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TO CHEMOTHERAPY

AUTHOR(S): R. A. Tobey, M. D. Enger, J. K. Griffith, C. E. Hildebrand

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Los Alamos Los Alamos National Laboratory
Los Alamos, New Mexico 87545

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A POTENTIALLY NOVEL APPROACH TO CHEMOTHERAPY

Robert A. Tobey, Toxicology Group, Life Sciences Division, Los Alamos National Laboratory, University of California, P.O. Box 1663, MS 880, Los Alamos, New Mexico 87545

M. Duane Enger, Genetics Group, Life Sciences Division, Los Alamos National Laboratory, University of California, P.O. Box 1663, MS 886, Los Alamos, New Mexico 87545

Jeffrey K. Griffith, Genetics Group, Life Sciences Division, Los Alamos National Laboratory, University of California, P.O. Box 1663, MS 886, Los Alamos, New Mexico 87545

C. Edgar Hildebrand, Genetics Group, Life Sciences Division, Los Alamos National Laboratory, University of California, P.O. Box 1663, MS 886, Los Alamos, New Mexico 87545

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Robert A. Tobey, M. Duane Enger, Jeffrey K. Griffith, and C. Edgar Hildebrand

Toxicology and Genetics Groups, Life Sciences Division, Los Alamos National Laboratory,
University of California, Los Alamos, New Mexico 87545

SUMMARY

A number of toxic chemical and physical agents elicit the induction of a series of protein species, some of which react with the agents and render them non-toxic. A few of the induced species (e.g., metallothionein) are rich in thiol groups which might be expected to react with alkylating agents and render them non-toxic. If a safe means could be found for selectively enhancing the synthesis of alkylating agent-reactive species in normal but not tumor cells, such a procedure would have ramifications in the area of cancer chemotherapy. In this report, we have utilized a variety of trace elements (Zn, Se, Cu, As) as inducers of synthesis of protective species in line CHO Chinese hamster cells and in a number of derived variants to determine whether this type of approach can be utilized to increase resistance to alkylating agent toxicity. Our results indicate that Zn, Se and Cu elicit a protective response (increased survival, monitored by colony-forming ability) against the toxic effects of iodoacetate or melphalan, and, at least in the case of zinc, at levels which are physiologically reasonable. Arsenite appears to be a marginally effective inducer in the CHO cell and an ineffective inducer in the Cd^r20F4 variant cell. The increased survival is not attributable to metallothionein inducibility, decreased availability of the alkylating agent in the medium or decreased uptake of the drug into the trace element-pretreated cells. The protective responses induced by zinc or selenite alone are additive in cells receiving both trace elements prior to exposure to alkylating agent which suggests that different domains of response are elicited by the two metals. Based upon reported differences in inducibility of protective proteins between

normal and tumor cells, a possibility is raised for a novel approach to alkylating agent chemotherapy, somewhat analogous to the protocol utilized in high dose methotrexate therapy.

INTRODUCTION

Alkylating agents represent an extremely useful class of anticancer drugs which are effective against a wide range of tumors. A major problem associated with their employment, however, is their toxicity for hematopoietic tissue which results in cumulative myelosuppression.^{1,2} To enhance the usefulness of alkylating agents in a clinical setting, it would be helpful if a technique could be developed which would increase selectively the resistance of normal cells over that of tumor cells in a manner analogous to the protocol utilized in high dose methotrexate therapy.^{3,4}

A possible approach to achieving this goal involves inducible protective proteins. A number of toxic physical and chemical agents (including alkylating agents) elicit the induction of a series of protein species,⁵⁻¹⁰ some of which react with certain agents and render them non-toxic. A few of the induced species (e.g., metallothionein) are rich in sulfhydryl groups which might be expected to react with alkylating agents and play a role in their detoxification. If a means could be found to induce the synthesis of alkylating agent-reactive species safely and prior to exposure to the drug, then those induced species would be capable of increasing cellular resistance to alkylating agent toxicity.

As part of a long-term program to investigate inducible protective proteins, we have examined the capacity of several trace elements to serve as inducers of a protective response in line CHO Chinese hamster cells and several cadmium-resistant variants against subsequently-administered alkylating agents such as lodonacetate and melphalan. Zinc, selenium, copper and arsenic all were able to increase the resistance of cells (monitored as colony-forming ability) to alkylating-agent toxicity, and there were indications that different agents elicited different domains of response. Thus, it appears that resistance to alkylating agents can be modulated by manipulation of the level of available trace

elements. Based upon reported differences in inducibility of protective proteins in normal and in tumor cells, a novel approach to alkylating agent chemotherapy is proposed.

