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A FLUORESCENT ANALOGUE OF METHOTREXATE AS A PROBE FOR FOLATE ANTAGONIST MOLECULAR RECEPTORS

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Abstract—A dansyl-L-lysine analogue of methotrexate, N^{α}-(4-amino-4-deoxy-10-methylpteroyl)-N^{ε}-(5-[*N*,*N*-dimethylamino]-1-naphthalenesulfonyl)-L-lysine, is a potent inhibitor of murine L1210 dihydrofolate reductase. The dansyl fluorescence emission was enhanced approximately 3-fold with a 10 nm blue shift upon binding to L1210 dihydrofolate reductase. The fluorescent analogue was only 10-fold less potent than methotrexate in inhibiting the growth of methotrexate-sensitive and -resistant L1210 cells and competes effectively for [^{3}H]methotrexate transport with a K_i of 7.02 μM, a value virtually identical to the K_t for methotrexate in both cell lines. In addition, strong dansyl fluorescence was found to be associated with dihydrofolate reductase from methotrexate-resistant, dihydrofolate reductase-overproducing L1210 cells following incubation of viable cells with the fluorescent methotrexate analogue for 4 hr. The results demonstrate that the dansyl-L-lysine analogue of methotrexate was rapidly transported into L1210 cells where it formed a high-affinity, fluorescent complex with intracellular dihydrofolate reductase.

Dihydrofolate reductase (DHFR)§ is the major intracellular receptor for the chemotherapeutic action of 4-amino analogues of folic acid, such as methotrexate (MTX). The binding of MTX to DHFR has been well characterized from X-ray crystallographic studies of enzymes from *Escherichia coli* [1], *Lactobacillus casei* [2] and chicken liver [3]. A specific charge interaction between the α-carboxylate of the L-glutamate moiety of MTX and an invariant arginine residue has been implicated to be important in the binding of MTX to DHFR. The importance of a free α-carboxylate on the MTX molecule for binding has been further substantiated from the studies with α- and γ-carboxyl modified MTX derivatives [4–6]. These studies indicate that the presence of certain groups on the γ-carboxylate of MTX does not alter significantly the binding of these MTX derivatives to DHFR as evidenced from the inhibition studies. For example, the MTX-γ-benzylamide derivative, despite the bulkiness of the benzyl ring, was shown to be a good inhibitor of L1210 DHFR [5]. However, the α-carboxyl substituted derivatives were found to be considerably less inhibitory as compared to MTX.

We have reported recently the synthesis and properties of lysine and ornithine analogues of MTX [7, 8]. These compounds possess the essential free α-carboxyl group required for binding to DHFR and, in addition, contain a terminal amino group instead of a carboxylate group. In spite of the charge difference, both analogues are potent inhibitors of DHFR. Furthermore, the presence of a free amino group provides an excellent attachment point for additional substituent groups. As a result, we have synthesized two fluorescent analogues of MTX using the lysine and ornithine analogues and dansyl chloride [8, 9], and have devised a high performance liquid chromatographic procedure for their purification [9]. The dansylated derivatives of the lysine and ornithine analogues of MTX are highly potent inhibitors of DHFR, despite the presence of the bulky naphthalene substituent [8]. Binding of either fluorescent analogue to avian or to *L. casei* DHFR results in a 10–15 nm blue shift of the ligand emission maximum and a 2- to 5-fold enhancement of the fluorescence emission [10].

The synthesis of a fluorescein conjugate of MTX (MTX-F) has been reported previously in which fluorescein-diaminopentane was linked to the α- and/or γ-carboxyl group(s) of MTX using a carbodiimide coupling procedure [11]. The MTX-F conjugate has been used in recent years as an intracellular marker for DHFR although its transport into cells is extremely slow [12, 13]. An important consequence of carbodiimide coupling during the synthesis of MTX-F is the possible generation of the α-carboxyl conjugate and also racemization of the glutamate moiety. During the synthesis of α- and γ-glutamate

