Cytotoxicity of Antifolate Inhibitors of Thymidylate and Purine Synthesis to WiDr Colonic Carcinoma Cells

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ABSTRACT

We have studied the cytotoxicity of 5,10-dideazatetrahydrofolate (DDATHF) and of D-1694 to human WiDr colonic carcinoma cells as a model system for the effects of pure inhibitors of either the de novo purine synthesis pathway or thymidylate synthesis. The growth of this cell line was inhibited by very low concentrations of either agent and the lethality of DDATHF and D-1694 was completely prevented by continuous exposure to either hypoxanthine or thymidine, respectively, indicating that these compounds were very potent metabolic inhibitors, each specific for one of these pathways. D-1694 was highly cytotoxic (>3 logs of kill) after a 4-h exposure to 1 µM drug, or a 24-h exposure to very low concentrations of DDATHF and D-1694 was completely prevented by continuous exposure to DDATHF as an inhibitor of purine synthesis, with a resultant decrease in the efficiency of “thymineless death” (1–6). The increased cell kill by MTX in the presence of a source of purines (1) has been confirmed by other studies in mouse L1210 and human CEM cells (2, 3), but the impact of inhibition of purine synthesis on the effectiveness of the cytotoxicity of thymidylate starvation in MTX-treated cells has been the subject of considerable discussion due to the multiple direct and indirect effects of the drug.

In recent years, new classes of folate antimetabolites have been developed that have no effect on dihydrofolate reductase. Two recently developed classes of antifolates have proven to be inhibitors of thymidylate synthase and of the first folate-dependent enzyme of de novo purine synthesis, GART. The novel GART inhibitor, DDATHF (7, 8) as well as the second-generation quinazoline thymidylate synthase inhibitor D-1694 (9), have both been advanced to clinical trial (10–13). The patterns of toxicity observed appear clinically manageable, and an encouraging level of therapeutic activity was observed with both drugs in heavily pretreated patient populations (10–13). Preclinical experiments with D-1694 (9) and DDATHF (7, 8) have indicated that they were highly specific for their primary targets, thymidylate synthase and GART, respectively. The polyglutamate metabolites of both have been shown to rapidly accumulate (9, 14) and to be substantially more potent inhibitors of these two target enzymes (9, 15).

INTRODUCTION

Antifolates inhibit to dihydrofolate reductase, such as MTX3, have been shown to result in inhibition of both thymidylate synthase and de novo purine synthesis in cultured cells (as well as other folate-dependent processes in media not supplemented with amino acids). The action of such drugs against both purine and thymidylate synthesis pathways has complicated interpretation of the contribution of each activity to the cytotoxicity of MTX. Classic studies by Borsa and Whitmore (1) drew the conclusion that the cytotoxicity of MTX to L-cells resulted from inhibition of thymidylate synthesis and that the concurrent inhibition of purine synthesis tended to prevent efficient cell killing. Conceptually, this effect has been attributed to the accumulation of cells in G1 as a result of inhibition of RNA synthesis, although this effect has been difficult to distinguish from an early S block (2, 3). As a result of such inhibition of cell cycle progression, the entry of MTX-treated cells into S phase is thought to be limited by inhibition of RNA synthesis, with a resultant decrease in the efficiency of “thymineless death” (1–6). The increased cell kill by MTX in the presence of a source of purines (1) has been confirmed by other studies in mouse L1210 and human CEM cells (2, 3), but the impact of inhibition of purine synthesis on the effectiveness of the cytotoxicity of thymidylate starvation in MTX-treated cells has been the subject of considerable discussion due to the multiple direct and indirect effects of the drug.

In recent years, new classes of folate antimetabolites have been developed that have no effect on dihydrofolate reductase. Two recently developed classes of antifolates have proven to be inhibitors of thymidylate synthase and of the first folate-dependent enzyme of de novo purine synthesis, GART. The novel GART inhibitor, DDATHF (7, 8) as well as the second-generation quinazoline thymidylate synthase inhibitor D-1694 (9), have both been advanced to clinical trial (10–13). The patterns of toxicity observed appear clinically manageable, and an encouraging level of therapeutic activity was observed with both drugs in heavily pretreated patient populations (10–13). Preclinical experiments with D-1694 (9) and DDATHF (7, 8) have indicated that they were highly specific for their primary targets, thymidylate synthase and GART, respectively. The polyglutamate metabolites of both have been shown to rapidly accumulate (9, 14) and to be substantially more potent inhibitors of these two target enzymes (9, 15).

