EQUILIBRATIVE-SENSITIVE NUCLEOSIDE TRANSPORTER
FUNCTION AND REGULATION IN GEMCITABINE SENSITIVITY
AND RESISTANCE:

IS THERE A POTENTIAL THERAPEUTIC BENEFIT FOR
PANCREATIC CANCER?

by

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A thesis submitted in conformity with the requirements
for the Degree of Master of Science,
Graduate Department of Pharmacology,
University of Toronto

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Equilibrative-Sensitive Nucleoside Transporter (es-NT) Function and Regulation in Gemcitabine Sensitivity and Resistance: Is There a Potential Therapeutic Benefit for Pancreatic Cancer?

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Abstract

Salvage of preformed nucleosides requires transport across the plasma membrane by specific transport proteins and subsequent conversion to their ribo- and deoxyribonucleotide forms. In mammalian cells, plasma membrane transport occurs by sodium-dependent (concentrative) and sodium-independent (equilibrative) mechanisms. These transport systems are also the route of cellular uptake for many synthetic nucleoside analogue agents used in cancer treatment, including gemcitabine (a novel deoxycytidine analogue). This thesis examines the *in vitro* effects of gemcitabine on cytotoxicity and the modulation of these effects by the es-nucleoside transporter and two DNA synthesis inhibitors (5-fluorouracil and tomudex), with a specific focus on pancreatic cancer.

Cytotoxicity was assessed by clonogenic assay in one human bladder (MGH-U1) and three human pancreatic cancer cell lines (PANC-1, HS-766T, PK-8). Basal levels of es-NT were quantified in all four cell lines by flow cytometric analysis. Combination experiments were carried out to determine if upregulating the es-NT modulates increases in sensitivity to gemcitabine. In two pancreatic cell lines (PANC-1, HS-766T), treatment with 5-FU followed by gemcitabine yielded increased cytotoxicity. This effect was also seen in the HS-766T cell line when pre-treated with tomudex. For these concentrations of 5-FU and tomudex, es-NT content was found to be increased over basal levels.
Acknowledgements

I would first like to thank my supervisor, Dr. Malcolm Moore for his constant encouragement, guidance and support during the past two years. Your comments, criticisms and suggestions were instrumental in my development as an independent, critical, research scientist.

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LIST OF ABBREVIATIONS

\(\alpha\text{-MEM}\) \quad \alpha\text{-modification of minimal essential medium}

\(2\text{CdA}\) \quad 2\text{-chlorodeoxyadenosine}

\(5\text{-FU}\) \quad 5\text{-Fluorouracil}

\(5\times8\) \quad 5\text{-}(SAENTA-x_8)-Fluorescein

\(\text{ara-C}\) \quad Cytosine arabinoside

\(\text{cib}\) \quad Concentrative and insensitive to NBMPR, accepts broad array of nucleosides as permeants

\(\text{cif}\) \quad Concentrative and insensitive to NBMPR, accepts formycin B as a permeant

\(\text{cit}\) \quad Concentrative and insensitive to NBMPR, accepts thymidine B as a permeant

\(\text{cs}\) \quad Concentrative and sensitive to NBMPR

\(\text{csg}\) \quad Concentrative and sensitive to NBMPR, accepts guanosine as a permeant

\(\text{CDP}\) \quad Cytidine diphosphate

\(\text{CTP}\) \quad Cytidine triphosphate

\(\text{dCK}\) \quad Deoxycytidine kinase

\(\text{dCMP}\) \quad Deoxycytidine monophosphate

\((\text{d})\text{CMPD}\) \quad (Deoxy)cytidine monophosphate deaminase

\(\text{dCTP}\) \quad Deoxycytidine triphosphate

\(\text{dFdC}\) \quad 2',2'-difluorodeoxycytidine (gemcitabine)

\(\text{dFdCDP}\) \quad 2',2'-difluorodeoxycytidine diphosphate

\(\text{dFdCMP}\) \quad 2',2'-difluorodeoxycytidine monophosphate

\(\text{dFdCTP}\) \quad 2',2'-difluorodeoxycytidine triphosphate

\(\text{dFdU}\) \quad 2',2'-difluorodeoxyuridine

\(\text{dFdUMP}\) \quad 2',2'-difluorodeoxyuridine monophosphate

\(\text{DNA}\) \quad Deoxyribonucleic acid

\(\text{dTMP}\) \quad 2'deoxythymidine-5'-monophosphate

\(\text{dTPP}\) \quad Deoxythymidine triphosphate

\(\text{dUMP}\) \quad 2'-deoxyuridine-5'-monophosphate

\(\text{ei}\) \quad Equilibrative and insensitive to NBMPR

\(\text{es}\) \quad Equilibrative and sensitive to NBMPR

\(\text{es-NT}\) \quad Equilibrative-sensitive nucleoside transporter

\(\text{FdUMP}\) \quad 5'-fluoro-2'-deoxurytidine-5'-monophosphate

\(\text{FdUTP}\) \quad 5'-fluorodeoxyuridine triphosphate

\(\text{FITC}\) \quad Fluorescein-5'-isothiocyanate

\(\text{FPGS}\) \quad Folyl polyglutamate synthetase

\(\text{FUMP}\) \quad Fluorouridine monophosphate

\(\text{GST}\) \quad Glutathione S-transferase

\(\text{HEPES}\) \quad N-(2-hydroxyethyl(piperazine)-N'-(4-ethanesulfonic acid))

\(\text{IC}_{50}\) \quad Concentration required to inhibit colony formation by 50%

\(\text{IC}_{90}\) \quad Concentration required to inhibit colony formation by 90%
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>LV</td>
<td>Leucovorin</td>
</tr>
<tr>
<td>MDR</td>
<td>Multi-drug resistance</td>
</tr>
<tr>
<td>MESF</td>
<td>Molecules equivalent soluble fluorescein</td>
</tr>
<tr>
<td>MRP</td>
<td>Multi-drug resistance associated protein</td>
</tr>
<tr>
<td>NBMPR</td>
<td>S-(p-nitrobenzyl)-6-thioinosine; nitrobenzyl mercaptopurine riboside</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>P-gp</td>
<td>P-glycoprotein</td>
</tr>
<tr>
<td>RFC</td>
<td>Reduced folate carrier</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RR</td>
<td>Ribonucleotide reductase</td>
</tr>
<tr>
<td>SAENTA</td>
<td>5'-S-(2-aminomethyl)-N6-(4-nitrobenzyl)-5'-thioadenosine</td>
</tr>
<tr>
<td>TS</td>
<td>Thymidylate synthase</td>
</tr>
<tr>
<td>TTP</td>
<td>Thymidine triphosphate</td>
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Chapter 1: INTRODUCTION
1.1 Introduction

There exist two pathways of DNA synthesis within human cells: the de novo and salvage pathways. Salvage of preformed nucleosides requires their transport across the plasma membrane and subsequent conversion to their ribo- and deoxyribonucleotide forms. The efficiency of the salvage pathway of DNA synthesis can have major effects on cellular sensitivity to a wide variety of antimetabolite drugs because most of these drugs, including nucleoside analogues, undergo a generic two-stage metabolism: transport into the cell ("salvage"), followed by phosphorylation to their active triphosphate forms. Nucleoside analogues taken up by the salvage pathway include cytosine arabinoside (ara-C), 2-chlorodeoxyadenosine (2CdA) and gemcitabine (dFdC). Therefore, low activity of this pathway may be a potential mechanism of drug resistance to these agents. This paper examines the in vitro effects of the novel nucleoside analogue gemcitabine as a single agent and in combination with two DNA synthesis inhibitors, 5-FU and tomudex, looking at cytotoxicity and the effects on the equilibrative-sensitive nucleoside transporter (es-NT). In addition, schedule dependence of drug administration was examined in order to provide a rationale for future clinical trials protocol design.

1.2 Pancreatic Cancer

Adenocarcinoma of the pancreas is the fifth leading cause of cancer-related deaths in North America exceeded only by lung, colorectal, prostate and breast cancers (Moore, 1996). Surgery is the only curative treatment currently
available; however, greater than 80% of patients with carcinoma of the exocrine pancreas are metastatic at diagnosis. Chemotherapy and radiation therapies most commonly play a palliative role in pancreatic cancer care and have not shown a significant impact on 5-year survival rates (Clark et al., 1996). At present, pancreatic cancer has the worst 5-year survival rate of any cancer - less than 5% of all pancreatic cancer patients survive five years (Clark et al., 1996; Moore 1996).

A variety of drug resistance mechanisms have been described in pancreatic tumours. These include P-glycoprotein (P-gp), multi-drug resistance-associated protein (MRP), glutathione S-transferases (GSTs) and metallothionein (Collier et al., 1994; Dietel, 1996; Holm et al., 1994; Miller et al., 1996; Ohshio et al., 1996; Verovski et al., 1996). P-gp is an integral membrane-bound efflux pump capable of removing a variety of structurally and functionally unrelated cytotoxic drugs including the anthracyclines, epidophyllotoxins, alkylating agents and vinca alkaloids, from tumour cells (Dietel, 1996; Holm et al., 1994; Verovski et al., 1996). Holm et al. (1994) found that by decreasing the level of mdr1 mRNA expression in a human pancreatic carcinoma cell line resistant to doxorubicin, one could inhibit the formation of P-gp and reduce the cell’s resistance to doxorubicin. GSTs function as enzymes of detoxification. By catalysing the conjugation of potentially mutagenic electrophilic compounds with reduced glutathione, cells may be protected from toxic cell damage (Dietel, 1996). The Pi class of GSTs (those with acidic isoelectric points) are expressed by a variety of human tumours at higher than normal values. It is this isozyme
that is more prominently expressed in the majority of pancreatic adenocarcinomas (Collier et al., 1994). Because a variety of cytotoxic drugs are metabolised by GSTs, elevated levels of this enzyme may potentially lead to tumour drug resistance.

Over the past ten years, many new drugs have been tested for activity against pancreatic cancer. Over forty phase II studies of new single agents and combination regimens have been reported (Moore, 1994). None has shown a response rate greater than 20%, the usual standard that must be met for further testing to be warranted (table 1.2a). In randomized trials, gemcitabine was the first and only chemotherapeutic agent that has been shown to have any meaningful impact on either survival or disease related symptoms in pancreatic adenocarcinoma. Much interest now exists in maximizing the benefit of gemcitabine against pancreatic cancer and other solid tumours.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Responses (%)</th>
<th>Median Survival (mos.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Best supportive care</td>
<td></td>
<td>3.9</td>
</tr>
<tr>
<td>Gemcitabine</td>
<td>5.4</td>
<td>5.7</td>
</tr>
<tr>
<td>5-FU</td>
<td>N/S</td>
<td>3</td>
</tr>
<tr>
<td>5-FU</td>
<td></td>
<td>5.1</td>
</tr>
<tr>
<td>5-FU</td>
<td>7*</td>
<td>4.5</td>
</tr>
<tr>
<td>5-FU, doxorubicin, cisplatin</td>
<td>15</td>
<td>3.5</td>
</tr>
<tr>
<td>5-FU, methyl CCNU</td>
<td>10</td>
<td>3.3</td>
</tr>
<tr>
<td>5-FU, methyl CCNU, streptozotocin</td>
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<td>5-FU, Mi-C, methotrexate, cyclophosphamide, vincristine</td>
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</tr>
<tr>
<td>Mi-C, 5-FU</td>
<td>8</td>
<td>4.1</td>
</tr>
<tr>
<td>FAM</td>
<td>14</td>
<td>6.1</td>
</tr>
<tr>
<td>FAM</td>
<td>14</td>
<td>2.7</td>
</tr>
<tr>
<td>Melphalan</td>
<td>2</td>
<td>1.8</td>
</tr>
<tr>
<td>SMF</td>
<td>34</td>
<td>4.2</td>
</tr>
<tr>
<td>SMF</td>
<td>4</td>
<td>4.2</td>
</tr>
<tr>
<td>SMF</td>
<td>14</td>
<td>4</td>
</tr>
<tr>
<td>SMF (modified)</td>
<td>15</td>
<td>3.1</td>
</tr>
<tr>
<td>SMF</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Cisplatin, Ara-C, caffeine</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

*The majority of patients in these studies did not have measurable disease.

5-FU, 5-Fluorouracil; CCNU, lomustine; Mi-C, mitomycin C; FAM, 5-FU, doxorubicin, mitomycin C; SMF, streptozotocin, mitomycin C, 5-FU; N/S, not significant.

Table 1.2a. Randomized trials in advanced pancreatic carcinoma (adopted from Moore, 1996; Buris et al., 1997)
1.3 Antimetabolites

The antimetabolite classes of chemotherapeutic agents generally function as inhibitors of macromolecule biosynthesis that block cell replication. These antimetabolites compete with endogenous substrates in cellular metabolic processes leading to what can be thought of as 'competitive starvation' (Kinsella and Smith, 1998). 5-FU, tomudex and gemcitabine are all members of this class of oncolytic agents which act at different points in the cellular metabolic process. Three important classes of antimetabolites are: Nucleoside analogues (ara-C, gemcitabine), anti-folates (methotrexate) and thymidylate synthase (TS) inhibitors (tomudex, 5-FU).

