Isothiocyanates inhibit proteasome activity and proliferation of multiple myeloma cells

Lixin Mi*, Nanqin Gan and Fung-Lung Chung

Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University, Washington, DC WA 20057, USA

*To whom correspondence should be addressed. Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University, 3800 Reservoir Road, LL 129, Box 571465, Washington, DC WA 20057, USA
Tel: +1 202 687 3648; Fax: +1 202 687 1068;
Email: lm293@georgetown.edu
Correspondence may also be addressed to Fung-Lung Chung.
Tel: +1 202 687 3021; Fax: +1 202 687 1068;
Email: flc6@georgetown.edu

Isothiocyanates (ITCs), including benzyl isothiocyanate (BITC), phenethyl isothiocyanate (PEITC) and sulforaphane, compounds found in cruciferous vegetable, are highly effective in inducing cell cycle arrest and apoptosis in a variety of cancer cells and animal models. Although some studies indicate that ITC-induced reactive oxygen species (ROS) generation may underlie apoptosis induction, our recent studies show that covalent binding to target proteins may be an important event triggering apoptosis. In this study, we report that BITC and PEITC significantly inhibit proteasome activity in a variety of cell types. Further studies show that ITCs inhibit both the 26S and 20S proteasomes, presumably through direct binding, and that this inhibition is unrelated to either ROS generation or ITC-induced protein aggregation. The potency of ITC-induced proteasome inhibition correlates with the rapid accumulation of p53 (tumor suppressor) and IκB nuclear factor-kappaB (nuclear factor-kappaB inhibitor). Finally, our results demonstrate that BITC and PEITC, the two strongest proteasome inhibitors, significantly suppress growth of multiple myeloma (MM) cells through induction of cell cycle arrest at G2/M phase and apoptosis. This study suggests that proteasome, like tubulin, is a potential molecular target of ITCs, thus providing a novel mechanism by which ITCs strongly inhibit growth of MM cells and new leads in identifying compounds with therapeutic and preventive efficacies for MM. It also supports the future studies of ITCs as therapeutic and preventive agents for MM.

Introduction

Multiple myeloma (MM), a neoplastic proliferation of plasma cells, is currently the second most prevalent blood cancer (10% of all blood cancers), after non-Hodgkin’s lymphoma, in the USA. The incidence of myeloma is 9.5 cases per 100 000 African-Americans and 4.1 cases per 100 000 Caucasian-Americans (1). Among African-Americans, myeloma is one of the top 10 leading causes of cancer death. Almost all patients with MM who survive initial treatment will eventually relapse and require further therapy (2). Since the Food and Drug Administration’s approval in 2003, Bortezomib, a proteasome inhibitor, has become a standard treatment of patients with relapsed and resistant MM (2,3). However, its clinical application is still plagued by relapse, drug resistance and adverse side effects (4). Compounds like tubulin, is a potential molecular target of ITCs, thus providing a novel mechanism by which ITCs strongly inhibit growth of MM cells and new leads in identifying compounds with therapeutic and preventive efficacies for MM. It also supports the future studies of ITCs as therapeutic and preventive agents for MM.

Abbreviations: BITC, benzyl isothiocyanate; GSH, glutathione; ITC, isothiocyanate; MM, multiple myeloma; NF-κB, nuclear factor-kappaB; NMPEA, N-methyl phenethylamine; PEITC, phenethyl isothiocyanate; ROS, reactive oxygen species; SFN, sulforaphane; UPS, ubiquitin–proteasome system.

Materials and methods

Cells and chemicals

The human MM cell lines U266 and RPMI-8226 (American Type Culture Collection Manassas, VA) were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) at 37°C in 5% CO2. HeLa and A549 cells (American Type Culture Collection) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Invitrogen) at 37°C in 5% CO2. Bortezomib and MG132 were purchased from LC Labs (Woburn, MA) and Biomol (Plymouth Meeting, PA), respectively. SFN was provided by Dr Stephen Hecht (University of Minnesota). PEITC, BITC, N-methyl phenethylamine (NMPEA), dimethyl sulfoxide, cycloheximide and all other reagents were the highest grade available from Sigma–Aldrich (St Louis, MO), unless otherwise noted.

