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PEITC by regulating Aurora Kinase A reverses chemoresistance in breast cancer cells

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Development of acquired chemoresistance renders a challenge in breast cancer therapy. Aurora kinases, a family of serine/threonine mitotic kinases play pivotal roles in the acquirement of chemoresistance. Aurora A is intricately associated with mitotic events and is overexpressed in different cancers including breast cancer. Amplification or overexpression Aurora A confers chemoresistance and are considered as a promising therapeutic target in cancers. Therefore, targeting Aurora A by natural means particularly by using Phenethyl isothiocyanate (PEITC), a natural isothiocyanate might be an effective strategy for reversing resistance towards chemotherapeutics. The present study investigated the modulatory role of PEITC on Aurora A and their downstream target proteins in breast adenocarcinoma cell line (MCF-7) and its paclitaxel-resistant counterpart; designated as MCF-$7^{Pacli/R}$. Paclitaxel resistance was warranted by P-gp1, MRP1, Ki-67 overexpression, rhodamine 123 accumulations and upregulation of Aurora-A along with phospho-IκBα. Multidrug resistance was confirmed by MTT assay. Western blotting, RT-PCR analysis revealed overexpression of Aurora-A in MCF-$7^{Pacli/R}$ cells; which was eventually diminished by PEITC. PEITC by targeting Aurora A and their downstream proteins (phospho-p53, phospho-IκBα) acted as a resistance-modifying agent and ultimately led to paclitaxel- induced apoptosis. These findings demonstrated that PEITC reverses chemoresistance by regulating Aurora A and restores chemosensitivity towards paclitaxel.

Keywords: Aurora-A, Chemoresistance, Paclitaxel, Phenethyl isothiocyanate, Threonine

The development of acquired chemoresistance is a major challenge to combat the recurrence of breast cancer in spite of the recent advancement in the treatment modality. Many molecular markers have been identified to date that is responsible for the multidrug resistance in cancer cells and its relapse[1-3]. Apart from the known molecular markers like P-glycoprotein 1 (P-gp1), Multidrug resistance-associated protein 1 (MRP1), ATP-binding cassette sub-family G member 2 (ABCG2) (drug efflux markers) responsible for decreasing efficacy of the drug, organic anion transporter (OAT), organic cation transporter (OCT) (SLC family of drug influx markers) and certain other markers belonging to the family of mitotic kinases like Aurora A and Aurora B (cell cycle regulatory kinase protein) are also contributing a major role in acquirement of chemoresistance[4-10]. Several mitotic and non-mitotic roles of Aurora- A have been identified that are directly associated with the resistance to chemotherapy[11-17]. In human, evolutionary conserved three members of Aurora family (Aurora A, B, and C) have been identified and interestingly they almost share the conserved catalytic domain, indicating their origin from a common ancestor gene[10,18]. Aurora-A, located on chromosome 20q13.2 is reported to be aberrantly expressed in breast cancer[19]. Majorly being

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*Abbreviation:*Aurora A, Aurora Kinase A; PEITC, Phenethyl isothiocyanate; P-gp1, P-glycoprotein 1; MRP1, Multidrug resistance- associated protein 1; ABCG2, ATP-binding cassette sub-family G member 2; OAT, Organic anion transporter; OCT, Organic cation transporter; SLC, Solute Carrier; Ki-67, Kiel-clone 67; BRCA1, Breast cancer type 1 Susceptibility protein; MDM2, Mouse double minute 2 homolog; NF-κB, Nuclear Factor kappa-light-chain-enhancer of activated B cells; IκBα, Nuclear Factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; MCF-7, Michigan Cancer Foundation-7; MCF-7Pacli/R, Paclitaxel resistant MCF-7; MEM, Minimum Essential Medium Eagle; FBS, Fetal Bovine Serum; 5-FU, 5-fluorouracil; MTT, 4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltet razolium Bromide; PBS, Phosphate Buffered Saline; DMSO, Dimethyl sulfoxide; ELISA, Enzyme-linked Immune Sorbent Assay; IC50, half maximal Inhibitory Concentration; PCR, Polymerase Chain Reaction; EtBr, ethidium bromide; PI, Propidium Iodide; BCIP/NBT, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium; TUNEL, Terminal deoxynucleotidyl transferase-mediated dUTP Nick-End Labeling.

a centrosomal protein, Aurora A functions primarily in centrosome maturation and separation. In addition to its involvement in the centrosome, some other noteworthy functions of Aurora A are mitotic entry and exit, bipolar spindle formation and cytokinesis[18,19]. Some of the notable downstream target proteins of Aurora A are p53, BRCA1 or Breast cancer type 1 susceptibility protein and IκBα[20-22]**.** Aurora A phosphorylates p53 at serine 315 and 215 residues, indicating its two different fates. Phosphorylation at serine 315 residue of p53 protein by Aurora A leads to MDM2-mediated proteasomal degradation. Phosphorylation, on the contrary at serine 215 residue of p53 results into lesser DNA binding activity of the tumor suppressor protein to the DNA responsive element of target gene[21,23]. Another important protein of concern is IκBα. Aurora A phosphorylates IκBα at serine 32 and 36 residue and releases NF-κB, which eventually leads to enhanced expression of P-gp1[20,24]. NF-κB, being a transcription factor conversely promotes survival and chemoresistance in solid tumor[25-27].

Therefore, targeting Aurora A would be a rational strategy in the treatment of breast cancer. Several small-molecule inhibitors of Aurora kinases are in trials, but they elicit serious toxicity within cells. Therefore, using natural products in cancer research is considered nowadays as a powerful approach as these natural compounds possess unique structures and mechanisms of actions[28]**.** These compounds having low cost, easy availability and minimal or no toxicity, are used in combination with chemotherapeutic drugs to minimize the toxicity. Accumulating epidemiological evidence indicates an inverse correlation between the intake of cruciferous vegetables and the occurrence of malignant tumors. Phenethyl isothiocyanate (PEITC), an important member of the family of cruciferous vegetables showed interesting anti-tumor properties on several cancer cell lines either alone or in combination with chemotherapeutics[29-33]. Therefore, the use of dietary phytochemicals like PEITC possibly is a relevant approach to restore chemosensitivity towards common chemotherapeutics and to minimize drug toxicity as well.

Materials and Methods

Development and Isolation of Cell Line

Human breast adenocarcinoma (MCF-7) cell line was obtained from National Centre for Cell Science (NCCS), Pune. A paclitaxel- resistant cell line was derived from the parental MCF-7 cells by treating those cells with increasing concentration of paclitaxel with an initial dose of 1 nM. Cells were maintained in each concentration for at least 6 passages and finally, a colony has been isolated at 120 nM. The isolated resistant subline has been designated as MCF-$7^{Pacli/R}$. Before experimentation, MCF-$7^{Pacli/R}$ cells were maintained in drug-free medium for at least one week.

Maintenance of Cell Lines

MCF-7 and its paclitaxel-resistant counterpart MCF-$7^{Pacli/R}$ were maintained in Minimum Essential Medium Eagle (MEM) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS) and antibiotics (gentamycin 40 µg, penicillin 100 units, streptomycin 10 µg/mL). Cells were maintained at 37°C in a humidified CO_2 incubator having 5% CO_2/95% air.