The recent development of antimetabolites that are such close approximations of pure inhibitors of thymidylate synthase and de novo purine synthesis that also cannot be incorporated into nucleic acids is of considerable interest from both therapeutic and theoretical standpoints. Perhaps two of the most important questions that are raised by the availability of these valuable standard compounds are whether an inhibitor of de novo purine synthesis is cytotoxic per se and what the efficiency of cell kill would be for such compounds relative to that of a pure inhibitor of thymidylate synthesis. We now report that DDATHF, as an inhibitor of purine synthesis, is indeed cytotoxic, albeit with characteristics that require definition of the concept of cytotoxicity, and that the efficiency of cell kill of WiDr cells by the thymidylate synthase inhibitor D-1694 is substantially greater than that of DDATHF. Our results also indicate that the processes leading to the loss of clonogenicity in WiDr cells treated with DDATHF are fundamentally different from those seen with folate antagonists that induce thymineless death.

MATERIALS AND METHODS

Materials. (6R) and (6S)-DDATHF were a generous gift of Dr. Chuan Shih of Eli Lilly Research Laboratories (Indianapolis, IN). These diastereomers were resolved by fractional crystallization and were determined to be >95% stereochemically pure with the use of chiral high-pressure liquid chromatography by Dr. Shih (8). Except where specifically noted, (6R)-DDATHF, the diastereomer with a configuration equivalent to that of the naturally occurring tetrahydrofolate cofactors (16) and that which has gone to clinical trial, was used in all experiments. D-1694 was also synthesized by Dr. Shih. Fetal bovine sera were purchased from Gemini Bio-Products, Inc. (Calabasas, CA).

Cell Culture. Human WiDr colon carcinoma cells were obtained from the American Type Culture Collection (Rockville, MD) and were maintained adherent to plastic dishes in RPMI 1640 medium supplemented with 10% fetal...
bovine serum at 37°C in 5% CO2. All experiments were carried out in RPMI 1640 medium supplemented with 10% diazoylated fetal bovine serum. Stock cultures were routinely found negative for Mycoplasma by screening for specific rRNA species (Genprobe, San Diego, CA). The plating efficiency of these cells was typically about 60% in medium supplemented with either serum.

**Growth Inhibition Studies.** Cells adapted to growth in 10% diazoylated fetal bovine serum were trypsinized and seeded into 60-mm culture dishes at 4.5 × 10^4 cells/dish (about 2.5% confluence). After 20-24 h at 37°C, fresh medium containing either PBS, DDATHF, or D-1694 was added. At least 4 dishes were trypsinized and cell count was determined at the time of addition of drug. In some experiments, incubation was continued for 96 h in the continuous presence of drugs. In other experiments, drug-containing medium was replaced with drug-free medium containing diazoylated serum after 4 h following a single wash with PBS. Incubation was continued, and total cell number was determined after 96-h total incubation time. Adherent cells were harvested by trypsinization and nonadherent cells by centrifugation; a single cell suspension of mixed adherent and nonadherent cells was counted electronically.

In other experiments, growth inhibition was studied at high initial density after seeding 2.5 × 10^5 cells in 35-mm dishes. Again, drug was added after 24 h of growth with duplicate dishes at each concentration. After 4-h incubation with drug, dishes were rinsed with PBS and drug-free medium containing diazoylated serum was added. Incubation was continued for 7 days with medium changes at 3-day intervals. Cells were fixed with methanol for 10 min and stained with Giemsa as a 10% solution in methanol for 1 h.

**Photomicroscopy.** WiDr cells were seeded into 75-cm^2 culture flasks at 2 × 10^5 cells/flask (~30% confluence) or 3 × 10^5 cells/flask (~4% confluence) and allowed to attach for 24 h. Cells were then exposed to either 100 μM DDATHF or 0.32 μM D-1694 for 4 h or to 60 μM MTX for 24 h. Treatment medium was withdrawn, flasks were rinsed with PBS, and fresh medium was added. Incubation was continued with medium changes at 3-day intervals. Photographs were taken with a Zeiss Axiovert 35 inverted light microscope using Kodak Tmax 400 film at ×320.

