Brief Articles

Synthesis and Biological Activity of a Gemcitabine Phosphoramidate Prodrug

Weidong Wu,[†,§] Jennifer Sigmond,[‡] Godefridus J. Peters,[‡] and Richard F. Borch*[,†]

Department of Medicinal Chemistry and Molecular Pharmacology and Cancer Center, Purdue University, West Lafayette, Indiana 47907, and Department of Medical Oncology, VU University Medical Center, Post Office Box 7057,1007 MB Amsterdam, the Netherlands

Received March 9, 2007

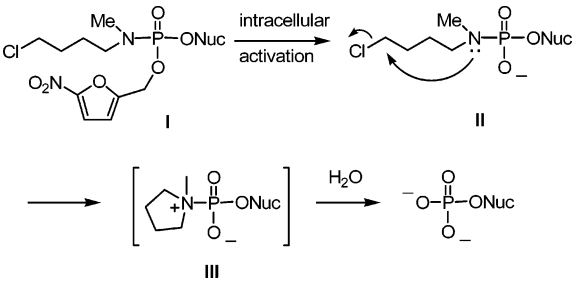
A gemcitabine (2′,2′-difluorodeoxycytidine, dFdC) phosphoramidate prodrug designed for the intracellular delivery of gemcitabine 5′-monophosphate was synthesized. The prodrug was about an order of magnitude less active than gemcitabine against wild-type cells, and the nucleoside transport inhibitor dipyridamole reduced prodrug activity. The prodrug was more active than gemcitabine against two deoxycytidine kinase-deficient cell lines. The results suggest that the prodrug is a potent growth inhibitor that can bypass dCK deficiency at higher drug concentrations.

Introduction

Nucleoside analogues represent an important class of anticancer and antiviral drugs.[1,2] The biological activity of these agents requires intracellular metabolism to nucleoside 5′-mono, di-, or triphosphates by kinase-mediated phosphorylation to bind to their molecular targets. However, inefficient intracellular phosphorylation of the nucleoside may reduce the efficacy of these agents. This may result either from the poor affinity of unnatural nucleoside to the kinase or from a decreased nucleoside kinase activity leading to drug resistance.[3] One strategy to solve this problem consists of developing prodrug approaches that would deliver nucleoside 5′-monophosphates intracellularly,[4] because the first phosphorylation step is rate limiting for many nucleoside analogs. Our laboratory has a long-standing interest in the development of nucleoside and other phosphoramidate prodrugs.[5−8] These prodrugs are designed to undergo intracellular activation to generate an unstable phosphoramidate anion intermediate, which in turn undergoes spontaneous cyclization and P−N bond cleavage by water to liberate the nucleoside monophosphate (Scheme 1). We have reported the synthesis and biological activities of a series of halobutyl phosphoramidate prodrugs of 5-fluoro-2′-deoxyuridine 5′-monophosphate.[5] It was found that these novel prodrugs undergo rapid conversion to the corresponding nucleotide and exhibit potent growth inhibition of both wild-type and thymidine kinase-deficient cells.[7] Recently a phosphoramidate prodrug of cytarabine (1-β-D-arabinosylcytosine, Ara-C) has also been synthesized and evaluated in comparison with cytarabine for growth inhibitory activity against wild-type, nucleoside transport-deficient, and nucleoside kinase-deficient CEM leukemia cell lines.[8]

We have now extended the phosphoramidate prodrug approach to gemcitabine (**1**, 2′,2′-difluorodeoxycytidine, dFdC), a deoxycytidine analog with a marked self-potentiating effect on several enzymes involved in DNA synthesis and repair[9−11]

Scheme 1



and with demonstrated clinical activity against various solid tumors.[12,13] Similar to the structurally and functionally related deoxycytidine analogue Ara-C, gemcitabine requires intracellular phosphorylation to mono-, di-, and triphosphates to be active, and the first step in phosphorylation catalyzed by deoxycytidine kinase (dCK) is the rate-limiting step and, thus, is essential for the activiation of gemcitabine.[14−16] The most frequently described form of acquired resistance to gemcitabine in vitro is dCK deficiency,[17,18] so intracellular delivery of gemcitabine 5′-monophosphate might be expected to overcome resistance in those tumors deficient in dCK. Similar to other nucleoside analogs, gemcitabine is hydrophilic and cannot traverse cell membranes by passive diffusion.[19] Specialized transport systems such as ENT are essential for gemcitabine cytotoxicity, and inhibition of nucleoside transport across the cell membrane results in resistance to gemcitabine.[20,21] An appropriately designed gemcitabine prodrug might also be able to enter cells by passive diffusion, thus circumventing the resistance caused by nucleoside transport deficiency. Herein we report the synthesis and in vitro studies of a phosphoramidate prodrug of gemcitabine.

