Inhibition of prenyltransferase activity by statins in both liver and muscle cell lines is not causative of cytotoxicity

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Abbreviations: HMGCR = 3-hydroxy-3-methylglutaryl-CoA reductase; FTase = farnesyl transferase; GGTase = geranylgeranyl transferase

Abstract

As inhibitors of 3-hydroxy-3-methylglutaryl-CoA reductase, statins are an important first-line treatment for hypercholesterolemia. However, a recognized side-effect of statin therapy is myopathy, which in severe cases can present as potentially fatal rhabdomyolysis. This represents an important impediment to successful statin therapy, and despite decades of research the molecular mechanisms underlying this side-effect remain unclear. Current evidence supports a role for reduced levels of mevalonate pathway intermediates, with the most accepted hypothesis being a reduction in isoprenoids formation, leading to faulty post-translational modifications of membrane-associated proteins. We have undertaken a comprehensive analysis of the impact of nine statins on two human cell lines; Huh7 hepatoma and RD rhabdomyosarcoma. In both cell lines, concentration-dependent inhibition of prenylation is observed for cerivastatin and simvastatin, which could be rescued with the pathway intermediate mevalonate; in general, muscle cells were more sensitive to this effect, as measured by the levels of unprenylated Rap1A, a marker for prenylation by geranylgeranyl transferase I. Concentration-dependent toxicity was observed in both cell lines, with muscle cells again being more sensitive. Importantly, there was no correlation between inhibition of prenylation and cell toxicity, suggesting they are not causally linked. The lack of a causal relationship was confirmed by the absence of cytotoxicity in all cell lines following exposure to specific inhibitors of geranylgeranyl transferase I and II, and farnesyl transferase. As such, we provide strong evidence against the commonly accepted hypothesis linking inhibition of prenylation and statin-mediated toxicity, with the two processes likely to be simultaneous but independent.

1. Introduction

Statins remain a front line treatment for the prevention of cardiovascular disease. As inhibitors of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), the rate limiting enzyme of cholesterol synthesis, they significantly reduce cholesterol production in the liver, and circulating cholesterol levels (Goldstein and Brown 1990; Istvan and Deisenhofer 2001). However, significant adverse-effects associated with statin usage either limit or preclude their utility in some individuals. The major statin-mediated adverse effects are myopathies, muscle related side-effects that can range from mild (muscle aches and cramps) to severe (rhabdomyolysis). While in some patients these myopathies are tolerable, in many cases they necessitate the withdrawal of treatment, and in some cases rhabdomyolysis can be fatal (Arora et al. 2006; Graham et al. 2004).

Cholesterol biosynthesis is one endpoint within the mevalonate pathway, which is responsible for the production of a number of biologically-important molecules, including cholesterol, ubiquinone, phosphodolichol and the isoprenoids farnesyl and geranylgeranyl pyrophosphate (FPP and GGPP; Fig. 1). As HMGCR sits within the shared portion of this pathway, its inhibition by statins potentially impacts upon all these biosynthetic endpoints (Takemoto and Liao 2001).

The mechanism that underlies statin toxicity is not fully understood, but is likely to be a direct consequence of the inhibition of the mevalonate pathway, rather than an indirect transcription-mediated effect (Howe et al. 2011), since mevalonate supplementation prevents toxicity both *in vitro* (Johnson et al. 2004) and *in vivo* (Westwood et al. 2008). Depletion of cholesterol is not thought to be a primary cause of myopathy as squalene synthase inhibitors, which block the first step in the cholesterol branch of the mevalonate

pathway are not myotoxic (Nishimoto et al. 2007; Nishimoto et al. 2003). In addition, insects and nematodes both lack the cholesterol biosynthetic arm of the mevalonate pathway, but faithfully reproduce the other biosynthetic endpoints seen in mammals; they thus represent ideal models to examine non-cholesterol-dependent effects of statins. Experiments in *Drosophila melanogaster* and *Caenorhabditis elegans* are able to replicate both some of the beneficial effects (for example, cardioprotection) and the adverse effects of statins, demonstrating that these endpoints are not reliant on the cholesterol-lowering effects of these drugs (Morck et al. 2009; Rauthan et al. 2013; Spindler et al. 2012). Taken together, this evidence is consistent with myopathic adverse endpoints being mediated through inhibition of one (or more) of the alternate biosynthetic endpoints of the mevalonate pathway. Evidence exists to support disruption of ubiquinone (Marcoff and Thompson 2007), dolichol-mediated N-linked glycosylation (Mullen et al. 2010; Siddals et al. 2004) and prenylation (Blanco-Colio et al. 2002; Guijarro et al. 1998; Itagaki et al. 2009; Matzno et al. 2005; Sakamoto et al. 2011; Satoh et al. 2001) following statin treatment, but it is as yet unclear as to which, if any, is the primary determinant of the observed human myopathies.

In the current work, we have used secondary cell lines as a tool to delineate the molecular mechanisms underlying statin-induced myopathy, and in particular the potential role of inhibition of prenylation. We have demonstrated that while liver and muscle cell lines differ in their sensitivity to statins both in terms of cell death and reduction in prenylation, these are not causally linked, since cells inhibited for prenylation do not show a reduced viability or morphological defects. As such, we provide strong evidence that statin-induced myopathy is not mediated via inhibition of prenylation, as commonly assumed.

2. Materials and Methods

2.1. *Materials* – Statins were obtained from the following sources: simvastatin (lactone), lovastatin, and fluvastatin from Calbiochem (Merck KGaA, Darmstadt, Germany); atorvastatin and rosuvastatin from Molekula (Dorset, UK); and cerivastatin, simvastatin (sodium salt) and pravastatin from Sequoia Research Products Limited (Pangbourne, UK). Mevalonate (lithium salt), and the prenyltransferase inhibitors GGTI-2133, FT-277 and perillyl alcohol were purchased from SigmaAldrich (Dorset, UK).

Primary antibodies were purchased from Santa Cruz Biotechnology (TX, USA) for Rap1A (C17), Rap1 (I21), HMGCR (H-300), GGTase-I (XX-12), GGTase-II (17-Q), FTase (H-300), from the Developmental Studies Hybridoma Bank (IA, USA) for MyoG (clone F5D) and MYH3 (F1-652) or from SigmaAldrich for β-actin (A5441). Appropriate secondary antibodies were purchased from Santa Cruz Biotechnology.

