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INCREASE IN ETOPOSIDE-INDUCED TOPOISOMERASE II-MEDIATED DNA BREAKS AFTER CELL SYNCHRONIZATION INDUCED BY LOW DOSES OF METHOTREXATE

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Etoposide (VP-16) induces the formation of DNA-topoisomerase II cleavable complexes, which are converted into DNA single-strand breaks (SSB) and double-strand breaks (DSB) upon treatment with denaturating agents [1-2]. VP-16 shares this mechanism of action with many intercalating agents, such as doxorubicin, daunorubicin, ellipticines and m-AMSA. Different from intercalating agents, VP-16 neither intercalates nor binds to DNA, resulting in a more suitable model for the study of the role of cleavable complexes formation in the antitumor activity of these drugs.

It has been recently found that proliferating cells are more susceptible to this mechanism [3]. In quiescent cells VP-16 does not cause any DNA damage, whereas in cells in the S phase a maximum topoisomerase II-mediated DNA damage has been reported [3]. The present study was designed to investigate the effects of VP-16 on the formation of DNA-topoisomerase II cleavable complexes according to cell cycle progression, from the beginning of S phase to mitosis. For this purpose, a synchronization method with subcytotoxic doses of methotrexate (MTX) was developed.

Materials and Methods

Cell culture and treatment. The human hystiocytic lymphoma cell line U937 was cultured in RPMI 1640 tissue culture medium, supplemented with 10% fetal calf serum, 100 IU penicillin/ml, 100 μg streptomycin/ml and 2 mM glutamine. MTX (Lederle, Catania, Italy) was sterilely dissolved in saline just before use. VP-16, a gift of Bristol, Latina, Italy was dissolved in dimethyl sulfoxide. The final concentration of dimethyl sulfoxide in the culture medium was always less than 0.1%. Cells for growth delay experiments were plated onto 25 cm^2-flasks at a density of $5x10^4$ cells/ml. Following drug treatments, the cell number was determined by multiple hemocytometer counts every two days for 14 days. Cells were maintained in the logarithmic phase by dilution in growth medium. Cell number values represent a mean from triplicate flasks and are subject to an average error of less than 10%. When the growth rates were equivalent, the fraction of cells present in the drug-treated cultures compared to the untreated cultures was determined.

Cell cycle analysis. Flow cytometry studies were performed on a FACStar cell sorter (Becton-Dickinson, USA). Cells were stained with propidium iodide in hypotonic solution [4]. The percentages of the cell cycle phases were calculated by the method of Dean [5].

Alkaline elution studies. Asynchronously proliferating U937 cells were labeled for 24 hr with 0.05 μCi/ml 3H-thymidine followed by a 16-24 hr chase in medium without 3H-thymidine before treatment with MTX. The uniform labeling of all DNA strands of synchronized U937 cells was ensured by adding 0.05 μCi/ml 3H-thymidine 8 hr before and during the 16-hr treatment with MTX. DNA SSB and DSB were assayed by the alkaline elution method according to Kohn et al [6,7].

Results and discussion

Treatment of U937 with 0.02 μM MTX for 16 hr arrested most cells at the G1-S boundary in a reversible manner. At the end of MTX treatment, 73.5% of cells were in G1-S early phase (G_1S_E), 14.5% in S middle (SM) and 12% in S late-G_2M (S_LG_2M); soon after MTX wash out, cells restarted cycling in a synchronous way: 2 hr after, 43% were in G_1S_E, 38% in SM and 19% in S_LG_2M; 4 hr after, 29.5% were in G_1S_E, 39% in SM and 31.5% in S_LG_2M; 6 hr after, 9% were in G_1S_E, 30.5%

in SM and 60.5% in $S_L G_2 M$; 24 hr after, the cell cycle distribution was equal to control. MTX treatment did not cause any detectable delay in cell growth. Since MTX treatment may induce the formation of both SSB and DSB (A. Lorico et al., manuscript in preparation), we have investigated whether this was the case in our cellular system. As can be seen in Table 1, 0.02 μM MTX for 16 hr did not cause any detectable SSB or DSB. VP-16 alone caused 531±94 SSB, 1827±341 DSB and 53.8% growth inhibition. The maximal increase in etoposide-induced cytotoxicity and SSB formation was observed when most cells were passing through the S phase of the cell cycle, i.e. when the interval between the end of MTX treatment and the beginning of VP-16 treatment was from 0 to 4 hr. DSB formation was not correlated either with cytotoxicity or with SSB, since it was maximal for intervals of 4 to 6 hr, when a high percentage of cells were passing through the $S_L G_2 M$ phase. Further experiments were performed to evaluate whether newly synthesized DNA (labeled during MTX treatment) was more vulnerable to VP-16-induced protein-linked DNA breakage than bulk DNA (labeled before MTX treatment). In contrast to a recent report [8], no significant changes were observed between newly synthesized and bulk DNA.

TABLE 1. Effect of MTX and VP-16 in combination on DNA SSB, DNA DSB and cell growth.

	DNA SSB* (rad equivalents)	DNA DSB* (rad equivalents)	Growth inhibition (% of control)
MTX	0	0	2
VP-16	531+94	1827+341	53.8
MTX→ VP-16†	782+172	1300+0	93
MTX→ 2 h Rec.→ VP-16†	949+122	1455+63	92.6
MTX→ 4 h Rec.→ VP-16†	800+108	2887+219	92.8
MTX→ 6 h Rec.→ VP-16†	558+50	2823+247	82.2
MTX→ 24 h Rec.→ VP-16†	496+136	1850+205	60

* Values are expressed as mean of three independent replicates ± standard deviation.
† Cells were exposed to 0.02 μM MTX for 16 hr; after MTX wash-out, 1 μM VP-16 was added for 2 hr after 0,2, 4,6 and 24 hr of recovery in drug-free medium.

In conclusion, in U937 cells, synchronized in S phase by low MTX exposure, VP-16 caused the formation of more DNA-topoisomerase II cleavable complexes and greater cytotoxicity than in unsynchronized cells. In cells synchronized in $S_L G_2 M$, an increase in VP-16-induced SSB and DSB was observed but without a remarkable increase in cytotoxic effects, thus indicating that when DNA-topoisomerase II complexes are formed in premitotic phase or in mitosis they do not necessarily produce cytotoxic effects.

MTX-induced reversible cell cycle arrest can be exploited not only for molecular pharmacology studies requiring synchronization, but may be potentially useful to increase the antitumoral activity of VP-16 or other anticancer agents acting as topoisomerase II inhibitors.

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