Pergamon

Biochemical Pharmacology, Vol. 50, No. 6, pp. 803–809, 1995.

0006-2952/95 $9.50 + 0.00

0006-2952(95)00203-0

STUDIES ON THE ANTITUMOR EFFECTS OF ANALOGUES OF 5,8-DIDEAZAISOFOLIC ACID AND 5,8-DIDEAZAISOAMINOPTERIN*

ROBERT L. HAGAN,†‡ JOHN B. HYNES,§ MEADE PIMSLER† and ROY L. KISLIUK‖

†Clinical Investigation Facility, David Grant Medical Center, Travis AFB, CA 94535; §Department of Pharmaceutical Sciences, Medical University of South Carolina, Charleston, SC 29425; ‖Department of Biochemistry, Tufts University School of Medicine, Boston, MA 02111, U.S.A.

(*Received* 30 *December* 1994; *accepted* 10 *March* 1995)

Abstract—Six new analogues of 5,8-dideazaisofolic acid and 5,8-dideazaisoaminopterin were synthesized in an effort to obtain enhanced antitumor activity. The modifications included the replacement of the 2-amino group by hydrogen or methyl as well as the inclusion of a methyl substituent at position 9. Based upon activity against L1210 leukemia cells in culture, three of the new analogues together with one compound described previously were evaluated for cytotoxicity *in vitro* using three human tumor cell lines (Colo 320 DM, Hep G2 and HL-60). The most effective compound was 2-desamino-N^9-methyl-5,8-dideazaisoaminopterin (**2c**) with the HL-60 cells being the most sensitive to its cytotoxic effects. These analogues were evaluated *in vitro* as inhibitors of dihydrofolate reductase (DHFR) and thymidylate synthase (TS) from human as well as bacterial (*Lactobacillus casei*) sources. All four of the 4-amino analogues were most effective toward *L. casei* DHFR compared with human DHFR, with 2-desamino-2-methyl-5,8-dideazaisoaminopterin (**2d**) and its 9-methyl derivative (**2e**) having 818- and 430-fold greater selectivity (*L. casei*/human). Most of the compounds studied were found to be only modest inhibitors of human TS (I_{50} values = 1.5 to 20 μM) and were therefore at least 40-fold less inhibitory than 10-propargyl-5,8-dideazafolic acid. Nevertheless, reversal of cytotoxicity studies with thymidine, hypoxanthine and folinic acid using the HL-60 cell line suggested that TS is the primary target for these analogues.

Key words: folic acid antagonists; quinazolines; thymidylate synthase; tumor cells; cytotoxicity

The compound IAHQ¶ (**1a**; Fig. 1), was found to possess modest antitumor activity against a variety of human and murine tumor cells in culture. *In vivo*, IAHQ 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 [1–5]. However, large doses of IAHQ were required to achieve therapeutic effectiveness in these animal models. This lack of potency was attributed to slow influx into target cells as demonstrated with [^{3}H]IAHQ in the presence of HCT-8 human colon adenocarcinoma cells in culture [6]. Enhancing the penetration of IAHQ into tumor cells, therefore, seemed paramount to improving the biological activity of this quinazoline antifolate.

Manipulation of the solubility characteristics of a drug is one possible way of affecting its disposition and biological activity. Generally, the introduction of amino, hydroxy, or mercapto substituents into a molecule leads to increased aqueous solubility; however, the converse is true with some heterocycles, such as quinazoline. The decreased water solubility of amino- or hydroxyquinazolines has been attributed to a stacking of the molecules into a stable lattice via intermolecular hydrogen bond formation [7]. Based on this solubility paradox, and the enhanced solubility and biological activity demonstrated in other 2-modified quinazoline antifolates [8, 9], IAHQ was targeted for similar structural changes. Modification of IAHQ at the 2-position of the quinazoline ring has resulted in analogues with enhanced *in vitro* activity. The analogues 2-desamino-5,8-dideazaisofolic acid (**1b**) and 2-desamino-2-methyl-5,8-dideazaisofolic acid (**1d**) were found to be 6- and 44-fold, respectively, more cytotoxic toward L1210 cells than the parent compound (**1a**) [10, 11]. This enhancement of cytotoxicity correlated well with an increased affinity for the reduced folate transporter, as measured by competition with [^{3}H]MTX for uptake into MOLT-4 cells [11].

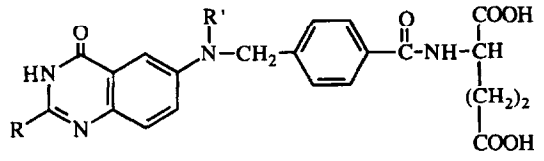
This paper represents an extension of earlier work and includes the preparation of the 9-methyl derivatives of **1b** and **1d**. In addition, four new analogues of 5,8-dideazaisoaminopterin (**2a**) modified at positions 2 and 9 were synthesized for the first time and fully characterized.

Based upon the cytotoxicity results obtained with **1b** and **1d** as well as the six new analogues mentioned above against L1210 leukemia cells in culture [12], four compounds were selected for further study. The Colo 320 DM colon carcinoma, Hep G2 hepatocellular carcinoma and HL-60 promyelocytic leukemia cell lines were used for *in vitro* cytotoxicity evaluations. The HL-60 cell line was also employed for cytotoxicity reversal studies. In addition, all eight analogues were evaluated as inhibitors of DHFR and TS from human and bacterial sources.

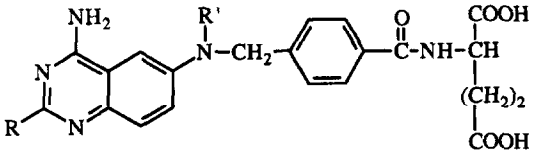
* This material has been presented in part (*Proc Am Assoc Cancer Res* **35**: 303, 1994). The views expressed in this manuscript are those of the authors and should not be construed as official views of the Department of the Air Force or the Department of Defense.