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§ Abbreviations: DHFR, dihydrofolate reductase (EC 1.5.1.3); MTX, methotrexate, 4-amino-4-deoxy-10-methylpteroylglutamic acid; MTX-F, fluorescein-diaminopentane-methotrexate; ALD, N^{α}-(4-amino-4-deoxy-10-methylpteroyl)-N^{ε}-(5-[*N*,*N*-dimethylamino]-1-naphthalenesulfonyl)-L-lysine; and ALF, N^{α}-(4-amino-4-deoxy-10-methylpteroyl)-N^{ε}-(4′-fluoresceinthiocarbamoyl)-L-lysine trihydrate.

peptides of MTX, Rosowsky and Yu [14] observed that the α-substituted isomer was the major product in direct condensation reactions using peptide bond forming reagents such as *N,N'*-dicyclohexylcarbodiimide. For these reasons, a direct reaction of dansyl chloride with the ε- or δ-amino groups on lysine or ornithine analogues of MTX provides fluorescent analogues of MTX with free α-carboxyl groups. The present study describes the interaction of the fluorescent compound N^{α}-(4-amino-4-deoxy-10-methylpteroyl)-N^{ε}-(5-[*N,N*-dimethylamino]-1-naphthalenesulfonyl)-L-lysine (ALD) with purified DHFR from murine L1210 cells and examines the cellular uptake of the derivative into MTX-resistant, DHFR overproducer L1210 cells.

MATERIALS AND METHODS

Reagents. Cell culture products were purchased from KC Biologicals (Lenexa, KS). MTX was a gift from Lederle Laboratories (Pearl River, NY). Sephadexes and AH-Sepharose-4B were obtained from Pharmacia (Piscataway, NJ) and Bio-Gel HPT from Bio-Rad Laboratories (Richmond, CA). Dihydrofolic acid was prepared from commercial folic acid by dithionite reduction [15] and stored at −20° as a suspension in 10^{-3} N HCl. NADPH was purchased from the Sigma Chemical Co. (St. Louis, MO). [3',5',7-^{3}H]MTX was obtained from Amersham (Arlington Heights, IL) and was purified before use as described previously [16]. All other chemicals were reagent grade or of the highest quality available.

Cells. MTX-sensitive L1210 cells (L1210/S) were grown in suspension culture as described previously [16]. The MTX-resistant L1210/R71 cell line with a 100-fold elevation of DHFR was initially provided by Dr. R. C. Jackson (Warner-Lambert, Ann Arbor, MI), and was maintained in culture medium containing 3 μM MTX. The L1210/R71 cells were cultured in the absence of MTX for at least three transfer generations before harvesting in order to eliminate MTX bound to DHFR. Both cell lines have population doubling times of 12–14 hr.

Enzyme purification. Frozen cells (5×10^{10}) were thawed, suspended in 100 ml of 10 mM Tris–Cl, pH 7.2, subjected to another freeze–thaw cycle, and centrifuged at 40,000 *g* for 30 min. The DHFR was purified to homogeneity by subjecting the cell supernatant fraction to MTX-affinity chromatography and gel filtration [17], followed by chromatography on a Bio-Gel HPT (hydroxylapatite) column to remove bound substrate. The purified enzyme exhibited a single protein band following electrophoresis on 10% polyacrylamide gels containing 0.1% sodium dodecyl sulfate [18]. Removal of substrate was evaluated by measuring the absorption spectrum of the enzyme (A_{280}/A_{260} of 2.2). The purified L1210 enzyme was stored at −20° and found to be stable for several months.

Standard enzyme assay. Dihydrofolate reductase activity was assayed spectrophotometrically. Initial rates were derived from the change in absorbance continuously recorded with a Cary model 219 spectrophotometer. The assay mixture, in a total volume of 1 ml, consisted of 0.05 M potassium phosphate buffer (pH 7.0), 0.14 mM NADPH, and 0.1 mM dihydrofolate. The standard unit of enzyme activity was determined from the change in absorbance at 340 nm using a $\Delta\varepsilon$ for the reaction of 12,300 M^{-1} at 22° [19]. Inhibition studies were carried out by preincubating the enzyme and the inhibitor in the assay buffer for 2 min at 22°, and residual enzyme activity was determined after the addition of NADPH and dihydrofolate. Remaining activity was expressed as percentage of activity compared to the activity of the enzyme obtained in the absence of inhibitor.

