

Fluvastatin Induces Apoptosis in Primary and Transformed Mast Cells

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Abbreviations

BMMC: bone marrow-derived mast cells

Di(OC₆)₃: 3,3'-Dihexyloxycarbonyl cyanine iodide

FPP: farnesyl pyrophosphate

GGPP: geranylgeranyl pyrophosphate

HMG CoA: hydroxymethylglutaryl co-enzyme A

HMGCR: HMG CoA reductase

LC3 II: light chain associated microtubule protein

MVA: mevalonic acid

PMC: peritoneal mast cell

ZA: Zaragozic acid A

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Abstract

Statin drugs are widely employed in the clinic to reduce serum cholesterol. Due to their HMG CoA reductase (HMGCR) antagonism, statins also reduce isoprenyl lipids necessary for the membrane anchorage and signaling of small G-proteins in the Ras superfamily. We previously found that statins suppress IgE-mediated mast cell activation, suggesting these drugs might be useful in treating allergic disease. While IgE-induced function is critical to allergic inflammation, mast cell proliferation and survival also impact atopic disease and mast cell neoplasia. In this study, we describe fluvastatin-mediated apoptosis in primary and transformed mast cells. An IC_{50} was achieved between 0.8-3.5 μ M in both cell types, concentrations similar to the reported fluvastatin serum C_{max} value. Apoptosis was correlated with reduced SCF-mediated signal transduction, mitochondrial dysfunction, and caspase activation. Complementing these data, we found that p53 deficiency or Bcl-2 overexpression reduced fluvastatin-induced apoptosis. We also noted evidence of cytoprotective autophagy in primary mast cells treated with fluvastatin. Finally, we found that intraperitoneal fluvastatin treatment reduced peritoneal mast cell numbers *in vivo*. These findings offer insight into the mechanisms of mast cell survival and support the possible utility of statins in mast cell-associated allergic and neoplastic diseases.

Significance Statement: Fluvastatin, a statin drug used to lower cholesterol, induces apoptosis in primary and transformed mast cells by antagonizing protein isoprenylation, effectively inhibiting SCF-induced survival signals. This drug may be an effective means of suppressing mast cell survival.

Introduction

Statin drugs were approved by the FDA in the early 1990's to combat the rising incidence of hypercholesterolemia in the United States. These drugs reduce serum cholesterol and low density lipoproteins, which have been linked to coronary heart disease and stroke (Khush and Waters, 2006). However, statins also have anti-inflammatory and anti-neoplastic effects that broaden their possible clinical utility. The intended pharmacological mechanism of statins is HMG-CoA reductase (HMGR) antagonism. HMGR is the rate-limiting step in cholesterol synthesis, yielding mevalonic acid. While mevalonic acid is processed to cholesterol, intermediate and side reactions in this cascade also generate the isoprenoids farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP). Isoprenylation, the process of conjugating FPP and GGPP to substrates, is required for subcellular localization of many proteins, particularly the Ras superfamily of small G-proteins. Since Ras members are critical to proliferation, migration, and cytokine production, there is great interest in determining if statins can be an efficacious therapy for inflammatory diseases in which G-protein pathways figure prominently (Takai et al., 2001; Zhang et al., 2013).

Epidemiological studies have noted that asthmatic patients prescribed statins experienced fewer emergency department visits for their allergic conditions as compared to their counterparts (Alexeeff et al., 2007; Huang et al., 2011; Wang et al., 2018). These reports prompted us to study statin effects on mast cells during the allergic response. Mast cell activation through the high affinity immunoglobulin E (IgE) receptor (FcεRI) is critical to allergic pathology. Antigen-mediated crosslinking of IgE bound to FcεRI induces release of preformed granules containing proteases and histamine, followed by secretion of arachidonic acid metabolites and cytokines. Collectively, these mediators induce cellular infiltration, vasodilation, and bronchoconstriction, leading to edema, dyspnea, tissue damage, and even systemic shock (Metcalf et al., 2009; Metcalf et al., 2016). We

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found that statins are powerful suppressors of IgE-mediated mast cell activation. Of the statins tested, fluvastatin was most effective in suppressing mast cell function *in vitro and in vivo* (Kolawole et al., 2016), with effects that could be tied to loss of GGPP (Fujimoto et al., 2009; Kolawole et al., 2016).

In addition to activation, mast cell numbers increase in allergic disease. This can be due to hyperplasia and recruitment of mast cell precursors (Elieh Ali Komi and Bjermer, 2019). Reducing mast cell numbers in diseases such as asthma and atopic dermatitis, in addition to inhibiting inflammatory function, could be a beneficial approach to treatment. Separate from their role in allergic disease, mast cells can form neoplasms. Aberrant mast cell proliferation ranges from slow-growing localized mastocytomas to systemic mastocytosis and aggressive mast cell leukemias. These have a very high incidence (>90%) of CD117 (c-Kit) D816V mutation that drives survival and proliferation (Castells and Butterfield, 2019). Mutant c-Kit is the target of clinical interventions but shows a high rate of drug-resistance (Jin et al., 2014). Therefore, therapies limiting D816V c-Kit signaling could be useful in mast cell neoplasias.

While short-term (<24 hour) fluvastatin exposure suppresses mast cell function, we noted that fluvastatin induces mast cell apoptosis within 4 days of culture. This report details the mechanism of fluvastatin-induced apoptosis, which we find occurs in both primary and transformed mast cells. We also noted evidence of cytoprotective autophagy that delays fluvastatin-mediated cell death. We provide *in vivo* support for these effects and offer mechanistic insights that support the possible clinical use of statins in mast cell-associated diseases.

Materials and Methods

Reagents and Cell Lines

Murine IL-3 and SCF were purchased from Shenandoah Biotechnology, Inc. (Warwick, PA).

Fluvastatin was purchased from Biovision, Inc. (Milpitas, CA). Mevalonic acid, SC66, bafilomycin A,

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chloroquine diphosphate and Akti-1/2 were purchased from Millipore Sigma (Burlington, MA). Geranylgeranyl pyrophosphate (GGPP) and farnesyl pyrophosphate (FPP) were purchased from Echelon Biosciences (Salt Lake City, UT). Zaragozic acid A (ZA), GGTI-286, and FTase II were purchased from Cayman Chemical (Ann Arbor, MI). FR180204, SCG772984, and raxoxertinib were purchased from Medchem Express LLC (Monmouth Junction, NJ). 10-DEBC HCl was purchased from Tocris Biosciences (Minneapolis, MN). The mast cells lines P815 and PDMC-1 were the kind gift of Dr. Thomas Huff (Virginia Commonwealth University). RBL-2H3 cells were purchased from ATCC (Rockville, MD)

Mice

C57BL/6J, 129/SvImJ, and p53 KO mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and bred in our facility. Bone marrow from H2K Bcl2-Tg mice was the kind gift of Kevin Bunting (Emory University). Mice were maintained at Virginia Commonwealth University (VCU) facilities in accordance with Institutional Animal Care and Use Committee guidelines and euthanasia criteria (IACUC).

