

Effect of Epidermal Growth Factor Receptor Inhibitor on Development of Estrogen Receptor–Negative Mammary Tumors

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Background: Although antiestrogens have been effective in preventing estrogen receptor (ER)-positive breast cancer, chemopreventive agents are still needed to prevent ER-negative breast cancer. Tyrosine kinase inhibitors are promising agents for the treatment and prevention of human cancers. ZD1839 (gefitinib or Iressa) is an orally active epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor that blocks signal transduction pathways in epithelial cells. We examined whether ZD1839 blocks signal transduction and prevents the development of ER-negative breast cancer. **Methods:** The ability of ZD1839 to block signal transduction in normal, immortalized, and malignant breast cells was assessed by western blotting with specific antibodies to detect phosphorylation of cytoplasmic signaling molecules. The effect of ZD1839 on growth of these breast cells was assessed with anchorage-dependent and anchorage-independent growth assays. Its effect on ER-negative mammary tumorigenesis was assessed in MMTV-erbB2 transgenic mice. All statistical tests were two-sided. **Results:** ZD1839 suppressed the phosphorylation of EGFR and mitogen-activated protein kinase in normal and malignant breast cells. ZD1839 treatment statistically significantly suppressed mammary tumorigenesis in MMTV-erbB2 transgenic mice; median time to tumor development was approximately 230 days in vehicle-treated mice and more than 310 days in mice treated with ZD1839 at 100 mg/kg ($P < .001$). ZD1839 reduced proliferation of normal breast cells by 20.3% (95% confidence interval [CI] = -13.7% to 44.2%) and of tumor cells by 42.0% (95% CI = 20.2% to 58.2%). ZD1839 also increased expression of the cell cycle regulator p27 in normal mammary tissue by 48.7% (95% CI = 27.0% to 74.2%) and in tumor tissue by 50.3% (95% CI = 35.8% to 66.7%). **Conclusion:** This study appears to provide the preclinical rationale for the development of these EGFR tyrosine kinase inhibitors for the prevention of human breast cancer. [J Natl Cancer Inst 2003;95:1825–33]

Breast cancer is a major public health concern in the United States and is the second highest cause of cancer death in women (1). These statistics highlight the need to further develop methods for the treatment and prevention of breast cancer. Results from the National Surgical Adjuvant Breast and Bowel Project's Breast Cancer Prevention Trial (P-1) (2) and more recent trials (3–5), such as the Multiple Outcomes of Raloxifene Evaluation (MORE) (3) and the International Breast Cancer Intervention

Study (IBIS-I) (5), demonstrated the effectiveness of antiestrogens such as tamoxifen and raloxifene in reducing the risk of breast cancer. Although these agents reduced the incidence of estrogen receptor (ER)-positive breast cancer, they did not reduce the incidence of ER-negative breast cancer. Thus, chemopreventive agents with the potential to prevent ER-negative breast cancers are urgently needed.

Several agents prevent ER-positive breast cancer in carcinogen-treated rats, including retinoids, vitamin D analogs, difluoromethylornithine, cyclooxygenase-2 inhibitors, and antiestrogens. We have recently shown that naturally occurring and synthetic retinoids prevent the development of ER-negative breast cancer in transgenic mice (6,7). However, the toxicity of these agents may limit their use in women. We have therefore investigated the cancer-preventing activity of other novel agents.

Some of the most promising agents for the prevention of ER-negative breast cancer are growth factor receptor tyrosine kinase inhibitors. The epidermal growth factor receptor (EGFR) is one of a family of four closely related receptors (EGFR or erbB1, HER2/neu or erbB2, HER3 or erbB3, and HER4 or erbB4) that use tyrosine kinase activity as the signal transduction trigger. The EGFR pathway contributes to a number of processes involved in tumor survival and growth, including cell proliferation and inhibition of apoptosis, angiogenesis, and metastasis, thus making it an attractive target for cancer prevention and treatment. ZD1839 (gefitinib or Iressa) is an orally active EGFR tyrosine kinase inhibitor that blocks signal transduction processes implicated in the proliferation and survival of cancer cells. Several *in vitro* and *in vivo* studies (8,9) have shown that ZD1839 can inhibit the growth of various types of cancer cells including breast cancer cells. ZD1839 also suppresses the growth of xenografts (8,9) and noninvasive human breast cancer or ductal carcinoma *in situ* (10).

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In this study, we investigated the ability of ZD1839 to inhibit the growth of normal, precancerous, and malignant human mammary epithelial cells and to prevent tumorigenesis in a model relevant to human ER-negative breast cancer. We also determined the effect of ZD1839 on signal transduction in ER-negative breast cells.

MATERIALS AND METHODS

Drugs and Growth Factors

ZD1839 was obtained from AstraZeneca (Macclesfield, U.K.). For tissue culture studies, ZD1839 was suspended in dimethyl sulfoxide at a final concentration of 1 μ M. For *in vivo* animal studies, ZD1839 was suspended in distilled water containing 1% Tween 80. Purified human recombinant EGF was obtained from Sigma Chemical Company (Carlsbad, CA).

Cell Lines

Normal human mammary epithelial cells (HMECs) were obtained from Cambrex (East Rutherford, NJ) and from Martha Stampfer (184 cells, Lawrence Berkeley National Laboratory, Berkeley, CA). The immortal cell line MCF10A was obtained from American Type Culture Collection (Manassas, VA), and the immortalized cell line 184B5 was obtained from Martha Stampfer. The breast cancer cell line MDA-MB-468 was obtained from American Type Culture Collection. Cells were grown in the following culture media: MEGM (Clonetics, San Diego, CA) for normal HMECs and 184 and 184B5 cells; DME/F-12 with 5% horse serum and supplements (1.4 $\times 10^{-6}$ M hydrocortisone, insulin at 10 μ g/mL, cholera toxin at 100 ng/mL, EGF at 20 ng/mL, penicillin at 100 U/mL, and streptomycin at 100 μ g/mL) for MCF10A cells; and improved minimum essential medium (high-zinc option; Life Technologies, Rockville, MD) supplemented with 10% fetal calf serum and penicillin (100 U/mL)/streptomycin (100 μ g/mL) (Life Technologies) for MDA-MB-468 cells. All cell lines used were ER-negative.

