Biochemical Pharmacology, Vol. 31, No. 17, pp. 2727–2732, 1982.
Printed in Great Britain.

0006-2952/82/172727–06 $03.00/0

THE EFFECT OF ALKYLATING AGENTS AND OTHER DRUGS ON THE ACCUMULATION OF MELPHALAN BY MURINE L1210 LEUKAEMIA CELLS *IN VITRO*

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(*Received* 29 *December* 1981; *accepted* 10 *March* 1982)

Abstract—The effect of cytotoxic and other drugs on the accumulation of melphalan by L1210 murine leukaemia cells was studied. We have confirmed that uptake is an active process competitively inhibited by L-leucine. In 36 experiments in amino acid-free medium the mean concentration of melphalan taken up was 225 pmoles/10^6 cells. High pressure liquid chromatographic analysis showed that the majority of the drug is present as free native melphalan. 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU) was the only drug that stimulated accumulation, but without significant effect on influx or efflux rates. Busulphan, chlorambucil, cyclophosphamide, interferon, methotrexate and prednisolone had no effect on accumulation after 30 min melphalan transport. Adriamycin, CCNU, methyl CCNU, mustine and vincristine all impaired melphalan accumulation as did the non-cytotoxic drugs aminophylline, chlorpromazine and ouabain. Adriamycin, aminophylline, chloropromazine, indomethacin and ouabain all reduced melphalan influx.

Melphalan (L-phenylalanine mustard) is actively taken up by murine leukaemia L1210 cells as it can proceed against a concentration gradient, is temperature sensitive and prevented by metabolic inhibitors [1]. It is also competitively inhibited by the presence of amino acids, particularly L-leucine [1]. Investigations by Vistica *et al.* have led to the suggestion that at minimum cytotoxic concentrations (6.8 μM), melphalan transport is divided equally between two amino acid transporting systems [2]. These are a leucine-preferring transport system (the L system) and a monovalent cation-dependent transporting system, the latter being similar to the sodium-dependent system used for the transport of 3 and 4 carbon aliphatic, hydroxyaliphatic and sulphur containing amino acids in the Ehrlich ascites tumour cell [3]. Similar melphalan transporting systems have been reported in other cell types [4, 5].

Melphalan has been used since its synthesis for treating multiple myeloma. However, on its own, a response to treatment was seen in less than half the patients and the median survival time from the beginning of treatment was only 18 months [6]. Treatment using melphalan with prednisolone improved the response rate and survival time of the patients designated "good risk" by Costa *et al.* [7]. The Medical Research Council showed that cyclophosphamide was as effective as melphalan [6], and Bergsagel *et al.* [8] showed that cross-resistance between alkylating agents did not always occur. This led to the theory that if different alkylating agents had different modes of action, then administering several may improve the treatment of multiple myeloma. However the results of combining several cytotoxic agents have in general been disappointing. Drug combinations of melphalan, cyclophosphamide, prednisolone and BCNU‡ used by Alexanian *et al.* [9], gave a slightly better survival time, but no improvement in response rate. Addition of vincristine to multi-drug regimens resulted in an increase in survival time and response rate [9, 10].

It is possible that this response may be in part due to changes in transport characteristics of melphalan, as Vistica has shown a close correlation between cytotoxicity of L1210 cells and uptake of melphalan at similar concentrations to those we have used [11].

Because more drug combinations are being used in cancer chemotherapy, we have investigated the effect of alkylating agents and other drugs on the accumulation of melphalan *in vitro*.

MATERIALS AND METHODS

Materials. L1210 murine leukaemia cells, newborn calf serum, HEPES buffer, RPMI 1640 medium, glutamine and fungizone were all supplied by Flow Laboratories Ltd. (Irvine, U.K.). Bovine serum albumin (obtained as serum fraction V), trypan blue dye, *N*-nitroso-*N*-methylurea, ouabain, mustine, allopurinol and indomethacin were purchased from the Sigma Chemical Company (Poole, U.K.). Diethylurea and 1,3-dimethylurea were obtained from the Aldrich Chemical Company (Gillingham,

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‡ Abbreviations used: ACNU, 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosourea hydrochloride; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; GANU, 1-(2-chloroethyl)-3-(β-D-glucopyranosyl)-1-nitrosourea; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid; HPLC, high pressure liquid chromatography; MCNU, 1-(2-chloroethyl)-3-(methyl-α-D-glucopyranos-6)-1-nitrosourea; methyl CCNU, 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea.

