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PRECLINICAL STUDY

Transcriptomic and proteomic profiling of KEAP1 disrupted and sulforaphane-treated human breast epithelial cells reveals common expression profiles

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Abstract Sulforaphane (SFN), an isothiocyanate found in cruciferous vegetables, is a potent inhibitor of experimental mammary carcinogenesis and may be an effective, safe chemopreventive agent for use in humans. SFN acts in part on the Keap1/Nrf2 pathway to regulate a battery of cytoprotective genes. In this study, transcriptomic and proteomic changes in the estrogen receptor negative, non-tumorigenic human breast epithelial MCF10A cell line were analyzed following SFN treatment or KEAP1 knockdown with siRNA using microarray and stable isotopic labeling with amino acids in culture (SILAC), respectively. Changes in selected transcripts and proteins were confirmed by PCR and Western blot in MCF10A and MCF12A cells. There was strong correlation between the transcriptomic and proteomic responses in both the SFN treatment ($R = 0.679$, $P < 0.05$) and KEAP1 knockdown ($R = 0.853$, $P < 0.05$) experiments. Common pathways for SFN treatment and KEAP1 knockdown were xenobiotic metabolism and antioxidants, glutathione metabolism, carbohydrate metabolism, and NADH/NADPH regeneration. Moreover, these pathways were most prominent in both the transcriptomic and the proteomic analyses. The aldo–keto reductase family members, AKR1B10, AKR1C1, AKR1C2 and AKR1C3, as well as NQO1 and ALDH3A1, were highly upregulated at both the transcriptomic and the proteomic levels. Collectively, these studies served to identify potential biomarkers that can be used in clinical trials to investigate the initial pharmacodynamic action of SFN in the breast.

Keywords Sulforaphane · Prevention · Keap1/Nrf2 pathway · Biomarker discovery · Microarray · SILAC

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Abbreviations

SFN	Sulforaphane
SILAC	Stable isotopic labeling with amino acids in culture
ER	Estrogen receptor
Nrf2	Nuclear factor-E2-related factor 2
Keap1	Kelch-like ECH-associated protein 1
ARE	Antioxidant response element
siRNA	Small interfering ribonucleic acid
AKR	Aldo–keto reductase
HSD	Hydroxysteroid dehydrogenase

Introduction

Breast cancer remains a significant worldwide public health concern despite advances in early detection and treatment. In the United States, breast cancer is currently the greatest contributor to cancer incidence and the second cause of cancer mortality in women [1]. Combating this disease before it ensues can reduce incidence and deaths considerably. The selective estrogen receptor (ER) modulators, tamoxifen, and raloxifene, are the only United States Food and Drug Administration approved chemoprevention drugs for women with elevated breast cancer risk. The perception of adverse side effects with these drugs [2] coupled with lack of well-developed chemopreventive options for the often more aggressive ER negative cancers calls for new molecular targets for breast cancer prevention.

A potential chemopreventive agent is sulforaphane (SFN), an isothiocyanate found in cruciferous vegetables with particularly high levels in 3-day old broccoli sprouts [3]. It is converted by hydrolysis of the glucosinolate, glucoraphanin, by the enzyme myrosinase. SFN is an attractive chemopreventive agent because it is safe and can be distributed widely as broccoli sprout extract (BSE) preparations [4, 5]. The best characterized mechanism through which SFN protects cells from endogenous and exogenous carcinogenic damage [6] is by induction of detoxication and antioxidant enzymes such as NAD(P)H:quinone oxidoreductase (NQO1), the aldo–keto reductase (AKR) family of enzymes, and heme oxygenase-1 (HMOX1) [7–10]. Enzyme transcripts are induced when the Nuclear factor-E2-Related Factor 2 (Nrf2) transcription factor binds to the antioxidant response element (ARE) at the regulatory regions of these genes [11]. Nrf2 is normally sequestered in the cytoplasm by an inhibitory interaction with Kelch-like ECH-Associated Protein 1 (Keap1). SFN interacts with critical cysteines in Keap1, thereby disrupting Keap1-facilitated ubiquitination and subsequent proteasomal degradation of Nrf2 [12] and allowing Nrf2 to translocate into the nucleus and modulate expression of its target genes. Other potential mechanisms of SFN action include antiproliferative effects, NF-κB DNA binding inhibition, apoptosis activation, and histone deacetylase inhibition [13, 14]. Based on its varied molecular targets, SFN has the potential to prevent breast cancer irrespective of ER status.

When 3-day-old BSE preparations were given to female rats treated with 7-12-dimethylbenz[a]anthracene, the number, size, and rate of mammary tumor development were significantly reduced [3, 15]. Upregulation of *Nqo1* and *Hmox1* transcripts, as well as NQO1 activity and HMOX1 protein levels was observed in rat mammary glands after SFN treatment [16]. Transcriptomic and proteomic studies analyzing SFN regulation have focused on rodent cells [8, 9, 17–21] and human cancer cells [22–26]. However, the effects of SFN on non-cancerous human cells are not known.

Standardized BSE preparations with defined concentrations of SFN and glucoraphanin have been developed and the metabolism and elimination pharmacokinetics of SFN have been measured [4, 27, 28]. However, there is a need for biomarkers that effectively define the pharmacodynamic action of SFN in human tissues. In this preclinical study, we treated the human ER negative [29] non-tumorigenic [30] MCF10A cell line with SFN to analyze global transcript and protein expression changes using microarray and SILAC technologies, respectively. To affirm the role of Nrf2 signaling in the pharmacodynamic action of SFN in non-cancerous human cells, siRNA against KEAP1 was utilized to provide a parallel genetic mechanism to increase Nrf2 signaling. Several genes and proteins with low constitutive expression, but with a broad dynamic range of induction following pharmacologic or genetic stimulation, were identified. Such properties define potentially useful biomarkers for evaluating the mechanism of action and optimizing the dose and schedule of broccoli sprout preparations in clinical trials, especially those targeting the breast.

Materials and methods

Chemicals

R,S-Sulforaphane was purchased from LKT Laboratories (St. Paul, MN). Acetonitrile (ACN) was from MP Biomedicals (Solon, OH).

