Cancer Chemother Pharmacol (2002) 50: 193–201
DOI 10.1007/s00280-002-0472-0

ORIGINAL ARTICLE

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Mechanisms of the apoptotic activity of Cl-F-araA in a human T-ALL cell line, CCRF-CEM

Received: 12 December 2001 / Accepted: 23 April 2002 / Published online: 23 July 2002

Abstract *Purpose*: The purpose of the present study was to characterize the mechanisms of the antitumor activity of 2-chloro-9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)adenine (Cl-F-araA) against lymphocytic leukemia. Recent evidence indicates that Cl-F-araA has more potent antitumor activity in vitro against human leukemia cell lines than against human solid tumor cell lines originating from different tissue. We analyzed the mechanism of action of Cl-F-araA using a human T-acute lymphocytic leukemia cell line, CCRF-CEM, in vitro and in vivo. *Results*: Cl-F-araA exhibited marked antitumor activity in vitro and in vivo, and this was correlated with its ability to induce apoptosis, particularly in vivo. To analyze the mechanisms of the apoptotic activity of Cl-F-araA, we sought to determine the effects of the drug on the levels of Bcl-2 family proteins (Bcl-2, Bcl-X_L, Mcl-1, Bax, Bak) and cell survival signals via Akt. Western blot analysis revealed that Cl-F-araA induced a dose- and time-dependent downregulation of Bcl-X_L and Mcl-1 proteins, and a dose- and time-dependent dephosphorylation of Akt and its downstream effectors (Bad, FKHRL1), particularly in vivo. In addition, there was a marked increase in the population of cells in G_1/S and early S phase. We therefore investigated the changes in the Cdc25A protein to characterize the mechanism involved in the G_1/S accumulation. Cl-F-araA induced a dose- and time-dependent downregulation of the Cdc25A protein whereas the Cdc25C protein remained unchanged. We further found that in combination with caffeine, Cl-F-araA potentiated apoptosis induction. *Conclusions*: Taken together, our findings suggest that Cl-F-araA may be an effective drug in vivo.

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Keywords Antimetabolite · Deoxyadenosine · Leukemia · Apoptosis · Bcl-2 · Akt

Introduction

2-Chloro-9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)adenine (Cl-F-araA) which is being developed for clinical use under the name clofarabine, is the latest analog of deoxyadenosine, similar to clinically useful drugs for the treatment of leukemia and lymphoma, such as fludarabine (2-fluoro-9-(β-D-arabinofuranosyl)adenine) and cladribine (2-chloro-2′-deoxyadenosine), which exhibit promising activity against a variety of tumor cell lines in vitro and in vivo [3, 17]. Of particular interest is the selective and marked antitumor activity of Cl-F-araA in vivo. We have previously demonstrated that Cl-F-araA has potent antitumor activity against human solid tumor xenograft models, particularly colon tumors [28]. Recently, Waud et al. have also demonstrated that this compound has excellent activity against renal tumors as well as colon tumors in vivo and has no cross-resistance to drug-resistant sublines [31].

Cl-F-araA has exhibited potent antitumor activity against human leukemic cell lines in vitro [3, 20, 21, 31, 32, 33]. However, there are no reports of the in vivo antileukemic activity of Cl-F-araA, except for a report by Carson et al. that Cl-F-araA is effective for the treatment of chronic lymphocytic leukemia (CLL) but is toxic to non-dividing lymphocytes and monocytes [3]. The reason for the lack of such reports may be the fact that there are very few good animal models that reflect clinical diseases.

Cl-F-araA is metabolized by 2′-deoxycytidine kinase and is finally phosphorylated to its corresponding triphosphate form in cells. The phosphorylated drug inhibits ribonucleotide reductase and DNA polymerases, thereby causing deoxyribonucleotide depletion and inhibition of DNA synthesis. Furthermore, the drug is used as a substrate by DNA polymerases and is incorporated into DNA causing premature chain termina-

tion. The mechanisms of action of Cl-F-araA in exerting its cytotoxic effect, including activation, inactivation and inhibition of target enzymes, have been extensively studied [13, 14, 21, 32, 33]. However, there are no reports of apoptosis induction by Cl-F-araA except for a recent report by Genini et al. indicating that the drug leads to the release of the proapoptotic mitochondrial proteins, cytochrome *c* and apoptosis-inducible factor, and the activation of the Apaf-1-mediated caspase pathway in vitro [8, 9]. Therefore, it is not completely clear as to how apoptosis is triggered.

We investigated the antitumor activity of Cl-F-araA in a human T-acute lymphocytic leukemia (T-ALL) cell line, CCRF-CEM, in vitro and in vivo, and in particular its ability to induce apoptosis. Our studies point to a relationship between antitumor activity and apoptosis induction by Cl-F-araA. In addition, we determined the ability of the drug to activate the S phase checkpoint and correlated this with its apoptosis-inducing activity.

Materials and methods

Reagents

Cl-F-araA was synthesized in our institute (Shizuoka, Japan). Bio/Por type F (PVDF) hollow fibers (internal diameter 1 mm with a molecular weight cutoff of Mr 1,000,000) were purchased from Spectrum (Rancho Dominguez, Calif.). Acetyl-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid-7-amino-4-methyl-coumarin (DEVD-AMC) was purchased from the Peptide Institute (Osaka, Japan). Okadaic acid was purchased from Wako Chemicals (Osaka, Japan). Caffeine anhydrous was purchased from Nacalai tesque (Kyoto, Japan). All other reagents were from Sigma Chemical Company (St. Louis, Mo.).

Tumors

CCRF-CEM cells were obtained from ATCC (Rockville, Md.) and routinely maintained as suspension cultures in RPMI-1640 supplemented with fetal bovine serum (FBS, 10%) and penicillin/streptomycin (100 IU/ml, 100 μg/ml) at 37°C in a humidified atmosphere of 5% CO_2 in air. All cell culture materials were purchased from Life Technologies (Grand Island, N.Y.). Male BALB/c-nu/nu (nude) mice at 5–6 weeks of age were obtained from Clea Japan (Tokyo, Japan)

Antiproliferative activity

Antiproliferative activity following treatment with Cl-F-araA in vitro and in vivo was determined by measurement of cell viability using the XTT assay as described previously [25]. Briefly, cells exposed to various concentrations of drugs were evaluated by uptake of XTT (Sigma) in conjunction with phenazine methylsulfonate (Sigma).

