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STUDIES ON THE MECHANISM OF ANTITUMOR ACTION OF 2-DESAMINO-2-METHYL-5,8-DIDEAZAISOFOLIC ACID

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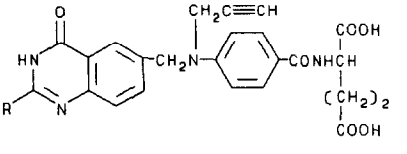
Abstract—The new folate analogue, 2-desamino-2-methyl-5,8-dideazaisofolic acid, **2c**, was synthesized and evaluated using a variety of biochemical and antitumor assays. For purposes of comparison, its 2-desamino, **2b**, and 2-amino, **2a**, counterparts, as well as N^{10}-propargyl-5,8-dideazafolic acid, **1a**, and the corresponding 2-desamino, **1b**, and 2-desamino-2-methyl, **1c**, modifications were included in these studies. Compound **2c** was found to be a potent inhibitor of the growth of L1210 and MCF-7 cells in culture, being only 2-fold and 5-fold less effective than **1c**, respectively. However, although analogue **2c** was 189-fold less inhibitory toward L1210 thymidylate synthase (TS) than **1c**, its cytotoxicity was reversed completely by thymidine alone which suggests that the compound behaves as a TS inhibitor in cells. Enzymatically synthesized polyglutamates of **2c** were substantially more inhibitory toward human TS than the parent compound. Compound **2c** was the most efficient substrate for mammalian folylpolyglutamate synthetase of the compounds studied having a V_{max}/K_m nearly 12-fold larger than **1c**. Both **1c** and **2c** were effective inhibitors of the uptake of [^{3}H]methotrexate into MOLT-4 cells, implying that each is efficiently transported into tumor cells. These results suggest that a weak inhibitor of TS *in vitro* can be a potent cytotoxic agent if it can readily gain entry into target cells and be converted to polyglutamated metabolites.

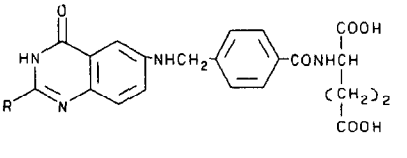
The folate analogue, N^{10}-propargyl-5,8-dideazafolic acid (CB3717), **1a** (Fig. 1) was first reported by Jones *et al.* in 1981 [1]. It was found to be a potent inhibitor of mammalian thymidylate synthase (TS)¶ and to possess a high degree of efficacy against L1210 leukemia in mice. Subsequently, **1a** was introduced into clinical trials [2]. Due to dose-limiting renal and hepatic toxicities, apparently related to solubility problems, clinical trials with CB3717 were discontinued [3].

Based on the premise that the presence of hydroxy, amino or mercapto substituents at the 2-position of the quinazoline ring decreases aqueous solubility due to the formation of intermolecular hydrogen bonds [4], Jones and coworkers synthesized 2-desamino-N^{10}-propargyl-5,8-dideazafolic acid, (2-desamino-CB3717), **1b**. This analogue proved to be 8-fold less inhibitory towards mammalian TS and 30-fold less inhibitory towards rat liver dihydrofolate reductase (DHFR) than **1a**, while exhibiting a 10-fold increase in cytotoxicity towards L1210 cells in culture. The enhanced cytotoxicity of 2-desamino-CB3717 was attributed to increased rate of uptake into cells [5].

Subsequently, other 2-substituted analogues of CB3717 were prepared, with 2-desamino-2-methyl-N^{10}-propargyl-5,8-dideazafolic acid (2-desamino-2-methyl-CB3717), **1c**, exhibiting highly promising



1a: R = NH_2, CB3717
b: R = H, 2-desamino-CB3717
c: R = CH_3, 2-desamino-2-methyl-CB3717



2a: R = NH_2, IAHQ
b: R = H, 2-desamino-IAHQ
c: R = CH_3, 2-desamino-2-methyl-IAHQ

Fig. 1. Structural formulas and designations of the analogues studied.