Cell culture

MCF10A and MCF12A (American Type Culture Collection, Manassas, VA) cells were cultured in (DMEM)/F12 minus L-lysine and L-arginine for SILAC. Medium was supplemented with 5% horse serum, 20 ng/ml epidermal growth factor, 0.5 μg/ml hydrocortisone, 100 ng/ml cholera toxin, and 10 μg/ml insulin at 37°C in a humidified environment with 5% CO_2. For light medium $^{12}C_6$ L-lysine:2HCL and

$^{12}C_6$ L-arginine-HCl were supplemented and for heavy medium $^{13}C_6$ L-lysine:2HCl and $^{13}C_6$ L-arginine:HCl were added (Cambridge Isotope Laboratories, Andover, MA). Cells were transfected with 30 nM KEAP1 or non-targeting control (NTC) siRNA (Dharmacon, Lafayette, CO) in heavy and light media, respectively, according to the Lipofectamine™ RNAiMax reagent (Invitrogen) reverse transfection protocol. Cells were treated with 15 μM SFN or ACN vehicle in heavy and light medium, respectively, 24 h after plating. RNA was collected 24 h and protein collected 48 h post-transfection and SFN treatment (Figs. 1, 2).

Microarray

Total RNA was isolated from cells using TRIZol reagent, and purified by the Qiagen RNeasy mini kit. RNA quality assessment was carried out according to previously published methods [31]. Agilent whole human genome chips (G4112F), with 41,000 unique probes representing 26,705 genes, were used according to the manufacturer's instructions. The SFN treatment and KEAP1 knockdown experiments each had four biological replicates. Data was imported into GeneSpring GX 11.5 (Agilent Technologies) and differentially expressed genes were identified by unpaired *t* test with a cut-off $P < 0.05$. Correction for false discovery rate (FDR) of 5% was made using the Benjamini–Hochberg procedure and a 1.5-fold change cut-off was implemented. The microarray data have been deposited in NCBI's Gene Expression Omnibus [32] and are accessible through GEO Series accession number GSE28813 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE28813). Agilent probe identification numbers and corresponding fold change values were exported to Ingenuity Pathway Analysis (IPA) software (Ingenuity® Systems, Redwood City, CA).

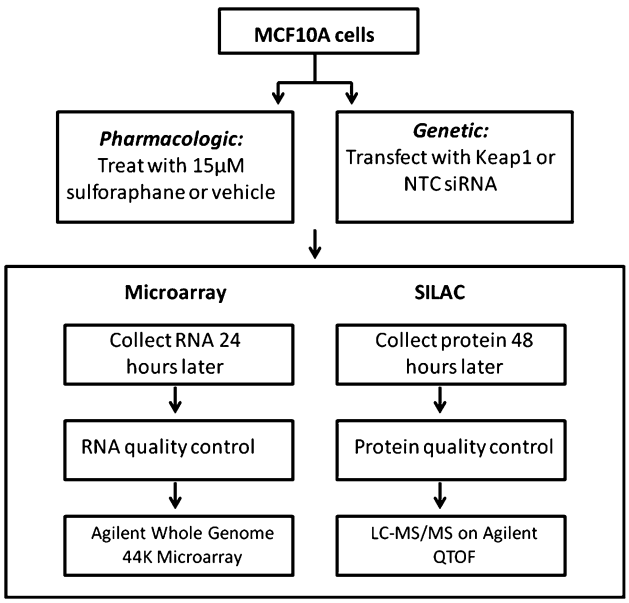


Fig. 1 Workflow for microarray and SILAC experiments. The vehicle used for SFN was acetonitrile. *NTC* non-targeting control, *LC–MS/MS* liquid chromatography tandem mass spectrometry, *QTOF* quadripole time of flight

Quantiative real-time polymerase chain reaction (qRT-PCR)

The qScript™ cDNA synthesis kit (Quanta BioSciences, Gaithesburg, MD) was used to convert 1 μg RNA to 50 ng/μl cDNA. TaqMan Gene Expression Assays and Master Mix (Applied Biosystems, Foster City, CA) were then used to amplify 10 ng/μl cDNA with *TBP* as the endogenous control. Fold-change values were determined using the $2^{-\Delta\Delta Ct}$ relative quantification method [33].

SILAC

Protein was extracted in 8 M urea (Thermo Scientific) and the in-gel trypsin digestion method for SILAC was followed according to previously published protocols [34]. Peptides were analyzed using the Agilent 6538-accurate-mass QTOF mass spectrometer. A technical replicate was run under the same conditions. The MS data were searched and quantified at an FDR of 1% using Spectrum Mill MS Proteomics Workbench (Agilent, Rev A.03.03) using the Human RefSeq 35 protein sequence database (34,906 sequences). Proteins with single unique peptide identification from Spectrum Mill were confirmed by manual inspection of MS/MS spectra. The complete set of raw data (.d files) generated from this study can be downloaded from ProteomeCommons.org Tranche using the following hash: 5/PnW2mDvgBj6k3slbmIwlSAbRR9/mKv67uSvVENhZKwLXvTSVJi3aIIXAPukCI0WtR/ZSaEW/E4g8958fyRGw0eXhAAAAAAAACJ9A==. The protein accession numbers and their corresponding protein fold changes were exported to IPA.

