DOI:10.1158/0008-5472.CAN-07-0340

Cancer Research

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Cancer Res 2007;67:6409-6416. Published online July 6, 2007.

DOI:10.1158/0008-5472.CAN-07-0340

Research Article

The Role of Protein Binding in Induction of Apoptosis by Phenethyl Isothiocyanate and Sulforaphane in Human Non–Small Lung Cancer Cells

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Abstract

Induction of apoptosis underlies a mechanism for inhibiting tumorigenesis by phenethyl isothiocyanate (PEITC) and sulforaphane (SFN). However, the upstream events by which isothiocyanates (ITC) induce apoptosis have not been fully investigated. As electrophiles, ITCs could trigger apoptosis by binding to DNA or proteins or by inducing oxidative stress. To better understand the molecular mechanisms of apoptosis by ITCs, we examined, as a first step, the role of these events in human non–small lung cancer A549 cells. PEITC was a more potent inducer than SFN; it induced apoptosis at 20 μmol/L, whereas SFN induced at 40 μmol/L but not at 20 μmol/L. To study binding with cellular proteins and DNA, cells were treated with ^{14}C-ITCs; the initial protein binding by PEITC was almost 3-fold than that of SFN. The binding by PEITC increased with time, whereas binding by SFN remained low. Therefore, 4 h after incubation proteins became the predominant targets for PEITC with a 6-fold binding than that of SFN. To characterize the chemical nature of binding by the ITCs, we used bovine serum albumin (BSA) as a surrogate protein. PEITC also modified BSA covalently to a greater extent than SFN occurring exclusively at cysteine residues. Surprisingly, neither PEITC nor SFN bound to DNA or RNA at detectable levels or caused significant DNA strand breakage. The levels of oxidative damage in cells, measured as reactive oxygen species, 8-oxo-deoxyguanosine, and protein carbonyls formation, were greater in cells treated with SFN than PEITC. Because PEITC is a stronger inducer of apoptosis than SFN, these results indicate that direct covalent binding to cellular proteins is an important early event in the induction of apoptosis by the ITCs. [Cancer Res 2007;67(13):6409–16]

Introduction

Cruciferous vegetable-derived phenethyl isothiocyanate (PEITC) and sulforaphane (SFN) are versatile cancer chemopreventive compounds showing activity to inhibit tumor growth during initiation, promotion, and progression phases (1). Evidence obtained from both *in vitro* cell culture and *in vivo* animal tissues supports that they are antiproliferative by inducing cell cycle arrest and apoptosis, and these functions are important for their antitumor activities (2–10). As electrophiles, PEITC and SFN may covalently bind to cellular proteins, DNA, and RNA. Although these bindings are likely to underlie their biological activities, only limited studies exist on the relationship between the interactions with the macromolecules in cells and their biological functions. Earlier studies showed that isothiocyanates (ITCs) block chemical-induced tumorigenesis by inhibiting cytochrome p450 enzymes for the activation of carcinogens, presumably by binding to the enzymes (11, 12). Benzyl ITC and PEITC form adducts with cytochrome p450 2E1, 2A6, and 2A13 (13, 14). Later, studies showed that SFN induces phase II enzymes through its binding to the cysteine residues of the Kelch-like ECH-associated protein 1 (Keap1; refs. 15–17).

We have investigated the chemopreventive effects of ITCs in lung cancer. Our recent studies showed that PEITC and SFN and their *N*-acetylcysteine conjugates significantly inhibited the progression of lung adenomas to adenocarcinomas in A/J mice treated with tobacco carcinogens (7). These results suggest that mechanisms other than phase II enzyme induction or phase I enzyme inhibition are likely to be involved in the inhibition of tumorigenesis by the ITCs. The lungs of ITC-treated mice show increased apoptotic cells and mitogen-activated protein kinase (MAPK) and activator protein 1 activities. The study of human non–small lung cancer A549 cells reinforced the *in vivo* observations that the apoptosis was induced by the PEITC conjugate, and the induction was significantly enhanced in the fast growing A549 cells transfected with c-jun or pretreated with 12-*O*-tetradecanoylphorbol-13-acetate (18).

Multiple signaling pathways have been shown to be associated with the apoptosis induced by ITCs (2, 6, 8, 9, 19, 20). Although several studies have indicated that oxidative stress plays an important role, apoptosis can also be elicited through direct binding to proteins and DNA. At present, it is unclear whether these bindings are involved in apoptosis induced by ITCs. As a prelude to identify the specific molecular targets of the ITCs, we determined their quantitative binding to proteins or DNA in cells and examined their relationships with apoptosis. The results indicate that covalent binding to cellular proteins, not DNA or oxidative damage, is likely an important early chemical event leading to the induction of apoptosis.

Materials and Methods

Materials. SFN was generously provided by Dr. Stephen Hecht (University of Minnesota). ^{14}C-SFN (50 mCi/mmol) was provided by Dr. Shantu Amin (Penn State University at Hershey). ^{14}C-PEITC (55 mCi/mmol) was purchased from American Radiolabeled Chemicals. All other

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi:10.1158/0008-5472.CAN-07-0340

DOI:10.1158/0008-5472.CAN-07-0340

reagents were the highest grade available from Sigma-Aldrich, unless otherwise noted.

Cell culture and treatment with ITCs. The human non–small cell lung carcinoma A549 cells were maintained in full serum medium (DMEM medium supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, 100 unit/mL penicillin, 100 μg/mL streptomycin; Invitrogen) at 37°C, 5% CO_2. For the material balance study, cells (~50% confluency) were treated as by spiked ^{14}C-PEITC and ^{14}C-SFN (1.2 mCi/mmol) at 30 μmol/L for 30 min, 1 h, 2 h, and 4 h. For all other experiments including apoptosis assays, reactive oxygen species (ROS) production, comet assay, 8-oxo-deoxyguanosine (8-oxo-dG), and protein carbonyl assay, cells (~50% confluency) were treated with nonradioactive PEITC or SFN at 20 μmol/L for up to 24 h.

