

## **Aborted Autophagy and Non-apoptotic Death Induced by Farnesyl Transferase Inhibitor and Lovastatin**

Jonathan W. Wojtkowiak,<sup>1</sup> Komal M. Sane,<sup>1</sup> Miriam Kleinman, Bonnie F. Sloane, John J.  
Reiners, Jr., and Raymond R. Mattingly

Wayne State University School of Medicine, Department of Pharmacology (J.W.W., K.M.S.,  
B.F.S., J.J.R., R.R.M.), Detroit, Michigan; and Wayne State University, Institute of  
Environmental Health Sciences (M.K., J.J.R.), Detroit, Michigan.

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*Address correspondence to:* Raymond R. Mattingly, WSU Department of Pharmacology, 540 East Canfield avenue, Detroit, MI 48201. Telephone: +1-313-577-6022. Fax: +1-313-577-6739. Email: [r.mattingly@wayne.edu](mailto:r.mattingly@wayne.edu)

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*Abbreviations:* AMC, 7-amino-4-methyl coumarin; AO, acridine orange; DEVDase, proteolytic activity toward the sequence Asp-Glu-Val-Asp; FACS, fluorescence-activated cell sorting; FPP, farnesyl pyrophosphate; FTI, farnesyl transferase inhibitor; GGPP, geranylgeranyl pyrophosphate; GGTI, geranylgeranyl transferase inhibitor; LAMP, lysosome-associated membrane protein; LC3, microtubule-associated protein light chain-3; MPNST, malignant peripheral nerve sheath tumor; PBS, phosphate-buffered saline.

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**Abstract:**

Exposure of the human malignant peripheral nerve sheath tumor cell lines STS-26T, ST88-14 and NF90-8 to nanomolar concentrations of both lovastatin and the farnesyltransferase inhibitor FTI-1, but not either drug alone, induced cell death. ST88-14 and NF90-8 cells underwent apoptosis, yet dying STS-26T cells did not. FTI-1 cotreatment induced a strong and sustained autophagic response as indicated by analyses of LC3-II accumulation in STS-26T cultures. Extensive colocalization of LC3 positive punctate spots was observed with both LAMP-1 and LAMP-2 (markers of late endosomes/lysosomes) in solvent, FTI-1 or lovastatin-treated STS-26T cultures, but very little colocalization in lovastatin/FTI-1 cotreated cultures. The absence of colocalization in the cotreatment protocol correlated with loss of LAMP-2 expression. Autophagic flux studies indicated that lovastatin/FTI-1 cotreatment inhibited the completion of the autophagic program. In contrast, rapamycin induced an autophagic response that was associated with cytosclerosis but maintenance of viability. These studies indicate that cotreatment of STS-26T cells with lovastatin and FTI-1 induces an abortive autophagic program and non-apoptotic cell death.

## Introduction:

Prenylation entails the covalent addition of either farnesyl pyrophosphate (FPP) or geranylgeranyl pyrophosphate (GGPP) onto the cysteine of a “CaaX” box prenylation motif (Goldstein and Brown, 1990). The discovery that the function of oncogenic Ras proteins required prenylation of the nascent polypeptides led to the development of inhibitors of protein farnesylation, termed farnesyl transferase inhibitors (FTIs). Two FTIs, R115777/Zarnestra/tipifarnib and SCH66336/Sarasar/Ionafarnib, have progressed to phase III clinical trials (Adjei, 2005). Limitations to their efficacy as treatments for cancer may stem from the ability of certain farnesyl transferase substrates, for example K-Ras and N-Ras, to become alternatively prenylated by GGPP in the presence of an FTI (Whyte et al., 1997).

One approach to increasing the efficacy of prenylation inhibition could be to combine two classes of prenylation inhibitors with the goal of effectively blocking both protein farnesylation and alternative geranylgeranylation of substrates (Wojtkowiak et al., 2009). Statins are used clinically to reduce levels of plasma cholesterol by blocking metabolic flux through the mevalonate pathway. This pathway contains a key branch point in which FPP can supply the cell with cholesterol or prenyl diphosphates used for protein prenylation (Goldstein and Brown, 1990). Many benefits of statins are observed in patients with coronary heart disease (Cannon et al., 2004), and it is possible that some of these cardiovascular benefits may be due to the blockade of protein prenylation in addition to the suppression of cholesterol synthesis (Mattingly et al., 2002).

We previously reported that N-Ras, and to a lesser extent K-Ras, are the predominant active Ras isoforms expressed in human malignant peripheral nerve sheath tumor (MPNST) cell lines (Mattingly et al., 2006). Both of these Ras isoforms must be prenylated in order to

associate with membranes and function effectively. We also previously reported that lovastatin alone, and FTI-1 alone (which is an FTI based on an FPP analog) had little effect on Ras prenylation or the growth of MPNST NF90-8, ST88-14 and STS-26T cell lines (Clark et al., 2007; Wojtkowiak et al., 2008). In contrast, a strong synergistic cytotoxic response and block of Ras prenylation were observed when the two compounds were used in cotreatment protocols (Clark et al., 2007; Wojtkowiak et al., 2008). Interestingly, although the NF90-8 and ST88-14 cell lines died by a caspase-dependent apoptotic pathway in the cotreatment protocol (Wojtkowiak et al., 2008), preliminary studies indicated that STS-26T cells died by what appeared to be a non-apoptotic mechanism. Since MPNSTs are not amenable to currently available therapeutics, investigation of new drug approaches to induce targeted cytotoxicity is needed (Dilworth et al., 2006; Dilworth et al., 2008).

Macroautophagy (hereafter referred to as autophagy) is a normal physiological process that is both constitutive and inducible. Autophagy is characterized by the encapsulation of cytoplasm and entire organelles within a double membrane vacuole termed the autophagosome. Autophagosomes can fuse directly with lysosomes to form autolysosomes, or with other endocytic vesicles to form amphisomes, which may subsequently fuse with lysosomes (Berg et al., 1998). Once fusion with lysosomes occurs, the contents of the autophagosome or amphisome undergo proteolytic degradation. As such, autophagy is thought to be the preferred mechanism for removal of long-lived proteins, protein aggregates and aged/damaged organelles. Stressors such as nutrient and energy deprivation, reactive oxygen species, hypoxia, and a variety of cytotoxic agents are common inducers of autophagy (Klionsky, 2007). Comparisons of autophagic proficient and deficient cell lines indicate that stress-induced autophagy often plays a pro-survival function (Debnath et al., 2005; Elliott and Reiners, 2008; Scarlatti et al., 2009). Nevertheless, numerous examples have been published

in which autophagy appears to contribute to cell death by a non-apoptotic mechanism, which has been termed Class II death (Bursch, 2001; Kessel et al., 2007; Scarlatti et al., 2009).

Recent studies indicate that statins (Araki and Motojima, 2008), the geranylgeranyl transferase inhibitor GGTI-2Z (Sane et al., 2010) and the FTI lonafarnib (Pan et al., 2008) are capable of inducing autophagy in a variety of cell types. In the current study we explored the possibility that the different modes of cell death noted in our studies of MPNST cell lines might reflect differential effects of FTI-1 and lovastatin cotreatment on the induction and development of the autophagic response. Indeed, FTI-1 and lovastatin cotreatment induced a very strong and protracted autophagic response in the STS-26T cell line that reflected an aborted autophagic program. This aborted autophagy was associated with non-apoptotic, type II cell death. Our studies suggest that an incomplete autophagic response can lead to cell death, and that prenylated proteins may play a major role in the autophagic process.

