Advances in Brief

Treatment with Farnesyl-Protein Transferase Inhibitor Induces Regression of Mammary Tumors in Transforming Growth Factor (TGF) α and TGFα/*neu* Transgenic Mice by Inhibition of Mitogenic Activity and Induction of Apoptosis[1]

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Abstract

Mouse mammary tumor virus-transforming growth factor α (MMTV-TGFα) and MMTV-TGFα/*neu* transgenic mice develop mammary tumors after a long latency and therefore provide useful model systems for breast cancer with its recognized activation of receptor tyrosine kinase signaling. We used these mice to study the antitumor effect of L-744,832 (FTI), a potent and selective inhibitor of farnesyl-protein transferase, and hence of Ras function. A total of 55 mice were assigned randomly to treatment with FTI or vehicle, and one-half of the mice were crossed over after initial treatment to the opposite group. L-744,832 induced reversible regression of mammary tumors that was paralleled by a decrease in serum levels of TGFα secreted by the tumor cells. There was no difference in response to treatment with FTI between MMTV-TGFα mice, in which tumorigenesis was accelerated by multiparity or the chemical carcinogen 7,12-dimethylbenzanthracene, and MMTV-TGFα/*neu* mice. The tumor histological type had no impact on FTI sensitivity. For mechanistic analyses, tumor excision biopsies were obtained from 12 mice before and after treatment with L-744,832. In these samples, tumor regression was paralleled biochemically by inhibition of mitogen-activated protein kinase activity and biologically by an increase in G_1-phase and decrease in S-phase fractions, as well as induction of apoptosis. These results suggest that the potential clinical use of FTI could be expanded to include cancers harboring activated receptor tyrosine kinases as well as those containing activated Ras.

Introduction

Activation of oncoprotein signaling pathways is believed to play an important role in human cancer (1). RTKs[3] and a large number of other proto-oncoproteins, which have been implicated in the signaling pathways of these receptors, control normal cell proliferation, differentiation and apoptosis. These processes are dysregulated as a consequence of oncogenic activation (2). Members of the EGFr family, such as EGFr (HER1) and Neu (erbB-2, HER2), are examples of RTKs activated in breast cancer by amplification or mutation. Activation of these receptors as well as overexpression of their ligands, such as TGFα, have been implicated in mammary carcinogenesis (3, 4). The proto-oncogenes of the *ras* family encode the Ras proteins, which act as signal transducers downstream of RTKs, in the Ras-MAP kinase pathway (2, 5). Mutated cellular *ras* genes can transform cells *in vitro*, and activation of cellular Ras by mutation or gene amplification has been found in ~30% of human cancers but rarely in breast cancer (6). Recent data indicate that activation of the Ras-MAP kinase pathway plays a role in breast cancer (7), consistent with the large number of studies demonstrating RTK activation in these tumors (3).

Ras is posttranslationally modified in a series of steps in which farnesyl-protein transferase catalyzes the first step, by adding a 15-carbon farnesyl group to the COOH-terminal tet-

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[3] The abbreviations used are: RTK, receptor tyrosine kinase; EGFr, epidermal growth factor receptor; TGF, transforming growth factor; hTGF, human TGF; s-hTGF, serum hTGF; FTI, farnesyltransferase inhibitor; MAP, mitogen-activated protein; DMBA, 7,12-dimethylbenzanthracene; AUC, area under the tumor growth curve; MGR, mean growth rate.

rapeptide of the Ras precursor. Farnesylation causes translocation of Ras to the cell membrane, which is required for its full biological activity (8). This observation was used to develop a new class of drugs, FTIs (9). Peptidomimetic FTIs were shown to selectively inhibit farnesylation *in vitro* and *in vivo*, thereby preventing Ras processing and reverting specifically the Ras-transformed phenotype in fibroblasts (10, 11). Treatment of spontaneous mammary and salivary tumors in H-*ras* and N-*ras* transgenic mice with the FTI, L-744,832, resulted in regression of the tumors (12) because of induction of apoptosis and/or arrest in the G_1 phase of the cell cycle (13, 14). A wide range of cancer cell lines responded to treatment with L-744,832 with inhibition of growth and increased sensitivity to Taxol and epothilones, regardless of whether they harbored *ras* mutations (15, 16).

The study of RTKs in mammary carcinogenesis was greatly facilitated by the development of transgenic mice in which expression of TGFα (17–19) or c-*neu* (20) was targeted to the mammary glands. These mice developed focal mammary tumors after a long latency. The latency was reduced significantly by either multiparity (21) or treatment of the mice with the carcinogen DMBA (22), or expression of both transgenes in bigenic MMTV-TGFα/*neu* transgenic mice (23). Biochemically, these tumors were characterized by activation of mitogenic signaling: overexpression of TGFα, EGFr, and, in the bigenic mice, Neu, probably involving Neu-EGFr transactivation (17, 23).

To examine the sensitivity to L-744,832 of tumors with an activated RTK pathway but wild-type Ras, we measured the effect of L-744,832 on the growth of tumors in three different groups of mice: multiparous MMTV-TGFα mice; DMBA-treated, virgin MMTV-TGFα mice; and virgin, bigenic MMTV-TGFα/*neu* mice. L-744,832 induced regression of mammary tumors in all three groups. Tumor regression was reversible and paralleled by a decrease in serum levels of TGFα. Analysis of tumor biopsies obtained before and after treatment with L-744,832 demonstrated that growth inhibition was paralleled by inhibition of MAP kinase activity, an increased G_1 fraction, and induction of apoptosis.

