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Comparative studies of galactose-6-mustard and L-phenylalanine mustard on cell growth and cell cycle kinetics *in vitro*

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The effect of galactose-6-mustard (G-6-M) on cell growth and cell cycle kinetics was studied in murine P388 leukemia and Chinese hamster ovary (CHO) cells *in vitro* and compared with the effect of L-phenylalanine mustard (L-PAM). The IC_{50} values of G-6-M for the P388 and CHO cells were 10 and 100 μM, respectively. No difference of the IC_{50} value of L-PAM (2 μM) between the two cell lines was found. The effect of G-6-M and L-PAM on cell kinetics was similar for the two cell lines at IC_{50} doses. The relative cell outflow from the G_2 stage was inhibited to a higher extent than the relative cell outflow from the S phase. The relative cell outflow from the G_1 stage was only partly inhibited. These results are discussed in relation to growth conditions, differences in DNA repair capacity, and cellular uptake of G-6-M between P388 and CHO cells.

Key words: Cell cycle kinetics, IC_{50}, galactose-6-mustard, L-phenylalanine mustard.

Introduction

In spite of the large efforts made to develop new anticancer compounds during the last three decades alkylating agents still have a strategic role in the treatment of human cancers. In clinical use the toxicity on the bone marrow is the limiting factor. By covalently linking alkylnitrosoureas to the C-2 position of glucose (streptozotocin and chlorozotocin) bone marrow toxicity can significantly be reduced without changing the antitumor effect. This has been shown for L1210 leukemia of mice,[1–3] and for melanoma and hematological malignancies in man.[4,5] The reduced marrow toxicity of glucose nitrosoureas has been correlated with less alkylation of the transcriptionally active chromatin of bone marrow cells whereas the alkylation of tumor cell DNA involved in transcription is similar to that of the more myelotoxic analogs.[6,7]

Bifunctional nitrogen mustard compounds covalently linked to the C-2 or C-6 of glucose also have strong activity against murine ascitic P388 leukemia with reduced bone marrow toxicity. In contrast, placement of the mustard moiety on the C-1 of either D-glucose or L-glucose resulted in myelotoxic compounds.[8]

Galactose-6-mustard (G-6-M) has recently been shown to possess an antitumor effect superior to that of glucose-6-mustard and with good bone marrow sparing properties.[9] G-6-M is as potent as L-phenylalanine mustard (L-PAM) on murine ascites P388 leukemia but has less effect on peripheral leukocyte counts and neutrophil counts in mice and also a lower toxicity towards human bone marrow granulocyte-macrophage colony forming units (CFU-GM).[9]

The aim of this paper is to investigate whether the lymphoid tumor cells P388 and the fibroblast-like tumor cell (CHO) respond differently with respect to G-6-M toxicity and whether the nature of growth inhibition differs between these cell lines.

Material and methods

Cells and growth conditions

Murine P388 leukemia cells and Chinese hamster ovary (CHO) cells were grown in minimal essential medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 IU penicillin and 100 μg/ml streptomycin and HEPES at 37°C in 75 cm^2 tissue culture flasks (Falcon). Under these conditions, P388 cells grow in suspension and the CHO cells as monolayer cultures. Stock cultures were

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subcultivated every 2–3 days to maintain a high growth fraction of the cultures. When running the experiments, the cells were cultivated in multidisks (Costar 12-well as described previously).[10] At times indicated, the CHO cells were removed from the bottom of the disks by treatment with 0.02% (w/v) trypsine in Tris-buffer.

Compounds

Lyophilized G-6-M was obtained from Astra, Södertälje, and diluted with 0.9% (w/v) of sodium chloride (pH 4.1) just before the experiment to form a concentrated stock solution. L-PAM (Burroughs and Wellcome Foundation, Beckenham, Kent) was dissolved in 92% ethanol with 2% HCl and diluted with 60% propylenglycol in sterile water with 1.2% dipotassium hydrogen phosphate to obtain a stock solution.

Treatment with G-6-M and L-PAM

In order to have exponentially growing cells at the time when the drug was added, the cells were preincubated at 37°C without test compound for 12 h. The cells were then further incubated up to 72 h in the presence of the drugs. However, since G-6-M is stable for only a few hours during these conditions, the incubation with G-6-M should be regarded as a pulse treatment. L-PAM is stable over the incubation time of 72 h.

Cell counting

The cells were counted in a Coulter Counter.