MATERIALS AND METHODS

The cells utilized in this report were all derived from line CHO Chinese hamster cells and were selected by exposing the cells to ever-increasing concentrations of CdCl_2 (0.2 to 30 μM), after which cultures exhibiting the control (untreated) growth rate were cloned.¹¹ The derived variants which were resistant to 2, 20 or 30 μM CdCl_2 were designated $\text{Cd}^r2\text{C10}$, $\text{Cd}^r20\text{F4}$ and $\text{Cd}^r30\text{F9}$, respectively, and they were shown to maintain their resistance to cadmium during a six-month period of maintenance in Cd-free medium.¹¹ Suspension cultures of cells were grown in F-10 medium supplemented with 15% neonate calf serum, penicillin and streptomycin.

Stock solutions of ZnCl_2 , K_2SeO_3 and CuSO_4 were prepared in 0.01 N HCl, sterilized by filtration through a 0.22 μ filter, dispensed into polyethylene tubes and stored at either -20°C (ZnCl_2 and K_2SeO_3) or at room temperature (CuSO_4); dilutions were prepared in distilled water. Sodium arsenite was weighed out immediately prior to use, dissolved in distilled water and sterilized by filtration. Solutions of iodoacetate were prepared immediately prior to utilization by dissolving the alkylating agent in serum-free medium, sterilization by filtration and dilution in sterile serum-free medium. Melphalan (L-PAM, sarcolysin, L-phenylalanine mustard), NSC 8806, was generously provided by Dr. David Abraham, Investigational Drug Branch, National Cancer Institute, Bethesda, Maryland, U.S.A. Solutions of melphalan were prepared immediately prior to use by dissolving the drug in acidic ethanol (70% ethanol in 0.05 N HCl) prior to dilution in serum-free medium and sterilization by filtration.

Because of the sensitivity of line CHO and its derivatives to serum-free medium, dilutions of alkylating agent were added directly to cells in complete (serum-containing) medium for the exposure studies. The presence of serum did not adversely affect melphalan activity, based upon observed linear decreases in survival over the exposure periods

utilized and a minimal experiment-to-experiment variability in survival measurements. In this regard, O'Neill et al.¹² have demonstrated that the mutagenicity and cytotoxicity resulting from exposure of CHO cells to a variety of alkylating agents is not affected by the presence of serum. We have noted, however, that the absolute survival rate varies somewhat with the batch of serum employed (unpublished preliminary observations); a single batch of serum was utilized for all of the experiments described in this report.

For survival studies, aliquots of alkylating agent-exposed cells were removed from suspension, pelleted by centrifugation, resuspended in drug-free medium, counted in a Coulter counter, diluted and plated into 60 mm tissue culture dishes. Following a one-week incubation period, the dishes were washed, fixed and stained with crystal violet prior to determination of colonies (50 or more cells). Plating efficiencies (P.E.) ranged from 80% to 96%, with no significant differences in P.E. observed between trace element-treated control cultures except for the one high level of NaAsO_2 cited in Table 3. It should be emphasized that the utilization of suspension cultures for exposure of cells to trace elements and alkylating agents circumvented the need to trypsinize the cells, thereby eliminating possible trypsin specific-effects on survival which would greatly complicate interpretation of data.

The technique utilized to monitor metallothionein synthesis rate¹³ is a variation on a general analytical approach utilized by Steinberg et al.¹⁴ In our procedure, Sephadex G-75 column chromatography of cytoplasmic fractions pulse-labeled with [^{35}S]cysteine was employed to separate the [^{35}S]-labeled metallothionein species from larger, ^{35}S -containing cytoplasmic components and from unincorporated amino acid. The relative rate of metallothionein synthesis was then calculated as one hundred times the fraction of ^{35}S cpm in the metallothionein peak relative to the counts in non-metallothionein proteins which eluted prior to metallothionein.

