A New Fluorescent Dihydrofolate Reductase Probe for Studies of Methotrexate Resistance*

Andre Rosowsky, Joel E. Wright, Howard Shapiro, Peter Beardsley, and Herbert Lazarus
From the Sidney Farber Cancer Institute and Harvard Medical School, Boston, Massachusetts 02115

(Received for publication, April 23, 1982)

A new fluorescent methotrexate analogue (PT430) was synthesized as a reporter ligand for dihydrofolate reductase. The analogue was prepared by attachment of lysine in place of the glutamate side chain of methotrexate and conjugation to fluorescein isothiocyanate via the ε-amino group of lysine. Spectrophotometric evidence in L1210/R6 cells to be about one-tenth as potent as methotrexate against either Lactobacillus casei or L1210 mouse leukemia enzyme; competitive radioligand binding assays using tritiated methotrexate gave similar results. In assays of L1210 cell proliferation in culture, on the other hand, PT430 was 100-fold less toxic than methotrexate. In dilute solution, the fluorescence intensity of PT430 was 5-fold lower than that of equimolar fluorescein and diminished with decreasing pH. On complexation with dihydrofolate reductase, however, fluorescence intensity was enhanced 3- to 5-fold depending on the pH. Measurement of fluorescence increase with added ligand provided data for the determination of the stoichiometric ratio, dissociation constant, and extent of fluorescence enhancement. Specificity of PT430 for methotrexate binding sites was indicated by the observation of decreased fluorescence uptake in PT430-treated L1210 cells in the presence of methotrexate. Fluorescence uptake occurred faster, and to a greater extent, in methotrexate-resistant dihydrofolate reductase overproducing L1210/R6 cells than in the methotrexate-sensitive parent line. Therefore, PT430 may be used as a flow cytometry probe to detect methotrexate resistance based on dihydrofolate reductase overproduction.

*A new fluorescent methotrexate derivative (MTX-F, Fig. 1) prepared by Gapéski, et al. (1) has provided a valuable tool for the study of intracellular dihydrofolate reductase (EC 1.5.1.3) in cells that overproduce this enzyme (2-9). The usefulness of MTX-F as a dihydrofolate reductase probe in these mutants prompted our interest in developing new compounds that could extend the application of this concept to clinically relevant forms of methotrexate resistance. We sought to develop fluorescent substances combining a strong affinity for dihydrofolate reductase with the following properties: 1) uptake should be measurable, not only in dihydrofolate reductase overproducing mutants, but also in methotrexate-sensitive parent cell lines and even in transport resistant sublines. 2) Incubation periods of less than 6 h in the presence of 10-60 μM concentrations of the drug should give measurable fluorescence. 3) Attachment of the fluorophore should unambiguously involve the terminal position of the amino acid and not the α-carboxyl group.

In this paper, we report the synthesis and characterization of one such compound, PT430 (Fig. 1, Structure 1), and present data on its binding to dihydrofolate reductase from L1210 cells and Lactobacillus casei, its rapid dose- and time-dependent uptake by both methotrexate-sensitive L1210 cells and dihydrofolate reductase overproducing L1210/R6 cells (10), and the inhibition of its uptake by methotrexate pretreatment.

MATERIALS AND METHODS

Cell Lines—Parental methotrexate-sensitive L1210 cells and the methotrexate-resistant L1210/R6 line obtained from Dr. F. M. Huennekens ( Scripps Clinic and Research Foundation, La Jolla, CA) were grown under standard culture conditions. The L1210/R6 line was previously shown to have an 80-fold elevation of dihydrofolate reductase content but essentially normal methotrexate transport properties (10). For use in cytotoxicity assays and flow cytometry studies, methotrexate-sensitive L1210 cells were grown at 37 °C in Spinner’s or Eagle’s MEM in a humidified atmosphere with 5-7% CO₂. The growth medium contained 10-15% FBS, streptomycin (50-100 μg/ml), penicillin (100 units/ml), and 0.05 mg 2-mercaptoethanol. Uptake experiments with L1210 and L1210/R6 cells were performed at 37 °C in α-MEM containing 10% FBS without antibiotics, in a 5% CO₂, humidified atmosphere. For use in flow cytometric assays, the FBS was dialyzed against Earle’s balanced salt solution prior to use; in the cytotoxicity studies, whole FBS was used. Cells for all experiments were in logarithmic growth phase.