Bone marrow-derived mast cell (BMMC) and peritoneal mast cell (PMC) cultures.

BMMC were derived from bone marrow extracted from femurs and tibias that was depleted of red blood cells and placed in complete RPMI (cRPMI) 1640 medium (Invitrogen Life Technologies, Carlsbad, CA) containing 10% heat-inactivated FBS, 2mM l-glutamine, 10 U/mL penicillin, 10 µg/mL streptomycin, 1mM sodium pyruvate, and 10mM HEPES (Biofluids, Rockville, MD), supplemented with SCF-containing supernatant from BHK-MKL cells and IL-3 from WEHI-3B cells for 21-28 days. Final concentrations of SCF and IL-3 were adjusted to 15 ng/mL and 5 ng/mL, respectively, as measured by ELISA. PMC were expanded from peritoneal lavage cells cultured as described (Caslin et al., 2018) using the same media as stated for BMMC, with selection against adherent cells for 7-14

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days. At the time of use, BMMC and PMC were >95% mast cells, based on dual staining for Fc ϵ RI and c-Kit, as assessed by flow cytometry.

Human mast cells

MC isolation from human skin was performed, as previously described (Kambe et al., 2001). Briefly, MCs were dispersed from the skin, enriched, and then cultured in X-VIVO 15 medium (Lonza, Walkersville, Md) containing recombinant human stem cell factor (100 ng/mL; Swedish Orphan Biovitrum, Stockholm, Sweden). Without other cytokines or serum, non-MC populations do not survive, leaving MCs of greater than 98% purity after approximately 3 weeks.

Flow Cytometric analysis

All flow cytometric analysis was done using a BD FACSCaliber or BD FACSCelesta (BD Biosciences, San Jose, CA). Cell surface staining was conducted using antibodies against the following mouse proteins: CD117 (c-KIT), CD25, CD2, CD16/CD32, (eBiosciences, Waltham, MA). For Propidium Iodide-DNA (PI-DNA) staining, cells ($1-2 \times 10^5$ per sample) were placed in 200 μ l Fixation Buffer (36 % ethanol, 12.8 % fetal calf serum, and 50.8 % 1X phosphate-buffered saline (PBS)) for at least 4 hours at 4°C, washed with PBS, and resuspended in 200 μ l Staining Buffer (PBS containing 100 μ g/ml RNase A and 50 μ g/ml PI) for 2 hours at room temperature in the dark. For staining with Di(OC $_6$) $_3$ (3,3'-Dihexyloxacarboncyanine iodide; Enzo Life Sciences, Farmingdale, NY), a final concentration of 1nM Di(OC $_6$) $_3$ was added to culture media for 30 minutes at 37°C in the dark prior to flow cytometry analysis. Caspase 7/9 activity DEVD assays were performed according to the manufacturer's instructions (Thermo Fisher, Waltham, MA).

Western Blotting:

Western blots were conducted using standard protocols. Nitrocellulose membrane (0.45 μ M) was purchased from Bio-Rad (Hercules, CA). The following primary antibodies were used at a 1:1000

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dilution of the stock concentration: LC3I/II, p62, GAPDH, ERK1/2, phospho-ERK1/2, AKT, phospho-AKTser473, p85, phospho-p85 (Cell Signaling, Danvers, MO). Western blot membranes were blocked with TBS-bovine serum albumin (BSA) and incubated with primary antibodies for 1-3 days in TBS/0.1% Tween (TBS-T 0.1%) and (0.5% BSA Blocker™), all from Thermo Fisher (Waltham, MA). Western blots were analyzed using infrared-conjugated secondary antibodies on an Odyssey Imaging System (LI-COR, Lincoln, NE).

Fluvastatin use *in vivo*

Female C57BL/6J mice received 10mg/kg fluvastatin or vehicle (DMSO diluted in PBS to match fluvastatin solvent) daily by intraperitoneal injection for 4 days. Mice were sacrificed and peritoneal lavage was harvested and analyzed for mast cells by staining for CD45+ cells and gating on FcεRI+/c-Kit+ cells. Cross sections of small intestine were also analyzed by histology, using staining with pinacyanol erythrosinate to identify mast cells.

Statistics

Data shown in each figure are the mean ± standard deviations (SD). Graphical and statistical analyses were conducted on Graph Pad-Prism 7. For comparisons of two samples, a Student's t-Test was applied unless otherwise stated. ANOVA analysis with Bonferroni post-hoc testing was used to compare 3 or more sample sets. Significance was assigned as follows: *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001.

Results

Fluvastatin induces mast cell apoptosis.

While investigating the ability of fluvastatin to suppress IgE-mediated mast cell activation, we found that apoptosis occurred after culturing cells with the drug for more than 2 days. Figure 1A shows that approximately 60% of C57BL/6J BMDC were apoptotic after 4 days of culture in 1μM fluvastatin. A

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dose response of fluvastatin was conducted at 96-hours, which found that nearly all cells exhibited DNA fragmentation at concentrations of 5 μ M or greater (Figure 1B). In addition to DNA fragmentation assessed by PI-DNA staining, we also noted a significant increase in caspase-3/7 activation (Figure 1C).

Because we previously found that mast cells from the 129S1/SvImJ background were fluvastatin-resistant (Kolawole et al., 2016), we compared the IC₅₀ for apoptosis of BMMC from this strain to C57BL/6J. As shown in Figure 1D, C57BL/6J BMMC exhibited an IC₅₀ of 0.8 μ M, while 129S1/SvImJ BMMC were 3-fold more resistant. Both values are within the 2.5 μ M serum C_{max} of fluvastatin in patients taking the drug (Siekmeier et al., 2001). By comparison, atorvastatin and simvastatin induced C57BL/6 BMMC apoptosis at higher concentrations of 20 μ M and 6.5 μ M, respectively (Supplemental Figure 1). Interestingly, BMMC treated with fluvastatin undergo apoptosis with no discernable cell cycle arrest, even in the 24 hours preceding detectable apoptosis. Instead, all phases of the cell cycle were reduced by fluvastatin, with only the apoptotic fraction increasing (Supplemental Figure 2).

We corroborated the BMMC data with similar testing of C57BL/6J PMC. These cultures showed a greater sensitivity to fluvastatin than BMMC, with an IC₅₀ of approximately 0.2 μ M and 90% apoptosis at a 0.5 μ M dose (Figure 1E). Further, human skin-derived mast cells from 3 donors also showed varying degrees of apoptosis after 4 days of fluvastatin treatment (Figure 1F). Human mast cells appeared to be more resistant than BMMC or PMC, with consistent apoptosis detected at 10 μ M fluvastatin. These data support the conclusion that fluvastatin induces apoptosis of primary mouse and human mast cells.

