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TRANSPORT OF METHOTREXATE IN L1210 CELLS

MECHANISM FOR INHIBITION BY *p*-CHLOROMERCURIPHENYLSULFONATE AND *N*-ETHYLMALEIMIDE

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Summary

Methotrexate transport in L1210 cells is highly sensitive to inhibition by *p*-chloromercuriphenylsulfonate (CMPS) and, to a lesser extent, by *N*-ethylmaleimide. A 50% reduction in the methotrexate influx rate occurred upon exposure of cells to 3 μM CMPS or 175 μM *N*-ethylmaleimide, while complete inhibition was achieved at higher levels of these agents. Dithiothreitol reversed the inhibition by CMPS, suggesting that a sulfhydryl residue is involved. This residue is apparently not located at the substrate binding site of the transport protein, since methotrexate failed to protect the system from inactivation by either CMPS or *N*-ethylmaleimide, and the transport protein retained the ability to bind substrate (at 4°C) after exposure to these inhibitors (at 37°C). Methotrexate efflux was also inhibited by CMPS (50% at 4 μM), indicating that both the uptake and efflux of methotrexate in L1210 cells occur via the same transport system. High concentrations of CMPS (greater than 20 μM) increased the efflux rate, apparently by damaging the cell membrane and allowing the passive diffusion of methotrexate out of the cell.

Introduction

Metabolic inhibitors including azide, cyanide, and iodoacetate stimulate steady-state levels of methotrexate in mouse leukemia cells [1—5]. The basis

Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; CMPS, *p*-chloromercuriphenylsulfonic acid.

for this enhancement is not understood, but it has been suggested to result from the inhibition of an energy-dependent efflux route for methotrexate [1,2]. Alternatively, increased uptake could result from an inhibitor-induced elevation in the intracellular concentration of AMP or P_i, possible coupling anions for this process [2,5,6]. Other metabolic inhibitors, e.g., CMPS and *N*-ethylmaleimide, produce opposite effects; these thiol reagents inhibit the uptake process and stimulate the release of intracellular methotrexate [1,3]. Since low levels of CMPS rapidly inactivate methotrexate transport [3], and since the cell membrane is probably impermeable to this agent [7], it was concluded that CMPS inhibits the transport system by reaction at the outer surface of the cell [3]. Protection experiments suggested further that the binding site on the carrier protein might be involved in the CMPS reaction, and have led to an attempt to label the methotrexate carrier with *N*-[^{14}C]ethylmaleimide [8]. In the present study, the mechanism of inhibition and possible use of sulfhydryl reagents in labeling the methotrexate carrier protein have been re-evaluated. With the use of a recently developed assay for substrate binding to the methotrexate carrier protein [9] and protection experiments, the reaction of CMPS and *N*-ethylmaleimide with the transport system has been shown to occur at a site not directly involved in substrate binding. It was also observed that the effect of CMPS treatment on methotrexate efflux is a complex process, with either inhibition or stimulation being observed depending upon the experimental conditions.

Materials and Methods

Chemicals. [3′,5′,9′-^{3}H(n)]Methotrexate (250 mCi/mmol) was obtained from Amersham/Searle and purified by thin-layer chromatography on cellulose sheets (Kodak 6064), using 0.1 M K^+-Hepes, pH 7.4, as the solvent. CMPS, *N*-ethylmaleimide and Hepes were purchased from Sigma. Hg^{2+} was removed from CMPS by passage of the reagent through a column of Dowex chelating resin (Sigma) as described by Will and Hopfer [10].

Cells. L1210 cells were grown as described previously [4], centrifuged at $900 \times g$ (5 min, 4°C), washed with Hepes buffer (160 mM Hepes plus 1 mM $MgCl_2$ adjusted to pH 7.4 with KOH), and suspended in the same buffer to a density of $2 \cdot 10^7$ cells per ml.

Pretreatment with inhibitors. Cells (1.0 ml, final volume) were admixed with the indicated amounts of CMPS or *N*-ethylmaleimide, incubated for 5 min at 37°C, and washed with 5 ml of Hepes buffer prior to determination of methotrexate transport or binding activity.

