Received: 16 March 2018 | Accepted: 18 May 2018

DOI: 10.1002/jcb.27157

RESEARCH ARTICLE

WILEY Journal of Cellular Biochemistry

Computational and biochemical studies of isothiocyanates as inhibitors of proteasomal cysteine deubiquitinases in human cancer cells

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Funding information
NCI, NIH, Grant/Award Numbers: R21CA184788, P30 CA022453

Abstract

Isothiocyanates (ITCs) are natural chemoprotective products found abundantly in cruciferous vegetables. However, the cancer-relevant targets and molecular mechanisms of ITCs remain unclear. We hypothesize that ITCs, as electrophiles, can interact with the catalytic triads (CYS, HIS, and ASP) of the proteasomal cysteine deubiquitinases USP14 and UCHL5, ultimately inhibiting their activities. In the current study, we exploited this possibility by performing both computational docking and biochemical validation assays using human breast and prostate cancer cell models. Docking results suggest that benzyl isothiocyanate, phenethyl isothiocyanate, and DL-sulforaphane are more potent inhibitors of UCHL5 than USP14, and these ITCs could interact with the catalytic triads of UCHL5 and USP14. Indeed, ubiquitin vinyl sulfone assay confirmed the inhibitory activity of each ITC on the ubiquitin-binding activity of UCHL5 and USP14. We also found that inhibition of USP-14 and UCHL5 activities by the ITCs caused increased levels of USP14 and UCHL5 proteins, but not the third 19S-deubiquitinating enzyme (DUB), POH1/RPN11, suggesting feedback loop activation and further supporting that ITCs are inhibitors of proteasomal cysteine DUBs. Associated with DUB inhibition by ITCs, ubiquitinated proteins were significantly increased, accompanied with induction of apoptosis, inhibition of proliferation and suppression of cell invasion. Our findings of ITCs as proteasomal cysteine DUB inhibitors should provide insightful information for designing, discovering and developing potent, specific 19S-DUB inhibitors for cancer therapies.

KEYWORDS

benzyl isothiocyanate (BITC), drug discovery, phenethyl isothiocyanate (PEITC), sulforaphane (SFN), ubiquitin-proteasome system, UCHL5, USP14

Zainab Sabry Othman Ahmed and Xin Li contributed equally to this study.

1 | INTRODUCTION

Apoptosis is a physiological process required for regulating the number of cells in the tissue of organisms.[1] Failure of apoptosis could lead to the development of various diseases, such as cancers, because of the uncontrolled cell proliferation and consequent tumor growth.[2] Therefore, induction of apoptosis has become one of the most effective strategies for cancer treatment.[3,4]

It has been well documented that cancer cell apoptosis could be induced by targeting the ubiquitin-proteasome system (UPS),[5,6] which controls many cellular processes and is considered as the major regulator of protein degradation in eukaryotes.[7,8] UPS-mediated protein degradation contains 2 distinct steps: ubiquitination and 26S proteasome-mediated degradation. In the ubiquitination step, multiple ubiquitin molecules are linked to a protein substrate by several ubiquitin-conjugating enzymes (E1, E2, and E3). The complex is then recognized and degraded by the 26S proteasome. The 26S proteasome consists of a 20S barrel-shaped structure known as a central catalytic core, which is responsible for the proteolytic activity of the proteasome and that is capped on each end by a 19S regulatory particle.[7,9,10] The 19S regulatory particle has at least 19 subunits and composed of a lid and a base,[11] which prevents the erroneous degradation of nonubiquitinated cellular proteins and gives the proteasome high specificity.[7] The 20S catalytic core consists of 28 subunits arranged in 4 stacked heptameric rings. The outer 2 rings composed of 7 different α subunits have a regulatory function, while the inner 2 rings are composed of 7 different β subunits possessing catalytic activity (β1 has caspaselike, β2 has trypsinlike, and β5 has chymotrypsinlike activity).[12]

Ubiquitination is reversible through deubiquitinating enzymes (DUBs),[13] which are responsible for removal of ubiquitin molecules from the substrate protein before its degradation by cleaving the isopeptide bond at the C-terminal of ubiquitin.[14-17] There are approximately 100 DUBs encoded by the human genome[13] that are divided into 6 subfamilies.[13,18] Three of these DUB enzymes are attached to the 19S regulatory particle: USP14/Ubp6 and UCHL5/UCH37 belong to the cysteine protease class and their activity is toward the distal tip of the ubiquitin chain while POH1/RPN11 belongs to the metalloprotease class, which can cleave the bottom of the ubiquitin chain.[15-17] These proteasome-associated DUBs play an important role in ubiquitin homeostasis.[19] Expression of DUBs was reported to be overexpressed in different tumor tissues.[7,20] This made them an important target in the treatment of many cancers including prostate and breast cancers.[21] Currently, only 1 USP14-selective inhibitor, VLX1570, is being tested in a phase 1/2 multiple myeloma clinical trial.[20] Therefore, discovering potent, specific 19S-DUB inhibitors should have a major impact on improving current cancer therapies.

Consuming cruciferous vegetables has been linked to decreased risk of malignancies.[22] The cancer-preventative effect of cruciferous vegetables is associated with the presence of isothiocyanates (ITCs), such as benzyl isothiocyanate (BITC), phenethyl isothiocyanate (PEITC), and sulforaphane (SFN).[23,24] However, the exact molecular mechanism of ITCs as anticancer compounds is still unclear.[25] It is reported that these compounds could induce apoptosis and inhibit tumor cells in vitro and in vivo.[26,27] It is also reported that some ITCs could inhibit several DUBs through the formation of an adduct with a cysteine residue of the active site.[28-31] However, whether and how ITCs inhibit proteasomal DUBs remain to be further examined.

In this study, we intended to investigate if all 3 ITCs, BITC, PEITC, and SFN, could inhibit the 2 proteasomal cysteine DUBs, USP14, and UCHL5, and the association of the DUB inhibition by ITCs with levels of cell growth and cell death in both breast cancer and prostate cancer cell models.

