TITLE

A NOVEL GERANYLGERANYL TRANSFERASE INHIBITOR IN COMBINATION WITH LOVASTATIN INHIBITS PROLIFERATION AND INDUCES AUTOPHAGY IN STS-26T MPNST CELLS


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RUNNING TITLE

Running head: GGTI-2Z and lovastatin induce autophagy in STS-26T MPNSTs

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Abbreviations used are: FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; GGMP, geranylgeranyl monophosphate; FTase, farnesyl transferase; RabGGTase, Rab geranylgeranyl transferase; FTI, farnesyl transferase inhibitor; GGTI, geranylgeranyl transferase inhibitor; MPNST, malignant peripheral nerve sheath tumor; iSC, immortalized Schwann cells; NF1, neurofibromatosis type I; GI50, concentration of drug for 50% inhibition of growth.

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ABSTRACT

Prenylation inhibitors have gained increasing attention as potential therapeutics for cancer. Initial work focused on inhibitors of farnesylation (FTIs), but more recently geranylgeranylation inhibitors (GGTIs) have begun to be evaluated for their potential antitumor activity in vitro as well as in vivo. In this study, we have developed a non-peptidomimetic GGTI, termed GGTI-2Z, which in combination with lovastatin inhibits geranylgeranyl transferase I (GGTase I) as well as GGTase II/RabGGTase, without affecting farnesylation. The combination treatment results in a G₀/G₁ arrest and synergistic inhibition of proliferation of cultured STS-26T malignant peripheral nerve sheath tumor (MPNST) cells. We also show that their anti-proliferative activity 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 GGPP derivative that may work through a new mechanism involving the induction of autophagy, and that in combination with lovastatin it may serve as a valuable paradigm to develop more effective strategies in this class of antitumor therapeutics.
INTRODUCTION

Lipid modifications of proteins are known to facilitate their membrane association and cellular localization as well as protein-protein interactions, which are related to their functions including cell survival, proliferation, differentiation and invasion. Most GTPases, like Ras, contain a C-terminal CaaX motif that undergoes prenylation catalyzed by prenyl transferase enzymes including farnesyl transferase (FTase) or geranylgeranyl transferase I (GGTase I), followed by serial modification via several other enzymes (Konstantinopoulos et al., 2007). The substrate specificities of FTase and GGTase I are not mutually exclusive and therefore, cross-prenylation can occur. For example, inhibition of N- and K-Ras farnesylation using a farnesyl transferase inhibitor (FTI) leads to alternative geranylgeranylation by GGTase 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 Rab geranylgeranyl transferase (RabGGTase or GGTase II) (Leung et al., 2006).

Several different strategies have been explored to develop inhibitors of GGTase (GGTIs). Peptidomimetic inhibitors and small molecule inhibitors of GGTase I developed in different laboratories have shown promise as antitumor agents in vitro as well as 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 GGTase 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 (HMG-CoA) reductase of the mevalonate pathway of lipid synthesis, which further leads to inhibition of downstream isoprenoids farnesyl pyrophosphate (FPP) 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 μM and 20 μM respectively (Hamadmad and Hohl, 2007; Kou et al., 2009). One approach to achieving effective GGTase 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 GGTI 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 since a lower concentration of an isoprenoid-competitive GGTI can better target GGTase 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, in order to determine whether it effectively blocks protein geranylgeranylation in a MPNST cell line. We tested this compound either alone or in combination with lovastatin. We demonstrate that GGTI-2Z, in combination with lovastatin, causes a G_0/G_1 cell cycle arrest, inhibits proliferation and induces autophagy in cultured STS-26T MPNST cells.
METHODS

Synthetic Chemistry

(2E,6E,10E)-Ethyl 7,11,15-trimethyl-3-(trifluoromethylsulfonyloxy)hexadeca-2,6,10,14-tetraenoate (compound 2)

In an argon-flushed flask, (6E, 10E)-ethyl 7,11,16-trimethyl-3-oxohexadeca-6E,10E,14-trienoate (compound 1; (Gibbs et al., 1999) 3200 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; 0.5M in toluene, 11.5 mmol, 23 ml) was added drop wise. The solution was warmed to 0°C for 5 minutes and then re-cooled to -60°C for 30 minutes. 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 x 75 ml) and water (4 x 100 ml), dried over MgSO4, and concentrated. Purification by flash chromatography (95:5 hexane/ethyl acetate) gave 3130 mg (70%) of triflate compound 2 as a pale yellow 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).