Materials and Methods

Animals. The generation and characterization of MMTV-TGFα and MMTV-TGFα/*neu* mice have been described previously (17, 20, 23). A total of 69 mice with 75 tumors were used, from three groups: group 1, 32 multiparous (having had two litters) MMTV-TGFα mice; group 2, 17 virgin, DMBA-treated MMTV-TGFα mice; and group 3, 20 virgin, bigenic MMTV-TGFα/*neu* mice. Fifty-five of the mice received the 35-day treatment as described below for determination of growth rate, serum TGFα, and histology, whereas 14 mice were used for isolation of pre- and posttreatment biopsies as described below. All three groups of mice used in the study have been shown to have decreased mammary tumor latency compared with virgin MMTV-TGFα mice (17, 22, 23). The animals were maintained in accordance with the Vanderbilt University Animal Care Committee guidelines.

DMBA Treatment. Virgin MMTV-TGFα female mice were given six weekly doses of 1 mg/kg DMBA (Sigma Chemical Co., St. Louis, MO) dissolved in corn oil and administered by orogastric tube from 8 weeks of age.

Compound. The structure of the FTI L-744,832 was described previously (12). For injection, L-744,832 was dissolved in 89 mM sodium chloride, 17 mM sodium citrate to give an isotonic solution (pH 5.4). The vehicle was an isotonic 94 mM sodium chloride, 17 mM sodium citrate solution (pH 5.4).

FTI Treatment. Animals were palpated for tumors twice weekly and entered the study when tumors achieved a volume of ~300 mm^3. The mice were assigned randomly to treatment with either 40 mg/kg FTI or vehicle, both given as daily 100 μl s.c. injections for 35 days or until the tumor reached the maximum tolerable size. One-half of the mice were crossed over after primary treatment to the opposite group. For tumor biopsy studies, mice were treated with FTI for 1 ($n = 5$) or 3 ($n = 9$) weeks.

Tumor Volume. Tumors were measured twice weekly with calipers. The data were analyzed as described (24). Two perpendicular measurements, width (W) and length (L), were made in millimeters, where $W \leq L$, and the tumor volume was calculated with the formula: $(W^2 \times L)/2$. Tumor volume was plotted as a function of treatment time in days. The AUC for a particular tumor was calculated with the formula:

$$\mathrm{AUC} = [(\mathrm{vol}_1 + \mathrm{vol}_2)/2] \times (\mathrm{day}_2 - \mathrm{day}_1)$$

MGR was calculated with the following formula:

$$\mathrm{MGR} = [(\mathrm{sumAUC}_n) - (\mathrm{vol}_1 \times (\mathrm{day}_n - \mathrm{day}_1)]/(\mathrm{day}_n - \mathrm{day}_1)^2$$

Statistical Analysis. This study was a higher-order Balaam's design (25) to determine whether treatment produced a beneficial effect on the average MGR of the tumors. There were four groups of mice in the study; treatment alone, vehicle alone, treatment followed by vehicle, and vehicle followed by treatment. Tests of hypotheses concerning treatment effect, as well as the carry-over effect, were made using the restricted/residual maximum likelihood-based mixed effect model to adjust the intracorrelation effect for the mice that had multiple tumors (four mice had two tumors, and one mouse had three tumors). Because no statistically significant carry-over effect or interaction effect was found in the study, the tests concerning the correlation between treatment effect and other variables, *e.g.*, histological diagnosis, were computed by the similar mixed effect model. Analysis of pre- and posttreatment parameters were done with Wilcoxon Matched-Pairs test. All tests were two-sided, and differences with a $P < 0.05$ were considered statistically significant.

Histology. At the time of sacrifice, biopsies of tumor tissue were fixed in 4% formaldehyde. H&E-stained paraffin sections were prepared by standard methods and examined for histological diagnosis.

Serum TGFα. The MMTV-TGFα transgene encodes hTGFα. Peripheral blood samples were obtained from the mice by retroorbital bleeding before treatment and at the time of sacrifice. Sera were assayed for hTGFα by radioimmunoassay as described previously (21).

H-*ras* Mutation. Genomic DNA was extracted from samples of DMBA-induced tumors with phenol and chloroform by standard methods (26). The presence of DMBA-induced

mutation in the H-*ras* gene (codon 61) was assayed for by allele-specific hybridization modified from protocols described previously (27, 28). Mouse genomic DNAs harboring DMBA-induced mutations (kindly provided by Dr. S. Sukumar, Johns Hopkins Oncology Center, Baltimore, MD) were assayed as positive controls.

Biopsies. For mechanistic analyses of the effect of L-744,832 on the tumor cells, excision biopsies were obtained from 14 mice before and after FTI treatment. Biopsies were either processed immediately or frozen in liquid nitrogen and stored at −80°C. FTI treatment was initiated upon sufficient healing of the incision and subsidence of edema. The biopsies were verified histologically, and two mice were excluded: one with a responding tumor that had no detectable tumor cells in posttreatment biopsy, and one with a progressing tumor showing extensive necrosis in the posttreatment biopsy.

Flow Cytometric Analysis. Tumor biopsies (2–3 mm in diameter) were transferred immediately to HBSS containing 2 mg/ml Collagenase A (Boehringer Mannheim, Indianapolis, IN.), minced, and passed through 18- and 21-gauge needles. The cell suspension was pelleted, resuspended in PBS, passed through a 95 μm nylon mesh, and passed six times through a 21-g needle. The cells were then fixed by adding ethanol to a 67% (v/v) final concentration and stored at 4°C. Corresponding biopsies were analyzed on the same day. Stored cells were pelleted, resuspended in PBS containing 0.1% RNase (Amersham, Arlington Heights, IL.), 10 μg/ml propidium iodide (Sigma), and incubated for 30 min at room temperature in the dark. Flow cytometry was performed with a FACScalibur using CellQuest software (Becton Dickinson, San Jose, CA).

Apoptosis. Paraffin sections of tumor biopsies were analyzed for apoptotic cells using a commercially available kit (Apoptag; Oncor, Gaithersburg, MD) and evaluated by light microscopy. Positive cells were counted in five representative fields. The total number of cells per field was counted, and the percentage of apoptosis was calculated.