Immunoblot analysis

Protein lysates were resolved on 4–20% gradient polyacrylamide gels and transferred to nitrocellulose. Membranes were blocked in Odyssey® blocking buffer (LI-COR Biosciences, Linocln, Nebraska), and then incubated with the following primary antibodies: 1:750 mouse anti-NQO1, 1:1500 rabbit anti-GAPDH (Cell Signaling Technology, Boston, MA); 1:750 mouse anti-AKRIC1, 1:1000 l rabbit anti-AKRIC3, 1:750 mouse anti-AKRB10, 1:500 mouse anti-GCLC, 1:2000 rabbit anti-BETA-ACTIN (Abcam, Cambridge, MA); 1:1000 rabbit anti-ALDH3A1 and 1:750 rabbit anti-KEAP1 (Proteintech group, Chicago, IL); 1:1250 mouse anti-SQSTM1 (Santa Cruz Biotechnology, Santa Cruz, CA). The blots were then incubated with IRDye®

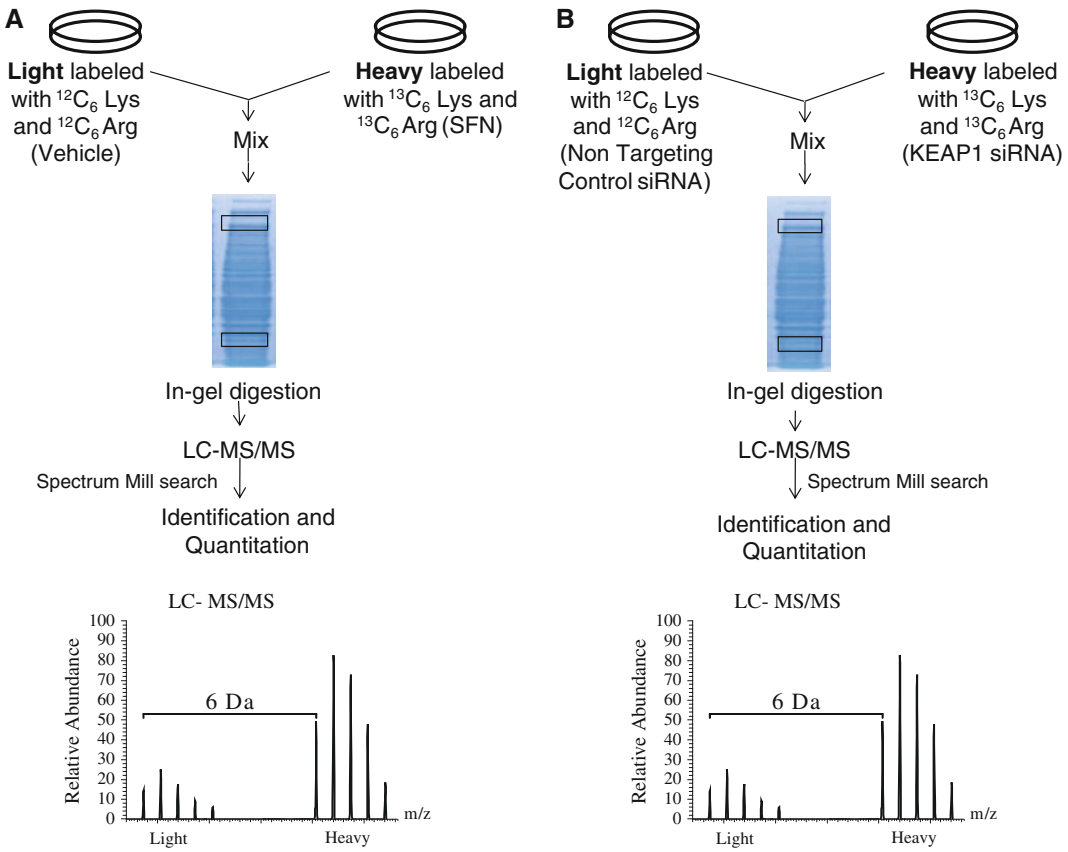


Fig. 2 Detailed workflow for SILAC experiments. The vehicle used for SFN was acetonitrile. *Lys* lysine, *Arg* arginine. Prototypical MS traces from LC–MS/MS are shown indicating the 6 Dalton (Da) shift between light and heavy labeled amino acid isotopes

fluorescent secondary antibodies and scanned with the Odyssey® Infrared Imaging System (LI-COR). The infrared fluorescence densitometry ratios for treated samples compared to controls were determined for three biological replicates and normalization was to GAPDH or BETA-ACTIN.

Results

MCF10A cells were treated with SFN or *KEAP1* siRNA to provide pharmacologic and genetic means to alter Nrf2 signaling; global gene and protein expression were then analyzed by microarray and SILAC, respectively (Figs. 1, 2). *KEAP1* transcripts were knocked down by 81% in the MCF10A cells (Online Resource (OR) 1, Table 3) while KEAP1 protein levels decreased 79% (Fig. 4; OR1, Table 4). For the microarray analyses there were 6,378 transcripts significantly regulated by SFN above and below the chosen 1.5-fold change cut-off and 1,710 transcripts significantly regulated by KEAP1 knockdown. The overlap between these two experiments was 879 transcripts. The main focus for the microarray pathway analyses was those transcripts shown to be regulated by both SFN treatment and KEAP1 knockdown. The top pathways to emerge for this subset of genes were xenobiotic metabolism and antioxidants, glutathione metabolism, carbohydrate metabolism, and NADH/NADPH regeneration.

The SILAC analysis indicated a normal distribution with the majority of proteins minimally regulated and a small percentage of proteins upregulated and downregulated above and below a 1.5-fold change cut-off (Fig. 3). With SFN treatment, of the 666 proteins that were detected by the mass spectrometer, 96 proteins were upregulated and 26 were downregulated above and below a 1.5-fold change cut-off, respectively. For KEAP1 knockdown, of the 1,102 proteins that were detected, 50 were upregulated and 76 were downregulated. The overlap for these two experiments, within the 1.5-fold change cut-off, was 29 proteins. Pathway analysis of the genetic and pharmacologic SILAC experiments yielded xenobiotic metabolism and antioxidants, glutathione metabolism, carbohydrate metabolism, and NADH/NADPH regeneration as top regulated pathways, in agreement with the microarray analysis. The members of the top gene and protein IPA-based functional groups are summarized in Table 1.

