Biochemical Pharmacology, Vol. 41, No. 8, pp. 1173–1177, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

TEMPERATURE DEPENDENCE OF MELPHALAN EFFLUX KINETICS IN CHINESE HAMSTER OVARY CELLS

DIANA A. BATES*† and JASON H. T. BATES‡

*Département de Chime, Université du Québec à Montréal, Montréal, Québec H3C 3P8; and
‡Meakins-Christie Laboratories, McGill University, Montréal, PQ H3A 2B4, Canada

(*Received* 31 *May* 1990; *accepted* 11 *October* 1990)

Abstract—The temperature dependence of the kinetics of efflux of melphalan from Chinese hamster ovary (CHO) cells was studied from 4° to 47°. Time courses for melphalan efflux showed an initial rapid phase of efflux followed by a plateau. The data for melphalan concentration (c) versus efflux time (t) were described by the equation $c(t) = A + B \exp(-kt)$, where A is the final steady-state melphalan concentration, B is the total change in melphalan concentration from time zero until steady-state conditions are reached, and k is the rate constant for the efflux process. The plateau level obtained was not dependent on temperature and corresponded to 22 ± 3.2% of the drug remaining in the cells after efflux. The time for melphalan efflux to reach the plateau level was dependent on temperature. This was reflected by an increase in the rate constants for melphalan efflux with increasing temperature from 30° to 47°. The rate constant for melphalan efflux at 37° was 0.045 ± 0.002 min^{-1}. Efflux of melphalan occurred much more slowly at lower temperatures such as 4° and 20°. An Arrhenius plot for melphalan efflux showed a linear and decreasing trend at temperatures between 30° and 47° with an activation energy of 1.046×10^3 J/mol.

There is potential for the combination of systemic chemotherapy with clinical hyperthermia in the treatment of human cancers [1]; however, it is important to study the interactions between heat and anticancer agents *in vitro* before these combined modalities can be used successfully in the clinic. Melphalan (4-[bis(2-chloroethyl)-amino]-1-phenylalanine) is a chemotherapeutic agent used in the treatment of malignant melanoma and multiple myeloma cancers [2]. Thermal enhancement of melphalan cytotoxicity has already been demonstrated *in vivo* in murine tumours at 43° [3] and *in vitro* in Chinese hamster ovary (CHO§) cells at temperatures from 38° to 45° [4, 5]. The mechanisms involved in the thermal enhancement are presently unknown.

Cellular uptake of melphalan occurs by an active, carrier-mediated process involving two separate amino acid transport systems [6, 7], but there is little known about the mechanism of melphalan efflux. It has been shown that efflux from lymphoblasts occurs by a mechanism differing from that for the influx process [8]. Begleiter and coworkers [9] have also shown that melphalan efflux is greater from drug-resistant mutants of CHO cells than from drug-sensitive lines. It is postulated that changes in drug efflux could explain, in part, the decreased cytotoxicity of melphalan in drug-resistant cells.

The present study describes the kinetics of melphalan efflux from CHO cells over the temperature range 4° to 47° and also considers the relevance of temperature-induced changes in melphalan efflux with respect to the thermal enhancement of melphalan cytotoxicity.

MATERIALS AND METHODS

Tissue culture. CHO cells (AuxB1) [10] were grown in monolayer in Minimum Essential Medium Alpha (MEM Alpha) (Gibco Canada, Burlington, Ontario) plus 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin (50 units/mL)-streptomycin (50 μg/mL) (Flow Laboratories, Mississauga, Ontario), in 25 cm^2 tissue culture flasks (Falcon, Becton-Dickinson Canada Inc., Mississauga, Ontario) in a humidified atmosphere of 5% CO_2 in a water-jacketed incubator at 37°. The cells were grown to confluence and were then incubated for 24 hr with fresh culture medium. For experimental studies, cells were harvested using citrated phosphate-buffered saline (0.14 M NaCl, 0.01 M sodium phosphate, 0.015 M sodium citrate), washed by centrifugation, and resuspended in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 10 mM glucose.

Measurement of melphalan efflux. [^{14}C]Melphalan (L-*p*-(di-2-chloroethyl[^{14}C]amino)phenylalanine) with a specific activity of 43.8 μCi/mg was a gift from Mr. Maurice Leaffer of SRI International, Menlo Park, CA, U.S.A. The radiochemical purity was 97% as determined by thin-layer chromatography on silica gel 60 (Merck, Darmstadt, F.R.G.) in *n*-butanol:acetic acid:water (7:2:1). Melphalan was freshly prepared immediately before each experiment and kept on ice at all times. It was dissolved in ethanol (92%) containing HCl (2%, w/v) and then diluted to the appropriate concentration in PBS containing 1% BSA and 10 mM glucose, at pH 7.3.

