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Identification of Potential Protein Targets of Isothiocyanates by Proteomics

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Abstract

Isothiocyanates (ITCs), such as phenethyl isothiocyanate (PEITC) and sulforaphane (SFN), are effective cancer chemopreventive compounds. It is believed that a major mechanism for the cancer preventive activity of ITCs is through induction of cell cycle arrest and apoptosis. However, the upstream molecular targets of ITCs have been underexplored until recently. To identify proteins that are covalently modified by ITCs, human non-small cell lung cancer A549 cells were treated with ¹⁴C-PEITC and ¹⁴C-SFN and the cell lysates were extracted for analysis by 2-D gel electrophoresis and mass spectrometry. After superimposing the colloidal Coomassie blue protein staining pattern with the pattern of radioactivity obtained from X-ray films, it was clear that only a small fraction of cellular proteins contained radioactivity, presumably resulting from selective binding with PEITC or SFN via thiocarbamation. More than 30 proteins with a variety of biological functions were identified with high confidence. Here we report the identities of these potential ITC target proteins and discuss their biological relevance. The discovery of the protein targets may facilitate studies of the mechanisms by which ITCs exert their cancer preventive activity and provide molecular basis for designing more efficacious ITC compounds.

Introduction

Cell culture, animal, and epidemiological studies all support isothiocyanates (ITCs) as promising cancer chemopreventive agents.^{1–4} Tumorigenesis studies in animals indicate that their anti-carcinogenic activity covers all three major stages of tumor growth: initiation, promotion, and progression.^{5, 6} The induction of cell cycle arrest and apoptosis is considered an important mechanism for the cancer preventive activity of ITCs because it may selectively eliminate the initiated cells.^{7–11} However, the upstream molecular targets of ITCs remain mostly unknown.

Identifying the molecular targets of ITCs is an important step toward understanding the mechanisms of their biological effects and designing and screening more efficacious ITC analogs in cancer prevention and therapy. Studies have indicated that ITCs induce reactive oxygen species (ROS), probably via glutathione depletion and inhibition of mitochondria

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activity.^{12, 13} However, owing to their electrophilic nature, ITCs can readily bind covalently with some amino acid residues. For example, ITCs react with thiol-containing cysteines and ϵ -amino containing lysines form thiocarbamates and thiourea, respectively (Figure 1). Although thiocarbamation is reversible and alkylation is irreversible, the rate of thiocarbamation is at least 1000 times faster than alkylation.¹⁴ Since cysteines are often located in the catalytic sites of enzymes, binding to cysteine residues by ITCs may significantly influence enzymatic activity, redox status, and signaling transduction. In fact, earlier studies have implicated binding to cytochrome P450 enzymes and Kelch-like ECH-associated protein 1 (Keap1) in the cancer chemoprevention activities of ITCs.^{15, 16} In a previous study using human lung cancer A549 cells treated with radiolabeled phenethyl isothiocyanate and sulforaphane (¹⁴C-PEITC and ¹⁴C-SFN, see Figure 2), we found binding to glutathione is a major initial event for both PEITC and SFN yet binding to proteins by PEITC is almost 3-fold greater than that of SFN.¹⁷ The level of protein binding by PEITC increased with incubation time, whereas binding by SFN remains consistently low. After 4 hours of incubation, proteins became the predominating targets of PEITC with a 6-fold greater binding compared to SFN and nearly 90% of the PEITC taken up by cells was bound to proteins, indicating that binding to cellular proteins by ITCs is a major event. More importantly, this study also showed that the protein binding affinities of PEITC and SFN correlate with their potency of apoptosis induction.¹⁷

To decipher the potential protein binding targets of ITCs, in this study we treated A549 cells with ¹⁴C-PEITC or ¹⁴C-SFN. The cell lysates obtained after incubation were analyzed by two-dimensional (2-D) gel electrophoresis and the radioactive protein spots were identified using mass spectrometry.¹⁸ More than 30 proteins were identified as potential ITC targets by the method. Among them, tubulin (a major constituent of microtubules) and proteasomes (protein complexes that are essential in degradation of intracellular proteins) were further confirmed as ITC binding targets using a variety of assays in separate studies.^{19–21} Here we report, in addition to tubulin and proteasome, all other potential ITC targets identified in the proteomic study and discuss their biological relevance.

Experiments and Methods

Cells and chemicals—The human lung cancer cell line A549 (ATCC, Rockville, MD) was grown and maintained in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) at 37 °C in 5% CO₂. SFN was provided by Dr. Stephen Hecht (University of Minnesota). ¹⁴C-SFN (50 mCi/mmol) was a gift from Dr. Shantu Amin (Penn State University at Hershey). ¹⁴C-PEITC (55 mCi/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO). The synthesis methods for these two radiolabeled compounds were reported previously.^{22, 23} Sequencing-grade trypsin was obtained from Promega (Madison, WI). PEITC and all other reagents were the highest grade available from Sigma-Aldrich (St. Louis, MO), unless otherwise noted.