1.4 Cytidine Analogues

Cytidine analogues are compounds that closely resemble the endogenous nucleoside cytidine. These agents require transport into the cell and phosphorylation to their active, triphosphate forms at which point they compete with deoxycytidine triphosphate (dCTP) for incorporation into DNA (White et al., 1987; Wiley et al., 1985). These drugs may also exert their cytotoxic effects by acting at various points along the DNA synthesis pathway. Cytosine arabinoside (ara-C) for example, is a competitive inhibitor of DNA polymerase. Ara-C has been the prototypical drug for this class of chemotherapeutic agents for some time.
1.4.1 Gemcitabine

Gemcitabine (2',2'-difluorodeoxycytidine (dFdC); Gemzar™) is a cell cycle dependent (S-phase specific) deoxycytidine analogue and oncolytic agent of the antimetabolite class. It has two fluorine atoms substituted for the hydrogens of the 2-carbon atom in the sugar moiety of its deoxycytidine parent (figure 1.4a).

Gemcitabine was initially synthesized as a potential anti-viral agent and proved to be quite active in vitro against both DNA and RNA viruses. Its unfavourable therapeutic index when administered on a daily schedule, however, precluded any further development of gemcitabine as an anti-viral drug (Guchelaar et al., 1996).

Gemcitabine is currently used in the palliative care of non-small cell lung cancer and is the only agent that has shown any clinical benefit toward pancreatic cancer. As pancreatic cancer has one of the poorest prognoses of all types of cancer, considerable interest lies in discovering ways to improve the efficacy of gemcitabine, both as a single agent and in combination therapy.

1.4.1.1 Mechanism of Action of Gemcitabine

Gemcitabine, like most antimetabolite drugs, must first be transported into the cell and then be phosphorylated to its active, triphosphate form. Transport of gemcitabine occurs via the nucleoside transporter, of which there exist multiple forms (section 1.6, Heinemann et al., 1995). Transport of nucleosides into the cell becomes saturated at extracellular nucleoside concentrations of 1 \( \mu \text{M} \) (Wiley et al., 1985; White et al., 1987; Pressacco et al., 1995). Once inside the cell,
**Figure 1.4a.** Chemical structures of deoxycytidine, cytosine arabinoside (Ara-C) and gemcitabine.
numerous enzymatic reactions lead to the formation of gemcitabine triphosphate (dFdCTP) (figure 1.4b). Gemcitabine is phosphorylated to its monophosphate form 2',2'-difluorodeoxycytidine monophosphate (dFdCMP) by the enzyme deoxycytidine kinase (dCK). Phosphorylation of gemcitabine (by dCK) is also a saturable process, however, uptake of gemcitabine into the cell is the rate-limiting step of its metabolic process because when gemcitabine transport is saturated, dCK phosphorylation is not (Guchelaar et al., 1996). The specific enzyme that phosphorylates dFdCMP to its diphosphate form (dFdCDP) has not yet been identified, but is assumed to be a base-specific (deoxy)cytidine monophosphate ((d)CMP) kinase that lacks specificity for the carbohydrate (Plunkett et al., 1995). dFdCDP is converted to the triphosphate form (dFdCTP) by the ubiquitous non-specific enzyme, nucleoside diphosphate kinase (Plunkett et al., 1995; Heinemann et al., 1995; Guchelear et al., 1996). Incorporation of dFdCTP into DNA is most likely the major mechanism by which gemcitabine exerts its cytotoxic actions. After incorporation of dFdCTP on the end of the elongating DNA strand, one additional deoxynucleotide is added before the DNA polymerases are unable to continue DNA synthesis. This action, termed “masked-chain termination”, locks the drug into place within the DNA as proofreading enzymes are unable to remove dFdCTP from this position (Plunkett et al., 1995; Huang et al., 1991).

Elimination of gemcitabine occurs via three routes (figure 1.4b): (1) transport out of the cell via equilibrative nucleoside transporters. (2) Deamination of gemcitabine by cytidine deaminase into 2',2'-difluorodeoxyuridine
Figure 1.4b. Metabolic scheme of gemcitabine. dFdC, gemcitabine; dFdCDP, gemcitabine diphosphate; dFdCDP, gemcitabine diphosphate; dFdCTP, gemcitabine triphosphate; dCMP, deoxycytidine monophosphate; dFdU, difluorodeoxyuridine; dFdUMP, difluorodeoxyuridine monophosphate.
(dFdU). (3) Deamination of dFdCMP by dCMP deaminase into 2',2'-difluorodeoxyuridine monophosphate (dFdUMP). Both dFdUMP and dFdU are ultimately broken down into uracil and difluorodeoxyribose (Plunkett et al., 1995; Guchelear et al., 1996).

Gemcitabine, along with its metabolites, interact with a variety of cellular metabolic regulatory processes. These interactions serve to enhance the overall inhibitory actions of gemcitabine on cell viability. Termed 'self-potentiation', these interactions are not evidenced to such an extent in other anticancer drugs (Plunkett et al., 1995; Heinemann et al., 1992; Gandhi et al., 1991). The pathways of gemcitabine self-potentiation and the sites of action of gemcitabine and its metabolites are summarized in figure 1.4c. The six major mechanisms of gemcitabine self-potentiation are as follows: (1) dFdCDP is an inhibitory substrate for ribonucleotide reductase (RR), the enzyme that is responsible for producing deoxynucleotides required for both DNA synthesis and repair. (2) The resulting decrease in cellular deoxynucleotides, in particular deoxycytidine triphosphate (dCTP), is essential in that dFdCTP directly competes with dCTP for incorporation into DNA by DNA polymerases. This decrease in cellular dCTP, therefore, increases gemcitabine nucleotide incorporation into DNA. (3) dCK phosphorylation of gemcitabine is inhibited by deoxycytidine and dCTP (Heinemann et al., 1988; Bouffard et al., 1993). Therefore, when cellular dCTP pools are lowered, the rate of gemcitabine phosphorylation is increased resulting in greater dFdCTP accumulation for incorporation into DNA. These higher levels of dFdCTP would also act to maintain inhibition of ribonucleoside diphosphate
Figure 1.4c. Self-potentciating mechanisms of gemcitabine. The numbered pathways are discussed sequentially in the text. The dashed lines indicate inhibitory reactions. RR, ribonucleotide reductase. ---● indicates an inhibitory pathway.
reductase. (4) dCTP is also a required cofactor in the activity of dCMP deaminase, the rate-limiting enzyme of gemcitabine nucleotide elimination (Heinemann et al., 1992; Xu et al., 1992). When the cellular levels of dCTP decline as a result of ribonucleotide reductase inhibition by dFdCDP, dCMP deaminase activity also decreases accordingly. This results in a lower rate of removal of gemcitabine nucleotides from the cell and most likely contributes to the retention of the active nucleotides in tumour cells. (5) dFdCTP also functions to inhibit the dCMP deaminase reaction, adding an additional mechanism that contributes to the prolonged retention of gemcitabine nucleotides found in tumour cells (Heinemann et al., 1992; Grunewald et al., 1992). (6) dFdCTP inhibits CTP synthetase at high concentrations, thereby blocking the synthesis of dCTP as well (Heinemann et al., 1995). This action decreases the available supply of CDP as a substrate for ribonucleotide reductase, which puts further stress on the other normal pathways of dCTP formation.

1.4.1.2 Drug Resistance and Gemcitabine

Resistance to gemcitabine may be due to several mechanisms. dCK deficiency, leading ultimately to decreased dFdCTP formation; increased elimination of gemcitabine or its monophosphate through increased deamination; increased dCTP pools, resulting in increased feedback inhibition of dCK; and finally decreased influx or increased efflux of gemcitabine (Van Haperen et al., 1995; Van Haperen et al., 1994).
Van Haperen et al. (1995) developed a gemcitabine-resistant human ovarian cancer cell line in which they found a 10-fold reduction in dCK activity as compared to the normal, parent cell line. This resistant cell line was found to be cross-resistant to antimetabolites known to depend on dCK for activation (ara-C, 2-CdA), dFdU (the deamination product of gemcitabine) as well as to cisplatin, doxorubicin and vincristine (Van Haperen et al., 1995). This may have implications in future gemcitabine combination therapy.

Various lines of evidence have shown an association between increased cytidine deaminase activity and cellular resistance to cytidine analogues. Firstly, cytidine deaminase irreversibly catalyses the deamination of its physiologic substrates (cytidine and deoxycytidine) as well as nucleoside analogue agents, including gemcitabine (Camiener, 1967; Chabot et al., 1983; Bouffard et al., 1993). This deamination causes a marked decrease in anti-tumour activity (Creasey et al., 1966; Muller and Zahn, 1979). Second, in leukaemia patients, high levels of cytidine deaminase activity have been correlated with resistance to treatment with ara-C (Steuart and Burke, 1971; Onetto et al., 1987). Third, inhibition of cytidine deaminase in experimental tumours and cell lines has been associated with an increased susceptibility to killing by ara-C (Honma et al., 1991; Riva et al., 1992). None of these studies, however, has demonstrated a direct association between cytidine deaminase activity and drug resistance. Tobias and Blau (1996) performed in vitro studies to determine whether forced expression of a gene encoding for the enzyme cytidine deaminase can confer resistance to the cytidine analogues ara-C and gemcitabine. They found that by
overexpressing cytidine deaminase they could confer at least a 2-fold resistance to ara-C and gemcitabine as compared to cells expressing normal levels of this enzyme.

Sliutz et al. (1996) linked a heat shock protein (hsp70) overexpression with gemcitabine resistance. Heat shock protein expression is induced in response to adverse changes in the cellular environment (Lindquist and Craig, 1988). Hsp70 overexpression has been reported to induce cytoprotection in vivo and in vitro under a wide variety of adverse conditions (Sliutz et al., 1996). In this study, the cytoprotective effect of hsp70 against gemcitabine was moderate, with a factor of 2-3 fold. It was shown that by depleting cellular levels of hsp70 using quercetin, a natural flavonoid known to inactivate the heat shock transcription factor, sensitivity to gemcitabine increased. This increase was quercetin dosage-depndant.

Mackey et al. (1998) showed that nucleoside transporter activity was a prerequisite for growth inhibition by gemcitabine in vitro. They evidenced two reasons for this: 1) nucleoside transport-deficient cells were highly resistant to gemcitabine and 2) treatment of cells that exhibited only equilibrative nucleoside transporter activity with nitrobenzylthioinosine (NBMPR) or dipyridamole (equilibrative transport inhibitors, section 2.6) increased gemcitabine resistance.

1.4.1.3 Phase I Studies (Toxicities)

Various dosing schedules of gemcitabine were evaluated during many phase I studies. Dose-limiting toxicities of gemcitabine have been found to be
schedule dependent (Eckardt et al., 1995). Poplin et al. (1992) studied two different therapy regimens: 5-90 mg/m² twice weekly as a 30-minute infusion and 30-150 mg/m² twice weekly as a bolus injection during 5 minutes. The maximum tolerated doses of gemcitabine for these schedules were 65 and 100 mg/m² respectively. The dose limiting toxicity was myelosuppression for both schedules and non-haematological toxicities, including nausea, vomiting and malaise, were mild (Poplin et al., 1992).

From other phase I studies, gemcitabine (10-1000 mg/m² as a 30-minute intravenous infusion weekly for 3 weeks every 4 weeks) was found to have myelosuppression as its major dose-limiting toxicity. Other haematological toxicities included anaemia and thrombocytopenia. Non-haematological toxicities found were nausea, vomiting and malaise, which were all mild. The maximum tolerated dose of gemcitabine was assessed to be 790 mg/m² for this dosing schedule (Abbruzzese et al., 1991; Rosso and Martin, 1994; Pollera et al., 1994). In studies of previously untreated patients with pancreatic adenocarcinoma, high-dose gemcitabine (1200, 1500 or 1800 mg/m² given weekly for 3 weeks every 4 weeks at a constant infusion rate of 10mg/m²/min) elicited fever, myelosuppression, nausea, vomiting and confusion as its dose-limiting toxicities (Tempero et al., 1994).