2 | MATERIALS AND METHODS

2.1 | Cells and materials

Human prostate cancer 22Rv1 cells (a generous gift from Dr. Asfar Azmi of Karmanos Cancer Institute at Wayne State University) were grown in RPMI 1640 Medium (Gibco), and breast cancer MDA-MB-231 and MDA-MB-468 cell lines (from ATCC, American Type Culture Collection, Manassas, VA) were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco, Hanover Park, IL), all supplemented with 10% FBS (J R Scientific, Woodland, CA) and, 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco, Hanover Park, IL). Cell culture experiments were performed at 37°C and 5% CO_2. BITC (252492 Aldrich, St. Louis, MO), PEITC (253731 Aldrich, 99%, St. Louis, MO), and DL-SFN (S4441 Sigma, St. Louis, MO; ≥90% high performance liquid chromatography [HPLC]) were all dissolved in dimethyl sulfoxide (DMSO; Thermo Fisher Scientific, Hanover Park, IL) at 50 mM concentration as a stock solution and stored at −20°C and diluted with cell culture medium when used. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Thermo Fisher Scientific (Hanover Park, IL); crystal violet powder was from Sigma-Aldrich and a 0.5% solution of crystal violet was prepared by using distilled water and methanol (Thermo Fisher Scientific, Hanover Park, IL). Ubiquitin vinyl sulfone (Ub-VS; 250 μM) was obtained from Boston Biochem. UCHL5 polyclonal antibody, PSMD14/POH1 polyclonal antibody, and HRP-conjugated beta-actin monoclonal antibody were all purchased from Proteintech (Rosemont, IL). USP14 (D8Q6S)

rabbit mAb was from Cell Signaling Technology (Danvers, MA); Ubiquitin (P4D1, sc8017) mouse monoclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA); purified mouse anti-human poly (ADP-ribose) polymerase (PARP) was purchased from BD Pharmingen. The protein assay kit was from Bio-Rad Laboratories, Inc (Hercules, CA).

2.2 | In silico docking and analysis

Computational analysis was performed through molecular docking using the AutoDock Vina software.[32] The Protein Data Bank (PDB) files 3IHR and 2AYO were chosen as suitable crystal structures representing UCHL5 and USP14, respectively. To prepare these protein crystal structures for AutoDock Vina, the UCSF Chimera software was used. Specifically, the 'Dock Prep' function was used to delete any solvents, resolve incomplete side chains, and add hydrogens and appropriate charges while retaining the highest-occupancy set of alternate locations.[33] Molecular design limited (MDL) Molfile files were created in ChemSketch for BITC, PEITC, and SFN.[34] These Molfiles were converted to PDBQT file-type for use with AutoDock Vina.

On inputting the files into the software, a search area was defined to maximize the accuracy of the docking analysis. The search area was 30 Å × 30 Å box centered around 3 residues, which represented the catalytic active site of both 3IHR and 2AYO. Output of the software includes an estimation of the free energies of binding and an output files for visualization. Both the free energy and the protein-ligand interactions were considered for evaluation of potential inhibition.

2.3 | Whole cell protein extract

The treated cells were harvested by a scraper, centrifuged at 1200 rpm for 5 minutes and then suspended in ice-cold cell lysis buffer for 30 minutes. The lysis buffer was prepared using 1 mM Tris-HCL (pH 8.0), 1 M NaCl, 10% NP-40, 0.5 M EDTA in distilled water, and Halt protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific) was added to the whole lysis buffer in 1:100 ratio. Cells were then centrifuged at 12 000 rpm for 15 minutes. The clear supernatant was used as a cell protein extract.

2.4 | Ubiquitin vinyl sulfone assay

This assay was used to detect the inhibitory effect of ITCs on 19S-associated USP14 and UCHL5. MDA-MB-231 breast cancer cells or 22Rv1 prostate cancer cells were seeded in 60-mm dishes and incubated at 37°C and 5% CO_2. When the cell confluence reached approximately 70%, the cells were either untreated or treated with DMSO or a selected ITC at different concentrations for 3 hours. Cell lysates (25 μg per sample) were mixed with Ub-VS (1 μM) in vitro for 30 minutes at 37°C in DUB buffer [50 mM Tris-HCL (PH 7.5), 250 mM sucrose, 5 mM of $MgCl_2$, 1 mM of Phenyl methane sulfonyl fluoride (Sigma-Aldrich), 2 mM adenosine triphosphate (Thermo Fisher Scientific), and 0.2 mM DTT (1,4-dithiothreitol; Sigma-Aldrich) in ddH_2O], followed by sodium dodecyl sulfate gel electrophoresis and immunoblotting with USP14 and UCHL5 antibodies.

2.5 | Cell viability analysis

MTT assay was used to determine cell proliferation. MDA-MB-231 breast cancer cells and 22Rv1 prostate cancer cells were seeded in a 96-well plate at 2×10^3 cells/well. Cells (in triplicates) were either untreated or treated with either DMSO or various concentrations of ITCs. Twenty-four hours after treatment, cells were incubated with 10 μM MTT reagent for 4 hours at 37°C and then the dye was solubilized by using 100 μL DMSO for 30 minutes. The optical density (OD) of each well was measured with a spectrophotometer at 750 nm and the calculation of mean values of OD for each concentration was performed.

2.6 | Western blot analysis

MDA-MB-231 and MDA-MB-468 breast cancer cells and 22Rv1 prostate cancer cells were seeded in 60-mm dishes. When the confluence of cells was 70%, cells were either untreated or treated with DMSO or 25 μM of BITC, PEITC, or SFN. Cells were harvested at the indicated time points and equivalent amounts of protein extracts (40 μg) were separated by 10% of sodium dodecyl sulfate polyacrylamide gel electrophoresis (Bio-Rad, Hercules, CA) under reducing conditions using 105 V for 120 minutes. The proteins were then electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes (Milpore Corp, Billerica, MA) using the X Cell II Blot Module (Invitrogen Life Technologies). After blocking the nonspecific binding sites by incubation for 1 hour with tris-buffered saline (TBS)-containing 5% fat-free milk and 0.1% Tween 20, the membranes were incubated with the primary antibodies overnight at 4°C. Immunologic detection was performed using the following primary antibodies: anti-UCHL5 polyclonal antibody (1:500 dilution), anti-USP14 (D8Q6S), rabbit mAb (1:500 dilution), PSMD14/POH1 polyclonal antibody (1:500 dilution), Ub (P4D1): sc8017 mouse monoclonal antibody (1:1000 dilution), and purified mouse anti-human PARP (1:1000 dilution). The membranes were then incubated for 2 hours with horseradish peroxidase–conjugated secondary antibodies (1:2000 dilution with the blocking buffer). The antigen-antibody complexes were detected with the electrochemiluminescence (ECL) chemiluminescence detection

system (Amersham Bioscience, Piscataway, NJ). The membranes were then reprobed with a monoclonal antibody raised against β-actin (horseradish peroxidase [HRP]-conjugated beta-actin monoclonal antibody, 1:10 000 dilution) as an internal control for protein loading and normalization between samples. Films were exposed, and blots were scanned, and the optical densities of the positive signals were quantified.

2.7 | Crystal violet assay

This assay was used to determine the cell viability of MDA-MB-231 and 22Rv1 prostate cancer cells that were treated with ITCs. The cells were seeded in a 12-well plate at 40% to 50% confluence and, when the cell confluence became 80% to 90%, the cells were either untreated or treated with DMSO or the indicated concentrations of BITC, PEITC, or SFN. After 24-hour treatment, the medium was removed, and the cells were fixed by using 70% ethanol for 10 minutes; ethanol was aspirated, and cells were washed twice with 1X PBS and then 500 μL of 0.5% crystal violet staining solution was added to each well and kept overnight at room temperature. Scanning of the plate was performed.

