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Mechanism of Inhibition of MMTV-neu and MMTV-wnt1 induced mammary oncogenesis by RAR α agonist AM580

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Abstract

We hypothesized that specific activation of a single retinoic acid receptor, RAR α without direct and concurrent activation of RAR β and γ , will inhibit mammary tumor oncogenesis in murine models relevant to human cancer. Fifty uniparous MMTV-neu and 50 nuliparous MMTV-wnt1 transgenic mice were treated with RAR α agonist, (retinobenzoic acid, Am580) added to the diet for 40 (neu) and 35 weeks (wnt1), respectively. Among the shared anti-tumor effects was the inhibition of epithelial hyperplasia, a significant increase ($p < 0.05$) in tumor-free survival, and a reduction in tumor incidence and in growth of established tumors. In both models the mechanisms responsible for these effects involved inhibition of proliferation and survival pathways, and induction of apoptosis. The treatment was more effective in the MMTV-wnt1 model in which Am580 also induced differentiation, both *in vivo*, and in 3D cultures. In these tumors Am580 inhibited the wnt-pathway, measured by loss of nuclear β -catenin, suggesting partial oncogene-dependence of therapy. Am580-treatment increased RAR β and lowered the level of RAR γ , an isotype whose expression we linked to tumor proliferation. The anti-cancer effect of RAR α together with the newly discovered pro-proliferative role of RAR γ , suggests that specific activation of RAR α and inhibition of RAR γ might be effective in breast cancer therapy.

Keywords

RAR α ; RAR γ ; AM580; MMTV-neu; MMTV-wnt1; breast cancer; differentiation

Introduction

Vitamin A maintains the differentiated state of adult epithelia (Wolbach and Howe 1978). Its functional derivative, retinoic acid (RA), acts by binding to retinoid (RAR) and rexinoid (RXR) family of nuclear receptors which belong to a superfamily of ligand-dependent transcription factors. RARs and RXRs (each a family of 3 genes, α , β and γ , which give rise to multiple isoforms) function cooperatively through heterodimerization, actively repressing

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Conflict of interest

The authors declare no conflict of interest.

or activating target gene transcription (Chambon 1996). Retinoids have been shown to suppress cancer cell growth and prevent mammary cancer in carcinogen rodent models (Moon and Mehta 1990, Sporn et al 1976), but success in clinical trials of breast cancer has been minimal at best (Astrom et al 1990, Hong et al 1990, Lotan 1995, Shin et al 2001, Wu et al 2001). The only exception is the remarkable efficacy of atRA in acute promyelocytic leukemia (APL) where durable remissions and increased survival have been achieved (Collins 2008, Patatianian and Thompson 2008, Warrell et al 1993). In this cancer, atRA specifically targets a fusion protein (PML-RAR α) which, when present, functions by blocking normal differentiation (Wolf and Smas 2000). This suggests that once the functions of specific RARs in the context of defined oncogenic environment become identified, retinoids might become more broadly effective.

One such recently reported example shows that treatment of MMTV-neu mice with atRA was not only ineffective but was counterproductive, increasing mammary tumor incidence and growth (Schug et al 2007). The authors concluded that the outcome (cell growth arrest and differentiation versus cell proliferation) is determined by a balance between two atRA chaperones CARBP-II and FABP5; predomination of the latter leads to activation of PPAR β/δ and cancer cell proliferation and survival.

In keeping with this idea and supported by a recent publication (Purton et al 2006), we propose that RAR γ , under certain conditions has a pro-proliferative role and that its activation by atRA might be responsible for the limited success of retinoid treatment in human cancers. Thus, rather than using atRA, an activator of all 3-RAR isotypes, we tested the RAR α agonist Am580, which has a 10 fold greater affinity for RAR α than β , is almost inactive against RAR γ (Delescluse et al 1991) and is resistant to degradation by CYP26A1-P450 (Osanai and Petkovich 2005, White et al 1997), for its effect on mammary carcinomas in two transgenic mouse models, MMTV-neu and MMTV-wnt.

Neu (HER2/ErbB2) encodes a tyrosine kinase receptor which is amplified in ~30% of human breast cancer and is associated with poor prognosis (Slamon et al 1987). The activation of neu leads to a cascade activation of kinases such as the src family, focal adhesion kinase (FAK), PI3K/Akt, MAPK and possibly others, inducing cell growth and tumor formation (Dankort and Muller 2000, Marcotte and Muller 2008). The MMTV-wnt1 is believed to target the mammary stem/progenitor compartment as evidenced by tumors in which both epithelial and myoepithelial cells are present (Li et al 2003). Wnt gene encodes a family of secreted glycoproteins that bind to specific membrane receptors. Activation of the wnt pathway leads to stabilization of β -catenin and its translocation to the nucleus where it interacts with TCF/LEF transcription factor inducing gene expression, including genes involved in cell proliferation and survival such as c-myc (Nelson and Nusse 2004). The wnt family pathways, which are important in mammary development and renewal of stem cell in the adult tissue (Brennan and Brown 2004, Clevers 2006), are often perturbed in tumors. Aberrant activation of the wnt pathway and epigenetic downregulation of wnt inhibitors has been reported in many tumors, including breast carcinomas (Bukholm et al 2000, Lin et al 2000, Ryo et al 2001, Veeck et al 2008a, Veeck et al 2008b). The effect of RA signaling on wnt, although complex suggest some inhibitory effect (Easwaran et al 1999).

We found that Am580 treatment strongly inhibited the neu and wnt1 induced mammary gland hyperplasia, the incidence of microscopic tumors, tumor growth and, in the neu model, strongly inhibited lung metastases, leading to a significant increase in tumor-free survival in both models. In addition to inhibiting tumor cell proliferation and increasing apoptosis, mechanisms responsible for the AM580 inhibition of oncogenesis in both models, Am580 also induced differentiation, but only in MMTV-wnt1. This difference provides a potential explanation for the increased magnitude of the tumor-free survival found in MMTV-wnt1.

Overall, our data suggest that in 2 different models of mammary oncogenesis, by targeting specific RARs, it is possible to achieve anti-tumor effects which are partially universal and partially oncogene-specific. These effects are, most likely, the result of activation of an inhibitory RAR α and simultaneous reduction in RAR γ , which we showed to be pro-proliferative. We propose that treatment of breast cancer patients selected on the basis of oncogene-activated pathway with RAR-specific retinoids might overcome the current difficulties with retinoid therapies.

Results

Am580 treatment reduces tumorigenesis in wnt1 and neu transgenic models

We used an RAR α agonist retinobenzoic acid Am580 (Kagechika et al 1988), a compound with a 10X fold lower affinity for RAR β (Delescluse et al 1991) and no detectable affinity toward RAR γ , to treat transgenic mice with mammary cancer induced by wnt1 or neu (Muller et al 1988, Tsukamoto et al 1988). We hypothesized that by evading RAR γ activation, we will lessen skin toxicity (Chapellier et al 2002) and avoid the potential pro-proliferative tumor effects of RAR γ (Purton et al 2006).

A dose of 0.3mg/kg/day of Am580, (suggested by Dr. Koichi Shudo, who supplied the drug) was tested by treating 10 FVB mice for 4 months; no overt toxicity was found in liver, lungs, kidney, and spleen, (Results not shown). This dose of Am580, contained in the diet, was used to treat MMTV-neu and MMTV-wnt1 transgenic mice. (Control mice were fed the same diet without Am580). The mice were palpated twice weekly, the tumor appearance was recorded and the data was used to generate Kaplan Meier tumor free survival curves. Figs. 1A and B show that tumor-free survival was significantly (log-rank test) increased in both MMTV-neu ($p < 0.05$) and MMTV-wnt1 ($p < 0.01$) mice treated with Am580; a dose of 0.1mg/Kg/day produced similar but somewhat weaker effects (Results not shown). The effect was more profound in the MMTV-wnt1 model in which 50% of the untreated mice had palpable tumors at ~22 weeks, while in the treated group as late as week 35, fewer than 50% mice had tumors (Fig. 1B). The difference between the control and treated MMTV-neu mice was smaller, but even in this group the treatment increased the % of tumor-free mice from <10% in control to 30% in the treated group (Fig.1A). The pronounced effect of Am580 past week 35 in the neu-model might be due to the unique biology of the later appearing tumors or longer duration of the treatment.

