Induction of Apoptosis in Neurofibromatosis Type 1 Malignant Peripheral Nerve Sheath Tumor Cell Lines by a Combination of Novel Farnesyl Transferase Inhibitors and Lovastatin


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ABSTRACT

Neurofibromatosis type 1 (NF1) is a genetic disorder that is driven by the loss of neurofibromin (Nf) protein function. Nf contains a Ras-GTPase-activating protein domain, which directly regulates Ras signaling. Numerous clinical manifestations are associated with the loss of Nf and increased Ras activity. Ras proteins must be prenylated to traffic and functionally localize with target membranes. Hence, Ras is a potential therapeutic target for treating NF1. We have tested the efficacy of two novel farnesyl transferase inhibitors (FTIs), 1 and 2, alone or in combination with lovastatin, on two NF1 malignant peripheral nerve sheath tumor (MPNST) cell lines, NF90-8 and ST88-14. Single treatments of 1, 2, or lovastatin had no effect on Ras prenylation or MPNST cell proliferation. However, low micromolar combinations of 1 or 2 with lovastatin (FTI/lovastatin) reduced Ras prenylation in both MPNST cell lines. Furthermore, this FTI/lovastatin combination treatment reduced cell proliferation and induced an apoptotic response as shown by morphological analysis, procaspase-3/-7 activation, loss of mitochondrial membrane potential, and accumulation of cells with sub-G, DNA content. Little to no detectable toxicity was observed in normal rat Schwann cells following FTI/lovastatin combination treatment. These data support the hypothesis that combination FTI plus lovastatin therapy may be a potential treatment for NF1 MPNSTs.
creasing the intrinsic rate of Ras-GTP to Ras-GDP (Eccleston et al., 1993). Germline mutations of the NF1 gene result in reduced Nf expression and a loss of Ras-GAP activity. The consequence of losing Ras-GAP activity is aberrant Ras signaling that can potentially lead to the development of NF1 (Busu et al., 1992; Feldkamp et al., 1999). Our laboratory and others have previously targeted downstream signaling partners of Ras by treating MPNST cell lines with MEK inhibitors (Tang et al., 1998; Chadee and Kyriakis, 2004; Mattingly et al., 2006; Roth et al., 2007). We have shown that PD184352 (Mattingly et al., 2006) (CI-1040) induced apoptosis in MPNST cell lines, confirming the dependence of the Ras-MAPK pathway in this disease.

Ras proteins are translated as inactive precursor molecules that must undergo a series of post-translational modifications before becoming fully functional (Gibbs et al., 2001). The first necessary step is the covalent addition of a prenyl group, either a 15C farnesyl or a 20C geranylgeranyl group, to the C-terminal “CaaX” box (Basso et al., 2006).

Reducing the prenylation of proteins to treat NF1 has been recognized as a potential therapeutic approach. For example, the farnesyl transferase inhibitor (FTI) BMS-186511 (Mazieres et al., 2003) reduces proliferation of MPNST cell line ST88-14 (Yan et al., 1995), and FTI L-739,749 reduces proliferation of Nf-deficient mouse Schwann cells (Kim et al., 1997). A phase I clinical trial using FTI tipifarnib to treatplexiform neurofibromas was tolerated well in children, yet no objective responses were achieved (Widemann et al., 2006). Although this study has advanced to an ongoing phase II trial (NCT00029354), it is likely that further development of this treatment approach will be required.

Our laboratory is interested in using FTIs and lovastatin, an inhibitor of the hydroxymethylglutaryl (HMG)-CoA reductase, to reduce prenylation of proteins as a potential therapy for numerous diseases. We have previously reported that lovastatin, in combination with FTI 3-allylfarnesol, induces relocation of RhoB from the membrane fraction to the cytosolic fraction following treatment in A10 vascular smooth muscle cells. The translocation of RhoB from the membrane to the cytosol is the result of inhibiting RhoB prenylation (Mattingly et al., 2002). A prodrug analog of 3-allylfarnesol phosphate was also shown to inhibit RhoB prenylation in STS-26T MPNST cells when used in combination with lovastatin, resulting in reduced cell proliferation (Clark et al., 2007).

