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ORIGINAL ARTICLE

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The effect of 9-β-D-arabinofuranosyl-2-fluoroadenine and 1-β-D-arabinofuranosylcytosine on the cell cycle phase distribution, topoisomerase II level, mitoxantrone cytotoxicity, and DNA strand break production in K562 human leukemia cells

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Abstract Antimetabolites and topoisomerase (topo) II-reactive drugs are frequently combined in the therapy of acute leukemia. The two types of agents are thought to be synergistic in their actions against malignant blasts but the mechanism for this synergism is incompletely described. This study sought to determine whether the combination of two rather than one antimetabolite with the topo II-reactive intercalator mitoxantrone would be greater than the effect of the single antimetabolite ara-C on mitoxantrone's cytotoxic actions. We also aimed to determine a mechanism for synergism should it occur. The model system used was K562 human leukemia cells. The second antimetabolite selected was F-ara-A, the active form of fludarabine. The resultant combination (F-ara-A, ara-C, and a topo II-reactive drug) is one currently being tested against acute myelogenous leukemia in clinical trials. F-ara-A itself had little effect on the cytotoxicity or the topo II-mediated DNA cleaving actions of mitoxantrone, while ara-C potentiated these actions as it does those of other topo II-reactive drugs. Surprisingly F-ara-A enhanced the actions of ara-C on mitoxantrone-associated cytotoxicity by at least an order of magnitude. The effect of the addition of F-ara-A to ara-C on mitoxantrone-induced DNA cleavage was considerably smaller, but present. Antimetabolite treatment did not increase the amount of topo II within cells measured directly by immunoblotting or indirectly by quantifying the maximum number of topo II – DNA complexes stabilized by mitoxantrone. Rather, the antimetabolites altered the distribution of the cells in the cell cycle. Antimetabolite treatment caused a large increase in S-phase cells, a phase in which cells are more sensitive to topo II-reactive drugs than the associated topo II-mediated DNA cleavage would predict. Therefore, it is likely that this shift in the distribution of the cells within the cell cycle accounts for both the enhanced cytotoxicity of mitoxantrone in antimetabolite pretreated cells and the discrepancy between the magnitude of antimetabolite action on topo II-mediated DNA cleavage.

Key words Antimetabolite · Acute leukemia · Topoisomerase II

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Introduction

Progress in the treatment of acute myelogenous leukemia (AML) has rested on the novel use of drugs in combination. Among the most successful combinations have been those that have wedded antimetabolite and topoisomerase-directed drug therapies. Arabinosylcytosine (ara-C) has been the single most successful agent in the treatment of AML [23]. The cytotoxicity of ara-C depends on its phosphorylation to the active 5′-triphosphate, ara-CTP [4, 26]. Strong correlations have been established between the ability of leukemia cells to accumulate and retain ara-CTP during therapy and the response to single-drug ara-C therapy [21, 22]. These observations have stimulated the evaluation of strategies designed to increase cellular levels of ara-CTP [18]. The most successful of these utilizes fludarabine to activate ara-C phosphorylation, a strategy which augments ara-CTP concentrations in K562 cells [15] and in AML blasts [16, 17] and is associated with an improved complete remission rate [11, 12].

Topoisomerase (topo) II-reactive drugs such as the anthracyclines, the anthracenediones, and the amsacrine analogs also have been shown to induce remissions in leukemia. These drugs stabilize a complex between topo II and DNA, which appears to initiate a cascade

of events that leads to apoptosis and cell death [7]. While the antimetabolites and the topo II-directed agents have been combined for many years, the mechanism of their synergistic effect is not clear. Previously, using human leukemia HL-60 cells, we have shown that ara-C enhances the cytotoxic actions of amsacrine and etoposide, two topo II-directed drugs. This enhanced cytotoxicity is not due to increased topo II-reactive drug uptake, to increased amounts of extractable topo II, or to increased sensitivity of the enzyme to the drug. Furthermore, ara-C has almost no effect on the amsacrine resistance of one cell line whose resistance is thought to be due to the presence of a mutant drug-resistant form of topo II [1, 19, 32]. Thus, it appears that the antimetabolite does not act on topo II per se but rather on an intracellular target that could influence the action of topo II-directed drugs.

