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**STATINS INHIBIT THE DIMERISATION OF  $\beta$ -SECRETASE VIA BOTH ISOPRENOID- AND CHOLESTEROL-MEDIATED MECHANISMS**

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**SHORT TITLE:** Statin inhibition of BACE dimerisation

## SYNOPSIS

We have previously reported that protein lipidation in the form of palmitoylation and farnesylation is critical for the production of  $\beta$ -amyloid, the dimerisation of  $\beta$ -secretase and its trafficking into cholesterol-rich microdomains. As statins influence these lipid modifications in addition to their effects upon cholesterol biosynthesis, we have investigated the effects of lovastatin and simvastatin at a range of concentrations chosen to distinguish different cellular effects upon  $\beta$ -amyloid production and  $\beta$ -secretase structure and its localisation in bHEK cells. We have compared the changes brought about by statins to those brought about by the palmitoylation inhibitor cerulenin and the farnesyltransferase inhibitor CVFM. The statin-mediated reduction in A $\beta$  production correlated with an inhibition of  $\beta$ -secretase dimerisation into its more active form at all concentrations of statins investigated. These effects were reversed by the administration of mevalonate, showing that these effects were mediated via hydroxymethylglutaryl-CoenzymeA-dependent pathways. At low (1 $\mu$ M) statin concentrations, reduction in A $\beta$  production and inhibition of  $\beta$ -secretase dimerisation was mediated by inhibition of isoprenoid synthesis. At high (>10 $\mu$ M) concentrations of statins, inhibition of  $\beta$ -secretase palmitoylation occurred, which we demonstrated to be regulated by intracellular cholesterol levels. There was also a concomitant concentration-dependent change in  $\beta$ -secretase subcellular trafficking. Significantly,  $\beta$ -amyloid release from cells was markedly higher at 50 $\mu$ M simvastatin than at 1 $\mu$ M, whereas these concentrations resulted in similar reductions in total  $\beta$ -amyloid production, suggesting that low-dose statins may be more beneficial than high doses for the therapeutic treatment of Alzheimer's disease.

## KEYWORDS

Alzheimer's disease, amyloid- $\beta$ , dimerisation, palmitoylation, prenylation, trafficking

## ABBREVIATIONS USED IN THE TEXT

A $\beta$ :  $\beta$ -amyloid; AD: Alzheimer's disease; APP: amyloid precursor protein; ATORVA: atorvastatin; BACE:  $\beta$ -site amyloid cleaving enzyme (BACE); bHEK: HEK 293 transfected with the *Asp-2* gene plus a polyhistidine tag; CMD: cyclo- $\beta$ -methyl dextrin; ELISA: enzyme-linked immunosorbent assay; GPI: glycosyl-phosphatidylinositol; HmGCo-A: hydroxymethylglutaryl Coenzyme A; HRP: horse

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radish peroxidase; IP: isoprenoid; LOVA: lovastatin, MA: mevalonate; PA: palmitic acid; PBS: phosphate-buffered saline; SIMVA: simvastatin; TGN: trans-Golgi network

## INTRODUCTION

Patients on long-term statin treatment have been shown to have a 70% lower risk of developing Alzheimer's disease (AD) [1,2], and a recent study has shown that atorvastatin (ATORVA) improved the cognitive ability of patients with mild-to-moderate AD [3]. We have previously reported that the production of  $\beta$ -amyloid ( $A\beta$ ), whose aggregation and deposition as senile plaques is a pathological hallmark of AD [4], is reduced by lovastatin (LOVA) *in-vitro*, which can be reversed by addition of exogenous cholesterol [5]. Inhibition of the final step of cholesterol biosynthesis using BM15.766 reduces  $A\beta$  production [6], suggesting that statins may reduce  $A\beta$  production by lowering cholesterol levels.

However, statins have other cellular effects, the most relevant being an anti-inflammatory effect mediated by in part by inhibition of the synthesis of the isoprenoids farnesyl and geranylgeranyl pyrophosphate [7]. There is an inflammatory response in a significant number of AD cases [8]. Non-steroidal anti-inflammatories have been shown to reduce the incidence of AD by 40-50% and improve cognitive ability [9], however such treatments can be prohibitively toxic [10].  $A\beta$  stimulates the release of soluble inflammatory markers from microglia [11], and statins have been shown to reduce microglia activation [7]. Statin-mediated inhibition of isoprenoid synthesis reduces the release of  $A\beta$  from cells but increases the amount remaining within the cell [12].

Several studies have shown that statin actions on  $A\beta$  release is likely to be mediated by  $\beta$ -secretase.  $\beta$ -secretase ( $\beta$ -site amyloid-cleaving enzyme, BACE) is a 140kDa membrane-associated homodimer [13,14] containing extensive N-glycosylation sites which undergo successive processing and sulphation within the ER and Golgi apparatus [15]. BACE is palmitoylated on cysteine residues 478, 482 and 485, which promote its recruitment into cholesterol-enriched microdomains (rafts) [16]. Inhibiting the palmitoylation of these residues with the fatty acid synthase inhibitor cerulenin prevents BACE dimerisation and changes its intracellular trafficking, resulting in a decrease in  $A\beta$  production [17]. There is also substantial evidence that BACE associates with further proteins, for example BRI3 [18] and nicastrin [19], an integral component of the  $\gamma$ -secretase complex.

BACE is localised primarily to the acidic intracellular compartments such as the endosome and trans-Golgi network (TGN) [20], however a small yet significant amount also associates with APP within cholesterol rafts within the cell and the plasma membrane [21]. BACE does not contain a glycosylphosphatidylinositol (GPI) anchor, an important modification of proteins which associate with cholesterol-rich rafts, but BACE does associate with GPI-coupled proteins which may stabilise BACE within these cholesterol-rich rafts [22].

Though BACE does not contain the necessary isoprenylation consensus sequences, the demonstration by Cole and colleagues that reduction in isoprenoid synthesis by statins reduces A $\beta$  production [12] suggests that isoprenoids may still be involved in the production of A $\beta$  by BACE. We recently demonstrated that inhibiting protein farnesylation using the farnesyltransferase inhibitor CVFM resulted in a concentration-dependent decrease in A $\beta$  production, inhibition of BACE dimerisation and alteration in BACE subcellular localisation. This may involve an as-yet unidentified 53kDa isoprenylated protein which co-purifies with BACE [17].

Statins reduce both cholesterol and isoprenoid synthesis via their inhibition of hydroxymethylglutaryl-coenzyme A (HmGCoA) reductase. HmGCoA reductase catalyses the conversion of HmGCoA to mevalonate (MA), a common intermediate of both isoprenoid and cholesterol synthesis. Concentrations in the range of 1-5 $\mu$ M inhibit isoprenoid biosynthesis [23] whereas reduction of cholesterol biosynthesis requires concentrations in the region of 20-40 $\mu$ M [5]. We have shown that 40 $\mu$ M LOVA inhibits the palmitoylation of the CLR sequence of the BACE C-terminal tail [24]. The marked concentration differences in statin actions may be of relevance for the therapy of AD. We have investigated the concentration-dependence of statin-mediated A $\beta$  production in an *in-vitro* model system, and compared the effects of LOVA and simvastatin (SIMVA) to the effects of cerulenin and CVFM. The results are presented here.

## MATERIALS AND METHODS

Unless otherwise stated, all chemicals were obtained from Sigma-Aldrich (Poole, Dorset, UK). Statistical analysis was performed using the InStat statistical package

(GraphPad, San Diego, USA) using one-way ANOVA followed by Tukey post-hoc comparison test, comparing treated cells with untreated (media only).