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¶ Abbreviations: TS, thymidylate synthase; DHFR, dihydrofolate reductase; FPGS, folylpolyglutamate synthetase; GAR Tfase, glycinamide ribonucleotide transformylase; AICAR Tfase, 5-aminoimidazole-4-carboxamide ribonucleotide transformylase; MTX, methotrexate; Hx, hypoxanthine; TdR, thymidine. The designations for the six analogues studied are presented beneath their respective structures in Fig. 1.

activity. This analogue was 2-fold less inhibitory towards TS than the parent 2-amino compound, **1a**, but 40-fold more cytotoxic towards L1210 cells in culture [6]. Jackman and coworkers demonstrated the reversal of 2-desamino-2-methyl-CB3717 cytotoxicity by thymidine (TdR), implicating TS as its primary site of action. The 2-desamino and 2-desamino-2-methyl analogues of CB3717 were converted to polyglutamated forms by the enzyme folylpolyglutamate synthetase (FPGS), with the polyglutamated metabolites being superior to the monoglutamate counterparts in terms of TS inhibition [7, 8]. This evidence emphasizes the importance of FPGS in the mechanism of action of such compounds.

The folate analogue 5,8-dideazaisofolic acid (IAHQ), **2a**, has demonstrated modest activity against a variety of human tumor cell lines in culture. It also was active against the colon 38 tumor in mice, the CX-1 human colon tumor xenograft in the nude mouse, and a human osteogenic sarcoma xenograft in hamsters [9–13]. However, large doses of IAHQ were required to achieve therapeutic effectiveness in these animal models. This lack of potency was attributed to its slow rate of influx into target cells as demonstrated using [^{3}H]IAHQ and HCT-8 human colon adenocarcinoma cells in culture [14].

Although the affinity of IAHQ towards TS was found to be approximately 100-fold less than that of CB3717, the two compounds had similar levels of cytotoxic potency *in vitro*. In addition, the cytotoxicity of IAHQ toward HCT-8 cells was reversed by TdR, indicating that its primary target was also TS [9]. Recently, 2-desamino-5,8-dideazaisofolic acid (2-desamino-IAHQ), **2b**, was prepared in an effort to achieve enhanced antitumor efficacy [15]. A 3-fold decrease in DHFR inhibition and a 19-fold decrease in TS inhibition were observed with 2-desamino-IAHQ, as compared to the parent compound, **2a** [15]. However, 2-desamino-IAHQ was found to be 6-fold more effective than IAHQ in inhibiting the growth of L1210 cells in culture [15]. We now report the synthesis and preliminary biological evaluation of 2-desamino-2-methyl-5,8-dideazaisofolic acid, **2c**, as a logical extension of our earlier work. This analogue was evaluated for inhibition of the enzymes TS and DHFR, the growth of L1210 and MCF-7 cells in culture, as well as for substrate activity for FPGS and the ability of the compound to bind to the reduced folate transporter in MOLT-4 cells. Compounds **1a–c** as well as **2a** and **2b** were included in this study in order to assess the effects of various chemical modifications at position 2 of the quinazoline ring upon biochemical and antitumor properties of these interesting analogues.*

* After the completion of this work, the IC_{50} for **2c** against TS (L1210) and the IC_{50} toward L1210 leukemia cells in culture were presented in a poster. The results reported were in good agreement with those obtained in the present study: Marsham PR, Hughes LR, Hayter AJ, Oldfield J, Jackman AL, O'Connor BM, Bishop JAM and Calvert AH, Quinazoline antifolate thymidylate synthase inhibitors: Potent cytotoxic agents containing heterocyclic isosteres of the para-aminobenzoate unit, In: *Chemistry and Biology of Pteridines 1989*, (Eds. Curtius H-C, Ghisla S and Blau N), pp. 1048–1051. Walter de Gruyter, Berlin, 1990.

