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

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ABSTRACT

Background
Isothiocyanates, a family of phytochemicals found in cruciferous vegetables, have cytotoxic effects against several types of tumor cells. Multiple myeloma is a fatal disease characterized by clonal proliferation of plasma cells in the bone marrow. The growing body of preclinical information on the anti-cancer activity of isothiocyanates led us to investigate their anti-myeloma properties.

Design and Methods
We evaluated the anti-myeloma activity of the isothiocyanates, sulforaphane and phenethyl isothiocyanate, on a panel of human myeloma cell lines as well as primary myeloma tumor cells. Cell viability, apoptosis, cell cycle alterations and cell proliferation were then analyzed in vitro and in a xenograft mouse model *in vivo*. The molecular sequelae of isothiocyanate treatment in multiple myeloma cells were evaluated by multiplex analyses using bead arrays and western blotting.

Results
We observed that sulforaphane and phenylethyl isothiocyanate have activity against myeloma cell lines and patients' myeloma cells both *in vitro* and *in vivo* using a myeloma xenograft mouse model. Isothiocyanates induced apoptotic death of myeloma cells; depletion of mitochondrial membrane potential; cleavage of PARP and caspases-3 and -9; as well as down-regulation of anti-apoptotic proteins including Mcl-1, X-IAP, c-IAP and survivin. Isothiocyanates induced G_2/M cell cycle arrest accompanied by mitotic phosphorylation of histone H3. Multiplex analysis of phosphorylation of diverse components of signaling cascades revealed changes in MAPK activation; increased phosphorylation of c-jun and HSP27; as well as changes in the phosphorylation of Akt, and GSK3α/β and p53. Isothiocyanates suppressed proliferation of myeloma cells alone and when co-cultured with HS-5 stromal cells. Sulforaphane and phenylethyl isothiocyanate enhanced the *in vitro* anti-myeloma activity of several conventional and novel therapies used in multiple myeloma.

Conclusions
Our study shows that isothiocyanates have potent anti-myeloma activities and may enhance the activity of other anti-multiple myeloma agents. These results indicate that isothiocyanates may have therapeutic potential in multiple myeloma and provide the preclinical framework for future clinical studies of isothiocyanates in multiple myeloma.

Key words: isothiocyanates, sulforaphane, phenethyl isothiocyanate, PEITC, multiple myeloma, bone marrow microenvironment, signaling pathways.

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The online version of this article has a Supplementary Appendix.

Introduction

Multiple myeloma (MM) is a B-cell malignancy characterized by clonal proliferation of plasma cells in the bone marrow. The behavior of MM cells is determined not only by their genetic features but also by their interactions with the bone marrow microenvironment via diverse adhesion molecules and/or cytokines.[1] The use of novel targeted agents (such as thalidomide, bortezomib and lenalidomide), alone or in combination with novel or high-dose conventional therapies, has improved the clinical outcome of MM patients. However, the disease remains incurable and the pursuit of novel therapeutic agents is, therefore, critically important.

A large proportion of conventional and novel anti-cancer agents are derived from natural products. For instance, natural products such as resveratrol, epigallocatechin-3-gallate, and curcumin have been shown to have anti-MM activity in preclinical models.[2-4] This growing body of preclinical data led us to investigate whether other natural products could also exhibit anti-MM properties.

In this study, we evaluated the anti-MM activity of the isothiocyanate (ITC) compounds, sulforaphane (SFN) and phenethyl isothiocyanate (PEITC). We studied this particular class of compounds since epidemiological studies suggest that increased dietary intake of cruciferous vegetables is associated with a reduced incidence of cancer.[5] The anti-carcinogenic activity of cruciferous vegetables has been attributed to ITC, which are formed by hydrolysis of their precursor compounds glucosinolates.[6] Several studies in tumor models have shown that these ITC have anti-tumor activities *in vitro* and/or *in vivo*.[7-10]

ITC inhibit phase I enzymes, protect against DNA adduct formation, and induce phase II detoxification enzymes.[11] Previous studies indicated that the anti-cancer activity of ITC may be attributed, at least in part, to modulation of signal transduction pathways such as MAP kinases and cdk-mediated cell cycle arrest.[8,12] Signaling molecules modulated include Chk2, cdc2, cyclin B1, Cdc25B and Cdc25C and the serotonin receptor.[13,14] ITC have also been implicated in histone deacetylase inhibition, tubulin polymerization disruption, Cox-2 inhibition, and decreased expression of the estrogen receptor α.[7,15-18]

In this study, we demonstrate the cytotoxic effects of SFN and PEITC against a panel of human MM cell lines, including cells resistant to conventional or novel anti-MM agents as well as primary MM tumor cells. Furthermore, we also observed that SFN and PEITC have anti-MM activity using a xenograft mouse model of human MM cells. ITC induced G_2/M cell cycle arrest and subsequent induction of apoptosis. Multiplex analysis of phosphorylation status of members of diverse signaling pathways revealed changes in the activation of MAPK pathway, increased phosphorylation of c-jun and HSP27, as well as changes in phosphorylation of Akt, and GSK3α/β and p53. We show that co-culture of MM cells with bone marrow stromal cells does not confer resistance to ITC. Furthermore, SFN exhibits synergistic effects when combined with bortezomib, dexamethasone, doxorubicin, and melphalan; whereas PEITC shows synergistic effects combined with lenalidomide, bortezomib, and melphalan. These preclinical studies provide the framework for clinical evaluation of ITC as novel MM therapeutics.

Design and Methods

Primary cells and cell lines

A panel of MM cell lines (RPMI 8226-S, also referred to as RPMI-S, RPMI-Dox40, RPMI-MR20, RPMI-LR5, OPM-1, OPM-2, MM.1S, and MM.1R cells); the non-transformed human liver epithelial cell line THLE-3; the human bone marrow stromal cell line HS-5 (ATCC; Manassas, VA, USA); and primary MM cells purified from bone marrow aspirates of MM patients or healthy donors by positive selection using CD138 monoclonal antibody-conjugated magnetic beads were processed and cultured *in vitro*, as described in more detail in the *Online Supplementary Appendix*.

Reagents

SFN and PEITC were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions of ITC were dissolved in dimethylsulfoxide (DMSO); control cells were treated with DMSO alone (final concentration <0.1%). Bortezomib was obtained from Millennium Pharmaceuticals Ltd. (Cambridge, MA, USA). Lenalidomide was provided by Celgene (Summit, NJ, USA). Dexamethasone, melphalan, and doxorubicin were obtained from Sigma-Aldrich.

