Seeman, N. C., Maestre, M. F., Ma, R.-I., & Kallenbach, N. R. (1985) in *The Molecular Basis of Cancer* (Rein, R., Ed.) Progress in Clinical and Biological Research 172A, pp 99-108, Alan Liss, New York.
Seeman, N. C., Chen, J.-H., & Kallenbach, N. R. (1989) *Electrophoresis 10*, 345-354.
Sigal, N., & Alberts, B. (1972) *J. Mol. Biol. 71*, 789-791.
Tullius, T. D., & Dombroski, B. (1985) *Science 230*, 679-681.
Tullius, T. D., & Dombroski, B. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 5469-5473.
Wells, A. F. (1977) *Three-dimensional Nets and Polyhedra* John Wiley & Sons, New York.
Wemmer, D. E., Wand, A. J., Seeman, N. C., & Kallenbach, N. R. (1985) *Biochemistry 24*, 5745-5749.
Williams, R. (1979) *The Geometrical Foundation of Natural Structure*, Dover, New York.

Synthesis and Biological Evaluation of a Fluorescent Analogue of Folic Acid†

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ABSTRACT: A fluorescein derivative of the lysine analogue of folic acid, N^{α}-pteroyl-N^{ϵ}-(4′-fluoresceinthiocarbamoyl)-L-lysine (PLF), was synthesized as a probe for dihydrofolate reductase (DHFR) and a membrane folate binding protein (m-FBP). Excitation of PLF at 282 nm and at 497 nm gave a fluorescence emission maximum at 518 nm. Binding of PLF to human DHFR or human placental m-FBP results in approximately a 20-fold enhancement in the magnitude of the fluorescence emission, suggesting that the ligand interacts with a hydrophobic region on these proteins. Additional evidence suggests that an energy transfer may occur between the pteridine and the fluorescein moieties. PLF binds to the active site of human DHFR since methotrexate (MTX) competes stoichiometrically and the denatured enzyme in the presence of PLF did not exhibit fluorescent enhancement. The dissociation constant for the fluorescein derivative with respect to human DHFR is 115 nM as compared to 111 nM for folic acid. The K_i value for the competitive inhibition of human DHFR by the fluorescent analogue of folic acid is 2.0 μM compared to 0.48 μM for folic acid. PLF was reduced to N^{α}-(7,8-dihydropteroyl)-N^{ϵ}-(4′-fluoresceinthiocarbamoyl)-L-lysine (H_2PLF) and assayed by the enzymatic conversion to the tetrahydro derivative. The K_m value for human DHFR for the dihydrofolate analogue is 2.0 μM. The K_D value for H_2PLF to human DHFR is 47 nM as compared to 44 nM for dihydrofolate. The K_D values for both H_2PLF and PLF indicate that the fluorescein moiety does not significantly affect folate binding in enzyme binary complexes. PLF also binds to a m-FBP on a human leukemia CCRF/CEM-FBP overproducing cell line, resulting in a fluorescence enhancement that is completely abolished in the presence of excess folic acid. There is an absence of fluorescence enhancement when PLF is added to CCRF/CEM cells or CCRF/CEM-7A cells, which overproduce the carrier protein involved in the uptake of reduced folates and MTX, indicating the specificity of PLF toward the m-FBP. The binding of PLF to CCRF/CEM-FBP cells is similar to that of folic acid as demonstrated by [^{3}H]folic acid competition for binding and by a K_D determination of 1.6 nM. CCRF/CEM-FBP cells can be separated from parental CCRF/CEM and from CCRF/CEM-7A cells by fluorescence-activated cell sorting using this fluorescent analogue of folic acid.

Fluorescent analogues of the classical folic acid antagonist methotrexate (MTX)[1] have previously been synthesized and evaluated as probes of dihydrofolate reductase (DHFR)[1] structure and function (Freisheim et al., 1986). X-ray crystallographic studies on DHFR from *Lactobacillus casei* and *Escherichia coli* (Matthews et al., 1977, 1978) and human sources (Oefner et al., 1988; Davies et al., 1990) reveal certain invariant residues in the primary sequence of DHFR which have important functions in substrate and inhibitor binding. An invariant arginine residue in bacterial (Arg 57) and vertebrate DHFRs (Arg 70) is involved in a charge interaction with the α-carboxylate group of the L-glutamate of MTX. The

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[1] Abbreviations: PLF, N^{α}-pteroyl-N^{ϵ}-(4′-fluoresceinthiocarbamoyl)-L-lysine; DHFR, dihydrofolate reductase; FBP, folate binding protein; MTX, methotrexate [(4-amino-4-deoxy-N^{10}-methylpteroyl)glutamic acid]; H_2PLF, N^{α}-(7,8-dihydropteroyl)-N^{ϵ}-(4′-fluoresceinthiocarbamoyl)-L-lysine; H_4PLF, N^{α}-(5,6,7,8-tetrahydropteroyl)-N^{ϵ}-(4′-fluoresceinthiocarbamoyl)-L-lysine; MTX-F, 1-[[(4-amino-4-deoxy-N^{10}-methylpteroyl)-γ-L-glutamyl]amino]-5-[(4′-fluoresceinthiocarbamoyl)amino]pentane; DNS-Cl, 5-(*N*,*N*-dimethylamino)-1-naphthalenesulfonyl chloride; Tris, tris(hydroxymethyl)aminoethane; NADPH, reduced nicotinamide adenine dinucleotide phosphate; FAB-MS, fast atom bombardment mass spectrum; DMF, dimethylformamide; DEPC, diethyl phosphorocyanidate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HBSS, HEPES balanced saline solution; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; NMR, nuclear magnetic resonance; APA, 4-amino-4-deoxy-N^{10}-methylpteroic acid.

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importance of this free α-carboxyl group has been illustrated by studies with α- and γ-substituted modifications of MTX (Montgomery et al., 1979; Piper et al., 1982; Rosowsky et al., 1981a,b). Lysine and ornithine analogues of MTX have been synthesized (Kempton et al., 1982; Kumar et al., 1983a). These analogues possess the essential free α-carboxyl groups for binding to DHFR, and even though they have a positive charge as compared to the glutamate moiety, both analogues are potent inhibitors of DHFR. An advantage of these analogues is that the free amino group provides an excellent attachment point for further substitutions. Although MTX binds in an inverted orientation to DHFR (Matthews et al., 1978) as compared to that of the substrate, the side chains are bound similarly (Birdsall et al., 1977; Oefner et al., 1988; Davies et al., 1990). Therefore, in developing a fluorescent analogue of folic acid, the terminal L-glutamate was substituted by L-lysine and a fluorescein group attached to the free ε-amino group.