Physical Measurements—The 520-550 nm emission of fluorescein-labeled cells was measured with a flow cytometer (11) using 488 nm argon ion laser illumination at 100-milliwatt power. A forward scatter signal was used to gate an orthogonal channel peak detector. Fluorescence distributions were accumulated on a Gilford 240 spectrophotometer equipped with a flow cytometer (12) using the DNA fluorochrome H33342 (Hoechst, Frankfurt, West Germany) and krypton ion laser illumination at 350 nm (20-milliwatt power). Fluorescence spectra of solutions of 1 were recorded on a thermostatted Perkin Elmer MFP4 spectrophotometer using 3-mm disposable cuvettes (Evergreen Scientific, Los Angeles, CA). Infrared spectra were obtained using the Perkin-Elmer Model 137B double beam instrument. Functional enzyme inhibition assays were performed on a Gilford 240 spectrophotometer equipped with a thermostatted programmable cell changer assembly. Scintillation counting was performed in Biofluor (New England Nuclear) using a Beckman LS-7000 counter. In the cytotoxicity assays, cells were counted with the aid of a Coulter Model F cytophotometer. Column chromatography was run at 30 °C on a water-jacketed glass column (25 x 30 cm) packed with N,N-dicylamethanoethylcellulose (DEAE-cellulose, Whatman DE-52) and connected to a solvent minipump. Effluents were collected with the aid of a Gilson Model PC-80K microfractionator. TLC was performed on 0.25-mm Analtech GHLF silica gel plates.
Reagents and Enzymes—Dihydrofolate reductase (0.43 units/mg) from methotrexate-resistant Lactobacillus casei was provided by the New England Enzyme Center, Boston, MA. Partially purified dihydrofolate reductase from L1210 mouse leukemia was as described previously (12). The enzymes were kept at -20 °C prior to use. NADPH was purchased from Sigma, fluorescein isothiocyanate, and fluorescein from Aldrich, and [3',5',7',F]HMTOX (hereafter referred to as [F]HMTOX) from Amersham. The [F]HMTOX was purified by ion exchange chromatography (13). The preparation of the precursor N'- (4-amino-4-deoxy-N'-methylpteroyl)-L-lysine trihydrate (Fig. 1, Structure 2) was described previously (14). MTX, as the disodium salt formulated for clinical use, was supplied by the National Cancer Institute, Bethesda, MD.

N'- (4-amino-4-deoxy-N'-methylpteroyl)-L-lysine trihydrate (Fig. 1, Structure 2) was likewise pooled and evaporated, and the ammonium carbonate eluted with 1.0 M ammonium carbonate. Fractions were analyzed by TLC using n-butanol:acetic acid:water (3:1:1) as the developing solvent. Early fractions containing starting material (Rf, 0.23) were removed in vacuo by entrainment with two 400-ml portions of water. The residues were dissolved in 20 ml of water, and the solution lyophilized to give an orange solid which was left exposed to the ambient atmosphere until a constant weight was reached: 50 mg (59% yield based on recovered 21); m.p. > 360 °C; IR (potassium bromide, cm⁻¹) 3100-3400, 2900, 2230-2390, 1550-1650, 1500, 1460, 1360, 1180-1220, 1140.

C₅₆H₆₅N₁₃O₆S·3H₂O

Calculated: C 56.24 H 4.94 N 15.62 S 3.78
Found: C 56.67 H 5.00 N 15.39 S 3.78

Competitive Inhibition Assays—The ability of PT430 to compete with dihydrofolate for the active site of Lactobacillus casei or L1210 leukemia-derived dihydrofolate reductase was determined by the standard spectrophotometric method (15) based on the change in UV absorbance at 340 nm when NADPH is oxidized to NADP. Addition of PT430 was repeated to measure fluorescence increase as a function of added ligand (Fig. 2) was calculated by linear regression after conversion to nanomolar concentration units.

