Brief Articles

Synthesis and Cytotoxic Activity of Two Novel 1-Dodecylthio-2-decyloxypropyl-3-phosphatidic Acid Conjugates with Gemcitabine and Cytosine Arabinoside

Richard L. Alexander,[†] Susan L. Morris-Natschke,[‡] Khalid S. Ishaq,[‡] Ronald A. Fleming,[§] and Gregory L. Kucera*,[#]

Department of Physiology and Pharmacology, Wake Forest University School of Medicine, Medical Center Boulevard, Winston-Salem, North Carolina 27517, Department of Medicinal Chemistry and Natural Products, School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, Kucera Pharmaceutical Company, 111 N. Chestnut Street, Suite 104, Victoria Hall, Winston-Salem, North Carolina 27101, and Department of Internal Medicine, Section on Hematology/Oncology, Wake Forest University School of Medicine, Medical Center Boulevard, Winston-Salem, North Carolina 27517

Cytosine arabinoside (ara-C) and gemcitabine (dFdC) are two standard chemotherapy drugs used in the treatment of patients with various cancers. To alter the pharmacokinetic and pharmacodynamic properties of these molecules, we conjugated a synthetic phospholipid to both ara-C and dFdC and investigated their chemotherapeutic potential. The dFdC conjugate had greater cytotoxic activity compared with the ara-C conjugate and demonstrated notable cytotoxicity against all human cell lines tested.

Introduction

The deoxycytidine analogue cytosine arabinoside (ara-C) is effective against several types of hematological cancers such as leukemia and lymphoma[1] but is not as effective against solid tumors.[2] Once inside the cell, ara-C is phosphorylated by deoxycytidine kinase to yield ara-C monophosphate (ara-CMP), which is further phosphorylated to the active form, ara-C triphosphate (ara-CTP),[3] which is a potent inhibitor of DNA synthesis causing DNA chain termination and DNA strand breaks.[4]

In comparison, gemcitabine (dFdC) is a deoxycytidine analogue[5] that shows activity against leukemias[6] and various solid tumors.[7,8] Like ara-C, gemcitabine is also phosphorylated by deoxycytidine kinase to the monophosphate dFdC-MP.[9] The drug is further phosphorylated to the active form dFdC triphosphate (dFdC-TP), which can be incorporated into DNA and inhibit DNA polymerase, ultimately promoting apoptosis.[10]

To increase the efficacy of ara-C, Ryu et al. linked ara-C with natural phospholipids.[11] These compounds demonstrated promising in vitro and in vivo results; however, they were subject to degradation in the GI tract by phospholipase A_2 and other lipases that degrade ester-containing lipid molecules.[12] However, previous animal model experiments using 1-dodecylthio-2-decyloxypropyl-3-phosphatidic acid as a carrier for AZT

demonstrated oral bioavailability,[13] and previous structure–activity relationship studies showed that the phospholipid molecule chosen had a favorable toxicity profile in vitro.[14]

To increase the uptake and efficacy of ara-C and dFdC, we chose to conjugate these drugs to a synthetic C-1 thioether, C-2 oxyether phospholipid, which would not be subject to metabolism at the C-1 and C-2 alkyl side chains in the GI tract. To our knowledge, this is the first report of the synthesis of a phospholipid conjugated to gemcitabine. The resulting conjugates have shown promising results in vitro in MTS assays. Furthermore, on the basis of our data, we believe the lipid–dFdC conjugate is a drug that is superior to the lipid–ara-C conjugate and should be further investigated.

Chemistry

The phospholipid was synthesized as previously published (see Supporting Information for Scheme 1).[14,15] Briefly, 3-mercapto-1,2-propanediol (**1**) was reacted with bromododecane in ethanol/KOH to yield 1-*S*-dodecyl-2,3-glycerol (**2**). The primary OH was protected by reacting **2** with triphenylmethyl chloride in pyridine. Next, the product **3** was refluxed with bromodecane and sodium hydride in tetrahydrofuran (THF) to give **4**. The triphenylmethyl protecting group was removed using *p*-toluenesulfonic acid in $CHCl_3$/MeOH, and the resulting dialkylglycerol **5** was reacted with phosphorus oxychloride in pyridine and THF to yield 1-*S*-dodecyl-2-*O*-decylthioglycero-3-phosphatidic acid (**6**).

