In summary, using protein fluoresence quenching and competitive displacement of ANS, we have assessed organic anion binding to human hepatic GSH *S*-transferases. The cationic, neutral and acidic forms showed comparable binding properties. Bilirubin and BSP were bound with much lower affinity than by rat ligandin, whereas ICG and hematin were bound with comparable high affinity as by rat ligandin.

Liver Research Laboratory HAJIME TAKIKAWA
Medical and Research Services YUICHI SUGIYAMA
Wadsworth Veterans ANDREW STOLZ
Administration Hospital MOTONOBU SUGIMOTO
and the UCLA School of Medicine NEIL KAPLOWITZ*
Los Angeles, CA 90073, U.S.A.

REFERENCES

1. W. B. Jakoby, *Adv. Enzymol.* **46**, 383 (1978).
2. N. Kaplowitz, *Am. J. Physiol.* **239**, G439 (1980).
3. Y. Sugiyama, M. Sugimoto, A. Stolz and N. Kaplowitz, *Biochem. Pharmac.* **33**, 3511 (1984).
4. K. Kamisaka, W. H. Habig, J. N. Ketley, I. M. Arias and W. B. Jakoby, *Eur. J. Biochem.* **60**, 153 (1975).
5. P. C. Simons and D. L. Vander Jagt, *J. biol. Chem.* **255**, 4740 (1980).
6. D. L. Vander Jagt, V. L. Dean, S. P. Wilson and R. E. Royer, *J. biol. Chem.* **258**, 5689 (1983).
7. M. Warholm, C. Guthenberg and B. Mannervik, *Biochemistry* **22**, 3610 (1983).
8. H. Jensson and B. Mannervik, *Acta chem. Scand.* **B36**, 205 (1982).
9. U. K. Laemmli, *Nature, Lond.* **227**, 680 (1970).
10. J. N. Ketley, W. H. Habig and W. B. Jakoby, *J. biol. Chem.* **250**, 8670 (1975).
11. Y. Sugiyama, T. Yamada and N. Kaplowitz, *Biochem. J.* **199**, 749 (1982).
12. Y. Sugiyama, T. Yamada and N. Kaplowitz, *J. biol. Chem.* **258**, 3602 (1983).
13. Y. Sugiyama, A. Stolz, M. Sugimoto and N. Kaplowitz, *J. Lipid Res.* **25**, 1177 (1984).
14. G. Scatchard, *Ann. N.Y. Acad. Sci.* **51**, 660 (1949).
15. C. F. Chignell, in *Methods in Pharmacology* (Ed. C. F. Chignell), Vol. 2, p. 33. Appleton-Century-Crofts, New York (1972).

* Address reprint requests to: N. Kaplowitz, M.D., W111C Wadsworth Veterans Administration Hospital, Los Angeles, CA 90073.

Biochemical Pharmacology, Vol. 35, No. 2, pp. 356–360, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00

Transport of a fluorescent antifolate by methotrexate-sensitive and methotrexate-resistant human leukemic lymphoblasts

(*Received* 16 *May* 1985; *accepted* 15 *July* 1985)

Interest in fluorescent inhibitors of dihydrofolate reductase (DHFR*) has grown over the decade since the original report [1] of the synthesis of MTX-F from methotrexate (MTX), fluorescein isothiocyanate (FITC), and 1,5-pentanediamine. It was shown [2, 3] that MTX-F could serve as an intracellular marker for DHFR, and this led to impressive uses of the compound in studies of DHFR overproduction in MTX-resistant cells by flow cytometry [4–8]. A compound of related but simpler structure, PT430 (Fig. 1), was synthesized in our laboratory from FITC and the lysine analogue of MTX [9, 10]. other fluorescent DHFR ligands were synthesized independently by reaction of the lysine and ornithine analogues of MTX with dansyl chloride [11–14]. Flow cytometric studies have been performed with PT430 as the fluorescent marker [9, 10, 15, 16], but have not been reported for the dansyl derivatives.

In this paper, we present an improved synthesis of PT430 and describe flow cytometric measurements of its uptake by MTX-sensitive human leukemic lymphoblasts (CEM cells) [17] and a resistant subline (CEM/MTX) with normal DHFR levels but a marked defect in MTX transport [18]. The influence of MTX on PT430 uptake by these cells is also compared. Our results demonstrate that cells with a defect in their transport mechanism for MTX can be distinguished from otherwise normal cells by flow cytometric analysis following incubation with PT430.

