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# Antiproliferative activity and mechanism of action of fatty acid derivatives of arabinofuranosylcytosine in leukemia and solid tumor cell lines

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**Abstract**

1-β-D-Arabinofuranosylcytosine (ara-C) is a deoxycytidine analog with activity in leukemia, which requires phosphorylation by deoxycytidine kinase (dCK) to allow formation of its active phosphate 1-β-D-arabinofuranosylcytosine triphosphate, but can be deaminated by deoxycytidine deaminase. Altered membrane transport is also a mechanism of drug resistance. In order to facilitate ara-C uptake and prolong retention in the cell, lipophilic prodrugs were synthesized. Fatty acid groups with a varying acyl chain length and number of double bonds were esterified at the 5′ position on the sugar moiety of ara-C. The compounds were tested in two pairs of ara-C resistant leukemic cell lines (murine L1210 and rat BCLO and their resistant variants L4A6 and Bara-C, respectively) and two pairs of cell lines with a resistance to gemcitabine, another deoxycytidine analog (human ovarian cancer A2780 and murine colon cancer C26-A and their resistant variants AG6000 and C26-G, respectively). L4A6, Bara-C and AG6000 have varying degrees of decreased dCK activity, while the mechanism for C26-G is not yet clear. In the parent cell lines, ara-C was more active, but in the resistant variants several of the analogs were more active, while the degree of cross-resistance varied. In AG6000 with a total dCK deficiency, all compounds were inactive. Structure–activity relation analysis showed that ara-C derivatives with shorter acyl chains and more double bonds were more active in the parental and drug resistant cells. Further mechanistic studies were performed with the elaidic acid derivative of ara-C (CP-4055). CP-4055 inhibited deamination of dCyd partly and induced DNA synthesis inhibition effectively in C26-A and C26-G cells, but the retention of inhibition was much longer for CP-4055 than for ara-C. In contrast to ara-C, CP-4055 inhibited RNA synthesis for 60% after drug exposure. In conclusion, CP-4055 seems to be a promising prodrug, whose effects were different and longer lasting than for the parent drug.
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*Keywords:* Ara-C; Ara-C derivative; Drug sensitivity; Deoxycytidine deaminase; Drug accumulation; Drug retention; DNA damage; RNA damage

## 1. Introduction

Ara-C, a deoxycytidine analog, is the major drug in the treatment of acute leukemia [1]. Ara-C requires phosphorylation by dCK in order to be active, and can be inactivated by deamination by deoxycytidine deaminase (dCDA) to 1-β-D-arabinofuranosyluracil (ara-U) [2]. In its active form, 1-β-D-arabinofuranosylcytosine triphosphate (ara-CTP) is a potent inhibitor of DNA synthesis by inhibition of DNA polymerase, which is still a reversible effect, however, after long incubation ara-CTP can be incorporated into DNA, which causes irreversible DNA damage [3].

The inherent or acquired resistance of tumors to cytostatic agents is a major clinical problem. Although the treatment of acute myelogenous leukemia with ara-C is relatively successful, the eventual formation and/or selection of resistant tumor cells allow uncontrolled regrowth of the tumor resulting in failure to cure [4–6]. Moreover, solid tumors are not sensitive to ara-C [7].

Like other nucleoside analogs, ara-C is hydrophilic and cannot easily traverse cell membranes by passive diffusion [8,9]. Specialized transport systems are required for the passage of nucleoside analogs in or out of cells [10,11]. Membrane efflux pumps have activities directed outward

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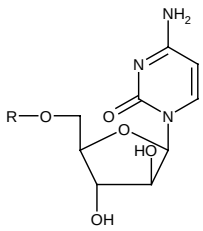


| Compound | double bonds | chain length | Structure R |
|---|---|---|---|
| CP-4055 | 1 | 18 | |
| CP-4056 | 0 | 18 | |
| CP-4057 | 1 | 20 | |
| CP-4091 | 1 | 20 | |
| CP-4092 | 2 | 18 | |
| CP-4093 | 0 | 16 | |
| CP-4094 | 1 | 16 | |
| CP-4095 | 3 | 18 | |
| CP-4096 | 0 | 20 | |
| CP-4097 | 1 | 22 | |
| CP-4098 | 3 | 18 | |
| Ara-C | 0 | 0 | H |

Fig. 1. Chemical structures of the fatty acid derivatives.

of the cell. The MRP family of membrane efflux pumps has nine members, all with different drug specificities [12]. Recently, the structurally related drug gemcitabine has been identified as a substrate for MRP5 efflux pump [13], however, whether ara-C is a substrate for a membrane efflux pump is not known yet. Altered nucleoside transport activity as a mechanism of drug resistance has been described for ara-C in leukemia cells [14,15].