2.8 | Wound healing assay

This assay was used to determine the effect of ITCs on cell invasion and proliferation. MDA-MB-231 breast cancer cells were seeded in a 6-well plate, and at approximately 80% confluence, cells were scratched with a sterile 200 μL micropipette tip and washed twice with 1X PBS and were either untreated or treated with DMSO or the indicated concentrations of SFN for 48 hours. Morphological evaluation was performed using a Zeiss (Thornwood, NY) Axiovert 25 phase contrast microscope, and the same fields of the wound margin were photographed at 0 and 48 hours at 50× magnification. Aspiration of the medium was performed, and the cells were then fixed using 70% ethanol for 10 minutes. The ethanol was then aspirated, and the cells were washed twice with 1X PBS and stained with 500 μL of 0.5% crystal violet staining solution to stain the viable invading cells. Imaging of cells after staining was performed at 50× magnification.

3 | RESULTS

3.1 | Computational modeling of ITCs exhibits their ability to interact with 19S-associated DUBs USP14 and UCHL5

We hypothesize that ITCs as electrophiles can interact with the catalytic triads (CYS, HIS, and ASP) of the proteasomal cysteine deubiquitinases USP14 and UCHL5, leading to inhibition of their activities. To test this hypothesis, docking studies were conducted between USP14 or UCHL5 (as receptors) and BITC, PEITC, or SFN (as ligands). Protein Data Banks 2AYO (USP14) and 3IHR (UCHL5) were selected for docking. Using AutoDock Vina, predictions of the interactions between the DUBs and the ligands were generated. Increased specificity was achieved through targeting the reported active site residues of USP14 (CYS114, HIS435, and ASP451)[35] and UCHL5 (CYS-88, HIS164, ASP179).[36] Through the AutoDock-specific algorithm, a predicted interaction is outputted to a file in addition to a free energy score. The files were then visualized in PyMol. Figure 1 displays the generated conformation of interaction between each of the 3 ITC ligands and the active site residues of USP14 (A-C) and UCHL5 (D-F). The free energy scores of the conformations of interactions are summarized in the legend of Figure 1.

Figure 1A depicts the potential interactions between BITC and USP14. Histidine 434 (HIS-434) could interact with BITC, through addition of histidine's nitrogen to BITC's unsaturated carbon, leading to thiol reduction of BITC. Alternatively, the unsaturated carbon of BITC might undergo addition to the thiol of BITC to form a covalent adduct. While docking did not display any specific interaction with the cysteine residue (CYS-113), there was a predicted conformation for Aspartic acid 451 (ASP-451). ASP-451 may either bind to BITC alone or in combination to what was described above. The free oxygen in aspartic acid may interact with the unsaturated carbon of BITC. In addition, hydrogen bonding could stabilize interaction between BITC and the active site of USP14. Figure 1B displays the predicted interaction between PEITC and USP14, which has a virtually identical interaction mechanism to the depiction of the BITC-USP14 complex (Figure 1A). The free energy scores do not have a significant difference between BITC (−3.5 kcal/mol) and PEITC (−3.7 kcal/mol). Figure 1C has a lower predicted free energy score (−3.0 kcal/mol). The potential covalent interactions of SFN with the active site of USP14 seem to occur with the nitrogen of the HIS-434 residue and the unsaturated carbon of SFN. SFN does not seem to have any interaction with CYS-114, but ASP-451 could be able to form covalent adducts with SFN.

The ITC compounds may interact stronger with UCHL5 than to USP14 because more favorable bonding interactions were found on analyzing the general decrease of free energy across the 3 compounds corresponding to Figure 1D-F. Figure 1D suggests interaction of BITC with the active site CYS-88 of UCHL5 through 2 formed bonds. Through Michael addition, the thiol of cysteine and BITC may form covalent interaction similar to the interaction between DUBs and K48-/K63-linked diubiquitinated proteins.[37] Figure 1E demonstrates interaction of PEITC with

