ROLES OF NUCLEAR FACTOR-ERYTHROID 2-RELATED FACTOR 2 IN
GLUTAMATE CYSTEINE LIGASE INDUCTION BY ORGANOSELENIUM
COMPOUNDS

A Thesis in
Molecular Medicine
by
Sans Wellington Emmert

Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Doctor of Philosophy

May 2008
The thesis of Sans Emmert was reviewed and approved* by the following:

John P. Richie, Jr.
Professor of Public Health Sciences & Pharmacology
Thesis Advisor
Chair of Committee

Karam El-Bayoumy
Professor of Biochemistry and Molecular Biology

Thomas Spratt
Associate Professor of Biochemistry and Molecular Biology

Kent E. Vrana
Elliot S. Vesell Professor and Chair of Pharmacology

Craig Meyers
Professor of Microbiology and Immunology
Co-Chair of the Graduate Program in Molecular Medicine

*Signatures are on file in the Graduate School
ABSTRACT

Levels of dietary selenium are inversely associated with cancer risk in humans, and selenium has played a major role in the field of chemoprevention. In particular, the organoselenium agents 1,4-phenylenebis(methylene)selenocyanate (p-XSC) and its glutathione conjugate, p-XSeSG, are effective in preventing cancers at numerous sites, including the lung in rodent models. In the A/J mouse lung, p-XSC is highly effective in inducing glutathione (GSH), but mechanisms are not well understood. Glutathione is the most abundant antioxidant in animals, and is an important mechanistic factor in chemoprevention. Glutamate cysteine ligase (GCL) is the rate-limiting enzyme responsible for GSH production. Transcriptional regulation of GCL occurs via antioxidant response elements (ARE) in the promoter regions of its genes. Induction of the ARE is in turn regulated by stabilization and nuclear translocation of the transcription factor, Nuclear factor-erythroid 2-Related Factor 2 (Nrf2). Furthermore, the extracellular signal-regulated kinase (ERK) pathway has been shown to play a role in Nrf2 stabilization. Potent chemopreventive phytochemicals, such as sulforaphane (SFN), have been shown to act largely through the Nrf2/ARE pathway.

The potential relevance of Nrf2 to GSH induction led to the hypothesis that Nrf2 activation may be involved in organoselenium-mediated induction of GSH, and this was investigated in vivo and in several cell culture models. Here we show that dietary p-XSC induces Nrf2, p-ERK, GCL and GSH in the lung of the
Fisher rat, suggesting involvement of Nrf2 and the ERK pathway in GSH induction. In addition, GSH and GCL were induced by p-XSeSG in the Fisher rat. In cell culture studies we observed that p-XSC and p-XSeSG activate an ARE luciferase reporter in an Nrf2-dependent manner. It was also shown that p-XSeSG induced GSH \textit{in vitro}, and is less toxic than p-XSC. The dependence of GCL induction by p-XSeSG upon Nrf2 was confirmed in wildtype and Nrf2-mutanted Mouse Embryonic Fibroblasts (MEF). These results suggest that p-XSC acts through the Nrf2 pathway \textit{in vivo}, and that p-XSeSG may be the metabolite responsible for such activation, thus offering p-XSeSG as a less toxic, yet highly efficacious inducer of GSH.

Isothiocyanate compounds such as SFN are among the most potent Nrf2 inducers known. We hypothesized that substitution of sulfur with selenium in the isothiocyanate functional group of SFN would result in an isoselenocyanate compound (SFN-isoSe) with enhanced Nrf2 induction capability. Here we show that SFN-isoSe activates an ARE-luciferase reporter in HepG2 cells more potently than SFN. It was also found that SFN-isoSe induces GCL and GSH in MEF cells in an Nrf2-dependent manner. Finally, we provide evidence that SFN-isoSe is more effective in killing HepG2 cancer cells, yet is less toxic to non-cancer MEF cells, than is SFN. These data support our hypothesis, and suggest that SFN-isoSe may be a highly effective chemoprotective agent \textit{in vivo}.

Taken together, these results support the hypothesis that Nrf2 is required for organoselenium-mediated induction of GSH, and suggest that the induction of the Nrf2 pathway by organoselenium compounds may represent a general...
mechanism of chemoprevention. Induction of GSH, with low associated toxicity, by agents like p-XSeSG may prove useful for treatments of conditions such as HIV and chronic inflammatory conditions. Isoselenocyanates, such as SFN-isoSe, may be ideal candidates for future studies as chemopreventive and/or chemotherapeutic agents due to their ability to induce Nrf2 with low toxicity in normal cells and high efficiency at killing cancer cells.
# TABLE OF CONTENTS

**LIST OF FIGURES**..............................................................................ix

**LIST OF TABLES**.................................................................................xi

**ACKNOWLEDGEMENTS**.................................................................xi

Chapter 1. General Introduction.................................................................1
   Selenium and Chemoprevention............................................................1
   Glutathione............................................................................................5
   Antioxidant Response Element Pathway..................................................9
   Isothiocyanates, Selenium and Induction of the ARE Pathway..............12
   Hypotheses..........................................................................................14
   References...........................................................................................15

Chapter 2. Roles of Nuclear factor-erythroid 2-Related Factor 2 in Glutamate-Cysteine Ligase Induction in the Rat Lung by 1,4-phenylenebis(methylene)seleno-cyanate and its Glutathione Conjugate........22
   Abstract...............................................................................................22
   Introduction..........................................................................................23
   Materials and Methods..........................................................................26
   Results..................................................................................................30
   Discussion............................................................................................37
Chapter 3. Induction of Glutamate Cysteine Ligase by 1,4-phenylenebis(methylene)selenocyanate and its Glutathione Conjugate is Dependent upon Nuclear factor-erythroid 2-Related Factor 2.................47

Abstract.................................................................47
Introduction............................................................48
Materials and Methods.............................................51
Results.................................................................55
Discussion............................................................72
References............................................................77

Chapter 4. Enhanced Nrf2-Dependent Induction of Glutathione in Mouse Embryonic Fibroblasts by an Isoselenocyanate Analog of Sulforaphane ........................................81

Abstract.................................................................81
Introduction............................................................82
Materials and Methods.............................................85
Results.................................................................89
Discussion............................................................102
References............................................................106

Chapter 5. General Summary........................................112
Selenocyanates and Related Agents.............................................112
Isoselenocyanates and Isothiocyanates.........................................118
Overall Conclusions..................................................................119
Future Experiments....................................................................120
References..................................................................................121
LIST OF FIGURES

Figure 1. Chemical Structures of Organoselenium Compounds……5
Figure 2. A schematic of regulation of glutathione biosynthesis……9
Figure 3. Nrf2/ARE Signaling Pathway……………………………………..11
Figure 4. Chemical Structures of Isothiocyanates and
Isoselenocyanates…………………………………………………………….13
Figure 5. Relative Nuclear Nrf2 expression in lung and liver of rats fed
organoselenium compounds……………………………………………35
Figure 6. Relative Nuclear p-ERK expression in lung and liver of rats fed
organoselenium compounds………………………………………………37
Figure 7. HepG2 ARE-Reporter cell Viability upon p-XSC or p-XSeSG
Treatment…………………………………………………………………………57
Figure 8. Luciferase Activity in HepG2 ARE-Reporter cells upon p-XSC or p-
XSeSG Treatment……………………………………………………………………59
Figure 9. Glutathione Levels in HepG2 ARE-Reporter cells upon p-XSC or p-
XSeSG Treatment………………………………………………………………63
Figure 10. Luciferase Activity and Nuclear Nrf2 levels in HepG2 ARE-
Reporter cells upon p-XSC or p-XSeSG Treatment……………………………65
Figure 11. Effects of siNrf2 on Luciferase Activity, Glutathione and Nuclear
Nrf2 in HepG2 ARE-Reporter cells upon p-XSC or p-XSeSG Treatment..67
Figure 12. Viability of Mouse Embryonic Fibroblasts upon p-XSC or p-
XSeSG Treatment………………………………………………………………69
Figure 13. GCL Induction in Mouse Embryonic Fibroblasts upon p-XSeSG Treatment.................................................................71

Figure 14. Luciferase Activity and Viability in HepG2 ARE-Reporter cells upon SFN or SFN-isoSe Treatment..............................................91

Figure 15. Nuclear Nrf2 Expression in Mouse Embryonic Fibroblasts upon SFN or SFN-isoSe Treatment.................................................95

Figure 16. GCL Expression in Mouse Embryonic Fibroblasts upon SFN or SFN-isoSe Treatment..........................................................97

Figure 17. Time-Course of Relative Glutathione Levels in Mouse Embryonic Fibroblasts upon SFN or SFN-isoSe Treatment...................99

Figure 18. Glutathione in Mouse Embryonic Fibroblasts upon SFN or SFN-isoSe Treatment...............................................................101

Figure 19. Schematic of in vivo and in vitro effects of p-XSC or p-XSeSG.................................................................................117
LIST OF TABLES

Table 1. Effects of Organoselenium Compounds on Bodyweight.....31
Table 2. Concentrations of glutathione in blood and tissues of rats fed organoselenium compounds.................................................................32
Table 3. Relative GCL expression in lung or liver of rats fed organoselenium compounds.................................................................34
Table 4. Luciferase Activity in HepG2 ARE-Reporter cells upon Organoselenium Treatment.................................................................61
Table 5. Luciferase Activity and Viability in HepG2 ARE-Reporter cells upon isoselenocyanate or isothiocyanate treatment.................................93
ACKNOWLEDGEMENTS

I first acknowledge my wife Jane Emmert for her incredible care and support throughout this process.

John P. Richie, a great friend, mentor and scientist.

Karam El-Bayoumy, one of the founders of the field of chemoprevention and the developer of \( p \)-XSC.

Shantu Amin and Dhimant Desai for synthesis of organoselenium compounds and many useful discussions.

Thomas Kensler for kindly providing Nrf2 deficient mouse embryonic fibroblasts.

John Pezzuto for kindly providing HepG2 ARE-reporter cells.
Chapter 1

General Introduction

Selenium and Chemoprevention

Selenium is an essential trace element that is obtained in the diet from plants, which have incorporated inorganic soil selenium mostly into proteins containing selenomethionine. Selenium’s essential nature in biological systems is partly due to its role in selenoproteins, in which selenocysteine, the 21st amino acid, has been specifically incorporated by a special serine tRNA. Examples of well characterized selenoproteins include glutathione peroxidases and thioredoxin reductases, both part of the antioxidant defense system (Tapiero, Townsend et al. 2003). Less than optimal activity of antioxidant-associated selenoproteins has been correlated with many chronic degenerative conditions such as atherosclerosis, arthritis, male infertility, lowered immune function, central nervous system pathologies, accelerated aging disorders, and specific cancers (Rayman 2000). Endemic deficiency of selenium leads to conditions such as cardiomyopathic Keshan disease, and osteoarthropathic Kashin-Beck disease (Tapiero, Townsend et al. 2003). Excess intake of selenium, generally through consumption of selenium accumulator plants in geographic regions of high soil selenium, is toxic to animals and humans. Symptoms of toxicity, as
occurs in particular regions of China, require selenium intakes on the order of
greater than 2 mg/day (Patrick 2004). Toxicity depends on the form of selenium,
and inorganic compounds such as selenite or selenate are more toxic than
organic forms. Chronic doses above 5 ppm of inorganic selenium in the diet
cause liver toxicity and are teratogenic (Tapiero, Townsend et al. 2003).
Epidemiological studies show that normal serum selenium levels (below toxic
levels, yet above those seen in deficiency conditions) are inversely associated
with risk of many cancers including prostate, lung, stomach and colorectal
(Patrick 2004). A meta-analysis of data from studies relating cancer risk to serum
levels of vitamin E, β-carotene, retinal, and selenium, revealed that selenium was
the factor consistently providing the most protective effect (Comstock, Bush et al.
1992). It has also been determined, through analyses of many animal studies
that chemoprotective effects occur at serum selenium levels higher than are
normally required for maximal activity of at least the well-characterized
selenoproteins, such as glutathione peroxidase. These and other findings have
led to research into the prospect of supplementing the diet with selenium as a
means of lowering cancer risk.

Chemoprevention can be defined as a therapeutic effort to block, slow, or
reverse carcinogenic processes, and often employs naturally occurring agents
such as phytochemicals, but may also include synthesized compounds.
Selenium, in both organic and inorganic forms, has played a major role in the
field of chemoprevention, particularly after the reporting of an almost 50%
reduction in morbidity and mortality by major cancers following dietary
supplementation with selenized brewer’s yeast (Clark, Combs et al. 1996). Data from this study continues to be analyzed and additional trials using supplemental selenium-enriched yeast, such as the large Prevention of Cancer by Intervention by Selenium (SELECT), are ongoing in efforts to discern mechanisms of selenium-mediated chemoprevention. Selenomethionine was thought to be the major selenium species in enriched yeast, and this was part of the reason that pure selenomethionine was chosen for use in the ongoing SELECT phase III trial, which was initiated by the NCI in 2001. This trial is examining the effects of diets supplemented with Vitamin E and/or selenium on incidence of prostate cancer and related biomarkers (Ip, Dong et al. 2002). Advanced selenium speciation analysis of selenium-enriched yeast has revealed that selenomethione actually accounts for only 20-42% of total selenium-containing materials, another 20-25% included selenocystine, Se-methylselenocysteine, and selenoethionine, and the remainder consisted of unknown forms (Bird, Ge et al. 1997). Clearly, much work remains to identify the active components of selenium-enriched yeast and to understand the mechanisms by which they exert chemopreventive effects.

Various synthetic selenium compounds have been developed with goals of lowering toxicity while enhancing efficacy when compared to other more commonly used chemopreventive agents such as selenium-enriched yeast or selenomethionine (El-Bayoumy and Sinha 2004). Aromatic selenium compounds were developed in part due to the bio-stability of selenium bonded to benzene. The organoselenium agents 1,4-phenylenebis(methylene)selenocyanate (p-XSC) and its glutathione conjugate, p-XSeSG have proven to be very effective in
preventing a variety of carcinogen-induced cancers in several animal models (Tanaka, Makita et al. 1997; Rao, Wang et al. 2001; Richie, Kleinman et al. 2006). Chemical structures of $p$-XSC and related organoselenium compounds are shown in Figure 1. Limited information regarding chemopreventive mechanisms of these aromatic selenium compounds is available. For example, in a DMBA-rat mammary tumor model, both $p$-XSC and $p$-XSeSG were shown to inhibit DMBA-DNA adduct formation, and $p$-XSC was shown to inhibit cytochrome p450 as well as induce certain Phase II DMBA activating enzymes (El-Bayoumy and Sinha 2004). It has also been recently reported that elevated lung glutathione levels coincide with $p$-XSC-mediated protection from NNK-induced lung tumors in an A/J mouse model, (Richie, Kleinman et al. 2006). Induction of glutathione may be an important mechanism of chemoprevention by $p$-XSC and possibly other organoselenium compounds (Huber and Parzefall 2007).
Glutathione (GSH) is the most important and abundant endogeneous antioxidant in mammals. It is found in a range of 1-10 millimolar concentrations in most tissues and is involved in a plethora of physiological processes. GSH is a tripeptide of glycine, glutamate, and cysteine with a non-typical peptide bond between glutamate and cysteine. The ATP-activated $\gamma$-carboxyl of glutamate is
involved in peptide bond formation with cysteine’s amino group, followed by a
more typical condensation of γ-glutamylcysteine with glycine. GSH synthesis
does not occur on ribosomes, but rather is performed enzymatically by sequential
action of the rate-limiting glutamate-cysteine ligase (GCL) followed by glutathione
synthetase in ATP-dependent reactions. The γ-peptide bond is thought to provide
protection from peptidases. The sulfhydryl group of the cysteine moiety of GSH
offers reactivity, but GSH does not have the toxicity associated with cysteine
alone.

Glutathione can easily be oxidized to glutathione disulfide (GSSG) and the
proportion of GSH to GSSG is considered to be an indicator of a cell’s redox
state and also provides a redox buffer system for protection against oxidative
insult. Glutathione disulfide is converted back to GSH by glutathione reductase,
with NADPH acting as reducing equivalent donor, and the typical ratio of GSH to
GSSG in a non-stressed cell is maintained on the order of 100:1. This ratio is
cell-type specific, but values below normal are generally considered to be
apoptosis promoting, while higher values offer an environment more conducive to
proliferation and differentiation (Schafer and Buettner 2001).

