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ORIGINAL ARTICLE

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A novel phospholipid gemcitabine conjugate is able to bypass three drug-resistance mechanisms

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Abstract We have previously synthesized a phospholipid–gemcitabine conjugate and a phospholipid–cytosine arabinoside conjugate that we tested in different human cancer cell lines. The gemcitabine conjugate was more cytotoxic to the cancer cells tested than the cytosine arabinoside (ara-C) conjugate. The focus here was to elucidate the mechanism of action of the conjugate molecule and its ability to bypass certain drug-resistance mechanisms. In contrast to gemcitabine, the gemcitabine conjugate did not enter the cell via the human equilibrative nucleoside transporter (hENT1). Additionally, the gemcitabine conjugate was not a substrate for the multidrug resistance efflux pump, MDR-1, even though the molecule is more lipophilic. Finally, we showed that deoxycytidine kinase (dCK) was not required for the activation of the gemcitabine conjugate. As expected, cells overexpressing dCK were more sensitive to gemcitabine whereas cells overexpressing dCK were not more sensitive to the gemcitabine conjugate. Taken together, these results suggest that the gemcitabine conjugate may be therapeutically superior to gemcitabine due to the conjugate's ability to bypass three resistance mechanisms that often render gemcitabine ineffective as an anticancer agent.

Keywords Resistance · Gemcitabine · Phospholipid · Conjugates · Chemotherapy · Drug delivery

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Introduction

Gemcitabine is a deoxycytidine analog [27] that shows activity against different solid tumors [2]. Gemcitabine is translocated across the cellular membrane by nucleoside transporters [13] and it is phosphorylated by deoxycytidine kinase (dCK) to gemcitabine monophosphate [3]. The drug is further phosphorylated to the active form gemcitabine triphosphate, which can be incorporated into DNA and inhibit DNA polymerase, ultimately leading to apoptosis [4, 15, 27]. Gemcitabine may have other mechanisms of action [25–27], including inhibition of ribonucleotide reductase, which causes inhibition of DNA synthesis [11].

Over time, tumor cells can develop different resistance mechanisms to gemcitabine which can render gemcitabine ineffective [6, 16, 30]. Defective dCK activity is a common mechanism of resistance to gemcitabine both in vitro and in vivo (for review see references 2, 8, 17, 31). In addition, increased degradation of gemcitabine by cytidine deaminase can lead to resistance [12, 24]. Resistance mechanisms ultimately lead to decreased amounts of active drug at the desired target site. These mechanisms, when present in patients, are a cause of low survival rates and poor prognostic outcome. Therefore, there is an urgent need for compounds that can bypass these mechanisms of resistance.

With this problem in mind, we chose to chemically link gemcitabine to a synthetic C-1 thioether molecule,

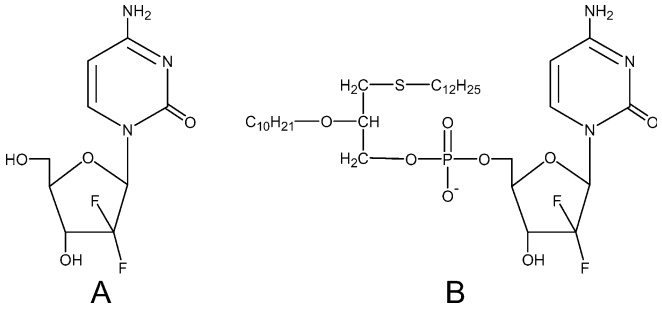

Fig. 1 The chemical structures of gemcitabine (A) and the gemcitabine conjugate (B)

C-2 oxyether phospholipid (Fig. 1). This new chemical entity would not be hydrolyzed at the C-1 and C-2 alkyl side chains in the gastrointestinal tract, and the conjugate would alter the pharmacology of the parent drug, gemcitabine [1]. Previous investigators have conjugated nucleoside analogues to phospholipids that contained ester linkages. These prodrugs are subject to degradation in the gastrointestinal tract by phospholipase A_2 and other lipases that degrade ester-containing lipid molecules [20, 32]. However, previous animal model experiments using 1-dodecylthio-2-decyloxy-propyl-3-phosphatidic acid as a carrier for AZT have demonstrated oral bioavailability [14], and previous structure-activity relationship studies have shown that the phospholipid carrier molecule has a favorable toxicity profile in vitro [19].

