PEITC: A resounding molecule averts metastasis in breast cancer cells \textit{in vitro} by regulating PKC$\delta$/Aurora A interplay

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HIGHLIGHTS

- PEITC-mediated PKC$\delta$ activation disrupts nuclear lamin expression
- Activated PKC$\delta$ facilitates nuclear accumulation of phospho-Nrf2 (Ser-40).
- PKC$\delta$ imparted a negative impact on functional expression of Aurora A.
- Consequentially, repression of Aurora A effectuated in suppression of PLK1.
- Altered STKs begat apoptosis & suppressed metastasis in breast-cancer cells.

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ABSTRACT

Background/aim: Intricate association and aberrant activation of serine/threonine kinase (STK) family proteins like Polo-like kinase (PLK1) and Aurora kinase (Aurora A abruptly regulate mitotic entry whereas activation of PKC$\delta$), another important member of STK family conversely induces apoptosis which is preceded by cell cycle arrest. These STKs are considered as major determinant of oncogenicity. Therefore, the contributory role of Aurora A/PLK-1 axis in mitotic control and PKC$\delta$ in apoptosis control and their reciprocity in cancer research is an emerging area to explore. The present study investigated the intricate involvement of STKs in breast cancer cells (MCF-7 and MDA-MB-231) and their disruption by PEITC.

Methods: Both MCF-7 and MDA-MB-231 cells were checked for clonogenic assay, cell-cycle analysis and the results were compared with normal MCF-10A, Western blotting, TUNEL & DNA-fragmentation assay, wound healing, transwell migration assays in presence and absence of PEITC.

Results: PEITC was found to increase the expression of PKC$\delta$ with subsequent nuclear translocation. Nuclear translocation of PKC$\delta$ was accompanied by inhibition of nuclear lamin vis a vis phosphorylation of Nrf2 at Ser 40 alongside nuclear accumulation of phospho-Nrf2. Activated PKC$\delta$ furthermore exerted its apoptotic effect by negatively regulating Aurora A and consequently PLK1; indicating activation of PLK1 by Aurora A. Involvement of PEITC induced PKC$\delta$ activation and Aurora A inhibition was ascertained by using Rottlerin/Aurora A Inhibitor.

Discussion & conclusion: Natural isothiocyanates like PEITC efficiently altered the functional abilities of STKs concerning their entangled functional interplay. Such alterations in protein expression by PEITC was chaperoned with inhibition of the aggressiveness of breast cancer cells and ultimately induction of apoptosis.

1. Introduction

Several serine/threonine kinases are reported to be aberrantly expressed in many cell types and ultimately pre-disposed to cancer in most of the cases [1, 2]. Mitosis, a highly orchestrated self-reproducing process of somatic cells, involves several serine/threonine kinases of which Aurora Kinase A (Aurora A) and Polo-like kinase-1 (PLK1) are noteworthy [3]. Spatio-temporal distribution of these serine/threonine kinases regulate cell cycle checkpoint, centrosome duplication & maturation, mitotic entry & exit, cytokinesis, and programmed cell death [4]. Protein Kinase C delta or PKC$\delta$, a member of the novel PKC family functions as a double edged sword, and balances cell death and cell survival [5]. Functional behaviour of PKC$\delta$ depends on differential phosphorylation status of the protein [6]. Depending on the stimuli and/or cell type, PKC$\delta$ has several phosphorylatable

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sites which contribute to the activation of PKCs. Thr505 (activation loop), Ser643 (turn motif), and Ser662 (hydrophobic motif) are conserved sites among all PKCs [7]. The phosphorylation of Ser643 and Ser662 seems to be important for PKCδ’s catalytic maturation. PKCδ has eight Tyr residues (located at position 52, 155, 187, 311, 332, 512, 523, and 565), that can be phosphorylated by tyrosine kinases. Two important tyrosine phosphorylation sites of PKCδ (Tyr-155 and PKCδ Tyr-311) are involved in the inhibitory effect of PKCδ on cell proliferation and are associated with PKCδ-mediated proinflammatory signaling and the initiation of cytoxicic/apoptotic pathways [8, 9]. Phosphorylation at specific tyrosine residues (Y64 and Y155) essentially directs nuclear translocation of PKCδ to induce apoptosis [10]. PLK1 another master regulator of mitosis supports centrosomal maturation, assembly of bipolar spindle, entry into mitotic phase, breakdown of nuclear envelope (NEBD), cohesion of sister chromatids, attachment of kinetochore to microtubule, mitotic exit and cytokinesis [9]. Phosphorylation on a conserved threonine residue in the T-loop of the kinase domain (T210) by Aurora A during cell cycle progression, particularly at G2 phase is a major prerequisite of PLK1 activation [11, 12]. Aurora A mainly residing in the centrosome and involved in centrosome duplication, maturation and confirmation of bipolar spindle microtubule have been documented to be overexpressed/amplified in various breast cancer cell types, including triple negative breast cancer cells or TNBC [13, 14, 15, 16, 17, 18]. The Aurora A/PLK1/Bora axes is considered as key determinant of mechanisms underlying.