2.2. *Cell culture* **–** The human hepatoma cell line Huh7 (Nakabayashi et al. 1982) was a kind gift from Steve Hood (GlaxoSmithKline, Ware, UK) whereas the human rhabdomyeloma cells RD (McAllister et al. 1969) were purchased from the American Tissue Culture Collection (CCL-136). Both were cultured in Dulbecco's Modified Eagle Medium with 2 mM L-glutamine, 4.5 g/L glucose, 100 units/mL each penicillin and streptomycin, and 10% foetal bovine serum (FBS), at 37˚C and 5% CO_2. RD cell differentiation was initiated by switching to medium containing 1% FBS and culturing for 5 days.

Cells were seeded into appropriate vessels 24 h (Huh7 and undifferentiated RD cells (RD-U)) or 5 days (differentiated RD cells, RD-D) prior to treating with statins or inhibitor for 48 h or 72 h; treatment was carried out in serum-free medium and appropriate vehicle

controls (serum-free medium or 0.1% dimethylsulfoxide (DMSO) in serum-free medium) were included for each treatment. Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT) assay: treated cells and controls were incubated with 0.5 mg/mL MTT for 2½ h and the resultant formazan salt dissolved in DMSO and its absorbance measured at 540 nm. Results are expressed as a percentage of vehicle control; each data point represents the mean of a minimum of three independent experiments of 6 wells per experiment, with error bars representing the standard error of the mean (SEM). Curves were plotted by non-linear regression and compared through a two-way ANOVA with Tukey's multiple comparison test, using GraphPad Prism (v6, GraphPad Software Inc., La Jolla, USA).

Protein analysis – Total protein was extracted from treated and control cells using RIPA buffer (phosphate buffered saline plus 1% nonidet P40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, with complete protease inhibitor cocktail (EDTA-free; Roche) as previously described (Plant et al. 2009) and the concentration measured by Lowry assay (Lowry et al. 1951). Proteins were separated by SDS-PAGE (15 μg per well) and blotted onto PVDF membrane before immunodetection using primary antibodies raised against a variety of proteins; details of antibody concentrations are given in Supplementary Table 1. Secondary antibodies were linked to horseradish peroxidise and detection was using the Enhanced Chemiluminescence (ECL) Plus reagent (GE Healthcare, Bucks, UK). Computer based densitometry of gels was undertaken using a GeneGenius Bioimaging System (Syngene, Frederick, MD). All experiments were repeated on at least three independent occasions, and densitometric readings normalised against β–actin. Statistical significance

was examined through a two-way ANOVA with Tukey's multiple comparison test, using GraphPad Prism (v6, GraphPad Software Inc., La Jolla, USA).

2.3.

3. Results

3.1. *Molecular markers differ between muscle and liver cell lines.*

In the present study we have used the Huh7 hepatoma cell line to represent the target cells for the therapeutic effects of statin therapy, and the RD rhabdomyosarcoma cell lines as a surrogate target cell for the myopathic adverse endpoint. Naïve RD cells (RD-U) can be induced to differentiate into a more muscle-like phenotype (RD-D) by growth in medium containing reduced serum levels. Differentiation is complete within 5 days, with expression of muscle-specific markers myogenin (MyoG) and embryonic muscle myosin heavy chain 3 (MYH3) being observed at both the protein and transcript levels (Fig 2. and Supplemental Fig. S1). Protein levels of MyoG and MyH3 were 1.5-fold and 1.4-fold higher in RD-D cells compared to RD-U cells, respectively. In addition, myotube formation was observed after 72 hours of differentiation (Supplemental Fig. S1).

HMGCR, the target enzyme for statins, was expressed in all three cell lines: Huh7 cells showed the highest level of expression, being approximately 1.3-fold higher than both RD-U and RD-D cell lines expression. Finally, the levels of geranylgeranyl transferase I (GGTase-I), GGTase-II and farnesyl transferase (FTase) were assessed through detection of their specific β-subunits: All cell lines expressed all three prenyltransferase enzymes, with no significant differences in expression levels observed.

3.2. Muscle cells are more sensitive to statin toxicity than liver cells.

Following initial characterisation of the model cell lines, a comprehensive comparison of statin toxicity was undertaken (Fig. 3). All statins elicited concentration-dependent toxicity in each cell line, although in some cases (*e.g.* pravastatin) the concentrations required to elicit statistically significant toxicity in any cell line were very high (mM). RD cells (undifferentiated or differentiated) were, generally, more sensitive to statin-mediated toxicity than Huh7: A two-way ANOVA determined that RD-U cells were significantly more sensitive than Huh7 cells to the toxic effects of cerivastatin, simvastatin (lactone and acid) and fluvastatin ($p<0.001$ in all cases), atorvastatin ($p<0.01$), and lovastatin (acid) and pravastatin ($p<0.05$). RD-D cells were significantly more sensitive to simvastatin (acid; $p<0.001$), cerivastatin, atorvastatin and rosuvastatin (all $P<0.01$). In general, there was no significant difference in sensitivity of RD-U and RD-D cells, with the exceptions of cerivastatin ($p<0.01$) and the lactone form of simvastatin ($p<0.05$); in both cases differentiation reduced statin sensitivity.

IC50 values for each cell line were derived for all statins where limiting solubility was not a confounding factor. For cerivastatin, IC50 values for Huh7, RD-U and RD-D were >100μM, 2.1 ± 0.5μM and 4.4 ± 1.9μM, respectively; for simvastatin (acid form) >100 μM, 2.4 ± 0.4μM and 0.3 ± 0.2μM, respectively; and for fluvastatin 8.5 ± 5.5μM, 6.0 ± 1.8μM and 2.6 ± 2.6μM, respectively.

3.3. Lack of correlation between statin-dependent inhibition of prenylation and cell toxicity.

Three statins were chosen for further analysis: Cerivastatin was the first statin withdrawn from the market for unacceptable toxicity and caused significant toxicity in all cell lines; simvastatin (acid form) represents the most commonly prescribed statin, and also caused toxicity in all three cell lines; pravastatin was significantly better tolerated by all three cell

lines, with toxicity not evident until millimolar concentrations were used. To examine the potential role of GGTase-I-mediated prenylation in statin-mediated toxicity we used geranylgeranylation of Rap1A as a marker for this enzyme activity. Under normal conditions almost all Rap1A protein is prenylated in all model cells lines, with no unprenylated Rap1A detectable (Fig. 4). Exposure of all three cell lines for 48 hours to cerivastatin (1μM) or simvastatin (10μM) reduced the level of Rap1A prenylation significantly, causing an average 7-fold increase in the ratio of unprenylated:total RAP1A ratio. This phenotype was rescued by supplementation with 100μM mevalonate, indicating it was specifically due to HMGCR inhibition. By contrast, exposure of cells to pravastatin (10μM) for 48 hours had no appreciable impact on prenylation of Rap1A, in any of the cells lines examined.

