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Original Paper

Influence of the Schedule of Exposure on the Cytotoxic Effect of Melphalan on Human 8226 and A2780 Cells

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Melphalan was investigated for antitumoral activity using two schedules of exposure (solid versus sequential exposure) in two human cancer cell lines (8226 and A2780). Sequential exposure of melphalan was more effective than solid exposure at the same total dose. The IC_{50} values averaged 8.2 (solid exposure) and 0.16 μg/ml (sequential exposure) for 8226 cells (myeloma), and 7.5 (solid) and 0.53 μg/ml (sequential) for A2780 cells (ovarian carcinoma). Intracellular melphalan accumulation, determined by high-performance liquid chromatography, showed that the area under the intracellular concentration of melphalan versus time curve (between 0 and 30 h) was significantly higher after sequential doses (9.4 μg/ml × h) than after solid dose (6.6 μg/ml × h). Moreover, intracellular/extracellular concentration ratios indicated that melphalan uptake followed a passive transport system. The increase of both duration of exposure (11 h after solid exposure versus 20 h after sequential doses) and intracellular concentrations 5–6 h after the beginning of the experiment (approximately 3 times higher after sequential doses) indicate sequential administration of melphalan could be more effective than solid exposure. © **1999 Elsevier Science Ltd. All rights reserved.**

Key words: melphalan, schedule of exposure, tumour cell culture, cytotoxic activity, intracellular concentrations
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INTRODUCTION

THE SELECTION of an optimal dose and schedule of administration for cancer chemotherapeutic agents is particularly important as their therapeutic index is usually low. Infusional schedules for administration represent a rational method of delivery of many antineoplastic agents [1, 2]. It is founded on the pharmacokinetic principles of phase-specificity, schedule-dependency, plasma half-life and stability in solution. The use of continuous infusion or frequent fractionated-dose delivery in clinical trials have been demonstrated to increase the antitumour activity of several drugs (for example, cytarabine in the treatment of acute leukaemia, bleomycin in cervical carcinoma, 5-fluorouracil in colon carcinoma, methotrexate in acute lymphocytic leukaemia), and to decrease or alter the toxicity of a number of cytotoxic agents (for example doxorubicin and cardiotoxicity, 5-fluorouracil and myelosuppression, cis-dichloro-diamino-platinum and renal toxicity, bleomycin and pulmonary toxicity) [1, 3–5].

Melphalan was introduced into clinical use in the late 1950s and has since been established as an agent with a wide spectrum of antitumour activity [6]. Melphalan is an alkylating agent of the bischloroethylamine type which exerts a cytotoxic effect through formation of interstrand or intrastrand DNA cross-links, or DNA–protein cross-links, via the two chloroethyl groups of the molecule. It is extensively used in the treatment of multiple myeloma, ovarian cancer, breast cancer, neuroblastoma and localised soft tissue sarcoma [7, 8].

Based on considerations of drug pharmacology and on issues related to our understanding of tumour cell growth patterns, melphalan seems to be a good candidate for intermittent and frequent bolus infusion or continuous infusion:

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melphalan has a short half-life; a small volume of distribution; its mechanism of action is not cycle specific and is time-dependent; and its uptake is a saturable process [9]. However, continuous infusion of melphalan has not been investigated in clinical use because this agent is not stable in solution. Stability is a crucial component to the concept of infusional chemotherapy and melphalan stability has been found to be limited to 1.5 h [10]. However, we have previously shown that melphalan in 3% sodium chloride is 4- to 8-fold more stable than in 0.9% sodium chloride. In these conditions, melphalan is stable during the time necessary to realise a continuous infusion [11].

As a first step in investigation of an eventual optimal schedule of melphalan administration in clinical practice, the aim of the present study was to determine the impact of solid versus sequential exposure of melphalan on the proliferation of two human cancer cell lines, and the intracellular kinetics of melphalan.

