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Suppression of inflammatory cascade is implicated in methyl amooranin-mediated inhibition of experimental mammary carcinogenesis

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Abstract

Breast cancer represents the second leading cause of cancer-related deaths among women worldwide and preventive therapy could reverse or delay the devastating impact of this disease. Methyl-amooranin (methyl-25-hydroxy-3-oxoolean-12-en-28-oate, AMR-Me), a novel synthetic oleanane triterpenoid, reduced the incidence and burden of 7,12-dimethylbenz(*a*)anthracene (DMBA)-induced mammary tumors in rats through antiproliferative and proapoptotic effects. Since chronic inflammation plays an important role in the pathogenesis of breast cancer and several synthetic oleanane compounds are known potent anti-inflammatory agents, we aim to investigate anti-inflammatory mechanisms of AMR-Me by monitoring various proinflammatory and stress markers, such as cyclooxygenase-2 (COX-2) and heat shock protein 90 (HSP90), and nuclear factor- κ B (NF- κ B) signaling during DMBA mammary tumorigenesis in rats. Mammary tumors were harvested from a chemopreventive study in which AMR-Me (0.8 –1.6 mg/kg) was found to inhibit mammary carcinogenesis in a dose-response manner. The expressions of COX-2, HSP90, NF- κ B and inhibitory κ B- α (I κ B- α) were determined by immunohistochemistry and reverse transcription-polymerase chain reaction. AMR-Me downregulated the expression of intratumor COX-2 and HSP90, suppressed the degradation of I κ B- α , and reduced the translocation of NF- κ B from cytosol to nucleus. Our present study provides the first *in vivo* evidence that NF- κ B-evoked inflammatory cascade is a major target of AMR-Me in breast cancer. Our current results together with our previous findings suggest that disruption of NF- κ B signaling contributes to anti-inflammatory, antiproliferative and apoptosis-inducing mechanisms involved in AMR-Me-mediated chemoprevention of rat mammary carcinogenesis. These encouraging mechanistic results coupled with a safety profile should facilitate the clinical development of AMR-Me as breast cancer chemopreventive drug.

Keywords

DMBA; breast tumor; inflammation; oleanane triterpenoid; COX-2; HSP90; NF- κ B

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INTRODUCTION

Breast cancer represents one of the leading causes of death in women worldwide [1]. Approximately 233,000 new breast cancer cases and nearly 40,000 deaths are estimated to occur in women in the United States in 2013 alone [2]. Genetic risk factors, including germ line mutation of breast cancer predisposition genes *BRCA-1* and *BRCA-2*, account for approximately 5–10% of breast cancer cases. The majority of breast cancers (approximately 90%) originate from somatic genetic abnormalities, such as mutations in oncogenes and loss of tumor suppressor genes. Based on available evidence, exposure to environmental pollutants (e.g., polycyclic aromatic hydrocarbons), obesity, diabetes, and other life-style factors play an important role in the increased prevalence of breast cancer [3–6]. Although current treatment modalities, including surgery, radiotherapy, chemotherapy, hormone therapy, and monoclonal antibody immunotherapy, are effective for early-stage local and regional disease, these approaches fail to eradicate advanced-stage metastatic breast cancer. In view of these limitations, preventive control approaches using pharmacologic and natural agents with acceptable efficacy and toxicity could be considered viable strategy in reducing the morbidity and mortality of this devastating disease [7–10]. According to a recent consensus, preventive therapy needs to be integrated into wider strategies of breast cancer risk reduction, including avoidance of obesity and increase of physical activity [11].

Emerging studies show that natural products, including plant-derived constituents, serve as possible starting material for designing novel cancer preventive and anticancer drugs [12,13]. A growing number of natural products, isolated phytochemicals, and related compounds have been shown to inhibit the process of breast tumorigenesis *in vitro* and *in vivo* through suppression of proliferation, cell cycle progression, differentiation and angiogenesis, induction of cell death (apoptosis and autophagy), diminution of oxidative stress and inflammation, and modulation of several cell signaling pathways [14–20]. The potential utility of several dietary supplements, herbal extracts and pure phytochemicals in breast cancer prevention and treatment are being tested in clinical intervention trials [21]. Terpenoids (also known as terpenes or isoprenoids), the largest group of phytochemicals present in various vegetables, fruits, and medicinal plants, are classified according to the number of isoprene units [22]. More than 20,000 triterpenoids are known to exist in nature and some of them exhibit anti-inflammatory and antitumorigenic properties [23–26]. Oleanolic acid, an oleanane-type pentacyclic triterpenoid, exists in dietary, medicinal, and other plants in nature in the form of either free acid or saponin glycosides and possesses variety of biological activities [27,28]. Oleanolic acid has been found to influence multiple intracellular signaling pathways and exert antitumor and chemopreventive activities in various cancers, including breast cancer [29–31]. To improve pharmacological potency, a series of synthetic oleanane triterpenoids have been synthesized using oleanolic acid as a starting compound [32]. Several synthetic oleanane triterpenoids represent the most potent anti-inflammatory and anticarcinogenic triterpenoids known to mankind [23]. Accumulating studies show that oleanolic acid and its synthetic derivatives inhibit proliferation and induce apoptosis of various cancer cells *in vitro* and display cancer preventive or therapeutic effects in animal models of breast, colon, liver, lung, pancreas, prostate, and skin cancer [23,33]. At least two recently completed Phase I clinical trials (NCT00322140 and NCT00508807)

evaluated the antitumor activities of several synthetic analogs of oleanolic acid (<http://www.clinicaltrials.gov/>).

