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Enhanced Nrf2-dependent induction of glutathione in mouse embryonic fibroblasts by isoselenocyanate analog of sulforaphane

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ABSTRACT

Epidemiological and laboratory studies have highlighted the potent chemopreventive effectiveness of both dietary selenium and cruciferous vegetables, particularly broccoli. Sulforaphane (SFN), an isothiocyanate, was identified as the major metabolite of broccoli responsible for its anti-cancer properties. An important mechanism for SFN chemoprevention is through the enhancement of glutathione (GSH), the most abundant antioxidant in animals and an important target in chemoprevention. Enhancement of GSH biosynthetic enzymes including the rate-limiting glutamate cysteine ligase (GCL), as well as other Phase II detoxification enzymes results from SFN-mediated induction of the nuclear factor-erythroid 2-related factor 2 (Nrf2)/antioxidant response elements (ARE) signaling pathway. While isothiocyanate compounds such as SFN are among the most potent Nrf2 inducers known, we hypothesized that substitution of sulfur with selenium in the isothiocyanate functional group of SFN would result in an isoselenocyanate compound (SFN-isoSe) with enhanced Nrf2 induction capability. Here we report that SFN-isoSe activated an ARE-luciferase reporter in HepG2 cells more potently than SFN. It was also found that SFN-isoSe induced GCL and GSH in MEF cells in an Nrf2-dependent manner. Finally, we provide evidence that SFN-isoSe was more effective in killing HepG2 cancer cells, yet was less toxic to non-cancer MEF cells, than SFN. These data support our hypothesis, and suggest that SFN-isoSe and potentially other isoselenocyanates may be highly effective chemoprotective agents in vivo due to their ability to induce Nrf2 with low toxicity in normal cells and high efficiency at killing cancer cells.

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Epidemiological studies highlight the substantial chemopreventive effectiveness of cruciferous vegetable intake in relation to many cancers including lung, breast, colon and prostate.^{1–4} Sulforaphane (SFN) was isolated from broccoli, a cruciferous vegetable widely consumed by Western societies, and identified as a potent inducer of phase II detoxification enzymes, such as quinone reductase, glutathione S-transferases, and glutamate cysteine ligase.^{5,6} SFN, a member of the family of chemopreventive agents whose functional group is an isothiocyanate and one of the most intensively studied chemopreventive phytochemicals, has also been found to act through other pathways, such as the induction of apoptosis in cancer cells.^{7,8} Other isothiocyanates derived from phytochemicals such as phenethyl isothiocyanate (PEITC) and benzyl isothiocyanate (BITC) are also potent chemopreventive agents and inducers of Phase II genes.^{9,10}

Selenium has also played a major role in the field of chemoprevention, particularly after the reporting of an almost 50% reduction in morbidity and mortality by major cancers following dietary sup-

plementation with selenized brewer's yeast.¹¹ Chemopreventive effectiveness depends on the molecular form of administered selenium, and certain plants or yeast grown in selenium-enriched media generate a range of active compounds, such as selenomethionine and selenocysteine.¹² Broccoli grown in soil enriched in selenium resulted in better inhibition of colon cancer in rats but also an 80% reduction in glucosinolate production when compared with normal broccoli.¹³ It is unknown whether isoselenocyanate precursors may be generated in place of isothiocyanate precursor analogs under such conditions.

Glutathione is the most important and abundant endogenous antioxidant in mammals and its induction represents an important objective in chemoprevention.^{14,15} Synthesis of the catalytic subunit of the rate-limiting enzyme for glutathione synthesis, glutamate cysteine ligase (GCL), is regulated partly by the presence of antioxidant response elements (ARE) in upstream promoter regions of the gene.¹⁶ Enhanced nuclear translocation and subsequent binding of the nuclear factor-erythroid 2-related factor 2 (Nrf2) transcription factor to ARE-containing promoters activates a variety of chemoprotective Phase II genes, including many in the glutathione homeostasis pathways.¹⁷ Dietary administration of broccoli seeds, a potent source of SFN, resulted in elevated GCLC

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in the stomach and small intestine of wildtype but not Nrf2^{-/-} mice, demonstrating in vivo dependence of SFN-mediated glutathione induction upon Nrf2.⁶

