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Effect of Resveratrol on Growth of 4T1 Breast Cancer Cells *in Vitro* and *in Vivo*

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In vitro,* resveratrol inhibited growth of 4T1 breast cancer cells in a dose- and time-dependent manner. *In vivo,* however, resveratrol had no effect on time to tumor take, tumor growth, or metastasis when administered intraperitoneally daily (1, 3, or 5 mg/kg) for 23 days starting at the time of tumor inoculation. Resveratrol had no effect on body weight, organ histology, or estrous cycling of the tumor-bearing mice. Resveratrol, therefore, is a potent inhibitor of 4T1 breast cancer cells *in vitro;* is nontoxic to mice at 1–5 mg/kg; and has no growth-inhibitory effect on 4T1 breast cancer *in vivo.

***Key Words:* resveratrol; breast; neoplasms; 4T1 cells.**

Trans (*t*)-resveratrol (3,4′,5-trihydroxystilbene) is present naturally in grapes, fruits and a variety of medicinal plants (1, 2). It functions as a phytoalexin that protects plants against fungal infections (1). Because of its high concentration in grape skin, a significant amount of resveratrol is present in wines, especially red wines (3). It has been suggested that resveratrol may be partially responsible for the beneficial effect of red wines in protecting against coronary heart disease (e.g., the French Paradox) (4, 5).

Recently, resveratrol has also been shown to have cancer chemopreventive activity (6) and to inhibit the *in vitro* growth of a number of human cancer cell lines, including breast cancer cell lines such as MCF-7, MCF-10, T47D, MDA-MB-231, and the highly invasive MDA-MB-435 (7–12). Resveratrol inhibits the growth of both estrogen receptor (ER)-positive (MCF-7) and ER-negative (MCF-10, MDA-MB-231) breast cancer cells (8). The exact mechanism by which resveratrol inhibits the growth of breast cancer cells, is not clear. Lu *et al.* (10) reported that resveratrol acted as an estrogen antagonist in inhibiting the growth of MCF-7 cells, while Clement *et al.* (7) and Nakagawa *et al.* (12) demonstrated that resveratrol inhibited the growth of T47D and MCF-7 cells by inducing apoptosis. So far, there has been no report of the effect of resveratrol on the *in vivo* growth of breast cancer cells.

In the current study, we investigated the effect of resveratrol on the growth of a murine breast cancer cell line (4T1) both *in vitro* and *in vivo. In vitro,* resveratrol inhibited 4T1 cell proliferation in a dose- and time-dependent manner. However, *in vivo,* resveratrol at doses (1–5 mg/kg) that had previously been shown to inhibit the growth of ascites hepatoma (13) and Lewis lung carcinoma (14), had no effect on tumor growth and metastatic spread of 4T1 breast cancer cells.

MATERIALS AND METHODS

Chemicals. *t*-Resveratrol was purchased from Pharma Science (Montreal, Quebec, Canada). A stock solution of resveratrol was made in ethanol at 200 mM and was stored protected from light at −20°C. Working dilutions were directly made in the tissue culture medium. The control vehicle used was the tissue culture medium containing amounts of ethanol equivalent to those present in resveratrol. Etoposide (VP16) was purchased from the Sigma Chemical Co. (St. Louis, MO).

Culture and treatment of cells. 4T1 mammary carcinoma cells (obtained from F. Miller, Michigan Cancer Foundation) are an ERα, ERβ positive cell line. They are subpopulations of cells from a spontaneous mammary tumor in a C_3H/BALBc mouse, which can be maintained in culture and grown in syngeneic BALB/c mice where it metastasizes to the lungs (15). 4T1 cells were cultured in DMEM (without phenol red, Gibco-BRL, Grand Island, NY) supplemented with 10% charcoal stripped fetal bovine serum (Hyclone, Logan, UT), 1 mM non-essential amino acids, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. 4T1 cells (1×10^5/ml) in logarithmic growth phase were treated with various concentrations of resveratrol (10–50 μM), etoposide (2–50 μM) or vehicle (ethanol, 0.015% equivalent to the ethanol concentration in 30 μM resveratrol) alone as control. At 1–3 days after incubation, the number of viable cells was determined by trypan blue stain using a hemacytometer as previously described (16).

