Allyl isothiocyanate inhibits invasion and angiogenesis in breast cancer via EGFR-mediated JAK-1/STAT-3 signaling pathway

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Received: 23 March 2023 / Accepted: 22 May 2023 / Published online: 13 June 2023
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Abstract
Angiogenesis, invasion, and metastasis are the main events of cancer cells. JAK-1/STAT-3 is a key intracellular signaling transduction pathway, which controls the growth, differentiation, apoptosis, invasion, and angiogenesis of various cancer cells. The present study explored the impact of allyl isothiocyanate (AITC) on the JAK-1/STAT-3 pathway in DMBA-induced rat mammary tumorigenesis. The mammary tumor was initiated through a single dose of 25 mg DMBA/rat by a subcutaneous injection administered near the mammary gland. We observed decreased body weight and increased the total number of tumors, tumor incidence, tumor volume, well-developed tumor, and histopathological abnormalities in DMBA-induced rats that were modulated after being treated with AITC. Staining of mammary tissues showed a high accumulation of collagen in DMBA-induced rats and it was normalized by the AITC treatment. Moreover, DMBA-induced mammary tissues showed up-regulated expressions of EGFR, pJAK-1, pSTAT-3, nuclear fraction of STAT-3, VEGF, VEGFR2, HIF-1α, MMP-2, and MMP-9 and the down-regulated expressions of cytosolic fraction of STAT-3 and TIMP-2. Oral administration of AITC on DMBA-induced rats inhibits angiogenesis and invasion by modifying these angiogenic and invasive markers. The finding of the present study was further confirmed by molecular docking analysis that shows a strong binding interaction between AITC with STAT-3 and cocrystal structure of STAT-3 glide energy of −18.123 and −72.246 (kcal/mole), respectively. Overall, the results suggested that AITC inhibits activation of the JAK-1/STAT-3 pathway, which subsequently prevents angiogenesis and invasion. It was recommended that AITC might develop a beneficial effect against breast cancer.

Keywords 7,12-Dimethylbenz(a)anthracene · JAK-1 · STAT-3 · Angiogenesis · Invasion · Allyl isothiocyanate

Abbreviations
JAK Janus kinase
STAT Signal transducer and activator of transcription
EGF Epidermal growth factor
EGFR Epidermal growth factor receptor
AITC Allyl isothiocyanate
H&E Hematoxylin and eosin
MT Masson’s tri-chrome
PR Picrosirius red
RT Reverse transcription
PCR Polymerase chain reaction
EtBr Ethidium bromide
ECM Extracellular matrix
BM Basement membrane
ROS Reactive oxygen species

Introduction
Epidermal growth factor (EGF) is a polypeptide that has mitogenic properties. EGF performs via epidermal growth factor receptor (EGFR) and affects cell proliferation-inducing by mammary cells. EGF is the main target for developing anti-tumor agents, which catalyze the transfer of phosphate molecules from ATP to an active form of tyrosine kinase to trigger a cascade of molecular mechanisms that inhibit cell apoptosis, enhance invasion, and promote angiogenesis responses (Yarden and Sliwkowski 2001). Activation of EGFR promotes the downstream signal transduction pathway, including the JAK/STAT (Krasinskas 2011; Ciardiello and Tortora 2001). JAK-1/STAT-3 is an important intracellular signal transduction pathway in the development of various cancers.
pathway in mammary cancer that controls cell growth, invasion, differentiation, apoptosis, and angiogenesis. In response to cytokines (IL-5 and IL-6) and growth factors (interferons and EGF), STAT-3 is phosphorylated by receptors of JAK to form a dimer and translocated into the cell nucleus and it stimulates the expression of genes responsible for cell proliferation, angiogenesis, and invasion (Banerjee and Resat 2016; Zhang et al. 2003).