Cell growth inhibition. Cells at an initial concentration of 5×10^4 cells/ml were grown in medium containing the indicated concentrations of MTX or ALD in 24-well cluster dishes and incubated at 37° in a 5% CO_2 atmosphere. After 48 hr, cells were counted using a model Z_F Coulter counter (Coulter Electronics, Hialeah, FL). The IC_{50} is defined as the drug concentration which reduces the number of cells in 48 hr to 50% of the untreated control value.

Transport determinations. Transport of [^{3}H]MTX was measured in RPMI 1640 without folic acid following the detailed procedures previously described [16]. [^{3}H]MTX influx velocity was measured prior to saturation of intracellular binding sites. Uptake rates were determined from samples taken 30, 60, 90 and 120 sec after the addition of [^{3}H]MTX (K_t studies) or the simultaneous addition of [^{3}H]MTX and ALD (K_i studies). Since L1210/S cells exhibit Michaelis–Menten saturation kinetics for MTX influx [20], K_t represents the concentration of substrate at which the rate of influx is half the maximum velocity. K_t and V values were obtained by Lineweaver–Burk analysis. K_i values for ALD were determined from Dixon plots of the reciprocal rate of [^{3}H]MTX transport as a function of ALD concentration.

ALD binding in cell extracts. Cells were harvested at a density of 10^6 cells/ml, centrifuged at 500 *g* for 5 min (4°), washed with RPMI 1640 without folic acid (4°), and resuspended in folate-free medium at a density of 10^7 cells/ml. The cell suspension was incubated at 37° for 5 min prior to the addition of ALD. After 4 hr, the cells were centrifuged at 500 *g* for 5 min (4°), washed twice by resuspending in 100-ml aliquots of 0.15 M NaCl (4°), and finally resuspended in 10 mM Tris–Cl, pH 7.2, at 2.5×10^8 cells/ml. The cells were subjected to three freeze–thaw cycles utilizing a dry ice–acetone mixture and centrifuged at 40,000 *g* for 30 min. Cell extracts were applied to a Sephadex G-50 (Fine) column (1.5×100 cm) at 4°, and eluted with 50 mM Tris–Cl, pH 7.2. Fractions were monitored for absorbance at 280 nm and for DHFR activity. Fluorescence was measured in DHFR peak fractions.

Fluorescence detection. Fluorescence was recorded using a Perkin–Elmer MPF-44A fluorescence spectrophotometer operated in the ratio mode. Excitation was at 328 nm, slit widths were set at 6 nm and sample sensitivity at 1. The spectra reported are uncorrected and are represented in terms of arbitrary fluorescence units. The sample buffer was 50 mM Tris–Cl, pH 7.2, and the temperature was maintained at 20°.

RESULTS

Inhibition of L1210 *dihydrofolate reductase by ALD.* Purified, homogeneous, substrate-free L1210 DHFR at a concentration of 8×10^{-8} M was used to compare the effectiveness of MTX and ALD as enzyme inhibitors. As indicated in Fig. 1, ALD was a potent inhibitor of L1210 DHFR, with 50% inhibition occurring at 9.2×10^{-8} M. This value is 2.3 times the concentration of the stoichiometric inhibitor MTX necessary for 50% inhibition of DHFR.

Fluorescence enhancement upon binding ALD by dihydrofolate reductase. The fluorescence enhancement observed when purified DHFR binds to ALD in 50 mM Tris–Cl, pH 7.2, at 20° is shown in Fig. 2. Fluorescence emission was recorded as increasing quantities of DHFR were added to 4.6 μM ALD. A maximum fluorescence enhancement at 545 nm of approximately 3-fold was observed with a 2-fold molar excess of ALD. In addition, a 10 nm blue shift in the fluorescence emission wavelength was observed for the enzyme–ALD complex as compared to ALD alone, suggesting that ALD is bound to DHFR in a relatively hydrophobic environment.

Cellular uptake of ALD. Kinetic properties of the MTX transport system in L1210/S and L1210/R71 cells are summarized in Table 1. The K_t and V values for MTX transport were virtually identical for the sensitive and resistant cell lines. K_i values for ALD were also identical for each cell line and indicate an affinity for the transport system comparable to that of MTX.

ALD demonstrated significant *in vitro* growth inhibitory activity against both L1210/S and L1210/-R71 cell lines, as indicated in Fig. 3. The IC_{50} values for ALD in the sensitive and resistant cell lines were approximately 10-fold greater than those for MTX.