In the present work, we extended these studies in several ways, with a clear eye toward clinical application. First, we employed K562 cells, a line derived from a patient with chronic myelogenous leukemia that exhibits nucleotide analog metabolism resembling that observed in AML blasts during therapy [16]. Second, the topo II-reactive drug chosen was mitoxantrone which is currently being combined with fludarabine and ara-C in the treatment of AML [13]. Third, we used the nucleoside of fludarabine, 9-β-D-arabinofuranosyl-2-fluoroadenine, (F-ara-A) in combination with ara-C in a fashion that mimicked the administration of this couplet in clinical trials. This was done to evaluate whether F-ara-A might also be effective in enhancing the actions of topo II-directed drugs. If so, this might suggest a future direction for clinical studies.

Materials and methods

Cells

K562 human leukemia cells were obtained from ATCC (American Type Tissue Culture Collection, Rockville, Md.). The cells were grown continuously in exponential growth phase in RPMI-1640 medium (GIBCO, Grand Island, N.Y.) supplemented with 10% fetal bovine serum at 37 °C in an atmosphere containing 5% CO_2. L1210 murine leukemia cells were grown in RPMI-1640 plus 10% fetal calf serum, and served as internal standards in alkaline elution experiments [32]. All cells were Mycoplasma free (ATCC).

Drugs

Mitoxantrone was obtained from the National Cancer Institute and stored as a 0.01 *M* stock solution in 0.01 *N* HCl. Amsacrine [4′-(9-acridinylamino) methanesulfon-*m*-anisidide] was obtained from the National Cancer Institute and stored as a 0.01 *M* stock solution in DMSO. F-ara-A was obtained by dephosphorylating fludarabine (F-ara-A-monophosphate) (kindly provided by Berlex Laboratories, Richmond, Calif.) with *E. coli* alkaline phosphatase (Sigma Chemical Co., St. Louis, Mo.). Ara-C(1-β-D-arabinofuranosylcytosine) was obtained from Sigma. Both antimetabolites were stored as 1 m*M* stock solutions in phosphate-buffered saline (PBS).

Cytotoxicity

K562 cells were treated with or without 100 μM F-ara-A for 1 h at 37 °C. The cells were washed with PBS, centrifuged, and resuspended in fresh medium. The cells were then treated with or without 0.1 μM ara-C for 18 h or 10 μM ara-C for 3 h, washed with PBS, and incubated for an additional hour with mitoxantrone (0.2 μM). After all drug treatments, cells were washed twice with PBS and resuspended in fresh medium for each experiment. To determine the effect of reversing the sequence of drug administration on cytotoxicity, in some experiments, cells were treated for 1 h with mitoxantrone, followed by the indicated concentration of F-ara-A and then ara-C. Cytotoxicity was quantified using the soft agar colony-formation method of Chu and Fisher [5].

Analysis of DNA strand breaks and crosslinks with protein

The methodology of DNA alkaline elution has been previously described [24]. Briefly, K562 cells were radiolabeled with 0.03 μCi/ml [2-^{14}C]thymidine (New England Nuclear, Boston, Mass) for 48–72 h and then grown in radiolabel-free medium for about 6 h prior to any drug treatment. L1210, internal standard cells, were labeled with 0.1 μCi/ml [methyl-^{3}H] thymidine for 24 h and then grown in radiolabel-free medium for at least 2 h before each experiment. The results of the experiments are expressed as rad equivalents, the amount of X-radiation that produces an effect comparable to that observed in drug-treated cells.

Effect of ara-C and F-ara-A on topo II level

Following incubation with the antimetabolites alone or in combination, cells were sonicated with 40 bursts (Fisher Scientific Sonic Dismembrator, Pittsburgh, Pa.) in alkylation buffer (6 *M* guanidine-HCl, 250 m*M* Tris, pH 8.5, 10 m*M* Na_2EDTA) and 1% 2-mercaptoethanol to which the protease inhibitor phenylmethylsulfonyl fluoride was added to a final concentration of 1 m*M* immediately before use. The cell lysates were then subjected to electrophoresis in an SDS-PAGE system, the protein was transferred to nitrocellulose paper, and immunoblotting was performed as previously described [25]. The bands were detected using a monospecific antihuman topo II antibody (from Dr. Leroy Liu, Robert Wood Johnson Medical School, UMDNJ) and the immunoreactivity quantified using a Phosphorimager (Molecular Dynamics, Sunnyvale, Calif.).