MATERIALS AND METHODS

Drugs and chemicals

Melphalan (L-PAM) (Glaxo-Welcome, Paris, France), tetrazolium dye (MTT) and phosphate buffered saline (PBS) (Sigma, St Quentin Fallavier, France), RPMI 1640 medium and fetal calf serum (Polylabo, Paris, France), L-glutamine and L-alanine (Merck, Darmstadt, Germany) were used in this study. All other reagents were of analytical grade and were obtained from Carlo Erba (Milan, Italy) or Prolabo (Paris, France).

Cell culture and culture conditions

The human myeloma 8226 cell line was obtained from the American Type Culture Collection (Rockville, Maryland, U.S.A.). The human ovarian adenocarcinoma A2780 was a kind gift from Dr Canal, Centre Claudius Régaud, Toulouse, France. Cells were maintained in culture flasks at 37°C in a humidified atmosphere containing 5% CO_2, in RPMI-1640 medium supplemented with 10% fetal calf serum, antibiotics and glutamine (complete medium) and used in the exponential phase of growth. The viability of the cells was assessed by their ability to exclude 0.5% trypan blue dye. Cell density in culture flasks was determined by a Coulter counter (Model Z1, Hialeah, Florida, U.S.A.).

MTT cytotoxic assay

In order to determine the effect of various schedules of exposure on the cytotoxicity of melphalan, preconfluent cell cultures were treated as follows. Cells derived from the solid tumour A2780 were detached with trypsin–EDTA (0.25–0.02%, w/v) in PBS, washed twice with PBS, resuspended in complete culture medium to obtain a suspension, counted and then seeded at a final density of 20×10^3 cells/well in 24-well plates in a final volume of 1000 µl. The cells were then allowed to attach for 24 h at 37°C. For the myeloma-derived cell line 8226, cells were resuspended in complete medium, counted and seeded at a density of 20×10^3 cells/well.

Melphalan was reconstituted in a water/ethanol/propyleneglycol mixture (35:5:60, v/v/v), diluted in culture medium and added to wells. For solid exposure, doses of melphalan were added only at the start of the study and the culture was continued for 96 h. For sequential exposure, 1/9 of the total dose was added at the beginning of the study and every 1.5 h during the subsequent 12 h and the culture was continued for 84 h. The total administered dose in the two arms of the study was the same.

Drug cytotoxicity was quantified using the MTT assay after 96 h [12, 13]. Metabolic reduction of the tetrazolium salt MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide] led to formation of MTT-formazan. MTT (20 µl of 5 mg/ml sterile PBS) was added to each well after 96 h and plates were incubated for 4 h at 37°C. The blue formazan crystals formed were dissolved in a mixture of isopropanol and hydrochloric acid 1 M (96-4; v/v). The plates were then gently agitated for 10 min and the absorbance measured at 570 nm on a microculture plate reader (Dynatech MR5000, France). The IC_{50} values were defined as the concentration of drug resulting in 50% survival of the treated cells as compared with controls and were calculated using a program implemented on EXCEL 5.0 software. Three different experiments were performed.

Cell counting and viability

Cell counting and viability of the cells were performed as follows: in 24-well microtitre plates, cells were seeded (10^6 8226 cells/well) and treated with melphalan according to the two schedules of exposure described above. For solid exposure, the dose was 200 µg for 10^6 cells; for sequential exposure, doses were fractionated as follows: 22.2 µg for 10^6 cells every 1.5 h during 12 h. Wells without melphalan were used to assess growing cells during the study. After different times of incubation (15 and 30 min, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 and 15 h), cell density was determined by a Coulter counter (Model Z1, Hialeah, Florida, U.S.A.) and their viability was assessed by their ability to exclude 0.5% trypan blue dye. Only viable cells were taken into account in our results.

Intracellular melphalan concentrations

Samples containing 10^6 8226 cells in 1 ml of complete medium were treated with melphalan according to the two schedules of exposure (as described above). For solid exposure, the dose was 40 µg for 10^6 cells; for sequential exposure, doses were fractionated as follows: 4.4 µg for 10^6 cells every 1.5 h during 12 h. Cells were then incubated.