(2Z,6E,10E)-Ethyl 3,7,11,15-tetramethylhexadeca-2,6,10,14-Tetraenoate (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 drop wise. 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 ether, and washed with 10% HCl (10 ml), aqueous NaHCO3 (15 ml) and brine, dried over MgSO4 and concentrated. Purification by flash chromatography (hexanes/ethyl acetate 95:5) gave 313 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 with tetramethyltin utilized in our communication.
(Zahn et al., 2001) on 2Z-GGPP (compound 9). $^1$H NMR (300 MHz, CDCl$_3$): δ 1.55 (t, $J=6.9$ Hz, 3H), 1.87 (m, 9H), 1.94 (s, 3H), 2.15 (s, 3H), 2.27-2.4 (m, 10H), 2.9 (t, $J=7.8$ Hz, 2H), 4.41 (q, $J=6.9$ Hz, 2H), 5.44 (m, 3H), 5.92 (s, 1H).

(2Z,6E,10E)-3,7,11,15-tetramethylhexadeca-2,6,10,14-tetraen-1-ol (2Z-geranylgeraniol; 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. 30 ml of water was added, and the aqueous solution was extracted with ethyl acetate (2 x 20 ml). The combined organic layers were washed with brine (2 x 20 ml) and dried over MgSO$_4$. Concentration followed by flash chromatography (hexanes/ethyl acetate 4:1) afforded alcohol compound 4 (210 mg, 76%) as a colorless oil. $^1$H NMR (300 MHz, CDCl$_3$): δ 1.64 (m, 15H), 1.95 (m, 12H), 4.03 (d, $J=7.2$ Hz 2H), 5.03 (s, 3H), 5.34 (t $J=7.25$, 1H).

4-Chlorobutyl(methyl)phosphoramidic dichloride (compound 5)

A solution of 4-chloro-N-methylbutanamine (2.4g, 15.6 mmol) in CH$_2$Cl$_2$ (21 ml) was cooled to 0°C. POCl$_3$ (1.4mL, 10.4 mmol) was added followed by a solution of triethylamine (4.3 ml, 31.2 mmol) in CH$_2$Cl$_2$ (7.75 ml, 4M). 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 CH$_2$Cl$_2$ (50 ml). The aqueous layer was extracted with CH$_2$Cl$_2$ (2 x 50 ml) and the combined organic layers dried over MgSO$_4$. Concentration followed by flash chromatography (hexanes/ethyl acetate 4:1) gave compound 5 (1.57 g, 42%) as an oil. $^1$H NMR (300 MHz, CDCl$_3$): δ 1.67 (m, 4H), 2.74 (m, 3H), 3.18 (m, 2H), 3.48 (m, 2H). $^{31}$P NMR (CDCl$_3$, 121 MHz): -7.8 ppm;
(5-Nitrofuran-2-yl)methyl 4-chlorobutyl(methyl)phosphoramidochloridate (compound 6)

Lithium bis(trimethylsilyl)amide ((Me₃Si)₂NLi; 3.16 ml, 1 M solution in THF, 3.16 mmol) was added dropwise to a solution of 2-hydroxymethyl-5-nitrofuran (452 mg, 3.16 mmol) at -78°C. After 10 min at -78°C, a solution of compound 5 (750 mg, 3.16 mmol) was added. The reaction was warmed to -65°C within 1 h, then quenched with 20 ml saturated ammonium chloride solution. It was extracted with CH₂Cl₂ (2 x 20 ml) and dried over Na₂SO₄. It was purified by flash chromatography (hexanes/ethyl acetate 9:1) to give compound 6 (692 mg, 63%). ¹H NMR (300 MHz, CDCl₃): δ 1.78(m, 4H), 2.7 (m, 3H), 3.10 (m, 2H), 3.50 (m, 2H), 5.12 (m, 2H), 6.75 (s, 1H), 7.29 (s, 1H). ³¹P NMR (CDCl₃, 121 MHz): -8.2 ppm.

