**Naturally Occurring Isothiocyanates Inhibit Deubiquitinating Enzymes**

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**SUPPLEMENTARY MATERIALS AND METHODS**

**Materials**

All chemicals and reagents were from Sigma Aldrich unless otherwise stated. Solvents (except DMSO) were from Fisher (Pittsburg, PA). Other reagents used in this study: G5 isopeptidase inhibitor 1 (50-230-7928, Calbiochem); PEITC (Acros Organics); Bortezomib (Millennium Pharmaceuticals); Mini-Complete and PhosSTOP inhibitory cocktails (Roche Applied Science); Bortezomib (LC laboratories); Alamar Blue® (Invitrogen); USP9x, USP7(catalytic domain), UCH-L3, Ubiquitin-AMC, Suc-Leu-Leu-Val-Tyr-AMC, RAP80 UIM Domains Agarose AM-120, and 20S human proteasome (Boston Biochem.); normal goat IgG SC-2028 (Santa Cruz); DMEM, glutaMAX, penicillin/streptomycin (Gibco); trypsin (0.25%), DPBS and RPMI (Hyclone); Bradford dye and Chill-out wax (BioRad); dithiothreitol (GoldBio Tech); Protein G magnetic beads and ECL II (Pierce); Blue Biofilm (Denville Scientific); PVDF (Millipore); TAMRA-ubiquitin propargylamide and Cy5-ubiquitin vinyl methyl ester (UbiQ); HA-ubiquitin vinylsulfone and HA-ubiquitin vinyl methyl ester were synthesized using standard methods previously described([1](#_ENREF_1)). The plasmid encoding the HA-1-75Ub-Intein-chitin binding domain fusion protein was a gift from Prof H. Ploegh of the Whitehead Institute.

**Antibodies**

The following antibodies were used: anti-K48-linked ubiquitin, clone APU2 and anti-K63-linked ubiquitin, clone APU3 (Millipore); anti-PARP 9542, anti-cAbl 2862, anti--tubulin 2156, anti-Mcl-1 D35A5, anti-Flag 2368, (Cell Signaling Technologies); anti-actin clone AC-40 A3853, anti-GAPDH clone G9295, and anti-HA, Clone 3F10 (Roche); anti-ubiquitin, clone 6C1.17 (BD Pharmingen); anti-HSP70, anti-USP7, anti-UCH37, anti-USP24 and anti-USP9x (all rabbit monoclonal) (Abcam); HRP conjugated secondary antibodies (Abcam)

**Vehicle**

All compounds were dissolved in DMSO and further diluted with culture medium before use for tissue culture assays (final DMSO concentrations were less than 0.1%). For *in vitro* assays, the final DMSO concentration was 1%.

**Tissue culture assays and preparation of cell lysates**

B16/F10 and MCF7 cells were purchased from ATCC. BaF3 and BaF3/p210([2](#_ENREF_2)) cells were provided by Dr. Nathaniel Gray (Harvard Medical School, Boston, MA; obtained 2013), K562 cells were provided by Jeffrey Strovel (Avalon Pharmaceuticals; obtained in 2011), HeLa cells were provided by Benjamin F. Cravatt (The Scripps Research Institute; obtained in 2010), NIH/3T3 cells were provided by Dr. Rubio Ren (Brandeis University; obtained in 2012) and COS1 were provided by the Dr. Daniel Oprian (Brandeis University; obtained 2010). Cell lines were authenticated (9-Marker STR May 2015). The genetic profiles of K562, MCF-7, HeLa, BaF3, BaF3/p210 and NIH/3T3 cells were identical to reported genetic profiles. COS1 cells were confirmed to be of African green monkey in origin and free of all interspecies contamination.

Cells were cultured in DMEM (HeLa, COS1, NIH/3T3, B16-F10, and MCF-7) or RPMI (BaF3, BaF3/p210 and K562) supplemented with 10% heat inactivated FBS (DBS was used for NIH/3T3 cells), 1X glutaMAX, and 1% penicillin/streptomycin at 37 oC in a 5% CO2 humidified atmosphere. BaF3 cells were also supplemented with 1 ng/mL recombinant mouse interleukin-3 (rmIL-3, R&D Systems).

