INVESTIGATION OF THE MECHANISM OF ACTION OF ISOTHIOCYANATE DERIVED ISOSELENOCYANATES AND THE CHEMOPREVENTIVE POTENTIAL OF PHENYLBUTYL ISOSELENOCYANATE

A Dissertation in Pharmacology by Melissa A. Crampsie

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Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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Abstract

Goals of Dissertation Research
Lung cancer is currently the leading cause of cancer deaths among men and women and has a survival rate of only 16%. Because nearly 90% of lung cancer cases are attributed to smoking tobacco and tobacco related lung cancer has a latency period of 20-30 years from time of exposure to invasive disease, intervention before invasive disease with a chemical agent, termed chemoprevention, is a well suited approach for this particular disease. Research suggests that many natural compounds, often found in the human diet, have chemopreventive properties and are actively being explored and/or can make excellent lead compounds. Our lab, using rational drug design techniques, developed a novel isoselenocyanate compound known as phenylbutyl isoselenocyanate (ISC-4) based on naturally occurring compounds found in cruciferous vegetables. The central hypothesis of this research was that ISC-4, based on its design, would function as a potent and effective anti-cancer agent and could be used as a chemopreventive agent.

Specific Aims

Specific Aim 1: To determine the effects of replacing the sulfur atom of a panel of phenyl alkyl isothiocyanates with selenium to form a new panel of phenyl alkyl isoselenocyanates (Chapter 2). A structure activity study was performed on the panel of parent isothiocyanate compounds and the resulting panel of isoselenocyanate compounds to determine if mechanistic characteristics were retained and to ascertain a reason for increased activity.

Specific Aim 2: Testing the hypothesis that ISC-4 has potential as a chemopreventive agent (Chapter 3). This drug was found to potently target and inhibit carcinogen bioactivation by cytochrome P450 enzymes, including the tobacco specific nitrosamine procarcinogen, N-nitrosyl ketone (NNK). When bioactivated, NNK can lead to mutations in DNA and ultimately result in tumorigenesis. A series of experiments were conducted to assess chemopreventive properties including bioavailability, anti-initiation, and DNA adduct studies, all of which were needed as proof of concept before performing an animal bioassay.
Specific Aim 3: Testing ISC-4 as a chemopreventive agent in a lung cancer chemoprevention bioassay (Chapter 4). Using an A/J mouse model, ISC-4 at varying doses and along with its sulfur analog PBITC were tested for the ability to inhibit NNK induced lung tumorigenesis over a 24 week period of time.

Original Breakthroughs and Findings

Isoselenocyanate (ISC) compounds were actually found to have increased activity with cellular thiols compared to their corresponding sulfur analogs leading to the hypothesis that specific protein modifications and redox activity in the cell are responsible for the increased activities seen with the ISC compounds. As Phase I cytochrome P450s were identified early as a significant protein target of ISC-4, the cancer chemopreventive properties of this compound were explored. ISC-4 was found to be orally bioavailable, able to transcriptionally induce Phase II detoxification enzymes, and inhibit DNA adduct formation in lung which showed that ISC-4 had anti-initiation properties that warranted further investigation as a lung cancer chemopreventive agent. In a large scale lung cancer chemoprevention bioassay using A/J mice, ISC-4 was able to inhibit NNK induced lung tumorigenesis by 86% when fed in the diet at 0.38 µmol per gram. ISC-4 treated mice also had a lower percentage of adenomas compared to hyperplasia in the lung, which suggests ISC-4 may have anti promotion or progression properties as well. However, when compared to its sulfur analog PBITC, ISC-4 is not as well tolerated and therefore may be more suited in anti-progression and chemotherapy, at least for lung cancer.
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Chapter 1A

Lung Cancer Risk, Etiology, and Chemoprevention

Melissa A. Crampsie
According to the American Cancer Society, lung cancer is the leading cause of cancer death among men and women worldwide with only a 16% five year survival rate (2010). This year 222,500 new cases will be diagnosed with 157,000 deaths. Approximately 90% of lung cancer cases are attributed to smoking tobacco, while other causes include asbestos, pollution, and radon (ACS 2010). Thus, there is a great need to optimize prevention, detection, and treatment of lung cancer due to smoking. Moreover, former smokers are at a higher risk for lung cancer than individuals that never smoked (Van't Westeinde and van Klaveren). In a comprehensive review by Hecht et al., the role of cigarette smoking in lung carcinogenesis is discussed (Hecht, Kassie et al. 2009). Smoking tobacco results in 60+ carcinogens entering the lung which can lead to DNA adducts. If these DNA adducts are not repaired in time, they can result in mutations which can accumulate. The addition of co-carcinogens (promotion) from continuous smoking can lead to genetic instability and via progression, ultimately neoplasia. In humans, smoking is strongly related to two types of histologically different lung cancers, small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC most commonly manifests as adenocarcinoma, large cell carcinoma, and squamous cell carcinoma and accounts for >80% of all lung cancers.

In the United States, about 25% of the population are tobacco smokers, a figure that has remained fairly constant for the past 10-20 years (WHO 2011). Smokers are exposed to multiple classes of carcinogens that can result in DNA damage, miscoding, and carcinogenesis. The classes of carcinogens found in tobacco smoke are listed in Table 1 (Hecht 1999). The most well studied tobacco carcinogens known to induce pulmonary adenomas and adenocarcinomas are the poly aromatic hydrocarbons (PAHs) and the tobacco specific nitrosamines (TSNAs). The TSNA 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is an extremely lung specific carcinogen.
in that it is the only tobacco carcinogen known to induce lung tumors in all three commonly used rodent models (mouse, rat, and hamster), and that lung tumors are induced irrelevant to the route of administration.

Table 1 – Carcinogens found in tobacco smoke

<table>
<thead>
<tr>
<th>Carcinogen Class</th>
<th># Found in Tobacco Smoke</th>
<th>Examples of Pulmonary Carcinogens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly Aromatic Hydrocarbons</td>
<td>10</td>
<td>Benzo[a]pyrene</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Benzo[b]fluoranthene</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Benzo[j]fluoranthene</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Benzo[k]fluoranthene</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dibenzo[a,i]pyrene</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Indeno[1,2,3-cd]pyrene</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dibenzo[a,h]anthracene</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5-Methylchrysene</td>
</tr>
<tr>
<td>Aza-arenes</td>
<td>3</td>
<td>Dibenzo[a,h]acridine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7H-Dibenzo[c,g]carbazole</td>
</tr>
<tr>
<td>N-Nitrosamines</td>
<td>7</td>
<td>N-Nitrosodiethylamine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4-(Methylamino)-1-(3-pyridyl)-1-butanone</td>
</tr>
<tr>
<td>Aromatic Amines</td>
<td>3</td>
<td>Aniline</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4-toluidine</td>
</tr>
<tr>
<td>Heterocyclic Aromatic Amines</td>
<td>8</td>
<td>2-amino-9H-pyrido[2,3-b]indole</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine</td>
</tr>
<tr>
<td>Aldehydes</td>
<td>2</td>
<td>Acetaldehyde</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Formaldehyde</td>
</tr>
<tr>
<td>Other Organic Compounds</td>
<td>15</td>
<td>1,3-Butadiene</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ethyl carbamate</td>
</tr>
<tr>
<td>Inorganic Compounds</td>
<td>7</td>
<td>Nickel</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chromium</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Arsenic</td>
</tr>
</tbody>
</table>
Figure 1 – DNA adducts formed from ultimate carcinogens of NNK metabolism. A).

Pyridyloxobutyl (POB) DNA adducts from Phase I metabolism of NNK and pyridylhydroxybutyl (PHB) DNA adducts from Phase I metabolism of the reduced form of NNK known as NNAL. B). Methyl DNA adducts formed from Phase I metabolism of NNK. dCyd: deoxycytidine, dGuo: deoxyguanosine, Gua: Guanine, Cyt: Cytosine, dThd: deoxythymidine.
NNK is a procarcinogen that is bioactivated upon entering the liver to form products that result in DNA adducts, usually at adenine, guanine, or thymine residues. The adducts can be either big bulky pyridyloxobutyl or pyridylhydroxybutyl adducts or methyl adducts depending on the bioactivation pathway (Figure 1A and 1B, for the specific pathways see Chapter 3). Because of the specificity and potency at which NNK induces adducts and tumorigenesis, it is very commonly used in animal studies of tobacco induced lung cancer. A/J mice, a strain which spontaneously develops lung tumors due to ki-ras mutations, develop lung tumors at a rate of 100% only 16 weeks after given NNK, regardless of how it is administered. Models such as this allow for timely and cost effective ways to study lung tumorigenesis, since cigarette smoke itself is considered to be a weak carcinogen in most mouse models thus requiring quite large sample sizes for proper statistical analyses (Witschi 2005).

Because the latency period between smoking and lung cancer development can be from 20-30 years, chemoprevention may be a promising option for people at high risk for developing tobacco related lung cancer. Chemoprevention is defined as the use of natural or synthetic agents applied at any time during the multi-stage carcinogenesis process before the start of invasive disease in order to inhibit, delay, or reverse carcinogenesis (Hursting, Slaga et al. 1999). The multi-stage carcinogenesis process begins when a normal cell’s DNA becomes damaged due to either an endogenous (e.g. reactive oxygen species) or exogenous (e.g. UV light) agent. This first step is known as initiation. If an initiated cell is stimulated to divide without repair, it can lead to a mutation. Cells continuously exposed to DNA damaging agents and/or proliferation stimuli which do not undergo apoptosis or repair can progress to a preneoplastic state in which additional genetic alterations can ultimately result in a neoplastic cell. This is known as promotion and progression. Typically a cell requires 3 to 12 independent events to progress from
normal to neoplastic. Figure 1 illustrates the multi-step carcinogenesis process and the potential for chemopreventive intervention at various points in the process.

**Figure 2** – The multistage carcinogenesis process. Normal cells are exposed to DNA damaging agents such as carcinogens which can damage DNA causing the cell to become initiated. Co-carcinogens and promotion agents can lead to an increase in cell proliferation which in unrepaired initiated cells results in mutations and genetic instability which is characteristic of a preneoplastic cell. Once the cell accumulates enough alterations that it becomes invasive, it is considered neoplastic.
Chemopreventive agents inhibit, delay, or reverse the process of carcinogenesis. Therefore, agents that have anti-initiation properties and/or anti-promotion/progression properties may be chemopreventive. To evaluate anti-initiation it is necessary to test a compound’s ability, either directly at the protein level or by altering gene expression to inhibit bioactivation of the carcinogen, induce detoxification of carcinogen, to neutralize or scavenge electrophilic species which can damage DNA, and enhance DNA repair. These properties can also be looked at simultaneously by analyzing DNA adduct formation of animals treated with one or multiple carcinogens and looking at ability of a chemopreventive agent to inhibit DNA adduct formation. Bioactivation is a result of the body’s attempt to prepare a xenobiotic or endogenous substance to be conjugated to facilitate excretion of that substance by unmasking or creating a polar or reactive group. These reactions are termed Phase I reactions and occur mainly in the liver, but can occur in other tissues, and are represented mainly by CYP450 enzymes (abbreviated CYP for humans, CyP for rodents). Conjugation or detoxification enzymes are referred to as Phase II enzymes and are mostly transferases that conjugate a polar moiety to a reactive group of a compound which increases the water solubility of the substance and facilitates excretion from the body. Phase II enzymes include UDP-glucuronyl transferase (UGT), glutathione –S-transferase (GST), N-acetyl transferase (NAT), and sulfotransferases. Anti-promotion properties include scavenging of reactive oxygen species (which can be pro-mitogenic), inhibition of cell proliferation, inducing differentiation and apoptosis, decreasing inflammation, and altering gene expression of mitogenic pathways. Anti-promotion strategies are especially relevant in lung cancer related to smoking tobacco since it has been shown that Akt, a pro-mitogenic and pro-survival kinase is activated in non-small cell lung cancer (NSCLC) as an early event (Tsao, McDonnell et al. 2003) and that tobacco components, including nicotine and
NNK, stimulate Akt dependent proliferation and NFkB dependent survival in NSCLC (Tsurutani, Castillo et al. 2005).

Chemopreventive drug development has the goal of identifying safe and effective chemopreventive agents for clinical use (Kelloff, Boone et al. 1996). These agents are intended for chronic use in relatively healthy individuals, therefore any toxicity, even mild, is problematic. Thus, it is important to properly identify subjects who are likely to develop neoplasia, identify key pathways or events involved in carcinogenesis, and properly characterize existing and new chemopreventive agents. In 2007, a study looked at airway epithelial gene expression in smokers to develop a gene set to use as a biomarker for diagnosis of lung cancer. Optimization of a biomarker could allow for identification of smokers at high risk for developing lung cancer who may benefit from chemoprevention (Spira, Beane et al. 2007). Designing a given chemopreventive strategy for a cancer requires an in depth knowledge of the carcinogenesis process underlying the neoplasia. For lung cancer, cells are initiated by exposure to carcinogens breathed in from the environment. Tobacco smoke is a known inducer of airway inflammation, neutrophil recruitment, and immune suppression, which can affect multiple signaling pathways and transcription factors. Thus characterization of existing and new chemopreventive agents will help elucidate important mechanisms of action for chemopreventive effects and toxicity. From this, we can potentially design more effective compounds or combination treatments with less toxicity or adverse effects. Consumption of certain fruits and vegetables has been long associated with chemopreventive properties. Attempts to isolate and modify the compounds responsible for chemopreventive effects of these plants have been made. In 1987, the NCI began a chemoprevention drug development program. A list of chemopreventive agents reaching clinical trials that are sponsored by the NCI are listed in Table 2.
Table 2 – Chemopreventive agents in clinical trials sponsored by the NCI

<table>
<thead>
<tr>
<th>Agent</th>
<th>Mechanism of Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retinoids</td>
<td>Ant-proliferation and progression</td>
</tr>
<tr>
<td>DFMO</td>
<td>Alkylation of ODC</td>
</tr>
<tr>
<td>NSAIDs</td>
<td>Inhibition of prostaglandins (anti-inflammatory)</td>
</tr>
<tr>
<td>Oltipraz</td>
<td>Induction of Phase II enzymes</td>
</tr>
<tr>
<td>DHEA</td>
<td>Inhibition of G6PDH</td>
</tr>
<tr>
<td>S-Allyl 1 Cysteine</td>
<td>Electrophile detoxification</td>
</tr>
<tr>
<td>Curcumin</td>
<td>Anti-inflammatory and antioxidant, anti proliferative, inhibition of CYP450, enhances GST activity</td>
</tr>
<tr>
<td>Genistein</td>
<td>Anti-estrogenic, inhibition of PTKs, inhibition of CYP450, scavenging ROS, induction of cell cycle arrest and apoptosis</td>
</tr>
<tr>
<td>Indole 3-carbinol</td>
<td>Induction of Phase I and II enzymes</td>
</tr>
<tr>
<td>Perillyl alcohol</td>
<td>Inhibition of isoprenylation of small guanine nucleotide binding proteins, induction of Phase I and Phase II enzymes</td>
</tr>
<tr>
<td>PEITC</td>
<td>Inhibition of CYP450, induction of Phase II enzymes, inhibition of cell proliferation, induction of apoptosis</td>
</tr>
<tr>
<td>EGCG</td>
<td>Inhibition of lipid peroxidation and free radical formation, inhibition of cellular proliferation, modulation of Phase I and induction of Phase II enzymes, inhibition of interaction of tumor promoters, hormones and growth factors with receptors</td>
</tr>
<tr>
<td>Ursodiol</td>
<td>Unknown</td>
</tr>
<tr>
<td>Vitamin D3 analogs</td>
<td>Inhibition of proliferation and DNA synthesis, modulation of signal transduction by calcium and PKC, modulation of oncogene expression, inhibition of ODC, inhibition of lipid peroxidation and angiogenesis, induction of differentiation, TGF beta expression, and apoptosis</td>
</tr>
<tr>
<td>p-XSC</td>
<td>Antioxidant, anti-inflammatory, UGT induction, apoptosis induction</td>
</tr>
</tbody>
</table>
Chapter 1B

Selenium and Selenocompounds in Chemoprevention

Melissa A. Crampsie
Selenium as a trace nutritional element was first mentioned as protective against cancer almost 40 years ago due to the observation that populations with low selenium intake and low plasma selenium levels have increased incidence of many cancers (Reid, Duffield-Lillico et al. 2002) including lung cancer. Since then it has been well reported that the form of selenium is very important to effectiveness and toxicity of the compound. This is important because generally anti-carcinogenic and chemopreventive effects require supra-nutritional doses of selenium compounds (higher than nutritional values). The NCI lists about 13 upcoming clinical trials which involve elemental, inorganic, or organic selenium compounds in cancer prevention. Elemental selenium may be important for chemoprevention simply because of the importance of an organism’s “selenium status”, particularly its effects on selenoproteins.

Selenoproteins incorporate selenium in the form of selenocysteine (Sec), which has been dubbed the 21st amino acid. Selenocysteine is incorporated into proteins when the UGA stop codon is recoded. For this to occur the mRNA needs a certain stem loop secondary structure in the 3’ UTR known as selenocysteine insertion sequence (SECIS) which codes for amino acids on the C terminal side of the UGA and also guides Sec elongation factor SelB. SelB recruits a special tRNA specific for Sec (Behne and Kyriakopoulos 2001; Rayman 2005). The -SH group of cysteine becomes a Se-H group in selenocysteine and the pKa for Sec is 5.2 compared to 8.3 for cysteine, thus at physiological pH the selenol of Sec is mostly anionic selenolate whereas a cysteine residue would typically be protonated making selenoproteins much more reactive at physiological pH (Johansson, Gafvelin et al. 2005). The redox potential is also much more negative for selenylsulfide-containing peptides vs. disulfide-containing peptides. Of the 25 known human selenoproteins, only 18 have been characterized, most of which are involved in redox reactions with Sec essential for activity. Table 1 shows a list of characterized
selenoproteins and associated reaction. Those who have characterized the selenoproteome have found that selenoproteins can be put in two groups: 1) those with Sec together with a Cys residue or 2) those that have a Sec on a C terminal extension to other domains (conversion of a stop codon to selenocysteine TGA gave protein selenoprotein function) (Gladyshev, Jeang et al. 1996). Selenium can also be incorporated non-specifically into proteins when selenomethionine is abundant compared to methionine, however no major effects on function have been documented. Increases in nonspecific incorporation of selenium, however, can result in a decrease in the concentrations and effects of selenoproteins (Waschulewski and Sunde 1988). Lastly, there are selenium binding proteins, which are thought to be responsible for transport of selenium throughout the body (Behne and Kyriakopoulos 2001).