To determine if ALD was transported into cells or simply binding to the cell surface, L1210/R71 cells were incubated (37°) with 17 μM ALD for 4 hr,

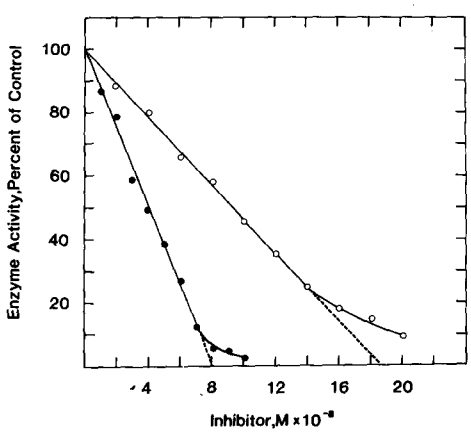

Fig. 1. Inhibition of L1210 DHFR with MTX and ALD. Purified DHFR (8×10^{-8} M) was incubated with appropriate amounts of inhibitor in 0.05 M potassium phosphate buffer (pH 7.0) for 2 min at 22°. Remaining DHFR activity was determined as described in Materials and Methods. Key: inhibition of DHFR by MTX (●—●) and by ALD (○—○).

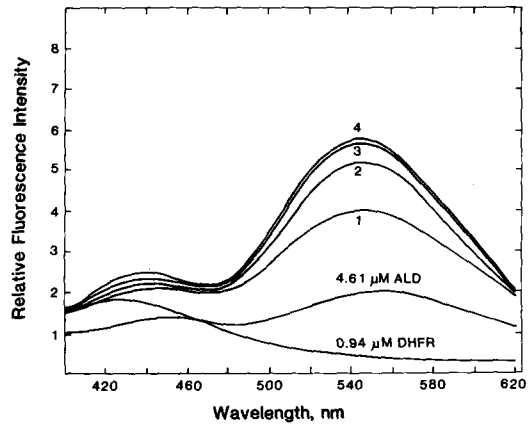

Fig. 2. Fluorescence emission spectra obtained by successive additions of purified L1210 DHFR to ALD (4.6 μM). Curve 1, ALD plus 0.9 μM DHFR; curve 2, ALD plus 1.9 μM DHFR; curve 3, ALD plus 2.4 μM DHFR; and curve 4, ALD plus 3.3 μM DHFR. Excitation at 328 nm.

thoroughly washed, disrupted, and the cell extract applied to a gel filtration column (Fig. 4). The enzyme peak from untreated L1210/R71 cells (Fig. 4A) contained 9.4 units of DHFR activity/10^9 cells, while the extract from ALD-treated cells (Fig. 4B) contained 2.9 units/10^9 cells, indicating that sufficient ALD had entered the cells during the 4-hr incubation to significantly inhibit DHFR. Fluorescence emission spectra obtained from fractions containing DHFR activity are shown in Fig. 5. The emission spectrum of the DHFR peak from ALD-treated L1210/R71 cells (Fig. 5, curve 3) clearly corresponds to the spectra obtained from ALD bound to purified L1210 DHFR (cf. Fig. 2).

DISCUSSION

Previous results from our laboratory [8–10] have shown that ALD, a fluorescent analogue of MTX, exhibits fluorescence properties characteristic of the dansyl substituent. The analogue has an excitation maximum at 328 mm and exhibits a broad fluorescence emission in the 540–600 nm range. In addition, ALD is an effective inhibitor of *L. casei* and avian DHFRs.

In the present study, the amount of ALD required to produce 50% inhibition of L1210 DHFR has been used to assess the inhibitory effectiveness of the compound relative to MTX. By this means, ALD is nearly as potent an inhibitor of L1210 DHFR as is

Table 1. Kinetic properties of the MTX transport system in L1210/S and L1210/R71 cells*

Cell line	K_t(MTX) (μM)	V(MTX) (nmoles/min/ 10^9 cells)	K_i(ALD) (μM)
L1210/S	7.25	0.642	7.02
L1210/R71	7.37	0.596	7.02

* Experimental details are described in the text.

