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Potentiation of etoposide-induced cytotoxicity and DNA damage in CCRF-CEM cells by pretreatment with non-cytotoxic concentrations of arabinosyl cytosine

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Summary. Pretreatment of the human lymphoblastoid cell line CCRF-CEM with 0.02 μM arabinosyl cytosine (ara C) enhances both the cytotoxic and the DNA-damaging effects of etoposide. This concentration of ara C is itself non-cytotoxic and results in no detectable DNA damage as measured by alkaline elution. Ara C pretreatment results in the synchronisation of cells, a 24-h pretreatment resulting in the accumulation of cells in the early S phase. The sensitivity of cells to etoposide-induced cytotoxicity was increased 2.5 times and DNA damage was enhanced 1.66 times by this pretreatment. Maximal potentiation of etoposide-induced DNA damage (2.06-fold increase) was observed after 48 h continuous treatment with ara C, but no further enhancement of cytotoxicity occurred. Cell-cycle analysis demonstrated that 48 h ara C treatment resulted in the accumulation of cells in the late S/G_2M phase. Cells returned to a normal cell-cycle distribution within 24 h of the removal of ara C, and the potentiation of etoposide activity was then reduced to a 1.3- to 1.4-fold level. DNA damage induced by etoposide following ara C pretreatment was qualitatively identical to that produced by etoposide alone, suggesting a mechanism involving topoisomerase II. To investigate this possibility, we measured topoisomerase II protein levels by immunoblotting. Measurement of topoisomerase II levels in whole-cell lysates of ara C-pretreated cells showed a 3- to 5-fold increase in topoisomerase levels relative to total protein content. This suggests that elevated enzyme levels may be responsible for the increased sensitivity of ara C-pretreated cells to etoposide.

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Abbreviations: ara-C, cytosine β-D-arabinofuranoside; *m*-AMSA, 4′-(9-acridinylamino)-methanesulfon-*m*-anisidide; DMSO, dimethylsulphoxide; MTT, 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide; SSBs, single-strand breaks

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Introduction

Etoposide and intercalating agents such as *m*-AMSA, daunorubicin and doxorubicin are effective drugs in the treatment of a number of human cancers [7, 25]. They are used both as single agents and in combination chemotherapy. These agents have proved to be particularly effective in the treatment of both murine and human leukaemia when used in combination with the DNA-synthesis inhibitor arabinosyl cytosine [11, 24, 31, 36]. Murine studies demonstrated that the potentiation of cytotoxicity by ara C was schedule-dependent, but the mechanism resulting in schedule-dependent synergy was not understood [11, 31].

It is now widely accepted that topoisomerase II is one of the main targets of the epipodophyllotoxins (etoposide and teniposide), amsacrines (*m*-AMSA), anthracyclines (doxorubicin and daunorubicin) and ellipticines [7, 25, 33, 37, 42]. Topoisomerase II alters DNA conformation by passage of a DNA segment through a transient double-stranded break [26]. This reaction is inhibited by the antitumour drugs, resulting in the stabilisation of an abortive reaction intermediate, the 'cleavable complex' [4, 11, 25]. The frequency of cleavable complex formation produced by the drugs often correlates with their cytotoxicity [34, 35], although the cleavable complex alone does not result in a loss of viability [3, 12, 38]. It has been demonstrated that cytotoxicity can be abrogated without reduction in the frequency of cleavable complex formation by depletion of calcium ions or treatment with 2,4-dinitrophenol (DNP). Similarly, novobiocin treatment and, in some circumstances, inhibition of protein or DNA synthesis can decrease the cytotoxicity of etoposide [3, 8, 21, 38, 43].