Drug sensitivity assays

The inhibitory effect of ITC was evaluated through a series of assays including: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St Louis, MO, USA) colorimetric survival assay;[8] viability assessment with CellTiterGlo assay (CTG; Promega); flow cytometry-based evaluation of MM cell proliferation, both in the presence and absence of BMSCs, by labeling of MM cells with carboxyfluorescein diacetate succinimidyl ester (CFSE), annexin V-FITC apoptosis assay for quantification of apoptosis; histone H3 phosphorylation, and flow cytometric analysis of DNA content of nuclei labeled with propidum iodide (PI) for evaluation of cell cycle changes. In addition, flow cytometric analysis of mitochondrial membrane potential of ITC-treated *versus* control MM cells was done using the JC-1 fluorescent probe. Detailed information on these assays is included in the *Online Supplementary Appendix.*

Molecular profiling analyses and functional assays

The molecular sequelae of ITC treatment of MM cells were evaluated by western blotting[8] and by multiplex evaluation of total and phosphorylated levels of proteins using the xMAP luminex platform (Luminex, Austin, USA). Multiplex luminescence assay for caspase-8/9 and fluorescent caspase-3/7 activity was performed with caspase-GloT™ 8 reagent or caspase-Glo™ 9 reagent and fluorescent caspase-3 substrate (Z-DEVD)2-R110 (Promega, Madison, WI, USA), according to the manufacturer's instructions, and as detailed in the *Online Supplementary Appendix*.

In vivo anti-tumor activity of isothiocyanates

Sublethally irradiated (150 rad) CB17/SCID mice were injected subcutaneously (24 h post-irradiation) with MM.1S cells ($2.5x10^6$/mouse) re-suspended in phosphate-buffered saline (PBS). Following engraftment, tumor-bearing mice were randomly assigned to three groups (eight mice each) receiving oral gavage every day for 5 days a week throughout the duration of the experiment with: group 1: control (0.2 mL PBS); group 2: SFN (100 mg/kg); and group 3: PEITC (60 mg/kg). Mice were monitored every 2-3 days for changes in tumor burden measured by calipers and for body weight and sacrificed, at the end of the study, in accordance with institutional guidelines. Mice were housed in the Animal Research Facility of the Dana-Farber Cancer Institute and experiments were performed in accordance with approved protocols.

Statistical analysis

The statistical significance of differences in drug-treated and control cultures was determined using Student's t test. Data are presented as mean ± standard deviation (SD). The minimal level of significance was $P<0.05$. Results from MTT viability assays are expressed as fraction of cells affected in drug-treated *versus* untreated cells. The IC_{50} of each ITC was evaluated using the CalcuSyn software (Biosoft, Ferguson, MO, USA). To evaluate whether the interaction between each ITC and other (novel or conventional) anti-MM agents was additive or synergistic, the CalcuSyn software (Biosoft, Ferguson, MO, USA) was used to perform isobologram analysis and calculate the combination index (CI), according to the Chou-Talalay method.[19] When the CI is 1, the effects are considered additive, whereas CI less than 1 indicates synergism and CI greater than 1 indicates antagonism. Tumor burden measurements in the *in vivo* studies were analyzed using the Mann-Whitney rank sum test overall survival was analyzed by the Kaplan-Meier survival analysis.

Results

Dose-dependent inhibition of survival of multiple myeloma cells by isothiocyanates

First, we evaluated the effect of ITCs on survival of MM cell lines. Cell viability was assessed by MTT assay for control cells (DMSO) and cells treated with SFN or PEITC (1.56-50 μM) at 24 and 48 h. SFN and PEITC significantly decreased survival of MM.1S (Figure 1A) and OPM1 cells (Figure 1B) in a concentration- and time-dependent manner. We extended our studies to a panel of other MM cell lines sensitive and resistant to conventional and novel anti-MM agents including RPMI-S, RPMI-Dox40, RPMI-MR20, RPMI-LR5, MM.1R, and OPM2 cells, and similarly observed concentration-dependent anti-MM effect (*Online Supplementary Figures 1A,B*). In most cell lines, IC_{50} values were 2-3 times lower for PEITC than SFN.

To determine the effect of ITC on primary MM cells, affinity-purified $CD138^+$ cells from bone marrow samples from eight MM patients were treated for 48 h with ITCs. As is shown in Fig. 1C and 1D, a dose-dependent response was observed (IC50 values in the range of 5-72 μM for SFN and 6-37 μM for PEITC). Similarly, to examine the effect of both ITCs on non-malignant cells, purified $CD138^+$ cells from bone marrow of healthy donors as well as peripheral mononuclear cells from healthy donor were treated with SFN (*Online Supplementary Figures 2A,C*) and PEITC (*Online Supplementary Figures 2B,D*) for 48 h. We also observed that the IC_{50} for the bone marrow stromal cells HS-5 was 262 μM for SFN and 105 μM for PEITC, while was non-transformed human hepatocyte cell line THLE-3 177 μM for SFN and 89 μM for PEITC (*Online Supplementary Figures 2E, F*). Therefore, neither of these cells showed decreased viability at the IC_{50} concentrations tested in MM cell lines. These results suggest that MM cell lines and primary MM tumor cells are more susceptible to the cytotoxic effect of both ITCs.

Isothiocyanates induce cell cycle arrest

Concentration- and time-dependent effects of ITC on cell proliferation were evaluated by measuring cell cycle profiles using flow cytometry. Both ITC induced G_2/M arrest of MM.1S cells at 12 h and 24 h (Figure 2A,B). Prolonged treatment (48 h) or higher concentrations of ITC increased the sub-G1 fraction. Accumulation of cells in G_2/M phase was detected in PEITC-treated OPM1 cells at a lower concentration (5 μM) than SFN-treated cells, while prolonged treatment increased the sub-G1 cell fraction at 24 and 48 h (*Online Supplementary Figures 3,B*). Both MM.1S and OPM1 cells responded to the highest concentration of PEITC with pronounced DNA fragmentation.

Expression patterns of cell cycle-related proteins were studied in MM.1S cells following 12 h treatment with SFN and PEITC (Figure 2C). Cyclin B1 is expressed in G_2 and M phases of cell cycle, and low concentrations of SFN slightly increased its expression (the relative intensity of cyclin B1 bands of SFN treatment compared to control were 1.3 and 1.6 for MM.1S and OPM1), correlating with an increase in G_2/M cell fraction. The decrease of cyclin B1, p-cdc2, p53, p21, and p27 in MM1.S cells treated with 10 μM PEITC reflects the pronounced early induction of cell death (Figure 2B). To determine whether the G_2/M arrest occurs in the G_2 or mitosis phase, we assessed activation of phosphorylated histone 3 p-H3 mitotic marker. The proportional increase of p-H3 levels was in accord with ITC-induced mitotic arrest (Figure 2C and 2D), while the expression of total H3 was not affected by treatment with ITCs (Figure 2C).Treatment of OPM1 cells with PEITC showed distinct results including increased p21 expression, late decrease in 14-3-3ε expression, and a concentration-dependent decrease in CDC25C levels (*Online Supplementary Figure 3C*). In addition, a more pronounced fragmentation of β-catenin was revealed in OPM1 cells than in MM1.S cells. These distinct results likely reflect the dissimilar cytogenic backgrounds of these cell lines. Overall, ITC modulated the cell cycle, induced mitotic arrest, and triggered MM cell death.

