16	13	23	29	31			
	gem	4.7	31.4	3.5	380	391			
	NBMPR	43.7	64.5	8.3	713	671			
L.3.6 pL	passage	36	39	30	36				
	gem	2.5	1.8	2.9	2.94				
	NBMPR				3.9				
L.3.6 sL	passage	47	49	55	58				
	gem	1.7	1.3	2.6	1.2				
	NBMPR	2.6	0.7	1.5	1.5				
MiaPaCa2	passage	6	9	11	22	23			
	gem	35.4	40.0	29.8	40.1	23.2			
	NBMPR	49.0	40.4	41.0	40.4	59.3			
MPanc96	passage	47	10	9	11	18	21	13	
	gem	2203	3908	806	12173	1399	2088	447	
	NBMPR	125		862	5171	1835	851		
Panc1	passage	5	6	13	16	33	27	31	42
	gem	508	607	542	518	175	310	948	172
	NBMPR				6361	8348	9876	9557	9002
SU86.86	passage	10	14	17	28				
	gem	151	14.3	26.1	218				
	NBMPR		140	122	352				
SW1990	passage	37	40	53	69				
	gem	5.3	20.5	9.2	34.6				
	NBMPR			19.9	58.9				

* EC_{50} values were calculated for cell lines without NBMPR (gem) and with 20 nM NBMPR (NBMPR).

† Cell passages are given in chronological order. In cases where cell line passage goes down, an earlier passage of that cell line was retrieved and grown from frozen stock.

†† EC_{50} values for Panc 2.03, passage 13, and Panc 5.04, passage 37, are reported for completeness but were not used in further calculations. The MTT used in these two experiments was expired and therefore these values are not reliable.

The EC_{50} value was determined for each cell line after exposure to a series of gemcitabine concentrations with and without 20 nM NBMPR as described in the Materials and Methods.

All cell lines are available through ATCC with the exception of L.3.3 L.3.6 pL, L.3.6 sL, and MPanc96

Determination of TaqMan Control Gene. TaqMan control plates were analyzed as described in the Materials and Methods. Eight cell lines were randomly picked and used to test the variation in expression of typical housekeeping genes among the pancreatic cancer cell lines. An internal positive control (IPC) is included on each plate and can be used to visualize the amount of variation in expression that is due to sample volume differences caused by pipetteing errors. Panc1, MPanc96, L.3.3, and L.3.6 sL were used on control plate 1 and Panc 4.21, Panc 02.03, SU 86.86, MiaPaCa2, were used on control plate 2. Panc1 was used as the calibrator sample for plate 1 and Panc 4.21 was used as the calibrator sample for plate 2. The housekeeping gene with the smallest variation in expression will be used as the control gene in subsequent TaqMan assays. As shown in Fig 3.5, 18S showed the smallest variation across experiments and across cell lines. Therefore, 18S was used as the control gene in all subsequent TaqMan analysis.

Nucleoside Transporter mRNA Expression in Pancreatic Cancer Cell Lines. ENT1, CNT1 and CNT3 mRNA expression levels were determined in our panel of 16 pancreatic cancer cell lines by quantitative RT-PCR. As shown in Table 3.5, there is a wide degree of variability of nucleoside transporter expression both between and within cell lines. Panc 8.13 and MPanc96 expressed relatively higher mRNA levels of all three nucleoside transporters. However, some cell lines, such as L.3.3, L.3.6 pL, and SW1990 cells expressed comparatively low nucleoside transporter mRNA levels. We also determined the mRNA levels of ENT1, CNT1, and CNT3 within each cell line. ENT1 had the highest mRNA expression in most cell lines. Many cell lines, such as Panc1, expressed predominantly or exclusively ENT1. In contrast a number of cell lines expressed the concentrative nucleoside transporters predominantly. MiaPaCa2 expressed

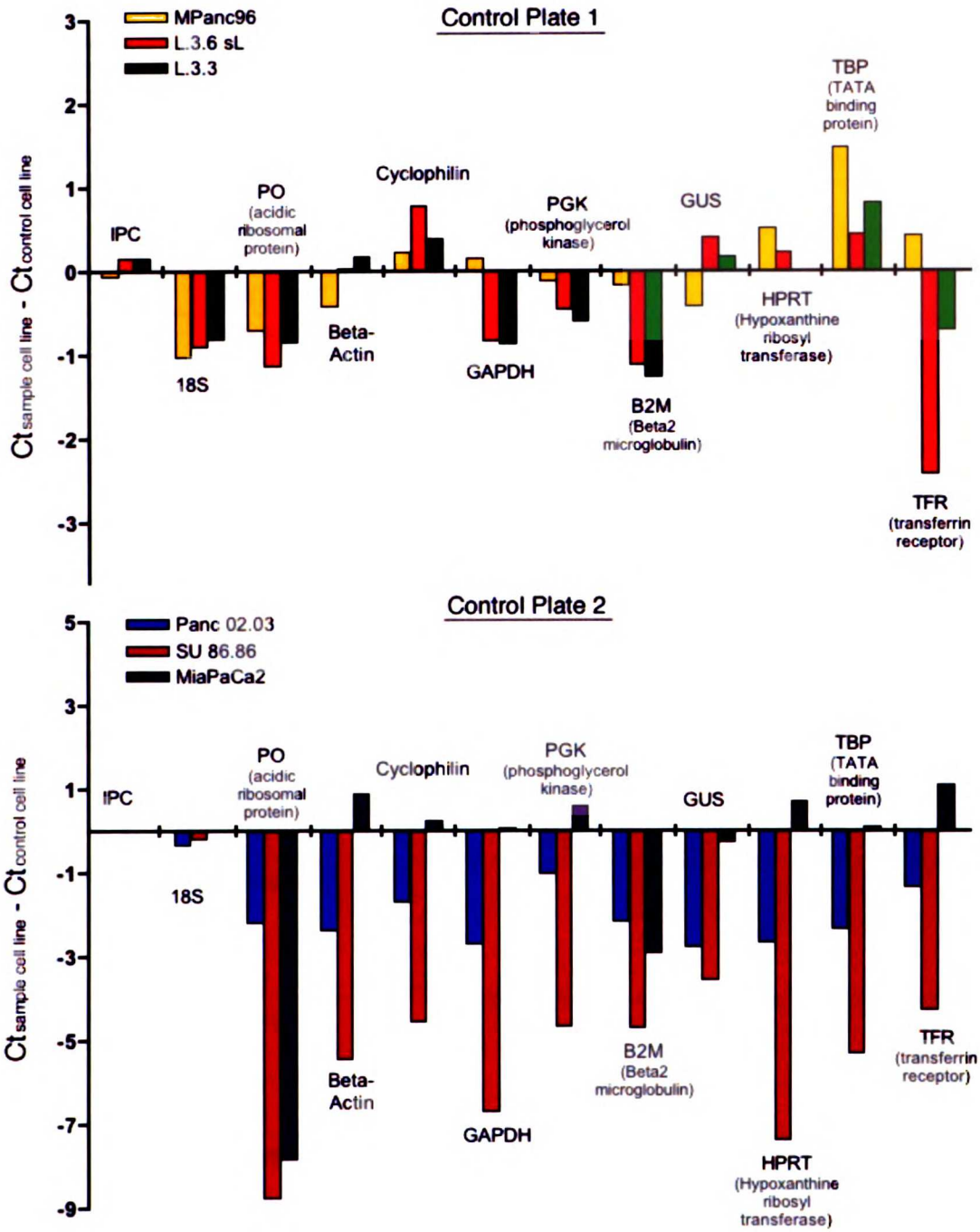

Figure 3.5 Determination of TaqMan control gene. Two control plates were analyzed according to the Materials and Methods. The ΔC_T ($C_{T,\text{sample cell line}} - C_{T,\text{control cell line}}$) of each gene in each cell line is shown above. Panc1 was used as the control cell line for plate 1 and Panc 4.21 was used as the control cell line for plate 2. Of the 11 housekeeping genes, 18S had the least amount of variation across experiments and across cell lines. IPC is an internal positive control which can be used to estimate the amount of variation due to sample volume errors.

Table 3.5 *Relative expression of nucleoside transporters in a panel of pancreatic cancer cell lines* *						
Cell line		**Transporter expression for each cell passage†**				
Panc 2.03	passage	6	10	16		
	ENT1	16.892	8.845	3.208		
	CNT1	2.021	0.156	0.078		
	CNT3	0.030	0.013	0.032		
Panc 2.13	passage	31	1	4	8	12
	ENT1	4.117	39.714	17.814	80.351	92.941
	CNT1	0.001	0.046	0.031	0.032	0.079
	CNT3	0.032	0.081	0.045	1.336	2.600
Panc 3.27	passage	37	43	69	75	
	ENT1	57.743	5.931	0.670	5.716	
	CNT1	0.125	0.017	0.000	0.008	
	CNT3	0.053	0.383	0.038	0.960	
Panc 5.04	passage	6	17	19		
	ENT1	0.264	1.355	12.713		
	CNT1	0.039	0.126	12.596		
	CNT3	0.010	0.057	0.823		
Panc 8.13	passage	13	12			
	ENT1	111.810	6.844			
	CNT1	0.446	0.004			
	CNT3	1.634	0.184			
Panc 10.05	passage	6				
	ENT1	2.517				
	CNT1	0.004				
	CNT3	0.055				
CaPan2	passage	4	6	24		
	ENT1	7.235	7.151	1.158		
	CNT1	0.026	0.019	0.004		
	CNT3	0.000		0.000		
HPAF II	passage	6	8	12	14	22
	ENT1	0.354	0.121	0.819	0.201	0.177
	CNT1	0.014	0.005	0.007	0.014	0.015
	CNT3	0.534	0.118	1.469	0.286	0.225

Cell line		Transporter expression for each cell passage[†]			
L.3.3	passage	10	16		
	ENT1	0.009	0.015		
	CNT1	0.002	0.001		
	CNT3	0.003	0.150		
L.3.6 pL	passage	30	36	39	
	ENT1	0.000	0.000	0.000	
	CNT1	0.014	0.008	0.004	
	CNT3	0.000	0.009	0.000	
L.3.6 sL	passage	19	49	58	55
	ENT1	0.000	0.001	0.000	0.001
	CNT1	0.001	0.591	0.041	0.333
	CNT3	0.014	0.022	0.040	0.054
MiaPaCa2	passage	4	6	9	
	ENT1	0.000	0.001	0.001	
	CNT1	0.015	0.393	0.433	
	CNT3	0.000	0.000		
MPanc96	passage	9	11	14	
	ENT1	0.194	29.890	11.698	
	CNT1	0.001	0.626	0.159	
	CNT3	0.038	5.296	0.281	
Panc1	passage	5	6	7	13
	ENT1	45.831	93.587	58.010	84.540
	CNT1	0.002	0.004	0.003	0.004
	CNT3	0.000	0.000	0.000	
SU86.86	passage	19	28		
	ENT1	6.014	0.388		
	CNT1	0.011	0.001		
	CNT3	0.043	0.004		
SW1990	passage	37			
	ENT1	0.305			
	CNT1	0.003			
	CNT3	0.000			

* Relative expression (X 10^{-6}) of each gene was calculated as $2^{-\Delta Ct}$ where $\Delta Ct = (Ct_{target} - Ct_{18S})$

† Cell passages are given in chronological order. In cases where cell line passage goes down, an earlier passage of that cell line was retrieved and grown from frozen stock.

RNA expression levels were determined for ENT1, CNT1, and CNT3 as described in the Materials and Methods.

All cell lines are available through ATCC with the exception of L.3.3 L.3.6 pL, L.3.6 sL, and MPanc96.

CNT1 exclusively. Finally, a few cell lines expressed both concentrative and equilibrative nucleoside transporters. For example, MPanc96 cells express appreciable levels of ENT1, CNT1, and CNT3.

3.3 Discussion

Numerous assays have been developed to determine the cytotoxity of xenobiotics by measuring the survival or proliferation of cells. These methods include measuring the incorporation of radiolabeled nucleotides (e.g. [^{3}H]-thymidine or [^{125}I]-iodo-deoxyuridine) in proliferating cells, measuring the release of ^{51}Cr-labeled protein after cell lysis, counting cells after the exclusion/inclusion of dye, or counting cell colonies that form on soft agar plates. The human tumor clonogenic assay (HTCA), described by Salmon and Hamburger, is one of the most reliable methods of determining cell viability and is also useful as a clinical predictor. [17-19] In this assay, cell colonies are allowed to form on soft agar plates in the presence of a particular concentration of the drug tested. Colonies are stained with a reporter dye (e.g. Crystal Violet) and colonies can be counted with a dissecting microscope or suspended in media, cultured for two weeks, and counted with a particle counter. The treated cells are compared to control cells that have not been exposed to drug to determine percent growth inhibition. Although this assay has a slow turnaround, a low efficiency and is labor intensive, it is extremely accurate in determining the cytotoxicity of a given drug concentration since it is a direct measure of tumor growth and does not rely on a surrogate marker for cell proliferation.

The HTCA assay can also be used as a clinical predictor. For this, the blood concentration at the start of the ß-phase of elimination following an IV bolus dose of the

standard clinical dose is determined. Cells are deemed sensitive to a drug when 50-70% inhibition of colony forming activity is obtained after a 1-hour exposure of cells to this concentration of the drug. This sensitivity at one hour is used to predict clinical effect. [20-23] Roper and Drewinko compared the HTCA assay to numerous other proliferation assays, including dye exclusion, ^{51}Cr release assay, [^{3}H]-thymidine incorporation, and measurement of doubling time, and determined that the HTCA assay was the best indicator of cell death. [24] Recently, Kawada *et al.* compared the HTCA assay to a colorimetric MTT assay. [25]

The MTT assay uses a surrogate marker to estimate cell proliferation. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) is a water soluble tetrazolium salt which forms a yellow solution when dissolved in PBS. Dehydrogenase enzymes found in the active mitochondria of living cells metabolize this yellow solution to a form a dark blue formazan crystal (Fig. 3.6). [26] These formazan crystals are only partially soluble in water and largely impervious to cell membranes, however they dissolve readily in acidified isopropanol. Cell number is measured by incubating cells with the yellow MTT solution. The formation of dark blue crystals is proportional to the number of living cells present. These crystals are dissolved with acidified isopropanol and the resulting color intensities are measured with a microplate reader. Dead cells do not interfere with absorbances readings even at concentrations of 1×10^{6}.