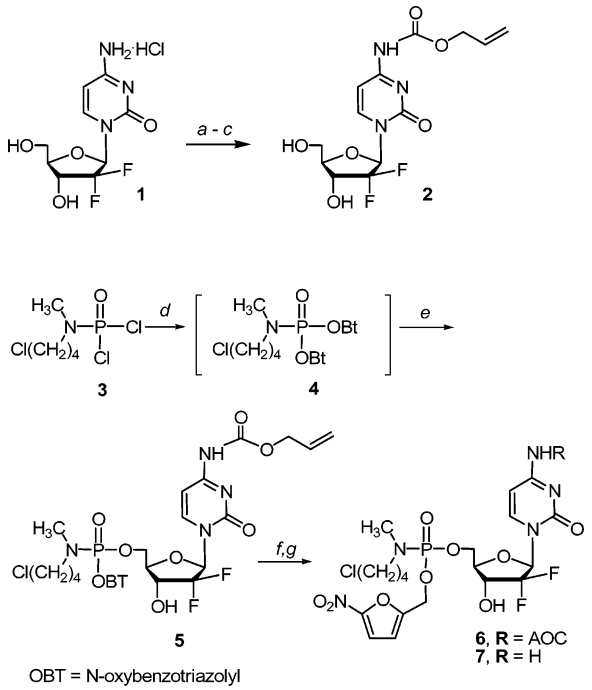
Results and Discussion

Chemistry. To avoid potential side reactions and improve solubility, the exocyclic amine moiety of gemcitabine was protected with the allyloxycarbonyl group (AOC),[22] as reported previously for the synthesis of the Ara-C prodrug.[8] Thus, N^4-AOC-2′,2′-difluorodeoxycytidine **2** was prepared in almost quantitative yield, following the procedure used to prepare N^4-

* To whom correspondence should be addressed. Phone: 765-494-1403. Fax: 765-494-1414. E-mail: borch@purdue.edu.
† Purdue University.
‡ VU University Medical Center.
§ Current address: Lasergen, Inc., 8052 El Rio Street, Houston, TX 77054.

10.1021/jm070269u CCC: $37.00
Published on Web 06/29/2007

Scheme 2[a]



[a] Reagents and conditions: (a) $(Me_3Si)_2NH$, $(NH_4)_2SO_4$, dioxane, 2 h, reflux; (b) AOCCl, *N*-methylimidazole, CH_2Cl_2, 4 h, rt; (c) Et_3N, MeOH, overnight, rt; (d) HOBT, pyridine, THF, 4 h, rt; (e) **2**, *N*-methylimidazole, pyridine, 20 h, rt; (f) 5-nitrofurfuryl alcohol, DMAP, THF, overnight, rt; (g) $Pd(PPh_3)_4$, *p*-$C_6H_4SO_2Na$, THF/H_2O (2:1), 1 h, rt.

AOC-2′-deoxycytidine[23] (Scheme 2). Compound **2** was then converted to prodrug **7** according to a previously reported procedure that features phosphorylation of the nucleoside using a highly reactive phosphoramidic bis(1-benzotriazolyl) ester.[24] Selective phosphorylation of **2** on the 5′-OH was achieved with phosphorylating agent **4**, generated in situ from phosphoramidic dichloride **3**[25] and 1-hydroxybenzotriazole (HOBT), to give benzotriazolyl phosphoramidate **5** in 45% yield. The OBT moiety in **5** was displaced by 5-nitrofurfuryl alcohol in the presence of 4-dimethylamino-pyridine (DMAP) to give phosphoramidate **6** in 59% yield. Finally, compound **6** was deprotected[8,22] to give the desired gemcitabine phosphoramidate prodrug **7** as a mixture of diastereomers in 70% yield.