Table 1 – Selenoproteins relevant to cancer (Rayman 2005)

<table>
<thead>
<tr>
<th>Selenoprotein</th>
<th>Function/Reaction</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iodothyronine deiodinases D1-3</td>
<td>Conversion of T4 to T3 and degradation of T3 and T4</td>
<td>Kohrle 2000</td>
</tr>
<tr>
<td>Thioredoxin reductases (1-3)</td>
<td>NADPH reduction of thioredoxin and other substrates, reduction of nucleotides in DNA synthesis, regeneration of antioxidant systems, maintenance of intracellular redox state, cell viability and proliferation, regulation of gene expression by redox control of transcription factors, overexpressed in cancer cells and repressed by p53</td>
<td>Allan et al. 1999, Gladyshev et al. 1998</td>
</tr>
<tr>
<td>Selenoprotein P</td>
<td>Found in plasma, antioxidant and transport</td>
<td>Burk et al. 2003,</td>
</tr>
</tbody>
</table>
functions, scavenger of peroxynitrite, downregulated in tumors  
Arteel et al. 1999

<table>
<thead>
<tr>
<th>Selenophosphate synthetase 2</th>
<th>Conversion of selenide to selenophosphate</th>
<th>Johansson et al. 2005</th>
</tr>
</thead>
</table>

There have been many studies linking selenoproteins to cancer and chemoprevention. It has been shown that the selenium status of an organism contributes to selenoprotein expression and activity. Thus, individuals with low selenium levels or intake have reduced selenoprotein expression and activity compared to normally supplemented individuals (Hoffmann 2007; Hoffmann, Hoge et al. 2007). Another study showed that the binding affinity of selenium to a selenium binding protein SBP2 is a major determinant in differential selenoprotein mRNA translation and sensitivity to nonsense mediated decay (Squires, Stoytchev et al. 2007). Another study showed that selenium itself may be regulating selenoprotein activity and mRNA levels in rat livers (Hadley and Sunde 2001). Epidemiological studies have shown that individuals with low baseline selenium exhibit decreased incidence of overall cancer when supplemented with selenium (Reid, Duffield-Lillico et al. 2002). Proposed mechanisms of chemoprevention by selenoenzymes reviewed by Rayman include reduction of DNA damage, oxidative stress, and inflammation due to antioxidant functions. The glutathione peroxidase (GPx) and thioredoxin reductase (TR) enzymes have been found to be increased in many cancer cells (Gladyshev, Factor et al. 1998; Brown and Arthur 2001) so the exact role that these proteins are playing in cancer development is unclear. Upregulation of antioxidant and reducing enzymes may be a
defense to combat increased oxidative and nitrosative stress. Thus selenoprotein roles in carcinogenesis may be more complex than it appears at first glance.

Selenium as an important element in the molecular formula of chemoprevention agents has been well reported and reviewed extensively (Ip 1998; Brown and Arthur 2001; Rayman 2005; Zeng and Combs 2008). There are many inorganic and organic selenium compounds that are in development as chemopreventive agents and many are defined by chemopreventive index (CI) that is the maximum tolerable dose divided by the effective dose that produces 50% inhibition of tumor yield (MTD/ED50). Agents with higher CI values are considered safer and more suitable for use as chemopreventive agents (Sinha and El-Bayoumy 2004). At physiological levels, elemental selenium has anti-mutagenic properties, however at greater concentrations selenium can be genotoxic (Shamberger 1985). Selenium compounds are therefore administered as ppm Se. The types of selenium compounds in development now are typically low molecular organic and inorganic compounds. It would be erroneous to say that because these compounds incorporate selenium, the mechanism of action and toxicity profile will be the same as elemental selenium. The position of Se in the molecule, bioavailability, metabolism and metabolites, as well as other structural features dictates the overall properties of a compound. However, as discussed above, selenium has interesting and unique chemical properties. Two possible mechanisms for the chemopreventive effects of low molecular weight selenium compounds have been proposed and reviewed as of 2008 by Brigelius-Flohe, to understand the potential health effects of Se compounds. First, selenium compounds such as selenomethionine, selenite, and phenylenebis(methylene) selenocyanate (p-XSC) have been shown to directly modify cellular thiols which have a wide range of effects including cell signaling, DNA repair, protein degradation, and gene expression changes. Selenocompounds
may also contribute to the overall selenium status of the organism and thus contribute to selenoprotein expression and activity. Figure 1 shows the proposed pathways for metabolism of biologically important selenomolecules.

Figure 1 – Proposed pathways of metabolism of biologically important selenomolecules.

Methylselenocysteine and selenomethionine are consumed through foodstuffs and are converted enzymatically to the anticancer metabolites hydrogen selenide and methylselenol which are both associated with a variety of anticancer effects at nutritional and supranutritional levels. Selenium can also be incorporated into proteins via selenocysteine incorporation.
Methylated selenium compounds are also substrates for methylation enzymes, which can methylate histones and may affect gene expression. Also, some selenium compounds can induce expression of ROS. Drake has proposed that selenium’s chemopreventive effects may be due to its role as a prooxidant (Drake 2006), a supposition that is supported by Sinha and El-Bayoumy (2004) who suggest that apoptosis is a critical cellular event in cancer chemoprevention by selenium compounds and that this occurs due to increased oxidative stress. This is further supported by a study that showed apoptosis induced by selenomethionine and methionase was found to be superoxide-mediated and p53-dependent in human prostate cancer cells (Zhao, Domann et al. 2006). Finally, both selenoprotein deficiency and high levels of selenium compounds can inhibit hepatocarcinogenesis in transgenic mice, which indicates increased oxidative stress as a possible mechanism of action of chemoprevention since the selenoproteins looked at in this study have antioxidant function and the selenium compounds induced oxidative stress (Novoselov, Calvisi et al. 2005).

Unfortunately, despite the clear evidence selenium plays an important role in many forms of carcinogenesis, selenium and selenium compounds have recently gained negative attention. In 2001 patients began enrolling in the SELECT trial which was to test selenized yeast and vitamin E each alone and in combination for having chemopreventive effects for prostate cancer. Unfortunately, early results showed small increases in prostate cancer cases and diabetes causing the study to be terminated (Ledesma, Jung-Hynes et al.). However, several articles have pointed out that the negative result of the SELECT trial does not nullify the potential effects of other selenium compounds (Dunn, Richmond et al.; El-Bayoumy 2009; Micke, Schomburg et al. 2009). The results do highlight the need for any drug to have substantial preclinical testing before being brought to a large clinical trial setting. Of the selenium agents currently being
studied, many fail to be useful chemopreventive agents because either their CI is too low, or those with high CI values may not be effective. Therefore, our lab saw an opportunity to identify and/or develop less toxic and more effective selenium compounds, a goal that is being tackled by other labs as well. (Roy, Ghosh et al.).
Chapter 1C

Development of Isoselenocyanates from Isothiocyanates

Melissa A. Crampsie
It has been found that consumption of cruciferous vegetables from the genus *Brassica*, such as broccoli, cauliflower, cabbage, and watercress, is associated with a decreased risk of developing several types of cancers including lung, stomach, colon, and breast in humans (Verhoeven, Goldbohm et al. 1996; Zhao, Seow et al. 2001; Seow, Yuan et al. 2002; Fowke, Chung et al. 2003). The effect is attributed to a class of electrophilic chemicals known as isothiocyanates (ITCs) which are stored in cruciferous vegetables as their glucosinolate precursors (Carlson, Daxenbichler et al. 1981; Cinciripini, Hecht et al. 1997; Drewnowski and Gomez-Carneros 2000; Fahey, Zalcmann et al. 2001). When the vegetables are chewed in the mouth, the glucosinolates are broken down to ITCs and thiocyanates by myrosinase (Figure 3), an enzyme also found in these plants (Tookey H.L. 1980).

**Figure 1** – Formation of isothiocyanates and thiocyanates from glucosinolates and myrosinase (Tookey et al. 1980)
The R group of ITCs can vary significantly and often plays a key role in the properties of the compound. Over 90 naturally occurring isothiocyanates have been described, but generally eight are consumed by humans (Table 1) ((IARC) 2004).

**Table 1 - Naturally Occurring Isothiocyanates Consumed by Humans**

<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulforaphane</td>
<td><img src="structure1.png" alt="Sulforaphane structure" /></td>
<td>Broccoli, Brussel Sprouts</td>
</tr>
<tr>
<td>Allyl ITC</td>
<td><img src="structure2.png" alt="Allyl ITC structure" /></td>
<td>Mustard, horseradish, wasabi</td>
</tr>
<tr>
<td>4-pentenyl ITC</td>
<td><img src="structure3.png" alt="4-pentenyl ITC structure" /></td>
<td>Brassica Vegetables</td>
</tr>
<tr>
<td>3-butenyl ITC</td>
<td><img src="structure4.png" alt="3-butenyl ITC structure" /></td>
<td>Brassica Vegetables</td>
</tr>
<tr>
<td>Iberin</td>
<td><img src="structure5.png" alt="Iberin structure" /></td>
<td>Brassica Vegetables</td>
</tr>
<tr>
<td>Phenyl ethyl ITC (PEITC)</td>
<td><img src="structure6.png" alt="Phenyl ethyl ITC structure" /></td>
<td>Watercress, root species</td>
</tr>
<tr>
<td>Benzyl ITC (BITC)</td>
<td><img src="structure7.png" alt="Benzyl ITC structure" /></td>
<td>Watercress</td>
</tr>
<tr>
<td>6-methylsulfanylhexyl ITC</td>
<td><img src="structure8.png" alt="6-methylsulfanylhexyl ITC structure" /></td>
<td>Wasabi</td>
</tr>
</tbody>
</table>

Isothiocyanates are often responsible for the pungent odor and taste of the foods they are found in such as wasabi, mustard, and horseradish. In the 1970s, the phenyl alkyl isothiocyanates (BITC and PEITC) were found to inhibit cytochrome P450 activity and ultimately carcinogen metabolism/bioactivation and therefore became of great interest in the newly emerging field of
chemoprevention (Wattenberg 1975; Sporn, Dunlop et al. 1976). The naturally occurring aryl alkyl ITCs, as well as many synthetic analogues, were tested vigorously against a variety of carcinogens in several models of chemoprevention; however, most of the studies focused on metabolism, DNA adduct formation, and pre-initiation effects of the compounds (Chung, Wang et al. 1985; Morse, Eklind et al. 1989; Smith, Guo et al. 1990; Morse, Eklind et al. 1991; Jiao, Eklind et al. 1994; Stoner and Morse 1997; Sticha, Staretz et al. 2000). From these studies, it was found that the aryl alkyl isothiocyanates were especially effective at inhibiting metabolism of the nicotine derived tobacco specific nitrosamine 4-(methylnitrosamo)-1-(3-pyridyl)-1-butanone (NNK). Dosing of these compounds found that ITCs are relatively nontoxic and were able to be administered intragastrically to mice at concentrations up to 5 µmol/mouse/day when dissolved in corn oil. It was also found that increasing the chain length of the phenylalkyl ITCs increased the potency of the compound’s ability to inhibit tumor multiplicity and tumor incidence (Table 2).
Table 2 – Chain length effects of ITCs on NNK induced tumorigenesis in A/J mice

(Morse, Eklind et al. 1989)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>Tumors/mouse</th>
<th>% of mice with tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn oil + NNK</td>
<td>39</td>
<td>9.2 ± 0.5</td>
<td>100</td>
</tr>
<tr>
<td>PITC + NNK</td>
<td>30</td>
<td>9.8 ± 0.9</td>
<td>100</td>
</tr>
<tr>
<td>BITC + NNK</td>
<td>29</td>
<td>10.4 ± 0.7</td>
<td>100</td>
</tr>
<tr>
<td>PEITC + NNK</td>
<td>28</td>
<td>3.3 ± 0.4</td>
<td>93</td>
</tr>
<tr>
<td>PPITC + NNK</td>
<td>30</td>
<td>0.4 ± 0.1</td>
<td>37</td>
</tr>
<tr>
<td>PBITC + NNK</td>
<td>28</td>
<td>0.4 ± 0.1</td>
<td>32</td>
</tr>
</tbody>
</table>

Studies of ITCs have shown that the isothiocyanate (N=C=S) group has an essential role for inhibiting nitrosamine-induced lung tumorigenesis (Jiao, Smith et al. 1996). Many studies have been done to analyze the mechanism of action for chemopreventive effects of ITCs and two excellent reviews by Hecht (1999) and Conaway (2002) summarize these findings. ITCs modulate Phase I and Phase II enzymes in ways that inhibit carcinogen binding to DNA and enhance cellular antioxidant capacity and detoxification. Specifically, in rodents ITCs are strong inhibitors of Cyp2b1. In human primary hepatocyte cultures, ITCs decreased expression of CYP3A4 and also decreased activities of CYP1A1/2 and CYP2B1/2, enzymes that are important in the bioactivation of NNK and PAHs and generation of ROS.
ITCs also induce activity of important Phase II detoxification enzymes such as glutathione $S$-transferase (GST), quinone reductase (QR), sulfatase, and UDP glucuronyl transferase (UGT). This is important because ITCs are also themselves detoxified from cells by conjugation with glutathione, a spontaneous reaction which is catalyzed by GST, but has been shown to be reversible under physiological conditions (Meyer, Crease et al. 1995; Shapiro, Fahey et al. 1998). The detoxification pathway has been studied in great detail and shown in Figure 2.

(Shapiro, Fahey et al. 1998)

**Figure 2** – Metabolism and detoxification of ITC compounds. Isothiocyanates will react with glutathione both spontaneously and enzymatically by GST. The resulting conjugate has the glutamic acid and lysine enzymatically cleaved and the resulting cysteine residue is N-acetylated. The N-acetyl conjugate is excreted typically in the urine.
First, the ITC is conjugated to glutathione via a dithiocarbamate bond. The glutamic acid is then enzymatically cleaved, followed by the glycine residue. The remaining cysteine residue is subsequently acetylated via N-acetyl transferase and the ITC is excreted as the N-acetyl cysteine conjugate in the urine. The reactivity to glutathione, ironically, is also responsible for the rapid accumulation and sequestration of ITC inside the cell at much higher concentrations than outside the cell, usually observed within 4 h of treatment (Zhang 2001).

The ITC sulforaphane, also found in cruciferous vegetables, has been shown to be electrophilic and form thioacyl adducts on sensor cysteine residues of Keap1, a protein that keeps the transcription factor Nrf2 in the cytosol. Thioacyl adduct formation inactivates Keap1 allowing Nrf2 to translocate to the nucleus and bind antioxidant response elements (AREs) in DNA and leads to transcription of antioxidant and phase II genes (Hong, Freeman et al. 2005). Studies have also shown that ITCs induce MAP kinase pathway which is associated with induction of apoptosis, induction of p53, and cell cycle arrest in a variety of cancer cells such as prostate, leukemia, and colon.

Since it was discovered that ITCs react so readily with sulfhydryl groups, many more recent studies have begun to look at the sulfhydryl reactivity of isothiocyanates using glutathione, an abundant free thiol found at concentrations of 2-10 mM inside the cell, and protein cysteine residues as targets of isothiocyanates, which may account for the their effects (Zhang 2001; Mi and Chung 2008; Mi, Xiao et al. 2008). As stated earlier, it was found that increasing the chain length phenylalkyl isothiocyanates increases the potency of inhibition of carcinogen metabolism and tumorigenesis induced by NNK. In terms of sulfhydryl reactivity, it has been found that increasing the chain length decreases the reactivity of the isothiocyanate moiety to sulfhydryl groups (Jiao, Eklind et al. 1994). Therefore, it was hypothesized that it was
the reduction in reactivity, possibly to the detoxifying glutathione, that was responsible for the increase in potency and activity seen with the longer chain synthetic ITCs.

Our lab began to hypothesize that aryl alkyl isothiocyanates were excellent lead compounds for both chemoprevention and chemotherapeutic approaches for development of new compounds due to their low toxicities and known mechanisms of action. The idea to replace sulfur with selenium in these compounds came from several observations. First, there is a great need for less toxic, more effective selenium compounds in the chemoprevention field due to the promise selenium has as described previously. Because selenium and sulfur are in the same column of the periodic table, they have similar oxidation states and covalent bonding properties, thus replacement would not be chemically difficult. Selenium is a larger atom than sulfur, surrounded by more electrons, so it was hypothesized that the selenium atom would be less electron withdrawing to the vicinal carbon atom, making it less electrophilic and therefore less reactive to -SH groups. Because ITCs were shown to increase potency with decreasing reactivity it was hypothesized that replacement of sulfur with selenium would cause reactivity to alter as well. Therefore a panel of phenyl alkyl isoselenocyanates was synthesized from the naturally occurring (BITC and PEITC) and synthetic (PBITC and PHITC) ITC compounds currently available to create ISC-1, ISC-2, ISC-4, and ISC-6 (Figure 3).
Figure 3 – Panel of ITC and ISC compounds for development. Isothiocyanate: benzyl isothiocyanate (BITC), phenylethyl isothiocyanate (PEITC), phenylbutyl isothiocyanate (PBITC), phenylhexyl isothiocyanate (PHITC). Isoselenocyanates: benzyl isoselenocyanate (ISC-1), phenylethyl isoselenocyanate (ISC-2), phenylbutyl isoselenocyanate (ISC-4), phenylhexyl isoselenocyanate (ISC-6). Other selenium compounds developed are allyl isoselenocyanate and selenium sulforaphane.
Chapter 2

Selenium replacement of sulfur in isothiocyanates: sulfhydryl reactivity and its implications

Melissa A. Crampsie, Dhimant Desai, Julian Spallholz, Shantu Amin, and Arun K. Sharma

This chapter represents the contribution of M.A.C to a manuscript in preparation. M.A.C. designed and performed the majority of the experiments and wrote the manuscript. D.D and A.K.S. synthesized the compounds used in the study. J.S. (Texas Tech) designed and performed the redox cycling experiment. S.A and A.K.S. contributed to the experimental design and provided editorial input and revisions.
2.1 Introduction

Naturally occurring phenyl alkyl ITCs (BITC, PEITC) and longer chain synthetic phenyl alkyl ITCs (PBITC, PHITC) have been shown to inhibit carcinogenesis induced by the tobacco specific nitrosamine procarcinogen NNK in mice and rats due to the direct inhibition of bioactivating CYP450 enzymes (Smith, Guo et al. 1990; Hecht, Morse et al. 1991; Morse, Eklind et al. 1991; Guo, Smith et al. 1993; Stoner and Morse 1997; Talalay and Fahey 2001). Furthermore, these studies show that ITC chain length plays a vital role in the ability to inhibit tumorigenesis specifically that potency increases with increasing chain length of up to eight carbons. It was originally discovered that increasing the chain length on the ITC reduces the reactivity of the isothiocyanate group to sulfhydryl (-SH) groups (Jiao, Eklind et al. 1994). This is important because it has been shown that ITCs are detoxified from cells by conjugation to glutathione (GSH), both spontaneously and enzymatically by glutathione-S-transferase (GST) isoforms to form dithiocarbamates and detoxified as the N-acetyl cysteine (NAC) conjugates (Meyer, Crease et al. 1995; Shapiro, Fahey et al. 1998; Zhang 2001; Conaway, Yang et al. 2002). The increase in potency seen with increasing chain length was therefore attributed to a decrease in the ability to be detoxified and having a longer half life in cells.

ITCs are not just detoxified by GST isoforms, but also are able to induce expression of them and other Phase II detoxification enzymes via the Nrf2-antioxidant response element (ARE) pathway (Ernst, Wagner et al.; Hecht 1999; Conaway, Yang et al. 2002). Aryl alkyl ITCs also cause induction of apoptosis of proneoplastic and neoplastic cells (Hwang and Lee; Nakamura, Kawakami et al. 2002; Lee and Cho 2008; Mi and Chung 2008). Interactions with thiols, such as GSH and cysteine residues of proteins, have been studied with ITC compounds and it has been shown that ITCs reduce intracellular GSH levels, cause thiocarbamoylation of proteins such as tubulin (Mi and Chung 2008; Mi, Xiao et al. 2008), and have also been shown to
generate ROS (Trachootham, Zhou et al. 2006). However, it is unclear which mechanism plays the most important role in their chemopreventive and/or chemotherapeutic action.