To direct conjugation to the 5′-OH, the other reactive groups were chemically protected (see Supporting In-

* To whom correspondence should be addressed. Phone: (336) 716-6348. Fax: (336) 716-0255. E-mail: gkucera@wfubmc.edu.
† Department of Physiology and Pharmacology, Wake Forest University School of Medicine.
‡ University of North Carolina at Chapel Hill.
§ Kucera Pharmaceutical Company.
# Department of Internal Medicine, Wake Forest University School of Medicine.

Table 1. Effects of dFdC and ara-C Conjugates on Different Cell Lines

	IC$_{50}$ ± SD, $n = 3$ (μM)	
	ara-C	ara-C conjugate
HL-60 (human, promyelocytic leukemia)	0.76 ± 0.16	67.3 ± 26
	IC$_{50}$ ± SD, $n = 3$ (μM)	
	dFdC	dFdC conjugate
HL-60 (human, promyelocytic leukemia)	0.33 ± 0.07	1.8 ± 0.57
CEM-SS (human T-4 lymphoblastoid clone)	0.03 ± 0.02	0.6 ± 0.13
HeLa (human, epitheliod carcinoma, cervix)	0.008 ± 0.0012	0.69 ± 0.33
HT-29 (human, colon, adenocarcinoma)	0.043 ± 0.015	0.75 ± 0.07
MCF7/WT-2′ (human, breast carcinoma)	1.66 ± 1.1	1.73 ± 1.1
U373-MG (human, glioblastoma)	0.13 ± 0.03	0.29 ± 0.043
BxPC3 (human, pancreas, adenocarcinoma)	0.16 ± 0.02	1.59 ± 0.41
SK-LU-1 (human, lung, adenocarcinoma)	0.055 ± 0.01	1.12 ± 0.17
SCC-25 (human, squamous cell carcinoma, tongue)	0.047 ± 0.026	0.59 ± 0.39
BG-1 (human, breast, adenocarcinoma)	0.68 ± 0.37	1.84 ± 0.73
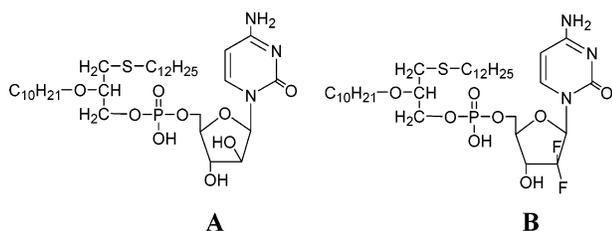
Figure 1. Chemical structures of the two compounds synthesized: (A) ara-C conjugate; (B) dFdC conjugate.

formation for Scheme 2). The ara-C **7** was refluxed with benzoic anhydride in ethanol to form the amide derivative **8**.[16] The 5′-OH was protected using *tert*-butyldimethylsilyl chloride (TBDMS) in dimethyl formamide (DMF) to give **9**.[17] Next, the 3′-OH and 2′-OH were protected using acetic anhydride in pyridine.[18] Finally, the TBDMS group of **10** was removed using tetrabutylammonium fluoride in THF.[17]

The protected ara-C **11** was reacted with the phosphatidic acid **6** using 2,4,6-triisopropylbenzenesulfonyl chloride in pyridine at 40−50 °C to generate **12**. The amide and ester protecting groups were removed using 2 N NH$_3$−MeOH to yield the final lipid−ara-C conjugate molecule **13**.[18] The dFdC conjugate was synthesized in the same manner as described above. Both conjugate molecules can be seen in Figure 1. For synthetic schemes and NMR chemical shifts, please see Supporting Information.

