

Reduction of Intracellular pH as a Strategy to Enhance the pH-Dependent Cytotoxic Effects of Melphalan for Human Breast Cancer Cells

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Abstract The microenvironment within solid tumors is slightly acidic, and manipulation of this extracellular acidity to cause intracellular acidification might be used to increase selective antitumor effects of some anticancer drugs. Potential mechanisms include inhibition of repair of DNA damage and inhibition of repopulation of tumor cells between successive courses of chemotherapy. Here, we evaluate the influence of extracellular pH (pHe) and of two agents that lead to intracellular acidification (cariporide and S3705) on toxicity of melphalan for two human breast cancer cell lines (MDA-MB231 and MCF7). Both the total number and number of colony-forming cells were evaluated during and after three sequential weekly drug treatments. Our results indicate the following: (a) Slow or absent repopulation after the first course of treatment that is influenced minimally by pHe. (b) Rapid repopulation after the second course of treatment that may be inhibited at low pHe. (c) Effects of low pHe following treatment with melphalan to increase cell kill. (d) Small effects of incubation in cariporide and S3705 at low pHe to increase the net cell kill after treatment with melphalan. Although these results add to evidence that manipulation of intracellular pH within the acidic environment of solid tumors can influence the effects of chemotherapy, they are too small and inconsistent to warrant clinical evaluation.

The extracellular pH (pHe) within the microenvironment of solid tumors is low (median pHe typically 6.9-7.0) compared with that in normal tissues (pHe typically ~7.4). The cytotoxicity of many chemotherapeutic drugs is influenced by the pH of the environment. Passive diffusion of drugs into cells occurs mainly when they are uncharged. Thus, the cellular uptake of drugs with acidic or basic groups depends on the pHe, whereas their trapping in a charged form inside cells depends on intracellular pH (pHi). Basic drugs, such as doxorubicin (1, 2), mitoxantrone (3, 4), and vinblastine (5), have decreased cytotoxic effects against cells at low pHe due to decreased cellular uptake. This puts them at a therapeutic disadvantage within the acidic milieu of solid tumors. In contrast, passive cellular uptake of acidic drugs, such as chlorambucil and melphalan, is increased when the pHe is more acidic (6-9). However, low pHe may also impair energy-dependent cellular uptake of acidic drugs, such as methotrexate.

Increased cell killing by melphalan at low pHe has been shown in A549, KHT, and L1210 cells and in KHT, RIF-I, and

SCCVII/Ha tumors (10-12). Siemann et al. (10) showed that tumor cells were ~1.3 times more sensitive to melphalan when the environment during the treatment was acidified from pHe 7.4 to 6.6. This pHe-dependent sensitivity was enhanced when the cells were treated with melphalan at low pHe in the presence of nigericin, an ionophore, which causes reduction in pHi. However, these authors also observed that nigericin and melphalan were synergistic in killing cells even at pHe 7.4. The mechanisms underlying this interaction are not clear.

A process that may limit the effectiveness of chemotherapy is proliferation of surviving tumor cells between courses of treatment, known as repopulation (13, 14). Repopulation is known to occur during fractionated radiation therapy where it limits the ability to obtain local control (15, 16). It is likely to be even more important between courses of chemotherapy that are given typically to patients at 3- to 4-week intervals, unlike daily fractions of radiation. This treatment interval is necessary to allow the recovery of bone marrow through repopulation from surviving bone marrow stem cells. Studies in mice and in multicellular spheroids have shown increasing proliferative rates of tumor cells following chemotherapy (17-19), and our laboratory has provided evidence that this may persist during successive courses of chemotherapy (14). Strategies that inhibit repopulation of tumor cells without inhibiting the regeneration in normal tissues would have potential for improving the effectiveness of chemotherapy.

Cariporide and S3705 are two new compounds that block, respectively, the Na⁺/H⁺ antiport and the Na⁺-dependent Cl⁻/HCO₃⁻ exchanger, which maintain pHi in the face of an acid load. These agents have shown selective cytostatic effects on cells grown at low pHe (20). Cariporide and S3705 are not cytotoxic and they are not, therefore, likely to be useful as

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antitumor agents on their own. However, under extracellular acidic conditions that occur in solid tumors, these agents might be used to cause tumor-selective intracellular acidosis. This strategy might sensitize tumor cells to the effects of some types of chemotherapy, e.g., by inhibiting repair of sublethal damage, and/or might inhibit the repopulation of surviving tumor cells between courses of chemotherapy. These effects would be expected to be relatively tumor-specific because of the low pHe within the microenvironment of solid tumors.

