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Degradation of melphalan in vitro: rationale for the use of continuous exposure in chemosensitivity assays*

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Summary. The hydrolysis of melphalan in cell culture medium at 37° C has been studied. Degradation of melphalan proceeded via monohydroxy-melphalan (MOH) to dihydroxymelphalan [$M(OH)_2$] with a half-life of 66 min for melphalan and 58 min for MOH. The half-life for melphalan was similar to the terminal half-life of the drug in vivo. The effect of the two metabolites, MOH and $M(OH)_2$, on the chemosensitivity of K562 leukaemia cells during continuous exposure to melphalan was also examined. $M(OH)_2$ had no potentiating effect on melphalan cytotoxicity at concentrations up to 100 μg/ml. MOH also had little effect on cell kill at concentrations higher than those commonly achieved during in vitro chemosensitivity assays. The LD_{50} for 1 h exposure to melphalan was twice that for continuous exposure: this also suggests no interference by MOH and $M(OH)_2$. These data suggest that continuous exposure of melphalan in in vitro chemosensitivity assays is probably preferable to the arbitrary 1 h drug exposure time commonly employed.

Introduction

The optimum exposure for a drug when tested in an in vitro chemosensitivity assay should be that which best reflects the pharmacokinetic properties and the concentration of the drug in the in vivo situation. Varied drug exposure times have therefore been used in experiments using established cell lines according to the stability, schedule dependency and other properties of the drug under investigation [13, 15, 16]. However, in work using human primary tumour samples for the prediction of clinical response, where a large number of drugs must be tested simulataneously, such varied exposure times are not feasible [3]. Workers in this area have, therefore, tended to use either 1 h or continuous drug exposure.

Melphalan is one of the drugs which is often incubated with cells for 1 h, as it is unstable and schedule-independent (i.e. not phase-specific) [4]. However, we recently found that the half-life of the drug at 37° C in cell culture medium [6] was very similar to its terminal half-life in vivo in man [1, 2, 7, 9, 19]. It may be better, therefore, to incubate melphalan continuously in in vitro chemosensitivity assays, but this depends on whether the degradation products of the drug interfere with the cell kill caused by the parent compound.

This work describes experiments to further investigate the degradation of melphalan in medium and determine whether the two known degradation products MOH[1] and $M(OH)_2$ [17] interfere with the cytotoxicity of melphalan in the differential staining cytotoxicity (DiSC) assay [5] and a colony-forming assay.

Materials and methods

K562 human leukaemia cells were maintained in liquid culture with RPMI-FBS as described previously [5].

For the DiSC assay, asynchronous cells were suspended in fresh RPMI-FBS at a concentration of 10^4 cells/ml and incubated for 4 days with the drug conditions indicated in "Results". At the end of this time, the DiSC assay was performed using procedures described previously [5].

For the colony-forming assay, cells were plated at 10^3 cells/ml in an upper agar layer of 0.3% agar in RPMI-FBS over a base layer of 0.5% agar in the same medium in a 35-mm plastic petri dish. Colonies containing 40 or more cells were counted at 10–14 days. Cloning efficiency averaged 24%.

Drug exposure was either continuous (for the colony-forming assay, the drug was incorporated into the upper agar layer at twice the required final concentration) or 1 h. In this latter case, cells were incubated with the drug at 37° C and washed twice with fresh medium, thus reducing the final drug concentration to a calculated 0.16% of the initial value.

