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14. ABSTRACT

This grant program encompasses two complimentary projects. The hypothesis that leukemia can be treated effectively by inhibition of putative cancer stem cells will be tested in project #1. This will be done by application of inhibitors of stem cells as a novel approach for eradication of leukemia tumor cells. Parthenolide (PTL)-based drugs and related drugs that inhibit nuclear factor kappa B (NF-κB) will be used. The effects of these drugs will also be tested on normal hematopoietic cells. In project 2, studies will investigate how standard therapies effect normal CNS stem cells, and will attempt to develop less toxic regimens for the treatment of brain cancers. To this end, studies will determine whether parthenolide or related drugs cause CNS damage in animals treated with these substances, and will assess whether parthenolide can function as a chemosensitizing agent for various conventional chemotherapy drugs.

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Introduction

This grant is comprised of two complementary projects. For the purposes of this report, progress for each project will be described separately below.

Body

Project 1

The objective of this project is to develop a novel therapeutic agent that specifically targets human leukemia stem cells (LSC). While the concept of a critical leukemia stem cell in myeloid disease has been postulated for over three decades, to date no therapeutic agent has been identified that specifically and preferentially ablates LSC in vivo. Thus, the central premise of this grant is that direct targeting of LSC will yield more effective therapy for leukemia. Previously, we demonstrated that parthenolide (PTL) is highly cytotoxic to LSC in vitro, but does not significantly affect normal hematopoietic stem cells (HSC). However, solubility of PTL is limiting; thus we have generated a PTL analog, dimethyl amino parthenolide (DMAPT), that is much more soluble in water and retains the anti-leukemic activity of PTL. Using this agent, the tasks below were specified:

SOW task #1: To demonstrate that a parthenolide analog can be used for preclinical and clinical applications related to treatment of chronic leukemia (Months 1-36).

Studies completed, please see progress reports from years 1-3.

SOW task #2: To demonstrate that a parthenolide analog can function as a chemosensitizing agent to enhance ablation of chronic leukemia cells (Months 37-60).

In order to identify optimal drug combinations to employ with parthenolide, we have performed a series of studies designed to determine the central molecular properties of parthenolide. Our rationale has been that if we can understand the specific pathways modulated by parthenolide, then we can make rational predictions regarding how to use this compound in combination with conventional agents to achieve chemosensitization. To this end, we have exploited the chemistry described in the year 3 report, where we generated a modified version of parthenolide, known as melampomagnalide B (MMB). This stereoisomer of parthenolide is useful because of the hydroxyl group on carbon 14, which permits the chemical addition of other moieties. As described last year, one option is to add a biotin group to created a version of MMB that can then be used for biochemical studies and identification of protein targets. Briefly, these efforts entail incubating leukemia cells with MMB-biotin, followed by lysis and cellular

fractionation studies to isolate plasma membrane, cytoplasm, cytoskeletal, and nuclear compartments. Each fraction is then subjected to biochemical "pull downs" using streptavidin beads. Proteins targeted by MMB (i.e. via covalent interaction) are isolated from the beads and then subjected to mass spectrometry to identify individual binding partners. This work has yielded 312 individual proteins that are directly bound to MMB. Interestingly, these proteins fall into several distinct signaling pathways, the most prominent of which is glutathione metabolism. This finding led us to evaluate the role of glutathione metabolism perturbation in more detail.

We performed a detailed biochemical analysis of glutathione pathway components and activity in human leukemia cells. Our findings (Figure 1) show