The sensitivity of cells to topoisomerase II poisons varies with fluctuations in the level of the topoisomerase II

protein [6, 19, 27, 40], and drug resistance is often associated with reduced levels of the enzyme [13, 15, 20]. Enzyme levels vary not only according to cell type but also as a function of the proliferative status of the cell [29, 39, 41] and the position of the cell in the cell cycle [6, 27, 30]. Using an immunoassay, Woessner et al. [44] have demonstrated that topoisomerase II (p170) levels increase as cells progress from the G1 phase through synthesis to the G_2/M phase and then rapidly decline as cells complete mitosis. If the cell-cycle position of tumour cells could be modulated in vivo such that a greater proportion of the cells are in the S phase, then the sensitivity of these cells to etoposide could be increased. Synchronisation of tissue-culture cells in vitro by centrifugal elutriation and serum starvation has been demonstrated to enhance the DNA-damaging and cytotoxic effects of intercalating agents [6, 12, 30]. Lorico et al. [27] found that synchronisation of human lymphoma cells (U937) with low-dose methotrexate, which resulted in S-phase block, increased topoisomerase II levels and concomitantly increased the etoposide sensitivity of the cells.

In the present study we determined the effects of non-cytotoxic levels of ara C on cell-cycle distribution, topoisomerase II levels and etoposide activity. An understanding of the interactions of the two drugs at the molecular level may be useful in improving the scheduling and dosage of these agents in the clinic.

Materials and methods

Materials. All drugs were prepared immediately before use. Etoposide (Bristol Myers) was prepared at a concentration of 10 mM in DMSO. The final DMSO concentration used to treat cells was always less than 0.1%, which did not affect cell viability. Cytosine β-D-arabinofuranoside (Sigma) was dissolved in chilled serum-free medium at a concentration of 1 mM. Immobilon-P PVDF membrane for Western blotting was obtained from Millipore, and alkaline phosphatase assay reagents were supplied by Promega. All other chemicals used were of analytical grade or above.

Cell culture and cytotoxicity assays. CCRF-CEM, a human lymphoblastoid cell line, was maintained in RPMI 1640 medium supplemented with 10% foetal calf serum and glutamine to 2 mM; under these conditions the doubling time was approximately 23 h.

For assessment of cytotoxicity, log-phase cells were suspended at 1×10^5 cells/ml in full medium. Drugs were incubated with cells for the stated times, after which the cells centrifuged, washed twice in drug-free medium and finally resuspended in medium at 3.5×10^4 cells/ml. In all experiments control cells received an appropriate amount of drug vehicle. Following pretreatment with ara C for 24 and 48 h, cell numbers and viability were determined by trypan blue exclusion. It was found that no cell doubling occurred during ara C treatment. Therefore, for cytotoxicity and DNA damage assays, control cells were suspended at 0.5 and 0.25×10^5 cells/ml, respectively, such that both control and pretreated cell solutions contained 1×10^5 cells/ml for etoposide treatment. A tetrazolium dye reduction assay (MTT assay) was used to measure cytotoxicity. The assay was based on that originally described by Mossman [32] except that DMSO was used as the solvent. Cells (7×10^{31}) were plated in 200 µl complete medium/well. Plates were incubated for three doubling times in 5% CO_2 at 37° C. Each drug response was evaluated in three independent experiments and the mean IC_{90} value (the dose that reduced viability by 90%) was determined. Fractional viability was determined by division of the A550 value for treated cells by the control value for samples that received ara C pretreatment the control used was ara C alone. The A550 readings obtained for ara C (0.02 µM) in pretreated and control cells were always approximately equal.

Alkaline elution. DNA-protein cross-linking and drug-induced single-strand breaks (SSBs) were measured by DNA alkaline elution (pH 12.1) as described by Kohn et al. [22]. Early logarithmic-phase cells (1×10^5 cells/ml) were labeled for 24 h with 0.015 µCi [^{14}C]-thymidine/ml (specific activity, 56 mCi/mmol; Amersham, UK), centrifuged and resuspended in fresh medium at 1×10^5 cells/ml for at least 1 h before being subjected to drug treatment. Cells were treated with drugs for the periods described and were either analysed immediately or incubated at 37° C for various periods before elution analysis.

