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IN VIVO ANTITUMOR ACTIVITY AND METABOLISM OF A SERIES OF 5-DEAZAACYCLOTETRAHYDROFOLATE (5-DACTHF) ANALOGUES

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Abstract—This study compares the antitumor activity and metabolism of the purine *de novo* biosynthesis inhibitor 5-deazaacyclotetrahydrofolate and a series of analogues. All compounds have similar IC_{50} values for inhibition of MCF-7 cell growth, activity of glycineamide ribonucleotide transformylase, and methotrexate uptake by MOLT-4 cells, the latter a measure of cellular uptake potential. Only 5-deazaacyclotetrahydrofolate and the 2′-fluoro and 3′-fluoro analogues demonstrated significant inhibition of colon 38 adenocarcinoma or HCT-116 colon carcinoma growth *in vivo*. This correlated with the K_m of these compounds for folylpolyglutamate synthetase. 5-Deazaacyclotetrahydrofolate and 2′-fluoro-5-deazaacyclotetrahydrofolate which displayed the strongest antitumor activity were detectable in colon 38 tumor tissue 24 hr after dosing and were present nearly exclusively as the polyglutamated species. These results indicate that polyglutamation represents a critical step in the *in vivo* antitumor activity of these compounds.

The activity of 5-deazaacyclotetrahydrofolate (5-DACTHF†) as an inhibitor of purine *de novo* biosynthesis and potential antitumor agent has been reported previously [1, 2]. 5-DACTHF represents one of several compounds designed to test the potential of purine *de novo* biosynthesis as a target for antineoplastic chemotherapy. This effort has been stimulated by the observations of both elevated purine synthesis and elevated levels of purine biosynthetic enzymes in neoplastic cells [3–5]. Indications that one of the mechanisms of action of methotrexate is due to inhibition of purine *de novo* synthesis [6] also adds to the potential of purine *de novo* biosynthesis as a target for chemotherapy. A considerable number of the compounds reported to inhibit purine *de novo* biosynthesis have structures based upon that of N^{10}-formyltetrahydrofolate, the cofactor for the two folate-requiring steps in the purine *de novo* biosynthetic pathway [6]. These compounds include homofolate [7], reduced homofolate analogues [8, 9], 5,10-dideazatetrahydrofolate (DDATHF) and its analogues [10–12], 10-alkyl-5,10-dideaza analogues of tetrahydrofolate [13, 14], 10-thia-5,8-dideazafolate [15] and 5DACTHF [1, 2].

5-DACTHF is an inhibitor of glycineamide ribonucleotide (GAR) transformylase and 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase with respective IC_{50} values of 3 and 94 μM [1]. 5-DACTHF is also an excellent substrate for folylpolyglutamate synthetase (FPGS) and is found primarily as polyglutamate derivatives in MCF-7, MOLT-4 and L-cells in culture as well as murine liver, colon 38 adenocarcinoma and P388 leukemia *in vivo* [2]. Polyglutamation of 5-DACTHF leads to its improved activity as an inhibitor of GAR transformylase [1] and is presumed to result in increased cellular drug retention with intracellular concentrations several hundred fold above extracellular levels being reported [2, 16]. The latter is typical of antifolates where polyglutamation is thought to allow for retention against a concentration gradient [17, 18]. The relative absence of 5-DACTHF compared to polyglutamate metabolites 24 hr following drug administration in the biological systems mentioned above supports this hypothesis [2].

This paper reports on the *in vivo* antitumor activity of a series of recently prepared 5-DACTHF analogues [19] against the murine colon 38 adenocarcinoma and human HCT-116 colon carcinoma and addresses the role of polyglutamation in the activity of this class of compounds.

MATERIALS AND METHODS

Chemicals

5-DACTHF and the 2′-fluoro (2′-F-5-DACTHF),

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† Abbreviations: AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; DDATHF, 5,10-dideazatetrahydrofolate; GAR, glycineamide ribonucleotide; PicA, tetrabutylammonium phosphate; TFA, trifluoroacetic acid; 2′-F-5-DACTHF, 2′-fluoro-5-deazaacyclotetrahydrofolate; 3′-F-5-DACTHF, 3′-fluoro-5-deazaacyclotetrahydrofolate; 5-DACTHF, 5-deazaacyclotetrahydrofolate; 5,10-DDACTHF, 5,10-dideazaacyclotetrahydrofolate; FPGS, folylpolyglutamyl synthases; conjugase, folypolyglutamyl hydrolase; and 10-S-5,10-DDACTHF, 10-thia-5,10-dideazaacyclotetrahydrofolate.