‡ Corresponding author: Dr. Robert L. Hagan, 60th Medical Group, Clinical Investigation Facility, 101 Bodin Circle, Travis AFB, CA 94535-1800. Tel. (707) 423-7284; FAX (707) 423-7267.

¶ Abbreviations: IAHQ, 5,8-dideazaisofolic acid; MTX, methotrexate; DHFR, dihydrofolate reductase; TS, thymidylate synthase; TdR, thymidine; Hx, hypoxanthine; THF, tetrahydrofuran; and DMF, dimethylformamide. Designations for the analogues studied are presented beneath their respective structures in Fig. 1.



Compound	R	R'
1a	NH_2	H
1b	H	H
1c	H	CH_3
1d	CH_3	H
1e	CH_3	CH_3



Compound	R	R'
2a	NH_2	H
2b	H	H
2c	H	CH_3
2d	CH_3	H
2e	CH_3	CH_3

Fig. 1. Structural formulas and designations of analogues.

MATERIALS AND METHODS

Chemical characterization and procedures

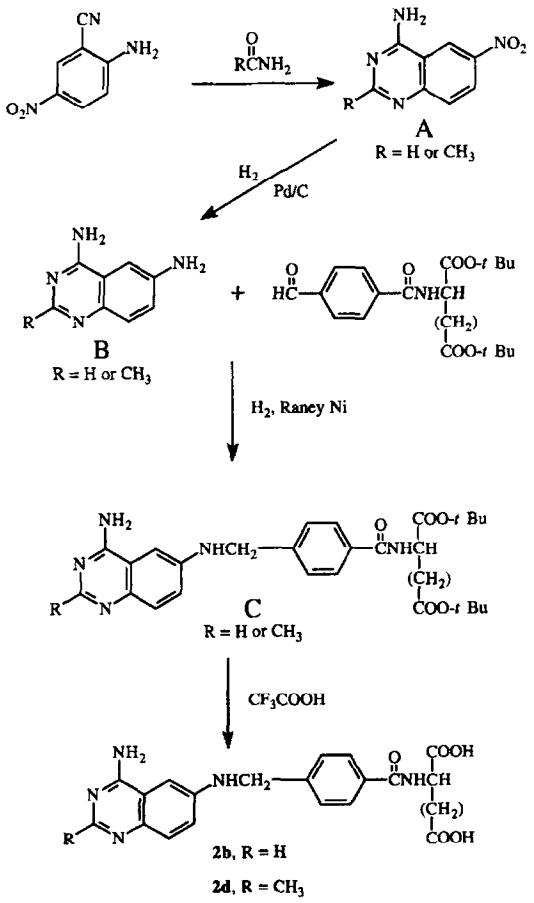
Melting points were determined on a Mel-temp apparatus and are uncorrected. Elemental analyses were performed by Atlantic Microlabs, Norcross, GA. All analytical samples gave combustion values of C, H, and N within ±0.4% of the theoretical values. Solvation due to H_2O was confirmed by the presence of a broad peak centered at approximately 3.4 ppm in the ^1H 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 silica gel medium (Eastman Kodak 13181). The free acids were assayed on Eastman Kodak 13254 cellulose plates (5% NH_4HCO_3). Preparative column chromatographic separations were performed using either Baker 60-200 mesh silica gel or Avicel® microcrystalline cellulose (E. Merck & Co., Darmstadt, Germany). High resolution ^1H NMR spectra were acquired on a Bruker AM-300 spectrometer at the Chemistry Department, University of South Carolina, Columbia, SC. ^1H 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. Fast atom bombardment mass spectra were obtained with a VG 705Q analytical spectrometer at the Chemistry Department, University of South Carolina, Columbia, SC, by Dr. Michael Walla. Ultraviolet-visible spectral characterizations were performed using a Hewlett-Packard 8451A diode array spectrophotometer.

Catalytic hydrogenations and reductive condensation reactions were conducted using a Parr 3911 shaker-type hydrogenation apparatus. The intermediate di-*tert*-butyl-*N*-(4-formylbenzoyl)-L-glutamate was prepared as described previously [13]. Raney nickel (Raney 30) was obtained as a gift from the Davison Chemical Division, W.R. Grace & Co.

Synthesis

The syntheses of compounds **1b** and **1d** have been described previously [10, 11]. The synthetic route to **2a–d** is shown in Scheme 1. The methylation reaction yielding compounds **1c** and **1e** is analogous to the methylation in the final step of Scheme 1 and is described below for **2b** and **2d.**

4-Amino-6-nitroquinazoline (Compound A, R = H) (Scheme 1). A mixture of 16.3 g (100 mmol) of 2-amino-5-nitrobenzonitrile and 100 mL (113 g, 2.50 mol) of formamide was heated at 170° for 3 hr. The reaction mixture was allowed to cool to room temperature, then poured into ice-H_2O and stirred for about 1 hr. The solids were collected by vacuum filtration, washed with cold H_2O, and dried at 50° for 18 hr to yield 16.1 g (85.0%) of product. Observed m.p. = 315–322°; literature m.p. = 320–320.5° [14]; TLC R_f = 0.50 (THF:hexanes, 6:4). NMR (Me_2SO-d_6) δ 7.79 (d, 1, H_8, $J_{8,7}$ = 9.0 Hz), 8.15–8.63 (m, 4, H_2, H_7 and NH_2; amine exchanges with D_2O), 9.33 (app d, 1, H_5, $J_{5,7}$ = 2.7 Hz).



Scheme 1. Synthetic route to 2-desamino and 2-desamino-2-methyl analogues of 5,8-dideazisoaminopterin.

