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# TOXICITY OF STATINS ON NEURONAL CELLS: POSSIBLE REVERSIBILITY BY MITO-Q

by
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# ABSTRACT
# OF THE THESIS OF

Hala Ahmad Sardouk for Master of Science
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Title: Toxicity of Statins on Neuronal Cells: Possible Reversibility by Mito-Q

Statins are among the most commonly prescribed drugs to patients with cardiovascular events. They are cholesterol-lowering drugs that competitively inhibit HMG-CoA reductase, the rate-limiting enzyme in the mevalonate pathway. They exhibit a pleiotropic role in different tissues and body organs. On the other hand, statin toxicity involves renal, liver, muscle, and neurological events. Studies however on its neurotherapeutic effects have been controversial being neuroprotective in some and neurodegenerative in others. In addition to their hypocholesterolemic effects, statins decrease downstream biomolecules of important cellular functions such as isoprenoids, and ubiquinone.

In this study, we compared the direct effect of 5 different statins on 2 neuronal cell lines: rat PC12 and human SH-SY5Y. The following statins were used in this study: one natural product mevastatin and 4-synthetic (atorvastatin, rosuvastatin, pitavastatin, and fluvastatin). The viability of statin-treated (1-15 μM; 24 and 48 hours) cells was evaluated using (trypan blue exclusion/MTT assays). Changes in reactive oxygen species and ATP levels in treated cells were determined using NBT and luciferase assays respectively. The possible protective effect of pre-/ or co-MitoQ treatment with statins was investigated. The level of cholesterol and the direct inhibitory effect of statins on purified HMG-CoA reductase activity was determined.

Compared to SH-SY5Y, PC12 was insensitive to statins, hence we opted to limit the study to human cells treated with statins, for 24 and 48 hours at a concentration lower than the estimated $IC_{50}$. We report in the statin-treated SH-SY5Y a significant dose and time-dependent a) decrease in viability (85-90% at 48hrs); b) increase in ROS (50-75%); and c) a decrease in ATP (60%-99%). MitoQ pre- and co-treatment with statins exhibited no protective effect. Catalase also did not prevent or protect against cell death. Preliminary determination of cholesterol level showed an increase in statin-treated cells that may result from induced LDL receptors expression favoring cholesterol uptake, in response to HMG-CoA reductase inhibition.

Statins' neurotoxicity in-vitro is not limited to the inhibition of HMG-CoA reductase enzyme, but to statin-related factors including differences in their physicochemical properties, size, and hydrophobicity. The sensitivity of SH-SY5Y cells to the different

statins varied with lipophilicity in the following order with the most potent being fluvastatin> pitavastatin> atorvastatin > rosuvastatin the least effective. The structural differences of the various statins supported the difference in the pharmacokinetic response of these drugs on SH-SY5Y neuronal cells. While most of the literature claim indirect and direct protective effect for statins, we hereby show a toxic effect on statin-treated (48 hours) human neuronal cells. These findings may underlie some of the reported side effects in patients on statins such as dementia, cognitive decline, and Alzheimer's disease.

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# ABBREVIATIONS

| | |
|---|---|
| ABC | ATP-Binding Cassette |
| AD | Alzheimer’s Disease |
| Ala | Alanine |
| AMP | Adenosine monophosphate |
| AMPK | AMP Regulated Kinase |
| APOE | Apolipoprotein E |
| ARG | Arginine |
| ATP | Adenosine triphosphate |
| BAX | Bcl-2-associated X protein |
| BBB | Blood Brain Barrier |
| BCL-2 | B-cell Lyphoma-2 |
| BCRP | Breast Cancer Resistance Protein |
| BSA | Bovine Serum Albumin |
| $Ca^{2+}$ | Calcium ion |
| cAMP | cyclic-Adenosine Monophosphate |
| CLOGP | Calculated Log-P |
| CNS | Central Nervous System |
| $CO_2$ | Carbon Dioxide |
| CoA | Co-enzyme A |
| CSF | Cerebrospinal Fluid |
| CYP | Cytochrome |
| DAOY | desmoplastic cerebellar medulloblastoma cell line |
| $ddH_2O$ | Double distilled water |
| DMEM | Dulbecco's Modified Eagle's medium |
| DMSO | Dimethyl sulfoxide |

| | |
|---|---|
| DNA | Deoxyribonucleic acid |
| ELISA | Enzyme-Linked Immunosorbent Assay |
| ER | Endoplasmic Reticulum |
| ETC | Electron Transport Chain |
| FBS | Fetal Bovine Serum |
| FDA | Food and Drug Administration |
| FPP | Farnesyl pyrophosphate |
| GGPP | Geranylgeranyl pyrophosphate |
| GLUT4 | Glucose transporter type 4 |
| GPP | Geranyl pyrophosphate |
| GSH | Glutathione |
| GTP | Guanosine triphosphate |
| $H_2O_2$ | Hydrogen peroxide |
| HDL | high density lipoprotein |
| HMG-CoAR | Hydroxy methylglutaryl-CoA Reductase |
| HMG-CoA | Hydroxy methylglutaryl-CoA |
| HPLC | High-performance liquid chromatography |
| $IC_{50}$ | Inhibitory Concentration |
| Insig | Insulin-Induced Genes |
| IRS-1 | Insulin Receptor Substrate-1 |
| Ki | Inhibitory Constant |
| KOH | Potassium Hydroxide |
| LDL | Low Density Lipoprotein |
| LDL-C | LDL-Cholesterol |
| LDLR | LDL-Receptor |
| LEU | Leucine |
| LRP | LDL Receptor-Related Protein |

