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APHIDICOLIN POTENTIATES APOPTOSIS INDUCED BY ARABINOSYL NUCLEOSIDES IN HUMAN MYELOID LEUKEMIA CELL LINES

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Abstract—We investigated the effect of aphidicolin, an inhibitor of DNA polymerase α and δ, on the induction of apoptosis by arabinosyl nucleosides in a human promyelocytic leukemia cell line, HL-60. Pretreatment of HL-60 cells with aphidicolin (2 μM) significantly increased the number of morphologically apoptotic cells induced by 1-β-D arabinofuranosylcytosine (ara-C) during 4 hr of incubation. This is consistent with the appearance of DNA fragmentation as determined quantitatively by diphenylamine or by agarose gel electrophoresis. The inhibition of cell growth on day 3 after drug exposure was correlated with the degree of apoptosis. Such synergistic interaction between aphidicolin and ara-C has also been observed in other human myeloid leukemia cell lines, U937 and KG-1. In addition, the induction of apoptosis by 9-β-D arabinofuranosyladenine or 9-β-D arabinofuranosylguanine is augmented by aphidicolin.

Arabinosyl nucleosides have a role in chemotherapy because of their anticancer or antiviral activity. One of these, 1-β-D arabinofuranosylcytosine (ara-C†), is used widely for the treatment of acute leukemia [1, 2]. Another drug, 9-β-D arabinofuranosyladenine (ara-A) has antiviral activity [3], although destruction by adenosine deaminase limits its clinical applications. Therefore, the synthesis of a monophosphate form, ara-AMP, has been considered for clinical use [3]. Finally, several investigators have reported the usefulness of 9-β-D arabinofuranosylguanine (ara-G) against T cell lymphoproliferative disorders [4, 5].

These nucleosides must be phosphorylated to their triphosphates with deoxycytidine kinase, adenosine kinase or deoxyguanosine kinase to exert their toxicity [6–8]. Ara-CTP and ara-ATP are known to work as inhibitors of DNA polymerase α or to be incorporated into elongating DNA strands [9, 10], thus inhibiting DNA synthesis. However, the reason why the inhibition of DNA synthesis caused by these analogues results in cell death is not yet fully understood. Apoptosis has recently been recognized as a mode of cell death, especially under physiological conditions [11]. Several anticancer agents including glucocorticoid [12], methotrexate [13], hydroxyurea [14] and inhibitors of topoisomerases [15, 16] can induce apoptosis. Ara-C is one of these agents [17], although, again, the precise mechanism responsible for the apoptosis remains unclear.

In an effort to clarify the mechanism by which ara-C induces apoptosis, we examined the effect of aphidicolin, which has been reported to inhibit the incorporation of ara-C into DNA [18]. It was found that aphidicolin enhanced, rather than inhibited, apoptosis induced not only by ara-C but also by ara-A and by ara-G, as determined morphologically and by the degree of DNA fragmentation. Our data indicate the possibility that arabinosyl nucleoside analogues induce apoptosis without being incorporated into DNA, at least in human myeloid leukemia cell lines.

MATERIALS AND METHODS

Materials. All chemicals except ara-G (Calbiochem, La Jolla, CA, U.S.A.) and proteinase K (Merck, Darmstadt, Germany) were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). [5-^{3}H]Ara-C (33 Ci/mmol) came from Amersham Radiochemicals (Tokyo, Japan).

Cell lines. A human promyelocytic leukemia cell line, HL-60, and myeloid leukemia cell lines, U937 and KG-1, were obtained from the Japanese Cancer Resources Bank (Tokyo, Japan). Cells were cultivated in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, L-glutamine (2 mM), penicillin (100 U/mL) and streptomycin (100 μg/mL). The cultures were maintained at 37° in humidified atmosphere of 95% air and 5% CO_2.

Experimental design. Cells in their late logarithmic phase were diluted to 3×10^5/mL with fresh medium, and first incubated with aphidicolin (2 μM) for 1 hr. Then an arabinosyl nucleoside at the indicated concentration was added to the culture without washings. After further incubation for 4 hr, cells were stained by the May–Giemsa method, and more than 500 cells were examined by light microscopy to determine the percentage of apoptotic cells. The remaining cells were washed twice with phosphate-

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† Abbreviations: ara-C, 1-β-D arabinofuranosylcytosine; ara-A, 9-β-D arabinofuranosyladenine; ara-G, 9-β-D arabinofuranosylguanine; PBS, phosphate-buffered saline.

buffered saline (PBS), pH 7.4 and resuspended in fresh medium at a concentration of 2×10^5/mL. Three days later, viable cells were enumerated by the Trypan blue dye exclusion test. Cell growth was expressed as a percentage of the number of cells in untreated control cultures. In another set of experiments, drug-treated cells were used for further analysis described below after two washings with PBS.

