Cancer Chemother Pharmacol (1994) 34: 385–392

Cancer Chemotherapy and Pharmacology

Effect of pharmacologic doses of zinc on the therapeutic index of brain tumor chemotherapy with carmustine

Norbert Roosen[1], François Doz[2]*, Kathleen L. Yeomans[1], Dolores V. Dougherty[2], Mark L. Rosenblum[1]

[1] Department of Neurological Surgery, Henry Ford Hospital, and Midwest Neuro-Oncology Center, Henry Ford Health Sciences Center, Detroit, MI 48202, USA

[2] Brain Tumor Research Center, Department of Neurological Surgery, University of California, San Francisco, California, USA

Received: 22 October 1993 / Accepted: 11 March 1994

Abstract. To evaluate the potential differential effect of pretreatment with pharmacologic doses of the trace element zinc on the chemosensitivity of glioma cells and bone marrow cells for carmustine (BCNU), we performed in vitro and in vivo studies of zinc toxicity as well as of the combined treatment with zinc and the anticancer drug. We studied the in vitro effects on established human and rat glioma cell lines using a microcolorimetric growth assay and on murine bone marrow using a clonogenic assay for committed progenitor cells of the granulocyte-monocyte lineage. Zinc exposures of up to 100 μ*M* for 120 h did not influence the growth of six of seven human glioma cell lines. Only U87MG demonstrated statistically significant toxicity during high zinc exposure (100 μ*M* over 120 h). Dose-response growth curves generated for BCNU did not show protection against the anticancer agents by a 48-h pretreatment with different zinc concentrations. The clonogenic capacity of bone marrow cells was slightly reduced by in vitro culture for 24 and 48 h. Although this effect appeared to be more prominent in the presence of zinc supplementation, overall a statistically significant inhibition was seen only after exposure to a concentration of 100 μ*M* zinc over 48 h. As compared with chemotherapy alone, in vitro pretreatment with 50 μ*M* zinc over 48 h followed by chemotherapy resulted in an increased number of colony-forming unit-granulocyte monocyte (CFU-GM): CFU-GM increased by a factor of 2 for BCNU (60 μ*M*×2 h). This statistically significant in vitro chemoprotection would translate into a dose-protection factor of 1.5, i.e., for the same level of myelosuppression, zinc pretreatment would allow administration of a 50% increased dose of BCNU. The in vivo studies were performed in an s.c. xenograft model of the human glioma cell line U87MG in athymic mice. The maximal tolerable pretreatment with zinc was determined to be a 10-day course of daily i.p. injections of 10 mg/kg $ZnCl_2$. The subsequent i.p. administration of the dose lethal to 10% of the mice (LD_{10}) and of a 1.5×LD_{10} dose of BCNU resulted in less bone marrow toxicity in pretreated animals than in non-zinc-pretreated mice as determined in a CFU-GM assay. Glioma colony-forming efficiency (CFE) assays, on the other hand, did not show any zinc-related difference in the BCNU sensitivity of U87MG. Our in vitro and in vivo results suggest the potential usefulness of high-zinc pretreatment for improving the therapeutic index of BCNU chemotherapy for gliomas.

Key words: Brain tumor – Glioma – BCNU – Zinc – Bone marrow – CFU-GM – Therapeutic index

Introduction

One reason for the low efficacy of brain tumor chemotherapy is its unfavorable therapeutic index. Indeed, most commonly used antiglioma drugs have considerable side effects that preclude administration of higher and more cytotoxic drug doses to patients. The goal of our research has been to develop a method by which normal tissues can be protected from the side effects of anticancer drugs, thereby increasing the therapeutic index for these agents.

In the present studies we evaluated the effect of various in vitro pretreatment schedules with zinc on murine bone marrow cells as well as on the 9L rat gliosarcoma and several human glioma cell lines, and we determined the in vitro pretreatment conditions required to obtain maximal chemoprotection of bone marrow cells as compared with glioma cells against the nitrosourea compound carmustine (BCNU). In addition, we investigated the effect of in vivo high-dose zinc pretreatment on bone marrow and glioma chemosensitivity for subsequent single-dose BCNU administration.

* *Present address:* Service d'Oncologie Pédiatrique, Institut Curie, Paris, France

Correspondence to: Dr. Mark L. Rosenblum, c/o Editorial Office (Attention: S. Whitehouse), Department of Neurological Surgery, Henry Ford Hospital, 2799 West Grand Boulevard, Detroit, MI 48202, USA

Materials and methods

Glioma cell lines. The following human glioma cell lines, kept in continuous culture at the Brain Tumor Research Center (BTRC) of the University of California at San Francisco (UCSF), were used for the in vitro experiments: U87MG, U251MG, SF126, SF188, SF763, and SF767. The 9L rat gliosarcoma cell line was also studied. All cells were maintained in minimal essential medium (MEM) with Earle's balanced salt solution and glutamine (Cell Culture Facility, UCSF) supplemented with 10% fetal bovine serum (FBS; Cell Culture Facility, UCSF). For experiments, gentamicin (Cell Culture Facility, UCSF) was added to this medium (final concentration, 0.5 mg/ml). All cell incubations for routine passaging of cultures and for proliferation assays were done at 37° C in a water-saturated 95% air and 5% CO_2 mixture.

Microcolorimetric glioma cell proliferation assay. Glioma cells were harvested during the late-exponential growth phase. After trypsinization with 0.05% trypsin/0.53 m*M* ethylenediaminetetraacetic acid (EDTA; Gibco BRL Life Science Technologies) in Hanks' balanced salt solution (HBSS) without calcium and magnesium salts (Cell Culture Facility, UCSF), cells were counted using a Neubauer hemocytometer. Viability was assessed by trypan blue exclusion (Gibco BRL Life Science Technologies). The 60 central wells of 96-microwell plates (Falcon) were plated with 300 cells in 200 µl medium/well, except for 1 column of control wells, which contained only medium. Zinc supplementation was achieved by adding the appropriate amount of a $ZnCl_2$ stock solution to the tissue culture medium. The $ZnCl_2$ stock solution was prepared with distilled water and diluted to supplement the medium with 12.5, 25, 50, or 100 µ*M* zinc for the zinc toxicity studies and with 25, 50, or 100 µ*M* zinc for the zinc pretreatment studies. The 96-well plates were incubated for 24, 48, or 120 h. The medium was then changed to non-zinc-supplemented medium; zinc toxicity experiments were terminated after 120 h incubation. For zinc pretreatment studies, BCNU (Bristol-Meyers Co.) was added after 48 h of zinc preincubation and cells were exposed for 2 h to concentrations of 0, 20, 40, and 60 µ*M* BCNU. Cells were rinsed twice with HBSS, and 200 µl regular non-zinc-supplemented medium was added to each well. The cells were incubated again.