For the preparation of cell lysates, non-adherent cells were harvested by centrifugation (600 g) followed by aspiration of the media, washing 2 X with DPBS, centrifugation, and resuspension in lysis buffer [10 mM HEPES (pH 7.9), 5 mM MgCl2, 140 mM KCl, 1% NP40, protease inhibitors and Phosphatase Inhibitor Cocktail II]. Cells were lysed using 3 X freeze thaw cycles then centrifuged at 20,000 rpm (microcentrifuge, Eppendorf 5417 C) for 10 min. Protein concentration was determined using Bradford assay with IgG as standard. Where indicated, the whole cell lysate was analyzed. Whole cell lysates were prepared by adding 0.1% SDS to the cell pellet together with supernatant followed by sonication for a total of 30 s (in 10 s bursts). Samples were centrifuged at 20,000 rpm from 10 min (Eppendorf microcentrifuge 5417 C). Protein was analyzed by western blot as delineated below (6 g total protein for K48-linked ubiquitin (1:9,000 antibody dilution); 30-40 g protein loaded for PARP (1:1500), K63-linked ubiquitin (1:1,500), Mcl-1 (1:1,000), FLAG (1:6,000), K48-linked Ubiquitin (1:20,000), or cAbl (1:1,000). 10-20 ug protein loaded for ubiquitin (1:14,000). Signals were normalized to actin (1:10,000 for 6 g lysate; 1:30,000 for 30-40 g lysate), -tubulin (1:8,000), GAPDH (1:35,000) or HSP70 (1:2,000).

For UbiquitinG76V-GFP assay (plasmid from Addgene, number 11941, from the lab of Nico Dantuma), cells were transfected using Mirus 2020 (Madison, WI) as per the manufacturer’s instructions and confluence at transfection was approximately 75%. Transfected cells were harvested 1.5 days (total time including addition of compound) post transfection. Prior to harvesting, medium was replaced with fresh medium containing either 0.1% DMSO or 0.1% DMSO plus compound. Cells were harvested after 8 h by aspiration of media, trypsinization, resuspension in complete media, centrifugation 700 g, and washing 3 times in DPBS.

**FACS analysis**

FACS was carried out on a Beckman FACS-Calibur. For COS1 cells, media was removed and trypsin was added. Harvested cells were placed in FACS buffer (0.5% FBS in PBS with 3 g/mL propidium iodide) 30 s prior to analysis. All data were analyzed using FlowJo V10, from TreeStar (Ashland, OR). Approximately 2500 cells were sorted per replicate. Cells were sorted by propidium iodide dye exclusion to give a “viable population”. GFP positive cells within this group were identified relative to untransfected controls. Then the geometric mean of the whole GFP positive population within the viable population was calculated. Typical transfection efficiencies for UbiquitinG76V-GFP were 60-70% for COS1, based on GFP positive cells.

**20S Proteasome assay**

Compounds (or DMSO vehicle) were pre-incubated with human 20S proteasome (1.22 g/mL) in 50 mM potassium phosphate pH 7.6, 50 mM NaCl, 1 mM DTT at 25 oC for 30 minutes after which time substrate (Suc-Leu-Leu-Val-Tyr-AMC, 100 M or 10 M) was added and release of AMC was measured using a Bio-tek plate reader for 30 min (EX:380 nm; EM: 460 nm). The K0.5 value was experimentally determined to be 12 ± 2 M (Hill coefficient = 2), in good agreement with literature values([3](#_ENREF_3)) . The concentration of inhibitor required to inhibit the enzyme by 50% was calculated as described. Alternatively, rabbit reticulocyte lysate (Promega, L4151, Madison WI) was diluted 40 fold into proteasome assay buffer and treated with compound at 25 oC for 1 h prior to addition of Suc-Leu-Leu-Val-Tyr-AMC (10 M). Apparent K0.5 of this lysate for Suc-Leu-Leu-Val-Tyr-AMC was 28 ± 3 M when fit to a Hill equation (Hill coefficient = 1.3).

