

9-*cis*-Retinoic Acid Suppresses Mammary Tumorigenesis in C3(1)-Simian Virus 40 T Antigen-transgenic Mice¹

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ABSTRACT

Retinoids have been investigated as potential agents for the prevention and treatment of human cancers. These compounds play an important role in regulating cell growth, differentiation, and apoptosis. 9-*cis*-Retinoic acid (9cRA) is a naturally occurring ligand with a high affinity for both the retinoic acid receptors and the retinoid X receptors. We hypothesized that treatment with 9cRA would prevent mammary tumorigenesis in transgenic mice that spontaneously develop mammary tumors. To test this hypothesis, C3(1)-SV40 T antigen (Tag) mice, which develop mammary tumors by the age of 6 months, were treated daily p.o. with vehicle or two different dose levels of 9cRA (10 or 50 mg/kg) from 5 weeks to 6 months of age. Tumor size and number were measured twice each week, and histological samples of normal and malignant tissue were obtained from each mouse at time of sacrifice. Our results demonstrate that 9cRA suppresses mammary tumorigenesis in C3(1)-SV40 Tag-transgenic mice. Time to tumor development was significantly delayed in treated mice; median time to tumor formation for vehicle-treated mice was 140 days versus 167 days for mice treated with 50 mg/kg 9cRA ($P = 0.05$). In addition, the number of tumors per mouse was reduced by

>50% in mice treated with 9cRA (3.43 for vehicle, 2.33 for 10 mg/kg 9cRA, and 1.13 for 50 mg/kg 9cRA, $P \leq 0.002$). Histological analysis of the mammary glands from vehicle and treated mice demonstrated that 9cRA treatment also did not affect normal mammary gland development. Immunohistochemical staining of normal and malignant breast tissue and Western blot analysis demonstrated that SV40 Tag expression was not affected by treatment with retinoids. Single doses of 10 and 50 mg/kg resulted in peak plasma concentrations of 3.4 and 6.71 μM , respectively. Daily doses of 9cRA for 28 days resulted in plasma concentrations of 0.86 and 1.68 μM , respectively, concentrations consistent with that seen in humans treated with 9cRA in clinical trials. These results demonstrate that 9cRA suppresses mammary carcinogenesis in transgenic mice without any major toxicity and suggest that retinoids are promising agents for the prevention of human breast cancer.

INTRODUCTION

Breast cancer is the most common cancer among women and is the leading cause of cancer death in women between the ages of 15 and 54 (American Cancer Society). These statistics demonstrate an urgent need to develop effective methods to treat breast cancer or prevent its development. Recent results of the breast cancer prevention trial [from the NSABP cooperative group (P-1 trial)] demonstrate that the antiestrogen tamoxifen causes a 49% reduction in the incidence of breast cancer in high risk women (1). In other studies, raloxifene, a second generation-selective estrogen receptor modulator was also found to reduce breast cancer incidence (2). However, although these studies demonstrate that antiestrogens reduce the incidence of breast cancers by ~50%, there remains a need to reduce breast cancer incidence further. Therefore, we have investigated the activity of other promising chemopreventive agents to suppress breast carcinogenesis in preclinical models. Some of the most promising chemopreventive agents include retinoids, vitamin D analogues, dehydroepiandrosterone derivatives, monoterpenes, and difluoromethylornithine. Of these, retinoids have previously been shown to suppress cancer development in animals and humans.

Retinoids are vitamin A analogues that play an important role in regulating cell growth, differentiation, and apoptosis (3, 4). These hormones function by binding to specific RARs³ (RAR α , β , and γ) and specific RXRs (RXR α , β , and γ) (5). Retinoid receptors are nuclear DNA-binding proteins that regulate gene expression by binding to retinoic acid-responsive elements in the promoters of target genes. The RAR and RXR

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³ The abbreviations used are: 9cRA, 9-*cis*-retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor; Tag, T antigen; ER, estrogen receptor; DMBA, 7,12-dimethylbenz[*a*]anthracene; NMU, *N*-nitroso-*N*-methylurea; MAPK, mitogen-activated protein kinase.

proteins are expressed differently during development and differentiation (6), and various isoforms of these proteins can heterodimerize to produce a variety of complexes to regulate different sets of retinoid-induced genes.

In vitro studies have shown that retinoids can inhibit the growth and invasion of cancer cells and induce apoptosis. Several laboratories, including ours, have found many different cell lines to be significantly growth inhibited by retinoids (7–10). The retinoid used in the present study, 9cRA, has been found to inhibit proliferation, induce differentiation, and induce apoptosis in a variety of cell lines including the breast cancer cell line MCF-7 and the human leukemia cell line HL-60 at micromolar concentrations (9–15). This retinoid binds both RAR and RXR receptors and thus is able to activate both RAR- and RXR-dependent pathways.

In humans, retinoids have been used in clinical trials for the treatment of leukemia (16, 17) and squamous cell carcinomas of the skin and cervix (18–20). Retinoids have also been used in the prevention of cervical, head and neck, and lung cancers (21–23). All-*trans*-retinoic acid was effective against the development of primary cervical cancers (24), whereas isotretinoin (13-*cis*-retinoic acid) was effective against second primary tumors of head and neck squamous cell carcinoma (25). Retinyl palmitate significantly delayed development of second primaries of lung cancer (26), and polyphenolic acid was shown to prevent second primary tumors of the liver (27). These studies suggest that retinoids may be useful for the prevention of breast cancer.

