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Proteomic Identification of Binding Targets of Isothiocyanates: A Perspective on Techniques

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Abstract

Intake of cruciferous vegetable is inversely associated with the risk of several cancer types. Isothiocyanates (ITCs) are believed to be important constituents contributing to these cancer-preventive effects. Although several mechanisms, including induction of apoptosis, have been proposed for the anti-carcinogenesis activities of ITCs, detailed upstream triggering events are still not fully understood. Identification of ITC binding targets in cellular proteins is crucial for not only mechanistic studies but also future drug screening and design. In this review, we summarize recent progress in discovery of ITC protein targets from a technical perspective. The advantages and limitations of each method are discussed to facilitate future studies on target discovery of ITCs and perhaps other compounds.

Keywords

isothiocyanates; proteomics; mass spectrometry; target identification; covalent modification

1. Introduction

Naturally occurring isothiocyanates (ITCs), including benzyl ITC (BITC), phenethyl ITC (PEITC), and sulforaphane (SFN), are effective cancer chemopreventive compounds (1, 2). Mounting evidence from animal studies indicates their anti-carcinogenic activity in all three major stages of tumor growth: initiation, promotion, and progression. Epidemiological studies have also shown that dietary intake of ITC is associated with reduced risk of human cancers (1, 2). Current literature indicates that induction of cell cycle arrest and apoptosis are important for the cancer preventive activity of ITCs (1-3). However, the detailed mechanisms, especially the earliest interactions between ITCs and their molecular targets, are still unclear (4).

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ITCs may react with nucleophilic amino acid residues in cellular proteins. The potential binding sites include thiol-containing cysteine, amine-containing lysines, arginine, and proline, and hydroxyl-containing serines, threonine and tyrosine. Among these sites, cysteines, especially the ionized cysteines (thiolate), are the strongest in proteins and represent the most likely binding sites of ITCs (5). Cysteines, being nucleophilic, redox-active, and metal binding active, are both structurally and functionally important components in many enzymes (6). Modifications of cysteine, through disulfide formation, oxidation, glutathionation, thioester formation, or direct covalent binding, may have critical biological consequences. For example, modification of reactive cysteines in Keap1 by SFN has been implicated as a trigger for Nrf2 activation (7). Therefore, identification of binding targets of ITCs is a key step in elucidating mechanisms by which these compounds induce various downstream activities. This knowledge may also guide the screening and design of anti-cancer compounds with more efficacy and fewer side effects. However, the target identification is complicated and sometimes can be difficult due to multiple reasons. For example, modified proteins are likely to account for only a small fraction of the whole proteome. Searching for such proteins and pinpointing the exact site of modification can be as challenging as searching for a needle in a haystack. To track down a rare modification such as that by ITCs *in vivo* requires approaches with sufficient sensitivity and specificity. Also, ITC-modified proteins may undergo a series of transformations. For example, tubulin forms aggregates as a result of the structural misfolding induced by ITC modification and is consequently degraded through the ubiquitin-proteasome system in living cells (8, 9). As we discuss later, proteasome-mediated degradation may be a common event for several potential ITC target proteins.

Fortunately, the unique chemical property of ITCs and their rapid formation of covalent bond with potential targets allow us to trace and identify the modified proteins with the following approaches (Figure 1): First, target proteins were labeled with ITCs and identified using proteomic techniques; second, ITC-induced functional and structural changes of the target proteins were demonstrated using appropriate biological assays *in vitro* and *in vivo*; third, the ITC affinity to target proteins was determined and ITC binding sites in the target proteins confirmed. In this review, we summarize recent progress in ITC protein target discovery from a technical angle. Each method in different stages of the target identification is analyzed in terms of advantage and disadvantage.