| | |
|---|---|
| LXR | Liver X Receptor |
| Lys | Lysine |
| MCT | Monocarboxylated Transporter |
| MITOQ | Mitoquinone |
| mRNA | Messenger Ribonucleic acid |
| MRP | Mutli Drug Resistant Associated Protein |
| MTT | 3-[4, 5-dimethylthiazol-2, 5-diphenylTetrazolium bromide] |
| Mw | Molecular Weight |
| NAC | N-acetyl Cysteine |
| NADPH | Nicotinamide Adenine Dinucleotide phosphate |
| NBP | Neuroblastoma Cell Line |
| NBT | Nitroblue tetrazolium chloride |
| OATP | Organic Anion Transporter Protein |
| OHC | Hydroxy-Cholesterol |
| PAMPA | Parallel Artificial Membrane Permeation Assay |
| PBS | Phosphate Buffered Saline |
| PC12 | Rat pheochromocytoma |
| PD | Parkinson Disease |
| Pe | Effective permeability |
| PGC-1 | Peroxisome Proliferator Activated Receptor Gamma Co-Activator |
| PKA | Protein Kinase A |
| PPI-1 | Phosphoprotein Phosphatase Inhibitor |
| PRIMO | Prediction of Muscular Risk in Observational conditions |
| PS | Penicillin-Streptomycin |
| PTP | Permeability Transition Pore |
| RHOCK | Rho-associated coiled-coil forming protein serine/threonine kinase |
| RME | Receptor Mediated Endocytosis |

| | |
|---|---|
| ROS | Reactive Oxygen Species |
| RXR | Retinoid X Receptor |
| S100B | S100 Calcium Binding Protein B |
| SAMS | Statin Associated Muscle Side Effect |
| SCAP | SREBP Cleavage Activating Protein |
| SEM | Standard Error of the Mean |
| SER | Serine |
| SH-SY5Y | Human neuroblastoma cell line |
| SLC | Solute Carrier |
| SOD | Super Oxide Dismutase |
| SRE | Sterol Responsive Element |
| SREBP | Sterol Regulatory Binding Protein |
| TG | Triglycerides |
| UQ | Ubiquinone |
| Val | Valine |
| VLDL | Very Low-Density Lipoprotein |

To the memory of my beloved father

**Ahmad Sardouk (1965, 2020)**

# CHAPTER I

# INTRODUCTION

## A. Cholesterol

Cholesterol was first identified in human bile and gallstones in 1769 by Poulletier de la Salle. It was purified and its structure was determined in 1815 by the chemist Chevreul who named it ‘‘cholesterin” [1]. Since then, the biosynthetic pathway was investigated, determined, and mechanisms regulating its metabolism elucidated.

Cholesterol is a lipophilic molecule found in all mammalian cells [2] and an important component of the cell membrane that maintains its integrity and fluidity [3]. In addition, it is the precursor of many biomolecules including steroid hormones; bile acids; and vitamin D [4]. This versatile molecule is also linked to a diversity of biological roles such as neuronal myelination as well as signaling mediating cell-to-cell recognition, adhesion, and communication being a component of lipid rafts [5, 6].

Cholesterol pool is contributed endogenously (~70%) via the de novo biosynthesis that occurs in all cells, and exogenously (~30%) of dietary source being abundant in certain foods, such as meat, fish, eggs, and cheese [7]. All human cells are capable of de novo cholesterol synthesis, but much of its production takes place in the hepatocytes and enterocytes [8]. Cholesterol synthesis involves many steps, a simplified scheme is presented in figure 1, referred to as the mevalonate pathway. In brief, the pathway begins with the condensation of three acetyl-CoA molecules to form HMG-CoA that gets reduced into mevalonate. This reaction is catalyzed by the rate-limiting enzyme: HMG-CoA

reductase (HMG-CoAR). Mevalonate is then converted to isoprene pyrophosphate ($C_5$) unit that condenses to geranyl pyrophosphate ($C_{10}$) and farnesyl pyrophosphate ($C_{15}$) and ultimately to form squalene ($C_{30}$) [9]. The final steps of this pathway include the cyclization of squalene to form the four-ring steroid structure lanosterol that is further modified (oxidation, demethylation, etc.) to produce cholesterol [3]. It is worth noting that some of the mevalonate pathway intermediates geranyl-PP and farnesyl-PP have been recognized for their important role in regulating many biological processes ranging from intracellular signaling to inflammatory responses [10]. Other metabolites of the mevalonate pathway include heme A and ubiquinone (coenzyme Q10) an electron carrier in the electron transport chain, and dolichol that is required for glycoprotein synthesis.

