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Simultaneous disruption of estrogen receptor and Wnt/ β -catenin signaling is involved in methyl amooranin-mediated chemoprevention of mammary gland carcinogenesis in rats

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Abstract

Methyl-amoorain (methyl-25-hydroxy-3-oxoolean-12-en-28-oate, AMR-Me), a novel synthetic oleanane triterpenoid, exerts a striking chemopreventive effect against 7,12-dimethylbenz(a)anthracene (DMBA)-induced rat mammary tumorigenesis through antiproliferative and proapoptotic actions. Nevertheless, the underlying mechanisms of action remain to be established. As estrogen receptor (ER) and canonical Wnt/ β -catenin signaling are involved in the development and progression of breast cancer, the current study was designed to investigate the effects of AMR-Me treatment on the expressions of ER- α , ER- β , β -catenin and cyclin D1 in rat mammary tumors induced by DMBA. Mammary tumor samples were harvested from an 18-week chemopreventive study in which AMR-Me (0.8–1.6 mg/kg) was shown to inhibit mammary carcinogenesis in a dose–response manner. The expressions of ER- α , ER- β , β -catenin, and cyclin D1 were determined by immunohistochemistry and reverse transcription-polymerase chain reaction. AMR-Me downregulated the expression of intratumor ER- α and ER- β and lowered the ratio of ER- α to ER- β . AMR-Me also reduced the expression, cytoplasmic accumulation, and nuclear translocation of β -catenin, the essential transcriptional cofactor for Wnt signaling. Furthermore, AMR-Me modulated the expression of cell growth regulatory gene cyclin D1, which is a downstream target for both ER and Wnt signaling. AMR-Me at 1.6 mg/kg for 18 weeks did not exhibit any hepatotoxicity or renotoxicity. The results of the present study coupled with our previous findings indicate that simultaneous disruption of ER and Wnt/ β -catenin signaling possibly contributes to antiproliferative and apoptosis-inducing effects implicated in AMR-Me-mediated chemoprevention of DMBA-induced breast tumorigenesis in rats. Our results also suggest a possible crosstalk between two key regulatory pathways, namely ER and Wnt/ β -catenin signaling, involved in mammary carcinogenesis and the value of simultaneously targeting these pathways to achieve breast cancer chemoprevention.

Keywords

Mammary carcinogenesis; DMBA; Oleanane triterpenoid; Estrogen receptors; Cyclin D1; β -Catenin

Introduction

Breast cancer is a leading cause of death among women between the ages of 40 and 55 worldwide with an estimated 1.4 million women diagnosed with this disease annually [1]. About 233,000 new breast cancer cases and approximately 40,000 deaths are estimated to occur in women in the United States in 2013 [2]. Elevated lifetime exposure to endogenous or exogenous estrogen has been recognized as the single most important risk factor in the occurrence of breast cancer [3]. The principal mechanism of estrogen action in breast tissue is mediated through binding to nuclear estrogen receptors (ERs) [4]. Two isoforms of ER, namely ER- α and ER- β , have been identified. ERs represent a family of proteins that function as ligand-activated transcription factors that bind to promoter regions of ER-regulated genes. The development of healthy mammary glands requires estrogen activation of ERs that triggers specific signaling pathways responsible for mammary cell proliferation and differentiation. Nevertheless, altered ER signaling is implicated in the abnormal cell proliferation as well as the development and progression of breast cancer [5, 6]. Especially, the activation of ER- α is believed to promote the growth of breast tumors. On the other hand, the role of ER- β in breast cancer is not well understood [7]. Interestingly, it has been found that normal proliferating mammary epithelial cells in rats rarely express ER- α , whereas ER- β is the predominant form in the normal rat mammary gland [8, 9]. Accumulating evidence suggest that 70 % of breast cancers express ERs [10]. Since alterations in ER signal transduction pathways are believed to contribute to the development and progression of breast cancer, interference with activated ER signaling with anti-estrogenic drugs could be a viable approach in preventing and treating estrogen-responsive neoplastic diseases of the breast.