In this article, we describe experiments that have examined the effects on cultured human breast cancer cells that were treated with the anticancer drug melphalan in media adjusted to different pHe values in the range 6.8 to 7.4. Melphalan is an amino acid and alkylating agent for which there is evidence of pH-dependent effects against rodent cell lines (10–12). We have assessed the ability of cariporide and S3705 to influence cell killing following sequential treatments with melphalan and their potential to inhibit repopulation of tumor cells as a function of pHe. These *in vitro* experiments were designed to model processes that might occur within the more complex microenvironment of solid tumors.

Materials and Methods

Cells. Experiments were done using the human breast cancer cell lines, MDA-MB231 and MCF7 (purchased from the American Type Culture Collection, Manassas, VA). Cells were maintained in α -MEM with 10% fetal bovine serum. The cells were checked periodically to ensure absence of *Mycoplasma* by staining the cells with Hoescht 33258. New cultures were reestablished from frozen stock at ~3-month intervals. In experiments where cells were grown at different pHe values, the cells were maintained in pH-adjusted media; this was prepared by mixing α -MEM with 10% fetal bovine serum, 25 mmol/L HEPES, and the appropriate amount of HCl or NaOH. The medium was allowed to equilibrate in 95% air and 5% CO₂ and its pH was repetitively readjusted during a 1-week period.

Reagents. Cariporide and S3705 were supplied by Aventis (Frankfurt, Germany) and were dissolved in PBS. Melphalan, purchased from Sigma (Oakville, ON, Canada), was dissolved in methanol.

Determination of number of clonogenic cells during and after treatment. Cells were seeded into 25 cm² flask and allowed to attach overnight in α -MEM with 10% fetal bovine serum. Following overnight incubation, the medium was replaced with medium adjusted to pHe 7.4. The pH-adjusted media was changed every 2 days until day 7. On day 7, the cells were exposed to 10 μ mol/L melphalan for 1 hour in α -MEM (at pHe 7.4) and then washed twice with PBS. Before and following drug treatment, a flask was selected randomly and its cells were trypsinized, counted, and plated to determine the clonogenic ability of the cells. After washing, the cells were incubated in medium adjusted to different pHe values in the presence or absence of 80 μ mol/L cariporide and 40 μ mol/L S3705. On day 14 (i.e., 7 days after treatment with the anticancer drugs), the cells were trypsinized, counted, and replated to determine the proportion of clonogenic cells. Colonies were counted 10 days after plating the cells. The results are expressed as "clonogenic cell number," which is the product of the total number of cells and their plating efficiency.

In further experiments, cells were grown in medium adjusted to different pHe values in the range 7.4 to 6.8 in the presence or absence of 80 μ mol/L cariporide and 40 μ mol/L S3705 since day 1. The cells were treated thrice weekly with exposures to melphalan (10 μ mol/L for 1 hour). The pHe was maintained at 7.4 during every treatment with melphalan to avoid confounding effects related to changes in drug uptake. The medium was replaced with medium of

the same pHe lacking cariporide and S3705 1 day before each treatment with chemotherapy (days 6, 13, and 20). The cells were washed twice with PBS after each exposure to chemotherapy and were again incubated with medium adjusted to different pHe values in the presence or absence of 80 μ mol/L cariporide and 40 μ mol/L S3705. The pH-adjusted media was changed every 2 days throughout the experiment. Before and immediately after each chemotherapy treatment, one of the flasks was randomly selected and cells were trypsinized, counted, washed, and plated to determine the clonogenic ability of the cells.

Statistical analysis. All of the figures are plotted using Microsoft Excel and the data in the figures are the means \pm SE from experiments that have been repeated at least thrice. Levels of survival after each weekly treatment of melphalan were compared by ANOVA using Excel data analysis software.

