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Mechanisms of Simvastatin Myotoxicity: The Role of Autophagy Flux Inhibition

Running Title: Autophagy Flux and Myotoxicity

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34 **Abstract**

35 Statins are some of the most widely used drugs worldwide, but one of their major side
36 effects is myotoxicity. Using mouse myoblast (C2C12) and human alveolar rhabdomyosarcoma
37 cell lines (RH30) in 2-dimensional (2D) and 3-dimensional (3D) culture, we investigated the
38 mechanisms of simvastatin's myotoxicity. We found that simvastatin significantly reduced cell
39 viability in C2C12 cells compared to RH30 cells. However, simvastatin induced greater
40 apoptosis in RH30 compared to C2C12 cells. Simvastatin-induced cell death is dependent on
41 Geranylgeranyl pyrophosphate (GGPP) in C2C12 cells, while in RH30 cells it is dependent on
42 both Farnesyl pyrophosphate (FPP) and GGPP. Simvastatin inhibited autophagy flux in both
43 C2C12 and RH30 cells and inhibited lysosomal acidification in C2C12 cells, while autophagy
44 inhibition with Bafilomycin-A1 increased simvastatin myotoxicity in both cell lines. Simvastatin
45 induced more cell death in RH30 cells compared to C2C12 in 3D culture model with similar
46 effects on autophagy flux as in 2D culture. Overall our results suggest that simvastatin-induced
47 myotoxicity involves both apoptosis and autophagy, where autophagy serves a pro-survival role
48 in both cell lines. The sensitivity to simvastatin myotoxicity is different in 2D versus 3D culture,
49 demonstrating that the cellular microenvironment is a critical factor in regulating simvastatin-
50 induced cell death in myoblasts.

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56 INTRODUCTION

57 The statin drugs ('statins') are competitive inhibitors of HMG-CoA (3-hydroxy-3-
58 methylglutarylcoenzyme A) reductase, and thus attenuate cholesterol and isoprenoid biosynthesis
59 in the mevalonate (MA) pathway (Endo et al., 1977). They are used clinically as lipid-lowering
60 drugs that prevent and treat cardiovascular diseases including atherosclerosis, coronary artery
61 disease, and stroke (Grundy and Vega, 1985; Illingworth and Sexton, 1984; Tikkanen and
62 Nikkila, 1987). The MA pathway is an essential contributor to mammalian cell homeostasis, as it
63 is involved in the regulation of a multitude of cellular processes that require cholesterol and the
64 isoprenoid intermediates (Cartocci et al., 2017; Hashemi et al., 2017). Cholesterol is the final
65 sterol product of the MA cascade but several upstream isoprenoid metabolites including Farnesyl
66 pyrophosphate (FPP) and Geranylgeranyl pyrophosphate (GGPP) are necessary for the
67 prenylation of monomeric small GTPase proteins (e.g. Rho, Ras, Rac, Cdc42, Rab, Rap)
68 (Hashemi et al., 2017; Sheikholeslami et al., 2019). These prenylated GTPases are critical cell
69 signaling molecules involved in many basic cellular processes including proliferation, growth,
70 migration, cytoskeletal dynamics, vesicular trafficking, barrier integrity, and smooth muscle
71 contraction, to name a few. Thus, the MA pathway is tightly regulated to maintain these precise
72 cellular functions under varied conditions in many cell types critical to health and disease (Jiao et
73 al., 2017; Yeganeh et al., 2014).

74 Statins are generally well-tolerated medications, however, there are side effects associated
75 with these compounds which are dose-dependent. One of the most important and clinically
76 relevant side effects is skeletal muscle myopathy which occurs in 1–5% of patients who take
77 statins. Rarely, this can lead to lethal rhabdomyolysis if it is not diagnosed promptly (Ballantyne
78 et al., 2003; Graham et al., 2004; Staffa et al., 2002; Thompson et al., 2003). According to recent

79 investigations, statin-related muscle disorders are potentially dependent on the inhibition of FPP
80 and GGPP (Bhardwaj et al., 2013; Matzno et al., 1997). Treatment of C2C12 cells with GGPP
81 can reverse the inhibitory effects of statins on myotube formation (Baba et al., 2008). In support
82 of these findings, there is evidence that statin-induced muscle toxicity is connected to the
83 inhibition of protein geranylgeranylation (Johnson et al., 2004), where prenylation of small
84 GTPases is essential to their signaling function, including RAP GTPase. This reaction
85 exclusively requires geranylgeranylation of RAP1A small Rho-GTPase protein, which is
86 catalyzed by the prenyltransferases (Crick et al., 1997). Although these investigations have been
87 illuminating regarding statin-induced muscle toxicity, the exact mechanisms underlying this
88 phenomenon remain incompletely understood.

89 Macroautophagy (hereafter listed as autophagy) is a multi step “self-eating” physiological
90 process that regulates cellular response to stress (Amiri et al., 2019). Autophagy can be involved
91 in both survival and death mechanisms based on the type of the cells and stimuli (Hombach-
92 Klonsch et al., 2018; Mokarram et al., 2017). Once it has been induced, tightly regulated
93 sequential steps direct the formation of a bilayer vesicle called the autophagosome to consume
94 cytoplasmic cargo (Klionsky et al., 2016; Mehrbod et al., 2019). This cargo is then ubiquitinated
95 and recognized by autophagy receptors like p62. The cargo receptor later binds to the cargo and
96 LC3-II, a component of the autophagosome membrane, which facilitates the isolation of the
97 cargo and its delivery to lysosomes.

98 Autophagy can be involved in regulation of programmed cell death I (apoptosis) under
99 different scenarios: i) as a positive controller (autophagy increases apoptosis), ii) as a negative
100 controller (autophagy decreases apoptosis), or iii) parallel to apoptosis (autophagy does not
101 change cellular apoptosis) (Ghavami et al., 2010a; Ghavami et al., 2010b; Ghavami et al., 2012a;

102 Ghavami et al., 2011; Ghavami et al., 2014; Ghavami et al., 2012b; Liu et al., 2017; Song et al.,
103 2017). Investigators have used autophagy and apoptosis cross regulation to develop new
104 therapeutic approaches for cancer. For example, different autophagy inducers and inhibitors have
105 been used with chemotherapy agents, and radiotherapy to increase the efficiency of cancer
106 therapy in some patients (Hombach-Klonisch et al., 2018; Mokarram et al., 2017).