Several *in vivo* models exist for the study of breast cancer, including chemically induced mammary tumor models in rats using 7,12-dimethylbenz(a)anthracene (DMBA) and NMU to induce mammary tumors, as well as several transgenic mouse models (28–31). The ability of retinoids to prevent mammary tumorigenesis has been extensively investigated with the chemical carcinogenesis models. Moon *et al.* (32–34) found that retinyl acetate and *N*-(4-hydroxyphenyl)-retinamide (fenretinide) reduced tumor incidence and multiplicity and also increased the latency of 7,12-dimethylbenz(a)anthracene or NMU-induced mammary cancers. Similar results were found in the NMU-induced model using an RXR-selective retinoid, LGD1069 (35, 36). 9cRA has also been shown to inhibit mammary carcinogenesis induced by NMU in Sprague Dawley rats (37). Although previous studies have shown that retinoids suppress mammary carcinogenesis in chemically treated rats, the relevance of these carcinogen-induced models to human breast cancer has been questioned. The tumors that arise in these animals contain mutations in *ras* genes (28), which are a rather uncommon event in human breast cancer (38).

We have investigated the ability of 9cRA to inhibit mammary tumorigenesis in a transgenic mouse model, the C3(1)-SV40 Tag mouse model. These mice express the SV40 Tag in breast tissue and develop tumors that histologically are similar to high grade human breast cancer (39). In addition, the SV40 Tag causes inactivation of the *p53* and *Rb* genes, tumor suppressor genes that are commonly mutated in human breast cancer (40, 41). Another attractive feature of the SV40 Tag transgenic mouse model is that the tumors that develop arise through a multistep process, similar to that occurring in humans. Thus, female transgenic mice develop mammary hyperplasia by

3 months of age, which is followed by the development of carcinoma *in situ* lesions, which in turn is followed by invasive adenocarcinoma (reaching 100% incidence by 6 months). In addition, some of the mice develop metastases to the lungs. All these features make this an attractive model to investigate the efficacy of chemopreventive agents. Using these SV40 Tag transgenic mice, we investigated whether 9cRA would affect mammary tumor development in this model. Our data demonstrate that 9cRA suppresses mammary tumorigenesis in these transgenic mice and suggest that 9cRA may be useful for the prevention of breast cancer.

MATERIALS AND METHODS

Transgenic Mice. Female C3(1)-SV40 Tag-transgenic mice (obtained from the Animal Production Program, National Cancer Institute, Frederick, MD) were housed in the animal facilities at the University of Texas Health Science Center in San Antonio. Animals were obtained at 5 weeks of age and treated with retinoids daily until the age of 6 months. Virgin animals were used because the development of mammary tumors does not depend on pregnancy. Animals were fed a controlled diet of Teklad LM-485 Mouse/Rat Diet (Harlan Teklad, Madison, WI).

Treatment and Data Collection. 9cRA (Panretin, LGD1057, M_r 300; Ligand Pharmaceuticals, Inc., San Diego, CA) was suspended in purified sesame oil (Croda, Inc., Mill Hall, PA). 9cRA was administered p.o. to animals by a 20-gauge gavage needle in a volume of 0.1 ml containing either 10 or 50 mg/kg 9cRA. The animals were treated daily with either sesame oil (vehicle) or 10 or 50 mg/kg 9cRA from an age of 5 weeks until the age of 6 months. Tumor measurements were made biweekly with electronic calipers (Mitutoyo, Utsonomiya, Japan), and tumor volume was determined by multiplying the square of the width by the length and dividing by 2. Individual tumor size and tumor location for each animal were recorded. Weights of all mice were recorded monthly.

At the time of sacrifice, each tumor was resected and separate portions were (a) processed for histological analysis, (b) used to isolate protein for Western blot analysis, and (c) explanted into tissue culture to prepare *in vitro* tumor cell lines. These cell lines were grown in DMEM containing 10% FBS, 1% glutamine, 1% penicillin/streptomycin, and 1% Fungizone.

Histological Analysis. Tumor samples were fixed in 10% neutral buffered formalin (10% formaldehyde, phosphate-buffered) overnight and then embedded in paraffin. Tissue sections were then mounted on slides and processed for either H&E staining or immunohistochemical staining. H&E staining was conducted by cutting 4- μ m tissue sections and deparaffinizing in xylene. Sections were rehydrated in ethanol and running water and then incubated with hematoxylin for 7 min. Samples were destained in running water and then fixed in acidified alcohol and ammonia. Slides were then incubated in eosin for 2 min, rinsed in alcohol and xylene, and mounted for evaluation.

For immunohistochemical studies, tissue sections were cut at 4 μ m and mounted onto slides. Slides were deparaffinized, and then endogenous peroxidase was blocked with 0.1% sodium azide in 3% hydrogen peroxide in 1 \times auto buffer. Slides were

then rinsed in PBS, and nonspecific binding was blocked with 10% albumin. SV40 Tag was stained using mouse anti-SV40 large Tag monoclonal antibody (1:50; PharMingen, San Diego, CA) followed by a biotinylated rabbit antimouse IgG antibody (1:100). The slides were then incubated with streptavidin-horseradish peroxidase at 1:100, and peroxidase activity was visualized using DAB chromagen intensified with 0.2% osmium tetroxide. Counterstaining was done with 1% methyl green.