Western Blot Analysis

After plating, cells were starved in serum-free medium for 24 hours and were treated with either vehicle (dimethyl sulfoxide) or 1 μ M ZD1839 in serum-free medium for 8 hours. The cells were then stimulated with EGF (still in the presence of dimethyl sulfoxide or ZD1839) for the indicated times. Whole-cell protein extracts were collected at 0, 5, 10, 15, and 30 minutes after EGF stimulation (0 time samples were collected immediately before addition of EGF). Protein extracts were electrophoresed on a sodium dodecyl sulfate–polyacrylamide gel and transferred to a nitrocellulose membrane (Hybond ECL; Amersham Life Sciences, Arlington Heights, IL). The following primary antibodies were used for western blot analysis. Phosphorylated EGFR (product 2234, 1:1000 dilution), total EGFR (product 2232, 1:1000 dilution), and phosphorylated mitogen-activated protein kinase (MAPK; product 9101, 1:1000 dilution) were obtained from Cell Signaling Technology (Beverly, MA). Total MAPK (product 06–182, 1:1000 dilution) antibody was obtained from Upstate Biotechnology (Lake Placid, NY). Actin antibody (product MAB1501, 1:100 000 dilution) was obtained from Chemicon (Temecula, CA). Anti-rabbit or anti-mouse antibody

(1:4000 dilution; Amersham, Piscataway, NJ) was used as the secondary antibody. Blots were developed with the enhanced chemiluminescence system (Amersham), and the expression levels were measured with a densitometer (Alpha-Innotech, San Leandro, CA). Relative expression levels of phosphorylated EGFR and phosphorylated MAPK were determined after normalizing for protein loading by dividing the level of phosphorylated proteins by the level of actin for each sample.

Cell Growth Assays

Proliferation assays to measure the effect of ZD1839 on anchorage-dependent cell growth. Normal or immortal breast cells (1 $\times 10^6$ cells) were plated in quadruplicate cultures and then treated with ZD1839, as indicated. For extended treatments, cells were incubated with ZD1839 for 0–12 days with fresh drug and medium replaced every 2 days. Cell proliferation was measured with the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI). Relative cell growth was then assessed by measuring absorbance at 620 nm with a spectrophotometric plate reader. Cells grown in medium alone were used as controls. Experiments were carried out for 4, 8, 10, and 12 days because of the different growth rates of the different cell lines.

Soft agar assay for anchorage-independent growth. Approximately 5 $\times 10^4$ MDA-MB-468 cells were suspended in 4 mL of 0.35% SeaPlaque agarose (FMC BioProducts, Rockland, ME) supplemented with complete culture medium. This suspension was layered over 1.5 mL of 0.7% agar/medium base layer in one well of a six-well plate and treated with 1 μ M ZD1839 for 14 days; colonies with a diameter larger than 0.05 mm were then counted.

Transgenic Mice

Female MMTV-erbB2 transgenic mice (The Jackson Laboratory, Bar Harbor, ME) were used for this experiment. The MMTV promoter from the mouse mammary tumor virus long terminal repeat causes the erbB2 gene to be expressed in the mammary gland. The mice were housed in the institutional animal facilities, and appropriate animal treatment guidelines were followed. Animals were obtained at 10–12 weeks of age, given a pituitary isograft to chronically stimulate the MMTV promoter [as described by Lydon et al. (11)], and treated with ZD1839 as described below. Animals were fed a controlled diet of AIN-76A Purified Diet (Harlan Teklad, Madison, WI).

Treatment and Data Collection

Mice were treated with ZD1839 at 10 mg/kg (n = 18) or 100 mg/kg (n = 19) suspended in distilled water containing 1% Tween 80 or with vehicle for 6 days/week from age 3 months to 12 months. ZD1839 or vehicle (n = 19) was administered in 0.1 mL by gastric gavage with a 20-gauge gavage needle. Tumors were measured twice a week with electronic calipers (Mitutoyo, Utsunomiya, Japan), and tumor volume was determined by multiplying the square of the width (*w*) by the length (*l*) and dividing by two (i.e., $w^2l/2$). Individual tumor size and tumor location for each animal were recorded twice a week. Weights of all mice were recorded weekly. Animals were killed when they developed tumors of 1000 mm³ or more or at the end of the experiment. Two hours before killing, the mice were injected intraperitoneally with bromodeoxyuridine (Amersham) (3

mg/mL in phosphate-buffered saline; 100 μ L/10 g body weight). The end of the experiment was defined as the time when all vehicle-treated mice had developed a tumor (at 330 days of age or 240 days of treatment). At that time, all remaining mice (vehicle-treated and ZD1839-treated) were killed, and tumors were resected. The primary end point of the study was time to tumor formation. After the mice were killed, each tumor was resected, and separate portions were processed for histologic analysis, explanted into tissue culture to prepare *in vitro* tumor cell lines, or frozen for future use in biomarker studies. Explanted tumor cells were grown in Dulbecco's modified Eagle medium containing 10% fetal bovine serum, 1% glutamine, 1% penicillin/streptomycin, and 1% Fungizone (Invitrogen, Carlsbad, CA).

Histology and Biomarker Analysis

Histology was performed as previously described (12). Briefly, samples were fixed in neutral-buffered 10% formalin (i.e., phosphate-buffered 10% formaldehyde) overnight and then embedded in paraffin. Tissue sections were then mounted on slides and processed for staining with hematoxylin–eosin.