Here, we describe our efforts to characterize the effectiveness of two novel FTase inhibitors, 1 (Clark et al., 2007) and 2, on human NF1 MPNST cell lines, NF90-8 and ST88-14. The prodrug structures are shown in Fig. 1. Prodrug 1 is highly lipophilic, so analog 2 was prepared in which a carboxylate side chain, which would be ionized at physiologic pH, replaced the N-methyl group on the prodrug moiety. The entire prodrug moiety is released from the inhibitor following activation inside the cell (Clark et al., 2007), so this modification will have no effect on inhibitor affinity. Our objective was to determine the efficacy of these compounds on Ras prenylation and cell proliferation when used in combination with lovastatin. The data show reduction of Ras prenylation in both cell lines with cell cycle G1 arrest and increased caspase activity following FTI/lovastatin combination treatment but lack of toxicity in normal rat Schwann cells.

![Fig. 1.](image1) The synthesis of prodrug FTI 1, which was previously reported as compound 5c, has been described previously (Clark et al., 2007). Prodrug FTI 2 was synthesized via an analogous route using amino ester 3 instead of methyl chlorobutylamine to generate the methyl ester of FTI 2, followed by selective hydrolysis to the free acid. Compounds 1 and 2 differ at the prodrug moiety, which is removed following entrance into the cell and prodrug activation. The prodrug moiety should not affect the inhibitory function of the farnesyl phosphate analog released. FTI 4 is an analog of compound 1 that is inactive as an FTI (Clark et al., 2007).

### Materials and Methods

#### Compounds and Reagents

The synthesis of prodrug 1 has been reported (Clark et al., 2007). Prodrug 2 was synthesized via an analogous route using amino ester 3 instead of methyl chlorobutylamine, followed by selective hydrolysis of the methyl ester (Fig. 1).

Lovastatin (Sigma-Aldrich, St. Louis, MO) aliquots were prepared in dimethyl sulfoxide and stored at −80°C. JC-1 and Hoechst 33342 (Invitrogen, Carlsbad, CA) were prepared in dimethyl sulfoxide and stored at 4°C. MitoTracker Orange CM-H2TMRos (Invitrogen) aliquots were prepared in dimethyl sulfoxide and stored at −20°C.

#### Cell Culture

NF90-8 and ST88-14 MPNST cell lines were generously donated by T. Glover (University of Michigan, Ann Arbor, MI). Cells were maintained as adherent cultures in RPMI 1640 (Invitrogen) with 5% fetal bovine serum (HyClone Laboratories, Logan, UT), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Primary normal rat Schwann cells were isolated from the sciatic nerves of neonatal Sprague-Dawley Rats and grown on poly-d-lysine-coated coverslips as described previously (Skoff et al., 1998). These cells were grown in Eagle’s medium with 10% calf serum before experimental manipulations. Normal, spontaneously immortal rat Schwann cell clones (iSCs) isolated from sciatic nerves were a generous gift from E.M. Shooter (Stanford University, Stanford, CA) and described previously (Bolin et al., 1992). These cells were maintained in minimal essential medium supplemented with 10% horse serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. All cells were maintained in a humidified incubator under 5% CO2. For all experiments, cells were plated 24 h before drug treatment. Immedi-
ately before drug treatment, the medium was replaced with fresh growth medium for the duration of the experiment.

**Western Analysis.** Lysates were prepared from monolayers of cells in 2× Laemmli sample buffer by boiling for 5 min and cleared by centrifugation (Mattingly et al., 2001). Samples were then separated on polyacrylamide-SDS gels and electrophoretically transferred to nitrocellulose. Ras was detected with a 1:250 dilution of anti-pan Ras monoclonal antibody (BD Transduction Laboratories, San Jose, CA). Caspase-3 was detected with a 1:1000 dilution of anti-caspase-3 polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) as described previously (Menard et al., 2005). Retinoblastoma protein (pRb) phosphorylated Ser780 was detected with a 1:1000 dilution of a mouse monoclonal antibody (Cell Signaling Technology Inc., Danvers, MA). The nitrocellulose membrane was stripped and reprobed for total pRb with a 1:2000 dilution of the E7 monoclonal antibody (Developmental Studies Hybridoma Bank, Iowa City, IA).

**Proliferation Assay.** NF90-8 cells were plated at 20,000 cells/35-mm dish, and ST88-14 cells were plated at 20,000 cells/60-mm dish 24 h before drug treatment. Fresh medium was added before the drug treatment as described in the text. Attached cells were trypsinized and combined with media containing detached cells. The cells were collected by centrifugation for 5 min at 1000 g and counted via a hemacytometer.