Our lab has modified both the naturally occurring and synthetic phenyl alkyl ITCs by isostERICALLY replacing sulfur (S) with selenium (Se) to make isoselenocyanates (ISCs) (\(-\text{N}=\text{C} = \text{Se}\)): ISC-1, ISC-2, ISC-4, and ISC-6 (Sharma, Sharma et al. 2008) (Table 1). The rationale behind this was twofold: (i) enhance the half life of the compound by reducing the reactivity towards nonspecific thiols and (ii) add additional redox cycling and anti-cancer properties of selenium. The selenium is a larger atom with more labile electrons in the same column of the periodic table as sulfur. The replacement of S with Se was an attempt to further reduce the reactivity of the electrophilic carbon in isothiocyanate (\(-\text{N}=\text{C} = \text{S}\)) group without altering the general reaction mechanism, thus making the ITC compounds even less reactive towards GSH and therefore more potent. Secondly, selenium deficiency has been associated with an increase in cancer risk, and supplementation with certain selenium compounds has been shown to be chemopreventive (Brown and Arthur 2001; Novoselov, Calvisi et al. 2005; Rayman 2005; Brigelius-Flohe 2008; Zeng and Combs 2008). With the idea of increasing potency of the ITC compounds as well as creating less toxic organoselenium agents for chemoprevention the, ISC panel was created. We have found these compounds to be more potent in cancer cell viability assays and in a xenograft mouse model inhibiting tumor growth (Nguyen, Sharma et al.; Sharma, Kline et al.; Sharma, Sharma et al. 2008; Sharma, Sharma et al. 2009). In the present study, a structure activity investigation of ISCs and ITCs in terms of thiol reactivity was carried out to determine a possible mechanistic reason for the increased potency of ISCs seen in cell and animal models.
2.2 Materials and Methods

2.2.1 Chemicals and reagents

Porcine tubulin (α and β heterodimer) was purchased from Cytoskeleton Inc. (Denver, CO). N-acetyl cysteine (NAC), glutathione (GSH), 5,5'-dithio-bis 2-nitrobenzoic acid (DTNB), and guanine-HCl were purchased from Sigma Aldrich (St. Louis, MO). 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) and phenazine methosulfate (PMS) powder were purchased from Promega (San Luis Obispo, CA).

2.2.2 ITC vs. ISC pseudo first order kinetics

A stock solution of each ITC and ISC compound in acetonitrile (10 mM) was prepared. GSH solution (10 mM) was freshly prepared by dissolving 30.7 mg of GSH (reduced form) in a 10 ml mixture of 0.2 M phosphate buffer (pH 7.4) and methanol (1:1). 10 µl of ITC or ISC solution was added to 1 ml of GSH solution at room temperature and the product formation was monitored by UV in a Cecil 2041 spectrophotometer. The change of the absorbance at wavelength 270 nm for ITCs and 300 nm for ISCs was measured and analyzed using GraphPad Prism software using the equation y=ymin + span*(1-exp(-k*x)) to determine rate of non-enzymatic conjugate formation. Each drug was tested in triplicate to determine rates.

2.2.3 Cell culture

A549 human lung adenocarcinoma cells were cultured in RPMI 1640 media supplemented with 10% Fetal Bovine Serum (Atlanta Biologicals, Lawrenceville, GA). Cells were incubated at 37 °C and 5% CO2, and passaged every two to four days. For cell lysate, cells were treated with compound for 24 h in 100 or 150 mm plates. Media was aspirated and cells
washed 2x with PBS. Ice cold RIPA buffer was added to each plate and incubated on ice for 10 min. Lysed cells were then scraped with a rubber policeman and collected in 15 ml conical tubes. Tubes were spun at 4000 rpm for 10 min at 4 °C and the supernatant was kept for analysis.

2.2.4 GSH depletion in cells

A549 cells were plated in white 96 well plates at 10,000 cells per well and cultured for 24 h. Each ITC or ISC compound was dissolved in DMSO for a stock solution of 10 mM. Cells were treated with a final concentration of 10, 15, or 20 µM drug in quadruplicate and assayed for intracellular levels of glutathione at time points up to 4 h with GSH-Glo assay kit (Promega, San Luis Obispo, CA) via luminescence per the manufacturer’s instructions. In short, media was removed from the cells and luciferin-NT added to each well and incubated for 30 min to form luciferin. Detection agent was added and the plate read using SpectraMax Plate Reader.

2.2.5 MTS assay to determine IC50 values of panels

A549 cells were plated in clear 96 well plates at 5000 cells per 100 µl 10% FBS supplemented media per well and cultured for 24 h. Cells were then treated with DMSO or drug at final concentrations ranging from 500 nM to 100 µM for 48 h. Each drug was dissolved in 100 µl of serum free media or 100 µl media supplemented with 10% FBS and added to the cells to give a final volume in the wells of 200 µl. Plates were then assayed by adding 20 µl of MTS/PMS (2 mg/ml and 0.042 mg/ml, respectively). Plates were incubated in the dark at 37 °C for 2 h and then read at 492 nm by a SpectraMax plate reader. Each concentration was done in replicates of n = 6 and the entire assay was repeated twice for a total n=12. IC50 values were calculated using Graphpad 4.0 nonlinear regression curve fitting.
2.2.6 Ellman assay

GSH, NAC, or porcine tubulin was dissolved in 0.1 M sodium phosphate (pH 7.4). FBS was assayed without dilution. DTNB was dissolved in 4 M Guanidine-HCl at 1 mM to make Ellman’s reagent and then adjusted to pH 7.4. Free -SH standard curves were constructed using NAC or GSH standard solutions from 1 mM to 50 µM. 10 µl of sample (GSH, tubulin, FBS) was added to 1 µl of drug dissolved in DMSO and incubated at room temperature for 30 min in the dark. Reactions were analyzed using drug:sulfhydryl ratio of 1:1, 0.5:1, or 0.25:1. For cell lysate, 10 µl of sample was used. 90 µl of Ellman’s reagent was added to each sample and incubated at room temperature for 15 min. All reactions were measured in triplicate. The wells were then read at 412 nm absorbance using a Spectramax Plate Reader.

2.2.7 Western blot analysis

A549 cell lysates from treated cells were assayed for total protein concentration using a BCA assay kit (Pierce, Rockford, IL). 60 µg of protein from each sample was mixed with 4X sample buffer and heated at 95 °C for 5 min and loaded onto a 12 well Nu-Page 10% Tris-Glycine gel. Proteins were separated by electrophoresis at 200 V for 45 min. Proteins were transferred to a PVDF membrane via iBlot (Invitrogen, Carlsbad, CA) and blocked with 5% non fat milk for 1 h. Membrane was incubated with β-tubulin primary antibody (Cell Signaling, Danvers, MA) at 1:1000 dilution overnight shaking at 4 °C. The membrane was washed 3x with TBS-Tween and incubated with HRP-conjugated goat-anti rabbit secondary antibody for 1 h (Cell Signaling). The blot was imaged using chemiluminescence and Kodak imager. The loading control was β-actin (Cell Signaling). Band intensity was quantified using ImageJ software and results from three separate blots were averaged to obtain the results.
2.2.8 Chemiluminescence (CL) assay

One ml of the cocktail solution (sodium phosphate (0.05 M), lucigenin (20 µg/ml), and reduced glutathione, (4 mg/ml) pH 7.4) was added to a 10 × 50 mm polypropylene tube used for the CL assay. Each ITC or ISC was added last to initiate the reaction. CL using lucigenin as the detector of superoxide (O$_2^-$) was counted in repetitive integrated 30 s increments over time using a chemiluminometer (model 535, Los Alamos Diagnostics) to which was attached a circulating water bath (model 2209, LKB) that held the tube at 36°C.

2.2.9 Flow cytometry to determine apoptosis, cell cycle, and ROS

A549 cells were plated in 12 well plates at 100,000 cells per well and cultured for 24 h. Cells were then treated with 10 mM stock solutions of drug dissolved in DMSO at varying concentrations and time points. For cell cycle analysis, cells were treated with drug for 4 h and then drug was taken and fresh media was added, but cells were not collected until 24 h after initial treatment. Cells were washed with PBS, collected, and stained with Annexin-V and 7-AAD for apoptosis (BD Biosciences, San Jose, CA), propidium iodide for cell cycle analysis (BD Biosciences), or dye for superoxide and total ROS (Enzo Scientific, Farmingdale, NY) and analyzed using flow cytometry. Each run counted 10,000 cells.
2.3 Results:

2.3.1 ITC and ISC reactivity towards glutathione

To determine the rates of reactions of ITC and ISC compounds towards GSH, the rate of conjugate formation was monitored by UV. The absorbance of the ISC-GSH conjugates occurred at 300 nm and ITC-GSH conjugates occurred at 270 nm. None of the parent compounds had detectable absorbances at either of these wavelengths. The results from pseudo first order kinetic experiments with 100-fold excess GSH showed that the rate of reaction with GSH decreased with increasing chain length for both ITC and ISC compounds (Table 1). When ITCs were compared to ISC, ISC compounds reacted more quickly with GSH than the corresponding ITC compound for all chain lengths. The reaction rates were supported using the Ellman assay (Figure 1A). GSH was mixed at a 1:1 ratio with either ITC or ISC compounds and incubated for 30 min, which gave time for the reactions to reach equilibrium. These results showed the equilibrium concentrations of the GSH conjugate were lower as chain length increases. However, no clear relationship between sulfur and selenium compounds could be ascertained. The equilibrium concentrations of the conjugates appeared to be similar for both ITC and corresponding ISC compounds.
<table>
<thead>
<tr>
<th>Isothiocyanate</th>
<th>Reactivity $(GSH \times 10^2)/sec$</th>
<th>LogP</th>
<th>Isoselenocyanate</th>
<th>Reactivity $(GSH \times 10^2)/sec$</th>
<th>LogP</th>
</tr>
</thead>
<tbody>
<tr>
<td>BITC</td>
<td>$8.05 \pm 0.63$</td>
<td>3.0</td>
<td>ISC-1</td>
<td>$16.19 \pm 4.82$</td>
<td>2.1</td>
</tr>
<tr>
<td>PEITC</td>
<td>$2.23 \pm 0.11$</td>
<td>3.4</td>
<td>ISC-2</td>
<td>$7.40 \pm 0.58$</td>
<td>2.5</td>
</tr>
<tr>
<td>PBITC</td>
<td>$1.23 \pm 0.09$</td>
<td>4.2</td>
<td>ISC-4</td>
<td>$5.40 \pm 0.34$</td>
<td>3.3</td>
</tr>
<tr>
<td>PHITC</td>
<td>$0.96 \pm 0.05$</td>
<td>5.2</td>
<td>ISC-6</td>
<td>$4.35 \pm 0.07$</td>
<td>4.3</td>
</tr>
</tbody>
</table>

Mean ± SD
Figure 1A – ITC and ISC Binding to Glutathione (in vitro)

% Free SH of Control

1:1 Compound:GSH (30 min)

BITC  PEITC  PBTC  PHITC  ISC-1  ISC-2  ISC-4  ISC-6

Figure 1B – Time Dependent GSH depletion in A549 cells

% Intracellular GSH to Control

Treatment (15 µM)

30 min  1hr  2hr

BITC  PEITC  PBTC  PHITC  ISC-1  ISC-2  ISC-4  ISC-6
Figure 1C – Dose Dependent GSH depletion in A549 cells

Figure 1D – Dose Dependent GSH:GSSG ratio in A549 cells
Figure 1 – Glutathione reactivity and depletion in vitro and in vivo. A) ITC and ISC compounds were mixed with GSH at a 1:1 ratio and incubated for 30 min (n=4, mean ± SE). Ellman assay was performed to determine the amount of free -SH in the solution. B) Time dependent GSH depletion in A549 lung adenocarcinoma cells by ITC and ISC compounds at 15 µM C) Dose dependent GSH depletion in A549 cells at 2 h. D) Dose dependent alterations of the glutathione ratio in A549 cells at 2 h. For all GSH experiments, values are reported as mean ± SE, n=4.

<table>
<thead>
<tr>
<th>Table 2 - 48 h IC$_{50}$ (µM) in A549 Cells (MTS)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>BITC</td>
</tr>
<tr>
<td>PEITC</td>
</tr>
<tr>
<td>PBITC</td>
</tr>
<tr>
<td>PHITC</td>
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<tr>
<td>ISC-1</td>
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<td>ISC-2</td>
</tr>
<tr>
<td>ISC-4</td>
</tr>
<tr>
<td>ISC-6</td>
</tr>
</tbody>
</table>
2.3.2 Glutathione depletion in A549 cells by ITC and ISC compounds

To determine if the rate of GSH reactivity in vitro corresponds to its depletion in cells, A549 cells were treated with each compound at 15 µM for up to 2 h. Both GSH levels and GSH ratio in cells were determined (Figure 1B-D) using menadione as a positive control. For all compounds, GSH levels were depleted in a dose and time dependent manner (Figure 1B and 1C). At 1 h, levels of GSH were lower in cells treated with ISC compounds compared to ITC, but at 2 h the opposite was seen. These results suggest that over time, GSH is depleted more rapidly with ISC compounds, but the total depletion is not as extensive as with the ITC compounds. For this particular cell line, of the ITCs PEITC was able to deplete GSH levels and the GSH ratio to the greatest extent (Figure 1C-D), showing that even for both the ITCs and ISCs rate of reactivity with GSH in vitro does not necessarily correlate with the extent of depletion in vivo.

2.3.3 Induction of apoptosis

The panels of compounds were tested for their ability to induce apoptosis in A549 lung adenocarcinoma cells to determine if rate of reactivity with or depletion of GSH in cells had a correlation with cell death. The flow cytometry results (Figure 2A-B) show that ISC compounds induce higher levels of apoptosis at 12.5 µM compared to the corresponding ITC compounds. MTS assays were used to find the IC₅₀ values of the panels. ISC compounds were found to be more cytotoxic, showing lower IC₅₀ values than the corresponding ITCs. ISC-4 was the most cytotoxic drug of the panels. Interestingly, the IC₅₀ values change depending on whether serum or serum free media is used to dissolve the drug prior to treatment of cells (Table 2). We believed this to be due to reaction with proteins found in FBS because recent evidence suggests that naturally occurring ITCs may bind to proteins such as tubulin to cause cell cycle arrest and
induce apoptosis (Mi, Xiao et al. 2008). Based on this, we decided to look at protein binding capabilities of ISC compounds.

**Figure 2** – Induction of Apoptosis by ITC and ISC compounds. A) Flow cytometry plots (below) of Annexin-V and 7-AAD staining of A549 lung adenocarcinoma cells treated with 12.5 µM compound. B) Graph of apoptosis (above) as measured by flow cytometry (10,000 cells counted per treatment group). Experiment was done n=2
2.3.4 Protein modification of -SH groups by ITC and ISC compounds

We first wanted to assess the binding of ITC and ISC compounds to FBS, since a difference in IC\textsubscript{50} values are seen when drugs are dissolved in serum free media compared to serum supplemented with FBS. A time course assay was done to assess binding over time. The -SH concentration of FBS used in the assay was determined to be 400 µM. Therefore drug was added at 1:1 ratio; at a final concentration of 400 µM. Aliquots were taken out every 5 min for the first half hour and then every 30 min for 6 h to determine total protein binding by the ITC and ISC compounds. Six representative time points are shown in Figure 3A to illustrate binding over time. As seen with GSH, both ITCs and ISCs modify -SH residues of proteins in the FBS and the equilibrium concentration of the conjugates appeared to decrease with increasing chain length for both sulfur and selenium compounds. As with the GSH conjugation, it is again difficult to pinpoint a significant difference for total conjugate formation when comparing sulfur to selenium. It appears equilibrium concentrations are similar, although for ISC-6 the conjugate equilibrium concentration appears consistently higher than for PHITC. As seen by the graphs, approximately 5% to 30% of the protein -SH groups are modified in as quickly as 5 min and these changes persist for at least 6 h. The binding to FBS proteins explains why there is a difference seen in IC\textsubscript{50} values depending on whether the drugs are mixed with serum free or serum supplemented media.

In cell lysate samples from cells treated for 4 h with each compound, total sulfhydryl (free and protein) levels were depleted to a greater extent with the ISC compounds as compared to the ITC compounds (Figure 3B). Coupled with the results from the GSH depletion study, it can be concluded that the thiol depletion seen in the cells are not due to GSH depletion because ISC compounds do not deplete GSH to the extent that ITC compounds do, so it may be appropriate to state that the reduced level of -SH seen is due to a decrease in protein -SH groups.
Figure 3A – ITC and ISC Binding to -SH Residues of FBS

Figure 3B – ITC and ISC Binding to -SH in cells

Figure 3 – Ellman Assay of FBS and A549 cells treated with ITC or ISC. A) Ellman assay of FBS treated with 400 µM ITC or ISC over 6 h (n=3, reported as average of ratios). B) Ellman assay of A549 cell lysate from cells treated with 10 µM ITC or ISC for 4 h (n=3, mean ± SE)
2.3.5 Protein modification of tubulin

One of the protein targets of ITC compounds that has been identified is tubulin. Since our results suggest that ISC compounds may prefer binding to protein sulfhydryls over free sulfhydryls, the ability of ISC compounds to modify tubulin was analyzed. Porcine tubulin was measured for free -SH concentration and was found to have 160 µM free -SH. ITC and ISC drugs were therefore mixed with tubulin at 40 µM, 80 µM, and 160 µM. Once again, Figure 4A shows that protein -SH modification was dose dependent, and once again there was no ascertainable difference of equilibrium conjugate concentrations between sulfur and selenium compounds. Cells were treated with 10 µM compound for 12 h and tubulin degradation was determined by western blot (Figure 4B-C). This concentration causes apoptosis to occur with minimal necrosis for all the compounds in the panel. Although both ITC and ISC compounds were able to modify -SH groups in purified tubulin in vitro, only BITC, PEITC, and PBITC compounds resulted in tubulin degradation at the concentration tested as determined by the western blot analysis. These results suggest that ITC and ISC may have different protein targets possibly based on their lipophilicities. Protein targets may also be affected by the reactivity of the electrophilic Carbon (C) in the –N=C=X (X = S, Se) group. PHITC and ISC compounds possess a less electrophilic C compared to BITC, PEITC, and PBITC which may in turn affect reactivity with specific proteins.
Figure 4A – ITC and ISC modification of porcine tubulin in vitro
Figure 4 – Effects of ITCs and ISCs with tubulin. A) Purified porcine tubulin was mixed at varying ratios of ITC or ISC compounds for 30 min and quantified for -SH concentration using the Ellman assay (n=3, reported as average of ratios). B) Representative western blot image of B-tubulin from A549 cells treated with 10 µM compound for 24 h. C) Quantification of western blot bands (average from three blots, mean ± SE).
2.3.6 Induction of cell cycle arrest

Cell cycle arrest has been implicated in the reactivity of isothiocyanates with tubulin and other proteins. We tested our panel in their ability to arrest cell cycle in A549 cells. Figure 5A and 5B show ITCs were capable of inducing G2/M cell cycle arrest, especially the longer chain ITCs PBITC and PHITC and the effect is dose dependent. ISC compounds, however lacked the ability to induce G2/M arrest, even when tested at higher doses (Figure 5B), suggesting that replacement of sulfur with selenium in ITCs results in differences in protein targets and/or difference of mechanism of action for induction of apoptosis.

Figure 5A – Cell Cycle Analysis of cells treated with ITCs and ISCs
Figure 5B – Cell cycle analysis of cells treated with PBITC and ISC-4

![Cell cycle analysis graph](image)

**Figure 5** – Ability of ITC and ISC compounds to induce cell cycle arrest. A) A549 cells treated with 10 µM ITC or ISC were stained with propidium iodine to determine phase of cell cycle (n=2, reported as range). B) Dose dependent cell cycle arrest caused by PBITC but not ISC-4 (n=2, reported as range).
2.3.7 *Chemiluminescence assay*

Selenium compounds have been shown to redox cycle. To determine if ITC or ISC panel could redox cycle in the presence of -SH, namely GSH, we conducted an experiment that measure superoxide formation upon incubation with GSH and lucigenin (Figure 6A). Interestingly, the ISC compounds were capable of redox cycling more efficiently as compared to the corresponding ITC compounds. The compounds with two carbons in their chain lengths (PEITC and ISC-2) showed the highest ability of redox cycling suggesting chain length plays an important role in this mechanism.

