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ORIGINAL ARTICLE

Anti-Estrogenic and Anti-Cell Proliferative Effect of Allyl Isothiocyanate in Chemoprevention of Chemically Induced Mammary Carcinogenesis in Rats

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Abstract

The anti-estrogenic and anti-cell proliferative effect of allyl isothiocyanate (AITC) was carried out by analyzing the status of sex hormones and its receptors and cell proliferative markers in chemically induced mammary carcinogenesis in rats. Mammary tumor was induced by a single dose of DMBA (25 mg/rat) and MNU (50 mg/kg bw) injected subcutaneously near mammary gland. RT-PCR, western blotting and immunohistochemical analysis of mammary tissues show an upregulation of ER-α, PR, aromatase, PCNA, cyclin D1 and AgNORs staining and down regulation of p53 expression as well as plasma estradiol, prolactin and testosterone levels increased in DMBA and MNU-induced tumor bearing rats. Oral administration of AITC at a dose of 20 mg/kg bw restored the levels of sex hormones and its receptors, aromatase, cell proliferative markers and AgNORs staining near to normal levels. Molecular docking studies also supported these findings. The results suggest that anti-estrogenic and anti-proliferative effect of AITC prevent the development of DMBA and MNU-induced mammary carcinogenesis in rat.

Keywords 7,12-dimethylbenz(a)anthracene · Allyl isothiocyanatep · Cell proliferation · Chemoprevention · Mammary carcinogenesis · N-methyl-N-nitrosourea

Introduction

Breast cancer is the major health concern among women folk and continues to be the leading cause of morbidity and mortality worldwide [1]. Sex hormones play a vital role in the process of mammary carcinogenesis. Prolonged exposure of estrogen and its related factors such as early menarche, late menopause, nulliparity, frequent use of oral contraceptive and hormone replacement therapy are considered to be the major risk factors [2]. Estrogen and progesterone actions are mediated via nuclear receptor known as estrogen receptors (ER) α and β and progesterone receptors (PR) A and B respectively promote the proliferation of both normal and cancerous mammary gland epithelium by altering the intracellular signaling pathways [3]. Aromatase inhibition is an important target for reducing cell proliferation in estrogen dependent breast cancer [4]. Prolactin is a lactogenic hormone and its cleavage product, activates uncontrolled proliferation of epithelial cells in mammary gland [5].

Cell proliferation process plays an important role in multistep carcinogenesis. p53 is a tumor suppressor protein controlling cell proliferation, growth and transformation. It has been strongly implicated in the process of carcinogenesis and apoptosis [6]. Proliferating cell nuclear antigen (PCNA), a highly conserved nuclear protein of DNA polymerase-δ, act as a useful marker to assess tumor cell proliferation and progression [7]. Cyclin D1 contributes to mammary carcinogenesis, presumably by increasing cell proliferation and differentiation [8].

7,12-dimethylbenz(a)anthracene (DMBA) and N-methyl-N-nitrosourea (MNU) are chemical carcinogens induce breast cancer. DMBA metabolite, diol epoxide is a strong carcinogen interacts with DNA of proliferating cells in the terminal end buds of mammary epithelium inducing neoplastic transformation [9]. MNU is a highly specific carcinogen and does not require metabolic activation to form DNA mutation and also estrogen dependent, invasive and metastasize. The

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mechanism of cancer induction of DMBA and MNU are different but both producing adenocarcinoma. The tumor growth is hormone dependent and upregulate the expression of estrogen and progesterone receptors [10].

The chemopreventive efficacy of phytochemicals have been studied by using in vitro and in vivo models [11]. Allyl isothiocyanate (AITC) found in some brassica vegetables emerging as a promising chemopreventive agent. The anti-tumor [12] and anti-proliferative [13] properties of AITC were studied in various cancer cell lines and found that the putative mechanisms are inhibition of cytochrome P450 enzymes and activation of phase II enzymes. Our previous study also reported that AITC found to be effective in prevention of mammary tumorigenesis in DMBA induced female Sprague-Dawley (SD) rats [14]. The present study aimed to investigate the anti-estrogenic and anti-proliferative effect of AITC in chemoprevention of DMBA and MNU induced mammary carcinogenesis in female SD rats by analyzing the sex hormone in plasma, hormone receptors and cell proliferative markers in mammary tissues.

Materials and Methods

Chemicals

AITC, DMBA and MNU were purchased from Sigma-Aldrich Chemicals Pvt. Ltd., Bangalore, India. Primary antibodies for ER-α, PR, aromatase, p53, PCNA, cyclin D1 and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All the other chemicals used were of analytical grade.

Animal Model

Female SD rats (weighed 100–120 g), six to seven weeks of age were purchased from National Institute of Nutrition, Hyderabad, India. After getting proper approval from the Institutional Animal Ethics Committee for the Control and Supervision of Experimental Animal (CPCSEA approval no: 983) guidelines, the rats were maintained in the Central Animal House, Rajah Muthiah Medical College and Hospital, Annamalai University, Chidambaram, Tamilnadu, India. The rats were acclimatized under the controlled conditions of temperature (24 ± 2 °C), humidity (50 ± 10%) and 12 h light/dark cycle with provided feed and water ad libitum.

Experimental Design

After dose fixation, total numbers of 60 rats were divided into six groups consisting of 10 rats each. Group I rats were served as untreated control. Groups II & IV, III & V rats were received a single subcutaneous injection of DMBA (25 mg/rat) and MNU (50 mg/kg bw) respectively near to the mammary gland at the end of the first week. Groups II & III rats received no other treatment. Groups IV & V rats were administered orally AITC (20 mg/kg bw) by intubation once in a day by starting one week before the exposure of the carcinogen and were continued till the end of experimental period. Group VI rats were received oral administration of AITC (20 mg/kg bw) throughout the experimental period (16 weeks). The dose of AITC 20 mg/kg bw was decided based on our previous dose dependent study [14].

Evaluation of Hormone Profile

Hormones such as plasma estradiol, progesterone, prolactin and testosterone were measured using enzyme-linked immunosorbent assay kits as per the manufacturer's instructions.

Histological Analysis

For histological analysis, mammary tissues were excised and immersed in 10% formalin solution for fixation, dehydrated with graded ethanol solutions from 50% to 100% and then embedded in paraffin. Paraffin-embedded mammary tissue sections (3–5 μm) were cut using a microtome then rehydrated with xylene and dehydrated with graded ethanol. Argyrophilic Nucleolar Organizer Regions (AgNORs) staining was performed according to the method of Ploton et al. [15]. Images were captured by Nikon Coolpix 4500 microscope at 40x magnification. Mammary tissues AgNORs dots/nuclei was analysed by using standard quantification software ImageJ.

Reverse Transcription-Polymerase Chain Reaction Analysis

The total RNA was isolated from the mammary tissues using Trizol reagent according to the method of Chomczynski and Sacchi [16]. The diluted RNA sample was quantified spectrophotometrically by measuring the absorbance at 260 nm. The purified RNA was reverse transcribed by using reverse transcriptase enzyme into a single strand cDNA. The cDNA was amplified with respective primers of ER-α, PR, aromatase, p53, PCNA and cyclin D1 as mentioned below, GAPDH used as an internal standard.

ER-α forward primer: 5'-AATTCTGACAATCG ACGCCAG-3′ and reverse primer: 5'-GTGCTTCA ACATTCTCCCTCCTC-3′, PR forward primer: 5'-CCCACAGGAGTTTGTCAAGCTC-3′ and reverse primer: 5'-TAACTTCAGACATCATTTCCGG-3′ for the amplification was carried out in an automated thermal cycler as follows: the reaction was performed with 35 cycles of denaturation for

1 min at 95 °C, annealing for 1 min at 62 °C, extension for 2 min at 72 °C and then terminated with a 5 min extension at 72 °C. Aromatase forward primer: 5'-GCTTCTCATCGCAGAGTATCCGG-3′ and reverse primer: 5'-CAAGGGTAAATTCATTGGGCTTGG-3′ for the amplification was carried out in an automated thermal cycler as follows: the reaction was performed with 35 cycles of denaturation for 30 s at 95 °C, annealing for 30 s at 60 °C, extension for 30 s at 72 °C and then terminated with 5 min extension at 72 °C.

p53 forward primer: 5'-GATTCTTTCTCCTCTCCTAC-3′ and reverse primer: 5'-TGTAGATGGCCATGGCACGG-3′ for the amplification was carried out in an automated thermal cycler as follows: 35 cycles of denaturation for 1 min at 95 °C, annealing for 1 min at 53 °C, extension for 1 min at 72 °C with initial denaturation at 95 °C for 5 min and final extension for 5 min at 72 °C. PCNA forward primer: 5'-GCCCTCAA AGACCTCATCAA-3′ and reverse primer: 5'-GCTCCCCA CTCGCAGAAAAC-3′ for the amplification was carried out in an automated thermal cycler as follows: 25 cycles of denaturation for 1 min at 95 °C, annealing for 30 s at 60 °C, extension for 1 min at 72 °C with initial denaturation at 72 °C for 30 s and final extension for 7 min at 72 °C. Cyclin D1 forward primer: 5'-TGGAGCCCCTGAAGAAGAG-3′ and reverse primer: 5′- AAGTGCGTTGTGCGGTAGC-3′ for the amplification was carried out in an automated thermal cycler as follows: the reaction was performed with 45 cycles of denaturation for 45 s at 94 °C, annealing for 30 s at 45 °C, extension for 30 s at 72 °C and then terminated with 7 min extension at 72 °C.