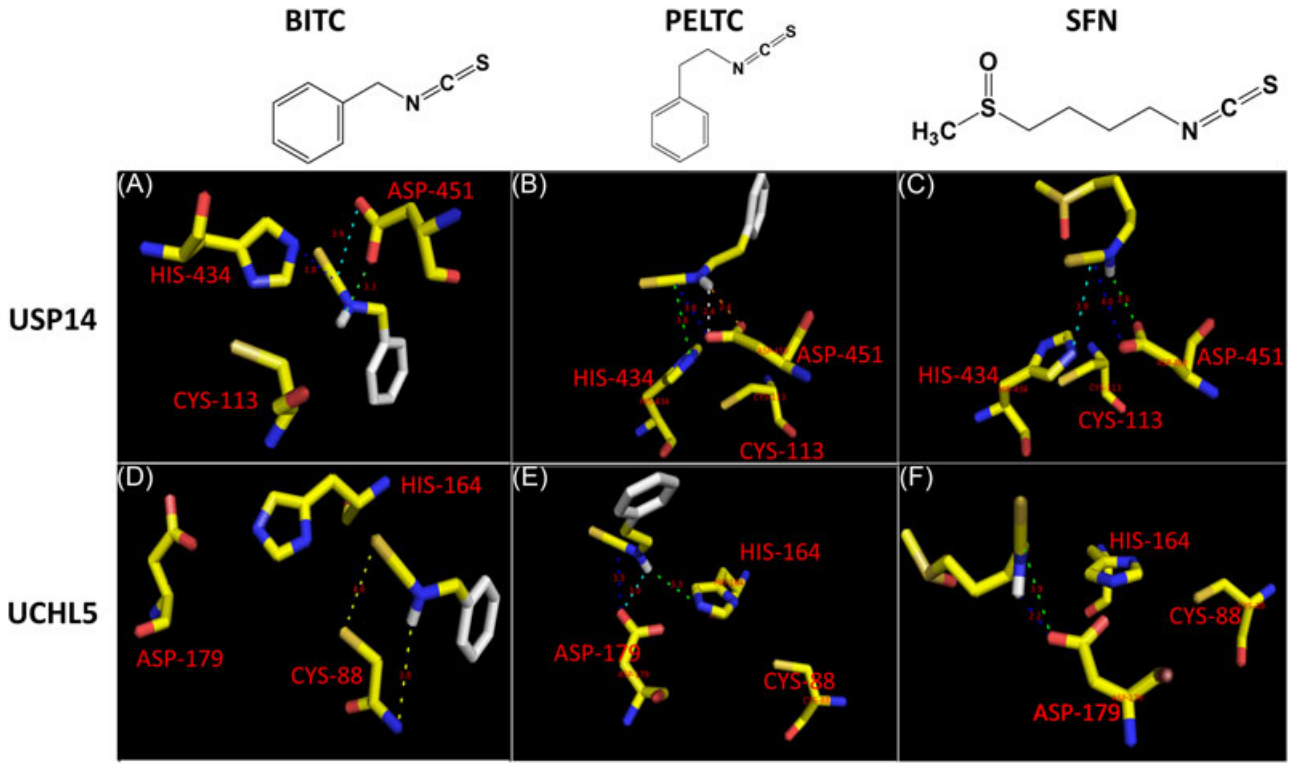

FIGURE 1 Determination of interactions of BITC, PEITC, and SFN with 19S-associated DUBs USP14 and UCHL5 by computational docking. Structures of BITC, PEITC, and SFN are shown at the top. Docking studies were conducted between USP14 (A-C) and UCHL5 (D-F) with BITC, PEITC, and SFN (left, middle, and right columns, respectively). The generated conformations (docking modes) between the active site amino acid residues of USP14 or UCHL5 and the ligands (ITCs) have been displayed. The free energy scores of the reactions of BITC, PEITC, and SFN with USP14 were −3.5, −3.7, and −3.0 kcal/mol, respectively, while that with UCHL5 were −5.0, −5.0, and −3.9 kcal/mol, respectively. BITC, benzyl isothiocyanate; DUB, deubiquitinating enzyme; ITC, isothiocyanate; PEITC, phenethyl isothiocyanate; SFN, sulforaphane

HIS-164 and ASP-179 of the active site of UCHL5. SFN seems to only interact with ASP-179 of UCHL5 through a similar addition reaction (Figure 1F). In summary, docking results suggest that these 3 ITC compounds could be inhibitors of USP14 and UCHL5.

3.2 | Inhibition of active site–directed labeling of proteasomal deubiquitinases by BITC, PEITC, and SFN in breast and prostate cancer cells

To confirm the predicted USP14-/UCHL5-inhibitory activities of BITC, PEITC, and SFN (Figure 1), we performed a competition assay between these ITCs and the ubiquitin active site probe Ub-VS. Both MDA-MB-231 breast cancer (Figure 2A,B) and 22Rv1 prostate cancer (Figure 2C-E) cell lines were either untreated or treated with the solvent DMSO, BITC, PEITC, or SFN at the indicated concentrations for 3 hours. Protein lysates were used to incubate with 1 μM Ub-VS for 30 minutes, followed by sodium dodecyl sulfate gel electrophoresis and immunoblotting with USP14 and UCHL5 antibodies. As controls, lysates from cells untreated or treated with DMSO without incubation with Ub-VS exhibited the lower bands of USP14 and UCHL5 expression at molecular weight 60 and 37 kD, respectively, while the addition of Ub-VS (MW approximately 10 kD) to these preparations generated new bands of approximately 70 and 47 kD (representing USP14-Ub-VS and UCHL5-Ub-VS conjugates), detected by anti-USP14 and anti-UCHL5, respectively (lanes 2 vs 1; Figure 2A-E). In contrast, we observed lower levels of USP14-Ub-VS conjugates and higher levels of USP14 in lysates of MDA-MB-231 cells treated with BITC or PEITC at 25 and 50 μM, compared with the DMSO-treated preparation (Figure 2A, upper panel, lanes 3-6 vs 2), indicating a dose-dependent inhibition of USP14 by BITC or PEITC. Similarly, BITC and PEITC also inhibited the Ub-binding activity of UCHL5 in a dose-dependent manner (Figure 2A, lower panel, lanes 3-6 vs 2). Please note that the basal level of the USP14 protein is much higher than UCHL5 in these cells. When lysates of MDA-MB-231 cells treated with 25 μM of SFN were used in the Ub-VS assay, inhibition of both USP14 and UCHL5 by SFN was observed (Figure 2B, lanes 3 vs 2).