Quantitation of DNA fragmentation. Cells (5×10^6) were lysed by the addition of 1 mL of ice-cold buffer containing 5 mM Tris–HCl, 1 mM EDTA and 0.5% Triton X-100, pH 8.0, before centrifugation for 20 min at 27,000 *g*. The DNA count in the supernatant (DNA fragments) and pellets (intact chromatin) was quantified with the use of diphenlyamine reagent [19]. DNA fragmentation was expressed as the percentage of total DNA in each specimen that resisted sedimentation at 27,000 *g*.

DNA purification and agarose gel electrophoresis. Cells were first incubated in the lysis buffer (150 mM NaCl, 25 mM EDTA, 100 μg/mL proteinase K and 0.2% sodium dodecyl sulfate) at 60° overnight. Then, the DNA was extracted twice with phenol/chloroform (1:1) and once with chloroform, and precipitated with 66% ethanol in the presence of 0.1 M CH_3COONa. The resultant fraction was treated with RNase (50 μg/mL) for 1 hr at 37°, followed by the same extraction and precipitation procedures. The DNA was dissolved in 10 mM Tris, 1 mM EDTA, pH 8.0, and approximately 10 μg DNA was placed on 1.8% agarose gel containing 0.5 μg/mL ethidium bromide. Electrophoresis was carried out at 1 V/cm for 10 hr, and photographs were taken under UV light [20].

Incorporation of [5-^{3}H]ara-C into DNA. Cells (10^6) were exposed to [5-^{3}H]ara-C (2 μg/mL) adjusted to final concentrations of 0.08 to 10 μM with an unlabeled drug. After two washings, their DNA was extracted by the method described above. The DNA was filtered on Whatman GF/C filters, and the radioactivity was measured [21].

Ara-CTP generation. After drug exposure and two washings with ice-cold PBS, cells were extracted with 0.4 N perchloric acid, followed by neutralization with 2 M KOH. Ara-CTP was separated and quantified by HPLC on a Partisil 10-SAX anion exchange column eluted with 0.5 M KCl/0.25 M KH_2PO_4, pH 3.0 at a flow rate of 1.5 mL/min [22].

RESULTS

Inhibition of [^{3}H]ara-C incorporation into DNA by aphidicolin

We first evaluated the effect of aphidicolin on the incorporation of various concentrations of [^{3}H]ara-C into DNA in HL-60 cells. The concentration of 2 μM for aphidicolin was chosen, since our preliminary experiments showed that this was the lowest concentration which inhibited [^{3}H]thymidine incorporation into DNA by more than 95% (data not shown). As shown in Fig. 1, aphidicolin significantly decreased ara-C incorporation, especially at lower concentrations; namely, the inhibition was 85% at 0.08 μM versus 60% at 10 μM. The decreasing inhibitory effect of aphidicolin on incorporation with increasing concentrations of ara-C seen in this study is consistent with the results of an earlier observation [18].

Augmentation of ara-C-induced apoptosis by aphidicolin

The number of HL-60 cells exhibiting morphological changes characteristic of apoptosis increased following incubation with ara-C for 4 hr in a dose-dependent manner (Table 1). These cells were readily determined by staining the cells with May–Giemsa as nuclear condensation and apoptotic

