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Retinoic acid and ascorbic acid act synergistically in inhibiting human breast cancer cell proliferation

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Abstract

Background: Breast cancer is an increasingly common malignancy. Several vitamins such as retinoic acid (RA), ascorbic acid (AA), vitamin D and vitamin E are known to prevent the development and progression of breast cancer.
Objective: We sought to determine whether RA and AA together (RA+AA) acted synergistically in blocking the proliferation of human breast cancer cells. To elucidate the mechanism by which RA+AA inhibited breast carcinoma proliferation, we then evaluated the gene expression profiles of the treated and untreated cells by radioactive cDNA microarray analysis.
Methods: We cultured the human breast cancer cell line MCF-7 for 3 days with 100 nM RA and/or 1 mM AA, counted the cell numbers and harvested the total RNAs for cDNA microarray analysis.
Results: RA, AA and RA+AA reduced MCF-7 cell proliferation by 20.7%, 23.3% and 75.7% relative to the untreated cell proliferation, respectively. The synergistic ratio of RA and AA was 1.72. The MCF-7 gene expression profiles showed that 29 genes were up-regulated and 38 genes were down-regulated after RA+AA treatment. The nature of these genes suggests that the mechanism by which RA and AA act synergistically in inhibiting human breast cancer cell proliferation may involve the expression of genes that induce differentiation and block proliferation, and the up-regulation of antioxidant enzymes and proteins involved in apoptosis, cell cycle regulation and DNA repair.
Conclusion: Combined treatment with RA and AA inhibits the proliferation of human breast cancer cells by altering their gene expression related to antioxidation processes as well as the proliferation inhibitory pathway.

Keywords: Retinoic acid; Ascorbic acid; Anti-proliferation; Human breast cancer; cDNA microarray; Combined treatment of vitamins

1. Introduction

Breast cancer is an increasingly common malignancy. Of all the cancers that affect women, breast cancer occurs with a particularly high incidence rate and is associated with a considerable mortality [1]. Various exogenous and endogenous factors are associated with the risk of developing breast cancer. In particular, exposure to estrogens and the presence of estrogen receptors (ERs) appear to influence the development of breast cancer [2]. Therefore, some clinical trials have sought to prevent breast cancer by applying hormone-related bioactive food components and other chemoprevention agents [1]. Moreover, epidemiologic studies have shown that a high dietary intake of fruits and vegetables protects against carcinogenesis in general, with at least seven prospective studies investigating the relationship between vitamin intake and the risk of developing breast cancer [2]. Vitamins that appear to protect from breast cancer include provitamin A (β-carotene), the vitamin A derivative retinoic acid (RA), vitamin C, vitamin D (1,25-dihydroxyvitamin D3) and vitamin E (α-tocopherol) [3,4].

Vitamin A was the first vitamin to be studied with respect to carcinogenesis. In the late 1950s and throughout the 1960s, a number of reports demonstrated that vitamin A deficiency increased the number of spontaneous and

Abbreviations: RA, retinoic acid; AA, ascorbic acid; RA+AA, combination of retinoic acid and ascorbic acid; ROS, reactive oxygen species; FBS, fetal bovine serum.

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chemically induced tumors in experimental animals [5–11]. Moreover, retinoids, which are naturally occurring and hormonally active vitamin A metabolites, have been shown to have antitumor activities both in vitro and in vivo [2]. Apart from its role in vision, the active metabolite of vitamin A, RA, appears to play an important role in development, cellular differentiation and the control of cell division. In addition, many researchers have examined the ability of the retinoids all-*trans*-RA, 9-*cis*-RA and *N*-(4-hydroxyphenyl)retinamide (fenretinide) to protect against breast cancer and have reported that these compounds prevent breast cancer cell proliferation and promote the apoptosis of these cells [12]. Rousenauer et al. [13] suggested that retinoid-induced growth inhibition of breast cancer cell could be mediated by binding to estrogen receptor (ERα).

L-Ascorbic acid (AA) (C6H8O6), which goes by the common name of vitamin C, is an essential nutrient involved in many biochemical functions. AA participates in collagen synthesis, functions of the immune system, amino acid metabolism, the synthesis of certain hormones and the metabolism of minerals and other vitamins [14,15]. The biochemical roles of AA are related to its ability to act as an electron donor or reducing agent. In its role as a free radical scavenger, AA is believed to protect cellular biopolymers, including genetic material, and as such could protect against the initiation and progression of carcinogenesis.