An additional advantage of prodrug conjugates is that they may offer an opportunity to bypass resistance mechanisms often observed in the treatment of cancer. The development of drug resistance is an important clinical problem [7], and the construction of chemically modified drugs offers an opportunity to modulate this property. In order to determine if the gemcitabine conjugate would offer a clinical advantage with respect to bypassing resistance mechanisms, we first compared the phospholipid–gemcitabine conjugate with gemcitabine. The conjugate had a different pharmacological profile with a delayed cytotoxicity compared to gemcitabine, and the conjugate bypassed three multidrug resistance mechanisms including MDR-1, the loss of dCK, and the loss of certain nucleoside transporters. Taken together, these findings suggest that the phospholipid–gemcitabine conjugate may have improved efficacy in cases of gemcitabine resistance and therefore the gemcitabine conjugate merits further investigation.

Materials and methods

Reagents and statistical analysis

Tissue culture media and reagents were purchased from Invitrogen, Life Technologies unless otherwise stated. Gemcitabine was purchased from the Wake Forest University Baptist Medical Center Pharmacy. Doxorubicin and etoposide were purchased from Sigma-Aldrich (St. Louis, Mo.). Phenazine methosulfate (PMS) was purchased from Sigma-Aldrich and MTS was purchased from Promega Corporation (Madison, Wis.). MCF-7 (human breast carcinoma) cells transfected with the MDR-1 gene (BC-19), and the pcDNA 3.1 empty vector were kind gifts from Dr. Charles S. Morrow, Department of Biochemistry, Wake Forest University School of Medicine, as previously described [33]. The dCK peptide antibody and the pcDNA 3.1-myc-dCK expression vector were kind gifts from Dr. Iannis Talianidis, Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology, Crete, Greece, and have been described previously [10]. The gemcitabine conjugate was synthesized as previously described [1]. GraphPad Prism (GraphPad Software) was used for all graphs and statistical analysis.

Cell culture

CEM-SS (human T-4 lymphoblastoid clone) and HL-60 (human, promyelocytic leukemia) cells were maintained in RPMI-1640 medium supplemented with 10% FBS. U373-MG cells (human glioblastoma) were maintained in minimum essential medium supplemented with 10% FBS. MCF7/WT-2′ and BC-19 cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/F-12 with 10% FBS, and 10 μg/ml insulin. SCC-25 cells (human squamous cell carcinoma, tongue) were maintained in DMEM with 10% FBS, and 400 ng/ml hydrocortisone. All cells were maintained in log phase and kept in a humidified atmosphere of 5% CO_2/95% air at 37°C. All media also contained penicillin (100 U/ml) and streptomycin (100 μg/ml).

MTS assay

The CellTiter 96 aqueous non-radioactive cell proliferation assay (Promega Life Sciences) was used according to the manufacturer's instructions. Briefly, different numbers of cells (250–5000) were plated on Costar 96-well cell culture cluster plates (Fisher Scientific) and different concentrations (0.38 n M to 100 μM) of gemcitabine or gemcitabine conjugate were added. Gemcitabine was dissolved in water, and the gemcitabine conjugate was sonicated in medium to form lipid aggregates. The cells were exposed for 48, 72, 96, 120, 144, or 168 h and a mixture of MTS and PMS was added. The plates were incubated for 4 h, and the results were read (490 nm) on a precision microplate reader (Molecular Devices, Sunnyvale, Calif.). The optical density of drug-treated and untreated control cells were compared and the data were plotted in GraphPad Prism. Non-linear regression analysis was used to determine IC_{50} values.

Western blot analysis

Cells were harvested by scraping into medium, washed in ice-cold PBS, and the cell pellets were frozen at −80°C until analysis. At the time of analysis, the cell pellets were suspended in ice-cold NP40 lysis buffer (50 m*M* Tris, pH 8.0, 5.0 m*M* EDTA, 150 m*M* NaCl, and 0.5% NP40) containing a cocktail of protease inhibitors, including phenylmethylsulfonyl fluoride (0.1 mg/ml), and the mixture was sonicated for 15 s. Aliquots representing equal amounts of protein from each lysate were separated on a 16% SDS-PAGE and analyzed by Western blotting using a rabbit anti-human caspase-3 polyclonal antibody (Stressgene), a dCK peptide antibody described above, or a mouse anti-myc monoclonal antibody (Cell Signaling Technology, Beverly, Mass.). Primary antibodies were detected using a peroxidase-conjugated secondary antibody and enhanced chemiluminescence according to the manufacturer's instructions (Amersham, Pharmacia Biotech). Equivalent loading and protein transfer were confirmed by Western blotting using a mouse anti-human β-actin antibody (Sigma) and by staining the membranes with Ponceau S (Sigma). Etoposide was dissolved in dimethyl sulfoxide and the final concentration was less than 0.4%. Dimethyl sulfoxide at this concentration did not result in caspase-3 activation (data not shown).

