Pergamon

Biochemical Pharmacology, Vol. 47, No. 6, pp. 1067–1078, 1994.
Elsevier Science Ltd
Printed in Great Britain.
0006-2952/94 $6.00 + 0.00

0006-2952(93)E0067-H

EFFECT OF PURINE SYNTHESIS INHIBITION ON WiDr SPHEROIDS *IN VITRO* OR ON WiDr OR COLON 38 TUMORS *IN VIVO*

COMPLETE GROWTH INHIBITION BUT NOT REGRESSION

MARILYN JANSEN, MICHAEL DYKSTRA, JACQUELINE I. LEE, JEREMY STABLES, PETER TOPLEY, VINCENT C. KNICK, ROBERT J. MULLIN, DAVID S. DUCH and GARY K. SMITH*

The Wellcome Research Laboratories, Research Triangle Park, NC 27709, U.S.A.

(*Received* 14 *May* 1993; *accepted* 4 *October* 1993)

Abstract—Clinical responses for anticancer agents are based upon tumor regression. We have investigated the potential of glycineamide ribonucleotide transformylase (GAR TFase) inhibitors to produce regressions in multiple preclinical models of colon carcinoma. The growth of multicellular tumor spheroids of WiDr human colon carcinoma was inhibited by the GAR TFase inhibitors 5-deazaacyclotetrahydrofolate (5-DACTHF), its 2′-fluoro, 3′-fluoro, 10-deaza, and 10-thia analogs as well as 5,10-dideazatetrahydrofolate, but none of the compounds caused spheroid regressions. By contrast, complete spheroid disruption was observed with exposure to etoposide, *m*-AMSA (amsacrine), piritrexim, or 2-desamino-2-methyl-10-propargyl-5,8-dideazafolate (DMPDDF). Light microscopy of the spheroids treated with either 5-DACTHF or DMPDDF suggested that the reason for the difference is extensive cell kill throughout the spheroid in the presence of DMPDDF compared with little or no kill, over that found in controls, with 5-DACTHF. Treatment of spheroids with 5-DACTHF in the presence of 1 μM hypoxanthine resulted in no significant reversal of growth inhibition; 50% reversal required 10 μM hypoxanthine. The spheroid studies were extended to *in vivo* studies examining the effects of 5-DACTHF on established WiDr and colon 38 tumors. The results showed that, in contrast to melphalan, which produced cures and tumor regressions, 5-DACTHF produced reversible growth inhibition with no significant regression of tumors. The results predict that clinical response, typically measured by tumor regression, may be rare following single agent therapy with inhibitors of *de novo* purine biosynthesis.

Key words: purine synthesis, 5-deazaacyclotetrahydrofolate; 5,10-dideazatetrahydrofolate; 2-desamino-2-methyl-10-propargyl-5,8-dideazafolate; etoposide; piritrexim; colon cancer; multicellular tumor spheroids

De novo purine biosynthesis is a potential target for cancer chemotherapy, and potent new inhibitors of the pathway are under development [1–8]. These new compounds are clean inhibitors of GAR TFase† [1–8] in constrast to previously investigated antipurines, such as 6-mercaptopurine, which enter DNA and RNA as well as inhibit purine synthesis [9, 10]. Thus, it is expected that the effects of the GAR TFase inhibitors on cancer cells should be due solely to purine depletion. It has been found that in the absence of salvageable purines, inhibition of GAR TFase in cancer cells does deplete cellular purines and inhibit cell growth [1–8]. However, against MOLT-4 T-cell leukemia, the growth inhibition produced little actual cell kill when compared with that of TS inhibition [8]. In addition, as expected for an inhibitor of purine synthesis, the inhibition of cell growth was blocked by the presence of 50–100 μM hypoxanthine in the growth medium [1, 4], although the effects of lower levels of hypoxanthine, similar to those found in human blood plasma [11–14], are not known. To understand better the clinical potential of GAR TFase inhibitors, it is important to determine (i) whether sufficient cell kill is produced by the compounds to yield tumor regressions, and (ii) whether circulating purine levels will influence the antipurine effects.

The *in vivo* multicellular spheroid tumor model presents an excellent opportunity to test the effects of agents on tumor growth. Spheroid technology allows the growth of tumor cells in a three-dimensional array having characteristics similar to tumors *in vivo* but with the experimental control only available *in vitro* [15–19]. The similarities

* Corresponding author: Dr. Gary K. Smith, The Wellcome Research Laboratories, 3030 Cornwallis Road, Research Triangle Park, NC 27709. Tel. (919) 315-4179; FAX (919) 315-8375.

† Abbreviations: GAR TFase, glycineamide ribonucleotide transformylase; 5-DACTHF, 5-deazaacyclotetrahydrofolate; DDACTHF, 5,10-dideazaacyclotetrahydrofolate; DDATHF, 5,10-dideazatetrahydrolfolate; DMPDDF, 2-desamino-2-methyl-10-propargyl-5,8-dideazafolate; TS, thymidylate synthase; and *m*-AMSA, amsacrine.

between spheroids and tumors include cell-to-cell contacts, a viable rim and necrotic core, low internal oxygen tensions, and restricted drug penetration.

In the present study, we chose the spheroid model of WiDr human colon carcinoma tumors to test the effects of purine synthesis inhibition on tumor growth and regression. Since the WiDr cells grow *in vivo* as well as *in vitro*, we extended the spheroid results to WiDr tumors *in vivo*. Finally, the *in vivo* results were confirmed in the mouse tumor colon 38. The data *in toto* demonstrated that GAR TFase inhibitors largely produced only reversible inhibition of cell growth with no tumor regression and that this inhibition of growth was the same in the presence of physiological hypoxanthine levels. However, since the effects were solely growth inhibition, the results suggest that clinical regressions with this class of inhibitors may be rare.

MATERIALS AND METHODS

Chemicals. Etoposide (VePesid) was obtained from Bristol Myers. *m*-AMSA was a gift of the National Cancer Institute and was prepared as a 10 mM stock in 100% DMSO. Piritrexim (Burroughs Wellcome) was used as a 5 mM stock in 0.01 N NaOH. DMPDDF was a gift of Dr. T. R. Jones and was prepared as a 1 mM stock in 0.001 N NaOH. DDATHF (Southern Research Institute) was a 1 mM stock in 0.001 N NaOH. 5-DACTHF (543U76) and the 2′-fluoro, 3′-fluoro, 10-deaza-10-thia and 10-deaza-10-methylene (DDACTHF) analogs (299U88, 339U88, 674U88, and 662U88, respectively) were prepared as previously described [4, 7]; stock solutions were 1 mM in 0.01 N NaOH. Hypoxanthine (Sigma, St. Louis, MO) was prepared as a 10 mM stock in 0.01 N NaOH. [8-^{14}C]-Hypoxanthine (56 mCi/mmol) was obtained from Moravek Biochemicals, Brea, CA. Working stock solutions of all compounds were diluted in Dulbecco's phosphate-buffered saline before addition to cultures.

Cell monolayer and spheroid culture. The human colon carcinoma cell line WiDr was obtained from the American Type Culture Collection (ATCC; Rockville, MD) and adapted to grow on 10 nM leucovorin as the folate source [20]. Cell monolayer and spheroid cultures were maintained in folate-free RPMI-1640 (Life Technologies, Inc., Grand Island, NY) containing 10% charcoal dialyzed fetal bovine serum (JRH Biosciences, Lenexa, KS), 10 nM (6*R,S*)-calcium leucovorin (Burroughs Wellcome) and the antibiotics penicillin (50 IU/mL)-streptomycin (50 μg/mL) at 37° in a 95% humidified air–5% CO_2 atmosphere. Spheroid size was measured as a cross-sectional area with an Artek 980 counter attached through the camera side port of a Nikon Diaphot inverted microscope. Successive measurements of experimental spheroid area were compared with that at day zero, and growth was expressed as a percentage of day zero (size prior to drug exposure). Using a template of standardized areas, a linear relationship between diameter and square root of the cross-sectional area was determined. The diameter of individual spheroids was interpolated from this standard curve.