Rhodamine 123 Accumulation Assay

A fluorescent dye, Rhodamine 123 is one of the substrates for P-gp1. For confirming resistance towards paclitaxel and enhanced P-gp1 activity Rhodamine 123 assay kit was used. Briefly, MCF-7 and MCF-$7^{Pacli/R}$ cells were incubated with Rhodamine 123 (5 µM) for 30 min at 37°C in the presence or absence of 1 µM Zosuquidar (SIGMA # SML1044), a specific P-gp1 inhibitor. Treated cells were thereafter washed with Phosphate Buffered Saline (PBS), lysed and Rhodamine 123 was quantitated using spectrofluorimeter (Varian) with excitation and emission wavelength of 485 nm and 535 nm, respectively.

Determination of Multidrug Resistance

MTT (4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltet razolium Bromide) assay was performed to verify whether the isolated paclitaxel-resistant cell was resistant to multiple drugs. Cells at a density of 1×10^4 were seeded in each of the 96 well plates and exponentially growing cells were treated in triplicate with logarithmic doses of either paclitaxel or doxorubicin or 5-fluorouracil (5-FU) for 24 h. MTT solution 50 µL (1.2 mg/mL in water) was added to each of the 96 wells and incubated for 5 h. Finally, after centrifugation, DMSO was added to dissolve MTT-formazan product (purple colored) and estimated by measuring absorbance at 570 nm in an ELISA plate reader.

Computation of IC_{50} value by MTT assay

MTT assay was furthermore carried out for

determination of (a) IC_{50} value (the half- maximal inhibitory concentration) of PEITC and (b) reversal of acquired paclitaxel resistance in presence of PEITC.

(a) Exponentially growing parental and resistant cells (1×10^4/well) seeded in 96 well plates were treated with increasing concentration of PEITC (Sigma-Aldrich: catalogue no# 253731-5G) for 24 h. MTT assay was carried out following the protocol mentioned previously.

(b) Both MCF-7 and MCF-$7^{Pacli/R}$ were seeded in 96 well plates (1×10^4/well). Confluent cells were treated with or without PEITC along with logarithmic doses of paclitaxel for another 24 h. MTT assay was carried out.

Western Blotting

Whole-Cell lysate was prepared using the lysis buffer. Protein concentrations were quantified following Lowry's method. Equally loaded proteins from cell lysates were then electrophoresed on SDS-polyacrylamide gel using electrophoresis buffer (Tris: 25 mM, glycine: 192 mM, SDS: 20%) and separated proteins were electro-transferred to nitrocellulose membranes using transfer buffer (Tris: 250 mM, glycine: 192 mM, methanol: 10%) and blocked in 5% BSA. The membranes were then incubated with primary antibody to react overnight at 4°C; thereafter washed with TBST (Tris Buffered Saline with Tween20) thrice followed by treatment with alkaline phosphatase-conjugated anti-mouse IgG or anti-rabbit IgG (1:1000 dilutions in TBS) depending on the specificity of primary antibodies. Membranes were washed properly with TBST and treated with BCIP/NBTto visualize the proteins. The experiment was repeated three times.

The antibodies used were: MRP1 (GeneTex, GTX116046, 1:1000) P-gp1 (GeneTex, GTX108370, 1:1000), ABCG2 (GeneTex, GTX100437, 1:1000) Ki-67 (GeneTex, GTX26526, 1:1000), phospho-Aurora A (Thr288, 1:1000) from BioLegend, phospho-p53 (S315, 1:1000) GTX50164, p53 (GTX70218, 1:1000), β-actin (GTX629630, 1:1000), Phospho-IκBα (Ser32) antibody from CST (1:1000). Enzyme commission (EC) number of Aurora Kinase A enzyme is EC:2.7.11.1.

Semi-quantitative Reverse Transcription PCR analysis (RT-PCR)

Isolation of total cellular RNA was performed using the RNA queous 4PCR kit (Ambion/Applied Biosystem) according to the manufacturer's instructions. cDNA was synthesized from 2 μg of total RNA using the RetroScript kit (Ambion/Applied Biosystem). The cDNA was amplified by PCR using forward and reverse primer sequences of Aurora A (Aurora A: Forward primer 5′-AATTGCAGATTTTGGGTGGT-3′; Reverse primer 5′-AAACTTCAGTAGCATGTTCCTGTC-3′. β-actin/ACTB gene (Forward primer 5′-CTGGAAC GGTGAAGGTGACA-3′; Reverse primer 5′-AAGGGA CTTCCTGTAACAACGCA-3′) was used as loading control. PCR product was analyzed by electrophoresis on 2% agarose gel and visualized by staining with ethidium bromide (EtBr) under Gel Documentation System.

TUNEL assay

Harvested cells were smeared on the poly-L lysine coated slides and fixed with paraformaldehyde. After fixation of cells, the glass slides were washed with PBS (twice) followed by addition of 50 μL of the labeling reaction mixture (prepared as per information provided with the TaKaRa *in situ* Apoptosis Detection kit) (Catalogue no# MK500) and incubated afterward in a humidified chamber at 37°C for 90 min. The reaction was terminated by washing with PBS and finally, the slides were mounted and detected under a fluorescence microscope.

Morphological examination of cells by Propidium Iodide (PI)

Treated cells were harvested, washed with PBS and centrifuged and to the pellet PI (Sigma-Aldrich: catalogue no# P4170-10MG) was added (final: 50 μg/mL). Cells were then incubated in the dark at RT for 10 min. Treated cells along with control one was spread over slides, covered with coverslips, and examined under the fluorescent microscope. Number of apoptotic and normal cells was counted in each slide. The ratio of apoptotic cell to normal cell was calculated and the apoptotic index was plotted graphically.

Statistical analysis

Statistical analysis was carried out using GraphPad Prism Software. A *P*-value <0.005 was considered statistically significant.

Results

Confirmation of acquired chemoresistance towards paclitaxel

During the development of paclitaxel– resistant subline, a stepwise increasing dose of the drug has been used. In each of the incremental stages, gradual