2. Protein Target Identification

Two proteomic techniques have been used in the discovery of ITC target proteins. The first approach is to label target proteins with radioactive ITCs in cultured cells and purify target proteins in the cell lysate using two-dimensional gel electrophoresis (2D-GE). The visualized radiolabeled proteins are located and identified by mass spectrometry (Figure 2A). The other method is to “pull down” target proteins from cell lysate using affinity chromatography. This technique relies on the design of hybrid probes with ITC functional group at one end and a crosslinking probe at the other. The ITC functional group serves as the “bait” for target proteins while the crosslinker is required for facile and selective solid-phase target purification (Figure 2B).

2.1 Radio-labeling and two-dimension gel electrophoresis

Two-dimensional gel electrophoresis (2D-GE) has been frequently used to separate complex protein mixtures such as cell lysates. Mass spectrometry (MS) has become the premier technology for identifying proteins after 2D-GE. The combination of 2D-GE and MS has been used not only to reveal protein expression in cells (10) but also to identify drug targets. For example, ¹⁴C-labeled acetaminophen has been used in target discovery in liver proteins (11, 12). Inspired by this pioneering work, we treated human non-small cell lung cancer

A549 cells with 20 μ M ^{14}C -PEITC or ^{14}C -SFN for 1 h (8). The α -carbon in the phenethyl group was labeled as ^{14}C as a tracer to indicate ITC-conjugated proteins (Figure 3A). After treatment, cytoplasmic proteins were fractionated from the whole cell lysate and separated by 2D-GE. The gels were dried and exposed to radiosensitive films. The ITC-modified proteins were located through the spots present on the autoradiographic image. After overlapping this image and the image of coomassie blue staining of the same gel, only a small fraction of protein spots were found to carry radioactivity, suggesting that ITC binding targets are specific. These spots were excised from the coomassie stained gel and tryptically digested. The peptides were identified using matrix-assisted laser desorption-ionization (MALDI)-MS. Approximately 30 proteins were identified as potential binding targets of ITCs (unpublished results). To validate, we demonstrated that tubulin, one of proteins identified, was covalently bound by ITCs resulting in its conformational and functional changes. These changes were found to correlate well with ITC-induced cell cycle arrest and apoptosis (8). In addition to tubulin, we recently showed that proteasome, which has been identified using 2D-GE, is another ITC binding target and the binding by BITC and PEITC correlates with proteasome activity inhibition and growth inhibition in multiple myeloma (13). Although binding to these potential targets by ITCs have been shown to have distinctive protein functional effects, it is likely that the anti-carcinogenic activity of ITCs is collectively contributed by binding to multiple target proteins and multiple signaling pathways.

The radiolabeling 2D-GE approach has the advantages of high reliability and reproducibility due to the maturity of 2D-GE. Immobilized pH gradient strips with a broad range can be used for comprehensive proteomic separation while strips with a narrow *pH* range (i.e. 3.9-5.1) can improve local resolution. The resolution of the second dimension can also be improved for proteins with different molecular weight ranges through adjusting gel density. For example, a 4-20 % Bis-Tris gradient gel can be constructed to separate proteins between 5-200 kDa. However, 2D-GE has its limitations. First, the availability of radiolabeled compounds is a potential limitation. However, previously published synthesis methods on radioactive ITCs, including a variety of phenyl-containing ITCs (14) and SFN (15), can be instructive for generating isotope-labeled ITCs. Synthesis of ^{14}C PEITC is briefly illustrated in Figure 3A. Second, proteins with extreme *pI* values, such as less than 3 or more than 10, cannot be effectively resolved by isoelectric focusing. Third, reproducibility of 2D-GE may be affected by the lengthy sample preparation procedures. Also, the sensitivity of the technique relies on the radioactivity of the probe. To generate clear ^{14}C autoradiographic images, films were kept exposed for more than a month. More importantly, the radioactivity is proportional to the level of protein adducts, which is dependent on the protein expression level and ITC's protein affinity. Thus, low copy number or low binding affinity proteins, are less likely to be recognized compared to abundant proteins with higher binding affinities.

