Sensitization of Non-small Cell Lung Cancer Cells to Cisplatin by Naturally Occurring Isothiocyanates

ARTICLE *in* CHEMICAL RESEARCH IN TOXICOLOGY · AUGUST 2010

Impact Factor: 3.53 · DOI: 10.1021/tx100187f · Source: PubMed

Published in final edited form as:
Chem Res Toxicol. 2010 August 16; 23(8): 1307–1309. doi:10.1021/tx100187f.

Sensitization of non-small cell lung cancer cells to cisplatin by naturally occurring isothiocyanates

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Abstract

We show that naturally occurring isothiocyanates (ITCs) sensitize human non-small cell lung cancer cells to cisplatin. Moreover, structure of the ITC side chain moiety is important for sensitization. In NCI-H596 cells, 20 μM benzyl isothiocyanate (BITC) and phenethyl isothiocyanate (PEITC) enhance the efficacy of various concentrations of cisplatin, but sulforaphane (SFN) does not. Reducing the concentration of BITC and PEITC to 10 μM still allows for sensitization of cells to cisplatin. Neither cellular platinum accumulation nor DNA-platination account for this increased cytotoxicity. BITC and PEITC deplete β-tubulin, but SFN does not; this correlates with and may be important for sensitization.

Lung cancer is difficult to treat; thus, it is important to develop new strategies to combat this disease in the clinic. The platinum anticancer agent cisplatin (*cis*-diamminedichloroplatinum(II)) (Figure 1) is commonly used to treat lung and other types of cancer, alone and in combination with other drugs (1). Isothiocyanates (ITCs), which are contained in cruciferous vegetables as glucosinolates and released via hydrolysis by the enzyme myrosinase (2,3), are organic compounds with a –N=C=S functional group that can react with cellular nucleophiles (2). ITCs are known cancer chemopreventive agents (4,5).

We here show that dietary ITCs enhance the efficacy of cisplatin when used against human non-small cell lung cancer cells. Moreover, we show that the structure of the ITC side chain moiety is important for sensitization. To our knowledge, we are the first to use ITCs and cisplatin together to treat lung cancer. In 2001, Tanaka *et al.* demonstrated that simultaneous administration of synthetic 2-(4-hydroxyphenyl) ethyl ITC and cisplatin increases apoptosis in head and neck cancer cells (6). Later, Sedlak and coworkers used the synthetic ITC ethyl 4-isothiocyanatobutanoate to sensitize A2780 ovarian cancer cells and its cisplatin-resistant variant to cisplatin (7–9). More recently, Guerrero-Beltran *et al.* demonstrated the protective effects of SFN against cisplatin-induced nephrotoxicity (10).

The arylalkyl ITCs benzyl isothiocyanate (BITC) and phenethyl isothiocyanate (PEITC) and the alkyl ITC sulforaphane (SFN) (Figure 1), all of which are naturally-occurring ITCs, were used in this study. Human NCI-H596 non-small cell lung cancer cells were pre-treated with DMSO (vehicle control) or ITCs for 1 h; culture medium was then removed and replaced with fresh medium with or without cisplatin. Cells were then washed and allowed to recover in the absence of drug for 48 h, after which time cytotoxicity was measured using a tetrazolium salt viability assay (11,12). We demonstrate that 1 h pre-treatment with 20 μM BITC or PEITC sensitizes human NCI-H596 non-small cell lung cancer cells to various concentrations of cisplatin, while 20 μM SFN does not (Figure 2a). Thus, pre-treatment with

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20 μM BITC or PEITC would allow for ~50% less cisplatin to be used to obtain the same cytotoxicity as cisplatin alone. To determine if a lower concentration of BITC and PEITC could be used to sensitize NCI-H596 cells to cisplatin, cells were pre-treated with 10 μM BITC or PEITC. We show that this lower concentration of BITC and PEITC also sensitizes NCI-H596 cancer cells to 30 μM cisplatin (Figure 2b). Based on our data, it is likely that the effects of ITCs and cisplatin are more synergistic in nature than additive.