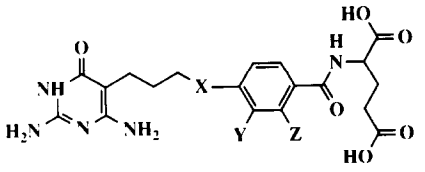

Fig. 1. Structure of 5-DACTHF and its analogues.

	X	Y	Z
5-DACTHF	NH	H	H
2'-F-5-DACTHF	NH	H	F
3'-F-5-DACTHF	NH	F	H
10-S-5,10-DDACTHF	S	H	H
5,10-DDACTHF	CH_2	H	H

3'-fluoro (3'-F-5-DACTHF), 5,10-dideaza 5,10-DDACTHF) and 10-thia-5,10-dideaza (10-S-5,10-DDACTHF) analogues (Fig. 1) were synthesized according to methods described elsewhere [1, 19]. Pteroyltri-γ-glutamic acid was purchased from Dr. Charles Baugh, University of South Alabama, Mobile, AL. Low UV tetrabutylammonium phosphate (PicA) was purchased from the Waters Division of the Millipore Corp. (Milford, MA). All other chemicals were of reagent grade.

Tissue culture

Human HCT-116 colon carcinoma cells were obtained from ATCC and maintained in folate-free RPMI 1640 medium (Gibco) with 10% dialyzed fetal bovine serum supplemented with 10 nM calcium leucovorin. Growth inhibition IC_{50} values were determined following 72 hr of drug exposure.

Tumor growth and measurement

Colon 38. 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 with a passage time of 21–28 days. Tumor growth was monitored with electronic digital calipers (L. S. Starrett Co., Athol, MA) which 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 [20]. With multiple tumors, tumor burden was calculated as the sum of individually measured tumors. Tumors were typically measured twice weekly. Animals were maintained on conventional mouse diet (RHM 3000, Agway Inc., Syracuse, NY) and housed in Microisolator cages (LabProducts, Inc.), at 72 ± 2°F with a 12-hr light/dark cycle. Pretreatment plasma folate levels were approximately 100 ng/mL based upon microbiological analysis.* All animal handling was performed in a laminar flow hood (Lab Products) using sterile technique.

HCT-116 subrenal assay. Human HCT-116 was maintained in culture as described above. Prior to growth as a xenograft the culture was verified to be free of bacteria, mycoplasma, and advential viral pathogens. For initiation of *in vivo* growth, 2×10^7 cells were harvested by centrifugation ($250 \times g$ for 5 min) and were then mixed with 60 μL of 60 mg/ml fibrinogen. A fibrin clot was formed by the addition of 7 μL of 50 U/mL thrombin followed by a 30-min incubation at 37°. Clots were then cut into fragments of approximately 1.0 mm^3 containing 1×10^6 cells. Fragments were implanted by published procedures [21] under the renal capsule of 18–22 g male CD-1 athymic mice (Charles River Laboratories) anesthesized with 75 mg/kg pentobarbitol. Animals were housed and handled as described above. Length and width of implanted fragments were measured at the time of surgery and again at autopsy with a dissecting microscope calibrated so that 10.0 ocular units = 1.0 mm. Tumor volume was obtained by modifying the formula of a prolate spheroid ($1/6\ (l \cdot w^2)$) to adjust for variations in depth of the tumor due to growth or treatment: tumor volume = $1/6\ (l \cdot w \cdot d)$. Tumor depth was determined as a fraction of width by measurement of histological cross-sections of the tumors. Tumor doublings were calculated from changes in the relative tumor volume. Growth inhibition (G_i) was determined from doublings of treated (D_t) and control (D_c) tumors as: $G_i = (D_c - D_t)/D_c \cdot 100$. Mice were dosed by the intraperitoneal route, adjusted according to individual body weight. 5-DACTHF and related compounds were prepared as sterile, neutral solutions in endotoxin free Dulbecco's phosphate-buffered saline so that the appropriate dose would be delivered when mice were given 20.0 mL/kg.

Conjugase preparation

Folylpolyglutamate hydrolase (conjugase) was prepared from rat plasma according to previously described methods [22]. The crude plasma activity was purified further by means of ammonium sulfate precipitation. A 60–80% fraction, which contained the bulk of activity, was dialyzed extensively against 50 mM sodium phosphate, pH 7.0. Conjugase activity was measured by the release of folic acid from pteroyltri-γ-glutamate. A 250-μL reaction mixture contained 2.5 μmol of sodium phosphate buffer, pH 7.0, 0.08% sodium ascorbate (w/v), 0.08% mercaptoethanol (v/v) and 10 μg of pteroyltri-γ-glutamate. The buffer and conjugase were brought to 37°, and the reaction was initiated by the addition of substrate. The 15-min reaction was stopped by heating in a 95° bath. Following centrifugation for 15 min at 12,000 *g* the supernatant was analyzed for folic acid by HPLC, employing the mobile phase described below containing 25% methanol.

