Cancer Chemother Pharmacol (1987) 19: 25–29

Cancer Chemotherapy and Pharmacology

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The role of methionine in methotrexate-sensitive and methotrexate-resistant mouse leukemia L1210 cells*

Kevin J Scanlon[1], Mohammed Kashani-Sabet[1], Arlene R. Cashmore[2], Michele Pallai[1], Barbara A. Moroson[2], and Maria Saketos[1]**

[1] Section of Biochemical Pharmacology, Department of Medical Oncology, City of Hope National Medical Center, Duarte, CA 91010, USA

[2] Department of Pharmacology, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06510, USA

Summary. A mouse L1210 leukemia cell line was made 25-fold resistant to methotrexate (MTX) and had altered methionine transport and metabolism. L1210 cells resistant to methotrexate also had a 50-fold decrease in the exogenous methionine requirement for optimal cell growth compared to the parent cells. This change in methionine requirement was associated with differences in methionine metabolism between MTX-sensitive and MTX-resistant cell lines. Analysis of amino acid transport systems revealed different K_t and Vmax properties of methionine and nonmetabolizable amino acid analogues. There was a greater than twofold decrease in the initial sodium-dependent uptake of methionine in the resistant cells. Amino acid competition experiments revealed altered substrate specificities in the resistant cells. The cellular alterations occurring upon resistance may result from methotrexate-membrane interactions, and have been previously observed in cisplatinum-resistant cells. Thus modulation of methionine metabolism may provide the biochemical basis for MTX and cisplatinum collateral resistance.

Introduction

Exogenous methionine has been shown to be an absolute requirement for both L1210 and human tumors [9]. The ASC system has been shown to make a major contribution to the concentrative capacity of the neutral amino acid transport systems in mammalian cell lines [3]. Differences in substrate specificity for the ASC system have been demonstrated in mouse L1210 cells [6] and human K562 cells [17] sensitive and resistant to cisplatinum, and also between normal rat hepatocytes and methionine-auxotrophic hepatoma cells (HTC) [7]. Stimulated normal human lymphocytes and human leukemic K562 cells are also auxotrophic for methionine [9], and MTX inhibits the uptake of methionine into these cells [13, 14]. Drug-membrane interactions with L1210 cells are not unique: homofolate, cisplatin, and adriamycin have recently been demonstrated to influence cell growth by interacting at the membrane [6, 8, 12, 15, 17]. In fact, cisplatinum-resistant cells exhibit decreased amino acid transport and altered substrate specificities in addition to possessing a lowered exogenous methionine requirement for cell growth compared to sensitive cells. Since these drug-membrane interactions occur in the methionine-auxotrophic L1210 cells, the effect of MTX on methionine uptake in MTX-sensitive and -resistant L1210 cells (L1210S and L1210MTX) has been studied, since it may point to an additional mechanism of resistance for MTX in L1210 cells [1]. Finally, since MTX and cisplatinum have been shown to be collaterally resistant [16, 18], it would be of interest to compare the changes in amino acid transport systems when cells develop resistance to MTX or cisplatinum.

Materials and methods

Chemicals. L-(1-^{14}C)-Methionine (50 mCi/mmol), (1-^{14}C)-aminoisobutyric acid (AIB, 51.6 mCi/mmol), (G-^{3}H)-hypoxanthine (4.3 Ci/mmol), L(-4,5^{3}H(N))-lysine (80.5 Ci/mmol), and L-(G^{3}H)-threonine (20 Ci/mmol) were purchased from Amersham, Arlington Heights, Illinois. Alpha-(1-^{14}C)-methylaminoisobutyric acid (MeAIB, 51.6 mCi/mmol) was purchased from New England Nuclear, Boston, Massachusetts. MTX was obtained from Lederle Laboratories, Pearl River, New York.

Cells and growth media. Stock suspension cultures of L1210 cells were grown in Fischer's medium supplemented with 10% horse serum, as previously described [6]. The L1210MTX cells were established by cloning in soft agar with 10^{-7} *M* MTX and had a stable resistance.