Immunohistochemical staining for erbB2, ER- α , and p27 was performed by a modified avidin–biotin complex technique as previously described (7). Briefly, tissue sections on glass slides were deparaffinized in xylene and rehydrated. Antigen retrieval was performed by microwaving the samples in 0.01 M citric acid, and endogenous peroxidase activity was blocked by incubation in 30% methanolic hydrogen peroxide. Nonspecific binding was blocked with 20% normal goat serum. Sections were then incubated at 23 °C for 4 hours with one of the following antibodies: rabbit anti-c-erbB2 polyclonal antibody (Neomarkers, Fremont, CA) diluted 1:50 in phosphate-buffered saline (PBS), rabbit anti-ER- α polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:2000 in PBS, or rabbit anti-p27 polyclonal antibody (Abcam Limited, Cambridge, MA) diluted 1:200 in PBS. After three washes in PBS, sections were incubated with biotinylated goat anti-rabbit immunoglobulin G antibody for 30 minutes at 23 °C and then incubated with the ABC kit (Vector Laboratories, Burlingame, CA) for 30 minutes in the dark. Sections were then incubated with 3-amino-9-ethylcarbazole (2 mg/mL; Sigma Chemical Co., St. Louis, MO) to visualize the peroxidase complex. Levels of p27 were evaluated by visual assessment with a semiquantitative scoring system that rated the staining intensity (from 0 to 3), as previously described (13).

Staining for bromodeoxyuridine was performed with the DAKO ARK (Animal Research Kit; DAKO, Copenhagen, Denmark) system. Briefly, tissue sections were cut, mounted onto slides, and deparaffinized. Endogenous peroxidase was blocked with 30% hydrogen peroxide. Slides were then rinsed, and nonspecific binding was blocked (A/B Blocking Kit; Vector Laboratories, Burlingame, CA). Bromodeoxyuridine was stained with a mouse anti-bromodeoxyuridine monoclonal antibody (clone Bu20a; DAKO). The slides were then incubated with streptavidin–horseradish peroxidase, and peroxidase activity was visualized with diaminobenzidine chromagen intensified with 0.2% osmium tetroxide. Counterstaining was done with Harris acidified hematoxylin. The stained sections were reviewed and scored with an ocular grid. The percentage of positive cells was determined with tissue samples from four

mice from each group by counting the numbers of positive and negative cells in 10 high-powered fields (counting approximately 4000 tumor cells or 1000 normal cells per slide) in each treatment group, and results were expressed as an average percentage with 95% confidence intervals (CIs).

Statistical Analysis

Two outcome measures were considered in the animal studies: tumor-free survival and tumor multiplicity. Tumor-free survival was measured from time of initiation of treatment to the time of first appearance of a tumor (defined as a palpable mass of ≥ 100 mm³). Tumor-free survival curves were estimated by the Kaplan–Meier product limit method and compared with the generalized Wilcoxon test (14). Tumor multiplicity was determined by counting the total number of tumors occurring in each animal up to the time they were killed. Multiplicity was summarized by means and 95% confidence intervals, and multiplicity between groups (number of animals = 18 or 19 per group) was compared by one-way analysis of variance (15) and Student's *t* test. Student's *t* test was used to determine the statistical significance of the difference in biomarker expression. Relative changes in biomarker levels were backtransforming differences in means (and 95% confidence intervals of differences in means) of log-transformed data. Analyses were performed with SAS, version 8.1 (SAS Institute, Cary, NC). All statistical tests were two-sided.

RESULTS

ZD1839 and the Phosphorylation of EGFR and MAPK

In Vitro

To investigate the effect of ZD1839 on signal transduction in breast cells, we measured the expression of phosphorylated and total EGFR and MAPK proteins in HMECs and MDA-MB-468 cells by western blot analysis. Cells were treated with ZD1839 for 8 hours and then stimulated with EGF for 5, 10, 15, or 30 minutes before analysis. In both cell lines, EGFR phosphorylation was completely blocked, but the expression of total EGFR did not change compared with that in untreated control cells (Fig. 1). In both cell lines, MAPK phosphorylation was markedly inhibited but the expression of total MAPK did not change (Fig. 2). Although ZD1839 totally blocked phosphorylation of EGFR in these cells, it only partially blocked MAPK phosphorylation. Thus, other signal transduction pathways may still be able to activate MAPK in these cells, even if EGFR kinase activity is completely blocked.

ZD1839 and the Growth of Normal, Precancerous, and Malignant Breast Cells *In Vitro*

We next determined the effect of ZD1839 on the growth of normal breast cells (184 cells and HMECs), immortalized human breast epithelial cells (184B5 and MCF10A cells), and breast cancer cells (MDA-MB-468 cells). ZD1839 at 1 μ M completely inhibited the proliferation of 184, HMEC, 184B5, and MCF10A cells (Fig. 3, A) and also inhibited the anchorage-independent growth of MDA-MB-468 breast cancer cells (Fig. 3, B). Thus, ZD1839 at 1 μ M is able to suppress the growth of ER-negative normal (184 cells and HMECs), immortalized (184B5 and MCF10A cells), and malignant breast cells (MDA-MB-468 cells).