Responses to cisplatin can be either p53-dependent or -independent (13). The NCI-H596 non-small cell lung cancer cell line harbors a G245C mutation in its p53 DNA-binding domain. To determine if p53 status is important for the sensitization of cells to cisplatin by ITCs, we used a non-small cell lung cancer cell line with wild-type p53 status. In Figure S1 of Supporting Information we show that pre-treatment with 20 μM BITC or PEITC sensitizes NCI-H1299 non-small cell lung cancer cells with tetracycline induced wild-type p53 to 60 μM cisplatin. Thus, it appears p53 status is not important for sensitization.

Cisplatin accumulates in the cell (12,14) and is known to react with DNA; the 1,2-GG intrastrand cross-link is the primary lesion formed (15). Cellular platinum accumulation has been shown to correlate with cisplatin cytotoxicity (12). Thus, we studied platinum accumulation in H596 cells pretreated with DMSO (vehicle control) or ITCs to determine if increased levels of platinum correlate with the increased cytotoxicities. Cellular platinum accumulation is reported in ng Pt per mg protein (Table 1). Pre-treatment with an ITC did not increase platinum accumulation in NCI-H596 cancer cells. Interestingly, cells pre-treated with DMSO (control) and then treated with 15 μM cisplatin accumulated a greater level of platinum than cells pre-treated with 20 μM PEITC and then treated with 15 μM cisplatin ($p<0.05$). These results show that cellular platinum accumulation measured after treatment with cisplatin does not account for the increased cell death.

Cisplatin can bind to the thiol group of intracellular glutathione (GSH) and this reaction is considered important for the inactivation of cisplatin (16). However, Gibson and coworkers recently suggested that binding of cisplatin to GSH is not the most important cellular interaction for inactivation (17). It has been demonstrated that ITCs also react with GSH (18,19); thus, treatment of cells with ITCs could reduce the level of GSH in a cell, preventing formation of the cisplatin-GSH complex, consequently increasing the cytotoxicity of cisplatin. Using Ellman's assay (18,19), we measured relative GSH levels (compared to controls) in NCI-H596 cancer cells after treatment with ITCs (Figure 3). GSH levels after 1 h exposure were lower in cells treated with 20 μM BITC or PEITC than cells treated with DMSO vehicle control ($p<0.5$); there was no significant difference between cells treated with DMSO or 20 μM SFN. After removal and 2 h recovery, GSH returned to control levels. Although GSH levels recover over this 2 h period, it is possible that the initial depletion of intracellular GSH by BITC or PEITC is important for sensitization. Buthionine sulfoximine (BSO), an inhibitor of GSH synthesis, has been shown to reduce intracellular GSH levels, resulting in increased cytotoxicity of cisplatin (20). However, it is important to note that GSH levels in the NCI-H596 cells after 1 h treatment with BITC or PEITC do not differ significantly from the level measured in cells treated with SFN. Thus, depletion of GSH by ITCs is probably not an important event for sensitization.

Because it is ultimately the reaction of cisplatin with DNA that is considered important for cytotoxicity, DNA-platination was measured and is reported in ng Pt per ng DNA (Table 1). The values obtained after pre-treatment with DMSO, 20 μM BITC or 20 μM PEITC and then treatment with 15 μM cisplatin are higher than that obtained for cells exposed to culture medium alone (control; cells not exposed to platinum drug) ($p<0.05$). The value obtained for DNA-platination after pre-treatment with 20 μM SFN then treatment with 15 μM cisplatin has a large standard deviation and thus does not differ significantly from the culture medium

control. However, because DNA-platination measured after pre-treatment with DMSO then treatment with 15 μM cisplatin does not differ from that measured after pre-treatment with 20 μM BITC or PEITC, it can be concluded that DNA-platination does not account for the increased cell death. It should be mentioned that although the cytotoxicity of cisplatin is attributed to its covalent modification of DNA, its reaction with proteins may also play an important role in its mechanism of action (21). Mechanisms other than DNA binding for the cytotoxicity of cisplatin have been discussed recently (22,23).

