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GRANT NUMBER DAMD17-94-J-4100

TITLE: Regulation of Retinoid Responsiveness in Mammary Carcinoma Cells

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REPORT DATE: September 1996

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1996	Annual (1 Sep 95 - 31 Aug 96)

4. TITLE AND SUBTITLE
Regulation of Retinoid Responsiveness in Mammary Carcinoma Cells

5. FUNDING NUMBERS
DAMD17-94-J-4100

6. AUTHOR(S)
David A. Talmage, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Columbia University
New York, New York 10032

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 2170-25012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

The data presented in this annual report (for award DAMD17-94-J-4100) support our hypothesis for a mechanism of retinoid-induced growth arrest of human breast cancer cells. Specifically, we believe that retinoids inhibit human breast cancer cell proliferation by inducing a state of functional mitogen deprivation. Kinetic and quantitative changes in cell cycle progression and gene expression following retinoic acid treatment of the estrogen dependent, T-47D breast cancer cell line, are consistent with retinoic acid inducing a block in the treated cells' ability to respond to external mitogenic signals. The earliest measurable effect of retinoic acid was repression of the induction of immediate early response gene expression by epidermal growth factor. This was followed by decreased cyclin D1 expression and a failure of treated cells to enter the S phase of the cell cycle. Our interpretation of these data is that retinoic acid induces a state of functional mitogen deprivation in breast cancer cells. Preliminary data indicate that this effect involves disruption of signaling by the novel lipid kinase, phosphatidyl inositol 3-kinase. By manipulating the expression of the cellular retinoic acid binding protein, type II, the cellular retinol binding protein and the nuclear retinoic acid receptor alpha, we also have shown that expression of these mediators of vitamin A action is sufficient to exert growth inhibitory effects on human breast cancer cells in culture.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer	18
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

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David Ardo 10/31/96

PI - Signature Date

Table of Contents

Introduction.

The anti-tumorigenic action of retinoids can be accounted for both by inhibition of cell proliferation and induction of cell differentiation (Lotan, 1996). Links between dietary vitamin A status and breast cancer development has resulted in clinical trials to evaluate the efficacy of retinoids in the prevention and therapy of breast cancer (Costa, 1993). The ability of retinoic acid (RA) to affect a target cell depends on the action of cytoplasmic retinoid-binding proteins and nuclear, ligand-dependent transcription factors (primarily the RARs) (Gudas et al., 1994; Mangelsdorf et al., 1994). Expression and activity of these retinoid binding proteins and nuclear receptors may be critical in regulating the biological response and tissue sensitivity to retinoids at the cellular and molecular level (Gudas et al., 1994). Understanding the cellular and molecular mechanism of retinoid action could provide a basis for more effective retinoid therapy of breast cancer and a greater understanding of the role of retinoids in the etiology of breast tumor development.

Retinoids inhibit the proliferation of hormone-dependent, but not hormone-independent human breast carcinoma (HBC) cell lines (Liu et al., 1996; Wilcken et al., 1996). Proliferation of normal and malignant mammary epithelial cells is regulated by the interplay of hormonal and growth factor signalling pathways that regulate cell cycle progression (Dickson and Lippman, 1995). Two HBC cell lines are used in our study. The steroid hormone-dependent T-47D cell line, originated from a ductal carcinoma and the hormone-independent MDA-MB-231 line, originated from an adenocarcinoma. The experiments in this project will provide important new information on the biological response of HBC cells to retinoids. We hypothesize that RA inhibits HBC cell proliferation by inducing a state of functional mitogen deprivation.

Summary of previous findings.

Task 1. Previously, we used northern blot analysis to compare the expression cellular retinol- and retinoic acid binding proteins and nuclear retinoid receptors between T-47D (RA-sensitive) and MDA-MB-231 (RA-insensitive) cells. Our work focused on the role of RARa, CRABP-II and CRBP in mediating the antiproliferative effects of retinoids because: (a) RARα and CRABP-II expression vary most between RA-sensitive and RA-resistant cells; (b) in vivo RARα expression is inversely related to the severity of neoplastic breast disease; (c) we have established that RARα can impart RA sensitivity on RA-resistant cells and inhibits transformation by blocking immediate early gene expression (Talmage and Lackey, 1992; Talmage and Listerud, 1994); (d) CRBP expression varies during hormonally regulated mammary development (Morrison and Leder, 1994) and we have demonstrated that similar regulation alters retinol (ROL) responsiveness in the cervix (Tannous-Khuri et al., 1994; Tannous-Khuri and Talmage, 1997).

Task 2. RA treated T-47D cells arrest in the G1 phase of the cell cycle. A more limited response was seen in cells treated with the RA precursor, retinol (ROL). Arrest was associated with decreased expression of a number of genes whose products regulate cell cycle progression. We utilize these genes as molecular markers to explore the mechanistic details of retinoid induced growth arrest.

Task 3. Cytokines, such as interferon-gamma (IFNγ) have attracted attention for their potential to synergize with retinoids as anti-tumor agents. In earlier studies, we found that IFNγ alone had no effect on MDA-MB-231 cell proliferation, but at high concentrations did slow T-47D cell growth. IFNγ synergized with retinol and with low concentrations of RA to rapidly and totally arrest T-47D cell proliferation. No synergy between IFNγ and RA was seen in MDA-MB-231 cells.

Task 4. Previously we reported the construction of appropriate sense and anti-sense expression vectors to allow us to manipulate expression of CRABP I and II, CRBP, and RARs α, β and γ. These have been transfected, alone and in various combinations into T-47D and MDA-MB-231 cells. Preliminary results from three of these transfectants will be reported below.

Methods and Results, Year 2.

Task 1. Task 1 was completed during the first year of this project.