Dose-dependent induction of apoptosis in multiple myeloma cells by isothiocyanates

We further addressed whether reduced survival of MM cells triggered by ITC correlates with apoptosis. As shown in Figure 3A and *Online Supplementary Figure S4A*, significant time- and concentration-dependent increases of the total percentage of dead cells, i.e. early apoptotic cells (annexin V^+/PI^-) plus necrotic cells (annexin V^+/PI^+), was observed in both MM.1S and OPM1 cells treated with SFN. In addition, a significant increase of late apoptotic/necrotic populations in PEITC-treated MM.1S cell line was observed at 48 h (Figure 3B). As for MM.1S, treatment of OPM1 cells with PEITC significantly increased the percentage of early apoptotic cells, as well as the late apoptotic/necrotic population (*Online Supplementary Figure S4B*).

To define the mechanism of ITC-induced apoptosis in MM cells better, we used western blotting to examine the effect of SFN (5, 10, and 20 μM) and PEITC (2.5, 5, and 10 μM) treatment of MM cells after 24 h. As shown in Figure 3C and *Online Supplementary Figure S4C*, concentration-dependent apoptosis triggered by both SFN and PEITC was confirmed by cleavage of poly (ADP-ribose) polymerase (PARP), caspase-3 and (primarily with SFN for MM.1S) caspase-9. The activities of caspase-3 and caspase-9 were significantly increased in MM.1S cells treated with higher doses of SFN and PEITC, whereas caspase-8 activity was not increased at 12 h (*Online Supplementary Figure S5A,B*). Moreover, modest up-regulation of the pro-apoptotic protein apoptosis-inducing factor (AIF) was observed after treatment with both ITC in MM.1S and OPM1 cells.

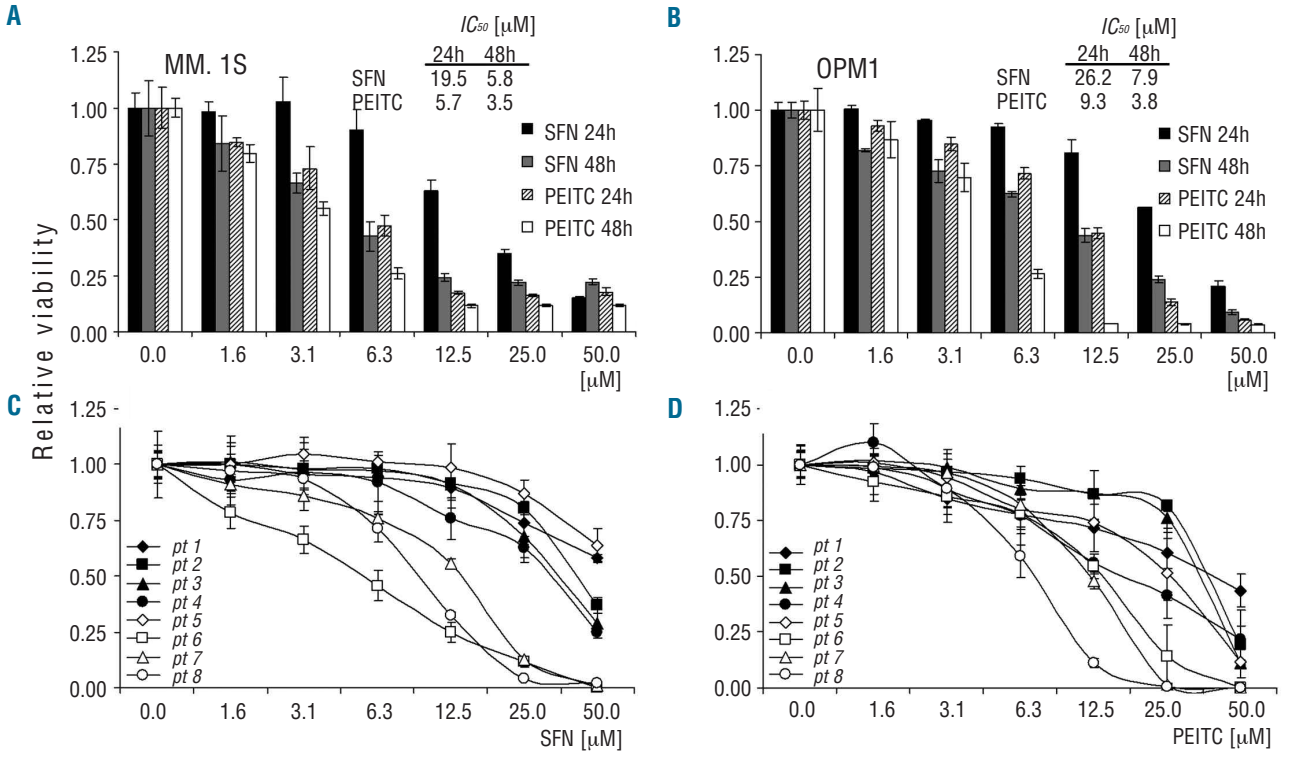

Figure 1. Cytotoxic effects of SFN and PEITC in MM cells. (A) MM.1S cells were treated with SFN (0-50 μM) and PEITC for 24 and 48 h in 96-well plates. Each treatment was performed in quadruplicate and assessed by MTT. (B) OPM1 cells were treated with SFN and PEITC for 24 and 48 h and cytotoxicity was assessed by MTT. Freshly isolated bone marrow CD138+ tumor cells from eight patients with MM were cultured with SFN (C) and PEITC (D) for 48 h. Cytotoxicity was analyzed using a CellTiterGlo assay and measured with a luminometer.

Conversely, down-regulation of the anti-apoptotic proteins Mcl-1, X-linked inhibitor of apoptosis (X-IAP), as well as other IAP members such as c-IAP-1 and survivin, was evident in PEITC-treated, but not in SFN-treated, MM.1S cells. SFN decreased Mcl-1, X-IAP, c-IAP-1, and survivin in OPM1 cells at 24 h (*Online Supplementary Figure S4C*), with similar but less pronounced changes in MM.1S cells (Figure 3C). Treatment with ITC also induced expression (primarily in MM.1S) of heat shock proteins Hsp-70 and Hsp-90 in a concentration-dependent manner; as well as decreased (primarily with PEITC) IRF-4, a member of the interferon regulatory factor family of transcriptional regulators involved in plasma cell differentiation.

To determine whether ITC modulated mitochondrial membrane potential in MM cells, the lipophilic cation JC-1 dye was used. The extent of ψ_m modulation triggered by treatment with SFN and PEITC (*Online Supplementary Figure S6*) was reflected by the elevated levels of JC-1 monomers and occurred in a time- and concentration-dependent manner. Overall, these results indicate that the cytotoxic activity of SFN and PEITC against MM cells is hallmarked by induction of apoptotic cell death, and modulation of mitochondrial transmembrane potential, and that PEITC is more potent than SFN.

