Anti-tumor activity and signaling events triggered by the isothiocyanates, sulforaphane and phenethyl isothiocyanate, in multiple myeloma

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Citation: Jakubikova J, Cervi D, Ooi M, Kim K, Nahar S, Klippel S, Cholujova D, Leiba M, Daley JF, Delmore J, Negri J, Blotta S, McMillin DW, Hideshima T, Richardson PG, Sedlak J, Anderson KC, and Mitsiades CS. Anti-tumor activity and signaling events triggered by the isothiocyanates, sulforaphane and phenethyl isothiocyanate in multiple myeloma. Haematologica 2011;96(8):1170-1179. doi:10.3324/haematol.2010.029363

Supplementary Material and Methods

Culture of MM cell lines, primary MM tumor cells, stromal cells, and normal cells

The chemosensitive cell line RPMI 8226-S and its sublines resistant to doxorubicin (RPMI-Dox40), mitoxantrone (RPMI-MR20), and melphalan (RPMI-LR5) were kindly provided by Dr. William S. Dalton (Lee Moffitt Cancer Center, Tampa, FL, USA). Human MM cell lines OPM-1 and OPM-2 were obtained from Dr. Teru Hideshima and the DSMZ (*Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany*), respectively. Dexamethasone-sensitive MM.1S and dexamethasone-resistant MM.1R cells were kindly provided by Dr Steven Rosen (Northwestern University, Chicago, IL, USA). The non-transformed human liver epithelial cell line THLE-3 and the human stromal cell line HS-5 were obtained from ATCC (Manassas, VA, USA). All MM cell lines, THLE-3 cell line and the human stromal cell line HS-5 were cultured in RPMI 1640 (Cellgro, Mediatech, VA, USA) and Dulbecco's modified Eagle medium (DMEM; Cellgro, Mediatech, VA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Harlan, Indianapolis, IN, USA), 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine (GIBCO, Grand Island, NY, USA) at 37°C in 5% CO_2, respectively.

Plasma $CD138^+$ cells were purified from freshly isolated bone marrow of MM patients or healthy donors by positive selection using CD138 monoclonal antibody-conjugated magnetic beads according to the manufacture's instructions (Miltenyi Biotec Inc., Auburn, CA, USA). Fresh peripheral blood mononuclear cells were obtained from four healthy volunteers by Ficoll-Hypaque (Pharmacia, Piscataway, NJ, USA) density sedimentation. Cells were cultured in RPMI 1640 containing 20% heat-inactivated FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine, and maintained at 37°C in 5% CO_2. Approval for these studies was obtained from the Dana-Farber Cancer Institute Institutional Review Board. Informed consent was obtained from all patients and healthy volunteers in accordance with the Declaration of Helsinki protocol.

Drug viability assays

The inhibitory effect of ITC, alone or in combination with conventional or novel anti-MM agents, on survival of MM cells was determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay (MTT; Sigma-Aldrich, St Louis, MO, USA). Formazan crystals were dissolved with addition of 200 μL of DMSO, and absorbance was measured at 540 and 690 nm in a microplate reader (Dynatech Lab Inc., Chantilly, VA, USA). The concentration of drug that inhibited cell survival by 50% (IC_{50}) was determined using Calcusyn software (Biosoft, Ferguson).

Purified MM patients' cells were plated in 384-well plates at a density of 10,000 cells per well, and treated with increasing concentration of ITC for 48 h at 37 °C. Viability was assessed using the CellTiterGlo (CTG; Promega) assay. CTG was added for 30 min, and plates were read with a Luminoskan luminometer (Labsystems, Franklin, MA, USA).

Cell proliferation analysis

To study the effect of ITC on proliferation of MM cells cultured alone or together with HS-5 bone marrow stromal cells, MM cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE), a cytoplasmic dye that is equally diluted between daughter cells during cell division. Flow cytometry was performed to evaluate changes in mean fluorescence intensity of CFSE signal as a surrogate marker of cell proliferation. Briefly, MM cells were labeled with 1 μM CFSE (Molecular Probes, Eugene, OR, USA) for 10 min at 37°C in serum-free RPMI in the dark. The reaction was stopped by adding RPMI 1640 medium supplemented with 2% FBS, and cells were washed three times with 10% FBS RMPI 1640. Cells were then seeded in 96-well plates in medium alone or together with unlabeled stromal HS-5 cells seeded 24 h prior to co-culture. After 12, 24 and 48 h of culture, propidium iodide (PI; Sigma-Aldrich, St. Louis, MO, USA) was added at a final concentration of 2 μg/mL to exclude necrotic cells; and the fluorescence intensity in these 96-well plates was analyzed using a FACS Canto II flow cytometer (Becton Dickinson).

Detection of apoptosis

Apoptotic cells were quantified using the Annexin V-FITC Apoptosis assay. Briefly, both suspended and adherent cells

were collected and washed twice with cold phosphate-buffered saline (PBS). Cells (3×10^5) were resuspended in 100 μL of manufacturer-supplied 1X binding buffer and mixed with 5 μL of annexin V-FITC (BD Biosciences Pharmingen) and 5 μL of PI. After 15 min incubation in the dark at room temperature, cells were analyzed by a FACS Canto II flow cytometer (Becton Dickinson) using a 96-well format.

Cytofluorimetric analysis of mitochondrial potential

The mitochondrial membrane potential of ITC-treated and control MM cells was studied using the JC-1 fluorescent probe, a mitochondria-selective probe that forms aggregates in normal polarized mitochondria resulting in an orange emission. Its monomeric forms, present in cells with depolarized mitochondrial membranes, emit only green fluorescence. Briefly, 3×10^5 cells were incubated in 200 μL of PBS/0.2% bovine serum albumin (BSA) containing 4 μM of JC-1 (Molecular Probes, Eugene, OR, USA) for 30 min at 37 °C. After 30 min incubation in the dark at 37 °C, cells were analyzed using a FACS Canto II flow cytometer (Becton Dickinson) and a 96-well format.