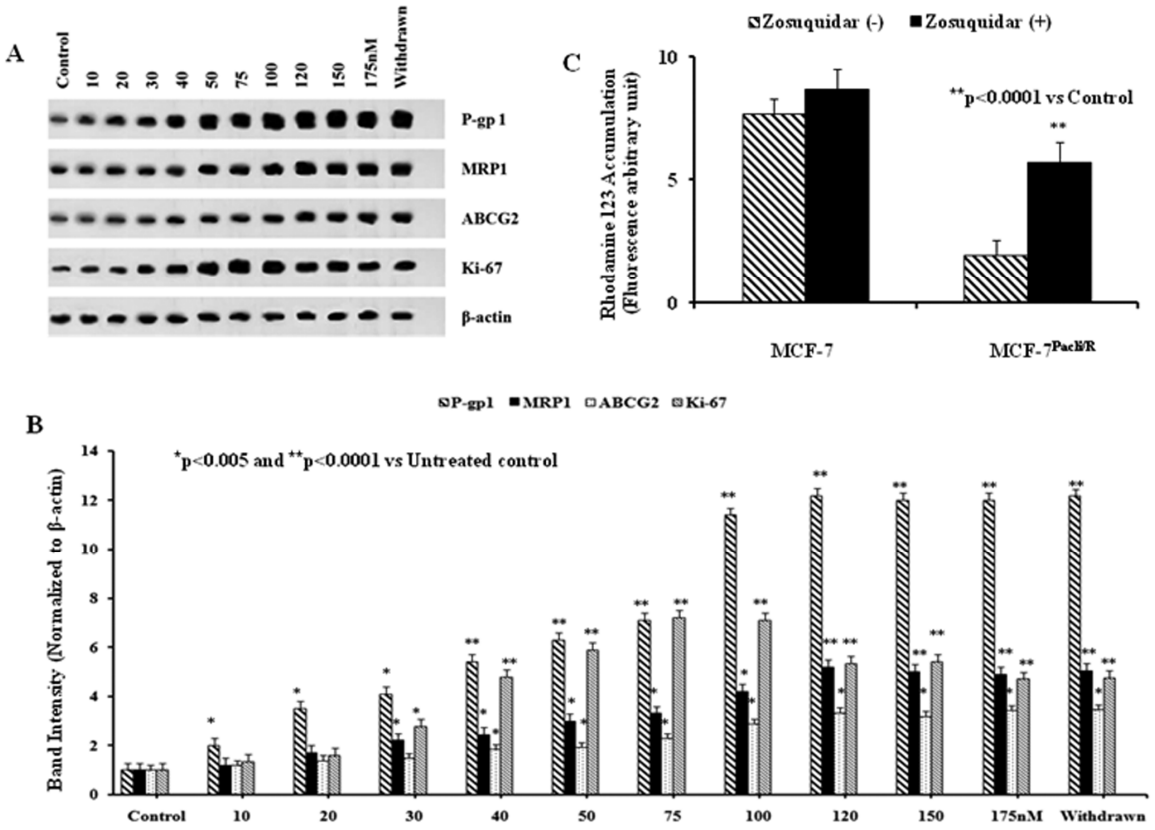

Fig. 1 — Confirmation of acquired paclitaxel resistance in MCF-$7^{Pacli/R}$ subline. (A) Protein expressions of drug efflux pumps (P-gp1, MRP1, ABCG2) and proliferative marker (Ki-67) during the developmental process of paclitaxel– resistant cell line, treated with stepwise increasing doses of paclitaxel. β-actin was used as a loading control; (B) Corresponding band intensities were plotted graphically after calculating intensities using Image Master Software. Intensities were normalized to β-actin. *P* values were calculated and have been displayed; and (C) Rhodamine 123 accumulation in parental MCF-7 and MCF-$7^{Pacli/R}$. Cells were exposed to Rhodamine 123 (5 μM) for 30 min at 37°C in the absence of Zosuquidar, a specific inhibitor of P-gp1 or pretreated with Zosuquidar (1 μM) for 30 min at 37°C, followed by Rhodamine 123 treatment. Accumulation was quantified spectrofluorimetrically. Data were represented as fluorescence arbitrary units. Values represent the mean ± SD of three independent experiments (n=3). *P* values were significant as calculated and were shown in the figure

elevations in the expressions of the members of ATP-binding cassette (ABC) transporter family proteins (MRP1, P-gp1, ABCG2) were observed. Protein expression of proliferative (Ki-67) marker has also been checked. Paclitaxel concentration was increased gradually up to 120 nM and cells were finally isolated at that concentration. Paclitaxel dose was increased beyond 120 nM but the expressions of drug efflux pumps remain unaltered from 120 nM onwards. Cells were allowed to grow in drug– free medium for 4 weeks for ascertaining resistance in an isolated subline of MCF-7 and thereafter designated as MCF-$7^{Pacli/R}$ (Fig. 1A). Protein expressions of efflux pumps and proliferative markers as obtained from western blotting were quantified using software and plotted graphically (Fig. 1B). To ensure acquired chemoresistance towards paclitaxel, P-gp1 activity was measured by Rhodamine 123 assay. Rhodamine 123 is the substrate for P-gp1; as the protein expels out the dye. Results revealed that the accumulation of Rhodamine 123 in MCF-$7^{Pacli/R}$ was very poor in comparison to parental MCF-7; reflecting P-gp1 mediated efflux of Rhodamine in resistant counterpart. However, the accumulation of Rhodamine in MCF-$7^{Pacli/R}$ was found to be increased when the resistant subline was treated with Zosuquidar (1 μM), a specific P-gp1 inhibitor, for 30 min at 37°C (Fig. 1C).

To confirm the resistance of MCF-$7^{Pacli/R}$ towards other chemotherapeutics, a multidrug resistance assay was performed using MTT assay. Absorbance was taken at 570 nm. Results showed resistance towards the respective chemotherapeutic drugs (Paclitaxel, Doxorubicin, and 5-Fluorouracil) in

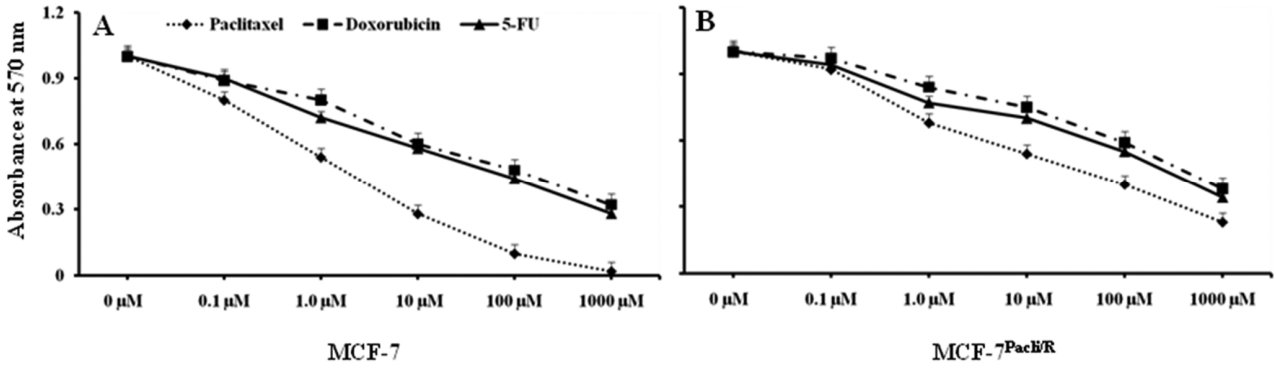

Fig. 2 — Acquired resistance towards different chemotherapeutic drugs as obtained from MTT assay after treatment for 24 h. MTT reduction assay was carried out after treatment of (A) MCF-7 and (B) MCF-$7^{Pacli/R}$ with different concentrations of paclitaxel, doxorubicin , and 5-Flurouracil (5-FU). Absorbance was taken at 570 nm. Values represent mean ± SE (n=3)