2. Materials and methods

2.1. Maintenance of cell lines

Human breast adenocarcinoma cell line MCF-7 and triple negative metastatic breast adenocarcinoma cell line MDA-MB-231 were maintained in Minimum Essential Medium Eagle (MEM) supplemented with 10% heat inactivated Fetal Bovine Serum (FBS) and antibiotics (gentamicin 40 μg/ml, penicillin 100 units, streptomycin 10 μg/ml). Human normal breast epithelial cell line MCF-10A was a kind gift of Dr Amit Pal, Scientist F (ICMR-NICED, Kolkata, INDIA). MCF-10A was maintained in DMEM/F12 supplemented with serum (5%) (EGF 20 ng/ml final); Hydrocortisone (0.5 mg/ml final); Cholera Toxin (100 ng/ml final); Insulin (10 μg/ml final) and Pen/Strep. All the cells were maintained at 37 °C in a humidified CO2 incubator having 5%CO2/95% air.

2.2. MTT assay

To examine the PEITC (SIGMA# M2003-1G) mediated repression of proliferative potential of breast cancer cells, MTT assay was performed following the standard laboratory protocol [26]. Briefly, cells were seeded in 96-well plate, treated with PEITC, and incubated for 24 h. MTT (SIGMA# M2003-1G) solution was added (6 mg/5ml) and kept for another 5 h. After centrifugation, 170 μl supernatants were removed followed by the addition of DMSO (170 μl) to dissolve MTT-formazan product (purple colored). Coloured products were measured at an absorbance of 570 nm in an ELISA plate reader.

2.3. Cell cycle analysis

Confluent cells were treated with Sp/M PEITC for 24 h. Both treated and control cells were harvested, counted and equal numbers of cells (2 × 10^5) were taken for each of the experimentation. Harvested cells were washed with cold PBS (pH 7.4) and fixed with 70% chilled ethanol according to the standard laboratory protocol [28]. Cells were incubated in ice for 30 min and properly fixed cells were centrifuged at 1500 rpm for 5 min at 4 °C to remove residual ethanol. Cell pellets were suspended in 1 ml DNA binding solution containing 200 μg/ml RNase A (SIGMA# R4875-100MG) and 50 μg/ml Propidium Iodide (PI; SIGMA# P4170-10MG) and incubated in dark for 30 min. Finally cells were analysed using a FACScan flow cytometer (Beckton Dickinson), and CellQuest software. Fluorescence was captured for each determination on FL2H channel with logarithmic amplification by counting 10,000 cells.

2.4. Clonogenic assay

MCF-7 and MDA-MB-231 cells were seeded in six-well plates and treated with PEITC for 0 h, 12 h and 24 h. Medium was removed following treatment and washed with PBS, trypsinized and plated at a low density (1000 cells/well in six-well plates). PEITC treated cells for 0 h served as a control. The cells were allowed to grow for next 15 days and the medium was refreshed at every three days interval. Finally, the colonies were stained with crystal violet (SIGMA# C0775-25G) and the colony numbers were counted under microscope.

2.5. Western blotting: cytosolic and nuclear fraction

Western Blotting was carried out as per standard laboratory protocol followed previously [29]. In brief, collected cells were lysed; proteins were isolated and quantified using Lowry's method. Equally loaded proteins were separated in SDS-polyacrylamide gel. The separated proteins thereafter were transferred to a nitrocellulose membrane by electrophoresis. The antibodies used were: PKCδ (abcam: ab182126), phospho PKCδ-Tyr155 (Santacruz Biotechnology, sc-23770R), Total PLK1 (abcam: ab17056), pPLK1-Thr210 (abcam: ab39068), Aurora A (abcam: ab61114), pAurora A-Thr288 (abcam: ab83968) and PARP1 (abcam: ab194586), Cleaved PARP1 (abcam: ab4830) and β-actin (GeneTex: GTX629630). For the determination of expression of Lamin B1 (abcam: ab65986), Nrf2 (abcam: ab137550) phospho Nrf2-Ser40 (abcam: EP1809Y) and TBP (abcam: ab28175) nuclear proteins were isolated following the standard laboratory method [30], followed by western blotting as mentioned above. BCIP/NBT was used to visualise the proteins. Band intensities were quantified using Image J Software.

2.6. Aurora A activity assay

Treated and untreated cells were harvested; lysed and whole cell proteins were isolated as per standard lab protocol. Aurora A proteins were purified from cell lysate using Dynabeads™ Protein G Immunoprecipitation Kit (Invitrogen; Cat#10007D). Purified Aurora A was used to perform Aurora A activity assay using CycLex Aurora Family Kinase Assay Screening kit (MBL: Cat#CY1174). The activities of Aurora A as recorded from spectrophotometric reading (OD at 450nm) were represented graphically.

2.7. Isolation of cytosolic and membrane fraction

Both MCF-7 and MDA-MB-231 cells were washed twice in ice cold PBS, harvested and centrifuged at 500× g for 10 min. The resulting cell
pellets were briefly sonicated in a buffer comprising 20 mM Tris-HCl, pH 7.5, with 5 mM EDTA, 10 mM EGTA, 0.3% β-mercaptoethanol and protease inhibitor cocktail followed by centrifugation at 100,000 g for 1 h. The yielded supernatant (cytosolic fraction) was stored at −80 °C.