L1210 cells resistant to cisplatinum (L1210DDP) were characterized by a 10-fold resistance to the drug [6]. The dose to inhibit 50% of cell growth (ED_{50}) was determined by continuously exposing exponentially growing L1210 cells to MTX (for 48 h) at concentrations of 1–400 nMol. Controls (100% cell growth) consisted of L1210S and L1210MTX cells grown in the absence of MTX. Cells grown in the presence of drug were compared to the controls [60]. Exogenous methionine requirements were determined by growing L1210S and L1210MTX cells in methionine-depleted Fischer's medium supplemented with 15% dialyzed horse serum and various concentrations of methionine (0–100 mg/ml). Experiments were performed in triplicate and counted 48 h later. Optimal conditions for exponentially growing L1210 cells were characterized for Fischer's medium with various concentrations of methio-

* This work is supported by the American Cancer Society Grant (CH-265), USPHS CA 08010 and CA 16359, and the Chemotherapy Foundation, Inc.

** Kevin J. Scanlon is a Scholar of the Leukemia Society of America

Offprint requests to: Kevin J. Scanlon

nine in 15% dialyzed horse serum. The maximum growth at the optimal methionine concentration was then individually normalized to 100% for each cell line [6]. The generation time of L1210S and L1210DDP cells was established as 12 (±1) h and that of the L1210MTX cells as 15 (±2) h. Cell volume analysis of L1210MTX by a Coulter Counter channelyzer revealed a heterogeneous cell population in which the mean cell volume was 25% smaller than the L1210S cells.

Methionine metabolism. L1210S and L1210MTX cells (5×10^6 cells/ml) were preincubated for 15 min in Fischer's medium depleted of methionine and containing 15% dialyzed horse serum. (1-^{14}C)-Methionine (500 μ*M*) was then added to the cell incubation. The uptake of methionine and its metabolism into acid insoluble material were measured over 60 min as previously described [6].

Incubation media for amino acid transport studies. The incubation medium (IM-1) for determining sodium-dependent amino acid transport was Earle's balanced salt solution (GIBCO). The incubation medium (IM-2) for determining sodium-independent transport was Earle's balanced salt solution substituting 116 m*M* choline chloride for 116 m*M* sodium chloride. The osmolality of IM-1 and IM-2 was 300 mosm with a pH of 7.4 [17].

Transport measurements. Cells were harvested during logarithmic growth by centrifugation at 500 g, washed once with each specific incubation medium, and resuspended to a final density of 5.0×10^6 cells/ml. Labeled compounds (methionine, MeAIB, AIB, threonine, hypoxanthine, or lysine) were added at the zero timepoint. At time intervals, 200 μl samples were withdrawn and immediately separated from the incubation medium by centrifugation through a silicone-mineral oil gradient onto 7% perchloric acid and counted as previously described [9]. The data presented in the charts were the means of at least three separate experiments. The standard error was less than 10% of the mean for L1210MTX cells and less than 5% of the mean for L1210S cells.

Source of enzymes. Crude enzyme extracts of L1210S and L1210MTX cells were prepared as previously described [12]. The activity of dihydrofolate reductase (EC 1.5.1.3), serine transhydroxymethylase (EC 2.1.2.1), and thymidylate synthase (EC 2.1.1.45) was measured as previously described [14, 12, and 2 respectively]. Thymidine kinase (EC 2.6.1.75) activity was measured by the method of Chello and Jaffe [2]. 5.10-Methylenetetrahydrofolate (CH_2FH_4) reductase (EC 1.1.1.68) was assayed as previously described [12]. Methionine synthetase (EC 2.1.1.13) activity was determined according to the procedure of Coward et al. [4].