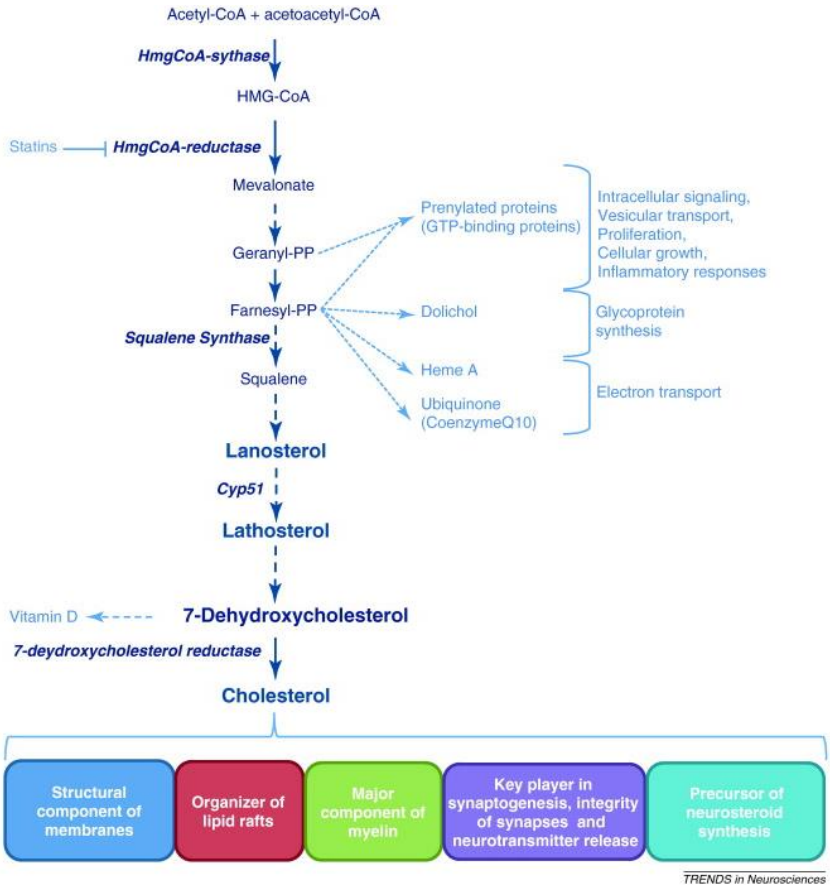



**Figure 1. Cholesterol biosynthetic pathway [11]**

Due to its lipophilic nature, cholesterol is transported in circulation via lipoprotein complexes that are characterized by a hydrophobic inner core (cholesterol esters and triglycerides (TG)) and a hydrophilic outer surface (phospholipids and unesterified cholesterol) [12]. Of the many types of lipoproteins the liver synthesizes and secretes, very-low-density lipoprotein (VLDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL) [13]. The VLDL carries TGs and cholesterol (dietary, biosynthesized) and distributes TGs to all tissues including muscle and adipocytes [13]. The remnant VLDL transforms in circulation, by the exchange of apoproteins, into LDL the main carrier of cholesterol [14] referred to as “bad cholesterol”. The importance of LDL in the

pathogenesis of atherosclerosis and hence the increased risk of cardiovascular disease was documented and correlated [15]. Reducing low-density lipoprotein cholesterol (LDL-C) is one main target of drug industries in treating heart disease [16]. On the other hand, HDL levels, referred to as “good cholesterol”, exert a protective role against the development of atherosclerosis by transporting cholesterol from vascular tissue back into the liver contributing thus to the decrease in cholesterol level and in preventing the development of atherosclerotic plaque [13, 17].

Over the years, several drugs were developed with a blood cholesterol-lowering therapeutic potential [18, 19], targeting cholesterol biosynthesis (inhibition) by statins [20], or absorption (ezetimibe), and bile acid-reabsorption (sequestrant resins) [21]. Statins were the most potent having a dual effect 1) inhibiting HMG-CoA reductase thereby reducing endogenous cholesterol synthesis and 2) increasing plasma membrane expression of LDL receptors that favors the uptake of cholesterol from the circulation into the cells [22].

### B. HMG-CoA Reductase

HMG-CoAR catalyzes the rate-limiting step of the cholesterol biosynthetic pathway and the main target of statins. It is an oxidoreductase that utilizes two NADPH to reduce HMG-CoA to mevalonate and free CoA [23].

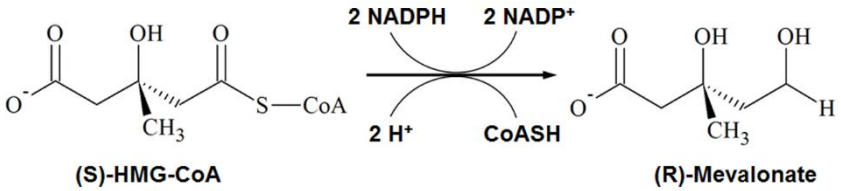



**Figure 2. Reaction catalyzed by HMG-CoAR [24]**

The human HMG-CoAR gene is located on chromosome 5 (5q13.3-q14) that encodes two isoforms produced by the alternative splicing of exon 13. The full-length transcript is composed of 20 exons [24]. It translates into a single polypeptide chain of 888 amino acids and is divided into three domains: The N-terminal membrane anchor domain, a linker, and a catalytic domain (figure 3) [25]. The hydrophobic N-terminal domain anchors the enzyme to the endoplasmic reticulum (ER) with eight membrane-spanning segments involved in sterol regulated degradation by the proteasome [23, 26-28]. The hydrophilic (cytoplasmic) catalytic domain harbors residues of the active and cofactor binding site as well as a flap domain that creates the substrate-binding site with a lid that covers the active site following substrate binding. In addition, the flap domain has a crucial role in enzyme catalysis and regulation as well as in statin binding [29].

