A Novel Geranylgeranyl Transferase Inhibitor in Combination with Lovastatin Inhibits Proliferation and Induces Autophagy in STS-26T MPNST Cells


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ABSTRACT

Prenylation inhibitors have gained increasing attention as potential therapeutics for cancer. Initial work focused on inhibitors of farnesylation, but more recently geranylgeranyl transferase inhibitors (GGTIs) have begun to be evaluated for their potential antitumor activity in vitro and in vivo. In this study, we have developed a nonpeptidomimetic GGTI, termed GGTI-2Z [(5-nitrofuran-2-yl)methyl-(2E,6E,10E)-3,7,11,15-tetramethylhexadeca-2,6,10,14-tetraenyl 4-chlorobutyl(methyl)phosphoramidate], which in combination with lovastatin inhibits geranylgeranyl transferase I (GGTase I) and GGTase II/RabGGTase, without affecting farnesylation. The combination treatment results in a G2/M arrest and synergistic inhibition of proliferation of cultured STS-26T malignant peripheral nerve sheath tumor cells. We also show that the antiproliferative activity of drugs in combination occurs in the context of autophagy. The combination treatment also induces autophagy in the MCF10.DCIS model of human breast ductal carcinoma in situ and in 1c1c7 murine hepatoma cells, where it also reduces proliferation. At the same time, there is no detectable toxicity in normal immortalized Schwann cells. These studies establish GGTI-2Z as a novel geranylgeranyl pyrophosphate derivative that may work through a new mechanism involving the induction of autophagy and, in combination with lovastatin, may serve as a valuable paradigm for developing more effective strategies in this class of antitumor therapeutics.

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ABBREVIATIONS: GGTI, geranylgeranyl transferase inhibitor; GGTI-2Z (compound 7), [(5-nitrofuran-2-yl)methyl-(2E,6E,10E)-3,7,11,15-tetramethylhexadeca-2,6,10,14-tetraenyl 4-chlorobutyl(methyl)phosphoramidate]; GGPP, geranylgeranyl pyrophosphate; GGMP, geranylgeranyl monophosphate; FTase, farnesyl transferase; RabGGTase, Rab geranylgeranyl transferase; FTI, farnesy transferase inhibitor; GGTase, geranylgeranyl transferase; MPNST, malignant peripheral nerve sheath tumor; iSC, immortalized Schwann cells; NF1, neurofibromatosis type I; Gsp, concentration of drug for 50% inhibition of growth; compound 1, (2E,6E,10E)-ethyl 7,11,15-trimethyl-3-trifluoromethylsulfonyloxyhexadeca-2,6,10,14-tetraenolate; compound 2, (2E,6E,10E)-ethyl 7,11,15-trimethyl-3-(trifluoromethyl)sulfonyloxyhexadeca-2,6,10,14-tetraenolate; compound 3, (2Z,6E,10E)-ethyl 3,7,11,15-tetramethylhexadeca-2,6,10,14-tetraenolate; compound 4, (2Z,6E,10E)-ethyl 7,11,15-tetramethylhexadeca-2,6,10,14-tetraenoate; compound 5, 4-chlorobutyl(methyl)phosphoramic dichloride; compound 6, (5-nitrofuran-2-yl)methyl 4-chlorobutyl(methyl)phosphoramic dichloride; 2Z-GGMP, (compound 8); 2Z geranylgeranyl monophosphate; 2Z-GGPP, (compound 9); 2,6-dichloro-4-methylpyrimidine; TAM, trastuzumab/mam; TCH, tetrahydrofuran; GFP, green fluorescent protein; HEK, human embryonic kidney; E64D, I-3-trans-carboxyran; LC3, microtubule-associated protein-1 light chain 3; LAMP-2, lysosomal-associated membrane protein 2; TEA, triethylamine.
occur. For example, inhibition of N-Ras and K-Ras farnesylation using a FTase inhibitor (FTI) leads to alternative geranylgeranylation by GTTase I (Lerner et al., 1997). On the other hand, Rab GTPases have a CXC or a CC motif at their C termini, which is geranylgeranylated by RabGGTase or GTTase II (Leung et al., 2006).

Several different strategies have been explored to develop inhibitors of GTTase (GGTIs). Peptidomimetic inhibitors and small-molecule inhibitors of GTTase I developed in different laboratories have shown promise as antitumor agents in vitro and in vivo (Vogt et al., 1996; Peterson et al., 2006). For instance, geranylgeranylation inhibition was found to regress breast tumor xenografts in vivo via nuclear accumulation of hypophosphorylated p27Kip1 (Kazi et al., 2009). Although GGTIs were expected to target more proteins in a cell than FTIs, the toxicity of GTTase I inhibition and genetic ablation was surprisingly less significant than initially expected (Sun et al., 2003).