### **Cell culture incubations**

We used a cell-line of HEK293 transfected with the *Asp-2* gene plus a poly-histidine coding tag (bHEK) [5,15] (a kind gift from GlaxoSmithkline, Harlow, UK) cultured in DMEM supplemented with 2% v/v **Ultrosor® G** (Pall BioSeptra, Cergy-Saint-Christophe, France). **Ultrosor® G** is free of cholesterol, palmitate and isoprenoid precursors, requiring cells to rely upon their own *de-novo* synthesis for these components. Other workers have studied the processing of BACE in transfected human 293 cells [24,27,28]. Cells expressing BACE were selected using geneticin (250µg/ml, Invitrogen, Paisley, UK).

The effects of LOVA and SIMVA were studied at 1, 10 & 50 µM. Farnesyltransferase activity in the cells was inhibited by the addition of CVFM (0.1µM, Alexis Corp, Nottingham, UK). Palmitoyltransferase activity was inhibited by the addition of cerulenin (5µg/ml). For MA reincorporation experiments, cells were co-incubated with 50µM SIMVA and 0 – 400µM MA. This has been shown to maintain isoprenoid levels whilst reducing cholesterol synthesis [12,29] and to return Aβ production back to those observed in untreated cells [30].

### **Enzyme-Linked Immunosorbent Assay (ELISA) detection of Aβ**

Aβ production was measured independently in three compartments: released, remaining and total [29]. Released Aβ consisted of Aβ secreted into the culture media. Remaining Aβ was obtained by removing media from the cells, washing once with phosphate-buffered saline (PBS, Invitrogen) and lysing with RIPA buffer [17], diluted 1:10 in PBS, pH 7.4, for 30 mins at 4°C. Total Aβ production was obtained by lysing cells into the culture media by the addition of 1xRIPA (final concentration 0.1X) to the culture media and incubating for 30 mins at 4°C. Prior to assay, all samples were centrifuged for 10 mins at 14,000 rpm in order to pellet insoluble material.

Aβ was quantitated using a double-antibody Aβ ELISA [11] using a combination of rabbit-anti-NTA4 [20,24,25] (2µg/ml), monoclonal anti-6E10 (1:2000, IDLabs,

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Glasgow, UK) and anti-mouse IgG, horse radish peroxidase (HRP) conjugate (1:500, AmershamBiosciences, Chalfont St. Giles, UK). The ELISA detected all species of A $\beta$  monomers, and is linear in the A $\beta$  concentration range of 1-100ng/ml [17,26]. The specificity of the ELISA for soluble monomeric A $\beta$  over oligomeric A $\beta$  was confirmed by using an A $\beta$  oligomer ELISA [11] to measure A $\beta$  oligomers in the synthetic A $\beta$ <sub>1-40</sub> samples used to construct the calibration curve for the A $\beta$  monomer ELISA. The A $\beta$  oligomer ELISA utilised anti-6E10 as a capture antibody in concert with biotinylated anti-6E10 to detect oligomers within samples. No oligomers were detected in any of the A $\beta$  standards used (data not shown), demonstrating that the combination of anti-NTA4 and anti-6E10 antibodies were specific for monomeric A $\beta$ . Samples (50 $\mu$ l) were applied in triplicate and incubated for 1.5 hours. Colour was developed by the addition of TMB peroxidase substrate (Europa Bioscience, Ely, UK). Absorbance at 420nm was measured using a MultiSkan EX 96-well plate reader (ThermoElectron Corporation, Basingstoke, UK). Concentrations were calculated from the average of three experiments all performed in triplicate and expressed as A $\beta$  concentration in ng/ml using standard concentrations of synthetic A $\beta$ <sub>1-40</sub>.

### **Cytotoxicity assay**

The cytotoxicity of inhibitors was measured in the bHEK cells using the LDH Cytotoxicity Kit (Roche Diagnostics, Lewes, UK). Results were calculated from the average of three experiments all performed in triplicate and were expressed as percentage cell death.

### **BACE and $\gamma$ -secretase activity assays**

BACE and  $\gamma$ -secretase activities were measured using the fluorescence-based  $\beta$ -secretase and  $\gamma$ -secretase assay kits (R&D Systems, Abingdon, UK). Results were expressed as specific activities (F.U./hour/mg protein), with protein concentrations measured using the BioRad D<sub>c</sub> Protein Assay Kit (BioRad Laboratories, Hemel Hempstead, UK).

### **Cellular cholesterol assay**



Total cellular cholesterol levels were measured using the fluorescence-based Amplex total cholesterol assay (Molecular Probes, Invitrogen). Total cellular cholesterol content was calculated and expressed as  $\mu\text{g}$  total cholesterol/mg protein.

### **Western blotting analysis of BACE**

BACE protein was detected in protein lysates using Western blotting as previously described [15] with appropriate modifications using our own anti-BACE antibody which detects both monomeric (70kDa) and dimeric (140kDa) forms of BACE [13,17,20]. As palmitoyl and farnesyl moieties are attached to proteins via reducible thioester bonds, samples were prepared by diluting samples 1:1 in both reducing (Laemmli sample buffer, buffer R) and non-reducing (buffer NR comprising Laemmli sample buffer without  $\beta$ -mercaptoethanol) buffers, followed by boiling for 5 minutes. Protein lysate samples (30 $\mu\text{l}$ ) were electrophoresed on a 4-12% NuPage Bis/Tris gel (Invitrogen), then transferred to PVDF membrane (Waters Ltd., Elstree, UK). BACE was detected using a combination of a rabbit anti-BACE peptide antibody (1:2000), mouse-anti-rabbit IgG, HRP conjugate (1:5000) (AmershamBiosciences) and electrochemiluminescence (ECL) detection (AmershamBiosciences) using Hyperfilm M (AmershamBiosciences). Molecular weights of visualised proteins were calculated using a calibration plot constructed using standards of known molecular weight and expressed as  $\text{kDa} \pm 9.3\%$ , calculated as the average error between actual molecular weights of the standards used and their observed molecular weights calculated from the resultant calibration line.

### **Confocal microscopy**

The subcellular localisation of BACE was visualised using dual-label confocal microscopy as previously described [5] using a combination of rabbit-anti-BACE (1:200) and a premixed secondary antibody complex consisting of biotinylated mouse-anti-rabbit IgG (1:500, Dako, Ely, UK) and streptavidin-linked Rhodamine (1:500, Dako). Cholesterol was detected using the cholesterol binding agent filipin (50 $\mu\text{g}/\text{ml}$ ). Fluorescence was observed using a Leica 510 Confocal Microscope using a x40 objective lens, with BACE observed at 543nm (red fluorescence) and cholesterol observed at 488nm (green fluorescence).

### **Incorporation of palmitoyl and farnesyl moieties**

*Incorporation of palmitoyl using [<sup>3</sup>H]-palmitate into BACE*

In order to assess the effects of LOVA and cholesterol upon the incorporation of palmitoyl into BACE, the incorporation of [<sup>3</sup>H] (9,10n)-palmitic acid (PA) using a method based upon that of Benjannet et al. [16] was employed. bHEK cells ( $5 \times 10^6$  cells/flask) were incubated for 16 hours with [<sup>3</sup>H]-PA (AmershamBiosciences) (1mCi/ml) in the presence and absence of LOVA. Additional cells were treated with 50 $\mu$ M LOVA and 200 $\mu$ g/ml cholesterol in cyclo- $\beta$ -methyl dextrin (CMD) plus [<sup>3</sup>H]-PA for 16 hours. BACE was isolated using nickel affinity purification as previously described [13]. Samples prepared in sample buffers R & NR were electrophoresed as above and stained for protein using the PlusOne silver staining kit (AmershamBiosciences). Proteins in which [<sup>3</sup>H]-PA was incorporated were visualised using Amplify fluorographic reagent (AmershamBiosciences) followed by exposure to HyperFilm MP (AmershamBiosciences) for 4 days at -70<sup>o</sup>C.