2.2 Affinity chromatography

Recently, several laboratories have reported that macrophage migration inhibitory factor (MIF), a pro-inflammatory cytokine, can be covalently modified by ITCs, including BITC and PEITC through binding to N-terminal catalytic proline residues (16-18). Taking advantage of the strong interaction between biotin and streptavidin, Cross *et al.* designed a biotin-labeled pentyl-ITC (Bio-ITC, see Figure 4A) for detection and purification of ITC binding targets (16). Lysates extracted from HeLa cells were labeled with Bio-ITC *in vitro*. To detect ITC-conjugated target proteins, lysates were separated on 1-D SDS-PAGE gels and blotted using either streptavidin-conjugated horseradish peroxidase or anti-biotin antibody. To purify ITC binding targets, cell lysates were loaded onto affinity columns with streptavidin-Sepharose beads (Figure 4B), comparing the proteins recovered to those from extracts of cells treated prior to lysis with natural ITCs. Mass spectrometry analysis

identified MIF, a small protein with 12 kDa, as the ITC target. Previously, this laboratory used a similar method to show protein kinase MEKK1 was also modified by ITCs (19). The synthesis of biotin-labeled ITCs for these two studies is illustrated in Figure 3B.

To identify MIF as a target of PEITC, another group used a hybrid probe called amino-PEITC (2-(3-(2-aminoethyl)phenyl)ethyl isothiocyanate) featuring PEITC on one end and a *meta*-aminoethyl group (18). The synthesis of this probe is illustrated in Figure 3C. After the *meta*-aminoethyl group of the amino-PEITC was crosslinked with *N*-hydroxysuccinimide ester groups on solid-phase resin (Figure 5A), beads were incubated with lysates of Jurkat T-lymphoma cells (Figure 5B). ITC-modified proteins that coupled to the resin were eluted and separated by SDS-PAGE. Macrophage inhibitory factor was identified as one of the proteins coupled to the resin and therefore modified by PEITC using MS.

Due to its high specificity, affinity chromatography can be optimized to concentrate the eluents so that target proteins with a low copy number and low binding affinity can be enriched. Targets with different affinities can also be fractionated by using gradient elution steps. Increasing the sample purity and quantity through fractionation facilitate greater accuracy in the MS identifications. However, the current technique has also its limitations. For example, we have shown that ITCs with different side groups demonstrated differential binding affinity to tubulin (4, 8, 13), suggesting hydrophobicity, size, and rigidity of the side group may play a role in ITC binding to target proteins. Therefore, it is a reasonable assumption that using an ITC probe with a bulky side chain may affect its binding specificity and affinity. To contravene this limitation, Cross *et al.* (16) used a competition assay to reduce the non-specific binding. The other lab (18) improved binding specificity through comparison of protein bands pulled down by Affi-gel beads with and without ITC groups. More importantly, because the probes containing the ITC-functional group are exposed to extracted cell lysate, in which a significant amount of free thiols either forms disulfide or are oxidized during cell lysis, much less target proteins are likely to be identified through affinity chromatography as compare to a method involving the modification of target proteins *in vivo*.

3. Target Validation

3.1 Protein functional inhibition

After tubulin was identified as one of the 30 potential ITC binding targets, a functional assay was used to verify this target (8). Microtubules are important components of cytoskeletons, forming metaphase spindle for cell division and serving as “highways” for intracellular transportation of vesicles, granules, and organelles (21). The dynamic equilibrium between tubulin (dimer) and microtubules (polymer) is essential for a variety of cellular functions and is also a valid target for cancer therapy. Taxanes, which stabilize microtubules, and colchicine analogues and vincas, compounds that destabilize polymerization, constitute an important category of the current chemotherapy arsenal. To verify tubulin as an ITC target, we treated purified tubulin with 30 μ M of BITC, PEITC, SFN, and *N*-methylphenethylamine (NMPEA), a structural analog of PEITC that does not contain the ITC functional group. Tubulin treated with DMSO or NMPEA rapidly formed tubulin polymers at 37 °C. However, BITC and PEITC significantly inhibit tubulin polymerization and SFN also showed moderate inhibition. Immunofluorescent staining of tubulin also showed that ITCs disrupt microtubule networks *in vivo*. Taken together, these results indicate that ITCs inhibit tubulin biological function. Further studies showed that disruption of microtubules by ITCs correlates well with the induction of G₂/M arrest, apoptosis, and cell growth inhibition, suggesting that ITC-binding to tubulin is biologically significant (8, 9).