Statins, the cholesterol-lowering drugs, inhibit the rate-limiting enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase of the mevalonate pathway of lipid synthesis, which further leads to inhibition of downstream isoprenoids farnesyl pyrophosphate and geranylgeranyl pyrophosphate (GGPP). Several statins by themselves have been shown to inhibit geranylgeranylation of different target proteins, including Rac1 and Rap1, in diverse cellular systems at concentrations of 10 and 20 μM, respectively (Hamadmad and Hohl, 2007; Kou et al., 2009). One approach to achieving effective GTTase I inhibition includes simultaneously targeting the prenylation pathway at two distinct steps, one to deplete the cellular GGPP pools via statin treatment and the other using a GGPP competitive GTTI that will be more effective when competition from the endogenous GGPP is alleviated (Konstantinopoulos et al., 2007). The underlying rationale is that the two compounds demonstrate modest action by themselves, but when combined they exhibit much more pronounced effects because a lower concentration of an isoprenoid-competitive GTTI can better target GTTase I in an environment with reduced endogenous GGPP (Wojtkowiak et al., 2009). We have previously demonstrated the utility of this approach for the inhibition of cellular farnesylation (Wojtkowiak et al., 2008).

In the current study, we tested a novel compound, GGTI-2Z, which is a GGPP derivative designed to block geranylgeranylation in an isoprenoid-competitive manner, to determine whether it effectively blocks protein geranylgeranylation in a malignant peripheral nerve sheath tumor (MPNST) cell line. We tested this compound either alone or in combination with lovastatin. We demonstrate that GGTI-2Z, in combination with lovastatin, causes G0/G1 cell-cycle arrest, inhibits proliferation, and induces autophagy in cultured STS-26T MPNST cells.

**Materials and Methods**

**Synthetic Chemistry**

**Compound 2.** In an argon-flushed flask, compound 1 (Gibbs et al., 1999) (2300 mg, 9.58 mmol) was dissolved in 65 ml of dimethylformamide (DMF) (Fig. 1). The solution was cooled to −60°C, and potassium bis(trimethylsilyl)amide ([Me3Si]2NK; 0.5 M in toluene, 11.5 mmol, 23 ml) was added dropwise. The solution was warmed to 0°C for 5 min and then recooled to −60°C for 30 min. A slurry of 2-[N,N-bis(trifluoromethylsulfonyl)amide]-5-chloropyridine (4500 mg, 11.5 mmol) in 8 ml of DMF was added to the potassium enolate solution. The reaction was allowed to warm from −60°C to room temperature over 3 h. It was then taken up in 100 ml of ether, washed with 10% aqueous citric acid (2 × 75 ml) and water (4 × 100 ml), dried over MgSO4, and concentrated. Purification by flash chromatography (95:5 hexanes/ethyl acetate) gave 3130 mg (70%) of compound 3 as a colorless oil.1H NMR (300 MHz, CDCl3): δ 1.25 (t, J = 7.05 Hz, 3H), 1.56 (m, 9H), 1.60 (s, 3H), 1.97 (m, 8H), 2.25 (m, 2H), 2.89 (t, J = 7.5 Hz, 2H), 4.15 (q, J = 7.125 Hz, 2H), 5.06 (m, 3H), 5.87 (s, 1H).

**Compound 3.** In a flame-dried, argon-flushed flask were placed triflate compound 2 (750 mg, 1.6 mmol), Pd(PPh3)2Cl2 (11.2 mg, 0.016 mmol), and 15 ml of tetrahydrofuran (THF). The reaction mixture was heated to reflux at 70°C, and (CH3)3In (Perez et al., 2001) (15 ml, 1.5 mmol in THF) was added dropwise. After 4 h, 2 ml of MeOH was added, and the reaction mixture was concentrated. The reaction mixture was next taken up in 30 ml of ether, washed with 10% aqueous citric acid (2 ml), distilled over MgSO4, and concentrated. Purification by flash chromatography (95:5 hexanes/ethyl acetate) gave 3130 mg (59%) of compound 3 as a colorless oil. Note that this procedure using trimethylindium (Perez et al., 2001) affords superior results to the Stille coupling procedure (95:5 hexanes/ethyl acetate) given 3130 mg (59%) of compound 3 as a colorless oil. Note that this procedure using trimethylindium (Perez et al., 2001) affords superior results to the Stille coupling procedure (95:5 hexanes/ethyl acetate).

**Fig. 1.** Synthesis of prodrug GGTI-2Z (compound 7) and structures of 2Z-GGMP (compound 8) and 2Z-GGPP (compound 9). TEA, triethylamine; DCM, dichloromethane.
**Compound 4.** To the solution of ester compound 3 (313 mg, 0.94 mmol) in 7 ml of toluene was added diisobutyl aluminum hydride (1.0 M solution in toluene, 2.82 ml, 2.82 mmol) under argon at −78°C. The reaction was stirred at −78°C for 1 h. The reaction was quenched by adding 30 ml of ethyl acetate and allowed to warm to room temperature. Thirty milliliters of water was added, and the aqueous solution was extracted with ethyl acetate (2 × 20 ml). The combined organic layers were washed with brine (2 × 20 ml) and dried over MgSO4. Concentration followed by flash chromatography (hexane/ethyl acetate 4:1) afforded alcohol compound 4 (210 mg, 76%) as a colorless oil. 1H NMR (300 MHz, CDCl3): δ 1.61 (4H, 7.2 Hz, 2H), 1.78 (4H, 2.7 Hz, 2H), 2.7 (m, 3H), 3.10 (m, 2H), 5.03 (dd, 4H), 5.34 (t, 1H). J = 7.5, 1H).