Inhibition of GGT-I-mediated prenylation by statins was concentration dependent in all three model cells (Fig. 5). Both RD-U and RD-D cell lines showed greater sensitivity to statin-mediated inhibition of Rap1A prenylation than Huh7 cells, with a significant increase in the unprenylated:total RAP1A ratio detectable at 0.3μM (cerivastatin) and 3μM (simvastatin) but at 1μM (cerivastatin) and 10μM (simvastatin, acid form) in muscle and liver cell lines, respectively. This is consistent with our MTT data where RD cells were significantly more sensitive to statin-induced cytotoxicity than Huh7 cells. However, in all three cell types unprenylated Rap1A was detected at approximately 10-fold lower concentrations of cerivastatin than simvastatin; this is at variance with our MTT data, where IC50s were either similar for the two statins (RD-U), or lower for simvastatin (acid form) than cerivastatin (RD-D). Thus, whereas both statins caused a reduction in cell

viability and protein prenylation, the lack of concordance in concentration-response data is strongly supportive that these two phenomena are not causally linked.

To further examine this apparent lack of concordance between statin-mediated inhibition of prenylation and cell death, we next used a pharmacological inhibitor of prenyltransferases, GGTI-2133. This compound inhibits GGTase-I *in vitro* with an IC_{50} of 38 nM. Western blot analysis confirmed that GGTI-233 inhibited RAP1A prenylation in a concentration dependent manner, causing increased levels of unprenylated Rap1A in all three cell lines. Huh7 cells were the most sensitive, with a significant change in Rap1A prenylation status observed at the lowest concentration examined (0.1μM; Fig. 6B). In contrast, both muscle cell lines were more resistant, with significant changes in the unprenylated:total RAP1A ratio not observed until 1 μM. GGTI-2133 had no negative impact on either cell viability (Fig. 6C) or cell morphology (Fig. 6D and Supplemental Fig S2) at any concentration tested (maximal 50μM), while 10μM cerivastatin caused significant cell death and morphological changes indicative of apoptosis. To support that lack of toxicity with GGTI-2133 reflected a target-specific, rather than chemical-specific effect, two alternate prenyltransferase inhibitors were examined. RD-D cells exposed to FTI-277 (maximal 10μM) or perillyl alcohol (maximal 500μM) at concentrations producing greater than 99% inhibition of FTase and GGTase-II, respectively, also failed to significantly impact on cell viability. Lack of toxicity with three different prenyltransferase inhibitors at concentrations exceeding 10x IC_{50} for their target prenyltransferase strongly supports the hypothesis that inhibition of prenyltransferases themselves is not directly toxic to cells.

4. Discussion

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In order to understand both the mechanisms of statin toxicity and the differences between skeletal muscle and liver as sites of toxicity and therapy respectively, we have used cell lines that represent the target tissues for therapeutic and adverse effects. Huh7 cells are a hepatoma cell line commonly used for *in vitro* assessment of toxicity and its aetiology (Al-Salman and Plant 2012; Elphick et al. 2012; Kolodkin et al. 2013; Kolodkin et al. 2010; Lin et al. 2012). RD cells were derived from a human rhabdomyosarcoma and represent a mixture of spindle cells and larger multinucleated cells; these cells can also be induced to differentiate into myotube-like structures by exposure to low serum levels (Knudsen et al. 1998; Rossi et al. 2010). All three cell phenotypes (Huh7, RD-U and RD-D) expressed the target protein for statins, HMGCR, with levels highest in liver cells. Interestingly, HMGCR levels decrease during RD cell differentiation, while muscle-specific markers increase; this may reflect the lower requirements for synthesis of new membranes and generally lower metabolic status of differentiated muscle cells, which although not entirely quiescent, have much lower rates of cell division than in their undifferentiated state. As such, Huh7 and RD cells represent good model cell lines for examining the effect of statins on liver and muscle, respectively.

Since one of the principle branches of the mevalonate pathway is that of protein prenylation, and there has been much interest in this as a possible mechanism in statin-mediated toxicity. We examined the levels of prenyltransferases in Huh7 and RD cells, finding all three prenyltransferases present at equivalent levels in each cell line. We have used Rap1A, a member of the Ras-related family of G-proteins that requires prenylation for correct membrane insertion, as a marker of GGTas-I activity (Qian et al. 1998; Wasko et al.

2011). Under normal culture conditions, RAP1A is fully prenylated in all cell models, despite the significantly lower level of HMGCR present in RD cells. This suggests that in these cell lines, the ability to process prenylated proteins is not limited by rate of production of isoprenoids, or the level of the catalysing prenyltransferase.

Reported serum concentrations of statins during standard therapy are reported to be between 2-15nM, with Cmax concentrations of 8-40nM (Bjorkhem-Bergman et al. 2011). The concentrations used within the current study therefore include clinically-relevant concentrations, although it should be noted that the exact relationship between serum concentration and liver/muscle concentration is unclear. All nine of the statins tested showed some degree of concentration-dependent toxicity, mainly at doses that are close to that observed in clinical therapy. The exception was pravastatin, where toxicity only occurred at doses in the millimolar range, which is far above the expected therapeutic concentration. The latter observation is likely to be a mixture of both reduced toxic liability and restricted cellular access; pravastatin is the least lipophilic of all the statins and its uptake is highly dependent on the expression of SLCO1B1 (OATP1B1). SLCO1B1 is mainly expressed in the liver (Obaidat et al. 2012), but its expression in cultured hepatoma cells is severely limited (Cui et al. 2003; Rodrigues et al. 2009). Rosuvastatin also utilises SLCO1B1 for uptake, but may utilise a broader range of transporters, which may explain why it still exhibits toxicity (Obaidat et al. 2012). Other statins varied in their cytotoxicity, with cerivastatin and simvastatin showing the greatest toxicity, consistent with the published literature (Joshi et al. 1999; Serajuddin et al. 1991). It should be noted that the highly lipophilic lactone forms of simvastatin and lovastatin showed similar or lower toxicity than their acid forms. This is consistent with a requirement to metabolise these lactone pro-