MAP Kinase Activity. Protein was extracted from tumor biopsies by sonication in Ripa buffer (PBS+1% NP40, 0.5% sodium deoxycholate, and 0.1% SDS). Homogenates were clarified by centrifugation at 18,500 × *g* for 30 min at 4°C. Extracts were diluted with water to yield equal protein concentrations, dissolved in Laemmli sample buffer, and boiled for 5 min. For Western blotting, 20-μl aliquots of tumor extracts containing 20 μg of protein were resolved by 10% SDS-PAGE. Extracts from quiescent and EGF-stimulated MK cells were included as controls. The gels were transferred to nitrocellulose, and the membranes were blocked in 50 mM Tris (pH 8.0), 150 mM NaCl (wash solution) containing 5% BSA and 0.2% Tween 20 for 1 h at room temperature. Blots were probed with rabbit polyclonal antibodies to either MAP kinase or the dually phosphorylated, active form of MAP kinase (Promega) diluted 1:1000 in wash solution containing 1% BSA and 0.05% Tween 20 for 2 h. Blots were washed five times for 5 min with wash solution containing 1% BSA, 1% Tween 20 and 0.2% SDS. Blots were developed using alkaline phosphatase-conjugated goat anti-rabbit antibodies (Sigma) with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium detection. For in-gel kinase assays, 20 μg of protein were resolved by 10% SDS-PAGE as described above, except that the resolving gels contained 5 mg/ml myelin basic protein (Boehringer Mannheim). Gels were washed four times for 15 min each with 20% isopropanol in 50 mM Tris (pH 8.0) and 5 mM β-mercaptoethanol (buffer A) to remove the SDS. Gels were washed twice for 30 min in 6 M guanidine-HCl in water to denature the proteins. Proteins were renatured by washing the gels for 16 h in buffer A containing 0.04% Tween 20, changing the buffer six to seven times. Gels were washed two times for 15 min in 50 mM HEPES (pH 7.2), 5 mM $MgCl_2$, and 0.1 mM EGTA (phosphorylation buffer). Kinase assays were initiated with the addition of phosphorylation buffer containing 10 μM ATP and 100 μCi of [γ-^{32}P]ATP in a volume of 20 ml/gel. Gels were washed with 5% TCA and 1% sodium PP_i until the washes were free of radioactivity. Gels were then equilibrated with water, dried, and quantitated using a PhosphorImager (Molecular Dynamics). Identical in-gel kinase assays were performed without substrate proteins in the gel to distinguish substrate phosphorylation from autophosphorylation.

Results

L-744,832 Induced Reversible Regression of TGFα and TGFα/*neu* Mammary Tumors. To test the effect of L-744,832 in the MMTV-TGFα mammary tumor model system, we used three different groups of mice in which the tumor latency period was reduced compared with virgin transgenic mice: group 1, multiparous MMTV-TGFα mice; group 2, DMBA treated, virgin MMTV-TGFα mice; and group 3, virgin, bigenic MMTV-TGFα/*neu* mice. Mice were assigned randomly to daily s.c. injections of either L-744,832 at a dose of 40 mg/kg or an isosmotic vehicle. The dose of L-744,832 was identical to the effective dose required to obtain complete regression of mammary tumors in H-*ras* transgenic mice in previous experiments (12–14). Tumors in the majority of animals treated with L-744,832 regressed in size during the treatment period (Fig. 1, *a*, *c*, and *e*), in contrast to the vehicle-treated tumors in which all progressed (Fig. 1, *b*, *d*, and *f*). No new tumors appeared in the mice during L-744,832 treatment, as opposed to the vehicle-treated group in which 10 of 38 mice developed secondary tumors during treatment.

A MGR was calculated for each individual tumor and averaged for tumors in each particular tumor induction group (multiparous, DMBA, and α/*neu;* Ref. 24). The average MGR for all tumors treated with L-744,832 (all FTIs) was -7.4 ± 1.6 mm^3 per day, whereas that of all vehicle-treated tumors (all vehicles) was 19 ± 3.4 mm^3 per day, demonstrating a highly significant statistical difference ($P = 0.0001$; Fig. 2*a*). There was no statistically significant difference between the responses to L-744,832 in the multiparous, DMBA-treated, and TGFα/*neu* mice as expressed by the average MGRs.

The purpose of using mice from three particular tumor induction groups (multiparous, DMBA, and α/*neu*) was to investigate a possible difference in the response to L-744,832 among these groups. Treatment of mice with DMBA was shown previously to specifically induce activating mutations in the 61st codon of the H-*ras* gene (27, 29). From a hypothetical point of view, the presence of activating H-*ras* mutations in a subgroup of the tumors in this study would be of direct relevance. We were, however, not able to demonstrate any mutations in H-*ras*