(5-Nitrofuran-2-yl)methyl-(2Z,6E,10E)-3,7,11,15-tetramethylhexadeca-2,6,10,14-tetraenyl 4-chlorobutyl(methyl)phosphoramidate (GGTI-2Z; compound 7).

To a solution of compound 6 (129 mg, 0.375 mmol), 2Z-geranylgeraniol compound 4 (120 mg, 0.413 mmol) and TEA (68 μl, 0.487 mmol) in CH₂Cl₂ (2 ml) was added TiCl₄ (1M solution in CH₂Cl₂, 37.5 μl, 0.0375 mmol) at room temperature. The reaction mixture was stirred for 1 h, filtered, concentrated, and following flash chromatography (hexanes/ethyl acetate 1:1) afforded 7 (63 mg, 28%) as colorless oil. ¹H NMR (500 MHz, CDCl₃): δ 1.58 (s, 9H), 1.66 (m, 5H, N-CH₂-CH₂ under CH₃), 1.75 (m, 5H, CH₂-CH₂-Cl under CH₃), 1.95 (m, 4H), 2.05 (m, 8H), 2.6 (m, 3H), 3.03 (m, 2H), 3.55 (t, J=6Hz, 2H), 4.46 (m, 2H), 4.96 (d, J=8.5Hz, 2H), 5.08 (m, 3H), 5.34 (t, J=7.5, 1H), 6.6 (d, J=4Hz, 1H), 7.2 (d, J=3.5Hz, 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, 120.04 (d, P-C, J=6.9Hz), 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)) and 2Z GGPP (compound 9; (Zahn et al., 2001)) were evaluated against mammalian GGTase I (Hartman et al., 2005) in a modified version of the previously published FTase biochemical continuous fluorescent assay (Reigard et al., 2005). For all assays, the excitation wavelength used was 340 nm, and emission was monitored at 500 nm. The inhibitor assay mixture consisted of assay buffer (52 mM Tris-HCl pH=7.0, 5.8 mM dithiothreitol, 12 mM MgCl₂, and 12 M ZnCl₂), 0.25% detergent (0.4% n-dodecyl-D-maltoside in 52 mM Tris-HCl, pH=7.0), 0.7 M dansyl-GCVLL, 1.35 M GGPP, varying analog concentrations (0.1-5.0 M) and 0.02 M GGTase I. The first five assay components are added to a 750 μl quartz cuvette for a total volume of 500.5 μl in the order listed; the reaction is initiated by addition of enzyme, and change in fluorescence intensity is monitored over 300 s. Inhibitor data is expressed as a V/S plot and the IC₅₀ is determined utilizing GraphPad Prism software. Using this procedure, we demonstrated that 2Z-GGMP (compound 8) inhibits GGTase I with an IC₅₀ value of 21 nM, and that 2Z-GGPP (compound 9) exhibited the same IC₅₀ value (100 nM) 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 aliquots were prepared in dimethyl sulfoxide (DMSO) and stored at -80°C. HA14-1 (Ryan Scientific Inc., Isle of Palms, 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’GATCCGGCTGCATGAGCTGCAAATGTGTGCTGTCCTG3’ and a reverse primer with the sequence 5’AATTCAGGACAGCACACACATTTGCAGCTCATGCAGCAGCCG3’ 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 (Wojtkowiak 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 penicillin and 100 µg/ml 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 (Karmanos Center Institute, Detroit, MI) and maintained as a monolayer in DMEM/F12 containing 5% horse serum, 20 ng/ml EGF, 0.5 µg/ml hydrocortisone, 10 µg/ml insulin, 50 U/ml penicillin, and 50 µg/ml streptomycin at 37°C and 5% CO₂.

Western Blot Analysis

Lysates were prepared from monolayers of cells in 2x Laemmli 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-RhoA, 1:500 dilution of anti-Rab5 and 1:600 dilution of anti-unprenylated Rap1 antibodies, and caspase-3 antibody at 1:1000 dilution (Santa Cruz Biotechnology). LC3-I and -II were detected using 1:2000 dilution of anti-LC3 (gift from Dr. David Kessel, Wayne State University).