2-Methyl-4-amino-6-nitroquinazoline (*Compound A*, $R = CH_3$). A mixture of 10.0 g (61.3 mmol) of 2-amino-5-nitrobenzonitrile and 100 g (1.69 mol) of molten acetamide was stirred at 175–180° for 24 hr. The slightly cooled mixture was poured over crushed ice to precipitate the product. The nitroquinazoline was collected by vacuum filtration and washed with water and diethyl ether. Drying under vacuum at 65° for 18 hr yielded 8.27 g (66.7%) of product. Observed m.p. = >315° dec.; literature m.p. = 331–333° [15]; TLC R_f = 0.23 ($CHCl_3$:MeOH, 98:2); NMR (Me_2SO-d_6) δ 2.46 (s, 3, CH_3), 7.71 (d, 1, H_8, $J_{8,7}$ = 9.18 Hz), 8.10–8.25 (br s, 2, NH_2, exchanges with D_2O), 8.43 (dd, 1, H_7, $J_{7,8}$ = 9.20 Hz, $J_{7,5}$ = 2.53 Hz), 9.29 (app d, 1, H_5, $J_{5,7}$ = 2.49 Hz).

4,6-Diaminoquinazoline (*Compound B*, $R = H$). A suspension of 3.80 g (20.0 mmol) 4-amino-6-nitroquinazoline in 125 mL of 2-methoxyethanol was hydrogenated in the presence of 500 mg of 10% Pd/C at low pressure until H_2 uptake ceased. The reaction mixture was diluted with 200 mL of 2-methoxyethanol, heated to 110° to dissolve the product, then filtered through a Celite bed to remove the catalyst. After removing the solvent under reduced pressure, the product was triturated with diethyl ether and collected by vacuum filtration. A yield of 2.5 g (78.1%) was obtained after drying *in vacuo* at 65° for 18 hr. Observed m.p. = >279° dec; TLC R_f = 0.49 (EtOAc:DMF, 7:3); NMR (Me_2SO-d_6) δ 5.26 (br s, 2, 6-NH_2), 6.85–7.13 (m, 4, H_5, H_7 and 4-NH_2), 7.33 (d, 1, H_8, $J_{8,7}$ = 7.8 Hz), 8.02 (s, 1, H_2); Elemental analysis ($C_8H_8N_4 \cdot 0.23\ H_2O$) C,H,N: found 33.33, calculated 34.10.

2-Methyl-4,6-diaminoquinazoline (*Compound B*, $R = CH_3$). A suspension of 1.50 g (7.30 mmol) of 2-methyl-4-amino-6-nitroquinazoline, in 250 mL of 2-methoxyethanol was hydrogenated at low pressure in the presence of 0.75 g of 10% Pd/C until H_2 uptake ceased. The resulting solution was filtered through a Celite bed to remove the catalyst, and then evaporated to dryness under reduced pressure. The product was stirred in diethyl ether overnight and then collected by vacuum filtration and washed with diethyl ether. Upon drying under vacuum at 60°, 1.12 g (87.5%) of a light tan powder was obtained. Observed m.p. = 235–238°; literature m.p. = 244–246° [16]; TLC R_f = 0.42 ($CHCl_3$:MeOH, 9:1); NMR (Me_2SO-d_6) δ 2.32 (s, 3, CH_3), 5.23 (s, 2, 6-NH_2, exchanges with D_2O), 7.00 (app d, 1, H_5, $J_{5,7}$ = 2.34 Hz), 7.09 (dd, 1, H_7, $J_{7,8}$ = 8.84 Hz, $J_{7,5}$ = 2.44 Hz), 7.12 (s, 2, 4-NH_2, exchanges with D_2O), 7.32 (d, 1, H_8, $J_{8,7}$ = 8.79 Hz).

2-Desamino-5,8-dideazaisoaminopterin di-tert-butyl ester (*Compound C*, $R = H$). A solution of 0.806 g (5.03 mmol) of 4,6-diaminoquinazoline and 1.97 g (5.03 mmol) of di-*tert*-butyl-*N*-(4-formylbenzoyl)-L-glutamate [13] in 70% acetic acid was hydrogenated in the presence of Raney Ni (*ca.* 500 mg) until H_2 uptake ceased. The catalyst was removed by filtration through a Celite bed and the filtrate was basified in an ice bath to pH 8.5 by slow addition of concentrated NH_4OH. The product was collected by vacuum filtration and washed with cold H_2O and hexanes. The product was dissolved in $CHCl_3$ and stirred in a slurry of 20 g silica gel. The slurry was poured onto a 10 × 2 cm bed of silica gel and eluted in a stepwise fashion with $CHCl_3$:MeOH (95:5) to $CHCl_3$:MeOH (9:1) (1% increments, 150 mL each). Pure fractions were combined and the solvent was removed under reduced pressure. The impure fractions were pooled and applied to a 1 × 25 cm bed of silica gel. Stepwise elution as before with $CHCl_3$:MeOH yielded 0.119 g of the final compound. The combined yield was 1.04 g (38.7%) of light green crystals. Observed m.p. = 124–125°; TLC R_f = 0.47 ($CHCl_3$:MeOH, 95:5); NMR (Me_2SO-d_6) δ 1.37 (s, 9, $C(CH_3)_3$), 1.40 (s, 9, $C(CH_3)_3$), 1.82–2.08 (m, 2, glu β-CH_2), 2.32 (t, 2, glu γ-CH_2, J = 7.38 Hz), 4.32 (m, 1, glu α-CH), 4.43 (app d, 2, NHC$\underline{H}_2$, J = 5.76 Hz), 6.65 (app t, 1, N$\underline{H}$CH_2, exchanges with D_2O), 7.02 (s, 1, H_5), 7.23 (d, 1, H_7, $J_{7,8}$ = 8.91 Hz), 7.26 (br s, 2, NH_2, exchanges with D_2O), 7.42 (d, 1, H_8, $J_{8,7}$ = 8.91 Hz), 7.51 (d, 2, 3′, 5′, J_0 = 8.13 Hz), 7.81 (d, 2, 2′, 6′, J_0 = 8.07 Hz), 8.10 (s, 1, H_2), 8.54 (app d, 1, CONH, J = 7.47 Hz); Elemental analysis ($C_{29}H_{37}N_5O_5 \cdot 0.7\ H_2O$) C,H,N.

