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CHARACTERISTICS OF MEMBRANE TRANSPORT OF METHOTREXATE BY CULTURED HUMAN BREAST CANCER CELLS

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Abstract—Methotrexate, a folic acid analogue, enters cells using a high-affinity carrier system that is shared with naturally occurring reduced folates. Methotrexate transport by MCF-7 cells, a hormonally responsive line of human breast cancer cells, exhibited a high-affinity carrier system that displayed Michaelis–Menten kinetics [$K_m = 8.22 \pm 0.62$ μM; $V_{max} = 12.22 \pm 2.8$ nmoles · min^{-1} · (g cell protein)$^{-1}$]; was competitively inhibited by leucovorin and aminopterin, but not by folic acid; and was temperature-sensitive ($Q_{10} = 2.25 \pm 0.32$). Initial uptake rates were not affected by ouabain or sodium azide, but efflux of intracellular drug was inhibited markedly by sodium azide, suggesting the presence of an energy-dependent active efflux mechanism. At extracellular methotrexate concentrations in excess of 10 μM, a second, low-affinity uptake component could be identified that may represent a lower affinity membrane carrier or passive diffusion. Examination of hormonal influences on methotrexate transport revealed that growth of MCF-7 cells in serum-free medium induced a significant increase in the transport K_m value (15.93 ± 1.6 μM) compared to the K_m of 8.22 μM for cells grown in fetal calf serum. This change in affinity of the transport carrier could be reversed by the additon of insulin, but not of estradiol, to the culture medium. Methotrexate transport by human breast cancer cells displayed characteristics that were similar to those of transport reported for human leukemia cells but that have not been documented previously for cells derived from a human solid tumor. In addition, the drug transport carrier was subject to modulation by insulin but not by estrogen.

Methotrexate (2,4-diamino-N^{10}-methyl pteroyl glutamic acid, MTX) has been a useful agent for the treatment of breast cancer and other human solid tumors for many years. Murine and human leukemia cells actively transport this drug, using a high-affinity carrier system shared with naturally occurring reduced folates [1–4]. Indeed, alterations in membrane transport are a well-established mechanism of drug resistance [5, 6] in experimental tumors.

Although the principles derived from the study of membrane transport of methotrexate in murine tumors have played an important role in the design of clinical treatment protocols, little information is available concerning the characteristics of uptake of MTX by the cells of any human solid tumor. This paper presents the results of investigations of MTX membrane transport by MCF-7 cells, a cultured line of estrogen- and insulin-responsive human breast cancer cells. Our results demonstrate that MTX uptake by these cells displays characteristics of a high-affinity carrier system similar to those previously described for murine and human leukemia cells. In addition, a second low-affinity component of transport is demonstrated which may represent either a second membrane carrier or passive diffusion at high extracellular MTX concentrations. Further, we describe the effects of estrogen and insulin on drug transport in this line of hormonally responsive tumor cells.

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MATERIALS AND METHODS

Propagation of cells in culture. MCF-7 cells, a line of human breast cancer cells in continuous monolayer culture, were obtained from Dr Marc Lippman, National Cancer Institute, Bethesda, MD. The human derivation, hormonal responsiveness, and growth characteristics of these cells have been described [7, 8]. Cells were grown in 75 cm^2 plastic flasks in improved minimal essential medium (NIH Media Unit) supplemented with 10% fetal calf serum, L-glutamine (584 μg/ml), penicillin (124 μg/ml), and streptomycin (270 μg/ml) under an atmosphere of 95 per cent O_2–5% CO_2 at 37°. For transport experiments, cells were trypsinized and replicately plated in 25 cm^2 plastic flasks 2 days prior to each experiment. One hour prior to beginning each experiment, the medium was changed to improved minimal essential medium without glutamine, folic acid, or serum; transport experiments were performed in this medium. To determine the influence of hormones on MTX transport, cells were grown in improved minimal essential medium with 5% charcoal-treated fetal calf serum [7] and 5×10^{-7} M insulin. Two days prior to each transport experiment, cells were transferred to 25 cm^2 flasks and the medium was changed to improved minimal essential medium without serum or insulin. After 24 hr in this hormone-free medium, 5×10^{-7} M insulin or 1×10^{-8} M estradiol was added to one

group of cultures for 24 hr while control cultures received no added hormones, and transport experiments were then performed as described below. We used hormone concentrations that maximally stimulate cell growth in this cell line [9, 10].

