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Folic Acid Analogues Lacking the 2-Carbon Are Substrates for Folylpolyglutamate Synthetase and Inhibit Cell Growth

Recent reports from several laboratories have pointed to the fact that folylpolyglutamate synthetase (FPGS), the enzyme responsible for the conversion of endogenous reduced folates as well as a variety of folate antagonists to their γ-polyglutamylated forms,[1] has a high degree of side chain specificity but is relatively tolerant of structural changes in rings A and B, the "bridge region" and the phenyl ring (Figure 1). With regard to ring A, for example, it has been shown that replacement of the 2-amino group by hydrogen in the N^{10}-methyl-, N^{10}-ethyl-, N^{10}-allyl-, and N^{10}-propargyl-substituted derivatives of 5,8-dideazafolic acid results in a minimal change in either the K_m or V_{max} for γ-diglutamate formation by mouse liver FPGS.[2] Similar results were obtained for replacement of the 2-amino group by hydrogen in 10-oxafolic acid and 10-thiafolic acid,[3] and for replacement of the 2-amino group by hydrogen or methyl in aminopterin.[4] We postulated that since changes in the 2-substituent at in ring A are well tolerated, *the carbon itself at position 2 may not be absolutely required for binding to the enzyme.* To test this hypothesis we prepared the heretofore undescribed compound *N*-[4-[[(2-amino-3-carbamoylpyrazin-5-yl)methyl]-amino]benzoyl]-L-glutamic acid (**1**), which may be viewed as a folic acid analogue in which *both the 2-amino group and C_2 have been deleted.* Also synthesized was *N*-[4-[[(2-amino-3-carbamoylpyrazin-5-yl)methyl]formamido]-benzoyl]-L-glutamic acid (**2**), the corresponding ring-opened analogue of N^{10}-formylfolic acid. Compound **1** inhibited the growth of L1210 murine leukemic cells in culture with an IC_{50} of 5 μM and was comparable to folic acid as a substrate for FPGS, providing the first demonstration that an intact A ring in folate analogues is not an absolute requirement for FPGS substrate activity. We believe these results are a timely and potentially exploitable lead for the design of other biologically active folate analogues.

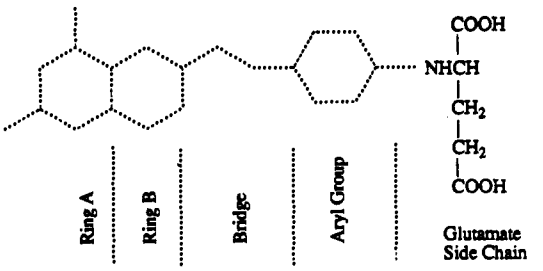
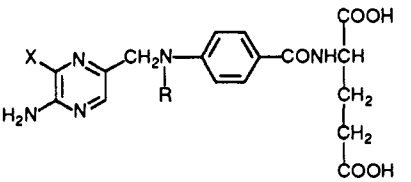


Figure 1. General structure of FPGS substrates.



1: X = $CONH_2$, R = H
2: X = $CONH_2$, R = CHO
3: X = CN, R = H

The starting material **3** was prepared previously in our laboratory by N-monoalkylation of di-*tert*-butyl *N*-(4-aminobenzoyl)-L-glutamate with 2-amino-5-(chloromethyl)pyrazine-3-carbonitrile followed by cleavage of the ester groups with TFA.[4,7] Conversion of **3** to the carboxamide **1** was accomplished in quantitative yield by allowing a solution of the nitrile in 1 N NaOH to stand at room temperature and monitoring the course of reaction by HPLC. Complete conversion was observed after 2.5 h, and the product was purified by ion-exchange chromatography (Dowex 50W-X2, H^+ form) using 3% NH_4OH as the eluent. Freeze-drying afforded analytically pure **1** as a sesquihydrate [HPLC 30 min (C_{18} silica gel, 5% MeCN in 0.1 M NH_4OAc, pH 5.0, 2 mL/min); UV λ_{max} (pH 7.4) 253 nm (ε 15 500), 289 (19 400), 357 (6710), λ_{max} (0.1 N HCl) 216 nm (ε 13 900), 246 (15 900), 294 (10 400), 358 (6620)].[8]