MTX-fluorescein (MTX-F)[1] has been synthesized previously (Gapski et al., 1975). It has been shown that MTX-F can act as an intracellular marker for DHFR (Whiteley & Russell, 1979; Henderson et al., 1980) and DHFR overproduction in MTX-resistant cells can be measured by fluorocytometry (Kaufman et al., 1978; Mariani & Schimke, 1984). Previous work from this laboratory has also demonstrated that fluorescent dansyl groups may also be attached to the amino groups of the lysine and ornithine analogues of MTX by using 5-(dimethylamino)naphthalene-1-sulfonyl chloride (DNS-Cl).[1] The detailed synthesis and high-performance liquid chromatography (HPLC)[1] purification procedures have been previously reported (Kumar et al., 1983a,b). The dansylated derivatives of the lysine and ornithine analogues of MTX exhibit fluorescence properties characteristic of the dansyl moiety with an excitation at 328 nm and an emission at 580 nm in aqueous media (Kumar et al., 1983c). Both DNS derivatives are very potent inhibitors of DHFRs from bacterial, avian, and human sources (Kumar et al., 1983a). The binding of these fluorescent analogues to any of these DHFRs results in a 10–15-nm blue shift of the ligand emission maxima and a 2–5-fold enhancement of the emission (Kumar et al., 1983c; Freisheim et al., 1983). The DNS derivative of the lysine analogue of MTX is also a potent inhibitor of murine L1210 DHFR and is only 10-fold less effective than MTX in inhibiting the growth of MTX-sensitive and MTX-resistant, DHFR-overproducing L1210 cells (Susten et al., 1984). This compound competes effectively for [^{3}H]MTX transport with a K_i of 7.02 μM, a value virtually identical with the K_t for MTX in both cell lines (Susten et al., 1984). The analogue is transported rapidly into DHFR-overproducing L1210 cells, where it forms a high-affinity fluorescent complex with intracellular DHFR. The lysine analogue of MTX has also been conjugated to fluorescein (Rosowsky et al., 1982, 1986). This compound, as well as being a fluorescent probe for MTX-resistant cells with elevated DHFR levels, can also be used to distinguish cells that have a defect in MTX transport (CEM/MTX) from MTX-sensitive human leukemic lymphoblasts (CEM cells) by flow cytometry (Rosowsky et al., 1986).

The rationale for the synthesis of the fluorescein derivative of the lysine analogue of folic acid, N^{α}-pteroyl-N^{ϵ}-(4′-fluoresceinthiocarbamoyl)-L-lysine (PLF),[1] was to design a specific probe for membrane folate binding proteins (m-FBP)[1] and other folate-dependent enzymes. This fluorescent folate analogue also binds well to DHFR as does N^{α}-(7,8-dihydropteroyl)-N^{ϵ}-(4′-fluoresceinthiocarbamoyl)-L-lysine (H_2PLF).[1] Use of a fluorescent substrate (H_2PLF) rather than an inhibitor (MTX-F) will allow the future development of a fluorescence assay for DHFR and the synthesis of one-carbon N^{α}-(5,6,7,8-tetrahydropteroyl)-N^{ϵ}-(4′-fluoresceinthiocarbamoyl)-L-lysine (H_4PLF)[1] analogues to probe other folate-dependent enzymes and transport systems. The studies described herein include the synthesis of PLF and H_2PLF, their interaction with DHFR and with a m-FBP, and their utility in the fluorescence-activated cell sorting of various cell subline clones expressing folate transport and binding proteins.

Experimental Procedures

Chemicals and Reagents. [3′,5′,7,9-^{3}H]Folic acid (40 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA). Radioactive folic acid was purified immediately before use by thin-layer chromatography (TLC)[1] using Kodak cellulose sheets with fluorescent indicator (Rochester, NY) according to Henderson et al. (1986). Radiochemical purity of [^{3}H]folic acid was >99% after rechromatography. Nonradioactive folate, folinic acid, ascorbic acid, and tris(hydroxymethyl)aminoethane (Tris)[1] were purchased from the Sigma Chemical Corp. (St. Louis, MO). Dihydrofolate was prepared from commercial folic acid by dithionite reduction (Blakley, 1960). Reduced nicotinamide adenine dinucleotide phosphate (NADPH)[1] was purchased from P-L Biochemicals. MTX was a gift from Dr. John A. R. Mead (Division of Cancer Treatment, NCI), and carboxypeptidase G_1 was a generous gift from Dr. Andre Rosowsky (Dana-Farber Cancer Institute, Boston, MA 02115). The buffer 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)[1] was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN) and guanidine hydrochloride was obtained from Research Organics (Cleveland, OH). All other chemicals and reagents were of the highest quality commercially available.

Synthesis and Purification of PLF. Melting points were determined on a Mel-temp apparatus and are uncorrected. Elemental analyses were performed by Galbraith Laboratories, Knoxville, TN, or Atlantic Microlab, Inc., Atlanta, GA. The UV spectrum of compound **4** (Figure 1) was determined by using a Cary 219 spectrophotometer. High-resolution ^{1}H nuclear magnetic resonance (NMR)[1] spectra were acquired on a Bruker AM-300 spectrometer in the Chemistry Department, University of South Carolina, Columbia, SC. NMR values for chemical shifts are presented in parts per million downfield from Me_4Si as the internal standard, and the relative peak areas are given to the nearest whole number. The fast atom bombardment mass spectrum (FAB-MS)[1] of compound **4** was determined on a Varian MAT 731 spectrometer. TLC analyses were performed by using silica gel media (Baker Si250F), cellulose (Kodak 13254), or reverse phase (Whatman KC18F). Fluorescein 5-isothiocyanate (FITC, "isomer I") was obtained from Molecular Probes, Inc. (Eugene, OR) while N^{ϵ}-[(*tert*-butyloxy)carbonyl]-L-lysine *tert*-butyl ester hydrochloride was purchased from Vega Biotechnologies, Inc. (Tucson, AZ). Pteroic acid was prepared by the enzymatic cleavage of folic acid using carboxypeptidase G_1 (McCullough et al., 1971) and was purified according to the literature method (Godwin et al., 1972). N^{10}-(Trifluoroacetyl)pteroic acid (**1**) was also prepared as described previously (Godwin et al., 1972).

(*A*) N^{α}-[N^{10}-(*Trifluoroacetyl*)*pteroyl*]-N^{ϵ}-[(*tert-butyloxy*)*carbonyl*]-L-*lysine tert-Butyl Ester* (**2**). A mixture containing N^{10}-(trifluoroacetyl)pteroic acid (**1**) (0.3 g, 0.675 mmol), N^{ϵ}-[(*tert*-butyloxy)carbonyl]-L-lysine *tert*-butyl ester hydrochloride (0.51 g, 1.51 mmol), and triethylamine (0.33 mL, 2.37 mmol) was prepared in 15 mL of anhydrous dimethylformamide (DMF)[1] and maintained at 0 °C in a N_2