Tumor angiogenesis is the formation of new blood vessels that are essential for tumor development and progression (Darakhshan et al. 2013). In the progression stage, malignant cells in primary tumors acquire the ability to penetrate neighboring tissues (invasion) and further enter into lymphatic and blood circulation (metastasis) anchored in different sites to form secondary tumors. Hence, cancer cells escape from the normal molecular restrictions that link nearby cells and remodel their cell–matrix and cell–cell adhesion molecule to gain invasive and metastatic capabilities (Pecorino 2012). HIF-1α is considered an important factor that is activated in hypoxic conditions and up-regulates various angiogenesis-related genes, such as VEGF, and its receptors like VEGFR2 (Pugh and Ratcliffe 2003). MMP-2 enzyme plays a vital function in the invasion of tissue basement membranes (Jeziorska and Motyl 2009). MMP-9 is another zinc-dependent peptidase that is activated upon cleavage by various kinds of extracellular proteases (Klein and Bischoff 2011). TIMP-2 inhibits angiogenesis, endothelial cell proliferation, and migration via MMP-dependent and independent-mediated endothelial cells (Bourboulia et al. 2011). The TIMP-2 expression is related to low cancer progression/recurrence or with a poor diagnosis confirming the triggering or the suppressive role of TIMP-2 (Têtu et al. 2006). Therefore, the functions of JAK-1/STAT-3 are essential targets for treatment approaches in cancer progression.

Natural dietary ingredients and phytochemicals are potential sources of JAK-1/STAT-3 inhibitors. Many studies have concluded that the intake of fruits and vegetables can reduce cancer incidence (Wang et al. 2016; Shuai and Liu 2003; Kisseleva et al. 2002). Allyl isothiocyanate (AITC) is a phytochemical present in several dietary sources having various pharmacological properties (Zhang 2010). Previously, we have reported the anti-oxidative, anti-inflammatory, and anti-cell proliferative properties of AITC in DMBA-induced rat mammary carcinoma through chemopreventive effects (Rajakumar et al. 2015, 2018a, 2018b, 2018c; Thangarasu et al. 2020). However, the action of AITC on the JAK/STAT signaling pathway of mammary cancer is still unclear. Hence, the present work explores the impact of AITC on the JAK-1/STAT-3 pathway for the inhibition of angiogenesis and invasion in DMBA-induced mammary tumorigenesis in rats.

**Materials and methods**

**Chemicals**

AITC and DMBA were purchased from Sigma–Aldrich Chemicals Pvt. Ltd., Bangalore, India. Primary antibodies for EGFR, JAK-1, pJAK-1, STAT-3, pSTAT-3, HIF-1α, VEGF, VEGFR2, MMP-2, MMP-9, TIMP-2, and β-actin were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. All additional chemicals were used of analytical grade.

**Animals**

Female SD rats (6 to 7 weeks old) were purchased from the NIN, Hyderabad, India. Ethical clearance was approved by the IAEC for the CPCSEA (approval no: 983) guidelines. The rats were kept in the Central Animal House, RMMCH, Annamalai University, Chidambaram, Tamilnadu, India. The rats were acclimatized in standard environments of humidity (50 ± 10%), 12 h light/dark cycle, and temperature (24 ± 2 °C). Feed and water were provided ad libitum.

**Experimental plan**

Totally 40 rats were divided into four groups of 10 rats in each group. The first group of rats were served as untreated control. Group II and Group III rats have injected with a