Reaction of **1** with 95% formic acid (room temperature, 7 days) and purification by ion-exchange chromatography (DEAE-cellulose, HCO_3^- form) using H_2O followed by 0.2 and 0.4 M NH_4HCO_3 as the eluents afforded a 65% yield of **2** as a hydrated partial ammonium salt [HPLC 7 min (C_{18} silica gel, 5% MeCN in 0.1 NH_4OAc, pH 7.5, 1 mL/min); NMR (D_2O with enough K_2CO_3 to dissolve the sample) δ 7.33 (d, J = 8 Hz, $C_{3'}$- and $C_{5'}$-H), 7.78 (d, J = 8 Hz, $C_{2'}$- and $C_{6'}$-H), 8.37 (minor) and 8.50 (major) (singlets, NCHO rotomers); UV λ_{max} (pH 7.4) 253 nm (ε 26 700), 357 (5980), λ_{max} (0.1 N HCl) 249 nm (ε 18 700), 359 (4520), λ_{max} (0.1 N NaOH) 253 nm (ε 27 500), 357 (6250)].[8] HPLC evidence for two other minor products was obtained, one of which appears to be formed from **1**, but no attempt was made to isolate and characterize these side products. The site of the N-formylation in **2** was determined to be N^{10} as opposed to the 2-amino group on the basis of the ^1H NMR spectrum, which showed the $C_{3'}$ and $C_{5'}$ protons as a doublet at δ 7.33, whereas the chemical

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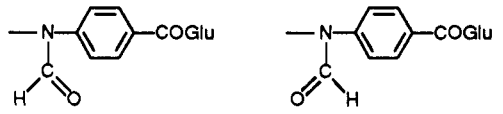
Table I. FPGS Susbstrate Activity of Folic Acid Analogues with an Open A Ring[a]

compound	K_m(apparent)	rel V_{max}	rel V_{max}/K_m (k')
folic acid	140 ± 47	1.0	1.0
methotrexate	166 ± 49	0.99 ± 0.16	0.90 ± 0.05
aminopterin	21 ± 4.8	1.59 ± 0.01	10.1 ± 4.2
1	109 ± 19	0.99 ± 0.10	1.52 ± 0.65
2	131 ± 3	1.84 ± 0.01	2.6 ± 0.4
3	163 ± 4	1.06 ± 0.23	0.70 ± 0.03

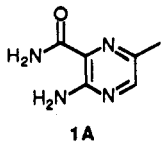
[a] FPGS substrate activity was determined as described earlier.[6,15] Data for methotrexate and aminopterin are from ref 15.

shift for the $C_{3'}$ and $C_{5'}$ protons in **3**, a typical example of a compound not formylated on N^{10}, was at δ 6.67. In agreement with its somewhat hindered nature, the *N*-formyl group in **2** appears to exist in two rotomeric forms,[9,10] giving rise to a pair of singlets at δ 8.50 (major rotomer, assumed to be **2B**) and δ 8.37 (minor rotomer, assumed to be **2A**). Steric considerations suggest that rotomer **2B** ought to be more abundant than rotomer **2A**.

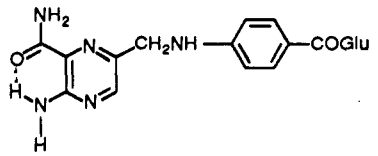


2A **2B**

Compounds **1–3** were tested as substrates for partly purified FPGS from mouse liver[5] as previously described.[6] As shown in Table I, the apparent K_m for the amino carboxamide **1** (109 ± 19 μM) was comparable to that of folic acid (140 ± 47 μM) but 5-fold higher than that of aminopterin (21 ± 4.8 μM). The apparent K_m for amino nitrile **3** (163 ± 4 μM) was also in the range of some very active compounds, such as methotrexate, whose apparent K_m as a substrate for mouse liver FPGS has been reported as 166 ± 49 μM.[6] The V_{max} of **1** and **3** was indistinguishable from that of folic acid. The relative first-order rate constant k', calculated as the ratio V_{max}/K_m(apparent), was 1.52 for **1** and 0.70 for **3**. Thus, the amino carboxamide is utilized more efficiently as a substrate than the amino nitrile, and the presence of an intact A ring is not essential for substrate activity. To our knowledge, **1** and **3** are the first examples of folate analogues lacking an intact A ring with substrate activity for FPGS. An interesting question with regard to the binding of **1** to FPGS is the conformation of the carboxamide group. As indicated in structures **1A** and **1B**, this molecule could conceivably bind to the enzyme in two conformations, one with the carboxamide oxygen mimicking the 4-oxo group of folic acid and the other with the carboxamide mimicking the 4-amino group of aminopterin and other classical antifolates. The latter possibility is intriguing because it affords the opportunity to form a "pseudo-ring" via intramolecular hydrogen bonding.