2-Desamino-2-methyl-5,8-dideazaisoaminopterin di-tert-butyl ester (*Compound C*, $R = CH_3$). A solution of 0.67 g (3.8 mmol) of 2-methyl-4,6-diaminoquinazoline and 1.5 g (3.8 mmol) of di-*tert*-butyl-*N*-(4-formylbenzoyl)-L-glutamate in 70% acetic acid was hydrogenated in the presence of Raney Ni (*ca.* 400 mg) until H_2 uptake ceased. The catalyst was removed by filtration through a Celite bed and the filtrate basified in an ice bath to pH 8.5 by slow addition of concentrated NH_4OH. The product was collected by vacuum filtration and washed with cold H_2O. Drying overnight *in vacuo* at 65° yielded 1.89 g (90.0%) of a light yellow powder. Observed m.p. = 120–123°; TLC R_f = 0.60 ($CHCl_3$:MeOH, 95:5); NMR (Me_2SO-d_6) δ 1.38 (s, 9, $C(CH_3)_3$), 1.40 (s, 9, $C(CH_3)_3$), 1.80–2.10 (m, 2, glu β-CH_2), 2.34 (m, 5, C_2-CH_3 and glu γ-CH_2), 4.35 (m, 1, glu α-CH), 4.40 (br s, 2, NHC$\underline{H}_2$), 6.57 (br s, 1, N$\underline{H}$CH_2, exchanges with D_2O), 7.00 (br s, 1, H_5), 7.23 (d, 1, H_7, $J_{7,8}$ = 9.20 Hz), 7.36 (d, 1, H_8, $J_{8,7}$ = 9.27 Hz), 7.41 (br s, 2, NH_2, exchanges with D_2O), 7.49 (d, 2, 3′, 5′, J_0 = 7.98 Hz), 7.79 (d, 2, 2′, 6′, J_0 = 8.07 Hz), 8.56 (app d, 1, CONH, J = 7.95 Hz); Elemental analysis ($C_{30}H_{39}N_5O_5 \cdot 1.26\ H_2O$) C,H,N.

2-Desamino-5,8-dideazaisoaminopterin (**2b**). A 0.388-g (0.720 mmol) sample of 2-desamino-5,8-dideazaisoaminopterin di-*tert*-butyl ester was stirred in 10 mL of CF_3COOH at ambient temperature for 2 hr. The CF_3COOH was removed under reduced pressure to yield a dark green oil. Approximately 25 mL of H_2O was added to the flask, and the mixture was basified to pH 8.5 with concentrated NH_4OH. The product was precipitated by acidification to pH 2.5–3.0 with 0.5 N HCl, collected by vacuum filtration, and washed with cold H_2O. Drying *in vacuo* at 65° yielded 0.172 g (56.5%) of a dark green powder. Observed m.p. = >185° dec; TLC R_f = 0.32 (5% NH_4HCO_3); UV λ_{max} 230 nm (ε 1.80 × 10^4), 236 (ε 1.78 × 10^4), 242 (ε 1.78 × 10^4), 248 (ε 1.80 × 10^4), 252 (ε 1.85 × 10^4), 296 (ε 1.13 × 10^4), 360 (ε 3.75 × 10^3); NMR (Me_2SO-d_6) δ 1.85–2.13 (m, 2, glu β-CH_2), 2.34 (app t, 2, glu γ-CH_2), 4.43 (m, 3, NHC$\underline{H}_2$, and glu α-CH), 6.67 (app t, 1, N$\underline{H}$CH_2), 7.02 (s, 1, H_5), 7.25 (d, 1, H_7, $J_{7,8}$ = 8.22 Hz), 7.32 (s, 2, NH_2, exchanges with D_2O), 7.43 (d, 1, H_8, $J_{8,7}$ = 9.15 Hz), 7.52 (d, 2, 3′, 5′, J_0 = 7.59 Hz), 7.84 (d, 2, 2′, 6′, J_0 = 7.68 Hz), 8.13 (s, 1, H_2), 8.53 (d, 1, CONH, J = 7.11 Hz, exchanges with D_2O); FAB-MS 424 (M + 1); Elemental analysis ($C_{21}H_{21}N_5O_5 \cdot 0.75\ H_2O$) C,H,N.

2-Desamino-2-methyl-5,8-dideazaisoaminopterin (**2d**). A 1.50-g (2.70 mmol) sample of 2-desamino-,5,8-dideazaisoaminopterin di-*tert*-butyl ester was stirred in 20 mL of CF_3COOH at ambient temperature for 2.5 hr. The CF_3COOH was removed under reduced pressure and the

