A Novel Class of Monoglutamated Antifolates Exhibits Tight-binding Inhibition of Human Glycinamide Ribonucleotide Formyltransferase and Potent Activity against Solid Tumors1

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ABSTRACT

Tight-binding inhibition of recombinant human monofunctional glycaminide ribonucleotide formyltransferase by Lometrexol (6R,5,10-dideza-tetrahydrofroloate) requires polyglutamation. LY254155 and LY222306 differ from 5,10-dideza-tetrahydrofroloate in the replacement of the 1',4'-phynyl moiety by a 2',5'-thiophene and a 2',5'-furan, respectively. Compared to Lometrexol, the thiophene and furan analogues had 25- and 75-fold greater inhibitory potencies against human monofunctional glycaminide ribonucleotide formyltransferase (Ki = 2.1 and 0.77 nM, respectively). The binding affinities of the thiophene and furan analogues for membrane folate-binding protein from human KB cells were 6- and 50-fold weaker than Lometrexol, respectively. Both the thiophene analogue and 5,10-dideza-tetrahydrofroloate inhibited the in vivo growth of murine 6C3HED lymphosarcoma, murine C3H mammary carcinoma, and human xenograft HTXGC3, HC1, and VRCS colon carcinomas by 95-100%. The thiophene analogue was efficacious against human xenograft PANCl, a pancreatic carcinoma which was completely resistant to 5,10-dideza-tetrahydrofroloate. These novel antifolates represent the first monoglutamated tight-binding inhibitors of glycaminide ribonucleotide formyltransferase. By eliminating the need for polyglutamation, this class of antifolates may have clinical activity in the treatment of solid tumors expressing low levels of folylpolyglutamate synthetase or tumors resistant to antifolate therapy due to increased y-glutamyl hydrolase activity.

INTRODUCTION

GARFT is the first of two folate-dependent enzymes in the de novo purine biosynthetic pathway. Because of its role in de novo synthesis, it has become a target for cancer chemotherapy. Initial characterization of the mammalian enzyme was performed using GARFT purified from murine cell lines (1, 2). In humans, as in mice, GARFT activity resides at the carboxy-terminal region of a trifunctional protein which also has glycaminide ribonucleotide synthetase and aminoimidazole ribonucleotide synthetase activity (3). Using the deduced amino acid sequence from Escherichia coli, where GARFT was found as a single, independent activity (4), the human monofunctional GARFT domain was cloned and expressed in E. coli (5).

In the mid-1980s, the first selective inhibitor of GARFT, DDATHF, was synthesized (5, 6). The 6R diastereomer of DDATHF, Lometrexol, had in vivo activity against several solid murine and human xenograft tumors (7, 8). In contrast, methotrexate, an antifolate used clinically today, had little or no activity in this same panel. In addition to its inhibitory activity against GARFT, the antitumor activity of Lometrexol has been attributed to its transport properties (9) and its ability to serve as an excellent substrate for FPGS (2, 10). Many variations of DDATHF have been synthesized in an effort to determine the structural requirements which contribute to potent GARFT inhibition and in vivo antitumor activity (11, 12).

We report the initial characterization of monoglutamated analogues of DDATHF which exhibited tight-binding inhibition of recombinant hGARFT. One of these novel antifolates, the thiophene analogue LY254155, was also active against a broad panel of murine and human xenograft solid tumors.

MATERIALS AND METHODS

Materials. Recombinant hGARFT was obtained from Agouron Pharmaceuticals, Inc., San Diego, CA. 10F-DDAF, a,β-GAR, Lometrexol, LY243246, LY254155, LY309886, LY309887, LY222306, and their polyglutamated derivatives were synthesized at Lilly Research Laboratories, Indianapolis, IN. Dialyzed FBS was purchased from Hyclone, Logan, UT. RPMI 1640 was purchased from Whittaker Bioproducts, Walkersville, MD. Bio Rad protein assay dye reagent concentrate was obtained from Bio-Rad Laboratories, Melville, NY. 3H-labeled folic acid (histamine derivative; NEX-114) was acquired from Dupont NEN, Boston, MA. All additional chemicals were purchased from Sigma Chemical Company, St. Louis, MO. Mice were supplied from Charles River Laboratories, Portage, MI. The ENZFITTER microcomputer package was obtained from Biosoft, Ferguson, MO. JMP software was purchased from SAS Institute, Inc., Cary, NC.