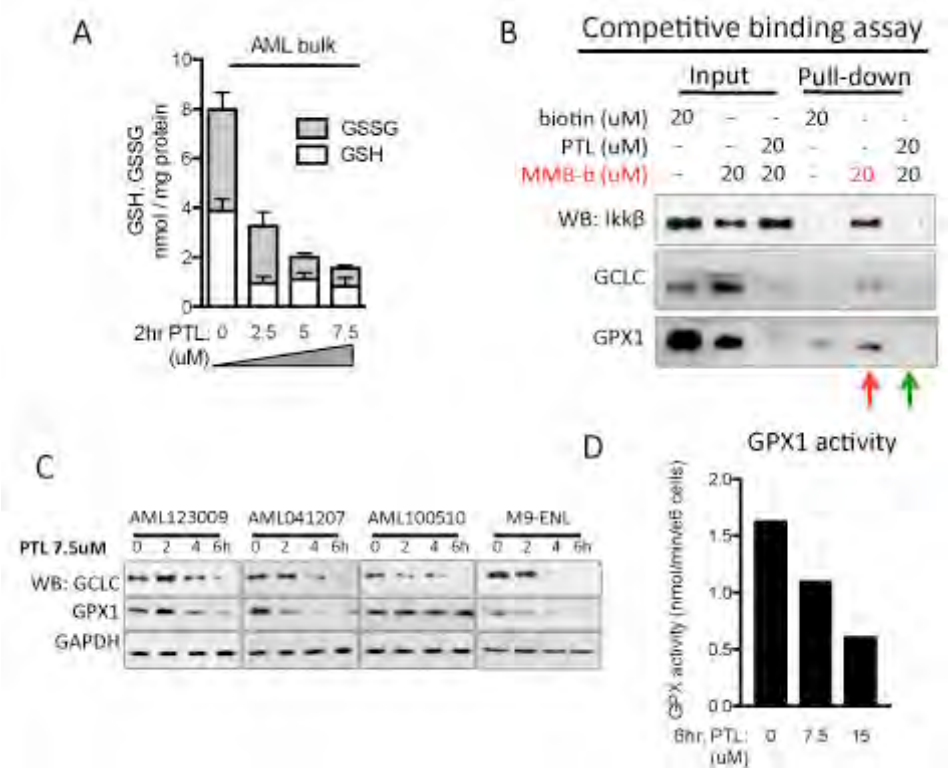

Figure 1: Analysis of glutathione regulation in leukemia cells
A) primary leukemia cells were evaluated with respect to reduced (GSH) and oxidized (GSSG) glutathione as a function of parthenolide dose. B) Biochemical pull down assays using biotinylated MMB (MMB-b). Red arrow indicates successfully pull down of GCLC and GPX1. Green arrow indicates completion for binding by pre-incubating cells with parthenolide. C) Western blot analyses of GCLC and GPX1 in leukemia cells treated with increasing doses of parthenolide. D) GPX activity level in leukemia cells treated with increasing doses of parthenolide.

several intriguing points. First, upon parthenolide treatment, cellular glutathione levels are dramatically suppressed (Fig 1A). Second, key enzymes for glutathione metabolism, GCLC and GPX1, are both direct targets of parthenolide (Fig 1B). Third, GCLC and GPX1 levels are reduced in leukemia cells upon treatment with parthenolide. Fourth, biochemical activity of GPX1 in reduced upon parthenolide treatment. Collectively, these data indicate that overall glutathione, and therefore oxidative balance, is very impaired by parthenolide treatment.

To further explore mechanisms controlling leukemia-specific activity of parthenolide, we compared glutathione levels in primary normal bone marrow cells to leukemia cells treated with parthenolide. Remarkably, the degree of glutathione depletion was much less in the normal cells (Figure 2). In addition, over time the normal cells were able to at least partially recover from the parthenolide insult, whereas the leukemia cell do not. We propose that the differentially cytotoxicity observed for parthenolide in leukemia vs. normal cells is based at least in part on the ability of glutathione systems in normal cells to respond more effectively. Going forward, we will develop rational drug combinations based on the unique ability of parthenolide to selectively modulate glutathione levels in leukemia cells.

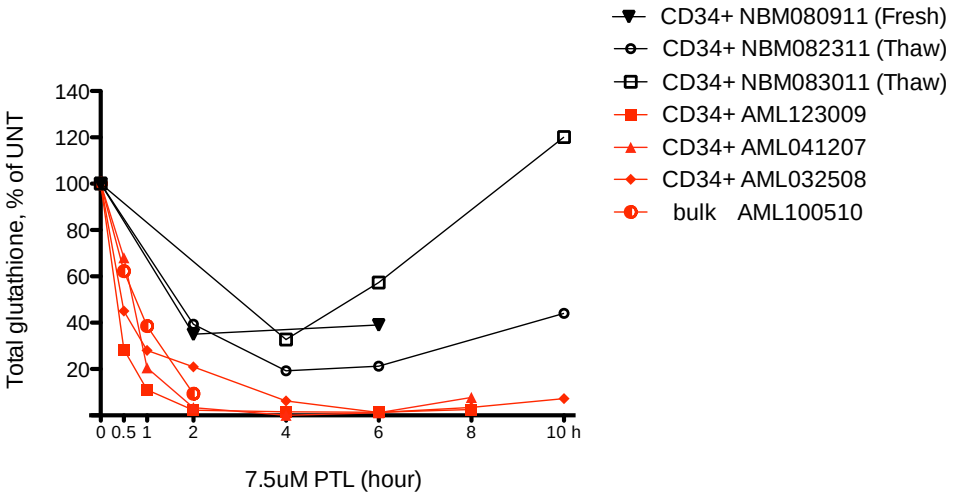

Figure 2: Glutathione levels in cells treated with parthenolide
Normal specimens (black lines) and leukemia specimens (red lines) were treated with parthenolide (PTL) and monitored for glutathione level as a function of time.