*Identification of incorporated isoprenyl using [<sup>3</sup>H]-mevalonate*

The nature of the isoprenylation of the 53kDa protein which co-purifies with BACE was characterised by assessing the incorporation of [<sup>3</sup>H]-isoprenoid ([<sup>3</sup>H]-IP) into this protein in the presence and absence of CVFM. Cells were incubated for 16 hours with 50 $\mu$ M LOVA, after which media was replaced with media supplemented with 50 $\mu$ M SIMVA plus [<sup>3</sup>H]-MA (200 $\mu$ Ci/ml, Tocris Bioscience, Bristol, UK) in the presence or absence of CVFM (0.1 $\mu$ M) as appropriate for a further 16 hours. Radiolabel incorporation was assessed as described above, with HyperFilm MP exposed to the gel for 5 weeks at -70<sup>o</sup>C.

**RESULTS****LOVA and SIMVA reduce the production of A $\beta$  by a mechanism independent of cholesterol**

Total A $\beta$  production was significantly reduced at all concentrations of statin investigated with the exception of 50 $\mu$ M LOVA (Figures 1A and B), which also gave an increase in cell death ( $3.9 \pm 1.95\%$  vs  $6 \pm 2.2\%$ ); however, this was not significant. LOVA (1 $\mu$ M and 10 $\mu$ M) reduced total A $\beta$  production by 58% and 67% respectively ( $p < 0.001$ ), with 50 $\mu$ M LOVA resulting in a 11% decrease in total A $\beta$  production ( $p < 0.05$ ). A $\beta$  release was not significantly affected, whereas A $\beta$  remaining within the cell decreased significantly by 81% and 95% at 1 $\mu$ M and 10 $\mu$ M respectively

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( $p < 0.001$ ). SIMVA reduced total A $\beta$  production at all three concentrations used, resulting in a 48% decrease in total A $\beta$  production at 50 $\mu$ M ( $p < 0.01$ ). In contrast to LOVA, SIMVA significantly increased the release of A $\beta$  at 10 $\mu$ M and 50 $\mu$ M, resulting in a 20% and a 66% increase in A $\beta$  release respectively ( $p < 0.05$  and  $p < 0.01$  respectively). A $\beta$  remaining within the cell decreased significantly at all three concentrations of SIMVA, decreasing by 66%, 79% and 85% respectively ( $p < 0.01$ ). Cerulenin and CVFM resulted in decreases in total A $\beta$  production of 57% and 54% respectively (Figure 1C) ( $p < 0.001$ ).

Reduction of total A $\beta$  production occurred at concentrations (1 $\mu$ M) of statins which did not change intracellular cholesterol levels as well as at concentrations at which intracellular cholesterol was significantly reduced (Table 1), suggesting that at low (1 $\mu$ M) concentrations, statins were exerting their effects upon A $\beta$  production via cholesterol-independent pathways. At higher (>10 $\mu$ M) concentrations, the cholesterol-dependent pathways may become more important, as intracellular cholesterol was significantly reduced at this concentration for both statins. Cholesterol reduction was concomitant with a statin concentration-dependent change in BACE trafficking (Figure 2). In untreated cells, BACE was co-localised with cholesterol within the Golgi apparatus and associated vesicles. Staining was punctate, with discrete areas of intense staining concurrent with cholesterol raft localisation. There was very little staining associated with other compartments within the cell. With increasing LOVA concentration, the punctate staining associated with cholesterol raft localisation was replaced with diffuse staining throughout the membrane. Staining associated with BACE became more pronounced in intracellular locations with increasing LOVA concentration. Co-localisation of BACE with non-raft associated cholesterol increased with increasing LOVA concentration, **resulting in an increase in orange/yellow staining in merged immunofluorescence images (Figure 2B & C)**. Staining associated with cholesterol decreased with increasing LOVA concentration, **which coincided with the measured decrease in total cellular cholesterol (Table 1)**. **This decrease in green fluorescence associated with cholesterol staining resulted in the staining associated with BACE within membranes and intracellular regions becoming more orange in appearance at 50 $\mu$ M LOVA (Figure 2D)**. Treatment of the cells with SIMVA, cerulenin or CVFM (data not shown) produced similar effects.

There was no correlation between A $\beta$  production and the in-vitro activities against synthetic substrates of either BACE or  $\gamma$ -secretase released from cells with detergent after incubation with statins (Table 2), suggesting that statins are not competitive inhibitors of BACE. There was also no correlation between A $\beta$  production and cell death, as there was no significant increase in cell death at any of the concentrations of statin investigated (data not shown).

### **LOVA and SIMVA inhibit the dimerisation of BACE**

As statins are also able to inhibit both palmitoyl transfer to BACE [24] and farnesyl biosynthesis, their effects upon BACE dimerisation were assessed using Western blotting (Figure 3). In cells grown in **Ultrosor® G** media alone, BACE consisted of two proteins of 145.5kDa and 73.7kDa, corresponding to the predicted molecular weights of the dimeric and monomeric forms of glycosylated and lipidated BACE as reported previously [13,14,17]. Treatment of the cells with either LOVA or SIMVA resulted in a single monomeric BACE protein of 73.7kDa (samples prepared with reducing agent, buffer R) or 65.5kDa (samples prepared in the absence of reducing agent, buffer NR) at 1, 10 and 50 $\mu$ M. Treatment of cells with cerulenin (73.7 and 67.5kDa for buffers R and NR respectively) and CVFM (73.7 and 69.3kDa) gave similar results. The differences in mobilities produced by reducing agent in the sample buffer may be caused by release of covalent modifications linked via thioester bonds or the reduction of internal disulphide bonds.

### **Mevalonate addition reverses statin-induced reduction of A $\beta$ levels and promotes the dimerisation of BACE**

The inhibition of A $\beta$  production by 50 $\mu$ M SIMVA was overcome by the addition of MA in a dose-dependent manner (Figure 4), with almost 100% A $\beta$  production restored at 400 $\mu$ M MA. This was concomitant with the reappearance of the dimeric form of BACE (Figure 4). The addition of MA did not return total cellular cholesterol levels back to those observed in untreated cells, nor did they differ from those observed in cells incubated with 50 $\mu$ M SIMVA alone (Table 3). Therefore, the inhibition of A $\beta$  production and BACE dimerisation by statins was mediated via inhibition of HmGCoA-reductase-dependent isoprenoid synthesis.

**BACE palmitoylation is regulated by intracellular cholesterol levels**

Our previous studies have shown that palmitoylation is not dependent upon prior protein farnesylation [17], and it is possible that palmitoylation is mediated by alterations in cellular cholesterol levels. In cells grown in **Ultroser®** G media alone, [<sup>3</sup>H]-PA was incorporated into both dimeric and monomeric forms of BACE (Figure 5A), resulting in labelled proteins of 141kDa and 75kDa respectively. Boiling in the presence of the reducing agent β-mercaptoethanol removed all trace of radiolabelled protein, showing that [<sup>3</sup>H]-PA was attached via a reducible thioester bond.