Biological Activity. Prodrug **7** as a mixture of diastereomers was evaluated for growth inhibitory activity in the NCI 60-cell line in vitro assay. Although the diastereomers may be activated at different rates, the phosphoramidate (e.g., **II** in Scheme 1) is generated as a single enantiomer. The prodrug has a growth inhibitory profile that is similar to that of gemcitabine, although the prodrug is approximately an order of magnitude less potent than the parent nucleoside (Median GI_{50} is 1.3 μM for **7** compared with 0.14 μM for gemcitabine). When the average GI_{50} values for prodrug **7** and gemcitabine were each subjected to COMPARE analysis (https://itbwork.nci.nih.gov:8443/PrivateServer/CompareServer), the results for the two drugs were very similar as expected; 12 of the 15 most highly correlated agents for each drug were common to both drugs. Prodrug **7** was further tested in selected cell lines in which the enzymatic activity of dCK, known to activate dFdC by phosphorylation, was characterized. The differences in these cell lines' sensitivity to dFdC and prodrug **7** are shown in the upper panel of Table 1. Most of the cell lines tested are 3- to 8-fold less sensitive to the prodrug compared to the nucleoside. To investigate whether **7** uses nucleoside transporters, cells were also exposed to drug in the presence of dipyridamole, which inhibits the equilibrative nucleoside transporter.[26] Growth inhibitory effects of both dFdC and prodrug **7** are reduced significantly by dipyridamole in all wild-type cell lines, demonstrating that transporter-mediated uptake contributes to activity for both compounds. To test whether **7** might be able to bypass deoxycytidine kinase deficiency, two dCK-deficient cell lines were compared to the parental cell lines (Table 1, lower panel). In the wild-type cell lines (A2780 and CEM), the prodrug is less active than gemcitabine, and both become less active in the presence of dipyridamole. However, in the dCK-deficient variants (AG6000 and CEM/dCK-), the prodrug is about 4-fold more active than gemcitabine itself, while dipyridamole does not diminish the activity of the prodrug in these cell lines. These results show that dCK-deficient cell lines exhibit a high level of resistance to gemcitabine (13 000-fold for AG6000 vs A2780 and 385-fold for CEM-dCK- vs CEM, respectively), but are 1−2 orders of magnitude less resistant (625-fold for AG6000 vs A2780 and 6.5-fold for CEM-dCK- vs CEM, respectively) to the prodrug. The results suggest that high concentrations of the prodrug can bypass dCK deficiency and the nucleoside transporter and that the monophosphate after intracellular delivery can be further phosphorylated to the active triphosphate.

Table 1. Deoxycytidine Kinase Activity and Sensitivity of Cell Lines to dFdC and Prodrug **7** in the Presence and Absence of the Transport Inhibitor Dipyridamole[a]

cell line	dCK (nmol/h/mg protein)	dFdC IC_{50}, nM		prodrug IC_{50}, nM		IC_{50} ratio
dipyridamole (+/-)		-	+	-	+	-
A549	4.86	14	225[b]	79[c]	>500[b,c]	5.6
H292	7.05	13	210[b]	37[c]	330[b]	2.8
H460	3.17	10	103[b]	77[c]	>500[b,c]	7.7
SW1573	4.38	16	275[b]	92[c]	>500[b,c]	5.8
A2780	6.12	1.5	15[b]	8[d]	400[b,d]	5.3
AG6000	<1	20 000	20 000	5000[d]	6000[d]	0.25
CEM	22.7	130	1000[b]	2000[d]	4000[b,d]	15
CEM/dCK-	<1	50 000	100 000	13 000[d]	17 000[d]	0.26

[a] Statistical significance was evaluated using the one-tailed student's t-test for paired data. [b] Significant difference in IC_{50} ($p < 0.01$) in the presence vs absence of dipyridamole. [c] Significant difference in IC_{50} ($p < 0.05$) for the prodrug vs dFdC, both for the tests with and without dipyridamole. [d] Significant difference in IC_{50} ($p < 0.01$) for the prodrug vs dFdC, both for the tests with and without dipyridamole.

Conclusions

The phosphoramidate prodrug strategy for the intracellular delivery of nucleoside monophosphate has been applied to the preparation of a gemcitabine monophosphate prodrug. Although the prodrug is less active than gemcitabine in wild-type cell lines, it is more active than gemcitabine itself in the dCK-deficient variants, and its activity against these cell lines is retained in the presence of transport inhibitor dypiridamole. These results are consistent with a mechanism of activation involving intracellular delivery of gemcitabine 5′-monophosphate. Thus, neither deoxycytidine kinase nor the nucleoside transporter is essential for prodrug activity.