Nrf2 is normally sequestered in the cytoplasm by the actin-bound protein Keap1, a substrate adaptor for an E3 ubiquitin ligase, which targets Nrf2 for rapid turnover.¹⁸ Keap1 contains multiple reactive cysteine residues that, when modified directly or indirectly by a variety of inducers, reduces its affinity for and promotes nuclear translocation of Nrf2.¹⁹ The isothiocyanate group of SFN has been demonstrated in vitro to directly modify Keap1 through the formation of thionoacyl adducts.²⁰

Substitution of selenium for sulfur in a functional group has the potential to alter a compound's reactivity and/or target specificity. There is also the possibility that plants such as selenium-enriched broccoli may produce an isoselenocyanate analog of sulforaphane, namely; suforaphane isoselenocyanate (SFN-isoSe), and that this may partly account for enhanced chemopreventive properties. The chemical structures of SFN and SFN-isoSe are provided in Figure 1A. Therefore, it was decided to test the hypothesis that synthetic SFN-isoSe would, like SFN, induce the Nrf2/ARE pathway, including the downstream targets GCLC and glutathione levels, and perhaps could do so more effectively.

In the present study, we first report the synthesis of newly developed SFN-isoSe. SFN was synthesized as reported in the literature.²¹ The synthesis of SFN-isoSe (**3**) is shown in Figure 1B. The synthetic strategy involves the formylation of alkyl amine, 1-amino-4-(methylsulfinyl) butane (**1**).¹⁰ The desired key intermediate alkyl formamide (**2**) was prepared following general procedure reported by Elliott and Williams.^{21,22} Isoselenocyanate was synthesized using a modified procedure by Fernandez-Bolanos et al.²³ Alkyl formamides on treatment with triphosgene and selenium powder in the presence of triethylamine in one-pot dehydration furnished the desired alkyl sulfinyl isoselenocyanate in moderate yields as oil.^{24,25} The final products SFN-isoSe was purified by silica gel column chromatography and the pure compounds was characterized on the basis of the NMR and Mass spectra. The purity of SFN-isoSe was determined by analytical HPLC and found to be $\geq 97\%$.

HepG2 ARE-luciferase reporter cells were used to test the hypothesis that an isoselenocyanate analog of SFN, SFN-isoSe, would have enhanced ability to induce the ARE pathway. Both luciferase activity and cell viability were measured.^{26–28} The fact that selenocyanates are much more effective than their thiocyanate counterparts in chemoprevention studies,^{29,30} led to the hypothesis that perhaps even very potent Phase II inducers, such as sulforaphane, could be improved upon through substitution of selenium for sulfur in isothiocyanate functional groups. Other phytochemical-derived isothiocyanates, such as PEITC and BITC, are also potent chemopreventive agents in various animal models,^{10,31}

and inducers of Phase II genes,^{9,10} so these compounds and their isosteric selenium analogs, isoselenocyanate compounds were included in the present study.

Effects on viability were noticed at short exposure times, and preliminary time-course studies showed that a concentration range of 0–20 μM with a 6-h exposure provided useful data for comparing these compounds. Effects of SFN and SFN-isoSe on relative cell viability as determined by MTS assay are summarized in Figure 2A. At concentrations of 10 μM or below there was no effect by either compound on viability while, at 20 μM , a greater than 50% reduction in viability was observed for SFN-isoSe but not for SFN. It was determined in this study that not only SFN-isoSe, but isoselenocyanates in general, were more effective in killing HepG2 cancer cells than their isothiocyanate analogs. Cells demonstrated 100% viability following a 6-h exposure to isothiocyanates, SFN, PEITC, and BITC at concentrations up to 50 μM . In contrast, viability in cells treated with the isoselenocyanate analogs of SFN (SFN-isoSe), PEITC (phenethylisoselenocyanate [PEISC]), and BITC (benzylisoselenocyanate [BISC]) were reduced to 0%, 14%, and 0% of controls, respectively (data not shown). It is not known