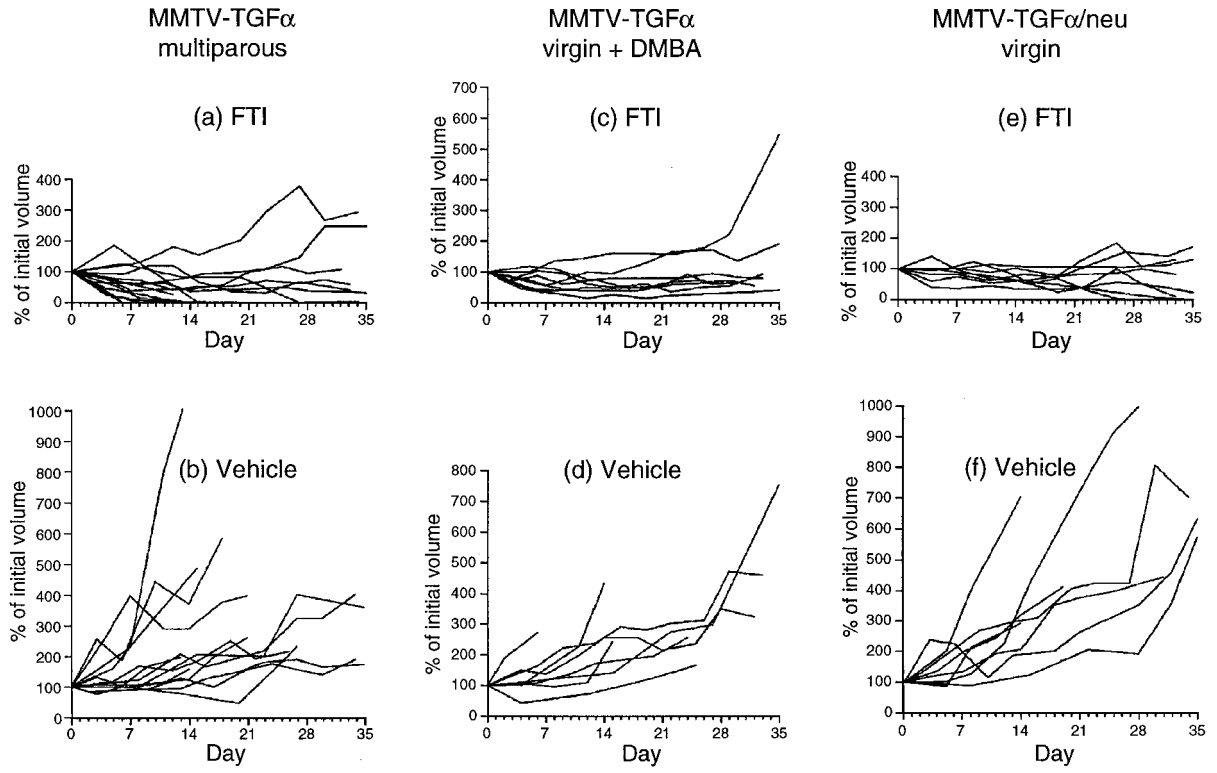

Fig. 1 Effect of L-744,832 on the growth of mammary tumors in multiparous (*a* and *b*), and DMBA-treated (*c* and *d*), virgin MMTV-TGFα mice, as well as virgin MMTV-TGFα/*neu* (*e* and *f*) mice. The volume of tumors in mice receiving treatment with L-744,832 (*a, c,* and *e*) or vehicle (*b, d,* and *f*) was plotted as a percentage of tumor volume at treatment initiation.

codon 61 in DNA isolated from the DMBA-induced mammary tumors.

After a primary course of treatment with either L-744,832 or vehicle, one-half of the tumors were assigned randomly to receive a succeeding course of the opposite treatment. MGRs for tumors treated with L-744,832 subsequent to treatment with vehicle were all reduced, whereas those of tumors treated with vehicle subsequent to FTI treatment all increased (Fig. 2*b*). These observations led us to conclude that the effect of L-744,832 on the tumors leading to regression was reversible.

The animals were sacrificed after termination of the treatment, and histological diagnosis was performed on 54 of the tumors. In agreement with previous characterizations of these mice (21, 23), the tumors fell into six diagnostic groups: simple mammary cyst, adenosis, cystic adenoma, solid adenoma, adenocarcinoma, and carcinosarcoma (Table 1). When comparing the histology of tumors treated with L-744,832 to that of vehicle-treated tumors, regressing tumors had the characteristics of a less active epithelial cell component: decreased cellularity, foci of cell dropouts, a reduced number of mitotic figures, and an increased number of apoptotic bodies, whereas the tumor-stroma was slightly more prominent than in the vehicle-treated tumors (data not shown). There were no statistically significant differences in the responses (MGR) to L-744,832, among tumors of different histological subtypes, when comparing either of the diagnostic groups to the others or when comparing carcinomas *versus* non-carcinoma tumors (hyperproliferative lesions and adenomas).

Reduction of Serum TGFα in Animals with Regressing Tumors. In the MMTV-TGFα mice, hTGFα is expressed by the transgene in mammary epithelial and tumor cells, and it can be measured specifically in serum samples by RIA (17, 21). To examine the effect of L-744,832 on the s-hTGFα in the tumor-bearing mice, serum samples were collected before and after treatment with L-744,832 or vehicle. Pretreatment s-hTGFα levels (normalized to tumor volume) were significantly higher ($P < 0.01$) in mice with solid carcinomas compared with those with noncarcinomas (adenosis or solid adenomas). Because one diagnostic criterium for carcinomas was increased cellularity, this finding supported the assumption that the tumor cells were the source of hTGFα (data not shown). In agreement with this hypothesis, we found that tumor regression in response to L-744,832 was paralleled by a decrease in s-hTGFα (Fig. 3*a*). In contrast, s-hTGFα levels increased both in mice with tumors resistant to L-744,832 (Fig. 3*b*) and in vehicle-treated mice (Fig. 3*c*).