Task 2. Retinoids arrest T-47D cell proliferation. Based on flow cytometry of propidium iodide stained cells, we concluded that RA-induced growth arrest of T-47D cells was associated with an accumulation of cells in the Go/G1 phase of the cell cycle. Arrest in Go/G1 typically is associated with either a block to transit through the restriction point in late G1, or to withdrawal of cells from the cell cycle. Withdrawal can reflect either quiescence, terminal differentiation or programmed cell death. The observation that RA-induced growth arrest was fully reversible, coupled with the lack of apparent fragmented nuclear DNA in the flow cytometry profiles, rules out either terminal differentiation or apoptotic cell death. To determine if RA-induced arrest occurred at the G1/S restriction point, or resulted from induction of quiescence, we measured the effect of RA on the expression of genes involved in progression through early G1 (the immediate early response genes, c-fos, c-jun and c-myc) or through mid-to-late G1 (cyclin D1). In addition we compared the temporal relationship between RA alteration of the expression of these genes, with that of mitogen deprivation (following serum withdrawal).

Accumulation of cells in Go/G1 phase and a concomitant decrease in DNA synthesis was first evident between 16 to 20 hours after RA addition, compared to equivalent changes seen 8 hours after serum withdrawal (Figure 1). The 8 hour delay in the RA response compared to mitogen withdrawal is consistent with the need for sufficient expression of RA-induced gene products that inhibit cell cycle progression. The decrease in the percent of treated cells in S phase was preceded by a fall in the expression of cyclin D1. Decreased D1 mRNA levels were apparent at 6 and 9 hours after serum withdrawal or RA addition, respectively. In contrast, to these delayed responses, when we measured mitogen induction of c-fos, c-jun and c-myc expression by northern blot analyses, we found that a 3-6 hour treatment with RA repressed immediate early gene (IEG) induction. This effect of RA required new protein synthesis since it was prevented by co-treatment with cyclohexamide.

We interpret these kinetic analyses as follows: RA-induces the expression of a gene or genes whose product inhibits cell cycle progression. This, as yet unidentified RA-induced gene inhibits the expression of the AP-1 (Fos/Jun) and/or Myc transcription factors that are required for expression of the G1 cyclins. Failure to express cyclin D1 prevents cells from progressing through G1 and results in cell cycle arrest.

We have demonstrated that the RA-induced block of mitogen-induced cell cycle progression is associated with an accumulation of cells with a G_0/G_1 DNA content and that RA repressed mitogen induction of c-fos, c-jun and c-myc immediate early response genes. The importance of these molecular markers for RA action is supported by the observation that in MDA-MB-231 cells which are resistant to the anti-proliferative action of retinoids, RA treatment had no effect on the level of mitogen-induced immediate early gene expression (data not shown).

Ligand binding to the epidermal growth factor receptor (EGFR) by EGF induces receptor dimerization with other erbB receptors such as erbB2 and erbB3. Following ligand-dependent activation, receptor tyrosine kinases autophosphorylate and the resulting cytoplasmic phosphotyrosines bind to and activate signaling molecules such as the Ras GTP-binding protein and phosphatidylinositol 3-kinase (PtdIns 3-kinase). Activation of each of these pathways leads to activation of cytoplasmic and nuclear serine/threonine protein kinases which alter the expression of genes intimately involved in cell cycle regulation. To investigate further which growth factor activated signaling pathways are inhibited by RA, we have begun to assay a series of protein kinases that are known to be activated by mitogenic stimuli. The results of these initial studies are shown in Figure 2. EGF induction of p70 S6 kinase, Jun N-terminal kinases (JNK) and p38 kinase was attenuated by RA pretreatment of T-47D cells. Surprisingly, no EGF stimulation of the mitogen activated protein kinase (MAPK) was seen..

JNK, p38 and $p70^{s6k}$ can be activated following receptor tyrosine kinase activation of PtdIns 3-kinase. RA inhibits transformation of rat fibroblasts by preventing oncogene signaling to the c-fos promoter (Talmage and Listerud, 1994). In these cells, RA inhibits c-fos transcriptional activation by preventing PtdIns 3-kinase-dependent activation of JNK (Chen et al., submitted). If RA inhibits growth of T-47D cells in a similar fashion, then inhibition of this pathway by other agents should also inhibit proliferation. Therefore, we compared the antiproliferative effects of wortmannin, a PtdIns 3-kinase inhibitor, and rapamycin an immunosupressive compound that can inhibit proliferation by preventing activation of $p70^{s6k}$ with the effect of RA on proliferation, cell cycle progression and IEG induction (Figure 3).

All three agents inhibit cell proliferation (Figure 3a) and arrest cells in G_0/G1 (Figure 3b). Wortmannin and RA, but not rapamycin, also prevent mitogen activation of IEG expression (Figure 3c). Therefore, repression of EGF-induced c-fos and c-jun gene expression distinguishes the cell cycle arrest and antiproliferative effect of RA and wortmannin from rapamycin. Since MDA-MB-231 cells are resistant to RA, we determined if these cells were also resistant to wortmannin-induced inhibition of proliferation. Wortmannin inhibited neither proliferation nor mitogen induction of IEGs in MDA-MB-231 cells (data not shown). Taken together, these data support a critical role for PtdIns 3-kinase in T-47D proliferation and demonstrate that this signaling pathway is sensitive to RA-inhibition. The resistance of the MDA-MB-231 cell line to the anti-

proliferative effects of RA might, in part, reflect the minor mitogenic role that PtdIns 3-kinase signaling plays in these cells.

Task 3. During year 1 of this project, we demonstrated that although interferon gamma can enhance the anti-proliferative response of T-47D cells to retinoids, this effect was limited to cells that are already sensitive to retinoids. Therefore it seemed unlikely that IFNγ could act by increasing retinoid signaling in resistant lines. No further experiments with IFNγ are planned.

Task 4. We have demonstrated that the inhibition of IEG expression and cell cycle progression correlate with the growth-inhibitory effect of both RA and ROL on T-47D cells (Figure 4). The antiproliferative effect of ROL is consistent with its role as a precursor for RA (Figure 4a). The lower sensitivity of T-47D cells to ROL compared to RA is consistent with the low uptake of ROL by these cells (data shown in last year's progress report). The magnitude of the effects of ROL of cell cycle changes (Figure 4b) and IEG expression (figure 4c) parallels the degree of ROL-induced growth inhibition further supporting a mechanistic link between the anti-proliferative effects of retinoids and modulation of immediate early gene expression.