Results and Discussion

The conjugates were tested for their solubility in aqueous solution. When sonicated and subjected to centrifugation for 20 min in a microfuge, approximately 50% of the ara-C conjugate formed lipid aggregates that were soluble in phosphate-buffered saline (PBS). On the other hand, the dFdC conjugate was about 80% soluble in the PBS under similar conditions. When the two conjugates were subjected to a 1-octanol and PBS mixture, the ara-C conjugate was found almost equally in both layers (partition coefficient of 1.17). Conversely, the dFdC conjugate favored the octanol layer with a partition coefficient of 3.47. Taken together, these results suggest that the dFdC conjugate forms a lipid aggregate in PBS that remains in solution better than the ara-C conjugate. Furthermore, the dFdC conjugate favors the octanol layer, which supports our hypothesis

that the dFdC conjugate is able to cross the lipid membrane of the cell better than the ara-C conjugate. These results would also suggest a reason for why the dFdC conjugate is more cytotoxic than the ara-C conjugate. The specific experimental conditions can be found in the Supporting Information.

Using an MTS assay, we confirmed that the dFdC conjugate was more cytotoxic than the ara-C conjugate in all cell lines tested. The resulting IC$_{50}$ values can be seen in Table 1. The ara-C conjugate did show cytotoxicity in HL-60 cells; however, it was much less cytotoxic in the other cell lines tested. In contrast, the dFdC conjugate was cytotoxic in every cell line tested. Therefore, we believe that the dFdC conjugate warrants further study into its metabolism and mechanism of action.

We also used electrospray ionization mass spectroscopy to investigate the metabolism of these two conjugate molecules. When the conjugates were incubated in media over 72 h, we observed <4% reduction of the parent conjugate. However, when the ara-C conjugate was incubated in the presence of HL-60 cells (50 μM) for 72 h, there was a slight reduction in the parent drug (10−20%). When the dFdC conjugate was incubated in the presence of HL-60 cells (10 μM) for 72 h, there was a statistically significant ($p < 0.05$, one-way ANOVA, with Dunnett's multiple comparison post test) reduction in the parent compound (50%). The specific experimental conditions can be found in the Supporting Information.

Conclusion

The synthetic ara-C and dFdC lipid conjugates show cytotoxic activity in several different tumor cell lines. The dFdC conjugate showed better activity than the ara-C conjugate across all cell lines tested (ara-C data not shown). These results are supported by the fact that the dFdC conjugate more easily formed soluble lipid aggregates in PBS than the ara-C conjugate. Additionally, the dFdC conjugate had a greater partition coefficient in octanol than the ara-C conjugate. These results suggest that the dFdC conjugate may be superior to the ara-C conjugate because it can more readily pass through the lipid membrane of the cell.

The results of the metabolism studies suggest that the presence of cells is necessary for the metabolism/activation of the compounds. Furthermore, the ara-C

molecule is incorporated very poorly into cells as shown by the slight reduction in the parent ara-C conjugate over time. Conversely, the dFdC conjugate was more readily taken up by the cells and metabolized. These results may also indicate why the dFdC conjugate is superior to the ara-C conjugate.

We also have hypothesized that the conjugate molecules take longer to enter the cell and be cleaved by a phospholipase C-like enzyme.[19] Although the ara-C and dFdC molecules are transported across the cell membrane via a transporter, we believe that the conjugates cross the membrane by passive diffusion because of the increased lipophilicity of the conjugate molecules compared to the nucleosides alone. On the basis of our initial data, the lipid−dFdC conjugate has altered pharmacokinetic properties compared with the parent nucleoside, may have possible clinical efficacy, and warrants further investigation.

Experimental Section

Proton NMR spectra were recorded on a Bruker-Spectrospin 300 MHz spectrometer. The chemical shifts are reported in parts per million from internal TMS. Melting points were determined on a Laboratory Devices Mel-Temp II melting point apparatus, and mass spectral analysis was performed on an Agilent 1100 series LC/MSD trap. High-resolution mass spectral analysis was performed on a JEOL, JMS-SX102A mass spectrometer. Silica gel 60 plates (Merck F_{254} or Whatman) were used for thin-layer chromatography. Purity of all samples was ascertained by thin-layer chromatography in the indicated solvent systems; compounds were visualized by UV light, iodine, H_2SO_4 spray, or phosphomolybdate spray. Column chromatography was performed with silica gel (70−230 mesh). All chemicals and solvents were purchased from Sigma-Aldrich and Fisher Scientific except dFdC, which was purchased from the Wake Forest University Baptist Medical Center Pharmacy. NMR data of the lipid intermediates were consistent with those previously reported.[14,15] All reactions were carried out under N_2 and extremely dry conditions. Aqueous solubility and partition coefficient measurements were tested as previously described.[20]

