# Trichostatin A Is a Histone Deacetylase Inhibitor with Potent Antitumor Activity against Breast Cancer *in Vivo*[1]

**David M. Vigushin,[2] Simak Ali, Paul E. Pace, Nina Mirsaidi, Kazuhiro Ito, Ian Adcock, and R. Charles Coombes**

Department of Cancer Medicine, Cancer Research Campaign Laboratories, Imperial College of Science, Technology, and Medicine, Hammersmith Hospital, London W12 0NN [D. M. V., S. A., P. E. P., N. M., R. C. C.], and Department of Thoracic Medicine, National Heart and Lung Institute, Imperial College of Science, Technology, and Medicine, London SW3 6LY [K. I., I. A.], United Kingdom

## ABSTRACT

***Purpose:*** **Trichostatin A (TSA), an antifungal antibiotic with cytostatic and differentiating properties in mammalian cell culture, is a potent and specific inhibitor of histone deacetylase (HDAC) activity. The purpose of this study was to evaluate the antiproliferative and HDAC inhibitory activity of TSA *in vitro* in human breast cancer cell lines and to assess its antitumor efficacy and toxicity *in vivo* in a carcinogen-induced rat mammary cancer model.**

***Experimental Design and Results:*** **TSA inhibited proliferation of eight breast carcinoma cell lines with mean ± SD $IC_{50}$ of 124.4 ± 120.4 nM (range, 26.4–308.1 nM). HDAC inhibitory activity of TSA was similar in all cell lines with mean ± SD $IC_{50}$ of 2.4 ± 0.5 nM (range, 1.5–2.9 nM), and TSA treatment resulted in pronounced histone H4 hyperacetylation. In randomized controlled efficacy studies using the *N*-methyl-*N*-nitrosourea carcinogen-induced rat mammary carcinoma model, TSA had pronounced antitumor activity *in vivo* when administered to 16 animals at a dose of 500 μg/kg by s.c. injection daily for 4 weeks compared with 14 control animals. Furthermore, TSA did not cause any measurable toxicity in doses of up to 5 mg/kg by s.c. injection. Forty-one tumors from 26 animals were examined by histology. Six tumors from 3 rats treated with TSA and 14 tumors from 9 control animals were adenocarcinomas. In contrast, 19 tumors from 12 TSA-treated rats had a benign phenotype, either fibroadenoma or tubular adenoma, suggesting that the antitumor activity of TSA may be attributable to induction of differentiation. Two control rats each had tumors with benign histology.**

***Conclusions:*** **The present studies confirm the potent dose-dependent antitumor activity of TSA against breast cancer *in vitro* and *in vivo*, strongly supporting HDAC as a molecular target for anticancer therapy in breast cancer.**

## INTRODUCTION

Reversible acetylation of $NH_2$-terminal lysine residues of core histones is important in the modulation of chromatin topology and regulation of gene transcription. Histone acetylation relaxes the normally tight supercoiling of chromatin, enhancing accessibility of DNA-binding transcriptional regulatory proteins to promoter regions. Conversely, HDAC[3] maintains chromatin in a transcriptionally silent state. Nuclear receptor coactivators including p300/CBP and pCAF have intrinsic histone acetyltransferase activity, whereas corepressor proteins such as mSin3, N-CoR, and SMRT exist as complexes with HDAC enzymes (1–3). Six HDAC enzymes have been identified in mammalian cells to date. HDAC1, HDAC2, and HDAC3, which are homologues of the yeast transcriptional regulator RPD3, bind the E2F transcription factor and repress transcription through an association with the retinoblastoma protein (4, 5). HDAC4, HDAC5, and HDAC6 are homologues of yeast HDA1 and form a second family of deacetylases that are specifically involved in cell differentiation (6, 7). Inhibiting HDAC activity causes transcriptional activation of certain genes, such as the cyclin-dependent kinase inhibitor $p21^{Waf1/Cip1}$ (8), but repression of others (9). mHDA1 and mHDA2, the murine homologues of yeast HDA1, are expressed in a differentiation-dependent manner, and induction of gene expression occurs in response to HDAC inhibition (6). The antifungal antibiotic TSA is a noncompetitive reversible inhibitor of HDAC activity in cultured mammalian cells and in fractionated cell nuclear extracts at low nanomolar concentrations. TSA has been shown to arrest cells in $G_1$ and $G_2$ phases of the cell cycle, induce differentiation, and revert the transformed morphology of cells in culture (reviewed in Ref. 10). We report on the potent activity of TSA as an inhibitor of proliferation and HDAC activity in human breast cancer cell lines and antitumor efficacy without measurable toxicity in the NMU carcinogen-induced rat mammary carcinoma model.