drugs into their active acid form prior to any toxic effect being observed. In all cases, there was more toxicity observed in the muscle than the liver cell line; these results correlate well with clinical data for these drugs as well as with results reported in the literature (Mullen et al. 2010; Mullen et al. 2011). One general conclusion is that the statins appear to be more toxic to the muscle cell line (especially once differentiated) compared to the liver cell line. This might reflect a cell line-specific effect and not be related to the tissue or origin, with examination of further cell lines experiments required to confirm this finding. There are, however, two hypotheses by which muscle cells may be more susceptible to the toxic effects of statins: First, the metabolic capacity of the liver is superior to that of skeletal muscle, and while hepatoma cell lines have diminished metabolic capacity, they may still possess an enhanced capability to clear the statins, effectively lowering the intracellular concentration (Plant 2004, 2007). Second, due to the role of the liver in response to toxic insult, its repair systems (e.g. regenerative capacity, antioxidant response etc.) are highly developed. It is possible that liver cells are fundamentally more robust to toxicity than muscle cells. To our knowledge this is the most comprehensive comparison of statins across cell types to date, and as such provides an important resource for further investigations into statin-mediated effects in muscle and liver.

We further examined the effects of cerivastatin, simvastatin and pravastatin on prenylation in each cell line. These statins represent the archetypal toxic statin (cerivastatin), the most commonly prescribed statin (simvastatin) and the statin the lowest toxic potential in our preliminary work (pravastatin). The concentration-dependent effect of cerivastatin, simvastatin (acid form) and pravastatin on prenylation of Rap1A broadly reflected their relative toxicity, with RD cells (both differentiated and undifferentiated) approximately 3-

10-fold more sensitive than Huh7 cells. Such observations seem to support the generally accepted paradigm that statin-mediated toxicity is prenylation-dependent (Guijarro et al. 1998; Matzno et al. 2005; Sakamoto et al. 2007; Sakamoto et al. 2011; Takemoto and Liao 2001). However, further analysis argues against such a conclusion: First, relative sensitivities are not conserved between cell lines, with cell lines approximately 10-fold more sensitive to prenylation inhibition by cerivastatin than simvastatin, but the opposite is seen with regards to relative cytotoxicity (compare Fig. 5 and Fig. 3). Second, the specific GGTase-I inhibitor GGTI-2133 caused no toxicity when used at concentrations providing greater than 99% inhibition of GGTase-I in either the liver or muscle cell lines; this was not through a lack of cellular uptake or activity since at these concentrations unprenylated Rap1A was readily detected in all cell types. These data do not preclude the possibility that toxicity is through disruption of FTase or GGTase-II mediated prenylation, but some preliminary comment can be made. GGTI-2133 inhibits GGTase-I with an IC_{50} of 38nM, but also FTase with a much higher IC50 (IC_{50} 6μM) (Johnson et al. 2004). At the highest concentration of GGTI-2133 used (10μM), 99% and 65% inhibition of GGTase-I and FTase-I activity would be achieved, respectively. Under these conditions, no impact on cell viability was observed in any cell line, suggesting that FTase-mediated prenylation is also not associated with toxicity. This is further supported through the use of the FTase inhibitor FTI-277, where no toxicity was observed at concentrations that would cause 99% inhibition of both GGTase-I and FTase (Lerner et al. 1995a). . Finally, a similar absence of cytotoxicity was observed with the general prenylation inhibitor perillyl alcohol (Hohl and Lewis 1995). Further experimentation would be required to confirm that under the experimental conditions used inhibition of GGTase-II and FTase-dependent prenylation

had been achieved, for example using Rab and Ras family members as specific target proteins of GGTase-II and FTase respectively (Lerner et al. 1995a; Zhang and Casey 1996). However, given the experimental conditions are those previously shown to disrupt these enzymes *in vitro*, it is probable that this has been achieved in the current study.

The evidence here supports the hypothesis that inhibition of prenylation by statins is not causative of the toxicity seen in muscle and liver cells. However, it cannot be discounted that there is a higher-order interaction occurring between different cell-types or organs, meaning that in vitro-in vivo extrapolation is compromised. To examine such a possibility would require animal experimentation, and the data provided herein supports such a course of action. Presuming that inhibition of prenylation and toxicity are causally disassociated, it is interesting to speculate on alternate mechanisms. As previously stated, the alternative metabolic endpoints from the mevalonate pathway are the formation of isoprenoids, ubquinone and dolichol. Given that mevalonate can rescue cells from statin-mediated toxicity, it is logical to hypothesise that one of the alternate metabolic fates is central to the observed toxicity. Evidence exists to support both disruption of ubiquinone (Marcoff and Thompson 2007) and dolichol-mediated N-linked glycosylation (Mullen et al. 2010; Siddals et al. 2004). At present these areas have been poorly examined compared to prenylation, and should not be more fully explored. Together, these data are consistent with the conclusion that statin-mediated inhibition of prenyltransferases is not responsible for the observed toxicity of statins.

5. Conclusion

In the present study we have carried out a comprehensive comparison of statin effects in two cell lines, representing liver and muscle phenotypes. Our data show that statins are

more potent toxins towards muscle compared to liver cell phenotypes, and that this is likely to be a consequence of reduced capacity in the mevalonate pathway in this cell type. In addition, we provide strong evidence to counter the assumption that statin-mediated inhibition of prenylation is responsible for the observed toxicity. In vivo analysis should now be undertaken to confirm these findings in a whole animal setting.

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Figure Legends

Figure 1: The mevalonate pathway. Principle products are in boxes. Key enzymes are shown in italics, with relevant inhibitors in square brackets. Multistep processes (for instance in the production of cholesterol from lanosterol) are indicated with dotted lines and arrows

Figure 2: Basal comparisons of Huh7 and RD cells. Total protein was extracted from Huh7, undifferentiated RD (RD-U) or differentiated RD (RD-D). Specific proteins were detected immunologically using antibodies and conditions as given in the methods. A representative blot is shown in (A), with quantitation of triplicate independent repeats provided in (B). Error bars = S.E.M and ***=$p<0.001$ for indicated comparison.

