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Differential Effects of Phenethyl Isothiocyanate and D,L-Sulforaphane on TLR3 Signaling

Jianzhong Zhu,*† Arundhati Ghosh,*† Elizabeth M. Coyle,*† Joomin Lee,‡ Eun-Ryeong Hahm,‡ Shivendra V. Singh,‡ and Saumendra N. Sarkar*†

Naturally occurring isothiocyanates (ITCs) from cruciferous vegetables are widely studied for their cancer chemopreventive effects. In this study, we investigated the effects of ITCs on TLR signaling, and found that the two most promising ITCs, phenethyl ITCs (PEITC) and D,L-sulforaphane (SFN), have differential effects on dsRNA-mediated innate immune signaling through TLR3. PEITC preferentially inhibited TLR3-mediated IFN regulatory factor 3 (IRF3) signaling and downstream gene expression in vivo and in vitro, whereas SFN caused inhibition of TLR3-mediated NF-κB signaling and downstream gene expression. Mechanistically, PEITC inhibited ligand (dsRNA)-dependent dimerization of TLR3, resulting in inhibition of signaling through IFN regulatory factor 3. In contrast, SFN did not disrupt TLR3 dimerization, indicating that it affects further downstream pathway resulting in NF-κB inhibition. To examine the biological significance of these findings in the context of antitumor activities of these compounds, we used two approaches: first, we showed that dsRNA-mediated apoptosis of tumor cells via TLR3 was inhibited in the presence of PEITC, whereas this response was augmented by SFN treatment; second, in a separate assay measuring anchorage-independent growth and colony formation by immortalized fibroblasts, we made similar observations. Again in this study, PEITC antagonized dsRNA-mediated inhibition of colony formation, whereas SFN enhanced the inhibition. These results indicate biologically relevant functional differences between two structurally similar ITCs and may provide important insights in therapeutic development of these compounds targeted to specific cancer. The Journal of Immunology, 2013, 190: 4400–4407.

Numerous studies have shown that naturally occurring isothiocyanates (ITCs) from cruciferous vegetables can suppress cancer initiation, promotion, and progression (1, 2). ITCs are characterized by the sulfur-containing functional group — N=C=S. They primarily exist as glucosinolates, which are hydrolyzed to ITC when the plant cells are disrupted releasing the enzyme myrosinases. ITCs have been shown to affect many cellular functions related to tumorigenesis including cell-cycle regulation, apoptosis, hormone signaling, angiogenesis, and so on (3, 4). Among the multitude of effects that these compounds exhibit on different tissues, the primary mechanistic focus has been on their role in activation of NF-κB, inhibition of NF-κB transcriptional activity, and induction of apoptosis (1–3). However, except for a few studies that focus on the TLR4 response to D,L-sulforaphane (SFN) (5, 6), to the best of our knowledge, the effect of ITC on TLR signaling largely remains unknown.

The innate immune system recognizes conserved components of invading microbes through pattern recognition receptors and helps to mount a response to protect the host (7). Activation of TLRs and other cytoplasmic receptors with their cognate ligands causes transcriptional induction of a set of genes, mainly via the NF-κB and/or IFN regulatory factor (IRF) family of transcription factors (8). Together, they trigger the initial host-defense response and shape the later adaptive immune response. TLR3 is one of the primary sensors of dsRNA (9–11). TLR3 is activated by either natural dsRNA from virus infection or synthetic dsRNA polyinosinic acid:polycytidylic acid [p(I):p(C)] applied externally. Upon dsRNA binding, TLR3 engages its unique adaptor Toll/IL-1R domain-containing adapter protein–inducing IFN-β to propagate the signal leading to NF-κB or IRF3-mediated upregulation of a series of proinflammatory and cytokine genes.