Nucleoside transporter inhibition

The nucleoside transporter assay was performed as previously described [21]. Briefly, cells in log phase were exposed to 10 μ*M* dipyridamole (Sigma) for 30 min in a humidified atmosphere of 5% CO_2/95% air at 37°C prior to drug treatment. Different concentrations (0.38 n*M* to 100 μ*M*) of drug were added to the cells for 72 or 96 h. The MTS assay (as described above) was used to measure IC_{50} values.

SCC-25 transfection

SCC-25 cells were transfected with either a pcDNA 3.1 empty vector or a pcDNA 3.1 vector that encoded the human dCK gene described above. The cells were transfected using FuGENE 6 (Roche Applied Biosciences) according to the manufacturer's instructions. Cells were then selected with 800 μg/ml geneticin (Invitrogen).

Results

Time-course and caspase-3 activation

We first analyzed the time-course of gemcitabine and the gemcitabine conjugate cytotoxicity using four different doses (50, 1.5, 0.4, and 0.02 μ*M*). U373-MG cells were used because our previous studies had shown that the IC_{50} values of gemcitabine and the gemcitabine conjugate were similar in this cell type [1]. The percent survival of each dose versus time was compared. As shown in Fig. 2, at low doses, the gemcitabine conjugate showed a delay in cytotoxicity when compared to gemcitabine. However, at higher doses, both gemcitabine and the gemcitabine conjugate were equally effective at killing U373-MG cells. Similar results were obtained when treating HL-60 cells in a similar fashion (data not shown).

To determine if both gemcitabine and the gemcitabine conjugate were inducing apoptosis, we measured caspase-3 cleavage as an indicator of apoptotic activity. HL-60 cells were exposed to either gemcitabine (10 μ*M*) or the gemcitabine conjugate (10 μ*M*) for different times. The results were compared with those in untreated control cells. Etoposide (100 μ*M*) was used as a positive control of caspase-3 activation [22]. As seen in Fig. 3, activation of caspase-3 was seen in cells treated with gemcitabine and those treated with the gemcitabine conjugate. These results are in agreement with those of previous studies showing that gemcitabine induces apoptosis, and suggest that the gemcitabine conjugate also induces an apoptotic mode of cell death [15].

Nucleoside transporter inhibition

Gemcitabine typically enters cells through human equilibrative nucleoside transporters such as hENT1 or hENT2 and human concentrative nucleoside transporters 1 and 2 (hCNT1 or hCNT2) with hENT1 and hCNT1 being the most efficient transporters for gemcitabine [5, 29]. Since gemcitabine requires one or more of these nucleoside transporters to enter the cell, we wanted to determine if the gemcitabine conjugate also relies on these transporters. Previous studies have shown that dipyridamole (10 μ*M*) can be used 30 min prior to drug treatment to specifically block the hENT1 transporters [5, 21]. First, cells were incubated in the presence or absence of dipyridamole for 30 min prior to drug treatment. Then, different doses of either gemcitabine or gemcitabine conjugate were added; the results are presented in Fig. 4. The IC_{50} value of gemcitabine in U373-MG cells incubated for 72 h with the drug was increased 23-fold ($P = 0.0003$, two-tailed *t*-test) by prior incubation with dipyridamole. However, the IC_{50} values of the gemcitabine conjugate in the presence or absence of dipyridamole were not significantly different ($P > 0.05$, two-tailed *t*-test). Taken together, these results suggest that in contrast to gemcitabine, the gemcitabine conjugate does not enter these cells via the hENT1 transporters that are inhibited by dipyridamole.

Lipophilicity and MDR-1

Drugs that are lipophilic such as doxorubicin (Adriamycin) are substrates for the MDR-1 efflux pump [23].