Chemicals. [3′,5′,9-^{3}H]MTX (sp. act. 10.9 Ci/mmole) was purchased from Amersham/Searle, Arlington Heights, IL. The drug was further purified using DEAE-cellulose chromatography with elution along a linear gradient of 0.05 to 0.4 M NH_4HCO_3, pH 8.3 [2]. Unlabeled MTX, leucovorin, and aminopterin were obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute, Bethesda, MD, and were purified as described above. Regular insulin was purchased from Eli Lilly & Co., Indianapolis, IN, and 17-β-estradiol from Steraloids, Inc., Wilton, NH. Purified synthetic MTX-γ-glutamate was provided by Dr C. M. Baugh, Department of Biochemistry, University of Southern Alabama, Mobile, AL. Aquasol liquid scintillation counting fluid was obtained from the New England Nuclear Corp., Boston, MA. All other chemicals were of reagent grade and were purchased from either the Fisher Scientific Co., Fair Lawn NJ, or the Sigma Chemical Co., St. Louis, MO.

Uptake of [^{3}H]MTX by MCF-7 cells. To determine uptake of [^{3}H]MTX, MCF-7 cells growing in 25 cm^2 flasks were exposed to 2 μM [^{3}H]MTX for various periods of time at 37°. Drug uptake was stopped by the rapid addition of 5 vol. of 4° phosphate-buffered saline (PBS) to the flasks. This was followed by three additional washes with 4° PBS, following which the cells were digested from the flask surface by the addition of 1 ml of 1 N NaOH. An aliquot of the digested cellular material was taken for liquid scintillation counting and another for protein assay according to the method of Lowry *et al.* [11]. The component of MTX uptake due to surface adsorption was determined by measurement of cellular MTX at 0°. Intracellular drug levels were calculated by subtraction of the absorbed drug from total cellular MTX as measured at 37°. At extracellular drug concentrations of 1–10 μM, surface adsorption was negligible but became significant at higher drug concentrations, accounting for 6 per cent of total cellular MTX at an [MTX]e of 15–50 μM. Initial influx kinetics were determined over a concentration range of 0.5 to 50 μM. Preliminary experiments demonstrated that MTX uptake was linear for at least 10 min at an [MTX]e of 1 μM and for at least 4 min at an [MTX]e of 50 μM. Therefore, during kinetic experiments, drug exposure was limited to only 3 min to assure linearity of uptake at all extracellular MTX concentrations.

Determination of dihydrofolate reductase (DHFR) content of MCF-7 cells. Following a 24-hr incubation with 2 μM [^{3}H]MTX, MCF-7 cells were washed, disrupted by three cycles of freeze-thawing, and spun at 100,000 *g* for 60 min. The supernatant fraction was then applied to a Sephadex-G75 column and the MTX–dihydrofolate reductase complex was eluted as described previously [12]. The amount of bound MTX was then determined by liquid scintillation counting. Assuming a binding stoichiometry of 1:1 for enzyme and inhibitor, the dihydrofolate reductase binding capacity of MCF-7 cells was determined to be 5.7 ± 0.85 nmoles/g protein. These results were confirmed using a competitive binding protein assay [13] for dihydrofolate reductase binding capacity.

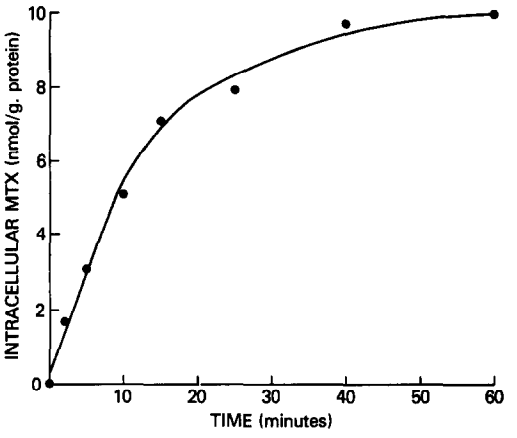

Fig. 1. Uptake of 2 μM [^{3}H]MTX by MCF-7 cells.