Since decreased sensitivity to ara-C might be the result of reduced drug accumulation, fatty acid ester derivatives of ara-C were synthesized (Fig. 1). These derivatives were intended to facilitate the accumulation and increase retention of ara-C in leukemic and solid tumor cells. The fatty acids vary in chain length and have a different number and position of double bonds. In all ara-C derivatives the fatty acid chain was coupled to the 5′ position on the sugar moiety. In the cell these derivatives need to be converted to ara-C in order to be phosphorylated. In this paper, we describe their cytotoxicity in ara-C and gemcitabine resistant cell lines, the modulation of dCDA activity and effects on DNA and RNA synthesis compared to ara-C.

## 2. Materials and methods

### 2.1. Materials

Dulbecco's Modified Eagle's Medium (DMEM) and RPMI1640 were purchased from Flow Laboratories and fetal calf serum (FCS) from Gibco, rat serum was purchased from Harlan/Olac, trichloroacetic acid (TCA), glutamine and gentamicin from Merck, trypsin and sulforhodamine B (SRB) from Sigma Chemical Co. Ara-C was obtained from Upjohn. All test compounds were provided by Clavis Pharma. [2-$^{14}$C]TdR, 62.8 mCi/mM, was purchased from Dupont de Nemours NEND and [5-$^{3}$H]UR, 27.0 Ci/mM, from Amersham. All other chemicals were of analytical grade and commercially available.

### 2.2. Preparation of the test samples

The ara-C derivatives were dissolved in DMSO to a final concentration of 20 mM. The compounds CP-4057 and CP-4096 were further diluted to 10 mM with DMSO because of poor solubility at 20 mM. Serial dilutions ranging from $8 \times 10^{-10}$ to $8 \times 10^{-3}$ M were prepared from these stock solutions in cell culture medium. Drug concentrations $>10^{-3}$ M could not be obtained due to precipitation of the compounds in the wells. Moreover, at a concentration of $2 \times 10^{-3}$ M cells are exposed to too high (>1%), i.e. too toxic concentrations of DMSO. The stock solutions of ara-C and its derivatives were stored at $-20^{\circ}$.

### 2.3. Cell culture

The *in vitro* experiments were performed in eight cell lines belonging to a standard panel in the testing laboratory, both of leukemia and solid tumor origin (Table 1). Murine leukemia L5 cells, a variant of L1210 and the ara-C

Table 1
Characteristics of the leukemia and solid tumor cell lines

| Parental | Resistant variant | Primary resistance | Resistance factor | Origin | Reference |
|---|---|---|---|---|---|
| L5 | L4A6 | Ara-C | 27,000 | Murine lymphocytic leukemia | [16] |
| BCLO | Bara-C | Ara-C | 100 | Rat acute myelogenous leukemia | [17,37] |
| C26-A | C26-G | Gemcitabine | 10 | Murine colon carcinoma | [20] |
| A2780 | AG6000 | Gemcitabine | 150,000 | Human ovarian carcinoma | [19] |

L5 is a variant of L1210.

resistant variant L4A6, were routinely cultured in suspension in RPMI1640 medium supplemented with 10% FCS, 60 μM 2-β-mercaptoethanol and 250 ng/mL gentamicin, as described previously [16] (Table 1). Rat leukemia BCLO cells were cultured in RPMI1640 medium, as described [17], supplemented with 3.2 mM L-glutamine, 10% FCS and 250 ng/mL gentamicin, the ara-C resistant variant Bara-C was cultured in the same medium as BCLO, but instead of 10% FCS, supplemented with 5% FCS and 5% rat serum. BCLO and Bara-C were generously provided by Dr. L. Colly (then at Department of Hematology, University Hospital Leiden, The Netherlands).

The solid tumor cell lines were A2780, a human ovarian cancer cell line [18], and its variant AG6000, which is highly resistant to gemcitabine [19] (Table 1). The murine colon cancer cell lines C26-A and C26-G were derived from the murine colon 26-A tumor and its variant colon 26-G with an *in vivo*-induced resistance to gemcitabine, respectively [20]. Solid tumor cells were grown in monolayers in DMEM supplemented with 5% FCS and 250 ng/mL gentamicin.

All populations were cultured at 37° in a humidified atmosphere of 95% air and 5% $CO_2$ and regularly screened for mycoplasma contamination by using a rapid detection system with a $^3$H-labeled DNA probe (Gen-Probe) and were found to be negative.

### 2.4. Chemosensitivity tests

Sensitivity to drugs was defined by the concentration of the drug causing a growth inhibition of 50% ($IC_{50}$) after 72-hr drug exposure of the cells.