2-D gel electrophoresis

The procedures used for 2-D gel electrophoresis were performed according to established protocols.²⁴ Cells at 50% confluency were treated with 20 μ M ¹⁴C-PEITC or ¹⁴C-SFN for 1 h. Cells were washed three times with PBS buffer before being lysed in 7 M urea, 2 M thiourea, 2% CHAPS, 1% ASB-14, 0.2% carrier ampholytes 4–7, 40 mM Tris, pH 6.8, 0.0002% bromophenol blue, protease inhibitor mix (GE Healthcare) on ice for 30 min. The cell lysate was centrifuged at 13,000 $\times g$ for 5 min to remove the insoluble material. The supernatant was adjusted to 1 μ g/KL protein concentration. For the first dimension, isoelectric focusing was conducted using an Ettan IPGphor system (GE Healthcare) according to the user manual. Samples were applied to 24 cm IPG drystrips with ranges of either pH 4–7 or pH 3–10 (Bio-Rad). After 12 h of in-gel rehydration at 50 V, Isoelectric

focusing (IEF) was performed in IEF Cells (Amersham) with the following voltage gradient: (i) 0–500 V gradient in 1 h, (ii) 500–10000 V gradient in 8 h and (iii) 10000 V constant until 80000 Vh. IPG strips were equilibrated in 50 mM BisTris-Cl, 6 M urea, 30% glycerol, 2% SDS, 1% DTT, 0.005% bromophenol blue, pH 6.5. Each strip was loaded on top of a 5–20% Bis-Tris SDS-polyacrylamide gel slab (20 × 26 cm, 1 mm thick), and electrophoresis was performed in 50 mM MOPS, 50 mM Bis-Tris, 0.1% SDS, 1 mM EDTA, pH 6.8, at a constant power of 30 W for 30 min and 100 W for 6 h or until the dye front reached the gel bottom. The proteins in the 2-D gel were stained with colloidal Coomassie blue,²⁵ and proteome images of the lysate labeled with radioactive ITCs were acquired using an Expression 1680 flatbed scanner (Epson). The radioactivity images of gels were obtained by exposing air-dried gels to BioMax MR X-ray film (Kodak) for up to 60 days at –80 °C.

In-gel digestion

Upon superimposing images of Coomassie blue staining and autoradiograph, protein spots with radioactivity were identified and excised manually. Gel pieces were destained in a solution of 50 mM NH_4HCO_3 in 50% (v/v) acetonitrile, with shaking, for 1 h and then dehydrated in 100% acetonitrile for 15 min. After removal of acetonitrile, dehydrated gel pieces were vacuum dried and rehydrated in 25 mM NH_4HCO_3 containing 5 ng/ μL trypsin (sequencing grade trypsin, Promega Corporation, Madison, WI, USA) at 4 °C for 30 min, followed by incubation at 37 °C overnight. Digested peptides were extracted in 70% acetonitrile/5% formic acid. The resulting peptide solution was mixed with an equal volume of 10 mg/ml R-cyano-4-hydroxycinnamic acid in 0.1% trifluoroacetic acid/50% acetonitrile prior to mass spectrometry (MS) analysis.

Mass spectrometry analysis

Samples were analyzed using matrix-assisted laser desorption/ionization-time-of-flight/time-of-flight (MALDI-TOF/TOF) MS and liquid chromatography-tandem mass spectrometry (LC-MS/MS) as previously described.^{26, 27} Tandem mass spectra were searched against the UniProt human protein database from the European Bioinformatics Institute (<http://www.ebi.ac.uk/integr8>). Peptides were considered legitimately identified if they met specific charge state and proteolytic cleavage-dependent cross correlation scores of 1.9 for $[\text{M}+\text{H}]^{1+}$, 2.2 for $[\text{M}+2\text{H}]^{2+}$ and 3.5 for $[\text{M}+3\text{H}]^{3+}$, and a minimum delta correlation of 0.08.