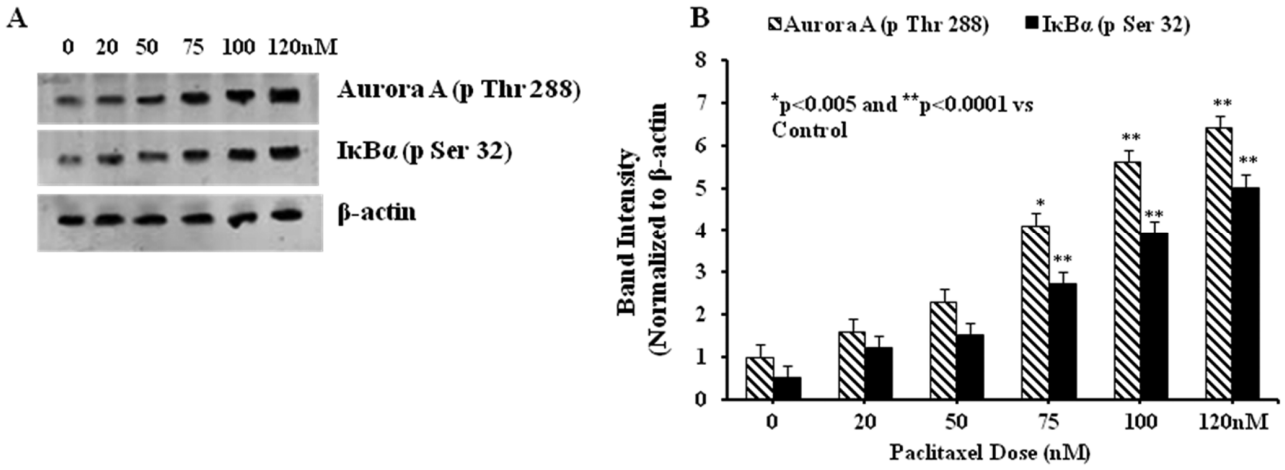

Fig. 3 — Expressions of phospho-Aurora A (Thr 288) and phospho-IκBα (Ser32) during each of the incremental doses of paclitaxel treatment. (A) Cells were harvested in each incremental dose and subjected to western blot analysis. β-actin was used to ensure equal protein loading; and (B) Band intensities of both phospho-Aurora A (Thr 288) and phospho-IκBα (Ser32) were calculated using Image Master Software. Results were an average of three independent experiments. Values represented mean ± SD (n=3). *P* values as represented are significantly different compared with untreated counterparts

resistant subline in comparison to parental one (Fig. 2A & B). Based on MTT results IC_{50} values were calculated and represented in a table (Table 1). The values are in agreement with the development of a paclitaxel– resistant subline, which is not only resistant to paclitaxel but also other chemotherapeutics. Next, it was examined whether Aurora– A, in addition to ABC transporters, has any contributory role in the development of paclitaxel resistance. For this purpose, protein expressions of phospho-Aurora A (Thr 288) were observed at each of the incremental steps. Expression of phospho-IκBα (Ser 32) was also checked furthermore as the protein gets phosphorylated by Aurora A and it releases from NFκB (Fig. 3A). Band intensities were calculated accordingly using Image Master Software and depicted an increasing trend for both phospho-Aurora A and phospho-IκBα with increasing concentrations of paclitaxel (Fig. 3B).

Table 1 — IC_{50} values of different chemotherapeutic drugs as obtained from MTT assay after treatment for 24 h

Drugs (µM)	MCF-7	MCF-$7^{Pacli/R}$
Paclitaxel	2	28
Doxorubicin	82	355
5-Fluorouracil	60	337

Determination of IC_{50} and IC_{30} value of PEITC in parental and resistant subline

Before look into the influence of PEITC in reversing chemoresistance, it is important to determine the IC_{50} and IC_{30} value of the compound in both parental and resistant sublines. For this purpose,

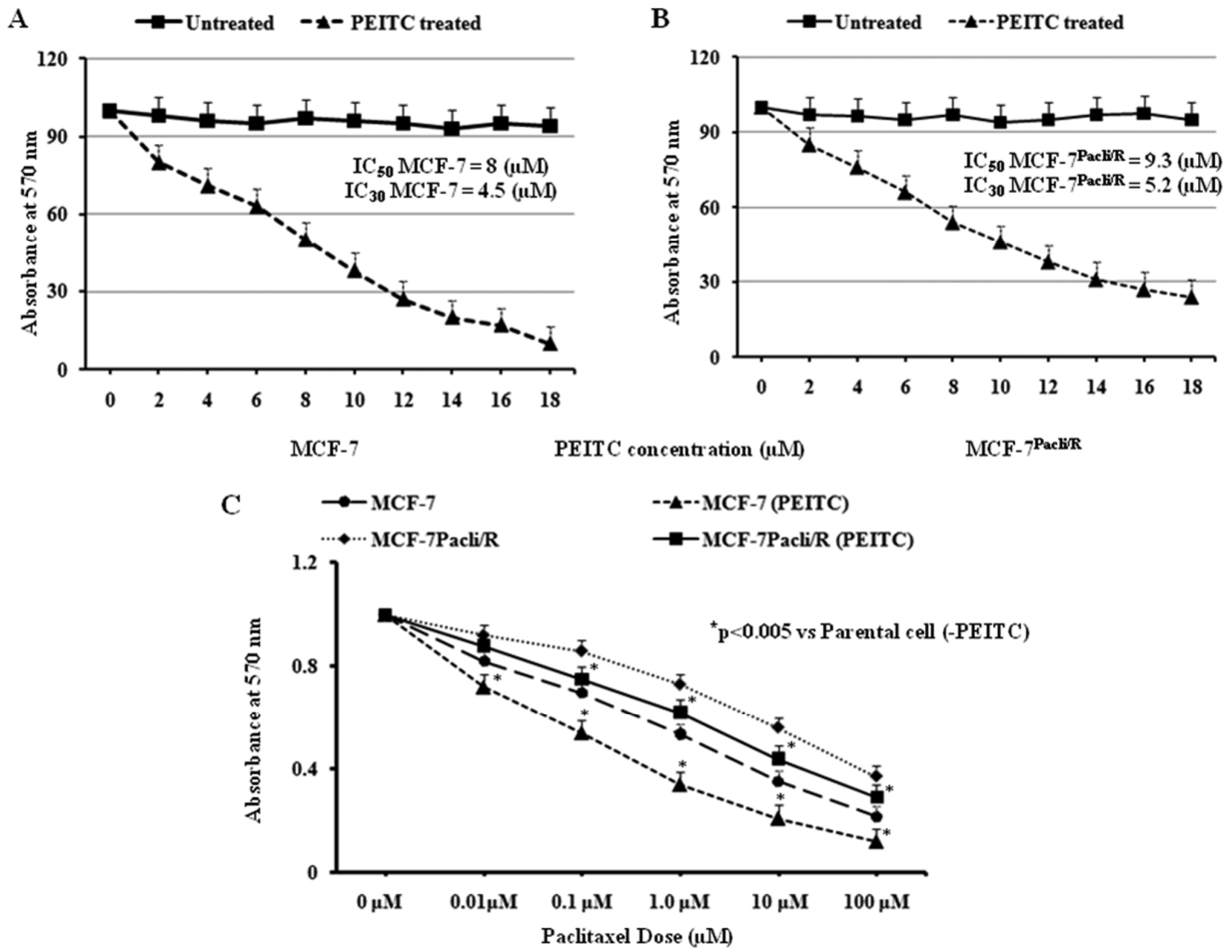

Fig. 4 — Determination of IC_{50} and IC_{30} values of PEITC and furthermore establishing PEITC as a resistance modifying agent. (A) MCF-7; (B) MCF-$7^{Pacli/R}$ cells were incubated with different concentrations of PEITC for 24 h. Cytotoxicity was measured by MTT reduction assay. The error bars indicated standard deviations of three samples; and (C) Both MCF-7 and MCF-$7^{Pacli/R}$ cells were treated with different logarithmic doses of paclitaxel for 24 h in the presence and absence of PEITC (5 µM). Cytotoxicity was measured by MTT reduction assay. The error bars indicated standard deviations of three samples. *P* values of PEITC treated cells were significant *vs* PEITC untreated controls

MTT assay was carried out after the treatment of cells with increasing concentrations of PEITC with a maximum concentration of 18 µM. The proliferation of MCF-7 and MCF-$7^{Pacli/R}$ were found to be inhibited significantly by PEITC in a concentration-dependent manner when cells were treated for 24 h. Based on MTT results, both IC_{30} and IC_{50} values were calculated (Fig. 4A & B) and an approximate dose of IC_{30} values was selected (5 µM) for experimental purpose and both parental and resistant cells were treated with that concentration.