Murine and Human Tumors. Murine C3H mammary carcinoma was obtained from The Jackson Laboratory, Bar Harbor, ME. Murine CA-755 adenocarcinoma, murine M-5 ovarian carcinoma, and human LX-1 lung carcinoma were obtained from the NCI, Bethesda, MD. Murine 6C3HED lymphosarcoma was obtained from EG&G Mason Research, Worcester, MA. Human colon carcinomas (HTXGC3, HC1, and VRCS) were obtained from Drs. Peter Houghton and Janet Houghton, St. Jude’s Children Research Hospital, Memphis, TN. Human PANC-1 pancreatic carcinoma and human KB epidermoid carcinoma cells were obtained from the American Type Culture Collection, Rockville, MD. Human CCRF-CEM lymphoblastic leukemia cells were obtained from St. Jude’s Children Research Hospital, Memphis, TN.

Dissolution of Folate Analogues. Stock solutions of Lometrexol were prepared directly in assay buffer which differed with type of assay. All other folate analogues were dissolved in 100% DMSO at 10, 0, or 1 µm and subsequently diluted into assay buffer. The final DMSO concentration never exceeded 0.5%. Vehicle controls confirmed that there was no effect of DMSO at this concentration.

Determination of Km and Michaelis Constants for hGARFT and Substrates. Initial rates of reaction were determined by monitoring the increase of absorbance resulting from formation of the product, 5,8-dideazafolate (ε = 18.9 mM-1 cm-1), at 295 nm using a Beckman DU 640 spectrophotometer. The assay was performed in 75 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.5), 18% glycerol, 45 mM KC1, and 40 mM a-thioglycerol at 25°C in a 0.5-ml volume. 10F-DDAF concentrations were determined spectrophotometrically (ε254 = 23.5 µM cm-1). The Km for 10F-DDAF (n = 12) was determined using four concentrations of 10F-DDAF in the presence of 10 µM a,β-GAR (β = 66%) and 10 µM hGARFT. Vmax for hGARFT and Km for...
\(\alpha,\beta\)-GAR (\(n = 7\)) were determined using six concentrations of \(\alpha,\beta\)-GAR in the presence of 20 \(\mu\)M 10F-DDAF and 10 \(\mathrm{nm}\) hGARFT. \(K_{\text{app}}\) and \(V_{\text{max}}\) values were calculated by the Enzyme Mechanism Analysis program of the Beckman DU 640 spectrophotometer, which uses a nonlinear least squares curve fitting algorithm (13).

**In Vitro Cytotoxicity and Reversal Assays.** Log-phase cultures of human leukemia CCRF-CEM cells (4.8 \(\times\) 10^6/well) were seeded in 24-well plates (Cambridge, MA). The cells were incubated for 72 h (37°C, 5% CO\(_2\) in air) and without combinations of 0 to 10 \(\mu\)M test compound, 300 \(\mu\)M AICA, 5 \(\mu\)M thymidine, and 100 \(\mu\)M hypoxanthine. After incubation, the number of cells was determined using a ZBI Coulter Counter, and IC\(_{50}\) for hGARFT and Michaelis constants for the substrates \(\alpha,\beta\)-GAR and 10F-DDAF were determined. The \(K_{\text{app}}\) and hGARFT were 20 \(\mu\)M and 10 \(\mathrm{nm}\), respectively. Initial estimates of \(K_{\text{app}}\) were determined using the Henderson equation (14), and final \(K_{\text{app}}\) values were determined by nonlinearly fitting the data to the Morrison equation (15) using the ENZFITTER microcomputer package.

**Kinetic Analysis of Tight-binding Inhibition of hGARFT.** Uninhibited and inhibited initial rates of reaction were determined by monitoring the increase of absorbance at 295 nm resulting from formation of the product, 5,8-dideazafolate \((\epsilon = 18.9 \, \text{mM}^{-1}\text{cm}^{-1})\), using a Beckman DU 640 spectrophotometer. Assays were performed in 75 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid \((\text{pH} 7.5)\), 18% glycerol, 45 mM KCl, and 40 mM \(\alpha\)-thioglycerol at 25°C in 0.5 ml. Initial velocity was assessed at seven concentrations of folate analogue and three concentrations of 10F-DDAF. Each 10F-DDAF concentration was determined spectrophotometrically \((\epsilon_{254} = 23.5 \, \text{mM}^{-1}\text{cm}^{-1})\). The concentrations of \(\alpha,\beta\)-GAR and hGARFT were 20 \(\mu\)M and 10 \(\mathrm{nm}\), respectively. Initial estimates of \(K_{\text{app}}\) were determined using the Henderson equation (14), and final \(K_{\text{app}}\) values were determined by nonlinearly fitting the data to the Morrison equation (15) using the ENZFITTER microcomputer package. 

\[ \text{Ki} = \frac{(IC_{50}/1 + [L]/K_s)}{K_s} \]

with \(1/K_s\) equal to the competition of inhibitor which decreased folate acid binding by 50%, \(L\) equal to the concentration of folate acid, and \(K_s\) equal to the equilibrium dissociation constant for folate acid.