2.3.8 *Induction of ROS species*

To confirm that ROS are being generated in cells upon treatment as is suggested by the chemiluminescence assay, cells were treated with each compound for 4 h with 15 µM drug and then analyzed for superoxide and other ROS species (Figure 6B and 6C). ISC compounds caused higher levels of total ROS species as compared to the ITC compounds as evidenced by the shift of cells when analyzed by flow cytometry. This correlates well with the higher ability of ISC compounds to redox cycle in the presence of -SH when compared to the corresponding ITC compounds.
6A

Redox Cycling

Chemiluminescence Units

Control BITC ISC-1 PCTC ISC-2 PBTC ISC-4 PHTC ISC-6

** p < 0.01

*** p < 0.001
Figure 6C – ROS staining of A549 cells treated with ITC or ISC

![Graph showing ROS staining of A549 cells treated with ITC or ISC.](image)

Figure 6 – ROS induction in A549 cells and redox cycling capabilities of ITCs and ISCs. A) Redox cycling capability of ITC and ISC compounds when mixed with GSH via superoxide formation detected by lucigenin (n=3, mean ± SE). B) Flow cytometry plots of A549 cells treated with 15 µM drug for 4 h stained for superoxide and total ROS. C) Quantification of ROS positive cells when treated with ITC or ISC compounds measured by flow cytometry, 10,000 cells counted per treatment (n=2, reported as range).
2.4 Discussion:

A new panel of isoselenocyanate compounds designed in our lab have been found to be more potent in inhibiting cell and tumor growth and inducing apoptosis as compared to corresponding isothiocyanates (Nguyen, Sharma et al.; Sharma, Kline et al.; Sharma, Sharma et al. 2008; Sharma, Sharma et al. 2009). These agents have also been shown to inhibit phase I and induce phase II enzymes similar to isothiocyanates (Emmert, Desai et al.; Crampsie MA In Press). The goal of the current study was to evaluate the underlying mechanisms responsible for better cytotoxic efficacy of ISC compounds as compared to the ITCs. Initial reactivity experiments to GSH of the ISC panel showed that, similar to ITCs, these compounds also reacted with GSH to form conjugates and that reactivity decreased with increasing alkyl chain length. Surprisingly, ISC compounds were found to be more reactive towards GSH than the corresponding ITC compound of the same chain length. The explanation for this may be that the C=Se bond is simply less stable than the C=S bond which makes it more reactive to -SH nucleophiles. Interestingly, even though these compounds were more reactive to GSH, they still tend to be more potent in cancer cell viability assays.

A549 lung adenocarcinoma cells were used to measure GSH depletion by the ITC and ISC panels. Depletion of GSH in cells for all compounds was both time and dose dependent. ISC compounds appear to deplete GSH levels more quickly, however the total depletion is not as high as it is with the ITC panel. It is possible that the depletion with ISCs is so transient that it was not detected, in which case it still could be said that the GSH depletion is more prolonged with the ITC panel than with the ISC panel.

As observed with the all of the in vitro studies of ITC and ISC reactions with -SH groups, the equilibrium conjugate concentrations appear to be in line with the reactivity rates. That is the
higher the reactivity of the compound with the \(-\text{SH}\) group, the higher the conjugate equilibrium concentration. Alkyl chain length may explain the differences in potency within the ITC panel and ISC panel, as increasing chain length appears to increase potency. This is probably due to the ability to detoxify the compounds out of the cell by conjugation with GSH, and also may be due to nonspecific binding ability of these compounds to proteins, such as serum albumin, causing the effective concentration of active compound at site of action to be reduced. Also, as hypothesized with ITC inhibition of CYP450 enzymes, a longer chain length may be contributing to the reactive isothiocyanate or isoselenocyanate group’s ability to access a critical cysteine \(-\text{SH}\) needed for enzyme activity. It is possible that the shorter chain compounds cannot access this residue as well. We have seen the same trend with the ISC panel using mouse liver microsomes as has been seen with the ITCs that increasing alkyl chain length results in a more potent inhibition of cytochrome P450 metabolism (data not shown).

It is also important to note that compound reactivity to GSH is not predictive of its cytotoxicity. Other studies have shown that reactivity with certain proteins may be more important (Mi and Chung 2008; Mi, Xiao et al. 2008). However, because of the broad reactivity spectrum of these compounds, identifying specific targets is difficult and specificity depends on a multitude of factors such as how well the compounds can enter the cell, the compound lipophilicity, its reactivity to thiols, and the stability of the conjugate. As seen with the protein modification studies, the in vitro reactivity to cysteine residues of the panels mimic the reactivity with GSH. However, the cell environment cannot be ruled out as playing an important role in how well compounds reach specific protein targets. This was observed in the current study: although both ITC and ISC can modify tubulin in vitro, the tubulin degradation and G2/M arrest was only seen with the ITC panel (at concentrations that result in apoptosis). Thus, ISC
compounds may exhibit a difference in protein binding preferences, or conjugation of ITC and ISC compounds with proteins may not produce the same effects on the protein itself. However, as mentioned earlier both ISC and ITC compounds are able to inhibit mouse microsome (cytochrome P450) metabolism of the tobacco procarcinogen NNK to ultimate carcinogens, showing there are examples of similar protein targets preferences and effects, likely due to the similarities in their overall structure.

Both ITCs and organoselenium compounds have been shown to generate ROS, therefore this mechanism of action was investigated for the ISC panel. As shown in the chemiluminescence assay, the ISC compounds have a much higher ability to redox cycle, in that they form higher amounts of superoxide and other ROS species when mixed with GSH. In A549 cells, treatment with the ITC and ISC panels at concentrations that result in apoptosis resulted in increases in both superoxide and hydroperoxides (total ROS) to a greater extent with the ISC panel compared to the ITC panel. Therefore, these compounds may cause redox dysregulation in cells as a result of increased ROS species which may be the reason for the increased potency seen with the ISC panel compared to the ITC panel. This hypothesis needs further testing, but would suggest that ISC compounds could selectively kill neoplastic or even preneoplastic cells due to a higher level of these species in cancer cells to start. The reason for the increased redox cycling capabilities of the ISC compounds compared to the ITC compounds is speculated in Figure 7A-B. When ITC or ISC compounds react with GSH or proteinyl cysteine (PSH) the resulting conjugate may be capable of resonance. The resonant structures result in a –SH group for ITCs and a –SeH group for ISCs. The –SeH group is well known for its redox cycling capabilities (Chaudiere, Courtin et al. 1992; De Silva, Woznichak et al. 2004; Spallholz, Palace et al. 2004) and is far more reactive than the corresponding –SH, which would only be slightly redox active.
Figure 7B summarizes our overall findings. In conclusion, ISC compounds were able to induce higher levels of apoptosis in A549 cells and deplete intracellular GSH levels to a greater extent than ITC compounds. However, the original hypothesis suggesting that selenium replacement of sulfur would result in decreased electrophilicity of the carbon atom proved incorrect. Our results show that although both ITCs and ISCs were able to bind similarly to proteins in vitro, that cellular effects in vivo are different. ISC compounds were found to have an increased ability to redox cycle compared to ITC compounds, a novel finding. Therefore redox activity may be the reason for differences in cellular protein targets as well as the increased potency seen with ISC compounds in both cancer cell and tumor assays. It remains unclear whether or not the ITC/ISC conjugate has the ability to modify thiols, which is why a dashed arrow is used in the figure. In order to discover the full potential of the ISC panel, it is vital to identify protein targets as well further investigate the redox effects in various cancer models.
Figure 7 – Redox cycling scheme of ITCs and ISCs. Upon entering cells, ITC or ISC can react with thiols forming conjugates. The conjugate compounds are capable of resonance to either a thiol (ITC) or selenol (ISC) compound. Selenols are known to exhibit redox cycling capabilities.
Chapter 3

ISC-4 Modulates Phase I and II Enzymes and NNK Induced DNA Adducts in A/J Mice

Melissa A. Crampsie, Nathan Jones, Arunangshu Das, Cesar Aliaga, Dhimant Desai, Philip Lazarus, Shantu Amin, and Arun K. Sharma

In Press: Cancer Prevention Research (2011). The text and formatting represent the final, copy-edited version of the journal article. M.A.C performed the majority of the experiments and wrote the manuscript. N.J. performed all of the RNA extraction, bioanalysis, and rt-PCR work and wrote this section of the manuscript. A.D. analyzed the selenium in the samples using the atomic absorption instrument. C.A. administered drug to the mice and performed the animal sacrifice. D.D. and A.K.S. synthesized the compounds used in the study. P.L., S.A., and A.K.S. provided experimental design, editorial input, and revisions to the manuscript.
3.1 Introduction

Lung cancer is one of the most preventable forms of cancer due to the fact that 90% of cases are attributed to smoking and/or chewing tobacco, thus the majority of prevention efforts are focused on smoking cessation. For those who cannot quit due to the addictive nature of nicotine, and for former smokers who may be at high risk for developing lung cancer, chemoprevention strategies may be the answer. Lung cancer development in smokers and former smokers can have a latency period of 10-30 years (Novello 2008), allowing for a significant time frame to intervene in the carcinogenesis process. Therefore, cancer chemoprevention, which seeks to arrest or reverse the disease process of carcinogenesis in its initiation, promotion and progression toward invasive malignancy, holds a great scientific promise. However, optimal prevention of lung cancer has not been achieved yet due to the lack of an effective and safe chemopreventive agent.

There is strong literature data demonstrating ITCs to be effective chemopreventive agents for specific human cancers (Chung, Jiao et al. 1997; Stoner, Adams et al. 1998; Yu, Mandlekar et al. 1998; Talalay and Fahey 2001; Chung and Saltz 2007; Traka and Mithen 2009). ITCs have been shown to exhibit their anticarcinogenic effects through dual mechanisms occurring at the level of initiation of carcinogenesis by blocking Phase I enzymes (cytochrome P450) that activate procarcinogens and also by inducing Phase II enzymes that detoxify electrophilic metabolites generated by Phase I enzymes (Zhang and Talalay 1994; Kassahun, Davis et al. 1997; Maheo, Morel et al. 1997). Specifically, they have been shown to be very effective in modulating tobacco carcinogen NNK metabolism and are potent inhibitors against NNK-induced lung tumorigenesis in A/J mice (Morse, Eklind et al. 1991; Jiao, Eklind et al. 1994). NNK requires metabolic activation by cytochrome P450 to exhibit its mutagenicity and possible
carcinogenicity (Hecht 1998) (Figure 1). Hydroxylation of the alpha carbons yields two reactive species, which alkylate DNA to produce pyridyloxobutyl (pob)-DNA or methyl-DNA adducts. This is believed to be an important mechanism of carcinogenesis in both rodents (Staretz, Foiles et al. 1997; Sticha, Kenney et al. 2002; Boysen, Kenney et al. 2003) and smokers (Foiles, Akerkar et al. 1991; Schlobe, Holze et al. 2002), since pob-DNA adducts have been detected in animals treated with NNK and in the lung tissue from smokers (Schlobe, Holze et al. 2002). Furthermore, the $O^6$-methyl guanine ($O^6$-MG) adducts have been determined to be critical for tumor formation in A/J mice treated with NNK and is less efficiently repaired in the presence of bulky pob-DNA adducts (Peterson and Hecht 1991; Peterson, Mathew et al. 1991). The chemopreventive efficacy, favorable mechanism of action, and safety profile of ITCs in general and towards NNK induced carcinogenesis in particular, makes them ideal lead compounds for structural optimization.
Figure 1 – Bioactivation and detoxification pathways of NNK. Bioactivation of NNK leads to DNA adduct formation via the keto acid pathway (methyl adduct formation) or the keto alcohol pathway (pyridyloxobutyl adduct formation). Detoxification of NNK occurs via glucuronidation of NNAL by UgtS.
Our lab has modified both naturally occurring and synthetic phenyl alkyl ITCs by isosterically replacing sulfur with selenium to make isoselenocyanate (ISC) compounds (Sharma, Sharma et al. 2008). The rationale for this modification was based on the observation that organoselenium compounds have been shown to be effective in retarding tumorigenesis of several cancer types (Clark, Combs et al. 1996; Reddy, Wynn et al. 1996; Combs GF Jr 1998; Jacobs, Jiang et al. 2004), in both animal models and epidemiological studies. Hence, ISC compounds combined the anticancer properties of both selenium and ITCs. Furthermore, compared to sulfur structural analogs, selenium compounds have been shown to be more potent anti-cancer agents (Ip 1992). We have also found the selenium compounds (ISCs) to be more potent in cell viability and animal bioassays for cancer as compared to the corresponding ITC derivatives (Sharma, Sharma et al. 2008). Extensive structure-activity studies on ITCs and newly generated ISCs have identified phenylbutyl isoselenocyanate (ISC-4) (Fig. 2A) as the most efficacious agent both in terms of potency and drug-likeness (Sharma, Sharma et al. 2008; Sharma, Sharma et al. 2009).

The suitability of ISC-4 as a chemopreventive agent was tested in an animal model of lung cancer using A/J mice. These mice are susceptible to Ki-ras mutations which lead to 20-40% of them to spontaneously develop lung adenomas by 20 weeks of age (Witschi 2005). Treatment with NNK leads to DNA adducts and 100% incidence of lung tumors in these mice only 16 weeks after carcinogen administration, regardless of the route of administration (Witschi 2005). To assess the chemopreventive potential of ISC-4, intragastric dosing of the drug was first established and then mice were analyzed for Phase I and II enzyme activity and gene expression after a single dose of the drug in a time dependent manner. Mice were also treated with the
procarcinogen NNK to determine if ISC-4 was able to inhibit DNA adduct formation in liver and lung.
3.2 Materials and Methods

3.2.1 Chemical and reagents

ISC-4 was synthesized following a method recently developed by Sharma et al. (Sharma, Sharma et al. 2008). $^{3}$H[NNK] was purchased from Moravek Biochemicals (Brea, CA). The deuterated pob adduct standards were a kind gift from Dr. Stephen Hecht (University of Minnesota Cancer Center, Minneapolis, MN). Glucose-6-phosphate (G-6-P), NADP+, G-6-P dehydrogenase, phosphodiesterase II, alkaline phosphatase, guanine, $O^{6}$-methyl guanine ($O^{6}$-MG), and 7-methylguanine (7-MG) were purchased from Sigma Aldrich (Milwaukee, WI).

3.2.2 Animal experiments

Animal experimentation was carried out according to protocols approved by the Institutional Animal Care and Use Committee (IACUC) at Penn State University. Female A/J mice were purchased at 6 weeks of age from The Jackson Laboratory (Bar Harbor, ME) and stratified into groups by treatment of ISC-4. Mice were given corn oil (vehicle control) or ISC-4 dissolved in corn oil at 2.5 ppm, 5.0 ppm, or 10.0 ppm (as selenium) per mouse (20 g average). Mice were sacrificed by CO$_2$ asphyxiation, organs harvested and immediately frozen on dry ice. Serum was collected from blood immediately by centrifugation. Tissue and serum samples were stored at -80 °C until analysis.

3.2.3 Selenium analysis

Liver or lung tissue (0.100 g to 0.400 g) was homogenized in 1.15% cold KCl (0.1 gm/ml) using a glass hand homogenizer. Exact amount of tissue homogenate or 200 µl serum was digested in a MARS Xpress microwave digestion system (CEM Corp., Mathews, NC) equipped with 55 ml Teflon PFA vessels and a turntable. The digestion was conducted in 50%
nitric acid and was diluted to 20% before Se analysis by Atomic Absorption Spectroscopy. An A Analyst 600 instrument from PerkinElmer with Graphite Furnace was used for total selenium analysis by measuring the absorbance peak area at 196 nm for each sample. Palladium matrix modifier was added along with each sample to the furnace. A reference standard solution of selenium dioxide was used to construct standard curve. Analysis was performed in duplicate for each sample and the average value was recorded. For each group or category at least three samples were analyzed and the results were expressed as mean ± SD (n=3).

3.2.4 Microsome and cytosol fraction preparation

Liver and lung microsomes or cytosol extracts were prepared as previously described (Guengerich 1994). Briefly, homogenate was centrifuged once at 10000xg to remove nuclear pellet, then at 105000xg for cytosol extract (supernatant). The remaining pellet was resuspended and spun at 105,000xg for microsomes (pellet). Fractions were stored at -80 °C until use. Protein concentrations for microsomes and cytosol fractions were determined using a BCA protein assay kit (Pierce, Rockford, IL).

3.2.5 Cyp activity assay with the substrate NNK

Microsomal Cyp activity was assayed in 150 µl 0.1 M Tris (pH 7.4), 1 mM EDTA, 20 mM MgCl₂, and 0.3 M KCl. Cyp activity was induced by an NADPH generating system (1 µg/µl G-6-P and NADP⁺ , 0.4 mU/µl G-6-P dehydrogenase). ³H[NNK] was added at 0.5 µCi/reaction. Non-radiolabeled NNK was added to 20 µM. Reactions were initiated by addition of microsomes (1 mg/ml) and incubated at 37 °C for 1 h. Reactions were terminated with cold 7.5 M NH₄Ac, vortexed, and placed on ice for 10 min. Tubes were centrifuged for 10 min at 14000 rpm.
Samples were filtered and analyzed by HPLC (Waters, Milford, MA) for oxidative metabolism of NNK using Radio Flow Detection (INUS Systems). A Phenomenex Max-RP C18 reverse phase column was used to separate metabolites. The HPLC conditions were 100% solvent A (25 mM sodium phosphate, pH 7.0)/0% B (Methanol) to 70% A/30% B with a linear gradient over 50 min. Cyp activity was calculated based on mean peak areas of metabolites formed in triplicate reactions.

3.2.6 Ugt activity assay with the substrate 4-methylumbelliferone (4-MU)

Mouse liver microsomes (10 µg protein) were assayed for Ugt activity in 100 µl reaction buffer (50 mM Tris pH 7.5, 10 mM MgCl₂, 10 µg/ml alamethicin, 4 mM UDPGA) using 4-MU as the substrate at 100 µM (liver) or 250 µM (lung). Reactions were initiated by addition of microsomes and incubated at 37°C for 15 min. Reactions were terminated with 100 µl cold acetonitrile. Tubes were centrifuged for 10 min at 14000 rpm. Samples were filtered and analyzed by HPLC (Waters) for glucuronidation of 4-MU. Glucuronide and parent compound were eluted isocratically at a flow rate of 1 ml/min with 80% A (3.5% triethylamine, pH 2.1 with perchloric acid)/20% B (acetonitrile) v/v using a Phenomenex Max-RP C18 reverse phase column and measuring fluorescence (365 nm/455 nm) and UV at 318 nm. Glucuronidation activity was determined by the ratio of the 4-MU glucuronide peak compared to the unconjugated 4-MU peak.
3.2.7 Ugt activity assay with the substrate 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL)

Mouse liver microsomes (50 μg protein) were incubated with alamethicin (10 μg/ml) on ice for 15 minutes. 25 μl reactions containing 5 mM NNAL were incubated at 37 °C for 1 h using the same conditions as for 4-MU. NNAL glucuronidation activity assays were analyzed using an Acquity UPLC system (Waters) with an ACQUITY UPLC BEH HILIC (2.1 mm × 100 mm, 1.7 μm particle size; Waters) column at 25 °C. UPLC was performed at a flow rate of 0.5 mL/min using the following conditions: 6.0 minutes in 10% solvent A, a linear gradient for 1.5 minutes to 100% solvent A, and 30 seconds in 100% solvent A, where solvent A is 5 mM NH₄Ac (pH 6.7) and 50% acetonitrile (v/v) and solvent B is 5 mM NH₄Ac (pH 6.7) and 90% acetonitrile (v/v). UV absorbance at 254 nm was used to detect NNAL and NNAL glucuronide. The amount of NNAL glucuronide formed was calculated based on the ratio of the NNAL-glucuronide peak compared to the unconjugated NNAL peak.