Multiplex analysis of isothiocyanate-induced changes in diverse signaling pathways

Multiplex analysis of ITC-induced changes in the phosphorylation state of members of diverse signaling pathways showed that both ITC triggered activation of the MAPK pathway in MM.1S and OPM1 cell lines, with a greater effect being triggered by PEITC than SFN (Figure 4 and *Online Supplementary Figure S7*). Specifically, ITC treatment induced increased phosphorylation of JNK and c-Jun in MM.1S and OPM1 cells. We also observed time-dependent activation of MEK1 and p38 kinase in MM.1S cells, with early activation of MEK1 by SFN compared to a transient, more pronounced activation of p38 kinase by PEITC. Similarly, activation of ERK1/2 peaked later after SFN than after PEITC treatment. A sustained increase in phosphorylation of JNK and c-Jun (at 2, 6 and 12 h) and p38 (at 2 and 6 h) was observed in OPM1 cells after PEITC treatment. Although activation of JNK, p38 and ERK1/2 was observed after SFN treatment of OPM1 cells, MEK1 and ERK1/2 activation was not observed in PEITC-treated cells.

An early transient induction of phosphorylation of Akt and GSK3α/β was observed, followed by significant decreases in phosphorylation of Akt and GSK3α/β by SFN and PEITC in both MM cell lines (Figure 4 and *Online Supplementary Figure S7*). In contrast, there were no significant changes in phosphorylation status of NFκB p65, IRS-1, STAT3, STAT6 and Src in MM.1S cells treated with either ITC. Early activation of p70 S6 kinase and p90 RSK was found in MM.1S cells treated with either ITC. Interestingly, both ITC induced significant dephosphorylation of p53 in OPM1 cells, while increased p53 phosphorylation was observed in SFN-treated MM1.S cells at 24 h. Finally, phosphorylation of HSP27 was evident in PEITC-treated OPM1 cells.

Next we confirmed the observed changes in the multiplex analysis using western blotting in MM cells cultured for 2 h with SFN and PEITC (*Online Supplementary Figure*

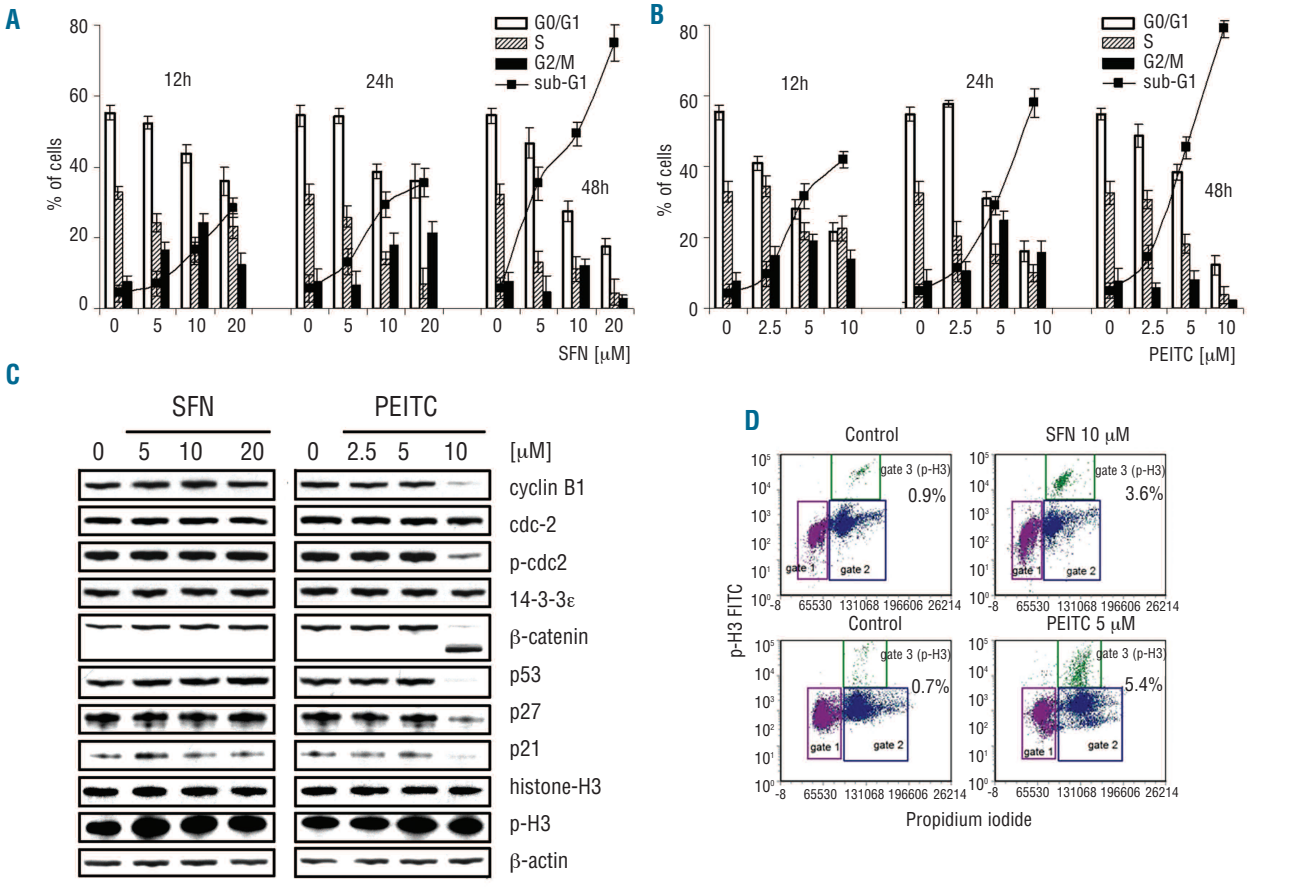

Figure 2. SFN and PEITC induce cell cycle arrest and increased the sub G_1 fraction in MM.1S cells. (A) MM.1S cells were cultured with SFN (5, 10 and 20 μM) for 12, 24 and 48 h. (B) MM.1S cells were treated with PEITC (2.5, 5 and 10 μM) for 12, 24 and 48 h. The distribution of cells in G_0/G_1, S and G_2/M phase and the percentage of the sub-G_1 fraction were obtained from analysis of side scatter (SSC) *versus* log FL3 dot plot using *de novo* FCS Express software. Three independent experiments were performed, and means ± SE are presented. (C) MM.1S cells were treated with SFN (5, 10 and 20 μM) and PEITC (2.5, 5 and 10 μM) for 12 h. Whole cell lysates (20 μg of proteins/lane) were immunoblotted using anti-cyclin B1, cdc-2, p-cdc-2, 14-3-3ε, β-catenin, p53, p27, p21, β-actin, histone H3, and p-H3 antibodies. (D) The dot plots show mitotic cells (gate 3, with percentage of total cells indicated in the upper right corners) detected by phosphorylated histone H3 in SFN (10 μM) and PEITC (5 μM) treated versus control MM.1S cells. Gate 1 contains cells in the the G_0/G_1 phase of the cell cycle; gate 2 contains cells in G_2/M phase and no fluorescence of phospho-H3; and gate 3 contains mitotic cells identified by tetraploid DNA content (G_2/M phase) and a high level of phosphorylated H3 (M phase).

