# 1-β-D-Arabinofuranosylcytosine-5′-alkylphosphonophosphates and diphosphates: new orally active derivatives of ara-C

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**Abstract** ara-Cytidine-5′-alkylphosphonophosphates and the corresponding -diphosphates were found to be cytostatically active in vitro against the human mammary epithelial cell line H184 A1N4 and the human mammary tumor cell line MaTu. Our results indicate that the replacement of the diphosphate by the phosphonophosphate group has no influence on antiproliferative activity in this case. The compounds were more active than the corresponding cytidine phospholipid conjugates and related compounds lacking a cytostatically active nucleoside, the ara-C prodrug Cytoros, and were slightly less active than ara-C. The cytostatic effect was prevented by 2′-deoxycytidine indicating their action as prodrugs of ara-C. In contrast to ara-C, they increase $[Ca^{2+}]_1$ in H184 A1N4 cells, pointing to a different mechanism of action in addition to their prodrug effect. In combination with phospholipid analogs, synergistic effects could be observed. Further studies within the disease-oriented in vitro Anticancer Screening Program of the National Cancer Institute show selectivity for certain cancer cell lines. The hexadecyl derivatives revealed a significant antitumor activity in vivo against the murine lymphatic leukemia P 388 cells being equally potent or even superior to ara-C. In contrast to ara-C, they were found to be orally active. Side effects measured as leukopenia and body weight reduction were less pronounced than with the parent drug.—**Brachwitz, H., J. Bergmann, I. Fichtner, Y. Thomas, C. Vollgraf, P. Langen, and W. E. Berdel.** 1-β-D-Arabinofuranosyl-cytosine-5′-alkylphosphonophosphates and diphosphates: new orally active derivatives of ara-C. *J. Lipid Res.* 1998. **39:** 162–172.

**Supplementary key words** ara-C derivatives • alkylphospholipids • calcium • antiproliferative activity • in vivo studies • in vitro studies • drug combination • phosphonophosphates

1-β-D-Arabinofuranosylcytosine (ara-C) has been shown to be an effective anticancer drug for the treatment of human acute myeloblastic and lymphoblastic leukemia (1). In clinical use, however, the compound is rapidly converted to the inert metabolite 1-β-d-arabinofuranosyluracil by the action of cytidine deaminase (2). In attempts to avoid deamination of ara-C and to enhance cellular uptake, several types of prodrugs have been synthesized. ara-Cytidine-phospholipid conjugates like 1-β-d-arabinofuranosylcytosine-5′-diphosphate-rac-1-S-octadecyl-2-O-palmitoyl-1-thioglycerol (Cytoros) have been prepared and have shown therapeutic efficacy exceeding the activity of ara-C in some cases (3). Recently, we have found that some analogs of the naturally occurring cytidinediphosphate-diacylglycerol (CDP-DAG) as well as similarly structured deoxycytidine, thymidine, and adenosine derivatives, possessing a special phospholipid component exhibit antitumor activity though lacking a cytostatically active nucleoside (4–7). Apparently, the effects are mainly or exclusively attributed to the phospholipid component. Structure–activity studies with these compounds have shown that derivatives containing one long-chain alkyl group in the glycerol were cytostatically active whereas diacyl-alkylacyl-, and dialkylglycero derivatives had no effect (6). Analogs in which the alkylglycerol group was substituted by a long-chain alkyl group have also proved to be effective, indicating that the presence of a glycerol backbone is not a necessary prerequisite for cytotoxicity of these compounds (5, 7). Further, by replacing the diphosphate group by a phosphonophosphate group, a clear increase in effectiveness could be observed (6, 7).

In this context it was of interest to prepare new ara-C

Abbreviations: ara-C, 1-β-D-arabinofuranosylcytosine; $[Ca^{2+}]_i$, concentration of intracellular calcium; $C_{16}$Cl-GPC, 1-O-hexadecyl-2-chloro-2-deoxyglycero-3-phosphocholine; $C_{16}$-NAc-PS, hexadecylphospho-*N*-acetyl-L-serine; $C_{16}$P-CMP, cytidine-5′-hexadecylphosphonophosphate; CDP-DAG, cytidine-5′-diphosphate-diacylglycerol; FAB MS, fast atom bombardment mass spectrometry; ESI MS, electrospray ionization mass spectrometry; TLC, thin-layer chromatography.

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phospholipid conjugates possessing cytostatically active phospholipid components in addition to the active nucleoside in order to obtain compounds with improved antitumoral effectiveness compared with the parent nucleoside. The rationale leading to this approach was to design compounds that might generate two cytotoxic principles with different target sites, mitogenic signal transduction (5) and nucleic acid metabolism (8), inside a neoplastic cell. Based on this concept we have synthesized some new ara-cytidine-5′-alkylphosphonophosphates and diphosphates in order to study their antiproliferative activity in vitro and in vivo and their influence on the cytosolic $Ca^{2+}$ concentration.

To study whether the cell growth inhibition potency of the new ara-C phospholipid conjugates as well as the parent nucleoside ara-C might be enhanced when used in combination with cytostatically active phospholipid analogs, we treated cells with the newly synthesized conjugates and ara-C simultaneously with the alkyllysophospholipid analog $C_{16}$Cl-GPC and two further phospholipid analogs $C_{16}$-NAc-PS and $C_{16}$P-CMP, which were also found to exhibit cytostatic activity (9–11).

The chemical structures of the new compounds including the ara-cytidine-5′-hexadecylphosphonophosphate (**1**), ara-cytidine-5′-octadecylphosphonophosphate (**2**), the corresponding diphosphate (**4**) and of the reference substances **3** (12), Cytoros (3), and ara-C are shown in **Fig. 1**.

## MATERIALS AND METHODS

### Compounds

ara-Cytidine (1-β-D-arabinofuranosylcytosine, ara-C) and ara-cytidine-5′-monophosphate (ara-CMP) were purchased from Sigma. The ara-cytidine-5′-monophosphomorpholidate (4-morpholine-N,N′-dicyclohexylcarboxamidinium salt) was prepared by condensation of ara-CMP with morpholine in the presence of dicyclohexylcarbodiimide by using the published procedure (12). The hexadecyl- and octadecylphosphates were obtained by reaction of the respective alcohols with phosphorus oxychloride in hexane in the presence of triethylamine, similar to the method previously described (13). The hexadecyl- and octadecylphosphonates were prepared by the Michaelis-Arbuzov reaction of alkylbromides with triethylphosphite via the diethyl alkylphosphonates (14) and bis(trimethylsilyl) alkylphosphonates (15).

