Biochemical Pharmacology, Vol. 39, No. 12, pp. 2019–2025, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

GROWTH INHIBITION BY HOMOFOLATE IN TUMOR CELLS UTILIZING A HIGH-AFFINITY FOLATE BINDING PROTEIN AS A MEANS FOR FOLATE INTERNALIZATION

GARY B. HENDERSON* and BRIAN P. STRAUSS

Department of Molecular and Experimental Medicine, Scripps Clinic and Research Foundation, La Jolla, CA 92037, U.S.A.

(*Received* 7 *August* 1989; *accepted* 21 *November* 1989)

Abstract—A subline (JT-1) of L1210 mouse leukemia cells that contains elevated levels of a high-affinity folate binding protein is sensitive to growth inhibition by homofolate. Inhibition was observed at nanomolar concentrations of folate or 5-formyltetrahydrofolate where the high-affinity binding protein is the predominant uptake route for folate compounds. At 1.0 nM folate, inhibition of growth by 50% occurred at 0.7 nM homofolate, and maximal inhibition exceeded 90% at homofolate concentrations above 10 nM. Homofolate also inhibited the uptake of 1.0 nM [^{3}H]folate by L1210/JT-1 cells in 72-hr cultures, and the extent of uptake inhibition by 1.0 and 20 nM homofolate was comparable to the inhibition of cell growth by the same concentrations of homofolate. At a growth-limiting concentration of 5-formyltetrahydrofolate (0.5 nM), half-maximal inhibition of L1210/JT-1 cell growth occurred at 1.0 nM homofolate. When excess concentrations of folate (5 μM) or 5-formyltetrahydrofolate (0.5 μM) were added to the medium, no growth inhibition was observed for homofolate at concentrations up to 100 μM. Parental cells lacking the folate binding protein did not respond to homofolate either at growth-limiting (0.5 μM) or excess (5.0 μM) levels of folate. Binding measurements showed that homofolate has a high affinity for the folate-binding protein (K_i = 0.03 nM) but interacts poorly with the reduced-folate transport system (K_i = 203 μM). These results indicate that homofolate inhibits the growth of L1210 cells when intracellular folates are acquired via the high-affinity folate binding protein. The basis for this inhibition appears to be competition by homofolate for substrate binding and internalization.

Tumor cells become resistant to methotrexate by a variety of mechanisms including decreased cellular uptake via carrier-mediated transport systems [1–3]. The usual function of these transport systems is to mediate the uptake of folate compounds, but flexibility in binding specificity is generally observed which allows most systems to also transport folate analogs such as methotrexate. As a consequence, resistance to methotrexate that occurs via decreased transport has a high likelihood of also reducing the uptake of folate compounds needed for cell growth. Survival of tumor cells, therefore, hinges on compromising the transport of methotrexate without eliminating the ability of cells to acquire sufficient folate cofactors for growth. A mechanism by which tumor cells might achieve these transport changes is to selectively down-regulate a system which transports methotrexate with a high efficiency and to utilize a second transport system which shows a lower transport efficiency for methotrexate.

Sublines of mouse L1210 cells have been isolated which grow at low concentrations of folate or 5-formyltetrahydrofolate and have acquired elevated levels of a high-affinity folate binding protein [4, 5]. Similar high-affinity binding proteins have been observed previously in KB [6, 7], MA104 [8], and various other [6] cells and have been implicated in the cellular internalization of folate compounds. Transport via this binding protein was proposed as the basis for growth of L1210 cells under low folate conditions [4]. The binding protein in L1210 cells exhibits a high affinity for folate (K_d = 0.07 nM), 5-methyltetrahydrofolate (K_i = 13 nM) and 5-formyltetrahydrofolate (K_i = 45 nM) relative to methotrexate (K_i = 325 nM) [4]. This preference for folate and reduced folate compounds relative to methotrexate is much different from the specificity of the reduced-folate carrier system of L1210 cells [2, 9, 10], which transports methotrexate and reduced-folate compounds with about equal facility (K_t = 1–5 μM) but is much less able to transport folate (K_t = 100–200 μM). These differences in specificity suggest that resistance to methotrexate should develop in sublines of L1210 cells which down-regulate the reduced-folate carrier system and acquire folate compounds by a compensating up-regulation of the high-affinity folate-binding protein system.

Antifolate compounds which can utilize or interfere with the activity of high-affinity folate binding proteins may be useful in killing tumor cells which rely on these binding proteins for folate acquisition. One possible folate analog with these characteristics is homofolate, which contains an additional methylene group between the C-9 and N-10 positions of folate [11]. Reduction of homofolate to tetrahydrohomofolate occurs via dihydrofolate reductase in mouse leukemia cells [12], and this leads to folate coenzyme analogs which inhibit purine [13] and pyrimidine [14] biosynthesis. The efficacy, however, of homofolate [12, 13, 15, 16], dihydrohomofolate [12, 16], tetrahydrohomofolate [12, 13, 16, 17], and

* Send correspondence to: Dr. Gary B. Henderson, Department of Molecular and Experimental Medicine, Scripps Clinic and Research Foundation, 10666 North Torrey Pines Road, La Jolla, CA 92037.

5-methyltetrahydrohomofolate [17] in killing either sensitive or methotrexate-resistant tumor cells is low compared to other antifolate compounds, presumably due to inefficient conversion to polyglutamate forms [18, 19]. In the present study, homofolate was examined as a possible means for inhibiting the growth of a subline of L1210 cells (L1210/JT-1) [4] under conditions where folate acquisition occurs via the high-affinity folate binding protein. Homofolate was found to be a potent growth inhibitor under these conditions and the mechanism of inhibition appears to involve competition for folate internalization via the high-affinity binding protein.