Live Cell Imaging Assays

HEK293 cells were plated into 35-mm culture plates 24 h prior to transfection with pRK7.GFP.H-Ras.CaaX using lipofectamine 2000 reagent (Invitrogen) as previously described (Norum et al., 2005). Four hours after transfection, fresh media were added along with vehicle or drug at appropriate
concentrations as stated in the figure legend. At the end of treatment, nuclei were stained using Hoechst 33342, followed by confocal live-cell imaging on the LSM-510 at 40x magnification. A similar protocol was used to study nuclear morphology of STS-26T cells with and without drug treatment.

**Immunofluorescence Assays**

STS-26T cells were plated onto glass cover-slips and treated as indicated in the figure legends. The cells were fixed and processed for confocal immunofluorescence analysis using anti-LAMP-2 mouse monoclonal (BD Biosciences) 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 using Volocity software 5.2.1 (Perkin Elmer).

**Cell Proliferation Assay**

STS-26T cells and iSC were plated at ~20,000 cells per 35-mm dish 24 h before drug treatment. At 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 via 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. 20 μl of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Invitrogen, Eugene, Oregon) stock solution (5 mg/ml in PBS) 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 using a plate reader (SpectraFluor Plus, Tecan, Salzburg, Australia) at 485 nm wavelength. After normalizing the absorbance values for media and vehicle controls, the data were analyzed using GraphPad Prism version 4.0c
(GraphPad Software Inc., San Diego, CA) by non-linear regression (curve fit) and plotting sigmoidal dose-response to obtain GI_{50} 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 using a FACScalibur instrument (BD Biosciences, San Jose, CA). A minimum of 10^4 cells per sample was analyzed to determine the percentage of apoptotic cells and cells in G_1, S and G_2/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 have demonstrated that the monophosphate derivatives of certain FPP 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 (IC$_{50}$ = 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 GGTase 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 hours 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; upper panel). The amount of unprenylated Rap1A in comparison to the total Rap1 levels (Fig. 2A; lower panel) 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. Sebti and colleagues have shown that GGTI treatment of pancreatic cancer cells results in an increase in RhoA expression levels (Delarue et al., 2007). In our study with GGTI-2Z and lovastatin combination, we saw a similar marked increase in the expression level of RhoA within 24 h...
compared to vehicle control, and this increase was maintained even at 48 h of treatment (Fig. 2B, lanes 4 and 8).

We also tested for inhibition of RabGGTase or GGTase II by looking for reduced prenylation of Rab5. Strikingly, we observed a clear up-shift due to appearance of unprenylated Rab5 upon combination treatment (Fig. 2C, lane 4) 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 Figure 3, we observed that in 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, as high as 6 μM GGTI-2Z plus 500 nM lovastatin combination 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 the figure, there was a dose-dependent as well as time-dependent inhibition of proliferation of these cells (Fig. 4A, 4B). In addition to proliferation, we examined the percent 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 (Figure 4D). The synergistic inhibition was indicated by the GI50 (concentration of drug for 50% inhibition of growth) 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 co-treatment with GGTI-2Z and lovastatin. We therefore next determined which point of the cell cycle these compounds targeted in order 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 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 if 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 to 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 Figure 6B, procaspase-3, which is the uncleaved form of caspase-3, was evident in all 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 Figure 6C. Conversely, with GGTI and/or lovastatin treatment, neither did we see any nuclear morphological changes (Figure 6C), nor did we see any appearance of DNA laddering (data not shown), which further confirms 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 following 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 phophatidylethanolamine (PE) attachment. 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 when compared with vehicle treatment and 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 due to an absence of autophagosome/lysosome fusion or depression of lysosomal protease activities. In order to distinguish between effects on synthesis and on degradation, we pretreated the cells with 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 to vehicle control, whereas the combination treatment again increases LC3-II accumulation either in the presence or absence of bafilomycin A1 (Fig. 7C).