GAPDH forward primer: 5'-TTCTTGTGCAGTGC CAGCCTCGTC-3′ and reverse primer: 5'-TAGGAACA CGGAAGGCCATGCCAG-3′ for the amplification was carried out in an automated thermal cycler as follows: the reaction was performed with 35 cycles of denaturation for 30 s at 95 °C, annealing for 30 s at 58 °C, extension for 30 s at 72 °C and then terminated with 5 min extension at 72 °C. The amplified PCR products were analyzed by electrophoresis in 2% agarose gels and visualized under UV illumination using ethidium bromide.

Western Blotting Analysis

Mammary tissues were homogenized in ice-cold RIPA buffer. The homogenate was centrifuged at 12,000 rpm for 15 min at 4 °C. The supernatant was collected and the protein concentration was measured by the method of Lowry et al. [17]. Total cellular proteins containing 50 μg were loaded and separated using 10% SDS polyacrylamide gel electrophoresis. The separated proteins were transferred into PVDF membrane. The membranes were incubated with the blocking buffer containing 5% BSA (Bovine Serum Albumin) for 2 h to reduce non-specific binding sites and then incubated with the specific primary antibodies for ER-α (mouse monoclonal; 1:1000), PR (mouse monoclonal; 1:1000), aromatase (rabbit polyclonal; 1:1000), p53 (mouse monoclonal; 1:1000), PCNA (mouse monoclonal; 1:1000), cyclin D1 (mouse monoclonal; 1:1000) and β-actin (rabbit polyclonal; 1:500) were diluted with 5% BSA in TBS (Tris-Buffered Saline) for overnight at 4 °C. Membranes were washed thrice with TBST (Tris-Buffered Saline Tween 20). The membranes were incubated with their corresponding secondary antibodies for 2 h at room temperature. Membranes were washed thrice with TBST. Protein bands were visualized by an enhancing chemiluminescence method using an ECL kit (GenScript ECL kit, USA). Bands were scanned using a scanner and quantitated by ImageJ, a public domain Java image processing software, Wayne Rasband, NIH, Bethesda, MD, USA, which control was set to 1.

Immunohistochemical Analysis

Paraffin embedded mammary tissue sections were processed and then immunostained with the primary antibodies for ER-α and PR at a concentration of 1 μg/mL with 3% BSA in TBS and incubated overnight at 4 °C. The slides were washed thrice with TBST and incubated with their respective HRP conjugated secondary antibodies, diluted 1:200 with 3% BSA in TBS and incubated for 2 h at room temperature. Sections were then washed with TBST and incubated with 0.02% diaminobenzidine containing 0.01% hydrogen peroxide for 5–10 min and counter staining was performed using hematoxylin and the slides were visualized under a light microscope.

Molecular Docking Study

Molecular docking study was done using Schrodinger suite 2014–2. The structure of AITC was retrieved from PubChem (www.ncbi.nlm.nih.gov/pccompound) and the structures of target proteins such as aromatase (PDB ID: 3EQM) and ER-α (PDB ID: 3ERT) were retrieved from the protein data bank (www.rcsb.org/pdb). Receptor grid generation and ligand docking were performed by employing Glide Xp docking algorithm.

Statistical Analysis

Statistical analysis was performed using SPSS 16 (SPSS, Inc., Chicago) statistical package. The data is expressed as mean ± standard deviation. One way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT) comparison method was used to correlate the difference between the variables. Data is considered statistically significant if p values are less than 0.05.

Results

Effect of AITC on Level of Sex Hormones in Plasma of Control and Experimental Rats

Table 1 shows the level of sex hormones in plasma of control and experimental rats. The level of sex hormone such as estradiol, prolactin and testosterone are significantly ($p < 0.05$) increased and the level of progesterone is significantly ($p < 0.05$) decreased in group II and III cancer-bearing rats when compared to the group I control rats. Whereas the levels of estradiol, prolactin and testosterone are significantly ($p < 0.05$) decreased and the level of progesterone is significantly ($p < 0.05$) increased in group IV and V AITC treated rats when compared to the group II and III cancer-bearing rats. However, there are no significant ($p < 0.05$) changes in the level of sex hormones in group VI AITC alone treated rats when compared to group I control rats.

Effect of AITC on mRNA and Protein Expression of ER-α, PR and Aromatase in Mammary Tissues of Control and Experimental Rats

Figures 1 and 2 show the RT-PCR and western blot analysis expression of ER-α, PR and aromatase in mammary tissues of control and experimental rats. The mRNA and protein expression of ER-α, PR and aromatase are significantly ($p < 0.05$) increased in group II and III cancer-bearing rats when compared to group I control rats. Whereas the mRNA and protein expression of ER-α, PR and aromatase are significantly ($p < 0.05$) decreased in group IV and V AITC treated rats when compared to the group II and III cancer-bearing rats. However, there are no significant ($p < 0.05$) changes in the mRNA and protein expression of ER-α, PR and aromatase in group VI AITC alone treated rats when compared to group I control rats.

Effect of AITC on the Protein Expression of ER-α and PR in Mammary Tissues of Control and Experimental Rats

Figures 3 and 4 (A-F) show the immunohistochemical analysis of ER-α and PR in mammary tissues of control and experimental rats. The protein expression of ER-α and PR are significantly ($p < 0.05$) increased in group II (B) and III (C) cancer-bearing rats when compared to group I (A) control rats. Whereas the protein expression of ER-α and PR are significantly ($p < 0.05$) decreased in group IV (D) and V (E) AITC treated rats when compared to the group II (B) and III (C) cancer-bearing rats. However, there are no significant ($p < 0.05$) changes in the expression of ER-α and PR in group VI (F) AITC alone treated rats when compared to group I (A) control rats.

Effect of AITC on Histological Analysis of AgNORs Staining in Mammary Tissues of Control and Experimental Rats

Figure 5a–f shows the histological analysis of AgNORs staining in mammary tissues of control and experimental rats. The group II (B) and III (C) cancer-bearing rats showed significantly ($p < 0.05$) increased AgNORs dots/nuclei, which is reduced in group IV (D) and V (E) AITC treated rats. The group I (A) control and group VI (F) AITC alone treated rats exhibited very low accumulation of AgNORs dots/nuclei.

