Growth Inhibition of Human Breast Cancer Cells by 1,25-Dihydroxyvitamin D₃ Is Accompanied by Induction of Apolipoprotein D Expression

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ABSTRACT

We have analyzed the effect of 1,25-dihydroxyvitamin D₃ on the expression of the gene encoding apolipoprotein D (apoD), a protein component of the human plasma lipid transport system that is overproduced by a specific subset of breast carcinomas. Northern blot analysis revealed that 1,25-dihydroxyvitamin D₃ strongly up-regulated apoD mRNA levels in T-47D human breast cancer cells in a time- and dose-dependent manner. The potency of this vitamin as an inducer of apoD expression was stronger than the effect observed for other steroid hormones such as androgens and progesterone, described previously as hormonal up-regulators of apoD expression in these cells. A time course study demonstrated that the induction of apoD mRNA reached a level of 5-fold over the untreated cells after 48 h of incubation in the presence of 10⁻¹⁰ M 1,25-dihydroxyvitamin D₃. A dose-response analysis showed that a 10⁻⁸ M concentration of this vitamin consistently induced a maximal accumulation of 7-fold over the control cells. Similar up-regulatory effects on the apoD gene expression were obtained by treatment of T-47D cells with 1,25-dihydroxyvitamin D₃ analogues, including MC 903, which is relatively devoid of hypercalcemic side effects in clinical applications. Western blot analysis revealed that the inductive effect of 1,25-dihydroxyvitamin D₃ was also reflected at the protein level as an increase of immunoreactive protein in the conditioned media of vitamin-treated cells. This increased expression of apoD was accompanied by an inhibition of cell growth and morphological changes in T-47D cells. By contrast, we did not detect any inductive effect of 1,25-dihydroxyvitamin D₃ on apoD gene expression in MDA-MB-231 cells, which are refractory to the growth-inhibitory effects of this compound. On the basis of these results, we propose 1,25-dihydroxyvitamin D₃ as an important regulator of the expression of the apoD gene in breast carcinomas. We also suggest that apoD may be of interest as a biochemical marker of the action of 1,25-dihydroxyvitamin D₃ derivatives in current studies using these compounds as inhibitors of breast cancer cell growth or as chemotherapeutic agents in the prevention of breast cancer.

INTRODUCTION

apoD³ is a component of the human plasma lipid transport system that was first isolated by McConathy and Alaupovic (1). This glycoprotein is mainly associated with high-density lipoprotein particles and consists of a single polypeptide chain of about Mᵋ 30,000, polymorphic in charge and with ability to form disulfide-linked complexes with other lipoproteins (2). Amino acid sequence analysis of apoD has revealed that this glycoprotein exhibits sequence similarity to members of the lipocalin family of proteins, the common function of which is to bind and transport small hydrophobic ligands in the plasma (3). The functional role of apoD in the metabolism of plasma lipoproteins remains unclear, but the finding of complexes with lecinthin-cholesterol acyltransferase has led to the proposal that apoD may be involved in the early steps of the cholesterol transport from peripheral tissue to the liver (4). However, recent studies from different groups appear to indicate that apoD is able to bind and transport a wide variety of ligands other than cholesterol, including heme-related compounds (5), progesterone and pregnenolone (6, 7), arachidonic acid (8), or odorant substances (9), thus extending its potential functional significance to a number of different biological processes.

