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# Potentiation of Etoposide Cytotoxicity against a Human Ovarian Cancer Cell Line by Pretreatment with Non-toxic Concentrations of Methotrexate or Aphidicolin

**Eugenio Erba, Soumitra Sen, Aurelio Lorico and Maurizio D'Incalci**

**Exposure of human ovarian cancer SW626 cell line to 0.08 μmol/l methotrexate or 25 μmol/l aphidicolin for 24 h caused no cytotoxiciy but enhanced etoposide cytotoxicity. Methotrexate or aphidicolin treatment induced a reversible blockade at the beginning of S phase which was reversed upon drug removal with a consequent wave of synchronisation. The enhancement of etoposide cytotoxicity was not due to higher etoposide intracellular uptake in the methotrexate or aphidicolin-pretreated cells. The topoisomerase II content in methotrexate or aphidicolin pretreated SW626 cells was higher than in control cells assessed by western blotting or flow cytometry. The higher etoposide cytotoxicity observed after synchronization with methotrexate or aphidicolin was apparently unrelated to the number of drug-induced DNA–topoisomerase II complexes evaluated as DNA double strand breaks or DNA–protein crosslinks. These data support the view that etoposide-induced DNA–topoisomerase II complexes are more cytotoxic in cells which are in S-phase.**
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## INTRODUCTION

IT HAS been recently reported by this laboratory that pretreatment of the human histiocytic U937 cell line with non-toxic concentration of methotrexate that did not cause any detectable cytotoxicity, significantly enhanced the cytotoxic potential of etoposide. It was proposed that the methotrexate pretreatment was inducing synchronisation of U937 cells and that the higher sensitivity to etoposide was related to a increased proportion of S-phase cells [1]. The studies with U937 cells also indicated that methotrexate pretreatment was able to enhance the ability of etoposide to induce DNA breaks, presumably due to a relative increase in intracellular DNA–topoisomerase II enzyme levels. However, the possibility that the enhanced cytotoxicity of etoposide was due to some unknown mechanism related to complex perturbations of biochemical process(es) induced by the antifolate could not be excluded. In order to evaluate whether the phenomenon seen could be generalised for the different inhibitors of DNA synthesis and also for cancer cells with different biological properties and sensitivities to etoposide, the present study was carried out. The aim was to ascertain whether etoposide cytotoxicity was enhanced by pretreatment with non-toxic concentrations of methotrexate or by aphidicolin, a drug causing specific and reversible inhibition of DNA polymerase α and δ [2]. The study involved utilisation of an epithelial cancer cell line SW626, derived from a human ovarian carcinoma, to investigate the mechanism of potentiation of cytotoxicity.

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## MATERIALS AND METHODS

### *Chemicals*

Methotrexate was obtained from the National Cancer Institute, Bethesda, Maryland. Aphidicolin-17-glycinate HCl was a

gift of ICI, UK. Etoposide was from Bristol Meyers. $^3$H-etoposide (specific activity 14.8 GBq/mmol) was purchased from Moravek, USA. Rabbit anti-human topoisomerase II antibody (gel purified) was kindly provided by Dr Leroy F. Liu, Johns Hopkins University, Baltimore. Bromodeoxyuridine (BrdU) and anti-BrdU were from Becton Dickinson. EDTA (disodium salt) and anti-mouse IgG developed in goat were from Sigma. Propidium iodide (PI) and normal goat serum were purchased from Calbiochem Corporation and Dakopatts, Denmark, respectively. RPMI 1640 cell culture medium was obtained from Gibco, while fetal bovine serum (FBS) (batch 9080802) was purchased from Flow. $^3$H-thymidine ($^3$HThd) (specific activity 3.07 GBq/mmol and 4.77 GBq/mmol) was from Amersham. Tetrapropyl ammonium hydroxide and proteinase K were purchased from Fluka, Milan and EM Laboratories, Germany, respectively.

*Cell and culture conditions*

Human ovarian carcinoma SW626 cells were grown in monolayer culture in RPMI 1640 medium supplemented with 10% FBS under standard culture conditions described elsewhere [3]. The cells were tested for mycoplasma presence every 3–4 weeks using the Hoechst 33258 test according to the ATCC quality control methods for cell lines.

