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Geranylgeranyltransferase I Inhibitor GGTI-2154 Induces Breast Carcinoma Apoptosis and Tumor Regression in H-Ras Transgenic Mice

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ABSTRACT

Treatment of H-Ras transgenic mice with the geranylgeranyltransferase I (GGTase I) inhibitor GGTI-2154 results not only in halting the growth of aggressive breast tumors but actually in inducing the regression (54 ± 3%) of all 19 tumors analyzed. The farnesyltransferase (FTase) inhibitor FTI-2148 induced an average of 87 ± 3% regression in the 13 tumors analyzed. GGTase I, but not FTase, is inhibited in breast tumors after treatment with GGTI-2154, whereas in tumors from mice treated with FTI-2148, only FTase is inhibited. The processing of the geranylgeranylated proteins RhoA, Rap1, and R-Ras, but not the farnesylated proteins H-Ras and HDJ-2, is inhibited in tumors obtained from mice treated with GGTI-2154. GGTI-2154 and FTI-2148 suppress constitutively activated phospho-Erk1/2 and phospho-Akt, induce apoptosis, and induce differentiation toward ductolobular breast epithelium. The data demonstrate that geranylgeranylated proteins are critical in H-Ras oncogenesis *in vivo* and give strong support for GGTase I as a target for anticancer drug discovery.

INTRODUCTION

The Ras superfamily of low molecular weight GDP/GTP-binding GTPases is critically involved in the regulation of many important biological events, such as the cell division cycle, cell survival and death, differentiation, and development (1, 2). These GTPases are also intimately involved in pathological conditions. For example, in oncogenesis, Ras and Rho proteins mediate malignant transformation, invasion, and metastasis (1, 2). These proteins require prenylation, a lipid posttranslational modification, for biological function (3). Some proteins such as H-Ras, N-Ras, and K-Ras are farnesylated, and others such as RhoA, Rap1, and R-Ras are geranylgeranylated, whereas RhoB is both farnesylated and geranylgeranylated (3). Interestingly, K-Ras, possibly N-Ras, but not H-Ras, can become geranylgeranylated if protein farnesylation is inhibited (4–8). The two enzymes responsible for this prenylation are farnesyltransferase (FTase) and geranylgeranyltransferase I (GGTase I), which attach covalently the 15-C farnesyl and 20-C geranylgeranyl lipids, respectively, to the cysteine sulfhydryl of proteins that end at their COOH terminal with the tetrapeptide consensus sequence CAAX, in which C is cysteine, A is an aliphatic residue, and X is any amino acid (3). FTase prefers to farnesylate proteins in which X is methionine or serine, whereas GGTase I geranylgeranylates proteins in which X is leucine (3).

The fact that prenylation is required for the cancer-causing activity of some low molecular weight GTPases prompted us and others to design FTase and GGTase I inhibitors (FTIs and GGTIs) as potential anticancer drugs (9–12). For example, the CAAM and CAAL peptide mimics FTI-2148 and GGTI-2154, inhibiting FTase/GGTase I with IC_{50} values of 1.4 nM/1700 nM and 5600 nM/21 nM, respectively (13). Inhibition of protein geranylgeranylation with GGTIs results in an arrest of the cell division cycle in the G_1 phase (14, 15). In contrast, treatment of cells with FTIs results in G_2-M accumulation, in a G_1 block or in no effect on cell cycle distribution, depending on the human cancer cell line (14, 16–18). Furthermore, in some cells, FTIs induce apoptosis by inhibiting the phosphatidylinositol 3′OH-kinase/Akt survival pathway (19, 20). Although the mechanism by which GGTIs induce apoptosis has yet to be uncovered, their ability to induce a G_1 block seems to be attributable to induction at the transcriptional level of the cyclin-dependent kinase inhibitor p21waf (15) and subsequent inhibition of cyclin-dependent kinases 4 and 2 and retinoblastoma protein hypophosphorylation (21).

The antitumor efficacy of FTIs has been demonstrated in many animal models (9–12), and some FTIs are presently in clinical trials (22, 23). Whereas in the nude mouse xenograft model, FTIs are mainly cytostatic, in some transgenic mouse models, they induce cytoreduction and tumor regression (reviewed in Ref. 24). For example, in mouse mammary tumor virus (MMTV)-*v*-Ha-Ras mice, in which oncogenic Ha-Ras drives mammary oncogenesis, FTI L-744,832 induces regression (25). Treatment with FTI L-744,832 also results in tumor regression in mice that express the transgenes Ha-*ras* and c-*myc* as well as those that express oncogenic Ha-*ras* and lack the tumor suppressor p53 (26), and those in which mammary tumors are driven by transforming growth factor-α (27). In contrast, FTI L-744,832 treatment of tumors that express N-Ras or K-Ras in the same MMTV mammary oncogenesis model results in tumor growth inhibition, not tumor regression (28, 29).

The antitumor efficacy of GGTIs in transgenic models has not been evaluated, and whether they will induce cytostasis or tumor regression or be effective at all in these models is not known. In this study, we have used the MMTV-*v*-Ha-Ras model and demonstrated that GGTI-2154 induced apoptosis, tumor regression, and differentiation as well as inhibited oncogenic and tumor survival pathways. Because Ha-Ras is exclusively farnesylated and because GGTI-2154 inhibited protein geranylgeranylation but not farnesylation, our results confirm that geranylgeranylated proteins mediate Ha-*ras* malignant transformation (1, 2) and validate GGTase I as an important anticancer drug discovery target.

MATERIALS AND METHODS

Animals. MMTV-*v*-Ha-ras transgenic mice (30) were obtained from Charles River Laboratories (Wilmington, MA). All animals were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and experimental protocols were reviewed by the Institutional Animal Care and Use Committee at the University of South Florida.

FTI and GGTI Compounds. The synthesis and biological characterization of FTI-2148 and GGTI-2154 have been described previously (13).

Antitumor Efficacy Study. All MMTV/v-Ha-ras transgenic mice were studied when they developed one or more tumors with tumor volumes between 100 and 6000 mm^3. GGTI-2154 and FTI-2148 were administered s.c. at 100 mg/kg/day with osmotic mini-pumps for 14 days, as described previously (13).

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The tumor volumes were monitored before and after administration of drugs by at least twice weekly caliper measurements (7, 13).