Preparation of cell lysate for material balance study. At each time point, A549 cells incubated with ^{14}C-PEITC or ^{14}C-SFN in serum-free medium were harvested and separated from medium according to a published method (21). The cell pellet was resuspended in 850 μL H_2O before adding 100 μL of 100% trichloroacetic acid (TCA). After a 30-min ice bath, the cell lysate was centrifuged at 4°C and 13,000×*g* for 15 min. The TCA precipitate was washed by 500 μL acetone before dissolving in 500 μL of 6 mol/L guanidine hydrochloride.

Extraction of DNA and RNA from the ITC-treated cells. DNA and RNA were extracted from A549 cells treated with ^{14}C-PEITC and ^{14}C-SFN according to the Trizol reagent protocol from Invitrogen.

Scintillation counting. Cell culture medium (200 μL), phthalate mixture (200 μL), TCA precipitation supernatant (200 μL), and protein solution (200 μL) were mixed individually with 15 mL Pico-Flour 40 scintillation cocktail fluid (Perkin-Elmer). The mixtures were counted for 1 min in a LS-6500 scintillation counter (Beckman-Coulter). The radioactivity of DNA and RNA was determined in a similar manner by mixing 200 μL DNA or RNA solution with cocktail fluid.

High performance liquid chromatography analysis of extracellular and intracellular ITCs and the metabolites. The method is in the supplemental materials. The ITC-Cys and ITC-reduced glutathione (GSH) conjugates were identified by comigrations with standards prepared by a previously published method (22, 23).

Binding studies of ITCs with deoxyribonucleosides or calf thymus DNA. A mixture containing of 1 mg 2′-deoxyadenosine, 1 mg 2′-deoxyguanosine, 1 mg deoxycytidine, 1 mg thymidine, and 10 μCi of either ^{14}C-PEITC or ^{14}C-SFN (final concentration, 0.5 mmol/L) in a total volume of 400 μL water was incubated at 37°C for 18 h. The mixture was analyzed by high performance liquid chromatography (HPLC) on a reverse phase column using UV and β-RAM detectors. Similarly, calf thymus DNA (400 μg, 1 mg/mL) was incubated with ^{14}C-PEITC or ^{14}C-SFN (final concentration, 1.5 mmol/L) at 37°C for 18 h and 10% cold TCA was added. The TCA precipitates were collected by a Whatman GF/C glass fiber filter and sequentially washed by 1 mL of 10% TCA and then rinsed once with 3 to 5 mL of 95% ethanol. The filter paper was placed in a scintillation vial containing 15 mL of scintillation cocktail and counted.

Measurement of pseudofirst order reaction rate constants between ITCs and GSH. The measurements of k_{obs} of PEITC and SFN were done according to a previously published method (24).

Binding of ITCs to bovine serum albumin and measurement of free thiols in bovine serum albumin. The number of free thiols (cysteines other than those engaged in disulfide bonds) bound to ITCs in bovine serum albumin (BSA) was determined using the Ellman Assay by titrating either ITC-treated or untreated BSA with 5,5′-dithiobis-2-nitrobenzoic acid (DTNB; ref. 25). The number of ITCs bound to each BSA was estimated from the labeled radioactivity. Briefly, BSA (1 mg/mL; ~15 μmol/L) in the phosphate buffer (0.1 mol/L potassium phosphate, pH 7.4) was reduced with 1.0, 5.0, and 10.0 mmol/L DTT at room temperature for 1 h. After extra DTT was removed by a two-in-series Sephadex G25 desalting column (GE Healthcare), BSA concentration was adjusted to 3 μmol/L. One and two microliters of the ^{14}C-PEITC and ^{14}C-SFN stock solutions (20 mmol/L with a specific radioactivity of 2.5 mCi/mmol) in DMSO were added into an aliquot of 283 μL of the reduced BSA solution, so that the final concentrations were 70 and 140 μmol/L (0.7:1 and 1.4:1 ratio of ITC/thiol (SH), respectively). As a positive control, 1 μL of 100 mmol/L iodoacetamide in H_2O was added to another aliquot of reduced BSA (final iodoacetamide concentration was 350 μmol/L; 3.5:1 ratio of iodoacetamide/SH). As a negative control, 2 μL of DMSO was added to an aliquot of reduced BSA. Another negative control was a solution containing 140 μmol/L *N*-methyl phenethyl amine at 1.4:1 ratio of *N*-methyl phenethyl amine/SH. All samples were incubated at room temperature in dark for 30 min before GuHCl and DTNB were added. The absorbance of each sample containing 4 mol/L GuHCl, 0.1 mol/L potassium phosphate, 1 mmol/L DTNB (10:1 ratio of DTNB/SH) at 412 nm was monitored continuously at room temperature by a Shimadzu UV-1700 spectrophotometer until it reached an equilibrium. The number of free thiols was estimated using the extinction coefficient of 13,600/cm mol/L.

The number of ITCs bound to BSA was determined by running the radiolabeled BSA through a two-in-series Sephadex G25 desalting column with a UV and a β-RAM detector. The number of bound ITCs was calculated from the integrated area under the radioactive BSA peak.

Apoptosis assays. Cells were treated with 20 or 40 μmol/L of PEITC or SFN for 24 h before lysed in assay buffer [50 mmol/L HEPES, 100 mmol/L NaCl, 10 mmol/L DTT, 1 mmol/L EDTA, 0.1% CHAPS (pH 7.4)]. Ten microliters of cell lysate supernatant was mixed with 70 μL assay buffer and 20 μL of 50 μmol/L Ac-DEVD-Rhodamine 110 substrate (Roche). The mixture was incubated at 37°C in dark for 1 h. The release of rhodamine from the substrate was monitored with excitation at 485 nm and emission at 528 nm every 10 min with a Bio-Tek Synergy HT fluorescent microplate reader.

For detection of poly(ADP-ribose)polymerase (PARP) cleavage, A549 cells treated with PEITC or SFN were lysed and the lysates were analyzed by Western blotting. Membranes were probed with an anti-PARP monoclonal antibody from BD Bioscience that recognizes both the 116-kDa intact form of PARP and the 85-kDa cleavage fragment.