The pellets were extracted on ice for 20 min in buffer containing 10 mM CHAPS, pH 8.0, 2 mM EDTA, 4 mM iodoacetate and protease inhibitor; centrifuged and the detergent-soluble membrane protein-containing supernatants were also stored at −80 °C.

2.8. TUNEL assay

Cells were exposed separately to PEITC (5 μM), Aurora A inhibitor I (0.01 μM) for 24 h. In another set of experiment, cells were pretreated with Rottlerin (10 μM for 1 h) followed by PEITC (5 μM for 24 h). Cells were harvested afterwards and proceeded for TUNEL assay as per information provided with the TaKaRa in situ Apoptosis Detection kit (Catalogue no# MK500). Experimental details were followed as done previously [27]. TUNEL positive cells were counted from random microscopic fields at 20× magnification (objective) via a vis counting of normal cells. Apoptotic index was calculated as the ratio of TUNEL positive cells and negative cells.

2.9. DNA fragmentation assay

Treated cells were harvested; pellets were lysed in lysis buffer (1% NP-40 in EDTA 20 mM, Tris. HCl 50 mM, pH 7.5) for 30 min, centrifuged at 14000 g for 5 min at 4 °C. Supernatant was collected and treated with RNase A (5 μg/ml) for 1 h at 37 °C and further digested with proteinase K (2.5 μg/ml) for 2 h at 56 °C. Equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) were added and vortexed for 30 s, then centrifuged at 14,000 g for 2 min. This step was repeated twice. Upper aqueous phase
was taken out in a separate microcentrifuge tube. Chloroform:isoamyl alcohol (24:1) was added to it, vortexed for 30 s and finally centrifuged at 4 °C, 14,000 × g for 2 min. After collecting the upper aqueous phase ammonium acetate (10 M) equal volume was added to it and DNA was precipitated with equal volume of chilled 70% ethanol. DNA was dissolved in loading buffer (Tris-HCl 50 mM, pH 7.5, EDTA 10 mM containing 0.25% Bromophenol Blue and 30% glycerol). Fragmented DNA was analyzed electrophoretically on agarose gel (1.5%, prepared in Tris-HCl 40 mM, pH 8, EDTA 10 mM containing EtBr 3 μg/ml).

2.10. In vitro wound healing assay

Effect of PEITC (5μM), Aurora A inhibitor I (0.01μM), Rottlerin (10μM) followed by PEITC (5μM) treatment on cell motility was assessed by wound healing assay following the method of Sarkar et al. [29]. In a different set of experiment cells were treated with a Nrf2 inhibitor (ML-385) of SIGMA make (Cat No# SML1833) at a concentration of 1.5 μM for 2 h followed by PEITC (5 μM) treatment for 24 h. The monolayer of confluent breast cancer cells were scratched in a straight line to create a “wound” with a sterile pipette tip. This point was considered as “0 h”, and the width of the “wound” was measured and photographed under an inverted microscope. Cells that were migrated towards the wounded regions following “24 h” treatment were photographed and the wound regions were measured. Wound healing was determined by calculating the reduction in the width of the wound after the indicated incubation time and expressed as percentage of wound closure.

2.11. Transwell migration assay

The transwell migration of cancer cells (following treatment schedule of wound healing assay) was accomplished by using a modified Boyden chamber assay. This assay was performed in a transwell chamber with polyethylene terephthalate (PET) filter membrane containing 8-μm pore insert (Greiner bio one). Serum free suspensions of cells (4 × 10^5 cells/well) were seeded into the upper chamber. In the lower compartment medium with 20% FBS (chemoattractant) was added. Cells were permitted to migrate for 24 h. Cells that were underlying on to the upper surface of the membrane were discarded. Migrated cells on the underside of the membrane was fixed with paraformaldehyde (4%) and stained with 0.1% crystal violet. Photograph of cells were taken under inverted microscope.

3. Results

3.1. PEITC affects proliferative potential and clonogenic ability of breast cancer cells

Cytotoxic effect of PEITC was assessed by MTT assay in MCF-10A, MCF-7 and MDA-MB-231 cells that were exposed to different

Figure 2. Intrinsic expressions of serine/threonine kinases in different (metastatic and non-metastatic breast cancer cell lines) along with normal breast epithelial cell line MCF-10A. (A) Expressions of PKCδ and phosphoPKCδ and (B) Membrane and cytosolic expressions of PKCδ in two different cell lines as determined by Western blot. The results were representative of three independent experiments. Corresponding band intensities were calculated using Image J Software and normalized with β-actin and represented graphically (C) Aurora A and pAurora A expressions pattern in whole cell lysates of MCF-10A, MCF-7 and MDA-MB-231 along with their corresponding band intensities obtained from western blotting and normalized by β-actin. (D) Aurora A activity assay was performed using CycLex Aurora Family Kinase Assay Screening kit and data was recorded spectrophotometrically at OD 450nm. Each experiment was done in triplicate. Values represented mean ± SD. *p represented p < 0.005 in comparison to MCF-10A.
concentrations of PEITC for 24 h. As indicated in Figure 1A, PEITC exerted concentration-dependent cytotoxic effects on both the cell lines; particularly that in non metastatic MCF-7 cells. However, PEITC did not elicit any cytotoxic effect in normal breast epithelial cell line MCF-10A. The percentage of viable MDA-MB-231 cells was not significantly reduced at a lower concentration (1 μM) of PEITC but at highest concentration (5 μM), used. The percentage of viable cells in MCF-7 as observed from Figure 1A declined to 28% (MCF-7) and 59% (MDA-MB-231) respectively upon 5 μM PEITC treatment.