**Compound 5.** A solution of 4-chloro-N-methylbutanamine (2.4 g, 15.6 mmol) in CH2Cl2 (21 ml) was cooled to 0°C. POCI3 (1.4 ml, 10.4 mmol) was added followed by a solution of triethylamine (4.3 ml, 31.2 mmol) in CH2Cl2 (7.75 ml, 4 M). It was left to warm gradually to room temperature over 3 h. The reaction mixture was quenched with saturated ammonium chloride (30 ml) and diluted with CH2Cl2 (50 ml). The aqueous layer was extracted with CH2Cl2 (2 × 50 ml), and the combined organic layers were dried over MgSO4. Concentration followed by flash chromatography (hexane/ethyl acetate 4:1) gave compound 5 (1.57 g, 42%) as an oil. 1H NMR (300 MHz, CDCl3): δ 1.67 (4H, 7.2 Hz, 2H), 2.74 (m, 3H), 3.18 (m, 2H), 3.48 (m, 2H). J = 7.8 Hz, 2H), 5.03 (s, 3H), 5.34 (t, 1H). J = 7.5, 1H). 31P NMR (CDCl3, 121 MHz): −7.8 ppm.

**Compound 6.** Lithium bis(trimethylsilyl)amide [(Me3Si2)NLi; 3.16 ml, 1 M solution in THF, 3.16 mmol] was added dropwise to a 3.55 (t, 1H) solution of compound 5 (750 mg, 3.16 mmol) at room temperature. The reaction mixture was stirred for 1 h, filtered, concentrated, and after the addition of THF (50 ml) and brine (20 ml). The combined organic layers were washed with saturated ammonium chloride solution. It was extracted with CH2Cl2 (2 × 20 ml) and dried over Na2SO4. It was purified by flash chromatography (hexane/ethyl acetate 1:1) to give compound 6 (692 mg, 63%). 1H NMR (300 MHz, CDCl3): δ 1.95 (m, 12H), 4.03 (d, 1H). J = 8.5 Hz, 2H), 5.03 (s, 3H), 5.34 (t, 1H). J = 7.5, 1H). 13C NMR (CDCl3, 125 Hz): 16.27, 17.94, 23.83, 25.38, 25.95, 26.86, 26.99, 29.70, 32.39, 33.45, 39.95, 44.91, 48.48, 59.44, 63.28 (d, P-C, 6.9 Hz, 2H), 7.2 (d, P-C, J = 3.5 Hz, 1H). 13C NMR (CDCl3, 125 Hz): 16.27, 17.94, 23.83, 25.38, 25.95, 26.86, 26.99, 29.70, 32.39, 33.45, 39.95, 44.91, 48.48, 59.44, 63.28 (d, P-C, J = 5.1 Hz), 112.30, 112.97, 120.74 (d, P-C, J = 6.9 Hz), 123.48, 124.32, 124.59, 131.55, 135.32, 136.25, 143.04, 153.55. 31P NMR (CDCl3, 121 MHz): −14 ppm. MS: ESI 621/623 + Na+ (Fig. 1).

**In Vitro GGTase I Inhibition Assay**

2Z-GGMP [compound 8; synthesized from 2Z-geranylgeraniol compound 4 in a similar manner to that described for 3-allylfarnesyl monophosphate (Clark et al., 2007)] was evaluated against mammalian GGTase I. The first five assay components were added to a 750-μl quartz cuvette for a total volume of 500.5 μl in the order listed; the reaction was initiated by the addition of enzyme, and change in fluorescence intensity was monitored over 300 s. Inhibitor data were expressed as a reaction velocity vs. substrate concentration plot, and the IC50 was determined by using GraphPad Prism software (GraphPad Software Inc., San Diego, CA). Using this procedure, we demonstrated that 2Z-GGMP (compound 8) inhibits GGTase I with an IC50 value of 21 nM, and 2Z-GGPP (compound 9) exhibited the same IC50 value (190 nM) as that previously reported (Zahn et al., 2001). Using the analogous FTase assay (Clark et al., 2007), we demonstrated that the IC50 of compound 8 for this enzyme is ≈ 10 μM.

**Reagents**

GGTI-2Z and lovastatin were prepared in dimethyl sulfoxide (DMSO) and stored at −80°C. HA14-1 (Ryan Scientific Inc., Mount Pleasant, SC) and lovastatin (Sigma-Aldrich, St. Louis, MO) aliquots were prepared and stored similarly. A plasmid (pRK7.GFP.H-Ras.CaaX) encoding green fluorescent protein (GFP) fused to the C-terminal 10 amino acids of rat H-Ras sequence, which encompasses its CaaX sequence, was constructed by subcloning into the pRK7.GFP plasmid (Yang and Mattingly, 2006), a forward primer with the sequence 5’-GATCCGCGTGCATGAGCCTAAATGGTGCTGTCTG3’ and a reverse primer with the sequence 5’-AATTCAGGACAGCA-CATTGGACCTAGCATGGCCG3’ using the sticky-end ligation method.