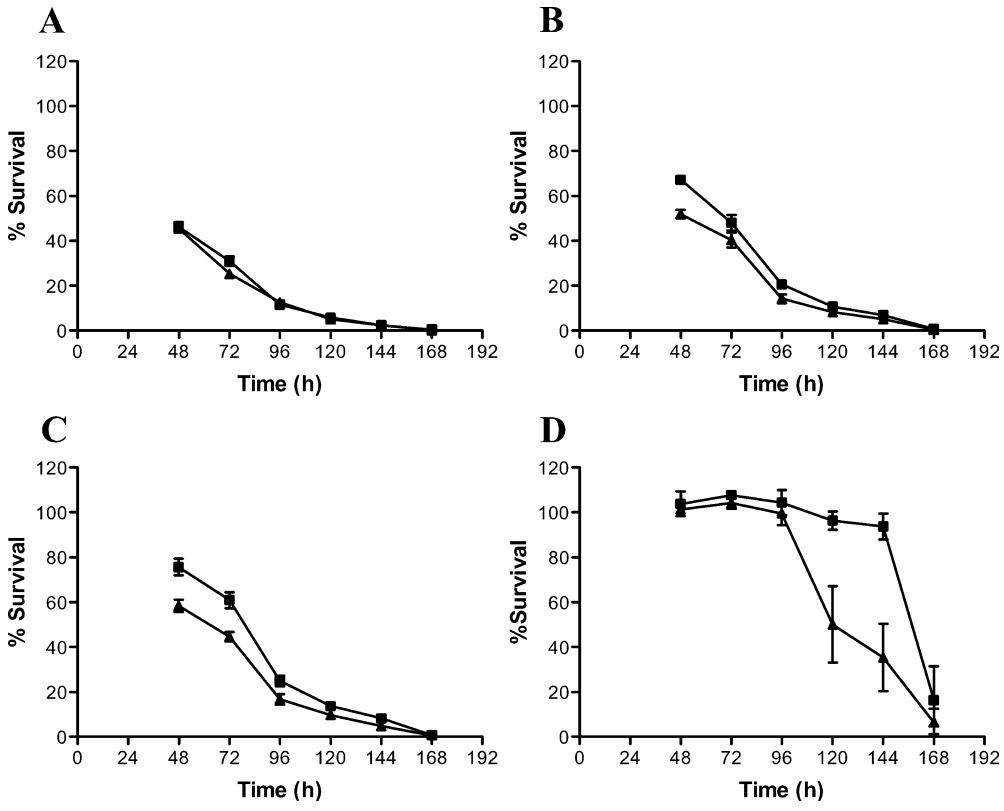

Fig. 2 U373-MG time-course. A time course experiment was done using four doses (**a** 50 μ*M*, **b** 1.5 μ*M*, **c** 0.4 μ*M*, **d** 0.02 μ*M*) of either gemcitabine (*triangles*) or gemcitabine conjugate (*squares*) in U373-MG cells. The percent survival was measured at each time-point (48, 72, 96, 120, 144, or 168 h) using the MTS assay. Each point represents the average ± SEM of three independent experiments

In order to test whether the gemcitabine conjugate was also a substrate for MDR-1, we compared the cytotoxicity of the gemcitabine conjugate in wild-type cells (MCF-7/WT-2′, breast cancer cells) to that observed in breast cancer cells that overexpress MDR-1 (BC-19) [33]. As shown in Fig. 5, there was no significant difference between the IC_{50} values of the gemcitabine conjugate in MCF-7/WT-2′ and BC-19 cells ($P > 0.05$, two-tailed *t*-test). In contrast, when the cells were treated with doxorubicin, there was a significant difference between the two IC_{50} values ($P < 0.05$, two-tailed *t*-test). These results indicate that the gemcitabine conjugate is not a substrate for the MDR-1 resistance mechanism.