The connection between apoD and breast diseases was established by our observation that apoD accumulates to extremely high concentrations (about 1000-fold higher than in plasma) in cyst fluid from women with gross cystic disease of the breast (7), a benign condition associated with an increased risk of subsequent breast cancer (10—12). These observations were further extended by the finding of a certain type of breast carcinomas that have the ability to produce and secrete this glycoprotein (13—16). Interestingly, analysis of the potential relationship between apoD levels in breast carcinomas and clinical outcome of patients has revealed that low apoD values are significantly associated with a shorter relapse-free survival and poorer survival (16). A hypothesis to explain why apoD is a marker of favorable clinical outcome in women with breast cancer is that these carcinomas possess the required degree of differentiation to synthesize this protein. In fact, apoD values are higher in well-differentiated carcinomas than in moderately or poorly differentiated tumors (16). These data, together with reports showing that apoD transcription in human fibroblasts occurs specifically in nonproliferating cultures (17), and that stimulation of apoD secretion by steroids in breast and prostate cancer cells coincides with inhibition of cell proliferation (18—21), have suggested that apoD expression is a marker of cell differentiation and growth arrest. Consistent with this proposal, we have recently shown that RA, a potent antiproliferative and differentiating agent, strongly induces the expression of apoD in breast cancer cells through a RA receptor α-dependent signaling pathway (22, 23). On this basis, and considering that 1,25-dihydroxyvitamin D₃ has been reported to have differentiating and growth-inhibitory effects on a variety of cancer cell types (24—29), we have examined the possibility that this hormone could also have a stimulatory effect on apoD gene expression in breast cancer cells. In this study, we report that 1,25-dihydroxyvitamin D₃ induces apoD expression in T-47D breast cancer cells in a time- and dose-dependent manner. Similar inductive effects were obtained by treatment of these cells with 1,25-dihydroxyvitamin D₃ analogues including KH 1060 and MC 903. In addition, we show that induction of apoD expression by 1,25-dihydroxyvitamin D₃ is accompanied by a marked inhibition of proliferation of breast cancer cells.

MATERIALS AND METHODS

Materials. All media and supplements for cell culture were obtained from Life Technologies Inc. (Gaithersburg, MD), except for FCS, which was from Boehringer Mannheim (Mannheim, Germany). All-trans-RA, the steroid hormones dihydrotestosterone, dexamethasone, and estradiol, and the fluorescent dye sulforhodamine B were from Sigma Chemical Co. 1,25-Dihydroxyvitamin D₃ and 9-cis-RA were kindly donated by Dr. M. Klaus (F. Hoffmann-La Roche, Ltd., Basel, Switzerland). The 1,25-dihydroxyvitamin D₃ analogues KH 1060 and MC 903 (calcipotriol) were synthesized in the Department of...
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Chemical Research, Leo Pharmaceutical Products (Ballup, Denmark). [32P]dCTP (3000 Ci/mmol), the random priming labeling kit, and the nylon membranes for RNA blots were from Amersham International (Buckinghamshire, United Kingdom).

Cell Culture and Cell Growth Assessment. Human breast cancer cells T-47D and MDA-MB-231 were obtained from the American Type Culture Collection (Rockville, MD) and routinely maintained in DMEM supplemented with 10% FCS and 100 μg/ml gentamicin. Cells were subcultured weekly by incubation at 37°C for 2 mm with 0.0125% trypsin in 0.02% EDTA, followed by the addition of complete medium, washing, and resuspension in fresh medium. Cell cultures were maintained at 37°C in a humidified atmosphere of 5% CO2. In experiments to test the effect of 1,25-dihydroxyvitamin D3 on cell number, T-47D cells were plated out in 24-well plates and allowed to adhere to substrate for 24 h in DMEM containing 10% FCS. Afterward, serum concentration was reduced to 1%, and 1,25-dihydroxyvitamin D3 was added at different concentrations. Cells were incubated for 7 days in the presence of 1,25-dihydroxyvitamin D3 with media changes every 2 days. At the end of the incubation period, the total number of cells was estimated by a fluorimetric protein assay (30). Briefly, cells were washed once with 1X PBS and fixed and permeabilized with pure ethanol at 4°C for 1 h. Sulfhodamine B fluorescent dye was added at a final concentration of 0.4% in 1% acetic acid, and the incubation proceeded for 15 min in the dark at room temperature. Finally, plates were washed extensively with 1% acetic acid, prepared for reading by the addition of 500 μl of 10 mm Tris-HCl (pH 10.4), followed by gentle agitation for 10 mm, and read in a Cytofluor 2350 system (Millipore Corp., Bedford, MA).