We have proposed that CRBP is necessary for efficient retinol utilization by target cells and that the lack of CRBP expression in T-47D cells might account for the minor effect of ROL on these cells compared to RA. Therefore, we reasoned that transfecting T-47D cells with an expression vector encoding CRBP should increase the ability of retinol to inhibit proliferation. We isolated and have completed proliferation assays on T-47D-CRBP expressing cells (Figure 5). Although the relative decrease in cell number in response to ROL treatment is not greater in the T-47D-CRBP cells compared to the parental line, both the magnitude and duration of decreased progression into S phase of ROL-treated T-47D-CRBP cells is greater than in ROL-treated parental cells (Figure 5b), and the total cell number of untreated T-47D-CRBP cells after 5 days is lower than in the untreated parental cells (Figure 5a). This slower rate of growth and prolonged response to ROL might reflect a CRBP-mediated change in the uptake and/or utilization of ROL from the media. This conclusion is supported indirectly by preliminary data indicating that EGF is unable to activate JNK and p38 kinases in T-47D-CRBP cells.

Studies with the RARα selective agonist, Am580, indicate that this receptor isoform mediates the anti-proliferative effect of RA (Cho et al., 1997). This conclusion is supported by demonstrating that pretreating T-47D cells with Ro41-5253, an RARα selective antagonist, abrogated the ROL-induced repression of c-fos induction in T-47D-CRBP cells and causes a superinduction of c-fos in the parental cells. Since the RA-resistant MDA-MB-231 cell line expresses only low levels of RARα we transfected these cells with a vector encoding the human RARα cDNA. These cells do not acquire RA sensitivity, indicating that the under-expression of RARα is not the sole cause of RA-resistance. MDA-MB-231 do not take up RA from the culture media efficiently. We also found that these cells do not express CRABP II (year 1, Task 1). Since CRABPs might influence the uptake and metabolism of serum retinoids, we transfected both MDA-MB-231 and RARα expressing MDA-MB-231 cells with CRABP II expression vectors. Despite expression of both the cytoplasmic retinoic acid binding protein and

the nuclear receptor, proliferation of these cells was still not affected by RA treatment (Figure 6). However, in a manner analogous to CRBP expression in T-47D cells, MDA-MB-231 cells that express CRABP II and RARα have reduced basal proliferation rates. In both instances, determination of whether these decreased rates of proliferation reflect increased uptake and utilization of serum retinoids awaits experimental confirmation.

Conclusion

The data presented in this annual report support our hypothesis for a mechanism of RA-induced growth inhibition of HBC cells. Kinetic and quantitative changes in cell cycle progression and gene expression following RA treatment are consistent with RA inducing a block to external mitogenic signals. The earliest measurable effect of RA was repression of the induction of immediate early gene expression, followed by decreased cyclin D1 expression and failure to enter S phase. Our interpretation of these data is that RA induces a state of functional mitogen deprivation in HBC cells. Preliminary data indicate that this effect might involve disruption of PtdIns 3-kinase signaling. By manipulating the expression of CRABP II, RARa and CRBP we have shown that expression of these mediators of retinoid action is sufficient to exert growth inhibitory effects in the presence of serum.

Future work: We have described molecular and biochemical effects of RA which correlate with RA-induced growth arrest of HBC cells. Our goal is to identify in more detail the mitogenic signalling pathways that are disrupted by RA treatment. We have recently demonstrated that RA treatment of serum-starved T-47D cells blocks EGF-induced tyrosine phosphorylation of the EGFR but does not affect EGFR protein level. We are currently investigating the potential role of PKCα in mediating the anti-mitogenic effects of RA, ROL and RAR-selective agonists (Cho et al., 1997). We will perform parallel experiments using T-47D-CRBP cells and MDA-MB-231-RARα-CRABP-II cells to determine the effects of constitutive expression of retinoid binding proteins and receptors on EGF-induced mitogenic signaling. To extend our observations on RA-inhibition of IEG expression, we are currently measuring IEG and AP-1 promoter activation (by conversion of chloramphenicol acetyl transferase) in RA-treated T-47D and MDA-MB-231 cells.

References.

Chen, Y., Z. Wang, M. Listerud, R. Freund and D.A. Talmage, Retinoic acid inhibits transformation by preventing phosphatidylinositol 3-kinase dependent activation of the c-fos promoter. Submitted.

Cho, Y.C., A.P. Tighe and D.A. Talmage, Retinoic acid induced growth arrest of human breast carcinoma cells requires protein kinase Cα expression and activity. Journal of Cellular Physiology, in press.

Costa, A. Breast Cancer Chemoprevention. European Journal of Cancer, 1993, 29A:1031-1039.

Dickson, R.B. and M.E. Lippman, Growth factors in breast cancer. Endocrine Reviews, 16:559-589, 1995.

Gudas, L.J., M.B. Sporn and A.B. Roberts.1994. in The Retinoids: Biology, Chemistry and Medicine. 2nd Ed. M. Sporn, A. Roberts and D.Goodman eds., Raven Press Ltd. New York pp. 443-520.

Han, E.K., A. Sgambato, W. Jiang, Y. Zhang, R.M. Santella, Y.Doki, A. Cacace, I. Schieren and I.B. Weinstein. Stable overexpression of cyclin D1 in a human mammary epithelial cell line prolongs the S-phase and inhibits growth, 1995, :953-961.

Liu, Y., M. Lee, H. Wang, Y. Li, Y. Hashimoto, M. Klaus, J.C. Reed and X. Zhang. Retinoic acid receptor β mediates the growth-inhibitory effect of retinoic acid by promoting apoptosis in human breast cancer cells. Molecular and Cellular Biology, 1996, 16:1138-1149.