1-O-hexadecyl-2-chloro-2-deoxyglycero-3-phosphocholine ($C_{16}$CI-GPC), the hexadecylphospho-*N*-acetyl-L-serine ($C_{16}$-NAc-PS) and the hexadecyl-5′-cytidine-phosphonophosphate ($C_{16}$P-CMP) have been synthesized as previously described (10, 11, 13).

### Analytical

UV spectra were recorded in $H_2O$ on a Shimadzu UV-1202 spectrophotometer. $^1$H NMR-spectra were recorded in DMSO-D6 on a Varian Gemini-300 spectrometer operating at 300 MHz; chemical shifts (δ) are reported in ppm. FAB (fast atom bombardment) and ESI (electrospray ionization) mass spectra were recorded in negative ion mode on a MAT 95 mass spectrometer. TLC (thin-layer chromatography) was carried out on Merck silicagel 60 $F_{254}$ plates using $CHCl_3$–MeOH–$H_2O$–acetic acid 50:30:8:4 (v/v/v/v) as eluent.

### Syntheses

*ara-Cytidine-5′-hexadecylphosphonophosphate (**1**).* A mixture of hexadecylphosphonate (297 mg, 0.97 mmol) and ara-cytidinemonophosphomorpholidate (4-morpholine-N,N′-dicyclohexylcarboxamidine salt with 1.5 mol of water, 765 mg, 1.12 mmol) was evaporated 3 times with 25 ml dry toluene in a rotary evaporator, dried in vacuum over $P_2O_5$, dissolved in 40 ml of dry pyridine and stirred for 6 days at room temperature. The progress of reaction was monitored by TLC. Pyridine was evaporated in vacuum to dryness. The residue obtained was dissolved in $CHCl_3$–MeOH–$H_2O$ (60 ml, 2:3:1, v/v/v), the pH was adjusted to 2–3 by addition of formic acid, and the aqueous phase was extracted twice with chloroform. The combined chloroform phases containing the crude product were dried over sodium sulfate and evaporated to dryness in a rotary evaporator. The crude product obtained was purified chromatographically on a CM-52 cellulose column (25 g, Na-form) using $CHCl_3$–MeOH mixtures with increasing polarity of the solvent according to the procedure described by Comfurius and Zwaal (16). Fractions containing the pure product were evaporated to dryness. The residue was crystallized by treatment with acetone, and the pure substance was obtained in the form of the disodium salt in an amount of 130 mg.

TLC: $R_f$ 0.45; UV $\lambda_{max}$ 272 nm; FAB MS: $[M–H]^-$ *m/z* 610, $[M–2H + Na]^-$ *m/z* 632; $^1$H NMR δ 0.87 (3H, t, $CH_3$, J = 6.7 Hz), 1.13 to 1.38 (28H, m, $CH_2 \times 14$), 1.44 (2H, $m_c$, P-$CH_2$), 3.83 to 4.10 (4H, m, H3′, H4′, H5′), 4.22 (1H, $m_c$, H2′), 5.75 (1H, d, H5, J = 7.3 Hz), 6.06 (1H, d, H1′, J = 4.3 Hz), 6.81 (2H, bs, $NH_2$), 7.64 (1H, d, H6, J = 7.3 Hz).

*ara-Cytidine-5′-octadecylphosphonophosphate (**2**).* As described for the synthesis of compound **1** octadecylphosphonate (318 mg, 0.95 mmol) and ara-cytidinemonophosphomorpholidate (4-morpholine-N,N′-dicyclohexylcarboxamidine salt with 1.5 mol of water, 750 mg, 1.09 mmol) were reacted at 35°C. The reaction mix-



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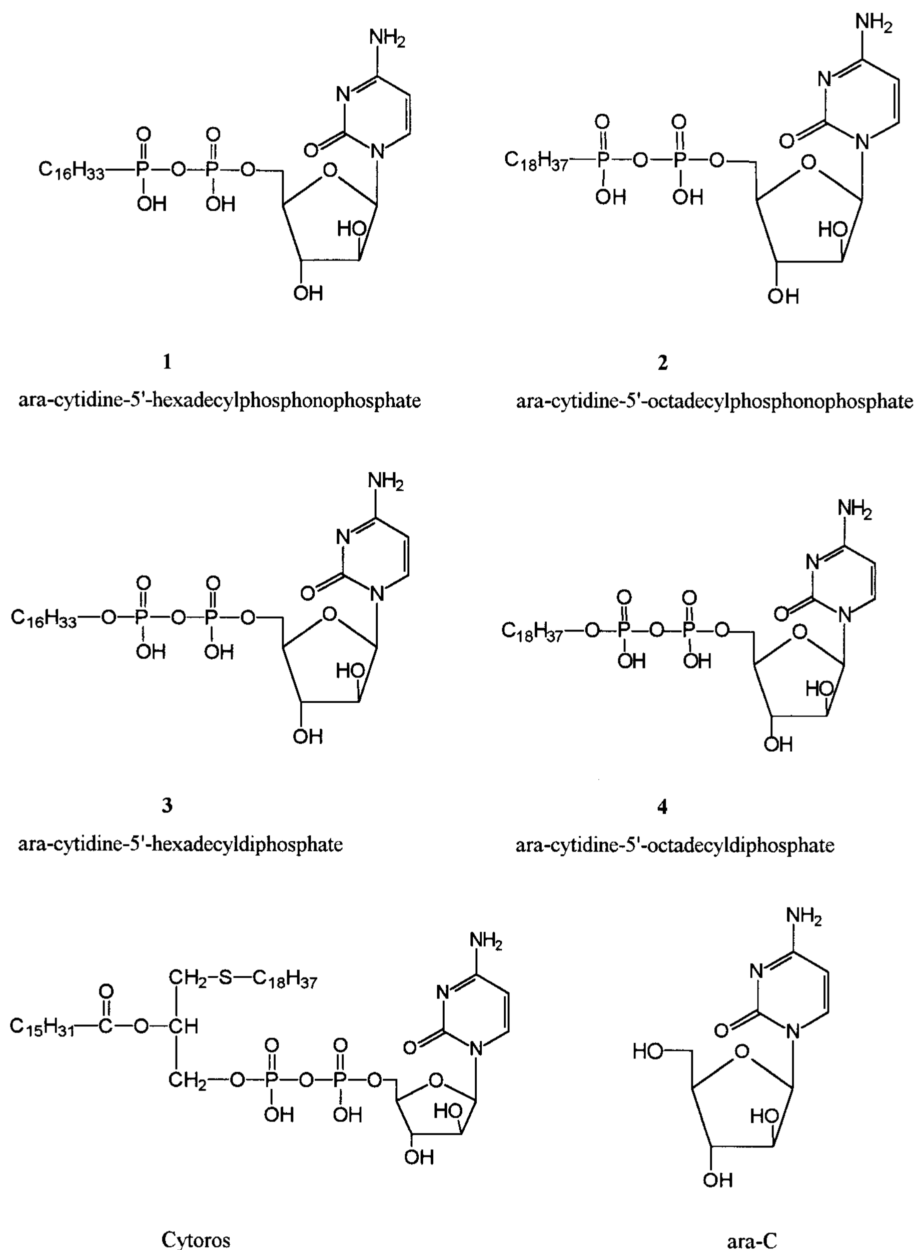