Results and Discussion

ITC binding to proteins is selective

A549 cells were treated with 20 μM of ^{14}C -PEITC or ^{14}C -SFN. This is a physiologically attainable concentration and apoptosis was significantly induced by PEITC, but not SFN, at this concentration.¹⁷ Previously, we found that up to 8% of PEITC and SFN were up-taken in A549 cells at 20 μM and serum concentration did not play a role in uptake of ITCs at this concentration.¹⁷ Cell lysate was fractionated and separated using 2-D gel electrophoresis. Protein images were obtained by Coomassie blue protein staining (Figure 3A and D) and autoradiograph (Figure 3B and E) using x-ray films. Results indicated that only a small fraction of cellular proteins are radioactive, presumably via covalent binding with ITCs, suggesting that binding to proteins by ITCs is a selective process. The observation that spots on the 2-D gels obtained from cells incubated with PEITC generally showed higher radioactivity than that with SFN is consistent with our previous notion that PEITC has higher protein affinity than SFN.¹⁷

Multiple proteins are identified in spots containing radioactivity

Radioactive protein spots were identified by superimposing the Coomassie stained 2-D gel image with that of autoradiograph (Figures 3C and F). Twenty-seven spots with the highest

radioactivity in the gel obtained from cells treated with ^{14}C -PEITC were excised. These slices were individually processed by in-gel trypsin-digestion and identified using MALDI-TOF/TOF and/or LC-MS/MS. Hundreds of peptides were identified within these gel slices with various levels of abundance, as indicated by the total number of identified peptides per given protein (spectral counting). We focused on 53 proteins (Table 1) that were identified by a minimum of three total peptides. Another 30 proteins identified by two peptides are listed in Supplemental Table S1. In the 21 most-radioactive gel spots excised from the 2-D gel of the SFN-treated cells, 49 proteins were identified by at least three total peptides (Table 2). Another 25 protein subunits were identified by two peptides (Supplemental Table S2).

The potential targets of PEITC and SFN are associated with a variety of functions

To understand the biological significance, the potential targets of both PEITC and SFN were compiled according to their sub-cellular organelles or protein pathways (Table 3). Because many of the peptides identified share common sequences with multiple protein isoforms (e.g. are not proteotypic), as shown in Table 1 and 2, the total number of potential target proteins is therefore reduced to 36. Some of the major ones are summarized below.

A. Cytoskeleton proteins—As we previously reported,¹⁸ cysteine-rich tubulin, including alpha and beta isoforms, but not the gamma isoform, was a binding target of ITCs. Benzyl ITC (BITC) and PEITC, but not SFN, significantly inhibit tubulin polymerization *in vitro* and disrupt microtubule *in vivo*. These effects correlated well with their 1) binding affinities on purified tubulin; 2) potencies in inducing tubulin conformational changes; and 3) activities of inducing G₂/M arrest and apoptosis. More importantly, BITC binding adduct on Cys347 of α -tubulin was identified *in vivo* by mass spectrometry,¹⁸ providing convincing evidence for binding to cellular tubulin by an ITC. Actin, a constituent for actin filaments, was also a likely binding target of ITCs. Although the binding sites are yet unknown, it has been reported that Cys285 and Cys374, two highly conserved cysteines in all eukaryote actin isoforms, are sensitive to oxidative stress.²⁸ Another study indicated that Cys374 of actin can be covalently modified by N-ethylmaleimide (NEM) and the modification blocks the inhibitory effects of profilin on actin polymerization.²⁹ Vimentin, a type III intermediate filament protein, has been found to be overexpressed in a variety of cancer cells and has been associated with metastasis and poor patient survival.^{30, 31} Cys328, the only cysteine residue and highly conserved in vertebrate vimentin, has been reported to be modified by cyclopentenone prostaglandin 15-deoxy- $\Delta^{12,14}$ -PGJ₂ (15d-PGJ₂) by Michael addition.³² Previously, we observed vimentin cleavage, which was likely caused by ITC-activated caspase-3.¹⁹

B. Redox regulating proteins—In this study, thioredoxin-1 and glutaredoxin-1 were also identified as potential ITC targets. Previously, ITCs, including allyl ITC (AITC), BITC, PEITC, and SFN, have been shown to significantly inhibit the activities of purified glutathione reductase and thioredoxin reductase in a time-dependent manner with IC₅₀s in the lower micromolar range.³³ Mammalian thioredoxin-1 contains 5 cysteines, including Cys32 and Cys35 which reside in the redox-regulatory domain. Although Cys62, Cys69, and Cys73 are non-active, their posttranslational modifications, including glutathionylation, thiol-oxidation, and S-nitros(yl)ation, are also important in the regulation and functions of thioredoxin-1.³⁴ Glutaredoxins function similarly to thioredoxins. Unlike thioredoxin, which relies on thioredoxin reductase for reduction, glutaredoxins are reduced by the oxidation of glutathione. Covalent modification of the two cysteines in the consensus sequence Cys-Pro-Tyr-Cys of glutaredoxins may inhibit their activity.³⁵