Isolation of RNA and Northern Blot Analysis. Approximately 5 × 10⁶ T-47D or MDA-MB 231 breast cancer cells were plated out in 6-well plates and treated with 1,25-dihydroxyvitamin D3, 1,25-dihydroxyvitamin D3 analogues, retinoids, and steroid hormones at the concentrations and for the times indicated. All of these compounds were dissolved in ethanol and added to the growth medium so that the final concentration of ethanol was 0.01% in both control and treated cultures. All procedures involving the use of 1,25-dihydroxyvitamin D3, 1,25-dihydroxyvitamin D3 analogues, and retinoids were carried out in subdued light. Total RNA from the cells was isolated as described by Chomczynski and Sacchi (31) and separated by electrophoresis in 1.2% agarose-formaldehyde gels, followed by blotting onto Hybond nylon filters. The integrity of the RNA in the different samples was ascertained by direct visualization of the stained gel and the nylon membrane under UV light. Filters were prehybridized at 42°C for 2 h in 50% formamide, 5 X SSC (1X SSC = 150 m NaCl, 15 mm sodium citrate, pH 7.0), 2 X Denhardt’s (1X Denhardt’s = 0.02% BSA, 0.02% polyvinylpyrrolidone, and 0.02% Ficoll), 0.1% SDS, and 0.1 mg/ml denatured herring sperm DNA in 0.1 M 1.25-dihydroxyvitamin D3, all-trans-RA, 9-cis-RA, estradiol, dihydrotestosterone (DHT), and dexamethasone (DEX). RNA was electrophoretically fractionated on a 1.2% denaturing agarose gel and transferred to a nylon membrane. The filters were hybridized with a 32P-labeled apoD probe under stringent conditions. Filters were subsequently hybridized to a human actin probe to ascertain the differences in RNA loading among the different samples. In B, suitably exposed autoradiograms were scanned by using a densitometer, and the signals obtained for apoD were corrected to the signals obtained for actin in the corresponding samples. The results are expressed as relative to the corresponding apoD mRNA levels in control cells.

RESULTS

Induction of apoD Gene Expression by 1,25-Dihydroxyvitamin D3 and 1,25-Dihydroxyvitamin D3 Analogues in Breast Cancer Cells. To evaluate the effect of 1,25-dihydroxyvitamin D3 on the expression of the apoD gene in human breast cancer cells, estrogen receptor-positive T-47D cells, which express functional 1,25-dihydroxyvitamin D3 receptor, were treated with 1,25-dihydroxyvitamin D3, and the levels of apoD mRNA were analyzed by Northern blot. As shown in Fig. 1, treatment of T-47D cells for 48 h with 10⁻⁷ M 1,25-dihydroxyvitamin D3 resulted in an accumulation of apoD mRNA of approximately 6-fold over control cells. To compare the effect of 1,25-dihydroxyvitamin D3 on apoD gene expression with other apoD inducers, T-47D cells were also incubated in the presence of RA, which we have described previously as a very strong inducer of apoD expression in estrogen receptor-positive breast cancer cells.

Fig. 1. Effect of 1,25-dihydroxyvitamin D3 and other hormonal regulators on apoD mRNA levels in T-47D human breast cancer cells. In A, RNA blot analysis was performed using 10 μg of total RNA from T-47D cells incubated for 48 h in the presence of 10⁻⁷ M 1,25-dihydroxyvitamin D3, all-trans-RA, 9-cis-RA, estradiol, dihydrotestosterone (DHT), and dexamethasone (DEX). RNA was electrophoretically fractionated on a 1.2% denaturing agarose gel and transferred to a nylon membrane. The filters were hybridized with a 32P-labeled apoD probe under stringent conditions. Filters were subsequently hybridized to a human actin probe to ascertain the differences in RNA loading among the different samples. In B, suitably exposed autoradiograms were scanned by using a densitometer, and the signals obtained for apoD were corrected to the signals obtained for actin in the corresponding samples. The results are expressed as relative to the corresponding apoD mRNA levels in control cells.

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and the steroid hormones dihydrotestosterone and dexamethasone, well known up-regulators of apoD expression in breast and prostate cancer cells (18–21). According to the results shown in Fig. 1, the potency of 1,25-dihydroxyvitamin D₃ as a stimulus for the expression of the apoD gene is intermediate between a maximal induction triggered by the same concentration of all-trans-RA or 9-cis-RA (about 14- and 12-fold, respectively) and the up-regulation observed for the steroid hormones dihydrotestosterone and dexamethasone (about 3-fold in each case; Fig. 1B). However, treatment of T-47D cells with other sex steroids such as estradiol or progesterone (Fig. 1 and data not shown) did not have any effect on apoD gene expression. Similarly, treatment of estrogen receptor-negative breast cancer cells (MDA-MB-231) with 1,25-dihydroxyvitamin D₃ did not have any up-regulatory effect on apoD mRNA levels (data not shown). This observation agrees very well with our previous observations showing that apoD mRNA is induced by RA only in estrogen receptor-positive breast cancer cells (22, 23).