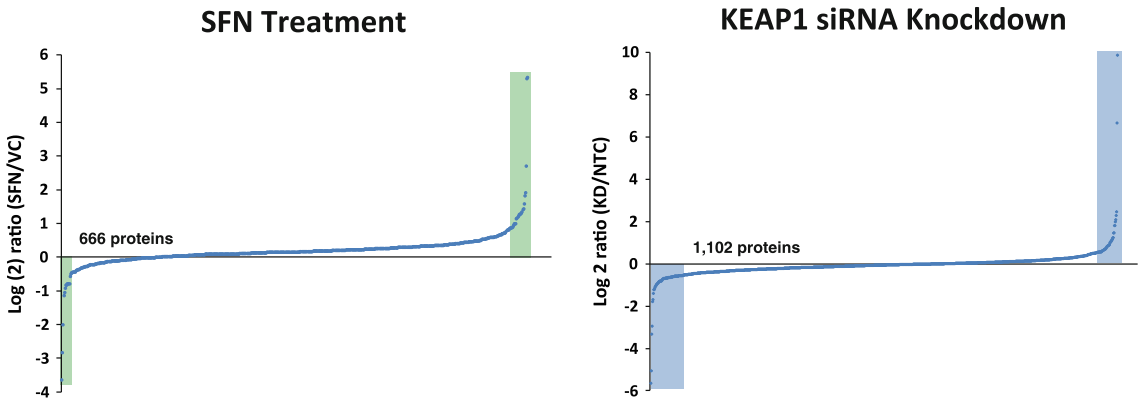


Fig. 3 *Left* distribution of fold changes in proteins determined by SILAC between vehicle and SFN treated MCF-10A cells. *Highlighted* are the 96 upregulated and 26 downregulated proteins above and below a 1.5-fold change cut-off, respectively. *VC* vehicle control. *Right* distribution of fold changes in proteins determined by SILAC between non-targeting control and KEAP1 knockdown siRNA treated MCF-10A cells. *Highlighted* are the 50 upregulated and 76 downregulated proteins above and below a 1.5-fold change cut-off, respectively. *NTC* non-targeting control, *KEAP1 KD* KEAP1 knockdown

The xenobiotic and antioxidant transcripts and proteins were the predominant group regulated by SFN treatment and KEAP1 knockdown

Key genes and proteins involved in xenobiotic metabolism were regulated by SFN treatment and/or *KEAP1* knockdown in both microarray and SILAC experiments included *AKR1* subfamily members, *NQO1*, *CBR1*, *ALDH3A1*, and *EPHX1* (Table 1). The antioxidant genes *TXNRD1*, *FTH*, *BLVRA*, and *TXN* were also coordinately regulated. The genes, *NQO1*, *AKR1B10*, *AKR1C1*, *AKR1C2*, *HMOX1*, *GPX2*, *TXNRD1*, *TXN*, *FTH*, *FTL*, *GSR*, and *PRDX1*, have been shown to have AREs [35–38]. Strikingly, the most highly upregulated transcripts and proteins were the AKR1 subfamily members. AKR1B10 was the most highly upregulated transcript with 302.9- and 69.4-fold increases by SFN treatment and KEAP1 knockdown, respectively (Table 1). While AKR1B10 was not observed by SILAC, Western blot analysis showed that this protein was dramatically upregulated by SFN treatment and KEAP1 knockdown (Fig. 4). AKR1C1 and AKR1C3 also had high transcript levels compared to the other xenobiotic metabolism and antioxidant genes. AKR1C3 was one of the most highly upregulated proteins in the SFN treatment SILAC experiment at 39.3-fold, and was upregulated by 10-fold in the KEAP1 knockdown SILAC experiment. The AKR1C1 and AKR1C2 family members were collectively referred to as AKR1C1/2 because the mass spectrometry and immunoblot techniques were not able to differentiate conclusively between them (OR1, Figure 1). AKR1C1 and AKR1C2 differ by only 7 amino acids [39]. The differences in nucleotide sequence enabled the design of specific primer probes used to detect AKR1C1 for the qRT-PCR experiment (OR1, Table 3). AKR1C1/2 was highly upregulated in the SFN treatment SILAC experiment but less so in the KEAP1 knockdown SILAC (Table 1). AKR1B1 was upregulated to lower levels compared to the other AKR1 subfamily members in both the microarray and the SILAC experiments.

Of the 43 transcripts regulated in this class, 14 were correspondingly altered by SFN treatment and/or KEAP1 knockdown using SILAC (Table 1; OR1, Tables 1 and 2). The transcript and protein levels correlated well in terms of the direction of the fold change. This was clearly seen with the AKR1 subfamily members, *NQO1*, *TXN*, *CBR1*, *ALDH1B1*, and *FTH1* for which the direction and magnitude of the fold change were well correlated. For *AKR1C1*, *ALDH3A1*, *EPHX1*, and *BLVRA*, although the magnitude of the fold changes for the microarray and SILAC were not strongly correlated, they were upregulated in all cases. There were few downregulated transcripts and in one case, *ALDH1B1*, both transcript and protein decreased. In addition to the AKR1 subfamily other families that were coordinately regulated by SFN treatment and/or KEAP1 knockdown included the ALDH, GST, FTH, UBE, HSP, and TXN families. Some transcripts and proteins that modulate the KEAP1/NRF2 pathway were regulated by SFN treatment or KEAP1 knockdown as well. *MAFG* transcript levels were upregulated by 2.3- and 2.9-fold in the SFN treatment and KEAP1 knockdown experiments, respectively (OR1, Table 3). *SQSTM1* was upregulated by 6.1- and 2.1-fold by SFN treatment and KEAP1 knockdown, respectively (OR1, Tables 1 and 2) in the microarray. SQSTM1 was also upregulated by 4.3-fold with KEAP1 knockdown in the SILAC and was shown to be upregulated by SFN treatment by Western blot analysis (Fig. 4).