Assay for proteasome activity

The assay was based on a previously published method (17). After treatment, cells were harvested and washed with proteasome lysis buffer (50 mM Tris–HCl, pH 7.8, 20 mM KCl, 5 mM MgCl2, 1 mM dithiothreitol, 1 mM ATP, 10% glycerol and 0.04% NP-40) by repeated pipetting, followed by incubation on ice for 20 min. Cell lysate with 30 μg proteins was incubated with 20 μM fluorogenic peptide substrates in 100 μl volume of 60 min at 37°C in the dark. Cold phosphate-buffered saline was used to dilute samples to 10 μl before fluorescence was measured by a Synergy 96-well plate reader (Bio-Tek, Winooski, VT) with an excitation filter of 365 ± 20 nm and an emission filter of 485 ± 25 nm.

Purified 20S proteasome extracted from rabbit erythrocytes (Boston Biochem, Cambridge, MA) was further purified by size exclusion column
was used. Briefly, cells were plated in 96-well plates at a density of 10^4 cells
Aqueous One Solution cell proliferation assay kit (Promega, Madison, WI)
The assay was based on a previously published method (14). Treated cells were
fixed with ice-cold 80% ethanol, treated with 500
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UV-1700 spectrophotometer. Cells treated with solvent dimethyl sulfoxide
Measurement of reactive oxidative species production
The assay was based on a previously published method (13). After treatment with
0.5% NP-40, on ice for 20 min before centrifugation at 15 000g for 10 min. The supernatants were collected as
peptide and incubating the gels at 37°C for 2 h at room temperature in the dark.
Native gel electrophoresis and substrate overlay assay
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SDS-PAGE. The results of substrate overlay assay (Figure 2A)
with propidium iodide
PC-3 (prostate cancer) and MCF-7 (breast cancer), with 10 μM BITC for 4 h. Results (Figure 1D) show that the chymotrypsin-like activity in
mean fluorescent intensity. We incubated PEITC, SFN and NMPEA individually
was used to estimate statistical significance.

Results

BITC and PEITC are effective proteasome inhibitors in a variety of cancer cells
To study whether ITCs (structures are shown in Figure 1A) inhibit UPS, we treated U266 cells with a series of concentrations of BITC,
PEITC and SFN for 4 h. Proteasome activities, including chymotrypsin-like, trypsin-like and caspase-like, were measured using fluorescent-
labeled peptides. The results (Figure 1B) show that both BITC and PEITC significantly inhibited all three proteasome activities in a
concentration-dependent fashion. SFN, however, was unable to inhibit proteasome activities at concentrations up to 30 μM. Instead, it
promoted both chymotrypsin-like and caspase-like activities at lower concentrations, consistent with a previous study that SFN enhances
proteasome activity in murine neuroblastoma cells (17). MG132, a known proteasome inhibitor, was more effective in inhibiting
chymotrypsin-like and caspase-like, but not trypsin-like, activities compared with BITC and PEITC. The inhibition was rapid—observed as
early as 30 min after PEITC treatment (Figure 1C). It was time
dependent within the first 4 h and the effects lasted up to 24 h.
NMPEA, a structural analog of PEITC without ITC functionality, did not inhibit proteasome activity under the same conditions, suggesting
that the ITC functional group is essential for inhibiting proteasome activity. The potency order of BITC > PEITC > SFN suggested that the side chain moiety dictates the inhibitory potency.

To study whether ITC-induced proteasome inhibition is cell type specific, we treated a variety of cell types, including HeLa (cervical cancer),
A549 (non-small cell lung cancer), HT-29 (colon cancer), PC-3 (prostate cancer) and MCF-7 (breast cancer), with 10 μM BITC for 4 h. Results (Figure 1D) show that the chymotrypsin-like activity in
all five cell lines was significantly (~50%) inhibited, indicating that ITC-induced proteasome inhibition is not cell type specific.

BITC inhibits both 20S and 26S proteasomes
Next, we studied the mechanisms of ITC-induced proteasome inhibition. The 26S and 20S proteasome complexes are responsible for
ATP-dependent and -independent protein degradation, respectively (5,6). The 26S complex is composed of one or two 19S regulatory
peptidylated p38 (Cell Signaling, Danvers, MA) and enhanced chemiluminescence-based detection (GE Healthcare).

