Mechanism of Action of 5,8-Dideazaisofolic Acid and Other Quinazoline Antifols in Human Colon Carcinoma Cells

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ABSTRACT

The clonal cytotoxic effects and mechanism of action of a new series of 2-amino-4-hydroxyquinazoline folate analogues (5,8-dideazafolates) have been assessed using the human colon tumor cell line HCT-8. Of these compounds only 5-methyl-5,8-dideazafolate was potentially more effective than a compound previously identified, 5,8-dideazaisofolate (H-338, NSC 289517). HCT-8 sublines resistant to methotrexate, 5-fluorodeoxyuridine, and H-338 were either minimally or not cross-resistant to the other agents. The cytotoxicity of H-338 was strongly dependent on the time of exposure; at exposure times shorter than 8 h it was essentially nontoxic. Thymidine alone, as well as leucovorin or folic acid, protected against the cytotoxic effects of H-338. This is consistent with thymidylate synthase (TS) as its only locus of action. Studies with dihydrofolate reductase and TS isolated from HCT-8 cells indicated that these quinazolines were weaker inhibitors of dihydrofolate reductase than was methotrexate, but they were not particularly potent TS inhibitors. However, synthetic poly-γ-glutamate derivatives of quinazolines showed dramatically increased TS, but not dihydrofolate reductase, inhibition. TS inhibition increased as the polyglutamate chain length increased. Using isolated HCT-8 folyopolyglutamate synthetase, all the parent quinazolines containing γ-glutamate were found to be substrates. With H-338, the results indicated that tetraglutamate or longer derivatives could be synthesized intracellularly. These results are consistent with our hypothesis that cytotoxicity by such quinazolines necessarily involves "lethal synthesis" from a prodrg; i.e., the nontoxic parent drug must be converted to polyglutamates before TS inhibition and subsequent cytotoxicity can occur.

INTRODUCTION

MTX4 is one of the most useful drugs in the treatment of human cancer (1). However, with MTX and other DHFR inhibitors which inhibit both purine and pyrimidine biosynthesis, there is evidence that the inhibition of purine biosynthesis contributes to gastrointestinal toxicity (2) without increasing tumor cell kill (3, 4). Antifols which inhibit pyrimidine biosynthesis alone may therefore have in vivo efficacy superior to that of MTX. In addition, natural and acquired resistance to MTX, which limits its clinical utility, might be overcome by antifols with mechanisms of action that are different from that of MTX and which do not potently inhibit DHFR.

The one folate-dependent enzyme the inhibition of which would induce pyrimidine starvation is TS, which catalyzes the ultimate step in thymidylate biosynthesis. TS is also an ideal target since it may be the rate-limiting enzyme in DNA synthesis (5). The earliest described TS inhibitors, 5-fluorouracil [as its metabolite, 5-fluorodeoxyuridylate (6)] and tetrahydrofolate (7), are not entirely TS specific inasmuch as the former is also incorporated into RNA (6) and the latter also inhibits purine biosynthesis (8). The 2-amino-4-hydroxy quinazolines (5,8-dideazafolates) have been suggested to inhibit TS more than DHFR (9). Recently, two such quinazolines which are specific inhibitors of TS have been described. Jones et al. (10) have rationally designed, synthesized, and extensively investigated the properties of 10-propargyl-5,8-dideazafolate (CB3717). This compound is the tightest binding folate analogue inhibitor of TS (Ki 4–20 nm) yet described (11–13). In a search for TS-specific inhibitors, we have investigated the properties of a series of such 2-amino-4-hydroxyquinazoline analogues of folic acid. Many such quinazolines were inhibitors of TS from Lactobacillus casei and the murine leukemia cell line, L1210 (14). On the basis of cytotoxicity to a human colon tumor cell line (HCT-8) as measured by outgrowth and efficacy in treating a murine colon tumor in vivo (15), one quinazoline, 5,8-dideazaisofolic acid (H-338, NSC 289517), was chosen for further studies. This analogue has an N-9, C-10 in the bridge connecting the heterocycle and benzoylglutamate (an "iso" bridge) rather than the normal C-9, N-10. Detailed studies of H-338 interactions with TS were performed in a heterologous system with LI 210 TS because of difficulties in obtaining HCT-8 thymidylate synthase (15). The conclusion of these studies is that H-338 may be converted intracellularly to polyglutamate forms, similar to those of naturally occurring folates (16), and these polyglutamate forms are specific, potent inhibitors of TS (15). Results from other laboratories are consistent with this hypothesis (17, 18).