Cell cycle analysis

Log-phase cells were treated with or without 100 μM F-ara-A for 1 h followed by the addition of either 10 μM or 0.1 μM ara-C for 3 h or 18 h, respectively. The cells were washed free of drug, resuspended in cold PBS, fixed by the addition of 2.3 ml 100% ethanol, and stored at 4°C overnight. The samples were then washed once with PBTB (PBS with the addition of 0.5% Tween-20 and 0.5% bovine serum albumin) and resuspended in 3 ml of PBTB. RNase was added to a final concentration of 0.01% and the cells were incubated at 37 °C for 30 min. Cells were then centrifuged and resuspended at 10^6 cells/ml in PBTB with 20 μl of 1 mg/ml propidium iodide in 95% ethanol. The distribution of the number of cells in the different

phases of the cell cycle was determined using a Coulter EPICS profile analyzer (Hialeah, Fl.).

Results

Cytotoxicity

Ara-C (0.1 μ*M* for 18 h) and F-ara-A (100 μ*M* for 1 h) alone were nontoxic (Table 1). Combining these two antimetabolites, with cells being incubated with F-ara-A before ara-C to maximize ara-CTP accumulation [15, 16], still did not reduce the colony-forming ability of K562 cells. Mitoxantrone (0.2 μ*M*) administered alone reduced the survival fraction of K562 cells to 0.063. When cells were incubated with 0.1 μ*M* ara-C for 18 h prior to mitoxantrone treatment, the cytotoxicity increased by 13-fold. In contrast, prior treatment of cells with F-ara-A did not increase the cytotoxicity of mitoxantrone. However, treatment of cells with F-ara-A followed by ara-C administered prior to mitoxantrone produced greater cytotoxicity than any of the other combinations.

Because ara-C alone produced greater enhancement of mitoxantrone cytotoxicity than did F-ara-A alone under the incubation conditions described above, we also evaluated whether the ara-C effect was dose and schedule dependent. A dosing scheme approximating the one used clinically [16] would increase the concentration of ara-C (10 μ*M*) for a shorter time period (3 h) while keeping the other drug concentrations and incubations the same. In contrast to results with the lower concentrations of ara-C incubated for longer times (Table 1), pretreatment with F-ara-A and ara-C at the higher concentration for a shorter time resulted in only a twofold increase in cytotoxicity over that seen with mitoxantrone alone (data not shown). Administration of mitoxantrone prior to F-ara-A and ara-C resulted in a decrease in cell killing, indicating that the sequence of the drug treatments was important (data not shown). Thus, the time of antimetabolite exposure, the sequence of drug administration, and the use of a schedule of ara-C and F-ara-A administration that was optimized for biochemical modulation all affected the overall potency of these drug combinations.

Table 1 The effect of Ara-C and/or F-ara-A on mitoxantrone- induced cytotoxicity in terms of the colony-forming ability of K562 cells treated with or without F-ara-A (100 μ*M* × 1 h). The cells were washed and resuspended in medium with or without ara-C (0.1 μ*M* × 18 h) followed by a 1-h incubation with mitoxantrone (0.2 μ*M*). The cells were then washed and resuspended in medium with soft agar

Treatment	Survival fraction[a]	Fold increase by antimetabolite pretreatment[b]	Expected[c]/ observed
Control	1.00		
Ara-C	0.960		
F-ara-A	0.890		
F-ara-A → Ara-C	0.970		
Mitoxantrone	0.063		
Ara-C → mitoxantrone	0.005	12.6	12.1
F-ara-A → mitoxantrone	0.073	0.86	0.77
F-ara-A → Ara-C → mitoxantrone	0.003	21.0	20.4

[a] Fraction of seeded cells forming colonies when corrected for colony-forming ability of untreated cells. The mean colony-forming efficiency was 0.92. Data represent the mean of two independent experiments with each point performed in triplicate in each experiment

[b] Result of dividing mitoxantrone alone survival fraction by survival fraction of antimetabolite treatment plus mitoxantrone