Methods for spheroid culture were adapted from previously reported techniques [15–17]. Spheroids were initiated in siliconized spinner flasks with WiDr cells from trypsinized monolayers in exponential growth. Spinner flasks (500 mL, Techne, Cambridge, U.K.) were siliconized for 30 min with 20% Surfacil (Pierce, Rockford, IL) in 100 mL total volume dry hexane, autoclaved and seeded with 2×10^6 cells in 250 mL growth medium. The flasks were stirred at 20 rpm. Spheroid drug treatment studies were performed on individual spheroids grown in 24-well plates (Costar, Cambridge, MA) that had been

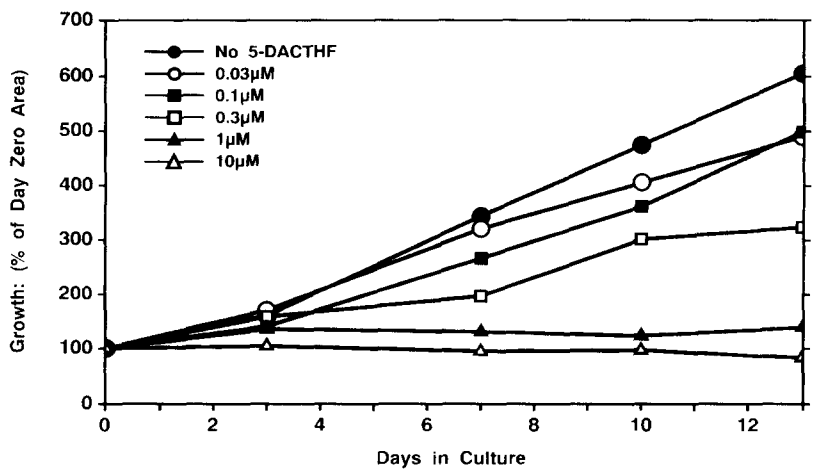


Fig. 1. Effect of continuous exposure to the purine synthesis inhibitor 5-DACTHF on WiDr spheroid growth. Individual spheroids were transferred to agarose-coated multiwell plates containing various concentrations of drug, and spheroid size was subsequently measured every 3–4 days. Values represent the mean spheroid area as a percentage of day zero of triplicate samples.

coated with 0.25 mL of 0.56% low melt agarose (Bio-Rad, Richmond, CA) prepared in growth medium. Individual spheroids (200–400 μm diameter) were transferred to the plates after 4–7 days growth in the spinner flask and maintained in 1 mL of growth medium. Growth medium in the multiwell plates was replaced weekly. Typically, drugs were added at day zero (day of transfer of the spheroid from spinner flask to multiwell plate) and medium was replaced at day 7. To prevent drug decomposition of 5-DACTHF and its analogs, medium and drugs were replaced every 48 hr. For experiments with [^{14}C]hypoxanthine, the growth medium and [^{14}C]hypoxanthine were replaced with fresh medium every 24 hr. The radioactive content of the spent medium was examined and quantitated with an HPLC system [21] that resolves uric acid, xanthine, hypoxanthine, guanine,and adenine (retention times 350, 450, 600, 1000, and 1150 sec, respectively). Elution of radioactivity from this HPLC system was monitored with a Beckman 171 radioisotope detector with liquid scintillation and Ecolite (+)® (ICN) liquid scintillant.

Light microscopy. Spheroids were placed in McDowell's and Trump's fixative [22] for several days at 4°. They were then embedded in methacrylate resin solution (Polysciences, Inc., Warrington, PA; JB-4 Plus Embedding Kit). Manufacturer's instructions were followed for embedding and polymerization. The spheroid blocks were then sectioned at 2 μM thickness. Finally, the sections were stained with hematoxylin-eosin and viewed by both phase contrast and bright field microscopy.

Tumor growth and measurement. Colon 38 adenocarcinoma was obtained from the NCI Tumor Repository and maintained in C57BL/6 mice (Charles River Laboratories Inc., Wilmington, MA) as subcutaneous axillary implants of 25–40 mg, with a passage time of 21–28 days. WiDr cells were obtained from the ATCC, maintained subcutaneously in the flank of male CD1 nude mice, and passed as 1 mm cubes. Animals were fed *ad lib.* with Agway PROLAB Rat/Mouse/Hamster 3000, which has a folic acid content of 1.67 mg/kg. Pretreatment plasma folate levels were 100 ng/mL [6].

Tumor growth was monitored with manual or electronic digital calipers (L. S. Starrett Co., Athol, MA) that were interfaced with a personal computer. Tumor weight in milligrams was calculated with use of the equation: [length (mm)·width^2 (mm)]/2 = weight in mg [23]. With multiple tumors, tumor burden was calculated as the sum of individually measured tumors. Tumors were typically measured twice weekly. Animals were maintained in Micro-isolator cages (LabProducts, Inc.), at 72 ± 2°F with a 12-hr light/dark cycle. All animal handling was performed in a laminar flow hood (LabProducts) using a sterile technique. Tumors were not examined for fibroid residue following 5-DACTHF therapy since the rapid tumor outgrowth following drug removal was indicative of temporary tumor inhibition rather than actual cell kill.

Experiments with SK-MEL-31 (melanoma), H69 (small cell lung carcinoma), OVCAR 5 (ovarian adenocarcinoma), and HT29 and DLD-1 (colorectal adenocarcinomas) were performed under contract at the Southern Research Institute, Birmingham, AL.

RESULTS

An *in vitro* method [15–17] using intact spheroids from the WiDr colon carcinoma cell line was established to assess the effects of inhibitors of *de*

Table 1. Spheroid size after 16 days continuous exposure to various agents*

Agent	WiDr monolayer IC_{50} (μM)	Minimum concentration for complete growth inhibition or destruction of WiDr spheroids (μM)	Size as % of day zero†
GAR TFase inhibitors			
5-DACTHF	0.045‡	1.0	64 ± 19
3′-Fluoro-5-DACTHF	0.109	0.3	93 ± 3
2′-Fluoro-5-DACTHF	0.045	1.0	101 ± 33
5,10-DDACTHF	0.047	1.0	103 ± 6
10-Thia-5,10-DDACTHF	0.025	1.0	81 ± 3
5,10-DDATHF	0.016‡	0.03	105 ± 2
Other agents			
DMPDDF	0.065	0.1	0
Piritrexim	ND	1.0	0
m-AMSA	ND	30	0
Etoposide	ND	4.4	0
No treatment			439 ± 51

* Individual spheroids were transferred to agarose-coated plates with or without various agents. Medium and drug were changed every other day with fresh agent added. After 16 days of continuous exposure, the spheroid area was measured. Values are means of triplicates of spheroid area as % of day zero ± SD. ND = not determined.

† At 1 μM drug.

‡ Data reproduced from Smith *et al.* [5].