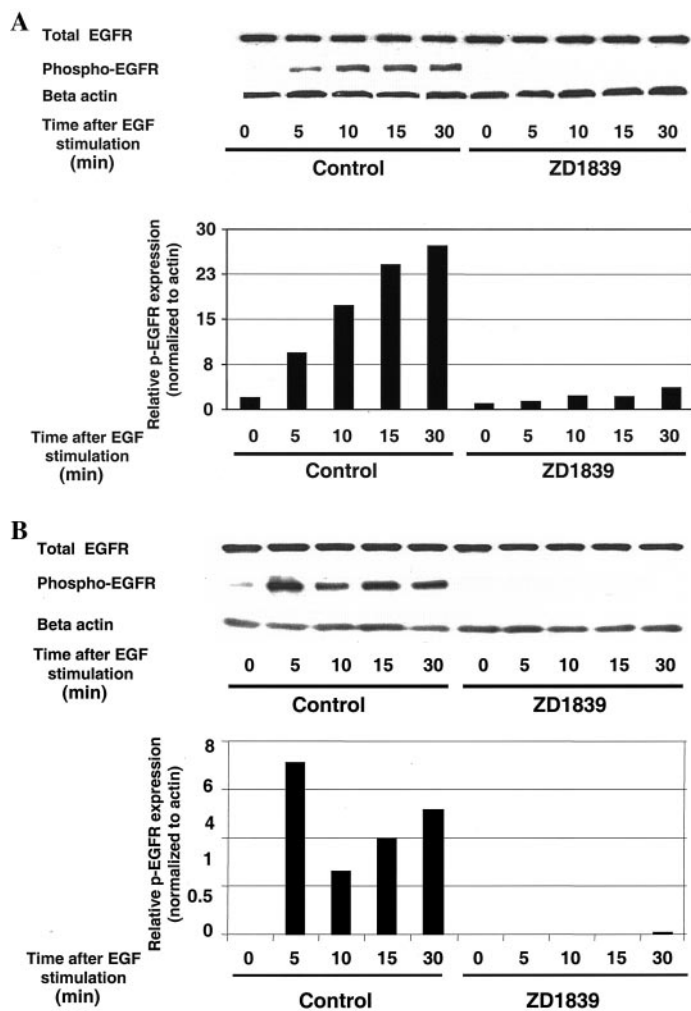


Fig. 1. ZD1839 and epidermal growth factor receptor (EGFR) autophosphorylation in human mammary epithelial cells (A) and MBA-MD-468 cells (B). **A) Upper panels**—Cells were treated with 1 μ M ZD1839 for 8 hours and then stimulated with 1 μ M EGF. Cells were harvested, as indicated, and protein extracts were prepared. Expression of total and phosphorylated EGFR was examined by western blot analysis. Actin expression was used as the loading control. **B) Lower panels**—The relative expression of phosphorylated EGFR (phospho-EGFR or p-EGFR) from the upper panel is shown (as measured by densitometric analysis and normalized to the expression of actin). Experiments were performed three times; data from a representative experiment are shown.

ZD1839 and Mammary Tumor Development

We next investigated the ability of ZD1839 to inhibit the development of ER-negative mammary tumors in MMTV-erbB2 transgenic mice. These mice carry the unactivated neu/c-erbB2 protooncogene under the transcriptional control of the MMTV promoter and develop focal tumors beginning at 6 months of age, with a median incidence at 230 days old (when the MMTV promoter is stimulated by a pituitary isograft). We have previously shown that the tumors arising in these mice are ER-negative (7).

MMTV-erbB2 mice were treated daily for 6 of 7 days with vehicle or ZD1839 at 10 mg/kg or 100 mg/kg from 3 to 12 months of age. The number and size of all mammary tumors were measured twice a week. The mice were observed daily for any apparent signs of toxicity, and they were weighed weekly. ZD1839 treatment did not affect the histologic appearance of

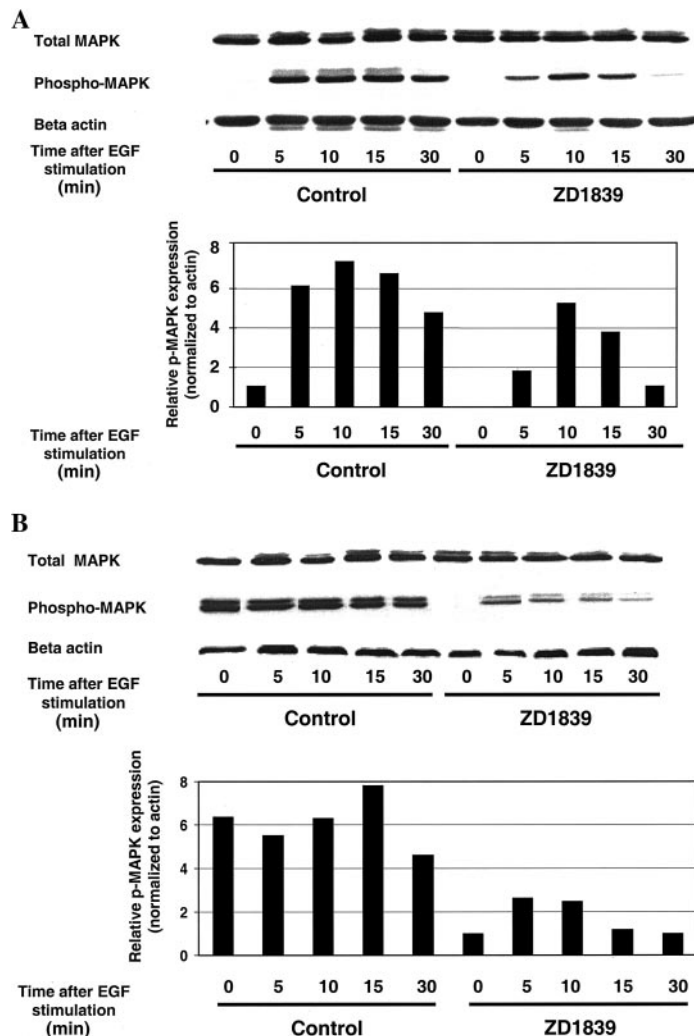
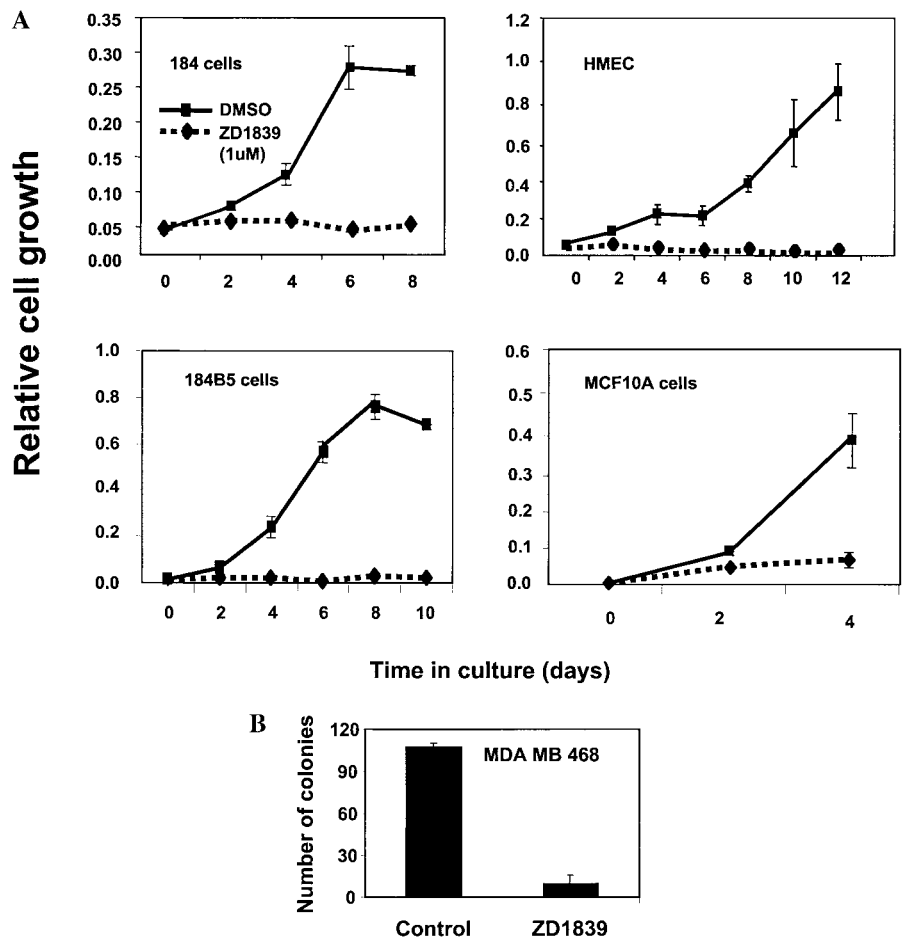