Furthermore, immunocytochemical staining of LC3 along with the lysosomal marker, LAMP-2, was also performed following treatments with the compounds as indicated (Fig. 7D). Vehicle or single compound treatments did not result in co-localization of the two proteins. In contrast, a very distinct punctate co-localization of LC3 and LAMP-2 as observed in cells following the combination treatment. Quantitative analysis of LC3-positive puncta, using two independent LC3 antibodies, revealed an approximately five-fold increase in the number of punctate structures upon combination treatment as compared to DMSO treatment (Fig. 7E). These results indicate that the autophagic process is both induced and proceeds to completion in STS-26T cells co-treated with GGTI-2Z and lovastatin.
GGTI-2Z and lovastatin also effectively inhibit proliferation of 1c1c7 cells and induce autophagy in DCIS cells

In order 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 Figure 1A and 1B). 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 Figure 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 Figure 1D).

Normal immortalized rat Schwann cells (iSC) are resistant to GGTI-2Z and lovastatin co-treatment

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).
DISCUSSION

Prenylation inhibitors of different classes have been tested pre-clinically as well as 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 Type I neurofibromatosis (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 substrate(s) 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 co-treatment 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 utilized 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, 2Z geranylgeranyl monophosphate compound
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 to evaluate cellular GGTase I inhibition. Additionally, 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 while farnesylation
remains unaffected. Furthermore, the fact that it did not have any effect on either morphology or
proliferation of normal iSC implies 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 GTPase 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, as 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 anti-
proliferative activity is consistent with 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 cell survival or a cell death
mechanism (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 GGTI/lovastatin combination treated cultures to see if autophagy occurred. Based on the analysis of LC3-I conversion to LC3-II via western blot, and the co-localization of LC3 with the lysosomal protein LAMP-2 via immunocytochemistry, we confirmed that autophagy was induced and driven to completion in STS-26T cultures co-treated 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 since the two enzymes share strikingly similar active sites (Lackner et al., 2005) and hence, 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 GGTase II to determine the $K_M$ value. An alternative explanation for the dual inhibition of GGTase I and RabGGTase-mediated prenylation is that GGTI-2Z 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 GGTI-2Z/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 the case of our study we saw that inhibition of Rab5 prenylation via GGTase 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 GGTI may target one or more other protein(s) that may potentially contribute to its action. Further studies would be required to better elucidate the role of Rab5 and other proteins in GGTI/lovastatin induced autophagy in MPNSTs.

In conclusion, we have developed a novel compound GGTI-2Z that blocks prenylation mediated by both GGTase I and RabGGTase, and exerts cytostatic activities in STS-26T MPNST cells in a caspase-3-independent manner. The action of GGTI-2Z is potentiated by low-dose statin combination treatment and strongly correlates with induction of autophagy. This combination treatment does not block proliferation of, or induce toxicity in normal, immortalized Schwann cells, but does have activity against two other transformed cell lines: 1c1c7 murine hepatoma cells and MCF10.DCIS cells that model human breast ductal carcinoma in situ. Further studies testing therapeutic efficacy of GGTI-2Z may serve to develop better understanding of geranylgeranylation inhibitors and to evaluate their potential in the context of cancer therapy as well as some Rab-associated protein-trafficking disorders.
ACKNOWLEDGEMENTS

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FOOTNOTES

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Reprint requests to: Raymond R. Mattingly; Address: 540 E. Canfield Ave, Scott Hall Rm. 6322, Detroit, MI 48201; Tel: 313-577-6022; Fax: 313-577-6739; Email: r.mattingly@wayne.edu
LEGENDS FOR FIGURES

Figure 1. Synthesis of prodrug GGTI-2Z (compound 7), and structures of 2Z-GGMP (compound 8) and 2Z-GGPP (compound 9).

Figure 2. Inhibition of prenylation in STS-26T cells by GGTI-2Z/lovastatin combination treatment. STS-26T cultures were treated as indicated for 24 h or 48 h. Whole-cell lysates were probed for prenylation status of Ras superfamily GTPases via Western analysis. A. Detection of Rap1A via an antibody directed toward the unprenylated form of Rap1A (middle panel), and detection of total Rap1 (lower panel). B. Detection of RhoA, which has been reported to be over-expressed following block of GGTase. C. Detection of Rab5. Unprenylated GTPases migrate more slowly on SDS-PAGE gels. β-tubulin was used as a loading control in all western blots. Results shown are representative of at least three independent experiments.

Figure 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.