## Methods

### Compounds

The synthesis of compound FTI-1 was previously described [(Wojtkowiak et al., 2008); compound was named **1**]. Aliquots of FTI-1 and lovastatin (Sigma-Aldrich, St. Louis, MO) were prepared in dimethyl sulfoxide [DMSO (Sigma-Aldrich)] and stored at -80°C. Rapamycin (Sigma-Aldrich) aliquots were prepared in DMSO and stored at -20°C.

### Cell culture and Transfection

STS-26T, NF90-8, and ST88-14 MPNST cell lines were generously donated by T. Glover (University of Michigan, Ann Arbor, MI). Maintenance of these cell lines was described previously (Dilworth et al., 2008; Wojtkowiak et al., 2008). Briefly, adherent cultures were maintained in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 5% FBS (Hyclone Laboratories, Logan, UT), 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen). Spontaneously immortalized normal Schwann cells (iSC) that were isolated from rat sciatic nerves were a gift from E.M. Shooter (Stanford University, Stanford, CA). This cell line was maintained in minimal essential medium (Invitrogen) supplemented with 10% horse serum (Hyclone Laboratories), 100 U/ml penicillin, and 100 µg/ml streptomycin. Murine hepatoma 1c1c7 cells that stably express GFP-LC3 were maintained in alpha minimal essential medium (Invitrogen) supplemented with serum and antibiotics as above plus 2 µg/ml G418 (Invitrogen). For experiments, 1c1c7 cells were plated in medium lacking G418. All cell lines were maintained in a humidified incubator under 5% CO<sub>2</sub>. For all experiments, cells were plated ~24 h prior to drug treatment.

The pCMV5 Flag vector with an insert encoding Rheb was a generous gift from J. Avruch (Harvard Medical School, Boston, MA) and was previously described (Long et al., 2005). Lipofectamine 2000 (Invitrogen) was used for transient transfection of STS-26T cells with pCMV5 Flag-Rheb.

### Western Blot Analysis

Whole cell lysates were prepared from a monolayer of cells in Insect Cell Lysis Buffer [(10 mM Tris pH 7.5, 130 mM NaCl, 1% Triton X-100, 10 mM NaF, 10 mM NaPi, 10 mM NaPPi) from BD Biosciences, San Jose, CA] containing 1x protease inhibitor cocktail [(aprotinin, bestatin hydrochloride, E64, leupeptin, and pepstatin A) from Sigma-Aldrich]. Samples were flash frozen in liquid nitrogen and stored at -80°C. Prior to use, samples were thawed on ice, sonicated, and cleared by centrifugation. The Bradford protein assay (Bio-Rad, Hercules, CA) was used to determine protein concentration and 25 µg of protein per sample was 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 Biosciences). The Flag tag epitope was detected with a 1:2000 dilution of anti-Flag monoclonal antibody (Sigma-Aldrich). Caspase-3 was detected with a 1:1000 dilution of anti-Caspase-3 polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) as described (Menard et al., 2005). Rab5 was detected with a 1:200 dilution of anti-Rab5 monoclonal antibody (Santa Cruz Biotechnology). LC3 expression was detected with a 1:2000 dilution of anti-LC3 polyclonal antibody (a generous gift from Dr. David Kessel, Wayne State University). LAMP-1 and LAMP-2 expression were detected with a 1:400 dilution of monoclonal antibodies (BD BioSciences). All membranes were stripped (Mattingly et al., 2001) and probed for β-tubulin expression with a 1:2000 dilution of the E7 monoclonal antibody (Developmental Studies Hybridoma Bank, Iowa City, IA).



### **Cell Proliferation Assay**

STS-26T cells were plated in 60-mm dishes ~24 h prior to drug treatment in fresh medium. Attached cells were trypsinized and combined with media containing detached cells. The cells were collected by centrifugation for 5 min at 1000g and counted with a hemacytometer using Trypan blue to distinguish dead from viable cells.

### **Flow Cytometry**

STS-26T cultures were harvested and processed for DNA analyses as described previously (Wojtkowiak et al., 2008). DNA content was analyzed using a FACScalibur instrument (BD Biosciences). A minimum of  $10^4$  cells/sample was analyzed to determine the percentage of cells with sub-G<sub>1</sub>, G<sub>1</sub>, S, and G<sub>2</sub>/M phase DNA content (MODFIT; Variety Software, Topsham, ME).

### **DEVDase Activity Assay**

Lysates of STS-26T cultures were prepared and used in DEVDase assays as previously described (Wojtkowiak et al., 2008). Changes in fluorescence over time were converted into picomoles of product by comparison with a standard curve made with 7-amino-4-methylcoumarin. DEVDase 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.

### **Colony Formation Assay**

STS-26T cells were plated at a density of  $2 \times 10^4$  per 35-mm culture plate ~24 h prior to drug treatment. After the 48-h treatment, cultures were trypsinized and  $3 \times 10^3$  cells were subcultured in triplicate in 60-mm plates containing fresh media without drugs. Colonies containing

4 or more cells after 48 h and 72 h of growth were counted in 10 randomly selected fields per plate.

### Immunofluorescence

Cultures were sequentially rinsed with PBS containing  $\text{Ca}^{2+}/\text{Mg}^{2+}$ , fixed in cold 100% methanol at  $-20^{\circ}\text{C}$  for 5 min, and then blocked in PBS supplemented with 2% bovine serum albumin (BSA) and 0.2% saponin. Cultures were subsequently incubated with primary antibodies (1:50 dilution in PBS supplemented with 2% BSA): anti-LC3 polyclonal antibody (Abgent, San Diego, CA), anti-LAMP-1 and LAMP-2 monoclonal antibodies (BD Biosciences). Secondary antibody incubation included 1:500 dilution of either Alexa-Fluor 488 anti-rabbit or Alexa-Fluor 555 anti-mouse antibodies in PBS supplemented with 2% BSA. Nuclei were stained with a 1:10,000 dilution of 4',6-diamidino-2-phenylindole (DAPI). All washes consisted of PBS supplemented with 0.2% saponin. Stained coverslips were mounted on slides using ProLong Gold antifade reagent (Invitrogen) and images were captured with either Leica TCS SP5 (Wetzlar, Germany) or Zeiss LSM 510 (Gottingen, Germany) confocal microscopes.

Colocalization profiling in STS-26T cultures was performed using Metamorph software. A single cell was selected per field and a line was drawn through the cell as indicated in the relevant figures. Pixel-by-pixel intensity of immunofluorescence was plotted against distance in  $\mu\text{m}$  along that line and is represented in the graphs shown.

Colocalization analyses of GFP-LC3 with LAMP-1 were performed with cultures of GFP-LC3 expressing murine hepatoma 1c1c7 cells grown on poly-L-lysine coated coverslips. Cultures were washed with PBS and fixed with 4% paraformaldehyde/PBS for 30 min at room temperature. Thereafter, the coverslips were washed 3x with PBS/0.1% saponin and incubated in blocking buffer (5% BSA/PBS/0.1% saponin) for 1 h at  $37^{\circ}\text{C}$ . The coverslips were washed and then incubated with 1:1000 1D4B rat anti-mouse LAMP1 antibody (Developmental Studies Hybridoma Bank, Iowa City, IA) in blocking buffer for 2 h at  $37^{\circ}\text{C}$ . The coverslips were washed

followed by incubation with 1:200 dilution of AlexaFluor 546 goat anti-mouse IgG (Molecular Probes, Eugene, OR) in blocking buffer for 1 h at 37°C. The coverslips were again washed three times and inverted onto glass slides with a drop of Slowfade solution (Molecular Probes, Eugene, OR) and sealed with acrylic nail polish. Images were captured with a Zeiss LSM 510 confocal microscope.

