In-vitro and in-vivo anti-cancer activity of a novel gemcitabine–cardiolipin conjugate

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Our objectives were to study the biological activity of a novel gemcitabine–cardiolipin conjugate (NEO6002) and compare that with gemcitabine. Cytotoxicity *in vitro* was determined against several gemcitabine-sensitive parental and gemcitabine-resistant cancer cell lines using the sulforhodamine B assay. The *in vivo* toxicity was examined by changes in body weight and hematologic indices of conventional mice. Immunodeficient SCID mice bearing P388 and BxPC-3 tumor xenografts were used to evaluate the in-vivo therapeutic efficacy. Both NEO6002 and gemcitabine showed pro-apoptotic and cytotoxic effects against all gemcitabine-sensitive cell lines tested. Unlike gemcitabine, the cytotoxicity of NEO6002 was independent of nucleoside transporter (NT) inhibitors, indicating a different internalization route of NEO6002. The conjugate demonstrated a favorable activity not only in ARAC-8C, a NT-deficient gemcitabine-resistant human leukemia cell line, but also in several other gemcitabine-resistant cell lines. At the in-vivo level, a comparative toxicity study showed a significant body weight loss and a decrease in white blood cell counts in gemcitabine-treated mice, whereas the influence of NEO6002 was mild. Treatment of NEO6002 at 27 μmol/kg increased the median survival of CD2F1 mice bearing P388 cells by up to 73%, while at the same doses and schedule of gemcitabine resulted in toxic deaths of all treated mice. At a dose of 18 μmol/kg, NEO6002 inhibited the growth of BxPC-3 xenografts by 52%, while only 32% of tumor inhibition was achieved with gemcitabine. We conclude that NEO6002 may be an effective chemotherapeutic agent with improved tolerability and can potentially circumvent NT-deficient, gemcitabine-resistant tumors. *Anti-Cancer Drugs* 17:53–61 © 2006 Lippincott Williams & Wilkins.

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Introduction

Gemcitabine [2′,2′-difluorodeoxycytidine (dFdC)] is a pyrimidine nucleoside anti-metabolite with broad-spectrum activity against human malignancies. It has been approved for the treatment of pancreatic cancer as monotherapy [1], and non-small cell lung [2], metastatic breast [3] and ovarian cancers [4] in combination with other chemotherapeutics. Gemcitabine is a cell cycle (S-phase)-specific chemotherapeutic reagent [5] that enters the cells through functional nucleoside transporters (NTs) [6]. At least seven different NTs have been documented in mammalian cells, including two Na^+-independent equilibrative NTs with broad nucleoside selectivity {*es* [nitrobenzylthioinosine (NBMPR)-sensitive] and *ei* [NBMPR-insensitive]} and five Na^+-dependent concentrative NTs (*cit*, *cif*, *cib*, *csg* and *cs*, each with specific nucleoside selectivity) [6,7]. Among those, equilibrative NTs are the main transporters for gemcitabine influx in cancer cells, probably because of their broad tissue expression [8]. Once internalized, gemcitabine is phosphorylated by deoxycytidine kinase (dCK) to inactive monophosphorylated dFdCMP, and further to active diphosphorylated dFdCDP and triphosphorylated dFdCTP forms [9]. dFdCDP inhibits ribonucleoside reductase which decreases the intracellular concentration of deoxynucleoside triphosphate (dNTP), depletes the negative feedback of dCK from dNTP [10] and potentiates the activity of gemcitabine by increasing its phosphorylation. dFdCTP is incorporated into DNA, resulting in the termination of DNA elongation which leads to cell cycle arrest and ultimately cell death [11]. Gemcitabine is inactivated by dephosphorylation and deamination through 5′-nucleotidase and cytidine deaminase, respectively [12].

The efficacy of gemcitabine is dependent on both its dose and schedule [13,14]. High-dose or prolonged infusion of gemcitabine is required to maintain the minimum effective plasma concentration to produce significant clinical response in patients. Currently, most dose regimes of gemcitabine monotherapy or combinatory therapy adopt 1000 mg/m^2 with weekly infusion for 30 min. Patients treated with this dose regime usually develop myelosuppression – the dose-limiting side-effect

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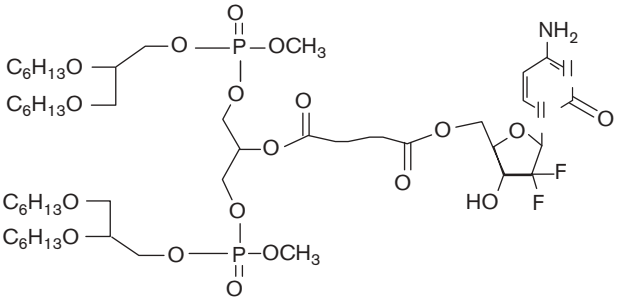
of gemcitabine. Furthermore, because of the nature of the heterogeneity and genetic instability of tumor cells, some patients develop resistance to gemcitabine [15]. Resistance to gemcitabine has been a major challenge to gemcitabine-based treatment regimes. One of the primary factors of resistance to gemcitabine is caused by dysfunction of NTs on tumor cell membranes [6]. Lack of efficient NTs on tumor cells makes it difficult to deliver enough gemcitabine intracellularly to kill the targeted cells.