Determination of growth inhibition in suspension growing leukemia cells was performed by a cell counting method as previously described [21]. The cells were plated in 24-well plates in different densities, depending on their doubling times, to enable log linear growth for 72 hr (L5, 50,000 cells/well; L4A6, 100,000 cells/well; BCLO, 150,000 cells/well; Bara-C, 175,000 cells/well) in duplicate. Drugs were added directly after plating the cells. Final concentrations of the drugs in the wells ranged from $2 \times 10^{-10}$ to $2 \times 10^{-3}$ M. After 72 hr, growth rate was determined by cell counting using a Sysmex Microcell counter. Growth inhibition curves were made relative to control of every assay. Two control values were always included; the number of cells plated at the day of drug administration (set at 0%) and that of cells after 72 hr not exposed to drugs (set at 100%). Straight lines connected the points, and the $IC_{50}$ values were determined from the interpolated graph. Each experiment was performed at least 3-fold.

The determination of the $IC_{50}$ in the monolayer solid tumor cells was performed using the SRB assay [22,23]. On day 1, 100 μL cell suspension was plated per well in 96-well plates (A2780, 6000 cells/well; AG6000, 18,000 cells/well; C26-A, 5000 cells/well; C26-G, 8000 cells/well) in triplicate, followed by 100 μL drugs at day 2. The final concentrations of the drugs in the wells ranged from $2 \times 10^{-10}$ to $2 \times 10^{-3}$ M. Cells were exposed to the drugs for 72 hr or for 4 hr, whereafter cells were washed and cultured in drug-free medium. Total culture time was always 72 hr, allowing at least two doubling times. After exposure, cells were precipitated with 50 μL ice-cold 50% (w/v) TCA and fixed for 60 min after which the SRB assay was performed as described [24]. Growth inhibition curves were made by calculation of the relative growth based on the optical densities of drug-treated and control wells of every SRB assay. The points were connected by straight lines and the $IC_{50}$ values were determined from the interpolated graph [24]. Each experiment was performed at least 3-fold.

### 2.5. Partial purification of dCDA

dCDA was partially purified from BxPC3 human pancreatic cancer cells, which has the highest dCDA activity tested in our panel of cell lines. Partial purification was performed essentially as described previously [25]. A pellet of $8.6 \times 10^7$ BxPC3 cells was suspended in 4 mL buffer (20 mM Tris (pH 7.5), 5 mM KCl, 1 mM DTT, 40 μL (10,000 U/mL) streptomycin, 160 μL protease inhibitor cocktail (25×). The cell suspension was sonificated five times for 5 s with an interval of 20 s. After centrifugation for 30 min at 20,000 *g*, $(NH_4)_3SO_4$ was added to the supernatant to a final concentration of 0.232 g/mL to get a 40% saturation. This was mixed for 1 hr and then centrifuged by an ultracentrifuge at 36,000 *g* for 20 min at 4°. To this supernatant $(NH_4)_3SO_4$ was added to a concentration of 0.327 g/mL to get a 55% saturation, mixed for 1 hr and centrifuged by an ultracentrifuge at 36,000 *g* for 20 min at 4°. The pellet was resuspended in 1 mL of 20 mM Tris (pH 7.5). The salt was removed by overnight dialysis against MQ-water. Since the BioRad protein assay is not sensitive enough to measure the amount of protein, the enzyme

activity was calculated per microliter of enzyme. To the enzyme solution glycerol was added and was stored at −20°.

### 2.6. dCDA assay

Activity of dCDA was determined as described earlier [26]. Enzyme activity was determined at 37° in 100 μL partially purified enzyme with 500 μM dCyd or ara-C as a substrate in a volume of 20 and 500 μL competitive compound (ara-C and CP-4055) in a volume of 20 μL. Tris–HCl was added to a final volume of 200 μL. A blank without substrate and inhibitor and a control with a substrate but without an inhibitor was included in every experiment. After 15- or 25-min incubation, the reaction was terminated by precipitating the proteins by addition of 40% (w/v) TCA. The supernatant was neutralized with trioctylamine/1,1,2-trichlorotrifluoroethane (v/v, 4:1). The upper, aqueous layer containing the substrate dCyd or ara-C and their respective products deoxyuridine or ara-U, was analyzed using reversed phase HPLC [26]. In order to measure dCDA activity in cell lines, we used 10,000 *g* supernatants as described previously [19–21].