C. Protein quality control system—Proteasome subunits of both 20S catalytic and 19S regulatory complexes were potentially targets of modifications by ITCs. Recently, we showed that BITC and PEITC significantly inhibit proteasome activity in a variety of cell types.²¹ Further studies show that ITCs inhibit both the 26S and 20S proteasomes, presumably through direct binding. The potencies of inhibition of proteasome activity by the ITCs correlated with their abilities to suppress growth of multiple myeloma cells through induction of cell cycle arrest at G₂/M phase and apoptosis.²¹ However, the exact ITC binding sites on proteasomes remain unknown. Another potential interesting ITC target protein is valosin-containing protein (VCP/p97), which transports degraded protein substrates from the endoplasmic reticulum to the cytoplasm via its ATPase activity.³⁶ Cys522 in the D2 domain of VCP has been identified as being sensitive to oxidative stress and the modification of Cys522 by electrophilic agents may irreversibly inhibit VCP activity.³⁷ Since VCP is overexpressed in a few cancer types³⁸ and has been associated with poor prognosis of cancer,³⁹ inhibition of VCP may have implication in cancer therapy.

D. Heat shock proteins (HSPs)—Hsp110, Hsp70, and Hsp27 were found to be potential ITC binding proteins. Hsp110 is another cysteine-rich protein containing 18 cysteines. Reports detailing the activities of these cysteines are rare. The reactive cysteines in Hsp70 are usually found in the vicinity of chaperone nucleotide binding sites such as the ATP-binding site.⁴⁰ Modifications of these sites by NEM have been found to block ATP binding, nucleotide exchange, and consequently inhibit its chaperone activity. Cys137 is the lone cysteine in Hsp27, an ATP-independent small molecular chaperone. It may facilitate dimerization through disulfide formation. A recent study indicated that the C137A mutation provides less protection than wild-type Hsp27 in preventing caspase-3 and caspase-9 activation and apoptosis induced by 1 mM H₂O₂ in CHO and HeLa cells.⁴¹ Additionally, two mitochondrial HSPs, Hsp60 and Hsp10, were also identified. They have been found to be sensitive to electrophilic modification.⁴² A study reported that covalent binding of epolactaene to Cys442 of Hsp60 inhibits its chaperone activity.⁴³ Binding of ITCs to this site may cause similar effects.

E. Mitochondrial proteins—Several studies have indicated that the mitochondria is a primary site for ITCs' action, as BITC and PEITC can induce mitochondrial membrane potential change, cytochrome *c* release, and caspase-9-dependent apoptosis.^{44–46} ITCs have been shown to induce the generation of superoxide radical and hydrogen peroxide through inhibition of Complex I and III activities.^{44, 45} However, the exact protein targets in the mitochondria remain elusive. This study reveals that an important potential ITC target is NADH dehydrogenase (Complex I), which catalyzes the transfer of electrons from NADH to coenzyme Q. It is the first enzyme of the mitochondrial electron transport chain and a potent source of reactive oxygen species (ROS). NEM or 5, 5'-dithio-bis(2-nitrobenzoic acid) (DTNB), both thiol-reactive, have been found to irreversibly modify the deactivated (D)-form of complex I at critical cysteine residue(s), consequently inactivating it. On the contrary, the activated (A)-form of complex I is inert to these agents.⁴⁶ Potentially, the key cysteines in NADH dehydrogenase may be modified by ITCs and the binding may be responsible for ITC-induced mitochondrial activities, including inhibition of respiratory chain and induction of ROS generation. Additionally, several subunits of cytochrome *c* oxidase (Complex IV) and ATP synthase (F₀F₁ Complex) were also identified as potential target proteins of ITCs, suggesting that direct binding of ITCs may underlie mitochondria-mediated apoptosis induction.⁴⁷

F. Signaling regulatory proteins—14-3-3 proteins play key roles in the regulation of cell cycle, apoptosis, and cell survival signaling. Particularly, the interactions between 14-3-3 and Raf are essential for the activation of mitogen activated protein kinase (MAPK)

through oncogenic Ras⁴⁸ and 14-3-3 overexpression transgenic mice develop a variety of tumors.⁴⁹ Therefore, the discovery of various 14-3-3 isoforms as potential binding targets in this study may be of significance. Previously, it was reported that cysteines in 14-3-3 zeta and theta isoforms are binding sites of S-nitrosylation,⁵⁰ suggesting that they are likely to be modified by other thiol-reactive agents, such as ITCs.

Conclusions

ITCs are effective cancer preventive agents. Their major anti-carcinogenic mechanisms can be generalized into direct and indirect effects.⁴ The direct effects refer to covalent binding of proteins at post-synthetic level, such as inhibition of cytochrome P450 enzymes¹⁵ and tubulin-related activity.^{18–20} And the indirect effects refer to alteration of gene expression at transcriptional level, such as induction of phase II and antioxidant enzymes through Keap1-Nrf2 pathway.¹⁶ Literature suggests that binding to target proteins is an important upstream event for both types of activity.⁴ Therefore, identification of target proteins is crucial for better understanding of the mechanisms underlying the cancer preventive activity of ITCs and for future design of compounds with better efficacy.

