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# Antitumor Activity and Pharmacology of 1-β-D-Arabinofuranosylcytosine-5′-stearylphosphate: An Orally Active Derivative of 1-β-D-Arabinofuranosylcytosine

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**The antitumor activity of 1-β-D-arabinofuranosylcytosine-5′-alkylphosphates (CnPCAs) against L1210 leukemia in mice after oral administration was demonstrated. The optimum length of the alkyl group on the phosphate moiety of CnPCA for exhibiting a high antitumor activity was found to be between tetradecyl (C14) and tricosyl (C23). The most active alkyl derivative in this system was found to be 1-β-D-arabinofuranosylcytosine-5′-stearylphosphate (C18PCA). The optimum and minimum effective doses of C18PCA were 100 and 6.25 mg/kg/day (q1d, day 1 to day 5), respectively. The maximum T/C% of C18PCA was approximately 220. The antitumor activity of C18PCA was not greatly dependent on the treatment schedule and route. Plasma concentration of 1-β-D-arabinofuranosylcytosine (ara-C) remained in the range of 0.4 to 0.75 μmol/ml for 24 h after oral administration of 100 mg/kg (170 μmol/kg) of C18PCA. These results indicate that C18PCA administered per orally is absorbed intact through the gastrointestinal tract and ara-C is released for a long period of time. C18PCA is regarded as an orally active depot form of ara-C.**

Key words: Ara-C — 1-β-D-Arabinofuranosylcytosine-5′-stearylphosphate — C18PCA — Oral activity — Pharmacokinetics

1-β-D-Arabinofuranosylcytosine (ara-C)[4] is an indispensable agent for the treatment of acute myelocytic or lymphocytic leukemias. It has often been utilized in combination chemotherapy against solid tumors as well as leukemias. In clinical use, however, the compound is rapidly converted to an inert metabolite, 1-β-D-arabinofuranosyluracil (ara-U), by the action of cytidine deaminase. On the basis of this evidence it has been suggested that ara-C should be poorly retained in blood and tissues. In practical use in clinical chemotherapy, repetitive dosage schedules or continuous intravenous infusion are conventionally essential. To overcome this disadvantage, various derivatives of ara-C have been synthesized and examined for antitumor activity. Although 2,2′-anhydro-1-β-D-arabinofuranosylcytosine (cyclo-C) and $N^4$-behenoyl-1-β-D-arabinofuranosylcytosine (BH-AC) have been used clinically,[1-4] some complicated parenteral procedure, such as continuous infusion, is still inevitable. On the other hand, it has been reported that the oral administration of ara-C is effective in combination with a cytidine-deaminase inhibitor, tetrahydrouridine.[5,6] The syntheses of ara-C derivatives which can be administered orally have also been reported.[7,8] The effects were, however, not adequate from a practical point of view. Therefore, the discovery of an orally active form of ara-C is urgently required. Recently, Hori *et al.*[9] reported that $N^4$-palmitoyl-1-β-D-arabinofuranosylcytosine (PL-AC), an analog of ara-C, exhibited marked activity against tumors in mice when given orally. We also previously synthesized a series of new phosphorylated ara-C derivatives, 5′-alkylphosphates (CnPCAs, Fig. 1), 5′-alkenylphosphates and 5′-arylphosphates of ara-C, and examined them for antileukemic activity against L1210 in mice by intraperitoneal administration.[10] Among 32 derivatives of ara-C, the compounds having a long alkyl group (C10 to C20) on the phosphate

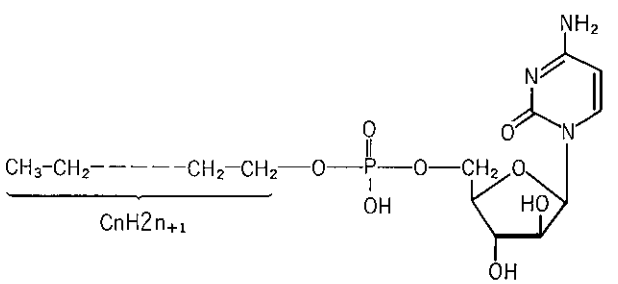


Fig. 1. Structure of 1-β-D-arabinofuranosylcytosine-5′-alkylphosphates. The abbreviation 'Cn' stands for the alkyl group bearing n methylenes substituted on the phosphate moiety of ara-CMP.

