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INHIBITION OF AN EPIGENETIC MODULATOR, HISTONE DEACETYLASE BY PEITC IN BREAST CANCER – A DETAILED MECHANISTIC APPROACH.

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ABSTRACT

Histone deacetylases (HDACs) are enzymes that modify the architecture of chromatin leading to decreased gene expression, the effect of which may be reversed by HDAC inhibition. The balance between acetylation and deacetylation of histones play a key role in many biological events including regulation of cell proliferation and cancer. There are certain inhibitors of HDACs which showed toxicity, therefore search of natural inhibitors is of tremendous importance. The aim of the present study is to investigate the effect of PEITC, a natural isothiocyanate found in cruciferous vegetables on HDAC and to elucidate the mechanism of action. We found that PEITC significantly inhibited the expression of class I HDACs (HDAC1 and HDAC2), both at protein and genetic level in two different human breast cancer cell lines: breast adenocarcinoma cell MCF-7, having wild type p53 and metastatic breast cancer cell MDA-MB-231, having mutated p53. Inhibition of HDAC 1 and 2 were associated with increased acetylation of lysine residues of histones H3 and H4. p53 and cMyc expressions are expected to play a role in regulation of telomerase activity. PEITC furthermore markedly inhibited the activity of telomerase and the expression of human telomerase reverse transcriptase (hTERT), which is the main determinant of the telomerase enzymatic activity. Transcriptional downregulation of hTERT mRNA by PEITC is mediated by repression of transcription factor c-Myc in both the cell lines. PEITC induced upregulation of p21 and p27 proteins independent of p53 status and this may be due to the downregulation of c-Myc expression. Elevated expressions of p21 and p27 were associated with concommitant downregulation of cyclin D level, a cell cycle regulatory protein, which is a probable cause of cell cycle arrest. PEITC therefore may be a potential compound that can be used as an antitumor agent in breast cancer irrespective of p53 status.

INTRODUCTION

Genetic instability is a key feature of cancer development which is often associated with the acquisition of mutations in oncogenes, tumor suppressor genes, and DNA repair genes[1]. Post-translational modifications of histones and chromatin-binding proteins provide a regulatory platform for gene transcription, DNA replication and repair of DNA damage[2,3]. Epigenetic modifications such as acetylation and deacetylation of histones influence architecture of chromatin. These modifications are mainly regulated by the opposing activities of histone acetyl transferases (HATs) and histone deacetylases (HDACs)[4]. Disruption of epigenetic processes like imbalance in the expressions of HDAC and HAT lead to altered gene function and ultimately malignant cellular transformation[5]. Chromosomal mapping and DNA sequencing studies have revealed a global loss of acetylated H4 at lysine 16 (H4K16ac) residue[6], which is mediated by HDACs resulting in gene repression[5]. Overexpression of HDACs is frequently observed in various types of cancer which lead to alteration in cellular epigenetic programming to promote cell proliferation and survival[7]. HDAC inhibitors are emerging as potent anticancer agents which can reactivate gene expression and restore the capability of malignant cells to undergo programmed cell death[8]. Most of the researches on HDAC inhibitors have focused on up regulation of p21 expression in a p53 independent manner via increased acetylation of the chromatin at sp1 sites in the p21 promoter region[9]. p21 being a key player in cell cycle process, is regulated by a variety of mechanisms and among them down modulation by c-Myc has been found to be an important event in a wide variety of tumor cases[10]. Studies indicated that c-Myc interacts with and antagonize the activity of transcriptional regulators such as sp1 and sp3 and thus suppress the transcription of p21[11]. The activity of the RNA dependent DNA polymerase enzyme telomerase is high in 90% of human

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cancers[12] including breast cancer[13,14]. Telomerase activation is essential for the stabilization of chromosomes in proliferating cells which ultimately leads to cellular immortality and oncogenesis[15]. Epigenetic modulation of the hTERT core promoter region may provide transcriptional regulation of TERT[16]. Several transcription factors like c-Myc and p21 plays a pivotal role in both the expression and indicated that the intake of cruciferous vegetables containing dietary isothiocyanates is inversely related with incidences of cancer[19]. Phenethylisothiocyanate (PEITC) belonging to isothiocyanate family act as growth inhibitor and induce apoptosis in various cancers including breast cancer[20,21]. Growth inhibitory effect of PEITC was reported to be related with arrest at different

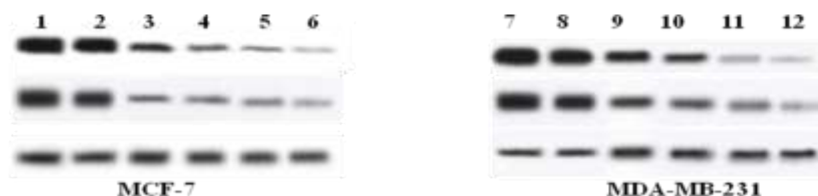

Fig. 1 (a).

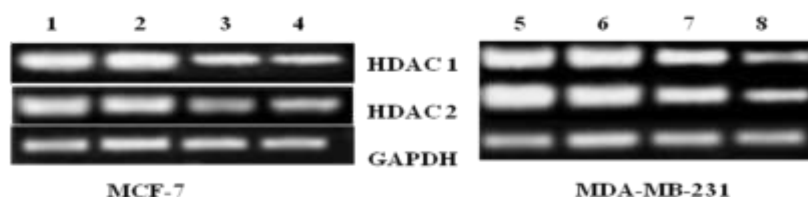

Fig. 1 (b).