Amooranin (25-hydroxy-3-oxoolean-12-en-28-oic acid, AMR) is an oleanane-type triterpenoid isolated from the stem bark of the Indian medicinal plant *Amoora rohituka* [34]. It exhibited cytotoxic and apoptosis-inducing effects in several human breast carcinoma, colon carcinoma and leukemia cells [35–39] and inhibited the growth of *N*-methyl-*N*-nitrosourea-induced mammary tumors in rats [34] and xenografted human colon carcinoma in mice [39]. Since the antineoplastic activity of plant-derived AMR was weak, new derivatives have been developed by structural modification of this compound in an attempt to obtain more potent candidates. A methyl derivative of AMR (methyl-25-hydroxy-3-oxoolean-12-en-28-oate, methyl amooranin or AMR-Me) has been found to exhibit significant cytotoxic activity against human breast cancer cells with superior potency to the parent compound AMR [35]. Subsequent investigations revealed that AMR-Me has growth-inhibitory and apoptosis-inducing effects against various human breast carcinoma and acute lymphoblastic leukemia cells [40–42] and antitumor efficacy in mice inoculated with Dalton's ascites lymphoma cells [41].

Recently, we have developed a novel method for synthesis of AMR-Me [43]. Our custom synthetic method eliminates the need for using plant material and hence the dependency to Mother Nature to obtain sufficient amount of AMR-Me to advance its development as anti-breast cancer drug. Using dimethylbenz(*a*)anthracene (DMBA)-induced rat mammary tumorigenesis, a classical animal model that mimics human breast cancer [44, 45], we have recently demonstrated that AMR-Me exhibits a striking inhibition of DMBA-induced mammary tumor incidence, total tumor burden, and average tumor weight through suppression of abnormal cell proliferation and induction of apoptosis mediated through modulation of proapoptotic protein Bax and antiapoptotic protein Bcl-2 [43]. We have further noticed that AMR-Me downregulates the expression of estrogen receptor (ER)- α , ER- β , and cyclin D1 and interferes with Wnt/ β -catenin signaling during mammary tumorigenesis in rats without any hepatotoxicity and renotoxicity [unpublished observation]. Nevertheless, additional mechanisms may be involved in AMR-Me chemoprevention of experimental mammary carcinogenesis. Since chronic inflammatory cascade is implicated in the development and progression of breast cancer [46–48] and synthetic oleanane triterpenoids are potent anti-inflammatory agents [23,33], we have postulated that AMR-Me exerts inhibition of DMBA-initiated mammary tumorigenesis at least, in part, by suppression of inflammatory stress response. Accordingly, our current study aims to investigate chemopreventive anti-inflammatory mechanisms of AMR-Me by monitoring various proinflammatory and stress markers, namely cyclooxygenase-2 (COX-2) and heat shock protein 90 (HSP90), and nuclear factor- κ B (NF- κ B), a cardinal regulator of inflammatory insult, during DMBA-evoked mammary gland carcinogenesis in rats.

MATERIALS AND METHODS

Materials

AMR-Me was synthesized as per our previous communication [43]. DMBA and paraformaldehyde were procured from Sigma-Aldrich (St. Louis, MO) and Ted Pella

(Redding, CA), respectively. Primary antibodies, such as COX-2, NF- κ B p65, and inhibitory κ B- α (I κ B- α), and ABC staining system were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). HSP90 was a product of Enzo Life Sciences (Farmingdale, NY). Quick RNA mini Prep kit was obtained from Zymo Research (Irvine, CA) and Verso cDNA synthesis kit was purchased from Thermo Fisher Scientific (Waltham, MA).