To ensure the cytotoxic effect of PEITC on the DNA content of the cell lines and to investigate the underlying molecular mechanism(s) associated with the noticeable growth inhibition, cell cycle distribution patterns were observed Flowcytometrically (Figure 1C). The patterns of distribution of cells at different phases (Histogram Data) were altered significantly after incubation with PEITC (5 μM) for 24 h (Figure 1B). An appreciable increase of sub G0/G1 cells were observed; from 4% to 31% (MCF-7) and 3.7%–15.4% (MDA-MB-231) cells. No such changes were observed in MCF-10A where sub G0/G1 cells were found to be 3.5% in untreated and 4.8% after PEITC treatment. This result indicated shifting of the G0/G1 population to the sub-G0/G1 phase, suggesting that apoptosis is possibly the predominating event upon PEITC treatment particularly with a more substantial effect on receptor positive breast cancer cells.

To further examine the long-term cytotoxicity of PEITC, clonogenic assays were performed. Since the concentration of PEITC used in this study didn’t affect the viability of MCF-10A cells, therefore further experimentation was carried out with MCF-7 and MDA-MB-231. Cells were treated with PEITC (5 μM) for 0 h, 12 h and 24 h followed by trypsinization, harvesting and plating. Colonies formed after 15 days, were stained with crystal violet and observed under microscope. PEITC rigorously abrogated the clonogenic ability of MCF-7 cells than the triple-negative, metastatic MDA-MB-231 cells as reflected in Figure 1D and corresponding graph in Figure 1E. Colony formation was inhibited to 25.5% (MCF-7) and 36.9% (MDA-MB-231) after 24 h of PEITC treatment in comparison to untreated cells. This finding particularized the effect of this compound on suppressing breast cancer cell proliferation.

3.2. Differential expression patterns of STKs in metastatic and non-metastatic breast cancer cells

To characterise PEITC-mediated inhibition of cellular proliferation and apoptotic signalling pathways, expression patterns of some of the serine/threonine kinases were noticed. However, prior to intervention studies, it is important to look into the basal levels of expression of different serine/threonine kinases (PKCδ, Aurora A and PLK1) in MCF-7 and MDA-MB-231 along with normal MCF-10A. It was evident from the result (Figure 2A) that the expression patterns of p-PKCδ (Tyr 155) as well as total PKCδ were more pronounced in non metastatic cell line rather than that in metastatic one. Original raw blots have been provided in Supplementary file S.1.A. Hardly any expression was observed in MCF-10A. Corresponding band intensities also revealed similar trend. Next we checked the differential

![Figure 3](image-url). Effect of PEITC on PKCδ and its nuclear substrate. (A) Effect of PEITC (5 μM) on phospho and total PKCδ were determined by western blotting. β-actin was used as loading control. (B) Corresponding differential fold change of phospho to total PKCδ was represented graphically. Each bar as depicted was mean ± SD (n = 3). The experiments were repeated thrice. *p represents p < 0.005 comparison to untreated. (C) The cytosolic and nuclear expression of catalytic domain of PKCδ, indicating activated PKCδ. (D) Impact of activated PKCδ on nuclear lamin expression. Cells were treated with either PEITC or Rottlerin pretreatment prior to PEITC. Lamin expression in the nuclear fraction was observed by Western blot analysis. To ensure equal protein loading, TBP was used as control.

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Figure 4. Effect of PKCδ activation on Nrf2, Aurora A and its substrate PLK1 (A) Breast cancer cells were treated with either PEITC (5 μM) or pretreated with Rottlerin 10 μM followed by PEITC treatment, harvested, fractionated into cytosolic and nuclear parts. Proteins from each fraction were subjected to western blotting using specific antibody for analyzing Nrf2 expressions. β-Actin and TBP (TATA binding protein) were used as loading controls for cytosolic and nuclear fractions respectively. (B) Upper Panel: Aurora A expression in breast cancer cells subjected to similar treatment condition as that of 4A. Lower Panel: Band intensities normalized to loading control (β-actin) was plotted graphically. Results were mean of three independent experiment mean ± SEM.*p represented p < 0.005 in comparison to untreated. (C) Aurora A activity in breast cancer cells subjected to similar treatment condition as that of A. Results were mean of three independent experiment mean ± SEM. Biswas et al. Heliyon 8 (2022) e11656.
distribution patterns of PKCδ in membrane as well as cytoplasmic fraction as translocation of PKCδ from the cytosol to a membrane location is an indication of the active form of enzyme. The result as depicted in Figure 2B evidenced minimal (MCF-7) or no (MDA-MB-231) expressions of PKCδ in the membrane fraction. No significant expressions were observed in MCF-10A (original blots are depicted in supplementary file: S.1 B). This finding indicated inactive form of the proapoptotic kinase in untreated breast cancer cells. Expression fold in cytoplasmic and membrane fraction after calculating band intensities also reflected similar trend. Expression patterns of mitotic serine/threonine kinases Aurora A/pAurora A, PLK1/ pPLK1 were examined. Expressions of phosphorylated forms were much pronounced particularly in metastatic cell line MDA-MB-231 (Figure 2C). Fold change values of Western blot results (Figure 2C), manifested a higher expression of the kinase in the metastatic MDA-MB-231 cells in comparison to MCF-7. Higher expressions of phospho-Aurora A (Thr 288) and phospho-PLK1 (Ser 210) in metastatic breast cancer cell line MDA-MB-231 in comparison to non-metastatic one; signified aggressive nature of the cells (Figure 2C). Supplementary file: S.2 depicted the original blots obtained from western blotting. Aurora A activity was also measured in all the three (MCF-10A, MCF-7 and MDA-MB-231) cells. The result (Figure 2D) demonstrated significantly high activity of Aurora A in metastatic MDA-MB-231 cell lines in comparison to non-metastatic MCF-7. Basal level of activity was detected in normal MCF-10A.

