Sulforaphane prevents acetaminophen-induced hepatic injury in mice.

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SULFORAPHANE PREVENTS ACETAMINOPHEN-INDUCED HEPATIC INJURY IN MICE

By

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B.S., St. John's University, 2006

A Thesis
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for the Degree of

Master of Science

Department of Pharmacology and Toxicology
University of Louisville
Louisville, KY

May 2011
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Thesis Approved on
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ABSTRACT

SULFORAPHANE PREVENTS ACETAMINOPHEN-INDUCED HEPATIC INJURY IN MICE

Robin H. Schmidt

11/29/2011

Sulforaphane (SFN), an isothiocyanate found in cruciferous vegetables, is known to confer antioxidant protection in vivo. Rather than directly reacting with free radicals, however, SFN works by inducing Nrf2, a transcription factor that binds to the promoter regions of several known antioxidant genes and enhances detoxification. Because oxidative stress is a major contributor to acetaminophen (APAP)-induced hepatotoxicity, SFN may defend the liver against APAP overdose by activating the Nrf2 pathway and increasing endogenous antioxidant response. To test this hypothesis, mice were pre-treated with SFN for four days, injected with APAP on the fifth day, and sacrificed shortly thereafter. APAP overdose caused massive hepatic injury, as shown by increases in serum liver enzyme activity and lipid peroxidation. APAP overdose also manifested as decreases in total glutathione and glutathione reductase activity. SFN administration clearly prevented these manifestations of liver injury, however: increases in serum liver enzyme activity and lipid peroxidation were blunted, while total glutathione and glutathione reductase activity remained similar to those of control animals. SFN treatment did not affect the catalytic activity of acetaminophen-metabolizing enzyme CYP2E1, but did increase nuclear accumulation of Nrf2, suggesting that SFN acts primarily through the Nrf2 pathway. In summary, these data support the hypothesis that sulforaphane attenuates acute acetaminophen-induced liver injury. This decrease in injury results from the increased availability of glutathione to react with toxic metabolites of acetaminophen.
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INTRODUCTION

The byproducts of hepatic drug metabolism are often oxidants or electrophiles which readily react with important biological molecules. Under ideal conditions, reactive metabolites are removed from the liver by a multitude of enzymes that have evolved for this purpose. Yet in certain situations these metabolites may accumulate. The resulting condition is broadly termed "oxidative stress" and leads to potentially dangerous alterations in hepatic structure and function.

Oxidative stress is demonstrably reduced in vitro with direct-acting antioxidants such as tocopherols and melatonin. In animal and human studies, however, the benefits of antioxidant use are far less apparent. The reason for this experimental incongruity is still unclear. One suggestion is that, in whole animal models, it is difficult to achieve adequate antioxidant concentrations at the desired site of action. Alpha-tocopherol, for example, tends to accumulate in cellular membranes, and therefore has limited ability to quench reactive molecules within the cytoplasm. Many free radical-scavenging agents are also hypothesized to act as pro-oxidants, which may explain the observation that high doses of antioxidant vitamins actually increase morbidity and mortality.

Many researchers are now looking toward the use of indirect antioxidants as a treatment for oxidative stress. In contrast to direct agents, such as the previously-mentioned antioxidant vitamins, indirect antioxidants do not interact with reactive metabolites or free radicals. Sulforaphane (SFN), an isothiocyanate derived from Brassica vegetables, is a prototypical indirect antioxidant. Rather than reducing reactive metabolites, SFN induces Nrf2, a transcription factor that regulates cellular defense against toxicity. The proposed mechanism of defense is an interaction between SFN and Keap1, a substrate adaptor for the E3 ubiquitin ligase Cul3. During homeostasis Nrf2 is found in the cytoplasm, where it binds Keap1. In the absence
of stressors, Nrf2 and Keap1 remain bound, and Keap1 will eventually facilitate proteasomal
degradation of Nrf2. Conversely, if electrophiles or oxidants are present, they will disrupt the
bond between Nrf2 and Keap1, allowing Nrf2 to translocate to the nucleus and bind antioxidant
response elements (AREs) in DNA.\cite{16,17} In particular, it is the electrophilic central carbon atom
of SFN's -N=C=S side chain which theoretically disrupts the Nrf2-Keap1 bond, as this carbon can
readily form thionoacyl adducts\cite{18,19}. Following alkylation, Nrf2 would then be free to bind AREs
which regulate the transcription of genes encoding cytoprotective enzymes, e.g., heme
oxygenase 1 (Fig. 1).