Role of PEITC as RMA

After calculating IC_{50} values, it was intended to observe whether PEITC could reverse paclitaxel-induced acquired chemoresistance. To achieve this goal, both parental and paclitaxel– resistant sublines were co-treated with PEITC and paclitaxel for 24 h followed by MTT reduction assay. Absorbance was taken at 570 nm and data were shown (Fig. 4C).

Table 2 — Reversal of acquired resistance in breast cancer cells by PEITC. Values are mean ± SD, (n=3)

Compound	MCF-7		MCF-$7^{Pacli/R}$	
	EC_{50} Pacli	RI	EC_{50} Pacli	RI
Compound (-)	2±0.2	-	28±1.8	-
PEITC	0.2±0.03	10	5.3±0.5	5.28

From the result obtained from MTT assay, the EC_{50} value of paclitaxel was calculated in PEITC treated and untreated cells. EC_{50} values of paclitaxel decreased significantly when both MCF-7 and MCF-$7^{Pacli/R}$ cells were treated with PEITC (Table 2). Reversal index (RI) which is the ratio of EC_{50} of the

chemotherapeutic drug alone to EC_{50} value of chemotherapeutic drug in presence of PEITC was then calculated. The values assertively indicated that PEITC was much more efficient in imparting sensitivity of resistant cells towards paclitaxel by reversing chemoresistance.

Influence of PEITC on the expression profile of Aurora –A in MCF-7$^{Pacli/R}$ subline

Since PEITC was found to reverse the resistance towards paclitaxel, therefore it was felt interesting to understand the underlying mechanism by which PEITC is performing its function. To accomplish the objective, expression of phospho-Aurora A (Thr 288) at protein as well as genetic levels were determined by western blot analysis and semi-quantitative Reverse Transcriptase-PCR technique, respectively. Results indicated a time– dependent inhibition of phospho-Aurora A in both the cell lines (Fig. 5A). Expression of mRNAs of Aurora A in presence of PEITC was found to diminish with time (Fig. 5B).

Effect on downstream signaling proteins of Aurora A

Next, it was attempted to find out whether PEITC, by inhibiting Auroras, could affect phosphorylation of p53 and IκBα, two important targets of Aurora A. Results revealed that untreated cells contained high levels of phospho-p53 at serine 315 residues. Treatment with PEITC reduced the phosphorylation status of the protein. The ratio of phospho p53 to total p53 was calculated in both parental and resistant cells (Fig. 6A). The results were analogous to those obtained after treatment with Aurora A inhibitor I (Sigma-Aldrich: catalogue no# SML0882-5MG) using a concentration of 3 nM (Fig. 6B).

Next objective of the study was to look into the expression pattern of IκBα (p Ser 32) as it is a target protein of Aurora A. Western blotting followed by calculation of band intensities of IκBα (p Ser 32) showed significant fold decrease after treatment with PEITC. The result was comparable to that in Aurora A inhibitor I treated cells (Fig. 6C). This result probably indicated that diminished expression of IκBα (p Ser 32) is due to inhibition of Aurora A. All these results cumulatively pointed out the efficacy of PEITC in targeting Auroras and their downstream target proteins.

PEITC augments apoptosis mediated by paclitaxel

It was thereafter intended to observe whether PEITC by regulating Auroras and reversing chemoresistance could induce apoptosis in MCF-7 and MCF-7$^{Pacli/R}$ cells. TUNEL assay was performed for the purpose. Briefly, exponentially growing cells were treated with 5 µM PEITC for 24 h. Cells were then harvested and smeared on poly-L lysine coated slides, stained with 50 µL labeling mixture for fluorescence microscopy and visualized at 20X magnification. Apoptotic cells (bright green colour) were identified in PEITC treated cells in both MCF-7 and MCF-7$^{Pacli/R}$ cells but the extent of programmed

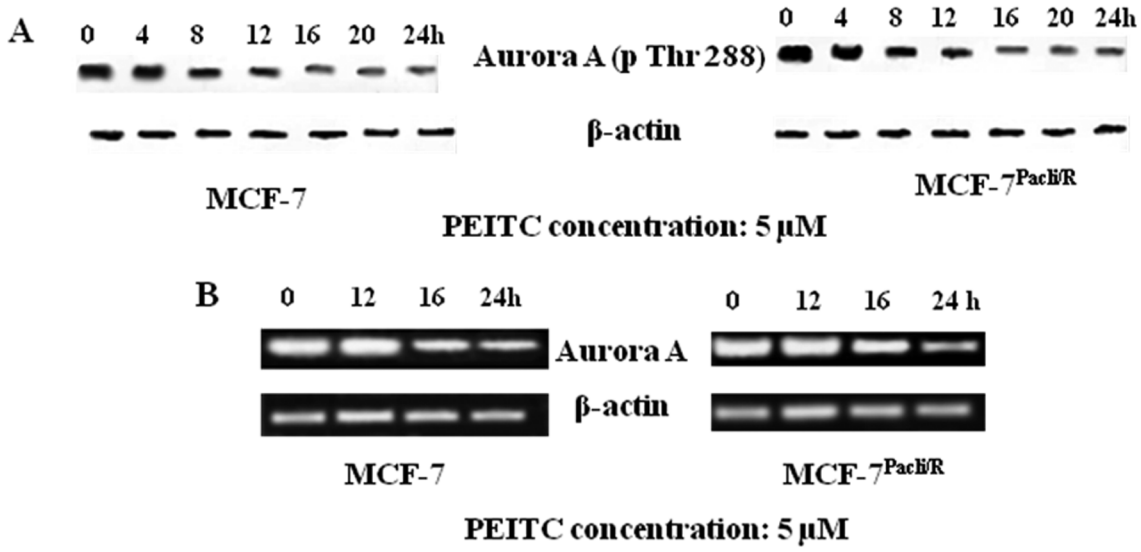

Fig. 5 — Differential expressions of Aurora Kinase A in the presence or absence of PEITC. MCF-7 and MCF-7$^{Pacli/R}$ cells were treated with PEITC (5 µM) for different time periods. (A) Expression profile of active form of Aurora A protein was determined using the anti-phospho-Aurora A (Thr 288) antibody; and (B) RT-PCR was performed to determine the mRNA expression in both the cells following the protocol mentioned in the materials and method section. β-actin was used as an internal loading control in both the experimental setup