Figure 4. A and B. Inhibition of proliferation of STS-26T cells following GGTI-2Z/lovastatin treatment. Samples were collected every 24 h post-treatment for analysis of cell number. Data represent means ± S.D. of three independent experiments. C. Number of non-viable cells was analyzed with respect to total number of cells to calculate percent viability at the given time points. D. Cells were treated with the
compounds at several different concentrations as shown, alone or in combination and the data were tested for synergy using isobologram analysis. Data represent means ± S.D. of two independent experiments.

Figure 5. GGTI-2Z/lovastatin treatment arrests STS-26T cells in G0/G1. STS-26T cells were treated as shown. Cultures were harvested 48 h post-treatment for DNA content by staining with propidium iodide. Histograms represent 10^4 events, and the cell cycle profile was determined using Modfit. Results are representative of two independent experiments.

Figure 6. Lack of apoptosis in STS-26T cells following GGTI-2Z/lovastatin treatment. HA14-1 treated cells were used as a positive control. A. STS-26T cells were treated as indicated in the figure. Data represent means of triplicate samples and are representative of two independent experiments. B. STS-26T cells were treated as indicated in the figure. Attached as well as detached cells were pooled, and whole-cell lysates were separated and probed for cleaved caspase-3. C. STS-26T cells were treated for 48 h with the indicated concentrations of lovastatin and/or GGTI-2Z or for 30 min with HA14-1. Nuclei were stained using Hoechst 33342 and live-cell imaging was performed on LSM510 at 40x magnification.

Figure 7. Induction of autophagy in STS-26T cells by GGTI-2Z/lovastatin treatment. STS-26T cells were treated with indicated concentrations of GGTI-2Z and lovastatin alone or in combination. Results are representative of three independent experiments. A. Treatments were for 24 or 48 h as shown. Whole cell lysates were then probed for LC3 and β-tubulin. B. Cells were subject to 2 h pretreatment with protease inhibitors, 10 μM pepstatin A and 10 μM E64D, as indicated. Whole cell lysates were then probed for LC3 and β-tubulin. C. Cells were subject to 48 h drug treatment, with addition of 50 nM bafilomycin A1 for the last 2 h of the incubation, as indicated. Whole cell lysates were then probed for LC3 and β-tubulin. D. STS-26T cells were treated as indicated for 48 h followed by methanol fixation. Cells were then stained for LC3 and LAMP-2. Nuclei were stained using DAPI and cells were visualized under the LSM-
510 at 40x magnification. E. Quantitative analysis of LC3-positive puncta treated with either DMSO or the drug combination for 48 h.
Figure 1

1. KHMD, Ar(OTf)$_2$
   
2. DMF, 70%

3. Me$_3$In, Pd(PPh$_3$)$_2$Cl$_2$
   
4. THF, 75%

5. DIBAL-H
   
6. Toluene, -78°C
   52%

7. CH$_3$P$_2$Cl + H$_2$N-CH$_2$-CH$_2$Cl
   
8. TEA
   DCM, 42%

9. O$_2$N
   
10. LiHMDS, THF, 63%

11. TEA, TiCl$_4$
    
12. DCM, 28%

Figure 2.

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Figure 3.

GFP.H-Ras.CaaX  Nuclear DAPI  Overlay

DMSO

Lovastatin 500 nM + FTI-1 500 nM

Lovastatin 500 nM + GGTI-2Z 3 μM

Lovastatin 500 nM + GGTI-2Z 6 μM
Figure 4.
Figure 5.

- **DMSO**
  - G1: 61.41%
  - G2/M: 8.98%
  - S: 29.61%

- **Lovastatin 1 μM**
  - G1: 58.11%
  - G2/M: 10.26%
  - S: 31.63%

- **GGTI-2Z 3 μM**
  - G1: 60.10%
  - G2/M: 8.75%
  - S: 31.14%

- **Lovastatin 1 μM + GGTI-2Z 3 μM**
  - G1: 79.24%
  - G2/M: 5.52%
  - S: 15.23%
**Figure 7.**

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**D**

**E**

- DMSO
- Lov 500 nM
- GGTI-2Z 3 μM

**LC3**
**Lamp-2**
**DAPI**
**Time: 48 h**

**LC3-positive puncta**

- DMSO
- Lov 500 nM + GGTI-2Z 3 μM

**Treatment**

- 0
- 100
- 200
- 300