We have recently shown natural variations of TLR3-mediated NF-κB activation between primary and metastatic head and neck cancer cells leading to their differential sensitivity to p(I):p(C)–stimulated cell death (12). These findings have opened possibilities of differentially modulating TLR3-mediated IRF3 and NF-κB signaling for therapeutic purpose. Toward this we have previously identified and characterized small molecules that differentially modulate TLR3-mediated NF-κB and IRF3 activation (13). In this study, we have used naturally occurring ITCs to study their effect on TLR3 signaling. We show that phenethyl ITC (PEITC), a constituent of watercress, potently inhibits IRF3 signaling, whereas a similar ITC, SFN (a synthetic racemic analogue of broccoli constituent 1,sulforaphane), preferentially blocks NF-κB. Besides TLR3-mediated gene inductions, we also show the biological importance of these findings by

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demonstrating differential effects of these compounds on p(l)pc(C)-stimulated apoptosis and anchorage-independent growth inhibition.

Materials and Methods

Cell culture and reagents

HEK293 cells and HEK293-derived TLR3-expressing stable cells (Wt11), RL24 cells, HT1080 cells, RAW264.7 cells, SV40 T Ag-expressing FVB mouse embryonic fibroblasts (MEFs), and head and neck squamous cancer cells PCI15A were cultured in DMEM containing 10% FBS and penicillin/streptomycin. Prostate cancer LNCaP cells were cultured in RPMI 1640 supplemented with 10% FBS, 2.4 mg/ml glucose, 1 mmol/l sodium pyruvate, and antibiotics. Synthetic dTTP(d)RNA poly(I):(C) was obtained from GE Healthcare and dissolved in PBS. TLR3 signaling was induced by adding poly(I):(C) stock solution to the media as described earlier (13). Human IFN-α was from PBL IFN source (Piscataway, NJ). ITCs including PEITC, erucin (ERU), erysolin (ERY), and sulforaphane (SFN) were purchased from LKT Laboratories (St. Paul, MN) and dissolved in DMSO. Anti-FLAG beads were from Sigma-Aldrich (St. Louis, MO); anti-Cleaved-PARP was from Cell Signaling Technology (Beverly, MA); anti-hemagglutinin (HA), anti-α-tubulin, and anti-β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA); and anti-TLR3 was from Imgenex (San Diego, CA). Anti–IFN-stimulated gene 54 (ISG54), ISG56, ISG60, anti-DRBP76, anti-IRF3, and anti-SV40 large T Ag Abs have been described previously (13, 14).

Analysis of TLR3 oligomerization

TLR3 was PCR amplified from template plasmid pcDNA3-FLAG-human TLR3 using the primers: 5′-CACAGCTCTCTTTGTTGGAGCTGCAGTTGCT-3′ and 5′-TTAATTCCTCTTCTGAGATGAGTTTTTGTTCA-3′ with myc tag sequence (with myc tag sequence between TLR3 coding sequence and the stop codon). The PCR product was generated by LR recombination between pENTR/HA-TLR3-myc and pENTR CMV/HA-TLR3-myc. The expression vector pLenti CMV/HA-TLR3-myc was generated by LR recombination between pENTR/HA-TLR3-myc and pENTR CMV Puro DEST (Addgene) using Gateway LR Clonase II enzyme mix (Invitrogen) according to manufacturer’s guidelines. Wt11 cells, which stably express FLAG-TLR3, were transfected with pLenti CMV/HA-TLR3-myc and selected with puromycin (1 μg/ml). The selected cells expressed FLAG-tagged TLR3, as well as HA/myc-tagged TLR3.

Transfection and reporter assays

HEK293 cells were seeded into flat-bottom, white-well, 96-well cell culture plates at a density of 4 × 10^4 cells/well in 100 μl media. The cells were transfected the next day by Fugene 6 (Roche) with IFN-β luciferase reporter (10 ng/well) and β-actin Renilla luciferase reporter (0.3 ng/well), together with IKe, TB1K, IRF3-5D mutant, or vector control (20–40 ng/well). The total DNA per well was normalized to 50 ng by adding empty pcDNA3.1 vector. Wt11 cells were similarly transfected with NF-κB luciferase reporter (10 ng/well) and β-actin Renilla luciferase reporter (0.3 ng/well). Twenty-four hours posttransfection, the cells were treated with PEITC and SFN, respectively, for 8 or 1 h followed by 7-h 50 mM NaCl, 1.5 mM MgCl2, 12.5 mM β-glycerophosphate, 2 mM EGTA, 10 mM NaF; 2 mM DTT, 1 mM Na3VO4, 1 mM PMSF, and 1 mM PMSF (poly(I):(C)) stimulation. Luciferase activities were measured using the Dual-Glo luciferase assay system (Promega, Madison, WI).