### **Induction of autophagy by culturing in leucine-free medium**

STS-26T cultures were washed 3x with PBS and refed with a leucine-free starvation media consisting of Earle's balanced salts, 1x vitamin mix (Invitrogen), 1x non-essential amino acids, 1% dialyzed fetal bovine serum (Thermo Scientific), 1 mM MgCl<sub>2</sub>, 1.8 mM CaCl<sub>2</sub>, 0.2 mg/L  $\alpha$ -lipoic acid, and 1 mM HEPES, pH 7.25. Cultures were generally processed for either western blot analyses or immunocytochemistry 6 h after medium switch.

## Results:

### ***Suppression of prenylation by FTI-1 and lovastatin cotreatment***

Our laboratory previously reported that the NF90-8, ST88-14 and STS-26T MPNST cell lines express predominantly N-Ras, lesser amounts of K-Ras, and no detectable H-Ras (Mattingly et al., 2006). We also reported that Ras prenylation in the NF90-8 and ST88-14 cell lines was suppressed by low  $\mu$ M FTI-1 and lovastatin cotreatment (Wojtkowiak et al., 2008). Ras prenylation in the STS-26T cell line was not notably affected by DMSO, 500 nM lovastatin, or 500 nM FTI-1 treatment (Figure 1A); however, within 24 h of 500 nM FTI-1 plus 500 nM lovastatin cotreatment a slower moving band, indicative of non-prenylated Ras, was detected by western blot analyses (Figure 1A). It should be noted that total Ras protein expression was decreased at 24 h, which could suggest protein instability following inhibition of prenylation (Figure 1A).

The protein targets responsible for the observed effects of FTIs remain unknown, probably at least in part due to the large number of farnesylated proteins in the cell. The exclusively farnesylated small GTPase Rheb, for example, has been suggested to be a potential target of FTI therapy (Basso et al., 2005). In order to determine if FTI-1 plus lovastatin cotreatment is capable of reducing Rheb prenylation, we transiently transfected Flag-Rheb into STS-26T cells. As observed with Ras prenylation, FTI-1 plus lovastatin cotreatment increased the accumulation of non-prenylated Rheb within 24 h when compared to cultures treated with just FTI-1 or lovastatin (Figure 1B). A screening approach has provided evidence that FTIs that inhibit proliferation may also reduce prenylation of Rab proteins, in addition to other farnesylated proteins (Lackner et al., 2005). We therefore tested whether FTI-1 and lovastatin cotreatment affected Rab5 prenylation. As observed with Ras prenylation, the cotreatment protocol induced the appearance of the more slowly migrating, non-prenylated form of Rab5 and also caused a large decrease in the total expression of the protein (Figure 1A).

### ***Anti-proliferative effects of FTI-1 and lovastatin cotreatment***

We subsequently investigated the effects of FTI-1 plus lovastatin cotreatment on STS-26T proliferation and viability. Neither STS-26T proliferation nor viability was affected by single exposure to DMSO, 500 nM FTI-1, or 500 nM lovastatin (Figure 2A). However, cell proliferation was greatly reduced within 48 h of FTI-1 and lovastatin cotreatment. Significant cytotoxicity was also observed within 72 h of cotreatment (Figure 2B). We observed very few dying cells with morphological characteristics of apoptosis (e.g., shrunken nuclei or blebbed cells).

Fluorescence-activated cell sorting (FACS) was used to determine the cell cycle profile of cultures following treatment with prenylation inhibitors (Figure 2C). Cultures treated with either FTI-1 or lovastatin alone exhibited distribution profiles similar to cultures treated with just solvent. In contrast, cultures cotreated with 500 nM FTI-1 and 500 nM lovastatin for 48 h exhibited marked gains and losses in G<sub>1</sub> and S phase cells, respectively (Figure 2C).

Previous analyses indicated that the NF-90-8 and ST88-14 MPNST cell lines died by apoptosis following FTI-1 and lovastatin cotreatment (Wojtkowiak et al., 2008). Preliminary DNA analyses and morphological observations of STS-26T cells, however, suggested that they die by an alternative pathway under this cotreatment protocol. Further, only a very minor population of cells with sub-G<sub>1</sub> DNA contents (which would be consistent with an apoptotic population) accumulated in cotreated cultures (Figure 2C). In order to obtain a quantitative appraisal of apoptosis, we monitored DEVDase activities (a measure of activation of procaspases-3 and -7) in STS-26T cultures. Exposure of STS-26T cultures to lovastatin or FTI-1 either alone or in combination did little in terms of DEVDase activation (Figure 3A). However, exposure to the Bcl-2 antagonist and respiratory chain uncoupler HA14-1 rapidly induced DEVDase activation (Figure 3B). Western blot analyses of pro-caspase-3 processing confirmed the differential abilities of HA14-1 and FTI-1 and lovastatin to activate pro-caspase-3 in STS-

26T cultures (Figure 3C). In contrast to what was observed with STS-26T cells, pro-caspase-3 was readily cleaved in NF90-8 cells following FTI-1 and lovastatin cotreatment (Figure 3C).

### ***Induction of autophagy by FTI-1 and lovastatin cotreatment***

Exposure to statins (Araki and Motojima, 2008) or the FTI lonafarnib (Pan et al., 2008) has been reported to induce autophagy in a variety of cell types. Although autophagy is generally perceived as being a pro-survival process, it has been linked to caspase-independent cell death (Bursch, 2001; Kessel et al., 2007; Scarlatti et al., 2009). The induction of autophagy is characterized by the accumulation of autophagosomes, which can be monitored by following the posttranslational addition of phosphatidylethanolamine to the cytosolic protein LC3-I to form LC3-II (Mizushima and Yoshimori, 2007). We observed that FTI-1 plus lovastatin cotreatment resulted in a substantial and sustained accumulation of LC3-II in STS-26T cells within 48 h (Figure 4A). In contrast, little LC3-II accumulation occurred in the NF90-8 cell line, and an intermediate response occurred in the ST88-14 cell line (Figure 4A). Treatment of STS-26T cultures with either FTI-1 or lovastatin alone for 48-66 h induced only marginal accumulations of LC3-II compared to the robust response seen with the cotreatment protocol (Figure 4B).

We previously reported that the normal Schwann cell line iSC is refractory to the cytostatic and cytotoxic effects of FTI-1 and lovastatin cotreatment (Wojtkowiak et al., 2008). FTI-1 and lovastatin cotreatment induced only a very small accumulation of LC3-II in iSC cells relative to what was observed in the STS-26T cell line (Supplemental Figure 1).

### ***Suppression of 'autophagic flux' by FTI-1 and lovastatin cotreatment***

LC3-II accumulation can reflect the aggregate effects of induced autophagosome synthesis, decreased fusion of constitutively synthesized or induced autophagosomes with lysosomes, and/or suppression of LC3-II proteolytic turnover in the autolysosomes. In order to

determine which of these processes contributes to the observed LC3-II accumulation in STS-26T cultures, we performed an analysis of 'autophagic flux'. Pepstatin A and E64D inhibit lysosomal/autolysosomal aspartate and cysteine cathepsins, respectively, and should suppress autolysosomal proteolysis of LC3-II. If autophagosome-lysosome fusion and the capacity for degradation of LC3-II in the autolysosome are not impaired, pepstatin A and E64D cotreatment should suppress LC3-II degradation, and lead to an accumulation of LC3-II in excess of what is observed in cultures not treated with the cathepsin inhibitors. Conversely, the failure of cathepsin inhibitor treatment to increase LC3-II contents beyond what is observed following drug treatment alone would be consistent with drug treatment reducing autophagosome degradation due to either a block in fusion, or reduced capacity for proteolysis. Furthermore, comparisons of LC3-II contents in cathepsin inhibitor-treated control cultures versus cathepsin inhibitor + drug-treated cultures provides information related to autophagosome formation.