Native gel electrophoresis and substrate overlay assay
The assay was based on a previously published method (18). Samples with 50
ng proteins were analyzed on mini gels using a Mini-Protein gel apparatus (Bio-Rad, Hercules, CA). Non-denaturing gels consisted of 2.5% stacking and
intracellular ROS generation was measured using FACScalibur flow cytometer (BD Biosciences).

Measurement of reactive oxidative species production
The assay was based on a previously published method (19). Briefly, HeLa cells with 1 and 10
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of the 3-(4,5-dimethylthia-

Aqueous One Solution cell proliferation assay kit (Promega, Madison, WI) was used. Briefly, cells were plated in 96-well plates at a density of 10^4 cells
for 2 h at room temperature in the dark.

Cell lysate preparation and western blotting
The assay was based on a previously published method (14). Treated cells were
iced cold. After electrophoresis, protease activity was detected in non-de-
agarose gels by overlaying the gels with 20 mM Tris–HCl, pH 7.8, 5 mM
MgCl2, 10 mM KC1, 1 mM dithiothreitol, 2 mM ATP and 200 μM fluorescent
peptide and incubating the gels at 37°C for 30-60 min. The fluorescent gels were
transilluminated by a ultraviolet light and photographed. The density of the
fluorescent bands was determined by ImageJ software.

Native gel electrophoresis and substrate overlay assay
The assay was based on a previously published method (18). Samples with 50
ng proteins were analyzed on mini gels using a Mini-Protein gel apparatus (Bio-Rad, Hercules, CA). Non-denaturing gels consisted of 2.5% stacking and
4.5% resolving gels cast in 90 mM Tris, pH 8.3, 1.6 mM borate and 0.08 mM
ethylenediaminetetraacetic acid. Samples were electrophoresed for 280 Vh in
an ice cold room. After electrophoresis, protease activity was detected in non-de-

Cell cycle analysis
The assay was based on a previously published method (14). After treatment with
a variety of agents, cells were further stained with 10
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of propidium iodide

Cell proliferation assay
The assay was based on a previously published method (14). CellTiter 96 Aqueous One Solution cell proliferation assay kit (Promega, Madison, WI) was used. Briefly, cells were plated in 96-well plates at a density of 10^4 cells per well (200 μl). Twenty-four hours after plating, cells were treated with
different doses of each ITC for 24 h. Then, 20
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ATP-dependent and -independent protein degradation, respectively (5,6). The 26S complex is composed of one or two 19S regulatory
particles and a 20S catalytic particle. To study which complexes were affected by ITC treatment, we treated HeLa cells with 20 μM BITC and
purified both 26S and 20S from cell lysates using native gel electrophoresis (18). The results of substrate overlay assay (Figure 2A)
show that chymotrypsin-like activity in both the 26S and 20S complexes was significantly inhibited by BITC treatment. The inhibition occurred as rapid as 30 min. The inhibition of the 26S complex, indi-
cicated by the densitometry, was more substantial than that of 20S, suggesting that the functions of both the 19S regulatory particle and 20S catalytic particle were affected by ITC treatment.

ITCs inhibit proteasome activity through direct binding
To further study the interaction between ITCs and proteasome activity, we incubated PEITC, SFN and NMPEA individually with purified
20S proteasome at room temperature for 4 h. The results (Figure 2B) show that similar to the cultured cell data, PEITC significantly in-
hibited all three activities of purified proteasome, suggesting that ITC-induced proteasome inhibition is through direct binding. Further-
more, both SFN and NMPEA had little effect on proteasome activity, suggesting that both the ITC moiety and side-chain structure play
important roles in ITC activity.