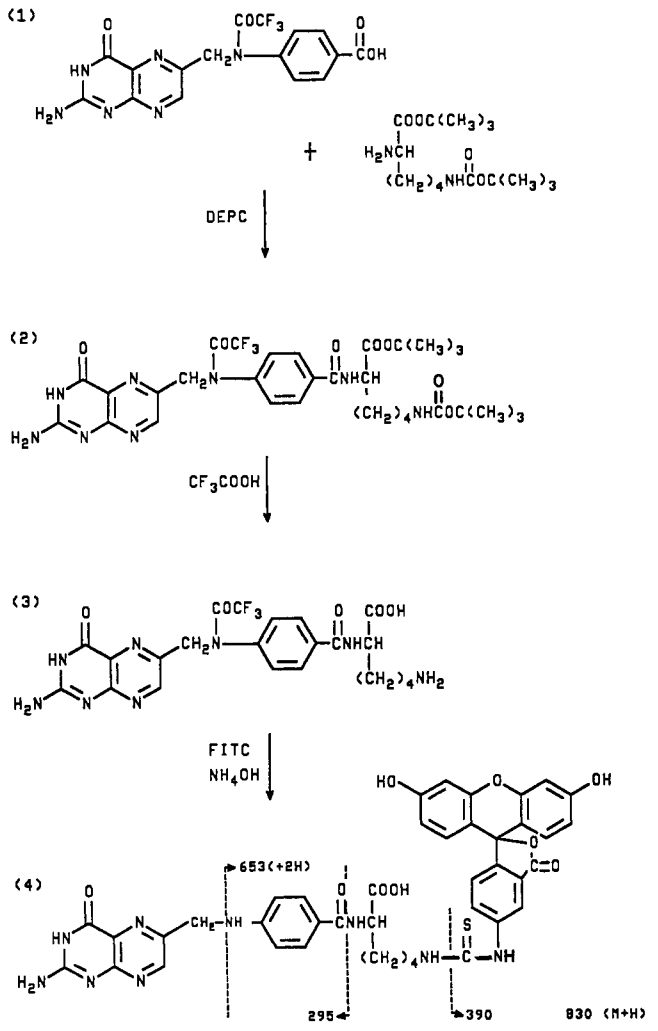

FIGURE 1: Synthetic pathway, structure, and fast atom bombardment mass spectral fragmentation pattern of PLF.

atmosphere. To this mixture was added a solution of diethyl phosphorocyanidate (DEPC)[1] (0.39 g, 2.37 mmol) in 2 mL of DMF dropwise with stirring over a period of 10 min. The mixture was stirred at 0 °C for an additional 2.5 h and then at ambient temperature for 18 h. The solvent was removed under vacuum, and the residue was triturated with H_2O to give a solid which was separated by filtration, washed with H_2O, and dried under vacuum. The solid was then stirred with silica gel in $CHCl_3$ and applied to a silica gel column (2.4 × 24 cm). After elution of the colored impurities with $CHCl_3$–CH_3OH, 96:4, the pure product was eluted by $CHCl_3$–CH_3OH (4:1). Fractions shown to be homogeneous by TLC were combined and evaporated to dryness and then dried under vacuum over P_2O_5 for 60 h to give 0.40 g (74%) of the desired compound: mp 199–200.5 °C dec; TLC (silica gel, $CHCl_3$–CH_3OH, 85:15) R_f 0.22; ^1H NMR [300 MHz, $(CH_3)_2SO$-d_6] δ 1.23–1.52 [m, 22, $2C(CH_3)_3$ + CH_2CH_2], 1.73 (br, s, 2, $CHCH_2$), 2.89 (br s, 2, CH_2NH), 4.24 (m, 1, $CHCH_2$), 5.12 (s, 2, CH_2[9]), 6.78 (app t, 1, CH_2NH), 6.95 (br s, NH_2, partially exchanged with H_2O), 7.62 (d, 2, 3′, 5′, J_0 = 7.77 Hz), 7.89 (d, 2, 2′, 6′, J_0 = 8.19 Hz), 8.63 (s, 1, H_7), 8.68 (d, 1, CONH, J_0 = 7.92 Hz). Anal. Calcd for $C_{31}H_{39}F_3N_8O_7 \cdot 6H_2O$: C, 46.50; H, 6.42, N, 13.99. Found: C, 46.54; H, 5.19, N, 13.67.

(*B*) *N^{α}-[N^{10}-(Trifluoroacetyl)pteroyl]-L-lysine* (**3**). Compound **2** (0.35 g, 0.437 mmol) was dissolved in a mixture of 12 mL of $CHCl_3$ and 6 mL of CF_3COOH and stirred at ambient temperature for 1 h. The resulting solution was then poured into 90 mL of cold ether. The precipitate was separated by filtration, washed with ether, and dried under vacuum over P_2O_5 for 48 h to give 0.29 g (92%) of **3**: mp 140–148 °C dec with preliminary softening; TLC (cellulose, 5% NH_4HCO_3) R_f 0.75; ^1H NMR [300 MHz, $(CH_3)_2SO$-d_6] δ 1.28–1.92 [m, 6, $(CH_2)_3$], 2.78 (m, 2, $CH_2NH_3^+$), 4.36 (m, 1, $CHCH_2$), 5.13 (s, 2, CH_2[9]), 7.13 (br s, 2, NH_2), 7.53–7.85 (m, 5), 7.91 (d, 2, 2′, 6′, J_0 = 8.37 Hz), 8.64 (s, 1, H_7), 8.73 (d, 1, CONH, J_0 = 7.68 Hz). Anal. Calcd for $C_{22}H_{23}F_3N_8O_5 \cdot 1CF_3COOH \cdot 4H_2O$: C, 39.89; H, 4.46; N, 15.51. Found: C, 39.76; H, 4.27; N, 15.21.

(*C*) *N^{α}-Pteroyl-N^{ϵ}-(4′-fluoresceinthiocarbamoyl)-L-lysine* (**4**). All of the following operations were carried out under a N_2 atmosphere with protection from light. To a mixture of **3** (0.30 g, 0.415 mmol) and 3.7 mL of triethylamine in 30 mL of anhydrous DMF was added a solution of fluorescein 5-isothiocyanate (0.18 g, 0.461 mmol) in 15 mL of DMF. After the resulting green solution was stirred at ambient temperature for 4 h, the TLC (silica gel, 3:1:1 1-butanol–acetic acid–H_2O) indicated the disappearance of **3** (R_f 0.26) and the presence of a new spot (R_f 0.74). The solution was evaporated under vacuum at 45 °C and the residue triturated with ether to give a dark yellow solid that was separated by filtration, washed with ether, and dried over P_2O_5 under vacuum. The crude solid (0.50 g) was dissolved in 35 mL of 1 N NH_4OH, and the resulting dark red solution was stirred at ambient temperature for 3 h. The solution was evaporated under vacuum at 45 °C, 50 mL of H_2O was added, and the process was repeated. The resulting solid was dissolved in 50 mL of 1.5 M $(NH_4)_2CO_3$ and purified on a DEAE-cellulose column (Whatman DE-52, 2.8 × 24 cm) by elution with 1.5 M $(NH_4)_2CO_3$. Fractions that were pure by TLC analysis (cellulose, 5% NH_4HCO_3) (R_f 0.07) were pooled, and the H_2O was removed under vacuum. Residual $(NH_4)_2CO_3$ was removed by entrainment by using four 50-mL portions of H_2O. The reddish orange powder was dried under vacuum and then left exposed to the atmosphere until a constant weight was obtained to give 0.21 g (48%): mp > 360 °C; TLC (reverse phase, CH_3OH–H_2O, 4:1) R_f 0.84; HPLC on a Waters μBondapak C_{18} column (Kumar et al., 1983b) single peak, retention time 107 min; UV λ_{max} (0.1 M phosphate buffer, pH 7.0) 237 (ϵ 59 551), 277 (44 382), 497 (54 494), and shoulder at 350; λ_{max} (0.1 M NaOH) 240 (ϵ 64 949), 368 (12 371), 493 (65 979), and shoulder at 281; FAB-MS (Figure 1) 830 (M + 1), 655 [(653 + 2H), 9–10 bond cleavage], 390 (lysine–thiocarbonyl bond cleavage), 295 (amide bond cleavage). Anal. Calcd for $C_{41}H_{35}N_9O_9S \cdot 14H_2O$: C, 45.51; H, 5.87; N, 11.65; S, 2.96. Found: C, 45.60; H, 4.40; N, 11.74; S, 3.10.