product basified to pH 9 with 1 N NaOH, and then acidified to pH 3–4 with 1 N HCl. The resulting thick suspension was allowed to stir overnight. The product was collected by centrifugation, washed twice with H_2O, then dried *in vacuo* for 18 hr at 50°, upon which 0.754 g (63.9%) of a greenish yellow powder was obtained. Observed m.p. = 241–244°; TLC R_f = 0.25 (5% NH_4HCO_3); UV λ_{max} 210 (ϵ 1.74 × 10^4), 232 (ϵ 2.31 × 10^4), 240 (ϵ 2.34 × 10^4), 244 (ϵ 2.34 × 10^4), 252 (ϵ 2.37 × 10^4), 292 (ϵ 1.11 × 10^4), 368 (ϵ 3.78 × 10^3); NMR (Me_2SO-d_6) δ 1.82–2.18 (m, 2, glu β-CH_2), 2.33 (t, 2, glu γ-CH_2, J = 7.26 Hz), 2.36 (s, 3, CH_3), 4.41 (br s, 3, glu α-CH and NHC<u>H</u>$_2$, separates to m and br s with D_2O exchange), 6.64 (app t, 1, N<u>H</u>CH_2), 7.05 (s, 1, H_5), 7.24 (d, 1, H_7, J = 11.01 Hz), 7.38 (d, 1, H_8, $J_{8,7}$ = 8.94 Hz), 7.51 (d, 2, 3′, 5′, J_0 = 8.13 Hz), 7.58 (br s, 2, NH_2 exchanges with D_2O), 7.84 (d, 2, 2′, 6′, J_0 = 8.16 Hz), 8.52 (d, 1, CONH, J = 7.7 Hz, exchanges with D_2O); FAB-MS 436 (M − 1), 438 (M + 1); Elemental analysis ($C_{22}H_{23}N_5O_5 \cdot 0.6\ H_2O$) C,H,N.

2-Desamino-9-methyl-5,8-dideazaisoaminopterin **(2c).** To a stirred solution of 0.150 g (0.350 mmol) of 2-desamino-5,8-dideazaisoaminopterin (**2b**) in 12 mL DMF was added 0.8 mL glacial acetic acid, 1.5 mL of 37% HCHO solution, and 0.075 g (1.19 mmol) $Na(CN)BH_3$. After 18 hr stirring at ambient temperature, the solvent was removed under reduced pressure and the resulting oil washed twice with EtOH. The product was suspended in 30 mL H_2O, basified to pH 9 with 1 N NaOH, and then acidified after 20 min to pH 3–4 with 1 N HCl. The product was collected by centrifugation, washed twice with H_2O, and then dried *in vacuo* for 18 hr at 40° to give 142 mg (92.8%) of a yellow powder. Observed m.p. = 225–228° dec.; TLC R_f = 0.46 (5% NH_4HCO_3); UV λ_{max} 206 nm (ϵ 2.727 × 10^4), 246 (ϵ 3.056 × 10^4), 308 (ϵ 1.211 × 10^4), 386 (ϵ 4.072 × 10^3); NMR (Me_2SO-d_6) δ 1.83–2.20 (m, 2, glu β-CH_2), 2.33 (t, 2, glu γ-CH_2, J = 7.37 Hz), 3.15 (s, 3, N^9-CH_3), 4.38 (m, 1, glu α-CH), 4.83 (s, 2, NCH_3C<u>H</u>$_2$), 7.33 (d, 2, 3′, 5′, J_0 = 8.19 Hz), 7.40 (s, 1, H_5), 7.53 (br s, 2, NH_2, exchanges with D_2O), 7.64 (app dd, 1, H_7, $J_{7,8}$ = 9.60 Hz), 8.37 (s, 1, H_2), 8.55 (d, 1, CONH, J = 7.71 Hz, exchanges with D_2O); FAB-MS 436 (M − 1), 438 (M + 1); Elemental analysis ($C_{22}H_{23}N_5O_5 \cdot 1.75\ H_2O$) C,H,N.

2-Desamino-2,9-dimethyl-5,8-dideazaisoaminopterin **(2e).** This compound was prepared by the methylation of **2d** using the previously described procedure to yield 140 mg (68.0%) of a greenish yellow powder. Observed m.p. = 219–224°; TLC R_f = 0.45 (5% NH_4HCO_3); UV λ_{max} 206 nm (ϵ 2.554 × 10^4), 248 (ϵ 3.139 × 10^4), 306 (ϵ 1.297 × 10^4), 390 (ϵ 3.905 × 10^3); NMR (Me_2SO-d_6) δ 1.83–2.18 (m, 2, glu β-CH_2), 2.33 (t, 2, glu γ-CH_2, J = 7.14 Hz), 2.36 (s, 3, C^2-CH_3), 3.06 (s, 3, N^9-CH_3), 4.37 (m, 1, glu α-CH), 4.73 (s, 2, NCH_2), 7.24 (s, 1, H_5), 7.32 (d, 2, 3′, 5′, J_0 = 8.34 Hz), 7.36 (s which becomes d upon D_2O exchange, 1, H_7), 7.43 (d, 1, H_8, $J_{8,7}$ = 9.12 Hz), 7.56 (br s, 2, NH_2, exchanges with D_2O), 7.81 (d, 2, 2′, 6′, J_0 = 8.10 Hz), 8.50 (d, 1, CONH, J = 7.68 Hz, exchanges with D_2O); FAB-MS 452 (M + 1); Elemental analysis ($C_{23}H_{25}N_5O_5 \cdot 0.75\ H_2O$) C,H,N.

2-Desamino-9-methyl-5,8-dideazaisofolic acid **(1c).** This compound was prepared from **1b** in a fashion analogous to that used for **2c.** It was dried for 18 hr *in vacuo* at 40° to yield 61.0 mg (59.2%) of an orange powder. Observed m.p. = 217–219°; TLC R_f = 0.60 (5% NH_4HCO_3); UV λ_{max} 206 nm (ϵ 2.285 × 10^4), 238 (ϵ 2.978 × 10^4), 306 (ϵ 1.548 × 10^4), 370 (ϵ 3.352 × 10^3); NMR (Me_2SO-d_6) δ 1.85–2.15 (m, 2, glu β-CH_2), 2.34 (app t, 2, glu γ-CH_2), 3.15 (s, 3, N^9-CH_3), 4.38 (m, 1, glu α-CH), 4.76 (s, 2, NCH_2), 7.19 (app d, 1, H_5), 7.30 (d, 3, H_7, and 3′, 5′, J_0 = 7.86 Hz), 7.50 (d, 2, H_8, $J_{8,7}$ = 9.00 Hz), 7.81 (app t, 3, H_2, and 2′, 6′), 8.55 (d, 1, CONH, J = 7.71 Hz), 11.94 (br s, 1, lactam NH); FAB-MS 437 (M − 1); Elemental analysis ($C_{22}H_{22}N_4O_6 \cdot 0.38\ H_2O$) C,H,N.