Melphalan degradation in previous experiments had been measured using an HPLC system already described [8], but the mobile phase could not quantify the MOH and $M(OH)_2$ peaks. A new mobile phase was therefore devel-

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Abbreviations used: DiSC assay, Differential Staining Cytotoxicity assay [5]; MOH, monohydroxymelphalan; $M(OH)_2$, dihydroxymelphalan; AUC, area under the concentration-versus-time curve; MOH-mix, mixture of melphalan, MOH and $M(OH)_2$ enriched in MOH; $M+M(OH)_2$, mixture of melphalan and $M(OH)_2$; SDS, sodium dodecyl sulphate; HPLC, high-performance liquid chromatography; NS, 150 m*M* sodium chloride solution; RPMI-FBS, RPMI 1640 medium containing 10% fetal bovine serum, fungizone, gentamicin and sodium bicarbonate [5]

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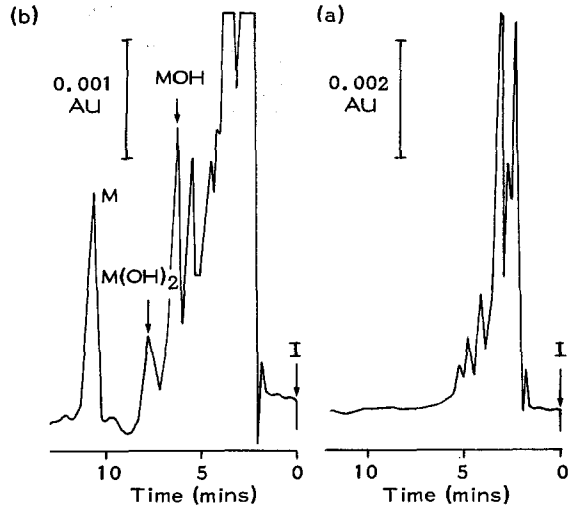

Fig. 1. HPLC traces showing (a) an extract from medium and (b) an extract from medium containing 20 μg/ml melphalan after incubation for 180 min at 37° C. *I*, point of injection; *M*, melphalan; *MOH*, monohydroxymelphalan; *M(OH)$_2$*, dihydroxymelphalan. The chromatograms run from right to left

oped for this work, consisting of 10 m*M* SDS and 11 m*M* sulphuric acid in 67% methanol water (SDS:conc. sulphuric acid:methanol:water 6.49:2.5:1500:750, w/w/v/v). This separated the melphalan, MOH and $M(OH)_2$ peaks from those due to the medium (Fig. 1).

Melphalan (Alkeran, Wellcome Laboratories, Beckenham, Kent) was dissolved in the solvent supplied, and then diluted with NS to 1 mg/ml. This solution was then added to RPMI-FBS to give a final solution containing 20 μg/ml melphalan and was kept at 37° C. At intervals samples were taken, the protein precipitated by the addition of 2 parts of methanol and microcentrifugation, and the resulting supernatant analysed by HPLC.

MOH-mix 1 was made for the DiSC assays by heating 1 mg/ml melphalan in NS for 6 h at 49° C. MOH-mix 2 was subsequently made for the colony-forming assay experiments by heating the same solution for 85 min at 59° C to produce a mix that contained a higher melphalan concentration, so that allowance could be made for diffusion of the drug into the feeder layer. $M(OH)_2$ (1 mg/ml) was made by heating a melphalan solution overnight [10]. $M+M(OH)_2$ was then made by adding appropriate amounts of melphalan and $M(OH)_2$ together to give the same concentrations of melphalan and $M(OH)_2$ as were found in the MOH-mix. Different concentrations of MOH-mix and $M+M(OH)_2$ were made by diluting with NS so that the proportions of melphalan, MOH and $M(OH)_2$ remained constant.

For curve fitting and calculating AUCs it was assumed that melphalan degraded directly to MOH and then to $M(OH)_2$ [8]. Peak height versus time results of the HPLC assay of melphalan to MOH and $M(OH)_2$ were fitted simultaneously by the non-linear regression computer program NONLIN [14] using the apparent first-order rate constants k_1 and k_2. The equations used were:

$$[\text{melphalan}] = Ae^{-k_1(t+t_0)} \quad (1)$$

$$[\text{MOH}] = \frac{k_1 AR_1 \{e^{-k_1(t+t_0)} - e^{-k_2(t+t_0)}\}}{(k_2 - k_1)} \quad (2)$$

