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# Phenethyl isothiocyanate and paclitaxel synergistically enhanced apoptosis and alpha-tubulin hyperacetylation in breast cancer cells

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## Abstract

Combination of phenethyl isothiocyanate (PEITC) and paclitaxel (taxol) has been shown to work synergistically to increase apoptosis and cell cycle arrest in breast cancer cells. In this report, we further explored the mechanisms for the synergistic activity of PEITC and taxol in MCF7 and MDA-MB-231 (MB) breast cancer cell lines. By Western blotting analysis, treatment of MCF7 cells with both PEITC and taxol led to a 10.4-fold and 5.96-fold increase in specific acetylation of alpha-tubulin over single agent PEITC and taxol, respectively. This synergistic effect on acetylation of alpha-tubulin was also seen in MB cells. The combination of PEITC and taxol also reduced expressions of cell cycle regulator Cdk1, and anti-apoptotic protein bcl-2, enhanced expression of Bax and cleavage of PARP proteins. In conclusion, this study provided biochemical evidence for the mechanism of synergistic effect between the epigenetic agent PEITC and the chemotherapeutic agent taxol.

## Introduction

Epigenetic modification of DNA and histone proteins by methylation and deacetylation plays a key role in carcinogenesis [1-5]. Methyltransferase inhibitors and histone deacetylase (HDAC) inhibitors are novel anti-cancer agents. Two DNA methyltransferase inhibitors, azacitidine and decitabine, and two histone deacetylase inhibitors, vorinostat and romidepsin, have been in clinical use [6-12]. Belinostat was reported to induce durable remission in refractory peripheral T-cell lymphoma [13].

Breast cancer is the most commonly diagnosed cancer and the second leading cause of death among women. Taxanes are a class of major chemotherapeutic agents for breast cancer therapy. Paclitaxel (taxol) is a widely used chemotherapy drug in the treatment of breast cancer and other solid tumors [14-16]. Taxol inhibits microtubule disassembly when it binds to assembled tubulin,

making the microtubules locked in polymerized state [17]. Thus the taxol -exposed cells are in cell cycle arrest [18-21]. Another effect of taxol is that it inhibits the anti-apoptosis protein Bcl-2, and induces apoptosis in cancer cells [22]. Even though taxol is a highly effective anti-neoplastic agent, the toxicity of taxol, particularly at a higher dosage, limits its prolonged use in patients [15,23,24]. Further research is being done to increase therapeutic efficacy and minimize toxicity. Radiation and targeted therapy has been used effectively for breast cancer therapy [25-28]. Novel anti-cancer agents with novel mechanisms of actions and new formulations are being actively sought [29-31].

Phenethyl isothiocyanate (PEITC) belongs to the family of isothiocyanates, which are initially found in a wide variety of cruciferous vegetables. Natural ITCs are released when the vegetables are cut or masticated. Phenethyl isothiocyanate (PEITC) regulates epigenetic process. PEITC has been shown to be a HDAC inhibitor in prostate cancer, leukemia, and myeloma cells [32-35]. PEITC was also shown to inhibit leukemia development in mice. PEITC was shown to have dual functions and can induce DNA hypomethylation as well as histone hyperacetylation

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[34,36]. Our group has recently shown that combination of PEITC and taxol has synergistic inhibitory effects on breast cancer cell growth [37]. The combination synergistically increased apoptosis and cell cycle arrest in breast cancer cells. In this report, we further explored the mechanisms for the synergistic activity of PEITC and taxol.

## Materials and methods

### Chemicals and cell cultures

As described in previous reports [33-35], PEITC (phenethyl isothiocyanate) was purchased from LKT Labs and dissolved in 70% methanol and 30% deionized water to a stock concentration of 10 mM. Paclitaxel (taxol) powder (Sigma Chemical Co.) was dissolved in DMSO and stored as a stock concentration of 200 nM.

Maintenance and culture of the MCF7 and MDA-MB-231 (MB) cell lines were described in a prior report [37]. Briefly, the cells were seeded at  $0.4 \times 10^6$  per ml and  $0.2 \times 10^6$  per ml, respectively, of PRMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 IU of penicillin/ml and 100  $\mu$ g of streptomycin/ml, and maintained at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. At the specified time points, the cells were harvested. Cell number and viability were determined from at least triplicate cultures by the trypan blue exclusion method.