The canonical Wnt or Wnt/ β -catenin pathway plays an important role in mammary gland development as well as tumorigenesis through its involvement in cell adhesion, signal transduction, and regulation of cell-context-specific gene expression [11, 12]. β -Catenin represents the cardinal regulator of this pathway. In the absence of a Wnt signal, β -catenin is associated with a multiprotein destruction complex consists of glycogen synthase kinase, adenomatous polyposis coli, casein kinase-1 α , and axin which leads to its phosphorylation and subsequent ubiquitination and degradation by proteasome [13, 14]. In the presence of appropriate Wnt signal, the destruction complex becomes inactive, resulting in stabilization and accumulation of β -catenin in the cytoplasm. Subsequently, β -catenin translocates to the nucleus, binds to T cell factor/lymphoidenhancer factor, and activates transcription of target genes, including *cyclin D1*, *c-myc*, *matrix metalloproteinase 7*, and *vascular endothelial growth factor*, which play important roles in breast cancer pathogenesis [15, 16]. Mounting evidence suggests that dysregulation of Wnt/ β -catenin signaling through stabilized and nucleus-bound β -catenin leads to mammary tumor initiation and progression in various genetically engineered animal models [11, 12, 17]. A substantial accumulation of β -catenin in the nucleus and/or cytoplasm has been detected in human breast cancer samples and this feature has been associated with poor prognosis [18–20]. The elevated levels of nuclear and/or cytoplasmic β -catenin in human breast tumors have been correlated with the expression of its target gene *cyclin D1* [18, 21, 22]. Moreover, an increased frequency of cytosolic and nuclear β -catenin accumulation has been observed in ductal carcinoma in situ and basal-like in situ breast tumors, suggesting that Wnt/ β -catenin pathway activation may be an early event in human breast cancer [19, 23, 24]. Since aberrant activation of Wnt/ β -catenin

signaling is involved in the pathogenesis of mammary carcinoma, it can be a potential target for prevention and novel therapy of human breast cancer [17].

Terpenoids, also known as terpenes or isoprenoids, represent the largest group of phytochemicals present in various fruits, vegetables, and medicinal plants. Several terpenoids resemble the structure of human hormones. Naturally occurring terpenoids, including monoterpenes, diterpenes, triterpenes, and tetraterpenes, and their synthetic analogs have exhibited promising results in the chemoprevention as well as therapy of breast cancer [25–28]. Amoorain (AMR), a triterpene acid (25-hydroxy-3-oxoolean-12-en-28-oic acid) isolated from the stem bark of the Indian medicinal plant *Amoora rohituka*, displayed cytotoxic and proapoptotic effects in several human mammary carcinoma cells [29–31] and significantly inhibited the growth of *N*-methyl-*N*-nitrosourea-induced mammary tumors in rats [32]. One of the analogs of AMR, methyl-25-hydroxy-3-oxoolean-12-en-28-oate (amooranin-methyl ester or AMR-Me, Fig. 1), has been found to possess significant antiproliferative effect against MCF-7 breast cancer cells with superior potency to the parent compound AMR [29]. A follow-up study confirmed the potent antineoplastic effect of AMR-Me against MCF-7 cells through induction of apoptosis mediated by two distinct mitogen-activated protein kinase (MAPK) signaling pathways, namely p38 MAPK and c-jun *N*-terminal kinase (JNK) [33]. AMR-Me has also been found to inhibit phosphatidylinositol 3-kinase/Akt signaling in hormone-dependent MCF-7 cells and abrogate activation of nuclear factor-kappaB in hormone-independent MDA-MB-231 cells [34].