Western Blot Analysis of Tumors. Analysis of tumor powders was done by grinding the tumor and suspending in 5% SDS. Samples were heated for 5 min at 95°C and spun in a microfuge to pellet debris. Protein extracts in the supernatant were run on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose membrane (Hybond ECL; Amersham Life Sciences, Arlington Heights, IL). Membranes were probed with mouse anti-SV40 large Tag monoclonal antibody and then with antimouse IgG conjugated to horseradish peroxidase (Amersham Life Sciences). SV40 Tag protein was visualized using ECL (Amersham Life Sciences).

Cell lines derived from tumor explants were treated with either DMSO (vehicle) or 9cRA for 0, 6, 24, and 48 h. Cells were pelleted and lysed in 50 mM Tris (pH 6.8), 2% SDS. Protein extracts were run on a 10% SDS-polyacrylamide gel and analyzed as before.

Plasma Concentration of Retinoids. After blood was collected in a heparinized tube, the plasma was separated by centrifugation and transferred into an opaque tube. Samples were stored under nitrogen gas at -70°C until ready for analysis. Plasma from three or four mice at each time point was pooled to provide a sufficient volume for analysis of 9cRA concentration. The plasma was extracted with 5 volumes of cold methanol, centrifuged, and dried in a vacuum. Residues were reconstituted in mobile phase (70% acetonitrile/30% 10 mM ammonium acetate, adjusted to pH 3 with glacial acetic acid) and injected into a Zorbax SB C₁₈ column (5 μm, 4.6 × 250 mm, MAC-MOD Analytical, Inc., Chaddsford, PA) at 40°C using a 1-ml/min flow rate with UV detection at 330 nm. Standards were prepared by spiking control plasma and were analyzed as above to generate a standard curve. Peak areas of the samples were quantified using linear regression (weighted 1/X) of the standard curve. The area under the plasma time curve from 0 to the last time point was determined by linear trapezoidal approximation.

Statistical Analysis of Results. Two outcome measures, tumor-free survival and tumor multiplicity, were considered in this study. Tumor-free survival was defined from time of birth to first appearance of a tumor. Tumors were defined as palpable masses ≥100 mm³. Tumor-free survival curves were estimated by the Kaplan-Meier product limit method and compared by the generalized Wilcoxon test. Similar analysis was performed using a log rank test that yielded smaller *P* values than the generalized Wilcoxon test for each curve. However, to avoid confusion, we report *P* values from the more conservative generalized Wilcoxon test. (In our experience, the generalized Wilcoxon test is the most appropriate test for these kinds of experiments. The log rank test is appropriate when differences between tumor-free survival curves can be described by proportional hazards. For nonproportional hazard differences between survival curves, such as those in our experiments, the general-

ized Wilcoxon test is most appropriate.) We also evaluated tumor multiplicity by counting the total number of tumors occurring in each animal up to the time of sacrifice. Tumor multiplicity was summarized by means and standard errors, and compared by Wilcoxon rank sum tests. Tumor incidence at a particular time is often also used as an end point in chemoprevention studies. Because tumors develop in these animals over a wide time period, we instead have reported tumor-free survival as a function of time. Incidence at any time point can be obtained from these curves by subtracting the proportion free of tumors at any given time from 1.

RESULTS

SV40 Tag Model of Breast Cancer. C3(1)-SV40 Tag mice develop tumors which display a multistage pattern of tumor progression. Within their mammary glands, the mice develop dysplastic lobules, carcinoma *in situ* lesions, and invasive adenocarcinomas. By the age of 6 months, 100% of the mice develop invasive cancer. These different stages of tumor development are shown in Fig. 1, which shows dysplastic lobules within the ducts of normal mammary glands, ducts swollen with transformed cells (carcinoma *in situ* lesions), and poorly differentiated carcinomas which invade the surrounding stroma.

9cRA Inhibits Development of Mammary Carcinogenesis. To determine whether 9cRA suppresses tumor formation in the C3(1)-SV40 Tag mice, mice were treated with either vehicle or two different doses of 9cRA. The mice were treated daily from the age of 5 weeks to months after which time the experiment was ended. To measure the chemopreventive efficacy of 9cRA, the number and size of all mammary tumors were measured twice weekly as described in "Materials and Methods." The mice were observed daily for any apparent signs of toxicity. Any mice that did not develop tumors at the end of the experiment were followed until tumor development occurred. All animals eventually developed tumors by 200 days of age.

Fig. 2 shows a plot of the proportion of animals free of tumor *versus* age in days. Mice treated with vehicle show a median time to tumor development of only 140 days. Tumor development began at 110 days, and by 180 days (6 months) all vehicle mice had developed tumors. The time to tumor development curve of the mice treated with low dose 9cRA (10 mg/kg) showed no significant difference to the curve of the control mice. However, the mice treated with high dose 9cRA (50 mg/kg) showed a delay in the time to tumor development with the median time to tumor development delayed to 167 days.

