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Inhibiting geranylgeranylation blocks growth and promotes apoptosis in pulmonary vascular smooth muscle cells

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Stark, William W., Jr., Michelle A. Blaskovich, Bruce A. Johnson, Yimin Qian, Anil Vasudevan, Bruce Pitt, Andrew D. Hamilton, Saïd M. Sebti, and Paul Davies. Inhibiting geranylgeranylation blocks growth and promotes apoptosis in pulmonary vascular smooth muscle cells. *Am. J. Physiol.* 275 (*Lung Cell. Mol. Physiol.* 19): L55–L63, 1998.—The activity of small GTP-binding proteins is regulated by a critical step in posttranslational processing, namely, the addition of isoprenoid lipids farnesyl and geranylgeranyl, mediated by the enzymes farnesyltransferase (FTase) and geranylgeranyltransferase I (GGTase I), respectively. We have developed compounds that inhibit these enzymes specifically and in this study sought to determine their effects on smooth muscle cells (SMC) from the pulmonary microvasculature. We found that the GGTase I inhibitor GGTI-298 suppressed protein geranylgeranylation and blocked serum-dependent growth as measured by thymidine uptake and cell counts. In the absence of serum, however, GGTI-298 induced apoptosis in these cells as measured by both DNA staining and flow cytometry. The FTase inhibitor FTI-277 selectively inhibited protein farnesylation but had a minor effect on growth and no effect on apoptosis. To further investigate the role of geranylgeranylated proteins in apoptosis, we added the cholesterol synthesis inhibitor lovastatin, which inhibits the biosynthesis of farnesyl and geranylgeranyl pyrophosphates. This also induced apoptosis, but when geranylgeraniol was added to replenish cellular pools of geranylgeranyl pyrophosphate, apoptosis was reduced to baseline. In contrast, farnesol achieved only partial rescue of the cells. These results imply that geranylgeranylated proteins are required for growth and protect SMC against apoptosis. GGTase I inhibitors may be useful in preventing hyperplastic remodeling and may have the potential to induce the apoptotic regression of established vascular lesions.

pulmonary vasculature; prenyltransferase inhibitors; lovastatin

SMOOTH MUSCLE HYPERPLASIA is a major complication in pulmonary hypertension and other vascular pathologies. Thus a rational approach to reduce the smooth muscle in the vessel wall is to target the growth mechanisms active during the progressive stage of lesion development. Because smooth muscle growth is mediated through small GTP-binding (G) proteins such as Ras (9, 10), and potentially Rho, Rac, and Cdc42 (26), a novel approach is offered by agents that we have recently developed. These agents work by inhibiting the posttranslational processing of small G proteins in which lipid is added to the proteins to facilitate insertion into the plasma membrane and permit normal activity (18, 19, 28, 34). The first, and most critical, in a series of processing steps (7) transfers a farnesyl (3, 8, 11) or geranylgeranyl (5) lipid from the cholesterol intermediates farnesyl pyrophosphate or geranylgeranyl pyrophosphate (GGPP) (6), respectively, to cysteines at the carboxy-terminal tetrapeptide CAAX, where C is cysteine, A is aliphatic, and X is any amino acid (2). When X is methionine or serine, the cysteine will be farnesylated (2, 25, 29); when X is leucine, it will be geranylgeranylated (2). The two enzymes responsible for these modifications are farnesyltransferase (FTase) (20, 29) and geranylgeranyltransferase I (GGTase I) (13, 24). Our agents are CAAX peptidomimetics that selectively inhibit these two enzymes (18, 19, 35).

Previous work has demonstrated that FTase-specific CAAX peptidomimetics inhibit the processing of H-Ras (18, 19, 34, 35), disrupt oncogenic Ras signaling through mitogen-activated protein kinase (MAPK) (12, 18, 19), reverse oncogenic H-Ras transformation (14, 16), inhibit the growth of H-Ras-transformed cells in culture (13), slow the growth of H-, K-, and N-Ras-transformed cells in nude mice (17, 33), and accumulate inactive Ras-Raf complexes in the cytoplasm (18).

Much less is known about the effect of GGTase I-specific CAAX peptidomimetic inhibitors. Recently, we have shown that inhibition of GGTase I, not FTase, in fibroblasts results in a reduction in receptor tyrosine phosphorylation (21) and blockade at the G_1 phase of the cell cycle (35). However, little is known about the effects of CAAX peptidomimetic inhibitors in smooth muscle. Because our research focuses on smooth muscle involvement in pulmonary hypertension, we used cultures of smooth muscle cells from the pulmonary microvasculature to evaluate the effects of these agents. The present paper documents that inhibition of GGTase I, not FTase, selectively inhibits growth and promotes apoptosis in smooth muscle cells. This offers a potential strategy for preventing and reversing hyperplasia in vivo.

METHODS

Cell Culture

Rat pulmonary arterial microvascular smooth muscle cells (SMC) were isolated by the method of Johnson et al. (15) and grown in DMEM, fetal bovine serum (20%), HEPES (25 mM), penicillin (100 U/ml), and streptomycin (100 µg/ml) (serum-supplemented, SS). Cells stained positively for the smooth muscle isoforms of α-actin and myosin (15) were routinely subpassaged on plastic with 0.05% trypsin, fed every 2–3 days, and used between *passages 4* and *15.*

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Stock Solutions

All stock solutions [FTase inhibitor (FTI)-271, FTI-277, GGTase inhibitor (GGTI)-298, lovastatin, farnesol (FOH), and geranylgeraniol (GGOH)] were made in 10 mM dithiothreitol-DMSO.