**Recombinant USP9x assays**

The hydrolysis of Ub-AMC was measured by monitoring the production of AMC at 37 oC in a black 96 well plate using a Bio-tek plate reader (fluorescence excitation wavelength = 380 nm, emission wavelength = 460 nm). Assay buffer contained 50 mM HEPES, pH 7.6, 100 mM NaCl, 0.75 mM BME. The fluorescence intensities were quantified with the appropriate AMC standard curves. The Michaelis-Menton parameters were determined by varying Ub-AMC from 220-1870 nM. The values of *Km* and *kcat* 520 nM and 0.35 ± 0.01 s-1, respectively, in reasonable agreement with previous reports([4](#_ENREF_4)) and the manufacture’s data. The enzyme lost activity over the course of the assay. Inhibition assays were typically performed by monitoring the hydrolysis of Ub-AMC (315 nM) by USP9X (0.7 nM) for 1 h. The enzyme was not stable for the duration of the experiment, complicating analysis. The progress curves were fit to a competitive inhibition mechanism including the enzyme decomposition term using Dynafit (Biokin)([5](#_ENREF_5))

E + S 🡸🡺 ES

ES 🡺 E + P

E 🡸🡺 EI

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**Data analysis**

All data were fit using Graphpad Prism (La Jolla, CA).

For the analysis of the kinetics of USP9x initial rate data were fit to the Michaelis Menten equation

RATE = *Vmax\**[S]/(*KM*+[S])

As previously reported, the initial rate data for the chymotrypsin site of the 20S proteasome were fit to a Hill equation([3](#_ENREF_3))

RATE = *Vmax\**[S]n/(*K0.5*n + [S]n)

Where n is the Hill coefficient.

The values of *EC50* were calculated using the following equation:

Effect = 1/(1+([Compound]/*EC50*))

Where relevant, all data points were analyzed for significance using the two-tailed Student’s t-test function on Graphpad Prism.

**HA-Ub-VS and HA-Ub-VME activity profiling**

Cell pellets were typically stored at -80 oC until required, at which time they were thawed on ice. Cell lysis was performed in lysis buffer using a Dounce homogenizer (10 strokes, with grinding, on ice). Crude lysate was centrifuged at 17000 g for 10 min at 4 oC, after which time the concentration of the lysate was adjusted to 1.5 mg/mL. Lysate was treated with the stated concentration of inhibitor (or 1% DMSO control) for 10-15 min, then treated with HA-UbVS or HA-UbVME (1.5-0.7 M) for the stated time. Aliquots (9 L) were removed at the stated times and immediately quenched in (2X final concentration) reducing (dithiothreitol) loading buffer and frozen (-20oC) until required. Western blot analysis was carried out using standard methods. Samples were resolved by SDS-PAGE, transferred to PVDF [(0.45 m) (Towbin buffer, tank apparatus, 90 V 1 h, then overnight at 30 V, 4 oC)] then blocked in 10% milk in TBS-T HS (100 mM Tris HCl, pH 7.6, 500 mM NaCl, 0.5% Tween-20) for at least 2 h at RT. Afterward, membrane was washed in TBS-T HS then probed with anti HA-HRP (1:18000) for 1.30 h at RT. Membrane was washed 2 times in TBS-T HS (15 min) then once in TBS (15 min) and exposed to ECL II and visualized using blue biofilm. When required, membranes were stripped in 100 mM glycine, pH 4, 500 mM NaCl, 1% SDS, 5 mM BME, at 55 oC for 20 min. For all HA-UbVS/VME experiments, samples in loading buffer were heated only to 37 oC prior to loading on a gel and blotting with anti-HA. In select instances, these data were replicated by blotting for the target enzyme (UCH37, USP7, USP24 or USP9x) and observing the amount of HA-UbVS or HA-UbVME DUB complex formed. For recovery experiments, a lysate of 12 mg/mL was treated with saturating PEITC or BITC(250 M) and incubated for 10 min. Afterward, the lysate was diluted to 0.6 mg/mL (final concentration of inhibitor 25 M) in lysis buffer (final volume 100 L), then HA-UbVS was added. Aliquots (15 L) were removed at the stated time (5-120 min) and immediately quenched in 2x (final concentration) reducing loading buffer.