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[2] To whom requests for reprints should be addressed, at Department of Cancer Medicine, Cancer Research Campaign Laboratories, 5th Floor MRC Cyclotron Building, Imperial College of Science, Technology and Medicine, Hammersmith Hospital, Du Cane Road, London W12 0NN, United Kingdom. Phone: 44-208-383-8370; Fax: 44-208-383-5830; E-mail: d.vigushin@ic.ac.uk.

[3] The abbreviations used are: HDAC, histone deacetylase; TSA, trichostatin A; NMU, *N*-methyl-*N*-nitrosourea; $IC_{50}$, 50% inhibitory concentration; PMSF, phenylmethylsulfonyl fluoride; ER, estrogen receptor; hER, human ER.

## MATERIALS AND METHODS

**Chemicals.** TSA was obtained from Sigma-Aldrich Company Ltd. (Dorset, United Kingdom). For cell proliferation and *in vitro* enzyme inhibition studies, a 10 mM solution of TSA in absolute ethanol was prepared and stored at −20°C until use. A stock solution of 2 mg/ml TSA in DMSO was used for *in vivo* experiments.

**Cell Proliferation Assay.** Stock cultures of breast cancer cell lines MCF-7, T-47D, ZR-75-1, BT-474, MDA-MB-231, MDA-MB-453, CAL 51, and SK-BR-3 (American Type Culture Collection, Rockville, MD) were grown in DMEM containing 10% (v/v) FCS, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% $CO_2$ humidified atmosphere. Cells were counted in a hemocytometer after detachment using 0.25% (w/v) trypsin in Dulbecco's PBS without $Ca^{2+}$ or $Mg^{2+}$ (DPBS; Sigma-Aldrich) containing 0.02% (w/v) EDTA. Viability was determined by trypan blue exclusion. For each cell line, cells were seeded in 96-well microtiter plates at optimal densities determined in prior experiments to ensure exponential growth for the duration of the assay. After a 24-h preincubation, growth medium was replaced with experimental medium containing TSA at final concentrations ranging from $10^{-12}$ M to $10^{-5}$ M in log dilutions and 0.1% (v/v) ethanol, or growth medium containing 0.1% (v/v) ethanol as a vehicle control. After 96 h incubation, cell proliferation was estimated using the sulforhodamine B colorimetric assay (11), and the results are expressed as the mean ± SD for six replicates as a percentage of vehicle control (taken as 100%).

**Immunodetection of Acetylated Histone H4.** Immunodetection of acetylated histone H4 in cell lines by Western blotting was performed as described (10, 12), with the following modifications. Each cell line (~$1 \times 10^5$ cells) was treated with 2 μM TSA in 0.1% ethanol or with 0.1% ethanol as vehicle control for 24 h at 37°C. Cells were lysed directly in 200 μl of boiling Laemmli sample buffer containing 10% (v/v) 2-mercaptoethanol. Protein concentration of each sample was determined using the Bio-Rad (Bradford) Protein Assay kit (Bio-Rad Laboratories, Ltd., Hertfordshire, United Kingdom). Each sample (20 μg protein) was then separated by 4–12% gradient SDS-PAGE (NuPAGE; Invitrogen, Groningen, the Netherlands). Proteins were transferred by electroblotting onto nitrocellulose membrane (Immobilon-NC HAHY; Millipore, Hertfordshire, United Kingdom), and acetylated histone H4 was detected with a rabbit polyclonal antihuman acetylated H4 antibody (Upstate Biotechnology, Lake Placid, NY) diluted 1:1000 in 3% nonfat dried milk powder in 150 mM NaCl, 10 mM Tris (pH 8.0). A goat antirabbit IgG F(ab′)$_2$ secondary antibody-alkaline phosphatase conjugate (Sigma-Aldrich) was visualized using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium color substrate.

