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Biochemical Pharmacology, Vol. 43, No. 5, pp. 1154–1158, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

Modulation of melphalan uptake in murine L5178Y lymphoblasts *in vitro* by changes in ionic environment

(*Received* 29 *August* 1991; *accepted* 5 *November* 1991)

Abstract—The alkylating agent melphalan is actively transported in mammalian cells by two amino acid transport carriers: the sodium-dependent carrier with substrate preference for alanine-serine-cysteine (system ASC), and a sodium-independent carrier with preference for leucine (system L). The effect of altering the ionic environment of murine L5178Y lymphoblasts was investigated in order to determine not only the direct effects of hydrogen and calcium ions on these transport systems, but also the indirect effects of agents or modulators known to alter intracellular calcium. Melphalan transport followed a bell-shaped distribution curve over a pH range from 3 to 9 with a pH optimum of 4.3 and 4.6 for transport by systems ASC and L, respectively. Those agents that could cause a decrease in cytosolic calcium such as the calcium channel blockers verapamil, diltiazem and nitrendipine, the calcium chelator (ethyleneglycol-bis-(β-aminoethylether) *N,N,N',N'*-tetraacetic acid (EGTA) and reduction of pH were found to augment melphalan uptake, whereas conditions that would elevate intracellular calcium such as the calcium ionophore A23187, the calcium channel agonist (−) Bay K 8644, elevation of extracellular calcium and the calcium pump inhibitor trifluoperazine were all found to decrease melphalan uptake. These findings suggest that modification of ionic environment directly or indirectly by agents known to alter intracellular calcium can modulate melphalan uptake.

Melphalan influx is an active process mediated by two separate neutral amino acid transport carriers [1, 2], whereas melphalan efflux apparently occurs by passive diffusion [3]. One amino acid transport carrier (system ASC*) is sodium dependent, whereas the other (system L) is sodium independent. However, the effect of altering the concentration of other extracellular ions on melphalan uptake has not been investigated. In addition, the calcium channel blocker verapamil has been reported to enhance the cytotoxicity of melphalan against murine bone marrow and fibrosarcomas, and this appeared to be due, at least in part, to increased melphalan uptake [4]. Accordingly, the following investigation was undertaken to evaluate the direct effects of manipulation of the ionic environment, as well as the indirect effects of agents or modulators of intracellular calcium, on melphalan uptake by murine L5178Y lymphoblasts.

* Abbreviations: ASC, alanine-serine-cysteine; L, leucine; EGTA, ethyleneglycol-bis-(β-aminoethylether) *N,N,N',N'*-tetraacetic acid; PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide; BCH, 2 aminobicyclo-[2.2.1]heptane-2-carboxylic acid; and BSS, balanced salt solution.

Materials and Methods

Drugs and chemicals. Melphalan [L-*p*-(di-2-chloro[^{14}C]-ethylamino)phenylalanine], specific activity 9.9 or 14.2 mCi/mmol, was synthesized by M. Leaffer of the Stanford Research Institute, Menlo Park, CA; the radiochemical

purity was 97% as determined by thin-layer chromatography as described previously [5], and the compound was obtained as a gift from Dr. R. Engle, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. A23187 was a gift from the Calbiochem-Behring Corp., La Jolla, CA; racemic BAY K 8644 and its purified enantiomers were gifts of Dr. A. Scriabine from Miles Institute for Preclinical Research, New Haven, CT; trifluoperazine was a gift from Smith Kline & French Canada Limited, Missausaugga, Ontario; and nitrendipine was provided by Dr. G. Glavin, Department of Pharmacology, University of Manitoba, and Dr. D. Frankel from Miles Laboratories, Limited, Rexdale, Ontario.

Buffers and solutions. Dulbecco's phosphate-buffered saline (PBS), obtained as a 10-fold concentrated stock solution from Gibco Laboratories, Life Technologies Inc., Chagrin Falls, OH, represented the standard transport medium and was routinely supplemented with 0.1 g/L $CaCl_2$ and 0.1 g/L $MgCl_2 \cdot 6H_2O$. Sodium ion was replaced with Tris to provide sodium-free Hanks' solution.

Racemic Bay K 8644 and its purified isomers were dissolved in 100% ethanol and diluted in PBS so that the final concentration of ethanol did not exceed 1%. Since BAY K 8644 is light-sensitive, stock solutions were stored in opaque vials and experiments were performed in darkness. Nitrendipine was dissolved in dimethyl sulfoxide (DMSO) and then diluted in PBS so that the final concentration of DMSO was less than 1%; since nitrendipine is also light-sensitive, studies were performed in darkness. Verapamil and diltiazem were dissolved in saline.