Determinations of Cellular Growth and Proliferation

Cell growth. SMC were plated out at 8,000 cells per well on a 96-well plate in SS and allowed to attach overnight. The cells were then washed once with 1× Hanks' balanced salt solution (HBSS), and the cells were synchronized by treatment with basal medium (DMEM, 0.1% BSA, 100 U/ml penicillin, and 100 µg/ml streptomycin) (BM) overnight. The cells were rewashed with HBSS and treated with 200 µl of SS or inhibitors in SS. Treatment media were refreshed 24 h later. After 48 h, each well was spiked with 10 µl of a 20 µCi/ml solution of [^{3}H]thymidine (NEN) in SS to give a final concentration of 1 µCi/ml. These were incubated at 37°C for 4 h, the radiolabel was removed, the cells were washed twice with HBSS, and 200 µl of 0.05% trypsin were added. The cells were incubated at 37°C for 30 min, the solution was mixed 20 times by pipette aspiration, and 150 µl were transferred to Beckman Ready-Caps. These were dried, and the radioactivity incorporated into the cells was determined by a scintillation counter. Uptake was normalized to total cellular protein.

Cell proliferation. SMC were plated at 32,000 cells per well on a 24-well plate in 1 ml of SS and allowed to attach overnight. For treatment, cells in separate plates were washed once with HBSS and treated with either SS, FTI-277 (5 µM) in SS or GGTI-298 (10 µM) in SS. To determine the number of attached cells, trypsin was added, and total cell number was counted using a Brightline hemocytometer. This value was considered baseline. Cell number was determined at 1, 2, and 3 days. Fresh drug was added daily. Each treatment was assessed in triplicate, and each experiment was repeated a minimum of five times. Results are presented as a percentage of baseline.

Determination of the Effect of FTI and GGTI on Apoptosis

SMC were plated out at 400,000 cells per flask in T-25 flasks in SS and allowed to attach overnight. The cells were then washed once with HBSS. SS, BM, or inhibitors in SS or BM were added. On the basis of previous studies, treatment media were replenished at 24 h; at 48 h, attached and detached cells were collected, fixed in 1% paraformaldehyde containing 10 mg/ml of the DNA-binding fluorochrome Hoechst 33342 for 30–60 min, and stored in 1× PBS without Ca^{2+} or Mg^{2+} in the dark at 4°C. Apoptotic morphology was determined using epifluorescent illumination on a Nikon MikroPhot photomicroscope at a ×60 objective magnification. Cells were scored as apoptotic based upon the degree of chromatin condensation and nuclear breakdown by a non-blinded reviewer. Each experiment was repeated a minimum of three times.

Flow Cytometry

After 48 h of treatment, cells were collected as above, resuspended in ice-cold 70% ethanol, and allowed to fix overnight at 4°C. The cells were then resuspended in 1% glucose in PBS solution containing 1.67 µg/ml of RNase A and 50 µg/ml propidium iodide and stained for 30 min at room temperature. DNA content was measured using a Becton Dickinson flow cytometer.

Ras and Rap1A Processing Assay

Cells were plated in SS at 1.2 × 10^6 cells per T-75 flask and allowed to attach overnight. Cells were washed with 1× HBSS and treated identically to the cells used to determine apoptotic morphology. After 48 h of treatment, attached cells were lysed in HEPES buffer (30 mM HEPES, pH 7.5, 10 mM NaCl, 1% Triton X-100, 10% glycerol, 5 mM $MgCl_2$, 25 mM NaF, 1 mM EGTA, 5 µg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 5 µg/ml aprotinin, and 25 µg/ml antipain). Equal amounts of protein were separated with 12.5% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with isoform-independent antibodies against Ras (Y13-259, ATCC) or Rap1A (SC-65, Santa Cruz Biotechnology, Santa Cruz, CA). Antibody reactions were visualized using either peroxidase-conjugated goat anti-rat IgG (for Y13-259) or peroxidase-conjugated goat anti-rabbit IgG (for SC-65) and an enhanced chemiluminescence detection system (Renaissance, NEN, Boston, MA).

Statistics

Data were analyzed using a one-way ANOVA ($P < 0.05$) with the Student-Newman-Keuls post hoc test for pairwise comparisons ($t < 0.05$). Figures 1–7 present means ± SD.

RESULTS

Development of Peptidomimetic Inhibitors of Prenyltransferases

Recently, we designed potent and selective inhibitors of FTase based on the carboxy-terminal tetrapeptide CAAX. FTI-276 is a cysteine-valine-isoleucine-methionine peptidomimetic in which reduced cysteine is coupled to methionine by 2-phenyl-4-aminobenzoic acid (19). The peptidomimetic inhibited FTase [mean inhibitory concentration (IC_{50}) = 500 pM] selectively over the closely related enzyme GGTase I (IC_{50} = 50 nM) (18). On the other hand, GGTI-297, a cysteine-valine-leucine-leucine peptidomimetic in which a reduced cysteine is coupled to leucine by 2-naphthyl-4-aminobenzoic acid, was more selective for GGTase I (IC_{50} = 54 nM) over FTase (IC_{50} = 190 nM) (35). This in vitro selectivity was reproduced in cultured NIH/3T3 cells in which the processing of H-Ras (exclusively farnesylated) and Rap1A (another small G protein that is exclusively geranylgeranylated) was inhibited by the methyl ester of FTI-276 (FTI-277) (18, 19, 35) and GGTI-297 (GGTI-298) (21, 35), respectively.

Effects of Peptidomimetics on Protein Processing

To confirm that FTI-277 and GGTI-298 were selective inhibitors of protein prenylation in SMC, we analyzed their effects on the processing of Ras and Rap1A by Western blotting of lysates harvested from attached cells following 2 days of treatment with FTI-277 or GGTI-298. We electrophoresed the cell lysates and then immunoblotted them with anti-Ras or anti-Rap1A antibodies as described in METHODS. Figure 1

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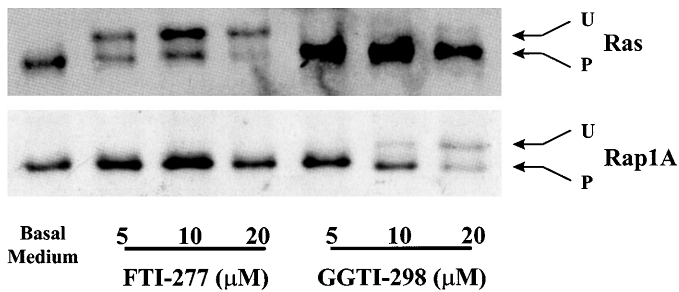