**In Vivo Inhibition of Tumor Growth in Mice.** Malignant tumors were evaluated in the following strains of female mice: 6C3HED lymphosarcoma and C3H mammary carcinoma in C3H mice and CA-755 adenocarcinoma and M-S ovarian carcinoma in C57BL/6 mice. All human xenograft tumors were assessed in female CD-1 nude mice. Within an experiment, 10 mice were inoculated at each dosing level. Tumors were implanted s.c. (18) 1, 5, or 7 days prior to dosing of compound, depending on tumor type. Dosing schedules varied within and between tumor types and were chosen to optimize antitumor efficacy while minimizing toxicity. Compounds were administered i.p. in units of mg of compound per kg of mouse weight. At the end of treatment, tumor weight was estimated using an electronic caliper interfaced to a microcomputer (19), and average tumor weight was calculated for each dosing level and a no-compound control group. Percentage inhibition of tumor growth was calculated as

\[ \frac{\% \text{ inhibition of tumor growth}}{100} = \frac{1 - \frac{\text{average tumor weight experimental group}}{\text{average tumor weight control group}}}{100} \]

Percentage inhibition was not calculated at a particular dosing level if mortality exceeded 20%. Cumulative dose was calculated by multiplying the daily dose by the number of days doses were administered. Using 6C3HED lymphosarcoma-bearing C3H mice on a 1-, 4-, and 7-day dosing regimen, a therapeutic index was calculated for the thiophene analogue by dividing the dose which caused 50% mortality by the dose which inhibited tumor growth by 50%.

**RESULTS**

**Kinetic Parameters of Recombinant hGARFT.** The \(V_{\text{max}}\) for hGARFT and Michaelis constants for the substrates \(\alpha,\beta\)-GAR and 10F-DDAF were determined. The \(K_{\text{app}}\) for \(\alpha,\beta\)-GAR (4.4 \(\pm\) 0.7 \(\mu\)M; SEM) was determined using 20 \(\mu\)M 10F-DDAF and six concentrations of \(\alpha,\beta\)-GAR (\(n = 7\)). The \(V_{\text{max}}\) calculated using the above conditions was 26.4 \(\pm\) 1.6 \(\mu\)M/min/mg (\(n = 7\)). The \(K_{\text{app}}\) for 10F-DDAF (0.74 \(\pm\) 0.06 \(\mu\)M; \(n = 12\)) was determined using 10 \(\mu\)M \(\alpha,\beta\)-GAR and four concentrations of 10F-DDAF. The concentration of \(\alpha,\beta\)-GAR was chosen to avoid substrate inhibition which occurs at \(\alpha,\beta\)-GAR concentrations greater than 20 \(\mu\)M (11).

**In Vitro Inhibition of Human Leukemic Cell Growth.** Like DDATHF (20), the thiophene analogue consists of two diastereomers which differ in chirality at carbon 6 (Fig. 1). The growth of human CCRF-CEM leukemia cells was potently inhibited by the thiophene analogue \((IC_{50} = 2.3 \, \text{nm} \, n = 1)\) and its diastereomers. Diastereomer A had an IC\(_{50}\) against CCRF-CEM cells of 1.3 \(\text{nm} \, n = 1)\) and diastereomer B had an IC\(_{50}\) of 2.9 \(\text{nm} \, n = 1)\). The IC\(_{50}\) of Lometrexol and the furan analogue were 15.2 \(\pm\) 2.6 \(\text{nm} \, n = 3)\) and 27.1 \(\pm\) 2.7 \(\text{nm} \, n = 4)\), respectively.