While GSH is well known for its role in maintaining the ferrous state of
heme, GSH also provides reducing equivalents for glutaredoxin in DNA
synthesis, and conjugates hydrogen peroxide in a reaction catalyzed by
 glutathione peroxidase, the aforementioned selenoprotein. Glutathione is
involved in conjugation of electrophiles in reactions catalyzed by glutathione S-
transferases, but can also react non-enzymatically with certain free radicals and
reactive oxidative species, thus GSH plays a prominent role in detoxification
and/or excretion of carcinogens. Glutathione can also conjugate with cysteine
residues of redox sensitive proteins in a process known as glutathionylation, and
this has been shown to regulate function of proteins such as glutathione
transferases, actin and Ras (Gallogly and Mieyal 2007). Because of its
widespread physiological roles, particularly in antioxidant defense systems, GSH
is relevant to a multitude of human diseases.

Defects in GSH metabolism, including polymorphisms in GCL genes, are
linked to aging effects, progression of HIV, cystic fibrosis, heart disease,
diabetes, neurodegenerative diseases and cancer (Townsend, Tew et al. 2003;
Wu, Fang et al. 2004). Aging humans show a progressive and linear decline in
plasma GSH:GSSG levels beginning after approximately age 45 (Richie,
Skowronski et al. 1996; Jones, Mody et al. 2002). This finding lends support to
the free radical theory of aging in the sense that diminishing GSH-mediated
defenses may allow elevated levels of reactive oxidative species and subsequent
damage to proteins, lipids, and DNA. Aging is a risk factor for many GSH-
associated degenerative conditions including neurodegenerative diseases, such
as Parkinson’s disease (Maher 2005), and is also the most important risk factor
for cancer. Glutathione may be considered a first line of defense against cancer
initiation due to its major role in detoxification of carcinogens and protection from
DNA-damaging reactive oxidative species. Therefore, an important goal of
chemoprevention would be to maintain tissue GSH at youthful levels (Townsend,
Tew et al. 2003; Huber and Parzefall 2007).
Glutathione levels can be regulated by GCL activity, cysteine availability, and efflux of GSSG from cells, however GCL activity is the most important factor. Activity of GCL is determined for the most part by GCL mRNA levels and feedback inhibition of GCL by GSH itself (Huang, Moore et al. 1988). GCL is a heterodimer of subunits produced by independent genes. The 73 kDa catalytic subunit, GCLc, can act alone, but regulatory function is added by its dimerization with a 31 kDa modulatory subunit, GCLm. In resting cells, levels of GCLc are generally found in much greater quantity than GCLm (Krzywanski, Dickinson et al. 2004). Transcription of both modulatory and catalytic subunits of GCL are regulated in part by the presence of Antioxidant Response Elements (ARE) in upstream promoter regions of each gene (Wild, Moinova et al. 1999). A schematic of the GSH biosynthetic process is shown in Figure 2.
Antioxidant Response Element Pathway

The transcription factor, Nuclear factor-erythroid 2-Related Factor 2 (Nrf2) has come under intense scrutiny as protector of endogenous and exogenous insult, and mediator of chemoprevention. Nrf2 has been shown to be involved in the induction of many anticarcinogenic/antioxidant genes such as glutathione S-transferases, UDP-glucuronosyltransferases, epoxide hydrolase,

Figure 2: A schematic of regulation of glutathione biosynthesis.
NAD(P)H:quinone oxidoreductase 1, and glutathione reductase (Zhang and Gordon 2004; Zhang 2006). Enhanced nuclear translocation and subsequent binding of Nrf2 to ARE-containing promoters activates a variety of chemoprotective Phase II genes, including many in the glutathione homeostasis pathways (Zhang and Gordon 2004). Nrf2 knockout mice are more sensitive to toxic agents and carcinogens, and less protected by many chemopreventive compounds, than their wildtype counterparts (Zhang 2006). In addition to induction of Phase II detoxification and antioxidant genes, Nrf2 has also been shown to activate genes involved in apoptosis, inflammatory responses and the ubiquitin pathway (Thimmulappa, Mai et al. 2002). It is unclear, however, how Nrf2 mediates processes leading to apoptosis in cancer cells in contrast to inducing Phase II protective genes in normal cells.

Nrf2 is normally sequestered in the cytoplasm by the actin-bound protein Keap1, a substrate adaptor for an E3 ubiquitin ligase, which targets Nrf2 for rapid turnover (Zhang, Lo et al. 2005). Keap1 contains multiple reactive cysteine residues that, when modified directly or indirectly by a variety of inducers, reduce its affinity for and promote the nuclear translocation of Nrf2 (Zhang and Hannink 2003). Phosphorylation of Nrf2, mediated by Extracellular signal-Regulated Kinase (ERK) and c-Jun-NH$_2$-kinase pathways, has also been shown in vitro to disrupt its association with Keap1 (Xu, Yuan et al. 2006). Neither post-translational modifications of Nrf2, or induction of kinase pathways, by ARE inducers, have been observed in vivo. A general mechanistic schematic of Nrf2-mediated ARE induction is shown in Figure 3. Many compounds of diverse
Chemical class and structure have been demonstrated to induce the Nrf2/ARE pathway, but despite this wide diversity, most have at least the theoretical capacity to modify sulfhydryl groups of proteins. The most widely studied of these inducers is sulforaphane.

Figure 3: Nrf2/ARE signaling pathway.

 Isothiocyanates, Selenium and Induction of the ARE Pathway
Epidemiological studies highlight the substantial chemopreventive effectiveness of cruciferous vegetable intake in relation to many cancers including lung, breast, colon and prostate (Lin, Probst-Hensch et al. 1998; Spitz, Duphorne et al. 2000; Ambrosone, McCann et al. 2004; Joseph, Moysich et al. 2004). Glucosinolate, a precursor of sulforaphane (SFN), was isolated from broccoli, a cruciferous vegetable widely consumed by Western societies. SFN was since identified as, and remains to this day to be, the most potent natural inducer of phase II detoxification enzymes, such as quinone reductase, glutathione S-transferases, and GCL (Zhang, Talalay et al. 1992; McWalter, Higgins et al. 2004). Sulforaphane belongs to a family of chemopreventive agents whose functional group is an isothiocyanate. Sulforaphane has since become one of the most intensively studied chemopreventive phytochemicals, and has been found to act through additional pathways, such as the induction of apoptosis in cancer cells (Gamet-Payrastre, Li et al. 2000; Choi, Lew et al. 2007). Chemical structures of sulforaphane and related compounds are shown in Figure 4. Dietary administration of broccoli seeds, a potent source of glucosinolates, resulted in elevated GCLc in the stomach and small intestine of wildtype but not Nrf2 knockout mice, demonstrating in vivo dependence of SFN-mediated glutathione induction upon Nrf2 (McWalter, Higgins et al. 2004). The isothiocyanate group of SFN has been demonstrated in vitro to directly modify Keap1 through the formation of thionoacyl adducts (Hong, Freeman et al. 2005).
Because of the high chemopreventive potential of both cruciferous vegetables and selenium compounds, it was hypothesized that growth of such vegetables in a high selenium environment may enhance their effectiveness. Broccoli grown in enriched selenium and fed to rats resulted in better inhibition of colon cancer, but contained 80% less glucosinolate when compared with normal broccoli (Finley, Sigrid-Keck et al. 2005). It is unknown whether isoselenocyanate precursors are generated in place of isothiocyanate precursor analogs under such conditions.
Preliminary studies with a synthetic isoselenocyanate analog of sulforaphane (SFN-isoSe) indicate that it is more effective than sulforaphane at inducing apoptosis in a variety of melanoma cell lines yet is not toxic to normal fibroblasts, however the mechanisms by which this differential behavior occurs is unknown (Amin, Desai, Robertson, et al., manuscript in preparation).

It is unknown whether, and to what extent, selenium compounds require Nrf2 in their chemopreventive/antioxidant function. The synthetic organoselenium compound Ebselen, a glutathione peroxidase mimetic and weak \textit{in vitro} inducer of Nrf2, has been shown in a cell culture model to directly modify Keap1 (Sakurai, Kanayama et al. 2006). It is not known if the promising chemopreventive organoselenium agents, \textit{p}-XSC and \textit{p}-XSeSG, require Nrf2 in their ability to induce GSH \textit{in vivo}. It is also unknown whether selenium analogs of isothiocyanates can induce the Nrf2 pathway. Therefore, the following hypotheses were tested.

**Hypotheses**

It is hypothesized that Nrf2 is involved in \textit{p}-XSC-mediated induction of GSH \textit{in vivo}. Specific aims include determination of whether feeding of \textit{p}-XSC or \textit{p}-XSeSG will result in concerted induction of nuclear Nrf2, GCLc protein expression, and elevated GSH in the rat lung.
It is further hypothesized that *in vitro* studies will confirm dependence of GSH induction by chemopreventive organoselenium compounds upon Nrf2 induction. A second specific aim includes testing if treating ARE-reporter cells with *p*-XSC, *p*-XSeSG and related compounds will result in luciferase activation, elevated nuclear Nrf2 and GCLc protein levels, and also induction of GSH. Furthermore, it is hypothesized that specific knockdown of Nrf2 in these treated cells will result in reductions in luciferase activity, GCLc protein levels, and GSH.

A third specific aim involves testing if treating mouse embryonic fibroblast (MEF) cells from wildtype and Nrf2 knockout (Nrf2 -/-) mice with *p*-XSC, *p*-XSeSG and related compounds will result in elevated nuclear Nrf2, GCLc protein levels, and GSH in wildtype cells, but not Nrf2 -/- cells.

A fourth specific aim involves testing if treating HepG2 ARE-Reporter and mouse embryonic fibroblast (MEF) cells from wildtype and Nrf2 knockout mice with SFN, SFN-isoSe and related compounds will result in luciferase activation and elevated nuclear Nrf2, GCLc protein levels, and GSH in wildtype cells but not Nrf2 knockout (Nrf2 -/-) cells.

**References**

Ambrosone, C. B., S. E. McCann, et al. (2004). "Breast cancer risk in premenopausal women is inversely associated with consumption of


Richie, J. P., Jr., W. Kleinman, et al. (2006). "The organoselenium compound 1,4-phenylenebis(methylene)selenocyanate inhibits 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol-induced tumorigenesis and


Roles of Nuclear factor-erythroid 2-Related Factor 2 in Glutamate-Cysteine Ligase Induction in the Rat Lung by 1,4-phenylenebis(methylene)selenocyanate and its Glutathione Conjugate

Keywords: Lung cancer, chemoprevention, glutathione, selenium, p-XSC, p-XSeSG, selenomethionine, antioxidant response element (ARE), Nrf2, GCLc, Fisher 344 rat, ERK pathway

Abstract

The organoselenium agents 1,4-phenylenebis(methylene)selenocyanate (p-XSC), and its glutathione conjugate, p-XSeSG, are effective in preventing cancers in rodent models, but mechanisms are not well understood. In the A/J mouse lung, p-XSC is also highly effective at inducing glutathione (GSH), the most abundant antioxidant in mammals and an important factor in chemoprevention. Glutamate cysteine ligase catalytic subunit (GCLc) is the rate-limiting enzyme responsible for GSH production. Transcriptional regulation of GCLc occurs via antioxidant response elements (ARE) in the promoter region of
its gene. Induction of the ARE is in turn regulated by stabilization and nuclear translocation of the transcription factor, Nuclear factor-erythroid 2-Related Factor 2 (Nrf2). Furthermore, the extracellular related kinase (ERK) pathway has been shown to play a role in Nrf2 stabilization.

Here we show that dietary \( p \)-XSC induces Nrf2, p-ERK, GCL and GSH in the lung of the Fisher Rat, suggesting involvement of Nrf2 and the ERK pathway in GSH induction. Feeding of \( p \)-XSC at a dose of 10 ppm (as selenium) for two weeks resulted in significant (\( p < 0.05 \)) inductions in nuclear p-ERK (220%), nuclear Nrf2 (610%), and GCLc (55%) protein expression, as well as a 230% induction in total GSH in the lung. Similarly, 10 ppm \( p \)-XSeSG induced GCLc (88%) and total GSH (200%). Selenomethionine, a widely studied chemopreventive organoselenium agent, was also fed at 10 ppm, but this resulted in no significant inductions in p-ERK, Nrf2, GCLc or GSH. These results suggest that \( p \)-XSC acts through the Nrf2 pathway \textit{in vivo}, and that \( p \)-XSeSG may be the metabolite responsible for such activation, thus offering \( p \)-XSeSG as a less toxic, yet highly efficacious inducer of GSH. The induction of GSH via the Nrf2 pathway by organoselenium compounds may represent a general mechanism of chemoprevention. Induction of GSH, and potentially other ARE-regulated Phase II genes, with low associated toxicity, by agents like \( p \)-XSeSG may prove useful for chemoprevention as well as for treatment of conditions such as HIV and chronic inflammatory conditions.

**Introduction**
Lung cancer is the second most common cancer and is the leading cause of cancer deaths in the world. Because of difficulties associated with smoking cessation, early detection, and chemotherapeutic effectiveness, research into chemoprevention has become a priority. Selenium, in both organic and inorganic forms, has played a major role in the field of chemoprevention, particularly after the reporting of an almost 50% reduction in morbidity and mortality by major cancers, including lung cancer, following dietary supplementation with selenized brewer’s yeast (Clark, Combs et al. 1996). Various synthetic selenium compounds have been developed with goals of lowering toxicity while enhancing efficacy when compared to other chemopreventive agents under investigation such as selenium-enriched yeast or selenomethionine (El-Bayoumy and Sinha 2004).

The organoselenium agents, \( p \)-XSC and its glutathione conjugate, \( p \)-XSeSG have proven to be very effective in preventing a variety of carcinogen-induced cancers, including lung cancer, in several animal models (Tanaka, Makita et al. 1997; Rao, Wang et al. 2001; Richie, Kleinman et al. 2006). It has been recently reported that elevated lung GSH levels coincide with \( p \)-XSC-mediated protection from NNK-induced lung tumors in an A/J mouse model, (Richie, Kleinman et al. 2006). Although the mechanisms by which this occurs are unknown, induction of GSH may be an important mechanism of chemoprevention by \( p \)-XSC, and possibly by \( p \)-XSeSG. The most likely cause of induced GSH is through enhanced protein expression of the catalytic subunit of the rate-limiting enzyme of \textit{de novo} glutathione synthesis, glutamate-cysteine
ligase (GCL) (Huang, Moore et al. 1988). The 73 kDa catalytic subunit, GCLc, can act alone, but regulatory function is added by its dimerization with a 31 kDa modulatory subunit, GCLm. In resting cells, levels of GCLc are generally found in much greater quantity than GCLm (Krzywanski, Dickinson et al. 2004). Transcription of both modulatory and catalytic subunits of GCL are regulated in part by the presence of Antioxidant Response Elements (ARE) in upstream promoter regions of each gene (Wild, Moinova et al. 1999). A schematic of the GSH biosynthetic process is shown in Figure 2.

The transcription factor, Nuclear factor-erythroid 2-Related Factor 2 (Nrf2) has come under intense scrutiny as a protector of endogenous and exogenous insult, and mediator of chemoprevention. Enhanced nuclear translocation and subsequent binding of Nrf2 to ARE-containing promoters activates a variety of chemoprotective Phase II genes, including many in the glutathione homeostasis pathways (Zhang and Gordon 2004). Nrf2 is normally sequestered in the cytoplasm by the actin-bound protein Keap1, a substrate adaptor for an E3 ubiquitin ligase, which targets Nrf2 for rapid turnover (Zhang, Lo et al. 2005). Keap1 contains multiple reactive cysteine residues that, when modified directly or indirectly by a variety of inducers, reduce its affinity for and promote the nuclear translocation of Nrf2 (Zhang and Hannink 2003). Phosphorylation of Nrf2, mediated by extracellular signal-regulated kinase (ERK) and c-Jun-NH₂-kinase pathways, has also been shown to disrupt its association with Keap1 (Xu, Yuan et al. 2006). Neither post-translational modifications of Nrf2 nor induction of
kinase pathways have been observed \textit{in vivo}. A schematic of Nrf2-mediated ARE induction is shown in Figure 3.