### 3.3. Loss of nuclear lamin B1 by PEITC via activation of PKCδ

To further understand whether these STKs are involved in PEITC-mediated inhibition of cell proliferation and induction of apoptosis, Western blot analysis was employed at the outset. Exposure of MCF-7 and MDA-MB-231 cells to PEITC resulted in time-dependent increase in levels of phospho-PKCδ (Tyr 155) as well as total PKCδ; specifying an apoptotic trend; with maximum effect at 24 h (Figure 3A). Supplementary file: S.3 showed original bands obtained from western blotting. Calculation of fold change values of phospho to total PKCδ also signified similar trend of fold increase by PEITC (Figure 3B). Next we examined whether nuclear translocation of PKCδ was taking place in breast cancer cells undergoing PEITC induced apoptosis. The result confirmed increased expression of phospho-PKCδ (Tyr 155), not only in the cytosolic fraction but also in the nuclear one owing to PEITC treatment (Figure 3C). Original blots of these findings have been represented in supplementary file: S.4. This finding clearly gave a clue of increased expression and functional activation of PKCδ upon PEITC treatment.

Since, nuclear lamin B1 protein represents an important target for the proapoptotic function of PKCδ, expression pattern of nuclear lamin B1 was observed in presence and absence of PEITC. The result clearly showed an inverse association of expression of lamin with that of PKCδ (Figure 3D). Original image has been given in supplementary file: S.5. To determine the relative influence of PKCδ activity, on lamin B1 degradation, an indicator of apoptotic event, Rottlerin (10 μM), a specific PKCδ inhibitor was added in cultures for 1 h prior to PEITC treatment. Surprisingly, Rottlerin treatment prior to PEITC treatment failed to reduce the expression of nuclear lamin B1. This experimental outcome confirmed about the activation of PKCδ by PEITC, which subsequently decreased lamin B1 expression probably by degrading the protein and thus driving cells towards apoptosis.

### 3.4. Involvement of PEITC mediated PKCδ activation on Nrf2 regulation and other serine/threonine kinases

Since Nrf2, an essential activator of antioxidant defence enzymes is reported to be phosphorylated by PKCδ, we investigated the expression pattern of phospho and total Nrf2 in cytosolic and nuclear fraction of MCF-7 (Data not shown) and metastatic MDA-MB-231 cells (Figure 4A). PEITC-mediated upregulation of PKCδ escalated Nrf2 phosphorylation at Ser 40 residue as well as nuclear accumulation of phospho-Nrf2. Total Nrf2 expression was found to be increased alongside the cytoplasmic fraction, but not in the nuclear one. Inhibition of PKCδ by Rottlerin on the contrary, significantly reduced the expression level of phospho-Nrf2 as well as Nrf2 accumulation in the nucleus; even after subsequent treatment with PEITC. Original findings have been displayed in supplementary file: S.6.A. This result substantiated the role of PKCδ in Nrf2 activation and nuclear accumulation due to PEITC treatment.

Our next objective was to explore whether PKCδ is deploying its proapoptotic role by acting as a negative regulator of mitotic serine/threonine kinases (Aurora A). Western blot analysis was performed using antibody against Aurora A (Figure 4B; upper panel) in untreated, PEITC treated and in Rottlerin treated cells prior to PEITC treatment. Original findings have been displayed in supplementary file: S.6.B. The densitometric results of western blotting of Aurora A over β-actin showed decreased expression of Aurora A by PEITC as opposed to control, in both MCF-7 and MDA-MB-231 (Figure 4B; lower panel). However, Rottlerin treatment prior to PEITC treatment failed to diminish the expression of Aurora A. This result in all likelihood gave an indication of inverse association of PKCδ with Aurora A.

Moreover, Aurora A activity as measured using a kit also gave out similar trend upon similar treatment condition (Figure 4C). Rottlerin treatment ahead of PEITC treatment brought back Aurora A activity significantly. This result also explains PKCδ driven inhibition of enzymatic activity of Aurora A.