To study uptake and intracellular distribution of labeled iodoacetic acid, cultures of CHO or $\text{Cd}^{120\text{F4}}$ cells, grown in suspension culture in the presence or absence of $100\ \mu\text{M}$

ZnCl₂ for 9 h, were exposed to 100 μ M ³H-iodoacetic acid (100-300 mCi/mmol, New England Nuclear Corp., Boston, Mass., U.S.A.) for 3 h. Cells were harvested by centrifugation and washed twice with Hank's balanced salt solution. Aliquots of cells and supernatant solutions were removed at each step for analysis of ³H-iodoacetate levels. Washed cell pellets were resuspended at 1.7×10^7 cells/ml in 0.01 M Tris·Cl (pH 7.4), 0.01 M KCl, 0.0015 M MgCl₂, and 0.02 M 2-mercaptoethanol, after which the cells were lysed by addition of 1/10 volume of 10% NP-40, and cytoplasmic and nuclear fractions were obtained as detailed elsewhere.¹³ Fifty- μ l aliquots of cytoplasmic and nuclear fractions, removed for monitoring of ³H-iodoacetate levels, were first digested with 0.5 ml Protosol (New England Nuclear Corp.) at 37°C for 12-16 h, then counted in 10 ml of Econofluor (New England Nuclear Corp.).

To measure binding of ³H-iodoacetic acid to cytoplasmic components, aliquots of cytoplasmic fractions were prepared for polyacrylamide gel electrophoresis as described previously.¹⁵ In some experiments, unlabeled cytoplasmic extracts were mixed with labeled ³H-iodoacetic acid (100 μ M) and then prepared for analysis via sodium dodecyl sulfate polyacrylamide gel electrophoresis. Samples were analyzed as described elsewhere.¹⁵

RESULTS

Our initial attempts to devise a means for increasing the resistance of cells to alkylating agents centered on the expectation that metallothionein might play a role in the detoxification of these agents.^{16,17} Metallothionein (MT) species represent a class of low molecular weight, cysteine-rich proteins which appear to play a role in regulation of zinc and copper metabolism;^{18,19} they are also involved in detoxification of harmful trace elements such as cadmium and mercury.^{20,21} Given the thiol-rich nature of MT,²² the expected reactivity of alkylating agents with the sulfhydryl moieties of metallothionein and the observation that metallothionein synthesis is induced in hepatic tissue of rats treated with alkylating agents,⁶ we decided to explore the possibility that synthesis of that protein species could serve as a detoxification mechanism for alkylating agents.

The availability of a series of cultured cell variants of line CHO which differed greatly in their ability to induce synthesis of metallothionein following treatment with ZnCl_2 ^{16,17} provided a means to test directly the correlation between metallothionein inducibility and resistance to alkylating agent toxicity. The approach utilized was to compare the cytotoxicity resulting from exposure to an alkylating agent in both induced and non-induced cultures of each cell type.

Figure 1 presents data on the inducibility of metallothionein by zinc in each of the cells. All three cadmium-resistant variants responded to treatment with ZnCl_2 by elaborating synthesis of metallothionein while zinc was without apparent effect on MT synthesis in the parental CHO cell. If MT actually plays a major role in reducing alkylating-agent cytotoxicity, then the zinc-pretreated 2C10, 20F4 and 30F9 cells, which already possess high levels of metallothionein when the alkylating agent is first added, should exhibit enhanced resistance relative to their non-induced counterparts. In similar fashion, CHO cells should not benefit from pretreatment with zinc, since ZnCl_2 failed to induce MT synthesis in that cell. Any variation from that pattern (e.g., lack of enhancement of survival in zinc-pretreated cadmium-resistant cells or increased survival of zinc-treated CHO cells) is an indication that MT does not play a major role in the detoxification of alkylating agents.

In Fig. 2 are presented the results obtained when each of the four cell variants was exposed to the alkylating agent, iodoacetate ($100 \mu\text{M}$) $\pm$ a nine-hour pretreatment period with $100 \mu\text{M}$ ZnCl_2 . After three hours in the presence of iodoacetate, two of the zinc pretreated cadmium-resistant cells, 30F9 (Fig. 2A) and 20F4 (Fig. 2B) exhibited a nearly ten-fold increase in survival. Note, however, that even though zinc pretreatment greatly enhanced the rate of synthesis of MT in 2C10 cells (Fig. 1), there was no evidence that the induced metallothionein reduced the toxicity of iodoacetate in this cell (Fig. 2C), a finding which is incompatible with the notion of a simple correlation between MT inducibility and resistance to toxicity. Even stronger evidence that the protective

response does not depend upon MT-inducibility is provided in Fig. 2D in which zinc pretreatment greatly enhanced the survival of CHO cells under conditions in which the amount of MT was essentially zero in both induced and uninduced cultures.