As one of the approaches to improve biological activity and potentially overcome the gemcitabine resistance, we have conjugated a cardiolipin moiety with gemcitabine [16]. In this study we have investigated in-vitro cytotoxicity of the gemcitabine–cardiolipin conjugate (NEO6002) against cancer cells and studied the mechanism of the internalization of NEO6002. In-vivo antitumor efficacy and toxicity were also examined and compared with gemcitabine (Gemzar).

Fig. 1



Chemical structure of NEO6002. Molecular weight: 1110.2

Materials and methods

Chemicals

Trichloroacetic acid (TCA), sulforhodamine B (SRB), NBMPR, dipyridamole, 100% ethanol and DMSO were purchased from Sigma (St Louis, Missouri, USA). Dextrose (5%) and saline were purchased from Abbott Laboratories (Abbott Park, Illinois, USA).

Synthesis and preparation of NEO6002

NEO6002 was synthesized by conjugating gemcitabine to the central hydroxyl group of the ether analog of cardiolipin via a succinate linker (Fig. 1). NEO6002 was dispersed into 5% dextrose from an ethanol stock solution followed by 30-s vortex. The dispersion was diluted into cell culture medium for in-vitro use or administered to animals directly. The final formulation of NEO6002 had a mean particle size of 286 nm. Gemcitabine hydrochloride (Gemzar; Eli Lilly, Indianapolis, Indiana, USA) was resuspended as per the manufacturer's protocol and used as comparison within 24 h after reconstitution.

Cell culture

Human lung A549, breast MX-1 and colon HT-29, and murine leukemia P388 cell lines were obtained from the National Cancer Institute (Fredrick, Maryland, USA). These cells were maintained in RPMI 1640 medium containing 10% heat-inactivated FBS (HI-FBS). Human leukemia CCRF-CEM and pancreatic BxPC-3 cancer cells were purchased from ATCC (Manassas, Virginia, USA), and maintained in RPMI 1640 medium containing 10% HI-FBS, 10 mmol/l HEPES, 1 mmol/l sodium pyruvate and 4.5 g/l glucose. ARAC-8C, a NT-deficient gemcitabine-resistant derivative of CCRF-CEM, was a gift from Dr Ullman (Oregon Health Sciences University, Portland, Oregon, USA) and cultured in DMEM containing 10% HI-FBS. Other gemcitabine-resistant cell lines were created in house by exposing cancer cells stepwise to a 2- to 5-fold increase of gemcitabine, starting from 0.05 nmol/l. The concentration was increased when the proliferation rate of gemcitabine-treated cells was similar to the untreated cells. The resistant cells were maintained in their final selection concentration of gemcitabine and transferred to drug-free medium 1 week before any experiments. All culture media contained penicillin 100 U/ml and streptomycin 100 µg/ml, and cells were incubated at 37°C in a 5% CO_2 incubator. The culture media and reagents were purchased from Invitrogen (Carlsbad, California, CA).

Cytotoxicity assay

The growth inhibition was determined based on the SRB assay [17]. In brief, different cancer cells (10 000–25 000 cells/well) were pre-incubated on 96-well microtiter plates overnight. Gemcitabine or NEO6002, freshly prepared as described above, was diluted in the culture medium to final concentrations of 5×10^{-2} to 5×10^{4} nmol/l. Cells were co-cultured with the drugs for 20 min, or 5, 24 or 48 h for time point experiments, or 48 h for cytotoxicity screening and NT inhibitor experiments. After the incubation, the cells were fixed with 10% TCA at 4°C for 1 h. The cytotoxicity was determined based on the total cellular protein levels. The 50% growth inhibition (GI_{50}) was estimated as the concentration of the drug that gives 50% growth inhibition, whereas the total growth inhibition (TGI) was the concentration to give 100% growth inhibition. For NT inhibitor experiments, NBMPR or dipyridamole was first dissolved in DMSO as stock solutions and further diluted in culture medium before use. CCRF-CEM or ARAC-8C cells (25 000 cells/well) were first incubated with 100 nmol/l NBMPR or 10 µmol/l dipyridamole for 1 h and further co-cultured with serial diluted NEO6002 or gemcitabine for an additional 48 h. The cytotoxicity of NEO6002 or gemcitabine in the presence of NT inhibitors was determined by the SRB assay and compared with the

pretreatment of control DMSO. The final DMSO concentration in each well was 0.1% (v/v). To determine the gemcitabine resistance, the cytotoxicity of gemcitabine or NEO6002 was determined in the parental or gemcitabine-resistant cells after 48 h of treatment. The folds of gemcitabine resistance were calculated as the GI_{50} ratios of the parental and the resistant cells.