Once palpable, the tumors in control and Am580 treated mice (MMTV-neu: 40 control and 33 treated; MMTV-wnt1: 35 control and 26 treated) were measured weekly and the mice

were sacrificed when tumors reached ~1.5gm. As shown in Fig. 1C and D, in which zero represents the time the tumor was first palpable, the Am580 treatment significantly ($p < 0.01$ and $p < 0.001$ for neu and wnt, respectively) retarded tumor growth in both transgenic models. In approximately 80% of the MMTV-wnt1 treated mice, the tumors grew to ~0.5 g, at which time they showed cycles of partial regression and re-growth (Fig. 1D), possibly due to development of cysts that burst and re-grow; in about 10% the palpable tumors completely regressed (Results not shown).

We found that neu-mice have relatively high incidence of lung metastases and thus examined whether Am580 treatment, in addition to inhibiting tumorigenesis, will also affect the incidence of lung metastasis. As shown in Fig. 1E, in a randomly selected group of 20 control and 20 treated mice, the treatment reduced the incidence of lung metastases from 75% down to 35%. Because, at the time of metastasis evaluation, the average size of the treated tumors was lower than control, it is impossible at this time to conclude that the observed metastasis reduction was a direct Am580 effect on this process.

Mechanisms that mediate the antitumor effects of Am580

To determine the mechanism through which Am580 reduces tumor growth and possibly increases tumor-free survival, we tested tumor sections for markers of proliferation and apoptosis. Sections from 10 control and 10 treated tumors were stained for pRb as a marker of proliferation, and for p27, which is known to increase during cell cycle arrest and has been shown to accumulate in response to RAR activation (Borriello et al 2006, Radu et al 2008). P27 is a regulator of pRb phosphorylation (Vidal and Koff 2000). As shown in Fig. 2A, in both models, the Am580 treated tumors have a significantly higher p27 ($p < 0.001$) levels and a significantly reduced level of pRb, ($p < 0.01$), suggesting a degree of proliferative arrest in both tumor models. However, while the reduction in pRb was observed in all the tested, Am580-treated wnt1 tumors sections, it was detected in only 6/10 sections from treated neu tumors, suggesting a more consistent effect on MMTV-wnt1 tumors.

We used caspase-3 activation and TUNEL assay as markers of apoptosis and Akt phosphorylation as a measure of pro-survival pathway. A significant increase ($p < 0.01$ and $p < 0.001$, for neu and wnt, respectively) in caspase-3 activation by Am580 (Fig. 2B), confirmed by TUNEL assay (Results not shown) was found in both tumor types. However, while in MMTV-neu, caspase-3 was distributed randomly across the tumor tissue, in MMTV-wnt1 tumors, it was more localized to lumens of acinar looking structures, suggesting a possibility of cavitation and thus, differentiation (Fig. 2B). This was confirmed in 3D cultures of MMTV-wnt1 cells that were incubated with 50nM AM580, or with medium alone for 13 days and stained for caspase-3. The treatment increased caspase-3 expression in all of the colonies, and in 30% of the colonies induced acinar-like cavitation (Fig.2D). We quantified the Am580 effect on apoptosis using EtBr staining (Gantenbein-Ritter et al 2008) and showed that the treatment caused a significant increase (Fig. 2E, t-test, $p < 0.001$).

To examine the effect of Am580 treatment on survival pathway, protein extracts of randomly selected 12 tumors/per group (neu-control and treated and wnt control and treated)

were prepared and analyzed for total Akt and P-Akt levels. Fig.2C, which shows 3 representative samples of these tumors, indicates that the treatment reduced P-Akt/Akt ratio by ~50%, suggesting that the survival of tumor cells might be compromised by the treatment.

Oncogene-dependent effect of Am580

As shown in Figs.1A and B, Am580 treatment had a more profound effect on tumor-free survival of MMTV-wnt1 mice, the effect being noticeable even in early appearing tumors. To test whether this was a reflection of Am580 treatment modifying more effectively the pre-malignant wnt-expressing glands, we examined whole mounts of those wnt and neu glands that, at the time of euthanasia for tumor removal, were deemed tumor free by palpation. Both MMTV-neu and MMTV-wnt1 glands showed signs of focal hyperplasia, appearing as small patches, or as large areas that sometimes involved the entire mammary gland (Fig. 3A, top). An analysis of the whole mounts (n=40 per group) indicated that Am580 treatment reduced substantially and equally the level of hyperplasia in both transgenic glands (Fig. 3A, bars; Fisher's Exact test, neu: * $p < 0.001$, wnt1: ** $p < 0.0001$). Analysis of whole mount glands also revealed the presence of small, microscopic tumors (Fig.3B top) which could not be detected by palpation; the total number of glands with small tumors (5 for wnt control, and 14 for neu control) and of glands with multiple small tumors (< 2mm in diameter) (0 for wnt and 4 for neu) was also significantly reduced by the Am580 treatment (Fig. 3B, bars; Fisher's Exact test, neu: * $p < 0.001$ and wnt: ** $p < 0.01$). The similarity in the level of hyperplasia reversion and the reduction, by Am580, of percent of mice with microscopic tumors in both tumor models suggest that these effects are, most likely, not responsible for the more profound tumor-free survival effect of wnt1-mice.

We repeatedly noticed that sections of wnt-induced tumors, and especially Am580-treated wnt tumors, appear to be more differentiated than the neu tumors. H&E sections of untreated MMTV-neu showed the typical, poorly differentiated solid nodules with little stroma (Fig. 3C, left upper panel), which were not changed by Am580 treatment, but for some necrotic areas (Figure 3C, left lower panel). In contrast, sections of untreated MMTV-wnt1 tumors (Fig. 3D, upper left panel) showed luminal and myoepithelial components with a large stromal compartment and areas of glandular differentiation. As indicated by large areas of acinar differentiation with cell remnants inside the lumen, likely the result of anoikis, differentiation was further enhanced by the Am580 treatment (Fig. 3D, lower left panel, arrows). Some MMTV-wnt1 tumors from treated mice showed multiple cysts of different sizes (Fig.3D). As already mentioned, the differentiation of MMTV-wnt1 tumor cells by Am580 in 3D cultures (Debnath et al 2003), induced lumen formation, most likely due to cavitation, in about 30% of the colonies, while no cavitation was detected in the control colonies. Moreover, this morphogenetic effect induced by the activation of RAR α by Am580 was reversed by the RAR α antagonists Ro-415253 (Results not shown), indicating the central role RAR α plays in the reversion of the transformed phenotype induced by wnt. Together, these results suggest that in the MMTV-wnt1 tumors, in addition to inhibiting proliferation, Am580 treatment also initiates a RAR α -dependent process of differentiation.

Tests of additional markers of differentiation confirmed these conclusions. For example, although the treatment reduced the overall level of CK-14, a basal cell marker, in MMTV-neu tumors (Figs. 4A and 4B), only in the MMTV-wnt1 tumors it led to its relocation to the basal surface of acini which appeared to have undergone cavitation (Fig. 4A). Similarly, Am580-treated MMTV-wnt1 tumors, which showed acinar differentiation (Fig.4C), had properly polarized CK-6, a luminal marker. Finally, CRBP-1, a RAR α target gene highly expressed in the luminal cells of the normal mammary gland, was expressed in both tumor types, (Figs.4A and 4B), but in MMTV-wnt1 treated with Am580, it appeared to be localized to cells surrounding acini-like structures (Fig. 4A). Although, not clearly evident from the immunohistochemical staining, the expression of the specifically localized CRBP1 in Am580-treated wnt tumors must be very high, as indicated by its increased level in the immunoblots (Fig. 4B).