Fig. 1. Farnesyltransferase inhibitor (FTI)-277 and geranylgeranyltransferase I inhibitor (GGTI)-298 inhibit processing of Ras and Rap1A in the presence of serum. Cells were treated with FTI-277 or GGTI-298, and lysates were immunoblotted with either anti-Ras or anti-Rap1A antibodies as described in METHODS. Bottom band, processed (P) form of the protein; top band, unprocessed (U) form. Data are representative of 2 independent experiments.

shows that, in the presence of serum, control cells treated with vehicle contained only fully processed Ras and Rap1A proteins. FTI-277 inhibited the processing of Ras at all concentrations but not completely, even at 20 μM, the highest concentration used (Fig. 1). FTI-277 did not inhibit the processing of Rap1A. In contrast, GGTI-298 did not inhibit Ras processing but inhibited

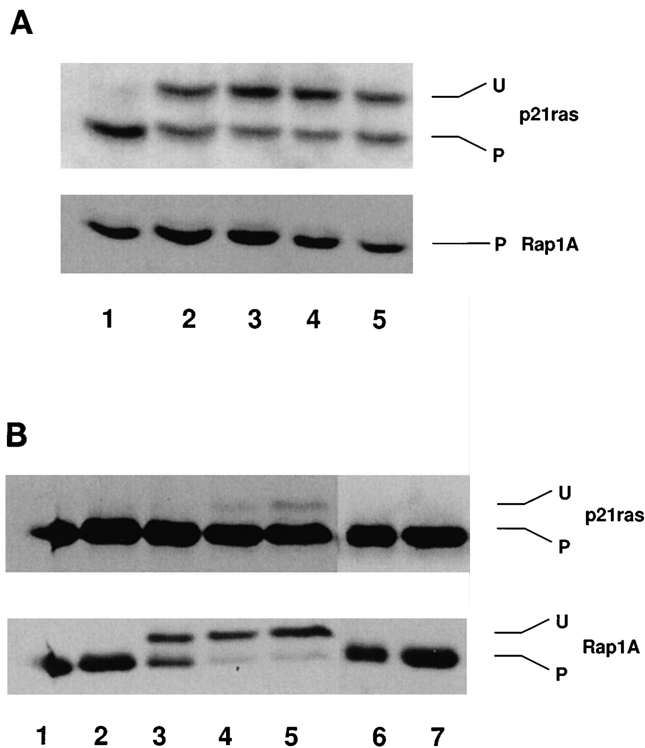

Fig. 2. Inhibiting protein prenylation blocks the processing of Ras and Rap1A in smooth muscle cells (SMC) under serum-free conditions. Cells were treated with either FTI-277, GGTI-298, or FTI-271 (a congener of GGTI-298 inactive as a prenyltransferase inhibitor), and the lysates were then immunoblotted with either anti-Ras or anti-Rap1A antibodies as described in METHODS. Bottom band, P form of the protein; top band, U form. *A*: *lanes 1–5,* FTI-277 at 0, 2.5, 5, 10, and 20 μM. *B*: *lanes 1–5,* GGTI-298 at 0, 2.5, 5, 10, and 20 μM, respectively; *lane 6,* FTI-271 (5 μM); *lane 7,* FTI-271 (10 μM). Data are representative of 3 independent experiments.

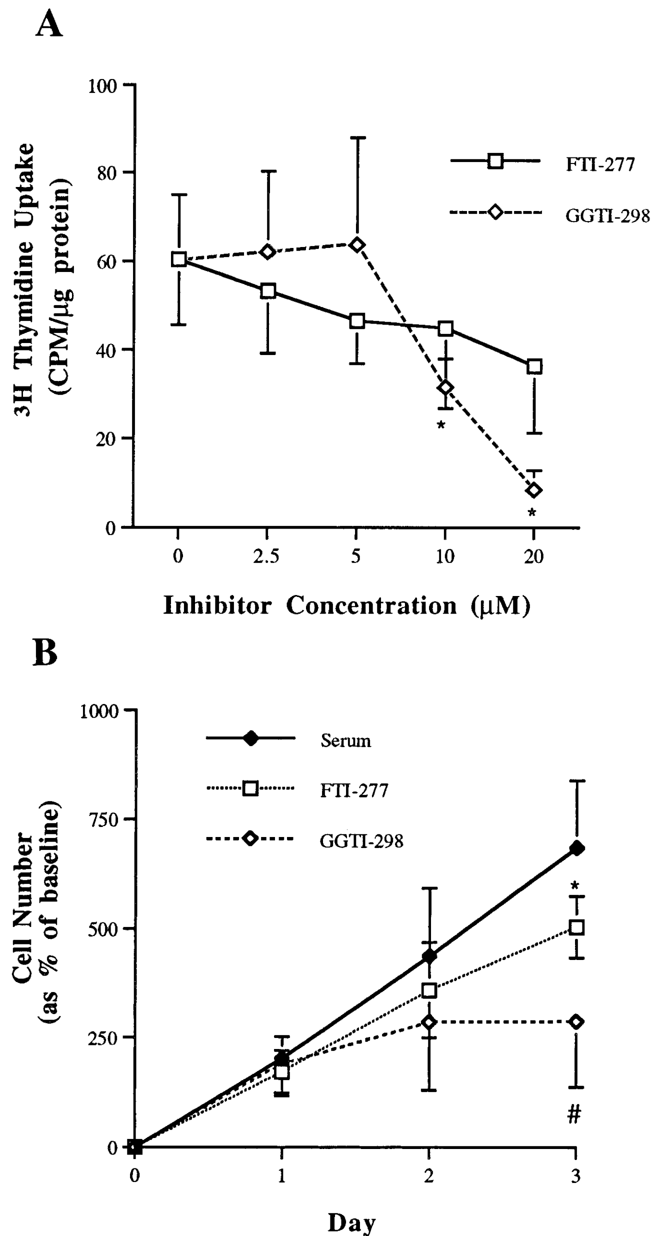

Fig. 3. GGTI-298 inhibits serum-stimulated growth in SMC. *A*: cells were treated with FTI-277 or GGTI-298 in the presence of serum, and [^{3}H]thymidine uptake was measured 48 h later, as described in METHODS. CPM, counts/min. Data are representative of 3 independent experiments [$*P = 0.02$ vs. basal medium (BM)]. *B*: cells were treated with FTI-277 (5 μM) or GGTI-298 (10 μM), and total cell number was determined, as described in METHODS ($*P < 0.05$ vs. BM; $^{\#}P < 0.001$ vs. BM).

