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Advances in Brief

GGTI-298 Induces G_0-G_1 Block and Apoptosis Whereas FTI-277 Causes G_2-M Enrichment in A549 Cells[1]

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Abstract

The mechanism by which the geranylgeranyltransferase I inhibitor GGTI-298 and the farnesyltransferase inhibitor FTI-277 inhibit human tumor growth is not known. Herein, we demonstrate that in the human lung adenocarcinoma A549 cells, GGTI-298 induced a G_1-G_0 block whereas FTI-277 induced an enrichment in the G_2-M phase of the cell cycle. Although FTI-277, GGTI-298, and compactin inhibited A549 cell growth, only GGTI-298 and compactin induced apoptosis as demonstrated by four criteria: 4′,6-diamidine-2-phenylindoledihydrochloride staining, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling, DNA fragmentation assay, and flow cytometry. Furthermore, the involvement of geranylgeranylated proteins in apoptotic pathways was confirmed by demonstrating that geranylgeraniol was able to block the ability of compactin to induce apoptosis. These results suggest that protein geranylgeranylation is critical for the control of programmed cell death and that, in A549 cells, farnesylated and geranylgeranylated proteins are involved in G_2-M and G_0-G_1, respectively.

Introduction

Prenylation of the oncoprotein Ras and other small G-proteins is critical to their cellular localization and function (1). Because Ras is one of the most frequently activated oncoproteins in human cancers, inhibitors of FTase,[3] an enzyme that prenylates Ras, are sought after as potential anticancer drugs (2). The recent demonstration that K_B-Ras can be geranylgeranylated *in vitro* (3) and that inhibitors of GGTase I inhibit K_B-Ras processing in whole cells (4) suggested that geranylgeranylation of K_B-Ras is a potential mechanism of resistance to FTase inhibitors. This has led to an interest in developing potent and selective inhibitors of GGTase I, in addition to FTase inhibitors (5, 6).

FTase and GGTase I are α/β heterodimeric enzymes that share the α subunit. FTase catalyzes the transfer of farnesyl from farnesylpyrophosphate to a cysteine at the carboxyl terminus of proteins ending in CAAX where A is any aliphalic amino acid and X is any amino acid except leucine or isoleucine (1, 2). GGTase I, on the other hand, transfers geranylgeranyl from geranylgeranylpyrophosphate to a cysteine in proteins with CAAX terminal sequences where X is leucine or isoleucine (1). FTase and GGTase I recognize and prenylate CAAX tetrapeptides depending on the nature of the COOH-terminal X amino acid. For example, the CAAX box of K_B-Ras, CVIM, is an excellent substrate for FTase (7). Consequently, CAAM tetrapeptides are potent competitive inhibitors of FTase *in vitro* (7). However, these tetrapeptides are inactive in whole cells because of low cellular uptake and susceptibility to proteases. We and others have developed CAAM peptidomimetics that are potent and selective inhibitors of FTase *in vitro*, antagonize oncogenic Ras processing and signaling in whole cells, and inhibit tumor growth in animals (2, 8, 9). Similarly, we have recently developed CAAL peptidomimetics that are potent inhibitors of GGTase I (4–6).

The mechanism of action of FTase and GGTase I inhibitors is unknown. Recently, we have shown that in NIH-3T3 cells and Rat-1 cells the GGTase I inhibitor GGTI-298, but not the FTase inhibitor FTI-277, blocked cells in G_0-G_1 (6). This was consistent with a recent report that showed that Rac1 and RhoA, two geranylgeranylated proteins, are important for the G_1-S-phase transition (10). Moreover, we have also recently reported that compactin, a competitive inhibitor of HMG-CoA reductase, triggered apoptosis in human lung A549 cells (11).

In this report we show that in A549 cells, GGTI-298 induces G_0-G_1 block and FTI-277 induces an enrichment in G_2-M. Furthermore, we demonstrate that GGTI-298 but not FTI-277 induces apoptosis in A549 cells. These results implicate geranylgeranylated proteins in G_1-S-phase and apoptosis and farnesylated proteins in G_2-M.

Materials and Methods

Synthesis of CAAX Peptidomimetics. The FTase-specific peptidomimetic FTI-277 (12) and the GGTase I-specific peptidomimetic GGTI-298 (5, 6) were synthesized by the routes described previously and dissolved in 10 mM DTT in DMSO before being added to the cells.