2-Desamino-2,9-dimethyl-5,8-dideazaisofolic acid **(1e).** This compound was obtained from **1d** in a fashion analogous to that used for **2c** to yield 316 mg (61.4%) of the final compound: Observed m.p. = softens at 165°, melts at 185–188°; TLC R_f = 0.63 (5% NH_4HCO_3); UV λ_{max} 230 nm (ϵ 1.708 × 10^4), 246 (ϵ 1.670 × 10^4), 252 (ϵ 1.667 × 10^4), 300 (ϵ 1.608 × 10^4), 372 (ϵ 3.063 × 10^3); NMR (Me_2SO-d_6) δ 1.88–2.03 (m, 2, glu β-CH_2), 2.27 (s, 3, C^2-CH_3), 2.29–2.36 (m, 2, glu γ-CH_2), 3.12 (s, 3, N^9-CH_3), 4.32 (q, 1, glu α-CH, J = 7.02 Hz), 4.72 (s, 2, NHC<u>H</u>$_2$), 7.15 (app d, 1, H_5, $J_{5,7}$ = 2.94 Hz), 7.25–7.31 (m, 3, H_7 and 3′, 5′), 7.41 (d, 1, H_8, $J_{8,7}$ = 9.00 Hz), 7.80 (d, 2, 2′, 6′, J_0 = 8.28 Hz), 8.36 (d, 1, CONH, J = 7.35 Hz); FAB-MS 453 (M + 1); Elemental analysis ($C_{23}H_{24}N_4O_6 \cdot 1.31\ H_2O$) C,H,N: N calculated 11.77, found 12.69.

Biological evaluation

Cytotoxicity and reversal studies. All compounds were evaluated previously against the L1210 murine leukemia cell line. Four compounds that exhibited the most promising activity against L1210 cells (**1d, 1e, 2c** and **2e**) were further evaluated using Colo 320 DM, Hep G2 and HL-60 cell lines. Cell lines were obtained from the American Type Culture Collection, Rockville, MD. Human tumor cells (2,500–10,000) in 0.1 mL of tissue culture medium (RPMI 1640, 10% fetal bovine serum, 1 mM glutamine, 100 μM sodium pyruvate, 200 U/mL penicillin/streptomycin, 25 mM HEPES buffer, pH 7.3) were plated in sterile, flat-bottomed 96-well microculture plates. After incubation for 24 hr (5% CO_2, 37°), an additional 0.1 mL of tissue culture medium containing various concentrations of the test compounds was added to each well. Cytotoxicity was assessed after an additional 5-day incubation period using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described in the literature [17]. Results are expressed as percent reduction in absorbance at 540 nm relative to growth controls cultured in the absence of cytotoxic agents. The means of octuplicate cultures were used in all calculations. Optimal cell numbers and incubation times were empirically determined for each cell line used. Reversal studies were conducted according to methods described previously [11].

Enzyme assays. DHFR was assayed spectrophotometrically at 340 nm in a solution containing 50 μM dihydrofolic acid, 80 μM NADPH, 0.01 M 2-mercaptoethanol, 1.0 mM EDTA and 0.05 M Tris–HCl at pH 7.4 and 30°. The reaction was initiated with an amount of enzyme yielding a change in optical density at 340 nm of 0.015/min. The *Lactobacillus casei* enzyme was 30% pure, while the pure recombinant human enzyme was provided by Dr. J. H. Freisheim [18]. TS was assayed according to the literature procedure [19]. Pure recom-

Table 1. Cytotoxic effects of selected analogues of 5,8-dideazaisofolic acid and 5,8-dideazaisoaminopterin against cultured human cell lines

Compound	IC_{50} (μM)			
	L1210*	Colo 320 DM	Hep G2	HL-60
1b	0.53			
1c	0.21			
1d	0.073	0.47	0.86	0.52
1e	0.060	0.32	0.48	0.22
2b	4.3			
2c	0.044	0.13	0.66	0.043
2d	1.5			
2e	0.18	2.44	15.9	0.23
MTX	0.004	0.048	0.064	0.013

* L1210 IC_{50} values for compounds **1b** and **1d** are from Hagan *et al.* [11]; L1210 IC_{50} values for compounds **1c, 1e, 2b–e** and MTX are from Hynes *et al.* [12].

binant *L. casei* and human enzymes were provided by Dr. D. V. Santi, University of California, San Francisco.

RESULTS AND DISCUSSION

The results presented herein represent the culmination of research efforts begun several years earlier with the intent of gaining a better understanding of quinazoline isofolate structure–activity relationships. The reversed-bridge compound IAHQ **(1a)** was the starting point for these investigations. The enchanced biological activity of **1b** and **1d** over the parent compound [10, 11] prompted the synthesis of other analogues of both IAHQ **(1a)** and 5,8-dideazaisoaminopterin **(2a)**, the 4-amino counterpart of IAHQ. Compounds **1b** and **1d** are, respectively, 2-desamino and 2-desamino-2-methyl analogues of **1a.** Another logical modification, methylation of the N^9-position, is represented by compounds **1c,e** and **2c,e.** Methylation of the bridge portion of classical folate antagonists has led to more desirable *in vivo* activity in some cases, the best example being the incorporation of a methyl group at the N^{10}-position of aminopterin, yielding MTX, a less toxic and more clinically useful therapeutic agent [20].