Incubation of cells with LOVA reduced the incorporation of [<sup>3</sup>H]-PA in a dose-dependent manner. [<sup>3</sup>H]-PA incorporation occurred into a single protein of 73kDa at 1μM LOVA (Figure 5A). As BACE dimerisation was inhibited at this concentration (Figure 3), this suggests that BACE palmitoylation is not inhibited at this concentration and does not require prior BACE dimerisation. [<sup>3</sup>H]-PA incorporation was reduced in monomeric BACE of 73kDa by 10μM LOVA, whereas [<sup>3</sup>H]-PA incorporation was totally abolished by 50μM LOVA. The addition of 200μg/ml cholesterol to cells treated with 50μM LOVA resulted in the incorporation of [<sup>3</sup>H]-PA into two proteins of 141kDa and 75kDa (Figures 5A), which corresponded to the appearance of both dimeric and monomeric BACE on Western blot (Figure 5B). The farnesyltransferase inhibitor CVFM had no effect upon palmitoylation of monomeric BACE, further supporting the hypothesis that statins inhibit BACE palmitoylation via their influence upon intracellular cholesterol levels and does not require prior BACE dimerisation. Cerulenin inhibited the palmitoylation of both forms of BACE.

**Statins inhibit farnesylation of a 53kDa protein**

In order to determine whether the isoprenylation present on the 53kDa protein is farnesyl or geranylgeranyl, the incorporation of [<sup>3</sup>H]-MA in the presence and absence of CVFM was investigated. As previously observed, there was significant isoprenyl incorporation into many bHEK proteins (Figure 6). Boiling protein samples in buffer R removed all [<sup>3</sup>H]-IP label, showing that the prenylation modifications were attached to proteins via thioester bonds. The isoprenylation of the 53kDa protein was inhibited by CVFM, showing that this protein is farnesylated. The farnesylated 53kDa protein

co-purified with BACE on a nickel affinity column, suggesting that the two proteins may be part of the same complex.

## DISCUSSION

As  $\beta$ -amyloid is implicated by the amyloid hypothesis as a causative agent in AD, BACE and  $\gamma$ -secretase offer potential targets for drugs for the disease. Recent evidence suggesting that statin use reduces the risk of AD makes it important to fully elucidate the mechanisms by which statins influence A $\beta$  production. The present study demonstrates that statins inhibit the dimerisation of BACE via HmGCoA-reductase-dependent pathways, involving both isoprenoid synthesis and intracellular cholesterol levels, which in turn regulates the palmitoylation of BACE. These data provide further evidence that physiologically active BACE exists as a complex of proteins, whose assembly is reliant upon protein lipidation reactions.

### **There is a dose-dependent effect of statins upon cellular cholesterol and isoprenoid levels**

The differences in the concentrations of statins required to inhibit both isoprenoid and cholesterol biosyntheses [20,25,30], in which we observed significant reduction in cellular cholesterol levels at LOVA and SIMVA concentrations greater than 10 $\mu$ M (Table 1), may reflect the differences in the way cellular levels of isoprenoids and cholesterol are maintained. There are two pools of cholesterol, free cholesterol and esterified cholesterol. Esterified cholesterol acts as a metabolic reserve of cholesterol which can be converted back to cholesterol by cholesterol esterase in times of low cellular cholesterol levels. This reduces the reliance of the cell upon *de-novo* cholesterol synthesis in order to maintain cellular cholesterol levels. In contrast, isoprenoids have no such metabolic reserve, and rely solely upon *de-novo* synthesis in order to maintain cellular isoprenoid levels. This would make cellular isoprenoid levels much more sensitive to HmGCoA reductase inhibition than cellular cholesterol levels, also resulting in preferential isoprenoid synthesis over cholesterol synthesis in the presence of MA, resulting in isoprenoid synthesis utilising the majority of the MA passing down this shared metabolic pathway. This is in accord with our results which show no effect of MA upon cellular cholesterol levels after 50 $\mu$ M LOVA incubation, and is also in agreement with studies by other laboratories which have shown that MA

reincorporation after statin inhibition of HmGCoA reductase preferentially increases isoprenoid synthesis over cholesterol synthesis [12,29].

### **Statins inhibit the dimerisation of BACE by inhibiting isoprenoid synthesis**

Our study is the first demonstration that BACE dimerisation can be inhibited by statins via HmG-CoA reductase-dependent isoprenoid biosynthesis. Blocking the dimerisation of BACE is an attractive therapeutic target, as the dimer is 30-fold more active at cleaving APP than the monomer [14] and so inhibiting BACE dimerisation would reduce A $\beta$  production markedly.

### **BACE palmitoylation is mediated via cholesterol**

LOVA treatment yielded dose-dependant concomitant changes in BACE subcellular trafficking and inhibition of palmitoylation, complete at 50 $\mu$ M LOVA, which were reversed by the addition of exogenous cholesterol (Figure 5), showing that both are connected to the cholesterol gradient in much the same way as the maturation of the glycosylation of BACE [31]. The palmitoylation of BACE does not require prior dimerisation, as monomeric BACE was palmitoylated in the presence of both 1 $\mu$ M LOVA and 0.1 $\mu$ M CVFM. It is also clear that palmitoylation is required, though not sufficient in itself, for dimerisation to occur, as inhibiting palmitoylation using cerulenin inhibits the dimerisation of BACE (figure 3) [17]. It is therefore likely that the cholesterol gradient may play a part in the dimerisation process, as reintroducing cholesterol after 50 $\mu$ M LOVA treatment results in the dimerisation of BACE and reincorporation of [ $^3$ H]-PA into both dimeric and monomeric forms of BACE. Palmitoylation is usually required by proteins to stabilise their association with membranes [32], an association that BACE requires for dimerisation to occur [14]. Therefore, in the temporal sequence of processing of BACE, palmitoylation occurs prior to BACE dimerisation and prosequence cleavage, which are driven by the cholesterol gradient. Inhibiting cellular cholesterol biosynthesis by LOVA lowers the intracellular cholesterol gradient [33,34], therefore reducing the cholesterol-driven palmitoylation and dimerisation of BACE.

### **BACE undergoes palmitoylation and associates with a farnesylated 53kDa protein in a sequential manner**

It is common for protein palmitoylation to require prior farnesylation [32], however BACE is not farnesylated [17]. There is mounting evidence that BACE exists as a complex within cholesterol-rich rafts [22], and though many associations with other proteins may be transitory in nature, some associations may be permanent. The association of BACE with a 53kDa farnesylated protein [17] would indirectly provide BACE with a farnesyl group necessary to stabilise BACE within its lipid raft localisation. We propose that BACE undergoes a timed sequence series of maturation steps prior to entry into the cholesterol-rich rafts, as summarised in Figure 7. After production within the endoplasmic reticulum and during its cholesterol gradient-driven processing through the TGN, BACE undergoes palmitoylation which is followed by dimerisation and association with a 53kDa farnesylated protein. This in turn stabilises BACE within the membrane, enabling it to undergo prosequence cleavage and subsequent recruitment into cholesterol-rich microdomains.

The model suggests several potential therapeutic targets which could be exploited in order to disrupt the assembly of the BACE complex. Inhibition of any of these steps would prevent the dimerisation of BACE and its association with cholesterol-rich rafts, all of which we have demonstrated experimentally. Such a strategy would channel APP processing via the non-amyloidogenic  $\alpha$ -secretase pathway. Transgenic BACE knock-out animal models show no pathological or behavioural abnormalities [35], which suggests that BACE is not necessary for survival. In contrast, BACE activity and expression increases as a consequence of natural ageing [36] and studies have shown a small but significant increase in BACE activity in patients with AD compared to non-disease controls [37]. As increased A $\beta$  load is associated with an increase in AD pathology [4], targeting the assembly of the BACE complex may be an effective therapeutic strategy.