Fig 1. Effect of PEITC on HDAC 1 and HDAC 2 at protein and genetic level in MCF-7 and MDA-MB-231.

1. (a) Cells were exposed to various concentrations 0 µM (lanes 1, 7); 0.5 µM (lanes 2, 8); 2.5 µM (lanes 3, 9); 5 µM (lanes 4, 10) and 7.5 µM (lanes 5, 11) of PEITC for 24 h. Lanes 6, 12 represent HDAC1 and 2 expressions in MCF-7 and MDA-MB-231 cells after treatment with TSA (1 µM). Proteins were analyzed by western blot analysis using specific antibodies against HDAC 1 and HDAC 2. β-actin was used as control to ensure equal loading of protein.
1. (b) Cells were incubated with PEITC 0 µM (lanes 1, 5); 2.5 µM (lanes 2, 6); 5 µM (lanes 3, 7) and 7.5 µM (lanes 4, 10) for 24 h to examine mRNA levels of HDAC 1 and 2. Total RNA was isolated and reverse transcribed. The resulting cDNAs were subjected to PCR with primers and the reaction products were subjected to electrophoresis using 2% agarose gel and visualized by EtBr staining. GAPDH was used as an internal control.

repression of telomerase by regulating the transcription of hTERT gene[15,17]. Telomerase inhibition therefore becomes an attractive target for cancer therapeutics. HDAC inhibitors are reported to de-repress hTERT gene in human cells and it was proposed that acetylation and deacetylation of histones are a common underlying feature to hTERT trans-activation and repression respectively in normal and malignant cells[18]. However the mechanisms underlying telomerase inhibition by HDAC inhibitors are controversial and scanty. Furthermore most of the studies were carried out using synthetic inhibitors. Epidemiological surveys phases of the cell cycle[22,23]. It was furthermore demonstrated in our laboratory that PEITC efficiently sensitized the effect of chemotherapeutic drugs via modulation of protein kinase C (PKC) and telomerase in cervical and prostate cancer cells[24,25]. PEITC functions as a potent disruptor of ER-α and provides a strong explanation for its the growth inhibitory activity in breast cancer cells[21]. Previous studies identified PEITC as an inhibitor of histone deacetylases (HDACs) in case of prostate cancer[26]. However, the mechanism underlying epigenetic modulation of cancer cells by PEITC needs to be elucidated.

The present study aims to evaluate the effect of PEITC on HDAC 1 and 2 in two different breast cancer cell lines MCF-7 and MDA-MB-231 and to elucidate the detailed mechanism involved therein.

Table 1. INHIBITION OF TELOMERASE ACTIVITY IN BREAST CANCER CELL LINES BY PEITC

Concentration	Telomerase inhibition in	
used (μM)	MCF-7	MDAMB-231
None	0	0
2.5	21.9±2.3*	18.7±2.0*
5.0	38.2±2.5*	42.8±3.5*
7.5	76.4±3.3*	64.2±3.6

The band intensity obtained from the lane with untreated control cells was considered to have 100 % telomerase activity and accordingly those obtained from lanes of treated ones were normalised.

*Telomeric repeat bands represent the mean intensity of several bands from three independent experiments ± S.D.

MATERIALS AND METHODS

Chemicals

MEM, DMEM, Ham's F12, fetal bovine serum (FBS), gentamycin, streptomycin, acrylamide, N, N'-methylene bis-acrylamide and sodium dodecyl sulphate (SDS) were procured from InVitrogen India Pvt Ltd. Ponceau S, dithiothreitol (DTT), bovine serum albumin (BSA), ethylene glycol-O,-O'-bis,(2-aminoethyl) N,N,N',N'- tetraacetic acid (EGTA), 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), RNase and propidium iodide (PI) were purchased from Sigma Chemical Co, St. Louis, MO, USA; Phenethylisothiocyanate (PEITC) was purchased from Fluka Chemicals, Sigma Aldrich, USA and Trichostatin A was purchased (TSA) from Sigma Aldrich, USA. Goat anti-mouse IgG-alkaline phosphatase conjugate and 5-bromo-4-chloro-3-indolyl phosphate/ nitro blue tetrazolium (BCIP-NBT) were purchased from Bangalore Genei, India. Nitrocellulose membrane was purchased from Hybond ECL, Amersham Biosciences, U.K. Telomerase detection kit was purchased from Allied Biotech, USA. The RNAqueous 4PCR kit, RETROscript kit and Super Taq Plus polymerase were purchased from Ambion/Applied Biosystem, Austin, Texas, USA. Antibodies used in this study viz. anti-HDAC1 (05-614), anti-HDAC2 (05-814), anti-acetyl histone H3 (07-352), anti-acetyl histone H4 (07-329), were purchased from Upstate, Millipore, Massachusetts, USA. Anti-hTERT mouse monoclonal [2C4] (ab5181), anti-p21 mouse monoclonal [EA10] (ab16767), anti-p27 KIP1 mouse monoclonal (ab54563), anti-cyclin D1 rabbit polyclonal [DCS-6] (ab10540), anti-cMyc mouse 35monoclonal [9E10] (ab32), anti-Sp1 rabbit polyclonal (ab13370) and anti-β-actin [AC-15] (ab6276) were purchased from abcam, Cambridge, Massachusetts, USA. Other reagents used were of analytical grade and procured locally.