Cell Culture. Human lung adenocarcinoma A549 cells (ATCC-CCL 185) were routinely grown at 37°C in RPMI 1640 supplemented with 5% FCS under standard conditions of humidity and a CO_2 atmosphere.

Cell Growth Analysis. Cell growth inhibition assays were performed as described previously (11). Cells were treated with inhibitors or vehicle every 24 h for 3 days. Compactin was obtained from Merck Research Laboratories (Rahway, NJ) and dissolved in ethanol before adding to the cells.

Flow Cytometry Analysis. After treatment of cells with compactin, FTI-277, or GGTI-298 for the lengths of time indicated in the legends, the cells were harvested and stained with propidium iodide and then flow cytometry analysis was done as described previously (11). The proportions of cells in G_0-G_1, S, and G_2-M-phase were calculated from their histograms using Cellfit software (Becton Dickinson, Yvry sur Seine, France).

DNA Fragmentation Assay. Cells (5.6×10^4) were seeded into 60-mm dishes and grown in 5% FCS-RPMI 1640 during 48 h. Cells were then treated every 24 h with inhibitors or vehicle for 48 and 72 h. Apoptosis was quantitated on adherent cells using the Cell Death ELISA detection kit (Boehringer Mannheim, Meylan, France). This method allowed detection of histone-associated DNA fragments demonstrating the internucleosomal degradation of genomic DNA occurring during apoptosis.

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[3] The abbreviations used are: FTase, farnesyltransferase; GGTase, geranylgeranyltransferase; FOH, farnesol; GGOH, geranylgeraniol; HMG, hydroxymethylglutaryl; DAPI, 4′,6-diamidine-2-phenylindoledihydrochloride; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling; FTI, farnesyltransferase inhibitor; GGTI, geranylgeranyltransferase inhibitor; TX100, Triton X-100.

TUNEL Analysis and DAPI Staining. *In situ* labeling of apoptosis-induced DNA strand breaks was carried out using the TUNEL technique (13). Briefly, cells (1.2×10^4) grown during 72 h on glass coverslips in culture medium were then treated with inhibitors or vehicle every 24 h for 2 days. Cells were then washed in PBS and fixed in paraformaldehyde (3% w/v in PBS) for 40 min at room temperature. Coverslips were then rinsed three times with PBS and permeabilized with 50% ethanol/50% acetone for 6 min at −20°C. Cell monolayers were rinsed three times with PBS and incubated for 1 h at 37°C in a humidified chamber with 50 μl of TUNEL reaction solution containing 0.3 units/μl TdT (Life Technologies, Inc., Cergy Pontoise, France), TdT buffer, 0.01 nmol/μl biotin 16 dUTP (Boehringer Mannheim), and 0.05 mg/ml BSA. Following three additional washes for 5 min, respectively, with PBS, 0.1% TX100 in PBS, and PBS, cells were incubated for 30 min with 50 μl of staining solution (5% nonfat milk, 4× SSC, 0.1% TX100; and 2.5 μg/ml streptavidin-FITC; Boehringer Mannheim) in a humidified chamber at room temperature and protected from light. Coverslips were rinsed with PBS and washed for 5 min with PBS, 0.1% TX100 in PBS, and PBS successively. For nuclear morphology studies, slides were then incubated for 10 min with 0.1 μg/ml DAPI (Boehringer Mannheim) at 37°C. Cells were viewed on a Zeiss microscope through a ×100 aperature oil immersion lens.

Western Blot Analysis of Ras, Rap1A, and Lamin B Processing. Cells treated as indicated in the figure legends were washed once with PBS and lysed in 50 mM HEPES (pH 7.5), 10 mM NaCl, 1% TX100, 10% glycerol, 5 mM MgCl2, 1 mM EDTA, 2 mM NaVO, 10 μg/ml soybean trypsin inhibitor, 25 μg/ml leupeptin, 5 μM pepstatin, 2 mM phenylmethylsulfonyl fluoride, and 6.4 mg/ml 104 phosphatase substrate (Sigma, St. Louis, MO and Saint Quentin Fallavier, France) during 30 min at 4°C. After centrifugation (12,000 rpm, 4°C, 10 min) equivalent amounts of protein were separated onto 12.5% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Nitrocellulose filters were blocked with 5% nonfat dry milk in Tris-buffered saline and 0.05% Tween 20 (Tris-buffered saline with Tween 20) and probed with either anti-H-Ras (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Rap1A (Santa Cruz Biotechnology), or anti-lamin B (Santa Cruz Biotechnology) IgG in 1% nonfat dry milk in Tris-buffered saline with Tween 20, and then with horseradish peroxidase-conjugated antibodies (Bio-Rad, Richmond, CA and Yvry sur Seine, France). Detection was performed with the enhanced chemiluminescence system (Amersham, Arlington Heights, IL and Les Ulis, France).