Lotan, R. Retinoids in cancer chemoprevention. FASEB Journal, 1996, 10:1031-1039.

Mangelsdorf, D., L. Umesono and R. Evans. 1994 in The Retinoids: Biology, Chemistry and Medicine. 2nd Ed. M. Sporn, A. Roberts and D. Goodman eds., Raven Press Ltd. New York pp. 319-349.

Talmage, D.A. and Lackey, R.S. Retinoic acid receptor-α suppresses polyoma virus transformation and c-fos expression in rat fibroblasts. Oncogene, 1992, 7:1837-1845.

Talmage, D.A. and Listerud, M.D. Retinoic acid suppresses polyoma virus transformation by inhibiting transcription of the c-fos protooncogene. Oncogene, 1994, 9:3557-3563.

Tannous-Khuri, L., Hillemanns, P., Rajan, N., Wright, T.C. and Talmage, D.A. Expression of cellular retinol- and cellular retinoic acid-binding proteins in the rat cervical epithelium is regulated by endocrine stimuli during normal squamous metaplasia. American Journal of Pathology, 1994, 144:148-159.

Tannous-Khuri, L. and Talmage, D.A. Cellular retinol-binding protein is required for retinol responsiveness in rat cervical epithelial cells. Expt. Cell Res. 1997, 230:38-44.

Watts, C.K.W., N.R.C. Wilcken, J.A. Hamilton, K.J.E. Sweeney, E.A. Musgrove, and R.L. Sutherland. Mechanisms of antiestrogen, progestin/antiprogestin, and retinoid inhibition of cell cycle progression in breast cancer cells. 1996. in Hormone Dependent Cancer. J.R.Pasqualini and B.S. Katzenellenbogen eds., Marcel Dekker, New York pp. 119-140.

Wilcken, N.R.C., B. Sarcevic, E. Musgrove, and R.L. Sutherland. Differential effects of retinoids and antiestrogens on cell cycle progression and cell cycle regulatory genes in human breast cancer cells. Cell Growth and Differentiation, 1996, 7:65-74.

Appendices.

Figures 1-6, Attached.

Figure Legends.

Figure 1.

a. T-47D cells were left untreated (10%), or treated with 10^{-6}M RA (RA) or serum-starved (0.5%). After the indicated times cells were harvested and assayed for cyclin D1 mRNA by northern blotting as previously described (Talmage and Listerud, 1994). Briefly, total RNA (15ug each) isolated from untreated, RA-treated and serum-starved cells was separated on 1.2% agarose gels, transferred to nylon membranes and hybridized to ^{32}P-labeled cRNA probe for cyclin D1. To determine that equal amounts of RNA were used, a parallel filter was probed with a cRNA probe for the ribosomal protein L30.

b. Temporal changes in T-47D cell cycle phase distribution following RA treatment (10^{-6} M) or serum deprivation (0.5% FBS). Exponential cultures of T-47D cells were plated in 10 cm dishes containing phenol red-free RPMI medium plus 5% FBS. At time zero cells were left untreated (control), treated with 10^{-6}M RA or serum starved (0.25% FBS). At the indicated times cells were trypsinized, collected and resuspended in 0.2mg/ml of propidium iodide. Cell cycle distribution was measured by fluorescence activated cell sorting (FACS) (Han et al., 1995). Raw data was analyzed with the Mod-Fit software program to yield percents of cells in G1, S or G2.

Figure 2. Serum starved T-47D cells were stimulated with 10 ng/ml EGF for 30 min following a 24 hr pre-treatment with 10^{-6} M RA, or ethanol control (C). Lysates (200ug protein) were immunoprecipitated with specific antibodies recognizing the indicated kinases. After washing, immune complexes were incubated with ^{32}P-γ–ATP and peptide substrates. Kinase activity was quantified by scintillation counting. The data are presented as the fold stimulation over activities in serum starved cells.

Figure 3.

a. A total of 10.5 x 10^4 cells were seeded in 60 mm dishes and treated with 10^{-7} M RA, 10^{-6} M wortmannin or 20 ng/ml rapamycin for 5 days. Media and experimental treatments were renewed every 72 hours. On day 5, cells were trypsinized and counted with a hemocytometer. Cell viability was determined by trypan blue exclusion (90+% at all times). Error bars show standard deviations.

b. Serum starved T-47D cells were stimulated to re-enter the cell cycle with 10 ng/ml EGF and 10% FBS, with or without 10^{-6} M wortmannin. Asynchronously growing T-47D cells were cultured in 5% FBS with or without 10^{-6} M RA or 20 ng/ml rapamycin. For the release experiment, cell cycle phase distribution was determined by FACS analysis after 24 hr. For RA or rapamycin treated cells, FACS analysis was performed after 44 hr. FACS analysis and cell cycle phase distribution were determined as described in the legend to Figure 1b.

. c. Northern blot analysis of total RNA (15ug each) isolated from serum-starved T-47D cells 30 min after stimulation with 10 ng/ml EGF. Cells were pretreated with 10^{-6} M

RA, 10^{-6} M wortmannin or 20 ng/ml rapamycin for 16 hr prior to EGF stimulation. Filters were probed with ^{32}P-cRNAs as described in the legend to Figure 1a.

Figure 4.

a. 15 x 10^4 cells were plated on 60 mm dishes and treated with 10^{-6} M RA or 10^{-6} M ROL. Cell numbers and viability were measured on days 0, 1, 2, 3 and 5 as described in the legend to Figure 3a.. Viability was 90+% at all times. Error bars show standard deviations.

b. Serum starved T-47D cells were stimulated to re-enter the cell cycle with 10 ng/ml EGF and 10% fbs at time 0. After 5 hr, 10^{-6} M RA or 10^{-6} M ROL was added and cell cycle phase distribution determined by FACS after 24 and 72 hr as described in the legend to Figure 1b.

c. Total RNA was isolated from serum starved T-47D cells 30, 60, 90 and 120 min after stimulation with 10 ng/ml EGF. Cells were treated with 10^{-6} M RA or 10^{-6} M retinol (ROL) for 16 hr prior to EGF stimulation. Northern blot analysis was performed as described in the legend to Figure 1a.