Effect of AITC on mRNA and Protein Expression of p53, PCNA and Cyclin D1 in Mammary Tissues of Control and Experimental Rats

Figures 6 and 7 show the RT-PCR and western blot analysis expression of p53, PCNA and cyclin D1 in mammary tissues of control and experimental rats. The mRNA and protein expression of p53 are significantly ($p < 0.05$) decreased and

Table 1 Effect of AITC on hormone profile in plasma of control and experimental rats

Groups	Estradiol (pg/mL)	Progesterone (ng/mL)	Prolactin (ng/mL)	Testosterone (ng/mL)
Control	31.56 ± 3.0^{a}	11.46 ± 0.8^{a}	14.59 ± 1.0^{a}	3.63 ± 0.3^{a}
DMBA	49.23 ± 3.7^{b}	6.84 ± 0.5^{b}	25.92 ± 1.7^{b}	9.16 ± 0.8^{b}
MNU	45.08 ± 3.3^{c}	8.51 ± 0.7^{c}	23.18 ± 1.7^{c}	7.97 ± 0.6^{c}
DMBA + AITC	35.21 ± 2.6^{a}	10.68 ± 0.9^{a}	16.12 ± 1.4^{a}	4.23 ± 0.3^{a}
MNU + AITC	34.16 ± 2.7^{a}	11.06 ± 0.9^{a}	15.61 ± 1.1^{a}	4.12 ± 0.3^{a}
AITC	32.86 ± 2.7^{a}	11.21 ± 0.9^{a}	14.89 ± 1.0^{a}	3.91 ± 0.3^{a}

Values are expressed as mean ± SD for ten rats in each group

Values not sharing a common superscript differ significantly at $p < 0.05$

[a] Comparisons were made between groups I, IV, V and VI

[b] Comparisons were made between group II

[c] Comparisons were made between group III

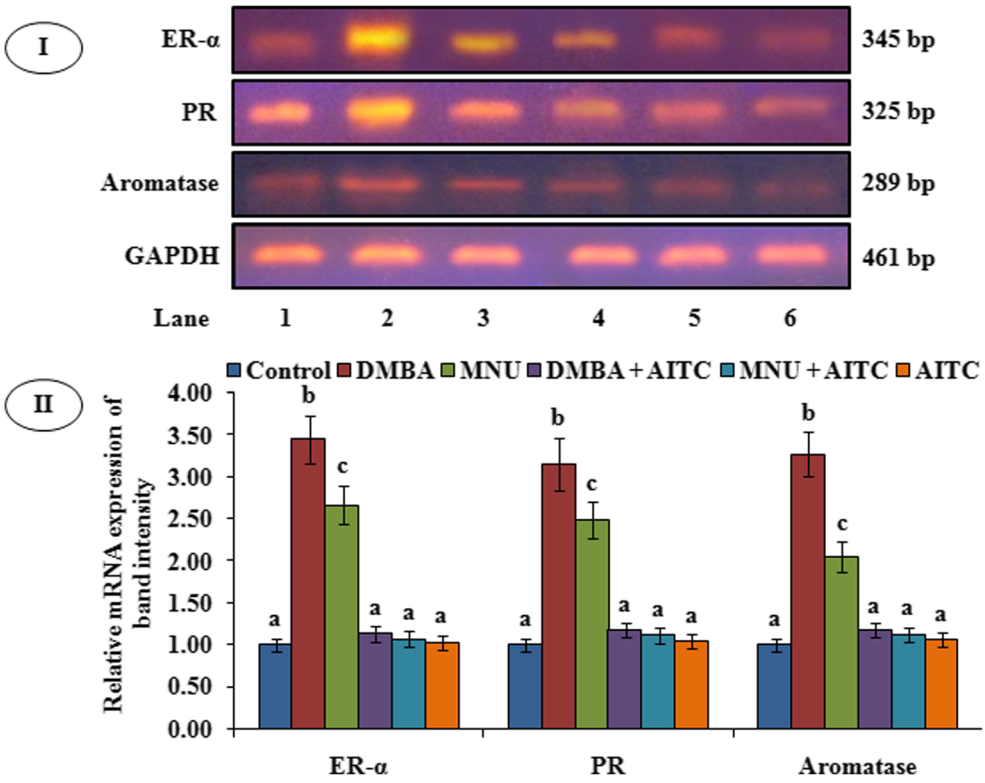

Fig. 1 RT-PCR analysis of ER-α, PR and aromatase in the mammary tissues of control and experimental rats. (I) Shows the band intensity quantified by densitometry and normalized to GAPDH loading control. Lane 1: control rats (group I); Lane 2: DMBA-induced rats (group II); Lane 3: MNU-induced rats (group III); Lane 4: DMBA-induced rats treated with AITC (group IV); Lane 5: MNU-induced rats treated with AITC (group V); Lane 6: AITC alone treated rats (group VI). (II) The representative graph shows the relative mRNA expression of fold changes in RT-PCR. Values are given as mean ± SD for groups of ten rats in each. Values not sharing a common superscript differ significantly at $p < 0.05$ (DMRT)

PCNA and cyclin D1 were significantly ($p < 0.05$) increased in group II and III cancer-bearing rats when compared to group I control rats. Whereas the mRNA and protein expression of p53 was significantly ($p < 0.05$) increased and PCNA and cyclin D1 are significantly ($p < 0.05$) decreased in group IV and V AITC treated rats when compared to the group

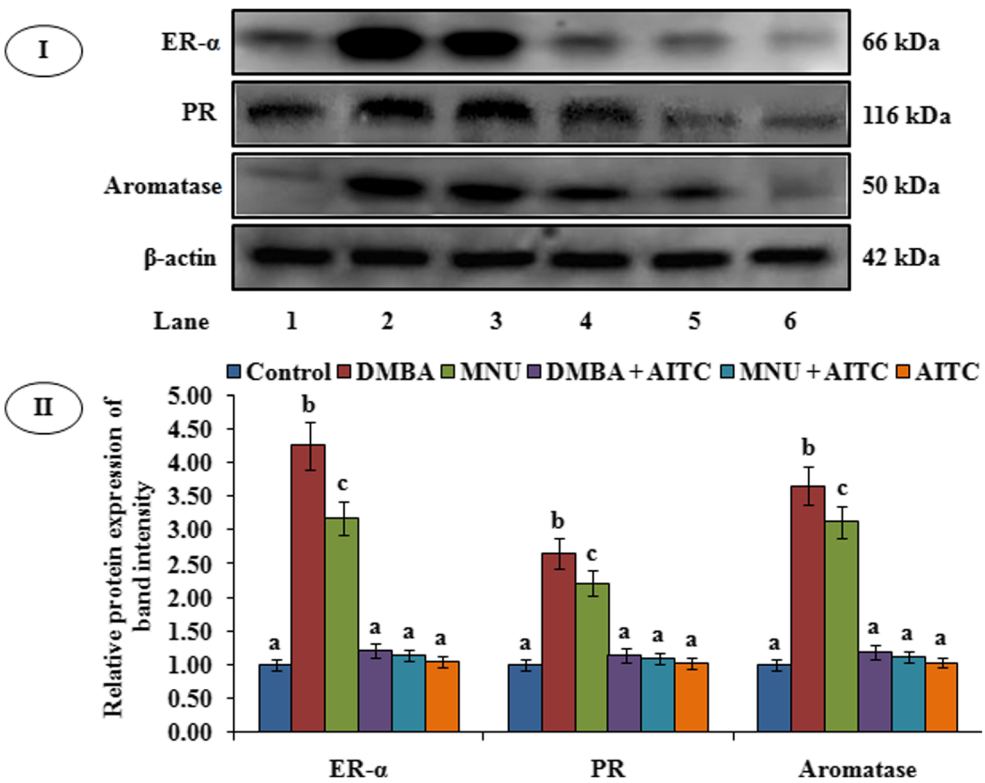

Fig. 2 Western blotting analysis of ER-α, PR and aromatase in the mammary tissues of control and experimental rats. (I) Shows the band intensity quantified by densitometry and normalized to respective β-actin loading control. Lane 1: control rats (group I); Lane 2: DMBA-induced rats (group II); Lane 3: MNU-induced rats (group III); Lane 4: DMBA-induced rats treated with AITC (group IV); Lane 5: MNU-induced rats treated with AITC (group V); Lane 6: AITC alone treated rats (group VI). (II) The representative graph shows the relative protein expression of fold changes in western blot. Values are given as mean ± SD for groups of ten rats in each. Values not sharing a common superscript differ significantly at $p < 0.05$ (DMRT)