Effects of GGTI-2154 and FTI-2148 on GGTase I and FTase in Tumor Biopsies. FTase and GGTase I enzymatic activities were determined in incisional biopsies taken from the same tumor at basal level before drug initiation and 4 days after s.c. implantation of mini-pumps delivering either GGTI-2154 or FTI-2148 (100 mg/kg/day). FTase and GGTase I are cytosolic enzymes, and, therefore, postmicrosomal fractions (60,000 × *g* supernatants) were first isolated as follows. Tumor biopsies were homogenized by a tissue tearor in ice-cold sonication buffer [50 mM Tris (pH 7.5), 1 mM EGTA, 1 mM DTT, 2 mM phenylmethylsulfonyl fluoride, 25 μg/ml leupeptin, and 10 μg/ml aprotinin]. The homogenates were then sonicated, spun at 12,000 × *g* and at 60,000 × *g*, and the resulting supernatants were assayed for FTase and GGTase I enzymatic activities, as described previously (6, 13).

Effects of GGTI-2154 and FTI-2148 on the Processing of R-Ras, RhoA, Rap1, H-Ras, and HDJ-2 in Tumor Biopsies. The farnesylated forms of H-Ras and HDJ-2 migrate faster than unfarnesylated forms in SDS-PAGE gels (3–6, 31, 32). Therefore, the effect of prenylation inhibitors on the processing of these proteins was determined by protein bandshift on whole-cell lysates. However, for some prenylated proteins such as RhoA, this bandshift is difficult to detect. Therefore, for the geranylgeranylated proteins, we prepared membrane and cytosolic fractions because in their prenylated forms they associate with cellular membranes. Membrane and cytosolic fractions were prepared by homogenizing tumor biopsies with a tissue tearor in ice-cold hypotonic buffer [10 mM Tris (pH 7.5), 5 mM $Mgcl_2$, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 25 μg/ml leupeptin]. The homogenates were then spun at 12,000 × *g*, and the resultant supernatant was spun at 100,000 × *g* for 30 min at 4°C to separate membrane from cytosolic fractions. The cytosolic and membrane fractions were lysed by a HEPES lysis buffer [30 mM HEPES (pH 7.5), 1% Triton X-100, 10% glycerol, 10 mM NaCl, 5 mM $MgCl_2$, 25 mM NaF, 1 mM EGTA, 2 mM Na_2VO_4, 10 μg/ml soybean trypsin inhibitor, 25 μg/ml leupeptin, 10 μg/ml aprotinin, 2 mM phenylmethylsulfonyl fluoride, and 6.4 mg/ml 2-nitrophenylphosphate]. The lysates were then separated by SDS-PAGE, transferred to nitrocellulose membranes, then immunoblotted with antibodies against Rap1 (121), RhoA (26C4), H-Ras (C-20), and R-Ras (C-19; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and HDJ-2 (KA2A5.6; NeoMarkers, Fremont, CA), as described previously (13).

Immunohistochemistry. Needle biopsies taken from the same tumor at basal level before drug initiation and 4 days after drug treatment were fixed in 10% neutral buffered formalin for 6 h. After fixation, the tissue samples were processed into paraffin blocks. Four micrometer-thick tissue sections were obtained from the paraffin blocks and stained with H&E (Richard-Allan Scientific, Kalamazoo, MI) using standard histological techniques. For each tumor sample, the mitotic rate was determined by counting the number of mitoses per high power field. Tissue sections were also subjected to immunostaining using the avidin-biotin peroxidase complex technique (Vectastatin Elite ABC kit; Vector Laboratories, Burlingame, CA), following the manufacturer's instructions. We used antibodies to phospho-Erk 1/2 (Thr 202/Tyr204; Cell Signaling Technologies) and phospho-Akt (Ser 473; Cell Signaling Technologies). The stains followed microwave antigen retrieval (four cycles of 5 min each on a high setting in 0.1 M citrate buffer) using a 1100 W Emerson Model AT 736 microwave oven. The stains were examined semiquantitatively using the Allred 8-unit system, which consists of the combination of a proportion score (0–5) added to the intensity score 0–3 (none, weak, moderate, strong; Ref. 33).

Detection of Apoptosis by *in Situ* Hybridization Using TUNEL Reaction. Apoptosis was determined by terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) using an *in situ* cell death detection kit (Roche Applied Science, Indianapolis, IN). Frozen sections were prepared from biopsies from the same tumor before and 4 days after initiation of GGTI-2154 or FTI-2148 drug treatment of the animals. The slides were fixed in paraformaldehyde [4% in PBS (pH 7.4)]. After rinsing with PBS and incubating with permeabilization solution, the tissues were cross-reacted with TUNEL reaction mixture for 60 min at 37°C in a humidified chamber. TUNEL results were visualized by incubating tissue samples with either converter alkaline phosphatase solution or with alkaline phosphate substrate solution (Vector Laboratories, Burlington, MA). The reactions were analyzed by light microscopy. The apoptotic rate was expressed as the number of apoptotic cells per two microscopic fields using a Leitz Laborlux D microscope and a ×25 ocular.

RESULTS

GGTI-2154 and FTI-2148 Induce Breast Tumor Regression in MMTV-*v*-Ha-Ras Transgenic Mice. Here, we report on the investigation of the ability of GGTI-2154 and FTI-2148, peptidomimetic inhibitors of GGTase I and FTase, respectively, to perturb breast cancer oncogenesis and tumor survival in a *ras* transgenic mouse model. To this end, MMTV-*v*-Ha-Ras transgenic mice bearing mammary carcinoma of sizes ranging from 100 to 6000 mm^3 were implanted s.c. with mini-osmotic pumps delivering either GGTI-2154 or FTI-2148 (100 mg/kg/day for 14 days). Comparisons between tumor sizes before initiation of treatment and on the day of treatment demonstrated that the tumors grew aggressively and some grew with a rate as high as 400 mm^3 per day