Protein binding in cells is important for the induction of apoptosis by ITCs (24). We previously showed that BITC > PEITC > SFN covalently modify cysteine residues in tubulin, resulting in tubulin conformational changes, disruption of microtubule polymerization and ultimately apoptosis in cancer cells (5,25). Tubulin was shown to be depleted in the soluble fractions of cell lysates of A549 non-small cell lung cancer cells treated with 20 μM BITC and PEITC, but not in those treated with 20 μM SFN (5,25). This depletion is due to precipitation followed by degradation of tubulin in cells after treatment in culture (5,25). Tubulin-binding agents are commonly used in combination with platinum anticancer agents to treat non-small cell lung cancer in the clinic (26,27). Paclitaxel (taxol), for example, is used with cisplatin and targets the β-tubulin subunit of α/β-tubulin heterodimers (26,27). We here show the depletion of β-tubulin in the soluble fraction of cell lysates of NCI-H596 cancer cells treated with ITCs in culture (Figure 4). After 1 h exposure, depletion was observed in cells treated with 20 μM BITC and PEITC, but not in cells treated with 20 μM SFN. After the ITCs were removed and cells were incubated with fresh medium for an additional 2 h, the β-tubulin levels in cells treated with BITC or PEITC remained depleted. Thus, depletion of β-tubulin by ITCs parallels their ability to sensitize H596 cancer cells to cisplatin, and this may be an important event for sensitization. A study by Donehower and coworkers showed that sequential treatment of taxol or vincristine then cisplatin enhances cytotoxicity in leukemia cells, compared to treatment with cisplatin alone (28). Although cytotoxicity was enhanced, DNA damage did not increase. This corroborates our DNA-platination results.

Using naturally occurring ITCs with cisplatin is an exciting new strategy for the treatment of lung cancer. BITC and PEITC are contained in foodstuffs (i.e., cruciferous vegetables) and thus may be a safer alternative to the tubulin binding agents currently used in the clinic, which have many toxic side effects (29,30). In a previous study, we safely gave a single dose of 40 mg PEITC orally to human subjects (31). Then, in a Phase I clinical study we gave subjects 120 mg PEITC per day for 30 days; subjects safely maintained ~1–2 μM ITC plasma concentrations (Liebes, L. *et al.*, unpublished results). We conclude that naturally occurring ITCs can sensitize human non-small cell lung cancer cells to cisplatin and this may offer a new strategy for treating lung cancer in the clinic.

MTS assay

Cytotoxicity was measured using the CellTiter 96 AQueous One MTS assay (Promega, Madison, WI). Human NCI-H596 non-small cell lung cancer cells (ATCC, Manassas, VA) were seeded in 96-well plates at a density of 10,000 cells per well the day prior to treatment. Cells were then incubated with culture medium (control) or pre-treated with DMSO or 10–20 μM ITCs in culture medium for 1 h at 37°C. Culture medium was then removed and cells were treated with 15–45 μM cisplatin or no drug in culture medium for 2 h at 37°C. Cells were then washed and allowed to recover and grow in fresh culture medium for 48 h (11,12). MTS reagent was then added to each well, cells were incubated for 2 h and $A_{490nm/650nm}$ was read using a Bio-Tek plate reader. For each condition, 6 wells per plate were allotted, and each reported percent viability is the average of at least three experiments with standard deviation. A two-sample t-test was performed assuming unequal variances in

Microsoft Excel and * indicates $p<0.05$ compared to the control, corresponding DMSO then cisplatin (DMSO/CDDP), and ITC alone.