ITCs inhibit proteasome inhibition is unrelated to protein aggregation
Previously, we found that BITC- and PEITC -induced tubulin-con-
taining aggregates-like protein aggregates that are insoluble in buffers that are often used to extract whole cell lysate (15,16). These buffers
usually contain 1% non-ionic detergents, such as NP-40, Triton X-100 or Tween 20. We also showed that 10 μM of colchicine, vinblastine or
taxol, three tubulin binding agents, can almost completely block the
binding of ITCs to tubulin and the consequential formation of the
protein aggregates (16). Among these three agents, colchicine and vinblastine effectively disrupt microtubules, whereas taxol promotes microtubule formation. To study if ITC-induced tubulin-containing
protein aggregates contribute to proteasome inhibition, we treated HeLa cells with 1 and 10 μM colchicine, vinblastine or taxol for

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1 h followed by treatment with 10 μM BITC for 4 h. The results (Figure 3A–C) show that 10 μM colchicine and vinblastine, but not taxol, alone caused moderate proteasome inhibition (<15%). However, pretreatments with these agents had little effects on proteasome inhibition by BITC, suggesting that BITC-induced proteasome inhibition may be unrelated to ITC-induced protein aggregation, supporting again that ITC direct binding may be the underlying mechanism of proteasome inhibition.

**ITC-induced proteasome inhibition is unrelated to ROS generation**

ITCs are known to induce ROS generation at least partially due to conjugation and depletion of intracellular glutathione (GSH) (19,20). Therefore, an important aspect of this mechanistic study is to understand the role of ROS in ITC-induced proteasome inhibition. To investigate this, we treated U266 cells with either 10 μM PEITC or up to 1 mM H₂O₂ for 4 h. Results (Figure 3D) show that up to 250 μM H₂O₂ did not significantly induce proteasome inhibition. H₂O₂ at 1 mM was needed to achieve comparable levels of proteasome inhibition to that of 10 μM PEITC. A separate experiment indicated that the ROS level in U266 cells treated with 10 μM PEITC was much less than that of treatment with 100 μM H₂O₂ at all three time points of 1, 4 and 24 h (data not shown). Next, we treated U266 cells with 10 μM BITC in the presence and absence of cell membrane-permeable polyethylene glycol-linked catalase, which is effective in quenching intracellular ROS (21). The results (Figure 3E) show that polyethylene glycol catalase, at both low and high doses, did not affect BITC-induced proteasome inhibition, confirming that ITC-induced ROS do not play a significant role in proteasome inhibition. Additionally, we treated U266 cells with up to 1 mM 3-amino-1,2,4-triazole (22), a specific catalase inhibitor, followed by treatment with 10 μM BITC. Results (Figure 3F) show that 3-amino-1,2,4-triazole alone failed to induce substantial proteasome inhibition and 3-amino-1,2,4-triazole did not aggravate proteasome inhibition by BITC. Lastly, we measured cellular GSH concentration levels after cells were treated with ITCs and NMPEA for 1 h. The results (Figure 3G) show that both concentrations of 10 and 20 μM BITC and SFN depleted GSH at a slightly higher rate than PEITC and BITC. The results agree with a previous study in which BITC and SFN at several concentration levels depleted GSH at similar rates (19). In an earlier study (13), we demonstrated that SFN conjugates faster to GSH than PEITC. The inconsistency between the potency of ITC depleting GSH and that of proteasome inhibition suggests that proteasome inhibition by ITCs is not linked to GSH depletion. Taken together, these data suggest that ITC-induced proteasome inhibition is unrelated to ITC-induced ROS generation.

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**Fig. 1.** ITCs significantly inhibit proteasome activity in a variety of cells. (A) Structures of BITC, PEITC, SFN and NMPEA, a structural analog of PEITC without ITC functionality. (B) U266 cells were treated with a series of concentrations of BITC, PEITC and SFN for 4 h. Chymotrypsin-like, caspase-like and trypsin-like proteasome activities were measured using Suc-LLVY-AMC, Z-LLE-AMC and Boc-LRR-AMC substrates, respectively. White bars: chymotrypsin-like activity; striped bars: trypsin-like activity; black bars: caspase-like activity. (C) Inhibiting proteasome activity by PEITC is time-dependent and long lasting. U266 cells were treated with 10 μM PEITC for up to 24 h. NMPEA was used as a negative control. (D) A variety of cancer cells, including HeLa, A549, HT-29, PC-3 and MCF-7, were treated with 10 μM BITC for 4 h. Chymotrypsin-like activity was measured in cell lysates before (white bars) and after (black bars) the treatment (upper panel). The ubiquitinated proteins in the same cell lysates were immunoblotted (lower panel). *P < 0.05; **P < 0.01.
Degradation of p53 and IκBα is inhibited in ITC-treated cells