U.K.). Unlabelled melphalan, busulphan and chlorambucil were generous gifts from Burroughs Wellcome and Co. (London, U.K.). Human lymphoblastoid interferon was kindly donated by Drs. Fantes and Johnson, Wellcome Research Laboratories (Beckenham, U.K.). BCNU and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) were donated by Bristol Myers Co. Ltd. (Slough, U.K.). Methyl CCNU and [^{14}C]melphalan (chloroethyl labelled, 40 μCi/mg) were kindly donated by Dr. R. Engle of the Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, Maryland, U.S.A. MCNU was donated by the Tokyo Tanabe Co. Ltd., ACNU by the Sankyo Co. Ltd., and GANU by Meiji Kaisha Ltd., all of Tokyo, Japan. [G-^{3}H]Melphalan (8 Ci/mmole), [^{14}C]carboxyinulin and [^{3}H]water were purchased from the Radiochemical Centre (Amersham, U.K.).

The melphalan concentration used throughout the experiments (6.8 μM) was chosen for two reasons, (a) because it is a clinically achievable plasma level, and (b) so that we could compare our results with those of Vistica *et al.* [2] who had used this concentration throughout his work. Dilutions of labelled and unlabelled melphalan were made in aqueous medium immediately before use in order to minimise hydrolysis. These dilutions produced a corresponding decrease in radioactivity taken up, demonstrating that both radioactive and nonradioactive melphalan showed similar transport characteristics. All other drugs were obtained from the hospital pharmacy as drugs for intravenous injection. Cyclophosphamide was activated by the method of Sladek [12]. However, calculations from the kinetics described suggest that only a small proportion of the cyclophosphamide was activated. Drug concentrations used were very approximately peak plasma concentrations found in man (and 10 times this value) when these could be ascertained; or 2.1 μg/ml (the concentration of melphalan used) multiplied by the very approximate ratio of i.v. injection size of the drug to 20 mg (typical melphalan injection size), and 10 times this value.

The silicone oil used in these experiments was obtained by mixing Dow Corning 550 and 200/1 cs silicone fluids 9:1 (v/v) (Hopkins and Williams, Swansea, U.K.) to give an approximate specific gravity of 1.045. All other reagents used were of analar grade.

Cell cultures. Cell cultures were maintained at 37° in RPMI 1640 (Roswell Park Memorial Institute) medium supplemented with 10% newborn calf serum, 2 mM glutamine, 20 mM HEPES buffer, fungizone (125 ng/ml), and gentamicin (40 μg/ml).

Estimation of intracellular water space was made with [^{3}H]water using the method of Wohlhueter *et al.* [13]. [^{14}C]Carboxyinulin was used as a measure of extracellular water space. The intracellular water space of our L1210 cells was 0.94 μl/10^{6} cells.

As some of the drugs tested were cytotoxic and present at high concentrations, it was possible that they could have damaged the cell membrane during the experiment. A trypan blue dye exclusion test was therefore undertaken at the end of each experiment, and with all the drugs tested no significant reduction in membrane integrity was observed.

Accumulation of melphalan by L1210 cells. Logarithmic phase (48 hr) L1210 cells (approximately 1×10^{6} cells/ml) were harvested by centrifugation at 120 *g* for 10 min and then washed in 'transport medium' comprising of Dulbecco's phosphate buffered saline supplemented with 0.1 mM bovine serum albumin and 0.1% glucose (pH 7.4). The cells were incubated in a water bath at 37° at a concentration of 2×10^{6} cells/ml in transport medium and any drugs (except melphalan) were then added. After 20 min incubation transport was initiated by addition of 6.8 μM labelled melphalan, and just prior to the selected time points, duplicate 0.5 ml aliquots of the incubation mixture were laid on silicone oil (0.5 ml) in a microcentrifuge tube maintained at 37°. Transport was then terminated by centrifugation of the cells through the oil at 12,500 *g* for 2 min in a 320a microcentrifuge (Burkhard, Uxbridge, U.K.). Initial rate of influx was calculated from the accumulation of melphalan in the first 2 min of transport during which time uptake was approximately linear ([14]; Fig. 1). Tips containing the cell pellets were cut off and the pellets dissolved by incubation at room temperature with 100 μl of 0.2 M NaOH and 100 μl of toluene for at least 1 hr. The NaOH was neutralised by addition of 20 μl of 1 M acetic acid, 3 ml of N.E. 260 scintillation fluid (Nuclear Enterprises, Edinburgh, U.K.) was added, and the samples counted on a Nuclear Enterprises 8312 scintillation counter. All data were corrected for the binding of melphalan to the cell surface by subtraction of the cell associated radioactivity measured within 1 min of melphalan addition at 0°. Experiments were completed in triplicate and transport