Recently, we have reported for the first time that AMRMe exerts a striking chemopreventive effect against 7,12-dimethylbenz(*a*)anthracene (DMBA)-induced rat mammary tumorigenesis [35], a classical animal model that mimics human breast cancer [36, 37]. As per our study, oral administration of AMR-Me dose-dependently reduced the incidence, total burden, and average weight of mammary tumors in DMBA-initiated rats. Additional results showed that AMR-Me suppressed abnormal cell proliferation, induced apoptosis, upregulated proapoptotic protein Bax, and downregulated antiapoptotic protein Bcl-2 in mammary tumors [35] and suppressed inflammatory cascade during mammary carcinogenesis [38]. Nevertheless, the antiproliferative and apoptosis-inducing mechanisms of AMR-Me during DMBA rat mammary carcinogenesis are not clearly understood. Since ERs are implicated in mammary cell proliferation [5, 6] and DMBA-induced rat mammary tumors express ERs [39], we have hypothesized that AMR-Me-mediated inhibition of mammary tumor cell proliferation could be achieved through interference with the expressions of ERs. Additionally, since upregulation of β -catenin has recently been detected in DMBA-initiated mammary tumors in rats [40], it is plausible that AMR-Me may trigger antiproliferative effect through crippling Wnt/ β -catenin signaling and thereby blocking the expression of target genes involved in promoting cell proliferation and apoptosis evasion. Accordingly, the current study was designed to extend our previous studies [35, 38] to retrospectively investigate the effects of AMR-Me treatment on the expressions of ER- α , ER- β , β -catenin, and cyclin D1 in rat mammary tumors induced by DMBA. The potential toxicity of AMR-Me has also been evaluated.

Materials and methods

Materials

AMR-Me has been synthesized as indicated in our previous report [35]. Paraformaldehyde and DMBA were purchased from Ted Pella (Redding, CA, USA) and Sigma-Aldrich (St. Louis, MO, USA), respectively. Primary antibodies, such as rabbit polyclonal ER- α (sc-542), ER- β (sc-8974), cyclin D1 (sc-753), and β -catenin (sc-7199), and rabbit ABC staining system (sc-2018) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Quick RNA mini Prep kit was procured from Zymo Research (Irvine, CA, USA) and

Verso cDNA synthesis kit was purchased from Thermo Fisher Scientific (Waltham, MA, USA).

Animals and experimental design

Mammary tumor samples for this study were harvested from our previously completed chemopreventive study [35] following an animal protocol approved by the Institutional Animal Care and Use Committee of Northeast Ohio Medical University (Rootstown, OH, USA). In short, virgin female Sprague–Dawley rats (Harlan Laboratories, Indianapolis, IN), at approximately 43 days of age, were randomized into six groups of 4–11 animals each. Two groups (groups A and B) were maintained on the basal diet (LabDiet, St. Louis, MO, USA) without any further treatment, whereas the remaining four groups (groups C, D, E, and F) were fed with AMR-Me through oral gavage three times a week (Monday–Wednesday–Friday). Three doses of AMR-Me were used for this work: 0.8 mg/kg (for group C), 1.2 mg/kg (for group D), and 1.6 mg/kg (for groups E and F). Following 2 weeks of aforementioned treatment, mammary carcinogenesis was initiated in all animals belonging to groups B, C, D, and E by a single administration of DMBA (50 mg/kg body weight) by oral gavage. Oral treatment of rats with AMR-Me in groups C, D, E, and F were continued for 16 consecutive weeks following DMBA administration (i.e., a total period of 18 weeks). All animals were sacrificed at 16 weeks post-DMBA treatment (i.e., 18 weeks following the start 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 assay. Representative liver and kidney samples were collected from normal (group A) and AMR-Me control (group F) rats and fixed in 4 % paraformaldehyde for histopathological assessment. Serial tissue sections, approximately 15- μ m thick, were prepared using a microtome and stored at –80 °C freezer. These tissue sections were used for immunohistochemical as well as histopathological study.

Immunohistochemical analysis

ER- α , ER- β , cyclin D1, and β -catenin expressions in mammary tumors were analyzed by immunohistochemistry. Briefly, frozen tissue sections were first thawed and later air dried for 30 min, subjected to antigen retrieval by submersion in 10 mM sodium citrate buffer (pH 6.0) heated up to 80 °C for 10 min. Endogenous peroxidases were blocked by 1 % H₂O₂ (5 min) which was followed by washing the sections with phosphate-buffered saline (PBS) for 5 min. Sections were then treated with blocking solution for 1 h followed by washing with PBS and incubation overnight (at 4° C) with primary antibody ER- α , ER- β , cyclin D1, or β -catenin (1:100). Following several washes, sections were treated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:200) for 30 min at room temperature and then with 3,3'-diaminobenzidine tetrahydrochloride solution to visualize the antigen–antibody complexes. Finally, sections were slightly counterstained with Gill's hematoxylin solution, air-dried and mounted using DPX (Electron Microscopy Sciences, Hatfield, PA, USA). The immunohistochemical slides were visualized under a light microscope (BX43, Olympus, Center Valley, PA, USA) and at least 1,000 tumor cells/animal were analyzed. Results were expressed as percentage of immunopositive cells.