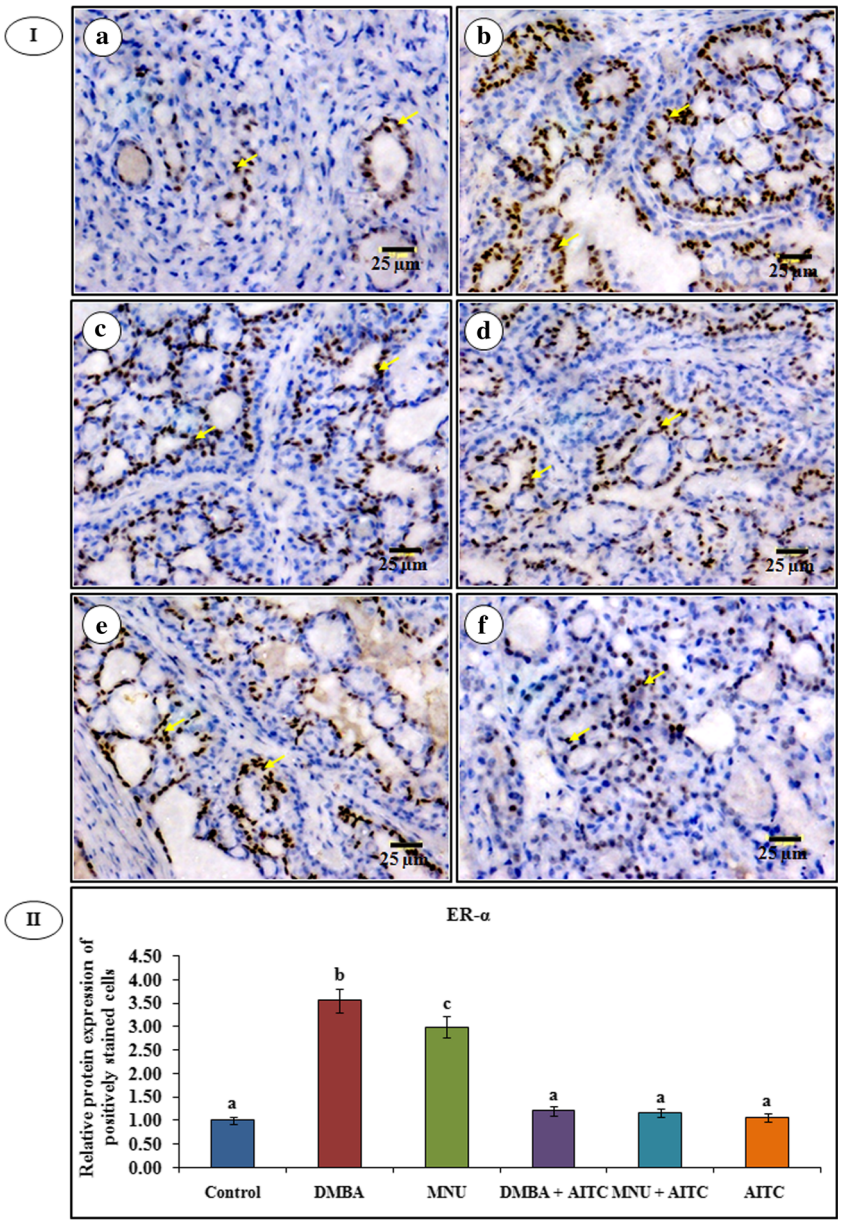

Fig. 3 Immunohistochemical analysis of ER-α status in the mammary tissues of control and experimental rats. (I) A photomicrographic image shows the immunohistochemical analysis of ER-α. **a** control rats (group I); **b** DMBA-induced rats (group II); **c** MNU-induced rats (group III); **d** DMBA-induced rats treated with AITC (group IV); **e** MNU-induced rats treated with AITC (group V); **f** AITC alone treated rats (group VI). Arrows indicate positively stained cells. (II) The representative graph shows the relative protein expression of fold changes in immunohistochemistry. Values are given as mean ± SD for groups of ten rats in each. Values not sharing a common superscript differ significantly at $p < 0.05$ (DMRT). 40× magnification

II and III cancer-bearing rats. However, there are no significant ($p < 0.05$) changes in the mRNA and protein expression of p53, PCNA and cyclin D1 in group VI AITC alone treated rats when compared to group I control rats.

Molecular Docking Study

Figure 8 (A1, A2, B1 and B2) shows the molecular docking studies of AITC binding with aromatase and cocrystal structure of aromatase, ER-α and cocrystal structure of ER-α. The results revealed that AITC exhibit a phenomenal binding interaction with aromatase and cocrystal structure of aromatase, ER-α and cocrystal structure of ER-α glide energy −18.363, −48.381, −19.532 and − 63.083 (kcal/mol) respectively. A1 represents AITC form hydrogen bond with Ala 438 and hydrophobic interactions with Ile 133, Ala 306, Phe 148, Leu 152 and Met 303 of aromatase. A2 represents AITC form hydrogen bond with Ala 438 and hydrophobic interactions with Met 311, Cys 437, Met 446, Ile 442, Gly 439, Phe 148, Ile 132, Leu 152, Phe 203, Ala 306 and Met 303 of cocrystal structure of aromatase. B1 represents AITC form hydrogen bond with Trp 383 and hydrophobic interactions of Met 357, Leu 387, Leu 354 and Ala 350 of ER-α. B2 represents AITC form hydrogen bond with Arg 394 and Glu 353 and hydrophobic interactions of Leu 354, Leu 391, Leu 349, Leu 387, Asp 351, Phe 404, Leu 346, Thr 347, Ala 350, Leu 428, Trp 383, Ile 424, Leu 384, Leu 525, Met 421, Met 343, Gly 521 and His 524 of cocrystal structure of ER-α.

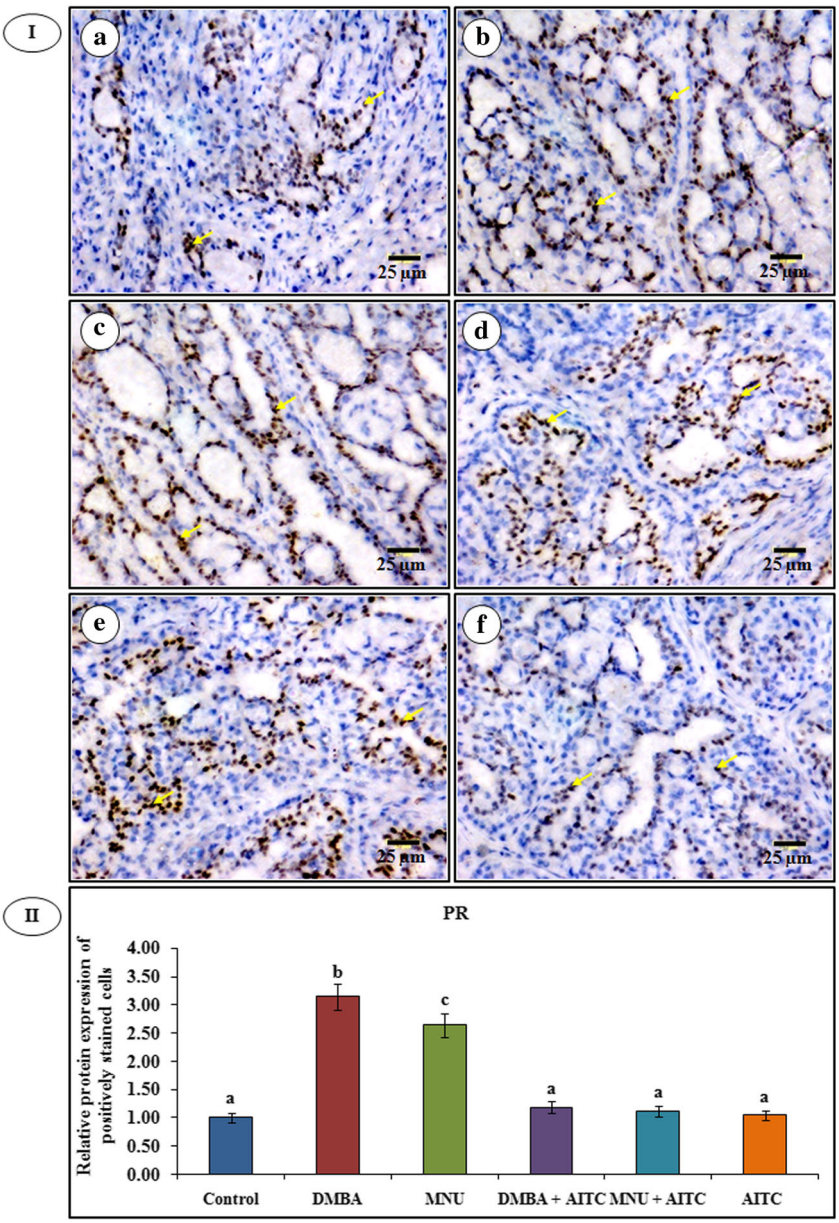

Fig. 4 Immunohistochemical analysis of PR status in the mammary tissues of control and experimental rats. (I) A photomicrographic image shows the immunohistochemical analysis of PR. **a** control rats (group I); **b** DMBA-induced rats (group II); **c** MNU-induced rats (group III); **d** DMBA-induced rats treated with AITC (group IV); **e** MNU-induced rats treated with AITC (group V); **f** AITC alone treated rats (group VI). Arrows indicate positively stained cells. (II) The representative graph shows the relative protein expression of fold changes in immunohistochemistry. Values are given as mean ± SD for groups of ten rats in each. Values not sharing a common superscript differ significantly at $p < 0.05$ (DMRT). 40× magnification