Cell cycle analysis

Changes in cell cycle status of ITC-treated cells were determined by flow cytometric measurement of DNA content of nuclei labeled with PI. Briefly, MM cells (3×10^5) were collected, washed twice with cold PBS and incubated in 0.05% Triton X-100 and 15 μL RNA-se A (10 mg/mL) for 20 min at 37 °C. Then cells were cooled and incubated on ice for at least 10 min before PI (50 μg/mL) was added. Finally, the stained cells were analysed using a FACS Canto II flow cytometer (Becton Dickinson) using a 96-well format.

Detection of histone H3 phosphorylation

Cells (1×10^6) were washed twice with cold PBS, fixed in 1% methanol-free paraformaldehyde in PBS at 0 °C for 3 min, washed in PBS/0.2%BSA, and suspended in 70% ethanol for at least 2 h at –20 °C. Cells were washed twice in 1% solution of BSA in PBS to suppress non-specific antibody binding. The cell pellet was then resuspended in 100 μL of 1% BSA containing 1:200 diluted rabbit polyclonal anti-phosphorylated histone H3 (pH3/Ser10; Upstate, Lake Placid, NY, USA) for 1 h at room temperature, washed twice with PBS/1% BSA, and incubated for 30 min at room temperature in the dark in 100 μL of 1:100 diluted, FITC-conjugated anti-rabbit Ig. After having been washed twice washing in PBS, cells were counterstained with 5 μg/mL of PI dissolved in PBS for 15 min at 4 °C. Cellular fluorescence was measured using a FACS Canto II flow cytometer (Becton Dickinson) using a 96-well format.

Flow cytometry measurements and data analysis

Flow cytometry measurements were performed with a FACS Canto II flow cytometer equipped with a 488 nm excitation laser. Fluorescence emission was measured using a bandpass filter set of 530, 585, 670 and 780 nm with respective photomultipliers FL1–FL4 required for fluorochromes. All fluorochromes were excited with a 488 nm laser, and data were collected through a respective photomultiplier, as follows: Annexin V-FITC and PI (FL1, FL3); CFSE and PI (FL1, FL3); JC-1 (FL1, FL2, ratio FL2/FL1); cell cycle (log FL3, sub G1; lin FL3, DNA cell cycle histogram; FL3 peak *versus* integral for doublets discrimination); and anti-pH3 and PI (FL1, lin FL3, FL3 peak and integral). Forward/side light scatter characteristics were used to exclude the cell debris from the analysis. For each analysis, 1×10^4 cells were acquired for analysis. Data were analysed with De Novo FCS Express software (De Novo software, Los Angeles, CA, USA). Cell cycle calculations were performed with MultiCycle AV DNA analysis (Phoenix Flow Systems, San Diego, CA, USA).

Multiplex total and phosphoprotein analysis

Multiplex evaluation of total and phosphorylated levels of proteins of interest was performed with the xMAP luminex platform (Luminex, Austin), which combines the principle of a "sandwich" immunoassay with fluorescent bead–based technology for analysis of up to 100 different analytes in a single microtiter well in 96-well plates. A panel of five non-phosphorylated proteins Akt, ERK1/2, IκB-α, JNK, p38 MAPK and a panel of 16 phosphoproteins: phosphorylated Akt (Ser473), c-Jun (Ser63), ERK1/2 (Thr202/Tyr204, Thr185/Tyr187), GSK-3alpha/beta (Ser21/Ser9), HSP27 (Ser78), IRS-1 (Ser636/Ser639), JNK (Thr183/Tyr185), MEK1 (Ser217/Ser221), NF-kappaB p65 (Ser536), p38 MAPK (Thr180/Tyr182), p53 (Ser15), p70 S6 kinase (Thr421/Ser424), p90RSK (Thr359/Ser363), Src (Tyr416), STAT3 (Ser727), STAT6 (Tyr641) were analyzed in a 96-well format using the Bio-Plex suspension array system, according to manufacturer' instructions (Bio-Rad Laboratories, Hercules, CA).

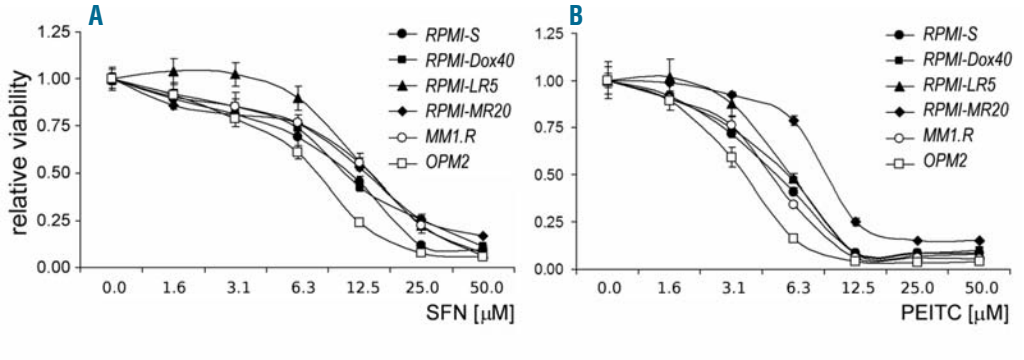
Multiplex luminescence assay for caspase-8/9 and fluorescent caspase-3/7 activity

MM.1S cells were seeded at 1×10^4 cells/well (in 50 μL of medium) in optical 96-well plates and treated with ITC (in 50 μL of medium) 24 h after plating. After 6, 12, and 24 h, caspase-Glo™ 8 reagent or caspase-Glo™ 9 reagent (Promega, Madison, WI, USA) was prepared by combining assay buffer with substrate; fluorescent caspase-3 substrate (Z-DEVD)2-R110 was mixed into the Caspase-Glo™ reagent at a final concentration of 50 μM. The combined reagent/substrate was then added (100 μL), and incubated for 1 h at room temperature; luminescence and fluorescence ($485_{Ex}/527_{Em}$) were measured using a multilabel reader Mithras LB940 (Berthold Technologies).