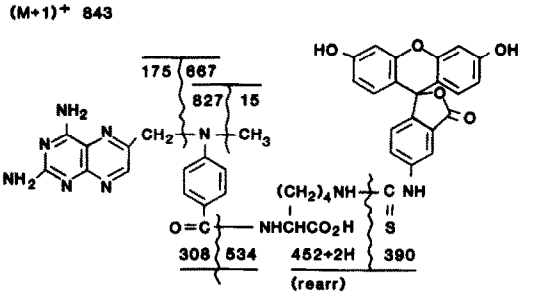

Fig. 1. Structure and fast atom bombardment mass spectral fragmentation pattern of PT430. Sites of fragmentation are shown by wavy lines.

Materials and methods

Spectra. Infrared spectra were obtained on a Perkin–Elmer model 781 double-beam spectrophotometer, and ultraviolet absorbance spectra were recorded on a Cary model 210 UV/visible instrument. Proton NMR spectra were obtained on a Varian T60A spectrometer. Fast atom bombardment mass spectra (FABMS) were determined on a Varian MAT 311A instrument through the courtesy of Dr. James Piper, Southern Research Institute, Birmingham, AL. Samples were dissolved in a mixture of *N,N*-dimethylformamide (DMF) and glycerol with 10% *p*-toluenesulfonic acid added to a final concentration of 10%. The probe tip was bombarded with Xe atoms of 8 keV energy, at a beam current setting of 1.2 mA [19].

N$^{\alpha}$-(4-*Amino*-4-*deoxy*-N^{10}-*methylpteroyl*)-N$^{\epsilon}$-(tert-*butyloxycarbonyl*)-L-*lysine* tert-*butyl ester.* To a suspension of 4-amino-4-deoxy-*N*^{10}-methylpteroic acid monohydrate

* Abbreviations: DHFR, dihydrofolate reductase (EC 1.5.1.3); MTX, methotrexate, 4-amino-4-deoxy-*N*^{10}-methylpteroyl-L-glutamic acid; MTX-F, fluorescein-diaminopentane-methotrexate; PT430, *N*$^{\alpha}$-(4-amino-4-deoxy-*N*^{10}-methylpteroyl)-*N*$^{\epsilon}$-(4′-fluoresceinthiocarbamyl)-L-lysine; FITC, fluorescein isothiocyanate; DEAE-cellulose, *N,N*-diethylaminoethylcellulose; FBS, fetal bovine serum; DME, Dulbecco's modified Eagle's medium; and PBS, phosphate-buffered saline.

(1.73 g, 5 mmoles) [20] in dry DMF (500 ml) at 35° were added *N,N*-diisopropyl-*N*-ethylamine (1.75 ml, 10 mmoles) and diethyl phosphorocyanidate (1.48 g, 10 mmoles) (Aldrich, Milwaukee, WI). The clear solution was kept for 3 hr before being added, with moisture exclusion, to a solution of N^{ε}-*tert*-butyloxycarbonyl-L-lysine *tert*-butyl ester hydrochloride (3.72 g, 22 mmoles) (Chemical Dynamics, South Plainfield, NJ) in dry DMF (20 ml) containing *N,N*-diisopropyl-*N*-ethylamine (1.92 ml, 11 mmoles). The mixture was kept at room temperature for 4 days and was then evaporated at 60° for 2.5 hr under reduced pressure. The oily residue was suspended in water (200 ml), and the pH was brought to 8 with concentrated ammonia. The precipitate was filtered and dried *in vacuo* prior to chromatography on a silica gel column with 3:1 $CHCl_3$–MeOH as the eluent. The major yellow band eluting after a minor impurity was collected, and most of the solvent was evaporated. Addition of water (400 ml) caused precipitation of a yellow solid, which was filtered and dried *in vacuo* at 25° overnight; yield 2.76 g (87%); m.p. 138–144° (lit. [11] m.p. 131–134°); IR: ν (KBr) 3330, 2980, 2940, 1640, 1610, 1510, 1390, 1370, 1250, 1210, 1160, 1060, 960, 840, 820, 770, 740 cm^{-1}; NMR: δ ($CDCl_3$ + 1% TMS) 0.7–2.1 [m, 24H, 2 C(CH_3)$_3$ + (CH_2)$_3$], 3.0 (m, 5H, CH_3N + CH_2NH), 4.6 (m, 3H, CH_2N + α-CH), 6.6–7.6 (2 m, 6H, C_6H_4 + 2 CONH), 8.5 (s, 1H, CH=N). Anal. Calc. for $C_{30}H_{43}N_9O_5 \cdot 1.5H_2O$: C, 56.59; H, 7.28; N, 19.80. Found: C, 56.96; H, 7.15; N, 19.83.