In searching for a possible mechanism for the observed oncogene-dependent difference of Am580 effect, we considered the published evidence implicating activated RAR in wnt-pathway inhibition (Mulholland et al 2005), predominantly through interference with β -catenin function (Easwaran et al 1999). Activation of β -catenin pathway is reflected, among others, in its accumulation in the nucleus (Polakis 2000). To test whether Am580 affected this pathway, cells obtained from MMTV-wnt1 tumors were cultured in 3D conditions and treated with either 50nM AM580, or vehicle control and stained for β -catenin. In the control colonies, which showed no signs of cavitation, β -catenin was localized to cell membrane, but also present in the nuclei (Fig.4D). Am580 treatment produced mostly cavitated colonies and profoundly reduced nuclear β -catenin (Fig.4D). This finding suggests that over-expressed wnt-driven pathways might be normalized by the Am580-treatment allowing tumor differentiation by Am580.

Thus, as evidenced by reduction in pRb, increase in p27, reduction in P-Akt and activation of caspase-3, Am580 induces cell cycle arrest and apoptosis in both tumor types. In addition, in MMTV-wnt1 tumors, the treatment induces differentiation as indicated by proper localization of CK-14, CK-6 and CRBP-1, and induction of cavitation of acinar structures. One of the induced gene, CRBP-1, is a known target of RAR, and p27 accumulation is highly sensitive to RAR activation (Radu et al 2008).

Is evading RAR γ activation the reason for anti-tumor effect of Am580?

To test the status of RARs and their signaling in tumors of Am580-fed mice, we measured RAR α , β , and γ protein content in extracts of tumors derived from MMTV-neu and MMTV-wnt1 mice. As shown in Fig.5A, the level of RAR β 2, a known retinoid target gene, was increased in both tumor types, indicating that RAR-mediated signaling was active. However, the RAR γ -protein level was strongly reduced in both tumor types treated with Am580 (Fig. 5A).

Since atRA treatment, which activates all 3-RARs, was shown to stimulate the growth of MMTV-neu tumors (Schug et al 2007), while we showed RAR α -agonist to be inhibitory and to reduce the level of RAR γ (Fig.5A), we wondered whether it was the RAR γ that, when activated by atRA, produced the deleterious effects. Immunohistochemistry of RAR γ in sections of MMTV-wnt1 tumors showed homogeneously distributed expression throughout

the tumor tissue (Fig.5B); in Am580 treated tumor sections, with the exception of strong staining in the normal skin adjacent to the tumor, there was almost no RAR γ staining. This suggests different regulation of RAR γ by Am580 in normal and tumor tissue.

We attempted to probe the role of RAR γ in wnt1 and neu tumor derived cells by knock down or overexpression experiments but so far, we did not find conditions which allowed for consistent RAR γ downregulation or overexpression. However, using immortalized human breast cell line, MCF-10, in which we overexpressed RAR γ (Fig.6A), we showed that RAR γ overexpression significantly increased the proliferation rate (Fig.6B) and, importantly, rendered the cells resistant to the well known atRA-mediated down-regulation of pRB and upregulation of p27 (Borriello et al 2006) (Fig.6C). Thus, like in wnt1 and neu tumors, the anti-proliferative effect of Am580 in human cells appears to be counteracted by over-expression of RAR γ . Thus, in addition to the published mechanism of atRA enhancement of neu-tumor growth through increase in FABP5 (Schug et al 2007), we identified RAR γ activity as another possible contributor to this effect. Importantly, we found that unlike atRA (Schug et al 2007), Am580 neither increased the level of FABP5 protein nor induced PPAR activation in a PPRE-luc assay, (Fig.S1 Supplement).

Discussion

This work was designed to determine whether different RAR isoforms differ in their ability to provide protection from oncogene induced mammary carcinogenesis and whether this effect is oncogene dependent. We conclude that activation of RAR α by a specific agonist, Am580, significantly diminished mammary gland tumorigenicity of two oncogenes, wnt1 and neu. Moreover, we found that inhibition of proliferation and induction of apoptosis occurred in both oncogene-induced tumors but that induction of differentiation was oncogene-specific and only detected in wnt1 induced tumors. This is important, because the overall effect on tumor incidence was more pronounced in the MMTV-wnt1 model, suggesting that treatment that combines growth inhibition, induction of apoptosis and differentiation might be most effective.

Our data is more relevant when considered in the background of the poor retinoid performance in clinical trials (Paik et al 2003), and the recent publication by Schug et al., (Schug et al 2007) indicating that treatment of MMTV-neu mice with atRA, a non-selective RAR activator, enhances neu-mammary tumor incidence and growth (Schug et al 2007). Treatment with atRA was shown to change the proportion of FABP5/CRABP-II causing PPAR β/δ activation at the expense of RARs and increased proliferation and cell survival, a finding not confirmed in cells treated with Am580 (Supplemental Fig.S1). Thus, although PPAR β/δ can bind several ligands (Berry and Noy 2007, Noy 2007), Am580 does not appear to function as its activator, a fact that provides a possible explanation for the pro-proliferative atRA effect and the inhibitory Am580 effect in MMTV-neu tumors. Another advantage of Am580 over atRA is its resistance to degradation by CYP26A1 P450 cytochromes involved in atRA catabolism to its oxo-derivatives (White et al 1997). These cytochromes are overexpressed in several tumor cell lines and human tumors leading to atRA resistance (Klaassen et al 2001, Ozpolat et al 2002, Van heusden et al 1998). Thus, the use

of a retinoid resistant to CYP26 regulation might help to overcome the development of retinoid resistance.

However, based on our own data and on published evidence (Purton et al 2006, Schneider et al 2000) we propose that active RAR γ is pro-proliferative and as such responsible for the enhancing atRA effect. Our findings that RAR γ over-expression interferes with atRA-induced expression of p27 and reduction in pRb (Fig.6C), support this notion. Moreover, we showed that RAR γ level is reduced in Am580-treated tumors while its level in the neighboring skin remains unaffected (Fig.5B) suggesting a tumor-specific-RAR γ regulation by RAR α -specific agonist. It also shows that treatment with RAR α agonist might both bypass RAR γ activation and reduce its level. We have not yet investigated the mechanism of RAR γ reduction by Am580. Published evidence shows that by inhibiting PI3K, retinoids lead to p38 activation which in turn phosphorylates RAR γ and leads to its degradation (Gianni et al 2002, Kim et al 2001). Whether this pathway mediates the loss of RAR γ in tumors from Am580-treated mice remains to be seen. The observed pro-proliferative role of RAR γ is in agreement with the finding in SK-BR-3 human breast cancer cell line in which treatment with an RAR γ agonist, CD437, induced growth (Schneider et al 2000). Purton et al. (Purton et al 2006) have shown that the high RAR γ level present in primitive hematopoietic precursors wanes during cell maturation simultaneously with an increase in RAR α and β . Bone marrow of RAR γ knockout mice exhibited markedly reduced numbers of HSCs associated with increased numbers of more mature progenitor cells, suggesting that RAR γ in these cells serves self-renewal and anti-differentiation functions (Purton et al 2006). Although, it is too early to extrapolate from hematopoietic to mammary biology, it remains theoretically possible that during cycles of proliferation and involution of the mammary epithelium a similar regulation in the expression of RARs exists in the stem/progenitor cells compartment. atRA treatment, by activating RAR γ , could perturb the equilibrium between progenitors and differentiated cells, increasing tumorigenicity. The observed ability of Am580 to reverse mammary gland hyperplasia, lends support to this hypothesis. It should be noted, however, that growth inhibitory effects of RAR γ have been described (Su and Gudas 2008, Walkley et al 2007) suggesting that individual RARs might have unique roles in specific tissues.