After 7 days of incubation, cells in the control wells had reached a subconfluent state and the assay was terminated using MTT (3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, Sigma Chemical Co.) [32]. MTT was dissolved in HBSS, and a total volume of 50 µl was added to each 200-µl well to obtain a final MTT concentration of 1 mg/ml. After 4 h incubation, the medium was discarded and the microtiter plates were allowed to dry overnight. Thereafter, 100 µl mineral oil (Sigma Chemical Co.) was added to each well. Overnight, the 96-well plates were gently shaken to dissolve the formazan crystals in the mineral oil. Subsequently, the plates were read with a spectrophotometer at 540 nm (Titertek). The readings were averaged and the standard deviation was calculated. Next, readings were normalized versus the control wells and expressed as a percentage.

Colony-forming unit-granulocyte monocyte assay of murine bone marrow. Mouse studies were approved by the institutional Committees on Animal Research at UCSF and at Henry Ford Hospital. Bone marrow was obtained from *nu/nu* BALB/c athymic mice (National Cancer Institute). Mice were housed in specifically designated units with a 12-h/12-h light/dark cycle. The mice had access to sterile, regular rodent food and water ad libitum. In the morning, mice were killed by cervical dislocation and the femurs were sterilely resected. Each femur was flushed with 1 ml MEM supplemented with 0.05% gentamicin to collect the bone marrow cells. The cells of several femurs were pooled.

Cell counts were made with Unopette (Becton-Dickinson), and trypan blue exclusion was used to assess viability. Appropriate dilutions were made and cells either were directly plated (for the in vivo studies) or were treated with zinc and/or BCNU according to the experimental protocol. In general, bone marrow cells were suspended in MEM supplemented with 0.05% gentamicin, 20% FBS, 25% mouse-lung conditioned medium (MLCM), and 15% agarose (2% in phosphate-buffered saline). A total volume of 250 µl of this cell dilution was plated onto an agar underlayer in four-well plates (Nunc). For control plates, 50,000 bone marrow cells/well were plated; for treatment plates, appropriately increased numbers of cells were seeded. Colony-forming unit-granulocyte monocyte (CFU-GM) scored after 10 days' incubation were identified as cell aggregates of more than 20 cells. The quantity of CFU-GM was calculated as the number of colonies divided by the number of cells plated.

For in vitro zinc toxicity studies, pooled bone marrow cells were incubated for 24 or 48 h in MEM supplemented with 20% FBS; 0.05% gentamicin; and 0, 25, 50, or 100 µ*M* zinc. Zinc pretreatment studies involved 0, 50, or 100 µ*M* zinc preincubation for 48 h followed by a 2-h treatment with 0, 20, 40, or 60 µ*M* BCNU. Ratios between zinc-preincubated and non-zinc-preincubated CFU-GM values for each BCNU treatment concentration were calculated.

MLCM was produced by challenging DBA/2 mice (Simonson Laboratory, Gilroy, Calif.) with 100 µl i.p. lipopolysaccharide (100 µg/ml) from *Salmonella typhosa* (Sigma Chemical Co.). At 4 h after the injection, mice were killed and the lungs were sterilely resected and rinsed with MEM supplemented with 0.05% gentamicin. Thereafter, the lungs were fragmented into small pieces and incubated in MEM supplemented with gentamicin for 2 days. Subsequently, the tissue fragments were centrifuged at 3,000 rpm for 30 min and the supernatant was collected. This was dialyzed against 0.9% NaCl for 24 h at 4° C (using MW12000 dialysis tubing; Arthur H. Thomas Co., Philadelphia, Pa.]). The resulting MLCM fluid was aliquoted into 3-ml vials by filtration through a 0.22-µm filter and stored at –20° C. A titration experiment was performed for every batch of MLCM to determine the optimal concentration necessary for growth of CFU-GM. This MLCM concentration was typically 20%–25%.

In vivo study of zinc toxicity. Athymic mice (*nu/nu*) of BALB/c genetic background (National Cancer Institute) were housed and fed as described above. Zinc was injected i.p. as daily doses of 1, 2, 5, 10, or 20 mg/kg $ZnCl_2$ over 10 days, with ten mice being included in each treatment group. Daily weight determinations and observation of the animals' behavior were performed. After 10 days, the animals were euthanized by cervical dislocation and the femoral bone marrow of each animal was processed for CFU-GM assay as described above.

In vivo CFU-GM assay of BCNU toxicity after zinc pretreatment. After determining daily i.p. injections of 10 mg/kg $ZnCl_2$ over 10 days as the maximal tolerable dosing schedule for zinc, this was used prior to subsequent BCNU treatment. BCNU was given as a single i.p. injection of 35 mg/kg, corresponding to the dose lethal to 10% of the animals (LD_{10}), and as 52.5 mg/kg ($1.5 \times LD_{10}$) to athymic mice carrying s.c. U87MG glioma xenografts; BCNU injection was performed on the day following the last zinc administration. Control animals were injected with a 1% ethanol solution in HBSS. Mice were euthanized the day after BCNU administration and the femoral bone marrow of each individual animal was harvested and processed for CFU-GM as described above. The mean CFU-GM value was calculated from at least four different wells for each experimental animal. Experimental groups consisted of at least 11 animals each. The results were normalized for the BCNU control animals within each pretreatment group.

In vivo colony-forming efficiency assay of BCNU toxicity after zinc pretreatment. After euthanasia of the s.c. xenograft-carrying athymic mice for bone marrow harvesting as described above, the U87MG xenografts were resected sterilely and processed for the colony-forming efficiency (CFE) assay according to previously published methods [40]. Briefly, the tumors were minced with crossed scalpels and treated with an enzyme solution containing collagenase, pronase, and DNAse in HBSS without Ca^{2+} and Mg^{2+} salts. The cell suspension was filtered through a nylon mesh, and a cell count was performed using trypan blue and a Neubauer hemocytometer. Appropriate numbers of single cells were then seeded into the wells of a six-well tissue-culture plate that contained a feeder layer of irradiated 9L cells in MEM supple-

mented with 10% FBS and 0.5 mg glutamine/ml. After a period of 14 days, the plates were fixed and stained with crystal violet in methanol and the colony number was determined. The CFE was calculated as the percentage of all cells plated that formed colonies (i.e., the number of colonies formed over the number of cells plated, expressed in percent). Each CFE value was the mean of at least six different determinations per experimental animal. At least nine experimental animals made up a treatment group. The results were normalized for the BCNU control groups within the two different zinc treatment groups.