Fluorescence Enhancement Studies. A Perkin-Elmer Model MPF-66 fluorescence spectrophotometer was used in the fluorescence enhancement and titration studies. This was operated in the integration mode with a Series 7000 professional computer. The excitation and emission wavelengths were set at 497 and 518 nm, respectively. Tris-HCl buffer (50 mM, 24 °C) was used in each determination. To avoid errors due to thermal denaturation of protein, fresh protein solution was used for each data point. The inner-filter effect was determined by varying PLF concentration and measuring the resultant fluorescence emission peak on excitation at 497 nm. Excitation and emission monochromators were set at 6.0 nm and the excitation and emission correction factors generated by following the manufacturers' instructions with rhodamine 101 as a quantum counter. The samples were scanned at a rate of 120 nm/min, and the absorbance of the samples did not exceed a value of 0.05 at the excitation

wavelength. Recombinant human DHFR is routinely purified in this laboratory from a high-level expression system in *E. coli* by affinity chromatography on MTX–Sepharose (Prendergast et al., 1988). Dihydrofolate bound to DHFR is removed by isoelectric focusing. Human placental m-FBP was purified by the method of Antony et al. (1981) as modified by Ratnam et al. (1989). The PLF concentration was held constant at 50 nM with incremental additions of recombinant hDHFR or human placental FBP. Fluorescence emission maxima were measured at 518 nm.

Fluorescence Titration Studies. Increasing amounts of PLF were added to 50 nM human DHFR, and the fluorescence emission was measured at 518 nm. PLF was reduced to H_2PLF by sodium dithionite according to Blakley (1960) with the modifications that 2 M urea was included in the incubation system and the time of reaction was extended to 10 h. Increasing concentrations of H_2PLF were incubated with 50 nM human DHFR and excited at 497 nm, and the fluorescence emission at 518 nm was recorded. The concentration of H_2PLF was determined by absorbance at 497 nm (ϵ = 54 500 M^{-1} cm^{-1}) and by enzymatic conversion to the tetrahydro derivative (H_4PLF) in the presence of human DHFR and NADPH, measuring the change in absorbance at 340 nm (Kempton et al., 1982). Between 80% and 90% of PLF was converted to H_2PLF. The enzyme assays as well as absorption spectra were performed on a Cary 219 recording spectrophotometer (Varian Instruments).

Cell Culture. CCRF/CEM 7A (Jansen et al., 1990) and CCRF/CEM-FBP (Jansen et al., 1989) human lymphoblastic leukemia cell lines both require folate-deficient RPMI 1640 (JRH Biosciences, Lenexa, KS) which is HEPES buffered. Media in both cases were supplemented with 5% dialyzed fetal calf serum (JRH Biosciences), 2 mM glutamine (Sigma), 100 units/mL penicillin, 100 μg/mL streptomycin sulfate (Irvine, Santa Ana, CA), and 0.25 nM, *d,l*-folinic acid (Sigma). Parental CCRF/CEM cells were grown in standard HEPES-buffered RPMI-1640 medium. Cell cultures were grown continuously at 37 °C in a humidified 5% CO_2 atmosphere. Cell doubling time is 23 h for CCRF/CEM, 32 h for CCRF/CEM-7A, and 24 h for CCRF/CEM-FBP cells.

[^{3}H]Folic Acid Displacement by PLF. CCRF/CEM-FBP cells were washed in cold (4 °C) HEPES balanced saline solution (HBSS)[1] which contains 107 mM NaCl, 20 mM Hepes, 26.2 mM $NaHCO_3$, 5.3 mM KCl, 1.9 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 7.0 mM D-glucose, pH 7.4 (Sirotnak et al., 1984). Cells were resuspended in HBSS at 4 °C, and various amounts of PLF were added together with 50 pmol of [^{3}H]folic acid which had been purified by TLC. Incubation for 20 min at 4 °C followed. The cells were then pelleted, and the supernatant was removed completely. The cell pellet was resuspended in a small amount of HBSS, and the radioactivity associated with the cell pellet was quantitated by liquid scintillation counting (Beckman LS 3801).

K_D for PLF for CCRF/CEM-FBP Cells. CCRF/CEM-FBP cells were washed twice with ice-cold HBSS (pH 7.4). Various concentrations of PLF (0.25–10 nM) were incubated for 15 min at 4 °C with 1 × 10^6 cells. The cells were then centrifuged at 2000 rpm for 5 min, and all of the residual supernatant was removed. The cell pellets were resuspended in HBSS (3 mL), and the fluorescence emission was measured at 518 nm after excitation at 497 nm. A correction for the fluorescence emission due to autofluorescence and light scattering of the cells was made by suspending 1 × 10^6 untreated cells and measuring the fluorescence promptly. This reduces the error due to settling of the cells. In addition, excess folic acid was added in a number of cases to examine the specificity of PLF binding to the FBP present in these cells.

Flow Cytometry. Cells were analyzed with an EPICS 753 flow cytometer and data analyzed with the EASY 2 computer system (Coulter Electronics, Hialeah, FL). Laser excitation was 500 mW at 488 nm, and emitted cellular fluorescence was measured through a 525-nm band-pass filter. Fluorescence histograms were gated on forward and 90° light scatter to eliminate cellular debris and aggregates. A total of 10 000 cells were analyzed for each sample. Cells (CCRF/CEM and CCRF/CEM-7A) were washed twice in ice-cold HBSS buffer, and 10 pmol of fluorescent probe was added to 1 × 10^6 cells. One picomole of PLF was added to CCRF/CEM-FBP cells. Binding was allowed to occur for 15 min at 4 °C. Unbound probe in the supernatant was removed by centrifugation for 5 min at 2000 rpm at 4 °C. Cell lines were compared on the basis of mean linear fluorescence.

Results

Synthesis and Characterization of PLF. The synthesis of PLF is summarized in Figure 1. The FAB-MS fragmentation pattern of PLF is in complete agreement with the expected structure (**4**, Figure 1). PLF was judged to be pure as indicated by a single elution peak from reverse-phase HPLC. The molecular weight of PLF is 1082 for a 14 H_2O hydration product, and the absorption maxima and extinction coefficients are as indicated in the previous section. PLF is freely soluble in 1.5 M $(NH_4)_2CO_3$. A solution of PLF in this solvent is stable if protected from light and maintained at 4 °C. PLF is also soluble in 0.1 N NaOH but only slightly soluble in 5% NH_4HCO_3, distilled H_2O, dimethyl sulfoxide, and 0.1 N HCl. The absorption spectrum revealed maxima at 282 nm and 497 nm with a shoulder at 346 nm in three different buffers, phosphate-buffered saline (pH 7.5), Tris-HCl (pH 7.5), and HBSS (pH 7.4). Excitation of PLF at 497 nm results in a fluorescence emission maximum at 518 nm. Also, excitation at 346 nm results in an emission maximum at 518 nm while excitation at 282 nm results in an emission maximum at 518 nm.