L-744,832 Inhibited MAP Kinase Activity and Increased G_1 Cell Cycle Fraction. Histological examination of vehicle-treated and FTI-treated tumors indicated that mitogenic activity was reduced in response to FTI. For a more detailed analysis of the effect of L-744,832 on mitogenic activity in the tumors, we examined protein extracts from tumor biopsies ob-

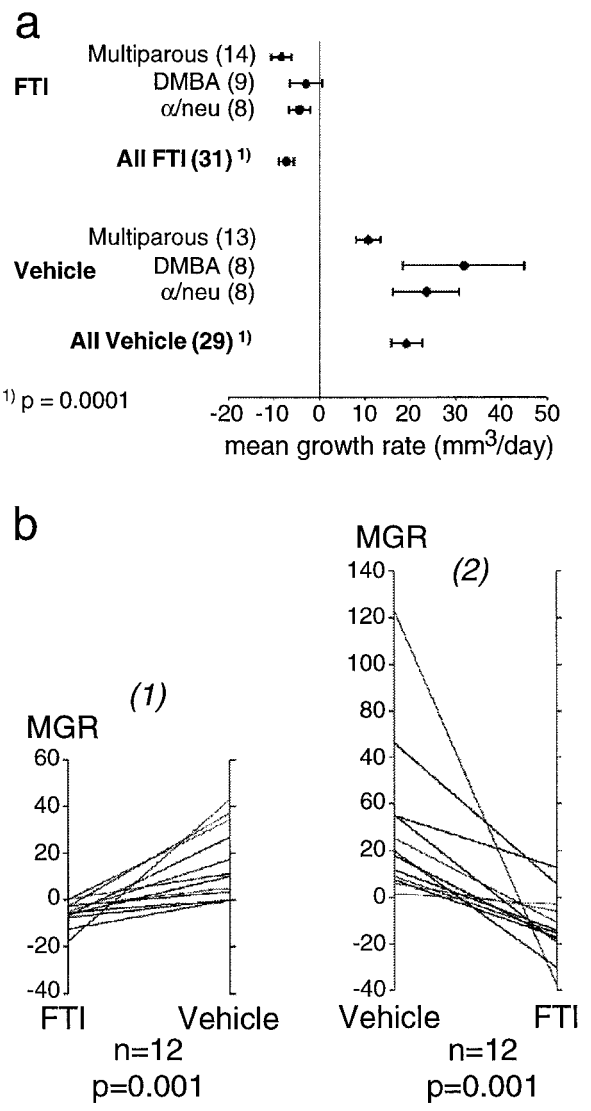

Fig. 2 Effect of L-744,832 or vehicle given as primary treatment or cross-over treatment on the growth of tumors in multiparous and DMBA-treated, virgin MMTV-TGFα mice, as well as virgin MMTV-TGFα/*neu* mice. *a*, MGRs were calculated for each individual tumor in the group, and the average MGR was plotted. *Bars*, SE. *FTI* or *Vehicle*, tumors receiving primary treatment with FTI or vehicle. *All FTI* and *All vehicle:* all tumors treated initially with FTI or vehicle, respectively. Numbers in parentheses, number of tumors. [1], statistical test performed as described in "Materials and Methods." *b*, MGR was calculated for individual tumors after primary treatment with L-744,832 *(1)* or vehicle *(2)* and after cross-over treatment in the opposite group and plotted as matched pairs. Statistical analysis was performed using the Wilcoxon Matched Pairs test.

tained from 12 mice before and after treatment with the compound. These tumors responded to treatment with L-744,832 with an average MGR similar to that of the FTI-treated tumors described above. Western blots probed with an anti-active MAP kinase antibody showed a weaker staining of posttreatment extracts, indicating a decreased MAP kinase activation (Fig. 4*a*). This was confirmed by quantitation of MAP kinase activity in the extracts using in-gel kinase assays, which showed a reduction of kinase activity by L-744,832, varying from 16–80% among the mice (Fig. 4*b*).

Next, we performed flow cytometric analysis on tumor biopsies obtained from six mice before and after treatment with L-744,832. We found an increased G_1 fraction and a concomitant decrease in S- and G_2-M fractions in posttreatment biopsies in all cases examined (Table 2A).

Induction of Apoptosis by L-744,832. Histological examination of tumors showed an increased number of apoptotic bodies in mice treated with L-744,832 in comparison with tumors from control animals. This was quantitated by performing the terminal deoxynucleotidyl transferase-mediated nick end labeling assay on paraffin sections of pre- and posttreatment biopsies and the percentage of apoptotic cells determined in representative fields (Table 2B). Treatment with L-744,832 led to an induction of apoptosis in all cases examined, thus confirming the histological observations.

Discussion

The MMTV-TGFα transgenic mouse model provides an effective means for studying the activity of antitumor drugs on mammary tumors with activated RTKs. In the present study, we used this model system to test the antitumor activity of a peptidomimetic FTI, L-744,832. It was found that treatment with L-744,832 induced regression of the majority of mammary tumors in mice from the three tumor induction groups studied: multiparous MMTV-TGFα mice, DMBA-treated MMTV-TGFα mice, and bigenic MMTV-TGFα/*neu* mice. The effect of L-744,832 treatment on tumor volume correlated with the level of s-hTGFα. Tumor regression was also paralleled by inhibition of MAP kinase activity, growth arrest in the G_1 phase of the cell cycle, and increased apoptosis. These results demonstrate antitumor effects of FTI in an *in vivo* model in which the tumors: (*a*) express wild-type *ras*, and (*b*) exhibit activated RTK signaling.

The FTIs were shown previously to exhibit potent antitumor activity in *in vivo* models of mutant-Ras neoplasia (12–14, 30–33). The antitumor effects of L-744,832 that we observed in the MMTV-TGFα and MMTV-TGFα/*neu* mice were in many respects similar to what was found previously in MMTV-H-*ras* transgenic mice (12, 13) but greater than those observed with FTI treatment of transgenic mice harboring an activated MMTV-c-*neu* gene (13). The microscopic appearance of regressing tumors in the TGFα and TGFα/*neu* mice and the H-*ras* mice were characterized by an inactive epithelial component with decreased cellularity and a reduced number of mitotic figures. In accordance with this, we detected a reduction in MAP kinase activity in biopsies obtained from tumors regressing after treatment with L-744,832, compared with pretreatment levels (Fig. 4). The biological significance of the observed reduction in MAP kinase activity could not be assessed directly in the *in vivo* experimental setup used in the study. However, the importance of the Raf/MEK/MAP kinase pathway in the transduction of mitogenic signals is well established (2, 5), and the sensitivity of cancer cell lines to L-744,832 was shown to correlate with its ability to inhibit MAP kinase activation by