Preparation of BCNU. BCNU stock solution was made at 54 m*M* in 100% ethanol. This and all further preparatory steps were carried out at 4° C. The stock solution was further diluted using 100% ethanol, and the final preparation of BCNU was done with HBSS immediately prior to its use. An appropriate volume of BCNU in ethanol/HBSS was added to either the bone marrow cells or the glioma cells for the in vitro studies; the final ethanol concentration was 1%, previously and also in the present control experiments determined to be without effect on proliferative or clonogenic potential under the conditions used. Cells were exposed to BCNU for 2 h. This treatment was terminated by two washings with HBSS. The cells were subsequently prepared for further incubation, i.e., glioma cells received new complete medium and bone marrow cells were processed for the CFU-GM assay. For in vivo studies, BCNU was prepared with ethanol and HBSS as described above.

Statistical analysis. The statistical significance of the in vitro experiments was evaluated using analysis of variance. We evaluated the results of the MTT assays for each cell line studied after obtaining the mean value ± SEM for 3 independent experiments involving 12 replicates each. The results of CFU-GM assays for zinc toxicity were expressed as mean values ± SEM for six independent experiments involving quadruplicate determinations. BCNU toxicity experiments were done as five independent experiments comprising quadruplicate determinations. For the zinc-BCNU experiments, ratios of the CFU-GM values at each BCNU concentration were calculated for 100 μ*M* zinc pretreatment versus no pretreatment and 50 μ*M* zinc pretreatment versus no pretreatment, respectively. The mean values ± SEM for these ratios were calculated. The in vivo CFU-GM experiments were evaluated using pairwise comparisons of two-sample *t*-tests. The CFE assays were evaluated with a Wilcoxon two-sample rank-sum test. The statistical significance level was set at 0.05; *P* values ranging between 0.10 and 0.05 were considered to be marginally significant.

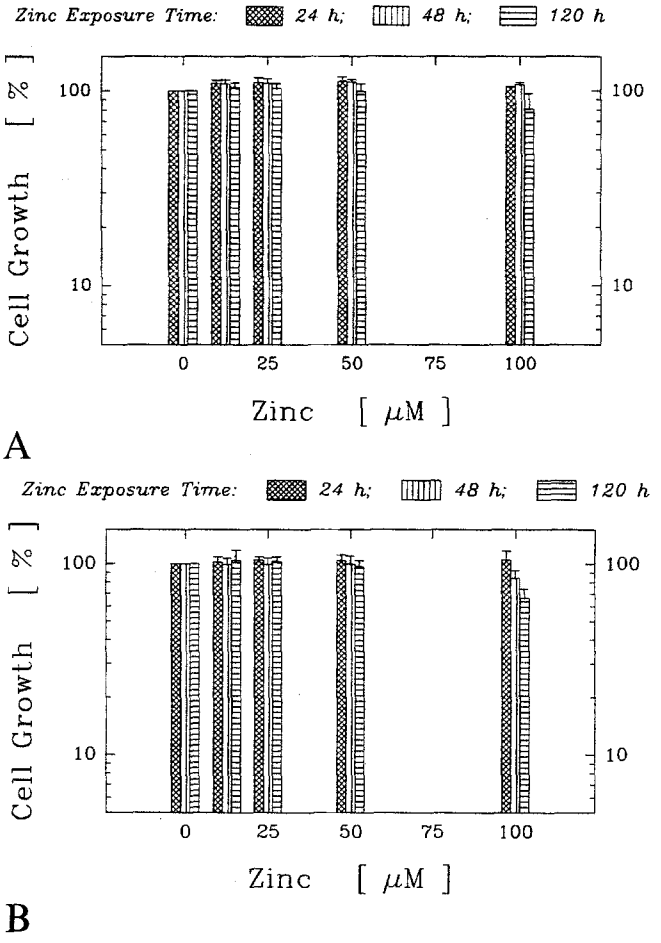

Fig. 1 A, B. Results of MTT growth assays for SF763 and U87. **A** The human glioma cell line SF763 does not demonstrate zinc toxicity except for a slight and insignificant decrease in growth after 120 h exposure to 100 μ*M* zinc. **B** The cell line U87 showed unequivocal growth inhibition at higher zinc concentrations. Data are expressed as mean values ± SEM for three separate experiments

Results

In vitro MTT assays for the zinc and zinc/BCNU chemosensitivity of glioma cell lines

The growth of 9L, U87MG, U251MG, SF763, and SF767 was not affected by exposure to up to 50 μ*M* zinc supplementation for up to 5 days. At higher concentrations a slight growth-inhibitory effect was seen only in cell lines U87MG, SF126, and SF763 during the longer exposure periods. Only U87MG seemed somewhat more sensitive to zinc-mediated growth inhibition, but statistical significance was reached only in comparisons of control versus 100 μ*M* zinc exposure for 120 h ($P < 0.01$; Fig. 1).

BCNU caused growth inhibition at 20, 40, and 60 μ*M* exposure for 2 h. SF767 was the most sensitive cell line and SF763 the most resistant (Fig. 2). 9L, U87MG, U251MG, SF126, and SF188 showed intermediate sensitivity. The chemosensitivity profile for each cell line tested was independent of the zinc pretreatment conditions. No protection against BCNU toxicity as a function of the zinc pretreatment concentration was detected.

Murine bone marrow CFU-GM assays for in vitro zinc and zinc/BCNU studies

We performed six experiments to determine the effect of zinc preincubation on CFU-GM. The CFU-GM value obtained after immediate plating in MLCM containing agar medium was 0.178% ± 0.076%. Preincubation without zinc supplementation resulted in a decreased CFU-GM yield after 1 and 2 days (0.143% ± 0.066% and 0.118% ± 0.076%, respectively). This decrease, however, was not statistically significant. Preincubation with increasing zinc concentrations for either 1 or 2 days also resulted in a decreased CFU-GM yield, which was not statistically significant as compared with the control values for the same preincubation period. The lowest CFU-GM value (0.078% ± 0.053%) was obtained at zinc concentrations of 100 μ*M* and 2 days' preincubation (Fig. 3). This was the only statistically significant value as compared with the results obtained after immediate plating ($P < 0.05$) despite the obvious trend toward a decrease in CFU-GM after longer incubation at higher zinc concentrations. Notwithstanding their variability, these data