Animals. Animal studies were conducted in accordance with the NIH Guides for the Care and Use of Laboratory Animals, and

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were approved by the Institutional Animal Care and Use Committee of the Stratton VA Medical Center (Albany, NY). Thirty-two female BALB/c mice (Taconic Laboratories, Germantown, NY), 17 weeks of age, housed 4/cage alongside singly housed male mice to enhance estrous cycling, were used. Animals were divided into four treatment groups (8/group). Animals were kept on lighting schedules (12 h light/12 h dark) with food and water freely available.

Tumor. 4T1 mammary carcinoma cells were maintained *in vitro* until passage into the animal. Tumor cells were inoculated subcutaneously at 1×10^5 viable cells in the right hind flank. Animals received a daily intraperitoneal (ip) injection of either culture medium or resveratrol (1, 3, or 5 mg/kg). Tumors were measured daily (length, width, height) by the same individual using calipers. Tumors were excised from animals 23 days after inoculation, at an average size of 2500 mm^3, weighed and half of the tumor fixed in 10% formalin and the other half frozen (−80°C). Lungs were collected (absence/presence of metastases noted, number of metastases recorded) and fixed in Bouin's solution (0.9% picric acid, 9% formaldehyde, 5% acetic acid; Sigma). Kidneys, liver, mammary glands, uteri and ovaries were removed, weighed, and stored at −80°C for histopathological examination.

Fertility phase determination. Daily vaginal smears were done using sterile saline washings, stained with Diff Quik (J. T. Baker, Phillipsburg, NJ) and were read by one individual using standard criteria (17). Slides from each mouse were read in a daily sequence 24 h apart to determine the progression of cycling and classify smears as proestrus, estrus, metestrus, or diestrus. Estrous cycle stage was determined daily, from 4 days prior to tumor inoculation until the day of sacrifice.

Statistical analyses. All statistical analyses were performed using a commercially available statistical software (SPSS, Chicago, IL). Numerical values for cell numbers and tumor volumes were analyzed by one-way analysis of variance (ANOVA). Numbers of lung metastases were analyzed by χ^2 analysis. For all analyses, a difference was considered to be significant at $P < 0.05$.

RESULTS

Effect of Resveratrol on the Growth of 4T1 Cells in Vitro

When 4T1 cells were incubated with resveratrol, there was a dose-dependent inhibition of the growth of breast cancer cells. This was clearly evident as early as 1 day after incubation (Fig. 1A). Complete inhibition of 4T1 cells was noted at a resveratrol concentration of 30 μM. Based on the results at day 3, the estimated median effective concentration (ED_{50}) of resveratrol was approximately 13 μM. This value is similar to those reported for THP-1 human monocytic (16) and HL60 human promyelocytic leukemia cells (7, 18). However, compared to etoposide (Fig. 1B), resveratrol was less effective than etoposide in inhibiting the *in vitro* growth of 4T1 cells.

Effect of Resveratrol on the Growth of 4T1 Cells in Vivo

As shown in Fig. 2, resveratrol when given by daily ip injections at doses of 1, 3, or 5 mg/kg, had no effect on the growth of 4T1 mammary tumor in BALB/c female mice. Resveratrol also had no effect on the development of lung metastases (Fig. 3).

Resveratrol at the doses used, was not toxic to the animals as we observed no differences in animal body weights and behaviors. Histologic examination of tissue sections (liver, kidneys, uterus, ovaries, mammary glands) from control and resveratrol-treated animals also showed no detectable differences. Treatment with resveratrol did not alter uterine weights or estrous cycles as the resveratrol-treated animals continued to cycle with the same frequency as the control animals (data not shown).

