SUPPLEMENTAL MATERIAL

ALLYL ISOTHIOCYANATE ARRESTS CANCER CELLS IN MITOSIS, AND MITOTIC ARREST IN TURN LEADS TO APOPTOSIS VIA BCL-2 PHOSPHORYLATION

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Supplementary Methods:

Western blot analysis: Cells harvested after treatment with solvent or an test agent were washed with ice-cold PBS and suspended in 1x cell lysis buffer (Cell Signaling), supplemented with 2 mM phenylmethanesulfonyl fluoride and a proteinase inhibitor cocktail (Roche). Cell lysis was enhanced by sonication. Protein contents were measured using a bicinchoninic acid assay kit (Pierce). The samples (20-40 μg each) were resolved by SDS-PAGE (8-12%), which was followed by blotting to polyvinylidene fluoride membranes. The membranes were then probed by specific antibodies and the bands were visualized using an Amersham ECL Plus Kit (GE Healthcare).

Reaction of porcine tubulin with allyl isothiocyanate (AITC) and in-solution digestion for identification of AITC modified cysteine residues: Purified porcine tubulin purchased from Cytoskeleton, Inc. (0.25 mg/ml in PBS containing 1% acetonitrile) was treated with AITC (30μM) at 37°C for 1h. A cold acetone precipitation was performed to remove unreacted AITC and salts - six sample volumes of cold acetone was added to each sample and incubated at -20°C for 1hr. The protein precipitates were recovered by centrifugation (10,000 x g for 10 min), and the pellet was washed twice with cold 90% acetone and air-dried for 15 min. The pellet was resuspended in ammonium bicarbonate and acetonitrile (50mM/10% acetonitrile) and proteins were digested by the addition of trypsin (Sequencing Grade, Promega) at a ratio of 1:10 (w/w, trypsin/protein) at 37°C for 2 h. After incubation, the reactions were quenched by the addition of formic acid to a final concentration of 2%. The sample was dried and reconstituted with 2% formic acid.

LC-MS/MS Analysis: The tryptic digests were analyzed by liquid chromatography, nanoelectrospray-tandem mass spectrometry (nanoLC-ESI-MS/MS) using a nanoACQUITY UPLC (Waters) coupled through a nebulization-assisted nanospray ionization source to a Q-ToF Premier mass spectrometer (Waters/Micromass). The LC consisted of a trap column (Symmetry C18, 5μ, 180μ x 20 mm, Waters), followed by separation on an analytical column (Atlantis C18, 3μ, 100μ x 100 mm, Waters). Samples were loaded, trapped, and washed at a flow rate of 3 μL/min with 98% solvent A (0.1% aqueous formic acid)/2% solvent B (acetonitrile containing 0.1% formic acid) for 5 min. Peptides were eluted with a gradient of 98% A/2% B to 80% A/20% B for 10 min at 0.4 μL/min, 35% A/65% B for 115 min at 0.4 μL/min, and then 10% A/90% B at 0.8 μL/min for 10 min. The mass spectrometer was programmed (Data Dependent Acquisition experiment, DDA) to monitor ions within a m/z range of 300-1500, and ions with +2 to +5 charges only were selected for MS/MS using the preset DDA collision energy parameters.

Database Search and Peptide and Protein Identification: MS/MS spectra were processed and transformed to the PKL file format using Proteinlynx Global Server (PLGS) v2.3 (Waters/Micromass) and the default

parameters of MaxEnt3 (Waters/Micromass). The PKL file was used to search a protein database of porcine tubulin alpha and beta (ABB58919 and P02554, respectively) that was created in PLGS using sequences obtained from GeneBank and UniProt, respectively. The search parameters were as follows: trypsin as the proteolytic enzyme with 4 possible missed cleavages; AITC modification of cysteine (+99.014) and oxidation of methionine as variable modifications; the allowable mass error was 100 ppm for peptides and 100 mDa for fragment ions; peptide charge was set to 2+ and 3+. Tubulin peptides modified by AITC at cysteine residues were identified by PLGS and confirmed by manual inspection of the MS/MS fragment ion spectra.

Supplementary Figure Legends

Supplementary Figure S1: Effect of AITC and hydroxyurea (HU) on JNK isoforms in UM-UC-3 cells.

A, Human bladder cancer UM-UC-3 cells were treated with AITC for 3 h and whole cell lysates were analyzed by Western blot analysis for expression level of JNK1, JNK2 and JNK3. B. and C, UM-UC-3 cells were treated with or without HU for 16 h and then treated with AITC with or without HU for 3 h (B) or 24 h (C), from which whole cell lysates were prepared for analysis of JNK1 and JNK2 by Western blotting. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control.

Supplementary Figure S2: Effect of JNK inhibitor XI, BI-87G3 on AITC-induced Bcl-2 phosphorylation.

UM-UC-3 cells were pretreated with JNK inhibitor XI, BI-87G3 (2-[5-nitrothiazol-2-ylthio)benzo[d]thiazole) for 1 h and then cotreated with AITC and the JNK inhibitor for 24 h, followed by Western blot analysis of Bcl-2 and p-Bcl-2 (Ser70). α-Tubulin and β-tubulin were also measured to rule out any effect of the JNK inhibitor on their expression. GAPDH was used as a loading control.

Supplementary Figure S3: Identification of cysteine residues of porcine tubulin modified by AITC using nanoLC ESI-MS/MS.

The tandem MS spectra of tryptic peptides modified by AITC are shown in Figure S3-A to S3-G. The sequence-specific "b" and "y" ions are indicated on the spectra and the numbering system used for the peptides is for porcine tubulin alpha and beta (accession numbers ABB58919 and P02554, respectively). Immonium ions are indicated on the spectra using the three-letter amino acid code. AITC-modified cysteine residues are as indicated.

S3-A, MS/MS spectrum of tubulin alpha: ^{125}LADQC$_{AITC}$TGLQGFLVFHSFGGGTGSGFTSLLMER^{156} from precursor ion at m/z 1144.88 $[M+3H]^{3+}$. S3-B, MS/MS spectrum of tubulin alpha: ^{340}SIQFVDWC$_{AITC}$PTGFK^{352} from precursor ion at m/z 813.88 $[M+2H]^{2+}$. S3-C, MS/MS spectrum of tubulin alpha: ^{374}AVC$_{AITC}$MLSNTTAIAEAWAR^{390} from precursor ion at m/z 953.96 $[M+2H]^{2+}$. S3-D, MS/MS spectrum of tubulin beta: ^{3}EIVHIQAGQC$_{AITC}$GNQIGAK^{19} from precursor ion at m/z 622.32 $[M+3H]^{3+}$. For this peptide, b and y ions containing the modified cysteine were not found, and the assignment of the modification to Cys-12 was made based on the m/z of the parent ion. S3-E, MS/MS spectrum of tubulin beta: ^{217}LTTPTYGDLNHLVSATMSGVTTC$_{AITC}$LR^{241} from precursor ion at m/z 884.45 $[M+3H]^{3+}$. S3-F, MS/MS spectrum of tubulin beta: ^{298}NMMAAC$_{AITC}$DPR^{306} from precursor ion at m/z 554.22 $[M+2H]^{2+}$. S3-G. MS/MS spectrum of tubulin beta: ^{351}TAVC$_{AITC}$DIPPR^{359} from precursor ion at m/z 535.80 $[M+2H]^{2+}$.