Equilibrative-Sensitive Nucleoside Transporter and Its Role in Gemcitabine Sensitivity

David R. Rauchwerger, Patricia S. Firby, David W. Hedley, and Malcolm J. Moore

ABSTRACT

Salvage of preformed nucleosides requires transport across the plasma membrane by sodium-dependent (concentrative) and sodium-independent (equilibrative) mechanisms. These transport systems are also the route of cellular uptake for nucleoside analogues, including gemcitabine (2′,2′-difluorodeoxycytidine), a deoxycytidine analogue used in the treatment of pancreatic cancer. To determine whether gemcitabine cytotoxicity is influenced by the equilibrative-sensitive nucleoside transporter (es-NT), basal levels of the es-NT were quantified in three human pancreatic cancer cell lines (PANC-1, HS-766T, and PK-8) and one human bladder cancer cell line (MGH-U1) by flow cytometric analysis, and the results were compared with gemcitabine cytotoxicity assessed by clonogenic assay. To determine whether the salvage pathway of DNA synthesis can be up-regulated by inhibiting de novo DNA synthesis, combination experiments were carried out using the thymidylate synthase (TS) inhibitors 5-fluorouracil or raltitrexed with gemcitabine in a concurrent and sequential fashion. No relationship between basal es-NT and gemcitabine cytotoxicity was demonstrated. For two pancreatic cell lines, sequence-dependent effects of the combination of TS inhibitors and gemcitabine were seen with maximum effect when the TS inhibitors preceded gemcitabine. This was also associated with a significant increase in es-NT levels caused by the TS inhibitors. Thus, modulation of the es-NT by pretreatment with TS inhibitors may have the potential to improve the therapeutic benefit of gemcitabine.

INTRODUCTION

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 (1). Surgery is the only curative treatment currently available; however, >80% of patients have unresectable disease 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 (2). At present, pancreatic cancer has the worst 5-year survival rate of any cancer; <5% of all pancreatic cancer patients survive 5 years (1, 2). In randomized trials, gemcitabine was the first and only chemotherapy agent that has been shown to have any meaningful impact on either survival or disease-related symptoms in pancreatic adenocarcinoma (3).

Gemcitabine (Gemzar) is a cell cycle-dependent (S-phase-specific) deoxycytidine analogue of the antimetabolite class. It must first be transported into the cell and then be phosphorylated to its active, triphosphate form. Transport of gemcitabine occurs via the NT of which there exist multiple forms (4). Once inside the cell, numerous enzymatic reactions lead to the formation of gemcitabine triphosphate. Incorporation of gemcitabine triphosphate into DNA is most likely the major mechanism by which gemcitabine exerts its cytotoxic actions.

Cells can synthesize nucleotides either through de novo synthesis or via reutilization of nucleotides and nucleobases derived from either the intracellular turnover of nucleic acids and nucleotides or from extracellular sources. In the latter case, known as the salvage pathway, nucleosides and nucleobases must first be transported across the cell membrane by specific transport proteins. Transport inhibitors, such as dipyridamol and dilaze, can enhance the effectiveness of various chemotherapeutic agents, including 5-FU and methotrexate, by modulating either drug influx or efflux and by interfering with the salvage pathways of DNA synthesis. The therapeutic effects of combining a transport inhibitor with an inhibitor of de novo nucleotide biosynthesis to develop a chemotherapeutic regimen in which both the de novo and salvage pathways of DNA synthesis are blocked has been widely explored (5–7). In addition to the endogenous nucleosides, nucleoside analogues are also taken up into the cell via these specific transport proteins (8–10). Therefore, combining a nucleoside analogue with agents that increase NT expression at the cell surface has the potential for increased cell kill. 5-FU and raltitrexed, two antimetabolite de novo DNA synthesis inhibitors, are two such agents (11, 12).

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 (13, 14). Nucleoside transport also plays an important role in a variety of physiological processes including vasoregulation, neurotransmission, platelet aggregation, and lipolysis (15, 16). There is also growing evidence that nucleoside transport processes of intracellular membranes play a role in the intracellular distribution of nucleosides (17).

Sodium-dependent mechanisms of nucleoside transport are limited to specialized cells such as intestinal and renal epithelia, choroid plexus, liver, splenocytes, macrophages and leukemic cells (17–21). These transporters generally mediate influx only and act via active transport processes, depending on cellular ATP for their function.