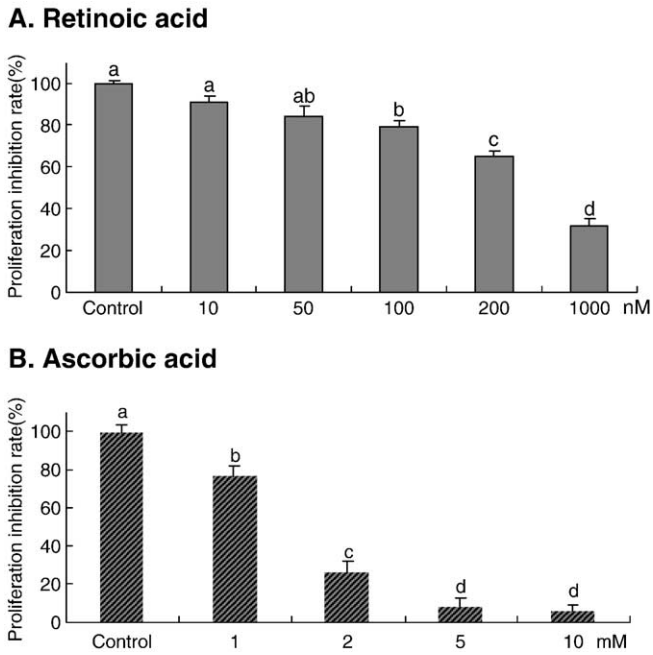

Fig. 1. Effect of various doses of RA and AA on MCF-7 cell growth. The data were expressed as a percentage of the control cell growth, which is set at 100%. Statistically significant differences in cell growth were determined by Duncan's multiple test after ANOVA. The same superscript letter (a, b, c, d) indicates no significant differences ($P<.05$). Cell numbers were decreased by RA or AA treatment in a dose-dependent manner and were significantly lower in cultures treated with at or above 100 nM RA (A) or treated with at or above 1 mM AA (B).

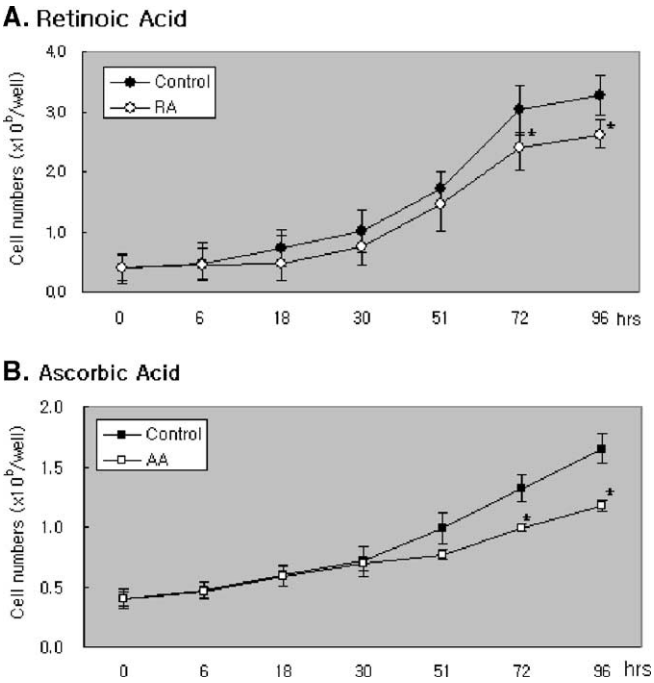

Fig. 2. Time-dependent effect of RA and AA on MCF-7 cell growth. The data were expressed as the number of cells ($\times 10^5$ per well). Cell growth rate was decreased by RA or AA treatment in a time-dependent manner and were significantly lower in cultures treated with RA (A) or AA (B) after 72 h of incubation.

Researchers believe that breast carcinogenesis is due to oxidative damage of mitochondrial DNA by reactive oxygen species (ROS) arising from catechol estrogen redox cycling, which is a process in estrogen metabolism [16,17]. Thus, AA could prevent the initiation of breast carcinogenesis by neutralizing free radicals before they can damage DNA and initiate tumor growth.

Antioxidants such as vitamin A, vitamin C and vitamin E have been shown in some short-term intervention studies with humans to protect against oxidative DNA damage. Experimental data also suggest that these antioxidants can interact such that they protect each other from degradation and/or promote their regeneration [18]. For example, AA is known to induce the regeneration of α-tocopherol and to convert the β-carotene radical back to its reduced form [18]. In addition, RA inhibits malondialdehyde formation [19] and its antioxidant activity is strengthened by the inhibition of ROS that is generated by the iron–ascorbate system by employing Fenton's reaction [20].