Fluvastatin-induced apoptosis targets the geranylgeranyl lipid pathway.

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A simplified depiction of cholesterol and isoprenoid biosynthesis is shown in Figure 2A. To determine the mechanism by which fluvastatin induces apoptosis, we first examined the effect of cholesterol blockade with zaragozic acid A (ZA), which antagonizes squalene synthase, a reaction downstream from the HMGCR statin target and proximal to cholesterol. As shown in Figure 2B, ZA treatment had no effect on BMMC survival and did not enhance fluvastatin-induced cell death. By contrast, adding mevalonic acid (MVA), the HMGCR product, reversed the effects of fluvastatin (Figure 2C). This indicates that fluvastatin-induced death is not an off-target drug effect. The 15-carbon isoprenoid lipid farnesyl pyrophosphate (FPP) was unable to reverse apoptosis, but the 20-carbon geranylgeranyl pyrophosphate (GGPP) provided significant protection from fluvastatin effects (Figure 2C). These data were complemented by using farnesyl transferase (FT) or geranylgeranyl transferase (GGT) inhibitors (Figure 2D). While FT inhibition had no effect, GGT blockade mimicked fluvastatin effects. Collectively, these data support the hypothesis that fluvastatin-induced apoptosis results from HMGCR blockade that reduces downstream geranylgeranyl pyrophosphate.

Fluvastatin Decreases SCF-mediated Survival Signals in BMMC.

Isoprenoid lipids such as GGPP are critical for subcellular localization of small G proteins in the Ras superfamily, which control activation of MAPK and other pathways (Wang and Casey, 2016). Hence we suspected that statin-mediated GGPP reduction induced apoptosis by inhibiting growth factor signals. Since c-Kit function is critical for primary and neoplastic mast cell survival, we investigated how fluvastatin affects c-Kit signaling. BMMC were cultured for 24 hours in cRPMI supplemented with SCF and IL-3 at 10 ng/mL, plus either vehicle (DMSO) or fluvastatin (1 μ M). Cells were then starved of IL-3+SCF for four hours prior to SCF re-stimulation. Western blots for phosphorylated (p)ERK1/2 and pAKT were performed. A representative blot and quantification of pERK1/2: total ERK1/2 ratio, measured as fold increase from the unstimulated group, showed fluvastatin significantly reduced ERK

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phosphorylation (Figures 3 A,B). By comparison, SCF-mediated AKT phosphorylation was unaffected. To further test the correlation of decreased ERK activity with apoptosis, we determined the effect of ERK blockade. As shown in Figure 3C, three ERK inhibitors mirrored fluvastatin effects, inducing BMMC apoptosis. These data support the theory that fluvastatin effects on GGPP suppress the c-Kit-Ras-ERK cascade, resulting in mast cell apoptosis.

Apoptosis is dependent on p53 expression and mitochondrial stability

Loss of c-Kit signaling can elicit p53 expression, which has been shown to occur in neoplastic mast cells expressing oncogenic c-Kit (Dos Santos et al., 2013; Bandara et al., 2018). Therefore, we measured fluvastatin-induced apoptosis in p53 “knockout” (KO) BMMC and their background- and age-matched wild type (WT; C57BL/6) counterparts. BMMC were cultured in either vehicle (DMSO) or fluvastatin (10 μ M) in cRPMI with IL-3 and SCF at 10 ng/mL over a 72-hour time course. We found that p53 KO BMMC demonstrated significantly less fluvastatin-induced apoptosis than WT cells (Figure 4A). Since p53 activation can induce mitochondrial damage, and statins have been shown to induce mitochondrial dysfunction (Ramachandran and Wierzbicki, 2017), we determined the effect of overexpressing the anti-apoptotic mitochondrial protein Bcl-2. We cultured age- and strain-matched Bcl-2 transgenic and C57BL/6 WT BMMC with fluvastatin over a 72-hour time course and found that Bcl-2 overexpression significantly protected mast cells from apoptosis (Figure 4B). These results indicate a role for the p53-mitochondrial pathway in fluvastatin-mediated apoptosis, perhaps due to decreased c-Kit survival signals that can be compensated by overexpressing Bcl-2.

Fluvastatin induces cytoprotective autophagy

In addition to apoptosis, statins or growth factor blockade can induce autophagy (Parikh et al., 2010; Zhang et al., 2013). One measure of autophagy is the accumulation of cleaved light chain associated microtubule protein (LC3 II) and degradation of ubiquitin-binding protein p62, as detected by Western

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blotting. Because mast cells undergo constitutive autophagy (Ushio et al., 2011), we used chloroquine to raise lysosomal pH and inhibit LC3 II and p62 degradation during autophagy. Autophagy inhibition alone and in combination with fluvastatin treatment demonstrated changes from baseline autophagy, via measurement of LC3 II and p62 accumulation. As shown in Figures 5A and 5B, fluvastatin significantly increased the accumulation of LC3 II and p62 in the presence of chloroquine. We also noted a modest increase in LC3 I in the presence of fluvastatin ($p < .05$), although the importance of this change is unclear. Thus fluvastatin appears to increase mast cell autophagy. To determine how autophagy affects fluvastatin-induced apoptosis, we blocked autophagy with chloroquine or bafilomycin A and measured sub-diploid DNA content. BMDC treated for 48 hours with fluvastatin showed modest but significant cell death at this early time point that was greatly enhanced by chloroquine or bafilomycin A (Figure 5C). These data suggest that fluvastatin induces cytoprotective autophagy that delays mast cell apoptosis.

Fluvastatin suppresses mast cell survival *in vivo*

To examine fluvastatin effects on mast cell survival *in vivo*, mice were injected with fluvastatin (10mg/kg) or vehicle (DMSO diluted in PBS) daily for 4 days, via intraperitoneal route. As shown in Figure 6, fluvastatin did not reduce total peritoneal cells, but greatly reduced the proportion and total number of mast cells found in peritoneal lavage. Because we have previously found fluvastatin inhibits mast cell migration to factors such as SCF (Kolawole et al., 2016), it seems unlikely that this loss of mast cells is due to emigration to other sites. For example, we noted no increase in small intestine mast cell numbers (not shown). These data support the hypothesis that fluvastatin suppresses mast cell survival *in vivo*.

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Fluvastatin induces apoptosis in transformed mast cells.

Mastocytomas have a high incidence of c-Kit mutation that promotes their proliferation and survival (Carter et al., 2014). Because we found that fluvastatin inhibits c-Kit signaling in BMMC, we next determined if these effects extended to neoplastic mast cells. Mouse PDMC-1 and P815 and rat RBL-2H3 mastocytoma cells were cultured in vehicle (DMSO) or fluvastatin at a range of concentrations for 96 hours and analyzed with PI-DNA staining. This yielded IC₅₀ values of 1.5-3.5 μM, with nearly all cells killed above the 7 μM dose (Figure 7A and data not shown). Due to their intermediate IC₅₀ and ease of culture, we continued analyzing the P815 cell line.