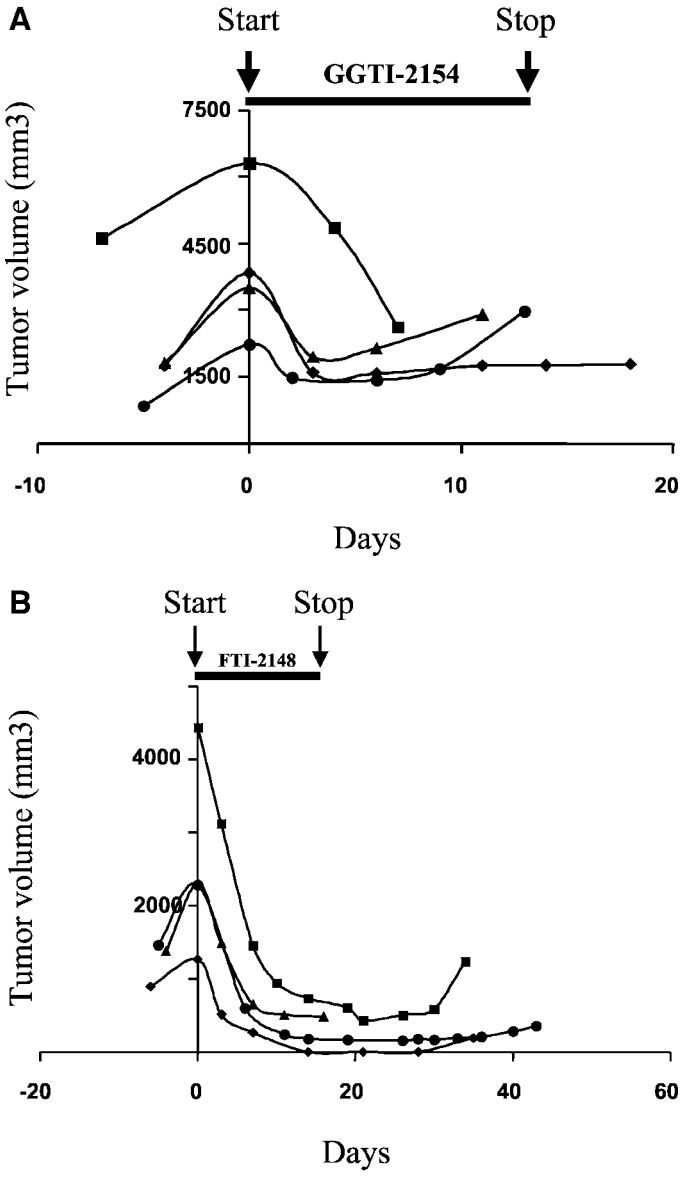

Fig. 1. GGTI-2154 and FTI-2148 induce mammary tumor regression in MMTV-*v*-Ha-Ras transgenic mice. MMTV-*v*-Ha-Ras mice bearing mammary carcinomas received s.c. implants of 14-day osmotic mini-pumps delivering GGTI-2154 (*A*) or FTI-2148 (*B*) at 100 mg/kg/day, as described in "Materials and Methods." Tumor sizes were determined before, during, and after treatment and plotted for each individual tumor.

Table 1 *Antitumor efficacy of GGTI-2154 in MMTV/v-Ha-ras tumors*

MMTV-*v*-Ha-ras transgenic mice bearing tumors of sizes ranging from 331 to 6295 mm^3 received s.c.implants of osmotic mini-pumps delivering GGTI-2154 (100 mg/kg/day for 14 days).

Tumor no.	To[a] (mm^3)	Ts[b] in mm^3 (days)	% tumor regression[c]	Days before tumor regrowth[d]
1	622	124 (19)	80	26
2	373	100 (26)	73	28
3	908	663 (5)	27	13
4	1458	838 (5)	43	7
5	2463	1335 (5)	46	9
6	331	129 (13)	61	16
7	6295	2624 (7)	58	>7[e]
8	425	135 (6)	68	9
9	2222	1421 (6)	36	9
10	500	211 (3)	58	6
11	721	299 (10)	59	>10[e]
12	600	409 (3)	32	6
13	683	188 (10)	72	>10[e]
14	267	126 (6)	53	10
15	3485	1940 (3)	44	6
16	5339	1913 (6)	64	11
17	2572	1680 (3)	35	6
18	3828	1568 (6)	60	11
19	1467	766 (7)	48	14
Average ± SE			54 ± 3	>11 ± 1

[a] To, tumor size at the day of initiation of drug treatment.
[b] Ts, smallest tumor size after drug treatment. The days at which the tumors were smallest (day 0 is day of drug initiation) are indicated in parentheses.
[c] % tumor regression = 100 − (Ts/To × 100).
[d] The number of days it took tumors to regrow after drug initiation.
[e] The animal was sacrificed because of tumor ulceration.

Table 2 *Antitumor efficacy of FTI-2148 in MMTV/v-Ha-ras tumors*

MMTV-*v*-Ha-ras transgenic mice bearing tumors of sizes ranging from 101 to 4444 min^3 received s.c. implants of osmotic mini-pumps delivering FTI-2148 (100 mg/kg/day for 14 days).

Tumor no.	To[a] (mm^3)	Ts[b] in mm^3 (days)	% tumor regression[c]	Days before tumor regrowth[d]
1	2287	168 (30)	93	40
2	469	164 (11)	65	19
3	1124	0 (14)	100	>41[e]
4	1925	0 (14)	100	>41[e]
5	663	88 (21)	87	26
6	4444	427 (21)	90	30
7	425	29 (3)	93	>41[e]
8	1349	55 (19)	96	>41[e]
9	2313	484 (16)	79	>16[e]
10	707	225 (16)	68	>16[e]
11	1266	189 (35)	85	>35[e]
12	1072	52 (35)	95	>35[e]
13	101	28 (10)	82	>35[e]
Average ± SE			87 ± 3	>32 ± 3

[a] To, tumor size at the day of initiation of drug treatment.
[b] Ts, smallest tumor size after drug treatment. The days at which the tumors were smallest (day 0 is the day of drug initiation) are shown in parentheses.
[c] % tumor regression = 100 − (Ts/To × 100).
[d] The number of days it took tumors to regrow after drug initiation.
[e] The animal was sacrificed because of tumor ulceration.