MATERIALS AND METHODS

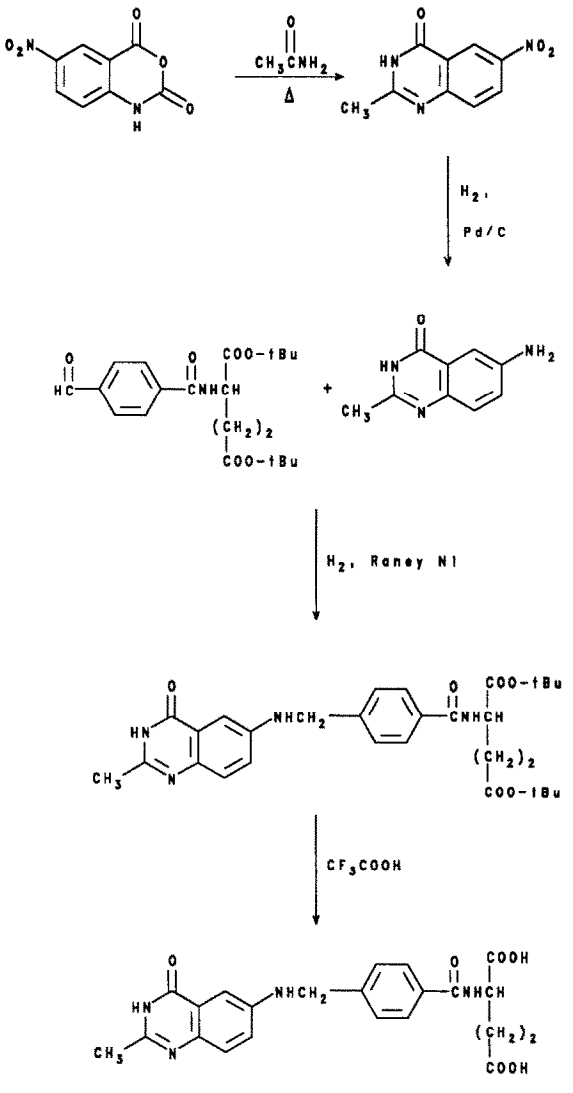
Miscellaneous

Melting points were determined on a Mel-temp apparatus and are uncorrected. Elemental analyses were performed by Galbraith Laboratories, Knoxville, TN. The analytical samples gave combustion values of C, H, and N within ±0.4% of the theoretical values. Solvation due to H_2O was confirmed by the presence of a broad peak centered at approximately 3.4 ppm in the ^{1}H NMR spectrum, which was transformed into a sharp singlet (DOH) by the addition of D_2O. Each of the intermediates was free of significant impurities on TLC using Eastman Kodak 13181 silica gel plates. The free acid was assayed on Eastman Kodak 13254 cellulose plates (5% NH_4HCO_3). High resolution ^{1}H NMR spectra were acquired on a Bruker AM-300 spectrometer at the Chemistry Department, University of South Carolina, Columbia, SC. ^{1}H NMR spectra of quinazoline intermediates were acquired on a Varian EM-390 spectrometer. NMR values for chemical shifts are presented in parts per million downfield from tetramethylsilane as the internal standard. The relative peak areas are given to the nearest whole number. The FAB mass spectrum for **2c** was obtained with a VG 705Q analytical spectrometer at the Chemistry Department, University of South Carolina, Columbia, SC, by Dr. Michael Walla.

Synthesis (Scheme 1)

2-Methyl-6-nitro-4(3H)-quinazolone. The first synthetic step was accomplished by dissolving 5-nitroisatoic anhydride [6-nitro-2*H*-3,1-benzoxazine-2,4(1*H*)-dione] (20.81 g, 0.100 mol) in molten acetamide (100 g, 1.69 mol). The temperature was maintained at 160° for 2.5 hr. The slightly cooled mixture was poured over crushed ice and refrigerated for 18 hr to effect precipitation of the product. The product was collected by vacuum filtration and washed with copious amounts of ice-cold water and then diethyl ether. Drying *in vacuo* at 100° for 18 hr yielded 16.12 g (78.6%) of product. Observed m.p. = softens at 220°; melts at 261–264° (literature m.p. = 278–281°, corrected) [16]. TLC (chloroform : methanol, 4 : 1) R_f = 0.55. ^{1}H-NMR (90 MHz, DMSO-d^6) δ: 2.45 (*s*, 3H) CH_3; 7.73 (*d*, J = 8.1 Hz, 1H) H-8; 8.52 (*dd*, $J_{7,8}$ = 10.8 Hz, $J_{7,5}$ = 2.7 Hz, 1H) H-7; 8.79 (*app.d*, 1H) H-5.