Figure 3: Liver and muscle cell lines show differential sensitivity to statins. Huh7, undifferentiated RD (RD-U) or differentiated RD (RD-D) were exposed for 48 h with statins or appropriate vehicle controls (0.1% DMSO or medium alone). Cell viability was measured by MTT assay and is expressed as a percentage of vehicle control. Each data point represents the mean of three independent experiments. Error bars represent the standard error of the mean (SEM). Circular data points with solid lines = Huh7; square data points with hatched lines = RD-U; diamond data points with dotted lines = RD-D

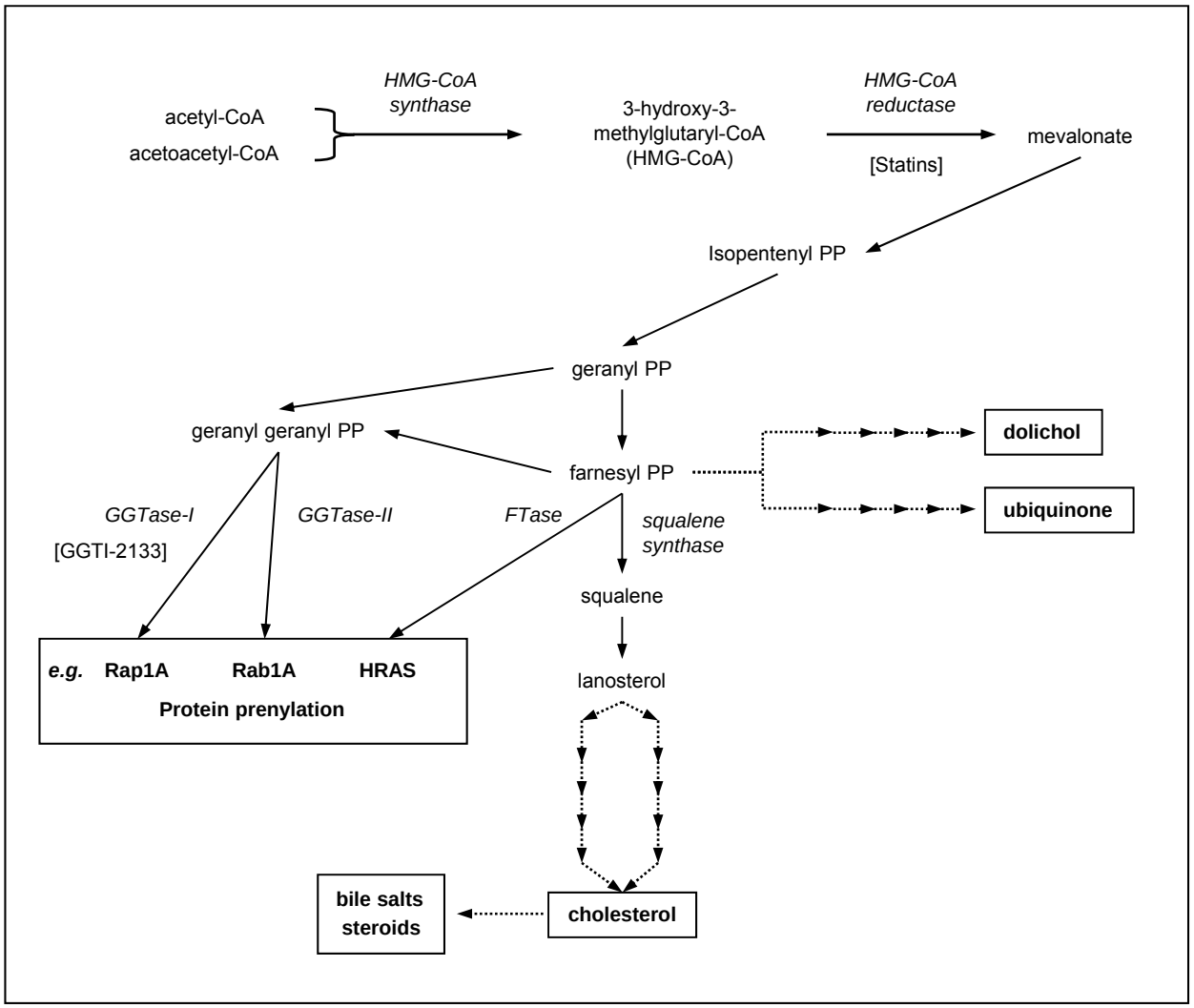
Figure 4: Effect of statin treatment on Rap1A prenylation. Total protein was extracted from Huh7, undifferentiated RD (RD-U) or differentiated RD (RD-D) exposed for 48 hours to statin (10 μM for simva- and pravastatin, or 1 μM cerivastatin), vehicle control (0.1%

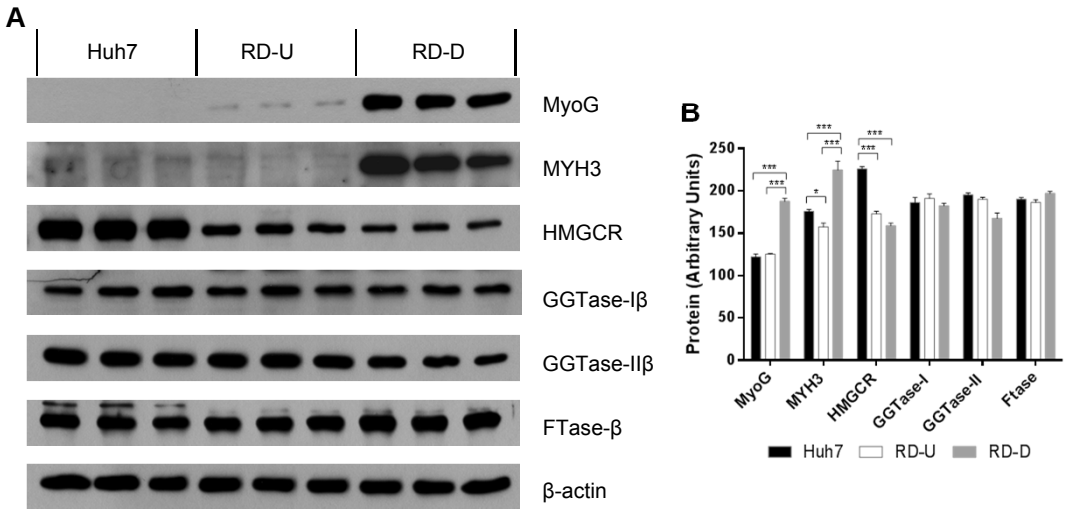
DMSO or serum free medium) or mevalonate (100 μM) plus statin. Protein was analysed by Western blotting using antibodies against the unprenylated form of Rap1A, total Rap1A protein and β-actin as a loading control. A representative blot is shown in (A), with the prenylated:total RAP1A ratio from three independent repeats provided in (B). Error bars = S.E.M, and ***=p<0.001 for indicated comparison.