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[4] Abbreviations used: ara-C, 1-β-D-arabinofuranosylcytosine; ara-U, 1-β-D-arabinofuranosyluracil; ara-CMP, 1-β-D-arabinofuranosylcytosine-5′-monophosphate; CnPCA, 1-β-D-arabinofuranosylcytosine-5′-alkylphosphate; C18PCA, 1-β-D-arabinofuranosylcytosine-5′-stearylphosphate, 4-amino-1-β-D-arabinofuranosyl-2 (1H)-pyrimidinone 5′-(octadecyl hydrogen phosphate); PL-AC, $N^4$-palmitoyl-1-β-D-arabinofuranosylcytosine.

moiety of 1-β-D-arabinofuranosylcytosine-5′-monophosphate (ara-CMP) were effective even at low doses such as 10 mg/kg. However, they were not superior to their parent compound ara-C in terms of life span of treated animals. In the course of further studies, we found that some CnPCAs bearing a long alkyl group exhibited a high degree of antileukemic activity after oral administration.

This report describes the structure-activity relationship of CnPCA in the case of oral administration to mice bearing L1210 leukemia. The antileukemic and pharmacological properties of 1-β-D-arabinofuranosylcytosine-5′-stearylphosphate (C18PCA) are also described. This compound was concluded to be one of the most promising, orally active derivatives of ara-C so far found.

## MATERIALS AND METHODS

**Animals and tumor** Male C57BL/6NJcl×DBA/2NJcl F1 (BD2F1) mice (7–10 weeks old) were used for the evaluation of antitumor activity against L1210 leukemia or for pharmacological study. The mice were purchased from CLEA Japan (Kanagawa) and fed on a pellet diet (CE-2; CLEA Japan), with water *ad libitum*. The parent line of L1210 leukemia was supplied by the Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo, and maintained by weekly intraperitoneal transfer of ascites cells into mice.

**Chemicals** Ara-C, ara-CMP and CnPCAs were prepared in our laboratories. Methods of synthesis and physicochemical properties of CnPCAs were described in our previous paper.[10] The abbreviation 'Cn' in the term of 'CnPCA' stands for the alkyl group bearing n methylenes substituted on the phosphate moiety of ara-CMP. For instance, C18 stands for stearyl group (carbon number is 18). Each drug was dissolved in sterilized phosphate-buffered saline (PBS) at a specified concentration. PL-AC was also prepared in our laboratories and suspended in sterilized saline containing 0.5% carboxymethylcellulose (Sigma Chemical Company, St. Louis, USA) at a specified concentration.

**Evaluation of antitumor activity** For the evaluation of antitumor activity against ascites L1210 leukemia, BD2F1 mice were intraperitoneally (ip) implanted with $1\times10^5$ viable cells of L1210 and administered ip or per orally (po) with 0.1 ml of drug solution or suspension per 10 g body weight of mouse. The treatment schedules and routes are described in "Results." Mean survival time (MST, days) of each group was calculated and antitumor activity was expressed in terms of T/C% as follows: T/C% = (MST of treated group/MST of control group) ×100%. The observation period was 30 days. The minimum effective dose and the optimum dose have been determined as the doses required to produce 130 T/C% and to give the maximum T/C%, respectively. Chemotherapeutic index is defined as the ratio of the optimum dose to the minimum effective dose.