The purpose of the present study was to determine the ability of SFN to prevent toxicity in an
established model of oxidative stress. Acetaminophen (APAP) was chosen because, though it is
safe within its intended dose range, it causes a concentration-dependent and easily reproducible
injury to hepatocytes at larger doses.\cite{20} APAP's toxicity is mediated in part by its oxidative
byproduct NAPQI, which can drastically deplete glutathione reserves.\cite{21,22} Despite substantial
evidence of SFN's protective properties, there are no FDA-approved therapies for oxidative injury
that incorporate indirect antioxidants. The results presented here further support the need for
indirect antioxidants in clinical practice.
MATERIALS AND METHODS

Animals and treatments

Male C57BL/6J mice (6-8 weeks old) were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were housed in a pathogen-free barrier facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, and procedures were approved by the local Institutional Animal Care and Use Committee. All diets were switched from standard laboratory chow to AIN-76A purified diet one week prior to the study, since impurities found in standard laboratory chow may create experimental variability. Food and tap water were allowed ad libitum.

In experiments designed to test the effects of SFN alone, mice were administered SFN (10 mg/kg i.g. diluted in water; LKT Laboratories, St. Paul, MN) or water for 4 days q.a.m. Groups of mice were selected to be euthanized at six or 24 hours after the last i.g. dosing. In experiments designed to test the protective effects of SFN on APAP overdose, mice were administered SFN (10 mg/kg i.g.) or water for 4 days q.a.m. On the following day, mice were injected with APAP (300 mg/kg i.p.; Sigma-Aldrich, St. Louis, MO). Six hours after APAP injection (24 h after last i.g. SFN or water), the mice were euthanized.

Regardless of experimental treatment, procedures used in harvesting tissue were the same. Mice were euthanized with ketamine/xylazine (100/15 mg/kg i.m.), and their blood and livers were collected for later analysis. Blood was collected from the vena cava just prior to sacrifice by exsanguination and citrated plasma was stored at -80°C for later analysis. Portions of liver tissue were snap-frozen in liquid nitrogen, frozen-fixed in OCT-Compound (Tissue-Tek OCT compound, Sakura Finetek, Torrance, CA), or fixed in 10% neutral buffered formalin for subsequent sectioning and mounting on microscope slides. Some snap-frozen liver was homogenized in 4.4% metaphosphoric acid for detection of glutathione.
Biochemical analyses and histology

Plasma levels of aminotransferases (ALT) and hepatic levels of triglycerides were determined using standard kits (Thermotrace, Melbourne, Australia). Paraffin-embedded sections of liver were stained with hematoxylin & eosin (H&E). Red blood cell extravasation in the livers was assessed by staining tissue sections for chloroacetate esterase, a marker for RBCs, using the naphthol AS-D chloroacetate esterase (CAE) kit (Sigma, St. Louis MO). Adducts of 4-hydroxynonenal (lipid peroxidation) were detected by immunohistochemistry as described previously.\(^{(23)}\)

Quantitation of glutathione content and glutathione reductase activity

Total cellular glutathione content was quantitated colorimetrically from liver samples using a modified version of the method described by Rahman.\(^{(24)}\) A 10% solution of tissue homogenate (w/v) was prepared in 4.4% metaphosphoric acid using an all-glass Tenbroeck homogenizer, and kept on ice. After standing for 20-40 min, the homogenate was centrifuged for 1 min (10,000 \( \times \) g), and the supernatant collected. Twenty \( \mu \)l of the supernatant were added to each well of a microtiter plate and incubated with 120 \( \mu \)l of a solution containing potassium phosphate buffer (pH 7.5), 10 mM EDTA, 2 mM 5,5’,dithiobis(2-nitrobenzoic acid) (DTNB) and 230 U/mL glutathione reductase (GR, EC 1.6.4.2, Sigma-Aldrich, St. Louis, MO). After allowing 60 seconds for conversion of GSSG to GSH, 60 \( \mu \)l of buffered NADPH solution was added, and the absorbance was read for 5 minutes at 412 nm during the linear portion of the reaction. Total glutathione was calculated from a standard curve prepared with purified GSH. Results were reported as nmol GSH per g tissue wet weight.