Results and Discussion

We recently demonstrated that the GGTase I inhibitor GGTI-298 blocks NIH-3T3 and Rat 1 cells in G_1 whereas the FTase inhibitor FTI-277 did not affect cell cycle distribution (6). Whether this G_1 block by GGTI-298 could lead to apoptosis or simply cytostasis is not known. In the present study, we sought to determine the effects of GGTI-298 and FTI-277 on cell cycle distribution, cell growth, and programmed cell death in the human lung adenocarcinoma cell line A549. A549 cells were incubated with vehicle, FTI-277, GGTI-298, and compactin (as a control) at various concentrations for 48 h, and the number of cells remaining after treatment was determined as described in "Materials and Methods." Fig. 1*A* shows that GGTI-298, FTI-277, and compactin all inhibited the growth of A549 cells with IC_{50} values of 4, 10, and 10 μM, respectively. Furthermore, a time-course study with a concentration of 10 μM of each inhibitor demonstrated that GGTI-298 blocked A549 growth whereas FTI-277 and compactin at the same concentration inhibited the growth only partially (Fig. 1*B*). To demonstrate that the inhibitors are able to affect protein prenylation in A549 cells, we treated these cells with vehicle, FTI-277, or GGTI-298 and immunoblotted the lysates with an anti-H-Ras antibody, an antibody to RaplA (a small G-protein that is only