The MTT assay has numerous advantages over the HTCA assay, including higher throughput and an expanded range of cell types that can be tested with the assay. Higher throughput is achieved by plating cells on 96- or 384- well plates, therefore several log concentrations of xenobiotic can be measured in one experiment. While HTCA is

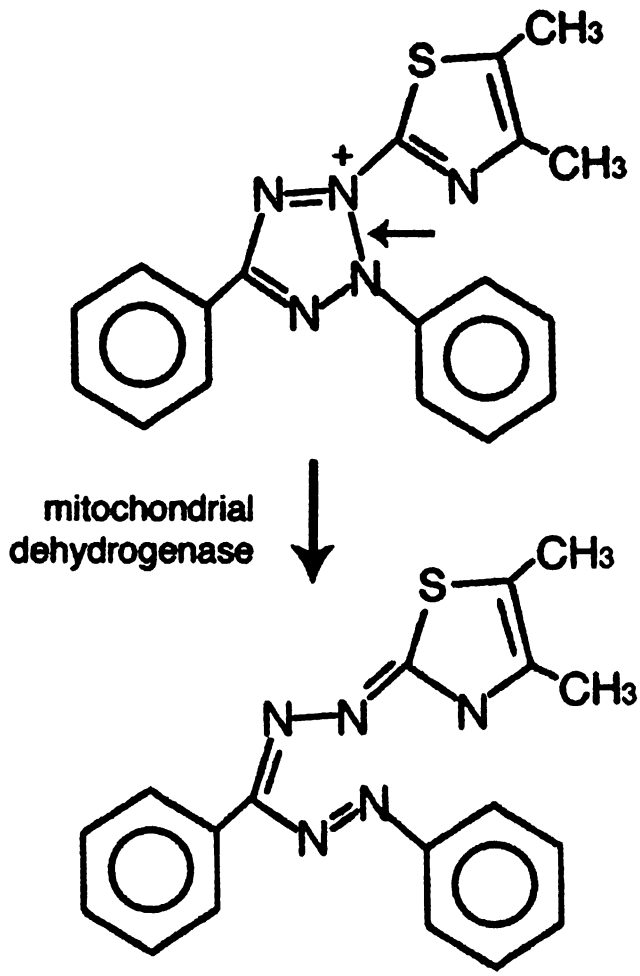

Figure 3.6 MTT conversion. Active mitochondria cleave the tetrazolium ring of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide). This metabolizes MTT from a yellow solution to a dark blue formazan crystal which can be used as a surrogate for the number of live cells.

generally employed to determine cytotoxicity, the MTT assay can be used to measure cytotoxicity, as well as cell activation and proliferation in response to cytokines, nutrients, and growth factors. [26-28] However, the assay does possess some disadvantages compared to the HTCA assay. Initial cell number must always be optimized in the MTT assay to ensure the linear relationship of absorbance to cell number. In addition, cells must possess active mitochondria. For example, the assay is not applicable for metabolically inactive cells such as red blood cells. [26] The study of Kawada *et al.* determined that there was a high degree of correlation ($r = 0.67$, $p < 0.01$) in the cytotoxicity indexes between the HTCA assay and the MTT assay. [25] They studied five lung cancer cell lines and 22 cytotoxic agents, including platinum analogs, anthracyclins/anthracenedione, and vinca alkaloids. Although there was generally a high degree of correlation between the two assays, MTT predicted a higher level of cytotoxicity for vinca alkaloids than HTCA predicted. However, for these drugs cells were exposed for one hour in the HTCA assay and for 96 hours in the MTT assay. This increased exposure is probably responsible for the increased prediction of cytotoxicity. In our studies, sensitivities were compared across all cell lines using the MTT assay. With the exception of optimal cell densities, conditions were held constant in all cell lines. Therefore, any overprediction inherent in the MTT assay will be consistent throughout the experiments.

In addition to characterizing the gemcitabine sensitivities of pancreatic cancer cell lines, we also determined the relative expressions of nucleoside transporters using quantitative RT-PCR. Quantitative RT-PCR is widely used to determine the relative expression of genes in cell samples. [29-31] PCR reactions generate copies of starting

DNA template in an exponential fashion until depletion of reagents or polymerase inhibitors cause the reaction to plateau. Quantitative RT-PCR is based upon the detection of fluorescent signal during the exponential phase in real time PCR experiments. In our studies, we used TaqMan reporter probes which are primer oligos conjugated at the 5' end to a fluorescent molecule and at the 3' end to a quencher molecule. Prior to the incorporation and extension phase of the PCR process, the probe does not fluoresce since the quencher is held in close proximity. During extension, the 5' nuclease activity of *Taq* polymerase cleaves the fluorophore from the quencher, the fluorophore fluoresces, resulting in signal detection. The cycle number where fluorescence crosses a set detection threshold is designated the cycle threshold (Ct). These Ct values are directly proportional to starting template and form the basis for calculating mRNA expression levels (Fig 3.7).

One of the assumptions of relative quantitative RT-PCR is that there is little variation across experiments in expression of the control gene used. One way to verify this assumption is to run control plates, which test the expression of 11 housekeeping genes commonly used as control genes. Fig 3.5 shows the results of this analysis, which analyzed the variation in expression of housekeeping genes in eight different cell lines. The internal positive control (IPC) is included to determine the amount of variation due to small volume differences caused by pipetteing errors. The IPC ΔCt is very small on both control plates indicating that the experimental procedures were accurately performed. Based on the data generated by the control plates, there was a wide degree of expression variation across most housekeeping genes in the pancreatic cancer cell lines.

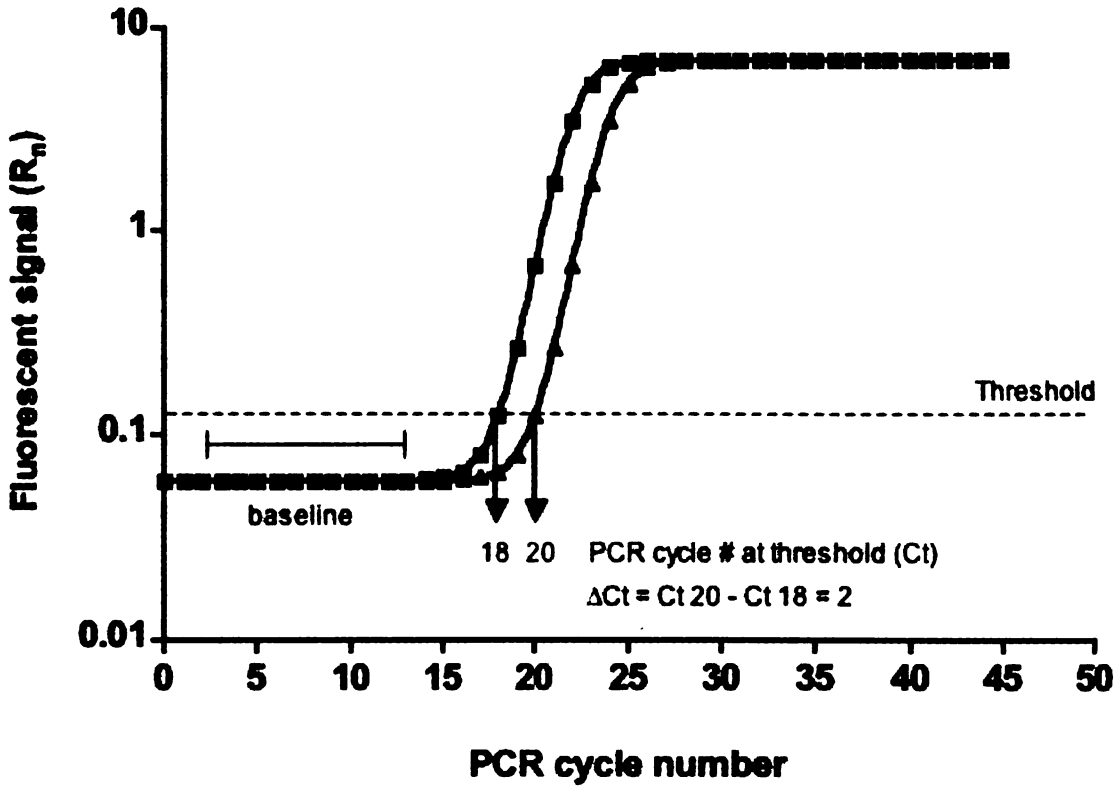

Figure 3.7 Hypothetical quantitative RT-PCR plot. At baseline values, signal is accumulating but fluorescence is below the limits of detection. The threshold is defined as 10 times the standard deviation around the average signal of the baseline. Ct values are the cycle number at which fluorescence crosses the threshold. Ct values for each sample are used to calculate the relative abundance of each gene for each sample. In this example, the first sample (square) crosses the threshold at cycle 18. The second sample (triangle) crosses the threshold at cycle 20. The difference between the two samples (ΔCt) is 20 – 18 = 2, or a 2 cycle difference between samples. Due to the exponential nature of the PCR reaction this 2 cycle difference can be transformed into a 2^(ΔCt), or 4-fold difference. Therefore, there is a 4-fold higher expression of sample one (square, Ct = 18) than of sample two (triangle, Ct = 20).

The only gene which appeared to have consistent expression was 18S. Therefore, 18S was used as the control genes in all future experiments. We used MTT assays to determine the sensitivity of pancreatic cancer cell lines to the nucleoside analog gemcitabine. We also determined the relative expression of nucleoside transporters, known to be essential in the cytotoxicity of gemcitabine, in these cells. In Chapter 4, I will discuss our analysis of the relationship between gemcitabine cytotoxicity and nucleoside transporter expression. Our studies demonstrate a complex interplay between concentrative and equilibrative nucleoside transporters. In particular, we observed that rather than mediating influx and sensitivity to gemcitabine, the equilibrative nucleoside transporter, ENT1, generally acts to mediate efflux and resistance to the drug in pancreatic cancer cell lines.

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CHAPTER 4

A COMPLEX INTERPLAY BETWEEN CONCENTRATIVE AND EQUILIBRATIVE NUCLEOSIDE TRANSPORTERS IN GEMCITABINE SENSITIVITY IN PANCREATIC CANCER CELL LINES

4.1 Introduction

Pancreatic ductal adenocarcinoma, a devastating and deadly disease, is one of the major causes of cancer death. In the United States, gemcitabine (2', 2'-difluorodeoxycytidine) is the only drug approved for use in pancreatic cancer, and has been shown to prolong survival in patients with early and late stages of pancreatic cancer. [1] Because of the great variation in response to gemcitabine, optimization of therapy is critical in the treatment of patients with pancreatic cancer. Gemcitabine is a prodrug that must be phosphorylated intracellularly to its active metabolites, gemcitabine di- and tri-phosphate. The first step of this activation pathway is entry into the cell via influx transporters. In particular, gemcitabine is transported across the plasma membrane by the concentrative nucleoside transporters, CNT1 and CNT3, and by the equilibrative nucleoside transporter, ENT1. [2, 3]

Studies in leukemic cell lines suggest that for gemcitabine to exert its cytotoxic effects, it requires a functional nucleoside transporter. [4] In 10 leukemia cell lines, Mackey *et al.* observed that a functional nucleoside transporter was necessary for the inhibitory effects of gemcitabine on cell growth. That is, cells lacking nucleoside transporters were resistant to the cytotoxic effects of gemcitabine. Further data suggested

that the cells were also resistant to gemcitabine when nucleoside transport activity was chemically inhibited.

Recently, conflicting results were obtained when two laboratories examined the relationship between the mRNA expression level of nucleoside transporters and sensitivity to gemcitabine in the NCI-60 panel of cell lines. Lu *et al.* did not find any association between the cytotoxicity of gemcitabine or other nucleoside analogs (cytarabine, cladribine, or fludarabine) and mRNA expression level of nucleoside transporters. [5] In contrast, Huong *et al.* found a positive correlation between CNT3 mRNA expression level and cytotoxicity of both cytarabine and gemcitabine in the NCI-60 panel. [6]

The general applicability of results obtained in previous studies to gemcitabine sensitivity in pancreatic cancer is questionable. First, results in leukemic cell lines cannot be extrapolated to pancreatic ductal adenocarcinoma cell lines, since these cell lines originate from distinct tissue types. Second, the NCI-60 panel consists of cell lines derived from a diverse range of tumor types none of which are derived from ductal adenocarcinomas of the pancreas. Thus studies in the NCI-60 panel cannot be directly extrapolated to pancreatic cancer. Moreover the use of DNA microarrays is not sufficiently accurate to assess expression levels of specific genes. Quantitative RT-PCR methods are much more sensitive for this purpose.

Because of the importance of gemcitabine in the treatment of pancreatic cancer we performed studies assessing the sensitivity of a panel of pancreatic cancer cell lines to gemcitabine. In particular, we determined the relative contribution of equilibrative and concentrative nucleoside transporters to gemcitabine sensitivity. We studied gemcitabine

cytotoxicity in 16 cell lines and quantitatively determined the mRNA level of the three relevant nucleoside transporters, ENT1, CNT1 and CNT3. Our studies demonstrate a complex interplay between concentrative and equilibrative nucleoside transporters. In particular, we observed that in addition to mediating influx and sensitivity to gemcitabine, the equilibrative nucleoside transporter, ENT1, can also mediate efflux and resistance to the drug in pancreatic cancer cell lines. Cells expressing high CNT1 and low ENT1 levels had greater gemcitabine sensitivity when compared to cells expressing low CNT1 and high ENT1 levels.

4.2 Materials And Methods

Cell Culture. Cell lines were provided by our collaborator, Martin McMahon. All cell lines are available for purchase from ATCC with the exception of L.3.3, L.3.6 pL, L.3.6 sL, and MPanc96. The following cell lines - CaPan2, HPAFII, L.3.3, L.3.6 pL, L.3.6 sL, MiaPaCa2, Panc1, and SW1990, were maintained in Dulbecco's Modified Eagles's Medium with 4 mM L-glutamine, 1.5 g/L sodium bicarbonate, and 4 g/L glucose (DME H-21), supplemented with 10 % fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin in 5% CO_2, 95% air. All other cell lines used RPMI 1640 medium with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, and 4.5 g/L glucose, 10 mM HEPES, 1 mM sodium pyruvate, and 10 units insulin/selenium/transferrin, supplemented with 15 % fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were passaged at 60-90% confluence using 0.25% trypsin.

Cytotoxicity Assay. Gemcitabine was obtained from the UCSF pharmacy. NBMPR (*S*-(4-nitrobenzyl)-6-thioinosine), dipyridamole, and MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) were purchased from Sigma Chemical Co.. Gemcitabine cytotoxicity, with and without ENT1 inhibitors, was assessed using the MTT assay. [7] Cultured cells were trypsinized, counted in a hemocytometer, and seeded into flat-bottomed 96-well plate (Costar) and cultured in a CO_2 incubator at 37°C for 24 hours. A portion of these cells were reserved for RNA extraction and quantitative RT-PCR. For each cell line, the optimal number of cells were seeded as determined by previous cell density studies (between 8-15 X 10^3 cells/well). Either gemcitabine alone (final volume 100 μL/well) or gemcitabine plus ENT1 inhibitor (final volume 150 μL/well) was added to each well, and the cells were cultured for 72 hours at 37°C. The ENT1 inhibitors, NBMPR and dipyridamole, are not cytotoxic at the concentrations used. For each 96-well plate, one row of cells was left untreated and used as a control. Cytotoxicity determination was completed by adding 20% by volume (either 20 or 30 μL) of MTT labeling reagent to each well. After incubating for 5 hours, the media was removed from each well, and 100 μL isopropanol (made to 0.1 N with HCl) was added to each well. The plates were shaken gently at room temperature for 20 minutes to allow complete dissolution of crystals. Absorbance was measured with a microplate reader at 580 nm with a reference wavelength of 670 nm. In each experiment, cells were plated in quadruplicate for a given concentration. All experiments were repeated at least 3 times.

The actual absorbance for each well was calculated by subtracting the background absorbance from the reading ($A=A_{580}-A_{670}$). These values were then expressed as percent

of control (absorbance of untreated cell growth=100%). The log of each gemcitabine concentration was plotted versus % control and the curve was fitted using PRISM 4 to the equation:

$$\%\ \text{control} = \%\ \text{control}_{min} + \frac{\%\ \text{control}_{max} - \%\ \text{control}_{min}}{1+10\text{^}((\text{LogEC}_{50} - \text{Log[gem]})*\text{HillSlope})}$$

Where % control_{max} is the maximum value of the % control, % control_{min} is the minimum value of the % control, HillSlope is the value of the Hill slope coefficient, and EC_{50} is the gemcitabine concentration that results in half maximal cytotoxicity.

Real-Time Quantitative Reverse Transcriptase-PCR (RT-PCR). Cells were reserved from each MTT assay for RNA isolation. RNA from each cell line was isolated using a RNeasy kit (QIAGEN, Valencia, CA) according to the protocol. Reverse transcription was carried out using TaqMan Universal Master Mix (Applied Biosystems, Foster City, CA). Primer and probe sets for each gene were Assays-on-Demand purchased from Applied Biosystems (Foster City, CA). Reactions were run on an ABI Prism 7700 and cycling conditions were: 50°C for 2 min, 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. The expression of specific transcripts relative to the control gene, 18S, was calculated by the following formula: Relative expression ($X\ 10^{-6}$) = $2^{-\Delta Ct}$ where $\Delta Ct = (Ct_{target} - Ct_{18S})$.

Correlation Analysis between Gene Expression and Gemcitabine Sensitivity. The relationship between expression levels of nucleoside transporters and gemcitabine EC_{50} was analyzed using the SPSS 13.0 statistical package. A linear regression was performed on each variable (log median of transporter expression) alone vs. log median of gemcitabine EC_{50}. In addition, multiple regression analysis was used to evaluate the variability of EC_{50} that can be predicted using combinations of each variable.