**TAMRA-Ub-PA and Cy5-Ub-VME activity profiling**

Performed as described above except after resolution of samples by SDS-PAGE, in-gel fluorescent scans were obtained using a GE Typhoon scanner.

**Cy5-Ub-VME activity profiling in tissue culture**

BaF3/p210 cells were treated with the stated concentration of inhibitor (or 0.1% DMSO control) for 2 or 4 h. Cells were harvested and washed two times with ice cold PBS. Cell pellets were then lysed with glass beads (vortexed, 5 X 3 sec bursts at 4 oC) in ice cold lysis buffer (75 mM K2HPO4, pH 7.5, 150 mM NaCl, 250 mM sucrose). The lysate was centrifuged at 17000 g for 10 min at 4 oC and the clarified supernatant (adjusted to the same protein concentration [0.4 to 0.6 mg/mL]) was incubated with Cy5-UbVME (250 nM) for 5 min at 37 oC. Aliquots (20 L) were removed at the stated times and immediately quenched in (2X final concentration) reducing (dithiothreitol) loading buffer. Samples were resolved by SDS-PAGE and in-gel fluorescent scans were obtained using a GE Typhoon scanner.

**Immunoprecipitations**

COS1 cells were transiently transfected with Addgene plasmid number 32978 (coding for 3XFLAG-tagged mouse Mcl-1, from the lab of Joseph Opferman) using Mirus 2020 (Madison, WI) as per the manufacturer’s instructions and confluence at transfection was approximately 60%. Prior to harvesting, the media were aspirated and replaced with fresh media containing either 0.1% DMSO or 0.1% DMSO plus compound and the cells were incubated at 37 oC for 2 h. The cells were harvested, washed in PBS and re-suspended in lysis buffer [50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% Triton, protease inhibitor cocktail and 20 nM bortezomib]. After lysis (3x freeze/thaw), protein concentrations were determined (Bradford Assay) and lysates were adjusted to the same concentration. 3×Flag–Mcl-1 was immunoprecipitated with anti-Flag M2 magnetic beads and eluted with 500 μg mL-1 3×Flag peptide (100 L) (Sigma) according to manufacturer’s instructions. Input (approx. 40 g), flowthrough (identical volume) and eluted proteins (15-20 L) were analyzed by SDS-PAGE and western blot.

BaF3/p210 cells were incubated with 5 M BITC or PEITC or with 0.1% DMSO (vehicle only) for 1 h at 37 oC. K562 cells were incubated with 5 M BITC or PEITC or with 0.1% DMSO for 2 h at 37 oC. The cells were harvested, washed in ice cold PBS and re-suspended in ice cold lysis/wash buffer [40 mM Tris pH 7.5, 140 mM NaCl, 10 mM glycerophosphate, 10 mM Na pyrophosphate, 0.5% NP40 and protease inhibitor cocktail]. After lysis (1 x freeze/thaw) lysates were adjusted to the same concentration (1 mg/mL). Poly-K63-linked ubiquitinated proteins were enriched with RAP80-UIM agarose (25 L re-suspended slurry) that was washed (4x800 L wash buffer) and pelleted between washes by centrifugation (2100g x 3 min). 650-750 g total protein incubated (2 h) with RAP80 UIM beads at 4 oC. After extensive washing, poly-K63-linked proteins were eluted by boiling in 1.5x loading buffer (DTT) for 8 min. Input (approx. 15 g), flowthrough (identical volume) and eluted proteins (15-20 L, identical volumes of eluent) were analyzed by SDS-PAGE and western blot. For Bcr-Abl immunoprecipitation, the PEITC, BITC (5 M) or DMSO treated cells were re-suspended in lysis/wash buffer [40 mM HEPES pH 7.2, 120 mM NaCl, 0.3% CHAPS, 10 mM NaF, 10 mM glycerophosphate, 10 mM sodium pyrophosphate, 10 mM sodium azide, 1 mM EDTA and 5 M PEITC or BITC or 0.1% DMSO]. After lysis (incubation on ice for 20 min), lysates were adjusted to the same protein concentration (1 mg/mL), pre-cleared (600 L lysate) with 10 L protein G (1 h at 4 oC). The beads were removed and the cleared lysate was incubated with anti-cAbl (1 L/100 L lysate) overnight at 4oC. cAbl was immunoprecipitated with protein G beads (40 L beads rotated for 4 h at 4 oC), the beads were washed extensively with lysis/wash buffer and eluted by boiling in 1x loading buffer (DTT) for 10 min. For IgG negative control, 1 g IgG/100 L lysate used in place of anti-cAbl. Input (approx. 15 g), flowthrough (identical volume) and eluted proteins (3-10 L, identical volumes of eluent) were analyzed by SDS-PAGE and western blot.