107 We have previously studied cell death mechanisms of statins in airway smooth muscle and
108 recently established a research program in developing new therapeutic approaches for
109 Rhabdomyosarcoma (RMS). Previous investigations have used C2C12 mouse myoblasts
110 (Jaskiewicz et al., 2019; Schirris et al., 2015a) as a model for investigation of statins myopathy.
111 In the current study, we aim to understand the myotoxic effects of statins, using the
112 rhabdomyosarcoma cell line (RH30) (Moghadam et al., 2018) as well as C2C12 cell lines to
113 address this clinically relevant question.

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121 **MATERIALS and METHODS**

122 *Chemicals and Antibodies*

123 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (M2128),
124 simvastatin (S6196), FPP (F6892), GGPP (G6025), cholesterol (47129), mevalonate (68519),
125 LC3 β antibody (L7543), and beta actin antibody (A2228) were purchased from Sigma/Aldrich
126 (Canada, Ontario) Dimethyl sulfoxide (DMSO) (4948-02) were purchased from VWR (Canada,
127 Ontario). SQSTM1/p62 antibody (5114) were purchased from cell singling (Canada Ontario).
128 Cleaved PARP (Asp214) (D64E10) XP $\text{\textcircled{R}}$ Rabbit mAb, LC3B (D11) XP $\text{\textcircled{R}}$ Rabbit mAb, and
129 SQSTM1/p62 (D5L7G) Mouse mAb were purchased from Cell Signaling Technology. Alexa
130 Fluor $\text{\textcircled{R}}$ 488 AffiniPure Donkey Anti-Rabbit IgG, and Alexa Fluor $\text{\textcircled{R}}$ 647 AffiniPure Donkey
131 Anti-Mouse IgG secondary antibodies and IgG-free bovine serum albumin (BSA) were
132 purchased from Jackson ImmunoResearch Inc. DAPI (4',6-Diamidino-2-Phenylindole,
133 Dihydrochloride) was purchased from Thermo Fisher Scientific. Bovine type 1 collagen (10
134 mg/mL) was purchased from Advanced BioMatrix Inc. Live/dead viability staining kit was
135 purchased from Millipore Sigma.

136

137 *Cell Lines and Cell Culture*

138 The human rhabdomyosarcoma cell line (RH30) [RC13, RMS 13, SJRH30] (ATCC $\text{\textcircled{R}}$
139 CRL-2061 TM) (Human muscle cancer cells) and mouse muscle cell line (C2C12) (ATCC $\text{\textcircled{R}}$
140 CRL-1772 TM) were used in this project. Cells were cultured in Roswell Park Memorial Institute
141 (RPMI- 1640) with L-glutamine and 25mM HEPES (BioWhittaker; Cat #: 12-115Q) and
142 Dulbecco's Modified Eagle's Medium (DMEM) (CORNING; Cat #: 50-003-PB) with 10% fetal
143 bovine serum (FBS) (Gibco TM ; Cat #: 16000044). RH30 cell lines were cultured in RPMI-1640
144 with L-glutamine and 25 mM HEPES media, and C2C12 cells were cultured in DMEM with
145 high glucose media. Both media were supplemented with FBS (10%), penicillin (1%), and

146 streptomycin (1%). Cells were grown to 35–40% confluency on a 100 mm cell culture plate, 6-
147 well plates, and 96-well plates. Cells were maintained in a humidified incubator with 95% air
148 and 5% CO₂ at 37 °C and were passed once every 2–3 days. Cell culture plastic ware, penicillin,
149 and streptomycin were purchased from VWR (Toronto, ON, Canada).

150

151 *MTT Assay*

152 The MTT assay was performed based on a protocol established in our group (Alizadeh et
153 al., 2017; Ghavami et al., 2004; Ghavami et al., 2010b; Ghavami et al., 2012a; Ghavami et al.,
154 2011; Ghavami et al., 2014). Briefly C2C12 (20,000 cells/mL) and RH30 (30,000 cells/mL) were
155 seeded in 96-well plates and treated with varying concentrations of simvastatin (Simva, 0-20
156 µM). At each time-point (24, 48, 72, and 96 hrs), 20 µL of (MTT, 5 mg/ml) was added to each
157 well. The cells were incubated at 37°C for 4 hours, after which the media was gently aspirated.
158 Then, 200 µL of DMSO was added to each well and mixed with the cells by pipetting to dissolve
159 the MTT formazan crystals. Lastly, the plates were incubated for 20 min at room temperature.
160 Absorbance was measured at 570 nm using a Synergy H1 Microplate Reader.

161

162 *Mevalonate Cascade Rescue Assay*

163 Rescue experiments were done according to previously reported protocols (Alizadeh et
164 al., 2017; Ghavami et al., 2012a; Ghavami et al., 2011; Ghavami et al., 2014). Briefly, cells
165 were seeded and grown in 96-well plates at a density of 2,000 cells per well, up to 50%
166 confluence. Cells were pre-treated with 5 mM mevalonate (MeV), 30 µM FPP, 30 µM GGPP,
167 and 50 µM cholesterol, and were incubated at 37°C for 4 hrs. These cells were then co-treated

168 with 10 μ M Simvastatin and incubated at 37°C for 96 hrs. Cell viability was then measured using
169 the MTT assay after 96 hrs, as described in the previous section.