#### **SIMVA increases the release of A $\beta$ from bHEK cells**

The decrease in A $\beta$  intracellular accumulation and production by low concentrations (<10 $\mu$ M) of statins was not unexpected. Previous studies have reported reductions in total A $\beta$  production and accumulation of upto 50% in HEK cells by concentrations of LOVA and SIMVA below 10 $\mu$ M [5,38], and upto a 40% reduction in intracellular accumulation of A $\beta$  by 4 $\mu$ M LOVA and SIMVA in isolated rat hippocampal neurones [29,39]. In contrast, Cole and colleagues have recently demonstrated that 10 $\mu$ M



LOVA increases the intracellular accumulation of A $\beta$ <sub>1-40</sub> by 400% in both HEK and SH-SY5Y cells [12]. It is unclear as to why this is the case, but it may represent methodological differences between studies.

However, we are the first to demonstrate an increase in A $\beta$  release in response to incubation with SIMVA. SIMVA at 10 $\mu$ M and 50 $\mu$ M decreased cellular cholesterol levels (Table 1) and increased A $\beta$  release from bHEK (Figure 1). This is in contrast with previous studies which have reported a 50-90% reduction in A $\beta$  release from cells exposed to 10 $\mu$ M SIMVA [3,29,38,39], however it is an effect we have previously observed [26]. This leakage of A $\beta$  that we observe most likely arises from the reduction in plasma membrane cholesterol [33,34], which would make it more permeable to small molecules such as A $\beta$  and thus result in the statin concentration-dependent increase in A $\beta$  that we observe.

#### **LOVA and SIMVA have different effects upon A $\beta$ production**

It is clear from our data that LOVA and SIMVA, though they both have the same effect upon A $\beta$  production, differ in the degree to which they have their effects. The percentage reductions in remaining and total A $\beta$  was less for SIMVA than for LOVA, suggesting that SIMVA is less effective at reducing A $\beta$  production than LOVA. Such a difference is not unexpected as different statins exhibit different abilities in inhibiting microglia activation [40]. Such differences between statins are not limited to *in-vitro* studies. Wolozin and colleagues reported that the prevalence of AD in patients receiving LOVA therapy was significantly reduced compared to the total patient population within their study whereas the prevalence of AD in patients receiving SIMVA did not differ from the total patient population [2].

The reasons for these differences are unclear. LOVA and SIMVA, which differ structurally by an additional methyl group, are transported into the cell via passive diffusion across the membrane, and have correspondingly similar IC<sub>50</sub> values for the inhibition of HMGCoA reductase [34]. Additionally, both statins are metabolised by CYP 3A4 [34].

Recently, it has become possible to obtain SIMVA over-the-counter without the need for a prescription. Taking into consideration both our data showing a greater effect of

LOVA upon A $\beta$  production compared to SIMVA and the reduction in the prevalence of AD by LOVA as reported by Wolozin and colleagues [2], it is likely that this change in prescription status for SIMVA will have little or no affect upon the incidence of AD in the general population, and that the use of LOVA would be more beneficial.

### **Low dose statin therapy may be beneficial for the treatment of AD**

Since the original retrospective reports showing a reduction in the incidence of AD in patients taking long-term statins, there has been much discussion regarding their use as an anti-AD treatment. Initial promising results have not been replicated in large-scale studies, with little effect seen on cognitive decline. A recent study has demonstrated a potentially beneficial effect of ATORVA upon the cognitive decline in patients with mild AD, however the difference observed was small [3]. The 80mg/day dosage of ATORVA used in these clinical studies is the maximal recommended dose for patients [29], and therefore represents statin dosage at the highest level. We have previously shown that statins at concentrations which reduce cholesterol production (20 $\mu$ M) reduce the total production of A $\beta$  in bHEK cells, yet increase its release from the cell [26]. Our present data confirm this finding whilst showing that lower doses may be just as effective at reducing total A $\beta$  production. At 1 $\mu$ M SIMVA, we observed a 42% decrease in total A $\beta$  produced, which differed from 50 $\mu$ M by a mere 5%, suggesting that low doses may be just as effective as high doses.

This benefit may not be solely limited to the low dose required, however. The amount of A $\beta$  released from cells increased by 66% at 50 $\mu$ M SIMVA, compared to a 20% increase at 1 $\mu$ M. The observed increase in release has ramifications for the use of statins in AD treatment. If this were to occur in the *in-vivo* human brain, this increase in A $\beta$  release at high statin concentrations may have detrimental consequences. An increase in A $\beta$  production under these conditions may accelerate the oligomeration of A $\beta$  into toxic aggregates, which may therefore be masking the beneficial effects of statins in previous clinical studies. The bioavailability of both LOVA and SIMVA is below 5%, whilst the bioavailability of ATORVA is 12% [34]. With the standard dose of ATORVA (40mg/day), this would result in a plasma concentration of approximately 1-5nM. Statins have a very high affinity for HMGCoA-reductase, in the region of 1.2 – 55nM [34], which places the maximal ATORVA concentration

within the upper end of this range. Therefore, use of low-dose statins may demonstrate a more beneficial effect in such trials.

The concentrations used in our study are in line with others who have used statins in their studies [3,5,12,23,29,30,38,39]. However, the differences in concentrations required to reduce cholesterol synthesis *in-vivo* and *in-vitro* may reflect differences in statin metabolism. *In-vitro*, alterations in plasma cholesterol levels require a number of weeks administration of statins. In contrast, the decreases in cellular cholesterol levels occur within a very short time frame. Both SIMVA and LOVA are prodrugs, administered in their lactone form which undergo subsequent reversible conversion within the body to their active open acid forms [34,41]. It is probable that these interconversions, though they may occur *in-vitro*, do not occur to a significant degree in comparison, and therefore much higher concentrations of statins are required in order to achieve physiological effects. It is therefore probable that the effective concentration of statin open acid forms used *in-vitro* may well be within the low nM range. It is also common to administer high doses of statin in *in-vivo* models in order to obtain a rapid decrease in cellular cholesterol levels [29]. Irrespective of these considerations however, the concentrations used in our study were chosen to allow the investigation of the cholesterol- and isoprenoid-effects of statins, and we have demonstrated that each of these lipid effects mediate the production of A $\beta$  via BACE.

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**TABLES**

Table 1. Effect of LOVA and SIMVA upon total cellular cholesterol concentration.

		Cholesterol concentration
Untreated		785.7 ± 56.2
1µM		694.9 ± 40.0 <sup>ns</sup>
10µM	Lovastatin	602.2 ± 23.5 <sup>*</sup>
50µM		682.4 ± 31.8 <sup>*</sup>
1µM		615.4 ± 75.9 <sup>ns</sup>
10µM	Simvastatin	590.2 ± 32.7 <sup>**</sup>
50µM		569.6 ± 24.7 <sup>**</sup>

Total cholesterol concentration, measured as free and esterified cholesterol, was calculated as the average of three experiments each performed in triplicates expressed as µg cholesterol/mg protein. ns = not significant; \* = p<0.05; \*\*\* = p<0.001.



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Table 2. Effect of statins, cerulenin and CVFM upon BACE and  $\gamma$ -secretase activity.