*Treatment protocols*

Exponentially growing cells were incubated with 0.08 μmol/l methotrexate or 25 μmol/l aphidicolin for 24 h under standard culture conditions. After treatment, the drug containing medium was removed, washed with phosphate-buffered saline (PBS) and fresh medium was provided. At different intervals synchrony was checked by flow cytometric procedures. Methotrexate concentrations and the exposure times have been optimised using RPMI 1640 medium with 10% FBS (cat.no.011-06290, batch no.30G8110S, Gibco). It can be expected that a different protocol should be followed using a different medium with different folate concentrations (i.e. the level of folic acid in human plasma are approximately 100-fold lower than those present in RPMI 1640 medium).

Etoposide was dissolved in very small amounts of dimethyl sulphoxide (DMSO) and diluted further in medium. The final DMSO concentration in medium after treatment was always less than 0.01%. The exponentially growing and the cells pretreated with methotrexate or aphidicolin were treated for 2 h at 37°C at different times from the end of pretreatment.

*Flow cytometric analyses*

Monoparametric cell cycle analysis using PI and biparametric PI/BrdU content analysis using the BrdU indirect immunofluorescent method [4] were performed by FACS Star Plus systems (Becton Dickinson) and the data were analysed with the Hewlett Packard Consort 30 computer system. The method of Krishan and Frei [5] was used to calculate the cell cycle phase distributions.

*Clonogenicity assay*

The exponentially growing cells as well as cells pretreated with methotrexate or aphidicolin were treated with etoposide for 2 h at 37°C. In the synchronised cells the treatment with etoposide was done at different times of recovery after pretreatment with methotrexate or aphidicolin. Clonogenicity was tested by seeding cells at a very low density in petri dishes under standard culture conditions. The mean (S.D.) plating efficiency of the control cells ranged from 53.03 (4.32)% to 62.4 (8.9)% which was normalised to 100%. Methotrexate-synchronised or aphidicolin-synchronised cells did not show plating efficiencies which were significantly different from the controls. They ranged between 56.1 (4.1) and 61.5 (6.3). Percentages of survival after drug treatment in synchronised or exponentially growing cells was calculated with respect to the control.

*DNA double strand breaks assay*

Partial labelling of DNA strands was ensured by incubating exponentially growing cells to 3.7 kBq/ml $^3$H-Thd added to the medium for 48 h. Cells were then washed and resuspended in ordinary medium and chased at least for 24 h prior to treatment with etoposide. During this 24 h, cells were pretreated with methotrexate or aphidicolin before etoposide treatment. A DNA double strand breaks (DNA-DSB) assay was performed with proteinase K at pH 9.6 (neutral elution) by the method of Kohn [6]. Drug-induced DNA-DSB was compared with the calibration curve obtained by irradiating cells by various doses (1500, 3000, 4500 and 6000 rads) of γ-rays. The results are expressed as DNA-DSB in rad equivalents.

*DNA protein crosslinks assay*

Partial labelling of DNA strands was ensured by incubating exponentially growing cells to 3.7 kBq/ml $^3$H-Thd added to the medium for 48 h. Cells were then washed and resuspended in ordinary medium and chased at least for 24 h before treatment with etoposide. During this 24 h, cells were pretreated with methotrexate or aphidicolin before etoposide treatment. At 6 h after methotrexate or aphidicolin pretreatment, cells were treated with etoposide. Drug-induced DNA–protein crosslinks (DPC) were detected by alkaline elution at pH 12.2 by the method of Zwelling *et al.* [7]. Drug-induced DPC were expressed as rad equivalents with respect to the crosslinks produced by 3000 rad γ-rays in the same set without drug treatment.

For detection of DPC to newly synthesised DNA, particularly after synchronisation, the cells were first synchronised with methotrexate or aphidicolin and then allowed to recover in synchroniser-free medium for 6 h. The newly synthesised DNA was labelled with $^3$H-Thd (specific activity 4.77 GBq/mmol) for 30 min using 74 kBq/ml $^3$H-Thd and etoposide treatment was done for 1 h in presence of $^3$H-Thd. Drug-induced DPC were expressed as rad equivalents with respect to the crosslinks produced by 2000 rad γ-rays in the same set without drug treatment. Exponentially growing cells were similarly labelled and etoposide-induced DPC were quantitated.