Transport and binding determinations. [^{3}H]Methotrexate transport at 37°C [5] and [^{3}H]methotrexate-binding activity at 4°C [9] were determined in Hepes buffer as described previously. Results are expressed in pmol transported/min per mg protein and pmol bound per mg protein, respectively. Protein concentrations were measured using the biuret reaction [11] using bovine serum albumin as the standard.

Results

Inhibition of methotrexate transport by CMPS and N-ethylmaleimide and the inability of substrate to protect against this inhibition

The concentration dependence for inhibition of methotrexate transport by CMPS and *N*-ethylmaleimide is shown in Fig. 1. The transport rate was reduced by 50% at 3 μM CMPS and by greater than 95% at reagent concentrations in excess of 20 μM. With *N*-ethylmaleimide, considerably greater levels (175 μM) were required to produce 50% inhibition. In addition, a progressive decrease in transport activity was observed as the *N*-ethylmaleimide concentration exceeded 100 μM. The latter effect could be attributed, at least in part, to a loss in cell integrity, since cell viability (as measured by trypan blue exclusion) decreased from 99% in control cells to 94% in cells exposed to 200 μM *N*-ethylmaleimide. No correponding reduction in viability was observed in cells exposed to 50 μM CMPS.

The addition of an excess amount (20 μM) of unlabeled methotrexate during treatment with the inhibitors did not affect the sensitivity of the transport system to inactivation by either CMPS or *N*-ethylmaleimide. This could be demonstrated for cells exposed to the inhibitors in either Hepes buffer (Fig. 1) or phosphate-buffered saline * (data not shown); the latter medium was employed in previous studies [8].

Effect of CMPS and N-ethylmaleimide on methotrexate-binding activity

Binding to the methotrexate transport protein, which can be measured without the subsequent translocation of substrate [9], was also examined in cells treated with the inhibitors. A double-reciprocal plot of bound methotrexate vs. methotrexate concentration (Fig. 2) disclosed (from the common *y*-intercept) that CMPS (at 20 μM) did not reduce total methotrexate-binding activity. An effect was observed on the dissociation constant (K_D) for methotrexate, which increased from 0.4 μM in the control to 1.1 μM in the CMPS-treated cells. Cells preincubated with *N*-ethylmaleimide also retained maximum binding activity, but in this case they exhibited only a small increase (to 0.5 μM) in the K_D value for methotrexate (Fig. 2).

Since the affinity of the carrier protein for the substrate was decreased upon treatment with CMPS and *N*-ethylmaleimide, it was possible that these agents inhibited methotrexate transport by simply increasing the K_t value for methotrexate. This was not the case, however, since the inhibition produced by 20 μM CMPS or 400 μM *N*-ethylmaleimide was not reduced when the [^{3}H]methotrexate concentration in the transport assay was increased from 2 to 20 μM.

Methotrexate efflux determinations

The effect of CMPS and *N*-ethylmaleimide on methotrexate efflux was also re-evaluated. Cells were preloaded with [^{3}H]methotrexate, exposed to an

* Composition of phosphate-buffered saline: 138 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$.