**Optimization of Detection of DDATHF Cytotoxicity.** In the first set of experiments, WiDr cells were seeded into 100-mm culture dishes at 3 × 10^4 or 3 × 10^5 cells per dish, allowed to attach for 24 h, then exposed to DDATHF for 4 h. Drug-containing medium was aspirated, dishes were washed once with PBS, and fresh drug-free medium was added. Incubation was continued with medium changes every 3 days. After 6 days, some of the dishes were removed and scored microscopically by counting the number of cell clusters containing ≥8 cells or ≥16 cells per cultured field at ×50. A minimum of 20 fields were counted on each of two dishes. In the same experiment, macroscopically visible, Giemsa-stained colonies were scored after 12 days of growth on another set of dishes. In another set of experiments, 3 × 10^5 cells were seeded into 100-mm dishes, allowed to attach for 24 h, and then treated with varying concentrations of DDATHF for 4 h as above. Colonies were stained with Giemsa and counted at 7-24 days.

**Cytotoxicity Studies.** WiDr cells were seeded into 100-mm dishes at densities ranging from 3 × 10^3 to 3 × 10^5 cells/dish, adjusting the cell inoculum to allow 1-300 colonies/dish. Three dishes were used for each condition and at least two experiments were performed under each set of conditions. After 24 h of incubation, cells were treated with drug for various times. Following drug exposure, dishes were rinsed once with PBS, fresh medium containing dialyzed serum was added, and macroscopically visible colonies were counted after 12-16 days of incubation. In some experiments, 32 μM hyoxanthine or 5.6 μM thymidine was added with drug and/or in the medium changes after drug. Drug-containing medium was replaced after 48 h in cultures scheduled for 72 or 96 h of exposure to drug. Plating efficiency was not detectably different in controls seeded at 3 × 10^5 and 3 × 10^3 cells/dish. Likewise, the survival of treated cultures which could be scored at both 3 × 10^5 and 3 × 10^3 cells seeded per dish or at both 3 × 10^5 and 3 × 10^3/dish were the same within the expected statistical limits in 9 of 10 evaluable data sets.

**Replating Studies.** WiDr cells were seeded into 60-mm dishes at ~1 × 10^6 cells/dish. Cells were exposed to either PBS, 100 μM DDATHF, or 1 μM D-1694 for 4 h beginning 24 h after seeding. Following exposure, cells were washed with PBS and cultured in drug-free medium for times varying from 0 to 10 days with medium changes at 2-day intervals. Total adherent cells were quantitated by trypsinization and electronic counting, diluted to a density allowing 1-300 colonies/dish, and replated into 100-mm dishes. Following 12 to 16 days of culture in drug-free medium, dishes were scored by counting visible Giemsa-stained colonies.

**RESULTS**

**Antiproliferative and Cellular Effects of DDATHF and D-1694 to WiDr Cells.** Previous work on the biological effects of DDATHF and D-1694 on malignant cells have primarily involved study of inhibition of the growth of leukemic cell lines (7-9, 14, 17-21). We chose to directly compare the antiproliferative and cytotoxic properties of these compounds for a human carcinoma cell. The WiDr colonic carcinoma cell line was chosen for these studies because it forms discrete, tightly attached colonies in medium supplemented with dialyzed serum. The growth of WiDr cells was inhibited 50% by concentrations of DDATHF and D-1694 of 14 ± 3.7 nM (n = 4) and 1.3 ± 0.43 μM (n = 3), respectively, when drug was continuously present during a 96-h period of growth (Fig. 1A). Thus, WiDr cells were found to be as sensitive to the antiproliferative effects of both of these compounds as previously reported for other tumor cell lines in culture (7-9, 17-21). If drug was present only for the first 4 h of a...
96-h growth period, the concentration of drug that reduced cell growth to 50% of control was observed to be $260 \pm 96 \text{ nm (} n = 4 \text{)}$ for DDATHF, while that for D-1694 was $12 \pm 3.5 \text{ nm (} n = 3 \text{)}$ (Fig. 1B). Hence, both drugs were potent cytostatic agents even after a brief exposure time, and the potency of the antiproliferative effects of either drug following a 4-h exposure decreased 10- to 20-fold relative to that seen upon continuous exposure. For these growth inhibition studies, initial cell densities were very low (1,800 cells/cm$^2$, or about 30% of confluence), so that differential were noted between the effects of DDATHF and D-1694 (Fig. 2). First, D-1694-treated cells lost adherence to the dishes, whereas DDATHF did not cause the cells to detach. Second, while D-1694 was as potent as a growth-inhibitory compound as predicted by low-density studies, DDATHF appeared to be rather ineffective as an antiproliferative agent. Other experiments did not detect additional loss of adherent cells at up to 1 mM DDATHF, using this experimental design.