Key Research Accomplishments

We have performed a detailed analysis of parthenolide mechanism in leukemia cells.

Conclusion

The differentially response of normal vs. leukemia cells with regard to glutathione metabolism may explain the leukemia-specific activity of parthenolide.

Reportable outcomes

Pending

Appendices

N/A

Project 2

The primary goal of this project is to investigate how standard therapies effect normal CNS stem cells and to develop less toxic regimens for the treatment of cancer:

SOW task #1: To determine whether parthenolide or parthenolide analogs cause CNS damage in animals treated with these substances, and to determine whether parthenolide or parthenolide analogs enhance the damage caused by cytarabine. (Months 1-24)

Our work on this task was described in the 2009 project report, with detailed data presented for our new findings on toxicity. In brief, we found that most cancer treatment regimens that we examine are toxic for progenitor cells and oligodendrocytes of the CNS. At this stage, we have demonstrated toxicity (both in vitro and in vivo) for BCNU, cisplatin, cyclophasphamide, vincristine, cytarabine, 5-fluorouracil and tamoxifen. We also have examined multiple regions of the CNS. We also found substantial toxicity of parthenolide for CNS progenitor cells.

We reported at this time that we can say that this task was effectively completed with the demonstration that the toxicity that we have identified is a problem that permeates through the world of cancer treatment and that it affects multiple subsystems within the CNS.

SOW task #2: Demonstrate that mice in which purified cells are more oxidized in vitro will exhibit more extensive damage from cytarabine, parthenolide (or parthenolide analogs) or the combination of these agents, than those in which purified cells are intrinsically more reduced (Months 25-48).

SOW task #3: To initiate identification of potential prognostic indicators to detect individuals at greater risk for adverse side effects of therapy with cytarabine, parthenolide (or parthenolide analogs) or the combination of these agents, and begin testing to provide proof of principle for protective strategies that involve administration of N-acetyl-L-cysteine (alone or in combination with Vitamins E and/or C) as an anti-oxidant to protect against oxidative damage (Months 49-60).

Task 2 and 3 are considered together because the interest in oxidative state that applies to both of these tasks links them together functionally.

Our report in 2010 showed that it is possible to protect against many aspects of chemotherapy-mediated damage with erythropoietin (EPO). This exciting possibility was the focus of last year's report because of its potential for clinical relevance as well as for its importance to how we approach the problem of

understanding the underlying mechanisms

The next goal we had to address in order to solve this problem has been to understand the dual faces of (i) the underlying mechanisms by which toxicity profiles change so as to make chemotherapy more or less toxic and (ii) the mechanisms by which EPO and/or anti-oxidants can be used to achieve protection. Such a mechanism-driven understanding is essential not just to move this work beyond a purely descriptive level but also be able to approach our goals in an efficiently rational manner.

The clue that appears to unify all components of this research is the role of oxidative status as both a predictor and an active determinant of outcome. Even our studies on cells derived from different strains of mice, characterized by having different redox states show that cells isolated from animals that are more oxidized are generally more vulnerable to physiological insults. This raises the possibility that a general principle might be at play that integrates vulnerability to multiple insults.

As discussed at the end of this report, our next goal is testing the hypothesis that EPO may work through physiological mechanisms more broadly relevant to understanding resistance to such side effects – but first we had to identify such mechanisms.

Background

As part of our investigations into how cell function is regulated by changes in oxidative status, we recently discovered a novel pathway by which oxidative increases caused by chemically diverse environmental toxicants induce pathway-specific disruption of receptor tyrosine kinase (RTK) signaling required for division of oligodendrocyte/type-2 astrocyte progenitor cells of the CNS (also referred to as oligodendrocyte precursor cells, and here abbreviated as O-2A/OPCs). Relatively small toxicant-induced increases in oxidative status (i.e, <20%) caused Fyn kinase activation, leading to activation of the c-Cbl ubiquitin ligase. Fyn/c-Cbl pathway activation caused specific reductions in levels of platelet-derived growth factor receptor-α (PDGRα) and other c-Cbl targets. Fyn and c-Cbl activation, with consequent pathway-specific suppression of RTK signaling, was induced by MeHg or lead concentrations that affect large segments of the population, as well as by paraquat, an organic herbicide. Due to the importance of RTK signaling in cellular function, redox/Fyn/c-Cbl pathway activation causes reductions in progenitor division and survival, thus demonstrating previously unknown roles of Fyn and c-Cbl in controlling vital progenitor cell functions.