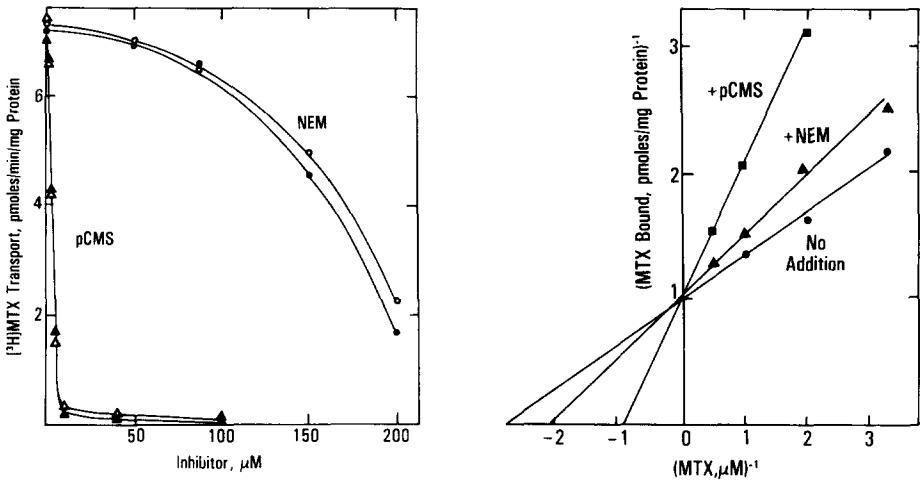

Fig. 1. Concentration dependence for inhibition of [^{3}H]methotrexate ([^{3}H]MTX) transport by CMPS and *N*-ethylmaleimide. Cells in 1.0 ml of Hepes buffer were treated (5 min, 37°C) with the indicated concentrations of CMPS (pCMS) or *N*-ethylmaleimide (NEM) in the absence (open symbols) or presence (filled symbols) of 20 μM unlabeled methotrexate, washed with 5 ml of buffer, and then analyzed for [^{3}H]methotrexate transport activity.

Fig. 2. Effect of CMPS (pCMS) and *N*-ethylmaleimide (NEM) on methotrexate (MTX) binding. Cells in 1.0 ml of Hepes buffer were exposed (5 min, 37°C) to no addition, or 20 μM CMPS, or 400 μM *N*-ethylmaleimide, washed with 5 ml of buffer and resuspended (at 4°C) in Hepes buffer prior to determination of methotrexate-binding activity. Data are plotted as the double reciprocal of methotrexate bound vs. methotrexate concentration.

amount of CMPS or *N*-ethylmaleimide that was sufficient to block methotrexate influx, and then examined for the time-dependent release of labeled methotrexate into the medium (Fig. 3). After correction for a small efflux component at 4°C (0.4 pmol/min per mg protein), CMPS (at 20 μM) was found to inhibit methotrexate efflux by 80%. Moreover, this level of inhibition remained relatively constant over the 25 min interval tested. In contrast, treatment with *N*-ethylmaleimide (at 400 μM) reduced the initial rate of methotrexate efflux by only 60%, and it led to an increase in the efflux rate as the incubation proceeded beyond 10 min.

Since previous studies [3] had shown that 50 μM CMPS induced the rapid release of methotrexate from L1210 cells suspended in phosphate-buffered saline, the effect of inhibitor concentration and buffer composition on the efflux rate was determined (Fig. 4). In Hepes buffer, methotrexate efflux rates were found to decrease at low levels of CMPS, with a 50% reduction occurring at an inhibitor concentration of 4 μM. However, the efflux rate increased as the concentration of the inhibitor exceeded 20 μM, until at 100 μM CMPS, there was no net effect on the efflux rate. An increase in the efflux rate was observed at 200 μM CMPS. With cells suspended in phosphate-buffered saline prior to efflux measurements (Fig. 4), the methotrexate efflux rate was again reduced by low levels of CMPS, but it increased much more rapidly in this buffer than in Hepes. At 100 μM CMPS, efflux rates in phosphate-buffered saline were approx. 3-fold higher than the untreated control, although essentially no effect was observed in Hepes buffer under the same

