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# Drug Concentration-Dependent DNA Lesions Are Induced by the Lipid-Soluble Antifolate, Piritrexim (BW301U)

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## SUMMARY

The lipid-soluble folate antagonist, 2,4-diamino-6-(2,5-dimethoxybenzyl)-5-methylpyrido[2,3-*d*]pyrimidine (piritrexim; BW301U), induced misincorporation of dUMP in human B (SB)- and T (MOLT-4)-lymphoblastoid cells, and in human promyelocytic leukemia cells (HL-60). Analysis by alkaline sucrose gradients and alkaline elution indicated that $^3$H-DNA that had been labeled for 15 min distributed into progressively smaller DNA fragment sizes in a drug concentration-dependent manner from 0 μM to 50 μM piritrexim. This phenomenon was observed regardless of the labeled nucleotide precursor employed for detection of newly synthesized DNA ([$^3$H]deoxyuridine, [$^3$H]deoxyadenosine, or [$^3$H]deoxycytidine). In contrast, formaldehyde denaturation and sedimentation of DNA in neutral denaturing sucrose gradients released only 3–4% of the newly synthesized DNA as 3S–6S fragments (80–200 nucleotides), whereas the remaining population of newly synthesized DNA pelleted to the bottom of the tube. Failure to detect DNA fragmentation under neutral conditions to the extent observed under alkaline conditions indicated the presence of apurinic and apyrimidinic sites in DNA—lesions which would be expected in DNA undergoing excision-repair of misincorporated dUMP. Cytotoxicity resulting from dUMP misincorporation was consistent with the enhanced toxicity of piritrexim which was observed when HL-60 cells or MOLT-4 cells were exposed concurrently to exogenous deoxyuridine. Deoxyuridine-enhanced toxicity was demonstrated to be concentration dependent for both cell lines when piritrexim concentrations were marginally toxic. The cytotoxic effect of dUMP misincorporation was further substantiated by the observation that MOLT-4 cells treated with 0.5 μM piritrexim alone eventually developed resistance to the drug, whereas treatment with both piritrexim and 10 μM deoxyuridine prevented the selection of piritrexim-resistant cells.

AP sites are among the most ubiquitous of DNA lesions and are a common consequence of many chemical (1–4) and repair processes (5) in DNA. AP sites have been demonstrated to be both mutagenic (6, 7) and recombinagenic (6, 8) and may underlie the mutagenic consequences of many base-modifying reagents (7).

Spontaneous events leading to AP sites in DNA occur on a regular basis. Lindahl and Nyberg (4) have estimated from arguments of chemical stability that up to 10,000 spontaneous depurination events occur per day in mammalian cells. Similarly, about 500 AP lesions are generated by spontaneous depyrimidination events (2), and spontaneous deamination of cytosine followed by DNA-glycosylase-mediated excision of the resulting uracil residue may generate up to 200 more apyrimidinic lesions per cell (3). Excision of uracil following misincorporation of dUMP in place of TMP in DNA is an additional source of apyrimidinic sites in replicating cells. Estimates based on reported levels of dUTP and TTP pool sizes (9, 10) predict that as many as 20,000 of these substitutions occur in mammalian cells during a single round of replication.

The occurrence of apyrimidinic sites in mammalian cell DNA is dramatically increased by antifolates which inhibit DHFR. Upon exposure to an antifolate, cellular dUMP pools increase by 300–1000-fold (10, 11), leading to detectable dUMP misincorporation (9, 12) as well as to the presence of alkaline labile AP sites in the DNA (12). Thus, although it has been generally assumed that antifolate cytotoxicity arises from thymidine, purine, and amino acid deficiency consequent to tetrahydrofolate depletion, it is also necessary to consider the possible cytotoxic and mutagenic potential exerted by these drugs through the amplification of AP lesions in DNA following the misincorporation of dUMP.

The studies presented in this paper confirm and extend previous reports of antifolate-induced dUMP misincorporation into DNA. However, whereas earlier studies documented this observation in cells exposed to methotrexate (9) or metoprine

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**ABBREVIATIONS:** AP, apurinic/apyrimidinic; DHFR, dihydrofolate reductase; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PBS, phosphate-buffered saline; EDTA, ethylenediaminetetraacetate; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; BSA, bovine serum albumin.

(12), this work demonstrates dUMP misincorporation in DNA of cells exposed to the lipid-soluble antifolate, 2,4-diamino-6-(2,5-dimethoxybenzyl)-5-methylpyrido[2,3-*d*] pyrimidine (piritrexim or BW301U) (13), a new second generation antifolate in clinical trials for use in cancer chemotherapy (14).[1] Because of the coincidental use of B-lymphoblastoid cells in both of the earlier reports of dUMP misincorporation (9, 12), a variety of cell types was used in our recent studies to ensure that dUMP misincorporation is a general effect of antifolate exposure. Here, results from experiments in three human blood cell lines, SB (B-lymphoblastoid cells), MOLT-4 (T-lymphoblastoid cells), and HL-60 (promyelocytic leukemia cells), are described. Antifolate exposure led to the concentration-dependent generation of alkaline labile AP sites in newly synthesized DNA and to an accumulation of small DNA fragments (3S–6S ssDNA), in addition to detectable levels of dUMP in DNA. This DNA damage resulting from dUMP misincorporation was found to be related to the cytotoxic effect of antifolates insofar as enhanced toxicity of piritrexim was achieved by concurrent exposure of cells to exogenous dU. The results suggest that repair of misincorporated dUMP may initiate a cascade of events at AP sites which subsequently augment, rather than reduce, the severity of the original lesion.

## Materials and Methods

**Cell culture.** MOLT-4 and SB cells were maintained at 300,000 cells/ml in RPMI 1640 media (Gibco) supplemented with 10% fetal calf serum and 25 units/ml of penicillin-25 μg/ml of streptomycin. HL-60 cells were maintained at 300,000 cells/ml in RPMI 1640 media supplemented with 10% fetal calf serum, 10 mM Hepes, and 50 units/ml of penicillin-50 μg/ml of streptomycin. All biochemical experiments with these cell lines were performed at a concentration of $1 \times 10^7$ cells/ml in RPMI 1640 media supplemented with 50 mM Hepes, 12.5 units/ml of penicillin-12.5 μg/ml of streptomycin, and 10% fetal calf serum which had been dialyzed versus 0.15 M NaCl (referred to as "experimental media").

**Pulse treatment of cells with piritrexim and $^3$H-labeled nucleoside.** Piritrexim was solubilized in water by titration with lactic acid and diluted to a final concentration of 1 mM. Less concentrated solutions were then prepared and neutralized to pH 7.2 to provide appropriate concentrations of piritrexim in experimental media.

