LY231514, a Pyrrolo[2,3-d]pyrimidine-based Antifolate That Inhibits Multiple Folate-requiring Enzymes

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ABSTRACT

N-[4-[2-(2-amino-3,4-dihydro-4-oxo-7H-pyrrolo[2,3-d]pyrimidin-5-yl)-ethyl]-benzoyl]-L-glutamic acid (LY231514) is a novel pyrrolo[2,3-d]pyrimidine-based antifolate currently undergoing extensive Phase II clinical trials. Previous studies have established that LY231514 and its synthetic γ-polyglutamates (glu$_3$ and glu$_5$) exert potent inhibition against thymidylate synthase (TS). We now report that LY231514 and its polyglutamates also markedly inhibit other key folate-requiring enzymes, including dihydrofolate reductase (DHFR) and glycinamide ribonucleotide formyltransferase (GARFT). For example, the K_i values of the pentaglutamate of LY231514 are 1.3, 7.2, and 65 nM for inhibition against TS, DHFR, and GARFT, respectively. In contrast, although a similar high level of inhibitory potency was observed for the parent monoglutamate against DHFR (7.0 nM), the inhibition constants (K_i) for the parent monoglutamate are significantly weaker for TS (109 nM) and GARFT (9,300 nM). The effects of LY231514 and its polyglutamates on aminoimidazole carboxamide ribonucleotide formyltransferase, 5,10-methylenetetrahydrofolate dehydrogenase, and 10-formyltetrahydrofolate synthetase were also evaluated. The end product reversal studies conducted in human cell lines further support the concept that multiple enzyme-inhibitory mechanisms are involved in cytotoxicity. The reversal pattern of LY231514 suggests that although TS may be a major site of action for LY231514 at concentrations near the IC$_{50}$, higher concentrations can lead to inhibition of DHFR and/or other enzymes along the purine *de novo* pathway. Studies with mutant cell lines demonstrated that LY231514 requires polyglutamation and transport via the reduced folate carrier for cytotoxic potency. Therefore, our data suggest that LY231514 is a novel classical antifolate, the antitumor activity of which may result from simultaneous and multiple inhibition of several key folate-requiring enzymes via its polyglutamated metabolites.

INTRODUCTION

Several novel folate-based antimetabolites are currently being actively investigated in clinical trials. These include lometrexol and LY309887,[2] which inhibit GARFT in the purine *de novo* biosynthetic pathway (1–3); edatrexate (4, 5) which acts on DHFR; and ZD1694 (Tomudex; Refs. 6 and 7), AG337 (Thymitaq; Ref. 8), and BW1843U89 (9), which specifically target TS.

LY231514 is a structurally novel antifolate antimetabolite that possesses the unique 6-5-fused pyrrolo[2,3-d]pyrimidine nucleus (10, 11) instead of the more common 6-6-fused pteridine or quinazoline ring structure (Fig. 1). Previous studies have demonstrated that LY231514 is one of the best substrates that is known for the enzyme FPGS ($K_m = 1.6$ μM and $V_{max}/K_m = 621$; Ref. 12). It is likely that polyglutamation and the polyglutamated metabolites of LY231514 play profound roles in determining both the selectivity and the antitumor activity of this novel agent (11, 12). Whereas LY231514 only moderately inhibited TS ($K_i = 340$ nM, recombinant mouse), the pentaglutamate of LY231514 was 100-fold more potent ($K_i = 3.4$ nM; Ref. 11), making LY231514 one of the most potent folate-based TS inhibitors known today (13).

Preliminary cell culture end product reversal studies in human CCRF-CEM and murine L1210 leukemia cells have demonstrated that thymidine (5 μM) alone was not able to fully reverse the cytotoxic action of LY231514 (11). Both thymidine (5 μM) and hypoxanthine (100 μM) were required to fully protect cells from the growth-inhibitory activity exerted by LY231514. This reversal pattern is significantly different from other TS inhibitors, such as ZD1694 (6) and BW1843U89 (9). Cell culture experiments showed that the antiproliferative activity of LY231514 was completely reversed by the addition of leucovorin (0.05–16 μM) in a competitive manner (11), suggesting that LY231514 competed with natural reduced folate cofactors both at transport and intracellular folate levels and acted as a pure folate antagonist.

Promising antitumor responses have recently been observed in the Phase I trials of LY231514. Moreover, patients who had previously failed to respond to ZD1694 and 5-fluorouracil/leucovorin treatment responded to LY231514 (14). This pattern of clinical response, together with the aforementioned observations of partial protection by thymidine in cell culture, suggest that inhibition of TS by LY231514 may not solely account for the overall antitumor effect of this novel antifolate. LY231514 and its polyglutamates may inhibit other folate-requiring enzymes, such as DHFR, or enzymes along the *de novo* purine biosynthetic pathway. LY231514 may thus act as a multitargeted antifolate, with multiple mechanisms of action affecting the intracellular folate pools and cellular pyrimidine/purine biosynthesis.

We now summarize our findings of LY231514 and its polyglutamates (glu$_3$ and glu$_5$) against various folate-requiring enzymes, including human TS, DHFR, AICARFT, 5,10-methylenetetrahydrofolate dehydrogenase, and 10-formyltetrahydrofolate synthetase activities of C1-S and murine GARFT. In addition, we report a detailed comparison of cell culture reversal patterns observed in several human cell lines between compounds LY231514 and ZD1694. Finally, we examine the role of polyglutamation and transport (via the RFC) in the cytotoxicity of LY231514.