Determination of the Pearson correlation for each transporter alone revealed that ENT1 was the only transporter that had an individual correlation between its expression level and gemcitabine EC_{50}. Therefore, all of the remaining multiple regression analyses contained ENT1 expression level as well as the expression level of at least one other nucleoside transporter (CNT1, CNT3, or both). In evaluating the degree to which an additional predictor significantly increases the amount of variability in EC_{50} that can be explained, the adjusted r^2 value was used. Adding predictors to a model will automatically improve the ability of the predictor to explain the dependent variable. The adjusted r^2 penalizes for each additional explanatory variable used. As such, the adjusted r^2 value is a more accurate estimate of the r^2 for the population.

Stable Expression of CNT3 in MDCK Cells. CNT3 was cloned by PCR amplification from a cDNA library made using the SuperScript First Strand cDNA Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) with human pancreas poly-A mRNA (Clontech, Palo Alto, CA) as template. Primers were designed to bases 89-114 (sense) and 2196-2170 (antisense) of the known mRNA sequence of human CNT3 (GenBank #AF305210). The PCR product was ligated into pGEM-T vector (Promega, Madison, WI) and then subcloned in-frame into pEGFP-C1 vector by adding a *Bgl II* site to the 5' end and a *Sal I* site to the 3' end. All plasmid construction and DNA sequences were confirmed by enzyme digestion and by automated sequencing at the Biomolecular Resource Center (BRC, UCSF, San Francisco, CA). MDCK cells were grown in MEM Eagle's media with Earle's BSS supplement, 5% heat inactivated FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere of 5% CO_2, 95% air at 37°C. Cells were transfected with 1 μg of CNT3-pEGFP-C1 or empty vector and 16

μg of Effectene (Qiagen, Valencia, CA). Cells were grown for 48 hours and then diluted into media supplemented with 700 μg/mL geneticin (Invitrogen, Carlsbad, CA). Clones were picked after two weeks growth in selection media and positive clones were chosen by Western blot, confocal microscopy, and functional uptake of ^{3}H-nucleoside.

[^{3}H]-Gemcitabine Uptake Assay. MPanc96, GFP-MDCK, and CNT3-MDCK cells were grown in their appropriate media. Cells were seeded at 5 X 10^{4} cells/well in 24-well Costar polycarbonate plates and grown for 24 hours. Uptake measurements were made as previously described. [8] Cells were exposed to 0.05 μM ^{3}H-gemcitabine (11 Ci/mMol, Moravek Biochemicals, Brea, CA) in sodium buffer (128 mM NaCl, 4.73 mM KCl, 1.25 mM, $CaCl_2$, 1.25 mM $MgSO_4$, 5 mM HEPES-Tris, pH 7.4) containing 0.5 μM unlabeled gemcitabine. Studies with ENT1 inhibitors also contained either 20 nM or 20 μM NBMPR in the reaction mix. Uptake of ^{3}H-gemcitabine was measured at room temperature after a 30-minute incubation for MPanc96 cells or a 60-minute incubation for MDCK cells. Cells were then rinsed three times in ice-cold Na^{+}-free buffer (128 mM Choline Chloride, 4.73 mM KCl, 1.25 mM, $CaCl_2$, 1.25 mM $MgSO_4$, 5 mM HEPES-Tris, pH 7.4). Cells were lysed in 500 μl of 1% SDS, and radioactivity was measured using a Beckman scintillation counter. Cells were plated in triplicate; each value represents mean ± standard error. Each experiment was carried out on two to three different passages of each cell line.

4.3 Results

Sensitivity of pancreatic cancer cells lines to gemcitabine. We examined a panel of 16 pancreatic cancer cell lines to determine the sensitivity of each cell line to gemcitabine. Sensitivity was determined by performing MTT cytotoxicity assays on

these cell lines to determine the concentrations of gemcitabine that inhibited cell growth by 50% (EC_{50}). In the experiments summarized in Table 4.1, cells were exposed to a range of gemcitabine concentrations for 72 hours and growth was then quantified via the MTT assay (see Material and Methods). Since preliminary studies suggested that EC_{50} values may vary with cell passage, each cell line was tested at least three times and the median value is reported. We found a wide range in the median EC_{50} values of the 16 pancreatic cancer cell lines with approximately 1400 fold difference between the most sensitive and the most resistant cell line (Table 4.1). This range is even more dramatic when each individual experiment is considered, in which case we observed a 10,000 fold difference in EC_{50} values between the most sensitive and the most resistant cell line.

Nucleoside transporter mRNA expression in pancreatic cancer cell lines. We examined the mRNA expression level of ENT1, CNT1 and CNT3 in a panel of 16 pancreatic cancer cell lines by quantitative RT-PCR. There is a wide degree of variability of nucleoside transporter expression both between and within cell lines (Fig. 4.1). Some cell lines, such as Panc 8.13 and MPanc96, expressed high levels of all three nucleoside transporters when compared across the cell lines studied. However, L.3.3, L.3.6 pL, and SW1990 cells expressed comparatively lower mRNA levels of all three nucleoside transporters. Many cell lines, such as Panc1, expressed predominantly or exclusively ENT1. In contrast a number of cell lines expressed the concentrative nucleoside transporters predominantly. MiaPaCa2 expressed CNT1 exclusively (Fig. 4.1). Finally, some cell lines expressed both concentrative and equilibrative nucleoside transporters. For example, MPanc96 cells express appreciable levels of ENT1, CNT1, and CNT3.

Table 4.1 *Effect of the equilibrative nucleoside transporter inhibitor NBMPR on the cytotoxicity of gemcitabine in a panel of pancreatic cancer cell lines*

Cell line	Median EC_{50}* (nM)		RI†
	gemcitabine	+ NBMPR	
Panc 2.03	42 (21-52)	289 (80-750)	6.9
Panc 2.13	11 (3.1-11)	46 (13-52)	4.4
Panc 3.27	11 (1.8-21)	13 (4.1-32)	1.2
Panc 5.04	20 (4.1-63)	291 (11.1-2800)	14.4
Panc 8.13	2002 (76-7800)	3780 (1714-10400)	1.9
Panc 10.05	1188 (380-1700)	2003 (1660-2300)	1.7
CaPan2	812 (480-2900)	2054 (1070-11200)	2.5
HPAF II	**7.4 (3.4-13)**	**4.1 (1.4-13)**	**0.6**
L.3.3	31 (4.7-390)	65 (8.3-710)	2.1
L.3.6 pL	2.6 (1.8-2.9)	3.9††	1.5††
L.3.6 sL	1.5 (1.2- 2.6)	1.5 (0.7- 2.6)	1.0
MiaPacCa2	35 (23- 40)	41 (40- 60)	1.2
MPanc96	**2088 (450- 12200)**	**862 (130- 5200)**	**0.4**
Panc1	310 (170- 610)	9000 (6400 - 9900)	29.1
SU86.86	26 (14- 150)	140 (120- 350)	5.4
SW1990	21 (9.2- 35)	39 (20- 59)	1.9

* The EC_{50} value was determined for each cell line after exposure to a series of drug concentrations with/without 20 nM NBMPR. As EC_{50} values varied with cell passage, the median EC_{50} and range of observed EC_{50}s are given. Each cell line was tested at least three times unless otherwise noted.

† Resistance Index : EC_{50} with inhibitor / EC_{50} without inhibitor.

†† L.3.6 pL was tested once with 20 nM NBMPR, therefore EC_{50} range is not given. However, a similar resistance index was achieved at higher concentrations of NBMPR (RI=1.2–1.5).

Cell lines that became more sensitive to gemcitabine with NBMPR treatment are in bold.

All cell lines are available through ATCC with the exception of L.3.3, L.3.6 pL, L.3.6 sL, and MPanc96.

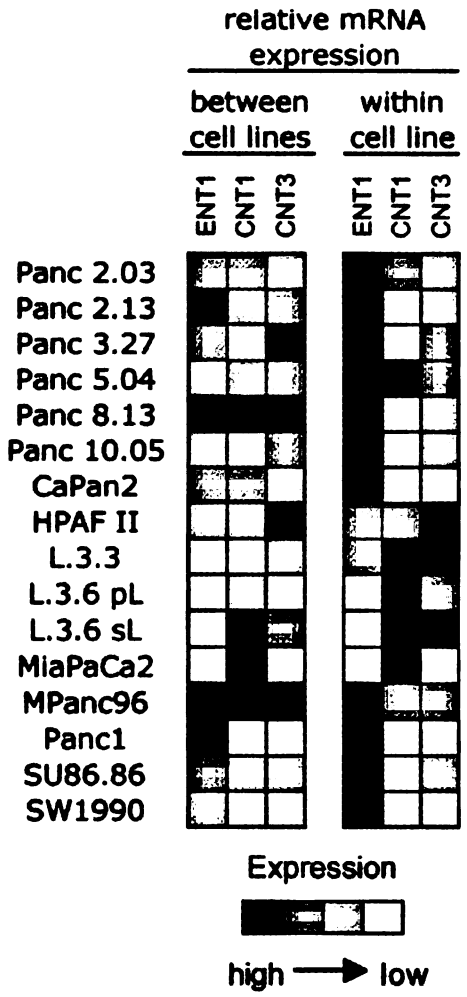

Figure 4.1 Expression profile of pancreatic cancer cell lines. ENT1, CNT1 and CNT3 mRNA expression levels were determined by quantitative RT-PCR as described in the Materials and Methods. The expression of each transporter is shown as belonging to either the first (□), second (▨), third (■), or fourth (■) quartile between and within cell lines. As shown in the between cell line panel, the cell lines L.3.3, L.3.6 pL, and SW1990 had lower levels of all three nucleoside transporters while Panc 8.13 and MPanc96 had higher levels of all three transporters. In addition, there was relative nucleoside transporter expression within each cell line. For example, Panc1 cells express exclusively ENT1 while MiaPaCa2 cells express exclusively CNT1. In contrast, MPanc96 cells express ENT1, CNT1, and CNT3.

Relationship between nucleoside transporter expression level and gemcitabine sensitivity in pancreatic cancer cell lines. To assess the relationship between sensitivity to gemcitabine and expression level of nucleoside transporters, the median log EC_{50} value versus the median log mRNA transporter expression for each cell line was plotted and a Pearson's correlation coefficient was calculated (Table 4.2 and Fig. 4.2A). We found a significant positive relationship between ENT1 expression level and EC_{50} (Fig. 4.2A). That is, higher ENT1 expression levels were associated with higher EC_{50} values, and thus greater resistance to gemcitabine. These data suggest that ENT1 contributes to resistance rather than sensitivity to gemcitabine. No significant correlations between the expression level of either CNT1 or CNT3 and EC_{50} values were observed (Table 4.2).

We also performed multiple regression analysis to evaluate the ability of each nucleoside transporter expression level to predict the variance in EC_{50}. This analysis revealed that, although ENT1 expression level is responsible for the majority of the variance in EC_{50}, the analysis is statistically improved by the addition of CNT1 expression level as a predictor (ENT1 alone, adjusted $r^2 = 0.41$; ENT1 and CNT1, adjusted $r^2 = 0.45$; $p=0.043$) (Table 4.2). Approximately 41% of the variance in EC_{50} can be predicted using ENT1 while 45% of the variance can be explained using both ENT1 and CNT1. The multiple regression analysis showed no such increase in prediction when CNT3 was considered along with ENT1 (ENT1 alone, adjusted $r^2 = 0.41$; ENT1 and CNT3, adjusted $r^2 = 0.40$; $p=0.73$). Finally, the combination of all three nucleoside transporters did not add any additional predictive ability over using just ENT1 and CNT1 (ENT1 and CNT1, adjusted $r^2 = 0.45$; ENT1, CNT1 and CNT3, adjusted $r^2 = 0.44$; $p=0.51$). Two cell lines, Panc 5.04 and Panc 8.13, did not generate reproducible EC_{50}

Table 4.2 *Correlation between nucleoside transporter RNA expression and gemcitabine sensitivity in a panel of pancreatic cancer cell lines*

Transporter	R	R Square	Adjusted R Square	p
ENT1	**0.65**	**0.42**	**0.41**	**<0.0001**
CNT1	0.07	0.01	-0.02	0.612
CNT3	0.25	0.06	0.04	0.078
ENT1 & CNT1	**0.69**	**0.47**	**0.45**	**<0.0001**
ENT1 & CNT3	0.65	0.43	0.40	<0.0001
ENT1, CNT1, & CNT3	0.69	0.48	0.44	<0.0001

Pancreatic cancer cells were tested for gemcitabine sensitivity (EC_{50}) and for nucleoside transporter RNA expression across several passages per cell line. The median log EC_{50} values were plotted vs. the median log transporter expression (weighted for number of experiments) and the data was analyzed to determine the Pearson correlation coefficient.

ENT1 was the only single transporter to show a correlation to gemcitabine EC_{50}. We then performed multiple regression analysis to determine whether additional factors would improve the correlation. For this analysis, the adjusted R squared value should be used. The R squared value is the proportion of the variance in EC_{50} that can be predicted from the independent variables (expression). The adjusted R squared penalizes for each additional explanatory variable used.

Analyses with significant correlation, or that improve the correlation, to EC_{50} values are shown are in bold.

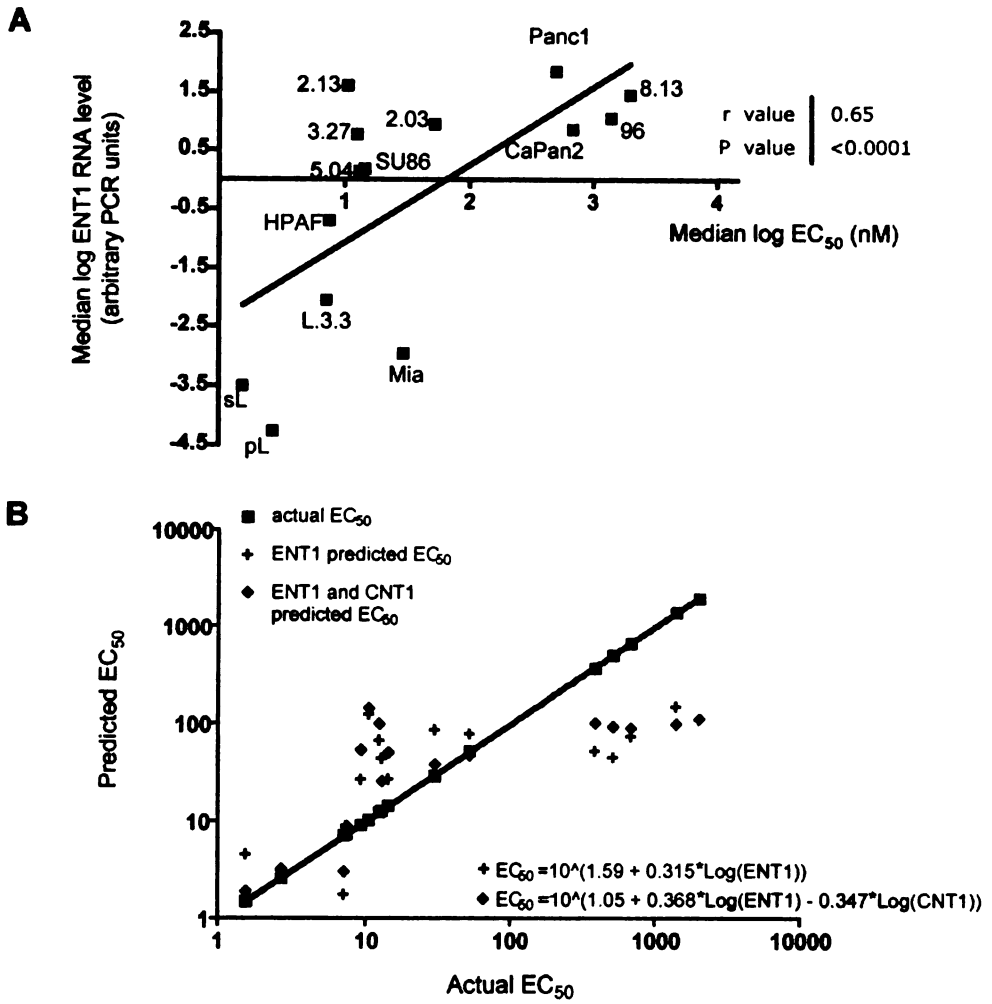

Figure 4.2 Correlation between potency of gemcitabine and nucleoside transporter expression. The median log EC_{50} values were plotted vs. the median log transporter expression (weighted for number of experiments) for each cell line and this data was analyzed to determine the Pearson's correlation coefficient. Each experiment was carried out in at least two different passages of the cell line. (A) A significant correlation was found for the expression of ENT1 and EC_{50} values. There was no correlation between either CNT1 or CNT3 expression and EC_{50} values. The Pearson's correlation and significance for each transporter is given in Table 4.2. (B) The relationship between expression levels of nucleoside transporters and gemcitabine EC_{50} was analyzed as described in the Materials and Methods. The results from this analysis showed that there was a correlation between ENT1 expression and gemcitabine EC_{50}. Furthermore, this correlation was improved when ENT1 and CNT1 were both considered as variables. The actual EC_{50} values obtained experimentally were graphed vs. actual EC_{50} (■), vs. predicted EC_{50} values using only ENT1 (✚), and vs. predicted EC_{50} values using ENT1 and CNT1 (◆). In general, the addition of the CNT1 term to the EC_{50} equation improves the predicted result.

values. There was little change in the overall analysis without these two cell lines (ENT1 alone, adjusted $r^2 = 0.40$; ENT1 and CNT1, adjusted $r^2 = 0.44$), although the predictive ability of adding CNT1 to the model is now significant at a level of $p=0.068$.