### 2.7. Determination of the effect on DNA and RNA synthesis

Inhibition of DNA and RNA synthesis was studied using 96-wells filter plates essentially as described previously [27,28]. One hundred thousand C26-A and C26-G cells/well were plated in a volume of 100 μL, whereafter 100 μL of drug-containing medium was added resulting in final concentrations of 2.5 μM ara-C and 25 μM CP-4055 for the thymidine (TdR) incorporation into DNA assay and for the uridine (UR) incorporation into RNA assay. Drug concentrations were selected after preliminary experiments with more concentrations to determine a concentration giving partial inhibition. As a control untreated cells were incubated with [$^{14}$C]TdR or [$^{3}$H]UR. For the blank [$^{14}$C]TdR or [$^{3}$H]UR was added immediately before harvesting. After 4-hr incubation, drug-containing medium was removed, cells were washed and incubated with drug-free medium for 0, 1, 2 and 4 hr. One hour before the end of drug exposure, 10 μL of [2-$^{14}$C]thymidine ([$^{14}$C]TdR, 62.8 mCi/mM) for the TdR incorporation assay and 10 μL of [5-$^{3}$H]uridine ([$^{3}$H]UR, 27.0 Ci/mM) for the UR incorporation assay was added. The incubation was terminated by suction through the filters. Filters were washed four times with 200 μL 8% TCA, followed by four washes with 200 μL PBS. Filters were removed and transferred separately to counting vials. The [$^{14}$C]TdR samples could be counted directly, but [$^{3}$H]UR samples were solubilized by shaking in 500 μL 2 M NaOH for 3 hr. Five milliliters of Optima Gold (Packard) was added and disintegrations were counted for 5 min.

### 2.8. Statistical analysis

Statistical evaluation was performed using the computer program SPSS (version 7.5, SPSS, Inc.). Differences in inhibition of dCDA activity were evaluated using a Student's *t* test. For the correlation, we calculated both Pearson's and the non-parametric Spearman's correlation coefficients.

## 3. Results

### 3.1. Chemosensitivity tests

The growth inhibitory effects of the compounds in the leukemia cells are listed in Table 2. Ara-C was the most active compound in L5 cells, but in BCLO P-4094 and P-4098 were 5- and 3.5-fold more potent than ara-C, respectively. In order of decreasing potency P-4098, P-4094, P-4057 and CP-4055 were the most active compounds in L5 cells, with $IC_{50}$ values in the same range as ara-C. CP-4096 was the least active compound in L5 and BCLO cells.

The growth inhibitory effects of the compounds in the solid tumor cells are also listed in Table 2. Ara-C is 175-fold less active in C26-A cells than in L5 cells, however, A2780 cells are equally sensitive to ara-C as L5 cells. Ara-C was more active than its derivatives in both C26-A and A2780 cells. In order of decreasing potency P-4094, CP-4055 and CP-4057 were the most active compounds in C26-A cells and P-4094, P-4095 and P-4098 were the most active compounds in A2780 cells.

To estimate the retention of the drug in the cell, C26-A and C26-G cells were not only exposed to drugs for 72 hr but also for 4 hr, followed by culture in drug-free medium to a total of 72 hr. An $IC_{50}$ for 4-hr exposure could be calculated. These cell lines were chosen because the analogs were designed to improve treatment results in solid tumors by facilitating a better uptake in tissues

Table 2
$IC_{50}$ values of ara-C and its derivatives in parental cell lines

| Compound | Leukemia cells | | Solid tumor cells | |
|---|---|---|---|---|
| | L5 | BCLO | C26-A | A2780 |
| Ara-C | 0.004 ± 0.002 | 0.88 ± 0.23 | 0.7 ± 0.2 | 0.003 ± 0.002 |
| CP-4055 | 0.05 ± 0.02 | 0.87 ± 0.6 | 1.8 ± 0.2 | 0.30 ± 0.09 |
| P-4056 | 0.12 ± 0.01 | 1.72 ± 0.65 | 22.8 ± 13.6 | 3.4 ± 2.2 |
| CP-4057 | 0.04 ± 0.02 | 1.23 ± 0.88 | 1.8 ± 0.4 | 3.9 ± 3.1 |
| P-4091 | 0.05 ± 0.01 | 2.24 ± 1.88 | 4.3 ± 2.8 | 1.2 ± 0.6 |
| P-4092 | 0.09 ± 0.05 | 2.25 ± 1.88 | 5.2 ± 1.9 | 0.4 ± 0.06 |
| P-4093 | 0.021 ± 0.005 | 1.49 ± 1.25 | 3.8 ± 2.2 | 0.3 ± 0.2 |
| P-4094 | 0.006 ± 0.002 | 0.17 ± 0.03 | 1.6 ± 0.6 | 0.012 ± 0.009 |
| P-4095 | 0.03 ± 0.006 | 0.76 ± 0.5 | 2.2 ± 0.2 | 0.09 ± 0.03 |
| CP-4096 | 1.3 ± 0.5 | 26.5 ± 8.5 | 68.0 ± 23.1 | 27.4 ± 10.2 |
| P-4097 | 0.16 ± 0.06 | 1.55 ± 0.74 | 8.0 ± 3.0 | 4.3 ± 1.1 |
| P-4098 | 0.004 ± 0.003 | 0.25 ± 0.03 | 4.5 ± 2.9 | 0.12 ± 0.07 |