DEVD-AMC cleavage

DEVD-specific caspase activity was determined by cleavage of DEVD-AMC used as a substrate as described previously [18]. Briefly, cells were treated with various concentrations of the drug for 24 h and the hydrolytic activity of DEVD was measured by incubating cell extracts in 20 m*M* PIPES-NaOH buffer (pH 7.2) including 50 μ*M* DEVD-AMC, 0.25% nonident P-40 (NP-40), 5 m*M* dithiothreitol (DTT), 1 m*M* EDTA and 0.1% CHAPS. After 2 h, the reaction was stopped by the addition of 0.75 *M* acetic acid. Fluorescence (excitation 380 nm, emission 460 nm) was compared with a standard curve of AMC.

Terminal deoxynucleotidyl transferase (TdT) nick end-labeling (TUNEL) assay

Cells were processed according to the manufacturer's directions using an ApopTag Direct Kit (Intergen, Purchase, N.Y.) as described previously [10]. Briefly, cells were fixed in 1% formaldehyde solution for 15 min on ice and suspended in 70% ethanol. The cells were resuspended in 50 μl TdT reaction buffer and incubated at 37°C for 30 min. After stopping the TdT reaction, the cells were incubated with 10 μg/ml propidium iodide (PI) and 10 μg/ml RNase A (type 1-A) for 15 min at room temperature in the dark. Bivariate analysis of apoptosis and DNA content was performed with an EPICS ELITE flow cytometer (FCM; Coulter, Hialeah, Fl.). The resulting bivariate plots allowed the detection of apoptotic events occurring in all phases of the cell cycle. The control sample was used to define normal levels of green fluorescence (i.e. basal levels of apoptosis), while cells with increased fluorescence (R1 region) were considered to be apoptotic. The data from 10,000–20,000 cells were collected and analyzed using the Multi 2D program (Phoenix, San Diego, Calif.). The cell cycle distribution was calculated using the Multicycle program (Coulter).

Western blot analysis

Cells treated with Cl-F-araA were solubilized in ice-cold lysis buffer containing 50 m*M* HEPES-NaOH (pH 7.4), 25 m*M* NaCl, 1% NP-40, 1 m*M* DTT, 1 m*M* sodium fluoride, 10 m*M* *β*-glycerophosphate, 2 m*M* sodium orthovanadate, 1 m*M* EDTA, 1 m*M* phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin and 5 μg/ml leupeptin for 20 min on ice. Cell debris was removed by centrifugation at 10,000 *g* for 10 min at 4°C. Protein concentrations were determined using a Bio-Rad protein assay kit (Bio-Rad, Tokyo, Japan).

Aliquots of cell extracts were subjected to SDS-PAGE and transferred to a PVDF membrane (Millipore, Tokyo, Japan). Membranes were blocked for 1 h at room temperature in a buffer containing 5% nonfat milk powder in TBS-T (10 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl and 0.05% Tween 20) and then incubated with anti-poly(ADP-ribose) polymerase (PARP) monoclonal antibody (Pharmingen, San Diego, Calif.), anti-procaspase 9 monoclonal antibody (Medical & Biological Laboratories, Nagoya, Japan), anti-procaspase 3 monoclonal antibody (Transduction Laboratories, Lexington, Ky.), anti-Bcl-2 monoclonal antibody (Genosys Biotechnologies, Cambridge, UK), anti-Bcl-X_L polyclonal antibody (Transduction Laboratories), anti-Mcl-1 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, Calif.), anti-Bax polyclonal antibody (Pharmingen), anti-Bak polyclonal antibody (Pharmingen), anti-^{473}Ser-Akt polyclonal antibody (Cell Signaling Technology, Beverley, Mass.), anti-Akt polyclonal antibody (Cell Signaling Technology), anti-*β*-actin monoclonal antibody (Sigma), anti-^{136}Ser-Bad polyclonal antibody (Upstate Biotechnology, Lake Placid, N.Y.), anti-Bad polyclonal antibody (Santa Cruz Biotechnology), anti-$p27^{Kip1}$ polyclonal antibody (Santa Cruz Biotechnology), anti-FKHRL1 polyclonal antibody (Upstate Biotechnology), anti-^{32}Thr-FKHRT1 polyclonal antibody (Upstate Biotechnology), anti-Cdc25A polyclonal antibody (Santa Cruz Biotechnology), and anti-Cdc25C polyclonal antibody (Santa Cruz Biotechnology).

After washing, membranes were incubated with anti-rabbit or anti-mouse IgG antibody conjugated with horseradish peroxidase (Amersham, Little Chalfont, UK) and detected using an enhanced chemiluminescence system (Amersham). Films were scanned and analyzed using NIH image software. All bands were normalized to *β*-actin in treated and untreated groups, and then the ratios of each protein in the treated groups to that in the untreated groups were determined and are shown under each band.

Hollow fiber assay

Hollow fibers were rehydrated, sterilized and loaded with a suitable concentration of cells (1×10^6 cells/ml, 2-cm fibers) as described previously [12]. They were transferred to culture dishes containing complete medium and incubated overnight at 37°C in an atmosphere containing 5% CO_2 prior to implantation in mice. They were implanted subcutaneously on day 1 into the flank of nude mice ($n=4$) using a sterile trocar (two fibers per site per mouse), and 6 days later (day 7), Cl-F-araA dissolved in a 25% PEG 400 aqueous solution was administered orally once a day for five consecutive days (days 7–11). The fibers were recovered from the mice 2 days (day 13) after the last treatment. A colorimetric assay based on XTT was used to determine the numbers of cells in the fibers. The chemosensitivity was expressed in terms of the rate of absorbance of the fibers prior to treatment (day 7). The studies were performed with cells in the linear part of the growth curve. Statistical analyses were carried out using Dunnett's test with the significance level at $P<0.05$.