Experimental Regimen and Tissue Harvesting

Mammary tumor samples for all assays in this study were harvested from our previously completed chemopreventive study in which female Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN) subjected to oral feeding of AMR-Me at 0.8, 1.2 and 1.6 mg/kg body weight exhibited 20, 54 and 54% inhibition of occurrence of DMBA-induced mammary tumors, respectively [43]. The entire animal experiment was carried out at the Northeast Ohio Medical University (Rootstown, OH) following an animal protocol approved by the Institutional Animal Care and Use Committee. Briefly, rats (at approximately 43 days of age) were randomly divided into four groups of 7–11 animals each. Two animal groups (groups A and B) were maintained on the basal diet (LabDiet, St. Louis, MO) without any additional treatment, whereas the remaining three groups (groups C, D, and E) were fed with AMR-Me (0.8, 1.2 and 1.6 mg/kg, respectively) through oral gavage three times a week (Monday-Wednesday-Friday). Following two week of aforementioned treatment regimen, mammary carcinogenesis was initiated in all animals in groups B, C, D and E by a single oral administration of DMBA (50 mg/kg body weight). Oral treatment of rats with AMR-Me (in groups C, D, and E) were continued for 16 consecutive weeks following DMBA treatment (i.e., a total period of 18 weeks). All animals were sacrificed at 16 weeks following DMBA treatment (i.e., 18 weeks following the beginning of the experiment). Mammary tumor tissues from various DMBA-treated animals (groups B, C, D and E) were harvested and either fixed in 4% paraformaldehyde for immunohistochemical analysis or immediately flash frozen in liquid nitrogen for gene expression study. Serial tissue sections (approximately 15- μ m thick) were cut using a microtome under freezing condition, stored at -80°C freezer, and subsequently used for immunohistochemical assay.

Immunohistochemical Assessment

Intratumor expressions of COX-2, HSP90, NF- κ B and I κ B- α were analyzed by immunohistochemistry following our established methods (49). In short, the tissue sections were first hydrated in 1X phosphate-buffered saline (PBS) for 5 min and followed by incubation in 10 mM sodium citrate buffer (pH 6.0) for 10 min at 80°C for antigen retrieval. All subsequent steps were carried out at room temperature. Endogenous peroxidases were blocked by 1% H_2O_2 in PBS for 5 min followed by washing the sections with PBS for another 5 min. Tissue sections were then treated with blocking solution for 1 h followed by washing with PBS and incubation overnight (at 4°C) with primary antibody HSP90, COX-2, NF- κ B or I κ B- α (1:100). Following several washes, sections were first treated with horseradish peroxidase-conjugated secondary antibody (1:200) for 30 min at room temperature and then with 3,3'-diaminobenzidine tetrahydrochloride solution to visualize brown antigen-antibody complexes. Finally, sections were counterstained with Gill's hematoxylin solution, air dried and mounted using DPX (Electron Microscopy Sciences, Hatfield, PA). The immunohistochemical slides were visualized under a light microscope

(BX43, Olympus, Center Valley, PA) and 1,000 tumor cells/animal were analyzed. Results were expressed as percentage of immunopositive cells.

Reverse Transcription-Polymerase Chain Reaction

Total RNA from 20 mg of tumor sample was extracted using Quick RNA mini Prep kit following the manufacturer's instructions. The transcriptional expression levels of COX-2 and NF- κ B were determined by reverse transcription-polymerase chain reaction (RT-PCR) using the cDNA verso kit with a temperature scale of 42 °C for 30 min for reverse transcription, and 32 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s. The RT-PCR was carried out using the primers: COX-2-F – 5'-ATCCTGAGTGGGATGACGAG-3', COX-2-R – 5'-CTGCTTGACAGCGATTGGA-3'; NF- κ B-F – 5'-GATAAAATCCTCGGGGTCCTAC –3', NF- κ B-R – 5'-GCTGCTATGTGTAGAGGTGTCG –3'; and 18s RNA-F – 5'-AAGCATTTGCCAAGAATGTTTT-3'; 18s RNA-R – 5'-AAATCGCTCCACCAACTAAGAA-3'. These primer sequences were designed using Primer3 online program [50] and synthesized by Eurofins MWG Operon (Huntsville, AL). Finally, all PCR products were analyzed on 1% agarose gel and visualized by ethidium bromide staining.

Statistical Analysis

The data are expressed as mean $\pm$ standard error of the mean (SEM) and analyzed by commercial software SigmaStat 3.1 (Systat software, Inc., San Jose, CA). One-way ANOVA followed by appropriate post hoc test (Student-Neuman-Keuls) was used to determine whether significant difference existed among various experimental groups. For all comparisons, *P* values less than 0.05 were considered statistically significant.