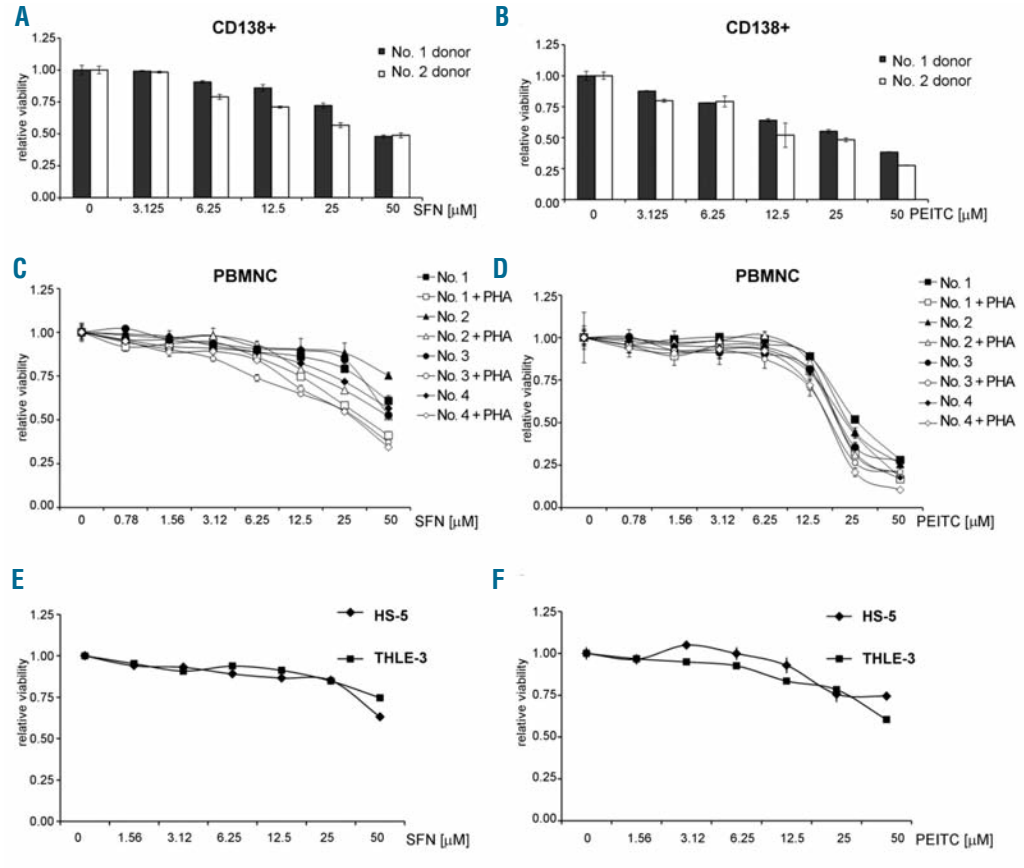
Western blotting analysis

After treatment with drugs, cells were washed twice with ice-cold PBS, resuspended in 100 μL of ice-cold cell lysis buffer (1% Nonidet P-40, 50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, 2 mM PMSF, 1 mM sodium vanadate, 1 mM sodium fluoride and 1 x protease mixture), and then incubated on ice for 20 min. After centrifugation for 10 min at 10,000 x g, supernatants were collected. Protein concentrations were measured using a Bradford protein assay kit. Equivalent amounts of protein (20 μg) were mixed with 4X SDS-PAGE sample buffer (Invitrogen, Carlsbad, CA, USA) and 10X reducing agent (0.5 M dithiotreitol, Invitrogen, Carlsbad, CA, USA) and separated by SDS-PAGE. Resolved proteins were transferred to a nitrocellulose membrane (Bio-Rad) using a semi-dry transfer system. Membranes were blocked for 1 h at room temperature with 5% non-fat dry milk in Tris–buffered saline (TBS), pH 7.4, con-

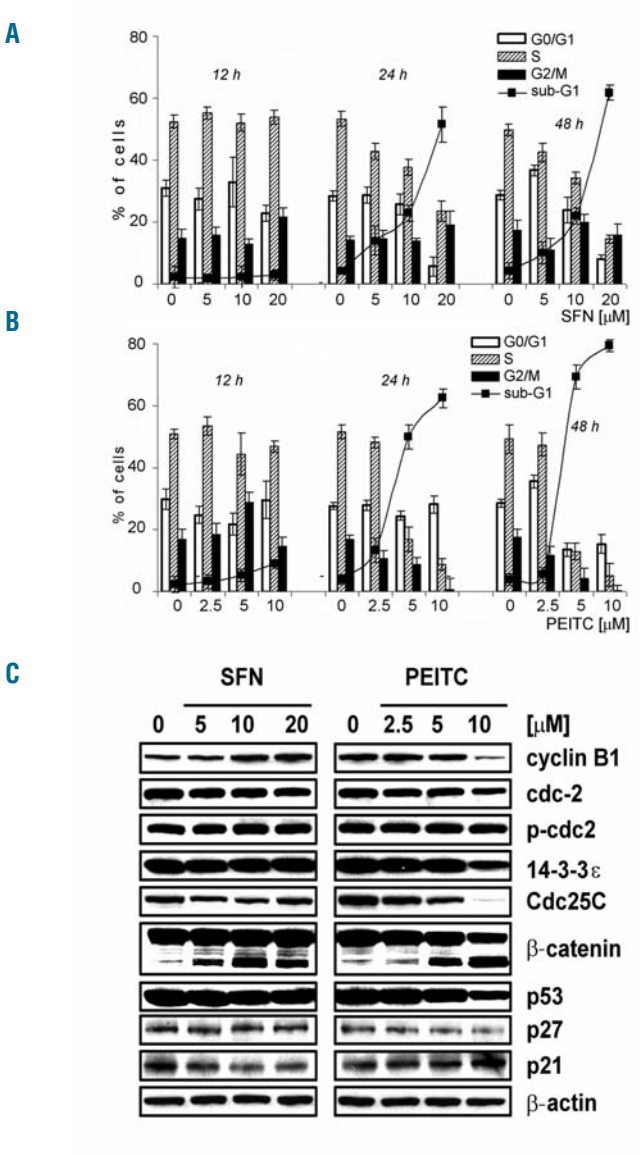
taining 1% Tween 20 (TBS-T), followed by overnight incubation at 4 °C with a 1:1000 dilution of the respective primary antibodies: anti–phospho (p)-Akt, Akt, p-GSK3α/β, p-p90RSK, p-p70S6K, p-cJun, ERK1/2, p-ERK1/2, JNK, and p-JNK, cyclin B1, cdc-2, p-cdc-2, CDC25C, 14-3-3ε, β-catenin, p53, p27, p21, PARP, caspase-3, caspase-9, AIF, X-IAP, c-IAP, Mcl-1, Hsp-90, Hsp-70, survivin, IRF4 and β-actin (Cell Signaling Technology). Membranes were washed in TBS-T, and incubated with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit secondary antibodies (Cell Signaling Technology, Danvers, MA, USA) for 1 h at room temperature. Proteins were visualized with an enhanced chemiluminescent (ECL) system (Amersham Bioscience, Little Chalfont, UK).



