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COMPARATIVE STUDIES OF TOTAL CROSS-LINKING, CELL SURVIVAL AND CELL CYCLE PERTURBATIONS IN CHINESE HAMSTER CELLS TREATED WITH ALKYLATING AGENTS *IN VITRO*

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Abstract—The toxicities of 4-hydroperoxycyclophosphamide (4-OOH CY), phosphoramide mustard (PM), melphalan (MEL) and busulphan (BU) have been compared in Chinese hamster cells, V-79-753B. The initial total amount of cross-linking was a determining factor for the clonogenic survival of cells treated with MEL or PM. Although 4-OOH CY generated cross-links in this cell line, this damage did not account for the toxicity of the compound. There was no evidence for cross-link formation in cells treated with BU, even at a dose of the drug (1000 μg/ml) that was too toxic to measure clonogenic survival. Comparison for the four compounds at equitoxic doses showed that both PM and MEL caused the arrest of the cell cycle at G_2 which persisted after drug removal. This was accompanied by a decline in the population growth rate and a decrease in total cell count. In contrast, both BU and 4-OOH CY caused a temporary arrest of the cell cycle G_2, 24 hr after drug removal. However, the cell cycle distribution returned the control values within 3–4 days after treatment. Both BU and 4-OOH CY showed little effect on the initial growth rate of the cells.

It is concluded that the initial amount of cross-links contributes to the toxicity of PM and MEL. However, it is unlikely that the generation of cross-links is of major importance for the toxicity of either 4-OOH CY or BU.

Melphalan (MEL) and cyclophosphamide (CY) are major constituents of combination chemotherapy for solid neoplasms [1–3], lymphoproliferative disorders and leukaemias [4, 5]. Whilst MEL does not require metabolic activation, CY is activated in the liver to 4-hydroxycyclophosphamide (4-OH CY) which accounts for the bulk of the cytotoxicity of the drug [6] and is subsequently catabolized to phosphoramide mustard (PM), the ultimate alkylating species [7]. Bifunctional alkylating agents such as MEL and PM can cause cross-links in cellular DNA which may be repaired once the drugs are removed [8]. It has been suggested that persistence of DNA–DNA interstrand cross-links with time is an important factor for determining cell death following treatment of cells with PM [9] and MEL [10] and that such damage is directly related to perturbations in the cell cycle leading to the arrest of cells at G_2 [11] and a decrease in cell survival in a dose-dependent relationship [12].

In contrast, there is only limited evidence that busulphan (BU), an agent used at a single intermittent high dose to patients with chronic granulocyte leukaemia [13, 14] exerts its toxicity by the formation of DNA–DNA interstrand cross-links. Bedford and Fox (1983) [15] claimed that the differential toxicity of BU in the Yoshida sensitive and resistant sarcoma was due to differences in the repair of DNA–DNA cross-links in the two cell lines. However, Ball and Roberts did not find any differences between sensitive and resistant Yoshida cells when they measured a number of parameters related to the repair of DNA damage [16].

In assessing the potential role of DNA–DNA, and DNA–protein cross-links as contributory factors which may influence cell killing by alkylating agents, the most appropriate comparison must be the ability of single clonogenic cells to proliferate and form viable foci after the insult, leading to the repopulation of the cell culture or tumour. In this respect, Chinese hamster cells provide a useful mechanistic tool since the plating efficiency is almost 100%. Thus each cell can be regarded as a stem cell, capable of infinite division. Concomitant examination of cell-cycle perturbations and cell growth curves provides additional information concerning the ability of the cell population as a whole to overcome a chemical insult in conditions where the absolute level of clonogenic survival is measurable.

This study was designed to compare the toxicity of BU, MEL, PM and 4-hydroperoxycyclophosphamide (4-OOHCY), a stable active derivative of cyclophosphamide, in relation to total DNA–DNA, DNA–protein interstrand cross-links, clonogenic survival, cell cycle perturbations and cell growth.

Whilst the authors appreciate that the alkaline elution technique used herein enables DNA cross-links to be measured at pharmacological doses of alkylating agents it should be emphasized that such measurements take no account of specific molecular interactions. Since DNA damaging agents are likely

to produce multiple lesions the contribution of each type of lesion towards cell killing cannot be assessed by these measurements.