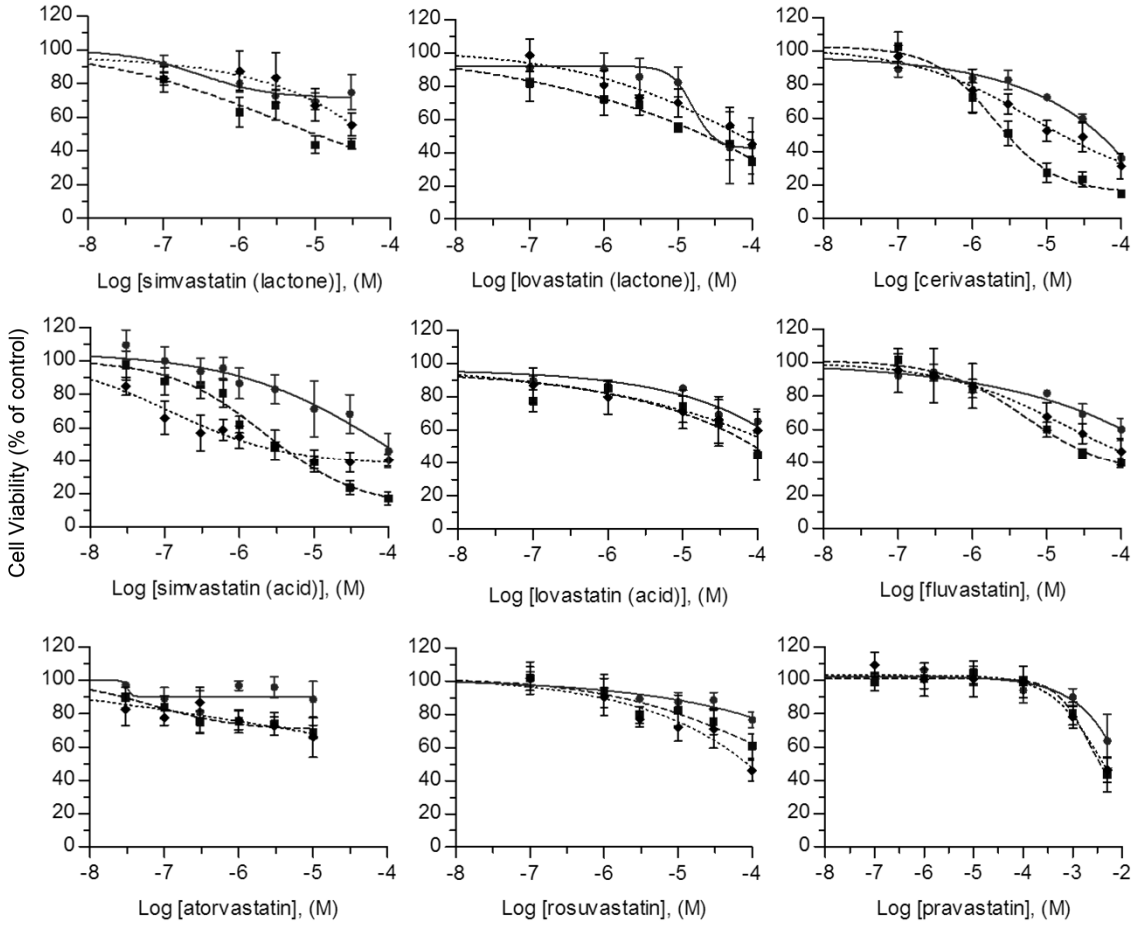
Figure 5: Sensitivity of Rap1A prenylation to HMGCR inhibition varies between statins and cell lines. Total protein was extracted from Huh7, undifferentiated RD (RD-U) or differentiated RD (RD-D) cells, following treatment for 48 h with varying concentrations of simvastatin (left panels) or cerivastatin (right panels). Protein was analysed by Western blotting using antibodies to the unprenylated form of Rap1A, total Rap1A protein and β-actin as a loading control. A representative blot is shown in (A), with the prenylated:total RAP1A ratio from three independent repeats provided in (B). Error bars = S.E.M, and *=p<0.05, **=p<0.01, ***=p<0.001 versus vehicle control.

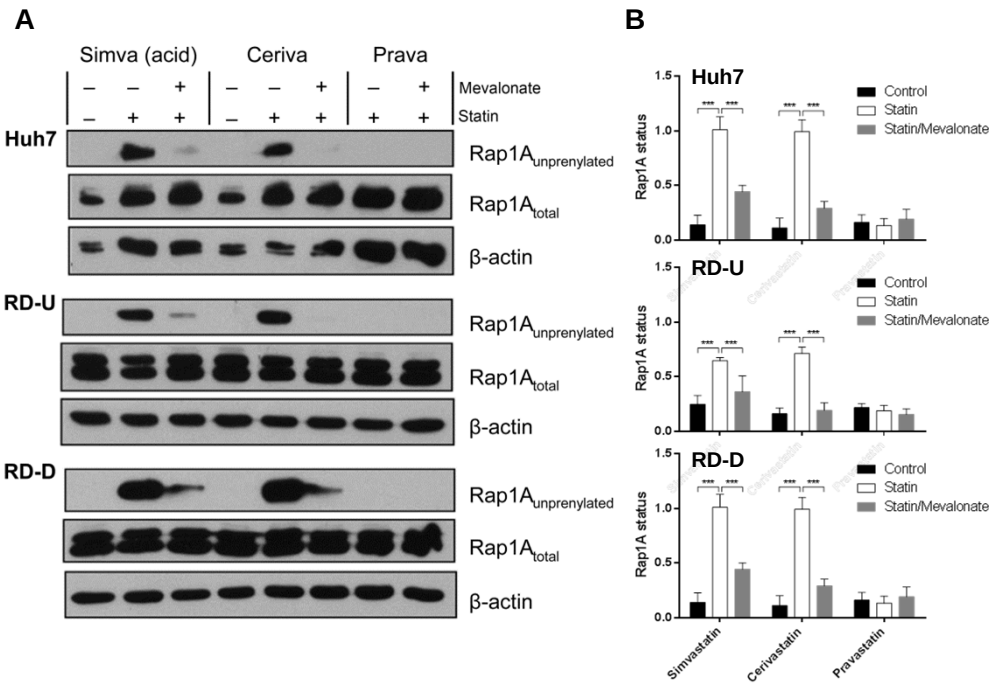
Figure 6: Prenyltransferase inhibitors do not impact on cell viability. Total protein was extracted from Huh7, undifferentiated RD (RD-U) or differentiated RD (RD-D) exposed for 72h to the GGTase-I inhibitor GGTI-2133. GGTase-I mediated prenylation was assessed by Western blotting using antibodies against Rap1A (unprenylated), total Rap1A and β-actin . A representative blot is shown in (A), with the prenylated:total RAP1A ratio from three independent repeats provided in (B). Cell viability was assessed by MTT assay (C), and morphological changes assessed by light microscopy at 400x magnification (D). Finally,