The equations that these analyses generated for the prediction of EC_{50} as a function of nucleoside transporter expression level are shown in Fig. 4.2B. We used these equations to generate a predicted EC_{50} value for each cell line and compared these to the actual experimental values. In general, using both the ENT1 and the CNT1 term to calculate the EC_{50} improved the predicted result. That is, these values were closer to the actual values obtained than the values that were predicted using only ENT1. This model shows an inverse relationship between the slopes associated with ENT1 and CNT1 expression levels. That is, high ENT1 and low CNT1 expression levels predict greater resistance to gemcitabine.

Effect of ENT1 inhibitors on gemcitabine cytotoxicity in pancreatic cancer cells lines. We determined the sensitivity of each cell line to gemcitabine in the presence of the ENT1 inhibitor NBMPR. We found that there were three classes of response to inhibition with NBMPR, expressed as the resistance index (RI = EC_{50} with inhibitor/EC_{50} without inhibitor) (Table 4.1). Some cell lines became more resistant when ENT1 was inhibited (RI greater than 2). Many cell lines showed no difference in sensitivity when treated with NBMPR (RI≈1). Most interestingly, two cell lines, HPAF II and MPanc96, became more sensitive to gemcitabine when treated concomitantly with ENT1 inhibitors (RI less than 1).

Effect of clinically approved ENT1 inhibitor dipyridamole on gemcitabine sensitivity. Based on the effect of NBMPR on gemcitabine sensitivity (RI greater than 2,

RI≈1, and RI less than 1) we selected three pancreatic cancer cell lines to test the effects of the clinically approved nucleoside transporter inhibitor, dipyridamole. The sensitivity of MiaPaCa2 cells was unaffected by NBMPR (RI = 1.2); Panc1 cells became more resistant to gemcitabine in the presence of NBMPR (RI = 29.1); and NBMPR enhanced the sensitivity of Mpanc96 to gemcitabine (RI = 0.4). Each of these cell lines respond to the clinically approved ENT1 inhibitor, dipyridamole, in a similar fashion as they did to NBMPR (Fig 4.3). In this study, the MTT assay was performed with cells treated with gemcitabine alone, gemcitabine plus NBMPR (20 nM), or gemcitabine plus dipyridamole (200 nM). The top panel (Fig. 4.3A) shows the effect of these inhibitors on MiaPaCa2 cells ($EC_{50,Gem}$ = 42 nM, $EC_{50,NBMPR}$ = 33 nM, and $EC_{50,Dip}$ = 39 nM). Fig. 4.3B shows the inhibitory effects of ENT1 inhibitors on Panc1 cells ($EC_{50,Gem}$ = 380 nM, $EC_{50,NBMPR}$ = 14200 nM, and $EC_{50,Dip}$ = 4800 nM). MPanc96 cells were made more sensitive to gemcitabine by the addition of either NBMPR or dipyridamole (Fig. 4.3C, $EC_{50,Gem}$ = 1044 nM, $EC_{50,NBMPR}$ = 125 nM, and $EC_{50,Dip}$ = 337 nM).

Gemcitabine resistance mediated through ENT1 efflux transport. The cell line MPanc96 became more sensitive to gemcitabine by inhibiting ENT1 with either the classical ENT1 inhibitor NBMPR or with dipyridamole, a clinically approved ENT1 inhibitor (Fig. 4.3C). We investigated whether this increased sensitivity to gemcitabine was due to the ability of ENT1 to act as an efflux transporter. Consistent with ENT1 acting as an efflux pump, we found that MPanc96 cells treated with NBMPR reached a significantly higher intracellular concentration of ^{3}H-gemcitabine than MPanc96 cell without NBMPR ($p<0.01$, Fig. 4.4A). As proof of concept, we performed experiments in MDCK cells stably transfected with the concentrative nucleoside transporter CNT3

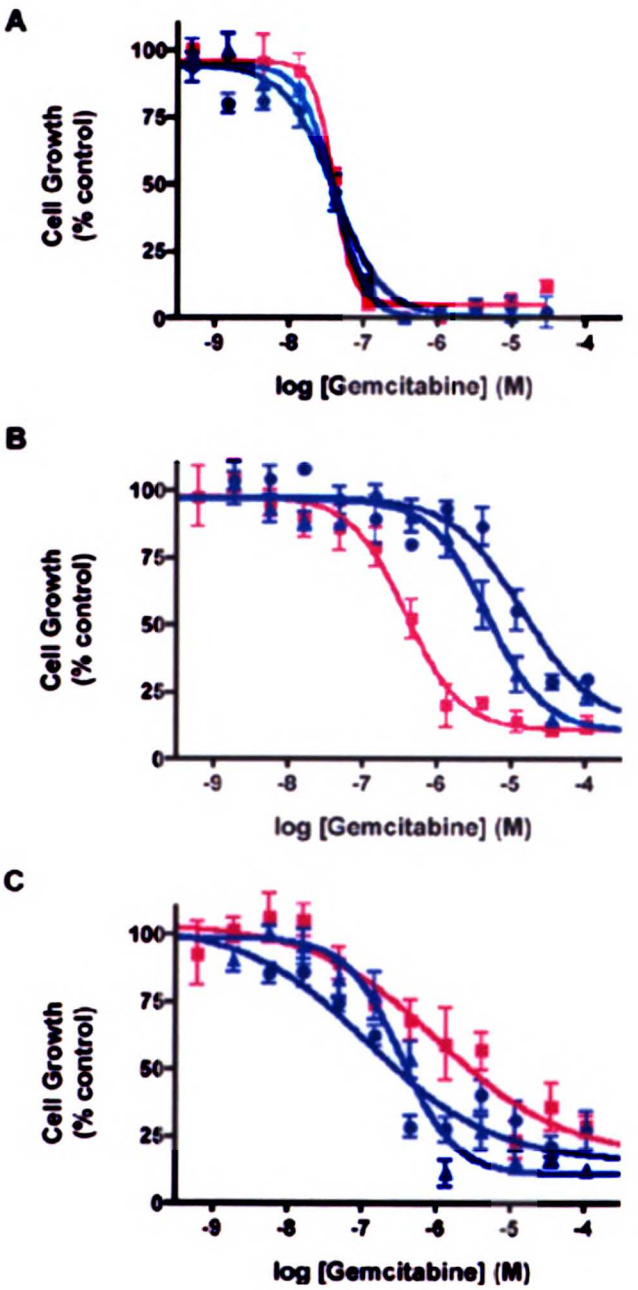

Figure 4.3 Effect of ENT1 inhibitors on gemcitabine cytotoxicity in three representative cell lines. Effect of ENT1 inhibitors on gemcitabine cytotoxicity in (A) MiaPaCa2, (B) Panc1, and (C) MPanc96 cell lines. Data shown are representative experiments of the MTT *in vitro* growth assays performed on each pancreatic cancer cell line. Cells were exposed to a range of gemcitabine concentrations – 6.3 pM to 30 μM (MiaPaCa2) and 0.63 nM to 3 mM (Panc1 and MPanc96). Cells were treated with gemcitabine alone (■), with gemcitabine plus 20 nM NMBPR (●) or with gemcitabine plus 200 nM dipyridamole (▲). Each concentration was tested in quadruplicate; points represent the mean ± standard error.

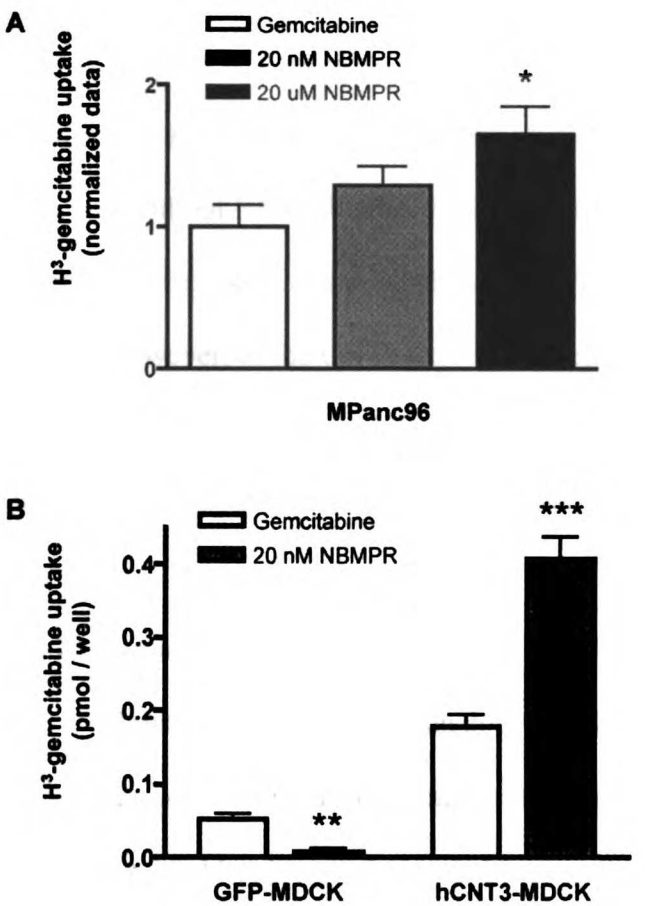

Figure 4.4 Uptake of ^{3}H-gemcitabine in cells expressing both equilibrative and concentrative nucleoside transporters. (A) Effect of ENT1 inhibitor on ^{3}H-gemciatbine uptake in MPanc96 cells. Uptake was measured with gemcitabine alone (white bar), with 20 nM NBMPR (gray bar), or with 20 μM NBMPR (black bar). For each condition, the data has been normalized to the uptake with gemcitabine alone. Uptake of ^{3}H-gemcitabine (0.05 μM ^{3}H- gemcitabine plus 0.5 μM unlabeled gemcitabine) was measured at room temperature after a 30-minute incubation. Cells were plated in triplicate; each value represents mean ± standard error. *, $P < 0.01$ (significantly different from gemcitabine alone). (B) Uptake of ^{3}H-gemcitabine in stable MDCK cells expressing endogenous ENT1 and either GFP-tagged empty vector or GFP-tagged CNT3. Uptake was measured with gemcitabine alone (white bar), or with 20 nM NBMPR (gray bar). Uptake of ^{3}H-gemcitabine (0.05 μM ^{3}H- gemcitabine plus 0.5 μM unlabeled gemcitabine) was measured at room temperature after a 60-minute incubation. Data shown is representative of experiments carried out on two to three different passages of each cell line. Cells were plated in triplicate; each value represents mean ± standard error. **, $P < 0.001$; ***, $P < 0.0005$ (significantly different from gemcitabine alone).

tagged with GFP. Previous studies in this lab show that the GFP tag does not interfere with the activity of concentrative nucleoside transporters. [8, 9] MDCK cells expressing endogenous ENT1 and stably transfected with either empty vector or CNT3 were tested with and without NBMPR (Fig. 4.4B). In cells expressing only ENT1 (GFP-MDCK), gemcitabine uptake was inhibited by NBMPR, consistent with ENT1 acting as an influx pump. However, in the CNT3 expressing cells (GFP-CNT3), gemcitabine uptake was increased by the addition of NBMPR, demonstrating that in the presence of concentrative nucleoside transporters, ENT1 acts as an efflux pump.

4.4 Discussion

Unlike many nucleoside analogs which mainly show activity in treating various leukemias, gemcitabine is a potent cytotoxic agent with a broad spectrum of activity in pancreatic, bladder, lung, ovarian and breast cancer. [10] Although gemcitabine has been shown to prolong survival in patients with early and late stages of pancreatic cancer [1], great variation in response exists to gemcitabine therapy, including tumors that are intrinsically resistant to the drug. [11-13] Determining the underlying causes for such resistance and optimizing gemcitabine therapy are critical in improving treatment of patients with pancreatic cancer.

Gemcitabine, 2', 2'-difluorodeoxycytidine, is a hydrophilic prodrug and as such must be transported into the cell before it can be phosphorylated into the active gemcitabine di- and tri-phosphate compounds. Nucleoside transporters have been shown to mediate this first step in the gemcitabine activation pathway. [4] Gemcitabine is transported specifically by the equilibrative transporter, ENT1, and by the concentrative

transporters, CNT1 and CNT3. The relative affinities (Km) of gemcitabine in oocytes expressing CNT1 and ENT1 are 24 μM and 160 μM, respectively. [2] Preliminary data in our laboratory suggest that gemcitabine also interacts with CNT3 (K_i = 100 μM). Gemcitabine is not transported by CNT2, a purine-selective transporter, and only very weakly interacts with ENT2 (Km = 750 μM). [2] Therefore, we focused on CNT1, CNT3, and ENT1 due to the relative affinities of these transporters for gemcitabine and the fact that all three are present in normal pancreas (unpublished data in this laboratory) and in pancreatic cancer cell lines.

We examined a panel of 16 pancreatic cancer cell lines to determine their sensitivity to gemcitabine and whether sensitivity was associated with expression of nucleoside transporters. Our studies revealed three major findings. First, there is a great degree of variation in response to gemcitabine across these cell lines. Second, in marked contrast to previous studies, we observed that ENT1 expression level was associated with reduced gemcitabine sensitivity ($r = 0.65$, $p<0.0001$) and that the expression levels of ENT1 and CNT1 were the best predictors of gemcitabine cytotoxicity (multiple regression analysis, $r = 0.69$, $p<0.0001$). Third, we observed that certain cell lines became more sensitive to gemcitabine after inhibiting the activity of the equilibrative transporter, ENT1. In these cell lines both equilibrative and concentrative nucleoside transporters were expressed. We propose a model in which equilibrative and concentrative nucleoside transporters work in concert to modulate the intracellular concentrations of gemcitabine. In this model, when equilibrative and concentrative nucleoside transporters are both present, ENT1 acts primarily as an efflux pump, extruding gemcitabine from the cell and lowering the intracellular concentrations of the

drug (Figure 4.5). This model was validated in a stably transfected cell line (Figure 4.4B). Below we discuss each of our findings.