(Fig. 1). Fig. 1*A* shows that treatment with GGTI-2154 not only halted this aggressive growth but resulted in rapid tumor regression within 3 days of initiation of drug treatment. Table 1 shows that all 19 breast tumors regressed after treatment with GGTI-2154. The tumors were reduced in size by an average of 54 ± 3%. Fig. 1*A* and Table 1 also show that the tumors resumed growth and that the average number of days before tumor regrowth was >11 ± 1 days. It is important to note here that tumors started to grow during GGTI treatment. Although the mechanism by which this occurs is presently unknown, one possibility is that this may be attributable to either intrinsic or acquired resistance. Fig. 1*B* shows that treatment with FTI-2148 also resulted in breast tumor regression. However, this tumor regression was more pronounced than that seen with GGTI-2154, and of 13 tumors analyzed, the average tumor regression was 87 ± 3% (Table 2). Furthermore the average number of days before tumor regrowth was longer with FTI-2148 (>32 ± 3 days; Table 2).

GGTI-2154 Inhibits GGTase I But Not FTase, Whereas FTI-2148 Inhibits FTase But Not GGTase I, in Breast Tumors from Mice Treated with GGTI-2154 and FTI-2148, Respectively. To determine whether GGTI-2154 reaches and inhibits its biochemical target GGTase I selectively, we evaluated FTase and GGTase I enzymatic activities in incisional biopsies taken from the same tumor at basal level before drug initiation and 4 days after s.c. implantation of mini-pumps delivering either GGTI-2154 or FTI-2148, as described in "Materials and Methods." Fig. 2 *top panel* shows that mice treated with GGTI-2154 reduced the levels of GGTase I enzyme activity by 50–60%. In contrast, GGTI-2154 treatment in the same tumors did not inhibit FTase enzymatic activity (Fig. 2 *top panel*). Fig. 2 *bottom panel* shows that treatment of mice with FTI-2148 resulted in 85–88% inhibition of FTase with no inhibition of GGTase I

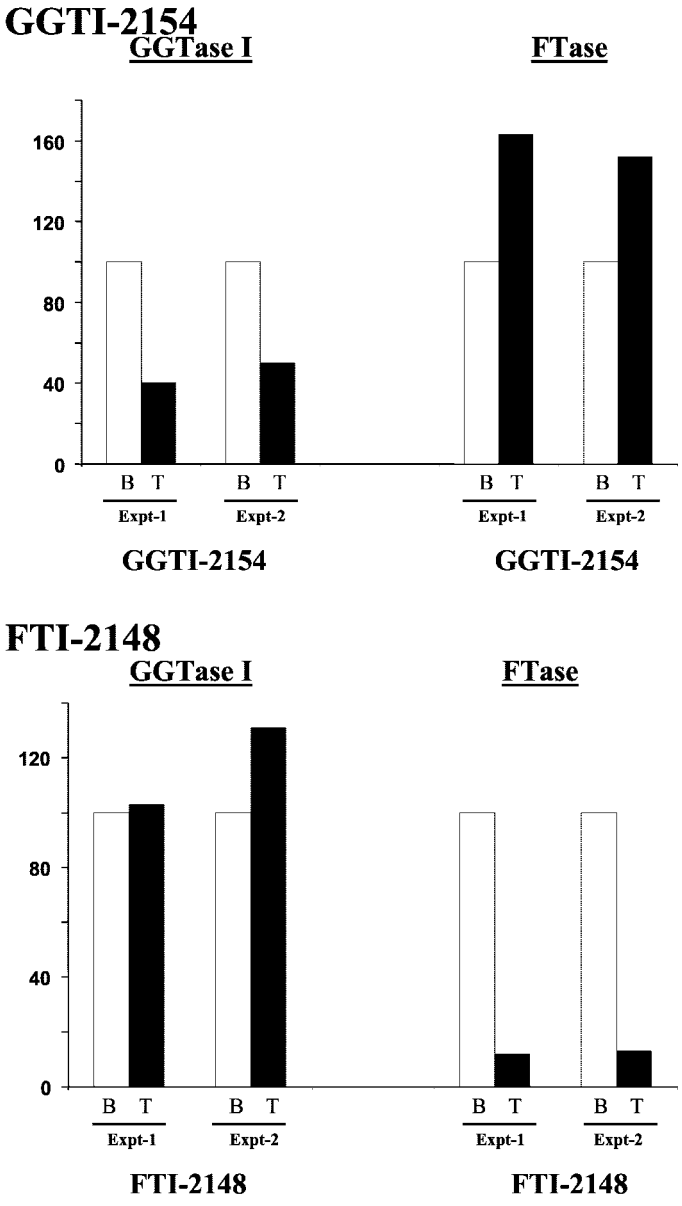

Fig. 2. GGTI-2154 inhibits GGTase I but not FTase, whereas FTI-2148 inhibits FTase but not GGTase I. Incisional biopsies from each tumor before (*B*) and 4 days after (*T*) treatment initiation with either GGTI-2154 (*top panel*) or FTI-2148 (*bottom panel*) were collected and processed for GGTase I or FTase enzymatic activities, as described in "Materials and Methods."

