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Synthesis and biological evaluation of gemcitabine–lipid conjugate (NEO6002)

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Abstract—A novel gemcitabine–lipid conjugate **5** was synthesized and tested for its in vivo efficacy and toxicity. Compound **5** was tested in BxPC-3 human pancreatic tumor model in SCID mice and exhibited promising activity and lower toxicity when compared with Gemzar®.

Gemcitabine (2′,2′-difluorodeoxyribofuranosylcytosine, dFdC) **1** is a difluorinated analogue[1] of deoxycytidine and currently marketed as Gemzar® for the treatment of non-small cell lung and pancreatic cancer. It is a potent antitumor agent in murine and human xenograft solid tumor models.[2] The drug acts as antimetabolite, inhibiting ribonucleotide reductase and DNA synthesis. It is activated inside the cell by deoxycytidine kinases to its active forms, the diphosphate and triphosphate of gemcitabine (dFdCDP, dFdCTP).[3] However, the compound has a narrow therapeutic index due to rapid deamination by an endogenous enzyme, deoxycytidine deaminase, to its corresponding inactive uracil derivative (dFDU).[4] The other limitations include the cellular mechanism of anticancer drug resistance, inadequate uptake of drug by lymphoid and hematopoietic tissues, toxicity, oral bioavailability, short-drug half-life, and prevention of extracellular drug metabolism.[5]

Methods, that have been employed to circumvent the problems associated with gemcitabine and other anticancer nucleoside-based drugs, include the development of lipid–nucleosides conjugates, prodrugs, and liposome preparations. Some of these methods include the synthesis of derivatives[6a] of gemcitabine as prodrugs such as elaidic acid (5′)-gemcitabine ester **2a** and elaidic acid (N^4)–gemcitabine amide **2b** and amino acid esters.[6b]

Alexander et al.[7] reported the development of lipid–nucleoside conjugates, which comprise an alkyl phospholipid moiety covalently conjugated with nucleoside drugs such as gemcitabine **3**, and demonstrated to have cytotoxicity against most cell lines (Fig. 1).

GemMP[10],[8] a novel multimeric prodrug of gemcitabine monophosphate, is a potent cytotoxic agent that serves to induce apoptosis in association with increased FAS expression in cultured thyroid cancer cell lines. GemMP[10] has shown to decrease tumor cell growth at concentrations ranging from 1 to 50 nM.

To improve the half-life and reduce the toxicity of gemcitabine, we chose to conjugate gemcitabine with ether analogue of cardiolipin. Cardiolipin (CL) **4** constitutes a class of complex phospholipids that occur mainly in heart and skeletal muscles and that usually associated with membranes of subcellular fractions showing high metabolic activity, for example, mitochondria.[9] CL plays an important role in many biological processes and is currently utilized in many areas of biochemical and biomedical research.[10] Our novel approach was to conjugate the ether analogue of synthetic cardiolipin with gemcitabine via a succinate linker as prodrug. We speculate that the succinate ester group would be hydrolyzed in vivo by the action of esterases which are prevalent in cells. In this manner there will be a prolonged release of the drug.

In this letter, we report the synthesis of gemcitabine–lipid conjugate (Fig. 1) **5**(NEO6002) and the evaluation