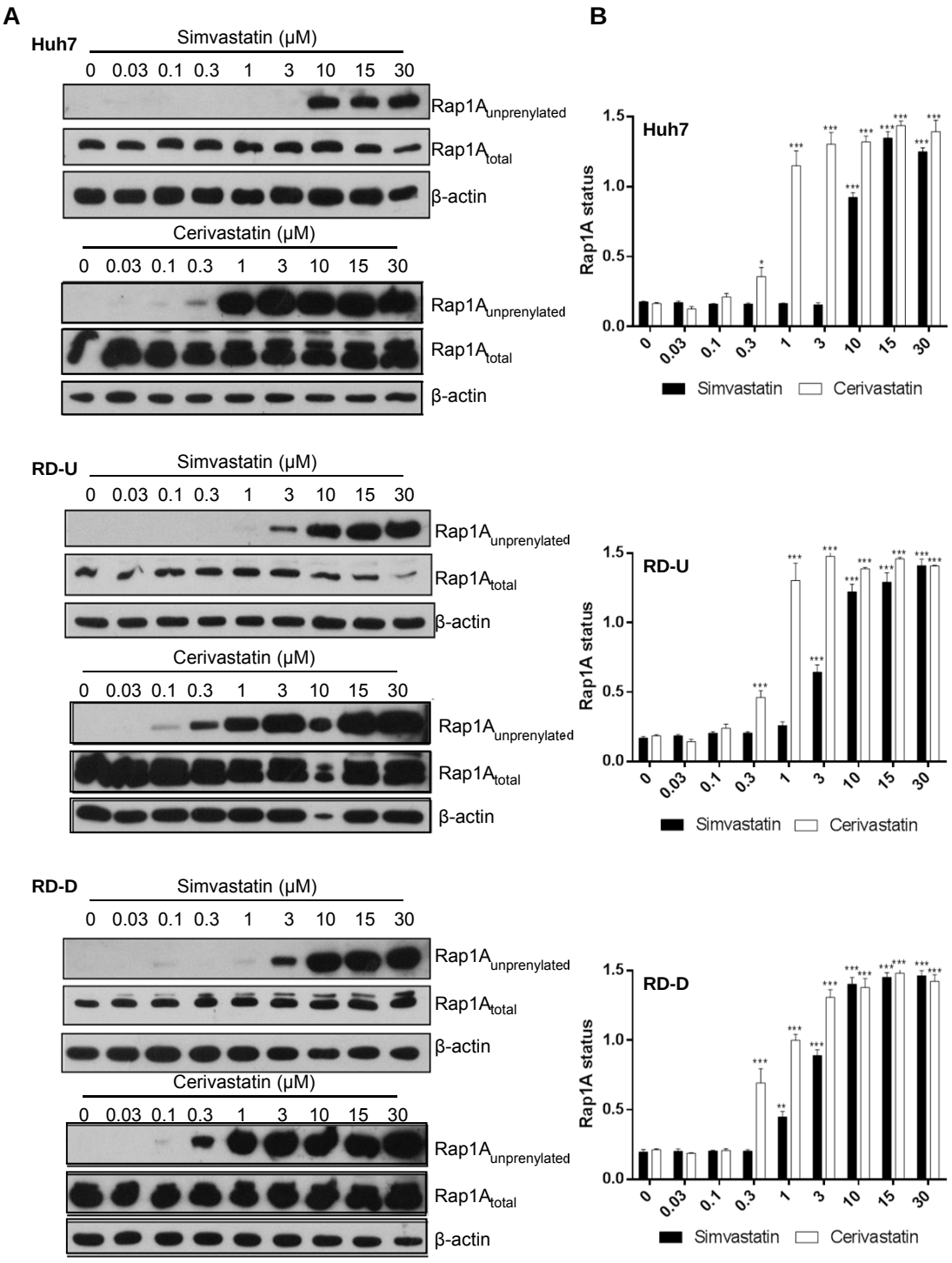
RD-D cells were exposed to the alternate prenyltransferase inhibitors FTI-277 and perillyl alcohol at the indicated concentrations for 72 h and cell viability assessed by MTT assay (E). Error bars = S.E.M, n=3, and **=$p<0.01$, ***=$p<0.001$ versus vehicle control.