Experimental Section

Materials and Methods. Routine ^1H NMR spectra were recorded using standard methods and are referenced to tetramethylsilane unless otherwise specified. ^{31}P NMR spectra were acquired using broadband ^1H decoupling and are referenced using 1% triphenylphosphine oxide in benzene-d_6 as a coaxial insert (triphenylphosphine oxide/benzene-d_6 has a chemical shift of +25.17 ppm relative to 85% phosphoric acid). Elemental analysis was performed by the Microanalysis Laboratory in the Department of

Chemistry, Purdue University. Mass spectra were obtained by the Mass Spectrometry Laboratory at Purdue University. Analytical TLC on silica gel was performed on polyester plates coated with silica gel 60 F_{254} and was visualized by UV light or using one of the following stains: (i) 5% phosphomolybdic acid in ethanol or (ii) 1% 4-(*p*-nitrobenzyl)pyridine (NBP) in acetone followed by heating and treatment with 3% KOH in methanol. Flash silica gel chromatography was performed on silica gel 60 (230−400 mesh).

All anhydrous reactions were carried out under argon using oven-dried flasks. Anhydrous solvents were either distilled from appropriate drying agents or obtained from commercial source. Dulbecco's modified Eagle's medium (DMEM) was obtained from Flow Laboratories (Irvine, Scotland). Both (nondialyzed) fetal calf serum (FCS) and Hank's balanced salt solution (HBSS) were purchased from Gibco Europe (Paisly, U.K.). Roswell Park Memorial Institute (RPMI) 1640, L-glutamine and 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid (HEPES) were obtained from BioWhittaker (Verviers, Belgium). Gemcitabine (Gemzar, dFdC) was provided by Eli Lilly Research Laboratories (Indianapolis, IN, U.S.A.). The CEM/dCK- cell line was a gift from Prof. Dr. Jan Balzarini, Rega Institute, Leuven, Belgium. Unless otherwise specified, all other chemicals were of analytical grade and commercially available.

In Vitro Assays. Four non-small-cell lung cancer (NSCLC) cell lines were used in this study: A549, H292 (NCI-H292), H460 (NCI-H460), and SW1573. Leukemic and ovarian cancer cell lines were also used: CEM and A2780, respectively, and the corresponding dCK-deficient variants CEM/dCK- and AG6000.[27] SW1573 cells as well as the ovarian cancer cells were cultured in DMEM medium containing 10% FCS and 20 mM HEPES. The other NSCLC cell lines and the leukemic cell lines were cultured in RPMI medium containing 10% FCS and 20 mM HEPES. Cells were maintained at 37 °C under an atmosphere of 5% CO_2.

Details of the methodology for the NCI in vitro screen are described at http://dtp.nci.nih.gov/branches/btb/ivclsp.html. Growth inhibitory effects of dFdC and prodrug **7** were evaluated in the other cell lines using the SRB assay. NSCLC cells and ovarian cancer cells (5000 cells/well) were seeded in 96-well flat-bottomed Greiner plates and were exposed to various drug concentrations for 72 h, whereafter, the standard SRB assay was performed.[28] Cells of CEM variants (100 000 cells/well) were evaluated with the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay, as described in Rots et al.[29] The final concentration of the deoxynucleoside transporter inhibitor dipyridamole was 1 μM. The optical density (OD) was measured at 540 or 492 nm by means of an automated spectrophotometric microplate reader (Titertek Multiskan MCC/340 of Tecan Spectrafluor, Austria). Relative growth was calculated as $[(OD_{treated} - OD_{zero})/(OD_{control} - OD_{zero})] \times 100\%$. The OD_{zero} depicts the cell number at the time of drug addition, the $OD_{control}$ reflects the cell number of untreated wells, and the $OD_{treated}$ reflects the cell number in treated wells on the day of the assay.

Deoxycytidine Kinase Assay. Radioactive assays were performed for measuring dCK activity as previously described using [^{3}H]-CdA[30] as the substrate. CdA is a very specific dCK substrate and not a substrate for other nucleoside kinases, for example, TK2.