170

171 *Immunoblotting*

172 Western blotting analysis of C2C12 and RH30 cell lysates was used to assess markers of
173 autophagy (LC3 (1:2500), p62 (1:1000)) as has been described in our previous studies (Ghavami
174 et al., 2010b; Ghavami et al., 2012a; Ghavami et al., 2011; Ghavami et al., 2014). Cells were
175 grown to 40-50% confluency in 100 mm dishes and either treated with 10 μ M Simva or with a
176 drug vehicle control (DMSO). At the appropriate time point, cells were collected, and protein
177 extracts were made using NP-40 Lysis Buffer (0.5% (v/v) Nonidet P-40, 20 mM Tris-HCl (pH
178 7.5), 0.5% (v/v) PMSF, 100 μ M β -glycerol 3-phosphate and 1.5% (v/v) protease inhibitor
179 cocktail). Once the protein concentration was known, samples were prepared for western blotting
180 with a total protein concentration of 1 μ g/ μ L (15 μ L of each sample was used). After
181 electrophoresis, the membranes were developed for LC3 β and p62 proteins.

182

183 *Measurement of Apoptosis with Flow Cytometry*

184 We measured apoptosis using the Nicoletti method. C2C12 and RH30 cells were cultured
185 in 6-well plates and treated with either Simva (5 or 10 μ M) or with a drug vehicle control
186 (DMSO) for 48 hrs. After drug treatment, cells were detached using EDTA buffer and
187 centrifuged at 1,500 \times g for 5 min at 4°C. Cells were washed with cold PBS before they were
188 permeabilized and stained with a hypotonic Propidium Iodide (PI) buffer (0.1% Triton X-100,
189 1% sodium citrate, 0.5 mg/ml RNase A, 40 μ g/ml propidium iodide). Samples were then
190 incubated for 1 hour in the dark at 4°C to prevent photo-bleaching. Flow cytometry was carried

191 out at 460 nm for 10,000 cells. Residual debris were gated out accurately to obtain accurate data.
192 The resulting histogram was analyzed to determine the percentage of normal and apoptotic
193 nuclei; the nuclei of apoptotic cells were located on the left side of the G1 peak as they have less
194 DNA compared to the nuclei of healthy G0/G1 cells. For each sample, the sub-G1 peak was
195 measured and statistically compared with other samples to determine significance (Hashemi et
196 al., 2007; Moghadam et al., 2018).

197

198 *Live Cell Imaging*

199 LC3 is a specific marker for autophagosomes, which are key structures in the process of
200 autophagy. LC3-GFP is a fusion of green fluorescent protein (GFP) and LC3, and it can behave
201 in the same manner as endogenous LC3. LC3-GFP is localized on the autophagosome membrane
202 and emits green light when excited. In a normal cell, LC3 is dispersed evenly through the
203 cytosol. However, when autophagic flux is initiated, LC3 is recruited to autophagosome
204 membranes, resulting in sharp green puncta and LC3-GFP-containing cells. To confirm
205 autophagy findings seen in western blots, C2C12 or RH30 cells were grown in 6-well plates and
206 transfected with a plasmid containing LC3-GFP (Addgene #24920) using Qiagen's Effectene
207 reagent, as per manufacturer's instructions. After transfecting cells for 18 hrs, the cells were
208 treated with either Simva (10 μ M), Bafilomycin-A1 (Baf-A1, 100 nM), Simva + Baf-A1, or a
209 vehicle control (DMSO). After 24 hrs, the cells were incubated with LysoTracker red dye
210 (Molecular Probes; LysoTracker Red DND-99; L7528) at a concentration of 50 nM to detect
211 lysosomal activity and counterstained with Hoechst. Cells were stained for 30 min in a 37°C
212 incubator. After the 30 min period, the cells were washed with PBS and fresh media was added.
213 The cells were imaged on an epifluorescence microscope. Images were analyzed to determine the

214 percentage of cells with distinct LC3-GFP puncta and to see if green and red puncta co-
215 localization had occurred, which was assumed to signify the fusion of autophagosomes with
216 lysosomes (Field et al., 2018; Moghadam et al., 2018).

217

218 *Transmission Electron Microscopy (TEM)*

219 TEM was used to evaluate autophagy in both C2C12 and RH30 cell lines. The TEM
220 protocol is the same as previously described¹. Briefly, cells were grown in 10 mm dishes
221 (300,000 cells/dish) and treated with either DMSO (drug vehicle control) or 10 μ M Simva for 48
222 hrs. At the time point, cells were detached with EDTA and centrifuged at 1,500 \times g. The samples
223 were then fixed with 3% glutaraldehyde in PBS (pH 7.4) for 3 hrs at room temperature. The
224 samples were later treated post-fixation with 1% osmium tetroxide in PBS for 2 hrs, followed by
225 an alcohol dehydration series, and then embedded in Epon and stained with uranyl acetate. They
226 were counterstained with lead citrate for 3 min sequentially and finally washed with water for 1
227 min and dried. The samples were imaged on a Philips CM10 at 80kV on ultra thin sections
228 (100nm on 200 mesh grids) (Alizadeh et al., 2018a; Moghadam et al., 2018).

229

230 *Culturing C2C12 and RH30 cells in 3D cultures*

231 C2C12 and RH30 cells were grown in 3D culture according to the same protocol
232 explained in previous publications (Moghadam et al., 2018). Briefly, cells were grown in culture
233 medium (DMEM or RPMI with 10% FBS and 0.5% Pen-Strep) until they reached 80%
234 confluency. Cells were detached with Trypsin-EDTA, spun down, and re-suspended in fresh
235 media. Collagen and media were gently added to the cell suspension at 4°C to reach a final
236 collagen concentration of 3 mg/mL and a cell density of 2 million cells/mL. Then, 20 μ L of this

237 solution was added to cylindrical wells with 5 mm diameter and 1 mm depth in PDMS holders
238 which were placed in 12 well-plates and put in a 37°C incubator for 45 min to cure the collagen.
239 Afterwards, 2 mL of media was added to each well and they were incubated overnight. The next
240 day, cells were treated with either DMSO or Simva (Moghadam et al., 2018).

241

242 *Live-Dead Assay in 3D Culture*

243 Cells were grown and treated as explained above. Live-dead assay solution was prepared
244 as per the supplier's instructions, where 5 µL of calcein and 20 µL of ethidium homodimer-1
245 were added to 10 mL of DPBS. After treatment, media was removed, and the live-dead solution
246 was added to the wells. After incubating for 2 hrs at room temperature in the dark, the solution
247 was removed and the cells were gently rinsed with DPBS three times, each time with 5 min
248 incubation. Stained cells were then imaged on a confocal microscope and quantified (Moghadam
249 et al., 2018).