At different times (T0, T5 min, T1, T3, T6, T9, T12, T13.5, T17 and T24 h), the samples were centrifuged at 1000*g* for 5 min and the pellets were washed twice with cold PBS. After a final centrifugation at 1000*g* for 5 min, the supernatant was removed and melphalan concentrations were determined in both cells and culture medium. An aliquot (20 µl) of the culture medium was directly injected on to the column. Cells were lysed using 2 ml of a chloroform-methanol mixture (4:1; v/v), after centrifugation, the organic phase was evaporated to dryness under a nitrogen stream. The residue was reconstituted in 200 µl of mobile phase and injected on to the column.

To elucidate the mechanism whereby melphalan penetrates into 8226 cells, the intracellular concentration of this drug was measured after 1 h cell exposure to different concentrations of melphalan (0.1, 0.25, 0.5, 1, 2.5, 5.0 and 10 µg/ml in RPMI medium containing 10^6 cells/ml), using the method described above. As it has been reported that melphalan could be actively transported into cells by the high-affinity L-amino acid transport system [9], we attempted to induce an uptake competition between two L-amino acids and melphalan. L-glutamine (0.5 µg/ml in RPMI), L-alanine

(0.5 μg/ml in RPMI), or the association of both compounds (0.25 μg of each in 1 ml RPMI) were added with melphalan (0.5 μg/ml in RPMI) in wells containing 10^6 cells. After 1 h exposure, intracellular concentrations of melphalan were determined.

Each determination was performed in replicate ($n = 4$).

Assay procedure

Intracellular melphalan concentration was determined by a high-performance liquid chromatography (HPLC) method. The chromatographic system consisted of a Shimadzu LC9-A (Tokyo, Japan) solvent pump, a 50 μl sample loop, a guard column, an Ultrasphere ODS column (5 μm, 150, 4.6 mm i.d.) and a Shimadzu RF 10AL fluorimetric detector [14]. The excitation and emission wavelengths were 260 and 360 nm, respectively. A Shimadzu C-R6A Chromatopac was used for recording and integrating chromatograms. The elution was performed using a mobile phase consisting of water, methanol, acetic acid (49.5:49.5:1; v/v) at a flow rate of 2 ml/min. Linear detection response was obtained for concentrations ranging from 0.1 to 1000 μg/ml in 1 ml of cell homogenate. The intraday and interday variations were lower than 15%. The limit of quantitation was 1 μg/ml. Results are expressed as amount of melphalan for 10^6 cells and the area under the curve was determined by the trapezoidal rule using the PK-fit software [15]. Two experiments were carried out in duplicate and performed at laboratory temperature.

The stability of melphalan in RPMI 1640 medium at 37°C in a humidified atmosphere containing 5% CO_2, was determined using the HPLC method described above. Stability is defined as the time during which a drug retains its integrity in terms of quantity and chemical identity. It can be affected by several environmental factors such as temperature and pH. RPMI samples spiked with 10 and 200 ng/ml of melphalan were prepared; the stability was assessed after 1, 3, 6, 9, 12, 24 and 30 h of storage. Rapid degradation occurred with an half-life value of approximately 3 h.

Statistical analysis

Results were expressed as mean values ± standard deviations (S.D.). To compare the results obtained using the two schedules of administration, a Student's *t*-test was used. A threshold of <0.05 was considered as significant.

RESULTS

Influence of schedule on melphalan cytotoxicity

MTT cytotoxic assay. For this cytotoxic assay, the duration of the experiment was 96 h and doses were variable. The sequential dose schedule was more effective than the solid exposure for both 8226 and A2780 cells ($P<0.001$) (Table 1).