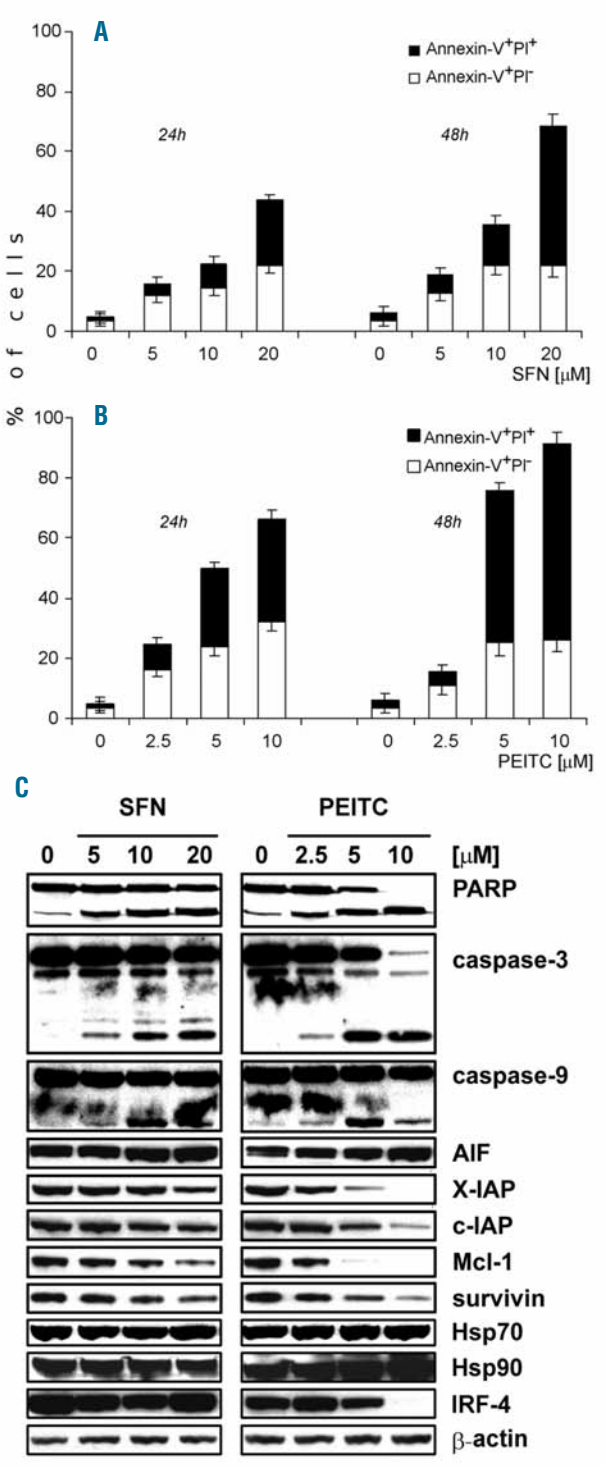
Online Supplementary Figure 1. Cytotoxic effects of SFN and PEITC in MM cell lines. (A) The MM cell lines RPMI 8226-S, RPMI-Dox40, RPMI-LR5, RPMI-MR20, MM.1R, and OPM2 were cultured with SFN (0-50 μM) or (B) PEITC (0-50 μM) for 48 h in 96-well plates. Each treatment was performed in quadruplicate and assessed by MTT.



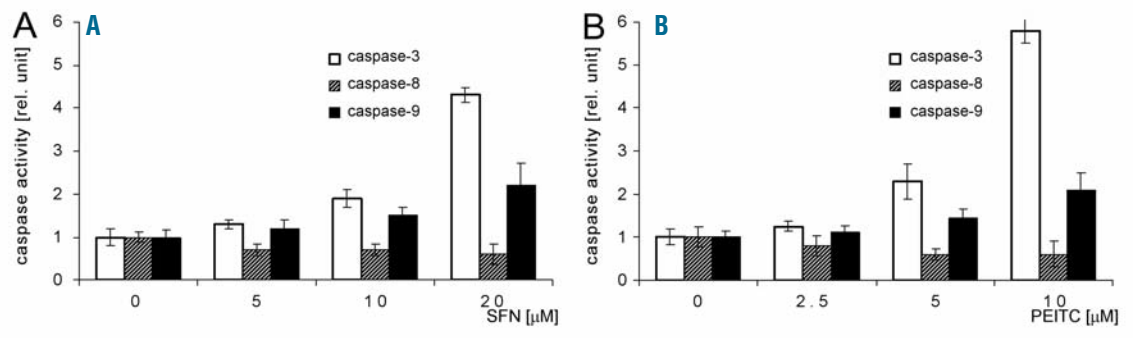
Online Supplementary Figure 2. The effect of SFN and PEITC on healthy cells. (A) CD138+ purified cells from healthy donors were cultured with SFN (0-50 μM) and (B) PEITC (0-50 μM) for 48 h. Cytotoxicity was analyzed using CellTiterGlo assay and measured with a luminometer. (C) Fresh peripheral blood mononuclear cells (PBMNCs) were obtained from four healthy volunteers by Ficoll-Hypaque, split in half and phytohemagglutinin (PHA; 2 μg/mL) was added to one half. PBMNCs were treated with SFN (0-50 μM) and (D) PEITC (0-50 μM) for 48 h and viability was assessed by the MTT assay. (E) The human bone marrow stromal cell line HS-5 and the non-transformed human liver epithelial cell line THLE-3 were similarly cultured with SFN (0-50 μM) and (F) PEITC (0-50 μM) and viability was analyzed by MTT assay.