Cellular platinum accumulation and DNA-platination

NCI-H596 cells at 40–50% confluency in a 10 cm dish were exposed to culture medium alone, or DMSO or 20 μM ITCs in culture medium for 1h. Culture medium was then collected and replaced with fresh medium with or without 15 μM cisplatin and cells were incubated for an additional 2 h. Culture medium and cells were then collected and cells were washed twice with PBS. For cellular platinum accumulation studies, cell pellets were suspended in 200 μL distilled and deionized water, sonicated, and protein concentrations were measured using Protein Assay (Bio-Rad, Hercules, CA). For DNA-platination, DNA was isolated using a modified Marmur's procedure (32). Precipitated DNA was then washed twice with ice-cold 80% ethanol and quantified by absorbance at 260 nm and purity checked using a Thermo Scientific NanoDrop. Samples were then digested in nitric acid for 48 h at 70°C and cellular platinum accumulation and DNA-platination were determined using inductively coupled plasma mass spectrometry (ICP-MS) (Syracuse University Engineering) (11). Each reported value is the average of three experiments with standard deviation. A two-sample t-test was performed assuming unequal variances in Microsoft Excel.

Measurement of GSH

NCI-H596 non-small cell lung cancer cells at 40–50% confluency in a 10 cm dish were exposed to DMSO or 20 μM BITC, PEITC or SFN in culture medium for 1 h. For each condition two plates were used; one set was collected after 1 h incubation, the other set after an additional 2 h incubation in fresh ITC-free medium. Cells were washed twice with PBS, resuspended in 200 μL water, sonicated, and protein concentrations were measured using Protein Assay. Cell lysates were treated with trichloroacetic acid (5% final concentration), put on ice for 10 min and then centrifuged at ~21,000×g at 4°C for 10 min. Ellman's assay was performed as described in the literature (18). Data shown are the relative free GSH levels in cells after treatment compared to their corresponding DMSO control and are the average of three experiments with standard deviation. A two-sample t-test was performed, assuming unequal variances, in Microsoft Excel and * indicates $p<0.05$.

Western blot of β-tubulin

NCI-H596 cells at 40–50% confluency in a 10 cm dish were exposed to culture medium alone, or DMSO or 20 μM ITCs in culture medium. Culture medium was collected after 1 h and cells were either collected or culture medium was replaced and those cells were returned to the incubator for an additional 2 h; culture medium and cells were then collected. Cells were washed twice with PBS and then lysed in RIPA buffer. Soluble fractions were separated from insoluble, protein concentrations were measured using Protein Assay, protein was resolved on 4–12% Bis-Tris gels (Invitrogen, Carlsbad, CA) and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA). Membranes were incubated with a β-tubulin antibody (1:200, H-235, Santa Cruz Biotechnology, Santa Cruz, CA), and with a β-actin antibody (1:5,000, AC-15, Santa Cruz Biotechnology) for loading control. Experiment was done in triplicate and shown is a representative image.

See Supporting Information for complete Experimental Section.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Dr. York Tomita (Georgetown University) for the stably transfected NCI-H1299 non-small cell lung cancer cells with tetracycline inducible wild-type p53 and Dr. Jessie Wu (Georgetown University) for helpful discussions pertaining to this work. We would also like to thank Dr. James C. Dabrowiak (Syracuse University) for his helpful comments on this manuscript. This research was funded by Ruth L. Kirschstein National Research Service Award T32 CA9686 (A. J. D.) and NIH grant number CA100853.

Abbreviations

DMSO	Dimethyl sulfoxide
CDDP	cisplatin, *cis*-diamminedichloroplatinum(II)
ITCs	isothiocyanates
BITC	benzyl isothiocyanate
PEITC	phenethyl isothiocyanate
SFN	sulforaphane
GSH	glutathione
ICP-MS	inductively coupled plasma mass spectrometry
Pt	platinum