Drug uptake. Suspension cultures of murine leukemia L5178Y lymphoblasts were grown at 37° in Fischer's medium (Gibco Laboratories) supplemented with 10% horse serum, penicillin and streptomycin, as described previously [5, 6]. Cell volume and cell counts were determined using a Coulter model Z_{B1} electronic particle counter calibrated with paper mulberry spores (mean cell diameter, 12.5 μm). The volume (mean ± SEM) of cells suspended in transport medium was 956 ± 26 femtoliters in agreement with previous reports [1, 2], and this value may be used to express uptake data as cell/medium ratios; cell volume was not altered significantly by the experimental conditions. Drug uptake studies were performed as described previously [5, 6] by addition of [^{14}C]melphalan to 2 to 4 × 10^6 cells/mL suspended in PBS unless otherwise stated. The viability of the cells as determined by trypan blue dye exclusion after exposure to the various conditions was 95% or greater. Incubations were terminated by rapid chilling to 4° and centrifugation through a layer of either 0.25 M sucrose in Hopkin's vaccine tubes or SF120 oil in Eppendorf tubes. The washed cells were solubilized in 0.5 N NaOH and radioactivity was determined by liquid scintillation spectrometry.

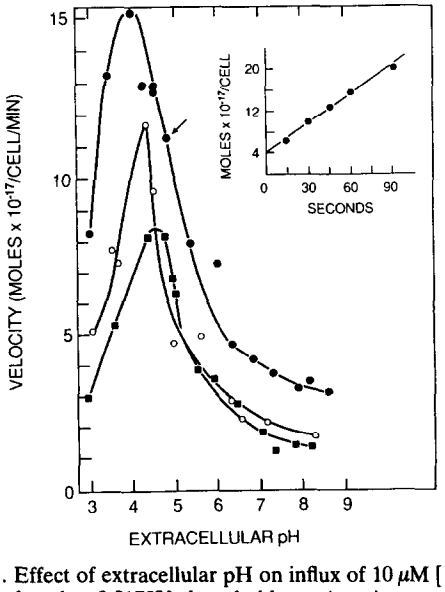

Fig. 1. Effect of extracellular pH on influx of 10 μM [^{14}C]-melphalan by L5178Y lymphoblasts *in vitro* at 37°. Melphalan influx by both amino acid systems was determined in PBS (●), that by system L in sodium-depleted medium (■), and that by system ASC in medium containing 5 mM BCH (○). The influx velocity data were obtained by linear regression analysis of a series of time courses of drug influx over 90 sec over a pH range of 3 to 9. The inset shows a typical time course at pH 5; the influx velocity calculated by linear regression analysis was plotted as a function of pH (as indicated by the arrow).

Results

Effect of extracellular pH on melphalan transport. Melphalan influx rates were determined over a pH range of 3 to 9, and velocity of drug influx was plotted as a function of pH (Fig. 1). A time course over 90 sec was linear suggesting that unidirectional drug influx was being measured (inset to Fig. 1). Melphalan transport mediated by system L was evaluated by measuring influx in sodium-free medium since that system is sodium independent. Melphalan influx mediated by system ASC was determined by measuring transport in the presence of a saturating concentration of 2-aminobicyclo[2.2.1]heptane-2-carboxylic acid (BCH) (a specific inhibitor of system L). Melphalan influx by either system L or system ASC alone or combined followed a bell-shaped distribution curve as a function of pH. The pH optimum for transport by systems ASC and L was 4.3 and 4.6, respectively. There was approximately a 4- to 6-fold increase in influx velocity as pH was reduced below physiological levels to reach the pH optimum for each amino acid transport system.

Effect of EGTA on melphalan uptake. The effect of the calcium chelator ethyleneglycol-bis-(β-aminoethylether) *N,N,N',N'*-tetraacetic acid (EGTA) on melphalan uptake was studied (Table 1). After a 10-min preincubation in PBS with or without 1 mM EGTA, melphalan uptake was measured at 10 or 30 min. At 10 min, drug uptake (mean ± SEM) by control cells was 7.1 ± 0.5 attomoles/cell, and that in the presence of 1 mM EGTA was 9.5 ± 0.6 attomoles/cell; this 33% increase was statistically significant ($P = 0.014$). Similarly, at 30 min, melphalan uptake by control cells was 8.6 ± 1.0 attomoles/cell, and that in the presence of 1 mM EGTA was 13.5 ± 0.9 attomoles/cell; this 57% increment in uptake was also statistically significant ($P = 0.004$).