We now report further results with H-338 as well as results with new quinazoline analogues which were screened by a clonogenic cytotoxicity assay utilizing HCT-8 cells (19). We have investigated their mechanism of action in HCT-8 cell cultures and in enzyme studies using enzymes derived from these same cells. Data from these homologous systems support our hypothesis that these quinazolines are converted to polyglutamate derivatives intracellularly through the action of cellular folyopolyglutamate synthetase. Quinazoline polyglutamate derivatives inhibit thymidylate synthase which leads to decreased thymidine nucleotide pools, cessation of DNA synthesis, and ultimately to cell death.

MATERIALS AND METHODS

Cells and Cell Culture. HCT-8 cells (20) were cultured as described previously (19). LoVo cells (21), obtained from Dr. B. Drewinko (M. D. Anderson Hospital and Tumor Institute, Houston, TX), and SW-620 cells (22), obtained from Dr. D. Houlton (Scott and White Clinic, Temple, TX), were carried in RPMI 1640 supplemented with 10% fetal calf serum. LoVo and SW-620 cells were subcultured similarly to HCT-8 cells. The doubling times of HCT-8, LoVo, and SW-620 cells were 18, 36, and 24 h, respectively. Cell lines were checked periodically for Mycoplasma contamination and were always negative.

Sublines of HCT-8 resistant to MTX, FdUrd, and H-338 were
selected by stepwise increases in drug concentration. The MTX-resistant subline had a 15-fold increase in DHFR specific activity compared to the parental line. Control mixing experiments demonstrated there was no activator or inhibitor of DHFR present in the resistant line. This DHFR was not altered with respect to its inhibition by MTX, H-338, or H-407. Additional mechanisms of MTX resistance have not been examined. The FdUrd-resistant line (23) is defective in folate transport. 

Enzyme Assays. Folylpolyglutamate synthetase was assayed by a method (24) which depends on the separation of \([^{1}H]glutamate\) ligated onto a folate-type substrate from unreacted \([^{1}H]glutamate\) by chromatography on DEAE-cellulose. Control experiments showed that all quinazolines were retained on the DEAE-cellulose column during the wash to remove unligated \([^{1}H]glutamate\); thus, polyglutamates of these quinazolines would also be retained and measured quantitatively. The following reaction conditions were determined as optimal for use with the enzyme partially purified from HCT-8 cells: 100 mM Tris-Cl, pH 8.85 (25°C); 100 mM 2-mercaptopentanol; 20 mM MgCl2; 10 mM ATP; 35 \(\mu\)M (R,S)-\([^{1}H]H_{4}PteGlu\); 4 mM L-[\(^{3}H\)]H_{4}PteGlu (2 \times 10^{6} \text{cpm}/\mu\text{mol} at 27% counting efficiency); and 20 mM KCl. \(\text{H}_{4}\text{PteGlu}\) was omitted when substrate activity of a quinazoline was tested. One unit of activity indicates the incorporation of 1 pmol \([^{1}H]glutamate\)/h into polyglutamates of \(\text{H}_{4}\text{PteGlu}\) at 37°C.

TS (EC 2.1.1.45) was assayed by the displacement of tritium from [5-\(^{3}H\)]UMP into \(\text{H}_{2}\text{O}\) (25, 26). One unit of TS activity synthesizes 1 nmol thymidylate/min. DHFR (EC 1.5.1.3) was assayed by measuring the decrease in absorbance with time at 340 nm (27) that occurs when NADPH and dihydrofolate are converted to NADP and tetrahydrofolate, respectively. Activity is expressed in IU using a molar extinction coefficient of 12,000 (27). Inhibition of TS or DHFR was measured by adding varying amounts of quinazoline to standard assays. The results (not shown) were virtually identical to those obtained using the human enzymes from three distinct cell lines and appear identical.