Discussion

Estrogen plays a vital role in promoting cell growth, differentiation and function of the mammary gland [18]. Estrogen mediates its action through classical pathway via nuclear receptors ER-α and ER-β. ER-α act as a ligand-activated transcription factor regulate the expression of estrogen responsive genes. In this study, both DMBA and MNU-induced tumor bearing rat showing increased level of circulatory estradiol, decreased level of progesterone and high expression of ER-α and PR in mammary tumor tissues. Previous studies were also reported the similar results [19, 20]. However, AITC administration reduces the level of estradiol, down regulates the expression of ER-α and PR and improves the level of progesterone indicates that AITC inhibit the synthesis of estrogen and expression of its receptors on target organ as evidenced by the results of RT-PCR, western blotting and immunohistochemical analysis. Kang et al. [21] reported that isothiocyanate such as PEITC and BITC suppress ER-α in MCF7 and T-47D breast cancer cells by inhibiting promoter activity of estrogen response elements. Molecular docking study also reveals that the antagonistic effect of AITC with ER-α. Previous study also reported that phytochemicals bind to human ER leading to selective estrogen receptor modulation [22]. Prolactin induces cellular proliferation, survival and motility in mammary glands [23]. In this study, we found that an increased level of prolactin in tumor bearing rats which is coincide with the study of Arivazhagan and Sorimuthu Pillai

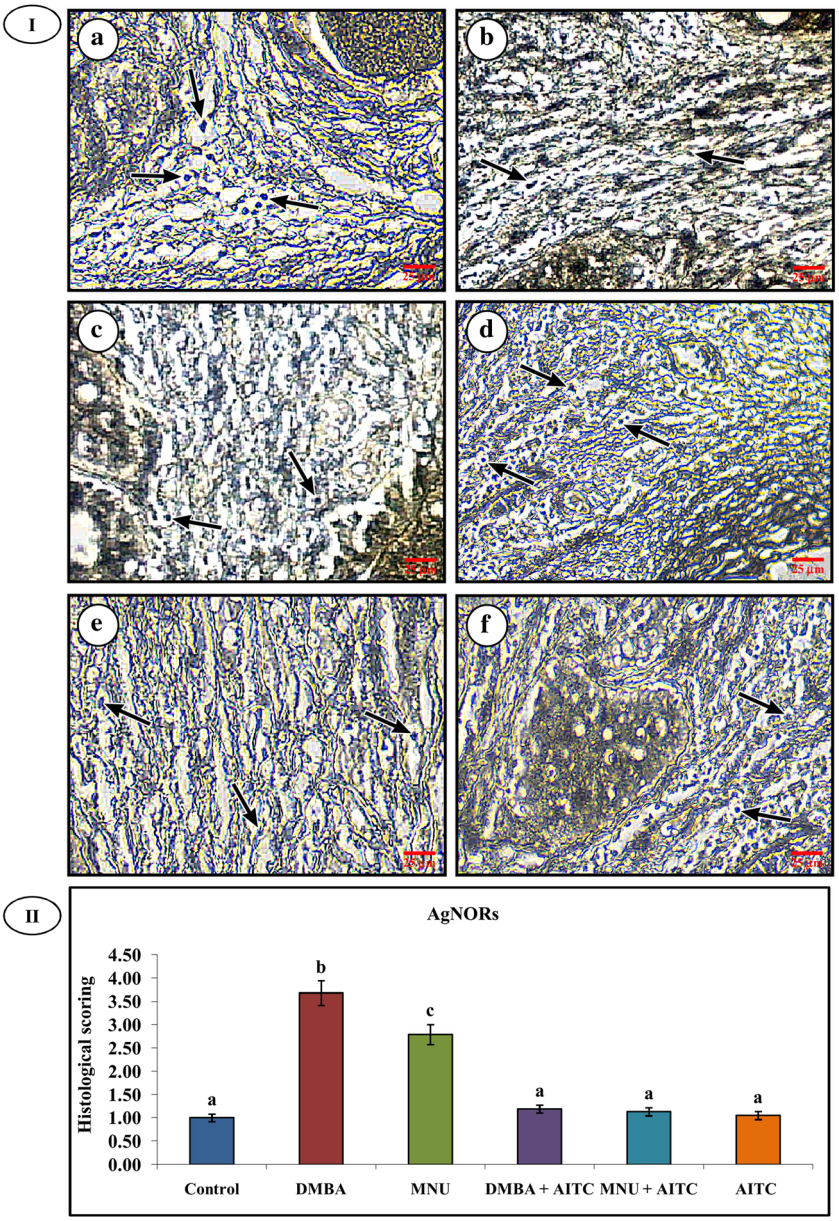

Fig. 5 Pathological analysis of AgNORs staining in the mammary tissues of control and experimental rats. (I) A photomicrographic image shows the pathological analysis of AgNORs staining. **a** control rats (group I); **b** DMBA-induced rats (group II); **c** MNU-induced rats (group III); **d** DMBA-induced rats treated with AITC (group IV); **e** MNU-induced rats treated with AITC (group V); **f** AITC alone treated rats (group VI). (II) The representative graph shows the histological scoring of AgNORs dots/nuclei in pathological analysis. 40× magnification. Arrows indicate AgNORs black dots

[9]. However, AITC administration reduce prolactin level significantly may be due to anti-estrogenic effect on mammary epithelium.

Upregulated expression of aromatase was observed in women breast tumor [24]. Aromatase inhibitors are normally used for the treatment of estrogen-dependent ER positive breast cancer [25]. In this study, the expression of aromatase and the level of testosterone were increased in cancer bearing rats and AITC administration reduces aromatase expression and the level of testosterone which indicates its aromatase inhibition potential. Previous data suggest that melatonin down-regulates the expression of aromatase through its modulatory activity of cAMP [24] and tangeretin reduces testosterone in cancer bearing rats [9]. In-silico docking study also reveals that AITC found to be a strong docking ligand to aromatase. Previous findings provide valuable information on the binding process of flavonoid compounds to the binding site of aromatase [26].

Cell proliferation plays a crucial role in initiation, promotion and progression of mammary carcinogenesis. The exploration of natural or dietary compounds that inhibit abnormal proliferation in tumor cells has immense value in chemoprevention of breast cancer [27]. Nucleolar organizer regions that bind to silver ions through a set of acidic, non histone proteins are chromosomal loops of DNA involved in the ribosomal synthesis [28]. Malignant cells exhibit a larger number of AgNOR patterns indicating increased cell proliferation [29].