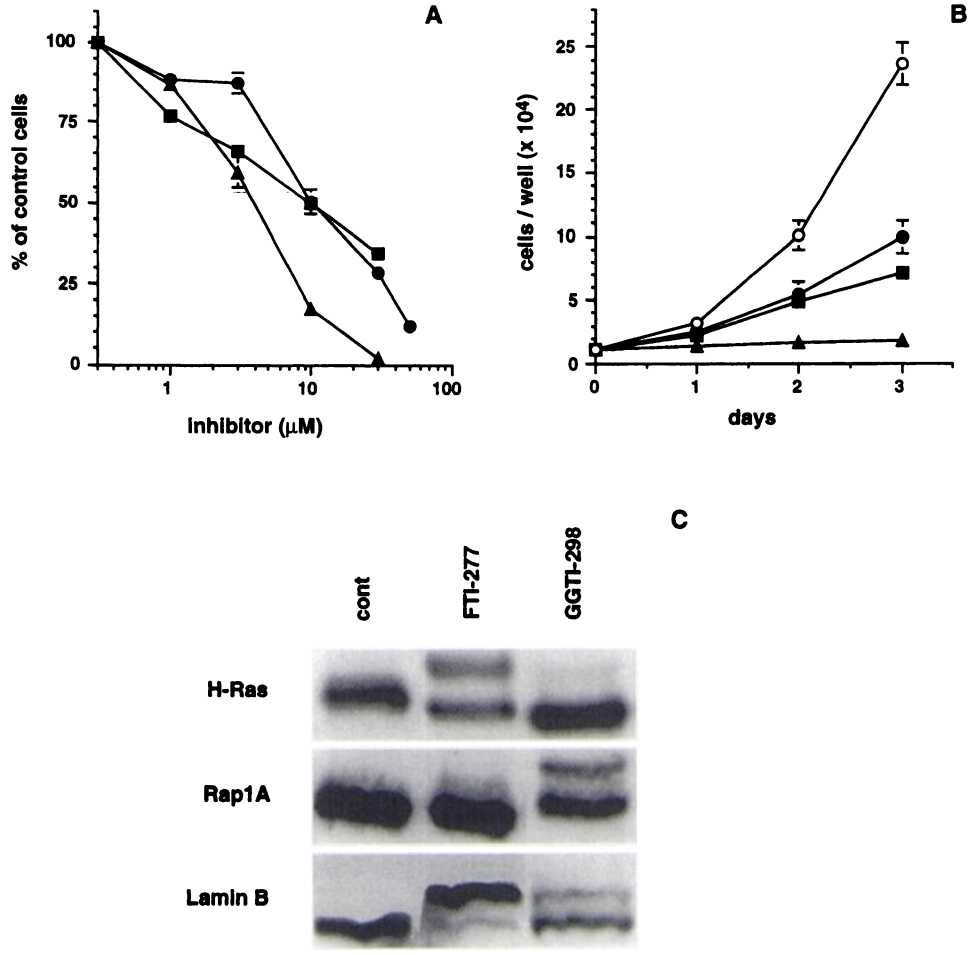

Fig. 1. Inhibition of A549 cell proliferation by FTI-277, GGTI-298, and compactin. *A*, Cells were counted after a 48-h incubation with increasing amounts of FTI-277 (■), GGTI-298 (▲), or compactin (●). Results are expressed as percentage of control cells, and each value is the mean of two separate experiments performed in triplicate. *B*, Cells were counted after 1, 2, or 3 days of incubation without (○) or with 10 μM of FTI-277 (■), GGTI-298 (▲), and compactin (●). Results are expressed as cells per well ($\times 10^4$), and each value is the mean of two separate experiments performed in triplicate. *C*, Cells were treated with vehicle, FTI-277 (5 μM), or GGTI-298 (15 μM) for 48 h. Then the cell lysates were analyzed by Western blotting with anti-H-Ras, anti-Rap1A, or anti-lamin B antibodies. Data are representative of four independent experiments. *Bars*, SE.