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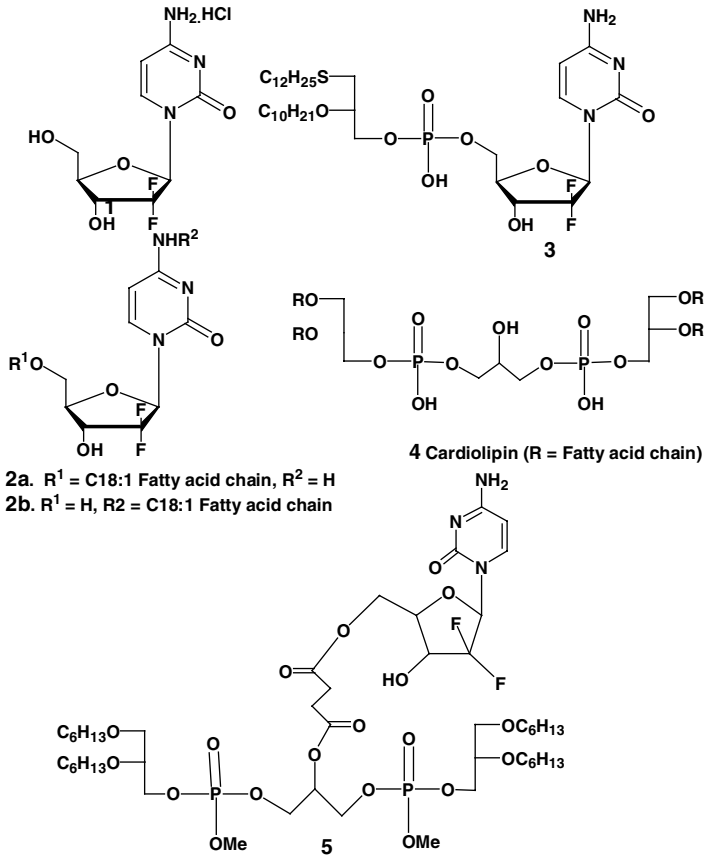
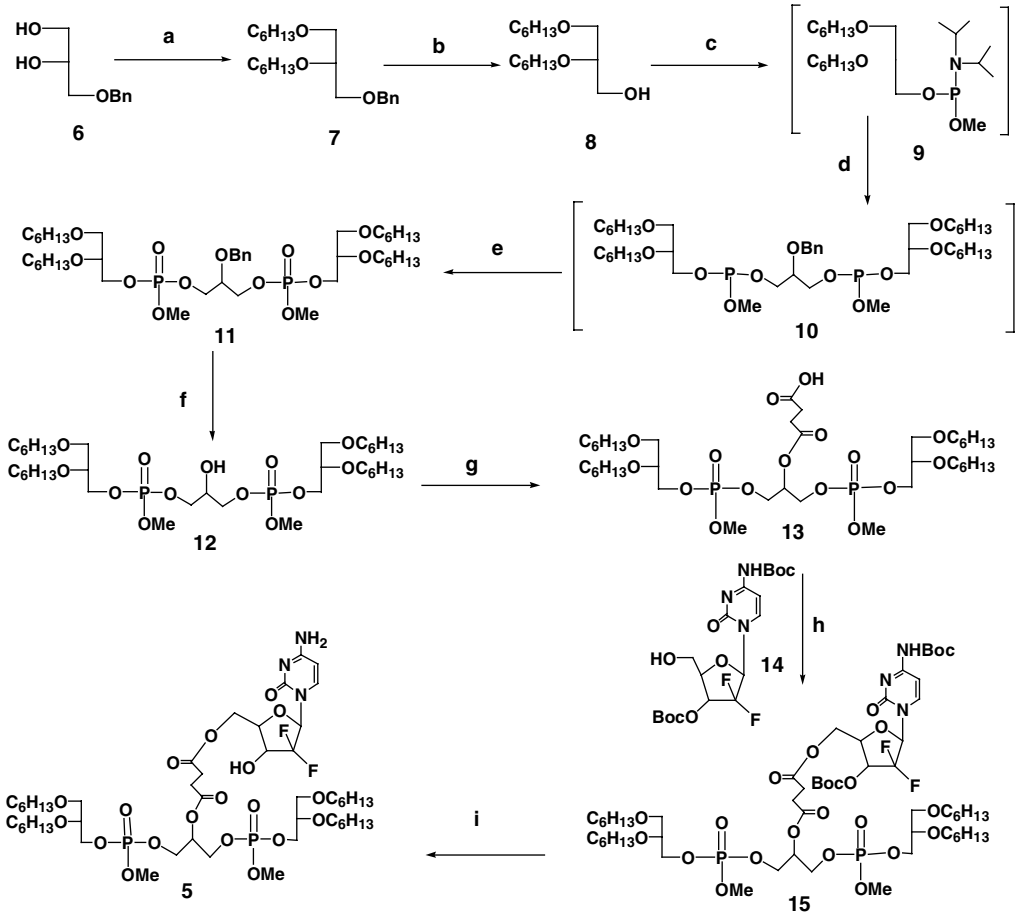


Figure 1. Structures of gemcitabine, gemicatabine derivatives, cardiolipin and gemcitabine–lipid conjugate.