Fig. 2. ZD1839 and mitogen-activated protein kinase (MAPK) phosphorylation in human mammary epithelial cells (A) and MBA-MD-468 cells (B). **A) Upper panels**—Cells were treated with ZD1839 for 8 hours and then stimulated with EGF. Cells were harvested, as indicated, and whole cell protein extracts were prepared. Expression of total and phosphorylated MAPK was examined by western blot analysis. Actin expression was used as the loading control. **B) Lower panels**—The relative expression of phosphorylated MAPK (phospho-MAPK or p-MAPK) is shown (as measured by densitometric analysis and normalized to the expression of actin).

normal mammary glands or breast tumor tissues (Fig. 4, A). As shown in Fig. 4, B, the mammary glands in these mice are ER-negative. Although a few normal mammary duct cells express ER, the tumors that arise in these mice are uniformly ER-negative.

The ability of ZD1839 to inhibit tumor development was investigated. The mice were monitored for tumors from 3 to 12 months of age daily, and tumor size was measured with calipers twice weekly. Median time to tumor development for vehicle-treated mice was 140 days of treatment (at 230 days of age). In mice treated with ZD1839 at 100 mg/kg, the median time to tumor development was statistically significantly delayed to more than 220 days of treatment, that is, to more than 310 days of age (Fig. 5; $P < .001$ by the generalized Wilcoxon test, compared with the median time to tumor development for vehicle-treated animals).

Fig. 3. ZD1839 and cell proliferation in normal, precancerous, and malignant estrogen receptor (ER)-negative breast cells. **A)** Anchorage-dependent cell growth. Human mammary epithelial cells (HMECs) and 184, 184B5, and MCF10A cells (10^6 cells/mL) were treated with $1 \mu\text{M}$ ZD1839, and cell proliferation was measured by CellTiter 96TM Aqueous Cell Proliferation Assay (Promega). Error bars show 95% confidence intervals. **B)** Anchorage-independent cell growth. Soft agar assay was performed to measure colony formation. MDA-MB-468 cells (5×10^4 cells) were plated in agar and treated with $1 \mu\text{M}$ ZD1839 for up to 2 weeks. Colonies larger than 0.05 mm in diameter were then counted. Each treatment was done in triplicate, and the results are shown as the average number of colonies. Error bars show 95% confidence intervals.



A dramatic effect was also seen on tumor multiplicity (number of tumors per mouse). Vehicle-treated mice developed an average of 1.6 tumors per mouse (95% CI = 1.3 to 1.9), mice treated with ZD1839 at 10 mg/kg developed 1.4 tumors per mouse (95% CI = 1.1 to 1.7), and mice treated with ZD1839 at 100 mg/kg developed 0.7 tumors per mouse (95% CI = 0.5 to 0.9) (Table 1). The difference between the vehicle-treated mice and mice treated with ZD1839 at 100 mg/kg was statistically significant (difference = 0.9 tumors per mouse; 95% CI = 0.6 to 1.3 tumors per mouse; $P < .001$, Student's *t* test).

Drug treatment was well tolerated, with no toxicity for 3 months. After 3 months, mice receiving ZD1839 at 100 mg/kg began to develop mild cutaneous toxicities, including slight hair loss and eye irritation (as shown by swelling around the eye). These toxicities evolved over approximately 6 months (starting from 3 months of treatment and continuing until 9 months of treatment), with 50% of mice treated with ZD1839 at 100 mg/kg showing these symptoms. No toxicities were observed in vehicle-treated mice and mice treated with ZD1839 at 10 mg/kg. Because these toxicities were mild and the mice did not appear to be in any distress, all mice continued to receive treatment until developing tumors (or until the end of the experiment, which was the point at which all vehicle-treated mice had developed a tumor).

ZD1839 Treatment and c-erbB2 Transgene Expression

To determine whether the tumor-suppressive effects of ZD1839 were caused by inhibiting the expression of the c-erbB2

transgene, we investigated the expression of c-erbB2 in normal and malignant mammary tissues by use of immunohistochemistry and western blotting. As shown in Fig. 6, c-erbB2 expression was similar in normal and tumor tissue after treatment with vehicle or ZD1839. We obtained the same result by western blot analysis (data not shown). Therefore, ZD1839 did not affect c-erbB2 transgene expression.