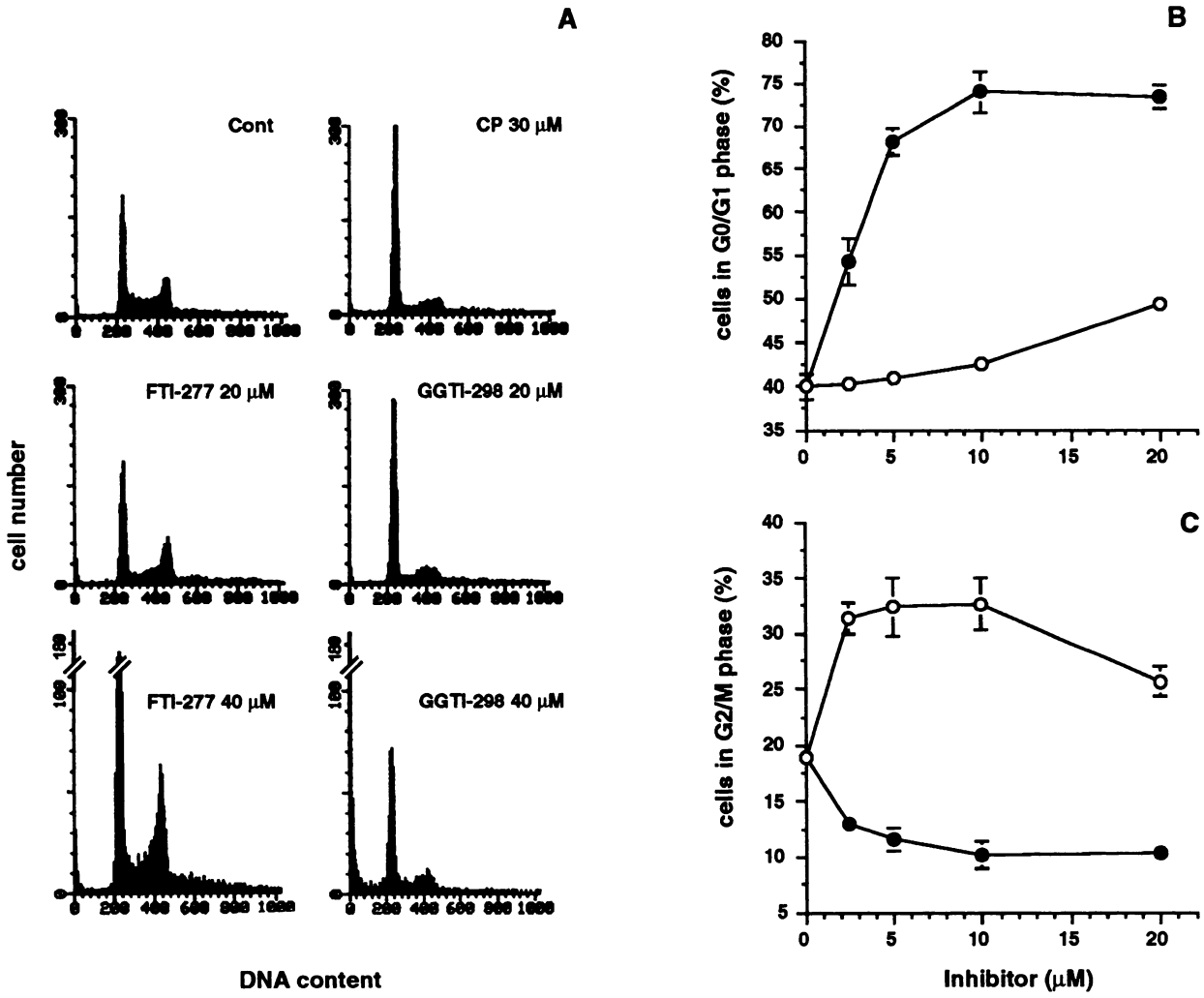

Fig. 2. Flow cytometry analysis of A549 cell cycle distribution. *A*, DNA content of A549 cells. Cells (7.5×10^4) were seeded into 100-mm dishes and 3 days later cells were treated every 24 h for 48 h without (*Cont*) or with FTI-277, GGTI-298, or compactin (*CP*) at various concentrations. Proportions of cells in G_0-G_1 (*B*) or in G_2-M (*C*) were calculated from their DNA content histograms after a 48-h treatment with increasing amounts of FTI-277 (○) and GGTI-298 (●). Each value is the mean of three separate experiments performed in duplicates. *Bars*, SE.

geranylgeranylated) or an antibody to lamin B which is only farnesylated. Fig. 1*C* shows that FTI-277 inhibited the processing of lamin B selectively whereas GGTI-298 inhibited more efficiently the processing of Rapl A. Fig. 1*C* also shows that A549 cells expressed H-Ras protein and that the prenylation of H-Ras was partially inhibited by FTI-277 but not by GGTI-298. The prenylation of N-Ras was blocked by FTI-277 but not by GGTI-298, whereas the prenylation of K-Ras was resistant to both inhibitors (data not shown).

We next evaluated the ability of FTI-277 and GGTI-298 to alter A549 cell cycle distribution by flow cytometry. Fig. 2*A* shows that A549 control cells displayed a typical distribution of 36% in G_0-G_1, 44% in the S-phase, and 20% in G_2-M. Treatment of cells with 30 μM compactin and 20 μM GGTI-298 increased the number of cells in G_0-G_1 to 70% whereas the number of cells in the S-phase decreased to 15% (Fig. 2*A*). Interestingly, treatment of A549 cells with FTI-277 led to an enrichment in G_2-M rather than in G_0-G_1 (Fig. 2*A*). A concentration-dependent investigation demonstrated that the ability of GGTI-298 to induce a G_0-G_1 block was maximal at 10 μM (Fig. 2*B*), whereas the ability of FTI-277 to induce a G_2-M enrichment was maximal at 5 μM (Fig. 2*C*). Furthermore, flow cytometry data also showed that at 40 μM for 48 h, GGTI-298 induced the appearance of a population of A549 cells that contain hypodiploid DNA corresponding to fragmented DNA (Fig. 2*A*). This hypodiploid population of cells also appeared when A549 cells were treated for 72 h with either 20 μM GGTI-298 or 50 μM compactin (data not shown). In contrast, treatment of A549 cells with concentrations of FTI-277 as high as 40 μM did not induce DNA fragmentation (Fig. 2*A*).