The primary purpose of this study was to test the hypothesis that the transcription factor Nrf2 is involved in organoselenium-mediated GSH induction \textit{in vivo}. The major specific aim is to determine if the organoselenium compounds \textit{p}-XSC and \textit{p}-XSeSG induce nuclear Nrf2 in the rat lung concurrently with increased protein expression of GCLc and GSH levels. Selenomethionine was included in the study due to its relevance to chemoprevention, and use in ongoing clinical trials, such as those related to prostate cancer (Ip, Dong et al. 2002).

\textbf{Materials and Methods}

\textbf{Reagents}

Organoselenium compounds were obtained courtesy of Dhimant H. Desai and had been synthesized as described previously (Sohn, Desai et al. 2005). Structures of \textit{p}-XSC, \textit{p}-XSeSG and selenomethionine are shown in Figure 1. Antibodies (Nrf2, p-ERK, GCLc, GCLm, Actin, LaminA) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

\textbf{Animals and diet preparation}
Pathogen-free male Fisher 344 rats 4 weeks of age were purchased from Charles River Breeding Laboratory (Kingston, NY). Following one week of quarantine, rats were weighed and placed into 4 groups of 6 such that average weights per group were as similar as possible. Rats were also weighed following one week of feeding and again at time of sacrifice. A semipurified diet (AIN-93A) containing 0.1 ppm of selenium as sodium selenite was used throughout the study. Levels of selenium (10 ppm) for incorporation into the diet were based on previous studies in our laboratories that employed p-XSC and p-XSeSG. The levels of selenium from selenomethionine were also 10 ppm. The organoselenium compounds were blended into the AIN-93A diets by Harlan Teklad (Ijamsville, MD), and stored at 4°C. At 6 weeks of age, rats were fed either the control diet or diets supplemented with various forms of selenium for 2 weeks.

Necropsy and tissue harvesting and processing

Animals were euthanized by CO₂ inhalation. Blood was collected into EDTA tubes by cardiac puncture, and lungs and livers were harvested, rinsed in saline and blotted dry, then divided into portions and snap-frozen in liquid nitrogen. Nuclear and cytoplasmic extracts were obtained from frozen tissue using reagents supplied by Marligen (Madison, WI) according to the
manufacturer’s protocol. Briefly, 0.15 g of frozen tissue was placed into 1 ml of ice-cold cytoplasm extract buffer containing EDTA and protease inhibitors, then homogenized by hand in glass homogenizers.

**Glutathione Assay**

Frozen lung and liver tissue were thawed, blotted dry, and weighed. Tissue (0.1 g) was placed in 3 ml of 5% metaphosphoric acid and homogenized by hand in glass homogenizers. Precipitated protein was removed by centrifugation. Free glutathione in cellular extracts was determined using the previously described enzymatic recycling method using Elman’s reagent (Tietze 1969) with modifications (Richie, Skowronski et al. 1996). Absorbency over time was measured using a Bio-Tek Synergy HT plate reader (Bio-Tek Instruments, Winooski, VT). Tissue GSH levels are normalized to tissue weight, while GSH in blood samples was normalized to hemoglobin levels.

**Western Blotting**

Western blotting for nuclear Nrf2 and LaminA, or cytoplasmic GCLc, GCLm or Actin, was performed according to manufacturer’s instructions using the Criterion™ Cell system (Bio-Rad, Hercules, CA). Protein of cellular extracts was determined by BCA assay according to the manufacturer’s protocol (Pierce
Protein Research Products, Thermo Scientific, Rockford, IL). Absorbency was measured using a Bio-Tek Synergy HT plate reader (Bio-Tek Instruments, Winooski, VT). Samples were normalized in Laemli’s buffer. Cytoplasmic or nuclear protein (50 or 20 μg per lane, respectively) were loaded in 4-20% gradient polyacrylamide gels (TRIS-HCL) immediately following boiling for 5 minutes in Laemli’s buffer. Resolved protein was then transferred to nitrocellulose membranes using a CAPS discontinuous buffer system and Trans-Blot® SD transfer cell according to manufacturer’s instructions (Bio-Rad tech note 2134). Membranes were blocked for 1 hour at room temperature in 5% non-fat dry milk/TBST, and probed overnight at 4°C with primary antibody diluted in TBST at concentrations recommended by the manufacturer (Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were incubated in HRP-conjugated secondary antibodies (Pierce Protein Research Products, Thermo Scientific, Rockford, IL), diluted 1:1000 in TBST, for 1 hour at room temperature and developed using SuperSignal West Dura Extended Duration Substrate reagents according to the manufacturer’s protocol (Pierce Protein Research Products, Thermo Scientific, Rockford, IL), prior to exposure. Membranes were re-probed for Actin (cytoplasmic marker) or LaminA (nuclear marker) to insure equal loading and purity of cytoplasmic or nuclear extracts. Bands were quantitated with the BioSpectrum AC Imaging System (UVP BioImaging Systems, Upland, CA), and data was normalized to corresponding actin or LaminA values.

Statistical Analysis
Where error bars are shown, data was obtained from experiments using 6 animals per group. Data are reported as mean ± standard deviation. Significance was assessed using either the Student’s t-test or ANOVA where appropriate. Differences between data sets were considered statistically significant if p < 0.05.

Results

As shown in Table 1, bodyweights from each group did not differ significantly between controls and organoselenium diets, which is consistent with observations from previous studies. These data suggests that the doses of the organoselenium compounds are not toxic over the time period of the study.
To investigate the effects of organoselenium compounds on glutathione levels, glutathione was measured in whole blood and lung and liver homogenates. Results are presented in Table 2. Feeding p-XSC results in significant 37% (p = 0.01) and 230% (p = 0.0005) increases over controls in whole blood and lung glutathione levels, respectively. Whole blood GSH levels were significantly induced 24% (p = 0.04), and lung GSH was enhanced 200% by

Table 1: Effects of Organoselenium Compounds on Bodyweights.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Baseline</th>
<th>1 Week</th>
<th>2 Weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>106 ± 7.79</td>
<td>138 ± 10.6</td>
<td>180 ± 13.4</td>
</tr>
<tr>
<td>p-XSC</td>
<td>105 ± 11.1</td>
<td>137 ± 15.9</td>
<td>175 ± 21.4</td>
</tr>
<tr>
<td>p-XSeSG</td>
<td>104 ± 8.81</td>
<td>135 ± 11.1</td>
<td>176 ± 13.8</td>
</tr>
<tr>
<td>Selenomethionine</td>
<td>105 ± 7.83</td>
<td>128 ± 12.7</td>
<td>167 ± 17.8</td>
</tr>
</tbody>
</table>

Values are mean ± standard deviation (n = 6)
Selenomethionine resulted in a significant 34% induction in whole blood GSH (p = 0.003), but no significant change in lung GSH was observed. No significant changes were detected in liver GSH by any of the organoselenium compounds tested.

Table 2: Concentrations of glutathione in blood or tissue of rats fed organoselenium compounds.

<table>
<thead>
<tr>
<th></th>
<th>Glutathione</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diet</td>
<td>Whole Blood</td>
<td>Lung</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(μmol/g hemoglobin)</td>
<td>(μmol/g tissue)</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>8.17 ± 1.24</td>
<td>1.95 ± 0.13</td>
</tr>
<tr>
<td>p-XSC</td>
<td></td>
<td>11.2 ± 1.76*</td>
<td>4.39 ± 0.74*</td>
</tr>
<tr>
<td>p-XSeSG</td>
<td></td>
<td>10.1 ± 1.75*</td>
<td>3.73 ± 0.44*</td>
</tr>
<tr>
<td>selenomethionine</td>
<td></td>
<td>11.1 ± 0.89*</td>
<td>2.32 ± 0.27</td>
</tr>
</tbody>
</table>

Values are mean ± standard deviation (n = 6)
*Significant difference (p < 0.05) between control and experimental groups.
To determine if the rate-limiting enzyme responsible for *de novo* glutathione synthesis, GCL, is induced by organoselenium compounds, protein levels of modulatory and catalytic subunits were compared by western blotting of cytoplasmic lung and liver extracts. Results are presented in Table 3. Protein levels of GCLc, were significantly induced 55% in the lung by *p*-XSC (*p* = 0.007), and 88% by *p*-XSeSG (*p* = 0.01). Selenomethionine resulted in no significant induction of GCLc relative to controls. In the lung, no significant difference in relative GCLm protein expression was observed by any organoselenium compound. Likewise, in the liver, no significant difference in relative protein expression of either GCLc or GCLm was observed by any organoselenium compound.
To investigate the effects of organoselenium compounds on nuclear Nrf2 levels, western blotting was applied to nuclear extracts of lung and liver homogenates from rats fed control, $p$-XSC, $p$-XSeSG, or selenomethionine containing diets. Results are shown in Figure 5. Feeding of $p$-XSC resulted in a significant 6.1-fold ($p = 0.0002$) increase in nuclear Nrf2 in the lung relative to

<table>
<thead>
<tr>
<th>Diet</th>
<th>Lung</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GCLc</td>
<td>GCLm</td>
</tr>
<tr>
<td>Control</td>
<td>1.00 ± 0.12</td>
<td>1.00 ± 0.22</td>
</tr>
<tr>
<td>$p$-XSC</td>
<td>1.55 ± 0.29*</td>
<td>1.09 ± 0.08</td>
</tr>
<tr>
<td>$p$-XSeSG</td>
<td>1.88 ± 0.55*</td>
<td>1.19 ± 0.23</td>
</tr>
<tr>
<td>Selenomethionine</td>
<td>1.53 ± 0.31</td>
<td>1.00 ± 0.19</td>
</tr>
</tbody>
</table>

Values are mean ± standard deviation (n = 6)

*Significant difference ($p < 0.05$) between control and experimental groups.
controls. No significant changes in lung nuclear Nrf2 were observed by \( p\)-XSeSG or selenomethionine. Likewise, in the liver, no significant effects on relative nuclear Nrf2 levels were observed by any organoselenium compound.

Figure 5: Relative Nuclear Nrf2 expression in lung or liver of rats fed organoselenium compounds. Protein expression of nuclear Nrf2, normalized to nuclear Lamin A, in lung and liver as determined by western blotting and quantitation of nuclear tissue extracts.
Because Nrf2 normally resides in the cytoplasm, it is important that nuclear extracts are not contaminated by cytoplasmic protein. Therefore, western blots of nuclear and cytoplasmic samples were simultaneously probed for the nuclear marker, LaminA and the cytoplasmic marker, GAPDH. No cross contamination was detected, even at saturating exposure levels, suggesting high efficacy of the nuclear and cytoplasmic extraction process.

The ERK pathway has been shown to be involved in Nrf2 stabilization in vitro. To investigate the in vivo effects of organoselenium compounds on nuclear p-ERK levels, western blotting was applied to nuclear extracts of lung and liver homogenates from rats fed control, $p$-XSC, $p$-XSeSG, or selenomethionine containing diets. Results are shown in Figure 6. Feeding of $p$-XSC resulted in a significant 2.2-fold increase in nuclear p-ERK in the lung relative to controls ($p = 0.04$). In contrast, no significant changes in lung nuclear p-ERK were observed by $p$-XSeSG or selenomethionine. Furthermore, no significant induction of nuclear p-ERK was observed in the liver by any organoselenium compound.
Figure 6: Relative Nuclear p-ERK expression in lung or liver of rats fed organoselenium compounds. Protein expression of nuclear p-ERK, normalized to nuclear Lamin A, in lung and liver as determined by western blotting and quantitation of nuclear tissue extracts.

Discussion
These results extend the previous finding of GSH induction by \textit{p}-XSC in the A/J mouse lung to the rat lung. The data also suggests that the induction of GSH by \textit{p}-XSC in the rat lung results from elevated expression of GCLc. Due to the presence of ARE sites in the promoter of GCLc, it is likely that the observed induction of nuclear Nrf2 by \textit{p}-XSC in the rat lung is responsible for the concurrent GCLc induction. Furthermore, because \textit{p}-ERK has been shown \textit{in vitro} to stabilize Nrf2 and promote its nuclear translocation, the observed induction of \textit{p}-ERK by \textit{p}-XSC in the rat lung may be partly responsible for enhancing nuclear Nrf2 levels. Taken together, these data support our primary hypothesis of Nrf2 involvement in \textit{p}-XSC-mediated GSH induction.

It is thus reasonable to propose the following mechanistic pathway. The ERK pathway is activated in the lung by \textit{p}-XSC leading to increased nuclear levels of phosphorylated ERK, which in turn phosphorylates Nrf2 leading to its increased stability and accumulation in the nucleus. Elevated nuclear Nrf2 is then available to bind ARE sites in the promoter of the GCLc gene, leading to its increased transcription and eventual enhanced protein expression and accumulation in the cytoplasm. A significant, more than doubling of lung GSH over controls is the final result of the enhanced GCL activity.

It may be important that \textit{p}-XSC was found to induce GSH in yet another species. The previous observation that \textit{p}-XSC results in elevated GSH in lungs of A/J mice is tempered by the fact that this strain is highly susceptible to spontaneous lung tumors (Richie, Kleinman et al. 2006). The genetic defects responsible for this susceptibility are unknown, but disregulated GSH metabolism
could conceivably be involved. The present finding that \( p \)-XSC induces GSH in the lung of the Fisher rat, a species without susceptibility to lung tumors, thus generalizes the \textit{in vivo} potential of \( p \)-XSC as a chemopreventive and GSH-inducing agent.

It is interesting that nuclear \( p \)-ERK was observed in parallel with nuclear Nrf2 induction by \( p \)-XSC, as this effect has not previously been observed \textit{in vivo}. Phosphorylation of Nrf2, mediated by extracellular signal-regulated kinase (ERK) and c-Jun-NH\(_2\)-kinase (JNK) pathways, has been shown \textit{in vitro} to disrupt the association of Nrf2 with Keap1 leading to elevated nuclear Nrf2 levels (Xu, Yuan et al. 2006). Furthermore, transfection of ERK or JNK into PC-3 cells resulted in elevated nuclear Nrf2 and activation of heme oxygenase-1, an ARE regulated gene (Xu, Yuan et al. 2006). Neither post-translational modifications of Nrf2, such as phosphorylation, or induction of ERK or JNK kinase pathways, has been observed \textit{in vivo}, and involvement of JNK was not explored in this study.

It is shown here, for the first time in \textit{any} species, that \( p \)-XSeSG induces lung GSH, and to a similar extent as \( p \)-XSC. Furthermore, like \( p \)-XSC, GCLc was significantly induced in the rat lung by \( p \)-XSeSG. These data suggest that \( p \)-XSC and \( p \)-XSeSG may act via similar pathways with the exception that induced \( p \)-ERK and Nrf2 was not observed in the case of \( p \)-XSeSG. Although it is possible that Nrf2-independent pathways are operative in \( p \)-XSeSG-mediated GSH induction, other explanations are more likely. It is expected that \( p \)-XSeSG is metabolized differently \textit{in vivo} than \( p \)-XSC. As \( p \)-XSeSG is thought to be the major metabolite of \( p \)-XSC (El-Bayoumy and Sinha 2004), and because
glutathiolated compounds are usually rapidly excreted, it is expected that less of
the agent would be incorporated into tissue. One possibility is that p-ERK and
Nrf2 were actually induced by p-XSeSG feeding, but the pulse was missed in
these experiments. Rats feed nocturnally, and it is possible that a blunted or
short-lived induction of nuclear Nrf2 in the lung by p-XSeSG may have dissipated
by the time of sacrifice. It is fully expected that a short pulse of Nrf2 induction,
following feeding of an inducer, would result in a more sustained induction of
GCLc and GSH. Future experiments may require higher doses and/or various
times of sacrifice after feeding p-XSeSG to better explore the possible induction
of nuclear p-ERK and Nrf2 in the rat lung.