In-vivo toxicity

Multiple-dose toxicity studies were conducted on normal female CD2F1 mice (5–6 weeks old). Mice were randomized into groups of eight mice based on body weight and injected i.v. with NEO6002 or gemcitabine at 18 μmol/kg/day for 5 consecutive days or at 27 and 36 μmol/kg/day for 6 days. Mice in the control group were given an equal dose volume of vehicle (5% dextrose and 5% ethanol). The toxicity was evaluated by the mortality, body weight loss and peripheral blood cell counts at the end of the study. Blood samples were collected from the orbital sinus into K_2-EDTA-coated tubes. All laboratory examinations were conducted at Antech Diagnostics (Chicago, Illinois, USA).

Tumor models

The mouse leukemia model was established with female CD2F1 mice (5–6 weeks old). Each mouse was injected with log-phase P388 cells (1×10^5 cells in 0.2 ml PBS) via lateral tail veins on day 0. Next day, mice were randomly divided into groups of five based on their body weight and treated i.v. with NEO6002 (18 or 27 μmol/kg) or gemcitabine (9 or 18 μmol/kg). Mice in the control group were administrated with control vehicle (5% dextrose and 5% ethanol). Mice were observed once a day for signs of moribundity and mortality for 60 days. Body weights were recorded during dosing and twice a week thereafter.

The human pancreatic tumor xenograft was established with female CB-17 SCID mice (4–5 weeks old) by s.c. injection of 2×10^6 BxPC-3 cells at the right flank region. Mice were randomized into eight mice per group and treated with NEO6002, gemcitabine or control vehicle i.v. when the tumors reached a volume of 80–160 mm^3. Tumor length (L) and width (W) were measured with a digital caliper twice a week. The tumor volume (V) and tumor growth (% of initial tumor volume) were calculated as follows: V (mm^3) = $L \times (W/2)^2 \times \pi$ (where $\pi = 3.14$) and tumor growth (% initial tumor volume) = $100 \times (V_1/V_0)$. V_1 represents the tumor volume at any given day and V_0 is the tumor volume on the day of treatment initiation.

All animal studies were conducted following the *Guidance for the Care and Use of Laboratory Animals* published by National Institutes of Health and the protocols were approved by the Institutional Animal Care and Use Committee.

Statistical comparison

Student's t-test was used for the statistical comparisons and it was considered significant if $P < 0.05$.

Results

NEO6002 induced rapid cytotoxicity in cancer cells

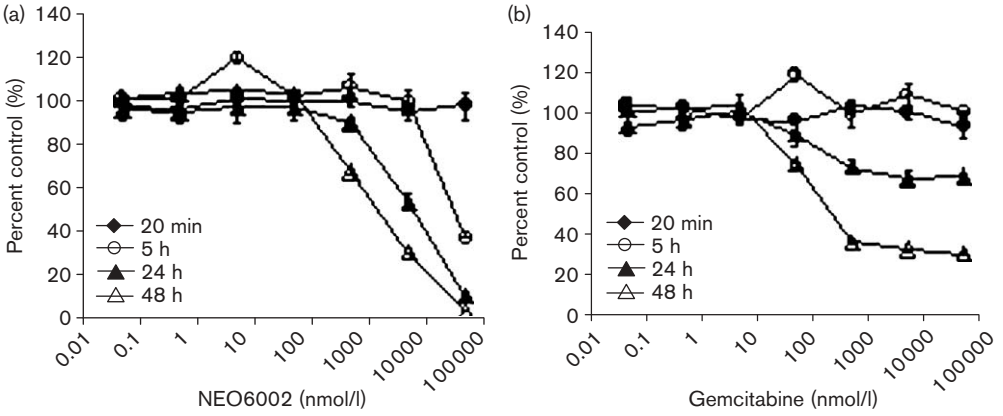
Both NEO6002 and gemcitabine showed dose-dependent cytotoxicity against the cancer cell lines tested, including A549, BxPC-3, HT-29, MX-1, CCRF-CEM and P388 cells. The GI_{50} and TGI values of gemcitabine and NEO6002 after 48 h of treatment are summarized in Table 1. Although the potency of NEO6002 was not as high as gemcitabine based on the GI_{50} values after 48 h of incubation, the effectiveness of NEO6002 and gemcitabine were nevertheless diverse at different time points. As shown in Fig. 2(a), NEO6002-induced cytotoxicity was detected in A549 cells as early as 5 h after treatment, whereas the activity of gemcitabine was not measurable until 24 h after treatment (Fig. 2b). At 24 h, the potency of NEO6002 was more pronounced than gemcitabine, but the activity of gemcitabine became higher than NEO6002 after 48 h of incubation. In addition, gemcitabine at the maximum concentration (50 000 nmol/l) did not reach TGI in the adherent cells tested, while the TGI of NEO6002 ranged from 7331 to 16948 nmol/l after 48 h of treatment (Table 1). The cardiolipin control at 50 000 nmol/l did not cause any significant cytotoxicity (data not shown).