ZD1839 Treatment and Cell Proliferation in Mammary Tissue

To further investigate the mechanism of the tumor-suppressive effects of ZD1839, we examined the effect of ZD1839 on cell proliferation in normal and cancerous cells from these mice, as measured by bromodeoxyuridine incorporation with a bromodeoxyuridine-specific antibody. As shown in Fig. 7, A, ZD1839 treatment caused a 20.3% (95% CI = -13.7% to 44.2%) relative reduction in the proliferation of normal breast cells, although this difference did not reach statistical significance ($P = .16$ by Student's *t* test). In tumors, 10.3% (95% CI = 8.0% to 12.6%) of the cells from vehicle-treated mice stained positive for bromodeoxyuridine, and 6.0% (95% CI = 4.0% to 8.0%) of the cells from mice treated with ZD1839 at 100 mg/kg stained positive for bromodeoxyuridine. Thus, ZD1839 at 100 mg/kg induced a 42.0% (95% CI = 20.2% to 58.2%) relative reduction in proliferation ($P = .005$, Student's *t* test). These results suggest that the ability of ZD1839 to prevent breast cancer development was associated with reduced proliferation in normal and malignant breast cells.

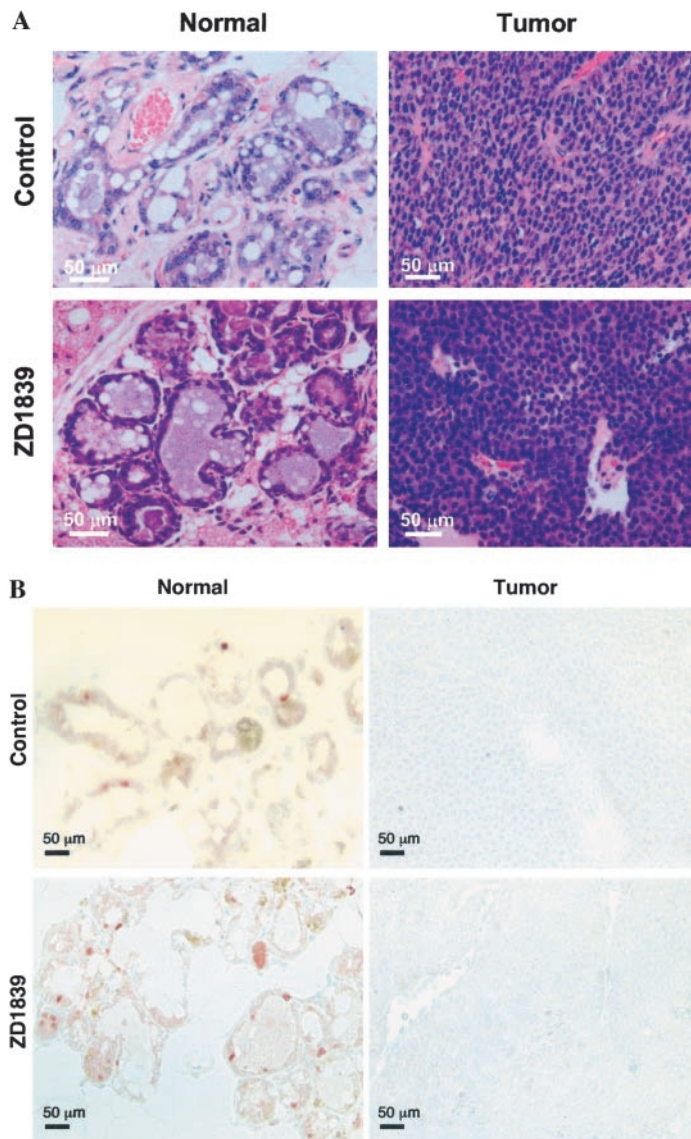


Fig. 4. ZD1839 and mammary tumor growth. Beginning at 3 months of age, MMTV-erbB2 transgenic mice were treated daily for 6 days/week by oral gavage with vehicle or ZD1839 at 10 mg/kg or 100 mg/kg. Tumors were measured twice weekly, mice were weighed weekly, and symptoms of toxicity were recorded daily. After mice were killed, tumors were resected, fixed, and embedded in paraffin. **A**) Histologic features of normal mammary tissue and tumors from vehicle-treated and ZD1839-treated mice. Tissue sections were stained with hematoxylin–eosin, and representative fields containing normal and tumor samples are shown. **B**) Expression of estrogen receptor α (ER- α) in normal mammary tissue and tumors from vehicle- and ZD1839-treated mice. Tissue sections were stained for ER- α with polyclonal anti-ER- α antibody. Representative fields containing normal and tumor samples are shown.

ZD1839 Treatment and the Expression of p27 in Mammary Tissue

It has previously been shown (16) that ZD1839 treatment leads to increased expression of the cell cycle inhibitor p27 and cell cycle blockade in cancer cells. Therefore, we measured the expression of p27 in mammary tissues by immunohistochemical staining for p27. Treatment with ZD1839 at 100 mg/kg increased the expression of p27 in normal mammary tissue (48.7%, 95% CI = 27% to 74.2%) and tumor tissues (50.3%, 95% CI = 35.8% to 66.7%) (both $P < .001$, Student's t test; Fig.

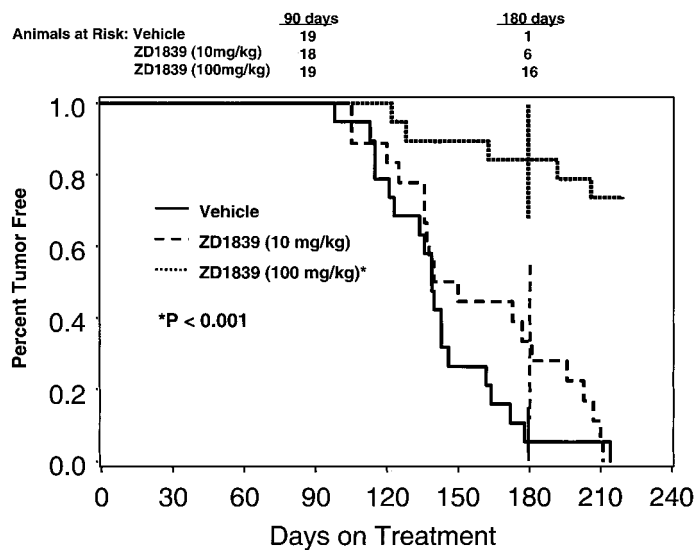


Fig. 5. Kaplan–Meier plot of the proportion of animals free of tumor versus days of treatment. MMTV-erbB2 transgenic mice were treated with vehicle or with ZD1839 at 10 mg/kg or 100 mg/kg by oral gavage from the age of 3 months until all vehicle-treated animals developed tumors at 220 days of treatment or 310 days of age. Tumors were measured twice weekly. Error bars show 95% confidence intervals at 90 and 180 days. $*P < .001$ (two-sided generalized Wilcoxon chi-square test) for comparison between groups treated with vehicle and with ZD1839 at 100 mg/kg.