**Flow Cytometry.** NF90-8 and ST88-14 cells were treated and collected for DNA analysis as described previously (Reiners et al., 1999). DNA content was analyzed using a FACS calibur instrument (BD Biosciences, San Jose, CA). A minimum of 10^4 cells/sample was analyzed to determine the percentage of apoptotic cells and cells in G1, S, and G2/M phase (MODFIT; Variosity Software, Topsham, ME).

**DEVDase Activity Assay.** Lysates of NF90-8 and ST88-14 cultures were prepared and used in DEVDase assays as described previously (Caruso et al., 2004). Changes in fluorescence over time were converted into picomoles of product by comparison with a standard curve made with 7-amino-4-methylcoumarin. DEVDase-
Fig. 4. FTI/lovastatin combination treatment of NF90-8 cells increases G1 and apoptotic cells. NF90-8 cultures were treated 24 h after plating according to the condition in figure. Cultures were harvested after 48 h of treatment for DNA content stained with propidium iodide. Histograms represent 10⁴ events, and the cell cycle profile was determined using MODFIT. FTI 1 (500 nM) in combination with 500 nM lovastatin increased the number of cells in G1 and greatly increased the presence of cells with sub-G1 DNA content.
specific activities are reported as nanomoles of product per minute per milligram of protein. The bicinchoninic acid assay, using bovine serum albumin as a standard, was used to estimate protein concentrations.

**Mitochondrial Membrane Potential and Nuclear Morphology.** NF90-8 cells were plated at 100,000 cells/100-mm dish 24 h before drug treatment. At the end of the drug treatment, mitochondrial membrane potential ($\Delta \Psi_{m}$) was assayed by two independent methods. In one series of experiments, JC-1 was added directly to the medium at a final concentration of 5 $\mu$M and incubated for 10 min at 37°C. Hoechst 33342 (excitation, 350 nm; emission, 461 nm) was added directly to the medium at a final concentration of 500 nM to reveal nuclear morphology and coincubated with the MitoTracker Orange and will exhibit reduced fluorescence emission.

**Our laboratory has previously reported that N-Ras is the predominant active Ras isoform expressed in the human-derived MPNST cell lines NF90-8 and ST88-14 (Mattingly et al., 2016).**

<table>
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<th>DNA Analysis of NF90-8 MPNST Cells</th>
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* Statistical significance was determined between the FTI/lovastatin-treated cultures and the DMSO controls. Significance was set at $p < 0.05$. 

**TABLE 2** ST88-14 cell cycle analysis

ST88-14 cells were treated as indicated above for 24 or 48 h. Data represent the mean ± S.D. of three independent experiments.

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* Statistical significance was determined between the FTI/lovastatin-treated cultures and the DMSO controls. Significance was set at $p < 0.05$. 

**Results**

Our laboratory has previously reported that N-Ras is the predominant active Ras isoform expressed in the human-derived MPNST cell lines NF90-8 and ST88-14 (Mattingly et al., 2016). We have confirmed these findings in our current study. Next, we investigated the effects of FTIs on cell cycle progression and apoptosis in these cell lines. Our data show that FTIs induce a G1 cell cycle arrest, which is accompanied by a decrease in the S phase and an increase in the G2/M phase (Table 1). This arrest is likely due to increased apoptosis, as evidenced by the decrease in the percentage of cells in the G1 phase and the increase in the percentage of cells in the sub-G1 phase (Table 2). In addition, we observed a decrease in mitochondrial membrane potential (ΔΨm) in cells treated with FTIs, indicating that these compounds may induce mitochondrial dysfunction and caspase-dependent apoptosis. These findings suggest that FTIs may have potential therapeutic applications in the treatment of MPNSTs.
al., 2006). N-Ras can be alternatively prenylated with a geranylgeranyl pyrophosphate in the presence of FTIs (Whyte et al., 1997). Therefore, we tested whether our novel FTIs (1 and 2) could reduce Ras prenylation in MPNST cell lines NF90-8 and ST88-14 (Fig. 2). Inhibition of prenylation is indicated by the slower mobility band or up-shift by Western analysis. Single 1 µM treatments of 1, 2, or lovastatin had little to no detectable effect on Ras prenylation in either cell line. However, a reduction of Ras prenylation was observed in the NF90-8 cells following combination treatment of 1 µM 1 or 2 with 1 µM lovastatin. Ras prenylation was also reduced in ST88-14 FTI/lovastatin combination-treated cells compared with the single treatments of 1, 2, or lovastatin. The observed reduction of Ras prenylation in ST88-14 cells was moderate compared with that occurring in NF90-8 FTI/-lovastatin-treated cells.