Results

Effect of extracellular pH on number of clonogenic cells following a single treatment with melphalan. A single 1-hour treatment with 10 μ mol/L melphalan had a relatively small effect on the survival of both cell lines when assessed by a colony-forming assay done immediately after incubation with the drug. The surviving fractions of MDA-MB231 and MCF7 cells were 0.42 (\pm 0.07) and 0.62 (\pm 0.07), respectively. MDA-MB231 cells cultured at pHe 7.4 following treatment with melphalan showed no significant change in the number of clonogenic cells 7 days later so that any repopulation was balanced by loss of cells due to delayed damage when cells were left *in situ* (Fig. 1A). In contrast, melphalan-treated cells that were incubated at pHe 7.0 showed a reduced number of clonogenically viable cells after 7 days. Following treatment with melphalan, MCF7 cells showed either a small increase (pH 7.4) or no change in the number (pH 7.0) of clonogenically viable cells after 7 days (Fig. 1B). The presence of cariporide and S3705 (which would be expected to lower the pHi) had no significant effects on the number of clonogenic cells of either cell line after 7 days of incubation (Fig. 1A and B).

Effect of extracellular pH on number of cells during and after multiple treatments with melphalan. The total number and number of clonogenic MDA-MB231 cells during and after three exposures to 10 μ mol/L melphalan at 7-day intervals are shown in Fig. 2A and B.

As indicated in Fig. 1A, following the first treatment of melphalan (day 7), there was no evidence of repopulation when cells were cultured at pHe 7.4 (days 7–14), whereas cells cultured at pHe 6.8 between treatments continued to die during the 7-day period. The second and third treatments with melphalan led to greater fractional cell kill than the first treatment when the pHe was maintained at 7.4 ($P = 0.05$), but not when the pHe between treatment intervals was 6.8, perhaps due to the slowed cellular proliferation at lower pHe values. After the second treatment with melphalan on day 14, the number of clonogenic cells was observed to increase (days 14–21) at both pHe values. Although the number of clonogenic cells fluctuated during these experiments, the total number of cells did not change rapidly, presumably because lethally damaged cells are removed quite slowly.

Similar results were observed when MCF7 cells were grown at pHe 7.4 and 6.8, and received multiple treatments of melphalan (data not shown). Minimal repopulation occurs

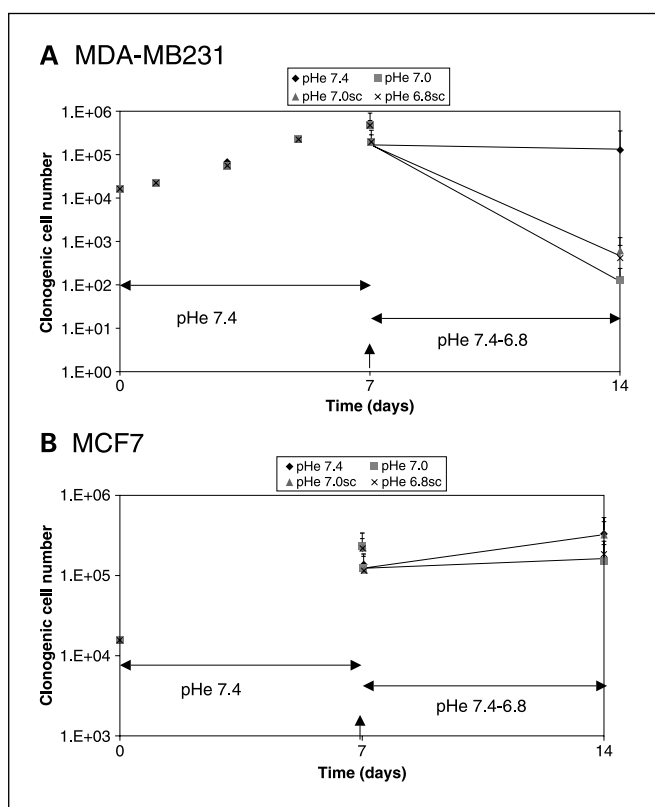


Fig. 1. Effect of pH on the number of clonogenic cells following treatment with melphalan. MDA-MB231 (A) and MCF7 cells (B) were incubated at pHe 7.4 before and during the 1 hour treatment with 10 $\mu\text{mol/L}$ melphalan (arrow). Following treatment, the cells were cultured at different pHe values in the absence or presence of cariporide and S3705 (sc). Points, mean of at least three experiments; bars, SE.

following the first treatment, but after the second treatment on day 14 repopulation of the cells was observed with a doubling time of 2.4 and 4.0 days (days 14-21) at pHe 7.4 and 6.8, respectively.