It has been shown that proteasome inhibitors induce accumulation of wild-type p53, the most-studied tumor suppressor protein, and IκBα, a natural inhibitor of nuclear factor-kappaB (NF-kB), because they are known UPS substrates (2,5). To investigate the functional consequences of ITC-induced proteasome inhibition, we treated A549 cells with 10 μM BITC for up to 2 h. Cell lysate was extracted and separated using native gel electrophoresis. The proteasome activity was determined by the substrate overlay assay (see Materials and methods). The panel below is the densitometry analysis of the results. White bars: 26S; black bars: 20S. (B) Purified proteasome was inhibited by PEITC, but not SFN and NMPEA. Purified 20S proteasome from rabbit erythrocytes was incubated with PEITC, SFN and NMPEA for 4 h at room temperature in the dark before the activity was determined using the fluorogenic substrates. *P < 0.01.

Discussion

The approval of Bortezomib for the clinical treatment of MM has led to a surge in the research and development of proteasome inhibitors (2–6). The current pool of proteasome inhibitors includes numerous compounds with diverse structures. What is common in these compounds is that they are all short peptides capped with an electrophilic C-terminus (5,23,24). The C-terminal modifications include boronic acid (such as Bortezomib), aldehyde (MG132), epoxyketone (epoxymycin), vinyl sulfone (z-Leu-Leu-Leu-vinyl sulfone) and others (5,23,24). The findings in this study may suggest ITCs as new additions to the family of electrophilic proteasome inhibitors. Compared with peptidal proteasome inhibitors, dietary ITCs have advantages of improved uptake rates and levels due to their smaller size.

In our earlier proteomic study using radiolabeled ITCs and 2-dimensional gel electrophoresis (14), some proteasome subunits have been identified in gel spots containing radioactivity, suggesting that proteasome is a potential target of ITCs. These subunits include proteasome subunit alpha type 3 (PSA3), proteasome subunit alpha type 4 (PSA4), proteasome subunit beta type 9 (PSB9), 26S protease regulatory subunit 6A (PR56A) and 26S protease regulatory subunit 8 (PR58). In this study using functional assays, we showed that ITCs inhibit proteasome activity in cells and purified proteasome. Proteasome inhibition by ITCs may contribute to ITC-induced cell cycle arrest and apoptosis.

Our study indicates that ITCs inhibit proteasome activity presumably through direct binding. Previously, we identified an in vitro adduct of BITC with Cys303 in β-tubulin and an in vivo adduct of BITC with Cys347 in α-tubulin. The ITC-binding affinities correlate well with not only cell cycle arrest and apoptosis induction in A549 cells (13) but also tubulin precipitation and degradation in a variety of cells (14–16). Recently, BITC has been shown to form a covalent adduct with the N-terminal proline of macrophage migration inhibitory factor and
inhibit the migration inhibitory factor-mediated inflammatory response (25–27). These results, together with previous studies on cytochrome P450 (28) and Keap-Nrf2 (29), support the notion that the binding of ITCs to nucleophilic residues in target proteins constitutes an important mechanism for their downstream molecular/cellular effects. A similar mechanism by which peptidal electrophiles inhibit proteasome activity was reported: the electrophilic cap binds with the \( -\text{hydroxyl of the N-terminal threonine within the active site of the catalytic subunits to form a covalent and (in most cases) irreversible bond (5,23). Additionally, X-ray structure reveals that the cysteine 118 of } \beta_3\text{ subunit, which protrudes into the } \beta_2\text{ active site, is also susceptible to electrophilic attack (24). In this study, we found that ITCs are able to inhibit proteasome activity, including both 26S and 20S complexes. More importantly, ITCs can directly inhibit the activity of the purified proteasome, suggesting that the proteasome, like tubulin, may serve as a molecular target of ITCs. The notion of direct binding is also supported by the observation that BITC-induced proteasome inhibition is unrelated to ITC-induced protein aggregation and ROS generation. Further investigations are needed to identify the binding site(s). Another major finding is that not all ITCs are equal. Results showed that the ITC functional group is required for proteasome inhibition and the side chain also affects the scale of inhibition. Interestingly, the potency order of inhibiting proteasome is consistent with that of inducing cell cycle arrest at G2/M phase, apoptosis and ultimately cell growth inhibition. The structure–activity relationship among three ITCs, which has been also observed in our previous studies (13–16), confirming that binding to target proteins for hydrophobic ITCs such as BITC and PEITC is an important biological event and suggesting that the activity of ITCs can be optimized through structure design of the agents. Contrary to BITC and PEITC, SFN has been...
demonstrated to upregulate proteasome activity and expressions of some proteasome subunits, such as PSMB5 and PSMB6, through activation of Keap1–Nrf2 pathway (17,30). Since previous studies have shown that SFN induces direct and indirect antioxidant response in cells through mild oxidative stress (29,31), the findings in the current study may suggest that stress on proteasome activity is a triggering event leading to proteasome activity enhancement by SFN. However, whether SFN inhibits proteasome activity, especially at early time points, requires further studies.