Cell cycle composition

The cell cycle composition was determined as described previously.[11] In brief, ethanol fixed cells were washed and incubated in Tris-NaCl-EDTA buffer (Tris 0.1 M, NaCl 0.01, EDTA 0.05 M, pH 7.5) containing RNase (1 mg/ml) and pepsine (5.0 g/l in 0.055 M HCl, 37°C for 10 min) in order to obtain cell nuclei. The nuclei were stained with ethidium bromide (25 μM) and analyzed in a flow cytofluorometer (PHYWE). The percentages of G_1, S phase and G_2 + M cells were calculated assuming a Gaussian distribution of the G_1 and G_2 maxima. The remaining part of the histogram belongs to the S phase cells. A high correlation between the percentage of S phase cells calculated from the DNA histogram and from autoradiography has been found.[12]

Calculation of cell cycle flow

The calculation of the relative outflow from the various cell cycle stages has been described elsewhere.[12] The calculation is based on the number of cells in the cell cycle phases, and the total number of cells at the beginning and at the end of the time interval studied. The relative outflow is defined as the total outflow of cells in relation to the number of cells in that particular compartment and is given as percentage per hour. For the P388 cells, the cell flow was calculated in the time intervals of 0–6, mean value of 6–10 and 10–24, 24–48 and 48–72 h. For CHO cells the corresponding intevals were 0–24, 24–48 and 48–72 h.

Mitotic index

Smears were prepared and fixed in ethanol:acetone (3:1) and then stained in Ham's hematoxylin and eosin as described earlier.[13] A total of 1000 cells were counted in each preparation.

Incorporation of [^{3}H]thymidine into cells

DNA synthesis was estimated by adding [^{3}H]thymidine ([^{3}H]TdR) (specific activity, 740 MBq/mol or 75 kBq/ml) for 30 min to cells treated for different times with G-6-M. The acid-insoluble material, obtained after washing 5 times with 2 ml of phosphate buffered saline and 5 times with 2 ml of ice cold 5% trichloroacetic acid, was solubilized in 1 ml of 0.5 M NaOH at 80°C for 20 min. The alkali soluble material obtained was neutralized with 100 μl of 0.5 M HCl before being counted in a scintillator.

Results

Cell growth after treatment with G-6-M and melphalan

The effect on cell growth of G-6-M and L-PAM was compared at 48 h. A 10-fold difference in toxicity was observed for G-6-M between P388 cells and

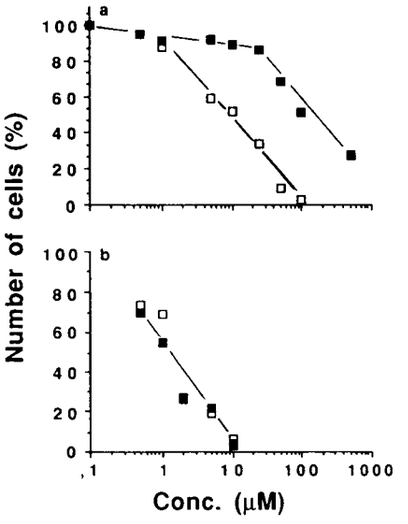

Figure 1. Number of cells 48 h after treatment of P388 cells (□) and CHO cells (■) with various concentrations of (a) G-6-M and (b) L-PAM.

CHO cells (IC_{50} of 12 and 100 μM, respectively; Figure 1a). In contrast, the IC_{50} values for L-PAM for these two cell lines were identical (2 μM) (Figure 1b).

In order to study the kinetics of growth inhibition, both cell lines were exposed to G-6-M at 0.5 × IC_{50}, 1 × IC_{50} and 2.5 × IC_{50}, and to L-PAM at 1 × IC_{50} and followed for 72 h (Figure 2). The growth rate of both P388 and CHO cells after treatment with G-6-M was reduced in a dose-dependent way up to 72 h (Figure 2a and c). Cell loss was obtained only in P388 cells, when treated at 2.5 × IC_{50} (30 μM). The growth rate of P388 and CHO cells after treatment with 10 μM L-PAM was reduced in a similar way up to 72 h (Figure 2b and d).

Cell cycle composition of G-6-M and L-PAM treated cells

The cell cycle composition of non-treated P388 cells was almost unchanged during growth (Figure 3a), while for the CHO cells the proportion of the G_1 cells increased with a corresponding decrease in the proportion of S phase and G_2 + M cells (Figure 3c). The mitotic index for both cell lines was about 1–1.5% (Figure 4a and b).