Our further studies have demonstrated that all increases in oxidative status appear to converge on activation of the redox/Fyn/c-Cbl pathway. This occurs

whether the oxidative increases are due to genetic mutation, exposure to physiological stressors, chemotherapeutic agents or even to due epigenetic regulation of redox control during development.

It is at this point that our parallel research efforts become directly relevant to our attempts to understand how to predict vulnerability to the chemotherapy and how to protect against these toxic insults. As we found that differences in oxidative status appear to be critical modulators of the vulnerability of cells to toxic insults, and all stimuli that increase oxidative status cause activation of the redox/Fyn/c-Cbl pathway, this raises the necessary question of whether changes in this pathway are relevant to understanding differences in vulnerability to chemotherapeutic agents.

To determine whether changes in vulnerability to chemotherapeutic agents have a mechanistic basis in activation of the redox/Fyn/c-Cbl pathway, we examined the combination of BCNU together with the environmental toxicant methylmercury (MeHg). We chose to initially focus on BCNU, a nitrosourea chemotherapeutic agent used in the treatment of several different kinds of cancers, because of our prior studies showing that it is highly toxic for progenitor cells of the CNS. We chose MeHg as an example of the general problem of exposure to environmental toxicants for several reasons: (i) Unlike changes in oxidative status caused by mutation, use of environmental toxicants enables us to precisely control the exposure dose and thus the degree of oxidative change. In contrast, genetic changes do not allow control of dosage. (ii) There is a great deal of knowledge about the types of environmental toxicants to which many people are exposed, and much less knowledge about the genetic alterations that cause differences in oxidative status. Thus, we can be much more confident that we are working with stimuli that may have broad applicability among patient populations. (iii) Environmental toxicants are also much more relevant clinically than pharmacological modulators that increase oxidative status as while such pharmacological modulators are useful tools in the laboratory, there is little exposure to them in the population at large. Thus, any results obtained through pharmacologically induced increases in redox status would sill have to be tested with other stimuli.

By taking the above path, we can first test the general hypotheses that relevant insults that increase oxidative status increase vulnerability and that the redox/Fyn/c-Cbl pathway is important in understanding the mechanisms underlying

Results

Our investigations have not only demonstrated that the redox/Fyn/c-Cbl pathway is an essential component of the mechanism by which increases in oxidative status increase vulnerability to chemotherapeutic agents, but that this pathway is also an essential component of the more complex problem of synergistic toxicity

reactions. This is a particularly important advance, as very little is known about the mechanisms relevant to understanding situations in which exposure to two agents that cause little or no harm by themselves are able to cause extensive damage when applied together. While we had not anticipated that we could make such a large step forward, this provides a major advance in planning on how to solve the problem of vulnerability.

We first discuss the results themselves, and then will consider the implications of these findings.

MeHg and BCNU cause synergistic increases in O-2A/OPC death

We initiated studies on synergistic toxicity reactions by extending upon our previous findings that O-2A/OPCs are targets of both MeHg and chemotherapeutic agents. The vulnerability of O-2A/OPCs to MeHg is of particular interest due to both the sensitivity of these cells to environmentally relevant levels of this toxicant and the association between mercury exposure and decreases in conduction velocity in the auditory brainstem response of MeHg-exposed children and rats, a physiological alteration that is generally considered to be indicative of myelination abnormalities. The vulnerability of CNS cells to clinically relevant levels of exposure to chemotherapy is also of particular interest due to the adverse effects of systemic chemotherapy on cognition, CNS function and pathological changes visualized by non-invasive imaging. We focused initial investigations on BCNU (carmustine), which is primarily used for treating brain cancers and Hodgkin's lymphoma and is associated with CNS toxicity both clinically and experimentally but is also of interest because of its activity as an inhibitor of glutathione reductase.

We first found that exposure to low levels of MeHg markedly increased vulnerability of O-2A/OPCs to BCNU. After conducting an 8-point dose-response analysis of O-2A/OPCs to MeHg, we then exposed cells to BCNU in the presence or absence of MeHg at its LD_{10} concentration (which was ~20 nM). The additive hypothesis would predict that the additional presence of BCNU at its LD_{10} concentration (of 10.2μM) would cause killing of 20% of cells; instead, the presence of both agents instead killed 65% of cells, a 225% increase over that predicted if increases in death simply additive. In addition, the presence of 20 nM MeHg reduced the concentration of BCNU required to achieve 50% killing from 22.3±0.2μM to 8.6±0.1μM, a concentration even less than the LD_{10} value for BCNU alone (i.e, 10.2±0.2μM). Moreover, when combined with exposure to 20 nM MeHg, the concentration of BCNU required to kill 10% of O-2A/OPCs was reduced to ~3μM BCNU. To place these levels of vulnerability in context, clinically relevant CSF concentrations of BCNU are 8-10μM for low-dose applications, and can be up to 100-fold or more higher in high-dose applications. Thus, the background presence of 20nM MeHg markedly increased vulnerability to BCNU.