The ability of FTI-277 and GGTI-298 to induce apoptosis of A549 cells was investigated next. A549 cells were treated with vehicle, FTI-277 (20 μM), GGTI-298 (20 μM), or compactin (50 μM) for 48 h and then stained with DAPI or labeled using the TUNEL technique as described in "Materials and Methods." Fig. 3*A* (*left panels*) shows that after DAPI staining control A549 cells displayed low intensity staining of the nucleus. Treatment of these cells with GGTI-298 and compactin caused the cells to undergo the morphological changes characteristic of apoptosis, *i.e.*, the chromatin appeared to be condensed and stained intensely with DAPI. In contrast, cells treated with FTI-277 showed very little increase in staining above control cells (Fig. 3*A*). Furthermore, TUNEL labeling confirmed that apoptosis in A549 cells was induced by GGTI-298 and compactin but not FTI-277 (Fig. 3*A*, *right panels*). To quantitate more precisely this apoptotic response in A549 cells, we used an ELISA method that allowed detection of histone-associated DNA fragments demonstrating the internucleosomal degradation of genomic DNA occurring during apoptosis. Fig. 3*B* shows that treatment of A549 cells for 48 h with GGTI-298 (20 and 30 μM) and compactin (30 and 50 μM) induced a

strong response. FTI-277 (30 μM) also induced a weak response but this effect was both not concentration dependent and much smaller than that for GGTI-298 and compactin (Fig. 3*B*).

The above results showed that GGTI-298 but not FTI-277 caused G_0-G_1 arrest and apoptosis, suggesting that a geranylgeranylated protein plays a critical role in biochemical events involved in G_1-S transition and apoptosis. To confirm that inhibition of protein geranylgeranylation and not farnesylation is important for apoptosis, we used an alternative method to the inhibitors (14, 15). We determined whether the addition of FOH or GGOH can prevent the G_0-G_1 block and apoptosis induced by compactin (an inhibitor of HMG-CoA reductase which lowers cellular pools of farnesylpyrophosphate and geranylgeranylpyrophosphate and hence inhibits the processing of both geranylgeranylated and farnesylated proteins). Fig. 4 shows that treatment of A549 cells with compactin induced a G_0-G_1 block and apoptosis. Treatment of cells with mevalonic acid, which replenishes cellular pools of both farnesylpyrophosphate and geranylgeranylpyrophosphate, prevented compactin-induced G_0-G_1 block and apoptosis. Cotreatment of cells with compactin and GGOH also prevented the effects of compactin. As predicted GGOH also restored the prenylation of Rap 1A but not that of H-Ras. Addition of FOH caused only a weak inhibition of compactin-induced apoptosis. Fig. 4 also shows that FOH restored partially H-Ras but not Rap1A prenylation after compactin addition.