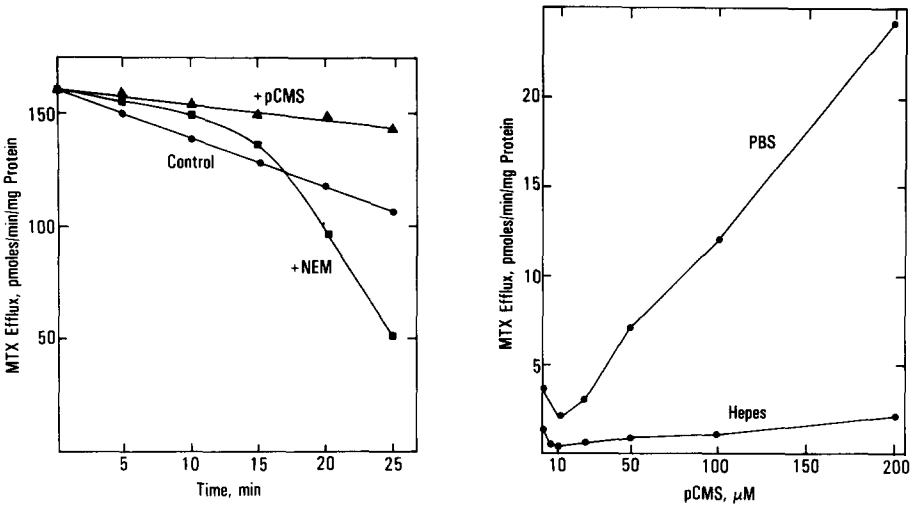

Fig. 3. Effect of CMPS (pCMS) and *N*-ethylmaleimide (NEM) on methotrexate (MTX) efflux. To preload with labeled substrate, cells ($6 \cdot 10^8$) were suspended in 40 ml of Hepes buffer containing 2.0 μM [^{3}H]methotrexate and 0.5 mM glucose, incubated for 20 min at 37°C, washed, and resuspended in 40 ml of Hepes buffer. Cells (1.0 ml) were then treated for 5 min at 37°C with no addition, or 20 μM CMPS, or 400 μM *N*-ethylmaleimide, washed to remove the inhibitors, and resuspended in 1.0 ml of buffer. After incubation for the indicated time interval at 37°C, the cells were collected by centrifugation and analyzed for radioactivity.

Fig. 4. Effect of buffer composition on the methotrexate (MTX) efflux rate in cells treated with various concentrations of CMPS. Preloading cells with [^{3}H]methotrexate and treatment with CMPS were performed as described in the legend to Fig. 3. The treated cells were suspended in either Hepes buffer or phosphate-buffered saline (PBS) and analyzed (at 37°C) for the release of substrate over a 20 min time interval.

TABLE I

EFFECT OF BUFFER CONSTITUENTS ON THE RATE OF METHOTREXATE EFFLUX IN CONTROL AND CMPS-TREATED CELLS

Cells were preloaded with [^{3}H]methotrexate and treated with 200 μM CMPS in Hepes buffer (160 mM Hepes plus 1 mM $MgCl_2$ adjusted to pH 7.4 with KOH) as described in the legend to Fig. 3. The cells were then resuspended in the indicated buffers containing the prescribed additions and analyzed for methotrexate efflux rate (see Fig. 3). The efflux buffers also contained $MgCl_2$ at a final concentration of 1.0 mM.

Buffer	Concentration (mM)	Addition	Concentration (mM)	Efflux rate (pmol/min per mg protein)	
				Control cells	CMPS-treated cells
K^+-Hepes	160	none	—	1.4	2.0
K^+-Hepes	160	$CaCl_2$	2	2.2	4.6
K^+-Hepes	150	potassium phosphate	10	2.3	3.6
K^+-Hepes	20	KCl	140	2.7	7.2
Na^+-Hepes	20	NaCl	140	2.3	11.8
Phosphate-buffered saline	—	—	—	2.5	21.7

TABLE II

REVERSAL OF CMPS INHIBITION OF METHOTREXATE TRANSPORT BY DITHIOTHREITOL

Cells in either Hepes buffer or phosphate-buffered saline were incubated for 5 min at 37°C with either no addition or the indicated concentration of CMPS, washed, and resuspended in Hepes buffer. Dithiothreitol (5 mM) or buffer alone were then added and the cells were incubated for an additional 5 min at 37°C prior to determination of methotrexate transport activity.

Pre-treatment buffer	CMPS concentration (μM)	Reversal by dithiothreitol (%)
Hepes	20	95
	100	70
	200	60
Phosphate-buffered saline	20	46
	100	25
	200	12

conditions. An analysis of the ionic components of phosphate-buffered saline revealed that Na^+ was the principal ion responsible for the CMPS-dependent enhancement of methotrexate efflux rates in phosphate-buffered saline (Table I).

Reversal by dithiothreitol

If CMPS reacts specifically with a cysteine residue in the transport system, then the inhibition produced by this mercurial should be reversible by thiols such as dithiothreitol. While cells pretreated with 20 μM CMPS (in Hepes buffer) transported methotrexate at a rate that was 5% of control values (see Fig. 1), subsequent exposure to 5 mM dithiothreitol (which alone has no effect on methotrexate trasnport) reversed this inhibition by 95% (Table II). At higher concentrations of CMPS, however, the restoration of methotrexate transport by dithiothreitol became progressively less efficient.