**Immunoprecipitation of Human Estrogen Receptor α and Western Blot Analysis.** MCF-7 cells (~$2.5 \times 10^6$) at 70–80% confluence in 15-cm plates were treated with 0.5 μM TSA in 0.1% ethanol or 0.1% ethanol as vehicle control for 24 h at 37°C. After washing with chilled DPBS, cells were harvested in DPBS, pelleted by centrifugation (1000 × *g* for 5 min at 4°C), and resuspended in 100 μl of high salt buffer [400 mM KCl, 20 mM Tris (pH 7.4), 2 mM DTT, and 20% (v/v) glycerol] containing a freshly added mixture of protease inhibitors (1 mM PMSF and 0.5 μg/ml each of leupeptin, pepstatin A, chymostatin, antitrypsin, and aprotinin). Whole cell extracts were prepared by three cycles of freezing (−80°C) and thawing (0°C), clarified by centrifugation at 15,000 × *g* for 15 min at 4°C, and the supernatants were stored at −80°C (13). Protein concentration of each extract was determined using the Bio-Rad Protein Assay.

Each whole cell extract (500 μg protein) was suspended in 1 ml of buffer A [400 mM NaCl, 50 mM Tris acetate (pH 7.5), 1 mM DTT, 1% (v/v) Triton X-100, 1 mM PMSF, and 0.5 μg/ml each of leupeptin, pepstatin A, chymostatin, antitrypsin, and aprotinin] and precleared with addition of 2 mg of Protein G-Sepharose 4B (Sigma-Aldrich; previously equilibrated in buffer A), followed by incubation on a rotating platform for 45 min at 4°C. After centrifugation at 15,000 × *g* for 15 s at 4°C, 2 μg of a mouse monoclonal anti-hERα antibody (clone B10; Ref. 13) were added to the supernatant, and the sample was incubated for 30 min at 4°C. Then 4 mg of buffer A-washed Protein G-Sepharose 4B were added, and samples were rotated at 4°C for 60 min, followed by four washes with buffer A containing 0.2% (w/v) SDS (13). Retained immune-complexes were eluted by boiling in Laemmli sample buffer, and the protein concentration of each sample was determined using the Bio-Rad Protein assay. Each sample (50 μg protein) was separated by 4–12% gradient SDS-PAGE, and proteins were transferred by electroblotting onto nitrocellulose membranes. hERα was detected with a mouse monoclonal anti-hERα (clone B10) primary antibody 1 μg/ml in 5% nonfat dried milk powder in 150 mM NaCl, 10 mM Tris (pH 8.0), 0.05% (v/v) Tween 20 by overnight incubation at 4°C, followed by a goat antimouse IgG secondary antibody-alkaline phosphatase conjugate (Sigma-Aldrich). Immunodetection of acetylated hERα was performed in a parallel incubation using a rabbit polyclonal anti-acetylated lysine antibody (a generous gift from C. Crane-Robinson) diluted 1:1000 in 5% nonfat dried milk powder in 150 mM NaCl, 10 mM Tris (pH 8.0), and 0.05% (v/v) Tween 20 by overnight incubation at 4°C, followed by goat antirabbit IgG F(ab′)$_2$

*Table 1* Effect of TSA on breast cancer cell proliferation and HDAC activity

| Cell type | Cell proliferation[a] $IC_{50}$ (nM) | HDAC[b] $IC_{50}$ (nM) |
|---|---|---|
| T-47D | 26.4 | 1.6 |
| MCF-7 | 27.7 | 1.6 |
| BT-474 | 33.3 | 2.4 |
| SK-BR-3 | 45.3 | 2.2 |
| ZR-75-1 | 102.6 | 2.6 |
| CAL 51 | 144.4 | 1.6 |
| MDA-MB-453 | 307 | 1.9 |
| MDA-MB-231 | 308.1 | 0.6 |
| Mean ± SD | 124.4 ± 120.4 | 1.8 ± 0.6 |

[a] The effect of TSA on cell proliferation was determined using the sulforhodamine B assay as described in "Materials and Methods."

[b] HDAC activity in cellular extracts was measured by incubation with [$^3$H]acetate-labeled histone H4 peptide substrate (~$1.5 \times 10^6$ cpm) in a reaction volume of 200 μl, and released [$^3$H]acetate was quantified by scintillation counting. $IC_{50}$ were determined graphically using nonlinear regression to fit inhibition data to the appropriate dose-response curve as described in "Materials and Methods."