**Cell Culture**

STS-26T MPNST cells and normal, spontaneously immortalized rat Schwann cells (iSC) were obtained and maintained as described previously (Wojtowik et al., 2008). The murine hepatoma 1c1c7 cell line was obtained from Dr. J. Whitlock, Jr. (Stanford University, Palo Alto, CA) and cultured in α-minimal essential medium containing 5% fetal bovine serum with 100 units/ml of penicillin and 100 μg/ml of streptomycin. Derivatives of 1c1c7 cells that stably expressed GFP-LC3 were generated by transfection of an expression plasmid obtained from N. Mizushima (Tokyo Medical and Dental University, Tokyo, Japan). These cells have been stably transfected to express a GFP fusion construct of LC3. The MCF10 DCIS cell line was obtained from the Cell Lines Resource (Karmans Cancer Institute, Detroit, MI) and maintained as a monolayer in Dulbecco’s modified Eagle’s medium/F12 containing 5% horse serum, 20 ng/ml epidermal growth factor, 0.5 μg/ml hydrocortisone, 10 μg/ml insulin, 50 U/ml penicillin, and 50 μg/ml of streptomycin at 37°C and 5% CO2.

**Western Blot Analysis**

Lysates were prepared from monolayers of cells in 2× Laemml sample buffer by boiling for 5 min and cleared by centrifugation (Mattingly et al., 2001). Samples were then separated on SDS-polyacrylamide gels and electrophoretically transferred to nitrocellulose. Membranes were then probed with 1:200 dilution of anti-LC3 (gift from Dr. David Kessel, Wayne State University, Detroit, MI).

**Live-Cell Imaging Assays**

Human embryonic kidney (HEK) 293 cells were plated into 35-mm culture plates 24 h before transfection with pRK7.GFP.H-Ras.CaaX by using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) as described previously (Norum et al., 2005). Four hours after transfection, fresh media were added along with vehicle or drug at the appropriate concentrations as stated in the figure legends. At the end of treatment, nuclei were stained with Hoechst 33342, followed by confocal live-cell imaging on a LSM-510 microscope (Carl Zeiss Inc., Thornwood, NY) at ×40 magnification. A similar protocol was

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used to study the nuclear morphology of STS-26T cells with and without drug treatment.

**Immunofluorescence Assays**

STS-26T cells were plated onto glass coverslips and treated as indicated in the figure legends. The cells were fixed and processed for confocal immunofluorescence analysis by using anti-LAMP-2 mouse monoclonal (BD Biosciences, San Jose, CA) and anti-LC3 rabbit polyclonal antibodies at 1:50 dilution followed by appropriate fluorescently coupled secondary antibodies. The number of LC3-positive puncta were quantified by using Velocity software 5.2.1 (PerkinElmer Life and Analytical Sciences, Waltham, MA).

**Cell Proliferation Assay**

STS-26T cells and iSC were plated at ~20,000 cells per 35-mm dish 24 h before drug treatment. At the appropriate time points, attached cells were trypsinized and combined with media containing detached cells. The cells were collected by centrifugation for 5 min at 50g and counted with a hemacytometer.

**MTT Assay**

Cells were plated at a density of 2500 cells per well containing 200 μl of growth media with inhibitors or vehicle in 96-well plates and cultured for 72 h. Twenty microliters of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Invitrogen) stock solution (5 mg/ml in phosphate-buffered saline) was then added, and the plates were incubated for 4 h. The medium was removed, and the formazan precipitate formed was dissolved in 150 μl of DMSO. Absorbance values were measured with a plate reader (SpectraFluor Plus; Tecan, Salzburg, Australia) at 495-nm wavelength. After normalizing the absorbance values for media and vehicle controls, the data were analyzed with GraphPad Prism version 4.0c by nonlinear regression (curve fit) and plotting sigmoidal dose-response to obtain GI50 (concentration of drug for 50% inhibition of growth) values, which were further plotted on an isobologram for synergy analysis.

**Flow Cytometric Analysis**

STS-26T cells were treated and collected for DNA analysis as described previously (Mattingly et al., 2006). DNA content was analyzed with a FACScalibur instrument (BD Biosciences). A minimum of 105 cells per sample was analyzed to determine the percentage of apoptotic cells and cells in G1, S and G2/M phases (Modfit; Variety Software, Topsham, ME).

**DEVDase Activity Assay**

Lysates of STS-26T cells were prepared and used for DEVDase assays as described previously (Wojtkowiak et al., 2008). Changes in fluorescence over time were converted to picomoles of product by comparison with a standard curve made with 7-amino-4-methylcoumarin. DEVDase-specific activities are reported as nanomoles of product made per minute per milligram of protein. The bicinchoninic acid assay, using bovine serum albumin as a standard, was used to estimate protein concentrations.

