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Phenylalkyl isoselenocyanates vs phenylalkyl isothiocyanates: thiol reactivity and its implications

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Abstract

Phenylalkyl isoselenocyanate (ISC) compounds were recently designed in our laboratory by incorporating the anticancer element selenium into a panel of phenylalkyl isothiocyanates (ITCs), known to have anticancer properties. A structural activity investigation was carried out to compare the ISC and ITC panels. Cell viability assay and Annexin V staining for apoptosis showed ISC compounds to be more potent in killing A549 lung adenocarcinoma cells. Both ITCs and ISCs were able to deplete reduced glutathione (GSH) in cells, ISCs more rapidly, but ITCs to a greater extent. ISC compounds had a higher rate of reaction to thiol (-SH) groups as determined by pseudo first order kinetics than the corresponding carbon chain length ITC. The equilibrium concentrations of the GSH and protein thiol conjugates did not differ significantly when comparing sulfur to selenium compounds of the same carbon chain length, and did follow the same trend of displaying decreasing reactivity with increasing carbon chain length for both ITCs and ISCs. Furthermore, only ITCs were able to induce cell cycle arrest, suggesting that protein targets inside the cell may differ for the S and Se panels. Finally, the panels were tested for their ability to redox cycle when reacted with GSH to form superoxide and other reactive oxygen species (ROS). ISC compounds showed a much greater ability to redox cycle than corresponding ITCs, and were able to induce higher levels of ROS in A549 cells. Also, the direct proapoptotic effects of ISCs and ITCs were inhibited by GSH and potentiated by depletion of intracellular GSH by buthionine sulfoximine. In conclusion, our studies suggest that the redox-cycling capabilities of ISCs and thus generation of higher levels of ROS may be contributing to the increased cytotoxicity of ISC compounds in A549 cells, compared to that of the corresponding ITCs.

Keywords

Isoselenocyanates; Isothiocyanates; Thiol reactivity; Chemiluminescence; Redox cycling; ROS

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Conflict of Interest Statement The authors declare that there is no conflict of interest.

1. Introduction

Isothiocyanates (ITCs¹) are naturally occurring compounds derived from glucosinolates found in cruciferous vegetables such as broccoli and cauliflower. It has been found that consumption of cruciferous vegetables is associated with a decreased risk of developing several types of cancer including lung, stomach, colon, and breast cancer in humans [1]. Naturally occurring phenylalkyl ITCs (BITC, PEITC) and longer carbon chain synthetic phenylalkyl ITCs (PBITC, PHITC) have been shown to inhibit tumorigenesis induced by the tobacco specific nitrosamine procarcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in mice and rats due to the direct inhibition of bioactivating cytochrome P450 (CYP) enzymes [2–7]. Furthermore, these studies show that the carbon chain length plays a vital role in ITCs ability to inhibit tumorigenesis, specifically, that inhibition increases with increasing chain length up to eight carbons. It was originally discovered that increasing the carbon chain length of the ITC reduces the reactivity of the isothiocyanate group to thiol (-SH) groups [8]. This is important because it has been shown that ITCs are detoxified in cells by conjugation to glutathione (GSH), both spontaneously and enzymatically by glutathione-S-transferase (GST) isoforms to form dithiocarbamates and the detoxified *N*-acetyl cysteine (NAC) conjugates [9–12]. The increase in activity seen with increasing carbon chain length was therefore attributed to a decrease in the ability to be conjugated and excreted from the cell, thus resulting in a longer half-life in cells. ITCs, which are detoxified by GST isoforms, are also able to induce the expression of these enzymes along with other Phase II detoxification enzymes via the Nrf2-antioxidant response element (ARE) pathway [12–14]. Arylalkyl ITCs also cause induction of apoptosis of preneoplastic and neoplastic cells [15–18]. 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 thiocarbamylation of proteins such as tubulin [18,19], and generate ROS [20]. Therefore, both the thiol conjugation and ROS generation, may play important role in their chemopreventive and/or chemotherapeutic action.