**Determination of ara-C and C18PCA in mouse plasma by HPLC** BD2F1 mice were administered intravenously (iv) with 50 mg/kg of C18PCA or po with 200 mg/kg of C18PCA. Plasma was collected periodically at specified intervals after the administration. Equal amounts of plasma (0.4 ml/head) obtained from 4 mice per group were mixed separately. To each mixture, 3 volumes of methanol (MeOH) was added for deproteinization. After centrifugation, the supernatant obtained (5.6 ml) was concentrated *in vacuo* and the residue was dissolved in 1.4 ml of 0.1 *M* Tris-HCl buffer (pH 7.5). This solution was analyzed by high-performance liquid chromatography (HPLC, ALC/GPC 204, Waters, USA). A reversed-phase column of μBondapak C18 (Waters) was employed. The elution solvents used were MeOH:0.1 *M* Tris-HCl, pH 7.5 (9:2) for C18PCA and PIC-B7 (Waters) for ara-C analysis.

**Radioimmunoassay of ara-C in mouse plasma** Male BD2F1 mice (5 mice per group) were administered po with 100 mg of C18PCA (170 μmol) or the same amount of PL-AC (180 μmol) per kg of body weight. Blood samples were obtained by cardiac puncture using heparinized syringes. Zinc sulfate was added to the blood at a final concentration of 10 m*M*. After centrifugation, HCl was added to the plasma to give a final concentration of 0.1 *N*. Individual plasma samples were directly analyzed for ara-C content by means of the sensitive and specific radioimmunoassay described in our previous paper.[11]

## RESULTS

**Antitumor activities of CnPCA by oral administration** BD2F1 mice bearing L1210 leukemia were administered po with CnPCA daily from day 1 to day 5. As shown in Table I, with increasing number of carbon atoms in the alkyl group of CnPCA, the activity at the dose of 100 mg/kg/day gradually increased and reached the maximum level when the number of carbon atoms in the alkyl group reached 18. Further increase in the carbon number led to a decrease of activity. The optimum length of the alkyl group was found to be between 14 and 23 carbon atoms. C18PCA exhibited the highest degree of activity at any dose below 100 mg/kg/day. Oleylphosphate of ara-C which possesses an alkenyl group (with 18 carbon atoms in the alkenyl group and one double bond) exhibited approximately the same degree of activity as that of C17PCA (data not shown). The result indicates that C18PCA is a potential candidate for an orally active antitumor agent. Thus, we examined its properties and pharmacology in detail.

Table I. Antitumor Activity of 5′-Alkylphosphates of Ara-C against L1210 Leukemia in Mice

| Compound | Alkyl group on the phosphate moiety of ara-CMP | T/C% Dose (mg/kg/day) 25 | 50 | 100 | 200 | 400 |
|---|---|---|---|---|---|---|
| ara-C | | | | 122 | 134 | 167 |
| ara-CMP | | | 108 | 121 | 157 | 173 |
| C1PCA | Methyl | | | 113 | 148 | 165 |
| C10PCA | *n*-Decyl | 100 | 104 | 108 | 136 | 139 |
| C11PCA | *n*-Undecyl | | 112 | 127 | 131 | 178 |
| C14PCA | *n*-Tetradecyl | 110 | 162 | 188 | 205 | 234 |
| C15PCA | *n*-Pentadecyl | 120 | 170 | 195 | 239 | 246 |
| C16PCA | *n*-Hexadecyl | 130 | 173 | 188 | 237 | >327[a] |
| C17PCA | *n*-Heptadecyl | 152 | 187 | 223 | >254[a] | 85 |
| C18PCA | *n*-Stearyl | 169 | 214 | >300[a] | 153 | 93 |
| C20PCA | *n*-Eicosyl | 160 | 190 | 226 | >299[a] | 111 |
| C23PCA | *n*-Tricosyl | 151 | 165 | 181 | 210 | 228 |

BD2F1 mice (3 or 7 per group) bearing L1210 leukemia ($1\times10^5$ cells) were po administered at the indicated dose daily from day 1 to day 5.
*a*) The 30-day survivors were counted.