RESULTS

Cytotoxicity of Quinazolines to Human Colon Tumor Cell Lines. Based on past results (15), new quinazolines analogues with potential increased cytotoxic effect were synthesized, tested in a clonogenic assay against HCT-8 cells, and compared to the previously described 5,8-dideoxaazafolate (H-338). The results (Table 1) showed that rearrangement in the "bridge" connecting the heterocycle and benzoylglutamate (H-353) was without much effect. The 10-oxa compound (H-375) was only 5- to 10-fold more activity was recovered.
Inhibition of HCT-8-derived TS and DHFR were determined as described in "Materials and Methods." Values presented are averages of at least two determinations.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R₁</th>
<th>Z</th>
<th>R₂</th>
<th>HCT-8 cell ED₅₀ μM</th>
<th>TS ED₅₀ μM</th>
<th>DHFR ED₅₀ μM</th>
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<tr>
<td>H-338</td>
<td>H</td>
<td>NH</td>
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<td>L-Glutamate 6</td>
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<td>H</td>
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<td>NH</td>
<td>L-Glutamate 2</td>
<td>8</td>
<td>0.05</td>
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<tr>
<td>H-375</td>
<td>H</td>
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<td>L-Glutamate 5</td>
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<td>14</td>
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<td>L-Glutamate 8</td>
<td>4</td>
<td>12</td>
</tr>
<tr>
<td>H-379</td>
<td>CH₁</td>
<td>CH₁</td>
<td>NH</td>
<td>L-Glutamate 1.3</td>
<td>16</td>
<td>0.35</td>
</tr>
<tr>
<td>H-407</td>
<td>CH₁</td>
<td>NH</td>
<td>CH₁</td>
<td>L-Glutamate 10</td>
<td>7</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Table 1 Cytotoxic and enzyme-inhibitory effects of quinazoline analogues of folic acid

(H-360) or "iso" compound (H-382) resulted in decreased cytotoxic effects. Methyl substitutions at positions 5 and/or 9 of the "iso" derivative were without effect (H-322, H-412, H-413) as was chlorination at position 5 (H-407). The 5-methyl-substituted normal bridge compound (H-379) showed a slight increase in inhibitory potency. Thus, none of the analogues was dramatically more potent than H-338.

H-338 had similar potency against two other human colon tumor cell lines, SW-620 (ED₅₀ 5.5 μM by clonogenic assay) and LoVo (ED₅₀ 4 μM by outgrowth). Because of technical problems with SW-620 and LoVo (see "Materials and Methods"), either outgrowth or clonogenic assays were used. HCT-8 cells, however, gave similar results in both assays (ED₅₀ 6 μM, clonogenic assay; ED₅₀ 2 μM, outgrowth), indicating the validity of the comparison.

Cytotoxicity of H-338 to HCT-8 Sublines Resistant to Antimetabolites. HCT-8 cell sublines resistant to MTX, fluorodeoxyuridine, and H-338, developed by stepwise increases in drug concentration, were tested for cross-resistance to these same drugs (Table 2). An HCT-8 subline which was 830-fold resistant to MTX by virtue of elevated DHFR was only 12-fold cross-resistant to H-338. An H-338-resistant subline was as sensitive as the parental line to MTX. Both MTX and H-338 were as potent against a 330-fold FUdR-resistant subline as they were against parental HCT-8 cells.