Although it is well known that a combination of antioxidants could provide greater antioxidant protection than single antioxidants on their own, studies examining the effect of combining these vitamins have been relatively rarely performed. Chorvatovicova et al. [21] showed that vitamin E and vitamin C act synergistically in decreasing the number of micronuclei in rat and bone marrow cells in guinea pigs. A few studies have also examined the synergism with which vitamin E and RA [22] and RA and vitamin D derivatives [23,24] act to prevent mutations from

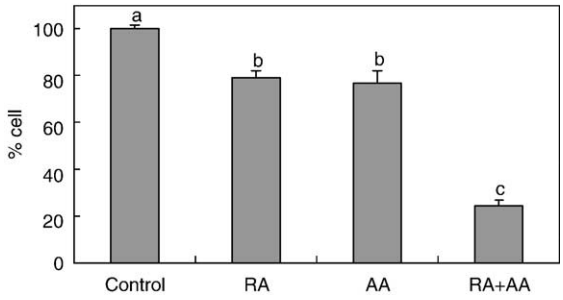

Fig. 3. Synergistic effect of RA+AA on MCF-7 cell proliferation. The cells were treated for 72 h with vehicle (control) or 100 nM RA plus 1 mM AA. The data are expressed as a percentage of the control cell growth, which was set at 100%. RA+AA was considerably more effective in inhibiting MCF-7 cell proliferation (24.3% of control cell growth) than RA alone (79.3%) or AA alone (76.2%). The synergistic ratio was 1.72.

being introduced. However, it is not known whether RA and AA can also act synergistically to influence the proliferation of human cancer cells.

Here, we assessed the effect of RA and AA in blocking the proliferation of the human breast cancer cell line MCF-7. We found there was a synergistic effect when the two vitamins were combined and then subjected combined RA and AA (RA+AA)-treated MCF-7 cells to cDNA microarray analysis to evaluate the molecular mechanism(s) by which this synergism is achieved.

Table 1
cDNA microarray analysis of genes that are up-regulated in human breast cancer cells by a combination of RA and AA

Genes	*Z*-transformed		
	RA	AA	RA+AA
Nuclear receptor subfamily 3, group C, member 1	−0.52	0.03	6.65
RAD51 (*S. cerevisiae*) homolog C	−0.13	0.44	6.50
MINOR mRNA	0.14	0.29	6.09
Galactosyltransferase-associated protein kinase p58 (GTA)	0.27	0.39	4.24
Hexokinase 1	0.44	−0.47	4.10
Down syndrome candidate region 1	−0.20	−0.69	3.89
Forkhead transcription factor HFH-4	0.19	2.01	3.88
PotassiumchannelKv2.1 mRNA, complete cds	0.97	−0.73	3.69
Glutathione peroxidase 2 (gastrointestinal)	−0.68	1.09	3.44
superoxide dismutase 1, soluble (amyotrophic lateral sclerosis 1	−0.93	−1.10	3.43
GATA-binding protein 1 (globin transcription factor1)	−1.09	−1.33	3.37
Human squamous cell carcinoma of esophagus mRNA for GRB-7	−0.13	−0.76	3.24
Fibronectin 1	−0.18	0.35	3.08
Endothelin 1	−0.52	−0.24	2.91
Burkitt lymphoma receptor 1, GTP-binding protein	0.41	1.27	2.84
Burkitt lymphoma receptor 1, GTP-binding protein; CXCR5	−0.18	0.41	2.65
Monokine induced by γ interferon	0.42	0.57	2.55
26S proteasome component TBP1(PSMC3: proteasome)	0.18	0.42	2.53
Protein kinase C, θ type	0.50	0.69	2.43
GDNF family receptor α1	−0.87	0.22	2.43

Analysis of the median densitometric signal intensity revealed that 67 genes differed on MCF-7 cells treated with RA+AA and controls by a *Z* ratio of ≥2 at a descriptive $P \leq .05$.

Table 2
cDNA microarray analysis of genes that are down-regulated in human breast cancer cells by a combination of RA and AA

Genes	*Z*-transformed		
	RA	AA	RA+AA
Human insulin-like growth factor binding protein 5	−1.57	2.23	−3.73
Cytochrome P450, subfamily XIX	−7.00	−2.31	−3.70
Tumor necrosis factor receptor superfamily, member 5	−2.05	2.17	−3.56
CD34 antigen	−0.23	1.19	−3.39
Vascular cell adhesion molecule 1	−1.17	−0.13	−3.33
Sulfotransferase mRNA, complete cds	−2.98	−3.60	−3.27
Nuclear transcription factor Y, α	−0.60	0.04	−2.95
Endothelin 3	0.29	1.77	−2.93
Tyrosine phosphatase	−0.08	−0.84	−2.92
40-kDa protein kinase related to rat ERK2	−0.22	1.52	−2.90
OS-9 precursor mRNA, complete cds	2.63	−1.10	−2.87
Cdc42-interacting protein 4(CIP4)	3.10	−2.22	−2.86
Nucleoside-diphosphate kinase	0.18	−1.38	−2.86
VRK1,complete cds	−1.99	−2.18	−2.61
Ninjurin1 mRNA, complete cds	−2.42	−2.04	−2.58
Tyrosine-protein kinase receptor eck	1.68	−2.45	−2.47
RAB6, member RAS oncogene family	1.64	−1.49	−2.43
Cyclin G-associated kinase (GAK)	−1.63	0.38	−2.40
Ubiquitin-conjugating enzyme E2A (RAD6 homolog)	1.06	−2.11	−2.40
Complement component (3d/Epstein–Barr virus) receptor 2	−6.97	−0.40	−2.30

Analysis of the median densitometric signal intensity revealed that 67 genes differed on MCF-7 cells treated with RA+AA and controls by a *Z* ratio of ≤−2 at a descriptive $P \leq .05$.