Another important aspect of our findings is the observed partial oncogene-dependent responsiveness to Am580 treatment regarding tumor incidence (Kaplan-Meyer Figs.1A and B) and tumor growth (Figs.2B and C); both were more profoundly affected in the MMTV-wnt1 model. This suggests that for better responses to retinoid therapy in breast cancer patients they might have to be stratified according to oncogenic pathways that are activated. We propose that induction of differentiation contributes to the increased responsiveness of wnt-1 tumors to Am580. Among the indicators of Am580-induced differentiation are formation of acini-like structures with signs of anoikis of the cells in the lumen, more normal-looking acini, proper localization of cytokeratins and redistribution of CRBP1 (Figs. 3 and 4) Thus, although not causing full differentiation, partial differentiation by Am580 might explain its stronger anti tumor effect on wnt1-induced tumors. We postulate that normalization of the mammary morphology (differentiation) in these tumors is affected through the inhibition by Am580 of the wnt-pathway, as determined by relocalization of β -catenin from the nucleus to the membrane (Fig.4D)

Taken together, our data document that two mammary carcinoma models, MMTV-neu and MMTV-wnt1 are responsive to Am580 treatment and that this response is only partially oncogene-dependent. The fact that Am580, a RAR α agonist insensitive to P450 cytochrome degradation is effective in 2 cancer models relevant to the human disease and in a human breast cell line, has potentially important clinical implications. Based on these observations and the novel role of RAR γ , we suggest that better understanding of the specific roles of individual RARs in cancer cell differentiation, proliferation and apoptosis will help develop rational chemopreventive and, possibly, chemotherapeutic retinoid-based approaches to breast cancer. Combinations of selective RAR α agonists with RAR γ antagonists may prove to be one such successful approach.

Materials and Methods

AM580 treatment

Four months old uniparous (1 pregnancy/lactation cycle) MMTV-neu and 6 weeks old nulliparous MMTV-wnt1 female mice (50 mice/group) were treated with the RAR α agonist AM580 (0.3 mg/kg body weight per mouse per day) in the diet (Purina 5053) prepared by Purina-TestDiet, Richmond, IN, by adding 1.5mg AM580 per kg of Purina 5053 diet. Mice that developed tumors within the first month of treatment were removed from the study. Mice were palpated twice a week and tumor appearance was recorded. Once palpable, the size of the tumors was measured weekly. Tumor-free survival was calculated from Kaplan-Meier curves and statistical significance was determined by the Log-rank test for the survival studies and t-test for the tumor growth studies. Metastasis was evaluated by removing the lungs of all the anesthetized mice, selecting randomly 20 mice per group and inspecting the lung surface for lesions using a stereoscope (Nikon SMZ800 stereoscope X3 to 5).

Mammary Gland Whole Mounts

Mammary glands were excised, fixed in Carnoy's fixative and stained in carmine alum solution as described in <http://mammary.nih.gov/tools/histological/Histology/index.html>.

Immunohistochemistry

Tumor samples were fixed in 10% buffered formalin for 24hrs, transferred to 70% ethanol and kept at 4°C until use. Sections were prepared from 10 tumors/group, subjected to standard antigen retrieval and incubated with p27 (BD Transduction Labs, NJ, USA), pRB, activated caspase-3 (Cell Signaling, MA, USA), CK-14 (Santa Cruz Biotechnology), CK-6 (Covance, NJ, USA) or RAR γ antibodies (Abcam, MA, USA), final concentration of 1 μ g/ml, overnight at 4°C. The sections were processed using VectaStain ABC Elite Kit (Vector Laboratories, CA, USA), the signal was detected using Metal Enhanced DAB Substrate Kit (Pierce Laboratories, IL, USA) and the sections counterstained with Harris Hematoxylin Solution (Sigma Diagnostics, MO, USA).

Immunoblotting

Tumor homogenates (total protein 25 to 50 μ g) boiled in Laemmli buffer with β -mercaptoethanol were electrophoresed on 10-15% SDS polyacrylamide gels, then

transferred to PVDF Membranes (Hybond-P, Amersham Biosciences, NJ, USA), blocked with 5% non-fat dried milk and incubated with the primary antibody in 3% non-fat dry milk solution diluted as specified; anti-RAR α , β and γ 1:500 (Abcam), anti-Akt and p-Akt 1:1000 (Cell Signaling), anti-CK-14 and CK-18 1:1000 (Santa Cruz Biotechnology), anti-FABP5 1:500 (BioVendor, NC, USA), anti-Tubulin and anti-Vinculin (1:3000) (Sigma). Some membranes were stripped and re-probed.

Cell lines and 3D cultures

MMTV-wnt1 cell lines were generated from fragments of tumors from untreated MMTV-wnt1 mice after digestion with collagenase (1.5mg/ml collagenase, 25mg/ml BSA in PBS with Ca²⁺ and Mg²⁺) at 37°C for 30-45min with gentle agitation. The 3D cultures were prepared and maintained as describe by Debnath et al. (Debnath et al 2003). MCF-10A cells generously provide by Dr. Brugge and maintained as described by Debnath et al. (Debnath et al 2003) (for details see Supplementary data). The apoptotic index was performed by etidium bromide (Gantenbein-Ritter et al 2008) (for details see Supplementary data).

Proliferation assay

MCF-10A (2×10^4) control cells or overexpressing RAR γ were seeded in triplicates in 6-well culture dishes. After 24hrs, cells were washed with PBS, incubated in 2ml of DMEM-F12 culture medium, detached and counted every 24hrs. Statistical significance was determined by t-test. pRB and p27 expression was tested by immunofluorescence analysis of control MCF-10A monolayers and monolayers stably transfected with pSG5-RAR γ expression vector, using the same antibodies described for the IHC analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Astrom A, Pettersson U, Krust A, Chambon P, Voorhees JJ. Retinoic acid and synthetic analogs differentially activate retinoic acid receptor dependent transcription. *Biochem Biophys Res Commun.* 1990; 173:339–345. [PubMed: 2175182]
- Berry DC, Noy N. Is PPARbeta/delta a Retinoid Receptor? *PPAR Res.* 2007; 2007:73256. [PubMed: 18274629]
- Borriello A, Cucciolla V, Criscuolo M, Indaco S, Oliva A, Giovane A, et al. Retinoic acid induces p27Kip1 nuclear accumulation by modulating its phosphorylation. *Cancer Res.* 2006; 66:4240–4248. [PubMed: 16618747]
- Brennan KR, Brown AM. Wnt proteins in mammary development and cancer. *J Mammary Gland Biol Neoplasia.* 2004; 9:119–131. [PubMed: 15300008]