MIF is an important cytokine that regulates the inflammatory response and is critically involved in the development of arthritis and glomerulonephritis and has been implicated in tumor growth and angiogenesis (22). It is also a tautomerase with a catalytically active N-terminal proline. ITCs have been shown to inhibit tautomerase activities of both recombinant MIF protein and endogenous MIF in HeLa cells (16-18). Binding of MIF to its receptor CD74, an upstream event leading to inflammatory response, has also been shown to be suppressed by ITCs (16). Additionally, BITC was found to inhibit MIF-mediated glucocorticoid overriding activity and inhibit MIF-induced AKT phosphorylation (18). Taken together, ITC-induced inhibition of MIF activity has been associated with anti-inflammation and anti-carcinogenesis (16-18).

3.2 Protein conformational changes

Functional inhibition of a protein usually stems from a change in its conformation. To study secondary structural changes, the ellipticity of tubulin was measured using circular dichroism before and after BITC treatment (8). The results indicated that BITC decreases the helical content of tubulin. Intrinsic fluorescence intensity of eight tryptophan residues in both α - and β - tubulin was also monitored during ITC treatment. The intensity was significantly suppressed by BITC and PEITC, moderately by SFN, but not by DMSO and NMPEA. We also found that the binding affinities to tubulin by ITCs follow the order of BITC>PEITC>SFN, the same relative potencies of inducing tubulin conformational and functional changes. The findings indicate that: 1) structural changes is consistent with that of functional changes; 2) binding to tubulin correlates with tubulin-related events; and 3) there is an interesting structure-activity relationship.

In the study of MIF, structural changes after ITC treatment were also systematically measured (17, 18). First, an ITC-induced conformational change of MIF was indicated by a gradual decrease in the affinity between MIF and a monoclonal antibody specific to the intact form of the protein (18). An NMR study found that BITC binding to proline 1 within MIF induces significant changes to the surrounding residues and the active site of the protein; a clear signature of tertiary structural changes (17). Because homotrimer formation is required for MIF tautomerase activity, a series of experiments, including analytical ultracentrifugation, size exclusion chromatography, and light scattering were performed. The results of these monomer-oligomer equilibrium studies indicated that it is unlikely that binding by BITC has any effects on the quaternary structure of MIF (17).

3.3 Turnover of target protein

Consistent with their binding affinities, BITC and PEITC, but not SFN, induce rapid, significant, and selective degradation of both α - and β -tubulin in a variety of cell types (8, 9). These results indicate that the tubulin degradation occurs in two sequential steps (8, 9, 23). First, ITC-bound tubulin forms aggresome-like protein aggregates that are insoluble in lysis buffers containing Nonidet P-40 or Triton X-100. In addition to tubulin, chaperone proteins such as Hsp27, proteasome subunits, and ubiquitinated proteins have also been identified in these aggregates (9, 23). Tubulin precipitation depends on both treatment time and concentration of ITCs and its potency follows the order of BITC>PEITC>SFN, suggesting that precipitation is a result of tubulin misfolding following ITC binding. This notion is also supported by the observations that tubulin precipitation can be blocked by pretreatments using tubulin binding agents, such as colchicine, vinblastine, and taxol, which have been shown to reduce ITC binding affinity to tubulin (9). In the second step, precipitated tubulin is likely to be degraded by the ubiquitin-proteasome system because it can be effectively blocked by proteasome inhibitors (MG-132, proteasome inhibitor I, epoxomicin, and *clasto*-lactacystin β -lactone), an inhibitor for ATP synthesis (2-deoxyglucose), and an inhibitor for ubiquitin-activating enzyme E1 (PYR-41) (9). Using

RT-PCR, we also demonstrated that ITCs neither promote nor inhibit transcriptional levels of tubulin, confirming that the turnover of tubulin is regulated by ITCs through post-synthetic modification (9).