Rap1A processing at 10 μM and maximally at 20 μM (Fig. 1).

Similar results were seen in cells treated under serum-free conditions (Fig. 2), except that the blots presented evidence that GGTI-298 inhibited Ras processing to a small extent at 10 and 20 μM. Its inhibition of Rap1A processing was, as in SS, much greater and was evident at 5 μM and maximal at 20 μM (Fig. 2*B*). The fact that higher concentrations of FTI-277 did not further inhibit Ras processing and that GGTI-298

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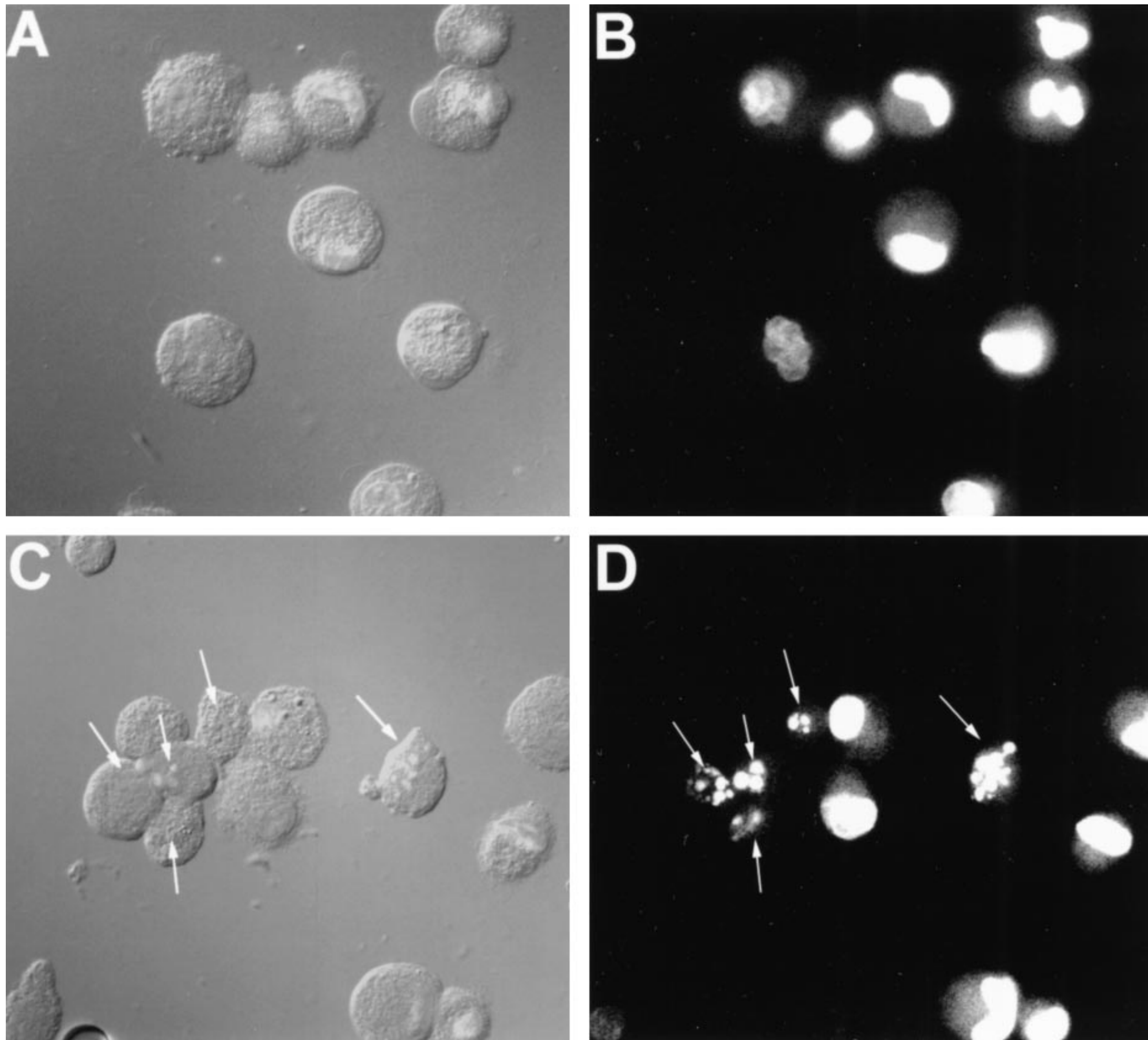

Fig. 4. Morphological assessment of apoptosis in SMC. Photomicrograph of cells incubated in BM alone (*A, B*) or BM with GGTI-298 (*C, D*) for 48 h and viewed under a ×60 objective lens. *A* and *C*: diffraction interference contrast images. *B* and *D*: same cells stained with the DNA-binding fluorochrome Hoechst 33342, as described in METHODS. Cells considered apoptotic show areas of highly fluorescent, condensed chromatin (arrows) and partial loss of the nuclear membrane.

partially inhibited Ras processing suggests that the pool of Ras proteins in SMC can be both farnesylated and geranylgeranylated. This is consistent with our previous results and those of others demonstrating that the K_B isoform of Ras (K_B-Ras) can be both farnesylated and geranylgeranylated (13, 19, 27).