Measurement of reactive oxidative species production. Cells were incubated with 5 μmol/L dihydroethidium (DHE) and 10 μmol/L 5-(and-6)-carboxy-2′,7′-difluorodihydro-fluorescein diacetate (both from Invitrogen and dissolved in DMSO) at 37°C for 30 min and then treated with 20 μmol/L PEITC and SFN for additional 0, 30 min, 1 h, and 2 h at 37°C (26, 27). The intracellular ROS generation was measured using FACScalibur flow cytometer (BD Biosciences). Cells treated with 100 μmol/L H_2O_2 for 30 min were used as a positive control. Cells harvested before ITC treatment were used as the baseline control.

Measurement of 8-oxo-dG DNA adduct in genomic DNA. A549 cells were treated by 20 μmol/L PEITC or SFN for 0, 30 min, 1 h, 2 h, 4 h, and 24 h. DNA was isolated by Marmur method (28). 8-Oxo-dG in DNA was analyzed by a published method (29). Cells treated with 100 μmol/L H_2O_2 for 30 min were used as a positive control. Cells harvested before ITC treatment were used as the baseline control.

Determination of protein carbonyls by ELISA. A modified method by Alamdari et al. (30) was used. The detailed method is in the supplemental materials.

Single-cell gel electrophoresis (comet assay). The alkaline comet assay was done as described previously with minor modifications (31). Briefly, a half million A549 cells in 1 mL PBS buffer were treated with either DMSO control, 20 μmol/L PEITC, 20 μmol/L SFN, 20 μmol/L H_2O_2, or 40 μmol/L H_2O_2 at 37°C for 30 min before embedding in 0.75% low-melting point agarose. Slides were analyzed using an Olympus BX-51 fluorescence microscope equipped with ND50 and ND25 neutral density filters and a cooled QImaging Micropublisher 5.0 RTV 5-megapixel digital video camera. Fifty comets on each slide, coded and blindly scored, were acquired using the LAI HCSA (v. 2.3.5) automatic image analysis system (Loats Associates, Inc.). To quantify the induced DNA damage, percentage of DNA in tail was used as the variable.

Identification of ITC binding sites by mass spectrometry. The detailed method is in the supplemental materials.

Results

PEITC is a more potent inducer of apoptosis than SFN. At 20 μmol/L PEITC induced apoptosis in A549 cells, as indicated by

DOI:10.1158/0008-5472.CAN-07-0340

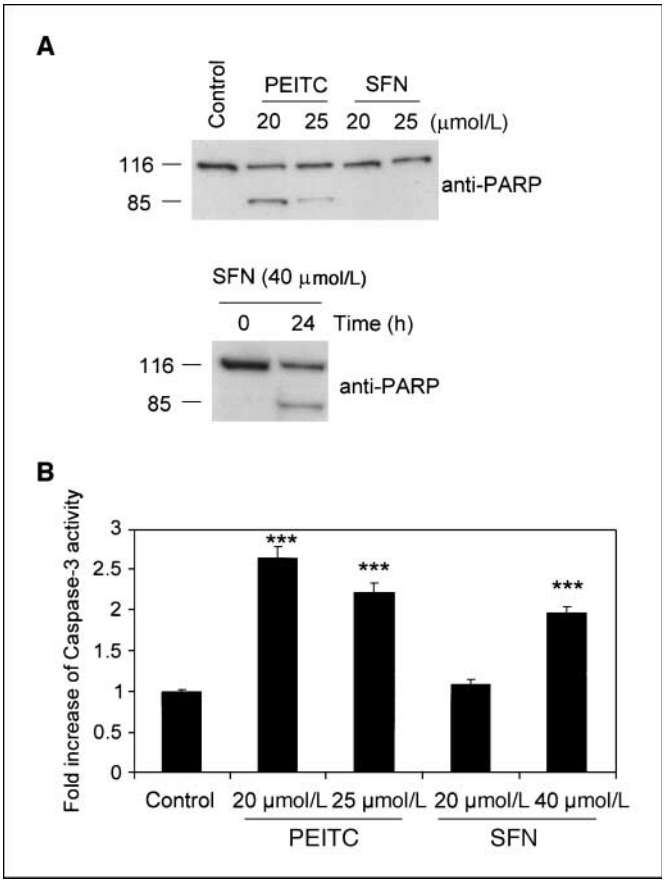

Figure 1. PEITC is a more potent apoptosis inducer than SFN in A549 cells. *A,* cells were treated with PEITC for 24 h and collected for Western blot to detect cleavage of PARP (*top*). Cells were treated with 40 μmol/L SFN under the same conditions for 24 h (*bottom*). *B,* caspase-3 activity in cells treated with 20 and 25 μmol/L of PEITC and SFN for 24 h. ***, $P < 0.001$.

PARP cleavage and caspase-3 activity (Fig. 1*A* and *B*). At the same concentration, however, SFN did not induce these activities; SFN only induced apoptosis at 40 umol/L (Fig. 1*A*). Therefore, PEITC is a stronger inducer of apoptosis than SFN in A549 cells. The decrease in PARP cleavage at 25 μmol/L PEITC may be due to increased necrotic cells.

Intracellular uptake of PEITC and SFN. Figure 2*A* shows the total ITC uptake during incubation of A549 cells with ^{14}C-PEITC or ^{14}C-SFN. The amount of radioactivity/ITC initially absorbed by cells was comparable for PEITC and SFN that accounts for a 7% to 8% of the total input (~1 mmol/L of intracellular concentration) close to an earlier calculation made by using the cyclocondensation assay (21). The difference of radioactivity in cells became wider with incubation as the percentage of radioactivity from PEITC remained constant up to 4 h, whereas that from SFN declined to <2%.