N^{α}-(4-*Amino*-4-*deoxy*-N^{10}-*methylpteroyl*)-L-*lysine*. The *tert*-butyl ester obtained in the preceding experiment (2.23 g, 3.5 mmoles) was dissolved in trifluoroacetic acid (25 ml) at room temperature and kept for 2 hr before being evaporated under a stream of nitrogen. The residue was triturated with ether (100 ml), filtered, and taken up in water (100 ml). The pH was adjusted to 9 with concentrated ammonia, the solution was filtered, and the filtrate was concentrated to a volume of 5 ml under reduced pressure. Absolute ethanol (200 ml) and triethylamine (5 ml) were added, and the precipitate was collected, suspended again in alcohol, filtered, and dried *in vacuo* at room temperature for 3 hr; yield 1.58 g (89%); TLC: R_f 0.12 (Baker Si250F silica gel, 3:1:1 *n*-BuOH–AcOH–H_2O). IR and NMR spectra were as previously reported [10]. Anal. Calc. for $C_{21}H_{27}N_9O_3 \cdot 3H_2O$: C, 49.70; H, 6.54; N, 24.84. Found: C, 49.94; H, 6.40; N, 24.84.

N^{α}-(4-*Amino*-4-*deoxy*-N^{10}-*methylpteroyl*)-N^{ε}-(4′-*fluoresceinthiocarbamyl*)-L-*lysine PT*430). A suspension of 4-amino-4-deoxy-N^{10}-pteroyl-L-lysine trihydrate (0.435 g, 0.856 mmole) in dry DMF (8 ml) at room temperature was added to a solution of FITC, isomer I (0.343 g, 0.881 mmole) (Sigma, St. Louis, MO). The mixture was triturated in an ultrasonic bath until the solid became finely dispersed, and stirring was continued magnetically for 2.5 days. After rotary evaporation, the last traces of DMF were removed by entrainment with two 25-ml portions of 5% ammonium hydroxide. The product was purified on a DEAE-cellulose column (Whatman DE-52, 2.8 cm i.d. × 20 cm) by elution with 1.5 M ammonium carbonate. Samples of 7 ml each were monitored by TLC, appropriately pooled, and evaporated. Residual ammonium carbonate was removed by entrainment with three 700-ml portions of distilled water, the residue was redissolved once more in distilled water, and the solution was freeze-dried for 2 days to obtain PT430 as an orange powder (0.414 g, 54% yield); m.p. > 360°; TLC: R_f 0.59 (Baker Si250F silica gel, 3:1:1 *n*-BuOH–AcOH–H_2O); IR: ν (KBr) 3650–2700, 1700–1520, 1500, 1460, 1420–1350, 1210, 1140–1070 cm^{-1}; UV λ_{max} (0.1 N HCl) 227, 287, 445 nm (shoulders at 309, 352, 490 nm); λ_{max} (0.1 N NaOH) 240, 284, 368, 498 nm (shoulders at 256, 310 nm); FABMS: *m/e* 843 (M + 1), 827 (M − CH_3), 667 ($C_{35}H_{33}N_4O_8S$), 534 ($C_{27}H_{24}N_3O_7S$), 454 ($C_{21}H_{28}N_9O_3$), 390 ($C_{21}H_{12}NO_5S$), 308 ($C_{15}H_{14}N_7O$), 175 ($C_7H_7N_6$). Anal. Calc. for $C_{24}H_{38}N_{10}O_8S \cdot 3H_2O$. C, 56.24; H, 4.94; N, 15.62; S, 3.57. Found: C, 56.04; H, 4.82; N, 15.48; S, 3.74.