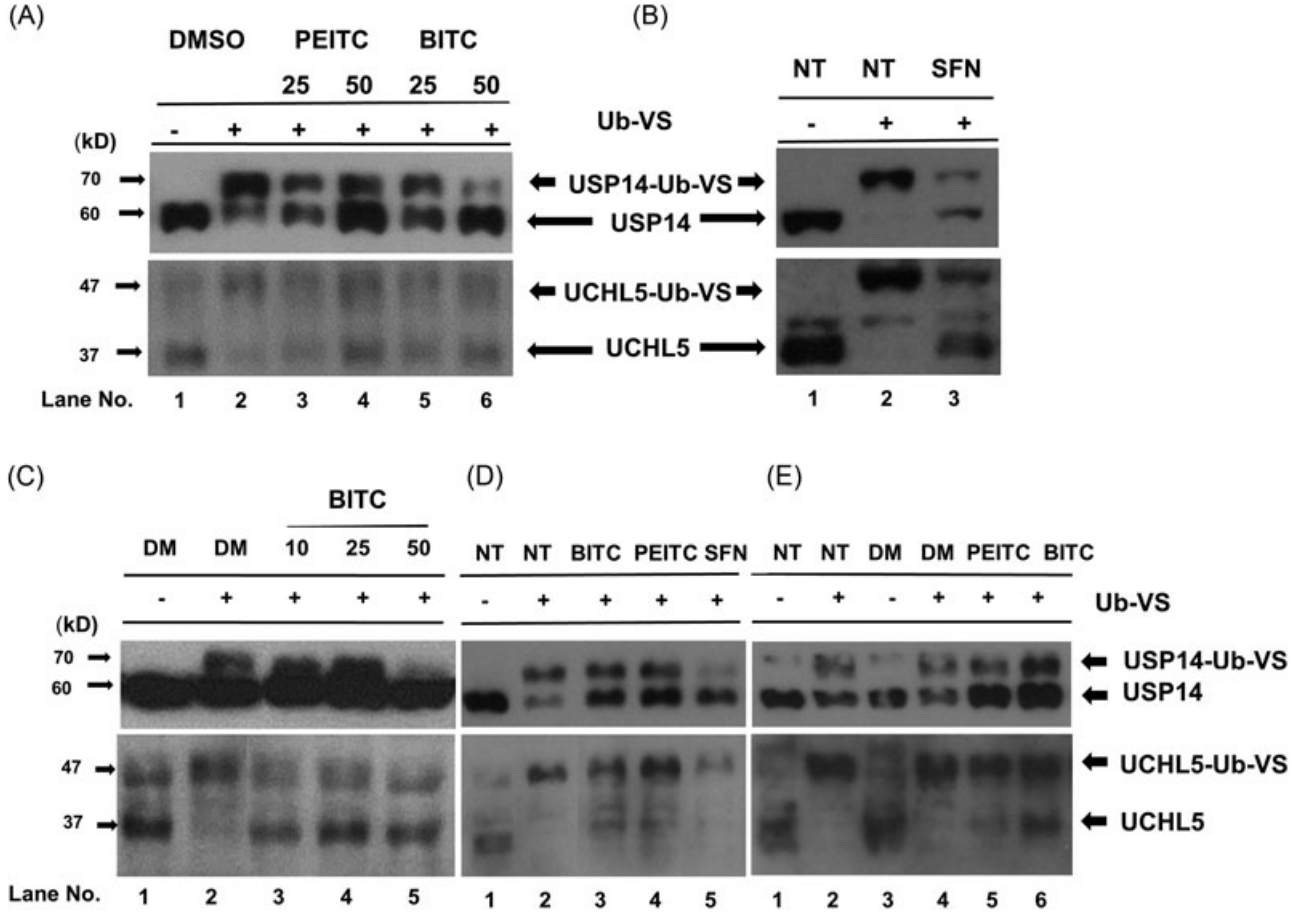

FIGURE 2 Inhibition of active site-directed labeling of proteasomal DUBs by BITC, PEITC, and SFN. Breast cancer MDA-MB-231 cells were either not treated (NT) or treated for 3 hours with either DMSO, PEITC, or BITC at the indicated concentrations (A), or SFN at 25 μM (B). Prostate cancer 22Rv1 cells were either not treated (NT) or treated for 3 hours with DMSO (DM) or BITC at the indicated concentrations (C), or 20 μM of the indicated ITC (D,E). After that, 25-μg cell lysates were subsequently labelled with Ub-VS (1 μM) in vitro for 30 minutes at 37°C followed by SDS gel electrophoresis and immunoblotting with USP14 and UCHL5 antibodies to evaluate the inhibitory activity of these ITCs on the 19S-associated deubiquitinases USP14 and UCHL5. The upper bands represent the active USP14 or UCHL5 bound by Ub-VS (USP14-Ub-VS and UCHL5-Ub-VS conjugates), while the lower bands are unbound USP14 or UCHL5. DMSO, dimethyl sulfoxide; DUB, deubiquitinating enzyme; BITC, benzyl isothiocyanate; PEITC, phenethyl isothiocyanate; SDS, sodium dodecyl sulfate; SFN, sulforaphane; Ub-VS, ubiquitin vinyl sulfone

We also used prostate cancer cells to confirm the biochemical inhibition of USP14 and UCHL5 by the ITCs. 22Rv1 prostate cancer cells were treated with 10-50 μM of BITC, followed by Ub-VS assay. We observed a dose-dependent inhibition of UCHL5 by BITC (Figure 2C, lower panel, lanes 3-5 vs 2); however, BITC, only at 50 μM, caused inhibition of USP14 (Figure 2C, upper panel, lanes 3-5 vs 2). Indeed, in 2 other independent experiments, 22Rv1 cells were treated with 20 μM of BITC, PEITC, or SFN for 3 hours. We found that SFN almost completely inhibited USP14′s activity (Figure 2D, upper panel, lanes 5 vs 2), but only decreased levels of UCHL5-Ub-VS conjugates without causing an increase in unmodified UCHL5 protein (Figure 2D, lower panel, lanes 5 vs 2). Both BITC and PEITC increased levels of unmodified USP14 and UCHL5 protein expression without decreasing (or even increasing) levels of Ub-VS-bound DUBs (Figure 2D-E). These data suggest that ITC treatment could also increase protein levels of these DUBs (see Figures 4 and 5).

3.3 | ITCS exhibit antiproliferative activity in prostate and breast cancer cells

So far, we have shown that ITCs are inhibitors of 19S-associated DUBs USP14 and UCHL5 by both computational modeling (Figure 1) and Ub-VS assay (Figure 2). Because levels of expression and activity of USP14 and UCHL5 are associated with cancer cell growth and metastasis,[38-41] we predict that inhibition of these DUBs by ITCs should result in suppression of tumor cell proliferation. To test that, cell proliferation was determined by MTT assay. Prostate cancer 22RV1 cells were either untreated or treated with the control solvent DMSO or the indicated doses of BITC (Figure 3A), PEITC (Figure 3B), or SFN (Figure 3C) for