| S. No | Gene name | Forward primer | Reverse primer |
|-------|-----------|----------------|---------------|
| 1     | HIF-1α    | 5′-TGC TAATGCCACCACCTACC-3′ | 5′-TGACTCCTTTTCTGCTCTG-3′ |
| 2     | VEGF      | 5′-CTTGGTCTGCTGTTCGCT-3′ | 5′-ACTTGGTAGGATTCTGCG-3′ |
| 3     | VEGFR2    | 5′-ACCAGGAGAGGAGGCTACT-3′ | 5′-GACACACTCCTGCTGCT-3′ |
| 4     | MMP-2     | 5′-GCTCGTCATCTCGTGAAT-3′ | 5′-GACATGCCGTTATCGGGA-3′ |
| 5     | MMP-9     | 5′-GGTGGTGGCGGTGCCGAC-3′ | 5′-ACATGAGCCGTTCCGCA-3′ |
| 6     | TIMP-2    | 5′-GTGTGCAATGCGACACGTA-3′ | 5′-ATGTTCAAGAAACTCCTGTT-3′ |
| 7     | GAPDH     | 5′-TTCTGTGCACTGCCAGCCTC-3′ | 5′-TAGGAACAGGAAGGCAATGCGA-3′ |
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single subcutaneous dose of DMBA (25 mg/rat) near the mammary gland after the completion of the first week. Group II rats received no additional treatment. Group III rats received oral administration of AITC (20 mg/kg body weight (bwt)) through intubation once a day. It began one week before carcinogen exposure and it continued until the completion of the study (16 weeks). Group IV rats were administered AITC (20 mg/kg bwt) orally for 16 weeks. The initial and final bwt of control and experimental rats were measured during the investigational period. At the end of the 16th week, rats were photographed and sacrificed by cervical decapitation. The mammary tumor was removed and tumor volume was measured using the formula \[ V = \frac{4}{3} \pi \left( \frac{D_1}{2} \right) \left( \frac{D_2}{2} \right) \left( \frac{D_3}{2} \right) \], where D1, D2, and D3 are the three diameters (in mm) of the tumor. Subsequently, mammary tissue was preserved in 10% formalin and stored at -80°C for histopathological analysis.

**Histopathological analysis**

Mammary tissues were removed and immersed in 10% formalin, dehydrated with 50%–100% ethanol solutions, and embedded in paraffin. Then, paraffin-fixed mammary tissue sects. (3–5 µm) were cut using a microtome, rehydrated using xylene and graded series of ethanol, and stained with hematoxylin and eosin (H&E). Deposition of collagen was detected by Masson’s tri-chrome (MT) and picrosirius red (PR) staining methods. Images were taken by Nikon Coolpix 4500 camera, fitted with a microscope of 40× magnification. The deposition of mammary tissue collagen was quantified by the standard quantification software ImageJ.

**Reverse transcription-polymerase chain reaction analysis**

The whole RNA was isolated from the mammary tissues with Trizol reagent by the Chomczynski and Sacchi (1987) method. The whole RNA was prepared without protein and DNA contamination and quantified spectrophotometrically by determining the absorbance at 260 nm. The reverse transcription (RT) and polymerase chain reaction (PCR) kits were obtained from Invitrogen. The purified RNA was reverse transcribed into a single-strand cDNA using the reverse transcriptase enzyme. The cDNA was amplified with relevant primers of HIF-1α, VEGF, VEGFR2, MMP-2, MMP-9, and TIMP-2, as mentioned in Table 1. RT-PCR was conducted under the following conditions: initial denaturation at 95 °C for 2 min, followed by 40 cycles of cyclic denaturation at 94 °C for 15 s, annealing at 59°C for 1 min, and extension at 72 °C for 15 s. GAPDH was used as an internal standard. The amplified PCR products were analyzed by electrophoresis in 2% agarose gels and visualized under UV illumination using ethidium bromide (EtBr).

**Western blotting analysis**

Mammary tissue was homogenized with chilled RIPA buffer and centrifuged (12,000 rpm) at 4 °C for 15 min. The supernatant was removed from a tissue homogenate and the concentration of protein was quantified using the method of Lowry et al. (1951). 50 µg of the total proteins were separated through 10% SDS-PAGE and transferred onto a PVDF membrane. The membrane was then kept with blocking buffer (5% BSA) for 2 h and incubated overnight with specific primary antibodies for EGFR, JAK-1, pJAK-1, STAT-3, pSTAT-3, HIF-1α, VEGF, VEGFR2, MMP-2, MMP-9, and TIMP-2.