STS-26T cultures treated with the cathepsin inhibitors prior to DMSO, 500 nM FTI-1, or 500 nM lovastatin exposure exhibited greater accumulations of LC3-II than cultures not pretreated with protease inhibitors (Figure 4C). These results indicate that autophagosomes are generated, fuse with lysosomes, and undergo degradation in control (DMSO) cultures and in those exposed to either lovastatin or FTI-1 alone. Furthermore, the increased levels of LC3-II in the cultures exposed to FTI-1 or lovastatin in the presence of cathepsin inhibitors, relative to the cultures exposed to DMSO in the presence of cathepsin inhibitors, suggest that FTI-1 and lovastatin induce autophagosome synthesis. In contrast, protease inhibitor pretreatment did not enhance LC3-II accumulation in STS-26T cultures cotreated with lovastatin and FTI-1 (Figure 4C). This result is suggestive of a block in autophagosome-lysosome fusion or a lysosomal proteolytic deficiency. It should be noted that the amount of LC3-II in cultures cotreated with FTI-1 and lovastatin (with and without protease inhibitors) appears markedly greater than the sum of the LC3-II content in cultures exposed to FTI-1 in the presence of cathepsin inhibitors

plus that in cultures exposed to lovastatin in the presence of cathepsin inhibitors (Figure 4C). This result is consistent with FTI-1 plus lovastatin cotreatment stimulating autophagosome synthesis beyond that induced by exposure to either FTI-1 or lovastatin alone.

We also evaluated autophagic flux through the alternative approach of cotreatment with bafilomycin A1, which inhibits lysosomal vacuolar H<sup>+</sup> ATPase and blocks autophagosome-lysosome fusion and degradation. Bafilomycin A1 enhanced LC3-II accumulation in vehicle-treated cells, in cells shifted to leucine-free media [a condition known to rapidly induce autophagy (Mordier et al., 2000)], as well as in cells treated with GGTI-2Z/lovastatin [previously shown to induce autophagy in STS-26T cells (Sane et al., 2010)] (Figure 4D). In contrast, bafilomycin A1 failed to enhance LC3-II accumulation in FTI-1/lovastatin treated cells. Hence, a second approach verified that cotreatment of STS-26T cells with FTI-1 and lovastatin either blocked autophagosome-lysosome fusion, or inhibited proteolysis in the autolysosome.

The mTOR signaling pathway is a potent regulator of cell proliferation and a known negative regulator of autophagy. Rapamycin inhibits mTOR activity and often induces autophagy (Meijer and Codogno, 2009). It has also been reported to be an inhibitor of MPNST proliferation (Bhola et al., 2009). Concentrations of rapamycin  $\geq 0.1 \mu\text{M}$  were nearly as effective as 500 nM lovastatin + 500 nM FTI-1 cotreatment in inhibiting STS-26T proliferation (Figure 5A). However, colony-forming assays indicated that rapamycin was purely cytostatic, and that its effects were reversible upon replating treated cells in fresh medium without drug (Figure 5B). We confirmed that cytostatic concentrations of rapamycin induced autophagy in STS-26T cultures, although the magnitude of LC3-II accumulation was consistently less than that observed following combined lovastatin and FTI-1 treatment (Figure 5C).

### ***Suppression of autophagosome – lysosome fusion by FTI-1 and lovastatin cotreatment***



A defect in autophagosome/amphisome fusion with lysosomes could explain the inability of protease inhibitors and bafilomycin to increase the amount of LC3-II observed in FTI-1+ lovastatin cotreated cultures. In order to monitor autophagosome development and fusion, we took advantage of the fact that LC3-II containing autophagosomes appear punctate when stained with an antibody to LC3. As anticipated, very weak punctate LC3 staining was observed in solvent-treated cultures (Figures 6A and 7A, left hand columns). Both the numbers and intensities of LC3 positive punctate spots were dramatically increased by shifting cultures to a leucine-free medium or by cotreatment with lovastatin and FTI-1 (Figures 6A and 7A).

Analyses of LC3 colocalization with the late endosome/lysosome marker LAMP-1 indicated a high degree of colocalization in leucine starved, and FTI-1 or lovastatin-treated cultures (Figure 6A, colocalization is indicated by orange/yellow color in the merged images of red and green fluorescence channels and by the coincident pattern of valleys and peaks in the pixel-by-pixel analysis shown in the graphs). In contrast, although there was some colocalization of LC3 and LAMP-1 staining in cultures cotreated with lovastatin and FTI-1, most of the LC3 staining did not colocalize with that of LAMP-1 (Figure 6A). Similar results were obtained for LC3 and LAMP-2. Specifically, there was significant colocalization of LAMP-2 with LC3 in leucine-starved, DMSO, lovastatin or FTI-1-treated cultures, but virtually no colocalization in cultures cotreated with lovastatin and FTI-1 (Figure 7A). Indeed, relative to the other treatment groups, overall LAMP-2 staining was notably less in cotreated cultures.

The confocal studies illustrated in Figures 6A and 7A indicate that the autophagosomes generated in lovastatin/FTI-1 cotreatment protocols fail to fuse with LAMP-1-positive and LAMP-2-positive vesicles (e.g., late endosomes/lysosomes). LAMP-1 and -2 expression levels in STS-26T cultures were not affected by exposure to FTI-1 or lovastatin over a 48 h period (Figure 6B and 7B). Whereas FTI-1 and lovastatin cotreatment did not alter LAMP-1 expression (Figure

6B), the expression of LAMP-2 was significantly reduced in cotreatment protocols, with the appearance of a faster mobility form suggesting that LAMP-2 may be undergoing degradation (Figure 7B).

We next determined whether FTI-1 and lovastatin cotreatment suppression of autophagosome-lysosome fusion was restricted to STS-26T or also occurred in a different cancer cell type: murine 1c1c7 hepatoma cells. Cultures of 1c1c7 cells that stably express GFP-LC3 exhibited diffuse GFP green fluorescence, with very few punctate green fluorescent spots (e.g., autophagosomes), under control conditions following exposure to solvent (Supplemental Figure 2). However, the GFP-LC3 coalesced into numerous highly fluorescent punctate green spots within 6 h of shifting 1c1c7 cultures to a leucine-deficient medium. Most of these spots colocalized with Lamp-1, as indicated by the large number of yellow spots in the merged colored images, or the near identical spatial patterns for the individual LAMP-1 and GFP-LC3 images (Supplemental Figure 2). Singular treatments with either 500 nM FTI-1 or 500 nM lovastatin for 48 h induced a modest accumulation of punctate green fluorescent spots, some of which colocalized with LAMP-1 (Supplemental Figure 2). Co-treatment with 500 nM FTI-1 and 500 nM lovastatin for 48 h induced a much greater accumulation of punctate fluorescent green spots (Supplemental Figure 2). However, there was very little, if any, colocalization of GFP-LC3 with LAMP-1. Hence, FTI-1 and lovastatin cotreatment suppresses autophagosome-lysosomes fusion in 1c1c7 cultures.

## Discussion:

Appreciation of the abilities of FTIs or statins to induce autophagy is a recent development. Exposure of U2OS cultures to micromolar concentrations of lonafarnib or manumycin A, two FTIs with different modes of action, induces autophagy (Pan et al., 2008) as do the statins fluvastatin and simvastatin in some (Araki and Motojima, 2008), but not all (Martinet et al., 2008), types of cultured cells. In the case of the statin study, it was concluded that the induction of autophagy reflected an inhibition of prenylation since the effects of statin treatment could be blocked by the addition of mevalonate, but not cholesterol. In the current study, exposure of MPNST STS-26T cells to nanomolar concentrations of FTI-1 plus lovastatin, but not to either agent alone, induced cell cycle arrest, a dysfunctional autophagic program, and non-apoptotic death. The induction of these processes by combined but not single treatments with FTI-1 or lovastatin occurred with concentrations of agents sufficient to suppress the prenylation of both Rheb and Ras. Whereas Rheb is exclusively farnesylated (Castro et al., 2003), N-Ras and K-Ras undergo alternative geranylgeranylation if farnesylation is blocked (Whyte et al., 1997). Because STS-26T cells express primarily N-Ras, and to a lesser extent K-Ras (Mattingly et al., 2006), we conclude that FTI-1 and lovastatin cotreatment effectively suppresses both farnesylation and alternative geranylgeranylation pathways in STS-26T cells and that both forms of prenylation may have a role in the regulation of autophagy. The ability of the FTI-1 and lovastatin cotreatment to reduce prenylation of Rab5 is also consistent with such a dual mechanism of action.