Table 2. OLIGONUCLEOTIDE USED IN RT-PCR.

Name		Sequence of Primers
HDAC 1	Sense	5'-ACC-GGG-CAA-CGT-TAC-GAA-T-3'
	Antisense	5'-CTA-TCA-AAG-GAC-ACG-CCA-AGT-G-3'
HDAC 2	Sense	5'-TCA-TTG-GAA-AAT-TGA-CAG-CAT-AGT-3'
	Antisense	5'-CAT-GGT-GAT-GGT-GTT-GAA-GAA-G-3'
hTERT [a]	Sense	5'-AGC-CAG-TCT-CAC-CTT-CAA-CC-3'
	Antisense	5'-GTT-CTT-CCA-AAC-TTG-CTG-ATG-3'
c-Myc	Sense	5'-AAG-ACT-CCA-GCG-CCT-TCT-CTC-3'
	Antisense	5'-AAG-ACT-CCA-GCG-CCT-TCT-CTC-3'
Sp-1	Sense	5'-ACA-GGT-GAG-VTT-GAC-CTC-AC-3'
	Antisense	5'-GTT-GGT-TTG-CAC-CTG-GTA-TG-3'
GAPDH [b]	Sense	5'-CGG-AGT-CAA-CGG-ATT-TGG-TCG-TAT-3'
	Antisense	5'-AGC-CTT-CTC-CAT-GGT-GGT-GAA-GAC-3'

[a]hTERT: human telomerase reverse transcriptase
[b]GAPDH:glyceraldehydes 6 phosphate dehydrogenase

Table 3. EFFECT OF PEITC ON CELL CYCLE DISTRIBUTION IN MCF-7 CELLS.

Concentration used (µM)	% Cells			
	SubG1	G0/G1	S	G2/M
None	2.75±0.95	54.23±2.5	18.56±2.0	24.46±1.6
0.5	3.32±0.94	58.36±3.0	16.85±1.8	21.47±2.1
2.5	3.87±0.82	63.2±2.2	14.02±2.4	18.91±2.3
5.0	5.95±0.96	70.28±3.1	10.31±1.1	13.46±2.0*
7.5	8.24±1.2*	79.17±2.7*	2.75±1.2	9.84±2.9*

Cells were treated with different concentrations of PEITC for 24 h. Cells were then collected, harvested and processed for analysis of cell cycle distribution by flow cytometry following staining with propidium iodide. Data are means ± SE (n = 3).
*$p < 0.05$, significantly different compared with control (paired sample t Test using SPSS software).

Maintenance of cell lines

Two human breast cancer cell lines: MCF-7 cells (ER+, EGFR-, HER2- having wild type p53) and highly metastatic breast cancer cell line MDA-MB 231 cells (ER-, PR-, HER2 -but EGFR over-expressing having mutated p53) were routinely maintained in our laboratory. MCF-7 cell was maintained in MEM and MDA-MB-231 cell was maintained in DMEM. Normal breast epithelial cell line MCF-12F was used in this study as a control and was maintained in a 1:1 mixture of DMEM and HAM's F12 medium with 0.4 mM Ca^{++}, 20 ng/ml epidermal growth factor. Cells were grown at $37^{0}C$ in the presence of 15% fetal bovine serum and antibiotics in a humidified atmosphere of 5% CO_2 /95% air.

PEITC Treatment

Confluent cells (MCF-7, MDA-MB-231 and MCF-12F) were treated with 0.5, 2.5, 5.0 and 7.5 µM PEITC for 24 h. Cells treated with 1 µM TSA for 24 h was used as positive control.

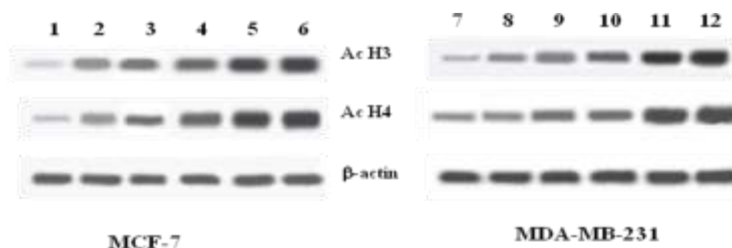

Fig. 2.

Fig. 2. Effect of PEITC on the acetylation of histones H3 and H4 in MCF-7 and MDA-MB-231 cells.
Both the cells were treated with various concentrations 0 µM (lanes 1, 7); 0.5 µM (lanes 2, 8); 2.5 µM (lanes 3, 9); 5 µM (lanes 4, 10) and 7.5 µM (lanes 5, 11) of PEITC for 24 h. Lanes 6 and 12 represent the expressions of histones in MCF-7 and MDA-MB-231 after treatment with TSA (1 µM) for 24 h. The histone fractions were isolated and 20 µg of the protein were electrophoresed on 15% SDS-PAGE gel and, blotted and probed with antibodies against H3 and H4. β-actin was used as loading control.