When cells suspended in phosphate-buffered saline were pretreated similarly with CMPS, dithiothreitol was found to be considerably less effective in reversing the inhibition (Table II). Thus, after exposure to 20 μM CMPS in phosphate-buffered saline, the system regained transport activity to only 46% of control values, whereas 200 μM CMPS produced an essentially irreversible inhibition of the transport process.

Discussion

The methotrexate transport system of L1210 cells is highly susceptible to inhibition by CMPS, *N*-ethylmaleimide and other sulfhydryl reagents [1,2,8] (see also Fig. 1). Since CMPS is relatively impermeable to cells membranes [7], this reagent (and presumably the others as well) appears to react with a protein on the external membrane surface. The reaction also appears to involve a sulfhydryl group, since the inhibition of methotrexate transport by CMPS can be reversed by dithiothreitol (Table II). The location of this reactive sulfhydryl residue has not been determined, although the present results suggest that it is not the substrate binding site on the methotrexate

carrier protein as suggested previously [8]. This conclusion is based upon the observation that unlabeled methotrexate present during cell treatment with either CMPS or *N*-ethylmaleimide does not protect the transport system against inactivation (Fig. 1). Binding of methotrexate to the carrier protein (at 4°C) is also not blocked by pretreatment of the cells with these agents (Fig. 2). The methotrexate carrier, however, appears to be the site of reaction with both CMPS and *N*-ethylmaleimide, since the latter agents reduce the affinity of the carrier for methotrexate (Fig. 2). Alternatively, the reactive sulfhydryl group could reside on a second component of the transport system that, by virtue of an interaction with the carrier protein, can affect its K_D for methotrexate.

CMPS produces varying effects on methotrexate efflux depending on the conditions employed. At low concentrations, CMPS reacts specifically with the transport system to produce a nearly complete block in the efflux process (cf. Figs. 3 and 4). At higher concentrations, however, increased efflux rates are observed (Fig. 4), particularly in the presence of Na^+ (Table I). A possible explanation for this increase is that CMPS damages the cell membrane and promotes the passive diffusion of methotrexate out of the cell. This possibility is supported by the fact that dithiothreitol totally reverses the inhibition of methotrexate transport achieved at low concentrations of CMPS (20 μM), but not at higher levels (200 μM) of the mercurial (Table II). Moreover, when cells are treated with CMPS in phosphate-buffered saline, conditions which lead to the most rapid rate of methotrexate efflux (Fig. 4), dithiothreitol is much less effective in reversing the effects of CMPS (Table II). It is of interest that CMPS treatment enhances the passage of Na^+ across the cell membrane of rat intestinal cells [10].

It is generally accepted that L1210 cells contain only a single high-affinity system for the uptake of methotrexate [12]. The present results suggest further that these cells also contain a single efflux route and that influx and efflux occur via the same carrier protein. These conclusions are based upon the observations that CMPS blocks both methotrexate influx and efflux (cf. Figs. 1, 3 and 4), and that the latter processes show the same sensitivity to inhibition (50% at about 5 μM) by CMPS (cf. Figs. 1 and 3). Kinetic evidence has been obtained previously suggesting that L1210 cells possess multiple routes for methotrexate efflux [13,14], but from the present results, this appears unlikely unless the multiple efflux routes each have the same high sensitivity to CMPS.

The methotrexate transport system of L1210 cells contains a sulfhydryl residue that is not only highly reactive, but is also essential to the functionality of the methotrexate transport system. It seems plausible, therefore, that this residue may perform a critical function in vivo, perhaps in the regulation of the system by cyclic AMP [4]. Under conditions in which it would be desirable to restrict the cellular entry of folate compounds, the thiol group could be modified by an ATP-dependent protein kinase. As a consequence, the transport protein would assume a conformation that prevents substrate translocation (but not binding) across the membrane. Enhancement of transport could be achieved by reversing the process via a specific thioesterase. The operation of a regulatory system of this type would both spare cells from

the burden of accumulating large amounts of unneeded pteroylpolyglutamates, and also allow an efficient distribution of folate compounds within a heterogeneous cell population.

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