Our laboratory has modified both the naturally occurring and synthetic phenylalkyl ITCs (R-N=C=S) by isosterically replacing sulfur (S) with selenium (Se) to make corresponding isoselenocyanates (ISCs; R-N=C=Se): ISC-1, ISC-2, ISC-4, and ISC-6 [21] (Table 1). The rationale behind this replacement was: (i) enhancement of the half-life of the compound by reducing the reactivity towards nonspecific thiols (i.e. non-target proteins) and (ii) addition of redox cycling and anti-cancer properties of selenium. Selenium, in the same column of the periodic table as sulfur, is a larger atom with more labile electrons. Selenium deficiency has been associated with an increase in cancer risk, and supplementation with certain selenium compounds has been shown to be chemopreventive [22–26]. The replacement of S in ITCs by Se was an attempt to add properties of selenium into the electrophilic isothiocyanate (-N=C=S) group to generate isoselenocyanates with a similar electrophilic (-N=C=Se) group, thus resulting in a panel of more potent compounds with similar reactivity pattern towards GSH. We have found these newly developed ISC compounds more potent in inducing cell death in the *in vitro* cancer cell viability assays and to inhibit tumor growth in several xenograft mouse models [21,27–29]. Furthermore, we have shown that ISC-4 transcriptionally induces Phase II enzymes, including GST and UGT isoforms, in mice [30]. In the present study, a structure-activity investigation of ISCs and ITCs in terms of thiol

¹**Abbreviations:** ITCs, isothiocyanates; ISCs, isoselenocyanates; BITC, benzyl isothiocyanate; PEITC, phenethyl isothiocyanate; PBITC, phenylbutyl isothiocyanate; PHITC, phenylhexyl isothiocyanate; ISC-1, benzyl isoselenocyanate; ISC-2, phenethyl isoselenocyanate; ISC-4, phenylbutyl isoselenocyanate; ISC-6, phenylhexyl isoselenocyanate; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; GSH, glutathione; GST, glutathione-S-transferase; NAC, *N*-acetyl cysteine; ROS, reactive oxygen species; -SH, thiol; -SeH, selenol; CL, chemiluminescence; UGT, UDP-Glucuronosyltransferase; 7-AAD, 7-Aminoactinomycin D; PSSG, glutathione.

reactivity was carried out to determine a possible mechanistic reason for the increased potency of ISCs.

2. Materials and Methods

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). GSH-Glo assay kit (V6911), 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). The LIVE/DEAD viability/cytotoxicity kit for mammalian cells (L-1224) was procured from Life Technologies (Grand Island, NY). The total ROS/Superoxide detection kit (ENZ-51010) was purchased from Enzo Scientific (Farmingdale, NY).

2.2. Cell culture

Human lung adenocarcinoma A549 cells were cultured in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA). Cells were incubated at 37 °C and 5% CO₂, and passed every two to four days. The cells were routinely screened for mycoplasma using Hoechst 33258 staining.

2.3. Cell lysate preparations

A549 cells were treated with compounds for 24 h in 100 mm plates. The whole-cell extracts were prepared by subjecting ITC- or ISC- treated cells to lysis in ice-cold RIPA buffer supplemented with protease and phosphatase inhibitor cocktails. Lysates were spun at 22,000 *g* for 10 min to remove insoluble material. Supernatant was collected and stored at -80° C until analysis.

2.4. Measurements of “pseudo first order” kinetics

The 10 mM stock solutions of each ITC and ISC compound were prepared in acetonitrile. A GSH solution (10 mM) was freshly prepared by dissolving 30.7 mg of GSH in a 10 ml mixture of 0.2 M phosphate buffer (pH 7.4) and methanol (1:1). Ten μ l of ITC or ISC solution was added to 1 ml of the GSH solution at room temperature and the product formation was monitored by UV absorption using 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 and the equation $y = y_{min} + \text{span} * (1 - \exp(-k * x))$ to determine rate of non-enzymatic conjugate formation. Each drug was tested in triplicate to determine rates, and represented as mean $\pm$ SD.