HPLC

HPLC analysis of pteroyltri-γ-glutamate, folic acid, 5-DACTHF and its analogues was performed isocratically on a Waters (Milford, MA) C18 μBondpak column with a mobile phase containing 5 mM PicA, 10 mM ammonium phosphate and methanol. The methanol content of the mobile phase was a function of the age of the column and the compound being analyzed. Detection of the above compounds was accomplished by UV absorbance with an LDC Spectromonitor III variable

* Knick V and Mullin R, unpublished data.

wavelength detector (Laboratory Data Control, Rivera Beach, FL). Data were collected and analyzed with a DS-80 microcomputer (Digital Specialties, Chapel Hill, NC).

HPLC analysis of the polyglutamates of 5-DACTHF, 2'-F-5-DACTHF, and 10-S-5,10-DDACTHF was performed on a Waters C18 Novapak 4 μm column, with UV absorbance monitored at 300 nm by a Perkin–Elmer LC-235 diode array detector. Polyglutamate peaks were identified by comparison of retention times to polyglutamate standards (donated by Mary H. Hanlon, Burroughs Wellcome Co.) which were prepared with *Escherichia coli* FPGS. Optimum separation of 2'-F-5-DACTHF and 10-S-5,10-DDACTHF and their polyglutamates was obtained using a 1 mL/min, 1600 sec linear gradient from 17.5 to 35% acetonitrile in 10 mM ammonium phosphate with 5 mM low UV Pic A; the same gradient was used for 5-DACTHF and its polyglutamates except that the initial acetonitrile concentration was 10%. The column eluent was mixed with Radiomatic Flo-Scint IV (Radiomatic Instruments) at 4.5 mL/min, and radioactivity was detected with a Flo-One radioactivity flow detector (Radiomatic Instruments, Tampa, FL). The output from each detector was captured and analyzed as described previously [23]

Sample extraction

Analysis of plasma and colon 38 adenocarcinoma tissue for the presence of 5-DACTHF and its analogues required extraction of these compounds prior to detection. The methods employed represent adaptations of methods previously described for 5-DACTHF [2] and tissue folates [24]. Blood samples were collected via the vena cava with syringes rinsed with a 10 mg/mL heparin solution prior to use. Plasma was separated by centrifugation at 4° at 2000 *g* for 10 min and stored at −70° under N_2. Prior to analysis, plasma (25–100 μL) was brought to 900 μL with N_2-gassed water and then acidified with 100 μL of 10% trifluoroacetic acid (TFA). After a 5-min incubation on ice, samples were clarified by centrifugation (12,000 *g* for 5 min). Drug was then extracted with a C8 reversed phase Bond Elute cartridge (Analytichem International) which was prewashed sequentially with 1 mL of acetonitrile, 1 mL of water and 1 mL of 0.1% TFA. Following sample application the cartridge was washed with 5 mL of 0.1% TFA. Compounds were eluted with 1 mL of 0.1% TFA in acetonitrile, dried under N_2 at 35°, and dissolved in HPLC mobile phase for analysis.

For analysis of tumor tissue, samples were quickly removed, weighed, and rinsed with ice-cold phosphate-buffered saline. Tissues were transferred to a sealable container and minced in 6 mL of 95° 50 mM sodium phosphate, 50 mM mercaptoethanol, pH 7.0. The sample containers were sealed and incubated at 95° for 10 min to allow for protein precipitation and inactivation of endogenous conjugase. Tissues were then cooled and homogenized with a Polytron (Brinkmann, Westbury, NY).

Following centrifugation of the homogenate at 20,000 *g* for 20 min, up to 5 mL of tissue supernatant was adjusted to pH 4.5 with 1 N acetic acid and applied to the Dowex column prepared as previously described [24]. The columns were then washed with 2 ml of 10 mM ammonium acetate, pH 4.5, and the sample eluted with 4 mL of 50 mM sodium phosphate, 50 mM mercaptoethanol pH 7.3. Eluants from Dowex chromatography were prepared for Bond Elute chromatography [2] by adjustment to pH < 2.0 with 10% TFA. After a 5-min incubation at 4°, samples were filtered through a 0.45 micron filter and were processed with C8 cartridges according to the methods described above for plasma analysis. Control tissues from untreated animals were routinely carried through this process to establish background for each compound studied. Results were adjusted for the recovery of each compound added to plasma or tissue from untreated animals and carried through the entire analytical process on the same day as actual sample analysis.