**In Vitro Reversal of Growth Inhibition.** Hypoxanthine (100 \(\mu\)M), a purine salvage metabolite, completely protected CCRF-CEM cells from the cytotoxicity of both diastereomers of the thiophene analogue, while 5 \(\mu\)M thymidine alone did not (Fig. 2). Hypoxanthine and thymidine combined had the same effect as hypoxanthine alone. Therefore, as seen with the racemic thiophene analogue (21) and diastereomer B were more inhibitory to cell growth than a purine salvage metabolite, completely protected CCRF-CEM cells from the cytotoxicity of both diastereomers of the thiophene analogue, while 5 \(\mu\)M thymidine alone did not (Fig. 2). Hypoxanthine and thymidine combined had the same effect as hypoxanthine alone. Therefore, as seen with the racemic thiophene analogue (21) and diastereomer B, diastereomer A and B inhibited de novo purine biosynthesis. AICARFT, the second folate-dependent enzyme of de novo purine synthesis, catalyzes the conversion of AICAR to formamidomimidazole-carboxamide ribonucleotide. AICA can be converted to AICAR by the salvage pathway enzyme phosphoribosyltransferase. Differences between the reversal of growth inhibition by AICA were observed for diastereomers A and B. In the presence of 300 \(\mu\)M AICA and concentrations of diastereomer A greater than 15 \(\mu\)M, CCRF-CEM cells proliferated to 75% of the level of the no-inhibitor control culture (Fig. 2, top). However, at identical concentrations of diastereomer B and AICA, CCRF-CEM cells proliferated to 50% of the no-inhibitor control level (Fig. 2, bottom). Thus, high concentrations of diastereomer B were more inhibitory to cell growth than diastereomer A, indicating that diastereomer B was a less pure inhibitor of hGARFT than A, perhaps due to greater inhibition of AICARFT.

**Wide-binding Inhibition of Monofunctional hGARFT by the Thiophene Analogue.** Initial estimates of the inhibitory potencies of antifolates to hGARFT using steady-state Michaelis-Menten kinetics
(results not shown) indicated that the monoglutamated thiophene analogue was a tight-binding inhibitor with a $K_i$ in the low nm range. Under the conditions used to generate velocity versus inhibitor concentration curves ($10 \text{ nM hGARFT}$ and 25 to 400 nm thiophene analogue), it became apparent that enzyme-bound inhibitor would be a significant fraction of the total inhibitor concentration. Therefore, analysis of the kinetic data using the Michaelis-Menten equation was not appropriate (22, 23). For this reason, data was nonlinearly fit to the Morrison equation (15) using the ENZFITTER microcomputer package (Fig. 3A). The Morrison equation does not assume that enzyme-bound inhibitor is a negligible fraction of the total inhibitor concentration. Using a fixed enzyme concentration of 10 nm, the ENZFITTER program was able to iteratively solve for $K_{i\text{app}}$ using least squares regression analysis. $K_i$ was then determined graphically (Fig. 3B) using the relationship

$$K_i = \left(\frac{K_{i\text{app}}}{1 + S/K_m}\right)$$

with $S$ equal to the concentration of 10F-DDAF and $K_m$ equal to the Michaelis constant for 10F-DDAF (0.74 $\mu$m). Using this analysis, 5 independent determinations yielded a $K_i$ value against hGARFT of $2.1 \pm 0.2$ nm for the thiophene analogue (Table 1).

Affinity of Lometrexol, Its Polyglutamates, and Analogues for hGARFT and mFBP. $K_i$ values for inhibition of hGARFT and dissociation binding constants to mFBP from KB membranes were generated for Lometrexol, its polyglutamates, and analogues (Table 1).

The polyglutamates of Lometrexol bound to recombinant human monofunctional GARFT with increasing affinity as the length of the polyglutamate chain increased. The pentaglutamate of Lometrexol demonstrated a 6-fold decrease in affinity for mFBP ($K_i = 1.7 \text{ nM}$) compared to Lometrexol. The diastereomers of the thiophene analogue had small but statistically significantly ($P < 0.02$) different affinities for hGARFT (16).

The furan analogue exhibited the highest affinity for human monofunctional GARFT ($K_i = 0.77 \text{ nM}$) of the monoglutamated inhibitors. Notably, the furan analogue bound very weakly to mFBP ($K_i = 103.9 \text{ nM}$).

**In Vivo Inhibition of Tumor Growth by the Thiophene Analogue and DDATHF.** The increased in vivo antitumor potency of the thiophene analogue compared to Lometrexol against C3H mammary carcinoma is shown in Table 2. By inspection, an approximate 10-fold increase in potency for the thiophene analogue compared to Lometrexol was observed. A therapeutic index was determined for the thiophene analogue using murine 6C3HED lymphosarcoma bearing C3H mice on a 1-, 4-, and 7-day dosing regimen. Dividing the dose which caused 50% mortality (70 mg/kg/day) by the dose which inhibited tumor growth by 50% (1.63 mg/kg/day) yielded a therapeutic index of 42.9.