Table 1 Functional groups for transcriptomic and proteomic experiments

Gene symbol	Description	SFN fold change		KEAP1 knockdown fold change	
		Microarray	SILAC	Microarray	SILAC
Xenobiotic metabolism and antioxidants					
AKR1B1	Aldo–keto reductase family 1, member B1	3.4	–	2.3	2.3
AKR1B10	Aldo–keto reductase family 1, member B10	302.9	–	69.4	–
AKR1C1/2	Aldo–keto reductase family 1, member C1/2	14.8	30.7	34.7	9.2
AKR1C3	Aldo–keto reductase family 1, member C3	27.0	39.3	16.0	10.0
NQO1	NAD(P)H:quinone oxidoreductase	4.4	3.7	6.7	4.4
ALDH3A1	Aldehyde dehydrogenase 3 family, member A1	4.1	6.2	26.3	5.4
ALDH3A2	Aldehyde dehydrogenase 3 family, member A2	-	–	1.9	–
ALDH1B1	Aldehyde dehydrogenase 1 family, member B1	−1.7	–	−2.1	−1.5
TXNRD1	Thioredoxin reductase 1,	10.2	–	7.3	2.8
TXN	Thioredoxin	2.0	1.7	1.5	2.0
TXNDC13	Thioredoxin domain-containing protein 13 precursor	−1.6	–	1.5	–
TMX1	Thioredoxin-related transmembrane protein 1 precursor	-	2.2	-	–
PRDX1	Peroxiredoxin 1	1.6	–	1.5	–
EPHX1	Epoxide hydrolase 1, microsomal (xenobiotic)	4.0	2.5	3.3	1.7
HMOX1	Heme oxygenase (decycling) 1	9.6	–	2.5	–
SULT1A1	Sulfotransferase family, cytosolic, 1A, phenol-preferring, member 1	1.7	–	1.5	–
SULT1A2	Sulfotransferase family, cytosolic, 1A, phenol-preferring, member 2	4.4	–	2.3	–
SRXN1	Sulfiredoxin 1 homolog (S. cerevisiae)	6.0	–	4.0	–
LTB4DH	Leukotriene B4 12-hydroxydehydrogenase	10.8	–	3.9	–
GPX2	Glutathione peroxidase 2 (gastrointestinal)	2.7	–	9.1	–
GPX8	Glutathione peroxidase 8	−1.7	–	−2.6	–
MGST1	Microsomal glutathione S-transferase 1	1.5	–	1.7	-
GSTM1	Glutathione S-transferase M1	1.7	–	1.9	–
GSTM4	Glutathione S-transferase M4	1.6	–	1.7	–
BLVRB	Biliverdin reductase B (flavin reductase (NADPH))	3.3	–	1.5	–
BLVRA	Biliverdin reductase A	1.5	4.6	-	–
UGT1A6	UDP glucuronosyltransferase 1 family, polypeptide A6,	26.5	–	13.0	–
CBR1	Carbonyl reductase 1	1.6	1.6	1.6	–
CBR3	Carbonyl reductase 3	1.9	–	4.1	–
DNAJB4	DnaJ (Hsp40) homolog, subfamily B, member 4	5.4	–	2.7	–
DNAJB9	DnaJ (Hsp40) homolog, subfamily B, member 9	2.1	–	1.5	–
FTH1	Ferritin, heavy polypeptide 1	2.1	1.5	2.1	–
FTHL12	Ferritin, heavy polypeptide-like 12	2.9	–	2.5	–
FTHL17	Ferritin, heavy polypeptide-like 17	2.7	–	2.0	–
FTL	Ferritin, light polypeptide	13.8	–	5.4	–
UBE2H	Ubiquitin-conjugating enzyme E2H (UBC8 homolog, yeast)	2.3	–	1.7	–
UBE2S	Ubiquitin-conjugating enzyme E2S	−1.6	–	-	–
UBE2K	Ubiquitin-conjugating enzyme E2 K (UBC1 homolog, yeast)1	−2.1	–	−2.4	–
HSPB8	Heat shock 22 kDa protein 8	8.2	–	2.1	–
HSPA1A	Heat shock 70 kDa protein 1A	1.8	–	−1.9	–
HSPC105	NAD(P) dependent steroid dehydrogenase-like	1.5	–	1.9	–
AHSA1	Activator of 90 kDa heat shock protein ATPase homolog 1	-	2.5	-	–
ABCC2	ATP-binding cassette, sub-family C (CFTR/MRP), member 2	5.3	–	4.0	–
AOX1	Aldehyde oxidase 1	3.7	–	−2.2	–

Table 1 continued

Gene symbol	Description	SFN fold change		KEAP1 knockdown fold change	
		Microarray	SILAC	Microarray	SILAC
Glutathione metabolism					
GCLC	Glutamate-cysteine ligase, catlytic subunit	4.5	2.9	3.3	2.4
GCLM	Glutamate-cysteine ligase, modifier subunit	2.6	2.7	3.3	2.4
GSR	Glutathione reductase	2.9	1.7	2.2	–
GLRX	Glutaredoxin 1	3.9	2.4	2.0	–
GLS	Glutaminase	2.7	–	1.7	–
GGT1	Gamma-glutamyltransferase 1	1.7	–	2.2	–
GGTLA4	Gamma-glutamyltransferase-like activity 4	1.8	–	2.5	–
Carbohydrate metabolism and NAD(P)H generation					
PGD	Phosphogluconate dehydrogenase	2.1	2.8	2.1	1.6
G6PD	Glucose-6-phosphate 1-dehydrogenase	2.5	2.1	1.9	2.7
UGDH	UDP-glucose dehydrogenase	2.7	2.5	1.9	1.9
TALDO1	Transaldolase	1.5	–	1.9	1.6
TKT	Transketolase isoform 1	-	1.5	2.1	1.5
HDK1	Hexokinase domain containing 1	146.9	–	29.6	–
HK1	Hexokinase-1 isoform HKI-ta/tb	-	1.5	-	–
PGAM1	Phosphoglycerate mutase 1	−1.6	1.6	-	–
NDUFA4	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 4	-	1.5	-	–
MT-CO2	Cytochrome c oxidase subunit II	-	–	-	−1.5
COX4I1	Cytochrome c oxidase subunit IV isoform 1 precursor	-	–	-	−1.5

Microarray and SILAC results for SFN treatment and KEAP1 knockdown in MCF10A cells. Transcripts that were either not significantly altered in the microarray or were not regulated above or below the 1.5-fold change cut off are denoted by a dash (-). For the SILAC results proteins that were not detected by the mass spectrometer, were not statistically significant or were not regulated above or below the 1.5-fold change cut-off are denoted by a double dash (–)