Cell culture. CEM and CEM/MTX cells [17, 18] were maintained at 37° with 8% humidified CO_2 in Dulbecco's modified Eagle's medium (DME) supplemented with 10% fetal bovine serum (FBS), L-glutamine (293 μg/ml), penicillin (100 units/ml), and streptomycin (100 μg/ml). Doubling times were determined by daily counts beginning at a density of 1×10^5 cells/ml until a plateau was reached. In cytotoxicity assays, an initial inoculum of 5×10^5 cells/ml was used, and cells were counted after 48 hr. The IC_{50} is defined as the drug concentration needed to reduce the number of cells in 48 hr to 50% of untreated controls. The DHFR content and MTX sensitivity of the two cell lines were measured and found to be unchanged from earlier values [18].

Flow cytometry. A Coulter Electronics Epics V instrument was used for these experiments, excitation being provided by a 488 nm, 100 mW argon laser. Fluorescence intensity was standardized in each run by centering 1/8-bright Fluorospheres (Coulter Corp., Hialeah, FL) on channel 175 (gain *ca.* 600 mV) and frequently rechecking their position. Output data were processed with the aid of a computer program for the Easy I system to obtain values of mean fluorescence per cell from populations of 20,000 cells per time point.

*PT*430 *uptake.* One day prior to each experiment, 7×10^7 cells were removed from suspension culture and plated onto 175-cm^2 plastic tissue culture flasks (Falcon, Oxnard, CA). On the day of the experiments, the suspended dead cells and debris were decanted, and the viable, attached cells were resuspended in fresh medium by vigorous agitation. An aliquot of 5×10^7 cells was pelleted and resuspended in 3.5 ml of DME supplemented with L-glutamine and antibiotics as before, but also containing 10% dialyzed FBS, 10 μM thymidine, and 100 μM deoxyinosine (hereafter called "uptake medium"). After 20–30 min of incubation at 37°, a 2.97-ml portion of the cells, suspended in uptake medium at a density of 2×10^6 cells/ml, was added to 30 μl of stock PT430 solution at 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, or 6.0 mM. Aliquots of 500 μl were withdrawn at 10-min intervals over 60 min and were quenched in 5 ml of ice-cold phosphate-buffered saline (PBS). In another experiment, uptake of 15 μM PT430 was measured at intervals of 10, 20, 30, 60, 90, 120, 150, 180, 240, 300, and 360 min. The cells were pelleted, and the supernatant fraction was discarded. They were then resuspended at a density of 1×10^6 cells/ml in ice-cold PBS and assayed by flow cytometry, with 20,000 cells being sorted per time point. Mean fluorescence intensities for each population of 20,000 cells were determined, and initial uptake velocities were calculated by least-squares analysis. Although reproducible under stated conditions of instrument standardization, such velocity values, including V_{max}, are necessarily expressed in the relative units mean fluorescence/time. *Ratios* of these values for the two cell lines, however, are absolute, unitless constants. Mean values from at least three experiments on different days were analyzed by the Lineweaver–Burk method [21].

Inhibition of PT430 uptake by MTX was measured by a modification of the assay described above, with 0, 20, 33, 50, 67, 100, or 150 μM MTX being added to the original PT430 stock solution.

Results and discussion

The present synthesis of PT430 represents a substantial improvement over the one reported earlier [10]. Our yield of N^{α}-(4-amino-4-deoxy-N^{10}-methylpteroyl)-N^{ε}-(*tert*-butyloxycarbonyl)-L-lysine *tert*-butyl ester from 4-amino-4-deoxy-N^{10}-methylpteroic acid was 87%, whereas other workers had reported a yield of only 56% [11]. The difference probably reflects the fact that we carried out the