Effect of extracellular calcium on melphalan uptake. The effect of increasing the extracellular calcium concentration on melphalan uptake by L5178Y cells was investigated (Table 1). After a 10-min preincubation in either PBS or PBS with 2.5 mM $CaCl_2$, 1 μM [^{14}C]melphalan was added and drug uptake measured. [^{14}C]Melphalan uptake (mean ± SEM) by cells exposed to 2.5 mM $CaCl_2$ was 10.1 ± 0.8 attomoles/cell compared to 15.4 ± 0.7 attomoles/cell for cells incubated in PBS with a calcium concentration of 0.9 mM; this 34% decrease in melphalan uptake was highly significant ($P < 0.001$).

Effect of calcium channel blockers on melphalan uptake. The effects of a structurally diverse group of calcium channel blockers, verapamil, diltiazem and nitrendipine, on uptake of melphalan by L5178Y lymphoblasts were evaluated (Table 1). In each case, melphalan uptake was increased approximately 25%, the effects were statistically significant, and the relative activity of the three drugs was verapamil > diltiazem > nitrendipine.

Effect of the calcium ionophore A23187 on melphalan transport. The effect of the calcium ionophore A23187 on

Table 1. Effects of agents that modulate calcium flux on melphalan uptake by L5178Y lymphoblasts *in vitro*

Agent	Concn (μM)	Melphalan uptake* (attomoles/cell) Control	Agent	P†
EGTA	1000	7.1 ± 0.5	9.5 ± 0.6	0.014
EGTA‡	1000	8.6 ± 1.0	13.5 ± 0.9	0.004
$CaCl_2$	2500	15.4 ± 0.7	10.1 ± 0.8	< 0.001
Verapamil	0.1	10.9 ± 0.1	13.8 ± 0.9	0.029
Diltiazem	0.5	11.9 ± 0.8	15.0 ± 0.7	0.025
Nitrendipine	10	14.3 ± 0.4	18.3 ± 0.7	< 0.001
BAY K 8644				
Racemic mixture	0.1	9.3 ± 0.5	6.9 ± 0.6	0.018
	1		7.1 ± 0.2	0.004
(−) Isomer	0.5	15.6 ± 0.4	10.7 ± 0.1	< 0.001
(+) Isomer	0.5		16.7 ± 0.3	0.045
	5.0		20.6 ± 0.1	< 0.001

* Approximately 2 to 4 × 10^6 cells/mL were preincubated for 10 min at 37°, in PBS with or without a calcium modulator; 1 μM [^{14}C]melphalan was added at 0 time and uptake was measured at 10 min unless otherwise indicated. Radioactivity was determined as described in the text; data are the means ± SEM of 4–8 determinations.

† Data were analyzed by an unpaired, two-tailed *t*-test comparing the significance of the difference of the mean uptake levels.

‡ Uptake was measured at 30 min.

melphalan uptake was investigated (Fig. 2, top panel). After a 10-min preincubation, a time course of melphalan uptake by L5178Y cells was performed in the presence and absence of 1 μM A23187. Over the first 5 min, there was a reduction in [^{14}C]melphalan uptake by cells incubated in the presence of A23187; however, after 10 min, the difference in uptake appeared to decline.

Effect of trifluoperazine on melphalan transport. The effect of the calmodulin-dependent calcium pump inhibitor [7] trifluoperazine on melphalan uptake was determined (Fig. 2, bottom panel). A time course showed decreased uptake by cells incubated in the presence of 10 μM trifluoperazine, a concentration known to inhibit the cell membrane calcium pump [7].

Effect of BAY K 8644 on melphalan uptake. The effect on melphalan uptake of BAY K 8644, a racemic mixture with both calcium channel agonist and antagonist activity, was studied. Cells were preincubated for 10 min in the presence or absence of 1 μM BAY K 8644 prior to the addition of 1 μM [^{14}C]melphalan. A time course of the effect of the racemic mixture of BAY K 8644 on melphalan transport was obtained (Fig. 3). Melphalan uptake was reduced over the entire 30-min time course.