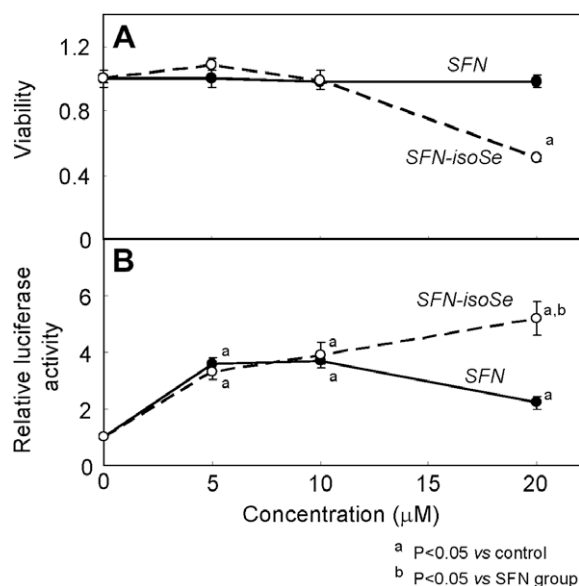


Figure 2. Effects of SFN or SFN-isoSe on HepG2 ARE-luciferase activity and viability. HepG2 cells at 50% confluence were provided fresh media containing vehicle control or 5, 10 or 20 μM of SFN or SFN-isoSe. After 6 h, cells were assayed for viability by MTS absorbance at 490 nm (A), then washed with PBS, lysed and luciferase activity was determined using Promega's Luciferase Assay System and single tube luminometer (B). Data are normalized to control values and represent the mean $\pm$ SD of three independent experiments.

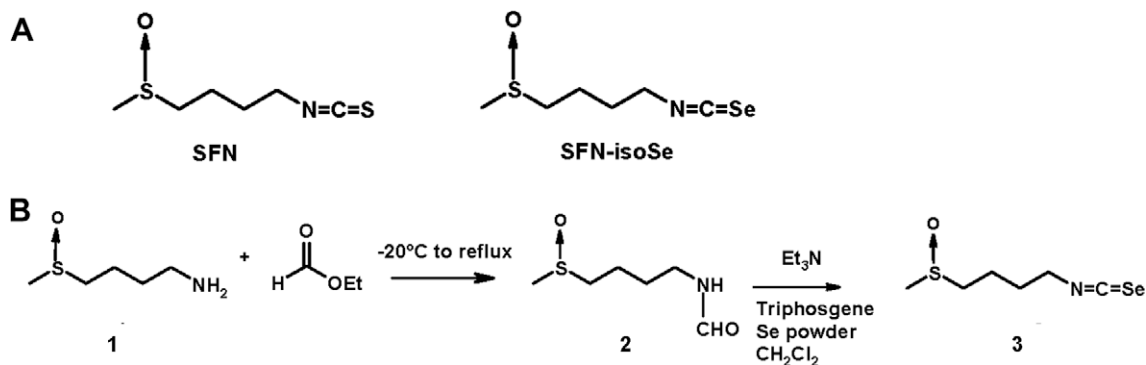


Figure 1. Sulforaphane (SFN) and suforaphane isoselenocyanate (SFN-isoSe): structures and synthesis.

how phytochemicals like SFN induce apoptosis in abnormal cancer cells, but it has been suggested that the transcription factors NF- κ B and AP-1 are involved.³² Studies are ongoing in our laboratory, to examine the involvement of these transcription factors in isoselenocyanate-mediated apoptosis of cancer cells.

Relative luciferase induction is plotted in Figure 2B following exposure of HepG2-luciferase reporter cells to varying concentrations of SFN or SFN-isoSe.³³ As with viability, the effects on ARE activation by the two compounds are identical up to 10 μ M, but deviate substantially at higher concentration. At 20 μ M, SFN-isoSe results in a fivefold luciferase induction, more than twice that observed for SFN. The ARE induction by SFN-isoSe increases in a dose-dependent manner even while cell viability is greatly reduced. In fact, when ARE reporter activity was normalized to the proportion of viable cells, luciferase induction mediated by SFN-isoSe was 4-fold greater than that induced by SFN at the same concentration. Compared with other isoselenocyanates tested, this phenomenon appears unique to SFN-isoSe since for both BISC and PEISC, decreased viability in the 10–20 μ M range was accompanied by an even larger decrease in ARE induction (data not shown). Peak luciferase activity and concentration at maximal induction were not remarkably different between the isothiocyanates PEITC and BITC and their isosteric selenium analogs PEISC and BISC.

Wildtype and Nrf2 deficient MEF cells were used to test the hypothesis that SFN-isoSe has enhanced ability to induce the Nrf2 transcription factor in non-cancer cells when compared with SFN. Figure 3 shows that nuclear Nrf2 levels were significantly induced over controls 3.5-fold for SFN and 4.5-fold for SFN-isoSe, regardless of concentration, in wildtype cells.