No detectable adducts with DNA and RNA. Neither DNA nor RNA extracted from the ^{14}C-ITC–treated cells showed a detectable amount of radioactivity (<30 dpm in 200 μg DNA and <40 dpm in 400 μg RNA with a baseline radioactivity of ~20–50 dpm), equivalent to levels below 4 to 8 adducts per 10^7 deoxynucleotides. To confirm, we incubated ^{14}C-PEITC and ^{14}C-SFN with 500 μg of calf thymus DNA; and the resulting DNA was again found to contain no detectable radioactivity. Furthermore, reactions of the radioactive ITCs with individual deoxynucleosides (dG, dC, dA, and T) at 37°C for up to 18 h yielded no radioactive products detectable by HPLC.

Proteins are the major targets of PEITC and SFN in cells, and PEITC binds more extensively than SFN. In contrast to DNA and RNA, a substantial amount of radioactivity was found with proteins. Figure 2*B* and *C* shows the amount of radioactivity in proteins and in the supernatant that contains soluble small metabolites, mostly the GSH conjugate (see below). Whereas the initial total uptake, i.e., the sum of protein-bound and small metabolites, between PEITC and SFN was similar, the protein binding by PEITC (7.1 nmol/mg protein) was almost thrice that of SFN (2.6 nmol/mg protein). As incubation time increased, protein binding by PEITC also increased but the small metabolites decreased, suggesting a shift in equilibrium from the GSH conjugate to protein binding. However, the protein binding by SFN remained low throughout the incubation even with a rapid decline in small metabolites as the GSH conjugate. At the end of the 4-h incubation, the amount of PEITC bound to protein reached 15.5 nmol/mg protein versus 2.4 nmol/mg protein for

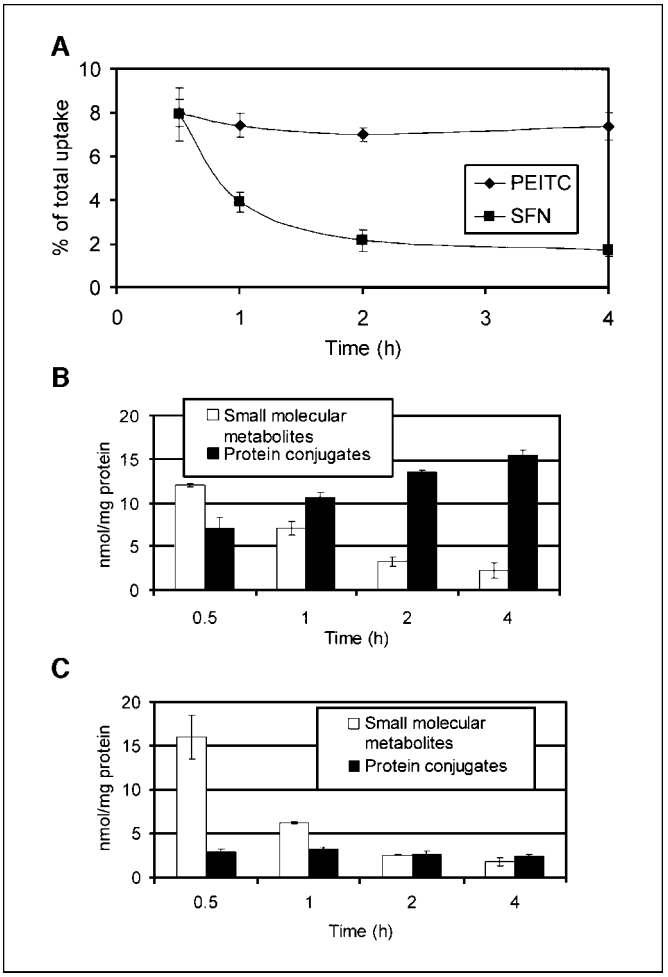

Figure 2. Uptake of PEITC and SFN in A549 cells incubated with ^{14}C-PEITC and ^{14}C-SFN. Up to 7.4% of total amount of ITCs is present intracellularly as soluble metabolites and protein conjugates within the first 30 min of incubation. The total radioactivity from PEITC remains fairly constant inside cells after 4 h of incubation, whereas it declined rapidly in cells treated with SFN. Amount of radioactive ITC compounds in A549 cells bound to protein (*solid column*) and as small metabolites in soluble fraction (*open column*) after incubation with (*A*) ^{14}C-PEITC and (*B*) ^{14}C-SFN. PEITC binds considerably more to proteins than SFN, and its binding to proteins increases with time.