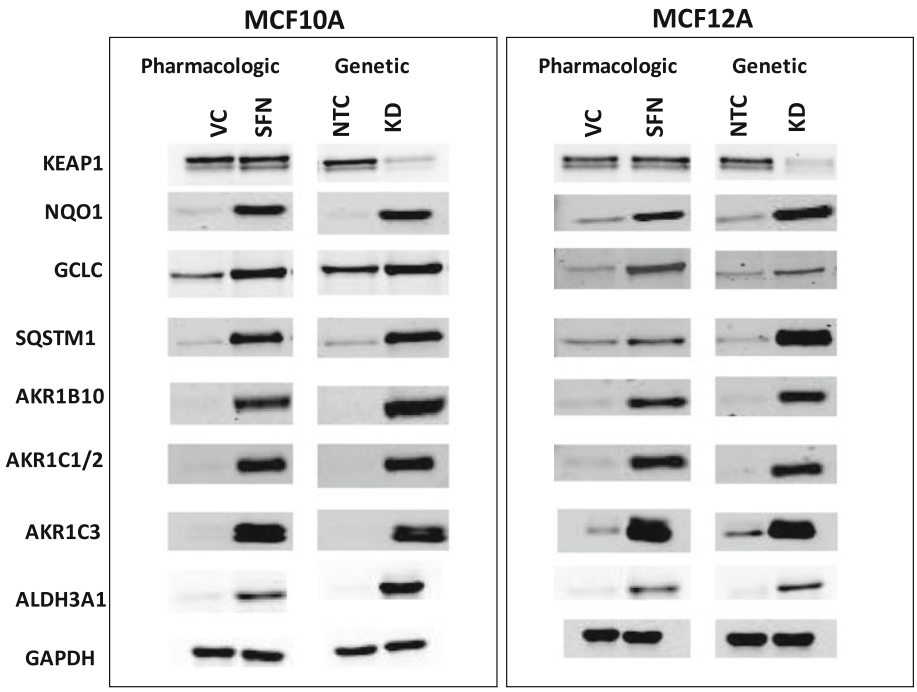


Fig. 4 Western blots for proteins of interest from microarray and SILAC studies showing elevation of protein levels with SFN treatment and KEAP1 knockdown

Expression of glutathione and carbohydrate metabolism transcripts and protein

Half of the transcripts associated with glutathione metabolism were also upregulated at the protein level. *GSR*, *GCLC*, and *GCLM* promoter regions have been shown to contain functional AREs [37]. These genes were also regulated at the protein level, with GCLC and GCLM upregulated by both SFN treatment and KEAP1 knockdown and GSR regulated by SFN treatment only. GCLC and GCLM transcripts and protein were upregulated to similar levels as seen with previous studies [10, 40]. *GLRX* was the only other transcript that was upregulated at the protein level, and it is only regulated by SFN treatment.

Carbohydrate metabolism and NADH/NADPH regeneration are key functions that can be regulated by the KEAP1/NRF2 pathway by genetic and pharmacologic intervention. *G6PD*, *PGD*, and *UGDH* are typically observed in microarray analyses following activation of the Nrf2 pathway [8, 10, 22, 40, 41]. In our study, these enzymes exhibited correlated changes in transcript and protein levels in terms of both direction and magnitude. Within the carbohydrate metabolism and NADH/NADPH regeneration classes different aspects of carbohydrate metabolism were represented. G6PD, PGD, TALDO1, and TKT are key enzymes of the pentose phosphate pathway, while PGAM1, HK1, and HDK1 are involved in glycolysis. HKD1 is the second most highly upregulated transcript in the microarray with a fold change of 146.9 with SFN treatment. Unlike the most highly upregulated transcript AKR1B10, HKD1 did not show elevated protein level in the SILAC. The mitochondrial electron transport chain proteins NDUFA4, MT-CO2, and COX4I1 were regulated exclusively in the SILAC experiments.

Correlation between microarray and SILAC responses

A Spearman rank order correlation analysis between the microarray and the SILAC results indicated a strong correlation for the SFN treatment ($R = 0.679$, $P < 0.05$) and KEAP1 knockdown ($R = 0.853$, $P < 0.05$) experiments in those instances where $\geq$1.5-fold changes were observed. A selected number of genes were validated by qRT-PCR in both MCF10A and MCF12A cells, a second non-malignant human breast epithelial cell line (OR1, Table 3). There was good correlation in the MCF10A cells between the SFN treatment and the KEAP1 knockdown microarray experiments ($R = 0.734$, $P < 0.0001$). There was also very good correlation between MCF10A microarray and qRTPCR data for SFN treatment ($R = 0.953$, $P < 0.0001$) and KEAP1 knockdown ($R = 0.977$, $P < 0.0001$). MCF10A microarray and MCF12A qRTPCR were also well correlated for SFN treatment ($R = 0.762$, $P < 0.0001$) and KEAP1 knockdown ($R = 0.782$, $P < 0.0001$). Finally, the qRT-PCR data for MCF10A and MCF12A correlated well for SFN treatment ($R = 0.821$, $P < 0.0001$) and KEAP1 knockdown ($R = 0.798$, $P < 0.0001$).