### Western blotting

MCF and MB cells were treated with PEITC and/or paclitaxel at various concentrations for 48 hours. The cell lysates were used for Western blot analysis as described previously [38,39]. The protein content of the lysates was determined using the BioRad Protein Assay Kit (BioRad, Hercules, CA), with a BSA standard. The antibodies against the following proteins were used for immunoblotting: PARP-1, BCL-2, Bax, Cdk-1, Cyclin B1,  $\alpha$ -tubulin,  $\beta$ -tubulin,  $\beta$ -actin, acetyl- $\alpha$ -tubulin, HDAC6, acetyl-H3, and Acetyl-H4 [34,35,40]. Secondary antibodies were chosen according to the primary antibodies used (goat anti-rabbit or anti-mouse IgG antibody linked to HRP, Santa Cruz). The proteins were visualized through the ECL system. The protein was quantified using the  $\beta$ -actin protein as the loading control.

### Confocal immunofluorescence

Immunostaining of cells for confocal immunofluorescence microscopy was done according to the published methods [35]. Briefly the MCF and MB cells grown on chamber slides were treated for 48 hours without or with PEITC, the cells were then fixed, permeabilized, blocked in BSA and incubated with a mouse anti-acetyl- $\alpha$ -tubulin (Sigma-Aldrich) for 1 h. A fluorescein-conjugated goat anti-mouse IgG was used as secondary antibody. The DNA was counterstained with propidium iodide (PI) to

visualize the nuclei of the cells. Images were captured using an MRC 1024 ES confocal laser scanning microscopy system.

## Results

### PEITC and taxol increased acetylation of alpha-tubulin in breast cancer cells

Alpha-tubulin has been shown to be acetylated by HDAC6 [41]. When the cells were treated with the combination of PEITC and taxol, the acetylation of alpha-tubulin was significantly increased in both MCF and MB cells in comparison with that in single agent treated cells (Figure 1). When the acetylation level was corrected for the amount of total alpha-tubulin present in the specimen, there was a 16% and 28% respective increase in the specific acetylation level (SAL) of acetylated alpha-tubulin (acetyl-alpha-tubulin per unit of total alpha-tubulin) in MCF cells treated with PEITC or taxol alone (Figure 1). There was a 167% increase in SAL in MCF cells treated with both PEITC and taxol. Therefore, the combination led to a 10.4-fold and 5.96-fold increase in SAL over single agent PEITC and taxol, respectively. This synergistic effect on acetylation of alpha-tubulin was also seen in MB cells (Figure 1). Interestingly, taxol alone also enhanced acetylation of alpha-tubulin in both cell lines. The combination also decreased expression of beta-tubulin more than each agent alone.

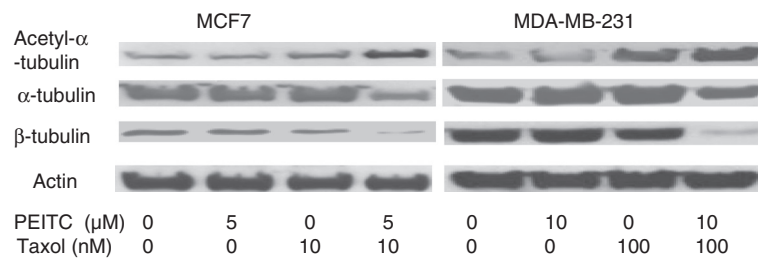
To directly visualize the activity of PEITC on breast cancer cells in live cell culture, we next studied the level and distribution of acetylated alpha-tubulin by immunostaining. The cells were visualized with confocal fluorescent microscopy. The cytoplasmic level of acetylated alpha-tubulin clearly increased in both MCF and MB cells after treatment with 5  $\mu$ M of PEITC for 48 hours, which can be directly visualized under confocal fluorescent microscope (Figure 2).

### Effect of combination of PEITC and taxol on cyclin B1 and CDK1 expression

Cyclin B1 and CDK1 are major cell cycle regulatory proteins for the G2 to M phase progression [42]. To explore the involvement of the major cell cycle regulatory proteins, the level of cyclin B1 and CDK1 expression was studied. Their expressions were characterized with Western blotting. When compared with single agent PEITC and taxol, the combination of both agents reduced the expression of CDK1 more significantly than either agent alone (Figure 3). In the mean time, the cyclin B1 expression was minimally decreased, indicating a less significant effect from the treatment.