Table 1. *Influence of the schedule of administration on melphalan cytotoxicity in various cancer cell lines (MTT cytotoxic assay)*

	IC_{50} (μg/ml) ± S.D. ($n = 3$)			
Cell line	Solid exposure	Sequential exposure	Ratio*	*P* values†
8226	8.2 ± 1.2	0.16 ± 0.03	51	<0.001
A2780	7.5 ± 0.9	0.53 ± 0.08	15	<0.001

*IC_{50} (solid exposure)/(sequential exposure). †Student's *t*-test. Data represent the mean values for three experiments carried out in duplicate; S.D., standard deviation.

Cell counting and viability. The influence of the schedule of exposure on melphalan cytotoxicity was determined on 8226 cells by cell counting. Viability was determined with the trypan blue dye test. For this cytotoxic assay, duration of the experiment was variable and melphalan doses were stable. The results are presented in Figure 1. After solid exposure, melphalan was more effective than sequential doses during the first 9 h of the experiment. Subsequently, the sequential doses induced an important enhancement of the cytotoxicity and a better result than the solid exposure.

Intracellular melphalan concentrations

The influence of the schedule on melphalan concentration in 8226 cells is presented in Figure 2. A few minutes after the solid exposure, melphalan concentrations averaged 2.1 μg/10^6 cells, then the intracellular concentration decreased with a mean half-life of 2.3 h. Ten hours after the beginning of exposure, concentrations were lower than the limit of detection of the method. After sequential exposure, intracellular concentrations of melphalan increased rapidly to reach a steady state (concentrations averaging 0.7 μg/10^6 cells). This concentration was maintained during the 12 h of sequential exposure, then decreased from 12 to 20 h with an elimination half-life of 2.2 h. The area under concentrations versus time curve was significantly higher after sequential exposure (9.4 μg × h/ml) than after solid exposure (6.6 μg × h/ml). The mean ratio (intracellular concentrations/concentrations in the supernatant) was 0.083 ± 0.017. When the concentrations of melphalan in the supernatant were plotted against melphalan intracellular concentrations (Figure 3) a statistically significant straight line could be fitted with a coefficient of correlation of 0.96 (6 df, $P<0.001$) and a slope of 2.62. These results indicate a passive uptake of melphalan into the cells.

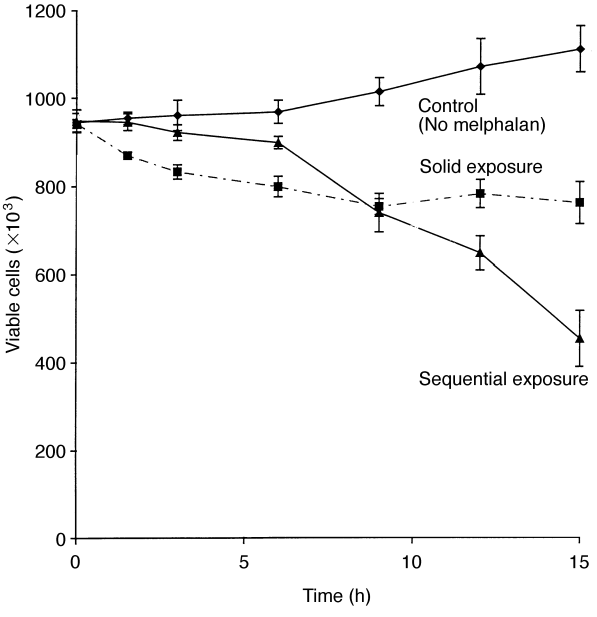

Figure 1. Survival of 8226 cells after the two shedules of melphalan exposure. Cell counting assay. (■) solid melphalan administration: 200 μg/ml at T0 in 1 ml of RPMI containing 10^6 cells; (▲) sequential melphalan administration, 22.22 μg/ml every 1.5 h during 12 h; (◆) cell control. Each point represents the mean value for three different experiments performed in triplicate.