$$[M(OH)_2] = AR_2 \{1 + \frac{k_2 e^{-k_2(t+t_0)}}{(k_1 - k_2)} - \frac{k_1 e^{-k_2(t+t_0)}}{(k_1 - k_2)}\} \quad (3)$$

where the concentration of a species is denoted by [ ], t_o is the apparent time that the solution of melphalan had decayed before the experiment started (this allows for the observation we made that melphalan powder contains approximately 7% MOH). *A* is the projected melphalan concentration at time $t = 0 - t_0$ and R_1 and R_2 are the proportional detector responses for equal quantities of MOH and $M(OH)_2$ respectively, taking the detector response of melphalan as 1. In practice, peak heights of melphalan, MOH and $M(OH)_2$ at time t (e.g., all the data points in Fig. 2) were fed into the computer which calculated *A*, t_0, k_1, k_2, R_1 and R_2. Actual concentrations of MOH and $M(OH)_2$ were then calculated by multiplying the peak heights by the detector response to melphalan and either R_1 or R_2.

AUCs from $t = 0$ to ∞ were calculated, using the parameters calculated above averaged over four experiments, using the following equations:

$$\text{AUC melphalan} = \frac{M_0}{k_1} \left(\text{or } \frac{M_0(1 - e^{-k_1 t})}{k_1} \text{ for } t = 0 \text{ to } t\right) \quad (4)$$

$$\text{AUC MOH} = \frac{Ak_1}{k_2 k_1} \left\{\frac{e^{-k_1 t_0}}{k_1} - \frac{e^{-k_2 t_0}}{k_2}\right\} \quad (5)$$

where M_0 is the concentrations of melphalan at $t = 0$. The MOH AUC in MOH-mix was calculated as a proportion due to the melphalan using Eq. 5, plus a proportion due to the initial MOH present (MOH_0) using Eq. 6:

$$\text{AUC MOH} = \frac{MOH_0}{k_2} \quad (6)$$

Results

Figure 1 shows the elution pattern of melphalan and its hydrolysis products using the mobile phase that we developed to separate MOH and $M(OH)_2$ from the peaks due to

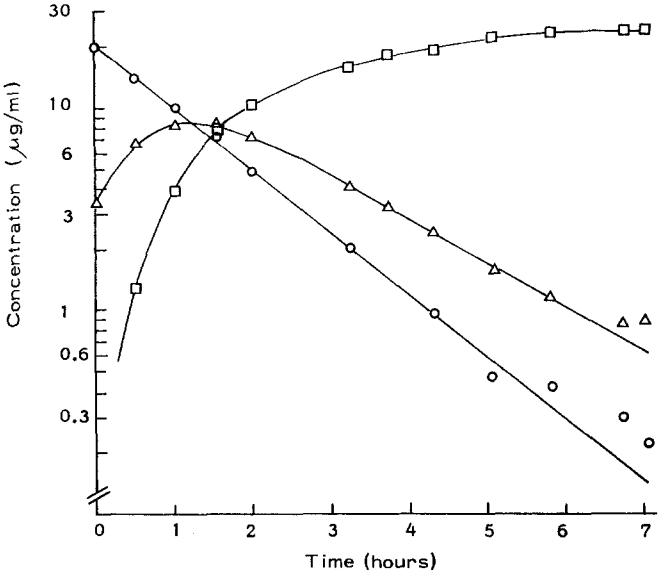

Fig. 2. Degradation of 20 μg/ml melphalan in RPMI 1640 medium at 37° C. Computer-calculated lines of best fit are drawn. ○, Melphalan; △, MOH; □, $M(OH)_2$

Table 1. Kinetics of melphalan and MOH hydrolysis in medium

Melphalan (20 μg/ml) was incubated at 37°C. Parameters (mean ± SD) were calculated from the computer-fitted curves of 4 experiments, of which Fig. 2 is representative