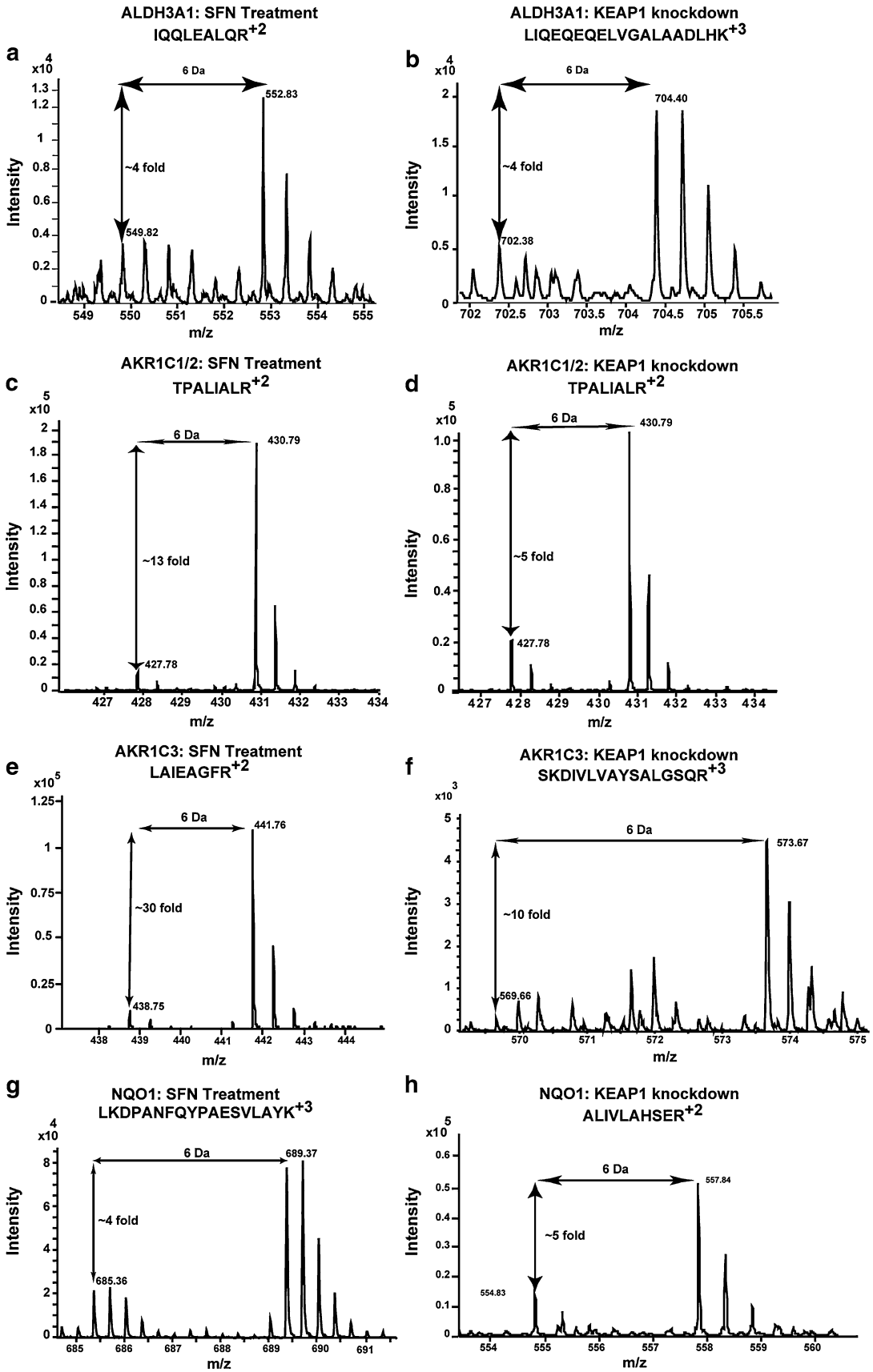
Correlation between Western blot and MS spectra

Western blots of MCF10A and MCF12As reproduced the protein fold changes observed in SILAC experiments. The proteins, NQO1, AKR1C1/2, AKR1C3, AKR1B10, SQSTM1, GCLC, and ALDH3A1, were all shown to be upregulated, as seen by SILAC, whereas KEAP1 was shown to be downregulated (Fig. 4; OR1, Table 4). The MS spectra for four proteins of interest, ALDH3A1, AKR1C1/2, AKR1C3, and NQO1, are shown in Fig. 5 and the SILAC fold changes are represented with arrows. ALDH3A1 and SQSTM1 were not detected in the SFN treatment SILAC and AKR1B10 was not detected in either SILAC experiment at a FDR of 1%, however, they were all shown to be upregulated by Western blot (Fig. 4). The MS spectra for these proteins at a FDR of 5% were searched for and corresponding peaks for ALDH3A1 (Fig. 5a) were found but SQSTM1 and AKR1B10 were not.

Discussion

Although several clinical trials evaluating SFN are in progress, there has been little characterization of its pharmacodynamic action in humans. Few studies have looked at KEAP1/NRF2-mediated gene regulation in normal human cells using the strategy of knocking down KEAP1 [10, 42]. In one of the two published studies using this approach, MacLeod et al. [10] employed microarray analysis in human keratinocytes after KEAP1 knockdown and SFN treatment. Many of the genes that were regulated in their study were also regulated in ours study including *AKR1B1*, *AKR1B10*, *AKR1C1/2*, *AKR1C3*, *NQO1*, *LTB4DH*, *GCLC*, *GCLM*, *GSR*, *G6PD PGD*, *HMOX1*, *SRXN1*, *TXNRD1*, *FTL*, *FTH*, and *MAFG*. These transcripts have also been shown to be upregulated in other microarray experiments using pharmacologic and/or genetic regulation of the KEAP1/NRF2 pathway in rodent tissues [8, 10, 22, 40, 41]. Whereas microarray analyses are common, quantitative proteomic experiments have not, to our knowledge, been used as an unbiased approach to study the proteins regulated by SFN treatment or in response to KEAP1 knockdown in non-cancerous human cells. There is one

Fig. 5 MS spectra from SILAC analyses of candidate biomarker ▶ proteins: **a** ALDH3A1: SFN treatment, **b** ALDH3A1: KEAP1 knockdown, **c** AKR1C1/2: SFN treatment, **d** AKR1C1/2: KEAP1 knockdown, **e** AKR1C3: SFN treatment, **f** AKR1C3: KEAP1 knockdown, **g** NQO1: SFN treatment, and **h** NQO1: KEAP1 knockdown


a
ALDH3A1: SFN Treatment
IQQLEALQR+2
x10 4
6 Da
552.83
~4 fold
549.82
Intensity
m/z
b
ALDH3A1: KEAP1 knockdown
LIQEQEQELVGALAADLHK+3
x10 4
6 Da
704.40
~4 fold
702.38
Intensity
m/z
c
AKR1C1/2: SFN Treatment
TPALIALR+2
x10 5
6 Da
430.79
~13 fold
427.78
Intensity
m/z
d
AKR1C1/2: KEAP1 knockdown
TPALIALR+2
x10 5
6 Da
430.79
~5 fold
427.78
Intensity
m/z
e
AKR1C3: SFN Treatment
LAIEAGFR+2
x10 5
6 Da
441.76
~30 fold
438.75
Intensity
m/z
f
AKR1C3: KEAP1 knockdown
SKDIVLVAYSALGSQR+3
x10 3
6 Da
573.67
~10 fold
569.66
Intensity
m/z
g
NQO1: SFN Treatment
LKDPANFQYPAESVLAYK+3
x10 4
6 Da
689.37
~4 fold
685.36
Intensity
m/z
h
NQO1: KEAP1 knockdown
ALIVLAHSER+2
x10 5
6 Da
557.84
~5 fold
554.83
Intensity
m/z