Experiments illustrated in Fig. 3 with a more clinically-relevant alkylating agent, melphalan, revealed a generally similar phenomenon, with all four cultures which were pretreated with ZnCl_2 exhibiting a nearly ten-fold enhancement in survival relative to the non-induced controls. Taken together, the data in Figs. 1-3 indicate the existence of a zinc-inducible protective response which is not dependent on metallothionein synthesis.

A further indication that resistance and MT synthesis are not related in simple fashion is provided by the data presented in Fig. 4 in which a comparison was made between proteins which bind ^3H -iodoacetate in induced and non-induced cultures of CHO and 20F9 cells. Analysis of ^3H -iodoacetic acid-binding components from metallothionein-deficient CHO cells or metallothionein induction-proficient 30F9 cells exposed to labeled iodoacetic acid in culture or in extracts ($\pm\text{Zn}$ pretreatment) indicates that metallothionein is not a major ^3H -binding component. Furthermore, since ^3H -iodoacetic acid is not observed to be associated with such a low molecular weight component in CHO cells, the protection against ^3H -iodoacetic acid-mediated cytotoxicity can not be ascribed to the presence of MT. Independent experiments demonstrated that the level of metallothionein synthesis in the cultures exposed to ^3H -iodoacetate in Fig. 4 were as expected from the data presented in Fig. 1.

The induction of the protective responses presented in Figs. 2 and 3 was obtained with $100\ \mu\text{M}\ \text{ZnCl}_2$, a level far removed from normal physiological conditions. The effect of varying the level of zinc utilized as an inducer on the survival of melphalan-treated CHO cells is shown in Table 1. While the $100\text{-}\mu\text{M}$ level of zinc yielded the greatest enhancement of survival, there was still a significant increase in resistance obtained in cells which had received only $20\ \mu\text{M}\ \text{ZnCl}_2$; a concentration of $0\ \mu\text{M}$ zinc is only slightly above the level of Zn normally found in human serum,²³ indicating that the protective response can be elicited under nearly normal physiological conditions.

To eliminate the possibility that the protective response was due to a zinc-induced process which rendered the alkylating unavailable to the cells (e.g., facilitated binding of the agent to serum proteins outside the cell, extracellular precipitation of the agent, etc.), an experiment was performed to assess the effect of removing the zinc from the medium prior to addition of the alkylating agent. A culture of CHO cells was split into three parallel cultures, and 100 μ M Zn Cl₂ was added to two of these. Nine hours later, the cells in one of the zinc-treated cultures were resuspended in normal medium, after which melphalan was added to all three cultures. The results presented in Fig. 5 reveal that removal of the inducer prior to addition of melphalan had little or no effect on the enhanced resistance. This observation indicates that the increased resistance of zinc pretreated cells is not the result of a zinc-induced, extracellular inactivation of the alkylating agent. The results instead suggest the existence of an induced product whose functional activity is not immediately lost when the inducer is removed from the culture medium.

While the preceding experiment ruled out the possibility of a zinc-induced decrease in availability of alkylating agent, another possible explanation for the results is that the transport of agent into the cell is reduced in the presence of high levels of zinc, with the result that the intracellular level of alkylating agent is lower in induced than in non-induced cells. Measurement of ³H-iodoacetic acid incorporation into induced or uninduced CHO or 20F4 cells (Table 2) indicated that pretreatment with zinc enhanced the incorporation of ³H-iodoacetic acid into CHO cells but not into 20F4 cells. While further studies will be required to delineate the factors responsible for the different patterns of iodoacetic acid uptake into the two cells, it is clear that the protection mediated by zinc cannot be ascribed to a simple reduction in uptake or binding of ³H-iodoacetic acid by zinc-pretreated cells.

Thus far, major attention has been focused on the effects of zinc as an inducer of a protective response against alkylating-agent toxicity, but what about other trace elements

suspected of inducing synthesis of batteries of proteins? To investigate the possibility that other trace elements could serve as inducers, cultures of CHO cells were exposed to different levels of selenium (potassium selenite, K_2SeO_3), copper (cupric sulfate, $CuSO_4$) or arsenic (sodium arsenite, $NaAsO_2$) for 9 h prior to addition of 8 μM melphalan; non-metal treated parallel cultures served as controls. At the end of a 3-h period of exposure to melphalan, cells were plated in normal medium to allow colonies to develop from the surviving cells. The results presented in Table 3 indicate that all three trace elements induce a protective response in CHO cells, with selenite the most effective element and arsenic the least effective in inducing protection. Preliminary experiments, which require confirmation, indicate generally similar quantitative protective responses induced by selenite and copper, but no enhancement induced by arsenite in 20F4 cells.