*Drug retention study*

Exponentially growing cells as well as synchronised cells, 6 h after synchronisation with methotrexate or aphidicolin, were incubated with 2.5 μmol/l [$^3$H-(G)] etoposide (Moravek, Brea, California) for 2 h under standard culture conditions with gentle agitation. At the end of the incubation, the medium containing labelled etoposide was removed, the cells washed with ice cold PBS and fresh culture medium was provided.

For uptake studies, the cells were washed and lysed immediately after the 2 h incubation with $^3$H-etoposide and the total intracellular radioactivity was measured by liquid scintillation spectrometry (LSS). For efflux studies at specific points of recovery, the medium was removed completely, cells were detached, lysed and the radioactivity retained in the cells was measured by LSS. The values initially calculated as intracellular $^3$H-etoposide concentration (cpm)/million cells were converted

in cpm /$\mu^3$ because the synchronised cells had a bigger diameter. For comparison of the efflux rates, the relative percentages of $^3$H-etoposide retained at different recovery times were calculated keeping the uptake value as 100%.

*Quantitation of DNA–topoisomerase type II*

*Immunoblotting.* After drug treatment, SW626 cells were washed three times in cold PBS in the presence of 1 mmol/l benzamidine, 1 mmol/l phenylmethylsulphonyl fluoride (PMSF), 10 μg/ml soybean trypsin inhibitor and 50 μg/ml leupeptin. Whole cell lysates were prepared by lysing the cells with 2% sodium dodecyl sulphate (SDS) in PBS at 68°C for 5 min. The lysates (4-2-1 x $10^5$ cell equivalents/lane) were separated in 7.5% SDS–polyacrylamide gel and the proteins were transferred to a nitrocellulose filter by electroblotting. Blots, blocked with 1% BSA in TBST (100 mmol/l Tris, pH 7.5, 150 mmol/l NaCl, 0.05% Tween 20) and probed with anti-human recombinant topoisomerase II antiserum (kindly donated by Dr L. Liu, Johns Hopkins University). Following three 5 min washes in TBST, the blots were incubated with peroxidase-conjugated goat anti-rabbit IgG (ICN Immunologicals, Lisle, Illinois). Diaminobenzidine was subsequently used for the detection of topoisomerase II.

*Flow cytometry.* At specific times after pretreatment with synchronising agents, the cells were harvested, washed with cold PBS, and nuclear preparations were made by incubating cells in a hypotonic buffer (1 mmol/l $KH_2PO_4$, 5 mmol/l $MgCl_2$, 150 mmol/l NaCl, 1 mmol/l EGTA, 0.1 mmol/l DTT, 0.2 mmol/l PMSF and 0.3% Triton X) for 10 min at 4°C and fixed in 50% methanol. Staining for nuclear topoisomerase II was done strictly by the method of Smith and Makinson [8]. In short, the fixed nuclear preparations were incubated with rabbit anti-human topoisomerase II antibody (1:25) for 120 min, washed and incubated for a further 120 min with swine anti-rabbit IgG conjugated with FITC (1:20). Reaction was stopped with addition of 5 μg/ml PI and the samples were analysed by FACS Star Plus after 120 min.

## RESULTS

Pretreatment of SW626 cells with either 0.08 μmol/l methotrexate or 25 μmol/l aphidicolin for 24 h clearly increased the cytotoxic potential of etoposide as assessed by colony formation assay. Methotrexate or aphidicolin did not affect the colony formation indicating that the concentrations of the drugs were not cytotoxic *per se*. The enhancement of etoposide cytotoxicity