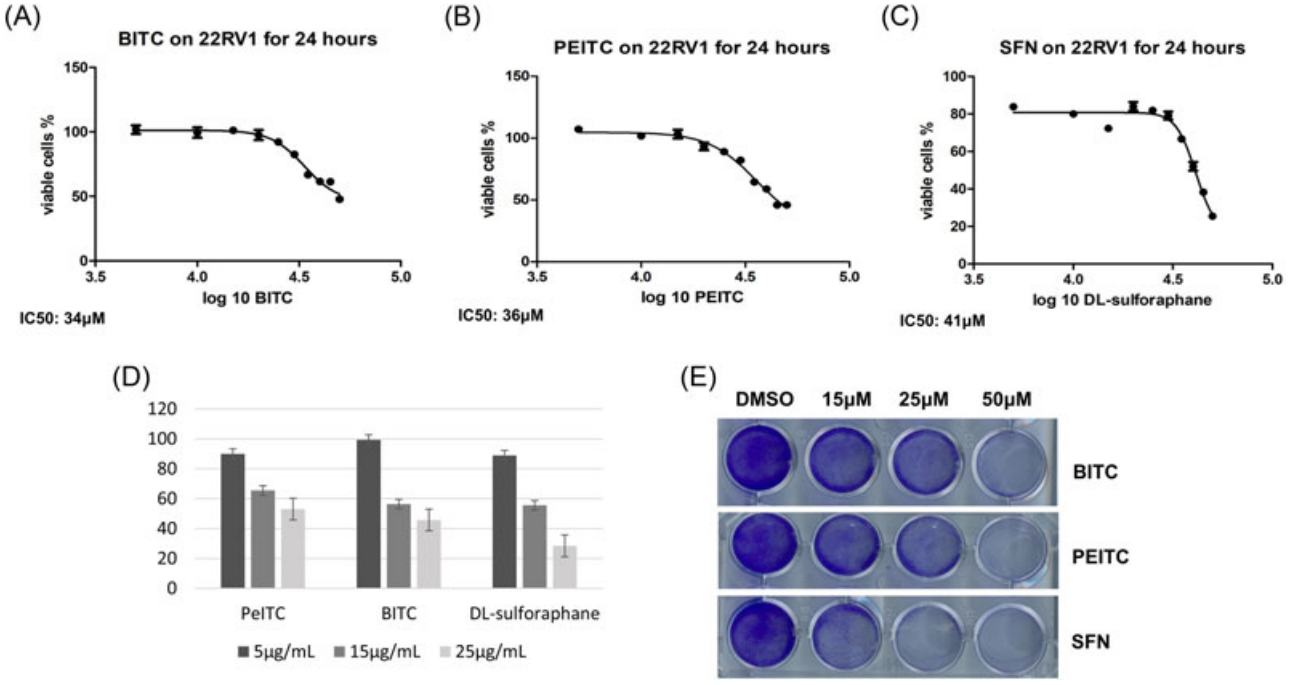

FIGURE 3 The cytotoxic effect of ITCs on prostate and breast cancer cells. Prostate cancer 22Rv1 (A-C) and breast cancer MDA-MB-231 (D) cells were seeded in a 96-well plate and either untreated or treated with the control solvent DMSO or different concentrations of BITC, PEITC, or SFN. Twenty-four hours after treatment, MTT assay was performed and the IC_{50} for each drug was determined. (E) MDA-MB-231 cells were treated with either DMSO or the indicated concentrations of BITC, PEITC, or SFN for 24 hours, followed by crystal violet dye staining. BITC, benzyl isothiocyanate; DMSO, dimethyl sulfoxide; IC, the half maximal inhibitory concentration; ITC, isothiocyanate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PEITC, phenethyl isothiocyanate; SFN, sulforaphane

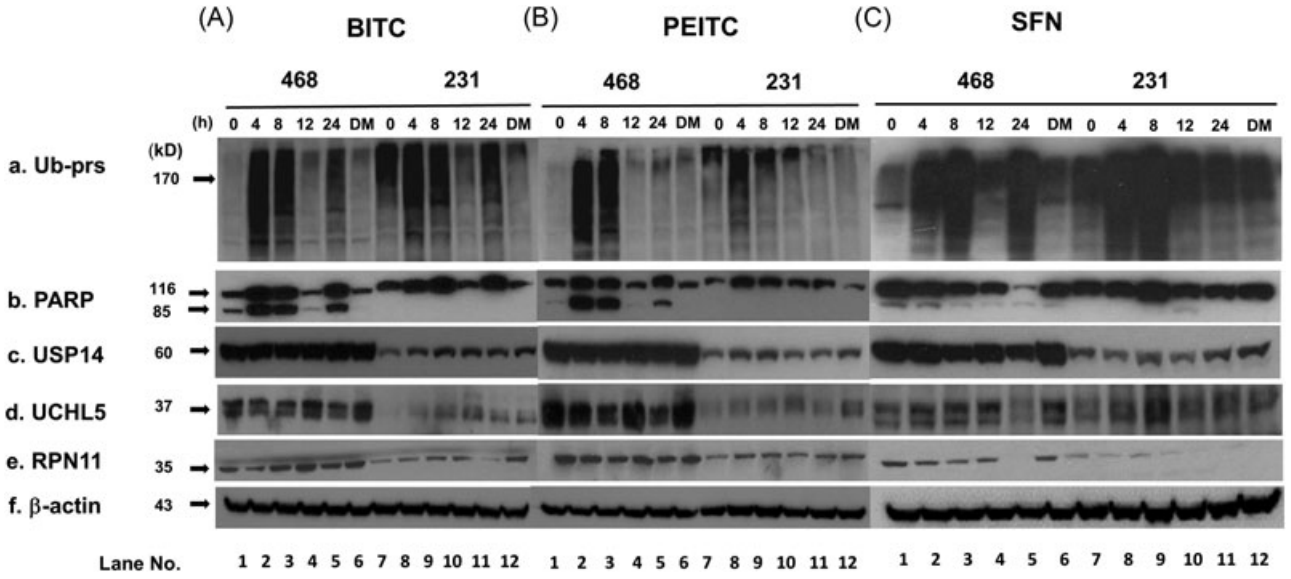

FIGURE 4 Effects of ITCs on levels of ubiquitinated proteins, apoptosis and 19S-DUBs in breast cancer MDA-MB-468 and MDA-MB-231 cells. MDA-MB-468 and MDA-MB-231 cells were either treated with DMSO (DM) or 25 μM of BITC (A), PEITC (B) or SFN (C) for the indicated hours (h). The cells were harvested, and protein lysates were extracted for electrophoresis, immunoblotting for ubiquitinated proteins (Ub-Prs, with anti-ubiquitin), PARP, USP14, UCHL5, and RPN11 as well as β-actin (as an internal control for protein loading and normalization between samples). BITC, benzyl isothiocyanate; DUB, deubiquitinating enzyme; DMSO, dimethyl sulfoxide; ITC, isothiocyanate; PARP, poly (ADP-ribose) polymerase; PEITC, phenethyl isothiocyanate; SFN, sulforaphane; Ub-Prs, ubiquitinated proteins