Biochemical Modulation of H-338 Cytotoxicity. H-338 and its polyglutamates interact with L1210 thymidylate synthase in the presence of the nucleotide substrate dUMP (15). Exposure of cells to dUrd might allow expansion of the dUMP pool, increase the binding of quinazoline, and thus potentiate H-338 cytotoxicity. H-338 (6 and 10 μM, giving 12 and 71% cell kill, respectively, after a 72-h exposure) and dUrd (50 and 100 μM) were tested in three schedules: (a) simultaneous presence of H-338 and dUrd; (b) 18 h pretreatment with dUrd, then removal of dUrd and exposure to H-338; and (c) 18 h pretreatment with dUrd, then H-338 added directly to the dUrd-containing medium. No increase in cytotoxicity was noted under any of these conditions. Either dUMP pools were already at saturating levels for TS in these cells or dUMP levels were regulated such that no expansion occurred. dUrd was also unable (11) to potentiate the cytotoxicity of 2-amino-4-hydroxy-10-propargylquinazoline (CB3717)

Time Dependence of H-338 Cytotoxicity. The cytotoxic effect of H-338 was strongly dependent on the time of exposure (Fig. 1). At exposure times of 24 h or less, this drug was only weakly cytotoxic. There was no increase in cell kill noted between a 72-h and continuous (9–10-day) exposure. The exposure times and ED₅₀ values were: 2 h (1000 μM, estimated), 4 h (300 μM, estimated), 8 h (64 μM), 24 h (13 μM), 72 h (5.4 μM), and continuous exposure (5.2 μM).

Protection of HCT-8 Cells against the Cytotoxic Effects of H-338. A determination was made of which folates or folate-dependent metabolites would protect against the cytotoxic effects of H-338 in the clonogenic assay (Fig. 2A). HCT-8 cells were exposed to H-338 in the presence or absence of (a) dThd, (b) hypoxanthine, (c) equimolar combination of dThd and hypoxanthine, or (d) leucovorin. Cytotoxicity of H-338 was completely prevented by either 0.5 μM LV or 0.5 μM dThd alone while hypoxanthine was without effect (Fig. 2A). The combination of dThd and hypoxanthine protected only as well as dThd alone. In a parallel control experiment, the lethal effects of MTX at 75 or 92% cell kill (0.045 and 0.09 μM, respectively, in a 72-h exposure) were completely prevented by 0.5 μM LV and only partially by the highest concentration (50 μM each) of the combination of dThd and hypoxanthine (data not shown). Higher concentrations of dThd were not tested because of dThd toxicity.

The threshold for protection by dThd or LV was determined (Fig. 2B). dThd at 3 × 10⁻⁸ M offered no protection against 10 μM H-338 (93% cell kill), but protection was concentration dependent.

Table 2 Drug sensitivities of H-338 cells resistant to H-338, methotrexate, or FdUrd

<table>
<thead>
<tr>
<th>HCT-8 cell line</th>
<th>MTX ED₅₀ (μM)</th>
<th>FdUrd ED₅₀ (μM)</th>
<th>H-338 ED₅₀ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitive*</td>
<td>0.024</td>
<td>0.003</td>
<td>6</td>
</tr>
<tr>
<td>MTX-R²</td>
<td>0.02</td>
<td>ND</td>
<td>70</td>
</tr>
<tr>
<td>H-338(R)</td>
<td>0.017</td>
<td>ND</td>
<td>90</td>
</tr>
<tr>
<td>FdUrd(R)</td>
<td>0.02</td>
<td>1.0</td>
<td>3.4</td>
</tr>
</tbody>
</table>

* Measured by clonogenic assay, but results in outgrowth are comparable.
* Measured by outgrowth. This line does not clone.
* ND, not determined.
* Measured by clonogenic assay.
* Measured by outgrowth because this line clones poorly.

Fig. 1. Time dependence of H-338 cytotoxicity to HCT-8 cells. HCT-8 cells were exposed to the indicated concentrations of H-338 for the times designated. Percentage of survival was determined by the clonogenic assay ("Materials and Methods"). Bars, SD.
QUINAZOLINE ANTIFOLS AND HUMAN COLON CANCER CELLS

Inhibition of HCT-8 Thymidylate Synthase by Polyglutamate Derivatives of Quinazolines.