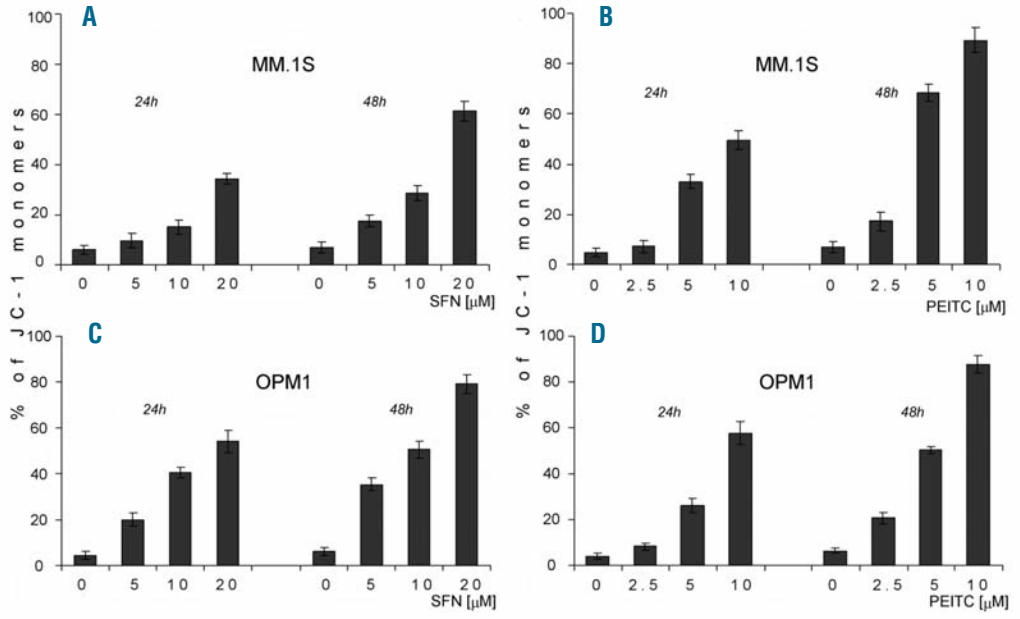
Online Supplementary Figure 3. SFN and PEITC induce cell cycle arrest and increased sub G_1 fraction in OPM1 cells. (A) OPM1 cells were cultured with SFN (5, 10 and 20 μM) for 12, 24 and 48 h. (B) OPM1 cells were exposed to PEITC (2.5, 5 and 10 μM) for 12, 24 and 48 h. The distribution of cells in G_0/G_1, S and G_2/M phase was analyzed by flow cytometry and MultiCycle AV DNA analysis. The percentage of sub-G_1 phase was defined by flow cytometry using De Novo FCS Express software. Three independent experiments were performed, and the means ± SE are presented. (C) OPM1 cells were exposed to SFN (5, 10 and 20 μM) and PEITC (2.5, 5 and 10 μM) for 12 h. Whole cell lysates were subjected to western blot analyses using anti-cyclin B1, -cdc-2, -p-cdc-2, -CDC25C, 14-3-3ε, β-catenin, p53, p27, p21, and β-actin. Data presented are representative of two independent experiments.