[c] Expected survival is the product of the survival fractions of each of the two or three treatment components

Analysis of DNA strand breaks and crosslinks with protein

Analysis of DNA integrity was assayed by alkaline elution to assess the ability of the antimetabolites to enhance the mitoxantrone-induced DNA strand breaks. Mitoxantrone produced DNA single-strand breaks in a dose-dependent fashion at concentrations up to 0.3 μ*M* (Fig. 1A). Although treatment with 0.1 μ*M* ara-C alone for 18 h produced no DNA cleavage (data not shown), ara-C prior to mitoxantrone increased the strand cleavage frequency produced by mitoxantrone alone (by approximately two-fold; Fig. 1A). F-ara-A (100 μ*M* for 1 h) alone did not cause DNA strand breaks (data not shown), but when used prior to mitoxantrone, DNA cleavage was increased by 20% when compared with mitoxantrone alone. Combining these two antimetabolites (F-ara-A prior to ara-C) with mitoxantrone resulted in DNA strand cleavage that was essentially equal to the additive actions of the two antimetabolites on mitoxantrone-induced DNA cleavage. Consistent with the cytotoxicity data, higher concentrations of ara-C (10 μ*M*) for a shorter time (3 h), whether alone or in combination with F-ara-A, did not enhance the effect of mitoxantrone on DNA cleavage (Fig. 1B).

The mechanisms by which the antimetabolites enhance mitoxantrone's ability to produce DNA cleavage could be either through induction of novel sites of DNA cleavage (e.g. by increasing the amount of topo II) or through the recruitment of topo II cleavage sites at lower mitoxantrone concentrations (enhanced topo II drug sensitivity). In the first case, the maximum frequency of DNA cleavage (a saturable quantity) should increase following antimetabolite treatment. In the second case, saturation levels will remain unaltered. To make this distinction, DNA–protein crosslinking rather than cleavage was measured because the drug-induced lesion frequencies that can be measured using this technique are greater and are not prone to artifactual underestimation for technical reasons (i.e. DNA