Folylpolyglutamyl synthetase

FPGS was partially purified and assayed as previously described [1]. 5-DACTHF and 2'-F-5-DACTHF at 1 and 5 μM and 10-S-5,10-DDACTHF at 5 and 25 μM were incubated in a modified FPGS reaction [25] with 8 U/mL hog liver FPGS for 16 hr at 37°. The assay was modified using 100 mM Tris, pH 8.6 (37°) and 20 mM KCl in a total volume of 500 μL in argon-saturated amber microtubes. Reactions were stopped and prepared for HPLC by placing in a boiling water bath for 5 min, freezing at −20° overnight, centrifuging for 10 min at 2000 *g*, and filtering the supernatant through Millipore 0.45 μ HV filters into amber microtubes. The samples were layered with argon and stored at −80° until analyzed by HPLC.

RESULTS

In vivo *antitumor activity of 5-DACTHF and analogues*

To measure antitumor activity of 5-DACTHF and analogues, mice were implanted with fragments, ~20–40 mg, of colon 38 adenocarcinoma and therapy was initiated three days later. All drugs were administered i.p. as neutral solutions to groups of animals (N = 8) twice daily for 10 days. 5-DACTHF and its analogues were administered at three dose levels in an attempt to employ each compound near the maximum tolerated dose to allow for comparison at equitoxic doses. Toxicity for these compounds was manifested by general weight loss with no other overt signs of toxicity. Toxicity for 10-S-5,10-DDACTHF, 5,10-DDACTHF and 3'-F-5-DACTHF was not observed at doses of up to 300 mg/kg b.i.d. × 10 and the doses employed were based upon available compound. The antitumor activity of 5-DACTHF and analogues in this system can be seen in Fig. 2. Compared to control tumor growth, statistically significant ($P < 0.05$) antitumor activity was seen at 20, 30, and 40 mg/kg of 5-DACTHF, 35 and 50 mg/kg of 2'F-5-DACTHF and 250 and 300 mg/kg of 3'F-5-DACTHF. At the highest concentrations administered the antitumor activities of 5-DACTHF, 3'F-5-DACTHF and 2'F-5-DACTHF did not differ significantly from each other