activation step at room temperature instead of 0° and that coupling was allowed to proceed for 4 days instead of 18 hr. Deprotection was accomplished in 89% yield with neat trifluoroacetic acid at room temperature for 2 hr. Once again, the yield was higher than was reported [11] for cleavage with aqueous trifluoroacetic acid in anisole. In the final step, condensation of the deprotected lysine derivative with FITC proceeded in 54% yield. Thus, the present route afforded PT430 in 42% overall yield and allowed a 10-fold scale-up of the synthesis, which previously had been limited to the production of PT430 in 50 mg batches. The product was rigorously characterized by microchemical and spectral analysis. In particular, fast atom bombardment mass spectrometry ruled out any possibility that FITC had attacked a ring nitrogen instead of the ε-amino group of the lysine moiety. The appearance of a significant m/e 534 fragment (Fig. 1) can only be explained if the fluoresceinthiocarbamyl group is attached to lysine. The lactone structure shown is one of the three familiar forms for the fluorescein moiety. In solution at pH 7.4, the ring-opened form, containing two ionized carboxyl groups, would be expected to predominate.

Comparative measurements of the ability of CEM and CEM/MTX cells to take up PT430 were performed by flow cytometry. The uptake medium contained dialyzed serum in order to eliminate possible competition for transport by reduced folates, which are present in whole serum. To ensure that removal of the reduced folates did not damage the cells, we also included thymidine and deoxyinosine in the medium. Histograms from a representative experiment in which the cells were incubated with 15 μM PT430 at 37° for 1 hr are shown in Fig. 2, where each histogram represents 20,000 cells. Higher fluorescence is evident in the CEM cells than in the CEM/MTX cells at each 10-min time point, reflecting decreased capacity for PT430 transport in the cells that have a defect for MTX transport. Inspection

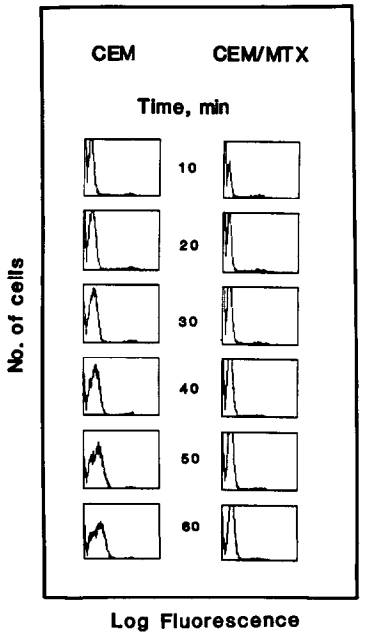

Fig. 2. Histograms of flow cytometric assays showing differential uptake of PT430 (15 μM) by CEM and CEM/MTX cells after 10–60 min at 37°.

of the two series of histograms also reveals an interesting property of the CEM cells that illustrates the great advantage of flow cytometry as an analytic tool for the detection of heterogeneity in populations of cells. The fluorescence distribution is broader in the CEM cells than in the CEM/MTX cells from the start, and by 40 min becomes bimodal, indicating two populations of cells with respect to PT430 uptake. One possible explanation for this finding is that the CEM line already consists of some cells with an inherently low capacity for PT430 transport, and that these cells served as the progenitors for the MTX/CEM subline. Another possibility is that PT430 uptake may be more pronounced in cell populations that are passing through DHFR-rich phases of the cell cycle [5, 20]. Since bimodality was not observed in the PT430 uptake histograms for CEM/MTX cells, which exhibit the same cytokinetic behavior as CEM cells, the former explanation seems more likely.

A plot of fluorescence for CEM and CEM/MTX cells incubated for 6 hr with 15 μM PT430 is presented in Fig. 3. Uptake was exponential in both cell lines, with an initial linear phase lasting about 1 hr. The data could be fitted to the kinetic equation $U = b(1 - e^{-kt})$, where U is the mean fluorescence per cell in the population, expressed in mV of photomultiplier output, k is the exponential coefficient (min^{-1}), and b is the asymptotic value of fluorescence at infinite time. Values of k for the CEM and CEM/MTX cells were calculated to be 2.9×10^{-3} and 1.0×10^{-2} min^{-1}. Initial velocities, calculated from the formula $V_i = kb$, were 0.69 and 0.43 mV/min. Thus, when uptake was measured at 15 μM PT430, there was a 3.4-fold increase in the first-order constant and a 40% decrease in initial velocity in the transport-defective CEM/MTX cells in comparison with the parent line. In our earlier study of [^{3}H]MTX uptake by CEM and CEM/MTX cells [18], a decrease in V_i of about 80% was observed in the resistant cells at a drug concentration of 0.5 μM. In other experiments using 4 μM [^{3}H]-MTX, we have found V_i to be decreased by 60% in the CEM/MTX cells.* It thus appears that, qualitatively at least, PT430 uptake differences shown by the two cell lines are comparable to the differences that we, and more recently other investigators [22], have observed in direct measurements of [^{3}H]MTX uptake.