250

251 *Immunofluorescence (IF) in 3D Culture*

252 IF was used to confirm apoptosis and autophagy findings by evaluating cleaved-PARP,
253 LC3, and p62 levels. C2C12 and RH30 cells were treated with DMSO or 10 µM Simva for 96 or
254 48 hrs, respectively. At the appropriate time point, media was removed, and cells were fixed by
255 incubating them with 4% paraformaldehyde in PBS for 15 min at room temperature.
256 Paraformaldehyde was then removed, and cells were washed 3 times with PBS. Cells were
257 blocked with blocking buffer (5% goat serum, 0.3% Triton-X in RBS) at room temperature.
258 After 60 to 120 min, blocking buffer was removed and the appropriate primary antibody—p62,
259 LC3, or c-PARP—was diluted 1:300 in Antibody Buffer (1% BSA, 0.3% Triton-X in PBS) and

260 incubated with the samples overnight at 4°C. The next day, the antibody solution was removed,
261 and cells were washed three times with PBS. Cells were then incubated with the appropriate
262 fluoro-conjugated secondary antibody which was diluted 1:400 in Antibody Buffer. Samples
263 were incubated in the dark for 2 hrs at room temperature, and then washed with PBS. Finally, the
264 cells were incubated with DAPI solution for 1 hr in the dark. After washing three times with
265 PBS, cells were immediately taken for imaging (Moghadam et al., 2018).

266

267 *Statistical Analysis*

268 All results were presented as mean \pm SD, and the differences between the groups were
269 tested by one-way ANOVA or two-way ANOVA analysis (non-parametric, Brown–Forsythe
270 test), using GraphPad Prism 7.0. The confidence interval in each analysis was 95%, and $p < 0.05$
271 was considered statistically significant.

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278 **RESULTS**

279 *Mevalonate Cascade Inhibition Induces Cell Death in Both RH30 and C2C12 Cells*

280 We previously showed that the MA cascade inhibitor simvastatin induces cell death in a
281 broad range of primary cells (primary airway mesenchymal cells, and primary atrial fibroblasts)
282 (Ghavami et al., 2010b; Ghavami et al., 2011; Ghavami et al., 2014) and tumor cell lines

283 (Alizadeh et al., 2018b; Alizadeh et al., 2017; Sheikholeslami et al., 2019) including breast
284 (MCF-7, MDA-MB231), brain (U87, U251), and lung (A549, H1965), as well as
285 medulloblastoma brain tumor cell lines (Daoy, D283, and D341 cells). Lovastatin and
286 mevalonate cascade inhibitors (GGTi-298, 6-Fluoromevalonate) also inhibit ovarian cancer
287 tumor growth (Kobayashi et al., 2017; Kobayashi et al., 2015).

288 We now demonstrate that simvastatin induces dose- (0-20 μ M) and time- (0-96 hr)
289 dependent cell death in both RH30 (Fig. 1 A-D) and C2C12 cells (Fig. 1 E-H). In RH30 cells,
290 simvastatin (20 μ M) significantly induced cell death ($p < 0.05$) in 24 hrs (Fig. 1 A), simvastatin
291 (10, 20 μ M) significantly induced cell death ($p < 0.0001$) in 48 hrs (Fig. 1 B), simvastatin (5,10,
292 20 μ M) significantly induced cell death ($p < 0.05$, $p < 0.0001$) in 72 and 96 hrs (Fig. 1 C&D).
293 Interestingly, simvastatin (0.5-20 μ M) induced significant cell death ($p < 0.001$, $p < 0.0001$) in all
294 time points (24-96 hr) in C2C12 cells (Fig.1 E-H). The morphology of RH30 cells treated with
295 simvastatin (10 μ M) is shown in Fig. 1J and compared with RH30 time-matched control.
296 Simvastatin at concentrations of ≥ 2.5 μ M induced significant cell death in C2C12 cells as
297 compared to RH30 cells ($p < 0.01$).

298

299 *Prenylation Precursors Differentially Control Simvastatin-Induced Cell Death in C2C12 and*
300 *RH30 Cells.*

301 We know that mevalonate (MEV) can reverse statin-induced cell death in many cell
302 models, and GGPP is the major regulator of prenylation events among the isoprenoid
303 intermediates. We now show that MA (5 mM) significantly ($p < 0.0001$) inhibits simvastatin-
304 (10 μ M) induced cell death in both C2C12 (Fig. 2 A) and RH30 (Fig. 2E) cells. While GGPP (30

305 μM) significantly ($p < 0.0001$) inhibited simvastatin-induced cell death in both C2C12 (Fig. 2B)
306 and RH30 (Fig 2F) cells, it was more effective in rescuing RH30 cells than C2C12 cells (Fig.
307 2J). We found that FPP (30 μM) did not significantly inhibit simvastatin-induced cell death in
308 C2C12 cells (Fig. 2C), but it did significantly ($p < 0.01$) inhibit simvastatin-induced cell death in
309 RH30 cells (Fig. 2G). FPP was also more effective in rescuing RH30 cells against simvastatin-
310 induced cell death (Fig. 2K). Furthermore, cholesterol (50 μM) did not significantly inhibit
311 simvastatin-induced cell death in either C2C12 (Fig. 2D) or RH30 (Fig. 2I).

312

313 *Mevalonate Cascade Inhibition Induces Apoptosis in Both C2C12 and RH30 Cells*

314 Mevalonate cascade inhibition can induce apoptosis in many cell models (Alizadeh et al.,
315 2018b; Alizadeh et al., 2017; Ghavami et al., 2010b; Ghavami et al., 2012b). In this study, we
316 show that simvastatin induces dose- (5, 10 μM) and time-dependent (48, 72 hr) apoptosis in both
317 C2C12 (Fig. 3A-C) and RH30 (Fig. 3D-F) cells ($p < 0.01$, $p < 0.0001$). We also show that
318 simvastatin significantly induces greater apoptosis in RH30 cells as compared to C2C12 cells
319 (Fig. 3G) ($p < 0.01$).