Because induction of the ARE pathway by isoselenocyanates had not been previously reported, it was unknown if downstream ARE-regulated genes would also be induced. Therefore, we examined the ARE-regulated GSH synthetic pathway in wildtype and Nrf2^{-/-} MEF cells.^{34–37} Cytoplasmic extracts of 10 or 20 μ M of SFN or SFN-isoSe treated wildtype or Nrf2^{-/-} cells were probed by western analysis for GCLC, the rate-limiting enzyme in glutathione synthesis. Figure 4 shows that relative GCLC protein expression was significantly enhanced 3.2-fold by 20 μ M SFN and 4.1-fold by 20 μ M SFN-isoSe over controls in wildtype cells, and that no such increase was seen in Nrf2^{-/-} cells. Furthermore, the induction by 20 μ M SFN-isoSe is significantly 30% greater than that by 20 μ M SFN.

We also examined if SFN and SFN-isoSe would induce glutathione levels in MEF cells in a Nrf2-dependent fashion. Because isothiocyanates such as SFN deplete glutathione in the short term, glutathione was measured in wildtype or Nrf2^{-/-} MEF cells as a

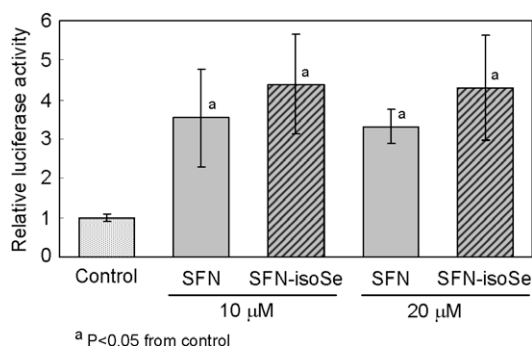


Figure 3. Effects of SFN or SFN-isoSe treatment on nuclear Nrf2 expression in mouse embryonic fibroblasts. Wildtype MEF cells at 50% confluence were provided fresh media containing vehicle control or 10 μ M or 20 μ M of SFN or SFN-isoSe. After 24 h, cells were washed with PBS, trypsinized and collected. Nuclear extracts, obtained using Pierce's NE-PER kit, were subjected to western blotting for Nrf2, quantitated and normalized to Lamin A protein levels. Data are normalized to control values and represent the mean $\pm$ SD of three independent experiments.

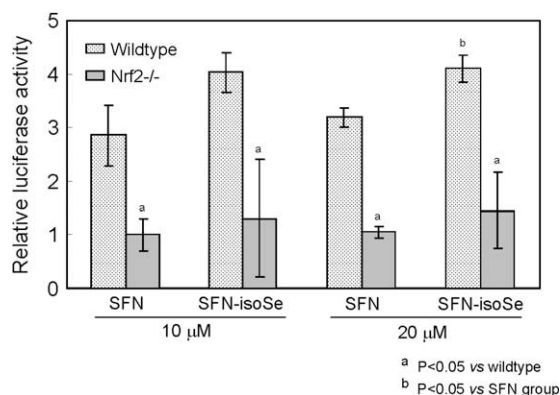


Figure 4. Effects of SFN or SFN-isoSe on GCLC expression in mouse embryonic fibroblasts. Wildtype or Nrf2^{-/-} MEF cells at 50% confluence were provided fresh media containing vehicle control or 10 μ M or 20 μ M of SFN or SFN-isoSe. After 24 h, cells were washed with PBS, trypsinized and collected. Cytoplasmic extracts, obtained using Pierce's NE-PER kit, were subjected to western blotting for GCLC, quantitated and normalized to Actin protein levels. Data are normalized to control values and represent the mean $\pm$ SD of three independent experiments.

function of exposure time at concentrations of 10 and 20 μ M SFN or SFN-isoSe (Fig. 5A and B). After 6 h exposure, both SFN and SFN-isoSe deplete glutathione to 40% of vehicle-treated controls at 10 μ M, and to 20% of controls at 20 μ M, regardless of the presence of functional Nrf2.