A concentration-response study was undertaken in which uptake of 1 μM [^{14}C]melphalan was measured in the absence and presence of a racemic mixture of BAY K 8644 at concentrations ranging from 0.1 to 10 μM. Significant reductions of melphalan uptake were evident at concentrations of 0.1 and 1 μM BAY K 8644 compared to control cells (Table 1). There was no effect of 10 μM BAY K 8644 on melphalan uptake (data not shown).

BAY K 8644 is a racemic mixture in which the (−) isomer acts as a calcium channel agonist and the (+) isomer acts as a calcium channel antagonist [8, 9]. Accordingly, the effect of each isomer on melphalan uptake was investigated (Table 1). In the presence of 0.5 μM (−) BAY K 8644 melphalan uptake (mean ± SEM) was 10.7 ± 0.1 attomoles/cell and that in control cells was 15.6 ± 0.4 attomoles/cell; this 30% decrease in drug uptake was highly significant ($P < 0.001$). Conversely, melphalan uptake was increased significantly in the presence of the (+) isomer of BAY K 8644. Melphanan uptake in the presence of 0.5 μM (+) BAY K 8644 was 16.7 ± 0.3 attomoles/cell, and this 7% increase was statistically significant ($P = 0.045$); uptake in the presence of 5 μM (+) BAY K 8644 was 20.6 ± 0.1 attomoles/cell, and this 32% increase in drug uptake was highly significant ($P < 0.001$).

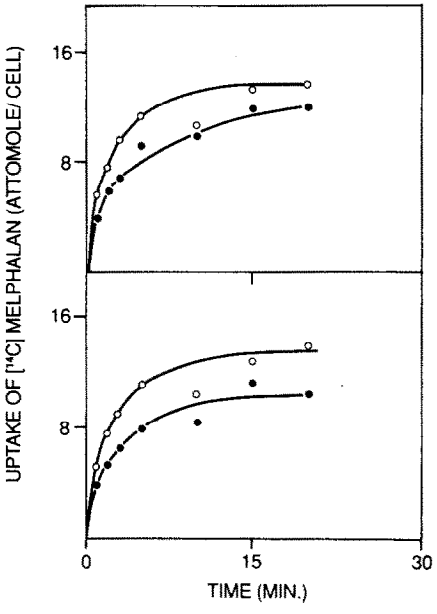

Fig. 2. Time course of the uptake of 1 μM [^{14}C]melphalan by L5178Y lymphoblasts incubated *in vitro* at 37° in the presence (●) and absence (○) of 1 μM A23187 (top panel), or in the presence (●) and absence (○) of 10 μM trifluoperazine (bottom panel).

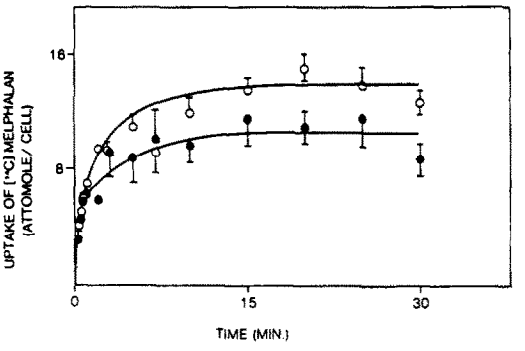

Fig. 3. Time course of the uptake of 1 μM [^{14}C]melphalan by L5178Y cells incubated *in vitro* at 37° in the presence (●) and absence (○) of 1 μM BAY K 8644 (racemic mixture). Values are means ± SEM, N = 4.

Discussion

The regulation of amino acid transport by systems L and ASC, which mediate melphalan transport, is poorly understood [10]. Intracellular calcium plays an intricate role in cellular activation and regulation of a variety of cellular functions [11, 12]. Calcium-dependent hormonal regulation of amino acid transport by system A has been reported [13, 14]. This study was undertaken to see if ions known to modulate other transport systems could affect melphalan uptake by L5178Y lymphoblasts.

Alterations in the level of extracellular calcium had a marked effect on melphalan uptake (Table 1). Chelation of extracellular calcium by EGTA produced a 33–57% increment in melphalan uptake, whereas increasing the concentration of extracellular calcium to 2.5 mM resulted in a 34% decrement in drug uptake (Table 1).