Contents of the cuvettes were then replaced with fresh buffer (2 ml) containing 37 nm Lactobacillus casei dihydrofolate reductase, and addition of PT430 was repeated to measure fluorescence increase due to binding to the enzyme. The intensity versus concentration data were plotted, and initial and final linear regions of the titration curves (Fig. 3) were extrapolated to their intersection to obtain the equivalence point. Titration data were fitted to the equation,

\[
\frac{1}{\Delta F} = \frac{K_D}{F(R_o - (\Delta F/\gamma))} + \frac{1}{F}
\]

where \( R_o \) is the concentration of PT430 added prior to complexation, \( K_D \) is the dissociation constant and is defined by the equation,

\[
K_D = \frac{(R_o - ER) (E_0 - ER)}{ER}
\]

in which the term \( ER \) is the concentration of the enzyme-ligand

Spectrofluorometric Titrations—The concentration and pH dependence of fluorescence of PT430 were determined using 1.0 μM stock solutions in 0.1 M sodium phosphate buffers at pH 6.0, 6.5, 7.0, and 7.4. Small aliquots (15-20 μl) of stock solutions were added consecutively to cuvettes containing 2.0 ml of the buffer alone, and the intensity of the emission maximum at 518 nm was measured using an excitation beam of 488 nm. The change of fluorescence intensity as a function of added ligand (Fig. 2) was calculated by linear regression after conversion to nanomolar concentration units.

FIG. 2. Change of fluorescence intensity upon addition of PT430 to 0.1 M sodium phosphate buffers at pH 6.0 (●), pH 6.5 (○), pH 7.0 (□), and pH 7.4 ( ▲).

FIG. 3. Change of fluorescence intensity upon addition of PT430 to Lactobacillus casei dihydrofolate reductase at pH 6.0 (●), pH 6.5 (○), pH 7.0 (□), and pH 7.4 ( ▲).

FIG. 1. Structures of MTX, MTX-F, 1(PT430), and N'- (4-amino-4-deoxy-N'-methylpteroyl)-L-lysine (2).

\[
\begin{align*}
\text{MTX} & : R' = \text{COH} \\
\text{MTX-F} & : -\text{CONH(CH₂)₃NHCSNH} \\
(1) & : -\text{CH₂CH₂NH₂} \\
(2) & : -\text{CH₂CH₂NHCSNH}
\end{align*}
\]
complex; \( \Delta F \) is the fluorescence increment for each addition of PT430 and is calculated by subtracting the fluorescence intensity of PT430 in buffer alone from that in the solution containing the enzyme; \( F \) is the asymptotic value of \( \Delta F \) at infinite \( R_s \). The term \( \gamma \) is defined as,

\[
\gamma = \beta' - \beta
\]

(3)

where \( \beta' \) and \( \beta \) are the slopes of the linear concentration-intensity functions for \( ER \) and \( R_s \), respectively. Empirical values of \( \gamma \) were estimated using the relationship,

\[
\gamma = \frac{F}{E_0}
\]

(4)

and the concentration of enzyme-ligand complex was calculated at each point from the equation,

\[
ER = \frac{\Delta F}{\gamma}
\]

(5)

These estimates were refined using the iterative method of Winer et al. (17), which allows computation of \( K \) from Equation 1 as the slope of the double reciprocal plot (inset, Fig. 4).

Flow Cytometry—Suspension cultures containing \( 5 \times 10^4 \) MTX-sensitive L1210 cells in 5 ml of medium containing 15% FBS were treated with 50 \( \mu \)l of PT430 at concentrations of 0, 1.0, 2.0, and 3.0 \( \mu M \) in 0.9% NaCl, pH 7.4, and incubated at 37 °C. Aliquots of 1 ml were drawn at intervals of 2, 4, and 6 h, and pelleted. The pellet was washed with ice-cold Earle's solution, centrifuged again, and resuspended at 4 °C in 1 ml of fresh medium. Flow cytometry was performed as described above. Histograms of the number of cells as a function of mean fluorescence per cell at each dose and time were obtained. A total of 2 \times 10^3 cells were used for each histogram. To a 0.5-ml aliquot of cell suspension at each concentration and time point were added 14 \( \mu l \) of 100 \( \mu g \) ml \(^{-1} \) Hoechst 33342. These cells were incubated for an additional 50 min at 37 °C and then assayed directly for DNA content by flow cytometry using 350 nm, 20-milliwatt illumination from the krypton ion laser. The luminescence of the DNA-bound fluorochrome was monitored at 450-500 nm. In a separate experiment designed to evaluate prolonged incubation at lower doses, cells were incubated with 0, 0.1, 1.0, and 10 \( \mu M \) PT430 for 48 h at 37 °C. To show specificity of the uptake of methotrexate binding agent, cells and resistant L1210/R6 cells were incubated concurrently at 37 °C prior to flow cytometric assay. Finally, MTX-sensitive L1210 and resistant L1210/R6 cells were incubated concurrently at 37 °C, pH 7.4, with 15, 30, and 60 \( \mu M \) PT430 and assayed by flow cytometry to determine whether uptake would be greater in the dihydrofolate reductase overproducing line. Cytograms showing the relationship between forward light scattering (cell size) and fluorescence intensity per cell, for 10^3 cells, were also recorded.