Treatment of WiDr cells with 0.32 $\mu$M D-1694 for 4 h caused drastic changes in morphology. By 24 h after removal of drug, the majority of cells underwent a striking megaloblastosis, with both cytoplasmic and nuclear enlargement (Fig. 3, A and B). After 43 h, treated cells were substantially enlarged (Fig. 3, C and D). At 48 h after treatment, cellular structure was disordered and individual cells had lost adherence to the dish (Fig. 3, F). By 53 h, most cells had detached (Fig. 3, H). In contrast, the majority of cells treated with 100 $\mu$M DDATHF for 4 h completed one successful mitotic event by 24 h after removal of drug (Fig. 4, E and F), after which growth was arrested (Fig. 4, E and F). Even 10 days after DDATHF treatment (Fig. 4H), cellular structure did not undergo the changes seen in D-1694-treated cells (Fig. 3). However, in any given field, multiple grossly abnormal cells were found (Fig. 4, F and G), some of which appeared to detach from the plate, while others persisted for at least 10 days (Fig. 4H). When DDATHF-treated cultures were trypsinized and adherent cells were quantitated by electronic counting, it was apparent that the number of cells adherent to the dish increased by 25–50% during the first 24 h after treatment with 10–100 $\mu$M DDATHF, then total adherent cells remained constant until outgrowth of surviving cells was observed (see below).

**Cytotoxicity of DDATHF.** Because WiDr cells did not detach from the dish after treatment with concentrations of DDATHF as much as 500 times higher than antiproliferative levels (Figs. 1, 2, and 4), it became of some significance to determine whether this compound was actually cytotoxic. Utilizing the formation of colonies from individual WiDr cells as an assay of cell kill, the effectiveness of DDATHF as a cytotoxic agent was again found to be very dependent upon experimental conditions (Fig. 5). When colonies were scored 6 days after a 4-h drug exposure, few macroscopically visible colonies were seen at DDATHF concentrations $\geq 3 \mu$M (untreated single WiDr cells would form colonies containing 100–300 cells in this period). Microscopic examination of these plates, however, revealed small groups of cells adherent to the dishes after exposure to even high concentrations of DDATHF. The frequency of occurrence of microscopic clusters of $\geq 8$ and $\geq 16$ cells at concentrations of DDATHF $\geq 10 \mu$M (Fig. 5) raised the question of whether these cells would eventually form colonies. Further incubation of groups of dishes which were then scored macroscopically after 12 days showed that substantial numbers of treated cells recovered and were clearly viable. A comparison of the number of colonies macroscopically visible at 12 days and cell clusters at 6 days made it apparent that colonies were deriving from a large number of cells that would not even be judged viable on a criterion of clusters $\geq 8$ cells at 6 days. In order to determine the appropriate conditions under which no further recovery of DDATHF-treated cells occurred, WiDr cells were treated with 30, 80, or 160 $\mu$M drug for 4 h and macroscopically visible colonies were scored after 7–24 days. The results of these experiments (Fig. 6A) indicated that all cells that were able to form colonies had done so by 12–18 days after removal of DDATHF. Given that DDATHF is highly polyglutamated (14) and that these metabolites are potent inhibitors of GART (15) which efflux from cells poorly or not at all (14), it seemed plausible that these adherent cells were still viable, but were merely in a growth-inhibited state due to inhibition of de novo purine synthesis and to the resultant limited supply of purines. In order to address this possibility directly, in another set of experiments, hypoxanthine was added 18 days after a 4-h treatment with DDATHF to circumvent any sustained inhibition of de novo purine synthesis and to allow any viable cells in the adherent population to form colonies. Twelve days later, when any cells capable of outgrowth should have formed macroscopic colonies, there was no difference in the frequency of colony formation in the presence or absence of hypoxanthine (Fig. 6B). We concluded from these experiments that DDATHF was indeed cytotoxic as judged by decreases in clonogenicity of treated cultures and by the failure of persistently adherent cells to form colonies even after the growth-inhibitory effect of DDATHF had been reversed. However, if cloning efficiency was scored prematurely, the antiproliferative effects of DDATHF would cause an overestimation of cell kill.