2. Materials and methods

2.1. Cell cultures

The human breast cancer cell line MCF-7 was obtained from the American Tissue Culture Collection (Rockville, MD) and cultured in RPMI (GIBCO BRL, Grand Island, NY) containing 10% fetal bovine serum (FBS) and 1% antibiotics at 37°C in a humidified atmosphere. The cells were grown in a monolayer culture, harvested at 50–70% confluence by brief exposure to trypsin, followed by trypsin neutralization with RPMI containing FBS and antibiotics.

2.2. Analysis of cell proliferation

Fifty thousand cells were seeded per well, and after 24 h the culture medium was renewed and cell attachment was verified at zero time. The medium was then supplemented with 10, 50, 100, 200 or 1000 nM RA dissolved in absolute ethanol, or 1, 2, 5 or 10 mM AA dissolved in phosphate-buffered saline, and the cells were incubated for a further 3 days. Control cultures were cultured without RA and AA treatment. According to the results from cell proliferation, a minimal dose with proliferation-inhibiting effect of each

vitamin was determined. In addition, cells were treated with 100 nM RA and 1 mM AA to evaluate the time-dependent effect or synergistic antiproliferation effects.

To analyze the time-dependent effect of RA or AA on MCF-7 growth, the cells were treated with 100 nM RA or 1 mM AA for 6, 18, 30, 51, 72 or 96 h. The effect of RA+AA was assessed by culturing the cells for 3 days with 100 nM RA and 1 mM AA. The cell numbers were then determined by using a Coulter counter.

2.3. Microarray analysis

The human cDNA microarray we used consisted of the 1152 genes that represented families of genes involved in differentiation, development, proliferation, transformation, cell cycle progression, cell growth and maintenance, and immune responsiveness, as well as for genes encoding transcription and translation factors and oncogenes.

Total RNAs isolated using TRIZOL from RA- and/or AA-treated MCF-7 cells and untreated MCF-7 cells were used to synthesize ^{32}P-labeled cDNAs by reverse transcription. The cDNAs were prehybridized in hybridization buffer containing Microhyb (Research Genetics), denatured human Cot 1 DNA (Life Technologies), and poly (dA) (Pharmacia, NJ). After 4 h of prehybridization at 42°C, hybridized arrays were then washed three times and exposed to phosphorimager screens for 1–3 days. The screens were scanned in a FLA-2000 Phosphorimager (Fujifilm, Japan) at 50-μm resolution. Array images were cropped, aligned in L process V1.96 (Science Lab), and then counted in ArrayGauge V 1.21 (Fujifilm). Raw intensity values were exported to EXCEL for data analysis. More detail on the array method that we used was described in our previous study [25].

Color overlay images were produced in ArrayGauge V 1.21 (Fujifilm). Cluster analysis was performed on Z-transformed microarray data by using two programs available as shareware from Michael Eisen's laboratory (http://rana.lbl.gov). The clustering of changes in gene expression was determined by using public domain cluster based on pairwise complete-linkage cluster analysis. Gene expression raw data, log values, and *Z* scores were calculated by using the means and S.D.'s.