		BACE activity (S.A. $\pm$ S.D.)	$\gamma$ -secretase activity (S.A. $\pm$ S.D.)
Untreated		34288 $\pm$ 1824	759.8 $\pm$ 39.6
1 $\mu$ M		22707 $\pm$ 1143 <sup>***</sup>	729.6 $\pm$ 8 <sup>ns</sup>
10 $\mu$ M	Lovastatin	47685 $\pm$ 1571 <sup>***</sup>	925.5 $\pm$ 26.8 <sup>**</sup>
50 $\mu$ M		27508 $\pm$ 1394 <sup>***</sup>	812.4 $\pm$ 10.1 <sup>ns</sup>
1 $\mu$ M		30186 $\pm$ 171 <sup>**</sup>	1192.1 $\pm$ 54.5 <sup>***</sup>
10 $\mu$ M	Simvastatin	29702 $\pm$ 1437 <sup>**</sup>	629.2 $\pm$ 2.58 <sup>**</sup>
50 $\mu$ M		40326 $\pm$ 1632 <sup>***</sup>	1231.9 $\pm$ 35.1 <sup>***</sup>
5 $\mu$ g/ml	Cerulenin	30329 $\pm$ 1901 <sup>ns</sup>	1377.8 $\pm$ 85.6 <sup>***</sup>
0.1 $\mu$ M	CVFM	31692 $\pm$ 678 <sup>ns</sup>	738.6 $\pm$ 10.8 <sup>ns</sup>

Specific activity, calculated from three independent experiments in which each experiment was performed in triplicate, is expressed as fluorescent units/hour/mg protein. ns = not significant; \*\* =  $p < 0.01$ ; \*\*\* =  $p < 0.001$ .

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Table 3. Effect of co-incubation of 50µM SIMVA and 0-400µM MA upon total cellular cholesterol levels.

MA concentration (µM)	Cholesterol concentration (percentage of untreated ± S.D.)	vs untreated <sup>a</sup>	vs 50µM SIMVA <sup>b</sup>
Untreated	100 ± 5.63	-	-
0	72.5 ± 2.97	p<0.001	-
50	55.6 ± 2.84	p<0.001	n/s
100	75.6 ± 2.00	p<0.001	n/s
250	53.5 ± 1.85	p<0.001	n/s
400	78.2 ± 6.82	p<0.001	n/s

Total cholesterol concentration, measured as free and esterified cholesterol, was calculated as the average of triplicates and expressed as percentage of total cellular cholesterol concentration in untreated cells, which was taken as 100%. In all incubations, cells were co-incubated with 50µM SIMVA and 0 – 400µM MA, with the exception of untreated cells, which were incubated in **Ultroser®** G media alone.

<sup>a</sup>Cellular cholesterol levels in cells co-incubated with 50µM SIMVA and increasing concentrations of MA were compared with cholesterol levels in cells grown in **Ultroser®** G media only to determine whether increasing concentrations of MA were able to reverse the reduction of total cellular cholesterol levels by 50µM back to those levels observed in untreated cells. <sup>b</sup>Cellular cholesterol levels in cells pretreated with 50µM SIMVA and increasing concentrations of MA were compared to cellular cholesterol levels in cells pretreated with 50µM SIMVA alone to determine whether total cellular cholesterol levels differed at any of the concentrations of MA investigated. n/s = not significant; \*\*\* = p<0.001.

**FIGURE LEGENDS**

Figure 1. *LOVA and SIMVA reduce total A $\beta$  production.* Cells were incubated with 0, 1, 10 and 50 $\mu$ M statin, 5 $\mu$ g/ml cerulenin or 0.1 $\mu$ M CVFM for 16 hours prior to assay. A $\beta$  was measured in the removed media (released), lysed cells with media (total), and lysed cells (remaining) using an A $\beta$  ELISA assay and expressed as ng/ml  $\pm$  S.D., with statistical analysis of untreated- vs. statin-treated cells performed using one-way ANOVA with Tukey post-hoc comparisons. Results were calculated as the average of three independent experiments, with each experiment assayed in triplicate. (A) Effect of LOVA upon A $\beta$  release (top panel), A $\beta$  remaining within the cell (middle panel) and total A $\beta$  production (bottom panel). \* =  $p < 0.05$ ; \*\*\* =  $p < 0.001$ . (B) Effect of SIMVA upon A $\beta$  release (top panel), A $\beta$  remaining within the cell (middle panel) and total A $\beta$  production (bottom panel). \* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ; \*\*\* =  $p < 0.001$ . (C) Effect of CVFM and cerulenin upon A $\beta$  release, A $\beta$  remaining within the cell and total A $\beta$  production. \*\*\* =  $p < 0.001$ . Black bars: untreated cells; white bars: 0.1 $\mu$ M CVFM; hashed bars: cerulenin.

Figure 2. *LOVA changes subcellular trafficking of BACE.* Cells were incubated with 0, 1, 10 & 50 $\mu$ M LOVA, 5 $\mu$ g/ml cerulenin or 0.1 $\mu$ M CVFM, after which BACE expression was detected using confocal microscopy using a combination of anti-BACE (1:200), biotinylated mouse-anti-rabbit IgG (1:500) and streptavidin-linked rhodamine (1:500). Cholesterol localisation was determined using the cholesterol marker filipin (50 $\mu$ g/ml). Fluorescent staining was observed with a Leica 510 confocal microscope using an excitation wavelength of 543nm for BACE (red fluorescence) and an excitation wavelength of 488nm for cholesterol (green fluorescence). BACE and cholesterol co-localisation was observed as orange/yellow fluorescence. In untreated cells (panel A), BACE was co-localised with cholesterol within the Golgi apparatus and associated vesicles (arrows). Staining was punctate, corresponding to cholesterol raft localisation. There was no staining within other intracellular areas of the cell (\*). With increasing concentrations of LOVA (panels B and C), BACE localisation became much more general throughout the cell (\*), with staining within the membrane becoming much more diffuse (arrows). This resulted in an increase in co-localisation with cholesterol (orange/yellow staining). **At 50 $\mu$ M LOVA, BACE localisation became much more diffuse throughout the membrane, with staining present in intracellular areas (\*). However, due to the decrease in**

staining associated with cholesterol arising from decreased cellular cholesterol, this staining was predominantly orange in appearance, with discrete areas of yellow staining in a very small number of areas (arrows) (panel D). These changes were in accord with changes in BACE localisation elicited by cerulenin and CVFM (data not shown). Panel A: untreated cells; B: 1 $\mu$ M LOVA; C: 10 $\mu$ M LOVA; D: 50 $\mu$ M LOVA. Scale bar = 10 $\mu$ m.

Figure 3. *LOVA and SIMVA inhibit BACE dimerisation.* Cells were incubated as per Figure 1, and BACE was detected using Western blotting with the following antibody combination: anti-BACE (1:2000), overnight 4°C; mouse-anti-rabbit IgG Horseradish peroxidase conjugate (1:5000), 1 hour RT. Molecular weights of detected proteins were calculated using a calibration line constructed using standards of known molecular weight and expressed as kDa. Samples were loaded in couplets, the first lane of each comprising sample boiled for 5 minutes in reducing sample buffer, and the second lane of each couplet boiled for 5 minutes in non-reducing sample buffer. Lanes 1&2: untreated cells; 3&4: 1 $\mu$ M LOVA; 5&6: 10 $\mu$ M LOVA; 7&8: 50 $\mu$ M LOVA; 9&10: 1 $\mu$ M SIMVA; 11&12: 10 $\mu$ M SIMVA; 13&14: 50 $\mu$ M SIMVA; 15&16: 5 $\mu$ g/ml cerulenin; 17&18: 0.1 $\mu$ M CVFM.