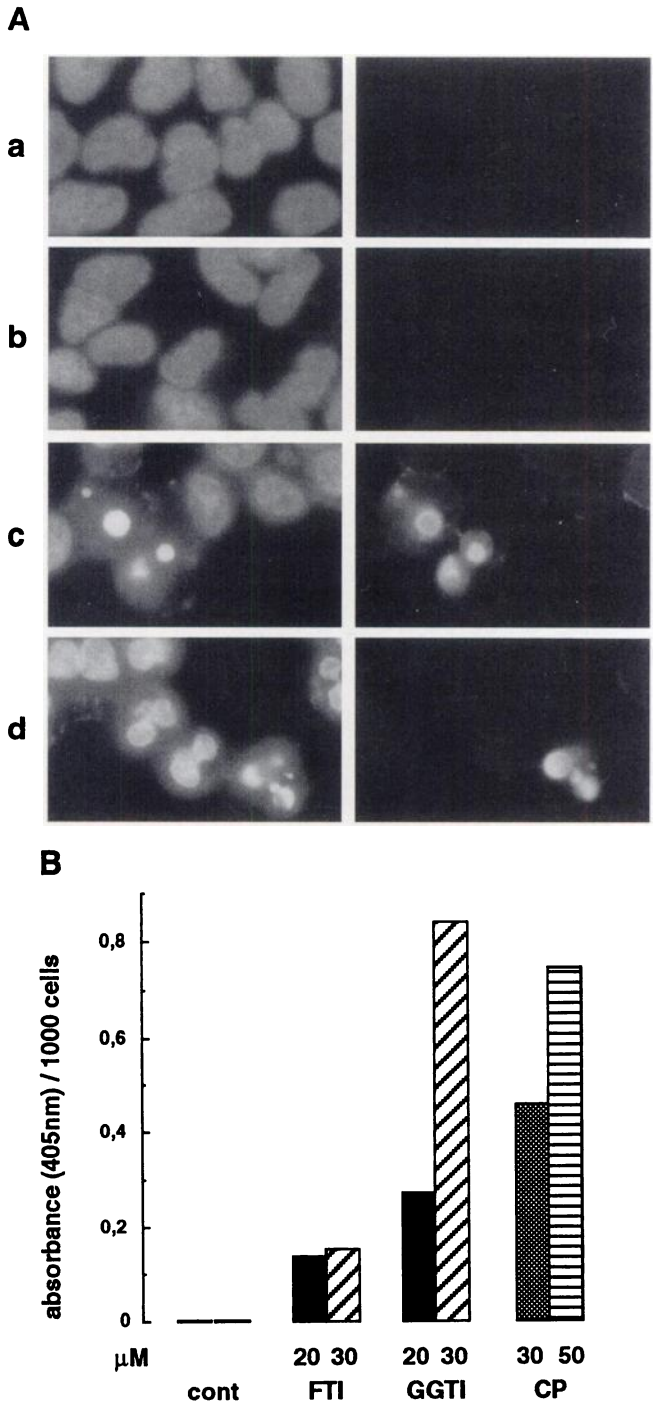

Fig. 3. Incubation with compactin and GGTI-298 causes apoptosis of A549 cells. *A*, Cells were treated without (*a*) or with 20 μM FTI-277 (*b*), 20 μM GGTI-298 (*c*), or 50 μM compactin (*d*) for 48 h. Cells were stained with DAPI (*left*) or labeled by TdT (TUNEL; *right*). *B*, Cells were treated with the inhibitors for 48 h and then DNA degradation was analyzed on 1000 adherent cells using the Cell Death ELISA detection kit as described in "Materials and Methods."

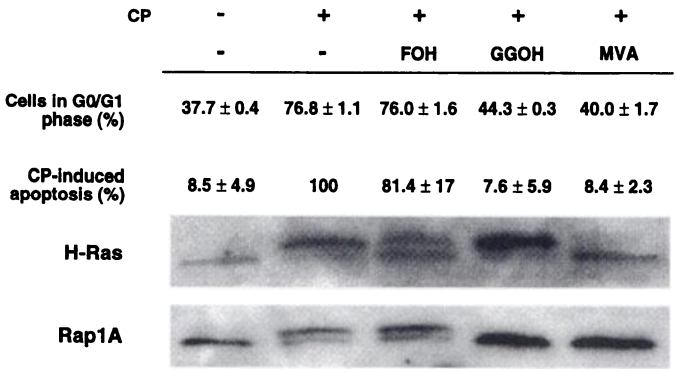

Fig. 4. Reversion by GGOH of compactin-induced G_0-G_1 block, apoptosis, and prenylation. Cells seeded at 5.6×10^4 in 60-mm dishes were allowed to attach for 48 h and then treated without or with FOH (20 μM), GGOH (20 μM), or mevalonic acid (*MVA*; 2 mM) in the presence or absence of 50 μM compactin (*CP*). Forty-eight h later the percentage of cells in G_0-G_1 was determined by flow cytometry, and the proportions of cells in G_0-G_1 were calculated from their DNA content histograms. Apoptosis analysis was performed with the Cell Death ELISA detection kit (Boehringer Mannheim) and results are expressed as percentage of compactin-induced apoptosis. Each value is the mean ± SE of two to three separate experiments performed in duplicate. The effects of compactin in combination with or without FOH (20 μM), GGOH (20 μM), or mevalonic acid (*MVA*; 2 mM) on the prenylation of H-Ras and Rap 1A was determined by Western blotting as described in Fig. 1*C* and "Materials and Methods." The data are representative of two independent experiments.

The present report provides data that implicate geranylgeranylated proteins in the regulation of pathways that trigger programmed cell death in A549 cells. This was accomplished by showing that the GGTase I inhibitor GGTI-298 induces apoptosis and that compactin-induced apoptosis can be prevented by GGOH, an alcohol known to restore protein geranylgeranylation (5, 6, 14, 15). This effect was selective to geranylgeranylation because the FTase inhibitor FTI-277 did not induce apoptosis. The ability of GGTI-298, but not FTI-277, to induce apoptosis may be related to its ability to block cells in G_0-G_1. The ability of GGTI-298 to block cells in G_0-G_1 was not limited to A549 cells, but was also demonstrated in NIH 3T3 cells and Rat-1 cells (6), in other human tumor cell lines, and in pulmonary artery smooth muscle cells.[4]

Thus, the results presented in this article suggest apoptosis as a mechanism by which GGTase I inhibitors inhibit tumor growth. Apoptosis, however, cannot account for the mechanism by which FTase inhibitors inhibit tumor growth. Furthermore, the data also suggest that in A549 cells, geranylgeranylated proteins are critical for G_1-S-phase transition whereas farnesylated proteins are important for G_2-M transition.