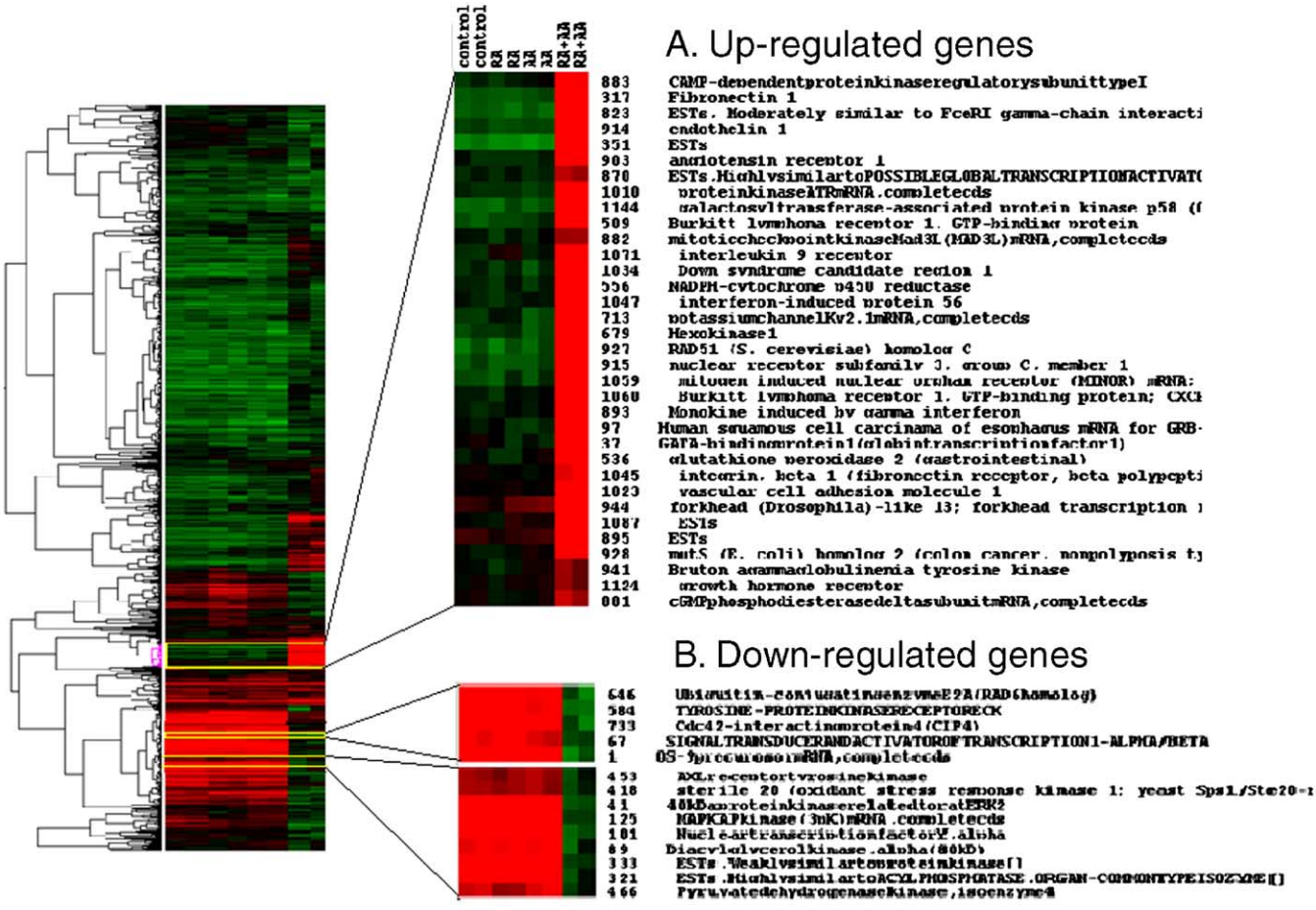

Fig. 4. Clustergram of gene expression that is up-regulated (A) and down-regulated (B) in MCF-7 cells by RA+AA. Cluster analysis was performed on *Z*-transformed microarray data using separate programs that were available as shareware from Michael Eisen's laboratory. Each gene is represented by a single row of colored boxes, while each experimental sample is represented by an individual column. These genes clustered could be grouped into four categories of differentiation, proliferation inhibition, antioxidation mechanisms and cell cycle regulation on the basis of their cellular functions.

2.4. Statistical methods

Data analysis of microarray was done using EXCEL (Microsoft). The values from cDNA microarray were normalized to Z scores with Z transformation by subtracting the average gene intensities and dividing by the respective S.D. Gene expression differences between vitamin treatment and control group were calculated by comparing the Z score differences of the same genes. These manipulations generated the Z ratio value and provided us with an effective method for comparing microarray experiments. Scatter plots of intensity values were produced using Sigma Plot (Sigma Plot). Correlations between duplicate spots had R^2 values of .9661 and .9865.

All other data are expressed as means ± S.D. After ANOVA, Duncan's multiple range test was performed to determine the significance of the differences in the means of cell numbers among vitamin-treated cultures. The differences were considered significant at the 5% level. The SPSS software 11.0 was used for the statistical analyses.

3. Results

3.1. Effect of RA and AA on cancer cell proliferation

We studied the individual abilities of RA and AA to block the proliferation of the human breast cancer cell line MCF-7 by treating the cells with five different concentrations (10, 50, 100, 200 and 1000 nM) of RA or four different concentrations (1, 2, 5 and 10 mM) of AA for 3 days (Fig. 1). Proliferation inhibition rates were expressed as a percentage of the vehicle-treated cell growth, which was set at 100%. Cell growth was significantly lower in cultures treated with RA at concentrations at or above 100 nM (Fig. 1A). At 100 nM, the proliferation inhibition rate was 79.3%, namely, 20.7% lower than the control cell growth. The RA-induced decrease in cell numbers was dose-dependent. Similar dose-dependent inhibition of MCF-7 cell growth was also observed upon AA treatment, with significant inhibition of growth already being observed at 1 mM AA (76.6% of the control cell growth).