cell death differs with cell type. PEITC treated MCF-

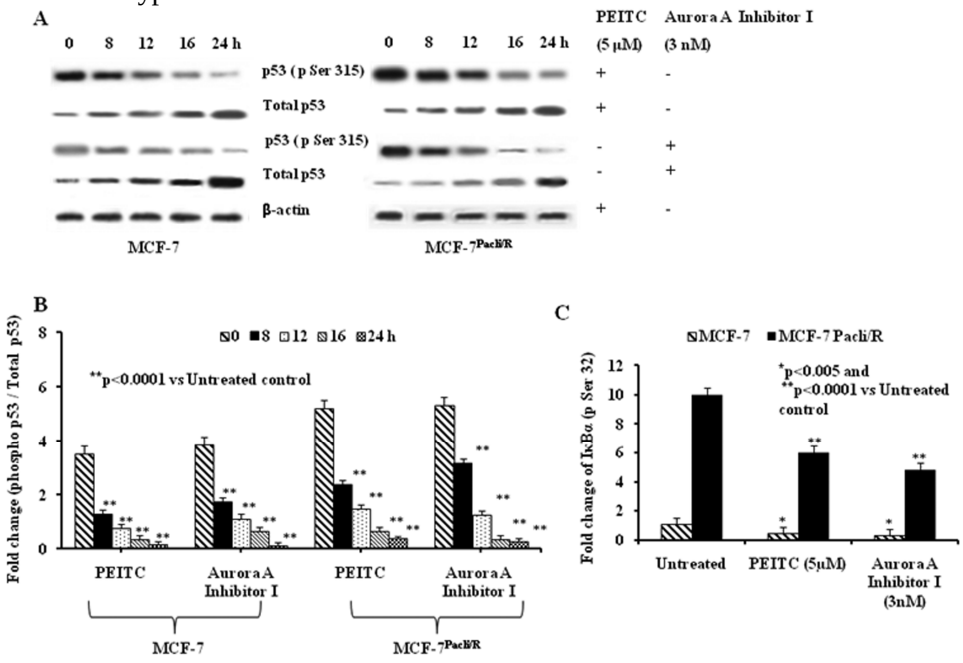

Fig. 6 — Expressions of downstream target proteins of Aurora A in the presence and absence of PEITC. The treatment duration was 16 h. (A) Expression of phospho-p53 (Ser 315) residue and total p53 in whole cell lysate. β-actin was used as an internal loading control. (B) Band intensities were calculated and comparative band intensities (ratio) of phospho as well as total p53 proteins as obtained in presence of either PEITC or Aurora-A Inhibitor I was calculated and plotted graphically. Values were the average of three independent experiments. p values were calculated *vs* untreated cells and have been depicted. (C) Determination of fold change of phospho-IκBα (Ser 32) after treatment with either PEITC or Aurora A inhibitor I. Values were mean of three independent experiments. *P* values *vs* control were calculated and have been illustrated

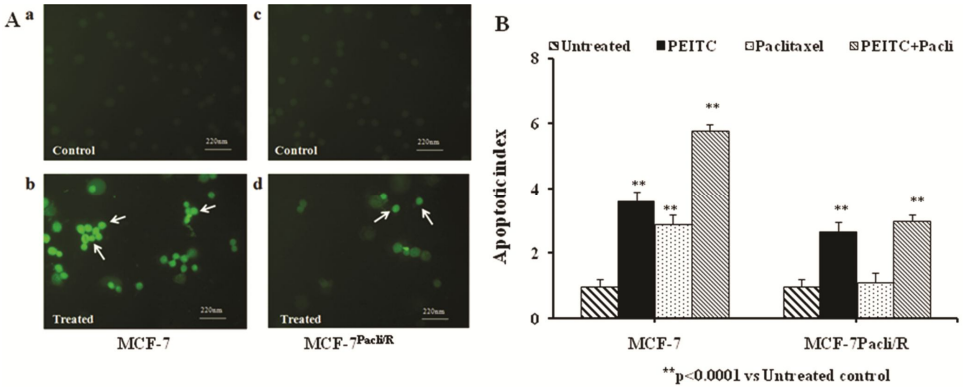

Fig. 7 — PEITC induced paclitaxel mediated apoptosis. (A) Cells (MCF-7 and MCF-$7^{Pacli/R}$) were incubated with PEITC (5 μM) for 24 h, and then harvested cells were processed and finally stained with a labeling reaction mixture that can label nicked end of DNA formed during apoptosis. Green fluorescence indicated apoptotic cells marked by a number of white arrows. Cells were visualized under fluorescence microscopy at 20X magnification; and (B) Apoptotic indices for MCF-7 and MCF-$7^{Pacli/R}$ cells treated with either PEITC (24 h) and/or paclitaxel (24 h). For each treatment, 100 cells were scored at random under the fluorescent microscope and classified into apoptotic and non-apoptotic cells based on their characteristic features after staining with Propidium Iodide (PI). The ratio of apoptotic to non-apoptotic cells was designated as the apoptotic index. Values represented mean ± SD (n=100). Values were significant $^{**}P$ <0.0001 *vs* untreated control

7 cells underwent apoptosis as observed in the figure compared to the untreated MCF-7 cells. Conversely, very few MCF-$7^{Pacli/R}$ cells were directed to apoptosis when cells were treated with PEITC (Fig. 7A). Apart from the TUNEL assay, apoptotic cells were stained with Propidium Iodide (PI). In brief, cells were treated with either PEITC (5 μM), or paclitaxel (0.25 μM) or a combination of both. Harvested cells

were stained with PI and observed under the fluorescent microscope. The apoptotic index value was then calculated under a fluorescent microscope after PI staining. The result established that in combination treatment, PEITC promotes paclitaxel-induced apoptosis in both resistant and parental breast adenocarcinoma cells (Fig. 7B).

Discussion

Paclitaxel is considered as a first-line chemotherapeutic agent for the treatment of breast cancer[34,35]. Nonetheless, the efficacy of this drug is limited by the resistance, inevitably acquired due to long-term exposure[7]. Among several factors associated with acquired chemoresistance, Aurora kinases (particularly, Aurora A) are the most relevant ones. Aurora kinases play crucial roles in mitotic regulation[18]. Therefore the management of drug–resistance by targeting Aurora kinases is an important approach for successful chemotherapy. The present study dealt with the development of a drug resistant subline from the parental MCF-7 using paclitaxel, a widely used chemotherapeutic for breast cancer. The resistant subline was initially characterized by observing the expressions of signature markers of chemoresistance like MRP1, P-gp1, ABCG2 and proliferative markers like Ki-67. The upregulation of these drug efflux transporters in resistant subline reinforced the assumption of acquired chemoresistance. Interestingly, Aurora A was found to be upregulated with incremental doses of paclitaxel; indicating its contributory role in the acquirement of chemoresistance. Aurora A phosphorylates IκBα at Ser 32, which in turn releases NF-κB that can translocate to the nucleus. That transcription factor directly influences P-gp1 overexpression and thus confers chemoresistance[20,24]. Present findings are also in agreement with previous results as the uprising trend of phospho IκBα (Ser 32) was observed during the development of chemoresistant subline. Measurement of P-gp1 activity and its inhibition by specific inhibitor Zosuquidar was carried out by Rhodamine 123 assay. The spectrofluorimetric study revealed a decrease in fluorescence spectra with paclitaxel, explaining the decreased accumulation of the drug within the cell at the isolated resistant subline. This result is in support of the previous work of Shatha Abu Hammad, 2013[36], where confirmation has been done by fluorescent cell imaging. All these cumulative results along with multi drug resistance assay strengthened the notion of acquired chemoresistance in the isolated subline (MCF-7$^{Pacli/R}$) of MCF-7.

Plant-derived natural compounds are reported to enhance the cytotoxic effects[37] of the chemotherapeutic drugs when used in combination. These compounds can, therefore, be used along with chemotherapeutic drugs for reversing multidrug resistance[38]. Herbal drugs are much cheaper and safer for which about 80% of the world's population relies on herbal medicines as primary health care support[39].These compounds possess strong antioxidant properties[40,41].

