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Synthesis and Biological Evaluation of a Cytarabine Phosphoramidate Prodrug

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Abstract: Recently, we reported a novel approach for the intracellular delivery of the anticancer nucleotide 5-fluoro-2′-deoxyuridine 5′-monophosphate (FdUMP) using phosphoramidate-based prodrugs. These phosphoramidate prodrugs contain an ester group that undergoes intracellular activation, liberating phosphoramidate anion, which in turn undergoes spontaneous cyclization and P−N bond cleavage to yield the nucleoside monophosphate quantitatively. This approach has now been extended to cytarabine [1-β-D-arabinofuranosylcytosine (Ara-C)], an anti-cancer nucleoside that is limited in its utility because of poor intracellular transport characteristics and weak activity as a substrate for tumor cell kinases. The cytarabine phosphoramidate prodrug **1** has 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. The prodrug was comparable in growth inhibitory activity (IC_{50} = 32 nM) to cytarabine (IC_{50} = 16 nM) in wild-type CCRF-CEM cells following drug treatment for 72 h. The nucleoside transport-deficient CEM/AraC8C exhibited a high level of resistance (6400-fold) to cytarabine but was more sensitive (210-fold resistant vs CCRF-CEM cells) to prodrug **1**. Similarly, the deoxycytidine kinase-deficient cell line (CEM/dCK−) was highly resistant to cytarabine (13900-fold) but more sensitive (106-fold resistant vs CCRF-CEM cells) to prodrug **1**. These results indicate that prodrug **1** is significantly more potent than cytarabine against transport- and kinase-deficient cell lines and are consistent with a mechanism involving intracellular delivery of cytarabine 5′-monophosphate.

Keywords: Prodrug; cytarabine; phosphoramidate; drug resistance

Introduction

Nucleoside analogues represent an important drug class in the anticancer chemotherapeutic area.[1,2] The biological activity of these agents requires initial intracellular conversion to 5′-mononucleotides by kinase-mediated phosphorylation. Thus, the development of drug resistance resulting from decreased nucleoside kinase activity has reduced the efficacy of these agents.[3] We[4,5] and others[6−9] have attempted to address this problem by developing prodrug approaches that would deliver nucleoside 5′-monophosphates intracellularly (see ref 10 for an excellent review). Cytarabine [1-β-D-

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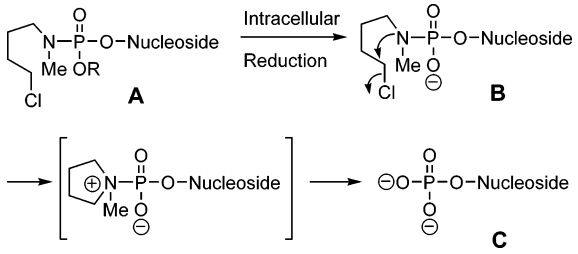
arabinofuranosylcytosine (Ara-C)] would appear to be an excellent candidate for such a prodrug approach. To exert its cytotoxic effects, cytarabine is converted initially to the 5′-phosphate via rate-limiting phosphorylation catalyzed by deoxycytidine kinase (dCK) and then ultimately to its 5′-triphosphate Ara-CTP. Ara-CTP presumably acts both by inhibiting the binding of 2′-deoxycytidine triphosphate to DNA polymerase[11,12] and by incorporation into elongating DNA strands, resulting in defective ligation or incomplete synthesis of DNA fragments.[13,14] It is well-established that tumor cells deficient in dCK are highly resistant to cytarabine,[15] so intracellular delivery of Ara-CMP might be expected to circumvent resistance in these cells. Many nucleoside analogues, including cytarabine, enter cells via specific nucleoside transporters in the plasma membrane. Nucleoside transporters are essential for cytarabine cytotoxicity in human tumor cells, and nucleoside transport-deficient cells are known to be highly resistant to cytarabine.[16−18] An appropriately designed cytarabine prodrug might also enter cells by passive diffusion, thus circumventing the resistance caused by nucleoside transport deficiency.