Inner-Filter Effect. If a significant part of the total absorption is due to the fluorescent probe, whose concentration is varied through the course of the experiment, the primary absorption effect will distort the concentration dependence of the fluorescence, leading to an incorrect value for the equilibrium constants (Birdsall et al., 1983). In the case of PLF there is a direct linear relationship between varying PLF concentrations and fluorescence (data not shown) indicating the absence of primary absorption effects at the concentrations of PLF used in these experiments.

Fluorescence Enhancement Studies. The fluorescence emission at 518 nm was measured by adding incremental amounts of human recombinant DHFR to 50 nM PLF with excitation at 497 nm. The maximum enhancement of fluorescence emission of PLF was approximately 20-fold (Figure 2). A similar fluorescence enhancement was observed when PLF bound to the m-FBP purified from human placenta (Figure 2). When the excitation wavelength was set at 282 nm, there was also an enhancement of fluorescence emission at 518 nm on binding to human DHFR. An MTX competition study with PLF demonstrated a reduction in fluorescent emission as a function of increasing MTX concentrations (Figure 3). Therefore, at high MTX concentrations, the amount of PLF binding to human DHFR (50 nM) was minimal and no fluorescence enhancement was observed. Also, no fluorescence enhancement was observed when human DHFR (100 nM) was first denatured by treatment with 6 M

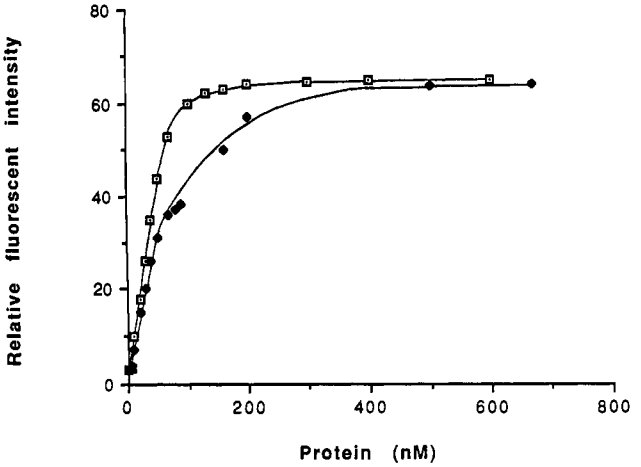

FIGURE 2: Fluorescence enhancement on binding PLF to hDHFR (◆) and purified placenta m-FBP (⊡). The PLF concentration was fixed at 50 nM and the fluorescence emission measured at 518 nm on the addition of incremental amounts of each protein.

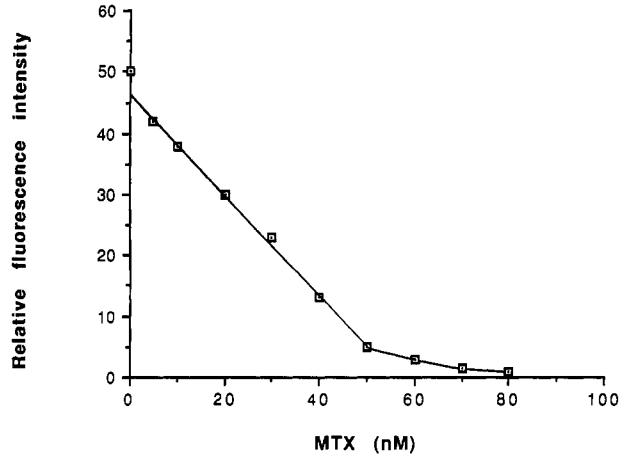

FIGURE 3: Competition for binding of PLF to human DHFR by MTX. DHFR (50 nM) and increasing amounts of MTX were incubated followed by the addition of 100 nM PLF. The decrease in fluorescence emission at 518 nm was measured.

guanidine hydrochloride for 30 min followed by the addition of 100 nM PLF (data not shown).

PLF Inhibition Constant for Human DHFR. A K_i value for the competitive inhibition of human DHFR by PLF was determined by introducing increasing amounts of PLF to the assay mixture containing different fixed amounts of dihydrofolate. The absorbance decrease at 340 nm, indicating the conversion of dihydrofolate to tetrahydrofolate, was measured. The value for the K_i is 2.0 μM as determined by a Dixon plot. A K_i value for human DHFR inhibition by folic acid was determined in a similar fashion and was found to be 0.48 μM (data not shown), a value identical with that reported by Stone et al. (1984) for chicken liver DHFR.

Fluorescence Titration Studies. The fluorescence emission at 518 nm was measured when varying concentrations of PLF were added to 50 nM human DHFR. From this saturation curve a Scatchard analysis resulted in a K_D of 115 nM. The K_D for folic acid for human DHFR is 111 nM (Huang et al., 1989). PLF can be reduced to H_2PLF with sodium dithionite (Blakley, 1960) in the presence of 2 M urea for 10 h. Varying concentrations of H_2PLF were added to 50 nM human DHFR, and the fluorescence emission was measured. From the resulting saturation curve, Scatchard analysis gave a K_D of 47 nM, which is in good agreement with the K_D value of 44 nM for dihydrofolate (Huang et al., 1989). H_2PLF acts as a

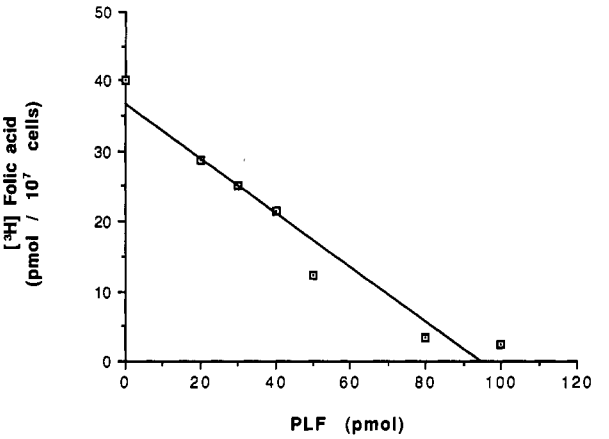

FIGURE 4: Competition between [^{3}H]folic acid and PLF for binding to FBP on the surface of CCRF/CEM-FBP cells.

substrate for human DHFR as indicated by the decrease in absorbance at 340 nm in the presence of NADPH. The K_m value for H_2PLF was determined to be 2.0 μM (data not shown). The k_{cat} value (V_{max}/[concentration of DHFR]) for H_2PLF is 1.8 s^{-1} compared to 7.3 s^{-1} for dihydrofolate (Prendergast et al., 1988).

Whole Cell Studies. When the concentration of PLF is increased, bound [^{3}H]folic acid is displaced from CCRF/CEM FBP cells (Figure 4). In addition, when excess folic acid is added together with PLF, there is no fluorescence enhancement. Likewise, when the PLF probe (100 nM) is added to CCRF/CEM-7A cells, which overexpress the reduced folate/MTX transporter, there is no fluorescence enhancement. The K_D value for PLF binding to FBP on CCRF/CEM-FBP cells was determined to be 1.6 nM (data not shown).