Figure 5.

a. 15 x 10^4 cells were plated on 60mm dishes and treated with 10^{-6}M ROL. Cell numbers and viability were measured on days 0 and 5 as described in the legend to Figure 3a. Viability was 90+% at all times. The horizontal dashed line represents cell number on day 0. Error bars show standard deviations.

b. Temporal changes in T-47D and T-47D-CRBP cell cycle phase distribution following ROL treatment (10^{-7}M). At the indicated times, FACS analysis and cell cycle phase distribution were determined as described in the legend to Figure 1b.

Figure 6.

15 x 10^4 cells were plated on 60mm dishes and treated with 10^{-6}M RA. Cell numbers and viability were measured on days 0 and 5 as described in the legend to Figure 3a. Viability was 90+% at all times. Error bars show standard deviations.

Figure 1a.

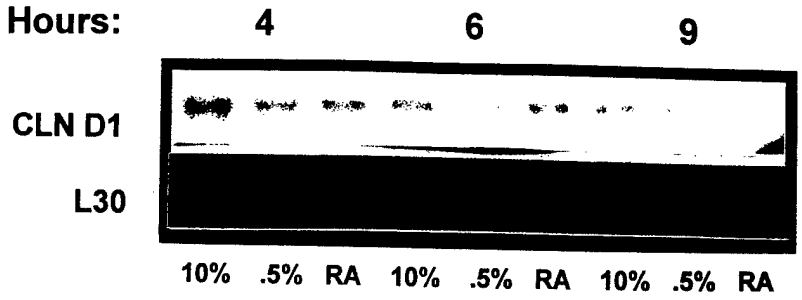


Figure 1b.