Scheme 1



Our laboratory has a long-standing interest in the development of nucleoside phosphoramidate prodrugs.[4,5,19] These prodrugs are designed to undergo intracellular activation to release an unstable phosphoramidate anion intermediate (**B**, Scheme 1); this intermediate undergoes spontaneous cyclization followed by P−N bond cleavage by water to liberate the nucleotide. Recently, we reported the synthesis and biological activity of a series of halobutyl phosphoramidate prodrugs of fluorodeoxyuridine monophosphate.[5] It was found that these novel prodrugs undergo rapid and quantitative conversion to the corresponding nucleotide and exhibit potent growth inhibition of both wild-type and thymidine kinase-deficient cells. Herein, we report the synthesis and *in vitro* studies of a halobutyl phosphoramidate prodrug of cytarabine.

Experimental Section

Materials and Methods. NMR spectra were recorded using a 250 MHz Bruker spectrometer equipped with a 5 mm multinuclear probe. ^{1}H chemical shifts are reported in parts per million using tetramethylsilane as an internal reference. ^{31}P NMR spectra were obtained using broadband ^{1}H decoupling, and chemical shifts are reported in parts per million using 1% triphenylphosphine oxide in benzene-d_6 as a coaxial insert (25.17 ppm relative to 85% phosphoric acid). Silica gel grade 60 (230−400 mesh) was used to carry out all chromatographic purifications. Thin-layer chromatography plates were visualized using UV or using one of the following stains: (i) 2% *p*-anisaldehyde, 21% sulfuric acid and 1% acetic acid in ethanol or (ii) 1% 4-(*p*-nitrobenzyl)pyridine (NBP) in acetone followed by heating and then treatment with 3% KOH in methanol. HPLC analysis was carried out using a Beckman System Gold equipped with a model 168 detector set to 250 nm, a model 126 solvent module, and an econosphere C18 column (5 μM, 4 mm ×

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250 mm). Mass spectral analyses were obtained from the mass spectrometry laboratory at Purdue University. All anhydrous reactions were carried out under argon, using flame-dried flasks, and all organic solvents were distilled prior to use. Diisopropylethylamine, acetonitrile, methylene chloride, and pyridine were distilled from calcium hydride prior to use. Cytosine arabinoside was coevaporated with anhydrous pyridine prior to use. *N*-Methyl-*N*-(4-chlorobutyl)-amine hydrochloride and 5-nitrofurfuryl alcohol were dried by coevaporation with anhydrous acetonitrile. All chemical reagents were purchased from Aldrich-Sigma.

***In Vitro* Growth Inhibition Assay.** Stock solutions of the drug were prepared in absolute ethanol, and serial dilutions of the drug were prepared. CEM, CEM/dCK−, and CEM/Arac8c cells were suspended in RPMI medium supplemented with 10% FBS, 0.1% gentamicin, and 1% sodium pyruvate. The cells were suspended in their respective media to give 10 mL volumes of cell suspension at a final density of 3−6 × 10^4 cells/mL. Appropriate volumes of the drug solution were transferred to the cell suspensions, and incubation was continued for 72 h. The cells were spun down and resuspended in fresh drug-free medium, and final cell counts were determined. The data were analyzed by sigmoidal curve fitting of the cell count versus drug concentration, and the results are expressed as the IC_{50} (the drug concentration that inhibits cell growth to 50% of the control value).

***N*⁴-(Allyloxycarbonyl)cytosine Arabinoside (2).** Diallyl pyrocarbonate (215 mg, 1.1 mmol) was dissolved in 1,4-dioxane (10 mL) and the mixture added to a solution of cytosine arabinoside (250 mg, 1.04 mmol) in doubly distilled H_2O (2 mL) at room temperature. The reaction mixture was heated at reflux for 4 h. The solvent was removed under reduced pressure, and the residue coevaporated with anhydrous pyridine (3 × 5 mL). The residue was subjected to chromatography (15% MeOH/$CHCl_3$) to give **2** (236 mg, 70%) as a white foam: R_f = 0.38 (15% MeOH/$CHCl_3$); ^{1}H NMR (DMSO-d_6) δ 10.75 (s br, 1H), 8.04 (d, 1H, J = 7.5 Hz), 7.00 (d, 1H, J = 7.5 Hz), 6.04 (d, 1H, J = 3.9 Hz), 5.95 (m, 1H), 5.46 (m, 1H), 5.39 and 5.25 (dd, 1H), 5.32 and 5.21 (dd, 1H), 5.11 (m, 1H), 4.62 (d, 2H, J = 5.3 Hz), 4.04 (m, 1H), 3.91 (m, 1H), 3.81 (m, 1H), 3.60 (m, 2H); MS (ESI) m/z 328 [(M + H)$^+$].