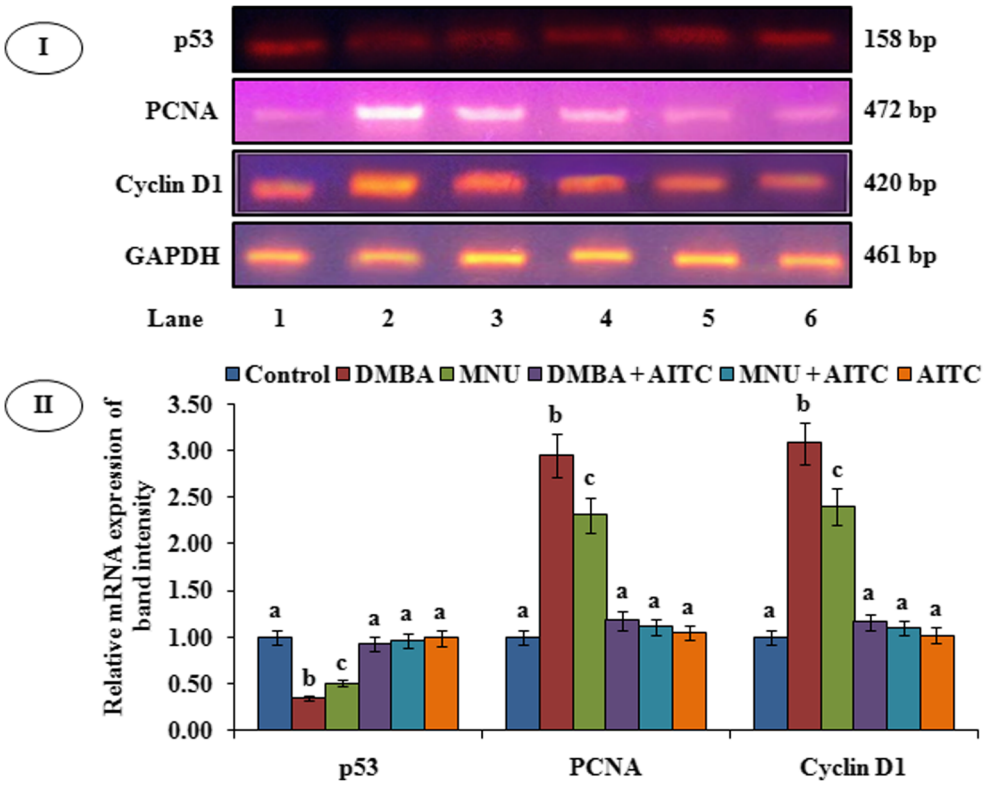

Fig. 6 RT-PCR analysis of p53, PCNA and cyclin D1 in the mammary tissues of control and experimental rats. (I) Shows the band intensity were quantified by densitometry and normalized to GAPDH loading control. Lane 1: control rats (group I); Lane 2: DMBA-induced rats (group II); Lane 3: MNU-induced rats (group III); Lane 4: DMBA-induced rats treated with AITC (group IV); Lane 5: MNU-induced rats treated with AITC (group V); Lane 6: AITC alone treated rats (group VI). (II) The representative graph shows the relative mRNA expression of fold changes in RT-PCR. Values are given as mean ± SD for groups of ten rats in each. Values not sharing a common superscript differ significantly at $p < 0.05$ (DMRT)

p53, a tumor suppressor protein regarded as a key factor regulate the balance between cell survival and cell death, via regulation of both the G1 and G2/M stage of cell cycle [30]. Activated p53 involved number of cellular processes such as DNA repair, cell cycle arrest, inhibition of cell proliferation and induction of apoptosis [31]. AITC triggers sub-G1 and

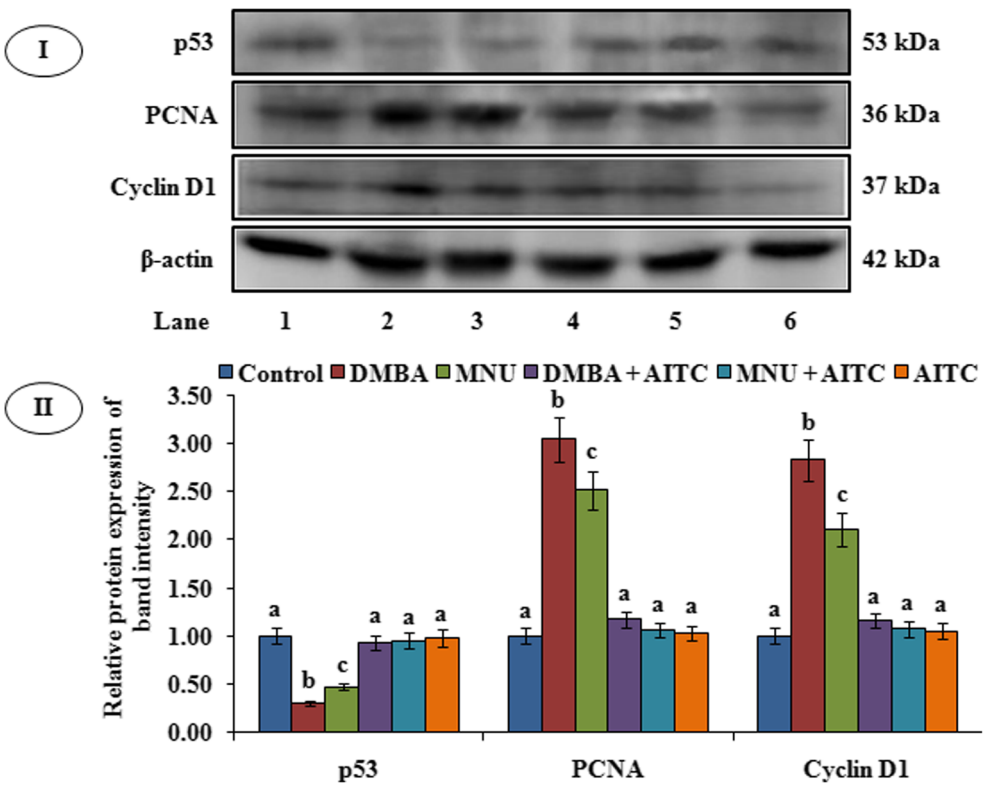

Fig. 7 Western blotting analysis of p53, PCNA and cyclin D1 in the mammary tissues of control and experimental rats. (I) Shows the band intensity were quantified by densitometry and normalized to respective β-actin loading control. Lane 1: control rats (group I); Lane 2: DMBA-induced rats (group II); Lane 3: MNU-induced rats (group III); Lane 4: DMBA-induced rats treated with AITC (group IV); Lane 5: MNU-induced rats treated with AITC (group V); Lane 6: AITC alone treated rats (group VI). (II) The representative graph shows the relative protein expression of fold changes in western blot. Values are given as mean ± SD for groups of ten rats in each. Values not sharing a common superscript differ significantly at $p < 0.05$ (DMRT)

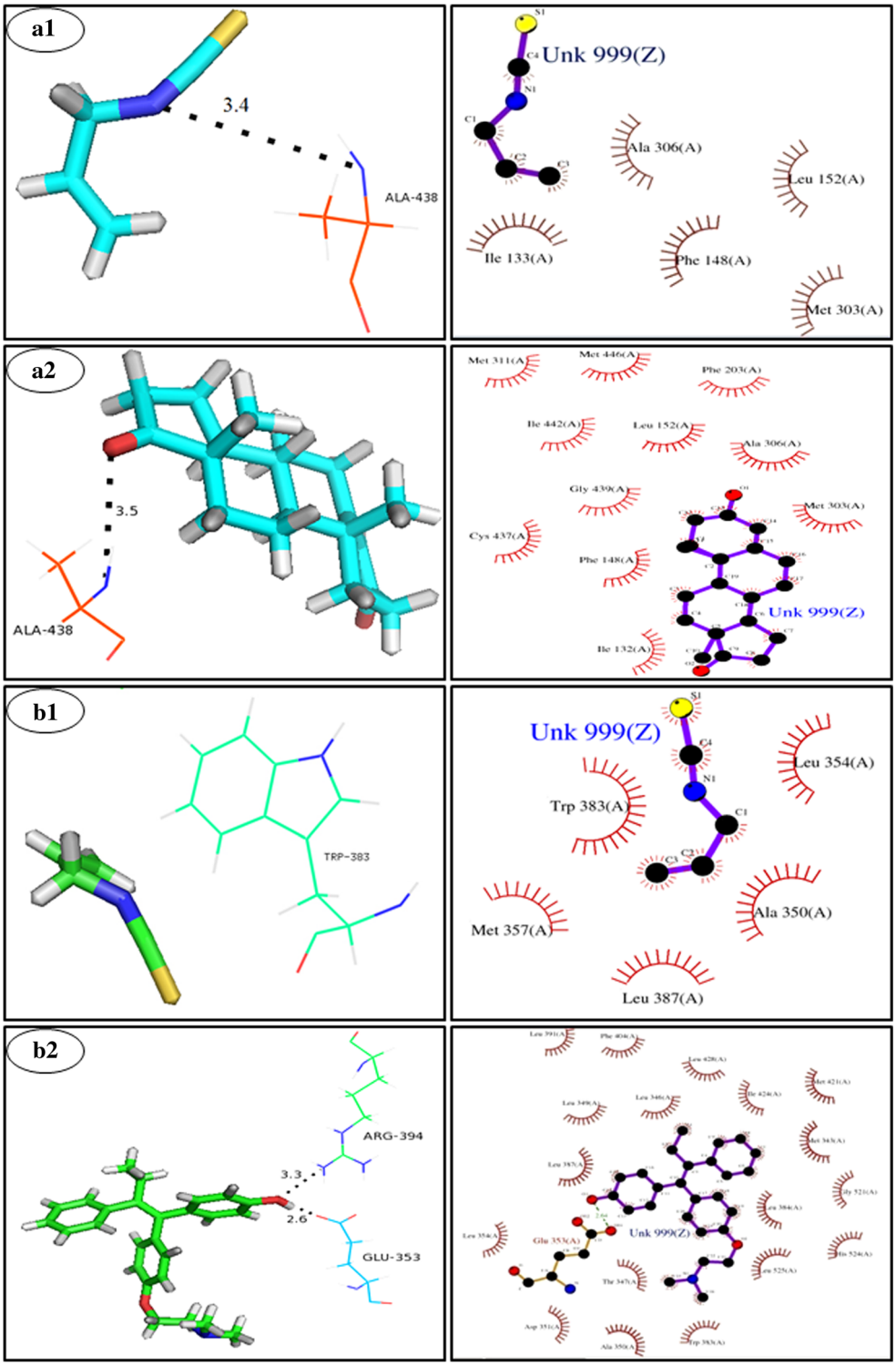

Fig. 8 Representation of binding mode of AITC with aromatase (A1), cocrystal structure of aromatase (A2), ER-α (B1) and cocrystal structure of ER-α (B2)