**RESULTS AND DISCUSSION**

The high-yield preparation of \( N^\prime-(4\text{-amino-4-deoxy-N^10-methylpteroyl})L\)-lysine (2) developed previously in our laboratory (14) provides convenient access to PT430 via condensation with fluorescein isothiocyanate. The reaction proceeds cleanly in dry \( N, N\)-dimethylformamide containing excess triethylamine at room temperature over two days. Purification by ion exchange chromatography on DEAE-cellulose with 1.0 M ammonium carbonate gave a homogeneous fluorescent solid with a high specific binding affinity for dihydrofolate reductase. Its elemental analysis was consistent with Structure 1, and IR absorptions at 3100-3400 (NH), 1640 (thioamide II band), 1305 (amide III band), and 1140 (C=S) cm \(^{-1} \) indicated that the expected aliphatic thiourea group was present (18). This route avoids the production of mixtures of \( \alpha \)- and \( \gamma \)-isomers, which are extremely difficult to separate. Formation of such mixtures is inherent in the reported DCC-mediated synthesis of MTX-F (1). Furthermore, the diethylphosphorylcytidine coupling method that we have used (14) precludes racemization, which has been shown to occur when MTX is exposed to DCC coupling conditions (19). The presence of an unsubstituted \( \alpha \)-carboxyl group and elimination of the lipophilic pentamethylene spacer should facilitate uptake into cells by reducing the likelihood of retention in the lipid bilayer of the cell membrane.

Competitive enzyme inhibition and radioligand binding data are shown in Table 1. The spectrophotometric assay results indicate that PT430 is 20-fold less active than MTX as an inhibitor of dihydrofolate reduction by the *Lactobacillus casei* enzyme. This reflects the 17-fold greater ability of \(^{[3]}H\) MTX to compete for the binding site of the enzyme, as shown in the radioligand binding assay. In a similar fashion, with dihydrofolate reductase from L1210 cells, PT430 showed an affinity for the binding site 12-fold lower than that of MTX in the spectrophotometric assay and 7-fold lower in the radioligand binding assay. Gapski et al. (1) reported spectrophotometric data for MTX-F and MTX using the same enzymes. From Lineweaver-Burk plots, they obtained \( K \) values whose ratios \((K_{\text{MTX/F}}, K_{\text{MTX}})\) indicate MTX-F to be 3-fold less inhibitory than MTX toward the *L. casei* enzyme and 1.5-fold less active in the L1210 enzyme assay.

In view of the results discussed above, one might expect cytotoxicity to limit the usefulness of PT430 as a vital fluorochrome. Cytotoxicity results, however, show that this is not the case, since the 48-h ID \(_{50} \) value for PT430 is 100-fold greater than that of MTX (Table I). The apparent discrepancy between the cytotoxicity of PT430 and its dihydrofolate reduc-

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**Table 1**

**Cytotoxicity of PT430 and MTX toward L1210 mouse leukemia cells in culture**

<table>
<thead>
<tr>
<th>Compound</th>
<th>L1210 cytotoxicity ((ID_{50} \mu M))</th>
<th>Enzyme inhibition ((ID_{50} \mu M))</th>
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<tbody>
<tr>
<td>PT430 (1)</td>
<td>3.0</td>
<td>203</td>
</tr>
<tr>
<td>MTX</td>
<td>0.03</td>
<td>12</td>
</tr>
<tr>
<td>Ratio: MTX</td>
<td>100</td>
<td>17</td>
</tr>
</tbody>
</table>

| | Spectrophotometric | Radioligand |
| | L. casei | L. casei | L. casei |
| PT430 (1) | | | |
| MTX | | | |
| Ratio: MTX | | | |

\(^a\) Concentration giving 50% reduction in cell proliferation over 48 h.