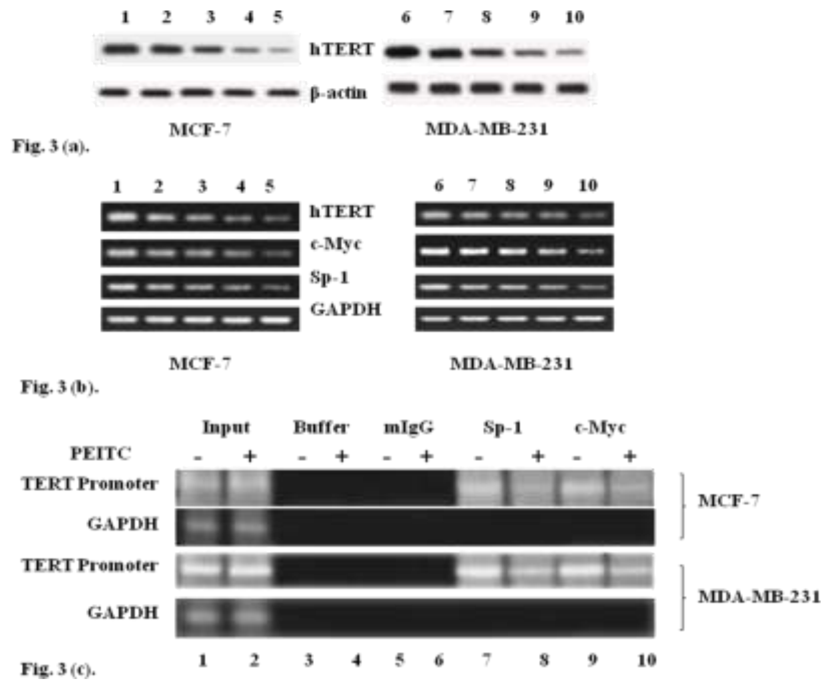

Fig. 3. Effect of PEITC on the protein and mRNA expressions of hTERT in MCF-7 and MDA-MB-231 cells. 3.(a). Western blot analysis was carried out to determine the expressions of hTERT protein in breast cancer cells. Lanes 1 and 6 represent the constitutive expressions of hTERT protein in MCF-7 and MDA-MB-231 cells respectively without any treatment. Cells were treated with PEITC at concentrations 0.5 µM (lanes 2, 7); 2.5 µM (lanes 3, 8); 5 µM (lanes 4, 9) and 7.5 µM (lanes 5, 10) for 24 h. β-actin was used as loading control. 3. (b). Expression of mRNAs were carried out following RT-PCR technique. RNAs were isolated after treatment of cells with different concentrations of PEITC as that mentioned in Fig 3.(a). RT-PCR was performed using indicated primers. The amplified PCR products were run in a 2% agarose gel and visualized by EtBr staining. GAPDH was used as a house-keeping control gene. 3. (c). Detection of c-Myc and hTERT gene promoter complex in response to PEITC by ChIP (Chromatin immunoprecipitation) analysis. MCF-7 and MDA-MB-231 cells were treated with (lanes 2, 4, 6, 8 and 10) or without (lanes 1, 3, 5, 7 and 9) PEITC (7.5 µM) for 24 h followed by ChIP with control or specific antibodies. The DNA hTERT promoter and human GAPDH gene were detected by PCR using specific primers. mIgG indicates mouse IgG. The data were representatives of two or more experiments from independent immunoprecipitations.

Western blot analysis

Cells after treatment with PEITC following treatment protocol was harvested, washed in wash buffer pH 7.5 of final working concentration (HEPES-KOH 10mM, $MgCl_2$ 1mM, KCl 10mM, DTT 1mM) followed by lysis in lysis buffer of final working concentration (Tris.HCl 10µM pH 7.5, $MgCl_2$ 1mM, β-mercaptoethanol 0.065%, CHAPS 0.5% and glycerol 10%). The lysates were then centrifuged at 12,000 rpm for 20 min and protein concentration was quantified following Lowry's method. Equally loaded proteins from cell lysates were then electrophoresed on 10% SDS-polyacrylamide gel using electrophoresis buffer (Tris 25mM, glycine 192mM, SDS 10%) and separated proteins were electro-transferred to nitrocellulose membranes using transfer buffer (Tris 250 mM, glycine 192 mM, methanol 10%) which was followed by proper washing and blocking with 5% BSA. Determination of expressions of HDAC 1, HDAC 2, hTERT, p21, p27 and cyclin D1 were performed after incubating membranes with specific antibodies as described previously. Membranes were thereafter washed properly with TBST and incubated with alkaline phosphatase conjugated anti-mouse IgG (1:1000 dilutions in TBS). Expressions of proteins were finally detected by incubating membranes with the substrate BCIP/NBT.

Determination of hyperacetylated histones (H3 and H4)

Cells after treatment with different concentrations of PEITC for 24 h was harvested and washed with PBS. Cells were pelleted and resuspended in 1 ml ice-cold lysis buffer (10 mM Tris.HCl, pH 6.5, 50 mM sodium bisulfite, 1% Triton X-100, 10 mM $MgCl_2$ and 8.6% sucrose) before homogenization with two dounce strokes. Nuclei were centrifuged at 1200 x g for 5 min and washed 3 times with 1 ml of lysis buffer. The final wash was performed with 1 ml of Tris.EDTA solution (10 mM Tris.HCl, pH 7.4, 13 mM EDTA). Nuclei was pelleted and resuspended in ice-cold water. Sulfuric acid was added to the samples to a final concentration of 0.2 M, vortexed and incubated on ice followed by centrifugation. The supernatant was precipitated with acetone and protein was collected by centrifugation, air dried, and resuspended in water. Proteins were then electrophoresed on SDS-polyacrylamide gel followed by electroblotting on nitrocellulose membrane which was then soaked in a blocking buffer containing bovine serum albumin. After proper washing the membrane was incubated with antibodies against antiacetylated H3 antiserum and antiacetylated H4 antiserum and protein expression was determined following the method as described previously.