To determine GR activity, liver tissue was homogenized in a phosphate buffer (pH 7.5) containing 1 mM DTT and centrifuged for 10 min (10,000 \( \times \) g) to clear the homogenate. Twenty \( \mu \)l of each sample were then added to each well of a microtiter plate and incubated for 60 sec with a solution containing phosphate buffer, 2 mM GSSG, and 1 mM NADPH. The absorbance was read for 5 min at 412 nm, and enzyme activity was calculated from a standard curve of purified GR. The protein concentration of the homogenate was subsequently measured.
according to the method of Bradford. (25) After adjusting for protein concentration, glutathione reductase activity was reported as units of activity/mg protein.

**Microsomal isolation and cytochrome P450 activity determination**

Expression and activity data were obtained for cytochrome P450 2E1 (CYP2E1), the microsomal enzyme that generates NAPQI. Microsomes were isolated according to a protocol adapted from those of Cox (26) and Falkner. (27) Briefly, liver tissue was kept on ice and homogenized with a Teflon pestle in a buffer containing 50 mM Tris-HCl (pH 7.4), 225 mM mannitol and 75 mM sucrose. The homogenate was spun at 15,000 x g for 20 minutes, after which the supernatant was collected and spun at 436,000 x g for an additional 20 min. The pellet remaining was next resuspended in a buffer of 250 mM mannitol, 0.5 mM EGTA, and 5 mM HEPES (pH 7.4) and centrifuged again at 9,000 x g for 30 min. The supernatant resulting from this centrifugation was collected, assessed for protein concentration, and stored at -80° until kinetic analysis.

CYP2E1-mediated conversion of p-nitrophenol to 4-nitrocatechol was measured by spectrophotometer as described previously. (28; 29) Isolated microsomes were added to each well of a microtiter plate along with an assay buffer containing 100 mM sodium phosphate (pH 6.8), 0.1 mM p-nitrophenol, and 1 mM NADPH. After incubating for 10 min at 37°, the absorbance was read at 480 nm for 12 min and concentration values were interpolated from a standard curve of 4-nitrocatechol dissolved in sodium phosphate buffer. Activity was determined from the change in 4-nitrocatechol concentration per unit time.

**Immunoblots**

All buffers used for protein extraction contained protease, tyrosine phosphatase, and serine/threoneine phosphatase inhibitor cocktails (Sigma, St. Louis, MO). Nuclear proteins were isolated according to the method of Dignam et al. (30) A sample of liver tissue (50-100 mg) from each animal was homogenized in a pre-chilled Dounce glass tissue grinder containing 500 μL hypotonic buffer (1.5 mM MgCl₂, 1 mM KCl, .5 mM DTT, 10 mM HEPES, pH 7.6). The
homogenate was placed on ice for 15 minutes, then vigorously mixed with 30 μL of a 10% NP-40 solution and centrifuged for 30 sec at 16,000 x g. The supernatant (cytosolic fraction) was transferred to a separate tube and the pellet was mixed with 70 μL of resuspension buffer (50% v/v glycerol, 420 mM NaCl, .25 mM EDTA, .5 mM DTT, 10 mM HEPES, pH 7.6). After the resuspended samples were agitated on ice for 15 min, they were then spun for 5 min at 16,000 x g, and the supernatant (nuclear fraction) was collected and stored at -80°C.

Nuclear and cytosolic proteins were loaded onto SDS-polyacrylamide gels of 10% (w/v) acrylamide followed by electrophoresis and Western blotting onto PVDF membranes (Hybond P, GE Healthcare, Piscataway, NJ). Primary antibodies against Nrf2 and GAPDH (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were used. Bands were visualized using an ECL kit (Pierce, Rockford, IL) and Hyperfilm (GE Healthcare, Piscataway, NJ).