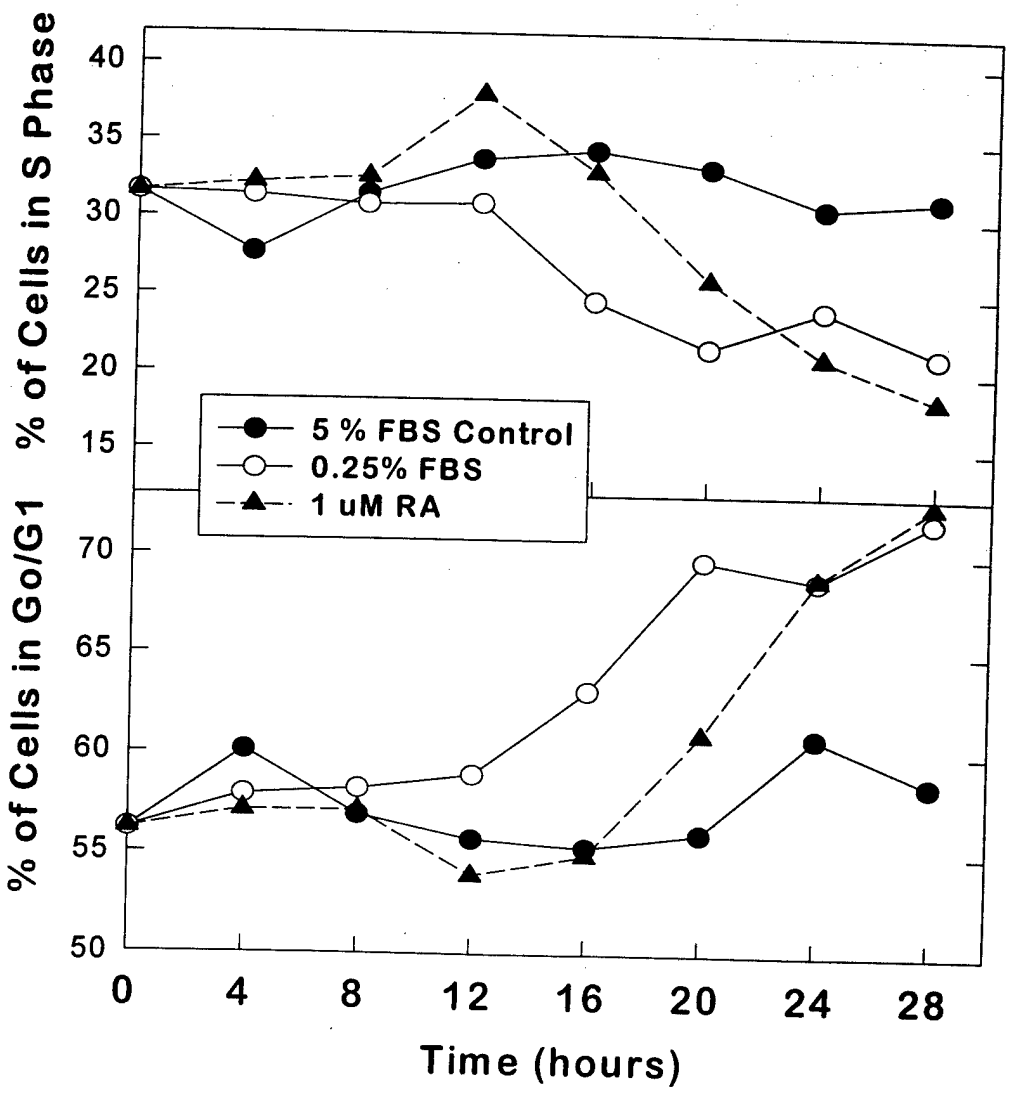


Figure 2. RA EFFECT ON EGF-STIMULATED MAPK AND JNK PATHWAYS

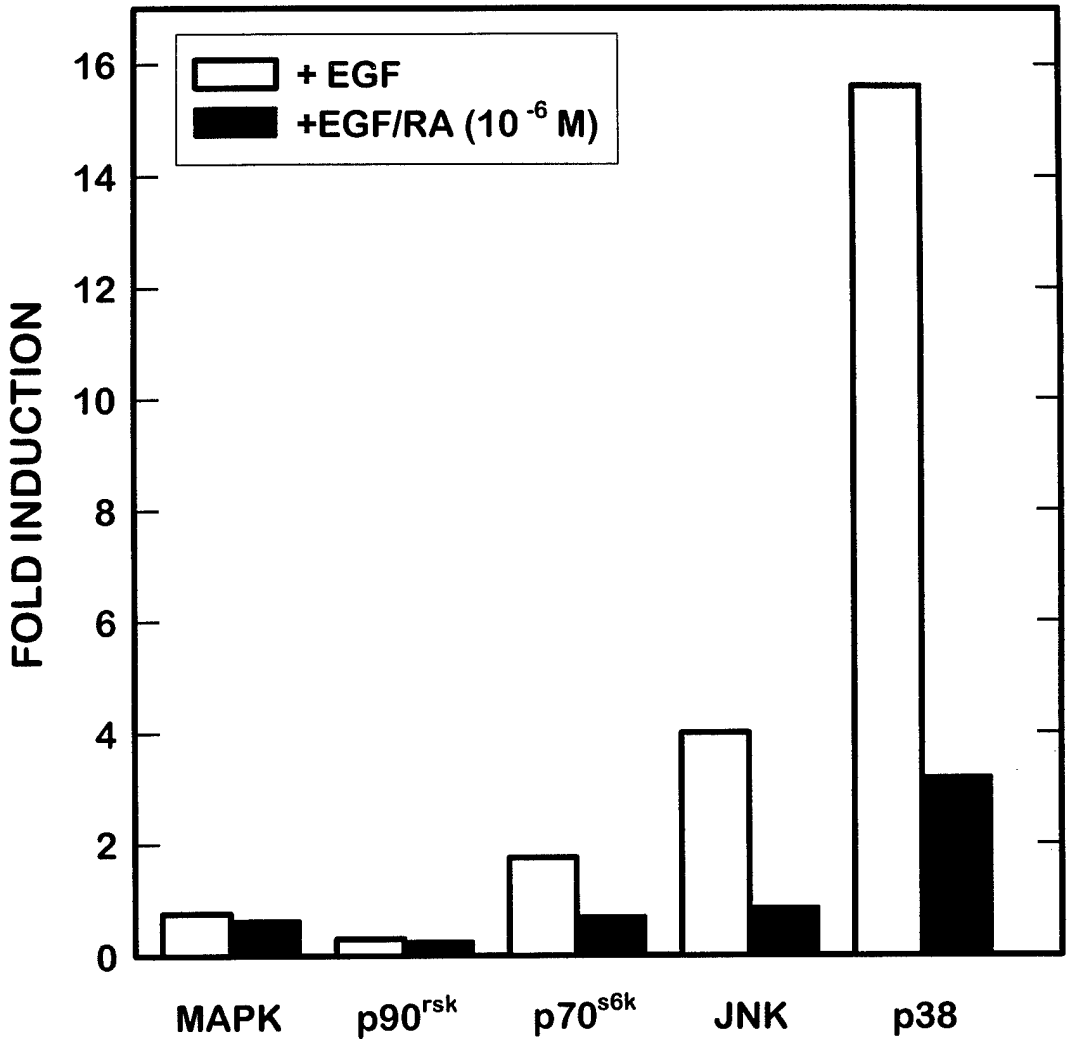


Figure 3a.

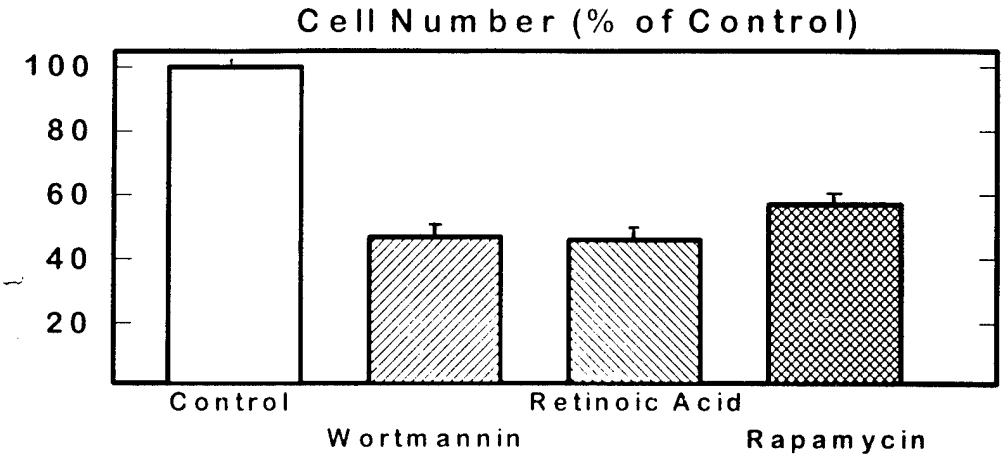


Figure 3b.

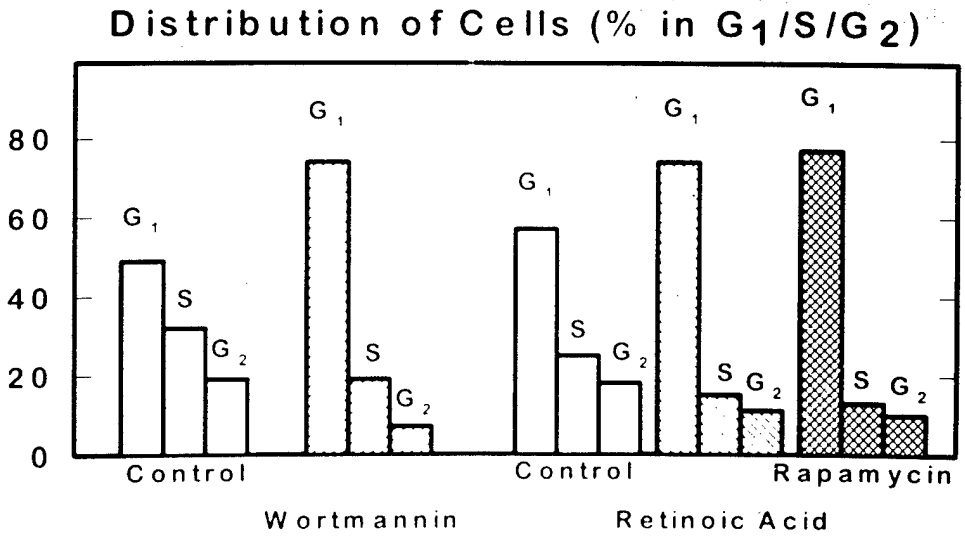


Figure 3c.