### Effect of combination of PEITC and taxol on Bax and Bcl-2 expression

Bax and Bcl-2 have opposing effects on apoptosis. Bax promotes apoptosis while Bcl-2 is an anti-apoptosis



**Figure 1 Synergistic effect of phenethyl isothiocyanate (PEITC) and paclitaxel (taxol) on acetylation of alpha-tubulin and expression of tubulins in breast cancer cells.** The MCF7 cells were treated with 5  $\mu$ M of PEITC and 10 nM of taxol alone or in combination. Due to higher resistance of MDA-MB-231 cells, PEITC at 10  $\mu$ M and taxol at 100 nM were used. Proteins were immunoprecipitated with appropriate antibodies and were detected by Western blotting as described in Materials and methods.  $\beta$ -actin level was used as an internal loading control for protein amount. The relative expression level over control cells was calculated after scanning of the density of each protein band with a densitometer. A representative blot from three or more independent experiments was shown.

protein. The levels of the two proteins in the breast cancer cell lines were examined through Western blotting analysis. When compared with single agent PEITC and taxol, the combination of both agents reduced Bcl-2 expression and increased Bax expression more than either agent alone (Figure 4).

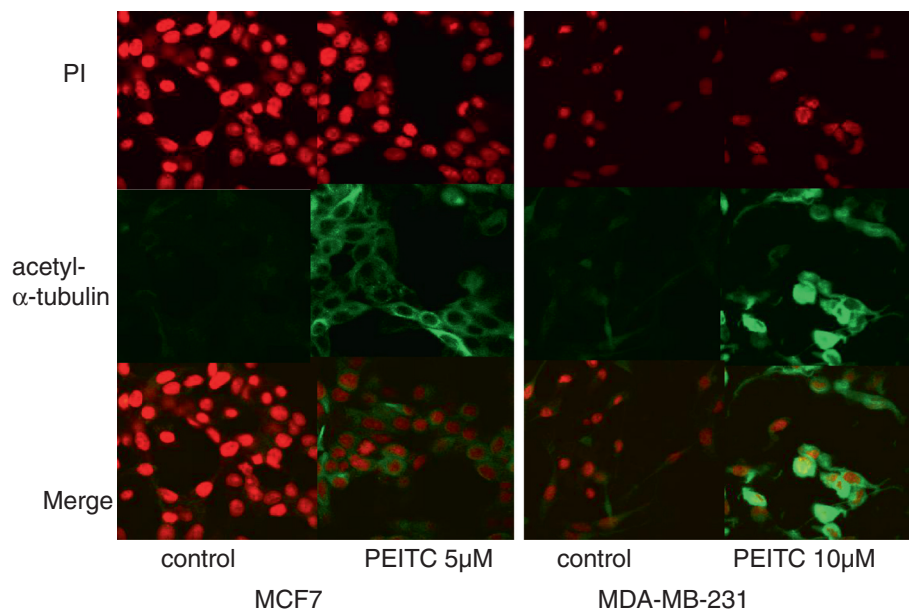
#### Effect of combination of PEITC and taxol on PARP cleavage

PARP proteins are important downstream components of the apoptosis pathways. Cell cycle arrest usually triggers the apoptosis machinery which leads to cellular apoptosis and cell death. The PARP protein cleavage in MCF and MB cells was examined. When compared with

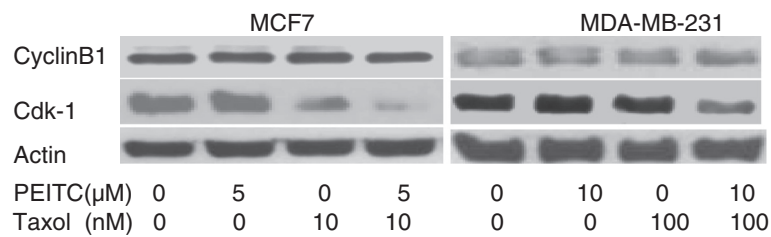
single agent PEITC and taxol, the combination of both agents increased the PARP-1 cleavage (thus an increase in the degradation products as described [43]) more than either agent alone in both cell lines (Figure 4).

#### Discussion

It has been shown that tubulin acetylation primarily occurs on assembled microtubules [44]. PEITC has been previously found to directly bind to alpha- and beta-tubulins, thus inhibiting microtubule polymerization in prostate cancer cells [45]. In this study, PEITC was shown, for the first time, to induce hyperacetylation of alpha-tubulin in two different breast cancer cell lines. It is possible that PEITC can inhibit the synthesis of alpha-



**Figure 2 Acetyl-alpha-tubulin immunofluorescence in the cytoplasm of MCF7 and MDA-MB-231 breast cancer cells.** The MCF7 cells and MDA-MB-231 cells were treated with or without PEITC. The cells were stained with anti-acetyl-alpha-tubulin. Fluorescin-labeled secondary antibody was then added. The cells were visualized under confocal fluorescent microscope. The nuclei were counter stained with propidium iodide (PI). In the treated cells, acetylated alpha-tubulin was seen in the cytoplasm.