MATERIALS AND METHODS

Cell growth and labelling

Asynchronous stock cultures of Chinese hamster cells, V-79-753B, were propagated as monolayers in Earle's based Minimal Essential Medium (MEM) containing 15% foetal calf serum, 20 mM HEPES buffer and 100 IU/ml penicillin and 100 μg/ml streptomycin. The routine handling of these cells has been reported elsewhere [17]. Cultures to be used for experiments were seeded in 175 cm^2 or 25 cm^2 plastic flasks (Nunc, Denmark) at concentrations of 10^6 cells/175 cm^2 flask and 2×10^5 cells/25 cm^2 flask 2 days before the start of each experiment and the cells grew with a doubling time of 9.5 hr. DNA was labelled by a 20 hr incubation with [2-^{14}C]thymidine (52.0 mCi/mmol; 0.02 μCi/ml) or with [6-^{3}H]thymadine (26.5 Ci/mmol; 0.2 μCi/ml; Amersham International, U.K.), diluted in the growth medium. At the end of the labelling period the growth medium was replaced with fresh growth medium containing unlabelled thymidine for 1 hr prior to the start of the experiment to ensure that all the labelled thymidine was incorporated into mature DNA. All cultures were in log phase at the time of exposure to the compounds.

Drug treatment

PM and 4-OOH CY were gifts from Boehringer Ingelheim Hospital Division (U.K. and Eire). MEL (Alkeran*) was obtained from Wellcome Laboratories (U.K.) who also generously donated the BU (Myleran*).

For experiments using the alkaline elution technique monolayers of cells pretreated with [2-^{14}C]-thymidine were exposed to each of the drugs for 1 hr at 37°. MEL was dissolved in 2% acid [5NHCl]-ethanol and diluted at least 1:1000 in Dulbecco's phosphate-buffered saline 'A' (PBSA) prior to administration. BU was dissolved in DMSO and diluted at least 1:100 in PBSA. PM and 4-OOH CY were dissolved directly into PBSA. All drug solutions were made fresh before each experiment. After drug treatment the drugs were removed from the cells and the monolayers washed with PBSA. For experiments in which the drug(s) effects were followed with time, fresh growth medium was added and the cultures incubated at 37° for the times indicated in the text. At the end of the incubation time, monolayers were trypsinized, counted and resuspended in PBSA (0°) at a final concentration of 2×10^5 cells/ml and held on ice.

Cells that had been labelled with [6-^{3}H]thymidine were trypsinized as above and resuspended in PBSA (0°) at a final concentration of 8×10^5 cells/ml. Cells labelled with [6-^{3}H]thymidine were irradiated at 0° with a dose of 3.5 Gy at a dose rate of ~4 Gy min^{-1} using a Cobalt-60 source. Cells labelled with [2-^{12}C]-thymidine were irradiated with 5Gy.

Alkaline elution

This was essentially the method of Murray and Meynes [18] which was based on the methods of Kohn (1979) [19]. The major difference from Kohn's method was that the filters were polycarbonate (Nucleopore, 2 μm pore size, 25 mm dia.) instead of PVC. Our preliminary experiments showed that the inclusion of a proteinase K digestion [19] before addition of the EDTA solution (see below) made little difference to the elution of DNA from cells treated with melphalan. This could indicate that melphalan did not cause DNA–protein cross-links. However, this would be in marked contrast to other reports, albeit using different cell lines [9, 11]. Alternatively, polycarbonate filters may not retain DNA cross-linked to protein, in which case our data reflects predominantly DNA-interstrand cross-links. In our hands PVC filters gave very variable results.

In brief, the method was as follows. Polycarbonate membranes were held in modified "Swinnex" (Millipore) holders. For each filter, 5×10^5 ^{3}H-labelled standard cells were mixed with 2×10^5 ^{14}C-labelled experimental cells in 8 ml of PBS (0°). The cells were collected on a pre-cooled membrane, and immediately lysed with 5 ml of lysing solution (2 M NaCl, 0.02 M EDTA, 0.2% Sarkosyl, pH 10.0) which was allowed to drain through by gravity. Then 2 ml of EDTA solution (0.02 M, pH 10.0) was added and allowed to drain through before addition of the elution buffer (0.02 M EDTA free acid + tetrapropylammonium hydroxide; pH 12.2). The flow rate was 1.5 ml/hr (via a Watson Marlow 10-channel pump) and 16 1-hr fractions were collected for each filter. All manipulations were carried out in subdued light, and the elution was continued in the dark. All of each fraction was counted in 12 ml of scintillation fluit (ACS, Amersham, containing 0.7% acetic acid).