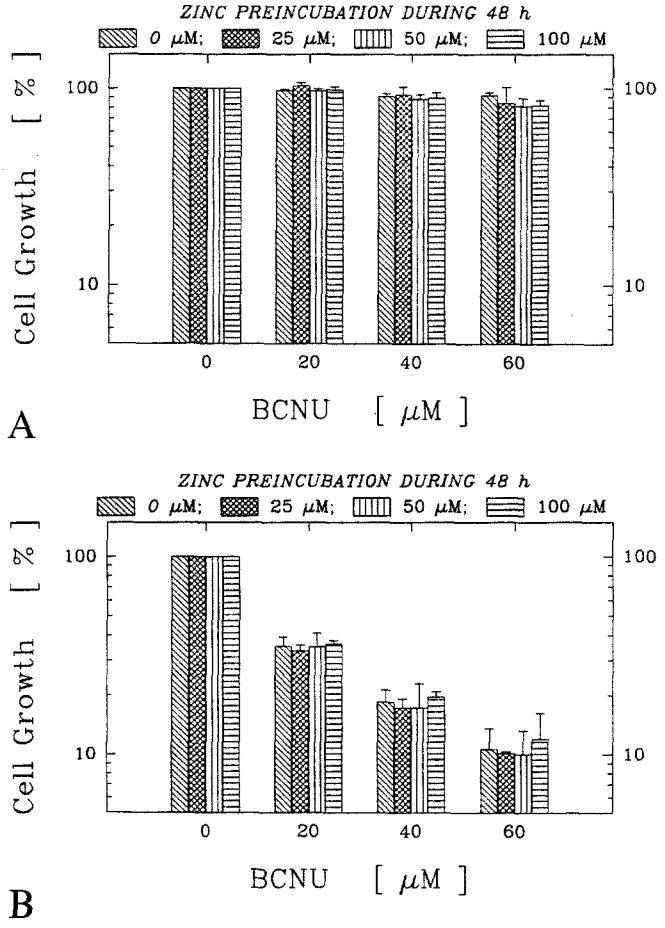

Fig. 2 A,B. Zinc preincubation for 48 h does not result in any change in the BCNU chemosensitivity of the glioma lines SF763 **A** and SF767 **B**. Data are expressed as mean values ± SEM for three independent experiments

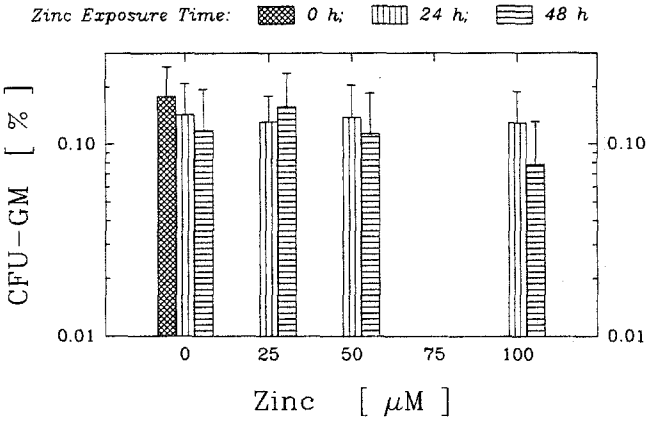

Fig. 3. The results of CFU-GM assay of murine bone marrow suggest some zinc toxicity after 24 h and 48 h exposure in vitro. Although these results are suggestive, the only statistically significant toxicity was found after 48 h of 100-μ*M* zinc supplementation. Data are expressed as mean values ± SEM for six independent experiments

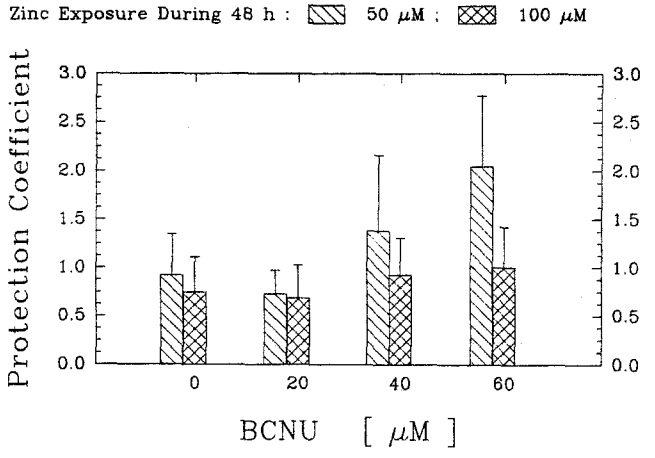

Fig. 4. Bone marrow CFU-GM results, expressed as chemoprotection coefficients (i.e., the ratio between CFU-GM of zinc-pretreated versus non-zinc-pretreated bone marrow for a given BCNU exposure; see text), indicate a zinc-related protection against BCNU cytotoxicity. Pretreatment with 50 μ*M* zinc for 48 h confers statistically significant protection against 60 μ*M* BCNU ($P < 0.05$). Data from five independent experiments

suggest that preincubation for 1 and 2 days tends to reduce progressively the number of CFU-GM found and that zinc concentrations of up to 50 μ*M* preincubation supplementation do not influence the CFU-GM yield.

Five experiments showed that pretreatment with 50 μ*M* zinc supplementation for 48 h resulted in chemoprotection against BCNU bone marrow toxicity as compared with regular, non-supplemented incubation conditions (Fig. 4). This difference in the CFU-GM ratio was obvious at 60 μ*M* BCNU. At the higher zinc supplementation of 100 μ*M*, the yield was somewhat lower, but not as low as the control results. The chemoprotection coefficient obtained at 60 μ*M* BCNU in comparing CFU-GM at 50 μ*M* zinc versus no supplementation was 2.05 ± 0.73 ($P < 0.05$).

In vivo zinc toxicity in nu/nu mice

Marked weight loss was found after 10 days of daily i.p. injections of 20 mg/kg $ZnCl_2$, whereas no change was detected with the lower doses of zinc. In addition, no significant effect of zinc on CFU-GM was found at any of the dose levels used (Fig. 5). Therefore, a 10-day course of 10 mg/kg $ZnCl_2$ per day i.p. was chosen as the maximal tolerable schedule for subsequent in vivo chemoprotection studies.

In vivo murine bone-marrow CFU-GM assay for zinc/BCNU studies

The CFU-GM assay data reveal a dose-response effect of BCNU (Fig. 6). The percentage of CFU-GM surviving BCNU administration after zinc pretreatment was higher than that obtained without this pretreatment. This was the case for LD_{10} BCNU as well as for 1.5×LD_{10} BCNU, i.e., 56.5% ± 27.8% versus 32.9% ± 15.4% and 46.9% ± 18.4% versus 33.5% ± 13.9% (both: $P < 0.02$).