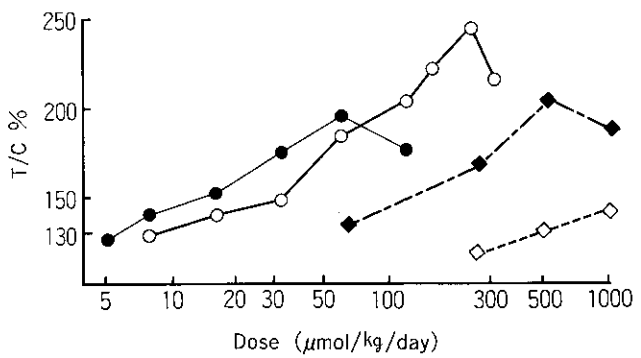


Fig. 2. Effect of the administration route on therapy of L1210 leukemic mice with C18PCA or ara-C. BD2F1 mice (5 per group) were implanted ip with $1\times10^5$ cells of L1210 leukemia and administered with C18PCA, ip, (●); C18PCA, po, (○); ara-C, ip, (◆); or ara-C, po, (◇), daily from day 1 to day 5.

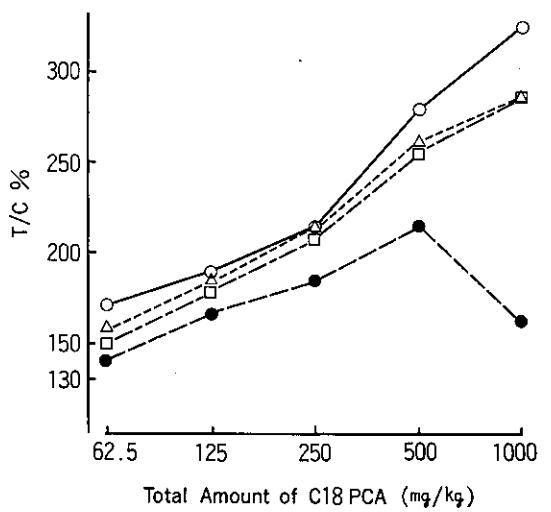


Fig. 3. Effect of the oral administration of C18PCA in various schedules of treatment. BD2F1 mice (5 per group) were implanted ip with $1\times10^5$ cells of L1210 leukemia and administered po with the total amount of C18PCA indicated in the schedule of q1d, on day 1 to day 5, (○); q2d, on days 1, 3, and 5, (△); q4d, on days 1 and 5, (□); and once on day 1, (●).

**Effect of the administration route on therapy of L1210 leukemic mice with C18PCA or ara-C** BD2F1 mice bearing L1210 leukemia were administered ip or po with C18PCA or ara-C daily from day 1 to day 5. As shown in Fig. 2, the minimum effective doses of C18PCA(ip), C18PCA(po), ara-C(ip) and ara-C(po) were determined to be 6.5, 12, 75 and 1000 μmol/kg/day, respectively. The optimum dose of each drug was observed to be 70 for C18PCA(ip), 250 for C18PCA(po) and 600 μmol/kg/day for ara-C(ip). The chemotherapeutic indices of C18PCA(ip), C18PCA(po) and ara-C(ip) were 11, 21 and 8, respectively.

**Effect of the oral administration of C18PCA in various schedules of treatment** BD2F1 mice bearing L1210 leukemia were divided into four groups (Group 1 to 4). Group 1 was further divided into five subgroups and administered po with 62.5 to 1000 mg/kg of C18PCA once on day 1. Groups 2, 3 and 4 were administered