RNA isolation and real-time RT-PCR
Message levels of select genes were detected by real-time reverse-transcriptase PCR. Total RNA was extracted from liver tissue samples by a guanidium thiocyanate-based method (Tel-Test, Austin, TX). RNA concentrations were determined spectrophotometrically, and cDNA was synthesized from 1 μg total RNA using an MMLV reverse transcriptase kit (Quanta Biosciences, Gaithersburg, MD). The cDNA was added to a mixture containing pre-made primers and probes (Applied Biosystems, Foster City, CA) and a ready-to-use reaction buffer (Quanta). Amplification reactions were carried out in the StepOne™ sequence detection system (Applied Biosystems). The comparative C_T method was used to determine fold differences between samples and the calibrator gene, β-actin.

Statistical analyses
Results are reported as means ± SEM (n = 4-7). ANOVA with Bonferroni's post-hoc test (for parametric data) or Mann-Whitney Rank Sum test (for nonparametric data) was used for the determination of statistical significance among treatment groups, as appropriate. A p value less than 0.05 was selected before the study as the level of significance.
RESULTS

SFN treatment protected mice from necrosis caused by APAP

Elevated plasma levels of transaminases (e.g., ALT and AST) indicate damage to cell membranes and may be evidence of widespread liver dysfunction. As expected, APAP overdose caused an average elevation in circulating ALT of >200-fold, and an elevation in AST of ~100-fold, compared with controls. However, pretreatment with sulforaphane attenuated the increase in transaminases. On average, sulforaphane-treated mice had roughly half the transaminase activity of mice that did not receive sulforaphane (Fig. 2). Transaminase data was paralleled by hepatic histology, as assessed by hematoxylin and eosin staining (Fig. 3A). APAP caused necroinflammatory liver damage at pericentral regions of the liver in all mice, but the size of the necrotic areas was visibly diminished by SFN. APAP overdose also caused an increase in hemorrhagic necrosis, which is seen after chloroacetate esterase staining as an infiltration of red blood cells into necrotic areas (Fig. 3B). SFN reduced the extravasation of red blood cells from pericentral foci, consistent with the observed changes in ALT, AST, and cellular morphology.

SFN decreased indices of oxidative stress

Glutathione is the first line of defense against APAP toxicity: APAP's toxic metabolite NAPQI is primarily removed by conjugation with GSH. In an experiment comparing the effects of SFN alone to control treatment, SFN was found to increase total hepatic glutathione (>1.2x on average; Fig. 4). A similar effect on glutathione was seen after APAP overdose. In control mice that were fasted overnight, glutathione content was measured at ~3400 nmol/gram of tissue. After APAP injection, glutathione was reduced by half (~1500 nmol/g). With previous SFN administration, however, glutathione depletion was blunted (~2500 nmol/g) (Fig. 5, GSH+GSSG).
Glutathione reductase activity was also significantly reduced by APAP. Enzyme activity in control mice averaged 35 units/g protein, but APAP overdose reduced this activity to ~27 U/g. SFN pretreatment maintained the catalytic activity of GR at 35 U/g even after APAP (Fig. 5, GR). Lipid peroxidation strongly correlates with the availability of glutathione. As expected, the lipid peroxidation product 4-hydroxynonenal was detected over a substantial area of liver tissue after APAP intoxication (Fig. 6). Though the characteristic brown stain indicating membrane lipid oxidation was present in all animals after APAP injection, this staining was more diffuse in mice that were pretreated with SFN. Lipid peroxidation, like necrosis, was largely confined to pericentral areas of the liver.

**SFN did not inhibit CYP2E1, but induced Nrf2**

The microsomal drug-metabolizing enzyme Cytochrome P450 2E1 (CYP2E1), found abundantly in the liver, is the primary P450 isozyme involved in NAPQI formation. Correspondingly, many of the substances that prevent APAP intoxication work by inhibiting CYP2E1 and therefore reducing the formation of NAPQI. At this dose of SFN, however, no inhibition of CYP2E1 was observed (Fig. 7). The average activity of CYP2E1 protein in water-treated mice was 0.57 nmol/mg/min. In comparison the average CYP2E1 activity of SFN-treated mice was 0.54 nmol/mg/min, which was not statistically different.

Though SFN had no apparent effect on CYP2E1, it caused a robust increase in Nrf2 protein (Fig. 8). Six hours after SFN treatment, Nrf2 was present in both nuclear and cytosolic cell fractions at levels roughly 2x greater than control. The ratio of nuclear Nrf2 to cytosolic Nrf2 was unaffected by treatment.