MATERIALS AND METHODS

Materials. LY231514 and ZD1694 were prepared according to published methods and procedures (7, 11). The syntheses of the γ-glutamyl derivatives of LY231514 were by the method of Pawelczak *et al.* (15). For *in vitro* studies,
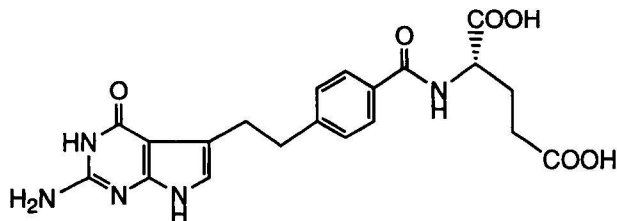
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Fig. 1. Structure of LY231514. *N*-[4-[2-(2-amino-3,4-dihydro-4-oxo-7H-pyrrolo[2,3-d]pyrimidin-5-yl)ethyl]-benzoyl]-L-glutamic acid.

compounds were dissolved in either DMSO or 5% sodium bicarbonate at an initial concentration of 1–50 mM, and dilutions were made in either enzyme assay buffer or cell culture medium (RPMI 1640 with 10% dialyzed FCS). The final DMSO concentration never exceeded 0.5%. Vehicle controls confirmed that there was no effect of DMSO at this concentration. A water-soluble form of the disodium salt of LY231514 was used in some investigations. The recombinant enzymes used were all obtained in purified form from the following sources: rhTS from Dr. D. V. Santi (University of California at San Francisco, San Francisco, CA; Ref. 16); trifunctional mGARFT from Dr. R. G. Moran (Medical College of Virginia, Richmond, VA; Ref. 17); rhDHFR from Dr. M. Ratnam of Medical College of Ohio, Toledo, Ohio (18) and Anatrace Co. (Maumee, OH). Two forms of rhCl-S were obtained from Dr. R. E. Mackenzie (McGill University, Montreal, Quebec, Canada; Ref. 19): (*a*) the M_r 101,000 full-length enzyme of C1-S containing 5,10-methylenetetrahydrofolate dehydrogenase, 5,10-methenyltetrahydrofolate cyclohydrolase and 10-formyltetrahydrofolate synthetase (EC 6.3.4.3) activities; and (*b*) the protein domain of C1-S containing the 5,10-methylenetetrahydrofolate dehydrogenase (EC 1.5.1.5) and 5,10-methenyltetrahydrofolate cyclohydrolase activities (the M_r 35,000 truncated version of C1-S, which contained only the dehydrogenase and cyclohydrolase activities). Human AICARFT was purified as described by Rayl *et al.* (20). 10-Formyl-[6R,S]-5,6,7,8-tetrahydrofolate was prepared by a method similar to that of Rowe (21). 10-Formyl-5,8-dideazafolic acid and α,β-glycinamide ribonucleotide were prepared as described previously (2). 6R-MTHF for use in the TS assay was obtained from Eprova AG (Schaffhausen, Switzerland); the trihydrochloride salt of [6R,S]-5,6,7,8-tetrahydrofolate and the magnesium salt of [6R,S]-5,10-methylene-5,6,7,8-tetrahydrofolate for use in the C1-S dehydrogenase assay were obtained from Dr. B. Schircks Laboratories (Jona, Switzerland). 5-Aminoimidazole-4-carboxamide ribonucleotide, AICA, folic acid, folinic acid, 7,8-dihydrofolate, NADPH, hypoxanthine, methotrexate, MTT, and thymidine were purchased from Sigma Chemical Company (St. Louis, MO). Dialyzed fetal bovine serum was purchased from Hyclone (Logan, UT). Regular and folate-free RPMI 1640 with 25 mM HEPES buffer were purchased from Whittaker Bioproducts (Walkersville, MD). The ENZFITTER microcomputer package was obtained from Biosoft (Ferguson, MO). CCRF-CEM cells were obtained from St. Jude Children's Research Hospital (Memphis, TN). HCT-8 cells were purchased from the American Type Culture Collection (Rockville, MD). CR15, a 5,10-dideazatetrahydrofolic acid-resistant CCRF-CEM subline, was described by Pizzorno *et al.* (22). ZR-75-1 human breast carcinoma cell sublines with differing folate transport properties were generously provided by Dr. K. Cowan (NCI, Bethesda, MD; Ref. 23). The GC3/C1 cell line was developed by Dr. J. Houghton (St. Jude Children's Research Hospital, Memphis, TN; Ref. 24). CCRF-CEM, HCT-8, CR15, ZR-75-1, MTX[R]ZR-75-1, and GC3/C1 cells were routinely cultured in RPMI 1640 medium containing L-glutamine and 25 mM HEPES buffer and supplemented with 10% dialyzed FCS. ZR-75-1 cells expressing FBP-α (MTX[R]BB3-FR+ and 2FR+AA6) were cultured in folic acid-free RPMI 1640 containing L-glutamine, 25 mM HEPES buffer, 2 nM folinic acid, and 10% dialyzed FCS.

Enzyme Assays and Methods. TS activity was assayed using a spectrophotometric method described by Greene *et al.* (25), which involved monitoring the increase in absorbance at 340 nm resulting from formation of the product, 7,8-dihydrofolate. The assay buffer contained 50 mM *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid, 25 mM MgCl$_2$, 6.5 mM formaldehyde, 1 mM EDTA, and 75 mM 2-mercaptoethanol, pH 7.4. The concentrations of deoxyuridylate monophosphate, 6R-MTHF, and hTS were 100 μM, 30 μM, and 30 nM (1.7 milliunits/ml), respectively. (One milliunit of enzyme activity is defined as 1 nmol of product produced per min.) At the

6R-MTHF concentration, an uninhibited reaction and six concentrations of inhibitor were assayed. K_i apparent ($K_{i\,app}$) values were determined by fitting the data to the Morrison equation (26) using nonlinear regression analysis with the aid of the program ENZFITTER. K_i values were calculated using the equation: $K_{i\,app} = K_i (1 + [S]/K_m)$, where [S] is equal to 30 μM and K_m is equal to 3 μM.