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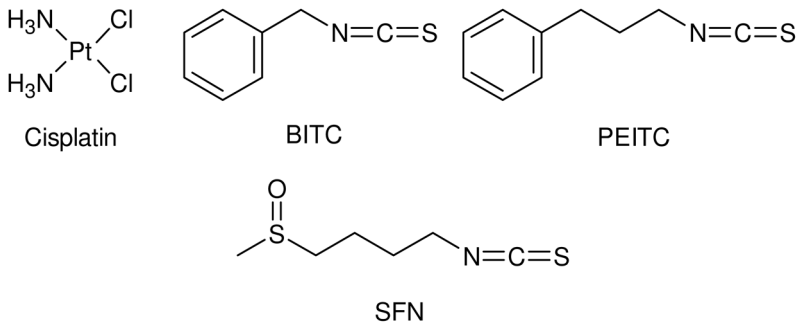

Figure 1.
Structures of cisplatin, benzyl isothiocyanate (BITC), phenethyl isothiocyanate (PEITC) and sulforaphane (SFN).

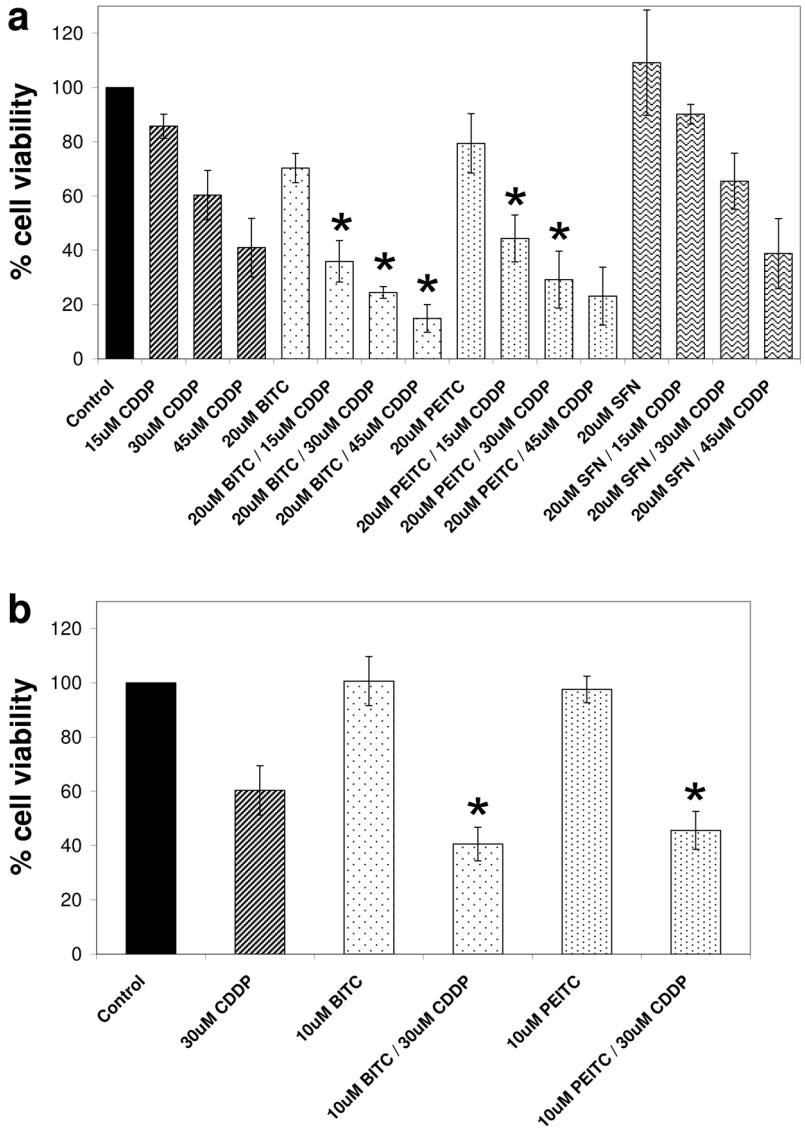

Figure 2.