Similarly to the above results, background exposure to BCNU at its LD_{10} concentration markedly increased the vulnerability of O-2A/OPCs to MeHg. For example, the LD_{50} for MeHg alone was 55.0±0.9nM and the LD_{10} was 21.4±0.6nM. In contrast, when applied in combination with the LD_{10} concentration of BCNU, the LD_{50} of MeHg was now 18.6±0.3nM, less than the LD_{10} of MeHg applied on its own.

Mathematical confirmation that co-exposure to BCNU + MeHg caused synergistic increases in toxicity was carried out by determining the LD_{50} for each drug and then evaluating the effects of the combination of MeHg and BCNU by using an isobologram analysis. Concentrations of the two drugs (alone or in combination) that yielded 50% killing were plotted as an isobologram, which enables evaluation of the outcome of exposure to two compounds independently of the underlying mechanisms of action. In an isobologram, a concave curve indicates synergy, a straight line indicates addition, and a convex curve indicates antagonism. The concave curve of the isobologram plot demonstrated synergistic effects of MeHg and BCNU on O-2A/OPCs.

Co-exposure to BCNU and MeHg causes greater-than-additive changes in oxidative status.

The frequent relationship between cell death and oxidative stress, and the ability of both MeHg and BCNU to make cells more oxidized, made it of interest to determine whether exposure to the combination of these agents also caused non-additive alterations in the oxidative status of O-2A/OPCs. Although increases in cell death were themselves greater-than-additive, this observation does not distinguish between the possibilities, for example, that death occurred because the magnitude of change induced by exposure to both agents simply exceeded a threshold at which cell death is initiated, or whether supra-additive changes in oxidative status were induced. In these experiments cells were exposed to various concentrations of either agent alone, or to combinations of MeHg and BCNU, and oxidative status was determined by analysis with dihydrocalcein-AM (DHC), a cell-permeant indicator of levels of reactive oxidative species that fluoresces when oxidized (and that has the additional advantage of not itself altering O-2A/OPC function; unpublished observations).

Co-exposure to BCNU + MeHg caused greater-than-additive changes in oxidative status in O-2A/OPCs. For example, exposure to 10 nM MeHg or 5μM BCNU caused DHC fluorescence to increase by 8.5±2.5% and 6.1±2.4% of control values, respectively, but exposure to both agents together increased fluorescence by 41.8±1.3% of the control group ($p<0.01$**)**. Similarly, treatment with 20nM MeHg + 10μM BCNU caused increases in DHC fluorescence that were greater than two-fold larger than those induced by exposure to either agent alone.

Co-exposure to BCNU and MeHg causes greater-than-additive changes in Fyn activation and c-Cbl phosphorylation

The observation that MeHg + BCNU caused synergistic increases in both cell death and oxidative status raises the question as to whether these changes are functionally connected, a question we first addressed by examining the effects of exposure to these substances on the sequential activation of Fyn kinase and the c-Cbl ubiquitin ligase. Analysis of these enzymes was prompted by our recent discovery of the redox/Fyn/c-Cbl pathway, which converts changes in oxidative status into accelerated degradation of RTKs essential for cell division and survival.

Molecular analysis revealed that co-exposure to MeHg and BCNU was associated with greater-than-additive increases in levels of activated Fyn and phosphorylated c-Cbl. In these experiments, O-2A/OPCs were exposed to MeHg for 24 hr and to BCNU for 1 additional hr before cells were lysed (as contrasted with the analysis of cell death 48hrs after the addition of BCNU). For example, the increase in Fyn activation caused by exposure to the combination of 20 nM MeHg (LD_{10}) + 10μM BCNU (LD_{10}) was 73% greater than that predicted by simple addition of the effects of exposure to each compound by itself ($p<0.01$). As we found for exposure to MeHg alone, Fyn activation was regulated as found previously, and was blocked by PP1 (a Src family kinase inhibitor) and by the anti-oxidant and glutathione pro-drug N-acetyl-L-cysteine (NAC). In contrast, Fyn activation was not prevented by NH_4Cl, a weak base that inhibits lysosomal activation and c-Cbl-induced degradation of proteins that are its targets.