DOI:10.1158/0008-5472.CAN-07-0340

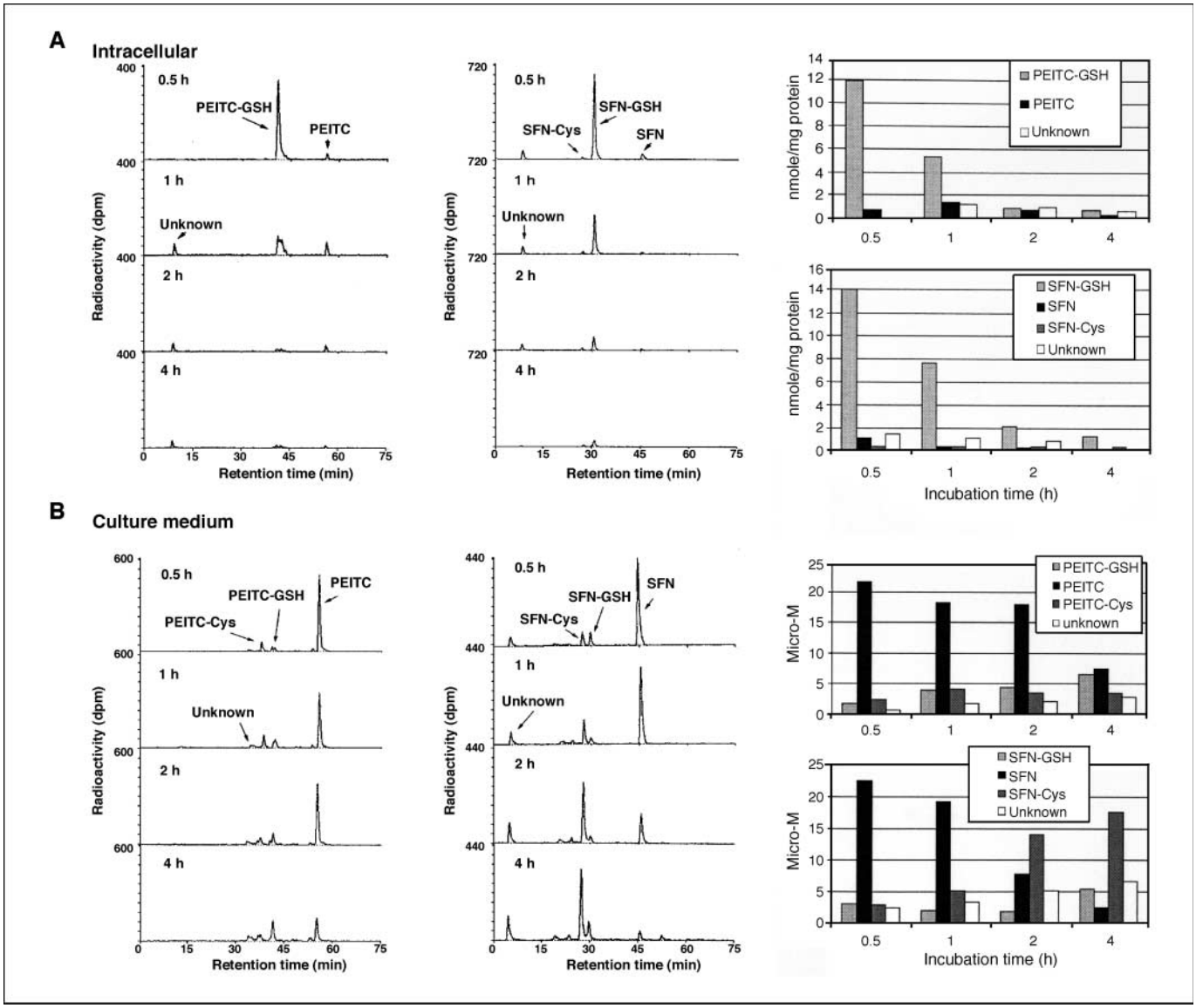

Figure 3. The amounts (nmol/mg protein) of the small metabolites as analyzed by HPLC radioflow detection. The identities of the metabolites were determined based on coelution with the synthetic UV standards. *A,* the supernatant of TCA precipitation of cell lysates. *B,* the cell culture medium.

SFN — a 6-fold difference. These results clearly showed that protein binding eventually becomes the predominant reaction with PEITC that accounts for 87% of the total uptake but only 12% for SFN.

GSH conjugates are initial predominant intracellular small metabolites. Analysis of cell lysate of the ITC-treated cells showed that initially most of the intracellular radioactivity comes from the soluble fraction (supernatants of TCA precipitation) as small metabolites, not as the protein conjugates (Fig. 2*B* and *C*). HPLC analyses showed that, after 30 min of incubation, PEITC-GSH or SFN-GSH accounts for >80% of the radioactivity in the soluble fraction (Fig. 3*A*). Only a small amount of the parent ITC compounds was present in the cells. A small, yet detectable, amount of SFN-Cys was found in the cells treated with SFN. Clearly, conjugation with GSH represents a major reaction for the ITCs once they enter the cells, consistent with that reported by Zhang et al. (32). The intracellular concentration of the GSH conjugates dropped quickly, however, partly due to exportation of the GSH and cysteine conjugates by membrane proteins as indicated by increasing amount of these metabolites in the culture medium (Fig. 3*B*), and the shift of binding toward cellular proteins (Fig. 2*B*). Interestingly, at the later incubation times SFN-Cys became the major species in the culture medium, whereas PEITC-Cys remained as a minor metabolite. These results show that not only the human lung cells are capable of degrading the GSH conjugates via the mercapturic acid pathway, but also there is a major difference in the metabolism of the GSH conjugate of PEITC versus SFN.

SFN reacts with GSH at a faster rate than PEITC but PEITC binds more to BSA than SFN. To explain the differences in their chemical interactions with GSH and proteins in cells, we carried out reactions with BSA as a surrogate protein and with GSH. Their pseudo first-order reaction rate constants (k_{obs}) with GSH were determined. The reaction rate constants are 7.1×10^{-2} S^{-1} for SFN and 4.1×10^{-2} S^{-1} for PEITC, in agreement with the observation that more GSH conjugate is formed in cells with SFN than PEITC shortly after incubation. The faster reaction rate of

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Table 1. Number of free thiols in BSA modified by ITC compounds (Ellman assay) and number of ITC compounds bound to BSA (radioactivity)

DTT-reducing agent	Total no. free thiols	Autoxidation*	No. free thiols modified (no. ITC compounds bound)					
			PEITC		SFN		Iodoacetamide †	*N*-methyl phenethyl amine ‡
			70 μmol/L	140 μmol/L	70 μmol/L	140 μmol/L	350 mmol/L	140 μmol/L
1 mmol/L	15.4	1.1	9.5 (9.3)	13.2 (13.1)	6.1 (6.4)	10.2 (9.9)	12.2	1.1
5 mmol/L	29.8	2.4	13.6 (14.0)	23.8 (24.6)	10.8 (10.1)	18.9 (18.2)	18.2	2.4
10 mmol/L	33.9	2.9	13.4 (15.2)	28.9 (27.9)	10.6 (10.0)	19.9 (19.7)	26.3	2.9

NOTE: Two assays were done: one used DTNB to measure unbound thiols (Ellman assay) after BSA reaction with the ITCs and the other used ^{14}C-PEITC and ^{14}C-SFN to directly measure the number of ITC compounds bound to BSA (see Materials and Methods).

*The numbers represent the background conversion of free thiols to disulfides during incubation.

† Iodoacetamide that alkylates free thiols in proteins was used as a positive control.

‡ *N*-methyl phenethyl amine, a structural analogue of PEITC without the ITC functionality, was used as a negative control.