Histopathological study

Histopathological evaluation of liver and kidney sections were performed by hematoxylin and eosin (H&E) staining as we described previously [35].

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 expression levels of ER- α , ER- β , and β -catenin were monitored 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: ER- α -F—5'- ATCTCCACGATCAAGTTCACCT-3', ER- α -R—5'- CGACATTCTTGCATTTTCATGTT-3'; ER- β -F—5'- CAA AGAGAGCTCCCAGAACCTA-3', ER- β -R—5'- AATGAG CTGATTGTCAATGTGG-3'; β -catenin-F—5'-GCTTGTGGCCATCTTTAAATC-3', β -catenin-R—5'- ACAGTTTTGAACAAGTCGCTGA-3'; and 18 s RNA-F—5'- AAGCATTTGCCAAGAATGTTTT-3'; 18 s RNA-R—5'- AAATCGCTCCACCAACTAAGAA-3'. These primer sequences were designed utilizing the Primer3 online program and synthesized by Eurofins MWG Operon (Huntsville, AL, USA). The PCR products were analyzed on 1 % agarose gel and visualized by ethidium bromide staining.

Statistical analysis

All data are presented as mean $\pm$ standard error of the mean (SEM). Significant differences among various groups were detected by one-way ANOVA. Post-hoc analysis was performed by the Student–Neuman–Keuls test. For all comparisons a *P* value less than 0.05 was considered to be statistically significant. All analyses were performed using commercial software SigmaStat 3.1 (Systat software, Inc., San Jose, CA, USA).

Results

Effects of AMR-Me on ER- α and ER- β expressions during DMBA-induced mammary tumorigenesis

Since ER status is a significant classifier of breast cancer, expressions of ER- α , and ER- β in DMBA-induced mammary tumors in rats in the presence or absence of AMR-Me treatment were investigated by immunohistochemistry. The protein expression of ER- α or ER- β was detected predominantly in the nuclei of epithelial cells. The frequency and intensity of ER- α -immunopositive cells were extremely high in tumors from DMBA-treated animals (Fig. 2A–a). On the other hand, a dose-dependent decrease in the expression of ER- α was noticed in tumor sections obtained from animals treated with 0.8 mg/kg (Fig. 2A–b) or 1.2 mg/kg of AMR-Me (Fig. 2A–c) compared to DMBA control. Like ER- α , a substantial expression of ER- β was observed in tumor samples of rats exposed to DMBA alone (Fig. 2A–d). Although the expression of ER- β was not altered by 0.8 mg/kg AMR-Me (Fig. 2A–e), 1.2 mg/kg AMR-Me displayed considerable attenuation of ER- β immunopositivity (Fig. 2A–f). The quantitative analyses of immunopositive cells revealed a significant ($P < 0.001$) reduction of ER- α - (Fig. 2B) and ER- β -positive cells (Fig. 2C) in tumor samples from rats that received 1.2 mg/kg AMR-Me compared to DMBA control. Both doses of AMR-Me reduced the ratio of ER- α to ER- β . However, the results was statistically significant ($P < 0.05$) in the group that received 1.2 mg/kg AMR-Me (Fig. 2D). To determine whether the effects of AMR-Me on ERs were at transcriptional level, mRNA levels of ER- α and ER- β were measured by RT-PCR technique. We found that AMR-Me decreased ER- α and ER- β mRNA expressions in mammary tumors in a dose-responsive fashion (Fig. 3).