After treatment of P388 cells with 12 μM G-6-M a transient accumulation of cells in G_2 was found (data not shown). For cells treated with 30 μM (2.5 × IC_{50}), the accumulation in G_2 was prolonged (Figure 3b). The mitotic index was close to control after 12 μM G-6-M, while it reduced to a minimum value at 24 h after the higher dose (Figure 4a). The mitotic index was normal at 48 h. Similar changes in the cell cycle composition, including the mitotic index, were observed in CHO cells treated with G-6-M. The effects of L-PAM on the cell cycle distribution of P388 and CHO cells (data not shown) at 10 μM as well as the mitotic index (Figure 4b) were similar to the effect of G-6-M, except that the cell cycle composition was normal at 72 h after treatment with L-PAM.

Cell cycle progress after treatment with G-6-M and L-PAM

The relative outflow from the S phase and G_2 + M of non-treated P388 cells during growth declined continuously, while the outflow from G_1 was more or less constant (Figure 5). The main effect of 12 and 30 μM of G-6-M on P388 cells was the markedly reduced relative outflow from G_2 + M; at 30 μM it was completely stopped for 24 h. This

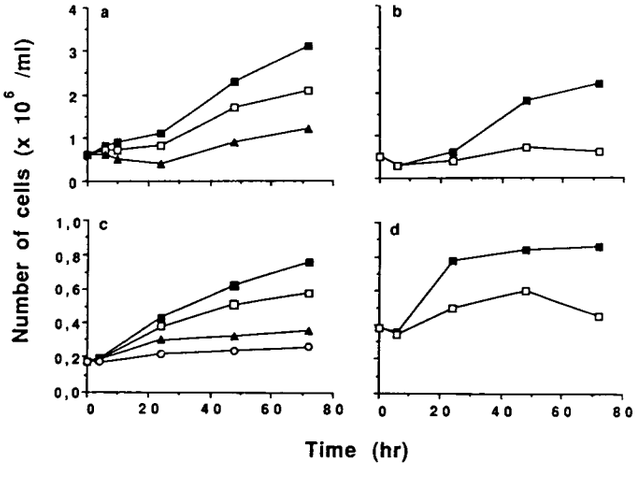

Figure 2. Cell growth of P388 (a and b) and CHO (c and d) cells after treatment with G-6-M and L-PAM. Mean values of two to three experiments: (a) ■, control; □, 12 μM G-6-M; ▲, 30 μM G-6-M. (b) ■, control; □, 10 μM L-PAM. (c) ■, control: □, 50 μM G-6-M; ▲, 100 μM G-6-M; ○, 250 μM G-6-M. (d) ■, control; □, 10 μM L-PAM.

