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MULTIPLE FOLATE ENZYME INHIBITION: MECHANISM OF A NOVEL PYRROLOPYRIMIDINE-BASED ANTIFOLATE LY231514 (MTA)

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INTRODUCTION

Since the late 1950 s, extensive research efforts have been devoted to the discovery and development of antifolate antimetabolites as chemotherapeutic agents for the management of neoplastic diseases. However, it was in the last 10 to 15 years, due to the rapid advances of medicinal chemistry, x-ray protein crystallography, molecular biology, pharmacology and clinical medicine, that a significant number of new generation antifolates were brought forward for clinical development. Several folate-based antimetabolites are currently being investigated in clinical trials. These include Lometrexol (6R-5,10-dideazatetrahydrofolic acid, DDATHF) (1-3), LY309887 (4) and AG2034 (5), which exhibit potent and selective inhibition of glycinamide ribonucleotide formyltransferase (GARFT, EC 2.1.2.2) of the purine *de novo* biosynthetic pathway; trimetrexate (6), edatrexate (7, 8) and PT523 (9) which act on dihydrofolate reductase (DHFR,

Abbreviations: r, recombinant; h, human; m, murine; TS, thymidylate synthase; DHFR, dihydrofolate reductase; GARFT, glycinamide ribonucleotide formyltransferase; AICARFT, aminoimidazole carboxamide ribonucleotide formyltransferase (EC 2.1.2.3); C1-S, C1 tetrahydrofolate synthase; D/C, the protein domain of C1-S containing the 5; 10-methylenetetrahydrofolate dehydrogenase (EC 1.5.1.5) and 5,10-methenyltetrahydrofolate cyclohydrolase activities; D/C/S, the full length enzyme of C1-S containing 5; 10-methylenetetrahydrofolate dehydrogenase, 5; 10-methenyltetrahydrofolate cyclohydrolase and 10-formyltetrahydrofolate synthetase activities (EC 6.3.4.3); FPGS, folylpolyglutamate synthetase; HEPES, N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]; MTT,3-[4; 5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide; DDATHF, 5; 10-dideazatetrahydrofolic acid; Lometrexol, 6R-DDATHF; ME, mercaptoethanol; NADPH, β-nicotinamide adenine dinucleotide phosphate; reduced form; ATP, adenosine 5′-triphosphate; 6R-MTHF, 6[R]-5; 10-methylene-5,6; 7,8-tetrahydrofolate; LY231514, N-[4-[2-(2-amino-3; 4-dihydro-4-oxo-7H-pyrrolo[2,3-d]pyrimidin-5-yl)ethyl]-benzoyl]-L-glutamic acid; MTA, multitargeted antifolate.

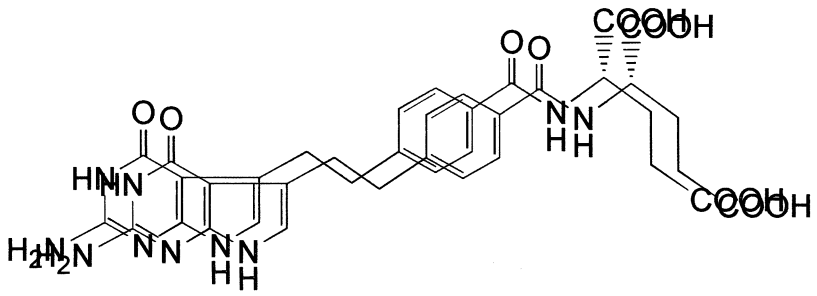

FIG. 1. The structure of N-[4-[2-(2-amino-3,4-dihydro-4-oxo-7H-pyrrolo[2,3-d]pyrimidin-5-yl)ethyl]-benzoyl]-L-glutamic acid, LY231514.

EC 1.5.1.3); Tomudex® (10, 11), AG337 (12), BW1843U89 (13) and ZD9331 (14) which target specifically at the enzyme thymidylate synthase (TS, EC 2.1.1.45) of the pyrimidine biosynthesis.