- Bukholm IK, Nesland JM, Borresen-Dale AL. Re-expression of E-cadherin, alpha-catenin and beta-catenin, but not of gamma-catenin, in metastatic tissue from breast cancer patients. *J Pathol.* 2000; 190:15–19. seecomments. [PubMed: 10640987]
- Cardiff RD, Wellings SR. The comparative pathology of human and mouse mammary glands. *J Mammary Gland Biol Neoplasia.* 1999; 4:105–122. [PubMed: 10219910]
- Chambon P. A decade of molecular biology of retinoic acid receptors. *Faseb J.* 1996; 10:940–954. [PubMed: 8801176]
- Chapellier B, Mark M, Messaddeq N, Calleja C, Warot X, Brocard J, et al. Physiological and retinoid-induced proliferations of epidermis basal keratinocytes are differently controlled. *Embo J.* 2002; 21:3402–3413. [PubMed: 12093741]
- Clevers H. Wnt/beta-catenin signaling in development and disease. *Cell.* 2006; 127:469–480. [PubMed: 17081971]
- Collins SJ. Retinoic acid receptors, hematopoiesis and leukemogenesis. *Curr Opin Hematol.* 2008; 15:346–351. [PubMed: 18536573]
- Dankort DL, Muller WJ. Signal transduction in mammary tumorigenesis: a transgenic perspective. *Oncogene.* 2000; 19:1038–1044. [PubMed: 10713687]
- Debnath J, Muthuswamy SK, Brugge JS. Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures. *Methods.* 2003; 30:256–268. [PubMed: 12798140]
- Delescluse C, Cavey MT, Martin B, Bernard BA, Reichert U, Maignan J, et al. Selective high affinity retinoic acid receptor alpha or beta-gamma ligands. *Mol Pharmacol.* 1991; 40:556–562. [PubMed: 1656191]
- Easwaran V, Pishvaian M, Salimuddin Byers S. Cross-regulation of beta-catenin-LEF/TCF and retinoid signaling pathways. *Curr Biol.* 1999; 9:1415–1418. [PubMed: 10607566]
- Gantenbein-Ritter B, Potier E, Zeiter S, van der Werf M, Sprecher CM, Ito K. Accuracy of three techniques to determine cell viability in 3D tissues or scaffolds. *Tissue Eng Part C Methods.* 2008; 14:353–358. [PubMed: 18800876]
- Gianni M, Kopf E, Bastien J, Oulad-Abdelghani M, Garattini E, Chambon P, et al. Down-regulation of the phosphatidylinositol 3-kinase/Akt pathway is involved in retinoic acid-induced phosphorylation, degradation, and transcriptional activity of retinoic acid receptor gamma 2. *J Biol Chem.* 2002; 277:24859–24862. [PubMed: 12032135]
- Hong WK, Lippman SM, Itri LM, Karp DD, Lee JS, Byers RM, et al. Prevention of second primary tumors with isotretinoin in squamous-cell carcinoma of the head and neck. *N Engl J Med.* 1990; 323:795–801. [PubMed: 2202902]
- Kagechika H, Kawachi E, Hashimoto Y, Himi T, Shudo K. Retinobenzoic acids. 1. Structure-activity relationships of aromatic amides with retinoidal activity. *J Med Chem.* 1988; 31:2182–2192. [PubMed: 3184125]
- Kim AH, Khursigara G, Sun X, Franke TF, Chao MV. Akt phosphorylates and negatively regulates apoptosis signal-regulating kinase 1. *Mol Cell Biol.* 2001; 21:893–901. [PubMed: 11154276]
- Klaassen I, Brakenhoff RH, Smeets SJ, Snow GB, Braakhuis BJ. Metabolism and growth inhibition of four retinoids in head and neck squamous normal and malignant cells. *Br J Cancer.* 2001; 85:630–635. [PubMed: 11506507]
- Li Y, Welm B, Podsypanina K, Huang S, Chamorro M, Zhang X, et al. Evidence that transgenes encoding components of the Wnt signaling pathway preferentially induce mammary cancers from progenitor cells. *Proc Natl Acad Sci U S A.* 2003; 100:15853–15858. [PubMed: 14668450]
- Lin SY, Xia W, Wang JC, Kwong KY, Spohn B, Wen Y, et al. Beta-catenin, a novel prognostic marker for breast cancer: its roles in cyclin D1 expression and cancer progression. *Proc Natl Acad Sci U S A.* 2000; 97:4262–4266. [PubMed: 10759547]
- Lotan R. Retinoids and apoptosis: implications for cancer chemoprevention and therapy. *J Natl Cancer Inst.* 1995; 87:1655–1657. [PubMed: 7473809]
- Marcotte R, Muller WJ. Signal transduction in transgenic mouse models of human breast cancer--implications for human breast cancer. *J Mammary Gland Biol Neoplasia.* 2008; 13:323–335. [PubMed: 18651209]

- Moon RC, Mehta RG. Cancer chemoprevention by retinoids: animal models. *Methods Enzymol.* 1990; 190:395–406. [PubMed: 2087191]
- Mulholland DJ, Dedhar S, Coetzee GA, Nelson CC. Interaction of nuclear receptors with the Wnt/beta-catenin/Tcf signaling axis: Wnt you like to know? *Endocr Rev.* 2005; 26:898–915. [PubMed: 16126938]
- Muller WJ, Sinn E, Pattengale PK, Wallace R, Leder P. Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-neu oncogene. *Cell.* 1988; 54:105–115. [PubMed: 2898299]
- Nelson WJ, Nusse R. Convergence of Wnt, beta-catenin, and cadherin pathways. *Science.* 2004; 303:1483–1487. [PubMed: 15001769]
- Noy N. Ligand specificity of nuclear hormone receptors: sifting through promiscuity. *Biochemistry.* 2007; 46:13461–13467. [PubMed: 17983246]
- Osanai M, Petkovich M. Expression of the retinoic acid-metabolizing enzyme CYP26A1 limits programmed cell death. *Mol Pharmacol.* 2005; 67:1808–1817. [PubMed: 15703382]
- Ozpolat B, Mehta K, Tari AM, Lopez-Berestein G. all-trans-Retinoic acid-induced expression and regulation of retinoic acid 4-hydroxylase (CYP26) in human promyelocytic leukemia. *Am J Hematol.* 2002; 70:39–47. [PubMed: 11994980]
- Paik J, Blaner WS, Sommer KM, Moe R, Swisshlem K. Retinoids, retinoic acid receptors, and breast cancer. *Cancer Invest.* 2003; 21:304–312. [PubMed: 12743994]
- Patatanian E, Thompson DF. Retinoic acid syndrome: a review. *J Clin Pharm Ther.* 2008; 33:331–338. [PubMed: 18613850]
- Polakis P. Wnt signaling and cancer. *Genes Dev.* 2000; 14:1837–1851. [PubMed: 10921899]
- Purton LE, Dworkin S, Olsen GH, Walkley CR, Fabb SA, Collins SJ, et al. RARgamma is critical for maintaining a balance between hematopoietic stem cell self-renewal and differentiation. *J Exp Med.* 2006; 203:1283–1293. [PubMed: 16682494]
- Radu M, Soprano DR, Soprano KJ. S10 phosphorylation of p27 mediates atRA induced growth arrest in ovarian carcinoma cell lines. *J Cell Physiol.* 2008; 217:558–568. [PubMed: 18615582]
- Ryo A, Nakamura M, Wulf G, Liou YC, Lu KP. Pin1 regulates turnover and subcellular localization of beta-catenin by inhibiting its interaction with APC. *Nat Cell Biol.* 2001; 3:793–801. [PubMed: 11533658]
- Schneider SM, Offterdinger M, Huber H, Grunt TW. Activation of retinoic acid receptor alpha is sufficient for full induction of retinoid responses in SK-BR-3 and T47D human breast cancer cells. *Cancer Res.* 2000; 60:5479–5487. [PubMed: 11034091]
- Schug TT, Berry DC, Shaw NS, Travis SN, Noy N. Opposing effects of retinoic acid on cell growth result from alternate activation of two different nuclear receptors. *Cell.* 2007; 129:723–733. [PubMed: 17512406]
- Shin DM, Khuri FR, Murphy B, Garden AS, Clayman G, Francisco M, et al. Combined interferon-alfa, 13-cis-retinoic acid, and alpha-tocopherol in locally advanced head and neck squamous cell carcinoma: novel bioadjuvant phase II trial. *J Clin Oncol.* 2001; 19:3010–3017. [PubMed: 11408495]
- Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science.* 1987; 235:177–182. [PubMed: 3798106]
- Sporn MB, Dunlop NM, Newton DL, Smith JM. Prevention of chemical carcinogenesis by vitamin A and its synthetic analogs (retinoids). *Fed Proc.* 1976; 35:1332–1338. [PubMed: 770206]
- Su D, Gudas LJ. Gene expression profiling elucidates a specific role for RARgamma in the retinoic acid-induced differentiation of F9 teratocarcinoma stem cells. *Biochem Pharmacol.* 2008; 75:1129–1160. [PubMed: 18164278]
- Tsukamoto AS, Grosschedl R, Guzman RC, Parslow T, Varmus HE. Expression of the int-1 gene in transgenic mice is associated with mammary gland hyperplasia and adenocarcinomas in male and female mice. *Cell.* 1988; 55:619–625. [PubMed: 3180222]
- Van heusden J, Wouters W, Ramaekers FC, Krekels MD, Dillen L, Borgers M, et al. All-trans-retinoic acid metabolites significantly inhibit the proliferation of MCF-7 human breast cancer cells in vitro. *Br J Cancer.* 1998; 77:26–32. [PubMed: 9459142]