**Comparison of Cytotoxities of DDATHF and D-1694.** Having determined conditions under which definitive measurements of cell kill due to DDATHF could be obtained, we were then able to compare...
Fig. 3. Morphological changes in WiDr cells following exposure to D-1694. Cells were exposed to either PBS (A, C, E, G) or 0.32 μM D-1694 (B, D, F, H) for 4 h beginning 24 h after seeding into 75-cm² culture flasks at 2 × 10⁶ cells/flask. Following exposure to drug, cells were washed with PBS and cultured in drug-free medium. Photographs of equivalent fields were taken at × 320 at 24 (A, B), 48 (C, D), 48 (E, F), or 53 h (G, H) following removal of drug-containing medium.

cytotoxicities due to either DDATHF or D-1694. Although both were extremely potent cytostatic agents, major differences were found in their cytocidal properties (Fig. 7). After 24 h, less than 1 in every 10³ cells exposed to 0.04 μM D-1694 could form progeny. Only 1 in every 10⁵-10⁶ cells survived treatment with D-1694 at 1 μM for 24 h, a level of survival which is often found equivalent to mutant frequencies at a single genetic locus. On the other hand, very high concentrations of DDATHF and long exposures were required to kill ≥2 logs of cells (Figs. 7 and 8). In a series of experiments, 100 μM DDATHF for 72 h only reduced survival to 1–6 × 10⁻³, the highest level of cell kill observed with this drug.

The cytotoxic effects of exposure to a 1 μM concentration either of D-1694 or of the diastereomers of DDATHF were compared as a function of time (Fig. 8). The presence of hypoxanthine (32 μM) during and after drug completely reversed the cytotoxicity caused by (6R)-DDATHF for up to 96 h of exposure and, likewise, 5.6 μM thymidine prevented the cytotoxicity of D-1694. This suggests that the cytocidal as well as the antiproliferative effects of the drugs are specific, each for one of these pathways. At this concentration, D-1694 was found to be 3.5–4 orders of magnitude more effective as a cytocidal agent than (6R)-DDATHF at any time point. After 96 h of exposure to 1 μM (6R)-DDATHF, 7–10% of the population remained viable; whereas a maximum of 0.003% of cells treated with 1 μM D-1694 survived 96 h of exposure. The cytotoxicity of the 6S diastereomer of DDATHF was somewhat higher than that of the 6R diastereomer, and there was a trend indicating that long-term exposure to the 6S diastereomer was not completely reversible by the simultaneous presence of hypoxanthine. The time course with which maximum cytotoxicity was reached for D-1694, or for either isomer of DDATHF, was nearly identical. Exposure to (6R)-DDATHF, (6S)-DDATHF, or D-1694 for longer than one generation time (22 h) did not significantly increase cytotoxicity (Fig. 8).

Given that thymidine prevented the cytotoxicity of D-1694 and a source of purine, such as hypoxanthine, completely circumvented the cytotoxic effects of (6R)-DDATHF, it was apparent that these compounds could be used to terminate the cytotoxic action of these inhibitors on thymidylate and purine synthesis, respectively. To determine the timing with which commitment to death occurred in cells...
following the onset of continuous inhibition of thymidylate or purine synthesis, hypoxanthine or thymidine was added to cell cultures following varying lengths of exposure to 1 \( \mu \)M DDATHF or D-1694 (Fig. 8). With either 1 \( \mu \)M D-1694 or DDATHF, cytotoxicity was not appreciable for up to 24 h of continuous exposure when reversing agent was added to the medium after drug was removed, yet this length of exposure to either drug without subsequent reversing agent was maximally cytotoxic. Hence, essentially all of the cytotoxicity of short exposures to either DDATHF or D-1694 developed because of events occurring after the point of removal of extracellular drug. For example, 99.99% of the cell population treated for 8 h with 1 \( \mu \)M D-1694 were killed if allowed to recover in the absence of thymidine, but this exposure was without effect if thymidine was added to cultures after drug. After interruption of thymidylate synthesis or de novo purine synthesis for 24–96 h, an increasing fraction of WiDr cells underwent events that irreversibly committed them to death, although the slopes of the cytotoxicity curves between 24 and 96 h were much steeper for D-1694 (0.75 logs of kill/24 h) than for DDATHF (0.22 logs/24 h).