Table 1 Histological diagnosis of transgenic mammary tumors

	TGFα multiparous		TGFα virgin, DMBA treated		TGFα/*a/neu* virgin	
	No.	Percentage	No.	Percentage	No.	Percentage
Cyst	4	17%	1	7%		
Adenosis	4	17%	1	7%	1	6%
Cystic adenoma	8	35%	3	20%	2	13%
Solid adenoma	1	4%	5	33%		
Adenocarcinoma	6	26%	4		13	81%
Carcinosarcoma			1	33%		
No. tumors examined	23		15		16	

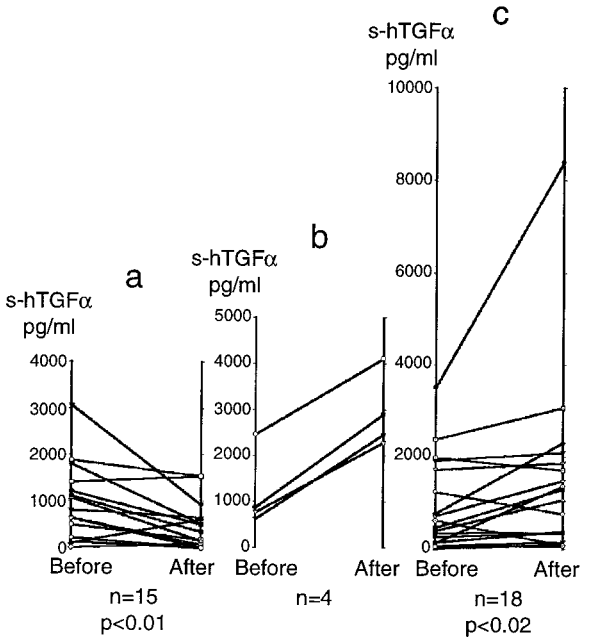

Fig. 3 Effect of L-744,832 on serum levels of hTGFα. Pre- and post-treatment s-hTGFα levels were plotted as matched pairs in three groups of mice: *a*, tumors regressing on treatment with L-744,832 (FTI); *b*, tumors progressing on L-744,832 treatment; and *c*, tumors progressing on vehicle treatment. Statistical comparison was performed using the Wilcoxon Matched Pairs test.

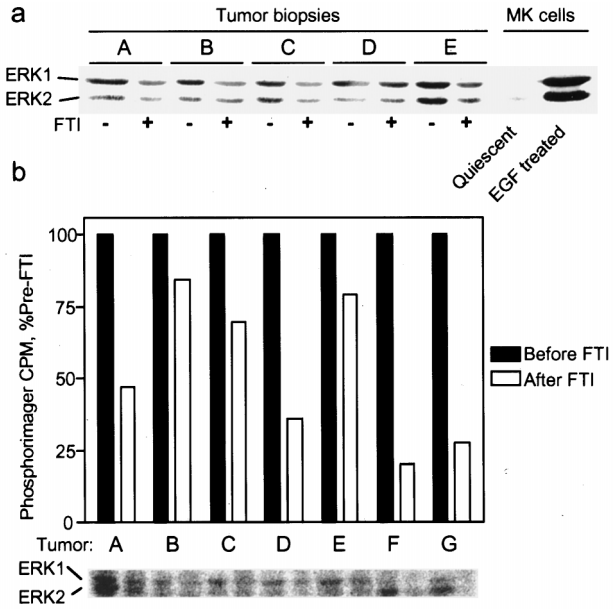

Fig. 4 MAP kinase activity in mammary tumor biopsies obtained before and after treatment of mice with L-744,832. *a*, example of a Western blot probed with an anti-active MAP kinase antibody (see "Materials and Methods"). − and +, before and after FTI treatment, respectively, of EGF-stimulated BALB-MK mouse keratinocyte cell line (38). *b*, kinase activity in protein extracts from tumor biopsies before and after treatment with L-744,832 was quantitated by PhosphorImager analysis (*upper panel*) of in-gel kinase assays (*lower panel*). *ERK*, extracellular signal-regulated kinase.

EGF (15). The present data are, to our knowledge, the first to demonstrate inhibition of MAP kinase activity by FTIs *in vivo*.

Biologically, tumor regression was paralleled by an increased G_1 cell fraction and decreased S- and G_2-M cell fractions and by induction of apoptosis (Table 2). Inhibition of Ras activity was shown previously to activate an apoptotic response *in vitro* (13, 34, 36), and recently published data demonstrated that L-744,832 inhibited the growth *in vivo* of H-*ras*- and N-*ras*-bearing tumors by induction of apoptosis or by cell cycle alterations, depending upon the presence of other oncogenic genetic alterations (14, 37). This suggests that the biological effects of FTIs leading to tumor regression are similar in the TGFα mice and the H-*ras* and N-*ras* mice.

The three tumor induction groups used in our study (multiparous, DMBA, and α/*neu*) responded to L-744,832 in a similar manner. Despite differences in tumor latency and prevalence of carcinomas among the TGFα and the TGFα/*neu* mice (Table 1), we did not observe any differences in tumor growth response or levels of s-hTGFα. The response to L-744,832 was similar in tumors with different histological diagnoses.

These observations indicate that despite differences in the etiology and pathogenesis of these mammary tumors, blocking protein farnesylation has similar consequences with regard to the growth of established tumors. It is important to keep in mind that besides Ras, farnesyltransferase modifies several other cellular proteins, which therefore are affected by FTI, including *rho*B, nuclear lamins A and B, and transducin (14, 15, 37). Thus, it is likely that the actions of FTI may be due to its effects on biochemical signaling pathways other than those involving Ras.