Effect of Peptidomimetics on Cell Growth and Proliferation

Because small G proteins such as Ras, Rho and Rac, which are farnesylated and geranylgeranylated, have been shown to be involved in G_1 to S transition in the cell cycle (26, 35), inhibition of the function of these proteins in SMC could block proliferation. To test this, we treated cells with a range of concentrations of FTI-277 or GGTI-298 and measured the effect on serum-stimulated [^{3}H]thymidine uptake after 2 days. GGTI-298 induced significant decreases in thymidine uptake at 10 and 20 µM. FTI-277 did not have a significant effect at any concentration (Fig. 3*A*). To test the possibility that, in the presence of GGTI-298, proteins that mediate growth could be alternatively farnesylated, we added FTI-277 and GGTI-298 together at concentrations that caused a substantial inhibition of protein processing (5 and 10 µM, respectively). This combination did not decrease thymidine uptake below the level seen with GGTI-298 (10 µM) alone (data not shown).

These results suggested that GGTI-298 had an antiproliferative effect in serum-stimulated SMC. We therefore determined the effect of FTI-277 (5 µM) and GGTI-298 (10 µM) on cell number. GGTI-298 significantly inhibited cell proliferation at 3 days, whereas FTI-277 only reduced the cell number at this time (Fig. 3*B*).

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Effect of Peptidomimetics on Apoptosis

Reduction in cell number and thymidine uptake could be due to a negative effect on growth or to a loss of cells through programmed cell death. To detect apoptosis, cells were harvested at 48 h, stained with Hoechst 33342, and examined microscopically under epi-illumination. Normal cells exhibited a distinct nuclear membrane enclosing diffusely distributed, moderately fluorescent chromatin (Fig. 4). Cells considered apoptotic showed areas of brightly fluorescent, condensed chromatin and partial loss of the nuclear membrane. In cells at later stages of apoptosis, the nuclear membrane was lost and the chromatin dispersed into several small aggregates. Cells exhibiting this morphology were already present at 24 h (data not shown) but were fewer than at 48 h.

Counts of the number of cells displaying normal or apoptotic morphology revealed that, following incubation in SS, the incidence of apoptosis (apoptotic cells as a percentage of the total number of cells) was low and was not increased by GGTI-298, FTI-277, or a combination of the two. In serum-free medium, however, a different picture was evident. Serum deprivation alone did not increase the incidence of apoptosis nor did treatment with FTI-277. Treatment with GGTI-298, however, increased apoptosis significantly and in a concentration-dependent manner (Fig. 5*A*).

The specificity of GGTI-298-induced apoptosis was investigated in separate experiments using a closely related compound that is inactive as a prenyltransferase inhibitor, FTI-271 (previously known as compound 4b) (31). At concentrations of 5 and 10 µM, FTI-271 had no effect on the processing of Ras or Rap1A (Fig. 2*B*). Furthermore, it failed to induce apoptosis in the cells (Fig. 5*B*).

To confirm the effects of the prenyltransferase inhibitors on apoptosis, the distribution of DNA in the cells was assessed by flow cytometry following treatment with FTI-277 or GGTI-298, each at 5 and 10 µM as described under METHODS. In serum-free medium alone, the majority (87.5%) of cells were diploid (Fig. 6 and Table 1). This profile is characteristic of cells in the G_0/G_1 phase of the cell cycle. FTI-277 (5 and 10 µM) had no effect on this distribution (Table 1). GGTI-298 (10 µM), however, induced a subdiploid peak (26%), indicative of the DNA breakdown associated with apoptosis. This appearance of a subdiploid peak was accompanied by a decrease in the population of diploid cells (Fig. 6 and Table 1). Again, the inactive compound FTI-271 had no effect on apoptosis as measured by flow cytometry (data not shown).

Effect of Lovastatin on Apoptosis

We used an alternative approach to demonstrate that inhibition of protein geranylgeranylation induces apoptosis in SMC. We have recently shown that, in lovastatin-treated NIH/3T3 cells, GGOH and FOH can selectively reverse inhibition of protein geranylgeranylation and farnesylation, respectively (21, 22, 35). Treatment of SMC with lovastatin (30 µM) inhibited

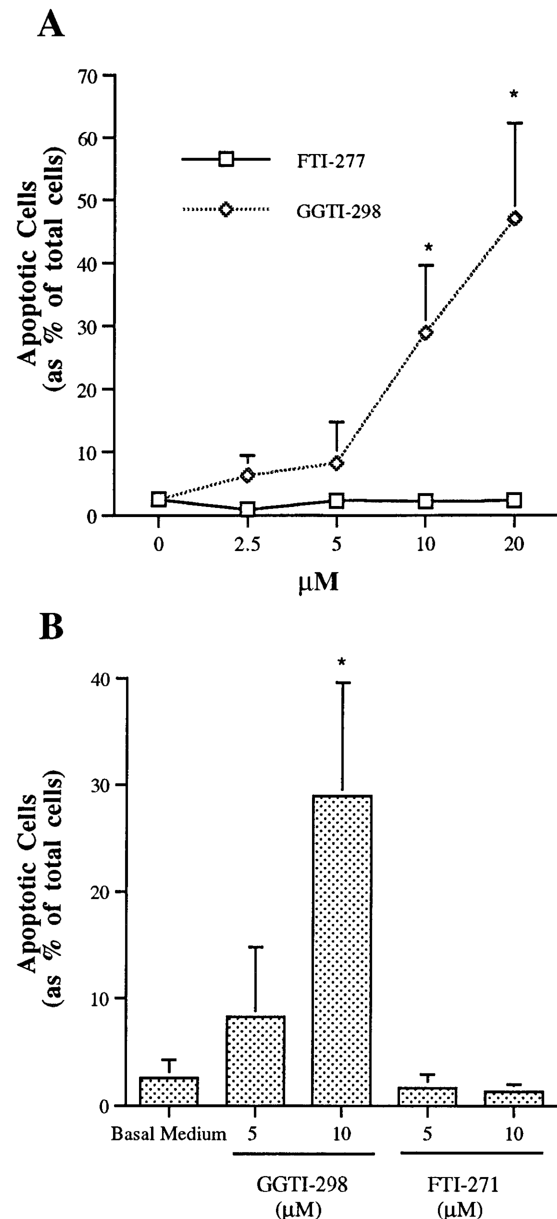

Fig. 5. GGTI-298 induces apoptosis in SMC. *A*: cells were treated for 48 h with a range of concentrations of FTI-277 or GGTI-298, and apoptosis was defined microscopically as in METHODS (*$P < 0.001$ vs. BM; mean ± SD). *B*: cells were treated with GGTI-298 or FTI-271 (either 5 or 10 µM) for 48 h, and then the incidence of apoptosis was determined microscopically (*$P = 0.032$ vs. BM; mean ± SD).