DHFR activity was assayed spectrophotometrically by monitoring the disappearance of the substrates NADPH and 7,8-dihydrofolate (combined $\epsilon = 12 \times 10^3$ M^{-1} cm^{-1}) at 340 nm. The reaction took place at 25°C in 0.5 ml of 50 mM potassium phosphate buffer, which contained 150 mM KCl and 10 mM 2-mercaptoethanol, pH 7.5, and 14 nM (0.34 milliunit/ml) DHFR. The NADPH concentration was 10 μM, and 7,8-dihydrofolate was varied at 5, 10, or 15 μM. At each 7,8-dihydrofolate concentration, an uninhibited reaction and seven concentrations of inhibitor were assayed. The ENZFITTER microcomputer program was used to obtain $K_{i\,app}$ values by fitting the data to the Morrison equation by nonlinear regression analysis. K_i values were calculated using the equation: $K_{i\,app} = K_i (1 + [S]/K_m)$, where [S] is equal to the concentration of 7,8-dihydrofolate used and K_m of 7,8-dihydrofolate is 0.15 μM (27, 28).

GARFT activity was assayed spectrophotometrically as described previously (2) by monitoring the increase of absorbance resulting from formation of the product 5,8-dideazafolate at 295 nm. The reaction solvent contained 75 mM HEPES, 20% glycerol, and 50 mM α-thioglygerol, pH 7.5, at 25°C. The concentrations of substrates and enzyme used were 10 μM α,β-glycinamide ribonucleotide, 0–10 μM 10-formyl-5,8-dideazafolic acid, and 10 nM (1.9 milliunits/ml) GARFT. K_i values were calculated using the Enzyme Mechanism program of the Beckman DU640 spectrophotometer, which uses nonlinear regression analysis to fit data to the Michaelis-Menten equation for competitive inhibition.

AICARFT inhibition assays were carried out at room temperature by monitoring the formation of [6S]-5,6,7,8-tetrahydrofolate from 10-formyl-[6R,S]-5,6,7,8-tetrahydrofolate at A_{298}. All solutions were purged with N$_2$ gas prior to use. The reaction solution contained 33 mM Tris-Cl, pH 7.4, 25 mM KCl, 5 mM 2-mercaptoethanol, 0.05 mM AICA ribonucleotide, and 16 nM (2.0 milliunits/ml) of AICARFT. 10-Formyl-[6R,S]-5,6,7,8-tetrahydrofolate concentrations of 0.037, 0.074, and 0.145 mM were used (0.61, 1.23, and 2.45 times its K_m value, respectively). LY231514 was tested as an inhibitor at 0.080–0.800 mM (four concentrations). When the tri- and pentaglutamates of LY231514 were used as inhibitors, the concentrations were 0.0005–0.009 mM (eight concentrations). Enzyme assays were initiated by the addition of enzyme. Data was analyzed using the ENZFITTER program for competitive inhibition.

The method of Tan *et al.* (29) was used for assaying the activities of C1-S. This involves quenching the reaction by acid and subsequent spectrophotometric quantitation of the amount of 5,10-methenyltetrahydrofolate produced at 350 nm. Accordingly, the dehydrogenase assay was conducted in a pH 7.3 reaction mixture containing 0.084 M potassium phosphate, 0.12 M 2-mercaptoethanol, 0.17 mM NADP, 5.75–168 μM [6R,S]-5,10-methylene-5,6,7,8-tetrahydrofolate, and 0.96 nM (0.7 milliunit/ml) protein domain of C1-S containing the 5,10-methylenetetrahydrofolate dehydrogenase (EC 1.5.1.5) and 5,10-methenyltetrahydrofolate cyclohydrolase activities. The synthetase assay was conducted in a pH 8.0 reaction mixture containing 0.1 M triethanolamine, 0.14 M 2-mercaptoethanol, 0.05 M KCl, 0.04 M sodium formate, 1.0 mM MgCl$_2$, 1.0 mM ATP, 62.5–2000 μM [6R,S]-5,6,7,8-tetrahydrofolate, and 1.4 nM (0.7 milliunit/ml) full-length enzyme of C1-S containing 5,10-methylenetetrahydrofolate dehydrogenase, 5,10-methenyltetrahydrofolate cyclohydrolase and 10-formyltetrahydrofolate synthetase (EC 6.3.4.3) activities. The concentrations of LY231514 and its polyglutamates studied in each activity were from about 0.5 × K_i to 3 × K_i. All reactions were conducted at ambient temperature (23°C) in a final volume of 0.475 ml and quenched with 0.025 ml of 0.4 M HCl. Activity data collected with a range of substrate and drug concentrations were fit to the Michaelis-Menten equation for competitive inhibition by nonlinear regression with the aid of the GRAFIT computer program (30).

In Vitro **Cell Culture Studies.** Dose-response curves were generated to determine the concentration required for 50% inhibition of growth (IC$_{50}$). Test compounds were dissolved initially in DMSO at a concentration of 4 mg/ml and further diluted with cell culture medium to the desired concentration. CCRF-CEM leukemia cells in complete medium were added to 24-well Cluster plates at a final concentration of 4.8 × 10^4 cells/well in a total volume

of 2.0 ml. Test compounds at various concentrations were added to duplicate wells so that the final volume of DMSO was 0.5%. The plates were incubated for 72 h at 37°C in an atmosphere of 5% CO_2 in air. At the end of the incubation, cell numbers were determined on a ZBI Coulter counter. Control wells usually contained 4×10^5 to 6×10^5 cells at the end of the incubation. For several studies, IC_{50}s were determined for each compound in the presence of either 300 μM AICA, 5 μM thymidine, 100 μM hypoxanthine, or combination of 5 μM thymidine plus 100 μM hypoxanthine.

For adherent tumor cells, we used a modification of the original MTT colorimetric assay described by Mosmann (31) to measure cell cytotoxicity. The human tumor cells were seeded at 1×10^4 cells in 100 μl of assay medium/well in 96-well flat-bottomed tissue culture plates (Costar, Cambridge, MA). The assay medium contained folic acid-free RPMI 1640 supplemented with 10% FCS and either 2 nM folinic acid or 2.3 μM folic acid as the sole folate source. Well 1A was left blank (100 μl of growth medium without cells). Stock solutions of antifolates were prepared in Dulbecco's PBS at 1 mg/ml, and a series of 2-fold dilutions were subsequently made in PBS. Ten-μl aliquots of each concentration were added to triplicate wells. Plates were incubated for 72 h at 37°C in a humidified atmosphere of 5% CO_2-in-air. MTT was dissolved in PBS at 5 mg/ml, 10 μl of stock MTT solution were added to each well of an assay, and the plates were incubated at 37°C for 2 additional h. Following incubation, 100 μl of DMSO were added to each well. After thorough formazan solubilization, the plates were read on a Dynatech MR600 reader, using a test wavelength of 570 nm and a reference wavelength of 630 nm. The IC_{50} was determined as the concentration of drug required to inhibit cell growth by 50% compared to an untreated controls.