Insight into the differential behavior of p-XSC and p-XSeSG in vivo may be
gained through in vitro observations. Both p-XSC and p-XSeSG activated an
ARE-luciferase reporter in HepG2 cells, albeit in different manners (Chapter 3). A
peak induction of 3-fold occurs at a p-XSC concentration of 10 μM, while the
same concentration of p-XSeSG resulted in no significant induction at all.
However, increasing the concentration of p-XSeSG caused a dose-dependent
luciferase induction, which ultimately surpassed the maximum induction
attainable by p-XSC by nearly 2-fold. Nevertheless, the effects of p-XSeSG are
attenuated when compared to p-XSC, and 6-fold higher concentrations of p-
XSeSG were required to achieve the peak ARE induction caused by p-XSC.
Translating these observations into an in vivo setting predict that higher doses of
p-XSeSG may be required to result in the same degree of Nrf2 induction by p-
XSC. In additional, in vitro studies with wildtype and Nrf2-knockout mouse
embryonic fibroblasts established that $p$-XSeSG-mediated induction of GCLc was absolutely dependent upon Nrf2 (Chapter 3). These data further support the hypothesis that Nrf2 may actually be involved in $p$-XSeSG-mediated induction of GCLc *in vivo*. If $p$-XSC and/or $p$-XSeSG induce the Nrf2 pathway, then other ARE-regulated genes should be activated in the lung. In an A/J mouse study, others have shown $p$-XSC-mediated induction of total glutathione S-transferase (GST) activity in the lung (Prokopczyk, Rosa et al. 2000), and certain GST subunits are known to be ARE-regulated.

Feeding of selenomethionine resulted in no significant change in lung GSH, GCLc, nuclear Nrf2 or p-ERK. Selenomethionine has not been shown by others to induce the Nrf2 pathway, and mechanisms of chemoprevention by selenomethionine are rather thought to be through induction of apoptosis and cell-cycle arrest (Nelson, Goulet et al. 2005; Connelly-Frost, Poole et al. 2006). In tumor cells, selenium is actually thought to *inhibit* the Nrf2-regulated peroxiredoxin 1 pathway (Kim, Baek et al. 2007). Although we found no p-ERK induction by selenomethionine, others have shown activation of the ERK pathway *in vitro* (Nelson, Goulet et al. 2005).

Our study was designed with the intention of comparing effectiveness of organoselenium compounds by feeding 10 ppm as selenium of each compound, as this dose was previously shown to be effective for $p$-XSC in A/J mice (Richie, Kleinman et al. 2006). In our rat study, no induction of liver GSH was observed by any organoselenium compound, including selenomethionine. However, in a
Dalton’s lymphoma bearing mouse model, a dose of 10 ppm selenomethionine did induce hepatic GSH (Mukhopadhyay-Sardar, Rana et al. 2000).

Future studies are required to determine the level of dependence upon nuclear Nrf2 of \( p \)-XSC or \( p \)-XSeSG-mediated GSH induction in the lung. Similar feeding experiments with Nrf2-knockout mice would be most useful, and simultaneous feeding of ERK or JNK pathway inhibitors may help establish the dependence of Nrf2 induction upon p-ERK or JNK. As mentioned previously, additional experiments should be performed to examine metabolic kinetics and to establish appropriate doses of \( p \)-XSeSG. Perhaps a bolus dose, administered by gavage, followed by a time course analysis of p-ERK, Nrf2, GCLc and GSH would better address temporal concerns and cause/effect hypotheses. Finally, additional studies should be performed to explore if \( p \)-XSC and/or \( p \)-XSeSG are potent inducers of other ARE regulated Phase II chemoprotective genes in the rat lung and other tissues.

In summary, we have shown induction of the Nrf2 transcription factor in \( p \)-XSC-mediated induction of glutathione in the rat lung, and also presented evidence for involvement of the ERK pathway in this process. These results suggest that \( p \)-XSC acts through the Nrf2 pathway \textit{in vivo}, and that \( p \)-XSeSG may be the metabolite responsible for such activation, thus offering \( p \)-XSeSG as a less toxic, yet highly efficacious inducer of GSH. Induction of GCLc, and possibly other Nrf2-mediated Phase II genes, may represent a general mechanism of chemoprevention by \( p \)-XSC and related organoselenium compounds, and future studies are planned to examine effects in other relevant
tissues such as prostate, tongue and colon. Induction of GSH, with low associated toxicity, by agents like \( p \)-XSeSG may prove useful for treatment of conditions such as HIV and chronic inflammatory conditions.

References


Chapter 3

Induction of Glutamate Cysteine Ligase by 1,4-phenylenebis(methylene)selenocyanate and its Glutathione Conjugate is Dependent upon Nuclear factor-erythroid 2-Related Factor 2

Keywords: \( p\)-XSeSG, Nrf2, MEF, \( \gamma\)-GCL, chemoprevention, glutathione, \( p\)-XSC organoselenium, Antioxidant response element (ARE), HepG2-ARE

Abstract

Levels of dietary selenium are inversely associated with cancer risk in humans, and selenium has played a major role in the field of chemoprevention. In particular, the organoselenium agents 1,4-phenylenebis(methylene)selenocyanate (\( p\)-XSC) and its glutathione conjugate, \( p\)-XSeSG, are effective in preventing cancers at numerous sites including the lung in rodent models. In the A/J mouse lung, \( p\)-XSC is highly effective in inducing glutathione (GSH), but mechanisms are not well understood. Glutathione is the most abundant antioxidant in animals and is an important mechanistic factor in chemoprevention. Glutamate cysteine ligase (GCL) is the rate-limiting enzyme responsible for GSH production. Transcriptional regulation of GCL occurs via
antioxidant response elements (ARE) in the promoter regions of its genes. Induction of the ARE is in turn regulated by nuclear translocation of the transcription factor, Nuclear factor-erythroid 2-Related Factor 2 (Nrf2).

The potential relevance of Nrf2 to GSH induction led to the hypothesis that Nrf2 activation may be involved in organoselenium-mediated induction of GSH, and this was investigated in several cell culture models. Here we report that p-XSC and p-XSeSG activate an ARE luciferase reporter in HepG2 cells in an Nrf2-dependent manner, and that p-XSeSG is less toxic than p-XSC and also induces GSH in these cells. Treatment of HepG2 ARE reporter cells with 100 μM p-XSeSG resulted in significant (p < 0.05) inductions in ARE luciferase activity (640%), nuclear Nrf2 (540%) and GSH (150%). The dependence of GCL induction by p-XSeSG upon Nrf2 was confirmed in wildtype and Nrf2 deficient Mouse Embryonic Fibroblasts (MEF). Treatment of MEF cells with 100 μM p-XSeSG resulted in significant (p < 0.05) inductions in GCLc (2-fold) and GCLm (60-fold) in wildtype, but not Nrf2 deficient cells. These results provide supporting evidence that p-XSC may act through the Nrf2 pathway in vivo, and that p-XSeSG may be the metabolite responsible for such activation, thus offering p-XSeSG as a less toxic, yet highly efficacious inducer of GSH. The induction of GSH via the Nrf2 pathway by organoselenium compounds may represent a general mechanism of chemoprevention.

Introduction
Chemoprevention can be defined as a therapeutic effort to block, slow or reverse carcinogenic processes, and usually employs naturally occurring agents such as phytochemicals, but may also include synthesized compounds. Selenium, in both organic and inorganic forms, has played a major role in the field of chemoprevention, particularly after the reporting of an almost 50% reduction in morbidity and mortality by major cancers following dietary supplementation with selenized brewer’s yeast (Clark, Combs et al. 1996). Various synthetic selenium compounds have been developed with goals of lowering toxicity while enhancing efficacy when compared to other popular chemopreventive agents such as selenite or selenomethionine (El-Bayoumy and Sinha 2004). The organoselenium agents 1,4-phenylenebis(methylene)selenocyanate (p-XSC) and its glutathione conjugate, p-XSeSG have proven to be effective in preventing a variety of carcinogen-induced cancers in several animal models (Tanaka, Makita et al. 1997; Rao, Wang et al. 2001; Richie, Kleinman et al. 2006).

It has been reported that elevated lung GSH levels coincide with p-XSC-mediated protection from NNK-induced lung tumors in an A/J mouse model, although the mechanism by which this occurs is unknown (Richie, Kleinman et al. 2006). Glutathione is the most important and abundant endogeneous antioxidant in mammals, and induction of supra-normal levels likely is part of an agent’s chemopreventive potential (Townsend, Tew et al. 2003; Huber and Parzefall 2007).
Both modulatory and catalytic subunits of the rate-limiting enzyme for GSH synthesis, Glutamate-Cysteine Ligase (GCLm and GCLc, respectively), are regulated in part by the presence of Antioxidant Response Elements (ARE) in upstream promoter regions of each gene (Wild, Moinova et al. 1999). Enhanced nuclear translocation and subsequent binding of the nuclear factor-erythroid 2-related factor 2 (Nrf2) transcription factor to ARE-containing promoters activates a variety of chemoprotective Phase II genes, including many in the GSH homeostasis pathways (Zhang and Gordon 2004).

Nrf2 is normally sequestered in the cytoplasm by the actin-bound protein Keap1, a substrate adaptor for an E3 ubiquitin ligase, which targets Nrf2 for rapid turnover (Zhang, Lo et al. 2005). Keap1 contains multiple reactive cysteine residues that, when modified directly or indirectly by a variety of inducers, reduce its affinity for and promote the nuclear translocation of Nrf2 (Zhang and Hannink 2003). The synthetic organoselenium compound Ebselen has been shown in a cell culture model to directly modify Keap1 (Sakurai, Kanayama et al. 2006). Phosphorylation of Nrf2, mediated by extracellular signal-regulated kinase and c-Jun-NH₂-kinase pathways, has also been shown to disrupt its association with Keap1 (Xu, Yuan et al. 2006).

We recently reported that enhanced GSH levels concurrent with elevated nuclear Nrf2 and cytoplasmic GCLc protein expression in lung tissue of Fisher rats fed p-XSC. The primary objective of the studies reported here was to determine if and to what extent p-XSC and p-XSeSG depend on nuclear translocation of Nrf2 in their ability to influence glutathione levels in several cell
culture models. Benzyl selenocyanate (BSC) and phenylethyl selenocyanate (PESC), additional organoselenium compounds with close structural relationships to p-XSC, have also been tested in animal models of cancer with varying degrees of success (Kawamori, El-Bayoumy et al. 1998). Therefore, a secondary aim of the present study was to examine relationships of chemical structure of these organoselenium compounds to their proposed ability to activate ARE-regulated genes.

**Materials and Methods**

**Reagents**

Chemical structures of p-XSC and related compounds are shown in Figure 1. Organoselenium compounds were obtained courtesy of Dhimant H. Desai and had been synthesized as described previously (Sohn, Desai et al. 2005). Antibodies (Nrf2, GCLc, GCLm, Actin, LaminA) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

**Cell Culture**

HepG2 human hepatoma cells stably transfected with ARE-luciferase plasmid were obtained courtesy of Muriel Cuendet (Cuendet, Oteham et al. 2006). Cells were maintained in F-12 media (Invitrogen) supplemented with 10%
fetal bovine serum, antibiotics, MEM amino acid solution, and insulin (1.45 μg/ml).

Wildtype and Nrf2 deficient (Nrf2 -/-) mouse embryonic fibroblasts (MEF) were obtained courtesy of (Wakabayashi, Dinkova-Kostova et al. 2004). Cells were maintained in Iscov’s MDM (Gibco12440) supplemented with 10% fetal bovine serum, and antibiotics.

**Viability Assay**

Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reduction assay, according to the manufacturer’s instructions (CellTiter 96™, Promega). Absorbency at 490 nm was measured using a Bio-Tek Synergy HT plate reader.

**Luciferase Assay**

Treated HepG2 ARE reporter cells were washed in PBS, lysed, and luciferase activity of resulting extracts was measured according to the Luciferase Assay System protocol (Promega) using a single tube luminometer.

**Glutathione Assay**
Aliquots of cells were washed in PBS and lysed in 5% metaphosphoric acid. Precipitated protein was removed by centrifugation. Free glutathione in cellular extracts was determined using the previously described enzymatic recycling method using Elman’s reagent (Tietze 1969) with modifications (Richie, Skowronski et al. 1996). Absorbency over time was measured using a Bio-Tek Synergy HT plate reader (Bio-Tek Instruments, Winooski, VT). Glutathione levels are normalized to protein levels as determined by BCA assay.

**Cellular Extracts and Western Blotting**

Cellular extracts were obtained using NE-PER® Nuclear and Cytoplasmic Extraction Reagents according to the manufacturer’s protocol (Pierce). Western blotting for nuclear Nrf2 and LaminA, or cytoplasmic GCLc, GCLm or Actin, was performed according to manufacturer’s instructions using the Criterion™ Cell system (Bio-Rad, Hercules, CA). Protein of cellular extracts was determined by BCA assay according to the manufacturer’s protocol (Pierce Protein Research Products, Thermo Scientific, Rockford, IL). Absorbency was measured using a Bio-Tek Synergy HT plate reader (Bio-Tek Instruments, Winooski, VT). Samples were normalized in Laemli’s buffer. Cytoplasmic or nuclear protein (50 or 20 μg per lane, respectively) were loaded in 4-20% gradient polyacrylamide gels (TRIS-HCL) immediately following boiling for 5 minutes in Laemli’s buffer. Resolved protein was then transferred to nitrocellulose membranes using a CAPS
discontinuous buffer system and Trans-Blot® SD transfer cell according to manufacturer's instructions (Bio-Rad tech note 2134). Membranes were blocked for 1 hour at room temperature in 5% non-fat dry milk/TBST, and probed overnight at 4°C with primary antibody diluted in TBST at concentrations recommended by the manufacturer (Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were incubated in HRP-conjugated secondary antibodies (Pierce Protein Research Products, Thermo Scientific, Rockford, IL), diluted 1:1000 in TBST, for 1 hour at room temperature and developed using SuperSignal West Dura Extended Duration Substrate reagents according to the manufacturer’s protocol (Pierce Protein Research Products, Thermo Scientific, Rockford, IL), before exposure. Membranes were re-probed for Actin (cytoplasmic marker) or LaminA (nuclear marker) to insure equal loading and purity of cytoplasmic or nuclear extracts. Bands were quantitated with the BioSpectrum AC Imaging System (UVP BioImaging Systems, Upland, CA), and data was normalized to corresponding actin or LaminA values.

**RNA Interference**

HepG2 ARE reporter cells were grown to 60% confluence in 6 cm dishes then transfected with non-targeting siRNA-Control (siControl) or siRNA-Nrf2 (siNrf2, sc-37030) using manufacturer-recommended reagents and protocols (Santa Cruz Biotechnology, Santa Cruz, CA). Thirty hours after siRNA transfection, siControl and siNrf2 cells were exposed to vehicle control, 10 μM p-
XSC, or 100 μM \( p \)-XSeSG added to the cell media. Following 18 hours of drug exposure, cells were assayed for viability and luciferase activity and cytoplasmic or nuclear extracts were subjected to western blotting.

**Statistical Analysis**

Where error bars are shown, data was obtained from at least 3 independent experiments. Data are reported as mean ± standard deviation. Significance was assessed using either the Student's t-test or ANOVA where appropriate. Differences between data sets were considered statistically significant if \( p < 0.05 \).