Sodium-independent, equilibrative nucleoside transport processes mediate the facilitated diffusion of nucleosides across plasma membranes and are widely distributed in different cell types (22). They function bidirectionally in the transmembrane flux of nucleosides in accordance with the concentration gradient. Equilibrative NTs are classified into two subtypes, based on their sensitivities to inhibition by NBMPR and dipyridamol. NBMPR-sensitive (es) transporters bind NBMPR with high affinity, whereas NBMPR insensitive (ei) transporters are unaffected, even by micromolar concentrations of NBMPR. Both display broad substrate specificity for purine and pyrimidine nucleosides. It has been shown previously that depleting the endogenous intracellular nucleotide pools using DNA synthesis inhibitors, such as 5-FU or raltitrexed, can increase es-NT abundance at the cell surface (11, 12).

The transport process is followed by phosphorylation of the nucleosides by kinases. Computer and kinetic analyses have suggested that the transport of nucleosides is rate-limiting at low (<1 μM) concen-
trations of extracellular nucleosides (8, 9). Once transport becomes saturated, the kinases become the rate-limiting step in nucleotide salvage. Nucleoside levels of this magnitude (1 μM) occur in human serum (23), indicating that nucleoside transport (and cell surface NT activity) may be an important step in the utilization of nucleosides by cells for the salvage pathway of DNA synthesis.

Gemcitabine has been shown to be a substrate for four of the NTs found in humans (es, et, cit, and cib; Ref. 21). The major mediators of gemcitabine uptake, however, are most probably the equilibrative NTs because human cit and cib activity has only been demonstrated in kidney, liver, intestinal epithelium, myeloid leukemic cell lines, freshly isolated myeloblasts, and the CaCo-2 colon cancer cell line (18, 20, 24, 25). In addition, Mackey et al. (21) demonstrated that NT activity was a prerequisite for growth inhibition by gemcitabine in vitro.

Pressacco et al. (11) and Cass (12) have shown previously that TS inhibition leads to increased numbers of es and cit transporters at the cell surface. In this report, we chose to examine the relationship between gemcitabine cytotoxicity and the es transporter, hypothesizing that with increased es transporter activity, gemcitabine will show increased cytotoxicity.

MATERIALS AND METHODS

Chemicals. Unless otherwise specified, all reagents were purchased from the Sigma Chemical Company (Oakville, Ontario, Canada). 5-(SAENTA-x8)-Fluorescein and NBMPR were the gifts of Dr. A. Paterson (University of Alberta, Edmonton, Alberta, Canada) and Dr. J. Wiley (Sydney, Victoria, Australia). Gemcitabine was a gift from Eli Lilly Pharmaceuticals (Indianapolis, IN). Raltitrexed (Tomudex) was a gift from Zeneca Pharma (Mississauga, Ontario, Canada). Trypsin was purchased from Fisher Scientific Canada (Whithy, Ontario, Canada). All growth media were supplied by the media department of the Ontario Cancer Institute (Toronto, Ontario, Canada).

Cell Culture. PANC-1 and HS-766T cell lines were originally purchased from the American Type Culture Collection (Rockville, MD). MGH-U1 cells were a gift from Michigan General Hospital; Dr. M. Tsao (Ontario Cancer Institute, Toronto, Ontario, Canada) provided PK-8 cells. All cell lines were maintained as monolayer cultures, and growth medium was supplemented with 0.1% streptomycin, 0.1% penicillin, and 10% FBS. The human bladder cancer cell line MGH-U1 was cultured in α-MEM at 37°C in 5% CO2. The human pancreatic cancer cell line PK-8 was cultured in RPMI 1640 supplemented with HEPES buffer (10 mM) at 37°C in a 5% CO2 humidified atmosphere. PANC-1 and HS-766T were cultured in Dulbecco’s H21 Modified Eagles Medium and maintained at 37°C in 10% CO2.

Drug Cytotoxicity. Cell survival after drug exposure was assessed by the clonogenic assay. Drugs were added to cells growing exponentially 24 h after plating of 1–5 × 103 cells. Cells were then incubated for 7 (MGH-U1), 10 (PANC-1, PK-8), or 14 days (HS-766T), and the resulting colonies were then counted. For single-agent studies, cells were exposed to drug for 24 h. The IC50 and IC90 values were estimated for each drug in all cell lines by plotting fractional survival versus drug concentration. For combination studies, cells were exposed to gemcitabine for 24 h just prior to, at the same time as, or immediately after treatment with either 5-FU or raltitrexed for 24 h.