report of an unbiased proteomic study with isobaric tag for relative and absolute quantitation (iTRAQ) analyzing KEAP1/NRF2 pathway regulation in rodent cells [21]. Two proteome-based studies of SFN action have focused on cancer cell lines [25, 26]. We chose the SILAC strategy for our quantitative proteomic experiment because it allowed comprehensive in vivo labeling of the proteome of cultured cells that could couple global protein expression with a transcriptomic analysis [34]. A straightforward and efficient labeling process allows SILAC experiments to be highly reproducible. Another major benefit of SILAC is virtually no physico-chemical difference between the labeled and natural amino acid isotope, allowing the labeled cells to function identically to the control cells. Apart from *LTB4DH*, *HMOX1*, *SRXN1*, and *MAFG*, all the transcripts commonly regulated in our study and the Macleod study [10] were also upregulated in our SILAC experiment. IPA analysis showed that familiar cytoprotective pathways were regulated in both the transcriptomic and the proteomic data sets further highlighting the fact that well-known KEAP1/NRF2-modulated genes were regulated at both levels. This result provided internal validation for our approach. Another form of validation was the observed upregulation of *MAFG* transcripts and SQSTM1 transcripts and proteins, which serve to positively modulate NRF2 signaling. Small Maf proteins are required for the upregulation of cytoprotective transcripts [11]. The *SQSTM1* gene has a functional ARE and positively modulates the KEAP/NRF2 pathway [43]. ALDH1 activity is a marker of stem cells in normal and malignant human mammary cells [44]. ALDH1B1 has recently been associated with stem cells in normal and cancerous colon tissue [45]. It was downregulated at the transcript level and by Keap1 knockdown at the protein level in our study. SFN has previously been shown to downregulate ALDH positive breast cancer stem cells [46]. ALDH3A1 and A2 which are cytoprotective in normal tissues were upregulated in our studies. The carbohydrate metabolism gene and protein expression correlated very well. NADH is produced from glycolysis and is an essential cofactor for many of the enzymes in the xenobiotic metabolism and antioxidant class. The electrons carried by NADH are fed into the mitochondrial electron transport chain to ultimately produce ATP. The mitochondrial electron transport chain proteins NDUFA4, MT-CO2, and COX4I1 were regulated exclusively at the protein level in our study. NADPH is produced from the pentose phosphate pathway and is also an important coenzyme for xenobiotic metabolism and antioxidant enzymes. NADPH is required for the regeneration of reduced glutathione, GSH, by GSR.

For the microarray, the overlap between the pharmacologic and the genetic experiments was 51% of all the genes regulated by KEAP1 knockdown but only 14% of the genes regulated by SFN treatment. These results indicate that many SFN-regulated transcripts were not regulated through the KEAP1/NRF2 pathway. These are expected since SFN has been shown to affect a number of pathways beyond KEAP1/NRF2 [13]. The number of proteins detected by mass spectrometry in the SILAC experiments was strikingly lower than the number of transcripts differentially regulated in the microarray experiment by SFN. Some proteins may have undergone post-translational modifications leading to diminished identification of proteins. It is most likely that many of the transcripts regulated by SFN treatment were translated to low abundance proteins not detected by mass spectrometry. Incomplete recovery of proteins from all cell compartments is an additional concern.

The AKR1 subfamily were the most highly upregulated family of genes and proteins. In a small clinical trial subjects received a glucosinolate-rich broccoli soup had high levels of AKRC1 and AKR1C2 in their gut mucosa [47]. In this study, as well as other preclinical studies [10, 48], or KEAP1 disruption [10, 42] in cell lines, members of the AKR family were notably highly induced and suggested to be good biomarker candidates. Our studies confirmed that AKR1B1, AKR1B10, AKR1C1, and AKR1C3 were upregulated by the KEAP1/NRF2 pathway at both the transcript and the protein levels. Based on their dynamic upregulation and low basal expression AKR1 family members are potential biomarkers for SFN action in normal breast epithelial cells. AKR1B10 and AKR1B1 are aldose reductases enzymes that generally reduce carbonyls, including cytotoxic α,β-unsaturated carbonyls, to alcohols using NADPH as a cofactor [49]. Members of the AKR1C subfamily of enzymes are hydroxysteroid dehydrogenases (HSD) and have the ability to reduce steroids [50]. AKR1C1 and AKR1C2 reduce progesterone to weak androgens that have been shown to have anti-cancer effects in the breast [39, 51]. AKR1C1 and AKR1C2 protein levels are decreased in breast cancer cell lines and tissue compared to normal cells [52, 53]. The AKR enzymes have been implicated in carcinogenesis [50, 54–59], with AKR1C3 and AKR1B10 overexpressed in breast cancers [59, 60].

MCF10A and MCF12A cells are well-established cell culture models for non-malignant human breast epithelial cells [30, 61] and show marked upregulation of cytoprotective enzymes as a result of SFN treatment or KEAP1 knockdown. Nevertheless, it is important to establish the context in which cytoprotective genes and proteins can be used as biomarkers. In a chemoprevention trial, biomarkers will be used to determine whether the dose of chemopreventive agent administered can reach the putative target cells and upregulate genes and proteins that protect them. Biomarkers that can effectively reflect the pharmacodynamic action of an agent must have specificity and sensitivity. The

biomarker measurements should also be robust and reliable and have a highly dynamic range with little baseline activity. Detection of upregulation of genes and proteins is usually the focus for biomarker discovery because measuring an increase in expression above baseline in biological samples is typically easier and more reliable than measuring a decrease in expression. Based on our preclinical studies AKR1C1, AKR1C2, AKR1C3, AKRB10, NQO1, and ALDH3A1 fulfill these criteria and are candidate biomarkers to assess the pharmacodynamic action of SFN in human breast tissue.

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