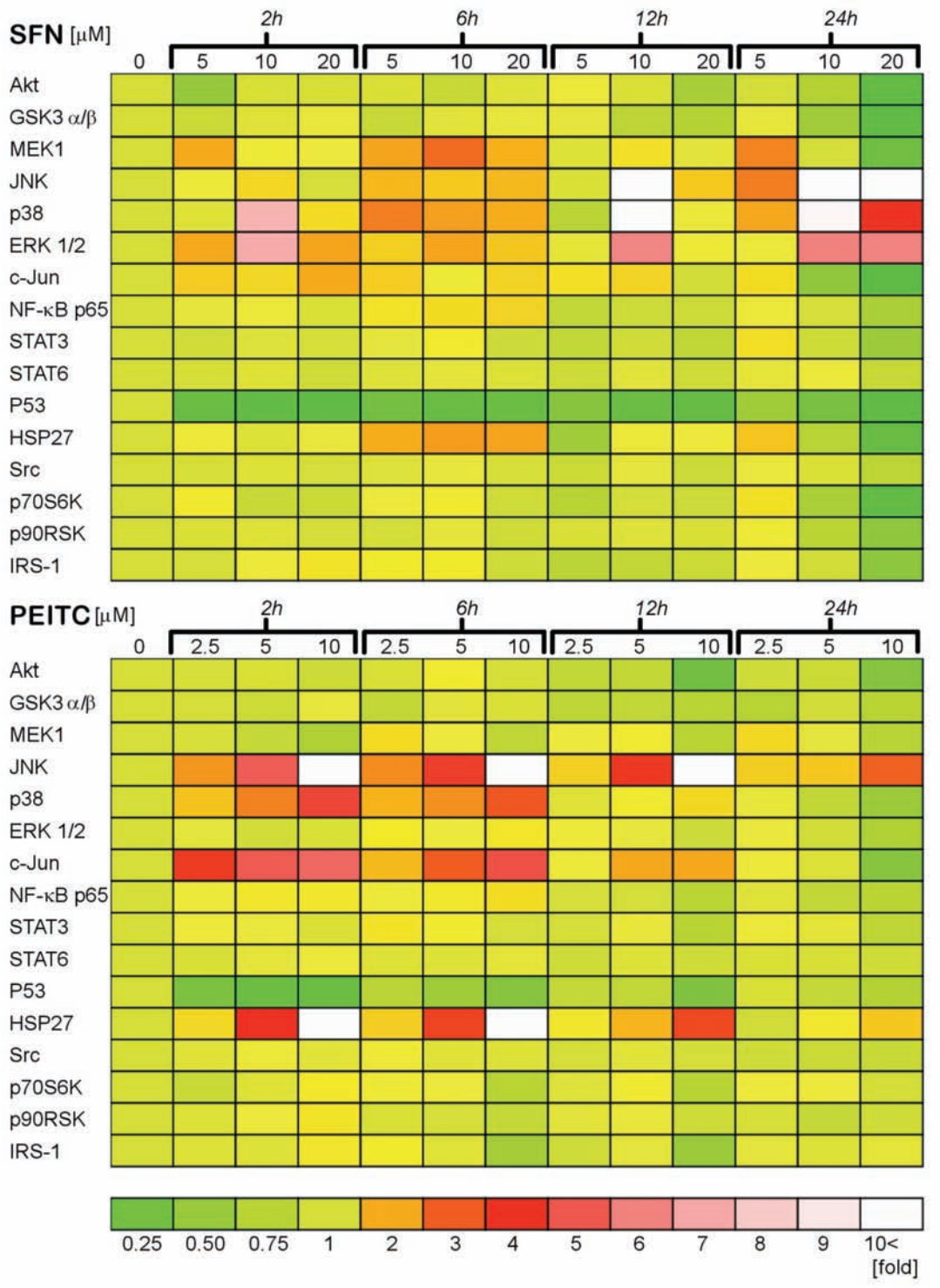
Online Supplementary Figure 4. SFN and PEITC induce apoptosis in OPM1 cells. (A) Effect of SFN on induction of apoptosis and necrosis was quantified by flow cytometry after staining with annexin V-FITC and PI. OPM1 cells were treated with SFN (5, 10 and 20 μM) or DMSO control for 24 and 48 h. (B) OPM1 cells were exposed to either DMSO (control cells) or different concentrations of PEITC (2.5, 5 and 10 μM) for 24 and 48 h. Percentages of apoptotic (Annexin V-FITC$^+$/PI$^-$) and late apoptotic/necrotic (Annexin V-FITC$^+$/PI$^+$ double positive) cells were analyzed by a FACS Canto II flow cytometer. (C) OPM1 cells were cultured with DMSO (control), SFN (5, 10 and 20 μM) or PEITC (2.5, 5 and 10 μM) for 24 h. Western blot analysis assessed PARP fragmentation, caspase-3 cleavage, caspase-9 cleavage, as well as expression of AIF, X-IAP, c-IAP-1, Mcl-1, Hsp-70, Hsp-90, survivin, and IRF4; β-actin served as the control. The data presented are representative of two independent experiments.



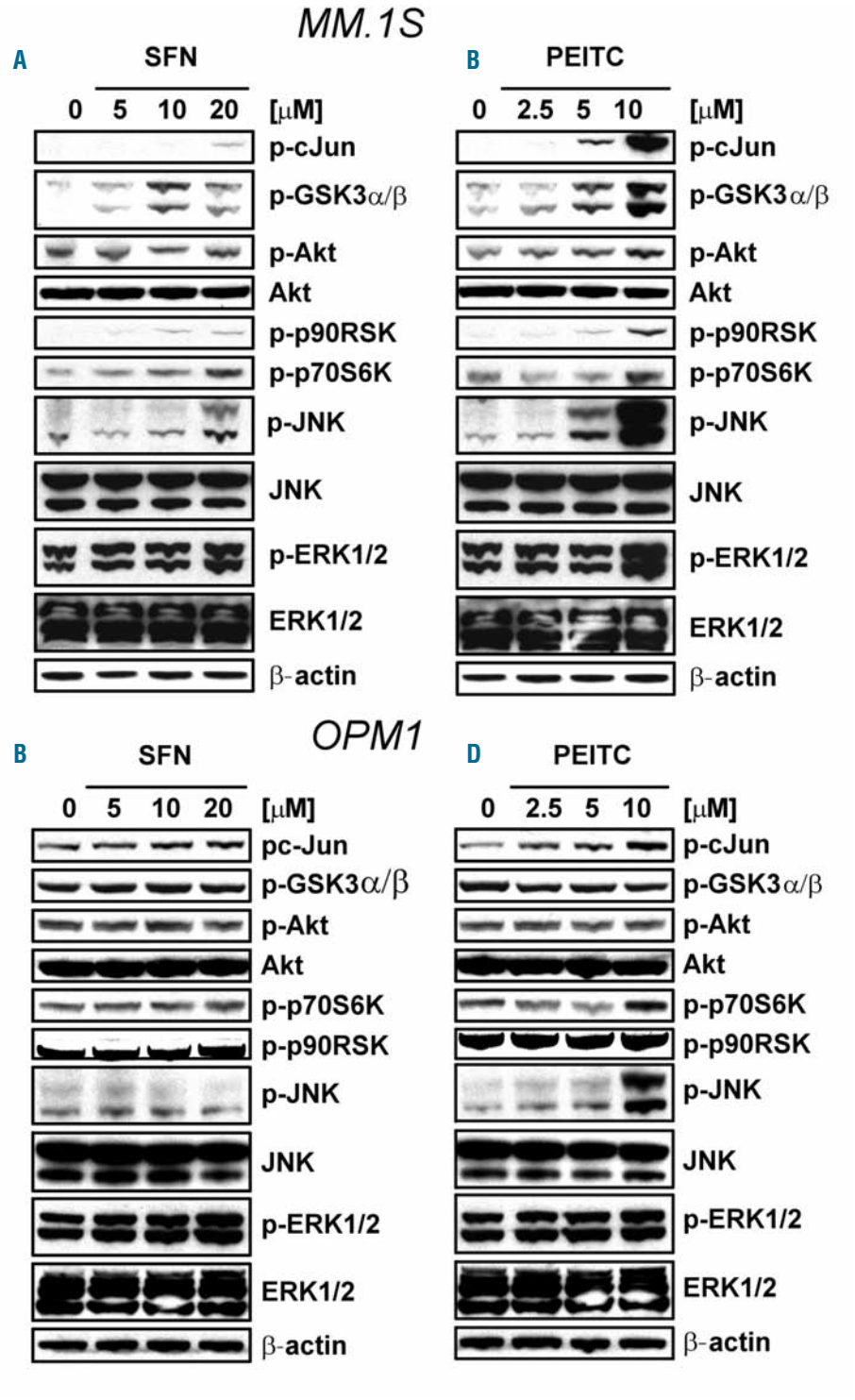
Online Supplementary Figure 5. SFN and PEITC induce activation of caspases in MM.1S cells. (A) MM.1S cells were treated with SFN and (B) PEITC for 12 h. The combined reagent/substrates (caspase-GloT™ 8 reagent or caspase-GloT™ 9 reagent mixed with fluorescent caspase-3 substrate) were added to replicate wells in 96-well plates for 1 h, and luminescence and fluorescence (485Ex/527Em) were measured.