3.2.8 Gst activity assay

Cytosolic Gst activity was assayed by diluting cytosol extracts to 1 mg/ml with Dulbecco’s PBS and measuring the rate of GSH conjugation with monochlorobimane (MCB) (excitation/ emission: 380 nm/460 nm). 10 μl of 10 mM GSH was added to 100 μl of cytosol in 96 well black wall plates. 100 μl of 0.3 mM MCB was added to start reaction. The reactions (n = 3) were kinetically monitored by Spectromax spectrophotometer at 37 °C until a fluorescence plateau. All samples were measured in triplicate. Gst conjugation rates were determined by measuring the time for all the GSH to be conjugated to MCB and finding the v50 using the Boltzman equation (Graphpad 5.0).
3.2.9 RNA extraction and cDNA synthesis

Total RNA was extracted from liver and lung tissue samples using RNeasy Mini Kit (Qiagen) according to manufacturer’s protocol. Samples were subjected to on-column DNase I digestion during extraction to prevent confounding of the results by genomic DNA contamination. RNA concentrations were determined using a Nanodrop ND-1000 spectrophotometer, and RNA purity was assessed by absorbance ratios A260/A280 (> 1.9). RNA integrity was determined using an Agilent 2100 Bioanalyzer with Agilent RNA 6000 Nano chips. RNA integrity of all samples was greater than 5.0. Reverse transcription was performed using Superscript First Strand cDNA synthesis kit (Invitrogen) with 1 µg of starting RNA per sample. A negative control without RNA and a negative control without enzyme were analyzed in parallel.

3.2.10 Relative expression levels of Cyp, Ugt, and Gst genes using real time qPCR

Real-time PCR was used to determine the relative expression levels of Cyps (2a4, 2c29, 2f2, 2s1, 3a11, 4b1, and 8b1), Gsts (Gsta3, Gsta4, Gstm1, Gstm2, Gstm3, and Gstp1), and Ugts (1a1, 1a5, 1a6a, 1a9, 1a10, 2a3, 2b1, and 2b5) in liver and lung tissue using Taqman gene expression assays (Applied Biosystems) utilizing predesigned primers. cDNAs were run in quadruplicate and amplified in a 10 µl reaction containing 5 µl 2x Taqman Universal PCR Master Mix, 0.5 ml 20x primer/probe mix, and a 25ng RNA equivalent of cDNA. Relative quantification of expression was calculated using the \( \Delta \Delta Ct \) method. Relative quantification (RQ) was determined with the formula \( 2^{(-\Delta \Delta Ct)} \). Expression levels in each sample were normalized to three separate internal control genes (ACTB, HPRT, and TBP), and final RQ values were calculated by taking the geometric mean of the individual RQ values. Several
studies have shown that normalization to multiple internal control genes reduces systematic error and allows small changes in gene expression to be detected more accurately (Der veaux, Vandesompele et al.; Thellin, Zorzi et al. 1999; Bustin 2000; Suzuki, Higgins et al. 2000; Warrington, Nair et al. 2000; Vandesompele, De Preter et al. 2002; Hellemans, Preobrazhenska et al. 2004).

3.2.11 DNA adduct analysis

For DNA adduct studies, mice were fed control diet (AIN-76A) or diet supplemented with 0.57 µmol/g diet ISC-4 for two weeks. Mice were then given a single intraperitoneal (IP) dose of 10 µmol NNK in saline and sacrificed either 4 h (methyl adducts) or 24 h (pob adducts) after NNK. DNA was extracted from lung or liver tissues by phenol-chloroform extraction method and dissolved in TE buffer. DNA was quantified by a Nanodrop ND-1000 spectrophotometer. For pyridyloxobutyl (pob) adducts, 100 µg of DNA in calcium chloride buffer was hydrolyzed with deuterated standards for 30 min at 90 °C and then enzymatically digested to nucleosides with micrococcal nuclease, phosphodiesterase II, and alkaline phosphatase at 37 °C overnight. The samples were then purified on Sep Pak C18 cartridges (Phenomenex) and analyzed by HPLC-Mass Spectrometry. Samples were normalized first by internal deuterated standards and then by total nucleoside content. For methyl adduct analysis, 200 µg – 300 µg of DNA was hydrolyzed in 0.1 N HCl for 30 min at 100 °C. Samples were filtered and analyzed by HPLC on a strong cation exchange column (Phenomenex) using UV and fluorescence detection (Waters). Adducts were eluted isocratically with 100 mM ammonium phosphate buffer, pH 2.0. Peaks were quantified using guanine, 7-MG, and O6-MG standard curves.
3.2.12 Data analysis and statistics

Statistical analyses were performed using GraphPad Prism version 5.0. Mean values for activity assays or gene expression were compared across the treatment or time groups using 1-way analysis of variance (ANOVA) with significant $p$-value $< 0.05$. A student’s t-test was used to compare individual treatment groups or time point groups to the control group when ANOVA values approached but did not reach significance (ANOVA $p$-value 0.05-0.10).
3.3 Results

3.3.1 ISC-4 is orally bioavailable in A/J mice

ISC-4 was given to A/J mice intragastrically to determine its oral bioavailability by measuring selenium levels in serum and target organs. To determine an effective and tolerable dose, ISC-4 was administered to animals at increasing doses of 0.675, 1.25, and 2.5 µmol per mouse (n = 6 per group). At 2.5 µmol (30 mg/kg), half of the mice died within 24 h, so no doses higher than this were tested. At 1.25 µmol (15 mg/kg) and 0.675 µmol (7.5 mg/kg) mice appeared as healthy as the corn oil treated control mice. Three mice from each group were analyzed for selenium content after 24 h. Liver and serum from each mouse was analyzed separately, but lungs were pooled. Selenium content in serum, lung, and liver increased in a dose dependent manner (Figure 2B). This indicated that selenium content from ISC-4 was being absorbed into the blood and reaching target tissues. For a complete time course study, mice (n=3) were dosed with 1.25 µmol ISC-4 and sacrificed at 0, 2, 4, 8, 16, 24, 36, and 72 h (Figure 2C). The time course study showed that selenium levels peaked first in serum (max. mean 1892 ng/g) at about 4 h post ISC-4 administration, followed by liver between 4-8 h (max. mean 1322 ng/g) and lung at about 8 h (max. 878 ng/g) post administration. Selenium levels began to fall to near normal levels between 24-72 h but did remain slightly elevated when compared to the zero time point even up to 72 h. (serum p = 0.058, liver p = 0.0072).
Figure 2 – Phenylbutyl isoselenocyanate (ISC-4) and its intragastric (IG) administration in A/J mice. A) structure of (ISC-4). B) selenium levels in serum, liver, and lung tissue of A/J mice treated with corn oil (vehicle), 0.675 µmol, or 1.25 µmol ISC-4 after 24 h (n=3, mean ± SD). C, selenium levels in A/J mice from serum, liver, and lung tissue taken at 2, 4, 8, 16, 24, 36, and 72 h after 1.25 µmol dose of ISC-4 (n=3, mean ± SD).
3.3.2 Cytochrome P450 activity is decreased in liver and lung of mice treated with ISC-4

Cyp450 activity was analyzed by incubating liver or lung microsomes from mice (n=3) at each time point with \(^3\)H[NNK] and measuring the metabolic profile (Figure 3A, 3B) by HPLC. Our results indicate that Cyp450 activity is decreased after oral administration of ISC-4 in both the liver and lung as evidenced by the decreased levels of keto acid, keto alcohol, and NNK-N-oxide (lung only) metabolites formed. Inhibition of metabolites occurred almost immediately after administration of ISC-4 (as early as 0.5 h in liver) and metabolites remained inhibited up to 24 h in liver and up to 12 h in lung. Inhibition of Cyp enzymes lasted longer in the liver which correlates with the higher selenium levels seen in the liver as compared to the lung (Figure 1C). Our results also indicate that conversion of NNK to NNAL, a detoxification pathway facilitated by a carbonyl reductase, remains unchanged or slightly elevated at each time point. To determine the concentration of ISC-4 required for microsomal Cyp450 inhibition, control liver microsomes were incubated with ISC-4 in DMSO at a range of doses and formation of NNK metabolites were measured. Inhibition of Cyp450 oxidative metabolism began as low as 25 nM ISC-4 and was dose dependent (Figure 3C, 3D).
3A – Liver Cyp Activity over Time

3B – Lung Cyp Activity over Time
Figure 3 – Effects of ISC-4 on Phase I metabolism of NNK by A/J mice microsomes. A) metabolism of $^3$H[NNK] by liver microsomes at time points 0, 0.5, 2, 4, 8, 16, 24, and 72 h post ISC-4 administration (1.25 µmol in 100 µl corn oil IG) (n=3, mean ± SE). B) metabolism of $^3$H[NNK] by lung microsomes at time points 0, 4, 8, 12, 16, 24, and 72 h
post ISC-4 administration (1.25 μmol in 100 μl corn oil IG) (n=3, mean ± SE). C) effect of in vitro incubation of ISC-4 on control liver microsome metabolism of NNK to keto acid. D, effect of in vitro incubation of ISC-4 on control liver microsome metabolism of NNK to keto alcohol.

**Table 1 - Genotyped Mouse Phase I and Phase II Human Orthologs**

<table>
<thead>
<tr>
<th>Phase I - Cyp</th>
<th>Phase II - Ugt</th>
<th>Phase II - Gst</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Human</strong></td>
<td><strong>Murine</strong></td>
<td><strong>Human</strong></td>
</tr>
<tr>
<td>CYP2A6‡†, CYP2A7</td>
<td>Cyp2a4,Cyp2a5</td>
<td>UGT1A4*</td>
</tr>
<tr>
<td>CYP2F1‡</td>
<td>Cyp2f2</td>
<td>UGT1A1</td>
</tr>
<tr>
<td>CYP2S1</td>
<td>Cyp2s1</td>
<td>UGT1A6</td>
</tr>
<tr>
<td>CYP4B1</td>
<td>Cyp4b1</td>
<td>UGT2B4</td>
</tr>
<tr>
<td>NF (2D6 superfamily)†</td>
<td>Cyp2c29</td>
<td>NF</td>
</tr>
<tr>
<td>NF (3A5 superfamily)‡</td>
<td>Cyp3a11</td>
<td>UGT2B10*</td>
</tr>
<tr>
<td>CYP8B1</td>
<td>Cyp8b1</td>
<td>UGT2B15</td>
</tr>
</tbody>
</table>

**NF - no ortholog found**

‡ Isoforms found to bioactivate NNK to keto alcohol

† Isoforms found to bioactivate NNK to keto acid

Isoforms found to glucuronidate NNAL
3.3.3 *Ugt activity against 4-MU and NNAL*

In humans, NNAL is *N*-glucuronidated primarily by the hepatic UGT2B10 and *O*-glucuronidated by UGTs 1A9, 1A10, 2B7 and 2B17 (Ren, Murphy et al. 2000; Chen, Dellinger et al. 2008). Real-time PCR assays were performed for the mouse ortholog of 2B10, Ugt2b34 (see Table 1) and it was found to be expressed in mouse liver but not lung (results not shown). NNAL glucuronidation was measured using liver microsomes (Figure 4A), but NNAL glucuronidation activity could not be detected with mouse lung microsomes. Therefore the substrate 4-MU, which is glucuronidated by all human UGT1A enzymes as well as UGT2B enzymes except 2B4 and 2B10 (Stone, Mackenzie et al. 2003), was used as a test substrate to determine overall glucuronidation activity of mouse lung and liver microsomes (Figure 4B, 4C). For liver microsomes, glucuronidation activity was increased after 8 h for NNAL (*p*<0.0001), but not for 4-MU where glucuronidation activity appeared to decrease, though not significantly (One way ANOVA = 0.09). For lung microsomes, 4-MU glucuronidation was significantly increased at 24 h (*p*=0.0016) post ISC-4 administration.

3.3.4 *Gst activity against monochlorobimane*

Cytosolic fractions were incubated with monochlorobimane (MCB), a nonspecific substrate conjugated by all human GST isoforms except GSTT (Eklund, Edalat et al. 2002) (mouse ortholog Gstt) to assess Gst activity. Liver cytosol activity against MCB was 20-50 fold higher than in lung (Figure 4D, 4E). In liver, Gst activity was significantly increased at 16 h post ISC-4 administration (*p*=0.049) and 24 h post ISC-4 administration (*p*=0.0031). In lung,
however, there was no significant difference in cytosolic Gst activity at any of the time points (One way ANOVA = 0.5531)
Figure 4 – Effects of ISC-4 on Phase II metabolism in A/J mice. Mice were treated with 1.25 µmol ISC-4 dissolved in 100 µl corn oil IG. A) glucuronidation of NNAL by liver microsomes at 0, 4, 8, 16, 24, and 72 h after administration of ISC-4. B) glucuronidation of 4-MU by liver microsomes at 0, 2, 4, 8, 16, 24, and 72 h after administration of ISC-4. C) glucuronidation of 4-MU by lung microsomes at 0, 4, 8, 12, 16, 24, and 72 h after administration of ISC-4. D) glutathione conjugation of MCB by liver cytosol of A/J mice at 0, 2, 4, 8, 16, and 24 h after administration of ISC-4. E) glutathione conjugation of MCB by lung cytosol of A/J mice at 0, 4, 8, 12, 16, and 24 h after administration of ISC-4. All experiments were done in triplicate and reported as mean ± SE. * p< 0.05, ** p < 0.01, *** p < 0.001
3.3.5 Relative phase I and phase II mRNA expression in liver and lung tissue

In liver tissue, treatment with ISC-4 was found to significantly alter expression of several Gsts, Cyps, and Ugts (Figure 5A). The mean expression levels of Gsta4, Gstm1, Gstm3, and Gstp1 were increased in both the 8 h and 16 h treatment groups relative to control animals ($p=0.0002$, $p=0.0088$, $p=0.0003$, and $p=0.0009$, respectively). Expression levels reached as high as 1.6-fold higher than control animals for Gsta4, 6.9-fold higher for Gstm1, 10.8-fold higher for Gstm3, and 7.0-fold higher for Gstp1. There was no significant change in the expression of Gsta3 and Gstm2 in liver. The mean expression of Cyp2a4 and Cyp8b1 was found to be significantly increased 8 h after treatment relative to the control group (2.8-fold for Cyp2a4, $p=0.0426$; 4.5-fold for Cyp8b1, $p=0.0026$), though no increase was seen at 16 h. There was no significant change in the expression of Cyp2c29, Cyp2f2, Cyp2s1, Cyp3a11, and Cyp4b1 in either the 8 h or 16 h treatment groups. In terms of hepatic Ugt expression, Ugt1a6a was significantly higher at 8 h (5.1-fold, $p=0.0045$), and expression of Ugt2b5 reached as high as 2.3-fold higher than control animals at 16 h ($p=0.0020$). There was no significant change in the expression of Ugt1a1, Ugt1a5, Ugt2a3, or Ugt2b1. Ugt1a9 and Ugt1a10 were not expressed at detectable levels in any of the mouse liver samples examined.

Treatment with ISC-4 was also found to alter the expression levels of phase I and phase II genes in lung tissue (Figure 5B). The mean expression levels of Gstm1, Gstm3, and Gstp1 were found to be significantly higher in lung tissue of animals treated with ISC-4 ($p=0.0022$, $p=0.0004$, and $p<0.0001$, respectively). Expression levels reached as high as 1.5-fold higher than control animals for Gstm1, 2.5-fold higher for Gstm3, and 3.4-fold for Gstp1. No change in expression was seen for Gsta3, Gsta4, or Gstm2. Cyp expression in lung tissue was found to be significantly decreased for Cyp2f2, reaching as low as 1.8-fold lower than control animals at 16
A small decrease in Cyp2s1 expression was also observed and approached significance at 16 h ($p=0.0634$). No significant change in expression was observed for Cyp4b1 or Cyp8b1. Cyp2a4, Cyp2c29, and Cyp3a11 were not expressed at detectable levels in mouse lung. Ugt1a6a and Ugt1a9 were both found to be significantly higher in lung tissue from treated animals, reaching as high as 1.9-fold for Ugt1a6a and 2.8-fold for Ugt1a9 ($p=0.0132$ and $p=0.0150$, respectively). Expression of Ugt1a1 was not found to be significantly different, while Ugt1a5, Ugt1a10, Ugt2a3, Ugt2b1, and Ugt2b5 were not expressed at detectable levels.
Figure 5 – Effects of ISC-4 on Phase I and Phase II gene expression. Mice were treated with 1.25 µmol ISC-4 dissolved in 100 µl corn oil IG. A, fold change of liver Gst, Cyp, and Ugt mRNA isoforms in A/J mice at 8 and 16 h after administration of ISC-4 compared to corn oil control. B, fold change of lung Gst, Cyp, and Ugt mRNA isoforms in A/J mice at 8 and 16 h after administration of ISC-4 compared to corn oil control. All experiments were done in quadruplicate and reported as mean ± SE. * p< 0.05, ** p < 0.01, *** p < 0.001
3.3.6 Inhibition of DNA adduct formation in A/J mice by ISC-4

Both pob adducts resulting from the keto alcohol pathway and methyl adducts resulting from the keto acid pathway of NNK metabolism were analyzed. The levels of $O^{6}$-pob-dG in liver and lung, and levels of $O^{2}$-pob-dT in lung were decreased in mice fed with ISC-4 supplemented diet compared to control diet mice but the decreases were not significant (Table 2). Methyl adduct analysis showed both $O^{6}$-MG and 7-MG adducts could not be detected in the lung tissues of mice fed with ISC-4 supplemental diet treated with NNK, whereas adducts were detected in mice fed control diet (Table 3). Statistics were not possible because lungs in each treatment group were pooled (n=3 or 4). Livers from three mice in each group were analyzed for methyl adducts. Significantly lower amounts of $O^{6}$-MG were seen in mice fed ISC-4 diet than mice fed control diet ($p=0.033$). Lower levels of 7-MG were also seen however the difference was not statistically significant ($p=0.41$).
### Table 2 - Pyridyloxobutyl (pob) DNA adduct formation (fmol adduct/nmol G/T)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Liver</th>
<th>Lung</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>O₂-pob dT</td>
<td>O⁶-pob dG</td>
</tr>
<tr>
<td>NNK Treated /Control Diet</td>
<td>16.21 +/- 4.75</td>
<td>2.29 +/- 0.87</td>
</tr>
<tr>
<td>NNK Treated/ ISC-4 Diet</td>
<td>22.02 +/- 5.62</td>
<td>1.78 +/- 0.90</td>
</tr>
</tbody>
</table>

n.d. - not detected

### Table 3 - Methyl DNA adduct formation (pmol adduct/nmol G)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Liver</th>
<th>Lung (pool)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>O⁶MG</td>
<td>7MG</td>
</tr>
<tr>
<td>Untreated /Control Diet</td>
<td>n.d.</td>
<td>0.29 +/- 0.04</td>
</tr>
<tr>
<td>NNK Treated /Control Diet</td>
<td>5.18 +/- 1.33</td>
<td>3.28 +/- 1.39</td>
</tr>
<tr>
<td>NNK Treated/ ISC-4 Diet</td>
<td>1.75 +/- 1.31†</td>
<td>2.54 +/- 0.08</td>
</tr>
</tbody>
</table>

† p = 0.0031 vs. NNK treated/Control Diet

n.d. - not detected
3.4 Discussion

Here we report for the first time the novel compound ISC-4 when administered orally to mice results in elevated selenium levels in serum and tissue in a dose and time dependent manner as measured by atomic absorption. The highest tissue levels of selenium were obtained in serum where selenium reached a maximum at about 4 h, however it is unclear how much ISC-4 is free or bound in the serum. After a single dose of 1.25 µmol, selenium levels peaked in liver between 4-8 h and in lung at 8 h. Selenium levels begin to taper off at about 24 h for all tissues tested and may be at subclinical concentrations in its active form by this time point as suggested by the recovery of microsomal Cyp activity. Interestingly, levels of selenium do not return to control (0 h) values in serum or liver even after 72 h suggesting that ISC-4 has a long half life, remains bound to protein, or possibly that after ISC-4 is metabolized, selenium is recycled in cells as selenocysteine. The inhibition of microsomal enzyme activity as early as 0.5 h after oral dosing suggests that ISC-4 is acting directly with protein to inhibit activity. This was confirmed by direct incubation of drug with mouse liver microsomes. We predict that the reactive isoselenocyanate group of ISC-4 is most likely reacting with protein thiols, thereby inhibiting enzyme activity. The isoselenocyanate functional group has been found by our lab to be reactive to sulfhydryl (-SH) groups of thiols and therefore may bind nonspecifically to protein cysteine sulfhydryl groups (unpublished results). Our results show ISC-4 was able to inhibit microsomal metabolism using concentrations as low as 25 nM, as evidenced by the reduction in bioactivation of NNK. Similar results were also observed for Ugt enzyme activity (data not shown) with inhibition starting at about 500 nM. In the lung, the greatest inhibition of oxidative metabolites was at 8 h, when selenium levels measure the highest. The initial sharp decrease in Cyp450 activity in the liver correlated well with the time course of selenium levels in the liver.
Interestingly, the peak ISC-4 level in liver (4-8 h) was not the same as the time point when NNK metabolism was the lowest in the liver (2 h) suggesting that ISC-4 might be metabolized or degraded as an inactive selenium compound and then detoxified out of the liver which would explain why selenium levels keep rising but inhibition of microsomes decreases. In both liver and lung, metabolite levels begin to increase after 8 h but then decrease again after 16 h, suggesting ISC-4 may be having both a direct effect on these enzymes as well as an indirect effect through a transcriptional mechanism.