Typical elution profiles from a single experiment are shown in Fig. 1. The data show that a greater percentage of the DNA from unirradiated cells exposed to melphalan is retained on filters than that from control cells. Since retention is dependent on the molecular weight of the DNA this indicates that the DNA in melphalan-treated cells has a higher

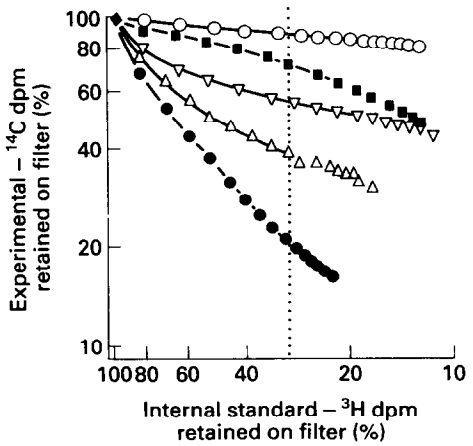

Fig. 1. Examples of typical elution curves; all from the same experiment. ^{3}H-TdR-labelled internal standard cells are received 3.5 Gray. ^{14}C-TdR labelled cells received ■, no drug; ○, 5 μg/ml melphalan; ●, no drug + 5 Gray; △, 2 μg/ml melphalan + 5 Gray; ▽, 5 μg/ml melphalan + 5 Gray.

molecular weight than that in control cells, due to the production of cross-links. The sensitivity of the technique is improved by inducing breaks in the DNA with radiation (5.0 Gy). This enables small changes in molecular weight to be measured more accurately and the response of cells to different concentrations of the drug to be assessed. Cells labelled with [^{3}H]thymidine and irradiated with 3.5 Gy provide an internal standard which enables comparison of elution profiles.

Cell survival studies

Cells were trypsinized, counted and plated onto 61 mm diameter glass Petri dishes in 2 ml of growth medium. After attachment, the medium was aspirated from the dishes and replaced with each of the four compounds in PBSA, at the concentrations indicated in the text. Cultures were incubated at 37° for 1 hr. At this time the drugs were removed from the dishes, the cells washed once with PBSA and the cultures replenished with 2 ml of fresh growth medium. All cultures were incubated for 6 days in an atmosphere of 5% CO_2 95% air at 37° to allow colony formation. At this time the colonies were fixed with ethanol, stained with methylene blue and counted. Cultures which had been sham-treated with PBSA were used as controls. Each survival curve was carried out at least three times. The bars on the data indicate the range of survival for all experiments.

Growth curves

Cultures were seeded in duplicate into 25 cm^2 plastic flasks at the concentrations indicated in the text. After attachment the medium was aspirated from the flasks and the cells treated with the compounds, dissolved in PBSA for 1 hr at 37°. At this time the cells were washed once with PBSA and fresh growth medium attached to each flask. Samples were trypsinized, at the times indicated, counted and prepared for cell cycle analysis.

Flow cytometry

Suspensions of single cells were centrifuged and the pellet resuspended in 0.1% tri-sodium citrate, 0.1% Triton X100, 0.05% ethidium bromide, 0.1% ribonuclease A to give a concentration of about 10^6 cells/ml. The DNA content of the cells was analysed on an Ortho Cytofluorograph 50H using 50 mw light at 488 nm from an argon ion laser and long pass filter with a cut off at 630 nm in front of the fluorescence detector.

The data was accumulated and analysed on the Ortho 2150 computer. The peak of the fluorescent signal was displayed against its area and the resulting cytogram was gated to display a histogram of the area of the signal generated by single cells only—'doublets' and cell clumps were excluded. The photomultiplier gain was adjusted so that the peak of the signal from control cells in G1 of the cell cycle was recorded in channel 300. This enabled a comparison to be made between histograms recorded on different days.

The histograms were stored on disc and were later displayed in a stack as seen in Figs. 7 and 8.