Logarithmically growing cells with viability >95% were pelleted by centrifugation at 230 × *g* for 10 min in a tabletop centrifuge, then gently resuspended at $1 \times 10^7$ cells/ml in experimental media containing the indicated concentrations of piritrexim. The cells were preincubated at 37° for 15 min and transferred to tubes containing labeled nucleoside which had been evaporated to dryness with nitrogen gas. After incubation for 15 min at 37°, DNA synthesis was terminated by addition of cold PBS (0.145 M NaCl, 10 mM $Na_2HPO_4$, 0.9 mM $CaCl_2$, 0.49 mM $MgCl_2$, pH 7.4) or cold PBS-20 mM $NaN_3$. The samples were then kept on ice.

Labeled nucleoside preparations used in these experiments were: [6-$^3$H]dU (100 μCi/ml final concentration from a stock solution at 24.5 Ci/mmol from New England Nuclear or Amersham), [5-$^3$H]dC (100 μCi/ml final concentration from a stock solution at 26 Ci/mmol from ICN and supplemented with 0.4 μM unlabeled dU), or [8-$^3$H]dA (100 μCi/ml final concentration from a stock solution at 15 Ci/mmol from ICN and supplemented with 4 μM unlabeled dU).

**Detection of misincorporated dUMP in DNA.** Cells ($1 \times 10^7$ cells/ml) that had been pulse labeled for 15 min with [$^3$H]dU in the presence of 0 μM or 10μM piritrexim were either lysed in 0.15 M NaCl, 1.5 mM $MgCl_2$, 50 mM Tris, pH 7.4, by expression through a 27 gauge needle (1-ml tuberculin syringe) or homogenized in a Thomas Teflon-pestle tissue grinder. The lysates were then centrifuged at 500 × *g* for 5 min. The pellets, containing nuclei, were resuspended in 75 mM NaCl, 0.75 mM $MgCl_2$, 5 mM EDTA, 2% SDS, and 50 mM Tris, pH 7.4, and left at room temperature overnight. An equal volume of 2 N NaOH, 0.2 N NaCl, and 0.2 M EDTA was then added to each sample followed by incubation at room temperature for 1 hr. The DNA in these samples was then analyzed by centrifugation through 5–20% (w/w) alkaline sucrose gradients. Following collection of fractions, an aliquot of each was removed for analysis of 5% TCA-precipitable $^3$H-label to determine the distribution of newly synthesized DNA in the gradients (see below). The remaining portions of each sucrose gradient fraction were then pooled for identification and quantification of [$^3$H]dU in DNA. Pooled fractions were then dialyzed versus 0.15 M NaCl and concentrated to 0.3–0.4 ml. The DNA in the concentrated samples was then precipitated with 2 volumes of 95% ethanol at −20° in the presence of 0.5 mg of sodium acetate. Digestion of the DNA to nucleosides, separation by thin layer chromatography on cellulose plates, and preparation for scintillation counting were performed as described previously (12).

**Alkaline sucrose gradient analysis.** Except as described above, piritrexim-treated cells were washed in cold PBS, lysed in 2% SDS buffered with either 10 mM Hepes, 1 mM EDTA, pH 7.2, or 100 mM NaCl, 32 mM $Na_2HPO_4$, 5 mM EDTA, pH 7.2, and digested with 100 μg/ml of Proteinase K for 3 hr at 37°. After addition of an equal volume (100 μl) of 2 N NaOH, 0.2 N NaCl, 0.02 M EDTA, the samples were incubated at room temperature for 1 hr and poured onto 11.0-ml linear 5–20% (w/w) or 10–30% (w/w) alkaline sucrose gradients [5% or 10% sucrose solution: 0.5 mM EDTA, 0.2 N NaOH, and 0.8 N NaCl; 20% or 30% solution: 0.5 mM EDTA, 0.8 N NaOH, and 0.2 N NaCl (15)]. Aliquots of 10 mg/ml of BSA were prepared similarly without Proteinase K digestion and poured onto 5–20% or 10–30% companion gradients. Separation in the 5–20% gradients was performed in a Beckman SW-41 rotor for 3.5 hr at 38,000 rpm (242,000 × *g* at $r_{max}$; 20°). Separation in the 10–30% gradients was carried out similarly, except that centrifugation was for 18 hr instead of 3.5 hr. All gradients were eluted into fractions of ~⅓-ml volume by pumping the gradient from the bottom of the tube. DNA in each fraction was monitored by TCA precipitation onto 2.3-cm Whatman No. 3 filter paper disks followed by scintillation counting (see below). The peak fractions containing BSA were detected by monitoring for absorbance at 230 nm.

**Neutral denaturing sucrose gradient analysis.** Piritrexim-treated cells were washed in cold PBS, lysed in 100 mM NaCl, 32 mM $Na_2HPO_4$, 5 mM EDTA, 2% SDS, pH 7.2, and digested with 100 μg/ml of Proteinase K for 3 hr at 37°. DNA in these samples was then denatured under alkaline conditions, as above, or under neutral denaturing conditions with addition of an equal volume of 37% formaldehyde, 45 mM $Na_2HPO_4$, pH 7.2 (made up immediately before use) followed by incubation for 45 min at 45° (16). After exhaustive dialysis versus 100 mM NaCl, 32 mM $Na_2HPO_4$, 5 mM EDTA, and 1% formaldehyde, pH 7.2, to remove alkali or excess formaldehyde while maintaining denaturing conditions, the samples were poured directly onto 11.0-ml 10–30% (w/w) linear neutral denaturing sucrose gradients containing the same buffer. As described above for the alkaline sucrose gradients, an aliquot of 10 mg/ml of BSA was prepared similarly for use as a sedimentation standard. Centrifugation was carried out in a Beckman SW-41 rotor for 18 hr at 38,000 rpm (20°). Fractionation was accomplished by pumping the gradient from a point ~0.5 cm above the bottom of the tube, taking care to avoid disturbing the large DNA pellet. The pellet was considered as a separate fraction (0) and analyzed accordingly. DNA in an aliquot of each fraction was TCA precipitated onto 2.3-cm Whatman No. 3 filter paper disks and prepared for scintillation counting (see below). BSA in identical companion gradients was detected by monitoring for absorbance at 230 nm.

**Quantification of radioactive DNA.** Washed cells or aliquots of sucrose gradient fractions were quantified on filter disks as described previously (17), except that cold 95% ethanol was used instead of ether.

[1] W. D. Brenkman, Jr., V. Currie, J. Laszlo, H. Iland, E. Morgan, M. O'Hehir, T. Williams, and C. W. Young, submitted for publication.