NEO6002 entered the cells through a NT-independent pathway

Gemcitabine has been shown to enter the cells through NTs. Tumor cells without functional NTs are resistant to gemcitabine, as demonstrated in NT-deficient cell lines or by blocking NT activity in normal cells [6,8,19]. To further explore whether NEO6002 bypassed NTs and therefore was potentially useful in NT-deficient gemcitabine-resistant tumors, we investigated the cytotoxicity of NEO6002 in CCRF-CEM and ARAC-8C cells in the presence of NBMPR (an *es* NT inhibitor) or dipyridamole (an *es*/*ei* NT inhibitor) [6]. ARAC-8C cells were originally isolated from CCRF-CEM by the selection of resistance to 8 μmol/l cytarabine [20] and later found to have cross-resistance to gemcitabine due to the absence of *es* NT expression [19].

When NT activity was blocked by NBMPR or dipyridamole, the growth inhibition curves of gemcitabine shifted toward the right in CCRF-CEM cells – an indication of decreased cytotoxicity (Fig. 3a). The potency of gemcitabine was significantly decreased in the presence of NBMPR and further reduced by dipyridamole, as compared with the control DMSO. When ARAC-8C cells were tested, the growth inhibition curves of gemcitabine were not significantly affected by the pre-incubation of NBMPR, but shifted slightly by dipyridamole (Fig. 3b). However, none of the NT inhibitors induced any

Fig. 2



Time-dependent cytotoxicity of NEO6002 and gemcitabine in A549 cells. Cells were treated with freshly diluted NEO6002 (a) or gemcitabine (b) at final concentrations of 5×10^{-2} to 5×10^{4} nmol/l for 20 min (●), or 5 (○), 24 (▲) or 48 h (Δ). The cytotoxicity was determined with the SRB assay as described in Materials and methods. The data is expressed as mean ± SEM from triplicate wells and the experiment was repeated 3 times.

Table 1 **GI_{50} and TGI of gemcitabine and NEO6002 in cancer cells**

Cell lines	Gemcitabine		NEO6002	
	GI_{50}(nmol/l)	TGI (nmol/l)	GI_{50}(nmol/l)	TGI (nmol/l)
A549	84	>50 000	523	10 988
BxPC-3	38	>50 000	528	7331
HT-29	374	>500 000	1513	16 948
MX-1	37	>50 000	200	7704
CCRF-CEM	12	195	502	7576
P388	4	162	126	8041

Cancer cells were treated with gemcitabine or NEO6002 in serial dilutions for 48 h and the cytotoxicity was determined. GI_{50} and TGI are the concentrations of the drugs that give 50% or total (100%) growth inhibition, respectively. The results are the average of at least three experiments. The upper limit of quantification for gemcitabine was 500 000 nmol/l for HT-29 or 50 000 nmol/l for the other cell lines.