RESULTS

AMR-Me Suppresses Elevated Expression of COX-2 during DMBA-Induced Mammary Tumorigenesis

As chronic inflammation plays a dynamic role in mammary carcinogenesis [46–48], the ability of AMR-Me in suppressing the induction of inflammatory marker COX-2 has been investigated in the present study. We evaluated the immunostaining of COX-2 protein in mammary tumor sections from rats exposed to DMBA in the presence or absence of AMR-Me treatment. As Figure 1A–a illustrates, a substantial expression of COX-2 was observed predominantly in the cytoplasm of tumor cells from DMBA control animals. AMR-Me at a dose of 0.8 mg/kg did not alter the magnitude of COX-2 immunopositivity compared to DMBA alone (Figure 1A–b). On the other hand, a drastic suppression of COX-2 expression was noticed following AMR-Me treatment at a dose of 1.2 mg/kg (Figures 1A–c and 1A–d). The percentage of COX-2-positive cells in all experimental animal groups is presented in Figure 1B. Our quantitative analysis reveals immunopositivity for more than 20% of mammary tumor cells in DMBA control animals. Although AMR-Me at 0.8 mg/kg reduced the percentage of COX-2-positive cells, the result did not reach the level of statistical significance. On the contrary, there was a significant (*P* < 0.001) inhibition of the percentage of COX-2-positive cells in rats fed with 1.2 mg/kg AMR-Me compared to DMBA control

group. Our immunohistochemical results have been confirmed by RT-PCR analysis. As depicted in Figure 1C, an elevated mRNA expression of COX-2 was observed in DMBA control group. A modest decrease in COX-2 expression by 0.8 mg/kg AMR-Me and a dramatic reduction of the same expression with AMR-Me at higher doses were noticed in DMBA-initiated animals.

AMR-Me Inhibits HSP90 Expression in Mammary Carcinogenesis

Since HSP90 has been found to be upregulated in breast cancer [51,52], protein expression of this molecular chaperon in DMBA-induced mammary tumors in rats in the presence or absence of AMR-Me treatment was determined by immunohistochemistry. Based on immunohistochemical profile as presented in Figure 2A–a, the expression of HSP90 in rat mammary tumor tissue was substantial in DMBA control animals. Similar results were observed in rats subjected to AMR-Me treatment at 0.8 mg/kg in conjunction with DMBA exposure (Figures 2A–b and 2A–c). Interestingly, the intratumor expression of HSP90 was markedly reduced in tumor samples obtained from animals treated with AMR-Me at 1.2 mg/kg compared to DMBA control (Figure 2A–d). The quantitative analyses of HSP90-immunopositive cells revealed a dose-responsive attenuation of this protein expression in tumor samples from rats from all DMBA-treated rats by AMR-Me treatment (Figure 2B). Nevertheless, the result was statistically significant ($P < 0.001$) in the group that received 1.2 mg/kg AMR-Me.

AMR-Me Attenuates Activation of NF- κ B during Mammary Tumorigenesis

It is well known that NF- κ B, a transcription factor, serves as a potential link between inflammation and cancer through regulation of a battery of proinflammatory genes [reviewed in ref. 53]. Since constitutive activation of NF- κ B contributes to the progression of breast cancer, we investigated the activation of NF- κ B in mammary tumor tissues harvested from rats exposed to DMBA in the presence or absence of AMR-Me treatment. As shown in Figure 3A–a, our immunostaining data demonstrate a high expression of NF- κ B p65 in nucleus and low expression of the same in cytoplasm in tumors of DMBA-initiated animals, indicating activation and subsequent translocation of NF- κ B p65 from the cytosol to the nucleus. Almost similar expression pattern of nuclear and cytoplasmic NF- κ B p65 was noticed following treatment with AMR-Me at 0.8 mg/kg (Figure 3A–b). Interestingly, AMR-Me at 1.2 mg/kg reversed NF- κ B p65 protein expression, i.e., it decreased nuclear NF- κ B expression and increased the expression of the same protein in the cytosolic (Figures 3A–c and 3A–d). This has been supported by our quantitative analysis of NF- κ B p65-positive cells that shows a significant ($P < 0.001$) drop in NF- κ B p65-positive cells in nuclei (Figure 3B) and a drastic increase ($P < 0.001$) in NF- κ B p65-positive cells in cytoplasm (Figure 3C) of AMR-Me (1.2 mg/kg) plus DMBA group compared to DMBA control. Accompanying RT-PCR analysis revealed a moderate decrease in the mRNA expression of NF- κ B due low dose of AMR-Me (0.8 mg/kg) and a drastic reduction in the same mRNA in rats subjected to medium (1.2 mg/kg) and high (1.6 mg/kg) doses of AMR-Me (Figure 3D).

Since degradation of I κ B- α represents an essential step for the activation of NF- κ B [54], we investigated whether suppression of DMBA-induced activation of NF- κ B by AMR-Me was due to the inhibition of I κ B- α degradation. As expected, very limited expression of cytosolic

I κ B- α was noticed in DMBA control animals (Figure 4A-a), indicating degradation of I κ B- α protein. AMR-Me suppressed DMBA-induced degradation of I κ B- α protein in cytosol in a dose-responsive manner. Immunostaining revealed a modest increase in cytosolic I κ B- α expression by 0.8 mg/kg AMR-Me (Figure 4A-b) and considerable upregulation of the same protein by 1.2 mg/kg AMR-Me (Figures 4A-c and 4A-d). This is supported by quantitative analysis of percentage of I κ B- α -positive cells that showed a statistically significant ($P < 0.001$) increment in the mammary tumor tissue obtained from rats treated with 1.2 mg/kg AMR-Me (Figure 4B).