$IC_{50}$: drug concentration resulting in 50% growth inhibition. Values are means in μM ± SEM of three to four experiments.

due to the presence of a lipophilic chain and a potential resistance to deamination (see below). Since AG6000 is completely resistant to all drugs and the leukemic lines have a low deaminase [30], we chose the colon cancer cell line pair. A low ratio of the 4 hr $IC_{50}$ and the 72 hr $IC_{50}$ ($IC_{50}$ ratio) is considered a measure of long retention of the drug in the cell. $IC_{50}$ ratios in C26-A cells for ara-C (180) were higher than for P-4098 (6.3), CP-4055 (78.4) and CP-4057 (67.2), while all values were generally lower than in C26-G cells (data not shown). This indicates a longer retention of the ara-C derivatives than ara-C, while the retention was also longer in the more sensitive C26-A cells.

The L5 variant L4A6 was 27,500-fold resistant to ara-C, however, the resistance factor of the ara-C derivatives in L4A6 was far lower (Table 3). Also in C26-G cells resistance factors of the ara-C derivatives was lower than of ara-C. In AG6000 cells, resistance factors were not calculated since $IC_{50}$ values for ara-C and its derivatives were immeasurably high.

The most active ara-C derivatives was P-4094 followed by CP-4055, P-4095 and P-4098, which are equally active in the seven tumor cell lines tested (mean $IC_{50}$: 0.76, 0.77 and 1.22 μM, respectively). In Fig. 2, a structure–activity relation between number of carbon atoms in the fatty acid chain and number of double bonds and mean $IC_{50}$ in the seven tumor cell lines is depicted. There is a clear relation between number of carbon atoms in the fatty acid chain and to a lesser extent with the number of double bonds and activity of the ara-C derivative. The shorter the acyl chain and the more double bonds the more active the compound.

### 3.2. Effect of fatty acid derivatives on dCDA activity

Apart from facilitating uptake, the analogs were designed for a potential resistance to deamination due to the presence of a lipophilic chain. The dCDA activities of the cell lines are listed in Table 4. dCDA activity is unmeasurably low in the leukemia L5, L4A6 and BCLO cells. However, in Bara-C cells the highest dCDA activity is found of the panel. In the solid tumor cells, dCDA activity in the resistant variants was lower than in the parental cells. No relation was found between dCDA activity and mean $IC_{50}$ of all derivatives tested (data not shown).

Further studies on the mechanism of action of ara-C derivatives were performed with CP-4055, the derivative containing the elaidic acid chain. CP-4055 was chosen because of its *in vitro* activity and its preferential retention in liver (data to be published separately). To study the effect of the fatty derivatives on deamination of dCyd and

Table 3
Resistance factors to ara-C and its derivatives in ara-C and gemcitabine resistant variants

| Compound | Leukemia cells | | Solid tumor cells | |
|---|---|---|---|---|
| | L4A6 | Bara-C | C26-G | AG6000 |
| Ara-C | 27,500 ± 14,289 | 77[a] | 6.2 ± 2.9 | n.d. |
| CP-4055 | 13,343 ± 11,330 | 181 ± 131 | 2.3 ± 0.9 | n.d. |
| P-4056 | 736 ± 171 | 123 ± 89 | 1.6 ± 1.2 | n.d. |
| CP-4057 | 1,824 ± 702 | 177 ± 144 | 2.0 ± 0.4 | n.d. |
| P-4091 | 3,450 ± 853 | 92 ± 75 | 5.8 ± 2.9 | n.d. |
| P-4092 | 3,243 ± 2,880 | 125 ± 97 | 4.1 ± 2.9 | n.d. |
| P-4093 | 7,429 ± 2,100 | 374 ± 349 | 5.0 ± 1.7 | n.d. |
| P-4094 | 12,815 ± 4,212 | 667 ± 167 | 2.2 ± 0.4 | n.d. |
| P-4095 | 2,191 ± 886 | 269 ± 231 | 2.4 ± 0.4 | n.d. |
| CP-4096 | 112 ± 37 | 6[a] | 0.5 ± 0.1 | n.d. |
| P-4097 | 1,967 ± 1,267 | 88[a] | 8.2 ± 1.8 | n.d. |
| P-4098 | 103,167 ± 98,417 | 71[a] | 1.8 ± 0.7 | n.d. |

Resistance factor: ratio of the $IC_{50}$ (resistant cells)/$IC_{50}$ (parental cells) in each separate experiment. Values are means ± SEM of three to four experiments.

n.d. not detectable; resistance factor could not be calculated since the $IC_{50}$ values in AG6000 were undetectably high.