2.5. GSH-Glo glutathione assays

A549 cells were plated in white 96-well plates at 10,000 cells per well and cultured for 24 h. A 10 mM stock solution of each ITC or ISC compound was prepared in DMSO. Cells were treated with a final concentration of 10, 15, or 20 μ M compounds in quadruplicate for various time periods. The intracellular level of glutathione was measured by using the GSH-Glo assay kit (Promega, San Luis Obispo, CA) as per the manufacturer's instructions.

2.6. Cell viability assays

Cell viability was measure by the MTS assay. A549 cells were plated in clear 96-well plates at 5000 cells per 100 μ l in 10% FBS supplemented media per well and cultured for 24 h. Cells were then treated with DMSO or a solution of compound in DMSO at final

concentrations ranging from 500 nM to 100 μ M for 48 h. Each compound was dissolved in either 100 μ l of serum-free media or 100 μ l media supplemented with 10% FBS and added to the cells to give a final volume of 200 μ l in the wells. Plates were then assayed by adding 20 μ l 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 using a Spectramax plate reader. Each concentration was done in replicates of n = 6 and the entire assays were repeated three times. IC₅₀ values were calculated using a Graphpad 4.0 nonlinear regression curve fitting.

2.7. Measurement of free thiols by 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 50 μ M to 1 mM. Ten μ l of either GSH or FBS was added to 1 μ l of each compound dissolved in DMSO and incubated at room temperature for 30 min in the dark. Reactions were analyzed using drug: thiol ratio of 1:1, 0.5:1, or 0.25:1. To perform Ellman assay in cell lysate, 10 μ l of lysates were used; 90 μ l of Ellman's reagent was added to each sample and incubated at room temperature for 15 min. The wells were then read at 412 nm absorbance using a Spectramax Plate Reader. All reactions were measured in triplicate or quadruplicate. Free thiol concentrations were determined by the standard curve and then normalized to the untreated control.

2.8. Chemiluminescence (CL) assay

Five hundred μ l of the cocktail solution of 0.05 M sodium phosphate, lucigenin (20 μ g/ml), and glutathione (1 mg/ml, pH 7.4) was added to a 10 $\times$ 50 mm polypropylene tube used for the CL assay. DMSO (100 μ l) was added to 500 μ l of the cocktail for a final cocktail volume of 600 μ l. A 10 mM stock solution of each of the ITC or ISC compounds was made in DMSO. A final 10 μ M of each compound was added to initiate the CL reaction. CL, using lucigenin as the detector of superoxide (O₂⁻), was counted for 5 min at room temperature in repetitive integrated 20 s increments using a chemiluminometer (Turner Biosystems Luminometer Model 1.2, 20/20N, Turner Biosystems, Sunnyvale, CA, USA). Samples were assayed in duplicates and averaged while the control assay of 150 μ l DMSO was performed in triplicate. CL from the control assay was subtracted from the sample assay to obtain net CL counts from the ITC and ISC compounds over 5 min.

2.9. Measurement of ROS and superoxide level

The ROS and superoxide levels were measured using a commercial kit according to the manufacturer's instructions (Total ROS/Superoxide Detection Kit; Enzo Life Sciences). In brief, 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 incubation times. After incubation, cells were stained with 500 μ l of ROS/Superoxide Detection Mix for 30 minutes at 37°C, and analyzed by using flow cytometry.

2.10. Cell cycle analysis by flow cytometry

A549 cells were plated in 12-well plates at 100,000 cells per well and treated with 10 mM stock solutions of drug at varying concentrations and incubation times. Both adherent cells and floating cells were collected following treatment and fixed with ethanol. Following fixation, the cells were stained with propidium iodide and their DNA content measured using a flow cytometer.

2.11. Flow cytometry evaluation of apoptotic cells

A549 cells were plated in 12-well plates at 100,000 cells per well and treated with 10 mM stock solutions of drug dissolved in DMSO at varying concentrations and incubation times. Apoptosis was assessed using PE Annexin V Apoptosis Detection Kit I (BD Biosciences, San Jose, CA) according to the instruction manual provided by manufacturer.