Nuclear fractionation, coinmunoprecipitation, and Western blotting

To isolate nuclear fractions, we suspended cells in the hypotonic buffer plus 0.1% Triton X-100, incubated on ice for 10 min, vortexed for 30 s, and centrifuged (12,000 × g for 10 min at 4°C). The supernatants were used as cytoplasmic fractions (CPs). The nuclei pellets were thoroughly washed and lysed in lysis buffer (20 mM HEPES [pH 7.4], 1% Triton X-100, 150 mM NaCl, 1.5 mM MgCl2, 12.5 mM β-glycerophosphate, 2 mM EGTA, 10 mM NaF; 2 mM DTT, 1 mM Na3VO4, 1 mM PMSF, and 1× protease inhibitors). For immunoprecipitation, the cleared supernatants were incubated with anti-FLAG beads overnight, washed three times with lysis buffer, and boiled in 1× SDS-PAGE loading buffer for elution. All the protein extracts were separated by SDS-PAGE and analyzed by immunoblotting as described.

Quantitative PCR analysis of gene expression

RNA was isolated from cells treated with ITC and/or stimuli by TRIzol (Invitrogen). cDNA was synthesized using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA) and subjected to real-time PCR using a CFX96 Real Time System (Bio-Rad) according to manufacturer’s instructions. PCR amplification was normalized to ribosomal protein L32. All quantitative PCR primer information is available in our previous published work (13, 15).

IL-8 ELISA

Cell supernatants were harvested and assayed for IL-8 protein amount by ELISA using the human IL-8 ELISA set reagent from BD Biosciences following manufacturer’s protocol as described previously (13).

Immunohistochemistry staining and quantitation

Immunohistochemistry was performed as previously described (16). In brief, prostate sections were quenched with 3% hydrogen peroxide and blocked with normal serum. The sections were then probed with anti-IFN-γ Ab, washed with TBS, and incubated with an HRP-conjugated anti-rabbit secondary Ab. The characteristic brown color was developed by incubation with 3.3’-diaminobenzidine. The sections were counterstained with Meyers hematoxylin (Sigma) and examined under a Leica microscope (Leica Microsystems, Bannockburn, IL). ISG54 expression was determined using Aperio Image Scope software, which automatically counts blue-negative and brown-positive stained cells and calculates the percentage (0–100) of positively stained cells, and is calculated using the following formula, H-score = % of negative cells × 0 + (% 1+ cells × 1) + (% 2+ cells × 2) + (% 3+ cells × 3).

Soft agar colony formation assay

One percent low-melting temperature agarose was melted in microwav oven and mixed with equal volume of 2× MEM (Invitrogen) containing 20% FBS and 2× antibiotics. The 0.5% agarose in 1× MEM plus 10% FBS was added to 6-well plates (2 ml/well) and left at room temperature for >30 min to allow agarose to solidify as base agar. FVB MEFs stably expressing SV40 large T and small T Ags in medium were mixed with above 0.5% agarose/MEM/FBS (volume ratio 2:3) to obtain 0.3% agarose and applied onto the top of 0.5% base agar in 6-well plates (1.6 × 10^5 cells in 2 ml agarose in each well). The plates were left at room temperature for >30 min to let the top agarose solidify before transferring into a cell culture incubator. Every 2 d, the (p(l):p(C) (50 μg/ml) and/or PEITC (5 μM), SFN (5 μM)-containing medium were added onto the surface of the top agar. After 3-wk incubation in CO2 incubator, the cell colonies were visualized for assessment of anchorage-independent cell growth.