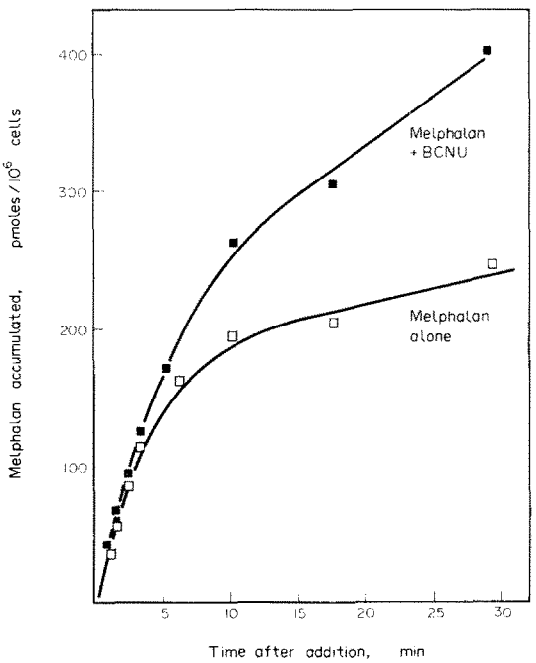

Fig. 1. The effect of BCNU on the accumulation of melphalan. Experimental conditions were those described for Table 1 except for the sample times. Ethanol (4 μl/ml) or BCNU (100 μg in 4 μl of ethanol/ml) were added to the cells 20 min before melphalan (6.8 μM). Each point represents the mean of three experiments.

Table 1. The effect of drugs on melphalan accumulation in L1210 cells

Drug	Concentration of drug	Melphalan accumulation: 2 min after addition (% control)	P	Melphalan accumulation: 30 min after addition (% control)	P
Adriamycin	40 μg/ml	74.0 ± 4.5	< 0.005	86.4 ± 5.8	< 0.05
Allopurinol	20 μg/ml	94.3 ± 7.9	NS*	95.4 ± 4.6	NS
Aminophylline	100 μg/ml	85.4 ± 3.5	< 0.005	82.2 ± 3.4	< 0.01
BCNU	100 μg/ml	128.4 ± 36.0†	NS	156.4 ± 26.2‡	< 0.001
Busulphan	4 μg/ml	96.7 ± 15.8	NS	96.0 ± 7.5	NS
CCNU	10 μg/ml	85.8 ± 3.7	NS	54.1 ± 4.5	< 0.001
Chlorambucil	10 μg/ml	91.5 ± 11.7	NS	115.8 ± 6.9	NS
Chlorpheniramine	2 μg/ml	99.0 ± 3.1	NS	95.6 ± 2.4	NS
Chlorpromazine	20 μg/ml	62.2 ± 5.3	< 0.001	69.0 ± 7.2	< 0.01
Cimetidine	20 μg/ml	93.9 ± 6.8	NS	95.1 ± 6.9	NS
Cyclophosphamide	100 μg/ml	91.5 ± 6.3	NS	88.9 ± 4.3	NS
Activated cyclophosphamide§	100 μg/ml	92.6 ± 13.2	NS	93.8 ± 9.0	NS
Cytosine arabinoside	8.2 μg/ml	86.0 ± 4.5	NS	92.4 ± 7.0	NS
Diazepam	10 μg/ml	91.3 ± 7.8	NS	97.3 ± 2.8	NS
Digoxin	10 ng/ml	101.0 ± 9.2	NS	102.3 ± 2.4	NS
Frusemide	800 ng/ml	86.7 ± 7.3	< 0.1	102.7 ± 2.0	< 0.1
Guanethidine	400 ng/ml	109.9 ± 7.7	NS	93.9 ± 3.6	NS
Hydrallazine	4 μg/ml	85.4 ± 13.7	NS	93.3 ± 14.4	NS
Imipramine	1 μg/ml	87.3 ± 11.6	NS	88.9 ± 16.8	NS
Immunoglobulin light chains‖	6.25 mg/ml	96.8 ± 7.8	NS	100.0 ± 9.8	NS
Indomethacin	10 μg/ml	87.2 ± 5.2	< 0.05	93.4 ± 5.0	NS
Interferon	15,000 units/ml	95.2 ± 11.2	NS	94.6 ± 8.9	NS
Lignocaine	40 μg/ml	84.2 ± 8.9	NS	87.8 ± 6.8	NS
Methotrexate	10 μg/ml	89.1 ± 9.7	NS	86.0 ± 4.1	< 0.1
Methyl CCNU	10 μg/ml	78.7 ± 5.6	< 0.05	59.9 ± 2.1	< 0.001
Metronidazole	50 μg/ml	105.4 ± 6.1	NS	96.5 ± 6.3	NS
Morphine	20 μg/ml	100.5 ± 17.0	NS	96.8 ± 2.0	NS
Mustine	20 μg/ml	88.6 ± 5.6	NS	64.8 ± 6.9	< 0.02
Ouabain	144 μg/ml	88.6 ± 6.3¶	< 0.02	68.3 ± 3.7	< 0.001
Phenobarbitone	200 μg/ml	90.0 ± 4.7	NS	96.4 ± 6.8	NS
Phentolamine	10 μg/ml	84.1 ± 17.7	NS	88.4 ± 11.7	NS
Prednisolone	320 ng/ml	95.2 ± 3.6	NS	94.6 ± 5.2	NS
Procainamide	40 μg/ml	92.7 ± 15.9	NS	93.0 ± 16.0	NS
Propranolol	2 μg/ml	96.6 ± 4.8	NS	95.2 ± 7.5	NS
Vincristine	10 μg/ml	94.7 ± 12.2	NS	80.8 ± 7.0	< 0.02