Combination with caffeine

CCRF-CEM cells were treated with 20 m*M* caffeine 30 min before the addition of Cl-F-araA at doses in the range 0.002–0.2 μ*M*. The cells were treated with both drugs for 15 h and the cleavage of PARP and the level of Cdc 25A were determined by Western blot analysis.

Results

Antileukemic activity of Cl-F-araA against CCRF-CEM cells in vitro and in vivo

Our recent studies suggest that Cl-F-araA has more potent antiproliferative activity against human leukemia cell lines (seven cell lines, log mean 50% growth inhibitory concentration, GI_{50}, 25 n*M*) than against human solid tumor cell lines (15 cell lines, log mean GI_{50} 160 n*M*) in vitro. To further characterize the nature of the antiproliferative activity of Cl-F-araA, we used the T-ALL cell line, CCRF-CEM.

Cl-F-araA showed concentration-dependent antiproliferative activity against CCRF-CEM cells in vitro, with a GI_{50} concentration of 20 n*M* (Fig. 1A). To determine whether Cl-F-araA could exhibit antileukemic activity in vivo, we used the hollow fiber assay established at the National Cancer Institute that is superior to xenograft models [12]. The hollow fibers containing CCRF-CEM cells were implanted into the flank of nude mice, and 6 days later Cl-F-araA (6.3–100 mg/kg per day) was administered orally once a day for five consecutive days. The hollow fibers were recovered from the mice 2 days after the last drug treatment, and viable cell numbers were determined by the XTT assay. As shown in Fig. 1B, Cl-F-araA exhibited dose-dependent antiproliferative activity against CCRF-CEM cells in hollow fibers, with a GI_{50} concentration of 6.3 mg/kg per day. Notably, at high doses (50 and 100 mg/kg per day) of Cl-F-araA, the cell numbers in fibers after Cl-F-araA treatment were decreased as compared to the initial cell numbers (day 7), suggesting that the drug may induce cell death in vivo.

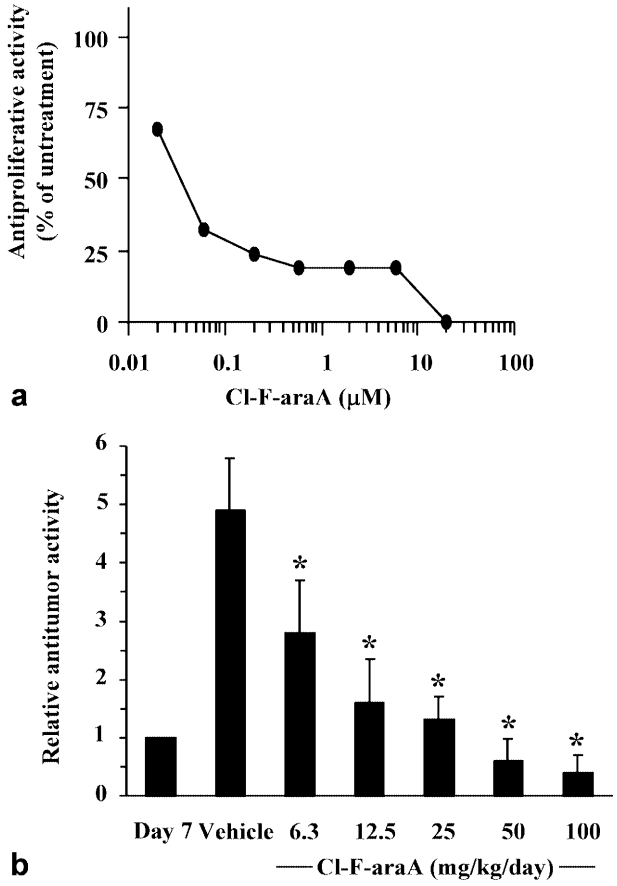
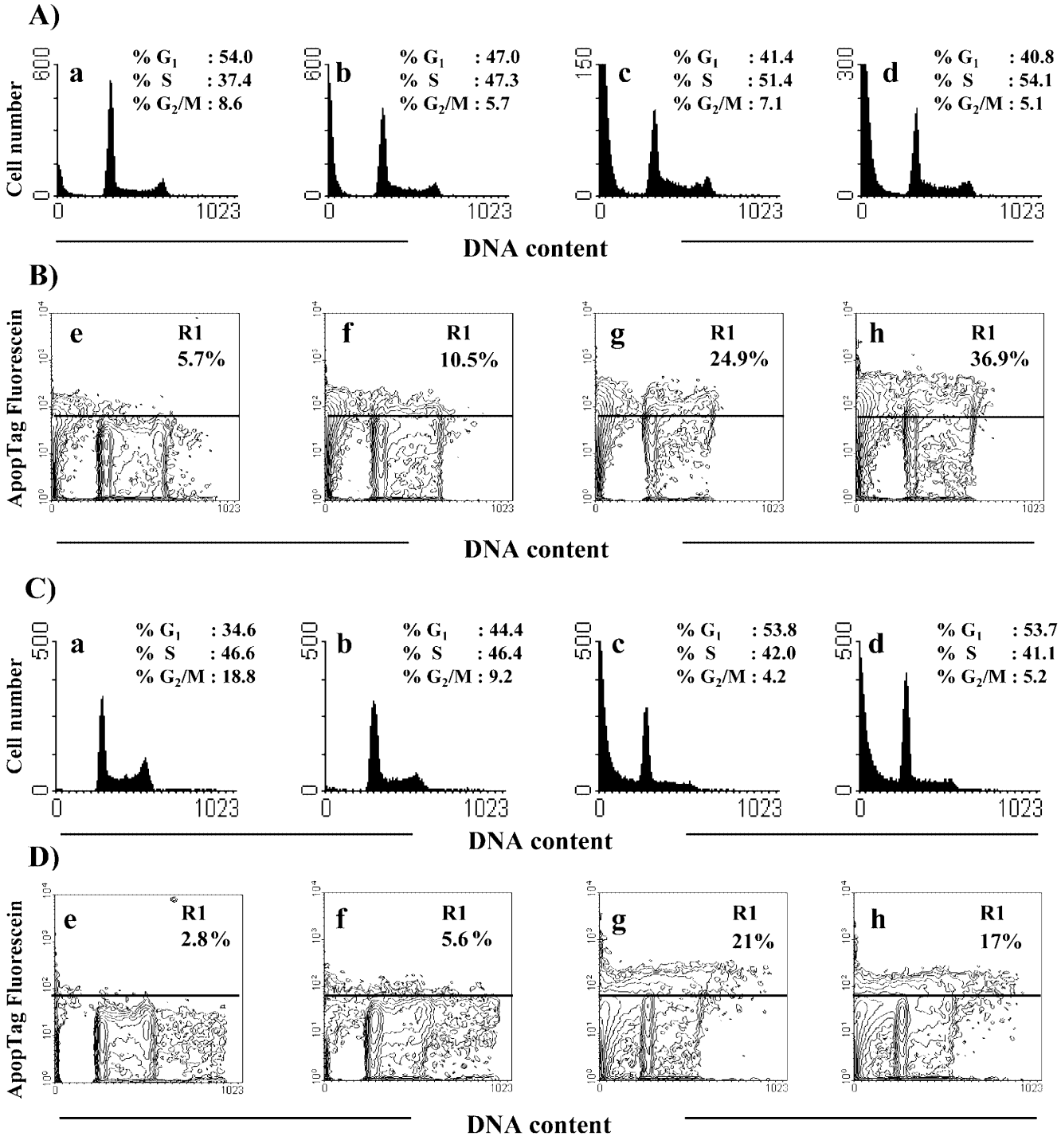