![Chemical structures of Lometrexol and analogues.](image-url)

**Fig. 1.** Chemical structures of Lometrexol and analogues. LY254155 and LY222306 differ from the lead compound in the replacement of the 1',4'-phenylene portion by a 2',5'-thiophene and a 2',5'-furan, respectively.

![Graphs showing inhibition of tumor growth.](image-url)

**Fig. 2.** Reversal of CCRF-CEM cytotoxicity produced by inhibition of de novo purine synthesis by the diastereomers of the thiophene analogue. Cells were exposed for 72 h to diastereomer A (top) or diastereomer B (bottom) alone ( ), diastereomer plus 5 $\mu$m thymidine ( ), diastereomer plus 5 $\mu$m thymidine plus 100 $\mu$m hypoxanthine ( ), diastereomer plus 5 $\mu$m thymidine plus 100 $\mu$m hypoxanthine and AICA ( ). The number of cells/culture was determined after incubation and is plotted as a percentage of a no-inhibitor control culture.
Inhibition of hGARFT and binding to mFBP by Lometrexol and analogues

Table 1

<table>
<thead>
<tr>
<th>Compound number</th>
<th>Compound name</th>
<th>hGARFT a,b</th>
<th>mFBP a,c</th>
</tr>
</thead>
<tbody>
<tr>
<td>LY249543</td>
<td>Lometrexol</td>
<td>59.7 (52.1; 67.3)</td>
<td>0.29 ± 0.05 (3)</td>
</tr>
<tr>
<td>LY255540</td>
<td>Diglu-6R-DDATHF</td>
<td>15.4 (13.5; 17.3)</td>
<td>0.25 ± 0.07 (2)</td>
</tr>
<tr>
<td>LY255337</td>
<td>Triglu-6R-DDATHF</td>
<td>13.3 (12.0; 14.7)</td>
<td>0.56 ± 0.09 (3)</td>
</tr>
<tr>
<td>LY266978</td>
<td>Tetraglu-6R-DDATHF</td>
<td>7.1 ± 2.2 (4)</td>
<td>0.62 ± 0.15 (3)</td>
</tr>
<tr>
<td>LY255542</td>
<td>Pentaglu-6R-DDATHF</td>
<td>5.3 (5.2; 5.4)</td>
<td>0.72 ± 0.16 (3)</td>
</tr>
<tr>
<td>LY243246</td>
<td>6S-DDATHF</td>
<td>17.9 (14.8; 21.0)</td>
<td>0.20 ± 0.07 (4)</td>
</tr>
<tr>
<td>LY254155</td>
<td>2’,5’-thiophene analogue</td>
<td>2.1 ± 0.2 (5)</td>
<td>1.7 ± 0.1 (5)</td>
</tr>
<tr>
<td>LY309886</td>
<td>Diastereomer A</td>
<td>1.2 ± 0.1 (3)</td>
<td>1.0 ± 0.3 (3)</td>
</tr>
<tr>
<td>LY309887</td>
<td>Diastereomer B</td>
<td>6.5 ± 1.1 (3)</td>
<td>1.7 ± 0.2 (3)</td>
</tr>
<tr>
<td>LY222306</td>
<td>2’,5’-furan analogue</td>
<td>0.77 (0.73; 0.81)</td>
<td>103.9 ± 13.0 (3)</td>
</tr>
</tbody>
</table>

a Values listed are the mean ± SEM of (n) replicate experiments or the mean of two experiments with individual values in parentheses.

b All Ks for hGARFT were generated using the Morrison equation (15).

c Folate-binding protein from membranes of human KB epidermoid carcinoma cells.

Table 2

<table>
<thead>
<tr>
<th>Cumulative dose (mg/kg)</th>
<th>% inhibition of tumor growth a</th>
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</thead>
<tbody>
<tr>
<td>LY254155</td>
<td>Lometrexol</td>
</tr>
<tr>
<td>5</td>
<td>50</td>
</tr>
<tr>
<td>10</td>
<td>71</td>
</tr>
<tr>
<td>20</td>
<td>90</td>
</tr>
<tr>
<td>40</td>
<td>94</td>
</tr>
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</table>

a LY254155 was dosed on days 1, 4, 7, and 10 (4 treatment days).

b Cumulative dose was calculated using the formula (daily dose × number of treatment days). Doses were administered in mg of compound per kg of mouse weight, and 10 mice were inoculated at each dosing level, including a no-compound control group.

c % inhibition of tumor growth was calculated using the formula [(1 - (average tumor weight experimental/average tumor weight control)) × 100].