**Results**

To determine if \( p \)-XSC and \( p \)-XSeSG activate the ARE pathway *in vitro*, HepG2-ARE-luciferase reporter cells were employed. Time-course studies of luciferase activation and viability were performed after exposure of HepG2 ARE-luciferase reporter cells to various concentrations of \( p \)-XSC and \( p \)-XSeSG. Different responses were observed when comparing these compounds, and a 22-hour exposure time was chosen to minimize toxic effects by \( p \)-XSC, while still allowing observation of luciferase induction as a function of drug concentration. Figure 7 shows effects upon cell viability from a 22-hour exposure to varying concentrations of \( p \)-XSC and \( p \)-XSeSG. With increasing concentration, viability is reduced by either compound, however at equimolar concentrations, \( p \)-XSC
reduced viability significantly greater than $p$-XSeSG for every concentration tested. At concentrations of 100 $\mu$M, approximately 80% of $p$-XSeSG treated cells remained viable while cells exposed to this same concentration of $p$-XSC exhibited a relative viability of 0%, suggesting that all cells had been killed. The concentration of $p$-XSC resulting in 50% viability was approximately 25 $\mu$M.
Figure 7: HepG2 ARE-Luciferase Viability upon p-XSC or p-XSeSG Treatment. Effects of varying concentration of p-XSC or p-XSeSG upon HepG2 ARE-luciferase cell viability. Cells at 50% confluence were provided fresh media containing vehicle control or 10, 25 or 100 μM of p-XSC or p-XSeSG. After 22 hours, cells were assayed for viability by MTS absorbance at 490 nm. Data are normalized to control values and represent the mean of 3 independent experiments ± standard deviation.
Both $p$-XSC and $p$-XSeSG activated the ARE-luciferase reporter in HepG2 cells. Fold induction as a function of drug concentration after 22-hour exposures are shown in Figure 8. A peak induction of 3-fold occurred at a $p$-XSC concentration of 10 $\mu$M with no increases being observed at higher concentrations. In contrast, 10 $\mu$M $p$-XSeSG resulted in no significant induction. However, increasing the concentration of $p$-XSeSG resulted in a higher luciferase activity than was attainable by $p$-XSC. Above 10 $\mu$M, $p$-XSeSG causes a dose-dependent induction and the highest concentration tested (100 $\mu$M) yielded a 5-fold luciferase activity over vehicle-treated control cells.
Figure 8: HepG2 ARE-Luciferase Activity upon p-XSC or p-XSeSG Treatment. Effects of varying concentration of p-XSC or p-XSeSG upon HepG2 ARE-luciferase reporter activity. Cells at 50% confluence were provided fresh media containing vehicle control or 10, 25 or 100 μM of p-XSC or p-XSeSG. After 22 hours, cells were washed with PBS, lysed and luciferase activity was determined using Promega’s Luciferase Assay System and single tube luminometer. Data are normalized to control values and represent the mean of 3 independent experiments ± standard deviation.
The organoselenium compounds BSC and PESC are structurally related to p-XSC, as shown in Figure 1, and have been evaluated as chemopreventive agents by others. Chemical structure may affect the ability of an organoselenium compound to induce ARE-regulated genes, so these additional compounds were tested along with p-XSC and p-XSeSG in HepG2 ARE-luciferase reporter cell culture. To avoid the potential for toxic effects, a 6-hour exposure time was used to compare luciferase induction by these compounds. It was found that BSC and PESC also induce the ARE reporter, and peak luciferase activity, relative viability and optimal concentrations (those required to achieve peak luciferase induction) for these compounds along with p-XSC and p-XSeSG data are reported in Table 4. None of the organoselenium compounds tested caused a reduction in cell viability within the 6-hour exposure time. Both BSC and PESC resulted in peak luciferase activity at concentrations of 10 μM as compared with p-XSC’s peak occurring at 20 μM. The magnitude of the peak luciferase activity is 3.7-fold for BSC in contrast with the approximately 2-fold increases seen by p-XSC and PESC. As stated previously, p-XSeSG-mediated luciferase activity does not peak, even at concentrations as high as 100 μM. A concentration of 50 μM p-XSeSG for 6 hours resulted in a 1.5 fold induction.
Table 4: HepG2 ARE-Luciferase Activity upon Organoselenium Treatment.

Peak HepG2 ARE-luciferase reporter activity, optimal concentration and corresponding viability after a 6-hour exposure to organoselenium compounds. Cells at 50% confluence were provided fresh media containing vehicle control or varying concentrations of organoselenium compounds. After 6-hours, cells were assayed for MTS viability, washed with PBS, lysed and luciferase activity was determined using Promega’s Luciferase Assay System. Data are normalized to control values and represent the mean of 3 independent experiments ± standard deviation.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Luciferase Induction (Fold)</th>
<th>Optimal Concentration (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-XSC</td>
<td>$2.0 \pm 0.1^a$</td>
<td>20</td>
</tr>
<tr>
<td>BSC</td>
<td>$3.7 \pm 0.3^a$</td>
<td>10</td>
</tr>
<tr>
<td>PESC</td>
<td>$2.2 \pm 0.2^a$</td>
<td>10</td>
</tr>
<tr>
<td>p-XSeSG</td>
<td>$1.5 \pm 0.3^a$</td>
<td>&gt; 50</td>
</tr>
</tbody>
</table>

$^a$ p < 0.05
Because the genes for the rate-limiting enzyme of glutathione synthesis are thought to be regulated in part by AREs, it was hypothesized that HepG2 ARE-luciferase cells would have elevated glutathione levels concurrent with reporter activation. Figure 9 shows relative glutathione levels in HepG2 ARE-luciferase cells when exposed for 22 hours to varying concentrations of \( p \)-XSC or \( p \)-XSeSG. Treatment with \( p \)-XSeSG results in significant increases in glutathione relative to vehicle treated control cells at all concentrations tested. Elevations of 120%, 155%, and 150% were seen at concentrations of 10, 25, and 100 \( \mu \)M respectively. No significant increases in glutathione levels were observed by \( p \)-XSC treatment at any concentration tested.
Figure 9: HepG2 ARE-Luciferase Glutathione Levels upon p-XSC or p-XSeSG Treatment. Effects of varying concentration of p-XSC or p-XSeSG upon HepG2 ARE-luciferase cell glutathione levels. Cells at 50% confluence were provided fresh media containing vehicle control or 10, 25 or 100 μM of p-XSC or p-XSeSG. After 22 hours, cells were washed with PBS, lysed in 5% metaphosphoric acid and glutathione was measured by enzymatic recycling method. Data are normalized to control values and represent the mean of 3 independent experiments ± standard deviation.
To support the hypothesis that Nrf2 is responsible for the ARE-luciferase inductions observed by \( p \)-XSC and \( p \)-XSeSG, nuclear extracts from treated HepG2 reporter cells were subjected to western analysis for Nrf2. A typical blot is shown in Figure 10 along with a bar graph showing nuclear Nrf2 quantitation in tandem with luciferase activation under several cell treatment conditions. Nuclear Nrf2 protein levels are significantly induced in a dose-dependent manner by \( p \)-XSeSG. Although the increase in nuclear Nrf2 levels induced by 10 \( \mu \)M \( p \)-XSC was not statistically significant, the induction of luciferase was. In addition, nuclear Nrf2 protein levels were generally associated with luciferase activation over all concentrations of organoselenium compounds. For example, nuclear Nrf2 protein levels were induced 5.4-fold by 100 \( \mu \)M \( p \)-XSeSG compared to the 6.4-fold increase in luciferase activity observed under the same conditions.
Figure 10: HepG2 ARE-Luciferase Activity and Nuclear Nrf2 upon p-XSC or p-XSeSG Treatment. Effects of p-XSC or p-XSeSG upon HepG2 ARE-luciferase activity and nuclear Nrf2 levels. Cells at 50% confluence were provided fresh media containing vehicle control or 10 μM of p-XSC or 50 or 100 μM p-XSeSG. After 22 hours, cells were washed with PBS, trypsinized and collected. A portion was lysed and luciferase activity was determined using Promega’s Luciferase Assay System. Nuclear extracts were obtained from remaining cells using Pierce’s NE-PER kit. Nuclear extracts were subjected to western blotting for Nrf2 and normalized to Lamin A. Data are normalized to control values and represent the mean of 3 independent experiments ± standard deviation.
It was hypothesized that experimental depletion of Nrf2 in cells in culture would inhibit organoselenium-induced luciferase activity and GSH levels. To test this, short interfering RNA targeting Nrf2 (siNrf2) or non-targeting control RNA (siControl) were transfected into HepG2 ARE-luciferase cells prior to an 18-hour treatment by vehicle control, 10 μM p-XSC or 100 μM p-XSeSG. Luciferase induction, nuclear Nrf2 protein and GSH levels were then assayed, and results are shown in Figure 11. The western blot reveals that nuclear Nrf2 protein levels were successfully reduced by siNrf2 in all conditions tested, and the average Nrf2 knockdown, as determined by blot quantitation, was approximately 50%. Luciferase activity was also lowered by siNrf2 for all conditions tested. Glutathione levels were unchanged by siNrf2 in vehicle treated control cells. However, the approximately 8-fold increase in GSH by 100 μM p-XSeSG in siControl cells was reduced to a 3-fold increase by siNrf2. As previously observed, 10 μM p-XSC did not result in large GSH increases, and this was marginally reduced by siNrf2.
Figure 11: Effects of siNrf2 on HepG2 ARE-Luciferase Activity, GSH and Nuclear Nrf2 upon $p$-XSC or $p$-XSeSG Treatment. Effects of short interfering RNA targeting Nrf2 upon $p$-XSC or $p$-XSeSG induced nuclear Nrf2, GSH levels and luciferase activity in HepG2 ARE reporter cells. Cells were grown to 60% confluence then transfected with non-targeting siRNA-Control (siControl) or siRNA-Nrf2 (siNrf2). Thirty hours after siRNA transfection, siControl and siNrf2 cells were exposed to vehicle control, 10 μM $p$-XSC, or 100 μM $p$-XSeSG in fresh media. After 18 hours of drug exposure, portions of cells were assayed for luciferase activity and GSH levels. Nuclear extracts were subjected to western blotting for Nrf2 using LaminA as a control marker.
To quantify the dependence of GSH induction by \( p\)-XSeSG on the Nrf2 transcription factor, wildtype or Nrf2-deficient (Nrf2 -/-) Mouse Embryonic Fibroblasts (MEF) were employed. The degree of toxicity to MEF cells was assessed using the MTS assay after a 24-hour exposure to various concentrations of \( p\)-XSC and \( p\)-XSeSG, and results are plotted in Figure 12. Cell viability was not affected by \( p\)-XSeSG, however \( p\)-XSC proved to be toxic to both wildtype and Nrf2 -/- cells, with relative viability being reduced to approximately 50% of vehicle treated control values at concentrations of 10 \( \mu \)M. Thus, additional experiments could not be performed with \( p\)-XSC, and only \( p\)-XSeSG was used in further experiments with MEF cells.
Figure 12: Viability of Mouse Embryonic Fibroblasts upon p-XSC or p-XSeSG Treatment. Effects of varying concentration of p-XSC or p-XSeSG upon wildtype or Nrf2 deficient MEF cell viability. Cells at 50% confluence were provided fresh media containing vehicle control or 5, 10 or 20 μM of p-XSC or p-XSeSG. After 24 hours, cells were assayed for viability by MTS absorbance at 490 nm.

Nuclear Nrf2 protein levels, as determined by western blotting of nuclear extracts, were significantly enhanced 2.3-fold by a 24-hour exposure to 100 μM
$p$-XSeSG in wildtype MEF cells ($p = 0.02$). A similar induction of mutated, and thus dysfunctional, Nrf2 was seen in Nrf2 -/- MEF cells.

It was hypothesized that the $p$-XSeSG-induced Nrf2 in wildtype MEF cells would result in enhanced protein expression of GCLm and/or GCLc, and that this would not be seen in Nrf2 -/- cells. Results of western blotting of cytoplasmic extracts for these subunits are shown in Figure 13. The modulatory and catalytic subunits of GCL were significantly enhanced 60-fold ($p = 0.05$) and 2-fold ($p = 0.02$) respectively in wildtype, but not Nrf2 -/-, MEF cells after a 24-hour exposure to 100 $\mu$M $p$-XSeSG.
Figure 13: GCL Induction in Mouse Embryonic Fibroblasts upon p-XSeSG Treatment. Effects of p-XSeSG upon cytoplasmic protein levels of GCLm and GCLc in wildtype or Nrf2 deficient MEF cells. Cells at 50% confluence were provided fresh media containing vehicle control or 100 μM p-XSeSG. After 24 hours, cells were washed with PBS, trypsinized and collected. Cytoplasmic extracts were obtained using Pierce’s NE-PER kit. Extracts were subjected to western blotting for GCLm, GCLc and normalized to Actin levels. Data are normalized to control values and represent the mean of 3 independent experiments ± standard deviation.
Discussion

We hypothesized that organoselenium compounds such as \( p \)-XSC and \( p \)-XSeSG exert their chemopreventive effects partly through activation of the Nrf2-mediated ARE pathway. Our recent \textit{in vivo} study (Chapter 2) found nuclear Nrf2 to be induced concurrently with the ARE-regulated GCLc gene in the lung, suggesting involvement of Nrf2, but not establishing dependence. It was thus decided to explore the dependence of GCLc induction upon Nrf2 using cell lines that others have successfully used to screen various inducers of Phase II chemoprotective genes for dependence upon Nrf2 (Wakabayashi, Dinkova-Kostova et al. 2004; Cuendet, Oteham et al. 2006).

Here we show that the chemopreventive organoselenium compounds \( p \)-XSC, \( p \)-XSeSG, BSC, and PESC all induced a HepG2-ARE luciferase reporter, suggesting they act through similar mechanisms and that Nrf2 is involved in the process. It had not been previously reported that \( p \)-XSC and related compounds could induce the ARE pathway, although others have shown organoselenium mediated induction of ARE-regulated genes such as certain glutathione S-transferase (GST) members. For example, in an A/J mouse study, others have shown \( p \)-XSC-mediated induction of total GST activity in the lung (Prokopczyk, Rosa et al. 2000).

The GSH conjugate of \( p \)-XSC, \( p \)-XSeSG has been less studied than \( p \)-XSC, but nevertheless has been shown to be an effective chemopreventive agent \textit{in vivo}. For example, in a rat model, \( p \)-XSeSG was found to inhibit COX
activity and to be effective against AOM-induced colon cancer with low toxicity (Rao, Wang et al. 2001). Our recent Fisher rat study (Chapter 2) found \( p\)-XSeSG to be an effective inducer of lung GCLc and GSH, but enhanced nuclear Nrf2 was not observed, thus indicating the need for \textit{in vitro} studies. Here we demonstrate convincingly that Nrf2 is required for \( p\)-XSeSG-mediated activation of \textit{in vitro} GSH synthesis, and that \( p\)-XSeSG is a more potent and less toxic inducer of the ARE-regulated genes, GCLc and GCLm, than is \( p\)-XSC. The addition of \( p\)-XSeSG to cell culture resulted in significantly elevated levels of nuclear Nrf2 protein in both HepG2 and MEF cells. Furthermore, luciferase activity in HepG2 ARE reporter cells is induced to levels that correlate well with nuclear Nrf2 levels, thus providing strong evidence that Nrf2 is the transcription factor responsible for induction of the luciferase reporter, and therefore is available for activation of other ARE regulated genes such as the subunits of GCL.

It has been established that the genes responsible for \textit{de novo} glutathione synthesis are regulated in part by the ARE pathway (Wild, Moinova et al. 1999). Indeed, we find that glutathione levels are significantly enhanced by \( p\)-XSeSG treatment in HepG2 cells, and demonstrated that both catalytic and modulatory subunits of the rate limiting enzyme of glutathione synthesis, GCLm and GCLc, are significantly enhanced by \( p\)-XSeSG in wildtype MEF cells. That nuclear Nrf2, GCL subunit protein expression and cellular GSH are concurrently induced upon \( p\)-XSeSG treatment does not prove causality. However, the effects of specific experimental manipulation of Nrf2, either by short interfering RNA in HepG2 cells
or through the use of Nrf2-deficient MEF cells, provide powerful evidence of such a relationship. It was hypothesized that depletion of available Nrf2 in HepG2 reporter cells would result in proportionally lowered ARE-luciferase activity and GSH levels. Short interfering RNA specifically targeting Nrf2 in HepG2 cells successfully resulted in an average 50% knockdown of nuclear Nrf2 protein levels, and that this brought about similar reductions in p-XSeSG-mediated luciferase activation and GSH levels. Glutathione in vehicle treated control cells were unaffected by the siNrf2-mediated lowering of nuclear Nrf2 protein which most likely indicates that basal GSH is not regulated by Nrf2, at least in HepG2 cells.