Ras isoforms are known to regulate cell processes such as survival, growth, and proliferation. Since our FTI/lovastatin combination treatment reduced Ras prenylation in NF90-8 and ST88-14 cells (Fig. 2), we next determined the effect of this treatment on cell proliferation. The results from proliferation experiments with NF90-8 cells are shown in Fig. 3, A and B. Single treatments of 500 nM 1, 2, or lovastatin alone had no effect on cell proliferation compared with cultures that were treated with DMSO. However, combination treatments of 500 nM 1 plus 500 nM lovastatin were cytostatic after 24 h of treatment and reduced total cell number below initial plating after 72 h of treatment (Fig. 3A). A similar cytostatic response was observed following treatment with 500 nM 2 plus 500 nM lovastatin (Fig. 3B). Inhibition of proliferation also occurred in the presence of lower concentrations of lovastatin (33 and 100 nM) in combination with 500 nM 1 or 2. Synergism of the FTI compounds with 33 nM lovastatin is notable because this dose of lovastatin is pharmacologically achievable in humans treated with anticholesterol doses of statins (Thibault et al., 1996). Proliferation data for ST88-14 cells treated with nanomolar combinations of FTI/lovastatin are shown in Fig. 3, C and D. Single treatment with DMSO, 1, 2, or lovastatin had no effect on cell proliferation. However, when 500 nM 1 or 2 were used in combination with 500 nM lovastatin, we observed a reduction of ST88-14 proliferation. Unlike the NF90-8 cells, lower doses of lovastatin in combination with 500 nM 1 or 2 had less effect on cell proliferation.

![Fig. 5](https://i.imgur.com/5.png)  
**Fig. 5.** Reduction of pRb hyperphosphorylation in MPNST cells lines by FTI/lovastatin combination treatment. ST88-14 and NF90-8 MPNST cultures were treated as indicated for 48 h. Whole-cell lysates were probed for pRb phosphorylated at Ser780 by Western blot analysis. Blots were stripped and reprobed for total pRb and β-tubulin. Clear reduction of pRb phosphorylation was observed following treatment with FTI plus lovastatin. *, hyperphosphorylated pRb.

We subsequently investigated the effects of FTI/lovastatin treatment on NF90-8 and ST88-14 cell cycle progression. Figure 4 shows fluorescence-activated cell sorting analysis of NF90-8 cells treated with 500 nM 1, or with 500 nM lovastatin, or a combination of the two drugs for 48 h. Single treatments with these compounds yielded cell cycle profiles comparable with the DMSO control, which was also similar to untreated controls (Table 1; Supp. Fig. 1). The combination treatment results in an increased percentage of cells in G1 and a significant percentage of cells undergoing apoptosis, compared with the control cultures. These data coincide with the proliferation data in Fig. 3A, where we observed a cytostatic effect of 500 nM 1 plus 500 nM lovastatin at 24 h and a cytotoxic effect at 48 h. Similar results were observed with 500 nM 2 in combination with 500 nM lovastatin (Table 1; Supp. Fig. 1). As a control for these experiments, we also tested compound 4 (Fig. 1), an analog of compound 1 that is inactive as an FTI and has no effect on the proliferation of the spontaneous MPNST cell line, STS-26T [Compound 5d in Clark et al. (2007)]. The cell cycle distribution of NF90-8 and ST88-14 cells was not affected by treatment with 1 µM 4 alone or in combination with 1 µM lovastatin (data not shown). Cell cycle progression of ST88-14 cells was also analyzed at 24 and 48 h following treatment with either 500 nM 1 or 500 nM 2 with or without 500 nM lovastatin (Table 2; Supp. Fig. 2). We observed a moderate G1 cell cycle arrest at 24 h that was maintained at 48 h following FTI/lovastatin combination treatment. Although we observed an increased percentage of apoptotic cells at 48 h, the effect was more modest than that observed in NF90-8 cultures.