PEITC, a natural isothiocyanate found in cruciferous vegetables has been established as a potent anticancer agent due to its wide spectrum of biological activities. Compelling evidences, indicated the involvement of PEITC in reversing resistance towards chemotherapy by regulating several molecular pathways[32,42,43]. Before treatment with PEITC, IC_{30} and IC_{50} values were determined. To assess the role of PEITC in reversing chemoresistance, MTT assay was performed. The result showed that EC_{50} of MCF-7 cells to paclitaxel was much lower than that of MCF-7$^{Pacli/R}$ cells indicating that MCF-7$^{Pacli/R}$ cells had significant paclitaxel resistance. PEITC was highly effective in reversing acquired chemoresistance as fold reversal index increased 5 times in the presence of PEITC.

For identifying the key molecular event underlying chemoresistance, the focus was kept on Aurora A. Elevated expression at the protein level and mRNA level was detected in MCF-7$^{Pacli/R}$ cells which were associated with the accomplishment of chemoresistance. Phosphorylation of Aurora A at Thr 288 within the kinase activation loop (catalytic domain) increases kinase activity. Western blot results demonstrated that in untreated cells expression of phospho-Aurora A was appreciably high and PEITC efficiently diminished phospho-Aurora A expressions in a concentration–dependent manner which was also reflected in RNA expression study, done by RT-PCR. These findings cumulatively explained the down regulation of Aurora A by PEITC at the transcription level, which was paralleled to significant inhibition of phosphorylated proteins. Functional activities of Aurora A were reported previously to be regulated at the transcriptional level and/or at the level of phosphorylation of protein[44]. The present finding, therefore, justified the use of PEITC in regulating Auroras. PEITC induced Aurora A inhibition

furthermore led to subsequent inhibition of phospho-p53 (Ser 315) and phospho-IκBα (Ser 32). The results were furthermore validated with specific inhibitors of Aurora A[21,45]. Collectively these results supported the hypothesis that restoration of chemosensitivity of MCF-7$^{Pacli/R}$ cells induced by PEITC was associated with regulation of Aurora– A and its downstream proteins.

Some apoptotic parameters like TUNEL assay and apoptotic indices were studied to ascertain whether PEITC induced restoration of chemosensitivity and subsequent cytotoxicity due to the regulation of Aurora A is occurring through the apoptotic pathway. It was observed that treatment with PEITC increases the frequency of apoptotic cells which were evident in both TUNEL assay and in PI stained cells (photograph not shown). PEITC treated cells were when subjected to paclitaxel treatment, frequency of apoptotic cells was much higher which was reflected in calculating apoptotic index values as plotted in the graph mentioned in the result section. It was apparent that combination treatment gave rise to a better impact on both MCF-7 and MCF-7$^{Pacli/R}$. Present findings corroborated with several other studies where PEITC was found to induce apoptosis in cancer cells[46,47].

Taken together these results established the role of PEITC in reversing acquired chemoresistance by targeting Aurora A. Regulation of Aurora A by PEITC suggestively attributes its role in inducing paclitaxel mediated apoptosis. Further mechanistic studies are needed to understand the molecular interaction of PEITC with Aurora– A.

Conclusion

The result affirmed the contributory role of Aurora-A in acquirement of paclitaxel resistance in breast cancer cells. PEITC by regulating Aurora-A reverses chemoresistance and compels cells to undergo apoptosis. Therefore PEITC may be considered as a potential chemosensitizing agent.

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Conflict of Interest

All authors declare no conflict of interest.

References

1 Velaei K, Samadi N, Barazvan B & Rad JS, Tumor microenvironment-mediated chemoresistance in breast cancer. *Breast*, 30 (2016) 92.

2 Leary M, Heerboth S, Lapinska K & Sarkar S, Sensitization of drug resistant cancer cells: A matter of combination therapy. *Cancers* (*Basel*), 10 (2018) 483.

3 Chambers CS, Viktorová J, Řehořová K, Biedermann D, Turková L, Macek T, Křen V & Valentová K, Defying Multidrug Resistance! Modulation of Related Transporters by Flavonoids and Flavonolignans. *J Agric Food Chem*, (2019) doi: 10.1021/acs.jafc.9b00694.

4 Zou Z, Yuan Z, Zhang Q, Long Z, Chen J, Tang Z, Zhu Y, Chen S, Xu J, Yan M, Wang J & Liu Q, Aurora kinase A inhibition-induced autophagy triggers drug resistance in breast cancer cells. *Autophagy*, 8 (2012) 1798.

5 Li Q & Shu Y, Role of solute carriers in response to anticancer drugs. *Mol Cell Ther*, 2 (2014) 15.

6 Choudary I, Barr PM, & Friedberg J, Recent advances in the development of Aurora kinases inhibitors in hematological malignancies. *Ther Adv Hematol*, 6 (2015) 282.

7 Němcová-Fürstová V, Kopperová D, Balušíková K, Ehrlichová M, Brynychová V, Václavíková R, Daniel P, Souček P, Kovář J, Characterization of acquired paclitaxel resistance of breast cancer cells and involvement of ABC transporters. *Toxicol Appl Pharmacol*, 310 (2016) 215.

8 Nyquist MD, Prasad B & Mostaghel EA, Harnessing solute carrier transporters for precision oncology. *Molecules*, 22 (2017) 539.

9 Estrada-Tejedor R & Ecker GF, Predicting drug resistance related to ABC transporters using unsupervised consensus self organizing maps. *Sci Rep*, 8 (2018) 6803.

10 Magnaghi-Jaulin L, Eot-Houllier G,Gallaud E & Giet R, Aurora A protein kinase: to the centrosome and beyond. *Biomolecules*, 9 (2019), 28.

11 Courtheoux T, Diallo A, Damodaran AP, Reboutier D, Watrin E & Prigent C, Aurora A kinase activity is required to maintain the spindle assembly checkpoint active during pro-metaphase. *J Cell Sci*, 131 (2018), doi:10.1242/jcs.191353

12 Zhang K, Chen J, Chen D, Huang J, Feng B, Han S, Chen Y, Song H, De W, Zhu Z, Wang R & Chen L, Aurora-A promotes chemoresistance in hepatocelluar carcinoma by targeting NF-B/microRNA-21/PTEN signaling pathway. *Oncotarget*, 5 (2014) 12916.

13 Wu X, Liu W, Q, Chen C, Chen Z, Xu Z, Li W, Liu F & Yao X, Inhibition of Aurora B by CCT137690 sensitizes colorectal cells to radiotherapy. *J Exp Clin Cancer Res*, 33 (2014) 13.

14 Zhang Y, Jiang C, Li H, Lv F, Li X, Qian X, Fu L, Xu B & Guo X, Elevated Aurora B expression contributes to chemoresistance and poor prognosis in breast cancer. *Int J Clin Exp Pathol*, 8 (2015) 751.

15 Sun JM, Yang LN, Xu H, Chang B, Wang HY & Yang G. Inhibition of Aurora A promotes chemosensitivity *via* inducing cell cycle arrest and apoptosis in cervical cancer cells. *Am J Cancer Res*, 5 (2015) 1133.

16 Yu J, Zhou J, Xu F, Bai W & Zhang W, High expression of Aurora-B is correlated with poor prognosis and drug resistance in non-small cell lung cancer. *Int J Biol Markers*, 33 (2018) 215.

17 Wu J, Cheng Z, Xu X, Fu J, Wang K, Liu T, Wu C, Kong X, Yang Q, Yan G & Zhou H, Aurora-A induces

chemoresistance through activation of the AKT/mTOR pathway in endometrial cancer. *Front Oncol*, 9 (2019) 422.