DISCUSSION

In our earlier study, we showed that oral administration of novel oleanane-type triterpenoid AMR-Me inhibited environmental carcinogen DMBA-induced rat mammary tumorigenesis, reduced the expression of intratumor proliferating nuclear antigen, and augmented programmed cell death (apoptosis) through mitochondrial apoptotic pathway [43]. Further investigation revealed that AMR-Me also suppressed the elevated expressions of ER- α , ER- β , cyclin D1 and β -catenin during DMBA mammary carcinogenesis [unpublished observation]. Emerging studies have established a vital role of chronic, unresolved inflammation in the promotion and progression of breast cancer [46–48]. Accumulating results from a large number of observational studies have provided convincing evidence that anti-inflammatory mechanisms play an important role in the prevention and intervention of a variety of cancers, including cancer of the breast [55–57]. Accordingly, the present work, an extension of our previous study, was designed to investigate the efficacy of AMR-Me to block DMBA-induced inflammation-related signaling pathways by utilizing mammary tumor samples from our previously completed chemopreventive study [43].

During the last several decades, numerous studies have indicated a connection between the pathogenesis of breast cancer and prostaglandin (PG) synthesis cascade regulated by COX family of PG synthases [58,59]. The COX family of enzymes consists of two distinct isozymes: COX-1 (PG endoperoxide synthase 1) and COX-2 (PG endoperoxide synthase 2). Whilst COX-1 is constitutively expressed, the expression of COX-2 is induced by various stimuli, including shear stress, cytokines, growth factors and oncogenes. COX-2, an important component of the inflammatory response and an early response gene, has a profound role in oncogenesis. Multiple evidence suggests that PGs produced by COX-2 are involved in various crucial steps in malignancy, such as cell proliferation, mutagenesis, resistance to apoptosis, immune suppression, angiogenesis and invasion [60]. It has been observed that PGE₂ potentiates local biosynthesis of estrogen via upregulation of aromatase transcription and represents a crucial link between the prostaglandin cascade and deregulation of estrogen biosynthesis in breast tumor [61]. Although COX-2 remains undetectable in normal breast tissue by immunohistochemistry, elevated COX-2 protein levels have been detected in approximately 40% of human breast carcinoma samples [59]. Interestingly, COX-2 mRNA expression in breast tumors varies between 50 and 100% [58]. It has been observed that COX-2 over-expression in breast cancer correlates with poor prognostic parameters, including large tumor size, high grade, high proliferation, hormone receptor negativity, over expression of human epidermal growth factor receptor 2, distant metastasis and reduced survival [58,59]. A recent meta-analysis have shown similar COX-2

protein expression in ductal carcinoma *in situ* as well as invasive breast cancer, suggesting the involvement of COX-2 in early mammary carcinogenesis [62]. Finally, the use of COX inhibitors has been associated with reduced breast cancer risk [63]. All these studies strongly support a pro-tumorigenic role of COX-2 in breast cancer and support the validity of COX-2/PG signaling as chemopreventive target in mammary neoplasia. In our study, analysis of COX-2 protein expression by immunohistochemistry in mammary tumor samples revealed reduced level of expression in AMR-Me plus DMBA group compared to DMBA control. These results are supported by data on mRNA expression of COX-2 in several experimental groups. Collectively, our results for the first time show AMR-Me-mediated suppression of COX-2 at transcriptional and translational levels in chemically induced mammary carcinogenesis. We propose that the chemopreventive efficacy of AMR-Me in experimental breast carcinogenesis could be, in part, due to inhibition of COX-2 expression and thereby reduction of PG production. Our findings with AMR-Me are in consistent with various synthetic COX-2 inhibitors showing chemopreventive efficacy in several chemical mammary carcinogenesis models [64–67]. The precise molecular mechanism by which AMR-Me inhibits COX-2 during mammary tumorigenesis is not known at the present time. It has been postulated that cyclooxygenase activity may lead to the metabolic activation and metabolism of DMBA through the cytochrome P-450 system [68]. Hence, continuous presence of AMR-Me before and after DMBA exposure may influence critical steps involved in the initiation and promotion phases of mammary tumorigenesis. It is noteworthy that oral treatment of AMR-Me did not produce any adverse side effects, including weight loss, hepatotoxicity and nephrotoxicity [43, unpublished observation]. Nevertheless, since clinical development of COX-2 inhibitors as chemopreventive drugs have been hampered due to serious cardiovascular events [69,70], potential cardiovascular toxicity of AMR-Me should be investigated.