Cell cycle analysis was performed using a pragmatic approach which gives a rapid analysis on the computer (Ormerod, Payne and Watson, in preparation).

RESULTS

The data in Fig. 2 show survival curves for Chinese hamster cells treated with each of the four compounds. With the exception of BU, each of the drugs produced exponential cell killing. PM was approximately 20 times less toxic than 4-OOH CY, which exhibited similar toxicity towards Chinese hamster cells as MEL. Interestingly, there was a shoulder to the survival curve of cells exposed to BU [20].

Examination of the initial amount of cross-linkage generated by each of the four compounds showed that there was an increase in this parameter when cells were treated with MEL or PM (Fig. 3) which correlated with a decrease in cell survival (Fig. 4). However, in cultures treated with 4-OOH CY, despite the substantial increase in total cross-links at very high drug doses (Fig. 3) for any given degree of cell killing, the degree of cross-linking was always markedly less than for PM or MEL (Fig. 4). When cells were exposed to BU there was no detectable cross-linking (Fig. 3), even at a concentration of 1000 μg/ml which reduced cell survival to a level that was not measurable using this assay (i.e. <0.001%).

In view of the short doubling time of this cell line (9.5 hr) studies on the time course of repair of cross-links were limited in most experiments to 8 hr after drug removal. Cultures were treated with doses of PM, 4-OOH CY or MEL that produced similar initial amounts of cross-linkage. In the case of BU, 1000 μg/ml was chosen to determine whether detectable cross-link formation might be measurable only at

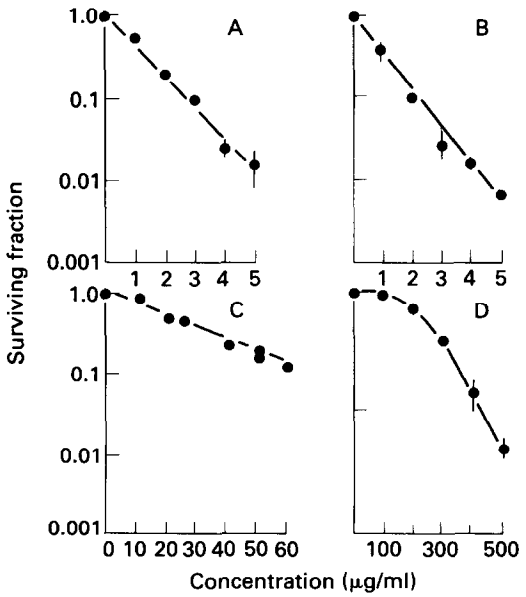

Fig. 2. The effect of alkylating agents on the clonogenic survival of Chinese hamster cells, line V-79-753B. A, 4-hydroperoxycyclophosphamide; B, melphalan; C, phosphoramide mustard; D, busulphan.

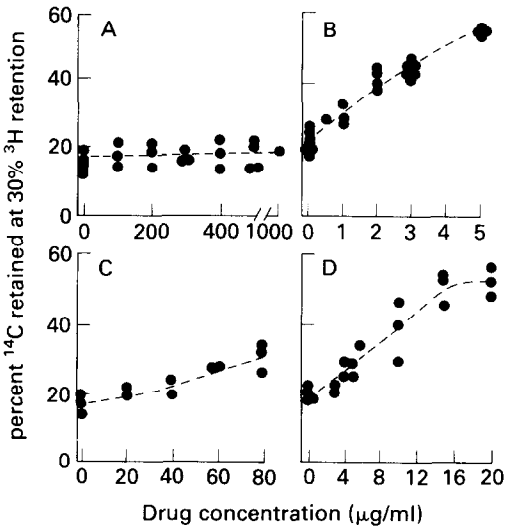

Fig. 3. The effect of alkylating agents on cross-linking in Chinese hamster cells, line V-79-753B. A, busulphan; B, melphalan; C, phosphoramide mustard; D, 4-hydroperoxycyclophosphamide. Samples exposed to each of the compounds were irradiated within 5 min after drug removal.