\(^b\) Spectrophotometric assay (\(\Delta A_{450}\)) of concentration giving 50% reduction in rate of conversion of dihydrofolate to tetrahydrofolate.

\(^c\) Radioligand assay of concentration inhibiting binding of \(^{[3]}H\) MTX to enzyme by 50% (14, 16).

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**Fig. 4.** Plot of fluorescence enhancement (\(\Delta F\)) versus concentration of added PT430 \((R_s)\). Circles represent experimental data, whereas the **plotted curve** was derived from Equations 1-5 using the constants given in Table II. Inset, double reciprocal plot of fluorescence enhancement versus uncomplexed PT430 at pH 6.5, slope = \(K_n\), intercept = 1/\(F\).
tase binding affinity may reflect decreased uptake, which may be due in part to the inherent inability of the compound to undergo intracellular conversion to γ-polyglutamates. Once it enters cells, MTX is converted to γ-polyglutamate conjugates (30) that contribute to cytotoxicity by helping to maintain a critical level of nonbound MTX above the amount needed just to saturate the active site of the enzyme, and also by binding directly to the enzyme (21). Since PT430 clearly cannot form such γ-polyglutamates, a higher extracellular concentration of the molecule may be required to achieve the critical intracellular level than with MTX.

The titration of dihydrofolate reductase with PT430 (Fig. 3) demonstrates the usefulness of this compound in elucidating the binding properties of the enzyme. Concentration-intensity plots reveal a linear increase in fluorescence throughout the concentration range (Fig. 2). Within the pH range 6.0 to 7.4, slopes increased in direct proportion to pH. In the presence of enzyme, fluorescence was enhanced up to 4.6-fold, depending on the pH at which the measurement was made. Values of $K_d, n$, and $γ$ at each pH, calculated from the titration data with the aid of Equations 1-5 are given in Table II. Fig. 4 shows the close correspondence between the calculated fluorescence enhancement data (curve) and the experimental values obtained at pH 6.5 (points).

Similar titrations, using a different fluorescent ligand (MTX-F) (3, 24), have been reported to fit a form of Equation 1 taken from Winer and Schwert (22) as follows:

$$\frac{1}{\delta \lambda} = \frac{K_d}{F} + \frac{1}{F}$$

(6)

The term $C$ was stated to represent the "concentration of ligand present" (3). It should be emphasized, however, that $C$ must be the uncomplexed ligand concentration $R_0 - ER$, and that failure to make this correction results in overestimation of $K_d$ by a factor of $R_d/[R_0 - ER]$ (23). The results reported for MTX-F with Equation 6 used for the calculation were as follows: $K_d = 50 \text{ nM}$ for L1210/R6 dihydrofolate reductase at pH 7.0 in 1.0 M potassium phosphate without NADPH added (3); $K_d = 30.9 \text{ nM}$ for L1210/R8 dihydrofolate reductase at pH 7.0 in 0.1 M potassium phosphate with no NADPH added; and $K_d = 39.8 \text{ nM}$ in the same buffer with 1 equivalent of NADPH present (24).