Table 4. EFFECT OF PEITC ON CELL CYCLE DISTRIBUTION IN MDA-MB-231 CELLS.

Concentration used (μM)	% Cells			
	SubG1	G0/G1	S	G2/M
None	2.22±0.95	51.64±2.3	15.61±2.1	30.53±1.4
0.5	2.89±0.96	56.46±2.0	14.03±2.0	26.62±1.9
2.5	4.52±0.88	61.35±1.9	12.79±3.2	21.34±1.9
5.0	5.25±0.97	64.24±3.1	11.86±2.4	18.65±1.2
7.5	7.96±1.1*	70.26±3.7*	9.24±2.4	16.54±1.1

Cells were treated with different concentrations of PEITC for 24 h. Cells were then collected, harvested and processed for analysis of cell cycle distribution by flow cytometry following staining with propidium iodide. Data are means ± SE (n = 3).
*$p < 0.05$, significantly different compared with control (Paired Samples T-Test using SPSS software).

Detection of telomerase activity

The Quantitative Telomerase Detection Kit was used for determining the activity of telomerase following the instructions of manufacturer (Allied Biotech, USA) as described by Chakraborty *et al.* [27]. Briefly, exponentially growing cells were treated with PEITC of different concentrations as that used for HDAC inhibition for 24 h. Cells were harvested, lysed in lysis buffer (provided in the kit) and kept in ice for 30 min. Cell extract (1 μl), 2 × QTD premix and PCR qualified water (provided in the kit) was thereafter mixed thoroughly and incubated in the thermal cycler for allowing telomeric substrate to extend by the cell extract at 25^0C for 20 min. Initial PCR activation step was continued for 10 min at 95°C followed by amplification of the telomeric product by PCR (95°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec; 35–40 cycles) using the reverse primer, provided in the kit. PCR products were resolved on 10% non-denaturing PAGE using double dye bromophenol blue and xylene cyanol. Gel was washed properly with 0.5 × TBE buffer and soaked in the same buffer containing SYBR green stain (10,000 × concentrate stock in DMSO) diluted up to 10,000-fold to make a final 1 × stock. Finally gel was observed under the Gel Documentation System. Intensities of clearly separated bands from each lane were measured and inhibition of telomerase activity was calculated accordingly.

RNA extraction and semiquantitative reverse transcription-PCR analysis

Total RNA from the treated cells was isolated by using a RNAqueous 4PCR kit (Ambion/Applied Biosyatem) following the manufacturer's instructions. Complementary DNA was synthesized from 2 μg of total RNA using RetroScript kit

(Ambion/Applied Biosystems). The complementary DNA was then amplified by PCR for 30 cycles with an initial hot start followed by denaturation at 940C for 30 s, annealing at 550C for 30 s, extension at 550C for 30 s and final extension at 720C for 90 s using forward and reverse primer sequences. For the positive control, a constitutively expressed ‘housekeeping’ gene , glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was taken. Amplification products obtained by PCR were electrophoretically separated on 2% agarose gel and visualized by ethidium bromide staining under the Gel Documentation System.

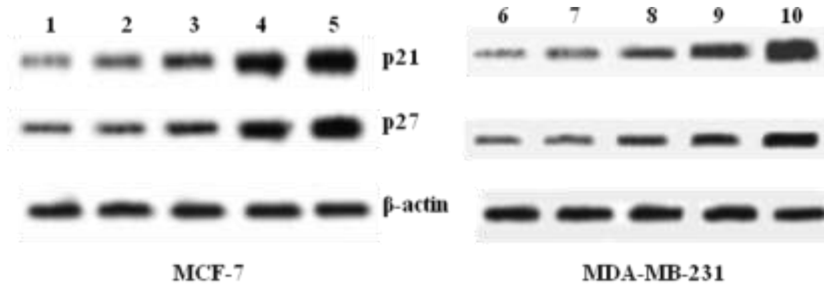

Fig. 4 (a).

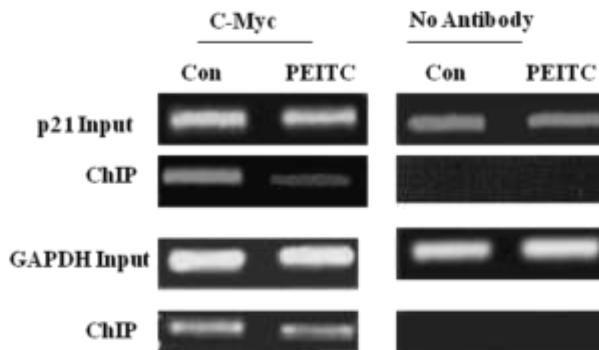

Fig. 4 (b).

Fig. 4. Regulation of cell cycle proteins by PEITC in MCF-7 and MDA-MB-231 cells.