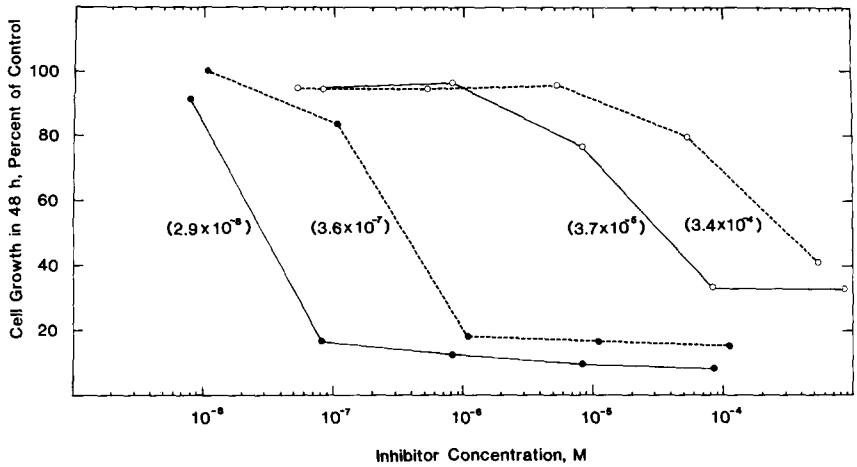

Fig. 3. Growth inhibition of L1210 cells by MTX and ALD. Key: (●—●) L1210/S plus MTX; (●- - -●) L1210/S plus ALD; (○—○) L1210/R71 plus MTX; and (○- - -○) L1210/R71 plus ALD. Numbers in parentheses indicate IC_{50} values.

MTX. Actual K_i determinations for tight-binding inhibitors are complex [21, 22]. The L1210 DHFR–MTX binary complex has a K_d of 3.7×10^{-9} M [23]. NADPH enhances DHFR binding to MTX; K_i values of 5.3×10^{-12} M [21] and 2.6×10^{-11} M [23] have been reported for the ternary complex. Previous studies in our laboratory have shown the binary complex of ALD with bacterial and avian DHFR to have K_d values of approximately 10^{-10} M [10]. Assuming a K_d value of the same order of magnitude for L1210 DHFR–ALD complex and enhancement of binding in the presence of NADPH, the K_i of the ternary complex would be similar to that for MTX. The inhibition studies demonstrate that replacement of the L-glutamate moiety of MTX by lysine and introduction of a dansyl group at the ε-amino position does not alter significantly the ability of these compounds to inhibit DHFR.

The fluorescence emission of ALD was enhanced on binding to L1210 DHFR with a blue shift of *ca.* 10 nm in the emission maximum (Fig. 2). This enhancement in the fluorescence intensity, as well as a shift in the emission maximum, is similar to the effects observed with free ALD in nonpolar solvents [10], suggesting the contribution of hydrophobic interactions at the binding site.

The fluorescein derivative of MTX (MTX-F) synthesized by Gapski *et al.* [11] is transported into L1210 cells extremely slowly, requiring several days at 37° for significant uptake of the compound [13].

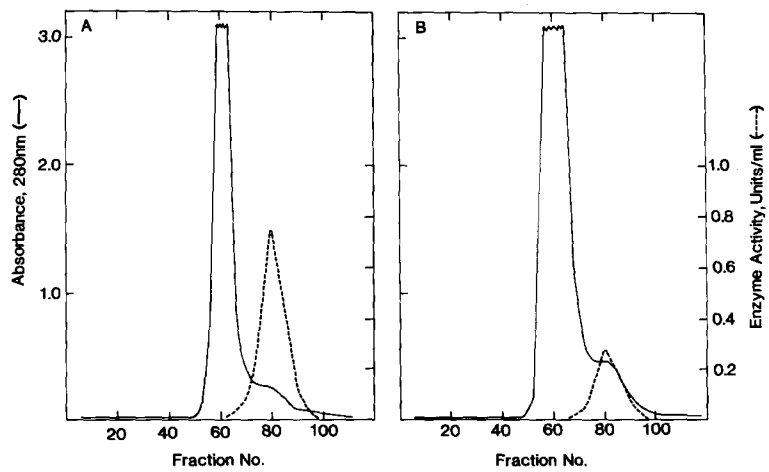

Fig. 4. Gel filtration of L1210/R71 cell extracts on Sephadex G-50. Crude cell extracts were applied to a 1.5 × 100 cm Sephadex G-50 (F) column, equilibrated with 0.05 M Tris–Cl. pH 7.2. Effluent fractions (1 ml) were monitored for absorbance at 280 nm (——) and DHFR activity (- - - -). (A) Extract from 0.94×10^9 cells. Cells were incubated at 37° for 4 hr in RPMI 1640 without folic acid. (B) Extract from 1.22×10^9 cells. Cells were incubated at 37° for 4 hr in RPMI 1640 without folic acid containing 17 μM ALD.