HSPs belong to the molecular chaperon family of proteins and are best known for their rapid and abundant induction by cellular stress. Five main HSP families, each encoding a structurally dissimilar group of HSPs, include HSPA (HSP70), HSPB (small HSP family), HSPC (HSP90), HSPD (HSP60) and HSPH (large HSP) [71]. HSP90, a highly abundant 90 kDa protein that exists as a homodimer, plays an extensive role in chaperoning the structures of about 200 client proteins, many of which are involved in growth control as well as mammary tumor cell proliferation [72]. Emerging studies indicate that HSP90 may regulate inflammatory cascade through modulation of cytokines and several cell signaling pathways [73–75]. It is interesting that breast cancer cells share similar gene expression profiles with heat shocked cells in that both express abundant levels of HSPs [76]. HSP90 appears to play a key role in facilitating transformation by stabilizing the mutated and over-expressed oncoproteins found in mammary tumors and permitting the activation of growth stimulatory and transforming signaling pathways in the absence of growth factors [77]. Substantial experimental data supports inhibition of apoptosis by HSP90 mediated through multiple cytosolic and mitochondrial pathways [reviewed in ref. 78]. Elevated expressions of HSP90 have been associated with poor prognosis in patients with breast cancer [51,52]. The interaction of HSP90 with numerous oncogenic pathways makes it an attractive target for anticancer therapy and several promising HSP90 inhibitors are undergoing clinical trials for the treatment of various oncologic diseases, including breast cancer [79–81]. We have used

immunohistochemical technique to determine the protein expression of HSP90 during DMBA-induced rat tumorigenesis in the presence or absence of AMR-Me. Based on high level of intratumor HSP90 in DMBA group, we can envisage that DMBA exerts some form of heat shock response possibly due to inflammatory stress in tumor cells which may lead to abnormal cellular proliferation and inhibition of programmed cell death as we observed previously [43]. Our data showing significantly reduced level of HSP90 in AMR-Me plus DMBA group suggest that AMR-Me is likely to impede mammary tumor cell growth and survival pathways by downregulation of HSP90 expression. This study also underscores the value of inhibiting HSP90 to achieve chemoprevention of mammary carcinogenesis. Consistent with our present finding, a previous study showed that isoflavone-deprived soy peptide is capable of inhibiting DMBA-initiated mammary tumorigenesis in rats through downregulation of HSP90 [82]. Additionally, a novel HSP90 inhibitor – CH5164840 – has been recently found to exhibit potent antitumor efficacy in BT-474 mammary tumor xenograft model [83].

NF- κ B, a cardinal regulator of the inflammatory pathway, plays a pivotal role in the development and progression of breast cancer. Mammalian NF- κ B represents a family of transcription factors consist of five members, namely RelA (p65), c-Rel, RelB, NF- κ B1 (p50/p105) and NF- κ B2 (p52/p100) [84]. The predominant cytoplasmic inactive form of NF- κ B is a p50-p65 dimer which binds to I κ B- α . In the classical or canonical pathway which is typically triggered by pro-inflammatory cytokines such as tumor necrosis factor- α and interleukin-1 β , I κ B- α is phosphorylated by I κ B kinase- β (IKK- β) followed by ubiquitination and eventual degradation of I κ B- α by the 26S proteasome. Consequently, the released NF- κ B dimer translocates into the nucleus, binds specific DNA sequences, and activates transcription of a battery of target genes involved in inflammation (e.g., COX-2), cell proliferation (e.g., cyclin D1) and anti-apoptosis (e.g., Bcl-2). Several lines of evidence suggest a strong association between NF- κ B-driven inflammation and breast cancer [reviewed in refs. 47,85]. The constitutive activation of NF- κ B in a number of mammary cancer cells lines has been found to have profound consequences in the initiation and progression of breast cancer [86]. It has been shown that NF- κ B is required for the induction and maintenance of the epithelial-mesenchymal transition, a process that critically regulates mammary cancer progression [87]. Selective activation of NF- κ B subunits has been found in human breast cancer cells as well as human breast tumor samples [88]. According to a recent clinical study in invasive ductal carcinoma, NF- κ B p65 overexpression has been associated with advanced stage, large tumor size, high grade, and high Nottingham prognostic index which are poor prognostic factors linked to enhanced aggressiveness of the disease [89]. Suppression of constitutive activation of NF- κ B inhibits the oncogenic potential of transformed cells and accordingly suppression of this pro-inflammatory pathway with various agents, including anti-inflammatory triterpenoids, may provide opportunities for prevention as well as treatment of cancer [26,90,91]. In the present study, low expression of I κ B- α and NF- κ B p65 in cytosol and high expression of NF- κ B p65 in nucleus in mammary tumors from DMBA control animals indicate degradation of I κ B- α , liberating activated NF- κ B p65 to translocate to the nucleus. The results of the current study are consistent with an earlier study which showed that activation of NF- κ B plays early and crucial role in DMBA-initiated malignant transformation in rat mammary glands [92].