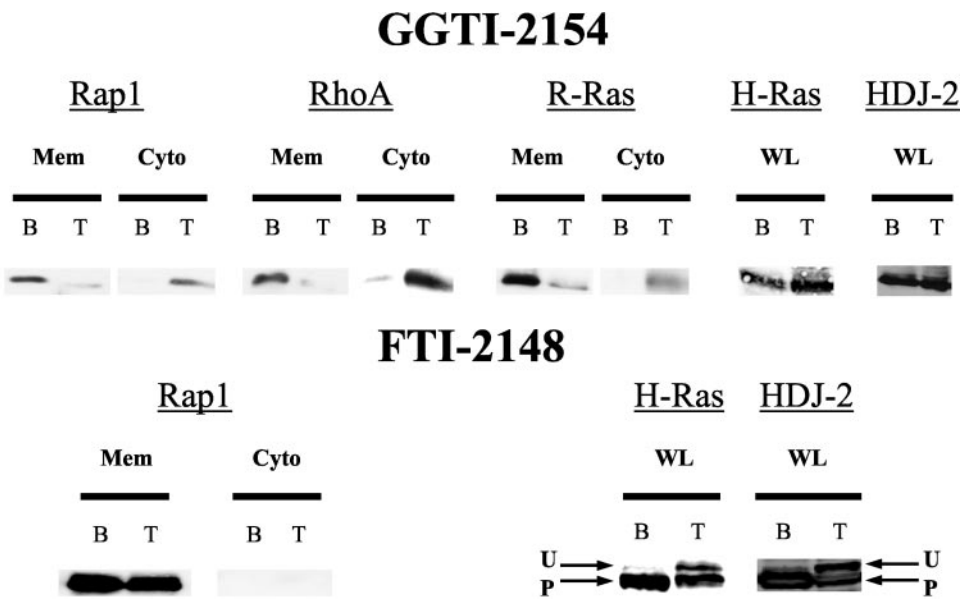

Fig. 3. GGTI-2154 inhibits protein geranylgeranylation but not farnesylation, whereas FTI-2148 inhibits protein farnesylation but not geranylgeranylation. Incisional biopsies from the same tumor before (*B*) and 4 days after (*T*) treatment initiation with either GGTI-2154 (*top*) or FTI-2148 (*bottom*) were collected and processed for SDS-PAGE Western immunoblotting, as described in "Materials and Methods." Inhibition of protein farnesylation was determined in whole lysates (*WL*) by bandshift of the farnesylated H-Ras and HDJ-2 proteins, in which the processed form (*P*) elutes faster than the unprocessed form (*U*). Because the bandshift is difficult to detect with geranylgeranylated proteins such as RhoA, Rap1, and R-Ras, inhibition of protein geranylgeranylation was evaluated by determining the proportion of these proteins associated with cellular membrane fractions (processed form) and cytosolic fractions (unprocessed form). Data are representative of two independent experiments. *Mem*, membrane; *Cyto*, cytosol.

enzymatic activity. Thus, under *in vivo* settings, GGTI-2154 inhibits GGTase I but not FTase, whereas FTI-2148 inhibits FTase but not GGTase I.

GGTI-2154 Treatment Inhibits the Processing of the Geranylgeranylated Proteins RhoA, Rap1, and R-Ras But Not the Farnesylated Proteins H-Ras and HDJ-2. To further confirm the *in vivo* selectivity of GGTI-2154, we determined its effects on the processing of RhoA, Rap1, R-Ras, H-Ras, and HDJ-2. To this end, incisional biopsies were obtained from the same tumor before treatment and 4 days after initiation of treatment. Membrane and cytosolic fractions were prepared from these biopsies and processed for SDS-PAGE Western immunoblotting, as described in "Materials and Methods." Fig. 3 *top panel* shows that Rap1, RhoA, and R-Ras proteins from the biopsies collected before treatment initiation (*B*, basal level) were present predominantly in the membrane fractions. Treatment (*T*) with GGTI-2154 resulted in a great decrease in Rap1, RhoA, and R-Ras associated with the membrane fractions and a corresponding increase in the cytosolic fractions. Fig. 3 *bottom panel* shows that unlike GGTI-2154, FTI-2148 treatment did not decrease the level of Rap1 associated with the membrane fractions and did not result in accumulation of Rap1 in the cytosol. We next determined the effects of GGTI-2154 and FTI-2148 on the farnesylation of H-Ras and HDJ-2 proteins. Because nonfarnesylated forms of proteins migrate slower than their farnesylated forms in SDS-PAGE, (3–6, 31, 32) and because this bandshift is much easier to detect with farnesylated than geranylgeranylated proteins, we determined the effects of FTI-2148 and GGTI-2154 on the processing of H-Ras and HDJ-2 on whole-cell lysates. Fig. 3 *bottom panel* shows that before treatment, H-Ras and HDJ-2 migrated as a single band corresponding to fully processed proteins. On treatment with FTI-2148, slower migrating bands appear, indicating that FTI-2148 partially inhibited the farnesylation of H-Ras and HDJ-2. In contrast, GGTI-2154 treatment did not result in a bandshift, indicating that H-Ras and HDJ-2 remained fully processed (Fig. 3 *top panel*). Taken together, the results from Figs. 2 and 3 demonstrate that *in vivo* treatment with GGTI-2154 results in inhibition of protein geranylgeranylation but not farnesylation, whereas FTI-2148 treatment results in inhibition of protein farnesylation but not geranylgeranylation. This is an important demonstration that indicates that the antitumor activity of GGTI-2154 is not attributable to cross-inhibition of FTase and that the antitumor activity of FTI-2148 is not caused by inhibition of GGTase I.

GGTI-2154 and FTI-2148 Treatment Results in Suppression of the Levels of Phospho-Akt and Phospho-Erk1/2 and Induction of Apoptosis. The ability of GGTI-2154 and FTI-2148 to induce regression of mammary carcinomas suggested that these agents may suppress oncogenic signal transduction pathways that the tumors rely on for survival. Therefore, we determined the effects of FTI-2148 and GGTI-2154 on the levels of phospho-Erk1/2 and phospho-Akt that are known to be constitutively activated by oncogenic Ha-Ras. Fig. 4, *A* and *B*, shows that pretreatment biopsies contained high levels of phospho-Akt and phospho-Erk1/2 and few apoptotic cells. Treatment with FTI-2148 or GGTI-2154 suppressed phospho-Akt and partially inhibited phospho-Erk1/2 levels and induced apoptosis in a large number of cells (Fig. 4, *A* and *B*). Table 3 shows quantification of the results in which a modification of the Allred 8-unit system was used as described in "Materials and Methods." We found that pretreated biopsies expressed high levels of pErk1/2 and pAKT with scores varying from 7.0 $\pm$ 0 to 7.7 $\pm$ 0.3 (with the highest possible score being 8; Table 3). Treatment with GGTI-2154 reduced the scores to 4.7 $\pm$ 0.3 (pErk) and to 2 $\pm$ 0 (pAKT), whereas treatment with FTI-2148 reduced the scores to 2.7 $\pm$ 0.7 (pErk) and to 0.7 $\pm$ 0.7 (pAKT; Table 3). Treatment with GGTI-2154 and FTI-2148 increased the number of apoptotic cells per two microscopic fields from 6.3 $\pm$ 1.5 to 258 $\pm$ 17 (GGTI-2154) and from 3.3 $\pm$ 1.5 to 96 $\pm$ 12 (FTI-2148; Table 3).