Consistent with the wide variation in patient response to gemcitabine, numerous studies have reported a wide range of sensitivities to gemcitabine in various cell lines. In a study of 10 human and murine leukemic and lymphomic cell lines expressing or lacking nucleoside transporters, Mackey *et al.* found a ~ 3200 fold difference in gemcitabine sensitivity. However, the general applicability of these studies to pancreatic cancer is questionable since results obtained in leukemic and lymphomic cell lines, which are not derived from epithelia, may not be applicable to adenocarcinomas such as pancreatic cancer. In our study of 16 pancreatic cancer cell lines we found that there was a 1400 fold difference in median EC_{50} values (median values of 1.5 – 2088 nM). In addition, depending on the passage, there was ~ 10,000 fold difference between the maximum and minimum EC_{50} (range of 1.23 - 2.58 nM for the most sensitive cell line and range of 100 - 12,000 nM for the most resistant cell line). Our data extend the observations of Rauchwerger *et al.*, who found an approximately 9000 fold difference in gemcitabine EC_{50} in three pancreatic cancer cell lines. [14]

Many studies have concluded that ENT1 is the primary nucleoside transporter responsible for sensitivity to chemotherapeutic nucleoside analogs such as cytarabine, cladribine, and fludarabine. For example, White *et al.* found that decreased ENT1 activity correlated with cytarabine resistance in acute myelogenous leukemia cells. [15] Wiley *et al.* and Gati *et al.* determined that resistance to cytarabine therapy was associated with a decrease in ENT1 activity in human leukemia cells. [16, 17] However, these studies have largely relied on leukemic cell lines, which express primarily ENT1

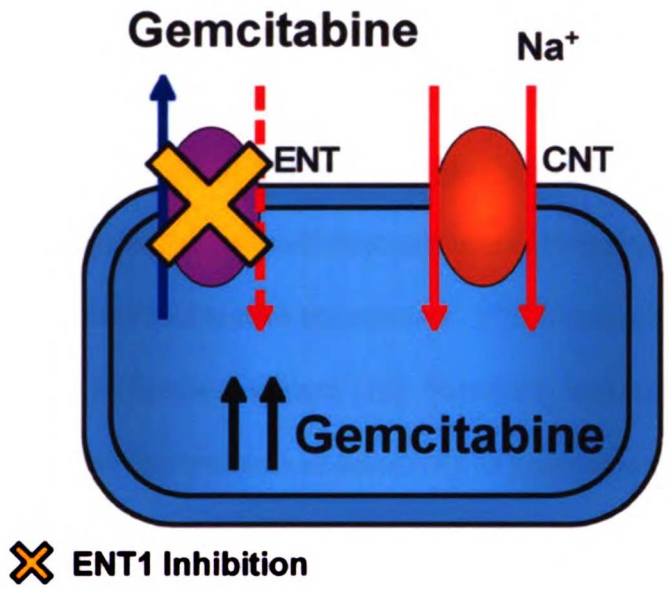

Figure 4.5 Schematic of a cell expressing both equilibrative and concentrative nucleoside transporters. In cells expressing both ENT1 and CNT (either CNT1, CNT3, or both) ENT1 can act predominantly as an efflux transporter. In these cells, blocking ENT1 with an inhibitor reduces the export of gemcitabine from the cell and increases the intracellular concentration of gemcitabine.

and two transporters which only poorly interact with gemcitabine, ENT2 and CNT2. [2, 18, 19] Molina-Arcas *et al.* observed abundant ENT1, ENT2, and CNT2 expression in normal leukocytes and in leukemic cell lines. However, CNT3 displayed variable expression across leukemic cell lines and was only expressed at very low levels when compared to the equilibrative transporters. CNT1 was not found in either normal leukocytes or leukemic cell lines. [18] Therefore, leukemias are not appropriate models for studying the determinants of sensitivity of pancreatic cancer to gemcitabine.

In our panel of pancreatic cancer cell lines, we found that higher ENT1 expression was associated with higher EC_{50} values, and thus greater resistance to gemcitabine. No significant correlation between either CNT1 or CNT3 expression and EC_{50} values was observed (Table 4.2, Fig. 4.2A). We also performed multiple regression analysis to evaluate the degree to which each nucleoside transporter expression level explained the variation in EC_{50}. As shown in Fig. 4.2B, we found that the most appropriate model for the prediction of gemcitabine EC_{50} used both ENT1 and CNT1 expression as predictors. This model predicts an inverse relationship between ENT1 and CNT1. That is, cell lines that had the highest expression of CNT1 relative to ENT1 expression were the most sensitive to gemcitabine. Although 45% of the variance in EC_{50} can be explained using both ENT1 and CNT1, it is important to note that there is at least one other unknown factor, and likely multiple factors, needed to account for the remaining 55% of the variance (see Fig. 4.2B). Increased resistance to gemcitabine has previously been associated with low expression of deoxycytidine kinase, high expression of cytidine deaminase, and high expression of ribonucleoside reductase. [20-22] It is possible that expression levels of these enzymes could help explain unaccounted variation in EC_{50}.

We found that the differential transporter expression patterns in cell lines were often predictive of the sensitivity to gemcitabine when treated concomitantly with the ENT1 inhibitor NBMPR (Table 4.1, Fig. 4.1). Notably, the cell lines HPAF II and MPanc96, which expressed relatively similar levels of equilibrative and concentrative transporters were more sensitive to gemcitabine treatment when equilibrative transport was blocked. We were intrigued by the increased sensitivity of these cells, as this response had not been previously reported in the literature. A study by Mackey *et al.* explored this type of response in CaCo-2 cells that had been shown to express both ENT1 and CNT3. [4] In their study gemcitabine sensitivity of CaCo-2 cells was unaffected by both NBMPR and dipyridamole treatment. Mackey *et al.* therefore concluded that efflux via ENT1 was a minor component of gemcitabine sensitivity since ENT1 inhibition did not cause CaCo-2 cells to become more sensitive to gemcitabine. However, in our studies, we found that response to ENT1 inhibition was predicated on sufficient expression of concentrative nucleoside transporter. In this case, nucleosides such as gemcitabine will accumulate against the concentration gradient; thus equilibrative transporters will dissipate this gradient and act as efflux pumps. As such, we further investigated ENT1 inhibition in the MPanc96 cell line with radio-labeled gemcitabine, which showed greater intracellular accumulation with increasing concentrations of inhibitor (Fig. 4.4A). In addition, as a proof of concept, MDCK cells stably transfected with CNT3 were inhibited with NBMPR. These cells also showed a greater accumulation of radio-labeled gemcitabine when inhibited (Fig 4.4B)..

Based on our studies, we propose a model diagramed in Fig. 4.5. If ENT1 and one or both of the concentrative nucleoside transporters are present in the cell at

sufficient levels, CNTs act as influx transporters, driving gemcitabine into the cell whereas ENT1 acts primarily as an efflux transporter. In these cases inhibition of ENT1 reduces the export of gemcitabine and leads to a greater accumulation of gemcitabine and increased cytotoxicity. It is possible then, that a gemcitabine/ENT1 inhibitor schedule could be used to treat patients whose tumors express both concentrative and equilibrative nucleoside transporters. To investigate the possibility of using an ENT1 inhibitor clinically, we tested the ENT1 inhibitor, dipyridamole, which has already been approved for use in patients. [23] We found that dipyridamole acted in the same manner as NBMPR, inducing greater sensitivity in cells that express both ENT1 and CNTs. These data suggest that in certain patients, dipyridamole could be used to enhance gemcitabine cytotoxicity thereby improving treatment efficacy.

Our data shows that sensitivity to gemcitabine is positively associated with CNT1 expression and negatively associated with ENT1 expression. In addition, we have shown for the first time that cells expressing ENT1 and sufficient levels of either CNT1 or CNT3 can be made more sensitive to gemcitabine with the inhibition of the equilibrative transporter. Both of these findings support our model that ENT1 acts predominantly as an efflux transporter in the presence of concentrative nucleoside transporters. It is interesting to note that gene variants of ENT1, CNT1, and CNT3 have been reported and that some of these variants alter transport function. [24-27] Understanding the expression levels as well as the genetic variants of nucleoside transporters in tumors will help optimize therapy. Further, the use of equilibrative nucleoside transporter inhibitors may be of value to certain patients with pancreatic cancer.

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CHAPTER 5

FURTHER STUDIES AND SUMMARY

5.1 Introduction

As discussed in Chapter 1 of this dissertation, numerous genes in the activation pathway of gemcitabine have been implicated in resistance to nucleoside analogs. Increased expression of cytidine deaminase (CD) and decreased expression of deoxycytidine kinase (dCK) have been associated with cytarabine and gemcitabine resistance. [1-6] The studies presented in Chapter 4 suggest that the relative expression of nucleoside transporters may be an important determinant of resistance or sensitivity to gemcitabine. Gemcitabine exerts its cytotoxic effects primarily through inhibition of DNA synthesis. Gemcitabine is metabolized intracellularly to dFdCTP, its active compound, which is incorporated into elongating DNA. This misincorporation initiates apoptotic responses designed to remove damaged cells. [7, 8] Therefore, sensitivity to gemcitabine depends not only on gemcitabine activation but also on properly functioning proliferation and apoptosis pathways.

Cellular alterations of the proliferation and/or apoptosis pathways are often responsible for the formation and progression of tumor cells, as well as their subsequent resistance to chemotherapeutics. [9, 10] The inability of a cell to undergo apoptosis is a critical mechanism of drug resistance. Numerous genes have been implicated in apoptosis dependent gemcitabine resistance, including signaling, cell cycle, and apoptosis genes. Transcription factors and extracellular matrix (ECM) proteins also appear to play a role in the resistance of pancreatic cancer to gemcitabine. The goal of the following

studies was to uncover genes that may be important determinants of response to gemcitabine therapy in pancreatic cancer. To this end we analyzed mRNA expression levels, generated by Stephan Gysin, of the pancreatic cancer cell lines examined in Chapter 4. We used the Significance Analysis of Microarrays or SAM program to determine genes that were differentially expressed in sensitive cell lines compared to resistant cell lines. We were particularly interested in differentially expressed genes that are involved in the gemcitabine activation pathway or in genes that affect the cell's ability to undergo gemcitabine induced apoptosis. The 16 pancreatic cancer cell lines were classified as resistant or sensitive to gemcitabine based on cytotoxicity studies presented in Chapters 3 and 4.

When considering genes that may be involved in sensitivity or resistance to gemcitabine, it is important to realize that several essential genes are misregulated in the majority of pancreatic cancer adenocarcinomas. Most notable of these are mutations in *p53* and *K-Ras*, and the constitutive activation of NF-κB. The tumor suppressor gene *p53* is inactivated in 40-75% of pancreatic ductal adenocarcinomas (PDACs). [11, 12] *K-Ras* activation mutations have been reported in 70-100% of PDACs; this is the highest incidence of *K-Ras* mutations in any human tumor type. [11, 13-15] The negative regulator of apoptosis NF-κB is constitutively activated in 70% of PDAC. [16] These aberrations act in concert in pancreatic cancer to prevent cell death and promote tumor growth, metastasis, and resistance to therapy.

Two pathways are present by which cells can induce apoptosis, the extrinsic and intrinsic apoptosis pathways (Fig. 5.1). Both pathways depend on the activation of cysteine-aspartic acid proteases, or caspases. The extrinsic pathway is governed by death

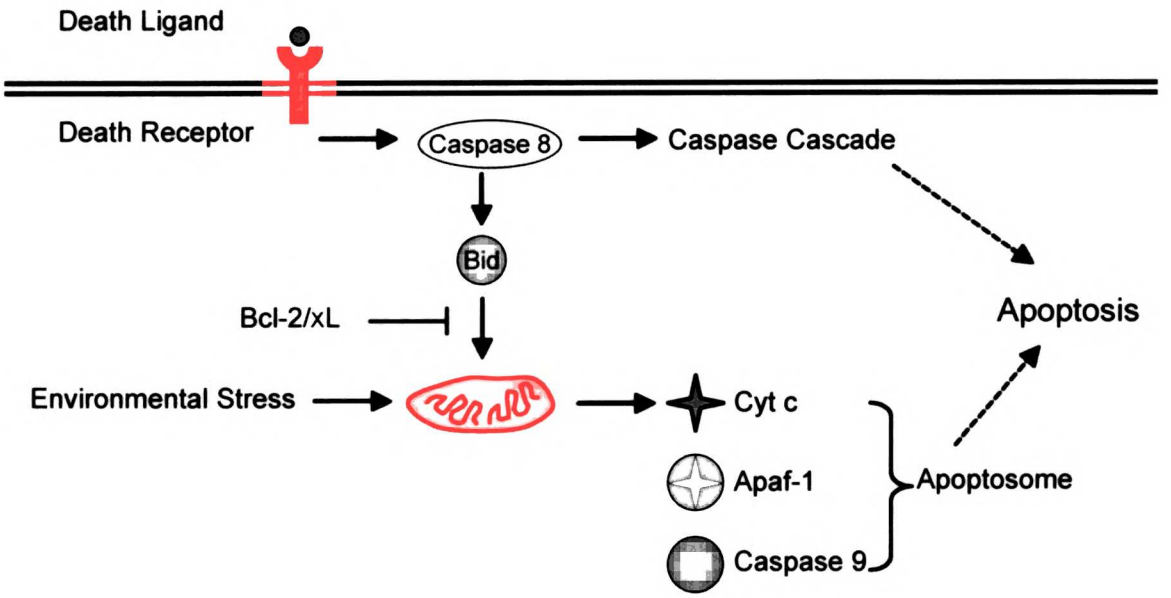

Figure 5.1 Extrinsic and intrinsic apoptosis pathways. The extrinsic apoptosis pathway is initiated upon ligation of one of the death ligands (e.g. TNF-α, TRAIL, or FasL) to the corresponding death receptor. This activates caspase 8 which initiates a caspase cascade resulting in apoptosis. The intrinsic pathway is initiated when cytochrome c (Cyt c) is released from mitochondria following environmental stress such as the removal of growth factors or cellular detachment from the extracellular matrix. Cytosolic cytochrome c, apoptotic protease factor 1 (APAF-1), and caspase 9 form a complex called the apoptosome which initiates an apoptotic caspase cascade. The Bcl-2 family member Bid acts as a link between the to pathways. Caspase 8 activates Bid which then releases cytochrome c into the cytosol. This action is inhibited by the anti-apoptotic proteins Bcl-2 and Bcl-XL.

receptors and their corresponding ligands TNF-α, TRAIL, and FasL. [17-19] Caspase-8 is activated upon death receptor and ligand binding, which then initiates a caspase cascade that induces apoptosis. The intrinsic pathway is regulated by the Bcl-2 family of pro- and anti- apoptotic genes and the permeabilization of the mitochondrial membrane. [18, 20, 21] Cytochrome *c* is released from the mitochondria following the opening of the permeability transition pore (PTP). Cytosolic cytochrome *c* activates caspase-9, and initiates an apoptotic caspase cascade. Although mitochondria permeability is regulated by the Bcl-2 family members Bcl-2 and Bcl-xL, release of cytochrome *c* can also be triggered by the removal of growth factors or by cell detachment from the extracellular matrix (ECM). The Bcl-2 family member Bid is considered a link between the extrinsic and intrinsic pathways. [18, 20, 21] In addition to its role in the extrinsic pathway, caspase-8 also activates Bid which then releases cytochrome *c* from mitochondria and initiates apoptosis. Bid activation is inhibited by Bcl-2 and Bcl-xL. Aberrant regulation and/or activation of the extrinsic and intrinsic pathways have an important impact on gemcitabine sensitivity.

Wild-type *p53* plays an essential role in cell cycle regulation, senescence, and apoptosis. However, human cancers frequently have a mutated form of *p53* (m*p53*) which has lost tumor suppressor activity. [22] *p53* regulates both the death receptor and mitochondria apoptosis pathways by activating several pro-apoptotic genes such as Fas, Bax, and Apaf1. [23-26] Tolis *et al.* determined that in lung cancer cells with wt*p53*, exposure to gemcitabine caused cells to accumulate in the G1 phase and induced apoptosis. However, cells with m*p53* had no G1 accumulation and no subsequent apoptosis. [27] Similar results have been observed in colon and breast cancer cells. [28,

29] Pancreatic cancer cell lines with reintroduced wt *p53* displayed increased sensitivity to gemcitabine. [30, 31]

K-Ras activates the Raf/MEK/ERK (MAPK) kinase signaling pathway leading to the activation of numerous transcriptional factors and cell proliferation. [32] In CaPan-1 pancreatic cancer cells, inhibition of Ras inhibited cell growth through apoptosis induction. [33] The K-Ras inhibitor FTS (*S*-trans,*trans*-farnesylthisalicylic acid) has been shown to induce gemcitabine sensitivity in pancreatic and colon cancer cell lines previously resistant to gemcitabine. [34]

NF-κB is a transcriptional regulator and a negative regulator of apoptosis. NF-κB is activated by various cytokines and growth factors to promote proliferation by increasing the expression of apoptosis inhibiting genes. NF-κB activation stimulates the expression of FLIP (FLICE inhibitory protein) which inhibits the caspase-dependent apoptosis pathway. Activation of NF-κB also increases the expression of Bcl-xL and Bcl-2, both inhibitors of *p53* mediated apoptosis. Constitutive activation of NF-κB contributes to the resistance of pancreatic cancer to cell death. Inhibition of NF-κB stimulates apoptosis in pancreatic cancer cells by inhibiting FLIP, Bcl-2, and Bcl-xL. [35, 36] Furthermore, inhibition of NF-κB sensitizes pancreatic cancer cell lines to gemcitabine induced apoptosis. [37]

In chapters 3 and 4 we determined the gemcitabine sensitivity of 16 pancreatic cancer cell lines. We used these results to analyze microarray data to discover genes that were differentially expressed in sensitive and resistant cell lines. It is important to remember, however, that most of the cells analyzed have activated K-Ras and could also have activated NF-κB and/or inactive *p53*. Both proliferation and apoptotic genes may

influence a cell's resistance or sensitivity to gemcitabine. As such, it is important to remember the oncogenic background of these cells.