1.4.1.4 Phase II and III Studies

Phase II studies of the weekly schedule have demonstrated that gemcitabine has activity against non-small cell lung cancer, bladder cancer,
ovarian cancer, breast cancer and pancreatic cancer (table 1.4a). In pancreatic cancer, the efficacy of gemcitabine (1000 mg/m² weekly for 7 weeks followed by 1 week of rest and thereafter weekly for 3 weeks every 4 weeks) has been studied (Rothenberg et al., 1995). 17 of 63 patients (27%) in the study showed a positive clinical benefit (defined as at least 50% reduction in pain or at least 50% reduction in daily consumption of analgesics). Clinical benefit, defined as a composite measure of pain, analgesic consumption, performance status and weight gain (Hidalgo et al., 1999), has only recently been incorporated into the investigation of novel therapies in pancreatic cancer care and recognized as a valid end point for drug approval. In a phase III study (Moore, et al., 1995) in previously untreated patients, gemcitabine was compared to 5-FU. Here, 126 patients with advanced pancreatic cancer were randomized to gemcitabine at the aforementioned schedule or to 5-FU (600 mg/m² over 30 minutes, weekly). With gemcitabine, 24% of patients showed a clinical benefit versus 5% of those in the 5-FU arm of the study. Median survival of the two groups was also reported, and was found to be 5.65 and 4.41 months respectively, with 18% of gemcitabine patients alive at 1 year as compared to 2% of those who received 5-FU.

1.5 Thymidylate Synthase (TS) Inhibitors

Thymidylate synthase (TS) is the rate-limiting enzyme involved in the de novo synthesis of thymine nucleotides. Inhibition of this enzyme limits the formation of thymidine triphosphate (TTP), resulting in inhibition of DNA synthesis. TS has been an attractive target for anticancer drugs since the
<table>
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<tr>
<th>Study</th>
<th>Dosing Schedule</th>
<th>Prior Therapy</th>
<th>Response Rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Non Small-Cell Lung Cancer</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anderson <em>et al</em> (1994)</td>
<td>800-1000 mg/m²/wk x 3 wk</td>
<td>No</td>
<td>20 (16/79)</td>
</tr>
<tr>
<td>Abratt <em>et al</em> (1994)</td>
<td>1000-1250 mg/m²/wk x 3 wk</td>
<td>No</td>
<td>20 (15/76)</td>
</tr>
<tr>
<td>Shepherd <em>et al</em> (1993)</td>
<td>1250 mg/m²/wk x 3 wk</td>
<td>No</td>
<td>20 (19/93)</td>
</tr>
<tr>
<td>Fosella <em>et al</em> (1993)</td>
<td>1000-1750 mg/m²/wk x 3 wk</td>
<td>No</td>
<td>21 (4/19)</td>
</tr>
<tr>
<td>Negoro <em>et al</em> (1994)</td>
<td>1000-1250 mg/m²/wk x 3 wk</td>
<td>Not stated</td>
<td>30 (11/37)</td>
</tr>
<tr>
<td>Negoro <em>et al</em> (1994)</td>
<td>1000-1250 mg/m²/wk x 3 wk</td>
<td>Not stated</td>
<td>24 (9/37)</td>
</tr>
<tr>
<td>Lund <em>et al</em> (1992)</td>
<td>90 mg/m² twice weekly</td>
<td>No</td>
<td>13 (5/40)</td>
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<tr>
<td><strong>Ovarian Cancer</strong></td>
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<tr>
<td>Lund <em>et al</em> (1994)</td>
<td>800 mg/m²/wk x 3 wk</td>
<td>Yes</td>
<td>19 (8/42)</td>
</tr>
<tr>
<td><strong>Breast Cancer</strong></td>
<td></td>
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<tr>
<td>Carmichael <em>et al</em> (1993)</td>
<td>800 mg/m²/wk x 3 wk</td>
<td>Yes</td>
<td>29 (9/35)</td>
</tr>
<tr>
<td><strong>Pancreatic Cancer</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Casper <em>et al</em> (1991)</td>
<td>800-1250 mg/m²/wk x 3 wk</td>
<td>No</td>
<td>13 (5/39)</td>
</tr>
<tr>
<td>Carmichael <em>et al</em> (1993)</td>
<td>800-1000 mg/m²/wk x 3 wk</td>
<td>No</td>
<td>9 (2/23)</td>
</tr>
</tbody>
</table>

*Table 1.4a.* Phase II trials of gemcitabine.
introduction of the fluoropyrimidines 5-FU and 5-fluorodeoxyuridine by Heidelberger et al. (Heidelberger et al., 1957). TS inhibition by these compounds is dependent on their conversion to fluorodeoxyuridine monophosphate (FdUMP) and the subsequent formation of a ternary complex between the enzyme, FdUMP and the folate cofactor (Langenbach et al., 1972; Danenberg et al., 1974). In addition to their effects on TS, fluoropyrimidines can also inhibit cell growth by incorporation into RNA and DNA (Heidelberger et al., 1957). An alternative approach to the inhibition of TS has been directed toward the synthesis of analogues of the folate cofactor. These folate-based TS inhibitors are specific in their actions and do not possess any of the non-specific actions of the fluoropyrimidines (Cunningham et al., 1996; Blackledge, 1998).

Other factors also favour TS as a chemotherapeutic target. TS levels are increased in neoplastic cells, implying a greater dependency of tumour cells on de novo pyrimidine biosynthesis and therefore, enhanced sensitivity to TS inhibitors relative to normal cells (Hashimoto et al., 1988). In addition, folate-based inhibitors of TS may also gain selectivity because of the increased reduced folate transport and polyglutamation capacity in tumour cells (Sirotnak et al., 1984). These factors should result in increased concentration and retention of the inhibitors in tumour cells.

1.5.1 5-Fluorouracil

5-Fluorouracil (5-FU) is a fluorinated pyrimidine antimetabolite belonging to the class of anti-metabolites known as TS inhibitors. It was originally
synthesized in 1957 (Heidelberg et al., 1957). The fluorine substitution occurs at carbon 5 of the pyrimidine ring in place of a hydrogen (figure 1.5a).

5-FU is the therapeutic mainstay for colorectal cancer (Schnall and MacDonald, 1991). It is also used clinically in the treatment of breast, pancreatic and stomach cancers and squamous cell carcinoma of the head and neck. Response rates to 5-FU monotherapy in patients with advanced colorectal cancer are typically less than 20% (Bleiberg, 1997). Therefore, considerable interest exists in combining 5-FU with other chemotherapeutic agents in order to improve therapeutic outcomes. Thus far, efforts to improve efficacy have included modifying the route or schedule of administration and combining 5-FU with biochemical modulating agents, such as folinic acid. Gastrointestinal toxicities (diarrhea, mucositis) are dose-limiting in clinical use.

![Chemical structures of uracil and 5-FU](image)

Figure 1.5a. Chemical structures of uracil and 5-Fluorouracil.
1.5.1.1 Mechanism of Action of 5-FU

5-FU, like gemcitabine, is an S-phase specific agent. Therefore, drug cytotoxicity requires active DNA synthesis and is related to exposure time. As 5-FU is a modified nucleobase, it must be transported into the cell before it can exert its cytotoxic actions. This uptake occurs via various nucleoside transport proteins located at the cell surface (section 1.6; Wang et al., 1997). Once it enters the cell, three mechanisms exist by which 5-FU exerts its cytotoxic effects (figure 1.5b): (1) Inhibition of thymidylate synthase (TS) (Heidelberg et al., 1960a, 1960b). This is the primary mechanism of action of 5-FU. Thymidylate synthase catalyzes the methylation of 2'-deoxyuridine-5'-monophosphate (dUMP) to 2'-deoxythymidine-5'-monophosphate (dTMP) in a reaction which uses the reduced folate 5,10-methylene tetrahydrofolate as a cofactor. 5-FU is converted intracellularly to 5-fluoro-2'-deoxyuridine-5'-monophosphate (FdUMP). FdUMP forms a covalent ternary bond with thymidylate synthase and its reduced cofactor N^5,10^-methylene tetrahydrofolate. Binding results in enzyme inhibition and subsequent depletion of deoxythymidine triphosphate (dTTP), thereby preventing DNA synthesis and cell growth. (2) 5-FU or FdUMP may be converted to fluorouridine monophosphate (FUMP) which is further phosphorylated to the triphosphate, FUTP. FUTP may be incorporated into RNA producing non-functional RNA and defects in protein synthesis. (3) FdUMP may be phosphorylated to the 5'-fluorodeoxyuridine triphosphate (FdUTP) and incorporated directly into DNA, altering DNA stability (Cheng and Nakayama,
UK, uridine kinase; UMK, UMP kinase; UDK, UDP kinase; TK, thymine kinase. Numbered pathways correspond to those discussed in the text.
The DNA repair enzyme uracil DNA glycosylase functions to remove uracil from DNA and also excises FdUTP resulting in DNA single strand breaks and DNA fragmentation (Lonn and Lonn, 1986).

It has been speculated that many of the toxic effects of 5-FU (and its modulators) are due to the lack of specificity in their actions on thymidylate synthase (Mead, 1996) and thus a search for more specific TS inhibitors has been undertaken.

1.5.1.2 Drug Resistance and 5-FU

5-FU resistance has been primarily associated with insufficient inhibition of thymidylate synthase (Peters et al., 1995). In vitro and in vivo 5-FU resistance has also been associated with the following: (1) Decreased accumulation of FdUMP due to decreased 5-FU activation or increased 5-FU inactivation (Mulkins and Heidelberger, 1982). (2) Increased activity of thymidylate synthase (Chu et al., 1993; Johnston et al., 1995) and amplification of the thymidylate synthase gene (Clark et al., 1987; Berger et al., 1985). (3) Changes in nucleotide pools (Aronow et al., 1984; Kaufmann et al., 1984). (4) Point mutations in the gene encoding for thymidylate synthase, resulting in an enzyme with an altered structural form and lower affinity for both FdUMP and the cofactor $N^{5,10}$-methylene tetrahydrofolate. Binding of 5-FU in the absence of this cofactor results in an unstable binary complex. FdUMP then becomes a weak inhibitor of thymidylate synthase.
1.5.2 Tomudex (Raltitrexed)

The biosynthesis of thymidine monophosphate (TMP) requires 5,10-methylene tetrahydrofolate, which serves as a cofactor in the TS-catalysed transfer of a one-carbon unit to dUMP. Due to the limited success of fluoropyrimidine substrate analogues (such as 5-FU) in the treatment of colorectal and other cancers (Rustum and Creaven, 1988), analogues of the folate cofactor of TS were developed. Non-specific, non-TS effects of 5-FU (and its modulators) on RNA are believed, as previously noted, to account for some aspects of toxicity seen during therapy with these antimetabolites, such as mucositis and rash. A specific TS inhibitor, which does not require modulation and does not possess non-specific actions on RNA, presents an attractive research goal. Tomudex, a quinazoline folate analogue (figure 1.5c), is a new chemotherapeutic agent that aims to meet this research target. It entered phase I evaluation in Europe in 1991 (Jackman et al., 1995). Tomudex is a folate-based TS inhibitor and is an analogue of the folate cofactor for TS. It is a highly specific, laboratory-designed TS inhibitor that does not require modulation and which does not have non-specific effects on RNA (Cunningham et al., 1996; Blackledge, 1998).

![Chemical structure of Tomudex](image)

**Figure 1.5c.** Chemical structure of tomudex.
Tomudex has been approved for the treatment of advanced colorectal cancer and is currently being studied in a wide variety of malignancies including squamous cell head and neck carcinoma, prostate and gastric cancers and pancreatic cancer. In patients with advanced colorectal cancer, Tomudex has a response rate similar to 5-FU (Blieberg, 1997; Cunningham et al., 1996) when used as a single agent. In pancreatic cancer, studies have shown that Tomudex has a response rate of 12% (Cunningham et al., 1996). As with 5-FU, there is considerable interest in combining Tomudex with other chemotherapeutic agents in an attempt to improve therapeutic outcomes. Adverse effects of Tomudex include fatigue, leukopenia, thrombocytopenia and diarrhea.

1.5.2.1 Mechanism of Action of Tomudex

Tomudex is a mixed non-competitive inhibitor of TS (Duch et al., 1993). The mode of action of tomudex is specific and is based on competition with 5,10-methylene tetrahydrofolate for TS. Tomudex is transported into cells via a reduced folate carrier (RFC) and is rapidly and extensively polyglutamated by the enzyme folyl polyglutamate synthetase (FPGS) (figure 1.5d). Polyglutamation enhances TS inhibitory potency and increases the duration of TS inhibition in cells, which may improve antitumor activity (Cunningham et al., 1996). This polyglutamation could also contribute to increased toxicity due to drug retention in normal tissues.
Figure 1.5d: Mechanism of action of Tomudex. RFC, Reduced Folate Carrier; 5,10-CH₂FH₄, 5,10-methylene tetrahydrofolate; dThd, deoxythymine; TMP, thymine monophosphate; TTP, thymine triphosphate; TK, Thymidine Kinase.
1.5.2.2 Drug Resistance and Tomudex

Acquired resistance to tomudex may occur through induction of TS, disturbed RFC function leading to reduced drug uptake, reduction in FPGS activity and alterations in intracellular folate homeostasis (Van Triest et al., 1999; Lu et al., 1995; Drake et al., 1996; Jackman et al., 1995). In the latter case, cell lines having expanded folate pools showed decreased polyglutamation of tomudex thus curtailing its biological activity (Van Triest et al., 1999). This may indicate that a high folate status may preclude activation of folate-based TS inhibitors such as tomudex, to their active polyglutamate forms, whereas a low folate status may be a cause of toxicity observed in patients.