Results

Differential effects of ITC on TLR3 signaling

Activation of TLR3 signaling directly induces a number of type I ISGs, such as ISG56 family genes, independent of IFN signaling (10). A number of cell lines of epithelial origin induce ISGs through TLR3 signaling pathway, when synthetic dsRNA analogue p(l):p(C) is added to the culture medium, as opposed to inducing RIG-I-like receptor (RLR) signaling (11, 17–19). Specifically, in HEK293 cells that do not express TLR3, this signaling is dependent on exogenous expression of TLR3 (20). To investigate the effects of ITC on dsRNA signaling initially, we used an HEK293 cell–based ISG56-promoter reporter assay (13). Using this system, we tested effect of four naturally occurring ITCs: PEITC, ERU, ERY, and SFN (Fig. 1A). PEITC is an aromatic ITC, whereas the remaining compounds belong to thioalkyl class and differ by oxidation state of the sulfur atom. We found that PEITC and ERU inhibited IRF3-mediated transcriptional activation of ISG56-promoter by p(l):p(C), whereas SFN and ERY did not show significant changes (Supplemental Fig. 1). To see the inhibition of endogenous gene induction, we used quantitative RT-PCR to measure mRNA induction for ISG56 and IFNB from HEK293 cells stably expressing TLR3 (HEK293-TLR3, also
named as Wt11 cells). TLR3-mediated induction of both these genes, which are IRF3/IRF7 dependent, were inhibited by PEITC and ERU in a dose-dependent manner (Fig. 1B, 1C). At the protein levels, we also saw similar inhibition with PEITC and ERU for ISG56 and another ISG56 family protein ISG60 (Fig. 1D). On the other hand, SFN and ERY failed to cause inhibition of ISG56 or ISG60 induction (Fig. 1D, lanes 7–10). In contrast, when we tested for the induction of genes that are induced by TLR3 signaling primarily via NF-κB signaling pathway, they were significantly more inhibited by ERY and SFN. As shown in Fig. 2A and 2B, both IL-8 and TNF-α mRNA induction were inhibited by ERY and SFN. However, PEITC and ERU did not inhibit IL-8 mRNA, and only partially inhibited TNF-α mRNA, which may be because of the additional dependence of TNF-α promoter on IRF3 (21). Similar observations were made in experiments measuring IL-8 protein by ELISA (Fig. 2C) and NF-κB reporter assay (Fig. 2D). Two important conclusions are discernible from these observations: 1) PEITC and SFN have differential response on TLR3 signaling, and 2) oxidation state of the sulfur in thioalkyl series significantly changes its activity. Specifically, this study shows that sulfinyl analogue with SO moiety (SFN) and sulfonyl analogue with SO₂ moiety (ERY) behave differently than the thio compound ERU. For example, TLR3-mediated induction of ISG56 is significantly inhibited in the presence of PEITC and ERU, but not SFN and ERY. We also found that the activity of ERU is similar to that exhibited by the aromatic ITC compound PEITC.

Specific effects of PEITC on IRF3 signaling

In resting cells, IRF3 is predominantly present in the cytoplasm. Upon stimulation of various pattern recognition receptor signaling pathways, IRF3 is phosphorylated by IKK family kinases TBK1 or IKKε, leading to its nuclear translocation and transcriptional activation of IRF3-responsive genes (7). To investigate the mechanism of ITC-mediated inhibition of IRF3-responsive gene induction, we assayed IRF3 nuclear translocation (15). Nuclear fractions were prepared from HEK293-TLR3 cells treated with p(I):p(C) and various ITCs, and immunoblotted with anti-IRF3 and actin Abs. *p < 0.05, **p < 0.01, the ITC-treated samples versus p(I):p(C) control sample in (B) and (C) performed by one-way ANOVA analysis. NS, Nonspecific band.

