Published in final edited form as:

J Cell Biochem. 2018 November ; 119(11): 9006–9016. doi:10.1002/jcb.27157.

Computational and Biochemical Studies of Isothiocyanates as Inhibitors of Proteasomal Cysteine Deubiquitinases in Human Cancer Cells

Zainab Sabry Othman Ahmed^{1,2,#}, Xin Li^{1,3,#}, Feng Li^{1,4}, Hassan Ali Cheaito¹, Kush Patel¹, El-Sayed Mohammed Mosallam², Gehad Abd El-Fattah Hassan Elbargeesy², and Q. Ping Dou^{1,*}

¹Barbara Ann Karmanos Cancer Institute, Department of Oncology, Pharmacology and Pathology, School of Medicine, Wayne State University, Detroit, Michigan

²Department of Cytology and Histology, Faculty of Veterinary Medicine, Cairo University

³Department of Biotechnology, Guangdong Polytechnic of Science and Trade, Guangzhou, Guangdong, China

⁴Department of Food science, College of Food Science and Engineering, Shandong Agricultural University, Tai'an, China

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 (BITC), phenethyl isothiocyanate (PEITC) and DL-Sulforaphane (SFN) are more potent inhibitors of UCHL5 than USP14, and these ITCs could interact with the catalytic triads of UCHL5 and USP14. Indeed, Ub-VS 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-DUB, 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.

*Corresponding author: Dr. Q. Ping Dou, Barbara Ann Karmanos Cancer Institute, Departments of Oncology, Pharmacology and Pathology, School of Medicine, Wayne State University, 540.1 Hudson Webber Cancer Research Building, 4100 John R Road, Detroit, MI 48201, USA; Tel: 313-576-8301; Fax: 313-576-8307; doup@karmanos.org.

#Equal Contribution

Keywords

UBIQUITIN PROTEASOME SYSTEM; DRUG DISCOVER; USP14; UCHL5; BENZYL ISOTHIOCYANATE; PHENETHYL ISOTHIOCYANATE; SULFORAPHANE

Apoptosis is a physiological process required for regulating the number of cells in the tissue of organisms [Ellis, Yuan and Horvitz, 1991]. Failure of apoptosis could lead to the development of various diseases, such as cancers, because of the uncontrolled cell proliferation and consequent tumor growth [Evan and Vousden, 2001]. Therefore, induction of apoptosis has become one of the most effective strategies for cancer treatment [Kelloff et al., 2002; Woynarowska and Woynarowski, 2002].

It has been well documented that cancer cell apoptosis could be induced by targeting the ubiquitin proteasome system (UPS) [Shen et al., 2013; Dou and Zonder, 2014], which controls many cellular processes and is considered as the major regulator of protein degradation in eukaryotes [Schrader, Harstad and Matouschek, 2009; D'Arcy and Linder, 2012]. UPS-mediated protein degradation contains two 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 [Amerik and Hochstrasser, 2004; Lee et al., 2010; D'Arcy and Linder, 2012]. The 19S regulatory particle has at least 19 subunits and composed of a lid and a base [Lander et al., 2012], which prevents the erroneous degradation of non-ubiquitinated cellular proteins and gives the proteasome high specificity [D'Arcy and Linder, 2012]. The 20S catalytic core consists of 28 subunits arranged in 4 stacked heptameric rings. The outer two rings composed of 7 different α subunits have a regulatory function, while the inner two rings are composed of 7 different β subunits possessing catalytic activity ($\beta 1$ has caspase-like, $\beta 2$ has trypsin-like and $\beta 5$ has chymotrypsin-like activity) [Borissenko and Groll, 2007].

Ubiquitination could be reversed by deubiquitinating enzymes (DUBs) [Liao et al., 2017], 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 [Aressy et al., 2010; Tian et al., 2014; Sahtoe et al., 2015; Vogel et al., 2015]. There are approximately 100 DUBs encoded by the human genome [Liao et al., 2017] that are divided into six subfamilies [Fraile et al., 2012; Liao et al., 2017]. 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 towards the distal tip of the ubiquitin chain while POH1/RPN11 belongs to the metalloprotease class, which can cleave the bottom of the ubiquitin chain [Tian et al., 2014; Sahtoe et al., 2015; Vogel et al., 2015]. These proteasome-associated DUBs play an important role in ubiquitin homeostasis [D'Arcy, Wang and Linder, 2015]. Expression of DUBs was reported to be overexpressed in different tumor tissues [D'Arcy and Linder, 2012; Wang et al., 2016]. This made them an important target in the treatment of many