Treatment with GGTI-2154 Induces Tumor Differentiation, Degeneration, Ischemic Necrosis, and Apoptosis. As mentioned above, the fact that GGTI-2154 induces tumor regression suggests one mechanism for this cytoreduction is through apoptosis. However, cytoreduction can also occur through other mechanisms such as degeneration and coagulative (ischemic) necrosis. To examine these possibilities, we analyzed the effects of GGTI-2154 on these morphological characteristics, as described in "Materials and Methods." In Fig. 5, pretreatment biopsies reveal tumors with a high number of mitotic figures and only rare apoptotic bodies. In contrast, we observed that GGTI-2154 and FTI-2148 posttreatment tumor biopsies contained very few mitotic figures and many apoptotic bodies, highlighted by the TUNEL staining in Fig. 4. Quantification of the mitotic rate data demonstrated that GGTI-2154 treatment reduced the number of mitotic figures per 10 high power fields from 46 $\pm$ 11 to 3.3 $\pm$ 0.9 (Table 3). Similarly, FTI-2148 treatment reduced this number from 38 $\pm$ 2 to 1.7 $\pm$ 0.3. Treatment with GGTI-2154 and FTI-2148 also

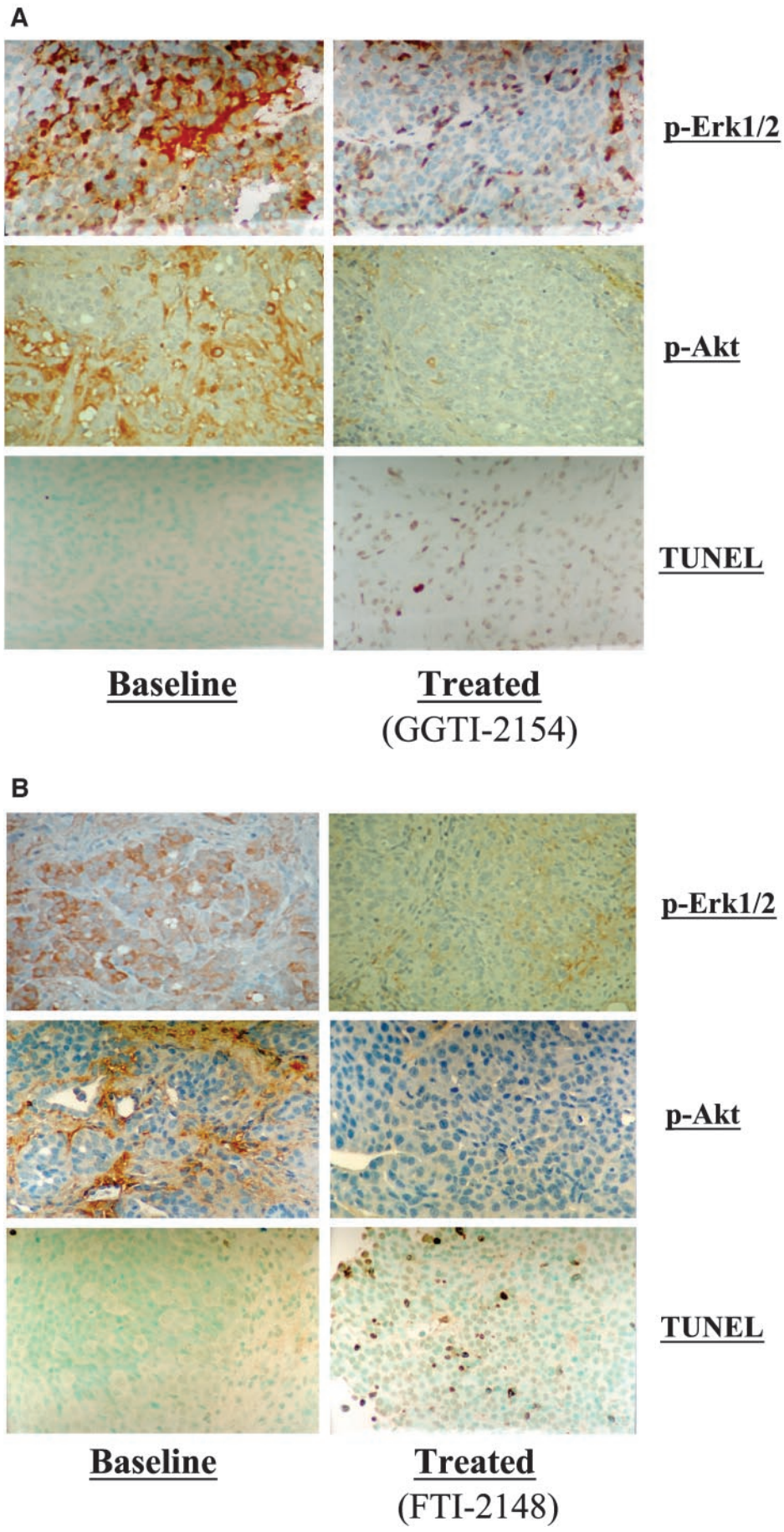

Fig. 4. GGTI-2154 and FTI-2148 inhibit the levels of constitutively activated phospho-Erk1/2 and phospho-Akt and induce apoptosis in breast tumors from MMTV-*v*-Ha-Ras transgenic mice. Needle biopsies from the same tumor before (baseline) and 4 days after (treated) initiation of treatment with either GGTI-2154 (*A*) or FTI-2148 (*B*) were collected and processed for immunohistochemistry using antibodies specific for phospho-Erk1/2 or phospho-Akt or TUNEL staining, as described in "Materials and Methods." Data are representative of two independent experiments.

Table 3 *Quantification of immunohistochemical and TUNEL analyses and of pathological findings*

Needle biopsies from the same tumors before and 4 days after initiation of treatment were collected and processed as described in "Materials and Methods."

		Untreated tumors (baseline)		Treated tumors	
Analysis	Exp.[a]	GGTI-2154	FTI-2148	GGTI-2154	FTI-2148
p-Erk1/2[b]	Exp. 1	8	8	5	4
	Exp. 2	8	8	4	2
	Exp. 3	7	7	5	2
	Avg. ± SE	7.7 ± 0.3	7.7 ± 0.3	4.7 ± 0.3	2.7 ± 0.7
p-AKT[b]	Exp. 1	7	7	2	2
	Exp. 2	8	7	2	0
	Exp. 3	7	7	2	0
	Avg. ± SE	7.3 ± 0.6	7.0 ± 0.0	2.0 ± 0.0	0.7 ± 0.7
TUNEL[c]	Exp. 1	4	1	279	101
	Exp. 2	6	3	270	82
	Exp. 3	9	6	225	104
	Avg. ± SE	6.3 ± 1.5	3.3 ± 1.5	258 ± 17	96 ± 12
Mitotic rate[d]	Exp. 1	28	37	2	1
	Exp. 2	44	35	3	2
	Exp. 3	66	41	5	2
	Avg. ± SE	46 ± 11	38 ± 2	3.3 ± 0.9	1.7 ± 0.3
Tobular/lobular formation[e]	Exp. 1	5%	5%	90%	30%
	Exp. 2	15%	10%	80%	20%
	Exp. 3	5%	5%	100%	40%
	Avg. ± SE	8% ± 3	7% ± 2	90% ± 6	30% ± 6

[a] Exp., experiment; Avg., average.