2-Methyl-6-amino-4(3H)-quinazolone. A suspension of 2-methyl-6-nitro-4(3*H*)-quinazolone (8.00 g, 39 mmol) in 150 mL of 2-methoxyethanol was reduced according to the literature method [17] in the presence of 700 mg of 10% Pd/C at low pressure until H_2 uptake ceased. The resulting solution was heated to 100° and filtered through a Celite bed to remove the catalyst. After removing the solvent under reduced pressure, the product was triturated with benzene and collected by vacuum filtration. A yield of 6.13 g (87.6%) was obtained after drying *in vacuo* at 100° overnight. Observed m.p. = 297–299° (literature m.p. = darkens at 300°; softens at 304°; melts at 314–315° [18]. TLC (THF : hexanes, 4 : 1) R_f = 0.46. ^{1}H-NMR (90 MHz, DMSO-d^6) δ: 2.23 (*s*, 3H) CH_3; 5.40 (*br.s*, 2H) NH_2, exchanges with D_2O; 7.11 (*m*, 3H) aromatic.



Scheme 1. Synthesis of 2-desamino-2-methyl-5,8-dideazaisofolic acid.

Di-tert-*butyl* 2-*desamino*-2-*methyl*-5,8-*dideazaisofolate*. A solution of 2-methyl-6-amino-4(3*H*)-quinazolone (1.03 g, 5.9 mmol) and di-*tert*-butyl *N*-(4-formylbenzoyl)-L-glutamate [9] (2.33 g, 5.9 mmol) in 70% acetic acid was hydrogenated in the presence of Raney nickel (*ca.* 500 mg) until H_2 uptake ceased. The catalyst was removed by filtration through a Celite bed and the filtrate was basified to pH 8.5 in an ice bath by slow addition of concentrated NH_4OH. Upon completion of the basification, the mixture was stirred for 45 min. The product was collected by vacuum filtration, washed with copious amounts of cold water and hexanes, and dried *in vacuo* at 65° overnight to yield 2.62 g (79.3%). Observed m.p. = 176–178°. TLC (chloroform:methanol, 95:5) R_f = 0.57. ^{1}H-NMR (300 MHz, DMSO-d^6) δ: 1.38 (*s*, 9H) $C(CH_3)_3$; 1.40 (*s*, 9H) $C(CH_3)_3$; 1.84–2.10 (*m*, 2H) glu β-CH_2; 2.24 (*s*, 3H) CH_3; 2.33 (*t*, J = 7.43 Hz, 2H) glu γ-CH_2; 4.32 (*m*, 1H) glu α-CH; 4.41 (*app.d*, J = 5.61 Hz, 2H) NCH_2; 6.80 (*t*, J = 5.97 Hz, 1H) $NHCH_2$; 6.98 (*app.d*, $J_{5,7}$ = 2.64 Hz, 1H) H-5; 7.14 (*dd*, $J_{7,5}$ = 2.75 Hz, $J_{7,8}$ = 8.81 Hz, 1H) H-7; 7.33 (*d*, $J_{8,7}$ = 8.76 Hz, 1H) H-8; 7.46 (*d*, J_o = 8.22 Hz, 2H) 3′,5′; 7.83 (*d*, J_o = 8.22 Hz, 2H) 2′,6′; 8.54 (*d*, J = 7.47 Hz, 1H) CONH; 11.85 (*s*, 1H) lactam NH. Elemental analysis: $C_{30}H_{38}N_4O_6 \cdot 0.5H_2O$; C,H,N.

2-*Desamino*-2-*methyl*-5,8-*dideazaisofolic acid* (**2c**). A sample of di-*tert*-butyl 2-desamino-2-methyl-5,8-dideazaisofolate (1.33 g, 2.4 mmol) was stirred in 45 mL of methylene chloride/trifluoroacetic acid (8:1) for 1.5 hr. The solvent was then removed under reduced pressure and the product suspended in 50 mL of water. This suspension was basified to pH 8.5 with concentrated NH_4OH, and then acidified to pH 2.5–3.0 with 0.5 N HCl. After stirring overnight, the product was collected by vacuum filtration and washed with copious amounts of cold water and hexanes. Drying *in vacuo* for 10 hr at 65° yielded 1.01 g (92.2%) of the target compound (overall yield = 50.3%). Observed m.p. = 188–190°. TLC (5% NH_4HCO_3) R_f = 0.59. ^{1}H-NMR (300 MHz, DMSO-d^6) δ: 1.89–2.08 (*m*, 2H) glu β-CH_2; 2.24 (*s*, 3H) CH_3; 2.34 (*t*, J = 7.55 Hz, 2H) glu γ-CH_2; 4.32–4.41 (*m*, 3H) glu α-CH and NCH_2; 6.83 (*app.t*, 1H) $NHCH_2$; 6.97 (*app.d*, $J_{5,7}$ = 2.49 Hz, 1H) H-5; 7.14 (*dd*, $J_{7,5}$ = 2.43 Hz, $J_{7,8}$ = 8.82 Hz, 1H) H-7; 7.32 (*d*, $J_{8,7}$ = 8.79 Hz, 1H) H-8; 7.46 (*d*, J_o = 8.16 Hz, 2H) 3′,5′; 7.83 (*d*, J_o = 8.16 Hz, 2H) 2′,6′; 8.53 (*d*, J = 7.56 Hz, 1H) CONH; 11.83 (*s*, 1H) lactam NH. Elemental analysis: $C_{22}H_{22}N_4O_6 \cdot 1H_2O$; C,H,N. FAB/MS: 439 (M + 1); 437 (M − 1).