Fig. 1A, B. Antileukemic activity of Cl-F-araA against the human T-ALL cell line, CCRF-CEM. The antiproliferative activity of Cl-F-araA against CCRF-CEM cells was determined in vitro (**A**) and in vivo (**B**). **A** CCRF-CEM cells were treated with Cl-F-araA at doses in the range 0.02–2 μ*M* for 24 h. Cell viability was determined by XTT assay and is presented as a percentage of the viability of untreated cells. **B** Hollow fibers loaded with suitable concentrations of cells were subcutaneously implanted into the flank of nude mice, and 6 days later Cl-F-araA at doses in the range 6.3–100 mg/kg was orally administered once a day for five consecutive days. The fibers were recovered from mice 2 days after the last administration. The cell numbers are presented in relation to the rate of absorbance of the fibers prior to treatment. Asterisks show the significance level at $P<0.05$

Induction of apoptosis by Cl-F-araA

To determine whether Cl-F-araA would actually induce apoptosis in CCRF-CEM cells, we used a TUNEL/PI double-staining method. Cl-F-araA produced a dose-dependent increase in apoptotic cells, as defined by the sizes of the sub-G_1 and TUNEL-positive fraction which were 10.5% at 6.3 mg/kg per day, 24.9% at 12.5 mg/kg per day and 36.9% at 25 mg/kg per day (Fig. 2A, B). Even at 6.3 mg/kg per day, which was the lowest dose tested, a significant proportion of apoptotic cells were observed. At the higher doses of 50 and 100 mg/kg per day, apoptosis induction by Cl-F-araA could not be determined because a sufficient number of cells could not be recovered. Cl-F-araA also induced an increase in



the sub-G_1 peak and TUNEL-positive cells which were 5.6% at 0.02 μ*M*, 21% at 0.2 μ*M* and 17% at 2 μ*M* in vitro (Fig. 2C, D). Interestingly, at over 0.2 μ*M*, a further increase in apoptosis was not observed. At lower concentration (0.02 μ*M*), Cl-F-araA produced an increase in the number of G_1/S phase cells which was supported by a broad peak of G_1 phase cells compared with untreated cells, and a decrease in G_2/M phases (G_2/M 9.2% at 0.02 μ*M* vs 18.8% in untreated cells, Fig. 2C). An obvious increase in early S phase cells which was shown as an increase in the percentage of S phase cells (S phase 47.3% at 6.3 mg/kg per day vs 37.4% in untreated cells) and a decrease in G_2/M phases (G_2/M 5.7% at 6.3 mg/kg per day vs 8.6% in untreated cells) was observed in vivo, suggesting that the drug may activate the S phase checkpoint (Fig. 2A).

Activation of caspases by Cl-F-araA

To analyze the mechanisms of apoptosis induced by Cl-F-araA, we sought to determine whether the drug

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Fig. 2A–D. Induction of apoptosis by Cl-F-araA. Induction of apoptosis by Cl-F-araA in CCRF-CEM cells was investigated in vivo (**A**, **B**) and in vitro (**C**, **D**). Cell cycle distribution (**A**, **C** *x-axis* PI fluorescein, *y-axis* cell numbers) and apoptosis induction (**B**, **D** *x-axis* PI fluorescein, *y-axis* ApopTag fluorescein) were analyzed using a TUNEL/PI double-staining method. The cell cycle distributions (%G_1, %S and %G_2/M) shown in **A** and **C** were calculated using the Multicycle program. Cells in the R1 region in **B** and **D** were defined as apoptotic cells. **A, B** CCRF-CEM cells in hollow fibers were analyzed 2 days after the last administration of 6.3–25 mg/kg of Cl-F-araA (*a, e* vehicle; *b, f* 6.3 mg/kg; *c, g* 12.5 mg/kg; *d, h* 25 mg/kg). **C, D** CCRF-CEM cells were treated with Cl-F-araA at doses in the range 0.02–2 μ*M* for 24 h (*a, e* untreated; *b, f* 0.02 μ*M*; *c, g* 0.2 μ*M*; *d, h* 2 μ*M*)

could activate caspase 3 or caspase 9 which are key molecules for apoptosis induction. Cl-F-araA resulted in a concentration-dependent increase in DEVD hydrolysis activity (Fig. 3A) and a concentration- and time-dependent increase in PARP cleavage (Fig. 3B, C), suggesting that the drug could activate caspase enzymes in the cells. Furthermore, Western blot analysis indicated a drug-dependent disappearance of procaspases 3 and 9, suggesting that the drug could activate caspases 3 and 9 (Fig. 3B). These results are consistent with those of apoptosis induction shown in Fig. 2C, D.