S8). As for the multiplex analysis, the western blot analysis confirmed phosphorylation of JNK, increased phosphorylation of p70S6K, p90RSK, (primarily with PEITC in MM.1S) ERK1/2 and c-Jun in both cell lines treated with higher concentrations of ITC, and changes in Akt and GSK3α/β phosphorylation. However, the total Akt, ERK1/2, JNK and actin levels were not affected by SFN or PEITC treatment of MM cells, reflecting the results of multiplex analysis (*data not shown*). Overall, there is a high concordance between multiplex and western blot data.

Isothiocyanates inhibit proliferation of multiple myeloma cells cultured with stromal cells

We assessed whether MM cell sensitivity to ITC is attenuated by co-culture with bone marrow stromal cells. In order to quantify proliferation of MM cells cultured alone and co-cultured with HS-5 stromal cells, MM cells were stained with CFSE. Cell division was assessed by CFSE staining, while counterstaining with PI served to distinguish live from non-viable cells. As shown in *Online Supplementary Figure S9A*, viable stromal cells were $CFSE^-/PI^+$ and non-viable stromal cells were $CFSE^-/PI^+$ (left dot plot), while viable MM cells were $CFSE^+/PI^-$ and dead MM cells were $CFSE^+/PI^+$ (right dot plot). The dot plot of *Online Supplementary Figure S9B* shows unlabeled-HS-5 stromal cells cultured with CFSE-labeled MM cells and the adjacent histogram represents the fluorescent intensity of viable CFSE-labeled MM cells. There is an inverse correlation between CFSE-staining intensity and number of cell divisions (proliferation) of cells (the increase of proliferation correlates with a decrease of fluorescent intensity of CFSE). Both MTT assay and flow cytometry analysis confirmed that the viability of HS-5 cells was not affected by the concentrations of ITC used (*data not shown*). CFSE-labeled MM cells were cultured with increasing concentrations of SFN or PEITC in the presence or absence of HS-5 bone marrow stromal cells for 12, 24 and 48 h. Cell proliferation of both cell lines at 12 and 24 h was significantly inhibited by SFN and PEITC treatment in dose- and time-dependent manners (Figure 5A,B and *Online Supplementary Figure S10A*). Treatment of OPM1 cells with PEITC inhibited proliferation less than SFN treatment (*Online*

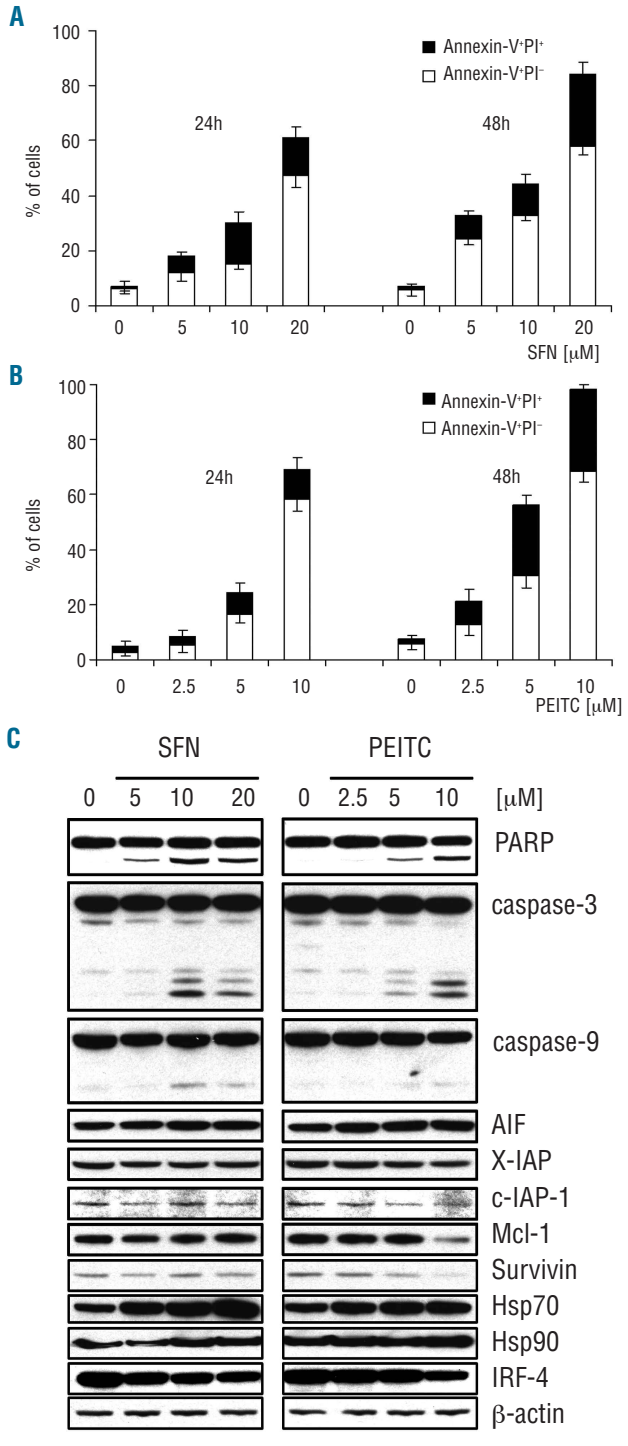

Figure 3. SFN and PEITC induce apoptosis in MM.1S cells. (A) Effect of SFN on induction of apoptosis and necrosis was quantified by flow cytometry after staining with annexin V-FITC and PI. MM.1S cells were treated with SFN (5, 10 and 20 μM) for 24 and 48 h or DMSO. (B) MM.1S cells were treated with either DMSO (control cells) or 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. Data are from two independent experiments and presented as means ± SE. (C) MM.1S cells were cultured with SFN (5, 10 and 20 μM) and PEITC (2.5, 5 and 10 μM) for 24 h. Whole cell lysates were subjected to western blot analyses using anti-PARP, -caspase-3, -caspase-9, -AIF, -X-IAP, -c-IAP-1, -Mcl-1, -Hsp-70, -Hsp-90, -survivin, and -IRF4. β-actin served as a loading control. Results are representative of two independent experiments.

Supplementary Figure S10B). The fraction of MM cells killed by ITC treatment (Fa – ratio of number of non-viable MM cells/number of all MM cells) was increased in a concentration-dependent manner. Interestingly, at several different doses and durations of SFN exposure, the fractions of non-viable MM-1S and OPM1 cells were higher in the presence of HS-5 stromal cells than in their absence (*Online Supplementary Figure S11A,C*). Furthermore, PEITC significantly increased the fraction of non-viable (CFSE$^+$/PI$^+$) MM.1S and OPM1 cells both alone and in the presence of stromal cells (*Online Supplementary Figure S11B,D*).