**Replating Experiments.** A common criterion of whether persistently adherent but nonproliferating cells are viable is whether they can reattach to a growth surface after trypsinization and subsequently grow. To apply this test, WiDr cells were treated with either DDATHF or D-1694 for 4 h, then the total cell number per dish was determined by trypsinization and cell counting. The ability of these trypsinized cells to replate and form colonies was determined. The results of these experiments (Fig. 9) indicated that, although essentially all DDATHF-treated cells did not detach from the dishes, >90% of them would not replate. This was consistent with the decrease in colony formation found after treatment of cells with the equivalent concentration of drug. Likewise, the ability of D-1694-treated cells to replate agreed with the survival of these cells as measured by colony formation. It was also noted that DDATHF-treated cells that would replate began to increase after only 3 days, an indication of recovery from growth inhibition.

**Comparison with Cytotoxicity of MTX.** WiDr cells were exposed to MTX and the cytotoxicity of such exposures was compared
were exposed to the indicated concentrations of DDATHF for 4 h beginning 24 h after
seeding into 100-mm dishes at either 3 x 10^5 or 3 x 10^6 cells/dish. Following exposure,
cells were washed with PBS and cultured in drug-free medium for the indicated periods
with medium changes at 3-day intervals. After 6 days, some dishes were scored micro-
scopically by counting the number of cell clusters containing ≥ 8 cells or ≥ 16 cells. After
12 days other dishes were scored by counting visible Giemsa-stained colonies. Points,
mean value of at least two dishes.

with those of DDATHF and D-1694. A 24-h exposure to MTX decreased the survival of WiDr cells, but the cell kill observed was limited to about 1.5–2 logs even at high concentrations (Fig. 10). The presence of hypoxanthine in the medium during MTX exposure resulted in a higher degree of cell kill, with an apparent plateau at 3.5–4 logs of cytotoxicity at the highest concentration used. Hence, the plateau of cell survival observed at high concentrations of these agents indicates that the relative efficiencies of cytotoxicity are: MTX < DDATHF < MTX + purines < D-1694. The morphological changes induced by MTX in the presence and absence of purines reinforced the concept of similarity, but not identity, of the action of MTX to that of DDATHF and of MTX plus hypoxanthine to that of D-1694. Thus, 48 h after the beginning of a 24-h exposure to MTX and hypoxan-
thine, WiDr cells underwent a megaloblastosis (Fig. 11F), similar to that seen after treatment with D-1694 (Fig. 3D), then individual cells rounded up and detached from the plate. However, the MTX plus hypoxanthine-treated cells did not detach from the dishes as completely as seen following D-1694. Likewise, a substantial fraction of cells treated with MTX alone remained adherent to the dishes, but others rounded up and detached. The morphology of the cells that remained adherent following treatment with MTX alone initially resembled that of DDATHF-treated cells (compare Fig. 4, E, F, and G with Fig. 11C) but later, cells treated with MTX alone appeared to begin the process of megaloblastosis (Fig. 11, D). The difference in the rate of development of megaloblastosis with MTX alone compared to that induced by MTX and hypoxanthine or with D-1694 was striking, and appeared to indicate that inhibition of purine synthesis docs, indeed, impair the development of thymine-less death.

DISCUSSION

The question of whether the cellular effects of DDATHF are limited
to cytostasis or extend to cytotoxicity is of primary importance for its
use as a chemotherapeutic agent. Two previous investigations have
addressed the cytotoxic activity of either (6R,S)-DDATHF (17) or of
the closely related compound 5-deazaacyclotetrahydrofolate (18). The
methods used (trypan blue dye exclusion, soft agar cloning, and cell
outgrowth after limiting dilution to less than one cell per microliter
well) have different limitations, with soft agar cloning and limiting
dilution outgrowth overpredicting cell kill with grossly cytostatic
drugs because of the limited period that culture medium will support
growth without medium changes. Trypan blue exclusion is usually
thought to detect only cells late in the stage of loss of membrane
integrity and, hence, probably underpredicts cytotoxicity. Neverthe-
less, both previous studies drew the conclusion that inhibitors of
purine synthesis were cytotoxic agents. In this study, the fact that large
numbers of single cells were still attached to the dishes even after
7–24 days after drug treatment raised concern that these cells were
quiescent but still viable. Our experiments applied the traditional and
very pragmatic criterion that a cell is alive if it can produce progeny
when given sufficient time to do so. After considerable attempts to
circumvent misinterpretation of cell stasis as cell death (e.g., Figs. 5
and 6), we drew the conclusion that the cells that lose clonogenicity
after DDATHF treatment met a reasonable criterion for dead cells.
Four observations argue that this compound is cytotoxic: (a) Growth-inhibited WiDr cells begin to recover by about 4 days after treatment