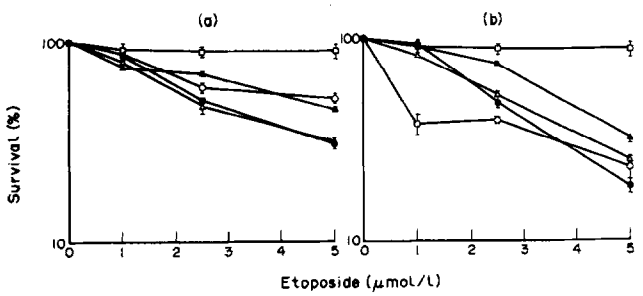


**Fig. 1. Influence of methotrexate (a) or aphidicolin (b) pretreatment on the cytotoxicity of etoposide. □ Etoposide alone or at ○ 2 h, ● 4 h, △ 6 h, ▲ 24 h after methotrexate or aphidicolin pretreatment. Cytotoxicity of etoposide at 2.5 and 5 μmol/l was significantly higher after pretreatment with methotrexate ($P < 0.01$) or aphidicolin ($P < 0.01$) (ANOVA and Tukey tests).**

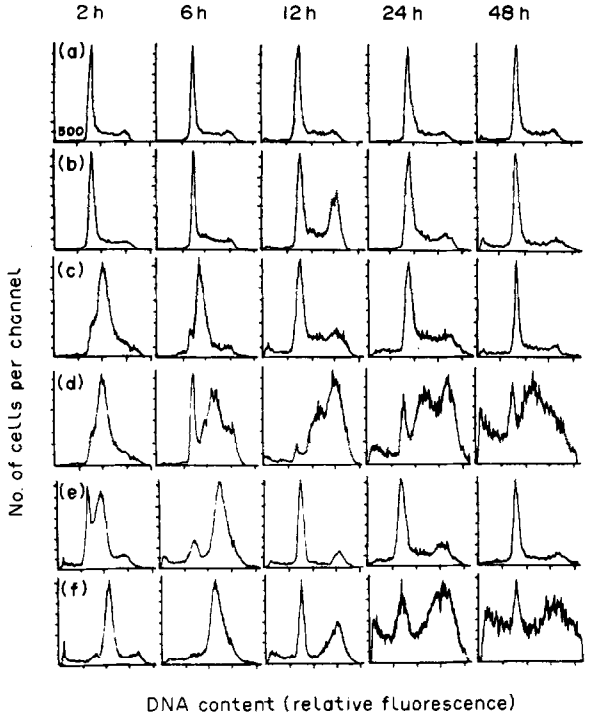


**Fig. 2. Influence of methotrexate or aphidicolin pretreatment on the cell cycle phase perturbations caused by 25 μmol/l etoposide treatment in SW626 cells by flow cytometry at different intervals after etoposide washout. (a) Control cells; (b) cells treated with 25 μmol/l etoposide for 2 h; (c) 0.08 μmol/l methotrexate for 24 h; (d) 0.08 μmol/l methotrexate for 24 h and after 2 h recovery time with 25 μmol/l etoposide for 2 h; (e) 25 μmol/l aphidicolin for 24 h; (f) 25 μmol/l aphidicolin for 24 h and after 2 h recovery time with 25 μmol/l etoposide for 2 h.**

in methotrexate or aphidicolin synchronised SW626 cells was evident for each etoposide concentration tested and when treated at 2, 4, 6 or 24 h after synchronisation (Figs 1a, b).

The potentiation of the cytotoxicity of etoposide on cells pretreated with non-toxic concentrations of methotrexate or aphidicolin was also assessed by studying the drug-induced cell cycle perturbations by flow cytometric methods. As shown in Fig. 2, methotrexate (panel c) and aphidicolin (panel e) caused a wave of synchronisation with a marked enrichment of S-phase cells from 2 to 6 h after drugs removal. The synchronisation produced by methotrexate or aphidicolin was no longer evident at 24 h of recovery time. A 2-h exposure with 25 μmol/l etoposide produced a moderate accumulation of cells in SL-$G_2$M phase of the cell cycle at 12 h recovery time (panel b). In methotrexate (panel d) or aphidicolin (panel f)-pretreated cells, etoposide showed a more marked and persistent accumulation in SL-$G_2$M phases and at 24 and 48 h after recovery from drug treatment, the majority of the cells were in the SM, SL-$G_2$M phases of the cell cycle.