Effect of AMR-Me on cyclin D1 expression during DMBA-induced mammary carcinogenesis

Figure 4 represents immunohistochemical data showing the expression of cell cycle specific gene cyclin D1 in mammary tumor sections from several experimental animals. Cyclin D1 was found to be highly expressed mainly in the nuclei of tumor cells in DMBA control rats (Fig. 4A-a). A marginal alteration in the expression of cyclin D1 was noticed in the group that received 0.8 mg/kg AMR-Me plus DMBA compared to DMBA alone (Fig. 4A-b). On the contrary, tumor sections from rats that received AMR-Me at 1.2 (Fig. 4A-c) or 1.6 mg/kg (Fig. 4A-d) displayed considerable reduction in the expression of cyclin D1. The quantitative analysis indicates a small increase (statistically insignificant) in the number of cyclin D1-immunopositive cells in the group treated with 0.8 mg/kg AMR-Me with respect to DMBA control (Fig. 4B). Interestingly, a significant ($P < 0.05$ or 0.001) drop in the number of cyclin D1-positive cells was recorded in DMBA-exposed rats treated with AMR-Me at 1.2 or 1.6 mg/kg in comparison with DMBA control, respectively (Fig. 4B).

Effect of AMR-Me on β -catenin signaling during mammary tumorigenesis induced by DMBA

The immunohistochemical profile indicates variation in the nuclear and cytosolic expressions of β -catenin in the tumor samples harvested from several groups of animals (Fig. 5). Elevated expression of both nuclear and cytosolic β -catenin-positive cells was recorded in rats subjected to DMBA mammary carcinogenesis (Fig. 5A-a and A-b). The rats treated with AMR-Me at 0.8 mg/kg in addition to DMBA had moderate decrease in the expression of nuclear as well as cytosolic expression of β -catenin compared to DMBA control (Fig. 5A-c). A substantial reduction in the expression of this protein in both nucleus and cytoplasm was achieved by AMR-Me at 1.2 mg/kg (Fig. 5A-d). The corresponding quantitative analysis as presented in Fig. 5B and C confirms our immunohistochemical results, indicating a significant ($P < 0.001$) decrease in nuclear and cytosolic β -catenin expression in rats treated with AMR-Me at 1.2 mg/kg plus DMBA compared to DMBA control. Tumor samples from the different experimental groups were also subjected to RT-PCR analysis. Our results show a high β -catenin mRNA level in DMBA control group, whereas a drastic decrease in the transcriptional expression of β -catenin gene was achieved with each experimental dose of AMR-Me (Fig. 6).

Effects of chronic AMR-Me treatment on liver and kidney of normal rats

The representative photomicrographs showing histopathological profiles of liver sections from rats in the presence or absence of AMR-Me treatment are presented in Fig. 7a and b. Hepatic sections of normal rats indicate normal appearance of hepatic parenchyma with regular hepatocellular architecture (Fig. 7a). No significant difference was detected in the liver sections obtained from rats subjected to AMR-Me treatment at a dose of 1.6 mg/kg, three times per week for 18 successive weeks (Fig. 7b). As depicted in Fig. 7c, the kidney section from normal group shows normal renal histology, including normal glomerulus, Bowman's capsule as well as distal and proximal convoluted tubes. Treatment of rats with AMR-Me did not induce histopathological changes in kidneys (Fig. 7d).

Discussion

All variables that modify the exposure to endogenous or exogenous estrogens are known to influence a woman's lifetime risk of developing breast cancer [41]. It has been estimated that 70 % of breast cancers express ERs [10]. Estrogen has been reported to play a pivotal role in estrogen-dependent breast cancer development and growth through ER-dependent cell proliferation [42]. Estrogen binds to ER α and ER β and induces transcription of estrogen-responsive genes resulting in promotion of tumor cell proliferation [9, 43]. Hence,