FTI-1 and lovastatin cotreatment induced a dramatic autophagic response in STS-26T cultures, and reductions in both the absolute amounts, and prenylated forms of both Rheb and Rab5. These reductions may have contributed to the mechanism by which FTI-1 and lovastatin cotreatment induced autophagy. Specifically, the prenylated form of Rheb is a potent activator

of mTOR complex 1 (Finlay et al., 2007), which is a suppressor of autophagy (Meijer and Codogno, 2009). This activation occurs via association of Rheb with mTOR in the late endosome (Flinn et al., 2010). Rab5 is essential for the conversion of early endosomes into late endosomes, and conditions which suppress this conversion inhibit mTOR complex 1 activation [(Flinn et al.); references therein]. Hence, reductions in Rab5 and Rheb content and prenylation could collectively, as well as individually, mute/eliminate mTOR's suppressive effects on the initiation of autophagy. Interestingly, the autophagic program induced in STS-26T cultures by FTI-1 and lovastatin cotreatment did not proceed to completion. STS-26T cells developed autophagosomes following either cotreatment with FTI-1 and lovastatin, or after being shifted to a leucine-free medium. In the latter treatment protocol, autophagosomes presumably fused with lysosomes to form autolysosomes since LC3 staining colocalized with LAMP-1 and LAMP-2 staining in punctate structures. However, a similar colocalization did not occur in cultures cotreated with FTI-1 and lovastatin. Indeed, in these latter cultures there was very little LC3 colocalization with any of the lysosomal markers. Hence, cotreatment suppressed autophagosome/amphisome fusion with lysosomes. The observed effects of combination treatment were not unique to STS-26T cells. Similar results were obtained in comparably treated murine hepatoma 1c1c7 cultures.

Multiple mechanisms are most likely responsible for the suppression of autophagosome-lysosome fusion observed in STS-26T cultures cotreated with FTI-1 and lovastatin. The *Rab* family of small GTPase proteins, which are generally involved in membrane transport and fusion events, must be geranylgeranylated to function and localize appropriately. There is evidence that FTI treatment can impair the prenylation of some Rab proteins (including Rab 5 and Rab7) via suppression of Rab geranylgeranyl transferase (Lackner et al., 2005). The early endosomal protein Rab5 has been shown to play a role in lysosomal biogenesis (Hirota et al., 2007), and the late endosomal marker protein Rab7 facilitates the fusion of autophagosomes/amphisomes

with lysosomes (Gutierrez et al., 2004). Nevertheless, we have previously shown that the combination of GGTI-2Z and lovastatin, which also effectively blocked Rab5 prenylation, induced a complete (not aborted) autophagic program in STS-26T cells (Sane et al., 2010). The ability of GGTI-2Z and lovastatin to induce autophagy also occurred without any decrease in cell viability (Sane et al., 2010), consistent with the cell killing in the current study being more closely associated with the failure to complete the autophagic program.

An alternative mechanism for the suppression of autophagosome-lysosome fusion could be a depletion of LAMP-2. For example, autophagosomes accumulate in LAMP-2 defective HeLa cells because of suppressed autophagosome-lysosome fusion (Gonzalez-Polo et al., 2005), whereas LAMP-2 depletion in pancreatic acinar cells leads to accumulation of autophagosomes coupled with non-apoptotic cell death (Fortunato et al., 2009). In the case of LAMP-2 deficient hepatocytes, there is reduced capacity for proteolysis by the lysosomes due to altered trafficking and impaired processing of procathepsin D (Eskelinen et al., 2002). We found that combined FTI and statin treatment markedly reduced LAMP-2, but not LAMP-1, in STS-26T cultures.

Western blot analysis of LC3-II indicated induction of autophagy in the three MPNST cell lines used in this study following FTI-1 and lovastatin cotreatment. Whereas the response in STS-26T cells was extremely robust, that in ST88-14 and NF90-8 cells was more modest. It should be noted that the concentrations of FTI-1 and lovastatin used in this study are pro-apoptotic when used in cotreatment protocols with the ST88-14 and NF90-8 cell lines (Wojtkowiak et al., 2008). It is likely that the profound increase in LC3-II levels seen in treated STS-26T cells may be facilitated by the absence of an apoptotic response in that cell line.

Autophagy can play either a pro-survival or pro-death role (Scarlatti et al., 2009). In its pro-death mode, autophagy may collaborate with and facilitate other death mechanisms. Alternatively, it may directly mediate cell death. In the case of the current study, STS-26T cells died with non-apoptotic features following FTI-1 and lovastatin cotreatment. STS-26T cells cotreated with FTI-1 and lovastatin exhibited an autophagic program that was severely aborted in its latter stages. Hence, cell death was not the consequence of a completed autophagic program. At issue is whether the observed non-apoptotic cell death was a consequence of autophagy being blocked/aborted. A precedent for this type of event is provided by the report that tumor cell killing can be enhanced by using chloroquine derivatives that inhibit therapy-induced autophagy (Amaravadi et al., 2007). Chloroquine is a lysosomotropic agent that aborts the autophagic program by raising lysosomal pH and suppressing autophagic protein degradation. These findings demonstrate a possible requirement for prenylated proteins to fulfill a complete autophagic response and raise the issue of whether an aborted autophagic program is actually responsible for the 'autophagic death' reported in some studies.

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## Authorship Contribution

<sup>1</sup>J.W.W. and K.M.S. contributed equally to this work.

*Participated in research design:* Wojtkowiak, Sane, Sloane, Reiners, Mattingly

*Conducted experiments:* Wojtkowiak, Sane, Kleinman, Reiners

*Contributed new reagents or analytic tools:* n.a.

*Performed data analysis:* Wojtkowiak, Sane, Kleinman, Reiners

*Wrote or contributed to the writing of the manuscript:* Wojtkowiak, Sane, Sloane, Reiners,  
Mattingly



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## Footnotes

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## Figure Legends:

**Figure 1.** Suppression of prenylation by FTI-1 and lovastatin cotreatment. STS-26T cells were plated 24 h prior to drug treatment. Cultures were treated as indicated in the figure for 24 h prior to being harvested and processed for western blot analyses of Ras and Rab5 (A) and Rheb (B) prenylation status. Unprenylated Ras, Rab5 and Rheb migrate more slowly on SDS gels and appear as an upper band. Cultures in panel B were transiently transfected with Flag-Rheb for 24 h, prior to treatment. Analyses are of 25  $\mu$ g of protein per lane. Similar results were obtained in two additional experiments for Ras and Rab5 prenylation and one additional experiment for Rheb prenylation.

**Figure 2.** Cytostatic and cytotoxic effects of FTI-1 and lovastatin cotreatment. STS-26T cells were plated 24 h prior to being treated with solvent, lovastatin, FTI-1, or a combination of FTI-1 plus lovastatin. Cultures were harvested at various times after treatment for assessment of cell numbers (A) and viability (B), or 48 h after treatment for FACS analyses of DNA contents (C). Data in A and B are the mean  $\pm$  S.D. of triplicate samples and are representative of three independent experiments. The data in panel C represent  $10^4$  gated events, and are representative of three independent experiments.