1A



1B

The finding that **1** is a substrate for FPGS is of interest in light of recent data on folic acid analogues in which regions of the molecule other than the glutamate side chain have been altered with retention of excellent FPGS substrate activity. For example, N-[4-[[3-(2,4-diamino-1,6-dihydro-6-oxopyrimidin-5-yl)propyl]amino]benzoyl]-L-glutamic acid, which may be viewed as a ring-opened analogue of tetrahydrofolic acid with C_7 deleted, is reported to have K_m and V_{max} values very similar to those of tetrahydrofolic acid.[9] In other studies, it has been found that either scission of ring B or replacement of N^{10} and the phenyl ring by a simple hydrocarbon chain is also compatible with FPGS substrate activity.[12] From these findings and the present results, it would appear that FPGS is a remarkably nonselective enzyme in that it can react with molecules vastly different from natural substrates. Even the presence of an intact glutamate moiety is not inviolable in this regard. For example, two analogues modified in the side chain are reported to have modest activity as substrates, namely *N*-(4-amino-4-deoxy-N^{10}-methylpteroyl)-2-aminopimelic acid[6] and γ-fluoromethotrexate.[13] However, the tolerance of FPGS for structural alteration of the amino acid side chain is very limited, whereas its tolerance for changes in the rest of the substrate molecule is surprisingly lenient. The apparent promiscuity of FPGS vis a vis its substrates is unusual, and suggests a high degree of conformational flexibility among the residues that comprise the active site. The basis for this flexibility will undoubtedly become better understood when more is known about the three-dimensional structure of the enzyme and the mechanistic details of its catalytic action. It is of interest to consider the possibility that drugs directed toward targets other than the enzymes of the folate pathway might be designed to have FPGS substrate activity, which would increase their cellular retention just as polyglutamylation increases retention of antifolates and phosphorylation increases retention of nucleosides.

Table II. Inhibition of L1210 Cell Growth by Folic Acid Analogues with an Open A Ring[a]

compound	IC_{50}, μM	*n*
1	5.5 ± 0.4	2
2	85	1
3	217 ± 43	2

[a] *n* = number of experiments; a typical IC_{50} value for the much more potent agent methotrexate against L1210 cells is 0.0046 μM.[16]

In addition to being assayed for FPGS substrate activity, compounds **1** and **3** as inhibitors of L1210 cell growth in culture.[14] As shown in Table II, the IC_{50} of **1** in a 72-h incubation was 5.5 μM. By contrast, the IC_{50} of the amino nitrile **3** and the N^{10}-formyl derivative **2** was 217 and 85

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(14) Growth inhibition assays were carried out with exponentially growing L1210 mouse leukemia cells in 24-well plates, at an initial cell density of (2.0–2.5) × 10^4/mL. Each well contained 1.35 mL of RPMI-1640 medium supplemented with 10% dialyzed fetal bovine serum, and the test compound was added as a solution in 0.15 mL of phosphate-buffered saline. Cells were incubated at 37 °C in a 5% CO_2 humidified atmosphere and were counted after 72 h with a Coulter counter, Model Z_F (Coulter Electronics, Hialeah, FL).
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μM, respectively. The lower activity of 3 was consistent with less efficient polyglutamylation (Table I), but obviously could reflect other factors such as transport and binding to a putative biochemical target. We are unaware of any folic acid analogues that possess this level of in vitro antitumor activity when ring A is opened.