Microarrays allow investigators to examine the expression changes of thousands of genes between two different biological states in a single experiment. However, proper statistical methods must be employed to determine the significance of these changes while accounting for the large number of samples. For example, a p=0.01 might be significant in an experiment analyzing a small number of genes. However, in an array that contains 22,000 genes that same significance level would identify 220 genes as significant purely by chance. Although the Bonferroni correction is often used when multiple comparisons are encountered, this correction is too strict to be used on such a large data set. The Bonferroni correction spreads the total significance desired in the experiment over the number of multiple tests. This dramatically increases the risk of missing real significant differences when it is applied to the large number of samples present on microarrays. In our example, a significance of p=0.01 would be spread over 22,000 genes. For a single gene to be considered significant, the test statistic could not exceed 0.01/22,000 or 4.5×10^{-7}, a level so stringent that no gene would be identified. Although the Bonferroni correction is applicable for small numbers of multiple comparisons, this correction is not reasonable for the large numbers of multiple tests encountered in analysis of expression arrays.

Recently, Tusher *et. al.* developed a method of analyzing array data that accounts for the problem of multiple comparisons without the extreme strictness of the Bonferroni correction called the Significance Analysis of Microarrays or SAM. [38] SAM uses permutations of the repeated measurements to calculate the false discovery rate (FDR),

an estimation of the percentage of genes identified by chance. The local FDR, the FDR associated with a specific gene, is analogous to the p-value of a simple t-test and indicates the significance associated with that gene. We examined expression changes between pancreatic cancer cell lines that were determined to be resistant and those that were determined to be sensitive based on experiments outlined in Chapters 3 and 4. The results of this analysis will provide a list of candidate genes that may be involved in the sensitivity or resistance of pancreatic cancer cells to gemcitabine.

5.2 Materials and Methods

RNA isolation and amplification was performed by Stephan Gysin as described previously. [39]

Hybridization and array reading. Amplified RNA was hybridized to a Human Genome U133A 2.0 Array (Affymetrix, Santa Clara, CA) by the Gladstone Core Facility (San Francisco, CA). Subsequent array readings were also performed by the Gladstone Core Facility.

Classification of cell lines. Cell lines were classified as either resistant or sensitive based on the median EC_{50} value given in Table 4.1. Cell lines with median $EC_{50} > 50$ nM (CaPan2, MPanc96, Panc1, Panc 10.05) were considered resistant, whereas cell lines with $EC_{50} < 50$ nM were considered sensitive (Panc 2.03, Panc 2.13, HPAFII, L.3.6 pL, L.3.6 sL, MiaPaCa2, SU86.86, and SW 1990). Three cell lines, Panc 5.04, Panc 8.13, and L.3.3 were not classified as either resistant or sensitive and were not used in subsequent studies. The cell lines Panc 5.04 and Panc 8.13 did not give consistent EC_{50} values. There was an extremely wide range of EC_{50}s for these two cell lines and they spread equally above and below the 50 nM cutoff for resistant vs. sensitive

determination. The cell line L.3.3 exhibited sensitive behavior (median EC_{50} ~ 7 nM) at earlier passages but was resistant at later passages (median EC_{50} ~ 390 nM). We felt that these three cell lines could not reliably be classified as either resistant or sensitive and were, therefore, not used for the analysis of microarray data. Of the remaining cell lines used, there was a statistical difference between the EC_{50} values of resistant and sensitive cell lines ($p<0.005$) confirming that the cell lines were classified correctly.

Analysis of microarray data. Expression levels read from GeneChip arrays are deposited at the website: cc-jainlab-sv1.ucsf.edu:8080/MagellanBeta/. The expression level for each gene was downloaded for the following samples: CaPan2, MPanc96, Panc1, Panc 10.05 (resistant pancreatic cancer cell lines), Panc 2.03, Panc 2.13, HPAF II, L.3.6 pL, L.3.6 sL, MiaPaCa2, SU86.86, and SW 1990 (sensitive pancreatic cancer cell lines). The Significance Analysis of Microarrays or SAM program was used to determine genes that were significantly up or down regulated between cell groups. For each analysis, the default value, 1234567, was used for the random number seed. SAM analysis was performed first to detect any change ($R>0$). The analysis was then performed again to detect only genes which had at least a 1.5 fold change ($R>1.5$) Genes with significant differences in expression and at least a 1.5 fold expression change were examined to determine those that may play a role in gemcitabine sensitivity and resistance.

5.3 Results

SAM Analysis. Expression array data were analyzed to compare resistant vs. sensitive cells as described in the Materials and Methods. When analyzing expression

array data, SAM first calculates the standard deviation of the repeated measures for that gene. The program then assigns each gene a score on the basis of the change in the gene's expression relative to the standard deviation of measurements. For all genes reported here, the score refers to resistant cell lines. That is, the score value indicates the magnitude of the change in expression, and genes have either increased (+) or decreased (-) expression in resistant cell lines. A threshold (Δ) is set by the user and genes with a score above that threshold are deemed potentially significant. Permutations of the repeated measurements are used to calculate an estimation of the percentage of genes identified by chance, or the false discovery rate (FDR). The total false discovery rate of the entire data set can be set by adjusting the threshold (Δ). The local false discovery rate (local FDR), the FDR associated with a specific gene, is then generated for each potentially significant gene. Local FDR, analogous to the p-value of a simple t-test, indicates the significance associated with that gene.

The first analysis of sensitive vs. resistant cell lines which considered any significant fold expression difference and a false discovery rate (FDR) of 10% resulted in a total of 5803 significant genes (R>0; 5556 genes had increased expression and 247 genes had decreased expression in resistant cell lines). However, the number of significant genes was reduced to 176 when only genes which had at least a 1.5 fold change were reported (R>1.5, 110 genes had increased expression and 66 genes had decreased expression in resistant cell lines).

As delta is set by the user to calculate the overall FDR for the genes reported, it is not possible to have a FDR of exactly 10% in the case of R>1.5. In this case an overall FDR of 10.6% was used. This will not affect the analysis, however, since the local FDR

is calculated for each individual gene. The parameters for each analysis, as well as the overall results, are given in Table 5.1.

Table 5.1 *Number of genes differentially expressed: SAM Analysis*

	Resistant vs. Sensitive	
	R > 0	R > 1.5
FDR	10 %	10.6 %
delta	1.04	1.29
+ genes*	5556	110
- genes*	247	66

* Genes are considered to have increased (+) or decreased (-) expression in resistant cell lines

Table 5.2 lists all 176 genes differentially expressed between sensitive and resistant pancreatic cancer cell lines that had at least a 1.5 fold change and a FDR less than 10.6%. Differentially expressed genes that are thought to play a role in apoptosis, cell cycle regulation, or transcriptional pathways are of particular interest since aberrant cell growth and resistance to chemotherapeutics often arises from perturbations in these pathways. Genes implicated in these pathways are highlighted in Table 5.2.

Cytotoxicity Pathway Analysis. In addition to generating a list of possible genes influencing gemcitabine resistance using SAM, we also analyzed the microarray data to determine if any genes in the gemcitabine cytotoxicity pathway were differentially expressed. (Figure 5.2) As discussed in Chapter 1, gemcitabine uses the nucleoside salvage pathway to exert its cytotoxic effect (Chapter 1, Figure 1.2). Although several members of the gemcitabine activation pathway had been previously implicated in its resistance in pancreatic cancer, none of these genes were differentially expressed at a fold of at least 1.5 and with a local fdr < 10.6%.

Table 5.2 *Genes differentially expressed between* ***resistant and sensitive*** *pancreatic cancer cell lines* *

Probe Set ID[1]	Gene Symbol	Score (d)[2]	localfdr (%)[3]	Gene Title
222380_s_at	---	5.95	3.76	Similar to Microneme antigen
217191_x_at	---	5.93	3.76	---
217586_x_at	---	5.18	3.30	---
222338_x_at	---	4.61	2.67	Hypothetical LOC401499
217135_x_at	---	4.37	2.41	---
213235_at	---	4.15	2.23	---
222152_at	---	4.10	2.20	Similar to Microneme antigen
217619_x_at	---	3.98	2.15	Hypothetical LOC389372
216858_x_at	---	3.88	2.14	---
216715_at	---	3.05	4.88	---
213786_at	---	2.99	5.31	---
213757_at	---	2.96	5.62	---
211691_x_at	---	2.78	7.57	Ornithine decarboxylase antizyme 4
216524_x_at	---	2.76	7.89	MRNA; cDNA DKFZp564E233 (from clone DKFZp564E233)
214949_at	---	-5.13	3.14	Similar to family with sequence similarity 9, member C
202604_x_at	**ADAM10**	**-5.20**	**3.06**	**a disintegrin and metalloproteinase domain 10**
205746_s_at	**ADAM17**	**-5.47**	**3.03**	**a disintegrin and metalloproteinase domain 17 (tumor necrosis factor, alpha, converting enzyme)**
205401_at	AGPS	-4.61	4.41	alkylglycerone phosphate synthase
215741_x_at	AKAP8L	2.79	7.48	A kinase (PRKA) anchor protein 8-like
212211_at	ANKRD17	-4.28	5.59	ankyrin repeat domain 17
204833_at	APG12L	4.35	2.39	APG12 autophagy 12-like (S. cerevisiae)
219336_s_at	ASCC1	2.90	6.20	activating signal cointegrator 1 complex subunit 1
201971_s_at	ATP6V1A	-5.83	4.22	ATPase, H+ transporting, lysosomal 70kDa, V1 subunit A
201355_s_at	BAZ2A	3.07	4.70	bromodomain adjacent to zinc finger domain, 2A
201083_s_at	**BCLAF1**	**-4.86**	**3.71**	**BCL2-associated transcription factor 1**
219591_at	BM88	3.91	2.14	BM88 antigen
204676_at	C16orf51	3.54	2.51	chromosome 16 open reading frame 51
215218_s_at	C19orf14	2.95	5.72	chromosome 19 open reading frame 14

Probe Set ID[1]	Gene Symbol	Score (d)[2]	localfdr (%)[3]	Gene Title
218081_at	C20orf27	3.80	2.17	chromosome 20 open reading frame 27
207504_at	CA7	3.08	4.58	carbonic anhydrase VII
214316_x_at	**CALR**	**2.77**	**7.69**	**Calreticulin**
212757_s_at	CAMK2G	3.17	4.03	calcium/calmodulin-dependent protein kinase (CaM kinase) II gamma
208853_s_at	**CANX**	**-4.44**	**4.98**	**calnexin**
207686_s_at	**CASP8**	**-4.97**	**3.44**	**caspase 8, apoptosis-related cysteine protease**
206788_s_at	CBFB	-5.90	4.67	core-binding factor, beta subunit
211559_s_at	**CCNG2**	**-4.00**	**6.86**	**cyclin G2**
219375_at	CEPT1	-5.03	3.31	choline/ethanolamine phosphotransferase 1
218914_at	CGI-41	2.80	7.39	CGI-41 protein
201825_s_at	CGI-49	3.00	5.27	CGI-49 protein
205723_at	CNTFR	2.80	7.30	ciliary neurotrophic factor receptor
219547_at	COX15	2.75	7.95	COX15 homolog, cytochrome c oxidase assembly protein (yeast)
212180_at	**CRKL**	**-4.11**	**6.33**	**v-crk sarcoma virus CT10 oncogene homolog (avian)-like**
208867_s_at	CSNK1A1	-4.51	4.75	Casein kinase 1, alpha 1
209258_s_at	CSPG6	3.19	3.88	chondroitin sulfate proteoglycan 6
214974_x_at	CXCL5	-5.48	3.05	chemokine (C-X-C motif) ligand 5
205998_x_at	CYP3A4	4.50	2.55	cytochrome P450, family 3, subfamily A, polypeptide 4
218774_at	DCPS	3.35	3.11	decapping enzyme, scavenger
218013_x_at	DCTN4	-4.23	5.80	dynactin 4 (p62)
212514_x_at	DDX3X	-5.23	3.02	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, X-linked
219664_s_at	DECR2	3.55	2.48	2,4-dienoyl CoA reductase 2, peroxisomal
209177_at	DKFZP564J0123	3.48	2.66	hypothetical protein DKFZP564J0123
213546_at	DKFZp586I1420	3.28	3.42	hypothetical protein DKFZp586I1420
202516_s_at	DLG1	-4.75	3.99	discs, large homolog 1 (Drosophila)
202867_s_at	DNAJB12	3.08	4.60	DnaJ (Hsp40) homolog, subfamily B, member 12
212491_s_at	DNAJC8	2.77	7.67	DnaJ (Hsp40) homolog, subfamily C, member 8
219061_s_at	DXS9879E	3.46	2.70	DNA segment on chromosome X (unique) 9879 expressed sequence

Probe Set ID[1]	Gene Symbol	Score (d)[2]	localfdr (%)[3]	Gene Title
218524_at	**E4F1**	**2.74**	**8.09**	**E4F transcription factor 1**
210883_x_at	EFNB3	3.19	3.87	ephrin-B3
201768_s_at	ENTH	-4.94	3.51	enthoprotin
205767_at	**EREG**	**-4.67**	**4.25**	**epiregulin**
202851_at	FLJ11506	3.44	2.78	hypothetical protein FLJ11506
218884_s_at	FLJ13220	-5.29	2.98	hypothetical protein FLJ13220
220967_s_at	FLJ14129	3.58	2.43	hypothetical protein FLJ14129
201514_s_at	G3BP	-4.44	5.00	Ras-GTPase-activating protein SH3-domain-binding protein
206383_s_at	G3BP2	-4.56	4.60	Ras-GTPase activating protein SH3 domain-binding protein 2
212379_at	GART	-5.25	3.00	phosphoribosylglycinamide formyltransferase
204659_s_at	GFER	4.07	2.18	growth factor, augmenter of liver regeneration (ERV1 homolog, S. cerevisiae)
219015_s_at	GLT28D1	2.76	7.87	glycosyltransferase 28 domain containing 1
201673_s_at	GYS1	-4.70	4.14	glycogen synthase 1 (muscle)
221745_at	HAN11	-5.68	3.52	WD-repeat protein
220085_at	HELLS	3.00	5.23	helicase, lymphoid-specific
203674_at	HELZ	-5.58	3.22	helicase with zinc finger domain
52159_at	HEMK1	3.37	3.02	HemK methyltransferase family member 1
220042_x_at	HIVEP3	2.76	7.84	human immunodeficiency virus type I enhancer binding protein 3
211142_x_at	HLA-DOA	2.80	7.30	major histocompatibility complex, class II, DO alpha
213470_s_at	HNRPH1	-4.19	5.98	heterogeneous nuclear ribonucleoprotein H1 (H)
219865_at	HSPC157	2.73	8.27	HSPC157 protein
208931_s_at	**ILF3**	**-4.14**	**6.21**	**interleukin enhancer binding factor 3, 90kDa**
205981_s_at	**ING2**	**3.82**	**2.16**	**inhibitor of growth family, member 2**
205561_at	KCTD17	2.75	7.92	potassium channel tetramerisation domain containing 17
206747_at	KIAA0514	3.43	2.80	KIAA0514
212840_at	KIAA0794	-5.64	3.40	KIAA0794 protein
220368_s_at	KIAA2010	-5.67	3.48	KIAA2010
220782_x_at	KLK12	3.46	2.71	kallikrein 12
216100_s_at	LAP1B	-4.19	5.99	lamina-associated polypeptide 1B
221822_at	LOC112869	2.83	6.97	hypothetical protein BC011981
210822_at	LOC283345	2.78	7.64	RPL13-2 pseudogene
216490_x_at	LOC442175	4.40	2.44	similar to dJ408B20.3
213713_s_at	LOC89944	3.08	4.64	hypothetical protein BC008326