One mechanism that could underlie a G1 arrest would be if pRb phosphorylation was reduced. Therefore, we investigated the phosphorylation pattern of pRb in the NF90-8 and ST88-14 cell lines (Fig. 5). Single treatments of 1 µM 1, 2, or lovastatin did not reduce hyperphosphorylation of pRb. However, combining 1 µM 1 or 2 with 1 µM lovastatin significantly reduced the phosphorylated pRb signal. Total pRb expression was also reduced in 1 or 2 plus lovastatin-treated cultures. As a further control for these experiments, we also tested the inactive control compound 4. No effect on pRb phosphorylation was observed in either cell line following treatment with 1 µM 4 alone or in combination with 1 µM lovastatin (Fig. 5).

In addition to cell cycle arrest, FTI/lovastatin treatment induced an increase in the number of cells with sub-G1 DNA

![Figure 5](https://i.imgur.com/5.png)

**Fig. 5.** Reduction of pRb hyperphosphorylation in MPNST cells lines by FTI/lovastatin combination treatment. ST88-14 and NF90-8 MPNST cultures were treated as indicated for 48 h. Whole-cell lysates were probed for pRb phosphorylated at Ser780 by Western blot analysis. Blots were stripped and reprobed for total pRb and β-tubulin. Clear reduction of pRb phosphorylation was observed following treatment with FTI plus lovastatin. *, hyperphosphorylated pRb.
content, which would be consistent with an induction of apoptosis. Figure 6, A and B, is a morphological analysis of both cell lines treated with 500 nM 1 or 2 with 500 nM lovastatin. NF90-8 and ST88-14 cells treated singularly with 1, 2, or lovastatin at $t = 24$ and 48 h have a flat, elongated morphology associated with healthy, proliferating cells. However, following 24 h of 500 nM 1 or 2 with 500 nM lovastatin combination treatments, both cell lines became more refractile. By 48 h of treatment, cells were shrunken, rounded, and blebbled.

The morphological characteristics of FTI/lovastatin-treated MPNSTs, coupled with increased numbers of cells with sub-G$_1$ DNA contents, suggested the occurrence of apoptosis. To monitor for apoptosis, we used N-acetyl-Asp-Glu-Val-Asp-amino-4-methylcoumarin to assay for the activity of caspases-3 and -7 (Fig. 7) (Caruso et al., 2004). Treatment with 500 nM 1, 2, or lovastatin resulted in no detectable activation of DEVDase. However, high amounts of active DEVDase were observed in NF90-8 cells (Fig. 7, C and D), and moderate amounts were observed in ST88-14 (Fig. 7, A and B) cells, following treatment with 500 nM 1 or 2 and 500 nM lovastatin. In addition, 100 nM 1 with 500 nM lovastatin also activated DEVDase in NF90-8 cells. NF90-8 cells treated with 500 nM 1 plus 500 nM lovastatin also exhibited a time-dependent increase of cleaved procaspase-3 (Fig. 7E). The kinetics of procaspase-3 cleavage correlated with the time-dependent increases in DEVDase (Fig. 7C).

Changes in $\Delta\Psi_m$ often precede/accompany the activation of procaspases and are generally indicative of an activation of the intrinsic apoptotic pathway (Kroemer et al., 1997). We employed two methods to monitor $\Delta\Psi_m$. The first method involved live cell microscopy using MitoTracker Orange (Fig. 8A). NF90-8 cells treated with DMSO, 500 nM 1, or 500 nM lovastatin exhibited tubular mitochondrial staining at 24 and 48 h with little to no detectable chromatin condensation. However, following 24 h of combination treatment with 500 nM 1 and 500 nM lovastatin, the cells exhibited less red fluorescence. Within 48 h of treatment, both the cells and

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**Fig. 6.** Morphological analysis of NF90-8 and ST88-14 cells following FTI/lovastatin combination treatment. ST88-14 (A) and NF90-8 (B) cells were treated as indicated at $t = 0$, and differential interference contrast images were captured at the 0-, 24-, and 48-h time points.
nuclei had shrunken, and there were dramatic reductions in red fluorescence (i.e., reduced $\Delta \Psi_m$).