Experiments were carried out to determine when, during culture, RA and AA started to exert their growth inhibitory effects on MCF-7 cells; the cells were incubated with 100 nM RA or 1 mM AA for 6, 18, 30, 51, 72 or 96 h (Fig. 2). Statistical analysis revealed that, in comparison to vehicle-treated cells, RA and AA induced significant differences in cell numbers after 51 h of incubation, as only the 72- and 96-h time points showed statistically significant differences between vehicle- and vitamin-treated cell cultures.

3.2. Effect on cancer cell proliferation of combining RA and AA

To determine whether treating cancer cells with both RA and AA would have a synergistic or additive effect on MCF-7 proliferation, cell cultures were treated for 3 days with 100 nM RA and 1 mM AA. As shown in Fig. 3, RA+AA was considerably more effective in inhibiting

Table 3
Functional classification of up-regulated genes in combination of RA+AA-treated MCF-7 cell

Differentiation-related gene	Anti-proliferation-related gene
Nuclear receptor subfamily 3, group C, member 1	Nuclear receptor subfamily 3, group C, member 1
Hexokinase 1	Galactosyltransferase-associated protein kinase p58 (GTA); cell division cycle 2-like 1 (CDC2L1; CLK1)/p130PITSL cyclin-kinase
Forkhead transcription factor HFH-4 (HFH-4) mRNA	Down syndrome candidate region 1
GATA-binding protein 1 (globin transcription factor 1)	26S proteasome component TBP1 [PSMC3: proteasome (prosome, macropain) 26S subunit, ATPase, 3] (similar to RAM206, but PSMA2)
Fibronectin 1	Potassium channel Kv2.1 mRNA, complete cds
Burkitt lymphoma receptor 1, GTP-binding protein; CXCR5	Angiotensin receptor 1
Monokine induced by γ interferon	GDNF family receptor α1
Angiotensin receptor 1	
Antioxidant enzyme	**Apoptosis**
Glutathione peroxidase 2 (gastrointestinal)	Nuclear receptor subfamily 3, group C, member 1
NADPH-cytochrome P450 reductase	MINOR mRNA; chondrosarcoma, extraskeletal myxoid
Superoxide dismutase 1, soluble [amyotrophic lateral sclerosis 1 (adult)]	Galactosyltransferase-associated protein kinase p58 (GTA); cell division cycle 2-like 1 (CDC2L1; CLK1)/p130PITSL cyclin-kinase
	Monokine induced by γ interferon
Cell cycle regulator	**DNA repair modulator**
Galactosyltransferase-associated protein kinase p58 (GTA); cell division cycle 2-like 1 (CDC2L1; CLK1)/p130PITSL cyclin-kinase	RAD51 (*S. cerevisiae*) homolog C
Endothelin 1	
Protein kinase C, θ type	
Chimerin (chimaerin) 1	

MCF-7 cell proliferation (24.3% of control cell growth) than RA alone (79.3%) or AA alone (76.2%), which indicates the two vitamins act synergistically. The synergistic effect was estimated by calculating the synergistic ratio, which was 1.72.

3.3. Effect of RA+AA on the gene expression profile of MCF-7 cells

To elucidate the molecular mechanisms by which RA+AA exert their synergistic effect in inhibiting MCF-7 cell growth, we compared the gene expression profiles of vehicle-treated MCF-7 cells and MCF-7 cells treated with RA+AA, RA alone or AA alone by using a cDNA microarray containing 1152 human genes. This revealed that 29 genes (~2.5%) were up-regulated and 38 genes (~3.3%) were down-regulated by RA+AA. The 20 genes that show the biggest increases or decreases in expression are listed in Tables 1 and 2. Genes showing highly altered expression levels were aligned according to the magnitude of alteration. In particular, the nuclear receptor subfamily 3 exhibited the maximum up-regulation observed (Z ratio, 6.65), while human insulin-like growth factor-binding protein 5 exhibited the maximum down-regulation observed (Z ratio, −3.73).

These genes clustered in Fig. 4 could be grouped into several categories on the basis of their cellular functions in Table 3. These categories included genes involved in differentiation [such as nuclear receptor subfamily 3, group C, member 1, hexokinase 1, GATA-binding protein 1, fibronectin 1, Burkitt lymphoma receptor 1 (CXCR5), monokine induced by γ interferon and angiotensin receptor 1], proliferation inhibition (such as nuclear receptor subfamily 3, group C, member 1, galactosyltransferase-associated protein kinase p58, Down syndrome candidate region 1, 26S proteasome component TBP1, potassium channel Kv2.1 mRNA, angiotensin receptor 1 and GDNF family receptor α1), antioxidation mechanisms (such as glutathione peroxidase 2, NADPH-cytochrome P450 reductase and superoxide dismutase 1), apoptosis [such as nuclear receptor subfamily 3, group C, member 1, mitogen-induced nuclear orphan receptor (MINOR) mRNA, galactosyltransferase-associated protein kinase p58 and monokine induced by γ interferon], cell cycle regulation [such as galactosyltransferase-associated protein kinase p58, endothelin 1, protein kinase C, θ type and chimerin (chimaerin) 1] and DNA repair [such as RAD51 (*Saccharomyces cerevisiae*) homolog C]. A possible metabolic pathway by which RA+AA act synergistically to inhibit MCF-7 proliferation was deduced from our results and is presented in Fig. 5.