2.12. Viability/cytotoxicity assay

To measure cell viability, we used a Live and Dead viability/cytotoxicity kit (Life Technologies), which determines intracellular esterase activity and plasma membrane integrity [31]. Briefly, A549 cells (25,000 cells per well of 8-chambers slide) were preincubated with various concentrations of either GSH for 2 h, or BSO for 24h, and then either PBITC or ISC-4 (12.5 μ M) was added for 24 h at 37°C [31]. After incubation, cells were stained with the Live/Dead reagent (5 μ M ethidium homodimer and 5 μ M calcein-AM) and incubated at 37 °C for 30 min. Cells were analyzed under a fluorescence microscope (Labophot-2; Nikon).

3. Results

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 that of ITC conjugates at 270 nm. None of the parent compounds had detectable absorbance 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 carbon chain length for both ITC and ISC compounds (Table 1). When ITCs were compared to ISCs, ISC compounds reacted more quickly with GSH than the corresponding ITC compounds for all carbon 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 that the equilibrium concentrations of the GSH conjugate were lower as the carbon chain length increased. However, no significant difference between sulfur and selenium compounds was seen after 30 min incubation. The equilibrium concentrations of the conjugates appeared to be similar for both ITC and corresponding ISC compounds.

3.2. Glutathione depletion in A549 cells by ITC and ISC compounds

To determine if the rate of GSH reactivity *in vitro* corresponded to its depletion in cells, A549 cells were treated with each compound at 15 μ M for up to 2 h. Both GSH levels and the GSH: GSSG ratio in cells was determined (Figure 1B–D) using menadione as a positive control. For all compounds, GSH levels in cells 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 the ISC compounds compared to the ITC compounds, but at 2 h the opposite was seen. These results suggest that initially the GSH is depleted more rapidly with ISC compounds, possibly due to their higher reactivity, but the total depletion over time is not as extensive as with the ITC compounds. For this particular cell line, of the ITCs, PEITC and PBITC were able to deplete GSH to the lowest levels and decreased the GSH: GSSG ratio to the greatest extent (Figure 1C–D). The results show that for both the ITCs and ISCs, rates of reactivity with GSH *in vitro* does not necessarily correlate with the extent of GSH depletion *in vivo*.

3.3. Induction of apoptosis

The panel of compounds was tested for their ability to induce apoptosis in A549 lung adenocarcinoma cells to determine if the 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 μ M compared to the corresponding ITC compounds. MTS assays were used to find the cytotoxicity IC_{50} values of the panels. ISC compounds were found to be more cytotoxic, showing lower IC_{50} values than the corresponding ITCs (Table 2), that are consistent with our previous studies [21,27]. ISC-4 was the most cytotoxic compound of either panel. Interestingly, the IC_{50} 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 a 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 [19]. Based on this observation, we decided to look at protein binding capabilities of ISC compounds.

3.4. Protein modification at -SH groups by ITC and ISC compounds

We first assessed the binding of ITC and ISC compounds to proteins present in FBS. Since a difference in IC_{50} values was seen when drugs are dissolved in serum-free media compared to serum supplemented with FBS prior to treatment of cells, a time-course assay was done to assess binding to serum proteins over time. The -SH concentration of undiluted FBS used in the assay was determined to be 400 μ M using the Ellman assay. Based on this concentration drug was added at a 1:1 ratio, at a final concentration of 400 μ M. Aliquots were taken out every 5 min for the first half hour and then every 30 min for 6 h to determine total binding of the ITC and ISC compounds to protein. Six representative time points are shown in Figure 3A to illustrate the kinetics of protein binding. 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, equilibrium concentrations are similar for sulfur and selenium compounds, although for ISC-6 the conjugate equilibrium concentration appears consistently higher than for corresponding sulfur analog PHITC. As seen in Figure 3A, approximately 5% to 30% of the protein -SH groups are modified as rapidly as in 5 min and these changes persist for at least 6 h. The binding to FBS proteins by the S and Se panels explains why there is a difference seen in cell IC_{50} values depending on whether the compounds are mixed with serum-free or serum-supplemented media prior to treatment of cells. When an ITC or ISC compound is mixed with FBS-supplemented media, the -N=C=X (X = S or Se) functionality will have access to an abundance of protein thiols causing formation of protein conjugates and thus reducing the amount of parent drug available to reach and enter cells. 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 is not only 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.