The degree of enhancement in survival attributable to pretreatment with 30 μM selenite is approximately equivalent to that achieved with 100 μM zinc, raising the possibility that the same protective response is elicited by the two elements. If so, simultaneous treatment of a cell with both zinc and selenite prior to exposure to melphalan should result in an equivalent degree of protection to that achieved with either agent administered singly (i.e., no improvement in survival of cells receiving both elements). Alternatively, if the two metals are inducing different domains of response, the level of survival in a cell treated with both metals should increase to a level which is equal to (or, perhaps, greater than) the sum of the survivals afforded by each agent administered singly.

Figure 6 presents results obtained when cultures of CHO cells induced with selenite alone, zinc alone, or selenite plus zinc were treated with 8 μM melphalan; an uninduced culture which received only melphalan served as control. The cells which were induced with both metals exhibited an enhanced survival relative to cells treated with either singly-administered agent. The survival of the doubly-induced cells for the 2-h and 3-h samples was approximately equivalent to the sum of survivals achieved in the singly-

induced cells, suggesting that selenite and zinc induce different protective mechanisms whose effects are additive.

DISCUSSION

These results indicate the existence of a novel, trace element-inducible response which protects cells against alkylating-agent cytotoxicity. The protective mechanism does not appear to involve decreased extracellular availability of the alkylating agent (Fig. 5), reduced transport of the drug into cells (Table 2) or the induction of metallothionein (Figs. 1-4). In regard to the latter point, Cagen and Klaassen,²⁴ have arrived at a similar conclusion from studies which examined the binding of radiolabeled iodoacetate to metallothionein isolated from hepatic homogenates of rats which had been pretreated with zinc to boost the level of MT. Their findings suggest that while the sulfhydryl groups on MT can act as binding sites for alkylating agents, the preferential affinity of alkylating agents for another thiol-rich species, glutathione, indicates that MT does not represent a major mechanism for detoxification of alkylating agents. Thus, their in vivo results, which monitored binding affinity, are in excellent agreement with our in vitro findings which employed cytotoxicity as the endpoint, indicating that comparable mechanisms are operative in the two systems. Stated differently, the comparability of results suggests that our cultured cells can serve as a useful model system to provide information of relevance to more complex systems such as intact animals. Given the comparability of results generated in the two analytical systems, our observation (Table 1) that the protective response may be elicited at a level of zinc which is only slightly in excess of the normal level of zinc in human serum²³ suggests that if a similar phenomenon can be induced in humans, the level of inducer required should fall within a physiologically-reasonable range.

While we have not yet identified the mechanism responsible for the protective response, several speculative possibilities may be proposed for consideration. Zinc could cause an increase in glutathione content, a species known to interact readily with

alkylating agents. While we have not determined the levels of glutathione in induced and non-induced cultured cells, there was no evidence for a significant enhancement of glutathione levels in kidney and liver tissue from rats exposed to a high level of zinc in the studies of Klaassen and his associates.^{24,25}

Other candidates as trace element-inducible products include non-metallothionein proteins which might be capable of binding and inactivating alkylating agents or species which enhance alkylating agent catabolism. In regard to the first possibility, while there is no direct evidence which bears on this notion, stressful conditions which cause the induction of metallothionein also induce the synthesis of a series of proteins whose functions have not yet been established.⁷⁻¹⁰ The result of enhanced catabolism of alkylating agents would be to reduce the effective concentration of the active forms of the drugs, thereby preventing them from attacking sensitive intracellular targets.

Based upon the studies of Samson and Schwartz,²⁶ another possibility is that the trace elements are inducing an enhancement in DNA repair capacity. These investigators demonstrated an enhanced resistance to high levels of the alkylating agent, N-methyl,N'-nitrosoguanidine (MNNG) in CHO and cultured human skin fibroblasts which had been previously exposed to sublethal levels of MNNG prior to challenge with a normally toxic level of the drug. The protection did not appear to be attributable to changes in cell-cycle distribution, altered drug permeability or detoxification, leading Samson and Schwartz to speculate that the low levels of MNNG induced an adaptive DNA repair capability in the resistant cells. While the precise nature of the mechanism responsible for the increase in resistance might be open to question, it is interesting that both our studies and those of Samson and Schwartz involve an inducible (adaptive) response which greatly increases the resistance to alkylating agents, perhaps suggesting a similar or identical domain of responses induced by trace elements and by low levels of alkylating agents.