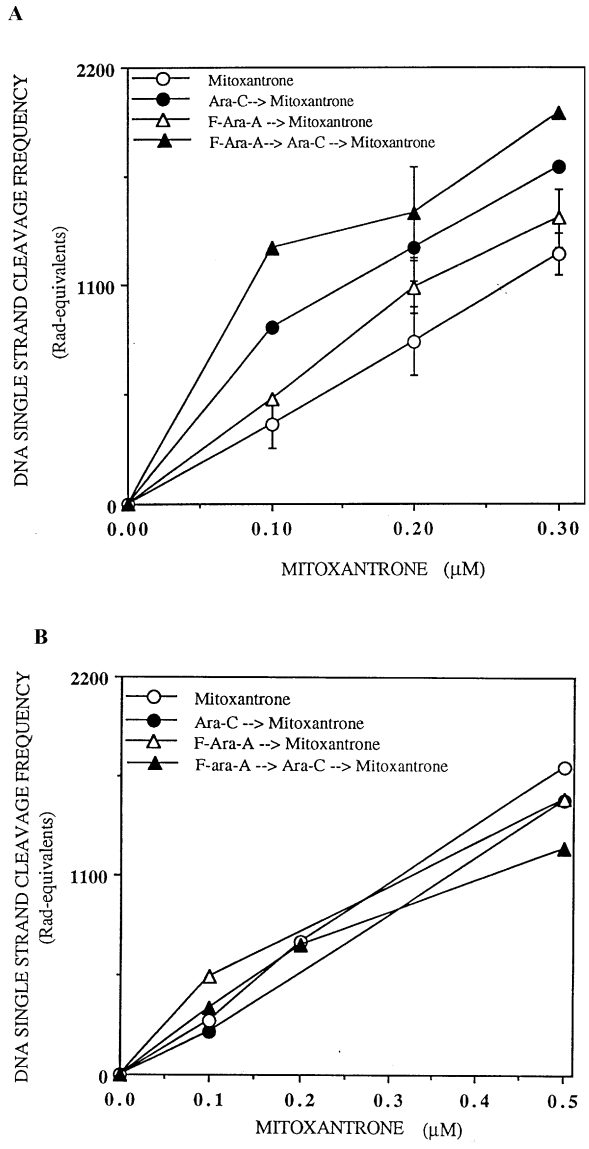

Fig. 1A,B Effect of F-ara-A and ara-C on mitoxantrone-induced single-strand cleavage in K562 cells. **A** K562 cells were treated with mitoxantrone for 1 h at the indicated concentrations following either no pretreatment (*open circles*), pretreatment with F-ara-A (100 μ*M*) for 1 h with an 18 h interval before mitoxantrone (*open triangles*), ara-C (0.1 μ*M*) for 18 h (*closed circles*), or F-ara-A followed by ara-C at the same concentrations and intervals as above (*closed triangles*). **B** The treatment strategy and symbols were identical except that the ara-C concentration was 10 μ*M* and the gap between F-ara-A treatment and mitoxantrone treatment was 3 h. DNA single-strand cleavage was quantified using alkaline elution with proteinase. Results are expressed as rad equivalents. Data points in **A** are mean ± SD for at least four independent determinations. A representative experiment is shown in **B**

exiting the filter during cell lysis due to high DNA break frequencies). To evaluate these possibilities, the DNA–protein crosslink frequency was measured at various mitoxantrone concentrations alone or following treatment with F-ara-A (100 μ*M* for 1 h) prior to an 18-h incubation with 0.1 μ*M* ara-C (Fig. 2). A similar

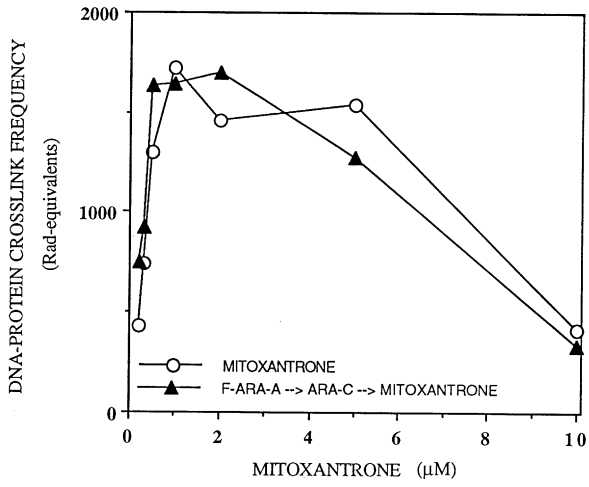

Fig. 2 Effect of F-ara-A and ara-C on mitoxantrone-induced DNA–protein crosslinks in K562 cells. Mitoxantrone-induced DNA–protein crosslinks in K562 cells were quantified in cells treated with F-ara-A (100 μ*M*) for 1 h and ara-C (0.1 μ*M*) for 18 h (*closed triangles*) or receiving no antimetabolite pretreatment (*open circles*). DNA–protein crosslinking frequency was quantified by alkaline elution and expressed in rad equivalents. This figure uses the means of data from two independent experiments

maximum frequency of mitoxantrone-induced DNA–protein crosslinking was observed following antimetabolite pretreatment, suggesting that the increased cytotoxicity (Table 1) and DNA strand cleavage (Fig. 1A) of this treatment sensitized the topo II already present in the cells rather than acting through an enhancement of the amount of topo II. The decline in the amount of topo II-mediated DNA crosslinking at higher mitoxantrone concentrations may be attributable to the interference by mitoxantrone in topo II–DNA binding [33].

Effect of ara-C and F-ara-A on reversal of mitoxantrone-induced strand breaks

In a preliminary study, Wang et al. [30] showed that in a biochemical system, the number of mitoxantrone-induced topo II-mediated DNA strand breaks is greater in DNA containing incorporated ara-C than in DNA containing no ara-C. These investigators also found that the mitoxantrone-induced topo II–DNA complexes are reversible in the presence of salt when the DNA contained no ara-C but that the reversal rate of topo II–DNA complexes in the DNA containing ara-C is reduced in the presence of salt. Those experiments were performed using isolated DNA. We performed similar experiments using intact cells to see whether the alterations in kinetics produced by ara-C incorporated into DNA could be reproduced in a living cell. The retention of mitoxantrone in cells is protracted [8, 28], and thus breaks reseal slowly following removal of mitoxantrone from the extracellular environment. For