3.5. Induction of cell cycle arrest

Cell cycle arrest has been implicated in the mechanism of action of ITCs. We tested our panels for their ability to arrest the cell cycle of A549 cells. Figures 4A and 4B show ITCs were capable of inducing G2/M cell cycle arrest, especially the longer chain analogs PBITC and PHITC, and the effect was dose dependent. ISC compounds, however lacked the ability to induce G2/M arrest, even when tested at higher doses (Figure 4B), suggesting that

replacement of sulfur with selenium in ITCs results in differences in protein targets and/or difference in apoptosis induction mechanism.

3.6. Effect of ISC and ITC compounds on redox cycling

Selenium compounds, as selenides, have been shown to redox cycle. To determine if ITC or ISC panel could redox cycle in the presence of -SH, namely GSH, we measured superoxide formation upon incubation with GSH and lucigenin (Figure 5A) by chemiluminescence assay. 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 (PEITC and ISC-2) showed the highest ability of redox cycling suggesting that chain length plays an important role in this mechanism.

3.7. 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 (15 μ M) for 4 h and then analyzed for superoxide and other ROS species (Figure 5B and 5C). ISC compounds caused higher levels of total ROS species as compared to the ITC compounds 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.

3.8 Glutathione inhibits ITC- and ISC-induced cell death

Our results show that cytotoxic potentials of ITCs and ISCs are associated with GSH depletion. We investigated whether GSH can modulate ITCs and ISCs' ability to induce apoptosis. A549 cells were pretreated with various concentrations of GSH for 2 h, after which either PBITC, or ISC-4 was added and cell death was assayed using the Live and Dead assay. As shown in Figure 6A, pretreatment with GSH inhibits the cytotoxic action of ISC-4. Interestingly, treatment with GSH did not rescue the cytotoxic effects of PBITC (Figure 6A, lower panels). Taken together our data suggest that the cytotoxic action of both ITCs and ISCs is associated with thiol interactions.

3.9. Downregulating endogenous GSH increases ITC- and ISC-mediated cell death

Because exogenous addition of GSH inhibited ISC-4-mediated cell death, we determined whether the cytotoxicity of ISC-4 can be enhanced by downregulating endogenous GSH. Endogenous GSH levels were decreased by treating cells with a selective inhibitor of GSH synthesis (BSO) for 24 h (Figure 6B). ISC-4 and PBITC induced cell death, as measured by Live and Dead assay, and BSO treatment increased ISC-4 and PBITC-induced cell death significantly (Figure 6B).

4. Discussion

A new panel of isoselenocyanate compounds developed recently in our laboratory have been found to be more potent in inhibiting cancer cell and tumor growth, and inducing apoptosis as compared to the corresponding isothiocyanates [21,27–29]. These agents have also been shown to inhibit phase I and induce phase II enzymes similar to isothiocyanates [30,32]. The goal of the current study was to evaluate the underlying mechanisms responsible for better efficacy of ISC compounds as compared to the ITC compounds. 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 compounds of the same carbon 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 with GSH, they still tend to be more cytotoxic 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 faster, but at equilibrium, depletion is not as extensive as with the ITC panel (Figure 1). 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 in 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 -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 the carbon chain length appears to increase cancer cell cytotoxicity. This is probably due to the reduced reactivity towards thiols thus enhancing the half-life of the compounds, and also that the increased lipophilicity with increasing alkyl chain length would allow the compound to enter the cells more efficiently. This may also be due to nonspecific binding ability of these compounds to proteins, such as serum albumin, causing the effective concentration of more reactive compounds at target sites to be reduced. Also, as hypothesized with ITC inhibition of CYP450 enzymes, a longer carbon chain length may be contributing for the reactive isothiocyanate or isoselenocyanate group to access a critical cysteine -SH needed for enzyme activity, possibly in a hydrophobic pocket, that the shorter chain compounds cannot access as well. We have seen a similar trend with the ISC panel using mouse liver microsomes. As with the ITC panel, increasing the 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 per se is not predictive of its cytotoxicity. Other studies have shown that reactivity with certain proteins may be more important [18,19]. However, because of the broad reactive spectrum of these compounds with thiols, 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 with thiols, and once conjugated, the stability of the conjugate. As seen with the protein modification studies, the *in vitro* reactivity of the panels mimics 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. Also, in this study, we only measured the spontaneous reaction rates between GSH and the compounds; however, the enzymatic conjugation by GST isoforms may also be contributing to differences in activity in certain cell lines, a possibility which needs further investigation. Furthermore, G2/M arrest was only seen with the ITC panel (at concentrations that result in apoptosis). Thus, incorporation of selenium into the ITCs may result either in a difference in protein binding preferences, or conjugation of the ITC and ISC compounds with protein 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 its reactive ultimate carcinogen, showing that there are examples of similar protein target preferences and effects, likely due to the similarity in their overall structure. Because ISC compounds failed to induce cell cycle arrest, ROS mechanisms were explored to explain increases seen in A549 cytotoxicity.