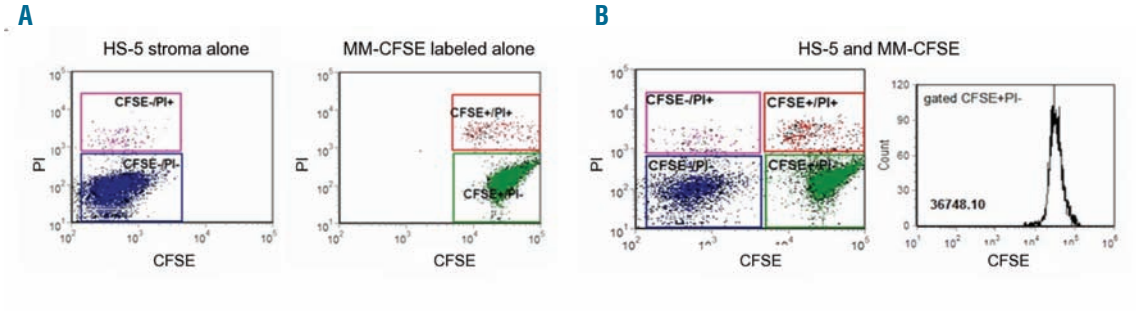
Online Supplementary Figure 6. SFN and PEITC induce mitochondrial membrane depolarization in MM.1S and OPM1 cells. (A) MM.1S cells were exposed to either DMSO (control), SFN (5, 10 and 20 μM) or (B) PEITC (2.5, 5 and 10 μM) for 24 and 48 h, stained with JC-1 dye, and analyzed using a FACS Canto II flow cytometer. The green fluorescence refers to the JC-1 monomers and the orange fluorescence corresponds to the formation of JC-1 aggregates. Effect of ITC treatment of MM.1S cells at 24 and 48 h on induction of mitochondrial membrane depolarization. (C) OPM1 cells were treated with SFN (5, 10 and 20 μM) at 24 and 48 h, stained with JC-1 dye and analyzed using a FACS Canto II flow cytometer. (D) Treatment of OPM1 cells with PEITC induced mitochondrial membrane depolarization at 24 and 48 h. Changes in the production of JC-1 monomers as a function of ITC concentration are shown.



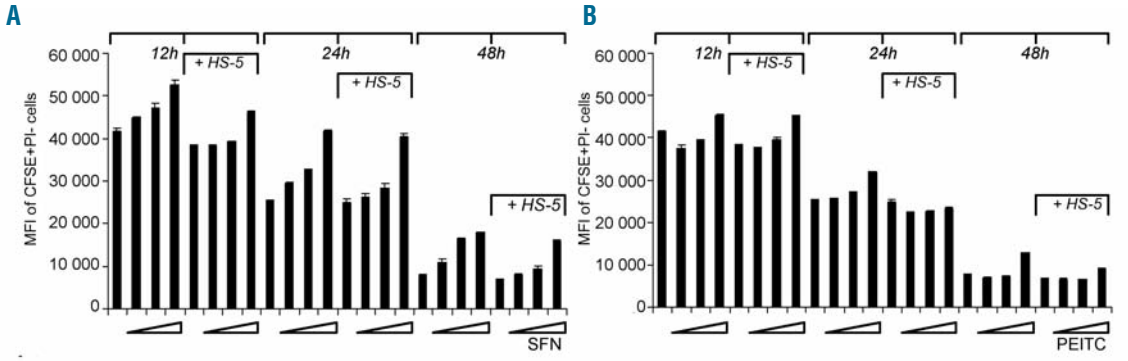
Online Supplementary Figure 7. Multiplex analysis of SFN- and PEITC- induced changes in phosphorylation state of members of signaling pathways in OPM1 cells. OPM1 cells were treated with different concentrations of SFN (5, 10 and 20 μM) and PEITC (2.5, 5 and 10 μM) for 2, 6, 12 and 24 h. Protein concentrations of whole cell lysates were measured using a Bradford protein assay kit and normalized to a panel of five total proteins. A multiplex panel of 16 phosphoproteins including Akt, c-Jun, ERK1/2, GSK-3α/β, HSP27, IRS-1, JNK, MEK1, NF-κB p65, p38 MAPK, p53, p70 S6 kinase, p90RSK, Src, STAT3, and STAT6 were analyzed in a 96-well format using the Bio-Plex suspension array system. Data are from two independent experiments; and fold changes in expression are depicted in a color-coded format, according to the scale included in the bottom part of the figure.