cancers including prostate and breast cancers [Lu and Luo, 2013]. Currently, only one USP14-selective inhibitor, VLX1570, is being tested in a phase 1/2 multiple myeloma clinical trial [Wang et al., 2016]. 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 [Gupta et al., 2014]. 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) [Cheung and Kong, 2010; Wang et al., 2014]. However, the exact molecular mechanism of ITCs as anticancer compounds is still unclear [Mi, Di Pasqua and Chung, 2011]. It is reported that these compounds could induce apoptosis and inhibit tumor cells *in vitro* and *in vivo* [Xiao and Singh, 2010; Moon, Brazeau and Morris, 2011]. It is also reported that some ITCs could inhibit several DUBs through formation of an adduct with cysteine residue of the active site [Yao and Cohen, 2002; Chen et al., 2013; Peng J et al., 2013; Lawson et al, 2015]. However, whether and how ITCs inhibit proteasomal DUBs remain to be further examined.

In this study, we intended to investigate if all three ITCs, BITC, PEITC and SFN, could inhibit the two 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.

MATERIALS AND METHODS

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) were grown in DMEM (Gibco™), all supplemented with 10% FBS (J R Scientific) and, 100 U/ml penicillin, and 100 µg/ml streptomycin (GIBCO). Cell culture experiments were performed at 37°C and 5% CO₂. Benzyl isothiocyanate (252492 Aldrich), Phenethyl isothiocyanate (253731 Aldrich, 99%) and DL-Sulforaphane (S4441 Sigma, 90% HPLC) were all dissolved in DMSO (Fisher Scientific) at 50 mM concentration as a stock solution and stored at -20°C and diluted with cell culture medium when used. 3-(4,5-dimethylthiazole-2-yl)-2,5-dihentetrazolium bromide (MTT) was purchased from Thermo Fisher; Crystal violet powder was from Sigma-Aldrich and a 0.5% solution of crystal violet was prepared by using distilled water and methanol (Fisher). 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™. USP14 (D8Q6S) rabbit mAb was from Cell Signaling Technology; Ubiquitin (P4D1, sc8017) mouse monoclonal antibody was from Santa Cruz Biotechnology; purified mouse anti-human PARP was purchased from BD Pharmingen™. The protein assay kit was from Bio-Rad Laboratories, Inc. (Hercules, CA).

IN-SILICO DOCKING AND ANALYSIS

Computational analysis was performed through molecular docking using the AutoDock Vina software [Trott and Olson, 2010]. The Protein Data Bank files (PDB) 3IHR & 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 utilized. 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 [Pettersen et al., 2004]. MDL Molfile files were created in ChemSketch for BITC, PEITC, and SFN [ACD/ChemSketch, version 2017]. These Molfiles were converted to pdbqt type of file for use with AutoDock Vina.

Upon inputting the files into the software, a search area was defined in order to maximize the accuracy of the docking analysis. The search area was 30 angstrom $\times$ 30 angstrom 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.

WHOLE CELL PROTEIN EXTRACT

The treated cells were harvested by a scraper, centrifuged at 1,200 rpm for 5 min 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), 1M NaCl, 10% NP-40, 0.5M EDTA in distilled water, and Halt™ protease and phosphatase inhibitor cocktail (Thermo Scientific) was added to the whole lysis buffer in 1:100 ratio. Cells were then centrifuged at 12,000 rpm for 15 min. The clear supernatant was used as a cell protein extract.

UBIQUITIN VINYL SULFONE (UB-VS) ASSAY

This assay was used to detect the inhibitory effect of isothiocyanates on 19S-associated USP14 and UCHL5. MDA-MB231 breast cancer cells or 22Rv1 prostate cancer cells were seeded in 60-mm dishes and incubated at 37°C and 5% CO₂. When the cell confluence reached about 70%, the cells were either untreated or treated with DMSO or a selected ITC at different concentrations for 3 hours. Cell lysates (25 μ g per sample) were mixed with Ub-VS (1 μ M) in vitro for 30 min at 37°C in DUB buffer [50 mM Tris-HCL (PH 7.5), 250 mM sucrose, 5 mM of MgCl₂, 1 mM of Phenyl methane sulfonyl fluoride (SIGMA ALDRICH), 2 mM ATP (ThermoFisher Scientific) and 0.2 mM DTT (1,4-Dithiothreitol, SIGMA ALDRICH) in dd H₂O], followed by SDS gel electrophoresis and immunoblotting with USP14 and UCHL5 antibodies.