In this study we identified more than 30 proteins in A549 cells as potential binding targets of both PEITC and SFN (Table 3), some of them are known to be modified covalently by other thiol-reactive compounds, suggesting they could be modified by ITCs. More importantly, our previous studies have provided strong evidence that tubulin is covalently modified *in vivo* by BITC, and that the modification not only causes tubulin conformation and functional changes but also correlates with cell cycle arrest at G₂/M phase and apoptosis induction in A549 cells.^{18–20} Additionally, the activity of proteasome, another protein listed in Table 3, has been shown to be directly inhibited by ITCs and the inhibition correlates with cell growth inhibition of multiple myeloma cells.²¹ These data suggest that proteins in Table 3 are potential ITC binding targets. However, in-depth investigations are needed to ascertain where and how ITCs bind to these proteins and, importantly, whether the binding has any biological consequences.

It is important to note that not all proteins in Table 3 are potential ITC binding targets. Although all gel slices analyzed by mass spectrometry contained radioactivity associated with ITCs, multiple proteins with different numbers of peptide were found in each gel slice. It is likely that some proteins identified in the same spot are not ITC binding targets. Additionally, even though proteins with high confidence, as indicated by three or more identified peptides, were chosen to be listed in Table 1–3, this index can be also affected by protein abundance. Therefore, the identities of *bona fide* ITC binding targets have to be further verified individually by *in vitro* and *in vivo* binding studies. Given the fact that the primary goal of this study was to search for initial clues of potential target proteins, no attempt was made to detect ITC adducts in any of the peptides. Even if some proteins are identified as binding targets, studies are needed to ascertain whether ITC binding induces changes of the protein function, the related signaling pathways, and ultimately the downstream cellular effects. It is conceivable that the biological and cellular effects of ITCs are a result of binding to, not a single, but multiple protein targets. In this study, we identified 30 potential protein targets for PEITC and 16 for SFN. However, only 10 of them, including tubulin, are shared by both PEITC and SFN. The difference in these targets between PEITC and SFN may contribute to their varied downstream activities. For example, in A549 cells PEITC at 10 μ M is shown to induce G₂/M phase arrest, whereas, at the same concentration SFN induces G₁ phase arrest.¹⁸ A greater number of targets were identified for PEITC than SFN, a result that is consistent with our previous studies demonstrating that PEITC is more potent in binding to cellular proteins, including tubulin, than SFN and ITCs with different side-chain groups have different binding affinities.^{17, 18} Importantly, our

results demonstrated that ITCs with greater binding affinities to tubulin are stronger inducers of apoptosis, suggesting an important structure-activity relationship among ITCs.^{18, 19}

Radiolabeling in identification of target proteins has a few advantages over the affinity chromatography in which customized hybrid ITC probes are used.^{51, 52} First and foremost, the binding occurs in living cells, not in cell lysates, in which a significant amount of free thiols either form disulfides or are oxidized during cell lysis required by the affinity column procedure. Therefore, the studies using radiolabeled ITCs are likely to be more physiologically relevant. Moreover, the hydrophobicity, size, and rigidity of the ITC side group may influence its binding to proteins as we have previously demonstrated,^{18, 19, 21} it is possible that the hybrid ITC probes with bulky side chains may alter its binding specificity and affinity. On the other hand, the lack of availability of the radiolabeled compounds may be a hindrance. In addition, the low detection sensitivity due to the weak radioactivity of ¹⁴C presents a bias against proteins with low abundance and/or low binding affinity. This could explain why certain ITC targets, such as macrophage migration inhibitory factor (MIF) recently identified by affinity chromatography,^{51, 52} was not detected in this study. In these studies, N-terminal proline is the only binding site of ITCs despite that two cysteine residues exist in MIF sequence. Lastly, since the stability of thiocarbamates is sensitive to buffer pH value, radiolabeled ITC adducts may be stripped away from its target proteins when buffers with alkaline pH are used during lengthy sample preparation for 2-D gel electrophoresis. In this study, we kept buffer pH under 7 and did not observe substantial loss of radioactivity.