Effect of Cl-F-araA on Bcl-2 family proteins

Bcl-2 family proteins are involved in mediating programmed cell death or apoptosis. Some of the members of this family act as inhibitors of apoptosis, while others promote cell death [6, 22, 23, 29, 34]. The balance between homo- and the heterodimers of bcl-2 family of proteins appears to be critical for the maintenance of survival and cell death [11, 19, 26, 27, 35]. Therefore, we investigated the effect of Cl-F-araA on the expression levels of Bcl-2, Bcl-X_L and Mcl-1 which act as death suppressors, and Bax and Bak which act as proapoptotic proteins. Western blot analysis revealed that a 24-h treatment with Cl-F-araA at concentrations at which both TUNEL-positive cells and cleavage of PARP were detected, resulted in a decrease the levels of Bcl-X_L (40% vs untreated cells at both 0.2 and 2 μ*M*) and Mcl-1 (30% at 0.2 μ*M* and 20% at 2 μ*M* vs untreated cells), whereas the levels of Bcl-2, Bax and Bak were not affected (Fig. 4A). In addition, Bcl-X_L and Mcl-1 were slightly decreased even as early as 4 h after Cl-F-araA treatment (Bcl-X_L 70% at 2 μ*M* and 60% at 20 μ*M*, Mcl-1 50% at both 2 and 20 μ*M*; Fig. 4B).

These results suggest that downregulation of Bcl-X_L and/or Mcl-1 proteins of the Bcl-2 family by Cl-F-araA might be the cause of apoptosis induction.

Inhibition of Akt activation by Cl-F-araA

Recently, many studies have demonstrated that Akt regulates cell survival through phosphorylation of downstream effectors such as Bad, FKHR and procaspase 9 proteins [1, 2, 7]. Previous studies have also shown that apoptosis induced by the PI3-kinase inhibitors can be attributed to the inhibition of Akt activation [24]. We therefore sought to determine whether Cl-F-araA might affect the phosphorylation level of the antiapoptotic kinase, Akt protein. Western blot analysis revealed that a 24-h treatment with Cl-F-araA resulted in a dose-

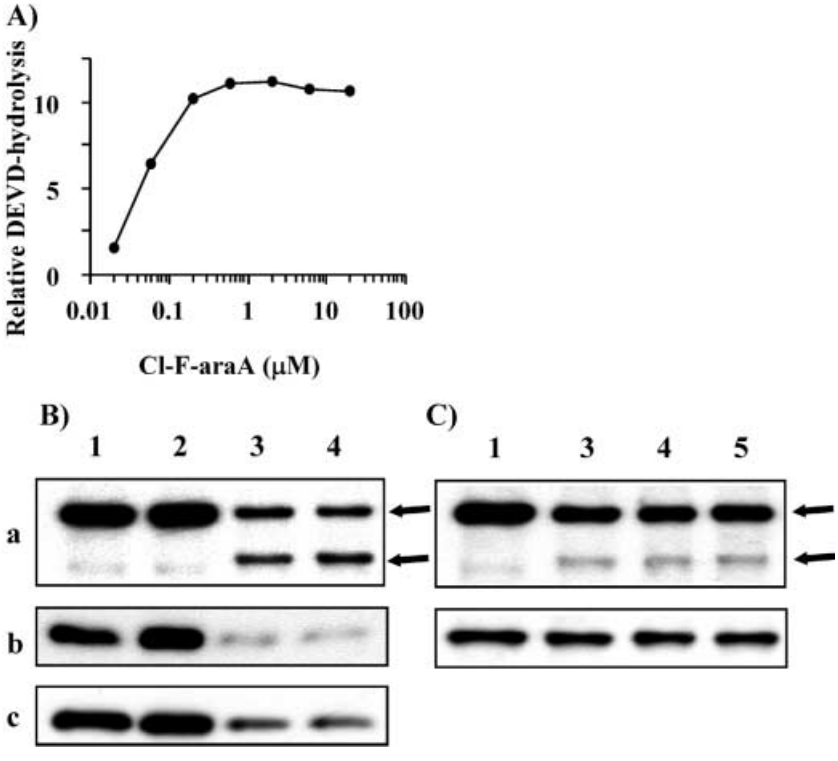

Fig. 3A, B. Activation of caspases by Cl-F-araA. **A** CCRF-CEM cells were incubated with Cl-F-araA at doses in the range 0.02–20 μ*M* for 24 h and DEVD-specific caspase activity was determined by cleavage of DEVD-AMC used as a substrate. **B, C** CCRF-CEM cells treated with 0.02–20 μ*M* Cl-F-araA for 24 h (**B**) or 4 h (**C**) were solubilized and subjected to Western blot analysis probed with anti-PARP (*a*), anti-procaspase 9 (*b*), anti-procaspase 3 (*c*) antibodies followed by anti-IgG antibody conjugated with horseradish peroxidase. The *two arrows* in **B** and **C** indicate uncleaved PARP (*upper arrows*) and cleaved PARP (*lower arrows*) (*lane 1* untreated, *lane 2* 0.02 μ*M*, *lane 3* 0.2 μ*M*, *lane 4* 2 μ*M*, *lane 5* 20 μ*M*)