In order to verify whether the enhancement of etoposide cytotoxicity could depend on changes in the rate of DNA synthesis in cells pretreated with methotrexate or aphidicolin we performed a DNA/BrdU biparametric analysis. The elaboration of flow cytometric data (see Fig. 3) showed that the rate of progression of BrdU-preselected SW626 cells through S phase, calculated as relative movement of the BrdU-positive S phase cells, was the same as in control cells or in cells pretreated with aphidicolin (Fig. 3) or methotrexate (data not shown).

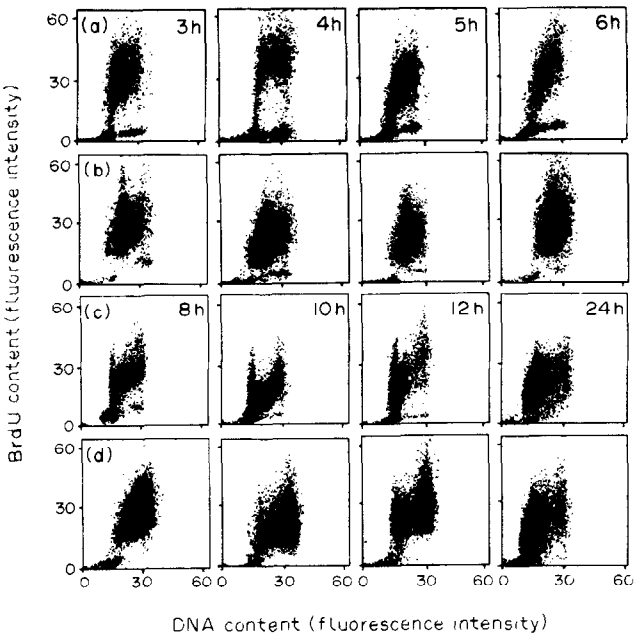


**Fig. 3. Biparametric DNA/BrdU analysis showing the movement of SW626 cells preselected with BrdU during the last 20 min of 24 h aphidicolin treatment and evaluated at different recovery time after aphidicolin treatment. (a) and (c) control cells; (b) and (d) aphidicolin-treated cells.**

We then asked the question whether the synergism could be due to a higher uptake or retention of etoposide in methotrexate or aphidicolin-pretreated SW626 cells. Since it is well established that the cells in S-phase have a greater cell volume, we have expressed etoposide intracellular concentration per $\mu m^3$, calculating the cell volume (in $\mu^3$) using a Coulter channelyser, in control or in methotrexate or aphidicolin-pretreated SW626 cells. As shown in Fig. 4, at the end of etoposide exposure and at subsequent time intervals the etoposide intracellular concentration was not significantly different in control cells and in methotrexate or in aphidicolin-pretreated cells.

A considerable amount of experimental data suggest that

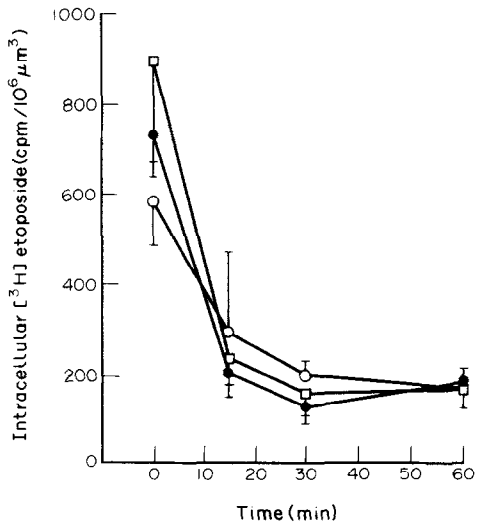


**Fig. 4. Intracellular concentration of $^3$[H]-etoposide in logarithmically growing SW626 cells (□) or in 0.08 μmol/l methotrexate (●) or 25 μmol/l aphidicolin (○) pretreated cells for 24 h and after 6 h recovery time treated with etoposide (48 μmol/l cold etoposide + 2 μmol/l $^3$H-etoposide).**