Unlike BMMC, P815 cells showed a significant loss of S phase and a G2 cell cycle arrest at the 24-hour time point preceding apoptosis (Figure 7B). Because fluvastatin-induced apoptosis was suppressed by Bcl-2 overexpression in BMMC, we investigated the effect of fluvastatin on mitochondrial stability in P815 cells. Cells were cultured for 48 hours in DMSO or fluvastatin and stained with Di(OC₆)₃, a dye with high affinity for the mitochondria. Decreased staining indicates loss of the trapped dye, consistent with mitochondrial membrane damage. P815 cells treated with fluvastatin exhibited diminished Di(OC₆)₃ staining compared to vehicle-treated cells (Figure 7C,D). Given this indication of mitochondrial damage, we next assessed the presence of active caspase 9, which can result from mitochondrial membrane instability. P815 cells treated for 24 hours with fluvastatin had increased active caspase 9 activity, as measured using a cleavable fluorescent caspase-9 peptide (Figure 7E). These data further support the theory that fluvastatin induces apoptotic cell death via a mitochondrial pathway.

We also examined changes in c-Kit expression and signaling on P815 mastocytoma cells. When gating on live cells, we found that c-Kit surface expression increased from 12-48 hours of culture in fluvastatin (Figure 8A). In contrast to increased c-Kit levels, constitutive phosphorylation of

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ERK and Akt was greatly diminished by fluvastatin treatment (Figure 8B). To link these changes to survival, we tested the effects of ERK or Akt inhibitors. While one of three ERK inhibitors induced P815 apoptosis when used at 5-10 μ M concentration, all three Akt inhibitors elicited significant cell death. These data support the theory that fluvastatin-induced apoptosis is linked to loss of survival signals provided by constitutively active c-Kit in mastocytoma cells, with particular importance for the Akt pathway. P815 cells appear to transiently increase c-Kit expression, perhaps as a compensatory mechanism that delays cell death.

Discussion

Mast cells primarily defend against bacterial and parasitic infections. But in allergic disease, environmental antigens elicit pathological mast cell activation when antigen aggregates receptor-bound IgE. There is an early and a late phase of mast cell activation that collectively cause vasodilation, capillary leakage, and recruitment and activation of other immune cells. The early phase of activation occurs when pre-formed granules containing histamine and proteases are released, causing increased vascular permeability and vasodilation. The late phase follows 4-6 hours later, elicited by cytokine and chemokine production (Metcalf et al., 2009; Metcalf et al., 2016). In addition to cell activation, mast cell hyperplasia occurs in allergic and neoplastic diseases (Elieh Ali Komi and Bjermer, 2019), hence reducing mast cell numbers is an important clinical goal.

Several studies have shown correlations between statin therapy and reduced emergency department visits by asthmatic/allergic patients (Alexeeff et al., 2007; Huang et al., 2011; Wang et al., 2018). HMGCR antagonism by statins reduces farnesyl and geranylgeranyl pyrophosphate, isoprenoid lipids that are coupled to Ras superfamily proteins (Wang and Casey, 2016). The loss of isoprenylation yields small G-proteins without membrane anchorage, diminishing signal transduction downstream of ligand-receptor interaction. Mechanisms possibly explaining statin anti-allergic effects include our recent demonstration that fluvastatin decreases MAPK and AKT signaling pathways activated by IgE crosslinking (Kolawole et al., 2016). In longer time courses, we noted that a significant proportion of BMNC died when treated with statins. While atorvastatin and simvastatin induced significant apoptosis, they did so at relatively high concentrations, hence we focused on fluvastatin. This was in keeping with our previous study of statin-mediated suppression of IgE signaling, in which fluvastatin was the most consistently effective of the statins tested (Kolawole et al.,

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2016). Because most patients use statins for long term therapy, statin-induced apoptosis may be clinically relevant. This possibility prompted our studies of apoptosis and autophagy.

This work shows that primary mast cells treated with fluvastatin undergo a p53-dependent apoptosis, but with no discernible cell cycle arrest. We noted selective loss of c-Kit-mediated signaling, with significant ERK blockade. This correlation between reduced ERK activation and apoptosis is supported by the literature. MAPK proteins are involved in labeling pro-apoptotic (BH3 only) proteins such as Bim for proteasome degradation in mast cells (Moller et al., 2005; Karlberg et al., 2010; Westerberg et al., 2012). Thus, decreased ERK/MAPK function is a plausible mechanism for apoptosis that should be overcome by Bcl-2 overexpression, as we found in Figure 4. We therefore propose that fluvastatin-induced apoptosis is driven by loss of survival signals from c-Kit and perhaps other growth factor receptors.

The timing of apoptosis, which peaked at 4 days of fluvastatin treatment, appears to be due in part to cytoprotective autophagy. Blocking autophagy accelerated fluvastatin-mediated cell death, suggesting that autophagy delays apoptosis. Interestingly, a recent study found that mast cells have a constant autophagic flux necessary for granule maintenance (Ushio et al., 2011), but how autophagy affects mast cell survival is poorly understood. Statins have been shown to alter autophagy and cause mitochondrial dysfunction (Parikh et al., 2010; Zhang et al., 2013). An apparent role for the mitochondrion, as evidenced by data from p53 KO and Bcl-2 Tg BMMC, offers a potential link to a form of autophagy called mitophagy. In this model, autophagy blockade causes the accumulation of damaged mitochondria that release reactive oxygen species (ROS) and have decreased oxidative phosphorylation (Youle and Narendra, 2011; Roberts et al., 2016; Springer and Macleod, 2016). This combination of poor functioning and increased ROS accelerates apoptosis, similar to the outcome we

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found. While our data are consistent with mitophagy, further study is needed to determine if this is the specific form of autophagy occurring.

Fluvastatin injections for 4 days greatly reduced peritoneal mast cell numbers without significant change in the total peritoneal population. Because mast cells comprise 1-3% of peritoneal cells (Morii et al., 2004), these data suggest that either mast cells are particularly sensitive to fluvastatin-mediated death or perhaps other cell types are repopulated more quickly. Because we previously found that fluvastatin inhibits mast cell migration (Kolawole et al., 2016), it is unlikely that drug treatment caused significant emigration out of the peritoneum. Fluvastatin has been shown to reduce adhesion molecule expression on endothelial cells and monocytes (Xenos et al., 2005) and to suppress eosinophil and leukocyte adhesion (Niwa et al., 1996; Fischetti et al., 2004; Robinson et al., 2009). Therefore, it is unlikely that fluvastatin selectively induced mast cell adhesion leading to reduced recovery in peritoneal lavage fluid. These data support the theory that fluvastatin may reduce mast cell numbers *in vivo*, an effect with clinical utility in both atopic and neoplastic conditions that warrants further investigation.