RESULTS

Methotrexate influx kinetics. Figure 1 illustrates the time course of uptake of [^{3}H]MTX by MCF-7 cells during a 60-min exposure to the drug. At an extracellular concentration of 2 μM, uptake appeared to be linear during the initial 10 min of drug exposure, following which the rate of drug accumulation slowed and approached a steady state.

Since there is negligible free intracellular drug prior to saturation of dihydrofolate reductase binding sites, study of initial influx kinetics is not complicated by backflux across the cell membrane or by intracellular metabolism. Preliminary experiments demonstrated drug uptake to be linear for at least 10 min at 1 μM and for at least 4 min at 50 μM. Therefore, initial influx kinetics were determined by exposing cells to various extracellular MTX concentrations for

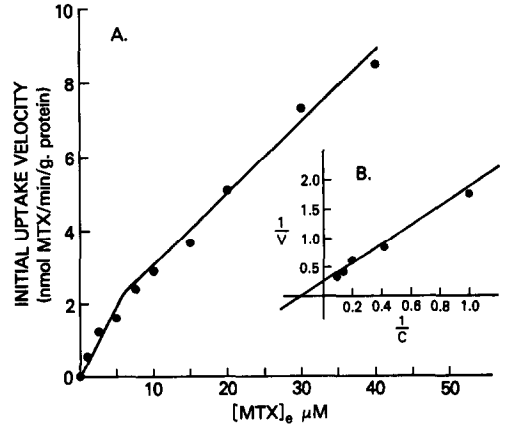

Fig. 2. Relationship between initial velocity of [^{3}H]MTX uptake and extracellular MTX concentration. (A) Velocity vs extracellular MTX concentration. (B) Double-reciprocal plot of velocity vs extracellular MTX concentration over the concentration range of 0.5 to 10 μM.

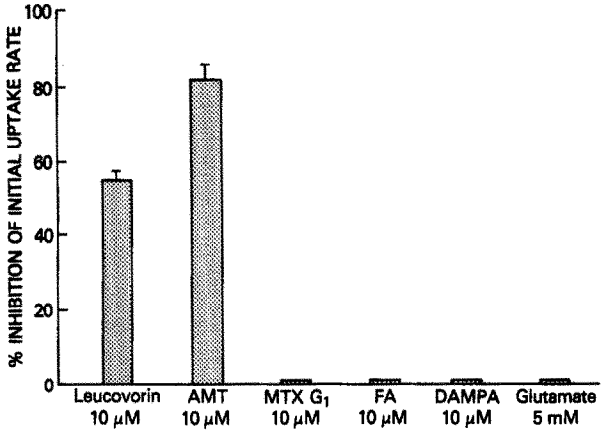

Fig. 3. Effects of physiologic folates and MTX analogues on initial rate of 2 μM [^{3}H]MTX uptake.

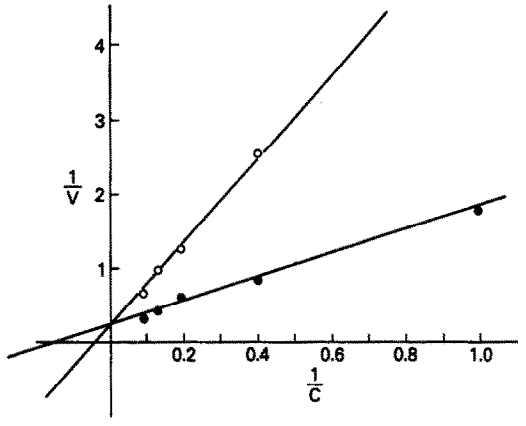

Fig. 4. Double-reciprocal plot of initial uptake velocity vs extracellular MTX concentration in the presence (○—○—○) and absence (●—●—●) of 10 μM leucovorin.