4. (a) Exponentially growing cells were exposed to PEITC of different concentrations for 24 h. Cells were harvested, total proteins were extracted and 50 µg proteins was subjected to western blotting using antibodies against p21 or p27. β-actin was used as control to ensure equal loading of protein. Lanes 1 and 6 denotes the expressions of p21 and p27 in untreated control cells. Lanes 2 and 7 denotes expression of proteins after treatment with PEITC (0.5 µM). Lanes 3 and 8 denotes after treatment with 2.5 µM; lanes 4 and 9 after treatment with 5 µM whereas lanes 5 and 10 designates after treatment with 7.5 µM PEITC. **4. (b)** c-Myc and P21 gene promoter complex were detected in response to PEITC. c-Myc antibody was used to immunoprecipitate soluble chromatins from MCF-7 cells cultured either with or without PEITC (7.5 µM) for 24 h. Immunoprecipitated DNAs were subjected to PCR using primers for the p21 and GAPDH genes as templates. The data were representatives of two or more experiments from independent immunoprecipitations. Con = Control.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assay was carried out using MCF-7 and MDA-MB-231 cells. Briefly, confluent cells were treated with or without 5 µM PEITC for 24 h. Cells were fixed with 1% formaldehyde for 10 min at room temperature after completion of treatment followed by neutralization after addition of glycine at a final concentration of 0.125 M for 5 min. Fixed cells were then harvested carefully and sequentially washed at room temperature for 15 min each with buffer I (Tris.HCl 10mM, pH 8.0, EDTA 10 mM, EGTA 0.5 mM, Triton X-100 0.25%) and buffer II (Tris.HCl 10 mM pH 8.0,

NaCl 200 mM, EDTA 1mM, EGTA 0.5 mM). Cells were then lysed in lysis buffer (EDTA 10 mm, SDS 1%, Tris.HCl 50 mM, pH 8, PMSF 0.2 mM, aprotinin 5 µg/ml, leupeptin 1 µg/ml, and sonicated salmon sperm DNA 5 µg/ml) for 30 min in ice. Samples were then sonicated for 5-30 s with 5 min intervals on ice. Centrifugation was then carried out for 10 min at 4^0C, supernatants were collected. 50 µl of the supernatant was removed and used as an input control during PCR. Sonicated supernatant was further diluted 10 fold in ChIP dilution buffer (Triton X-100 1%, EDTA 2mM, NaCl 150 mM, Tris.HCl 20 mM, pH 8.0, SDS 0.01%, aprotinin 550 µg/ml, leupeptin 150 µg/ml, PMSF 0.2 mM and sonicated salmon sperm DNA 5 µg/ml). Protein-chromatin complexes were immunoprecipitated overnight at 4^0C with rotation using the following antibodies: mouse control IgG ab18413, mouse monoclonal anti-cMyc [9E10], ab32, and rabbit polyclonal anti-Sp1 ab13370. The chromatin was collected on ImmunoPure plus Immobilized protein A/G sepharose beads. Beads containing protein-DNA complex was washed on a rotating platform for 5 min using following buffer: (a). low salt immune complex wash buffer (SDS 0.1%, Triton X-100 1%, EDTA 2mM, NaCl 150 mM, Tris.HCl 20mM pH 8), (b) high salt immune complex wash buffer (SDS 0.1%, Triton X-100 1%, EDTA 2mM, NaCl 500 mM, Na–deoxycholate 0.1% and Tris.HCl 20 mM pH 8), (c) LiCl buffer (LiCl 0.25 M, NP40 1%, Na-deoxycholate 1%, EDTA 1 mM, Tris.HCl 10 mM pH 8.0), (d) ice cold TE buffer (Tris.HCl 10 mM, EDTA 1 mM pH 8.0). Finally protein-DNA complex was extracted by elution buffer (SDS 1%, $NaHCO_3$ 0.1M) at 65^0C overnight and with 1/25 volume of 5M NaCl for 4-6 h at 65^0C. Samples were extracted twice with phenol/chloroform and precipitated overnight with ethanol. DNA fragments were recovered by centrifugation, resuspended in double distilled water and used for PCR amplification of the p21 and hTERT gene promoter. The primer sequence of p21 gene promoter was ACCGGCTGGCCTGCTGGAACT and TCTGCCGCCGCTCTCT CACCT and the sequence for hTERT promoter was GGCCGGGCTCCCAGTGGATTC and CAGCGGGGAGCGCGCGGCATCG. PCR products were separated on 2% gel and visualized by ethidium bromide staining.

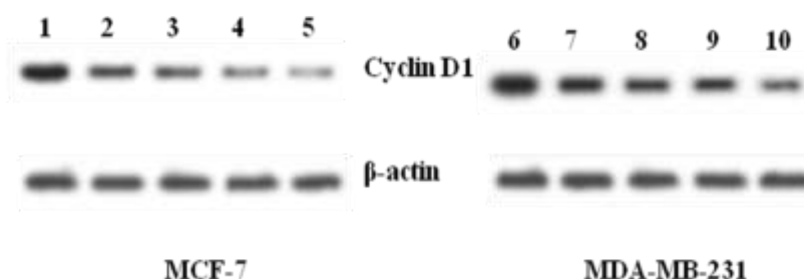

Fig. 5. Effect of PEITC on cyclin D1 expression in breast cancer cell lines MCF-7 and MDA-MB-231. Exponentially growing cells were treated with PEITC at concentrations 0 µM (lanes 1, 6); 0.5 µM (lanes 2, 7); 2.5 µM (lanes 3, 8); 5 µM (lanes 4, 9) and 7.5 µM (lanes 5, 10) for 24 h. Cells were harvested, total cell lysates were prepared and 50 µg from each of the proteins were subjected to western blot analysis using antibody specific for cyclin D1. β-actin was used as loading control. The results were representative of three independent experiments.