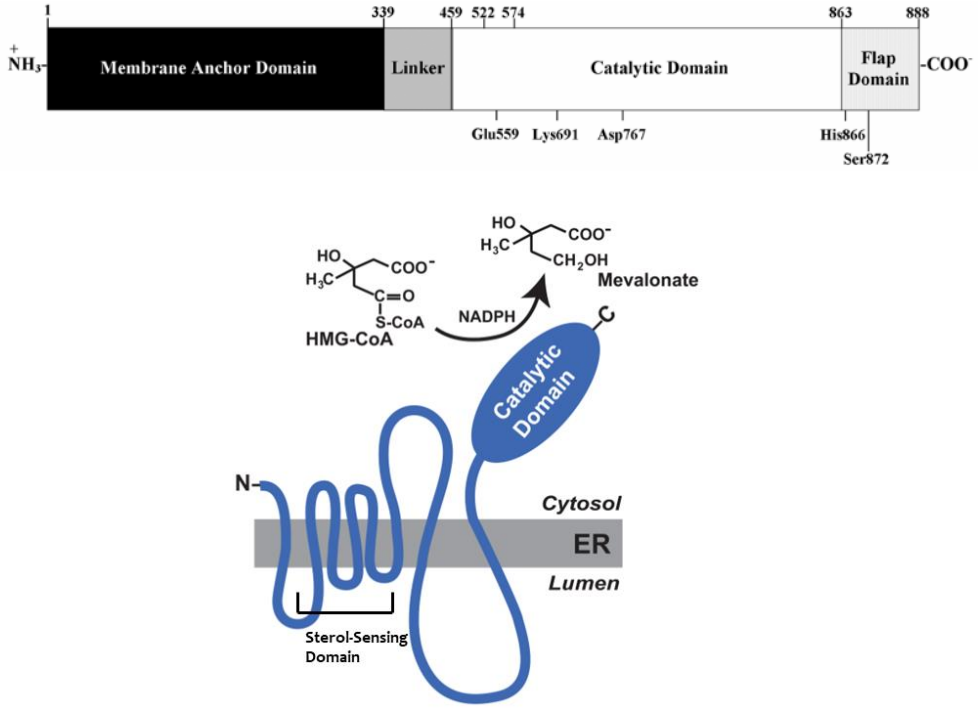



**Figure 3. Domain structure of HMG CoA reductase [26, 29].**

### *1. Regulation of HMG-CoAR:*

a. Transcriptional regulation:

The transcription of HMG-CoAR is regulated by a sterol regulatory element-binding protein (SREBP) (Figure 4). SREBPs are membrane-bound transcription factors linked to SREBP-cleavage activating protein (SCAP) in the ER. SCAP acts as a cholesterol sensor of intracellular cholesterol levels for SREBP cleavage and activation. When activated, mature SREBP controls the expression of HMG-CoAR, LDLR, and genes encoding enzymes of the mevalonate pathway.

Regulation is triggered by cholesterol levels mediated by insulin-induced genes (INSIG1 and INSIG2). When ER cholesterol levels are high (Figure 4A), SCAP interacts with INSIG1 and INSIG2 which prevent the translocation of SCAP/SREBP complex from the ER to the Golgi apparatus thus inhibiting the transcription of HMG-CoAR.

Conversely, when cholesterol levels are low (Figure 4B), the SCAP-INSIGs interaction is inhibited, and the SCAP/SREBP complexes transport to the Golgi where it is cleaved to be activated by proteases (S1P and S2P). The active form then translocates into the nucleus, binds the sterol responsive element (SRE) sequences within the regulatory region of targeted genes, and promote the transcription of the HMG-CoAR gene and other proteins involved in lipid synthesis [30-32]. Statins as well lead to the activation of SREBPs, increasing the transcription level of the LDLR mRNA hence the level of LDL receptors increase, elevating LDL clearance, and decreasing cholesterol level in circulation [33].

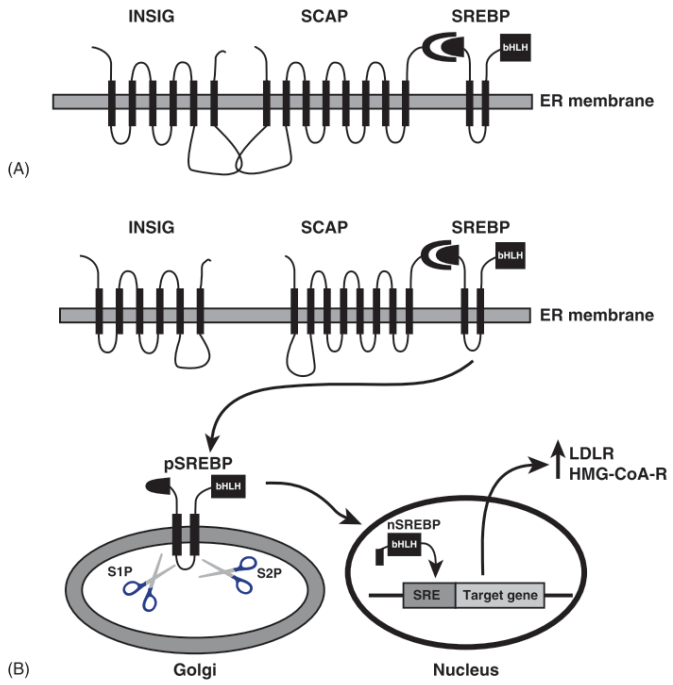



**Figure 4. Sterol dependent regulation of HMG-CoAR A) High cholesterol B) Low cholesterol [32]**

b. <u>Post-translational regulation:</u>

One of the post-translational modifications of HMG-CoAR includes enzymatic degradation stimulated by cholesterol and its oxidized derivatives as well as farnesol (dephosphorylated farnesyl pyrophosphate) [30, 34]. When the level of intracellular sterols is high, SCAP and HMG-CoAR compete to bind Insig. If SCAP binds to SREBP, the proteolytic release of SREBP is reduced thus inhibiting the transcription of HMG-CoAR. However, when HMG-CoAR binds to Insig, $Lys^{248}$ of the human HMG-CoAR is ubiquitinated promoting its proteolytic degradation [4]. On the other hand, low generation

of the by-products as lanosterol or 24,25-dihydro-lanosterol and geranylgeraniol attenuate the degradation of the enzyme, extending its half-life [35].