It is important to point out that not all cysteine residues are reactive toward ITCs. The reactivity of a cysteine depends on its protonation state, which usually depends on its micro-environment.⁴ Images of radiolabeling in this study confirm that binding to cellular proteins by ITCs is specific. Also, it should be noted that, while cysteine modification is the primary form,¹⁴ it is conceivable that some 2-D gel spots may contain proteins with modifications on lysine and other residues. Further studies are needed to pinpoint the binding sites and to investigate the biological consequences of these modifications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard abbreviations

AITC	allyl ITC
BITC	Benzyl ITC
DMEM	Dulbecco's modified eagle's medium
HSPs	Heat shock proteins
IEF	Isoelectric focusing
ITCs	isothiocyanates
Keap1	Kelch-like ECH-associated protein 1

MALDI-TOF/TOF	matrix-assisted laser desorption/ionization-time-of-flight/time-of-flight
MIF	macrophage migration inhibitory factor
ROS	reactive oxygen species
PEITC	phenethyl isothiocyanates
SFN	sulforaphane
VCP/p97	valosin-containing protein

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Figure 1.
Schematic modifications of cysteine and lysine residues in protein.

Figure 2.
Structures of ^{14}C - PEITC and ^{14}C -SFN.

Figure 3. The colloidal Coomassie Blue staining (A and D), radioautography (B and E), and superimposed images (C and F) of 2-D gel electrophoresis of the whole cell lysates of A549 treated with 20 μ M 14 C-PEITC (A, B and C) and 14 C-SFN (D, E, and F) for 1 h.

Table 1

Potential protein targets modified by PEITC.

#	Protein Name	UniProt #	Total Peptides Identified
1	Actin, aortic smooth muscle	P62736	62
2	Actin, cytoplasmic 1	P60709	54
3	Tubulin alpha-1 chain	P68366	44
4	Transitional endoplasmic reticulum ATPase	P55072	44
5	10 kDa heat shock protein, mitochondrial	P61604	35
6	ATP synthase beta chain, mitochondrial	P06576	32
7	Heterogeneous nuclear ribonucleoprotein K	P61978	31
8	Tubulin beta-2C chain	P68371	23
9	Heat shock 70 kDa protein 4	P34932	23
10	14-3-3 protein zeta/delta	P63104	22
11	Heterogeneous nuclear ribonucleoprotein F	P52597	22
12	Tubulin alpha-2 chain (Alpha-tubulin 2)	Q13748	20
13	Heat-shock protein beta-1	P04792	17
14	Calpactin I light chain	P60903	17
15	Tropomyosin 1 alpha chain	P09493	16
16	Tropomyosin alpha-3 chain	P06753	14
17	Tubulin alpha-3 chain	Q71U36	14
18	Vimentin	P08670	14
19	Cytochrome b5 type B	O43169	13
20	14-3-3 protein beta/alpha	P31946	12
21	14-3-3 protein theta	P27348	12
22	Myosin light polypeptide 6	P60660	12
23	Proteasome subunit alpha type 3	P25788	10
24	Myosin light polypeptide 6B	P60660	10
25	Tubulin beta-1 chain	Q9H4B7	10
26	Tubulin beta-2 chain	P07437	10
27	Ran-specific GTPase-activating protein	P43487	8
28	Succinyl-CoA ligase [GDP-forming] beta-chain, mitochondrial	Q96199	8
29	Ubiquitin carboxyl-terminal hydrolase isozyme L1	P09936	7
30	40S ribosomal protein S21	P63220	7
31	Ubiquitin carboxyl-terminal hydrolase 14	P54578	7
32	Tumor protein D54	O43399	6
33	Tubulin beta-3 chain	Q13509	6
34	ADAM 7	Q9H2U9	6
35	ATP synthase e chain, mitochondrial	P56385	5
36	Tenascin-X	P22105	5
37	Nuclear migration protein nudC	Q9Y266	5

#	Protein Name	UniProt #	Total Peptides Identified
38	F-box only protein 41	Q8TF61	5
39	Thioredoxin	P10599	4
40	Tropomyosin alpha-4 chain	P67936	4
41	14-3-3 protein epsilon	P62258	4
42	ATP synthase g chain, mitochondrial	O75964	4
43	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily E member 1	Q969G3	4
44	Pappalysin-1	Q13219	4
45	Splicing factor 3A subunit 3	Q12874	4
46	Heat-shock protein 105 kDa	Q92598	4
47	14-3-3 protein eta	Q04917	3
48	Diablo homolog, mitochondrial	Q9NR28	3
49	60S acidic ribosomal protein P2	P05387	3
50	NADH dehydrogenase 1 alpha subcomplex subunit 2	O43678	3
51	Ras-related protein Rab-15	P59190	3
52	26S protease regulatory subunit 6A	P17980	3
53	Mirror-image polydactyly gene 1 protein	Q8TD10	3

Table 2

Potential protein targets modified by SFN.