3 min, and drug accumulation was measured as described. Figure 2A displays the relationship between extracellular MTX concentration and initial influx velocity. The appearance of this curve suggested the presence of two influx components, one which was saturable at low extracellular drug concentrations and a second which was non-saturable over the concentration range studied. A double-reciprocal plot of uptake velocity against extracellular MTX concentration over the range of 0.5 to 10 μM (Fig. 2B) resulted in a linear relationship typical of Michaelis–Menten kinetics. The apparent K_m for this "high-affinity" transport route was 8.22 ± 0.62 μM and the V_{max} was 12.22 ± 2.8 nmoles·min^{-1}·(g cell protein)$^{-1}$(N = 9).

Effects of folate analogues on high-affinity transport. The effects of various natural folates and folate analogues on the high-affinity transport of MTX is shown in Fig. 3. Membrane transport of MTX was markedly inhibited by leucovorin and by aminopterin but not by equally low concentrations of folic acid, methotrexate-γ-glutamyl, 2,4-diamino-N^{10}-methylpteroic acid (DAMPA), a carboxypeptidase cleavage product of MTX, or by high concentrations of glutamic acid. A kinetic analysis revealed that this inhibition of uptake (Fig. 4) was competitive in nature, with a K_i for leucovorin of 2.95 ± 0.846 μM (assuming transport of both stereoisomers) and a K_i for aminopterin of 2.070 ± 0.624 μM. Although simultaneous exposure of MCF-7 cells to MTX and leucovorin resulted in inhibiton of MTX uptake, incubation of cells for 1 hr in the presence of 100 μM leucovorin prior to exposure to MTX resulted in a 52.24 ± 1.65 per cent increase in the initial transport rate, presumably due to heteroexchange with the intracellular reduced folate pool [14].

Effects of temperature and metabolic inhibitors on MTX transport. The high-affinity transport route is sensitive to changes in temperature. Decreasing the temperature to 27° inhibited initial MTX influx with

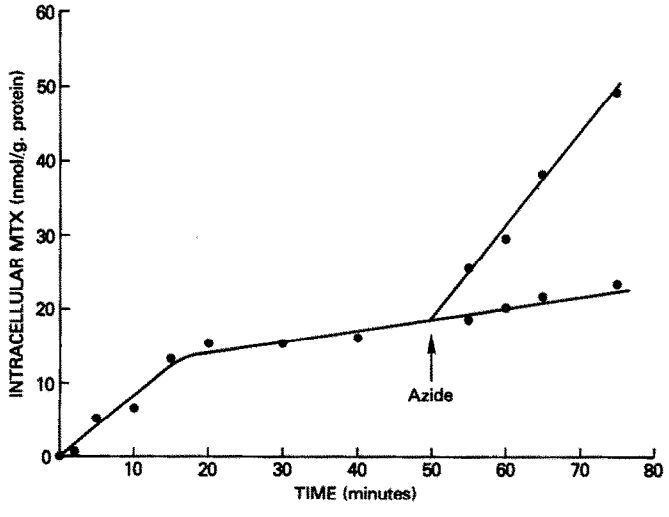

Fig. 5. Effect of 10 mM sodium azide on steady-state intracellular MTX level. MCF-7 cells were incubated with 2 μM [^{3}H]MTX for 50 min, after which 10 mM sodium azide was added to the medium of one group of cells while control cells remained in medium with 2 μM MTX.

Table 1. Influence of hormonal milieu on methotrexate transport*

Milieu	K_m (μM)	V_{max} [nM·min^{-1}·(g cell protein)$^{-1}$]
Fetal calf serum (N = 9)	8.22 ± 0.62	12.22 ± 2.8
Serum-free (N = 4)	15.93 ± 1.6	16.6 ± 4.2
+Estradiol (N = 4)	16.83 ± 1.05	14.34 ± 2.8
+Insulin (N = 4)	7.95 ± 1.7	12.45 ± 0.43

* Values are means ± S.E.M.

a calculated Q_{10} of 2.25 ± 0.32, whereas lowering the temperature to 4° virtually abolished drug accumulation. Sodium azide is a metabolic poison that inhibits oxidative phosphorylation and has been shown to inhibit MTX efflux in murine tumor cells [15]. Determination of MTX uptake in the presence of 10 mM sodium azide revealed no inhibition of initial uptake rate but a significant increase in the steady-state level of intracellular drug. When MCF-7 cells were pre-loaded with [^{3}H]MTX and, then, exposed to 10 mM sodium azide, there was also a progressive increase in the intracellular level of [^{3}H]MTX, as shown in Fig. 5. This suggests the presence of an energy-dependent active efflux mechanism in these cells which is inhibitied by sodium azide. Initial influx rates were unaffected by the addition of 100 μM ouabain to the medium, indicating that the influx carrier is independent of Na^+–K^+-dependent ATPase.