**Results**

**Inhibition of Geranylgeranylation of GTPases by GGTI-2Z and Lovastatin Combination.** Recently, we demonstrated that the monophosphate derivatives of certain farnesyl pyrophosphate analogs are potent FTIs, and that prodrugs derived from these analogs block protein farnesylation (Clark et al., 2007; Wojtkowiak et al., 2008). We have also synthesized and evaluated novel GGPP analogs and found several analogs that are in vitro inhibitors of GGTase I (Gibbs et al., 1999; Zahn et al., 2001; Maynor et al., 2008). In particular, we demonstrated that the 2Z-GGPP analog, compound 9, from which we synthesized the prodrug GGTI-2Z, is an excellent inhibitor of geranylgeranylation of dansyl-GCVLL peptide by GGTase I (Zahn et al., 2001). We have now demonstrated that the corresponding monophosphate compound 8 is the most potent GGPP-based GGTI yet reported (IC50 = 21 nM). In the cellular evaluation of GGTI-2Z, we sought to confirm whether the compound also inhibits geranylgeranylation in vivo. STS-26T cells were treated with 3 μM GGTI-2Z either alone or in combination with 500 nM lovastatin. DMSO treatment was used as a vehicle control. We first tested whether this novel GGTI could inhibit geranylgeranylation of Rap1A via GGTTase I. We achieved this by probing with an antibody that only recognizes the unprenylated form of Rap1A. GGTI-2Z alone was unable to inhibit Rap1A geranylgeranylation even after 48 h of treatment. A distinct band representing unprenylated Rap1A appeared within 24 h in whole-cell lysates treated with lovastatin alone, and this unprenylated Rap1A was strikingly increased upon treatment with a combination of GGTI-2Z and lovastatin (Fig. 2A). The amount of unprenylated Rap1A in comparison with the total Rap1 levels (Fig. 2A) increased over time with the combination treatment. This result suggests that GGTI-2Z, when combined with lovastatin, inhibits Rap1A geranylgeranylation.

Another geranylgeranylated protein that has been inhibited in the past by laboratories using other GGTI compounds is RhoA. Delarue et al. (2007) have shown that GGTI treatment of pancreatic cancer cells results in an increase in RhoA.
expression levels. In our study with the GGTI-2Z and lovastatin combination, we saw a similar marked increase in the expression level of RhoA within 24 h compared with vehicle control, and this increase was maintained even at 48 h of treatment (Fig. 2B).

We also tested for inhibition of RabGGTase or GGTPase II by looking for reduced prenylation of Rab5. Strikingly, we observed a clear up-shift caused by the appearance of unprenylated Rab5 upon combination treatment (Fig. 2C) as opposed to vehicle or single compound treatments.

Combination of GGTI-2Z and Lovastatin Does Not Inhibit FTase. Prenylation of Ras proteins helps target them to the plasma membrane where their site of action lies. These membrane proteins can be fluorescently tagged to visualize their cellular localization patterns in the presence or absence of prenylation inhibitors (Maurer-Stroh et al., 2007). We transfected HEK293 cells with a construct that encodes GFP fused to the CaaX motif of H-Ras (an exclusively farnesylated protein) and then treated the cells with our compounds alone or in combination. The nuclei were then stained followed by live-cell imaging via confocal microscopy for localization of GFP. As seen in Fig. 3, we observed that in the case of vehicle-treated cells GFP.H-Ras.CaaX localizes to the plasma membrane along with some intracellular expression that may represent the Golgi (Choy et al., 1999). Treatment with a low-dose combination of lovastatin and a FTI that we have previously shown to inhibit farnesylation (Wojtkowiak et al., 2008) inhibits the membrane localization and induces a diffuse cytosolic distribution of GFP.H-Ras.CaaX. In contrast, a combination of as high as 6 μM GGTI-2Z plus 500 nM lovastatin failed to prevent membrane localization of the GFP.H-Ras.CaaX protein. These data indicate that GGTase inhibition by GGTI-2Z and lovastatin does not inhibit prenylation of the exclusively farnesylated GFP.H-Ras.CaaX protein (Fig. 3).

GGTI-2Z in Combination with Lovastatin Inhibits Proliferation of STS-26T Cells without Significant Loss of Cell Viability. Next, we sought to test the effect of inhibition of geranylgeranylation by the two compounds on growth and proliferation of STS-26T cells. We treated the cells with the compounds alone or in combination and found that 1 μM concentration of GGTI-2Z or lovastatin alone had little effect on proliferation (Fig. 4A). However, 45 h of exposure of the cells to a combination of 3 μM GGTI-2Z and 1 μM lovastatin caused a significant inhibition of proliferation of the cells, and this inhibition was similar to the extent of 10 μM GGTI-2Z treatment alone (Fig. 4B). In addition, as shown in Fig. 4, there was a dose-dependent and a time-dependent inhibition of proliferation of these cells (Fig. 4, A and B). In addition to proliferation, we examined the percentage viability of the cells. At all the time points tested, there was little effect on cell viability with single or combination treatments (Fig. 4C). We further tested whether there was synergy between the two compounds when used in combination via an MTT assay. After 72 h of treatment, the data analysis showed that these compounds were indeed synergistic in their growth inhibitory effect (Fig. 4D). The synergistic inhibition was indicated by the GI50 values for the combination treatment lying below the theoretical line connecting the GI50 values for GGTI-2Z and lovastatin alone.