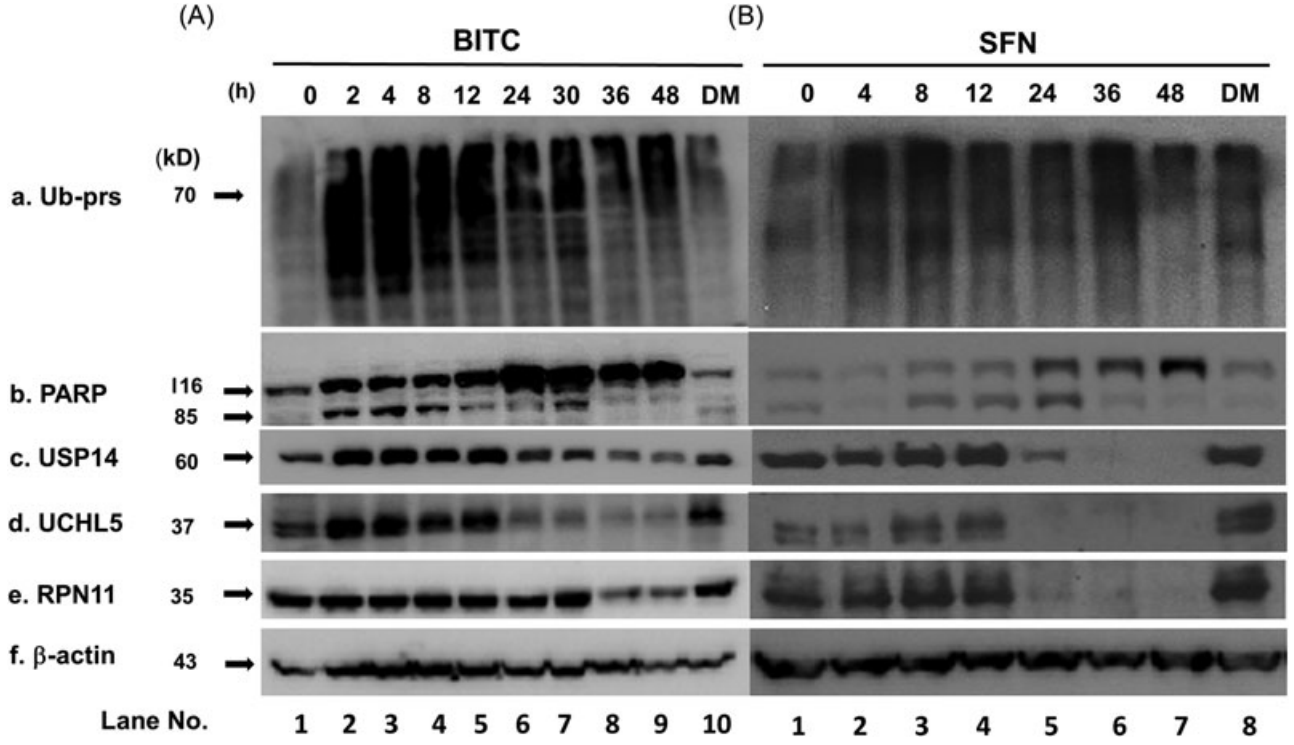

FIGURE 5 Effects of ITCs on levels of ubiquitinated proteins, apoptosis, and 19S-DUBs in prostate cancer 22Rv1 cells. 22Rv1 cells were treated with DMSO (DM) or 25 μM of BITC (A) or SFN (B) for the indicated hours (h), followed by Western blot analysis for ubiquitinated proteins (Ub-Prs), PARP, USP14, UCHL5, and RPN11 as well as β-actin (as an internal control). DMSO, dimethyl sulfoxide; BITC, benzyl isothiocyanate; SFN, sulforaphane

24 hours, followed by an MTT assay to determine the half maximal inhibitory concentration (IC_{50}) values. We found that BITC, PEITC and SFN exhibited antiproliferative activity in a dose-dependent manner; the IC_{50} of each ITC in 22Rv1 prostate cancer cells was approximately 34 μM for BITC (Figure 3A), approximately 36 μM for PEITC (Figure 3B), and approximately 41 μM for SFN (Figure 3C). Similarly, these 3 ITCs also caused a dose-dependent inhibition of proliferation in MDA-MB-231 breast cancer cells (Figure 3D).

We also performed the crystal violet staining assay to determine the effect of ITCs on cancer cell viability. MDA-MB-231 cells were treated with either DMSO or the indicated concentrations of BITC, PEITC, or SFN for 24 hours, followed by crystal violet dye staining. Dose-dependent inhibition by each of ITCs was seen with SFN being more potent than BITC and PEITC (Figure 3E).

3.4 | ITCS accumulate ubiquitinated proteins and induce levels of apoptosis and USP14/UCHL5 protein expression in breast cancer cells

If ITCs inhibit 19S-DUBs, we would expect increased levels of total ubiquitinated proteins (Ub-Prs). To test that, breast cancer MDA-MB-468 and MDA-MB-231 cell lines were treated with either DMSO or 25 μM of BITC (Figure 4A), PEITC (Figure 4B), or SFN (Figure 4C) for 4, 8, 12, and 24 hours, followed by Western blot analysis and immunoblotting with anti-ubiquitin. Accumulation of ubiquitinated proteins (Ub-Prs) was observed in both MDA-MB-468 and MDA-MB-231 cell lines after treatment with BITC, PEITC, or SFN (Figure 4A-C). There seemed to be 2 distinct peaks of accumulated poly-Ub-Prs, one at 4 to 8 hours and one at 24 hours. These 2 peaks were seen clearly in both 231 and 468 cell lines after BITC treatment (Figure 4A, panel a), and in 468 cells treated with SFN (Figure 4C, panel a). However, accumulation of Ub-Prs at only 4 to 8 hours was seen in 468 and 231 cells after PEITC treatment (Figure 4B, panel a), or in 231 cells after SFN treatment (Figure 4C, panel a).

We have shown the antiproliferative properties of ITCs (Figure 3). To determine whether these ITCs can also induce apoptosis, we probed levels of PARP and PARP cleavage fragment of p85, an indicator of cell apoptosis,[42] in 231 or 468 cells treated with BITC, PEITC, or SFN. PARP cleavage was clearly seen in the breast cancer cells, especially after treatment with BITC or PEITC (Figure 4A,B, panel b).

We found that under some Ub-VS conditions, ITCs could increase levels of unmodified DUBs (Figure 2D-E), suggesting that ITC treatment at least caused an increase in

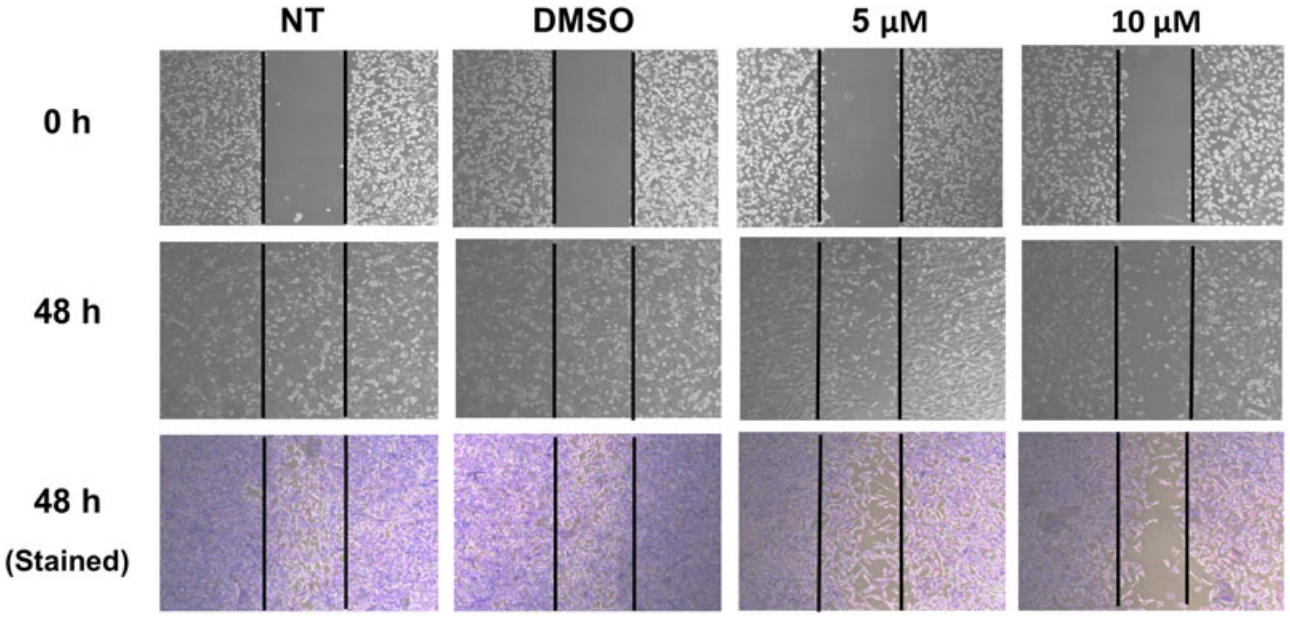

FIGURE 6 SFN inhibits invasion of breast cancer MDA-MB-231 cells. Wound-healing assay was performed by using MDA-MB-231 cells grown in a 6-well Matrigel-coated plate. A scratch was generated at 0 hours, and the cells were either not treated (NT) or treated with DMSO or SFN at 5 or 10 μM. Images were taken at both 0 and 48 hours by a Zeiss Axiovert 25 phase contrast microscope at 50× magnification. After 48 hours treatment the cells were also fixed and stained with crystal violet dye (Stained), followed by images taken (at 50× magnification). DMSO, dimethyl sulfoxide; SFN, sulforaphane