Effects of hormonal milieu on MTX transport. In many respects, cultured human breast cancer cells display characteristics of membrane transport of MTX that are similar to those described previously for murine and human leukemia cells. Unlike these other cell types, however, MCF-7 cells are hormonally responsive and contain receptors for insulin, estradiol, and other steroid hormones [8–10, 16]. The influence of insulin and estradiol deprivation and repletion on the kinetics of the high-affinity MTX transport system was studied, as described in Materials and Methods. As shown in Table 1, growth of MCF-7 cells in serum-free medium induced a significant increase ($P < 0.001$, Student's t-test) in the transport K_m as compared to cells growing in fetal calf serum. Supplementation of the serum-free medium with 5×10^{-7} M insulin for 24 hr prior to drug exposure restored this K_m value to one that closely approximated that seen in cells grown in fetal calf serum ($P < 0.025$ for insulin-supplemented vs serum-free medium, and $P > 0.50$ for insulin-supplemented vs fetal calf serum). Estradiol at 1×10^{-8} M, although a potent growth stimulant, produced no apparent effect on the K_m or V_{max} of MTX transport as measured 24 hr after addition of the hormone.

At MTX concentrations equal to or less than the K_m of transport, the insulin-induced decrease in K_m should result in the accumulation of substantially increased levels of free intracellular MTX. We compared MTX accumulation in cells grown in hormone-free medium with cells grown in medium supplemented with 5×10^{-7} M insulin. Cells were first exposed to 2 μM [^{3}H]MTX for 60 min at 37°. Following this initial uptake phase, they were washed and drug-free medium was added to the flasks for 120 min. Following efflux, both groups of cells contained equal amounts of MTX, reflecting binding to DHFR. Subtraction of plateau level from the peak level allowed calculation of the free intracellular MTX level. This was found to be 52 per cent greater in insulin-treated cells than in hormone-free cells.

DISCUSSION

These studies provide the first demonstration of carrier-mediated MTX transport in cells derived from a human solid tumor. At low extracellular MTX concentrations, drug uptake displayed saturable kinetics with K_m and V_{max} values similar to those reported for murine tumor cells and rat hepatocytes [1, 2, 17]. As extracellular MTX concentrations increased above 10 μM, a low affinity transport component became apparent, but, as in other studies [17], it could not be characterized in kinetic terms. This may represent a low-affinity membrane carrier or may reflect passive diffusion at high extracellular drug concentrations.

MTX transport along the high-affinity route was readily inhibited by the presence of leucovorin and aminopterin in the medium, but not by equally low concentrations of folic acid and was enhanced by preloading the cells with leucovorin. These characteristics of competition and heteroexchange strongly suggest that MTX uses a carrier-mediated transport route that is the same as that used by physiologic reduced folate cofactors. This system appears to display specificity for a single terminal glutamic acid moiety. Neither DAMPA, a metabolite which lacks the terminal glutamate, nor MTX-γ-glu, which contains an additional terminal glutamate in γ-carboxyl linkage, inhibits the initial uptake of [^{3}H]MTX in the concentrations studied. Although these MTX analogues could possess a lower affinity for the membrane carrier and thus not be inhibitory of MTX uptake under the experimental conditions chosen, it does not seem physiologically relevant to study their effects at much higher concentrations. Although a single terminal glutamate appears optimal for transport, it is not sufficient in that neither folic acid nor glutamic acid itself interferes with MTX uptake.