† Correspondence: Dr. Diana A. Bates, Département de chimie, Université du Québec à Montréal, CP 8888, succursale A, Montréal (Québec) H3C 3P8, Canada.

§ Abbreviations: BSA, bovine serum albumin; CHO, Chinese hamster ovary; E_a, activation energy; FBS, fetal bovine serum; k, rate constant; MEM, Minimum Essential Medium; and PBS, phosphate-buffered saline.

The final concentrations of ethanol and HCl did not exceed 0.005 and 0.0001% respectively.

Freshly harvested CHO cells (10^7/mL) were preloaded with [^{14}C]melphalan (5 μg/mL) for 15 min at 37° in PBS containing 1% BSA and 10 mM glucose at pH 7.3. The cells were then centrifuged at 0° (2 min, 1000 g) and washed three times with ice-cold PBS–1% BSA to remove extracellular drug. For efflux experiments, the cells were resuspended in ice-cold melphalan-free PBS–BSA–glucose, and 100-μL aliquots were put in glass tubes. This step was performed rapidly at 0°. No measurable melphalan efflux was detected up to 60 min at 0° (data not presented). A volume of 0.3 mL of PBS–BSA–glucose (prewarmed to the incubation temperature) was added immediately to each tube, and the cells were incubated at temperatures from 30° to 47° for 20 to 23 different times up to 100 min. At 4° and 20°, the incubations were performed up to 295 and 180 min respectively. The volume of the incubation solution was sufficiently large to minimise the problem of drug re-entry into the cells. To stop efflux, the cells were placed on ice and 0.7 mL of ice-cold PBS–1% BSA solution was added. The cell suspensions were centrifuged at 0° (1 min, 1000 g) and the supernatant and cell pellet were separated immediately. The liquid scintillation fluid Scintiverse II (Fisher Scientific) was added to the cell pellets (dissolved in 1% sodium dodecyl sulfate) and to the supernatants. The radioactivity was determined using a Tri-Carb liquid scintillation counter (model 2200CA, Canberra-Packard Canada Ltd., Montréal, Canada) equipped with a dpm calculation program.

Since many incubation times were investigated in each experiment, it was possible to perform experiments with cells from the same harvested population at only two or three different temperatures. Therefore, for each experiment, a control curve was always performed at 37° in order to be able to compare the efflux data from different harvested cell populations. Control curves at 37° were extremely reproducible. The zero time point represents the melphalan concentration, either in cell pellets or in the supernatants, prior to efflux. Thus, the efflux data obtained from the cell pellets were normalised so that the data start from 1.0 at time zero, and the data obtained from the supernatants were normalised so that the data asymptote to 1.0 as time tends to infinity (or steady state). The mean melphalan concentration in the cell pellets at time zero was 33.13 ± 3.39 (SD) pmol/10^6 cells. To determine the ratio of intracellular to extracellular melphalan concentrations at equilibrium, a mean cell volume of 1.194 ± 0.155 (SD) μl/10^6 cells was obtained by measuring the diameters of 50 cells on seven separate occasions under a phase contrast microscope (Leitz Wetzlar, F.R.G.) with a calibrated eyepiece microchrometer.

Thin-layer chromatography of [^{14}C]melphalan and the hydrolysed drug was carried out using plastic sheets precoated with Silica gel 60 (Merck). The radioactivity was characterised in the efflux supernatants obtained at 37° after removal of the cells by centrifugation. The solvent system used for the separation was n-butanol:acetic acid:water (7:2:1). Following the separation, the plates were cut into sequential 1-cm strips, and the radioactivity was determined using a liquid scintillation counter as described above.