this reason, resealing experiments were performed with amsacrine, which egresses rapidly from cells at 37 °C [31] thus allowing the actual reversal of topo II–DNA complexes to be the rate-limiting step in strand-break reversal. Removal of amsacrine by washing the cells twice in medium at 37 °C was followed by a rapid disappearance of single-strand breaks in both the cells treated with amsacrine alone and those pretreated with F-ara-A (100 μ*M* for 1 h) and ara-C (0.1 μ*M* for 18 h) prior to amsacrine treatment (Fig. 3). This finding suggests that antimetabolite pretreatment does not alter the affinity of topo II for DNA and/or that these antimetabolites do not interfere with topo II function upon removal of amsacrine.

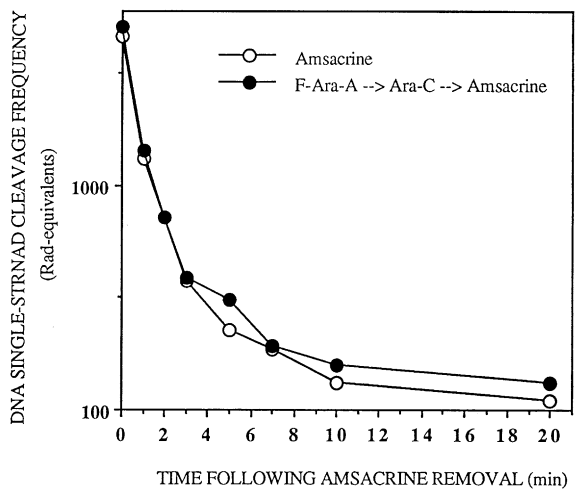

Fig. 3 Disappearance of amsacrine-induced DNA single-strand cleavage in K562 cells following drug removal. Control cells (*open circles*) were incubated with 0.7 μ*M* amsacrine for 1 h at 37 °C. Cells treated with F-ara-A (100 μ*M*) for 1 h and ara-C (0.1 μ*M*) for 18 h (*closed circles*) were exposed to a lower amsacrine concentration (0.35 μ*M*), as the amsacrine-induced cleavage frequency is higher in antimetabolite-pretreated cells. This assured that the point from which reversal would be measured would be the same in the pretreated and nonpretreated cells. Drug was removed by centrifugation and cells were resuspended in fresh medium at 37 °C. At various times following drug removal, DNA single-strand cleavage was quantified using alkaline elution with proteinase. Results are expressed as rad equivalents. Data points are from a representative experiment

Effect of ara-C and F-ara-A on topo II level

Immunoblotting to quantitate cellular levels of topo II was performed to ascertain the validity of the presumption that antimetabolites were not increasing the amount of topo II in cells (Fig. 2). As shown in Fig. 4, there were no marked differences between the amounts of topo II measured in the controls and the amounts in the cells pretreated with ara-C or F-ara-A alone or in combination ($P \geq 0.3$). This confirms [1] that the enhanced mitoxantrone-induced cytotoxicity and DNA cleavage seen in antimetabolite-treated cells was not due to an increase in the amount of topo II contained in the cell.

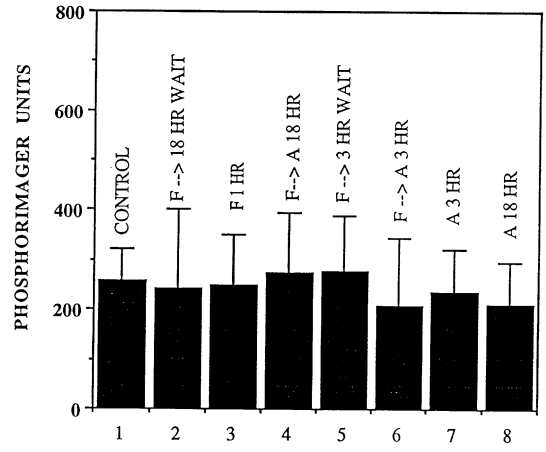

Fig. 4 Measurement of the 170 kDa Topo IIα levels in K562 cells treated with or without F-ara-A (100 μ*M*) for 1 h followed by ara-C either at (10 μ*M*) for 3 h or at (0.1 μ*M*) for 18 h. Immunoblotting was performed using an antihuman topo II polyclonal antibody and detected with ^{125}I-labelled protein A. The bands from four independent experiments were quantified using a Phosphorimager and the mean values are expressed in the Phosphorimager units