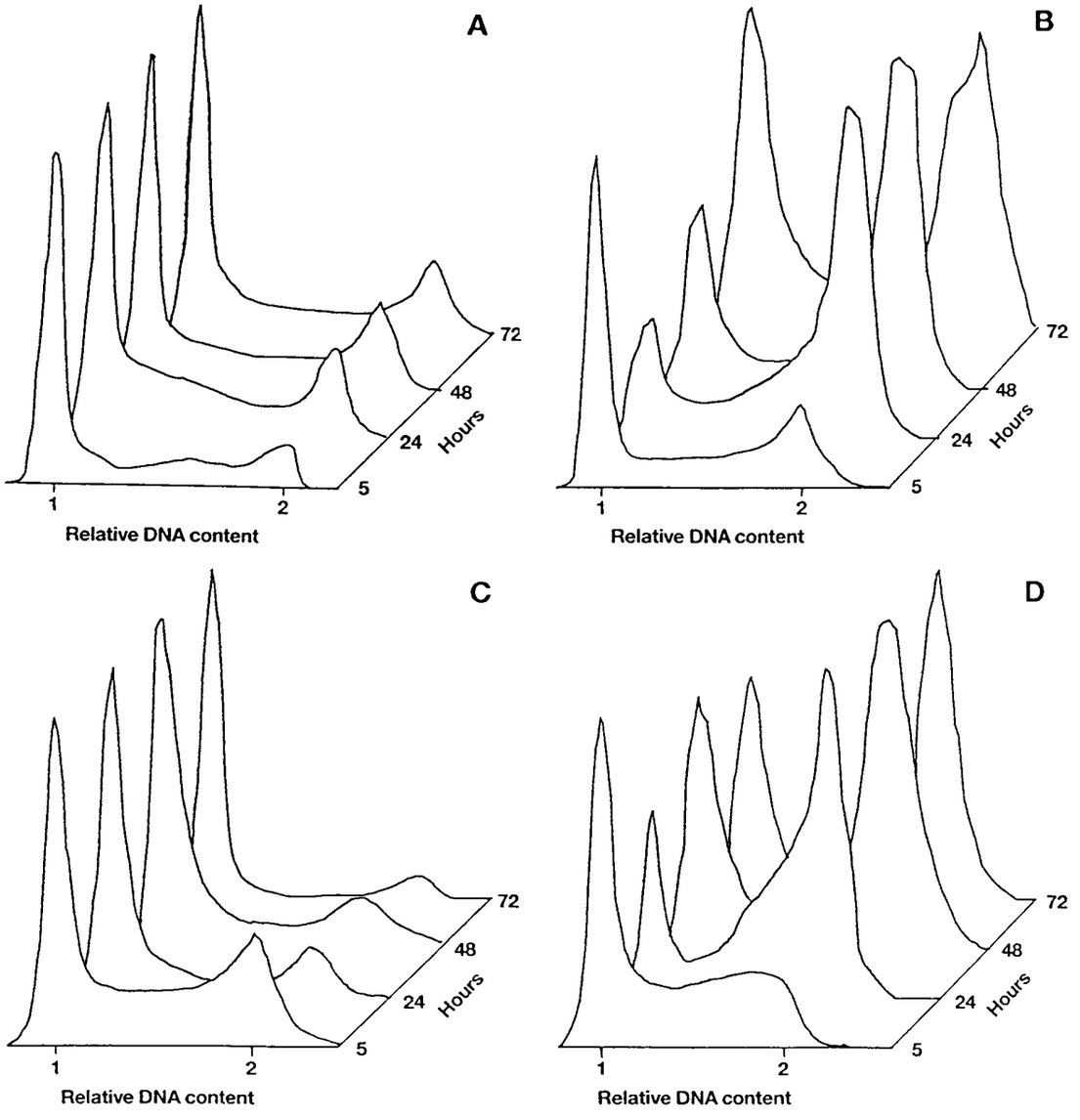

Figure 3. DNA histograms of (a) non-treated P388 cells, (b) P388 cells treated with 30 μM G-6-M, (c) non-treated CHO cells and (d) CHO cells treated with 250 μM G-6-M.

effect was maintained up to 72 h, although not completely. The relative outflow from the G_1 and S phase was inhibited up to 12 or 24 h depending on the dose given. After 24 h it was normal or elevated (Figure 5).

The effect of G-6-M on the flow of CHO cells through the cell cycle was similar to the effect found on P388 cells (Figure 6). A transient retardation of relative outflow from the G_1 and S phases up to 24 h was followed by normal values, while a marked reduction of the relative outflow from G_2 + M stages was maintained up to 72 h.

L-PAM affected the cell cycle flow of P388 and CHO cells in a way corresponding to G-6-M (Table 1).

Incorporation of [^{3}H]TdR into DNA

The incorporation of labeled TdR into DNA of P388 cells in S phase after treatment with G-6-M was not affected at 3 and 48 h, but dose-dependently reduced at 24 h (Figure 7a). Treatment with 10 μM L-PAM reduced the incorporation of [^{3}H]TdR at

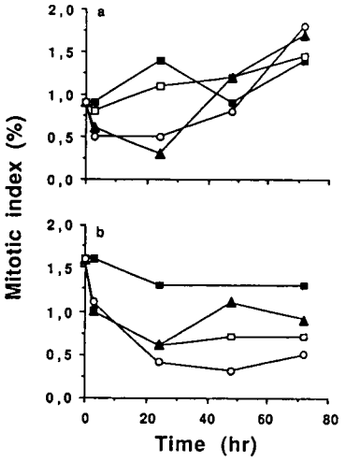

Figure 4. Mitotic index of (a) P388 and (b) CHO cells after treatment with G-6-M and L-PAM: (a) ■, control; □, 12 μM G-6-M; ▲, 30 μM G-6-M; ○, 10 μM L-PAM. (b) ■, control; □, 50 μM G-6-M; ▲, 250 μm G-6-M; ○, 10 μm L-PAM.