Results

L1210 cell growth in the presence of MTX

The ED_{50} for L1210S, L1210DDP, and L1210MTX cells was 6.6 nmol, 36 nmol, and 170 nmol, respectively (Fig. 1). This resistance to MTX was stable because the cells were grown in the absence of the drug, and as such retained their drug-resistant properties. The mechanism of MTX resistance may be due in part to changes in transport. MTX

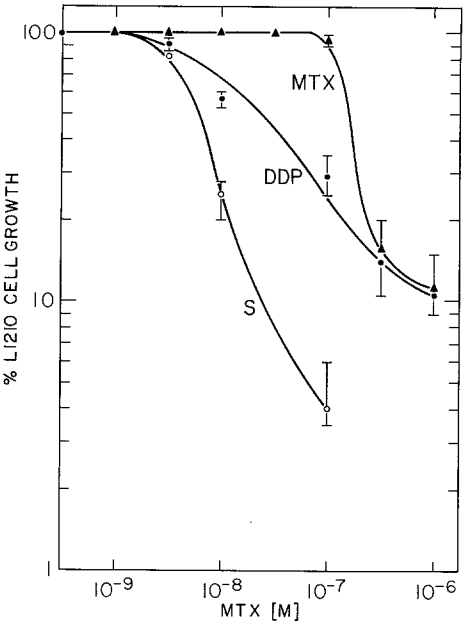


Fig. 1. Inhibition of L1210 cell growth by MTX. L1210S, L1210DDP, and L1210MTX cells were grown in various concentrations of MTX. After 72 h exposure to MTX the cell number for each concentration was determined. See Methods for details. The ED_{50} for MTX was 6.6 nmol, 36 nmol, and 170 nmol in L1210S, L1210DDP, and L1210MTX respectively

transport is decreased in L1210MTX cells compared to L1210S cells. This decrease was associated with a change in K_t from 4.7 μ*M* to 11.6 μ*M* in L1210S and L1210MTX cells respectively.

L1210S and L1210MTX exogenous methionine requirements

Cells grown in Fischer's medium and dialyzed serum containing varying concentrations of exogenous methionine had optimal cell growth at 100 mg/l of methionine for L1210S cells, while the concentration for optimal growth of L1210MTX cells was only 1 mg/l (Fig. 2). The cloning efficiency for L1210S and L1210MTX cells was 90% and 75%, respectively.

Metabolism of methionine in L1210S and L1210MTX cells

(^{14}C)-Methionine (500 μ*M*) uptake and metabolism was measured under tissue culture conditions (Fischer's medium) in L1210S and L1210MTX cells (Fig. 3). L1210S cells transported methionine two- to threefold better than L1210MTX cells; however, L1210MTX cells metabolized two times more methionine into acid-insoluble material than L1210S cells. Methionine transport, therefore, was more equivalent to methionine metabolism in the L1210MTX cells (Fig. 3).

Folate coenzyme studies

L1210S and L1210MTX crude supernatants were measured for enzymes of folate-dependent methionine biosynthesis (Table 1). Enzyme activity was measured in the initial rate of the reaction to ensure optimal enzyme activity. Thymidylate synthase and thymidine kinase, the two enzymes required for the synthesis of thymidylate, were not elevated in the resistant cells. The folate-dependent en-

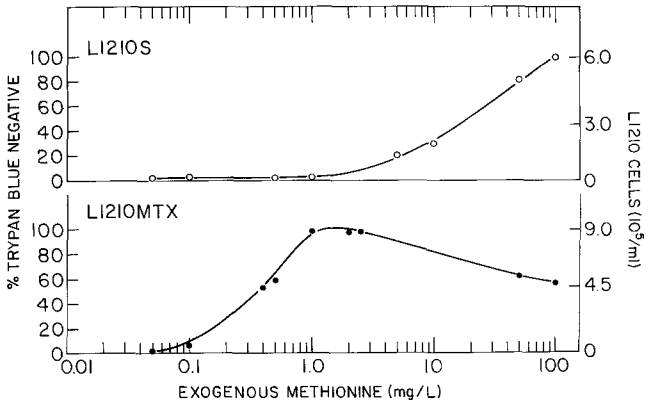


Fig. 2. Exogenous methionine requirement for optimal L1210S and L1210MTX cell growth. Cells were seeded at 50 cells/tube for L1210S and L1210MTX in Fischer's medium with various concentrations of exogenous methionine (0–110 mg/l). The colonies were counted on the 10th day. Experiments were done in triplicate, and the cloning efficiency for L1210S and L1210MTX cells under the optimal methionine concentrations was 90% and 75% respectively

zymes for methionine biosynthesis, including dihydrofolate reductase, were all elevated in the resistant cells (two- to threefold).