After drying, the filter disks were counted in Betafluor (National Diagnostics) in a Packard Tri-Carb 3255 scintillation counter.

When necessary, RNA was hydrolyzed with 1 N NaOH (100 μl/disk) at 37° for 30–60 min after removing the Betafluor with two 15-min acetone washes and one wash with 95% ethanol. RNA nucleotides were removed with two 15-min washes of 5% TCA and one wash with 95% ethanol. The filter papers were then recounted with Betafluor, as before.

**Alkaline elution.** Alkaline elution was performed essentially as described by Kohn *et al.* for newly synthesized DNA (18) with minimal protein adsorption (19). Polycarbonate filters (25 mm diameter, 2 μm pore size) from Nuclepore were fitted into "smokestack"-type filter holders (19) and rinsed twice with 10–15 ml of cold PBS-20 mM $NaN_3$. Cells in 10 ml of cold PBS-20 mM $NaN_3$ were then poured into the filter reservoir and the PBS-20 mM $NaN_3$ was drained by gravity. The retained cells were then rinsed twice with 10–15 ml of cold PBS-20 mM $NaN_3$, again drained by gravity, and then lysed with 5 ml of 2% SDS, 0.025 M EDTA, pH 9.7, followed by 1 ml of 2% SDS, 0.025 M EDTA, 10% glycerol, 10 mM UMP, pH 9.7. (UMP was included in order to remove any $^3$H-labeled nucleotides which may have been adventitiously bound to DNA.)

Protease treatment of filters was performed with a solution of 0.5 mg/ml of Proteinase K in 2% SDS, 0.025 M EDTA, pH 9.7. Each filter was first washed with 1 ml of the solution, then incubated with three successive 1-ml aliquots of the same solution for 20 min at 37° (warm room). The filters were drained by gravity after each incubation. After Proteinase K treatment, the filters were washed with 5 ml of lysis buffer (2% SDS, 0.025 M EDTA, pH 9.7) and finally with 3 ml of 0.02 M $Na_3$EDTA, 0.1% SDS, 10% glycerol, pH 9.7. (Glycerol was added to determine the included volume of our apparatus by monitoring the change in refractive index of the eluted fractions.)

DNA was eluted from the polycarbonate filter at ~1.5 ml/hr with 6 ml of 0.04 M EDTA adjusted to pH 12.3 with tetraethylammonium hydroxide. Fractions were collected every 20 min until 5 ml of eluant were collected. Each filter was then removed, cut into small pieces, and placed in a scintillation vial containing the remaining eluting buffer. After allowing >2 hr for the DNA which remained on the filters to denature and solubilize in the alkaline eluting buffer, Hydrofluor scintillation cocktail (National Diagnostics) was added, and the samples were counted in a Packard Tri-Carb 3255 scintillation counter.

## Results

**Piritrexim exposure leads to misincorporation of dU.** Treatment of B-lymphoblastoid cells (SB), T-lymphoblastoid cells (MOLT-4), and promyelocytic leukemia cells (HL-60) with the lipid-soluble antifolate, piritrexim, led to misincorporation of exogenous [$^3$H]dU into DNA. After preincubation of cells for 15 min at 37° with or without piritrexim followed by a 15-min pulse exposure to 100 μCi/ml of [$^3$H]dU, the newly synthesized DNA was analyzed for [$^3$H]dU and [$^3$H]Thy content (see Materials and Methods). The data in Table 1 show the amount of [$^3$H]Thy and [$^3$H]dU detected in the DNA of these three cell lines after exposure to 0 μM or 10 μM piritrexim and indicates the percentage of labeled [$^3$H]dU which was incorporated as [$^3$H]dUMP.

In spite of the active uracil excision capacity of mammalian cells, piritrexim induced detectable incorporation of dU into DNA. In all three cell lines, [$^3$H]dU was detected in DNA only when the cells had been treated with piritrexim, and the amount of [$^3$H]dU detected ranged from 9% to 26% of the total label in DNA. In MOLT-4 cells, [$^3$H]dU was detected in DNA of cells treated with as little as 1 μM piritrexim.[2] The fact that three different cell types showed a similar response indicates that dU misincorporation is most likely a general effect of antifolates in hematopoietic cells and is not a response specific to a particular cell type or cell line.

TABLE 1

**Detection of [$^3$H]dU in newly synthesized DNA of piritrexim-treated cells**

Cells were preincubated for 15 min at 37° with or without piritrexim at a concentration of $1 \times 10^7$ cells/ml, then incubated for 15 min more with 100 μCi/ml of [$^3$H]dU. DNA synthesis was stopped by washing the cells in cold PBS and keeping them at 4°. Isolated nuclei from these cells were lysed with buffered 2% SDS, and the nuclear DNA was alkaline denatured. After sedimentation through 5–20% alkaline sucrose gradients, pooled fractions were dialyzed versus 0.15 M NaCl and concentrated. The DNA was recovered by precipitation with ethanol, digested to nucleosides, and then separated by thin layer chromatography.

| | MOLT-4 | | SB | | HL-60 | |
|---|---|---|---|---|---|---|
| | 0 μM | 10 μM | 0 μM | 10 μM | 0 μM | 10 μM |
| [$^3$H]dU (dpm/μg DNA) | | 63 | | 293 | | 274 |
| [$^3$H]Thy (dpm/μg DNA) | 26661 | 659 | 24,677 | 846 | 38,760 | 1,095 |
| Percentage of label incorporated as [$^3$H] dUMP | 0 | 9 | 0 | 26 | 0 | 20 |

**Accumulation of small DNA fragments in alkaline sucrose gradients is drug concentration dependent.** Alkaline labile DNA was expected to accumulate as a consequence of dU incorporation because of the active repair mechanisms for excision of this misincorporated nucleotide. Indeed, piritrexim-induced accumulation of newly synthesized DNA fragments was found to be drug concentration dependent under alkaline conditions. This is shown in the experiments of Fig. 1, where cells were exposed to piritrexim, lysed, and treated with Proteinase K, then analyzed by sedimentation through 5–20% alkaline sucrose gradients.

Fig. 1A shows the gradient profiles of DNA from SB cells exposed to 0 μM, 0.5 μM, 5 μM, and 50 μM piritrexim. For the gradient analyses presented, [$^3$H]dU incorporation into DNA was inhibited by 0, 85, 97, and 99%, respectively. Similarly, the size distributions of DNA from MOLT-4 cells exposed to 0 μM, 0.5 μM, 1 μM, and 50 μM piritrexim are shown in Fig. 1B and correspond to inhibition levels of 0, 84, 90, and 99%, respectively, for the incorporation of [$^3$H]dU into DNA.