2′-Deoxy-2′,2′-difluoro-D-ribofuranosyl-*N*^{4}-(allyloxycarbonyl)-cytosine (2). A mixture of gemcitabine (2′-deoxy-2′,2′-difluorocytidine hydrochloride), **1** (300 mg, 1 mmol), hexamethyldisilazane (5 mL), and a catalytic amount of ammonium sulfate (5 mg) in dioxane (5 mL) was heated under reflux for 2 h. The reaction mixture was concentrated and coevaporated twice with dry toluene, and the residue was dissolved in CH_2Cl_2 (10 mL). To this solution were added *N*-methylimidazole (0.24 mL, 3 mmol) and allyloxycarbonyl chloride (0.32 mL, 3 mmol), and the resulting mixture was stirred at room temperature for 4 h. The reaction mixture was concentrated to a viscous oil, which was dissolved in methanol (20 mL) containing triethylamine (3 mL), and the resulting solution was stirred overnight at room temperature. Solvent was removed in vacuo, and the residue was purified by silica gel chromatography (CH_2Cl_2/MeOH, 20:1) to give N^4-AOC-protected gemcitabine **2** (345 mg, 99%) as a white foam. TLC R_f 0.45 (CH_2Cl_2/MeOH, 10:1); ^{1}H NMR (MeOH-d_4, 300 MHz) δ 8.25 (d, 1H, $J_{6,5}$ = 7.5 Hz, H-6), 7.28 (d, 1H, $J_{5,6}$ = 7.5 Hz, H-5), 6.19 (t, 1H, J = 6.9 Hz, H-1′), 5.92 (m, 1H, −CH═), 5.30 (m, 2H, ═CH_2), 4.63 (m, 2H, CH_2—CH═CH_2), 4.24 (m, 1H, H-3′), 3.91−3.76 (m, 3H, H-4′, H-5′ and H-5″); MS (ESI) 348 $[M + H]^+$; Anal. ($C_{13}H_{15}F_2N_3O_6$) C, H, N.

5′-[2′-Deoxy-2′,2′-difluoro-D-ribofuranosyl-*N*^{4}-(allyloxycarbonyl)cytosine] 1-benzotriazolyl *N*-Methyl-*N*-(4-chlorobutyl) Phosphoramidate (5). A solution of phosphoramidic dichloride **3**[25] (380 mg, 1.6 mmol) in tetrahydrofuran (2 mL) was added to a stirred solution of 1-hydroxybenzotriazole (432 mg, 3.2 mmol) and pyridine (0.26 mL, 3.2 mmol) in tetrahydrofuran (4 mL) at room temperature under argon. The reaction mixture was stirred for 4 h; at this time, ^{31}P NMR indicated that bisbenzotrizolyl phosphoramidate **4** was formed based on the appearance of its resonance at −11.94 ppm.[24] The mixture was centrifuged at 10 000 rpm for 10 min to precipitate the pyridine hydrochloride, and the supernatant was added to a stirred solution of N^4-AOC-protected gemcitabine **2** (280 mg, 0.8 mmol) in pyridine (4 mL) at room temperature. *N*-Methylimidazole (0.13 mL, 1.6 mmol) was added to the above solution and the reaction mixture was stirred for 20 h at room temperature under argon. Solvent was removed in vacuo, and the residue was purified by silica gel chromatography (CH_2Cl_2/EtOH, 30:1 to 20:1) to give benzotriazolyl phosphoramidate **5** (235 mg, 45%, mixture of diastereomers, as indicated by ^{1}H and ^{31}P NMR) as a white foam along with recovery of unreacted **2** (88 mg, 31%). TLC R_f 0.67 CH_2Cl_2/MeOH, 10:1); ^{1}H NMR (MeOH-d_4, 300 MHz) for diastereomers δ 7.93−7.37 (m, 5H, H-6 and Ph−H), 7.21 (dd, 1H, H-5), 6.08 (m, 1H, H-1′), 5.86 (m, 1H, −CH═), 5.26 (m, 2H, ═CH_2), 4.62 (m, 2H, CH_2—CH═CH_2), 4.52−4.00 (m, 4H, H-3′, H-4′, H-5′, and H-5″), 3.46 (m, 2H, $ClCH_2$−), 3.14 (m, 2H, −CH_2−N), 2.84 (dd, 3H, CH_3−N), 1.65 (m, 4H, −CH_2CH_2−); ^{31}P NMR (MeOH-d_4, 121 MHz) δ −11.80, −12.31 (1:1); MS (ESI) 648/650 $[M + H]^+$; HRMS (ESI) calcd for $C_{24}H_{30}ClF_2N_7O_8P$, 648.1550 $[M + H]^+$; found, 648.1570.