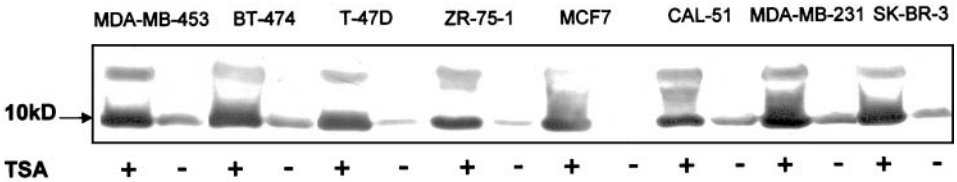


*Fig. 1* Western blot analysis of acetylated histone H4 in breast cancer cell lines. Cells ($\sim 1 \times 10^5$) were treated with 2 μM TSA in 0.1% (v/v) ethanol or 0.1% (v/v) ethanol as vehicle control for 24 h at 37°C. An acetylation-specific anti-histone H4 antibody was used to probe Western blots as described in "Materials and Methods."

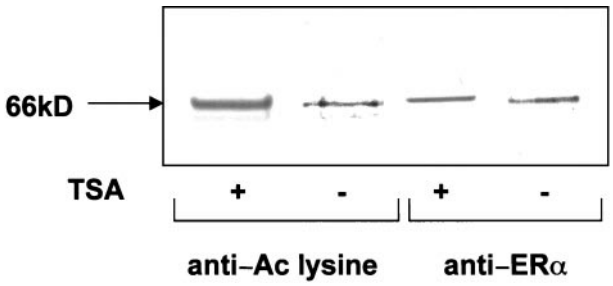


*Fig. 2* Immunodetection of acetylated hERα in MCF-7 breast carcinoma cells. Cells ($\sim 2.5 \times 10^6$) were treated with 0.5 μM TSA in 0.1% (v/v) ethanol or 0.1% ethanol (v/v) as vehicle control for 24 h at 37°C. hERα was immunoprecipitated from whole cell extracts; an antibody that recognize acetylated lysine residues (anti-AcLys) and another antibody to hERα (anti-ERα) were used to probe Western blots as described in "Materials and Methods."

secondary antibody-alkaline phosphatase conjugate (Sigma-Aldrich). Color was visualized using a 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate.

**HDAC Assay.** Total cellular extracts were prepared from each breast cancer cell line (14). All procedures were performed at 4°C. Briefly, $\sim 2.5 \times 10^6$ cells were washed with ice-cold DPBS and resuspended in 200 μl of lysis buffer [120 mM NaCl, 50 mM Tris (pH 7.5), 5 mM EDTA, and 0.5% (v/v) NP40] in the presence of freshly added protease inhibitors (2 μg/ml aprotinin, 10 μg/ml chymostatin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 0.5 mM PMSF). After disruption in a Dounce homogenizer and brief sonication, total cell lysates were cleared by two rounds of centrifugation at 15,000 × *g* for 10 min, and the supernatants were stored at −80°C.

*In vitro* HDAC activity was assayed as described previously (14, 15). Briefly, 20 μl of crude cell extract ($\sim 2.5 \times 10^5$ cells), in the presence of varying concentrations of TSA in 0.1% (v/v) ethanol or 0.1% (v/v) ethanol as vehicle control, were incubated for 60 min at 25°C with 1 μl ($\sim 1.5 \times 10^6$ cpm) of [$^3$H]acetyl-labeled histone H4 peptide substrate ($NH_2$-terminal residues 2–20) that had been acetylated with [$^3$H]acetic acid, sodium salt (3.7 GBq/mmol; New England Nuclear, Boston, MA) by an *in vitro* incorporation method (15). Each 200-μl reaction was quenched with 50 μl of 1 M HCl/0.16 M acetic acid and extracted with 600 μl of ethyl acetate, and released [$^3$H]acetate was quantified by scintillation counting.