the modulation of ER- α and ER- β may be important mechanism to suppress mammary carcinogenesis [44, 45]. Therapies using anti-estrogens capable of competing with estrogen for binding to ERs have been found effective in retarding estrogen-dependent breast tumor growth as well as preventing the occurrence of this tumor [46–48]. In addition to targeting the functions of estrogen, strategies to reduce ER protein level are also beneficial in controlling ER-positive breast tumors. We have investigated the expression of ER- α and ER- β in DMBA mammary carcinogenesis following AMRMe treatment by utilizing immunohistochemical technique. According to a prior study, immunohistochemistry is superior to the ligand-binding assay for assessing ER status in primary breast cancer and has an equivalent or even better ability to predict response to adjuvant endocrine therapy [49]. Our immunohistochemical results revealed significant expression of ER- α and ER- β in mammary tumors induced by DMBA. We have also noticed that administration of AMR-Me to rats before and after DMBA treatment reduced both ER- α and ER- β protein expression in tumors. Another important finding of this study represents AMR-Me-mediated reduction in the ratio of ER- α to ER- β . Previous report indicates an increase in the ER α :ER- β in breast cancer [44] and a high ER α :ER- β correlates with elevated level of cell proliferation during human breast tumorigenesis [50]. AMR-Me may compromise the responsiveness to endogenous estrogen by diminishing the expression of ERs and thereby providing less nuclear receptor sites available for estrogen binding during mammary carcinogenesis. Our RT-PCR gene expression studies support the results of immunohistochemical analyses. By inhibiting the transcription of both *ER- α* and *ER- β* gene, AMR-Me may reduce the protein levels of these receptors and finally impede ER signaling. The net effect could be a drastic reduction in intratumor proliferation as observed by the reduced expression of proliferating cell nuclear antigen using immunohistochemical technique [35]. There is another possibility that prior exposure of the mammary glands to AMR-Me may curtail the ability of ER-positive cells to respond to DMBA challenge. All these effects may account for the eventual development of fewer proliferated mammary tumors as we have demonstrated in our earlier study [35].

Estrogens are known to exert their oncogenic effects through regulation of ER-dependent and ER-independent pathways. Hence, the overall mammary tumor inhibitory effects of AMR-Me in DMBA-initiated, estrogen-dependent breast cancer model are likely attributed to AMR-Me disruption of ER signaling as well as non ER antiproliferative and proapoptotic effect of AMR-Me as we reported previously [35]. Interestingly, the parent compound AMR has been found to exhibit growth-inhibitory effect in ER-negative breast carcinoma cell line MDA-MB-468 [31]. Recently, similar results were reported for AMR-Me against ER-negative human breast adenocarcinoma MDA-MB-231 cells [34].

In addition to ER-mediated stimulation of cell growth and proliferation, estrogens deliver mitogenic signals for the growth of breast carcinoma that involve induction of G1/S transition in breast cancer cells through control of various crucial cell cycle regulators [51]. One of such regulatory proteins is cyclin D1, a known cofactor for ER action [52], contributes to breast tumorigenesis, presumably by increasing proliferation as well as influencing differentiation [53]. Cyclin D1 has been found to be overexpressed at the mRNA or protein levels in more than 50 % of the breast cancers and qualifies as one of the most commonly overexpressed proteins in breast cancer [54]. Cyclin D1, a well-established target for ER, is known to be overexpressed preferentially in ER-positive breast cancer [54]. In our study, cyclin D1 protein expression was detected in tumor sections from DMBA control and AMRMe-treated DMBA groups by immunohistochemistry. An elevated expression of intratumor cyclin D1 in DMBA control animals emphasizes the central role of this cell cycle regulatory protein in DMBA-induced mammary tumorigenesis in rats. This is in line with previous studies showing overexpression of cyclin D1 in rat mammary gland tumors induced by DMBA [56–59]. Similar upregulation of cyclin D1 protein in DMBA-induced

early stage mammary carcinogenesis in rats has been reported by several investigators [60, 61]. A drastic reduction of cyclin D1 protein expression following AMR-Me treatment was observed in our study. Based upon these results, reversal of carcinogen-induced dysregulation of a critical cell cycle checkpoint may represent one of the plausible cellular mechanisms of AMR-Me-mediated inhibition of mammary tumorigenesis. This finding also underlines the importance of targeting cell cycle progression protein cyclin D1 to achieve chemoprevention of breast cancer.