For measurements of DNA-protein cross-linking SSBs were introduced into cellular DNA by exposure to an X-ray source (3000 rad) and cells were held at ice-cold temperatures throughout determinations. Assays for DNA-protein cross-links and protein association of SSBs were conducted using 2 µm polyvinylchloride (PVC) filters (Millipore). Assays of total SSBs in the presence of proteinase K were conducted using 2 µm polycarbonate filters (Nucleopore). The DNA-protein cross-linking coefficient (*Pc*) was calculated as follows:

$$Pc = \frac{1}{\sqrt{1-r}} - \frac{1}{\sqrt{1-r^0}},$$

where r represents drug-treated and r^0, control DNA retained on the filter as measured from the linear part of the elution curve. DNA damage expressed in rad equivalents was calculated by multiplying Pc and the dose of irradiation given (3000 rad). The frequency of SSBs induced by etoposide was converted to rad equivalents using a calibration graph derived from the number of SSBs produced by a known X-ray dose.

Cell-cycle analysis. Logarithmically growing CCRF-CEM cells were treated with either ara C or drug vehicle as described for cytotoxicity studies. Following drug treatment, aliquots of 5×10^5 cells were collected by centrifugation and resuspended in ice-cold isotonic saline. They were then fixed by dropwise addition of 3 vol. 95% ethanol under vortexing. Fixed cells were stored at 4° C until analysis. For analysis of DNA content, cells were collected by centrifugation and resuspended in 0.5 ml propidium iodide solution (PI; 0.1% sodium citrate, 50 µg PI/ml, 7.5 µg heat-inactivated RNAse/ml, 0.002% NP40). Cells were incubated for 45 min in a dark environment at 20° C and were then analysed using a Beckton Dickinson FACSTAR fluorescence-activated cell sorter (FACS). The percentage of cells in each phase of the cell cycle was calculated with Beckton Dickinson DNA cell-cycle analysis software (Ver C) using the sum-of-broadened-rectangles model.

Western blotting. Cells were pretreated with ara C for the indicated times. Control cells were diluted as described for cytotoxicity assays such that their density was the same as that of ara C-pretreated cells for the

Table 1. Effect of ara C pretreatment time on etoposide cytotoxicity and DNA damage

Pretreatment time (h)	Etoposide IC_{90} (µM)	*P*	DNA SSBs etoposide (1 µM)	*P*
No ara C	37.0±4.8		157±35	
0	ND	ND	123±18	NS
2	39.5±3.5	NS	137±29	NS
6	36.9±0.2	NS	123±15	NS
12	39.1±2.5	NS	173±11	NS
24	14.5±0.5	*	261±53	*
48	14.4±3.7	*	323±44	*
24 + 24 drug-free	25.7±2.1	*	197±32	NS

Cells were pretreated with ara C (0.02 µM) for the times indicated before undergoing 60-min treatment with etoposide. Cytotoxicity was assessed using the MTT assay, and DNA damage, expressed as rad equivalents, was determined by alkaline elution. Data represent mean values ±SD (n = >3). Probability values (P) were determined using Student's two-sample t-test. NS, Not significant at the 5% level; ND, not determined
* P <0.0001

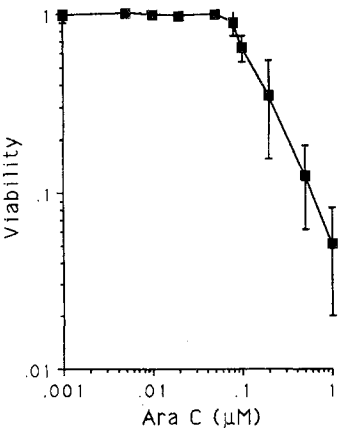

Fig. 1. Effect of ara C concentration on the viability of CCRF-CEM cells. Cells were treated with ara C for 30 h and their viability was measured by the MTT assay. All points represent mean values for 3 independent experiments, and bars indicate standard deviations (some points have error bars that are concealed by the symbol)