A similar phenomenon to Samson and Schwartz's cultured cell enhancement studies may be occurring in intact animals. Millar et al.^{27,28} reported a reduction in toxicity associated with administration of high doses of melphalan to sheep and mice if the animals were previously exposed to low doses of cyclophosphamide. It appears likely that the cyclophosphamide treatment induces an adaptive response in the animals which is analagous to the response obtained in cultured cells by Samson and Schwartz.²⁶ If this is correct, these in vivo studies provide yet another indication that the inducible protective responses are not an artifact arising only in cultured cells.

The studies described in this report suggest that inducible protective responses against alkylating-agent toxicity may indeed occur in humans and intact animals as well as in cultured cells, but if such a system is to be exploited successfully in cancer chemotherapy, there is also a requirement for obtaining a differential response between normal and tumor cells. For optimal effectiveness, a dramatic quantitative difference in response of the two types of cells would be required. Since the precise mechanism underlying the trace element-induced response is unknown, we can not yet specify the behavior of normal and tumor cells in a clinical setting; however, several observations from other laboratories suggest that clinically-exploitable differences may exist which justify continuing studies of the underlying mechanism.

If the protective response is triggered only when an intracellular "threshold" concentration of inducer is exceeded, then differences in the endogenous level of inducer in normal and tumor cells might be extremely important. For example, if the intracellular level of zinc were significantly lower in tumor cells than in their normal cell counterparts, it might be possible to utilize a level of zinc which would preferentially induce only the normal cells to respond.

Regarding this possibility, a number of studies²⁹⁻³⁵ have demonstrated reductions in the intracellular levels of zinc in tumor tissue (e.g., an approximately 45% reduction in the level of zinc is observed in tumors arising in mice fed diethylnitrosamine³⁵).

Further increasing the disparity in level of intracellular zinc between normal and tumor tissue is a tendency for the zinc lost from tumor tissue to migrate to normal (i.e., non-tumor-involved) tissue;³⁶⁻³⁸ the net result is an elevation in the zinc level of normal tissue of cancer patients to values in excess of those found in normal tissue from tumor-free individuals. Accordingly, it may be possible to provide a level of zinc to a patient which will specifically "trigger" a protective response in normal but not tumor tissue. Note, however, that a few reports have appeared in which the intracellular level of zinc in neoplastic tissue was equivalent to or slightly greater than that of normal tissue (reviewed by Pories et al.³⁹) which indicates that triggering of protective responses may not be feasible in all types of tumors.

That it is possible to utilize a trace element to induce a protective response in normal human cells under conditions where the response of human tumor cells is quantitatively greatly reduced has been demonstrated by Phillips.⁴⁰ Phillips showed that incubation of freshly-collected normal human lymphocytes with zinc transferrin (a zinc-binding serum glycopeptide⁴¹) resulted in induction of levels of metallothionein that were 3.6- to 21-times greater than the levels of MT induced in lymphocytes from leukemic patients. While the protective protein elicited in Phillips' study (i.e., synthesis of metallothionein) is clearly not responsible for the zinc-mediated reduction in alkylating-agent toxicity described in this report, Phillips' findings strongly suggest that conditions can be found in which a given level of trace element stimulates synthesis of an inducible protective protein to a much greater extent in normal cells than in tumor cells. If the trace element-inducible substance responsible for protecting cells against alkylating-agent toxicity behaves in similar fashion, pre-treatment of a patient with an appropriate level of trace element prior to initiation of alkylating-agent therapy may result in a selective sparing of normal tissue. Note that administration of zinc to animals results in elevated levels of inducible proteins such as metallothionein in the tissue of the treated animals,^{24,25,42,43} suggesting the feasibility of inducing protective proteins in humans.

It is obvious that much additional work will be required before the clinical relevance of this type of approach can be determined. If feasible, there are several advantages to utilization of a technique such as this one. In the first place, essential trace elements (e.g., zinc, selenium and copper) can be employed at subtoxic levels to elicit the protective response. The observation that the effects of selenite and zinc may be additive (Fig. 6) suggests the possibility of employing combinations of trace elements; utilization of low levels of a number of inducers as opposed to use of a high level of a single inducer should be technically easier to accomplish and less toxic. A second advantage is the rapidity with which such an approach could be applied to the clinic in the event that promising pre-clinical results were obtained; that is, the trace element technique could be utilized with a variety of existing alkylating agents which have already been approved for human therapy. Finally, if there is an identifiable biochemical species whose synthesis correlates with the protective response (e.g., increased enzymatic activity, elevated level of binding protein, etc.) whose induction can be quantitated precisely, it may be possible to monitor the induction capacity of a prospective patient by measuring the level of protective substance induced in cultured lymphocytes derived from a 5- to 10-ml aliquot of blood; this approach would allow identification of patients with a favorable prognosis for this type of therapy.