An interesting aspect of the uptake curves in Fig. 3, reminiscent of the behavior of MTX [18], is that the sensitive cells continued to take up drug long after a near plateau had been reached in the resistance cells. Since the two cell lines were found to contain the same amount of DHFR, 3.6 ± 0.2 pmoles/10^8 cells, it appears that the resistant cells are accumulating only as much PT430 as can become DHFR-bound. The sensitive cells, on the other hand, can concentrate the drug to a level several times higher than the level of enzyme. Consistent with this interpretation was the finding that the IC_{50} of PT430 for 48-hr treatment of CEM/MTX cells was 28 μM, while in CEM cells the IC_{50} was only 3.5 μM.

Michaelis–Menten constants (K_m) were obtained for PT430 uptake in CEM and CEM/MTX cells by determining V_i over a range of PT430 concentrations from 15 to 60 μM. Uptakes were linear for up to 60 min, allowing V_i to be derived directly from the slope at each concentration. From the Lineweaver–Burk plots shown in Fig. 4, K_m values of 58 and 97 μM, respectively, were obtained. There was thus a 40% decrease in K_m in the CEM/MTX cells in comparison with the parent line. V_{max} values, however, were identical for the two cells, 4.0 ± 0.1 mV/min. In other experiments,† we have found K_m values for MTX in CEM and CEM/MTX cells to be 2.3 and 12 μM, respectively, i.e. a 5.2-fold difference. Therefore, it appears that, while the trend in K_m values for PT430 is qualititatively consistent with that for MTX, the difference is smaller and cannot be used to quantitatively predict the extent of the transport defect for MTX.

It was of interest to examine what effect MTX would

* A. Rosowsky, J. E. Wright, C. A. Cucchi and E. Frei, III, manuscript in preparation.

† A. Rosowsky, J. E. Wright, C. A. Cucchi and M. Bijhani, unpublished results.

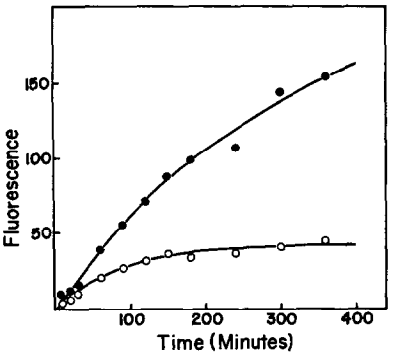

Fig. 3. Uptake of PT430 (15 μM) by CEM (●) and CEM/MTX (○) cells at 37°. The solid line was generated by first-order kinetic analysis using the experimental points shown. The squared regression coefficients were 0.99 for both curves.

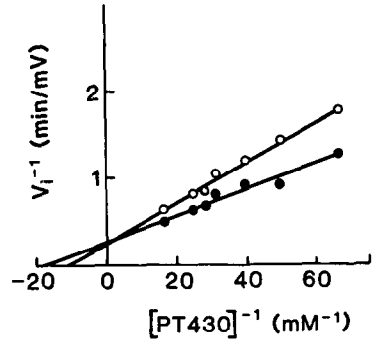

Fig. 4. Lineweaver–Burk plots of inverse concentration (μM^{-1}) versus inverse initial uptake velocity (min/mV) for CEM (●, N = 3) and CEM/MTX (○, N = 4) cells; S.D. ± 0.28 min/mV for both sets of points.

have on PT430 uptake in CEM and CEM/MTX cells. If PT430 and MTX share a common transport mechanism, one would expect that co-incubation of the cells with PT430 and increasing amounts of MTX would lead to a dose-dependent decrease in PT430 uptake with respect to MTX concentration. If, however, some fraction of the PT430 were taken up by a different mechanism, complete abolition of PT430 uptake by MTX might not be observed. As indicated in Fig. 5A, co-incubation of the CEM cells with 30 μM PT430 and zero to 0.67 μM MTX caused a decrease in V_i from 1.6 to 0.8 mV/min, but there was no further decrease as MTX was increased to 5 μM. This indicated that a component of PT430 uptake is not competitive with respect to MTX. When CEM/MTX cells were similarly co-incubated with PT430 and MTX (Fig. 5B), V_i decreased from 1.2 to 0.8 mV/min. The smaller change in V_i in the resistant cells reflected the fact that the V_i in the absence of MTX was smaller in these cells to begin with.