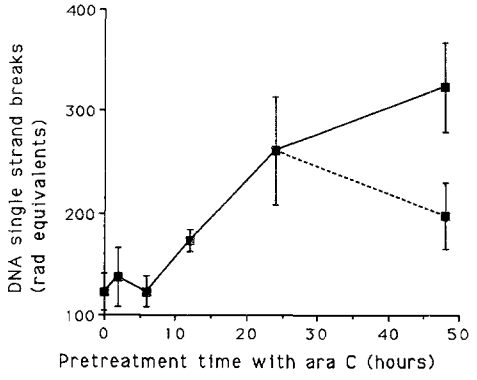

Fig. 2. Effect of ara C pretreatment time on etoposide-induced single-strand breaks. Cells were treated continuously with ara C (0.02 μM) for the indicated times and etoposide (1 μM) was then added for 1 h. DNA damage, expressed as rad equivalents, was assessed by alkaline elution in the presence of proteinase K. Points represent mean values for 3 independent experiments, and bars indicate standard deviations. The *dotted line* shows the effect of ara C removal after 24 h

experiment. Following treatment the cells were washed and resuspended in PBS containing protease inhibitors (1 mM) benzamidine, 1 mM PMSF, 10 μg soybean trypsin inhibitor/ml 50 μg leupeptin/ml at 1×10^8 cells/ml. Cells were then lysed by sonication and DNA was digested with DNAse I (100 μg/ml for 60 min). The protein content of the lysate was measured using a Bio-Rad protein assay kit. Protein samples were heated to 68° C for 5 min in SDS sample buffer and then electrophoresed overnight on 0.75-mm polyacrylamide slab gels [7.5% (w/v) separating gel, 4% (w/v) stacking gel] at 40 V. Proteins were transferred to Immobilon-P PVDF membrane at 400 mA for 2 h. Topoisomerase II was detected using an affinity-purified antibody to human p170 recombinant topoisomerase II (kindly provided by Dr. F. H. Drake, SmithKline Beecham Pharmaceuticals). Goat anti-rabbit alkaline phosphatase-conjugated secondary antibodies were used to detect the topoisomerase II as described elsewhere [10].

Results

Effect of ara C pretreatment on etoposide cytotoxicity

Table 1 shows the effect of ara C pretreatment on etoposide cytotoxicity. Pretreatment of CCRF-CEM cells with 0.02 μM ara C for up to 12 h did not affect the cytotoxicity of etoposide. In contrast 24- and 48-h pretreatments enhanced the activity of etoposide 2.5 times, reducing the IC_{90} value from 37.0 ± 4.8 μM to 14.5 ± 0.5 and 14.4 ± 3.7 μM, respectively. The enhancement of etoposide cytotoxicity by ara C was reduced to 1.4 times when cells were washed and reincubated in fresh medium for 24 h following ara C treatment. The concentration of ara C used in all drug-combination experiments 0.02 μM. Continuous incubation of CCRF-CEM cells with this concentration of ara C for 24, 30 or 48 h did not result in any loss of viability as measured both by trypan blue exclusion immediately after drug treatment (results not shown) and by the MTT assay (see Fig. 1). However, no cell doubling occured during drug treatment.

Effect of ara C pretreatment on etoposide-induced DNA damage

Measurement of DNA damage by alkaline elution demonstrated that the frequency of DNA SSBs and DNA-protein cross-links was increased by pretreatment with ara C (Table 2). Enhancement of DNA damage, like that of cytotoxicity, was dependent upon the duration of the pretreatment. Ara C pretreatments of less than 12 h did not significantly increase the SSB frequency, whereas cells treated with ara C for 24 and 48 h showed 1.66- and 2.06-fold increases, respectively, in the etoposide-induced SSB frequency (Table 1, Fig. 2). The enhancement of

Table 2. Effect of ara C pretreatment on etoposide-induced DNA damage

VP16 (μM)	SSBs			Protein cross-links		
	Pretreatment time			Pretreatment time		
	None	24 h	48 h	None	24 h	48 h
1	157 ± 35	261 ± 53	323 ± 44	162 ± 30	ND	282 ± 79
5	409 ± 32	583 ± 97	1230 ± 212	322 ± 20	435 ± 72	ND
10	593 ± 41	1565 ± 304	2025 ± 530	679 ± 122	1142 ± 507	1928 ± 153