[4]Unpublished results.

References

1. Casey, P. J. Biochemistry of protein prenylation. J. Lipid Res., *33:* 1731–1740, 1992.
2. Sebti, S. M., and Hamilton, A. D. Inhibition of Ras prenylation: a novel approach to cancer chemotherapy. Pharmacol. Ther., in press, 1997.
3. James, G. L., Goldstein, J. L., and Brown, M. S. Polylysine, and CVIM sequences of K-RasB dictate specificity of prenylation and confer resistance to benzodiazepine peptidomimetic *in vitro*. J. Biol. Chem., *270:* 6221–6226, 1995.
4. Lerner, E. C., Qian, Y. M., Hamilton, A. D., and Sebti, S. M. Disruption of oncogenic K-Ras4B processing and signaling by a potent geranylgeranyltransferase I inhibitor. J Biol. Chem., *270:* 26770–26773, 1995.
5. McGuire, T. F., Qian, Y. M., Vogt, A., Hamilton, A. D., and Sebti, S. M. Platelet-derived growth factor receptor tyrosine phosphorylation requires protein geranylgeranylation but not farnesylation. J. Biol. Chem., *271:* 27402–27407, 1996.
6. Vogt, A., Qian, Y. M., McGuire, T. F., Hamilton, A. D., and Sebti, S. M. Protein geranylgeranylation, not farnesylation, is required for the G_1 to S phase transition in mouse fibroblasts. Oncogene, *13:* 1991–1999, 1996.
7. Reiss, Y., Goldstein, J. L., Seabra, M. C., Casey, P. J., and Brown, M. S. Inhibition of purified p21ras farnesyl:protein transferase by Cys-AAX tetrapeptides. Cell, *62:* 81–88, 1990.
8. Gibbs, J. B., Oliff, A., and Kohl, N. E. Farnesyltransferase inhibitors-ras research yields a potential cancer therapeutic. Cell, *77:* 175–178, 1994.
9. Buss, J. E., and Marsters, J. C. Farnesyl transferase inhibitors: the successes and surprises of a new class of potential cancer chemotherapeutics. Chem. Biol., *2:* 787–791, 1995.
10. Olson, M. F., Ashworth, A., and Hall, A. An essential role for rho, rac, and cdc42 GTPases in cell cycle progression through G_1. Science (Washington DC), *269:* 1270–1272, 1995.
11. Miquel, K., Pradines, A., and Favre, G. Farnesol and geranylgeraniol induce actin cytoskeleton disorganization and apoptosis in A549 lung adenocarcinoma cells. Biochem. Biophys. Res. Commun., *225:* 869–876, 1996.
12. Lerner, E. C., Qian, Y. M., Blaskovich, M. A., Fossum, R. D., Vogt, A., Sun, J. Z., Cox, A. D., Der, C. J., Hamilton, A. D., and Sebti, S. M. Ras CAAX peptidomimetic FTI-277 selectively blocks oncogenic ras signaling by inducing cytoplasmic accumulation of inactive Ras-Raf complexes. J. Biol. Chem., *270:* 26802–26806, 1995.
13. Gavrieli, Y., Sherman, Y., and Ben-Sasson, S. A. Identification of programmed cell death *in situ via* specific labelling of DNA fragmentation. J. Cell Biol., *119:* 493–501, 1992.
14. Crick, D. C., Waechter, C. J., and Andres, D. A. Utilization of geranylgeraniol for protein isoprenylation in C6 glial cells. Biochem. Biophys. Res. Commun., *205:* 955–961, 1994.
15. McGuire, T. M., and Sebti, S. M. Geranylgeraniol and lovastatin: a novel approach to blocking oncogene H-Ras processing and signaling while restoring protein geranylgeranylation and preventing cytotoxicity. Oncogene, *14:* 305–312, 1997.