320 *Mevalonate Cascade Inhibition Induces Blockage of Autophagy Flux in Both C2C12 and RH30* 321 *Cells While Inhibiting the Acidification of Lysosomes in C2C12 Cells*

322 Statins can induce autophagy in different types of cells (Ghavami et al., 2011; Ghavami
323 et al., 2014; Ghavami et al., 2012b). Our current study shows that statins inhibit autophagy flux
324 in both RH30 and C2C12 cells. We show that simvastatin (10 μM) increases LC3 lipidation and
325 induced p62 accumulation in both RH30 and C2C12 cells (Fig. 4A). To further confirm our
326 results, using GFP-LC3 and lysotracker immunostaining, we show that simvastatin induced

327 significant increase of LC3 puncta in both C2C12 and RH30 (Fig. 4B-D) while in C2C12 cells
328 prevented acidification of lysosomes (Fig. 4B) (lack of lysotracker red activity in simvastatin-
329 treated cells). We further confirmed our results using the autophagy inhibitor Bafilomycin A1
330 (Baf-A1, 100 nM for 1 hr) and show that adding Baf-A1 does not significantly increase the
331 number of LC3 puncta in both C2C12 (Fig. 4 E&G) and RH30 cells (Fig 4 F&H). We also
332 confirmed increased numbers of autophagosomes in both C2C12 (Fig. 4I) and RH30 cells (Fig
333 4J). We then used Baf-A1 (4 nM) in presence and absence of simvastatin (10 μ M, 24 hrs) in
334 C2C12 and RH30 cells (Fig 4K). Immunoblotting results confirmed further inhibition of
335 autophagy flux in both C2C12 and RH30 cells (increase of LC3 β lipidation and decrease of p62
336 degradation) (Fig 4K). Further, inhibition of autophagy significantly increased simvastatin-
337 induced myotoxicity in both C2C12 and RH30 cells (Fig 4L&M).

338

339 *Simvastatin Induces Apoptotic Cell Death and Inhibits Autophagy in Both C2C12 and RH30* 340 *Cells in 3D Culture*

341 Cells cultured in 3D configurations using hydrogel biomaterials display a more
342 physiologically-relevant phenotype (Seyfoori et al., 2018). We recently showed that 3D-cultured
343 C2C12 and RH30 cells can be used to screen drugs (Moghadam et al., 2018). In this study, we
344 used this same 3D technique to evaluate the effect of simvastatin on C2C12 and RH30 cells. We
345 performed live/dead assays in 3D culture of C2C12 and RH30 cells and show that simvastatin (5,
346 10 μ M) induces both dose- and time- (48, 96 hrs) dependent cell death (Fig. 5 A-H). Also,
347 simvastatin induced significant ($p < 0.0001$) cell death in both C2C12 (Fig. 5 A, C, E&F) and
348 RH30 (Fig. 5 B, D, G, H) cells in the 3D model. However, when cultured in the 3D hydrogel,

349 simvastatin induced more cell death in RH30 cells as compared to C2C12 cells. This was
350 opposite to the effect we observed when such cells were culture in standard 2D conditions. In
351 addition, simvastatin (10 μ M) induces apoptotic cell death in both C2C12 and RH30 cells
352 (cleavage of PARP) in the 3D culture model (Fig. 5 I, J). We further investigated the effects of
353 simvastatin on autophagy in both C2C12 and RH30 3D culture cells. We showed that
354 simvastatin inhibits autophagy flux in 3D culture model (increase of LC3 puncta and lack of
355 localization with p62) in both C2C12 and RH30 cells (Fig. 5 K&L).

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362 **DISCUSSION**

363 Our previous studies have demonstrated that the HMG-CoA reductase inhibitor simvastatin
364 induces endoplasmic reticulum stress/unfolded protein response, autophagy, and apoptosis in
365 human airway smooth muscle (HASM) cells, human airway fibroblasts (HAF), and human atrial
366 fibroblasts through inhibition of GGPP biosynthesis (Ghavami et al., 2012a; Ghavami et al.,
367 2011; Ghavami et al., 2014). Previously, we also showed that simvastatin induces apoptotic cell
368 death in a wide variety of tumor cells (lung, brain, and breast) via inhibition of
369 geranylgeranylation of small Rho GTPases (Alizadeh et al., 2017) .

370 Statin-induced myotoxicity is a major concern for clinicians and basic scientists alike, and
371 several recent investigations have focused on the possible underlying mechanisms involved in
372 statin myotoxicity. In the current investigation, we used C2C12 as our non-cancerous cell line
373 and RH30 as a cancer skeletal muscle cell line to elucidate the mechanisms underlying
374 simvastatin-induced myotoxicity. Our experiments utilized both monolayer 2D and 3D cell
375 culture models, which are more physiologically relevant accounting in part for the cellular
376 microenvironment.

377 Previous investigations have demonstrated that statin-induced myotoxicity occurs via
378 vacuolation of skeletal muscle fibers, blebbing of sarcolemma, and cell necrosis (Sakamoto et
379 al., 2007). Inhibition of the mitochondrial complex III is involved in statin-induced myotoxicity
380 in C2C12 cells (Schirris et al., 2015b). Other reports indicated that mitochondria (Bouitbir et al.,
381 2012; Kwak et al., 2012; Schirris et al., 2015b), Ca²⁺ homeostasis (Sirvent et al., 2012), plasma
382 membrane mono-carboxylate transporter (Kobayashi et al., 2006), plasma membrane receptors
383 (Dricu et al., 1997; Siddals et al., 2004), and ubiquitin ligases (Cao et al., 2009) are statins'
384 primary targets for myotoxicity. Here we show that simvastatin induced cell death in both
385 C2C12 and RH30 cells. However, there were significant differences between C2C12 and RH30
386 in cell viability (MTT assay) after treatment with simvastatin in 2D monolayer cell culture
387 (simvastatin caused significantly greater reduction in C2C12 cell viability as compared to
388 RH30). Since the MTT assay is based on the measurement of mitochondrial reductase activity to
389 produce formazon, our results suggest that simvastatin-induced cell death may be dependent on
390 the decrease of reductase activity in C2C12 cell lines. Of note, statin-induced myotoxicity
391 (Graham et al., 2004) is augmented with the combination of drugs that block metabolic pathways
392 and decrease mitochondrial reductase activity in cells, such as cytochrome P450 and UDP-

393 glucuronyltransferase 1A1 and 1A3 systems (Prueksaritanont et al., 2002). Consistent with
394 previous studies, our results demonstrate that simvastatin has greater myotoxicity in C2C12
395 (non-cancerous muscle) cells than RH30 (skeletal muscle cancer) cells.