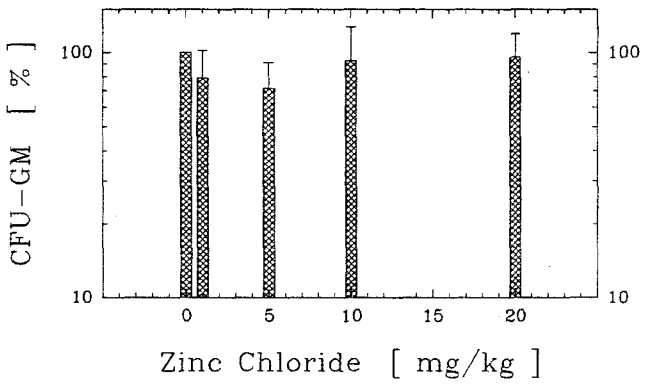

Fig. 5. In vivo pretreatment with daily i.p. injections of up to 20 mg/kg $ZnCl_2$ over 10 days does not significantly influence the CFU-GM yield. Data are expressed as mean values ± SD for 6 mice/experimental group

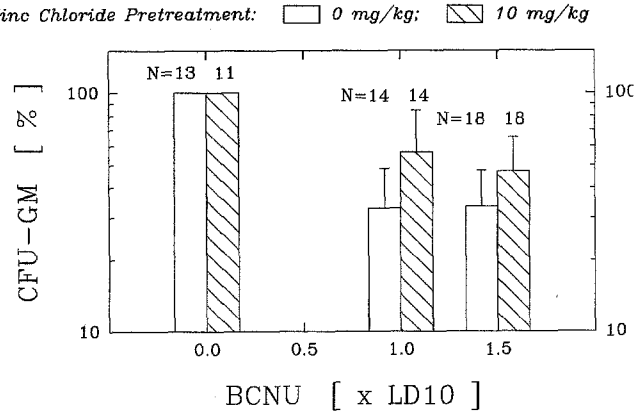

Fig. 6. In vivo high-zinc pretreatment results in an increased CFU-GM yield after BCNU administration. BCNU toxicity is significantly reduced in the zinc pretreatment group ($P < 0.001$). Data are expressed as the normalized mean value ± SD per experimental group

However, contrary to the 2-fold chemoprotection coefficient predicted according to the in vitro studies, the CFU-GM value recorded for the zinc-pretreated 1.5×LD_{10} BCNU group did not quite reach the same level as that obtained for the non-zinc-pretreated LD_{10} BCNU group, i.e., 46.9% ± 18.4% versus 32.9% ± 15.4% ($P < 0.05$).

In vivo U87MG-xenograft CFE assays for zinc/BCNU studies

No difference in the CFE results was observed between the high-zinc- and the non-zinc-pretreatment groups (Fig. 7). The BCNU dose response was the same in both pretreatment groups.

Discussion

Although knowledge of brain tumor biology and therapy is continuously evolving [1, 43], this scientific progress has

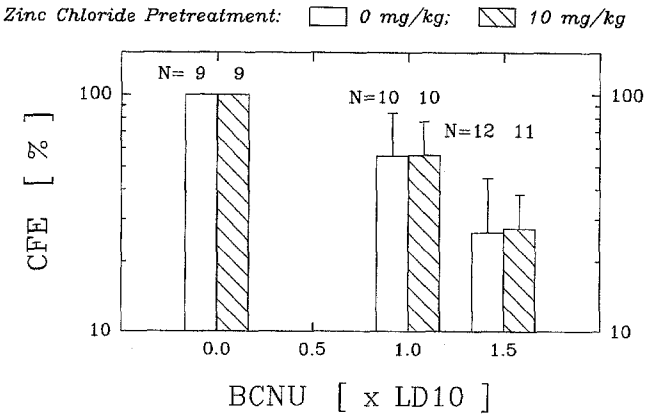

Fig. 7. High-zinc pretreatment in vivo does not cause any change in the BCNU chemosensitivity of s.c. U87MG xenografts in athymic mice as evaluated with a CFE assay. Data are expressed as the normalized mean value ± SD per experimental group

not yet resulted in an improved clinical prognosis for patients suffering from this disease. Patients with brain tumors infrequently live longer than 18 months, especially when diagnosed with glioblastoma or malignant astrocytoma, and a large number of patients die within 12 months despite aggressive treatment [42]. Part of the reason for this ineffectual treatment is the low therapeutic index of commonly used, newer or investigational chemotherapeutic drugs. Indeed, although in vitro studies of glioma cells often demonstrate a good dose-response curve and efficient cell kill [39], such results are usually obtained at drug exposures that are toxic for patients. An effective and easily tolerated method of normal tissue protection would substantially increase the therapeutic index of these anticancer agents. Although an improved therapeutic index may not lead to a cure of the disease – even autologous bone marrow transplantation has not achieved this goal [14] – it could result in more and/or longer remissions and certainly would make brain tumor chemotherapy more tolerable to patients.

The purpose of the present investigation was to increase the therapeutic index of brain tumor chemotherapy with BCNU by using zinc pretreatment to protect bone marrow selectively from the cytotoxicity of this commonly used anticancer agent for malignant glioma. Zinc was chosen because of (a) its ability to induce metallothionein (MT), a small cystein-rich protein that has been suggested to play an important role in cellular resistance against anticancer drugs [2, 23, 25]; (b) its specific properties with regard to the blood-brain barrier [22, 38]; and (c) its numerous other stabilizing and protective biological effects on normal cells [4, 8–10, 16, 24, 31, 36].

Indeed, zinc is an important trace element in biology and has a multitude of biological effects. It is involved in many enzymatic reactions either as a structural component of the enzyme or as a cofactor [8]. Transcription factors often have zinc-fingers necessary for protein-DNA interaction [36]. Zinc is necessary for membrane stability [4, 24] and important for protein kinase C activity [10, 16]. Furthermore, zinc protects cells from apoptosis [9, 31], which hypothetically is a mechanism of action of platinum com-

pounds and other anticancer drugs [3]. Furthermore, MT can be induced by zinc and has been implicated in cellular resistance against various anticancer agents, particularly platinum compounds, as well as alkylating agents. High intracellular MT concentrations have been detected in cells resistant to platinum compounds [23], but the importance of this finding for cisplatin resistance has been debated [13]. However, cells transfected with and hyperexpressing the MT gene are highly resistant against platinum compounds and other anticancer drugs [25]. Platinum compounds have been postulated to react with MT, thereby becoming inactivated [2]. Recently, Pattaniak et al. [37] demonstrated that the Pt atom from *cis*-dichlorodiammineplatinum(II) binds stoichiometrically to MT thiol groups and that zinc atoms are displaced. Also, MT is supposed to act as a free-radical scavenger [49].