Transport studies

Differences in sodium-dependent and sodium-independent methionine transport between L1210S and L1210MTX cells are depicted in Fig. 4. In L1210MTX cells, the decrease in sodium-dependent transport of methionine was associated with a concomitant decrease in K_t (Fig. 4A). The sodium-independent transport of methionine in L1210MTX cells was also associated with a decrease in K_t (Fig. 4B). MTX (10–25 μM) did not block methionine uptake into L1210MTX cells (unpublished data).

To define the selectivity of MTX-induced damage to the membrane and its consequences in resistant cells, other

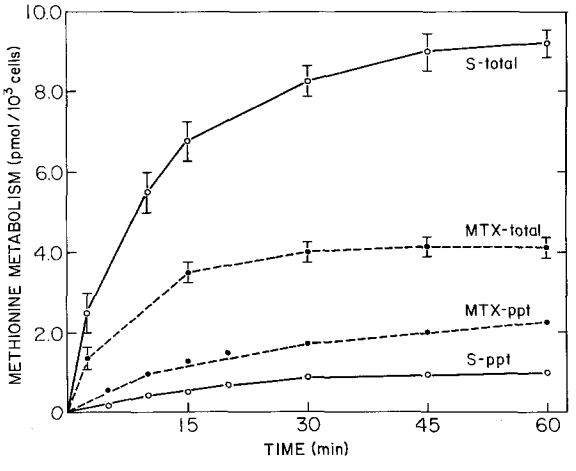


Fig. 3. Methionine transport and metabolism in L1210S and L1210MTX cells. L1210S and L1210MTX cells (5×10^6 cells/ml) were incubated with (^{14}C)-methionine (500 μM) and 15% dialyzed horse serum in Fischer's medium in order to measure total uptake into the cells and incorporation of labeled methionine into acid-insoluble material. See Methods for details

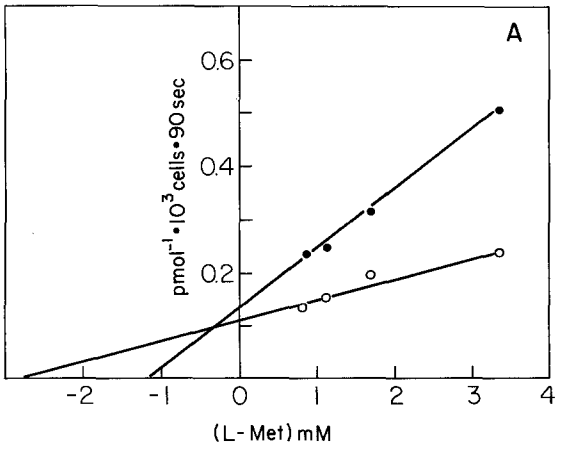
Table 1. Folate-dependent enzymes in MTX-sensitive (S) and MTX-resistant (MTX) L1210 cells

Enzyme	L1210S (μmol h^{-1})	L1210MTX (mg protein^{-1})
Dihydrofolate reductase	0.40	1.2
Serine transhydroxymethylase	0.04	0.10
Methylenetetrahydrofolate reductase	0.03	0.06
Methionine synthase	0.005	0.01
Thymidylate synthase	0.75	0.75
Thymidine kinase	0.03	0.03

Cell preparations were performed and enzyme measurements done in triplicate as described in methods

amino acids and nutrients were studied. In L1210MTX cells, the initial uptake of hypoxanthine (500 μM) and lysine (500 μM) was similar to that of the parent L1210 cell line (Fig. 5).