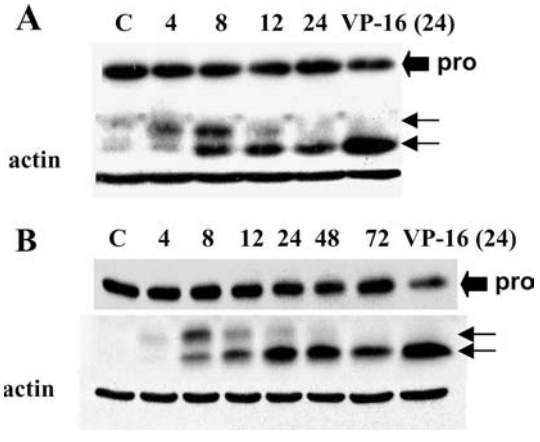

Fig. 3 Caspase-3 activation in HL-60 cells. Caspase-3 activation was measured by Western blotting following the treatment of cells with either gemcitabine (10 μ*M*) or gemcitabine conjugate (10 μ*M*). Etoposide (*VP-16*) was used as a positive control at 24 h. Gemcitabine (**a**) was tested at 0, 4, 8, 12, and 24 h, while the gemcitabine conjugate (**b**) was tested at 0, 4, 8, 12, 24, 48, and 72 h. These experiments were conducted in duplicate with similar results (*pro* proform of caspase-3)

Role of dCK and SCC-25 transfection

As stated above, gemcitabine enters the cell through a nucleoside transporter and it is phosphorylated by dCK,

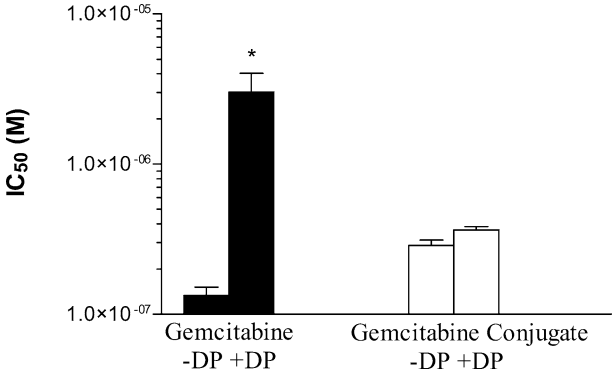

Fig. 4 Blockage of the nucleoside transporter. Dipyridamole (*DP*) experiments were conducted with U373-MG cells treated with gemcitabine or the gemcitabine conjugate for 72 h in the absence (−*DP*) or presence (+*DP*) of dipyridamole. Each bar is the average ± SEM of four independent experiments (*$P = 0.0003$)

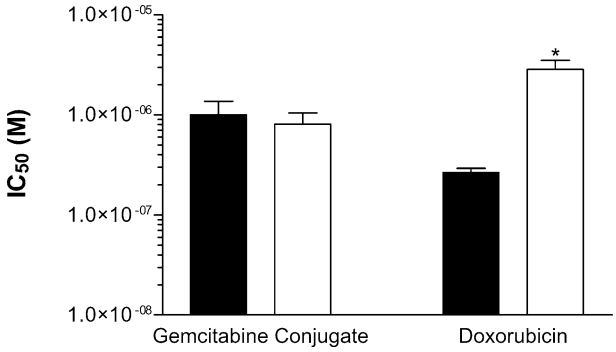

Fig. 5 MDR-1 resistance. MCF-7 (*black bars*) and BC-19 (*open bars*) cells were exposed to different concentrations of the gemcitabine conjugate or doxorubicin, and the IC_{50} values determined. Each bar represents the average ± SEM of four independent experiments (*$P < 0.05$, two-tailed *t*-test)

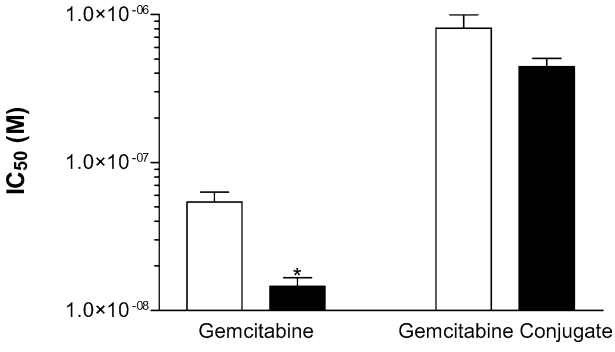

Fig. 7 Cytotoxicity data of transfected cells. IC_{50} values of gemcitabine and the gemcitabine conjugate in SCC-25 (*open bars*) and SCC-25/dCK (*black bars*) cells were compared at 72 h. Each bar represents the mean ± SEM of four independent experiments (*$P < 0.01$, one-way ANOVA with Dunnett's multiple comparison post test)