c-Cbl activation (as detected by phosphorylation analysis) was also synergistically induced by exposure to MeHg + BCNU. Exposure to 10 nM MeHg or 5μM BCNU (concentrations that did not induce death of O-2A/OPCs) caused 67% and 96% increases in c-Cbl phosphorylation, respectively. In contrast, exposure to both substances together caused a 504% increase in c-Cbl phosphorylation, more than 3 times the increase predicted if effects were solely additive. ($p<0.01$). Critically, and as predicted if c-Cbl phosphorylation was due to Fyn activation, increases in c-Cbl phosphorylation caused by exposure to MeHg + BCNU (or by exposure to single agents) were prevented by co-exposure of O-2A/OPCs to PP1 or NAC, but not by co-exposure to NH_4Cl**.**

Combined exposure of O-2A/OPCs to sublethal concentrations of MeHg + BCNU causes greater-than-additive suppression of PDGF-mediated signaling

We previously found that activation of the redox/Fyn/c-Cbl pathway was associated with reductions in levels of total and phosphorylated PDGFRα and with suppression of responses downstream of PDGFRα activation. We therefore next determined if the supra-additive effects of simultaneous exposure to MeHg + BCNU resulted in supra-additive changes in levels of total and phosphorylated

PDGFRα and/or in activation of critical signaling intermediaries downstream of PDGFRα activation.

We next found that co-exposure to MeHg + BCNU caused supra-additive reductions in the levels of both phosphorylated and total PDGFRα. For example, exposure to 20 nM MeHg decreased levels of PDGFRα phosphorylation by 28% and levels of total receptor by 23%, with similar levels of change caused by exposure to 10μM BCNU. In contrast, exposure to the combination of 20 nM MeHg + 10μM BCNU caused decreases in levels of PDGFRα phosphorylation by >95% and of total PDGFRα levels by 77% of control values. Combined exposure to MeHg + BCNU was so potent that even reducing levels of MeHg to 10nM and BCNU to 5μM was associated with a 45% decrease in levels of PDGFRα phosphorylation and a 60% decrease in levels of total PDGFRα levels.

Levels of phosphorylated Erk1/2 and Akt (both of which are increased following PDGFRα activation) were also were more reduced by combined exposure of O-2A/OPCS to MeHg + BCNU than to either agent alone, although the degree of total change was such that changes that the scope for exceeding additivity was less than in our other assays. For example, exposure to 20 nM MeHg alone was associated with a 35% fall in levels of phosphorylated Erk and a 40% fall in levels of phosphorylated Akt. A similar degree of change was associated with exposure to 10μM BCNU, with a 34% fall in levels of phosphorylated Erk and a 32% fall in levels of phosphorylated Akt. Exposure to the combination of 20 nM MeHg + 10μM BCNU, in contrast caused levels of phosphorylated Erk to fall by 95% and levels of phosphorylated Akt to fall by 90%. All of these levels of phosphorylation were so low that they may have represented the limits to which reduction is possible. More striking indications of greater-than-additive effects of co-exposure to MeHg + BCNU on Erk1/2 and Akt activation were provided by analysis of theoretical dose-equivalents. For example, exposure of O-2A/OPCs to LD_{20} concentrations of MeHg (30 nM) or BCNU (15 μM) caused ~40% reductions in levels of p-Akt. A theoretical dose equivalent analysis, in which cells were exposed to both agents at half of these concentrations (i.e., 15 nM MeHg + 7.5 μM BCNU) caused a >90% reduction in levels of p-Akt. This outcome is particularly striking in consideration of the fact that these concentrations of MeHg and BCNU were actually significantly lower than the real LD_{10} concentrations for each agent. Such an outcome underscores the importance of using multiple different types of analyses to examine this problem.

As predicted from their ability to prevent Fyn and c-Cbl activation, PP1 and NAC also rescued the suppression of PDGF signaling caused by combined exposure to MeHg + BCNU. For example, in cells that were exposed to 1mM NAC, together with 20nM MeHg + 10μM BCNU, we found that levels of p-PDGFRα were >15-fold higher than cells exposed to MeHg + BCNU alone (4.2±1.0%, vs. 64.9±11.3%, of control values, respectively. Similarly, in cells exposed to MeHg + BCNU, levels of total PDGFRα , pErk1/2 and pAkt, were decreased to 22.3±4.1%, 7.5±3.6% and 10.5±2.6% compared to the untreated group. In

contrast, in cells also exposed to NAC, these values were rescued to 63.9±9.2% of controls for PDGFRα, 75.5±11.4% for pErk, and 71.3±10.8% for pAKt. Similar protection was provided by exposure to PP1, which rescued levels of p-PDGFRα, PDGFRα, pErk1/2 and pAkt to 64.9±11.3%, 66.5±4.6%, 75.0±4.7% and 67.1±6.7% compared to the untreated group, respectively.