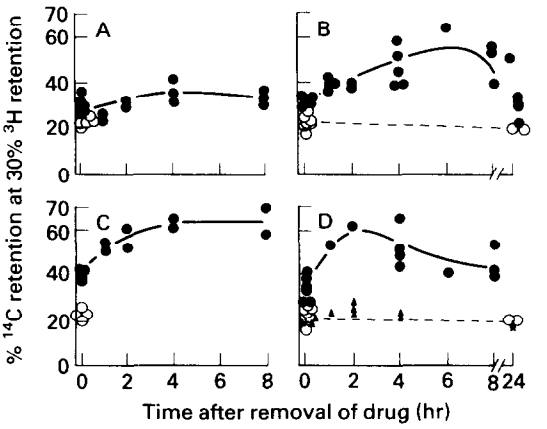

Fig. 5. The effect of crosslinking of the incubation period between exposure of Chinese hamster cells, V-79-753B to alkylating agents and lysis. A, busulphan (1000 μg/ml); B, melphalan (1 μg/ml); C, phosphoramide mustard (80 μg/ml); D, 4-hydroperoxycyclophosphamide (5 μg/ml, ●) (1 μg/ml, △). In each panel the open symbols represent data from control cells.

very high drug doses. Despite the high dose of BU used in these experiments no cross-linkage was measurable up to 8 hr after treatment. In contrast, in cultures treated with PM, 4-OOH CY or MEL there was an increase in cross-linking during the first 4–6 hr after drug removal, the degree of increase being similar in each case (Fig. 5). A low dose (1 μg/ml) of 4-OOH CY produced no detectable cross-linking even 4 hr after drug removal (Fig. 5). (It should not be overlooked that a repair of single-strand breaks would also increase molecular weight. However, we interpret the observed changes as being due to an increase in cross-links because of the long time course. Generally, simple single-strand breaks are repaired more rapidly. For example, in this cell line, radiation-induced strand breaks are repaired with a half-life of 17.5 min; B. C. Millar, unpublished data.)

Comparison of the growth of cells treated with equitoxic doses of the same drugs (Fig. 6) shows that in cultures treated with either 4-OOH CY or BU cell growth continued at a reduced rate compared with that of control cultures, whereas there was a decline in the total cell count in cultures treated with either MEL, beginning 2 days after drug removal, or PM, beginning 3 days after drug removal [21]. Thus MEL and PM must induce a much greater growth delay in this cell line than 4-OOH CY or BU. These data indicate that estimation of total cell numbers following treatment with cytotoxic compounds does not necessarily provide a reliable measurement of drug-induced toxicity.

To determine whether the drugs induced changes in the cell cycle, cultures were either untreated or

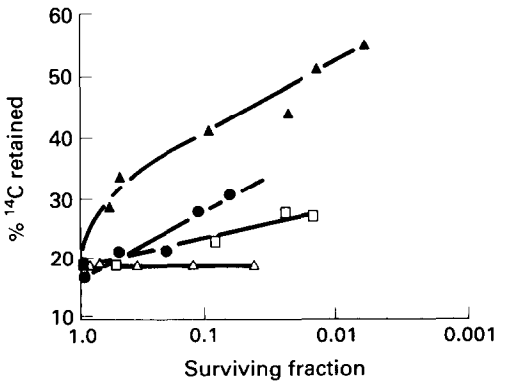

Fig. 4. The relationship between clonogenic survival and cross-link formation in Chinese hamster cells, line V-79-753B, exposed to alkylating agents. △, Busulphan; □, 4-hydroperoxycyclophosphamide; ▲, melphalan; ●, phosphoramide mustard.

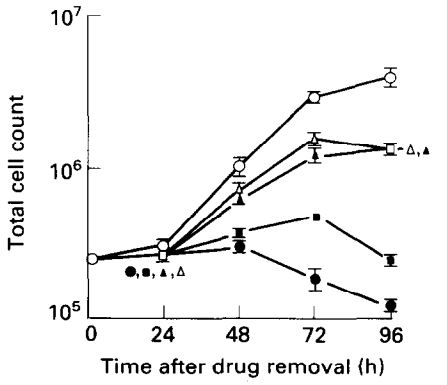

Fig. 6. The effect of alkylating agents on the growth of Chinese hamster cells, V-79-753B, at equitoxic concentrations. ○, control cells; ▲, 4-hydroperoxicyclophosphamide (3 μg/ml); △, busulphan (400 μg/ml); ■, phosphoramide mustard (60 μg/ml); ●, melphalan (2 μg/ml).

treated with 400 μg/ml BU, 3.0 μg/ml 4-OOH CY, 2.0 μg/ml MEL or 60 μg/ml PM; the doses of drug were selected to give approximately 10% survival. Cells were harvested and stained with ethidium bromide on successive days. The DNA histograms are shown in Fig. 7. Cells from the same cultures were used to produce growth curves (Fig. 6).