Data from primary cells prompted interest in statin effects on transformed mast cells. The P815 model used in our study conforms to several aspects of the mast cell leukemia diagnosis, including expression of the mutant c-Kit receptor, which causes constitutive growth factor-independent proliferation (Tsujimura et al., 1994). There has been considerable research employing statin therapy with various cancer types, including those with mutant K-Ras or B-Raf (Zhang et al., 2013). In particular, fluvastatin has been shown to effective against myeloid leukemias *in vitro* and *in vivo* (Dai et al., 2007; Sassano et al., 2007; Calabro et al., 2008; Williams et al., 2012; Henslee and Steele, 2018).

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The mechanism of fluvastatin-induced apoptosis in P815 cells was similar to primary cells, with indications of mitochondrial damage. But unlike primary cells, we noted a rapid increase in surface c-Kit expression following fluvastatin exposure. Because mutant c-Kit activates MAPK, Akt, and other survival signals (Agarwal et al., 2015), it was surprising that even with a 2-fold increase in c-Kit expression, ERK and AKT activation was consistently suppressed by fluvastatin. We used 3 different ERK and AKT inhibitors to test the importance of these pathways. While ERK blockade consistently induced BMMC apoptosis, only 1 of the 3 inhibitors induced P815 cell death under the same conditions. By contrast, all 3 Akt inhibitors elicited P815 apoptosis. These results suggest that Akt blockade may be a potent death-inducing fluvastatin effect in transformed mast cells, while ERK suppression may explain death in primary mast cells. While we do not fully understand the divergence of Kit-mediated signaling in primary and transformed mast cells, these data suggest that Akt blockade may be effective against mastocytomas.

P815 cells also demonstrated a transient G2/M arrest coupled with S phase reduction. These effects were distinct from primary mast cells, which showed no cell cycle changes preceding apoptosis. G2/M cell cycle arrest has been documented in transformed human mastocytomas by others, and may be a facet of their neoplastic phenotype. For example, Bandara et al. found G2 accumulation when sphingosine kinase 1 was inhibited (Bandara et al., 2018). Peter and co-workers noted similar effects with the multi-kinase inhibitor R763, which coincided with increased cell killing by midostaurin or dasatinib (Peter et al., 2018). Therefore, statins, which have an established history of safe use, could be considered as an adjuvant therapy for mast cell neoplasias.

In summary, we find that fluvastatin induces apoptosis in primary and transformed mast cells, likely due to a loss of c-Kit-mediated survival signals. Primary mast cells demonstrate a transient autophagy that may delay apoptosis, while transformed mast cells enter a G2/M cell cycle arrest

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preceding cell death. Because fluvastatin effects mapped to the geranylgeranyl pyrophosphate pathway involved in Ras family protein localization, our hypothesis is that statins reduce Ras family protein access or function, with subsequent loss of downstream survival signals from ERK and Akt. The identity of the affected Ras family proteins warrants further study. For example, c-Kit is a potent activator of the KRAS-PI3K-Akt pathway, which requires KRAS geranylgeranylation (Castellano and Downward, 2011; Akula et al., 2016). While we observed loss of Akt activation in P815 cells, fluvastatin did not suppress c-Kit-mediated Akt phosphorylation in BMMC. This suggests variation in the critical isoprenylation targets. Alongside studies showing reduced allergic disease among statin users (Alexeeff et al., 2007; Huang et al., 2011; Wang et al., 2018), our data support further study of statins and the isoprenoid pathway as a means to suppress mast cell survival where this is clinically beneficial.

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Authorship:

Participated in research design: Paez, Kolawole, Straus, Martin, Gewirtz, Schwartz, and Ryan.

Conducted experiments: Paez, Kolawole, Taruselli, Ajith, Dailey, Kee, Haque, Barnstein, McLeod, Caslin, Kiwanuka, Straus, Martin, Fukuoka, and Le.

Contributed new reagents or analytical tools: None

Performed data analysis: All authors

Wrote or contributed to the writing of the manuscript: All authors

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Footnotes:

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Figure Legends

Figure 1: Fluvastatin induces apoptosis in mouse BMMC and PMC. (A) C57BL/6 BMMC were cultured for the indicated times in 1 μ M fluvastatin and apoptosis was assessed by PI-DNA staining of sub-diploid DNA, as measured by flow cytometry. P values were calculated by comparing fluvastatin vs DMSO paired time points by 2-way ANOVA. **(B)** BMMC were cultured for 4 days in the indicated fluvastatin concentrations. Apoptosis was measured by PI-DNA staining. P values were calculated by comparing fluvastatin to the DMSO control by 2-way ANOVA. **(C)** BMMC were cultured as in (A) for 4 days and Caspase-3/7 activity was assessed by cleavage of fluorescent substrate and flow cytometry. **(D)** BMMC from the indicated strains were cultured in increasing concentrations of fluvastatin for 4 days, and IC₅₀ values were determined. **(E)** Primary PMC were cultured for 4 days in the indicated fluvastatin concentrations and apoptosis was assessed by PI-DNA staining. **(F)** Human skin-derived mast cells were cultured for 4 days in the indicated concentrations of fluvastatin, and apoptosis was assessed by PI-DNA staining. Concentrations of “0” indicate DMSO controls in each figure, which were matched to the highest DMSO concentration present in fluvastatin-treated cells. Data shown for all figures are from 6 samples/point from 1 of at least 3 experiments. ****p<.0001 by ANOVA.

Figure 2: Fluvastatin-induced apoptosis is linked to the geranylgeranyl pyrophosphate (GGPP) pathway. (A) Simplified version of cholesterol and isoprenoid synthesis pathways, with inhibitors indicated in grey font. **(B)** BMMC were cultured for 4 days with DMSO, fluvastatin, or zaragozic acid (ZA, 10 μ M). Apoptosis was assessed by PI-DNA staining and flow cytometry. **(C)** BMMC were cultured for 4 days with fluvastatin +/- mevalonic acid (MVA, 1mM), GGPP, or FPP (10 μ M each). Apoptosis was assessed as in (A). **(D)** BMMC were cultured for 4 days with DMSO, fluvastatin, farnesyl transferase inhibitor (Ftase II, 10 μ M), or GGT inhibitor (GGTI-286, 20 μ M). Apoptosis was

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assessed as in (A). Data shown are from 12 samples/point (A-B) or 9 samples/point (C), from 1 of at least 2 experiments that gave similar results. ** $p < .01$; **** $p < .0001$; NS not significant when comparing samples to fluvastatin (FLU) or DMSO controls as indicated, using one-way ANOVA.