Hypothetically, the chemical properties of MT might play a role in its detoxifying effect on highly reactive intermediates of compounds such as the nitrosoureas. Because of its above-described effects and because zinc is a nontoxic [7, 34], potent inducer of MT [18, 34], it is a good candidate for pretreatment to increase cellular resistance against cytotoxic drugs. Interestingly for neurooncology, there is a major barrier to zinc penetration into the brain; indeed, the blood-brain barrier is highly impermeable to zinc [38], and systemic administration of zinc does not induce MT in brain tissue as does intraventricular administration [22]. This finding is of some relevance because the blood-brain barrier in tissue adjacent to a tumor is not broken down to the same extent as in the tumor tissue itself [19] and because tissue adjacent to a tumor does contain a certain number of invading and infiltrating glioma cells [20]. It can therefore be hypothesized that these cells probably will not be influenced by systemic zinc pretreatment, regardless of the actual mechanism of action of zinc. Nevertheless, to obviate any concerns that zinc might adversely affect glioma cell chemosensitivity, we also investigated the effect of zinc supplementation on the in vitro as well as in vivo chemosensitivity of various glioma cell lines. Our results clearly show that there was no zinc-induced change in the BCNU chemosensitivity of any of the cell lines studied in vitro or in that of U87MG, which was additionally evaluated as a xenograft in vivo. Recently, MT induction in astrocytoma cells by zinc has been reported [26]. Our own preliminary immunohistochemistry studies of several other glioma cell lines showed MT induction by zinc (unpublished observations); however, the extent of MT induction was rather small. These findings together with the data from our MTT assays suggest that MT induction by zinc in glioma cells is not significant enough to cause a change in chemosensitivity.

Bone marrow is a major focus of cytotoxicity from anticancer therapy [21]. This includes anemia, thrombocytopenia, and leukopenia. Several assay systems are available to quantify myelotoxicity, ranging from peripheral blood counts to sophisticated stem cell assays. We chose to investigate CFU-GM in the present experiments because leukopenia is clinically important for most anticancer drugs and because a CFU-GM assay evaluating committed progenitor cells correlates well with global bone marrow quality [41].

The CFU-GM results of in vitro pretreatment studies show more variability than the MTT assays and are therefore somewhat more difficult to interpret. Nevertheless, preincubation of these bone marrow cells with zinc prior to exposure to 60 μM BCNU clearly resulted in an increased yield of CFU-GM as compared with control values. The chemoprotection coefficient, expressed as the ratio of the CFU-GM value obtained at a certain BCNU concentration after zinc preincubation over that obtained at the same BCNU concentration without zinc preincubation, was 2.05 ± 0.73 for 60 μM BCNU (five experiments). Although this result was somewhat variable throughout the five experiments, it was consistent enough overall to reach good statistical significance ($P < 0.05$). As compared with the clinically achievable concentration of 20 μM in humans [29], however, 60 μM BCNU is relatively high. Interestingly, these in vitro studies with BCNU showed the same amount of chemoprotection as both our previous in vivo study with carboplatin (CBDCA) [12] and the results recently reported for ziduvidine [17], i.e., a factor of approximately 2. This suggests the possibility of increasing the BCNU dose by 50% after high-zinc pretreatment without changing bone marrow cytotoxicity as compared with conventional non-zinc-pretreatment chemotherapy.

The in vivo studies were performed after we had evaluated an intense but practicable zinc pretreatment schedule and determined that a daily i.p. dose of 10 mg/kg $ZnCl_2$ over 10 days was well tolerated by mice. Then, BCNU hematotoxicity as assayed by CFU-GM was shown to be significantly lower after zinc pretreatment. The overall effect, however, was not quite as large as would be predicted by the in vitro results, i.e., the $1.5 \times LD_{10}$ BCNU dose after zinc pretreatment did not result in the same CFU-GM yield as did an LD_{10} BCNU dose without high-zinc pretreatment. This observation might have been the result of a suboptimal zinc pretreatment schedule. More studies with different variations of the zinc pretreatment schedule would be necessary to address this issue. On the other hand, a major difficulty with zinc is that the relationship between in vitro zinc levels and in vivo zinc loading is hard to determine (see below). Another factor to be considered is that the mice in this study can be assayed for CFU-GM only once due to the nature of the bone marrow harvesting process. As opposed to serial peripheral blood cell counts, in bone marrow CFU-GM experiments the animals cannot serve as their own controls. This causes increased variability in the results and necessitates larger sample sizes.

Despite the aforementioned limitations, the in vivo CFU-GM and CFE results unquestionably support the hypothesis of an improved therapeutic index for BCNU by zinc pretreatment. Our findings are similar to those of Shackelford and Tobey [45], who described a zinc-related decrease in the nitrogen mustard-induced mortality of non-tumor-bearing mice from 57% to 20%. Despite these results, however, these investigators could not show an important therapeutic benefit of their protocols in terms of the survival of tumor-bearing mice [46]. A major difference in our study was the use of a much more intensive and longer zinc pretreatment regimen and the use of the nitrosourea BCNU instead of the alkylating agent nitrogen mustard. Also, these authors focused on animal survival instead of

bone marrow toxicity, the latter probably being of more clinical relevance. Finally, although not formally statistically evaluated, their result was dependent upon the delivered dose of nitrogen mustard. Indeed, at a moderate dose of the alkylating agent their survival data suggest a therapeutic benefit, whereas this is not the case for the higher dose.

Although zinc pretreatment for selective chemoprotection of bone marrow is appealing, zinc therapy is unfortunately difficult to monitor. The zinc serum level is the parameter used most often to determine zinc status [27, 47]; MT determinations in plasma or in red blood cells have also been advocated for this purpose but are difficult and not widely done [18]. The normal zinc serum level is approximately 15 μM, or 1.00 μg/ml, but this level is highly variable [11, 51]. Our non-zinc supplemented medium for cell culture contains 4.13 μM zinc (atomic absorption spectrometry, Department of Clinical Laboratory Medicine, UCSF). Also, a major difference exists between extracellular and intracellular zinc concentrations. A significant proportion of the intracellular zinc pool is bound to subcellular structures resulting in an uneven distribution [5, 15, 52]. Because of the difficulties involved in correlating zinc supplementation with a specific zinc concentration range, we preferred to challenge glioma cells and bone marrow cells with different zinc concentrations and to focus on the maximal zinc concentrations that were well tolerated. This is the best in vitro approximation to similar in vivo studies with high-zinc supplementation.