5′-[2′-Deoxy-2′,2′-difluoro-D-ribofuranosyl-*N*^{4}-(allyloxycarbonyl)cytosine] 5-Nitrofurfuryl *N*-Methyl-*N*-(4-chlorobutyl) Phosphoramidate (6). A mixture of benzotriazolyl phosphoramidate **5** (79 mg, 0.12 mmol), 5-nitrofurfuryl alcohol (870 mg, 6 mmol), and 4-dimethylaminopyridine (58 mg, 0.48 mmol) in tetrahydrofuran (0.3 mL) was stirred overnight at room temperature under argon. The reaction mixture was purified by silica gel chromatography (CH_2Cl_2/MeOH, 20:1) to give **6** (47 mg, 59%, mixture of diastereomers as indicated by ^{1}H NMR and ^{31}P NMR) as a yellow foam. TLC R_f 0.20 (CH_2Cl_2/MeOH, 20:1); ^{1}H NMR (MeOH-d_4, 300 MHz) for diastereomers δ 7.99 (m, 1H, H-6), 7.35 (dd, 1H), 7.21 (m, 1H, H-5), 6.78 (dd, 1H), 6.20 (m, 1H, H-1′), 5.92 (m, 1H, −CH═), 5.26 (m, 2H, ═CH_2), 5.02 (d, 2H, −CH_2−), 4.61 (m, 2H, CH_2—CH═CH_2), 4.34−4.04 (m, 4H, H-3′, H-4′, H-5′, and H-5″), 3.50 (m, 2H, $ClCH_2$−), 3.03 (m, 2H, −CH_2−N), 2.63 (dd, 3H, CH_3−N), 1.66 (m, 4H, −CH_2CH_2−); ^{31}P NMR (MeOH-d_4, 121 MHz) δ −13.36, −13.69 (1:1); MS (ESI) 656/658 $[M + H]^+$; HRMS (ESI) calcd for $C_{23}H_{30}ClF_2N_5O_{11}P$, 656.1336 $[M + H]^+$; found, 656.1338.

5′-(2′-Deoxy-2′,2′-difluorocytidyl) 5-Nitrofurfuryl *N*-Methyl-*N*-(4-chlorobutyl) Phosphoramidate (7). Tetrakis(triphenylphosphine)-palladium(0) ($Pd(PPh_3)_4$, 8 mg, 0.007 mmol) was added to a solution of **6** (87 mg, 0.13 mmol) in tetrahydrofuran (1 mL), followed by the addition of *p*-toluenesulfinic acid sodium salt (25 mg, 0.14 mmol) in double distilled water (0.5 mL). The reaction mixture was stirred at room temperature for 1 h. Solvent was removed in vacuo, and the residue was purified by silica gel chromatography (CH_2Cl_2/MeOH, 20:1 to 8:1) to give phosphoramidate prodrug **7** (52 mg, 70%, mixture of diastereomers as indicated by ^{1}H NMR and ^{31}P NMR) as a white foam. TLC R_f 0.21 (CH_2Cl_2/MeOH, 10:1); ^{1}H NMR (MeOH-d_4, 300 MHz) for diastereomers δ 7.53 (m, 1H, H-6), 7.36 (m, 1H), 6.76 (m, 1H), 6.14 (m, 1H, H-1′), 5.83 (d, 1H, H-5), 5.01 (d, 2H, −CH_2−), 4.26−4.08 (m, 4H, H-3′, H-4′, H-5′, and H-5″), 3.50 (m, 2H, $ClCH_2$−), 3.01 (m, 2H, −CH_2−N), 2.60 (dd, 3H, CH_3−N), 1.66 (m, 4H,

$-CH_2CH_2-$); ^{31}P NMR (MeOH-d_4, 121 MHz) δ −13.49, −13.75 (1:1); MS (ESI) 572/574 $[M + H]^+$; HRMS (ESI) calcd for $C_{19}H_{26}ClF_2N_5O_9P$, 572.1125 $[M + H]^+$; found, 572.1118.