To further analyze this stimulatory effect of 1,25-dihydroxyvitamin D₃ on apoD expression in breast cancer cells, we also examined by Northern blot the dose-dependence and the time course of the 1,25-dihydroxyvitamin D₃-induced enhancement of apoD mRNA levels. As shown in Fig. 2A, treatment of T-47D cells for 48 h with concentrations of 1,25-dihydroxyvitamin D₃ ranging from 10⁻⁶ to 10⁻⁸ M resulted in the accumulation of apoD mRNA. A maximal induction of approximately 7-fold over the control cells was obtained when cells were incubated in the presence of 10⁻⁶ M 1,25-dihydroxyvitamin D₃ (Fig. 2B), whereas concentrations lower than 10⁻⁸ M did not produce significant accumulations of apoD mRNA. This represents a narrow range of 1,25-dihydroxyvitamin D₃ concentration for the up-regulation of the apoD gene, compared to other stimuli capable of stimulating apoD expression, such as retinoid derivatives, androgens, or glucocorticoids (18–22). The time course of the induction of the expression of the apoD gene by 1,25-dihydroxyvitamin D₃ is shown in Fig. 3A. There was a constant increase with time in the steady-state levels of apoD mRNA in T-47D cells treated with 10⁻⁷ M 1,25-dihydroxyvitamin D₃; the maximal effect was reached after 48 h of exposure. Densitometric analysis of the X-ray films revealed that the magnitude of the 1,25-dihydroxyvitamin D₃ induction of apoD mRNA was 5- and 4-fold after 48 and 72 h of treatment, respectively (Fig. 3B).

We next examined the possibility that 1,25-dihydroxyvitamin D₃ analogues could also stimulate the expression of apoD in breast cancer cells. To this purpose, T-47D cells were incubated for 48 h in the presence of 10⁻⁷ M concentrations of analogues with normal C20 stereochemistry (MC 903) or with altered C20 stereochemistry (KH 1060), and total cellular RNAs were purified and analyzed by Northern blot. As shown in Fig. 4, treatment of the T-47D cells with these compounds induced an expression of the apoD gene comparable to that observed with 1,25-dihydroxyvitamin D₃.

**Effect of Cycloheximide and Actinomycin D on apoD mRNA Induction by 1,25-Dihydroxyvitamin D₃.** As a preliminary step to investigate the mechanism of activation of the expression of the apoD gene by 1,25-dihydroxyvitamin D₃, we performed cell culture experiments in the presence of the protein synthesis inhibitor cycloheximide. As shown in Fig. 5, incubation of T-47D cells with cycloheximide (10 μg/ml, added 45 min before 1,25-dihydroxyvitamin D₃) extensively prevented the 1,25-dihydroxyvitamin D₃-induced accumulation of apoD mRNA. By contrast, and confirming previous observations (22), the induction of apoD mRNA by RA does not depend on new protein synthesis (Fig. 5). In an attempt to examine whether the stimulatory effect of 1,25-dihydroxyvitamin D₃ on apoD mRNA was due to an increase of the apoD mRNA half-life, T-47D cells were preincubated with 1,25-dihydroxyvitamin D₃, and then transferred to a medium containing actinomycin D, in the presence or absence of 1,25-dihydroxyvitamin D₃. Finally, total cellular RNAs were isolated at different times and analyzed by Northern blot. As shown in Fig. 6, there were not significant differences in the amount of apoD mRNA in T-47D cells. In A, T-47D cells were cultured for 48 h in the presence of different concentrations of 1,25-dihydroxyvitamin D₃, and total RNA was analyzed by Northern blot, as described in the legend to Fig. 1. Filters were hybridized consecutively with labeled probes for apoD and actin. In B, autoradiograms were scanned by densitometry, and the values of apoD mRNA in each sample were corrected for differences of total RNA/lane. The results are expressed as relative to the values of control cells.
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Enhancement of apoD Levels by Vitamin D₃ in T-47D Breast Cancer Cells. To evaluate the possibility that the stimulatory effect of 1,25-dihydroxyvitamin D₃ on apoD mRNA levels in breast cancer cells was also reflected at the protein level, we performed Western blot analysis of cell extracts from T-47D cells treated with 10⁻⁶ M 1,25-dihydroxyvitamin D₃ for 48 h. As illustrated in Fig. 7, the presence of an immunoreactive band of the expected molecular size (approximately M₉ 24,000) could be clearly detected in 1,25-dihydroxyvitamin D₃-treated T-47D cell extracts, compared to control cells. Also in agreement with the above-described mRNA analysis, the observed increase in apoD protein levels was time and dose dependent (data not shown).