Cells were pretreated with ara C (0.02 μM) for the indicated times before undergoing etoposide treatment. DNA damage, expressed as rad equivalents, was measured by alkaline elution. Data represent mean values ± SD ($n = >3$). Note that for a given etoposide concentration, the ratio of SSBs to DNA protein cross-links is approximately 1 : 1. This ratio is unchanged by ara C pretreatment

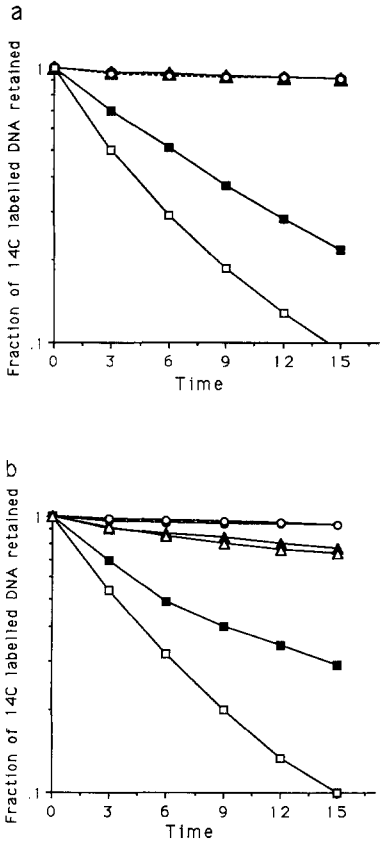

Fig. 3 a, b. Effect of ara C pretreatment (0.02 μM) on etoposide-induced lesions. **a** Protein association of single-strand breaks produced by a 1-h treatment with etoposide (1 μM). Note that the symbols for elution of control DNA and drug-treated DNA in the absence of proteinase K (PK) overlap. – – △ – –, Control; *solid symbols*, etoposide alone (■, + PK; ●, no PK); *open symbols*, pretreatment with ara C for 48 h followed by treatment with etoposide (□, +PK; ○, no PK). **b** Repair of etoposide (1 μM)-induced breaks following post-incubation (p.i.) at 37° C for the indicated times. ●, Control; ○, ara C pretreatment for 48 h (no etoposide); ■, etoposide treatment (no p.i.); □, pretreatment with ara C for 48 h + etoposide treatment (no p.i.); ▲, etoposide treatment (1 h p.i.); △, pretreatment with ara C for 48 h + etoposide treatment (1 h p.i.)

etoposide-induced SSBs was reduced to 1.25 times when cells were washed free of ara C and reincubated in fresh medium for 24 h (Fig. 2). The DNA-protein cross-link frequency was similarly increased by ara C pretreatment. The ratio of DNA-protein cross-links to SSBs produced by etoposide was approximately 1:1, and this ratio was not changed by ara C pretreatment (Table 2).

The etoposide-induced DNA damage remained qualitatively unchanged by pretreatment with ara C and was typical of a topoisomerase II-induced lesion. The elution of drug-treated DNA was identical to that of control DNA unless proteinase K digestion was performed, suggesting that all breaks were protein-concealed (Fig. 3a). Following drug removal, resealing of etoposide-induced breaks was equally rapid in control and pretreated cells (Fig. 3b). Ara C alone produced no detectable DNA damage. The fraction of DNA retained following 9 h elution was 0.93 for control cells and 0.94 for cells treated with ara C (0.02 μM) for 48 h (data shown in Fig. 3b). The elution of irradiated DNA (450 rad) was similarly unaffected by ara C pretreatment. The fraction of DNA retained following 9 h elution was 0.12 for control cells and 0.1 for cells pretreated with 0.02 μM ara C for 48 h (data not shown).