We prefer zinc to other trace metals (e.g., bismuth and selenium) that have been used for chemoprotection [6, 33, 44]. Studies using these other elements have shown protection against cisplatin's nephrotoxicity, with some reporting a decrease in hematologic toxicity as well [6, 33, 44]; however, these studies used peripheral blood counts and not clonogenic assays. The actual mechanism of action of these different trace elements is not yet clear at all. As discussed above, zinc has a multitude of effects and is involved in many biological processes. This is less the case for selenium and certainly for bismuth [30, 48]. A highly selective MT induction in normal tissue as opposed to no induction of MT in tumor tissue has been claimed to be a distinct advantage for bismuth as compared with a more generalized MT induction by zinc [28]. On the other hand, the relative importance of MT for cellular resistance against cytotoxic agents is being debated [13].

Our present findings together with previously published results [12, 17, 45, 46, 50] support the hypothesis of a selective protective action of high-zinc pretreatment against the side effects of anticancer drugs, specifically BCNU. The effect on BCNU is important because this nitrosourea compound is widely used as an antitumor agent in neurooncology. In addition to its myeloprotection, high-dose zinc pretreatment might also be effective against other side effects (nephrotoxicity, lung fibrosis, cardiac toxicity) because zinc reaches all organ systems (except the brain and testis, due to the capillary barriers in these two organs [22, 35, 38]). We are therefore continuing our efforts to investigate zinc's effects on the toxicity of anticancer drugs and are presently focusing on the mechanistic aspects of zinc's protective effects.

Acknowledgements. This work was accomplished at the University of California, San Francisco (UCSF), and at Henry Ford Hospital and was funded in part by a 1991–1992 REAC grant from UCSF and by American Cancer Society Grant DHP-6. Dr. N. Roosen was the recipient of a 1990–1991 National Cancer Institute/European Organization for Research and Treatment of Cancer Exchange Fellowship Award. The authors would like to acknowledge the statistical advice given by George Divine and Trey Spencer (Department of Biostatistics and Research Epidemiology, Henry Ford Hospital).

References

1. Apuzzo MLJ (Ed) (1990) Malignant cerebral glioma. American Association of Neurological Surgeons, Park Ridge, Illinois
2. Bakka A, Endresen L, Johnsen ABS, Edminson PD, Rugstad HE (1981) Resistance against *cis*-dichlorodiammineplatinum in cultured cells with a high content of metallothionein. Toxicol Appl Pharmacol 61: 215–226
3. Barry MA, Behnke CA, Eastman A (1990) Activation of programmed cell death (apoptosis) by cisplatin, other anticancer drugs, toxins, and hyperthermia. Biochem Pharmacol 40: 2353–2362
4. Bashford CL, Alder SM, Graham JM, Menestrina G, Pasternak CA (1988) Ion modulation of membrane permeability: effect of cations on intact cells and on cells and phospholipid bilayers treated with pore-forming agents. J Membr Biol 103: 79–94
5. Bettger WJ, O'Dell BL (1981) A critical physiological role of zinc in the structure and function of biomembranes. Life Sci 28: 1425–1438
6. Boogaard PJ, Slikkerveer A, Nagelkerke JF, Mulder GJ (1991) The role of metallothionein in the reduction of cisplatin-induced nephrotoxicity by Bi^{3+}-pretreatment in the rat in vivo and in vitro – are antioxidant properties of metallothionein more relevant than platinum binding? Biochem Pharmacol 41: 369–375
7. Brown MA, Thom JV, Cora P, Juarez J (1964) Food poisoning involving zinc contamination. Arch Environ Health 8: 657–660
8. Coleman JE (1992) Zinc proteins: enzymes, storage proteins, transcription factors, and replication proteins. Annu Rev Biochem 61: 897–946
9. Cotter TG, Lennon SV, Glynn JG, Martin SJ (1990) Cell death via apoptosis and its relationship to growth. Development and differentiation of both tumour and normal cells. Anticancer Res 10: 1153–1160
10. Csermely P, Somogyi J (1989) Zinc as a possible mediator of signal transduction in T lymphocytes. Acta Physiol Hung 75: 195–199
11. Cunnane SC (1988) Zinc: clinical and biochemical significance. CRC, Boca Raton, pp 11–57
12. Doz F, Berens ME, Deschepper CF, Dougherty DV, Bigornia V, Barker M, Rosenblum ML (1992) Experimental basis for increasing the therapeutic index of *cis*-diamminedicarboxylatocyclobutaneplatinum(II) in brain tumor therapy by a high-zinc diet. Cancer Chemother Pharmacol 29: 219–226
13. Eastman A (1991) Mechanisms of resistance to cisplatin. In: Ozols RF (ed) Molecular and clinical advances in anticancer drug resistance. Kluwer, Boston, pp 233–249
14. Finley JL, August C, Packer R, Fil AC Jr, Zimmerman R, Sutton L, Freid A, Rorke L, Bayever E, Kamani N, Kramer E, Cohen B, Sturgill B, Nachman J, Strendjord S, Turaki P, Frierdich S, Steeves R, Javid M (1990) High-dose multi-agent chemotherapy followed by bone marrow "rescue" for malignant astrocytomas of childhood and adolescence. J Neurooncol 9: 239–248
15. Forbes IJ, Zalewski PD, Giannakis C, Betts WH (1990) Zinc induces specific association of PKC with membrane cytoskeleton. Biochem Int 22: 741–748
16. Forbes IJ, Zalewski PD, Giannakis C, Petkoff HS, Cowled PA (1990) Interaction between protein kinase C and regulatory ligand is enhanced by a chelatable pool of cellular zinc. Biochim Biophys Acta 1053: 113–117