7, B). We found the same results by western blotting (data not shown). This increase in p27 is consistent with the reduced proliferation shown in Fig. 7, A. Thus, ZD1839 blocked EGFR and MAPK activation, induced p27, suppressed proliferation, and ultimately inhibited breast tumorigenesis.

DISCUSSION

We have demonstrated that the EGFR tyrosine kinase inhibitor ZD1839 inhibits ER-negative mammary tumorigenesis in MMTV-erbB2 transgenic mice. This suppressive effect of ZD1839 on breast tumor development was associated with reduced cell proliferation and increased expression of the cell cycle inhibitor p27. To our knowledge, this is the first report that

Table 1. Median time to tumor development and multiplicity in mice treated with ZD1839*

Treatment	Median age to tumor development, days	% of mice with tumors	No. of tumors per mouse (95% CI)
Vehicle (1% Tween 80)	230	100	1.6 (1.3 to 1.9)
ZD1839			
10 mg/kg	236	100	1.4 (1.1 to 1.7)
100 mg/kg	>310†	25	0.7 (0.5 to 0.9)

*Median age to tumor development, tumor incidence, and tumor multiplicity is shown. Mice were treated with vehicle or ZD1839, as indicated, and median time to tumor development, percentage of mice with tumors, and number of tumors per mouse (when mice were killed) were determined. Treatment with ZD1839 at 100 mg/kg statistically significantly delayed the median time to tumor development and decreased tumor multiplicity ($P < .001$; two-sided generalized Wilcoxon test).

†The median time of tumor development was not reached for this group.

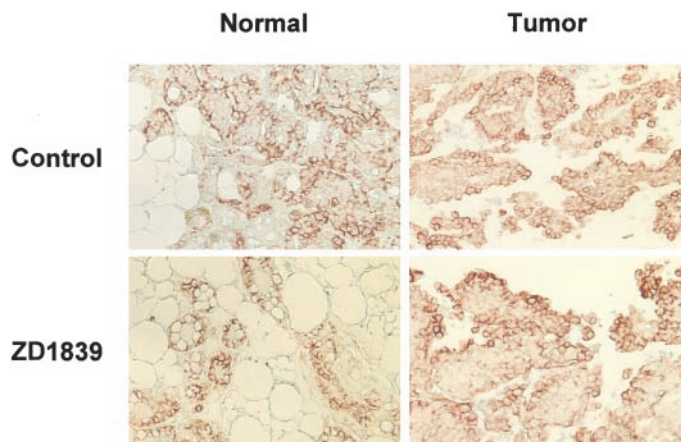


Fig. 6. ZD1839 and the expression of the erbB2 transgene in MMTV-erbB2 transgenic mice. Normal mammary glands and tumors were resected, fixed, and embedded in paraffin. Tissue sections were prepared for immunohistochemical staining and probed with anti-c-erbB2 antibody.

ZD1839 reduces the development of ER-negative breast cancer in mice.

The mechanism by which ZD1839 suppresses breast cancer development is unclear. ZD1839 has been shown to prevent autophosphorylation of EGFR in a number of cultured tumor cell lines, resulting in an inhibition of the activation of key downstream signaling molecules, such as MAPK and Akt (17–19). ZD1839 reduces breast cancer cell proliferation (9,20–22) and alters expression of cell cycle regulators, such as p27, thereby causing a cell cycle blockade (9). These previous results are consistent with the results in this article. We observed that ZD1839 completely blocked EGFR autophosphorylation and partially inhibited the downstream MAPK phosphorylation in normal, precancerous, and malignant breast cells. Thus, phosphorylated EGFR and phosphorylated MAPK could be used to assess the cellular response to EGFR blockade by ZD1839. Our study showed that treatment with ZD1839 was also associated with reduced proliferation and increased expression of p27 in normal and malignant breast cells. We observed increased p27 expression in normal mammary gland cells and in tumor cells from mice treated with ZD1839 at 100 mg/kg. Treatment with ZD1839 at 100 mg/kg also caused a decrease in proliferation in normal (by 20.3%, 95% CI = –13.7% to 44.2%) and malignant (by 42%, 95% CI = 20.2% to 58.2%) mammary cells. The modest reduction in proliferation in normal cells likely reflects the low basal level of proliferation seen in normal mammary glands. It is probable that ZD1839 also suppressed the growth of preinvasive breast cells (in hyperplasias or carcinomas *in situ*), which contributes to delayed development of invasive tumors. We are currently testing this hypothesis by analyzing mammary glands from vehicle- or ZD1839-treated mice at early time points when hyperplasias and carcinomas *in situ* are present.