the processing of Ras and Rap1A, although not completely (Fig. 7*A*), and induced apoptosis in 60% of the cells (Fig. 7*B*). Cotreatment of these cells with lovastatin and GGOH (25 µM) restored Rap1A, but not Ras, processing (Fig. 7*A*) and, in parallel, prevented lovastatin from inducing apoptosis (Fig. 7*B*). Cotreatment with lovastatin and FOH (25 µM) restored the processing of Ras completely but of Rap1A only partially. Whereas FOH reduced the incidence of lovastatin-induced apoptosis by one-half, 30% of the total number of cells remained apoptotic (Fig. 7*B*). These results were confirmed using flow cytometry (Fig. 7*C*). Lovastatin treatment increased the proportion of cells in the

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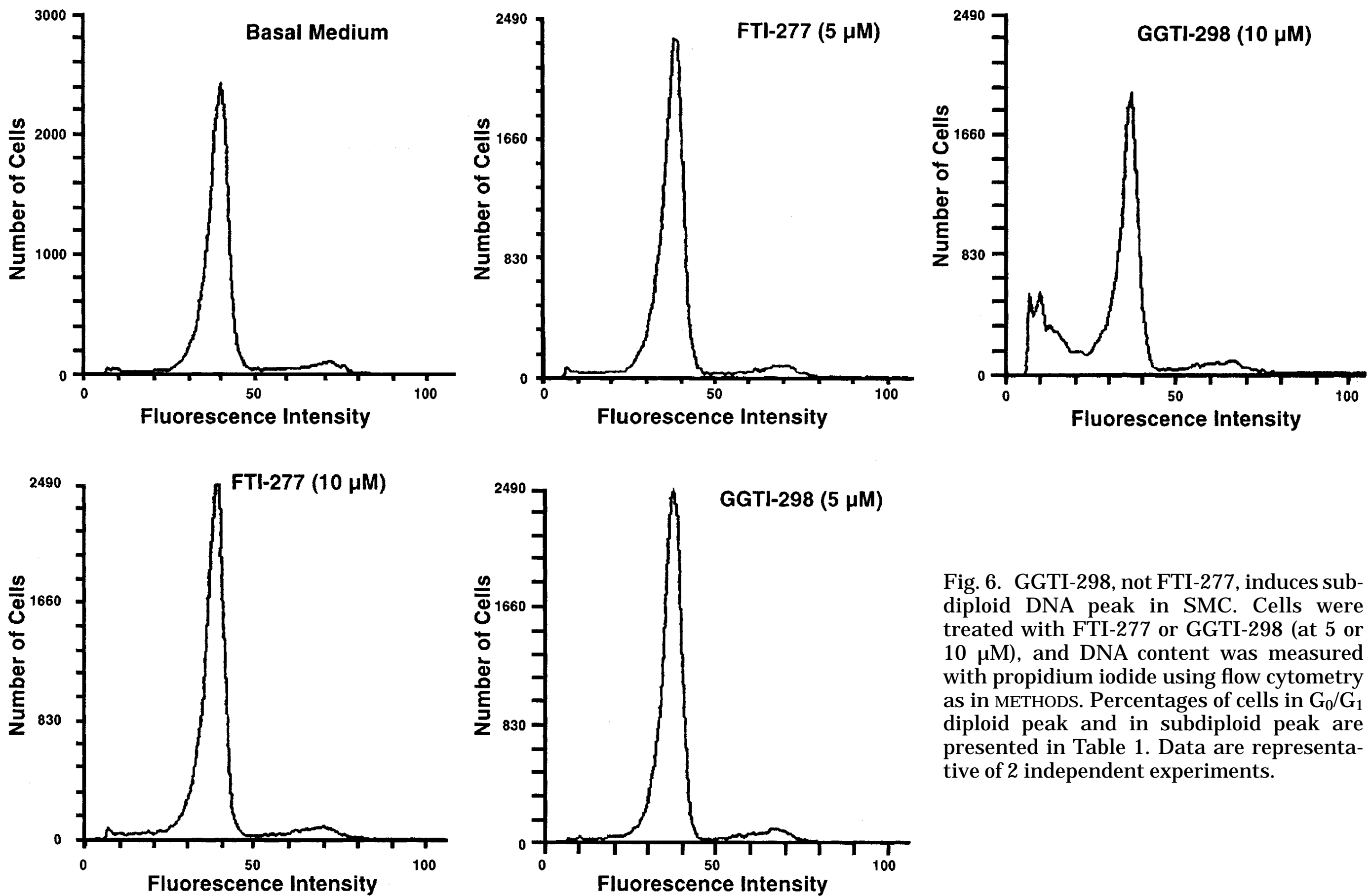

Fig. 6. GGTI-298, not FTI-277, induces subdiploid DNA peak in SMC. Cells were treated with FTI-277 or GGTI-298 (at 5 or 10 μM), and DNA content was measured with propidium iodide using flow cytometry as in METHODS. Percentages of cells in G_0/G_1 diploid peak and in subdiploid peak are presented in Table 1. Data are representative of 2 independent experiments.

subdiploid peak from 2 to 35% and decreased the proportion in the diploid peak from 88 to 54% (Table 1). GGOH completely reversed the apoptotic response, but with lovastatin + FOH treatment 24% of the cells were still apoptotic (Table 1).