CELL VIABILITY ANALYSIS

MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay was used to determine cell proliferation. MDA-MB-231 breast cancer cells and 22Rv1 prostate cancer cells were seeded in 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. 24 hours after treatment, cells were incubated with 10 μ M MTT reagent for 4 hours at 37 °C and then the dye was solubilized by using 100 μ L 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.

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 μ M of BITC, PEITC, or SFN. Cells were harvested at the indicated time points and equivalent amounts of protein extracts (40 μ g) were separated by 10% of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; Bio-Rad, Hercules, CA) under reducing conditions using 105V for 120 minutes. The proteins were then electrophoretically transferred onto PVDF membranes (Milpore corp., Billerica, MA) using the X Cell II Blot Module (Invitrogen Life Technologies). After blocking the non-specific binding sites by incubation for 1 hour with 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 ECL chemiluminescence detection system (Amersham Bioscience, Piscataway, NJ). The membranes were then re-probed with a monoclonal antibody raised against β -actin (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.

CRYSTAL VIOLET ASSAY

This assay was used to determine the cell viability of MDA-MB-231 and 22Rv1 prostate cancer cells which were treated with ITCs. The cells were seeded in 12-well plate at 40–50% confluence and, when the cell confluence became 80–90%, the cells were either untreated or treated with DMSO or the indicated concentrations of BITC, PEITC, or SFN. After 24 hours 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 1×PBS and then 500 μ l of 0.5% crystal violet staining solution was added to each well and kept overnight at room temperature. Scanning of the plate was performed.

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 at 6-well plate, and at ~80% confluence, cells were scratched with a sterile 200 μ l micropipette tip and washed twice with 1×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, USA) 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 1×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.

RESULTS

COMPUTER 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) with BITC, PEITC, and SFN (as ligands). PDBs 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) [Hu et al., 2005] and UCHL5 (CYS-88, HIS164, ASP179) [Maiti et al., 2011]. 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. Figures 1 displays the generated conformation of interaction between each of the three 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 oxygens in aspartic acid may interact with the unsaturated carbon of BITC. Additionally, 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 since more favorable bonding interactions were found upon analyzing the general decrease of free energy across the three compounds corresponding to Figure 1D–F. Figure 1D suggests interaction of BITC with the active site CYS-88 of UCHL5 through two 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 [Li et al., 2014]. Figure 1E demonstrates interaction of PEITC with 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 three ITC compounds could be inhibitors of USP14 and UCHL5.

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 μ M Ub-VS for 30 min, followed by SDS 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 kD and 37 kD, respectively, while addition of Ub-VS (MW ~10 kD) to these preparations generated new bands of ~70 kD 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 μ M, compared to 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 μ M 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).

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 μ M 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 μ M, caused inhibition of USP14 (Figure 2C, *upper*, lanes 3–5 vs. 2). Indeed, in two other independent experiments, 22Rv1 cells were treated with 20 μ M of BITC, PEITC or SFN for 3 hours. We found that SFN almost completely inhibited USP14's activity (Figure 2D, *upper*, lanes 5 vs. 2), but only decreased levels of UCHL5-Ub-VS conjugates without causing an increase in unmodified UCHL5 protein (Figure 2D, *lower*, 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 4–5).

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). Since levels of expression and activity of USP14 and UCHL5 are associated with cancer cell growth and metastasis [Rolén et al., 2006; Shinji et al., 2006; Mines et al., 2009; Chen et al., 2011], 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 24 hours, followed by a MTT assay to determine IC₅₀ values. We found that BITC, PEITC and SFN exhibited antiproliferative activity in a dose-dependent manner; the IC₅₀ of each ITC in 22Rv1 prostate cancer cells was: ~34 μ M for BITC (Figure 3A), ~36 μ M for PEITC (Figure 3B) and ~41 μ M for SFN (Figure 3C). Similarly, these three 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).