Substrate competition experiments were used to characterize the transport pathways for the ASC amino acid transport system (Fig. 6). MeAIB (200–600 μM) uptake



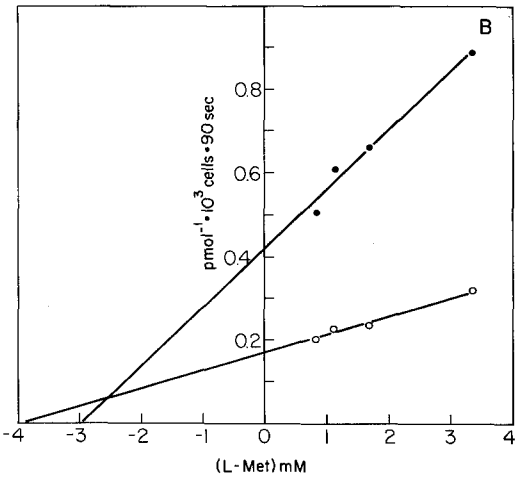


Fig. 4A, B. Initial sodium-dependent methionine uptake in L1210S and L1210MTX cells. The initial (1.5 min) uptake of methionine at various concentrations (250–1000 μM) was measured in L1210S (○) and L1210MTX (●) cells. **A** Initial sodium-independent uptake. **B** Initial sodium-dependent uptake. See Methods for details

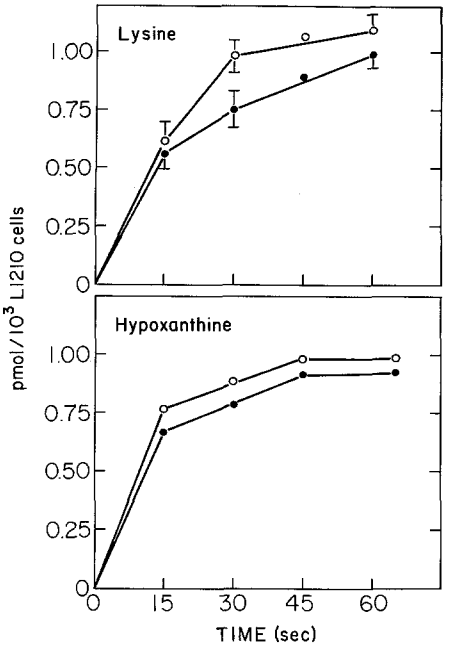


Fig. 5. Lysine and hypoxanthine uptake in L1210S and L1210MTX cells. The uptake of lysine at 500 μ*M* (top) and hypoxanthine at 500 μ*M* (bottom) was measured in L1210S (○) and L1210MTX (●) cells. See Methods for details

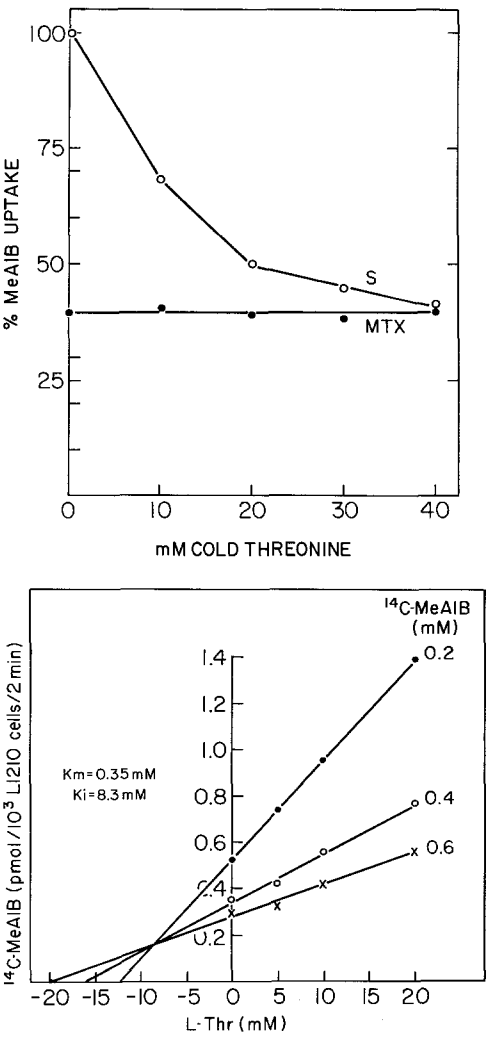


Fig. 6. Blocking of MeAIB uptake by unlabeled threonine in L1210S and L1210MTX cells. *Upper panel:* Uptake of labeled MeAIB was measured for 2 min at 37° in the presence of 10–40 m*M* unlabeled threonine in L1210S and L1210MTX cells. The L1210S cells were used as a control (100%) for only the sodium-dependent uptake of MeAIB in the presence of threonine. *Lower panel:* Uptake of labeled MeAIB [0.2 (●), 0.4 (○) and 0.6 (×) m*M*] in the presence of unlabeled threonine at 5, 10, and 20 m*M* in L1210S cells