Effect of ara C pretreatment on cell-cycle distribution

Treatment of cells with concentrations of ara C as low as 0.01 μM resulted in a marked perturbation of the cell-cycle distribution (Fig. 4a). Non-cytotoxic levels of ara C (0.01–0.05 μM) caused an accumulation of cells in the early to mid-S phase. Increasing the concentration of ara C resulted in a progressively earlier block in the cell and at levels of above 0.075 μM, cells were blocked at the G1/S boundary. Continuous treatment with ara C (0.02 μM) led to a gradual increase in the proportion of cells in the S phase, which reached a peak at approximately 24 h, at which time nearly 80% of the cells were in the S phase (Fig. 4b, c). Subsequent washing and reincubation of the cells in drug-free medium allowed the cells to proceed through the cell cycle; within 24 h the cell-cycle distribution had returned to that of control cells (Fig. 4b, c). Treatment of cells with ara C (0.02 μM) for 48 h did not result in a further increase in the number of cells in the S phase; indeed, a gradual release from the block occurred and cells accumulated in the late S/G_2M phase as shown in Fig. 4b.

Elevation of p170-α topoisomerase II levels in ara C-pretreated cells

The level of topoisomerase II in lysates of cells synchronised with ara C was measured by Western blotting (Fig. 5). The level of total intracellular protein doubled in cells that had been pretreated with ara C for 48 h as compared with untreated controls, as would be expected for a population of cells synchronised in the late S/G_2M phase. However, taking into account the general increase in protein content, the levels of topoisomerase relative to total protein increased 3 and 5 times in cells that had been pretreated for 24 and 48 h, respectively; this is equivalent to 4- and 8.6-fold increases per cell, respectively, as compared with controls.

Discussion

This study demonstrates that levels of ara C that produce no measurable DNA damage or cytotoxicity can significantly alter cell-cycle kinetics and increase the activity of etoposide. Levels of ara C as low as 0.02 μM resulted in cell-cycle synchronisation, blocking cells in the S phase. Following continuous treatment of the cells with ara C for 24 h, almost 80% of the cells accumulated in the early S phase of the cell cycle. Increasing the incubation time to 30 and 48 h resulted in an accumulation of cells in the mid-S and late S/G_2M phases of the cell cycle, respectively. Synchronisation of cells probably results from a reduced rate of DNA synthesis. Incorporation of ara C into DNA alters the reactivity of the 3′ terminus, slowing chain elongation [16, 23, 28].