The structurally diverse calcium channel blockers verapamil, diltiazem and nitrendipine all stimulated melphalan uptake by L5178Y lymphoblasts and their relative potency was verapamil > diltiazem > nitrendipine (Table 1). Verapamil has been shown to increase the cellular accumulation of two classes of chemotherapeutic drugs, the anthracycline antibiotics and the vinca alkaloids in multidrug-resistant cells, presumably by interacting with the cell membrane and blocking an active drug extrusion pump [15–17]. Verapamil has also been reported to potentiate melphalan uptake in murine fibrosarcoma and bone marrow, an effect that was apparently reversible, independent of external calcium and in bone marrow due, in part, to inhibition of melphalan efflux [4]. Although verapamil could have effects other than channel blockade, it would be difficult to explain why this group of structurally unrelated compounds could have similar effects on melphalan uptake unless it was in some way linked to their common function.

The calcium ionophore A23187, which promotes calcium ion flux across the plasma membrane [18], caused a reduction of melphalan uptake (Fig. 2, top panel). A23187 inserts into the plasma membrane to form a calcium channel resulting in a rapid influx of calcium [18, 19]. The effect of A23187 on melphalan uptake was somewhat transient, decreasing after 10 min; this finding is consistent with a previous report that the effect of A23187 is not as durable as activation of cells by an extracellular messenger [12].

Treatment of cells with the calmodulin inhibitor trifluoperazine appeared to reduce melphalan uptake (Fig. 2, bottom panel). This finding was consistent with the observation that tamoxifen, another calmodulin antagonist [20], also decreases melphalan uptake [21]. Trifluoperazine has been shown to inhibit the calmodulin-dependent calcium pump [7], an effect which would elevate intracellular calcium. A decrease in extracellular pH below the physiological range (from pH 7.4 to 6.0) has been shown to inhibit the influx of calcium through calcium channels [22].

BAY K 8644 is a racemic mixture consisting of a (−) isomer that acts as a calcium channel agonist, and a (+) isomer that functions as a calcium channel antagonist [8, 9, 23]. In the presence of 0.5 μM (−) BAY K 8644 melphalan uptake was reduced approximately 30%, whereas in the presence of 5 μM (+) BAY K 8644 there was a 32% increase in drug uptake (Table 1). This finding is consistent with other reports in the literature that (+) BAY K 8644 is approximately 10-fold less potent than the (−) isomer [8], and explains why the racemic mixture of BAY K 8644 containing equal amounts of each isomer resulted in a decrease of melphalan uptake (Table 1 and Fig. 3).

All of the above observations are consistent with the notion that melphalan uptake varies inversely with an expected rise in calcium concentration within the cell. Agents that impede calcium influx such as the calcium channel blockers, the calcium chelator EGTA and reduction of pH were found to augment melphalan uptake, whereas conditions and agents that elevate intracellular calcium such as the calcium ionophore A23187, a calcium channel agonist (−) BAY K 8644, reduction of extracellular sodium, elevation of extracellular calcium and a calcium pump inhibitor trifluoperazine all decreased melphalan uptake.

The two amino acid transport carriers, system L and system ASC, that mediate melphalan influx have not been investigated for their calcium sensitivity [1, 2] nor has the passive system that subserves drug efflux [3]. Studies using the patch clamp technique have demonstrated the presence of dihydropyridine-sensitive calcium channels in lymphocytes [24]. Acidosis has been shown to inhibit calcium influx through calcium channels [22]. Calcium pump and sodium–calcium exchange also appear to be present in most cells, including lymphocytes (Bose R, unpublished observation). These reports, together with our findings on modifying the ionic environment, as well as effects of modulators and agents that alter intracellular calcium, support the notion that changes of intracellular calcium may be an important regulator of melphalan transport.

Acknowledgements—The helpful advice of Dr. Deepak Bose is much appreciated. This work was supported by grants from the National Cancer Institute of Canada and Bristol-Myers Squibb, Wallingford, CT.

*The Manitoba Institute of Cell Biology and the †Department of Pharmacology & Therapeutics
University of Manitoba
Winnipeg, Manitoba
Canada R3E 0V9

LORRAINE MILLER*
ABDUL M. DEFFIE*
RATNA BOSE†

*Departments of Medicine and Pharmacology and the Interdepartmental Division of Oncology
University of Toronto
Toronto, Ontario
Canada M5G 1L4

GERALD J. GOLDENBERG‡

‡ Corresponding author: Dr. Gerald J. Goldenberg, Interdepartmental Division of Oncology, University of Toronto, 92 College St., Ontario, Canada M5G 1L4. Tel. (416) 978-3649; FAX (416) 978-8350.

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