18 Willems E, Dedobbeleer M, Digregorio M, Lombard A, Lumapat PM & Rogister B, The functional diversity of Aurora kinases: a comprehensive review. *Cell Div*, 13 (2018) 7.

19 Tang A, Gao K, Chu L, Zhang R, Yang J & Zheng J. Aurora kinases: novel therapy targets in cancers. *Oncotarget*, 8 (2017) 23937.

20 Katsha A, Soutto M, Sehdev V, Peng D, Washington MK, Piazuelo MB, Tantawy MN, Manning HC, Lu P, Shyr Y, Ecsedy J, Belkhiri A & El-Rifai W, Aurora kinase A promotes inflammation and tumorigenesis in mice and human gastric neoplasia. *Gastroenterol*, 145 (2013) 1312.

21 Sasai K, Treekitkarnmongkol W, Kai K, Katayama H & Sen S, Functional significance of Aurora Kinases–p53 protein family interactions in cancer. *Front Oncol*, (2016), 247.

22 Ertych N, Stolz A, Valerius O, Braus GH & Bastians H, CHK2–BRCA1 tumor-suppressor axis restrains oncogenic Aurora-A kinase to ensure proper mitotic microtubule assembly. *Proc Natl Acad Sci U S A*, 113 (2016) 1817.

23 Liu Q, Kaneko S, Yang L, Feldman RI, Nicosia SV, Chen J & Cheng JQ, Aurora-A abrogation of p53 DNA binding and transactivation activity by phosphorylation of serine 215. *J Biol Chem*, 279 (2004) 52175.

24 Briassouli P, Chan F, Savage K, Reis-Filho JS & Linardopoulos S, Aurora-A regulation of Nuclear Factor-κB signaling by phosphorylation of IκBα. *Cancer Res*, 67 (2007) 1689.

25 Dolcet X, Llobet D, Pallares J & Matias-Guiu X, NF-κB in development and progression of human cancer. *Virchows Arch*, 446 (2005) 475.

26 Dai Y, Lawrence TS & Xu L, Overcoming cancer therapy resistance by targeting inhibitors of apoptosis proteins and nuclear factor-kappa B. *Am J Transl Res*, 1 (2009) 1.

27 Antoon JW, White MD, Slaughter EM, Driver JL, Khalili HS, Elliott S, Smith CD, Burow ME & Beckman BS. Targeting NF-κB mediated breast cancer chemoresistance through selective inhibition of sphingosine kinase-2. *Cancer Biol Ther*, 11 (2011) 678.

28 Cragg GM & Pezzuto JM, Natural products as a vital source for the discovery of cancer chemotherapeutic and chemopreventive agents. *Med Princ Pract*, 25 (2016) 41.

29 Trachootham D, Zhou Y, Zhang H, Demizu Y, Chen Z, Pelicano H, Chiao PJ, Achanta G, Arlinghaus RB, Liu J & Huang P, Selective killing of oncogenically transformed cells through a ROS-mediated mechanism by β-phenylethyl isothiocyanate. *Cancer Cell*, 10 (2006) 241.

30 Huong LD, Shin JA, Choi ES, Cho NP, Kim HM, Leem DH & Cho SD, -Phenethylisothiocyanate induces death receptor 5 to induce apoptosis in human oral cancer cells *via* p38. *Oral Dis*, 18 (2012) 513.

31 Sakao K, Desineni S, Hahm ER & Singh SV, Phenethyl isothiocyanate suppresses inhibitor of apoptosis family protein expression in prostate cancer cells in culture and *in vivo*. *Prostate*, 72 (2012) 1104.

32 Gupta P, Wright SE, Kim SH & Srivastava SK, Phenethylisothiocyanate: a comprehensive review of anti-cancer mechanisms. *Biochim Biophys Acta*, 1846 (2014) 405.

33 Xiang J, Wu B, Zhou Z, Hu S, Piao Y, Zhou Q, Wang G, Tang J, Liu X & Shen Y, Synthesis and evaluation of a paclitaxel-binding polymeric micelle for efficient breast cancer therapy. *Sci China Life Sci*, 61 (2018) 436.

34 Gennari A, D'amico M & Corradengo D, Extending the duration of first-line chemotherapy in metastatic breast cancer: a perspective review. *Ther Adv Med Oncol*, 3 (2011) 229.

35 Elserafi MM, Zeeneldin AA, Abdelsalam IM, Nassar HR, Moneer MM & Buhoush WH, First-line paclitaxel and cisplatin used sequentially or in combination inmetastatic breast cancer: A phase II randomized study. *J Egypt Natl Canc Inst*, 30 (2018) 13.

36 Abu Hammad S & Zihlif M, Gene expression alterations in doxorubicin resistant MCF-7 breast cancer cell line. *Genomics*, 101 (2013) 213.

37 Bashyal P, Jung HY, Panday RP & Sohng JK, Comparative study on melanin production and collagen expression profile of polyphenols and their glycosides. *Indian J Biochem & Biophys*, 56 (2019) 137

38 Wang J, Seebacher N, Shi H, Kan Q & Duan Z, Novel strategies to prevent the development of multidrug resistance (MDR) in cancer. *Oncotarget*, 8 (2017) 84559.

39 Kumar K & Zakir M , Future prospects of fermentation in unanibased drugs. *Indian J Biochem Biophys*, 56 (2019) 347.

40 Turker AU, Yildirim AB & Tas I, *In vitro* adventitious plant regeneration of *Echium orientale* L., an endemic plant: The evaluation of biological activities and phenolic content. *Indian J Biochem Biophys*, 55 (2018) 264.

41 Mohon MSG, Ramakrishnan T, Mani V & Achary A, Protective effect of crude sulphated polysaccharide from Protective effect of crude sulphated polysaccharide from Turbinaria ornata on isoniazid rifampicin induced hepatotoxicity and oxidative stress in the liver, kidney and brain of adult Swiss albino rats. *Indian J Biochem Biophys*, 55 (2018) 237.

42 Singh SV & Singh K, Cancer chemoprevention with dietary isothiocyanates mature for clinical translational research. *Carcinogenesis*, 33 (2012) 1833.

43 Guo Z, Wang H, Wei J, Han L & Li Z, Sequential treatment of phenethyl isothiocyanate increases sensitivity of Temozolomide resistant glioblastoma cells by decreasing expression of MGMT *via* NF-κB pathway. *Am J Transl Res*, 11 (2019) 696.

44 Karthigeyan D, Prasad SB, Shandilya J, Agrawal S & Kundu TK, Biology of Aurora A kinase: implications in cancer manifestation and therapy. *Med Res Rev*, 31 (2011) 757.

45 Hsueh K, Fu S, Chang C, Chang Y & Lin C, A novel Aurora-A-mediated phosphorylation of p53 inhibits its interaction with MDM2. *Biochimica et Biophysica Acta*, 1834 (2013) 508.

46 Ramirez CN, Li W, Zhang C, Wu R, Su S, Wang C, Gao L, Yin R & Kong AN, *In vitro-in vivo* dose response of ursolic acid, sulforaphane, PEITC, and curcumin in cancer prevention. *AAPS J*, 20 (2017) 19.

47 Dai MY, Wang Y, Chen C, Li F, Xiao BK , Chen SM & Ta ZZ, Phenethyl isothiocyanate induces apoptosis and inhibits cell proliferation and invasion in Hep-2 laryngeal cancer cells. *Oncol Rep*, 35 (2016) 2657.