(a) Cytotoxicity of cisplatin (CDDP), ITC then CDDP (ITC/CDDP) or ITCs toward human NCI-H596 non-small cell lung cancer cells measured using a tetrazolium salt viability assay. Cells were incubated with culture medium (control) or pre-treated with DMSO (vehicle control) or 20 μM BITC, PEITC or SFN for 1 h, and then treated with 15–45 μM cisplatin or no drug for 2 h, and then allowed to grow for 48 h in 5% CO_2 at 37°C. (b) Cytotoxicity of CDDP, ITC/CDDP or ITCs toward human NCI-H596 non-small cell lung cancer cells measured using a tetrazolium salt viability assay. Cells were incubated with culture medium (control) or pre-treated with DMSO (vehicle control) or 10μM BITC or PEITC for 1 h, and then treated with 30 μM cisplatin in culture medium or no drug for 2 h, and then allowed to grow for 48 h in 5% CO_2 at 37°C. Each bar in (a) and (b) is the average of at least three experiments with standard deviation and statistical analyses was performed (*$p<0.05$ compared to control, corresponding DMSO/CDDP and ITC alone.

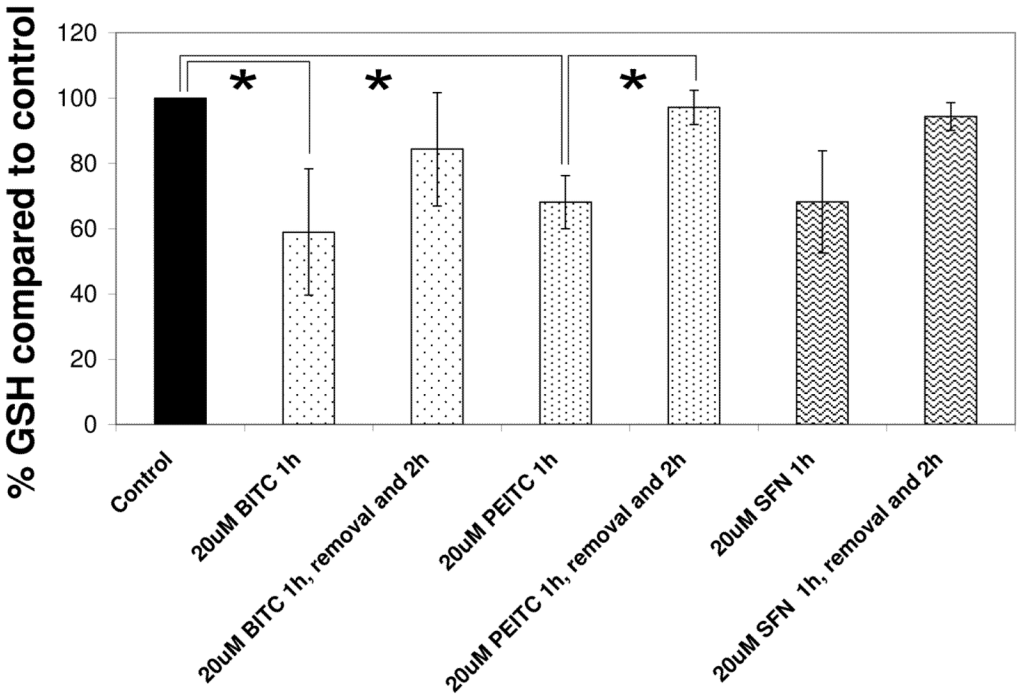

Figure 3.

Relative glutathione levels compared to controls (DMSO) in human NCI-H596 non-small cell lung cancer cells measured using Ellman's assay. Cells were pre-treated with DMSO or 20 μM BITC, PEITC or SFN for 1 h and then collected or returned to the incubator in fresh culture medium for an additional 2 h and then collected. Statistical analyses was performed and *$p<0.05$.