5-(SAENTA-x8)-Fluorescein Binding Assay. Binding of 5-(SAENTA-x8)-fluorescein was measured by flow cytometry as described previously (26).

RESULTS

Single-Agent Drug Cytotoxicity. Clonogenic survival was determined in MGH-U1, PANC-1, HS-766T, and PK-8 cells exposed to gemcitabine, 5-FU, or raltitrexed for 24 h (Fig. 1). As determined by IC50 concentration, sensitivity to gemcitabine was: MGH-U1 > HS-766T > PANC-1 >> PK-8; to 5-FU, it was: PANC-1 > MGH-U1 > HS-766T > PK-8; and to raltitrexed, it was: PANC-1 > PK-8 > HS-766T > MGH-U1 (Table 1). In some cell lines, 1 log of cell kill could not be achieved for certain drugs (i.e., gemcitabine in PK-8, 5-FU in MGH-U1 and PANC-1, and raltitrexed in three of the cell lines used).

Combination Drug Exposures. The effect of combining gemcitabine with either 5-FU or raltitrexed at a range of concentrations was studied in MGH-U1, PANC-1, and HS-766T cells. This was determined when cells were exposed concurrently or sequentially, with each exposure lasting 24 h. Pretreatment of the HS-766T cell line with either 5-FU or raltitrexed augmented the effects of single-agent gemcitabine treatment, whereas concurrent treatment or gemcitabine prior to the TS inhibitors did not (Figs. 2 and 3). In the PANC-1 cell line (Fig. 4), treatment with 5-FU prior to gemcitabine increased cytotoxicity over that seen with gemcitabine alone. All other sequences of TS inhibition and gemcitabine had no effect beyond that achieved by gemcitabine alone. In the MGH-U1 cell line, no augmentation in cytotoxicity was seen when either of the TS inhibitors was combined in any manner with gemcitabine.

5-(SAENTA-x8)-Fluorescein Binding Assay. Flow cytometric analysis was performed on all four cell lines to quantitate basal cell surface levels of the es-NT. 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 (Fig. 5). An es-NT saturating concentration of 5-(SAENTA-x8)-fluorescein was defined as that concentration where no further increase in cell-associated fluorescence occurred. This point 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 MESF from calibration curves (linear, \( r^2 > 0.99 \)) derived from beads containing known numbers of fluorescein molecules run through the flow cytometer prior to each experiment. As 5-(SAENTA-x8)-fluorescein binds to the es-NT on a 1:1 ratio, the MESF values for the specific binding of the probe correspond to the number of cell surface es transport sites/cell (26). 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).

**Modulation of the es-NT.** After treatment of the MGH-U1, PANC-1, and HS-766T cell lines with varying concentrations of gemcitabine, 5-FU, or raltitrexed, cells were again analyzed for es-NT content (Table 2). Treatment of the PANC-1 cell line with 1 \( \mu M \) gemcitabine and 30 \( \mu M \) 5-FU for 24 h 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 and 100 \( \mu M \) 5-FU and 100 and 1000 nm raltitrexed all resulted in a significant \(( P < 0.05, t\ test)\) increase in es-NT sites versus basal levels. All other drug exposures for these three cell lines showed no statistically significant difference in es-NT content compared with basal levels.

**DISCUSSION**

TS inhibitors, such as 5-FU and raltitrexed, block the formation of dTMP and deplete intracellular nucleotide pools so that actively proliferating cells then depend on salvage of preformed nucleosides from extracellular fluid. The DNA synthesis inhibitors 5-FU and raltitrexed have been shown previously to up-regulate the number of cell surface es-NTs (11, 12). An increase in these transporters has the potential to augment the effects of drugs like gemcitabine, which enters cells via this mechanism of nucleoside transport. It also has the capacity to demonstrate that an absence or lack of cell surface NTs will give rise to cells that are resistant to the effects of the nucleoside analogue drugs like gemcitabine.