GGTI-2Z in Combination with Lovastatin Arrests STS-26T MPNST Cells in G0/G1 Phase of the Cell Cycle. We observed significant inhibition of proliferation of STS-26T cells by cotreatment with GGTI-2Z and lovastatin. We

![Fig. 3. Lack of effect of GGTI-2Z/lovastatin treatment on membrane localization of a farnesylated GFP construct. HEK293 cells were transiently transfected with pRK7.GFP.H-Ras.CaaX plasmid, followed by treatment with prenylation inhibitors as shown for 16 h. FTI-1/lovastatin treatment inhibited H-Ras.CaaX localization at the plasma membrane, whereas GGTI-2Z/lovastatin treatment did not affect the localization even at 6 μM GGTI-2Z concentration. Results are representative of three independent experiments. DAPI, 4′,6-diamidino-2-phenylindole.](image-url)
therefore next determined which point of the cell cycle these compounds targeted to inhibit proliferation. We performed flow cytometry analysis of STS-26T cells treated with GGTI-2Z and lovastatin singly or in combination (Fig. 5). Our results showed that treatment with 3 μM GGTI-2Z or 1 μM lovastatin alone did not affect cell-cycle progression. Interestingly, the same concentrations of the drugs, when used in combination, induced a G0/G1 cell-cycle arrest and a subsequent reduction in the percentage of cells in G2/M and S phases.

GGTI-2Z Alone or in Combination with Lovastatin Does Not Induce Apoptosis in STS-26T Cells. Although analyses of cell viability by Trypan blue exclusion assay suggested no cytotoxicity by combined GGTI-2Z and lovastatin treatment, we wanted to re-examine whether the treatment induced any apoptosis in the cells. For this purpose, we used N-acetyl-Asp-Glu-Val-Asp-amino-4-methylcoumarin to assay the activity of caspases-3 and -7 (Wojtkowiak et al., 2008). HA14-1, a known inducer of apoptosis through inhibition of Bcl-2, was used as a positive control for this assay. When treated with HA14-1 for 2 h, STS-26T cells showed significant induction of apoptosis as demonstrated by a strong increase in DEVDase activation compared with untreated control cells. There was even stronger induction of caspase-like activity after 4 h of treatment with HA14-1. In contrast, treatment with the prenylation inhibitors alone or in combination did not yield any detectable DEVDase activation (Fig. 6A). As an additional test for caspase activation in these cells, we probed lysates of cells treated with the drugs alone or in combination for expression of cleaved caspase-3. As shown in Fig. 6B, procaspase-3, which is the uncleaved form of caspase-3, was evident in all of the cell lysates at 35 kDa. However, in contrast to HA14-1, the prenylation inhibitors did not induce any cleaved caspase-3, indicating lack of detectable apoptosis in these cells by this measure. Finally, we also performed nuclear morphology assays using Hoechst 33342 dye to monitor chromatin condensation as an indicator of apoptosis. Within 45 min of HA14-1 treatment, we observed nuclear condensation in the form of bright blue spots as seen in Fig. 6C. Conversely, with GGTI and/or lovastatin treatment, we did not see any nuclear morphological changes (Fig. 6C) or any appearance of DNA laddering (data not shown), which further confirms the lack of apoptosis in the cells.

GGTI-2Z and Lovastatin Combination Treatment Induces Autophagy in STS-26T Cells. We further investigated whether autophagy was involved in determining the response of the cells after prenylation inhibition. We achieved this by assaying LC3, the classical marker of autophagy (Klionsky et al., 2008). During the formation of autophagosomes, LC3-I is processed to LC3-II via phosphatidylethanolamine attachment. The presence of LC3-II is, therefore, associated with occurrence of autophagy. We observed a subtle increase with single treatments and a marked increase with combination treatment in the appearance of LC3-II in STS-26T cells within 24 h compared with vehicle treatment. This increase was sustained at 48 h (Fig. 7A). This result suggests that the drugs may be inducing or up-regulating the autophagic process in these cells.

Increase in LC3-II levels may be associated with either of two possible mechanisms: an increase in formation of autophagosomes or a decrease in processing/degradation of LC3-II caused by an absence of autophagosome/lysosome fusion or depression of lysosomal protease activities. To distinguish between the effects on synthesis and degrada-
tion, we pretreated the cells with the protease inhibitors E64D and pepstatin A. These compounds inhibit lysosomal proteases, which would prevent degradation of LC3-II in the autophagolysosome. The results showed that the protease inhibitors did induce a further increase in LC3-II levels (Fig. 7B), which is consistent with there being autophagosome and lysosome fusion and subsequent proteolytic processing in the autophagolysosome. We also used bafilomycin A1, which blocks the maturation of autophagosomes by its inhibition of the vacuolar ATPase. In this case, we treated the cells with the vehicle, single compounds, or combination treatment for 48 h, with bafilomycin A1 also present for the final 2 h of the incubation. The results show that the single treatments do not have much effect on autophagic flux compared with vehicle control, whereas the combination treatment again increases LC3-II accumulation either in the presence or absence of bafilomycin A1 also present for the final 2 h of the incubation. The results show that the single treatments do not have much effect on autophagic flux compared with vehicle control, whereas the combination treatment again increases LC3-II accumulation either in the presence or absence of bafilomycin A1 also present for the final 2 h of the incubation. The results show that the single treatments do not have much effect on autophagic flux compared with vehicle control, whereas the combination treatment again increases LC3-II accumulation either in the presence or absence of bafilomycin A1 also present for the final 2 h of the incubation. The results show that the single treatments do not have much effect on autophagic flux compared with vehicle control, whereas the combination treatment again increases LC3-II accumulation either in the presence or absence of bafilomycin A1 also present for the final 2 h of the incubation. The results show that the single treatments do not have much effect on autophagic flux compared with vehicle control, whereas the combination treatment again increases LC3-II accumulation either in the presence or absence of bafilomycin A1 also present for the final 2 h of the incubation.