Figure 3. Fluvastatin inhibits SCF-mediated ERK phosphorylation. (A and B) BMMC were cultured for 24 hours with DMSO or fluvastatin ($1\mu\text{M}$) prior to stimulation with 200ng/ml SCF for the indicated times. Image in (A) shows example Western blot data; (B) shows results from 3 populations. (C) BMMC were cultured for 4 days with fluvastatin ($2.5\mu\text{M}$) or the indicated ERK inhibitors ($10\mu\text{M}$, $10\mu\text{M}$, and $5\mu\text{M}$, respectively). Data are from 3 populations. **** $p < .0001$ by ANOVA.

Figure 4. Fluvastatin induces Bcl-2- and p53-dependent apoptosis. BMMC from C57BL/6, H-2^k-Bcl2 Tg, or p53 KO mice were cultured with Fluvastatin ($10\mu\text{M}$) for the indicated times. Apoptosis was determined by sub-diploid DNA content measured by PI-DNA stain using flow cytometry. Data shown are mean +/- SD from 6-9 samples from 1 of two separate experiments. Some error bars are too small to be shown. ** $p < .01$; **** $p < .0001$ by ANOVA.

Figure 5. Fluvastatin induces LC3 II and p62 accumulation in BMMC. (A) Example Western blot of BMMC cultured for 24 hours with DMSO or Fluvastatin ($10\mu\text{M}$) +/- chloroquine (CQ), used to enhance LC3 II and p62 detection. (B) Summary data from three BMMC populations treated as in (A) using 5 or $10\mu\text{M}$ fluvastatin compared to matched DMSO samples. * $p < .05$ by paired t-Test comparing each fluvastatin-treated sample to the DMSO control. (C) C57BL/6 BMMC were cultured with vehicle (DMSO) or fluvastatin (Fluva) +/- chloroquine or bafilomycin A (BafA) for 48-hours and apoptosis was measured by PI-DNA staining and flow cytometry. Data shown are from 6-9 samples/point.

**** $p < .0001$ by ANOVA.

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Figure 6. Fluvastatin reduces peritoneal mast cell number *in vivo*. C57BL/6 mice (4-6/group) were injected i.p. with 10mg/kg fluvastatin once daily for 4 days. Peritoneal lavage fluid was analyzed by flow cytometry to identify mast cells (FcεRI+, c-Kit+). *p<.05; ****p<.0001 by t-test.

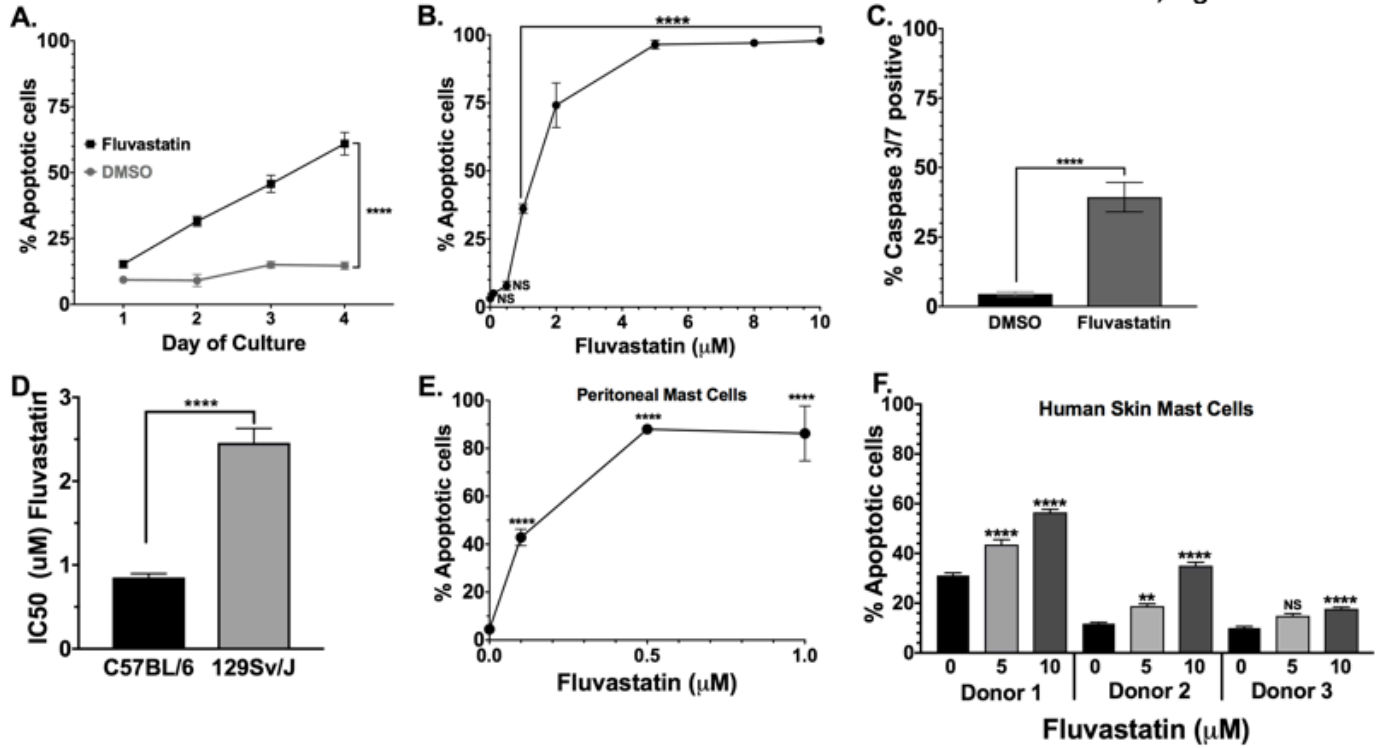
Figure 7. Fluvastatin induces apoptosis in transformed mast cells. (A) The indicated mastocytoma cell lines were cultured for 4 days in fluvastatin and IC₅₀ values were calculated as in Figure 1. Data are mean and SD of 8 samples, with p values calculated by one-way ANOVA. **(B)** P815 cells were cultured for 24 hours in fluvastatin (10μM) and cell cycle was measured using PI-DNA staining and flow cytometry. Data are mean and SD of 7-9 samples from 1 of 3 representative experiments, with p values calculated by t-Test. **(C and D)** Representative histogram and summary data of P815 cells stained with Di(OC₆)₃ after 48 hours of culture with fluvastatin (10μM). (D) shows mean and SD of 6 samples, with p value calculated by t-Test. **(E)** P815 cells were cultured for 48 hours and Caspase-9 activity was assessed by cleavage of fluorescent substrate and flow cytometry. Data are mean and SD of 12 samples from one of two representative experiments, with p value calculated by t-Test.

Figure 8. Fluvastatin increases c-Kit expression but inhibits signaling in mastocytoma cells.