1.6 Nucleoside Transporters

Cells can synthesise nucleotides either through de novo synthesis, or via reutilisation of nucleotides and nucleobases derived from either the intracellular turnover of nucleic acids and nucleotides, or from extracellular sources (Wohlhueter and Plagemann, 1980; Muller et al., 1983). In the latter case, known as the salvage pathway, nucleosides and nucleobases must first be transported across the cell membrane by specific transport proteins. These transport systems are of considerable pharmacological interest. Transport inhibitors, such as dipyridamole and dilazep, can enhance the effectiveness of various chemotherapeutic agents, including 5-FU and methotrexate, by modulating either drug influx or efflux and interfering with the salvage pathways of DNA synthesis (Lonn et al., 1989; Kang and Kimball, 1984; Marz et al., 1977;
Gaffen et al., 1989; Cabral et al., 1984; Chan, 1989; Chan and Howell, 1985; Plagemann and Kraupp, 1986). The therapeutic effects of combining a transport inhibitor with an inhibitor of de novo nucleotide synthesis in order to develop a chemotherapy regimen in which both the de novo and salvage pathways of DNA synthesis are blocked has been widely explored. The combination of 5-FU and dipyridamole as well as methotrexate and dipyridamole are two such examples. Studies have shown that dipyridamole (7.7 mg/kg/day as a continuous infusion over three days) enhances both methotrexate and 5-FU cytotoxicity (Remick et al., 1990; Willson et al., 1989).

In addition to the endogenous nucleosides, some drugs belonging to the nucleoside analogue class of oncolytic agents are also taken up into the cell via these specific nucleoside transport proteins (figure 1.6a). Therefore, combining a nucleoside analogue with agents that increase nucleoside transporter expression may lead to greater cell kill.

Two carrier mediated transport mechanisms for purine and pyrimidine bases have been described. In mammalian cells, plasma membrane transport occurs by both sodium-dependent (concentrative) and sodium-independent (equilibrative) mechanisms (Sundaram et al., 1998; Cass, 1995; Griffith and Jarvis, 1996). These protein mediated transport systems are also the route of cellular uptake for many synthetic nucleoside analogue agents including ara-C and gemcitabine (Wiley et al., 1985; White et al., 1987; Clumeck, 1993; Burke et al., 1998). In addition, through its effect on adenosine concentration at the cell surface, nucleoside transport plays an important role in a variety of physiological
Figure 1.6a. Structures of nucleosides and nucleoside analogues.
processes including coronary vasodilation, renal vasoconstriction, neurotransmission, platelet aggregation and lipolysis (Sundaram et al., 1998; Belardinelli et al., 1989; Jacobson et al., 1990). There is also growing evidence that nucleoside transport processes of intracellular membranes play an important role in the intracellular distribution of nucleosides and their analogues (Cass et al., 1998; Hogue et al., 1996; Mani et al., 1998).

Sodium-dependent mechanisms of nucleoside transport are limited to specialized cells such as intestinal and renal epithelia, choroid plexus, liver, splenocytes, macrophages and leukaemic cells (Belt et al., 1992; Cass, 1995; Griffith and Jarvis, 1996; Huang et al., 1994; Fang et al., 1996; Yao et al., 1996a; Yao et al., 1996b; Ritzel et al., 1997; Che et al., 1995; Wang et al., 1997a; Patil and Unadkat, 1997). These sodium-dependent, concentrative nucleoside transporters generally mediate influx only and act via active transport processes, thus depending on cellular ATP for their function. These transporters use the physiologic sodium gradient (extracellular to intracellular) generated by the ubiquitous Na⁺-K⁺-ATPase to move nucleosides intracellularly against their concentration gradient (Griffith and Jarvis, 1996; Cass, 1995). Based on functional studies, six subtypes (N1-N6) of sodium-dependent nucleoside transporters have been characterised in human cells and tissues (table 1.6a). They are subclassified according to substrate specificity and numbered sequentially in order of discovery (Wang et al., 1997; Cass et al., 1998). Cit (N1) are purine-selective and also accept uridine and formycin B as substrates. Cit (N2, N4) prefer pyrimidine nucleosides and also accept adenosine (N2 and N4
<table>
<thead>
<tr>
<th>NT Process</th>
<th>Permeant Selectivity</th>
<th>Sensitivity to NBMPR</th>
<th>Na(^+)- dependency</th>
<th>References</th>
</tr>
</thead>
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<tr>
<td></td>
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<tr>
<td><strong>Concentrative transporters</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>cif</em> (N1)</td>
<td>Purine nucleosides, uridine, formycin B</td>
<td>-</td>
<td>+</td>
<td>Ritzel <em>et al.</em> 1998; Wang <em>et al.</em> 1997b</td>
</tr>
<tr>
<td><em>cit</em> (N2)</td>
<td>Pyrimidine nucleosides, adenosine</td>
<td>-</td>
<td>+</td>
<td>Ritzel <em>et al.</em> 1997</td>
</tr>
<tr>
<td><em>cit</em> (N4)</td>
<td>Pyrimidine nucleosides, guanosine, adenosine</td>
<td>-</td>
<td>+</td>
<td>Gutierrez <em>et al.</em> 1992; Gutierrez and Giacomini 1993</td>
</tr>
<tr>
<td><em>cib</em> (N3)</td>
<td>Purine and pyrimidine nucleosides</td>
<td>-</td>
<td>+</td>
<td>Belt <em>et al.</em> 1993</td>
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<tr>
<td><em>cs</em> (N5)</td>
<td>Adenosine analogues, formycin B</td>
<td>+</td>
<td>+</td>
<td>Paterson <em>et al.</em> 1993</td>
</tr>
<tr>
<td><em>csg</em> (N6)</td>
<td>Guanosine</td>
<td>+</td>
<td>+</td>
<td>Flanagan and Meckling-Gill 1997</td>
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<td><strong>Equilibrative transporters</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td><em>es</em></td>
<td>Purine and pyrimidine nucleosides</td>
<td>+</td>
<td>-</td>
<td>Griffiths <em>et al.</em> 1997a</td>
</tr>
<tr>
<td><em>ei</em></td>
<td>Purine and pyrimidine nucleosides</td>
<td>-</td>
<td>-</td>
<td>Crawford <em>et al.</em> 1998; Griffiths <em>et al.</em> 1997b</td>
</tr>
</tbody>
</table>

**Table 1.6a.** Characterized human nucleoside transporter systems.
subtypes) and guanosine (subtype N4) as substrates. Cib (N3) is non-selective and accepts a broad range of purine and pyrimidine nucleosides as substrates (Cass, 1995; Belt et al., 1993; Cass et al., 1998). All of the above isoforms are resistant to inhibition by S-(p-nitrobenzyl)-6-thioinosine (NBMPR). Two additional isoforms, cs (N5) and csg (N6), that are sensitive to NBMPR inhibition, have recently been identified (Paterson et al., 1993; Flanagan and Meckling-Gill, 1997; Burke et al., 1998). These transport proteins accept adenosine analogues and guanosine respectively as substrates. The histological prevalence of these isoforms has yet to be identified.

Sodium-independent, equilibrative nucleoside transport processes mediate the facilitated diffusion of nucleosides across plasma membranes and are widely distributed in different cell types (Hammond, 1991). They also function bidirectionally in the transmembrane flux of nucleosides in accordance with the concentration gradient. This class of transporter displays low substrate specificity and affinity, and high substrate capacity. Sodium-independent nucleoside transporters are classified into two subtypes based on their sensitivities to inhibition by NBMPR and dipyridamole (table 1.6a). NBMPR sensitive (equilibrative sensitive (es)) transporters bind NBMPR with high affinity, whereas NBMPR insensitive (equilibrative insensitive (ei)) transporters are unaffected even by micromolar concentrations of NBMPR. Both display broad substrate specificity for purine and pyrimidine nucleosides. Equilibrative transporters are pharmacological targets for coronary vasodilator compounds such as dipyridamole and dilazep (Cass, 1995; Griffith and Jarvis, 1996) that
compete with permeant for the substrate-binding site (Jarvis et al., 1982; Jarvis, 1986). It has been previously shown that es transporter expression can be increased by depleting the endogenous intracellular nucleotide pools using DNA synthesis inhibitors such as 5-FU (Pressaco et al., 1995). The cell still has to maintain nucleotide levels in order to preserve the DNA synthesis process and must now scavenge those nucleotides from extracellular pools. By increasing the number of nucleoside transporters at the cell surface, it would follow that a greater number of nucleosides would enter the cell.

The transport process is followed by phosphorylation of the nucleosides by kinases which, contrary to the nucleoside transporters, have a high substrate affinity and low substrate capacity. Computer and kinetic analyses have suggested that the transport of nucleosides is rate-limiting at low (less than 1 μM) concentrations of extracellular nucleosides (Wiley et al., 1985; White et al., 1987). Nucleoside levels of this magnitude occur in human serum (Nottebrook and Then, 1977), indicating that nucleoside transport (and nucleoside transporter expression) may be an important, rate-limiting step in the utilisation of nucleosides by cells for the salvage pathway of DNA synthesis.

The recent cloning of the four major human nucleoside transporters (es, ei, cit (N2), cif) (Cass et al., 1998; Griffiths et al., 1997a; Crawford et al., 1998; Griffiths et al., 1997b; Ritel et al., 1997; Wang et al., 1997; Belt et al., 1993) has led to the construction and discovery of new molecular and immunological probes with which we are able to study nucleoside transporter distribution. This has the potential to lead to novel, transport-based strategies aiming to improve
the therapeutic indices of the nucleoside analogue class of chemotherapeutic drugs.

1.6.1 Gemcitabine and Nucleoside Transport

Gemcitabine has been shown to be a substrate for four of the nucleoside transporters found in humans (es, ei, cit, cib) (Mackey et al., 1998). The major mediators of gemcitabine uptake, however, are most probably the equilibrative nucleoside transporters because human cit activity has only been demonstrated in kidney, liver and intestinal epithelium (Ritzel et al., 1997; Patil and Unadkat, 1997) and that for human cib in myeloid leukemic cell lines, freshly isolated myeloblasts and the CaCo-2 colon cancer cell line (Belt et al., 1992; Belt et al., 1993).

To date, *in vitro* experimental evidence has shown that the efficiency of uptake of nucleoside analogues, such as gemcitabine, varies among cell lines that express only single nucleoside transporters. The efficiency of gemcitabine uptake in these cell lines as assessed through transport kinetics (\(K_m/V_{max}\) ratio) was as follows: es = cit > ei > cib >>> cif (Mackey et al., 1998). Mackey et al. (1998) also demonstrated that gemcitabine resistance increased when the various cellular nucleoside transport processes were inhibited or removed.
1.7 5-(SAENTA-x8)-Fluorescein Binding Assay

The recent synthesis of SAENTA, a derivatizable analogue of NBMPR that is a tightly-bound inhibitor of the es transport process (Agbanyo et al., 1990), has enabled the development of a method to allow the measurement of cell-surface es-NT content that requires 10-fold fewer cells than conventional [³H]NBMPR radioligand binding assays. Conjugates of SAENTA where the nucleoside analogue is linked to fluorescent reporting moieties, in this case fluorescein-5-isothiocyanate (FITC), have proven to be specific stains for cellular NBMPR binding sites (Jamieson et al., 1993). The interaction of fluorescein derivatives of SAENTA at the es-NT has been shown through mutual inhibition of NBMPR and SAENTA-fluorescein at binding sites in es-expressing cells (Buolamwini et al., 1994). Jamieson et al. (1993) showed that, when converted to molecules of equivalent soluble fluorescein, the fluorescence signal arising from 5-(SAENTA-x8)-fluorescein specifically bound to leukaemic myeloblasts correlated well (r = 0.98) with the number of es-NT sites assayed for concurrently by [³H]NBMPR equilibrium binding analysis. This enabled the cell-bound fluorescence output of 5-(SAENTA-x8)-fluorescein to be expressed in numbers of cell-surface es-NT sites per cell. The current use of 5-(SAENTA-x8)-fluorescein (figure 1.8a) in flow cytometric equilibrium binding assays, therefore, provides a simple and accurate means of determining the es-NT content of cells.
Figure 1.7a. Chemical structure of 5-(SAENTA-x8)-fluorescein showing the fluorescein and SAENTA moieties joined by an 8-atom linking structure.
1.8 Combination Chemotherapy

Combination chemotherapy aims to improve therapeutic effects versus single agent treatment. These improved effects can include an increased therapeutic index, increased drug efficacy and decreased drug toxicity. Combination chemotherapy may also play a role in the reduction or the delay of development of drug resistance in tumour cells. These cells are less likely to be resistant to multiple agents with differing mechanisms of action. Also, combining drugs that have different toxicity profiles may allow the use of each agent at its maximum tolerated dosage, thereby maximizing the effectiveness of each drug in the combination regimen.