N-[4-[2-(2-amino-3,4-dihydro-4-oxo-7H-pyrrolo[2,3-d]pyrimidin-5-yl)-ethyl]-benzoyl]-L-glutamic acid, LY231514 (Multitargeted Antifolate, MTA), is a structurally novel antifolate antimetabolite that possesses the unique 6-5 fused pyrrolo[2,3-d]pyrimidine nucleus instead of the more common 6-6 fused pteridine or quinazoline ring structure (15) (Fig. 1). As a "classical" antifolate, LY231514 was found to be one of the best substrates for the mammalian folylpolyglutamate synthetase (FPGS, EC 6.3.2.17) and it is believed that polyglutamation and the polyglutamated metabolites of LY231514 play profound roles in determining both the selectivity and the antitumor activity of this novel agent (15, 16). 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. Both thymidine (5 μM) and hypoxanthine (100 μM) were required to fully protect cells from the growth inhibitory activity exerted by LY231514 (15). This reversal pattern is significantly different from other known antifolates such as methotrexate, Tomudex® and the GARFT inhibitor DDATHF, and suggests that TS is only partially responsible for the antiproliferative action 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. This report summarizes our findings on the polyglutamation profile of LY231514, the activity of LY231514-polyglutamates (glu3 and glu5) against various folate-requiring enzymes, the cell culture rever-

sal and cross-resistance pattern of LY231514 and, finally, the effects of LY231514 on cellular folate and nucleoside triphosphate pools.

MATERIALS AND METHODS

Materials

Methotrexate, hypoxanthine and thymidine were obtained from Sigma Chemical Company, St. Louis, MO. Methotrexate polyglutamates were purchased from Dr. Schirks Laboratories, Jona, Switzerland. LY231514 and Lometrexol were prepared at Lilly Research Laboratories, Indianapolis, IN. The syntheses of the γ-glutamyl derivatives of LY231514 were by the method of Pawelczak *et al.* (17). L-[^{14}C(U)]-glutamate (NEC-290E) was acquired from Dupont NEN, Boston, MA. The Enzfitter microcomputer package was purchased from Biosoft, Ferguson, MO. Human CCRF-CEM lymphoblastic leukemia cells were obtained from St. Jude Children's Hospital, Memphis, TN. Human GC3/C1 cells were obtained from Dr. Janet Houghton of St. Jude Children's Hospital, Memphis, TN. MCF_{TDX}, H630, $H630_{TDX}$, $H630_{R10}$ cells were obtained from Dr. P. J. Johnston, Department of Oncology, Queen's University, Belfast, North Ireland. HCT-8 cells were purchased from the American Type Culture Collection, Rockville, MD. 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; trifunctional mGARFT from Dr. R. G. Moran of Medical College of Virginia, Richmond, VA; rhDHFR from Dr. M. Ratnam of Medical College of Ohio, Toledo, Ohio and Anatrace Co. (Maumee, OH). Two forms of rhC1 tetrahydrofolate synthase were obtained from Dr. R. E. Mackenzie of McGill University, Montreal, Canada: these are (A) the 101kD D/C/S full length trifunctional enzyme, containing the activities of the dehydrogenase, cyclohydrolase, and synthetase activities and (B) the D/C domain, the 35kD truncated version of C1-S which contained only the dehydrogenase and cyclohydrolase activities.

Determination of Kinetic Constants for Hog Liver FPGS and Substrate

Vmax and Michaelis constants for the conversion of folate analogues to their corresponding diglutamate forms were determined using FPGS purified from mouse (18) and hog liver. Purification of the hog liver FPGS was carried out through chromatographing column step as previously described by Cichowicz and Shane (19). The pooled activity peak was dialyzed against 100 mM Tris-HC1, 50 mM β-ME, 20% glycerol, pH 8.4, aliquoted, and stored at −70°C. An approximate 8000-fold purification was