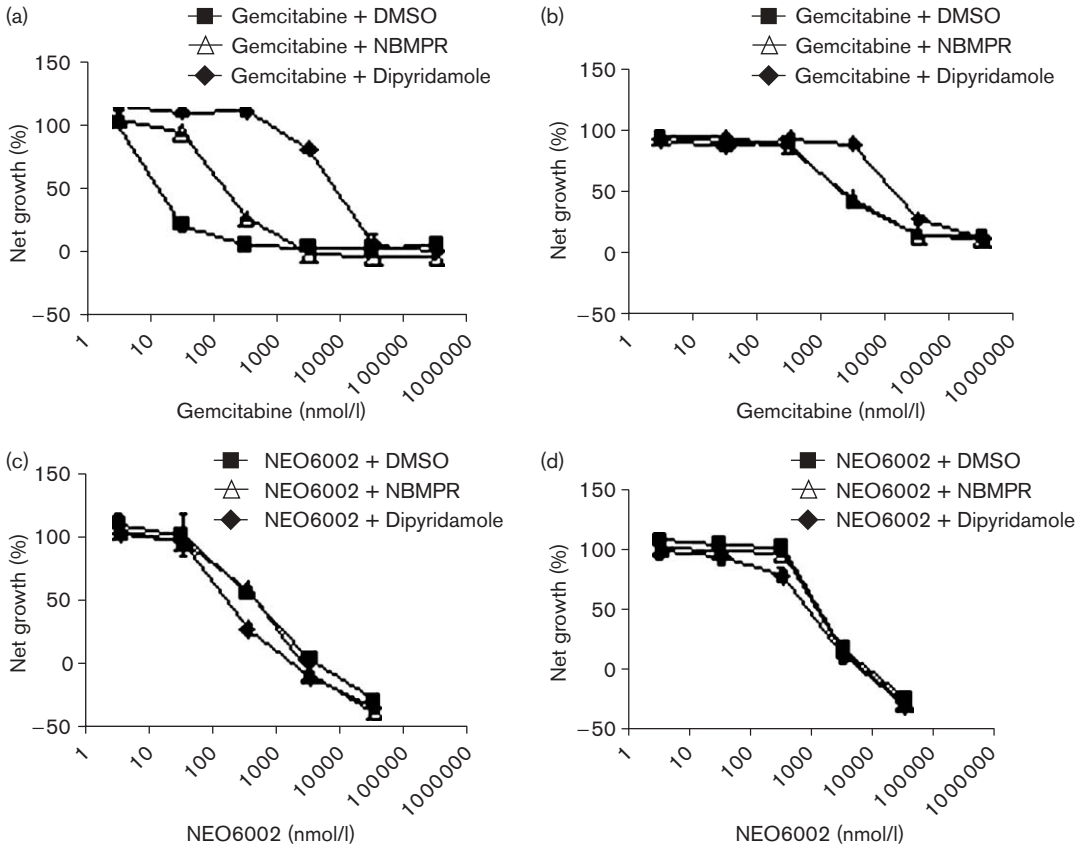
significant shift in the cytotoxicity curves of NEO6002 in CCRF-CEM cells (Fig. 3c) or ARAC-8C cells (Fig. 3d). All these results suggested that NEO6002 may enter the cells through an equilibrative NT-independent pathway.

When comparing the potency of gemcitabine between the parental and resistant cell lines, ARAC-8C cells had gained 235-fold resistance to gemcitabine as indicated by the increase of GI_{50} from 12 nmol/l in CCRF-CEM cells to 2739 nmol/l in ARAC-8C cells (Table 2). In contrast, ARAC-8C cells gained only 3-fold resistance to NEO6002 as the GI_{50} changed from 502 nmol/l in CCRF-CEM cells to 1413 nmol/l in ARAC-8C cells. In addition, NEO6002 killed ARAC-8C cells more effectively than gemcitabine since the GI_{50} of NEO6002 was lower than that of gemcitabine in ARAC-8C cells. To further explore the possibility of using NEO6002 in other gemcitabine-resistant cell lines, several resistant cell lines were generated. Two gemcitabine-resistant cell lines were created from P388 cells by selecting the cells in 200 (P388GR-200) or 25 000 nmol/l (P388GR-25000) of gemcitabine resulting in 5257- or 30 839-fold gemcitabine resistance, respectively (Table 2). Both P388GR-200 and P388GR-25000 cell lines responded better to NEO6002 than to gemcitabine. A similar trend with a lower GI_{50} of NEO6002 than gemcitabine was observed in the HT29GR-320 cell line which was generated by selecting the cells in 320 nmol/l gemcitabine.

In-vivo toxicity study

To test the toxicity of NEO6002 in animals, multiple-dose toxicity studies were conducted in CD2F1 mice. Three doses (18, 27 and 36 μmol/kg) were used. After multiple injections, all mice treated with gemcitabine at doses of 27 and 36 μmol/kg were found moribund, and had to be sacrificed on day 7. Mice treated with gemcitabine at 18 μmol/kg survived, but developed dehydration, a rough coat, hunched posture, ataxia and body weight loss. In contrast, NEO6002 at 18 and 27 μmol/kg did not show significant body weight loss or signs of toxicity. At 36 μmol/kg, 75% of mice survived after NEO6002 treatment. In hematology studies, noticeable decreases in white blood cells (WBCs) and lymphocytes were observed in mice treated with 18 μmol/kg of gemcitabine, but not in mice treated with the same dose of NEO6002. Both NEO6002 and gemcitabine-treated

Fig. 3



Equilibrative NT inhibitors had no effect on NEO6002-induced cytotoxicity. Gemcitabine-sensitive CCRF-CEM cells or gemcitabine-resistant ARAC-8C cells were pre-incubated with DMSO (■, as control), 100 nmol/l NBMPR (Δ) or 10 μmol/l dipyridamole (◆) for 1 h. After 1 h of pre-incubation, serially diluted gemcitabine or NEO6002 was added directly to the cells. The cytotoxicity was determined 48 h after the treatment using the SRB assay. (a) CCRF-CEM cells treated with gemcitabine. (b) ARAC-8C cells treated with gemcitabine. (c) CCRF-CEM cells treated with NEO6002. (d) ARAC-8C cells treated with NEO6002. The data is expressed as mean ± SEM from triplicate wells and the experiment was repeated at least 3 times.