Effects of cariporide and S3705 on MCF7 and MDA-MB231 cells between treatments with melphalan. In these experiments, cells were grown at different pHe values and exposed to cariporide and S3705 before the first treatment with melphalan and between treatments, although these agents were removed for 24 hours before each treatment with melphalan. For MDA-MB231 cells, the presence of these agents (a) stimulates the further loss of clonogenic cells after the first treatment with melphalan at pHe 6.8 (Fig. 3B) but not at pHe 7.0 (Fig. 3A) and (b) inhibits repopulation following the second treatment (days 14-21) at both pHe 7.0 and 6.8 (Fig. 3A and B).

The presence of cariporide and S3705 did not improve the efficacy of chemotherapy against MCF7 cells because the agents were not able to inhibit cellular proliferation between treatments (Fig. 4A and B). After 21 days, the number of clonogenically viable cells in the flasks that contained the agents is similar to those cultured in the absence of the agents.

Discussion

Several investigators have reported that increasing the acidity of the *in vitro* culture environment during treatment with melphalan increases its cytotoxicity (10, 11, 21). Although

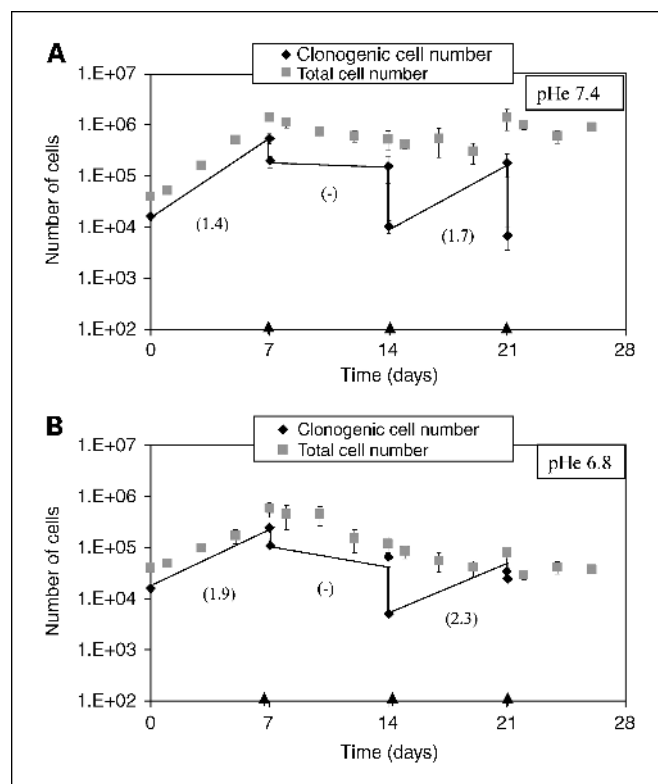


Fig. 2. Total number and number of clonogenic MDA-MB231 cells following multiple treatments with melphalan. Cells were treated with 1 hour exposures to 10 $\mu\text{mol/L}$ melphalan at pHe 7.4 every 7 days (arrows). Between treatments, cells were cultured at pHe 7.4 (A) or 6.8 (B). Points, mean of at least three experiments; bars, SE. Average doubling times in days are indicated in parentheses.

lowering the pHe of the environment increases the accumulation of melphalan in cells, this accumulation is not sufficient to account for the increased cell killing (8, 9). The results of Kuin et al. (12) suggest that a decrease in pHi as a result of the environmental acidity might be the cause of the enhanced cytotoxicity of melphalan. The decrease in pHi might affect the ability of the cell to repair its DNA during and after the treatment.