Flow Cytometry. The autofluorescence of the cell lines used in these studies grown in tissue culture is 10 times that of lymphocytes isolated from tissue. This autofluorescence can be reduced by lowering the photomultipler voltage. When 10 pmol of PLF is added to 1×10^6 CCRF/CEM and CCRF/CEM-7A cells, there is very little fluorescence enhancement compared to cells with no PLF added (Figure 5a,b). However, when 1 pmol of PLF is added to 1×10^6 CCRF/CEM-FBP cells, there is a relative fluorescence enhancement of 110-fold (Figure 5c). CCRF/CEM-7A cells (1×10^6) and 1×10^6 CCRF/CEM parental cells were washed in ice-cold HBSS and mixed with 1×10^6 washed CCRF/CEM-FBP cells. One picomole of probe was added and allowed to bind at 4 °C for 15 min. Unbound probe was removed by centrifugation and the supernatant aspirated. The resuspended cells were passed through the flow cytometer. The two peaks (Figure 5d) indicate a relatively nonfluorescent peak (CCRF/CEM-7A and parental CCRF/CEM cells) and a highly fluorescent cell population (CCRF/CEM-FBP).

Discussion

Both MTX and dihydrofolate have a requirement for the free α-carboxyl group of the terminal L-glutamate which is utilized in binding to the Arg 70 residue in human DHFR (Oefner et al., 1988; Davies et al., 1990). Most γ-carboxyl derivatives of MTX do not significantly alter the binding to DHFR (Rosowsky et al., 1981a,b). This laboratory has reported the synthesis and properties of lysine and ornithine analogues of MTX which possess the essential free α-carboxyl group and also a free amino group (Kempton et al., 1982; Kumar et al., 1983a). Fluorescent dansyl groups have been attached to these analogues at the primary aliphatic amino group, and purification and characterization of these analogues

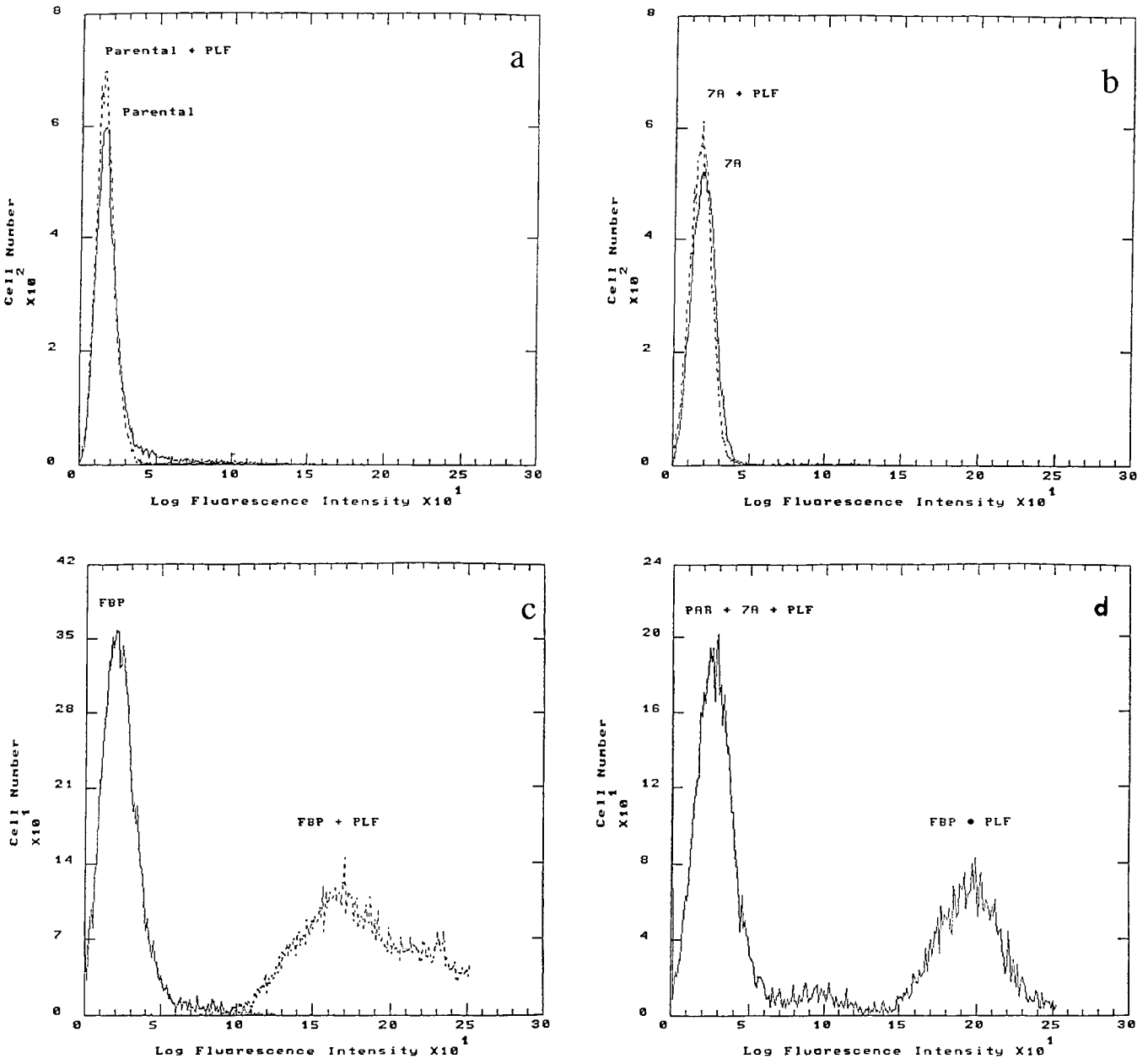

FIGURE 5: Fluorescence flow cytometry histograms. (a) Comparison of 1×10^6 CCRF/CEM cells with and without 10 pmol of PLF. (b) Comparison of 1×10^6 CCRF/CEM-7A cells with and without 10 pmol of PLF. (c) Comparison of the fluorescence enhancement when 1 pmol of PLF is bound to 1×10^6 CCRF/CEM-FBP cells. 1×10^6 cells without probe and with 1000-fold excess folic acid acted as controls. (d) Separation of parental CCRF/CEM and CCRF/CEM-7A cells from highly fluorescent CCRF/CEM-FBP cells.

have been reported (Kumar et al., 1983a–c; Freisheim et al., 1983).

In the design of PLF, lysine replaces the terminal L-glutamate moiety, thereby maintaining a free α-carboxyl group, and a fluorescein moiety is attached to the ϵ-amino group. As is the case with the dansyl fluorescent analogues of MTX, the ϵ-substituted fluorescein group of PLF does not interfere with the binding to DHFR. Table I indicates that the K_D value for the binding of PLF to human DHFR is 115 nM as compared to 111 nM for folic acid and the K_D value for H_2PLF binding to human DHFR is 47 nM as compared to 44 nM for dihydrofolate (Huang et al., 1989). The two buffer systems used in the determination of the dissociation constants are comparable in that they do not affect ligand dissociation in these binary complexes. The k_{cat} value for H_2PLF (1.8 s^{-1}) is 4-fold lower than that of dihydrofolate (7.3 s^{-1}), and the K_m for H_2PLF is approximately 100-fold greater than that of dihydrofolate for human DHFR (Table I). If

Table I: Summary of Michaelis, Inhibition, and Binding Constants for Human DHFR with Various Ligands

	FA	PLF	FAH_2	H_2PLF
K_m			0.022[a]	2.0[a]
K_D	0.111[b]	0.115[a]	0.044[b]	0.047[a]
K_i	0.48[a]	2.0[a]		

[a] Constants determined by using a Tris-HCl (50 mM) pH 7.5 buffer. All values shown are in μM. [b] K_D values determined in 50 mM Tris, 25 mM acetate, 2 mM MES, and 100 mM NaCl, pH 7.5, buffer (MATS). All values shown in are in μM.