A drug concentration-dependent increase in DNA fragments was observed in both cell types as demonstrated by the increase in the percentage of labeled DNA which sedimented near the top of the gradients as piritrexim concentrations were escalated. In the SB cells, there was also a shift in the bulk of the labeled DNA from fraction 17 in the 0 μM piritrexim profile to fraction 21 in the 5 μM piritrexim profile. This shift was not evident in the MOLT-4 cell profiles—probably because the piritrexim concentrations were too low (≤1 μM). At 50 μM piritrexim the position of the major peak became ambiguous with both cell types, and a substantial portion of the labeled DNA shifted to fractions 28–33 at the top of the gradient. These results are consistent with an antifolate concentration-dependent accumulation of AP sites in DNA which hydrolyze under alkaline conditions. Alternatively, however, the results could be explained by a drug concentration-dependent accumulation of small DNA fragments in response to antifolate treatment. Further experiments to distinguish between these two possibilities are described below.

**Accumulation of piritrexim-induced DNA fragments is independent of the precursor used to label DNA.** Since

[2] R. G. Richards and W. D. Sedwick, unpublished observation.

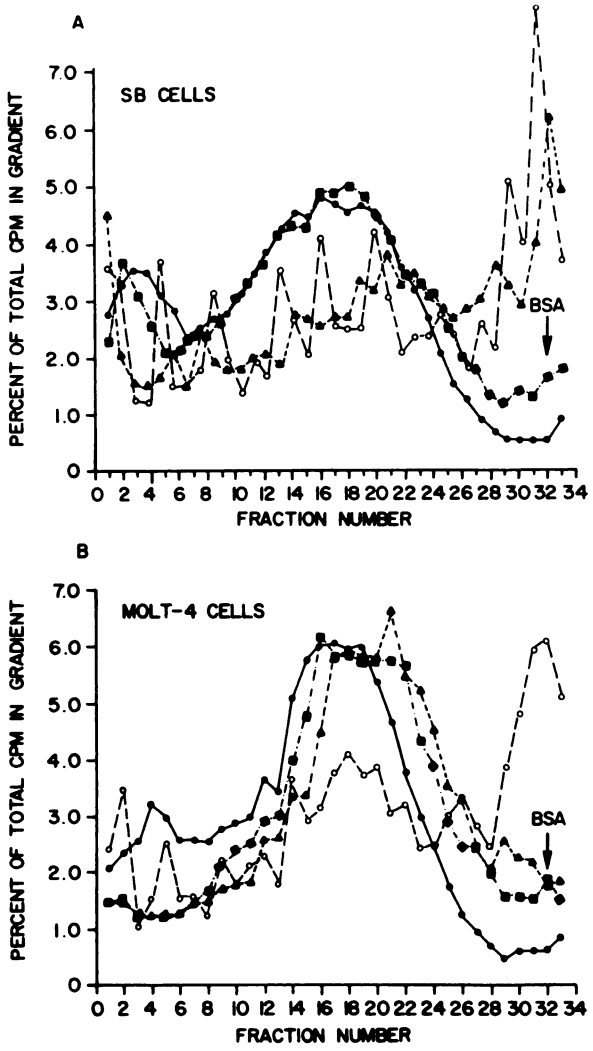


**Fig. 1.** Piritrexim induces concentration-dependent accumulation of [$^3$H]dU-labeled DNA under alkaline conditions. SB or MOLT-4 cells were preincubated for 15 min at 37° with piritrexim at a concentration of 1 × $10^7$ cells/ml, then incubated for 15 min more with 100 μCi/ml of [$^3$H]dU. The cells were lysed with 2% SDS buffered with 10 mM Hepes, 1 mM EDTA, pH 7.2, and treated with 100 μg/ml of Proteinase K for 3 hr at 37°. After alkaline denaturation with an equal volume of 2 N NaOH, 0.2 N NaCl, 0.02 M EDTA for 1 hr, the DNA of each sample was sedimented through 5–20% alkaline sucrose gradients. An aliquot of each gradient fraction was then TCA precipitated onto Whatman No. 3 filter paper disks and the amount of radioactivity was determined by scintillation counting. A. SB cells exposed to 0 μM (●), 0.5 μM (■), 5 μM (▲), and 50 μM (○) piritrexim. *BSA*, BSA marker (4.3 S). These concentrations of piritrexim inhibited [$^3$H]dU incorporation into DNA by 0, 85, 97, and 99%, respectively. The total cpm counted for analysis of these gradients (representing the sum of equal aliquots from gradient fractions) were 509,908, 79,387, 47,496, and 22,072, respectively. B. MOLT-4 cells exposed to 0 μM (●), 0.5 μM (■), 1.0 μM (▲), and 50 μM (○) piritrexim. *BSA*, BSA marker (4.3 S). These concentrations inhibited [$^3$H]dU incorporation into DNA by 0, 84, 90, and 99%, respectively. The total cpm counted for analysis of these gradients were 271,613, 66,696, 32,293, and 2,141, respectively.

[$^3$H]dU is incorporated into DNA as [$^3$H]dUMP as well as [$^3$H]TMP in cells exposed to an antifolate, we considered the possibility that removal of [$^3$H]uracil from DNA by uracil-DNA-glycosylate might give rise to artifacts in the DNA size distribution detected by labeling with [$^3$H]dU. Therefore, both [$^3$H]dA and [$^3$H]dC were also used to label DNA in the presence of exogenous dU at concentrations calculated to normalize intracellular dUMP levels (20).

In contrast to [$^3$H]dU incorporation into DNA, maximal inhibition of [$^3$H]dA incorporation into DNA was only ~50%. Thus, even though a large portion of [$^3$H]dA was converted to ATP and incorporated into RNA (90–95%), use of this labeled DNA precursor resulted in a 2-fold higher specific activity of the DNA isolated from cells treated with 50 μM piritrexim, facilitating more sensitive detection of DNA fragments at maximally inhibiting piritrexim concentrations. Enhanced visualization of dA-rich regions in DNA was also afforded by this precursor.