**Figure 3.** Pro-caspase activation by FTI-1 and lovastatin cotreatment. STS-26T cells were plated 24 h prior to drug treatments. (A) Cultures were treated with lovastatin, FTI-1, or a combination of lovastatin and FTI-1 for the indicated times prior to being harvested and processed for analyses of DEVDase activities. (B) Cultures were treated with 25  $\mu$ M HA14-1 for the indicated times prior to being harvested and processed for analyses of DEVDase activities. Data in panels A and B represent mean of triplicate replicants. Similar results were obtained in

two additional experiments. (C) STS-26T cultures were treated as indicated and then harvested and processed for western blot analyses of pro-caspase-3 cleavage. As positive controls, STS-26T cultures were treated with HA14-1 and NF90-8 cells were treated with lovastatin + FTI-1 prior to being harvested for analyses of pro-caspase-3 cleavage. Analyses are of 25  $\mu$ g of protein per lane.

**Figure 4.** FTI-1 and lovastatin cotreatment induce autophagy. (A) STS-26T, ST88-14, and NF90-8 MPNST cultures were treated with solvent or FTI-1 + lovastatin, and harvested at the indicated times for analyses of LC3 expression by western blot analyses. (B) STS-26T cultures were treated with lovastatin, FTI-1, or a combination of the two drugs for 16-66 h prior to being harvested for analyses of LC3. (C) STS-26T cultures were treated with or without 10  $\mu$ M E64D and 10  $\mu$ M pepstatin A for 2 h prior to the addition of FTI-1, lovastatin, or FTI-1 + lovastatin. Cultures were harvested 24 h later for western blot analyses of LC3 and estimates of 'autophagic flux'. Similar results were obtained in two additional studies. (D) STS-26T cells were subjected to 6 h of incubation in leucine free media; 48 h of DMSO, 500 nM GGTI-2Z or 500 nM FTI-1 either alone or in combination with 500 nM lovastatin as indicated; in the presence or absence of 50 nM bafilomycin A1 for the final 2 h of the culture. Whole-cell lysates were then separated on SDS-PAGE gels and analyzed for LC3 and  $\beta$ -tubulin expression.

**Figure 5.** Rapamycin induces autophagy and is cytostatic in STS-26T cells. (A) STS-26T cultures were treated as indicated in the figure and harvested for analyses of cell numbers. (B) STS-S6T cells were treated as shown in the figure for 48 h and then harvested, counted, and 3 x 10<sup>3</sup> of each condition were re-plated in fresh growth media without any inhibitors. After a further 48 or 72 h of culture, colonies containing 4 or more cells were counted in ten randomly selected fields of each culture. Data are the mean  $\pm$  S.D. of triplicate samples and are



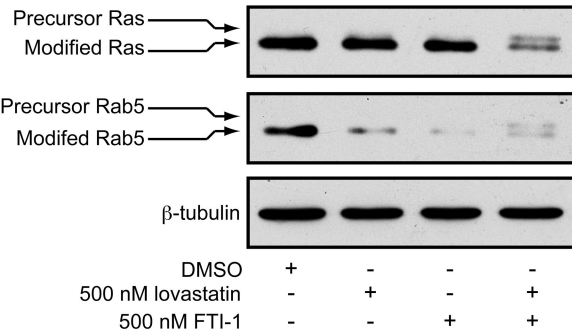
representative of two independent experiments. (C) STS-26T cultures were treated as indicated in the figure. Whole cell lysates were prepared and probed for LC3-II expression by western blot analysis.

**Figure 6.** Colocalization of LC3 and LAMP-1 in FTI-1 and lovastatin cotreated cultures. (A) STS-26T cultures were treated with DMSO, 500 nM lovastatin, 500 nM FTI-1 or FTI-1 plus lovastatin for 48 h prior to processing of cultures to analyze colocalization of LC3 with LAMP-1. Nuclei were stained with DAPI. Parallel cultures were shifted to a leucine-free media for 6 h in order to intentionally induce autophagy. Co-localization of LC3 with LAMP-1 is indicated by punctate orange/yellow fluorescence in merged images, or congruence of the patterns of valleys and peaks in overlaid scans of the red and green channels. (B) STS-26T cultures were treated with lovastatin, FTI-1 or lovastatin plus FTI-1 for either 24 or 48 h or shifted to a leucine-free media for 6 h prior to being processed for quantification of LAMP-1 expression by western blot analysis.

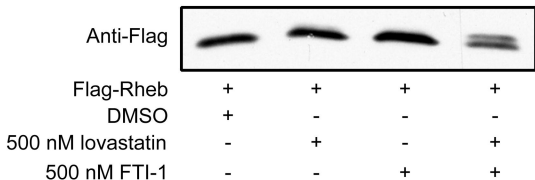
**Figure 7.** Colocalization of LC3 and LAMP-2 in FTI-1 and lovastatin cotreated cultures. STS-26T cultures were treated with DMSO, 500 nM lovastatin, 500 nM FTI-1 or FTI-1 plus lovastatin for 48 h prior to processing of cultures to analyze colocalization of LC3 with LAMP-2. Nuclei were stained with DAPI. Parallel cultures were shifted to a leucine-free media for 6 h in order to intentionally induce autophagy. Co-localization of LC3 with LAMP-2 is indicated by punctate orange/yellow fluorescence in merged images, or congruence of the patterns of valleys and peaks in overlaid scans of the red and green channels. (B) STS-26T cultures were treated with lovastatin, FTI-1 or lovastatin plus FTI-1 for either 24 or 48 h or shifted to a leucine-free media for 6 h prior to being processed for quantification of LAMP-2 expression by western blot analysis.

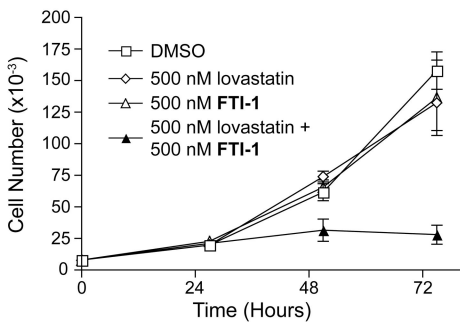
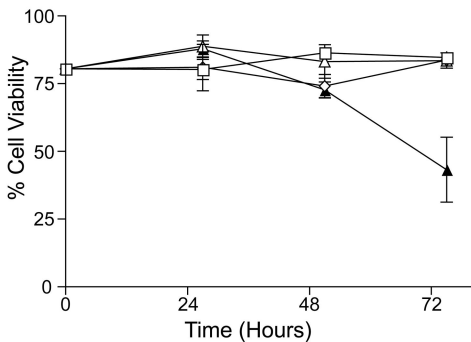
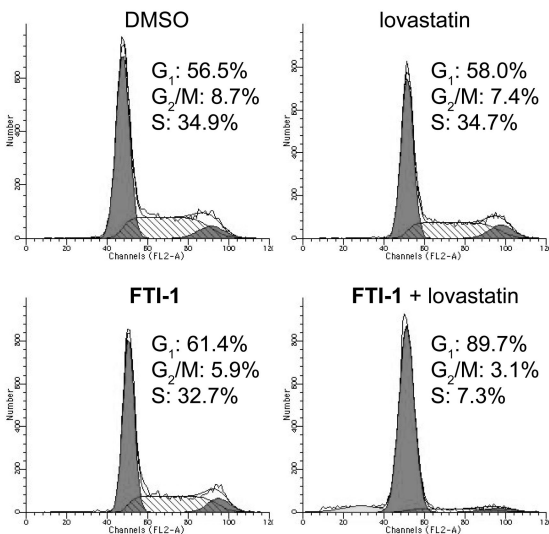
# Figure 1