[a] Experiment performed once.

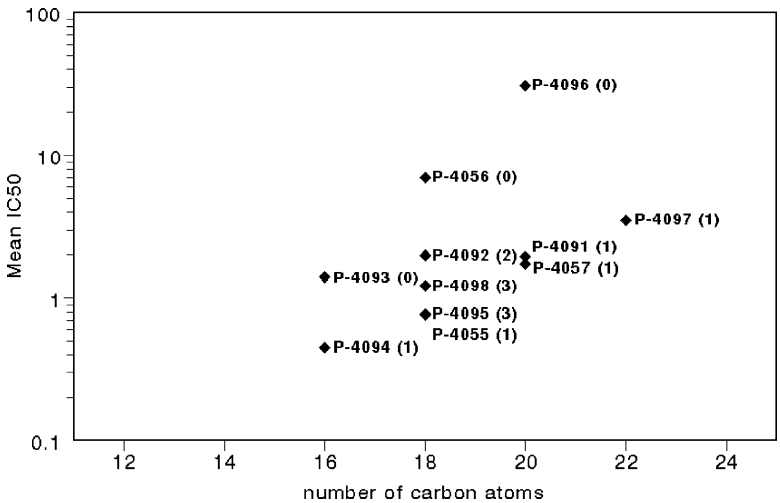


Fig. 2. Relationship between chain length, number of double bonds and mean $IC_{50}$ value of ara-C derivatives. The Pearson and Spearman's correlation coefficients for chain length (one, two and three double bonds combined) were 0.871 ($P = 0.005$) and 0.779 ($P = 0.023$), respectively; for chain length limited to one double bond, these values were 0.948 ($P = 0.014$) and 0.975 ($P = 0.05$), respectively.

Table 4
dCDA activities in the leukemia and solid tumor cell lines

| | dCDA activity | Reference |
|---|---|---|
| L5 | n.d. | [21] |
| L4A6 | n.d. | [21] |
| BCLO | n.d. | [21] |
| Bara-C | 2.27 ± 0.49 | [21] |
| A2780 | 0.9 ± 0.6 | [19] |
| AG6000 | 0.05 ± 0.03 | [19] |
| C26-A | 0.50 ± 0.30 | [20] |
| C26-G | 0.20 ± 0.10 | [20] |

Values are means in nanomoles per hour per $10^6$ cells ± SEM of three to four experiments.
n.d. not detectable.

ara-C, partially purified dCDA enzyme was incubated with dCyd or ara-C as a substrate and with a test compound. CP-4055 was not detectable in the chromatograms using the applied separation system. Neither the parent nucleoside nor its deaminated derivative were seen, thus indicating no detectable degradation to the parent nucleoside or subsequent deamination. This means that any observed effect would be a direct effect on dCDA. The relative enzyme activity is calculated from the amount of deaminated product of the substrate (dCyd; dUrd; ara-C; ara-U). Ara-C decreased deamination of dCyd to only 14.8%, while no ara-U was detected (Table 5). The fatty acid derivative CP-4055 inhibited deamination of dCyd to 40.3%. Ara-C was deaminated less effectively than dCyd. Although CP-4055 inhibited deamination of dCyd quite effectively, the derivative increased the deamination of ara-C to 142.9%.

### 3.3. Inhibition of DNA and RNA synthesis

Both C26-A and C26-G cells were used to study inhibition of DNA synthesis. After selection of a concentration resulting in a comparable partial inhibition of RNA and DNA synthesis, retention of the inhibition was investigated. In C26-A cells ara-C and CP-4055 inhibited DNA synthesis effectively after 4 hr of drug exposure (Fig. 3A). However, after 4 hr no more DNA inhibition was found in the ara-C-exposed C26-A cells, while DNA inhibition was retained in CP-4055-exposed cells. In C26-G cells the difference in retention of DNA inhibition between ara-C and CP-4055 is even more pronounced (Fig. 3B). In ara-C-exposed C26-G cells no more DNA inhibition was found after 2 hr, however, DNA inhibition in CP-4055-exposed cells remained unchanged after 4-hr retention.