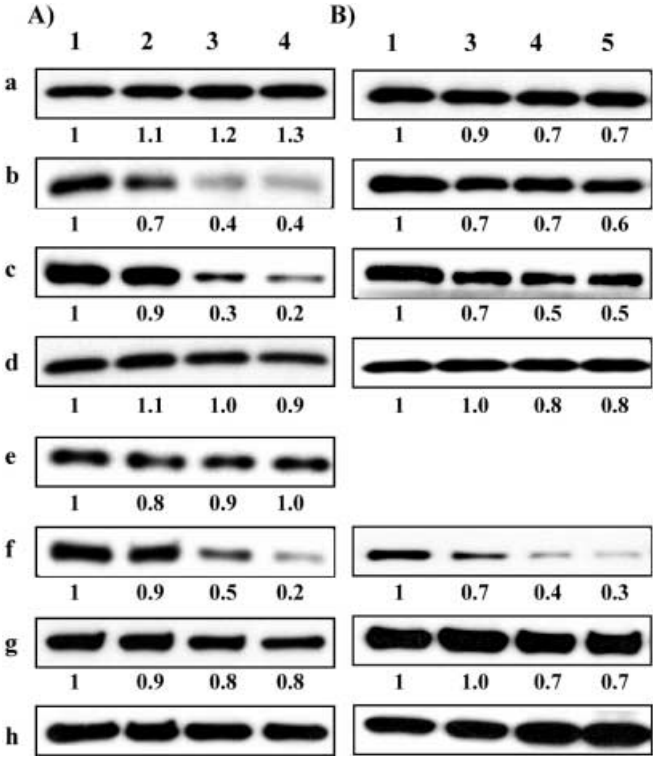

Fig. 4A, B. Effect of Cl-F-araA on Bcl-2 family and Akt proteins. CCRF-CEM cells were treated with 0.02–20 μ*M* Cl-F-araA for 24 h (**A**) or 4 h (**B**) and subjected to Western blot analysis probed with anti-Bcl-2 (*a*), anti-Bcl-X_L (*b*), anti-Mcl-1 (*c*), anti-Bax (*d*), anti-Bak (*e*), anti-^{473}Ser-Akt (*f*), anti-Akt (*g*) and anti-β-actin (*h*) antibodies followed by anti-IgG antibody conjugated with horseradish peroxidase. All bands were analyzed using NIH Image software and normalized to β-actin in the treated and untreated groups, and the ratio of each protein in the treated group to the untreated group was determined and is shown under each band (*lane 1* untreated, *lane 2* 0.02 μM, *lane 3* 0.2 μ*M*, *lane 4* 2 μ*M*, *lane 5* 20 μ*M*)

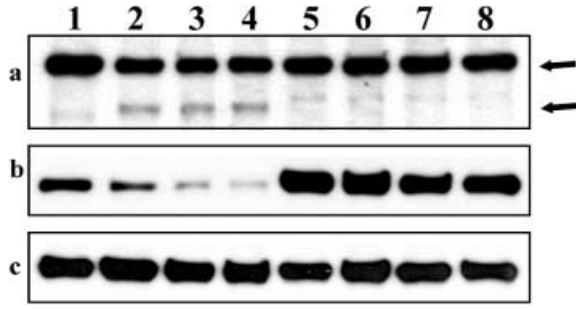

Fig. 5. Effect of okadaic acid on Cl-F-araA-induced cleavage of PARP. CCRF-CEM cells treated with 0.2–20 μ*M* Cl-F-araA for 4 h without (*lanes 1–4*) or with 0.5 μ*M* okadaic acid (*lanes 5–8*) were solubilized and subjected to Western blot analysis probed by anti-PARP (*a*), anti-^{473}Ser-Akt (*b*) and anti-Akt (*c*) antibodies followed by anti-IgG antibody conjugated with horseradish peroxidase. The *two arrows* indicate uncleaved (*upper arrow*) and cleaved (*lower arrow*) PARP (*lanes 1 and 5* untreated, *lanes 2 and 6* 0.2 μ*M*, *lanes 3 and 7* 2 μ*M*, *lanes 4 and 8* 20 μ*M*)

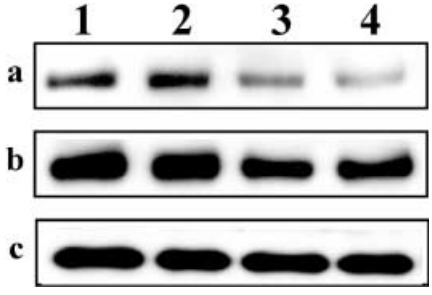

Fig. 6. Effect of Cl-F-araA on downstream effectors of Akt signals. Cells treated with 0.2–20 μ*M* Cl-F-araA for 4 h were solubilized and subjected to Western blot analysis probed by anti-^{32}Thr-FKHRL1 (*a*), anti-FKHRL1 (*b*) and anti-$p27^{Kip1}$ (*c*) antibodies followed by anti-IgG antibody conjugated with horseradish peroxidase (*lane 1* untreated, *lane 2* 0.2 μ*M*, *lane 3* 2 μ*M*, *lane 4* 20 μ*M*)

dependent decrease in the level of phospho-Akt (^{473}Ser-Akt) protein (90% at 0.02 μ*M*, 50% at 0.2 μ*M* and 20% at 2 μ*M* vs untreated cells), whereas Akt protein itself showed no significant change (Fig. 4A). In addition, we determined the time-dependent decrease in phospho-Akt protein. The dephosphorylation of Akt protein began at 0.5 h after treatment with 2 μ*M* Cl-F-araA (data not shown) and was virtually completed by 4 h (40% at 2 μ*M* and 30% at 20 μ*M*; Fig. 4B).

Effect of Cl-F-araA on downstream effectors of Akt signals

Phosphorylation of Akt has been shown to be regulated by a balance between phosphorylation by phosphoinositide-dependent kinases and dephosphorylation by protein phosphatase 2A [4, 16, 24, 30]. Therefore, we examined the effects of okadaic acid, a potent protein phosphatase 2A inhibitor. When added together with 0.2–20 μ*M* Cl-F-araA for 4 h, 0.5 μ*M* okadaic acid rescued the CCRF-CEM cells from Cl-F-araA-induced cleavage of PARP, with consequent hyperphosphorylation of Akt (Fig. 5, lane 2 versus 6, lane 3 versus 7 and lane 4 versus 8). To determine whether apoptosis induced by Cl-F-araA might be due to the inhibition of activation of Akt kinase, we investigated the effect of the drug on downstream effectors of the Akt signaling pathway. Western blot analysis indicated that a 4-h treatment with Cl-F-araA resulted in a dose-dependent decrease in phosphorylation of FKHRL1 (^{32}Thr-FKHRL1) protein, suggesting that Cl-F-araA inhibited the activity of Akt kinase (Fig. 6). However, the level of $p27^{Kip1}$ whose expression is partially regulated by FKHRL1 protein remained unchanged (Fig. 6). Phospho-Bad (^{136}Ser-Bad) protein, which is a key component linking survival signaling to the function of Bcl-X_L [7] could not be detected.