While the experiments employing partial knockdown of Nrf2 by RNA interference support the hypothesis that Nrf2 is involved in p-XSeSG-mediated GSH synthesis, such experiments do not prove total dependence. However, complete reliance of this process upon Nrf2 is supported by experiments with Nrf2-deficient MEF cells. Neither subunit of GCL was induced at all by p-XSeSG treatment of Nrf2 -/- cells despite elevated, albeit dysfunctional, nuclear Nrf2 protein levels and marked and significant enhancements of GCL subunits in their wildtype MEF counterpart.

It is important to address possible reasons for the differences observed between p-XSC and p-XSeSG-mediated responses in both HepG2 and MEF cells. Luciferase activity was induced by p-XSC in HepG2 ARE reporter cells, however concentration-response characteristics differ from those observed by p-XSeSG. At 10uM, the concentration at which p-XSC exhibits maximal ARE
induction, \( p \)-XSeSG results in no activation at all. However, induction by 100\( \text{uM} \) \( p \)-XSeSG surpasses the peak induction attainable by \( p \)-XSC by almost 2-fold. Viability data indicates that \( p \)-XSeSG is less toxic to HepG2 cells than is \( p \)-XSC, and it is likely that toxicity of \( p \)-XSC may diminish its ability to induce the HepG2 ARE-luciferase reporter. Glutathione was not significantly induced by \( p \)-XSC at any concentration tested, although the concentration-response curve paralleled that of ARE-luciferase activation. It is likely that toxic GSH depletion by \( p \)-XSC is responsible in part for limiting its ability to induce the ARE. Other ARE-inducing drugs such as sulforaphane have been demonstrated to deplete GSH in the short term (Singh, Srivastava et al. 2005). Furthermore, \( p \)-XSC was shown to reduce GSH to less than 50\% of control values in human prostate cancer cells (Pinto, Sinha et al. 2007). Studies of the \textit{in-vivo} metabolism of \( p \)-XSC indicate that the major pathway involves glutathiolation of \( p \)-XSC to \( p \)-XSeSG with the release of two molecules of hydrogen cyanide (El-Bayoumy and Sinha 2004). That \( p \)-XSeSG releases no cyanide and is pre-conjugated with glutathione may explain its lower toxicity and also its attenuated ability to induce the ARE. Glutathione conjugation may diminish a compound’s efficacy in cell culture models even if it is an active \textit{in vivo} metabolite. Our recent study with Fisher rats, as reported in the chapter 2, also found \( p \)-XSeSG-mediated effects on lung glutathione and GCLc induction to be somewhat attenuated as compared to \( p \)-XSC-mediated results when fed the same dose (10 ppm as selenium). Toxic effects of \( p \)-XSC in cell culture may be explained by release of cyanide during metabolic processes. However, the addition of relevant
concentrations of potassium cyanide to HepG2 ARE-luciferase reporter cell media results in neither a loss of viability nor induction of the luciferase reporter (data not shown). It is not known if potassium cyanide dissolved in the cell media provides an efficient means to deliver cyanide into cells. In vivo studies also show $p$-XSC to be more toxic than $p$-XSeSG, although the reasons for this are not known (Rao, Wang et al. 2001). It is likely that glutathione conjugation promotes more rapid excretion thus limiting toxicity when compared to $p$-XSC.

We have demonstrated a relationship between an organoselenium compound’s chemical structure and its ability to induce the HepG2 ARE reporter. Benzyl selenocyanate contains one selenocyanate adduct in contrast to $p$-XSC’s two, yet induces ARE-luciferase in HepG2 cells almost 2-fold greater than $p$-XSC at only one half the concentration. This suggests that one selenocyanate adduct may be sufficient for maximal ARE induction, but two adducts may be additive with respect to toxic effects. In contrast, in vivo studies have found $p$-XSC to be a more potent inhibitor of colon carcinogenesis in F344 rats than is BSC (Reddy, Wynn et al. 1996).

In summary, $p$-XSC and the structurally related compounds, BSC and PESC, were shown to activate an ARE reporter, suggesting that their chemopreventive mechanisms may be partly due to induction of Phase II genes and mediated by Nrf2. In addition, when combined with our in vivo data (Chapter 2), the organoselenium compound $p$-XSeSG appears to be a promising GCL inducing agent, which exhibits low toxicity and acts through the Keap1/Nrf2 pathway in the cell models discussed herein. In addition to its potential in
chemoprevention, $p$-XSeSG may prove useful as a glutathione-enhancing agent for treatment of conditions such as HIV, aging and chronic inflammatory conditions.

References


Pinto, J. T., R. Sinha, et al. (2007). "Differential effects of naturally occurring and synthetic organoselenium compounds on biomarkers in androgen
responsive and androgen independent human prostate carcinoma cells."


tumorigenesis induced by a mixture of benzo(a)pyrene and 4-
(methylnitrosamino)-1-(3-pyridyl)-1-butanone by the organoselenium
compound 1,4-phenylenebis(methylene)selenocyanate." Cancer Lett

 glutathione conjugate of 1,4-phenylenebis(methylene)selenocyanate, a
novel organoselenium compound with low toxicity." Cancer Res 61(9):
3647-52.

compounds for potential chemopreventive properties in colon cancer."

1,4-phenylenebis(methylene)selenocyanate inhibits 4-
(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced tumorgenesis and
enhances glutathione-related antioxidant levels in A/J mouse lung." Chem


Sakurai, T., M. Kanayama, et al. (2006). "Ebselen, a seleno-organic antioxidant,


Chapter 4

Enhanced Nrf2-Dependent Induction of Glutathione in Mouse Embryonic Fibroblasts by an Isoselenocyanate Analog of Sulforaphane

Keywords: chemoprevention, glutathione (GSH), selenium, isothiocyanate, isoselenocyanate, sulforaphane (SFN), SFN-isoSe, Antioxidant Response Element (ARE), Nrf2, γ-GCL, HepG2-ARE reporter cells, wildtype and Nrf2 deficient mouse embryonic fibroblasts (MEF).

Abstract

Levels of dietary selenium are inversely associated with cancer risk in humans, and selenium has played a major role in the field of chemoprevention. Epidemiological studies also highlight the substantial chemopreventive effectiveness of cruciferous vegetables, and particularly broccoli, in relation to many cancers. Sulforaphane (SFN), an isothiocyanate, was identified as the major metabolite of broccoli responsible for its chemopreventive properties. Glutathione is the most abundant antioxidant in animals and is an important mechanistic factor in chemoprevention. Glutamate cysteine ligase (GCL) is the rate-limiting enzyme responsible for GSH production. Transcriptional regulation
of GCL occurs via antioxidant response elements (ARE) in the promoter regions of its genes. Induction of the ARE is in turn regulated by stabilization and nuclear translocation of the transcription factor, Nuclear factor-erythroid 2-Related Factor 2 (Nrf2). Furthermore, the extracellular related kinase (ERK) pathway has been shown to play a role in Nrf2 stabilization. Sulforaphane has been shown to act largely through the Nrf2/ARE pathway.

Isothiocyanate compounds such as SFN are among the most potent Nrf2 inducers known. We hypothesized that substitution of sulfur with selenium in the isothiocyanate functional group of SFN would result in an isoselenocyanate compound (SFN-isoSe) with enhanced Nrf2 induction capability. Here we show that SFN-isoSe activates an ARE-luciferase reporter in HepG2 cells more potently than SFN. It was also found that SFN-isoSe induces GCL and GSH in MEF cells in an Nrf2-dependent manner. Finally, we provide evidence that SFN-isoSe is more effective in killing HepG2 cancer cells, yet is less toxic to non-cancer MEF cells, than is SFN.

These data support our hypothesis, and suggest that SFN-isoSe may be a highly effective chemoprotective agent in vivo. Isoselenocyanates, such as SFN-isoSe, may be ideal candidates for future studies as chemopreventive and/or chemotherapeutic agents due to their ability to induce Nrf2 with low toxicity in normal cells and high efficiency at killing cancer cells.

Introduction
Epidemiological studies highlight the substantial chemopreventive effectiveness of cruciferous vegetable intake in relation to many cancers including lung, breast, colon and prostate (Lin, Probst-Hensch et al. 1998; Spitz, Duphorne et al. 2000; Ambrosone, McCann et al. 2004; Joseph, Moysich et al. 2004). Glucosinolate, a precursor of sulforaphane, was isolated from broccoli, a cruciferous vegetable widely consumed by Western societies, and identified as the most potent inducer of phase II detoxification enzymes, such as quinone reductase, glutathione S-transferases, and glutamate cysteine ligase (Zhang, Talalay et al. 1992; McWalter, Higgins et al. 2004). Sulforaphane belongs to a family of chemopreventive agents whose functional group is an isothiocyanate. Sulforaphane has since become one of the most intensively studied chemopreventive phytochemicals, and has been found to act through additional pathways, such as the induction of apoptosis in cancer cells (Gamet-Payrastre, Li et al. 2000; Choi, Lew et al. 2007). Other isothiocyanates derived from phytochemicals such as phenethyl isothiocyanate (PEITC) and benzyl isothiocyanate (BITC) are also potent chemopreventive agents and inducers of Phase II genes (Jiao, Ho et al. 1994; Conaway, Wang et al. 2005).

Selenium has also played a major role in the field of chemoprevention, particularly after the reporting of an almost 50% reduction in morbidity and mortality by major cancers following dietary supplementation with selenized brewer’s yeast (Clark, Combs et al. 1996). Chemopreventive effectiveness depends on the molecular form of administered selenium, and certain plants or yeast grown in selenium-enriched media generate a range of active compounds,
such as selenomethionine and selenocysteine (Dumont, Vanhaecke et al. 2006). Broccoli grown in enriched selenium resulted in better inhibition of colon cancer in rats but also an 80% reduction in glucosinolate production when compared with normal broccoli, however it is unknown whether isoselenocyanate precursors are generated in place of isothiocyanate precursor analogs under such conditions (Finley, Sigrid-Keck et al. 2005). Glutathione is the most important and abundant endogeneous antioxidant in mammals, and induction of supra-normal levels likely plays a role in some of an agent's chemopreventive potential (Townsend, Tew et al. 2003; Huber and Parzefall 2007).

The catalytic subunit of the rate-limiting enzyme for glutathione synthesis, Glutamate-Cysteine Ligase (GCLc), is regulated partly by the presence of Antioxidant Response Elements (ARE) in upstream promoter regions of the gene (Wild, Moinova et al. 1999). Enhanced nuclear translocation and subsequent binding of the nuclear factor-erythroid 2-related factor 2 (Nrf2) transcription factor to ARE-containing promoters activates a variety of chemoprotective Phase II genes, including many in the glutathione homeostasis pathways (Zhang and Gordon 2004). Dietary administration of broccoli seeds, a potent source of sulforaphane, resulted in elevated GCLc in the stomach and small intestine of wildtype but not Nrf2−/− mice, demonstrating in-vivo dependence of sulforaphane-mediated glutathione induction upon Nrf2 (McWalter, Higgins et al. 2004).

Nrf2 is normally sequestered in the cytoplasm by the actin-bound protein Keap1, a substrate adaptor for an E3 ubiquitin ligase, which targets Nrf2 for rapid turnover (Zhang, Lo et al. 2005). Keap1 contains multiple reactive cysteine
residues that, when modified directly or indirectly by a variety of inducers, reduces its affinity for and promotes nuclear translocation of Nrf2 (Zhang and Hannink 2003). The isothiocyanate group of sulforaphane has been demonstrated in vitro to directly modify Keap1 through the formation of thionoacyl adducts (Hong, Freeman et al. 2005).

Substitution of selenium for sulfur in a functional group has the potential to alter a compound's reactivity and/or target specificity. There is also the possibility that plants such as selenium-enriched broccoli may produce precursors of SFN-isoSe, and that this may partly account for enhanced chemopreventive properties. Preliminary studies with a synthetic isoselenocyanate analog of sulforaphane (SFN-isoSe) indicate that it is more effective than sulforaphane at inducing apoptosis in a variety of melanoma cell lines yet is not toxic to normal fibroblasts, however the mechanisms by which this occurs are unknown (Desai and Robertson, manuscript in preparation).

Therefore, it was decided to test the hypothesis that synthetic SFN-isoSe would, like sulforaphane, induce the Nrf2/ARE pathway, including the downstream targets GCLc and glutathione levels, and perhaps could do so more effectively.

Materials and Methods

Reagents
Figure 4 shows chemical structures of isothiocyanate and isoselenocyanate compounds. Organoselenium compounds were obtained courtesy of Dhimant H. Desai and had been synthesized as described previously (Sohn, Desai et al. 2005). Antibodies (Nrf2, GCLc, GCLm, Actin, LaminA) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Culture

HepG2 human hepatoma cells stably transfected with ARE-luciferase plasmid were obtained courtesy of Muriel Cuendet (Cuendet, Oteham et al. 2006). Cells were maintained in F-12 media (Invitrogen) supplemented with 10% fetal bovine serum, antibiotics, MEM amino acid solution, and insulin (1.45 μg/ml).

Wildtype and Nrf2 deficient (Nrf2 -/-) mouse embryonic fibroblasts (MEF) were obtained courtesy of Dr. Thomas Kensler (Wakabayashi, Dinkova-Kostova et al. 2004). Cells were maintained in Iscov’s MDM (Gibco12440) supplemented with 10% fetal bovine serum, and antibiotics.

Viability Assay

Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reduction assay,
according to the manufacturer’s instructions (CellTiter 96™, Promega).

Absorbency at 490 nm was measured using a Bio-Tek Synergy HT plate reader.

**Luciferase Assay**

Treated HepG2 ARE reporter cells were washed in PBS, lysed, and luciferase activity of resulting extracts was measured according to the Luciferase Assay System protocol (Promega) using a single tube luminometer.

**Glutathione Assay**

Aliquots of cells were washed in PBS and lysed in 5% metaphosphoric acid. Precipitated protein was removed by centrifugation. Free glutathione in cellular extracts was determined using the previously described enzymatic recycling method using Elman’s reagent (Tietze 1969) with modifications (Richie, Skowronski et al. 1996). Absorbency over time was measured using a Bio-Tek Synergy HT plate reader (Bio-Tek Instruments, Winooski, VT). Glutathione levels are normalized to protein levels as determined by BCA assay.

**Cellular Extracts and Western Blotting**

Cellular extracts were obtained using NE-PER® Nuclear and Cytoplasmic Extraction Reagents according to the manufacturer’s protocol (Pierce). Western
blotting for nuclear Nrf2 and LaminA, or cytoplasmic GCLc, GCLm or Actin, was performed according to manufacturer’s instructions using the Criterion™ Cell system (Bio-Rad, Hercules, CA). Protein of cellular extracts was determined by BCA assay according to the manufacturer’s protocol (Pierce Protein Research Products, Thermo Scientific, Rockford, IL). Absorbency was measured using a Bio-Tek Synergy HT plate reader (Bio-Tek Instruments, Winooski, VT). Samples were normalized in Laemli’s buffer. Cytoplasmic or nuclear protein (50 or 20 μg per lane, respectively) were loaded in 4-20% gradient polyacrylamide gels (TRIS-HCL) immediately following boiling for 5 minutes in Laemli’s buffer. Resolved protein was then transferred to nitrocellulose membranes using a CAPS discontinuous buffer system and Trans-Blot® SD transfer cell according to manufacturer’s instructions (Bio-Rad tech note 2134). Membranes were blocked for 1 hour at room temperature in 5% non-fat dry milk/TBST, and probed overnight at 4°C with primary antibody diluted in TBST at concentrations recommended by the manufacturer (Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were incubated in HRP-conjugated secondary antibodies (Pierce Protein Research Products, Thermo Scientific, Rockford, IL), diluted 1:1000 in TBST, for 1 hour at room temperature and developed using SuperSignal West Dura Extended Duration Substrate reagents according to the manufacturer’s protocol (Pierce Protein Research Products, Thermo Scientific, Rockford, IL), before exposure. Membranes were re-probed for Actin (cytoplasmic marker) or LaminA (nuclear marker) to insure equal loading and purity of cytoplasmic or
nuclear extracts. Bands were quantitated with the BioSpectrum AC Imaging System (UVP BiolImaging Systems, Upland, CA), and data was normalized to corresponding actin or LaminA values.