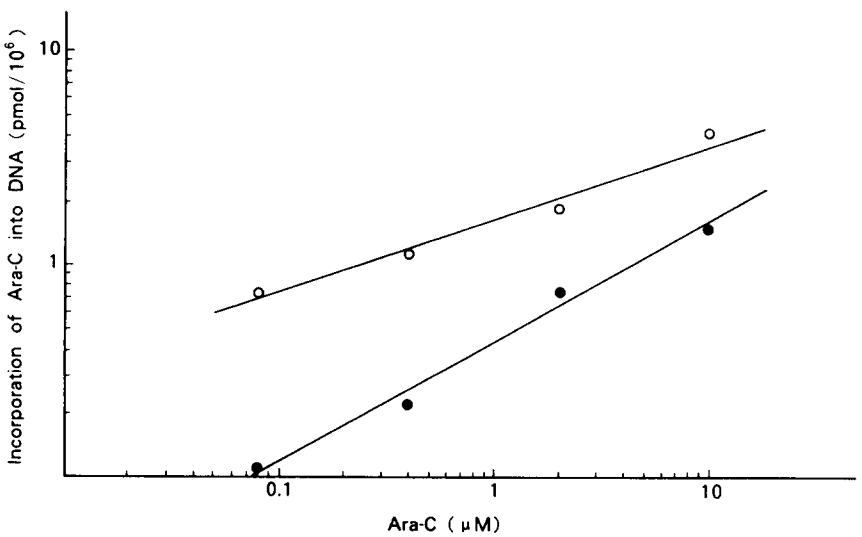

Fig. 1. Incorporation of [^{3}H]ara-C into DNA. HL-60 cells were incubated with (●) or without (○) aphidicolin (2 μM) for 1 hr, followed by further incubation with varying concentrations of [^{3}H]ara-C for 4 hr. The extent of ara-C incorporation into DNA was measured as described. The data represent the means of four experiments.

Table 1. Effect of aphidicolin on ara-C-induced DNA fragmentation and cytotoxicity in HL-60 cells

Ara-C (μM)	Aphidicolin	Apoptotic cells (%)	DNA fragmentation (%)	Cell growth (%)
None	(−)	3.1 ± 0.7 } *	8.7 ± 1.6 } †	
	(+)	5.6 ± 1.4 }	12.1 ± 4.7 }	91.7 ± 4.3
0.08	(−)	3.8 ± 1.5 } *	12.4 ± 0.8 } *	95.1 ± 0.5 } *
	(+)	27.1 ± 5.7 }	44.0 ± 4.8 }	53.8 ± 3.5 }
0.4	(−)	9.5 ± 1.2 } *	23.1 ± 3.3 } *	77.1 ± 6.7 } *
	(+)	44.7 ± 0.6 }	53.0 ± 3.6 }	45.9 ± 6.3 }
2.0	(−)	40.8 ± 1.1 } *	51.9 ± 4.6 } ‡	37.2 ± 5.4 } ‡
	(+)	62.5 ± 7.4 }	59.0 ± 4.6 }	33.3 ± 2.4 }
10.0	(−)	51.5 ± 2.5 } *	57.6 ± 4.0 } ‡	34.2 ± 2.7 } ‡
	(+)	60.4 ± 3.9 }	61.0 ± 9.0 }	31.0 ± 4.3 }

The data represent the means ± SD of more than four determinations.
The differences between the two groups are: *$P < 0.001$, †$P < 0.01$, ‡$P < 0.05$ by Student's *t*-test.

bodies (Fig. 2). DNA fragmentation, a biochemical hallmark of apoptosis, increased progressively (Table 1). Pretreatment of cells with aphidicolin apparently enhanced induction of apoptosis at 0.08 or 0.4 μM of ara-C. However, such an increase was not detectable at higher concentrations, since ara-C alone could induce substantial apoptosis.

Agarose gel electrophoresis

Agarose gel electrophoresis was performed to characterize further the pattern of DNA cleavage. DNA from control cells did not show any detectable level of DNA fragmentation. Treatment with ara-C at 0.4 μM for 4 hr revealed marginal DNA fragments at multiples of approximately 200 bp. This pattern became more prominent with increasing concentrations of ara-C (Fig. 3). In contrast, in the presence of aphidicolin, the cells revealed a distinct DNA ladder pattern even at the lowest concentration (0.08 μM) tested in the present study. Either aphidicolin (2 μM) or ara-C (0.08 μM) alone showed detectable DNA fragmentation at 24 hr of incubation (data not shown).

Effect of aphidicolin on ara-CTP production

Several agents, such as hydroxyurea or thymidine [22, 23], have been reported to show synergism with ara-C through enhanced synthesis of ara-CTP. This prompted us to determine whether aphidicolin could increase the synthesis of ara-CTP in HL-60 cells. As can be seen in Fig. 4, aphidicolin augmented ara-CTP production significantly at every concentration of ara-C ($P < 0.01$). However, augmentation of ara-CTP is not sufficient to explain the enhanced induction of apoptosis by aphidicolin. For example, cells treated with 0.4 μM ara-C alone generated more ara-CTP (17.9 ± 1.0 pmol/10^6 cells) than did those treated with 0.08 μM ara-C plus aphidicolin (9.4 ± 0.5 pmol/10^6 cells) (Fig. 4). Nonetheless, apoptosis was much more prominent in the latter cells (Table 1).