**Fig. 1.** The chemical structure of ara-cytidine-5′-hexadecylphosphonophosphate **1**, ara-cytidine-5′-octadecylphosphonophosphate **2**, the corresponding diphosphates **3** and **4**, Cytoros and ara-C.

ture was worked up and the product was isolated as described for compound **1.** Pure substance **2** was obtained in the form of the disodium salt in an amount of 125 mg.

TLC: $R_f$ 0.45; UV $\lambda_{max}$ 272 nm; ESI MS: $[M–H]^-$ *m/z* 638, $[M–2H + Na]^-$ *m/z* 660; $^1$H NMR δ 0.87 (3H, t, $CH_3$, J = 6.7 Hz), 1.18 to 1.38 (32H, m, $CH_2 \times 16$), 1.45 (2H, $m_c$, P-$CH_2$), 3.75 to 3.98 (4H, m, H3′, H4′, H5′), 4.31 (1H, $m_c$, H2′), 5.67 (1H, d, H5, J = 7.4 Hz), 6.06 (1H, d, H1′, J = 4.4 Hz), 6.79 (2H, bs, $NH_2$), 7.62 (1H, d, H6, J = 7.4 Hz).

*ara-Cytidine-5′-hexadecyldiphosphate (**3**).* As described for the synthesis of compound **1** and similar to the procedure of Hong et al. (12) hexadecylphosphate (313 mg, 0.97 mmol) and ara-cytidinemonophosphomorpholidate (4-morpholine-N,N′-dicyclohexylcarboxamidine salt with 1.5 mol of water, 765 mg, 1.12 mmol) were reacted at room temperature. The reaction was worked up and the product was isolated as described for compound **1.** Pure substance **3** was obtained in the form of the disodium salt in an amount of 100 mg.



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TLC: $R_f$ 0.46; UV $\lambda_{max}$ 272 nm; FAB MS: $[M-H]^-$ *m/z* 626, $[M-2H + Na]^-$ *m/z* 648; $^1H$ NMR δ 0.87 (3H, t, $CH^3$, J = 6.8 Hz), 1.15 to 1.38 (26H, m, $CH_2 \times 13$), 1.51 ($C\underline{H_2}$–$CH_2$–O–P), 3.62 to 3.98 (6H, m, $CH_2$–$C\underline{H_2}$–O–P, H3′, H4′, H5′), 4.20 (1H, dd, H2′, J = 3.5/2.7 Hz), 5.67 (1H, d, H5, J = 7.4 Hz), 6.06 (1H, d, H1′, J = 4.2 Hz), 6.81 (2H, bs, $NH_2$), 7.60 (1H, d, H6, J = 7.4 Hz).

*ara-Cytidine-5′-octadecyldiphosphate (**4**).* As described for the synthesis of compound **1** octadecylphosphate (339 mg, 0.97 mmol) and ara-cytidinemonophosphomorpholidate (4-morpholine-N,N′-dicyclohexylcarboxamidine salt with 1.5 mol of water, 765 mg, 1.12 mmol) were reacted at room temperature. The reaction mixture was worked up and the product was isolated as described for compound **1.** Pure substance **3** was obtained in the form of the disodium salt in an amount of 178 mg.

TLC: $R_f$ 0.46; UV $\lambda_{max}$ 272 nm; FAB MS: $[M-H]^-$ *m/z* 654, $[M-2H + Na]^-$ *m/z* 676; $^1H$ NMR δ 0.85 (3H, t, $CH_3$, J = 6.6 Hz), 1.14 to 1.38 (30H, m, $CH_2 \times 15$), 1.48 (2H, $m_c$, $C\underline{H_2}$–$CH_2$–O–P), 3.50 to 4.00 (6H, m, $CH_2$–$C\underline{H_2}$–O–P, H3′, H4′, H5′), 4.11 (1H, $m_c$, H2′) 5.70 (1H, d, H5, J = 7.3 Hz), 6.04 (1H, d, H1′, J = 4.0 Hz), 7.25 (2H, bs, $NH_2$), 7.58 (1H, d, H6, J = 7.3 Hz).

### In vitro studies

*Cell cultures.* We used the immortalized (by benzpyrene) but nontumorigenic human breast epithelial cell line H184 A1N4 (H184), obtained from Dr. M. Stampfer, Lawrence Berkeley Laboratory, University of California, Berkeley, CA and the human breast cancer cell line MaTu (17).

Both cell lines were grown as previously described (18) and cultured for 48 h. The compounds were added and the culture was continued further for 24 h. After harvesting, the cells were counted in a cell counter. Increase in cell number of the untreated controls was set as 100% growth. In some experiments cell proliferation was determined using the XTT assay (19), (Cell Proliferation Kit II, Boehringer, Mannheim).