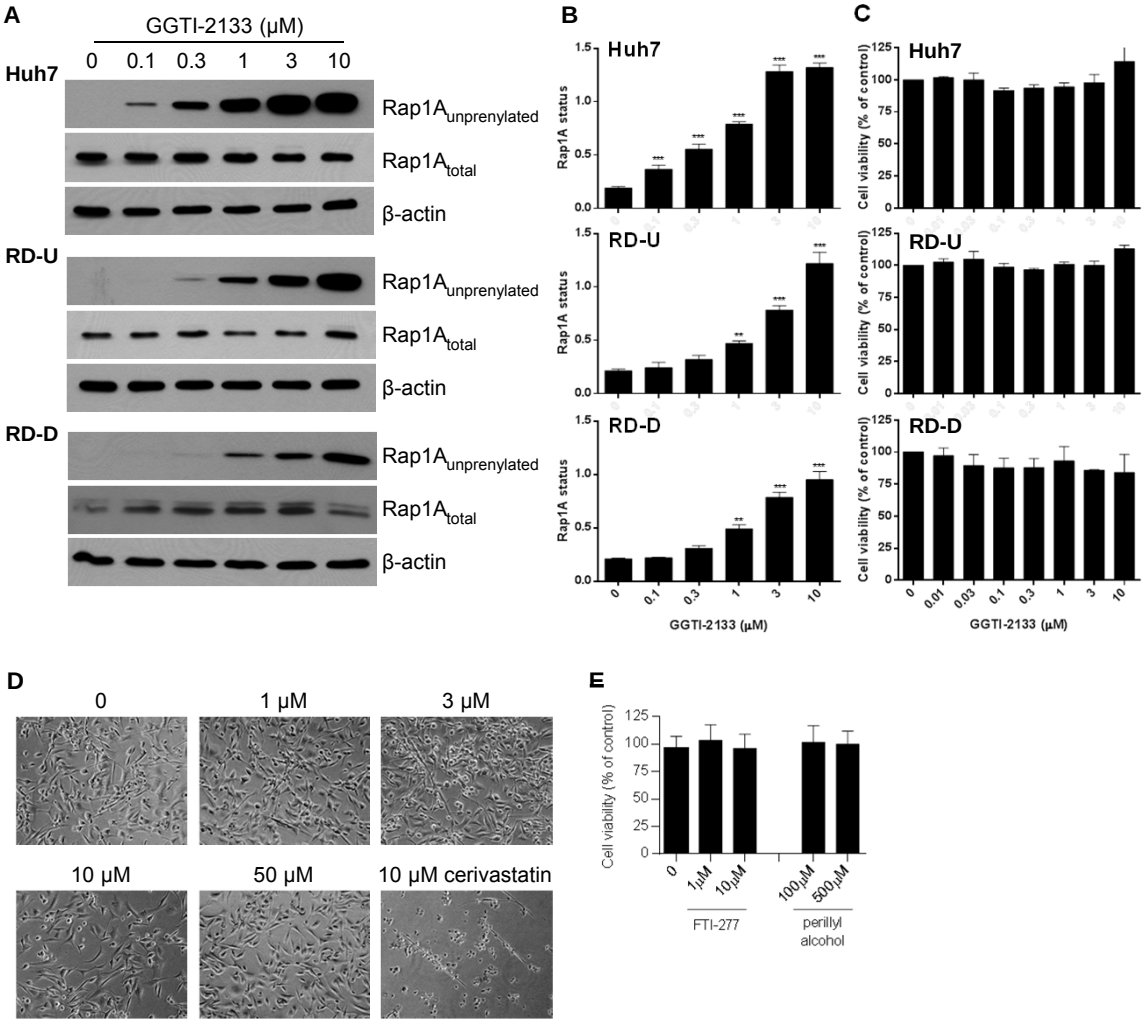

acetyl-CoA
acetoacetyl-CoA
HMG-CoA synthase
3-hydroxy-3-methylglutaryl-CoA (HMG-CoA)
HMG-CoA reductase
[Statins]
mevalonate
Isopentenyl PP
geranyl PP
geranyl geranyl PP
farnesyl PP
dolichol
ubiquinone
GGTase-I
[GGTI-2133]
GGTase-II
FTase
squalene synthase
squalene
lanosterol
e.g. Rap1A Rab1A HRAS
Protein prenylation
bile salts steroids
cholesterol


A
Huh7
RD-U
RD-D
MyoG
MYH3
HMGCR
GGTase-Iβ
GGTase-IIβ
FTase-β
β-actin
B
Protein (Arbitrary Units)
250
200
150
100
50
0
MyoG
MYH3
HMGCR
GGTase-I
GGTase-II
Ftase
Huh7
RD-U
RD-D


Cell Viability (% of control)
Log [simvastatin (lactone)], (M)
Log [lovastatin (lactone)], (M)
Log [cerivastatin], (M)
Log [simvastatin (acid)], (M)
Log [lovastatin (acid)], (M)
Log [fluvastatin], (M)
Log [atorvastatin], (M)
Log [rosuvastatin], (M)
Log [pravastatin], (M)


A
Simva (acid)
Ceriva
Prava
Mevalonate
Statin
Huh7
RD-U
RD-D
Rap1A unprenylated
Rap1A total
β-actin
B
Huh7
RD-U
RD-D
Control
Statin
Statin/Mevalonate
Rap1A status
Simvastatin
Cerivastatin
Pravastatin


A
B
Huh7
Simvastatin (μM)
0 0.03 0.1 0.3 1 3 10 15 30
Rap1A unprenylated
Rap1A total
β-actin
Cerivastatin (μM)
0 0.03 0.1 0.3 1 3 10 15 30
Rap1A unprenylated
Rap1A total
β-actin
Rap1A status
Simvastatin
Cerivastatin
RD-U
Simvastatin (μM)
0 0.03 0.1 0.3 1 3 10 15 30
Rap1A unprenylated
Rap1A total
β-actin
Cerivastatin (μM)
0 0.03 0.1 0.3 1 3 10 15 30
Rap1A unprenylated
Rap1A total
β-actin
Rap1A status
Simvastatin
Cerivastatin
RD-D
Simvastatin (μM)
0 0.03 0.1 0.3 1 3 10 15 30
Rap1A unprenylated
Rap1A total
β-actin
Cerivastatin (μM)
0 0.03 0.1 0.3 1 3 10 15 30
Rap1A unprenylated
Rap1A total
β-actin
Rap1A status
Simvastatin
Cerivastatin


A
GGTI-2133 (μM)
0 0.1 0.3 1 3 10
Huh7
Rap1A unprenylated
Rap1A total
β-actin
RD-U
Rap1A unprenylated
Rap1A total
β-actin
RD-D
Rap1A unprenylated
Rap1A total
β-actin
B
Huh7
RD-U
RD-D
Rap1A status
GGTI-2133 (μM)
C
Huh7
RD-U
RD-D
Cell viability (% of control)
GGTI-2133 (μM)
D
0
1 μM
3 μM
10 μM
50 μM
10 μM cerivastatin
E
Cell viability (% of control)
0
1μM
10μM
100μM
500μM
FTI-277
perillyl alcohol

Supplementary Data