RESULTS

Enzyme Inhibition Studies. The inhibition of rhTS by LY231514 and its polyglutamates is summarized in Table 1. The parent monoglutamate LY231514 inhibited rhTS with a K_i of 109 nM when the monoglutamated form of the substrate (6R-MTHF) (6[R]-5,10-methylenetetrahydrofolate) was used. This is in good agreement with the K_i value generated earlier for rmTS (K_i = 340 nM; Ref. 11). The longer-chain γ-glutamyl derivatives of LY231514 demonstrated significantly enhanced affinity to rhTS. The addition of two extra γ-glutamyl residues (glu$_3$) to LY231514 resulted in 68-fold reduction of the K_i value. Further extension of the glutamate tail (LY231514-glu$_5$) did not result in any significant enhancement of inhibitory potency toward rhTS. In comparison, ZD1694 was less dependent on polyglutamation. A 5-fold increase in affinity was observed for ZD1694 polyglutamates toward rhTS. It has been well recorded that mammalian TS showed a strong preference for polyglutamated folate substrates. A similar effect had been reported by Jackman et al. (6, 33) and Sikora et al. (32) in their studies of the quinazoline antifolates CB3717, ZD1694, and their polyglutamates by using partially purified L1210 murine TS. In both cases, the corresponding triglutamate derivatives demonstrated 87- and 56-fold reductions in K_i values, respectively, compared to the parent compounds. In a separate study by Cheng et al. (34), CB3717-glu$_3$ was approximately 20-fold more potent than the parent monoglutamate compound in inhibiting human TS isolated from HeLa S3 and KB cells. The quantitative differences in the
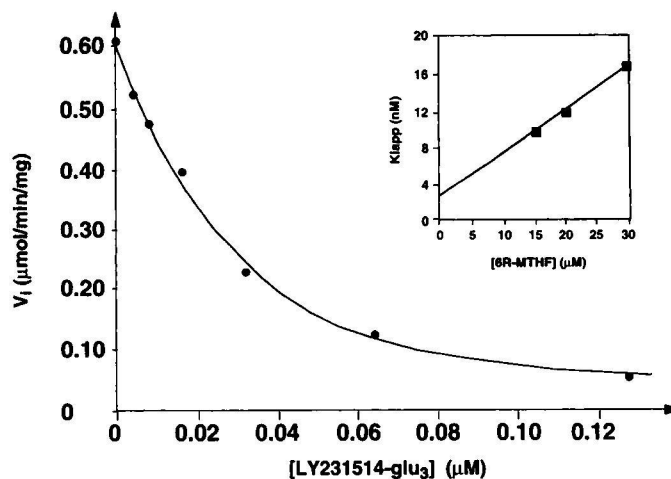

Fig. 2. Morrison Analysis of tight-binding inhibition of rhTS by LY231514-glu$_3$. A velocity *versus* inhibitor concentration curve is shown from a representative experiment illustrating the concentration-dependent inhibition of rhTS (29 nM) in the presence of 6R-MTHF (15 μM) and 100 μM deoxyuridylate monophosphate. *Inset*, $K_{i\,app}$ values were determined by the nonlinear fitting of data collected at three concentrations of 6R-MTHF to the Morrison equation using the ENZFITTER microcomputer package. The K_i value (1.3 nM) was determined from the slope of the graph $K_{i\,app}$ *versus* [6R-MTHF] using a K_m for 6R-MTHF of 3.0 μ[M.

reported degree of enhancement in potency as a result of polyglutamation are likely due to a combination of the variation in enzyme source used, as well as the inherent difficulty in obtaining K_i estimates for very tightly bound compounds. LY231514 and its polyglutamates inhibited rhTS in a competitive fashion with respect to the natural substrate [6R]-5,10-methylenetetrahydrofolate. The data of LY231514-glu$_3$ against rhTS is shown in Fig. 2. The K_i values reported in Table 1 are calculated assuming competitive inhibition for ZD1694.

LY231514 was found to be a very potent inhibitor when tested against recombinant human DHFR. Tight binding analysis showed that LY231514 inhibited rhDHFR in a competitive fashion with a K_i of 7.0 nM (Table 1). In contrast to rhTS, attachment of additional γ-glutamyl residues to LY231514 had little effect on the inhibition toward rhDHFR (the glu$_3$ and glu$_5$ of LY231514 exhibited identical K_i values against rhDHFR). The polyglutamates of LY231514 also showed a competitive inhibition pattern toward rhDHFR (data not shown). It was reported that CB3717 had a K_i of 250 nM on DHFR isolated from human KB/6B cells (34) and that ZD1694 inhibited rat liver DHFR with a K_i of 93 nM (6). Likewise, polyglutamation of CB3717 and ZD1694 did not enhance affinity to DHFR. In our hands, ZD1694 and its polyglutamates also inhibited rhDHFR but were 7-fold less potent than LY231514. The polyglutamates of ZD1694 showed slight enhancement of affinity toward rhDHFR.