To further ascertain the relationship of PKCδ with Aurora A, and PLK1 (a target serine/threonine kinase of Aurora A), Aurora A inhibitor I and PLK1 inhibitor (Poloxin) were used separately. Western blot (Figure 4D; lower panel) and corresponding densitometric results (Figure 4D; upper panel) of PEITC treated cells displayed a diminished protein level of Aurora A, which was accompanied by reduced expression level of PLK1. Treatment with Aurora A inhibitor I also showed similar inclination towards suppression of Aurora A with consequential reduction in PLK1 level. Supplementation of PEITC with Aurora A inhibitor I exhibited significant inhibition of both Aurora A as well as PLK1. Interestingly, Poloxin treatment (5 μM) reduced PLK1 expression in a definitive manner along with reduction in expression of Aurora A. These results signified a direct relation of Aurora A inhibition with that of PLK1 and vice versa upon PEITC treatment. Previous result (Figure 4B) denoted PKCδ mediated inhibition of Aurora A in presence of PEITC; yet we didn’t find any direct association between PKCδ and PLK1 (Data not shown). Taken together, these results gave a clue of PKCδ mediated inhibition of Aurora A by PEITC and subsequent suppression of PLK1.

### 3.5. Detection of characteristic hallmarks to identify apoptotic events

Our previous findings (Figure 1B) of increased sub G1 cell population and (Figure 3D) of PEITC induced PKCδ mediated decreased lamin B1 expression urged us to look into other apoptotic parameters to ascertain the event mediated by PKCδ. Apoptosis was then examined using definite determining parameters. Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick-end labeling (TUNEL) staining assay of MCF-7 (Figure 5A: Panel A) and MDA-MB-231 cells (5A: Panel B) depicted TUNEL positive cells (green fluorescence; white arrows) for PEITC treated as well as Aurora A Inhibitor I treated groups. Our results revealed that Rottlerin treatment prior to PEITC treatment markedly decreased the number of TUNEL-positive cells compared with the other.
two treatment groups. These results indicated that probably PKCδ is imparting a crucial role in apoptosis by inhibiting Aurora A. Furthermore, cells treated with PEITC at 5 μM following 24 h (Lanes 2 & 6 of Figure 5B) exhibited a typical ladder pattern [31]; implying an internucleosomal cleavage associated with apoptosis. The results were comparable with cells treated with Aurora A inhibitor I (Lanes 4 & 8 of Figure 5B); although pretreatment with Rottlerin intercepted the PEITC treated cells to undergo DNA fragmentation (Lanes 3 & 7 of Figure 5B). Original gel documentation images in DNA fragmentation assay obtained by Agarose gel electrophoresis has been depicted in supplementary file: S.7.A.

To further strengthen the notion, we further checked whether PEITC induced the cleavage of PARP1. As expected, PARP1 cleavage was observed in PEITC-treated MCF-7 and MDA-MB-231 cells. Pretreatment of Rottlerin further abrogated the cleavage of PARP1 (Figure 5C). Original gel electrophoresis images in DNA fragmentation assay obtained by Agarose gel electrophoresis has been depicted in supplementary file: S.7.B. These results cumulatively demonstrated the involvement of a negative association of PKCδ with Aurora A on PEITC treatment, which transmitted apoptotic events in cancer cells.

3.6. PEITC restrained the migratory ability of breast cancer cells by regulating serine/threonine kinase signaling

3.6.1. Wound healing assay

Our findings (Figure 4A) showed Rottlerin treatment prevented nuclear translocation of phospho-Nrf2; establishing an association of PKCδ mediated translocation. Our team in an earlier study observed that PEITC by activating nuclear translocation of Nrf2 quenches ROS and ultimately inhibits metastasis in breast cancer cells [29]; although mechanism of Nrf2 activation was not known at that time. Therefore, to reinforce the findings of Figure 4A, we subsequently observed the effects of PEITC on the motility of breast cancer cells. A scratch wound healing assay demonstrated that both the breast cancer cells, particularly MDA-MB-231 had high mobile capacities towards the wound when cells were retained untreated for 24 h (Figure 6: Panel A&B). Treatment with PEITC (5 μM) for 24 h, showed that the cells were migrated more slowly into the wound gap than the cells in the respective PEITC treated 0 h group. Similar findings were observed upon treatment of cells with Aurora A Inhibitor I (0.01 μM). Conversely Rottlerin pretreatment (10 μM for 1 h) followed by PEITC treatment failed to prevent the cell motility towards the wound gap (Figure 6A: Panel A&B). To further establish the antimetastatic role of nuclear Nrf2 we pretreated cells with ML-385 (1.5 μM) for 2 h followed by PEITC treatment for 24 h. ML-385 is a specific Nrf2 inhibitor that blocks the transcriptional activity of the transcription factor. The result interestingly showed a surge in cell motility in ML-385 group even after addition of PEITC. This finding justified the role of PKCδ mediated activation of Nrf2 in preventing migratory potential of breast cancer cells.

The area of the scratch was measured in the control and treated groups (0 h vs 24 h), and the quantitative data (percentage of wound closure) was calculated accordingly (Figure 6B). The results clearly explained lesser percentage of wound closure in PEITC and Aurora A Inhibitor I treated groups while Rottlerin treatment and ML-385 treatment impeded PEITC mediated delayed migration and thereby wound closure percentage. These results, collectively gave a probable insight of upregulation of PKCδ by PEITC, which by activating...
nuclear translocation of Nrf2, inhibiting Aurora A with subsequent inhibition of PLK1 eventually suppressed cell motility and cellular migration.