AMR-Me-mediated protection against I κ B- α degradation and interference with translocation of activated NF- κ B p65 to nucleus indicate that AMR-Me may block early critical events implicated in DMBA mammary tumorigenesis. Consistent with our results, a recent publication reported that AMR-Me inhibits constitutive activation of NF- κ B and abrogates epidermal growth factor-induced NF- κ B activation in MDA-MB-231 human breast adenocarcinoma cells [42]. It is well known that HSP90 facilitates activation of NF- κ B via IKK complex that phosphorylates I κ B- α [93]. In fact, HSP90 has been found to be associated with its cochaperon cdc37 and functions as a stabilizing factor of IKK through its interaction with cdc37 and the IKK- α and IKK- β kinase domains [94]. As mentioned earlier, our present study has witnessed down-regulation of HSP90 by AMR-Me during mammary carcinogenesis. Thus, we propose that inhibition of NF- κ B signaling could be achieved, in part, by inhibition of IKK-mediated I κ B- α phosphorylation via suppression of HSP90 expression by AMR-Me (Figure 5).

As per our previously published report [43], mammary tumor inhibitory effect of AMR-Me was devoid of any toxic effect as evidenced from unaltered food and fluid intakes, lack of behavioral changes, and uniform and consistent growth of rats received AMR-Me treatment (0.8-1.6 mg/kg, *per os*, 3 times/week) for 18 weeks. Moreover, histopathological assessment of liver and kidneys samples harvested from rats exposed to the highest dose of AMR-Me (1.6 mg/kg) did not reveal any hepatotoxicity or renotoxicity [unpublished observation]. According to a study conducted by another laboratory, the maximum tolerated dose of AMR-Me orally administered daily for 28 days to female BALB/c mice is 3 mg/kg. All these results suggest that AMR-Me possibly interferes key regulatory pathways involved in inflammation during rat mammary tumorigenesis without adverse effects.

Based on the results presented here, we conclude that novel oleanane triterpenoid AMR-Me suppresses the inflammatory cascade during DMBA-evoked rat mammary carcinogenesis by inhibiting inflammatory and stress mediators, such as COX-2, HSP90 and NF- κ B. We previously reported that AMR-Me exerts a striking chemoprevention of DMBA-induced mammary tumorigenesis in rats through antiproliferative and proapoptotic mechanisms [43]. It is likely that AMR-Me suppresses DMBA-driven mammary carcinogenesis by blocking degradation of I κ B- α , inhibiting activation and subsequent translocation of NF- κ B from cytosol to nucleus, hindering DNA-NF- κ B binding and finally disrupting the trans-activation of several genes regulated by NF- κ B. The NF- κ B-driven genes impacted by AMR-Me in rat mammary carcinogenesis include COX-2 as observed in this study as well as Bcl-2 [43] and cyclin D1 [unpublished observation]. Our present study, thus, provides the first *in vivo* evidence that NF- κ B-driven inflammatory cascade is a major target of AMR-Me in breast cancer. The results of the present study in conjunction with our previous findings suggest that disruption of NF- κ B signaling contributes to anti-inflammatory, antiproliferative and apoptosis-inducing mechanisms involved in AMR-Me-mediated chemoprevention of experimental rat mammary carcinogenesis (summarized in Figure 5). These encouraging mechanistic results coupled with a safety profile should facilitate the clinical development of AMR-Me as a breast cancer chemopreventive drug.

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Abbreviations

AMR	amooranin
AMR-Me	methyl-amooranin
DMBA	7,12-dimethylbenz(<i>a</i>)anthracene
COX-2	cyclooxygenase-2
HSP	heat shock protein
IκB-α	inhibitory κ B- α
IKK	I κ B kinase
NF-κB	nuclear factor- κ B
PBS	phosphate-buffered saline
PG	prostaglandin
RT-PCR	reverse transcription-polymerase chain reaction
SEM	standard error of the mean

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Figure 1.

Effects of AMR-Me on COX-2 expression in breast tumors induced by DMBA in female Sprague-Dawley rats. The rats were treated with various oral doses of AMR-Me (three times a week) 2 weeks prior to and 16 weeks following DMBA administration. All animals were sacrificed 18 weeks following the commencement of the study, tumor tissue was harvested and subsequently used for various assays. (A) Immunohistochemical localization of COX-2-positive cells in tumor samples. Arrows indicate immunohistochemical staining of COX-2 (magnification: $\times 200$). (a) Intense COX-2 immunoreactivity in DMBA control; (b) marginal decrease in COX-2 expression in AMR-Me (0.8 mg/kg) plus DMBA group; and (c and d) very limited expression of COX-2 in AMR-Me (1.2 mg/kg) plus DMBA group. (B) Quantitative analysis of COX-2-immunopositive cells during DMBA mammary carcinogenesis in rats in the presence or absence of AMR-Me treatment. Results are based on 1,000 cells per animal and 4 animals per group. Each bar represents the mean $\pm$ SEM ($n = 4$). * $P < 0.001$ compared to DMBA control. (C) COX-2 mRNA expression in mammary tumors isolated from rats exposed to DMBA in the presence or absence of AMR-Me treatment. Total RNA was extracted from tumor samples, subjected to reverse transcription, and resulting cDNA was used for RT-PCR analysis using specific primer sequence. The *18s RNA* was used as the loading control.