Figure 4. *MA reverses the effects of statins upon A $\beta$  production and BACE dimerisation.* Cells were incubated with 50 $\mu$ M SIMVA and 0 – 400 $\mu$ M MA for 16 hours prior to ELISA and Western blot. Total A $\beta$  production was measured using an A $\beta$  ELISA assay and expressed as ng/ml  $\pm$  S.D., with statistical analysis of untreated- vs. MA-treated-cells performed using one-way ANOVA with Tukey post-hoc comparisons. Results were calculated as the average of three independent experiments, with each experiment assayed in triplicate. \* = p<0.05; \*\* = p<0.01; \*\*\* = p<0.001; ns = not significant. BACE was detected using Western blotting as per Figure 3, using samples prepared by boiling for 5 minutes in non-reducing buffer only. Molecular weights were calculated using a calibration line produced from standards of known molecular weight and expressed as kDa. Upper arrow = 140kDa; lower arrow = 70kDa.

Figure 5. *BACE palmitoylation is mediated by cholesterol.* Cells were incubated for 16 hours in the presence of [<sup>3</sup>H]-PA (1mCi/ml) in normal media or media

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supplemented with 1, 10 & 50 $\mu$ M LOVA, 5 $\mu$ g/ml cerulenin or 0.1 $\mu$ M CVFM, or in media supplemented with 50 $\mu$ M LOVA and 200 $\mu$ g/ml cholesterol. (A) *Effect of statins and cholesterol upon the incorporation of [<sup>3</sup>H]-PA into BACE.* After incubation, cells were lysed, then BACE was purified using nickel affinity purification and subjected to SDS-PAGE. Radiolabel incorporation was visualised using fluorography, with gel exposure at -70<sup>0</sup>C for 4 days. Molecular weights of detected proteins were expressed as kDa. Each sample was prepared in either non-reducing (odd-numbered lanes) or reducing (even-numbered lanes) buffer, which were loaded in adjacent lanes, in order to see if [<sup>3</sup>H]-PA-label was attached via thioester linkages. The exceptions to this were cerulenin and CVFM treated samples, which were prepared in non-reducing sample buffer only. Lanes 1&2: untreated cells; 3&4: 1 $\mu$ M LOVA; 5&6: 10 $\mu$ M LOVA; 7&8: 50 $\mu$ M LOVA; 9&10: 50 $\mu$ M LOVA + 200 $\mu$ g/ml cholesterol in CMD; 11: 5 $\mu$ g/ml cerulenin; 12: 0.1 $\mu$ M CVFM. (B) *Effect of cholesterol upon BACE dimerisation.* BACE was detected using Western blotting as previously described, with samples prepared in non-reducing sample buffer. Molecular weights of detected proteins were calculated and expressed as kDa. Lane 1: untreated cells; 2: 50 $\mu$ M LOVA; 3: 50 $\mu$ M LOVA + 200 $\mu$ g/ml cholesterol.

Figure 6. A 53kDa protein which co-purifies with BACE is farnesylated. Cells were incubated for 16 hours with 50 $\mu$ M LOVA, followed by further overnight incubation with 50 $\mu$ M LOVA plus [<sup>3</sup>H]-MA (200 $\mu$ Ci/ml) in the presence and absence of CVFM (0.1 $\mu$ M). Cells were lysed and BACE purified using nickel affinity purification. Samples were prepared in non-reducing sample buffer only in order to ensure that any thioester-linked radiolabelled isoprenyl moieties were not removed. Samples were electrophoresed and radiolabel incorporation was determined as described in Figure 5, with gel exposure at -70<sup>0</sup>C for 5 weeks. Molecular weights of detected proteins were calculated and expressed as kDa. The figure is a typical result constructed from three experiments and is representative of results obtained. Lanes 1&2: all radiolabelled proteins from cells incubated in the absence of CVFM prepared in non-reducing and reducing buffer respectively; 3: purified BACE sample from cells incubated in the absence of CVFM; 4: purified BACE sample from cells incubated in the presence of 0.1 $\mu$ M CVFM.

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Figure 7. *Schematic representation of the palmitoylation of BACE, its association with farnesylated 53kDa protein and its stabilisation into its dimeric physiological form.*

Upon synthesis, BACE associates with the endoplasmic reticular membrane (1) where it undergoes cholesterol-driven palmitoylation (2), stabilising its association with the membrane. BACE then dimerises (3) upon association with farnesylated 53kDa protein (4), stabilising its dimeric structure within the membrane. BACE's prosequence is then cleaved (5), forming the physiological BACE complex, which then enters the cholesterol-rich rafts. Palmitoylation of BACE's C-terminal tail (closed circles) and association with the farnesylated 53kDa protein (open circles) provides the dual-lipidation common in lipid-raft-associated proteins.