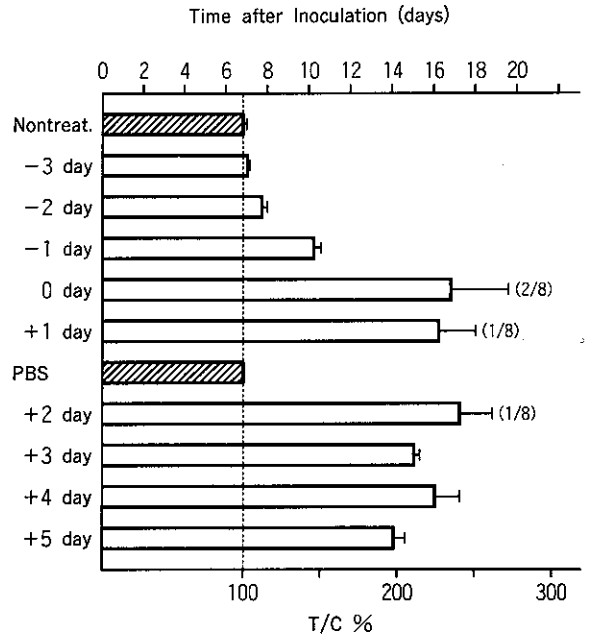


Fig. 4. Effect of single oral administration of C18PCA on the therapy of L1210 leukemia in mice. BD2F1 mice (8 per group) were inoculated ip with $1\times10^5$ cells of L1210 leukemia on day 0 and administered po with C18PCA (500 mg/kg) once on the indicated day prior (−3 to −1 day) or subsequent (+1 to +5 day) to tumor inoculation. The striped bar represents the group of mice treated with PBS on day 1 or the group without treatment. Numbers in parentheses indicate the number of survivors over that of treated mice on day 30 after implantation. Each bar represents the mean survival time±SE.

multiple doses of C18PCA (total amount of 62.5 to 1000 mg/kg), twice (q4d, on days 1 and 5), three times (q2d, on days 1, 3 and 5) and five times (q1d, from day 1 to day 5), respectively. As shown in Fig. 3, almost the same degrees of activity were observed among the multiple treatment groups at all doses, whereas single treatment (Group 1) showed a lower degree of activity than the other groups at the corresponding dose. It seems that the antitumor effect does not depend on the treatment schedule, but depends on the total amount of C18PCA administered, except in the case of Group 1. Even in the latter case (one treatment on day 1), C18PCA showed a marked antitumor effect. The maximum T/C reached 200% at the dose of 500 mg/kg.

**Sustained effect of C18PCA** The pronounced effectiveness of a single dose of C18PCA suggests that blood levels of the agent (and/or a metabolite) are sustained for a rather long period of time after administration. To investigate the sustained effect of C18PCA, 500 mg/kg of C18PCA was po administered to mice at a specified time prior to or subsequent to tumor inoculation. As can be seen in Fig. 4, day −3 (3 days prior to L1210 inoculation) and day −2 treatment groups showed almost no effect, while the day −1 group showed a moderate antitumor effect. C18PCA treatments subsequent to tumor inoculation showed almost the same degree of activity whenever the treatment was performed in the range of day 1 to day 5.

**Comparison of the antitumor activity of C18PCA with that of PL-AC** BD2F1 mice bearing L1210 leukemia

Table II. Antitumor Activity of C18PCA and PL-AC against L1210 Leukemia in Mice by Oral Administration

| Compound | Dose[a] | MST±SE[b] | T/C% | Tox.[c] | Surv.[d] |
|---|---|---|---|---|---|
| None | — | 8.5±0.27 | 100 | 0/20 | 0/20 |
| C18PCA(Na) | 12.5 (21) | 12.5±0.43 | 147 | 0/10 | 0/10 |
| | 25.0 (43) | 13.6±0.34 | 160 | 0/10 | 0/10 |
| | 50.0 (85) | 16.0±0.26 | 188 | 0/10 | 0/10 |
| | 100.0 (170) | 18.5±0.19 | 218 | 0/10 | 1/10 |
| | 200.0 (340) | 16.9±2.29 | 199 | 3/10 | 3/10 |
| | 400.0 (680) | 7.6±0.16 | 89 | 10/10 | 0/10 |
| PL-AC | 50.0 (90) | 12.0±0.54 | 141 | 0/10 | 0/10 |
| | 100.0 (180) | 12.2±0.33 | 144 | 0/10 | 0/10 |
| | 200.0 (360) | 14.0±0.63 | 165 | 0/10 | 0/10 |
| | 400.0 (720) | 17.7±1.28 | 208 | 0/10 | 0/10 |
| | 800.0 (1440) | 15.5±0.87 | 182 | 2/10 | 0/10 |