When drugs are combined, we aim to determine if the effect of the combination treatment is greater to, equal to or less than what would be expected from the single agents alone. There are three possible outcomes of any combination when each drug used has an effect on its own: synergy, additivity or antagonism. Several different mathematical models exist to quantify these effects. If, however, one drug exhibits no effect on its own yet enhances the effect of another drug, the outcome is referred to as potentiation.

Several factors must be considered when evaluating drug interactions as each drug in the combination has its own effect (concentration-response curve) and is influenced by various pharmacokinetic and pharmacodynamic factors. To study the effect of drug combinations, it is advisable to vary one variable (eg. concentration, exposure time) and measure one outcome (eg. cell kill) at a time. Following the quantitative determination of the synergy or antagonism of a drug
combination, biochemical or molecular biology techniques may be used to ascertain the mechanism behind the interaction seen (Rideout and Chou, 1991).

There have been several methods developed for the quantification of drug interactions including the isobologram method, fractional-product method and median-effect principle. The classical isobologram method is only valid for drugs whose effects are mutually exclusive. The fractional-product method is valid only for those drugs whose effects are mutually non-exclusive and have a hyperbolic concentration-response curve. Because of these limitations, these methods cannot be randomly used (Chou and Talalay, 1984). The median effect principle, however, is a more basic methodology that requires fewer assumptions and can be generalized to a greater number of situations including cancer treatment. Analysis with this method is based on the law of mass action and does not require prior knowledge of the specific mechanisms of action of the drugs involved.

Combining drugs also allows for the biochemical modulation of one drug's effects by the other. An example of this would be leucovorin (LV) given in combination with 5-FU which is now a standard treatment approach in colorectal cancer (Blaszkowsky, 1998; Labianca et al., 1996). For this paper, we will examine the modulating effects that 5-FU and tomudex have on gemcitabine toxicity. It is known that gemcitabine has activity against pancreatic cancer (section 1.2; Heinemann, et al., 1998; Peters et al., 1995; Bergman, 1996). It has also been previously shown that the equilibrative-sensitive nucleoside transporters can be upregulated by administering a DNA synthesis inhibitor such
as 5-FU or tomudex (Pressacco et al., 1995). We wish to examine the combination of 5-FU and tomudex with gemcitabine to determine if gemcitabine toxicity can be modulated. It is also important to note that the modulating agents used here also exhibit their own cytotoxicity through inhibition of the de novo pathway of DNA synthesis. Therefore, cytotoxic actions of the two drugs are acting on the different pathways of DNA synthesis, de novo and salvage pathways. These differing mechanisms of action make for an appealing combination approach.

Previous studies examining the interaction of gemcitabine and 5-FU have been conducted to date. These studies used clinical benefit response as an end point of efficacy in addition to objective tumour response. Hidalgo et al. (1999) and Cascinu et al. (1998) found that 50% of patients achieved a clinical benefit response. Half of the patients who achieved clinical benefit in the latter study did not achieve an objective tumour response. In a dose-escalation study of gemcitabine (starting at 700 mg/m²/week for 3 weeks of a 4 week cycle) and fixed dose 5-FU (200 mg/m²/day) by Cortes-Funes et al (1998), 55% of patients displayed a clinical benefit response and a median survival of 10.4 months was reported.
Chapter 2: RATIONALE, HYPOTHESIS AND OBJECTIVES
2.1 Rationale Behind Project

Pancreatic cancer is the fifth leading cause of cancer death in North America (Clark, et al., 1996; Schnall and Macdonald, 1996; Moore, 1996; Rothenberg, 1996; Robertson et al., 1996) and also has the worst five-year survival rate of any cancer (Clark et al., 1996; Moore 1996). For these reasons, as well as the resistance of pancreatic cancer to current chemotherapy, research to improve therapy is desperately needed. Recently, gemcitabine, a nucleoside analogue, has demonstrated more efficacy towards pancreatic tumours than other drugs, such as 5-FU, cisplatin and cytosine arabinoside (ara-C) (Heinemann, et al., 1988; Peters et al., 1995; Bergman, 1996).

As gemcitabine has been shown to exhibit efficacy in the treatment of pancreatic cancer, examining methods to modulate this effect would be of great interest and of potential clinical benefit. One method to accomplish this would be to manipulate cells so that greater amounts of the drug are taken up by increasing the number of nucleoside transport sites. More transporters should lead to increased uptake of the drug and possibly to increased efficacy. Therefore, the purpose of the work reported in this thesis was to define a new regimen of combination chemotherapy using gemcitabine and agents that upregulate nucleoside transporters, that has the potential to be used in the clinic for the treatment of pancreatic cancer.
2.2 Hypothesis

(1) The sensitivity of human pancreatic and bladder carcinoma cell lines to gemcitabine is related to their basal equilibrative-sensitive nucleoside transporter (es-NT) expression.

(2) Upregulation of es-NT in human pancreatic and bladder cancer cell lines by pre-treatment with the thymidylate synthase inhibitors 5-FU or tomudex will increase the efficacy of gemcitabine therapy.

2.3 Objectives

The experimental approach addresses the potential use of gemcitabine in combination with either 5-FU or tomudex and provides insight into the role of drug transport in cytotoxicity when the drugs are combined.

(1) Determine the sensitivity of one human bladder cancer (MGH-U1) and three human pancreatic cancer (PANC-1, PK-8, HS-766T) cell lines to gemcitabine, 5-FU and tomudex given alone and in combination. The MGH-U1 cell line was used as a method control as previous experiments demonstrating es-NT upregulation by 5-FU and tomudex were performed on this cell line.

(2) Determine the basal levels of the es-NT in all four human tumour cell lines.
(3) Determine the relationship between drug sensitivity and es-NT expression.

(4) Explore and gain insight into the relationship between sequence of drug administration, es-NT expression and cytotoxicity.
Chapter 3: MATERIALS AND METHODS
3.1 Cell Lines and Reagents

The cell lines used for these experiments were the human PANC-1, HS-766T and PK-8 pancreatic carcinoma cell lines and the human MGH-U1 bladder carcinoma cell line. PANC-1, HS-766T and MGH-U1 cell lines were originally purchased from the American Type Culture Collection (Rockville, MD, USA). PK-8 cells were provided by Dr. D. Hedley (Ontario Cancer Institute, Toronto, ON, Canada). Cells were maintained as monolayer cultures as follows: PANC-1 and HS-766T were cultured in Dulbecco’s Modified Eagle’s Medium, (Media Department, Ontario Cancer Institute, ON, Canada) in a humidified 10% CO₂ incubator at 37°C. PK-8 cells were cultured in RPMI 1640 growth medium, (Media Department, Ontario Cancer Institute, ON Canada). MGH-U1 cells were cultured in α-MEM growth medium (Media Department, Ontario Cancer Institute, ON, Canada). Both PK-8 and MGH-U1 cells were maintained in a humidified 5% CO₂ incubator at 37°C. All growth media were prepared at pH = 7.25, contained penicillin (100 mg/mL) and streptomycin (100 mg/mL) and was supplemented with 10% fetal Bovine Serum (Whitaker Bioproducts Inc., Walkersville, MD, USA). RPMI 1640 medium was also supplemented with HEPES buffer (10 mM). Cells were trypsinized (0.2% trypsin (Difco Laboratories, Detroit, MI, USA) in phosphate buffered saline (PBS)), washed and replaced when necessary.

Unless otherwise specified, all reagents were purchased from the Sigma Chemical Company (St. Louis, MO, USA). Gemcitabine was provided by Eli Lilly Pharmaceuticals (Indianapolis, IN, USA). Tomudex was provided by Zeneca Pharma (Mississauga, ON, Canada). Stock solutions of gemcitabine in PBS, 5-
FU in hydrochloric acid (0.1 M) and tomudex in PBS were prepared and stored at -20°C.

3.2 Cell Cytotoxicity Assay

Cytotoxicity was assessed by clonogenic assay. Cells were harvested during the logarithmic phase of cell growth, trypsinized, washed, counted and resuspended at concentrations of 1-5x10^5 cells/mL. 1 mL of cell suspension was plated in 25cm² flasks (Nunc, Roskilde, Denmark) containing 4 mL of the appropriate growth medium. Following a 24 hour incubation, 5 µL of drug solution was added to each flask for a 1:1000 dilution. PBS or hydrochloric acid (0.1 M) were added as vehicle controls for the appropriate experiments.

3.2.1 Single Agent Exposures

Following 24 hour exposures, cells were washed with PBS, trypsinized, centrifuged (1200 rpm) (Beckman Instruments Inc., Palo alto, CA, USA), resuspended in the appropriate growth medium, counted and 100 – 10,000 cells were plated in 60 mm² petri dishes. Cells were incubated for 7 days (MGH-U1), 10 days (PANC-1, PK-8) or 14 days (HS-766T). The resulting colonies were fixed and stained with a 1% (w/v) methylene blue solution (BIO RAD, Hercules, CA, USA).

Visible colonies (>40 cells/colony) were counted to determine the colony forming efficiency for each drug concentration. Fractional survival was calculated by dividing the number of colonies formed in drug-treated plates by
the number of colonies formed in the control plates. The IC$_{50}$ and IC$_{90}$, defined as the drug concentration that inhibited cloning efficiency (colony formation efficiency) by 50% or 90% respectively, were estimated for each drug in all cell lines by plotting fractional survival versus drug concentration.

3.2.2 Drug Combination Exposures

Cells were exposed to gemcitabine prior to, at the same time as, or following treatment with either 5-FU or tomudex for 24 hours. For concurrent drug combinations, the procedure was the same as for single drug exposures. For sequential drug combinations, following the first 24 hour exposure, medium was removed by aspiration, cells were washed twice with PBS and 5 mL of fresh medium was added prior to the addition of new drug, to which the cells were exposed for a further 24 hours.

3.3 5-(SAENTA-x8)-Fluorescein (5x8) Binding Assay

Es-specific binding of 5x8 was measured in all cell lines using a flow cytometric assay as described by Gati et al (1997). Briefly, cells were trypsinized, counted and resuspended in growth medium at a concentration of at least 2x10$^6$ cells/mL. Cell suspensions were then diluted with an equal volume of either PBS (for assay of total binding of the 5x8 probe) or PBS containing 10 µM NBMPR (for assay of non-specific binding of the probe) and kept at room temperature for at least 30 minutes. These suspensions were then added to graded concentrations of 5x8 (0-20 nM) in the absence or presence of 5 µM
NBMPR. 5x8 was allowed to equilibrate with cellular binding sites for 15 minutes before flow cytometric measurement of cell-associated fluorescence was carried out. Samples were analysed on an Epics XL II flow cytometer (Coulter Corporation, Hialeah, FL, USA). Data were gated on light scatter before recording of a fluorescence histogram composed of at least 10,000 cells. Fluorescence histograms for each condition were generated. Mean fluorescence intensity values for the fluorescence histograms were determined using Epics XL II software (Coulter Corporation, Hialeah, FL, USA). Es specific binding of 5x8 was calculated as the difference in mean fluorescence intensities between total and non-specific binding. To allow for day-to-day variation in the settings and performance of the flow cytometer, fluorescent beads containing known numbers of fluorescein molecules (rainbow calibration particles, RCP-30-5) (Spherotech, Libertyville, ILL, USA) were analyzed by the cytometer prior to each experiment. Calibration curves of molecules of equivalent soluble fluorescein (MESF) against mean fluorescence intensity were constructed following each assay to enable specific binding of the 5x8 probe to be converted to MESF.

Es transport sites were quantified using a modification of the above-described procedure. Three cell suspensions were used for each control or drug treatment. Cell suspensions were added to a single, es site-saturating concentration of 5x8 (determined from above assay) in the absence and presence of 5 μM NBMPR to assay for total and non-specific binding of the probe, and to PBS containing no 5x8 or NBMPR for assay of background autofluorescence of the cells and growth medium.
Chapter 4: RESULTS
4.1 Growth Curves

Figures 4.1a-d show that the MGH-U1, PK-8, PANC-1 and HS-766T cell lines exhibited the characteristic growth pattern of immortalized cell lines. This is an initial lag period, followed by a period of exponential growth followed by a stationary phase where cells are confluent and no cell division occurs and finally a loss in cell number and cell death. Doubling times were calculated using the linear portion of the curve where cells were growing exponentially. The doubling times of the MGH-U1, PK-8, PANC-1 and HS-766T cell lines were 14, 23, 28 and 40 hours respectively.