We also studied drug inhibition against the folate-requiring enzymes along the purine *de novo* biosynthetic pathway. LY231514 only moderately inhibited rmGARFT (K_i = 9.3 μM). Through earlier studies of 5,10-dideazatetrahydrofolates, it was discovered that GARFT inhibition is highly dependent upon the polyglutamation status of inhibitors (2). The triglutamate and pentaglutamate of LY231514 had significantly enhanced inhibitory activity against GARFT, with K_i values of 380 nM (24-fold) and 65 nM (144-fold), respectively. This makes the pentaglutamate of LY231514 a potentially potent inhibitor of purine *de novo* biosynthesis. In comparison, ZD1694 and its polyglutamates showed extremely weak inhibitory activity against GARFT. The K_i values of ZD1694, ZD1694-glu$_3$, and ZD1694-glu$_5$ were 424, 104, and 132 μM, respectively (Table 1). This result demonstrates that polyglutamyl derivatives of LY231514 are 300-2000-fold more effective than ZD1694 in inhibiting GARFT, an

Table 1 *Inhibitory activity of LY231514, ZD1694, and their polyglutamates against rhTS, rhDHFR, and rmGARFT*[a]

Compound	K_i value (nM $\pm$ SE; $n \geq 3$)		
	rhTS	rhDHFR	rmGARFT
LY231514	109 $\pm$ 9 ($n = 4$)	7.0 $\pm$ 1.9	9,300 $\pm$ 690
LY231514-(glu)$_3$	1.6 $\pm$ 0.1	7.1 $\pm$ 1.6	380 $\pm$ 92
LY231514-(glu)$_5$	1.3 $\pm$ 0.3	7.2 $\pm$ 0.4	65 $\pm$ 16
ZD1694	6.0 $\pm$ 0.9	45 $\pm$ 3	424,000 (336,000, 513,000)
ZD1694-(glu)$_3$	1.1 $\pm$ 0.3	37 $\pm$ 7	104,000 (81,000, 127,000)
ZD1694-(glu)$_5$	1.4 $\pm$ 0.1	30 $\pm$ 3	132,000 (124,000, 141,000)

[a] See "Materials and Methods" for assay procedures.

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Table 2 *Inhibitory activity of LY231514 and its polyglutamates against hAICARFT and the dehydrogenase and synthetase activities in C1-S*[a]

	K_i value (μM)		
Compound	5,10-methylenetetrahydrofolate dehydrogenase of C1 synthase	10-formyltetrahydrofolate synthetase of C1 synthase	AICARFT
LY231514	9.5 ± 0.9[b]	364	3.58
LY231514-(glu)$_3$	3.7	25	0.48
LY231514-(glu)$_5$	5.0	1.6	0.26

[a] See "Materials and Methods" for procedures.
[b] $\pm$ SD; $n = 3$.

important enzyme along the purine *de novo* biosynthetic pathway. The second folate-requiring enzyme along the purine *de novo* biosynthetic pathway is AICARFT, which uses the same folate cofactor as GARFT, 10-formyl-tetrahydrofolate, as the one carbon donor in purine biosynthesis. A similar trend of enhancement of affinity was observed for LY231514 and its polyglutamates toward hAICARFT. The K_i values observed were 3.58 μM, 480 nM (7.5-fold), and 265 nM (13.5-fold) for the mono-, tri-, and pentaglutamyl derivatives of LY231514, respectively (Table 2).

Finally, LY231514 and its polyglutamates were also found to be competitive inhibitors against both the 5,10-methylenetetrahydrofolate dehydrogenase and 10-formyltetrahydrofolate synthetase activities of C1-S (Table 2). The K_i values for the mono-, tri-, and pentaglutamyl derivatives of LY231514 were 9.5, 3.7, and 5.0 μM, respectively, for dehydrogenase and 364, 25, and 1.6 μM for synthetase. This demonstrates that the effect of polyglutamation of LY231514 on inhibition of dehydrogenase activity is marginal, but is quite significant for inhibition of synthetase activity. This observation is consistent with previous reports on the sensitivity of these two enzymes to polyglutamation status of their respective folate cofactors (35, 36). Based on the K_i values of LY231514 and its polyglutamates, the importance of C1-S as a potential target will be dependent upon the intracellular concentration of drug achieved (see below).

Cell Culture End Products Reversal Studies. Previous studies demonstrated that the antiproliferative activity of LY231514 was prevented by leucovorin but incompletely reversed by thymidine (10, 11). This suggested that aside from TS, additional enzymatic targets for this antifolate compound exist. We have now further characterized the reversal pattern of LY231514 and ZD1694 in various human tumor cell lines, including CCRF-CEM leukemia, GC3/C1 colon carcinoma, and HCT-8 ileocecal carcinoma. It was observed that 5 μM thymidine fully protected these cells from cytotoxicity with ZD1694 (Table 3). In sharp contrast, similar treatment with thymidine (5 μM) only increased the IC$_{50}$ of LY231514 *versus* CCRF-CEM cells by 5.5-fold, GC3/C1 by 18.7-fold, and HCT-8 by 15-fold. It is interesting to note that thymidine alone produced its greatest protective effect at or near the IC$_{50}$ of LY231514 (Fig. 3). In contrast, higher drug