3.6.2. Transwell migration assay
To further support the findings of wound closure, we performed transwell migration assay using breast cancer cells which were subjected to similar treatment condition. Likewise, PEITC significantly inhibited breast cancer cell migration upon 24 h treatment compared with that of untreated control cells [Figure 7: Panel A (MCF-7) & Panel B (MDA-MB-231)]. Results were analogous to the findings of wound healing assays. Quantification of cell migration through a physical barrier (insert) towards the lower chamber; as presented (percentage of migrated cells), showed similar trend as of representative images. Experiments were performed in triplicates (Figure 7B). These results along with wound healing results clearly specified the involvement of PEITC mediated PKCδ upregulation in restraining cell migration and invasion particularly by antagonizing the successive expressions of Aurora A and PLK1, and by

Figure 6. PEITC represses migratory properties of breast cancer cells. (A) Wound-healing assay, illustrating the inhibitory effect of PEITC (5 μM) on cell migration following treatment with the same for 24 h. Results were compared after treatment of cells with Aurora A Inhibitor I (0.01 μM). In two separate sets of experiments, cells were pretreated with (i) PKCδ inhibitor, Rottlerin (10 μM), (ii) Nrf2 inhibitor ML-385 (1.5 μM) followed by PEITC treatment to compare the results with cells treated only with PEITC. (Magnification of objective lens was 10×, Olympus, Tokyo, Japan). Images of wound closure after respective treatment duration were compared with that of 0h treatment. (B) Percentage of wound closure at indicated treatment conditions were calculated from the microscopic images obtained from the scratch wound assay. The values represented mean ± SD. The experiments were repeated thrice. *p < 0.005 and **p < 0.01 as represented, were significant in comparison to untreated cells.
positively influencing phosphorylation of Nrf2 and nuclear translocation of the same.

4. Discussion and conclusion

Apart from their intricate contribution in mitosis, mitotic STKs like Aurora A/PLK1 are overexpressed in variety of human cancers and are associated with high tumor grade, increased invasiveness, and poor prognosis \cite{32, 33, 34}. Aurora-PLK1 cascades are known as key signaling mediators in the regulation of mitosis \cite{35}. In \textit{in vivo} studies, similar phenotypic changes were observed in PLK1 and Aurora A knockout mice; suggesting an intersection in the signaling pathways between these two mitotic kinases, which work in an identical pathway \cite{36}. Functional activation of PLK1 by Aurora A and its cofactor Bora were reported earlier \cite{37, 38}. Apart from their mitotic role, recently Murga-Zamalloa and his team \cite{39} have reported that PI3K/AKT and Aurora A/PLK1 ultimately converges at FBW7 and therefore inhibition of Aurora A/PLK1 pathway is supposed to be synergistic. Functional role of Aurora A in the activation of EMT pathway and subsequent development of distant metastases in ER\textsuperscript{+} breast cancer cells was documented earlier and inhibition of Aurora A using Alisertib (specific Aurora A inhibitor) was found to reduce the expression of PLK1 as well and ultimately induced

Figure 7. Suppression of cellular invasion by PEITC. (A) Representative images of transwell migration of MCF-7 (Panel A) and MDA-MB-231 (Panel B) cells in each of the treatment condition (PEITC, Aurora A Inhibitor I, Rottlerin + PEITC, ML-385 + PEITC). Magnification of objective lens was 10x (Olympus, Tokyo, Japan). The cells migrated into the lower chamber were stained with crystal violet. The representative images were compared with that of 0h treatment. (B) Quantification of migrated breast cancer cells with indicated treatment conditions has been represented graphically as (percentage of migrated cells). Values were represented as mean ± SD. *p represented p < 0.005 in comparison to untreated.
PKCδ, another salient enzyme belonging to serine/threonine kinase was earlier reported to exert its proapoptotic functions in many cell types [41, 42, 43]. We in this particular study attempted to investigate the functional interaction of these serine/threonine kinases in lineage of cell proliferation and metastasis and potential role of PEITC in counteracting such interactions.

Our initial studies have found that natural isothiocyanate PEITC, negatively influenced proliferative potential and clonogenic ability of breast cancer cells without affecting the growth of normal MCF-10A cells. Increased cellular distribution at Sub-G1 phase of the cell cycle evidenced an indication of apoptosis by PEITC, particularly on receptor positive MCF-7 cell line against metastatic MDA-MB-231 cell line.

Based on these initial findings we further looked into the expression patterns of STks (PKCδ, Aurora A and PLK1) where differential expression patterns of p-PKCδ (Tyr 155) as well as total PKCδ were observed in these cell lines with a negligible expression in MDA-MB-231. Basal level of expression in MCF-10A was not remarkable rather indistinct. Membrane localization of PKCδ is essential for its DAG dependent activation which allows PKCδ to change its protein conformation for facilitating nuclear translocation and apoptotic function. Membrane localization of PKCδ was found to be weak in both MCF-7 and MDA-MB-231 cell lines; manifesting no apoptotic potential in untreated cells. This observation is in agreement with that of Assender et al. [44], where ER positive breast cancer cells were reported to express considerable amount of PKCδ and thus showed better endocrine response in comparison to ER negative cells. Expressions of active form of Aurora A and PLK1 were found to be high in both the cell lines with more significant expression in MDA-MB-231 cells. We then observed the effect of PEITC on PKCδ, which not only manifested upregulation of phospho-PKCδ (Tyr155) by PEITC but also nuclear translocation of the same; a characteristic indicator of intracellular apoptotic signaling.