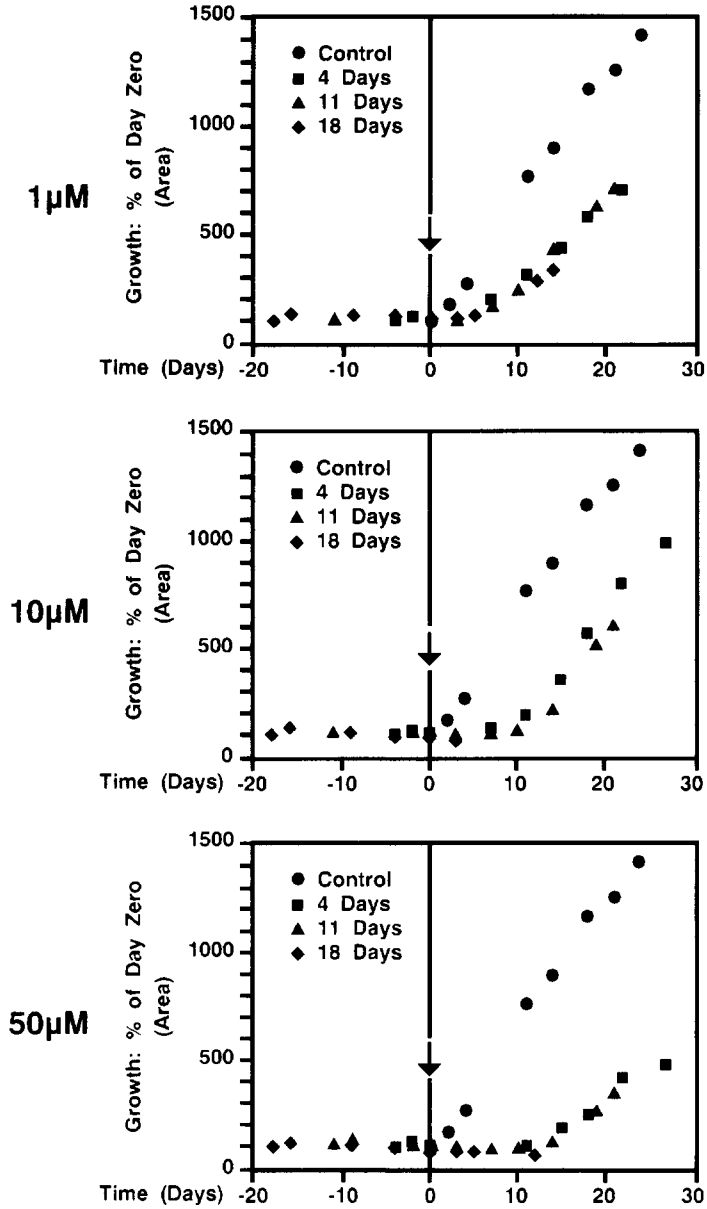


Fig. 2. Effect of exposure time and concentration of 5-DACTHF upon WiDr spheroid regrowth. Individual spheroids were transferred to agarose-coated multiwell plates containing 1, 10, or 50 μM 5-DACTHF. Spheroids were exposed to drug for 4, 11 or 18 days, after which the spheroids were washed with growth medium and transferred to fresh agarose-coated plates without drug. Values represent the mean spheroid area as a percentage of day zero of triplicate samples.

novo purine biosynthesis on growth. Comparable growth curves were achieved from spheroids established and grown in spinner flasks or established in spinner flasks and subsequently grown individually in 24-well agarose-coated plates. Spheroids grew at a rate of 0.054 mm/day in spinner flasks and 0.052 mm/day in agarose-coated wells. The effect of drug treatments on spheroids could be measured either on spheroids grown in the spinner flasks by random samples taken from flasks or more conveniently by treatment of individual spheroids in separate wells of a 24-well plate. The advantage of examining spheroids in multiwell plates is that individual spheroids can be followed with each being its own zero time control.

WiDr monolayers and spheroids were both inhibited by the purine *de novo* biosynthesis inhibitors 5-DACTHF, its analogs, and DDATHF (Fig. 1 and Table 1). All of these compounds are inhibitors of *de novo* purine biosynthesis at GAR TFase [1–8]. Figure 1 shows the effect of continuous 5-DACTHF exposure upon the spheroids; all other GAR TFase inhibitors behaved similarly. In all cases, the sole effect was growth

inhibition with no significant reduction in size. Similar results were obtained whether 10 nM leucovorin or 2 μM folic acid was used as the folate source (data not shown). Table 1 shows the concentrations of inhibitors that produce 100% growth suppression. The most potent spheroid growth inhibitor was DDATHF, which also was the most potent inhibitor of monolayer growth (Table 1). There was, however, no direct correlation between monolayer IC_{50} and spheroid growth inhibition. This poor correlation may be due to differences in the abilities of these drugs to penetrate the spheroid three-dimensional array to access interior cells versus direct application to monolayers. Concentrations above the 100% growth inhibitory concentration (up to 10 μM) also only produced growth suppression. At no concentration did significant regression in spheroid size occur. To test the viability of these growth-inhibited spheroids, individual spheroids were exposed to various concentrations of 5-DACTHF for 4, 11 or 18 days and then drug was removed. In all cases, following a lag period regrowth was observed after removal of the drug (Fig. 2). The length of dosing had no effect on the length of delay or rate of regrowth; however, the delay in regrowth was dependent on drug concentration. Spheroids exposed to 50 μM 5-DACTHF exhibited a greater delay than those exposed to 10 or 1 μM drug. Similar results were obtained with two other GAR TFase inhibitors, the 2′-fluoro or 3′-fluoro-analogs of 5-DACTHF (not shown).

By contrast to the purely growth inhibitory effects of exposure to purine synthesis inhibitors, complete spheroid disruption was induced by continuous exposure to agents with alternate mechanisms of action (Fig. 3 and Table 1). Continuous exposure to the topoisomerase II inhibitors etoposide (4.4 μM) and *m*-AMSA (30 μM) both caused disruption by day 13 and day 9 of treatment, respectively. The dihydrofolate reductase inhibitor piritrexim caused disruption at 1 μM by day 13 of treatment, and the TS inhibitor DMPDDF caused disruption at 0.03 μM by day 11 of treatment.

To investigate the reasons for these striking differences in spheroid response to the purine synthesis inhibitors and these other chemotherapeutic agents, spheroids were treated with either the *de novo* purine synthesis inhibitor 5-DACTHF or the TS inhibitor DMPDDF and examined by light microscopy. Drug exposures were terminated after 4 or 6 days, which is prior to spheroid disruption (Fig. 3). The treated spheroids were washed, resuspended in Trump's fixative for light microscopy, embedded in methacrylate resin, sectioned and analyzed. The results of the light microscopy experiment are shown in Fig. 4 for the 4-day exposure. The control spheroids and those treated with 5-DACTHF appeared normal; both contained viable rims 10–15 cells thick and necrotic core regions. In contrast, the spheroids treated with the TS inhibitor showed extensive cell kill throughout. By day 6, control and 5-DACTHF-treated spheroids were similar to the day-4 samples, but the spheroids treated with the TS inhibitor decomposed during fixation and embedding, presumably due to extensive cellular disruption. The results demonstrate that the difference between the purine synthesis inhibitors