Flow cytometric analysis of $\Delta \Psi_m$ with JC-1 was employed to quantify mitochondrial membrane potential (Fig. 8B). NF90-8 cells were treated with drugs and then incubated with JC-1, a compound that forms red fluorescent aggregates in cells having $\Delta \Psi_m$. After a loss of $\Delta \Psi_m$, JC-1 does not aggregate and fluoresce red. As observed with MitoTracker Orange, treatment with DMSO, 500 nM lovastatin, or 500 nM 1 did not affect $\Delta \Psi_m$ in NF90-8 cells. However, after...
FTI/lovastatin combination treatment, a progressive loss of ψm occurred from 24 to 72 h. It is noteworthy that a significant loss of ψm occurred at 24 h, a time that precedes the activation of DEVDase (Fig. 7C) and procaspase-3 cleavage (Fig. 7E).

We have demonstrated that treatment of human MPNST cell lines NF90-8 and ST88-14 with 500 nM 1 or 2 in combination with 500 nM lovastatin greatly reduced cell proliferation and induced an apoptotic response. We tested the effects of this FTI/lovastatin treatment on normal primary Schwann cells isolated from the sciatic nerve of rat pups. Normal primary rat Schwann cells were treated for 72 h, and differential interference contrast images were recorded (Fig. 9A). Once again, single treatments of DMSO, 500 nM 1 or 2, or 500 nM lovastatin had no detectable toxicity. However, in a stark contrast to the effect on MPNST cells, combination treatments of 500 nM 1 or 2 with 500 nM lovastatin had no observable toxicity on normal primary Schwann cells. To further examine whether the FTI/lovastatin treatment would be toxic, we tested our compounds on iSC (Bolin et al., 1992). Single treatments of 500 nM 1, 2, or lovastatin had no effect on proliferation (Fig. 9B). Combination treatments of 500 nM 1 or 2 with 500 nM lovastatin also did not significantly reduce proliferation of iSC cells.

**Discussion**

NF1 is a disease driven by the functional loss of Nf, a Ras-GAP protein (DeClue et al., 1991). The loss of Nf results in aberrant Ras signaling in cells derived from the neural crest. Ras must localize and attach to membranes where the protein functions and signals downstream. This membrane attachment requires numerous post-translational modifications. The initial step is the covalent addition of a 15C far-
addition, lovastatin reverted the learning disabilities of fractions (Mendola and Backer, 1990; Sebti et al., 1991). In nylation and increase the recovery of the protein in cytosolic shown that micromolar doses of lovastatin reduce Ras pre-
diphosphate synthesis (Morgan et al., 2003). Other labs have the initial steps of farnesyl diphosphate and geranylgeranyl cholesterol synthesis, HMG-CoA reductase is responsible for
lesterol synthesis (Wong et al., 2002). In addition to blocking the MPNST cell lines with lovastatin. Lovastatin is an inhib-
inhibitors. To augment the inhibition of FTase, we cotreated

of three independent experiments. nesyl or a 20C geranylgeranyl group to the cysteine located on the C-terminal CaaX box (Gibbs et al., 2001). This modi-
fication is critical for proper Ras function and has been in-
vestigated as a potential target for treating NF1 MPNST
cells.

We used novel farnesyl transferase inhibitors, 1 and 2, to reduce protein prenylation in MPNST cell lines NF90-8 and ST88-14. FTI 1 and 2 are farnesyl diphosphate-based FTase inhibitors. To augment the inhibition of FTase, we cotreated the MPNST cell lines with lovastatin. Lovastatin is an inhib-
itor of HMG-CoA reductase, the rate-limiting enzyme in cho-
lesterol synthesis (Wong et al., 2002). In addition to blocking cholesterol synthesis, HMG-CoA reductase is responsible for the initial steps of farnesyl diphosphate and geranylgeranyl diphosphate synthesis (Morgan et al., 2003). Other labs have shown that micromolar doses of lovastatin reduce Ras pre-
nylation and increase the recovery of the protein in cytosolic fractions (Mendola and Backer, 1990; Sebti et al., 1991). In addition, lovastatin reverted the learning disabilities of NF1−/− mice (Li et al., 2005). Recently, a clinical trial was opened to evaluate the safety of lovastatin in adults with NF1 (NCT00352599). In the current study, we used pharmaco-
logically achievable (Thibault et al., 1996) nanomolar doses of lovastatin, which alone had no effect on NF90-8 and ST88-14 MPNST cell line proliferation but showed synergy when used in combination with novel FTIs, 1 and 2.