In vivo anti-myeloma activity of isothiocyanates

The *in vivo* anti-tumor activity of SFN and PEITC was examined in a xenograft myeloma model established after s.c. injection of CB17/SCID mice with MM.1S cells. Tumor-bearing mice were treated with SFN, PEITC or with the respective vehicle (Figure 6). SFN and PEITC treatment caused no changes in weight compared to the weight of the vehicle-treated control mice (*data not shown*). SFN treatment significantly decreased tumor volumes compared to those in control mice. Moreover, treatment with PEITC decreased volumes to a greater extent than treatment by SFN (Figure 6A). The differences in tumor volumes between the vehicle and the SFN or PEITC treatment cohorts were statistically significant from day 9 of treatment (P<0.001 for all comparisons, Mann-Whitney rank sum test) until the end of the experiment. Moreover, Kaplan-Meier survival analyses showed that both SFN and PEITC administration was associated with statistically significant prolongation of overall survival in comparison with vehicle-treated control group, with the prolongation being more pronounced with PEITC administration (P<0.001 for all comparisons, log-rank test; Figure 6B). These results suggest that SFN delayed tumor growth and, importantly, that PEITC administration significantly decreased tumor burden during the whole treatment period.

Isothiocyanates enhance cytotoxicity of conventional and novel anti-multiple myeloma therapies

Combinations of novel and/or conventional anti-MM agents can achieve higher clinical response rates than single agents. We, therefore, next evaluated the response of MM.1S cells to treatment for 48 h with combinations of ITC with novel anti-MM agents (bortezomib, and lenalidomide) and conventional drugs (dexamethasone, doxorubicin and melphalan). The anti-MM activity of combined treatment was analyzed by MTT assays, and the presence of synergistic effects was evaluated using CalcuSyn software. Fa-CI plots and normalized isobolograms at different combination ratios were generated for the respective pairs of anti-MM drugs plus ITC (*data not shown*); the fractions affected and the combination indices for each of the combinations are summarized in Table 1. These analyses indicate that SFN had a synergistic effect when combined with bortezomib and showed slight/moderate synergism (defined according to criteria detailed by Chou-Talalay)[19] with lenalidomide. When PEITC was combined with bortezomib or lenalidomide, a synergistic effect was observed. All doses of SFN had synergistic effects when combined with conventional drugs, including dexamethasone, doxorubicin and melphalan. PEITC combinations with melphalan also had synergistic effects. Combinations of low doses of PEITC with dexamethasone

or doxorubicin had only moderate or slight antagonistic effects, while higher doses of PEITC had synergistic effects with dexamethasone or doxorubicin. The strong synergistic effect of SFN combined with bortezomib, as well as SFN or PEITC combined with melphalan, suggests a potential of these combinations for future clinical studies.

Discussion

ITC have anti-tumor activity in preclinical models of prostate, breast, stomach, and colon cancers.[7,10,20,21] In this study, we show that SFN and PEITC have significant dose- and time-dependent activity against various MM cell lines and primary MM cells. ITC were active against MM cell lines that are sensitive, as well as others that are resistant, to established anti-MM therapies. PEITC was more potent than SFN against all MM cell lines tested. In addition, ITC had IC_{50} against primary MM cells in the range of 6-37 μM (PEITC) and 5-72 μM (SFN). In our studies, both SFN and PEITC induced significant anti-tumor activity *in vivo* associated with significant decreased tumor burden and statistically significant prolongation of overall survival. PEITC administration was more efficacious than SFN. Despite the greater efficacy of PEITC observed in MM cell lines as well as in the animal model a slightly higher proportion of $CD138^+$ cells from MM patients was more sensitive to SFN treatment *in vitro*.

Plasma concentrations of SFN and its metabolites can reach 2.2 and 7.3 μmol/L after consumption of 100 g of standard and high-glucosinolate broccoli, respectively.[22] However, higher concentrations are achieved in tissues and it has been reported that the SFN concentration in the small intestine reached 3 and 13 nmol/g of tissue, which is equivalent to roughly 3-30 μM of total SFN.[23] The plasma levels of ITC reported so far in the literature (in the μM range, but often <10 μM) were achieved after administration of broccoli preparations (not a pure ITC preparation), containing doses of ITC substantially lower than the maximum tolerated ones.

Stromal cells in the bone marrow microenvironment interact with malignant plasma cells via cytokine production and/or cell-cell contact. This interaction stimulates proliferation and survival of MM cells and facilitates disease progression by attenuating the response of MM cells to conventional drugs.[1] We, therefore, evaluated whether ITC retain their tumor activity in the context of bone marrow stromal cells. We used a fluorescent cytoplasmic dye, CFSE, equally distributed between daughter cells after each cell division, to distinguish between CFSE-labeled MM cells and unlabeled stromal cells. Both ITC inhibited proliferation of MM cells, both alone and in the presence of the stromal cells.

In addition to the cytotoxic effect of ITC as single agents, synergy with established anti-MM agents was also observed. Our study revealed that SFN exhibited a synergistic cytotoxicity in combination with conventional anti-MM agents, including dexamethasone, doxorubicin, and melphalan. These results are compatible with prior observations that SFN enhanced doxorubicin-induced apoptosis, independently of p53 status;[24] and that the synthetic ITC ethyl 4-isothiocyanatobutanoate increases platinum accumulation in cisplatin-treated ovarian cancer cells, leading to depletion of glutathione and augmenting apoptotic induction.[25] PEITC combinations with conventional anti-MM drugs (dexamethasone, doxorubicin and melphalan) were generally synergistic, except for moderate or slight antagonistic effects of low-dose of PEITC when combined with dexamethasone or doxorubicin. Our study further shows that ITC are also synergistic with novel anti-MM agents such as bortezomib (in combination with SFN and PEITC) and lenalidomide (in combination with mainly PEITC). Both lenalidomide[26] and PEITC[27] have anti-angiogenic properties, but the molecular mechanisms of their *in vitro* synergy against MM cells is unclear. These studies provide the framework for clinical trials of combination ITC thera-