HMG-CoAR exist in two forms: phosphorylated (inactive) and dephosphorylated (active) forms (figure 5). Activation and inactivation of HMG-CoAR depend on the activity of HMG-CoAR phosphatase and AMP-regulated kinase (AMPK), respectively. When ATP levels decrease, AMP levels increase, activating a protein kinase that phosphorylates the enzyme at a $Ser^{872}$ (located within the flap domain). The phosphorylation down-regulates the HMG-CoAR catalytic activity since it decreases the affinity of the enzyme to NADPH and prevents the closure of a C-terminal region that facilitates the catalysis [31, 34]. Conversely, the subsequent dephosphorylation by phosphatases of this serine restores the catalytic activity of HMG-CoAR.

Hormones can also influence the phosphorylation state of HMG-CoAR thus its activity. Glucagon stimulates phosphorylation (inactivation), whereas insulin promotes dephosphorylation, activating the enzyme, and favoring cholesterol synthesis.

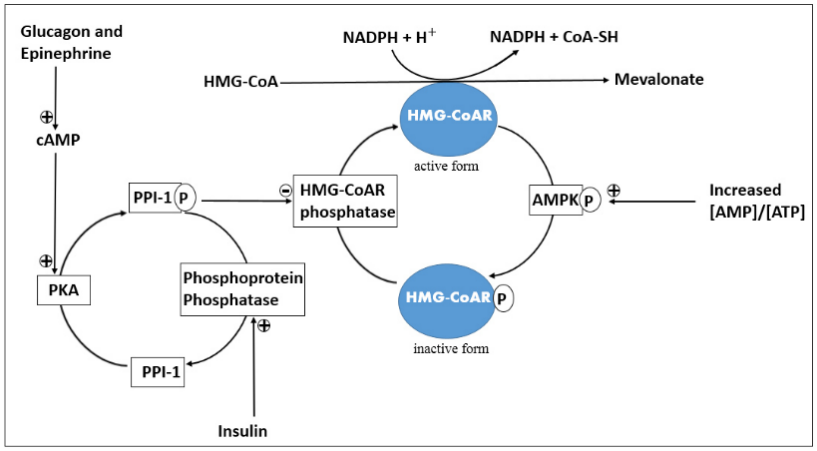



**Figure 5. HMG-CoA reductase regulation mevalonate pathway [34]**

cAMP: cyclic adenosine monophosphate; PKA: protein kinase A; PPI-1: phosphoprotein phosphatase inhibitor1; HMG-CoA: hydroxymethylglutaryl coenzyme A; HMG-CoAR: hydroxymethylglutaryl coenzyme A reductase; AMPK: adenosine mono-phosphate regulated kinase.

### *2. Direct inhibition of HMG-CoA reductase by statins:*

Statins act by reversibly and competitively inhibiting HMG-CoAR (figure 6) [36]. The active component of statins, modified 3,5-dihydroxy-glutaric acid moiety, binds to the active site and inhibits enzyme activity in a stereoselective process where the statin assumes a 3R,5R configuration [37]. The binding is facilitated by the flap domain that statin exploit creating a hydrophobic pocket. This enables the statins to extend to the active site effectively displacing the flap domain and inhibiting the enzyme.

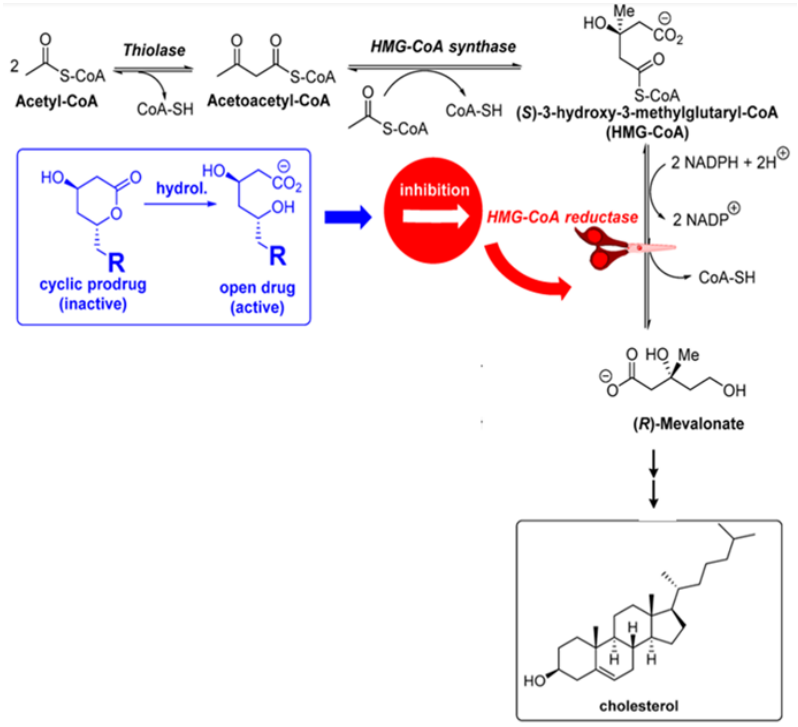



**Figure 6. Inhibition of the mevalonate pathway by statins [36]**

## C. Statins

### *1. History and discovery*

The clinical awareness regarding the importance of reducing cholesterol level has increased the search to identify drugs with hypocholesterolemic effect [38, 39]. The era of statins began after the discovery of penicillin (a group of antibiotics) from fungi by Dr. Alexander Fleming [40]. Antibiotics showed to inhibit several enzymes, not only in bacterial cells but also in mammalian cells [41]. The Japanese biochemist Akira Endo speculated that fungi similar to molds would produce an antibiotic that inhibits HMG-CoAR. Testing different fungal extracts, Endo hit the jackpot in a mold *(Penicillium*

*citrinum)* that grew in the rice fields near Kyoto that resulted in discovering a potent competitive inhibitor of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase. In the early 1970s, compactin or mevastatin was discovered that showed a structural similarity to the substrate of the reductase enzyme [18]. When tested on animals (dogs), compactin exhibited "toxic effects" due to the high concentration used (200 folds the current statin dosage used by patients) [42]. A decade after he successfully developed and marketed another statin, pravastatin [43].