(A) P815 cells were cultured for the indicated times in DMSO or fluvastatin, and c-Kit expression was measured by flow cytometry. (B) P815 cells were cultured for 24 hours in DMSO or fluvastatin (10μM) and lysates were probed for phosphorylated (p) and total ERK or AKT expression by Western blot. Bar charts show summary data from 6-7 samples/point. (C) P815 cells were cultured for 4 days with DMSO, fluvastatin (10μM), or the indicated ERK (10μM, 5μM, 10μM, respectively) or AKT (10μM) inhibitors, and apoptosis was measured by PI-DNA staining and flow cytometry **p<.01; ***p<.001; ****p<.0001 by ANOVA (A and C) or t-Test (B).

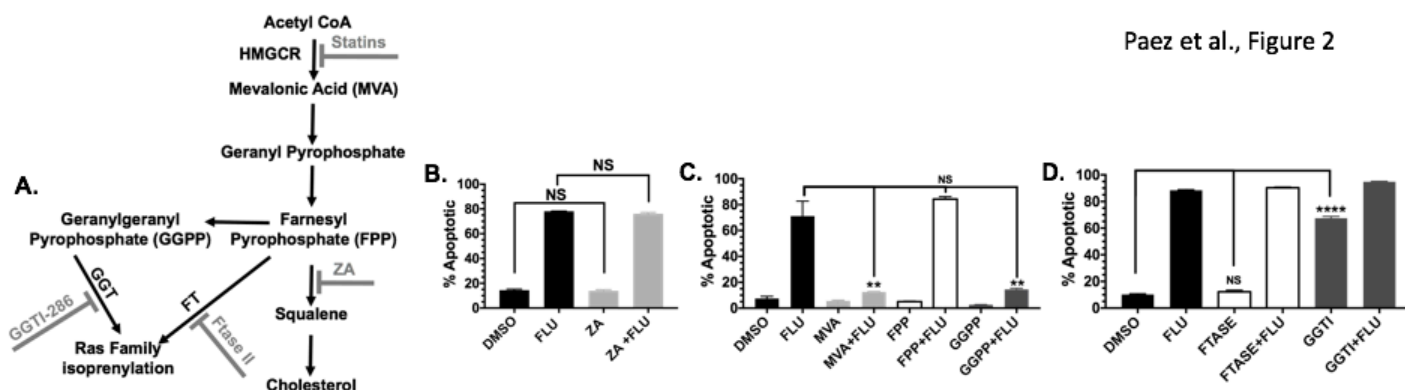
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Paez et al., Figure 1