The depletion of other ITC target proteins has also been observed (24-28). Although no direct evidence of binding by ITCs, previous studies showed that the levels of M3/6 and Cdc25c, two known phosphatases and potential ITC targets, were down-regulated by PEITC via a proteasome-dependent mechanism (24, 25). Also, protein kinase C (PKC), including PKC alpha and epsilon, another potential ITC target, has been reduced by PEITC (26). Very recently, oncoproteins Stat3 and mutant p53 have been shown to be depleted by treatments of BITC and PEITC (27, 28). It seems that rapid and significant protein level reduction is a common feature of potential targets of ITCs. The protein level change may be used as a biomarker, in addition to conformational and functional changes, to screen for potential ITC targets.

4. Binding site identification

Identifying and characterizing the ITC binding sites on target proteins is critical to deciphering the mechanisms of ITC-induced cellular effects. Not only the studies shed light onto mechanistic insights, but also provide molecular basis for drug optimization and screening.

4.1 Affinity quantification

Ellman assay, a biochemical assay that measures free thiols in purified proteins, can be used to measure the extent of ITC binding to cysteine residues within proteins (29). This assay works especially well with a target protein containing multiple cysteine residues, such as tubulin which contains 20 cysteines. Our studies showed that BITC and PEITC treatments result in the modification of 9-12 of the 15 free thiols in tubulin with the stoichiometric ratio 1:1 between ITC to cysteine residues. However, SFN treatment results in the modification of only 4 thiols under the same conditions. The relative affinity of these ITCs toward tubulin correlated with their downstream effects, suggesting that ITC binding to cysteines may underlie tubulin-related activities.

4.2 Mass spectrometry proof

MS is commonly used to identify posttranslational modifications of proteins as well as direct covalent interactions between proteins and small molecules (30, 31). It not only helps identify ITC binding sites but also serves as the “ultimate proof” of the conjugation between ITC and its target proteins.

As the first step to identify ITC binding sites on tubulin, purified porcine tubulin was treated with ITCs. The protein was then digested with trypsin and analysis using MALDI-MS/MS and nanospray reversed-phase liquid chromatography tandem MS (nanoRPLC-MS/MS) (8). The results showed a cysteine-containing β -tubulin peptide, $^{298}\text{NMMAACDPR}^{306}$ ($[\text{M}+\text{H}]^+$ of m/z 1008.4) was covalently modified by ITC, generating a mass shift of +149, 163, and 177 Da with BITC, PEITC, and SFN, respectively. Further analysis of the MS/MS spectra identified that Cys³⁰³ in the peptide fragment is the binding site of ITCs (8).

To determine if ITC covalently modified tubulin *in vivo*, A549 cells were treated with 20 μM BITC. Both the soluble and insoluble fractions of the cell lysate were analyzed using nanoRPLC-MS/MS (8). The results indicated that Cys347, a conserved residue in the α -tubulin isoform, is covalently modified by BITC (Figure 6). This is the first *in vivo* proof of the conjugation between ITC and a cellular protein. Because this adduct was found only in

the insoluble fraction, which contains the precipitated tubulin, the results further support that ITC binding to tubulin leads to protein misfolding and precipitation (8).

To identify ITC-cysteine adducts using MS could be challenging. One reason is that the thiocarbamation is reversible due to an increase in pH value and the presence of reducing agents, which cause ITC to be cleaved off the target protein. Therefore, sample preparation can be the key in successful identification of ITC binding adducts by MS. However, a previous study demonstrated that ITC adduct on MEKK1 is resistant to boiling in SDS and DTT (19).