of its biological activity of tumor growth inhibition in vivo, and prolongation of animals survival. The synthetic methodology (Scheme 1), which we have developed for the synthesis of cardiolipin, involves the application of phosphoramidite approach.[11] Alkylation of commercially available (*R*)-(+)-3-benzyloxy-1,2-propanediol **6** with 1-bromohexane in the presence of sodium hydride (60% in oil dispersion) in dimethyl formamide (DMF) followed by debenzylation via catalytic hydrogenolysis (H_2/Pd–C) provided 1,2-di-*O*-hexyl-*sn*-glycerol **8** in 89% yield. The next step involves the reaction of 1,2-di-*O*-hexyl-*sn*-glycerol **8** with the bifunctional phosphitylating reagent *N*,*N*-diisopropylmethyl phosphonamidic chloride in the presence of *N*,*N*-diisopropylethylamine (DIPEA) in dichloromethane at room temperature to give **9**, which was not isolated but subsequently reacted with 2-benzyloxy-1,3-propanediol in the presence of 1*H*-tetrazole to provide phosphite triester **10**. In situ oxidation of phosphite triester **10** with *m*-chloroperbenzoic acid (*m*CPBA) at −40 °C afforded 2-*O*-benzyl-1,3-bis-(1,2-di-*O*-hexyl-*sn*-glycero-3-phosph-oryl)glycerol dimethyl ether **11** as colorless oil in 79% yield after purification on silica gel column (hexane–ethyl acetate, 8:2–6:4). Hydrogenolysis of compound **11** with H_2/Pd–C at 50 psi for 2 h furnished 1,3-bis-(1,2-di-*O*-hexyl-*sn*-glycero-3-phosphoryl)glycerol dimethyl ether **12** as colorless oil in 96% yield. The central hydroxyl functionality of cardiolipin analogue **12** was reacted with succinic anhydride in the presence of triethylamine and 4-dimethylamino pyridine (DMAP) in 1,2-dichloroethane to provide 1,3-bis-(1,2-di-*O*-hexyl-*sn*-glycero-3-phosphoryl)-2-succinylglycerol dimethyl ether[12] **13** in 79% yield. Coupling of **13** with 4-*N*-3′-*O*-bis(*tert*-butoxycarbonyl)gemcitabine[13] **14** in the presence of *N*,*N*-dicyclohexyl carbodiimide (DCC) and DMAP in dichloromethane at room temperature for 8 h followed by purification on silica gel column (hexane–ethyl acetate, 7:3–4:6) afforded **15** in 84% yield. The protecting groups were removed using trifluoroacetic acid (TFA) in dichloromethane at room temperature. The reaction solution was neutralized with 5% aqueous sodium bicarbonate at 0 °C, extracted with dichloromethane and concentrated. Purification of the crude compound on silica gel column (chloroform–methanol, 98:2–96:4) afforded pure 5′-*O*-succinyl-[2-*O*-1,3-bis-(1,2-di-*O*-hexyl-*sn*-glycero)-3-phosphorylglycerol dimethyl ether]gemcitabine **5** as colorless viscous oil in 80% yield. The product was characterized[14] by ^{1}H NMR, ^{13}C NMR, IR, and Mass spectrometry. The purity was checked by HPLC and elemental analysis.

Toxicity and efficacy of compound **5** were evaluated and compared with Gemzar® in mice. In order to perform in vivo studies, compound **5** was formulated into a co-solvent system composed of 5% ethanol and 5% dextrose at a concentration 0.5, 1.0, 1.5, and 2.0 mg/ml.

Multiple dose toxicity of compound **5** and Gemzar® on non-diseased animals was conducted using CD2F1 mice. Mice were treated with compound **5** or Gemzar® for five



Scheme 1. Reagents and conditions: (a) 1-bromohexane, NaH, DMF, 60 °C, 24 h; (b) H_2/Pd–C, ethyl acetate, 4 h; (c) *N*,*N*-diisopropylmethyl phosphonamidic chloride, DIPEA, CH_2Cl_2, rt, 2 h; (d) 2-benzyloxy-1,3-propanediol, 1*H*-tetrazole, CH_2Cl_2, rt, 6 h; (e) *m*CPBA, −40 °C, 1 h; (f) H_2, Pd/C, 50 psi, 2 h; (g) succinic anhydride, Et_3N, DMAP, CH_2Cl_2, rt, 5 h; (h) **14**, DCC, DMAP, CH_2Cl_2, rt 8 h; (i) TFA, CH_2Cl_2, rt, 6 h; aq $NaHCO_3$.

or six daily doses and mortality of the mice were recorded accordingly. The dose, schedule, and animal death are summarized in Table 1. No mortality was seen in groups injected with 18 μmol/kg or 27 μmol/kg of compound **5**. However, all animals were moribund when they were injected with Gemzar® at a dose of 27 μmol/kg or 36 μmol/kg, while only 25% mice died at dose of 36 μmol/kg of compound **5**. This suggested that compound **5** is less toxic at equimolar dosage when compared with Gemzar®.