**SFN did not significantly increase the mRNA levels of antioxidant genes**

Because Nrf2 is a transcription factor for antioxidant-related genes, the mRNA levels of such genes should indirectly indicate the presence of Nrf2 in the nucleus. Two well-studied antioxidant genes were chosen as potential representatives of increased nuclear localization: heme oxygenase 1 (HO-1) and NAD(P)H:quinone oxidoreductase 1 (Nqo1).
Six hours after SFN there is an apparent trend toward increased transcription of Nqo1 and HO-1. This trend is not observed at 24 hours after SFN. At neither time was there a significant difference in transcription between treated subjects and controls.
DISCUSSION

Oxidative stress is a known mediator of several pathologies, such as drug-induced liver failure. In spite of this, it has been shown that compounds that directly interact with oxidant species are often ineffective, and at times may worsen clinical outcomes. Sulforaphane was used here to investigate the ability of indirect antioxidants to prevent liver injury due to acetaminophen overdose. In contrast to direct antioxidants, SFN prevented liver damage and decreased the levels of several biomarkers for oxidative stress.

As explained in the Introduction, SFN is hypothesized to act through Nrf2, a transcription factor for antioxidant genes. The immunoblot data presented here support this hypothesis. After SFN administration, Nrf2 levels increased in both the nucleus and the cytoplasm. The increase in nuclear Nrf2 may be due to SFN's purported ability to induce conformational change in Keap1, which would allow Nrf2 to move into the nucleus for the purposes of DNA binding. Additionally, the increase in cytosolic Nrf2 supports the finding of Purdom-Dickinson et al. that enhanced translation of Nrf2 is a central feature of the antioxidant response.\(^{(35)}\)

In parallel to its direct effect on Nrf2, SFN acted on some of the downstream targets of the transcriptional pathway. Mice treated with SFN had substantial augmentation of total cellular glutathione and glutathione reductase activity. These same mice were also protected from liver injury. Implied here is a specific, Nrf2-mediated mechanism by which SFN was protective. Maintenance of cellular thiols is especially important after acetaminophen overdose. SFN-treated animals maintained total cellular glutathione better than untreated animals; therefore, they were better able to remove the toxic APAP metabolite NAPQI before it could cause more extensive hepatic damage.

It must be noted that SFN, for all of its effects on Nrf2 and Nrf2's downstream targets, did not appear to influence mRNA levels of the antioxidant genes Nqo1 and HO-1. The reason for this is not immediately clear and might be attributed to several variables. Response to SFN has
been shown to differ according to species, target tissue, dose, and method of administration. Thimmulappa et al. reported an increase in hepatic Nqo1 mRNA after giving SFN i.g. at a dose of 9 μmol/mouse/day × 7 days.\textsuperscript{(36)} Robbins et al. reported no such increase in mRNA after 2 weeks of feeding a standardized diet which provided a dose of 18.96 μmol total glucosinolates/day.\textsuperscript{(37)} The dose chosen for this experiment – 10 mg/kg/day – is in between those chosen by Thimmulappa and Robbins, being equivalent to 56.4 μmol/kg/day. Due to the different times and routes of administration used in each study, however, it is difficult to extrapolate a cause for the variation in Nqo1 mRNA levels. Furthermore, at this time there are no published studies of hepatic mRNA expression using SFN at or near 10 mg/kg. In the future it will be necessary to test a wider assortment of genes over a range of doses so that SFN’s effects on gene expression may be better characterized.

Taken together, the results of this study indicate that SFN prevents acute liver injury after acetaminophen overdose. Specifically, SFN protected the liver by preventing the oxidative stress that invariably precedes APAP-induced hepatic failure. The data presented here support the role of alleviation of oxidative stress in APAP overdose and suggest a potential role for indirect antioxidants in clinical practice.
APAP overdose is the leading cause of fulminant hepatic failure,(38) yet there are few viable approaches to treating or preventing APAP-related liver injury. The FDA's approval in 1985 of oral n-acetylcysteine (NAC) remains an important medical breakthrough, but aside from the introduction of an injectable form of NAC in 2004, there have been no new drugs specifically indicated for APAP overdose in over 20 years. Meanwhile, the incidence of APAP-related toxicity shows no signs of decline,(39;40) so there is still a need for new and innovative therapies.