In Vitro Cytotoxicity Studies. The CEM-SS, HL-60, and BG-1 cells were maintained in RPMI-1640 medium supplemented with 10% FBS. HeLa cells were maintained in DMEM medium supplemented with 10% FBS. U373-MG and SK-LU were maintained in MEM medium supplemented with 10% FBS. BxPC3 were maintained in RPMI-1640 supplemented with 10% FBS, 10 mM HEPES, 1.5 g/L $NaHCO_3$, and sodium pyruvate. HT-20 cells were maintained in McCoy's 5A Medium Modified supplemented with 10% FBS. MCF7/WT-2′ cells were maintained in D-MEM/F-12 with 10% FBS and 10 μg/mL insulin. SCC-25 cells 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− streptomycin (1%). The CellTiter 96 aqueous nonreactive cell proliferation assay (Promega Life Sciences, Madison, WI) was used according to the manufacturer's details, and the results were read on a precision microplate reader (Molecular Devices, Sunnyvale, CA). The optical densities of the drug-treated cells were compared to those of untreated control cells, and the data were plotted in GraphPad Prizm. Nonlinear regression analysis was used to determine IC_{50} values. For a more detailed explanation of the biological assays, please see Supporting Information.

Supporting Information Available: Experimental procedures and synthetic schemes. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Mastrianni, D. M.; Tung, N. M.; Tenen, D. G. Acute myelogenous leukemia: current treatment and future directions. *Am. J. Med.* **1992**, *92*, 286−295.

(2) Chabner, B. A.; Myers, C. E. Clinical pharmacology of cancer chemotherapy. In *Cancer: Principles and Practice of Oncology*; DeVita, V. T., Jr., Hellman, S., Rosenberg, S. A., Eds.; Lippincott: Philadelphia, PA, 1989; pp 349−395.

(3) Kessel, D.; Hall, T. C.; Wodinsky, I. Transport and phosphorylation as factors in the antitumor action of cytosine arabinoside. *Science* **1967**, *156*, 1240−1241.

(4) Fram, R. J.; Kufe, D. W. DNA strand breaks caused by inhibitors of DNA synthesis: 1-beta-D-arabinofuranosylcytosine and aphidicolin. *Cancer Res.* **1982**, *42*, 4050−4053.

(5) Plunkett, W.; Huang, P.; Xu, Y. Z.; Heinemann, V.; Grunewald, R.; Gandhi, V. Gemcitabine: metabolism, mechanisms of action, and self-potentiation. *Semin. Oncol.* **1995**, *22*, 3−10.

(6) Grunewald, R.; Kantarjian, H.; Du, M.; Faucher, K.; Tarassoff, P.; Plunkett, W. Gemcitabine in leukemia: a phase I clinical, plasma, and cellular pharmacology study. *J. Clin. Oncol.* **1992**, *10*, 406−413.

(7) Lund, B.; Hansen, O. P.; Theilade, K.; Hansen, M.; Neijt, J. P. Phase II study of gemcitabine (2′,2′-difluorodeoxycytidine) in previously treated ovarian cancer patients. *J. Natl. Cancer Inst.* **1994**, *86*, 1530−1533.

(8) Casper, E. S.; Green, M. R.; Kelsen, D. P.; Heelan, R. T.; Brown, T. D.; Flombaum, C. D.; Trochanowski, B.; Tarassoff, P. G. Phase II trial of gemcitabine (2,2′-difluorodeoxycytidine) in patients with adenocarcinoma of the pancreas. *Invest. New Drugs* **1994**, *12*, 29−34.