Although a carrier-mediated mechanism for drug uptake appears to exist, the energy requirements of this carrier, if any, remain undefined. Although a Q_{10} of 2 is consistent with carrier-mediated transport, this value is substantially less than that demonstrated for murine leukemia cells [1, 2]. Further, the metabolic poisons sodium azide and ouabain have no effect

on MTX influx in this cell line, although azide clearly inhibits efflux. Warren *et al.* [3] have previously demonstrated similar energetics in a human lymphoblastoid cell line, and Bender [4] has described similar azide effects in other human leukemia cells. Thus, these and previous experiments suggest that, although MTX efflux may be an energy-requiring process in human cells, further experimentation is required to elucidate the energetics of MTX uptake by human tumor cells.

The interaction of drugs and hormones in cancer therapy is an area of considerable interest, with particular relevance to the therapy of breast cancer, which responds to both cytotoxic and hormonal agents. MCF-7 cells have been useful models for the study of hormonal effects on cell growth and macromolecular synthesis [18], as well as the combined effects of drugs and hormones on cell kill [19]. No previous studies, however, have directly addressed the issue of hormone effects on drug transport. Insulin is well known to stimulate amino acid transport in hepatocytes and hepatoma cells [20, 21], and the present studies indicate that this hormone has significant effects on the intramembrane MTX transport system of MCF-7 cells. In the absence of fetal calf serum, the affinity of the membrane carrier decreases by more than 2-fold. This change can be reversed by addition of insulin to the serum-free medium. Estradiol, although an equally potent stimulant of DNA synthesis and cell growth [9, 10], does not show similar transport effects under our experimental conditions, suggesting that the insulin effects are not simply a function of increased growth rate. Indeed, studies in L1210 murine leukemia suggest that the transport K_m for MTX does not change as the cell population passes from stationary to exponential phases of growth [22].

The biochemical events leading to the effects of insulin on membrane transport of MTX remain to be clarified. Changes in the plasma membrane lipid profile consequent to insulin-induced changes in cellular fat synthesis could be a mechanism of enhanced drug transport. Both decreases in membrane cholesterol/phospholipid ratio and increases in membrane lipid unsaturation have been associated with increases in membrane fluidity [23]. Burns *et al.* [24] have recently demonstrated that increased membrane fluidity results in a decrease in the K_m value for MTX transport in L1210 murine leukemia cells. Further, Monaco and Lippman [25] have shown that insulin produces increased lipid synthesis by MCF-7 cells, primarily as a result of enhanced formation of phospholipids. This effect was specific to insulin in that other growth-stimulating hormones, particularly estradiol, did not produce similar effects. Thus, insulin-induced changes in cellular lipid synthesis and perhaps in membrane lipid profile could result in changes in membrane fluidity and enhanced MTX transport. Recently, Pilch *et al.* [26] have demonstrated that transport of D-glucose by isolated rat adipocyte plasma membranes is enhanced both by increases in membrane lipid unsaturation and by insulin treatment of adipocytes prior to membrane purification. The insulin-induced stimulation of D-glucose transport could be blocked by incorporation of saturated fatty acids into the cell membranes. Because membrane lipid unsaturation is associated with increased membrane fluidity, these data support the hypothesis that insulin may mediate membrane transport processes by enhancing membrane fluidity. Further studies are needed to define more completely the mechanism of this drug–hormone interaction. Our findings suggest that hormonal manipulation may enhance drug accumulation and, potentially, cell kill. Indeed, Alabaster *et al.* [27] have recently demonstrated that the cytotoxic effects of MTX on MCF-7 cells can be enhanced by the addition of insulin to the culture medium. As we have demonstrated, this enhanced cytotoxicity may be related to an increased capacity of the cells to accumulate free intracellular MTX. Thus, the study of drug–hormone interactions in cultured human breast cancer cells may provide important clues to the optimization of drug therapy.

In summary, then, our studies have demonstrated that membrane transport of MTX by human breast cancer cells displays the characteristics of a high-affinity carrier system as described previously in murine leukemia cells. The presence of a second low-affinity component of the transport system, such as that noted in isolated rat hepatocytes, may be an important means of drug accumulation during high-dose MTX infusions. Finally, our data suggest that insulin has significant effects on the affinity of the transport carrier for MTX, an effect which must be taken into account particularly in *in vitro* experiments.

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