G2/M phase arrest by its regulator proteins CDK1, cyclin B and cyclin A [32]. In the present study, the expression of p53 was decreased in DMBA and MNU-induced rats [33]. Administration of AITC to carcinogens injected rats significantly augmented the expression of p53 [34]. The cellular and molecular responses in mouse lungs after treatment with benzyl isothiocyanate-N-acetylcysteine (BITC-NAC) and phenethyl isothiocyanate-N-acetylcysteine (PEITC-NAC) are known to be associated with oxidative stress. BITC-NAC and PEITC-NAC activate p53 activity in mouse lungs by inducing phosphorylation and subsequently expression of its effector genes, Bax and $p21^{WAF/CIP1}$ [35].

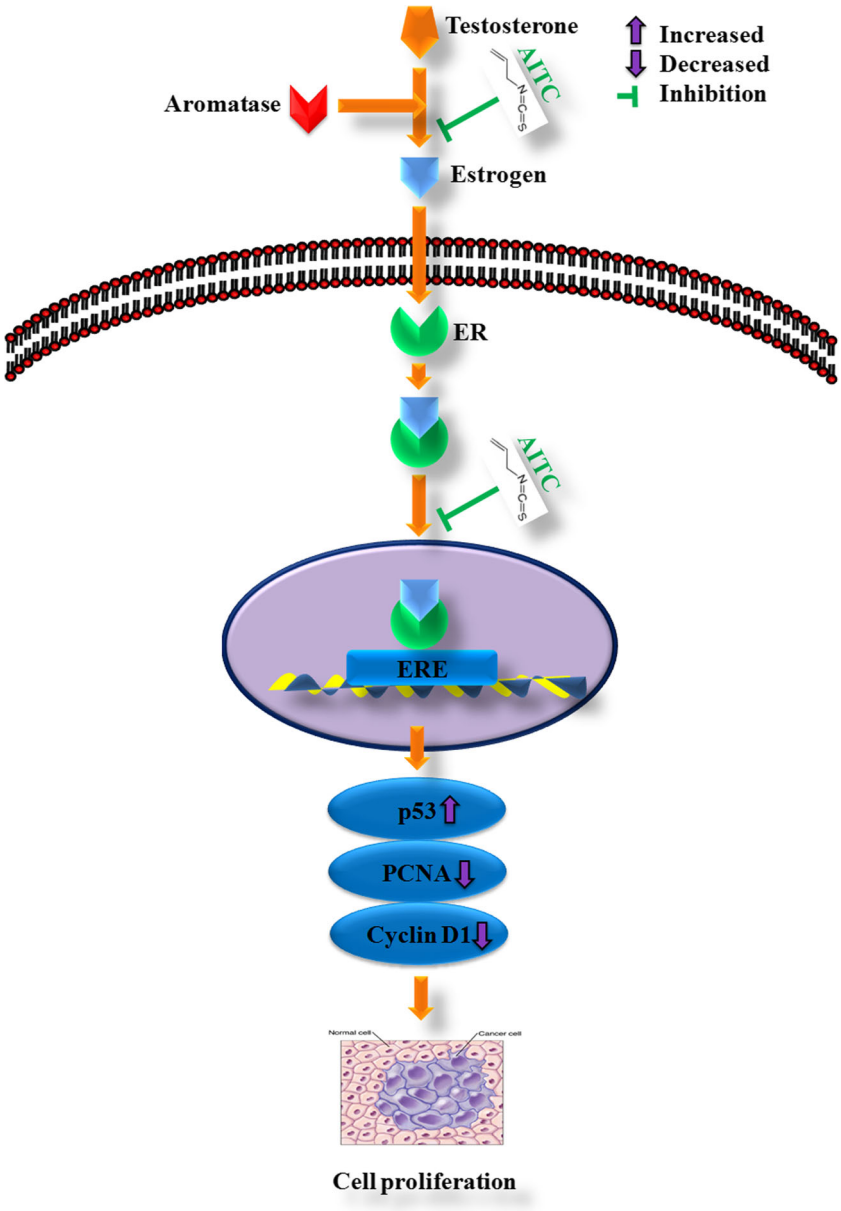

Fig. 9 Schematic representation of the possible mechanism of action of AITC during chemically induced mammary carcinogenesis through anti-estrogenic and anti-cell proliferative potential

PCNA, a 36-kDa nuclear protein essential for replication of DNA via DNA polymerase-δ, which acts as a candidate marker for cell proliferation in breast cancer [27]. In this study, an elevated expression of PCNA in tumors is in proportion with rate of tumor cell proliferation. Similar results were reported in DMBA [36] and MNU [37] induced mammary carcinogenesis in rats. The down regulation of PCNA expression in AITC treated rats suggest its anti-cell proliferative mechanism which has been implicated in the observed chemopreventive efficacy of AITC.

Cyclin D1, a cell cycle-regulatory nuclear protein controls cell proliferation by allowing cell proceed from G1 to S phase [38]. Cyclin D1 up regulated expression in tumor tissue of DMBA [39] and MNU [40] induced mammary tumors are find in line with the results of present study. Oral administration of AITC significantly reduce the expression of cyclin D1 which clearly demonstrates the anti-cell proliferative effect of AITC, which coincide with the study of sulforaphane, a natural analog of AITC block G1 phase of cell cycle associated with a down-regulation of cyclin D1 [41].

Conclusion

The mechanism of DMBA and MNU-induced mammary cancer are different but both produced same kind of estrogen dependent adenocarcinoma. The putative chemopreventive mechanism of AITC is mainly control the regulation of

estrogen synthesis, expression of estrogen receptors and cell proliferative markers as shown in the schematic diagram (Fig. 9). Anti-estrogenic and anti-cell proliferative effect of AITC inhibits neoplastic transformation and progression of mammary tumor. Molecular docking study also strengthens the study.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