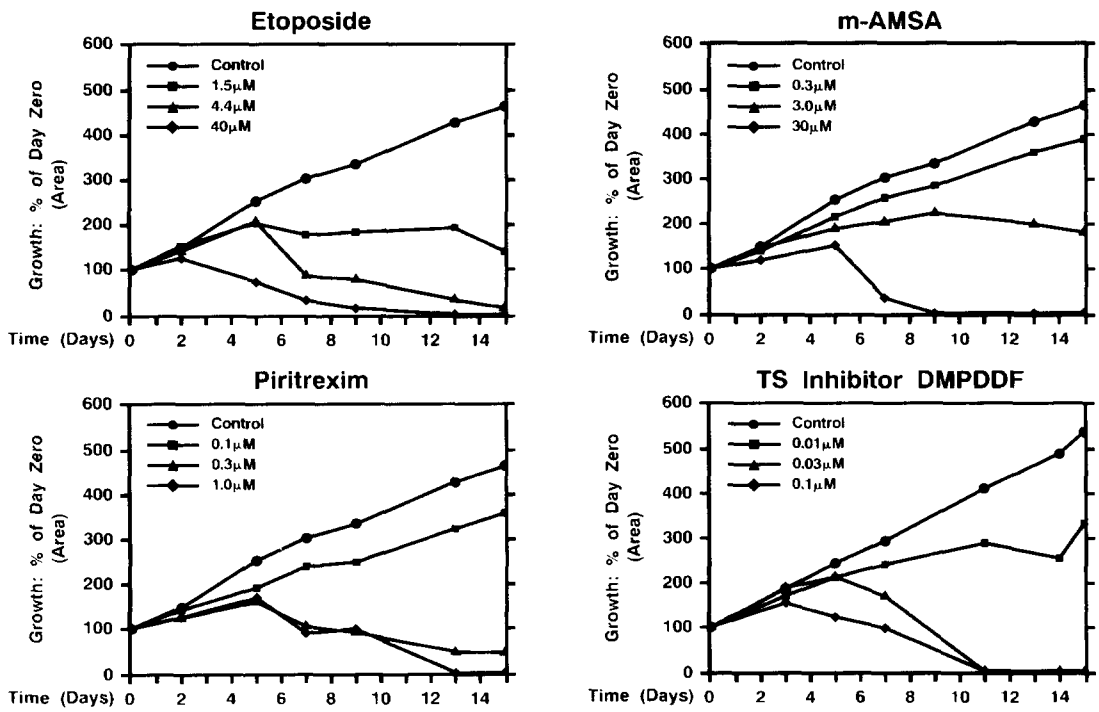
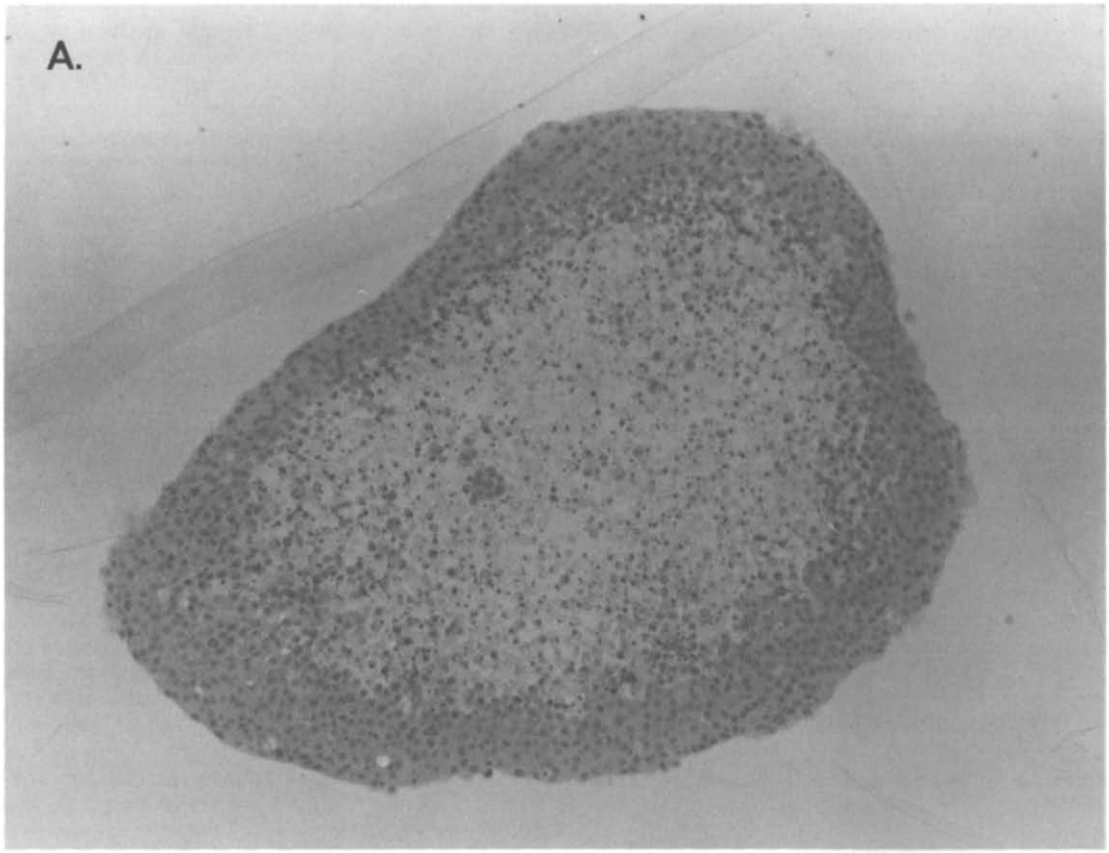
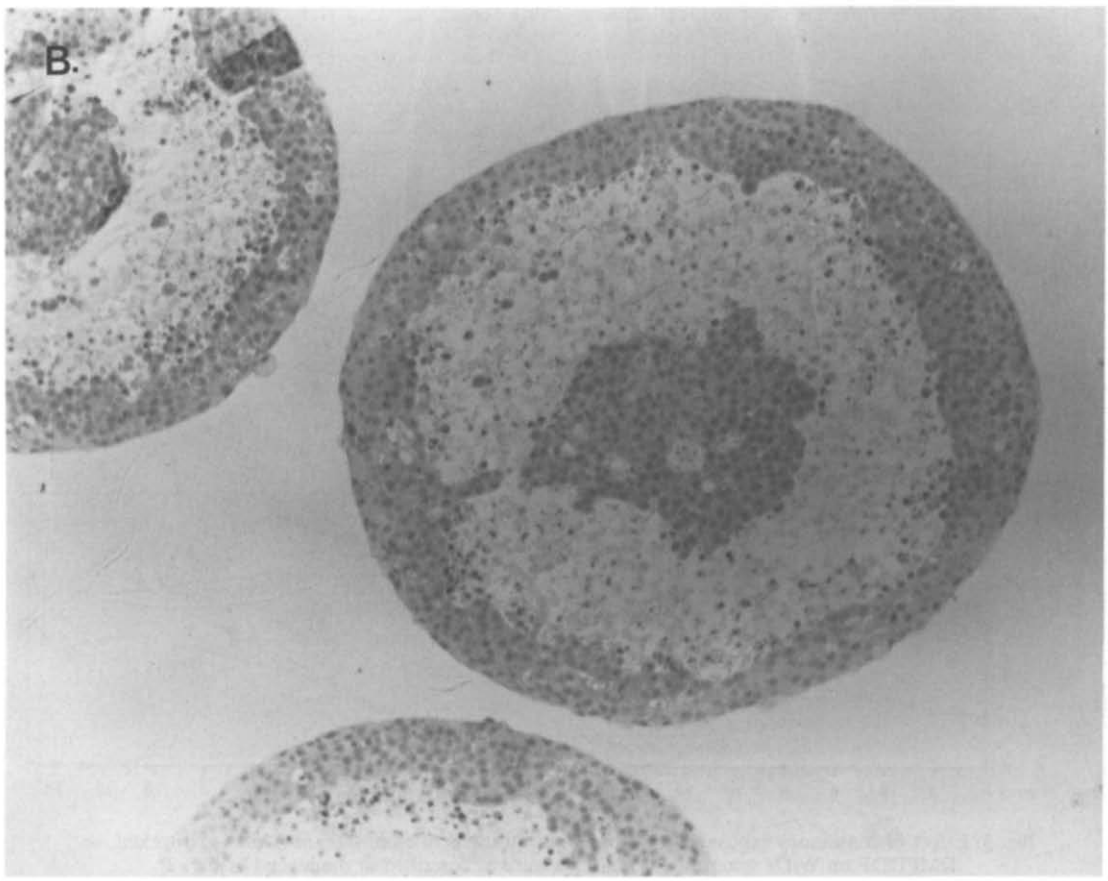


Fig. 3. Effect of continuous exposure to various concentrations of etoposide, *m*-AMSA, piritrexim, or DMPDDF on WiDr spheroids. Conditions were as described in the legend of Fig. 2.


A.


B.