DNA repair may occur for some time (>24 hours) after treatment with an alkylating agent, such as melphalan (22-24), and factors that modulate this process can therefore influence overall cell survival. Experiments were, therefore, conducted in which cells were maintained at different pHe following treatment with melphalan, both in the presence and absence of cariporide and S3705. The pHe was maintained at 7.4 during treatment with melphalan to avoid confounding effects related to changes in drug uptake. Our results suggest that lowering the pHe of the environment after the first melphalan treatment increases cell killing in MDA-MB231 (Fig. 1A) but not in MCF7 cells (Fig. 1B). We did not observe increased cell killing at low pHe following the second administration of melphalan (Figs. 2-4). It is possible that pH-dependent cell killing is overcome by the proliferation of the surviving cells following the second treatment.

Culturing melphalan-treated cells in the presence of cariporide and S3705 at low pHe did not lead to an increase in the sensitivity of the cells; this observation is perhaps surprising because cariporide and S3705 are known to lower pHi in such

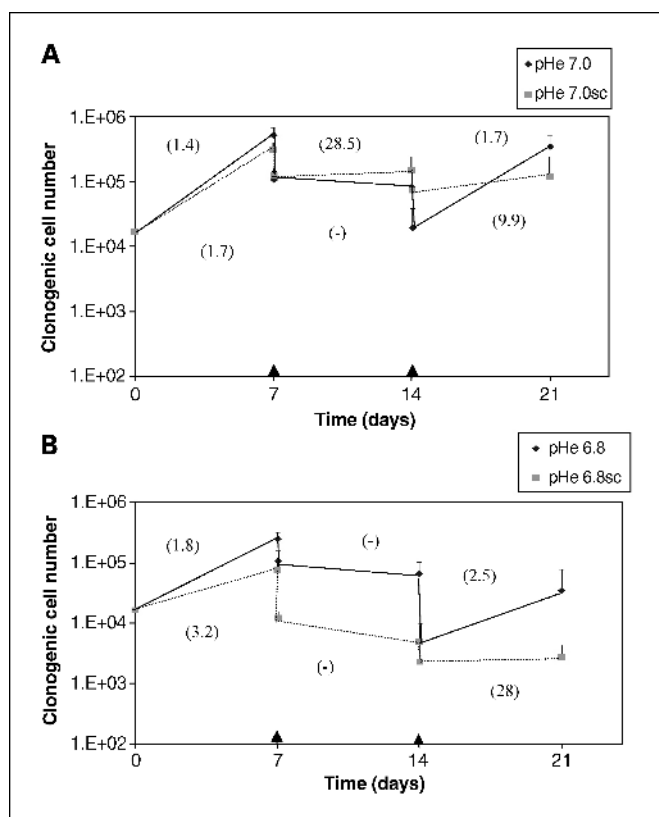


Fig. 3. The number of clonogenic MDA-MB231 cells as a function of pHe and of exposure to cariporide and S3705 following weekly treatment with melphalan. Cells were treated with 10 $\mu\text{mol/L}$ melphalan for 1 hour at pHe 7.4 every 7 days (arrows). Before and between these treatments, they were cultured at pHe 7.0 (A) or 6.8 (B) in the absence or presence of cariporide and S3705. Average doubling times in days are indicated in parentheses. Points, mean of at least three experiments; bars, SE.

cells (20). Laurecot and Kennedy (25) reported that culturing cells treated with cisplatin in the presence of 5-*N,N*-hexamethylene amiloride (an alternative inhibitor of the Na^+/H^+ antiport) and 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonic acid (an alternative but less potent inhibitor of the Na^+ -dependent $\text{HCO}_3^-/\text{Cl}^-$ exchanger than S3705) at low pHe decreased the sensitivity of the cells. They determined that this relative resistance was pH independent and was probably due to the presence of 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonic acid.

Modification of the toxicity of melphalan *in vivo* using amiloride and 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid was evaluated by Kuin et al. (12). These authors found that administering amiloride and 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid 1 hour before giving melphalan to mice increased delay to tumor regrowth (12). The tumor regrowth delay was further increased when *m*-iodobenzylguanidine or benzylguanidine, two mitochondrial inhibitors, and glucose, which cause extracellular acidosis, were administered simultaneously with amiloride and 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid before treatment with melphalan. These effects of amiloride and 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid might be specific to these agents but might also occur with the alternative inhibitors cariporide and S3705.