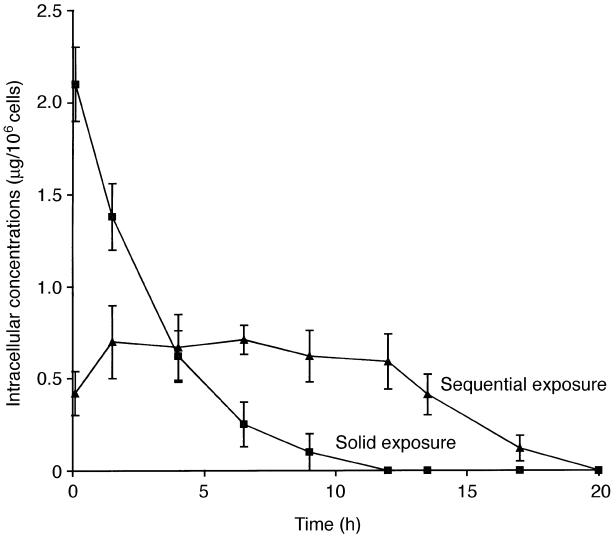

Figure 2. Intracellular melphalan accumulation in 8226 cells. (■) solid administration: 200 µg/ml at T0 in 1 ml of RPMI containing 10^6 cells; (▲) sequential administration: 22.22 µg/ml every 1.5 h during 12 h. Intracellular concentrations are expressed in ng/10^6 cells. Data represent the mean values for two experiments carried out in duplicate; error bars represent standard deviations (S.D.).

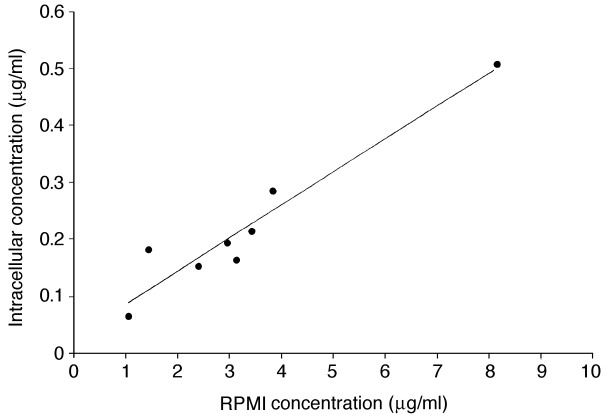

Figure 3. Relationship of intracellular (8226 cells) to extracellular concentrations of melphalan. Coefficient of correlation: 0.96 (6 df, $P<0.001$); slope: 2.62.

After 1 h of cell exposure to different concentrations of melphalan (0.1 to 10 µg/ml), no saturation process occurred, intracellular concentrations of the drug increasing in proportion to the initial concentration of melphalan added to the culture medium. When intracellular concentrations were plotted against initial doses, a statistically significant straight line could be fitted with a coefficient of correlation of 0.988 ($P<0.001$) without any plateau, indicating that no saturable transport system occurred for the entry of melphalan into cells.

Intracellular accumulation of melphalan, during exposure to this drug with or without L-amino acids (glutamine, alanine) was determined. The amino acids did not alter the intracellular concentrations of melphalan after 1 h, which shows that the penetration of melphalan into cells does not use the L-amino acids high affinity transport system (Table 2).

DISCUSSION

Alkylating agents are one of the most important classes of antitumour drugs [16]. Their antitumour effect when used either alone or in combination has been shown to vary according to the schedule of administration [17, 18]. Melphalan (L-phenylalanine mustard) is a bifunctional alkylating agent that is commonly administered orally or intravenously to treat a wide variety of malignancies, including ovarian cancer and multiple myeloma. This agent is a short-lived drug and displays a complex pharmacology [6]. Because melphalan is relatively unstable in aqueous solution, this drug is not currently used by continuous infusion [19].