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DISCUSSION

The results presented in the current study demonstrated that while it inhibited the *in vitro* growth of 4T1 breast cancer cells, resveratrol had no effect on the *in vivo* growth and metastasis of 4T1 breast cancer in mice. This observation cautions the extrapolation of *in vitro* findings to *in vivo* situations when *in vivo* studies are not actually carried out.

We investigated the *in vivo* effect of resveratrol at doses of 1–5 mg/kg per day. It is possible that our failure to demonstrate inhibition of 4T1 mammary tumor growth *in vivo*, may be due to an inadequate dose of resveratrol. However, resveratrol at these doses have previously been shown to be effective. Carbo *et al.* (13) reported that daily ip injections of resveratrol at 1 mg/kg for 7 days caused a significant decrease (25%) in the tumor cell content of an ascites hepatoma in rats. Likewise, Kimura and Okuda (14) demonstrated that daily ip injections of resveratrol at 2.5 or 10 mg/kg, prevented tumor growth (42% decrease in tumor volume and 44% decrease in tumor weight) and metastasis to lungs (56% decrease) of Lewis lung carcinoma in mice. In contrast, we observed that daily ip injections of resveratrol at 1–5 mg/kg for 23 days, neither inhibited the growth, nor prevented the metastatic spread of 4T1 breast cancer in mice.

Resveratrol, because of its structural similarity to the synthetic estrogen, diethylstilbestrol, was believed to be a phytoestrogen. Gehm *et al.* (19) reported that resveratrol stimulated the proliferation of ER-positive T47D breast cancer cells by acting as an agonist for the ER. In contrast, Clement *et al.* (7) reported that resveratrol inhibited the growth of T47D breast cancer cells by inducing Fas/Fas ligand-mediated apoptosis. *In vivo,* resveratrol has little or no estrogen agonism on reproductive and non-reproductive estrogen target tissues in growing rats. As a matter of fact, it could be an estrogen antagonist (20). Further studies by Bowers *et al.* (21) reveal that