Data analysis. The data analysis was performed independently using the data obtained from the cell pellets and from the supernatants. The efflux data for concentration of melphalan (c), either inside the cells or in the supernatant outside the cells, versus time (t), were fitted to the following equation:

$$c(t) = A + B\exp(-kt) \quad (1)$$

by systematically searching for the best-fit value of k. The parameter A is the final steady-state melphalan concentration, B is the total change in melphalan concentration from time zero until steady-state conditions are achieved, and k is the rate constant for the efflux processes. The sum of the parameters $A + B$ gives the initial melphalan concentration at time zero for the efflux process. For each value of k in the search, the corresponding best-fit values of A and B were found by multiple linear regression. After finding the best fit of Equation 1 to the data, confidence intervals on the fitted parameters were determined by Monte-Carlo simulation. That is, a set of noiseless data was generated by evaluating the fitted function at the sampling times of the original data. Two hundred sets of noisy data were then created by adding to the noiseless data 200 different sets of random numbers whose SDs were equal to those estimated for the noise in the original data set (obtained from the root mean squared residual between data and fit). The SDs of the parameter values estimated from the 200 simulated data sets were taken as estimates of the SDs of the parameters estimated from the original data set. The 95% confidence intervals were taken as twice the SDs.

Arrhenius analysis. The dependence of rate constants on temperature is described by the Arrhenius equation [11]:

$$k = A\exp(-E_a/RT), \quad (2)$$

where k represents the rate constant for the efflux process, A is the Arrhenius constant, E_a is the activation energy, R is the gas constant (8.3144 J/mol °K), and T is the absolute temperature in degrees Kelvin.

RESULTS

Figure 1 shows the temperature dependence of melphalan efflux from CHO cells during 100 min for temperatures ranging from 20° to 45°. Efflux experiments were also performed at 4° and 47° (data not presented). The data shown in Fig. 1a were obtained from the cell pellets and correspond to the residual intracellular melphalan concentration during the efflux process, whereas the data shown in Fig. 1b were obtained from the supernatants during the same experiments and correspond to melphalan concentrations appearing in the supernatants during efflux. Panels a and b of Fig. 1 show the experimental data points and panels c and d show the respective fitted curves obtained from the analysis of the data (Equation 1).