[b] Allred 8-unit system: combination of a proportion score (0–5) added to the intensity score (0–3); non, weak, moderate, strong; Ref. 33.

[c] Number of apoptotic cells/2 microscopic fields (choosing the areas with the most apoptotic cells) using a Leitz Laborlux D microscope and a ×25 ocular.

[d] Number of mitotic figures per 10 high power fields (using a Leitz Laborlux D microscope and a ×40 ocular).

[e] Percentage of the total tumor area exhibiting tubular and/or lobular architecture.

induced tumor necrosis, which was of the coagulative type as typically seen in ischemic conditions (Fig. 5). Interestingly, both GGTI-2154- and FTI-2148-treated tumors revealed a higher degree of differentiation compared with the biopsies before treatment. The latter were usually characterized by sheets of undifferentiated cells without any particular architectural arrangement. In contrast, GGTI-2154- and FTI-2148-treated specimens often revealed tubular and lobular formation reminiscent of the mammary ductolobular derivation (well differentiated tumors). Quantification of the results revealed that drug treatment increased the percentage of the total tumor area exhibiting tubular and/or lobular architecture from 8 ± 3 to 90 ± 6% (GGTI-2154) and from 7 ± 2 to 30 ± 6% (FTI-2148; Table 3).

DISCUSSION

Here, we have demonstrated that the potent and selective GGTase I inhibitor GGTI-2154 not only inhibited the growth of breast tumors but actually induced regression of these aggressively growing mammary tumors. The fact that GGTI-2154 induced regression of tumors that are driven by the farnesylated oncoprotein Ha-Ras and inhibited protein geranylgeranylation but not farnesylation indicates that geranylgeranylated proteins are important in Ha-ras-induced mammary oncogenesis in MMTV-*ν*-Ha-ras mice. This is consistent with previous studies in cultured fibroblasts that showed that dominant-negative forms of the geranylgeranylated proteins RhoA, Rac1, and Cdc42 interfere with the ability of Ras to induce malignant transformation (1, 2). Furthermore, RhoA, Rac1, and Cdc42 as well as other geranylgeranylated proteins such as R-Ras and TC-21 have also been shown to mediate oncogenesis, invasiveness, and metastasis (1, 2).

A recent study in nude mouse xenografts showed that treatment of animals with a combination of FTIs and GGTIs at doses that inhibited K-Ras prenylation was toxic and eventually led to animal death (34). The authors suggested that this toxicity may be attributable to GGTIs. However, in this study, the antitumor activity of the GGTI used as a single agent was not evaluated, making it difficult to assess the therapeutic index of this GGTI. Our previous studies with nude mouse xenografts showed that GGTIs can inhibit human lung cancer growth with no gross toxicity to the mice (7, 13). In the present study, at the dose used of 100 mg/kg/day, we have observed no weight loss or gross toxicity (loss of appetite or decreased activity) in all animals. The ability of GGTI-2154 to induce tumor regression at doses that do not induce animal death or gross toxicity is important. Thus, in two animal models, GGTIs have antitumor activity with no apparent gross toxicity.

Here, we have also demonstrated that treatment with a potent and selective FTase inhibitor, FTI-2148, induces regression by 87 ± 3% of mammary carcinomas in the MMTV-*ν*-Ha-Ras transgenic mouse model. FTI-2148 drug cessation resulted in tumor regrowth, but only after an average of >32 ± 3 days. Our results are consistent with those reported previously with the structurally unrelated FTI L-744,832 (25). GGTI-2154 was not as effective as FTI-2148 in that the average reduction in tumor size was 54 ± 3% and tumor regrowth occurred after >11 ± 1 days. The lower potency of GGTI-2154 suggests that geranylgeranylated proteins represent only one limb of the complex signal transduction pathways triggered by oncogenic Ha-Ras. That dominant-negative forms of RhoA, Rac1, and Cdc42 only partially reverse Ras-induced malignant transformation supports this interpretation (1, 2). Alternatively, inhibition of GGTase I may result in the induction of compensating oncogenic pathways that are independent of geranylgeranylation. This would also be consistent with the observation that in 12 of the 19 tumors analyzed, tumor regrowth occurred while the treatment of GGTI-2154 continued.

Among the most thoroughly studied pathways that are activated by oncogenic Ha-Ras are the Raf/Mek/Erk and the phosphatidylinositol 3′OH-kinase/Akt pathways (35). These pathways are believed to contribute, at least in part, to Ras induction of tumor survival and inhibition of apoptosis. Both FTI-2148 and GGTI-2154 were effective at suppressing phospho-Akt and phospho-Erk1/2 levels and at inducing apoptosis, suggesting that the ability of Ras to transduce oncogenic and tumor survival signals to Akt and Erk depends, at least in part, on geranylgeranylated proteins. However, GGTI-2154 was not as effective as FTI-2148 in inducing tumor regression, suggesting that, in addition to phospho-Akt and phospho-Erk1/2, other pathways driven by Ras that are insensitive to GGTI-2154 but sensitive to FTI-2148, are also involved in mammary tumor survival and oncogenesis.

The ability of GGTI-2154 to reduce tumor size reflects its proapoptotic and antiproliferative effect. GGTI-2154 suppressed the number of mitotic figures, consistent with the well documented effects of GGTIs on cell cycle events, including induction of p21waf, cyclin-dependent kinase inhibition, Rb hypophosphorylation, and G_1arrest (14, 15, 21). Furthermore, both GGTI-2154 and FTI-2148 induced apoptosis as documented by TUNEL staining and by the presence of apoptotic bodies on H&E stains. The large reduction in tumor size was not only because of programmed cell death but also because of other mechanisms involving degeneration and ischemic necrosis. Interestingly, our data also indicate that treatment with these compounds results in tumor differentiation toward ductolobular breast epithelium. This finding is particularly important because the outcome of patients bearing breast tumors depends not only on stage but also on the histological grade of the disease, with poorly differentiated tumors being highly aggressive and resistant to adjuvant therapy. With >60% of breast tumors being of high grade at diagnosis (36, 37), the availability of a drug capable of not only inhibiting tumor growth but also inducing tumor differentiation is highly desirable.