24 h to a value corresponding to the high dose of G-6-M (Figure 7a). The incorporation of labeled TdR into DNA of CHO cells after treatment with 250 μM G-6-M and 10 μM L-PAM was reduced at 3, 24 and 48 h after treatment, but reduced only at 24 h after treatment with 50 μM G-6-M (Figure 7b).

Discussion

Studies *in vivo* show that the antitumor activities of G-6-M and L-PAM are very similar (compared at LD_{10}) whereas the acute *in vivo* toxicities on bone marrow cells were significantly stronger for L-PAM than for G-6-M.[1] We have investigated whether the *in vitro* toxicity of G-6-M may vary for different tumor cell lines and studied the cell cycle specific effects of these compounds to see whether different toxic mechanism(s) exist in various cell lines. The well-known antitumor bifunctional alkylator L-PAM has been used as a reference compound.

The toxicity of G-6-M was about 8 times higher in P388 cells compared with CHO cells (IC_{50} 12 and 100 μM, respectively) while L-PAM did not show any cell-specific toxicity (IC_{50} 2 μM in both P388 and CHO cells). Since P388 cells are grown in suspension and CHO cells are grown as monolayers a difference in toxicity between these cell lines may be accounted for by different growth conditions as described previously.[14] However, the similar toxicity of L-PAM for P388 and CHO cells makes such an explanation less likely. Compounds which might be suspected to act in specific phases of the

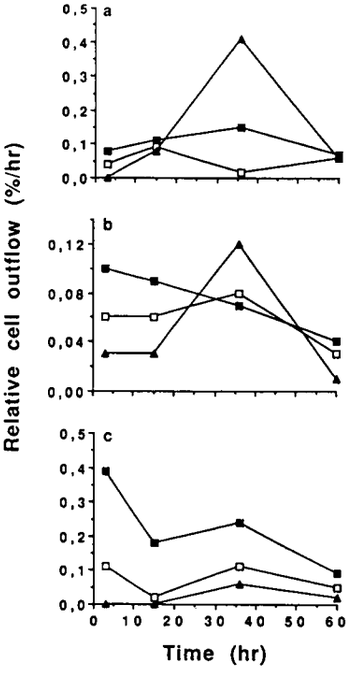

Figure 5. Relative outflow from G_1. S phase and G_2 + M of non-treated P388 cells (■), and cells treated with 12 μM (□) and 30 μM (▲) G-6-M.

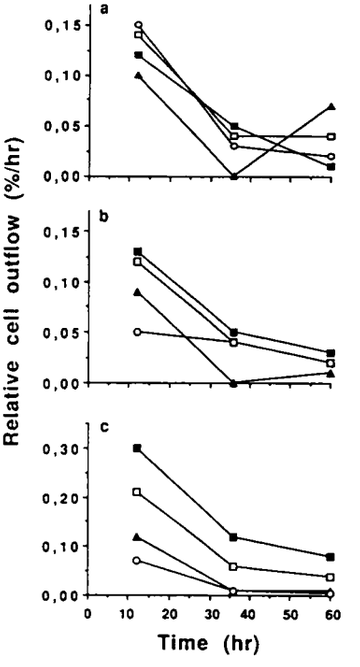

Figure 6. Relative outflow from G_1, S phase and G_2 + M of non-treated CHO cells (■), and cells treated with 50 μM (□), 100 μM (▲) and 250 μM (○) G-6-M.

Table 1. Relative cell outflow (%/h) from G_1, S phase and G_2 + M of P388 and CHO cells treated by 10 μM L-PAM (mean values of three experiments)

Cell line	Time (h)	G_1 (0 μM)	S (10 μM)	S (0 μM)	G_2 + M (0 μM)	G_2 + M (0 μM)	G_1 (10 μM)
P388	0–24	0.15	0.15	0.08	0.06	0.27	0.09
	24–48	0.16	0.16	0.12	0.12	0.42	0.08
	48–72	0.05	0.05	0.02	0.09	0.15	0
CHO	0–24	0.10	0.14	0.14	0.06	0.30	0.13
	24–48	0.03	0.06	0.05	0.06	0.34	0.03
	48–72	0.02	0	0	0.04	0.03	0.03

cell cycle can produce a higher toxicity in rapidly proliferating cells. However, since P388 and CHO cells grow at comparable growth rates this is not a reason for the differences in toxicities.