## A



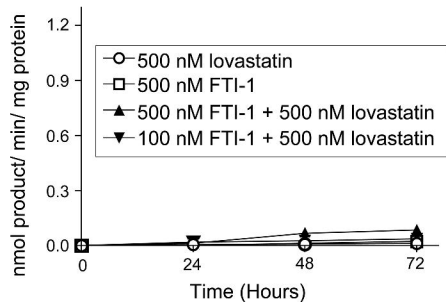
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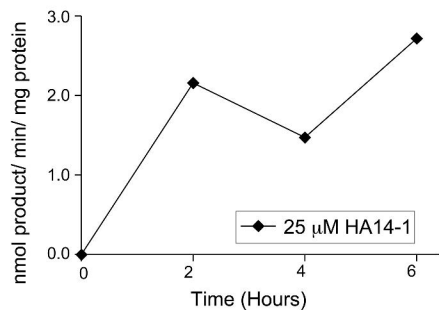
**Figure 2****A****B****C**

### Figure 3

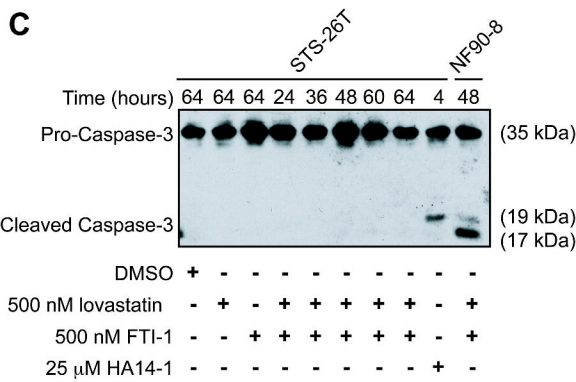
**A**



**B**

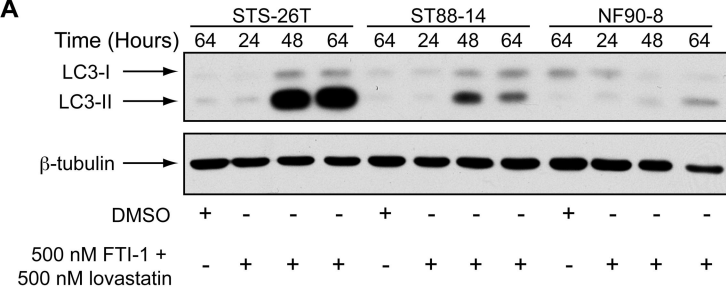


**C**

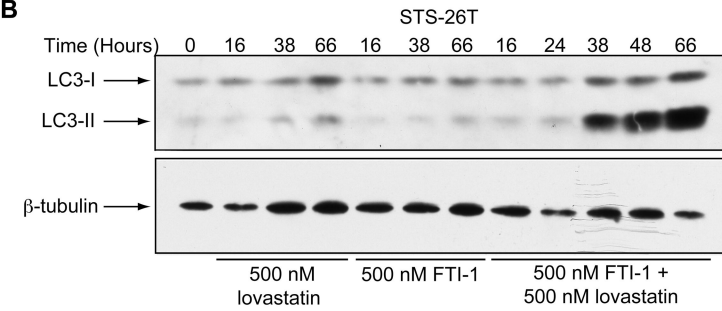


### Figure 4

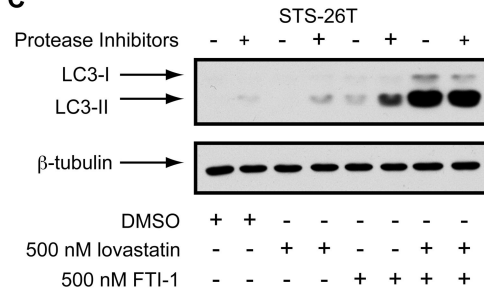
**A**



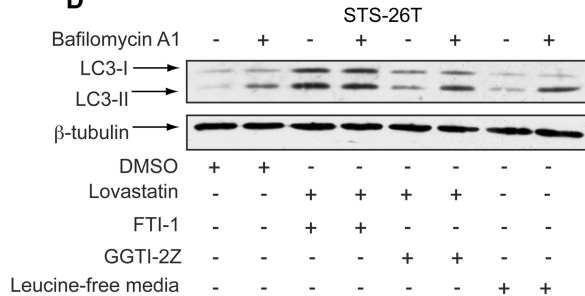
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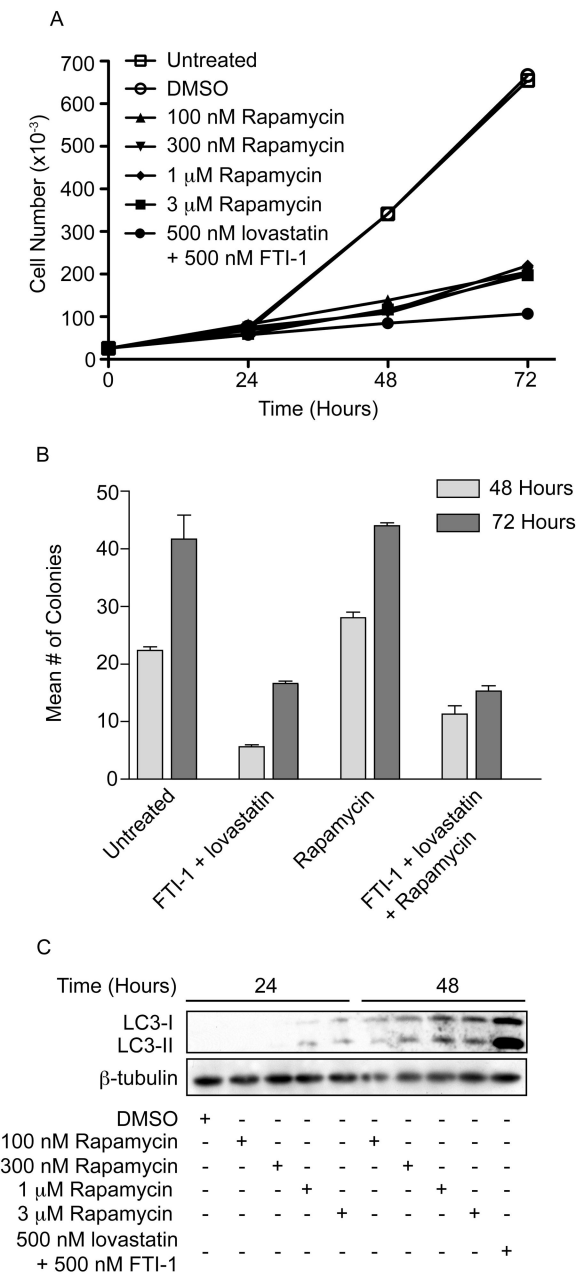
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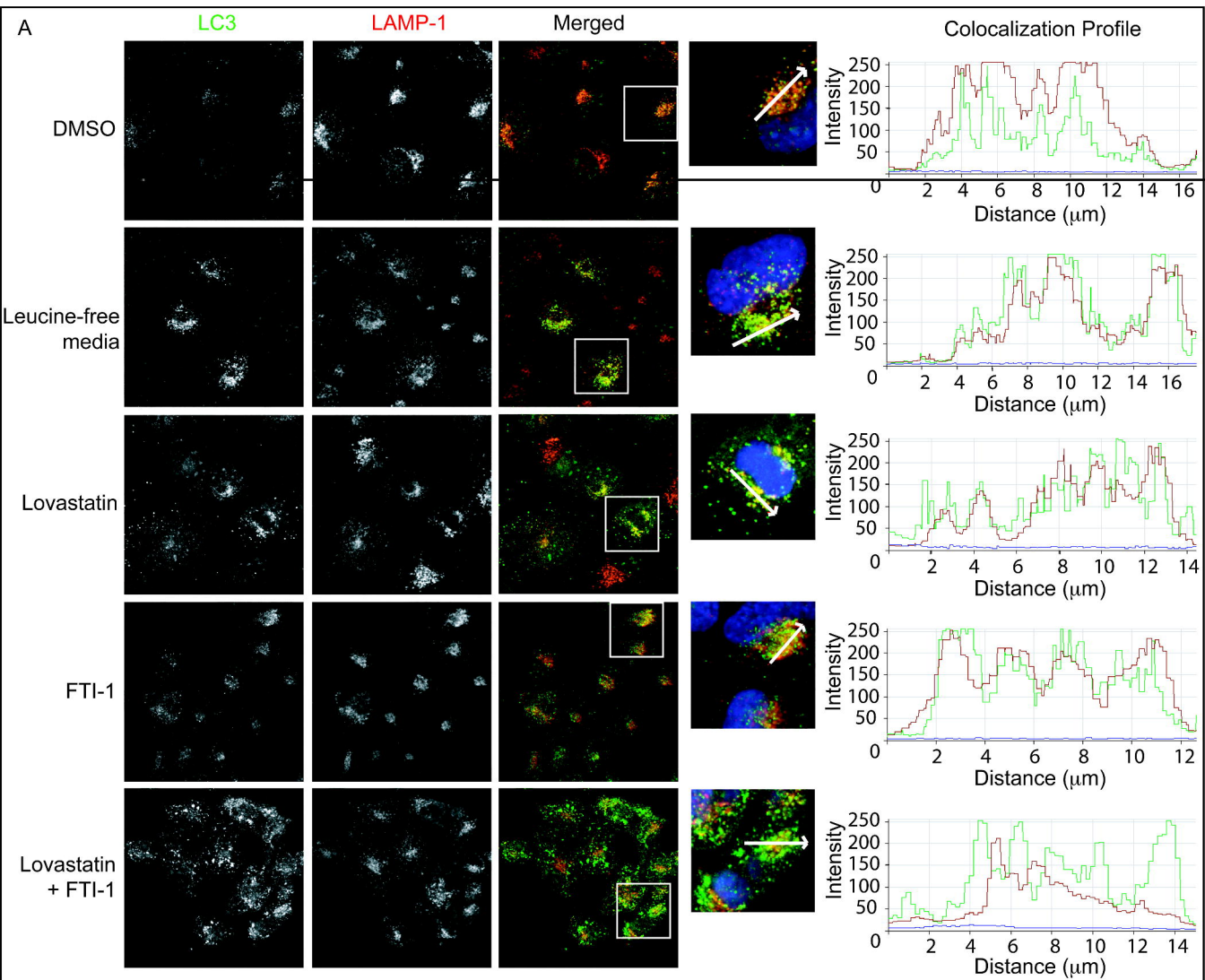
**D**