Both ITCs and organoselenium compounds have been shown to generate ROS, therefore this mechanism of action was investigated for the ISC panel. As shown by the chemiluminescence assay, the ISC compounds showed a much higher ability to redox cycle

compared to corresponding ITC compounds, in that they form greater amounts of superoxide and other ROS species per unit time when mixed with GSH. In A549 cells, treatment with the compounds at concentrations that trigger apoptosis resulted in increases in both superoxide and hydroperoxides (total ROS) to a greater extent with the ISC panel compared to the ITC panel. Furthermore, either supplementation of GSH or depletion of GSH was found to be associated with the cytotoxic response of PBITC and ISC-4 suggesting that these compounds may cause redox dysregulation in cells as a result of increased ROS species generation which may be the reason for the increased cytotoxicity 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 ROS in cancer cells compared to normal cells. Our suggested mechanism for the increased redox cycling capabilities of the ISC compounds compared to the ITC compounds is depicted in Figure 7. 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 [33–35] and is far more reactive than the corresponding –SH, which would only be slightly redox active. This model could also explain why alkyl chain length affects the cytotoxicity of these compounds, in that the chain length may affect the pKa of the thiol/selenol group formed in the conjugate. In fact, for both ITCs and ISCs the two-carbon-chain compounds, PEITC and ISC-2, had the highest level of redox cycling ability. PEITC has been investigated for ROS mechanisms by several laboratories [16,17,36]. The ROS mechanism may also explain the differing effects on protein thiols seen in the cell environment. The higher level of ROS produced by redox cycling may result in the production of the oxidized forms GSSG, PSSG or conjugates, thus resulting in different downstream effects for ITC vs. ISC compounds, a speculation that needs further testing.

In conclusion, ISC compounds were able to induce higher levels of apoptosis in A549 cells and deplete to a higher extent intracellular A549 GSH levels. However the original hypothesis suggesting that selenium replacement of sulfur would result in decreased electrophilicity of the carbon atom and hence a reduced reactivity towards thiols, proved incorrect based on the observed enhanced reactivity of ISC compounds with GSH compared to that of ITCs. Our results show that although both ITCs and ISCs were able to bind similarly to proteins in vitro (Fig.3), the in vivo cellular effects were different. We also report for the first time the ability for ITC and ISC compounds to redox cycle in the presence of GSH and oxygen; ISC compounds were found to have an increased ability to redox cycle compared to ITC compounds. In addition, we have found that depletion of GSH potentiates cytotoxic effects of ISC compounds to a much higher extent as compared to ITCs. Therefore, redox cycling and overall GSH depletion, but not the rate of reaction with GSH, may be the possible reasons for differences in cellular protein targets as well as the increased cytotoxicity seen with ISC compounds.

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Highlights

ISCs exhibited a higher rate of reaction to GSH than the corresponding ITCs.

ISCs induced higher levels of apoptosis and deplete GSH with different kinetics.

Results indicate that cellular protein targets may be different for ITCs and ISCs.