The lower antitumor effect of G-6-M compared with L-PAM on P388 *in vitro* is different from the similar effects observed *in vivo*.[1] However, this difference may well be a result of the fact that the animals receive a single injection of test compound whereas *in vitro* cells are continuously exposed to toxic compound for 48 h before growth inhibition was measured.

It has been shown[15] that the sensitivity to L-PAM is much higher in DNA repair deficient mutants of CHO cells. If the difference in sensitivity to G-6-M between P388 and CHO cells is due to a different capacity to repair DNA damage a corresponding difference to L-PAM should also be found. This however, is not the case.

The main effect of G-6-M was to reduce the progression through G_2. This effect was dose-dependent and evident already from the beginning of the treatment and maintained up to at least 72 h. In addition, the DNA synthesis was also affected, but only up to 24 h. The DNA synthesis was determined by two independent methods, i.e. incorporation of labeled TdR into DNA of S phase cells and by following the progress of cells through S phase. Since G-6-M and L-PAM are not involved in nucleotide metabolism,[16] it is not likely that changes in the size of the deoxythymidinetriphosphate (dTTP) pool occur and, thus, induce changes in the specific activity of labeled dTTP in the cell. Such changes affect the incorporation of labeled dTTP into DNA giving incorrect results.[17] The relative cell outflow calculation method is based on the assumption that no resting cells or dead cells are present. If this is the case, the relative cell outflow rate should be higher, since the calculation of the relative cell outflow of a cell cycle phase is based on the total number of cells in that compartment, including both proliferating/non-proliferating and dead cells. Thus, the results from the cell flow calculation are minimum values. The effect on the DNA synthesis and the G_2 blockage were in agreement with results usually obtained from alkylating agents.[16] In order to confirm that in our cell systems, we ran L-PAM in parallel and found almost identical results as found for G-6-M with respect to G_2 blockage and inhibited DNA synthesis. There is no reason to suspect that pulse incubation of L-PAM, as for G-6-M, would give rise to different results, since pulse incubation of L-PAM at similar concentration[19] leads to almost identical results as found for the prolonged incubation. However, equal alterations of cell cycle kinetics do not always mean identical mechanisms for cellular interactions.

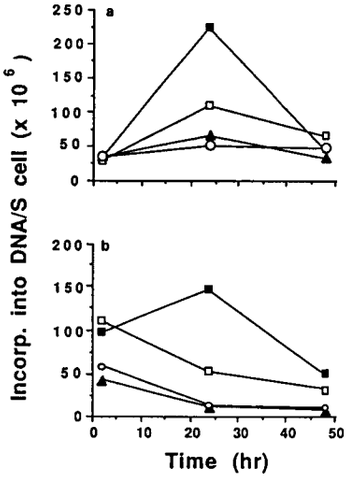

Figure 7. Incorporation of [^{3}H]TdR into DNA of (a) P388 and (b) CHO cells after treatment with G-6-M and L-PAM: (a) ■, control; □, 12 μM G-6-M; ▲, 30 μM G-6-M; ○, 10 μM L-PAM. (b) ■, control; □, 50 μM G-6-M; ▲, 250 μM G-6-M; ○, 10 μM L-PAM.

Although there is much evidence for DNA as the major target for alkylating agents,[18,19] results of Grunick *et al.*[20,21] and Ihlenfeldt *et al.*[22] show that the cell membrane is also affected by alkylating

agents. These authors found a reduced uptake of amino acids, glucose and thymidine and an inhibited Na^+K^+ pump, probably due to interaction between the Na^+K^+ ATPase and the drug. A similar mechanism of action of G-6-M would fully explain inhibition in both DNA synthesis rate by lack of thymidine and G_2 blockage. Studies by Baserga *et al.*[23] show that passage through the G_2 stage is more sensitive to lack of nutrients than the other cell cycle stages. However, it is not likely that the inhibition of the DNA synthesis in our cells is due to lack of thymidine, since the cells were grown in a thymidine-free medium and, thus, thymidine was synthesized by the cell (*de novo*). Any alteration in cellular uptake of thymidine would therefore not affect DNA synthesis.

Conclusion

The differences in sensitivity between P388 and CHO cells to G-6-M are not explained by growth conditions, differences in DNA repair or cell cycle specificity. We therefore suggest that this difference in sensitivity is due to alterations in net uptake of G-6-M, possibly reduced cellular uptake.

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