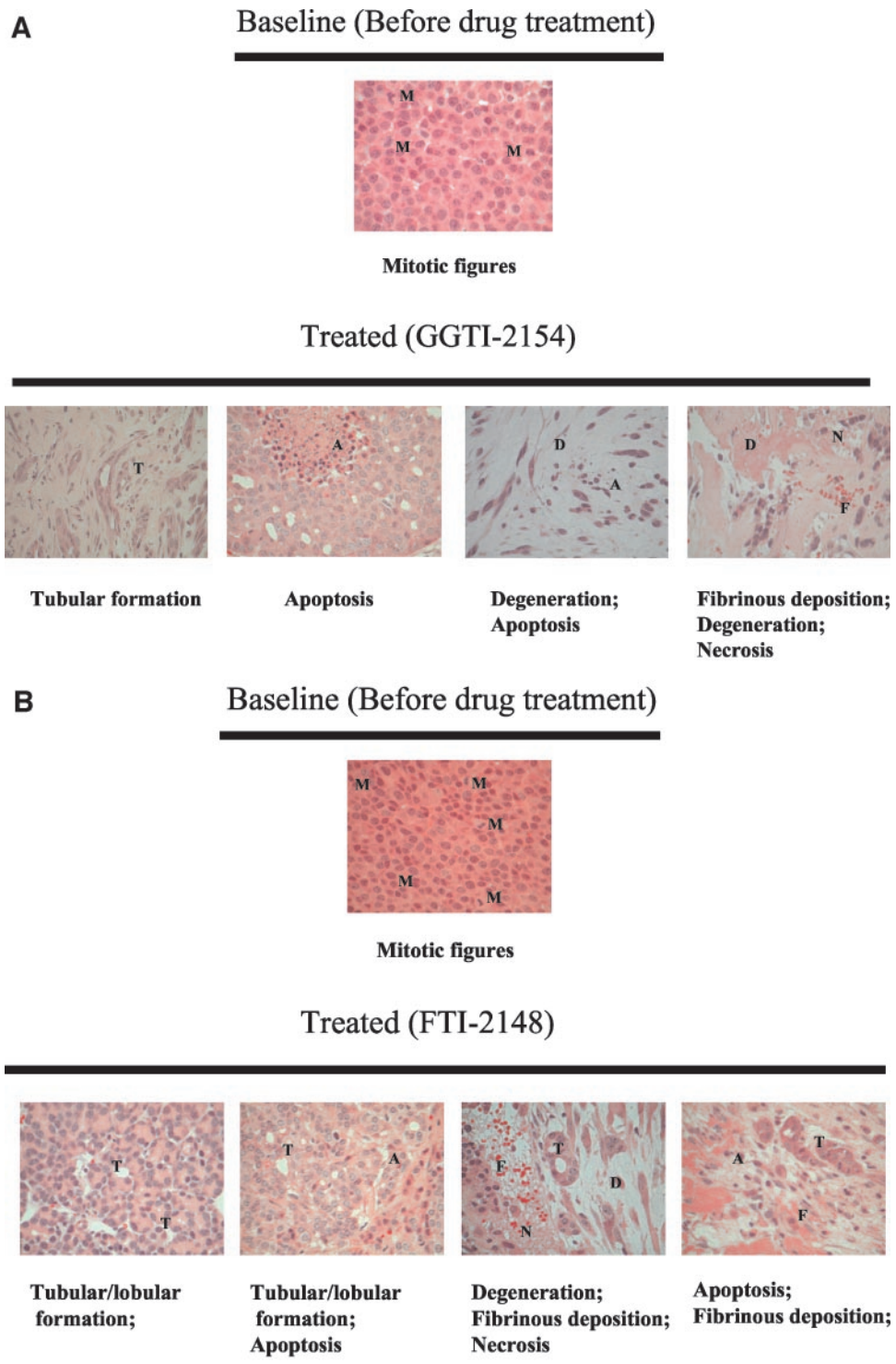

Fig. 5. GGTI-2154 and FTI-2148 treatment induces apoptosis, degeneration with coagulative (ischemic) necrosis, and differentiation in mammary carcinomas from MMTV-ν-Ha-Ras transgenic mice. Needle biopsies from the same tumor before (baseline) and 4 days after (treated) treatment initiation with GGTI-2154 (*A*) or FTI-2148 (*B*) were collected and processed for H&E staining, as described in "Materials and Methods." Data are representative of two independent experiments. *M*, mitotic figure; *T*, tubular/lobular formation; *A*, apoptosis; *F*, fibrinous deposition; *D*, degeneration; *N*, necrosis.

Previous studies showed that FTI L-744, 832 can induce mammary tumor regression in MMTV-based transgenic mice when these tumors are driven by oncogenic Ha-Ras but not oncogenic Ki-Ras (25, 28). In Ki-Ras-driven tumors, growth inhibition, but not tumor regression, was observed, suggesting, as a potential mechanism, the alternative prenylation of Ki-Ras by GGTase I when FTase is inhibited. It would be of great interest to determine whether GGTIs could induce regression in MMTV-Ki-Ras, for that would suggest that geranylgeranylated proteins are also important for K-Ras-induced mammary oncogenesis in this model. The role of geranylgeranylated proteins in mammary oncogenesis in the context of N-Ras, ErbB2, transforming growth factor-α, Ha-Ras/myc, and Ha-Ras/p53(−/−) can also be investigated, because these transgenic mice are available (24).

Taken together, our results clearly demonstrate that GGTI-2154 inhibits protein geranylgeranylation, not farnesylation, suppresses oncogenic and tumor survival pathways driven by the farnesylated protein Ha-Ras, and induces apoptosis and tumor regression in the MMTV-ν-Ha Ras breast cancer model. These results indicate that geranylgeranylated proteins play a pivotal role in farnesylated H-Ras-driven oncogenesis and give strong support for GGTase I as a cancer drug discovery target.

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