Decrease in phosphorylation of Bad protein in vivo

We sought to determine whether Akt signals could contribute to the survival of CCRF-CEM cells in vivo.

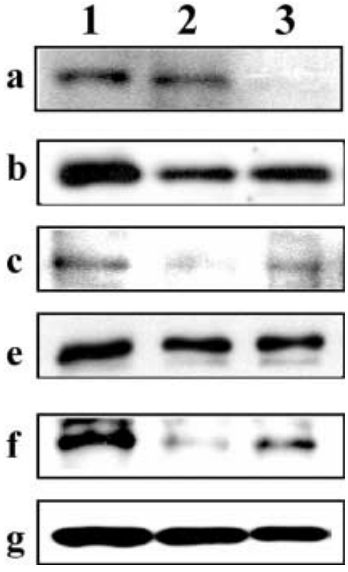

Fig. 7. Effect of Cl-F-araA on Bcl-2 family proteins and downstream effectors of Akt in vivo. CCRF-CEM cells in hollow fibers as presented in Fig. 1B were solubilized and subjected to Western blot analysis probed by anti-^{136}Ser-Bad (*a*), anti-Bad (*b*), anti-^{32}Thr-FKHRL1 (*c*), anti-FKHRL1 (*e*), anti-Mcl-1 (*f*) and anti-β-actin (*g*) antibodies followed by anti-IgG antibody conjugated with horseradish peroxidase (*lane 1* vehicle, *lane 2* 6.3 mg/kg, *lane 3* 12.5 mg/kg)

Upon treatment with Cl-F-araA, the level of ^{32}Thr-FKHRL1 protein decreased in a manner similar to that observed in vitro, whereas the level of FKHRL1 protein was unchanged (Fig. 7c, e). Unlike the situation in vitro, ^{136}Ser-Bad was detected in untreated cells in vivo. Its levels showed a marked decrease at 12.5 mg/kg of Cl-F-araA although the level of Bad protein was also slightly decreased (Fig. 7a, b). A decrease in MCl-1 protein was also observed in vivo (Fig. 7f).

Downregulation of Cdc25A protein

Cl-F-araA resulted in an increase in G_1/S phase and early S phase cells, which was accompanied by a decrease in G_2/M phase cells, suggesting that the drug may activate the S phase checkpoint (Fig. 2A, C). Activation of the S phase checkpoint allows cells to cease proliferating in order to provide time for DNA repair. Mailand et al. have demonstrated that activation of the S phase checkpoint involves activation of Chk1 protein kinase followed by rapid degradation of Cdc 25A which is required for progression from G_1 to S phase [15]. Therefore, we determined the changes in Cdc25A protein to characterize the accumulation of G_1/S and early S phase cells. Western blot analysis revealed that a 4-h treatment with 0.2 μM Cl-F-araA completely depleted Cdc25A protein, whereas Cdc25C protein remained unaffected, suggesting that Cl-F-araA could increase the population of early S phase cells by inactivation of Cdk2 via Cdc25A downregulation (Fig. 8A).

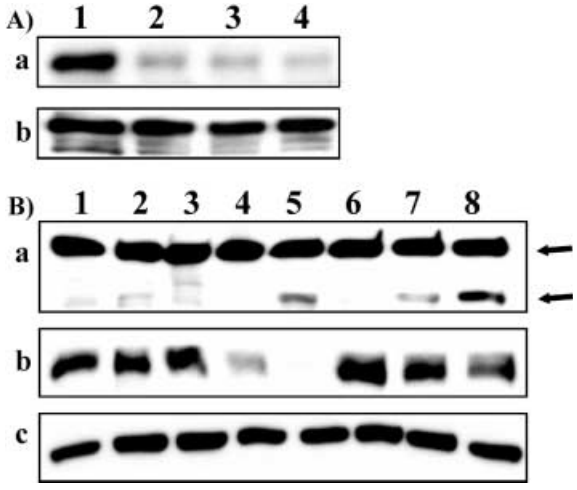

Fig. 8A, B. Potentiation of Cl-F-araA-induced apoptosis by caffeine. **A** Cells treated with 0.2–20 μM Cl-F-araA for 4 h were solubilized and subjected to Western blot analysis probed by anti-Cdc25A (*a*) and anti-Cdc25C (*b*) antibodies followed by anti-IgG antibody conjugated with horseradish peroxidase (*lane 1* untreated, *lane 2* 0.2 μM, *lane 3* 2 μM, *lane 4* 20 μM). **B** Cells were treated with 0.002–0.2 μM Cl-F-araA with (*lanes 6, 7 and 8*) or without (*lanes 3, 4 and 5*) 20 mM caffeine for 15 h and subjected to Western blot analysis probed by anti-PARP (*a*), anti-Cdc25A (*b*) and anti-β-actin (*c*) antibodies followed by anti-IgG antibody conjugated with horseradish. The *two arrows* indicate uncleaved (*upper arrow*) and cleaved (*lower arrow*) PARP (*lane 1* untreated, *lane 2* caffeine alone, *lanes 3 and 6* 0.002 μM, *lanes 4 and 7* 0.02 μM, *lanes 5 and 8* 0.2 μM)

Potentiation of Cl-F-araA-induced apoptosis by caffeine

We further investigated the effects of the combination of Cl-F-araA with caffeine in terms of inhibition of activated Chk1. A high concentration of caffeine (20 mM) was used as an inhibitor of activated Chk1 and was added 30 min before Cl-F-araA. The levels of Cdc 25A and cleavage of PARP were determined 15 h after the addition of Cl-F-araA at doses in the range 0.002–0.2 μM in combination with caffeine (Fig. 8B). Similar to the findings following a 4-h treatment with Cl-F-araA alone, downregulation of Cdc25A protein was observed in the absence of caffeine (Fig. 8B, lanes 3–5). As expected, the combination of Cl-F-araA with caffeine inhibited the downregulation of Cdc25A and increased the cleavage of PARP at 0.02 and 0.2 μM Cl-F-araA (Fig. 8B, lanes 7 and 8).