PEITC affects TLR3 oligomerization

To understand the mechanistic basis of this differential regulation, hereafter we focused on PEITC and SFN. In an attempt to identify the particular step in TLR3-IRF3 signaling pathway that was affected by PEITC, we used reporter assays. TLR3 signaling pathway
FIGURE 2. ITCs differentially affect TLR3-mediated NF-κB signaling and its downstream gene expression. **(A and B)** Quantitative RT-PCR for NF-κB-dependent gene IL-8 (A) and TNF-α (B) was performed as in Fig. 1. **(C)** HEK293-TLR3 cells in 24-well plates were treated with PEITC or SFN for 1 h, then stimulated with 50 μg/ml p(I):p(C) for another 24 h. Cell supernatants were harvested and IL-8 protein levels were detected by ELISA. **(D)** Wt11 cells were transfected with NF-κB luciferase reporter and β-actin Renilla luciferase reporter. Twenty-four hours posttransfection, the cells were treated with PEITC or SFN for 1 h followed by another 7-h stimulation with 50 μg/ml poly(I):C. The results were expressed as fold induction of NF-κB luciferase activity for poly(I):C-stimulated cells relative to those of nonstimulated controls after normalization to Renilla luciferase. *p < 0.05, **p < 0.01, the ITC-treated samples versus p(I):p(C) control sample performed by one-way ANOVA analysis.

FIGURE 3. PEITC inhibits TLR3-mediated IRF3 activation but does not affect IFN signaling. **(A)** HEK293-TLR3 cells were treated with PEITC or other ITCs for 1 h, then stimulated with 50 μg/ml p(I):p(C) for another 3 h. Cells were subjected to nuclear fractionation and the nuclear IRF3 detected by immunoblotting with the anti-IRF3 Ab. The effective nuclear and CP expression was evidenced by specific detection of DRBP76 and tubulin, respectively. The band intensities of nuclear IRF3 were normalized to DRBP76 band intensities and are shown on the top of each band. **(B)** HEK293-TLR3 cells were treated with PEITC or other ITCs for 1 h, then stimulated with 500 μg/ml human IFN-α for 20 h. Cell lysates were detected by immunoblotting for ISG60 and actin. The band intensities of ISG60 after normalization to actin were shown on the top of each band.

can be activated without dsRNA or virus infection by artificially expressing either of several signaling components, such as IKKe (22), TBK1 (23), or constitutively active IRF3 (IRF3-5D mutant) (24) using transient transfection. As shown in Fig. 4, IFNβ-promoter driven luciferase activity was strongly induced by expression of IKKe (Fig. 4A), TBK1 (Fig. 4B), and IRF3-5D (Fig. 4C). However, both PEITC and SFN did not show any inhibition of this induction at two different concentrations. This was consistent with our previous findings that PEITC did not affect TLR3-IRF3 signaling downstream of IRF3 indicated by the inhibition of IRF3 nuclear translocation; rather, it was further upstream.

TLRs are known to oligomerize upon ligand binding (25), and because of the presence of the reactive —N=C=S group present in the ITC, it was suggested that SFN might affect TLR4 oligomerization (5). To test dsRNA-mediated oligomerization of TLR3, we standardized an immunoprecipitation-based assay. Two differentially tagged TLR3 expression constructs, one with FLAG, another with HA were both stably expressed in HEK293 cells. Immunoprecipitation of FLAG-tagged TLR3 from p(I):p(C)-treated whole-cell lysates showed a signal-dependent enhancement in HA-tagged TLR3 coprecipitation (Fig. 4D, lanes 1 and 2 from left). However, treatment of cells with PEITC inhibited HA-TLR3 coprecipitation, whereas SFN treatment did not strongly inhibit the oligomerization (Fig. 4D, lanes 3–6 from left, Fig. 4E). These results indicated that unlike SFN, PEITC affected p(I):p(C)-mediated oligomerization of TLR3, which might be a possible mechanism for its inhibition of TLR3-IRF3 signaling.