- Veeck J, Bektas N, Hartmann A, Kristiansen G, Heindrichs U, Knuchel R, et al. Wnt signalling in human breast cancer: expression of the putative Wnt inhibitor Dickkopf-3 (DKK3) is frequently suppressed by promoter hypermethylation in mammary tumours. *Breast Cancer Res.* 2008a; 10:R82. [PubMed: 18826564]
- Veeck J, Noetzel E, Bektas N, Jost E, Hartmann A, Knuchel R, et al. Promoter hypermethylation of the SFRP2 gene is a high-frequent alteration and tumor-specific epigenetic marker in human breast cancer. *Mol Cancer.* 2008b; 7:83. [PubMed: 18990230]
- Vidal A, Koff A. Cell-cycle inhibitors: three families united by a common cause. *Gene.* 2000; 247:1–15. [PubMed: 10773440]
- Walkley CR, Olsen GH, Dworkin S, Fabb SA, Swann J, McArthur GA, et al. A microenvironment-induced myeloproliferative syndrome caused by retinoic acid receptor gamma deficiency. *Cell.* 2007; 129:1097–1110. [PubMed: 17574023]
- Warrell RP Jr, de The H, Wang ZY, Degos L. Acute promyelocytic leukemia. *N Engl J Med.* 1993; 329:177–189. [PubMed: 8515790]
- White JA, Beckett-Jones B, Guo YD, Dilworth FJ, Bonasoro J, Jones G, et al. cDNA cloning of human retinoic acid-metabolizing enzyme (hP450RAI) identifies a novel family of cytochromes P450. *J Biol Chem.* 1997; 272:18538–18541. [PubMed: 9228017]
- Wolbach SB, Howe PR. Nutrition Classics. *The Journal of Experimental Medicine.* 1978; 42:753–77. [PubMed: 19869087] Burt Wolbach S, Howe Percy R. Tissue changes following deprivation of fat-soluble A vitamin. *Nutr Rev.* 1925; 36:16–19. [PubMed: 342996]
- Wolf G, Smas CM. Retinoic acid induces the degradation of the leukemogenic protein encoded by the promyelocytic leukemia gene fused to the retinoic acid receptor alpha gene. *Nutr Rev.* 2000; 58:211–214. [PubMed: 10941258]
- Wu JM, DiPietrantonio AM, Hsieh TC. Mechanism of fenretinide (4-HPR)-induced cell death. *Apoptosis.* 2001; 6:377–388. [PubMed: 11483862]

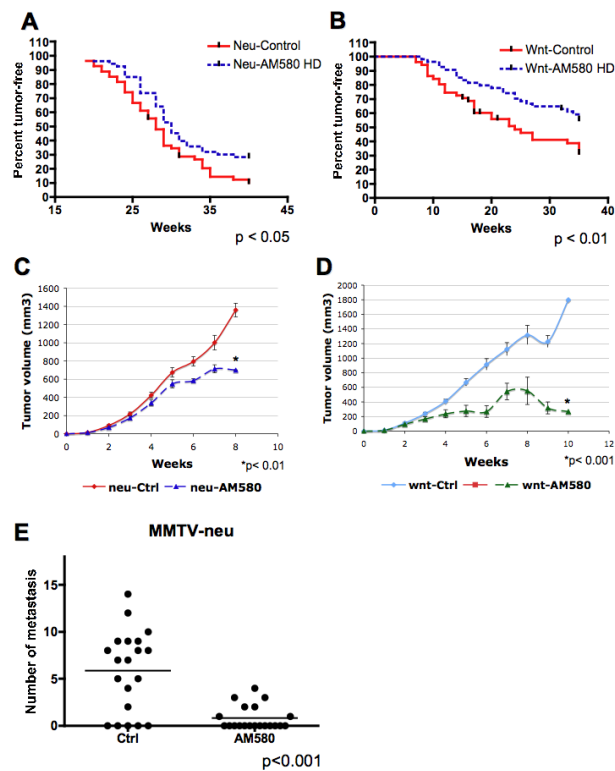
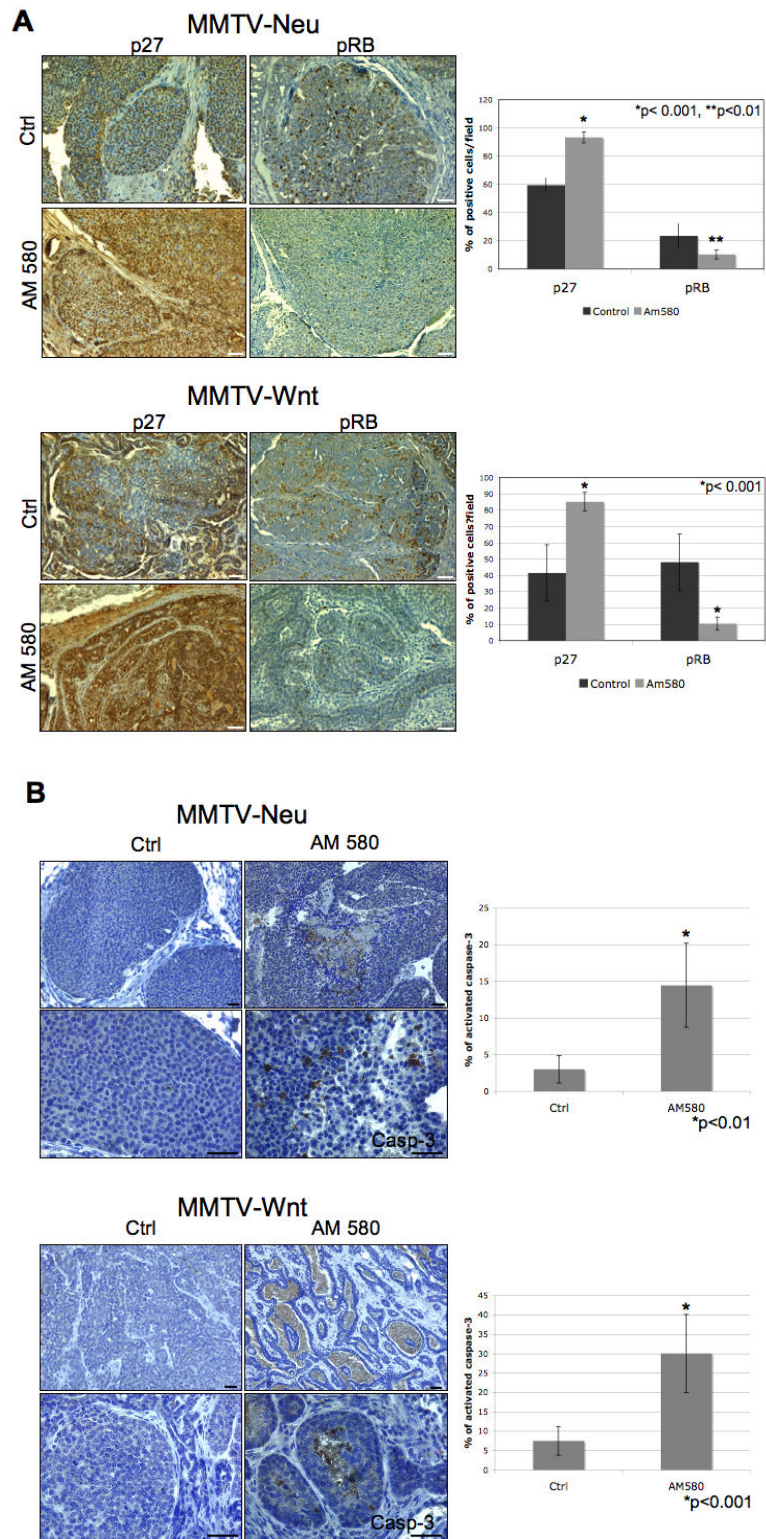