Table 1 shows median time to tumor development, tumor incidence, and number of tumors per mouse (tumor multiplicity) in mice treated with vehicle, low dose (10 mg/kg), or high dose (50 mg/kg) of 9cRA. Similar median time to tumor development was seen in the control mice and the mice treated with 10 mg/kg of 9cRA (~140 days). However, mice treated with the 50-mg/kg dose of 9cRA showed a significant delay in time to tumor development (167 days, *P* = 0.05 by the generalized Wilcoxon test). At the completion of the experiment, 100% of the mice treated with vehicle or low dose of 9cRA developed tumors; whereas 70% of mice treated with high dose of 9cRA developed

Fig. 1 Photomicrograph of different stages of tumor development in C3(1)-SV40 Tag mice. At the time of sacrifice, tumors were resected, fixed, and embedded in paraffin. Tissue sections were stained with H&E as described in "Materials and Methods." Selected fields containing representative stages in the tumorigenesis pathway are shown. Dysplastic lobules are terminal duct lobular units containing several acini of normal architecture. Many are lined by a single layer of epithelial cells with enlarged and hyperchromatic nuclei. $\times 200$. For carcinoma *in situ*, two ducts are markedly expanded by a solid proliferation of malignant cells. Mitotic figures and a few apoptotic nuclei are evident. $\times 400$. For invasive adenocarcinomas, both figures show invasive tumors with minimal gland formation, high nuclear grade, and abundant mitoses. $\times 400$.

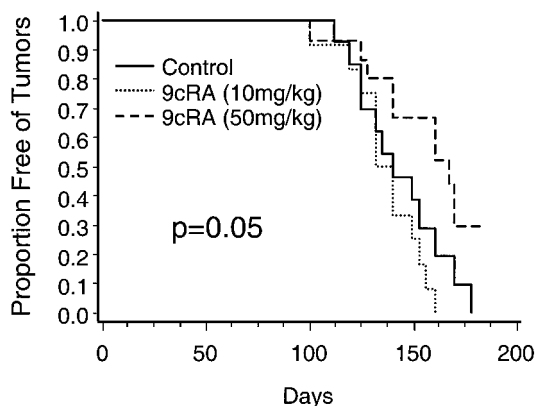
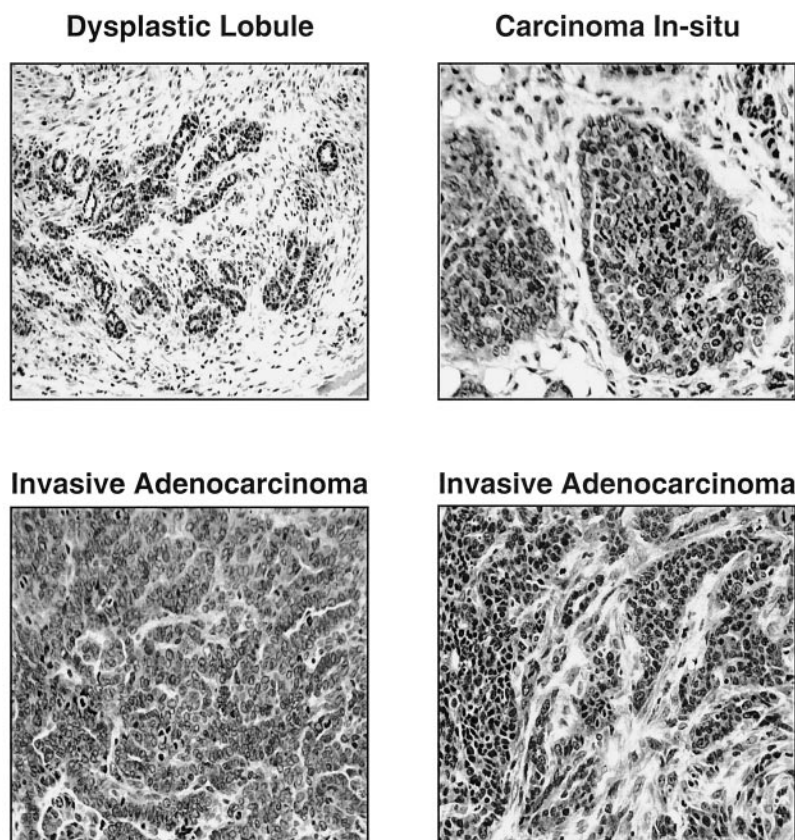


Fig. 2 Plot of animals free of tumor *versus* age in days for C3(1)-SV40 Tag mice treated with vehicle or 9cRA. Vehicle (sesame oil) or 9cRA (10 or 50 mg/kg) was administered p.o. to animals daily from the age of 5 weeks until 6 months of age. Tumor measurements were made biweekly. A Kaplan-Meier plot of the proportion of animals free of tumor *versus* time is shown. Statistical analysis was performed with the generalized Wilcoxon test.

tumors. Thus, 30% of the high dose 9cRA-treated mice failed to develop tumors by 6 months.