396 We also show that simvastatin induced apoptosis in a time- and dose-dependent manner in
397 both C2C12 and RH30 cells. Interestingly, RH30 was more susceptible to apoptosis than C2C12
398 in 2D monolayer culture model (Figure 3). The rate of simvastatin-induced apoptosis in RH30
399 cells was ~2-fold greater than the C2C12 cell line. Therefore, while simvastatin caused a
400 reduction in cell viability in C2C12 cells, these cells were also less susceptible to apoptosis than
401 RH30 cells. This is not unexpected given that in the broader statin-cancer literature, cancer cells
402 are predominantly more sensitive to statin-induced cell death than their normal or non-cancerous
403 controls. These results intriguingly show that simvastatin-induced apoptosis in skeletal muscle
404 cells does not correlate with loss of cell viability as measured by the MTT assay; this suggests an
405 effect mediated via other mitochondrial factors. For example, in our previous investigations we
406 showed that simvastatin-induced apoptosis is dependent on the release of Smac/Diablo and
407 Omi/Htr2 from mitochondria in HASM cells and HAF (Ghavami et al., 2010b) and is
408 independent of the release of cytochrome c from mitochondria.

409 The role of cholesterol biosynthesis in statin-induced cell death has been widely
410 investigated and those results are consistent with ours (Graham et al., 2004; Sakamoto et al.,
411 2007; Schirris et al., 2015b). Previous studies showed that statins reduce GGPP levels and
412 production of ubiquinones which are used as electron carriers in the electron transport chain
413 (Harper and Jacobson, 2007; Thompson et al., 2003). Consequently, the decrease of ubiquinone
414 production in cells leads to dysfunction of the electron transport chain, which reduces muscle
415 cell ATP levels, elevates free radical production, and induces apoptosis (Harper and Jacobson,

416 2007; Thompson et al., 2003). Further, the impaired geranylgeranylation of proteins may be a
417 root cause in statin-associated myopathy (Cao et al., 2009; Johnson et al., 2004; Mullen et al.,
418 2010), a concept contested by work carried out in rhabdomyosarcoma rather than normal skeletal
419 muscle cells (Gee et al., 2015). Therefore, we decided to investigate the MA pathway in both
420 C2C12 and RMS cells. In our study, co-treatment with MA or GGPP inhibited simvastatin-
421 induced cell death in the C2C12 cell line while co-treatment with cholesterol and FPP did not.
422 Takeda et al. demonstrated that the reduction of smooth muscle cell proliferation by simvastatin
423 was inhibited by GGPP, but not by FPP (Takeda et al., 2006). Their findings are compatible with
424 our results which show simvastatin signaling is dependent on GGPP in C2C12 cells. Our present
425 study also shows that in RH30 cells simvastatin-induced cell death is inhibited by MA and
426 GGPP, but not cholesterol (Fig 2). Unlike in C2C12 cells, we show that in RH30 cells
427 simvastatin-induced cell death was inhibited by FPP (Fig 2). These findings confirm that the
428 effect of simvastatin on cell death in both cells was mediated via inhibition of the MA pathway,
429 in particular, GGPP. In addition, we discovered that FPP may play an important role in
430 simvastatin-induced death mechanisms in RH30 cells.

431 GGPP and FPP are necessary for the prenylation of small Rho GTPase proteins including
432 Rho, Rac, Cdc42, Rab and Rac (Alizadeh et al., 2018b; Alizadeh et al., 2017; Ghavami et al.,
433 2010b; Ghavami et al., 2012a; Yeganeh et al., 2014). We show that simvastatin-induced cell
434 death is dependent on GGPP in C2C12 cells. This indicates that Rho, Cdc42, and Rac GTPases
435 may be involved in cell death induction mechanisms in C2C12 cells. Conversely, in RH30 cells
436 both FPP and GGPP mediate simvastatin-induced cell death, suggesting that Ras GTPases may
437 also be involved via farnesylation pathways.

438 Several recent investigations have shown that HMG-CoA reductase inhibitors such as
439 simvastatin either induce or inhibit autophagy in different cell models (Ghavami et al., 2014;
440 Hwang et al., 2015; Vilimanovich et al., 2015; Whitehead, 2016). There are two recent articles
441 that showed hydrophobic statins induced autophagy in A204 RMS cells (Araki and Motojima,
442 2008; Gee et al., 2015). But, the exact molecular mechanisms of the autophagy flux,
443 autophagosome fusion and degradation steps of autophagy have not been investigated in RMS
444 cells. Many studies demonstrated that the LC3-II/LC3-I ratio is often used to determine the
445 activation of autophagy (Mizushima et al., 2010). The present results show that simvastatin
446 increased the conversion of light chain 3 (LC3)-I to LC3-phosphatidylethanolamine conjugate
447 (LC3-II) in both C2C12 and RH30 cells by increasing the number of LC3 puncta
448 (immunofluorescence) and autophagosome formation (Figure 4). The protein p62 facilitates the
449 degradation of ubiquitinated protein aggregates by autophagy (Guo et al., 2013) and is a selective
450 substrate for autophagy and directly interacts with LC3 to mediate the degradation of
451 ubiquitinated protein aggregates by autophagy (Pankiv et al., 2007). Our results show that
452 simvastatin increases p62 accumulation in both RH30 and C2C12 cell lines, therefore,
453 simvastatin inhibits autophagy flux in both cells lines. Moreover, our results showed that
454 simvastatin induced acidification of lysosomes in RH30 cells, but in C2C12 cells simvastatin
455 inhibited acidification of lysosomes (Fig. 4B). Taken together, our data demonstrates that
456 simvastatin inhibits autophagy flux in a time-dependent manner in both non-cancer C2C12 and
457 RMS RH30 cells. Therefore, we conclude that simvastatin inhibits autophagy flux in both
458 C2C12 and RH30. In RH30 cells, autophagy inhibitory activity occurs via inhibition of
459 lysosomal acidification, however, further investigation is required to prove this hypothesis.
460 Further blockage of autophagy flux increases the myotoxicity of simvastatin in both C2C12 and