Table 2 Enhanced cytotoxicity of NEO6002 in gemcitabine-resistant cell lines

Cell lines	Gemcitabine		NEO6002	
	GI_{50}(nmol/l)	Fold resistance	GI_{50}(nmol/l)	Fold resistance
CCRF-CEM	12		502	
ARAC-8C	2739	235	1413	3
P388	4		126	
P388GR-200[a]	20118	5257	9827	80
P388GR-25000[a]	11 8024	30839	11 798	95
HT-29	374		1513	
HT29GR-320[a]	29846	80	4889	3

The cytotoxicity of gemcitabine or NEO6002 was compared between parental and resistant cell lines using the SRB assay as described in Materials and methods. The results are shown as an average of three experiments.
[a]The final concentration of gemcitabine (nmol/l) in the selection medium.

mice developed neutropenia, whereas no significant differences were found in red blood cell (RBCs) and platelet counts (Table 3).

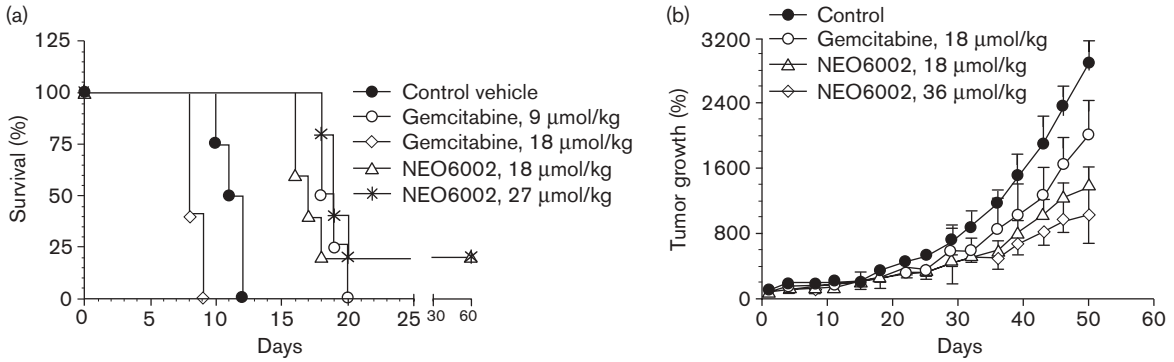
In-vivo efficacy studies

Human pancreatic BxPC-3 and murine P388 tumor models were chosen for further *in vivo* efficacy evaluation. A systemic leukemia model was established by i.v. injection of P388 murine leukemia cells (1×10^5) to female CD2F1 mice. Without treatment, the mice died after an average of 12 days. In contrast, treatment with NEO6002 at doses of 18 or 27 μmol/kg for 5 days increased median survival time by 55 to 73% (Fig. 4a), suggesting an anti-leukemia activity from NEO6002. At a

Table 3 Blood counts of mice treated with gemcitabine or NEO6002

	Control	Gemcitabine (18 μmol/kg)	NEO6002 (18 μmol/kg)
WBCs (10^3/μl)	3.4 ± 0.9	1.6 ± 0.4	3.5 ± 1.2
RBCs (10^6/μl)	10.7 ± 0.4	10.9 ± 0.6	11.3 ± 0.4
Neutrophils (/μl)	238 ± 56	27 ± 25	71 ± 47
Lymphocytes (10^3/μl)	2.8 ± 1.1	1.5 ± 0.3	2.8 ± 0.7
Platelet (10^3/μl)	513 ± 64	452 ± 88	417 ± 120

Fig. 4



In vivo efficacy studies of gemcitabine and NEO6002. (a) Percentage of survival versus time (days) for P388-bearing CD2F1 mice treated with gemcitabine at 9 (○) and 18 μmol/kg (◇) or NEO6002 at 18 (Δ) and 27 μmol/kg (*). Mice in the control group were treated with control vehicle (●). Treatments were started on day 1. (b) Tumor growth versus time (days) for SCID mice implanted with s.c. BxPC-3 human pancreatic cancer xenograft. Mice were treated with four weekly and three twice-a-week injections of gemcitabine at 18 μmol/kg (○) or NEO6002 at 18 (Δ) and 36 μmol/kg (◇) from day 1.

dose of 18 μmol/kg, however, gemcitabine resulted in toxic death of all treated mice. Even though the increase survival of gemcitabine at 9 μmol/kg was comparable to that of NEO6002 at 18 μmol/kg, one out of five mice treated with NEO6002 at 18 or 27 μmol/kg survived for more than 60 days in two separate experiments.