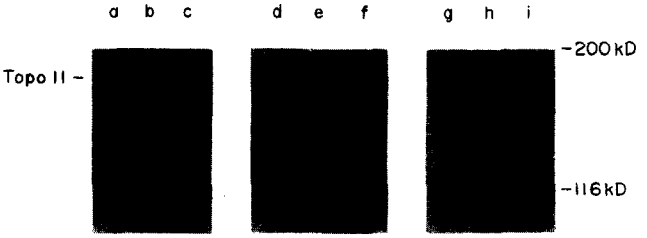


**Fig. 5. Immunoblotting of SW626 cell lysates after SDS-PAGE. Cells in exponential growth were treated with methotrexate or aphidicolin for 24 h, washed, resuspended in drug-free medium and analysed 6 h later. (a), (b) and (c) = control cells ($10^5$, $2 \times 10^5$, $4 \times 10^5$ cell equivalents, respectively); (d), (e) and (f) = cells treated with 25 μmol/l aphidicolin ($10^5$, $2 \times 10^5$, $4 \times 10^5$ cell equivalents, respectively); (g), (h) and (i) = cells treated with 0.08 μmol/l methotrexate ($10^5$, $2 \times 10^5$, $4 \times 10^5$ cell equivalents, respectively).**

etoposide cytotoxicity is related to its ability to induce the formation of DNA–topoisomerase II cleavable complexes. Previous studies by this laboratory suggested that methotrexate synchronised U937 cells had a higher topoisomerase II content 6 h following treatment, corresponding to the maximum potentiation of etoposide cytotoxicity. We therefore determined topoisomerase II content comparatively in logarithmically growing SW626 cells or in the same cells pretreated with methotrexate or aphidicolin. For these determinations, we used two independent methods such as western blotting and flow cytometric evaluation. In Fig. 5, cell lysates from SW626 cells prepared after 6 h from the end of the treatment with methotrexate or aphidicolin were analysed for topoisomerase II levels by immunoblotting. An approximately 4-fold increase in enzyme levels was observed after treatment with drugs, compared to control cells.

Figure 6 shows the biparametric analysis of DNA content versus topoisomerase II content using flow cytometric technique on exponential and synchronised SW626 cells. The fluorescence level related to the topoisomerase II content, evaluated after 6 h of recovery time from 24 h methotrexate (b) and aphidicolin (c) treatment, was increased with respect to the exponentially growing control cells (a).

It has been previously reported that a good relationship exists between etoposide induced DNA-DSB and its cytotoxic potential. Therefore it appeared a reasonable hypothesis that the potentiation of etoposide cytotoxicity in synchronised cells, which contained higher levels of topoisomerase II, could be due to a higher number of DNA-DSB. Table 1 shows the quantitative values of DNA-DSB produced by etoposide alone or by etoposide at 0, 2, 4, 6 or 24 h after synchronisation. The profile of etoposide-induced DNA-DSB in both the synchronised cell systems showed no significant change in the number of breaks and compares well with that induced by etoposide in exponentially growing unsynchronised cells. Also, the DPC were similar or even somewhat lower in methotrexate or aphidicolin-pretreated cells (data not shown). These data indicated that the higher etoposide cytotoxicity observed after methotrexate or aphidicolin pretreatment was apparently unrelated to the number of drug induced DNA–topoisomerase II complexes either evaluated as DNA-DSB or as DPC. These data were surprising in view of the finding that the levels of topoisomerase II were actually increased in methotrexate or aphidicolin-pretreated cells.

The alkaline elution assays were carried out labelling DNA with $^3$H-Thd before synchronisation. Therefore the DNA damage was assessed in parental DNA and not in the newly syn-

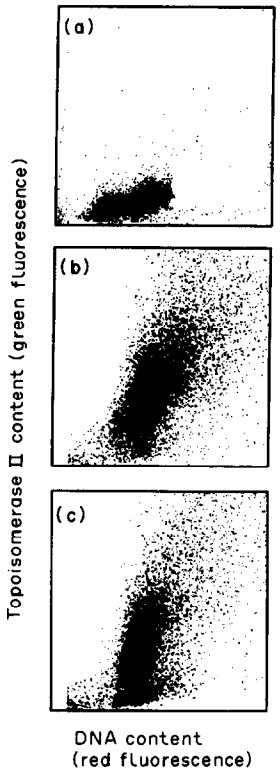


**Fig. 6. Flow cytometric biparametric DNA/topoisomerase II content analysis in logarithmically growing SW626 cells (a) or in methotrexate (b) or aphidicolin- (c) treated cells evaluated after 24 h treatment plus 6 h recovery time.**