**Statistical Analysis**

Where error bars are shown, data was obtained from at least 3 independent experiments. Data are reported as mean ± standard deviation. Significance was assessed using either the Student’s t-test or ANOVA where appropriate. Differences between data sets were considered statistically significant if p < 0.05.

**Results**

HepG2 ARE-luciferase reporter cells were used to test the hypothesis that an isoselenocyanate analog of SFN, SFN-isoSe, would have enhanced ability to induce the ARE pathway. Both luciferase activity and cell viability were measured. Effects on viability were noticed at short exposure times, and preliminary time-course studies showed that a concentration range of 0-20uM with a 6-hour exposure provided useful data for comparing these compounds. Figure 14 shows viability relative to controls as determined by MTS assay. Below 10 μM there is no effect by either compound. However, a difference was observed at 20 μM where SFN-isoSe reduces relative viability to 50% while SFN
had no measurable effect. This observation was not unique to SFN-isoSe, but rather was common to isoselenocyanates in general. Although SFN has been the most widely studied isothiocyanate, others such as benzyl isothiocyanate (BITC) and phenylethyl isothiocyanate (PEITC) have also been successful as chemopreventive agents in various animal models (Yang, Conaway et al. 2002; Conaway, Wang et al. 2005). Therefore, isoselenocyanate analogs of these compounds were also tested. Table 5 shows that for each of the isothiocyanates, SFN, PEITC, and BITC, viability was 100 % following a 6-hour exposure to 50 μM concentrations. In contrast, the isoselenocyanate analogs, SFN-isoSe, PEISC, and BISC viability were reduced to 0 %, 14 %, and 0 % of controls, respectively.
Figure 14: Effects of SFN or SFN-isoSe on HepG2 ARE-Luciferase Activity and Viability. Effects of varying concentration of SFN or SFN-isoSe upon HepG2 ARE-luciferase cell viability and luciferase activity. Cells at 50% confluence were provided fresh media containing vehicle control or 5, 10 or 20 μM of SFN or SFN-isoSe. After 6 hours, cells were assayed for viability by MTS absorbance at 490 nm, then washed with PBS, lysed and luciferase activity was determined using Promega’s Luciferase Assay System and single tube luminometer. Data are normalized to control values and represent the mean of 3 independent experiments ± standard deviation.
Relative luciferase induction is plotted in Figure 14b following exposure of HepG2-luciferase reporter cells to varying concentrations of SFN or SFN-isoSe. As with viability, the effects on ARE activation by the two compounds are identical up to 10 $\mu$M, but deviate substantially at higher concentration. At 20 $\mu$M, SFN-isoSe results in a 5-fold luciferase induction, more than twice that observed for SFN. The ARE induction by SFN-isoSe increases in a dose-dependent manner even while cell viability is greatly reduced. Compared with other isoselenocyanates tested, this phenomenon appears unique to SFN-isoSe. For example, with increasing concentrations of BISC and PEISC, as viability decreases within the 10-20 $\mu$M range, ARE induction precipitously dropped, in fact, to levels far below those that could be explained by loss of viability (data not shown). As shown in Table 5, peak luciferase activity and concentration at maximal induction are not remarkably different between the isothiocyanates PEITC and BITC and their isoselenocyanate analogs PEISC and BISC.
Table 5: Effects of ISCs and ITCs on HepG2 ARE-Luciferase Activity and Viability. HepG2 cells plated at 50% confluence were exposed to fresh media containing various doses of isothiocyanate or isoselenocyanate compounds for 6 hours. Luciferase activity and viability were measured and reported as maximal fold induction over controls and concentration of compound required for maximal luciferase induction. Concentrations resulting in 50% reduction in viability as well as viability at 50 μM concentration are also reported.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Maximal Induction</th>
<th>LD&lt;sub&gt;50&lt;/sub&gt;</th>
<th>Viability at 50 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fold Luciferase</td>
<td>Concentration (μM)</td>
<td>(μM)</td>
</tr>
<tr>
<td>SFN</td>
<td>3.7 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10</td>
<td>&gt; 50</td>
</tr>
<tr>
<td>SFN-IsoSe</td>
<td>5.2 ± 0.6&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>&gt; 20</td>
<td>20</td>
</tr>
<tr>
<td>PEITC</td>
<td>7.0 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10</td>
<td>&gt; 50</td>
</tr>
<tr>
<td>PEISC</td>
<td>5.6 ± 0.5&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>BITC</td>
<td>5.6 ± 0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5</td>
<td>&gt; 50</td>
</tr>
<tr>
<td>BISC</td>
<td>7.0 ± 0.7&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>5</td>
<td>30</td>
</tr>
</tbody>
</table>

<sup>a</sup> p < 0.05 vs. control
<sup>b</sup> p < 0.05 vs. analog
Wildtype and Nrf2-deficient MEF cells were used to test the hypothesis that SFN-isoSe would have enhanced ability to induce the Nrf2 transcription factor in non-cancer cells when compared with SFN. Western blots of nuclear extracts from cells treated with 10 or 20 μM SFN or SFN-isoSe for 24 hours were probed and quantified for Nrf2, then re-probed and normalized to Lamin A levels. Figure 15 shows that nuclear Nrf2 levels were significantly induced over controls 3.5-fold for SFN and 4.5-fold for SFN-isoSe, regardless of concentration, in wildtype cells.
Figure 15: **Nuclear Nrf2 Expression in Mouse Embryonic Fibroblasts upon SFN or SFN-isoSe Treatment.** Effects of SFN or SFN-isoSe upon wildtype MEF cell nuclear Nrf2 protein levels. Cells at 50% confluence were provided fresh media containing vehicle control or 10 μM or 20 μM of SFN or SFN-isoSe. After 24 hours, cells were washed with PBS, trypsinized and collected. Nuclear extracts were obtained using Pierce’s NE-PER kit. Nuclear extracts were subjected to western blotting for Nrf2, quantitated and normalized to Lamin A protein levels. Data are normalized to control values and represent the mean of 3 independent experiments ± standard deviation.

* p < 0.05
Due to the potential importance of glutathione in chemoprevention, and because \textit{de novo} glutathione synthesis is partly ARE-regulated, cytoplasmic extracts of 10 or 20 \( \mu \text{M} \) SFN or SFN-isoSe treated wildtype or Nrf2 -/- cells were probed by western analysis for GCLc, the rate-limiting enzyme in glutathione synthesis. Figure 16 shows that relative GCLc protein expression was significantly enhanced 3.2-fold by 20 \( \mu \text{M} \) SFN and 4.1-fold by 20 \( \mu \text{M} \) SFN-isoSe over controls in wildtype cells, and that no such increase was seen in Nrf2 -/- cells. Furthermore, the induction by 20 \( \mu \text{M} \) SFN-isoSe is significantly 30\% greater than that by 20 \( \mu \text{M} \) SFN.
Figure 16: **GCL Expression in Mouse Embryonic Fibroblasts upon SFN or SFN-isoSe Treatment.** Effects of SFN or SFN-isoSe upon wildtype or Nrf2 -/- MEF cell cytoplasmic GCLc protein levels. Cells at 50% confluence were provided fresh media containing vehicle control or 10 μM or 20 μM of SFN or SFN-isoSe. After 24 hours, cells were washed with PBS, trypsinized and collected. Cytoplasmic extracts were obtained using Pierce’s NE-PER kit. Extracts were subjected to western blotting for GCLc, quantitated and normalized to Actin protein levels. Data are normalized to control values and represent the mean of 3 independent experiments ± standard deviation.
It was hypothesized that SFN and SFN-isoSe would induce glutathione in MEF cells and that this process would be Nrf2-dependent. Because isothiocyanates such as SFN can deplete glutathione in the short term, glutathione was measured in wildtype or Nrf2 -/- MEF cells as a function of exposure time at concentrations of 10 and 20 μM SFN or SFN-isoSe. Relative glutathione levels of cells are plotted in Figure 17 for 10 and 20 μM concentrations. After 6 hours exposure, both SFN and SFN-isoSe deplete glutathione to 40% of vehicle-treated controls at 10 μM, and to 20% of controls at 20 μM, regardless of the presence of functional Nrf2. Cells deficient in Nrf2 and treated with SFN do not recover and glutathione levels continue to fall over a 24-hour period. On the other hand, SFN-isoSe treated Nrf2 -/- cells do show partial repletion of glutathione with 10 μM treated cells recovering to about 90% of control values and 20 μM treated cells reaching 70% after 24 hours. Wildtype MEF cells exposed to 10 μM SFN-isoSe exhibit a sharp increase in glutathione after 6 hours with supra-basal levels being reached by 9 hours and ultimately reaching 160% of controls by 24 hours. Ten μM SFN treated wildtype cells reveal a similar but attenuated pattern and basal glutathione levels are eventually reached by 24 hours. Increasing the concentrations to 20 μM reveals opposing behaviors between SFN and SFN-isoSe treatment of wildtype cells, particularly between the 9 and 24-hour time points, where SFN-isoSe causes a net increase in glutathione versus a net decrease for SFN.
Figure 17: Relative Glutathione Levels in Mouse Embryonic Fibroblasts upon Time-Course SFN or SFN-isoSe Treatment. Time course of effects of SFN or SFN-isoSe upon wildtype or Nrf2 -/- MEF cell relative GSH levels. Cells at 50% confluence were provided fresh media containing vehicle control or 10 μM or 20 μM of SFN or SFN-isoSe. After 0, 6, 9, or 24 hours, cells were washed with PBS and lysed in 5 % MPA. Lysates were clarified by centrifugation and GSH was assayed.
Absolute glutathione levels normalized to protein from wildtype or Nrf2 -/- MEF cells treated with SFN or SFN-isoSe for 24 hours are displayed in Figure 18. Basal glutathione levels in un-treated Nrf2 -/- cells are significantly 50% lower than values of un-treated wildtype cells. Glutathione levels are nearly doubled in wildtype cells by SFN-isoSe treatment regardless of concentration, but are unchanged by 10 μM SFN and significantly lowered by 20 μM SFN. SFN-isoSe does not induce glutathione in Nrf2 -/- cells.
Figure 18: Glutathione in Mouse Embryonic Fibroblasts upon SFN or SFN-isoSe Treatment. Effects of SFN or SFN-isoSe upon wildtype or Nrf2 -/- MEF cell GSH levels. Cells at 50% confluence were provided fresh media containing vehicle control or 10 μM or 20 μM of SFN or SFN-isoSe. After 24 hours, cells were washed with PBS and portions were lysed in HEPES buffer and protein was assayed. Remaining cells were lysed in 5 % MPA. Lysates were clarified by centrifugation and GSH was assayed. Data represent the mean of 3 independent experiments ± standard deviation.
Discussion

The discovery of the involvement of Nrf2 in GSH induction by organoselenium compounds such as p-XSC and p-XSeSG (Chapters 2 and 3), and the fact that selenocyanates are much more effective than their thiocyanate counterparts in chemoprevention studies (El-Bayoumy, Sinha et al. 2006), led to the hypothesis that perhaps even very potent Phase II inducers, such as sulforaphane, could be improved upon through substitution of selenium for sulfur in isothiocyanate functional groups. Other phytochemical-derived isothiocyanates, such as PEITC and BITC, are also potent chemopreventive agents and inducers of Phase II genes (Jiao, Ho et al. 1994; Conaway, Wang et al. 2005), so these compounds and their isoselenocyanate analogs were included in the present study.

Here we show that all isothiocyanates, and their corresponding isoselenocyanate analogs, induced luciferase activity in HepG2 ARE-reporter cells significantly over controls. Furthermore, the isoselenocyanates SFN-isoSe and BISC induced maximal luciferase induction 40% and 25%, respectively, greater than their isothiocyanate analogs (p < 0.05). Thus, the data presented herein confirmed our primary hypothesis that substitution of selenium for sulfur in the functional isothiocyanate group of SFN enhances its ability to induce the ARE pathway in vitro, and that this is also the case for BITC. These data suggest that isothiocyanates and isoselenocyanates may act through similar pathways and that Nrf2 is involved in the process, but also that enhancements of
chemopreventive mechanisms may be operative in the case of the isoselenocyanates.

Induction of the ARE by SFN-isoSe at concentrations of 20 μM is particularly interesting when compared to SFN because viability of HepG2 cancer cells is reduced by SFN-isoSe, but not by SFN, even while luciferase is more strongly induced. In fact, if ARE reporter activity were normalized to the proportion of viable cells, the SFN-isoSe-mediated luciferase induction would be 4-fold greater than that induced by SFN at the same concentration. These data suggest that SFN-isoSe may not only be a more efficient inducer of Phase II chemopreventive genes, but also may be a better post-initiation chemopreventive and/or chemotherapeutic agent by inducing apoptosis in cancer cells. It was determined in this study that not only SFN-isoSe, but isoselenocyanates in general, were more effective in killing HepG2 cancer cells than their isothiocyanate analogs and that this effect is most apparent in concentrations between 10-20 μM. This concentration range is also where SFN-isoSe had the best performance in inducing apoptosis in various melanoma cell lines (Desai, Robertson, Amin et al., manuscript in preparation). It is not understood how phytochemicals like SFN induce apoptosis in abnormal cancer cells, but it has been suggested that the transcription factors NF-κB and AP-1 are involved (Gopalakrishnan, Xu et al. 2006). Future studies are necessary to examine the involvement of these transcription factors in isoselenocyanate-mediated apoptosis of cancer cells.
Because Induction of the ARE pathway by isoselenocyanates had not been previously reported, it was unknown if downstream ARE-regulated genes would also be induced. Therefore, we examined the ARE-regulated GSH synthetic pathway in wildtype and Nrf2 -/- MEF cells. The hypothesis that SFN-isoSe would induce glutathione through the Nrf2 pathway was confirmed. The experiments with MEF cells revealed that a nearly two-fold glutathione induction over controls by SFN-isoSe is dependent upon Nrf2. That induced glutathione occurs coincidentally with elevated Nrf2 and GCLc in wildtype, but not Nrf2 -/-, MEF cells provides strong evidence that SFN-isoSe-mediated elevation of nuclear Nrf2 causes ARE-mediated transcriptional induction of GCLc, and ultimately leads to higher glutathione levels.

It is interesting that SFN-isoSe induces glutathione in MEF cells while SFN does not. This occurs despite induction of Nrf2 and GCLc by both compounds. Glutathione levels are influenced by depletion and synthesis, and GSH was depletion by both compounds in the short-term, followed by varying degrees of repletion or continued depletion at later times. Repletion in wildtype MEF cells treated with 10 μM SFN or SFN-isoSe can be explained by Nrf2 induction. SFN treated Nrf2 -/- cells, regardless of concentration, do not exhibit repletion, rather glutathione was further depleted with increasing exposure times. Sulforaphane has been demonstrated to accumulate in cells and deplete GSH in the short term (Singh, Srivastava et al. 2005). This is in contrast with SFN-isoSe treated Nrf2 -/- cells where repletion almost to basal levels occurs by 24 hours of exposure. This
may indicate an alternate pathway of glutathione induction, which is independent of Nrf2.

It is not known if isoselenocyanate precursors occur naturally in, as is the case with isothiocyanates like SFN and PEITC. However, selenium fertilization of broccoli has been shown to possess enhanced chemopreventive properties in some models (Davis, Zeng et al. 2002). But selenium fertilization of broccoli has also been shown to decrease production of glucosinolate, the precursor of SFN (Robbins, Keck et al. 2005). Taken together, these findings suggest that chemopreventive vegetables like broccoli may possess the means to synthesize isoselenocyanate precursors, and this possibility should be explored.