Since DHFR activity was relatively low in HCT-8 cells, many quinazolines were screened against DHFR isolated from the MTX-resistant HCT-8 line, which shows a 15-fold increase in DHFR activity. This DHFR displayed the same sensitivity to MTX, H-338, and H-407 as the DHFR of the parent line and thus was not altered, only elevated. The DHFR inhibition studies (Table 1) showed that the 2-amino-2-hydroxyquinazoline monoglutamates were generally far less potent than MTX ($I_{50} 1.3 \times 10^{-9} \text{M}$) or a 2,4-diaminoquinazoline (5-methyl-5,8-dideazaaminopterin; $I_{50} 6.5 \times 10^{-10} \text{M}$). For those 2-amino-4-hydroxy compounds where polyglutamates were available, these derivatives were no more potent inhibitors of DHFR than the monoglutamates. Again the lack of detectable $\gamma$-glutamyl hydrolase activity and the short time of assay indicate that this enzyme was not a factor in this result.

Quinazolines as Substrates for HCT-8 Cell Foly polyglutamate Synthetase. All of the quinazolines tested were substrates for FPGS, but their relative efficiencies varied (Fig. 3). The normal (C-9, N-10) bridge compound H-353 as well as its 5-methyl-substituted analogue H-379 (Fig. 3B) and the 5-methyl-substituted isoquinazoline H-322 (Fig. 3A) gave substantially better activity in the low concentration region (<10 $\mu$M) which might be anticipated to be achieved intracellularly. The 5-chloroisoquinazoline (H-407) and the 5,9-dimethylisoquinazoline (H-413) showed lower maximal activity than H-338. They also were significantly more active in the low concentration region ($\leq 10 \mu$M) which might be anticipated to be achieved intracellularly. The 5-chloroisoquinazoline (H-407) and the 5,9-dimethylisoquinazoline (H-413) showed lower maximal activity than H-338. They also showed better activity in the low concentration range. Substitution by a formyl group at either N-10 in the normal bridge compound (H-360) or N-9 in the isoquinazoline (H-382) caused a decrease in FPGS substrate activity relative to the parent. This effect may be specific for formyl substituents because the 9-methylisoquinazoline (H-412) gave much better activity than the 9-formyl analogue. Interestingly, the dimethyl-substituted compound (H-413), although less active than either of its monosubstituted analogues, still exhibited better activity than the 9-formyl isoquinazoline. The substitution of oxygen for nitrogen at position 10 (H-375) causes a fall in substrate activity over the entire concentration range tested compared to the parent. Although an insufficient quantity of the analogue of H-338 containing D-glutamate was available to test for substrate activity, it should not be a substrate (24).

The above analysis was based on the shape and height of the concentration dependence curves (Fig. 3). This qualitative approach was utilized because the synthesis of multiple polyglutamate products under some conditions means that Michaelis-Menten conditions are not maintained (24). Thus, such data cannot be used to generate Michaelis-Menten kinetic constants.

Inhibition of HCT-8 Dihydrofolate Reductase by Quinazolines. Since DHFR activity was relatively low in HCT-8 cells, many quinazolines were screened against DHFR isolated from the MTX-resistant HCT-8 line, which shows a 15-fold increase in DHFR activity. This DHFR displayed the same sensitivity to MTX, H-338, and H-407 as the DHFR of the parent line and thus was not altered, only elevated. The DHFR inhibition studies (Table 1) showed that the 2-amino-2-hydroxyquinazoline monoglutamates were generally far less potent than MTX ($I_{50} 1.3 \times 10^{-9} \text{M}$) or a 2,4-diaminoquinazoline (5-methyl-5,8-dideazaaminopterin; $I_{50} 6.5 \times 10^{-10} \text{M}$). For those 2-amino-4-hydroxy compounds where polyglutamates were available, these derivatives were no more potent inhibitors of DHFR than the monoglutamates. Again the lack of detectable $\gamma$-glutamyl hydrolase activity and the short time of assay indicate that this enzyme was not a factor in this result.