Table 2 Induction of G_1 cell cycle arrest and apoptosis in mammary tumor biopsies by L-744,832

A. Flow cytometric analysis			
	% cells in cell cycle fraction ± SE		
$n = 6$	G_1	S	G_2-M
Before FTI	67 ± 3.9	5.6 ± 1.4	22 ± 4.2
After FTI	80 ± 1.1	2.6 ± 0.6	15 ± 0.7
B. Apoptosis assay			
$n = 9$	% apoptotic cells		
Before FTI	0.16 ± 0.11		
After FTI	2.57 ± 0.31		

Our observations suggest that FTIs may be effective against tumors containing activated RTK signaling pathways, regardless of the presence or absence of mutationally activated Ras. These studies, along with those of others (13, 15, 16), indicate that FTIs may be effective against tumors containing a wide array of genetic alterations. Future studies should be directed toward determining the precise mechanisms of action of this promising class of anticancer compounds.

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References

1. Bishop, J. M. Molecular themes in carcinogenesis. Cell, *64:* 235–248, 1991.

2. Hunter, T. Oncoprotein networks. Cell, *88:* 333–346, 1997.

3. Dickson, R. B., Johnson, M. D., Mozeena, B., Shi, E., Kurebayashi, J., Ziff, B., Martinez-Lacaci, I., Amundadottir, L. T., and Lippman, M. E. Growth factors in breast cancer: mitogenesis to transformation. J. Steroid Biochem. Mol. Biol., *43:* 69–78, 1992.

4. Normanno, N., and Ciardiello, F. EGF-related peptides in the pathophysiology of the mammary gland. J. Mamm. Gland Biol. Neopl., *2:* 143–151, 1997.

5. Lowy, D. R., and Willumsen, B. M. Function and regulation of Ras. Annu. Rev. Biochem., *62:* 851–891, 1993.

6. Bos, J. L. *Ras* oncogenes in human cancer: a review. Cancer Res., *49:* 4682–4689, 1989.

7. Sivaraman, V. S., Wang, H., Nuovo, G. J., and Malbon, C. C. Hyperexpression of mitogen-activated protein kinase in human breast cancer. J. Clin. Invest., *99:* 1478–1483, 1997.

8. Maltese, W. A. Posttranslational modification of proteins by isoprenoids in mammalian cells. FASEB J., *4:* 3319–3328, 1990.

9. Gibbs, J. B., Oliff, A., and Kohl, N. E. Farnesyltransferase inhibitors: Ras research yields a potential cancer therapeutic. Cell, *77:* 175–178, 1994.

10. Kohl, N. E., Mosser, S. D., deSolms, J., Giuliani, E. A., Pompliano, D. L., Graham, S. L., Smith, R. L., Scolnick, E. M., Oliff, A., and Gibbs, J. B. Selective inhibition of ras-dependent transformation by a farnesyltransferase inhibitor. Science (Washington DC), *260:* 1934–1937, 1993.

11. James, G. L., Goldstein, J. L., Brown, M. S., Rawson, T. E., Somers, T. C., McDonell, R. S., Crowley, C. W., Lucas, B. K., Levison, A. D., and Marsters, J. C., Jr. Benzodiazepine peptidomimetics: potent inhibitors of Ras farnesylation in animal cells. Science (Washington DC), *260:* 1937–1942, 1993.

12. Kohl, N. E., Omer, C. A., Conner, M. W., Anthony, N. J., Davide, J. P., DeSolms, S. J., Giuliani, E. A., Gomez, R. P., Graham, S. L., Hamilton, K., Handt, L. K., Hartman, G. D., Koblan, K. S., Kral, A. M., Miller, P. J., Mosser, S. D., O'Neill, T. J., Rands, E., Schaber, M. D., Gibbs, J. B., and Oliff, A. Inhibition of farnesyltransferase induces regression of mammary and salivary carcinomas in *ras* transgenic mice. Nat. Med., *1:* 792–797, 1995.

13. Barrington, R. E., Subler, M. A., Rands, E., Omer, C. A., Miller, P. J., Hundley, J. E., Koester, S. K., Troyer, D. A., Bears, D. J., Conner, M. W., Gibbs, J. B., Hamilton, K., Koblan, K. S., Mosser, S. D., O'Neill, T. J., Schaber, M. D., Senderak, E. T., Windle, J. J., Oliff, A., and Kohl, N. E. A farnesyltranferase inhibitor induces tumor regression in transgenic mice harboring multiple oncogenic mutations by mediating alterations in both cell cycle control and apoptosis. Mol. Cell. Biol., *18:* 85–92, 1998.

14. Mangues, R., Corral, T., Kohl, N. E., Symmans, W. F., Lu, S., Malumbres, M., Gibs, J. B., Oliff, A., and Pellicer, A. Antitumor effect of a farnesyl protein transferase inhibitor in mammary and lymphoid tumors overexpressing N-ras in transgenic mice. Cancer Res., *58:* 1253–1259, 1998.

15. Sepp-Lorenzino, L., Ma, Z., Rands, E., Kohl, N. E., Gibbs, J. B., Oliff, A., and Rosen, N. A peptidomimetic inhibitor of farnesyl:protein transferase blocks the anchorage-dependent and -independent growth of human tumor cell lines. Cancer Res., *55:* 5302–5309, 1995.

16. Moasser, M. M., Sepp-Lorenzino, L., Kohl, N. E., Oliff, A., Balog, A., Su, D-S., Danishefsky, S. J., and Rosen, N. Farnesyl transferase inhibitors cause enhanced mitotic sensitivity to taxol and epothilones. Proc. Natl. Acad. Sci. USA, *95:* 1369–1374, 1998.

17. Matsui, Y., Halter, S. A., Holt, J. T., Hogan, B. L. M., and Coffey, R. J. Development of mammary hyperplasia and neoplasia in MMTV-TGFα transgenic mice. Cell, *61:* 1147–1155, 1990.