GGTI-2Z and Lovastatin Also Effectively Inhibit Proliferation of 1c1c7 Cells and Induce Autophagy in DCIS Cells. To test the effects of the drugs in cell lines that model other cancers, we performed cell proliferation and viability assays in a murine hepatoma cell line, 1c1c7. We found that GGTI-2Z and lovastatin combination also inhibits proliferation of these cells without significantly affecting their viability (Supplemental Fig. 1, A and B). This cell line stably expresses a GFP fusion construct of LC3. Upon combination treatment, there was increased appearance of LC3-positive vesicles, indicating induction of autophagy (Supplemental Fig. 1C). We also treated cultures of MCF10.DCIS cells with the drugs and assayed for LC3-II accumulation by Western blotting. The results showed increased LC3 processing, confirming autophagic induction in these cells as well (Supplemental Fig. 1D).

Normal Immortalized Rat Schwann Cells Are Resistant to GGTI-2Z and Lovastatin Cotreatment. We then tested whether GGTI-2Z and lovastatin in combination affected proliferation of iSC. As shown in Supplemental Fig. 2A, the compounds either alone or in combination had little to no effect on the proliferation of iSC. In addition, we found no detectable morphological changes in these cells upon treatment with the compounds (Supplemental Fig. 2B).
Prenylation inhibitors of different classes have been tested preclinically and clinically for therapy of many types of cancer. Statins were among the first of such compounds to be tested for their potential antitumor activity in preclinical models. Nevertheless, in general, statins have shown limited promise when combined with conventional chemotherapy (reviewed in Konstantinopoulos et al., 2007). Great effort has been focused on the development and testing of selective inhibitors of FTase. As previously mentioned, however, FTI-treated cells can bypass this inhibition for certain substrates (notably, K-Ras and N-Ras) through alternative geranylgeranylation. In addition, FTIs are capable of targeting prenylation of multiple proteins. There has been little success of these compounds when used alone in clinical trials.

In the past our group has tested prenylation inhibitors, including FTIs and statins, as potential therapies for neurofibromatosis type 1 (NF1) and other hyperproliferative disorders (Mattingly et al., 2002). We recently showed that a novel FTI compound, FTI-1, in combination with lovastatin induces apoptosis in two different NF1 MPNST cell lines (Wojtkowiak et al., 2008). Moreover, we observed little to no detectable toxicity of the treatment in normal iSC, indicating the potential use of this combination treatment for NF1 MPNSTs. The FTase substrates that are affected by FTI treatment to produce inhibition of cell proliferation and survival are still unclear. However, an interesting study involving a chemical genetics approach revealed RabGGTase or GGTase II as a target of FTIs (Lackner et al., 2005). This finding supported the idea that FTIs have many different targets that may be responsible for their activity and side effects and also identified a potential role for Rab proteins and RabGGTase in p53-independent apoptosis induced by FTIs.

In this study, we have developed a novel GGTI whose action is potentiated by cotreatment with lovastatin, resulting in inhibition of proliferation and cell-cycle arrest associated with induction of autophagy in STS-26T MPNST cell line. The strategy that we used to develop the GGPP-based inhibitor compound GGTI-2Z is analogous to that previously described for the development of FTIs (Maynor et al., 2008). Of all the GGPP analogs evaluated, compound 8 served as the best inhibitor with an impressive IC50 value of approximately 21 nM for GGTase I enzyme in vitro. Encouragingly, this analog also exhibited no significant binding to mammalian FTase, further confirming its promise as a tool for evaluating cellular GGTase I inhibition. In addition, as seen by immunocytochemistry, GGTI-2Z does not affect the membrane localization of a GFP construct that is exclusively farnesylated, suggesting that it acts to solely inhibit geranylgeranylation, whereas farnesylation remains unaffected. Furthermore, the fact that it did not have any effect on either morphology or proliferation of normal iSC implies a lower risk of toxicity to normal cells.

Our rationale for the combinatorial approach was to achieve more efficient GGTase I inhibition via GGTI-2Z by simultaneously depleting endogenous GGPP pools. Rap1A is a Ras family GTase that is known to be solely geranylgeranylated presumably by GGTase I (Casey et al., 1991). We observed inhibition of Rap1A prenylation, thus confirming our in vitro result that GGTI-2Z effectively inhibits GGTase I, although it does so only when combined with lovastatin. Conversely, lovastatin alone is sufficient to modestly inhibit Rap1A geranylgeranylation. These data suggest that Rap1A may not be a critical target for the inhibition of STS-26T cell proliferation, because even higher levels of lovastatin (up to 1 μM) do not affect cell-cycle distribution.