| Groups | Total number of tumors (n) | Tumor incidence (%) | Tumor volume (mm³)/rat |
|--------|---------------------------|---------------------|-----------------------|
| Control | (0)/10                    | 0                   | 0                     |
| DMBA   | (10)/10                   | 100                 | 21.86 ± 1.57a         |
| DMBA + AITC (20 mg/kg bwt) | (2)/10                | 20                  | 4.43 ± 0.33b          |
| AITC (20 mg/kg bwt) | (0)/10                   | 0                   | 0                     |

Tumor volume was measured using the formula \[ V = \frac{4}{3} \pi \left( \frac{D_1}{2} \right) \left( \frac{D_2}{2} \right) \left( \frac{D_3}{2} \right) \], where D1, D2 and D3 are the three diameters (in mm) of the tumor; (i) indicates total number of rats bearing tumors

Values are set as mean ± SD for 10 rats in each group

Values not sharing a common superscript differ substantially at \( p < 0.05 \)
MMP-9, TIMP-2, and β-actin at 4 °C. The membrane was three times washed with TBST and kept with its relevant secondary antibodies for 2 h at RT. Protein bands were three times washed with TBST and were observed using the ECL kit method. Bands were scanned via a scanner and quantities were determined by the ImageJ software.

Molecular docking analysis

AITC structure was retrieved from PubChem (www.ncbi.nlm.nih.gov/pccompound). The structure of target protein STAT-3 (PDB ID: 1BG1) was retrieved from the protein data bank (www.rcsb.org/pdb). The receptor grid generation and ligand docking were done using the Glide Xp docking algorithm.

Statistical analysis

The statistical analysis was performed using SPSS version 16. The values were represented as mean ± SD. One-way ANOVA, followed by the DMRT comparison system was used to compare differences among the variables. Data were considered statistically significant if p-values were < 0.05.

Results

Effect of AITC on body weight changes in control and experimental rats

Table 2 demonstrates the bwts of control and experimental rats. Initially, there were no significant (p < 0.05) bwts changes observed in control and experimental rats. Finally, we identified, significant (p < 0.05) reduction in the bwts of DMBA-induced cancer-bearing rats when compared with control rats. In contrast, treatment with AITC prevented the significant (p < 0.05) bwts reduction in DMBA-induced cancer-bearing rats. AITC alone group rats exposed growth in their bwts, but no significant (p < 0.05) variations when compared with control rats.

Effect of AITC on the total number of tumors, tumor incidence, and tumor volume in control and experimental rats

Table 3 shows the total number of tumors, tumor incidence, and tumor volume in control and experimental rats. This study identified increased tumor numbers (10) and 100% tumor incidence in DMBA-induced rats. AITC administration in DMBA-induced rats significantly (p < 0.05) reduced tumor numbers (2) and tumor incidence by 20%. Moreover, this study was identified that 21.86 mm³ of tumor volume in DMBA-induced rats. Oral administration of AITC in DMBA-induced rats significantly (p < 0.05) reduced the tumor volume to 4.43 mm³ (Table 3 and Fig. 1B).

Morphological appearance and histopathological changes of experimental rats

Figure 1 (A and C) shows the morphological appearance and histopathological structures of DMBA and DMBA + AITC treated rats. This study observed a well-developed tumor in DMBA-induced rats, which was reduced with AITC treatment. Additionally, DMBA-induced tumor-bearing rats showed loss of architecture with infiltrating malignant tumors. DMBA + AITC-treated rats indicated near-normal
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architecture of mammary tissues with an increased area of fibrosis.

**Effect of AITC on MT and PR staining in mammary tissue**

Figure 2a–d shows the pathological investigation of MT and PR staining of mammary tissue for collagen deposition. Collagen was increased significantly \((p < 0.05)\) in DMBA-induced rat mammary tissue and it decreased in DMBA + AITC-treated rats. Collagen deposition was normal among AITC-only administered and normal control rats.