Cells deficient in Nrf2 and treated with SFN do not recover as glutathione levels continue to fall over a 24-h period. On the other hand, SFN-isoSe treated Nrf2^{-/-} cells do show partial repletion of glutathione with 10 μ M treated cells recovering to about 90% of control values and 20 μ M treated cells reaching 70% after 24 h. Wildtype MEF cells exposed to 10 μ M SFN-isoSe exhibit a sharp increase in glutathione after 6 h with supra-basal levels being reached by 9 h and ultimately reaching 160% of controls by 24 h. Ten μ M SFN treated wildtype cells reveal a similar but attenuated pattern and basal glutathione levels are eventually reached by 24 h. Increasing the concentrations to 20 μ M reveals opposing behaviors between SFN and SFN-isoSe treatment of wildtype cells, particularly between the 9 and 24-h time points, where SFN-isoSe causes a net increase in glutathione versus a net decrease for SFN.

Absolute glutathione levels normalized to protein from wildtype or Nrf2^{-/-} MEF cells treated with SFN or SFN-isoSe for 24 h are displayed in Figure 5C. Basal glutathione levels in un-treated Nrf2^{-/-} cells are significantly 50% lower than values of un-treated wildtype cells. Glutathione levels are nearly doubled in wildtype cells by SFN-isoSe treatment regardless of concentration, but were unchanged by 10 μ M SFN and significantly lowered by 20 μ M SFN. SFN-isoSe did not induce glutathione in Nrf2^{-/-} cells.

The experiments with MEF cells revealed that a nearly twofold glutathione induction over controls by SFN-isoSe is dependent upon Nrf2. That induced glutathione occurs coincidentally with elevated Nrf2 and GCLC in wildtype, but not Nrf2^{-/-}, MEF cells provides strong evidence that SFN-isoSe-mediated elevation of nuclear Nrf2 causes ARE-mediated transcriptional induction of GCLC, and ultimately leads to higher glutathione levels.

It is interesting that SFN-isoSe induces glutathione in MEF cells while SFN does not. This occurs despite induction of Nrf2 and GCLC by both compounds. Glutathione levels are influenced by depletion and synthesis, and GSH was depletion by both compounds in the short term, followed by varying degrees of repletion or continued depletion at later times. Repletion in wildtype MEF cells treated with 10 μ M SFN or SFN-isoSe can be explained by Nrf2 induction. SFN treated Nrf2^{-/-} cells, regardless of concentration, do not exhibit repletion, rather glutathione was further depleted with

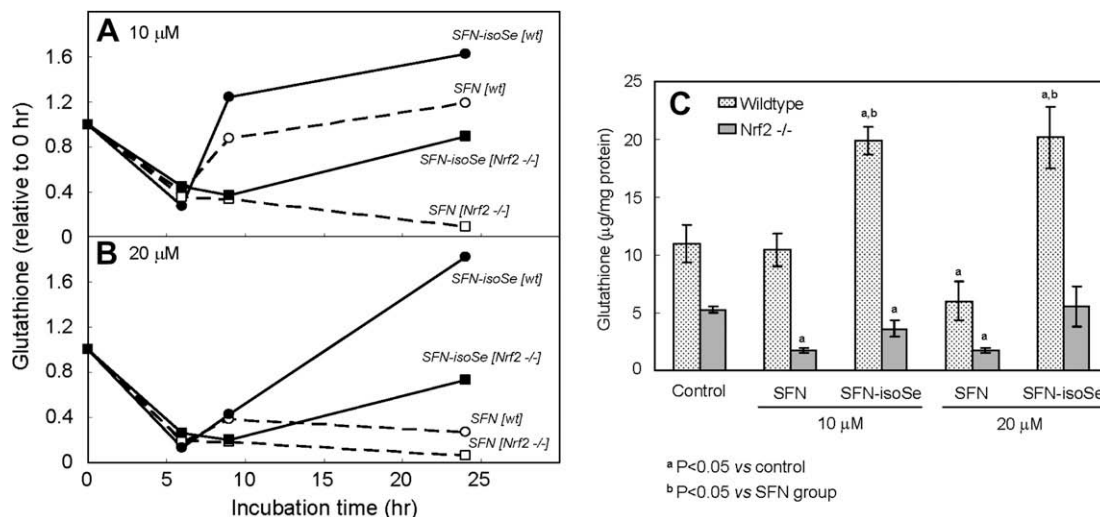


Figure 5. Effects of SFN or SFN-isoSe on glutathione levels in mouse embryonic fibroblasts. Wildtype or Nrf2^{-/-} MEF cells at 50% confluence were provided fresh media containing vehicle control or 10 μM or 20 μM of SFN or SFN-isoSe. After 0, 6, 9, or 24 h, cells were washed with PBS and lysed in 5% MPA. Acid soluble fractions were obtained by centrifugation and GSH levels were analyzed. Time course of relative GSH changes were assessed after administration of SFN or SFN-isoSe at concentrations of either 10 μM (A) or 20 μM (B). GSH concentrations in both SFN and SFN-isoSe treated cells after 24 h are plotted as the mean ± SD of three independent experiments (C).