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. To test that, breast cancer MDA-MB-468 and MDA-MB-231 cell lines were treated with either DMSO or 25 μ M of BITC (Figure 4A), PEITC (Figure 4B), or SFN (Figure 4C) for 4, 8, 12 and 24 hours, followed by Western blotting 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 two distinct peaks of accumulated poly-ubiquitinated proteins, one at 4–8 h and one at 24 h. These two 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 ubiquitinated proteins at only 4–8 h 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 anti-proliferative 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 [Decker and Muller, 2002], 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 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: (i) basal levels of USP14 and UCHL5 (as well as RPN11) proteins were higher in 468 than 231 cells (0 h/468 cells vs. 0 h/231 cells, *panels c–e*, Figure 4A–C); (ii) 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*); (iii) USP14 protein level was increased by BITC, PEITC, and SFN in 231 cells (Figure 4A–C, *panels c*).

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 h with 25 μ M of BITC to investigate the correlations of 19S-DUB inhibition and levels of ubiquitination, PARP cleavage, and three 19S-associated DUB proteins. Accumulation of ubiquitinated proteins started at 2 h and lasted up to 48 h while PARP cleavage was detected in 2 h and disappeared after 30 h (Figure 5A, *panels a–b*). Both USP14 and UCHL5 protein levels were increased between 2 to 12 h, 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-ubiquitinated proteins started at 2 h while PARP cleavage was between 8–24 h; all the three DUBs became undetectable after 24 or 36 h (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 DISCUSSION).

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 μ M 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 μ M SFN showed a partial inhibition, and those treated 10 μ M SFN showed greater inhibition, of the healing (Figure 6).

DISCUSSION

Previously, researchers have reported that BITC and PEITC effectively inhibit several DUBs including UCHL5, but not USP14 [Lawson et al., 2015]. The same group also reported that DUB targets of PEITC include about 10 DUBs, including UCHL5, but not USP14 [Lawson et al., 2017]. 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 three ITCs are weak inhibitors of USP14; these ITC could interact with one or two 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 μ M

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 [Lawson et al., 2015; Lawson et al., 2017].

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 one or two 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 [Lawson et al., 2015; Lawson et al., 2017].

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, ubiquitinated proteins 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 [Muchamuel et al., 2009; Farshi et al., 2015; Wang et al., 2016; Chen et al., 2017; Fang and Shen, 2017; Liao et al., 2017; Mofers et al., 2017; Soave et al., 2017]. The important role of 19S-DUBs in cancer has been strongly supported by the literatures [Borodovsky et al., 2001; Rolén et al., 2006; Shinji et al., 2006; Mines et al., 2009]. 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 work was partially supported by National Cancer Institute grant R21CA184788 (to Q. Ping 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 Q. Ping Dou).

References

- ACD/ChemSketch, version 2017.1.2. Advanced Chemistry Development, Inc.; Toronto, ON, Canada: viewed 2015, from <http://www.acdlabs.com>
- Amerik AY, Hochstrasser M. Mechanism and function of deubiquitinating enzymes. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*. 2004; 1695(1–3):189–207. [PubMed: 15571815]
- Aressy B, Jullien D, Cazales M, Marcellin M, Bugler B, Burlet-Schiltz O, Ducommun B. A screen for deubiquitinating enzymes involved in the G2/M checkpoint identifies USP50 as a regulator of HSP90-dependent Wee1 stability. *Cell Cycle*. 2010; 9(18):3839–3846.