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**Table 3 Inhibition of HCT-8 thymidylate synthase by polyglutamate derivatives of antifol**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$I_{50}$ (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-353</td>
<td>8</td>
</tr>
<tr>
<td>H-353(G1)</td>
<td>0.3</td>
</tr>
<tr>
<td>H-338</td>
<td>8</td>
</tr>
<tr>
<td>H-338(G1)</td>
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</tr>
<tr>
<td>H-338(G2)</td>
<td>0.25</td>
</tr>
<tr>
<td>MTX</td>
<td>70</td>
</tr>
<tr>
<td>MTX(G1)</td>
<td>2</td>
</tr>
<tr>
<td>MTX(G2)</td>
<td>0.3</td>
</tr>
</tbody>
</table>

* The subscript appended to the glutamate (G) in parentheses indicates the number of glutamates added to the parent drug, which already contains one glutamate.
unless it is verified with each quinazoline that only a single polyglutamate product is observed at both low and high substrate concentrations.

The lengths of polyglutamate products which could be synthesized from H-338 by HCT-8 FPGS was assessed by measuring substrate activity of synthetic H-338 polyglutamates (Table 4). Substrate activity increased as a function of concentration between 2 and 50 μM for each substrate tested. There was, however, a decrease in activity as the length of the initial substrate increased. The substrate activity of H-338(G2) indicates that products as long as H-338 (G3), a total of tetraglutamate, and perhaps longer could be synthesized in HCT-8 cells. The lack of demonstrable γ-glutamyl hydrolase (conjunctive) activity under the assay conditions confirms that this activity is genuine and not the result of hydrolysis of the synthetic polyglutamates and subsequent resynthesis.

**DISCUSSION**

The ability of several quinazolines to inhibit outgrowth in vitro of the HCT-8 human colon carcinoma cell line has been tested (15). However, theoretical and experimental considerations (38) indicate that inhibition of colony formation is the most significant end point for measuring cytotoxic effects. Therefore, a monolayer clonal growth assay (19) was used in the present studies. The relative potency of quinazolines tested by both methods was the same but, contrary to expectation, the ED50 values were somewhat higher in the clonal growth assays. A similar close correspondence between ED50 values for MTX under a variety of treatment schedules using outgrowth and clonal assays has been noted with CCRF-CEM human leukemia cells. Thus, the advantages of the clonal growth assay (38) may not pertain to the assay of the cytotoxicity of antifols in general or perhaps all antimitabolics.

The data on growth inhibition (Table 1) illustrate new structure-activity relationships for the 2-amino-4-hydroxy-quinazolines. Of the new compounds tested, only H-379 displayed cytotoxicity that might be significantly greater than H-338 and thus would bear further investigation. Other structural changes caused no change in cytotoxicity or were deleterious. The “iso” compound containing D- rather than L-glutamate could be much less effective because its transport is decreased, as is the case for 2,4-diaminoquinazolines containing d-glutamate (39) because it is a poor TS inhibitor. However, it may also be that the D-glutamate-containing 2-amino-4-hydroxyquinazoline is transported but, since it would not be a substrate for FPGS (24), the polyglutamate derivatives essential for cytotoxicity are never synthesized.

The relatively high ED50 values for the 2-amino-4-hydroxyquinazolines were not the result of intrinsic resistance of HCT-8 cells to antifols in general or quinazolines in particular. MTX (ED50 0.024 μM; see Table 3) and 5-methyl-5,8-dideazafolic acid (ED50 < 0.01 μM; data not shown) are both highly potent against this model tumor in vitro. Although the 2-amino-4-hydroxy compounds are not especially potent, their efficacy in vivo against certain tumors may be much higher than that of MTX as indicated by earlier studies (15) showing that optimal doses of H-338, but not MTX, delayed the growth of colon tumor 38 in mice and resulted in 6 of 20 long term survivors.

The isooquinazoline analogue of folic acid (H-338) is equally cytotoxic against several human colon tumors. LoVo, like HCT-8, is a well differentiated adenocarcinoma (21) while SW-620 is poorly differentiated (22). Thus, this class of quinazolines might be useful in treatment of different forms of colorectal cancer.

The 2-amino-4-hydroxy-quinazoline monoglutamates were more potent inhibitors of TS than was MTX, and they were much less inhibitory toward DHFR than was MTX (Table 1). The inhibitory power of these compounds against TS or DHFR did not, however, correlate with their potency against the HCT-8 cell line in vitro (Table 1). Presumably these differences in cytotoxicity are related either to transport or to ease of polyglutamate derivative formation.