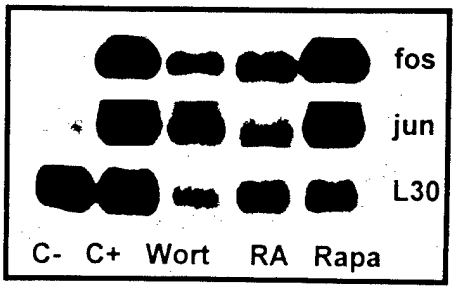
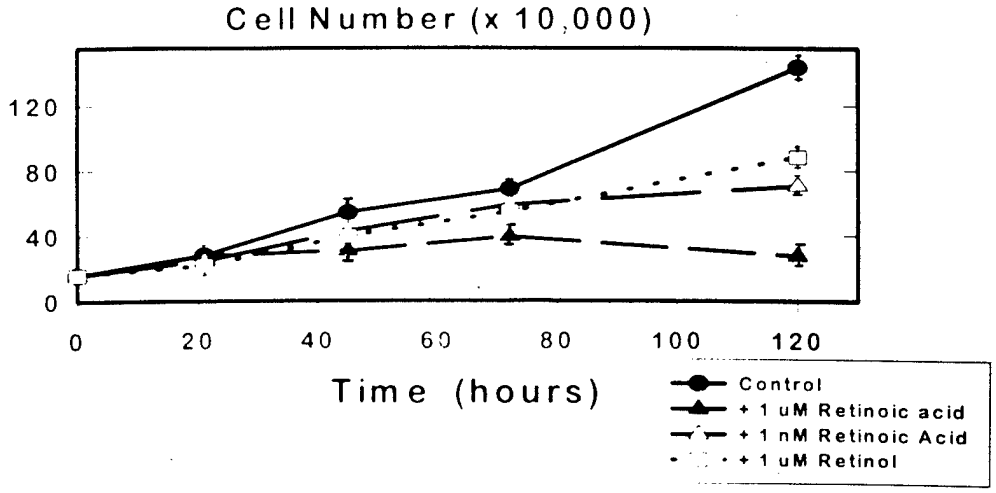


Figure 4a.



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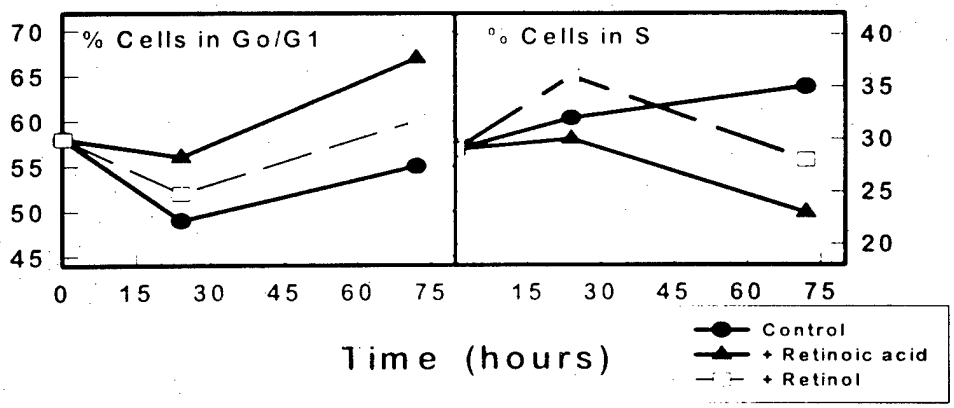

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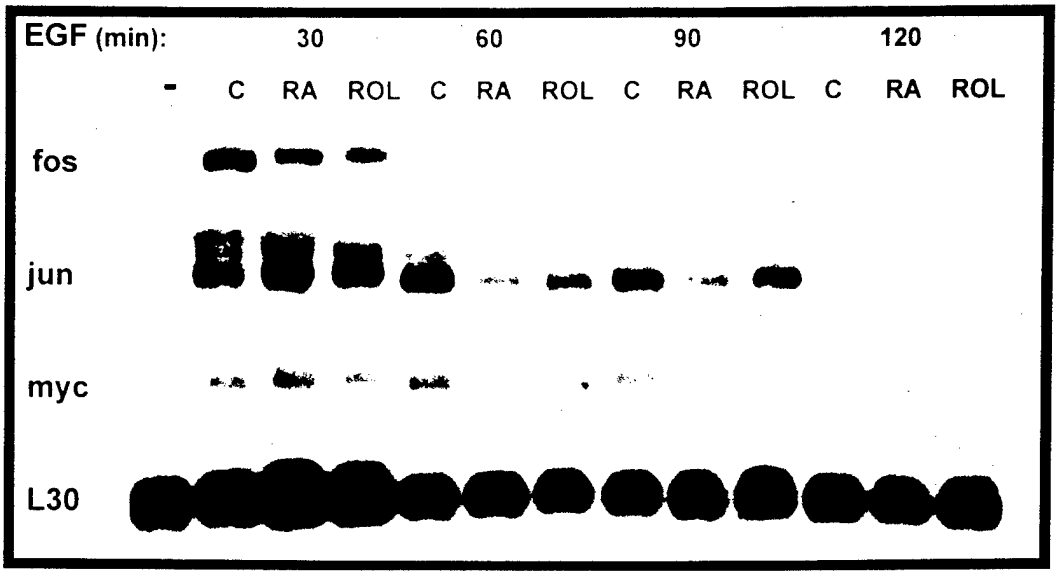

CRBP has modest effect on retinol responsiveness in human breast cancer cells

Figure 5a.