increasing exposure times. SFN has been demonstrated to accumulate in cells and deplete GSH in the short term.³⁸ This is in contrast with SFN-isoSe treated Nrf2^{-/-} cells where depletion to almost basal levels occurs by 24 h of exposure. This may indicate an alternate pathway of glutathione induction, which is independent of Nrf2.

In summary, it is not known if isoselenocyanate precursors occur naturally in cruciferous vegetables, as is the case with isothiocyanates like SFN and PEITC. However, broccoli fertilized with selenium has been shown to possess enhanced chemopreventive properties in some models.³⁹ But selenium fertilization of broccoli has also been shown to decrease production of the glucosinolate precursor of SFN.⁴⁰ The possibility that chemopreventive vegetables like broccoli can synthesize isoselenocyanate precursors needs to be explored.

It is not obvious how substitution of selenium for sulfur in an isothiocyanate functional group would change its reactivity and or target specificity, as the electronegativity of these elements are very similar. However, there exist clear examples in nature where selenium in place of sulfur greatly changes a protein's reactivity. For example, the ionized selenol of selenocysteine at physiological pH in active sites of selenoproteins accounts for their higher redox sensitivity.⁴¹ Furthermore, oxidized selenomethionine in proteins can be repaired non-enzymatically while oxidized methionine requires methionine sulfoxide reductases. The isothiocyanate group of sulforaphane has been demonstrated in vitro to directly modify Keap1 through the formation of thion-oacyl adducts and it is possible that isoselenocyanates may do this better.²⁰

Any compound under consideration as a chemopreventive agent must show minimal toxicity to normal cells. An overview of the data presented herein suggest that SFN-isoSe is more toxic to cancer cells than SFN, but less toxic to normal MEF cells, even in the absence of functional Nrf2. It was shown here that SFN-isoSe is less glutathione depleting, and therefore less toxic, than SFN to wildtype and Nrf2^{-/-} MEF cells. The zone between chemoprevention and chemotherapy becomes blurred when agents can kill cancer cells, even while inducing protective genes in normal cells. The need for such agents has clearly been suggested,⁴² therefore the synthetic isoselenocyanates, and particularly SFN-isoSe, are ideal candidates for future in vivo studies.

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- General synthesis: Proton NMR spectra were recorded in CDCl₃ using a Bruker 500 MHz instruments. The chemical shifts are reported in ppm downfield from TMS. MS were run on 4000 Q trap hybrid triple quadrupole/linear ion trap instrument (Applied Biosystems/MDS Sciex) at the proteomic facility in Penn State Cancer Institute at Penn State College of Medicine, Hershey, PA.