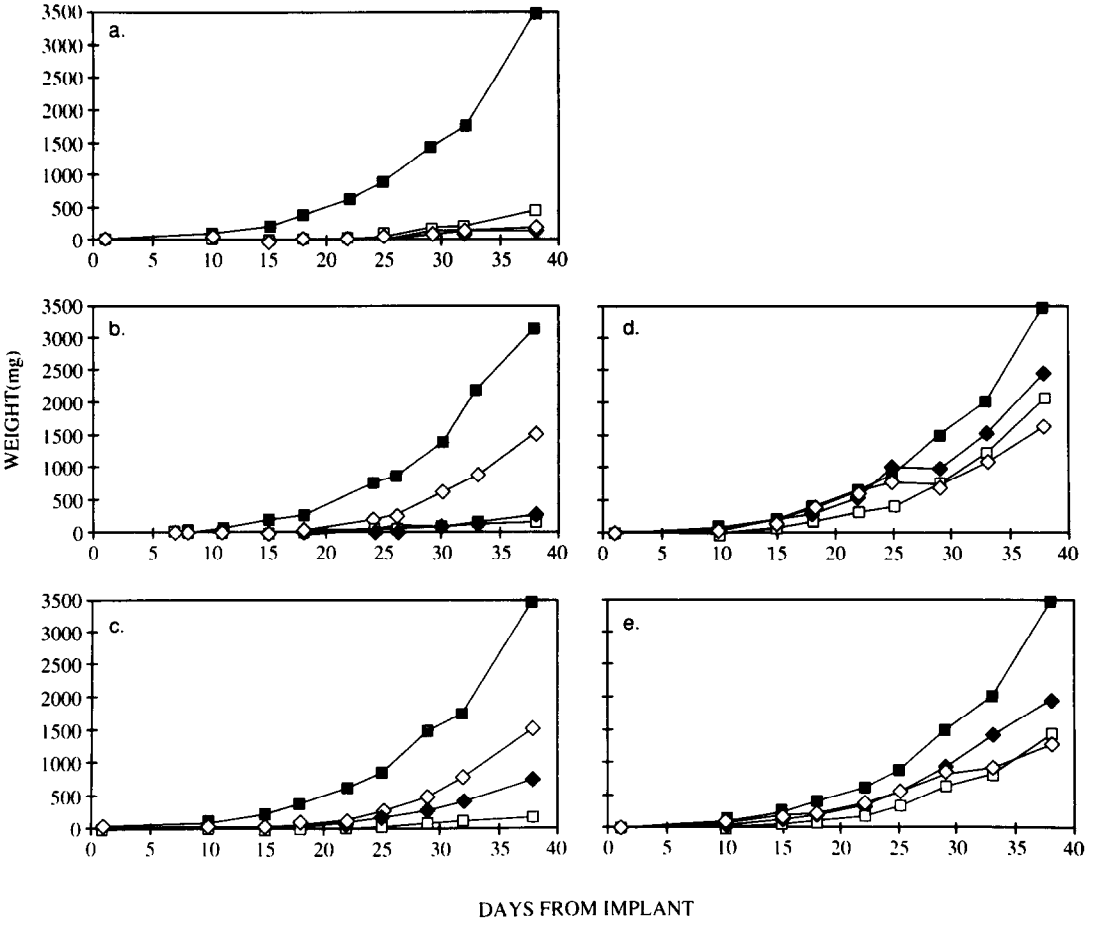

Fig. 2. Antitumor activity of 5-DACTHF and its analogues. Three days following transplantation all drugs were administered b.i.d. × 10 days. Tumor measurements were recorded on a twice weekly schedule. Untreated control tumor growth is presented in all panels as the closed squares. Dose levels for the various analogues are as follows: (Panel a) 5-DACTHF, (□) 40, (◆) 30, and (◇) 20 mg/kg. (Panel b) 2′-F-5-DACTHF, (□) 50, (◆) 35, and (◇) 20 mg/kg. (Panel c) 3′-F-5-DACTHF, (□) 300, (◆) 250, and (◇) 200 mg/kg. (Panel d) 10-S-5,10-DDACTHF, (□) 300, (◆) 250, and (◇) 200 mg/kg. (Panel e) 5,10-DDACTHF, (□) 250, (◆) 200, and (◇) 150 mg/kg.

but were significantly different from all doses of 10-S-5,10-DDACTHF and 5,10-DDACTHF. Neither 10-S-5,10-DDACTHF nor 5,10-DDACTHF had a significant antitumor effect at any of the drug levels tested. As a summary of overall antitumor activity, the time required to reach a mean tumor size of 100 mg for the treated and untreated animals can be seen in Table 1.

In the HCT-116 subrenal capsule assay, groups of mice (N = 4) were treated twice daily from days 3 through 7 and killed on day 10 post-implantation. 5-DACTHF was given at a maximally tolerated level of 50 mg/kg/dose in the athymic CD-1 mice; 2′-F-5-DACTHF, 3′-F-5-DACTHF, 10-S-5,10-DDACTHF and 5,10-DDACTHF were administered at the same dose to compare toxicity and efficacy to that of 5-DACTHF. Only 5-DACTHF, 2′-F-5-DACTHF, and 3′F-5-DACTHF produced statistically significant growth inhibition ($P < 0.05$, Kruskal–Wallis); 10-S-5,10-DDACTHF and 5,10-DDACTHF demonstrated no antitumor activity (Table 2). Toxicity, manifested as slight weight loss, was associated only with 5-DACTHF.

In light of *in vitro* data presented here (Table 2) and elsewhere [19], these *in vivo* results were somewhat unexpected. *In vitro* results indicated that this series of compounds was very similar in regard to a number of criteria presumed to be of importance for activity of these compounds. As monoglutamates all five compounds were potent inhibitors of GAR transformylase, having IC_{50} values between 1.6 and 3.6 μM. All five compounds were equally effective as inhibitors of methotrexate uptake having IC_{50} values between 0.8 and 1.2 μM in MOLT-4 cells and, as a result are thought to be equivalent substrates for the reduced folate transport system. In addition, the IC_{50} values for inhibition of cell growth by this series of compounds were between 23 and 61 nM for MCF- 7 cells [19] and between 54 and 110 nM for HCT-116 cells (Table 2). Since *in*

Table 1. *In vivo* antitumor activity versus colon 38 and *in vitro* polyglutamation of 5-DACTHF and its analogues

Drug	Antitumor activity* (days)	Polyglutamation		
		$V_{max}\%/K_m$†	K_m (μM)	V_{max} (%)
Untreated	10.4			
5-DACTHF (40 mg)	35.4	14.5	7.3	106
2′-F-5-DACTHF (35 mg)	30.2	25	4.3	107.3
3′-F-5-DACTHF (300 mg)	33.0	8.7	12.7	110
10-S-5,10-DDACTHF (300 mg)	16.6	4.7	26.8	125.5
5,10-DDACTHF (250 mg)	17.6	2.81	46.9	132

* Days from implant for mean tumor size (N = 8) to reach 100 mg.