An experiment was performed to determine whether inhibition of cell growth by 1 could be prevented with thymidine (TdR) and/or hypoxanthine (Hx). Incubation of L1210 cells for 72 h in the presence of 1 (5 μM) and TdR (5.6 μM) alone, Hx (100 μM) alone, or a combination of TdR (5.6 μM) and Hx (100 μM), afforded partial protection, suggesting that 1 possesses antifolate activity. However, the fact that *complete* protection was not observed at these normally protective concentrations of TdR and Hx indicates that growth inhibition by 1 may arise in part from interference with metabolic processes other than de novo thymidylate or purine nucleotide biosynthesis. At this time the specific enzyme or enzymes inhibited by polyglutamates of 1 are unknown.

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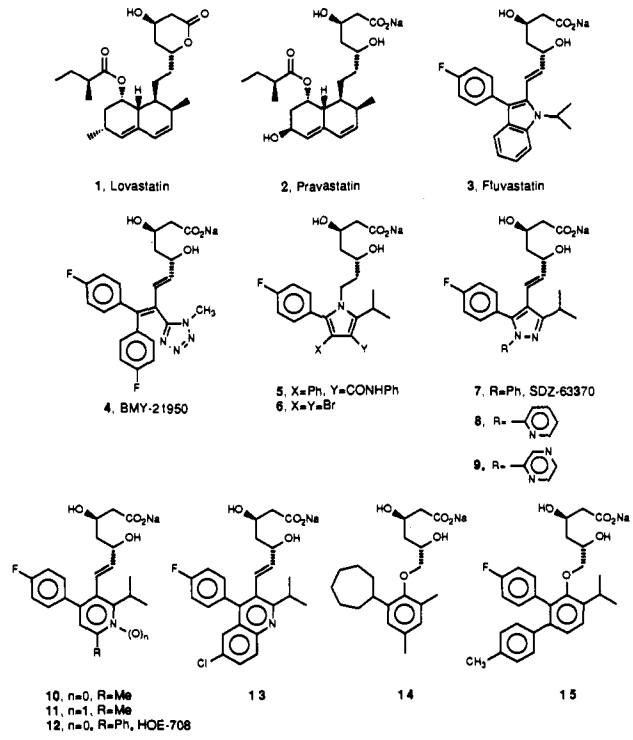
Relationship between Tissue Selectivity and Lipophilicity for Inhibitors of HMG-CoA Reductase

It is now well-established that inhibition of the enzyme HMG-CoA reductase (HMGR) is an effective means for lowering plasma total and LDL-cholesterol in hypercholesterolemic patients.[1] However, the long-term safety of these agents is still unproven. Although recent clinical experience with lovastatin (1) has indicated that it is well-tolerated in man,[2] some adverse reactions have been noted; particularly, elevated liver enzymes,[3] sleep disturbances,[4] and myositis.[5] Recently, there has been considerable controversy in the literature regarding both the nature and existence of tissue (liver) selectivity for various HMGR inhibitors, and whether confining their action to the liver would reduce the incidence of adverse reactions. The initial report[6] describing tissue selectivity for pravastatin (2) suggested that pravastatin and lovastatin were equipotent at inhibiting cholesterol biosynthesis in cultured rat hepatocytes, but pravastatin was 100 times less potent than lovastatin at inhibiting biosynthesis in human skin fibroblasts. This selectivity was further supported by ex vivo rat studies, which demonstrated that pravastatin inhibited cholesterol biosynthesis only in lipoprotein-producing organs (liver and intestine), whereas lovastatin and mevastatin (compactin) also inhibited cholesterol biosynthesis significantly in kidney, lung, spleen, prostate, and testis. The assertion that pravastatin is more tissue selective than lovastatin has been disputed, however, on the basis of measurements of peripheral drug distribution employing a bioassay[7] as well as the uptake and tissue distribution of radiolabeled drug.[8] More recently, other HMGR inhibitors have been reported to display liver selectivity.[9] It has been proposed that tissue selectivity is influenced primarily by the relative lipophilicity of the drugs, with the relatively more hydrophilic compounds showing higher liver selectivity.[10]

Since we had prepared HMGR inhibitors possessing considerable variation in structure and lipophilicity during the course of our program in this area, we decided to test this hypothesis directly. Thus, we compared a selection of potent inhibitors possessing a broad range of calculated lipophilicities (CLOGP) for their abilities to inhibit sterol synthesis in tissue cubes derived from rat liver, spleen, and testis. The results of these studies are the subject of this report.

Chart I



Chemistry

All of the inhibitors employed in this study (Chart I) have been reported previously.[11] Representative com-

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