5′-[*N*⁴-(Allyloxycarbonyl)cytosinearabinosyl] 5-Nitrofurfuryl *N*-Methyl-*N*-(4-chlorobutyl) Phosphoramidate (3). Phosphorus trichloride (1.14 mL, 2 M in CH_2Cl_2, 2.28 mmol) was cooled to −70 °C under argon. 5-Nitrofurfuryl alcohol (326 mg, 2.28 mmol) was dissolved in anhydrous CH_2Cl_2 (5 mL) and the mixture added slowly, followed by the dropwise addition of diisopropylethylamine (0.40 mL, 2.28 mmol). The reaction mixture was allowed to stir at −70 °C for 15 min. *N*-Methyl-*N*-(4-chlorobutyl)amine hydrochloride (360 mg, 2.28 mmol) was dissolved in anhydrous CH_2Cl_2 (2 mL) and the mixture added dropwise to the reaction mixture. Diisopropylethylamine (1.2 mL, 6.8 mmol) was added dropwise, and the reaction mixture was stirred at −70 °C for 25 min. Compound **2** (240 mg, 0.73 mmol) was coevaporated with anhydrous pyridine (6 × 5 mL), dissolved in anhydrous pyridine (5 mL), and cooled to −45 °C. The phosphoramidite reaction solution was added dropwise to the solution of **2** in pyridine until **2** had disappeared as determined by TLC (5% MeOH/$CHCl_3$). After 35 min, the reaction mixture was oxidized with *tert*-butyl hydroperoxide (0.48 mL, 4.6 M in decane) at −40 °C and warmed to 0 °C over the course of 30 min. Saturated NH_4Cl (5 mL) was added. The layers were separated, and the aqueous layer was extracted with $CHCl_3$ (5 × 10 mL). The organic layers were combined, dried over Na_2SO_4, and concentrated under reduced pressure. Column chromatography of the crude product (10% MeOH/$CHCl_3$) afforded **3** (249 mg, 54%) as a light yellow foam: R_f = 0.24 (10% MeOH/$CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 8.23 (d, 1H, J = 7.3 Hz), 7.23 (d, 1H, J = 1.3 Hz), 6.69 (m, 1H), 6.15 (1H), 5.91 (m, 1H), 5.34 (m, 3H), 5.02 (m, 2H), 4.55 (m, 4H), 4.23 (m, 2H), 3.55 (t, 2H, J = 5.7 Hz), 3.03 (m, 2H), 2.67 (d, 3H, J = 10.3 Hz), 1.73 (m, 4H); ^{31}P NMR ($CDCl_3$) δ −15.54 and −15.76; HPLC [gradient from 0 to 70% CH_3CN/H_2O (0.1% TFA) over the course of 30 min] 20.0 min, >95%; MS (ESI) for $C_{23}H_{31}ClN_5O_{12}P$ calcd 636.1474 [(M + H)$^+$], found 636.1474.

5′-Cytosinearabinosyl 5-Nitrofurfuryl *N*-Methyl-*N*-(4-chlorobutyl) Phosphoramidate (1). Tetrakis(triphenylphosphine)palladium (34 mg, 0.03 mmol) was added to a solution of phosphoramidate **3** (373 mg, 0.59 mmol) in THF (3 mL), followed by the addition of *p*-toluenesulfinic acid sodium salt (115 mg, 0.65 mmol) in doubly distilled H_2O (1.5 mL). The reaction mixture was allowed to stir at room temperature for 2.5 h and then passed through a pad of silica gel (20% MeOH/$CHCl_3$) to give a light yellow foam (275 mg, 85%): R_f = 0.20 (20% MeOH/$CHCl_3$); ^{1}H NMR (CD_3OD) δ 7.85 (m, 2H), 7.49 (m, 1H), 6.89 (m, 1H), 6.57 (m, 1H), 5.95 (m, 1H), 5.11 (m, 2H), 4.19 (m, 5H), 3.66 (m, 2H), 3.11 (m, 2H), 2.72 (m, 3H), 1.79 (m, 4H); ^{31}P NMR (CD_3OD) δ −14.18 and −14.37; HPLC [gradient from 0 to 70% CH_3CN/H_2O (0.1% TFA) over the course of 30 min] 21.4 min, 95%; MS (ESI) for $C_{19}H_{27}ClN_5O_{12}P$ calcd 552.1262 [(M + H)$^+$], found 552.1251