BD2F1 mice (10 or 20 per group) bearing L1210 leukemia ($0.9\times10^5$ cells) were po administered at the indicated dose daily from day 1 to day 5.

*a*) mg/kg/day (μmol/kg/day).

*b*) Mean survival time (days) was calculated without counting the number of survivors observed on day 30.

*c*) The number of toxic deaths over the number of treated mice at 8 days after tumor implantation.

*d*) The number of survivors over the number of treated mice observed on day 30.

were administered po with C18PCA or with PL-AC daily from day 1 to day 5. As shown in Table II, the optimum doses of C18PCA and PL-AC were 100 and 400 mg/kg/day, respectively. The maximum T/C% values of the two drugs were observed to be almost equivalent (approximately 200). PL-AC required a two- to four-fold greater amount administered as compared with C18PCA to exhibit the same degree of antitumor activity.

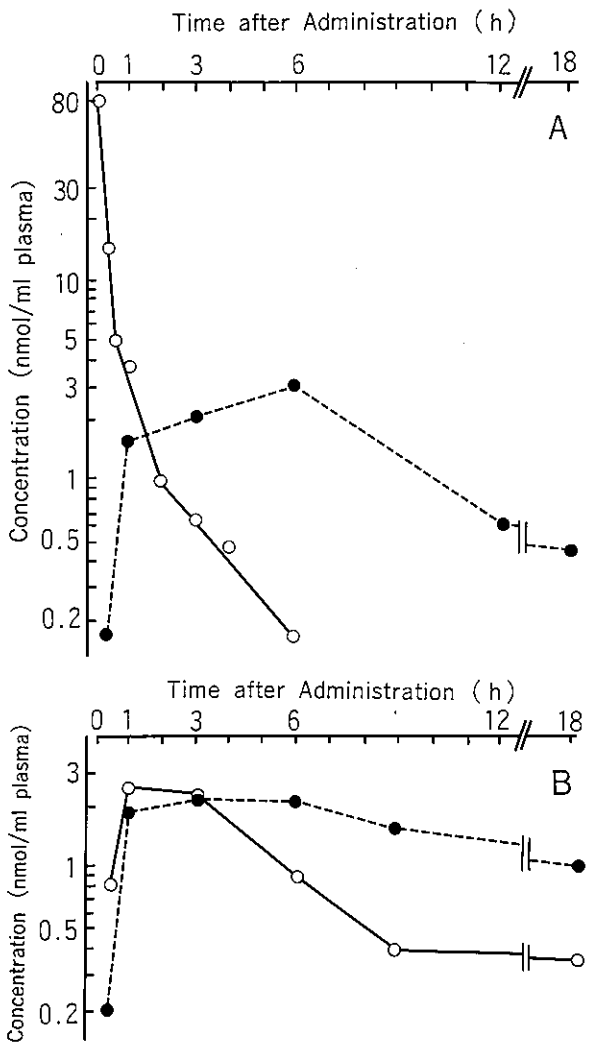


Fig. 5. C18PCA and ara-C levels in the plasma of mice administered with C18PCA. BD2F1 mice (4 per group) were administered iv with 50 mg/kg of C18PCA through the tail vein, (A); or po with 200 mg/kg of C18PCA, (B). Pooled plasma was deproteinized, dried and dissolved in 0.1 *M* Tris-HCl buffer. The plasma concentration of ara-C (●) and C18PCA (○) were analyzed by HPLC as described in "Materials and Methods."