***In Vivo Studies.*** Inbred virgin female (Ludwig/Wistar/Olac) rats bearing tumors induced with NMU were supplied by Harlan (UK) Ltd. (Oxfordshire, United Kingdom). All animals

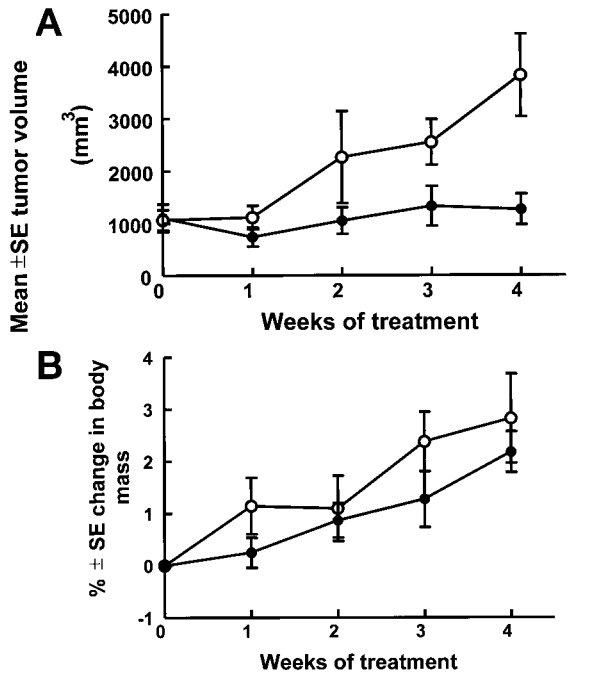


*Fig. 3* *A*, effect of TSA on growth of NMU-induced rat mammary tumors. Sixteen animals received 500 μg/kg TSA in 50 μl of DMSO, and 14 animals were given 50 μl of DMSO as vehicle control by s.c. injection daily for 4 weeks. Tumor measurements were obtained each week, and the tumor volume was calculated as described in "Materials and Methods." Results are expressed as the mean tumor volume ($mm^3$) for the TSA-treated (●) and vehicle control groups (○). *Bars*, SE. *B*, effect of TSA on body mass of rats bearing NMU-induced mammary tumors. Results are expressed as the percentage change from the mean body mass at the start of the experiment for TSA-treated (●) and vehicle control groups (○). *Bars*, SE.

were maintained and treated under Home Office license in accordance with the provisions of the Animals (Scientific Procedures) Act, 1986 of the United Kingdom. Experimental studies were conducted in the manner described previously (16) on adult rats bearing tumors measuring between 10 and 20 mm in diameter. Tumor dimensions were determined weekly by measuring two diameters at right angles with Vernier calipers. Tumor volume was estimated using the following formula:

$$1/6\pi[(d_1 \times d_2)^{3/2}]$$

where $d_1$ and $d_2$ are the two perpendicular diameters.

For the initial dose-ranging evaluation, 5 animals each

received a single dose of TSA ranging from 500 ng/kg to 5 mg/kg in 50 μl of DMSO by s.c. injection; one animal received 50 μl of DMSO by s.c. injection as a vehicle control.

Twelve rats were randomized to receive 500 μg/kg TSA in 50 μl DMSO, or 50 μl DMSO as vehicle control, by s.c. injection twice weekly for 4 weeks. In subsequent studies, 30 rats were randomized to receive TSA 500 μg/kg in 50 μl DMSO, or 50 μl DMSO as vehicle control, by s.c. injection daily for 4 weeks. Weekly tumor measurements, estimated tumor volumes, and body mass were recorded for each animal. Animals were sacrificed at the end of the 4-week study period; palpable tumors were resected and immediately snap-frozen in liquid nitrogen. Animals with tumors <2 cm in diameter or ulcerating tumors were withdrawn from study.

**Histopathology.** Tumors were fixed overnight in 10% (v/v) formalin in 0.9% NaCl solution before paraffin embedding and routine sectioning. Three representative H&E-stained sections were examined from each tumor. Tumors were classified according to the WHO classification of rat mammary tumors (17).

**Statistical Considerations.** The nonparametric Mann-Whitney *U* test was used for comparative statistical analysis of paired data and the Fisher's exact test for comparison of groups. Statistical significance was defined at the 5% level ($\alpha = 0.05$). The concentration of TSA that inhibited HDAC activity or cell proliferation by 50% ($IC_{50}$) was determined graphically in each case using nonlinear regression analysis to fit inhibition data to the appropriate dose-response curve (GraphPad Prism version 2.0; GraphPad Software, Inc., San Diego, CA).