- Borissenko L, Groll M. 20S proteasome and its inhibitors: crystallographic knowledge for drug development. *Chemical reviews*. 2007; 107(3):687–717. [PubMed: 17316053]
- Borodovsky A, Kessler BM, Casagrande R, Overkleeft HS, Wilkinson KD, Ploegh HL. A novel active site-directed probe specific for deubiquitylating enzymes reveals proteasome association of USP14. *The EMBO journal*. 2001; 20(18):5187–5196. [PubMed: 11566882]
- Chen X, Yang Q, Xiao L, Tang D, Dou QP, Liu J. Metal-based proteasomal deubiquitinase inhibitors as potential anticancer agents. *Cancer and Metastasis Reviews*. 2017; 36(4):655–668. [PubMed: 29039082]
- Chen YJ, Ma YS, Fang Y, Wang Y, Fu D, Shen XZ. Power and promise of ubiquitin carboxyl-terminal hydrolase 37 as a target of cancer therapy. *Asian Pacific Journal of Cancer Prevention*. 2013; 14(4):2173–2179. [PubMed: 23725108]
- Chen Z, Niu X, Li Z, Yu Y, Ye X, Lu S, Chen Z. Effect of ubiquitin carboxy-terminal hydrolase 37 on apoptotic in A549 cells. *Cell biochemistry and function*. 2011; 29(2):142–148. [PubMed: 21287580]
- Cheung KL, Kong AN. Molecular targets of dietary phenethyl isothiocyanate and sulforaphane for cancer chemoprevention. *The AAPS journal*. 2010; 12(1):87–97. [PubMed: 20013083]
- D'Arcy P, Linder S. Proteasome deubiquitinases as novel targets for cancer therapy. *The international journal of biochemistry & cell biology*. 2012; 44(11):1729–1738. [PubMed: 22819849]
- D'Arcy P, Wang X, Linder S. Deubiquitinase inhibition as a cancer therapeutic strategy. *Pharmacology & therapeutics*. 2015; 147:32–54. [PubMed: 25444757]
- Decker P, Muller S. Modulating poly (ADP-ribose) polymerase activity: potential for the prevention and therapy of pathogenic situations involving DNA damage and oxidative stress. *Current pharmaceutical biotechnology*. 2002; 3(3):275–283. [PubMed: 12164482]
- Ellis RE, Yuan J, Horvitz HR. Mechanisms and functions of cell death. *Annual review of cell biology*. 1991; 7(1):663–698.
- Evan GI, Vousden KH. Proliferation, cell cycle and apoptosis in cancer. *Nature*. 2001; 411(6835):342. [PubMed: 11357141]
- Fang Y, Shen X. Ubiquitin carboxyl-terminal hydrolases: involvement in cancer progression and clinical implications. *Cancer and Metastasis Reviews*. 2017; 36(4):669–682. [PubMed: 29080080]
- Farshi P, Deshmukh RR, Nwankwo JO, Arkwright RT, Cvek B, Liu J, Dou QP. Deubiquitinases (DUBs) and DUB inhibitors: a patent review. *Expert opinion on therapeutic patents*. 2015; 25(10):1191–1208. [PubMed: 26077642]
- Fraile JM, Quesada V, Rodríguez D, Freije JM, López-Otín C. Deubiquitinases in cancer: new functions and therapeutic options. *Oncogene*. 2012 May;31(19):2373. [PubMed: 21996736]
- Gupta P, Kim B, Kim SH, Srivastava SK. Molecular targets of isothiocyanates in cancer: recent advances. *Molecular nutrition & food research*. 2014; 58(8):1685–1707. [PubMed: 24510468]
- Hu M, Li P, Song L, Jeffrey PD, Chernova TA, Wilkinson KD, Cohen RE, Shi Y. Structure and mechanisms of the proteasome-associated deubiquitinating enzyme USP14. *The EMBO journal*. 2005; 24(21):3747–3756. [PubMed: 16211010]
- Kelloff GJ, Crowell JA, Steele VE, Lubet RA, Malone WA, Boone CW, Kopelovich L, Hawk ET, Lieberman R, Lawrence JA, Ali I. Progress in cancer chemoprevention: development of diet-derived chemopreventive agents. *The Journal of nutrition*. 2000; 130(2):467S–471S. [PubMed: 10721931]
- Lander GC, Estrin E, Matyskiela ME, Bashore C, Nogales E, Martin A. Complete subunit architecture of the proteasome regulatory particle. *Nature*. 2012; 482(7384):186. [PubMed: 22237024]
- Lawson AP, Long MJ, Coffey RT, Qian Y, Weerapana E, El Oualid F, Hedstrom L. Naturally occurring isothiocyanates exert anticancer effects by inhibiting deubiquitinating enzymes. *Cancer research*. 2015; 75(23):5130–5142. [PubMed: 26542215]
- Lawson AP, Bak DW, Shannon DA, Long MJ, Vijaykumar T, Yu R, El Oualid F, Weerapana E, Hedstrom L. Identification of deubiquitinase targets of isothiocyanates using SILAC-assisted quantitative mass spectrometry. *Oncotarget*. 2017; 8(31):51296. [PubMed: 28881649]
- Lee BH, Lee MJ, Park S, Oh DC, Elsasser S, Chen PC, Gartner C, Dimova N, Hanna J, Gygi SP, Wilson SM. Enhancement of proteasome activity by a small-molecule inhibitor of USP14. *Nature*. 2010; 467(7312):179. [PubMed: 20829789]