DISCUSSION

We have characterized recombinant human monofunctional GARFT and found its kinetic properties to be similar to previously reported values for human monofunctional and murine GARFT (3, 11, 24). Consistent with reports of others (11), we observed substrate inhibition of GARFT by α,β-GAR at concentrations greater than 20 μM. We have also investigated the kinetic properties of novel antifolate GARFT inhibitors and characterized the in vitro cytotoxicity and specificity of one of these.

Our results clearly demonstrate that polyglutamation is not required in vitro for tight-binding inhibition of human monofunctional GARFT by tetrahydrofolate analogues. LY254155 and LY222306, monoglutamated analogues of DDATHF, had 25- and 75-fold higher inhibitory potencies, respectively, for monofunctional hGARFT compared to Lometrexol. The exceptional activity of Lometrexol in a variety of solid murine and human xenograft tumor models has been attributed in part to its ability to be rapidly polyglutamated, resulting in increased cellular retention and higher affinity for GARFT (10, 11). Previous work has shown that inhibition of cell growth by Lometrexol against a CCRF-CEM cell line containing only 10% of the parental level of FPGS activity resulted in an approximate 10-fold decrease in in vitro cytotoxic potency (10). Thus, polyglutamation of Lometrexol may be required for in vitro cytotoxicity and in vivo antitumor activity. Monoglutamated analogues of Lometrexol which bind tightly to hGARFT, such as LY254155 and LY222306, may have increased antitumor activity against solid tumors expressing very low levels of FPGS (8, 10) or tumors resistant due to increased γ-glutamyl hydrodase activity (25). Preliminary data demonstrating that the human tumor xenograft PANC-1, which was unresponsive to Lometrexol but partially responsive to LY254155 (Table 3), has low levels of FPGS (26) are consistent with this hypothesis. Complete elimination of the need for, or ability to be polyglutamated, may be undesirable because the presence of high FPGS levels in some malignant cells may represent an important mechanism for selectivity between tumor and normal cells that typically have low FPGS. Furthermore, some polyglutamation may be desirable to enhance cellular retention as has been reported for methotrexate (27). Studies are planned which will address these possibilities.

Replacement of the 1’,4’-phenylene moiety of DDATHF by 2’,5’-thiophene did not change the selectivity of the thiophene analogue or its diastereomers as inhibitors of de novo purine biosynthesis. Thus, addition of thymidine to rescue cells blocked in the pyrimidine pathway had no effect on cell survival.

Inhibition of cell growth by high concentrations of DDATHF in the presence of 300 μM AICA has been attributed to inhibition of AICARFT, the second folate-dependent enzyme in the purine de novo pathway (2). Moreover, stereoselective differences in the potencies of (R)- and (S)-DDATHF to inhibit AICARFT (8, 28, 29) and in sensitivity to reversal by AICA have been reported (2, 8, 9). The diastereomers of Lometrexol may have increased cellular retention and higher affinity for GARFT (10, 11). Monoglutamated analogues of Lometrexol which bind tightly to hGARFT (10). Thus, polyglutamation of Lometrexol may be required for in vitro cytotoxicity and in vivo antitumor activity. Monoglutamated analogues of Lometrexol which bind tightly to hGARFT, such as LY254155 and LY222306, may have increased antitumor activity against solid tumors expressing very low levels of FPGS (8, 10) or tumors resistant due to increased γ-glutamyl hydrodase activity (25). Preliminary data demonstrating that the human tumor xenograft PANC-1, which was unresponsive to Lometrexol but partially responsive to LY254155 (Table 3), has low levels of FPGS (26) are consistent with this hypothesis. Complete elimination of the need for, or ability to be polyglutamated, may be undesirable because the presence of high FPGS levels in some malignant cells may represent an important mechanism for selectivity between tumor and normal cells that typically have low FPGS. Furthermore, some polyglutamation may be desirable to enhance cellular retention as has been reported for methotrexate (27). Studies are planned which will address these possibilities.