References

1. Siegel R, Ma J, Zou Z, Jemal A (2014) Cancer statistics. CA Cancer J Clin 64:9–29
2. Yager JD, Davidson NE (2006) Estrogen carcinogenesis in breast cancer. N Engl J Med 354:270–282
3. Mirzaie S, Chupani L, Asadabadi EB, Shahverdi AR, Jamalan M (2013) Novel inhibitor discovery against aromatase through virtual screening and molecular dynamic simulation: a computational approach in drug design. EXCLI J 12:168–183
4. Mense SM, Remotti F, Bhan A, Singh B, El-Tamer M, Hei TK, Bhat HK (2008) Estrogen-induced breast cancer: alterations in breast morphology and oxidative stress as a function of estrogen exposure. Toxicol Appl Pharmacol 232:78–85
5. Mertani HC, Garcia-Caballero T, Lambert A, Gérard F, Palayer C, Boutin JM, Vonderhaar BK, Waters MJ, Lobie PE, Morel G (1998) Cellular expression of growth hormone and prolactin receptors in human breast disorders. Int J Cancer 79:202–211
6. Storer RD, Kraynak AR, Mckelvey TW, Elia MC, Goodrow TL, Deluca JQ (1997) The mouse lymphoma L5178Y TK+/− cell line is heterozygous for a codon 170 mutation in the p53 tumor suppressor gene. Mutat Res 373:157–165
7. Kasala ER, Bodduluru LN, Madana RM, Athira KV, Gogoi R, Barua CC (2015) Chemopreventive and therapeutic potential of chrysin in cancer: mechanistic perspectives. Toxicol Lett 233: 214–225
8. Caldon CE, Sutherland RL, Musgrove E (2010) Cell cycle proteins in epithelial cell differentiation: implications for breast cancer. Cell Cycle 9:1918–1928
9. Arivazhagan L, Sorimuthu Pillai S (2014) Tangeretin, a citrus pentamethoxyflavone, exerts cytostatic effect via p53/p21 up-regulation and suppresses metastasis in 7,12-dimethylbenz(α)anthracene-induced rat mammary carcinoma. J Nutr Biochem 25:1140–1153
10. Allred CD, Allred KF, Ju YH, Clausen LM, Doerge DR, Schantz SL, Korol DL, Wallig MA, Helferich WG (2004) Dietary genistein results in larger MNU-induced, estrogen-dependent mammary tumors following ovariectomy of Sprague-Dawley rats. Carcinogenesis 25:211–218
11. Kwon KH, Barve A, Yu S, Huang MT, Kong AN (2007) Cancer chemoprevention by phytochemicals: potential molecular targets, biomarkers and animal models. Acta Pharmacol Sin 28:1409–1421
12. Wattenberg LW (1987) Inhibitory effects of benzyl isothiocyanates administered shortly before diethylnitrosamine or benzo[a]pyrene on pulmonary and forestomach neoplasia in A/J mice. Carcinogenesis 8:1971–1973
13. Xiao D, Srivastava SK, Lew KL, Zeng Y, Hershberger P, Johnson CS, Trump DL, Singh SV (2003) Allyl isothiocyanate, a constituent of cruciferous vegetables, inhibits proliferation of human prostate cancer cells by causing G2/M arrest and inducing apoptosis. Carcinogenesis 24:891–897
14. Rajakumar T, Pugalendhi P, Thilagavathi S (2015) Dose response chemopreventive potential of allyl isothiocyanate against 7,12-dimethylbenz(a)anthracene induced mammary carcinogenesis in female Sprague-Dawley rats. Chem Biol Interact 231:35–43
15. Ploton D, Menager M, Jeannesson P, Himber G, Pigeon F, Adnet JJ (1986) Improvement in the staining and in the visualization of the argyrophilic proteins of the nucleolar organizer region at the optical level. Histochem J 18:5–14
16. Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159
17. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with Folin phenol reagent. J Biol Chem 193:265–275
18. Santen RJ, Allred DC, Ardoin SP, Archer DF, Boyd N, Braunstein GD, Burger HG, Colditz GA, Davis SR, Gambacciani M, Gower BA, Henderson VW, Jarjour WN, Karas RH, Kleerekoper M, Lobo RA, Manson JE, Marsden J, Martin KA, Martin L, Pinkerton JV, Rubinow DR, Teede H, Thiboutot DM, Utian WH, Endocrine Society (2010) Postmenopausal hormone therapy: an Endocrine Society scientific statement. J Clin Endocrinol Metab 95:S1–S66
19. el-Aziz MA, Hassan HA, Mohamed MH, Meki AR, Abdel-Ghaffar SK, Hussein MR (2005) The biochemical and morphological alterations following administration of melatonin, retinoic acid and Nigella sativa in mammary carcinoma: an animal model. Int J Exp Pathol 86:383–396
20. Lakshmi A, Subramanian S (2014) Chemotherapeutic effect of tangeretin, a polymethoxylated flavone studied in 7,12-dimethylbenz(a)anthracene induced mammary carcinoma in experimental rats. Biochimie 99:96–109
21. Kang L, Ding L, Wang ZY (2009) Isothiocyanates repress estrogen receptor alpha expression in breast cancer cells. Oncol Rep 21:185–192
22. Powers CN, Setzer WN (2015) A molecular docking study of phytochemical estrogen mimics from dietary herbal supplements. In Silico Pharmacol 3:4
23. Clevenger CV, Furth PA, Hankinson SE, Schuler LA (2003) The role of prolactin in mammary carcinoma. Endocr Rev 24:1–27
24. Cos S, González A, Güezmes A, Mediavilla MD, Martínez-Campa C, Alonso-González C, Sánchez-Barceló EJ (2006) Melatonin inhibits the growth of DMBA-induced mammary tumors by decreasing the local biosynthesis of estrogens through the modulation of aromatase activity. Int J Cancer 118:274–278
25. Brueggemeier RW, Hackett JC, Diaz-Cruz ES (2005) Aromatase inhibitors in the treatment of breast cancer. Endocr Rev 26:331–345
26. Munusami P, Vasavi CS, Divya G (2014) Molecular docking studies on flavonoid compounds: an insight into aromatase inhibitors. Int J Pharm Pharm Sci 6:141–148
27. Al-Dhaheri WS, Hassouna I, Al-Salam S, Karam SM (2008) Characterization of breast cancer progression in the rat. Ann N Y Acad Sci 1138:121–131
28. Derenzini M, Ploton D (1991) Interphase nucleolar organizer regions in cancer cells. Int Rev Exp Pathol 32:149–192
29. Derenzini M, Pession A, Trerè D (1990) Quantity of nucleolar silver-stained proteins is related to proliferating activity in cancer cells. Lab Investig 63:137–140
30. Agarwal ML, Agarwal A, Taylor WR, Stark GR (1995) p53 controls both the G2/M and the G1 cell cycle checkpoints and mediates reversible growth arrest in human fibroblasts. Proc Natl Acad Sci U S A 92:8493–8497

31. Tor YS, Yazan LS, Foo JB, Wibowo A, Ismail N, Cheah YK, Abdullah R, Ismail M, Ismail IS, Yeap SK (2015) Induction of apoptosis in MCF-7 cells via oxidative stress generation, mitochondria-dependent and caspase-independent pathway by ethyl acetate extract of Dillenia suffruticosa and its chemical profile. PLoS One 10:e0127441
32. Chen NG, Chen KT, Lu CC, Lan YH, Lai CH, Chung YT, Yang JS, Lin YC (2010) Allyl isothiocyanate triggers G2/M phase arrest and apoptosis in human brain malignant glioma GBM 8401 cells through a mitochondria-dependent pathway. Oncol Rep 24:449–455
33. Rengarajan T, Nandakumar N, Balasubramanian MP (2013) D-Pinitol prevents rat breast carcinogenesis induced by 7, 12-Dimethylbenz [a] anthracene through inhibition of Bcl-2 and induction of p53, caspase-3 proteins and modulation of hepatic biotransformation enzymes and antioxidants. Biomed Prev Nutr 3:31–41
34. Gowtham Kumar S, Ramakrishnan V, Madhusudhanan N, Thiyagarajan G, Langeswaran VK, Balasubramanian MP (2011) Apoptosis induced in hepatoma cells (hepg2) by allyl isothiocyanate associated with expression of p53 and activation of caspase-3. IJBPR 2:19–26
35. Yang YM, Conaway CC, Chiao JW, Wang CX, Amin S, Whysner J, Dai W, Reinhardt J, Chung FL (2002) Inhibition of benzo(a)pyrene-induced lung tumorigenesis in a/J mice by dietary N-acetylcysteine conjugates of benzyl and phenethyl isothiocyanates during the postinitiation phase is associated with activation of mitogen-activated protein kinases and p53 activity and induction of apoptosis. Cancer Res 62:2–7
36. Bishayee A, Mandal A (2014) Trianthema portulacastrum Linn. Exerts chemoprevention of 7,12-dimethylbenz(a)anthracene-induced mammary tumorigenesis in rats. Mutat Res 768:107–118
37. Rajmani RS, Doley J, Singh PK, Kumar R, Barathidasan R, Kumar P, Verma PC, Tiwari AK (2011) Induction of mammary gland tumour in rats using N-methyl-N-nitroso urea and their histopathology. Indian J Vet Pathol 35:142–146
38. Yu Q, Geng Y, Sicinski P (2001) Specific protection against breast cancers by cyclin D1 ablation. Nature 411:1017–1021
39. Jang TJ, Kang MS, Kim H, Kim DH, Lee JI, Kim JR (2000) Increased expression of cyclin D1, cyclin E and p21(Cip1) associated with decreased expression of p27(Kip1) in chemically induced rat mammary carcinogenesis. Jpn J Cancer Res 91:1222–1232
40. Ashrafi M, Bathaie SZ, Abroun S (2012) High expression of cyclin D1 and p21 in N-Nitroso-N-Methylurea-induced breast Cancer in Wistar albino female rats. Cell J Fall 14:193–202
41. Chiao JW, Chung FL, Kancherla R, Ahmed T, Mittelman A, Conaway CC (2002) Sulforaphane and its metabolite mediate growth arrest and apoptosis in human prostate cancer cells. Int J Oncol 20:631–636