## RESULTS AND DISCUSSION

TSA was found to have potent antiproliferative activity in eight breast cancer cell lines using the sulforhodamine B assay (Table 1). After a 96-h incubation, the mean ± SD $IC_{50}$ was 124.4 ± 120.4 nM (range, 26.4–308.1 nM). Interestingly, the cell lines that express ERα (T-47D, MCF-7, BT-474, and ZR-75-1; immunohistochemical and reverse transcription-PCR, data not shown) were more sensitive to TSA (mean ± SD $IC_{50}$, 47.5 ± 36.9 nM) than the ERα-negative cell lines (SK-BR-3, CAL 51, MDA-MB-453, and MDA-MB-231; mean ± SD $IC_{50}$, 201.2 ± 129.3 nM; $P = 0.03$, Mann Whitney $U = 1$). However, total HDAC inhibitory activity of TSA was similar (mean ± SD $IC_{50}$, 2.4 ± 0.5 nM; range, 1.5–2.9 nM; Table 1), and TSA treatment induced pronounced histone H4 hyperacetylation in all of the breast cancer cell lines (Fig. 1). Differential sensitivity of the ERα-positive cell lines to TSA suggests that the mechanism of growth inhibition is different from cells that do not express functional ERα. Although the explanation for this observation is still unclear, one possibility is that inhibition of HDAC activity by TSA might induce hyperacetylation of the ER itself, such as in the DNA-binding domain, which could affect dimerization of the receptor. In preliminary experiments, hERα immunoprecipitated from lysates of MCF-7 cells grown in the presence and absence of TSA has been probed with an antibody to acetylated lysine residues in Western blot analysis and found to be hyperacetylated in response to TSA treatment (Fig. 2).

Initial toxicity and dose-ranging studies *in vivo* in rats bearing NMU-induced mammary carcinomas showed that TSA was well tolerated in single doses of up to 5 mg/kg administered by s.c. injection. Chemotherapeutic activity without toxicity was observed at doses of both 500 μg/kg and 5 mg/kg. Twelve animals were then randomized, 6 to receive 500 μg/kg TSA in 50 μl of DMSO and 6 to receive 50 μl of DMSO as vehicle control by s.c. injection twice weekly for 4 weeks. Three animals failed to complete the study, two in the TSA arm and one control; all had progressive disease and developed tumors >2 cm in diameter or ulcerating tumors. An initial tumor response was observed in 5 of the TSA-treated group. However, the response was short-lived, and subsequent disease progression occurred in all but one of the responding animals. There was no evidence of tumor regression in any control animal. The body mass of TSA-treated animals did not differ significantly from controls ($P > 0.05$; Mann-Whitney *U* test), and there was no evidence of systemic toxicity attributable to TSA.

*Table 2* Effect of TSA on growth of NMU-induced rat mammary carcinomas

Rats were given either drug or vehicle as described in "Materials and Methods" daily by s.c. injection for 4 weeks. Tumor growth was recorded every week by measuring two diameters at right angles using Vernier calipers, and tumor volume was estimated using the formula given in "Materials and Methods." Animals were categorized into three groups according to tumor response: (a) 50% or greater tumor regression; (b) <50% tumor regression; and (c) tumor progression.

| | Treatment groups | |
|---|---|---|
| | Control | TSA 500 μg/kg |
| No. of rats | 14 | 16 |
| No. of initial tumors >10 mm | 22 | 24 |
| No. of new tumors | 10 | 12 |
| No. (%) of rats with <50% tumor regression | 1 (8) | 5 (31) |
| No. (%) of rats with >50% tumor regression | 0 (0) | 7 (44) |
| No. (%) of rats with tumor progression | 13 (93) | 4 (25) |
| No. (%) of rats showing any regression | 1 (7) | 12 (75[a]) |

[a] Fisher's exact test: TSA *versus* control $P = 0.0002$.