In the human pancreatic (BxPC-3) xenograft model, the mice were initially treated four weekly followed by three twice-a-week injections of NEO6002 at doses of 18 and 36 μmol/kg or gemcitabine at a dose of 18 μmol/kg. As indicated in Fig. 4(b), the growth of established BxPC-3 tumor was inhibited by treatment with NEO6002 or gemcitabine at a dose of 18 μmol/kg. On day 50, the growth inhibition by NEO6002 was 52% ($P = 0.0001$) compared to that of control and 32% ($P = 0.0002$) by gemcitabine. At 36 μmol/kg, NEO6002 inhibited the growth by 64% ($P = 0.0001$).

Discussion

Gemcitabine is a small molecular compound which has hydrophilicity resulting in rapid clearance through glomerular filtration and a very short half-life in human plasma [21]. More importantly, gemcitabine can be inactivated by dCK found in plasma, the intestines, liver and kidneys, and only a small fraction of injected drug could reach the target tumor tissues [22]. Even at the target sites, the sensitivity of gemcitabine is often compromised as a result of multiple mechanisms that confer resistance to gemcitabine in some patients. The primary resistance to gemcitabine at the cellular level is caused by a less-effective intracellular concentration of dFdCTP or/and dFdCDP which may be the results of insufficient cellular uptake of gemcitabine, reduced activity of dCK [23], increased gemcitabine metabolism by deaminase [24] and increased expression of ribonucleotide reductase [25]. Lack of functional NT expression has been attributed not only to in-vitro gemcitabine resistance [6], but also to poor survival in gemcitabine-treated patients [26], posing a serious challenge to gemcitabine-based treatments. Furthermore, the use of gemcitabine could pose severe toxicity in some patients [27].

By conjugating cardiolipin to gemcitabine, we have created a novel class of compound that was characterized by the presence of diphosphatidylglycerol spaced by a succinate linker attached to the 5′-OH of gemcitabine [16]. The linker allows the facilitated hydrolytic release of gemcitabine. The two -OH groups from phosphate

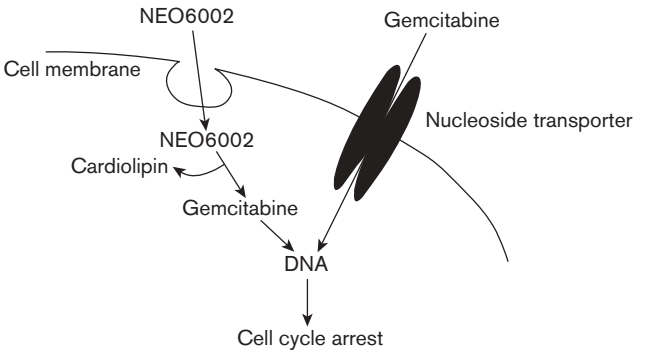
were replaced by two -OMe groups, and the four carbonyl groups between hydrocarbon and glycerol were removed. All these molecular features contribute to the increase of lipophilicity and stability, thus facilitating the entry of the drugs into cells by simple diffusion [28]. Another advantage of this group of conjugates is that they form lipid vesicles when dispersed into aqueous media from alcohol stock solutions. NEO6002 was selected from several similar compounds after screening for their ease of synthesis, formulation, chemical stability and in-vitro cytotoxicity against a group of cancer cell lines. In this study, NEO6002 was dispersed into a co-solvent system composed of 5% dextrose and 5% ethanol.

Even though both NEO6002 and gemcitabine were shown to have dose-dependent cytotoxicity against the cell lines tested *in vitro*, NEO6002 had advantages compared to gemcitabine. First, NEO6002 showed a strong cytocidal effect at higher concentrations, while gemcitabine was cytostatic after 48 h of incubation. Second, the activity of NEO6002 became detectable as early as 5 h after exposure to the cells, whereas gemcitabine did not show any significant effect until 24 h. Third, because of higher lipophilicity, NEO6002 may penetrate the cell membranes more efficiently than gemcitabine. Since NEO6002 is delivered in the form of lipid vesicle, endocytosis or passive diffusion could be involved in the internalization which can reach its effective intracellular concentration in a shorter time and is not saturated even in the presence of high concentrations such as 50 000 nmol/l. In contrast, the cytotoxicity of gemcitabine beyond 500 nmol/l in A549 cells reached a plateau after 48 h incubation; similar to what had been discovered in other non-small cell lung cancer cell lines [29]. This is probably due to the lack of available NTs in the presence of saturated concentrations of gemcitabine. In that case, the presence of NTs has become the rate-limiting factor for the sensitivity of gemcitabine. In order to exert its effect, gemcitabine has to be incubated with the cells for longer times and sometime this it cannot be achieved because of the short half-life of gemcitabine.