17. Gogu SR, Agrawal KC (1993) Modulation of drug-induced hematopoietic toxicity with zinc in murine bone marrow cells (MBC). Proc Am Assoc Cancer Res 34: 286
18. Grider A, Bailey LB, Cousins RJ (1990) Erythrocyte metallothionein as an index of zinc status in humans. Proc Natl Acad Sci USA 87: 1259–1262
19. Groothuis DR, Fischer JM, Lapin G, Bigner DD, Vick NA (1982) Permeability of different experimental brain tumor models to horseradish peroxidase. J Neuropathol Exp Neurol 41: 164–185
20. Halperin EC, Burger PC, Bullard DE (1988) The fallacy of the localized supratentorial malignant glioma. Int J Radiat Oncol Biol Phys 15: 505–509
21. Hoagland HC (1984) Hematologic complications of cancer chemotherapy. In: Perry MC, Yarbro JW (eds) Toxicity of chemotherapy. Grune & Stratton, Orlando, pp 434–448
22. Itoh M, Ebadi M, Swanson S (1983) The presence of zinc-binding proteins in brain. J Neurochem 41: 823–829
23. Kasahara K, Fujiwara Y, Nishio K, Ohmori T, Sugimoto Y, Komiya K, Matsuda T, Saijo N (1991) Metallothionein content correlates with the sensitivity of human small cell lung cancer cell lines to cisplatin. Cancer Res 51: 3237–3242
24. Kaszuba M, Hunt GRA (1990) Protection against membrane damage: a ^{1}H-NMR investigation of the effect of Zn^{2+} and Ca^{2+} on the permeability of phospholipid vesicles. J Inorg Chem 40: 217–225
25. Kelley SL, Basu A, Teicher BA, Hacker MP, Hamer DH, Lazo JS (1988) Overexpression of metallothionein confers resistance to anticancer drugs. Science 241: 1813–1815
26. Kikuchi Y, Irie M, Kasahara T, Sawada J, Terao T (1993) Induction of metallothionein in a human astrocytoma cell line by interleukin-1 and heavy metals. FEBS Lett 317: 22–26
27. King JC (1990) Assessment of zinc status. J Nutr 120: 1474–1479
28. Kondo Y, Satoh M, Imura N, Akimoto M (1992) Tissue-specific induction of metallothionein by bismuth as a promising protocol for chemotherapy with repeated administration of *cis*-diamminedichloroplatinum(II) against bladder tumor. Anticancer Res 12: 2303–2308
29. Kornblith PL, Smith BH, Leonard LA (1981) Response of cultured human brain tumors to nitrosoureas. Cancer 47: 255–265
30. Lambert JR (1991) Pharmacology of bismuth containing compounds. Rev Infect Dis 13(Suppl 8): S691-S695
31. Martin SJ, Mazdai G, Strain JJ, Cotter TG, Hannigan BM (1991) Programmed cell death (apoptosis) in lymphoid and myeloid cell lines during zinc deficiency. Clin Exp Immunol 83: 338–343
32. Naganuma A, Satoh M, Yokoyama M, Imura N (1983) Selenium efficiently depressed toxic side effect of *cis*-diamminedichloroplatinum. Res Commun Chem Pathol Pharmacol 42: 127–134
33. Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65: 55–63
34. National Research Council (1980) Recommended dietary allowances, 9th edn. National Academy of Sciences, Washington, D.C.
35. Onosaka S, Cherian MG (1982) The induced synthesis of metallothionein in various tissues of rats in response to metals. II. Influence of zinc status and specific effect on pancreatic metallothionein. Toxicology 23: 11–20
36. Pabo CO, Sauer RT (1992) Transcription factors: structural families and principles of DNA recognition. Annu Rev Biochem 61: 1053–1095
37. Pattaniak A, Bachowski G, Laib J, Lemkuil D, Shaw CF III, Petering DH, Hitchcock A, Saryan L (1992) Properties of the reaction of *cis*-dichlorodiammineplatinum(II) with metallothionein. J Biol Chem 267: 16121–16128
38. Pullen RGL, Franklin PA, Hall GH (1991) ^{65}Zn uptake from blood into brain in the rat. J Neurochem 56: 485–489
39. Rosenblum ML, Gerosa MA (1984) Stem cell sensitivity. In: Rosenblum ML, Wilson CB (eds) Brain tumor therapy. (Progress in experimental tumor research series, vol 28) Karger, Basel, pp 1–17
40. Rosenblum ML, Knebel KD, Wheeler KT, Barker M, Wilson CB (1975) Development of an in vitro colony formation assay for the evaluation of in vivo chemotherapy of a rat brain tumor. In Vitro 11: 264–273
41. Rowley SD, Zuehlsdorf M, Braine HG, Colvin OM, Davis J, Jones RJ, Saral R, Sensenbrenner LL, Yeager A, Santos GW (1987) CFU-GM content of bone marrow graft correlates with time to hematologic reconstitution following autologous bone marrow transplantation with 4-hydroperoxycyclophosphamide-purged bone marrow. Blood 70: 271–275
42. Salcman M (1990) Epidemiology and factors affecting survival. In: Apuzzo MLJ (ed) Malignant cerebral glioma. American Association of Neurological Surgeons, Park Ridge, Illinois, pp 95–109
43. Salcman M (ed) (1991) Neurobiology of brain tumors. (Concepts in neurosurgery series, vol 4.) Williams & Wilkins, Baltimore
44. Satoh M, Naganuma A, Imura N (1989) Optimum schedule of selenium administration to reduce lethal and renal toxicities of *cis*-diamminedichloroplatinum in mice. J Pharmacobiodyn 12: 246–253
45. Shackelford ME, Tobey RA (1992) Preliminary report on the use of zinc in vivo to protect against nitrogen mustard toxicity in female Balb/cJ mice. J Appl Toxicol 12: 291–293
46. Shackelford ME, Tobey RA (1992) Attempted use of zinc in vivo to protect against nitrogen mustard toxicity in tumor-free and in L1210 leukemia-bearing female B6D2F$_1$ mice. J Appl Toxicol 12: 295–300
47. Shapcott D (1982) Hair and plasma in the diagnosis of zinc deficiency. In: Prasad AS, Dreosti IE, Hetzel BS (eds) Clinical applications of recent advances in zinc metabolism. Alan R. Liss, New York, pp 121–139
48. Stadtman TC (1990) Selenium biochemistry. Annu Rev Biochem 59: 119–127
49. Thornalley PJ, Vasak M (1985) Possible role for metallothionein in protection against radiation-induced oxidative stress. Kinetics and mechanism of its reaction with superoxide and hydroxyl radicals. Biochim Biophys Acta 827: 36–44
50. Tobey RA, Tesmer JG (1985) Differential response of cultured human normal and tumor cells to trace element induced resistance to the alkylating agent melphalan. Cancer Res 45: 2567–2571
51. Versieck J, Cornelis R (1980) Normal levels of trace elements in human blood plasma or serum. Anal Chim Acta 116: 217–254
52. Zalewski PD, Forbes IJ, Giannakis C, Cowled PA, Betts WH (1990) Synergy between zinc and phorbol ester in translocation of protein kinase C to cytoskeleton. FEBS Lett 273: 131–134