Parameter	Melphalan	MOH
k^a (min^{-1})	0.0108 ± 0.0012	0.0120 ± 0.0009
half-life (min)	66.1 ± 7.8	58.1 ± 4.6
AUC (min · μg/ml)[b]	1877 ± 225	1940 ± 155

[a] k_1 for melphalan, k_2 for MOH
[b] From t = 0 to ∞ calculated using Eqs. 4 and 5

components in the media. In Fig. 2, the results of one of four studies of melphalan degradation in medium are presented. The curves are those calculated by computer, and fitted Eqs. 1–3 very well, with r^2 values always ≥0.989. The calculated terminal half-lives and AUCs for melphalan and MOH were found to be similar (Table 1). After incubation of melphalan overnight, $M(OH)_2$ free of both melphalan and MOH was obtained.

Figure 3a shows the effect on K562 cells in the DiSC assay of continuous incubation of pure $M(OH)_2$ compared to that of melphalan. Whereas melphalan showed an LD_{50} of 0.37 μg/ml, $M(OH)_2$ had no effect on K562 cells even at concentrations up to 100 μg/ml. Similarly in the colony-forming assay, $M(OH)_2$ at 50 μg/ml had no cytotoxic effect (cell survival was 109.6% of the control value; not illustrated).

The decay of melphalan in NS produced similar curves to those in Fig. 2 except that the half-life of MOH was calculated to be almost twice that of melphalan. This gave higher concentrations of MOH and so was a useful origin for solutions containing high concentrations of MOH for the cell survival experiments. (Pure MOH was not available due to the lack of isolation or synthesis facilities.) Proportions of melphalan:MOH:$M(OH)_2$ in the MOH-mix 1 (used in the DiSC assay) were approximately 1:20:70, and in the control M + $M(OH)_2$ 1:0.5:70. Proportions of melphalan:MOH:$M(OH)_2$ in the MOH-mix 2 (used in the colony-forming assay experiments) were approximately 1:6.2:7.5 and in the control M + $M(OH)_2$ 1:0.1:7.5.

The cell-killing effects of MOH-mix (containing a high concentration of MOH) and M + $M(OH)_2$ (low MOH) were then compared in both assays (Fig. 3b). Figure 3b also includes the results from Fig. 3a, and from this it can be seen that the addition of a 70-fold excess of $M(OH)_2$ did not cause any change in the LD_{50} for melphalan in the