H_2PLF follows the same catalytic pathway as dihydrofolate, H_2PLF will bind with the enzyme·$NADP^+$ complex, and NADPH primarily with the enzyme·H_2PLF complex (Tsay et al., 1990). The association rate constant (k_{on}) for NADPH may decrease if the fluorescein moiety interferes with NADPH binding. Alternatively, H_2PLF could bind to the human DHFR·NADPH complex and the k_{on} for H_2PLF may be

decreased. Since the K_D values are similar and the k_{on} may be decreased, it is also likely that one or more dissociation rate constants (k_{off}) will also be decreased. This could occur if the bulky, hydrophobic fluorescein moiety increased the time of release of the product, H_4PLF. To determine which of the possibilities outlined are responsible for the elevated K_m value for H_2PLF, a detailed kinetic investigation would be required.

The polarity of the environment surrounding the fluorescent group determines the fluorescence emission and its intensity as demonstrated by Chen (1967) using dansylamino acids, by Li et al. (1975) using 5-(*N*,*N*-dimethylamino)-1-naphthalenesulfonic acid, and by Kumar et al. (1983c) using the dansylated lysine and ornithine derivatives of MTX. There is a 20-fold enhancement of fluorescence on the binding of PLF to purified human placental FBP and to human DHFR (Figure 2). PLF binds to the MTX/dihydrofolate site of DHFR since MTX and PLF compete for binding (Figure 3) while the denatured form of human DHFR shows no binding of PLF or, alternatively, that which does bind binds in such a way that the fluorescein moiety is not in a hydrophobic environment. On binding of the pterin ring to the native enzyme the fluorescein group of PLF could interact with hydrophobic residues near the surface of the enzyme due to a folding of the molecule, as a result of the flexible nature of the $(CH_2)_4NH$ chain of lysine, resulting in fluorescence enhancement.

The efficiency of energy transfer is dependent on the distance between two fluors (Stryer & Haugland, 1967). In the case of PLF, $n = 4$ for the flexible methylene bridge, $(CH_2)_nNH$, and appears to allow efficient energy transfer. On excitation of PLF at 282, 346, and 497 nm there is an emission peak at 518 nm. Excitation of folic acid at 282 nm results in an emission at 346 nm. Folic acid also absorbs at 340 nm, and excitation at 346 nm results in a broad emission maximum at 460 nm. This emission maximum overlaps with that of the excitation maximum of fluorescein and explains how excitation at 282 nm results in an emission maximum at 518 nm. Fluorescein itself has absorbance at 282 nm, and there may be a direct energy transfer within the fluorescein moiety of PLF.

Kumar et al. (1983c) proposed that a hypochromic effect seen with 4-amino-4-deoxy-N^{10}-methylpteroic acid (APA)^1-Lys-DNS could be due to intramolecular stacking of the pterin and dansyl rings. The trimethylene bridged analogue, APA-Orn-DNS, has a minimal hypochromic effect. A space-filling model demonstrated that proper intramolecular stacking of the two ring systems does not occur in APA-Orn-DNS. The pterin and fluorescein rings of PLF may also be involved in intramolecular stacking as the methylene bridge $(CH_2)_nNH$, where $n = 4$, will allow proper intramolecular stacking of the pterin and fluorescein rings. The reduction of folic acid to dihydrofolate is performed by the addition of sodium dithionite to folic acid in a sodium ascorbate solution at pH 6.0 for 5 min (Blakely, 1960). In the case of PLF this reduction is possible only in the presence of a chaotropic agent (2 M urea) and with an extended reaction time of 10 h. In the absence of urea only 5% of the expected H_2PLF is produced. One explanation is that the hydrophobic interactions between the pterin and fluorescein rings are disrupted by urea and the pterin ring becomes available for reduction to the 7,8-dihydrofolate derivative.

A human T-cell derived leukemia cell line, CCRF/CEM, was used in the characterization of PLF binding to a m-FBP. Two different cell lines have been developed from the parental CCRF/CEM line. CCRF/CEM-7A cells overexpress the MTX/reduced folate transporter (Jansen et al., 1990), and the CCRF/CEM-FPB cell line overexpresses a high-affinity m-FBP (Jansen et al., 1989). The different mechanisms of entry of folate and reduced folates into various cells have been reviewed (Ratnam & Freisheim, 1990). PLF binds to CEM-FBP cells, resulting in an enhancement of fluorescence which is absent when excess folic acid is present. There is very little fluorescence enhancement when PLF is added to parental CEM or CEM-7A cells. In addition, [^{3}H]folic acid can be displaced from the surface of CEM-FBP cells by PLF (Figure 4). These observations together with a K_D determination of 1.6 nM for PLF for the CEM-FBP cells indicates that PLF is specific for the FBP under the conditions stated and that the binding of PLF to the CEM-FBP cells is the same as that for folic acid. Flow cytometry indicates that virtually no fluorescent enhancement occurs on the addition of PLF to CEM and CEM-7A cells (Figure 5a,b) but enhancement results on its addition to CEM-FBP cells (Figure 5c). The binding of PLF to the m-FBP on these cells is specific since excess folic acid prevents any fluorescence enhancement. This fluorescence enhancement may be used to separate cells which express a FBP from cells which have little or no FBP expression (Figure 5d). MTX-F has been used as an intracellular marker for human DHFR (Whiteley & Russell, 1979) and in studies of DHFR overproduction in MTX resistant cells (Haber & Schimke, 1981; Johnston et al., 1983; Mariani & Schimke, 1984). The lysine analogue similar to MTX-F has also been characterized (Rosowsky et al., 1982), and this analogue has been used to distinguish MTX-resistant cells (CEM-MTX), which have normal levels of DHFR but a dysfunctional MTX transporter, from parental CEM cells by flow cytometry (Rosowsky et al., 1986).

In conclusion, PLF, a fluorescent structural analogue of folic acid, has been synthesized and purified, and its physical characteristics have been determined. PLF binds in vitro to purified human DHFR and purified human placental FBP with a 20-fold fluorescence enhancement. The K_D values for PLF and H_2PLF are similar to those of folic acid and dihydrofolate, respectively (Huang et al., 1989). PLF also binds to a m-FBP on the cell surface in a similar fashion to that of folic acid, indicating that the fluorescein moiety does not interfere with binding. PLF can be used to clone cells expressing FBP's by flow cytometry, and a possible future use, along with the lysine analogue of MTX-F (Rosowsky et al., 1986), may be able to identify different types of transport of folate and reduced folates in normal and neoplastic tissues. PLF can be reduced to H_2PLF which is a substrate for human DHFR and is converted to H_4PLF. This property along with energy transfer between pterin ring and the fluorescein moiety may allow this fluorescent analogue to be used as a probe for a number of enzymes in one-carbon metabolism.

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References

Antony, A. C., Utley, C., Van Horne, C., & Kolhouse, J. F. (1981), *J. Biol. Chem. 257*, 10081–10089.