![Fig. 5.](image)

**Fig. 5.** Growth inhibition by DDATHF complicates quantitation of cytotoxicity. Cells
were exposed to the indicated concentrations of DDATHF for 4 h beginning 24 h after
seeding into 100-mm dishes at either 3 x 10^5 or 3 x 10^6 cells/dish. Following exposure,
cells were washed with PBS and cultured in drug-free medium for the indicated periods
with medium changes at 3-day intervals. After 6 days, some dishes were scored micro-
scopically by counting the number of cell clusters containing ≥ 8 cells or ≥ 16 cells. After
12 days other dishes were scored by counting visible Giemsa-stained colonies. Points,
mean value of at least two dishes.

![Fig. 6.](image)

**Fig. 6.** Recovery of WiDr cells from growth inhibitory effects of DDATHF does not
diminish drug-induced loss of colony formation. (A) Cells were exposed to the indicated
concentrations of DDATHF for 4 h beginning 24 h after seeding into 100-mm dishes at
3000 cells/dish. Following exposure, cells were washed with PBS and cultured in drug-
free medium for 7–24 days, then macroscopically visible staked colonies were scored.
Columns, mean value of three dishes. (B) After treatment with DDATHF at 30–160 μM as
indicated in (A), cultures were maintained for 18 days in drug-free medium, then 32 μM
hypoxanthine (Hx) was added to the medium of some dishes (Œ). Twelve days later,
macroscopically visible colonies were counted on these plates and on control plates (Œ),
which were maintained on unsupplemented medium for the additional 12 days. All media
were changed at least every 3 days. **Error bars** indicate standard deviations.
clearly indicate that the effect of either drug continues long after removal from the medium. This almost certainly reflects the metabolism of these drugs to poly-γ-glutamate derivatives, which are not easily lost from the cell (9, 14) but are active enzyme inhibitors (9, 15). Given the different time courses of cytotoxicity with end product added after drug removal or without end product in the medium changes (Fig. 8), we conclude that both drugs are metabolized quickly, with either maximal accumulation or maximum effect of accumulating drug occurring within 24 h. Likewise, for either DDATHF or D-1694, cell kill is negligible if reversing agent is added after up to 24 h of drug exposure (Fig. 8). Yet, for longer periods of exposure to either drug, commitment to cell death was found to progressively

with DDATHF (Fig. 9) and a growing WiDr cell easily forms visible colonies in 6 days, yet 99–99.9% of WiDr cells treated with high concentrations of DDATHF do not form colonies after almost 1 month of culture in the absence of drug (Fig. 6A). (b) In spite of the fact that hypoxanthine can prevent and, under some conditions, reverse the effects of DDATHF, colony formation experiments (Fig. 8) detect a hypoxanthine-irreversible decrease in viability following exposure times >24 h. (c) Cells adherent to culture dishes 18 days after a brief (4-h) exposure to DDATHF did not form colonies even in the presence of exogenous hypoxanthine (Fig. 6B). And, (d) The majority of DDATHF-treated cells that remained adherent to the dishes did not reattach when trypsinized and replated (Fig. 9), and the decrease in plating efficiency in such experiments agreed with the expected loss of colony-forming ability.

The cellular effects of D-1694 and of DDATHF have perplexing similarities and differences. End product reversal studies (Fig. 8)
accumulate, but at a substantial difference in rate for the two pathways. Also, D-1694 caused cells to detach from the growth surface (Figs. 2 and 3) but DDATHF did not (Fig. 4).

It is reasonable to conclude that the time course of cell kill with rescue agent added after drug is removed (Fig. 8) reflects the time course of the intrinsic lethality of complete interruption of the affected pathway. Hence, it seems that these cells can withstand inhibition of the supply of thymidylate or of newly synthesized purines for 20–24 h without effect, but longer periods of interruption of either pathway commits cells to death. This remarkable conclusion is reminiscent of the fact that thymine-requiring bacteria can survive for about one generation time without thymine in the medium before initiating thymine-less death (22, 23). Similar observations have been reported for mouse cells genetically deficient in thymidylate synthase (24). Studies on synchronized cell populations might allow a dissection of the events that are either causally involved in commitment to death or merely coincident with or resulting from cell death. For instance, the cellular and nuclear swelling seen in Fig. 3B are not sufficient to cause irreversible commitment to cell death, as indicated by the fact that the addition of thymidine at this stage will prevent the cytotoxicity of D-1694, but the irreversible changes causative of cell death have begun in the cells pictured in Fig. 3D.