Correlation between Growth Inhibition and apoD Expression Induced by 1,25-Dihydroxyvitamin D₃ in Breast Cancer Cells. Finally, in this work, we have studied whether the inductive effect of 1,25-dihydroxyvitamin D₃ on apoD gene expression was correlated with variations in the proliferation rate of T-47D breast cancer cells or in their status of differentiation. As shown in Fig. 8, treatment of T-47D cells in exponential growth phase with 1,25-dihydroxyvitamin D₃ resulted in a decrease in cell number that was concentration dependent. Thus, incubation of T-47D cells in the presence of a 10⁻⁶ M concentration of 1,25-dihydroxyvitamin D₃ caused a maximal growth inhibition, with an 80% decrease in cell number after 7 days, whereas concentrations of 1,25-dihydroxyvitamin D₃ lower than 10⁻⁹ M did not have a significant effect on cell proliferation. Similar results were obtained in previous studies directed to analyze the effect of 1,25-dihydroxyvitamin D₃ on proliferation of these breast cancer cells (32). Furthermore, we performed assays aimed to detect the putative...
appearance of a more differentiated phenotype in T-47D cells treated with 1,25-dihydroxyvitamin D3. We have shown previously that RA-treated T-47D cells are able to synthesize lipid and protein components characteristic of differentiated breast epithelial cells (22). However, and despite the fact that there was an appreciable change in morphology (Fig. 9), 1,25-dihydroxyvitamin D3-treated T-47D cells did not produce significant amounts of lipids or milk protein components compared to control cells (data not shown).

DISCUSSION

The present data show that the expression of apoD, a member of the lipocalin family of proteins that is overproduced by a subtype of breast carcinomas, is strongly induced by 1,25-dihydroxyvitamin D3 and 1,25-dihydroxyvitamin D3 analogues in T-47D breast cancer cells. The induction of the apoD gene is both time and dose dependent, but within the range of physiological concentrations of 1,25-dihydroxyvitamin D (24, 25). In addition, this up-regulation of apoD mRNA levels could be inhibited by cycloheximide, suggesting that new protein synthesis is necessary for the induction of the apoD gene expression. The increase by 1,25-dihydroxyvitamin D3 in the steady-state level of apoD mRNA was also reflected at the protein level as an increase of immunoreactive apoD in the 1,25-dihydroxyvitamin D3-treated breast cancer cells. On the basis of these results, 1,25-dihydroxyvitamin D3 should be included among the stimuli previously considered as major causative agents of apoD-increased expression in breast cancer cells, such as retinoids, androgens, and glucocorticoids (18–23). A comparison of the relative strengths of these compounds suggests that 1,25-dihydroxyvitamin D3 may be considered as an apoD gene inducer of intermediate potency, because the magnitude of this effect is higher than that resulting from treatment of human breast cancer cells with androgens and glucocorticoids but lower than the effects observed for retinoids (22, 23). Altogether, the data currently available on apoD regulation in breast cancer cells suggest that a complex interaction may exist in breast tumors between steroid hormones, retinoids, 1,25-dihydroxyvitamin D3, and their receptors to modulate apoD expression, thus resulting in the highly heterogeneous pattern of expression of apoD in breast tumor specimens (16, 33).