SFN may be due in part to its greater solubility than PEITC in aqueous medium.

BSA (69 kDa) contains 35 cysteines. BSA was first reduced by DTT before incubating with ^{14}C-PEITC or ^{14}C-SFN. The binding of ITCs to BSA was determined quantitatively by two complementary assays: one to determine how many free thiols (cysteines) are modified by ITC (Ellman assay) and the other, based on the radioactivity in BSA molecule, to quantify the number of ITC molecules that are bound to each BSA. The Ellman assay determined all the remaining unoccupied thiols that are accessible to DTNB. The decreased number of thiols after treatment of an ITC compound indicates the number of thiols that was modified. Table 1 shows that the number of free thiols was increased from 15.4 to 33.9 as the DTT concentration increased from 1 to 10 mmol/L. The reaction of the DTT (1 mmol/L) pretreated BSA with 70 and 140 μmol/L PEITC showed that 9.5 and 13.2 thiols were modified, respectively, compared with 6.1 and 10.2 thiols by SFN. As expected, phenethylamine, a structural analogue of PEITC that lacks the ITC functionality, did not modify the thiols in BSA. In the second assay, the same samples were quantified based on radioactivity for the number of ITC molecules bound to BSA. The numbers are in excellent agreement with the numbers of thiols modified (Table 1). These results indicate that the Cys residues in BSA are the major, if not the exclusive, sites of binding by ITCs under the conditions studied.

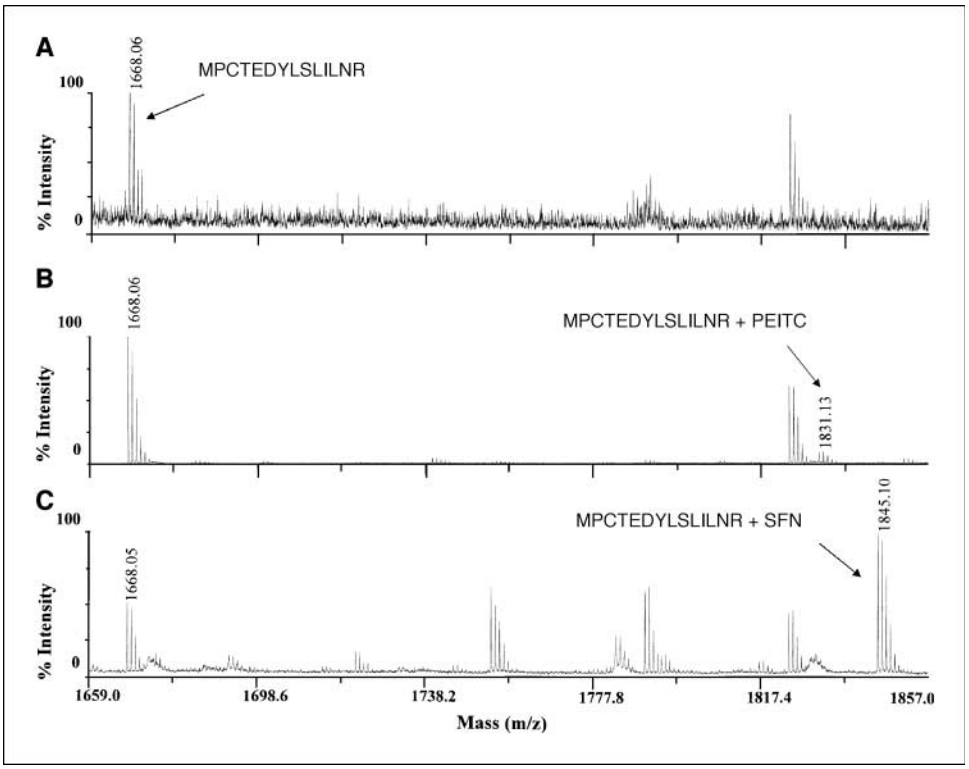

Figure 4. Modification of BSA by PEITC and SFN. Modification of the cysteine containing peptide from BSA showing (*A*) unmodified peptide at m/z 1,168.06; (*B*) modified with PEITC at m/z 1,831.13 (+163); and (*C*) modified with SFN at m/z 1,845.10 (+177). It should be noted that the intensity of the peaks does not quantitatively reflect the levels of modifications by PEITC and SFN.