4.2 Cytotoxicity Assays

Cells growing exponentially were treated with various concentrations of gemcitabine, 5-FU and tomudex for 24 hours and survival measured by the clonogenic assay in order to determine the IC_{50} and IC_{90} values. Following treatment, cells were washed free from drug and re-plated with fresh growth medium supplemented with 10% FBS. Due to the different growth rates, the MGH-U1, PK-8, PANC-1 and HS-766T cell lines were stained 7, 10, 10 and 14 days following re-plating, respectively. The cloning efficiencies of the MGH-U1, PK-8, PANC-1 and HS-766T cells were 21-60%, 24-44%, 36-59% and 18-50% respectively. Experiments in which there were less than 20 colonies on the control plates were discarded. Cell cytotoxicity was expressed as percent colony forming efficiency, calculated as the ratio of the number of colonies (≥40
cells/colony) on the drug treated plate divided by the number of colonies on the control plate.
Figure 4.1a. Growth Curve for the MGH-U1 cell line. Each point represents the mean ± standard deviation of three or more independent experiments.
Figure 4.1b. Growth Curve for the PANC-1 cell line. Each point represents the mean ± standard deviation of three or more independent experiments.
Figure 4.1c. Growth curve for the HS-766T cell line. Each point represents the mean ± standard deviation of three or more independent experiments.
Figure 4.1d. Growth Curve for the PK-8 cell line. Each point represents the mean ± standard deviation of three or more independent experiments.
4.2.1 Single Drug Exposures

Log concentration-response curves of all four cell lines following exposure to either gemcitabine, 5-FU or tomudex are illustrated in figures 4.2a-d. As indicated, all fits have a regression coefficient \( \geq 0.90 \). Relative sensitivities to individual agents at the IC\(_{50}\) versus the IC\(_{90}\) levels differed due to the slope of the log concentration-response curves and are shown in tables 4.2a-b. With the exception of the PANC-1 cell line, where a plateau was reached, an increase in drug concentration resulted in an increase in cell cytotoxicity. As illustrated in table 4.2a, at the IC\(_{50}\) concentration, sensitivity to gemcitabine for all cell lines was as follows: MGH-U1 (1.5 nM) > HS-766T (4 nM) > PANC-1 (40 nM) >> PK-8 (35 \(\mu\)M). At IC\(_{50}\) concentrations of 5-FU, cellular sensitivity was as follows: PANC-1 (230 nM) > MGH-U1 (500 nM) > HS-766T (2.75 \(\mu\)M) > PK-8 (4 \(\mu\)M). Sensitivity to tomudex at IC\(_{50}\) concentrations was as follows: PANC-1 (0.02 nM) > PK-8 (0.04 nM) > HS-766T (3 nM) > MGH-U1 (20 nM).

4.3 Combination Studies

The effect of combining gemcitabine with either 5-FU or tomudex at a range of concentrations was determined by exposing cells to the combination either concurrently for 24 hours, or sequentially, with each exposure lasting 24 hours. Table 4.3a illustrates the cytotoxicity of the agents used when acting alone (data estimated from log concentration-response curves, figures 4.2a-c).
Figure 4.2a. Log concentration-response curves following 24 hour exposure of the MGH-U1 cell line to (■) gemcitabine, (●) 5-FU and (▲) tomudex. Cytotoxicity was determined by the colony-forming assay. Each point represents the mean of three independent experiments; each line has an $r^2$ value $\geq 0.90$. 

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Figure 4.26. Log concentration-response curves following 24 hour exposure of the PANC-1 cell line to (■) gemcitabine, (●) 5-FU and (▲) tomudex. Cytotoxicity was determined by the colony-forming assay. Each point represents the mean of three independent experiments; each line has an $r^2$ value $\geq 0.90$. 
Figure 4.2c. Log concentration-response curves following 24 hour exposure of the HS-766T cell line to (■) gemcitabine, (●) 5-FU and (▲) tomudex. Cytotoxicity was determined by the colony-forming assay. Each point represents the mean of three independent experiments; each line has an $r^2$ value $\geq 0.90$. 

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Figure 4.2d. Log concentration-response curves following 24 hour exposure of the PK-8 cell line to (■) gemcitabine, (●) 5-FU and (▲) tomudex. Cytotoxicity was determined by the colony-forming assay. Each point represents the mean of three independent experiments; each line has an $r^2$ value $\geq 0.90$. 

% Survival

Concentration [µM]
Table 4.2a. Drug concentrations required to inhibit colony formation by 50% following 24 hour drug exposures. Values were estimated from the log concentration-response curves.

<table>
<thead>
<tr>
<th></th>
<th>MGHU1</th>
<th>PANC-1</th>
<th>HS-766T</th>
<th>PK-8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gemcitabine</td>
<td>1.5 nM</td>
<td>40 nM</td>
<td>4 nM</td>
<td>35 μM</td>
</tr>
<tr>
<td>5-FU</td>
<td>500 nM</td>
<td>230 nM</td>
<td>2.75 μM</td>
<td>4 μM</td>
</tr>
<tr>
<td>Tomudex</td>
<td>20 nM</td>
<td>0.02 nM</td>
<td>3 nM</td>
<td>0.04 nM</td>
</tr>
</tbody>
</table>

Table 4.2b. Drug concentrations required to inhibit colony formation by 90% following 24 hour drug exposures. Values were estimated from the log concentration-response curves. -- indicates IC$_{90}$ concentration was not achieved.

<table>
<thead>
<tr>
<th></th>
<th>MGH-U1</th>
<th>PANC-1</th>
<th>HS-766T</th>
<th>PK-8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gemcitabine</td>
<td>750 nM</td>
<td>4 μM</td>
<td>250 nM</td>
<td>--</td>
</tr>
<tr>
<td>5-FU</td>
<td>--</td>
<td>--</td>
<td>55 μM</td>
<td>65 μM</td>
</tr>
<tr>
<td>Tomudex</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>150 nM</td>
</tr>
</tbody>
</table>
### Table 4.3a. Concentration of agents used in combination experiments.

Numbers in parentheses indicate cell survival when used as a single agent (estimated from log concentration-response curves in figures 4.2a-c).

<table>
<thead>
<tr>
<th>Agent</th>
<th>MGH-U1 (µM% survival)</th>
<th>PANC-1 (µM% survival)</th>
<th>HS-766T (µM% survival)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gemcitabine</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.8 µM (10%)</td>
<td>1 µM (20%)</td>
<td>0.4 µM (8%)</td>
<td></td>
</tr>
<tr>
<td>1 µM (45%)</td>
<td>1 µM (35%)</td>
<td>1 µM (60%)</td>
<td></td>
</tr>
<tr>
<td>3 µM (40%)</td>
<td>3 µM (32%)</td>
<td>3 µM (50%)</td>
<td></td>
</tr>
<tr>
<td>10 µM (30%)</td>
<td>10 µM (31%)</td>
<td>10 µM (30%)</td>
<td></td>
</tr>
<tr>
<td>30 µM (17%)</td>
<td>30 µM (30%)</td>
<td>30 µM (17%)</td>
<td></td>
</tr>
<tr>
<td>100 µM (11%)</td>
<td>100 µM (30%)</td>
<td>100 µM (5%)</td>
<td></td>
</tr>
<tr>
<td><strong>5-FU</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 µM (80%)</td>
<td>1 µM (40%)</td>
<td>1 µM (95%)</td>
<td></td>
</tr>
<tr>
<td>10 nM (35%)</td>
<td>10 nM (40%)</td>
<td>10 nM (60%)</td>
<td></td>
</tr>
<tr>
<td>100 nM (17%)</td>
<td>100 nM (40%)</td>
<td>100 nM (25%)</td>
<td></td>
</tr>
<tr>
<td>1 µM (15%)</td>
<td>1 µM (40%)</td>
<td>1 µM (20%)</td>
<td></td>
</tr>
</tbody>
</table>

**Tomudex**

| 1 nM (80%) | 1 nM (40%) | 1 nM (95%) |
| 10 nM (35%) | 10 nM (40%) | 10 nM (60%) |
| 100 nM (17%) | 100 nM (40%) | 100 nM (25%) |
| 1 µM (15%) | 1 µM (40%) | 1 µM (20%) |
As shown in figures 4.3a-f, pre-treatment of the HS-766T cell line with either 5-FU or tomudex augmented the effects of sole gemcitabine treatment along with the PANC-1 cell line when pre-treated with 5-FU. The concurrent and gemcitabine pre-treatment regimens showed no added benefit over single agent gemcitabine treatment in these cell lines. In the MGH-U1 cell line, no augmentation in cytotoxicity was seen when 5-FU or tomudex was combined in any manner with gemcitabine.
Figure 4.3a. Combination studies of gemcitabine (0.8 μM) and 5-FU in the MGH-U1 cell line following both concurrent (24 hour) and sequential (24 hours per exposure) exposures. Results are expressed as % colony forming efficiency and are the mean ± S.D. of three or more independent experiments.
Figure 4.3b. Combination studies of gemcitabine (0.8 μM) and tomudex in the MGH-U1 cell line following both concurrent (24 hour) and sequential (24 hours per exposure) exposures. Results are expressed as % colony forming efficiency and are the mean ± S.D. of three or more independent experiments.
Figure 4.3c. Combination studies of gemcitabine (1 μM) and 5-FU in the PANC-1 cell line following both concurrent (24 hour) and sequential (24 hours per exposure) exposures. Results are expressed as % colony forming efficiency and are the mean ± S.D. of three or more independent experiments.
Figure 4.3d. Combination studies of gemcitabine (1 μM) and tomudex in the PANC-1 cell line following both concurrent (24 hour) and sequential (24 hours per exposure) exposures. Results are expressed as % colony forming efficiency and are the mean ± S.D. of three or more independent experiments.
Figure 4.3e. Combination studies of gemcitabine (0.4 μM) and 5-FU in the HS-766T cell line following both concurrent (24 hour) and sequential (24 hours per exposure) exposures. Results are expressed as % colony forming efficiency and are the mean ± S.D. of three or more independent experiments.
Figure 4.3f. Combination studies of gemcitabine (0.4 μM) and tomudex in the HS-766T cell line following both concurrent (24 hour) and sequential (24 hours per exposure) exposures. Results are expressed as % colony forming efficiency and are the mean ± S.D. of three or more independent experiments.
4.4 Equilibrative-Sensitive Nucleoside Transporter (es-NT) Quantitation

Flow cytometric analysis was performed on all four cell lines to quantitate basal cell-surface levels of the es-NT and to ensure that these cells did indeed contain es-NT. The total and non-specific binding curves of 5-(S-AENTA-xe)-fluorescein (5x8) in all four cell lines are illustrated in figures 4.4a-d. In every case there was found to be binding of the probe that was saturable and reversible when exposed in conjunction with the es transport inhibitor NBMPR. An es-NT saturating concentration of 5x8 was defined as that concentration where no further increase in cell-associated fluorescence occurred. This point, at the plateau of the binding curves, was where measurements of basal levels of es-NT were taken. All measurements from the flow cytometric analysis were reported as mean fluorescence intensities. These were converted into molecules equivalent soluble fluorescein (MESF) from calibration curves derived from beads containing known numbers of fluorescein molecules run through the flow cytometer before each experiment (figure 4.4e). As 5x8 binds to the es-NT on a 1:1 ratio, the MESF values for the specific binding of 5x8 correspond to the number of cell-surface es transport sites per cell. The cellular quantity of these cell surface es-NT (expressed as MESF) for the four cell lines used was as follows: PANC-1 (14148) > PK-8 (4792) ~ MGH-U1 (4281) > HS-766T (1216). This is illustrated in table 4.4a.
Figure 4.4a. Binding curves of 5x8 in the (■) absence and (○) presence of NBMPR in the MGH-U1 cell line. Specific binding may be calculated by subtracting non-specific binding (in the presence of NBMPR) from total binding (in the absence of NBMPR).
Figure 4.46. Binding curves of 5x8 in the (■) absence and (○) presence of NBMPR in the PANC-1 cell line. Specific binding may be calculated by subtracting non-specific binding (in the presence of NBMPR) from total binding (in the absence of NBMPR).
Figure 4.4c. Binding curves of 5x8 in the (■) absence and (●) presence of NBMPR in the HS-766T cell line. Specific binding may be calculated by subtracting non-specific binding (in the presence of NBMPR) from total binding (in the absence of NBMPR).
Figure 4.4d. Binding curves of 5x8 in the (●) absence and (○) presence of NBMPR in the PK-8 cell line. Specific binding may be calculated by subtracting non-specific binding (in the presence of NBMPR) from total binding (in the absence of NBMPR).
Figure 4.4e. Sample calibration curve for flow cytometric analysis using the rainbow calibration beads (RCP-30-5). $r^2 > 0.99$. 
Table 4.4a. Specific binding of SAENTA (molecules equivalent soluble fluorescein (MESF) per cell) and gemcitabine sensitivities for four human cancer cell lines prior to treatment with drug. These correspond to basal levels of cell surface es-NT and IC$_{50}$ values respectively.