Results

Chemistry. Initial attempts to synthesize the nucleoside phosphoramidate **1** were based on the direct phosphitylation of cytarabine using P^{III} chemistry as described previously.[5] However, these attempts were uniformly unsuccessful, presumably as a result of the very low solubility of cytarabine in organic solvents. Protective groups for cytarabine were then explored with the aim of increasing the lipophilicity of the nucleoside. We had shown previously that the nitrofuryl phosphoramidate moiety in the prodrug was stable to the deprotecting conditions required for the allyloxy group,[20] so the introduction of either an allyl or allyloxycarbonyl group at the cytosine NH_2 group was investigated. Reaction of

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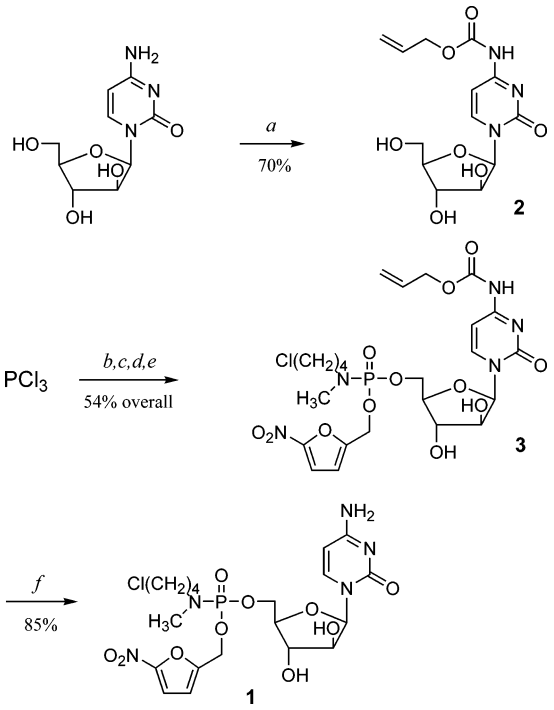
Scheme 2 [a]



[a] Reagents and conditions: (a) diallyl pyrocarbonate, dioxane/H_2O, reflux for 2 h; (b) 5-nitrofurfuryl alcohol, DIEA, CH_2Cl_2, −70 °C, 20 min; (c) *N*-methyl-*N*-(4-chlorobutyl)amine hydrochloride, DIEA, CH_2Cl_2, −70 °C, 20 min; (d) **2**, pyridine, −45 °C; (e) *tert*-butyl hydroperoxide, −45 to 0 °C, 30 min; (f) $Pd(PPh_3)_4$, *p*-$TolSO_2NA$, THF/H_2O, 2.5 h.

cytarabine with allyl bromide or allyl benzotriazolyl carbonate[21] resulted in a competitive reaction with the arabinose hydroxyl groups. However, reaction of cytarabine with diallyl pyrocarbonate[22] afforded *N*-allyloxycarbonyl derivative **2** (Scheme 2) in high yield. The conversion of **2** to **3** was accomplished without isolation of intermediates as shown in Scheme 2. Phosphorus trichloride was reacted sequentially with nitrofurfuryl alcohol and *N*-methyl-*N*-(4-chlorobutyl)-amine in the presence of Hunig's base at −70 °C to generate the monochlorophosphoramidite intermediate. This phosphoramidite solution was added to **2** in pyridine at −45 °C and then the solution oxidized with *tert*-butyl hydroperoxide to give **3** in 54% yield. The allyloxycarbonyl group was removed by treatment with $Pd(PPh_3)_4$ and sodium *p*-toluenesulfinate to give cytarabine phosphoramidate **1** in 85% yield.