**Figure 3 Combination of phenethyl isothiocyanate (PEITC) and paclitaxel (taxol) inhibits cell cycle regulator cyclin B1 and Cdk1 in MCF7 and MDA-MB-231 breast cancer cells.** The MCF7 cells were treated with 5 μM of PEI and 10 nM of taxol alone or in combination. Due to higher resistance of MDA-MB-231 cells, PEI at 10 μM and taxol at 100 nM were used. Cyclin B1 and Cdk1 were immunoprecipitated with appropriate antibodies and were detected by Western blotting as described in Materials and methods. β-actin level was used as an internal loading control for protein amount. A representative blot from three or more independent experiments was shown.

tubulin deacetylase HDAC6 [41] (data not shown). This may help to explain the previous findings that some HDAC inhibitors, such as TSA but not butyric acid, can cause alpha-tubulin hyperacetylation [46]. This study also provided evidence to illustrate the possible mechanisms for the synergistic anti-growth effect of PEITC and taxol to be due to hyperacetylation of alpha-tubulin. This synergism is best explained by the fact that taxol enhances tubulin acetylation by inhibiting depolymerization of microtubules and thus leads to availability of more substrates for acetylases, whereas PEITC decreases tubulin deacetylation.

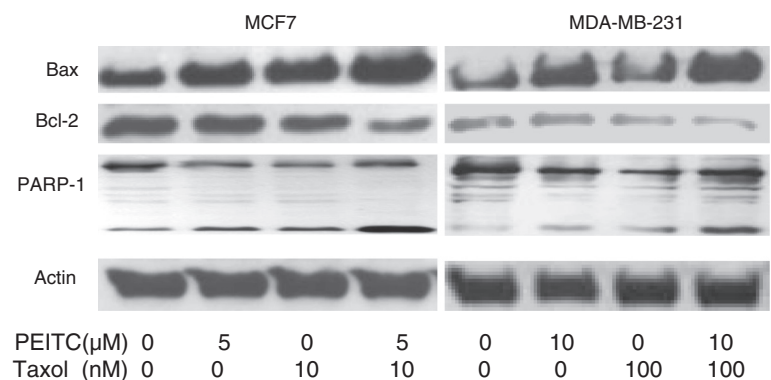
This study also showed that the combination of PEITC and taxol enhanced apoptosis by decreasing bcl-2 expression and by increasing BAX expression as well as degradation of PARP. The combination of the two agents also reduced CDK1 expression. These biochemical data provided the foundation of the mechanisms for the synergistic effects of the two agents on apoptosis and cell cycle arrest. The similar mechanism was also found to be responsible for PEITC inhibition of prostate cancer cells [32,35,47-49]. Further study of this effect on prostate cancer cells are ongoing in our laboratory.

Our lab and others have shown that PEITC has little toxic effects on normal cells [35,38,50]. However, taxol has significant toxicity at higher dosage and after prolonged use. We therefore hypothesize that by combining PEITC and taxol, it is possible to significantly reduce toxicity *in vivo* by reducing the dosage of taxol needed while maintaining clinical efficacy for breast cancer and possibly other solid tumors. This hypothesis will be tested first in mouse model carrying breast cancer xenografts.

The HDAC inhibitor vorinostat has been shown to up-regulate estrogen receptors and make breast cancer cells more sensitive to tamoxifen [51]. HDAC inhibitor was found to redirect the response of breast cancers cells to tamoxifen from cell cycle arrest to apoptosis [52]. Since PEITC is a HDAC inhibitor as well as a tubulin-targeting agent, it would be worthwhile to test the combination of PEITC and tamoxifen for therapy of hormone-refractory breast cancer.

### Conclusion

This study provided biochemical evidence for the mechanism of synergistic effect between the epigenetic agent



**Figure 4 Synergistic effect of phenethyl isothiocyanate (PEITC) and paclitaxel (taxol) on expressions of Bcl-2, Bax, PARP in MCF7 and MDA-MB-231 breast cancer cells.** The MCF7 cells were treated with 5 μM of PEITC and 10 nM of taxol alone or in combination. Due to higher resistance of MDA-MB-231 cells, PEITC at 10 μM and taxol at 100 nM were used. Cell lysates were immunoprecipitated with appropriate antibodies and were detected by Western blotting as described in Materials and methods. β-actin level was used as an internal loading control for protein amount. A representative blot from three or more independent experiments was shown.

PEITC and the chemotherapeutic agent taxol. This novel strategy deserves further study *in vivo* in animal models and may provide a new and enhanced treatment option for breast cancer patients.

#### Competing interests

The authors have no relevant competing interests.

#### Authors' contributions

All authors have contributed to data preparation, drafting and revising the manuscripts. All authors have read and approved the final manuscript.

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