One potential new therapy for APAP toxicity is the use of Nrf2 inducers. Numerous studies, several of which are referenced in this text, identify Nrf2 induction as a critical mediator of drug and xenobiotic metabolism. The experiments described here build on those earlier studies by demonstrating the application of the Nrf2 inducer SFN in a model of APAP overdose. The fundamental goal of this work was to determine the potential toxicologic utility of Nrf2 inducers such as SFN.

At this time there is no established protocol for administration of Nrf2 inducers in cases of liver injury. The ultimate development of new therapies for APAP-induced hepatic failure will require extensive additional research. These data, however, represent a small step toward the realization of a new approach to a widespread and costly public health problem. By demonstrating the efficacy of sulforaphane in vivo, this work provides further proof of concept for an Nrf2-based approach to APAP-induced liver injury.
Fig. 1: Simplified model of Nrf2 induction by sulforaphane
Sulforaphane's (SFN) electrophilic side chain alkylates cysteine residues on Keap1. The conformational change induced in Keap1 allows the release of its binding partner, Nrf2. Unbound Nrf2 translocates to the nucleus and binds to antioxidant response elements on DNA.
Fig. 2: Effect of acetaminophen and sulforaphane on plasma parameters
Male C57BL/6J mice were pretreated with water or sulforaphane for 4 days and injected with APAP or saline vehicle on day 5 as described in Methods. The plasma ALT and AST levels were analyzed. Data are means ± SEM (n = 4-6). 
\[ ^a p < 0.05 \] compared to saline injection; 
\[ ^b p < 0.05 \] compared to APAP alone.
Fig. 3: Effect of acetaminophen and sulforaphane on hepatic ultrastructure

Representative photomicrographs (200x) of hematoxylin and eosin (panel A) and chloroacetate esterase (panel B) are shown. Inflammation and coagulative necrosis of pericentral regions is observable in the APAP-treated groups.
Fig. 4: Effect of SFN on total hepatic glutathione
Male C57BL/6J mice were pretreated with water or SFN for 4 days and sacrificed 6h after last treatment, as described in Methods. Total glutathione was determined by spectrophotometry. Data are means ± SEM (n = 4). *p<0.05 compared to water alone.
Fig. 5: Effect of APAP and SFN on glutathione content and synthesis
Hepatic levels of total glutathione and glutathione reductase activity were determined by spectrophotometry. Data are means ± SEM (n = 4). \( ^a p < 0.05 \) compared to saline injection; \( ^b p < 0.05 \) compared to APAP alone.
Fig. 6: Effect of APAP and SFN on membrane lipid peroxidation
Adducts of 4-hydroxynonenal were detected by immunohistochemistry as described previously. (15)
Fig. 7: CYP2E1 activity

Activity of the microsomal enzyme CYP2E1 was determined by observing the conversion of p-nitrophenol to 4-nitocatechol. Data are reported as means ± SEM (n = 4).
Fig. 8: Nuclear accumulation of Nrf2
Male C57BL/6J mice were treated with water or SFN alone and immunoblots were performed as described in Methods. Representative immunoblots for Nrf2 at the 6 h time point are shown (top panel). Nrf2 protein levels were normalized to loading controls and quantified by densitometry (bottom panels).
REFERENCES


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ABSTRACTS


Tan M, Schmidt RH, Zhong H, States J, and Arteel GE (2011) Chronic Low-Dose Arsenic Exposure Enhances Hepatic Injury Induced by High Fat Diet in Mice. The Toxicologist 120:429

PRESENTATIONS

05/2009
Research Seminar – “Effects of Rapamycin on Hepatic Steatosis Caused by Ethanol”
University of Louisville Seminar in Pharmacology and Toxicology
Louisville, KY

09/2010
Presentation – “Sulforaphane prevents acetaminophen-induced hepatic injury in mice”
Ohio Valley Society of Toxicology Annual Meeting
Cincinnati, OH

10/2010
Poster Presentation – “Sulforaphane prevents acetaminophen-induced hepatic injury in mice”
Research! Louisville
Louisville, KY

03/2011
Poster Presentation – “Sulforaphane prevents acetaminophen-induced hepatic injury in mice”
Society of Toxicology Annual Meeting
Washington, DC