Table 2 In vivo inhibition of marine C3H carcinoma growth in C3H mice by LY254155 and Lometrexol

<table>
<thead>
<tr>
<th>Cumulative dose (mg/kg)</th>
<th>% inhibition of tumor growth a</th>
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<tr>
<td>5</td>
<td>50</td>
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<tr>
<td>10</td>
<td>71</td>
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a LY254155 was dosed on days 1, 4, 7, and 10 (4 treatment days).

b Cumulative dose was calculated using the formula (daily dose × number of treatment days). Doses were administered in mg of compound per kg of mouse weight, and 10 mice were inoculated at each dosing level, including a no-compound control group.

c % inhibition of tumor growth was calculated using the formula [(1 - (average tumor weight experimental/average tumor weight control)) × 100].
reomers of the thiophene analogue also demonstrated stereoselective differences in sensitivity to reversal by AICA. The greater reversal by AICA of the cytotoxic effects of diastereomer A compared to diastereomer B is reminiscent of the greater reversal by AICA observed for the S diastereomer of DDATHF compared to (R)-DDATHF (2). This result is consistent with inhibition of AICARFT and suggests that diastereomer B may be the more potent AICARFT inhibitor (Fig. 2). This hypothesis is currently under investigation in our laboratory. The pharmacological significance of this inhibition, however, may be small, because cytotoxicities in the presence of AICA occur at concentrations of thiophene analogue 5 × 10⁻⁶-fold higher than in the absence of AICA (IC₅₀, >15 μM and <3 nM, respectively). Furthermore, there is no stereoselective difference in the in vitro cytotoxicities of these diastereomers in the absence of AICA, suggesting they are equipotent at therapeutic concentrations. Thus, direct inhibition of AICARFT may have little relevance to in vitro cytotoxicity or in vivo efficacy of this compound.

The S and R diastereomers of DDATHF were originally designated diastereomers A and B, respectively, due to their elution order from a β-cyclodextrin bonded column (2). The elution order of the diastereomers of the thiophene analogue from bonded β-cyclodextrin, as well as the differing degrees of reversal by AICA, is consistent with A and B being the S and R diastereomers of the thiophene analogue, respectively. However, X-ray crystallography is required to definitively assign chirality. Another similarity between DDATHF and the thiophene analogue was the greater inhibition of hGARFT by diastereomer A in each case.

Lometrexol has been shown to be efficiently transported by the high capacity reduced folate carrier of CCRF-CEM cells (30-32). The potential in vitro cytotoxicities of the thiophene analogue and its diastereomers against human CCRF-CEM cells (IC₅₀, 1.3–2.9 nm) suggest that these compounds are also transported by the reduced folate carrier since CCRF-CEM cells do not express detectable levels of mFBP (33).

mFBP is a high affinity, low capacity transporter which mediates folate uptake via a process known as potocytosis in some cells (34). It is present in a limited number of normal human tissues, including choroid plexus, placenta, kidney, and small intestine, and is overexpressed in some tumors (35, 36). Lometrexol had very high affinity (Kᵢ = 0.29 nm) to mFBP from human KB cells, whereas the thiophene and furan analogues had 6- and 350-fold lower affinities, respectively. Pizzorno et al. (32) recently demonstrated that, at low concentrations of DDATHF (10 nm), mFBP-positive monkey kidney epithelial MA104 cells accumulated more DDATHF than mFBP-negative CCRF-CEM cells. At present, the role of mFBP in the tumor versus normal tissue selectivity or toxicity of these compounds is unclear. It is, therefore, difficult to predict whether high, low, or intermediate affinity to mFBP will result in better antitumor activity of GARFT inhibitors in vivo.

The pentaglutamate of Lometrexol had an 11-fold higher affinity for recombinant monofunctional hGARFT than Lometrexol. Other groups have reported approximately 100-fold higher affinities for the pentaglutamate using trifunctional murine and human GARFT (11, 10). These results suggest that the other two domains of the mammalian trifunctional enzyme (glycinamide ribonucleotide synthetase and aminoimidazole ribonucleotide synthetase) may participate in enhanced binding of polyglutamates. This is significant because natural reduced folate cofactors in the cell are predominantly polyglutamated.