Further studies were then undertaken to optimize the schedule of TSA administration. Thirty rats were randomized, 16 to receive 500 μg/kg TSA in 50 μl of DMSO and 14 to receive 50 μl of DMSO as vehicle control by s.c. injection daily for 4 weeks. The effect of TSA administration on the growth of NMU-induced tumors is depicted in Fig. 3*A*. TSA significantly inhibited tumor growth compared with control (Mann-Whitney $U = 4.5$, $P = 0.04$). Mean ± SE tumor volume of the control group increased by 260.4 ± 74.5% after 4 weeks, whereas the mean ± SE tumor volume of the TSA-treated animals was only 14.1 ± 26.6% greater after 4 weeks than at the commencement of the experiment. TSA treatment resulted in tumor regression in 12 of 16 rats compared with only 1 of 14 controls ($P = 0.0002$, Fisher's exact test; Table 2). Body mass of the TSA-treated rats did not differ significantly from controls (Mann-Whitney $U = 8.5$, $P > 0.05$; Fig. 3*B*), confirming the observation that TSA did not cause serious systemic toxicity.

NMU-induced rat mammary tumors rarely metastasize; nonetheless, most can be classified as malignant adenocarcinomas on the basis of abnormal cytological features and the pattern of local growth (18). Forty-one tumors from 26 animals in the present study were examined by histology. Fourteen