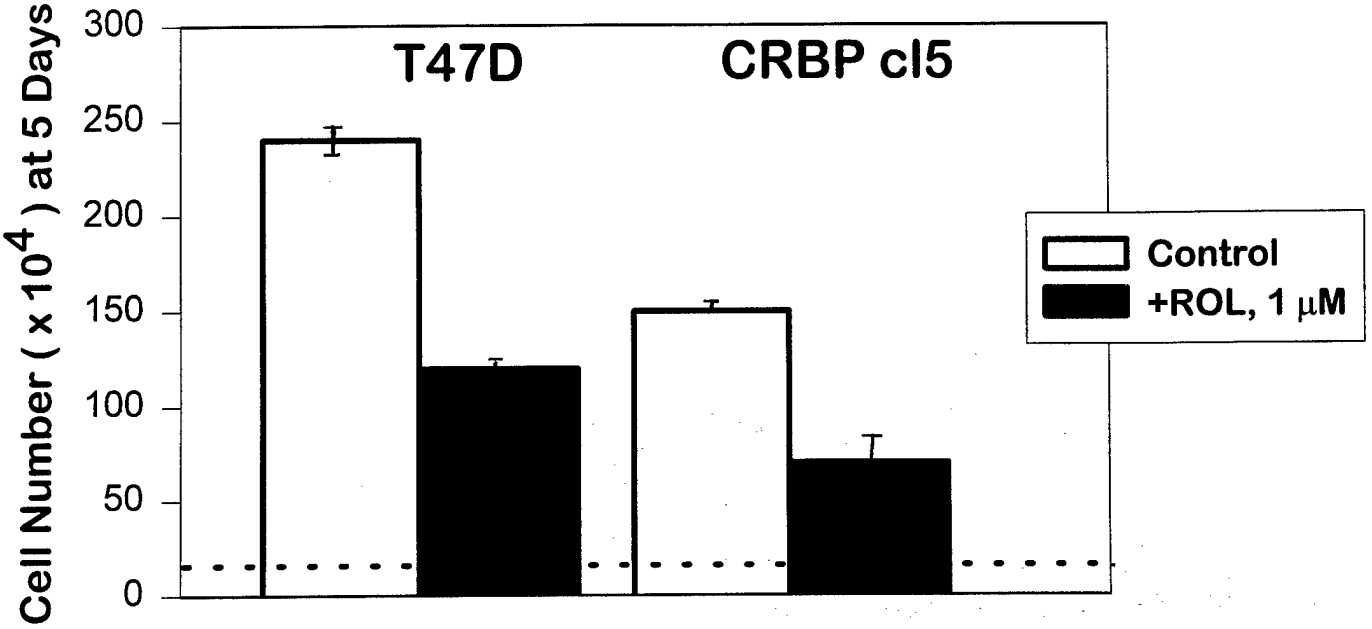

Figure 5b.

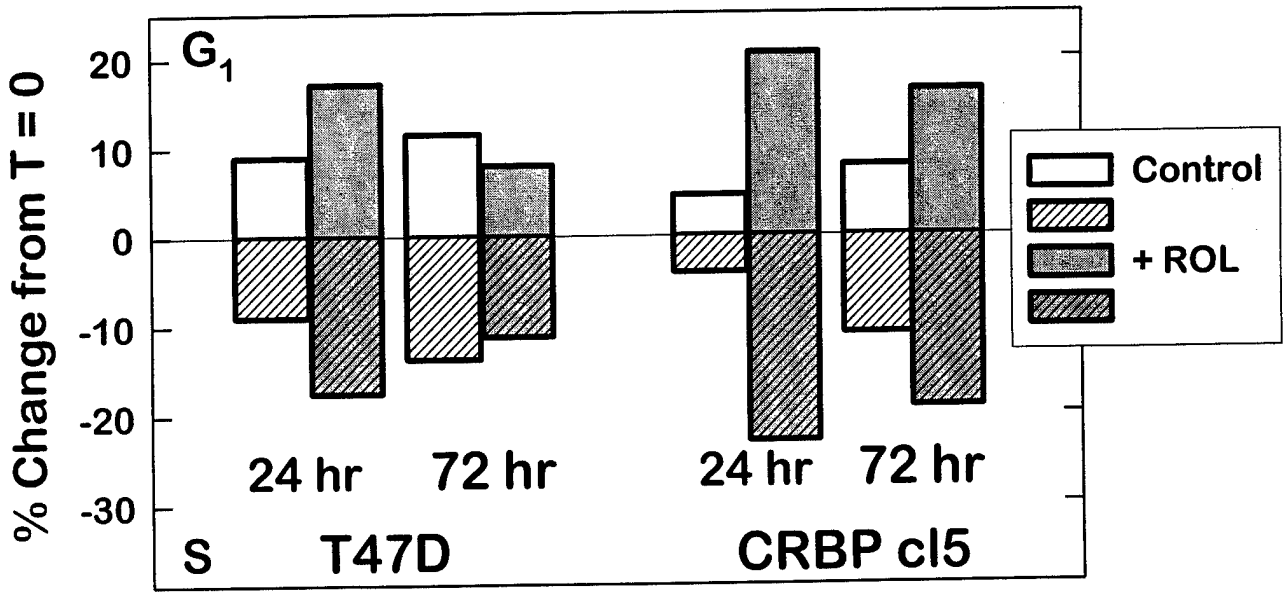

Figure 6. RARα and CRABP II Synergistically Inhibit Hormone-Independent Human Breast Cancer Cell Proliferation