*Measurement of $[Ca^{2+}]_i$ in H184 cells.* Intracellular calcium was determined using fura-2 fluorescence. Cells were loaded with 5 μM fura-2/AM in a HEPES-buffered saline solution for 30 min at 37°C (18). Thereafter the cell suspension was diluted 5-fold and incubated further for 10 min. After wash, cells were resuspended (1–2 × $10^6$ cells/ml) in buffer solution. Fluorescence was measured with a Shimadzu RF-5001 PC fluorescence spectrophotometer. The excitation wavelength alternated every 2 sec between 340 and 380 nm, and the emission was monitored at 510 nm. The fluorescence values are expressed as the ratios of the two excitation wavelength. Ratios were converted to $[Ca^{2+}]_i$ according to the formula described by Grynkiewicz, Poenie, and Tsien (20).

*NCI studies.* A cell panel consists of about 60 lines against which the compounds were tested at a minimum of five concentrations at 10-fold dilution. A 48-h continuous drug exposure protocol was used, and a protein assay, sulforhodamine B (SRB), was used to measure cell protein content and to estimate cell viability or growth when compared to controls according to published method (21).

### In vivo studies

The in vivo experiments were carried out with the P388 lymphatic leukemia; $10^6$ tumor cells/mouse (BDF 1, female) were transplanted i. p. on day zero. The evaluation parameter was the median survival time (MDST). It was compared to the control group and expressed as treated/control values in percent (T/C, two sample *t*-test). All animals dying before controls were registered as toxic deaths. Body weight change (BWC) as toxic parameter was registered between the first day of treatment and 7 days later and expressed as percentage. White blood cells (WBC) and thrombocytes (Thromb) were counted in peripheral blood 3 days after treatment.

## RESULTS

### In vitro studies

*Antiproliferative activity in H184 and MaTu cells.* **Figure 2** compares the concentration-dependent antiproliferative activity of ara-C and its phospholipid conjugates **1**–**4** in H 184 cells and MaTu cells. This figure shows that the maximum inhibition of cell proliferation induced by the compounds during the test period of 24 h was between 60% and 65% for H184 cells and 38% and 44% for MaTu cells as related to the untreated control. The difference between both cell lines is statistically significant for all compounds ($P < 0.01$). In order to compare the cytostatic efficiency of the analogs, we estimated the concentrations that produce a half-maximal inhibition of cell growth ($IC_{50}$). These values correspond to about 30% and 25% growth inhibition in H184 cells and MaTu cells, respectively, and are included in the legends in Fig. 2.

ara-C is significantly more active than the four ara-C phospholipid conjugates in both cell lines ($P < 0.05$). However, the new conjugates were found to be about 10 times more effective ($P < 0.001$) than the known thioether lipid conjugate Cytoros ($IC_{50}$ 8.5 ± 1.0 and 7.8 ± 0.7 for H184 cells and MaTu cells, respectively). Comparing the ara-C phospholipid conjugates among themselves, there is a significant difference observed



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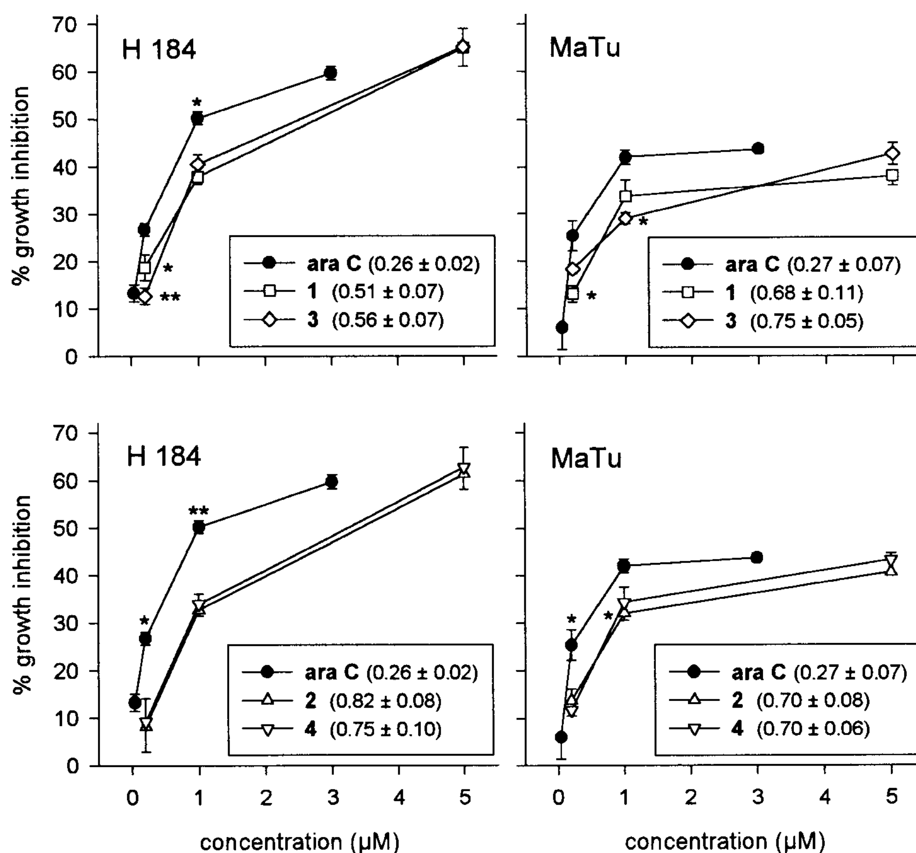


**Fig. 2.** Dose-dependent growth inhibition induced by ara-C and ara-C phospholipid conjugates **1–4** in H184 cells and MaTu cells. The values are expressed as % growth inhibition ± SEM related to the untreated control (n = 3). The asterisks indicate results significantly different from ara-C * $P < 0.05$ or ** $P < 0.01$ levels. Asterisks above ara-C values mean significant differences between ara-C and both of the test compounds. The values given in the boxes represent the $IC_{50}$ ± SEM.

between compounds **1** and **2** in H184 cells only ($P < 0.05$). As the antiproliferative activity of ara-C is inhibited by 2′-deoxycytidine, it was of interest to find out whether the ara-C phospholipid conjugates would behave in a similar way. **Figure 3** shows that the activity of the conjugates **1** and **3** is influenced by 20 μM 2′-deoxycytidine to a similar extent as ara-C.