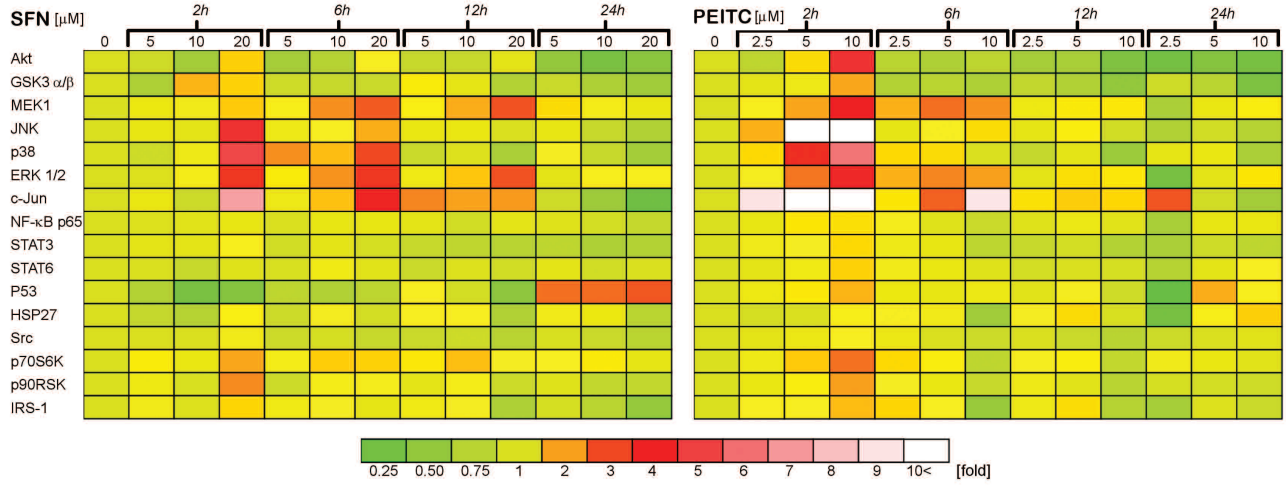

Figure 4. SFN and PEITC induced changes in phosphorylation of members from different signaling pathways in MM.1S cells. MM.1S cells were exposed to DMSO (control), SFN (5, 10 and 20 μM), or PEITC (2.5, 5 and 10 μM) for 2, 6, 12 and 24 h. Protein concentrations of whole cell lysates were measured using 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; fold change is depicted in a color-coded format, according to the scale included below.

pies in MM.

Our results show that the anti-MM effect of ITC involves induction of both apoptosis and cell cycle arrest. ITC-induced apoptosis in MM cells was detected by annexin V staining and DNA fragmentation, and further confirmed by proteolytic cleavage of PARP, up-regulation of the pro-apoptotic protein AIF, as well as down-regulation of the anti-apoptotic proteins Mcl-1, X-IAP, c-IAP and survivin. These events were associated with time- and concentration-dependent depolarization of mitochondrial membrane potential, suggesting that mitochondria are involved in ITC-induced cell death. This notion is further supported by our data that ITC induced concentration-dependent activation of caspase-3 and -9, while activation of caspase-8 was not detected.

In MM cells, the cell cycle arrest induced by ITC primarily involves G_2/M arrest, consistent with reports in other types of tumors.[28] This is associated with decreased expression of key G_2/M-regulating proteins including cyclin B1, p-cdc2, Cdc25C, as well as p53, p27, 14-3-3ε. Observed fragmentation of β-catenin by ITC might be associated with activity of caspase-3, which was shown previously.[29] The increase in cells positive for p-H3 histone with tetraploid DNA content further indicates that ITC can block cells in early mitosis, consistent with previous studies on ITC causing induction of aberrant mitotic spindles and disruption of mitotic microtubule polymerization *in vivo*.[7] Increased p21cip/waf1 was observed in PEITC-treated OPM1 cells, although to a lesser extent than in previous studies.[30]

How ITC modulate intracellular signaling molecules leading to apoptosis is not fully understood. To date multiple molecular targets of ITC have been proposed, such as inhibition of HDAC, phosphorylation of E4-BP1, covalent modification of tubulin, decreased expression of COX-2 and its downstream target Bcl-2 protein, and activation of heterogeneous nuclear ribonucleoprotein K (hnRNP K) and its target c-myc.[15,17,31,32] Recently, TGFβ1, EGF and insulin peptides, as well as serotonin receptors were described as novel targets of ITC.[14,33] Mechanistically, we here confirmed activation of MAPK pathways in MM triggered by ITC, as in other cell types,[9] including downstream targets ERK1/2, JNK, and Akt proteins. Recently, inhibition of cap-dependent translation through up-regulation of 4E-BP1 expression and inhibition of its phosphorylation were described as an important mechanism of PEITC-induced apoptosis.[31] Because this cap-dependent translation machinery is functionally controlled by the mTORC1 complex,[34] downstream target p70S6K was included in our phosphorylation panel. In addition to ribosomal protein S6 phosphorylation, p70S6K can phosphorylate IRS1 and impair the activation of PI3K and Akt by insulin.[35] Our multiplex phosphorylation profile showed that activation of this pathway is a very early response in both SFN- and PEITC-treated MM1.S cells. Activation of p90RSK, a downstream effector of ERK, which inhibits Bad-mediated apoptosis, was also observed.[36] The increased phosphory-

Table 1. SFN and PEITC enhance the effect of anti-MM agents. MM.1S cells were cultured for 48 h with DMSO (control), SFN (5 and 10 μM), or PEITC (2.5 and 5 μM) in combination with bortezomib (velcade), lenalidomide (CC-5013), dexamethasone (DEX), doxorubicin (DOX) and melphalan (MEL). Fractions-affected (Fa – ratio of number of non-viable MM cells/total number of MM cells) and combination indices (CI) of ITCs with bortezomib, CC5013, dexamethasone, doxorubicin and melphalan were calculated according to the Chou-Talalay method. Fractions-affected (Fa) for each single agents were as follows: bortezomib 2 nM: 0.16; lenalidomide 50 μM: 0.34; dexamethasone 12.5 nM: 0.17; doxorubicin 25 nM: 0.25; and melphalan 5 μM: 0.43). Fa values for SFN 5 and 10 μM were 0.22 and 0.57, respectively. For PEITC 2.5 and 5 μM, the Fa values were 0.27 and 0.50, respectively. All experiments were performed in triplicate.

	SFN	Fa	CI	PEITC	Fa	CI
Bortezomib [2 nM]	5	0.54	0.55	2.5	0.59	0.40
	10	0.94	0.30	5	0.81	0.37
Lenalidomide 50 [μM]	5	0.46	0.87	2.5	0.50	0.61
	10	0.65	0.81	5	0.65	0.70
Dexamethasone 12.5 [nM]	5	0.49	0.58	2.5	0.24	1.23
	10	0.84	0.54	5	0.62	0.71
Doxorubicin 25 [nM]	5	0.40	1.00	2.5	0.42	1.16
	10	0.85	0.70	5	0.78	0.56
Melphalan 5 [μM]	5	0.93	0.19	2.5	0.67	0.49
	10	0.98	0.20	5	0.95	0.13