**Proliferation assays**

Proliferation assays for adherent cells were conducted by plating cells (7,500 cells/mL, approx. 5% - 10% confluence) in 96 well plates together with compound or 0.1% DMSO. Cells were allowed to grow for 48 h and then Alamar Blue® was added and number of cells was measured by fluorescence on a microplate reader. Non-adherent cells were grown in 24 well plates. Every 24 h, cells were agitated to ensure complete mixing. When ready to harvest, cells were agitated and 100 L aliquots were removed to a non-adherent 96 well plate and incubated for 4 h prior to the addition of Alamar blue.

**Cytotoxicity and viability assays**

To quantify ATP generated by metabolically active cells, CellTiter-Glo luminescent cell viability assay (Promega) was used per the manufacturer's instruction. To determine cytotoxicity, LDH release was measured with the LDH Cytotoxicity Assay Kit (Pierce) according to the manufacture’s protocol. For 6 h assays, 96 well plates were seeded at 80,000 cells/mL for BaF3/p210 cells and at 30,000 cells/mL for K562 cells. After incubation at 37 oC for 6 h, cells were agitated and either 50 L was removed from every well and used in the LDH Cytotoxicty assay per the manufacturer’s protocol or 100 L was removed and used for the CellTiter-Glo assay per the manufacturer’s instructions. For 48 h assays, 7,500 cells/mL of both BaF3/p210 or K562 cells were incubated at 37 oC for 48 h (200 L/well). Prior to harvesting, wells were mixed by gentle pipetting and 100 L from each well was used for the CellTiter-Glo assay following manufacturer’s protocol. Cytotoxicity or viability were measured by fluorescence on a microplate reader.

**Activity profiling of reactive cysteines**

HeLa cell lysates were diluted to a 2 mg protein/mL solution in PBS and aliquoted into 0.5 mL volumes. Each sample (2 x 0.5 mL aliquots) was treated with either DMSO or PEITC (20 µM), followed by 100 µM of IA-alkyne. Click chemistry was performed by the addition of 100 µM of either the Azo-L (PEITC sample) or Azo-H (DMSO sample) tags (10 µL of a 5 mM stock), 1 mM TCEP (fresh 50X stock in water), 100 µM ligand (17X stock in DMSO:t-Butanol 1:4) and 1 mM CuSO4 (50X stock in water). Samples were allowed to react at room temperature for 1 h and then mixed together immediately following click chemistry. Streptavidin enrichment, on-bead trypsin digestion and subsequent release of labeled peptides with sodium dithionite was achieved using the exact protocol previously detailed([6](#_ENREF_6)). LC/LC-MS/MS analysis was performed on an LTQ-Orbitrap Discovery mass spectrometer (ThermoFisher) coupled to an Agilent 1200 series HPLC as previously described([7](#_ENREF_7)). The tandem MS data were searched using the SEQUEST algorithm using a concatenated target/decoy variant of the human IPI database. A static modification of +57.02146 on cysteine was specified to account for iodoacetamide alkylation and differential modifications of + 456.2849 (Azo-L modification) and + 462.2987 (Azo-H modification) were specified on cysteine to account for probe modifications. SEQUEST output files were filtered using DTASelect 2.0. Reported peptides were required to be fully tryptic and contain the desired probe modification and discriminant analyses were performed to achieve a peptide false-positive rate below 5%. Quantification of light/heavy ratios (R) was performed using the CIMAGE quantification package as previously described([8](#_ENREF_8)).