concentrations of LY231514 required the combination of both thymidine (5 μM) plus hypoxanthine (100 μM) to protect CCRF-CEM cells. Moreover, the combination of thymidine plus hypoxanthine totally reversed the cytotoxicity exerted by LY231514 in all three cell lines (IC$_{50}$ values > 40 μM; Table 3). Hypoxanthine (100 μM) or aminoimidazole carboxamide (300 μM) alone did not markedly influence cytotoxicity by LY231514 (except for HCT-8 cells, in which a 5-fold
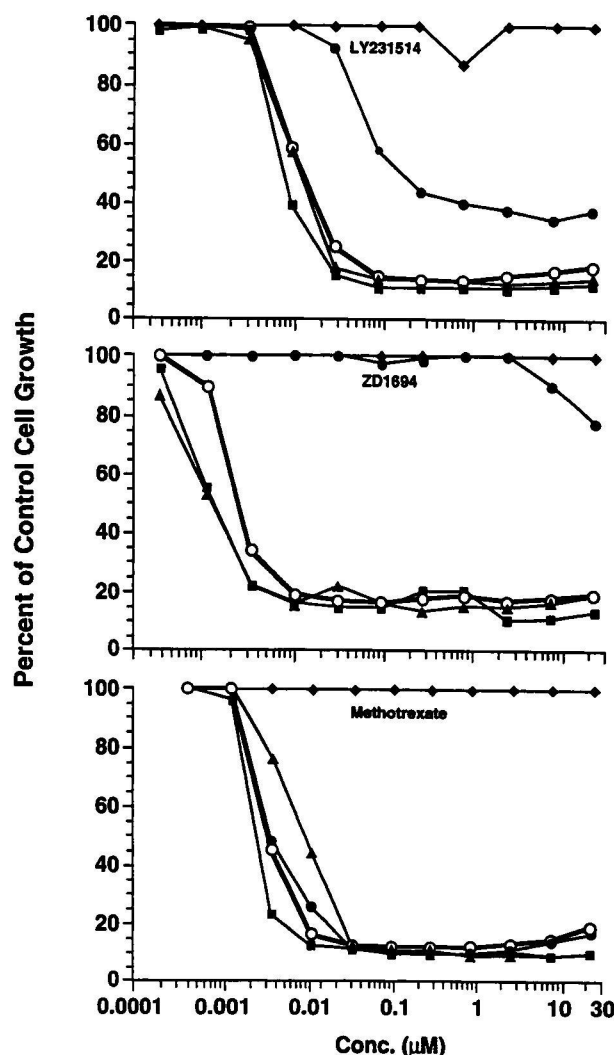

Fig. 3. End product reversal studies of LY231514, ZD1694, and methotrexate in CCRF-CEM human leukemia cells. The indicated concentrations of these compounds were incubated with cells for 72 h in the presence of a source of purines and/or thymidylate: no additions (○); 5 μM thymidine (●); 100 μM hypoxanthine (▲); 300 μM AICA (■); or 5 μM thymidine plus 100 μM hypoxanthine (◆). The reversal study of LY231514 was disclosed previously (11).

Table 3 *End products reversal studies with LY231514 and ZD1694*[a]

	IC$_{50}$ of compound (nM)			
Cell line	Alone	+5 μM dThd	+100 μM hypoxanthine	+dTHd and hypoxanthine
LY231514				
CCRF-CEM	25	138	32	>40,000
GC3/C1	34	637	34	>40,000
HCT-8	220	3104	1077	>40,000
ZD1694				
CCRF-CEM	15	>40,000	13	>40,000
GC3/C1	4	>40,000	4	>40,000
HCT-8	65	>40,000	44	>40,000

[a] Cytotoxicity determined by MTT analysis after 72 h exposure to drug.
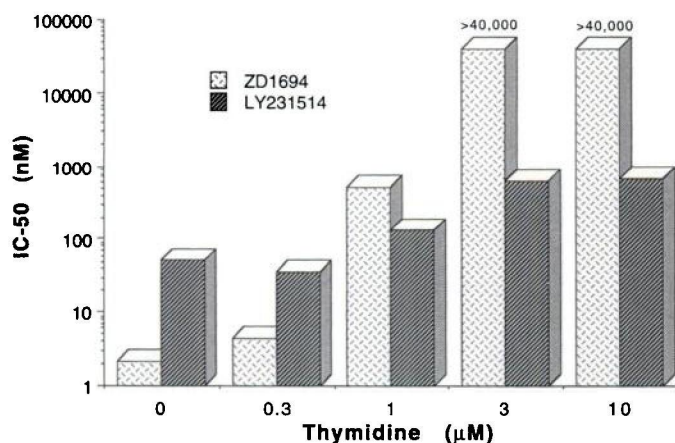SE of triplicate determinations did not exceed 10% of mean.

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Fig. 4. Effect of thymidine on cytotoxicity of LY231514 and ZD1694 against GC3/C1 human colon carcinoma cells.

Table 4 *Antiproliferative activity of various antifolates against a 5,10-dideazatetrahydrofolate-resistant CCRF-CEM subline (CR15) and relative efficiency as substrate for FPGS*

Compound	CCRF-CEM IC_{50} $(nM)^{a}$	CR15 IC_{50} $(nM)^{a,b}$	FPGS $(V_{max}/K_m)^c$
LY231514	25.4	>200,000	549
ZD1694	15.3	30,657	495
LY249543 (lometrexol)	9.7	>200,000	60
Methotrexate	4.2	336	4

[a] Cytotoxicity determined by MTT analysis after 72 h exposure to drug. SE of triplicate determinations did not exceed 10% of mean.

[b] CR15 cells, generously provided by Dr. G. P. Beardsley (Yale University, New Haven, CT), have markedly diminished capacity to accumulate 5,10-dideazatetrahydrofolic acid polyglutamates (their FPGS activity is approximately 10% of the wild type).

[c] Hog liver FPGS data taken from Ref. 17.

decrease in potency was observed with the addition of 100 μM of hypoxanthine). In GC3/C1 cells, the physiological concentration of thymidine in mouse plasma (1.0 μM; Ref. 24) was significantly more effective in reversing the cytotoxicity of ZD1694 than LY231514 (Fig. 4). The distinctively different reversal pattern exerted by thymidine on these two agents indicates that whereas TS may be the sole target for ZD1694, it is likely that there are other inhibitory sites for LY231514. The cell culture reversal pattern of LY231514 in CCRF-CEM cells was also distinctly different from that of methotrexate (no protection by thymidine alone; Fig. 3) and the GARFT inhibitor lometrexol (strong protection by hypoxanthine alone; data not shown; Ref. 3). These studies suggest that inhibition of DHFR and/or other enzymes along the purine *de novo* pathway may play major roles to the overall cytotoxic action of LY231514.