Inhibition of TS by the quinazolines increased dramatically as the length of the polyglutamate chain increased (Table 3).

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Figure 3. Substrate activity of quinazolines with HCT-8 folypolyglutamate synthetase. Substrate activity was measured as described in “Materials and Methods.” Activity is presented as a percentage of activity obtained with 10 μM H-338 which was included as an internal control in each experiment. Points are the averages of two separate experiments with each condition done in duplicate (i.e., n = 4). Bars, SD. The pattern observed with H-360 was the same as with H-338, that observed with H-413 was similar to that of H-407, that observed with H-412 was similar to that of H-375, and that observed with H-379 was similar to that of H-353.

**Table 4** Substrate activity of H-338 and its polyglutamates for HCT-8 folypolyglutamate synthetase

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Concentration (μM)</th>
<th>[3H]Glu incorporated/3 h (pmol)</th>
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<td>H-338</td>
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<td>50</td>
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<tr>
<td>H-338(G2)</td>
<td>2</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>130</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>224</td>
</tr>
</tbody>
</table>
This increased potency mirrors the fact that polyglutamate derivatives of the folate substrate for TS, methylenetetrahydrofolate, have $K_m$ values which generally decrease sharply with increasing chain length (reviewed in Ref. 16). Since our data indicate (Table 4) that HCT-8 FPGS can synthesize polyglutamates at least as long as H-338(G3), the inhibitor form which occurs intracellularly may be even more potent than those tested (Table 3).

All quinazolines containing L-glutamate would be polyglutamylated intracellularly (Fig. 3) producing metabolites more inhibitory to TS, and presumably more cytotoxic. There was, however, no correlation between the FPGS substrate activity of a quinazoline and its cytotoxicity. This series of quinazolines does offer insight into the folate substrate specificity of human FPGS which may be useful in designing new compounds with enhanced ability to form these cytotoxic metabolites. For example, a comparison of the structure-substrate activity profiles for the quinazolines tested indicates that 5-methyl substitution (H-322, H-379) is the best choice to enhance or maintain substrate activity relative to H-338. The 5-methylquinazoline H-379 is also a good substrate (and a better substrate than H-338) for rat liver FPGS. This observation is of interest because the natural folate substrate 5-methyltetrahydrofolate is a much poorer substrate than is H-338 for rat liver FPGS (24). In addition, 5-methyltetrahydrofolate can be converted only to a diglutamate product (42), while H-379 is readily converted to a tetraglutamate. These observations indicate that both the type of substitution and the heterocycle may dramatically alter FPGS substrate properties. Extrapolations between classes of antifols may thus be misleading.

The cytotoxic potency of the 2-amino-4-hydroxyquinazolines against the HCT-8 cell line thus appeared to be a cumulative function of three factors: transport; FPGS substrate activity; and TS inhibition by the parent quinazoline and its polyglutamate derivatives. Any worthwhile change in the quinazoline structure must, therefore, maximize these three factors or produce such a great positive change in one or two that it offsets the deleterious effects on the remaining factor(s).

Long exposure to H-338 was required in order to achieve cytotoxicity at physiologically achievable concentrations. The data did not differentiate between the possibilities that long exposure was required because H-338 was poorly transported or that, as we hypothesize, H-338 must be polyglutamylated before it becomes cytotoxic. However, it is clear from these cell culture results that tumor response in vivo is most likely following sustained exposure to high concentration of this drug.