DOI:10.1158/0008-5472.CAN-07-0340

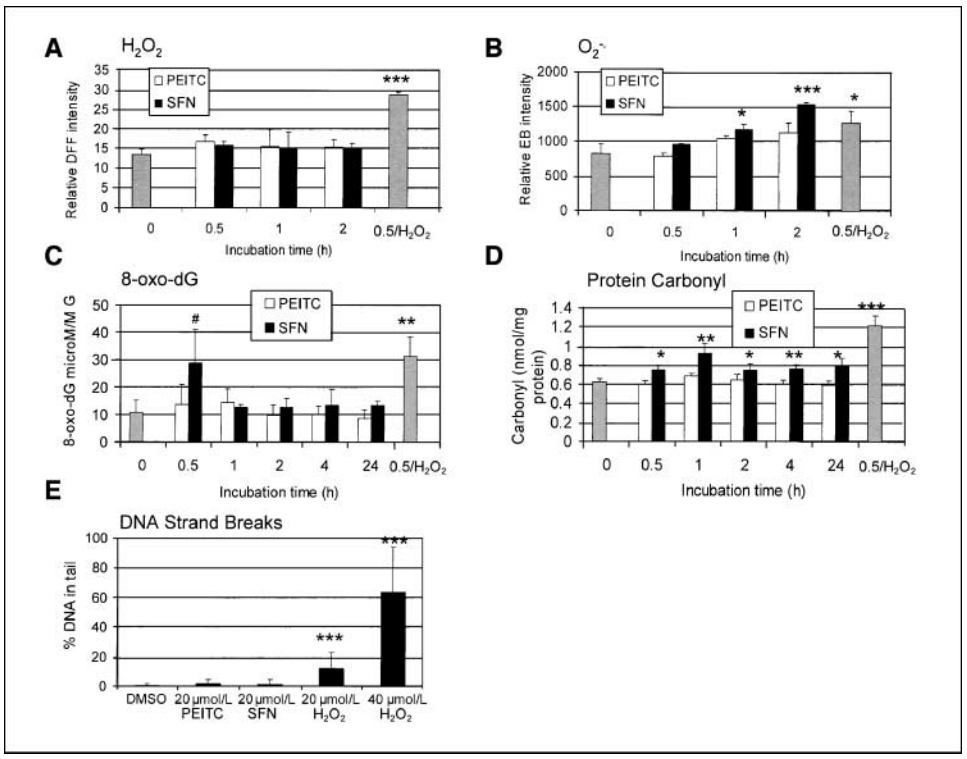

Figure 5. Formation of ROS, 8-oxo-dG, protein carbonyls, and DNA strand breaks in A549 cells treated with 20 μmol/L of PEITC and SFN. For ROS, the numbers are expressed as relative fluorescent intensity using difluoro-dihydrofluoresceine (*DFF*) or ethidium bromide (*EB*) for hydrogen peroxide (*A*) or superoxide anion (*B*), respectively. For 8-oxo-dG (*C*), the levels were determined by HPLC-EC detection and are expressed as μmol/mol G in DNA. For protein carbonyls (*D*), the levels were determined by an ELISA method and are expressed as nmol/mg protein. For DNA strand breaks, the levels were measured by the comet assay and are expressed as percentage of DNA in tail. Hydrogen peroxide was used as a positive control for all four assays. All values are obtained in at least triplicates and *p* values were calculated using Student's *t* test against control/untreated samples. #, $P = 0.057$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

To verify the results from Ellman assay that the ITCs are covalently bound to proteins via cysteines, the BSA treated with PEITC or SFN was digested by trypsin, and the resulting peptides were analyzed by MALDI-TOF MS (Fig. 4). The results showed a mass increment of m/e 163 and 177 for PEITC and SFN, respectively, verifying that both PEITC and SFN can covalently modify BSA, although the levels of modification cannot be determined by this method.

ROS, 8-oxo-dG, and protein carbonyl formation in cells treated with ITCs. To assess ROS generation by ITCs, the formation of superoxide radical O_2^- and H_2O_2 was measured by relative fluorescent intensity produced by EB and DFF, respectively, using flow cytometry. PEITC and SFN caused only a slight increase, if any, in H_2O_2 production in cells. Both compounds, however, induced the generation of O_2^- when compared with the untreated control cells (Fig. 5*A* and *B*) and SFN seemed to produce more O_2^- than PEITC. Although the staining-before-treatment approach (27) that we used here does not measure the dynamic balance of ROS between generation and consumption, it reflects the true level of generation by signal accumulation. However, the fluorescent signal may keep increasing, whereas the ROS generation rate starts to decline.

The formation of 8-oxo-dG is a marker of direct oxidative damage on DNA bases that could play a role in apoptosis (33). The formation of 8-oxo-dG was more than doubled from 10.8 to 28.9 μmol/mol dG in cells treated with 20 μmol/L SFN within 30 min, and the increased levels rapidly dropped to the control level after 1 h (Fig. 5*C*). In contrast, PEITC did not cause any significant increase in 8-oxo-dG at the time points examined. The increase in protein carbonyls serves as another marker of oxidative stress. Compared with the untreated control cells and cells treated with PEITC, cells treated with SFN showed a significant increase of protein carbonyls (up to ~30%) after 1 h of treatment (Fig. 5*D*). At all time points, SFN caused a higher level of protein carbonyl formation than PEITC. All of these findings indicate that SFN causes more oxidative damage in cells than PEITC, although the effect seems transient. The rapid decrease in 8-oxo-dG and protein carbonyl levels after 1 h may be due to the increased superoxide dismutase activity as reported previously (26).

No significant DNA strand breakage after cells treated with ITCs. To determine whether ITCs cause DNA strand breakage that is implicated in triggering apoptosis (34), we did comet assay in cells treated with 20 μmol/L PEITC or SFN at 37°C for 30 min. The results shown in Fig. 5*E* indicate that, unlike in the H_2O_2-treated cells, DNA strand breaks, including both single-strand and double-strand breaks, in cells treated with PEITC and SFN are comparable with the background levels. Only ~1% of cells shows tailed DNA in both cases even with the most sensitive assay conditions.

Discussion

The induction of apoptosis by ITCs is believed to underlie an important mechanism for their chemopreventive activities. Whereas PEITC and SFN induce apoptosis in cultured cancer cells (2–10) and, in a few cases, in tissues obtained from animals where tumor inhibition was observed (5, 7), little is known about the early chemical events that initiate apoptotic responses in cells by these compounds. In this study, three possible molecular events were examined: (*a*) covalent binding to proteins, (*b*) DNA adduct formation, and (*c*) oxidative stress. Several studies have shown that the oxidative stress, possibly mediated by conjugating with cellular GSH and/or by damaging mitochondria (35–37) contributes to the ITC-induced apoptosis (2, 26, 37). However, evidence also downplays the role of oxidative DNA damage (38). As electrophiles, PEITC and SFN are expected to react with DNA and proteins; however, no information is available about the covalent binding of ITCs with DNA and proteins in cells and its relationship with apoptosis. This study showed for the first time that proteins, not cellular DNA and RNA, are the major binding targets of the ITCs.