(9) Bouffard, D. Y.; Laliberte, J.; Momparler, R. L. Kinetic studies on 2′,2′-difluorodeoxycytidine (Gemcitabine) with purified human deoxycytidine kinase and cytidine deaminase. *Biochem. Pharmacol.* **1993**, *45*, 1857−1861.

(10) Huang, P.; Plunkett, W. Induction of apoptosis by gemcitabine. *Semin. Oncol.* **1995**, *22*, 19−25.

(11) Ryu, E. K.; Ross, R. J.; Matsushita, T.; MacCoss, M.; Hong, C. I.; West, C. R. Phospholipid−nucleoside conjugates. Syntheses and preliminary biological evaluation of 1-β-D-arabinofuranosylcytosine 5′-monophosphate-L-1,2-dipalmitin and selected 1-β-D-arabinofuranosylcytosine 5-diphosphate-L-1,2-diacylglycerols. *J. Med. Chem.* **1982**, *25*, 1322−1329.

(12) Lambert, D. M. Rationale and applications of lipids as prodrug carriers. *Eur. J. Pharm. Sci.* **2000**, *11,* S15−S27.

(13) Herrmann, D. B. J.; Opitz, H.-G.; Kucera, L. S. In vitro and in vivo evaluation of the new anti-AIDS compound Fozivudine. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **1997**, *356*, R44.

(14) Piantadosi, C.; Marasco, C. J., Jr.; Morris-Natschke, S. L.; Meyer, K. L.; Gumus, F.; Surles, J. R.; Ishaq, K. S.; Kucera, L. S.; Iyer, N.; Wallen, C. A.; et al. Synthesis and evaluation of novel ether lipid nucleoside conjugates for anti-HIV-1 activity. *J. Med. Chem.* **1991**, *34*, 1408−1414.

(15) Kucera, L. S.; Iyer, N.; Morris-Natschke, S. L.; Chen, S. Y.; Gumus, F.; Ishaq, K.; Herrmann, D. B. In vitro evaluation and characterization of newly designed alkylamidophospholipid analogues as anti-human immunodeficiency virus type 1 agents. *Antiviral Chem. Chemother.* **1998**, *9*, 157−165.

(16) Lin, T.-S.; Prusoff, W. H. 1-(5-Azido-5-deoxy-β-d-arabinofuranosyl) cytosine and 1-(5-Amino-5-deoxy-β-D-arabinofuranosyl) cytosine. In *Nucleic Acid Chemistry;* Townsend, L. B., Tipson, R. S., Eds.; John Wiley & Sons: New York, 1978; pp 261−272.

(17) Ryu, E. K.; Ross, R. J.; Matsushita, T.; MacCoss, M.; Hong, C. I.; West, C. R. Phospholipid−nucleoside conjugates. 3. Syntheses and preliminary biological evaluation of 1-β-D-arabinofuranosylcytosine 5′-monophosphate-L-1,2-dipalmitin and selected 1-β-D-arabinofuranosylcytosine 5-diphosphate-L-1,2-diacylglycerols. *J. Med. Chem.* **1982**, *25*, 1322−1329.

(18) Brachwitz, H.; Bergmann, J.; Thomas, Y.; Wollny, T.; Langen, P. Synthesis and antiproliferative potency of 9-beta-D-arabino-

furanosyl-2-fluoroadenine phospholipid adducts. *Bioorg. Med. Chem.* **1999**, *7*, 1195−1200.

(19) Kucera, G. L.; Goff, C. L.; Iyer, N.; Morris-Natschke, S.; Ishaq, K. S.; Wyrick, S. D.; Fleming, R. A.; Kucera, L. S. Cellular metabolism in lymphocytes of a novel thioether−phospholipid−AZT conjugate with anti-HIV-1 activity. *Antiviral Res.* **2001**, *50*, 129−137.

(20) Hong, C. I.; Nechaev, A.; Kirisits, A. J.; Vig, R.; West, C. R. Nucleoside conjugates. 13. Synthesis and antitumor activity of 1-β-D-arabinofuranosylcytosine conjugates of thioether lipids with improved water solubility. *J. Med. Chem.* **1993**, *36*, 1785−1790.