Our laboratory previously described N-Ras as the predomi-
ant active Ras isoform expressed in human-derived MPNST cell lines NF90-8 and ST88-14 (Mattingly et al., 2006). N-Ras is a member of a group of proteins that can undergo two types of prenylation, either farnesylation or

nylation and geranylgeranylation. This characteristic could allow an escape mechanism in which N-Ras could be alternatively prenylated with a geranylgeranyl pyrophosphate to maintain proper N-Ras localization and function in the presence of FTIs (Whyte et al., 1997). Indeed, NF1−/− hematopoietic cells confer a myeloproliferative disorder that is resistant to FTI L-744,832 (Kohl et al., 1995) treatment. This resistance occurs despite block of H-Ras prenylation and is proposed to be due to lack of efficacy against N-Ras and for K-Ras (Mahgoub et al., 1999). Since N-Ras is commonly overexpressed or mutated in cancer, designing a therapy that can reduce farnesylation and geranylgeranylation of N-Ras may be a logical approach.

Our data show that Ras prenylation was maintained follow-
ing single treatments of 1, 2, or lovastatin in both MPNST cell lines. It is possible that these agents singularly inhibited FTase and reduced farnesylation. However, N-Ras may have undergone a compensatory alternative geranylgeranylation. Combination treatment of 1 or 2 with lovastatin induced a near-complete inhibition of Ras prenylation in NF90-8 cells and a moderate inhibition in ST88-14 cells. The combination treatment may have provided a more effective inhibition of FTase, but it could also be that lovastatin may have reduced geranylgeranyl diphosphate pools in the cell and so impaired alternate prenylation of Ras (Morgan et al., 2005).

Combination treatment of 1 or 2 with lovastatin impaired Ras prenylation in both NF1 MPNST cell lines, but to different degrees. The degree to which Ras prenylation was suppressed correlated with the antiproliferative and proapoptotic effects of the combination treatment. Although inhibition of Ras preny-
lation correlated strongly with cellular response, Ras is not the exclusive target protein for FTIs (Lebowitz et al., 1997; Ashar et al., 2000; Clark et al., 2007). Approximately 0.5% of proteins in the cell must undergo prenylation to function properly. Known farnesylated proteins include the Ras proteins, RhoB, Rheb, CENP-E, CENP-F, and the nuclear lamins (Tamanoi et al., 2001). The large list of potential FTI targets increases the difficulty in identifying the true therapeutic target and which diseases would respond to an FTI-based therapy. Nevertheless, because Ras activation drives the NF1 phenotype, inhibition of Ras prenylation probably contributes to the efficacy of the combina-
torial drug treatment.

Combination FTI/lovastatin induced G1 arrest, coinciding with reduced pRb phosphorylation at 48 h. Total pRb content was also reduced in combination-treated cultures. Because pRb is a substrate for caspase-3 and -7 (Fattman et al., 1997), and combination treatment resulted in the activation of pro-
caspase-3 and -7, it is possible that pRb was cleaved by active caspases and degraded. This reduction of total pRb may also have contributed to reduced pRb phosphorylation and the observed G1 arrest. Induction of G1 arrest by prenylation inhibitors that include a lactone structure may be through a p21-dependent mechanism that includes inhibition of the proteasome (Efuet and Keyomarsi, 2006). The active compo-
unds in the current study, however, do not contain a lac-
tone moiety. They also induce a G1 arrest in STS-26T cells (Clark et al., 2007), which do not express p21 protein (Mattingly et al., 2006).

Theoretically, toxicity to normal cells could be a concern following FTI treatment due to the large number of proteins that are prenylated. FTIs in combination with lovastatin may increase the number of proteins with impaired prenylation.
However, we found a lack of detectable cytotoxicity in normal or immortalized rat Schwann cells following combined treatment. It is conceivable that the target protein of our FTIs responsible for the observed effects in the MPNST cell lines is not necessary for normal Schwann cell survival or proliferation.

NF1 MPNST cell lines have increased active Ras compared with non-NF1 cell lines. This makes Ras a rational therapeutic target for this disease. Ongoing clinical trials include one regarding lovastatin tolerance in adults with NF1 (NCT00352599) and the use of the peptide-competitive FTI tipifarnib on treating NF1 in children (Widemann et al., 2006) and NCT00293554. The novel FTIs described in this study are extremely effective against NF1 MPNST cell lines with a lack of toxicity against normal Schwann cells. We propose that a combination of FTI/statin treatment may be more efficacious in treatment of NF1 MPNSTs.

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References