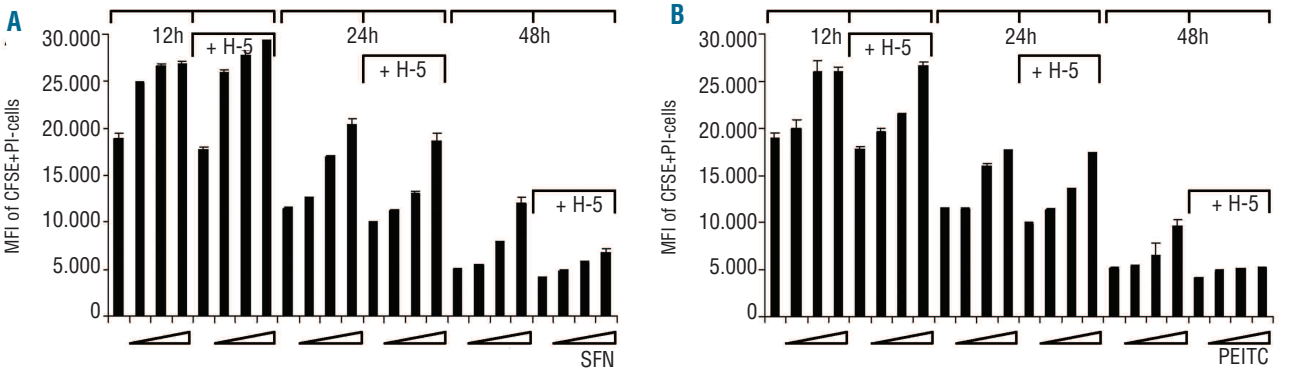

Figure 5. SFN and PEITC inhibit proliferation of MM.1S cells alone and in co-culture with HS-5 stromal cells. (A) CFSE-labeled MM.1S cells were cultured with SFN (5, 10 and 20 μM) for 12, 24 and 48 h in the presence or absence of HS-5 stromal cells. Fluorescence intensity of gated $CFSE^+PI^-$ -stained MM.1S is shown as a function of SFN concentration (5, 10 and 20 μM). (B) CFSE-labeled MM.1S cells were treated with PEITC (2.5, 5 and 10 μM) for 12, 24 and 48 h in the presence or absence of stromal HS-5 cells. Fluorescence intensity of $CFSE^+PI^-$-labeled MM.1S is shown as a function of the PEITC concentration (2.5, 5 and 10 μM). Data presented are from two independent experiments and are means ± SE.

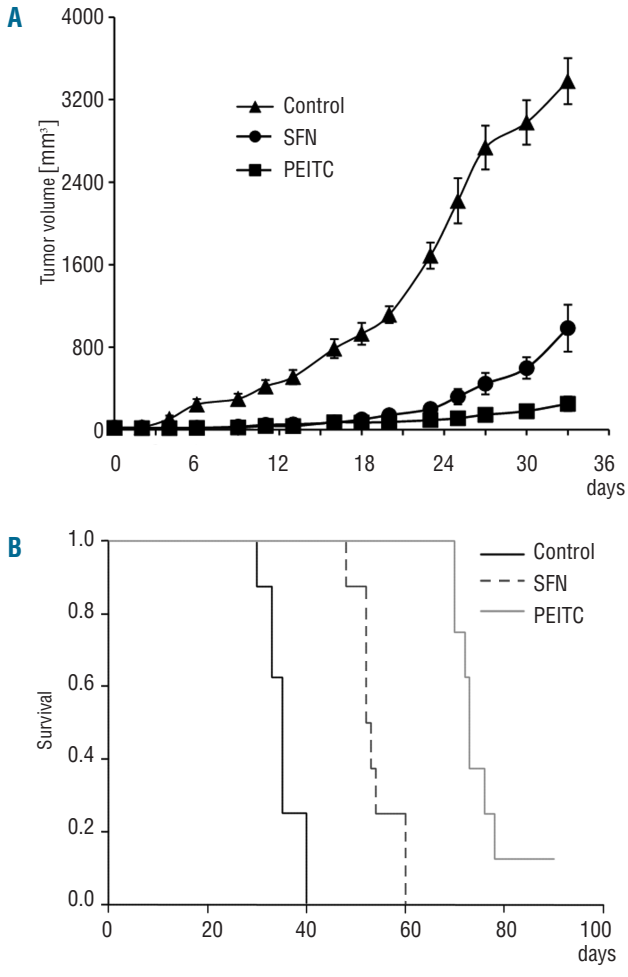

Figure 6. *In vivo* effect of SFN and PEITC in a xenograft model of human multiple myeloma. MM.1S myeloma cells ($2.5x10^6$ cells in 200 μL phosphate-buffered saline (PBS) were injected subcutaneously into the CB17/SCID mice model. Treatment was started when tumors became palpable. Tumor-bearing mice were randomly allocated into three groups (eight mice per group): animals received phosphate-buffered saline only (controls), SFN (100 mg/kg), or PEITC (60 mg/kg) orally by gavage every day for 5 days a week throughout the duration of the experiment. (A) Calliper measurements of tumor diameters were done every 2-3 days, and tumor volumes were calculated according to the formula of the volume of an ellipse: $V=4/3\pi \times (a/2) \times (b/2)^2$, where a and b correspond to the longest and shortest diameters of the tumor, respectively. Both SFN and PEITC significantly inhibited tumor growth ($P=<0.001$; Mann-Whitney rank sum test) Data are presented as the mean of data from eight animals ± SEM (n=8) (*$P<0.001$ compared with control and treatment). (B) Kaplan-Meier analysis of overall survival confirmed than both ITCs, (PEITC more that SFN) increased survival ($P<0.001$) compared with control mice.

lation of kinases suggests, at least in part, that phosphateses are potential targets of ITC. It has been previously reported that PEITC may induce JNK-mediated apoptotic signaling via suppression of the JNK specific M3/6 phosphatase activity in prostate cancer cells.[37] Recently it was also found that 14-3-3ε binds the evolutionarily conserved molecule Chibby when phosphorylated by Akt kinase, thereby resulting in sequestration of Chibby in the cytoplasm. Chibby and 14-3-3ε form a stable complex with β-catenin, thereby antagonizing β-catenin signaling.[38] The decrease in Akt activity in MM cells triggered by ITC may increase the amount of uncomplexed β-catenin that can activate early endoplasmic reticulum stress through eIF2a, CHOP, thereby leading to growth inhibition and triggering c-Jun dependent induction of p73 and MM cell apoptosis.[39]

Our observation that SFN and PEITC have *in vivo* anti-MM activity in a xenograft myeloma model indicate that pharmacologically achievable levels of ITC can affect the biological behavior of MM cells, thus supporting the relevance of our *in vitro* observations. For compounds with chemopreventive potential in healthy individuals, it is important that administration in cancer patients will not have harmful effects, e.g. antagonistic effect on activity of established anti-cancer therapies that these patients may receive. For example, the flavonoid quercetin inhibits bortezomib-induced apoptosis.[40] In contrast to quercetin, we have observed that chemopreventive ITC potentiate the anti-MM effects of bortezomib, and other conventional and novel anti-MM agents. These data provide the framework for future studies of ITC in combination with currently available anti-MM agents: the observed synergy may allow for ITC to be combined with lower doses of other anti-MM agents, thus decreasing the rates and severity of side effects while maintaining potent anti-MM activity. Importantly, our study raises the intriguing possibility that dietary supplementation with ITC during the treatment of MM patients could potentially improve therapeutic responses. In conclusion, our study indicates that ITC have potent anti-MM activities and may enhance the activity of other anti-MM agents, providing the preclinical framework for future clinical studies of ITC in MM.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

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