the rate-limiting step in the activation of this drug. We hypothesized that the gemcitabine conjugate enters the cell, and is cleaved to gemcitabine-MP and a glycerol backbone. This notion is derived from the fact that a similar phospholipid, AZT conjugate, releases AZT-MP and the glycerol backbone upon entering the cell [18]. To assess the involvement of dCK in the cytotoxicity of the gemcitabine conjugate, we transfected SCC-25 cells to stably express myc-tagged dCK. SCC-25 cells were used because this particular cell line has low levels of endogenous dCK. The expression level of the parental, empty vector, and the transfected cell lines can be seen in Fig. 6. As shown in Fig. 7, the IC_{50} values of gemcitabine in the parental SCC-25 cell line and the dCK-transfected cell line, SCC-25/dCK, were significantly different from each other ($P < 0.01$, one-way ANOVA with Dunnett's multiple comparison post test). However, there was no significant difference in the IC_{50} values of the gemcitabine conjugate between the SCC-25 and SCC-25/dCK cell lines ($P > 0.05$, one-way ANOVA with Dunnett's multiple comparison post test). The IC_{50} values of both drugs were not significantly different when tested in the empty vector (SCC-25/EV) and the parental cell line (data not shown; $P > 0.05$, one-way ANOVA with Dunnett's multiple comparison post test).

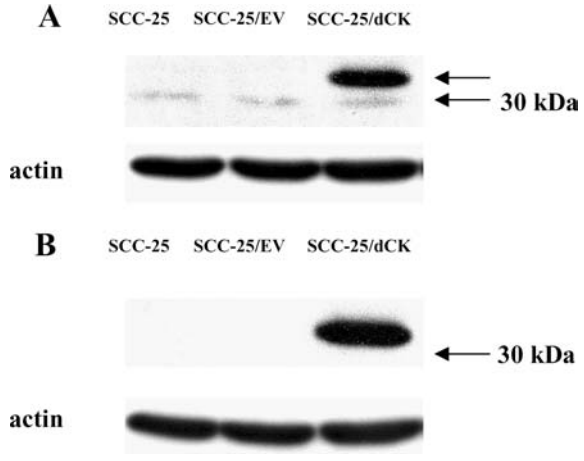

Fig. 6 SCC-25 transfected cell line. Western blot analysis of the amount of dCK protein present in SCC-25, SCC-25/EV (empty vector), and SCC-25/dCK (overexpress dCK) cell lines was done with either a dCK peptide antibody (**a**) or a myc tag antibody (**b**). The *lower band* in **a** is the basal dCK level and the *upper band* is the transfected dCK with a myc tag

Discussion

We previously synthesized a phospholipid gemcitabine conjugate molecule and found the molecule to be cytotoxic to different types of tumor cell lines [1]. We hypothesized that the gemcitabine conjugate would have a different pharmacological profile compared to gemcitabine. Our previous metabolism studies have shown that the gemcitabine conjugate is taken up by the cells within 24 h [1]. Therefore, we believe that the delayed action of the gemcitabine conjugate is likely due to the protracted release of the gemcitabine monophosphate from the lipid carrier.

In order to elucidate this temporal difference, we measured the percent survival of U373-MG and HL-60 cells treated with gemcitabine or the gemcitabine conjugate over the course of several days. At the lowest doses examined, there was a delay in the cellular response for the gemcitabine conjugate when compared to gemcitabine. However, the cytotoxicity profiles of gemcitabine and the gemcitabine conjugate were similar, if not identical, at all other doses examined. These results suggest that the conjugate molecule has the same cytotoxic effect as gemcitabine, but that the rate of killing at low doses is initially slower for the gemcitabine conjugate than for gemcitabine alone. Furthermore, our caspase-3 results show that the gemcitabine conjugate can induce apoptosis in HL-60 cells (Fig. 3). Caspase-3 is a known marker for apoptosis and gemcitabine is a known inducer of apoptosis in many tumor cell lines [15, 28]. Taken together, these results suggest that the

gemcitabine conjugate induces an apoptotic mode of cell death at levels comparable to gemcitabine.