Fig. 2A depicts the concentration-dependent accumulation of [$^3$H]dA-labeled DNA fragments from cells treated with 0 μM

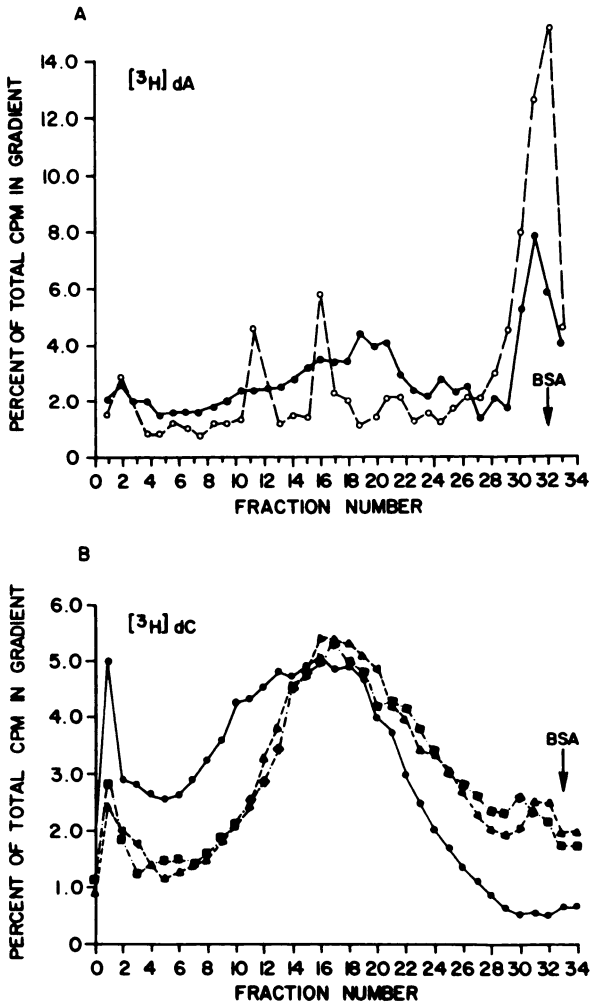


**Fig. 2.** Piritrexim induces accumulation of [$^3$H]dA- or [$^3$H]dC-labeled newly synthesized DNA fragments under alkaline conditions. MOLT-4 cells were exposed to piritrexim in the presence of unlabeled dU and either [$^3$H]dA or [$^3$H]dC, as described in Materials and Methods. After lysis with 2% SDS and Proteinase K treatment, the DNA was alkaline denatured and sedimented through 5–20% alkaline sucrose gradients. DNA in gradient fractions was then TCA precipitated onto filter paper disks and analyzed for radioactive label. A. [$^3$H]dA-labeled DNA of cells exposed to 0 μM piritrexim (●) or 50 μM piritrexim (○). *BSA*, BSA marker (4.3 S). The total cpm counted for analysis of these gradients were 95,661 and 34,592 for 0 μM and 50 μM piritrexim, respectively. B. [$^3$H]dC-labeled DNA of cells exposed to 0 μM piritrexim (●), 0.5 μM piritrexim (■), or 50 μM piritrexim (▲). *BSA*, BSA marker (4.3 S). The total cpm counted for analysis of these gradients were 87,202, 32,934, and 21,597 for 0 μM, 0.5 μM, and 50 μM piritrexim, respectively.

and 50 μM piritrexim in the presence of 4.0 μM exogenous dU. In contrast to the DNA fragments labeled with [$^3$H]dU precursor, the DNA appeared to be considerably more skewed to lower molecular weight sizes at both drug concentrations when the DNA was labeled with [$^3$H]dA. Since the labeling of DNA with [$^3$H]dU in the presence of unlabeled dA had no significant effect on the size distribution of the DNA compared to [$^3$H]dU, alone,[3] these results do not reflect a change which is simply due to the presence of exogenous dA. They may, therefore, more accurately reflect the impact of dUMP-substitution in A-T-rich regions of DNA where uracil-DNA-glycosylase-dependent excision-repair processes might be most effectively initiated.

Labeled dC was also used to afford visualization of DNA. In contrast to [$^3$H]dA, this labeled DNA precursor was not utilized in RNA synthesis. However, a large portion of [$^3$H]dC was expected to accumulate in antifolate-treated cells as dUMP through deinhibition of dCMP deaminase (20), potentially resulting in effects similar to those observed when [$^3$H]dU was used. In practice, incorporation of [$^3$H]dC into DNA at 50 μM piritrexim was found to be significantly less inhibited than [$^3$H]dU ([$^3$H]dC: 90%; [$^3$H]dU: 99%), providing greater sensitivity for detection of DNA fragments in drug-treated cells. Since label incorporated as dC cannot be removed by uracil-DNA-glycosylase, the [$^3$H]dC label not only increased the sensitivity of DNA fragment detection, but also provided better visualization of DNA asymmetries that might result from the distribution of label to dC-rich regions in DNA.

As shown in Fig. 2B, the size distributions of [$^3$H]dC-labeled DNA from 0 μM and 0.5 μM piritrexim-treated cells remained similar to that observed with [$^3$H]dU label (Fig. 1B). However, whereas 50 μM piritrexim treatment led to a significant increase in small DNA fragments when visualized with [$^3$H]dU, very little change was observed at this piritrexim concentration when [$^3$H]dC was employed to label DNA. A slight concentration-dependent accumulation of [$^3$H]dC-labeled DNA fragments could only be detected in the low molecular weight region of the gradient (fractions 31–34).

When DNA from piritrexim-treated cells was sedimented through 10–30% (w/w) alkaline sucrose gradients which gave better resolution in the lower molecular weight range (Fig. 3A), it became clear that smaller DNA fragments accumulated under alkaline conditions as a concentration-dependent response to piritrexim exposure. Moreover, alkaline elution analysis performed under conditions of minimal protein adsorption (Fig. 3B) indicated a concentration-dependent accumulation of [$^3$H]dC-labeled DNA fragments, as well. Since the elution rate of alkaline-denatured DNA under these conditions is largely dependent on DNA fragment size, this analysis provided independent confirmation of the drug concentration-dependent accumulation of small and/or alkaline labile DNA in the cell.

The differences in the DNA size distributions observed with the different labeled DNA precursors probably reflected the high AT/GC ratio of ~1.4 found in human blood cells (21), as well as the specificity of uracil-DNA-glycosylase-dependent DNA repair and the base composition of the resulting fragments. Interpretation of the size distribution of [$^3$H]dC-labeled DNA may be further complicated by differential equilibration of the [$^3$H]dC with dCTP and dUTP (TTP) endogenous nucleotide pools. In spite of the differences, however, all analyses

[3] R. G. Richards and W. D. Sedwick, unpublished observation.