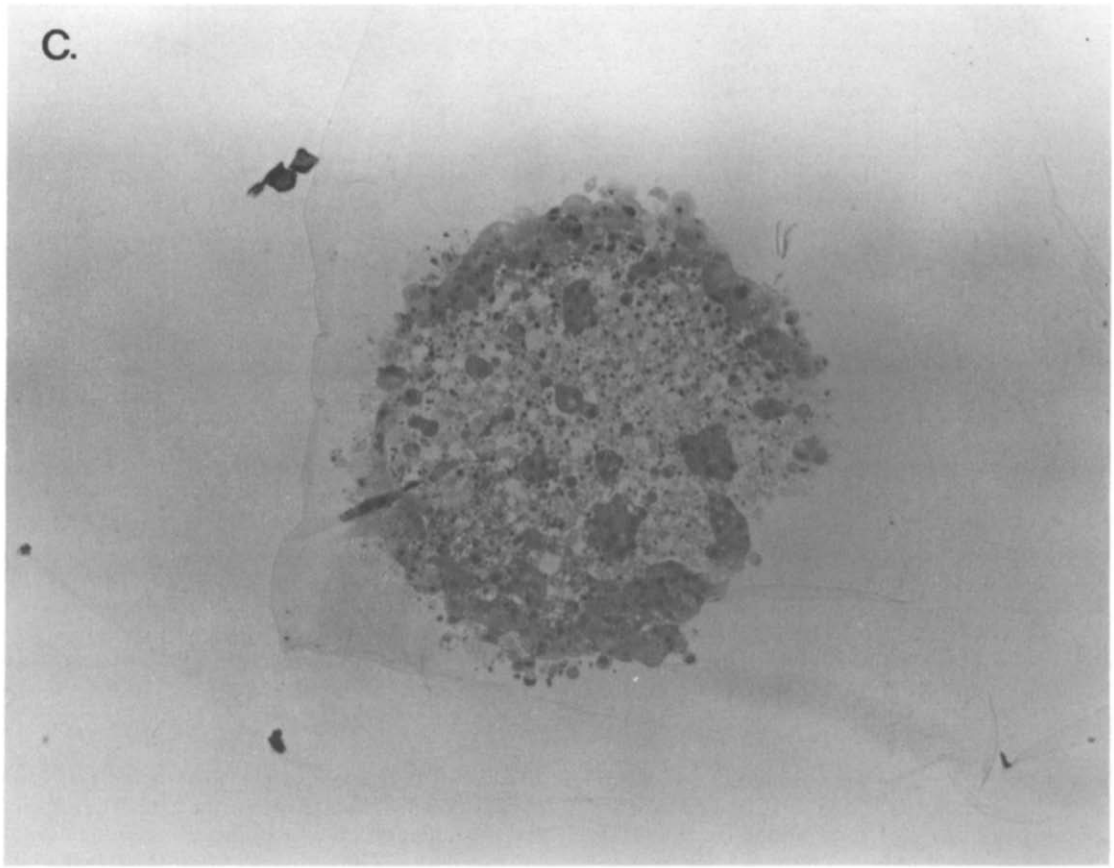


Fig. 4. Light microscopy of (A) control, (B) 5-DACTHF-treated, or (C) DMPDDF-treated spheroids. After 4 days on agarose-coated plates, spheroids were washed, fixed in Trump's fixative, embedded in methacrylate, sectioned, stained with eosin/hematoxylin, and viewed by phase contrast light microscopy. Film magnification = 25 times.

and the other agents is extent of cell kill; purine synthesis inhibition did not cause significant kill. The comparison with DMPDDF is especially relevant since the two types of compounds (GAR TFase and TS inhibitors) have similar structures and metabolic activation pathways. Thus, both types of compounds apparently are transported into cells similarly and are polyglutamated inside the cells to their active species [1–3, 5, 24–26]. Therefore, different effects of the two types of compounds (spheroid stasis vs kill) do not likely result from one type of compound being taken up by only the surface cells while the other type passes into the spheroid center.

For an inhibitor of purine synthesis to suppress tumor growth *in vivo*, circulating purine levels must be below those required to reverse the purine depletion caused by the inhibitor. In humans, circulating levels of purines are 0.5 to 1 μM, and the major circulating purine is hypoxanthine [11–14]. To study the ability of hypoxanthine to reverse growth suppression by an inhibitor of purine *de novo* biosynthesis, spheroids were exposed for 15 days to various concentrations of hypoxanthine with or without 10 μM 5-DACTHF (Fig. 5). [^{14}C]Hypoxanthine was used to monitor levels of hypoxanthine in the medium as a reflection of metabolism and to ensure that the compound was not depleted during the experiment. To ensure further that the hypoxanthine was not depleted, medium, hypoxanthine and 5-DACTHF were changed daily. The spent medium was counted for ^{14}C and also analyzed on HPLC for [^{14}C]hypoxanthine, -xanthine, -uric acid, -guanine and -adenine. The recovery of hypoxanthine in spent medium was found to be 93% at the lowest hypoxanthine concentration (0.5 μM) and 76% at the highest (20 μM) (Table 2). HPLC analysis demonstrated that 10–16% of the added hypoxanthine was converted to xanthine. No uric acid, adenine, or guanine were found. Thus, the protocol provided fairly constant hypoxanthine levels throughout the experiment, especially at the lower hypoxanthine concentrations. Presumably, the 24% loss of hypoxanthine at the highest hypoxanthine concentrations was due to spheroid outgrowth and, in turn, greater metabolism at these levels (see below).

In control experiments, it was found that hypoxanthine alone had no effect upon spheroid growth (Fig. 5). When spheroid growth was totally inhibited with 10 μM 5-DACTHF, 90% reversal of

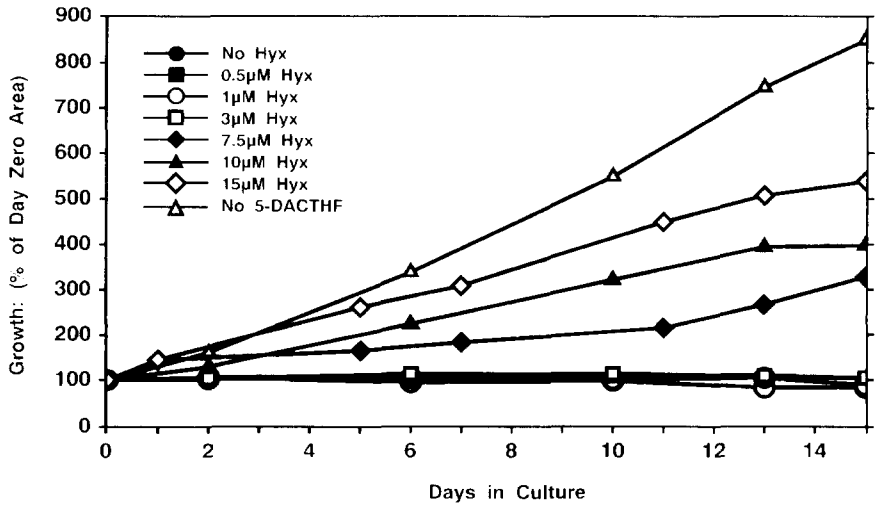


Fig. 5. Hypoxanthine reversal of 5-DACTHF-dependent inhibition of spheroid growth. Individual spheroids were plated in agarose-coated multiwell plates with or without 10 μM 5-DACTHF and various concentrations of hypoxanthine. Medium was changed every 24 hr with fresh additions of 5-DACTHF and hypoxanthine. Spheroid size measurements were taken every 24–48 hr. Results represent the mean spheroid area as a percentage of day zero of triplicate samples. Data are presented as a growth curve (size versus time) for the various hypoxanthine concentrations in the presence of 10 μM 5-DACTHF.

the growth inhibition was produced by 20 μM hypoxanthine; thus, the 5-DACTHF block was solely in purine synthesis. A hypoxanthine concentration of 10 μM was required to produce 50% reversal. By contrast, 1 μM hypoxanthine and below had no effect on the 5-DACTHF-induced growth suppression. These results suggest that at physiological levels of purines, purine synthesis inhibitors should efficiently inhibit tumor growth *in vivo*.

The results of the experiments with WiDr human colon tumor spheroids predict that inhibitors of GAR TFase will suppress *in vivo* tumor growth in humans or mice, but will provide little non-host-mediated tumor regression, and allow for tumor regrowth upon removal of drug.