A more dramatic effect was seen on tumor multiplicity (number of tumors per mouse). Whereas control mice developed an average of 3.43 (± 0.57) tumors per mouse, the 9cRA-treated

mice developed 2.33 (± 0.36 ; low dose of 9cRA) and 1.13 (± 0.29 ; high dose 9cRA) tumors per mouse. This difference in tumor multiplicity between the control and high dose treated animals was highly significant ($P \leq 0.002$ as assessed by ANOVA).

There was no significant difference in the average weight of the mice treated with either the vehicle or 9cRA from day 1 to 130 (after 130 days, the weights diverged in animals with large tumors; data not shown). These results suggest that the cancer-suppressive effect of 9cRA is not due to general weight loss. Cutaneous toxicities from the retinoids were observed in mice treated with 50 mg/kg 9cRA, including erythema of their ears (in 100% of the high dose-treated animals but not in the control and low dose-treated animals).

9cRA Does Not Affect Expression of the SV40 Tag Transgene. To determine whether the tumor-suppressive effects of 9cRA results were due to down-regulation of the expression of the SV40 transgene, several analyses were conducted. Western blot analysis to assess SV40 Tag protein levels was done as described above. The results are shown in Fig. 3A and demonstrate that tumors from both control and 9cRA-treated mice express similar levels of the transgene. These results were further supplemented by immunohistochemistry to detect SV40 Tag expression in normal and malignant mammary tissue from these mice. As shown in Fig. 3B, the transgene is expressed in the normal ducts of both groups of mice. In addition, tumors from both groups also express similar amounts of the SV40 Tag.

Table 1 Median time to tumor development, tumor incidence, and multiplicity

Treatment	No. of mice	Median time to tumor development (days)	Proportion of mice with tumors at 6 months (%)	No. of tumors/mouse
Sesame oil (control)	14	140	100	3.43 ± 0.57
9cRA (10 mg/kg)	12	136	100	2.33 ± 0.36 ^a
9cRA (50 mg/kg)	15	167	70	1.13 ± 0.29 ^a

^a $P \leq 0.002$ between control and 9cRA-treated groups.

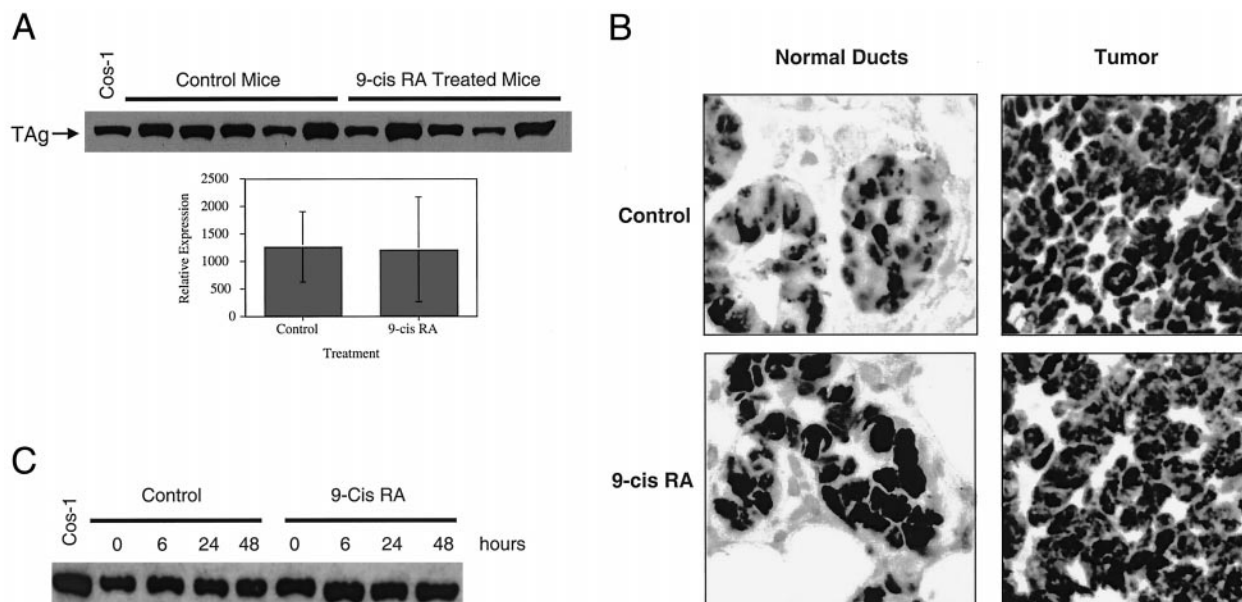


Fig. 3 A, expression of SV40 Tag in mammary tumors. Mammary tumor extracts were analyzed for expression of the SV40 Tag by Western blot analysis as described in "Materials and Methods." Protein extract from Cos cells was used as a positive control. An equal amount of protein (12.5 μ g) was loaded into each well. No difference in SV40 Tag expression was observed between vehicle- and 9cRA-treated mice. B, immunohistochemical staining for SV40 Tag. Tumors were resected, fixed, and embedded in paraffin. Tissue sections were prepared for immunohistochemical staining and probed with SV40 large Tag monoclonal antibody as described in "Materials and Methods." As shown, no difference in SV40 Tag expression was observed between vehicle- and 9cRA-treated tumor samples: In normal ducts, both figures demonstrate strong nuclear staining of most cells with SV40 Tag antibody. There is no difference in the intensity and number of tumor cells staining between the tumors from control and treated mice. C, effect of 9cRA on SV40 Tag expression in cell lines isolated from tumor explants in C3(1)-SV40 Tag mice. Cell lines started from tumor explants were treated with either DMSO (vehicle) or 9cRa for 0, 6, 24, and 48 h. Western blot analysis for SV40 large Tag was performed on whole cell lysates as described in "Materials and Methods." Protein extract from Cos cells was used as a positive control. 9cRA did not affect SV40 Tag expression in the cell lines.