*Cytostatic effects of the combination of ara-C and derivatives with cytostatically active phospholipid analogs.* **Table 1** shows the inhibitory effects of the ara-C phospholipid conjugates and ara-C on the growth rate of H184 cells as well as the effects of combined administration of these compounds with three different cytostatically active phospholipid analogs developed in our laboratory. The latter are the ether phospholipid analog $C_{16}$C1-GPC, the alkylphospho-L-serine $C_{16}$-NAc-PS, and the CDP-DAG analog $C_{16}$P-CMP, for which the antiproliferative activity was previously described (9–11). The H184 cells were treated alone with **1**, **2**, and ara-C and in combination with three phospholipid analogs in concentrations that induce 10% inhibition of cell growth when given alone (0.5 μM $C_{16}$C1-GPC, 10 μM $C_{16}$-NAc-PS, 5 μM $C_{16}$P-CMP). The XTT assay was used to evaluate growth inhibition. The $IC_{50}$ values in Table 1 indicate that simultaneous use of these different classes of agents, DNA-interactive ara-C and derivatives and ether phospholipids and related compounds, provides enhanced inhibition of H184 cell growth.

*Effects on $[Ca^{2+}]_i$.* $[Ca^{2+}]_i$ plays an important role in growth regulation (22) and is a messenger of cytotoxic effects (23). In previous studies (18, 24) we described multiple effects of alkyllysophospholipid analogs on the cytosolic $Ca^{2+}$ concentration in H184 cells. Therefore, we tested the ability of the compounds to change $[Ca^{2+}]_i$.

The results are presented in **Fig. 4**. All ara-C phospholipid conjugates increase $[Ca^{2+}]_i$, however, only at much higher concentrations than those that inhibited cell proliferation. There was no difference between the effects of the phosphonophosphates **1** and **2** and the diphos-



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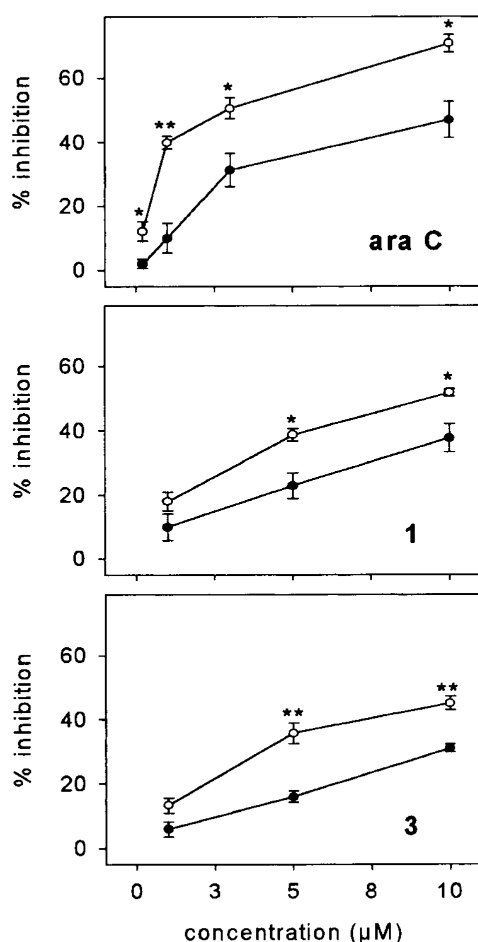


**Fig. 3.** Inhibition of the antiproliferative activity of ara-C and the ara-C phospholipid derivatives **1** and **3** by 2′-deoxycytidine (20 μM; 24 h) in H184 cells; ○ control; ● + 2′-deoxycytidine; each data point is the mean ± SEM of three separate experiments; $^*P < 0.05$; $^{**}P < 0.01$.

phates **3** and **4,** but the $[Ca^{2+}]_i$ increase depends on the length of the alkyl chain. Twenty μM of the $C_{16}$ analogs was without any effect. The same concentration of the $C_{18}$ analogs induced a high increase in $[Ca^{2+}]_i$ (0.8–1.0 μM). ara-C itself was not able to enhance $[Ca^{2+}]_i$.

*NCI studies.* Further, compounds **1–4** have been tested in vitro within the National Cancer Institute in an in vitro disease-oriented primary antitumor screen. **Figure 5** shows the $GI_{50}$ (concentrations resulting in 50% growth inhibition) mean graph for compound **1** representative for results of the NCI studies. Mean graphs facilitate visual scanning of data for potential patterns of selectivity for particular cell lines or for particular subpanels with respect to a selected response parameter. According to this graph compound **1** reveals cytotoxicity against two out of five leukemias, one ovarian and one non-small cell lung cancer cell line.

TABLE 1. Influence of the three phospholipid analogs rac-1-O-hexadecyl-2-chloro-2-deoxyglycero-3-phosphocholine ($C_{16}$C1-GPC), hexadecylphospho-N-acetyl-L-serine ($C_{16}$-NAc-PS), and hexadecyl-5′-cytidinephosphonophosphate ($C_{16}$P-CMP) on the growth inhibitory potency ($IC_{50}$) of ara-C and ara-C phospholipid conjugates **1** and **2**

| | $IC_{50}$ Values | | | |
|---|---|---|---|---|
| Compound | Control | +$C_{16}$C1-GPC (0.5 μM)[a] | +$C_{16}$-NAc-PS (10 μM)[a] | +$C_{16}$P-CMP (5 μM)[a] |
| | | μM | | |
| Ara-C | 0.42 ± 0.07 | 0.17 ± 0.01[b] | 0.19 ± 0.01[b] | 0.20 ± 0.04 |
| 1 | 3.02 ± 0.29 | 1.38 ± 0.25[c] | 1.39 ± 0.16[c] | 1.48 ± 0.24[c] |
| 2 | 3.01 ± 0.22 | 1.62 ± 0.34[c] | 1.39 ± 0.18[c] | 1.38 ± 0.22[c] |

Values are the mean ± SD of three separate experiments.
[a]Concentration causing about 10% growth inhibition.
[b]$P < 0.05$, [c]$P < 0.01$, significantly different compared to control.