Table 2. Recovery of hypoxanthine from WiDr spheroid medium*

Initial hypoxanthine (μM)	Purine recovered (μM)	
	Hypoxanthine	Xanthine
0.5	0.47 ± 0.06	0.08 ± 0.02
1.0	0.94 ± 0.09	0.15 ± 0.05
3.0	2.75 ± 0.37	0.27 ± 0.23
5.0	4.45 ± 0.29	0.62 ± 0.19
7.5	5.7 ± 0.8	0.93 ± 0.2
10.0	8.3 ± 0.16	1.1 ± 1.0
15.0	11.1 ± 0.68	1.7 ± 0.4
20.0	15.2 ± 0.17	2.2 ± 0.9

* Agarose-coated wells were seeded with 1 spheroid, 10 μM 5-DACTHF and 72,000 dpm/mL of [^{14}C]hypoxanthine plus enough unlabeled hypoxanthine to achieve the desired concentration. Values are means ± SD of at least three determinations.

We have reported previously that 5-DACTHF can inhibit colon 38 tumor growth in mice [6]. The effects of this compound on tumors *in vivo* were explored further here. The effects of 5-DACTHF on subcutaneous WiDr or colon 38 tumors in mice are shown in Figs. 6 and 7. Figure 6A shows the effects of 20 or 40 mg/kg of 5-DACTHF once or twice daily on days 15–24 on growth of subcutaneous WiDr tumors. A dose of 40 mg/kg twice daily was lethally toxic to all animals by day 26; 20 mg/kg twice daily was lethal to 40% of the animals. All other doses caused growth delay, but no regressions or cures. In other experiments (Fig. 6B), 20 and 40 mg/kg per day were given on days 1–47. Both doses effectively slowed growth over the entire period, but neither produced any tumor regressions or cures. By contrast, when melphalan (Fig. 6C) was given at 10 mg/kg per day on days 1, 5 and 9, tumor growth was inhibited, and 3 of 10 mice were tumor free at day 47. Thus, in contrast to 5-DACTHF, melphalan produced apparent cures. Figure 7A shows the effects of three dosing regimens with 40 mg/kg 5-DACTHF on subcutaneous colon 38 tumors. In these experiments dosing was commenced on day 3, 10 or 17 and continued for 10 days thereafter. In all cases, tumor growth was suppressed completely in the presence of drug; however, also in all cases, regrowth was apparent approximately 1 week after drug was removed. Further, in the experiments in which the dosing was commenced on days 10 or 17, after the tumors had attained a measurable size, no regressions occurred. (The lag period prior to resumption of tumor growth is similar to that observed following 5-DACTHF removal from the spheroids (Fig. 2) and presumbably is related to the time required for the drug to be eliminated from the tumor cells. Polyglutamation of 5-DACTHF by the

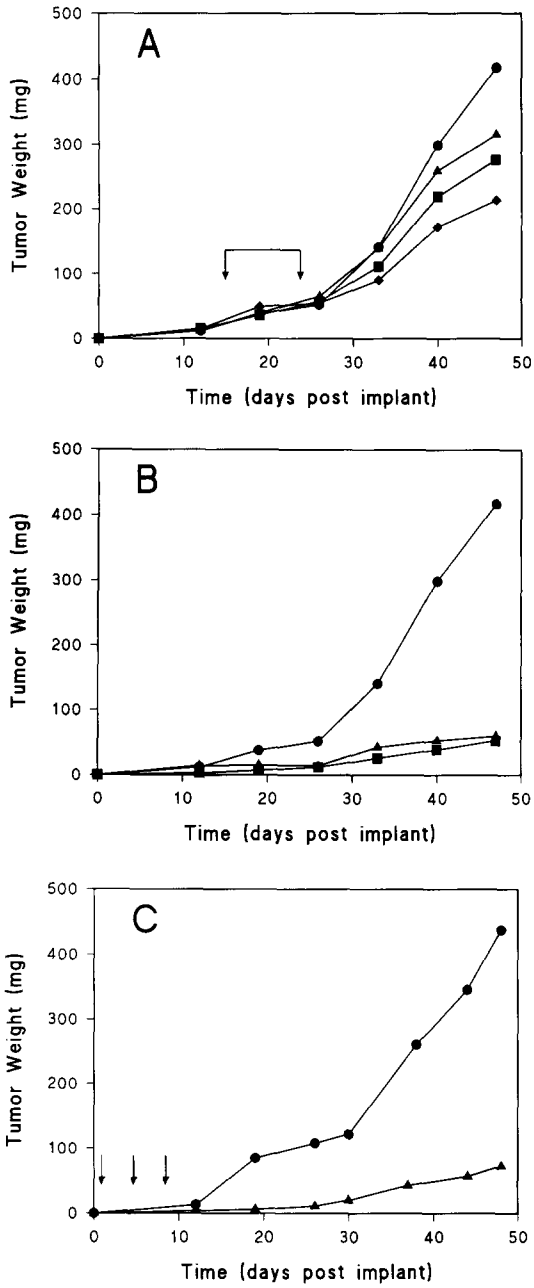


Fig. 6. Antitumor activity of 5-DACTHF or melphalan vs WiDr colon carcinoma. (A) 5-DACTHF was administered i.p. in neutralized saline solutions to 4 groups of 10 mice at (▲) 20 or (■) 40 mg/kg once daily or (◆) 20 or 40 (not shown) mg/kg twice daily. One group of 10 animals received saline alone (●). Arrows indicate dosing period. (B) 5-DACTHF was administered as in (A) at 20 or 40 mg/kg per day on days 1–47. (C) Melphalan was administered i.p. as in (A) at 10 mg/kg on days 1, 5, and 9.

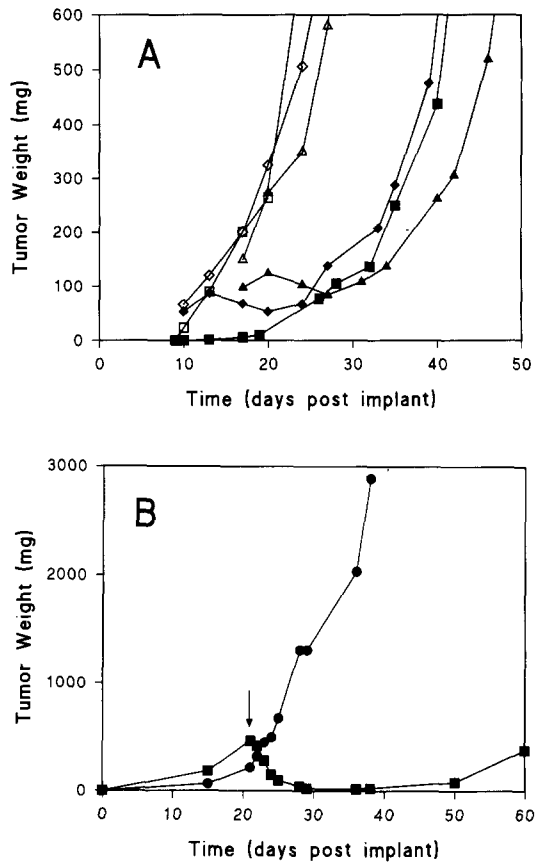


Fig. 7. Antitumor activity of 5-DACTHF or melphalan vs colon 38 adenocarcinoma. (A) Following tumor implantation, 5-DACTHF was administered in saline b.i.d. × 10 days at a dose of 40 mg/kg. Tumor measurements were recorded on a twice weekly schedule. Three experimental groups were examined; each comprised of control and drug-treated populations (N = 8). Drug administration commenced on either day 3 (■), 10 (◆), or 17 (▲) following tumor implantation. Untreated control populations are represented as the open symbols and treated populations as the corresponding filled symbols. (B) Following tumor implantation, melphalan was administered i.p. in neutralized saline solutions at 15 mg/kg on day 21 to one set of 10 animals (■) and saline alone was administered to another (●).

tumor cells [6, 24] would contribute to the length of time for this washout.) In contrast to the effects of 5-DACTHF, when mice bearing colon 38 tumors were dosed with melphalan on day 15 after tumor implantation alone, frank regression occurred in 8 of 8 tumors, and 5 of 8 mice were tumor free at 60 days (Fig. 7B).