Because tumors that arise in animals treated with 9cRA might still express SV40 Tag, analysis was also conducted on mammary tumor explants isolated from control mice and that had never been exposed to retinoids. As shown in Fig. 3C, levels of SV40 Tag in these tumor explants do not change with 9cRA treatment.

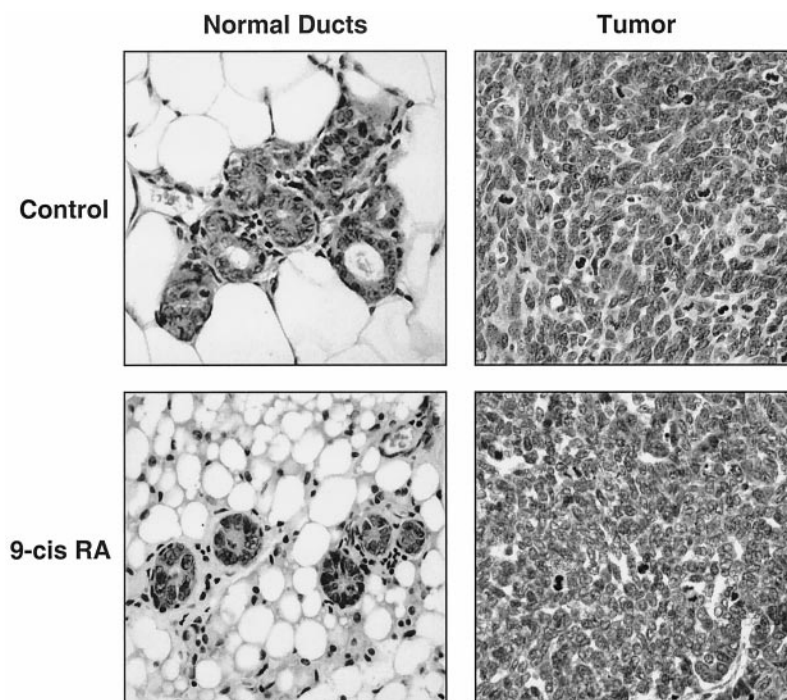
Histological analysis was also conducted using mammary tissue samples from control and 9cRA-treated mice to determine whether 9cRA affects the morphology of normal or malignant mammary glands in these mice. A representative example of the tumors from vehicle- and 9cRA-treated mice is shown in Fig. 4. Comparison of all tumor samples from vehicle- and retinoid-treated mice showed no significant difference in morphology or nuclear grade. These results suggest that 9cRA treatment does not induce differentiation of the tumor cells in these mice. However, a slight reduction in mitotic index was observed in the

retinoid-treated animals as compared with the control mice (data not shown).

9cRA Plasma Levels in Mice Treated with Vehicle or 9cRA. To determine the 9cRA plasma level that is associated with suppression of mammary carcinogenesis, we performed pharmacokinetic analysis after acute and chronic dosing in these animals. At time of sacrifice, blood was collected and plasma was isolated as described in "Materials and Methods." Plasma samples were then analyzed using reverse phase high performance liquid chromatography analysis to determine the plasma concentration of 9cRA.

Fig. 5A shows the average plasma concentration over time after a single oral dose of 9cRA (either 10 or 50 mg/kg). As shown in Table 2, the T_{max} for the 10-mg/kg dose was 60 min versus 30 min for the 50-mg/kg dose. Levels of 9cRA were undetectable after 6 h of time. Peak plasma concentrations

Fig. 4 Comparison of histological features of normal mammary tissue and tumors from control and 9cRA-treated mice. At time of sacrifice, tumors were resected, fixed, and embedded in paraffin. Tissue sections were stained using H&E as described in "Materials and Methods." Selected fields containing normal and tumor samples treated with vehicle and 9cRA are shown: In normal ducts, the terminal duct lobular units from both the control and treated mice demonstrate a small group of acini lined by benign epithelium. The tumors are high grade with minimal or no evidence of gland formation in both groups. The number of mitotic figures is slightly higher in the representative tumor from untreated mice than in with the treated one.



achieved after a single dose were 3.40 and 6.71 mM for 10 and 50 mg/kg, respectively, correspond with concentrations found to inhibit growth *in vitro*.

Fig. 5B shows the average plasma concentration over time after a dose of 9cRA in mice that were treated daily with either a 10-mg/kg (low dose) or a 50-mg/kg (high dose) of 9cRA for 28 days. As shown in Table 2, the maximum levels of retinoid concentration in the blood were at 30 min for both the low and high doses. Plasma concentrations of 9cRA after a single dose in these chronically treated mice were lower at maximal levels (0.86 and 1.68 mM for 10 and 50 mg/kg, respectively). Area under the curve values on day 28 were 86 and 78% lower than the day 1 values at 10- and 50-mg/kg dose levels respectively. These results suggest that chronic dosage of 9cRA induces enzymes that metabolize 9cRA.