Scientists at Merck & Company isolated from *Aspergillus terreus* their first statin lovastatin (Mevacor®) in 1976. Clinical trials demonstrated its cholesterol-lowering efficacy and safety. Shortly after lovastatin, other related drugs were developed modifying their chemical structure. The biological properties, efficacy, safety, and side effects have also been modified [44]. Currently, there are seven statins approved by the FDA including atorvastatin (Lipitor®), lovastatin (Mevacor®), fluvastatin (Lescol®, Lescol XL®), pravastatin (Pravachol), rosuvastatin (Crestor®), simvastatin (Zocor®), and pitavastatin (Livalo®) [20].

## *2. Properties of statins*

a. Structure

Structurally statins are composed of three moieties: an HMG-CoA like moiety (substrate analog); a hydrophobic ring structure; and ring(s) substituents that modify their solubility properties [45]. All statins share in common a conserved lactone ring (blue) yet

they can be subclassified into type 1 and type 2 statins (figure 7). In type 1 they have a decahydronaphthalene ring (black) and a butyryl side chain (red) whereas in type 2 statins the butyryl side chain is substituted with a fluorophenyl group (green) along with an N-heterocyclic five or six-membered ring structure [45, 46].

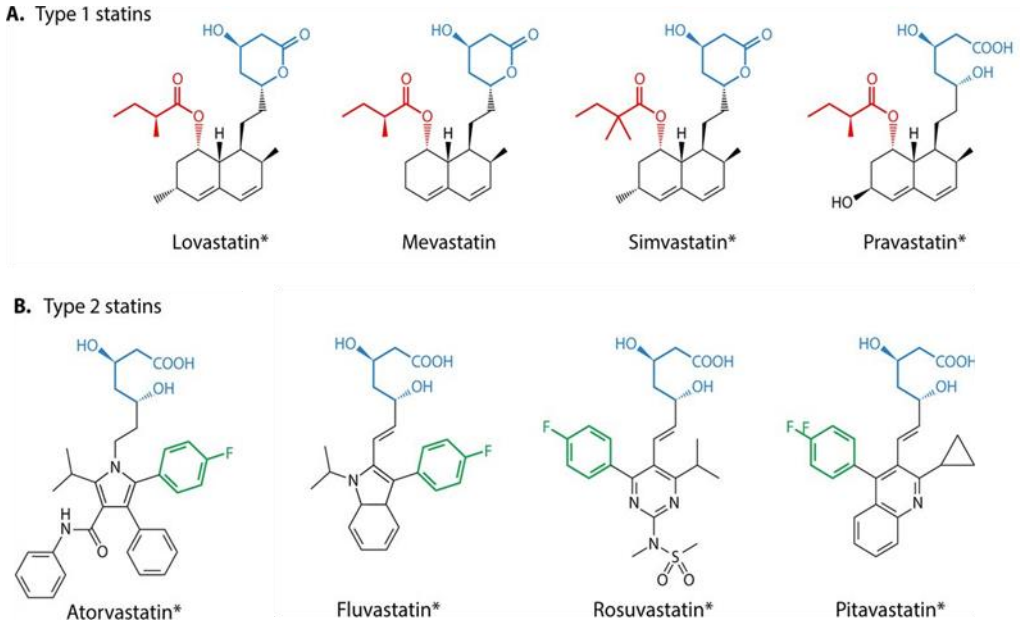



**Figure 7. Chemical structure of type I and type II statins [45]**

b. Solubility and binding properties

Statins are amphiphilic drugs possessing hydrophilic and hydrophobic regions [47]. The hydrophilicity of statins results from the conserved HMG-CoA like moiety containing a carboxy and a hydroxyl residue and may vary (increase, decrease) with ring substituents. The polar substituents on the ring(s) such as the hydroxyl, fluoro, carboxy side chains, amide, or sulphonamide increase the solubility of statin. On the other hand, the

hydrocarbon ring structure or the non-polar substituents (such as isopropyl, or phenyl groups) of some statin tend to decrease their water solubility [48].

Classified according to their solubility properties, rosuvastatin and pravastatin are clinically referred to as hydrophilic statins for having a methane sulphonamide and a hydroxyl group respectively that increase their water solubility. On the other hand, lovastatin, mevastatin, simvastatin, atorvastatin, fluvastatin, and pitavastatin are referred to as hydrophobic statins due to the hydrocarbon ring structure or the non-polar substituents (such as isopropyl, or phenyl groups) that increase their lipophilicity [46].

Structural differences among statins may account for variations in their potency of inhibition. The inhibitory concentration, $IC_{50}$, measures the efficacy of a drug in inhibiting its target, or more precisely, the concentration of the drug required to inhibit a biological product by 50% [49]. Studies on the purified human catalytic subunit of the HMG-CoAR showed that the median $IC_{50}$ values of some statins vary as such: mevastatin (23 nM), simvastatin (9 nM), atorvastatin (10 nM), fluvastatin (18 nM), pitavastatin (6.8 nM) and rosuvastatin (2 nM) [50]. The effectiveness of the statins in competitively inhibiting HMG-CoAR relates to the various tight interactions that tightly hold the statins to the enzyme hence decreasing the chance of the substrate-binding [51, 52]. Each of type 1 and type 2 statins adopts distinct structural conformations that maximize its contact with the hydrophobic pocket and hence affect their inhibitory action [53].