**AITC inhibits JAK/STAT signaling by preventing STAT-3 phosphorylation and nuclear translocation**

To investigate whether AITC affects the activation of JAK/STAT signaling in DMBA-induced mammary tumors, we analyzed protein expressions of EGFR, pJAK-1, tJAK-1, pSTAT-3, cytosolic and nuclear fraction of STAT-3 and tSTAT-3 in AITC treated cancer-bearing rats. Our results have shown that DMBA-induced rats significantly \((p < 0.05)\) increased the protein expression of EGFR, pJAK-1, pSTAT-3, and nuclear STAT-3 and decreased the protein expression of cytosolic STAT-3 compared to control rats. Oral administration of AITC inhibited JAK/STAT signaling by decreasing the expression of EGFR, and pJAK-1 and blocking STAT-3 phosphorylation and nuclear translocation compared with DMBA-induced cancer-bearing rats. Moreover, oral administration of AITC had no significant \((p < 0.05)\)
effect of tJAK-1 and tSTAT-3 compared to other groups (Fig. 3). This result suggested that AITC mediated effect on angiogenesis and invasion in mammary cancer involves the inhibition of STAT-3 activation, indicating that AITC might be a better compound in suppressing mammary tumor growth.

**AITC blocks angiogenesis formation by inhibiting JAK/STAT pathway**

Neovascularization plays a major role in tumor growth and is one of the most important downstream events triggered by the JAK/STAT pathway. To know whether AITC blocks angiogenesis formation via inhibition of the JAK/STAT pathway, we investigated the effect of AITC on angiogenic markers such as HIF-1α, VEGF, and VEGFR2 in DMBA-induced rats. Figure 4 shows the mRNA and protein expressions wherein HIF-1α, VEGF, and VEGFR2 were significantly ($p < 0.05$) increased in DMBA-induced tumor-bearing rats when compared with control rats. Oral administration of AITC significantly ($p < 0.05$) reduced the expression of HIF-1α, VEGF, and VEGFR2 compared to DMBA-induced rats. There were no significant ($p < 0.05$) modifications observed in the expression of angiogenic markers in AITC-only treated and normal control rats. These data suggested that AITC blocked angiogenesis formation in DMBA-induced tumor-bearing rats via prevention of the JAK/STAT signaling pathway.

**AITC impedes invasion by inhibiting JAK/STAT pathway**

Invasion is a crucial step in the development of tumor metastasis, which includes the secretion of substances that degrade the extracellular matrix (ECM), basement membrane (BM), and up-regulation of protein controlling cell motility and migration. To determine whether inhibition of the JAK/STAT pathway by AITC impedes invasion, we analyzed the expression of invasive markers like MMP-2 and
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MMP-9 and their inhibitor TIMP-2 in DMBA-induced rats. Figure 5 shows the mRNA and protein expressions wherein MMP-2 and MMP-9 were significantly (p < 0.05) increased and TIMP-2 was significantly (p < 0.05) reduced in DMBA-induced cancer-bearing rats when compared with control rats. Oral administration of AITC significantly (p < 0.05) modulated the expression of MMP-2, MMP-9, and TIMP-2 compared to DMBA-induced rats. There were no significant (p < 0.05) variations identified in the expression of invasive markers in AITC-only treated and normal control rats. These findings clearly showed that AITC impeded the invasion via inhibition of the JAK/STAT pathway.

AITC binds to STAT-3

To further confirm the inhibition of JAK/STAT signaling by AITC, we next investigated molecular docking studies of the binding interaction of AITC with STAT-3 and the cocrystal structure of STAT-3. Figure 6 (A1 and A2) indicates the molecular docking findings and the data confirmed that AITC reveals a strong binding interaction with STAT-3 and cocrystal structure of STAT-3 with glide energy of -18.123 and -72.246 (kcal/mole), respectively. A1 denotes that AITC forms a hydrogen bond with Gln 326 and hydrophobic interactions with Gln 247, Pro 336, Cys 251, and Ile 258 of STAT-3. A2 denotes that AITC forms a hydrogen bond with Asp 261, Cys 259, Asn 257, Ala 250, and Pro 333, and hydrophobic interactions with Ile 258, Glu 324, Leu 260, Gln 247, Cys 251, Pro 336, Gly 253, Pro 256, and Ser 514 of the cocrystal structures of STAT-3. Based on these glide energy, hydrogen bonds, and hydrophobic interactions, we detected that the AITC might have a better binding affinity with STAT-3 and the cocrystal structure of STAT-3 and ultimately inhibited JAK/STAT signaling pathway.