2-*Desamino*-2-*methyl*-5,8-*dideazaisofolic acid di- and triglutamyl conjugates*. 2-Desamino-2-methyl-5,8-dideazaisofolic acid (final concentration = 1 mM) was incubated for 48 hr at 37° in 2 mL of a solution which contained 20 mM sodium glutamate, 50 units of *Escherichia coli* folylpolyglutamate synthetase/dihydrofolate synthetase [19], 50 mM Tris–HCl, pH 8.6, 5 mM ATP, 50 mM KCl, 10 mM $MgCl_2$ and 70 mM 2-mercaptoethanol. The progress of the polyglutamylation reaction was observed by examination of aliquots of the reaction mixture on paired-ion HPLC (see below). Products were identified by their sequential appearance in the reaction mixture, increasing retention times on HPLC (263, 418, and 586 sec for **2c**, its di- and triglutamyl conjugates respectively), and by their UV spectra (captured on a Perkin–Elmer LC235 diode array detector) which were identical to that of **2c**. After 48 hr, 33% of the substrate had been converted to the diglutamate (**2c** plus one glutamyl residue) and 61% of the substrate was converted to the triglutamate (**2c** + two glutamyl residues). The synthesis was stopped by boiling the mixture for 5 min to precipitate the protein. After clarification by centrifugation at 8800 *g* for 10 min, the products of the enzymatic reaction were purified on a Waters 8 × 100 cm C18 novapak radial compression column. The mobile phase was 18% acetonitrile in 5 mM tetrabutyl ammonium phosphate, 10 mM $NH_4H_2PO_4$, pH 7, 1.5 mL/min. The product peaks were collected and diluted with water for use in TS inhibition studies.

Biological evaluation

Assays for inhibition of DHFR (WIL2), TS

(L1210), glycinamide ribonucleotide transformylase (GAR Tfase) (chicken liver), and 5-aminoimidazole-4-carboxamide ribonucleotide transformylase (AICAR Tfase) (chicken liver) were performed as described previously [20–23]. Thymidylate synthase from an SV 40-transformed human fibroblast cell line was cloned in *E. coli* and the protein was purified to homogeneity by affinity chromatography* [24]. It was assayed by the tritium-release method as described earlier [25], with 100 μM (6*R*,*S*)-tetrahydrofolate. Standard errors of the IC_{50} values were less than ±10%. Cytotoxicity studies using various cell lines in culture were performed according to the literature methods [26, 27]. Binding to the reduced folate transporter was assayed as the inhibition of uptake of [^{3}H]MTX into MOLT-4 cells and was determined as described previously [8]. In the absence of labeled drugs, this assay gives a prediction of transport potential. While this prediction has its limitations in not actually measuring velocity, in general compounds that bind well are also effectively transported. Studies on the reversal of cytotoxicity of **2c** were performed using leucovorin (100 μM), hypoxanthine (Hx) (50 μM), TdR (20 μM), or a combination of Hx and TdR in MCF-7 cells (see Table 2).