461 RH30 cells. These findings confirm the importance of autophagy flux inhibition in the
462 myotoxicity of statins. These results are inconsistent with our findings in HAF and HASM
463 (Ghavami et al., 2011; Ghavami et al., 2014). Also, Gu et al showed that simvastatin induces
464 autophagy in bronchial smooth muscle cells (BSMCs) and increases autophagy-related protein
465 Atg5, LC3B, and Beclin1 expression and autophagosome formation in lung tissue (Gu et al.,
466 2017).

467 The effect of chemical compounds on cells have mostly been performed using 2D cell
468 culture models, where cell-cell interaction, extracellular matrix, and cellular morphology
469 significantly differ from their natural structure in tissues (Levinger et al., 2014). These
470 differences highly influence cellular growth and their response to different chemical compounds
471 (Levinger et al., 2014). Three-dimensional (3D) culture models have been introduced for drug
472 assessment to improve the relation between cell cultures and cellular microenvironment
473 (Friedrich et al., 2009). Recently, 3D culture models have been used as clinically relevant models
474 for the study of cell death and autophagy (Gomes et al., 2015; Ma et al., 2011). We examined the
475 effects of simvastatin in RH30 and C2C12 3D culture models (Fig. 5), which showed that
476 simvastatin induces significantly greater cell death in RH30 cells as compared to C2C12 cells.
477 Whereas, the cell death effects of simvastatin were greater in C2C12 cells as compared to RH30
478 cells in the 2D cell culture model. This shows how the cell microenvironment and 3D structure
479 can affect fundamental cellular response(s) including to chemical compounds or drugs. We also
480 observed that simvastatin inhibits autophagy flux in 3D culture of RH30 and C2C12 cells
481 (absence of localization of p62 and LC3 puncta) which correlates with our 2D observations.
482 Overall, our results indicate that 3D culture models are important tools for screening cytotoxic

483 effects of chemical compounds as they can account for some of the effects of cellular matrix in
484 response to extracellular stress.

485 There are several limitations to this study that are important to mention. The dose of
486 simvastatin used in our experiments (10 μ M) is significantly higher than pharmacologic
487 concentrations found in human blood which is in the low nanogram/mL (nM) range (Ucar et al.,
488 2000). However, we don't know if statins accumulate in human skeletal muscle, and whether
489 they reach micromolar concentrations. Furthermore, simvastatin's half-life is approximately 2
490 hours in plasma, and results can vary according to which statin is selected. In addition, normal
491 human skeletal muscle behaves differently than rhabdomyosarcoma cell line, so the effects
492 observed could manifest differently in human normal skeletal muscle.

493 In conclusion, we found that simvastatin induces cell death in both RH30 and C2C12 cells
494 in both 2D and 3D culture. Our results showed that simvastatin significantly decreases cellular
495 viability in C2C12 cells compared to RH30 cells while it also significantly induces greater
496 apoptosis in RH30 cells compared to C2C12 cells. In addition, simvastatin inhibits autophagy
497 flux in both RH30 and C2C12 cells with differential effects on lysosomal acidification. We also
498 showed that simvastatin-induced cell death is dependent on both FPP and GGPP in RH30 cells
499 while it is only dependent on GGPP in C2C12 cells. Our current investigation provides solid
500 evidence that both autophagy and apoptosis are involved in statin-induced myotoxicity, and
501 further, autophagy flux inhibition varies between the non-cancerous and cancer muscle cell lines.

502

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511

512 **FIGURE LEGENDS:**

513 **Figure 1: Simvastatin induces cell death in RH30 and C2C12 cells.** (A-D). RH30 cells were
514 treated with simvastatin (0.5, 1, 2.5, 5, 10, 20 μ M) and cell viability was assessed 24, 48, 72 and
515 96 hrs after that by MTT assay. Control cells for each time point were treated with the solvent
516 control (DMSO). Results are expressed as a percentage of corresponding time point control and
517 represent the means \pm SD of 15 replicates in three independent experiments (*, $p < 0.05$; ****,
518 $p < 0.0001$). (E-H). C2C12 cells were treated with simvastatin (0.5, 1, 2.5, 5, 10, 20 μ M) and cell
519 viability was assessed 24, 48, 72 and 96 hrs after that by MTT assay. Control cells for each time
520 point were treated with the solvent control (DMSO). Results are expressed as a percentage of
521 corresponding time point control and represent the means \pm SD of 15 replicates in three
522 independent experiments (****, $p < 0.0001$). (I&J). **Simvastatin significantly decreased cell**
523 **viability in C2C12 compared to RH30 cells.** RH30 and C2C12 cells were treated with
524 simvastatin (0.5, 1, 2.5, 5, 10, 20 μ M) and cell viability was assessed 24, 48, 72 and 96 hrs after
525 that by MTT assay. Control cells for each time point were treated with the solvent control
526 (DMSO). Results are expressed as a percentage of corresponding time point control and
527 represent the means \pm SD of 15 replicates in three independent experiments (****, $p < 0.0001$).
528 (K&L). Phase contrast microscopy showed that simvastatin (10 μ M, 48 hrs) induces
529 morphological changes (cellular shrinkage) and decrease in the number of cells in RH30 cells.