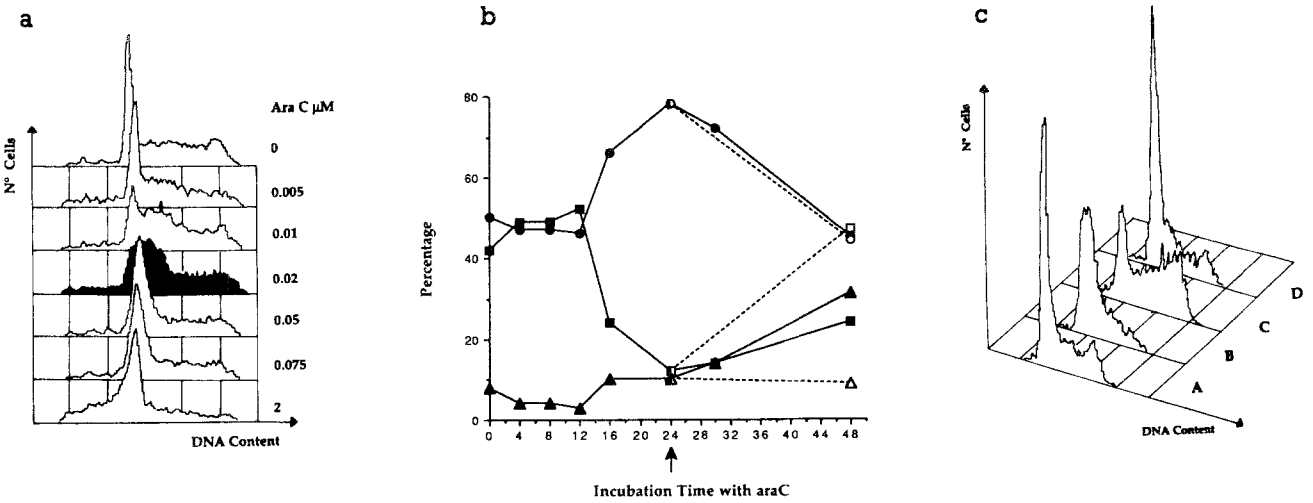

Fig. 4 a – c. Effect of incubation with ara C (0.02 μM) on cell-cycle distribution. **a** FACS analysis of the cell-cycle distribution of cells treated with a range of concentrations of ara C for 30 h. The distribution at 0.02 μM ara C is *shaded*. **b** Effect of treatment time on the percentage of cells in each stage of the cell cycle. ■, G_0/G_1; ●, S; ▲, G_2/M. *Dotted lines with open symbols* show the effect of removal of ara C after 24 h (*arrow*) and reincubation of cells at 37° C. **c** Representative DNA histograms used in the generation of plots of the type shown in Fig. 4b. *A*, Control cells; *B*, cells treated with ara C for 24 h; *C*, cells treated with ara C for 48 h; *D*, cells treated with ara C for 24 h and then reincubated in drug-free medium for 24 h at 37° C

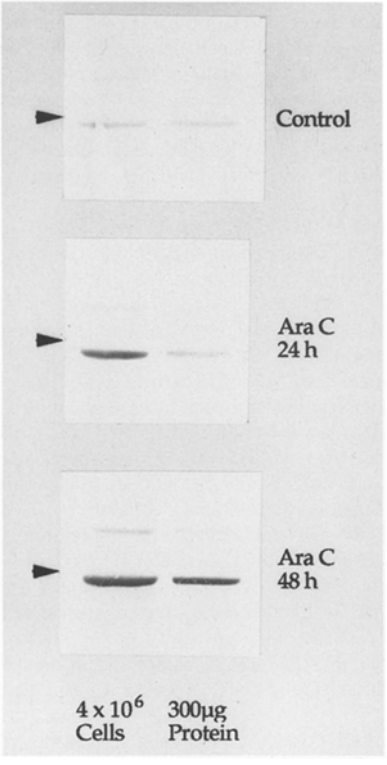

Fig. 5. Measurement of topoisomerase II p170-α levels by Western blotting. Western blots of DNA topoisomerase II in cell lysates of CCRF-CEM cells pretreated with ara C as indicated. Equal amounts of protein (300 μg) or equal numbers of cell lysates were separated on 7.5% SDS polyacrylamide gels and immunoblotting was carried out as described in Materials and methods. The *arrowheads* indicates the position of the 180-kDa molecular-weight marker

The enhancement of etoposide activity appears to correlate with the effect of ara C on cell-cycle kinetics. Short pretreatment times of up to 12 h did not affect etoposide-induced cytotoxicity or DNA damage. In contrast, 24- and 48-h incubations with ara C increased etoposide activity by greater than 2 times. The increased sensitivity of ara C-pretreated cells was probably partially due to elevated enzyme levels, which resulted in a concomitant increase in cleavable complex formation. We found a 3- to 5-fold increase in topoisomerase II protein in lysates of cells pretreated with ara C that might partially account for the heightened sensitivity of these cells to the action of etoposide. It has previously been demonstrated by Lorico et al. [27] that low-dose methotrexate induces an S-phase block in human lymphoma cells (U937) along with a concomitant increase in topoisomerase II levels and in the etoposide sensitivity of the cells. Similarly, Bakic et al. [1] demonstrated potentiation of *m*-AMSA activity by ara C in sensitive and resistant mutants of HL60 leukaemia cells; however, they found no increase in decatenating activity in nuclear extracts of HL60 human leukaemia cells. These authors suggested that the potentiation of *m*-AMSA activity was a result of changes in chromatin structure induced by ara C pretreatment. The apparent discrepancy between the findings of Bakic et al. and those presented herein may be due to the different cell lines used in the two studies; unlike CCRF-CEM cells, HL60 cells differentiate to mature myeloid cells in the presence of non-cytotoxic doses of ara C [5]. Alternatively, this discrepancy may result from the different methods used to measure topoisomerase levels. Topoisomerase II levels were assayed by Bakic et al. [1] as decatenating activity in 0.35 M salt extracts of HL60 nuclei. However, nuclear extracts may contain only a fraction of the topoisomerase activity measured in the whole cell [14, 15, 46]. Unless extracts are prepared in the presence of a wide range of protease inhibitors, the enzyme is rapidly degraded and consequently shows reduced activity [46]. Moreover, the extracts do not contain the nuclear matrix-bound enzyme, which in CCRF-CEM cells has been demonstrated to correlate with the sensitivity of these cells to etoposide [14, 15]. In our study, enzyme levels were measured by immunoblotting of whole-cell lysates, which were prepared by sonication in the presence of a wide range of protease inhibitors (see Materials and methods). This method results in disruption of nuclear matrices but does not cause any observable proteolysis of the enzyme.