The combination of GGTI-2Z/lovastatin synergistically inhibits proliferation of STS-26T cells. This antiproliferative activity is consistent with the induction of cell-cycle arrest in the G1 phase. Other GGTIs have also been shown to block
cell-cycle progression of several tumor cell lines and subsequently induce apoptosis (Vogt et al., 1997). However, with GGTI-2Z/lovastatin there was little effect on cell viability and we did not observe the classical apoptotic morphology or apoptotic markers in STS-26T cells.

Another potential determinant of cell survival is the phenomenon of autophagy. In recent years, autophagy has been discovered to be an important mechanism adopted by many different cell lines for determining their fate, and it is still a topic of debate whether autophagy is a mechanism of cell survival or cell death (Apel et al., 2009). Interestingly, there is increasing evidence suggesting that several cancer cells show up-regulation of the process leading to cell survival and cancer progression (Rubinsztein et al., 2007). Recently, three FTIs were found to induce autophagy in two different human cancer cell lines (Pan et al., 2008). In addition, some statins can induce autophagy in a cell type-specific manner owing to their ability to inhibit protein prenylation rather than cholesterol synthesis (Araki and Motojima, 2008). For instance, cerivastatin or simvastatin are capable of inducing autophagy in rhabdomyosarcoma cells (Araki and Motojima, 2008), whereas lovastatin or simvastatin fail to do so in hepatocytes (Samari and Seglen, 1998). We examined our cultures treated with the GGTI/lovastatin combination to determine whether autophagy occurred. Based on the analysis of LC3-I conversion to LC3-II via Western blot, and the colocalization of LC3 with the lysosomal protein LAMP-2 via immunocytochemistry, we confirmed that autophagy was induced and driven to completion in STS-26T cultures cotreated with GGTI-2Z and lovastatin.

One of our observations shows that the compounds inhibit prenylation of Rab5, a GGTase II or RabGGTase substrate. These data indicate that the compounds not only inhibit GGTase I, but also serve as substrates for GGTase II. This was not surprising because the two enzymes share strikingly similar active sites (Lackner et al., 2005), and, therefore, a compound designed to bind the GGPP binding pocket of either of the two can be expected to bind similarly to the other.
The preference of binding in that case will be determined by relative affinity of the compound for the enzymes. We have not yet tested the in vitro ability of GGTI-2Z to bind to GGTTase II to determine the $K_M$ value. An alternative explanation for the dual inhibition of GGTTase I and RabGGTase-mediated prenylation is that GGTTase II inhibits GGPP synthase, blocking the production of the GGPP substrate needed for both processes (Wiemer et al., 2007). In addition, there is a noticeable decrease in the expression level of Rab5 upon GGTTase-II/lovastatin treatment. Potentially, this RabGGTase inhibition and/or enhanced Rab5 turnover may contribute to the effects of the compounds on MPNSTs.

Rab proteins have been shown to play an important role in carcinogenesis (Cheng et al., 2004). Traditionally, Rab5 is known to have a well-established role in endocytosis and vesicular transport of proteins (Bucci et al., 1992). More recently, however, an interesting study in cell culture and fly models of Huntington’s disease suggested a role for Rab5 in the early stages of the process of macroautophagy that is independent of its endocytic function (Ravikumar et al., 2008). This study showed that Rab5 inhibition via expression of dominant-negative Rab5 results in a decrease in LC3-positive autophagic vacuoles and also enhances polyglutamine toxicity. In our study we saw that inhibition of Rab5 prenylation via GGTTase inhibition is correlated with an increase in LC3-II levels. Prenylation inhibitors may be capable of only partially blocking Rab5 activity, and thus, the partially prenylated and active Rab5 may still be sufficient for autophagic progression. Additional evidence that may support such a connection is that fluvastatin and pravastatin-induced RabGGTase inhibition causes vacuolation in rat skeletal myofibers (Sakamoto et al., 2007). Alternatively, the GGTTI may target one or more other proteins that may potentially contribute to its action. Further studies would be required to better elucidate the role of Rab5 and other proteins in GGTT/lovastatin-induced autophagy in MPNSTs.

In conclusion, we have developed a novel compound, GGTTase-II/lovastatin, that blocks prenylation mediated by both GGTTase I and RabGGTase and exerts cytostatic activities in STS-26T MPNST cells in a caspase-3-independent manner. The action of GGTTase-II/lovastatin is potentiated by a low-dose statin combination treatment and strongly correlates with the induction of autophagy. This combination treatment does not block proliferation or induce toxicity in normal, immortalized Schwann cells, but it does have activity against two other transformed cell lines, 1t1c7 murine hepatoma cells and MCF10.DCIS cells that model human breast ductal carcinoma in situ. Further studies testing therapeutic efficacy of GGTTase-II/lovastatin may serve to develop better understanding of geranylgeranylation inhibitors and evaluate their potential in the context of cancer therapy and some Rab-associated protein-trafficking disorders.

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