No clear RNA synthesis inhibition was found in ara-C-exposed C26-A and C26-G cells (data not shown). Only CP-4055 inhibited RNA synthesis in C26-A and C26-G cells after 4-hr drug exposure with 56 and 35%, respectively. After drug removal RNA synthesis recovered relatively quickly (data not shown).

Table 5
Effect of ara-C and its derivative CP-4055 on deamination of dCyd and ara-C by deoxycytidine deaminase

| Substrate (500 μM) | Test compound (500 μM) | Average product[a] (pmol/hr/μL enzyme) | Percent[c] | *P* |
|---|---|---|---|---|
| dCyd | – | 312.0 ± 5.2 | 100.0 | – |
| | Ara-C[b] | 46.1 ± 5.2 | 14.8 | <0.01 |
| | CP-4055 | 125.7 ± 18.8 | 40.3 | <0.01 |
| Ara-C | – | 199.7 ± 7.8 | 100.0 | – |
| | CP-4055 | 285.4 ± 13.8 | 142.9 | <0.01 |

Activity measured by area of the dUrd peak at 3.9 min calculated from the linear part of the curve from the assay at 15 and 30 min.
*P* values calculated by *t* test compared to dCyd or ara-C alone.
[a] Deaminated product; dUrd of dCyd and ara-U of ara-C.
[b] No ara-U detected.
[c] Activities with dCyd or ara-C as a substrate were set at 100%.

## 4. Discussion

In this paper, we describe cytotoxic activity and mechanism of action of ara-C derivatives in a panel of rodent and human leukemia and solid tumor cell lines. The compounds P-4094, CP-4055 and P-4095 showed the highest antiproliferative activity of the ara-C derivatives tested both in the parental cells and in the ara-C and gemcitabine resistant variants, although their $IC_{50}$ values were equally or slightly higher than the value of ara-C. However, CP-4055 showed a superior activity compared to ara-C in several human solid tumor and leukemia xenografts [29]. Although ara-C is not active in solid tumors [7], CP-4055 was even more effective in several melanoma xenografts than clinically established drugs previously tested in the same tumor models [29].

A clear structure–activity relationship was found. The cytotoxicity of the compounds correlated with the length and to a lesser extent with the number of double bonds of the acyl group. The shorter the chain and the more double bonds the more active the compounds. Probably shorter acyl chains are more easily de-esterified, while this property apparently prolonged retention compared to ara-C.

Ara-C derivatives were equal or less toxic in the parental leukemia cells than the parental drug, however, only in BCLO cells several ara-C derivatives display a lower $IC_{50}$ than ara-C. The resistance to ara-C in L4A6 and Bara-C is related to a decreased dCK activity [21]. In Bara-C cells the ara-C and gemcitabine resistance was related to a substrate-dependent dCK deficiency. No dCK activity was found with ara-C or gemcitabine as a substrate, but a normal activity with dCyd as a substrate [21]. Derivatives of ara-C were less active in solid tumor cells than the parental drug. Therefore, activity of these ara-C derivatives depends on phosphorylation by dCK, but not on a nucleoside transporter [29].

Since ara-C can be inactivated by deamination catalyzed by dCDA [2], the effect of the fatty acid derivatives on deamination of dCyd and ara-C was tested. The lower