Our results show that repopulation of surviving cells occurs *in vitro* after the second treatment with melphalan (days 14-21)

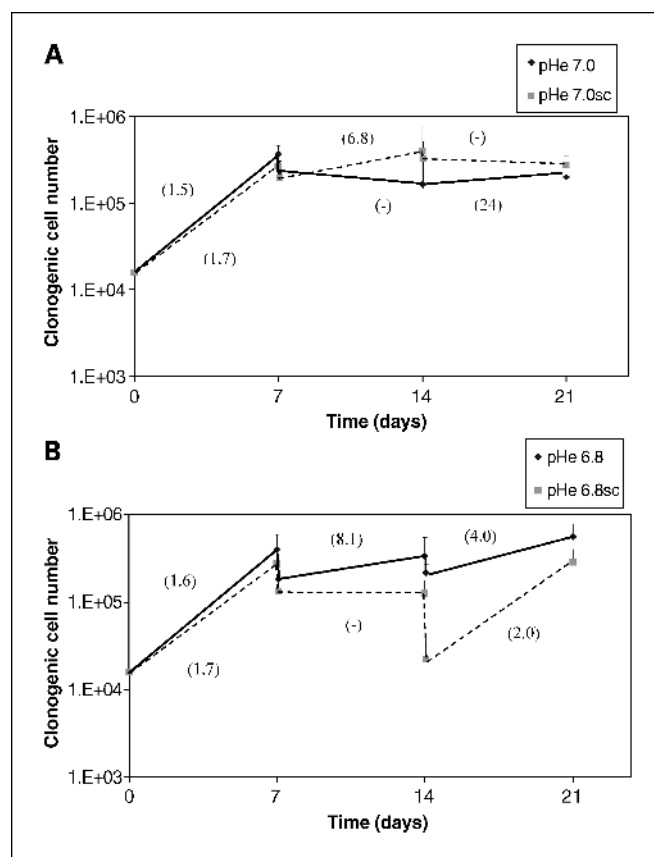


Fig. 4. The number of clonogenic MCF7 cells as a function of pHe and of exposure to cariporide and S3705 following weekly treatment with melphalan. Cells were treated with 10 $\mu\text{mol/L}$ melphalan for 1 hour at pHe 7.4 every 7 days (arrows). Before and between these treatments, they were cultured at pHe 7.0 (A) or 6.8 (B) in the absence or presence of cariporide and S3705. Average doubling times in days are indicated in parentheses. Points, mean of at least three experiments; bars, SE.

at all levels of pHe tested (Figs. 2–4). Most anticancer drugs, including melphalan, are more toxic to proliferating cells. Pu et al. (26) showed that cells arrested in the G_1 and G_2 phase are less sensitive to melphalan. Because low pHi may cause cells to accumulate at the G_1 phase, they may become less sensitive to melphalan compared with cells that were previously growing at pHe 7.4.

Our experiments suggest that S3705 and cariporide can increase net cell killing and inhibit repopulation of MDA-MB231 cells at low pHe during the intervals between treatments with chemotherapy. However, at pHe 7.0 (but not pHe 6.8), the inhibitors seemed to decrease the immediate cytotoxicity of melphalan. Even at low pH, the cells seem to proliferate in the absence of the inhibitors after the second exposure to melphalan (Figs. 2 and 3) and the inhibitors may then cause cytostatic effects and a net increase in cell kill.

Cariporide and S3705 did not increase net cell killing or inhibit repopulation of MCF7 cells. The number of clonogenically viable cells at day 21 in the flasks treated with cariporide and S3705 was similar to the number of cells in the untreated flasks at pHe 7.0 and 6.8 (Fig. 4A and B). MCF7 cells are known to have a greater capacity to regulate their pHi and lower sensitivity to the cytostatic effects of cariporide and S3705 (20, 27, 28), and this may contribute to their resistance to these compounds.

The present experiments were undertaken to provide an *in vitro* model to evaluate the therapeutic potential of new inhibitors of regulation of pHi on the net killing of tumor cells by melphalan. The effects are complex because reduced pHi can have both negative (decreased sensitivity to anticancer drugs because of

reduced cell proliferation) and positive effects (inhibition of repopulation, interference with DNA repair). Although our results suggest that modification of pHi can influence cell killing after multiple courses of melphalan, we judge these effects to be too small and inconsistent to have therapeutic application.

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