18. Jhappan, C., Stahle, C., Harkins, R., Fausto, R., Smith, G., and Merlino, G. TGFα overexpression in transgenic mice induces liver neoplasia and abnormal development of the mammary gland and pancreas. Cell, *61:* 1137–1146, 1990.

19. Sandgren, E. P., Lutteke, N. C., Palmiter, R. D., Brinster, R. L., and Lee, D. Overexpression of TGFα in transgenic mice: induction of epithelial hyperplasia, pancreatic metaplasia, and carcinoma of the breast. Cell, *61:* 1121–1135, 1990.

20. Guy, G. T., Webster, M. A., Schaller, M., Parsons, T. J., Cardiff, R. D., and Muller, W. J. Expression of the *neu* protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. Proc. Natl. Acad. Sci. USA, *89:* 10578–10582, 1992.

21. Halter, S. A., Dempsey, P. J., Matsui, Y., Stokes, M. K., Graves-Deal, R., Hogan, B. L. M., and Coffey, R. J. Distinctive patterns of hyperplasia in transgenic mice with mouse mammary tumor virus transforming growth factor-α. Am. J. Pathol., *140:* 1131–1146, 1992.

22. Coffey, R. J., Meise, K. S., Matsui, Y., Hogan, B. L. M., Dempsey, P. J., and Halter, S. A. Acceleration of mammary neoplasia in transforming growth factor α transgenic mice by 7,12-dimethylbenzanthracene. Cancer Res., *54:* 1678–1683, 1994.

23. Muller, W. J., Arteaga, C. L., Muthuswamy, S. K., Siegel, P. M., Webster, M. A., Cardiff, R. D., Meise, K. S., Li, F., Halter, S. A., and Coffey, R. J. Synergistic interaction of the *neu* proto-oncogene product and transforming growth factor α in the mammary epithelium of transgenic mice. Mol. Cell. Biol., *16:* 5726–5736, 1996.

24. Dexter, D. L., Diamond, M., Creveling, J., and Chen, S-F. Chemotherapy of mammary carcinomas arising in *ras* transgenic mice. Invest. New Drugs, *11:* 161–168, 1993.

25. Balaam, L. N. A two-period design with t^2 experimental units. Biometrics, *24:* 61–73, 1968.

26. Ausubel, F. M., *et al.* (eds.), Short Protocols in Molecular Biology. New York: John Wiley & Sons, Inc., 1992.

27. Kumar, R., Medina, D., and Sukumar, S. Activation of H-*ras* oncogenes in preneoplastic mouse mammary tissues. Oncogene, *5:* 1271–1277, 1990.

28. Wood, W. I., Gitschier, J., Lasky, L. A., and Lawn, R. M. Base composition-independent hybridization in tetramethylammonium chloride: a method for oligonucleotide screening of highly complex gene libraries. Proc. Natl. Acad. Sci. USA, *82:* 1585–1588, 1985.

29. Balmain, A., and Brown, K. Oncogene activation in chemical carcinogenesis. Adv. Cancer Res., *51:* 147–182, 1988.

30. Kohl, N. E., Wilson, F. R., Mosser, S. D., Giuliani, E. A., DeSolms, S. J., Conner, M. W., Anthony, N. J., Holtz, W. J., Gomez, R. P., Lee, T-J., Smith, R. L., Graham, S. L., Hartman, G. D., Gibbs, J. B., and Oliff, A. Protein farnesyltransferase inhibitors block the growth of *ras*-dependent tumors in nude mice. Proc. Natl. Acad. Sci. USA, *91:* 9141–9145, 1994.

31. Nagasu, T., Yoshimatsu, K., Rowell, C., Lewis, M. D., and Garcia, A. M. Inhibition of human tumor xenograft growth by treatment with the farnesyl transferase inhibitor B956. Cancer Res., *55:* 5310–5314, 1995.

32. Sun, J., Qian, Y., Hamilton, A. D., and Sebti, S. M. Ras CAAX peptidomimetic FTI 276 selectively blocks tumor growth in nude mice of a human lung carcinoma with K-*ras* mutation and p53 deletion. Cancer Res., *55:* 4243–4247, 1995.

33. Yonemoto, M., Satoh, T., Arakwa, H., Suzuki-Takahashi, I., Monden, Y., Kodera, T., Tanaka, K., Aoyama, T., Iwasawa, Y., Kamei, T., Nishimura, S., and Tomimoto, K. J104,871, a novel farnesyltransferase inhibitor, blocks ras farnesylation *in vivo* in a farnesyl pyrophosphate competitive manner. Mol. Pharmacol., *54:* 1–7, 1998.

34. Hung, W-C., and Chaung, L. Y. The farnesyltranferase inhibitor, FPT inhibitor III, upregulates Bax and Bcl-xs expression and induces apoptosis in human ovarian cancer cells. Int. J. Oncol., *12:* 137–140, 1998.

35. Bernhard, E. J., Kao, G., Cox, A. D., Sebti, S. M., Hamilton, A. D., Muschel, R. J., and McKenna, W. G. The farnesyltransferase inhibitor FTI-277 radiosensitizes H-ras-transformed rat embryo fibroblasts. Cancer Res., *56:* 1727–1730, 1996.

36. Lebowitz, P. F., Sakamuro, D., and Prendergast, G. C. Farnesyl transferase inhibitors induce apoptosis of Ras-transformed cells denied substratum attachment. Cancer Res., *57:* 708–713, 1997.

37. Lebowitz, P. F., Davide, J. P., and Prendergast, G. C. Evidence that farnesyltransferase inhibitors suppress Ras transformation by interfering with Rho activity. Mol. Cell. Biol., *15:* 6613–6622, 1995.

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