Table 1. Mortality of non-diseased CD2F1 mice treated with compound **5** or Gemzar®

Compds	Dose (μmol/kg)	Schedule (day)	% Mortality[a] Day 8
D5E5W[b]	(N/A)	1–6	0
Gemzar®	18	1–5	0
Gemzar®	27	1–6	100
Gemzar®	36	1–6	100
5(NEO6002)	18	1–5	0
5(NEO6002)	27	1–6	0
5(NEO6002)	36	1–6	25

[a] Values are of eight animals in each group (N/A = not applicable).
[b] 5% dextrose–5% ethanol.

The in vivo efficacy was evaluated with human pancreatic (BxPC-3) tumor model in SCID mice. Tumor cells (5×10^6) were implanted subcutaneously in each SCID mice. When the tumors reached a volume of 80–160 mm^3, they were randomly divided into six groups. Each of the four groups were administered with 9, 18, and 36 μmol/kg of compound **5** formulation, 9, 18 μmol/kg of Gemzar® and equal volume of control vehicle, respectively, on days 1, 7, 15, 22, 26, 29, and 33. The tumor volumes were measured with Vernier calipers. Percentage of tumor growth (% growth) was calculated as $100 \times (V_t/V_0)$, where V_t and V_0 represent the tumor volume at any given day and on the first day when the treatment started. The study was monitored up to 50 days where groups of mice treated with all doses of compound **5** and Gemzar® showed significant tumor growth inhibition compared to those in control group (Table 2).

In summary, we have designed and synthesized a novel gemcitabine–lipid conjugate that showed lower toxicity and promising efficacy in comparison to Gemzar®. This class of compounds with low toxicity may be further explored to circumvent the problems related with Gemzar®. Efficacy studies using compound **5** in other tumor models, mechanism of action and the

Table 2. Inhibition of BxPC-3 human pancreatic tumor growth by treatment with compound **5** or Gemzar®

Compds	Dose (μmol/kg)	% Growth[a] Day 50
D5E5W[b]	(N/A)	2884 ± 278
Gemzar®	9	1942 ± 438
Gemzar®	18	2007 ± 422
5(NEO6002)	9	1387 ± 354
5(NEO6002)	18	1393 ± 477
5(NEO6002)	36	1022 ± 357

[a] Values are means of eight animals in each group; standard deviations are given in parentheses (N/A = not applicable).
[b] 5% dextrose–5% ethanol.

pharmacokinetic studies will be the subject of further investigation.

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12. All new compounds were fully characterized by ^{1}H NMR, Mass spec, and TLC. Selected data of compound **13**: ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.26–4.14 (m, 8H); 4.09–4.07 (m, 2H); 3.79 and 3.77 (m, 6H); 3.63, 3.57, 3.52 and 3.46 (each m, 13H), 2.66 (m, 4H), 1.55 (m, 8H), 1.29 (m, 24H); 0.89 (t, J = 12 Hz, 12H).
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14. Compound **5**, ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.55 (m, 1H, H); 6.43 (d, J = 7.5 Hz, 1H); 5.93 (m, 1H); 5.22 (m, 1H); 4.71 (m, 1H); 4.54 and 4.51 (dd, J = 12 Hz and 5 Hz, 1H); 4.56–4.39 (m, 2H); 4.26–4.12 (m, 8H); 4.08–4.02 (m, 2H); 3.81–3.76 (m, 6H); 3.62–3.42 (m, 13H); 2.72 (m, 4H); 1.55 (m, 8H); 1.25 (m, 24H); 0.88 (t, J = 12.0 Hz, 12H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 171.53, 171.38, 165.77, 155.58, 141.11, 124.14, 122.07, 120.00, 95.51, 83.98, 78.69, 77.25, 77.07, 76.99, 71.77, 70.62, 69.34, 67.61, 64.76, 61.84, 54.60, 31.59, 29.87, 29.49, 25.67, 25.61, 22.55, 22.54, 13.97. IR (Neat, cm^{-1}): 3330, 3210, 2955, 2930, 2858, 1743, 1651. MS (ESI) *m/z* 1111 (M+1), 1133 (M+Na); Anal. Calcd for $C_{48}H_{87}F_2N_3O_{19}P_2$: C, 51.93; H, 7.90; F, 3.42; N, 3.79. Found: C, 51.88; H, 7.94; F, 3.69; N, 3.76.