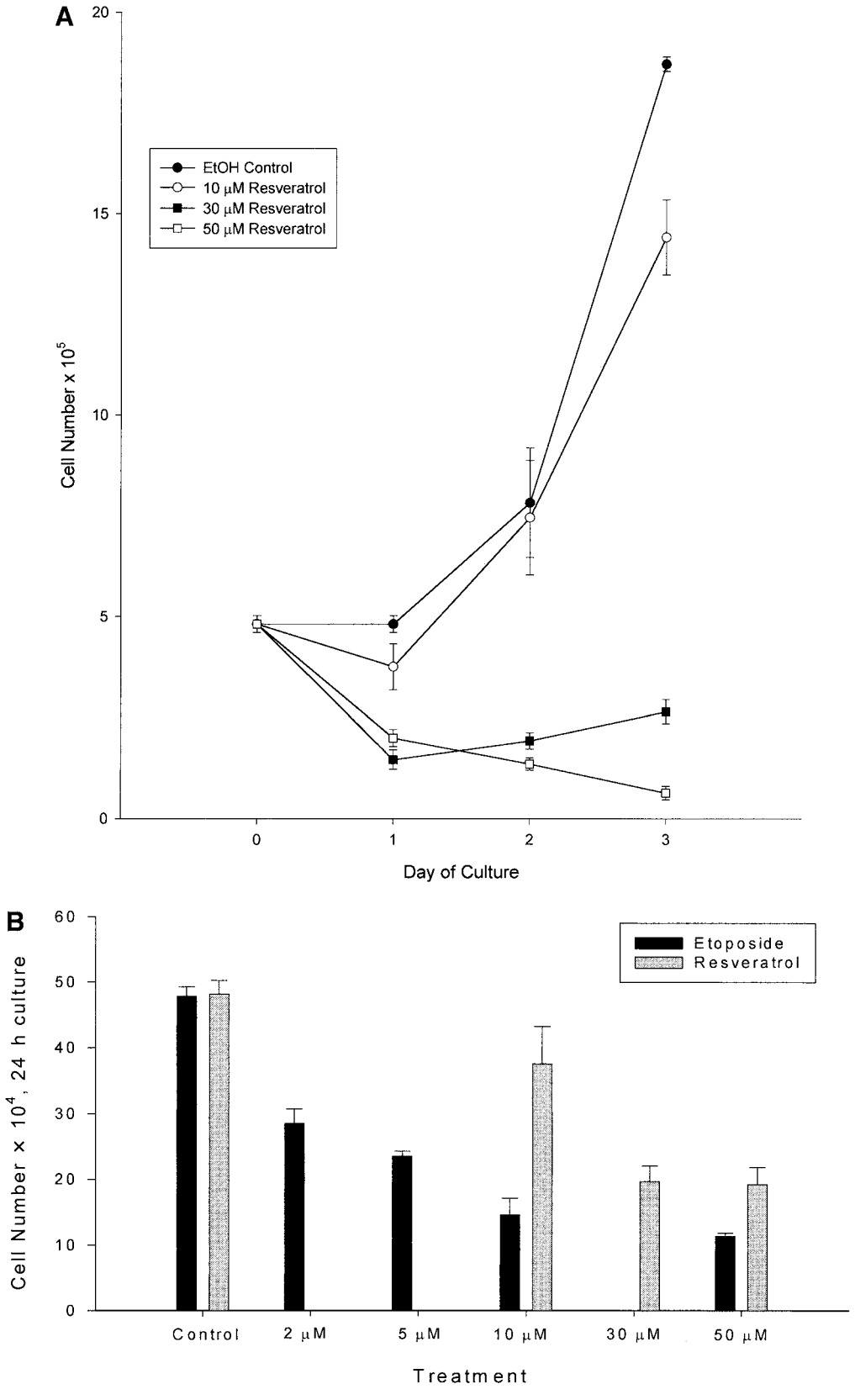

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FIG. 1. Effect of resveratrol on the *in vitro* growth of 4T1 breast cancer cells. (A) 4T1 cells (1×10^5/ml) were treated with or without resveratrol for 1–3 days and viable cells were then determined using trypan blue exclusion. Means ± SE, $n = 3$. (B) 4T1 cells (1×10^5/ml) were treated with either resveratrol (10, 30, 50 μM) or etoposide (2, 5, 10, 50 μM) for 24 h and viable cells were then determined using trypan blue exclusion. Means ± SE, $n = 3$.