Further evidence showing that NEO6002 entered the cells through an alternative route was its insensitivity to NT inhibitors. The failure of NBMPR or dipyridamole to block the cytotoxicity of NEO6002 in CCRF-CEM cells may be an indication that NEO6002 bypasses the NT pathway to enter the cells. A similar effect was observed in other cell lines, such as A549 and p388 cells (data not shown). It is not clear if NEO6002 completely lost its binding capacity to NTs or if the binding is too weak to compete with the non-specific hydrophobic interaction between NEO6002 and cellular membranes. Since NEO6002 may bypass the NT pathway and thus the availability of NT will no longer be the rate-limiting factor, NEO6002 may be a useful form of gemcitabine to treat NT-deficient gemcitabine-resistant tumors. By using ARAC-8C, a NT-deficient cell line, NEO6002, was found to have a superior activity over gemcitabine with a lower GI_{50}. Further incubation of ARAC-8C cells with NBMPR did not cause any changes of the gemcitabine-induced cytotoxicity, confirming an *es*-deficient phenotype of ARAC-8C cells. Interestingly, the growth inhibition curves of gemcitabine were slightly shifted by pre-treatment with dipyridamole, an *es/ei* NT inhibitor [6], suggesting the presence of *ei* or other dipyridamole-sensitive NTs in ARAC-8C cells. Similar inhibition of NBMPR or dipyridamole was observed in the P388 parental cell line, but not in the P388GR-200 cell line (data not shown). More importantly, other gemcitabine-resistant cell lines, such as P388GR-200, P388GR-25000 and HT29GR-320, also demonstrated a better response to NEO6002. Although the detailed mechanisms of the gemcitabine-resistance in P388GR and HT29GR cell lines are not yet understood, based on the results from NT inhibitor experiments in ARAC-8C cells, NT deficiency was likely to be one of the contributing factors.

Taken together, the results in cultured cells suggested that NEO6002 can overcome the gemcitabine resistance by entering the cells through a NT-independent pathway. A scheme is illustrated in Fig. 5. Once inside cells, NEO6002 may become hydrolyzed with intracellular esterases into gemcitabine, and exert its cytotoxicity by binding to DNA and inducing apoptosis. Gemcitabine enters the cells through NTs however, which can be blocked by the NT inhibitors such as NBMPR or dipyridamole. Therefore, NT deficiency in cancer cells then results in resistance to gemcitabine, but less to

Fig. 5



Schematic illustration of NT-independent entry of NEO6002 in cancer cells. Gemcitabine enters the cells through NTs, whereas NEO6002 may enter the cells through endocytosis or passive diffusion. Once inside the cells, NEO6002 can be hydrolyzed into gemcitabine resulting in cell cycle arrest.

NEO6002. Recently, a gemcitabine conjugated with a different lipid was found to bypass not only NTs, but also two other gemcitabine-resistance mechanisms in cells [30], suggesting that the conjugate is indeed an attractive therapeutic alternative for gemcitabine. NEO6002 can be particularly useful in humans where the expression of esterases in serum is low [31].

In addition to the cell level, we further tested NEO6002 in animals. The results of a multiple-dose toxicity study showed that mice treated with gemcitabine at higher than 27 μmol/kg were found moribund and mice treated with 18 μmol/kg gemcitabine developed dehydration with severe body weight loss. In contrast, the mice treated with 18 and 27 μmol/kg of NEO6002 did not show signs of toxicity. The blood count also showed a favorable result to NEO6002, suggesting that NEO6002 was less toxic than gemcitabine. The anti-tumor efficacy studies using murine leukemia and human pancreatic models were designed based on the toxicity results, and the higher toxicity of gemcitabine had restricted its frequency and dose of administration in animals. Interestingly, 20% of P388 leukemia mice survived long term after being treated with NEO6002 at both 18 and 27 μmol/kg. When administered with a less-frequent schedule to SCID mice bearing the BxPC-3 tumor xenograft, NEO6002 showed improved anti-tumor efficacy compared to gemcitabine. Further study will be required to test NEO6002 in animals carrying gemcitabine-resistant tumors.

In summary, a novel cardiolipin conjugate of gemcitabine (NEO6002) had potent anti-proliferative effects in cancer cell lines. In mice, NEO6002 was well tolerated, and showed anti-tumor activity in murine leukemia and human pancreatic tumors.

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