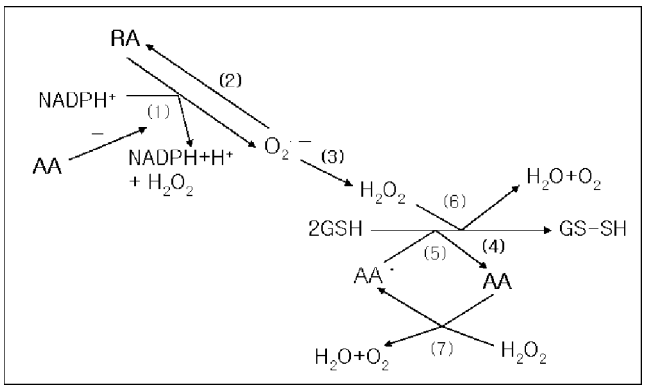

Fig. 5. Possible metabolic pathway that accounts for the synergistic effect of RA+AA in inhibiting MCF-7 cell growth. We found that compared to AA and RA alone, RA+AA elevated the expression of genes encoding NADPH-cytochrome P450 reductase, superoxide dismutase and glutathione peroxidase 2. On the basis of this, we deduced the possible mechanism by which RA and AA act synergistically to inhibit MCF-7 cell proliferation. AAsuper, dehydroascorbic acid; O_2^-, superoxide anion; H_2O_2, hydrogen peroxide; GSH, glutathione; GSSG, glutathione disulfide; 1, cytochrome P450; 2, NADPH-cytochrome P450 reductase; 3, superoxide dismutase; 4, glutathione peroxidase 2; 5, dehydroascorbic acid reductase; 6, catalase; 7, ascorbic acid peroxidase.

4. Discussion

Vitamin A, vitamin C and vitamin E have been proposed recently to stimulate tumor suppressor genes as well as to down-regulate oncogenes and block tumor-induced angiogenesis [26]. While it has been observed by several researchers that these vitamins act synergistically on cancer cells when they are combined in treatment protocols [27,28], the effect on tumor cell proliferation of combining RA and AA remains unknown. We addressed this issue here by using the MCF-7 human breast cancer cell line and indeed found that the two vitamins acted synergistically in suppressing tumor cell growth in vitro. We also evaluated the possible molecular mechanism behind this synergism by subjecting the vitamin-treated and untreated tumor cells to cDNA microarray analysis. This technology was employed because it identifies differentially expressed genes with high sensitivity and fidelity and can correctly predict the expression patterns of the corresponding proteins. Moreover, the microarray-based genomic survey is a high-throughput approach that allows parallel studies on the expression patterns of thousands of genes [29].

We found that on their own, RA and AA inhibited MCF-7 growth, but in combination, they reduced MCF-7 growth synergistically. That RA inhibits tumor cell proliferation and other tumor phenotypes has been reported many times before [9,12,13], and RA has also been used in combination with chemotherapeutic agents, where it has shown a synergistic effect on tumor growth [9]. However, the mechanism by which RA exerts its anticancer effects is not known, although a number of mechanisms have been proposed. One theory suggests that RA induces cell growth arrest and thereby inhibits cell proliferation. Supporting this is the study by Simeone and Tari [12], which suggested that the all-*trans*-RA-mediated regulation of cell proliferation results from direct and indirect effects on gene expression. Another proposed anticancer mechanism of RA

implicates the inhibition of a specific signaling pathway in neoplastic cells that involves inhibiting protein kinase C activity and the expression of c-*myc*, H-ras, a transcription factor (E2F) and inducing transforming growth factor-β, and p21 [11]. With regard to this, our study confirmed that the expression of the proliferation-related gene *myc* was down-regulated by RA , while the expression of the cell cycle arrest-related nek3 protein kinase was up-regulated. In addition, we found RA regulates the expression of transcription factors such as OS-9 precursor mRNA (OS-9), GA-binding protein transcription factor (GABPA), signal transduction-related molecules such as G-protein, α inhibiting activity polypeptide 2 (GNAI2), inositol 1,3,4-trisphosphate 5/6-kinase mRNA (ITPK1) and GTP-binding protein mRNA (RAB32), and various protein kinases, including mitogen-activated protein kinase 3 (MAP2K3) and extracellular signal-regulated kinase 3.