Lenferink et al. (23) demonstrated that blockade of the EGFR tyrosine kinase with AG-1478 suppresses tumorigenesis in MMTV-neu + MMTV-TGF- α bigenic mice. Their findings are consistent with those in this article. Both studies show that EGFR tyrosine kinase inhibitors suppress mammary tumor development. However, in their study, tumorigenesis was induced by overexpression of both transforming

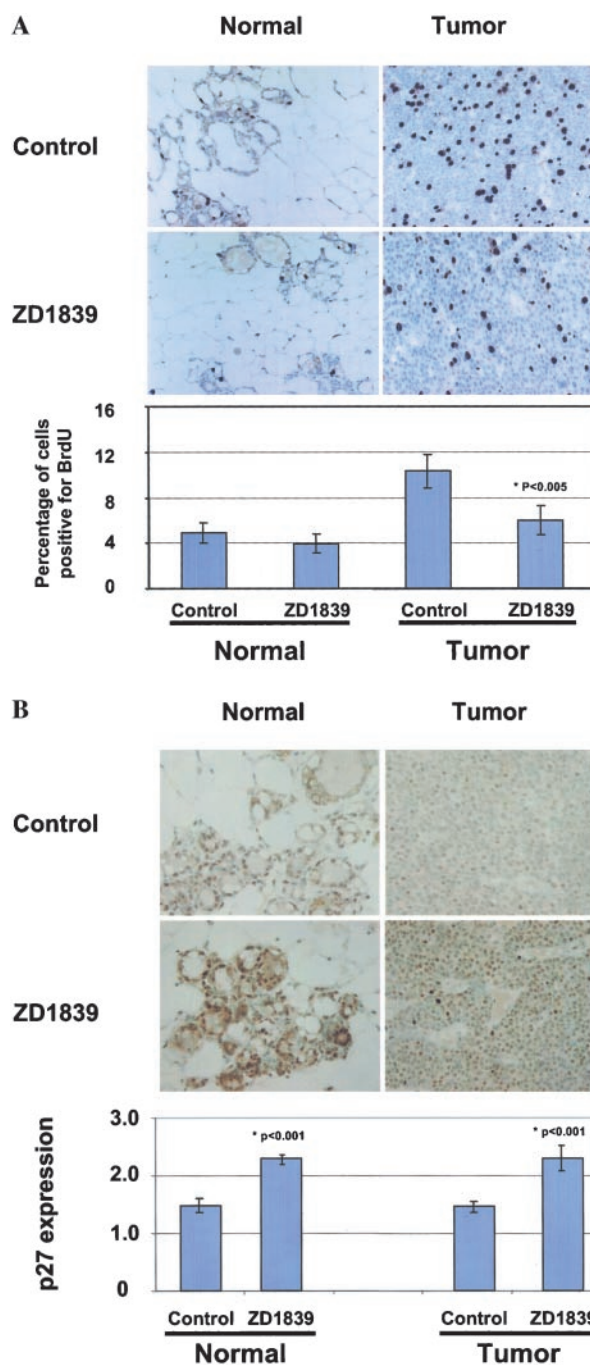


Fig. 7. A) Cell proliferation in normal mammary tissue and tumor tissue in MMTV-erbB2 transgenic mice that received bromodeoxyuridine (BrdU) by intraperitoneal injection, as described. After mice were killed, normal and malignant mammary tissues were resected, fixed, and embedded in paraffin. Tissue sections from vehicle-treated mice and mice treated with ZD1839 at 100 mg/kg were stained for BrdU with anti-BrdU antibody. Selected fields containing normal and tumor samples are shown. The percentage of BrdU-positive cells was lower in ZD1839-treated normal and tumor tissues than in corresponding vehicle-treated tissues ($P = .16$ for normal; $P = .005$ for tumor; two-sided Student's t test). Error bars show 95% confidence intervals. B) ZD1839 and p27 expression in MMTV-erbB2 mice. Normal and malignant mammary tissue were resected, fixed, and embedded in paraffin. Tissue sections from vehicle-treated mice and mice treated with ZD1839 at 100 mg/kg were prepared for immunohistochemical staining with anti-p27 antibody. The level of p27 is shown as the intensity score ($P < .001$ for both normal and tumor tissue compared with untreated corresponding tissues; two-sided Student's t test). Data are the means. Error bars show 95% confidence intervals.

growth factor α (TGF- α , the ligand for EGFR) and c-erbB2. It was not clear whether the preventive effect of the EGFR tyrosine kinase inhibitor was predominantly the result of blocking the activation of EGFR by increasing the expression of TGF- α or by blocking erbB2-dependent signals. Results of this study in mice expressing only one transgene, c-erbB2, demonstrated that ZD1839 was able to inhibit tumorigenesis induced by this single transgene.

Promising agents for prevention of ER-negative breast cancer include retinoids, cyclooxygenase-2 inhibitors, and now an EGFR tyrosine kinase inhibitor. Our previous work has demonstrated that a naturally occurring retinoid, 9-*cis*-retinoic acid, and the retinoid X receptor-selective retinoid LGD1069 prevent ER-negative mammary tumor development in mouse models (6,7,12). Howe et al. (24) showed that a selective cyclooxygenase-2 inhibitor, celecoxib, reduced the incidence of mammary tumors in MMTV-neu mice. In this study, we have shown that, in addition to retinoids and cyclooxygenase-2 inhibitors, the EGFR tyrosine kinase inhibitor ZD1839 may also be effective in preventing ER-negative mammary tumor development in MMTV-erbB2 mice. However, comparison of the activity of each of these agents in MMTV-erbB2 mice suggests that ZD1839 and LGD1069 are more effective than celecoxib at suppressing tumorigenesis.

ZD1839 may be most useful for prevention of breast cancer when combined with other chemoprevention agents such as antiestrogens. Antiestrogens such as tamoxifen and raloxifene have been shown to reduce the risk of ER-positive breast cancer. An increasing body of evidence demonstrates that growth factor networks are highly interactive with the ER signaling that controls breast cancer growth. We (in this article) and others (19,22) have shown that ZD1839 inhibits proliferation in both ER-positive and ER-negative breast cells. Recent studies from Wakeling et al. (25) demonstrated that ZD1839 blocks MAPK activity in tamoxifen-resistant MCF7 cells and that treatment of wild-type MCF7 cells with tamoxifen and ZD1839 prevents development of tamoxifen resistance. These data support the potential clinical utility of ZD1839 in treating tamoxifen-resistant breast cancer and also suggest that the combination of tamoxifen and ZD1839 may be particularly effective in reducing the risk of both ER-positive and ER-negative breast cancer. These studies appear to provide the preclinical rationale for the development of these EGFR tyrosine kinase inhibitors for the prevention of human breast cancer.

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NOTES

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