Various alterations in Wnt/ β -catenin pathway have been implicated in the development and progression of human breast cancer. In line with clinical situations, a significant body of preclinical data has shown that aberrant activation of Wnt/ β -catenin signaling by the virtue of overexpression, abnormal accumulation and/or nuclear translocation of β -catenin can lead to mammary tumorigenesis [11, 12, 17]. Although several studies have established the link between activation of Wnt/ β -catenin signaling and mammary tumor initiation and progression in various genetically engineered mouse models [11, 12, 17], only few reports are available on the status of β -catenin during carcinogen-induced mammary carcinogenesis in rodents. In one study, an upregulation of total and nuclear β -catenin protein was found in mammary tumors induced by DMBA in mice, indicating activation of this oncogenic signaling pathway [62]. Recently, a sequential increase in the nuclear expression of β -catenin in breast tumor cells as well as elevation of β -catenin level in mammary tissues has been detected in rats subjected to DMBA mammary carcinogenesis [39]. In our present study, we have used immunohistochemical technique to localize the expression of β -catenin in the nucleus and cytoplasm of mammary tumor samples from DMBA-exposed rats. A high frequency of tumor cells with nuclear and cytoplasmic β -catenin expression in DMBA control animals confirms the activation of Wnt/ β -catenin pathway at an early stage of DMBA mammary carcinogenesis in rats. Our data also revealed that AMR-Me treatment resulted in suppression of Wnt/ β -catenin signaling characterized by diminished expression of nuclear and cytosolic β -catenin. In consistent with these findings, our RT-PCR data show that AMR-Me administration markedly reduced the transcriptional expression of β -catenin. The expression of cyclin D1, a β -catenin-regulated gene, has also been found to be downregulated by AMR-Me in a same manner as β -catenin, suggesting β -catenin may be a potential target of AMR-Me in the chemoprevention of breast cancer. The inhibition of Wnt/ β -catenin signaling has been associated with inhibition of cellular proliferation and induction of apoptosis [63]. Thus, suppression of constitutive activation of Wnt/ β -catenin signaling could be a plausible mechanism of AMR-Me-mediated inhibition of cell proliferation and augmentation of apoptosis in DMBA-induced mammary tumorigenesis in rats as we previously observed [35]. Although several prior publications document that various natural and synthetic agents exert antiproliferative and proapoptotic effects through attenuation of β -catenin signaling in human breast cancer cells [64–68], our current study provides the first experimental evidence of modulation of Wnt/ β -catenin signaling in the chemoprevention of breast cancer in a preclinical animal model.

According to our previous study [35], mammary tumor inhibitory effect of AMR-Me has not been associated with any toxic effect of this synthetic triterpenoid. This is based on unaltered food and fluid intakes, lack of behavioral changes, and uniform and consistent growth of rats received AMR-Me treatment for 18 consecutive weeks. An earlier report also presented nontoxic potential of AMR-Me in mice [33]. In this study, we investigated potential organ-specific toxicity of AMR-Me in rats with particular emphasis on liver and kidney. Our histopathological analysis showed that oral administration of rats with AMR-Me at 1.6 mg/kg for 18 weeks neither inflict hepatotoxicity nor renotoxicity. Based upon aforementioned results, it is highly likely that AMR-Me interferes key regulatory pathways involved in breast cancer without adverse effects.

The data presented here allow us to conclude that novel oleanane triterpenoid AMR-Me downregulates the expression of ER- α and ER- β during DMBA-evoked mammary tumorigenesis in rats. AMR-Me also prevents cytosolic stabilization and accumulation as well as nuclear translocation of β -catenin, the essential transcriptional cofactor for canonical Wnt signaling. Additionally, AMRMe modulates the expression of cell growth regulatory gene cyclin D1, which is a downstream target for both ER and Wnt signaling. Previously, we have shown a significant mammary tumor-inhibitory effect of AMR-Me associated with antiproliferative and proapoptotic response under the same experimental conditions [34]. The results of the present study coupled with our previous findings, thus, portend that simultaneous disruption of ER and Wnt/ β -catenin signaling possibly contributes to antiproliferative and apoptosis-inducing effects implicated in AMR-Me-mediated chemoprevention of DMBA-induced breast carcinogenesis in rats. Our results also suggest a possible cross-talk between two key regulatory pathways, namely ER and Wnt/ β -catenin signaling, involved in breast carcinogenesis, and the value of simultaneously targeting these pathways to achieve breast cancer chemoprevention. These positive preclinical results together with a safety profile encourage the development of AMR-Me as a chemopreventive drug for reducing the risk of breast cancer.