The finding that there is a component of PT430 uptake which is unaffected by MTX, and is essentially the same in MTX-sensitive and MTX-resistant cells, shows that a portion of the PT430 uptake is independent of the MTX-specific transport mechanism. The fact that CEM/MTX cells were 11-fold resistant to PT430, but 200-fold resistant to MTX even though their DHFR content is the same, supports this view. We have observed previously that PT430 uptake by human head and neck squamous cell carcinoma in culture is diminished by pretreatment with *p*-chloromercuribenzoate, and that efflux of the compound from

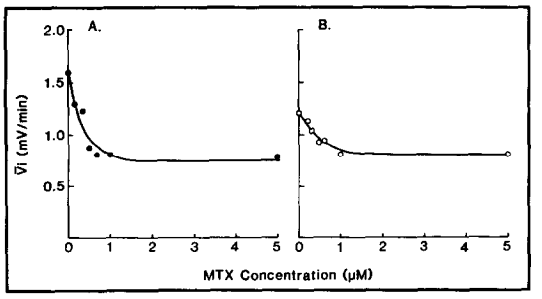

Fig. 5. Plots of PT430 influx velocity versus MTX concentration for CEM (panel A) and CEM/MTX (panel B) cells. Rates were measured by flow cytometry after incubation with 30 μM PT430 plus the concentrations of MTX shown on the abscissa.

preloaded cells is partly inhibited by sodium azide [16]. This suggests that a component of PT430 transport is carrier-mediated and requires energy, though it does not mean that the transport pathways for PT430 and MTX are necessarily the same. The data in Fig. 5 are consistent with the possibility that there are, in fact, different transport routes for the two compounds. It is of interest to note in this regard a recent report [23] that lipophilic antifolates may be taken up by a process which is more complex than passive transport but is different from the pathway used by MTX.

Two additional points should be made with regard to PT430 uptake by cells, regardless of whether they are MTX-sensitive or MTX-resistant. One is that we cannot at this time rule out the possibility that fluorescence observed in PT430-labeled cells represents, in part, binding to intracellular hydrophobic sites other than DHFR. This binding could lead to a fluorescence enhancement similar to that which occurs when PT430 is bound to DHFR. If there were fewer such sites in CEM/MTX cells than in CEM cells, decreased fluorescence would be observed. Thus, decreased fluorescence need not necessarily be due solely to an alteration in transport. The second point is that, when cells are incubated with both PT430 and MTX, the latter will bind preferentially to DHFR because of its lower K_m. This would decrease the amount of enzyme available for PT430 binding and lead to less fluorescence enhancement than would occur in the absence of MTX. Thus, the MTX-inhibited component of PT430 uptake may reflect competition not only at the transport level, but also at the level of the intracellular target for binding.

In summary, while it has been shown that PT430 can be used as a flow cytometric probe of DHFR overproduction in MTX-resistant cells [9, 10], the present study demonstrates that this compound may also be used to qualitatively distinguish cells with a defect in MTX transport from cells that are otherwise normal. PT430 fluorescence in cells with a defect in MTX transport reaches an early plateau, whereas cells with the same DHFR content but no defect in MTX transport are able to continue accumulating PT430 to levels several-fold higher. This behavior closely resembles that observed with MTX itself [18, 22]. However, the fact that MTX fails to completely block PT430 uptake in either cell type suggests that the latter compound may be another example of a lipophilic antifolate that can enter cells via a facilitated transport process not shared by MTX [23].

Acknowledgements—This work was supported in part by Grants CA25394 and CA19589 from the National Cancer Institute (DHHS); Biomedical Research Grant 118D from the American Cancer Society; and the Joseph Vaturi Respiratory Neoplasia Fund, Dana-Farber Cancer Institute.