Compared with ITC-cysteines adducts, binding of ITCs to amino-group containing residues, such as lysine and proline, is more stable (5). The *in vivo* adducts formed between ITCs, including PEITC and SFN, and lysine residues in albumin have recently been reported using LC-MS/MS (32). The identification of N-terminus proline to be the binding site of ITCs on MIF was accomplished in two labs using different methods. Cross *et al.* analyzed ITC-treated and trypsin-digested MIF using MS/MS. They also demonstrated modification of N-terminus proline by PEITC and SFN using intact, non-trypsinized MIF (16). In a separate study Brown *et al.* analyzed PEITC-treated recombinant human MIF without trypsin digestion by LC-MS/MS (18). A new peak with an increase in mass of 163 Da (PEITC molecular weight) was observed with PEITC-treated MIF, indicating that MIF is modified by the ITC with a stoichiometric ratio of 1:1. Individual mutation of Cys57, Cys60, and Cys81 on MIF with serine residues did not abolish the mass increment, therefore, excluding the possibility that these cysteines were modified by PEITC. However, the mass increment was completely abolished in a mutant MIF replacing N-terminal proline with alanine, indicating that the proline is the binding site of PEITC.

MS analysis, often with MALDI, of intact proteins can be used to provide rapid and sometimes accurate estimation of binding affinity of ITCs on target proteins (18). However, the accuracy of this determination is compromised for proteins with a large molecular weight (33). Comparatively, tandem MS analysis of trypsin-digested peptides yields satisfactory results regardless of protein size. It also can pinpoint the modified residue within a peptide of interest.

4.3 Side-directed mutagenesis

Besides being used as a function validation tool, site-directed mutagenesis is an important alternative approach for identifying ITC binding sites. The evidence comes from the observations that an ITC-induced downstream effect is diminished in a residue-specific mutation, but not in the wild-type (18, 34). For example, a recent study found that SFN inhibits UVB-induced skin carcinogenesis in SKH-1 mice through inhibition of AP-1 binding to TRE (12-O-tetradecanoylphorbol-13-acetate response element) (34). To study whether SFN blocks AP-1 as a result of binding to TRE through some key cysteines at the DNA binding domain, Cys154 of cFos and Cys272 of cJun were mutated to serines. The DNA binding assay results showed that SFN blocks the wild-type recombinant heterodimer AP-1 from binding to DNA while the blockade is voided in mutant AP-1, suggesting that these cysteines are potential sites for SFN binding. Another study identified TRPA1 (Transient receptor potential cation channel, subfamily A, member 1), a nonselective cation channel expressed by sensory neurons of the pain pathway, as a potential target of ITCs using site-directed mutagenesis (35). Three closely spaced N-terminal cysteines (Cys619, Cys639, and Cys663) of TRPA1 were mutated to either serine or alanine. The resulting triple mutant showed little response to ITC treatment compared with TRPA1 activation by ITCs, indicating that ITCs activate TRPA1 mainly through direct covalent modification of N-terminal cysteine residues within the channel protein.

Although site-directed mutagenesis is a popular technique in defining binding sites and functional roles for electrophiles, it also has limitations. First of all, it requires that mutation alone has little or no effect on the functional assay. Therefore, proper selection of the residue(s) to mutate may be critical to the success of the assay. Secondly, it does not provide definitive proof for the existence of covalent binding by ITC. Reactive oxygen species (ROS) induced by ITCs, besides ITC itself, can also modify target cysteine residues, especially those exposed to hydrophilic surfaces at the DNA binding domains. Thus, it is impossible to rule out the cause of indirect modifications. The most convincing evidence comes from studies using both MS and site-directed mutagenesis as complementary approaches for identifying ITC binding sites on proteins.