These results clearly signified proapoptotic function of PKCδ. To further establish the proapoptotic role of PKCδ, induced by PEITC, we examined nuclear lamin B1 expression, which reflected diminished level upon PEITC treatment and the expression pattern of the same was restored by Rottlerin, a specific PKCδ inhibitor. The result corroborated with previous other reports where PKCδ was considered as an apoptotic lamin kinase and thus phosphorylates nuclear lamin to undergo degradation; which ultimately facilitates apoptosis [45]. Another interesting data of Eitel et al. [46], suggested nuclear translocation and kinase activity of PKCδ as necessary prerequisite for induction of apoptosis. Previous studies have reported PKCδ mediated nuclear translocation of Nrf2 [47, 48]. Our team previously demonstrated facilitation of nuclear translocation of Nrf2 to nucleus from cytosol upon PEITC treatment which by activating diverse antioxidant enzymes reduced intracellular burden of reactive oxygen species (ROS) in breast cancer cells [29]. Based on these previous observations, we examined and confirmed the involvement of PKCδ in Nrf2 translocation as inhibition of PKCδ by Rottlerin significantly checked the nuclear translocation of this key molecular regulator of cellular antioxidant system.

Our result furthermore identified that PEITC induced functional activation of PKCδ hampered the expression and activity of Aurora A. The result exhibited that pretreatment with Rottlerin prevented PKCδ activation and consequently failed to inhibit Aurora A expression and activity subsequent to PEITC treatment. This interesting observation is in accordance with other previous studies which showed that silencing of PKCδ, led to activation of Aurora A via Wnt/β-catenin pathway [49]. Another finding was that PKCδ inhibitor mediated Aurora A inhibition in PKC dependent [50]. We further tried to explore whether PKCδ activation is imparting any negative effect on PLK1, but failed to authenticate any relation. Rather, the result apparently gave an indication of PKCδ activation by PEITC, which in turn inhibited Aurora A. Repression of Aurora A, resulted into concomitant suppression of PLK1.

We next studied some apoptotic parameters like TUNEL assay, DNA fragmentation assay, and PARP degradation to ascertain and confirm whether PEITC by activating PKCδ, and regulating Aurora A is subjecting breast cancer cells to undergo apoptosis. Result as obtained from TUNEL assay showed much higher frequency of apoptotic cells in PEITC treated group in comparison to untreated cells. The result was in correspondence with that of Aurora A inhibitor I treated cells. Surprisingly, Rottlerin treated cells prior to PEITC treatment, markedly decreased the frequency of TUNEL positive cells. These results justified our hypothesis that probably PKCδ activation by PEITC is propagating the apoptotic event in breast cancer cells by suppressing Aurora A activity. DNA ladder assay and PARP1 expression study also reflected the similar trend. PEITC mediated apoptosis in breast cancer cells were also observed earlier by our team [26, 27]. Present findings further validated the mechanisms of PEITC mediated apoptosis by activation of PKCδ and inhibition of Aurora A (two important serine/threonine kinases).

Anti-metastatic potential of PEITC in breast cancer cells as observed earlier in our laboratory evidenced downregulation of HIF1α due to potential activation of Nrf2 and subsequent induction of cellular antioxidant system [29]. The present result of wound closure of cells by PEITC further nourished the mechanism of PKCδ activation and corresponding translocation of Nrf2, which ultimately led to inhibition of migration of breast cancer cells as evident from the result of ML-385 treated cells; where PEITC failed to inhibit cells migration. Additionally, PKCδ mediated repression of Aurora A and consequent inhibition of PLK1 further exerted an additive effect in metastasis inhibition by PEITC. Several other literatures also documented anti-tumor potential and anti-metastatic potential of PEITC in breast cancer cells [51, 52, 53].

Considering these cumulative observations, it might be concluded that PKCδ had been one of the most important serine/threonine kinase contributing to Nrf2 activation and Aurora A inhibition in breast cancer cells. However, PKCδ didn’t impart any direct impact on PLK1, but through Aurora A. Regulatory interplay of these serine/threonine kinases may be considered as a crucial determinant of antiapoptotic and metastatic progression of breast cancer cells. PEITC efficiently disrupted such interplay by upregulating PKCδ with concomitant inhibition in lamin B1 expression, activating Nrf2 with a consequential downregulation of Aurora A and PLK1 which cumulatively thrived breast cancer cells to undergo apoptosis by inhibiting metastatic progression.

Declarations

Author contribution statement

Souvick Biswas: Performed the experiment; Analysed and interpreted the data; Wrote the paper.
Elizabeth Mahapatra; Salini Das; Madhumita Roy: Contributed reagents, materials, analysis tools or data.
Sutapa Mukherjee: Conceived and designed experiment; Analysed and interpreted the data; Wrote the paper.

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Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of interest’s statement

The authors declare no conflict of interest.

Additional information

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