protein levels of these DUBs. To prove that, we measured levels of USP14, UCHL5 and RPN11 (as a control) in breast cancer cells treated with each ITC in the kinetic experiment. We observed that: (a) basal levels of USP14 and UCHL5 (as well as RPN11) proteins were higher in 468 than 231 cells (0 hours/468 cells vs 0 hours/231 cells, panels c-e, Figure 4A-C); (b) UCHL5 protein level was increased by treatment of BITC, PEITC, or SFN in 231 cells and by SFN in 468 cells (Figure 4A-C, panels d); (c) USP14 protein level was increased by BITC, PEITC, and SFN in 231 cells (Figure 4A-C, panel c).

3.5 | ITCS accumulate ubiquitinated proteins and induce levels of apoptosis and USP14/UCHL5 protein expression in prostate cancer cells

Prostate cancer 22Rv1 cells were then treated for up to 48 hours with 25 μM of BITC to investigate the correlations of 19S-DUB inhibition and levels of ubiquitination, PARP cleavage, and 3 19S-associated DUB proteins. Accumulation of Ub-Prs started at 2 hours and lasted up to 48 hours while PARP cleavage was detected in 2 hours and disappeared after 30 hours (Figure 5A, panels a and b). Both USP14 and UCHL5 protein levels were increased between 2 to 12 hours, and then decreased; however, RPN11 was unchanged during the treatment (Figure 5A, panels c-e). When 22Rv1 cells were treated with SFN, accumulation of poly-Ub-Prs started at 2 hours while PARP cleavage was between 8 to 24 hours; all the 3 DUBs became undetectable after 24 or 36 hours (Figure 5B). Taken together, our results suggest that ITCs could bind to the active sites of USP14 and UCHL5, causing inhibition, and inhibition of these DUB activities by ITCs also lead to increased levels of expression of USP14 and UCHL5 proteins, representing activation of a feedback loop (see section 4).

3.6 | SFN inhibits the healing of the induced wound in MDA-MB-231 cells

We then determined whether an ITC could inhibit cancer cell invasion. MDA-MB-231 cells were seeded in a 6-well Matrigel-coated plate. A scratch was generated in each well (0-hour image), and then the cells were either untreated or treated with DMSO or 5 or 10 μM of SFN. Images were taken after 48 hours. We noticed that the untreated cells or cells treated with DMSO exhibited complete closure of the wound after 48 hours, while cells treated with 5 μM SFN showed a partial inhibition, and those treated 10 μM SFN showed greater inhibition, of the healing (Figure 6).

4 | DISCUSSION

Previously, researchers have reported that BITC and PEITC effectively inhibit several DUBs including UCHL5, but not USP14.[28] The same group also reported that targets of PEITC contain approximately 10 DUBs, including UCHL5,

but not USP14.[43] Whether the used ITCs could interact with the active site of UCHL5 remains unknown and the effect of the ITCs on USP14 needs to be confirmed independently. We were also interested in ITCs, proteasomes, and DUBs. In this study, we first docked BITC, PEITC, and SFN to USP14 (Figure 1). Our results suggest that the 3 ITCs are weak inhibitors of USP14; these ITC could interact with 1 or 2 amino acid residues of the catalytic triad of USP14, HIS-434, and/or ASP-451, but not the catalytic CYS-114 (Figure 1). The docking data was further confirmed by Ub-VS assay in which BITC only at 50 μM could inhibit the USP14′s activity (Figure 2). Our data may have provided an explanation for why USP14 was not found to be a target of ITCs in previous studies under their experimental conditions.[28,43]

We then docked BITC, PEITC, and SFN to UCHL5 (Figure 1). Free energy values suggested that these ITCs are more potent inhibitors of UCHL5 than USP14. Indeed, the docking modes suggested that the ITC could interact with at least 1 or 2 amino acid residues of the catalytic triad of UCHL5, CYS-88, HIS-164, and ASP-179. Specifically, interaction of ITC to the catalytic CYS-88 should cause inhibition (Figure 1D). Indeed, the Ub-VS assay confirmed inhibitory activity for each ITC on the ubiquitin-binding activity of UCHL5 (Figure 2). Our data further confirmed the previous reports that UCHL5 could be inhibited by the ITCs.[28,43]

Another new finding of our study is that ITCs not only inhibit the activity of USP14/UCHL5 (Figure 2), but also transiently increase protein levels of USP14 and UCHL5, but not RPN11, such as, in early hours of BITC treatment in 22Rv1 cells (Figure 5A). This suggests that inhibition of the proteasomal cysteine DUBs triggers a feedback reaction that increased levels of these DUB proteins to compensate for their activity inhibition. These results further support ITCs inhibiting USP14/UCHL5, but not RPN11.

Inhibition of these DUBs by ITCs should be functional. Indeed, we found that, associated with DUB inhibition, Ub-Prs were significantly increased, accompanied with induction of apoptosis (PARP cleavage), inhibition of proliferation, and suppression of cell invasion.

The most recent research has suggested that 19S proteasomal DUBs are novel anticancer targets.[13,20,44-49] The important role of 19S-DUBs in cancer has been strongly supported by the literatures.[38-40,50] Our study should provide insightful information for discovering potent, specific 19S-DUB inhibitors that will have a major impact on improving current cancer therapies.

ACKNOWLEDGMENTS

We thank Dr Asfar Azmi at Karmanos Cancer Institute for providing 22Rv1 cells. This study was partially supported by the National Cancer Institute grant R21CA184788 (to QP Dou) and National Institutes of Health grant P30 CA022453 (to the Karmanos Cancer Institute at Wayne State University) as well as Karmanos internal funds (to QP Dou).

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How to cite this article: Ahmed ZSO, Li X, Li F, et al. Computational and biochemical studies of isothiocyanates as inhibitors of proteasomal cysteine deubiquitinases in human cancer cells. *J Cell Biochem*. 2018;119:9006–9016. https://doi.org/10.1002/jcb.27157