L1210 cells were harvested as described in Materials and Methods and incubated at 37° for 20 min at 2×10^6 cells/ml in transport medium with one of the drugs. [^{14}C] or [^{3}H]melphalan was then added to initiate transport. At 2 and 30 min, duplicate aliquots were taken and melphalan accumulation terminated by centrifugation through silicone oil at 12,500 *g*. Radioactivity associated with the cell pellet was then counted. Each value is the mean (± S.D.) of 3 experiments and is expressed as a percentage of the quantity of melphalan accumulated in the absence of drug. All data were statistically evaluated by Student's two-tailed *t*-test.

* $P > 0.1$ by two-tailed *t*-test.

† $n = 9$.

‡ $n = 12$.

§ Activated by the method of Sladek [12]; however due to kinetics described, only a small proportion of the drug may be in the activated form.

‖ Human light chains for use in this experiment were concentrated from the urine of a patient with multiple myeloma and Bence Jones proteinuria using a minicon B15 urine concentrator. The concentration was that reported to totally inhibit the ouabain-sensitive ATPase of the plasma membranes from rat renal cortical tubules [18].

¶ $n = 6$.

terminated within 75 min of the removal of the cells from growth medium.

Loss of melphalan from L1210 cells. Cells were preincubated in transport medium at 37° for 20 min with the drug under test and then radioactive melphalan (6.8 μM) was added. After 10 min the cells were centrifuged at 600 *g*, washed in transport medium at 4° and then resuspended in transport medium at 37° in which, in some experiments, the drug under test was present. The radioactivity associated with 10^6 cells was then measured with time as previously described.

Resolution of free and bound melphalan. Protein bound and free intracellular melphalan were estimated by ultrasonicating the tips containing the cell pellets in 1 ml of a high pressure liquid chromatographic (HPLC) mobile phase (methanol:1.5 mM sodium lauryl sulphate (2:1; v/v) adjusted to pH 3.2 with sulphuric acid) used for the determination of melphalan by Bosanquet and Gilby [15, 21]. After ultrasonication, the cell debris was separated by centrifugation at 12,500 *g* in the microcentrifuge and the resulting pellet treated for radioactive counting as described previously. This data was taken as a

measurement of protein bound melphalan. The supernatant was used to estimate the intracellular free melphalan in two ways. A 200 μl aliquot was injected directly into the HPLC and assessed for unchanged melphalan (the system resolves melphalan from its hydrolysis products), and a 500 μl aliquot was added to 3 ml scintillation fluid and its radioactivity counted.