## In vivo studies

P388 leukemia cells were injected i. p. to female BDF 1 mice. Treatment with the indicated substances started at day one. Median survival time (MDST) of each group was used as therapeutic criteria, and treated to control (T/C) values were additionally calculated. BWC and thrombocytes were counted 3 days after start of treatment.

In experiment 1 (**Table 2**) we found that compound **1** caused a significant prolongation of survival time only with treatment for 4 days (group D). Experiment 2 shows that this compound is more active when given orally (group D and E) than ara-C i. p. (group F) and has fewer side effects (higher number of thrombocytes after treatment). In experiment 3 we could confirm that compound **1** is active when given orally and we found the maximally tolerable dose at 100–200 mg/kg. Also, at higher concentrations, ara-C is not active when given orally.

There is a therapeutic effect of compound **3** (**Table 3**) with treatment on days 1–4 (group D and E, experiment 1). In experiment 2 we found that **3** is equally active as ara-C (group D and E). In addition, compound **3** is also active when given orally (group F and G); and the 4- to 8-fold higher concentrations when given orally are better tolerated concerning the influence on both body weight or blood cells.

## DISCUSSION

The results demonstrate that the effects of the new ara-C phospholipid conjugates **1–4** confirm the rationale for their synthesis: to combine a cytostatically active phospholipid moiety and a cytostatic nucleoside (ara-C) in one and the same compound.



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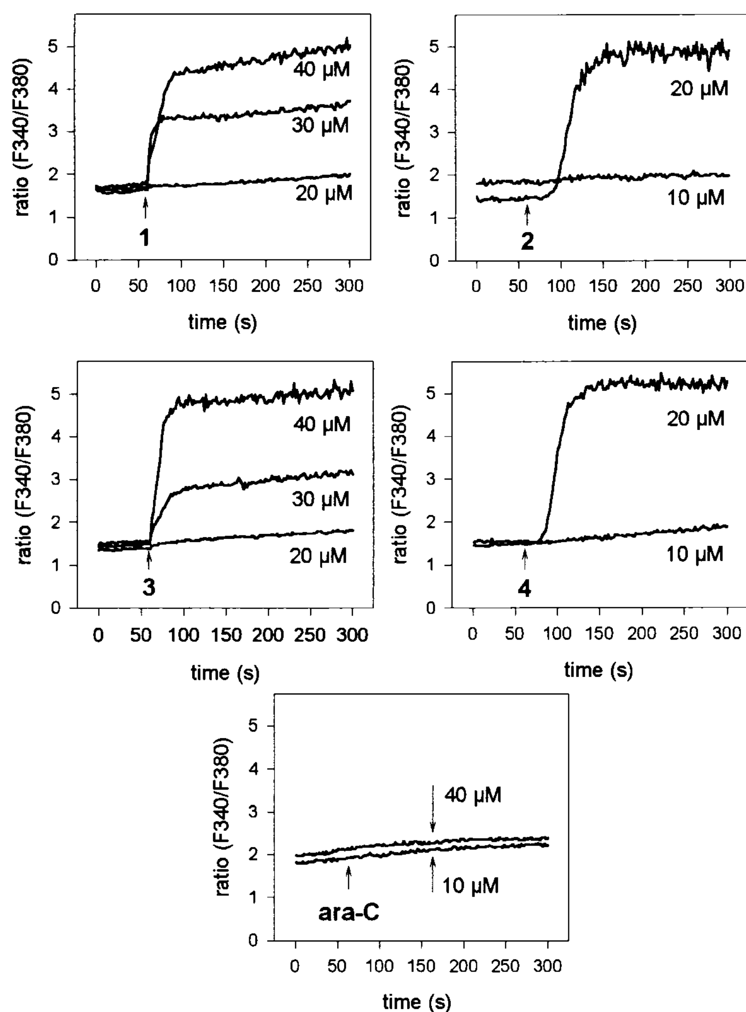


**Fig. 4.** Superimposed original traces of the increase in $[Ca^{2+}]_i$ induced by ara-C and ara-C phospholipid conjugates **1–4**, expressed as the ratio of the 340/380 fluorescence intensities. The traces are representative of three separate experiments.

In vitro the new compounds are about 10 times more effective than compounds containing the same phospholipid moiety in combination with nucleosides without cytostatic properties (e.g., cytidine, deoxycytidine, adenosine, thymidine) (6, 7). Their antiproliferative properties are reflected by dose–response properties that are similar to those we obtained with the parent substance ara-C under the same conditions. They are typical for cell cycle-specific compounds, i.e., in the dose–response curve the percentage of inhibition levels off after a steep initial increase (Fig. 2), because only those cells that were within the S-phase during the time of treatment were growth-inhibited (8). Moreover, the antiproliferative effects of the new compounds could be prevented as much as that of ara-C by the presence of 2′-deoxycytidine, indicating their prodrug properties (Fig. 3). Nevertheless, the new compounds still exhibit additional effects due to their structural relationship to phospholipids. In contrast to ara-C, in causing no elevation of the cytosolic calcium concentration $[Ca^{2+}]_i$, the new compounds are able to raise $[Ca^{2+}]_i$ (Fig. 4) in the same way as described for cytostatically active phospholipid analogs (25). This is a remarkable qualitative difference between the behavior of ara-C and the new ara-C derivatives **1–4**, probably indicating different mechanisms of action. That the increase of $[Ca^{2+}]_i$ by the compounds **1–4** takes place at drug concentrations much higher than those causing an inhibition of cell proliferation can be explained by the different experimental conditions used.

The overall cytostatic activities in vitro suggest that they act mainly as prodrugs of ara-C. Nevertheless, their effects, based on the presence of their phospholipid activity, may modulate the ara-C effect, e.g., in vitro (see below).