Enhancement by aphidicolin of ara-C-induced apoptosis in other human myeloid cell lines

To determine whether the observed synergism between aphidicolin and ara-C in terms of apoptosis induction is specific to HL-60 cells, we carried out the same experiments in two other cell lines of myeloid origin, U937 and KG-1. Table 2 demonstrates that such enhancement also occurred in both U937 and KG-1, although the magnitude of the increase and the dose–response were somewhat different. Again, the study by agarose gel electrophoresis was consistent with the DNA fragmentation assay (Fig. 5). Therefore, the enhancement by aphidicolin of ara-C-induced apoptosis is a rather common phenomenon, at least in human myeloid leukemia cell lines.

Effect of aphidicolin on ara-A- or ara-G-induced apoptosis in HL-60 cells

Finally, we evaluated the effect of aphidicolin on ara-A- or ara-G-induced apoptosis. As illustrated in Table 3, ara-A alone induced apoptosis when co-incubated with an inhibitor of adenosine deaminase, erythro-9-(2-hydroxy-3-nonyl) adenine (20 μM). Aphidicolin augmented ara-A-induced apoptosis in a similar fashion to ara-C-induced apoptosis. On the other hand, ara-G had a marginal effect on the induction of apoptosis as a single agent. This was presumably due to the low phosphorylation of this compound to ara-GTP in HL-60 cells (data not shown). Notably, the addition of aphidicolin had a significant effect on the generation of apoptosis even at 10 μM (Table 4).

DISCUSSION

Ara-C is an active agent in the treatment of acute myelogenous leukemia [1, 2]. Although ara-C is effective as a single agent, it is usually used in combination with other antileukemic drugs. In general, the mechanism by which these antileukemic drugs potentiate ara-C toxicity has been attributed to an enhanced generation of ara-CTP, an active metabolite of ara-C [24]. As the biochemical basis for the increase of ara-CTP synthesis, the following hypotheses have been postulated: (i) decreased dCTP pools [25, 26], (ii) increased TTP pools [27], (iii) decreased production of deoxycytidine [22], (iv) decreased deamination of ara-C [28], (v) block of ara-C efflux [29]. Different mechanisms, such as the promotion of single- or double-stranded DNA breaks [30] or an increase in the S-phase fraction [31], have