† V_{max} (% of velocity with 50 μM aminopterin)/$K_m$with hog liver FPGS [19]. Typically 0.8 to 1.0 nmol of aminopterin were polyglutamated in a 2-hr reaction of 200 μL.

Table 2. *In vivo* antitumor activity of a series of 5-DACTHF analogs against HCT-116 colon carcinoma xenografts in a subrenal capsule assay

Compound	IC_{50}† (nM)	Treatment effects*	
		Weight change‡ (%)	Growth Inhibition§ (%)
5-DACTHF	54	−7.9	42‖
2′-F -5-DACTHF	110	17.5	30‖
3′-F-5-DACTHF	58	13.3	16‖
10-S-5,10-DDACTHF	88	14.8	8
5,10-DDACTHF	78	16.4	0
Vehicle control	N/A¶	19.3	N/A

* Compounds were administered b.i.d. × 5, days 3–7.

† Tissue culture IC_{50}.

‡ Initiation of treatment, day 3, to sacrifice, day 10. Control animals averaged 22.9 g on day 3.

§ Determination from doublings of treated (D_t) and control (D_c) tumors as $(D_c - D_t)/D_c \cdot 100$. Control tumors had an estimated average volume of 2.8 mm^3 and underwent 3.5 doublings during study.

‖ Statistically significant inhibition vs control, $P < 0.05$.

¶ Not applicable.

vivo polyglutamation of 5-DACTHF has been reported [2] and is known to result in increased inhibition of GAR transformylase [1], these analogues have also been examined as substrates for hog liver FPGS. The ratio of V_{max} to K_m, for these compounds appears to correlate with the *in vivo* results. The basis for this correlation appears to be a function of K_m since the V_{max} values were largely unaffected by the modifications of 5-DACTHF presented here (Table 1).

Plasma drug levels

The inconsistency between the *in vivo* antitumor activity and the *in vitro* properties of these compounds raised questions concerning the availability of these compounds following drug administration. A time course of plasma drug levels was carried out following i.p. administration of 300 mg/kg of 3′-F-5-DACTHF or 250 mg/kg of 10-S-5,10-DDACTHF. These doses produced a statistically significant antitumor response for 3′-F-5-DACTHF and a nonsignificant response for 10-S-5-DACTHF. The results from this study can be seen in Fig. 3. Both compounds had plasma levels considerably higher than those reported following an effective dose of 5-DACTHF (50 mg/kg) [2]. These results indicated that the differences in *in vivo* antitumor response for this set of compounds were not a result of drug availability.

Tissue drug accumulation

As previously discussed for this series of compounds, the K_m values for FPGS appear to correlate with *in vivo* antitumor activity (Table 1). Polyglutamation of folate related compounds is generally thought to result in increased intracellular accumulation as well as retention. In the case of two GAR transformylase inhibitors, 5-DACTHF and DDATHF, polyglutamation also results in increased activity as inhibitors of GAR transformylase [1, 26]. The influence of polyglutamation is probably best reflected in the comparison of IC_{50} values for enzyme inhibition and cell growth for both 5-DACTHF and DDATHF. These observations indicate a 100-fold

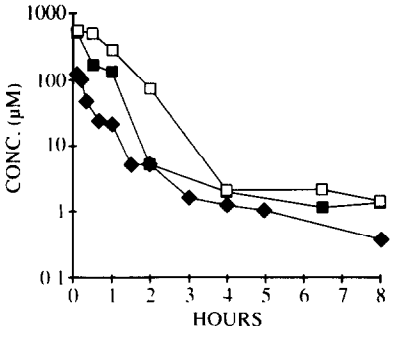

Fig. 3. Plasma levels for 3′-F-5-DACTHF and 10-S-5,10-DDACTHF. Animals were dosed with 300 mg/kg of each drug and plasma levels were determined according to methods described in Materials and Methods. Results are the average of data from three animals per time point. Key: (□) 3′-F-5-DACTHF, (■) 10-S-5,10-DDACTHF, and (◆) 5-DACTHF from [2].