Dana-Farber Cancer Institute and Departments of Pharmacology and Medicine
Harvard Medical School
Boston, MA 02115, U.S.A.

ANDRE ROSOWSKY*
JOEL E. WRIGHT
CAROL A. CUCCHI
KLAUS BOEHEIM
EMIL FREI, III

REFERENCES

1. G. R. Gapski, J. M. Whiteley, J. I. Rader, P. L. Cramer, G. B. Henderson, V. Neef and F. M. Huennekens, *J. med. Chem.* **18**, 526 (1975).
2. J. M. Whiteley and A. Russell, in *Chemistry and Biology of Pteridines* (Eds. R. L. Kisliuk and G. M. Brown), pp. 549–54. Elsevier/North Holland, New York (1979).
3. G. B. Henderson, A. Russell and J. M. Whiteley, *Archs Biochem. Biophys.* **202**, 29 (1980).
4. R. J. Kaufman, J. R. Bertino and R. T. Schimke, *J. biol. Chem.* **253**, 5852 (1978).
5. B. D. Mariani, D. L. Slate and R. T. Schimke, *Proc. natn. Acad. Sci. U.S.A.* **78**, 4985 (1981).
6. D. A. Haber and R. T. Schimke, *Cell* **26**, 355 (1981).
7. R. N. Johnston, S. M. Beverley and R. T. Schimke, *Proc. natn. Acad. Sci. U.S.A.* **80**, 3711 (1983).
8. B. D. Mariani and R. T. Schimke, *J. biol. Chem.* **259**, 1901 (1984).
9. A. Rosowsky, J. E. Wright, G. P. Beardsley and H. M. Shapiro, in *Chemistry and Biology of Pteridines* (Ed. J. A. Blair), pp. 475–9. Walter de Gruyter, Berlin (1983).
10. A. Rosowsky, J. E. Wright, H. Shapiro, P. Beardsley and H. Lazarus, *J. biol. Chem.* **257**, 14162 (1982).
11. A. A. Kumar, J. H. Freisheim, R. J. Kempton, G. M. Anstead, A. M. Black and L. Judge, *J. med. Chem.* **26**, 111 (1983).
12. A. A. Kumar, R. J. Kempton, G. M. Anstead, E. M. Price and J. H. Freisheim, *Analyt. Biochem.* **128**, 191 (1983).
13. A. A. Kumar, R. J. Kempton, G. M. Anstead and J. H. Freisheim, *Biochemistry* **22**, 390 (1983).
14. S. S. Susten, R. J. Kempton, A. M. Black and J. H. Freisheim, *Biochem. Pharmac.* **33**, 1957 (1984).
15. A. Rosowsky, J. E. Wright, G. P. Beardsley, H. Lazarus and H. M. Shapiro, *Proc. Am. Ass. Cancer Res.* **23**, 178 (1982).
16. J. E. Wright, A. Rosowsky, H. Lazarus, H. M. Shapiro and E. Frei, III, *Proc. Am. Ass. Cancer Res.* **23**, 179 (1982).
17. G. E. Foley, H. Lazarus, S. Farber, B. G. Uzman, B. A. Boone and R. E. McCarthy, *Cancer, N.Y.* **18**, 522 (1965).
18. A. Rosowsky, H. Lazarus, G. C. Yuan, W. R. Beltz, L. Mangini, H. T. Abelson, E. J. Modest and E. Frei, III, *Biochem. Pharmac.* **29**, 648 (1980).
19. M. Barber, R. S. Bardoli, G. J. Elliot, R. D. Sedwick and A. N. Tyler, *Analyt. Chem.* **54**, 645A (1982).
20. G. P. V. Reddy and A. B. Pardee, *Proc natn. Acad. Sci. U.S.A.* **77**, 3312 (1980).
21. M. Dixon, E. C. Webb, C. J. R. Thorne and K. F. Tipton, *Enzymes*, p. 60. Academic Press, New York (1979).
22. E. Mini, B. A. Moroson, C. T. Franco and J. R. Bertino, *Cancer Res.* **45**, 325 (1985).
23. D. W. Fry, J. A. Besserer, T. J. Boritzki, R. C. Jackson and E. F. Elslager, *Proc. Am. Ass. Cancer Res.* **26**, 340 (1985).

* To whom correspondence should be addressed.