MeHg + BCNU causes pathway-specific reductions in levels of other c-Cbl targeted RTKs

One of the critical features of c-Cbl mediated regulation of cell signaling is that only a subset of RTKs are targets of this ubiquitin ligase. Thus, a specific prediction of the hypothesis that oxidative increases cause suppression of Erk1/2 and Akt signaling indirectly by inducing RTK degradation is that oxidative activation of the redox/Fyn/c-Cbl pathway is associated with loss of specific RTKs that are targets of c-Cbl, but not of non-c-Cbl targets. For these reasons, analysis of multiple RTKs can provide additional evidence of c-Cbl activation.

As predicted by our previous analysis of the redox/Fyn/c-Cbl pathway, exposure of O-2A/OPCs to sublethal concentrations of MeHg + BCNU also caused marked reductions in levels of c-Met (the receptor for hepatocyte growth factor) and the epidermal growth factor receptor (EGFR), both of which are c-Cbl targets. EGFR receptor levels were reduced to 60.8±0.7% of control levels by MeHg exposure, to 63.8±1.3% by treatment with BCNU and to 12.4±1.5% of control levels in cells treated with MeHg + BCNU . c-Met levels were reduced to 57.0±3.6% in cells treated with MeHg and 50.1±10.6% in cells treated with BCNU, and to 18.6±7.6% in cells treated with MeHg + BCNU**.** Although these changes were not supra-additive in magnitude, this may have been because they were already close to the maximum changes possible. As for analysis of changes in levels of PDGFRα, co-exposure to NAC or PP1 prevented the reductions in levels of EGFR and c-Met.

Our previous studies demonstrated that activation of c-Cbl is not associated with degradation of TrkC (the receptor for neurotrophin-3), and analysis of this receptor thus provides a critical determinant of the pathway specificity of the effects of co-exposure to MeHg + BCNU. In contrast with effects on PDGFRα, c-Met and EGFR, levels of TrkC were unaffected by exposure to MeHg, BCNU or the combination of these agents, as predicted by the redox/Fyn/c-Cbl hypothesis. In addition, exposure to either agent alone, or to the combination of these stressors, had no effect on Erk1/2 phosphorylation induced by exposure of O-2A/OPCs to NT-3**.**

Genetic interference with Fyn or c-Cbl function protects cells from the combined toxicity of MeHg and BCNU

Our next experiments tested the hypothesis that Fyn and c-Cbl activity were necessary for the ability of MeHg + BCNU to cause greater-than-additive

reductions in cell survival and in levels of PDGFRα. These experiments were conducted by expression of the dominant-negative (DN) 70z mutant of c-Cbl or of small inhibitory RNAs (siRNA) for c-Cbl or Fyn in O-2A/OPCs prior to their exposure to MeHg + BCNU.

Effects of combined exposure to MeHg + BCNU on cell survival were effectively prevented by expression of DN-c-Cbl or of siRNA for c-Cbl or Fyn. For example, cell survival was not reduced by treatment with MeHg + BCNU in cells expressing DN-c-Cbl, but survival of cells infected with viral constructs encoding empty vectors was indistinguishable from uninfected cells exposed to these substances. Similarly, the survival of cells expressing c-Cbl-siRNA was 92.3±9.3% of control values, and was essentially unchanged from cells not exposed to MeHg + BCNU. Essentially identical results were obtained in cells expressing Fyn-siRNA or Fyn-scr-siRNA, with the former providing protection and the latter having no effect. In contrast, when cells were transduced with retroviral constructs expressing a nonfunctional siRNA containing a scrambled nucleotide substitution at the 19-nucleotide targeting sequence of the corresponding RNAi sequence for c-Cbl (c-Cbl-scr-siRNA) and then treated with MeHg + BCNU, cell survival was reduced to 30.9±5.0% of control cultures, a value no different from cells not expressing siRNA.