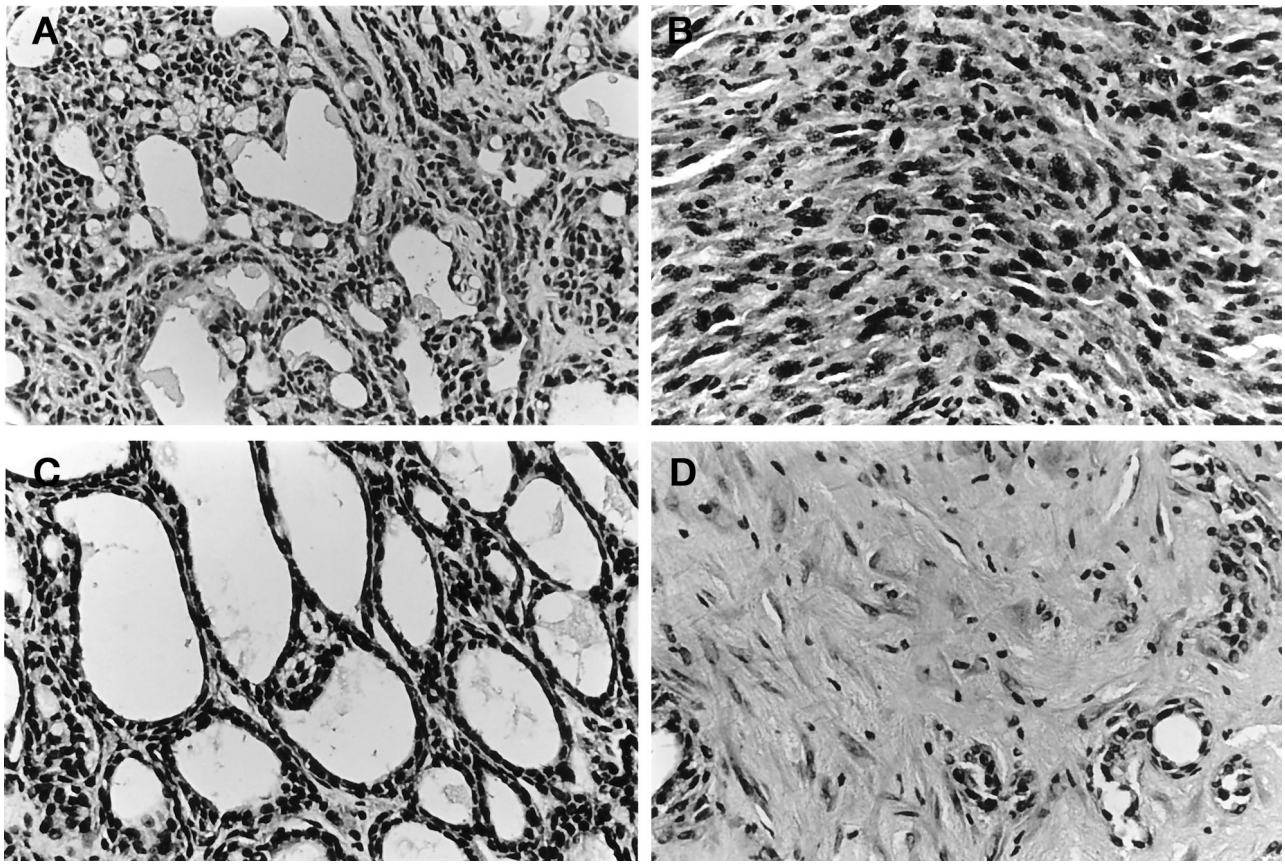


*Fig. 4* Photomicrographs of tumor sections stained with H&E. *A,* cribiform/papillary carcinoma in a control animal. Masses of proliferating epithelial cells surround acinar spaces of varying size and shape, some of which contain secretion. ×100. *B,* anaplastic carcinoma refractory to TSA treatment. Epithelial cells with high mitotic activity are arranged in irregular sheets with little stroma. Acini are absent. ×200. *C,* benign tubular adenoma in a tumor that responded to TSA. The tumor is composed of simple tubular structures separated by only a small amount of stroma. An occasional myoepithelial cell is present in some of the tubules. ×200. *D,* benign pericanalicular fibroadenoma in a trichostatin A-responsive tumor. Bands of fibrous tissue surround islets of secretory epithelium and myoepithelium with loss of lobular structure. ×200.

tumors from 9 control animals were adenocarcinomas of cribiform/papillary type (Fig. 4*A*). Two control rats each had tumors with benign histology. Six tumors from 3 rats refractory to TSA treatment were examined; 5 were adenocarcinomas, whereas a sixth tumor was an anaplastic carcinoma (Fig. 4*B*). However, 19 tumors from 12 other animals that responded to TSA had a benign histological phenotype, either tubular adenoma (Fig. 4*C*) or fibroadenoma (Fig. 4*D*). Although sequential tissue samples from individual animals were not obtained in the present study, the benign histology of most tumors in the TSA-treated group compared with the malignant phenotype of tumors in the majority of controls ($P < 0.0001$, Fisher's exact test), suggests that induction of differentiation is the predominant mechanism of antitumor action of TSA.

Our observations suggest that differential sensitivity of ERα-positive breast cancer cell lines in culture to the growth-inhibitory effects of TSA may be attributable to hyperacetylation of the ERα. Because NMU-induced rat mammary carcinomas express functional ERα (19) and most are hormone responsive (20), it is possible that the observed antitumor activity of TSA *in vivo* in this model may similarly be mediated by inhibition of ER functional activity. In future experiments, it will be important to establish whether the anti-estrogen tamoxifen in combination with TSA has greater antitumor efficacy than either agent alone in this model. Another important question to be addressed in future work is whether TSA treatment can prevent the establishment of rat mammary tumors after administration of NMU. Further studies are currently in progress to evaluate the antitumor efficacy of TSA in nude mice xenograft models of ERα-positive and ERα-negative breast cancer.

In the present studies, we have found that TSA is a potent inhibitor of breast cancer cell proliferation, inhibits HDAC activity in a dose-dependent manner, and induces pronounced histone H4 hyperacetylation. TSA has pronounced dose-dependent antitumor activity *in vivo* in the NMU-induced rat mammary carcinoma model when administered at a dose of 500 μg/kg by s.c. injection daily for 4 weeks. Furthermore, TSA does not cause any measurable toxicity in doses of up to 5 mg/kg by s.c. injection, a dose 10-fold higher than the effective antitumor dose in this model. Histological staining has shown a preponderance of cribiform/papillary adenocarcinoma in tumors that were resistant to TSA treatment and in control animals. However, in responding tumors, the predominant histological

phenotype was benign tubular adenoma or fibroadenoma, suggesting that the antitumor effects of TSA may have resulted from induction of differentiation. The present studies therefore confirm that TSA has potent dose-dependent antitumor activity against breast cancer *in vitro* and *in vivo*, strongly supporting HDAC as a molecular target for anticancer therapy in breast cancer. Together with the pronounced antitumor activity of TSA and striking absence of toxicity in this carcinogen-induced mammary cancer model, these observations provide a rational basis for further Phase I development of TSA in the treatment of human breast cancer. Because TSA also has potent cytostatic activity in a wide range of solid tumor and hematological cell lines in culture, further studies are needed to characterize the antitumor activity of TSA *in vivo* in other tumor types.

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