Role of Polyglutamation in Cytotoxicity. LY231514 has previously been demonstrated to be an exceptionally efficient substrate for FPGS (11, 12). To evaluate the role of FPGS in the cytotoxic activity of LY231514 and ZD1694, we used CR15 cells, a lometrexol-resistant CCRF-CEM subline. This subline has previously been shown to have a markedly diminished capacity to accumulate lometrexol polyglutamates and has approximately 10% of the FPGS activity of wild type cells (22). Impaired polyglutamation in CR15 cells was identified as the mechanism of resistance to lometrexol, and the cells have normal reduced folate transport activity and normal levels of the target enzyme, GARFT. We observed that CR15 cells were markedly cross-resistant to both LY231514 and ZD1694 (Table 4), suggesting that polyglutamation is a major determinant of cytotoxicity for both compounds. CR15 exhibited 7874-fold resistance to LY231514 and 2003-fold resistance to ZD1694. In comparison, methotrexate exhibited significantly less resistance in this polyglutamation-impaired subline compared to parent CEM cells.

Transport Mechanisms for Cytotoxic Activity. The roles of the RFC and FBP-α in the cytotoxic activity of LY231514 and ZD1694 were determined by using ZR-75-1 human breast carcinoma sublines that differ in expression of RFC and FBP-α (23). Wild-type ZR-75-1 cells express RFC as the major transport route for natural reduced folate cofactors and antifolate compounds and do not express detectable levels of FBP-α. The predominant role of RFC in transport of these compounds is illustrated by the fact that wild-type ZR-75-1 cells with or without transfected FBP-α were much more sensitive to drug cytotoxicity than sublines resistant to methotrexate through decreased RFC expression (Table 5). These results indicated that both LY231514 and ZD1694 are less dependent on FBP-α as the major route for internalization.

DISCUSSION

The antiproliferative activity of the "classical" antifolates depends not only on their ability to interact with intracellular folate-requiring enzyme target(s), but also on their cellular transport properties and their degree of polyglutamation. Polyglutamation, in particular, plays an essential role in determining the overall biochemical and pharmacological profiles of any given antifolate (37). The formation of polyglutamated metabolites of folates and antifolates results in the intracellular accumulation of polyglutamated metabolites to levels that are significantly higher than could otherwise be achieved at steady state by the parent compounds, and thus serves as an important cellular retention mechanism for folates and antifolates (38, 39). In addition, the resulting polyglutamates often demonstrate orders of magnitude of increased affinity toward certain target enzyme(s) (6, 32, 40). Polyglutamation may also lead to increased inhibition of other folate-dependent enzyme(s) for which the parent compounds had little or no apparent affinity.

A well-documented example of a classical antifolate is methotrexate, which was first identified as an extremely potent inhibitor of DHFR. Chabner *et al.* (41) demonstrated that methotrexate polyglutamates exhibit potent inhibition of both TS (42) and AICARFT (43), whereas the parent compound, methotrexate, had much less activity. Given the fact that methotrexate polyglutamates can accumulate in drug-sensitive cells to significantly high concentrations (estimated to be in the range of 1–10 μM in drug-sensitive cells; Refs. 38, 41, and 44–46), it is reasonable to assume that the polyglutamates of methotrexate can effectively inhibit several key enzyme systems (DHFR, TS, and AICARFT). Effective polyglutamation of methotrexate and accumulation of intracellular polyglutamates have transformed methotrexate into an agent that inhibited multiple enzymes of folate metabolism. It has been suggested that the increased activity of methotrexate polyglutamates toward other distal folate targets may be an important determinant both for its sensitivity and for selectivity in normal *versus* malignant tissues.

LY231514 is a novel pyrrolo[2,3-d]pyrimidine-based antifolate. Previous studies have demonstrated that LY231514 is one of the best

Table 5 *Antiproliferative activity of LY231514 and ZD1694 against ZR-75-1 human breast carcinoma cell lines with differing transport characteristics*[a]

Cell line	Transport	IC_{50} (nM) LY231514	IC_{50} (nM) ZD1694
Wild type	RFC+, FBP−	110.2	27.5
Wild type AA6-FR+	RFC+, FBP+	22.7	9.6
MTX^R	RFC−, FBP−	429.9	1763.2
MTX^R-BB3-FR+	RFC−, FBP+	1190.6	>20,000

[a] Cytotoxicity determined after 72 h drug exposure by MTT assay. Assay medium contained 2 nM folinic acid as the sole folate source. SE of triplicate determinations did not exceed 10% of mean. The ZR-75-1 sublines were generously provided by Dr. K. Cowan (National Cancer Institute, Bethesda, MD).

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substrates that is known for the enzyme FPGS (12). *In vitro* incubation (8–24 h) of LY231514 with hog liver FPGS effectively converted LY231514 to its longer-chain polyglutamates (glu$_4$ and glu$_5$; data not shown). Whereas the parent compound LY231514 demonstrated only a moderate level of inhibition (K_i = 340 nM) against TS, the pentaglutamate of LY231514 was 100-fold more potent (K_i = 3.4 nM) in inhibiting the rmTS and correlated better with its antiproliferative activity (IC$_{50}$ = 16 nM for CCRF-CEM cells) observed in whole cell assays (11). These data suggest that LY231514 is behaving very much like a classical antifolate, which depends highly on active membrane transport and polyglutamation as part of the activation and retention mechanism for achieving its therapeutic effects. It is thus reasonable to assume that LY231514 is a prodrug and that the polyglutamated metabolites are the responsible active species inside cells.

Two biochemically distinct transport systems, the high-affinity FBP-α and the lower-affinity RFC, have been implicated in tumor cell membrane transport of folates and antifolates (47–49). Because membrane transport is the first limiting step in the chemotherapeutic efficacy of folate analogues, and different expression levels of RFC and FBP-α are being recognized in normal and neoplastic tissues, a thorough knowledge of the relative role of each of these transport systems in the antitumor efficacy of candidate antifolates may be useful for the clinical development of novel antifolates. Westerhof *et al.* (50) have demonstrated that LY231514 and ZD1694 were efficiently transported via both transport pathways using a panel of murine L1210 leukemia cells with differing transport properties. To further investigate the mechanism(s) for LY231514 transport, we used a panel of ZR-75-1 human breast carcinoma sublines prepared by Dixon *et al.* (23) with different transport characteristics. The MTX-resistant ZR-75-1 cells that are deficient in RFC activity demonstrate 3.9-fold and 64.1-fold cross-resistance to LY231514 and ZD1694, respectively, compared to wild-type cells. Neither cell line contains detectable FBP-α. Expression of FBP-α in wild-type cells produced 4.8-fold and 2.9-fold increased sensitivity to LY231514 and ZD1694. Moreover, ZR-75-1 cells that express FBP-α but lack RFC activity (MTX^R-BB3-FR+) showed markedly decreased sensitivity toward both LY231514 and ZD1694, further suggesting a predominant role for RFC in transport of both antifolates. Differences in the involvement of FBP-α in antifolate growth-inhibitory activity between our studies and those of Westerhof *et al.* (50) may be related to the use of human *versus* murine tumor cell lines. In addition, we have recently noted that RFC is the preferential route of entry for antifolate compounds, even when mFBP-α is expressed to very high levels (51).