Cell cycle analysis

Previous studies [10] have shown that S-phase cells are maximally sensitive to topo II inhibitors without necessarily exhibiting an enhanced cleavability of their DNA. Thus, could the effect of antimetabolites be to produce a cell population with a lower tolerance to drug-induced topo II-mediated cleavage as a result of the creation of a biochemistry characteristic of cells within specific phases of the cell cycle? Cell cycle analysis was performed on K562 cells to determine whether treating the cells with antimetabolites could increase the accumulation of cells in S-phase and sensitize the population to the cytotoxic action of mitoxantrone (Table 1) to a greater extent than would be predicted by changes in DNA cleavage (Fig. 2). The 1-h treatment with F-ara-A resulted in only a small increase in cells in S-phase (Table 2). However, incubation of K562 cells in the presence of ara-C (0.1 μ*M* for 18 h) caused a substantial increase in the S-phase population (from 54% to 82%). Incubation of K562 cells with F-ara-A (100 μ*M* for 1 h) prior to ara-C further increased the S-phase fraction so that only 7% of cells were not in S-phase. In contrast, a higher concentration of ara-C for a shorter time (10 μ*M* for 3 h) did not have any effect on the cell cycle distribution compared with control (untreated) cell population. The concordance of this result with the cytotoxicity and DNA strand breaks at this ara-C concentration suggests a mechanistic link between the time-dependent recruitment of

Table 2 Effect of ara-nucleotides on cell cycle distribution. Cell cycle analysis of K562 cells treated with or without F-ara-A (100 μ*M* × 1 h). The cells were washed and resuspended in medium with or without Ara-C (10 μ*M* × 3 h) or (0.1 μ*M* × 18 h). The cells were then washed and resuspended in PBS, fixed, and stained with propidium iodide for cell cycle analysis. The results are expressed as the percentage of total cells

	G_0/G_1	S	G_2/M
0.1 μ*M* ara-C for 18 h			
Control	36	54	11
F-ara-A	30	62	9
Ara-C	9	82	9
F-ara-A → ara-C	4	93	3
10 μ*M* ara-C for 3 h			
Control	36	54	11
F-ara-A	33	59	9
Ara-C	43	54	3
F-ara-A → ara-C	45	51	5

cells into S-phase and the augmented cytotoxicity of topo II-reactive drugs in antimetabolite-pretreated cells.

Discussion

The mechanisms by which antineoplastic drugs selectively kill malignant cells are not known. While many of the actions of these drugs in cellular and biochemical systems can be measured, the precise manner in which these actions lead to cell death is not generally understood. It is therefore even more difficult to understand how one anticancer drug augments the actions of another, let alone of a third agent. All of the most effective anticancer drug regimens incorporate more than one and usually more than two drugs, and the success of such regimens in situations where single agents fail indicates that synergism is occurring, not only biochemically but also clinically. Thus, studies must be pursued to establish systems in which to examine the mechanisms of synergism in order to maximize the beneficial action of drugs in combination.

Mechanisms of two-drug synergism fall into four categories. First, one drug could alter the pharmacokinetics or cellular transport of another and thus functionally increase intracellular drug concentrations. An example of this is the increase in cellular levels of mdr-directed agents following treatment of cells with calcium-channel blockers that bind to the p-glycoprotein pump, thus blocking its actions to efflux mdr-directed agents [9]. Second, one drug can alter the chemistry or biochemistry of a second and make it more potent. An example of this is fludarabine's ability to increase intracellular ara-CTP levels by directly and indirectly potentiating the activity of deoxycytidine kinase, the enzyme responsible for phosphorylation of ara-C [15, 16]. Third, one drug could alter the cellular responses to another drug, making the cell less tolerant to the injury produced by the second agent. An example of this is the inhibition of DNA repair processes by antimetabolites when they are combined with DNA-damaging agents such as cisplatin [29]. Fourth, one drug could alter the cellular physiology in a more global fashion, leading to a cell more susceptible to injury by a second agent. The recruitment of cells into a susceptible phase of the cell cycle [10] is an example of the fourth type of synergy.