**PEITC affects ISG expression in vivo**

To investigate whether the ITC-mediated modulation of dsRNA signaling also occurs in cells with endogenous innate immune receptor expression, we used mouse macrophage cell line RAW246.7 cells. As expected, p(I):p(C)-stimulated induction of ISG54 (a member of the mouse Isg56 family) (26) was inhibited by PEITC, whereas SFN did not show any inhibition (Fig. 5A). Previously, ISG54 was found to be induced in a number of tissues in mice by p(I):p(C) stimulation or Sendai virus infection (27). We chose to use mouse prostate tissue to investigate in vivo downregulation of ISG54 expression by PEITC. This was due to the demonstrated importance of TLR3 signaling in prostate cancer (28). Analysis of ISG54 protein level in the prostate of C57/BL6 mice fed a control diet or PEITC-supplemented diet (3 μmol PEITC/g diet) for 19 wk (16) showed significantly reduced ISG54 expression in both stromal cells and epithelial cells (Fig. 5B, 5C). These results indicated...
that PEITC was capable of inhibiting endogenous ISG54 expression in vivo.

**Effects of PEITC and SFN on dsRNA-mediated cell death**

Both PEITC and SFN are known to cause apoptosis by multiple mechanisms in cancer cells (1, 2). Among various mechanisms, involvement of ROS production, mitochondrial antiapoptotic Bcl2 family proteins, NF-κB, and Nrf2 pathway have been implicated to induce apoptosis in different cancer cells. However, the contribution of IRF signaling in ITC-mediated apoptosis has not been described. We and others have shown that TLR3 activation by p(I):p(C) causes cell death through IRF3 pathway in a number of cancer cell lines (12, 29–31). As we observed inhibition of IRF3 signaling by PEITC, we hypothesized that PEITC might nega-

**FIGURE 4.** PEITC interferes with TLR3 oligomerization. HEK293 cells in 96-well plates were cotransfected with IFN-β firefly luciferase and β-actin *Renilla* luciferase, together with 20 ng IKKe (A), 20 ng TBK1 (B), and 40 ng IRF3-5D mutant (C) for 24 h. The transfected cells were treated with PEITC and SFN at the indicated concentrations for an additional 8 h. The results were expressed as fold induction of IFN-β luciferase activity for IKKe, TBK1, or IRF3-5D transfected cells relative to those of vector transfected controls after normalization to *Renilla* luciferase. The results were representative of two similar experiments. No significant difference between PEITC or SFN-treated samples and nontreated samples was found by one-way ANOVA analysis. (D) HEK293 cells stably expressing FLAG-TLR3 and HA-TLR3 were treated with PEITC and SFN at the indicated concentrations for 1 h and then stimulated with 50 μg/ml p(I):p(C) for another 3 h. Cell lysates were immunoprecipitated with anti-FLAG and the immunoprecipitates together with cell lysate control detected by immunoblotting for HA-TLR3. (E) Band intensities of each lane from the top panel in (D) were quantified and normalized corresponding with those of the bottom panel (anti-HA bands in whole-cell lysates) and plotted as relative intensities. The plotted graph represents the average band intensities plus SEs from two similar experiments. *p < 0.05, **p < 0.01, the ITC-treated samples versus p(I):p(C) control sample performed by one-way ANOVA analysis.

**FIGURE 5.** PEITC inhibits ISG 54 expression in vitro and in vivo. (A) Mouse macrophage RAW264.7 cells were treated with PEITC and SFN at the indicated concentrations for 1 h, then stimulated with 50 μg/ml p(I):p(C) for another 12 h. Cell lysates were detected by immunoblotting for ISG54 and actin. (B) Two representative immunohistochemical images depicting ISG54 expression in the prostate tissue sections from control and PEITC-treated normal mice (original magnification ×200). Scale bar, 100 μm. (C) Quantitative analysis of stromal and epithelial expression of ISG54 in the prostate tissue sections. At least 10 randomly selected fields from each section were analyzed. Results shown are mean ± SD (n = 5 for both control and PEITC treatment groups). Statistical significance was determined by two-sided Student t test.
tively impact p(I):p(C)–induced apoptosis in cancer cells. To test this hypothesis, we treated two well-characterized carcinoma-derived cell lines: prostate cancer (PCa) cell line, LNCaP, and head and neck squamous cell carcinoma (HNSCC) cell line, PCI15A either with p(I):p(C) alone or in combination with PEITC and SFN. As shown in Fig. 6A, treatment of LNCaP cells with p(I):p(C) induced ISG60 and mild apoptosis indicated by the appearance of cleaved PARP (Fig. 6A, lane 2), whereas treatment with PEITC alone resulted in strong induction of apoptosis (Fig. 6A, lane 3), as observed previously (32). However, when these were combined, ISG60 induction was inhibited by PEITC, and levels of cleaved PARP were reduced (Fig. 6A, lane 4). Surprisingly, a higher dose of 10 μM PEITC induced substantial cytotoxicity in LNCaP cells indicated by the loss of β-actin. In contrast, treatment of LNCaP cells with SFN alone resulted in lower levels of cleaved PARP, which was augmented by p(I):p(C) at low concentrations (Fig. 6A, lanes 7–10 from left, Fig. 6B). We saw a very similar pattern in PCI15A cells (Fig. 6C), where p(I):p(C) augmented SFN-mediated apoptosis induction, but it inhibited PEITC-mediated cell death, albeit weakly compared with LNCaP cells (Fig. 6D). Once again, these results indicated another important distinction between PEITC and SFN in terms of their effects on p(I):p(C)–induced apoptosis. SFN, which is known to inhibit NF-κB signaling (1), synergized with p(I):p(C)–mediated apoptosis that is IRF3 dependent. In contrast, PEITC affected p(I):p(C)–mediated apoptosis in an opposite manner, because of its inhibitory effects on IRF3 signaling.