RESULTS AND DISCUSSION

For the past several years we have attempted to enhance the antitumor activity of IAHQ by synthetic modification. The introduction of substituents at position 5 and/or position 9 proved to be of limited value in this regard [9]. With the recent disclosure of the potent antitumor effects of quinazolines modified at position 2 as compared with their 2-amino counterparts [6], we set out to synthesize and evaluate 2-desamino-2-methyl-5,8-dideazaisofolic acid, **2c**. The preparation of **2c** was readily accomplished in four steps in 50% overall yield and its structure was characterized by high resolution NMR spectroscopy and fast atom bombardment mass spectrometry. It was then subjected to a variety of biological evaluations in comparison with its 2-desamino, **2b**, and 2-amino, **2a**, counterparts, as well as the analogous set of three N^{10}-propargyl analogues, **1a–c**.

Table 1 shows the DHFR and TS inhibition data for compounds **1a–c** and **2a–c**. Included are results reported previously for **2a** and **2b** which were obtained under identical experimental conditions [15, 20]. It is apparent that all of the compounds studied were poor DHFR inhibitors compared to MTX (IC_{50} = 0.0038 μM), although compounds of the isofolate series were marginally more effective inhibitors than the corresponding members of the normal bridge N^{10}-propargyl series. Previous studies have shown that the normal bridge N^{10}-propargyl compounds, such as **1a–c**, are effective TS inhibitors [5, 7, 8]. In this study these compounds were found to be much more potent than their reverse bridge counterparts (Table 1). Thus, compound **1c** was 189-fold superior to **2c** as an inhibitor of TS. It should be noted that compound **2c** showed no inhibition of GAR Tfase at 100 μM and inhibited AICAR Tfase by only 24% at this concentration (data not shown).

Table 1 also compares the abilities of the compounds to bind to the reduced folate transporter. As discussed above, [^{3}H]IAHQ, **2a**, enters cells very slowly [14]. This low rate of entry correlates with its poor affinity for the transporter (K_i = 40 μM). It has been suggested that the low level of cytotoxicity of CB3717, **1a**, is due to impaired uptake into cells [5, 7, 8]. From the results shown in Table 1, it will be seen that **1a** bound weakly to the transporter (K_i = 28 μM) which supports the earlier contention. By contrast, the 2-desamino-2-methyl analogues of both IAHQ and CB3717, compounds **2c** and **1c**, respectively, bound very well to the transporter. In both cases the affinity increased approximately 20-fold with respect to the parent 2-amino compound. These results indicate that the presence of a 2-methyl substituent is sufficient to overcome the unfavorable interaction of quinazolines having a 2-amino-4(3*H*)-one configuration with the transporter. The results also suggest that both compounds, **1c** and **2c**, should be transported well into cells on this carrier. It can be seen in Table 1 that the 2-desamino compounds, **1b** and **2b**, had affinities for the transporter which were intermediate between their 2-amino and 2-desamino-2-methyl counterparts.

Inhibition of mammalian tumor cell growth is also presented in Table 1. It can be seen that **1c** was considerably more active than **1a** against both the murine and human lines, L1210 and MCF-7, respectively. This presumably reflects the enhanced binding and transport of **1c** by the reduced folate transporter [7, 8]. A similar phenomenon was observed with the transformation of **2a** to **2c**. In this instance cytotoxicity was enhanced 20-fold toward the human cell line and 44-fold toward the murine cell line. Thus, in both the normal bridge N^{10}-propargyl series and in the isofolate series, conversion of the 2-amino group to a 2-methyl group resulted in greatly enhanced activity against tumor cells in culture.

Table 2 shows the activities of compounds **1a–c** and **2a–c** as substrates for FPGS. Each of the isofolate analogues was a superior substrate for the hog liver FPGS than the corresponding normal bridge N^{10}-propargyl compound. For example, the V_{max}/K_m for **2c** was 12-fold larger than the corresponding value for **1c**. Indeed, the activity of **2c** approached that of the natural substrate (6*S*)-5,6,7,8-tetrahydrofolate (V_{max}/K_m = 100).

Compounds **1c** and **2c** are potent cytotoxic agents in tissue culture. In L1210 cells the activity of **2c** was only 2-fold less than that of **1c**, while in MCF-7 cells it was 5-fold weaker. It has been reported that **1c**, which is an excellent TS inhibitor *in vitro*, also acts as a TS inhibitor in tissue culture [7], since its activity is reversed by addition of TdR to the culture medium. Quite surprisingly, although **2c** is a weak inhibitor of TS *in vitro*, the compound behaved as a potent TS inhibitor when tested against cultured cells. This is shown in Table 3, where it can be seen that the activity of compound **2c** was reversed completely by 20 μM TdR in the medium or the combination of TdR plus Hx, but was not reversed by 50 μM Hx

* Dev I and Dallas W, personal communication, cited with permission.