It can be seen in Fig. 1a that the melphalan

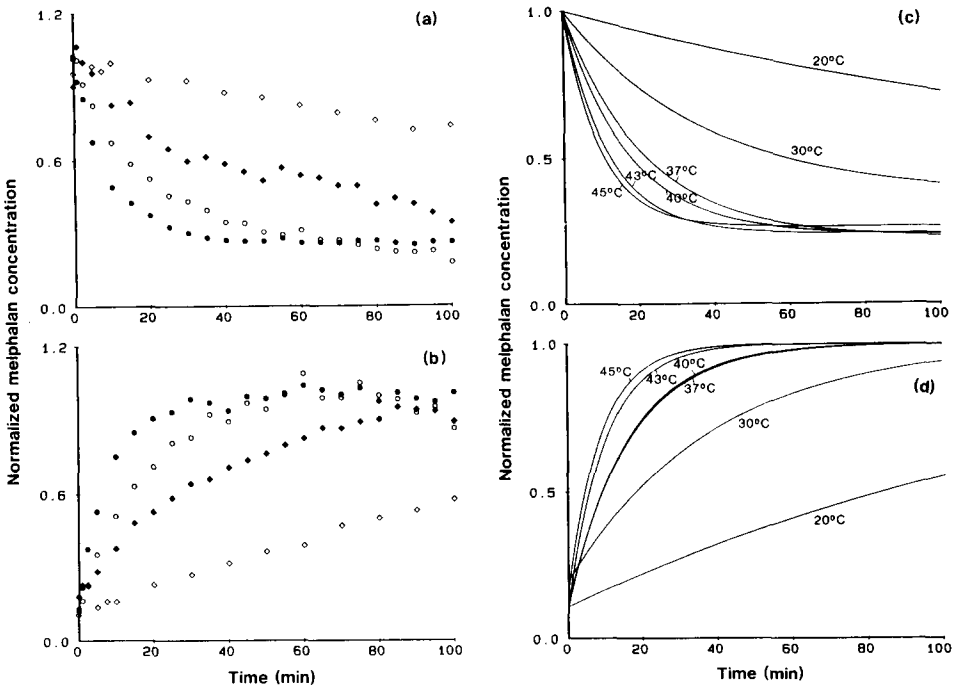

Fig. 1. Temperature dependence of melphalan efflux. CHO cells were preincubated with [^{14}C]melphalan (5 μg/mL) for 15 min at 37° and then resuspended in drug-free transport medium prior to efflux measurements. (a) Normalised intracellular melphalan concentration versus efflux time from 10^6 CHO cells at 20° (◇), 30° (◆), 37° (○), and 45° (●). The fitted curves (Equation 1) for the data set are shown as the solid lines in Fig. 1c at the temperatures indicated. The data have been scaled so that the fitted lines all start at 1.0 at time zero. (b) Corresponding normalised supernatant melphalan concentrations versus efflux time at 20° (◇), 30° (◆), 37° (○), and 45° (●). The fitted curves (Equation 1) for the data set are shown as the solid lines in Fig. 1d at the temperatures indicated. The data have been scaled so that the fitted lines asymptote to 1.0 as time tends to infinity. The data points are not shown at 40° and 43° in Figs. 1a and b to avoid overcrowding on the graphs. The data points in panels a and b represent the means of triplicate estimations. Experiments were repeated on 7 occasions to give 7 control curves at 37° and 1–2 curves at the other temperatures.

concentration in the cell pellet decreased with efflux time and tended toward a plateau level. The rate of decrease in melphalan concentration increased with temperature, thus diminishing the time required to reach the plateau level. For example, the time required to reach the plateau level at 37° was about 60 min, whereas the time at 45° was about 30 min. The corresponding data obtained from the supernatants showed an increase in the melphalan concentration in the supernatant with increasing efflux time (Fig. 1b). The melphalan concentration in the supernatant also tended towards a plateau level. The rate of efflux increased with temperature, thus diminishing the time required to reach the plateau level, as in Fig. 1a. The efflux times of 295 and 180 min were not sufficient to reach a plateau at 4° and 20° respectively. At 4°, only 5% of the drug had effluxed out of the cell by 295 min (data not shown).

The data for melphalan concentration versus efflux time, obtained from both the cell pellets and supernatants, at temperatures from 30° to 47° were well described by a curve of the form of Equation 1 (Fig. 1, c and d). The best-fit values for the rate constant k of the efflux process were determined for each temperature from 30° to 47°, as described in Materials and Methods. The efflux process had reached a steady state by 100 min at temperatures from 37° to 47°. Although the plateau level was not quite reached by 100 min at 30° (Fig. 1), the quality of the fits of Equation 1 to the data was still very good at this temperature. The rate constants were not determined at 4° and 20°, since the efflux process had not reached equilibrium within the time frame of the experiments.

A comparison was made of the rate constants obtained from both the cell pellets and the supernatants. Figure 2 shows a plot of k obtained from the intracellular melphalan concentrations (Fig. 1c) versus k obtained from the corresponding supernatant concentrations (Fig. 1d), for temperatures from 30° to 47°. The rate constants for the efflux process increased with increasing temperature. The points lie close to the dashed line of identity, indicating that the rate of disappearance of melphalan from the cells equalled its rate of appearance in the supernatant. The rate constants obtained from the cell pellets and from the supernatants were thus very comparable. The mean rate constant obtained at 37° was 0.045 ± 0.002 (SD) min^{-1}.

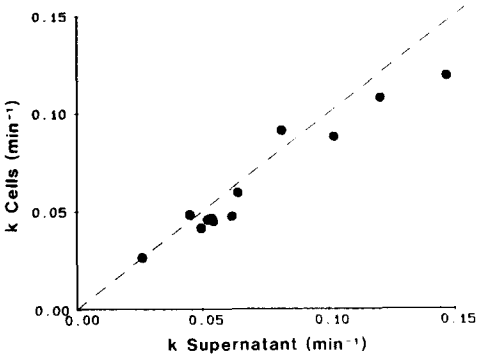

Fig. 2. Comparison of rate constants (k) for the efflux process obtained from the cell pellets and the supernatants. The values of k obtained from the intracellular melphalan concentration data (vertical axis) versus k obtained from the corresponding supernatant melphalan concentration data (horizontal axis) as a function of temperature from 30° to 47° are shown. The dashed line is the line of identity. The values for k were determined from the fitted curves shown in panels c and d of Fig. 1 (Equation 1) as described in Materials and Methods.

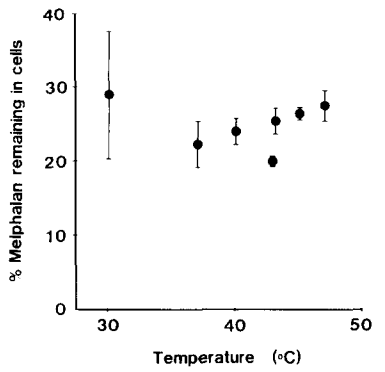

Fig. 4. Temperature dependence of the steady-state melphalan concentration. The fraction of melphalan ultimately remaining in the cells after efflux, $A/(A + B)$, was calculated from the fitted curves (Equation 1) for the data shown in Fig. 1c. The error bars are the 95% confidence intervals obtained for the parameters by Monte-Carlo simulation.