For both the Gst and Ugt activity assays, a delayed increase in activity was found after 8 h, which we hypothesized to be driven by an increase in gene expression. This is supported by the fact that ITCs are known to be detoxified by GST enzymes in humans and are able to induce them via a transcriptional mechanism involving the antioxidant response element and the transcription factor Nrf2 (Conaway, Yang et al. 2002; Owuor and Kong 2002; Hong, Freeman et al. 2005). Further studies are needed to determine if ISC-4 is also able to induce the Nrf2 pathway, but the expression results suggest it does induce expression of several cytoprotective Gst genes, which are under transcriptional control of Nrf2. The same Gsts induced in liver tissue were also found to be upregulated in lung tissue. The only exception was Gsta4, which was only modestly increased in the liver and showed no change in the lung. The increases in expression seen in lung tissue were smaller in magnitude than the increases seen in the liver, which correlate to the higher levels of selenium reached in the liver compared to the lung.

Delayed increases in activity of Ugt enzymes in mouse liver may have cytoprotective effects as well. Eight hours after treatment, there was a transient increase in the levels of two Phase I genes, Cyp2a4 and Cyp8b1, but by 16 h this effect had disappeared. Since Phase I expression levels returned to normal rather quickly while Phase II genes remained upregulated,
the potential negative effects of increased Cyp activity may be overcome by the sustained increase in Phase II genes. To explore Ugt activity the major detoxification metabolite of NNK, NNAL, was used. In humans, UGTs 1A4, 1A9, 2B7, 2B10, and 2B17 isoforms (see Table 3 for orthologs) all exhibit activity against NNAL. Glucuronidation of NNAL was significantly increased after 8 h, which may be explained by the transcriptional upregulation of the UGT2B10 ortholog Ugt2b34. Lung microsomes typically show poor activity against NNAL, which may be explained by the lack of 2B isoforms expressed in the lung, specifically 2B10. In A/J mouse lung no detectable expression of 2B orthologs tested in this study were found. We therefore used a second substrate, 4-MU, to explore glucuronidation activity in both liver and lung which is ubiquitously conjugated by most UGTs. Interestingly, in liver microsomes, no change in glucuronidation activity was seen over time against 4-MU. This data suggests that ISC-4 may be having an effect only on certain Ugt isoforms, in this case the 2b family, a possibility that needs to be further investigated. For lung microsomes, activity against 4-MU was significantly increased at 24 h, which may be explained by the upregulation seen in Ugt1a9, which is not expressed in liver. Some Ugts, such as Ugt1a6a were significantly upregulated in both tissues, while Ugt1a1 was unchanged in both tissues, which suggests that ISC-4-induced changes in Ugt expression could occur by the same regulatory mechanism in these two tissues for these particular enzymes.

The modulation of Phase I and II enzymes by ISC-4 led us to develop a bioassay to determine if ISC-4 could inhibit DNA adduct formation in vivo. Our results demonstrated that $O^6$-MG adduct formation was significantly inhibited in the liver of A/J mice and in lung these adducts were not detectable in mice treated with NNK and fed with the ISC-4 supplemented diet. The inhibition of methyl adducts formation in both liver and lung is most likely due to the
inhibition of Cyp enzyme activity and possibly to the upregulation of individual Ugts responsible for NNAL detoxification.

In conclusion, ISC-4 given to A/J mice appears to be bioavailable, causes increased selenium levels in tissue and serum, and results in modified activity and expression of both Phase I and II enzymes critical for bioactivation and detoxification of many carcinogens. ISC-4 fed to mice in the diet resulted in decreased DNA adducts from NNK critical for carcinogenesis. Taken together, ISC-4 may be a suitable chemopreventive agent due to its anti-initiation effects of inhibiting carcinogen metabolism and increasing detoxification.
Chapter 4

ISC-4 and PBITC inhibit NNK induced lung tumorigenesis in A/J mouse

Melissa A. Crampsie, Ugit Hossain Sk, Cesar Aliaga, Timothy Cooper, Shantu Amin, Arun K. Sharma

This chapter represents a manuscript in preparation for submission. M.A.C. performed animal work, prepared diets, processed all of the tissues, and wrote the manuscript. U.H.S. and A.K.S. helped prepare diets and assist with animal care and maintenance. C.A. injected mice with carcinogens and performed the animal sacrifice. T.M. reviewed all of the tissue slides for diagnosis. S.A. and A.K.S provided experimental design and editorial inputs and revisions.
4.1 Introduction

Tobacco will ultimately kill about half of that who consume it and is used by about 1 billion people worldwide (WHO 2011). Of the current chemoprevention agents being studied, the isothiocyanates (ITCs) are one of the most studied groups for lung cancer chemoprevention. Epidemiological studies have shown that people who consume higher amounts of cruciferous vegetables have lower incidences of cancers at specific organ sites (Beecher 1994; Verhoeven, Goldbohm et al. 1996; Talalay and Fahey 2001; Higdon, Delage et al. 2007), including lung. Both naturally occurring and synthetic ITCs have been tested for chemopreventive properties (Morse, Eklind et al. 1989; Hecht, Morse et al. 1991; Morse, Eklind et al. 1991; Guo, Smith et al. 1993; Jiao, Eklind et al. 1994; Jiao, Smith et al. 1996; Hecht 1999; Sticha, Kenney et al. 2002). They have been shown to be most effective against polyaromatic hydrocarbon (PAH) and tobacco specific nitrosamine (TSNA)-induced carcinogenesis in mouse models most likely due to inhibition of Phase I cytochrome P450 (CYP450) bioactivation of procarcinogens to ultimate carcinogens as well as induction of Phase II detoxification enzymes (Ernst, Wagner et al.).

Our lab modified a panel of naturally occurring and synthetic ITCs by replacing sulfur with selenium to create an ISC panel. Through our preliminary studies, phenylbutyl isoselenocyanate (ISC-4) emerged as the most favorable compound for further development. The sulfur analog of ISC-4 is phenylbutyl isothiocyanate (PBITC), which has been tested in only a handful of chemoprevention studies, but has been shown to be very effective (Morse, Eklind et al. 1989; Smith, Guo et al. 1990; Hecht, Morse et al. 1991; Morse, Eklind et al. 1991). Extensive studies with ISC-4 have shown that ISC-4 is able to potently inhibit Phase I Cyp activity against NNK, and induce Phase II detoxification (UDP-glucuronosyl transferases and glutathione-S-transferases) expression in A/J mice (Crampsie, et al). In this same study, ISC-4 was also able to
inhibit formation of methyl adduct formation in A/J mouse lung and liver resulting from NNK administration. Based on these results we decided to test ISC-4 and its corresponding sulfur analog PBITC in a lung cancer chemoprevention mouse bioassay.

For this study the A/J mouse model was used. These mice spontaneously develop lung adenomas at an incidence rate of 20-40% after only 20 weeks due to mutations in the Ki-ras oncogene (Witschi 2005), which has shown to be very important in human lung cancers (Okudela, Woo et al.). Upon a single intraperitoneal dose of the TSNA NNK, mice develop tumors at 100% incidence with 8-12 tumors per lung on average (Hecht 2008). NNK is bioactivated by Cyp450 enzymes to ultimate carcinogens via the keto acid and keto alcohol pathway. The unstable intermediates are reactive to DNA causing pyridyloxobutylation and methylation. The O\textsuperscript{6}-methylguanine adduct has been shown to be critical for lung carcinogenesis in these mice (Peterson and Hecht 1991). The adduction of DNA bases by metabolites of NNK is known as initiation. For this study, the chemopreventive agents were given both pre-initiation and post-initiation as part of the diet (Figure 1). Since neither of these compounds have been tested in a chronic feeding model it was important to test the long term effects of the drug on the mice before and after exposure to the carcinogen which would mimic what an actual patient receiving a chemopreventive agent would experience. Ideal chemopreventive agents are those that can be taken orally and have little to no side effects or toxicity, so multiple doses of ISC-4 were tested based on prior intragastric dosing results (Table 1).
Table 1 – A/J Mouse Bioassay Treatment Groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Name</th>
<th>NNK</th>
<th>Diet</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Untreated Control</td>
<td>Untreated</td>
<td>AIN-76</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>NNK Control</td>
<td>10 µmol</td>
<td>AIN-76</td>
<td>30</td>
</tr>
<tr>
<td>3</td>
<td>NNK:PBITC 3 µmol</td>
<td>10 µmol</td>
<td>3 µmol/g PBITC</td>
<td>20</td>
</tr>
<tr>
<td>4</td>
<td>NNK:PBITC 0.38 µmol</td>
<td>10 µmol</td>
<td>0.38 µmol/g PBITC</td>
<td>30</td>
</tr>
<tr>
<td>5</td>
<td>ISC-4 Control</td>
<td>Untreated</td>
<td>0.38 µmol/g ISC4</td>
<td>10</td>
</tr>
<tr>
<td>6</td>
<td>NNK:ISC-4 0.19 µmol</td>
<td>10 µmol</td>
<td>0.19 µmol/g ISC4</td>
<td>30</td>
</tr>
<tr>
<td>7</td>
<td>NNK:ISC-4 0.38 µmol</td>
<td>10 µmol</td>
<td>0.38 µmol/g ISC4</td>
<td>30</td>
</tr>
<tr>
<td>8</td>
<td>NNK:ISC-4 0.57 µmol</td>
<td>10 µmol</td>
<td>0.57 µmol/g ISC4</td>
<td>30</td>
</tr>
</tbody>
</table>

**Figure 1** – A/J mouse bioassay rationale and design. Mice were started on control or experimental diets for two weeks before NNK/saline injection. Mice were then continued on same diet for the remaining 22 weeks. This is a complete chemoprevention model.
4.2 Materials and Methods

4.2.1 Chemicals and reagents

PBITC was purchased from LKT Laboratories (St Paul, MN). ISC-4 was synthesized using a method developed in our lab (Sharma, Sharma et al. 2008). NNK was synthesized by our lab following a previously reported method (Hecht, Lin et al. 1983). All ingredients for AIN-76A control diet and experimental diets were purchased from Dyets Inc. (Bethlehem, PA).

4.2.2 Diet preparation and analysis

AIN-76A control diet or experimental diets were prepared with 5% corn oil or 5% corn oil mixed with ISC-4 or PBITC. Diet was prepared fresh every two weeks and stored at 4 °C. For ISC-4 diets, corn oil was mixed with 0.113, 0.226, or 0.339 g ISC-4 per 125 ml corn oil for the 0.19, 0.38, and 0.57 µmol/g diets, respectively. For PBITC diets, corn oil was mixed with 0.18 g or 1.43 g PBITC per 125 ml corn oil for the 0.38 µmol/g and 3 µmol/g diets, respectively. Food cups were weighed when filled and then weighed again after three days to assess animal intake of each diet.

4.2.3 A/J mouse bioassay

Animal experimentation was carried out according to protocols approved by the Institutional Animal Care and Use Committee (IACUC) at Penn State University. A/J mice were purchased at six weeks of age from Jackson Laboratories (Bar Harbor, ME). After quarantine for
a week mice were stratified into one of eight groups to give equal weight distribution between groups and then weighed once a week thereafter. Mice were started on control or experimental diet for two weeks. Mice were then given an intraperitoneal dose of saline (vehicle control) or 10 μmol NNK and continued on diets for another 22 weeks before sacrifice. Mice were sacrificed by either cervical dislocation (to avoid acidosis caused by carbon dioxide) or CO₂ asphyxiation when blood samples were needed. Blood was taken from six animals in each group in order to perform serum analysis. Lungs were taken out with the heart and tumors were immediately counted. Liver, kidney, colon, and pancreas were collected and portions taken for histology by placing slices in cassettes and fixing in 10% formalin. The remainders of the organs were snap frozen on dry ice and stored at -80 °C.

4.2.4 Lung tumor analysis

Whole lungs still attached to the trachea and heart were removed from each mouse. Lungs were rinsed with saline twice and placed under a dissecting microscope. Visible tumors on each lobe were counted and size recorded. Lungs were then portioned for histological analysis or snap frozen and processed as above.

4.2.5 Blood serum analysis

Blood samples were collected immediately by cardiac puncture from mice in 2 ml serum tubes (n=6 per group). Tubes were kept at room temperature and spun at 14000 rpm for 10 min. Serum was collected and then frozen at -20 °C until analysis.
4.2.6 Lung, liver, and kidney histology

Left lobe of lung, and slices of liver and kidney were placed in cassettes and fixed in 10% formalin for 48 h and then transferred to 70% ethanol. Samples from each group were paraffin embedded and slides were prepared. Tissue slices were stained with H&E stain and analyzed by a comparative medicine pathologist. Lung slides were analyzed for neoplasms. Liver and kidney slides were analyzed for histological abnormalities.

4.2.7 Statistics and analysis

For statistical analysis one way ANOVA and student’s t-test were employed using Graphpad 4.0 software, using an alpha of 0.05.
4.3 Results

4.3.1 ISC-4 and PBITC inhibit NNK induced lung tumorigenesis in A/J mice

The A/J mouse animal bioassay (Table 1) was designed to test the chemopreventive efficacy of ISC-4, a selenium compound developed in our lab which has been shown to inhibit Phase I Cyp activity, induce Phase II gene expression, and inhibit DNA adduct formation in mice. The experimental design (Figure 1) for the experiment was to administer the compound both pre initiation and post initiation for a total of 24 weeks. PBITC, the sulfur analog of ISC-4 was also used to compare efficacies of sulfur and selenium.

The results from the 24 week experiment show that both ISC-4 and PBITC are very effective at reducing both NNK induced tumor multiplicity and tumor incidence (Table 2). Untreated mice fed control diet had a basal incidence rate of 50% and a tumor multiplicity of 0.5 tumors per lung, while the positive control mice treated with NNK and fed control diet had tumors at 100% incidence and a tumor multiplicity of 13.9 tumors per lung. Mice treated with NNK and fed with experimental diet containing ISC-4 at 0.19 µmol/g diet, 0.38 µmol/g diet, and 0.57 µmol/g diet had tumor multiplicity reduced by 70%, 86%, and 85%, respectively. Mice fed with experimental diet containing PBITC at 0.38 µmol/g diet and 3 µmol/g diet had tumor multiplicity reduced by 80% and 94%, respectively.
Table 2 - Tumor Incidence and Multiplicity

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Incidence</th>
<th>Tumors/Lung</th>
<th>p &lt; 0.05*</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated: Control Diet</td>
<td>10</td>
<td>50</td>
<td>0.50 ± 0.53</td>
<td>2, 4, 6, 7, 8</td>
<td>na</td>
</tr>
<tr>
<td>NNK: Control Diet</td>
<td>30</td>
<td>100</td>
<td>13.93 ± 4.52</td>
<td>p &lt; 0.0001 all</td>
<td>na</td>
</tr>
<tr>
<td>NNK: PBTC 3 umol/g diet</td>
<td>20</td>
<td>55</td>
<td>0.80 ± 0.89</td>
<td>2, 4, 6, 7, 8</td>
<td>94</td>
</tr>
<tr>
<td>NNK: PBTC 0.38 umol/g diet</td>
<td>30</td>
<td>86.7</td>
<td>2.83 ± 2.46</td>
<td>1, 2, 3, 5, 6, 7</td>
<td>80</td>
</tr>
<tr>
<td>Untreated: ISC4 0.38 umol/g diet</td>
<td>10</td>
<td>60</td>
<td>0.90 ± 0.92</td>
<td>2, 4, 6, 7, 8</td>
<td>na</td>
</tr>
<tr>
<td>NNK: ISC4 0.19 umol/g diet</td>
<td>28</td>
<td>100</td>
<td>4.14 ± 1.78</td>
<td>all</td>
<td>70</td>
</tr>
<tr>
<td>NNK: ISC4 0.38 umol/g diet</td>
<td>29</td>
<td>75.9</td>
<td>1.96 ± 1.64</td>
<td>1, 2, 3, 4, 5, 6</td>
<td>86</td>
</tr>
<tr>
<td>NNK: ISC4 0.56 umol/g diet</td>
<td>30</td>
<td>76.7</td>
<td>2.06 ± 1.65</td>
<td>1, 2, 3, 5, 6</td>
<td>85</td>
</tr>
</tbody>
</table>

*Student's t-test

Table 3 - H&E staining of left lung lobes

<table>
<thead>
<tr>
<th>Group</th>
<th>% Adenoma</th>
<th>% Hyperplasia</th>
<th>% Adenocarcinoma</th>
<th>Lesions/lobe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated Control</td>
<td>100.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.2</td>
</tr>
<tr>
<td>NNK Control</td>
<td>96.6</td>
<td>3.4</td>
<td>0.0</td>
<td>2.9</td>
</tr>
<tr>
<td>NNK PBTC 3 umol/g</td>
<td>35.7</td>
<td>57.1</td>
<td>7.1</td>
<td>1.4</td>
</tr>
<tr>
<td>NNK PBTC 0.38 umol/g</td>
<td>69.6</td>
<td>30.4</td>
<td>0.0</td>
<td>2.6</td>
</tr>
<tr>
<td>Untreated ISC-4</td>
<td>33.3</td>
<td>66.7</td>
<td>0.0</td>
<td>1.0</td>
</tr>
<tr>
<td>NNK ISC-4 0.19 umol/g</td>
<td>65.2</td>
<td>26.1</td>
<td>8.7</td>
<td>2.3</td>
</tr>
<tr>
<td>NNK ISC-4 0.38 umol/g</td>
<td>41.2</td>
<td>52.9</td>
<td>5.9</td>
<td>1.7</td>
</tr>
<tr>
<td>NNK ISC-4 0.57 umol/g</td>
<td>45.5</td>
<td>54.5</td>
<td>0.0</td>
<td>2.2</td>
</tr>
</tbody>
</table>
4.3.2 *A/J* mice fed experimental diets have reduced formation of adenomas

H&E sections of left lobes of lungs from each group (n=5-10) were analyzed for neoplastic lesions. Lesions were classified as hyperplasia, seen in alveolar and/or airway cells, adenomas, both solid and papillary, or adenocarcinoma. The percentage of each lesion type is presented in Table 3. The NNK Control group had mostly adenomas, whereas the NNK treated groups fed experimental diets had reduced percentages of adenomas.