#	Protein Name	UniProt #	Total Peptides Identified
1	60 kDa heat shock protein, mitochondrial	P10809	80
2	Retinal dehydrogenase 1	P00352	55
3	Vimentin	P08670	40
4	Actin, aortic smooth muscle	P62736	35
5	Stress-70 protein, mitochondrial	P38646	34
6	Tubulin alpha-1 chain	P68366	31
7	Actin, aortic smooth muscle	P62736	27
8	T-complex protein 1 subunit beta	P78371	26
9	Protein disulfide-isomerase	P07237	24
10	Actin, cytoplasmic 1	P60709	24
11	14-3-3 protein epsilon	P62258	23
12	Transitional endoplasmic reticulum ATPase	P55072	21
13	Tubulin beta-2C chain	P68371	20
14	40S ribosomal protein SA	P08865	15
15	Proliferating cell nuclear antigen	P12004	14
16	Heterogeneous nuclear ribonucleoprotein H	P31943	14
17	Heterogeneous nuclear ribonucleoprotein K	P61978	14
18	Galectin-1 (Beta-galactoside-binding lectin L-14-I)	P09382	13
19	Glutathione S-transferase P	P09211	13
20	Tubulin beta-1 chain	Q9H4B7	12
21	Tubulin beta-2 chain	P07437	11
22	Histone-binding protein RBBP4	Q09028	9
23	Thioredoxin-dependent peroxide reductase, mitochondrial	P30048	9
24	Heat shock 70 kDa protein 1L	P34931	9
25	Tubulin-specific chaperone B	Q99426	8
26	14-3-3 protein sigma	P31947	8
27	Desmin	P17661	8
28	Heat shock 70 kDa protein 4	P34932	8
29	Tubulin alpha-3 chain	Q71U36	7
30	Nucleoside diphosphate kinase A	P15531	7
31	Eukaryotic translation initiation factor 5A-1	P63241	6
32	14-3-3 protein theta	P27348	6
33	Tubulin alpha-2 chain	Q13748	5
34	Calpain small subunit 1 (CSS1)	P04632	4
35	14-3-3 protein beta/alpha	P31946	4
36	Tenascin-X	P22105	4
37	Heterogeneous nuclear ribonucleoprotein F	P52597	4

#	Protein Name	UniProt #	Total Peptides Identified
38	Putative nucleoside diphosphate kinase	O60361	4
39	Tropomyosin 1 alpha chain	P09493	3
40	Plasma protease C1 inhibitor	P05155	3
41	ATP synthase beta chain, mitochondrial	P06576	3
42	Alpha-internexin	Q16352	3
43	Peripherin	P41219	3
44	Zinc finger protein 429	Q86V71	3
45	Histone H2A type 1-A	Q96QV6	3
46	Histone H4	P62805	3
49	Heat shock 70 kDa protein 1	P08107	3

Table 3

Potential ITC target proteins classified according to their sub-cellular organelles and biological functions. P: PEITC; S: SFN.

Organelles/pathways	Protein Name	Functions	ITCs
Cytoskeleton	1 Actin (Alpha- actin-2, Beta- actin)	A major constituent of actin microfilaments, which are involved in cell mechanics and various types of cell motility.	P S
	2 Tubulin (alpha-1, alpha-2, alpha-3, beta-1, beta-2, beta-2C, beta-3)	A major constituent of microtubules, which are involved in many cellular processes such as mitosis, cytokinesis, and vesicular transport.	P S
	3 Vimentin	A member of the intermediate filament family, which supports and anchors the position of the organelles in the cytosol.	P S
	4 Tropomyosin, alpha chain, alpha-3 chain, alpha-4 chain, beta	Binds to actin filaments in cells. Plays a central role, in association with the troponin complex.	P S
	5 Desmin	Class-III intermediate filaments.	S
Antioxidant response	6 Thioredoxin (ATL-derived factor) (ADF) (Surface associated sulphhydryl protein)	Participates in various redox reactions through the reversible oxidation of its active center dithiol to a disulfide and catalyzes dithiol-disulfide exchange reactions.	P
	7 Glutaredoxin-1 (Thioltransferase-1)	Has a glutathione-disulfide oxidoreductase activity in the presence of NADPH and glutathione reductase. Reduces low molecular weight disulfides and proteins.	P
	8 Retinal dehydrogenase 1	Binds free retinal and cellular retinol-binding protein-bound retinal. Can convert/oxidize retinaldehyde to retinoic acid.	S
	9 Protein disulfide- isomerase	A multifunctional protein catalyzes the formation, breakage and rearrangement of disulfide bonds.	S
Protein Quality Control	10 Proteasome, subunit alpha type 3, 26S protease regulatory subunit 6A, subunit 6B	A multicatalytic proteinase complex that regulates turnover of a majority of cellular proteins.	P
	11 Ubiquitin carboxyl-terminal hydrolase, isozyme L1, 14	Involves in the processing of ubiquitin precursors and of ubiquitinated proteins.	P
	12 F-box only protein 41	Component of the SCF (SKP1-CUL1-F-box protein)-type E3 ubiquitin ligase complex.	P
	13 VCP/p97	VCP binds ubiquitinated proteins and is necessary for the export of misfolded proteins from the ER to the cytoplasm, where they are degraded by the proteasome.	P S
	14 Hsp110, Heat shock 70 kDa protein (1, 4, 1L), Hsp27	Prevents the aggregation of denatured proteins in cells under severe stress and participates stress resistance and actin organization.	P S
Mitochondria	15 Chaperones Hsp60, Hsp10, GRP75	Prevent misfolding and promote the refolding and proper assembly of unfolded polypeptides generated under stress conditions in the mitochondrial matrix.	P S
	16 NADH dehydrogenase [ubiquinone] 1	Subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase (Complex I).	P
	17 ATP synthase (beta chain, e chain, g chain)	Mitochondrial membrane ATP synthase (F ₁ F ₀ ATP synthase or Complex V) produces ATP from ADP in the presence of a proton gradient across the membrane.	P
	18 Cytochrome b5 type B	A membrane bound hemoprotein which functions as an electron carrier for several membrane bound oxygenases	P
	19 Diablo homolog, mitochondrial	Promotes apoptosis by activating caspases in the cytochrome c/Apaf-1/caspase-9 pathway. Acts by opposing the inhibitory activity of apoptosis proteins (IAP).	P