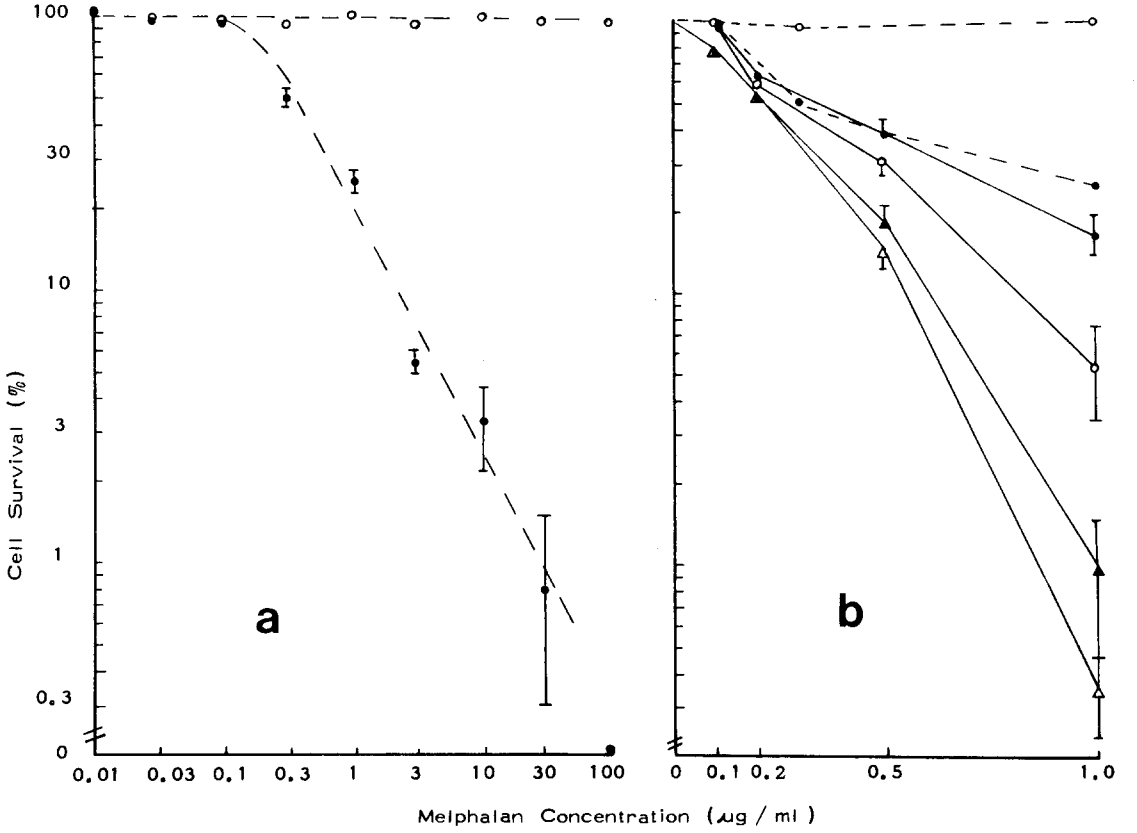

Fig 3. a Effect of continuous incubation with melphalan (●) and $M(OH)_2$ (○) on the survival of K562 cells in the DiSC assay. **b** Effect of continuous incubation with M + $M(OH)_2$ (●, ▲), and MOH-mix (○, △) on the survival of K562 cells. ○, ●, DiSC assay, MOH-mix 1; △, ▲, colony-forming assay, MOH-mix 2; – – – –, recapitulation of relevant information from **a**. Melphalan:MOH:$M(OH)_2$ ratios were: ○, 1:0.5:70; ●, 1:20:70; △, 1:0.1:7.5; ▲, 1:6.2:7.5. Addition of a 70-fold excess of $M(OH)_2$ did not cause any change in the LD_{50} for melphalan in the DiSC assay (● ——— ● vs ● ——— ●). In the colony-forming assay, MOH at 6× the melphalan concentration gave a small increase in cell kill (△ ——— △ vs ▲ ——— ▲); in the DiSC assay, a greater increase in cell kill due to MOH was seen at the higher concentrations tested, due to the use of MOH at 20× the melphalan concentration (○ ——— ○ vs ● ——— ●). Results are means ± SD of three experiments (SDs around 100% were too small to plot)

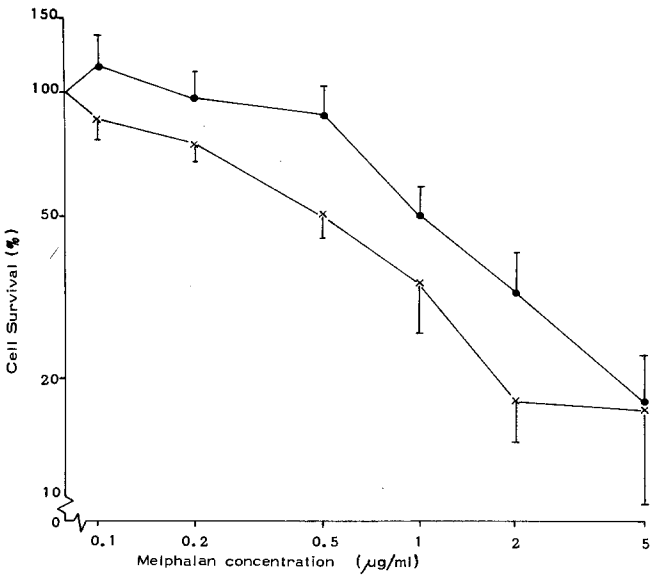

Fig. 4. Comparison of 1 h (●) with continuous (×) exposure of melphalan on the survival of K562 cells in the DiSC assay. Results are means ± SD of three experiments

DiSC assay. In the colony forming assay, MOH at 6× the melphalan concentration gave a small increase in cell kill; in the DiSC assay, a greater increase in cell kill due to MOH was seen at the higher concentrations tested, due to the use of MOH at 20× the melphalan concentration.