It should be emphasized that we recognize that tumor cell heterogeneity may be a limiting factor in this type of therapeutic approach. For example, even though all of the leukemic patients examined in the studies of Phillips exhibited a reduced capacity to induce metallothionein relative to normal controls when provided with zinc transferrin,⁴⁰ the difference in inducibility between the highest and lowest responder in the leukemic series was nearly seven-fold. Furthermore, examples of tumor lines in which inducible proteins such as metallothionein are readily expressed and subject to hormonal modulation have been reported,⁴⁴ which suggest that not all types of tumors will respond favorably in a clinical setting to the trace element stimulation approach to alkylating agent chemotherapy.

We are also not advocating a return to single agent therapy. Instead, we are suggesting that the effectiveness of alkylating agents in standard combination therapy regimens might be enhanced by utilization of the approach outlined in this report, provided, of course, that the efficacy of this approach is first established in pre-clinical studies.

The specific suggestions outlined in this report represent only one possibility for utilization of inducible biochemical processes. As our knowledge of the range of responses and associated mechanisms of protection increase, a variety of novel approaches to detoxification of hazardous physical and chemical agents may be possible which should allow us to: a) monitor and minimize the harmful effects of such agents following accidental exposure; and b) develop techniques for precisely controlling the behavior of selected toxic substances utilized in the treatment of disease.

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FIGURE LEGENDS

Figure 1. Metallothionein induction kinetics in CHO cells and in a series of CHO-derived, cadmium-resistant variant cells following exposure to zinc. ZnCl_2 ($100 \mu\text{M}$) was added to each culture at $t = 0$ and maintained throughout the course of the experiment.

Experimental details are provided in the Materials and Methods section and in reference 13. The symbols utilized are CHO (Δ), 2C10 ($\bullet$), 20F4 (∇) and 30F9 ($\blacksquare$). Assuming a maximum binding of 7 g atoms of Cd^{++} per MT molecule,²² MT is accumulated at a rate of 1.3×10^6 molecules per cell per hour in the maximally-induced (by Cd^{++} or Zn^{++}) 2C10 cell.

Figure 2. Survival of CHO and the Cd^r variant cells exposed to iodoacetate $\pm$ a 9-h pre-treatment period with zinc chloride. For each cell, a large culture was split into two smaller cultures, and zinc chloride ($100 \mu\text{M}$) was added to one of those. Nine hours later, both the zinc-pretreated culture (solid figures) and the non-treated control culture (open figures) received sodium iodoacetate ($100 \mu\text{M}$), and aliquots were removed from each culture at intervals thereafter for plating in regular F-10 medium to determine survivors via colony formation. The cells examined were (A) Cd^r 30F9, (B) Cd^r 20F4, (C) Cd^r 2C10, and (D) CHO.

Figure 3. Survival of CHO and the Cd^r variant cells exposed to melphalan $\pm$ a 9-h pre-treatment period with zinc chloride. Experimental details and symbols as in Fig. 2, except that melphalan ($8 \mu\text{M}$) was substituted for the iodoacetate.

Figure 4. SDS-polyacrylamide gel electrophoretic analysis of cytoplasmic components from ^3H -iodoacetic acid-treated cultured cells or cytoplasmic fractions. Lanes A and H represent molecular weight standards, with (a) representing phosphorylase b (92,500 MW), (b) ovalbumin (46,000 MW), (c) lactoglobulin a (18,367 MW), (d) cytochrome c (12,300 MW), and (e) insulin (5800 MW). MT denotes the mobility of metallothionein determined from other gels. Lanes B and C represent cytoplasmic fractions from CHO cells untreated (B) or pretreated with $100 \mu\text{M}$ ZnCl_2 (C) for 9 h prior to a 3-h exposure to ^3H -iodoacetic acid.

Lanes D and E represent cytoplasmic fractions from $\text{Cd}^{55}\text{30F9}$ cells untreated (D) or zinc-pretreated (E) for 9 h prior to exposure for 3 h to ^3H -iodoacetic acid. Lanes F and G represent the cytoplasmic fractions obtained from unlabeled $\text{Cd}^{55}\text{30F9}$ cells either untreated (F) or pretreated for 9 h with ZnCl_2 (G) prior to being mixed with ^3H -iodoacetic acid prior to analysis by SDS-PAGE.