Statins bind the active site of HMG-CoAR in a manner that is similar to the substrate-binding involving electrostatic, ion-dipole, and hydrogen bonds. A representative of the different interactions of atorvastatin with the HMG-CoAR is shown in figure 8.

Statins exploit the flexibility of the HMG-CoAR receptor, enticing it to fit their large, lipophilic ring systems and substituents [53]. Some specific statin-enzyme binding interactions that allow a stronger binding to HMG-CoAR are:

- Weak hydrophobic van der Waal's interactions between carbon-rich methyl-butyrate, isopropyl, and methyl substituents on the statin ring(s) and the hydrophobic amino acid residues Leu$^{562}$, Val$^{683}$, Leu$^{853}$, Ala$^{856,}$ and Leu$^{857}$ of the enzyme.
- The unique fluorophenyl groups of type 2 statins form an ion-dipole bond with the HMGCo-AR Arg$^{590}$ residue.
- The carboxyl group of the amide sidechain of atorvastatin, and the sulphonamide sidechain of rosuvastatin exhibit H-bond with Ser$^{565}$.

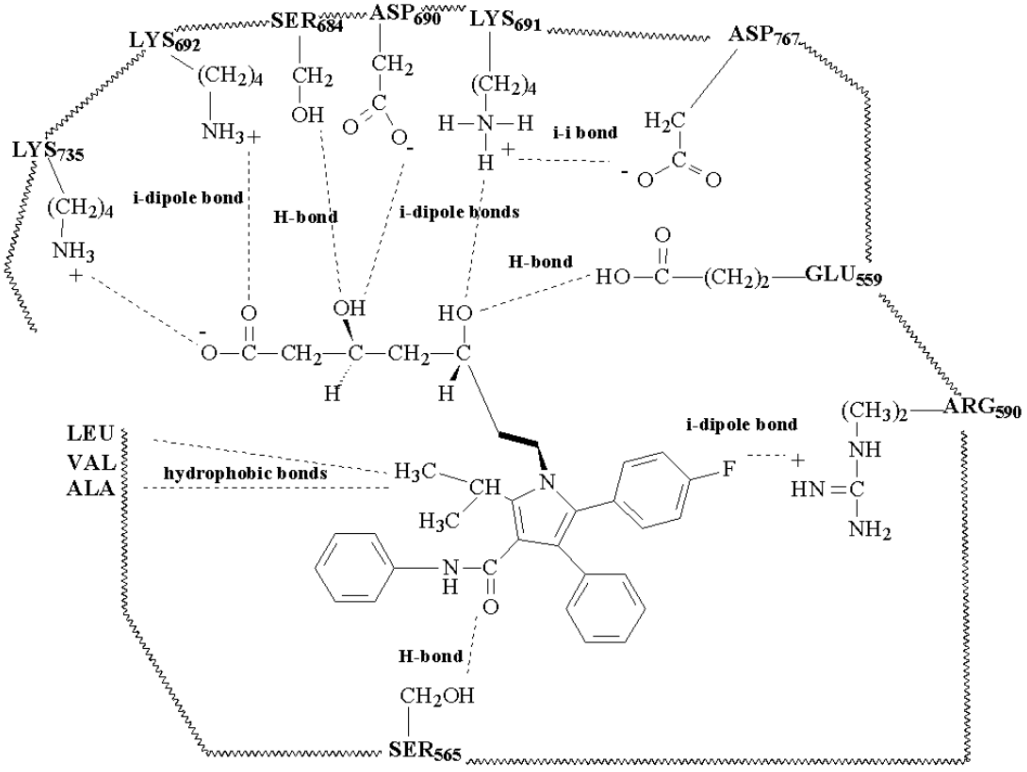



**Figure 8. HMG-CoAR binding interactions with atorvastatin [53].**

The distinct binding interactions of statins increase their binding affinity to the reductase enzyme. Type II statins are generally more potent than type I with an inhibition constant ($K_i$) ranging between 3.5 and 28 nM. This may be attributed to differences in the structure since type II as it has larger hydrophobic domains that account for more inhibitory binding interactions with HMG-CoAR [46, 53-55]. A clinical comparative study elucidated the relation between statins' efficiency to reduce cholesterol levels and their binding interaction to the HMG-CoAR residues. They concluded that atorvastatin and rosuvastatin have the most strongly binding interactions, while simvastatin and fluvastatin had the weakest [56]. This was in parallel with statin's efficacy in lowering LDL levels. Atorvastatin and rosuvastatin are the most potent in reducing LDL levels at 40 mg dosage to 50% and 60%; respectively [57].

c. Pharmacokinetic properties

i. Administration

Statins are administered orally as hydroxy acid (type II) or lactone form (type I). The hydroxy acid form is the active drug form that exists as an anion at the physiological pH (7.4) enabling the electrostatic binding to the enzyme at the cationic $Lys^{735}$. However the inactive lactone form of the drug is reversibly hydrolyzed chemically (pH) or enzymatically to their active dihydroxy-heptanoic acid form by carboxyesterases in the intestinal wall, the liver and in part in the plasma [45, 58]. At the physiological pH and above, the lactone form is unstable, and the equilibrium favors hydrolysis that yields the

hydroxy acid form (stable). At acidic conditions, the latter favors lactone formation. Both forms were however were observed in equilibrium in the systemic circulation (*in vivo)* [48].

ii. Absorption

Following oral administration, a drug crosses the intestinal wall and reaches the liver by portal blood flow. It then enters the systemic circulation to be distributed to various tissues of the body, including its site of action. Statins are rapidly absorbed reaching a peak plasma concentration ($C_{max}$) within 4 hours with a total bioavailability of about 20% (table 1) [59]. At the level of the intestine, statins enter the enterocytes via organic anion solute transporters [51]. The rate and extent of absorption are altered by many factors (increase, decrease, no effect) among them is food consumption. For example, lovastatin is absorbed more effectively when taken with food whereas the bioavailability of atorvastatin, fluvastatin, and pravastatin is reduced. No such effect is observed for pitavastatin, rosuvastatin, or simvastatin which are widely absorbed [48].