In the cultures treated with BU or 4-OOH CY there was an increase in the number of cells in G2 of the cell cycle with a corresponding decrease of the cells in G1 evident 24 hr after treatment. Between 3 and 4 days after the treatment the cell cycle, as evidenced from the DNA histograms, had returned to normal.

After treatment with either MEL or PM, there was a more dramatic change in the cell cycle. Cells accumulated in G_2 and then, with time, the DNA content of the cells in G_2 increased significantly to give a G_2/G_1 ratio of about 2.4 (as opposed to a ratio of 2.0 ± 0.1 usually observed). There were also indications that a new S phase was developing with DNA content corresponding approximately to tetraploidy. These effects were particularly marked in cells treated with MEL. Eventually these tetraploid cells disappeared from the culture and cells of normal ploidy grew out (Fig. 8).

Interestingly, when the surviving population from MEL treated cultures was allowed to grow to con-

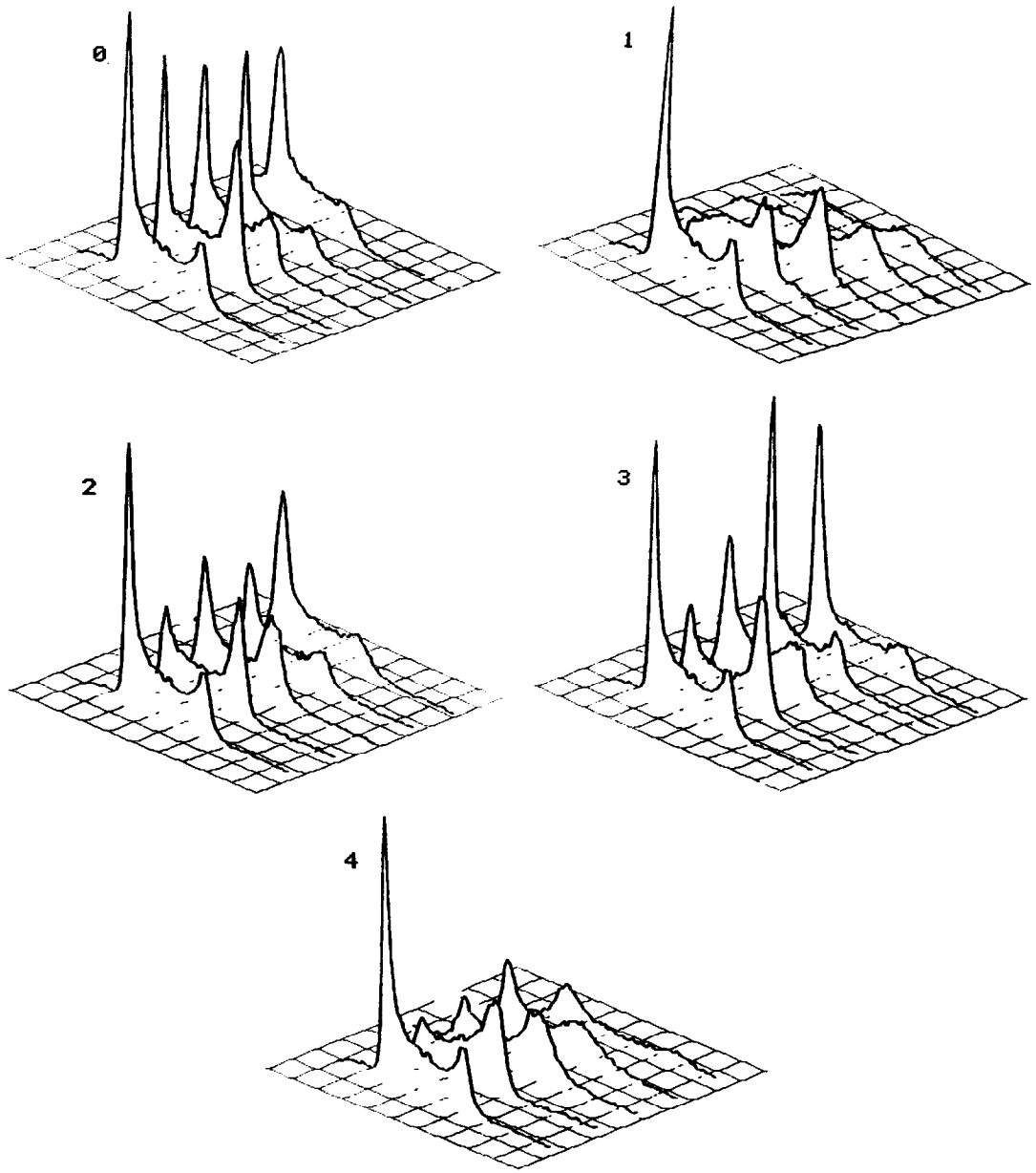

Fig. 7. DNA histograms. The abscissa of each figure represents DNA fluorescence, the ordinate the number of cells. The first histogram was recorded before treatment of the cells and the following histograms 1, 2, 3 and 4 days after treatment. 0: No treatment. 1: 2.0 μg/ml melphalan. 2:400 μg/ml busulphan. 3: 3.0 μg/ml 4-hydroperoxycyclophosphamide. 4: 60 μg/ml phosphoramide mustard.

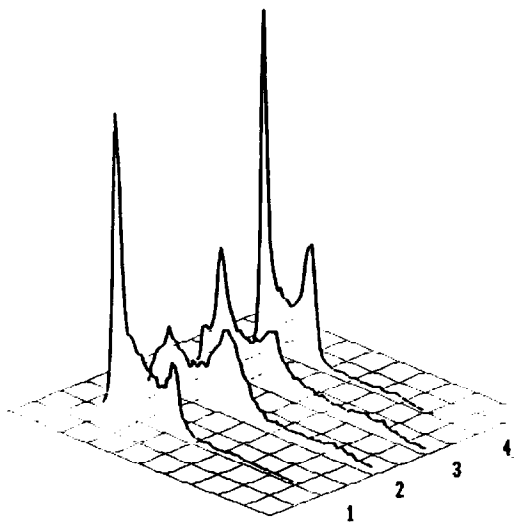

Fig. 8. DNA histograms as described in Fig. 6 recorded: 1: before treatment; 2: one day; 3: four days; 4: seven days; after treatment with 1.0 μg/ml melphalan.

fluence and passaged, re-examination of the cell cycle distribution showed that there was no evidence of a tetraploid population. Furthermore, the sensitivity of these cells to melphalan was similar to that in cultures which had not previously been exposed to the drug.

DISCUSSION

In this study we have attempted to compare four parameters of cell–drug interactions that are used to measure the toxicity of chemotherapeutic agents *in vitro*.