Table 1. Summary of inhibitory results for the folate analogues studied

Compound*	DHFR (WIL2, IC_{50}, μM)	TS (L1210, IC_{50}, μM)	L1210 cells (IC_{50}, μM)	[^{3}H]MTX uptake into MOLT-4 cells (K_i, μM)	MCF-7 cells (IC_{50}, μM)
1a	0.91	0.014	1.4	28	0.8
1b	7.0	0.13	0.27	10	0.035
1c	5.5	0.09	0.046	1.5	0.007
2a	0.11†	1.3†	3.2‡	40	0.75
2b	0.32‡	25‡	0.53‡	15	2.3
2c	3.1	17	0.073	1.8	0.037

* Compounds **1a**, **2a**, and **2b** were synthesized as described previously [1, 9, 15]. Samples of **1b** and **1c** were gifts from Dr. T. R. Jones, Agouron Pharmaceuticals, Inc., La Jolla, CA.
† From Hynes *et al.* [20].
‡ From Hynes *et al.* [15].

Table 2. Kinetic parameters for analogues studied as substrates for folylpolyglutamate synthetase

Compound	V_{max} (μmol/hr/mg)	K_m (μM)	V_{max}/K_m (rel)*
1a	20	38	3.2
1b	42	115	2.3
1c	56	54	6.5
2a	92†	21†	28†
2b	62	12	31
2c	104	8.6	76

* Values are relative to the substrate activity for (6*S*)-tetrahydrofolate ($V_{max}/K_m = 100$).
† From Cichowicz *et al.* [21].

Table 3. Reversal of the cytotoxicity of **2c** toward MCF-7 cells *in vitro*

Reversal agent*	% Inhibition	**2c** (μM)	Reversal
—	50	0.037	—
Leucovorin	0	100	Yes
Thymidine + hypoxanthine	0	100	Yes
Thymidine	0	100	Yes
Hypoxanthine	50	0.016	No

* When present, leucovorin, thymidine, and hypoxanthine concentrations were 100, 20 and 50 μM respectively.

alone. Thus, the primary target of **2c** appears to be TS. Since it is a weak TS inhibitor *in vitro*, the results suggest that it is activated intracellularly to a more potent TS inhibitor. A likely means of activation is polyglutamylation, since **2c** is a good substrate for FPGS and because other antifolate TS inhibitors are activated by polyglutamylation [7, 13]. We tested the effect of glutamylation of **2c** on its ability to inhibit TS. Because the solutions of the polyglutamated materials contained HPLC mobile phase, control assays were performed with matched amounts of mobile phase (and no inhibitors). It was found that the enzyme was not inhibited by the amounts of mobile phase present in the assays and that **2c** was equally inhibitory in the presence or absence of mobile phase. The K_i values, determined for **2c**, its diglutamyl, and triglutamyl conjugates, respectively, were 3.9, 0.2, and 0.12 μM. Thus, the addition of two glutamyl residues resulted in a 32-fold increase in potency against human TS.

The results of the TS, FPGS, transporter affinity, and cell culture data taken together appear to be required to understand the antitumor activity of **2c**. The enhanced affinity of both **1c** and **2c** for the reduced folate transporter over their respective 2-amino analogues, **1a** and **2a**, appears to result in

enhanced transport into cells as demonstrated by the nearly direct correlation between transporter affinity and cell growth inhibition (Table 1). Further, the excellent polyglutamylation potential of **2c** as compared to **1c** ($V_{max}/K_m = 76$ for **2c** vs 6.5 for **1c**) may result in more extensive intracellular production of polyglutamated metabolites of **2c** which cannot readily efflux from the cell. This predicted intracellular accumulation of **2c** polyglutamates may overcome the poor affinity of **2c** for TS and result in potent cytotoxicity. Thus, a relatively weak TS inhibitor *in vitro* may become a potent inhibitor of tumor cell growth if it can be effectively transported and activated by polyglutamylation. This infers that the *in vitro* assay of TS inhibition alone in the absence of tests for FPGS, transport and cell culture activity can be misleading and can be inadequate for predicting intracellular TS inhibition and antitumor activity.

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