Flow Cytometry analysis

Treated cells were harvested, centrifuged at 1200 rpm for 8 min, re-suspended in 1 ml PBS and again centrifuged at 1500 rpm for 5 min at 4^0C. From each pellet, $2x10^6$ cells were taken, suspended in 1ml cold PBS and centrifuged at 1500 rpm for 5 min at 4ºC. Pellets were suspended in 300 µl PBS and cold absolute ethanol (700 µl) was added drop wise with continuous vortexing. Suspension was incubated in ice for 30 min centrifuged to remove ethanol. 1ml DNA binding solution (200 µg/ml RNase + 50 µg/ml PI) was added to each pellet and incubated for 30 min in dark before analysis in Becton Dekinson Flow Cytometer.

Statistical Calculations

SPSS 10.0 (one way ANOVA followed by Dunett T-test) was used for statistical analysis. In Dunett t-test one group is treated as control and all other groups are compared with it.

RESULTS AND DISCUSSION

PEITC inhibits HDAC 1 and 2 expression in breast cancer cell lines.

Constitutive expressions of HDAC 1 and HDAC 2 were observed in two different breast cancer cell lines (MCF-7 and MDA-MB-231) and the results were compared with their normal counterpart MCF-12F. It was observed earlier that expressions of HDAC 1, 2 are high in these breast cancer cell lines compared to MCF-12F[27]]. Effect of PEITC on the expressions of HDAC 1 and 2 was examined in both the breast cancer cell lines. Cells were treated with different concentrations (0.5, 2.5 , 5 and 7.5 µM) PEITC for 24 h. HDAC 1 and HDAC 2 expressions were analyzed using western blot analysis. PEITC treatment inhibited the expressions of HDAC 1 and 2 in a dose dependent manner and particularly at highest concentration significant inhibition was observed in both MCF-7 and MDA-MB-231 cells. The results were represented in Fig. 1(a). Inhibition at this concentration was comparable with that of a known specific inhibitor of HDAC i.e. TSA (lanes 6 and 12) as represented in Fig. 1.(a). This result indicated that PEITC was potent enough to inhibit HDAC (1 and 2) expressions. It was furthermore examined whether PEITC regulates HDACs at transcription levels. Cells were incubated with PEITC (0, 2.5, 5 and 7.5 µM) for 24 h, RT-PCR analysis was performed and the results obtained were represented in Fig. 1(b). The results revealed reduced expressions of HDAC 1 and 2 mRNA by PEITC in both MCF-7 and MDA-MB-231 cells. However, the compound did not affect HDAC expressions in MCF-12F cells (data not shown). This result supported the fact that PEITC, a natural isothiocyanate inhibited HDAC 1 and 2 in breast cancer cells both at protein and at transcriptional level.

Effects of PEITC on histone acetylation:

The effects of PEITC on the intracellular level of histones (H3 and H4) were analyzed. Cells were treated with different concentrations of PEITC as described earlier and the western blot results of the extracted histone from the nuclei were represented in Fig. 2. The result showed increased level of acetylation of both H3 and H4 with increasing concentrations and maximum effect was observed at 7.5 µM treatment (lanes 5, 11). The results were similar to those mediated by TSA (1 µM), a known HDAC inhibitor (lanes 6, 12). PEITC previously has been described as an HDAC inhibitor resulting in to enhanced covalent acetylation of histones [26, 28]. Increased acetylation by PEITC subsequently lead to chromatin unfolding and would allow more accessibility for the transcription of genes associated with the hyperacetylated histones. Hyperacetylation of histones as a result of HDAC inhibition by specific inhibitors was reported earlier to be the most important mechanism of antitumor action[29, 30].

Effects of PEITC on telomerase activity, hTERT expression in breast cancer cells.

In order to determine whether or not the activity and the expression level of telomerase and hTERT would be modulated by PEITC, activity of the enzyme in both treated and untreated cells was performed using quantitative telomerase detection kit. The result revealed multiples of 6 bp interval ladder pattern in untreated control cells which suggest strong telomerase activity whereas treated cells inhibited telomerase activity with dose. Percentage inhibition of telomerase was calculated after measuring band intensity and the result was represented in Table 1. The effect of PEITC on the hTERT protein expression and gene transcription was examined by western blot and RT-PCR technique using specific primers as described in Table 2. The result as represented in Fig. 3(a) revealed concentration dependent hTERT inhibition in both MCF-7 and MDA-MB-231 cells. Fig. 3(b) indicated significant inhibition of hTERT mRNA by PEITC treatment. In addition, the mRNA levels of c-Myc and Sp-1, two transcription factors which play crucial roles in hTERT transcription were also down regulated by PEITC (Fig. 3b). These findings strongly correlated with other reports where specific HDAC inhibitors were reported to inhibit telomerase activity and hTERT gene transcription [31-33]. To determine the binding potentiality of transcription factors Sp-1 and c-Myc to the endogenous hTERT gene promoter, ChIP analysis was carried out in MCF-7 and MDA-MB-231 cells using specific anti-Sp-1 and anti-c-Myc antibody. Negative control for antibodies included diluent and mouse IgG. DNA from cell lysates prior to immunoprecipitation was considered as Input. The result as revealed in Fig. 3(c) showed that in untreated cells antibodies against Sp-1 and c-Myc were able to strongly precipitate hTERT promoter indicating the binding of these proteins. On the contrary, no amplified

product was detected with control IgG, verifying specificity of antibodies used in this study. Interestingly very poor signal of Sp-1 and c-Myc were detected after treatment with PEITC suggesting that both Sp-1 and c-Myc interacts with the hTERT promoter and increase its transcription. The result thus showed that PEITC induced hTERT gene inhibition by inhibiting the binding affinity of transcription factors Sp-1 and c-Myc to hTERT promoter, resulting in to transcriptional repression.