In this work, we have also found that this increased expression of the apoD gene in T-47D cells in response to 1,25-dihydroxyvitamin D3 treatment is accompanied by an inhibition of cell proliferation as well as by a marked change in the morphology of the cells. These results agree well with previous data from different groups showing that the biologically active 1,25-dihydroxyvitamin D metabolite, 1,25-dihydroxy1,25-dihydroxyvitamin D3, is an important modulator of cellular proliferation and differentiation for many normal and malignant cell types, including breast cancer cells (24–29). However, we did not observe any induction of apoD in response to 1,25-dihydroxyvitamin D3 treatment in estrogen receptor-negative MDA-MB-231 cells, which are not sensitive to the antiproliferative effects of 1,25-dihydroxyvitamin D3 (34). These observations, together with previous findings showing that apoD induction by RA is also comitant with cell growth inhibition and differentiation in estrogen receptor-positive breast cancer cells (22, 23), give additional support
to the proposal that apoD is a biochemical marker of growth arrest and cell differentiation in breast cancer cells. This proposal is also consistent with results indicating that apoD transcription is only detected in nonproliferating fibroblast cultures (17), as well as with clinical studies showing that apoD is overproduced by breast carcinomas with a high grade of differentiation (16). Nevertheless, it is remarkable that the status of functional differentiation elicited by 1,25-dihydroxyvitamin D3 in breast cancer cells seems to be distinct from the phenotypic changes caused by RA (22, 23), although in both cases differentiation is accompanied by an increase in the expression of the apoD gene. Thus, and despite a remarkable change in morphology in 1,25-dihydroxyvitamin D3-treated T-47D cells, we failed to see other signs of functional differentiation, such as the synthesis of lipid and protein components characteristic of fully differentiated and secretory breast epithelial cells.

In this work, we have also examined the possibility that some 1,25-dihydroxyvitamin D3 analogues presently used both in vitro and in vivo as inhibitors of breast cancer cell growth (35–38) could elicit a response of the apoD gene similar to that observed with 1,25-dihydroxyvitamin D3 in T-47D cells. Interestingly, compounds with a stereochemical configuration at C20 identical to that of 1,25-dihydroxyvitamin D3 such as MC 903, as well as 20-epi-1,25-dihydroxyvitamin D3 analogues like KH 1060, displayed also a potent inductive effect on apoD expression. Taken together, these findings strongly suggest that the mechanisms controlling apoD synthesis, growth arrest, and differentiation of breast cancer cells are not a specific response to 1,25-dihydroxyvitamin D3 and can be extended to structurally diverse 1,25-dihydroxyvitamin D3 analogues. This observation may be of interest if we consider that some of these derivatives possess potent effects as regulators of growth and differentiation of breast cancer cells but have a decreased risk of inducing calcemic side effects, which is currently a major limitation for the clinical usefulness of 1,25-dihydroxyvitamin D3 (24, 25).

At present, the molecular mechanisms responsible for the marked up-regulatory effect of 1,25-dihydroxyvitamin D3 and its synthetic analogues on apoD mRNA in T-47D cells are unknown. However, it is tempting to speculate that they are mediated by the nuclear VDR, which is a member of the steroid-thyroid hormone receptor superfamily (39). In support of this hypothesis, VDR is expressed at significant levels in T-47D breast cancer cells, whereas it is virtually undetectable in MDA-MB-231 cells (34), in which no effect on apoD expression is observed after 1,25-dihydroxyvitamin D3 treatment. Furthermore, the presence of high affinity intracellular VDRs has been clearly demonstrated in a significant percentage of human breast carcinomas (40, 41). Upon binding to its ligand, VDR could regulate the transcription of the apoD gene through binding to specific regulatory elements present in its promoter region. However, it is well established that besides genomic effects, 1,25-dihydroxyvitamin D3 is also responsible for changes in signal transduction pathways, including regulation of intracellular calcium concentrations, protein kinase C activity, or formation of cyclic nucleotides (25). These nongenomic changes could also have an effect on the observed induction of apoD expression in breast cancer cells. Structural and functional characterization of the promoter region of the apoD gene, now in progress, will be very useful to further clarify the precise role of 1,25-dihydroxyvitamin D3 and their derivatives in the induction of this gene in breast cancer cells. Finally, the identification of biochemical markers like apoD that are modulated by vitamin D derivatives and whose expression correlates with the cessation of proliferation and the induction of differentiation of breast cancer cells could be of interest in the context of present studies on the value of these compounds as new chemotherapeutic agents in the prevention of breast cancer.

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