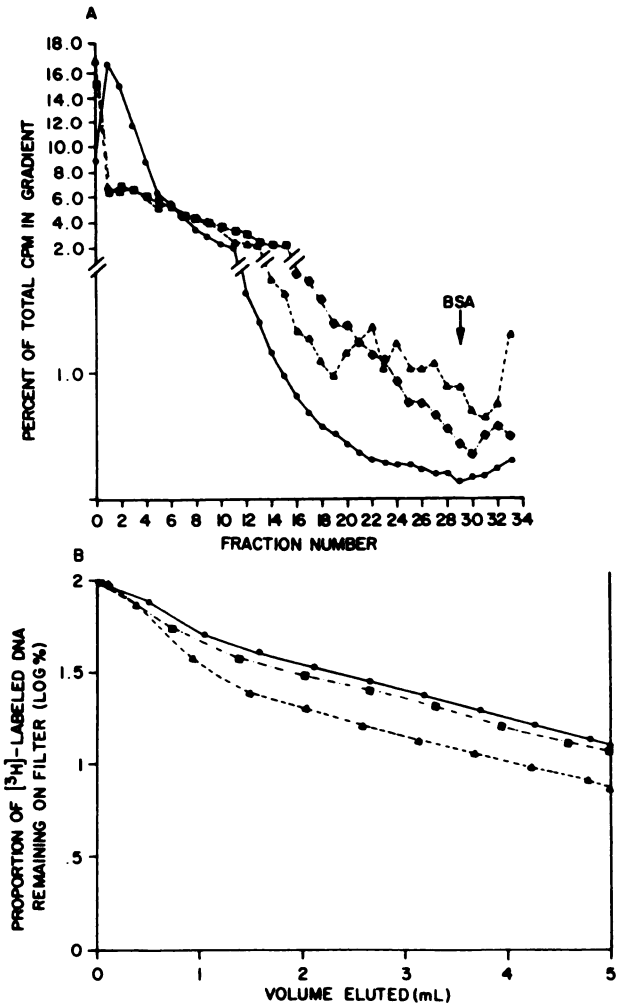


Fig. 3. Piritrexim-induced accumulation of [$^3$H]dC-labeled DNA fragments is concentration dependent. MOLT-4 cells were treated with 0 μM (●), 0.5 μM (■), or 50 μM (▲) piritrexim in the presence of unlabeled dU and [$^3$H]dC, as described in Materials and Methods. A. Sedimentation of the DNA in 10–30% (w/w) alkaline sucrose gradients. The total cpm counted for analysis of these gradients were 150,147, 43,454, and 22,580 for 0 μM, 0.5 μM, and 50 μM piritrexim, respectively. B. Alkaline elution of the DNA under conditions of minimal protein adsorption. The total cpm eluted were 214,970, 66,652, and 21,698 for 0 μM, 0.5 μM, and 50 μM piritrexim, respectively.

indicated that, under alkaline conditions, there was a concentration-dependent shift to smaller DNA fragment sizes when cells were exposed to piritrexim.

**Piritrexim causes accumulation of small DNA fragments in the cell.** Existing strand breaks as well as AP sites are visualized as DNA strand scissions by alkaline sucrose gradient and alkaline elution techniques. However, we were also interested in determining the extent to which DNA integrity was compromised under physiological conditions. For this study, MOLT-4 cells were exposed to piritrexim in the presence of [$^3$H]dC and unlabeled dU, then lysed with SDS. After the proteins were digested with Proteinase K, treatment of the DNA with 1 N NaOH was performed to denature DNA and hydrolyze the alkaline labile AP sites. Alternatively, the DNA was denatured under neutral conditions with 15% formaldehyde at 45°, leaving the AP sites intact. Following exhaustive dialysis versus a neutral buffer containing 1% formaldehyde to maintain the denatured state, the size distribution of the re-

sulting DNA fragments was determined by sedimentation through 10–30% (w/w) neutral denaturing sucrose gradients (Fig. 4).

After taking into account the fact that DNA sediments further in neutral denaturing sucrose gradients than in alkaline sucrose gradients (22), it became clear that alkaline-denatured DNA isolated from 0 μM, 0.5 μM, or 50 μM piritrexim-treated cells had essentially the same size distribution in 10–30% neutral denaturing sucrose gradients (Fig. 4A) as in 10–30% alkaline sucrose gradients (Fig. 3A). Denaturation of DNA with formaldehyde, however, led to substantially less DNA sedimenting as smaller fragments in 10–30% neutral denaturing sucrose gradients (Fig. 4B) than did denaturation of DNA with alkali (Fig. 4A), but the relative amount of smaller DNA fragments remained drug concentration dependent. It should also be noted that, whereas alkaline-denatured DNA showed fragments corresponding to a general distribution of DNA sizes, formaldehyde denaturation released DNA fragments of a distinct size class, having sedimentation coefficients which ranged from 3 S to 6 S (80–200 nucleotides). Since these DNA fragments could not have arisen by alkaline hydrolysis of DNA, we concluded that piritrexim exposure induced drug concentration-dependent accumulation of DNA fragments in the cell. The possible origin of these DNA fragments will be discussed below.