Acknowledgment. Financial support from the National Cancer Institute (Grant R01 CA34619) to R.F.B. and W.W. is gratefully acknowledged. Support from the Purdue Cancer Center Support Grant P30 CA23168 for services provided by the NMR and Mass Spectrometry Shared Resources is also appreciated.

Supporting Information Available: Table of GI_{50} values for the NCI 60-cell line panel. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Robins, R. K. The potential of nucleoside analogs as inhibitors of retroviruses and tumors. *Pharm. Res.* **1984**, 11−18.
(2) Hatse, S.; De Clercq, E.; Balzarini, J. Role of antimetabolites of purine and pyrimidine nucleotide metabolism in tumor cell differentiation. *Biochem. Pharmacol.* **1999**, *58*, 539−555.
(3) Jones, R. J.; Bischofberger, N. Minireview: Nucleotide prodrugs. *Antiviral Res.* **1995**, *27*, 1−17.
(4) Wagner, C. R.; Iyer, V. V.; McIntee, E. J. Pronucleotides: Toward the in vivo delivery of antiviral and anticancer nucleotides. *Med. Res. Rev.* **2000**, *20*, 417−451.
(5) Freel Meyers, C. L.; Hong, L.; Joswig, C.; Borch, R. F. Synthesis and biological activity of novel 5-fluoro-2′-deoxyuridine phosphoramidate prodrugs. *J. Med. Chem.* **2000**, *43*, 4313−4318.
(6) Freel Meyers, C. L.; Borch, R. F. Activation mechanisms of nucleoside phosphoramidate prodrugs. *J. Med. Chem.* **2000**, *43*, 4319−4327.
(7) Tobias, S. C.; Borch, R. F. Synthesis and biological studies of novel nucleoside phosphoramidate prodrugs. *J. Med. Chem.* **2001**, *44*, 4475−4480.
(8) Tobias, S. C.; Borch, R. F. Synthesis and biological evaluation of a cytarabine phosphoramidate prodrug. *Mol. Pharmaceutics* **2004**, *1*, 112−116.
(9) Hertel, L. W.; Boder, G. B.; Kroin, J. S.; Rinzel, S. M.; Poore, G. A.; Todd, G. C.; Grindey, G. B. Evaluation of the antitumor activity of gemcitabine (2′,2′-difluoro-2′-deoxycytidine). *Cancer Res.* **1990**, *50*, 4417−4422.
(10) Plunkett, W.; Huang, P.; Gandhi, V. Preclinical characteristics of gemcitabine. *Anti-Cancer Drugs* **1995**, *6 Suppl. 6*, 7−13.
(11) 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.
(12) van Moorsel, C. J.; Peters, G. J.; Pinedo, H. M. Gemcitabine: Future prospects of single-agent and combination studies. *Oncologist* **1997**, *2*, 127−134.
(13) Kroep, J. R.; Peters, G. J.; Nagourney, R. A. Clinical activity of gemcitabine as a single agent and in combination. In *Cancer Drug Discovery and Development: Deoxynucleoside Analogs in Cancer Therapy*; Peters, G. J., Ed.; Humana Press, Inc.: Totowa, NJ, 2006; pp 253−287.
(14) Heinemann, V.; Hertel, L. W.; Grindey, G. B.; Plunkett, W. Comparison of the cellular pharmacokinetics and toxicity of 2′,2′-difluorodeoxycytidine and 1-β-D-arabinofuranosylcytosine. *Cancer Res.* **1988**, *48*, 4024−4031.
(15) Heinemann, V.; Schulz, L.; Issels, R. D.; Wilmanns, W. Regulation of deoxycytidine kinase by deoxycytidine and deoxycytidine 5′-triphosphate in whole leukemia and tumor cells. *Adv. Exp. Med. Biol.* **1998**, *431*, 249−253.
(16) van der Wilt, C. L.; Kroep, J. R.; Bergman, A. M.; Loves, W. J.; Alvarez, E.; Talianidis, I.; Eriksson, S.; van Groeningen, C. J.; Pinedo, H. M.; Peters, G. J. The role of deoxycytidine kinase in gemcitabine cytotoxicity. *Adv. Exp. Med. Biol.* **2000**, *486*, 287−290.