The data generated from the melphalan experiments are not included to indicate that melphalan and 5-DACTHF are similar treatments. Rather, they are included to show that in both of the tumor models, with an effective drug, regressions and cures are achievable endpoints.

Several other human tumor xenografts were also tested as established subcutaneous tumors, as in Fig. 6A. These included SK-MEL-31 (melanoma), H69 (small cell lung carcinoma), OVCAR 5 (ovarian adenocarcinoma), and HT29 and DLD-1 (colorectal adenocarcinomas). In all cases, therapy was commenced at a tumor mass of 100–150 mg, and therapy lasted for 10 days. Dose levels of 5-DACTHF

of 40 mg/kg once daily or 10 or 20 mg/kg twice daily were used. In no case, was any significant regression or any cures observed. Therapy of the DLD-1 tumor was the most effective. Both 10 mg/kg twice daily and 40 mg/kg once daily arrested growth during therapy, but regrowth commenced again upon drug removal. No drug-induced arrest was observed in any other tumor.

These *in vivo* solid tumor experiments, *in toto*, are in excellent agreement with the WiDr spheroid experiments. They demonstrated that 5-DACTHF produces tumor growth inhibition but not tumor regression, whereas melphalan, a cytotoxic alkylating agent, produces both regressions and cures.

DISCUSSION

GAR TFase catalyzes the first 10-formyltetrahydrofolate-dependent step in *de novo* purine biosynthesis. Over the last 8 years, a number of GAR TFase inhibitors based upon the 10-formyltetrahydrofolate structure have been reported [1–8]. Enzyme inhibition by these compounds effectively inhibits cancer cell growth in culture and in animals [1–8, 27–29]. However, very little work has been reported to demonstrate any irreversibility of this growth inhibition. It has been shown that long-term cell culture treatment of HL-60 cells with DDATHF causes differentiation toward mature myeloid cells [27] and that continuous treatment of MOLT-4 T-cell leukemia with 5-DACTHF or DDATHF produces only reversible inhibition of growth for the first 48 hr, and at up to 96 hr less than one log of cell kill or irrevesible growth inhibition [8]. In comparison, a TS inhibitor produced rapid and extensive kill of MOLT-4 cells under similar conditions. Previously reported *in vivo* antitumor experiments were not designed to test the ability of these compounds to produce tumor regressions since drug treatment was initiated shortly after tumor implantation and prior to measurable tumor growth [2, 3, 5, 6, 28, 29]. The *in vitro* observations of limited and slow kill by inhibitors of *de novo* purine biosynthesis along with the lack of tumor regression data raised the question to us of whether these GAR TFase inhibitors will produce sufficient cell kill to irreversibly stop tumor growth or cause tumor regressions. Since tumor regression is a critically important measure of clinical response the major goal of the current study was to investigate the ability of GAR TFase inhibitors to produce regressions.

The WiDr spheroid model of solid tumor growth was a convenient system to begin to look for tumor regression and/or cell kill under the controlled conditions of the *in vitro* environment. In this test system, dihydrofolate reductase, TS and topoisomerase II inhibitors produced marked spheroid regressions. However, of the six GAR TFase inhibitors tested, including the clinical agent DDATHF, none produced regressions while all totally inhibited spheroid growth. Similarly, while a TS inhibitor produced extensive cell lysis throughout the spheroid, a representative GAR TFase inhibitor, 5-DACTHF, produced no significant cell kill over that found in controls. Finally, when the GAR TFase inhibitors were removed, these growth-inhibited spheroids regrew efficiently; thus, the growth inhibition was clearly reversible.

The observation that increased 5-DACTHF concentration, but not duration of exposure, delayed spheroid outgrowth (Fig. 2) is also best explained by reversible growth inhibition by the purine synthesis inhibitors. Presumably, by day 4 of treatment with 5-DACTHF, intracellular 5-DACTHF levels and its polyglutamates were in equilibrium with extracellular 5-DACTHF, and intracellular nucleotide levels were depleted to their minima as we have observed in other systems [8, 24]. Then if the inhibition is reversible, longer exposure of spheroids to a given 5-DACTHF dose would have no further effect on these metabolites or, in turn, on outgrowth delay. However, higher extracellular 5-DACTHF concentrations *would* extend delay, as we observed, by producing higher intracellular drug levels and, in turn, increasing the time to efflux the drug to below its effective intracellular level. If, on the other hand, drug exposure were lethal, increased exposure time at a given dose would be expected to result in increased cell kill. Then when drug was removed, spheroids exposed longer to the drug would have fewer live cells and a longer outgrowth delay. Thus, all of the spheroid results suport reversible inhibition by the GAR TFase inhibitors.

In vivo testing with solid tumor models was used to ask whether these inhibitors could produce regression or irreversible growth inhibition *in vivo*. WiDr tumors were used as one test system to extend the spheroid results *in vivo* directly, and the mouse colon 38 tumor was used to extend the results to another tumor and species. Again in both cases the GAR TFase inhibitor 5-DACTHF was able to produce inhibition of tumor growth while drug was present but could not produce regressions in either model. Further, as was seen with the spheroids, tumor outgrowth was observed following completion of therapy. In contrast, melphalan produced regressions and apparent cures in both models.

Considering that circulating purines in the mice could have interfered with any potential antitumor effects of the GAR TFase inhibitors, we returned to the spheroid model to investigate the effect of circulating purine levels on the activity of these inhibitors. We found that hypoxanthine levels up to and including physiological levels of 1–3 μM [11] had no effect upon the inhibition of spheroid growth or the lack of drug-induced spheroid regression. Thus, the lack of regressions *in vivo* is not likely due to partial drug reversal by circulating purines.

Our previous experiments with MOLT-4 and MCF-7 cells showed that these GAR TFase inhibitors can produce some cell kill, though the extent was low [8]. The results of the current experiments extend these findings to indicate that in three colon solid tumors in which complete growth suppression was accomplished *in vivo*, the compounds did not produce clinically significant cell kill. These results predict that in the clinic, responses as measured by tumor regressions may be rare. This prediction is currently being tested in the clinic in the form of DDATHF (lomotrexol) [30–32]. This compound

inhibited spheroid (Table 1) and cell growth [8] in a manner qualitatively similarly to the other GAR TFase discussed here. The Phase I DDATHF trials have stressed the importance of folic acid for the management of toxicity [30–32]. In these trials, a small number of responses have been reported; however, since not all Phase I patients receive optimal doses levels, Phase II and III data will be required to further test the clinical relationship between GAR TFase inhibition and tumor regression.

Acknowledgement—The authors wish to thank Drs. D. C. Jenkins and E. Rapson of Wellcome Research Laboratories, Beckenham, U.K., for help in establishing the spheroid model.