- Liao Y, Liu N, Hua X, Cai J, Xia X, Wang X, Huang H, Liu J. Proteasome-associated deubiquitinase ubiquitin-specific protease 14 regulates prostate cancer proliferation by deubiquitinating and stabilizing androgen receptor. *Cell death & disease*. 2017; 8(2):e2585. [PubMed: 28151478]
- Li G, Liang Q, Gong P, Tencer AH, Zhuang Z. Activity-based diubiquitin probes for elucidating the linkage specificity of deubiquitinating enzymes. *Chemical Communications*. 2014; 50(2):216–218. [PubMed: 24225431]
- Lu C, Luo J. Decoding the androgen receptor splice variants. *Translational andrology and urology*. 2013; 2(3):178. [PubMed: 25356377]
- Maiti TK, Permaul M, Boudreaux DA, Mahanic C, Mauney S, Das C. Crystal structure of the catalytic domain of UCHL5, a proteasome-associated human deubiquitinating enzyme, reveals an unproductive form of the enzyme. *The FEBS journal*. 2011; 278(24):4917–4926. [PubMed: 21995438]
- Mines MA, Goodwin JS, Limbird LE, Cui FF, Fan GH. Deubiquitination of CXCR4 by USP14 is critical for both CXCL12-induced CXCR4 degradation and chemotaxis but not ERK activation. *Journal of Biological Chemistry*. 2009; 284(9):5742–5752. [PubMed: 19106094]
- Mi L, Di Pasqua AJ, Chung FL. Proteins as binding targets of isothiocyanates in cancer prevention. *Carcinogenesis*. 2011; 32(10):1405–1413. [PubMed: 21665889]
- Mofers A, Pellegrini P, Linder S, D'Arcy P. Proteasome-associated deubiquitinases and cancer. *Cancer and Metastasis Reviews*. 2017; 36(4):635–653. [PubMed: 29134486]
- Moon YJ, Brazeau DA, Morris ME. Dietary phenethyl isothiocyanate alters gene expression in human breast cancer cells. *Evidence-based Complementary and Alternative Medicine*. 2011; 2011
- Muchamuel T, Basler M, Aujay MA, Suzuki E, Kalim KW, Lauer C, Sylvain C, Ring ER, Shields J, Jiang J, Shwonek P. A selective inhibitor of the immunoproteasome subunit LMP7 blocks cytokine production and attenuates progression of experimental arthritis. *Nature medicine*. 2009; 15(7):781.
- Peng J, Hu Q, Liu W, He X, Cui L, Chen X, Yang M, Liu H, Wei W, Liu S, Wang H. USP9X expression correlates with tumor progression and poor prognosis in esophageal squamous cell carcinoma. *Diagnostic pathology*. 2013 Dec.8(1):177. [PubMed: 24152793]
- Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, Ferrin TE. UCSF Chimera—a visualization system for exploratory research and analysis. *Journal of computational chemistry*. 2004; 25(13):1605–1612. [PubMed: 15264254]
- Ping Dou Q, A Zonder J. Overview of proteasome inhibitor-based anti-cancer therapies: perspective on bortezomib and second generation proteasome inhibitors versus future generation inhibitors of ubiquitin-proteasome system. *Current cancer drug targets*. 2014; 14(6):517–536. [PubMed: 25092212]
- Rolén U, Kobzeva V, Gasparjan N, Ovaa H, Winberg G, Kissel'jov F, Masucci MG. Activity profiling of deubiquitinating enzymes in cervical carcinoma biopsies and cell lines. *Molecular carcinogenesis*. 2006; 45(4):260–269. [PubMed: 16402389]
- Sahtoe DD, van Dijk WJ, El Oualid F, Ekkebus R, Ovaa H, Sixma TK. Mechanism of UCH-L5 activation and inhibition by DEUBAD domains in RPN13 and INO80G. *Molecular cell*. 2015; 57(5):887–900. [PubMed: 25702870]
- Schrader EK, Harstad KG, Matouschek A. Targeting proteins for degradation. *Nature chemical biology*. 2009; 5(11):815. [PubMed: 19841631]
- Shen M, Schmitt S, Buac D, Dou QP. Targeting the ubiquitin–proteasome system for cancer therapy. *Expert opinion on therapeutic targets*. 2013; 17(9):1091–1108. [PubMed: 23822887]
- Shinji S, Naito Z, Ishiwata S, Ishiwata T, Tanaka N, Furukawa K, Suzuki H, Seya T, Matsuda A, Katsuta M, Tajiri T. Ubiquitin-specific protease 14 expression in colorectal cancer is associated with liver and lymph node metastases. *Oncology reports*. 2006; 15(3):539–543. [PubMed: 16465409]
- Soave CL, Guerin T, Liu J, Dou QP. Targeting the ubiquitin-proteasome system for cancer treatment: discovering novel inhibitors from nature and drug repurposing. *Cancer and Metastasis Reviews*. 2017; 36(4):717–736. [PubMed: 29047025]
- Tian Z, D'Arcy P, Wang X, Ray A, Tai YT, Hu Y, Carrasco RD, Richardson P, Linder S, Chauhan D, Anderson KC. A novel small molecule inhibitor of deubiquitylating enzyme USP14 and UCHL5