<table>
<thead>
<tr>
<th></th>
<th>MESF / cell</th>
<th>Gemcitabine IC$_{50}$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MGH-U1</td>
<td>4281</td>
<td>4</td>
</tr>
<tr>
<td>PANC-1</td>
<td>14148</td>
<td>1.5</td>
</tr>
<tr>
<td>HS-766T</td>
<td>1216</td>
<td>35 000</td>
</tr>
<tr>
<td>PK-8</td>
<td>4792</td>
<td>40</td>
</tr>
</tbody>
</table>
Following treatment of the MGH-U1, PANC-1 and HS-766T cell lines with varying concentrations of either gemcitabine, tomudex or 5-FU, cells were analyzed for es-NT content. As shown in table 4.4b, treatment of the PANC-1 cell line with 1 μM dFdC and 30 μM 5-FU for 24 hours showed a significant (p < 0.05) increase in cell-surface es-NT content over basal levels. In addition, treatment of the HS-766T pancreatic carcinoma cell line with 30 μM and 100 μM 5-FU and 100 nM and 1000 nM tomudex all resulted in a significant (p < 0.05) increase in es-NT sites versus control levels. All other treatment groups for these three cell lines showed no difference in es-NT content compared with basal levels.
<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Treatment</th>
<th>Factor Increase in MESF over control</th>
</tr>
</thead>
<tbody>
<tr>
<td>MGH-U1</td>
<td>20 μM 5-FU</td>
<td>0.93</td>
</tr>
<tr>
<td></td>
<td>20 μM 5-FU x 48 hr</td>
<td>0.70</td>
</tr>
<tr>
<td></td>
<td>40 μM 5-FU</td>
<td>1.10</td>
</tr>
<tr>
<td></td>
<td>100 μM 5-FU</td>
<td>1.57</td>
</tr>
<tr>
<td></td>
<td>100 nM Tomudex</td>
<td>1.66</td>
</tr>
<tr>
<td></td>
<td>1 μM Tomudex</td>
<td>1.41</td>
</tr>
<tr>
<td></td>
<td>10 nM Gemcitabine</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td>800 nM Gemcitabine</td>
<td>1.03</td>
</tr>
<tr>
<td>PANC-1</td>
<td>30 μM 5-FU</td>
<td>**1.63</td>
</tr>
<tr>
<td></td>
<td>100 μM 5-FU</td>
<td>1.50</td>
</tr>
<tr>
<td></td>
<td>100 nM Tomudex</td>
<td>1.91</td>
</tr>
<tr>
<td></td>
<td>1 μM Tomudex</td>
<td>1.72</td>
</tr>
<tr>
<td></td>
<td>40 nM Gemcitabine</td>
<td>0.81</td>
</tr>
<tr>
<td></td>
<td>1 μM Gemcitabine</td>
<td>**1.36</td>
</tr>
<tr>
<td>HS-766T</td>
<td>30 μM 5-FU</td>
<td>**1.68</td>
</tr>
<tr>
<td></td>
<td>100 μM 5-FU</td>
<td>**1.70</td>
</tr>
<tr>
<td></td>
<td>100 nM Tomudex</td>
<td>**1.86</td>
</tr>
<tr>
<td></td>
<td>1 μM Tomudex</td>
<td>**1.40</td>
</tr>
</tbody>
</table>

**Table 4.4b.** Factor increase over control in specific binding of SAENTA after treatment with either 5-FU, tomudex or gemcitabine. All incubations were for 24 hours unless otherwise indicated. ** indicates statistically significant difference (p < 0.05, t-test) between treatment group and control.
Chapter 5: DISCUSSION
Characterizing the various factors that affect cellular differences in cytotoxicity to a chemotherapeutic agent can provide insight into how one might modulate these effects. This thesis focused on the nucleoside transporter. Specifically, the role that the es-NT plays in gemcitabine cytotoxicity. We hypothesized that the sensitivity to gemcitabine would be influenced by basal levels of es-NT. If this proved true, it would follow that manipulation of the amount of es-NT could alter cellular sensitivity to gemcitabine.

DNA synthesis inhibitors such as 5-FU and tomudex have been previously shown to upregulate the number of cell surface es-NT's (Pressacco et al., 1995a). These results point towards the usefulness of these agents as possible modulators of gemcitabine toxicity. We hypothesized that if we could increase the amount of es-NT on the cell surface, then greater amounts of gemcitabine would enter the cell leading to increased cytotoxicity. In examining this hypothesis, it is important to realize that both 5-FU and tomudex are cytotoxic antimetabolite drugs that will exert their own cytotoxic actions on de novo DNA synthesis in addition to any nucleoside transporter-related effects. These cytotoxic effects occur through different mechanisms than those caused by gemcitabine; TS inhibition versus incorporation into DNA. We therefore are interested in these drugs' effects on the es-NT and in their combined cytotoxic effects so that we may gain additional insight into this novel combination regimen.
5.1 Single Drug Cytotoxicity

The cytotoxicity of gemcitabine, 5-FU and tomudex in the MGH-U1, PANC-1, HS-766T and PK-8 cell lines was initially determined. Growth inhibition was found to be concentration dependent for all three drugs in all four cell lines. The log concentration-response curves for tomudex in the MGH-U1, PANC-1 and HS-766T cell lines as well as that for 5-FU in the HS-766T cell line appeared to reach a plateau. In addition, the log concentration-response curves for all three drugs in the PANC-1 cell line seemed to reach a plateau. The log concentration-response curves for gemcitabine and 5-FU continued to decline for all other cell lines, an indication that maximal cytotoxicity has not yet been attained. Due to this plateau effect mentioned earlier, IC$_{90}$ values were not obtained for all drugs in every cell line.

All the drugs used in this study belong to the anti-metabolite class of oncolytic agents. These agents all act as inhibitors of macromolecule biosynthesis which block cellular replication. Antimetabolites generally compete with endogenous substrates in cellular metabolic processes to accomplish this goal. Gemcitabine is in competition with dCyd, 5-FU with dUMP and tomudex with the endogenous cofactor for TS (5,10-methylene tetrahydrofolate). For the TS inhibitors 5-FU and tomudex, it is not surprising to observe a plateau in the log concentration-response curves. Once TS is completely inhibited, cytotoxic effects are at a maximum and additional drug present in the cell will not significantly enhance these effects. Gemcitabine must first be taken up into the cell via a saturable transport process. Once transport has reached saturating
levels, any additional drug present will not serve to increase cytotoxicity. Therefore, we would again expect a plateau to occur in the log concentration-response plots for gemcitabine. In those cell lines having greater amounts of functional transporters we would expect this plateau effect to occur farther along the log concentration-response curve. This trend, however, was not observed and could be accounted for by non-functional transporter present in the cells or by decreased dCK efficiency.

5.1.1 Gemcitabine

The log concentration-response curves for gemcitabine are shown in figures 4.2a-d. The MGH-U1 cell line was the most sensitive to gemcitabine at its IC\textsubscript{50} concentration (1.5 nM), having an IC\textsubscript{50} value 2.7, 26.7 and 2.3 x 10\textsuperscript{4} times lower than for the HS-766T, PANC-1 and PK-8 cell lines respectively. No IC\textsubscript{90} value was reached for the PK-8 cell line. For the other cell lines, the relative sensitivities to gemcitabine changed so that an IC\textsubscript{90} value of 250 nM for the HS-766T cell line was 3 and 16 fold lower than those for the MGH-U1 and PANC-1 cell lines respectively (tables 4.2a,b).

Gemcitabine has also been tested in several other human cell lines including ovarian (A2780, OVCAR-3), colon (WiDr, C26-10) and squamous cell carcinoma (UM-SCC-14C, UM-SCC-22B) cell lines. Table 5.1a compares gemcitabine sensitivities (24 hour exposure) in these cell lines with those in our cell lines at the IC\textsubscript{50} level. Aside from our gemcitabine resistant PK-8 cell line, the IC\textsubscript{50} values all fall in the nanomolar range.
<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Doubling Time (hrs)</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MGH-U1 (bladder)</td>
<td>14</td>
<td>1.5</td>
</tr>
<tr>
<td>C26-10 (colon)</td>
<td>23</td>
<td>22</td>
</tr>
<tr>
<td>PK-8 (pancreas)</td>
<td>23</td>
<td>35 000</td>
</tr>
<tr>
<td>A2780 (ovarian)</td>
<td>26</td>
<td>2</td>
</tr>
<tr>
<td>PANC-1 (pancreas)</td>
<td>28</td>
<td>40</td>
</tr>
<tr>
<td>UM-SCC-14C (squamous cell)</td>
<td>32</td>
<td>6</td>
</tr>
<tr>
<td>WiDr (colon)</td>
<td>32</td>
<td>51</td>
</tr>
<tr>
<td>OVCAR3 (ovarian)</td>
<td>37</td>
<td>2</td>
</tr>
<tr>
<td>HS-766T (pancreas)</td>
<td>40</td>
<td>4</td>
</tr>
<tr>
<td>UM-SCC-22B (squamous cell)</td>
<td>50</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 5.1a. Cell doubling times and sensitivities to gemcitabine after 24 hour exposure in several human tumour cell lines. Cell lines defined in text. Data for cell lines not from this work taken from Ruiz Van Haperen et al., 1994.
Gemcitabine is a cell cycle specific agent, with maximal cytotoxicity occurring during S-phase. We would therefore expect cell lines with shorter doubling times to be more sensitive to the cytotoxic actions of gemcitabine. The MGH-U1 cell line has the shortest doubling time, followed by the PK-8, PANC-1 and HS-766T cell lines. This, however, does not correspond with cell sensitivities at the IC_{50} values. This agrees with previous data from other human carcinoma cell lines mentioned earlier which also shows no correlation between cell doubling time and gemcitabine sensitivity (Ruiz Van Haperen et al., 1994) (Table 5.1a).

5.1.2 5-Fluorouracil

With the exception of the PANC-1 cell line, the log concentration-response curves of 5-FU continue to decrease at the highest drug concentrations used, indicating that maximal cell cytotoxicity has not yet been reached (figures 4.2a-d). 5-FU was most sensitive at its IC_{50} value of 230 nM in the PANC-1 cell line. This was 2.2, 12 and 17.4 fold lower than the IC_{50} values in the MGH-U1, HS-766T and PK-8 cell lines respectively. An IC_{90} value was only reached in the HS-766T and PK-8 cell lines and was 55 μM and 65 μM respectively (table 4.2a,b).

5-FU has been well studied in human colon tumour cells. A comparison of our bladder and pancreatic cell line sensitivities with several colon carcinoma cell lines may be seen in table 5.1b. The pancreatic cell lines used for this work are more sensitive to 5-FU than almost all of these colon tumour cell lines.
Table 5.1b. Cell doubling times and sensitivities to 5-FU and tomudex following 24 hour exposure in several human tumour cell lines. Cell lines defined in text. Data for cell lines not from this work taken from Van Triest et al., 1999.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Doubling Time (hrs)</th>
<th>IC₅₀ (µM)</th>
<th>IC₅₀ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MGH-U1 (bladder)</td>
<td>14</td>
<td>0.50</td>
<td>20</td>
</tr>
<tr>
<td>Colo201 (colon)</td>
<td>17.3</td>
<td>8.5</td>
<td>42</td>
</tr>
<tr>
<td>Colo205 (colon)</td>
<td>18</td>
<td>7.5</td>
<td>29</td>
</tr>
<tr>
<td>LoVo (colon)</td>
<td>21</td>
<td>3</td>
<td>6.5</td>
</tr>
<tr>
<td>Colo320 (colon)</td>
<td>22</td>
<td>4</td>
<td>48</td>
</tr>
<tr>
<td>PK-8 (pancreas)</td>
<td>23</td>
<td>4</td>
<td>0.04</td>
</tr>
<tr>
<td>HT29 (colon)</td>
<td>23</td>
<td>16</td>
<td>26</td>
</tr>
<tr>
<td>SW948 (colon)</td>
<td>26</td>
<td>4.7</td>
<td>6.9</td>
</tr>
<tr>
<td>PANC-1 (pancreas)</td>
<td>28</td>
<td>0.23</td>
<td>0.02</td>
</tr>
<tr>
<td>HS-766T (pancreas)</td>
<td>40</td>
<td>2.75</td>
<td>3</td>
</tr>
</tbody>
</table>
5-FU is also a cell cycle, S-phase specific cytotoxic agent and like gemcitabine, drug sensitivity in the four cell lines used and those listed in table 5.1b could not be explained by doubling times.