Pizzorno *et al.* (22) described the development and mechanisms of resistance of CCRF-CEM human lymphoblastic leukemia sublines resistant to increasing concentrations of lometrexol. The primary mechanism of resistance detected in these studies appears to be the significantly diminished accumulation of polyglutamate forms of the drug due to decreased FPGS activity. We used one of the lometrexol-resistant CCRF-CEM sublines (CR15; Ref. 22) to further test the role of polyglutamation in the growth-inhibitory activity of various antifolates, including LY231514. This line has normal levels of GARFT and normal reduced folate transport system. We observed that CR15 cells display >7874-fold cross-resistance to LY231514, 2003-fold cross-resistance to ZD1694, and 80-fold cross-resistance to MTX over a 72-h drug exposure period. The degree of resistance to methotrexate following chronic drug exposure was greater than that observed by Pizzorno *et al.* (22), and this may involve additional factors besides polyglutamation, such as levels of DHFR or intracellular pools of reduced folates. However, cross-resistance seems to correlate well with relative efficiency as substrates for FPGS for these antifolate compounds. Similarly, Jackman *et al.* (52) described an L1210 murine leukemia subline resistant to ZD1694 due to diminished FPGS levels

and subsequent inability to accumulate ZD1694 or MTX polyglutamates.

In addition to greater intracellular drug retention through polyglutamation, the cytotoxic activity of LY231514 polyglutamates appears to be enhanced due to increased affinity toward multiple folate-requiring enzymes. Our data now clearly demonstrate that polyglutamates of LY231514 effectively inhibited multiple folate-requiring enzymes, a phenomenon similar to what was observed for methotrexate. LY231514 polyglutamates exhibited tight binding inhibition toward rhTS and rhDHFR, with affinity in the low nanomolar range. LY231514-glu$_5$ also demonstrated high affinity toward GARFT (K_i = 65 nM), making it potentially an effective inhibitor of purine biosynthesis (2). The effective transport and excellent polyglutamation profiles of LY231514 suggested that significantly high levels of LY231514 polyglutamates could be achieved intracellularly. We have found that intracellular concentrations of LY231514 and its polyglutamates can reach to a level of 10–30 μM in CCRF-CEM cells when ^{14}C-labeled LY231514 was used.[3] These high intracellular drug concentrations could result in effective inhibition of multiple enzymes (TS, DHFR, and GARFT). At these high intracellular drug concentrations, other enzymes with K_i values in the micromolar range, including C1-S and AICARFT, may also be inhibited by LY231514 polyglutamates. This simultaneous inhibition of multiple folate-dependent enzymes (TS, DHFR, GARFT, C1-S, and AICARFT) would then lead to a major disturbance of intracellular reduced folate pools and result in significant decreases in pyrimidine and purine biosynthesis.

The cell culture end product reversal pattern of LY231514 was significantly different from those of ZD1694 and methotrexate (Table 3 and Fig. 3). The distinctively different reversal pattern exerted by thymidine indicated that although TS may be the sole target for ZD1694, it is likely that there are other important inhibitory sites for LY231514. The higher degree of protection by thymidine at low drug concentrations indicated that TS is a major target for LY231514. Addition of hypoxanthine together with thymidine fully reversed the cytotoxicity of LY231514, suggesting that at higher concentrations, inhibition of DHFR and/or purine *de novo* biosynthetic enzymes were responsible for other secondary cytotoxic actions of the drug. The reversal pattern of LY231514 was also significantly different from that of methotrexate (Fig. 3). Thymidine alone did not protect the cells from the cytotoxic effect of methotrexate at all drug concentrations. The affinity of methotrexate for DHFR (K_i = 5 pM) was several orders of magnitude higher than its affinity for TS (K_i = 0.047 μM for MTX-glu$_5$), suggesting that the primary intracellular target of methotrexate may still be DHFR and not TS.

Knowledge from *in vitro* studies of individual folate-dependent enzymes by antifolates have been incorporated into metabolic models that describe folate cycle kinetics in murine (53) and human (54, 55) systems for the purpose of evaluating multiple folate enzyme inhibition by methotrexate polyglutamates. A clear understanding of the relationship between the intracellular pools of reduced folates and LY231514 polyglutamates under various drug exposure and rescue conditions will be tremendously useful in assessing the relative significance of inhibiting each individual enzyme by LY231514 and its metabolites (41).

In summary, through enzymatic and cellular studies, we have demonstrated that as a result of polyglutamation, LY231514 can achieve high enough intracellular concentrations that it may drastically affect folate metabolism through blockade at TS, DHFR, and GARFT, and to a lesser extent at AICARFT and C1-S. The combined

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[3] R. M. Schultz, unpublished observation.

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effects of the inhibition exerted by LY231514 at each target gives rise to an unusual end product reversal pattern at the cellular level that is distinct from those of other inhibitors such as methotrexate and the quinazoline antifolates. This may explain the encouraging Phase I results of activity in advanced stages of colorectal and pancreatic cancer (14). A broad Phase II program is currently under way to investigate a variety of resistant solid tumors, including colorectal, breast, non-small cell lung, pancreatic, and other gastrointestinal tumors.

ACKNOWLEDGMENTS

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