Figure 2.

Expression of HSP90 during DMBA-initiated mammary gland tumorigenesis in rats in the presence or absence of AMR-Me. The rats were treated with various oral doses of AMR-Me (three times a week) 2 weeks prior to and 16 weeks following DMBA administration. All animals were sacrificed 18 weeks following DMBA treatment. The mammary tumors were subjected to immunohistochemical analysis using anti-HSP90 antibody. (A) Immunohistochemical localization of HSP90-positive cells in tumor samples. Arrows indicate immunohistochemical staining of HSP90 (magnification: $\times 200$). Various treatment groups are: (a) DMBA control; (b and c) AMR-Me (0.8 mg/kg body weight) plus DMBA; and (d) AMR-Me (1.2 mg/kg body weight) plus DMBA. (B) Quantitative analysis of HSP90-positive cells during DMBA mammary carcinogenesis in rats in the presence or absence of AMR-Me treatment. Results are based on 1,000 cells per animal and 4 animals per group. Each bar represents the mean $\pm$ SEM ($n = 4$). * $P < 0.001$ compared to DMBA control.

Figure 3.

Effect of AMR-Me on NF-κB p65 activation during DMBA-evoked mammary neoplasia in female Sprague-Dawley rats. The rats were treated with various oral doses of AMR-Me (three times a week) 2 weeks prior to and 16 weeks following DMBA administration. All animals were sacrificed 18 weeks following DMBA treatment. The mammary tumors were subjected to immunohistochemical analysis using anti-NF-κB p65 antibody. (A) Representative immunohistochemical localization of NF-κB p65 in nucleus (red arrows) and cytoplasm (black arrows) are depicted (magnification: $\times 200$). Various treatment groups are: (A) DMBA control; (b) AMR-Me (0.8 mg/kg body weight) plus DMBA; and (c and d) AMR-Me (1.2 mg/kg body weight) plus DMBA. Quantitative analysis of (B) nuclear and (C) cytoplasmic NF-κB-immunopositive cells in rat mammary tumors induced by DMBA in the presence or absence of AMR-Me treatment. Results are based on 1,000 cells per animal and 4 animals per group. Each bar represents the mean $\pm$ SEM ($n = 4$). * $P < 0.001$ compared to DMBA control. (D) Alteration of NF-κB mRNA expression in mammary tumors isolated from rats exposed to DMBA in the presence or absence of AMR-Me treatment. Total RNA was extracted from tumor samples, subjected to reverse transcription, and resulting cDNA was subjected to RT-PCR analysis using specific primer sequence. The 18s RNA was used as the loading control.

Figure 4.

Representative immunohistochemical expression of cytosolic IκB-α during DMBA-induced mammary gland carcinogenesis in rats in the presence or absence of AMR-Me. The rats were treated with various oral doses of AMR-Me (three times a week) 2 weeks prior to and 16 weeks following DMBA administration. All animals were sacrificed 18 weeks following DMBA treatment. The mammary tumors were subjected to immunohistochemical analysis using anti-IκB-α antibody. (A) Immunohistochemical localization of IκB-α-positive cells in tumor samples. Arrows indicate immunohistochemical staining of IκB-α in cytoplasm (magnification: $\times 200$). Various treatment groups are: (a) DMBA control; (b) AMR-Me (0.8 mg/kg body weight) plus DMBA; and (c and d) AMR-Me (1.2 mg/kg body weight) plus DMBA. (B) Quantification of cytoplasmic IκB-α-immunopositive cells in rat mammary tumors induced by DMBA in the presence or absence of AMR-Me treatment. Results are based on 1,000 cells per animal and 4 animals per group. Each bar represents the mean $\pm$ SEM (n = 4). * $P < 0.001$ compared to DMBA control.

Figure 5.

Schematic representation of the possible molecular mechanisms of AMR-Me-mediated chemoprevention of experimental mammary carcinogenesis. DMBA activates NF- κ B signaling pathway, which in turn, results in elevated expression of target genes, including COX-2, Bcl-2 and cyclin D1. Activation of all these molecular pathways contributes to sustained inflammation, accelerated proliferation, and evasion of apoptosis which consequently lead to the development of mammary tumor. AMR-Me blocks activated NF- κ B signaling cascade and thereby facilitates suppression of subsequent cellular events,

including anti-inflammatory, antiproliferative, and apoptosis-inducing effects, contributing to chemoprevention of mammary tumorigenesis. ↓, activation and ⊥, downregulation.