**Modulation of dsRNA-mediated inhibition of clonogenicity by ITC**

Among various assays available to measure in vitro cellular transformation, anchorage-independent growth of cells in soft agar is one of the hallmark characteristics of cellular transformation and uncontrolled cell growth. Because the ITCs are known for their...
cancer-chemopreventive functions, we performed soft agar colony formation (clonogenicity) assay using SV40 T Ag–transduced MEFs to assess how ITCs in combination with p(I):p(C) modulated cellular transformation. T Ag–transduced MEFs were responsive to p(I):p(C) by upregulating ISG54 expression (Fig. 7A). Culturing these cells in soft agar for 3 wk generated a large number of macroscopic colonies (Fig. 7B1). In the presence of 5 μM PEITC or SFN, the numbers of colonies were reduced significantly (Fig. 7B2, 7C, 7D). In the presence of 50 μg/ml p(I):p(C), there was a substantial reduction in colony number, indicating antigrowth properties of TLR3 signaling (Fig. 7B3, 6C, 6D). However, when p(I):p(C) was combined with either PEITC or SFN, we saw a striking difference between these two ITCs. Whereas SFN synergized with p(I):p(C), PEITC exhibited antagonistic effects (Fig. 7B4, 7C, 7D). In the presence of PEITC, the number of colonies was increased compared with only p(I):p(C)–treated samples. This result was consistent with our previous findings and indicated that p(I):p(C)–mediated inhibition of anchorage-independent cell growth is at least partially mediated through IRF3 signaling, and the inhibition of IRF3 signaling by PEITC resulted in partial rescue of this inhibition. In contrast, SFN probably promotes inhibition of NF-κB, which further enhances TLR3-IRF3–mediated inhibition of cell growth.

Discussion
In this study, we demonstrate differences in dsRNA signaling in the presence of two structurally similar, naturally occurring ITCs. Addition of p(I):p(C) to the medium engages TLR3 signaling in a large number of cells, resulting in activation of IRF3 and NF-κB. The other cytoplasmic dsRNA sensors RLRs are activated when the dsRNA ligand is delivered in the cytoplasm, experimentally by liposomal transfection of p(I):p(C), or in vitro transcribed dsRNA, or viral RNA (33). Although the mechanism of p(I):p(C) uptake by different types of cells is not completely worked out, there are evidences that cell-surface molecules such as CD11b (34), clathrin-mediated endocytosis (18), and lysosomal acidification (19) are uniquely important for this process. It is believed that p(I):p(C) taken up by these mechanisms binds and signals through TLR3 from the endosome. Specifically, for HEK293 cells, which normally do not express TLR3, there is no IRF3 or NF-κB activation by p(I):p(C) added to the medium through RLR signaling. However, TLR3 expression enables them to signal with p(I):p(C) (20). Because the majority of our studies were carried out in cells of epithelial origin by adding p(I):p(C) in the medium, we interpreted the results as ITC effects on TLR3 signaling. However, ITCs might have other effects on RLR signaling in a cell-type–specific manner, which we have not explored.