Discussion

Reactive oxygen species (ROS) generation and peroxidation of membrane lipids are related to the initiation and progression of cancer that affects the normal biochemical mechanism which further leads to the bwt reduction (Davis and Kuttan 2001). In the present study, the total bwt of DMBA-induced rats was reduced due to the changes in the
energy metabolism during tumor formation and development (Moselhy and Al mslmani, 2008). AITC-treated rats gradually improved bwt, which specified that the low level of ROS formation and lipid peroxidation could be attributed to the free radical scavenging activity of AITC (Rajakumar et al. 2015). AITC efficiently reduced the total number of tumors, tumor incidence, and tumor volume in cancer-bearing rats might be due to inhibitory action or anti-tumor activity. The anti-tumor activity of AITC has been reported against various cancer cell lines (Srivastava et al. 2003; Bhattacharya et al. 2012). Kumar et al. (2011) reported that AITC might disrupt the energy requirement of tumor tissue and lead to the suppression of tumor growth (Kumar et al. 2011).

Histopathological investigation of mammary tissues of cancer-bearing rats indicated carcinomas exhibited infiltrating malignant tumors. On the contrary, AITC-treated rats showed no sign of cellular proliferation and necrosis which was proved by the normal architecture of mammary tissue with an increased area of fibrosis. Hence, it proposes that the AITC has the potential to be safe and effective in cancer management and also evidences the anti-neoeplastic activity (Rajakumar et al. 2015). Collagen in tissues is a physical barrier against tumor invasion and metastasis (Fang et al. 2014). Collagen around normal epithelial cells in breast tissue is normally curly and smooth. In tumors, collagen gradually thickens, stiffens, and linearizes and it activates metastasis by fostering cell migration into the ECM (Wyckoff et al. 2007). In the present study, over-accumulation of collagen was detected in cancer-bearing rats, which is in agreement with earlier reports of pathological parameters in mammary tissues (Arivazhagan and Sorimuthu Pillai 2014). On the other hand, oral administration of AITC to DMBA-induced rats reduced the over-accumulation of collagen in mammary tissues.

EGFR plays a vital function in the control of cell growth, differentiation, proliferation, survival, and apoptosis during development and homeostasis (Normanno et al. 2006; Jorissen et al. 2003). EGFR homodimers are activated after binding with their ligand i.e. EGF, followed by transphosphorylation of several tyrosine kinase domains, and subsequent activation of STAT proteins (Spano et al. 2005). After activation, STAT-3 undergoes tyrosine phosphorylation, dimerization, DNA binding, and transcriptional activation of genes associated with cell division and survival. The interaction between JAK and STAT-3 mediates the transient phosphorylation of STAT-3.
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in normal cells, but in most cancer cells, which are constitutively phosphorylated (Leeman-Neill et al. 2009). The cascade effect of STAT-3 includes several molecules, such as HIF-1α and VEGF for angiogenesis and MMP-2 and MMP-9 for invasion (Jarnicki et al. 2010; Aggarwal et al. 2006). Phytochemicals that suppress the activation of STAT-3 have been considered potential agents for the inhibition and therapy of cancer (Wang et al. 2016). Lai et al. (2014) reported that AITC suppresses the invasion, migration, and metastasis of EGF-stimulated MAPK signaling pathways in human colon HT29 cells (Lai et al. 2014). Supportive with previous report, the present study revealed that AITC treatment abrogates the stimulation of STAT-3 phosphorylation, followed by nuclear translocation. Moreover, interactions of AITC with the STAT-3 dimerization site are validated by molecular docking analysis, which strengthens the result. It was found that AITC acts as a ligand for EGFR and it, therefore, inhibits STAT-3 phosphorylation, nuclear translocation, and its cascade activation.