**C18PCA and ara-C levels in the plasma of mice treated with C18PCA** BD2F1 mice were iv administered with 50 mg/kg of C18PCA through the tail vein, then killed periodically after drug administration, and their plasma collected. C18PCA and ara-C levels were analyzed by HPLC as described in "Materials and Methods." As shown in Fig. 5A, C18PCA rapidly diminished in the plasma: the plasma half life of C18PCA was ca. 10 min. On the other hand, the level of ara-C gradually increased and was maintained in the range of 0.5 to 3 nmol/ml for 1 to 12 h after iv administration. When mice were administered po with 200 mg/kg of C18PCA, intact C18PCA was detected in plasma as early as 30 min after the administration (Fig. 5B). The level of C18PCA reached the peak value (2.5 nmol/ml) at 1 h after the administration and thereafter decreased gradually to below 0.5 nmol/ml by 9 h, whereas the level of ara-C reached the peak level of 2.2 nmol/ml 1 h later than the peak time of C18PCA and the level was thereafter maintained in the range of 1 to 2.5 nmol/ml till 18 h after the administration.

**Plasma concentration of ara-C in BD2F1 mice administered with C18PCA or PL-AC** To investigate the difference between the activity of C18PCA and that of PL-AC, the measurement of ara-C concentration was performed in the plasma of mice administered po with C18PCA (170 $\mu$mol/kg) and with PL-AC (180 $\mu$mol/kg)

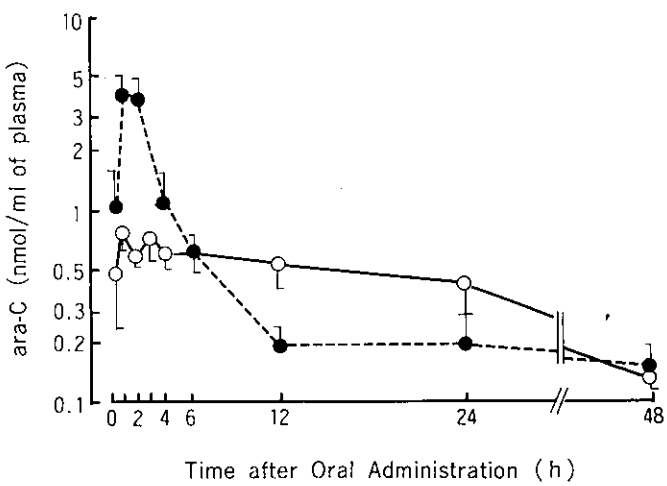


Fig. 6. Plasma concentration of ara-C in BD2F1 mice administered with C18PCA or PL-AC. Male BD2F1 mice (5 per group) were administered po with C18PCA (○) or with PL-AC (●). Heparinized blood was collected and zinc sulfate was added. After centrifugation, individual plasma obtained was directly analyzed for ara-C by radioimmunoassay as described in "Materials and Methods." Each point represents the mean ± SD.

by radioimmunoassay. As early as 15 min after po administration, ara-C was detected in plasma of each group (Fig. 6). The concentration of ara-C derived from PL-AC reached its peak level (4 nmol/ml) at 1 to 2 h after the administration and rapidly decreased thereafter. On the other hand, ara-C from C18PCA gave a level of 0.75 nmol/ml at 1 h after administration and a level above 0.4 nmol/ml was maintained until 24 h after the administration.