530

531 **Figure 2: Simvastatin induces cell death in RH30 and C2C12 cells is dependent on**
532 **mevalonate cascade isoprenoid mediators.** (A-H) 5 mM MA, 30 μ M GGPP, 30 μ M FPP, or
533 50 μ M cholesterol, were added to the cells 4 hrs prior to treatment with simvastatin (10 μ M, 96

534 hrs). Cell death was measured by MTT assay in C2C12 (A-D), and RH30 cells. For each
535 experiment control cells were treated with simvastatin solvent (DMSO) alone (control) or with
536 both DMSO and the appropriate solvent (i.e. ethanol for “mevalonate control). Mevalonate (A,
537 E) and GGPP (B, F) significantly inhibited simvastatin induced cell death in both C2C12 and
538 RH30 cells while FPP (C, G) only inhibited simvastatin-induced cell death in RH30 cells. Our
539 results also showed that cholesterol (D, H) is not involved in simvastatin induced cell death in
540 C2C12 and RH30 cells. Results are expressed as mean \pm SD of 15 replicate in 3 independent
541 experiments (* $p < 0.05$, *** $p < 0.001$, and **** $p < 0.0001$). (I&J) Our results also showed that
542 FPP (I) and GGPP (J) significantly rescues simvastatin induced cell death in RH30 cells
543 compared to C2C12 cells.

544

545 **Figure 3: Simvastatin induces dose and time depended apoptosis cell death in C2C12 and**
546 **RH30 cells.** Percent sub-G1 (A-C) C2C12, (D-F) RH30, abundance induced by simvastatin (5,
547 and 10 μM) or DMSO solvent control after 48 and 72 hrs. Results represent the means \pm SD of 9
548 replicates in three independent experiments. **** $p < 0.0001$; and *** $p < 0.001$ compared to time-
549 matched control. Representative figures of the flow cytometry histogram for C2C12 and RH30
550 are shown (A and D). Our results showed that simvastatin (10 μM) induced significant more
551 apoptosis in RH30 compared to C2C12 cells in 48 (G) and 72 (H) hours (** $p < 0.01$, and **** p
552 < 0.0001).

553

554 **Figure 4: Simvastatin inhibits autophagy flux inhibition in C2C12 and RH30 cells.** (A)
555 C2C12 and RH30 cells were treated with simvastatin (10 μM , 0-72 hours) and cell lysates were

556 collected. Immunoblotting for LC3 β and p62 were performed. The results showed that
557 simvastatin induced accumulation of LC3 β II and inhibits p62 degradation in both C2C12 and
558 RH30 cells. C2C12 and C2C12 cells were treated with simvastatin (10 μ M, 24 h). (B-D) using
559 immunocytochemistry LC3 puncta and changes in lysosomal activity (LysoTracker red staining)
560 has been investigated. The results showed that simvastatin increased LC3 puncta in both cell
561 lines. Our results also showed that simvastatin (10 μ M, 24 hours) increase lysosomal acidity in
562 RH30 while inhibits lysosomal acidification in C2C12 cells (B). C2C12 (E) and RH30 (F) cells
563 were treated with simvastatin (10 μ M, 24h) and Baf-A1 (100 nM, +3 hours) followed by
564 immunocytochemistry to evaluate LC3 puncta and changes in lysosomal activity (LysoTracker
565 red staining). The results showed that simvastatin increased LC3 puncta and decreased
566 LysoTracker red fluorescence intensity in C2C12 cells while increased LC3 puncta and increased
567 LysoTracker red fluorescence intensity in RH30 cells. On the other hand, Baf-A1 and
568 simvastatin + Baf-A1 did not significantly change LC3 puncta in both C2C12 and RH30 cells (G,
569 H) showing that simvastatin inhibited autophagy flux like Baf-A1. Transmission electron
570 microscopy showed that in treated C2C12 (I) and RH30 (J) cells there are accumulated
571 autophagosome-like structures compared to control and normal cells after 72 hours treatment.
572 Arrows show the autophagolysosomes containing the cargo (magnification \times 11,600). Autophagy
573 inhibition (Baf-A1, 4 nM, 24 hours) significantly increased simvastatin-induced cell death (10
574 μ M, 24 hours) in RH30 (L) and C2C12 (M) cell lines (** $p < 0.01$, Results represent the
575 means \pm SD of 15 replicates in three independent experiments). Baf-A1 (4 nM) and simvastatin
576 (10 μ M) combination did not increase accumulation of LC3 β -II and p62 in both RH30 and
577 C2C12 cells (K).

578

579 **Figure 5: Simvastatin induces apoptosis and autophagy in C2C12 and RH30 3D culture.**

580 (A&B). Bright field image of C2C12 (A) and RH30 (B) 3D culture which shows the morphology
581 of untreated and simvastatin treated cells (5, 10 μ M, 96 hours) in 3D culture. (C-H). Viability
582 assay was done by adding the live/dead solution to cells 48 and 96 hours after treatment with
583 simvastatin (0–20 μ M). Cells were incubated for 2 hours in the dark at room temperature, rinsed
584 three times with DPBS, and confocal microscopy was used to capture live/dead cell images in
585 C2C12 (C) and RH30 (D) cells. Quantification of live/dead assay was measured by calculating
586 the ratio of live: total cells which showed a significant decrease in viability of C2C12 (E&F) and
587 RH30 (G&H) cells treated with different concentrations of simvastatin. The data showed
588 simvastatin significantly induces cell death in both C2C12 and RH30 cells ($P < 0.0001$) while
589 simvastatin induces more cell death in RH30 compared to C2C12 cells. (I&J) IF labeling of
590 C2C12 cells (I) and RH30 cells (J) by cleaved PARP following treatment with simvastatin
591 (10 μ M, 48 hours) increased number of cells with cleaved PARP in simvastatin treated cells in
592 comparison to control cells which is the hallmark of increase of apoptosis in these cells. (K&L)
593 After treatment of C2C12 (K) and RH30 (L) cells with simvastatin (10 μ M, 48 h), cells were IF
594 labeled with autophagosome markers, LC3 and P62. Data showed that simvastatin increases LC3
595 puncta (green) which is not localized with p62 compared to corresponding time-matched control,
596 a hallmark of autophagy flux inhibition in C2C12 and RH30 3D culture

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