Effect of PEITC on cell cycle regulatory proteins:

Effect of PEITC on the activation of the inhibitors of cyclin dependent kinases such as p21 and p27 were investigated. It was observed that PEITC increased the protein level of p21 in both MCF-7 (wild type p53) and MDA-MB-231 (mutant p53). The result as depicted in Fig. 4(a) indicated that up-regulation of p21 was independent of p53 status. Another interesting observation was significant up-regulation of p27 proteins compared to untreated control cells. Alteration of cell cycle regulators were reported to be caused by change of protein stability via post-translational modifications[34]. It was observed earlier that recruitment of HDACs resulted in transcriptional repression whereas HDAC inhibition leads to transcriptional augmentation of p21 and p27 proteins[35]. Thus inhibition of HDAC by PEITC remarkably upregulated p21 and p27 expressions in breast cancer cell lines.

Transcription factor c-Myc gets overexpressed in tumor cells due to overexpression of HDAC and it has been shown that c-Myc interacts with p21 promoter resulting into transcriptional repression[36]. It was therefore examined whether PEITC could induce the release of c-Myc repression from p21 gene promoter. This was addressed by performing ChIP assay to determine whether c-Myc binding ability to the p21 promoter reduces in presence of PEITC. Both treated and untreated breast cancer cells were immunoprecipitated with antibody against c-Myc and were subjected to PCR amplification using primers specific for p21 promoter fragment. C-Myc was able to precipitate p21 promoter in untreated cells which indicated the binding of this protein with the promoter (Fig. 4b). Treated cells on the other hand exhibited very poor binding ability of c-Myc on the p21 promoter region. On the contrary no amplified product was detected with control IgG which verifies the specificity of c-Myc binding. This result strongly indicated that c-Myc plays a role in repression of p21 expression and down-regulation of this transcription factor by PEITC resulted in de-repression of p21 in breast cancer cells.

Effect of PEITC on cell cycle regulation

The possible effect of PEITC on cell cycle distribution by flow cytometric analysis was investigated thoroughly. Representative histograms for cell cycle distribution following exposure to various concentrations of PEITC were tabulated in Table 3 (MCF-7) and Table 4 (MDA-MB-231) cells. As shown in Table 3, treatment of MCF-7 cells with PEITC (7.5 μM) for 24 h resulted in a significant increase in cell population in the G0/G1 phase (79.17%) compared to untreated control cells (54.23%). Similar observations were found in case of MDA-MB-231 cells where PEITC (7.5 μM) increased G0/G1 cell population to 70.26% compared to untreated cells (51.64%). This increase was coupled with decreased percentage of cell population in S phase and subsequent G2/M phase. After treatment for 24 h, the percentages of cells in S phase in PEITC treated MCF-7 cell was 2.75%, whereas in control cells it was 18.56%. Similarly in case of MDA-MB-231 cells decrease in cell population at S phase after PEITC treatment was observed (9.24%) compare to untreated control cells (15.61%). These data suggested that PEITC exerted G0/G1 phase arrest attributing growth inhibition. These findings correlated with previous findings of up-regulated p21 and p27 expressions. p27 is a specific inhibitor of cyclin dependent kinases and is mainly involved in the G1/S transition of the cell cycle[37]. Cyclins and CDKs play an essential role in regulating cell cycle progression[38]. Perturbation of cyclins or CDKs thus may contribute to altered cell cycle distribution. Cyclin D1, a member of the cyclin family plays an important role in G1-S transition[38]. Since PEITC induced G0-G1 cell cycle arrest therefore expression of cyclin D1 was examined in both MCF-7 and MDA-MB-231 cells. As shown in Fig. 5 cellular protein expression of cyclin D1 down regulated after PEITC treatment in a concentration dependent manner suggesting that PEITC up-regulates p21 and p27 proteins thereby eventuating in cell cycle arrest at G0/G1 by inhibiting cyclin D1 expression.

In conclusion, PEITC, a natural isothiocyanate significantly inhibited HDAC 1 and 2, two important enzymes belonging to class I HDACs and induced hyperacetylation of histones leading to transcriptional de-repression in breast cancer cells. Inhibition of HDACs was associated with induction of

down-regulation of transcription factors c-Myc and Sp-1 which was correlated with specific inhibition of hTERT expression and telomerase activity. It was apparent that PEITC decreased c-Myc binding ability to the hTERT gene promoter region repressing genetic transcription and inhibit telomerase activity. Furthermore down-regulation of c-Myc results into release of c-Myc from the endogenous p21 promoter causing increased expression of p21. p21 and p27 up-regulation leads to cell cycle arrest at G0/G1 phase by inhibiting cyclin D1. Taken together, these data demonstrated that PEITC can be developed as an anti-tumor agent targeting HDAC activity and telomerase.

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