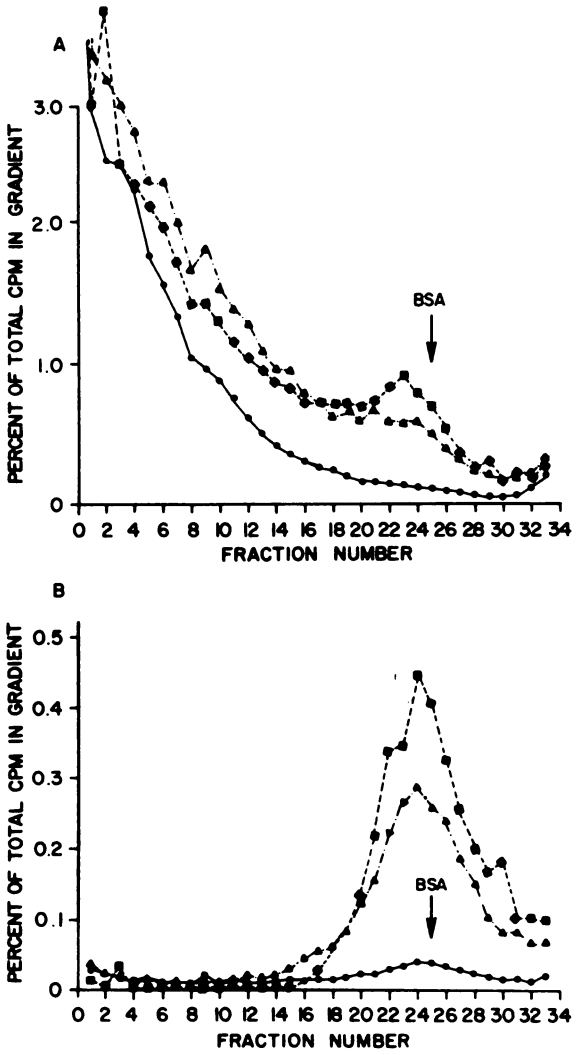


**Fig. 4.** Piritrexim induces the accumulation of DNA fragments in the cell. MOLT-4 cells were exposed to piritrexim and labeled with [$^3$H]dC as described in the legend to Fig. 3. After lysis with SDS followed by Proteinase K treatment, the DNA was alkaline denatured (A) or neutrally denatured with formaldehyde (B). The samples were exhaustively dialyzed versus 100 mM NaCl, 32 mM $Na_2HPO_4$, 5 mM EDTA, and 1% formaldehyde, pH 7.2 to remove alkali or excess formaldehyde while maintaining denaturing conditions. The size distribution of the DNA fragments was then determined by sedimentation through 10–30% (w/w) neutral denaturing sucrose gradients. Tritium-labeled DNA in the sucrose gradient fractions was TCA precipitated onto filter paper disks and analyzed for radioactivity. The total cpm counted for analysis of the gradients containing alkaline denatured DNA (A) were 704,992, 142,831, and 66,807 for 0 μM, 0.5 μM, and 50 μM piritrexim, respectively, whereas the total cpm counted for analysis of the gradients containing neutrally denatured DNA (B) were 615,392, 122,910, and 64,698 for 0 μM, 0.5 μM, and 50 μM piritrexim, respectively. ●, 0 μM piritrexim; ▲, 0.5 μM piritrexim; ■, 50 μM piritrexim. *BSA*, BSA marker (4.3 S).

**Exogenous dU enhances piritrexim toxicity.** For these experiments, HL-60 or MOLT-4 cells were grown in dialyzed fetal calf serum-containing media supplemented with and without piritrexim and exogenous dU. As shown in Fig. 5A, addition of 0 μM, 0.1 μM, 1.0 μM, or 10 μM dU, alone, elicited a transient stimulation in the growth of HL-60 cells which was probably due to perturbation of the nucleotide pools. By day 3, however, all control cell populations were within ±6% of cells which had not been treated with dU. In contrast, when cells were treated with piritrexim (Fig. 5B), cell death was augmented by exogenous dU in a concentration-dependent manner. This effect, however, could only be demonstrated within a narrow range of piritrexim concentrations which were marginally toxic.

As seen in Fig. 6, when MOLT-4 cells were treated with 0 μM, 0.5 μM, or 1.0 μM piritrexim and either 0 μM or 10 μM dU, addition of 10 μM dU to media containing nontoxic (0 μM) or toxic (1.0 μM in this experiment) concentrations of piritrexim (Fig. 6A) made only a small difference in the course of cell growth (0 μM) or drug toxicity leading to the death of the cell culture (1.0 μM). A significant difference was observed on day 6, however, when cells treated with 1.0 μM piritrexim and 10 μM dU exhibited a decrease in viability compared to the control cells with no dU. Nevertheless, cells in all drug-treated cultures were dead on day 20. In contrast, cultures grown in media containing a marginally toxic concentration of piritrexim (0.5 μM) were clearly more effectively killed in the presence of 10

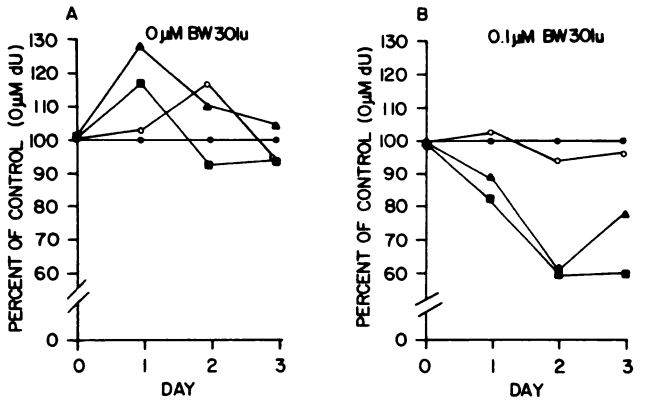


**Fig. 5.** Exogenous dU enhances toxicity of piritrexim. HL-60 cells were grown in RPMI 1640 growth media containing 10% dialyzed fetal calf serum with 0 μM or 0.1 μM piritrexim (BW301U) and either 0 μM (●), 0.1 μM (○), 1.0 μM (▲), or 10 μM (●) dU. The cells were seeded at $3.6 \times 10^5$ cells/ml with 97% viability by trypan blue exclusion. Cell counts were taken daily for 3 days. The data reflect a single determination for each experimental condition.

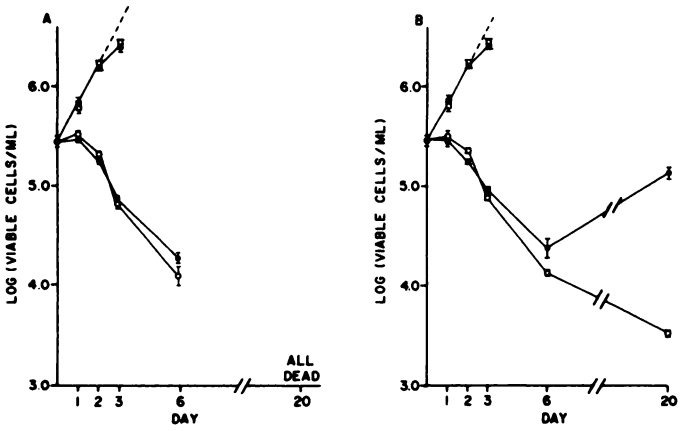


**Fig. 6.** Exogenous dU prevents selection of piritrexim-resistant cells. MOLT-4 cells were grown in RPMI 1640 growth media containing 10% dialyzed fetal calf serum with 0, 0.5, or 1.0 μM piritrexim and either 0 μM or 10 μM dU. The cells were seeded at a density of $2.93 \times 10^5$ cells/ml with a viability of 96% determined by trypan blue exclusion. Cell counts were taken on days 0, 1, 2, 3, 6, and 20. The error bars reflect the standard deviation for three determinations. A, 0 μM (■, □) and 1.0 μM (●, ○) piritrexim with 0 μM (■, ●) or 10 μM (□, ○) dU. B, 0 μM (■, □) and 0.5 μM (●, ○) piritrexim with 0 μM (■, ●) or 10 μM (□, ○) dU.

μM dU (Fig. 6B). Although dU initially rescued the cells on days 1 and 2, by day 3 and on successive days dU enhanced the rate of cell death compared to cultures with 0.5 μM piritrexim alone.

Especially interesting was the observation that cells treated without dU developed resistance and began to grow back by day 20, whereas dU supplementation sustained the toxic effect of piritrexim, as evidenced by the continued decrease in cell viability which ultimately led to the death of the culture. All cultures surviving the exposure to piritrexim alone went on to form cell lines that were stably resistant to piritrexim, whereas no such cell lines could be isolated from the cell cultures that had been supplemented with dU.