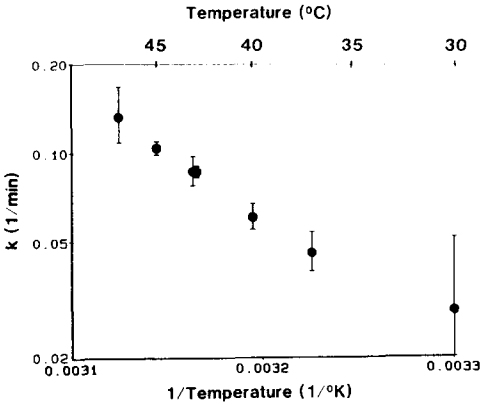

Fig. 3. Semi-logarithmic plot of k versus the inverse of the absolute temperature (Arrhenius plot). The values for k were determined from the fitted curves in panels c and d of Fig. 1 (Equation 1). The error bars are the 95% confidence intervals obtained for k by Monte-Carlo simulation as described in Materials and Methods.

The nature of the dependence of k with temperature is demonstrated in Fig. 3 in the form of an Arrhenius plot, which shows the logarithm of k versus the inverse of the absolute temperature. The Arrhenius plot shows a linear and decreasing trend at temperatures between 30° and 47°. The activation energy (E_a) was 1.046×10^3 J/mol.

Figure 4 shows how the fractional amount of melphalan ultimately remaining in the cells at steady state (that is, when net efflux had ceased to occur) varied with temperature. This fraction was evaluated from the fitted curve (Equation 1) as the ratio of the steady-state melphalan concencentration (A) to the total quantity of melphalan initially present in the cells ($A + B$). At 37%, the mean value for the percent melphalan remaining in the cells after efflux was $22.2 \pm 3.2\%$ (95% confidence interval). The differences between the individual temperatures were very small, suggesting that this value $[A/(A + B)]$ is unaffected by temperature from 30° to 47°, within the limits of the 95% confidence intervals. The ratio of the absolute concentration of melphalan outside the cell to that inside the cell at steady state had a mean value of $1.43 \times 10^{-5} \pm 0.27 \times 10^{-5}$ (SD).

Thin-layer chromatography studies were carried out to characterise the melphalan radioactivity in the supernatants obtained after preloading the cells during 15 min at 37° and after 15 and 30 min of efflux at 37°. Melphalan ($R_f = 0.75$) was clearly separated from the hydrolysed drug ($R_f = 0.05$) by the solvent system used. More than 70% of the radioactivity in the supernatants obtained after 30 min of efflux at 37° migrated as a single peak, corresponding to intact melphalan. Thus, during the period of time where efflux was occurring most rapidly and thus the melphalan concentration in the supernatant was near its maximum level, very little formation of hydrolysed melphalan was detectable.

DISCUSSION

The efflux of melphalan from CHO cells at 30° to 47° was well described by a single decaying exponential plus a constant (Equation 1) over its entire time-course, that is, until the intracellular drug concentration reached a plateau level. The corresponding appearance of melphalan in the supernatant outside the cells was described by a similar equation and the rate constants (k) for the efflux process obtained for the two complementary data sets were very similar. Furthermore, the final steady-state melphalan concentration inside the cells was more than four orders of magnitude greater than that outside the cells. This concentration difference is much greater than can be expected to be maintained by an active transport mechanism

into the cell [7], and is consistent with the idea that the drug ultimately remaining in the cells is firmly bound [8]. Thus, the intracellular concentration dynamics of melphalan during the initial decay phase must have been due entirely to efflux of the drug, with negligible influx occurring. These observations taken together support the hypothesis that efflux of melphalan from CHO cells occurs by passive diffusion. The value for the rate constant (k) obtained from the exponential fit (Equation 1) then relates directly to the diffusive rate constant. Other workers have reported that melphalan efflux occurs by a process different from the influx mechanism and have suggested that efflux occurs by simple diffusion or by a technically non-saturable carrier-mediated process [8]. The linear and decreasing nature of the Arrhenius plot is also consistent with the idea that melphalan efflux occurs by passive diffusion, since such a relationship is expected of a first-order process on thermodynamic grounds [11].