The data substantiate previous results with this cell line that 4-OOH CY is approximately 20 times more toxic towards Chinese hamster cells than PM [22]. This differential toxicity is likely to reflect differences in uptake of the two molecules. 4-OOH CY is actively transported into cells *in vitro* where it is catabolised to PM and acrolein via aldophosphamide, which is in equilibrium with 4-OOH CY [23]. In contrast, PM, which is charged at physiological pH, is likely to penetrate cells poorly. Although a much greater concentration of PAM than 4-OOH CY was required to produce a similar amount of cross-linkage, the time course of their appearance after drug removal was similar, in agreement with previously published reports [7, 24]. However, at equitoxic doses, 4-OOH CY caused significantly less cross-linking than PM (Fig. 4). Comparison of cell cycle data and cell growth at equitoxic doses of PM and 4-OOH CY provides further evidence that the type of damage or its repair by these agents is different. In cultures exposed to PM the cell cycle was arrested in G_2 and there was a decrease in the total cell count. In contrast, despite a temporary arrest of the cell-cycle at G_2, 24 hr after treatment with 4-OOH CY, the distribution of the cell-cycle returned to control values 3–4 days after treatment with this metabolite. Furthermore, there was no decrease in the total cell count, although the cells exhibited a reduced growth rate. The only metabolite of 4-OOH CY capable of alkylating DNA *in vivo*, thereby causing cross-links, appears to be PM ([25] and references therein). However, in view of the above differences in the effects of 4-OOH CY and PM, it seems that PM is not the only metabolite of 4-OOH CY that is involved in cell toxicity. Another metabolite, acrolein, accounts for the bulk of bladder toxicity *in vivo* [6]. Our results support the possibility that PM and acrolein act synergistically to cause cell death. This has been proposed previously from purely chemical considerations [6, 25].