(17) Bergman, A. M.; Pinedo, H. M.; Peters, G. J. Determinants of resistance to 2′,2′-difluorodeoxycytidine (gemcitabine). *Drug Resist. Updates* **2002**, *5*, 19−33.
(18) Gregoire, V.; Rosier, J. F.; De Bast, M.; Bruniaux, M.; De Coster, B.; Octave-Prignot, M.; Scalliet, P. Role of deoxycytidine kinase (dCK) activity in gemcitabine's radioenhancement in mice and human cell lines in vitro. *Radiother. Oncol.* **2002**, *63*, 329−338.
(19) Griffith, D. A.; Jarvis, S. M. Nucleoside and nucleobase transport systems of mammalian cells. *Biochim. Biophys. Acta* **1996**, *1286*, 153−181.
(20) Burke, T.; Lee, S.; Ferguson, P. J.; Hammond, J. R. Interaction of 2′,2′-difluoro-deoxycytidine (gemcitabine) and formycin B with the Na^+-dependent and -independent nucleoside transporters of Ehrlich ascites tumor cells. *J. Pharmacol. Exp. Ther.* **1998**, *286*, 1333−1340.
(21) Jansen, W. J.; Pinedo, H. M.; van der Wilt, C. L.; Feller, N.; Bamberger, U.; Boven, E. The influence of BIBW22BS, a dipyridamole derivative, on the antiproliferative effects of 5-fluorouracil, methotrexate and gemcitabine in vitro and in human tumour xenografts. *Eur. J. Cancer* **1995**, *31A*, 2313−2319.
(22) Hayakawa, Y.; Kato, H.; Uchiyama, M.; Kajino, H.; Noyori, R. Allyloxycarbonyl group: A versatile blocking group for nucleoside synthesis. *J. Org. Chem.* **1986**, *51*, 2400−2402.
(23) Heidenhain, S. B.; Hayakawa, Y. A convenient way to *N*-allyloxycarbonyl protected adenosine and cytidine derivatives. *Synlett* **1998**, 853−854.
(24) Wu, W.; Freel Meyers, C. L.; Borch, R. F. A novel method for the preparation of nucleoside triphosphates from activated nucleoside phosphoramidates. *Org. Lett.* **2004**, *6*, 2257−2260.
(25) Meyers, C. L.; Borch, R. F. A novel method for the preparation of nucleoside diphosphates. *Org. Lett.* **2001**, *3*, 3765−3768.
(26) Visser, F.; Vickers, M. F.; Ng, A. M.; Baldwin, S. A.; Young, J. D.; Cass, C. E. Mutation of residue 33 of human equilibrative nucleoside transporters 1 and 2 alters sensitivity to inhibition of transport by dilazep and dipyridamole. *J. Biol. Chem.* **2002**, *277*, 395−401.
(27) Ruiz van Haperen, V. W.; Veerman, G.; Eriksson, S.; Boven, E.; Stegmann, A. P.; Hermsen, M.; Vermorken, J. B.; Pinedo, H. M.; Peters, G. J. Development and molecular characterization of a 2′,2′-difluorodeoxycytidine-resistant variant of the human ovarian carcinoma cell line A2780. *Cancer Res.* **1994**, *54*, 4138−4143.
(28) Keepers, Y. P.; Pizao, P. E.; Peters, G. J.; van Ark-Otte, J.; Winograd, B.; Pinedo, H. M. Comparison of the sulforhodamine B protein and tetrazolium (MTT) assays for in vitro chemosensitivity testing. *Eur. J. Cancer* **1991**, *27*, 897−900.
(29) Rots, M. G.; Pieters, R.; Kaspers, G. J.; van Zantwijk, C. H.; Noordhuis, P.; Mauritz, R.; Veerman, A. J.; Jansen, G.; Peters, G. J. Differential methotrexate resistance in childhood T- versus common/preB-acute lymphoblastic leukemia can be measured by an in situ thymidylate synthase inhibition assay, but not by the MTT assay. *Blood* **1999**, *93*, 1067−1074.
(30) Kroep, J. R.; Loves, W. J.; van der Wilt, C. L.; Alvarez, E.; Talianidis, I.; Boven, E.; Braakhuis, B. J.; van Groeningen, C. J.; Pinedo, H. M.; Peters, G. J. Pretreatment deoxycytidine kinase levels predict in vivo gemcitabine sensitivity. *Mol. Cancer Ther.* **2002**, *1*, 371−376.

JM070269U