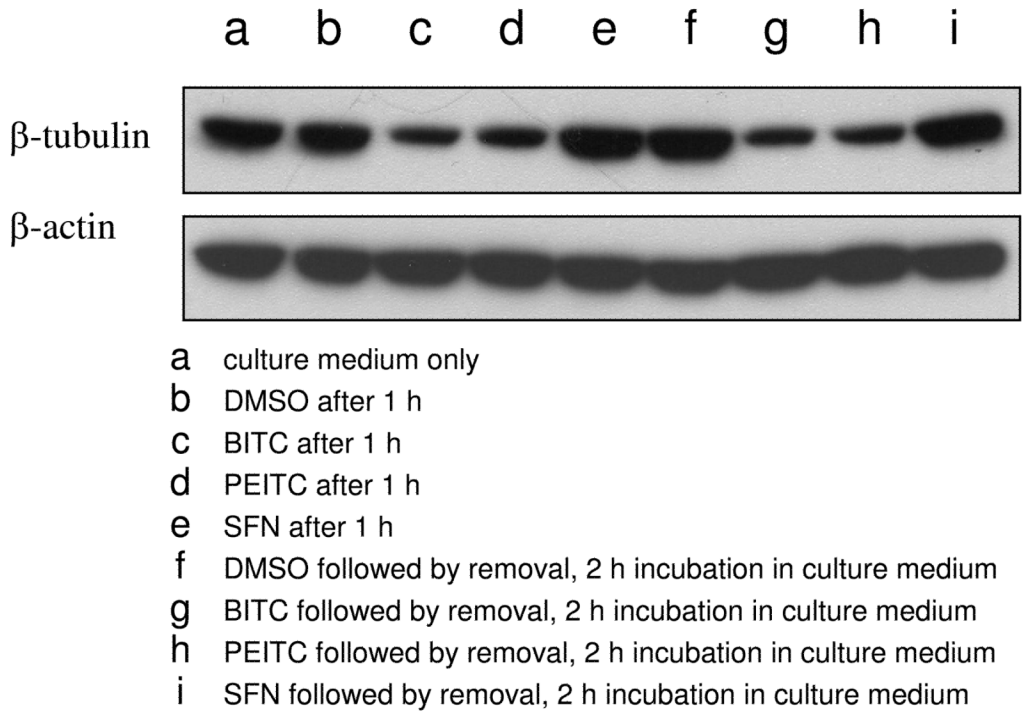

Figure 4.

Depletion of β-tubulin in human NCI-H596 non-small cell lung cancer cells, determined using Western blot. Cells were pre-treated with DMSO or 20 μM BITC, PEITC or SFN for 1 h and then collected or returned to the incubator in fresh culture medium for an additional 2 h and then collected. β-actin was used as a loading control.

Table 1

Cellular platinum accumulation (ng Pt per mg protein) and DNA-platination (ng Pt per ng DNA) in human NCI-H596 non-small cell lung cancer cells incubated in culture medium or pre-treated with DMSO (vehicle control), 20 μM BITC, PEITC or SFN for 1 h and then treated with 15 μM cisplatin (CDDP) or no drug for 2h.

Treatment[a]	ng Pt per mg protein	ng Pt per ng DNA
control (culture medium)[b]	$9.27 \pm 6.42 \times 10^{-02}$	$0.65 \pm 0.91 \times 10^{-05}$
DMSO/15 μM CDDP	50.12 ± 3.35	$4.06 \pm 0.92 \times 10^{-05}$
20 μM BITC/15 μM CDDP	43.83 ± 4.65	$4.28 \pm 1.67 \times 10^{-05}$
20 μM PEITC/15 μM CDDP	44.81 ± 2.29	$3.96 \pm 0.78 \times 10^{-05}$
20 μM SFN/15 μM CDDP	46.21 ± 3.84	$8.99 \pm 8.12 \times 10^{-05}$

[a] Platinum accumulation was measured in cells pre-treated with DMSO or 20 μM BITC, PEITC or SFN but not treated with cisplatin for additional controls and the values were 10^{-02} ng Pt per mg protein.

[b] Culture medium and other controls were used to determine sensitivity of the instrument and the value of 0 Pt; these samples contain no Pt.