Flow cytometric measurements of the uptake of PT430 by L1210 cells ($2 \times 10^9$ cells per experiment) showed the increase in mean fluorescence per cell to be both time- and dose-dependent. This conclusion was supported by histograms of fluorescence per cell versus number of cells (Fig. 5), and by measurements of mean fluorescence per cell calculated from quantitative output of the digital pulse height analyzer (Table III). These results show that PT430 is taken up rapidly by the MTX-sensitive parent line after 1 to 6 h of incubation with 10-30 μM PT430. Histograms showing influx of PT430 to be related to intracellular dihydrofolate reductase content are given in Fig. 6. In this experiment, equal numbers of L1210 and L1210/R6 cells were incubated for 4 h at 37 °C with 15, 30, and 60 μM PT430. Considerably greater uptake was seen in the resistant line at each concentration. Quantitation of mean fluorescence per cell gave the dose-response curve shown in Fig. 7. Kaufman et al. (2), using four Sarcoma 180 cell lines exposed to 10-30 μM MTX-F for 22 h followed by 5 min efflux into drug-free medium, reported saturation of uptake at the enzyme-bound level. Whiteley et al., working with L1210 and L1210/R8 cells rather than Sarcoma 180 cells, observed continuous increases in fluorescence uptake with no saturation after 6 h with 15 μM MTX-F (24) or 50 h with 10 μM MTX-F (3). The L1210/R8 line is similar to the L1210/R6 used in our experiments in that it contains approximately 100-fold elevated levels of dihydrofolate reductase (3). In the present study, neither L1210 nor L1210/R6 cells became saturated with PT430 after 1-6 h of incubation with 10-30 μM drug. As Hakala (26) has shown, Sarcoma 180 cells are unusual in lacking the ability to concentrate intracellular MTX much above the dihydrofolate reductase bound level. This property stands in contrast with the ability of L1210 cells to accumulate MTX well in excess of the enzyme, as reported by Hakala (26) and later confirmed in part and extended by Sioutnak and Donsbach (27). In view of this, the dose saturation effects reported for MTX-F in

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**TABLE II**

<table>
<thead>
<tr>
<th>pH</th>
<th>$K_o$</th>
<th>$n$</th>
<th>$γ$</th>
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<tr>
<td>6.0</td>
<td>1.4</td>
<td>1.2</td>
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<tr>
<td>6.5</td>
<td>2.5</td>
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</tr>
<tr>
<td>7.0</td>
<td>2.8</td>
<td>0.98</td>
<td>0.356</td>
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<td>7.4</td>
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<td>0.81</td>
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**TABLE III**

<table>
<thead>
<tr>
<th>Incubation time</th>
<th>PT430 concentration (μM)</th>
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<tbody>
<tr>
<td>h</td>
<td>0.1</td>
</tr>
<tr>
<td>2</td>
<td>8.3</td>
</tr>
<tr>
<td>4</td>
<td>8.8</td>
</tr>
<tr>
<td>6</td>
<td>8.5</td>
</tr>
<tr>
<td>48</td>
<td>6.4</td>
</tr>
</tbody>
</table>

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**Fig. 5.** Histograms of dose- and time-dependent uptake of PT430 by L1210 leukemia cells. Each curve was prepared from the flow cytometric assay of $2 \times 10^9$ cells.

**Fig. 6.** Flow cytometric measurements of the uptake of PT430 by L1210 leukemia cells. Each curve was prepared from the flow cytometric assay of $2 \times 10^9$ cells.
Sarcoma 180 cells would not necessarily be expected to occur under the same conditions in other cells. We have not studied the properties of PT430 in Sarcoma 180 cells and, therefore, cannot make a direct comparison at present.

A series of histograms was also obtained (Fig. 8) in which fluorescence was plotted against forward light scattering, indicative of cell size, for $10^7$ cells. Although it is apparent from these cytograms that a direct relationship exists between cell size and fluorescence, this phenomenon is not a major determinant of the observed fluorescence intensities.

Further evidence of the binding of PT430 to MTX-specific sites was obtained by preincubating L1210 cells with 90 $\mu$M MTX for 1 h, then adding 30 $\mu$M PT430 and resuming incubation for 1-6 h at 37 °C. Flow cytometric analysis showed inhibition of fluorescence uptake at each time point in the cells pretreated with MTX as opposed to nonpretreated controls (Fig. 9).

The results presented here exemplify the potential usefulness of PT430 in the rapid flow cytometric detection of dihydrofolate reductase overproduction in MTX-resistant cells, and demonstrate that binding involves MTX-specific sites. Because it incorporates the advantageous features of MTX-F in a new structure that (a) eliminates the need for a pentamethylene spacer, (b) avoids the problem of isomeric products, and (c) gives more rapid uptake into cells, PT430 is seen to be an improved enzyme-directed reporter ligand for the characterization of MTX resistance.

Acknowledgments—We are grateful to Dr. Michael Wick and Gerda Swedowski for the cytotoxicity data, Lisa Christiansen for operation of the flow cytometer, and Ann Camac for providing L1210 cells. The interest and encouragement of Dr. Emil Frei III are also acknowledged.

REFERENCES