Another mechanism suggested is that retinoid-induced growth inhibition of breast cancer cell could be mediated by binding to ERα through transcriptional cross-talk with the RA receptor (RAR) [13,30]. RAR has sequence homology with other members of the steroid receptor superfamily such as ER or retinoid X receptors [31]. These nuclear receptors are transcriptional factors that bind to RA response elements (RARE) in the promoter regions of target genes [32]. However, we failed to elucidate whether gene expression of ER, estrogen-responsive pS2 and vascular endothelial growth factor was regulated by combination of RA and AA, and whether these two vitamins induced growth inhibition in MCF-7 via ER.

The last suggested mechanism by which RA exerts its anticancer properties proposes that RA serves as an anti-carcinogen by protecting cells from oxidation-induced stress and toxicity [33]. Supporting this notion is that McCarthy et al. [34] have shown RA enhances the levels of glutathione *S*-transferase and quinine reductase, which are important phase II enzymes; they also showed that agents that elevate the levels of these enzymes act as blockers of tumor proliferation and can be categorized as chemopreventive agents. Moreover, Sultana et al. [20] reported that 13-*cis*-RA also induces the detoxifying enzyme system, as shown by elevated levels of glutathione *S*-transferase, quinine reductase and xanthine oxidase.

Notably, glutathione transferase is also needed for the antioxidant function of AA because it reduces the ascorbyl radical to AA, and the antioxidant property of AA is known be important in preventing human breast carcinogenesis. Moreover, ROS maybe generated by catechol estrogen redox cycling, which is a process in estrogen metabolism [17], and it has been suggested that ROS-induced mitochondrial DNA damage may play an important role in breast carcinogenesis [34,35]. In addition, Storz [36] reported that ROS within cells act as second messengers in intracellular signaling cascades that increase cell proliferation. In line with these observations, we observed that AA up-regulates the antioxidation-related gene encoding cytochrome P450 VIIA1 as well as signal transduction-related genes such as adenosine A2a receptor (ADORA2A), protein kinase, c-AMP-dependent, catalytic β (PRKACB), phospholipase C, δ1 (PLCD1), MAP kinase P38β and regulator of G-protein signaling 1 (RGS1).

While we found that RA or AA on their own failed to up-regulate antioxidant genes, it was notable that RA+AA enhanced glutathione *S*-transferase, NADPH-cytochrome P450 reductase and superoxide dismutase expression. These enzymes play important roles in protecting cells from oxidative stress and participate in the repair of oxidative damage. In addition, we discovered for the first time evidence of gene expression events that are related to the inhibitory effects of RA+AA on MCF- 7 proliferation, as we found that RA+AA modulates the cell cycle progression, differentiation, proliferation and apoptosis of MCF-7 cells in vitro. For example, the differentiation-related gene encoding nuclear receptor subfamily 3 (glucocorticoid receptor, or GR) was significantly up-regulated in the RA+AA-treated MCF-7 cells. GR directly interacts with the pro-apoptotic death-associated protein 3 and affects many different functions in the body, including development, differentiation, metabolism and immune responsiveness. Also up-regulated was the differentiation-related gene encoding Burkitt lymphoma receptor 1, a principal regulator of B-cell development, migration and localization, and monocytic cell differentiation. These results thus suggest that RA and AA exert their proliferation inhibitory effect through their antioxidant properties. Moreover, on the basis of these observations, we suggest that RA and AA in combination have synergistic effects on MCF-7 proliferation because AA slows RA degradation, thereby enhancing the cell proliferation inhibitory effects of RA (Fig. 5). In humans, vitamin A (retinol) is oxidized to retinal, which is in turn oxidized to RA. RA then undergoes cytochrome P450-dependent hydroxylation followed by oxidation to 4-oxo-metabolites that are conjugated with glucuronic acid and excreted into the bile. The ability of cytochrome P450 inhibitors (such as imidazoles) to suppress RA metabolism and delay RA plasma clearance has been demonstrated in animals and humans by administering RA together with cytochrome P450 inhibitors [36]. We hypothesize that AA inhibits the cytochrome P450-mediated catabolism of RA (i.e., the 4-hydroxylation of RA).

In summary, this is the first time the effect of combining RA and AA on breast cancer cell proliferation, differentiation, apoptosis and antioxidant-related gene expression has been studied. We found the two vitamins acted synergistically in inhibiting breast cancer cell proliferation by inducing the expression of molecules involved in differentiation, proliferation inhibition, apoptosis, antioxidation, cell cycle regulation and DNA repair. Further analysis of the concentrations of the proteins involved in RA+AA-mediated tumor growth inhibition will be needed, along with the assessment of other oxidative indices and the activity of antioxidant enzymes. Nevertheless, our

study here provides insights into the mechanism by which two vitamins can synergize in blocking the proliferation of tumor cells. This will ultimately aid the design of more effective anticancer treatments.

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