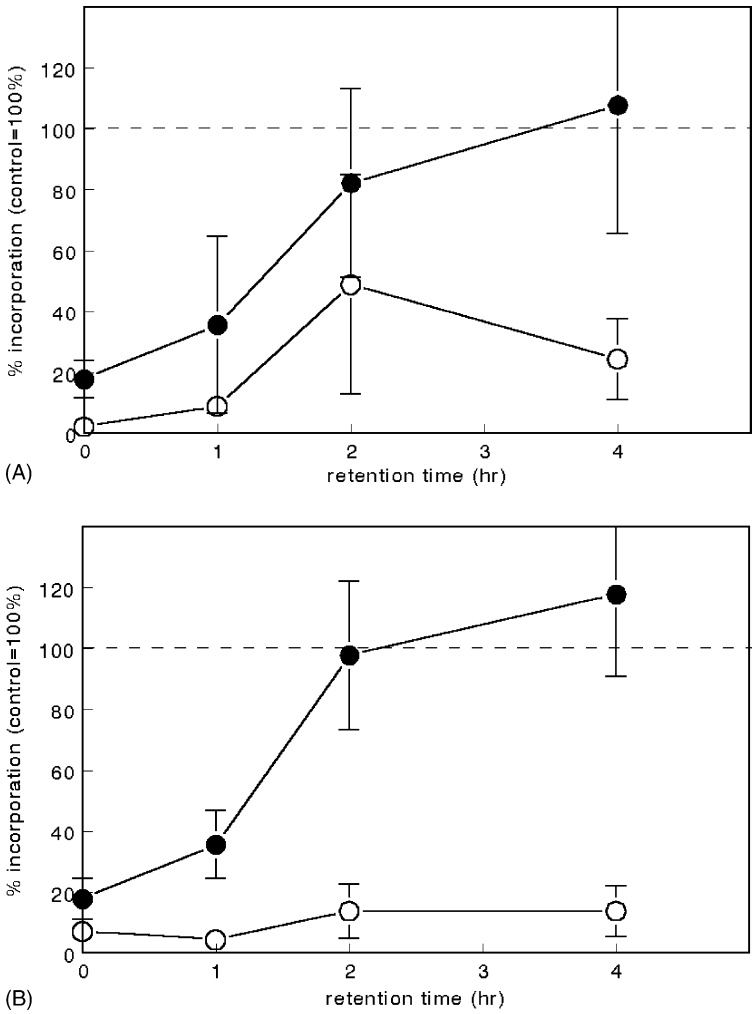


Fig. 3. Retention of DNA synthesis inhibition after exposure to 2.5 μM ara-C (●) or 25 μM CP-4055 (○) for 4 hr in C26-A (A) and C26-G (B) cells. The values represent means ± SEM of three separate experiments. The absolute thymidine incorporation into DNA of untreated C26-A and C26-G cells was 0.4 and 2.8 fmol/hr/$10^6$ cells, respectively.

deamination rate of ara-C compared to dCyd, is in agreement with a higher $K_m$ than for dCyd [30,31]. CP-4055 did not inhibit deamination of ara-C, however, deamination of dCyd was inhibited, in the cell leading to its accumulation, which can then inhibit ara-C deamination. Theoretically, inhibition of deamination of ara-C might result in higher drug levels and higher cytotoxicity. The role of dCDA in the development of resistance is not clear [32], although dCyd analogs are less effective in systems in which dCDA has a higher activity, e.g. through transfection of the gene [31], while addition of a dCDA inhibitor increased activity of ara-C and gemcitabine. In addition, our data indicate that the present compounds have a longer retention due to a decreased deamination. However, no relation between dCDA activity and mean $IC_{50}$ of all analogs tested was found.

The resistance factors of the ara-C derivatives in C26-G cells were lower than of the parental drug. No decreased dCK activity was found in C26-G cells, indicating another mechanism of resistance [20]. The derivatives seem to bypass the mechanism of resistance of the C26-G cells. Since inhibitors of nucleoside carrier transport decreased sensitivity of murine and human leukemia cells to ara-C, but not to CP-4055, increased nucleoside transport activity might be the mechanism of resistance to ara-C in C26-G cells [29].

All agents require phosphorylation to their active form ara-CTP [3]. However, ara-CTP was not detectable in C26-A and C26-G cells after 4-hr exposure to ara-C or CP-4055 (data not shown). In a previous study, ara-CTP pools were undetectably low in L4A6, BCLO and Bara-C cells, while pools of gemcitabine triphosphate were also detectable after exposure to the same concentrations of gemcitabine as ara-C [21]. The absence of detectable pools of ara-CTP might be explained by the high $K_m$ of ara-C for dCK [33]. However, sufficient ara-CTP seems to accumulate to inhibit DNA synthesis.

Incorporation of ara-C into DNA and its subsequent inhibition is considered an important mechanism of action, eventually leading to cell death [34]. After drug exposure, an equal extent of DNA synthesis inhibition was found in ara-C and CP-4055-exposed C26-A and C26-G cells.

However, DNA synthesis inhibition was retained much longer in CP-4055-exposed cells. Since DNA synthesis inhibition is an important mechanism of action of ara-C, the longer inhibition by CP-4055 might explain its higher activity *in vivo* than ara-C.

Since ara-C is not incorporated into RNA [35,36], no inhibition of RNA synthesis is found in C26-A and C26-G cells. However, CP-4055 inhibited RNA synthesis in C26-A cells and even more effectively in C26-G cells, but inhibition was retained shortly. Inhibition of RNA synthesis by ara-C has not been described previously. Probably exposure of cells to fatty acids might explain this effect.

In conclusion, CP-4055 seems to be a promising ara-C derivative, with a favorable cytotoxicity and low resistance factor for ara-C and gemcitabine resistant cells. Moreover, the drug has remarkable activity in solid tumor xenografts, a longer retention of its effect than ara-C and additional mechanisms of action and deserves evaluation in clinical trials, which were recently initiated.

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