Reductions in levels of PDGFRα induced by exposure to MeHg + BCNU also were prevented in cells expressing c-Cbl-siRNA or Fyn-siRNA. Quantitative analysis of 3 combined separate experiments showed that the levels of PDGFRα were 99.8±4.7%, 101.7±5.2% and 109.5±6.6% when cells transduced to express c-Cbl-siRNA were treated with MeHg, BCNU or both, respectively. In contrast, in cells transfected with c-Cbl-scr-siRNA, levels of PDGFRα were reduced to 76.9±1.2%, 75.3±1.1% and 18.5±5.8% of control values when cells were treated with MeHg, BCNU or both, respectively. Similar results were obtained in cells transfected to express Fyn-siRNA or DN-70z-c-Cbl, but not in cells transfected with empty vectors or in cells expressing Fyn-scr-siRNA.

c-Cbl and Fyn are required for multiple synergistic toxicity reactions

In our final experiments, we addressed the question of whether Fyn and c-Cbl were essential components of other types of synergistic toxicity reactions. To determine whether the principles under investigation were of possible relevance to interactions between other chemical toxicants (for which chemotherapeutic agents may be considered a special class of such substances) or between toxicants and other physiological stressors, we examined effects of sublethal levels of MeHg exposure on O-2A/OPC vulnerability to cisplatin, as well as to glutamate and OGD, two components of ischemic injury to the CNS. Glutamate toxicity for O-2A/OPCs is thought to be relevant to white matter damage in hypoxic birth injury, while OGD has been used to simulate brain ischemia in O-2A/OPC cultures to examine mechanisms of toxicity induced by brain ischemia.

Exposure to sublethal levels of MeHg, in combination with exposure to sublethal concentrations of cisplatin, glutamate or sublethal periods of exposure to oxygen-glucose deprivation (OGD) was associated with extensive cell death. For example, exposure to 0.1mM glutamate caused little or no cell death by itself, nor did exposure to 20 nM MeHg. In contrast, when cells were treated with MeHg 20nM for three days, and then with 0.1mM glutamate for another two days, there was an ~75% reduction in cell survival. Similarly, analysis of O-2A/OPC vulnerability to OGD showed that exposure to OGD for 1 hour or less did not cause cell death. In contrast, when cells were exposed to 20nM MeHg for three days and then to OGD for one hour, there was a >75% decrease in cell survival. MeHg exposure also increased vulnerability to clinically relevant levels of cisplatin, another chemotherapeutic agent to which O-2A/OPCs are vulnerable. Exposure to 0.1 μM cisplatin had no detectable effect on survival on its own, but was associated with highly significant ($p<0.01$) reductions in cell survival when combined with 10 or 20 nM MeHg**.**

As for the combination of MeHg + BCNU, expression of siRNA for c-Cbl was sufficient to protect O-2A/OPCs from combinations of MeHg with cisplatin, glutamate or OGD, thus indicating that c-Cbl is required for combinatorial toxicity outcomes in multiple circumstances. For example, O-2A/OPCs expressing c-Cbl siRNA - but not c-Cbl scr-siRNA - showed no response to 20 nM MeHg + 0.1 μM cisplatin, 20 nM MeHg + 100 μM glutamate, or 20 nM MeHg + 1 hr of OGD. In contrast, in cultures expressing c-Cbl scr-siRNA, all of these combined insults killed $\geq$ 70% of cells. Similar results were obtained by expression of Fyn-siRNA.

Implications for the next stage of this research

By identifying a molecular pathway that appears to play a central role in increasing the vulnerability of oxidized cells (in this case oxidized by MeHg exposure) to BCNU and MeHg, we have finally established a detailed mechanism that can be used as an appropriately mechanistic anchor from which we can investigate increases in toxicity caused by other contributions to an increased oxidative status (e.g., strain differences caused by genetic mutations or changes in gene expression patterns).

Perhaps even more exciting is that several other investigators have raised the possibility that EPO works in part of changing the activity

This now sets the stage for the next components of this work. Do anti-oxidants provide protection by preventing activation of the redox/Fyn/c-Cbl pathway? Does EPO work similarly and, if so, is this because of a promotion of Nrf2 activity? If this is a component of how EPO works, then can we replace EPO with a pharmacological activator of Nrf2 activity and thus achieve protection in this way?

Key Research Accomplishments

We have discovered a molecular pathway that appears to be required for the ability of increases in oxidative status (caused by exposure to the widespread environmental toxicant MeHg) to cause increases in vulnerability to MeHg and BCNU. This is one of the rare examples of a pathway that is involved in synergistic toxicity reactions, and is the only discovery of such a pathway that applies to chemotherapeutic agents. These findings now provide a mechanistic basis for completing the research of this project in a manner that will be most helpful in enabling us to develop means of protecting against the toxic side effects of chemotherapy.

Reportable outcomes

Pending

Conclusions

The redox/Fyn/c-Cbl pathway represents the first known mechanism that is essential for the increased vulnerability to chemotherapeutic agents caused in cells that are rendered more oxidized by an adjunctive physiological insult. These findings now provide a mechanistic basis for completing the research of this project in a manner that will be most helpful in enabling us to develop means of protecting against the toxic side effects of chemotherapy.

References

N/A

Appendices

N/A