5.1.3 Tomudex

The log concentration-response curves for tomudex reached a plateau in all cell lines except for PK-8, in which maximal cytotoxicity was never attained (figures 4.2a-d). At its IC₅₀ concentration, tomudex was most sensitive in the PANC-1 cell line (0.02 nM), which had an IC₅₀ value 2, 150 and 1000 fold lower than for the PK-8, HS-766T and MGH-U1 cell lines respectively. Only in the PK-8 cell line was an IC₉₀ value reached (150 nM) (tables 4.2a,b).

Like 5-FU, tomudex has been studied extensively in human colon cancer cells. Table 5.1b shows that all of the pancreatic cancer cell lines used in our study were more sensitive to tomudex than the colon carcinoma cells. This indicates that tomudex may prove to be a beneficial agent in chemotherapy of the pancreas. Once again, cell cytotoxicity for a cell cycle specific agent did not correlate to cell doubling times.

5.2 Drug Combinations

In designing a clinically useful combination therapy regimen, it is desirable that the drugs have different, non-overlapping toxicities and different mechanisms of action. Having non-overlapping toxicities will ideally allow us to use the maximum tolerated dose of each individual drug when designing the
combination dosing schedule. If the drugs had similar toxicities, we would have to roll back the dosage of each drug when combining them in order to make the regimen tolerable to the patient. Possessing different mechanisms and targets of action prolongs the development of resistance to the therapy because there exist two separate mechanisms and cellular processes to which resistance must develop against. For the combination regimens studied in this work (5-FU & gemcitabine; tomudex & gemcitabine) the drugs involved possessed non-overlapping toxicities and differing mechanisms of action. This is one reason that 5-FU and tomudex make good candidates for combination therapy with gemcitabine.

In order to establish a combination regimen showing increased efficacy against pancreatic tumours over gemcitabine alone, gemcitabine was combined with either 5-FU or tomudex both concurrently and sequentially in three human tumour cell lines (MGH-U1, PANC-1 and HS-766T). Cell cytotoxicity following these combination exposures was dependent upon the cell line, single agent concentrations used and sequence of drug administration. For all combination experiments, a constant concentration of gemcitabine was used within each cell line while varying the concentration of the DNA synthesis inhibitor (5-FU or tomudex) (Table 4.3a). This was done to determine at what concentration(s), if any, would 5-FU or tomudex increase cellular sensitivity to gemcitabine. Pressacco et al. (1995b) have previously reported that DNA synthesis inhibitors, including 5-FU and tomudex, will upregulate es-NT expression in the MGH-U1 cell line. Based on this observation, we hypothesized that if we could upregulate
the es-NT's by pre-treatment with either 5-FU or tomudex, we could increase
gemcitabine cytotoxicity.

5.2.1 Gemcitabine and 5-FU

A single concentration of gemcitabine (IC₈₀, IC₉₀, IC₉₂ concentration in the
PANC-1, MGH-U1 and HS-766T cell lines respectively) was combined with a
range of 5-FU concentrations from 0-100 μM (Table 4.3a). In the MGH-U1 cell
line, no increased effects were seen during any schedule of treatment with 5-FU
and gemcitabine, as the cell survival remained approximately the same as for
gemcitabine treatment alone (10%). At higher concentrations of 5-FU in the
PANC-1 and HS-766T cell lines (30, 100 μM), there appeared to be an
interaction between the two drugs when they were administered in sequence,
where 5-FU was given first (figures 4.3a,c,e).

5.2.2 Gemcitabine and Tomudex

A single concentration of gemcitabine (as above) was combined with
tomudex concentrations ranging from 0-1000 nM. In the MGH-U1 and PANC-1
cell lines, no increased effects were seen versus single agent gemcitabine
treatment with any of the schedules. At the upper range of concentrations of
tomudex in the HS-766T cell line (0.1, 1 μM), increased cell kill was observed
over gemcitabine alone when the cells were pre-treated with tomudex (figures
4.3b,d,f)
5.3  Equilibrative-Sensitive Nucleoside Transporter Expression

One way in which gemcitabine enters the cell is via the es-NT (Heinemann et al., 1995). Through flow cytometric measurements, we quantified the amount of cell-surface es-NT present per cell in each of the four cell lines and showed that: PANC-1 > PK-8 = MGH-U1 > HS-766T. We hypothesized that cell lines containing greater numbers of es-NT’s would show increased cytotoxicity to gemcitabine. This, however, did not prove to be the case, indicating that es-NT levels on their own do not provide a good measure for predicting gemcitabine cytotoxicity. Other factors are most probably playing a greater role in the determination of gemcitabine cytotoxicity than are the es-NT’s. These factors may include: another nucleoside transporter is playing a major role in gemcitabine uptake in some or all of the four cell lines used in this study; efflux of gemcitabine (through the es-NT and/or other mechanisms) may be occurring at different rates in the four cell lines used, with one cell line being more efficient than the others; and finally, the number of es transport sites may not necessarily correspond to their functionality. Functional studies of the es-NT in these cell lines are necessary to fully understand and characterize the relationship between nucleoside transport and gemcitabine cytotoxicity.

In addition to transport-related factors, other cellular determinants may be influencing gemcitabine sensitivity. Intracellular CTP pools – increased pools lead to increased competition with gemcitabine for incorporation into DNA; gemcitabine phosphorylation efficiency by deoxycytidine kinase – increased phosphorylation leads to increased gemcitabine triphosphate concentrations;
gemcitabine elimination efficiency by (d)CMP deaminase – increased elimination leads to decreased intracellular gemcitabine concentrations. Assaying for these cellular properties may lend us further insight into the mechanisms determining gemcitabine sensitivity.

Cell surface es-NT content in the MGH-U1, PANC-1 and HS-766T cell lines was also quantified after treatment with varying concentrations of 5-FU, tomudex or gemcitabine to ascertain whether increased cytotoxicity was a function of increased numbers of cell-surface es-NT’s and also to determine which, if any, of these drugs best modulates gemcitabine cytotoxicity. When gemcitabine was administered as a single agent, cytotoxicity among the four cell lines did not correspond to the relative basal amounts of es-NT in these cell lines. When gemcitabine was administered in a combination regimen where it sequentially followed either 5-FU or tomudex, the es-NT contribution to the modulation of gemcitabine toxicity became more pronounced. We found that in one of the pancreatic carcinoma cell lines (HS-766T) the amount of es-NT increased by a factor of 1.7 over basal levels when these cells were pre-treated with 30 μM and 100 μM 5-FU. When pre-treated with 100 nM and 1000 nM tomudex, the same cell line showed a 1.9 and 1.4 fold increase respectively in cell surface es-NT over basal levels. In the other pancreatic tumour cell line (PANC-1), the cell surface es-NT content increased by a factor of 1.6 over basal levels when the cells were pre-treated with 30 μM 5-FU, but was unaffected by tomudex pre-treatment (Table 4.4b). When pre-treated with 100 μM 5-FU, the PANC-1 cells did exhibit increased cell kill as compared to gemcitabine
monotherapy, but did not show a significant increase in cell surface es-NT. All of these increases were found to be statistically significant (p < 0.05). These concentrations of 5-FU and tomudex were analysed for increased es-NT content because the cytotoxic effects seen during their use in combination studies showed increased cell kill over single agent gemcitabine administration. This evidence lends support to our hypothesis that an increase in cell surface es-NT’s can positively modulate the in vitro cytotoxicity to gemcitabine in the pancreatic tumour cell lines tested.
Chapter 6: CONCLUSIONS AND FUTURE DIRECTIONS
6.1 Conclusions

Following single drug treatments for 24 hour exposures, one log of cell kill was not reached in all cell lines and the sensitivity to 5-FU, gemcitabine and tomudex varied in each cell line. In combination treatments, cell cytotoxicity was unaffected in the human bladder cancer cell line as compared to gemcitabine monotherapy. In the pancreatic carcinoma cell lines, cytotoxicity was influenced by the sequence of drug administration. Pre-treatment of these cell lines with high concentrations of either 5-FU or tomudex yielded increased cell kill as compared to gemcitabine alone.

Gemcitabine sensitivity among cell lines was found to be independent of cell surface es-NT content. Es-NT content was found to be increased in pancreatic cells that were treated with high concentrations of either 5-FU or tomudex for 24 hours. Human bladder carcinoma cells did not show this increase.

In pancreatic tumour cells, we have demonstrated that combinations of 5-FU and gemcitabine as well as tomudex and gemcitabine produce increased cell kill versus gemcitabine monotherapy in a sequence dependent fashion, where 5-FU or tomudex is administered prior to gemcitabine. This effect was observed in the PANC-1 cell line for 5-FU and in the HS-766T pancreatic carcinoma cell line for both DNA synthesis inhibitors used.

While es-NT content seems to play some role in the determination of gemcitabine toxicity, our results suggest that there are other major factors involved. Our studies of the equilibrative-sensitive nucleoside transporter lend us
insight, but do not provide us with the complete picture. The combination regimen of a DNA synthesis inhibitor such as 5-FU or tomudex, followed by gemcitabine seems to show promise in in vitro pancreatic cell line studies. Further exploration and analysis of these treatment regimens may lead to a refined dosing schedule that may be used in the clinic.

6.2 Future Directions

The drugs used for this work were all S-phase specific anti-metabolites and thus required that the cells be actively synthesizing DNA to exert their cytotoxic actions. If the duration of drug exposure is increased, more cells may be in S-phase and thus be susceptible to the actions of gemcitabine, 5-FU and tomudex. Additionally, a longer exposure period to these anti-metabolite drugs will reduce the concentration of drug needed to produce a fixed effect. Cytotoxicity was only assayed for 24 hour (maximum) drug exposures and it may thus be beneficial to examine longer exposures such as 36, 48 and possibly 72 hours.

For the studies in which we quantified the es-NT, we only examined 24-hour drug exposures. Studying longer drug exposures may give us insight into any time-dependence that exists in nucleoside transporter upregulation. Functional, radiolabel studies of the es-NT in the pancreatic cell lines to characterize parameters such as transport rates and efficiencies would also add important pieces to the nucleoside transport picture. Functional and molecular studies to determine what other nucleoside transporter types are present and
expressed in pancreatic cancer cells would enable us to gain greater knowledge into the drug-transporter relationship. This may be accomplished in part by utilizing the fact that cDNAs encoding four human nucleoside transporters have recently been cloned and functionally expressed: es, ei and two that show cit activity (Mackey et al., 1998). Gemcitabine sensitivity (and resistance), therefore, has the potential to be studied in pancreatic tumour cell lines that have been manipulated so they only express a single type of nucleoside transport protein.

This project focussed on the quantitative aspects of the equilibrative-sensitive nucleoside transporter. Studies examining other cellular processes including intracellular CTP pools, dCK and (d)CMPD expression and efficiency may provide added insight into the underlying mechanisms of gemcitabine cytotoxicity among pancreatic cell lines.
Appendix
Appendix A

Statistical Analysis:

<table>
<thead>
<tr>
<th>Treatment</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 µM 5-FU</td>
<td>0.3561</td>
</tr>
<tr>
<td>40 µM 5-FU</td>
<td>0.3998</td>
</tr>
<tr>
<td>100 µM 5-FU</td>
<td>0.1153</td>
</tr>
<tr>
<td>100 nM Tomudex</td>
<td>0.1007</td>
</tr>
<tr>
<td>1 µM Tomudex</td>
<td>0.1527</td>
</tr>
<tr>
<td>10 nM dFdC</td>
<td>0.4388</td>
</tr>
<tr>
<td>800 nM dFdC</td>
<td>0.4410</td>
</tr>
</tbody>
</table>

Table 1: Is there a statistically significant increase in the number of cell surface nucleoside transporters after treatment as compared to basal levels in the MGH-U1 cell line. **Bolded** figures indicate statistically significant differences.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 µM 5-FU</td>
<td>0.0281</td>
</tr>
<tr>
<td>100 µM 5-FU</td>
<td>0.0562</td>
</tr>
<tr>
<td>100 nM Tomudex</td>
<td>0.1027</td>
</tr>
<tr>
<td>1 µM Tomudex</td>
<td>0.0867</td>
</tr>
<tr>
<td>40 nM dFdC</td>
<td>0.0561</td>
</tr>
<tr>
<td>1 µM dFdC</td>
<td>0.0168</td>
</tr>
</tbody>
</table>

Table 2: Is there a statistically significant increase in the number of cell surface nucleoside transporters after treatment as compared to basal levels in the PANC-1 cell line. **Bolded** figures indicate statistically significant differences.
Table 3: Is there a statistically significant increase in the number of cell surface nucleoside transporters after treatment as compared to basal levels in the HS-766T cell line. **Bolded** figures indicate statistically significant differences.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 μM 5-FU</td>
<td>0.0028</td>
</tr>
<tr>
<td>100 μM 5-FU</td>
<td>0.0017</td>
</tr>
<tr>
<td>100 nM Tomudex</td>
<td>0.0040</td>
</tr>
<tr>
<td>1 μM Tomudex</td>
<td>0.0174</td>
</tr>
</tbody>
</table>
References