With a half-life of 66 min in RPMI-FBS, the melphalan AUC for continuous incubation would be approximately double that of a 1 h incubation of the drug (calculated from Eq. 4). Thus it would be expected that the LD_{50} of continuously exposured melphalan should be approximately half that of a 1 h exposure. A final experiment was therefore performed with the DiSC assay to test this and the results are presented in Fig. 4, from which an LD_{50} of 1.02 μg/ml was calculated for 1 h exposure and 0.50 μg/ml for continuous incubation.

Discussion

Melphalan, as a schedule-independent drug [4] has been incubated in cytotoxicity assays for 1 h by some authors and continuously by others [3, 5, 12, 18]. A wide variation in drug concentrations have been used ranging from 0.01 [3] to 12 μg/ml [18].

In this work we have been able to confirm our earlier results [6] that the half-life of melphalan in medium is similar to the terminal half-life of the drug in vivo, with values of 66.1 ± 7.8 min in medium compared with average values of 64–83 min in vivo after i.v. administration [1, 7, 9, 19]. This suggests that allowing melphalan to remain in contact with cells during a chemosensitivity assay may reflect the situation in vivo better than a short-term exposure.

The results of the degradation product studies (Fig. 3) show that a concentration of ≤ 50 μg/ml $M(OH)_2$ had no effect on K562 cells. The cytotoxicity of melphalan ($M + M(OH)_2$) was not greatly increased by a concentration of up to 10 μg/ml MOH in the DiSC assay (0.5 μg/ml melphalan point, Fig. 3b, melphalan:MOH ratio 1:20) or 6 μg/ml in the colony-forming assay (1.0 μg/ml point, melphalan:MOH ratio 1:6.2). MOH at this level of 6.0 μg/ml would produce an AUC for MOH of 530 min · μg/ml (calculated using Eq. 4) which could only be produced in a chemosensitivity assay by adding melphalan at an initial concentration of 5.4 μg/ml continuously (calculated using Eq. 5), or 11.4 μg/ml for 1 h. These values would exceed the peak plasma concentration in vivo after i.v. administration of melphalan [3], and also be greater than all but the highest melphalan concentration used in vitro. We therefore conclude that the hydrolysis products of melphalan will have a negligible effect on in vitro chemosensitivity assays. These findings confirm the preliminary observations of Goodman et al. [11] who suggested that, compared to melphalan, 20 times the concentration of MOH was required to kill the same proportion of cells and that $M(OH)_2$ was inactive.

The results presented in Fig. 4 confirm the suggestion calculated from Fig. 2 that the LD_{50} of a 1 h incubation ought to be approximately twice that of a continuous incubation. If MOH or $M(OH)_2$ had been significantly toxic, then we could have expected the difference in LD_{50} to be larger, as these degradation products would have been present for a long time with continuous drug exposure.

One aim for those working with in vitro chemosensitivity assays should be to produce a system that as far as possible mimics the action of the drugs in vivo. This study shows that for melphalan a continuous drug incubation should be optimal in this respect. For some drugs it could be argued that continuous incubation would reveal cytostatic rather than cytotoxic activity. In the case of melphalan, exposure is only short (a few hours) due to degradation, even with "continuous exposure" and so this is unlikely to be of concern. We further suggest that additional in vitro pharmacokinetic work should be undertaken to determine whether continuous exposure reflects the in vivo situation better for other non-schedule dependent drugs.

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