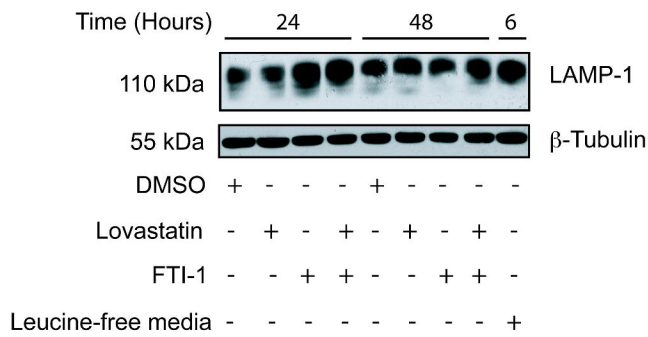
**Figure 5**



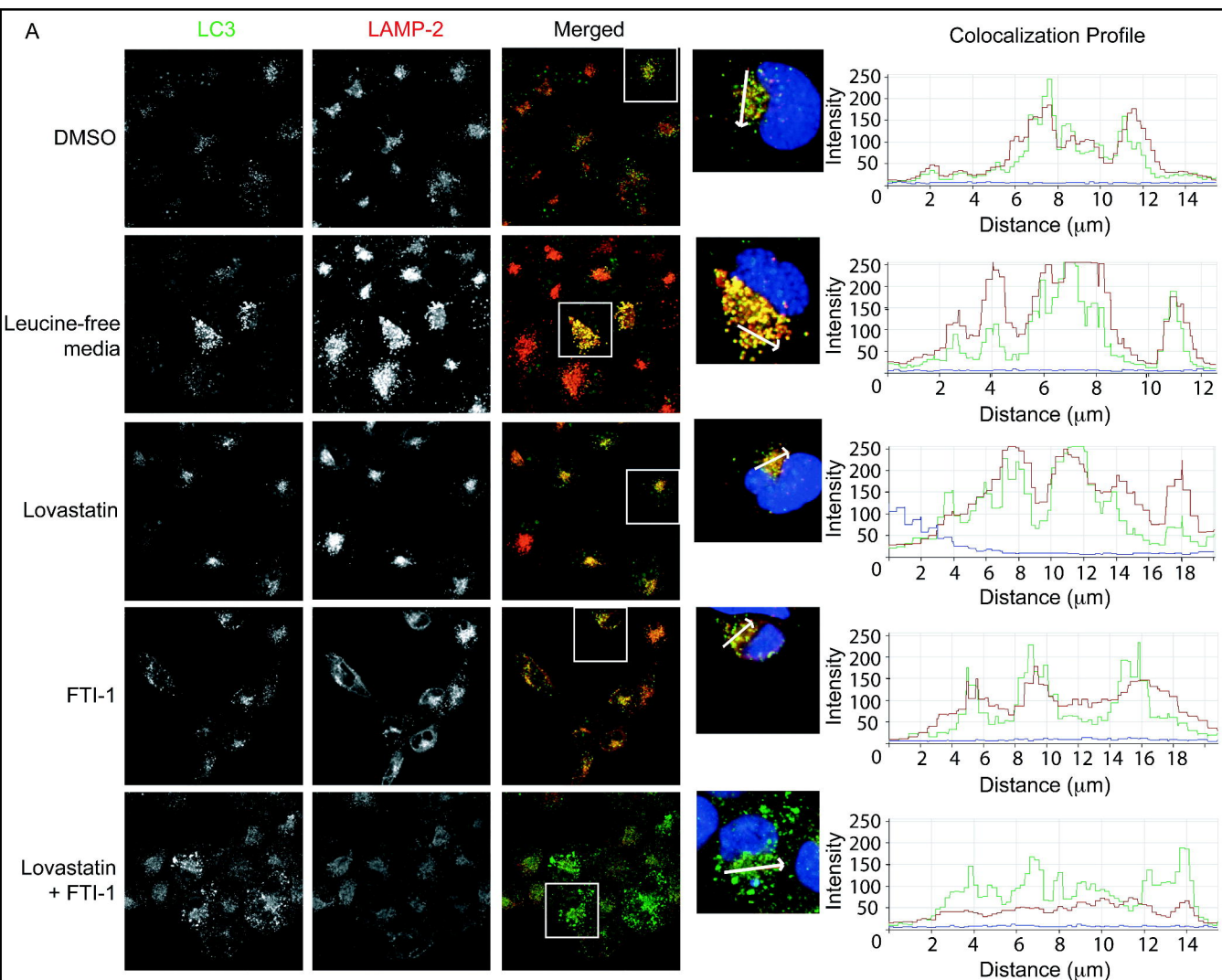
**Figure 6**



**B**



**Figure 7**



**B**