Angiogenesis is a key process for tumor development, invasion, and metastasis, and it is a possible target for cancer treatment (Raica et al. 2009). VEGF is an important survival mediator of vascular endothelial cells, which activate tyrosine kinase that subsequently binds to VEGFR. VEGFR2 is the main factor of VEGF-mediated angiogenesis (Korpanty et al. 2011). Previous study reported that decreased production of VEGF and VEGFR2 in AITC-treated mice, which indicates the anti-angiogenic effect of AITC in ascites tumor growth (Kumar et al. 2009). In this study, AITC treatment down-regulated VEGF and VEGFR2 expression in cancer-bearing rats, it may be due to its anti-angiogenic potential.
HIF-1 is the heterodimeric transcriptional mediator containing α and β sub-units (Yang et al. 2004). HIF-1α overexpression is related to tumor growth, mortality, treatment failure, and vascularity (Kubo et al. 2016). Previous study reported that sulforaphane, a natural analog of AITC, induced down-regulation of HIF-1α expression in human endothelial HMEC-1 cells (Bertl et al. 2006). Consistent with earlier report, our results suggested that AITC suppresses the HIF-1α overexpression in cancer-bearing rats.

In tumor invasion, tumor cells residing within a primary tumor invade the nearby stroma and adjacent normal tissue (Quail and Joyce 2013). MMP-2, an enzyme that damages ECM components, plays a crucial function in cell migration during pathological and physiological mechanisms (Jezierska and Motyl 2009). MMP-9 is another enzyme that performs a key function in cancer development, progression, invasion, and metastasis (Duffy et al. 2000). Inhibition of MMP expression or reduced enzyme quantity can be an important process that could serve as an early target for blocking cancer metastasis (Guruvayoorappan and Kuttan 2008). In the present study, we observed that AITC reduced MMP-2 and MMP-9 in DMBA-induced mammary cancer, which was coinciding with the previous report in human hepatoma SK-Hep1 cells (Hwang and Lee 2006). TIMP-2 plays a significant role in tumorigenesis and invasion. An elevated TIMP-2 level prevents pro-MMP-2 activation which reduces tumor angiogenesis and invasion (Munshi et al. 2004). Previous study reported that AITC treatment significantly improved the formation of TIMP and it is a suppressor of the MMP function in human endothelial HUVECs cells (Thejass and Kuttan 2007). In the present study, DMBA-induced rats showed decreased expression of TIMP-2. It significantly increased upon AITC treatment, which may be due to its anti-invasive potential.

**Conclusion**

EGFR and its downstream JAK/STAT pathway are possibly key processes in the pathogenesis of breast cancer. These processes have emerged as an effective target for both inhibition and therapeutics of breast cancer. Our work exhibited that AITC inhibits the EGFR-mediated JAK-1/STAT-3 signaling and downstream molecules associated with angiogenesis and invasion via suppression of STAT-3 phosphorylation and nuclear translocation (Fig. 7). This is confirmed through molecular docking analysis of the interaction of AITC with STAT-3 molecule. Thus, AITC might improve the positive effect against breast cancer.

**Acknowledgements** We gratefully acknowledged the financial assistance from the Indian Council of Medical Research (ICMR, Grant number-3/2/2/189/2013/NCD-III (OPA-27129)), in the form of a Senior Research Fellowship (SRF), New Delhi, India to the first author, Mr. T. Rajakumar.

**Author contributions** The authors declare that all data were generated in-house and that no paper mill was used. All authors contributed to the study’s conception and design. Material preparation, data collection, and analysis were performed by [Thangarasu Rajakumar] and [Pachaiappan Pugalendhi]. The first draft of the manuscript was written by [Thangarasu Rajakumar] and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

**Data availability** The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

**Declarations**

**Conflict of interest** The author declares that there are no conflicts of interest.

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