Comparison of the data for 4-OOH CY and PM with BU and MEL indicates similarities between PM and MEL and between 4-OOH CY and BU with respect to cell-cycle effects, inhibition of cell growth and cross-link formation. Unlike PM, MEL is actively transported into cells, principally by the high-affinity amino acid transport system for L-leucine [26]. In Chinese hamster cells the internal cellular concentration of MEL is approximately 6-fold greater than in the external medium (B. C. Millar, unpublished). Thus, the acute toxicity of MEL at low concentrations is explicable by the relative ease of incorporation of the drug. Given this difference in the uptake of MEL and PM, it is noteworthy that, of the drugs tested, these compounds were the most effective cross-linking agents for any given survival level (Fig. 4). Whilst it would be unjustifiable to compare these initial values, other than quantitatively, it is interesting that for both compounds both cell growth and cell-cycle distribution showed similar changes at equitoxic doses.

The appearance of the tetraploid population in cultures treated with MEL suggests that Chinese hamster cells may attempt futile DNA synthesis on a damaged template, since the surviving cells were essentially diploid. Previous studies have shown that MEL-induced cross-linking is dependent on both the drug concentration and the exposure time [11]. In Chinese hamster cells maximum cross-linking was detected 6 hr after removal of the drug, presumably due to the persistence of unreacted or partially reacted melphalan within the cells. After this time there was a small decrease in cross-links by 8 hr after drug removal. Although the amount of cross-links detected in MEL-treated cells 24 hr after drug removal reached a value similar to that seen immediately after MEL treatment, it is arguable that such measurements are misleading, since the doubling time of this cell line is 9.5 hr and the clonogenic survival about 60%, at the dose of MEL used in these repair studies. Our measurements take no account of cell loss due to lysosomal enzyme activity or the presence of reproductively dead cells.

Whilst some comparison can be made between the toxicity of BU and 4-OOH CY, the most notable difference was that clonogenic survival was not exponential at low doses of BU. The shoulder on the survival curve of cells exposed to BU cannot be explained by the poor incorporation of the drug at low doses, since labelling studies have shown that BU equilibrates in cells within 5 min of exposure to the drug (B.C. Millar, unpublished). It seems more probable that the mechanism(s) of action of this

compound are unlike those of the other compounds herein reported. Both BU and 4-OOH CY exhibited similar effects on cell cycle perturbation and cell growth. However, in cells treated with BU there was no evidence for DNA cross-links. We have no satisfactory explanation for the discrepancy between our results and those of Bedford and Fox [15] who presented alkaline elution data indicating that the BU caused DNA interstrand cross-links in Yoshida sarcoma cells. Previous workers have been unable to detect interstrand cross-linking by this drug, in experiments where other drugs did cause easily detectable cross-links (reviewed by Roberts) [27]. BU is a bifunctional alkylating agent and the early report of diguaninyl products in hydrolysates of DNA treated with BU [28] has been confirmed [29]. However, these may well have resulted from intrastrand cross-links. Using an *in vitro* bacteriophage system, Verly and Brakier [28] were unable to detect any evidence of DNA interstrand cross-linking by BU, using a number of criteria.

From our alkaline elution data we cannot rule out the possibility that BU causes a low level of particularly lethal cross-links. However, the data of Verly and Brakier [30], together with our data on cell survival and cell cycle effects, indicate that BU acts differently from a number of established cross-linking agents.

The evidence presented here supports the conclusion that, after treatment with either MEL or PM, the most important events are the persistence of DNA–DNA cross-links leading to the arrest and subsequent death of cells in G_2 of the cell cycle. In contrast, BU and 4-OOH CY kill cells by a different mechanism(s). The observed cytotoxicity of 4-OOH CY towards V79 cells cannot be predominantly due to catabolism of the drug to PM.

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