was blocked by unlabeled threonine (5–20 μ*M*) in L1210S cells (Fig. 6). In L1210MTX cells, sodium-independent MeAIB uptake was not inhibited by threonine (Fig. 6). In L1210S (but not L1210MTX) cells, the aforementioned competitive inhibition of MeAIB uptake by threonine occurred with a K_i of 8.3 m*M* (Fig. 6).

Discussion

Altered transport of MTX and elevated dihydrofolate reductase levels have previously been demonstrated as the mechanisms of MTX resistance in mammalian cells [1]. We isolated an L1210 cell line with both of these mechanisms of resistance. However, this cell line also displayed altered methionine transport and metabolism. Alone or in combination, thymidine (10 μ*M*) and hypoxanthine (10 μ*M*) rescued L1210MTX cells three- to eightfold better than L1210S cells from MTX cytotoxicity (unpublished data). This would suggest that MTX cytotoxicity in L1210S cells may be due to a lack of purines, pyrimidines, and other essential precursors. Thus, a study was initiated to elucidate the change in the role of methionine metabolism between L1210S and L1210MTX cells.

If MTX blocks methionine transport in L1210 cells, then cells resistant to MTX should have altered methionine requirements. L1210MTX cells possess a 50-fold lower exogenous methionine requirement than the sensitive cells. L1210 cells resistant to MTX have previously been shown to possess a four- to fivefold larger 5-methyltetrahydrofolate pool than the sensitive cells [10]. Studies of methionine metabolism showed that the aforementioned high exogenous methionine requirement in L1210S is associated with a large acid-soluble pool of methionine.

L1210 cells resistant to MTX had a much lower exogenous methionine requirement, a smaller acid-soluble pool, and a faster rate of methionine metabolism than L1210S. These changes in the methionine pool of resistant cells were associated with an elevation of the folate-dependent methionine biosynthetic pathway. Similar results have been reported in L1210 cells resistant to cisplatinum [6].

Moreover, changes occurred in the properties of amino acid transport. L1210MTX cells exhibited reduced transport properties for methionine in both the sodium-dependent and -independent components of methionine uptake. MTX effect on the membrane appears to be selective for the ASC-like amino acid transport system, since transport of hypoxanthine and lysine was unchanged between sensitive and resistant L1210 cells. Only in the L1210S cells was the sodium-dependent concentrative uptake of MeAIB inhibited by threonine (K_i = 8.3 m*M*). Similar results have been obtained in the methionine-auxotrophic hepatoma cell line [7]. However, MeAIB transport was not inhibited

by threonine in L1210MTX cells, and these resistant cells expressed similar properties to apparently normal rat hepatocytes [7]. We have previously shown the same qualitative alterations in amino acid transport between cisplatinum-sensitive and -resistant murine and human tumor cells [6, 17]. This similarity would seem to suggest a common site of action for both MTX and cisplatinum (i. e., directed at the amino acid transport systems). This hypothesis is supported by the establishment of collateral resistance between MTX and cisplatinum, shown not only in L1210DPP cells (Fig. 1), but also in cisplatinum-resistant P388 [16] and human squamous carcinoma cells [18].

In conclusion, MTX cytotoxicity may be due, in part, to methionine deprivation by the combined blocking of methionine uptake and inhibition of dihydrofolate reductase. Cells resistant to MTX have altered membrane transport systems and elevated methionine-dependent folate biosynthesis, rendering them less reliant on exogenous methionine and hence less susceptible to MTX inhibition of methionine transport. Moreover, methionine metabolism may represent the link between MTX and cisplatinum as established by their collateral resistance.

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Received June 16, 1986/Accepted October 1, 1986