All study compounds were evaluated against L1210 murine leukemia cells in culture [11, 12]. Compounds exhibiting the most promising activity against this well-established experimental line were subjected to further cytotoxicity tests using human cell lines, a colon carcinoma (Colo 320 DM), a hepatocellular carcinoma (Hep G2) and a promyelocytic leukemia (HL-60) (Table 1). Compounds **1d, 1e,** and **2c** exhibited similar cytotoxicities against L1210 cells, while **2c** was approximately 3- to 4-fold more effective, respectively, than **1d** and **1e** against Colo 320 DM. Analogue **1e** was slightly more cytotoxic towards the hepatocellular carcinoma Hep G2 than either **1d** or **2c,** and **2e** was much less effective on this cultured cell line. The promyelocytic leukemia HL-60 was most sensitive to the cytotoxic effects of **2c,** having an IC_{50} only 3.3-fold greater than MTX. Compounds **1e** and **2e** possessed similar inhibitory potencies toward HL-60, while **1d** was 2.4-fold less effective in inhibiting its growth. Based on the overall results presented in Table 1, the order of cytotoxic potency is **2c > 1e > 1d > 2e.**

Cytotoxicity reversal studies using the HL-60 cell line were carried out with compounds **1d, 1e, 2c, 2e** and MTX, and the results are presented in Table 2. Reversal of cytotoxicity by a particular metabolite indicates that an enzymatic target involved in the biosynthetic pathway of the metabolite is inhibited by the agent in question. Folinic acid (5-formyl-5,6,7,8-tetrahydrofolic acid, leucovorin) should reverse the cytotoxicity of all folate antagonists; this cofactor is a source of reduced folate, rescuing the cells from potentially lethal enzymatic inhibition. Hx reverses cytotoxicity resulting from inhibition of purine biosynthesis, while TdR circumvents a block of thymidylate synthesis.

Folinic acid reversed the cytotoxic effects of all compounds, whereas Hx alone had no effect on cytotoxicity, regardless of the agent in question. TdR completely reversed the cytotoxicities of **1d** and **1e,** but only partially reversed the effects of **2c, 2e** and MTX (see footnote, Table 2). This partial reversal by TdR is most likely due to the inhibition of purine biosynthesis by **2c, 2e** and MTX since Hx plus TdR reversed the cytotoxicities of all three compounds.

All test compounds were evaluated for *in vitro* enzyme inhibition using *L. casei* and human DHFR and TS (Table 3). MTX and 10-propargyl-5,8-dideazafolic acid were included in the evaluation for comparative purposes. The analogues of 5,8-dideazaisoaminopterin **(2b–e)** were more effective inhibitors of DHFR than were the

Table 2. Reversal of the cytotoxicities of selected compounds toward HL-60 cells *in vitro*

Compound	IC_{50} (μM)*†				
	Alone	Folinic acid (100 μM)	Hypoxanthine (50 μM)	Thymidine (20 μM)	Hypoxanthine + thymidine
1d	0.196	>20	0.175	>20	>20
1e	0.107	>20	0.144	>20	>20
2c	0.047	>20	0.035	0.743	>20
2e	0.475	>20	0.352	>20†	>20
MTX	0.009	>20	0.008	0.009	>20

* Higher values (e.g. >20) indicate reversal of cytotoxicity by addition of the indicated agent to the cell cultures. Data shown are from a representative experiment.

† Approximately 49 ± 6.2% inhibition was achieved with **2e** at 20 μM in the presence of added TdR. This value is therefore not statistically different from the extrapolated IC_{50}.

Table 3. Inhibition of dihydrofolate reductase and thymidylate synthase by selected analogues of 5,8-dideazaisofolic acid and 5,8-dideazaisoaminopterin

Compound	Dihydrofolate reductase			Thymidylate synthase		
	L. casei I_{50} (μM)	Human I_{50} (μM)	I_{50}, Human/ I_{50}, *L. casei**	*L. casei* I_{50} (μM)	Human I_{50} (μM)	I_{50}, Human/ I_{50}, *L. casei**
1b	>2.2 [0]†	>11		>90 [30]	>20 [9]	
1c	>2.2 [0]	2.0		1.8	5.0	2.8
1d	>2.2 [0]	>10 [28]		3.6	5.0	1.4
1e	>2.2 [0]	10		3.6	1.5	0.42
2b	1.4	45	32.1	>20 [6]	20	
2c	0.021	0.4	19.0	8.0	5.0	0.63
2d	0.11	90	818	>20 [8]	10	
2e	0.01	4.3	430	>20 [23]	3.6	
MTX/PDDF‡	0.01 (MTX)	0.02 (MTX)	2	0.036 (PDDF)	0.036 (PDDF)	1

* Relative I_{50} values are given for compounds if I_{50} values were within range of the assay.
† Numbers in brackets indicate percent inhibition at the indicated concentration.
‡ PDDF refers to 10-propargyl-5,8-dideazafolic acid, a potent selective inhibitor of TS.

4-oxo analogues (**1b–e**). Compounds **2b–e** also exhibited selective inhibition of *L. casei* DHFR over the human enzyme. Particularly noteworthy in this regard are the 2-desamino-2-methyl compounds **2d** and **2e**, being 818- and 430-fold, respectively, more inhibitory toward bacterial DHFR. For both *L. casei* and human DHFR, addition of a methyl group to the N^9-position enhanced inhibition, the largest effect occurring with the human enzyme where **2c** was 113-fold more inhibitory than **2b**. All compounds were relatively weak inhibitors of *L. casei* and human TS, being at least 50- to 100-fold less effective than 10-propargyl-5,8-dideazafolic acid. It is likely that metabolism to polyglutamate forms enhances their inhibitory potency for TS.