A potential problem of the use of gemcitabine as an anticancer agent is that the drug relies on nucleoside transporters to enter the cell and ultimately reach its target site [21]. This inherently can lead to tumor cell resistance due to a lack of nucleoside transporters. Therefore, we blocked the hENT1 transporters with dipyridamole to test whether the cytotoxic activity of the conjugate was affected by this inhibition. Our results indicated that gemcitabine is heavily dependent upon the nucleoside transporter (23-fold increase in U373-MG cells) while the gemcitabine conjugate did not rely on the transporter (no significant difference in U373-MG) to enter the cell. Furthermore, the IC_{50} value of the conjugate was lower than that of gemcitabine with dipyridamole in U373-MG cells. These findings confirm those of previous studies indicating that gemcitabine enters the cell through a nucleoside transporter [21] and strongly suggest that the gemcitabine conjugate does not rely on the hENT1 transporter to enter the cell.

We have hypothesized that the gemcitabine conjugate enters the cell through passive diffusion, in a manner similar to a steroid, and that the membrane may serve as a reservoir for the gemcitabine conjugate. While gemcitabine is transported across the cell membrane by a carrier-mediated process, the gemcitabine conjugate passively diffuses across the membrane and it is hydrolyzed within the cell. Furthermore, tumor cells resistant to gemcitabine due to a loss of nucleoside transporters may be more sensitive to the gemcitabine conjugate. In this case, the gemcitabine conjugate would have more clinical efficacy.

With the addition of a phospholipid to gemcitabine, the conjugate is rendered more lipophilic [1]. In addition, by making the molecule more lipophilic, it may become a substrate for MDR-1, which pumps highly lipophilic drugs such as doxorubicin from the cytoplasm. Using a MDR-1-transfected cell line, BC-19, we investigated the ability of the gemcitabine conjugate to serve as a substrate for this efflux pump by comparing the effect of MDR-1 expression on the drug's cytotoxic activity. Doxorubicin, a known substrate for MDR-1, was used as a positive control. As expected, expression of MDR-1 augmented the cytotoxicity of doxorubicin. In contrast, expression of MDR-1 did not affect the sensitivity of cells to the gemcitabine conjugate. Therefore, although we have made gemcitabine more lipophilic by the addition of a phospholipid moiety, the gemcitabine conjugate is not a substrate for MDR-1.

We next determined whether dCK was involved in the activation of the gemcitabine conjugate. Previous studies in our laboratory have indicated that a similar phospholipid, AZT conjugate, is cleaved to yield AZT monophosphate and the lipid carrier [18]. Additionally, previous investigators have found that a different phospholipid cytosine arabinoside (ara-C) conjugate is cleaved in a similar manner [29]. We hypothesized that the gemcitabine conjugate would be cleaved in a manner similar to the ara-C conjugate. Thus, we wanted to determine whether activity of the gemcitabine conjugate was dependent on it being phosphorylated by dCK. Indeed, gemcitabine is dependent upon dCK for activation, and phosphorylation by dCK is the rate-limiting step in the activation of gemcitabine [2]. We generated stably transfected SCC-25 cells that overexpressed myc-tagged dCK. The cytotoxicity of gemcitabine was greater in cells overexpressing dCK. This observation is consistent with previous reports [9]. However, cytotoxicity of the gemcitabine conjugate was unchanged in the presence of elevated dCK levels. These results suggest that, unlike gemcitabine which requires dCK for activation, the gemcitabine conjugate does not require dCK activity to exert its cytotoxic effect. This finding is consistent with a model in which the gemcitabine conjugate molecule is cleaved to yield gemcitabine-MP and the lipid carrier once it enters the cell, and this process bypasses the rate-limiting step in the activation of gemcitabine. Thus, the gemcitabine conjugate may have clinical utility in cases where resistance to gemcitabine is due to a loss of dCK. To date, we have not identified the enzyme responsible for this cleavage, but we believe that the protein is a phospholipase-C like enzyme. This conclusion is supported by the fact that the gemcitabine conjugate does not rely on dCK for activation and by the results of previous studies using a phospholipid AZT conjugate showing that the molecule is cleaved by a phospholipase C-like enzyme [18].

In this study, the cytotoxicity of a gemcitabine conjugate was compared with that of gemcitabine, and although the conjugate molecule is more lipophilic, it is not a substrate for MDR-1. Furthermore, the gemcitabine conjugate bypasses traditional resistance mechanisms such as the loss of the hENT1 transporters or the loss of dCK. Thus, we believe that the gemcitabine conjugate merits further study and could be clinically useful in cases of gemcitabine resistance.

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