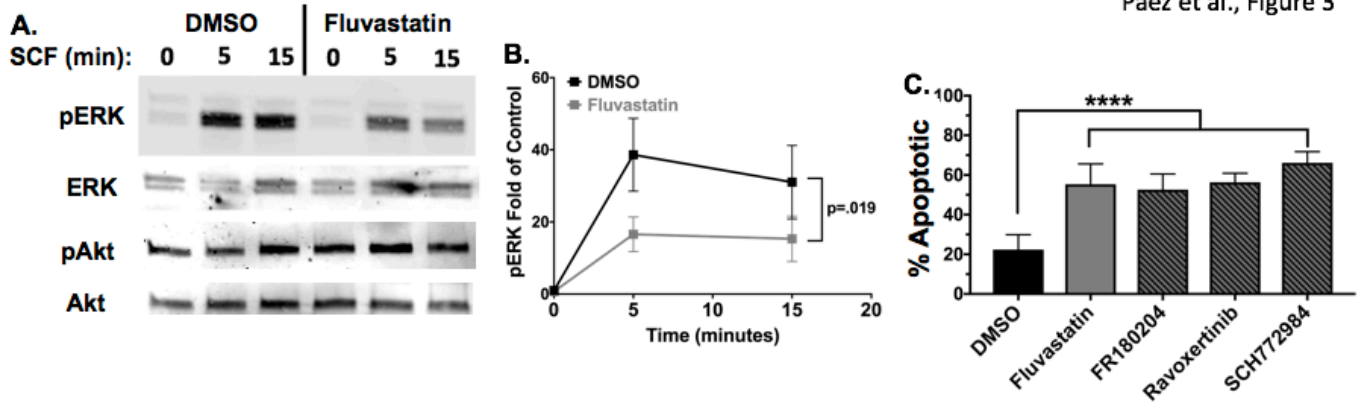


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Paez et al., Figure 2

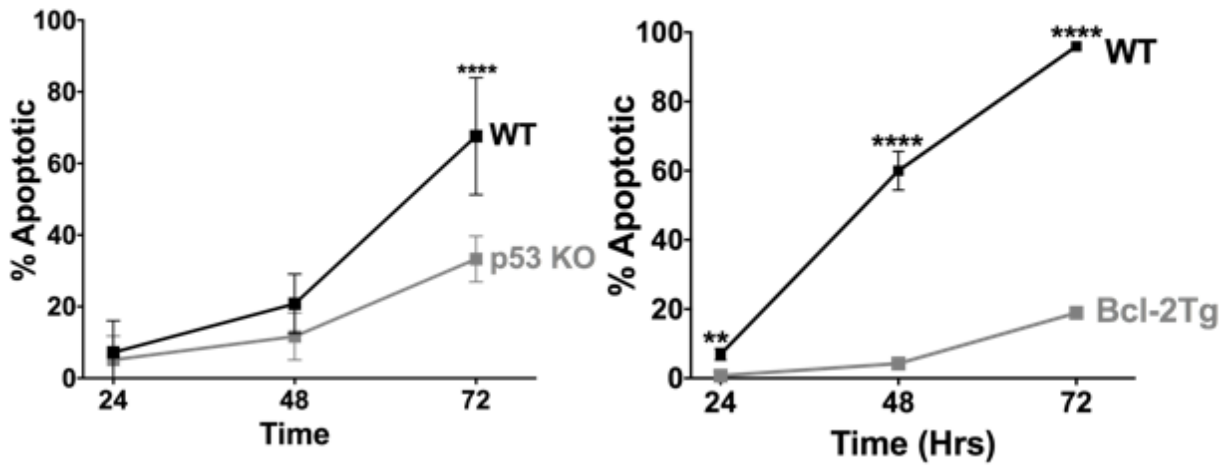


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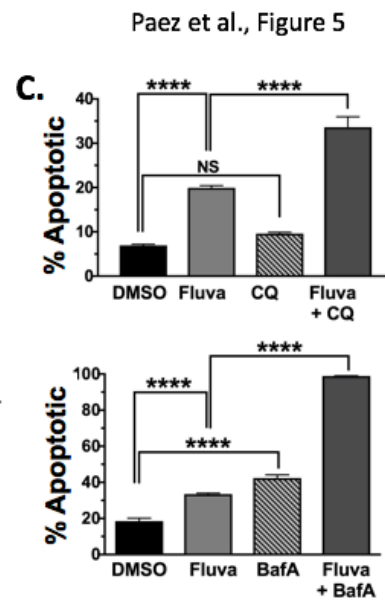
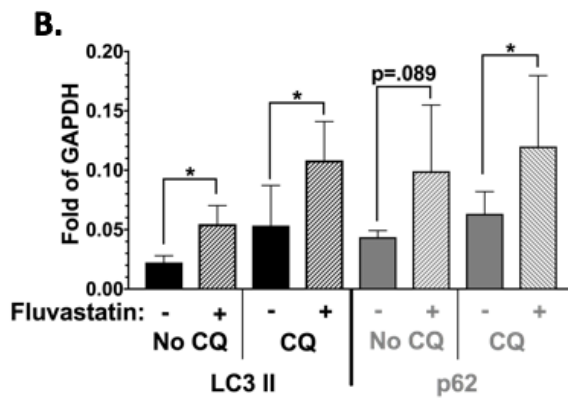
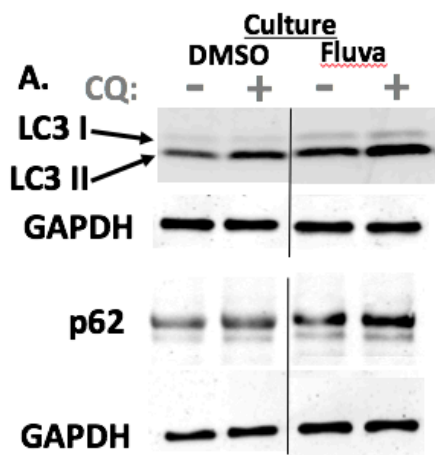


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Paez et al., Figure 4

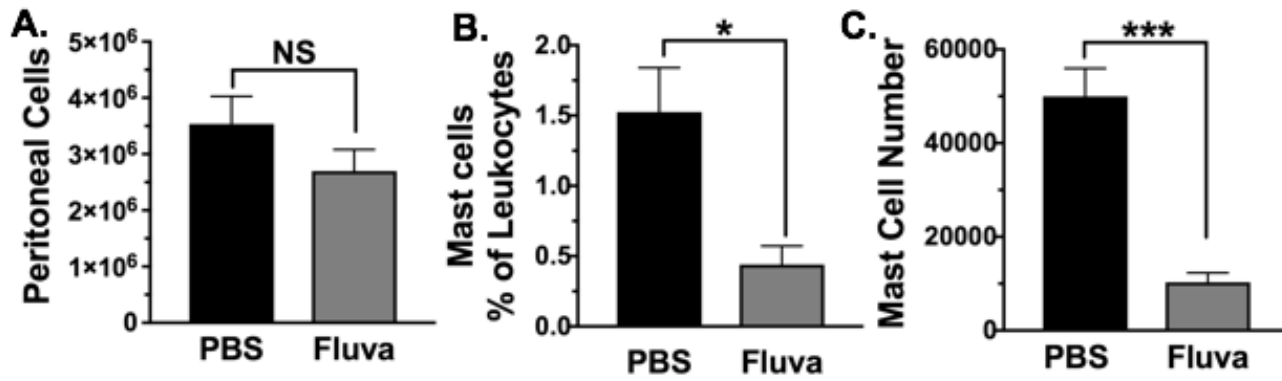


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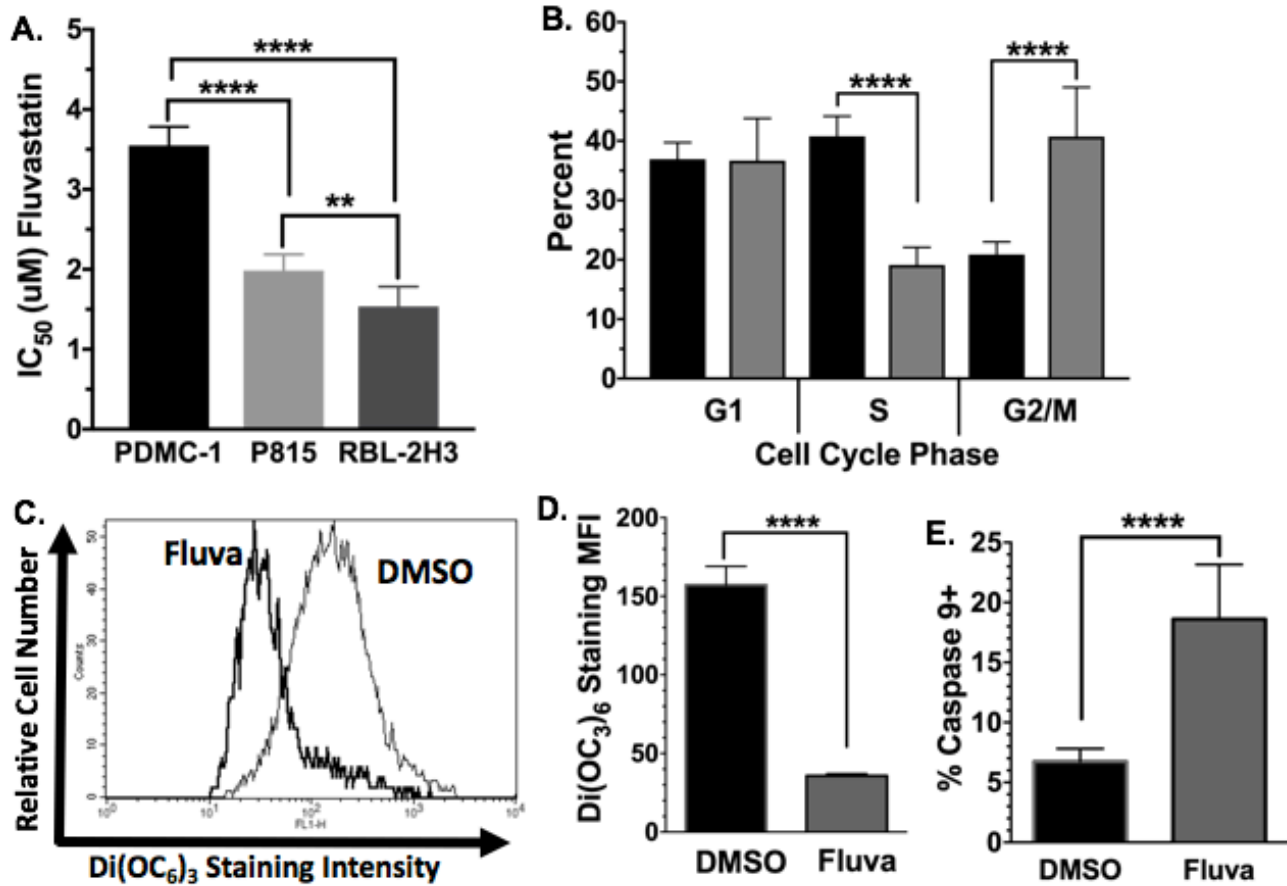


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Paez et al., Figure 6



Paez et al., Figure 7



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Paez et al., Figure 8