RESULTS

Over a series of 36 experiments, the mean quantity ± standard deviation of melphalan accumulated by 10^6 L1210 cells in 30 min at 37° was 225 ± 61 pmoles. In the first 2 min influx was linear at 58.2 ± 10.2 pmoles melphalan/10^6 cells/min. Figure 1 shows a typical example of this accumulation with time, the process was temperature sensitive, inhibited by leucine and after 30 min resulted in a cell–medium ratio of approximately 35.

Of the 40 drugs whose effects on melphalan were tested (Table 1), the only one whose presence resulted in a significant increase in melphalan accumulation was BCNU, although it had no significant effect on influx. After 30 min 54% more melphalan had accumulated in the presence of BCNU than in a control experiment ($P < 0.001$). The effect was seen at concentrations of 25 μg/ml BCNU and above.

BCNU also stimulated accumulation of melphalan significantly in a system containing 100 μM L-leucine, a competitive inhibitor of melphalan transporting systems ($P < 0.05$).

It was thought that differences in the rate of efflux of melphalan from the cells may account for the differences in the drug's accumulation in the presence of BCNU. However, experiments examining initial rate of loss of melphalan from cells charged with the radioactive drug showed no difference between control experiments and those with cells also preincubated with BCNU; in both cases the rate was approximately 5 pmoles/min/10^6 cells.

Increase in cell volume was also investigated as a possible cause of increased melphalan accumulation, but no significant change in cell size was observed.

Data for the increase in accumulation of melphalan were confirmed by measuring the intracellular free melphalan by HPLC as well as by radioactivity (Table 2). That the HPLC results were lower than those calculated by radioactivity could be explained by hydrolysis of melphalan during the experiment and subsequent processing of the cell pellets. However, the same percentage increase in melphalan accumulation was seen using both methods. It is apparent from Table 2 that after 2 min transport, both in the presence and absence of BCNU, only 4% of the intracellular melphalan present was protein-bound—a measure of how much drug had undergone alkylation. By 30 min this had only increased to 12%.

The effect of other nitrosoureas on melphalan accumulation was also tested. Both CCNU and methyl CCNU caused a significant drop of 50% on the quantity of melphalan accumulated ($P < 0.001$ in both experiments). Methyl CCNU also caused a significant drop in melphalan influx ($P < 0.05$). However, the water soluble nitrosoureas (GANU, ACNU and MCNU; data not shown), *N*-nitroso-*N*-methylurea, diethyl- and dimethylurea all showed no effect on melphalan accumulation at concentrations similar to those used for BCNU. In all experiments involving nitrosoureas no significant loss of viability of the cells was seen during the course of the experiment provided the concentration of ethanol present was kept below 1%.

The effects of other drugs at the concentrations shown can be seen in Table 1. Those which may be used in combination with melphalan which had no

Table 2. The effect of 100 μg/ml BCNU upon the uptake of melphalan in L1210 cells

	Melphalan accumulation pmoles/10^6 cells			
	2 min after addition		30 min after addition	
	Without BCNU	With BCNU	Without BCNU	With BCNU
Concentration of cell-associated melphalan measured by radioactivity	141.7 ± 14.5	160.4 ± 26.9	266.2 ± 12.7	467.9 ± 49.6
Intracellular free melphalan measured by radioactivity	150.6 ± 16.4	171.8 ± 29.2	231.4 ± 7.6	420.9 ± 65.8
Intracellular free melphalan measured by HPLC	107.6 ± 16.5	125.3 ± 24.8	158.8 ± 19.7	309.6 ± 53.3
Protein bound melphalan	6.4 ± 0.3	7.3 ± 0.4	31.7 ± 7.9	53.0 ± 6.1

The concentration of cell-associated melphalan was measured by the radioactivity of a cell pellet as described in the legend to Table 1. Intracellular and protein bound melphalan were measured in the supernatant and precipitate respectively resulting from ultrasonicating a cell pellet with HPLC mobile phase. Intracellular free melphalan was measured by both radioactivity and HPLC. Each value is the mean of three experiments (± S.D.).

significant effect on its accumulation at 30 min were busulphan, chlorambucil, cyclophosphamide, interferon, methotrexate and prednisolone. The presence of the other cytotoxic drugs; adriamycin, CCNU, methyl CCNU, mustine and vincristine, all resulted in a significant decrease in the accumulation of melphalan. In addition to reducing accumulation, adriamycin significantly reduced influx. A dose of 100 μg/ml cyclophosphamide had no significant effect on melphalan accumulation either before or after activation by the method of Sladek [12]. However, due to the kinetics described, only a small proportion of the drug was activated, and it is possible that the fully activated drug might have produced a different result.