The data presented are consistent with the view that compounds **1b–e** owe their cytotoxicity to inhibition of TS, whereas compounds **2b–e** are directed toward DHFR. Inhibition of TS or DHFR would lead to diminished thymidylate formation. This work illustrates that alterations at the 2- and 9-positions of IAHQ (**1a**) and 5,8-dideazaisoaminopterin (**2a**) can alter potency and selectivity.

Acknowledgements—This investigation was supported, in part, by USAF Surgeon General protocol 93-169 (R.L.H. and M.P.) and National Cancer Institute Grant CA10914 (R.L.K.). The technical assistance of Dr. Michael Walla and Helga Cohen, Department of Chemistry, University of South Carolina, is greatly appreciated. Ruben T. Sabater and Danilo D. Caraballo, Jr., of the Clinical Investigation Facility, David Grant Medical Center, provided expert assistance with the cell culture assays.

REFERENCES

1. Hynes JB, Yang YCS, McGill JE, Harmon SJ and Washtien WL, Improved synthesis and antitumor evaluation of 5,8-dideazaisofolic acid and closely related analogues. *J Med Chem* **27:** 232–235, 1984.
2. Fernandes DJ, Bertino JR and Hynes JB, Biochemical and antitumor effects of 5,8-dideazapteroylglutamate, a unique quinazoline inhibitor of thymidylate synthase. *Cancer Res* **43:** 1117–1123, 1983.
3. Hynes JB, Smith AB and Gale GR, Effects of 5,8-dideazaisopteroylglutamate (IAHQ) on L1210 leukemia in mice when given alone and in combination with methotrexate, probenecid, or verapamil. *Cancer Chemother Pharmacol* **18:** 231–234, 1986.
4. Tsang K-Y, Hynes JB and Fudenberg HH, Effects of 5,8-dideazaisofolic acid in human colon carcinoma cells. *Chemotherapy* **28:** 276–282, 1982.
5. McGuire JJ, Sobrero AF, Hynes JB and Bertino JR, Mechanism of action of 5,8-dideazaisofolic acid and other quinazoline antifols in human colon carcinoma cells. *Cancer Res* **47:** 5975–5981, 1987.
6. Sobrero AF, McGuire JJ and Bertino JR, Uptake and metabolism of 5,8-dideazaisofolic acid in human colon carcinoma cells. *Biochem Pharmacol* **37:** 997–1001, 1988.
7. Pfleiderer W, The solubility of heterocyclic compounds. In: *Physical Methods in Heterocyclic Chemistry* (Ed. Katritzky AR), Vol. 1, pp. 177–188. Academic Press, New York, 1969.
8. Jones TR, Thornton TJ, Flinn A, Jackman AL, Newell DR and Calvert AH, Quinazoline antifolates inhibiting thymidylate synthase: 2-Desamino derivatives with enhanced solubility and potency. *J Med Chem* **32:** 847–852, 1989.
9. Hughes LR, Jackman AL, Oldfield J, Smith RC, Burrows KD, Marsham PR, Bishop JAM, 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.
10. Hynes JB, Patil SA, Hagan RL, Cole A, Kohler W and Freisheim JH, Comparison of the biological effects of selected 5,8-dideazafolate analogues with their 2-desamino counterparts. *J Med Chem* **32:** 852–856, 1989.
11. Hagan RL, Duch DS, Smith GK, Hanlon MH, Shane B, Freisheim JH and Hynes JB, Studies on the mechanism of antitumor action of 2-desamino-2-methyl-5,8-dideazaisofolic acid. *Biochem Pharmacol* **41:** 781–787, 1991.
12. Hynes JB, Hagan RL, Shane B and Freisheim JH, Synthesis and biological evaluation of analogues of 5,8-dideazaisofolic acid (IAHQ) modified at positions 2, 4 and 9. *Adv Exp Med Biol* **338:** 417–420, 1993.
13. Hynes JB, Yang YCS, McCue GH and Benjamin MB, Improved synthetic routes to 5,8-dideazapteroylglutamates amenable to the formation of poly-γ-L-glutamyl derivatives. In: *Folyl and Antifolyl Polyglutamates* (Eds. Goldman ID, Chabner BA and Bertino JR), pp. 101–114. Plenum Press, New York, 1983.
14. Morley JS and Simpson JCE, The chemistry of simple heterocyclic systems. Part I. Reactions of 6- and 7-nitro-4-hydroxyquinazolines and their derivatives. *J Chem Soc* 360–366, 1948[1].

15. Berg SS, A search for new trypanocides. Part VI. Amidinophenyldiazoamino-quinolinium and -quinazolinium salts. *J Chem Soc* 4041–4045, 1961.
16. Berg SS and Parnell EW, Bisquarternary salts related to quinapyramine (antrycide). Replacement of the quinoline nucleus by other heterocycles. *J Chem Soc* 5275–5284, 1961.
17. Alley MC, Scudiero DA, Monks A, Hursey ML, Czerwinske MJ, Fine DL, Abbott BJ, Mayo JG, Shoemaker RH and Boyd MR, Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. *Cancer Res* **48**: 589–601, 1988.
18. Prendergast NJ, Delcamp TJ, Smith PL and Freisheim JH, Expression and site-directed mutagenesis of human dihydrofolate reductase. *Biochemistry* **27**: 3664–3671, 1988.
19. Wahba AJ and Friedkin M, The enzymatic synthesis of thymidylate. I. Early steps in the purification of thymidylate synthetase of *Escherichia coli*. *J Biol Chem* **237**: 3794–3801, 1962.
20. Burchenal JH, Kushida MN, Johnston SF, Cremer MA and Rhoads CP, Clinical experience with the 4-amino derivatives of pteroylglutamic acid and 2,6-diaminopurine in the treatment of neoplastic disease. *Am J Med* **7**: 240, 1949.