The results from the pharmacokinetic studies show that the dose of 50 mg/kg used in these studies can achieve peak plasma levels of up to 6 μ M, after a single dose, with levels of 1–2 μ M in chronically treated animals. These plasma levels are consistent with levels achieved in humans in recent Phase I clinical trials of 9cRA (42, 43). Such plasma levels represent total 9cRA, much of which is bound to plasma proteins. The biological effects of 9cRA are likely induced by intracellular 9cRA that binds to retinoid receptors. The concentration of intracellular 9cRA in the mammary epithelial cells is likely to be much lower than our reported plasma levels. However, to compare the present studies with those in humans, we have monitored total 9cRA levels.

DISCUSSION

The results reported here demonstrate that 9cRA suppresses the development of mammary tumors in C3(1)-SV40 Tag-transgenic mice. 9cRA treatment increased the median time

to tumor development and decreased tumor multiplicity in these mice. The suppressive effects of 9cRA were not due to inhibition of transgene expression because SV40 Tag expression was unchanged in normal and malignant tissue obtained from the mice treated with either vehicle or 9cRA. 9cRA suppressed tumor formation without inducing any apparent breast cell differentiation, because histological examination of the mammary tissue after treatment with 9cRA showed no morphological changes as compared with the tissue from vehicle-treated mice. This study is the first to demonstrate that 9cRA suppresses spontaneous mammary tumorigenesis in transgenic mice.

The suppression of the tumor development by 9cRA in the SV40 Tag mice is modest compared with the dramatic suppression by 9cRA previously reported by Anzano *et al.* (37) in NMU-treated rats. This difference in efficacy may occur because the tumors arise by different pathways in the two models. In the NMU rat model, the NMU carcinogen induces *ras* mutations that promote tumor formation. *ras* mutations activate the MAPK pathway that may be blocked by retinoids. Indeed retinoids are known to inhibit AP-1 transcription factors downstream of the *ras*-MAPK pathway (44–46). The tumors that arise in the NMU-treated rats are also ER positive, and the antiestrogen tamoxifen dramatically suppresses tumorigenesis in this model (37, 47). Thus, activation of the MAPK pathway and the dependence on estrogen signaling may cause NMU-induced tumorigenesis to be particularly sensitive to retinoids.

Our data suggest that SV40 Tag-induced tumorigenesis is also sensitive to retinoids, but less so than NMU-induced rat tumors. Tumorigenesis in SV40 Tag mice is caused by inactivation of the *p53* and *Rb* tumor suppressor genes. The present results indicate that cells transformed by *p53* and *Rb* inactivation are less sensitive to retinoids than are cells transformed by

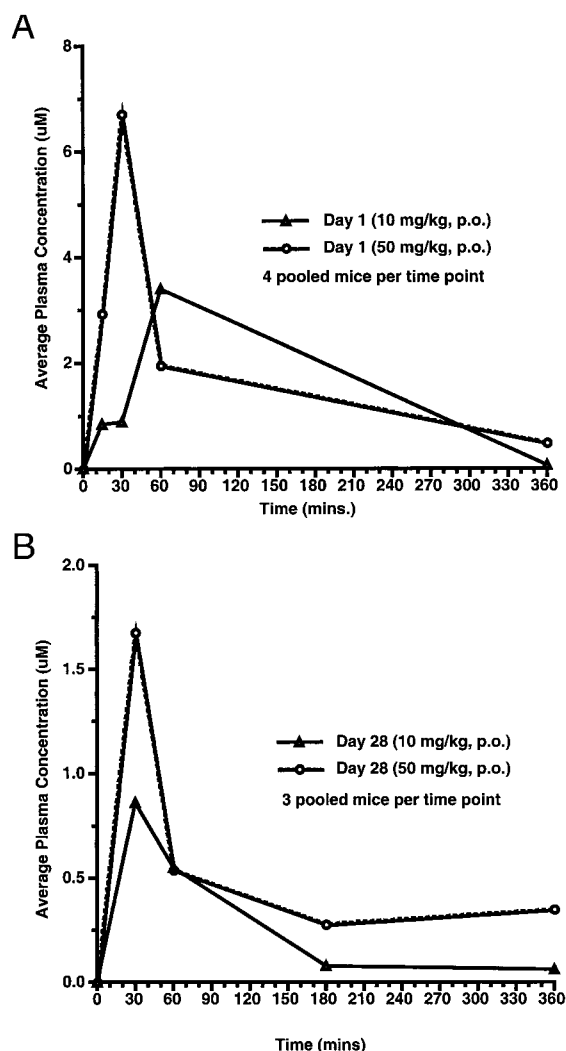


Fig. 5 A, Plasma concentrations of 9cRA in mice after a single dose. Mice were treated with a single dose of 9cRA, and blood was collected at 0, 15, 30, 60, and 360 min after treatment. Plasma concentrations of retinoids were measured as described in "Materials and Methods." Each point represents the plasma concentration of four pooled samples. B, plasma concentrations of 9cRA in mice after 28 days of chronic dosing. Mice were treated with either 10 or 50 mg/kg 9cRA for 28 days, and then blood was collected at 0, 30, 60, 180, and 360 min after a single dose. Plasma concentrations of retinoids were measured as described in "Materials and Methods." Each point represents the plasma concentration of three pooled samples.