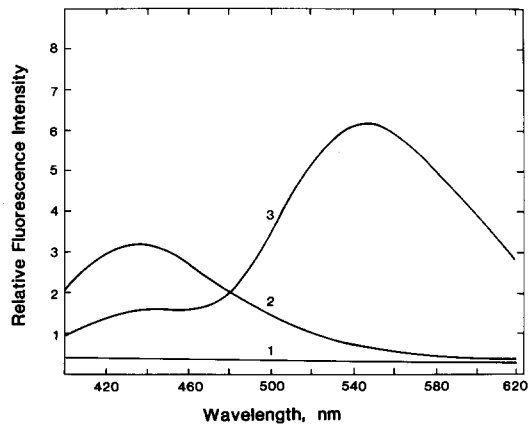

Fig. 5. Fluorescence emission spectra obtained from DHFR fractions of crude cell extracts following gel filtration. Curve 1, 0.05 M Tris–Cl, pH 7.2; curve 2, pooled fractions (79, 80, 81, cf. Fig. 4) from control L1210/R71 cells; and curve 3, pooled fractions (79, 80, 81) from ALD-treated L1210/R71 cells. Excitation at 328 nm.

This compound has been used by Kaufman *et al.* [12] to determine DHFR levels in enzyme overproducer MTX-resistant Sarcoma 180 cells using a fluorescence-activated cell sorter. Apparently, a small amount of MTX-F enters these cells producing measurable fluorescence upon binding to DHFR. The extremely slow uptake of MTX-F may be the result of either modification of the α-carboxyl group of the L-glutamate moiety of MTX with fluorescein-diaminopentane, or the inherent difficulty of this bulky substituent, attached to MTX, to penetrate the cell membrane.

Conversely, the α-carboxyl group in ALD synthesized in our laboratories is clearly unmodified, and the naphthalene portion of the dansyl moiety is considerably smaller than the fluorescein-diaminopentane group of MTX-F. Based on influx kinetics using ALD as a competitive inhibitor for the transport of [^{3}H]MTX, the K_i for ALD is 7.02 μM, indicating an affinity of the latter compound for the transport system comparable to that of MTX. Incubation of the fluorescent analogue with L1210 cells at 37° for only 4 hr, followed by thorough washing, cell disruption and gel filtration of the extract, demonstrated that: (1) DHFR was inhibited 70%, based on controls; and (2) dansyl fluorescence (λ_{max} = 545 nm) was associated with DHFR acitivity. These results indicate that ALD is rapidly transported into L1210 cells and is not simply binding to cell surface components.

ALD is 10-fold less potent than MTX as a growth inhibitor of L1210/S and L1210/R71 cells *in vitro*. It has been shown that intracellular MTX is converted to γ-polyglutamate conjugates [24] which bind to DHFR and accumulate in the intracellular compartment following DHFR saturation [25]. The higher extracellular concentrations of ALD required to inhibit the growth of L1210 cells may be related to the inability of ALD to form γ-polyglutamates, as well as to the DHFR inhibitory potency and transport characteristics of the compound.

Recently, Rosowsky *et al.* [26] reported the synthesis and characterization of a fluorescent analogue of MTX similar to ALD except that the dansyl moiety was replaced by a fluorescein derivative (ALF). ALF is 7- and 20-fold less potent than MTX as an inhibitor of L1210 and *L. casei* DHFRs, respectively, and is 100-fold less potent as a growth inhibitory agent of L1210 cells. The ALF compound appears to enter L1210 cells, based on flow cytometry experiments but, since influx kinetics were not performed, a direct comparison between ALF and ALD cannot be made.

Our results indicate the potential usefulness of ALD in the study of DHFR levels in MTX-sensitive and -resistant cell populations by using the fluorescence-activated cell sorter.

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