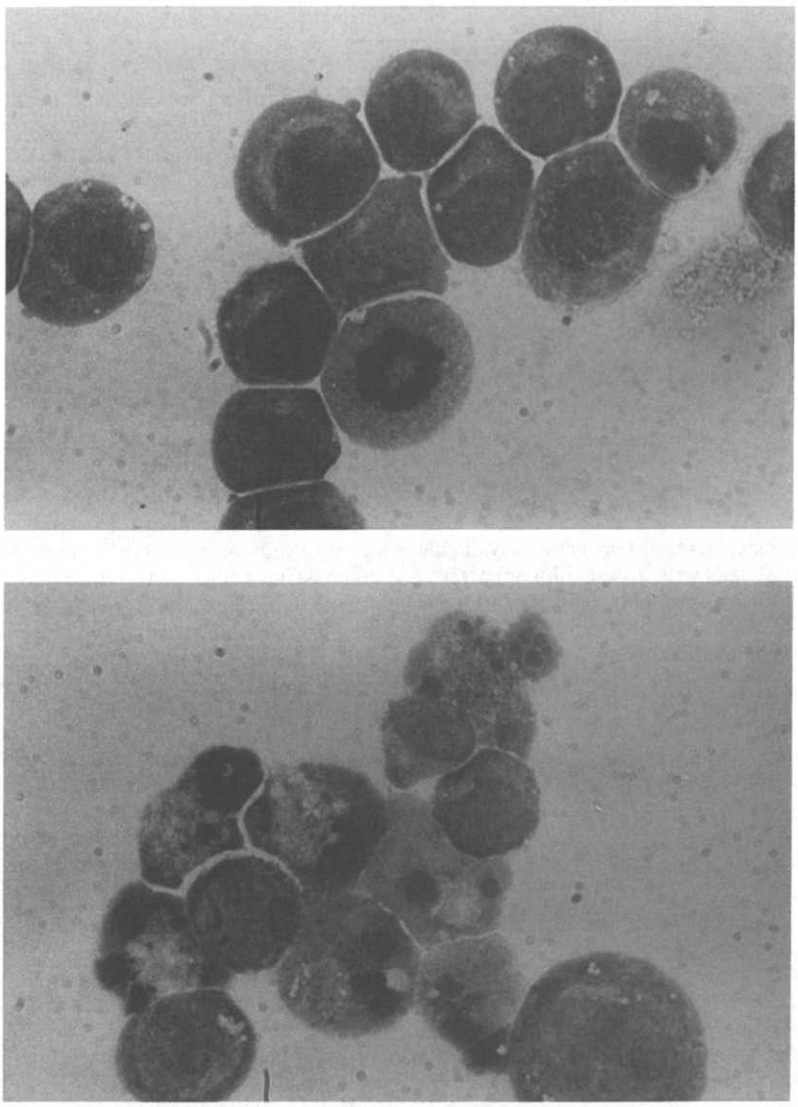

Fig. 2. May–Giemsa stained preparations of HL-60 cells. (A) Control HL-60 cells; (B) HL-60 cells treated with aphidicolin (2 μM) and ara-C (10 μM), showing the apoptotic morphology of chromatin condensation and the formation of apoptotic bodies (magnification $\times$1000).

also been suggested. The dose, schedule or sequence of the drugs is important to achieve greater cytotoxicity. Otherwise the same drugs in combination may work antagonistically [26, 29].

Aphidicolin is a potent competitive inhibitor of DNA polymerase α and δ [32]. Treatment of cells with aphidicolin would be expected to decrease the incorporation of ara-C into DNA. In fact, we and Kufe *et al.* [18] have shown that aphidicolin inhibits ara-C incorporation, the magnitude of inhibition depending on a function of ara-C concentration. In consequence, aphidicolin treatment abrogated ara-C cytotoxicity in a mouse lymphoid cell line, L1210 [18]. However, our present study demonstrates the opposite effect. The difference may be partly due to the cell lines used. Notably, human myeloid cell lines, such as HL-60 or KG-1, have been reported to be more susceptible to apoptosis induced by several anticancer drugs than Chinese hamster ovary cell line [15].

The mechanism by which aphidicolin potentiates apoptosis induced by arabinosyl nucleosides remains speculative. A previous study indicated that aphidicolin caused DNA damage as measured by an alkaline elution assay [33]. Considering the ability of ara-C to inhibit DNA ligase [34], one possible mechanism for the synergism may be the inhibition of DNA repair synthesis of pre-existing DNA damage caused by aphidicolin. Furthermore, a recent report of Mirzayans *et al.* [35] has shown that aphidicolin and ara-C elicited a synergistic inhibition of DNA repair synthesis in human fibroblasts exposed to ^{60}Co γ-radiation. An increase in ara-CTP production by aphidicolin is another possibility. However, the amount of ara-CTP generation did not parallel the degree of apoptosis. In any case, the

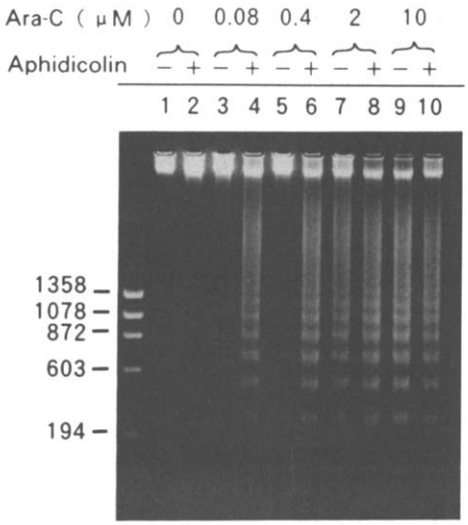

Fig. 3. Agarose gel analysis of DNA following treatment with ara-C in HL-60 cells. DNA samples extracted from HL-60 cells treated for 4 hr with 0, 0.08, 0.4, 2 or 10 μM ara-C are shown in lanes 1, 3, 5, 7 and 9, respectively. Those treated with aphidicolin (2 μM) and ara-C (0, 0.08, 0.4. 2 or 10 μM) are shown in lanes 2, 4, 6, 8 and 10, respectively. Ordinate demonstrates sizes of ø174 Hae III-digested fragments in base pairs.