ISCs showed a greater ability to redox cycle and induced greater levels of ROS.

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. Ellman assay was performed to determine the amount of free -SH in the solution (n=6). (B) Time -dependent GSH depletion in A549 lung adenocarcinoma cells by 15 μ M ITC and ISC compounds (n=3). (C) Dose-dependent GSH depletion in A549 cells at 2 h (n=3). (D) Dose-dependent alterations of the glutathione ratio in A549 cells at 2 h (n=3)

Figure 2. Induction of Apoptosis by ITC and ISC compounds. (A) Flow cytometry plots of Annexin-V and 7-AAD staining of A549 lung adenocarcinoma cells treated with 12.5 μ M compound. (B) Graph represents total Annexin V-positive cells (early and late apoptotic cells) as measured by flow cytometry. Representative histogram is shown from two independent experiments.

Figure 3.

Ellman Assay of FBS and lysate of A549 cells treated with ITC or ISC. Shown is the percentage of free SH compared to FBS control (without drugs). (A) Ellman assay of FBS treated with 400 μM ITC or ISC over 6 h ($n=3$). (B) Ellman assay of A549 cell lysate from cells treated with 10 μM ITC or ISC for 4 h.

Figure 4. Ability of ITC and ISC compounds to induce cell cycle arrest. (A) A549 cells treated with 10 μ M ITC or ISC were stained with propidium iodide to determine phase of cell cycle. (B) Dose-dependent cell cycle arrest caused by PBITC but not ISC-4.

Figure 5.

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 (n=2), ** $p < 0.05$, *** $p < 0.001$. (B) Flow cytometry plots of A549 cells treated with 15 μ M drug for 4 h, and stained for superoxide and total ROS as described under "Materials and Methods". (C) Quantification of ROS-positive cells when treated with ITC or ISC compounds, measured by flow cytometry (n=2).

Figure 6.

Effects of GSH and BSO on ISC-4 – and PBITC-induced cell death, respectively. (A) Glutathione inhibited ISC-4 or PBITC-induced cell death in a concentration-dependent manner. A549 cells were pretreated with the indicated concentrations of GSH for 2 h and then treated with either 12.5 μ M ISC-4 or PBITC for 24 h. (B) Depletion of endogenous GSH by pretreatment with BSO increases ISC-4 or PBITC-induced cell death. A549 cells were incubated with the indicated concentrations of BSO for 24 h and treated with either 12.5 μ M ISC-4 or PBITC for 24 h. After respective incubations, cells were stained with Live and Dead assay reagent for 30 min and then analyzed under a fluorescence microscope. Red color highlights dead cells, and green color highlights living cells. Percentages written under each panel are the values of dead cells.

Figure 7.
Proposed pathway for redox cycling capabilities of ITC and ISC compounds

TABLE 1
“Pseudo-first order” kinetic rates of ITC and ISC compounds with GSH (100-fold excess)

Isothiocyante	Reactivity (GSH×10 ⁻²)/sec	CLogP [*]	Isoselenocyanate	Reactivity (GSH×10 ⁻²)/sec	cLogP [*]
BITC	8.05±0.63	3.0	ISC-1	16.19±4.82	2.1
PEITC	2.23±0.11	3.4	ISC-2	7.40±0.58	2.5
PBITC	1.23±0.09	4.2	ISC-4	5.40±0.34	3.3
PHITC	0.96±0.05	5.2	ISC-6	4.35±0.07	4.3

* Calculated by molinspiration

TABLE 2

IC₅₀ (μM) of ITC and ISC derivatives for proliferation inhibition of A549 cells (48 h treatment), as determined with the MTS assay.

Compounds	Dissolved in serum-free medium (5% Total FBS in well)	Dissolved in medium with 10% FBS	Fold Difference
BITC	12.1	36.3	3.0
PEITC	18.8	39	2.1
PBITC	9.8	32.5	3.3
PHITC	7.7	38.5	5.0
ISC-1	8.1	27.4	3.4
ISC-2	8.8	25.0	3.0
ISC-4	3.1	23.5	10.8
ISC-6	5.2	29	3.5