thesised DNA. Considering that topoisomerase II is largely associated with newly synthesised DNA, we conducted a series of experiments to assess whether etoposide-induced DPC in newly synthesised DNA of cells previously synchronised with methotrexate or aphidicolin were higher than in logarithmically growing cells. For the determination of DPC in newly synthesised DNA, SW626 cells were exposed to a short pulse of $^3$H-Thd after synchronisation. As can be seen in Table 2, DPC to newly synthesised DNA in exponentially growing and synchronised cells 6 h after pretreatment with either methotrexate or aphidicolin did not show significant differences.

We then considered the possibility that DPC in newly synthesised DNA lasted longer in methotrexate or aphidicolin-treated SW626 cells. Figure 7 shows that the repair of DPC in synchronised cells was occurring at a similar rate.

*Table 1. DNA double strand breaks (DNA-DSB) (rad equivalents) caused by 100 μmol/l etoposide treatment in SW626 cells at different times of recovery after synchronisation with methotrexate or aphidicolin*

| Recovery time (h) | DNA-DSB | |
|---|---|---|
| | Methotrexate + etoposide | Aphidicolin + etoposide |
| 0 | 8889 (259) | 10567 (525) |
| 2 | 8132 (233) | 10235 (510) |
| 4 | 7630 (219) | 10908 (545) |
| 6 | 7592 (219) | 8354 (782) |
| 24 | 7156 (207) | 9044 (450) |

Mean (S.D.) of three experiments, in rad equivalents for DNA-DSB induced by various doses of γ rays.
100 μmol/l etoposide treatment in unsynchronised SW626 cells caused 9289 (689) rad equivalents DNA-DSB; this was not statistically different from those determined in methotrexate or aphidicolin synchronised cells.

*Table 2. Etoposide-induced DNA-protein crosslinks (DPC) in the newly synthesised DNA of exponentially growing cells and in cells after 6 h of recovery after synchronisation with methotrexate or aphidicolin*

| Etoposide concentration (μmol/l) | Unsynchronised | DPC | |
|---|---|---|---|
| | | Methotrexate-synchronised | Aphidicolin-synchronised |
| 50 | 415 ( 87) | 420 (32) | 344 (104) |
| 100 | 743 (258) | 596 (63) | 610 ( 50) |

Mean (S.E.) in rad equivalents.

## DISCUSSION

When human epithelial ovarian cancer SW626 cells are pretreated with non-toxic concentrations of methotrexate or aphidicolin, they become more sensitive to subsequent exposure to etoposide. The enhancement of etoposide cytotoxicity does not appear to be due to a different intracellular etoposide concentration, which was not markedly influenced by pretreatment with methotrexate or aphidicolin. We previously reported that etoposide cytotoxicity was enhanced in human histiocytic lymphoma U937 cells which were pretreated with low concentrations of methotrexate [1]. In U937 cells methotrexate caused a marked enrichment of S-phase cells, with an increase in the intracellular concentrations of topoisomerase II. It was therefore proposed that the increase in etoposide cytotoxicity was related to the higher number of etoposide-induced DNA–topoisomerase II complexes. In addition, a greater number of protein-linked DNA breaks was demonstrated after methotrexate pretreatment in this cell line.