Organelles/pathways	Protein Name	Functions	ITCs
	20 Cytochrome c oxidase polypeptide Va	Heme A-containing chain of cytochrome c oxidase, the terminal oxidase in mitochondrial electron transport.	P
	21 Cytochrome c oxidase copper chaperone	Copper chaperone for cytochrome c oxidase (COX). Binds two copper ions and delivers them to the Cu(A) site of COX.	P
	22 Mitochondrial import inner membrane translocase subunit TIM13	Mitochondrial intermembrane chaperone that participates in the import and insertion of some multi-pass transmembrane proteins into the mitochondrial inner membrane.	P
	23 Thioredoxin-dependent peroxide reductase, mitochondrial	Involves in redox regulation. Protects radical-sensitive enzymes from oxidative damage by a radical-generating system. Acts synergistically with MAP3K13 to regulate the activation of NF-kappa-B in the cytosol.	S
Cell signaling	24 14-3-3 protein, zeta/delta, beta/alpha, theta, epsilon, eta	A family of adapter proteins in the regulation of a large spectrum of both general and specialized signaling pathway. Bind to a large number of partners, resulting in the modulation of the activity of the binding partner.	P
	25 Proliferating cell nuclear antigen (PCNA)	Involves in the control of eukaryotic DNA replication by increasing the polymerase's processibility during elongation of the leading strand.	S
	26 Calpactin I light chain (S100 calcium-binding protein A10)	Functions as a regulator of protein phosphorylation in that the ANXA2 monomer is the preferred target (in vitro) of tyrosine-specific kinase.	P
	27 Ran-specific GTPase-activating protein	Inhibits GTP exchange on Ran. Acts in an intracellular signaling pathway which may control the cell cycle progression.	P
DNA/RNA regulation	28 Heterogeneous nuclear ribonucleoprotein F, H, K,	Plays a role in the regulation of alternative splicing events. Binds G-rich sequences in pre-mRNAs and keeps target RNA in an unfolded state.	P
	29 SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily E member 1	Involves in transcriptional activation and repression of select genes by chromatin remodeling (alteration of DNA-nucleosome topology).	P
	30 Nuclear migration protein nudC	Necessary for correct formation of mitotic spindles and chromosome separation during mitosis. Necessary for cytokinesis and cell proliferation.	P
	31 Splicing factor 3A subunit 3	Subunit of the splicing factor SF3A required for 'A' complex assembly formed by the stable binding of U2 snRNP to the branchpoint sequence (BPS) in pre-mRNA.	P
Others	32 Glutathione S-transferase P	Involves in conjugation of reduced glutathione to a wide number of exogenous and endogenous hydrophobic electrophiles.	S
	33 Succinyl-CoA ligase [GDP-forming] beta-chain	Carbohydrate metabolism; tricarboxylic acid cycle.	P
	34 Tumor protein D54 (hD54)	Regulates cell proliferation.	P
	35 Tenascin-X (TN-X)	Appears to mediate interactions between cells and the extracellular matrix. May play a role in supporting the growth of epithelial tumors.	P
	36 Pappalysin-1	Metalloproteinase which specifically cleaves IGFBP-4 and IGFBP-5, resulting in release of bound IGF.	P