achieved with a specific activity of 110 nmol/hr/mg when assayed with 200 μM MTX and 250 μM L-[^{14}C]-glutamate. Km and Vmax values were determined using 5 to 6 folate analogue concentrations in duplicate per experiment. The assay conditions were 100 mM Tris, 10 mM $MgCl_2$, 5 mM ATP, 20 mM KCl, 100 mg/ml BSA, 100 mM β-ME, 1 mM L-[^{14}C]-glutamate (4 μCi/μmol), pH 8.9, in a final vol of 0.25 or 1.0 ml at 37°C. The amount of protein added (1–6 μg) and incubation time (1–3 hr) varied to minimize the formation of higher polyglutamates at low substrate concentration, and were determined to be in the linear range. Reactions were halted by addition of ice-cold 10 mM L-glutamate, pH 7.5. Labeled and unlabeled substrate was separated from unincorporated [^{14}C]-glutamate by binding and elution from Waters Sep-Pak Plus C18 cartridges as previously described by Jansen *et al*. (20). Km and Vmax values were determined by non-linear fitting the data to a rectangular hyperbola using the Enzfitter microcomputer package.

In Vitro Polyglutamation and Separation of Polyglutamates by HPLC

Using the assay conditions listed above, the *in vitro* polyglutamation of selected folate analogues was examined. Reactions consisted of 1 or 20 μM folate substrate, 2 μg of protein, 0.25 ml final vol, and were incubated for 1, 8, or 24 hr aseptically. Reactions were halted by boiling for 3 min, and were centrifuged at 14,000 × *g* for 10 min to remove particulates prior to injection of 100 μl of sample. Folylpolyglutamates were separated and quantitated using a modification of the reversed-phase HPLC method described by Montero and Llorente (21). Our protocol utilized a 4.6 mm × 25 cm Beckman Ultrasphere IP 5 μM column, 1.5 ml/min flow rate, and the following gradient elution: (1) 100% A (0.1 M ammonium acetate pH 5.5 + 1% acetonitrile) for 5 min, (2) linear gradient to 9% B (acetonitrile) over 20 min (3) 5 min at 9% B, (4) linear gradient to 100% B over 5 min, (5) linear gradient to 100% A over 5 min, (6) 2 min at 100% A (total run time 42 min). The amount of polyglutamate product formed was calculated from the [^{14}C]-glutamate incorporated in the radioactive peaks, and was corrected for the increasing specific activity of increasing glutamate chain length.

Enzyme Assays and Methods

The Ki values for TS, DHFR, GARFT, AICARFT and C1 tetrahydrofolate synthase and the tight binding kinetic analysis for LY231514-glu3 for TS and DHFR were determined as described previously (22).

In Vitro Cell Culture Studies

Dose response curves were generated to determine the concentration required for 50% inhibition of cell 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 vol of 2.0 ml. Test compounds at various concentrations were added to duplicate wells so that the final vol of DMSO was 0.5%. The plates were incubated for 72 hr at 37°C in a 5% CO_2-in-air atmosphere. At the end of the incubation, cell numbers were determined on a ZBI Coulter counter. Control wells usually contained $4–6 \times 10^5$ cells at the end of the incubation. For several studies, IC_{50}s were determined for each compound in the presence of either AICA (300 μM), thymidine (5 μM), hypoxanthine (100 μM), or combination of thymidine (5 μM) plus hypoxanthine (100 μM).

For adherent tumor cells, we used a modification of the original MTT colorimetric assay described by Mosmann (23) 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-bottom tissue culture plates (Corstar, Cambridge, MA). Assay medium contained folic acid-free RPMI-1640 medium supplemented with 10% fetal calf serum 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 phosphate-buffered saline (PBS) at 1 mg/ml, and a series of 2-fold dilutions were subsequently made in PBS. Ten microliter aliquots of each concentration were added to triplicate wells. Plates were incubated for 72 hr 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 was added to all wells of an assay, and the plates were incubated at 37°C for two additional hours. Following incubation, 100 μl DMSO was 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 control.

Folate and Nucleotide Pool Studies

For the folate pools study, the CCRF-CEM cells ($2–5 \times 10^5$ per ml) were labeled with 100 nM of ^{3}H-folinic acid (20 Ci/mmol) for 16 hr, then the drugs were added and incubation was allowed for the indicated duration. The extraction and analyses of folate pools were conducted according to the methods published by Wilson and Horne (24). For the nucleotide pools study, CCRF-CEM cells were seeded in fresh complete medium at 3×10^5 cells per ml and cultured for 12 to 16 hr before drug

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