Discussion

Cl-F-araA has more potent antitumor activity against human leukemia cell lines than against human solid tumor cell lines in vitro. Therefore, we determined the susceptibility of a T-ALL cell line, CCRF-CEM, as a human leukemia model system in vivo. Our previous study had demonstrated that the maximum tolerated dose (MTD) of Cl-F-araA in nude mice in a 5-day daily

oral schedule is 100 mg/kg per day [28]. In this study using the hollow fiber model, more than 6.3 mg/kg of Cl-F-araA, which corresponds to 1/16th of the MTD, showed antileukemic activity resulting in a growth rate of CCRF-CEM cells less than half that following vehicle treatment. Therefore, CCRF-CEM cells were more sensitive than colon tumors which show the highest susceptibility (the lowest effective dose 25 mg/kg per day) among the solid tumors previously tested [28], We have also found that Cl-F-araA is highly selective against the human B-cell lymphoma cell line, Daudi, in the hollow fiber model (data not shown). In addition, 100 mg/kg of Cl-F-araA is effective in severe combined immunodeficiency (SCID) mice intravenously implanted with Daudi cells that develop disseminated lymphoma clinically resembling Burkett's lymphoma. Carson et al. have also demonstrated that Cl-F-araA eliminates 90% of CLL cells transplanted intraperitoneally into SCID mice [3]. Taken together, these results suggest that lymphoma may be highly sensitive to Cl-F-araA.

In addition, Carson et al. have demonstrated that Cl-F-araA induces apoptosis in lymphoma [3]. Recently, part of the mechanism has been identified by Genini et al. [8]. Cl-F-araA directly disrupts the integrity of the mitochondria resulting in the release of the proapoptotic mitochondrial protein, cytochrome *c*, an apoptosis-inducing factor in vitro. Of particular interest is the difference in the site of action between Cl-F-araA and fludarabine [8]. Therefore, we examined the level of Bcl-2 family proteins which are involved in mediating cell death or apoptosis. A 24-h treatment with Cl-F-araA at concentrations able to induce apoptosis resulted in decreases in the levels of both antiapoptotic proteins Bcl-X_L and Mcl-1. Both proteins were also slightly decreased even after a 4-h treatment. The ability of Cl-F-araA to induce apoptosis in CCRF-CEM cells was correlated with the decrease in both these antiapoptotic proteins. Importantly, Xie et al. have demonstrated a strong inverse correlation between cell survival and Cl-F-ara-AMP (Cl-F-araA monophosphate) incorporation into DNA of CCRF-CEM cells [32]. The incorporation of Cl-F-araAMP into DNA was markedly increased at concentrations between 0.01 μM and 0.3 μM. These results are consistent with our findings indicating a marked increase in apoptosis at 0.02 μM and 0.2 μM, and no further increase in apoptosis at concentrations above 0.2 μM. Therefore the decrease in both antiapoptosis proteins, Bcl-X_L and Mcl-1, may be a consequence of the incorporation of Cl-F-araAMP into DNA.

We further examined the inhibition of Akt, which acts as a key molecule in cell survival by mediating the phosphorylation of downstream effectors such as Bad, FKHR and procaspase 9 [1, 2, 7]. Cl-F-araA produced a dose- and time-dependent decrease in ^{473}Ser-Akt. Okadaic acid, a potent protein phosphatase 2A inhibitor, rescued Cl-F-araA-induced cleavage of PARP, by hyperphosphorylation of Akt. Cl-F-araA treatment resulted in a dose-dependent decrease in phospho-FKHRL1, but the level of $p27^{kip1}$ remained unchanged. Akt activation may contribute to survival of CCRF-CEM cells. However, the decreased phosphorylation of Bad and another substrate of Akt, ^{308}Thr-Akt, were not observed in untreated cells. Full activation of Akt needs both phosphorylations [4]. Therefore, it is not clear to what extent Akt activation contributes to the survival of CCRF-CEM cells in vitro. Interestingly, the level of ^{136}Ser-Bad decreased in a dose-dependent manner in vivo, suggesting that inhibition of Akt activation may play an important role in cell death induced by Cl-F-araA in vivo. In addition, a dose-dependent increase in apoptosis up to the highest dose investigated was observed in vivo, whereas no further increase was observed at 2 μM in vitro as shown in Fig. 2. These findings indicate that Cl-F-araA may be an effective drug in vivo. The mechanism involved in the inhibition of Akt signaling by Cl-F-araA metabolites remains to be determined.

Treatment with Cl-F-araA resulted in an increased population of G_1/S and early S phase cells. Recently, Mailand et al. have demonstrated that the ubiquitin- and proteasome-dependent protein degradation of Cdc25A, which is required for progression from G_1 to S phase of the cell cycle, involves activated Chk1 protein kinase, but not the p53 pathway [15]. As Western blot analysis demonstrated, Cl-F-araA produced a marked decrease in Cdc25A protein in CCRF-CEM cells which harbor a p53 mutation [5]. Similar results have also been observed in Daudi cells (data not shown). Interestingly, this downregulation of Cdc25A occurred at concentrations that did not induce apoptosis. If chk1 kinase can be selectively inhibited, as suggested by the combination with caffeine, a non-selective inhibitor, a more potent effect than that shown in Fig. 8B might be produced.

Taken together, Cl-F-araA exhibited potent antitumor activity in vitro and in vivo that may involve induction of apoptosis. Such apoptosis would be partly dependent upon the downregulation of the Bcl-2 family proteins Bcl-X_L and Mcl-1 and the dephosphorylation of Akt, particularly in vivo. In addition, a combinatorial effect with chk1 inhibitors could be expected.

Acknowledgements We thank Dr. Sreenath Sharma (Tokyo Laboratory, Kyowa Hakko Kogyo, Japan) for helpful comments. We also thank Ms. Kumiko Masunaga and Ms. Miyoko Suzuki for expert technical assistance.

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