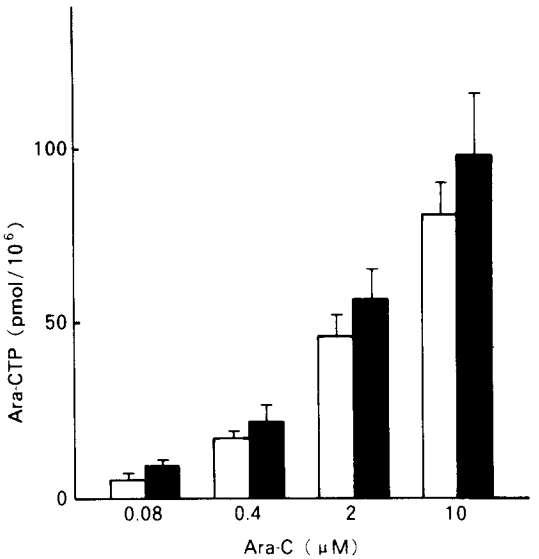

Fig. 4. Effect of aphidicolin on ara-CTP production. HL-60 cells were incubated with (closed bars) or without (open bars) 2 μM aphidicolin for 1 hr, followed by the addition of various concentrations of ara-C. After 4 hr, cells were extracted for ara-CTP determination by HPLC. The data represent the means ± SD of five separate experiments. The difference between ara-C levels in the presence and absence of aphidicolin is significant at every concentration of ara-C ($P < 0.01$).

exact explanation awaits further analysis of the nature of DNA damage by aphidicolin and/or arabinosyl nucleosides at the molecular level. Recent studies have demonstrated that ara-C induces c-*jun* [36] or c-*fos* [37], a family of early responsive genes, at the transcriptional level. This occurred within several hours of addition of ara-C, being independent of *de novo* protein synthesis. The exact role of the activation of these protooncogenes is unclear, but they seem to be an essential signaling pathway leading to apoptosis. In fact, Colotta *et al.* [38] showed that the reduction of expression of the c-*jun* or c-*fos* gene by antisense oligonucleotide in cells undergoing apoptosis resulted in prolonged survival. Whether or not pretreatment with aphidicolin affects the expression of these genes during the apoptotic process induced by arabinosyl analogues is now the subject of a study in progress in our laboratory.

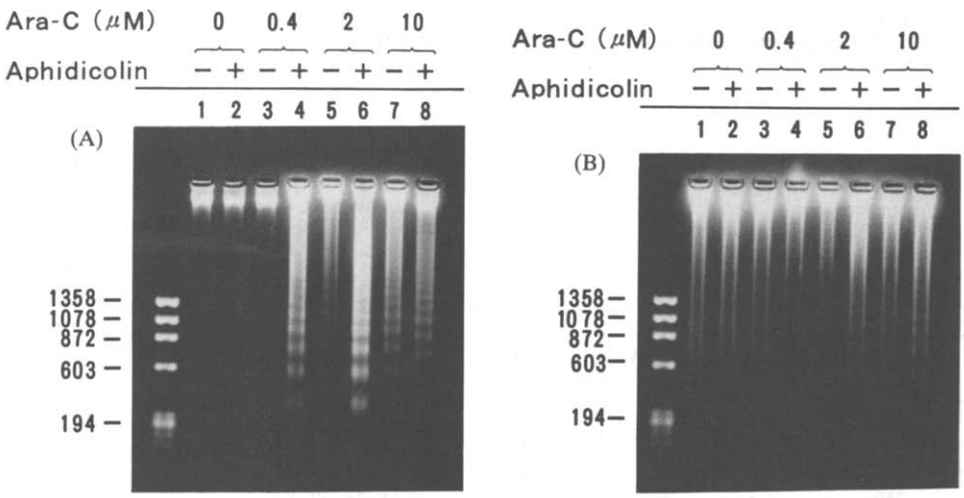

Fig. 5. Agarose gel analysis of DNA following treatment of ara-C in U937 and KG-1 cells. U937 (A) or KG-1 (B) cells incubated for 4 hr with 0, 0.4, 2 or 10 μM ara-C before isolation of DNA are shown in lanes 1, 3, 5 and 7, respectively. DNA from cells co-incubated with aphidicolin (2 μM) and ara-C are shown in lanes 2, 4, 6 and 8 in both panels (A) and (B).