## Discussion

Antifolates have played an essential role in cancer chemotherapy for more than 35 years (23). During this time, it has been generally assumed that the cytotoxic effect of antifolates results from metabolic imbalances attendant to DHFR inhibition. These include rapid inhibition of DNA synthesis due to reduction of thymidine levels, and later inhibition of RNA and protein biosynthesis resulting from purine and amino acid deficiency consequent to tetrahydrofolate depletion. We now know (9, 12), however, that dUMP misincorporation into DNA also occurs in response to antifolate exposure, and it has been our interest to determine the significance of this lesion with regard to antifolate cytotoxicity.

The studies presented in this paper confirm and extend the documentation of antifolate-induced dUMP misincorporation in DNA, and begin to assess its impact on DNA metabolism and cell viability. Demonstration of dUMP misincorporation with methothrexate (9), metoprine (12), and, most recently, with piritrexim (this work, and Refs. 24 and 25) indicates that this lesion is a consequence of DHFR inhibition. Moreover, the fact that dUMP misincorporation occurs in a variety of blood cells inluding B-cells (SB, WIL-2, and lymphoid line 8866), T-cells (MOLT-4), and promyelocytic leukemia cells (HL-60) indicates that dUMP misincorporation is a universal response of blood cells to DHFR inhibition by antifolates, and is not a response specific to B-lymphoblastoid cells.

The misincorporation of dUMP in DNA has major ramifications on DNA synthesis and protein-DNA interactions. Overall DNA synthesis is maximally inhibited at ~50% of the normal rate by antifolates, whereas inhibition of [$^3$H]dU incorporation into DNA approaches maximally limiting values of >97% (25)—the difference reflecting the excision of [$^3$H]uracil residues in DNA by uracil-DNA-glycosylase. Although this initiation of excision-repair results in the concentration-dependent generation of AP sites (this study—Figs. 1–3), further repair-motivated degradation leading to nicks and/or gaps in DNA appears to proceed at a substantially slower rate (this study—Fig. 4). Thus, despite the rapid excision of uracil, nuclear DNA appears to remain largely intact, as apyrimidinic DNA following a 30-min exposure with piritrexim.

AP sites are unstable in that they are potential points of DNA strand scission—not only by the action of AP endonucleases, but also as a consequence of protein-DNA or DNA-DNA crosslinking. At an AP site, the ribose sugar is in equilibrium between its closed furanose form and its open aldehyde form, the latter of which can be attacked at the carbonyl carbon by an amine. Reversible formation of a Schiff base through such a mechanism leads to DNA-protein crosslinks through amine-containing amino acid residues, or DNA-DNA crosslinks through amine-containing purine or pyrimidine bases. Such reactions result in DNA strand scission through β-elimination (26, 27), leaving a protein-DNA or DNA-DNA crosslink at the 3′-end of one fragment, and a phosphate group at the 5′-end of the other fragment.

Formation of a Schiff base and subsequent strand scission may be one mechanism for the piritrexim-induced accumulation of newly synthesized DNA fragments which were detected in our studies (Fig. 4). In a separate paper we present evidence from alkaline elution, alkaline cesium chloride gradient centrifugation, and nitrocellulose filter binding techniques showing that DNA-protein crosslinks are induced in a concentration-dependent manner by piritrexim (28).[4] These protein-DNA crosslinks probably occur through Schiff base formation, since sodium borohydride treatment yields consistently greater numbers of protein-DNA crosslinks. Thus, although these studies are not inconsistent with the argument that DNA fragments accumulate as products of aborted synthesis, which was suggested previously by Fridland (29), the results strongly support the hypothesis that significant fragmentation may also occur through Schiff base formation between primary amine constituents of proteins and DNA at AP sites.

When cells are treated with an antifolate, addition of exogenous dU leads to dUMP pool expansion greatly in excess of that seen with the antifolate, alone (12, 30), thus increasing the potential for dU misincorporation into DNA. Although exogenous dU may also effect the dUMP-driven depletion of tetrahydrofolate pools through thymidylate synthetase-dependent $N^5$,$N^{10}$-methylene tetrahydrofolate utilization, this possibility seemed unlikely since addition of hypoxanthine, adenosine, and amino acids had no effect on piritrexim toxicity, whereas the further addition of thymidine resulted in complete rescue.[5] Thus, if dU misincorporation into DNA is indeed a

[4] R. G. Richards, M. L. Gillison, and W. D. Sedwick, submitted for publication.
[5] R. G. Richards and W. D. Sedwick, unpublished observation.

major factor in the cytotoxicity of antifolates, it follows that exogenous dU might enhance piritrexim toxicity under conditions which eliminate the possibility of thymidine rescue.

In this regard, it is significant that, at antifolate concentrations which were just slightly greater than that needed to inhibit cell division, addition of exogenous dU led to more rapid cell kill in both HL-60 and MOLT-4 cells (Figs. 5 and 6). In both cell lines, enhanced cell kill with exogenous dU was observed in a narrow window between nontoxic and highly toxic concentrations of piritrexim (see Fig. 6, for example), and in MOLT-4 cells (at least) exogenous dU prevented selection of piritrexim-resistant cells. Because dU enhancement of piritrexim toxicity could only be demonstrated within a narrow range of marginally toxic piritrexim concentrations, the effective piritrexim concentrations differed between HL-60 and MOLT-4 cells and were sensitive to any changes in experimental conditions.

Although exogenous dU may have enhanced the toxicity of piritrexim by increasng the rate of tetrahydrofolate depletion, it appears more likely that increased dUMP misincorporation led to more rapid cell kill and irreversible toxicity. In addition to our experiments discussed above, this possibility is supported by the results of Hryniuk (31), who found that addition of hypoxanthine to methotrexate-treated mouse lymphoma L5178Y cells completely corrected suppression of DNA synthesis but only partially prevented cell kill. The correspondence between methotrexate cytotoxicity, accumulation of single-strand breaks in bulk DNA (32), and an increase in chromatin decondensation (33) also suggests that dUMP misincorporation may contribute to cell death. Such results support the premise that induction of dUMP misincorporation prohibits efficient replication and repair leading to an accumulation of DNA strand breaks which may ultimately be lethal to the cell.

Although it is unclear to what extent dUMP misincorporation is responsible for the cytotoxic effect of antifolates, alone, it is clear from our studies that a significant amount of DNA damage may result from this process. The generation of AP sites, protein-DNA or DNA-DNA crosslinks, and the fragmentation of DNA, in addition to the possible accumulation of DNA replication intermediates (29), represent potentially toxic lesions that need to be considered in the evaluation of biochemical events leading to cell death. Future studies need to define the relationship of these lesions to DNA repair, and to further assess their potential role in antifolate-induced cytotoxicity.

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