As prodrugs, the conjugates **1–4** ($IC_{50}$ 0.5–0.9 μM) are of somewhat lower activity than ara-C ($IC_{50}$ 0.15–0.2 μM). However, they proved to be more active than the ara-C prodrug Cytoros, an alkylmercaptoglycerophospholipid derivative of ara-C ($IC_{50}$ 8.5 ± 1.0 μM and 7.8 ±



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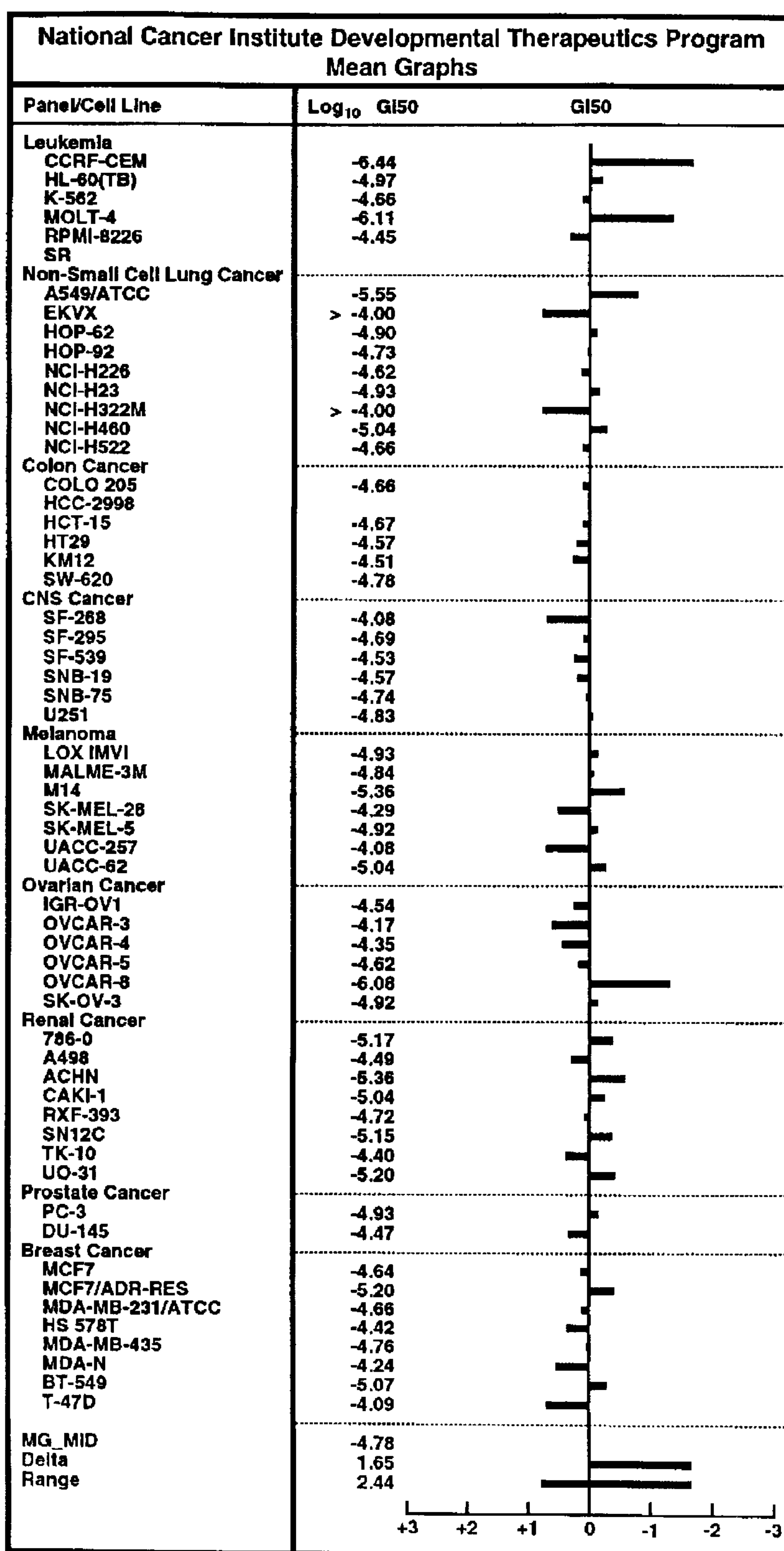


**Fig. 5.** Mean graph for $GI_{50}$ values (concentrations in 50% growth inhibition) for compound **1**. Bars extending to the right represent higher sensitivity of the cell line to the compound. As the bar scale is logarithmic, a bar 2 units to the right implies the compound achieved the $GI_{50}$ for the cell line at a concentration one-hundredth the mean concentration required over all cell lines, and thus the cell line is usually sensitive to that compound.



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TABLE 2. Antitumor activity of compound **1** against P388 leukemia in mice

| Group (n = 6) | Substance | Treatment | Dose | Toxic Deaths | BWC[a] d 1–7 | MDST ± SD[b] | T/C[c] | WBC[d] $10^5$/ml on day 4 | Thromb $10^6$/ml on day 4 |
|---|---|---|---|---|---|---|---|---|---|
| | | *days* | *mg/kg* | | % | *days* | % | | |
| Experiment 1 | | | | | | | | | |
| A | saline | 1–4, i.p. | | | 6 | 10.0 ± 1.0 | | | |
| B | **1** | 1, i.p. | 150 | 1 | −12 | 10.0 ± 3.0 | 100 | | |
| C | **1** | 1, i.p | 100 | | −3 | 10.0 ± 5.0 | 100 | | |
| D | **1** | 1–4, i.p. | 50 | | −9 | 13.0 ± 0.5 | 130[e] | | |
| E | **1** | 1–4, i.p. | 25 | | −4 | 10.5 ± 1.0 | 105 | | |
| Experiment 2 | | | | | | | | | |
| A | saline | 1–4, i.p. | | | 2 | 10.5 ± 0.5 | | 4.4 | 598 |
| B | **1** | 1–4, i.p. | 75 | | −11 | 13.5 ± 1.0 | 129[e] | 2.1[e] | 256[e] |
| C | **1** | 1–4, i.p. | 100 | | −10 | 7.0 ± 5.5 | 67 | 2.3[e] | 314 |
| D | **1** | 1–4, or. | 300 | 1 | −8 | 21.0 ± 5.0 | 200[e] | 1.6[e] | 576 |
| E | **1** | 1–4, or. | 200 | 2 | 8 | 17.5 ± 10.0 | 167[e] | 2.2[e] | 570 |
| F | ara-C | 1–4, i.p. | 300 | | −10 | 12.5 ± 5.0 | 119 | 1.2[e] | 445[e] |
| Experiment 3 | | | | | | | | | |
| A | saline | 1–4, or. | | | 4 | 10.0 ± 1.5 | | 3.7 | 538 |
| B | **1** | 1–4, or. | 100 | 2 | −9 | 14.0 ± 7.0 | 140 | 2.4 | 544 |
| C | **1** | 1–4, or. | 300 | 2 | −14 | 19.5 ± 5.5 | 195[e] | 1.0[e] | 385[e] |
| D | **1** | 1–4, or. | 400 | 4 | −14 | 14.0 ± 5.5 | 140[e] | 1.1[e] | 499 |
| E | ara-C | 1–4, or. | 400 | 4 | −5 | 10.5 ± 5.0 | 105 | 1.1[e] | 397[e] |