Polyglutamation of the thiophene analogue is not required to achieve tight-binding inhibition of monofunctional hGARFT. Nevertheless, it is an excellent substrate for FPGS from murine (37) and hog liver (38). The ability to be efficiently polyglutamated and the potentially increased inhibitory potency of polyglutamated forms of the thiophene analogue against hGARFT should enhance retention and cytotoxicity in cells expressing at least moderate levels of FPGS. The extent to which endogenous folates or antifolate drugs are polyglutamated is dependent on multiple factors including (39): the endogenous folate concentration; the antifolate concentration; the intracellular concentration of FPGS (8, 10, 40–42); substrate inhibition of FPGS observed with some diastereomers of antifolates (2, 6, 8); and levels of γ-glutamyl hydrolase and its specificity. Thus, Kᵢ values of these GARFT inhibitors in situ are likely to depend upon the final mix of polyglutamated endogenous folates within a specific tissue or tumor.

The thiophene analogue had equal or better in vivo antitumor activity compared to DDATHF in all of the tumor models tested. Of particular interest was the 80–94% inhibition of tumor growth achieved by the thiophene analogue against the human xenograft PANC-1 pancreatic carcinoma. This tumor model is among the most resistant of our solid tumor panel. The enhanced in vivo potency of the thiophene analogue compared to Lometrexol could be due to one or some combination of the following factors: increased inhibitory potency against GARFT, low tumor FPGS activity, or high tumor γ-glutamyl hydrolase activity. The therapeutic index of 42.9 calculated for the thiophene analogue, using 6C3HED lymphosarcoma-bearing C3H mice, was greater than the therapeutic indices of other commonly used oncolytic agents.

Clinical investigations with Lometrexol have demonstrated that some patients experience delayed, severe, cumulative toxicity (43). This toxicity has proven manageable through administration of folic acid or leucovorin, and current protocols are investigating the most appropriate regimens.

The potential exists for other GARFT inhibitors or possibly other antifolates to display similar clinical toxicities because the mechanisms responsible for the delayed and cumulative onset are poorly understood. Retention of polyglutamated forms of the drugs, transport and storage of drug in the liver, and the relative roles of the reduced folate carrier and isoforms of mFBP may all modulate drug retention and toxicity. Furthermore, nutritional status of the patient prior to therapy is sure to be of importance as folate status is often compromised in the elderly or ill patient (44). We are intensively investigating the multiple factors which affect folate accumulation of several antifolates and polyglutamation levels in vivo in tumors, liver, and normal tissues.

In summary, substitution of the 1',4'-phénylene moiety of DDATHF produced a novel class of tight-binding inhibitors of monofunctional hGARFT. The increased potency of these compounds may reduce the need for polyglutamation in vivo. These tight-binding GARFT inhibitors may be effective in solid tumors that have reduced FPGS activity or that have become resistant due to increased γ-glu-

Table 3 In vivo antitumor activity of LY254155 and DDATHF

<table>
<thead>
<tr>
<th>Tumor system</th>
<th>% Inhibition of tumor growth¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LY254155</td>
</tr>
<tr>
<td>6C3HED lymphosarcoma</td>
<td>++++</td>
</tr>
<tr>
<td>C3H mammary carcinoma</td>
<td>+++</td>
</tr>
<tr>
<td>M-5 ovarian carcinoma</td>
<td>–</td>
</tr>
<tr>
<td>CA-755 adenocarcinoma</td>
<td>++</td>
</tr>
<tr>
<td>Human xenograft</td>
<td></td>
</tr>
<tr>
<td>LX-1 lung carcinoma</td>
<td>+++</td>
</tr>
<tr>
<td>HXGC3 colon carcinoma</td>
<td>+++</td>
</tr>
<tr>
<td>HCl colon carcinoma</td>
<td>+++</td>
</tr>
<tr>
<td>VRC5 adenocarcinoma</td>
<td>+++</td>
</tr>
<tr>
<td>PANC-1 pancreatic carcinoma</td>
<td>++</td>
</tr>
</tbody>
</table>

¹ % inhibition of tumor growth at maximum dose which did not cause greater than 20% mortality. 
² ++++, 95–100% inhibition; ++, 80–94% inhibition; +, 60–79% inhibition; –, <60% inhibition.
tamyl hydrolase activity (25). One of these novel antifolates, the thiophene analogue, retained selectivity as an inhibitor of de novo purine biosynthesis, potently inhibited growth of human leukemia CCRF-CEM cells in vitro, and inhibited the growth of several solid murine and human xenograft tumors in vivo. These characteristics make members of this novel class of antifolates attractive candidates for clinical development.

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A Novel Class of Monoglutamated Antifolates Exhibits Tight-binding Inhibition of Human Glycinamide Ribonucleotide Formyltransferase and Potent Activity against Solid Tumors

Lillian L. Habeck, Tracy A. Leitner, Katherine A. Shackelford, et al.


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