Figure 1. Am580 effect on tumor-free survival and tumor growth in MMTV-neu and MMTV-wnt1 and metastasis in neu-mice

Fifty MMTV-neu (A) and MMTV-wnt1 (B) transgenic mice were treated with Am580 in the diet as described in Methods. Kaplan-Meier curves show significant (Neu: $p < 0.05$; Wnt: $p < 0.01$, Log-rank test) increase in tumor-free survival in Am580-treated transgenic mice. Tumor size (\pm SE) in MMTV-neu (C, ANOVA $p < 0.01$) and MMTV-wnt1 (D, ANOVA $p < 0.001$) was determined by twice weekly measurements; appearance of palpable tumors is considered time zero. The experiment was terminated when the untreated tumors were ~ 1.5 gm. (The variation in wnt-tumor volume with time is most likely due to development of fluid-filled cysts that burst and refill). E) Surface lung metastases were counted using a stereoscope at the end of the experiment described in C. Twenty randomly selected untreated and 20 Am580-treated MMTV-neu mice were evaluated (t-test, $p < 0.001$).



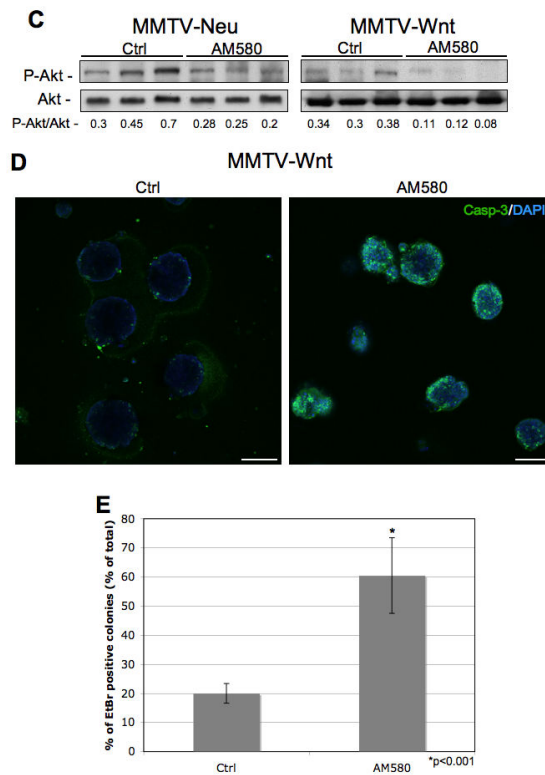


Figure 2. Effect of Am580 treatment on markers of cells cycle, apoptosis and survival

A) Ten sections each of neu and wnt1 tumors from untreated and Am580 treated mice were immunostained for p27 and pRB (scale bars, 100 μ m), and 3-400x fields in 10 sections each were used for the quantification shown in the bar graphs. (t-test, p27: p<0.001 and pRB: p<0.01). B) Sections of MMTV-neu and MMTV-wnt1 shown at 100X (upper panels) and 400X (lower panels) magnification, from untreated (left, top and bottom) or Am580 treated (right, top and bottom) tumors were stained for activated caspase-3. In wnt-1 section, caspase-3 appears concentrated in the center of an acinus which might be undergoing cavitation (lower right panel). The increase in activated caspase-3, quantified as in B, was statistically significant (t-test, neu: p<0.01, wnt1: p<0.001). C) Immunoblots of total Akt and P-Akt in lysates of tumors from 3 untreated and 3 Am580 treated neu transgenic mice (left panel), and 3 untreated and 3 Am580-treated wnt1 mice (right panel) (Reduction of P-Akt/Akt ratio by treatment ~50%). D) 3D cultures of MMTV-wnt1 cells (see Methods) grown for 13 days without or with 50nM AM580 were stained for caspase-3 as described in Methods. Note the cavitated acini and increased caspase-3 staining in the treated cultures. Scale bars, 50 μ m. E) Apoptosis of MMTV-wnt1 3D colonies in Matrigel, control (treated with DMSO (Ctrl)) or Am580-treated (50nM for 7 days), quantified by EtBr staining. Data of 3 independent experiments, each in quadruplicate, n=12 samples/group, t-test; p<0.001.