Among the two primary signaling pathways that are activated by dsRNA, the NF-κB is considered to be proinflammatory and growth promoting, whereas the IRFs are primarily involved in IFN induction and enhance growth inhibitory and apoptotic pathway. With respect to specific cancer, the balance between these two pathways has been shown to be changed and determine the outcome (35). What remains to be understood is how to specifically modulate this balance and use it to the therapeutic advantage for specific cancers. Results presented in this study provide us with new leads to develop such agents based on naturally occurring ITCs. We show that despite their structural similarities, SFN and PEITC can have quite distinct effects on TLR3 signaling and cell death induction.

TLR3 signaling has been characterized in a number of model cancer cell lines (12, 36–42) and has been shown to have anti-cancer activity in vivo in a prostate cancer model (28). The apoptotic activities of TLR3 ligand p(I):p(C) is dependent on IRF3 (26, 27, 43). In contrast, ITCs are also known to induce apoptosis in cancer cells by affecting multiple pathways. However, when in combination, ITCs differentially affected p(I):p(C)–mediated apoptosis. Whereas SFN augmented p(I):p(C)–mediated apoptosis, PEITC showed the opposite effect. Based on our results presented in Fig. 6 and supported by data in Fig. 7, we propose the following: IRF3 signaling positively contributes to dsRNA-induced cell death, and the inhibition of this pathway by PEITC leads to a reduction in net apoptosis by p(I):p(C). It has been shown that proapoptotic proteins Bak and Bax are involved in PEITC-mediated apoptosis in prostate cancer cells (44). Our observation that in combination with p(I):p(C), PEITC-mediated apoptosis is reduced may be because of the activation of the prosurvival NF-κB pathway by TLR3. In contrast, SFN enhances dsRNA-mediated apoptosis by inhibiting NF-κB, which primarily contributes to cellular survival. However, further experiments are needed to establish the biochemical details of PEITC on IRF3 signaling.

Evidence continues to accumulate to indicate that structural differences in ITCs can profoundly affect their activity. For example, autophagy induction by SFN serves to protect against apoptosis (45). To the contrary, autophagy contributes to cell death induction by PEITC (32). This study provides another example illustrating mechanistic differences in structurally related ITC compounds (e.g., SFN and PEITC or the thioalkyl series). We show there are profound differences between SFN and PEITC effects on TLR3 signaling when used individually or in combination with TLR3 ligand. These observations underscore caution in extrapolation of mechanistic results between structurally different ITC compounds.

The potential for using PEITC and SFN in tumor therapy has been demonstrated in a number of preclinical mouse models (16, 46–51). However, synthetic dsRNA analogues of TLR3 ligand are also being developed for various treatments including cancer (52). Our study provides a mechanistic basis for using them in combinatorial therapy. Accordingly, use of PEITC in combination with dsRNA may negatively affect their individual therapeutic potentials, whereas SFN and dsRNA combination may work synergistically to prevent tumor growth. In summary, our results show examples of mechanistic differences in the cellular response to two structurally similar ITCs and may provide important insights in therapeutic development of these compounds targeted to specific cancer.

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References


Supplementary Fig. 1. ITCs differentially affect p(I):p(C) induced ISG56 promoter activity in RL24 reporter cells. RL24 cells in flat-bottomed white-wall 96-well cell culture plates (4 x 10^4 cells in 100 μL media per well) were treated with ITCs at the indicated concentrations for 1 h, and then stimulated with 50 μg/mL poly(I):C for 8 h. Luciferase activities were measured using the Dual-Glo luciferase assay system. The results were expressed as fold induction of ISG56 luciferase activity for poly(I):C stimulated cells relative to those of non-stimulated controls after normalization to Renilla luciferase. * p < 0.05 and ** p < 0.01, the ITCs treated samples versus p(I):p(C) control sample performed by one-way ANOVA analysis.