Proteasome inhibitors modulate the activity of transcription factors such as p53 (32). Wild-type p53 expression is low in most cells under normal conditions and it has a short half-life. Its level is tightly regulated by Mdm2 (or Hdm2), the product of an inducible gene by p53. Mdm2, a RING finger-containing ubiquitin E3 ligase, binds to the N-terminal domain of p53 and labels it with ubiquitin before its degradation by the 26S proteasome. Rapid accumulation of p53, resulting from proteasome inhibition, triggers a variety of cellular responses including cell cycle arrest, apoptosis induction, DNA repair and differentiation (32,33). PEITC has been shown to induce p53 accumulation in a variety of cells and apoptosis induction by PEITC occurs through a p53-dependent pathway (34,35). Our results in this study show that p53 accumulation correlates well with the potency order of proteasome inhibition by ITCs, suggesting a possible link between these two events. However, it is unlikely that p53 plays a role in ITC-induced apoptosis in U266 cells because of its mutant p53 status. We believe that inhibition of NF-κB through accumulation of IkBα may contribute to apoptosis induction in U266 cells (36–38). NF-κB regulates various immune...
inflammatory responses and plays an important role in tumorigenesis by stimulating cell survival pathways, blocking apoptosis, inducing angiogenesis and increasing metastatic potential. IkB, the natural inhibitor of NF-κB, is a UPS substrate, so its level depends on proteasome activity. Inhibition of IkB degradation by proteasome inhibitors inhibits NF-κB activity through sequestering NF-κB in the cytoplasm. Additionally, proteasome inhibition blocks NF-κB activation through inhibiting proteasome-mediated cleavage of NF-κB family members p100 and p110 to the activated forms p50 and p52 (39). Both BITC and PEITC have been shown to inhibit NF-κB and inflammatory responses (40,41). In this study, we showed that treatment with BITC or PEITC resulted in delayed degradation of IkB. However, whether ITC-induced proteasome inhibition is responsible for NF-κB inhibition and apoptosis induction needs further investigation.

BITC and PEITC have been shown to induce apoptosis in leukemia and lymphoma cells (42–46). Currently, PEITC is being used in a clinical trial for the treatment of leukemia (47). These results are consistent with our finding that BITC and PEITC are potent inhibitors of MM cell growth. In fact, cells derived from hematologic cancers, such as MM and lymphoma, are among the most sensitive to proteasome inhibition (2,4,42). Previously, our laboratory studied the pharmacokinetics of orally administered PEITC in lung cancer prevention using F344 rats (48). The results indicate that PEITC can reach a peak concentration of 18.8 μM in blood after 2.9 h and PEITC concentration stays >5 μM for >12 h, suggesting that the effective concentrations for proteasome inhibition by BITC and PEITC are physiologically attainable. Additionally, it has been shown that dietary ITCs, including BITC and PEITC, have markedly low toxicity in a variety of animal studies (9). The findings in this study support the future investigation of ITCs as promising agents for prevention and treatment of MM.

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