**Cell transfection and RNA interference**

BaF3/p210 cells were transfected with siRNAs using the Amaxa Nucleofector II (Amaxa, Gaithersburg, MD) according to the manufacturer’s protocol. Briefly, 2 x 106 cells per sample were transfected with 1.5 M or 380 nM siRNA using Amaxa Solution-V and program X-001. Pre-designed ON-TARGET plus siRNA pools (non-targeting and targeting USP9x [Ms]) were obtained from Dharmacon. Transfected cells were incubated in RPMI under standard conditions for 24, 48 and 68 h prior to western blot analysis. K562 cells were also transfected with the Amaxa Nucleofector II (Amaxa, Gaithersburg, MD) according to the manufacturer’s protocol. Briefly, 1.5 x 106 cells per sample were transfected with 750 nM, 380 nM, or 120 nM siRNA using Amaxa Solution-V and program T-016. Pre-designed ON-TARGET plus siRNA pools (non-targeting and targeting USP9x [Hu]) were obtained from Dharmacon. Transfected cells were incubated in RPMI under standard conditions for 18, 24, or 48 h prior to western blot analysis. NIH/3T3/p210 cells were transfected with pre-designed ON-TARGET plus siRNA pools (Ms, Dharmacon) using Dharmafect 1 (GE Dharmacon, Lafayette, CO) according to the manufacturer’s protocol.  Briefly, 1.5x105 NIH/3T3/p210 cells were plated in each well of a 6 well plate.  24 h post plating cells were transfected with 25 nM siRNA obtained from Dharmacon.  24 h post transfection, the media was replaced with DMEM supplemented with 10% DBS, 1% penicillin/streptomycin, and 1x glutaMAX.  Transfected cells were split as necessary to maintain continual growth and harvested 96 h post transfection. The following siRNA pools (Ms or Hu, Target sequence) were used (On-Target plus, Dharmacon):

Ms si-USP9x no. 09, 5′-CAGCAAAACUGUUCGUCAA-3′;

Ms si-USP9x no. 10, 5′-GGGCUAACGAUCUCAUUUA-3′;

Ms si-USP9x no. 11, 5′-GCUAAUGUGUAAAUGGCAA-3′;

Ms si-USP9x no. 12, 5′-GAUGAGGCUUCAAGAUAUA-3′;

Hu si-USP9x no. 06, 5′-AGAAAUCGCUGGUAUAAAU -3′;

Hu si-USP9x no. 07, 5′-ACACGAUGCUUUAGAAUUU -3′;

Hu si-USP9x no. 08, 5′-GUACGACGAUGUAUUCUCA -3′;

Hu si-USP9x no. 09, 5′-GAAAUAACUUCCUACCGAA -3′;

si-non-targeting no. 1, 5’-UGGUUUACAUGUCGACUAA-3’

si-non-targeting no. 2, 5’-UGGUUUACAUGUUGUGUGA-3’

si-non-targeting no. 3, 5’-UGGUUUACAUGUUUUCUGA-3’

si-non-targeting no. 4, 5’-UGGUUUACAUGUUUUCCUA-3’

**Retroviral transduction**

MSCV-p210-IRES-GFP vector obtained from the Ren laboratory (Brandeis University, Waltham, MA([9](#_ENREF_9))) was used to produce retroviral pseudo-virus. Packaging was performed as previously described ([10](#_ENREF_10)) with the exception of Transit-2020 (Mirus Bio, Madison, WI) in place of CaPO4 for transfection. 1x105 NIH/3T3 cells were seeded in a 60mm plate 24 h prior to transduction. 1 ml of viral supernatant was diluted with 1ml of DMEM supplemented with 10% donor adult bovine serum (DBS), 1% penicillin/streptomycin, 1X glutaMAX, and 16 μg polybrene and added to the NIH/3T3 plate. 6 h post transduction, the media was replaced with fresh DMEM supplemented with 10% DBS, 1% penicillin/streptomycin, and 1X glutaMAX. 72 h post transduction, GFP expressing NIH/3T3 cells were sorted and collected on a FACS Aria Flow Cytometer.

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