Table 2. Melphalan uptake into 8226 *cell line with or without* L*-amino acids in* 1 *ml of RPMI medium containing* 10^6 *cells*

	Intracellular concentration (ng/ml) ± S.D.			
	Melphalan⋆	Melphalan + glutamine†	Melphalan + alanine‡	Melphalan + glutamine + alanine§
1 h	2.55 ± 0.23	2.32 ± 0.14	2.82 ± 0.32	2.75 ± 0.21
P values‖	NS	NS	NS	NS

S.D., standard deviation; NS, not significant; *n*, number of replicates. ⋆Melphalan (0.5 µg/ml), $n=3$. †Melphalan (0.5 µg/ml) + glutamine (0.5 µg/ml), $n=3$. ‡Melphalan (0.5 µg/ml) + alanine (0.5 µg/ml), $n=3$. §Melphalan (0.5 µg/ml) + glutamine (0.25 µg/ml) + alanine (0.25 µg/ml), $n=3$. ‖Student's *t*-test.

Bosanquet and associates [20] using *in vitro* chemosensitivity assays attempted to reproduce a system that mimics the action of this drug *in vivo*. This physicochemical study showed that, for melphalan, a continuous drug incubation should be optimal. These authors suggested that *in vitro* pharmacokinetic work should be undertaken to determine whether continuous exposure of melphalan would be preferable to the intravenous solid exposure commonly used. In a previous study [11], we optimised the stability conditions of melphalan. In 3% sodium chloride injection, this drug was stable for up to 6–12 h at room temperature, allowing a longer infusion time. Consequently, it was of interest to determine *in vitro* the optimal schedule of melphalan administration in order to increase the drug efficacy.

We used an *in vitro* model to compare the effects of the same dose of melphalan administered either as a solid or as sequential doses. The cytotoxic assay was performed using two different cell lines (8226 and A2780). The IC_{50} (bolus exposure)/(sequential exposure) ratio was more than three times higher for the 8226 than for the A2780 cell line. This result could be explained by a higher sensitivity of the human myeloma 8226 cell line to melphalan. Our data indicate that sequential exposure of melphalan was more effective than solid exposure using the same total dose. These results could be explained by: (1) an increase in the intracellular concentration of melphalan; (2) the duration of drug exposure; or (3) the mechanism of action of melphalan that is not cycle specific [21]. Therefore, intracellular concentrations of melphalan were determined using the 8226 myeloma cancer cell line. The area under the curve concentration of melphalan versus time was significantly higher after sequential doses than after solid exposure. According to Vistica and associates [9], this result could be explained by an active melphalan transport system, the drug being actively transported into cells by the high-affinity L-amino acid transport system, which also transports the amino acids glutamine and leucine.

A second transport system could contribute to melphalan uptake, particularly at low concentrations, which also carries alanine, serine, and cysteine. However, in the present study, alanine and glutamine did not modify melphalan uptake. Moreover, after 1 h of cell exposure to different concentrations of melphalan corresponding to the IC_{50} evaluated during the cytotoxic assay, no saturation process occurred, the intracellular concentrations being proportional to the initial concentration of the drug added to the culture medium. When intracellular concentrations of this drug were measured after different times of cell exposure (0–24 h), a linear relationship between intracellular concentrations and concentrations in the supernatant was also observed. These results suggest a passive uptake of melphalan rather than an active transport.

It has been reported that the extent of DNA cross-linking increases over 5–6 h following exposure to melphalan and that melphalan cytotoxic effects are related to its intracellular concentration and the duration of drug exposure [22, 23]. Thus, in the present study, the increase of both cell exposure (11 h after solid exposition versus 20 h after sequential doses) and intracellular concentrations 5–6 h after the beginning of the experiment (approximately 3-times higher after sequential doses) could explain the advantage of sequential doses. Moreover, the short elimination half-life (45 min), the small volume of distribution (20 l/m^2) and the low protein binding of melphalan are also in favour of this mode of exposure. Our results do not contradict those obtained by Teicher and associates [24], indicating that *in vitro* continuous administration of melphalan could be at least as effective as solid exposure of the same dose on drug toxicity on MCF7 breast cancer cells.

In conclusion, this *in vitro* study indicate that sequential exposure to melphalan may be superior to solid exposure.

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