Table 2. Effect of aphidicolin on ara-C-induced apoptosis in U937 and KG-1 cells

Ara-C (μM)	Aphidicolin	Apoptotic cells (%)		DNA fragmentation (%)	
U937					
None	(−)	2.3 ± 0.7	‡	6.0 ± 2.6	‡
	(+)	2.0 ± 0.9		6.7 ± 1.5	
0.08	(−)	2.6 ± 1.3	†	7.4 ± 0.4	†
	(+)	13.6 ± 3.7		13.7 ± 2.8	
0.4	(−)	2.4 ± 0.7	*	9.0 ± 0.7	†
	(+)	24.3 ± 1.0		32.0 ± 10.6	
2.0	(−)	9.2 ± 1.9	*	19.2 ± 1.3	*
	(+)	33.4 ± 1.8		50.0 ± 4.5	
10.0	(−)	22.3 ± 1.9	†	38.8 ± 4.4	*
	(+)	36.8 ± 1.4		52.6 ± 2.5	
KG-1					
None	(−)	1.1 ± 0.4	‡	7.6 ± 3.5	‡
	(+)	1.3 ± 0.7		9.1 ± 3.1	
0.4	(−)	ND		7.5 ± 2.1	†
	(+)	ND		13.7 ± 0.2	
2.0	(−)	5.6 ± 2.3	‡	11.3 ± 1.7	‡
	(+)	9.5 ± 2.2		15.2 ± 4.8	
10.0	(−)	8.0 ± 1.1	*	15.2 ± 1.8	†
	(+)	14.4 ± 1.1		26.1 ± 4.7	

The data represent the means ± SD of three determinations.
*P < 0.001, †P < 0.01, ‡P < 0.05.
ND, not determined.

Table 3. Effect of aphidicolin on ara-A-induced DNA fragmentation and cytotoxicity in HL-60 cells

Ara-A (μM)	Aphidicolin	Apoptotic cells (%)		DNA fragmentation (%)		Cell growth (%)	
2.0	(−)	3.6 ± 1.2	§	11.5 ± 1.3	*	93.9 ± 6.2	‡
	(+)	7.5 ± 3.7		30.1 ± 3.8		79.2 ± 8.0	
10.0	(−)	3.6 ± 1.5	*	16.2 ± 0.9	*	93.2 ± 5.9	†
	(+)	19.9 ± 3.2		36.7 ± 1.1		57.9 ± 8.1	
50.0	(−)	13.7 ± 5.2	*	30.9 ± 4.4	†	72.6 ± 7.5	*
	(+)	42.2 ± 2.9		58.8 ± 8.4		40.7 ± 1.4	

The cells were incubated with an adenosine deaminase inhibitor, erythro-9-(2-hydroxy-3-nonyl) adenine.
The data represent the means ± SD of three determinations.
*P < 0.001, †P < 0.01, ‡P > 0.01, §P < 0.05.

Table 4. Effect of aphidicolin on ara-G-induced apoptosis in HL-60 cells

Ara-G (μM)	Aphidicolin	Apoptotic cells (%)		DNA fragmentation (%)		Cell growth (%)	
10	(−)	2.8 ± 0.5	†	7.2 ± 1.7	†	102.3 ± 1.3	*
	(+)	8.1 ± 2.3		16.1 ± 0.5		95.2 ± 1.1	
50	(−)	3.8 ± 0.4	†	11.2 ± 2.9	†	101.9 ± 7.7	†
	(+)	13.2 ± 3.0		23.9 ± 1.7		78.9 ± 3.4	
250	(−)	9.3 ± 2.5	*	18.1 ± 4.3	†	86.4 ± 4.0	*
	(+)	29.8 ± 4.3		41.8 ± 4.2		58.1 ± 2.0	

The data represent the means ± SD of three determinations.
*P < 0.001, †P < 0.01.

Finally, our data provide a new insight into the synergy between inhibitors of DNA synthesis, including methotrexate and hydroxyurea, and ara-C. As mentioned above, the synergistic action was attributed to an increase in ara-CTP synthesis by these drugs. Several earlier reports, however, indicated that the degree of ara-CTP synthesis did not necessarily correlate well with that of cell killing. Therefore, these studies should be re-evaluated from the point of view of DNA damage and subsequent induction of apoptosis in leukemia cell lines and leukemic blasts freshly obtained from patients.

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