## DISCUSSION

In this study, we showed that alkylphosphates of ara-C bearing a long alkyl group (number of carbon atoms between 14 and 23) exhibited high antitumor activity against L1210 leukemia in mice after oral administration, and that the most active derivative was C18PCA. The activity of the series of alkylphosphates of ara-C increased with increasing length of the lipophilic group up to a maximum, and decreased with further increase in the length of the substituent (Table I). The structure-activity relationship of the series of compounds resembled that of $N^4$-acyl derivatives of ara-C,[1] though some differences in the characteristics of antitumor activity (such as minimum effective doses) were observed between the former and the latter. The minimum effective dose of C18PCA in oral administration was remarkably low (6.5 $\mu$mol/kg/day, q1d, day 1 to day 5). This is only about twice that obtained with intraperitoneal administration (Fig. 2). In general, it has been reported that the dose of an agent required to elicit an effect in oral administration is greater (ca. 3 to 10 times) than that required in intraperitoneal administration.[12, 13] The efficacy of C18PCA at low doses was also observed in the comparative study with PL-AC, i.e., C18PCA required only one-fourth to one-half of the amount of PL-AC to elicit the same degree of activity (Table II). These results suggest that C18PCA would show a high degree of bioavailability even if given orally. The antitumor activity of C18PCA did not depend greatly on the treatment schedules as far as tested, and no sustained effect of C18PCA was observed, either (Figs. 3 and 4). It is supposed that C18PCA administered po was retained in the gastrointestinal tract and gradually absorbed through the gut as if it had been continuously infused iv, and therefore the efficacy of C18PCA did not so much depend on the treatment schedule.

Analyses of the plasma levels of C18PCA and ara-C in mice administered iv with C18PCA by HPLC clearly proved that C18PCA was rapidly distributed to some depot sites and released as the active metabolite ara-C (Fig. 5A). The results obtained from another experiment on the oral administration of C18PCA proved that C18PCA was absorbed intact through the gastrointestinal tract (Fig. 5B). It is not clear whether C18PCA was converted to ara-C in the gastrointestinal tract. The results obtained above, however, suggest that at least a part of C18PCA was absorbed intact through the gastrointestinal tract, rapidly distributed into some depot sites and released as the active metabolite ara-C into the plasma over a long period of time.

Prolonged appearance of ara-C was also confirmed by radioimmunoassay in a separate experiment involving the oral administration of C18PCA (Fig. 6). In this experiment, mice were administered po with an effective dose of C18PCA (100 mg/kg or 170 $\mu$mol/kg) or an ineffective dose of PL-AC (100 mg/kg or 180 $\mu$mol/kg). PL-AC released a higher level of ara-C than that derived from C18PCA but did not maintain its level for a long period. In contrast, C18PCA released a relatively low level of ara-C in the range of 0.4 to 0.75 $\mu$mol/ml, but maintained the level for a long period. These results strongly suggest that the maintenance of a biologically effective level of ara-C for a long period of time is important for the exhibition of potent antitumor activity of ara-C, and that C18PCA would be an excellent depot form of ara-C which is available at relatively low doses.

In our previous paper, we showed that C18PCA did not exhibit remarkable cytotoxicity against leukemia cells in culture, but showed potent antitumor activity and toxicity in mice. It is supposed that C18PCA does not have direct biological activity, but exhibits its activity after conversion to an active metabolite ara-CTP through ara-C, ara-CMP or some other intermediates in mice. In any case, it would be expected that ara-C is the active intermediate. On the other hand, C18PCA might be supposed to be cleaved and converted to an inactive form (ara-U) through ara-C or 1-$\beta$-D-arabinofuranosyluracil-5′-monophosphate, or converted directly to another inactive form, 1-$\beta$-D-arabinofuranosyluracil-5′-stearylphosphate. C18PCA was, however, proved to be stable to cytidine deaminase in blood. When ara-C and C18PCA were incubated in the whole blood of a crab-eating monkey, which contained high activity of cytidine deaminase, ara-C was rapidly converted to ara-U ($t$ 1/2 = 12 min), but C18PCA was unchanged for 2 h (data not shown). Further studies on the distribution, metabolism and release of active metabolite(s) of C18PCA are in progress and the details will be reported in the following paper.

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