MATERIALS AND METHODS

Chemicals. [3′,5′,7,9-^{3}H]Folic acid (40 Ci/mmol) and [3′,5′,7-^{3}H]methotrexate (20 Ci/mmol) were obtained from Moravek Biochemicals (Brea, CA) and stored at −80°. Folic acid, [6*R*,6*S*]-5-formyltetrahydrofolate (folinate), methotrexate, and HEPES* were obtained from the Sigma Chemical Co. (St. Louis, MO). Homofolate was a gift of Dr. John H. Mangum, Brigham Young University.

Purification of labeled substrates. [^{3}H]Folate (24,000,000 cpm/nmol) was purified on Baker-flex cellulose sheets at 23° in 50 mM sodium HEPES, pH 7.5, as described previously [20] and stored in HEPES-buffered saline (HBS) containing 2% ethanol for up to 2 weeks at −20°. [^{3}H]Methotrexate (100,000 cpm/nmol) was purified by the same procedure and stored for up to 6 weeks at −20°.

Growth of cells. Parental L1210 mouse cells were grown in RPMI 1640 medium supplemented with 2.5% fetal bovine serum and 100 units each of penicillin and streptomycin. Folate-sufficient L1210 cells were grown in folate-free RPMI 1640 medium supplemented with 2.5% folate-depleted serum [4], antibiotics, and 500 nM folate. L1210/JT-1 cells were grown in folate-free RPMI 1640 medium supplemented with folate-depleted serum, antibiotics, and 1 nM folate. Folate-depleted L1210 and L1210/JT-1 cells were derived from folate-sufficient cells after growth for 3–4 generations (one transfer) in folate-free medium. Large cultures (500 mL) of L1210/JT-1 cells were grown in sealed 1-liter flasks with gentle shaking at 37°. Cells were harvested by centrifugation at 1000 *g* (5 min, 4°), washed with HBS, 20 mM HEPES, 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, pH 7.4, with NaOH, and suspended to a density of 3×10^7/mL.

The growth response of cells to various concentrations of homofolate or methotrexate was measured in 96-well plates by the procedure of Mosmann [21]. Each well contained 1000 cells and the desired additions in a final volume of 150 μL. Plates were developed with a tetrazolium dye assay after 4–5 days and were quantified in a plate reader at 570 nm [21]. Data points in growth studies represent the mean of three separate experiments performed in duplicate. Standard deviations were less than 20%.

* Abbreviations: HEPES, *N*-hydroxyethylpiperazine-*N*′-ethanesulfonate; and HBS, HEPES-buffered saline.

Transport and binding measurements. Methotrexate transport was measured in duplicate assay mixtures containing L1210/JT-1 cells (3×10^7), the desired additions, [^{3}H]methotrexate (100,000 cpm/nmol), and HBS in a final volume of 1.0 mL. After incubation for 4 min at 37°, the cells were chilled to 0°, diluted with 7 mL of ice-cold saline (160 mM NaCl, 1 mM sodium phosphate, pH 7.4), recovered by centrifugation at 1000 *g* (5 min, 4°), resuspended in 0.5 mL saline, and analyzed for radioactivity. Uptake at 0° served as the control.

Binding of folate was measured in duplicate assay mixtures containing L1201/JT-1 cells (3×10^7/mL), 5 nM [^{3}H]folate (24,000,000 cpm/nmol), the desired additions, and HBS in a final volume of 1.0 mL. After incubation for 10 min at 37°, the cells were chilled to 0°, diluted with 7 mL of ice-cold saline, recovered by centrifugation, and analyzed for radioactivity as described above. Uptake in samples containing 1 μM unlabeled folate served as the control. Protein concentrations were measured by the biuret reaction [22] using bovine serum albumin as the standard. Kinetic constants for transport or binding were the mean of three or more separate experiments whose standard deviations varied by less than 20%.

RESULTS

Effect of homofolate on cell growth. Parental L1210 cells require a relatively high extracellular concentration of folate (above 250 nM) to achieve optimal cell growth, and the basis for this requirement has been attributed to the relatively low capacity of these cells for transporting folic acid [2, 9, 10]. L1210/JT-1 cells, in contrast, have the capacity to grow at nanomolar concentrations of folate [4]. The latter cells contain elevated levels of a high-affinity folate binding protein which is more efficient than the existing reduced-folate transport system in retrieving folate compounds at low extracellular concentrations. Uptake at folate concentrations between 0.5 and 50 nM could be attributed to the high-affinity binding protein, whereas folate acquisition in the 100–2000 nM range occurred predominantly via the reduced-folate carrier system. The acquisition of a high-affinity binding protein system enabled L1210/JT-1 cells to grow at low concentrations of folate, and it also suggested that antifolate compounds which are analogs of folate might be able to inhibit the growth of these cells. One compound selected as a potential inhibitor of high-affinity binding protein function was homofolate.

Homofolate was examined for an effect on the growth of L1210/JT-1 cells over a range of extracellular concentrations of folate or 5-formyltetrahydrofolate. In medium containing folate at a concentration (1.0 nM) where uptake proceeds via the high-affinity folate binding protein (Fig. 1A), L1210/JT-1 cell growth was inhibited 50% at 0.7 nM homofolate, and inhibition exceeded 90% at higher concentrations of homofolate. In contrast, L1210/JT-1 cells grown at a high folate concentration (5 μM) where uptake proceeds primarily via the reduced-folate carrier system exhibited no growth inhibition by homofolate at concentrations up to 100 μM (Fib. 1B). Homofolate was not an effective inhibitor of