[a]Body weight change.
[b]Median survival time ± SD.
[c]Treated/control (>100 positive).
[d]White blood cells.
[e]Significantly different compared to control.

0.7 μm for H184 cells and MaTu cells, respectively). There is no major difference in the activity of ara-C and the new compounds on either the 'normal' (though immortal) H184 cells or MaTu cells (derived from a solid tumor). Such difference, however, cannot be expected as the effect of ara-C is mainly related to the growth rate of cells, not to their malignant properties.

There were no considerable differences in the antiproliferative activity between the phosphonophosphates **1** and **2** and the diphosphates **3** and **4** in both cell lines,

TABLE 3. Antitumor activity of compound **3** against P388 leukemia in mice

| Group (n = 6) | Substance | Treatment | Dose | Toxic Deaths | BWC[a] d 1–7 | MDST SD[b] | T/C[c] | WBC[d] | Thromb |
|---|---|---|---|---|---|---|---|---|---|
| | | *days* | *mg/kg* | | % | *days* | % | *$10^6$/ml* | |
| Experiment 1 | | | | | | | | | |
| A | saline | 1–4, i.p. | | | 5 | 10.0 ± 0.5 | | | |
| B | **3** | 1, i.p. | 300 | 1 | −4 | 10.5 ± 3.5 | 105 | | |
| C | **3** | 1, i.p. | 200 | | −7 | 11.0 ± 0.5 | 110[e] | | |
| D | **3** | 1–4, i.p. | 100 | 1 | −13 | 15.5 ± 5.0 | 155[e] | | |
| E | **3** | 1–4, i.p. | 50 | 2 | −13 | 13.5 ± 5.5 | 135 | | |
| Experiment 2 | | | | | | | | | |
| A | saline | 1–4, i.p. | | | 11 | 11.0 ± 0.5 | | 12.7 | 999 |
| B | ara-C | 1–4, i.p. | 200 | | −14 | 15.0 ± 4.5 | 136 | 0.3[e] | 105[e] |
| C | **3** | 1–4, i.p. | 25 | | 11 | 14.0 ± 0.5 | 127[e] | 9.2[ef] | 616[ef] |
| D | **3** | 1,4,9, i.p. | 200 | | −9 | 15.5 ± 0.5 | 141[e] | 4.8[ef] | 197[ef] |
| E | **3** | 1,4,9, i.p. | 100 | | 4 | 15.5 ± 1.0 | 141[e] | 5.8[ef] | 558[ef] |
| F | **3** | 1–4, or. | 200 | | −3 | 16.0 ± 4.0 | 145 | 1.2[ef] | 136[ef] |
| G | **3** | 1–4, or. | 100 | | −7 | 16.0 ± 3.5 | 145[e] | 4.6[ef] | 225[ef] |

[a]Body weight change.
[b]Median survival time ± SD.
[c]Treated/control (>100 = positive).
[d]White blood cells.
[e]Significantly different compared to control.
[f]Significantly different compared to ara-C.



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indicating that the biochemical stability against hydrolytic cleavage by phospholipase C of the P-containing linker between the ara-C moiety and the alkyl group (P-C or P-O-C) here does not play an essential role for the cytostatic properties in vitro. This is contrary to the properties of nucleoside-phospholipid conjugates without cytostatically active nucleoside components (mentioned above) (6, 7) bearing the same lipid moiety as the ara-C conjugates. In these cases, the phosphonophosphates were found to be more effective ($IC_{50}$ values 7–20 μM) than the corresponding diphosphates ($IC_{50}$ values 40–100 μM). But the overall effectiveness of the ara-C derivatives **1–4** is distinctively higher.

Cell lines other than H184 or MaTu, inhibited in their growth, were found in the in vitro Anticancer Screening Program of the American National Cancer Institute, e.g., leukemias, ovarian and non-small-lung cancer cells (Fig. 5).

Another property found for ara-C as well as for its new derivatives **1** and **2** is that their activity is enhanced by the combination with cytostatically active phospholipid analogs, such as $C_{16}$C1-GPC, $C_{16}$-NAc-PS, and $C_{16}$-P-CMP (Table 1), in concentrations of the latter achieving 10% inhibition only. This confirms other studies describing synergistic effects of phospholipid analogs with known cytostatic agents e.g., vinblastine (26), *cis*-diamminedichloroplatinum (27–29), cyclophosphamide (27, 30).

In vivo, the effects of the compounds **1** and **3** on the P388 leukemia cells are comparable to, or even stronger than, that of ara-C (Tables 2 and 3). Of greater importance, however, is the better tolerance of the conjugates **1** and **3** in comparison to ara-C. Weight losses were less pronounced than in leukopenia, an especially severe side effect of ara-C. Another advantage of the compounds over ara-C is that they are orally applicable. These in vivo properties of the new compounds, different from ara-C, should reflect altered pharmacokinetics but may also point to modified activity, probably based on the influence of the phospholipid content. This will be the subject of further studies.

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