The effects of ara C pretreatment on etoposide-induced DNA damage and cytotoxicity were complex. There was no direct relationship between enzyme levels, DNA damage and cytotoxicity. Both DNA damage and topoisomerase levels were higher in cells pretreated with ara C for 48 h than in those pretreated for 24 h but the cytotoxicity was not further potentiated by the longer pretreatment period. This finding is consistent with the results of previous studies using topoisomerase II-interactive drugs, which show that DNA damage is greatest in mitotic cells but that maximal sensitivity to the cytotoxic action of the drug occurs in S-phase cells [6, 12].

The increased sensitivity of S-phase cells to topoisomerase inhibitors is not well understood, but several lines of evidence suggest that events following cleavable complex formation are responsible for the enhanced sensitivity of these cells to the inhibitors [8, 18, 19, 45]. D'Arpa et al. [8] have presented a hypothesis that during the S phase, the replication machinery (DNA and RNA) collides with the cleavable complex and converts reversible SSBs into lethal lesions. This hypothesis is supported by the finding that inhibition of DNA or RNA synthesis can partially protect cells from the cytotoxic effects of *m*-AMSA [8]. In the present study, low-dose ara C treatment significantly increased the proportion of cells in the S phase without causing complete DNA-synthesis arrest. FACS analysis demonstrated that cells continue to synthesise DNA and progress to the G_2 phase in the presence of 0.02 μM ara C. Thus, collisions of the replication and transcription machinery with cleavable complexes may be increased by synchronisation of cells with low-dose ara C. Cells synchronised in the S phase have also been demonstrated to show increased sensitivity to topoisomerase inhibitor-induced chromosomal aberrations and arrest of cells in the G_2 phase [2, 9, 17]. S-phase treatments of mammalian cells with etoposide have been demonstrated to arrest the cells irreversibly in the G_2 phase [2, 17]. Thus, ara C pretreatment could also potentiate this form of DNA damage.

In conclusion, low-dose ara C pretreatment results in cell synchronisation along with a significant increase in the intracellular topoisomerase content and a concomitant increase in etoposide-induced DNA damage. The synchronisation of cells in the S phase may also indirectly potentiate the cytotoxic effect of topoisomerase inhibtors by enhancing the events that occur between cleavable complex formation and cell death. The present observations may be of clinical relevance. Both ara C and etoposide are widely used in cancer chemotherapy. The enhanced cytotoxicity of etoposide occurs at clinically relevant concentrations of this drug, whereas the ara C dose that effectively synchronises cells is considerably lower than that normally used in clinical practice. Whether this synergy leads to a clinical advantage is dependent on the relative effects of the combination on normal and tumour cells. However, it has previously been demonstrated in murine studies that ara C pretreatment increases the cytotoxicity of daunorubicin towards leukaemic colony-forming units whilst the cytotoxicity towards normal colony-forming units remains unchanged [11].

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