enhanced effectiveness with intact cells [19] where polyglutamation is known to occur [2]. A similar profile of enhanced activity against an intact cellular target has also been observed with the analogs of 5-DACTHF being studied here [19] and is an indication of the probable importance of polyglutamation for the activity of these compounds. Previous experimentation has indicated that following a single 50 mg/kg dose, polyglutamates of 5-DACTHF are detectable in colon 38 tissue 24 hr following administration [2]. To determine if this was also the case for this series of analogues, analytical methods based upon sample preparation by ion-exchange and reversed phase chromatography of tissue extracts followed by HPLC analysis were established to allow their detection by UV absorbance. Simple reversed phase sample preparation did not prove to be adequate for detection by UV absorbance due to high background interference. Methods were validated by following the recovery of 0.1 to 100 nmol of each of the compounds from colon 38 homogenates (71.3 ± 3.4% for 5-DACTHF, 72.8 ± 2.9% for 2′-F-5-DACTHF, 80.7 ± 3.3% for 3′-F-5-DACTHF, 63.3 ± 10.0% for 5,10-DDACTHF, and 86.7 ± 2.1% for 10-S-5,10-DDACTHF). These controls were analyzed at the same time as experimental tissues. Peaks were identified by their elution time compared to that of standard added directly to untreated sample extracts. Methanol content of HPLC mobile phase was adjusted for each of the compounds to allow for elution of the monoglutamate in less than 1 hr with maximum resolution of compound from background interference. 5-DACTHF, 2′-F-5-DACTHF and 3′-F-5-DACTHF were analyzed with mobile phases containing 20–25% methanol, while 10-S-5,10-DDACTHF and 5,10-DDACTHF required 28–30% methanol for elution.

Previous work [2] indicates that 1.5 hr after a 50 mg/kg dose all of the detectable 5-DACTHF present in colon 38 tissue is the monoglutamate parent drug, while at 24 hr plasma and tissue drug levels decline and the bulk of drug present in tumor tissue exists as the polyglutamated parent compound. As an extension of these earlier observations, colon 38 tumors from four animals were examined at 1.5 and 24 hr following a single 50 mg/kg dose of drug. Since analytical methods were based upon UV detection, the detection of a variety of polyglutamated species was deemed beyond the scope of this study and total polyglutamates were determined. Rat plasma conjugase was used to convert polyglutamates to the parent drug and the differential between analysis prior to and after conjugase digestion was taken to represent a summation of the polyglutamate pool. This allowed for the development of methods based upon the detection of a single species. At 1.5 hr samples were not analyzed prior to conjugase digestion. The results for this set of experiments are summarized in Table 3. All five compounds were present at 1.5 hr, indicating successful delivery to and uptake by the tumor tissue. The 24-hr data indicated that only 5-DACTHF and 2′-F-5-DACTHF were detectable and were present primarily as polyglutamated species. As an internal control, the results seen here for 5-DACTHF are very similar to those previously reported, which were obtained using radiolabeled compound [2].

In vitro *polyglutamation of 5-DACTHF and analogues*

The *in vivo* polyglutamation experiments described above failed to offer information concerning the extent of polyglutamation. In an effort to measure any differences in the potential for formation of higher order polyglutamates, two compounds with *in vivo* activity, 5-DACTHF and 2′-F-5-DACTHF, and one without *in vivo* activity, 10-S-5,10-DDACTHF, were examined *in vitro* as substrates for hog liver FPGS at drug concentrations roughly equal to the K_m and 1/5 of the K_m for each compound. The extent of polyglutamation was determined by HPLC analysis of the reaction products after an 18-hr incubation. The results of this study (Table 4) demonstrate that the two compounds with *in vivo* activity, 5-DACTHF and 2′-F-5-DACTHF, were better substrates for the synthesis of higher order polyglutamates than 10-S-5,10-DDACTHF. The pentaglutamate species was the predominant product with 5-DACTHF and 2′-F-5-DACTHF, while the triglutamate species was the predominant product observed for 10-S-5,10-DDACTHF.