Birdsall, B., Griffiths, D. V., Roberts, G. C. K., Feeney, J., & Burgen, A. S. V. (1977) *Proc. R. Soc. London, Ser. B. 196*, 251–265.

Birdsall, B., King, R. W., Wheeler, M. R., Lewis, C. A., Jr., Goode, S. R., Dunlap, R. B., & Roberts, G. C. K. (1983) *Anal. Biochem. 132*, 353–361.
Blakley, R. L. (1960) *Nature (London) 188*, 231–232.
Chen, R. F. (1967) *Arch. Biochem. Biophys. 120*, 609–620.
Davies, J. F., II, Delcamp, T. J., Prendergast, N. J., Ashford, V. A., Freisheim, J. H., & Kraut, J. (1990) *Biochemistry 29*, 9467–9479.
Freisheim, J. H., Kumar, A. A., Black, A. M., Anstead, G. M., Kempton, R. J., & Susten, S. S. (1983) *Chemistry and Biology of Pteridines* (Blair, J. A., Ed.) pp 469–473, de Gruyter and Co., Berlin and New York.
Freisheim, J. H., Price, E. M., Kumar, A. A., Susten, S. S., Smith, P. L., & Delcamp, T. J. (1986) *Biochem. Soc. Trans. 14*, 371–373.
Gapski, G. R., Whiteley, J. M., Rader, J. I., Cramer, P. L., Henderson, G. B., Neef, V., & Huennekens, F. M. (1975) *J. Med. Chem. 18*, 526–528.
Godwin, H. A., Rosenberg, I. H., Fereny, C. R., Jacobs, P. M., & Meienhofer, J. (1972) *J. Biol. Chem. 247*, 2266–2271.
Haber, D. A., & Schimke, R. T. (1981) *Cell 26*, 355–362.
Henderson, G. B., Russel, A., & Whiteley, J. M. (1980) *Arch. Biochem. Biophys. 202*, 29–34.
Henderson, G. B., Suresh, M. R., Vitols, K. S., & Huennekens, F. M. (1986) *Cancer Res. 46*, 1639–1643.
Huang, S., Delcamp, T. J., Tan, X., Smith, P. L., Prendergast, N. J., & Freisheim, J. H. (1989) *Biochemistry 28*, 471–478.
Jansen, G., Westerhof, G. R., Kathmann, I., Rademaker, B. C., Rijksen, G., & Schornagel, J. H. (1989) *Cancer Res. 49*, 2455–2459.
Jansen, G., Westerhof, G. R., Jarmuszewski, M. J. A., Kathmann, I., Rijksen, G., & Schornagel, J. H. (1990) *J. Biol. Chem. 256*, 18272–18277.
Johnston, R. N., Beverley, S. M., & Schimke, R. T. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 3711–3715.
Kaufman, R. J., Bertino, J. R., & Schimke, R. T. (1978) *J. Biol. Chem. 253*, 5852–5860.
Kempton, R. J., Black, A. M., Anstead, G. M., Kumar, A. A., Blankenship, D. J., & Freisheim, J. H. (1982) *J. Med. Chem. 25*, 475–477.
Kumar, A. A., Freisheim, J. H., Kempton, R. J., Anstead, G. M., Black, A. M., & Judge, L. (1983a) *J. Med. Chem. 26*, 111–113.
Kumar, A. A., Kempton, R. J., Amstead, G. M., Price, E. M., & Freisheim, J. H. (1983b) *Anal. Biochem. 128*, 191–195.
Kumar, A. A., Kempton, R. J., Anstead, G. M., & Freisheim, J. H. (1983c) *Biochemistry 22*, 390–395.
Li, Y.-H., Chan, L., Moody, R. T., Himel, C. M., & Hercules, D. M. (1975) *J. Am. Chem. Soc. 97*, 3118–3126.
Mariani, B. D., & Schimke, R. T. (1984) *J. Biol. Chem. 259*, 1901–1910.
Matthews, D. A., Alden, R. A., Bolin, J. T., Freer, S. T., Hamlin, R., Xuong, N., Kraut, J., Poe, M., Williams, M., & Hoogsteen, K. (1977) *Science (Washington, D.C.) 197*, 452–455.
Matthews, D. A., Alden, R. A., Bolin, J. T., Filmen, D. J., Freer, S. T., Hamlin, R., Hol, W. G. J., Kisliuk, R. L., Pastore, E. J., Plante, L. T., Xuong, N., & Kraut, J. (1978) *J. Biol. Chem. 253*, 6946–6954.
McCullough, J. L., Chabner, B. A., & Bertino, J. R. (1971) *J. Biol. Chem. 246*, 7207–7213.
Montgomery, J. A., Piper, J. R., Elliott, R. D., Temple, C., Jr., Roberts, E. C., & Shealy, Y. F. (1979) *J. Med. Chem. 22*, 862–869.
Oefner, C., D'Arcy, A., & Winkler, F. K. (1988) *Eur. J. Biochem. 174*, 377–385.
Piper, J. R., Montgomery, J. A., Sirotnak, F. M., & Chello, P. L. (1982) *J. Med. Chem. 24*, 559–567.
Prendergast, N. J., Delcamp, T. J., Smith, P. L., & Freisheim, J. H. (1988) *Biochemistry 27*, 3664–3671.
Ratnam, M., & Freisheim, J. H. (1990) in *Contemporary Issues in Clinical Nutrition* (Picciano, M. F., Ed.) Vol. 16, pp 91–120, Wiley–Liss, New York.
Ratnam, M., Marquardt, H., Duhring, J. L., & Freisheim, J. H. (1989) *Biochemistry 28*, 8249–8254.
Rosowsky, A., & Yu, C.-S., Uren, J., Lazarus, H., & Wick, M. (1981a) *J. Med. Chem. 24*, 559–567.
Rosowsky, A., Forsch, R., Uren, J., & Wick, M. (1981b) *J. Med. Chem. 24*, 1450–1455.
Rosowsky, A., Wright, J. E., Shapiro, H., Beardsley, P., & Lazarus, H. (1982) *J. Biol. Chem. 257*, 14162–14167.
Rosowsky, A., Wright, J. E., Cucchi, C. A., Boeheim, K., & Frei, E., III (1986) *Biochem. Pharmacol. 35*, 356–360.
Sirotnak, F. M., Moccio, D. M., & Yang, C. H. (1984) *J. Biol. Chem. 259*, 13139–13144.
Stone, S. R., Montgomery, J. A., & Morrison, J. F. (1984) *Biochem. Pharmacol. 33*, 175–179.
Stryer, L., & Haugland, R. P. (1967) *Proc. Natl. Acad. Sci. U.S.A. 58*, 719–726.
Susten, S. S., Kempton, R. J., Black, A. M., & Freisheim, J. H. (1984) *Biochem. Pharmacol. 33*, 1957–1962.
Tsay, J.-T., Appleman, J. R., Beard, W. A., Prendergast, N. J., Delcamp, T. J., Freisheim, J. H., & Blakley, R. L. (1990) *Biochemistry 29*, 6428–6436.
Whiteley, J. M., & Russell, A. (1979) in *Chemistry and Biology of Pteridines* (Kisliuk, R. L., & Brown, G. M., Eds.) pp 549–554, Elsevier/North-Holland, New York.