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EARLY DNA DAMAGE INDUCED IN CELLS EXPOSED TO N10-PROPARGYL 5,8-DIDEAZAFOLIC ACID (CB 3717) OR METHOTREXATE

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Methotrexate (MTX) and many other folate analogues inhibit dihydrofolate reductase enzyme, causing cellular deprivation of reduced folates and, consequently, a block of both purine and thymidine nucleotides synthesis [1]. A direct inhibitory effect of MTX on thymidylate synthase enzyme has also been described [2]. Though these metabolic effects lead to inhibition of DNA synthesis and therefore explain the cytostatic effect of MTX, they do not necessarily account for its cytocidal effect. Thymine-less death has been proposed to explain the mechanism of cytotoxicity of MTX [3]; the block of de novo synthesis of thymidylate causes growing cells to die, possibly because of misincorporation of uracil into DNA. Li and Kaminskas [4] reported that MTX caused single strand breaks in mature DNA of Ehrlich ascites tumor cells and found a good correlation between accumulation of breaks and cytotoxic effect of antifolates.

We have compared N^{10}-propargyl-5,8-dideazafolic acid (CB 3717), which directly inhibits thymidylate synthase [5], with MTX for its antiproliferative activity and mode of action on two different cell lines.

Materials and Methods

Cell culture. The murine fibroblast cell line NIH/3T3R is a secondary transfectant obtained from transfection of NIH/3T3 cultures with the T24 Ha-ras oncogene [6]. NIH/3T3R and the human melanoma cell line M14 were cultured in DMEM and RPMI 1640 respectively.

Colony-forming assay. Exponentially growing cells, detached from 25 cm^2-plastic flasks with 0.05% trypsin-0.02% EDTA in PBS, were plated into 6 cm -Petri dishes at a density of 500 to 1500 cells into each dish. Following drug treatment, cells were washed twice and the plates were incubated for 1 week in fresh medium. A colony was defined as more than 50 cells in close proximity.

Alkaline elution. DNA single-strand breaks (SSB), double-strand breaks (DSB) and DNA-protein cross-links (DPC) were assayed according to Kohn (7).

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 [8].

Results and discussion

CB 3717 was as active as MTX on both M14 and NIH/3T3R cell lines in inhibiting colony formation, but 20-100 times less potent. For both cell lines, the cytostatic effect increased with time of exposure, being already evident after 8 hr of treatment. Flow cytometry analysis of propidium iodide-stained nuclei showed an accumulation of cells in G1 phase of the cell cycle after 24 hr treatment with either drugs, probably because of inhibition of DNA synthesis and blockage at the G1-S boundary. In NIH/3T3R cells treated for 16 hr with 2 uM MTX or 200 uM CB 3717, we found SSB amounting to approximately 120 and 110 rad equivalents respectively (Table 1) and a considerable number of DSB, 1600 ± 436 and 1660 ± 168 respectively. The amount of DSB was far more than expected if they had been the result of the proximity of SSB on the two complementary DNA strands. No DPC were detected. When cells were incubated in drug-free medium for 6 hours, there was a further accumulation of SSB, possibly due to the effects of the drug retained intracellularly as polyglutamyl derivative (Table 1). Simultaneous treatment with cycloheximide, prevented DNA damage and cytotoxicity produced by both drugs (Table 1 and 2).

Table 1. Single-strand breaks in NIH/3T3R cells

	TOTAL DNA SSB (rad equivalents)*
MTX 2μM 16hr	118 (93-148)
MTX 2μM 16hr + 8hr recovery	184 (169-199)
MTX 2μM 16hr + cycloheximide 1.77μM 16 hr	0
MTX 2μM 16hr + thymidine 10μM 16hr	11 (9-12)
CB 3717 200μM 16hr	106 (82-148)
CB 3717 200 μM 16hr + 8hr recovery	172 (156-188)
CB 3717 200 μM 16hr + cycloh. 1.77μM 16 hr	17 (10-26)
CB 3717 200 μM 16hr + thymidine 10 μM	0
MTX 2 μM 16 hr†	23 (10-31)
CB 3717 200 μM 16 hr†	11 (5-15)

* mean and (range) of at least 3 values.
† drug treatment on confluent cells.

Table 2. Colony formation assay in liquid medium

	CONTROL	MTX 2μM 16 h	CB 3717 200μM 16 h
-----	257±15†	12±4	11±5
Thymidine 10 μM	235±10	254±18	241±23
Cycloheximide 0.35 μM*	160±22	136±15	155±14

*At this concentration, cycloheximide inhibited 3H-thymidine incorporation into DNA by 38%.
†Number of colonies per plate ± standard error.

Since after 16 hr treatment with MTX or CB 3717, cells were completely viable, as assessed by three different methods (data not shown), DNA damage does not appear to be the result of cell death with consequent DNA degradation, but an early event preceeding cell death.

In conclusion, the present study reinforces the relationship between DNA breakage and thymidine nucleotide deprivation. In fact, while MTX affects both thymidine nucleotide and purine synthesis, CB 3717 inhibits only thymidine nucleotide synthesis (5) and therefore the observed DNA damage is presumably related to thymidine starvation with consequent dUTP misincorporation into DNA.

This is the first report of DSB formation after MTX treatment, and it is in line with the observation that thymidylate synthase-negative mutants accumulate DSB when grown in medium without thymidine [3]. At present, however, the molecular mechanism by which this fragmentation occurs is still to be elucidated.

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