Of the other drugs tested, the presence of aminophylline, chlorpromazine or ouabain resulted in a significant drop in the influx and accumulation of melphalan and indomethacin significantly reduced melphalan influx.

DISCUSSION

In previous studies Begleiter [4], using L5178Y lymphoblasts, Goldenberg [5], using LPC-1 plasmacytoma cells, and Vistica and Rabinovitz [1], using L1210 cells, have all shown that melphalan is taken up in amino acid-free medium producing a cell–medium ratio of approximately 12:1. We, too, have demonstrated concentrative uptake of melphalan, but despite our cells having the same volume as Vistica's, we have shown far greater uptake of melphalan, giving a cell–medium ratio of approximately 35.

During the preparation of samples of cells for analysis by HPLC, the distribution of protein bound and free intracellular melphalan was estimated (Table 2). We have shown that the majority of intracellular melphalan present has an identical retention time to the intact drug, and is not bound to cell constituents. These data are similar to Goldenberg *et al.* [16] and Begleiter *et al.* [4] investigating L5178Y lymphoblasts. They showed that 95.8% of melphalan was present in the cell sap and that only 4.2% was protein bound after 10 min of melphalan transport.

The nitrosourea BCNU greatly enhanced melphalan accumulation 30 min after its addition. However, our data show that the drug has no significant effect on the rate of influx (Fig. 1) and efflux. It may be possible that the increased accumulation of melphalan may be due to increased binding to cell constituents caused by membrane-damage due to the high concentration of cytotoxic drugs present. However, both trypan blue dye exclusion (which showed no significant decrease in membrane integrity in the presence of BCNU) and HPLC studies (which showed the same fraction of melphalan present bound to protein both in the presence and absence of BCNU) indicate that this is not the case. It appears that in the presence of BCNU, the cells capacity to concentrate melphalan in its free unhydrolysed form is increased. This capacity is not shared by the other nitrosoureas tested or by dimethyl- or diethylurea.

Clinical trials made by Alexanian *et al.* [9] report a slight improvement in survival times in patients receiving an initial multi-drug treatment including BCNU and melphalan. Whether this is due to an increase in the quantity of melphalan accumulated is unknown. In attempting to make such a correlation it must be remembered that a high concentration of BCNU was used in our experiments, and also that when it is administered intravenously *in vivo* its uptake and metabolism occur very quickly [17]. Therefore, unless melphalan is present in the blood in the brief time when BCNU is present at high concentration, enhancement of melphalan accumulation is unlikely to occur. When L1210 cells are preincubated with some alkylating agents and other cytotoxic drugs at high concentrations impairment of melphalan uptake results. This may be due to damage of the transport system because of the high concentrations of the cytotoxic drugs used.

Ouabain, (a cardiac glycoside) is known to inhibit the Na^+, K^+-ATPase transporting system, and in its presence a decrease in melphalan influx and accumulation occurred. Inhibition of the ATPase results in a reduction of the concentration gradient of Na^+, and consequently reduces the potential of this ion across the membrane. As melphalan transport has been shown to be partially dependent on Na^+ [3], a decrease in the capacity to accumulate melphalan was expected. Surprisingly immunoglobulin light chains (Bence Jones protein), which have also been reported to inhibit the ouabain sensitive Na^+, K^+-ATPase in rat renal cortical tubules [18], did not inhibit melphalan uptake (Table 1).

Aminophylline is an inhibitor of phosphodiesterase and consequently leads to increased levels of cyclic-AMP in the cell. In its presence, the rate of melphalan influx is reduced, but whether this is due to an increase in cyclic-AMP concentration is not yet known. The cyclic nucleotide has been implicated in the control of the growth rates of some cells [19], and is involved in the regulation of transport of amino acids in some tissues [20].

We have shown that some cytotoxic drugs that are used in combination with melphalan *in vivo* impair its accumulation *in vitro*, and it may be, therefore, that the increase in cytotoxicity provided by a second drug may be negated by the reduction in melphalan accumulated.

Acknowledgements—This work was generously supported by a grant from the Leukaemia Research Fund. Our thanks go to Michelle Henfrey for her technical assistance and to Caroline Lewis for typing the manuscript. A grant from the Wessex Regional Health Authority for the scintillation counter is acknowledged.

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