Figure 5. Effect of melphalan on survival of induced CHO cells exposed in medium from which the ZnCl_2 inducer had been removed. ZnCl_2 ($100\ \mu\text{M}$) was added to two out of three parallel cultures of CHO cells. Nine hours later, the cells in one of the zinc-treated cultures was resuspended in regular F-10 medium, after which, melphalan ($8\ \mu\text{M}$) was added to all three cultures. Aliquots were withdrawn from culture at intervals and plated in drug-free medium to determine survivors via colony formation. Open circles represent the non Zn-induced control culture, solid circles represent the induced culture in which the ZnCl_2 was monitored throughout the entire course of the experiment and the squares represent the induced culture which was returned to inducer-free medium prior to addition of the melphalan.

Figure 6. Effect of utilization of multiple inducers (Zn and Se) on survival of CHO cells treated with melphalan. Four parallel cultures of CHO cells were set up, and ZnCl_2 ($100\ \mu\text{M}$) was added to one, K_2SeO_3 ($30\ \mu\text{M}$) was added to a second, both ZnCl_2 and K_2SeO_3 were added to a third, and a fourth culture received no inducer. Nine hours later, melphalan ($8\ \mu\text{M}$) was added to each culture, and aliquots were removed from each culture at intervals for plating in regular F-10 medium to determine survivors via colony formation. Open circles represent the uninduced control, solid circles represent the culture induced with zinc only, open squares represent the culture induced with selenite only and the solid squares represent the culture induced with both zinc and selenite.

TABLE 1
EFFECT OF CONCENTRATION OF ZnCl_2 USED AS AN INDUCER
TO ENHANCE RESISTANCE OF CHO CELLS
TO MELPHALAN-SPECIFIC CYTOTOXICITY

Concentration of ZnCl_2 (μM) ^a	Survival (%)	Relative enhancement of survival
0	0.58	--
20	2.6	4.5 fold
40	2.4	4.1 fold
60	1.6	2.8 fold
80	5.1	8.8 fold
100 ^b	9.5	16.4 fold

^a The cells were pretreated for 9 h with the appropriate level of ZnCl_2 , then for an additional 3 h with 8 μM melphalan prior to plating in normal (melphalan-free) medium for colony formation.

^b While a level of 100 μM ZnCl_2 could be tolerated by CHO cells for at least 48 h with no decrease in survival, slight increases in zinc level to concentrations of 125 μM or 150 μM resulted in decreases in survival (9 h exposure period) of 33% and 75%, respectively.

TABLE 2

EFFECT OF ZINC PRETREATMENT ON UPTAKE OF ^3H -IODOACETIC ACID
INTO CHO AND $\text{Cd}^{120}\text{F}_4$ CELLS^a

Cell	Treatment with 100 μM ZnCl_2 for 9 h	^3H cpm/ 10^6 cells		
		Cytoplasm	Nucleus	Total
CHO	-	6875	2733	9608
CHO	+	10269	3399	13668
$\text{Cd}^{120}\text{F}_4$	-	5620	3047	8667
$\text{Cd}^{120}\text{F}_4$	+	5047	2897	7944

^a See Materials and Methods section for experimental details.

TABLE 3

EFFECT OF SELENIUM (K_2SeO_3), COPPER ($CuSO_4$) OR ARSENIC ($NaAsO_2$)
 USED AS AN INDUCER TO ENHANCE RESISTANCE OF CHO CELLS
 TO MELPHALAN-SPECIFIC CYTOTOXICITY

Concentration of inducer (μM) ^a	Survival (%)	Relative enhancement of survival
K_2SeO_3		
0	0.98	---
10	0.95	0
20	6.0	6.1 fold
30	9.6	9.8 fold
$CuSO_4$		
0	3.7	---
75	8.5	2.3 fold
100	8.1	2.2 fold
150	11.4	3.1 fold
$NaAsO_2$		
0	4.1	---
2	6.1	1.6 fold
5	7.9	1.9 fold
10	3.0	---

^a The cells were pretreated for 9 h with the appropriate level of inducer, then for an additional 3 h with 8 μM melphalan prior to plating in normal (melphalan-free) medium for colony formation.

^b The plating efficiency of the arsenite-pretreated cells prior to addition of melphalan was lower than that of the control culture, indicating that this level of arsenic is, by itself, toxic.

ALBANY, N.Y.

a
b
c
d
-MT
e