- induces apoptosis in multiple myeloma and overcomes bortezomib resistance. *Blood*. 2014; 123(5):706–716. [PubMed: 24319254]
- Trott O, Olson AJ. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *Journal of computational chemistry*. 2010; 31(2):455–461. [PubMed: 19499576]
- Vogel RI, Coughlin K, Scotti A, Iizuka Y, Anchoori R, Roden RB, Marastoni M, Bazzaro M. Simultaneous inhibition of deubiquitinating enzymes (DUBs) and autophagy synergistically kills breast cancer cells. *Oncotarget*. 2015; 6(6):4159. [PubMed: 25784654]
- Wang D, Upadhyaya B, Liu Y, Knudsen D, Dey M. Phenethyl isothiocyanate upregulates death receptors 4 and 5 and inhibits proliferation in human cancer stem-like cells. *BMC cancer*. 2014; 14(1):591. [PubMed: 25127663]
- Wang X, Mazurkiewicz M, Hillert EK, Olofsson MH, Pierrou S, Hillertz P, Gullbo J, Selvaraju K, Paulus A, Akhtar S, Bossler F. The proteasome deubiquitinase inhibitor VLX1570 shows selectivity for ubiquitin-specific protease-14 and induces apoptosis of multiple myeloma cells. *Scientific Reports*. 2016; 6:26979. [PubMed: 27264969]
- Woynarowska BA, Woynarowski JM. Preferential targeting of apoptosis in tumor versus normal cells. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*. 2002; 1587(2–3):309–317. [PubMed: 12084473]
- Xiao D, Singh SV. p66Shc Is Indispensable for Phenethyl Isothiocyanate–Induced Apoptosis in Human Prostate Cancer Cells. *Cancer research*. 2010; 70(8):3150–3158. [PubMed: 20354186]
- Yao T, Cohen RE. A cryptic protease couples deubiquitination and degradation by the proteasome. *Nature*. 2002; 419(6905):403. [PubMed: 12353037]

Fig. 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.

Fig. 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 h with either DMSO, PEITC, or BITC at the indicated concentrations (A), or SFN at 25 μ M (B). Prostate cancer 22Rv1 cells were either not treated (NT) or treated for 3 h with DMSO (DM) or BITC at the indicated concentrations (C), or 20 μ M of the indicated ITC (D, E). After that, 25 μ g cell lysates were subsequently labelled with Ub-VS (1 μ M) in vitro for 30 min 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.

Fig. 3.

The cytotoxic effect of ITCs on prostate and breast cancer cells. Prostate cancer 22Rv1 (A, B, 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. 24 hours after treatment, MTT assay was performed and the IC₅₀ 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.

Fig. 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 μ M of BITC (A), PEITC (B) or SFN (C) for the indicated horus (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).

Fig. 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 μ M of BITC (A) or SFN (B) for the indicated hours (h), followed by Western blotting for ubiquitinated proteins (Ub-Prs), PARP, USP14, UCHL5 and RPN11 as well as β -actin (as an internal control).

Fig. 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 h, and the cells were either not treated (NT) or treated with DMSO or SFN at 5 or 10 μ M. Images were taken at both 0 and 48 h by a Zeiss Axiovert 25 phase contrast microscope at 50 $\times$ magnification. After 48 h treatment the cells were also fixed and stained with crystal violet dye (Stained), followed by images taken (at 50 $\times$ magnification).