It appears from our studies that DDATHF (and presumably any other “pure” inhibitor of de novo purine synthesis) induces tumor cell kill less efficiently than D-1694. Some of this difference is due to the fact that D-1694 is about 10-fold more potent of a metabolic inhibitor to whole cells, as judged by cell growth assays (Fig. 1). However, the apparent plateau of cytotoxicity at surviving frequencies of $10^{-2}$ to $10^{-3}$ with (6R)-DDATHF is not seen with D-1694 (Fig. 10). Borsa and Whitmore (1) found that the cytotoxicity of MTX was limited to about 1.5–2.0 logs of kill in standard medium even after exposure to high concentrations for extended periods, but that MTX became substantially more cytotoxic (to 3.5–4 logs of kill) when a purine was included in the medium to circumvent drug effects on de novo purine synthesis. Because the Borsa and Whitmore experiment was so crucial to the interpretation of our results, we repeated it, using the WiDr cell and found remarkably similar results (Fig. 10). Hence, the plateau seen for DDATHF cytotoxicity is consistent with the concept previously enunciated (1, 17) that the inhibition of purine synthesis by MTX may limit the cytotoxicity caused by inhibition of thymidylate synthesis by that agent. Previous studies have tried to directly test this idea by comparing the cytotoxicities of DDATHF and antifolates inhibitory to thymidylate synthase alone and in combination (17). However, the drugs used both relied on the same membrane transport and polyglutamation system for activity, so the interference by DDATHF noted in those studies might reflect any of a number of interactions, including competition for transport and anabolism. The plateau seen after a 24-h exposure to MTX, D-1694, and DDATHF for WiDr cells (Fig. 10) would indicate that a pure inhibitor of purine synthesis would be at least as cytotoxic as MTX, but that a pure inhibitor of thymidylate synthase would be substantially more cytotoxic than a pure inhibitor of purine synthesis or an agent active at both targets. However, the intrinsic cytotoxicity of an agent is of secondary interest compared with its preferential cytotoxicity toward neoplastic cells. Such selectivity cannot be determined from our studies and will only be discernible from the ongoing clinical trials with D-1694 and DDATHF.

The biochemical effects secondary to inhibition of thymidylate and purine synthesis have been extensively studied. Inhibition of thymidylate synthesis results in a diminished TTP pool and a substantial accumulation of dUTP (25). As a result, dUTP is misincorporated into both newly synthesized DNA and DNA patches that are being repaired, and the excision of dUMP residues by DNA-uracil glycosylase causes DNA single strand breaks in mature DNA and short Okazaki fragments (24–26). On the other hand, DDATHF depletes purine
nucleoside triphosphates and slows cell cycle traverse (17–20). It is not clear, at this point, whether DDATHF causes effects on DNA synthesis either directly or through depletion of ribonucleoside triphosphates. Thus, the results of the two studies of DDATHF effects on deoxynucleotide pools have differed (18, 19). Likewise, cell cycle traverse studies indicate that DDATHF blocks cells at the G1/S border in CEM cells (7), but HL60 cells seem to accumulate in late S (20). Hence, it seems that the detailed cellular and biochemical effects of DDATHF differ among cell types, yet the drug has a potent antiproliferative effect on all cells yet studied, including human hematopoietic stem cells (10–12). Previous studies have attributed the cytotoxicity of thymidylate synthase inhibitors and GART inhibitors to initiation of the process of apoptosis (17, 18). This concept is supported by the occurrence of intranucleosomal DNA ladders, usually considered definitive of apoptosis, in cells in which either enzyme is inhibited (17, 18, 27). However, the distinctly different morphologies of cells dying after D-1694 or DDATHF (Figs. 3 and 4) imply that it would be an oversimplification to think that both classes of agents are following exactly the same path to commitment to death. Likewise, the rates of irreversible commitment to cell death with DDATHF or D-1694 seen (Fig. 8) beginning after 24 h of drug exposure seems to be an important facet of the difference between inhibitors of purine synthesis and inhibitors of thymidylate synthesis. This is particularly the case given that inhibition of either pathway can be withheld for the same 24-h period without toxicity. Given that the concentration of drug used was sufficient to completely inhibit cell growth with either drug (and, presumably, completely inhibit the respective target enzymes), these similarities and differences would appear to hold the key to understanding why carcinoma cells die when faced with these antifolates.

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