Probe Set ID[1]	Gene Symbol	Score (d)[2]	localfdr (%)[3]	Gene Title
211536_x_at	**MAP3K7**	**-4.10**	**6.36**	**mitogen-activated protein kinase kinase kinase 7**
33850_at	MAP4	3.28	3.42	microtubule-associated protein 4
212271_at	**MAPK1**	**-4.66**	**4.26**	**mitogen-activated protein kinase 1**
202654_x_at	MAR8	-4.93	3.53	membrane-associated ring finger (C3HC4) 7
202569_s_at	MARK3	-4.74	4.03	MAP/microtubule affinity-regulating kinase 3
218411_s_at	MBIP	-4.71	4.12	MAP3K12 binding inhibitory protein 1
201151_s_at	MBNL1	-5.65	3.43	muscleblind-like (Drosophila)
209624_s_at	MCCC2	-4.44	4.99	methylcrotonoyl-Coenzyme A carboxylase 2 (beta)
200798_x_at	**MCL1**	**-4.69**	**4.17**	**myeloid cell leukemia sequence 1 (BCL2-related)**
211574_s_at	MCP	-4.09	6.40	membrane cofactor protein
219051_x_at	METRN	3.16	4.07	meteorin, glial cell differentiation regulator
211801_x_at	MFN1	-5.58	3.23	mitofusin 1
212340_at	MGC21416	3.16	4.10	hypothetical protein MGC21416
213105_s_at	MGC24381	3.39	2.95	hypothetical protein MGC24381
219709_x_at	MGC2494	4.47	2.51	hypothetical protein MGC2494
220352_x_at	MGC4278	3.99	2.15	hypothetical protein MGC4278
208141_s_at	MGC4293	3.39	2.96	hypothetical protein MGC4293
209845_at	MKRN1	3.87	2.14	makorin, ring finger protein, 1
213897_s_at	MRPL23	4.07	2.18	mitochondrial ribosomal protein L23
221155_x_at	MSCP	3.85	2.15	Mitochondrial solute carrier protein
211783_s_at	MTA1	-4.25	5.71	metastasis associated 1
212277_at	MTMR4	-5.17	3.09	myotubularin related protein 4
220319_s_at	**MYLIP**	**3.02**	**5.10**	**myosin regulatory light chain interacting protein**
217493_x_at	NCR2	2.99	5.34	natural cytotoxicity triggering receptor 2
204862_s_at	**NME3**	**3.17**	**4.02**	**non-metastatic cells**
209731_at	NTHL1	2.91	6.13	nth endonuclease III-like 1 (E. coli)
203978_at	NUBP1	3.52	2.55	nucleotide binding protein 1 (MinD homolog, E. coli)
218227_at	NUBP2	3.18	3.93	nucleotide binding protein 2 (MinD homolog, E. coli)
204766_s_at	**NUDT1**	**3.30**	**3.29**	**nudix (nucleoside diphosphate linked moiety X)-type motif 1**

Probe Set ID[1]	Gene Symbol	Score (d)[2]	localfdr (%)[3]	Gene Title
218609_s_at	**NUDT2**	**5.40**	**3.51**	**nudix (nucleoside diphosphate linked moiety X)-type motif 2**
216698_x_at	OR7E47P	3.07	4.70	olfactory receptor, family 7, subfamily E, member 47 pseudogene
201799_s_at	OSBP	-4.36	5.29	oxysterol binding protein
212720_at	PAPOLA	-4.38	5.21	poly(A) polymerase alpha
203557_s_at	PCBD1	3.31	3.25	6-pyruvoyl-tetrahydropterin synthase
215354_s_at	PELP1	2.73	8.23	proline-, glutamic acid-, leucine-rich protein 1
203335_at	PHYH	2.97	5.51	phytanoyl-CoA hydroxylase (Refsum disease)
215236_s_at	PICALM	-4.24	5.78	phosphatidylinositol binding clathrin assembly protein
210256_s_at	PIP5K1A	-4.99	3.40	phosphatidylinositol-4-phosphate 5-kinase, type I, alpha
201133_s_at	PJA2	-4.47	4.90	praja 2, RING-H2 motif containing
203515_s_at	PMVK	2.98	5.44	phosphomevalonate kinase
220113_x_at	**POLR1B**	**3.01**	**5.14**	**polymerase (RNA) I polypeptide B, 128kDa**
220894_x_at	PRDM12	2.86	6.64	PR domain containing 12
200604_s_at	**PRKAR1A**	**-4.78**	**3.92**	**protein kinase, cAMP-dependent, regulatory, type I, alpha**
219183_s_at	PSCD4	2.86	6.63	pleckstrin homology, Sec7 and coiled-coil domains 4
207782_s_at	**PSEN1**	**-4.86**	**3.69**	**presenilin 1 (Alzheimer disease 3)**
201233_at	PSMD13	4.43	2.47	proteasome (prosome, macropain) 26S subunit, non-ATPase, 13
212016_s_at	PTBP1	-4.90	3.59	polypyrimidine tract binding protein 1
219579_at	RAB3IL1	2.75	7.96	RAB3A interacting protein (rabin3)-like 1
203899_s_at	RCP9	3.06	4.77	calcitonin gene-related peptide-receptor component protein
215208_x_at	RPL35A	4.14	2.22	Ribosomal protein L35a
216976_s_at	RYK	-5.17	3.09	RYK receptor-like tyrosine kinase
212417_at	SCAMP1	-3.97	7.01	secretory carrier membrane protein 1
218748_s_at	SEC10L1	-6.30	9.13	SEC10-like 1 (S. cerevisiae)
200970_s_at	SERP1	-4.70	4.14	stress-associated endoplasmic reticulum protein 1
213936_x_at	SFTPB	4.47	2.51	surfactant, pulmonary-associated protein B

Probe Set ID[1]	Gene Symbol	Score (d)[2]	localfdr (%)[3]	Gene Title
221268_s_at	SGPP1	-6.02	5.68	sphingosine-1-phosphate phosphatase 1
218921_at	SIGIRR	3.01	5.17	single Ig IL-1R-related molecule
219185_at	SIRT5	3.08	4.59	sirtuin (silent mating type information regulation 2 homolog) 5 (S. cerevisiae)
217778_at	SLC39A1	3.10	4.44	solute carrier family 39 (zinc transporter), member 1
219215_s_at	SLC39A4	2.98	5.44	solute carrier family 39 (zinc transporter), member 4
202303_x_at	SMARCA5	-5.13	3.15	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 5
214544_s_at	SNAP23	-4.90	3.59	synaptosomal-associated protein, 23kDa
205482_x_at	**SNX15**	**2.78**	**7.57**	**sorting nexin 15**
217790_s_at	SSR3	-4.07	6.52	signal sequence receptor, gamma (translocon-associated protein gamma)
204701_s_at	STOML1	2.74	8.07	stomatin (EPB72)-like 1
204045_at	**TCEAL1**	**3.87**	**2.14**	**transcription elongation factor A (SII)-like 1**
213877_x_at	**TCEB2**	**4.56**	**2.61**	**transcription elongation factor B (SIII), polypeptide 2**
218548_x_at	TEX264	3.85	2.15	testis expressed sequence 264
218491_s_at	THY28	2.82	7.15	thymocyte protein thy28
205217_at	TIMM8A	3.32	3.22	translocase of inner mitochondrial membrane 8 homolog A (yeast)
214948_s_at	**TMF1**	**-4.21**	**5.90**	**TATA element modulatory factor 1**
217930_s_at	**TOLLIP**	**4.13**	**2.21**	**toll interacting protein**
218693_at	TSPAN15	3.09	4.57	tetraspanin 15
203273_s_at	TUSC2	2.92	6.03	tumor suppressor candidate 2
205300_s_at	U1 SNRNPBP	2.88	6.46	U11/U12 snRNP 35K
219192_at	UBAP2	2.90	6.24	ubiquitin associated protein 2
217826_s_at	UBE2J1	-4.09	6.44	ubiquitin-conjugating enzyme E2, J1 (UBC6 homolog, yeast)
209088_s_at	**UBN1**	**3.80**	**2.17**	**ubinuclein 1**
212008_at	UBXD2	-4.92	3.56	UBX domain containing 2
209136_s_at	USP10	-4.33	5.42	ubiquitin specific protease 10
222032_s_at	USP7	3.63	2.35	Ubiquitin specific protease 7 (herpes virus-associated)
201831_s_at	**VDP**	**-4.15**	**6.13**	**vesicle docking protein p115**
200628_s_at	**WARS**	**-4.17**	**6.08**	**tryptophanyl-tRNA synthetase**

215553_x_at	WDR45	3.76	2.19	WD repeat domain 45
218848_at	WDR58	3.40	2.89	WD repeat domain 58
217715_x_at	**ZNF354A**	**3.72**	**2.23**	**Zinc finger protein 354A**
218707_at	**ZNF444**	**2.83**	**7.01**	**zinc finger protein 444**

* Only genes with at least a 1.5 fold difference in expression are listed. Genes thought to be involved in apoptosis, cell cycle regulation, or transcription regulation are listed in bold.

[1] Probe Set ID is the gene identifier on the Affymetrix Human Genome U133A 2.0 Array

[2] Score represents the magnitude of the change in expression. Larger values are indicative of a larger change between the two categories. A positive score (shown in red) indicates increased expression in the resistant cell lines. A negative score (shown in green) indicates reduced expression in the resistant cell lines.

[3] Local FDR (%) is equivalent to a P-statistic. The local fdr percentage is a measure of the chance that a specific gene was falsely reported.

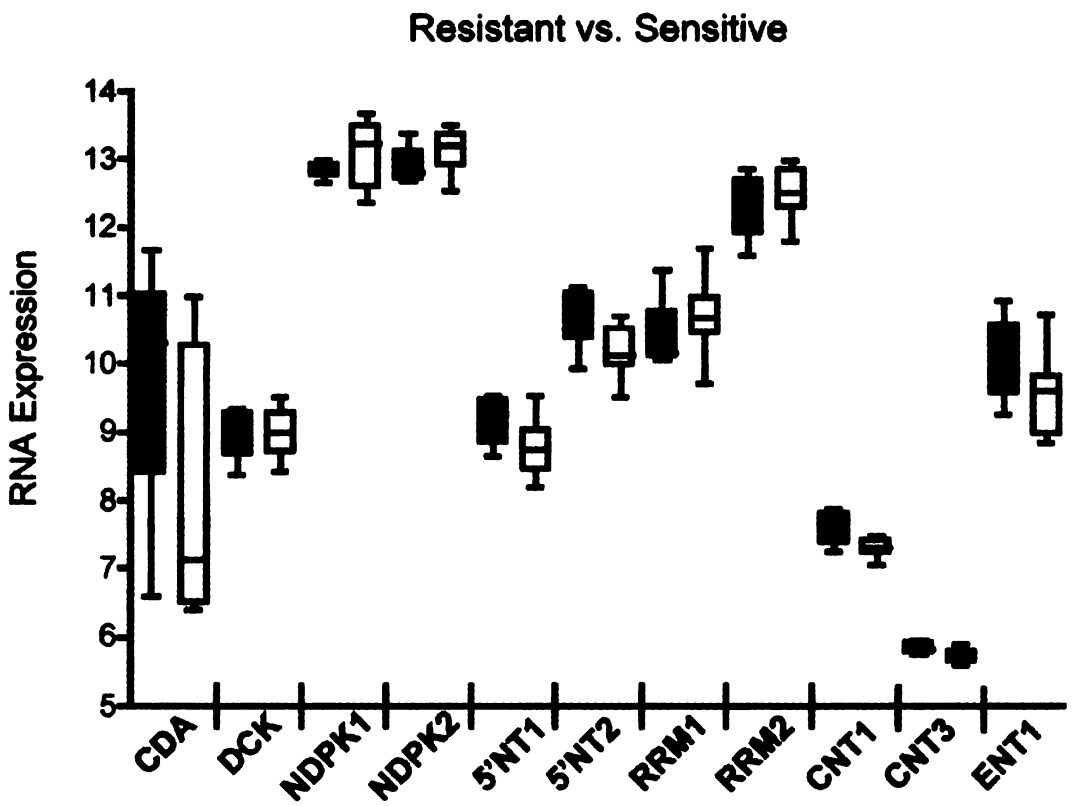

Figure 5.2 Differential expression of gemcitabine cytotoxicity genes. Genes involved in the cytotoxicity of gemcitabine were compared between resistant (gray bars) and sensitive (white bars) cell lines. CDA – cytidine deaminase, DCK - deoxycytidine kinase, NDPK1 and NDPK2 - nucleoside diphosphate kinase 1 and 2, 5'NT1 and 5'NT2 - cytoplasmic 5'-nucleotidase 1 and 2, RRM1 and RRM2 - ribonucleotide reductase subunits 1 and 2, CNT1 and CNT3 – concentrative nucleoside transporters 1 and 3, ENT1 – equilibrative nucleoside transporter 1. No genes were statistically differentially expressed at a fold $\geq$ 1.5 with local fdr < 10.6%.

5.4 Discussion

Analysis of resistant cell lines compared to sensitive cell lines gave a total of 5803 significant genes when any fold difference of expression was considered (Table 5.1). The number of significant genes was dramatically reduced when only genes with a fold greater than 1.5 (R>1.5) were considered. It appears that, although there are a large number of genes differentially expressed between resistant and sensitive cell lines that are significant, > 95% of these genes have minor differences in expression.

We examined the expression of genes in the gemcitabine activation pathway. Microarray analysis did not reveal any significant changes in the gemcitabine pathway genes between resistant and sensitive cell lines. This contradicts previous studies implicating dCK [2, 40], CD [5], RR [41-43], and nucleoside transporters [44] in gemcitabine resistance in other cell types. In addition, in Chapter 4 we found a correlation between ENT1 expression level and resistance to gemcitabine when expression was measured by TaqMan. However, as TaqMan is more sensitive than expression arrays, it may be that RNA expression microarrays are not sufficient to detect the changes in ENT1 expression that were observed when the more sensitive TaqMan assay was used. The expression changes of a gene, whether detected by TaqMan or with expression arrays, must be confirmed at the protein level before a definite conclusion can be made. As such, analysis of expression arrays between resistant and sensitive cells can be used to suggest genes that may play a role. However, it is not sufficient to prove a gene is involved in gemcitabine resistance.

Of the 176 genes determined by SAM analysis to be differentially expressed in gemcitabine resistant and sensitive pancreatic cancer cell lines, the genes listed in

Table 5.3 are known to play important roles in cell proliferation or apoptosis pathways. These genes, and their relationship to their possible function in gemcitabine sensitivity, are discussed in greater detail below.

Table 5.3 *Proliferation and apoptosis genes differentially expressed between* ***resistant and sensitive*** *pancreatic cancer cell lines*[*]

Gene Symbol	Score (d)[1]	localfdr (%)[2]	Gene Function
ADAM10	-5.20	3.06	Negative regulator of cell adhesion. Regulator of growth factors and cytokines.
ADAM17	-5.47	3.03	Involved in cell-cell signaling. Regulator of growth factors and cytokines as well as their receptors.
BCLAF1 (Btf)	-4.86	3.71	Transcriptional repressor of Bcl-2 and Bcl-xL
CALR (Calreticulin)	2.77	7.69	Regulator of calcium homeostasis and calcium dependent pathways.
CANX (Calnexin)	-4.44	4.98	Regulator of calcium homeostasis and calcium dependent pathways
CASP8	-4.97	3.44	Pro-apoptosis cysteine-aspartic acid protease (caspase).
CCNG2 (Cyclin G2)	-4.00	6.86	Cell cycle regulator.
ING2	3.82	2.16	Regulator of cell growth and senescence.
MAP3K7 (TAK1)	-4.10	6.36	Required for the activation of nuclear factor kappa B.
MAPK1 (ERK)	-4.66	4.26	Promoter of cell proliferation.
MCL1	-4.69	4.17	Apoptosis inhibitor.
PRKAR1A	-4.78	3.92	Regulatory subunit of cAMP-dependent protein kinase (PKA).
SIGIRR	3.01	5.17	Inhibitor of IL-1R.