REFERENCES

1. Beardsley GP, Moroson BA, Taylor EC and Moran RG, A new folate antimetabolite, 5,10-dideaza-5,6,7,8-tetrahydrofolate is a potent inhibitor of *de novo* purine synthesis. *J Biol Chem* **264**: 328–333, 1989.
2. Beardsley GP, Taylor EC, Grindey GB and Moran RG, Deaza derivatives of tetrahydrofolic acid. A new class of folate antimetabolite. In: *Chemistry and Biology of Pteridines* (Eds. Cooper BA and Whitehead VM), pp. 953–957. Walter de Gruyter, Berlin 1986.
3. Taylor EC, Hamby JM, Shih C, Grindey GB, Rinzel SM, Beardsley GP and Moran RG, Synthesis and antitumor activity of 5-deaza-5,6,7,8-tetrahydrofolic acid and its N^{10}-substituted analogues. *J Med Chem* **32**: 1517–1522, 1989.
4. Kelley JL, McLean EW, Cohn NK, Edelstein MP, Duch DS, Smith GK, Hanlon MH and Ferone R, Synthesis and biological activity of an acyclic analogue of 5,6,7,8-tetrahydrofolic acid, *N*-[4-[[3-(2,4-diamino-1,6 - dihydro - 6 - oxo - 5 - pyrimidinyl)propyl]amino] benzoyl]-L-glutamic acid. *J Med Chem* **33**: 561–567, 1990.
5. Smith GK, Banks SD, Bigham EC, Cohn NK, Duch DS, Edelstein MP, Ferone R, Hanlon MH, Heath LS, Humphreys J, Kelley JL, Knick V, McLean EW, Mullin RJ, Singer S and Wilson HR, *In vitro* and *in vivo* activity of the GAR transformylase inhibitor 5-deazaacyclotetrahydrofolate. In: *Chemistry and Biology of Pteridines* (Eds. Curtius H-Ch, Ghisla S and Blau N), pp. 1015–1022. Walter de Gruyter, Berlin, 1990.
6. Mullin RJ, Keith BR, Bigham EC, Duch DS, Ferone R, Heath LS, Singer S, Waters KA and Wilson HR, *In vivo* antitumor activity and metabolism of a series of 5-deazaacyclotetrahydrofolate (5-DACTHF) analogues. *Biochem Pharmacol* **43**: 1627–1634, 1992.
7. Bigham EC, Hodson SJ, Mallory WR, Wilson D, Duch DS, Smith GK and Ferone R, Synthesis and biological activity of open-chain analogues of 5,6,7,8-tetrahydrofolic acid—Potential antitumor agents. *J Med Chem* **35**: 1399–1410, 1992.
8. Smith GK, Duch DS, Dev IK and Kaufmann SH, Metabolic effects and cell kill of human T-cell leukemia by 5-deazaacyclotetrahydrofolate, a specific inhibitor of GAR TFase. *Cancer Res* **52**: 4895–4903, 1992.
9. Elion GB, Symposium on immunosuppressive drugs. Biochemistry and pharmacology of purine analogues. *Fedn Proc* **26**: 898–904, 1967.
10. Nelson JA, Carpenter JW, Rose LM and Adamson DJ, Mechanisms of action of 6-thioguanine, 6-mercaptopurine, and 8-azaguanine. *Cancer Res* **35**: 2872–2878, 1975.
11. Ontyd J and Schrader J, Measurement of adenosine, inosine, and hypoxanthine in human plasma. *J Chromatogr* **307**: 404–409, 1984.
12. Hamm CW, Kupper W, Bredehorst R, Hilz H and Bleifeld W, Quantitation of coronary venous adenosine in patients: Limitations evaluated by radio-immunoassay. *Cardiovasc Res* **22**: 236–243, 1988.
13. Gewirtz H, Brown P and Most AS, Measurement of plasma adenosine concentration: Methodological and physiological considerations. *Proc Soc Exp Biol Med* **185**: 93–100, 1987.
14. Kurtz TW, Kabra PM, Booth BE, Albander HA, Portale AA, Serena BG, Tsai HC and Morris RC, Liquid-chromatographic measurements of inosine, hypoxanthine, and xanthine in studies of fructose-induced degradation of adenine-nucleotides in humans and rats. *Clin Chem* **32**: 782–786, 1986.
15. Sutherland RM and Durand RE, Radiation effects on mammalian cells grown as an *in vitro* tumor model. *Curr Top Radiat Res* **11**: 87–139, 1976.
16. Sutherland RM and Durand RE, Growth and cellular characteristics of multicell spheroids. In: *Spheroids in Cancer Research* (Eds. Acker H, Carlsson J, Durand R and Sutherland RM), pp. 24–49. Springer, Berlin, 1984.
17. Sutherland RM, Cell and environment interactions in tumor microregions: The multicell spheroid model. *Science* **240**: 177–184, 1988.
18. Sutherland RM, Importance of critical metabolites and cellular interactions in the biology of microregions of tumors. *Cancer* **58**: 1668–1680, 1986.
19. Ladman AJ and Martinez AO, Cell contacts and surface features of three murine tumors grown as multicellular spheroids. *Eur J Cell Biol* **45**: 224–229, 1987.
20. Nair MG, Murthy BR, Patil SD, Kisliuk RL, Thorndike J, Gaumont Y, Ferone R, Duch DS and Edelstein MP, Folate analogues. 31. Synthesis of the reduced derivatives of 11-deazahomofolic acid, 10-methyl-11-deazahomofolic acid, and their evaluation as inhibitors of glycinamide ribonucleotide formyltransferase. *J Med Chem* **32**: 1277–1283, 1989.
21. McCairns E, Fahey D, Sauer D and Rowe PB, *De novo* purine synthesis in human lymphocytes. Partial copurification of the enzymes and some properties of the pathway. *J Biol Chem* **258**: 1851–1856, 1983.
22. McDowell EM and Trump BF, Histologic fixatives suitable for diagnostic light and electron microscopy. *Arch Pathol Lab Med* **100**: 405–414, 1976.
23. Corbet TH, Griswold DP, Roberts BJ, Peckham JC and Schabel FM, Evaluation of single agents and combinations of chemotherapeutic agents in mouse colon carcinomas. *Cancer* **40**: 2660–2680, 1977.
24. Hanlon MH, Ferone R, Mullin RJ and Keith BR, *In vivo* and *in vitro* metabolism of 5-deazatetrahydrofolate, an acyclic tetrahydrofolate analogue. *Cancer Res* **50**: 3207–3211, 1990.
25. Sirotnak FM, Otter GM, Piper JR and DeGraw JI, Analogs of tetrahydrofolate directed at folate-dependent purine biosynthetic enzymes. Characteristics of mediated entry and transport-related resistance in L1210 cells for 5,10-dideazatetrahydrofolate and two 10-alkyl derivatives. *Biochem Pharmacol* **37**: 4775–4777, 1988.
26. Hughes LR, Jackman AL, Oldfield J, Smith RC, Burrows KD, Marsham PR, Bishop AM, Jones TR, O'Connor BM and Calvert AH, Quinazoline antifolate thymidylate synthase inhibitors: Alkyl, substituted alkyl, and aryl substituents in the C2 position. *J Med Chem* **33**: 3060–3067, 1990.
27. Sokoloski JA, Beardsley GP and Sartorelli AC, Induction of HL-60 leukemia cell differentiation by the novel antifolate 5,10-dideazatetrahydrofolate acid. *Cancer Res* **49**: 4824–4828, 1989.

28. Shih C, Grindey GB, Houghton PJ and Houghton J, *In vivo* antitumor activity of 5,10-dideazatetrahydrofolic acid (DDATHF) and its diastereomeric isomers. *Proc Am Assoc Cancer Res* **29**: 283, 1988.
29. Taylor EC, New pathways from pteridines. Design and synthesis of a new class of potent and selective antitumor agents. *J Heterocycl Chem* **27**: 1–12, 1990.
30. Young CW, Currie VE, Muindi JF, Saltz LB, Pisters KMW, Esposito AJ and Dyke RW, Improved clinical tolerance of lometrexol with oral folic acid. *Proc Am Assoc Cancer Res* **33**: 406, 1992.
31. Cole JT, Gralla RJ, Kardinal CG and Rivera NP, Lometrexol (DDATHF): Phase I trial of a weekly schedule of this new antifolate. *Proc Am Assoc Cancer Res* **33**: 413, 1992.
32. Muggia F, Martin T, Ray M, Leichman CG, Grunberg S, Gill I, Moran R, Dyke R and Grindey G, Phase I clinical trial of weekly 5,10-dideazatetrahydrofolic acid (LY 26418, DDATHF-B). *Proc Am Assoc Clin Oncol* **9**: 74, 1990.