DISCUSSION

The antitumor activity and metabolism of a series of 5-DACTHF analogues are reported here. The compounds studied in this report were selected from a larger series on the basis of their similar properties following *in vitro* characterization [19]. The *in vivo* results with colon 38 adenocarcinoma and HCT-116 (Fig. 2, Table 2) indicated considerable differences both in toxicity and in antitumor activity. 5-DACTHF and the 2′-fluoro analogue were the most potent compounds tested here; both produced a significant reduction in tumor growth at the lowest comparative dose. The 3′-fluoro analogue of 5-DACTHF also had significant antitumor activity versus colon 38 but

Table 3. *In vivo* polyglutamation of 5-DACTHF and its analogues by colon 38

Drug	1.5 hr Total	24 hr	
		Monoglutamate	Total polyglutamates
5-DACTHF	15.0*	0.2	0.53
5-DACTHF†	5.4	0.1	0.6
2'-F-5-DACTHF	11.4	0.1	0.52
3'-F-5-DACTHF	8.8	ND‡	ND
10-S-5,10-DDACTHF	3.2	ND	ND
5,10-DDACTHF	3.2	ND	ND

* Concentration of drug in colon 38 tissue (nmol/g tissue).
† Values reported by Hanlon *et al.* from studies with [^{14}C]-5-DACTHF.
‡ Not detectable.

Table 4. *In vitro* polyglutamation of 5-DACTHF and its analogues

Drug	Substrate concn (μM)	% Distribution after 18-hr incubation					
		Glu_1	Glu_2	Glu_3	Glu_4	Glu_5	Glu_6
5-DACTHF	1	2*	0	0	20	73	6
	5	2	0	0	36	58	5
2'-F-5-DACTHF	1	0	0	0	8	84	8
	5	0	0	0	25	69	6
10-S-5,10-DDACTHF	5	14	0	39	33	13	0
	25	52	3	40	5	0	0

* Percentage of drug as indicated polyglutamate where Glu_1 is the parent compound.

only at doses greater than 250 mg/kg b.i.d. × 10, which represents 10-fold more compound than required with either 5-DACTHF or 2'-F-5-DACTHF.

Drug availability did not appear to be responsible for differences observed in the *in vivo* antitumor activity. This conclusion was reached based upon evaluation of plasma drug levels for active and inactive compounds (Fig. 3) and the levels of all compounds in tumor tissue 1.5 hr following drug administration (Table 3).

Both *in vitro* and *in vivo* data demonstrate the importance of polyglutamation for *in vivo* activity of these compounds. 5-DACTHF, 2'-F-5-DACTHF, and 3'-F-5-DACTHF, which were the best substrates for FPGS (Tables 1 and 4), were the most active as antitumor agents (Fig. 2 and Tables 1 and 2). In addition, 24 hr following drug administration only the two most active compounds *in vivo* were detectable in tumor tissue and appeared predominantly as polyglutamates (Table 3). *In vitro* studies on polyglutamation (Table 4) offer an explanation for the *in vivo* data. In addition to differences in the kinetics of addition of a single glutamate moiety, there also appear to be differences in the abilities of these compounds to act as substrates for the synthesis of higher order polyglutamates. At concentrations near their respective K_m values, over 90% of 5-DACTHF and 2'-F-DACTHF were converted to tetra- and pentaglutamates. Only 7% of the less active 3'-F-5-DACTHF was metabolized beyond the triglutamate level and 50% remained as the parent compound. Thus, only those compounds which are metabolized to higher order polyglutamates were capable of resulting in a significant antitumor response *in vivo*.

The importance of polyglutamation for similar compounds has been demonstrated previously. Increased inhibition of GAR transformylase with increasing polyglutamation of DDATHF [26] and 5-DACTHF [1] has been reported. Similar observations on the inhibition of GAR transformylase from bacterial, murine and human sources have been made for the polyglutamates of tetrahydrohomofolate [8]. Pizzorno *et al.* [27] have also demonstrated the importance of polyglutamation for the activation of DDATHF in the inhibition of CCRF-CEM cell growth.

In conclusion, the successful design of these compounds has required attention to several details. There is the obvious need for drug delivery. Based upon inhibition of methotrexate uptake [19], delivery of this series of compounds appears to be based upon the reduced folate transport system. The specificity of this transporter puts limits upon potential drug structure. Upon entry into the cell, two additional criteria must be met: the specificity of the target enzyme GAR transformylase and the specificity of FPGS. The latter activity has a dual role in the successful design of classical antifolates. Polyglutamation results in the increased inhibition of a number of folate-requiring enzymes and also

results in increased cellular retention of compounds [17, 18]. In meeting these requirements, 5-DACTHF, 2′-F-5-DACTHF and 3′-F-5-DACTHF are capable of producing a significant suppression of tumor growth of the colon 38 and HCT-116 tumor models used in this study. However the inability to generate cures with the above tumors following suppression of *de novo* purine biosynthesis is disappointing. These results lead us to question the value of purine *de novo* biosynthesis as an antitumor target. The presence of purine salvage activity in colon 38 [28] may offer an explanation for eventual recovery of these tumors following therapy.

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