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Fig. 1.
Chemical structure of AMR-Me (methyl-25-hydroxy-3-oxoolean-12-en-28-oate)

Fig. 2.

Expression of ERs (A), ER- α (a-c), and ER- β (d-f), during DMBA-induced rat mammary gland tumorigenesis 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 16 weeks following DMBA treatment. The mammary tumors were subjected to immunohistochemical analysis using anti-ER- α or anti-ER- β antibody. Arrows indicate immunohistochemical staining of ER- α or ER- β (magnification: $\times 200$). Various treatment groups are: DMBA control (a, d); AMR-Me (0.8 mg/kg body weight) plus DMBA (b and e); and AMR-Me (1.2 mg/kg body weight) plus DMBA (c and f). Quantitative analysis of ER- α -immunopositive cells (B), ER- β -immunopositive cells (C), and ER- α /ER- β ratio (D) during DMBA mammary carcinogenesis in rats in the presence or absence of AMR-Me treatment (0.8 or 1.2 mg/kg). Results are based on 1,000 cells per animal and four animals per group. Each bar represents the mean $\pm$ SEM ($n = 4$). B, C * $P < 0.001$ and D * $P < 0.05$ as compared to DMBA control

Fig. 3.

The electropherogram of *ER-α* and *ER-β* mRNA expressions in the 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

Fig. 4.

Expression of cyclin D1 during DMBA-mediated mammary gland tumorigenesis in rats in the presence or absence of AMR-Me. **A** Immunohistochemical detection of cyclin D1 in several rat groups. 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 16 weeks following DMBA treatment. The mammary tumors were subjected to immunohistochemical analysis using anti-cyclin D1 antibody. *Arrows* indicate immunohistochemical staining of cyclin D1 (magnification $\times 200$). Various treatment groups are: DMBA control (*a*); AMR-Me (0.8 mg/kg body weight) plus DMBA (*b*); AMR-Me (1.2 mg/kg body weight) plus DMBA (*c*); and AMR-Me (1.6 mg/kg body weight) plus DMBA (*d*). **B** Quantitative analysis of cyclin D1-positive cells during DMBA mammary carcinogenesis in rats in the presence or absence of AMR-Me treatment (0.8, 1.2, and 1.6 mg/kg). Results are based on 1,000 cells per animal and 4 animals per group. Each *bar* represents the mean $\pm$ SEM ($n = 4$). * $P < 0.05$ and ** $P < 0.001$ as compared to DMBA control

Fig. 5.

Expression β -catenin during DMBA-evoked mammary carcinogenesis in rats in the presence or absence of AMR-Me treatment. **A** Immunohistochemical detection of β -catenin in several rat groups. 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 16 weeks following DMBA treatment. The mammary tumors were subjected to immunohistochemical analysis using anti- β -catenin antibody. Representative immunohistochemical localization of β -catenin in nucleus (*white arrows*) and cytosol (*black arrows*) is depicted (magnification: $\times 200$). Various treatment groups are: DMBA control (*a* and *b*); AMR-Me (0.8 mg/kg body weight) plus DMBA (*c*); and AMR-Me (1.2 mg/kg body weight) plus DMBA (*d*). Quantitative analysis of nuclear (**B**) and cytoplasmic (**C**) β -catenin-immunopositive cells in rat mammary tumors induced by DMBA in the presence or absence of AMR-Me treatment (0.8 and 1.2 mg/kg). Results are based on 1,000 cells per animal and four animals per group. *Each bar* represents the mean $\pm$ SEM ($n = 4$). * $P < 0.001$ as compared to DMBA control

Fig. 6.

The electropherogram of β -catenin mRNA expression in the 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

Fig. 7. Histopathological profiles of liver (**a, b**) and kidney (**c, d**) of normal and AMR-Me-treated rats. Serial tissue sections were prepared from liver and kidney of normal rats (**a–c**) and normal rats treated with oral AMR-Me (1.6 mg/kg, three times per week) for 18 consecutive weeks (**b, d**). The sections were stained with H&E. Magnification: $\times 200$