It was reported in a previous study of melphalan efflux from L5178Y lymphoblasts that efflux followed a first-order process for at least 5 min with a rate constant of 0.13 ± 0.05 (SD) min^{-1} at 37° [8]. A rate constant of 0.045 ± 0.002 (SD) min^{-1} was obtained in this study at 37° in CHO cells. Previous studies also reported that 20–25% of the initial melphalan radioactivity remained in the cells in the form of bound and hydrolysed drug after a plateau was reached after 30–60 min at 37°, while 60–80% of the drug lost from the cells was present in the extracellular medium as free intact melphalan [8, 9, 12]. Our finding that $22 \pm 3.2\%$ (95% confidence interval) of the initial melphalan radioactivity remained in the cells after the plateau was reached is in close agreement with these studies. Furthermore our results showed that the amount of melphalan remaining inside the cells after efflux was apparently unaffected by temperature. However, the time of efflux required to reach the plateau decreased with temperature from 4° to 47°.

Our previous work has shown that both melphalan uptake (5 min) and equilibrium intracellular drug concentrations (20 min) increased with temperature from 31° to 45° in CHO cells [4, 5]. We report here that the rate of efflux of melphalan (in the absence of appreciable melphalan uptake) from cells is temperature dependent over the range employed in clinical hyperthermia. Despite the increased rate of melphalan efflux with increasing temperature, thermal enhancement of melphalan cytotoxicity occurs at temperatures from 38° to 45° in CHO cells [4, 5]. The steady-state intracellular drug concentration resulting from the efflux process alone (that is, in the absence of drug uptake) was unaffected by temperature. The melphalan which remains firmly sequestered inside the cell could be responsible for lethal injury to critical target sites by alkylation. Thus, the thermal enhancement of cytotoxicity could be explained by an increased rate of reaction of the sequestered intracellular drug with these sites.

Acknowledgements—This work was supported by the National Cancer Institute of Canada (D.A.B.) and the Medical Research Council of Canada (MRC) (J.H.T.B.). J.H.T.B. is a scholar of the MRC. The authors would like to thank Ewa Przybytkowski (M.Sc.) for criticism of the manuscript and Bastien Courtemanche and Nathalie Bernier for technical assistance.

REFERENCES

1. Hahn GM, Potential for therapy of drugs and hyperthermia. *Cancer Res* **391**: 2264–2268, 1979.
2. Furner RL and Brown RK, L-Phenylalanine mustard (L-PAM): The first 25 years. *Cancer Treat Rep* **64**: 559–574, 1980.
3. Joiner MC, Steel AA and Stephens IC, Response of two mouse tumours to hyperthermia with CCNU or melphalan. *Br J Cancer* **45**: 17–26, 1982.
4. Bates DA, Henritzy LL, Loutfi A and Mackillop WJ, The effect of hyperthermia on the cytotoxicity of melphalan in Chinese hamster ovary cells. *Cancer Lett* **34**: 145–155, 1987.
5. Bates DA and Mackillop WJ, The effect of hyperthermia on the uptake and cytotoxicity of melphalan in Chinese hamster ovary cells. *Int J Radiat Oncol Biol Phys* **16**: 187–191, 1989.
6. Goldenberg GJ and Begleiter A, Membrane transport of alkylating agents. *Pharmacol Ther* **8**: 237–274, 1980.
7. Vistica DT, Cellular pharmacokinetics of the phenylalanine mustards. *Pharmacol Ther* **22**: 379–405, 1983.
8. Begleiter A, Grover J and Goldenberg GJ, Mechanism of efflux of melphalan from L5178Y lymphoblasts *in vitro*. *Cancer Res* **42**: 987–991, 1982.
9. Begleiter A, Grover J, Froese E and Goldenberg GJ, Membrane transport, sulfhydryl levels and DNA cross-linking in Chinese hamster ovary cell mutants sensitive and resistant to melphalan. *Biochem Pharmacol* **32**: 293–300, 1983.
10. Ling V and Thompson LH, Reduced permeability in CHO cells as a mechanism of resistance to colchicine. *J Cell Physiol* **83**: 103–116, 1974.
11. Bromberg JP, Some theoretical approaches to chemical kinetics. *Physical Chemistry*, pp. 824–826. Allyn & Bacon, Boston, 1980.
12. Redwood WR and Colvin M, Transport of melphalan by sensitive and resistant L1210 cells. *Cancer Res* **40**: 1144–1149, 1980.