5. Perspectives

While ITCs are versatile cancer chemopreventive agents in both humans and animals, our knowledge of molecular mechanisms by which they exert anti-carcinogenesis effects is still limited. Identification of molecular targets of ITCs can provide useful information for mechanisms and future drug design. In this review, a number of major proteomic approaches for ITC target discovery are summarized. For example, 2D-GE and affinity chromatography are used in protein target identification; protein functional, conformational, and level changes can be used to confirm the interaction between ITC and target proteins; MS and site-directed mutagenesis are complementary for identifying and validating binding sites. Understanding the pros and cons of different methods would facilitate the discovery of protein targets of ITCs. Knowledge in functional biology is also valuable in providing clues for target discovery. For example, glutathione reductase (GR) and thioredoxin reductase (TrxR) are important redox homeostasis enzymes in cells. It is known that Cys58 and Cys63 are located in the catalytic sites of GR and must remain reduced before binding their substrate glutathione disulfide GSSG (36, 37). Similarly, there are two redox centers in TrxR, one containing Cys59 and Cys64 and the other Cys497 and SelenoCys498 (37, 38). These residues are all essential to its enzymatic activity and modification of these residues may inhibit activities of GR and TrxR. Taken together, the combination of proteomic approaches and functional biology offers a fruitful approach in protein target identifications of ITCs.

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Figure 1.
Thiocarbamylation between PEITC and a protein cysteine.

Figure 2.

Key steps in proteomic identification of ITC targets. A, cultured cells were treated with radiolabeled ITCs. The cell lysate was extracted and separated by 2D-GE. Protein and radioautography images were obtained by coomassie blue staining (blue) and X-ray film (red), respectively. Protein spots with radioactivity were cut and analyzed by mass spectrometry. B, ITC-carrying probes were added to cell lysates to bind proteins. Subsequently, affinity beads were crosslinked to the ITC probe-protein conjugates. ITC target proteins were separated by affinity chromatography before analyzed by mass spectrometry.

Figure 3.

Synthesis scheme for ITC probes: ¹⁴C-PEITC (A), Biotin-ITCs (B), and amino-PEITC (C). A, benzyl chloride reacted with radiolabeled potassium cyanide to form ¹⁴C-benzyl cyanide, which was further changed to ¹⁴C-phenethylamine. Finally, ¹⁴C-PEITC was obtained by a reaction of thiophosgene and ¹⁴C-phenethylamine. The asterisk indicates the labeling position. B, route I: norbiotinamine reacted with 1,1'-thiocarbonyldi-2,2'-pyridone (TCDP) to form norbiotinyl ITC (a), which was used in identifying MEKK1 as ITC target protein (ref. 19). Route II: Norbiotinamine reacted with succinimidyl 12-Cbz-aminododecanoate to form N-norbiotinyl-12-aminododecanoamide, which further reacted with TCDP to form 12-[(norbiotinylamino)carbonyl]dodecyl ITC, which was used in the ref. 16. C, at first, 1,3-bis(cyanomethyl)benzene was reduced by nickel borohydride to 1,3-bis(2-aminoethyl)benzene, of which one amino was protected by *tert*-Boc derivation and the other amino was transformed to ITC group by reacting with thiophosgene; and finally, 2-(3-(2-aminoethyl)phenyl)ethyl ITC was generated by removing *tert*-Boc in trifluoroacetic acid (TFA).

Figure 4. Affinity chromatography of ITC targets using biotinylated ITC (ref. 16). A, biotin-ITC, 12-((norbiotinylamino)carbonyl)dodecyl ITC, was used to react with the N-terminal proline of MIF. B, Streptavidin-coated beads were added to recognize and bind biotinylated MIF for separation.

Figure 5. Affinity chromatography of ITC targets using Affi-PEITC (ref. 18). A, Affi-Gel, a solid-phase resin containing reactive *N*-hydroxysuccinimide ester groups, reacted with the *meta*-aminoethyl group of amino-PEITC. B, Affi-gel beads coated with ITC targeted MIF for binding.

Figure 6.

Tandem mass spectrometry (MS/MS) spectrum of a tubulin alpha chain peptide isolated from the insoluble fraction of A549 cells treated with BITC (20 μ M) showing that a cysteine residue (Cys³⁴⁷) is covalently modified by BITC (ref. 8). A549 cells were treated and lysed. Tubulin-containing cellular fractions were identified by western blot and subjected to tryptic digestion prior to analysis using MS/MS. The BITC-cysteine adduct was identified by the presence of a mass of 149.03 Da added to this residue.