To confirm that GGTI-298 was not inducing apoptosis through a nonspecific inhibition of GGPP production, cells were treated with GGTI-298 (5 or 10 μM) and GGOH (25 μM). The addition of GGOH neither decreased GGTI-298-mediated inhibition of Rap1A processing nor decreased the incidence of GGTI-298-induced apoptosis (data not shown).

Table 1. *Effects of prenylation inhibitors on apoptosis*

Treatment	%Subdiploid	%Diploid
Control	2.3	87.5
FTI-277, μM		
5	3.8	88.2
10	4.0	88.0
GGTI-298, μM		
5	4.0	86.9
10	26.0	66.5
Lovastatin	35.4	54.0
Lovastatin + FOH	23.5	66.7
Lovastatin + GGOH	4.6	87.4

Values are percentages of cells containing subdiploid and diploid DNA. Smooth muscle cells were treated with either vehicle, farnesyltransferase inhibitor (FTI)-277 or geranylgeranyltransferase inhibitor (GGTI)-298 alone or in combination with farnesol (FOH) or geranylgeraniol (GGOH). DNA content was then determined by flow cytometry as described in METHODS and in Figs. 6 and 7.

DISCUSSION

The results presented here show that the prenyltransferase inhibitor GGTI-298 blocks serum-stimulated proliferation of pulmonary microvascular SMC in a nontoxic manner. In the absence of serum, however, GGTI-298 promotes apoptosis, characterized by classic microscopic morphology and the presence of a subdiploid DNA peak detected by flow cytometry.

This is the first report to demonstrate the effect of these prenyltransferase inhibitors on the proliferation and apoptosis of untransformed primary cells, specifically SMC from the lung. Previous work has shown that in mouse and rat cell lines the inhibition of GGTase I, but not FTase, arrests cells in the G_1 phase of their cycle (35). In the human tumor cell line A549, however, the inhibition of protein farnesylation results in a G_2/M enrichment (23).

Several isoprenylated proteins are potential targets for the GGTI-298-induced inhibition of serum-stimulated growth. These include members of the $p21^{Ras}$ superfamily that are essential for cell cycle progression (26), such as Rho A and Rac, which are uniformly geranylgeranylated (31), and Ras and Rho B, which can be geranylgeranylated or farnesylated (31, 36). Whereas Ras has been shown to be involved in the growth of

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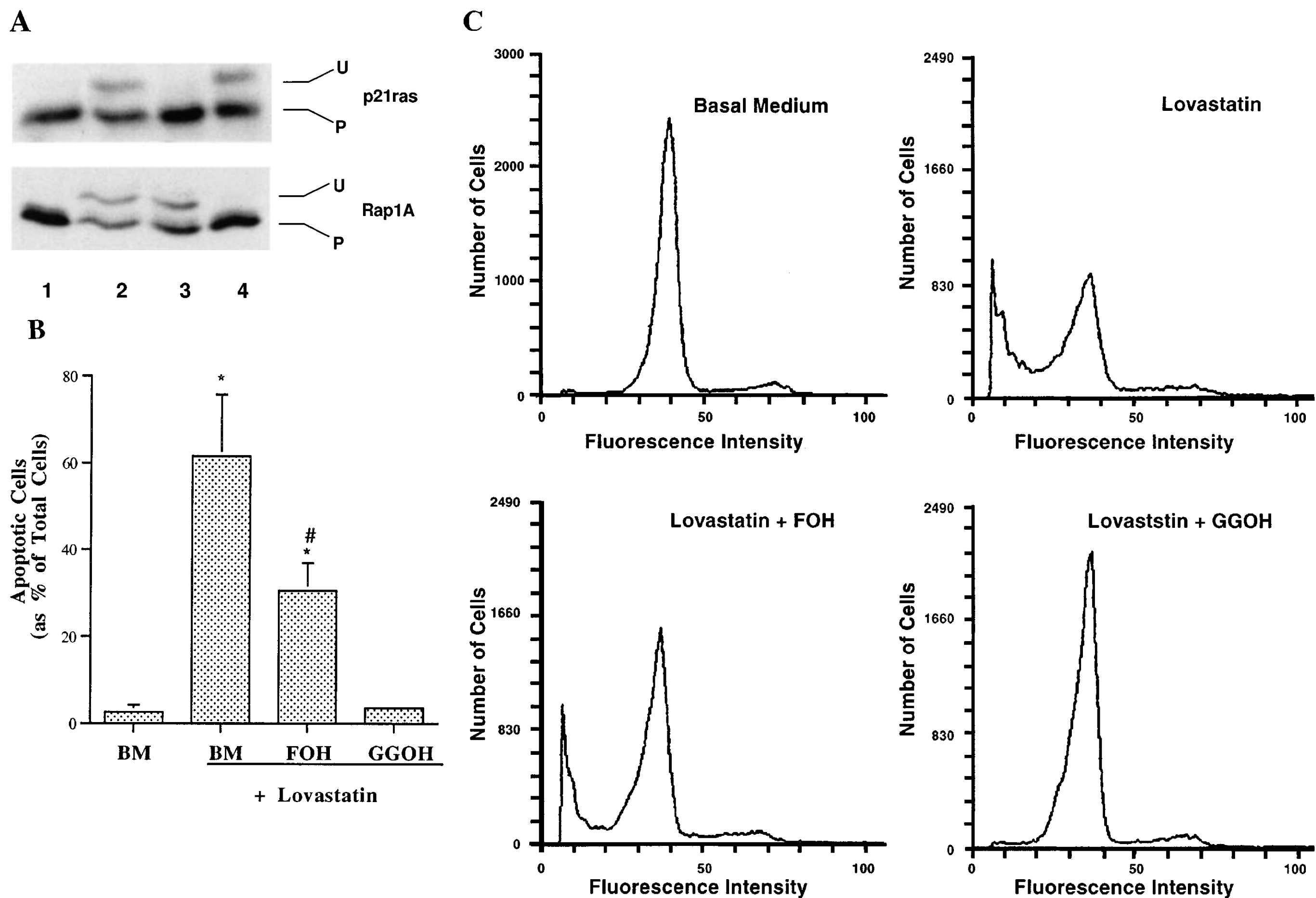