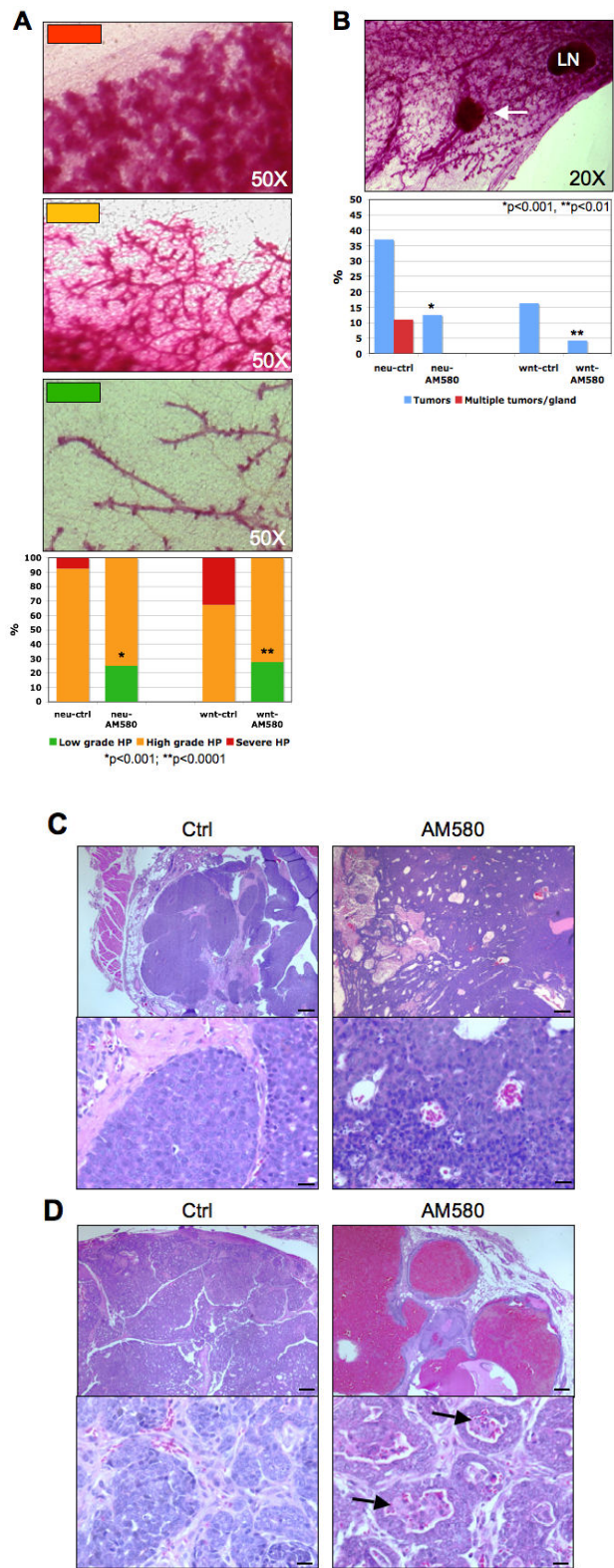


Figure 3. Am580 treatment reduces hyperplasia and microscopic neu and wnt tumors

A) Forty number four (#4) mammary glands (n=40), considered tumor-free by palpation at the time of euthanasia for tumor growth experiment, were retrieved, and examined as whole mounts for hyperplasia. The photographs show examples of severe (red barcode), high grade (orange) and low grade (green) hyperplasias. Bars; Reduction in the degree of hyperplasia in tumor-free neu and wnt glands treated with Am580, as percent of total glands tested. (Fisher's Exact test * $p < 0.00$, ** $p < 0.0001$, for neu and wnt glands, respectively). B) The same glands as in A but with microscopic tumors. The arrowhead - a microscopic tumor. LN=lymph node. Bars show the percent of glands with a single tumor and glands with multiple tumors (Fisher's Exact test neu: * $p < 0.001$, wnt1: * $p < 0.01$). C) Tissue sections of neu-tumors from untreated (left upper and lower panels) and Am580 treated (right upper and lower panels) mice stained with H&E. D) Sections of wnt-tumors from untreated mice (left top and lower panels) and Am580-treated (right top and lower panels). Top panels, scale bars 100 μ m; bottom, scale bars, 10 μ m. Sections of neu and wnt-1 tumors show previously described characteristic histopathology (Cardiff and Wellings 1999).

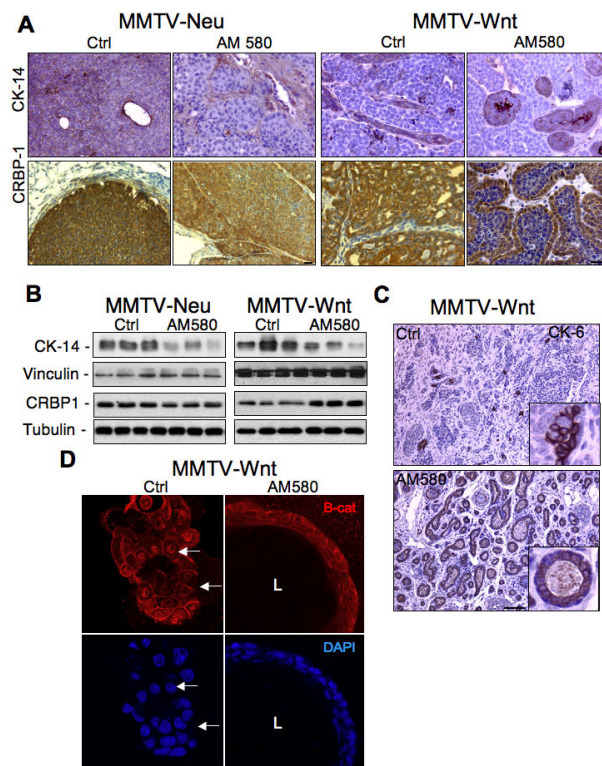


Figure 4. Am580 treatment induces differentiation of MMTV-wnt1 tumors

Immunohistochemistry (A and C, scale bar, 50 μ m) and immunoblotting (B) of tumors obtained from untreated or Am580 treated MMTV-neu or MMTV-wnt1 mice for markers of differentiation (CK-14, CK-6 and CRBP1). D) 3D cultures of MMTV-wnt1 cell line (passage #3), untreated, or treated with 50nM AM580, immunostained for β -catenin (red). Arrows indicate cells with nuclear staining of β -catenin. β -catenin is membrane localized in Am580 treated cells (upper right panel). Scale bar, 50 μ m.

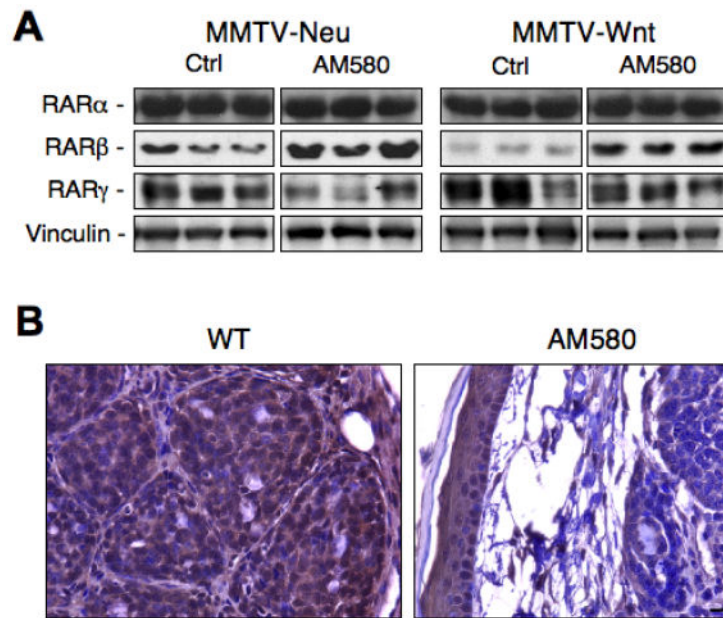


Figure 5. Am580 treatment reduces RAR γ

A) RAR α , β and γ level in tumor homogenates from untreated or Am580-treated MMTV-Neu and MMTV-Wnt1 mice determined by immunoblotting. Shown 3 representative sections of 10 total tested/group. B) Immunostaining of MMTV-wnt-1 tumor sections for RAR γ (untreated-left panel; Am580-treated, right panel). The tumor section from an untreated mouse has darkly stained nuclei (RAR γ). Scale bar, 50 μ m.

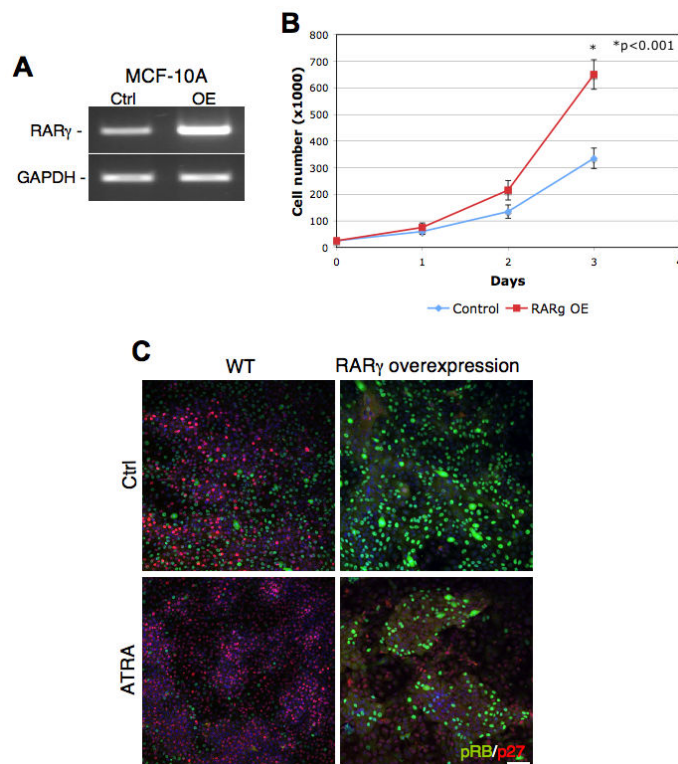


Figure 6. Pro-proliferative role of RAR γ in human MCF-10 cells

A) RT-PCR of RAR γ of MCF-10A cells stably transfected with RAR γ (OE, overexpression), or control (Ctrl) plasmids. B) Monolayers of MCF-10A cells (control or RAR γ OE, 4 wells/group) detached and counted daily for 3 days, ANOVA *p<0.001. C) MCF-10A cell cultures, untreated or treated overnight with 1 μ M atRA were analyzed by confocal immunofluorescence using anti p27 and anti-pRB antibodies. Scale bar, 100 μ m. Overexpressing RAR γ cultures, control and treated, have lower proportion of p27-positive and higher proportion of pRb positive cells than their untransfected counterparts.