DOI:10.1158/0008-5472.CAN-07-0340

The lack of any detectable binding of PEITC and SFN to DNA in A549 cells suggests that direct DNA adduct formation is an unlikely mechanism of apoptosis. However, it cannot be ruled out that the levels may be too low to be detected or unstable DNA adduct(s) are formed with transient half-life that is sufficient to trigger apoptotic response. The reactions of ^{14}C-PEITC and ^{14}C-SFN with deoxyribonucleosides revealed no product formation, reinforcing the findings in cells that although these compounds are strong electrophiles, they do not seem capable of forming adducts with DNA bases. These results were further confirmed by a lack of covalent binding by the ITCs with RNA in A549 cells. In contrast, the high binding affinity of PEITC and SFN toward proteins suggests that proteins are chemically one of the most accessible intracellular macromolecules for ITCs. The fact that PEITC, a stronger inducer of apoptosis than SFN, is also more extensively bound to proteins supports a role of proteins as the molecular targets in ITC-induced apoptosis. The differential binding between PEITC and SFN also indicates that their binding to proteins is highly selective and likely involves specific targets for different ITCs, depending on their structures. As a first step in our attempt to identify specific molecular target(s) of ITC, this study, therefore, provides new evidence that links ITC-protein binding to the functions of ITCs in cell growth regulation.

In cells, SFN is much more reactive toward GSH than PEITC. This may in part explain the greater oxidative damage induced by SFN than PEITC. Studies have shown that *N*-acetylcysteine reverses the apoptotic activity of ITCs, presumably by its virtue as an antioxidant. However, *N*-acetylcysteine also readily conjugates with ITC. It is, therefore, unclear whether its conjugation with ITC or antioxidant activity is important in its activity. The strongest evidence to date supporting oxidative stress in ITC-induced apoptosis comes from the study in which human prostate cancer cells transfected with catalase were found to be resistant to apoptosis by SFN (26). Our study showed that in A549 cells, however, SFN apparently induces more oxidative damage than PEITC, but it is a weaker inducer of apoptosis. Furthermore, we found that pretreated with antioxidants [vitamin E, phenyl-*N*-*tert*-butylnitrone, Mn(III)-tetrakis(4-benzoic acid) porphyrin, catalase, ebselen] did not abolish the apoptosis induced by PEITC in A549 cells.[3] These results suggest that the molecular mechanisms of apoptosis induction by ITC may be cell type–specific.

Although their initial cell uptake was similar, the final intracellular protein binding was considerably greater for PEITC than SFN. The increase in protein binding with PEITC may be explained by at least two factors: the dissociation of initial PEITC-GSH conjugate to free ITC inside cells that subsequently binds to proteins and the continued uptake of PEITC in culture medium. The rapid decline of intracellular PEITC-GSH conjugate suggests that a swift exchange from binding to GSH to proteins may have occurred as a major reaction. However, unlike PEITC, the amount of SFN binding to protein did not increase with time despite the fact that its GSH conjugate decreased rapidly, suggesting that factors other than the reactivity may be involved, such as the relative low hydrophobicity of SFN and the readily degraded intracellular SFN-GSH to SFN-Cys (Fig. 3*B*).

A predominant metabolic pathway of ITCs is via the mercapturic pathway initiated by GSH conjugation followed by enzymatic degradation to yield the *N*-acetylcysteine conjugate as the major end product (11). This metabolic fate argues against the possibility that the high levels of binding to proteins by ITCs come from some minor metabolites yet to be identified. Although the results of this study support that proteins are the main binding partners of the ITCs in cells for apoptosis, the specific protein targets and their chemical reactions are still not known. To better understand the nature of chemical interactions between the ITCs and proteins, we decided to first use BSA as a model protein. BSA is not known to be involved in apoptosis, but it is hoped that this study can guide future studies. The study with BSA provides strong evidence that PEITC covalently modifies proteins to a greater extent than SFN and the modifications seem to occur exclusively at the cysteine residues. Other possible important sites of ITC binding include amino group of lysine and hydroxyl group of serine (39, 40). Modification of thiols in proteins may have important consequences, as previous studies showed that the binding to cysteines in Keap1 by SFN constitutes a critical step in its induction of phase II enzymes mediated by Nrf2 translocation from cytoplasm to nucleus (15–17). A similar mechanism has also been reported with diallyl trisulfide, another promising anticancer compound found in garlic, targeting to β-tubulin (41).

Protein thiols are involved in numerous reactions, such as oxidation, glutathiolation, nitrosyaltion, disulfide interchange, and thioether and thioester formation. These posttranslational modifications are known to dictate their functions. In this context, it is tempting to consider a model in which the binding between ITCs and protein cysteine residues may trigger the cell signaling pathways that lead to apoptosis. Studies have shown that PEITC and SFN modulate multiple signaling pathways in various cell lines. Chief among them are the MAPK family of serine/threonine kinases that play an important role in cell proliferation and apoptosis in response to stimuli (8, 9, 20). PEITC and SFN have been shown to induce apoptosis cell death via a p53-dependent or p53-independent pathway (8, 42, 43). Together, these studies showed that ITCs can modulate the kinase pathways and transcription factors, often in a cell-specific manner. The persistent c-Jun-NH_2-kinase (JNK) activation in cells treated with PEITC was suggested as a result of inhibition of JNK phosphatases (44). It was postulated that the inhibition was caused by PEITC direct binding to the catalytic cysteine residue of JNK phosphatases or by modifying the active site via the oxidative species generated. Others also proposed that the direct binding to the active thiol by PEITC is responsible for the activation of JNK and tyrosine phosphorylation (6). However, in both cases, evidence for protein binding was not provided. A common feature in these models is that the modification of cysteine residues in the active sites may act as a switch of protein function, either as kinases or transcription factors, eventually leads to a chain of downstream events. The extensive covalent binding to cellular proteins by PEITC and SFN and its correlation with apoptosis shown in this study provides a compelling reason for identifying the specific ITC protein target(s) whose functions are to regulate cell growth.

Acknowledgments

Received 1/22/2007; revised 4/20/2007; accepted 5/3/2007.

Grant support: National Cancer Institute grant CA100853.

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We thank Dr. Karen Creswell and Michelle Lombard of the Lombardi Flow Cytometry and Cell Sorting Shared Resource, Georgetown University, for help in flow cytometry experiments; Jishen Pan for help on HPLC analysis of 8-oxo-dG; Wei Yu for help on protein carbonyl ELISA assay; and Karen Howenstein for the administrative and editorial assistance.

[3] Unpublished results.

DOI:10.1158/0008-5472.CAN-07-0340

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