The objective of the present investigation was to identify the way in which fludarabine and ara-C affect mitoxantrone-mediated cytotoxicity using K562 human leukemia cells as the model system. An 18-h continuous incubation with 0.1 μ*M* ara-C with or without F-ara-A was synergistic regarding cytotoxicity. The fact that a shorter duration of incubation with ara-C or F-ara-A did not alter the mitoxantrone-induced cytotoxicity suggested that initial accumulation of high concentrations of cytotoxic triphosphates, ara-CTP or F-ara-ATP may not be responsible for this effect. Rather, a secondary factor mediated through a constant presence of low levels of ara-CTP is affecting some process which in turn sensitizes the cells to mitoxantrone. Among the parameters studied, DNA single-strand cleavage frequency (Fig. 1) and recruitment of cells in S-phase (Table 2) were specifically affected by an 18-h incubation and not by a 3-h incubation of ara-C.

The next step was to understand the biochemistry producing this recruitment phenomenon. In our experiments, it was not due to increased amounts of topo II, although other investigators have reported increases in topo II associated with recruitment of cells into late S/G_2 [2, 3]. One reason for this may be that in the present studies, the increase in the S-phase population was due to a shift of the G_1 population into S-phase, an action known to be caused by ara-C both in vitro [2, 3] and in vivo [6, 27]. This was not a true recruitment from the quiescent cell population into the cell cycle, as such recruitment is associated with a major elevation in topo II protein levels [20]. An alternative mechanism could be based upon speculation that DNA containing ara-C displays a slower disappearance of drug-induced topo II-mediated DNA cleavage [30] than ara-C-free DNA. This could enhance cytotoxicity. However, we did not observe this in whole cells.

Clearly, the antimetabolites are sensitizing the cells in two ways; one related to topo II and one unrelated to it. The first way may involve modulations in topo II levels [3], although we did not demonstrate these in K562 cells. The second, and probably more crucial way, has to do with the increased sensitivity of S-phase cells to topo II-directed drugs. This effect may be related to topo II levels [10], but it may also be related to the critical nature of topo II's activity during this phase of cell cycle progression and to the consequences of inhibiting that action to cell viability.

Regardless of the precise mechanism by which this synergism occurs, parallels to the clinical effects are present. Fludarabine, the clinical form of F-ara-A, is an important addition to ara-C treatment of acute leukemia. While fludarabine itself does not greatly enhance the effects of mitoxantrone (Table 1, Fig. 1), it does potentiate the ability of ara-C to increase the effect of mitoxantrone in a way that is difficult to attribute solely to effects on topo II. This is particularly reflected in the difference between the actions of the antimetabolites on topo II-reactive drug action in elution assays and their actions in assays of cytotoxicity, the magnitude of which would not have been predicted by the results of elution assays. The schedule-dependent interaction of ara-C plus mitoxantrone further enhances the need for sequence-specific administration of these drugs in the clinic (present study; [14]).

In combination with fludarabine, ara-C administration in the clinical setting is for a briefer period of time [13] than that employed in the present study. However, when the major role of the antimetabolites is to sensitize cells to the actions of mitoxantrone rather than to kill cells directly, it is possible that the timing will not be identical in vitro and clinically. During therapy, ara-CTP accumulates in circulating leukemia blasts from patients at much higher levels than in K562 cells in vitro. Additionally, this cytotoxic metabolite is eliminated at a slower rate from circulating peripheral leukemia blasts [16] than from the K562 cells [15]. This provides a continuous exposure of cells to ara-CTP during therapy which may result in the required S-phase accumulation and sensitization of leukemia blasts to mitoxantrone-mediated cytotoxicity. Regardless of the mechanism once again we have demonstrated that noncytotoxic concentrations of antimetabolites can render a cell population more sensitive to the actions of topo II-reactive drugs than would be the case without antimetabolite pretreatment. These data suggest a sequence-specific schedule-dependent combination of these agents in a clinical regimen for treatment of myelogenous leukemias.

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