ras activation. Recent studies by us also suggest that tumors arising in the SV40 Tag mice are less dependent on estrogen. Antiestrogens fail to prevent tumor formation in the SV40 Tag mice, and the mammary tumors that arise in these mice are ER negative.⁴ Thus, the C3(1) SV40 Tag mice represent a model of ER-negative breast tumorigenesis. Such ER-negative cells may

⁴ K. Wu, J. E. Green, S. K. Mohsin, and P. H. Brown, unpublished observations.

Table 2 Pharmacokinetics of 9cRA

	Time (min)	Concentration Concentration	
		(μM) 10 mg/kg	(μM) 50 mg/kg
After a single dose ^a	0	0	0
	15	0.85	2.93
	30	0.88	6.71
	60	3.40	2.96
	360	0.06	0.49
$C_{p_{\max}}$ (μM)		3.40	6.71
T_{\max} (min)		60	30
AUC^b ($\mu\text{g}/\text{min}/\text{ml}$)		180.6	227.1
After chronic dosing ^c	0	0	0
	30	0.86	1.68
	60	0.55	0.54
	180	0.08	0.28
	360	0.06	0.35
$C_{p_{\max}}$ (μM)		0.86	1.68
T_{\max} (min)		30	30
AUC ($\mu\text{g}/\text{min}/\text{ml}$)		25.3	49.4

^a Average plasma concentration of 9cRA from four pooled mice per time point.

^b AUC, area under the curve.

^c Average plasma concentration of 9cRA from three pooled mice per time point.

be less sensitive to retinoids. Our data, showing modest suppression of mammary tumorigenesis by 9cRA, along with those of Anzano *et al.* (37) showing more dramatic suppression in NMU-induced rats, suggest that retinoids can suppress the development of both ER-positive and ER-negative tumors but that retinoids may more effectively suppress ER-positive, estrogen-dependent tumors.

The C3(1)-SV40 Tag model, in which SV40 Tag inactivates *p53* and *Rb*, is a model of human breast tumors that arise by *p53* or *Rb* inactivation. *p53* and *Rb* mutations are commonly seen in human breast cancers (39, 48). Inactivation of *p53* by SV40 Tag serves as a model of tumors that lose *p53* function, either through loss of *p53* expression or through the expression of a mutant *p53* that functions as a dominant negative mutant. However, some human breast cancers have gain-of-function *p53* mutations (49). Such mutations likely cause cancer through additional molecular mechanisms involving transcriptional regulation and induction of genomic instability (50). The model used in this study is not an accurate model for breast tumors arising as a result of such gain-of-function *p53* mutants. However, the C3(1) SV40 Tag mice represent a model of loss-of-function *p53* mutations and thus represent an advance in modeling human breast cancer. The present results suggest that retinoids will suppress the development of breast cancers that arise through *p53* mutation, at least those that have dominant negative or loss-of-function mutations.

Although retinoids have been shown to induce differentiation in mammary cells (51, 52), we did not observe a shift in the morphology of the mammary epithelial cells from 9cRA-treated mice as compared with vehicle-treated mice. The most likely effect of 9cRA is therefore an antiproliferative effect, leading ultimately to a delay in tumor development. Studies by Crist *et al.* (53) suggest

that retinoids may exert their effects on the earlier stages of tumorigenesis. We measured the incidence of dysplasia and carcinoma *in situ* in tumors arising in vehicle- or retinoid-treated mice and found no difference between the two groups in the incidence of these early lesions.⁵ Thus, their preliminary results suggest that retinoids are exerting their effects at all stages of tumor development. However, all of these premalignant lesions were observed in the context of invasive cancers that had developed in these mice. To definitively determine whether retinoids suppress the development of premalignant lesions, we will need to assess the effect of retinoids at earlier time points, before invasive tumors develop.

In the present study, mice were treated with 9cRA at an early age to prevent tumor development. This was necessary due to the very rapid tumorigenesis in the SV40 Tag mice. However, if retinoids are to be used in women for breast cancer prevention, it is likely that they will be first used in selected groups only. Because many retinoids are teratogenic, the presently available retinoids will likely be initially limited to postmenopausal women. To make retinoids more generally useful, drug development efforts are now focused on developing less toxic, nonteratogenic retinoids.

The ability of retinoids to suppress tumor development in this and other models of mammary tumorigenesis suggests that 9cRA or possibly other less toxic retinoids are promising agents for the prevention of breast cancer. Retinoids may be most useful when combined with antiestrogens to effectively prevent breast cancer development. Studies have already been conducted examining the effects of retinoids in combination with tamoxifen (37) and with other newer generation-selective ER modulators (54). Through continued studies of synthetic retinoids, it will be possible to identify retinoids capable of suppressing tumorigenesis while avoiding the toxicity of naturally occurring retinoids. Further research into the mechanisms by which retinoids suppress tumorigenesis will help to clarify the role of retinoids in chemoprevention of breast cancer.

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