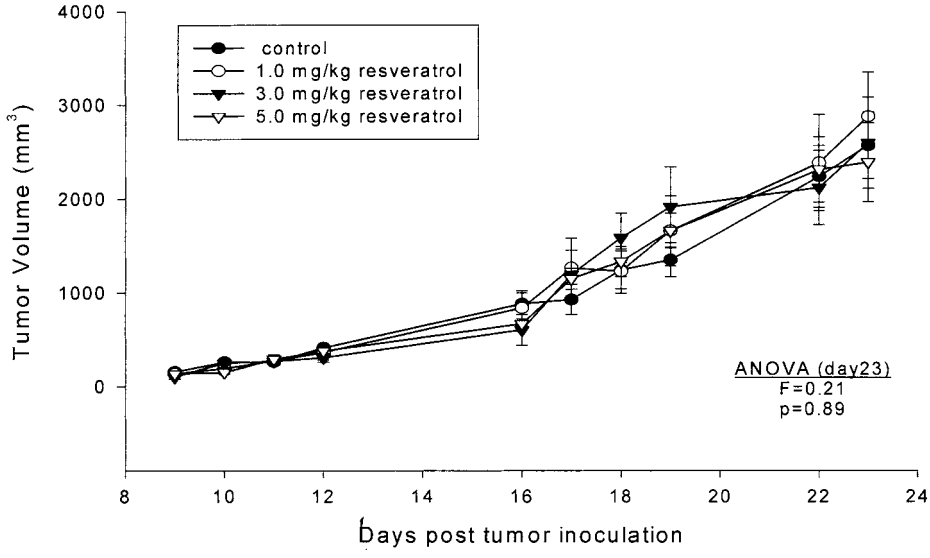

FIG. 2. Effect of resveratrol on 4T1 breast tumor growth *in vivo*. BALB/c female mice were injected subcutaneously with 4T1 cells (1×10^5) in the right hind flank and treated daily with intraperitoneal injections of varying doses (1–5 mg/kg) of resveratrol ($n = 8$/group) for 23 days. Tumor measurements were made daily by the same individual. Means ± SE.

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resveratrol exhibits estrogen antagonist activity for ERα with selected estrogen response elements (EREs), while it has no antagonist activity for ERβ. These data suggest that resveratrol differentially affects the transcriptional activities of ERα and ERβ in an ERE sequence-dependent manner. In the current study, we demonstrated that resveratrol had no effect on the estrous cycle frequency or uterine histology, suggesting that it has little or no estrogen agonism on the reproductive target tissues.

In recent years, there has been considerable interest in resveratrol as a potential cancer chemotherapeutic agent. However, most studies were performed using established cancer cell lines in *in-vitro* culture system. There is a paucity of studies demonstrating its *in vivo* anticancer effect. In view of the observations made in the current study, caution should be exercised in extrapolating the *in vitro* growth inhibitory effect to the *in vivo* anticancer effect of resveratrol.

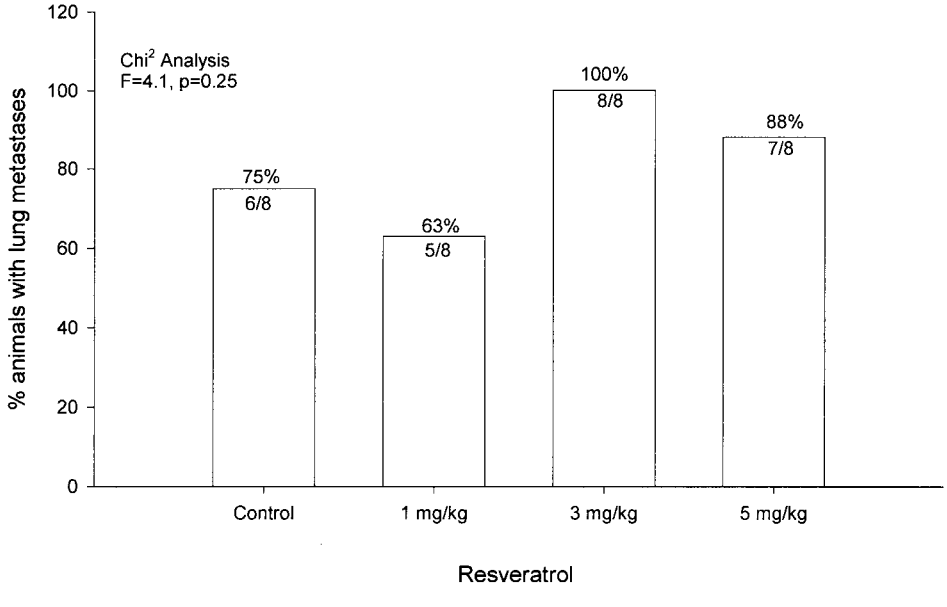

FIG. 3. Effect of resveratrol on the incidence of 4T1 breast tumor lung metastases. BALB/c female mice were injected with 4T1 cells (1×10^5) and treated daily with varying doses of resveratrol ($n = 8$/group) for 23 days as in Fig. 2. At sacrifice, lung metastases were counted. Means ± SE.

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