Biological Evaluation. The biological activity of prodrug **1** was compared with that of cytarabine using CCRF-CEM cells in a growth inhibition assay. Cells were treated with drug for 72 h at various concentrations and then counted. Growth inhibition dose−response curves were obtained by plotting cell count versus drug concentration, and IC_{50} values were obtained by fitting the experimental data to a sigmoidal curve. The results are shown in Table 1. Prodrug **1** was a low nanomolar inhibitor of CCRF-CEM (wild-type) cell proliferation but was 2-fold less potent than cytarabine in this assay. To compare the efficacy of prodrug **1** and cytarabine in transport- and kinase-deficient cells, similar growth inhibition assays were carried out using CEM/AraC8C (transport-deficient) and CEM/dCK− (deoxycytidine kinase-deficient) cells. Both of these cell lines are reported to be highly resistant to cytarabine,[16] and our results (Table 1) also show a high degree of resistance for cytarabine. However, prodrug **1** is 1−2 orders of magnitude more potent than cytarabine against the CEM/AraC8C and CEM/dCK− cell lines in this assay, but **1** still exhibits a significant degree of resistance (100−200-fold) compared to the wild-type CCRF-CEM cells.

Table 1. Inhibition of Cell Growth following Treatment with Cytarabine or **1** for 72 h

	IC_{50} (μM)[a]				
compound	CCRF-CEM	CEM/AraC8C	ratio	CEM/dCK−	ratio
Ara-C ($n = 5$)	0.016 ± 0.006	103 ± 13	6400	223 ± 18	13 900
1 ($n = 3$)	0.032 ± 0.007	6.8 ± 0.8	210	3.4 ± 0.4	106

[a] Mean ± the standard error.

Discussion

The one-pot conversion of nucleosides to the corresponding phosphoramidates[4,5] is a highly efficient method for the preparation of prodrugs derived from deoxyribonucleosides, but yields are poor when this method is applied to unprotected ribo- and arabinonucleosides. Poor yields result from both solubility problems and insufficient reaction selectivity for the ribonucleosides. These difficulties can be overcome by the introduction of protective groups that enhance lipophilicity and, in the case of ribonucleosides, mask the 2′- and 3′-hydroxyl groups.[23] The unique solubility problems of the cytidine analogues are best addressed by introduction of an *N*-alloc group; this protective group is readily cleaved without adverse effects on the acid- and base-sensitive nitrofuryl phosphoramidate moiety.

The very high potency of **1** against wild-type CCRF-CEM cells is gratifying but also somewhat surprising. These cells are highly sensitive to cytarabine in part because they possess an active transporter for the nucleoside. However, the prodrug presumably cannot exploit the transporter and enters the cell by passive diffusion only. Furthermore, two cytarabine phosphate prodrugs incorporating α-amino ester phosphoramidic acid groups are essentially inactive (IC_{50} > 100 μM) against the CCRF-CEM cell line, although the analogous FudR phosphoramidate prodrugs are active against this cell line.[7] The high potency of prodrug **1** against these cells may result from the presence of the nitrofuryl group and the unique mechanism of its activation.

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Prodrug **1** is 30- and 130-fold more potent than cytarabine against CEM/AraC8C and CEM/dCK− cell lines, respectively. However, the prodrug does not have activity against these cell lines comparable to its activity against the wild-type cells. The basis for this difference is not known. However, the development of stable resistance in these cell lines may induce other cellular changes that alter the response of these cells to the prodrug. It is interesting to compare these results with those of troxacitabine, a cytidine analogue containing a dioxolan ring in place of the natural furanose ring that has shown potent *in vivo* activity. Troxacitabine has IC_{50} values of 0.16, 1.18, and >100 μM against CCRF-CEM, CEM/AraC8C, and CEM/dCK− cell lines, respectively,[16] suggesting that troxacitabine is not dependent on the nucleoside transporter but is completely dependent on deoxycytidine kinase for growth inhibitory activity. Prodrug **1** is significantly more active than troxacitabine against the wild-type and kinase-deficient cell lines and only somewhat less active against the transport-deficient cell line, suggesting that neither drug requires the nucleoside transporter and that prodrug **1** does not require deoxycytidine kinase for activity.

In summary, the phosphoramidate synthesis that we recently reported for the delivery of FdUMP has been applied to the preparation of a cytarabine monophosphate prodrug. This prodrug is comparable in potency to cytarabine against wild-type CEM cells but is 1−2 orders of magnitude more potent than cytarabine against transport-resistant and deoxycytidine kinase-resistant cells. These results are consistent with a mechanism of activation involving intracellular delivery of cytarabine monophosphate.

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