- Thin-layer chromatography (TLC) was on aluminum-supported, pre-coated silica gel plates (EM Industries, Gibbstown, NJ). All starting materials and reagents were obtained from Aldrich Chemical Co. (Milwaukee, WI) and used without further purification.
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 24. **Synthesis of alkyl sulfinyl formamide (2):** Ethyl formate (0.9 g, 12 mmol) was added dropwise to 1-amino-4-(methylsulfinyl)butane (0.54 g, 4 mmol) at room temperature and the resulting mixture was refluxed for 6 h. The excess ethyl formate was removed under reduced pressure to yield the corresponding formamide as oil which was used in the next step without further purification.
 25. **Synthesis of sulforaphane isoselenocyanate (SFN-isoSe, 3):** To a refluxing mixture of the alkyl formamides (0.18 g, 1.5 mmol), triethylamine (0.64 g, 6.4 mmol) in CH₂Cl₂ (5 mL) and 4 Å molecular sieves was added dropwise a solution of triphosgene (0.8 mmol) in CH₂Cl₂ (2 mL) for a period of 1 h. After the addition was complete, the mixture was refluxed for an additional 2.5 h. Selenium powder (3.0 mmol) was then added and the resulting mixture was refluxed for additional 8 h. The mixture was cooled, filtered, and the solvent was evaporated to yield the crude mixture, which was chromatographed on a silica gel column by elution with hexane/ethyl acetate (1:1) to afford the desired product **3** as viscous oil (66 mg, 27% yield); ¹H NMR (CDCl₃): 3.72 (t, 2H, J = 6.5 Hz, N-CH₂), 3.10 (t, 2H, J = 7.0 Hz, SO-CH₂), 2.97 (s, 3H, S-CH₃), 1.98–2.07 (m, 4H, C-CH₂-CH₂-C), MS (m/e) 225 (MH⁺), 203, 145 (M⁺-Se), 118, 103.
 26. **Cell culture and viability assay:** HepG2 human hepatoma cells stably transfected with ARE-luciferase plasmid were obtained courtesy of Muriel Cuendet.²⁸ Cells were maintained in F-12 media (Invitrogen) supplemented with 10% fetal bovine serum, antibiotics, MEM amino acid solution, and insulin (1.45 µg/mL). Wildtype and Nrf2 deficient (Nrf2^{-/-}) mouse embryonic fibroblasts (MEF) were obtained courtesy of Dr. Thomas Kensler.²⁹ Cells were maintained in Iscov's MDM (Gibco12440) supplemented with 10% fetal bovine serum, and antibiotics. Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reduction assay, according to the manufacturer's instructions (CellTiter 96TM, Promega). Absorbency at 490 nm was measured using a Bio-Tek Synergy HT plate reader. Statistical significance was assessed using either the Student's *t*-test or ANOVA where appropriate. Differences between data sets were considered statistically significant if *p* < 0.05.
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 33. **Luciferase assay:** Treated HepG2 ARE reporter cells were washed in PBS, lysed, and luciferase activity of resulting extracts was measured according to the Luciferase Assay System protocol (Promega) using a single tube luminometer.
 34. **Glutathione assay:** Aliquots of cells were washed in PBS and lysed in 5% metaphosphoric acid. Precipitated protein was removed by centrifugation. Free glutathione in cellular extracts was determined using the previously described enzymatic recycling method using Elman's reagent³⁶ with modifications.³⁷ Absorbency over time was measured using a Bio-Tek Synergy HT plate reader (Bio-Tek Instruments, Winooski, VT). Glutathione levels are normalized to protein levels as determined by BCA assay. Statistical significance was assessed using either the Student's *t*-test or ANOVA where appropriate. Differences between data sets were considered statistically significant if *p* < 0.05.
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 37. **Cellular extracts and Western Blotting:** Cellular extracts were obtained using NE-PER[®] Nuclear and Cytoplasmic Extraction Reagents according to the manufacturer's protocol (Pierce). Western blotting for nuclear Nrf2 and LaminA, or cytoplasmic GCLC, GCLM or Actin, was performed according to manufacturer's instructions using the CriterionTM Cell system (Bio-Rad, Hercules, CA). Antibodies (Nrf2, GCLC, GCLM, Actin, LaminA) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Protein of cellular extracts was determined by BCA assay. Absorbency was measured using a Bio-Tek Synergy HT plate reader (Bio-Tek Instruments, Winooski, VT). Cytoplasmic or nuclear protein (50 or 20 µg per lane, respectively) was loaded in 4–20% gradient polyacrylamide gels (TRIS-HCL) immediately following boiling for 5 min in Laemli's buffer. Resolved protein were transferred to nitrocellulose membranes using a CAPS discontinuous buffer system and Trans-Blot[®] SD transfer cell (Bio-Rad tech note 2134). Membranes were blocked for 1 h at room temperature in 5% non-fat dry milk/TBST, and probed overnight at 4 °C with primary antibody diluted in TBST (Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were incubated in HRP-conjugated secondary antibodies (Pierce Protein Research Products, Thermo Scientific, Rockford, IL), diluted 1:1000 in TBST, for 1 h at room temperature and developed using SuperSignal West Dura Extended Duration Substrate reagents (Pierce Protein Research Products, Thermo Scientific, Rockford, IL). Membranes were re-probed for Actin (cytoplasmic marker) or LaminA (nuclear marker) to insure equal loading and purity of cytoplasmic or nuclear extracts. Bands were quantitated with the BioSpectrum AC Imaging System (UVP BioImaging Systems, Upland, CA), and data was normalized to corresponding actin or LaminA values. Statistical significance was assessed using either the Student's *t*-test or ANOVA where appropriate. Differences between data sets were considered statistically significant if *p* < 0.05.
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