Part of the rationale for developing quinazoline antifols is that they might be useful in treating drug-resistant tumors, especially those resistant to MTX (40) or to the class of drug most widely used in the treatment of colon cancer, fluoropyrimidines. Using HCT-8 sublines resistant to MTX, or fluorodeoxyuridine, or H-338 we found little evidence of cross-resistance between these antimetabolites. These results support our hypothesis that H-338 has a mechanism of action different from that of MTX and also suggest that H-338 might be a useful treatment for tumors with intrinsic or acquired resistance to MTX or fluorouracil. The slight cross-resistance of the MTX-resistant line to H-338 may be explainable by the binding of intracellular H-338 to the elevated DHFR (15) making it less available for TS inhibition. This cross-resistance to H-338 of cell lines which are MTX resistant by virtue of elevated DHFR has also been observed with two resistant murine cell lines, L1210 and L5178Y, and a human leukemia line, CCRF-CEM (43). Similar low level cross-resistance to CB3717 by MTX-resistant lines containing elevated DHFR has been observed (10, 13).

The results of the metabolite protection experiments with H-338 indicated that this agent acted mainly as an inhibitor of thymidylate synthase. However, the medium in which HCT-8 cells grow (RPMI 1640) contains all other folate-derived metabolites (glycine, serine, and methionine) in amounts adequate for normal growth, so effects of antifols on their biosynthesis would not be assessed. Supplementation of the medium with hypoxanthine did not affect H-338 cytotoxicity (Fig. 2A). Purines enter these cells, however, because purines participate in protection against MTX cytotoxicity (data not shown). Complete protection by dThd is specific and not a general pyrimidine effect since equivalent concentrations of dUrd did not affect cytotoxicity (see “Results” on metabolic modulation). Specific protection by dThd is consistent only with specific inhibition of TS. The reason that LV is able to protect against H-338 cytotoxicity is less apparent. Simple expansion of the folate pool might provide more methylenetetrahydrofolates, the TS cofactors, but methylenetetrahydrofolate itself is noncompetitive with respect to both H-338 and its triglutamate metabolite [see above (15)]. It might be, however, that the polyglutamate derivatives of methylenetetrahydrofolate are competitive rather than noncompetitive with H-338 and an increase in their concentration might relieve H-338 inhibition in that case. Such changes in mode of inhibition between mono- and polyglutamates are known to occur with thymidylate synthase (44). An alternate explanation is that H-338 is transported with low affinity by a high affinity leucovorin transport system and leucovorin effectively blocks H-338 transport. A third explanation is that LV is effectively transported while H-338 is not and the resulting high reduced folate concentrations competitively inhibit H-338 polyglutamate formation. If these derivatives are essential, as we postulate, inhibition of their formation would decrease cell kill. We are currently investigating the latter two possibilities using radiolabeled H-338. The protection afforded by folic acid could be for one of the same reasons as for LV. The higher levels required are consistent with the generally poor transport of folic acid and its slow reduction by DHFR. The protection by relatively low concentrations of folic acid illustrates clearly that DHFR was not appreciably inhibited under these circumstances.

Our results clearly show that the 2-amino-4-hydroxyquinazolines exert their cytotoxicity through specific inhibition of thymidylate synthase. The relatively high $I_{50}$ values for the monoglutamate against HCT-8 thymidylate synthase, the marked increase in inhibitory potency of synthetic polyglutamate derivatives, and the FPGS substrate activity of all L-glutamate-containing compounds are consistent with our hypothesis that polyglutamates are the actual intracellular thymidylate synthase inhibitors and are responsible for cell kill. The potential importance of polyglutamates of CB 3717 (a related quinazoline) in cytotoxicity has also been suggested (10–13, 41). Our results also point out the pitfalls of using enzyme data alone to screen new antifols containing a glutamate moiety, especially those active against enzymes other than DHFR. A monoglutamate may be nearly inactive as an enzyme inhibitor, but the synthesis of polyglutamates intracellularly may form extremely potent inhibitors leading to cytotoxicity.

* J. J. McGuire, unpublished observation.

* D. J. Fernandes and J. R. Bertino, unpublished observations.
Enzyme screening using only monoglutamate forms would thus be misleading.

ACKNOWLEDGMENTS

The authors wish to thank Pearl Hsieh for skillful technical assistance and Dawne Newcombe and Mae Brown for assistance in preparing this manuscript.

REFERENCES


Mechanism of Action of 5,8-Dideazaisofolic Acid and Other Quinazoline Antifols in Human Colon Carcinoma Cells


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