It is not obvious how substitution of selenium for sulfur in an isothiocyanate functional group would change its reactivity and or target specificity, as the electronegativity of these elements are very similar. However, there exist clear examples in nature where selenium in place of sulfur greatly changes a protein’s reactivity. For example, the ionized selenol of selenocysteine at physiological pH in active sites of selenoproteins accounts for their higher redox sensitivity (Copeland 2005). Furthermore, oxidized selenomethionine in proteins can be repaired non-enzymatically while oxidized methionine requires methionine sulfoxide reductases. The isothiocyanate group of sulforaphane has been demonstrated in vitro to directly modify Keap1 through the formation of thionoacyl adducts and it is possible that isoselenocyanates may do this better (Hong, Freeman et al. 2005).
Any compound under consideration as a chemopreventive agent must show minimal toxicity to normal cells. An overview of the data presented herein suggest that SFN-isoSe is more toxic to cancer cells than SFN, but less toxic to normal MEF cells, even in the absence of functional Nrf2. It was shown here that SFN-isoSe is less glutathione depleting, and therefore less toxic, than SFN to both wildtype and Nrf2 -/- MEF cells. The zone between chemoprevention and chemotherapy becomes blurred when agents can kill cancer cells, even while inducing protective genes in normal cells. The need for such agents has clearly been suggested (Stoner, Morse et al. 1997), therefore the synthetic isoselenocyanates, and particularly SFN-isoSe, are ideal candidates for future in vivo studies.

References


Gamet-Payrastre, L., P. Li, et al. (2000). "Sulforaphane, a naturally occurring
isothiocyanate, induces cell cycle arrest and apoptosis in HT29 human

1) and MAPK pathway by flavonoids in human prostate cancer PC3 cells."
Arch Pharm Res 29(8): 633-44.

Hong, F., M. L. Freeman, et al. (2005). "Identification of sensor cysteines in
human Keap1 modified by the cancer chemopreventive agent

Huber, W. W. and W. Parzefall (2007). "Thiols and the chemoprevention of

Jiao, D., C. T. Ho, et al. (1994). "Identification and quantification of the N-
acetylcycteine conjugate of allyl isothiocyanate in human urine after

polymorphisms in glutathione S-transferases M1 and T1, and prostate

genotype, broccoli, and lower prevalence of colorectal adenomas." Cancer
Epidemiol Biomarkers Prev 7(8): 647-52.

McWalter, G. K., L. G. Higgins, et al. (2004). "Transcription factor Nrf2 is
essential for induction of NAD(P)H:quinone oxidoreductase 1, glutathione
S-transferases, and glutamate cysteine ligase by broccoli seeds and isothiocyanates." J Nutr 134(12 Suppl): 3499S-3506S.


Sohn, O. S., D. H. Desai, et al. (2005). "Comparative excretion and tissue distribution of selenium in mice and rats following treatment with the chemopreventive agent 1,4-phenylenebis(methylene)selenocyanate."


Levels of dietary selenium are inversely associated with cancer risk in humans, and selenium has played a major role in the field of chemoprevention. In particular, the selenocyanate $p$-XSC and its glutathione conjugate and metabolite, $p$-XSeSG, are effective in preventing cancers at numerous sites including the lung in rodent models. Based on the previous finding that feeding of $p$-XSC resulted in elevated GSH in the lungs of A/J mice (Richie, Kleinman et al. 2006), and due to the possible relevance of induced GSH to chemopreventive potential (Huber and Parzefall 2007), it was decided to explore molecular mechanisms of how this may occur. A set of *in vivo* and *in vitro* experiments were designed to test the hypothesis that the transcription factor Nrf2 was involved.

In a Fisher rat study, it was discovered that feeding $p$-XSC, at a dose of 10 ppm as selenium in the diet for two weeks, resulted in significant inductions of 6.1-fold nuclear Nrf2, 2.2-fold nuclear p-ERK, and 1.6-fold cytoplasmic GCLc protein expression in the lung. These occurred concurrently with a significant 2.3-
fold induction in lung GSH. These data represent the following novel contributions:

1. That \( p \)-XSC induces GSH in the lung of the Fisher Rat, a species with no known defects in the GSH metabolic pathway or susceptibility to lung pathologies.
2. That \( p \)-XSC induces nuclear Nrf2 and cytoplasmic GCLc protein levels \textit{in vivo}, suggesting a concerted mechanistic pathway of GSH induction.
3. That nuclear p-ERK protein levels are induced by \( p \)-XSC concurrently with nuclear Nrf2 protein \textit{in vivo}, suggesting involvement of the ERK pathway in Nrf2 stabilization.

Taken together, these data support our hypothesis of Nrf2 involvement in \( p \)-XSC-mediated GSH induction and suggest that \( p \)-XSC feeding induces the ERK pathway in the rat lung leading to increased levels of phosphorylated ERK (p-ERK). In turn, p-ERK is available to phosphorylate Nrf2 leading to its increased stability and accumulation in the nucleus. Elevated nuclear Nrf2 is then available to bind ARE sites in the promoter of the GCLc gene, leading to its increased transcription and protein expression, and ultimately to a more than doubling of basal GSH levels. Concerted induction of elements of an Nrf2-mediated GSH synthetic process does not prove causality, so \textit{in vitro} studies were carried out to better demonstrate this possibility.
To further test the hypothesis of the involvement of nuclear Nrf2 in GSH induction by \( p \)-XSC, a HepG2 ARE-luciferase reporter cell line was employed. It was found that \( p \)-XSC significantly activated the HepG2 ARE-luciferase reporter 3-fold under optimum concentration and exposure time conditions, and that siRNA specifically targeting Nrf2 lessened this activation. However, \( p \)-XSC was also found to be cytotoxic in cell culture, thus limiting more detailed analyses. The glutathione conjugate of \( p \)-XSC, \( p \)-XSeSG, has been developed as a chemopreventive agent and is thought to be a major metabolite of \( p \)-XSC. Furthermore, it has been shown to be much less toxic than \( p \)-XSC \textit{in vivo}, and is similarly effective to \( p \)-XSC in chemoprevention in certain carcinogen-induced animal models of cancer (Rao, Wang et al. 2001; El-Bayoumy and Sinha 2004). Therefore, \( p \)-XSeSG was also employed in the Fisher rat study, and due to its low cytotoxicity, provided more \textit{in vitro} data than was obtainable with \( p \)-XSC.

In the Fisher rat study, it was discovered that feeding \( p \)-XSeSG, also at a dose of 10 ppm as selenium in the diet for two weeks, resulted in significant inductions of 1.9-fold cytoplasmic GCLc protein expression in the lung. This occurred concurrently with a significant 1.9-fold induction in lung GSH. However, no significant induction of nuclear Nrf2 or p-ERK was observed in the lung. These data represent the following novel contributions:

1. That \( p \)-XSeSG induces GSH in the rat lung, as it has not been previously examined whether \( p \)-XSeSG induced GSH in any tissue of any species.
2. That cytoplasmic GCLc are induced by \( p\)-XSeSG in the rat lung, suggesting that \( p\)-XSeSG may act, at least in part, through transcriptional regulation of GCLc in a manner similar to that observed by \( p\)-XSC.

These data suggest that \( p\)-XSC and \( p\)-XSeSG act via similar pathways with the exception of induced nuclear Nrf2 and p-ERK in the case of \( p\)-XSeSG. It is unknown why Nrf2 and p-ERK were not observed to be elevated at the time of sacrifice, but it is expected that \( p\)-XSeSG would be metabolized differently than \( p\)-XSC. As \( p\)-XSeSG is thought to be the major metabolite of \( p\)-XSC, and because glutathiolated compounds are usually rapidly excreted, it is expected that less of the agent would be incorporated into tissue. One possibility is that Nrf2 is actually induced by \( p\)-XSeSG feeding, but the pulse was missed in these experiments. Rats feed nocturnally, and it is possible that a blunted or short-lived induction of nuclear Nrf2 in the lung by \( p\)-XSeSG may have dissipated by the time of sacrifice. It is fully expected that a short pulse of Nrf2 induction, following feeding of an inducer, would result in a more sustained induction of GCLc and GSH. Further insight into the differential behavior of \( p\)-XSC and \( p\)-XSeSG \textit{in vivo} was gained through \textit{in vitro} observations.

As with \( p\)-XSC, \( p\)-XSeSG also activated the ARE-luciferase reporter in HepG2 cells, but in a very different manner. A peak induction of 3-fold occurs at a \( p\)-XSC concentration of 10 \( \mu \)M, while the same concentration of \( p\)-XSeSG resulted in no induction at all. However, increasing the concentration of \( p\)-XSeSG caused a dose-dependent luciferase induction, which ultimately surpassed the
maximum induction attainable by \( p \)-XSC by nearly 2-fold. Translating these observations into \textit{in vivo} behavior may imply that higher doses of \( p \)-XSeSG may be required to see the same degree of Nrf2 activation that is caused by \( p \)-XSC and/or that the metabolic kinetics may differ. In concert with \( p \)-XSeSG-mediated ARE-luciferase induction, it was found that nuclear Nrf2 and GSH were significantly elevated in HepG2 cells. Short interfering RNA specifically targeting Nrf2 in HepG2 reporter cells resulted in a 50% knockdown of nuclear Nrf2 protein levels, and this brought about similar reductions in \( p \)-XSeSG-mediated luciferase activation and GSH levels. Furthermore, complete dependence of \( p \)-XSeSG-mediated \( \gamma \)-GCL induction upon Nrf2 was established by experiments with wildtype and Nrf2-deficient MEF cells. Treatment of wildtype MEF cells with \( p \)-XSeSG resulted in significantly elevated levels in nuclear Nrf2 and protein expression of both subunits of \( \gamma \)-GCL. Neither subunit of GCL is induced at all by \( p \)-XSeSG treatment of Nrf2 -/- cells despite elevated, albeit dysfunctional, nuclear Nrf2 protein levels. These data represent the following novel contributions:

1. That \( p \)-XSC activates an ARE reporter in HepG2 cells in an Nrf2-dependent manner.
2. That \( p \)-XSeSG is much less cytotoxic than \( p \)-XSC \textit{in vitro}.
3. That \( p \)-XSeSG induces an ARE reporter, nuclear Nrf2 and GSH in HepG2 cells in an Nrf2-dependent manner.
4. That \( p \)-XSeSG induces nuclear Nrf2 in MEF cells, and coincident induction of GCL subunits is dependent upon Nrf2.
A summary schematic of the proposed *in vivo* and *in vitro* effects of *p*-XSC and *p*-XSeSG is shown in Figure 19. Taken together, the *in vitro* data support the overall hypothesis and suggest that *p*-XSC acts through the Nrf2 pathway *in vivo*, and that *p*-XSeSG may be the metabolite responsible for such activation, thus offering *p*-XSeSG as a less toxic, yet highly efficacious inducer of GSH.

Figure 19: A summary schematic of the proposed *in vivo* and *in vitro* effects of *p*-XSC and *p*-XSeSG.
Isoselenocyanates and Isothiocyanates

The discovery of the involvement of Nrf2 in GSH induction by organoselenium compounds such as $p$-XSC and $p$-XSeSG, and the fact that selenocyanates are much more effective than their thiocyanate counterparts in chemoprevention studies, led to the hypothesis that perhaps even very potent Phase II inducers, such as sulforaphane, could be improved by substitution of selenium for sulfur in isothiocyanate functional groups.

It was discovered that SFN-isoSe, the isoselenocyanate analog of SFN, was 2-4 times more potent at inducing the HepG2 ARE-luciferase reporter, even while being substantially more effective in killing these cancer cells, than was SFN at the same concentration. In fact, all isoselenocyanates tested were more efficient in killing HepG2 cancer cells than their isothiocyanate analogs. It was also found that SFN-isoSe induces nuclear Nrf2 and GCLc protein levels and glutathione in an Nrf2-dependent manner in non-cancer MEF cells. A nearly two-fold glutathione induction over controls was caused by SFN-isoSe, but not by SFN. This effect was seen in wildtype, but not Nrf2 knockout cells. Time course studies in MEF cells revealed that although both SFN and SFN-isoSe depleted GSH in the short-term, both wildtype and Nrf2 -/- MEF cells demonstrated GSH repletion in SFN-isoSe treated cells, but not in SFN treated cells. An exciting
consideration is that, in addition to potent Nrf2 induction, an Nrf2-independent pathway of glutathione induction may also be activated by SFN-isoSe. Glutathione repletion, even in the absence of functional Nrf2, suggests that SFN-isoSe may be a less toxic chemopreventive agent to normal cells than is SFN. These data represent the following novel contributions:

1. That SFN-isoSe activates an ARE-luciferase reporter in HepG2 cells more potently than SFN.
2. That SFN-isoSe, and isoselenocyanates in general, are more effective at killing HepG2 cancer cells than their isothiocyanate counterparts.
3. That SFN-isoSe induces nuclear Nrf2 in MEF cells, and coincident induction of GCLc and GSH is dependent upon Nrf2.
4. That SFN-isoSe induces GSH in MEF cells more effectively than SFN and is less toxic to Nrf2 -/- MEF cells.

Taken together, these data highlight selenium’s importance as a component of GSH induction pathways, and chemoprevention in general.

**Overall Conclusions**

In summary, we have shown induction of the Nrf2 transcription factor in p-XSC-mediated induction of glutathione in the rat lung, and also presented
evidence for involvement of the ERK pathway in this process. This may represent a general mechanism of chemoprevention by \( p \)-XSC and related organoselenium compounds, and future studies are planned to examine effects in other relevant tissues such as prostate, tongue and colon. It was also shown that the organoselenium compound \( p \)-XSeSG is a promising GCL and GSH inducing agent \textit{in vivo}, and which exhibits low toxicity and acts through the Nrf2 pathway in the cell models discussed herein. In addition to its potential in chemoprevention, \( p \)-XSeSG may prove useful as a glutathione-enhancing agent for treatment of conditions such as HIV, aging and chronic inflammatory conditions. Compounds under consideration as chemopreventive agents ideally would show minimal toxicity to normal cells, potent ability to induce Phase II enzymes, and efficiency in killing cancer cells. The evidence presented herein portrays the synthetic isoselenocyanates, and particularly SFN-isoSe, as ideal candidates for future studies as chemopreventive and/or chemotherapeutic agents.

\textbf{Future experiments}

Future \textit{in vivo} studies are required to determine the level of dependence upon nuclear Nrf2 of \( p \)-XSC or \( p \)-XSeSG mediated GSH induction in the lung and perhaps other tissues. Studies similar to the Fisher rat study described herein, but with wildtype and Nrf2 knockout
mice, would be most useful, and additional groups fed ERK pathway inhibitors together with organoselenium compounds may help establish the dependence of Nrf2 induction upon the ERK pathway. As mentioned previously, additional experiments should also be performed to examine metabolic kinetics and to establish appropriate doses of \( p \)-XSeSG. Perhaps a bolus dose of \( p \)-XSeSG, administered by gavage to fasted animals and followed by a time course analysis of \( p \)-ERK, Nrf2, GCLc and GSH, would better address temporal concerns and cause/effect hypotheses.

*In vivo* studies with isoselenocyanates, and particularly SFN-isoSe, should be performed to better compare efficiency of Phase II induction with their isothiocyanate analogs.

References


VITA

Sans Emmert

Experience

Laboratory Technician
1/2002-1/2003  Penn State College of Medicine, Hershey, PA
- Conduct animal studies relating to effects of amino acid stimulus of protein synthesis
- Perform western blots and radio labeled protein synthesis assays
- Perform enzyme activity assays
Co-authored manuscript entitled Oral Leucine Administration Stimulates Protein Synthesis in Rat Skeletal Muscle, J. Nutr. 135: 376-382, 2005

Independent Consultant/Inventor
9/1992-1/2002  Clients included TRW, Detroit Diesel, and Volkswagen, Conestoga, PA
- Licensed patent rights and provided consulting, research and prototype services as required for the development on a solid state in-line engine oil quality sensor
- Sole author of U.S. Patent 4,721,874 Apparatus and Method for Determining the Viscosity of a Fluid Sample
- Co-authored manuscript entitled An On-Line Oil Viscosity Sensor, Society of Automotive Engineers Technical Paper (Series 970848) and Sensors and Actuators 1997 (SP-1220)
Presented research and development efforts at the 1997 International Society of Automotive Engineers Convention at the Cobo Center in Detroit

Education

Millersville University of Pennsylvania, Millersville, PA
1/1999-12/2001
- Non-degree seeking, post-baccalaureate coursework in biology and chemistry
- GPA: 3.98/4.00
- Awarded the Groff academic scholarship

Millersville University of Pennsylvania, Millersville, PA
- Awarded B.S. in physics
- Graduated cum laude
GPA: 3.38/4.00