Fig. 7. Inhibition of geranylgeranylation induces apoptosis in SMC. *A*: cells were treated with lovastatin alone or in combination with farnesol (FOH) or geranylgeraniol (GGOH), and cell lysates were processed to determine the levels of processed and unprocessed Ras and Rap1A as described in METHODS. *Lane 1*, BM; *lane 2*, lovastatin (30 μM); *lane 3*, lovastatin + FOH (25 μM); *lane 4*, lovastatin + GGOH (25 μM). *B*: cells were treated as in *A*, and the incidence of apoptosis was determined microscopically (**P* < 0.001 vs. BM; #*P* < 0.05 vs. lovastatin; mean ± SD). *C*: cells were treated as above, and DNA content was determined with propidium iodide using flow cytometry. Percentages of cells in G_0/G_1 diploid peak and in subdiploid peak are also presented. Data are representative of 4 independent experiments (*A* and *B*).

vascular smooth muscle (10), the minor effect of FTI-277 on cell growth and proliferation at concentrations that significantly inhibit the processing of Ras suggests that farnesylated Ras is not critical for this process. The ineffectiveness of GGTI-298 on the inhibition of Ras processing in SS, despite its substantial inhibition of growth, suggests that geranylgeranylated isoforms of Ras are also not involved.

The deficiency of the combination FTI-277 and GGTI-298 to inhibit growth beyond that of GGTI-298 alone suggests that the signaling pathway in SMC utilized by serum principally requires geranylgeranylated proteins.

When SMC were serum deprived, the incidence of apoptosis remained low, which distinguishes SMC from other cell types that promptly enter apoptosis when deprived of essential growth factors (1), and suggests that SMC have an endogenous protective mechanism against serum deprivation. Although the exact nature of this antiapoptotic mechanism operating in serum-free conditions is unknown, the selective effect of GGTI-298 strongly suggests that it is mediated through geranylgeranylated proteins. Treatment with FTI-277 at concentrations (10 and 20 μM) that selectively inhibit Ras processing in SMC did not increase the incidence of apoptosis. GGTI-298 at both 10 and 20 μM inhibited geranylgeranylation in SMC and induced a large increase in the percentage of apoptotic cells.

Inhibition of the cholesterol synthesis pathway is known to block isoprenoid synthesis (11, 30) and prevent the addition of isoprenyl groups to cellular proteins (11, 22, 32). We therefore used the 3-hydroxy-3-methylglutaryl-CoA reductase inhibitor lovastatin as an intervention that would inhibit both farnesylation and geranylgeranylation. Lovastatin increased the incidence of apoptosis to 60% of the total number of cells. The addition of the alcohol GGOH, which restores geranylgeranylation and inhibits farnesylation (4), completely rescued cells (3% apoptosis), confirming a specific requirement for geranylgeranylation. FOH achieved a partial reduction in apoptotic cells, but 30% of the total number remained apoptotic. Under condi-

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tions in which lovastatin partially, but not completely, depletes the cellular pool of isoprenoids, FOH provides farnesyl groups that are utilized to prenylate Ras and also, potentially, to provide geranylgeranyl groups to partially restore geranylgeranylation (Fig. 7).

It is unclear at this time whether the geranylgeranylated protein that mediates this protection against cell death includes the K_B-Ras or one of the other geranylgeranylated small G proteins. GGTI-298 inhibited Ras processing to a small degree (Fig. 2), suggesting the presence of a geranylgeranylated isoform of this protein, consistent with earlier work on K_B-Ras by us and others (13, 19, 27). However, the fact that GGOH did not reverse the inhibition of prenylation of Ras by lovastatin suggests that even if Ras in these cells is geranylgeranylated, it will be farnesylated when GGTase I is inhibited. This argues against Ras being involved in GGTI-298- or lovastatin-induced apoptosis. Geranylgeranylated Rho family members, such as Rho, Rac, and Cdc42, are alternative candidates.

Despite the fact that GGTI-298 inhibited processing incompletely, it nevertheless completely prevented serum-stimulated cell proliferation after 2 days, demonstrating that its effect on growth is complete and manifested by the majority of the cells. In the absence of serum, GGTI-298 promoted apoptosis but only in a fraction of the cells. This illustrates the dissociation between growth and apoptosis in SMC and suggests that *1*) more complete inhibition of processing is necessary to induce apoptosis than to block growth; *2*) geranylgeranylated proteins regulating apoptosis are different from those regulating growth and are less sensitive to the effects of the drug; or *3*) antiapoptotic pathways are active in the resistant population of SMC.

The mechanism by which serum protects against GGTI-298-induced apoptosis is unknown. It may stimulate an antiapoptotic pathway, but one obvious candidate, the MAPK/extracellular response kinase (ERK) pathway, suggested elsewhere to be protective (37), can be ruled out. Neither positive stimulation of MAPK/ERK by platelet-derived growth factor in the absence of serum nor specific inhibition of MEK1, the upstream activator of MAPK/ERK, in the presence of serum altered the incidence of GGTI-298-induced apoptosis (results not shown).

In summary, the addition of FTI-277 or GGTI-298 to SMC inhibits the addition of farnesyl or geranylgeranyl groups, respectively, to cellular proteins. In SMC, GGTI-298 inhibits serum-stimulated cellular proliferation while promoting apoptosis in the absence of serum. The specificity of the apoptotic effect for geranylgeranylation is confirmed by the rescue of SMC from lovastatin-induced apoptosis by GGOH. Finally, these results suggest that inhibitors of GGTase I may be useful in vivo both to prevent ongoing smooth muscle hyperplasia and to reduce the established vascular lesions seen in pulmonary hypertensive remodeling.

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