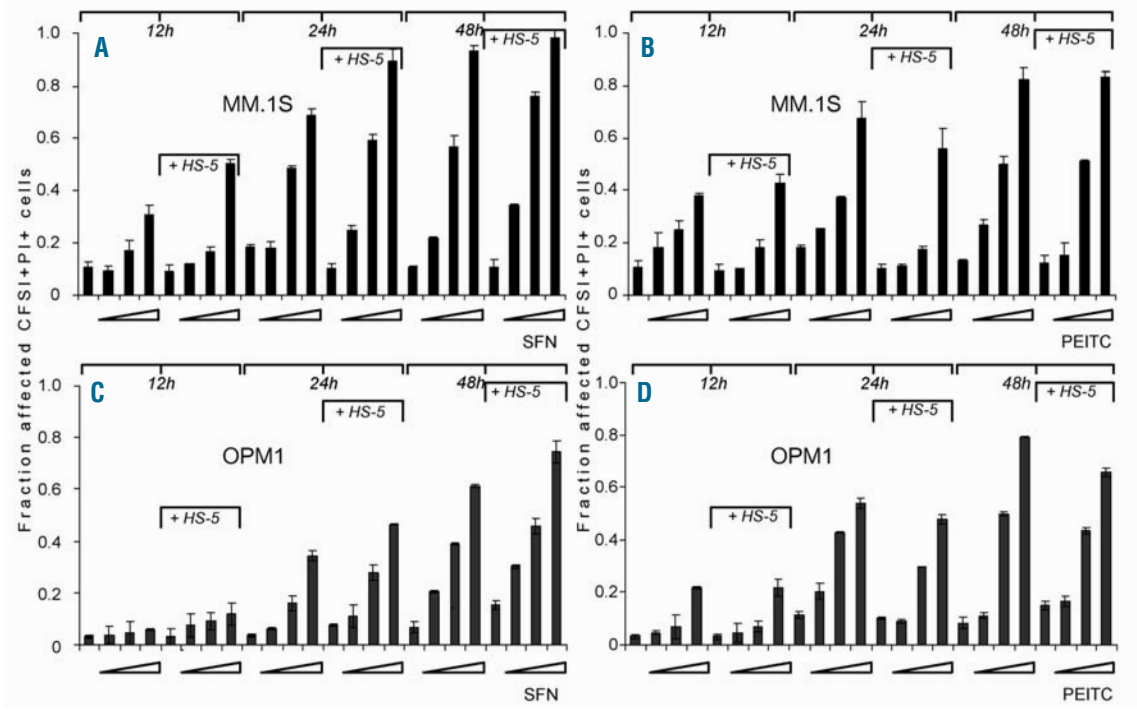
Online Supplementary Figure 8. SFN and PEITC induce phosphorylation of signaling molecules in MM cells. (A) MM.1S cells were cultured with SFN (5, 10 and 20 μM) and (B) PEITC (2.5, 5 and 10 μM) for 2 h. (C) OPM1 cells were cultured with SFN (5, 10 and 20 μM) and (D) PEITC (2.5, 5 and 10 μM) for 2 h. Western blot analysis was used to assess the effect of SFN and PEITC on expression or activation/phosphorylation of Akt, p-Akt, p-GSK3α/β, p-p90RSK, p-p70S6K, p-cJun, ERK1/2, p-ERK1/2, JNK, and p-JNK; β-actin served as a control for equal protein loading. Data are representative of two independent experiments.



Online Supplementary Figure 9. Representative dot plots of proliferation and viability analyses of MM cells alone or in co-culture with HS-5 stromal cells. (A) The left dot plot represents viable unlabeled-HS-5 stromal cells ($CFSE^-/PI^-$; lower-left quadrant) and nonviable stromal cells stained with propidium iodide ($CFSE^-/PI^+$; upper-left quadrant). The right dot plot shows viable MM cells labelled with CFSE ($CFSE^+/PI$-; lower-right quadrant) and double positive non-viable CFSE-labeled MM cells stained with propidium iodide ($CFSE^+/PI^+$; upper-right quadrant). (B) This representative dot plot shows unlabeled-HS-5 stromal cells cultured together with CFSE-labeled MM.1S cells. Events from the gate of viable CFSE-labeled MM.1S cells ($CFSE^+/PI^-$; lower-right quadrant) are presented in a histogram (right part of the panel) to depict fluorescence intensity of viable CFSE-labeled MM.1S. The number in the histogram is teh mean of fluorescence intensity. There is an inverse correlation between the MFI of CFSE and number of cell divisions (increase of proliferation correlates with decrease of MFI of CFSE).



Online Supplementary Figure 10. SFN and PEITC inhibit proliferation of OPM1 cells alone and in co-culture with HS-5 stromal cells. (A) CFSE-stained OPM1 cells were cultured with SFN (5, 10 and 20 μM) for 12, 24 and 48 h in the presence or absence of HS-5 stromal cells. Fluorescence intensity of gated $CFSE^+PI^-$ -stained OPM1 cells is shown as a function of SFN concentration (5, 10 and 20 μM). (B) CFSE-labeled OPM1 cells were treated with PEITC (2.5, 5 and 10 μM) for 12, 24 and 48 h in the presence or absence of HS-5 stromal cells. Fluorescence intensity of $CFSE^+PI^-$-labeled OPM1 is shown as a function of the PEITC concentration (2.5, 5 and 10 μM).



Online Supplementary Figure 11. SFN and PEITC inhibit viability of MM.1S and OPM1 cells alone and in co-culture with HS-5 stromal cells. (A) The values of the fraction of non-viable ($CFSE^+PI^+$) MM.1S cells within the entire population of MM.1S cells ($CFSE^+$) are presented as a function of the SFN concentration. (B) The values of the fraction of nonviable ($CFSE^+PI^+$) MM.1S cells within the entire population of MM.1S cells ($CFSE^+$) are presented as a function of the PEITC concentration. (C) The values of the fraction of nonviable ($CFSE^+PI^+$) OPM1 cells within the population of OPM1 cells ($CFSE^+$) are presented as a function of the SFN concentration. (D) The values of the fraction of non-viable ($CFSE^+PI^+$) OPM1 cells within the population of OPM1 cells ($CFSE^+$) are presented as a function of the PEITC concentration. Data are from two independent experiments, and results are means ± SE.