[1] Score represents the magnitude of the change in expression. Larger values are indicative of a larger change between the two categories. A positive score (shown in red) indicates increased expression in the resistant cell lines. A negative score (shown in green) indicates reduced expression in the resistant cell lines.

[2] Local FDR (%) is equivalent to a P-statistic. The local fdr percentage is a measure of the chance that that specific gene was called as significant accidentally.

Extrinsic Apoptosis Pathway. Caspase-8 is a major component of the extrinsic apoptosis pathway governed by the death receptors TNF-α, TRAIL, and FasL. [17-19] Caspase-8 inhibitors abolished gemcitabine induced apoptosis in NSCLC and pancreatic cancer cell lines. [45, 46] This suggests that caspase-8 is needed for gemcitabine sensitivity. That caspase-8 has a lower expression in pancreatic cancer cells resistant to gemcitabine is consistent with these observations (caspase-8: score = -4.97; local fdr = 3.44%).

MAPK Pathways Genes. Initially, the most surprising finding in Table 5.3 was that ERK expression is downregulated in pancreatic cancer cell lines resistant to gemcitabine (ERK: score = -4.66; local fdr = 4.26%). ERK is the final kinase activated in the Ras/Raf/MEK/ERK signaling pathway and regulates the expression of anti-apoptotic genes including Bcl-2, Bcl-xL, and Mcl-1. [32, 47] One would expect ERK expression to be higher in cells resistant to gemcitabine since ERK opposes apoptosis. That ERK expression promotes survival and acts to protect cells from apoptosis has been shown previously in studies where ERK inhibition triggered apoptotic cell death. [47, 48] It is possible, however, that ERK does not act to inhibit apoptosis in pancreatic cancer cell lines.

Recent studies suggest that gemcitabine apoptosis in pancreatic cancer is mediated not through the ERK MAP kinase pathway but through the p38 MAP kinase pathway. The p38 signaling pathway is activated by pro-inflammatory cytokines and environmental stress, resulting in growth arrest or apoptosis. [49, 50] This is particularly relevant in pancreatic cancer since most pancreatic tumors display an extensive network of fibrotic stroma that induces cellular stress and inflammatory cytokines. [51, 52]

Habiro *et. al.* found that gemcitabine dramatically increased the activation of p38 MAPK but not of other members of the MAPK superfamily, Akt, JNK, or ERK MAPK. [53] In addition, p38 inhibition inhibited gemcitabine induced apoptosis in pancreatic cancer cell lines. [53] Koizumi *et. al.* determined that suppression of p38 activity reduced apoptosis and increased cell survival of human pancreatic cancer cell lines. [54] These reports indicate that gemcitabine specifically activates p38 MAPK in human pancreatic cancer cell lines and suggests that p38 signaling is necessary for gemcitabine apoptosis. In light of these studies, the downregulation of ERK in gemcitabine resistant cell lines is not a contradiction, since ERK may not be essential to gemcitabine apoptosis in pancreatic cancer cell lines.

MAP3K, or TGF-β activated kinase (TAK1), expression is downregulated in gemcitabine resistant pancreatic cancer cell lines (TAK1: score = -4.10; local fdr = 6.36%). TAK1 is activated by TGF-β and goes on to activate multiple protein kinases including the p38 MAPK pathway mentioned above. [55-57] Although there are no studies investigating the role of TAK1 specifically in pancreatic cancer or in gemcitabine resistance, the studies of Habiro and Koizumi suggest that TAK1 activation of p38 would increase gemcitabine apoptosis in pancreatic cancer cell lines. [53, 54] This is consistent with the expression array data in which cells resistant to gemcitabine had lower TAK1 expression. Lower TAK1 expression would indicate reduced activation of p38 and reduced sensitivity to gemcitabine.

Two other MAP kinase pathway genes that were downregulated in resistant cells line are Mcl-1 and PRKAR1A (Mcl-1: score = -4.69; local fdr = 4.17%; PRKAR1A: score = -4.78; local fdr = 3.92%). Mcl-1 is an anti-apoptotic member of the Bcl-2 family

and is expressed in many pancreatic cancer tumor tissues. [58-60] Since Mcl-1 effects are anti-apoptotic, one would expect higher expression in resistant cells. However, it the balance of numerous Bcl-2 family members, both pro-apoptotic (such as Bcl-2, Bcl-xL and Mcl-1) and ant-apoptotic (such as Bax, Bak, Bid, Bad, and Bcl-xS) that ultimately determine the apoptotic fate of a cell. [18, 20, 21, 61]

PRKAR1A is a type 1 regulator subunit of cAMP-dependent protein kinase A (PKAR1α). PKA signaling can induce apoptosis by inactivating Bad through phosphorylation. Phosphorylated Bad can no long bind to and inhibit pro-apoptotic Bcl-2 and Bcl-xL. [62] As such reduced expression of PKAR1α might reduce PKA signaled apoptosis, consistent with reduced expression in resistant cells.

Calcium Binding Chaperones. The intracellular calcium content of the endoplasmic reticulum (ER) and the mitochondria determines the cell's sensitivity to apoptosis activation pathways. [63, 64] During normal calcium signaling, mitochondria take up increased cytoplasmic calcium. Elevated calcium levels in the cytoplasm, due to aberrant ER calcium regulation, can cause an overload of mitochondrial calcium, activating the permeability transition pore and releasing cytochrome *c*. [40] An increase or decrease in ER calcium store, or in release of these calcium stores, can alter cellular sensitivity to apoptosis.

Calreticulin and calnexin are the major calcium binding chaperones within the lumen of the endoplasmic reticulum (ER) and are involved in many essential cellular processes including signaling, apoptosis, and transcriptional activity. [65-67] Both molecules bind to calcium within the ER lumen to control the concentration of free calcium. Hong *et al.* found that calreticulin antibodies were overrepresented specifically

in the sera of pancreatic cancer patients compared to patients with lung cancer, colon cancer, or chronic pancreatitis or to normal healthy subjects. [68] HeLa cells overexpressing calreticulin showed increased sensitivity to drug induced apoptosis though overexpression of calnexin had no effect on cell death. [69] However, since calreticulin's role appears to be *p53* mediated, this increased sensitivity may not hold in pancreatic cancers expressing mutant *p53*. [70] This may be the case as our cell lines resistant to gemcitabine had increased calreticulin expression but decreased calnexin expression (calreticulin: score = 2.77; local fdr = 7.69%; calnexin: score = -4.44; local fdr = 4.98%). It is also possible that any calcium binding effects of increased calreticulin are masked by the corresponding decrease in calnexin expression.

Transcription Factors. Btf or BCLAF1 (BCL2-associated transcription factor 1) is a tumor suppressor gene that acts to repress the translation of anti-apoptotic genes such as Bcl-xL and Bcl-2. [71] Overexpression of Btf in HeLa cells resulted in increased apoptosis. This is consistent with reduced expression in resistant cell lines (Btf: score = -4.86; local fdr = 3.71%).

Cyclin G2 (CCNG2) is a regulator of cell cycle progression. Classical cyclins promote cell cycle progression, however, cyclin G2 acts to block entry into the cell cycle in a *p53*-independent manner. [72] Human colon cancer cell lines resistant to 5-FU displayed a decrease in apoptosis, an increase in aggressive growth and decreased expression of cyclin G2. Expression array analysis shows that cyclin G2 also has decreased expression in pancreatic cancer cell lines resistant to gemcitabine (cyclin G2: score = -4.00; local fdr = 6.86%).

Resistant cell lines have an increased expression of ING2, a member of the inhibitor of growth (ING) family (ING2: score = 3.82; local fdr = 2.16%). Increased expression of ING2 is associated with *p53*-dependent senescence, in which cells lose the ability to divide. However, replicative senescence can be bypassed by inactivation of *p53*. [73, 74] We would expect resistant cell lines to have decreased rather than increased ING2 expression if they possessed functioning *p53*. Since the majority of pancreatic cancers have inactivated forms of *p53* they may posses the ability to bypass senescence regardless of ING2 expression.

SIGIRR negatively regulates interleukin-1 (IL-1) signaling through the IL-1 receptor (IL-1R). IL-1R is a cytokine receptor whose ligands, IL-1α and IL-1β, are involved in immune and inflammatory response. [75, 76] IL-1α is the primary cytokine responsible for the constitutive NF-κB activation displayed by pancreatic cancer cell lines. Niu *et al.* determined that antibodies against IL-1α completely inhibited constitutive NF-κB activation in metastatic pancreatic cancer cell lines. [77] Consistent with these observation, overexpression of SIGIRR inhibited NF-κB activation. [76] It is unclear why SIGIRR is overexpressed in cell lines resistant to gemcitabine (SIGIRR: score = 3.01; local fdr = 5.17%).

Extracellular Matrix (ECM). The invasion and metastasis common to pancreatic cancer depends on the actions of matrix metalloproteinases (MMPs) and integrins on the extracellular matrix (ECM). MMPs act locally near the pancreatic cancer tumor to degrade the basement membrane and initiate invasion. The basement membrane is comprised of ECM proteins and forms a cellular support for both normal cells and tumors. MMPs degrade the extracellular matrix and allow micrometastases to enter the

bloodstream and attach at distant sites. [78] When normal cells are detached from the ECM, they undergo apoptosis due to cytochrome *c* release. [79] Vaquero *et. al.* studied two pancreatic cancer cell lines, MiaPaCa2 and Panc1, attached and detached from ECM proteins. They found that, although these detached cells released high levels of cytochrome *c*, they did not activate caspase-9 and the downstream caspase cascade. [80] Pancreatic cancers may have some defect in the caspase-9 apoptosis cascade that allows detached malignant cells to escape apoptosis.

The growth and survival of cells depends on attachment to the ECM through integrins, heterodimeric membrane proteins that mediate cell-cell and cell-matrix interactions. [81] Once at a new site, mobile pancreatic cancer cells require integrin to reattach to the ECM and reestablish cell proliferation. The activation of integrins correlates with the invasive ability of pancreatic cancer cells. [82]

ADAM10 and ADAM17, members of the A Disintegrin and Metalloprotease family of membrane shedding proteins, are cell surface glycoproteins that play roles in cell growth, differentiation, and motility. The role of ADAM10 and ADAM17 in pancreatic cancer cells is quite complex since these proteins contain both a metalloproteinase domain and a disintegrin domain. [83, 84] The metalloproteinase domain of ADAMs shed a variety of membrane bound molecules from the ECM into the cytosol, including growth factors, cytokines, and cytokine receptors. [85] ADAMs can bind directly to integrins and inhibit integrin mediated cell invasion. [86] Thus ADAMs stimulate proliferation through the activation of cytokines and growth factors but inhibit cell growth through their disintegrin activity.

No studies have yet been done regarding the role of ADAM10 and ADAM17 in pancreatic cancers or resistance to gemcitabine. In our microarray analysis, both ADAM10 and ADAM17 had decreased expression in cells resistant to gemcitabine (ADAM10: score = -5.20; local fdr = 3.06%; ADAM17: score = -5.47; local fdr = 3.03%) This suggests that the overall influence of ADAM10 and ADAM17 in these cell lines may be as a disintegrin. Cell lines with reduced expression of ADAM10 and ADAM17 would have a corresponding decrease in integrin binding and increase in cell proliferation.

Although not definitive, this microarray analysis identified a number of genes that may influence gemcitabine resistance in pancreatic cancer cell lines. We have used this analysis to generate a list of candidate genes that may be involved in gemcitabine resistance. We have also examined a number of differential expressed genes known to have an influence on cell proliferation or apoptosis, including transcription factors, MAPK and apoptosis pathway genes and genes essential to calcium binding and the integrity of the extracellular matrix. In particular, the analysis suggests that further studies on TAK1, ADAM10, and ADAM17 would be useful since there is evidence to suggest that that these genes play a role in pancreatic cancer resistance to gemcitabine but no explicit studies have proven this role.

Summary

I have sought in this dissertation to give an overview of my work regarding nucleoside analogs, nucleoside transporters, and pancreatic cancer.

Chapter 1 provided an overview of the endogenous nucleoside salvage pathway. Individual genes in the pathway were considered and evidence supporting their importance in the mechanism of action of nucleoside analogs was provided. A brief overview of the four clinically used nucleoside analogs, cladribine, fludarabine, cytarabine, and gemcitabine was given.

In **Chapter 2**, I presented my work investigating the genetic and functional variation in the concentrative nucleoside transporter, *CNT1* (*SLC28A1*). We determined the haplotype structure and functionally analyzed all coding region variants of CNT1 identified in ethnically diverse populations. A total of 58 coding region haplotypes were identified using PHASE analysis, 44 of which contained at least one amino acid variant. Over half of the coding region haplotypes were population specific. Using site-directed mutagenesis, 15 protein altering CNT1 variants, including one amino acid insertion and one base-pair deletion, were constructed and expressed in *X. laevis* oocytes. All variant transporters took up ^{3}H-thymidine with the exception of CNT1-Ser546Pro, a rare variant, and CNT1-1153del, a single bp deletion found at a frequency of 3% in the African American population. I determined that the anticancer nucleoside analog, gemcitabine, had a reduced affinity for CNT1-Val189Ile (a common CNT1 variant found at a frequency of 26%), in comparison to reference CNT1 (IC_{50} = 13.8 ± 0.60 μM for CNT1-reference and 23.3 ± 1.5 μM for CNT1-Val189Ile, $p<0.05$). We found that CNT1 is a

highly variable gene and that common genetic variants of CNT1 may contribute to variation in systemic and intracellular levels of anticancer nucleoside analogs.

Chapter 3 gives an in depth characterization of a panel of 16 pancreatic cancer cell lines. We used colorimetric cytotoxicity assays (MTT assay) to determine the EC_{50} of each cell line to gemcitabine. We also used TaqMan analysis to determine the relative expression of the three nucleoside transporters involved in gemcitabine uptake, ENT1, CNT1, and CNT3. **Chapter 3** provides an in depth explanation of the MTT and TaqMan assays and provides the EC_{50} values and nucleoside expression data generated from this characterization.

In **Chapter 4**, I analyzed the data generated in **Chapter 3**. For the first time, I found that ENT1 expression level was associated with reduced gemcitabine sensitivity. Multiple regression analysis revealed that using both ENT1 and CNT1 as predictors significantly improved the correlation between gemcitabine cytotoxicity and nucleoside transporter expression (ENT1 alone, adjusted $r^2 = 0.41$; ENT1 and CNT1, adjusted $r^2 = 0.45$; $p=0.043$). We found that ENT1 inhibitors effected a range of gemcitabine responses, from enhanced resistance to enhanced sensitivity, depending on the relative expression level of concentrative and equilibrative transporters. The observation that ENT1 can mediate both resistance and sensitivity to gemcitabine was confirmed in stably transfected cells. These results show for the first time, that ENT1 expressed in the absence of concentrative nucleoside transporters acts as a mediator of sensitivity, however, when expressed concomitantly with CNT1 or CNT3, ENT1 acts predominantly as an efflux transporter, mediating resistance to gemcitabine.

Finally, in **Chapter 5**, I used the resistance and sensitivity data generated in **Chapter 4** to analyze RNA expression array data to determine genes that are differentially expressed between pancreatic cancer cell lines resistant and sensitive to gemcitabine. This analysis yielded numerous apoptotic, cell cycle, and transcription genes that were differentially expressed in resistant cells. I hope that this analysis of resistant vs. sensitive cell lines can be used to generate new hypotheses and inform future studies investigating the mechanisms involved in gemcitabine resistance.

5.5 References

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