4.3.3 Food intake and weight gain of mice

Mice were weighed once a week throughout the bioassay. As seen in Figure 2A, mice treated with experimental diets did not gain weight or even lost weight in the first two weeks before NNK treatment, which may have been due to less eating due to the taste of the selenium and sulfur compounds. Therefore, the weights of the food cups were periodically checked to determine differences in eating patterns across groups. Since NNK treatment may result in a change in eating patterns, food cup weights were not measured until two weeks after NNK administration. At each weighing, diet intake did not differ significantly across groups (Figure 2B), with a one way ANOVA p = 0.75. After this point, all groups gained weight consistently. From the point of NNK treatment, the untreated control diet group and NNK control group gained the most weight, 6.3 g and 5.9 g, respectively whereas the high dose PBTC and ISC-4 diet groups only gained 4 g and 4.6 g, respectively. The rest of the groups had a weight gain of between 5-5.3 g.
Figure 2 – Body weight and food intake of A/J mice. A) Body weights of mice weighed once a week over 24 weeks of the bioassay (mean ± SD). B) Food intake per mouse based on weight of food cup, number of mice in cage, and days between weighing, (mean ± SD, one way ANOVA p = 0.75.)
4.3.4 Serum analysis of toxicity markers

Serum samples from each group (n=6 per group) were submitted to comparative medicine to test for markers of toxicity. Liver function was examined by analyzing the levels of two enzymes, alkaline phosphatase and serum glutamic pyruvic transaminase (SGPT). Kidney function was assessed by analyzing the levels of serum urea nitrogen, and creatinine. Other tissue function and toxicity were measured by analyzing the serum levels of the enzyme serum glutamic oxaloacetic transaminase (SGOT). Elevation of any of these markers indicates possible injury to tissues. The results from the serum tests indicate blood values were in normal range for all of the treatment groups except for the high dose of ISC-4 group (0.57 µmol/g diet). In this group, average creatinine levels were slightly below normal levels for mice (0.15 mg/dl), whereas the alkaline phosphatase (100.2 U/l) and SGPT (91.7 U/l) were slightly elevated above the normal range of values for mice (Table 4). This indicates that the dose of 0.57 µmol/g diet for ISC-4 may be approaching toxicity in a chronic feeding model, and doses higher than this would be ill advised.
<table>
<thead>
<tr>
<th></th>
<th>Creatinine (mg/dl)</th>
<th>Urea Nitrogen (mg/dl)</th>
<th>Alkaline Phosphatase (U/l)</th>
<th>SGOT (U/l)</th>
<th>SGPT (U/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated: Control Diet</td>
<td>0.27 ± 0.08</td>
<td>15.0 ± 4.5</td>
<td>71.5 ± 6.2</td>
<td>175 ± 48</td>
<td>72.7 ± 29.6</td>
</tr>
<tr>
<td>NNK: Control Diet</td>
<td>0.22 ± 0.11</td>
<td>17.0 ± 2.5</td>
<td>76.0 ± 16.6</td>
<td>205 ± 60</td>
<td>57.3 ± 12.2</td>
</tr>
<tr>
<td>NNK: PB 3 umol/g diet</td>
<td>0.22 ± 0.04</td>
<td>25.8 ± 8.0</td>
<td>85.8 ± 8.7</td>
<td>196 ± 51</td>
<td>64.8 ± 29.1</td>
</tr>
<tr>
<td>NNK: PB 0.38 umol/g diet</td>
<td>0.23 ± 0.05</td>
<td>18.3 ± 2.0</td>
<td>65.3 ± 22.2</td>
<td>201 ± 48</td>
<td>64.5 ± 23.5</td>
</tr>
<tr>
<td>Untreated: ISC 0.38 umol/g diet</td>
<td>0.2 ± 0.07</td>
<td>20.3 ± 5.5</td>
<td>77.5 ± 24.0</td>
<td>246 ± 109</td>
<td>76.3 ± 50.3</td>
</tr>
<tr>
<td>NNK: ISC 0.19 umol/g diet</td>
<td>0.22 ± 0.04</td>
<td>17.7 ± 3.8</td>
<td>83.8 ± 12.3</td>
<td>222 ± 176</td>
<td>55 ± 21.3</td>
</tr>
<tr>
<td>NNK: ISC 0.38 umol/g diet</td>
<td>0.18 ± 0.05</td>
<td>17.2 ± 2.8</td>
<td>87.8 ± 22.0</td>
<td>209 ± 69</td>
<td>62.2 ± 11.6</td>
</tr>
<tr>
<td>NNK: ISC 0.56 umol/g diet</td>
<td>0.15 ± 0.06</td>
<td>18.5 ± 4.2</td>
<td>100.2 ± 14.8</td>
<td>259 ± 154</td>
<td>91.7 ± 45.1</td>
</tr>
<tr>
<td>Normal Value Range</td>
<td>0.16-0.90</td>
<td>8 to 33</td>
<td>35-96</td>
<td>54-298</td>
<td>17-77</td>
</tr>
</tbody>
</table>
4.3.5 Liver and kidney histology

H&E sections of liver and kidney were analyzed for signs of toxicity (n = 6 per group). Each slide was scored with a number ranging from 0-4. Scores were as follows 0 - normal, 1 – minimal, 2 – mild, 3 – moderate, 4 – severe. Kidneys were scored based on proximal convoluted tubule (PCT) epithelial degeneration/necrosis, nephron degeneration/necrosis, interstitial nephritis, and tubulitis (Table 5). Livers were scored based on hepatitis, vacuolation, fibrosis, and hyperplasia (Table 6). No signs of toxicity were seen in the kidneys except in the high dose group of PBITC where a few of the samples exhibited minimal interstitial nephritis and nephron epithelial degeneration. In liver, all samples showed signs of hepatitis, ranging from minimal to mild. The NNK Control group had the highest degree of hepatitis (mild) and also exhibited minimal biliary hyperplasia. These results indicate that neither ISC-4 nor PBITC are adversely affecting liver or kidney at the doses tested. However, due to the results in the kidney, it may be prudent to say PBITC is approaching a maximal tolerated dose at 3 µmol/g diet.
Table 5 – H&E Stain of Kidney Samples from each group

<table>
<thead>
<tr>
<th>Kidney</th>
<th>PCT epithelial degeneration-necrosis score</th>
<th>Nephron epithelial degeneration-necrosis score</th>
<th>Tubular Cast score</th>
<th>Interstitial nephritis score</th>
<th>Interstitial nephritis type</th>
<th>Tubulitis score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Group 2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Group 3</td>
<td>0</td>
<td>0.5</td>
<td>0</td>
<td>0.67</td>
<td>Chronic</td>
<td>0</td>
</tr>
<tr>
<td>Group 4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Group 5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Group 6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Group 7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Group 8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 6 – H&E Stain of Liver Samples from each group

<table>
<thead>
<tr>
<th>Liver</th>
<th>Hepatitis score</th>
<th>Vacuolation score</th>
<th>Fibrosis score</th>
<th>Biliary hyperplasia score</th>
<th>Oval cell hyperplasia score</th>
<th>Foci of cellular alteration</th>
<th>Nodular regeneration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>0.83</td>
<td>1.50</td>
<td>0.00</td>
<td>0.17</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Group 2</td>
<td>2.00</td>
<td>1.80</td>
<td>0.00</td>
<td>0.80</td>
<td>0.20</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Group 3</td>
<td>1.33</td>
<td>1.33</td>
<td>0.17</td>
<td>0.33</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Group 4</td>
<td>1.50</td>
<td>1.50</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Group 5</td>
<td>1.00</td>
<td>2.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Group 6</td>
<td>1.33</td>
<td>1.17</td>
<td>0.00</td>
<td>0.17</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Group 7</td>
<td>1.17</td>
<td>0.83</td>
<td>0.00</td>
<td>0.33</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Group 8</td>
<td>0.67</td>
<td>0.67</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>
4.4 Discussion

ISC-4 has been previously shown to inhibit Phase I enzyme bioactivation of NNK and inhibit formation of DNA adduct formation in liver and lung of A/J mice treated with NNK. Based on these observations, a chronic feeding bioassay was developed to determine if ISC-4 would be effective as a chemopreventive agent. We predicted ISC-4 to reduce tumor incidence and multiplicity more potently than PBITC, the sulfur analog. Dosing was established based on previous experiments in our lab. We previously determined that a single 2.5 µmol intragastric dose is toxic to 50% of mice. Three doses of ISC-4 were chosen for several reasons. First we wanted to establish if there was a dose response relationship of ISC-4 in reducing lung tumor multiplicity. Second, mice eat a variable amount of food, 2-3 g per day so testing multiple doses allowed us to test a larger range of doses and effects. Third, we could assess higher doses for toxicity and this would give a better view of appropriate dosing for ISC-4. The lowest dose of ISC-4 was mixed at 0.19 µmol/g diet of AIN-76 control diet. Mice could be expected to receive anywhere between 0.4 and 0.6 µmol of ISC-4 per day if they ate as expected. The second dose was mixed at 0.38 µmol/g diet giving a dose between 0.8 and 1.2 µmol ISC-4 per day. The highest dose tested was mixed at 0.57 µmol/g diet giving a dose between 1.14 and 1.71 µmol ISC-4 per day. To compare ISC-4 and PBITC, PBITC was tested at a dose that was comparable to ISC-4, thus we chose the middle dose. Previous experiments investigating PBITC have only been in a pre-initiation model, but have been given at 5 µmol per day intragastrically for five days. Since PBITC is well tolerated at high doses we decided to use a high dose as well since we expected ISC-4 to perform as well as PBITC, but at a lower dose. We decided to use 3 µmol/g diet since our study was a chronic feeding study to avoid toxicity and to compare against the doses of ISC-4 being tested.
Our results show that both ISC-4 and PBITC were able to reduce tumor incidence and multiplicity significantly (70-94%). The reduction in tumorigenesis for ISC-4 appears to be dose dependent for the two lower doses, but not for the highest dose. Mice receiving the highest dose of ISC-4 gained less weight than the rest of the groups and also had serum values of toxicity markers that were out of normal range. Therefore, we conclude that this dose of ISC-4 may be inducing toxic effects. Based on food intake analysis, there was no significant differences in food cup weights across any of the groups, however, the food cups were not weighed the first two weeks of the study where the average weight actually decreased for Group 8 indicating that the mice either did not like the taste of the diet and therefore ate less of it, or that the diet was making them ill and they were not eating. The former is more likely, because after a few weeks the mice gained weight steadily, possibly once they got used to the taste of the diet, however their weight never caught up to the control group. The other possibility is that mice were taking food out of the food cup and burying it rather than eating it, which would also account for a reduction in the food cup weight without a concurrent weight increase in the mice. The tumorigenesis results support the conclusion that the mice being fed the highest dose of ISC-4 diet were not consuming as much food since the inhibition from the 0.38 µmol/g diet group was not significantly different from the high 0.57 µmol/g diet suggesting mice in both groups were possibly receiving similar doses of ISC-4, which would be possible if for example mice from Group 7 were consuming 3 g of food per day and mice from Group 8 were consuming only 2 g of food per day.

When comparing ISC-4 to PBITC at the same dose of 0.38 µmol/g diet, ISC-4 reduced tumor multiplicity, 1.96 vs. 2.83, and tumor incidence, 87% vs. 76%, by a greater amount, however the effects were not statistically significant (p = 0.12). PBITC at the dose of 3 µmol/g
diet was able to inhibit lung tumorigenesis by 94% and reduce the tumor incidence to 55%, very similar to the untreated control group. In fact, one way ANOVA analysis found no significant difference between Group 1 and Group 3 in tumor multiplicity.

Both ISC-4 and PBTC are known to potently inhibit Phase I cytochrome P450 enzymes, and the reduction in tumor multiplicity and incidence can be attributed to this effect. However, the histological analysis of the left lung lobes also points to anti-progression mechanisms for both compounds since in the NNK Control group the lesions found were mostly all adenomas, suggesting at the time the mice were sacrificed, most of the lesions would have progressed into adenomas left untreated. Mice fed experimental diets all exhibited greater levels of hyperplasia as compared to the NNK Control group, suggesting lesions were progressing more slowly.

In conclusion, ISC-4 and PBTC both performed remarkably well at inhibiting NNK induced lung tumorigenesis. Both are suitable as chemopreventive agents, however PBTC is a more suitable chemopreventive agent than ISC-4 due to its higher therapeutic index, a key requirement with any chemopreventive agent. This study warrants further study of PBTC as a chemopreventive agent against lung cancer. ISC-4 can also be pursued as a chemopreventive agent, but it may be more appropriate to focus on the anti-progression properties of this compound and to investigate this compound more as a chemotherapeutic agent, where toxicity is of less concern.
Chapter 5

The potential therapeutic value of ISC-4

Melissa A. Crampsie
5.1 Development and investigation of ISC-4 as an anti-cancer drug

Of the ISC compounds developed in our lab, ISC-4 emerged as the most effective, but more importantly, had the best “drug-like” profile. Drug likeness according to Lipinski’s rule of five includes having a favorable log P value (<5), an acceptable amount of hydrogen bond donors and acceptors, a molecular weight under 500, and rotatable bonds less than 10 (Lipinski, Lombardo et al. 2001). In addition to satisfying these conditions, ISC-4 is a relatively stable compound especially compared to ISC-1 and ISC-2, and also has essentially no distinctive odor. Of the ISC panel, it was therefore deemed as the most suited compound for further testing and development.

Initial screening studies with ISC-4 (along with the other ITCs and ISCs in the panel) led to several conclusions. First, that ISC compounds in general were outperforming their sulfur analogs, in a variety of cancer cell viability assays (Sharma, Sharma et al. 2008). Others have also reported the trend that the replacement of sulfur with selenium in compounds where this substitution is possible increases the effectiveness or activity of the compound (Emmert, Desai et al.; Madhunapantula, Desai et al. 2008). Second, ISC-4 was able to inhibit mouse and rat liver microsomal metabolism of the tobacco procarcinogen NNK to reactive metabolites, suggesting an inhibition of CYP enzymes, similar to the phenylalkyl ITC compounds from which they were derived. This observation also led me to focus my research on lung cancer, which only has a 16% survival rate and where 90% of the cases are a result of smoking tobacco.

The first part of this study as described in Chapter 2 aimed at performing a comprehensive structure activity analysis of the ITC and ISC panel developed in our lab. From this study we were able to conclude that both phenyl alkyl ITCs and ISCs were able to react substantially with -SH groups on both free small molecular weight thiols (i.e. glutathione) as
well as -SH groups found in proteins, preferentially over other H bonded groups such O-H and N-H₂. The original hypothesis that substitution of sulfur with selenium would result in a decrease of thiol reactivity when comparing the isoselenocyanate and the isothiocyanate proved to be incorrect. However, it was immediately apparent that the pattern of thiol reactivity was retained with chain length when comparing the sulfur panel to the selenium panel. The structure activity experimentation led to the following general conclusions:

- Isoselenocyanate compounds react more quickly to thiols than the corresponding isothiocyanate compounds. Chemically, this may be due the C=Se bond being a weaker bond than the C=S bond.
- Replacement of sulfur with selenium has the effect of decreasing the logP value of the molecule which may affect specific cellular targets of the sulfur compounds compared to the selenium compounds.
- Alkyl chain length between the phenyl ring and the isothio/seleno cyanate group affects thiol reactivity in the same manner, that is increasing chain length decreases not only the rate of reactivity with thiols, but also the equilibrium concentration of the thiol-drug conjugate for both sulfur and selenium compounds.
- Replacement of sulfur with selenium has a clear affect on protein targets in the cell. It was shown that while both ITC and ISC compounds were able to bind to cysteine residues in tubulin, that only ITCs were able to induce tubulin degradation and G2/M cell cycle arrest.
- While both ITCs and ISCs compounds were shown to be able to redox cycle, a novel discovery, ISCs were far better able to redox cycle compared to the
corresponding ITCs and were shown to induce higher levels of ROS in cancer cells. This may be a reason for the differences seen in protein targets and activities in other assays.

- Identification of specific protein targets of these compounds is key to development of these drugs as anti-cancer agents

Because ISC-4 was chosen for further development, it is important to identify targets in the cell, especially at the protein level and further research these pathways. Because we initially discovered that ISC-4 was very potent at inhibiting microsomal Cyp450 enzymes/protein we hypothesized that ISC-4 may have very promising potential as a chemopreventive agent. We found that ISC-4 inhibited microsomal Cyp450 enzyme activity similar to its sulfur analog PBITC, at around 250 nM. Therefore the second aim of my research was to explore the chemopreventive properties of ISC-4. Chapter 3 thoroughly details the process of rigorous testing of ISC-4 as a potential chemopreventive agent. This study was meant to be a proof of concept study to determine the feasibility of ISC-4 as a chemopreventive agent, because an actual chemopreventive bioassay is time consuming and requires a significant amount of animals and resources. The general conclusions from this research were very promising and as follows:

- A suitable effective and safe intragastric dose of 5 ppm Se as ISC-4 was established in A/J mice. An oral dose at this level was able to increase tissue and serum selenium levels in a time dependent manner suggesting to us that ISC-4 (or a selenium metabolite of ISC-4) was being absorbed from the gut and into the blood stream thus providing evidence of oral bioavailability, a necessity for a chemopreventive agent
• Selenium levels in tissues correlated with a change in microsomal Cyp activity in both liver and lung. ISC-4 administered orally to A/J mice resulted in a time dependent inhibition of microsomal liver and lung bioactivation of NNK that correlated well with the selenium levels in the tissues. Phase I inhibition is a well recognized mechanism of action for a multitude of chemopreventive agents.

• ISC-4 administration also resulted in a delayed increase in microsomal and cytosolic Phase II detoxification enzymes important in detoxifying carcinogens (Ugts, and Gsts), as well as detoxification of ITCs (Gsts), an important mechanism that has been identified for chemopreventive agents.

• Comprehensive real time quantitative PCR revealed that ISC-4 administration also resulted in transcriptional changes to key Phase I and Phase II enzymes which are most likely contributed to the activity changes seen in the enzymes. This observation points to the possibility that ISC-4 may be affecting the Antioxidant Response Element (ARE) which affects transcription factors like Nrf2 as well as other redox sensitive transcriptional pathways.

• Finally, mice fed with experimental diet containing ISC-4 for two weeks and then treated with NNK had a lower amount of DNA adduct formation in both liver and lung, a result that is strongly suggestive of anti-initiation chemopreventive properties of ISC-4.

From these studies we clearly felt that we had enough evidence to enter into an animal bioassay for chemoprevention. As described in Chapter 4, the bioassay was designed with A/J mice, a model of lung cancer using the tobacco carcinogen NNK. Several doses of ISC-4 were used since we had never established a safe and effective dose in a long term feeding study. We
also decided to include the sulfur analog PBITC, because we hypothesized that ISC-4, at equimolar and possibly even at lower doses, would be more effective at inhibition of tumors. The general conclusions from this experiment are as follows:

- Both ISC-4 and PBITC were able to significantly reduce the lung tumor multiplicity in A/J mice treated with the tobacco carcinogen NNK (anywhere from 70-94% inhibition).

- At equimolar doses (0.38 µmol/g diet), ISC-4 reduced tumor incidence and tumor multiplicity by a greater amount, however the results were not significant (76% vs 87% and 1.96 tumors/lung vs. 2.83 tumors/lung)

- PBITC is tolerated at a much higher dose than ISC-4. At 0.57 µmol /g diet ISC-4 began showing signs of toxicity as evidenced by some of the serum toxicity markers. PBITC can be administered at 3 µmol/g diet chronically with only slight signs of toxicity as evidenced by the kidney histology results. Therefore PBITC has a higher chemopreventive index compared to ISC-4. A high chemopreventive index is highly desirable for any chemopreventive agent.

- At 3 µmol/g diet, PBITC was able to induce nearly complete inhibition of NNK induced lung tumorigenesis. There was no significant difference of this group with the untreated control diet mouse group.