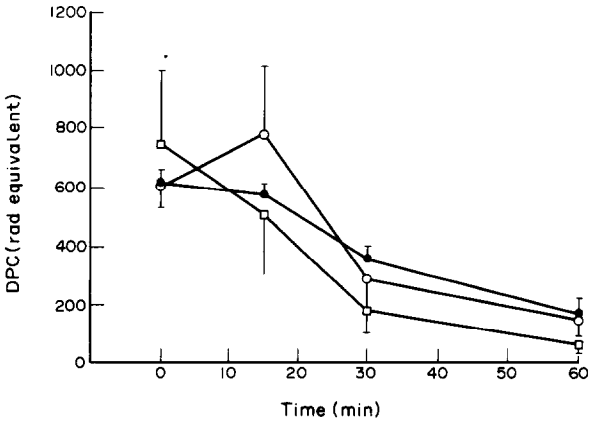


**Fig. 7. Influence of methotrexate or aphidicolin pretreatment on the disappearance of etoposide-induced DPC in SW626 cells. □ = exponentially growing cells treated for 2 h with 100 μmol/l etoposide; ○ = cells treated for 24 h with 0.08 μmol/l methotrexate and after 6 h recovery time treated for 2 h with 100 μmol/l etoposide; ● = cells treated for 24 h with 25 μmol/l aphidicolin and after 6 h recovery time treated for 2 h with 100 μmol/l etoposide.**

In SW626 cells methotrexate or aphidicolin pretreatment resulted in cell cycle synchronisation with a marked enrichment of S-phase cells. In addition both methotrexate and aphidicolin caused a clearcut increase in the levels of topoisomerase II assessed either by western immunoblotting or by flow cytometric methods. However, the number of etoposide-induced DNA-topoisomerase II complexes did not appear to be influenced by treatment with either synchronising agent. In fact, both the DNA-DSB or DPC caused by etoposide appeared similar in unsynchronised cells and in methotrexate or aphidicolin-pretreated cells. This was seen at different times after methotrexate or aphidicolin washout. Bearing in mind that topoisomerase II is associated with the replication fork [9] we have also evaluated etoposide-induced DPC in newly synthesised DNA in control SW626 cells or and SW626 cells pretreated with methotrexate or aphidicolin. However, in newly synthesised DNA the number of DPC caused by etoposide as well as their disappearance upon drug removal, did not appear to be influenced by the synchronising agents.

In our system it appears that the increase in the intracellular levels of topoisomerase II (at least of the form p170 [10] which reacted with the polyclonal antibody used by us), was not related to an increased number of DNA-topoisomerase II complexes as determined by DPC estimations.

The lack of correlation between drug-induced DNA–topoisomerase II complexes and cytotoxicity has been reported previously using different experimental systems. For example, Estey *et al.* [11] and Chow and Ross [12] investigated the DNA damage and cytotoxicity of 4′-(9-acridinylamino) methanesulfon-m-aniside (m-AMSA) and etoposide in relation to the cell cycle phases and found that the maximal cytotoxicity was evident in S-phase cells when there was also the maximal cellular concentration of topoisomerase II, whereas the maximal number of DNA-topoisomerase II cleavable complexes occurred in $G_2$-M phases. Chow and Ross [12] and Schneider *et al.* [13] reported that the cytotoxicity of m-AMSA and etoposide was reduced by concomitant treatment with cycloheximide whereas the number of DNA-topoisomerase II complexes was not decreased. More recently Chatterjee *et al.* [14] reported a clearcut dissociation between induction of DNA breakage and cytotoxicity of etoposide in a series of cell lines selected for alterations in poly(ADP-ribose) metabolism.

It appears therefore that our study as well as some reported in the recent literature support the view that the cytotoxicity of topoisomerase II inhibitors is not directly due to the formation of DNA–topoisomerase II complexes. The complexes probably activate several biochemical events which eventually lead the cell to death. The characterisation of these events in the cell cycle specific phases of cells is the object of investigation in several laboratories. The experimental system described here appears appropriate for these studies since the same amount of DNA damage (as measured by DSB or DPC) caused a much higher $G_2$ block and cytotoxicity in SW626 cells synchronised in S phase. The block in $G_2$ has been recently found to be associated with inhibition of p34 kinase activity, a protein complex which regulates the transition of cells from $G_2$ to mitosis. In addition it has been proposed that etoposide may induce chromosomal fragmentation and that the altered p34 kinase activity may be involved in this process. Fragmentation of internucleosomal DNA, typical of the phenomenon of apoptosis, has been recently reported to occur in cells exposed to etoposide [15]. Therefore it may be speculated that either the effects on p34 kinase or those leading cells to death (e.g. apoptosis) are enhanced when the cells are exposed to etoposide during the S-phase.


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