We have shown that in the four human cancer cell lines studied, there is no correlation between basal levels of cell surface es-NT and gemcitabine sensitivity as determined by IC50 concentrations (Table 3). We hypothesized that cell lines containing greater numbers of es-NTs would show increased cytotoxicity when treated with 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, such as other NTs, cellular efflux, and intracellular activation, are most probably playing a greater role in the determination of gemcitabine cytotoxicity. These other factors could also be examined in light of their effects on gemcitabine resistance, because an alteration in their levels may have a more pronounced effect on resistance than cytotoxicity (i.e., the presence of other types of NTs may not affect cytotoxicity; however, their absence may confer resistance). A study of the effects of gemcitabine in cell lines possessing no functional cell surface es-NTs has demonstrated that their presence is a requirement for gemcitabine toxicity (21). Therefore, studies examining the relationship between a decrease in functional es-NTs and the rise of gemcitabine resistance should now be undertaken. In addition, the number of es-NT sites may not necessarily correspond to their functionality. Functional studies of the es-NT in these cell lines are also necessary to fully understand and
characterize the relationship between NT and gemcitabine cytotoxicity and resistance.

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, raltitrexed, or gemcitabine to ascertain whether changes in cytotoxicity would correlate with up-regulation of es-NT. When gemcitabine was administered in a combination regimen to cell lines treated with 100 and 1000 nM raltitrexed, 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 tumor cell line (PANC-1), the cell surface es-NT content increased by a factor of 1.6 over basal levels when the cells were pretreated with 30 μM 5-FU but was unaffected by raltitrexed pretreatment (Table 2). When pretreated with 100 μM 5-FU, the PANC-1 cells did exhibit increased cell kill as compared with gemcitabine monotherapy but did not show a significant increase in cell surface es-NT. All of these increases, although small in magnitude, were found to be statistically significant (P < 0.05, t test) when compared with basal levels. These concentrations of 5-FU and raltitrexed were analyzed 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-NTs can positively modulate the in vitro cytotoxicity to gemcitabine in the pancreatic tumor cell lines tested. From the viewpoint of gemcitabine resistance, these findings also lend support to the assumption that decreased amounts of cell surface es-NTs lead to increased resistance to the cytotoxic effects of gemcitabine.

The relevance of NT to cancer treatment is an area that is rapidly expanding. There exist two aspects of nucleoside transport as it relates to chemotherapy: (a) reduced transporter expression may be associated with resistance to treatment with nucleoside analogue agents such as 1β-D-arabinofuranosylcytosine, 2-chlorodeoxyadenosine, and gemcitabine; and (b) increased cellular salvage capacity for preformed nucleosides from the extracellular fluid is believed to confer resistance to drugs of the antimetabolite class such as methotrexate and 5-FU, which inhibit the de novo process of DNA synthesis. This second aspect can be exploited by combining TS inhibitors with nucleoside analogues. We have shown that gemcitabine cytotox-

Table 2  Factor increase over control in specific binding of 5-(SAENTA-x 8 )-fluorescein after treatment with either 5-FU, raltitrexed, or gemcitabine

<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>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 μM raltitrexed</td>
<td>1.66</td>
</tr>
<tr>
<td></td>
<td>1 μM raltitrexed</td>
<td>1.41</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 μM raltitrexed</td>
<td>1.91</td>
</tr>
<tr>
<td></td>
<td>1 μM raltitrexed</td>
<td>1.72</td>
</tr>
<tr>
<td></td>
<td>40 μM 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 μM raltitrexed</td>
<td>1.86</td>
</tr>
</tbody>
</table>

Table 3  Number of cell-surface equilibrative-sensitive (es) transport sites per cell and gemcitabine sensitivities in all four human tumor cell lines studied

<table>
<thead>
<tr>
<th>Cell line</th>
<th>MESF/cell</th>
<th>Gemcitabine IC_{50} (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MGH-U1</td>
<td>4.281</td>
<td>4</td>
</tr>
<tr>
<td>PANC-1</td>
<td>14.148</td>
<td>1.5</td>
</tr>
<tr>
<td>HS-766T</td>
<td>1.216</td>
<td>35,000</td>
</tr>
<tr>
<td>PK-8</td>
<td>4.792</td>
<td>40</td>
</tr>
</tbody>
</table>
icy increased when administered immediately after a TS inhibitor in two of the human pancreatic cell lines studied. Further studies addressing the functional and molecular aspects of using agents to increase NT expression and their application in clinical use are warranted.

ACKNOWLEDGMENTS

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