- Besides anti-initiation properties, ISC-4 and PBITC also exhibited anti-progression properties as evidenced by the ratio of adenoma and hyperplasia areas as found by the lung histology results.

The results from the bioassay were very remarkable. The tumor inhibition was very significant for both ISC-4 and PBITC. Before starting the bioassay, we hypothesized that ISC-4
at the doses we tested would outperform even the high dose of PBITC. Our hypothesis was proved incorrect, but we still felt that ISC-4 performed well as a chemopreventive agent and was able to be administered at higher doses (as ppm Se) than other chemopreventive selenium compounds. However, based on our results we feel that because ISC-4 and PBITC performed so similarly, and PBITC has a greater chemopreventive index that it would be prudent to further develop PBITC over ISC-4 in a lung cancer chemoprevention setting. Chemopreventive agents need to be effective but it is imperative that they be safe as well. Even slight toxicity is unacceptable, and PBITC was shown by this study to be effective as well as nontoxic as evidenced by the histology and blood toxicity markers even at high doses. However, it is worth noting that the results warrant both ISC-4 and PBITC testing in other chemoprevention models. The effectiveness of ISC-4 and PBITC may change depending on the carcinogenesis pathway for the given cancer and that the mechanism of action for the carcinogenesis inhibition may not necessarily be identical for these two compounds, especially when considering anti-progression mechanisms that may need further investigation.

Chapters 2, 3, and 4 describe an important sequential and analytical process for further development of ISC-4. That is, identification of a specific target (ideally a protein or pathway) and then further investigation of the upstream or downstream pathways involved. In this particular case, the Cyp450 enzymes were identified as a target of interest which led to a successful investigation of the chemopreventive properties of ISC-4 for lung cancer. Recent research with ITCs has provided evidence that protein reactivity is playing a more important role than small molecular thiol reactivity in the activity of these compounds (Mi and Chung 2008; Mi, Xiao et al. 2008), however, low molecular weight thiol reactivity cannot be discounted as
contributing to the effects of ITCs or ISCs as shown in Chapter 2 with the formation of superoxide with when these compounds are mixed with GSH.

Although ISC-4 may not be as suitable as PBITC as a chemopreventive agent, it still exhibited anti-progression strategies and has been proven effective in cancer cell viability agents which may make it more suitable for development as a therapeutic agent. In fact many studies of the chemotherapeutic properties of ISC-4 are continuously being investigated in our lab and by others (Nguyen, Sharma et al.; Sharma, Kline et al.). Our lab has also begun preliminary studies of the effectiveness of ISC-4 at inhibiting A549 lung adenocarcinoma xenograft tumor growth with promising results (Figure 1). Mice (n=5) were injected on both flanks subcutaneously with about $1 \times 10^6$ A549 cells and then left to grow for 2 weeks. Mice were then treated 3 x weeks with 50 µl 0.76 µmol of ISC-4, PBITC, or vehicle control. Mice treated with ISC-4 had significantly lower tumor volumes than DMSO control or PBITC. A larger study is planned in the future.

**Figure 1** – Nude Mouse Lung Adenocarcinoma Xenograft using A549 cells. Mice were injected with ~5 million cells per side and treated 3x/week IP with ISC-4 or PBITC.
ISC-4 is an example of an isoselenocyanate (R-N=C=Se) and displays activity based on several possibilities: its reactivity with thiol compounds (both free and protein), its redox cycling capabilities, its ability to inhibit PI3K/Akt signaling pathway, and its contribution to the selenium status of an organism. None of these are mutually exclusive, and it could be functioning by all three mechanisms and possibly other unknown mechanisms. Because of its broad activity, it is important to identify therapeutic targets so to appropriately administer the compound. One of the most important ways to achieve this goal is to obtain the complete pharmacokinetic profile of this drug. Our lab is currently in the process of synthesizing the \(^{14}\text{C}\) radioisotope labeled ISC-4 to perform such studies. Swiss Webster mice will be used to perform the pharmacokinetic analyses to determine absorption properties, distribution, metabolism, and excretion (ADME). These studies will be critical to identify organ sites of accumulation, active metabolites, half life, toxicity, maximal plasma concentration, time of maximal plasma concentration, and more which can help target identification and even further compound optimization. Radio labeled ISC-4 will also be useful in cell culture studies where treatment of cells and cellular fractionation studies can determine where accumulation of the drug is occurring and what specific proteins are being targeted and/or being affected.

### 5.2.1 Reactivity with thiols:

The usefulness of radiolabeled ISC-4 in identifying protein and small molecular weight targets is great. In order to identify protein targets of ISC-4, cancer cells or normal cells would need to be treated with \(^{14}\text{C}\) ISC-4 at both a range of doses and across time points. The cells from each treatment would then be collected and homogenized and then run using two
dimensional gel electrophoresis, where the proteins are separated by both isoelectric point as well as molecular weight. The gels can then be imaged by autoradiography. The appearance of bands would indicate proteins that are bound to ISC-4, most likely through the adduction of a cysteine residue (but not necessarily). With this technique, the bands that appear can then be “picked” out of the gel and digested for analysis by mass spectrometry. Our facilities are well equipped for such experiments and it would be a great source of protein identification. For small molecular weight thiols, HPLC would be the choice of analysis. Our lab has a state of the art radio flow detector capable of detecting many isotopes. Portions of the cell homogenates from above could be extracted chemically and then the resulting fractions would be run on a C18 column to look for radiolabeled metabolites, which could be collected and identified by the use of mass spectrometry.

Using the above mentioned methodology, new protein or small molecular weight targets could be identified and further investigated, providing not only further insight to the mechanism of action of ISC-4, but also be a hypothesis generator for more experiments for this compound as an anti-cancer or chemopreventive agent. My focus on lung cancer was purely based on the ability of ISC-4 to inhibit the metabolism of a tobacco specific nitrosamine. Other areas of focus may be targeted by the identification of key thiol/selenol targets in specific carcinogenesis processes for a given cancer.

5.2.2 ROS generation and redox state alteration

Based on the structure activity results, it is clear that ITCs and ISCs preferentially interact with cell thiols (and most likely selenols). The thiol status of a cell, that is the amount of free -SH
groups as a part of the free cytoplasmic thiols as well as protein thiols as cysteine residues is a vital component of a cell’s overall redox status. The redox environment of the cell can be estimated by the redox state of the glutathione disulfide/glutathione couple (GSSG/2GSH for cell signaling or GSSG/GSH for protein glutathionylation) which represents the major redox buffering system of cells and it has been shown that changes in the reduction potential of this redox couple correlate with biological status of a cell (Schafer F.Q. 2001). Rahman and MacNee describe the GSH redox system as one of the most important antioxidant defense systems in mammalian species (1996). Typically cells with a more reducing environment have more free sulfhydryl groups in the form of protein sulfhydryls and GSH and are more prone to proliferate. As oxidative status increases and sulfhydryls are oxidized the cell progresses to differentiation and eventually to apoptosis and necrosis. Redox status has also been shown to vary throughout cell cycle and may even be necessary for cells to progress through the cell cycle with the use of ROS as second messengers.

ROS are able to induce cell signaling pathways similar to growth factors and hormones at low levels (Valko, Leibfritz et al. 2007), levels that would still enable overall cell redox status to be reducing. ROS have been shown to act as second messenger compounds and mediate signal cascades such as lipid and calcium signaling, activate receptor and non-receptor tyrosine kinases such as EGF, PDGF, VEGF, Src and JAK. ROS have also been shown to inactivate protein tyrosine phosphatases through target cysteine residues. ROS also play a role in regulation of serine/threonine kinases such as Akt, PKC, and MAP kinases. Finally, there are several redox sensitive transcription factors that are affected by ROS and cellular redox status including NFkB, AP-1, p53, NFAT, HIF-1 and others (reviewed by (Valko, Leibfritz et al. 2007), (Kamata and Hirata 1999), (Na and Surh 2006), (Huber and Parzefall 2007)). Thus the redox environment in
terms of ROS, RNS, reducing molecules, and antioxidants present in the cell all play an important role in the fate of the cell. In normal cells, equilibrium exists between cell proliferation and cell death whereas in cancer cells there is an imbalance. Oxidative stress in the form of reactive oxygen species (ROS) and reactive nitrogen species (RNS) have been shown to contribute to the carcinogenesis process via genotoxic effects resulting in oxidative DNA adducts (initiation) or through modification of gene expression and cell signaling (promotion and progression) (Klaunig and Kamendulis 2004).

Cancer cells have been found to have significantly affected levels of antioxidant system enzymes and molecules which may be a point of selectivity of cancer cells vs. normal cells for ISC-4, a hypothesis that needs further investigation. A study conducted by Saydam et al. (1997) looking at the differences between normal lung tissue and lung tumor tissue found that levels of several antioxidant enzymes in tumor tissues (n = 38) were significantly higher than those in normal tissues (n = 17). Reduced glutathione levels, but not oxidized glutathione levels, were found to be higher in normal tissues than those in tumor tissues as well (Saydam, Kirb et al. 1997). When ROS species were investigated using A549 adenocarcinoma cells, we found that with no treatment, about 30% of cells tested positive for elevated levels of ROS. If ISC-4 is able to further induce ROS by further depleting the already lessened amounts of GSH as well as by disabling antioxidant enzymes (most of which have a critical cysteine or selenocysteine residue) that combat ROS, the neoplastic cells may be more likely to enter into apoptosis than corresponding normal cells. A complete study of the effects of ISC-4 on reactive oxygen species in a panel of lung cancer cell lines as well as several normal cell lines (including epithelial and fibroblast cells) and the effects of ROS on induction of apoptosis would be prudent to thoroughly understand the merit of this hypothesis.
5.3.3 The contribution of ISC-4 to selenium status

The results from my research found that oral administration of ISC-4 to A/J mouse led to increased levels of selenium in serum and tissue. However, because atomic absorption was used which only is able to recognize the presence of elemental selenium, it remains unclear what form the selenium in the tissues and serum is in. Metabolism of ISC-4 may result in the recycling of selenium in some form to be used elsewhere in the body. If this is the case, ISC-4 will be contributing to overall selenium status of the organism it is given and may be forming other active selenium compounds. Selenium status of an organism helps regulate selenoprotein expression and activity, many of which have important metabolic and antioxidant functions, such as glutathione peroxidase. ISC-4 may also have direct effects on selenoprotein expression and activity as well due to the presence of selenocysteine. Selenoproteins may be contributing to the carcinogenesis process so it is important to relate expression and activity levels to treated and untreated mice and chemopreventive effects seen in these mice.

Based on other studies with ITCs, it is most likely that ISC-4 is at least partially excreted as an N-acetyl conjugate. However, longer chain ITCs were shown to be metabolized differently than shorter chain ITCs (Conaway, Jiao et al. 1999), which may mean other metabolites may exist for ISC-4. Experiments need to be designed to look at selenium status after treatment of ISC-4 on cancer cells or in animals and then investigation of selenoprotein activity and/or expression. If ISC-4 is only having an effect on selenium status of an organism, however, than similar cancer inhibitory results would be obtained by simply treating with a selenium supplement. Therefore, these kinds of experiments would best be performed in conjunction with thiol targeting and redox mechanism experimentation already discussed which are more likely to be important in the mechanism of action of ISC-4.
5.3 Summary and Conclusions

The research and discussion presented in this dissertation represents the investigation of ISC-4, a novel organoselenium compound developed using rational drug design methods, over the past 4 years. The research I have conducted has led to the following conclusions:

- ISC-4 is a more redox active molecule than its parent compounds and has a faster reaction rate to sulfhydryl groups.
- ISC-4 potently inhibits Phase I microsomal Cyp450 enzymes and induces expression of detoxifying Phase II enzymes, which makes it worthwhile compound for study in chemoprevention assays.
- ISC-4 was shown in an animal model to inhibit lung tumor formation when mice were treated with a carcinogen. It is an effective chemopreventive agent for lung cancer, however its relatively narrow chemopreventive window, when compared to its sulfur analog, may limit its feasibility in this setting.
- ISC-4 has been shown to be cytotoxic to cancer cells and induce apoptosis. In the animal bioassay it also showed anti-promotion/progression properties. Therefore ISC-4 may have promise as a chemotherapeutic agent.
APPENDIX

Table 1 - Table of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Meaning</th>
</tr>
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<tbody>
<tr>
<td>4-MU</td>
<td>4-methylumbelliferone</td>
</tr>
<tr>
<td>7-AAD</td>
<td>7-Aminoactinomycin</td>
</tr>
<tr>
<td>7-MG</td>
<td>7-methylguanine</td>
</tr>
<tr>
<td>A549</td>
<td>Lung cancer cell line</td>
</tr>
<tr>
<td>ACS</td>
<td>American Cancer Society</td>
</tr>
<tr>
<td>ACTB</td>
<td>Beta - actin</td>
</tr>
<tr>
<td>AIN</td>
<td>American Institute of Nutrition</td>
</tr>
<tr>
<td>Akt</td>
<td>Protein Kinase B</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance (Statistical)</td>
</tr>
<tr>
<td>ARE</td>
<td>Antioxidant response element</td>
</tr>
<tr>
<td>BCA Assay</td>
<td>Bicinchoninic acid protein assay</td>
</tr>
<tr>
<td>BITC</td>
<td>Benzyl isothiocyanate</td>
</tr>
<tr>
<td>CI</td>
<td>Chemopreventive Index</td>
</tr>
<tr>
<td>CL</td>
<td>Chemiluminescence</td>
</tr>
<tr>
<td>CO2</td>
<td>Carbon dioxide</td>
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<tr>
<td>CYP450</td>
<td>Cytochrome p450 enzymes</td>
</tr>
<tr>
<td>Cys</td>
<td>Cysteine</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTNB</td>
<td>5,5'-Dithio-Bis 2-Nitrobenzoic Acid</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>G1 phase</td>
<td>Gap 1 phase</td>
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<tr>
<td>G2 phase</td>
<td>Gap 2 phase</td>
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<td>G-6-P</td>
<td>Glucose-6-phosphate</td>
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<td>Glu</td>
<td>Glutamic Acid</td>
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<td>Gly</td>
<td>Glycine</td>
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<td>GPx</td>
<td>Gluathione peroxidase</td>
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<td>GSH</td>
<td>Glutathione</td>
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<td>GSSG</td>
<td>Oxidized glutathione</td>
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<td>GST</td>
<td>Glutathione –S-transferase</td>
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<tr>
<td>H&amp;E stain</td>
<td>Hematoxylin and eosin stain</td>
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<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>HPRT</td>
<td>Hypoxanthine-guanine phosphoribosyl transferase</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse radish peroxidase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Meaning</td>
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<tr>
<td>--------------</td>
<td>---------</td>
</tr>
<tr>
<td>IACUC</td>
<td>Institutional Animal Care and Use Committee</td>
</tr>
<tr>
<td>IC50</td>
<td>Inhibitory concentration that induces 50% of cell death</td>
</tr>
<tr>
<td>IG</td>
<td>Intragastric</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal</td>
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<tr>
<td>ISC</td>
<td>Isoselenocyanate (R-N=C=Se)</td>
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<td>ISC-1</td>
<td>Isoselenocyanate-1 or benzyl isoselenocyanate</td>
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<tr>
<td>ISC-2</td>
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<td>ISC-6</td>
<td>Isoselenocyanate-6 or phenyl hexyl isoselenocyanate</td>
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<tr>
<td>M phase</td>
<td>Mitotic phase</td>
</tr>
<tr>
<td>MAP</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MCB</td>
<td>monochlorobimane</td>
</tr>
<tr>
<td>MTD</td>
<td>Maximum tolerated dose</td>
</tr>
<tr>
<td>MTS</td>
<td>3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt</td>
</tr>
<tr>
<td>NAC</td>
<td>N-acetyl cysteine</td>
</tr>
<tr>
<td>NADP+</td>
<td>Nicotinamide adenosine diphosphate (oxidized)</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenosine diphosphate (reduced)</td>
</tr>
<tr>
<td>NAT</td>
<td>N-acetyl transferase</td>
</tr>
<tr>
<td>NCI</td>
<td>National Cancer Institute</td>
</tr>
<tr>
<td>NFkB</td>
<td>Nuclear Factor kappaB transcription factor</td>
</tr>
<tr>
<td>NNAL</td>
<td>4-(methyl nitrosamino)-1-(3-pyridyl)-1-butanol</td>
</tr>
<tr>
<td>NNK</td>
<td>N-nitrosyl ketone, or 4-(methyl nitrosamino)-1-(3-pyridyl)-1-butaneone</td>
</tr>
<tr>
<td>Nrf2</td>
<td>Nuclear factor like 2 transcription factor</td>
</tr>
<tr>
<td>NSCLC</td>
<td>Non small cell lung cancer</td>
</tr>
<tr>
<td>O2-pob dT</td>
<td>O-2 pyridyloxobutyl deoxythymine</td>
</tr>
<tr>
<td>O6-MG</td>
<td>O6-methyl guanine</td>
</tr>
<tr>
<td>O6-pob dG</td>
<td>O-6 pyridyloxobutyl deoxyguanine</td>
</tr>
<tr>
<td>PAH</td>
<td>Polycaromatic hydrocarbon</td>
</tr>
<tr>
<td>PBITC</td>
<td>phenyl butyl isothiocyanate</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCT</td>
<td>Proximal convoluted tubules (kidney)</td>
</tr>
<tr>
<td>PEITC</td>
<td>Phenyl ethyl isothiocyanate</td>
</tr>
<tr>
<td>PHITC</td>
<td>Phenyl hexyl isothiocyanate</td>
</tr>
<tr>
<td>PMS</td>
<td>Phenazine Methosulfate</td>
</tr>
<tr>
<td>pob</td>
<td>pyridyloxobutyl adducts</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Meaning</td>
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<td>--------------</td>
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<tr>
<td>PVDF</td>
<td>polyvinylidene fluoride</td>
</tr>
<tr>
<td>p-XSC</td>
<td>1,4-phenylenebis(methylene)selenocyanate</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>QR</td>
<td>Quinone reductase</td>
</tr>
<tr>
<td>RIPA buffer</td>
<td>RadiolimmunoPrecipitation Assay Buffer</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
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<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
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<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>RPMI media</td>
<td>Roswell Park Memorial Institute Media</td>
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<td>RQ</td>
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<tr>
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<td>Selenium binding protein 2</td>
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<tr>
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<td>SFN</td>
<td>Sulforaphane</td>
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<tr>
<td>SGOT</td>
<td>serum glutamic oxaloacetic transaminase</td>
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<tr>
<td>SGPT</td>
<td>serum glutamic pyruvic transaminase</td>
</tr>
<tr>
<td>-SH</td>
<td>Sulphydryl group</td>
</tr>
<tr>
<td>TBP</td>
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</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
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<tr>
<td>TR</td>
<td>Thioredoxin reductase</td>
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<tr>
<td>TSN</td>
<td>Tobacco specific nitrosamine</td>
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<td>UGT</td>
<td>UDP-glucuronyl transferase</td>
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<tr>
<td>UPLC</td>
<td>Ultra high performance liquid chromatography</td>
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<td>UTR</td>
<td>Untranslated region</td>
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<td>UV</td>
<td>Ultraviolet</td>
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</table>
References:


Curriculum Vitae

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Education:

The Pennsylvania State University College of Medicine, Hershey, Pennsylvania
August 2006-2011; Department of Pharmacology, Ph.D. Program

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Academic Associations and Societies

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Beta Gamma Sigma (2007-Present)
Penn State College of Medicine Graduate Student Association (Officer)

Manuscripts


Crampsie MA, Hossain Sk U, Aliaga C, Amin S, Sharma AK. Phenylbutyl isoselenocyanate (ISC-4) and phenylbutyl isothiocyanate (PBTC) inhibit 4-(methylNitrosamino)-1-(3-pyridyl)-1-butanone (NNK) induced lung tumorigenesis in A/J mice. In preparation for submission to Cancer Research