FIGURE 1

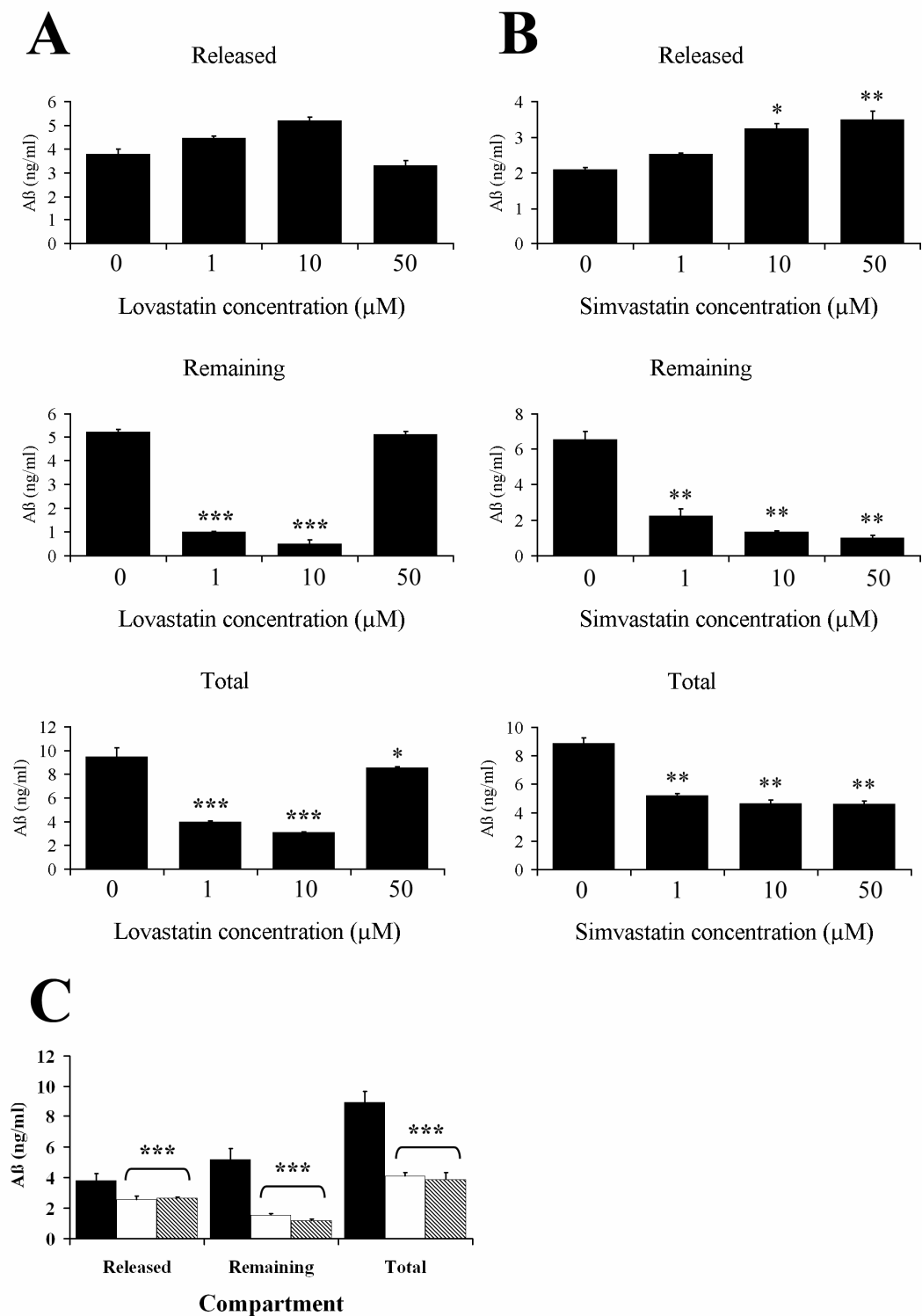


FIGURE 2

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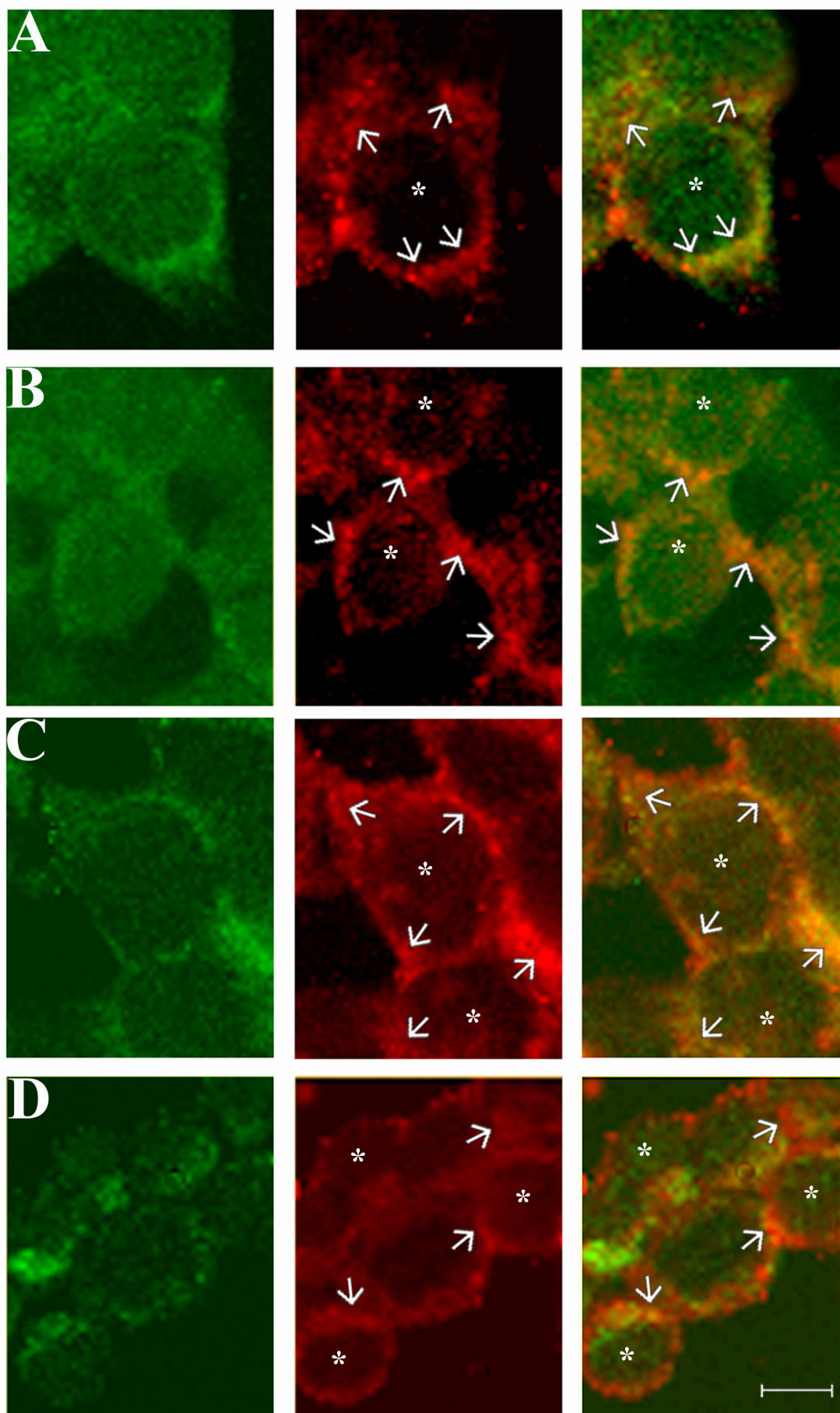




FIGURE 3

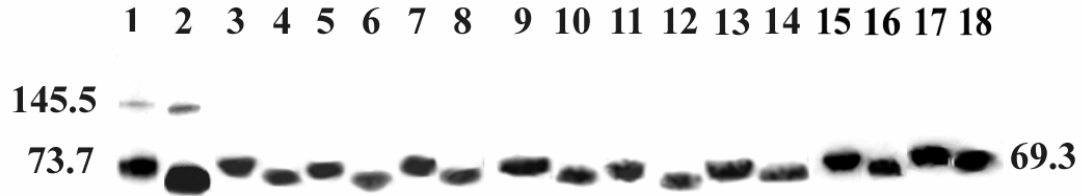


FIGURE 4

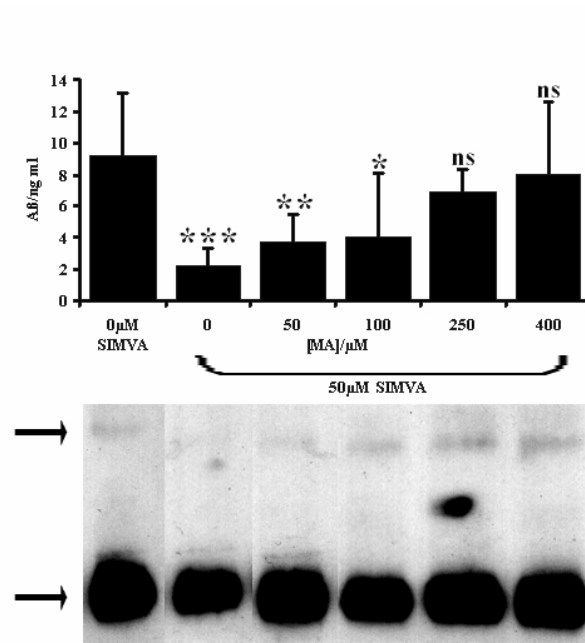


FIGURE 5

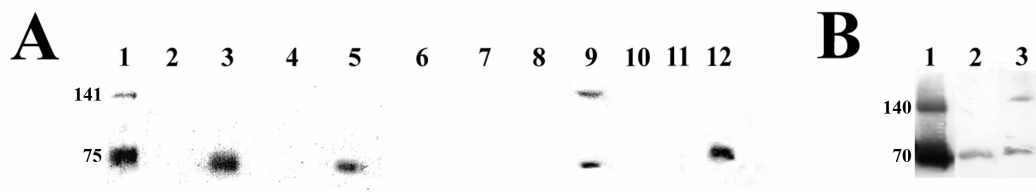


FIGURE 6

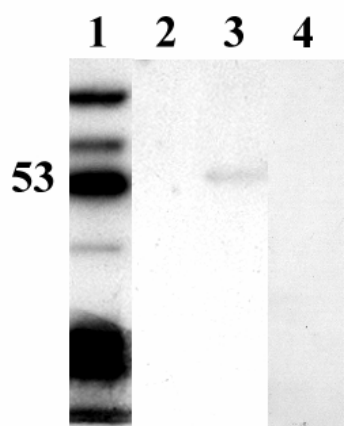


FIGURE 7