**Table 1. Pharmacokinetic properties of the different statins (modified) [59].**

| Drug Property | Atorvastatin | Fluvastatin | Lovastatin | Pitavastatin | Pravastatin | Rosuvastatin | Simvastatin |
|---|---|---|---|---|---|---|---|
| Year approved | 1996 | 1993 | 1987 | 2009 | 1991 | 2003 | 1991 |
| Generic available | Yes | Yes | Yes | No | Yes | No | Yes |
| Daily dose (mg) | 10–80 | 20–80 | 10–80 | 1–4 | 10–80 | 5–40 | 10–40 |
| Equipotent dose (mg) | 20 | >80 | 80 | 4 | 80 | 5 | 40 |
| Marketed drug form | Acid | Acid | Lactone | Acid | Acid | Acid | Lactone |
| log P (N-octanol/$H_2O$ partition coefficient) | 1.11 (lipophilic) | 1.27 (lipophilic) | 1.70 (lipophilic) | 1.49 (lipophilic) | −0.84 (hydrophilic) | −0.33 (hydrophilic) | 1.60 (lipophilic) |
| Oral absorption (%) | 30 | 98 | 31 | 80 | 37 | 50 | 65–85 |
| Bioavailability (%) | 14 | 29 | <5 | 51 | 17 | 20 | 5 |
| Effect of food on bioavailability | Decrease | Decrease | Increase | No effect | Decrease | No effect | No effect |
| Time to $C_{max}$ (hours) | 1–2 | 2.5–3 | 2 | 1 | 1–1.5 | 3–5 | 1–4 |
| Protein binding (%) | ≥98 | 98 | >95 | >99 | ~50 | 88 | 95 |

Statin can enter the hepatocytes by passive diffusion and/or by active transport mediated by solute carrier (SLC) membrane transporters and the organic anion transporting polypeptides (OATP), which is almost solely expressed on hepatocytes (figure 9) [59]. Statins' hepato-selectivity depends on their uptake by the active transporter system, whose affinity is related to their physicochemical properties. The uptake of statins by the liver varies ranging from 30 to 98% [52]. Nearly all statin are substrates of OATP1B1 (atorvastatin, rosuvastatin, pravastatin, fluvastatin, and pitavastatin). Pitavastatin can be uptaken by OATP1B1 (major) and OATP1B3 (minor) [60]. On the other hand, type I statins (simvastatin and lovastatin) can be passively up taken into the hepatocytes due to their acquired lipophilicity (as its ingested lactone form) that allows the facilitated diffusion through membranes [61].

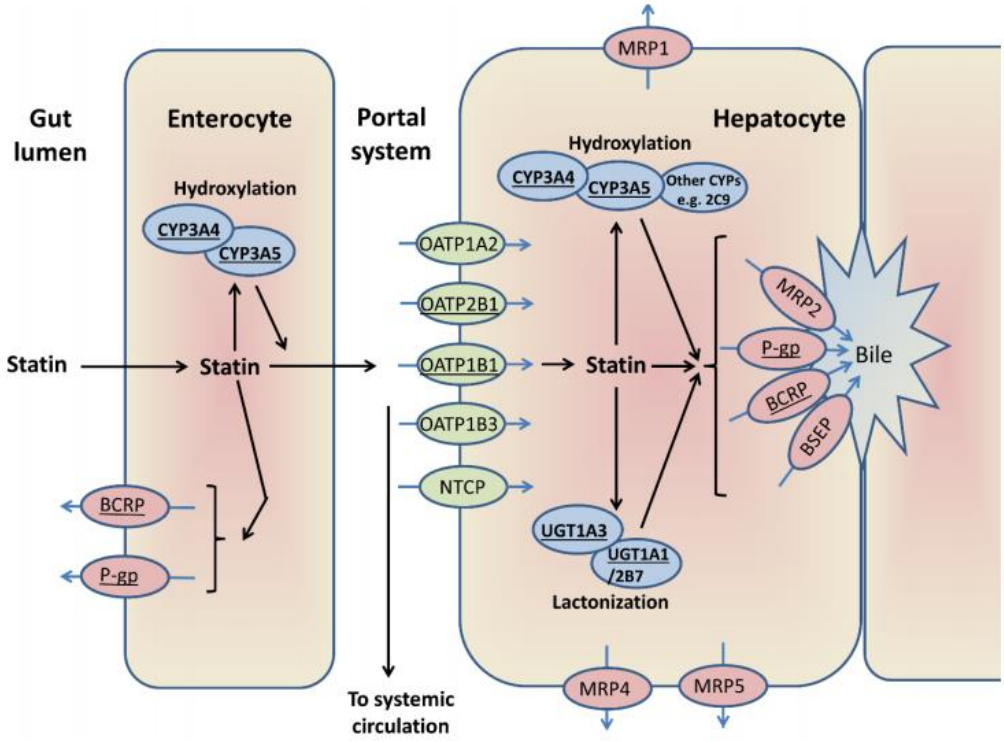



**Figure 9. A general schema for statin disposition [59].**

iii. Distribution

All statins, except for pravastatin, are distributed while bound to plasma proteins (albumin), thus their systemic exposure to the pharmacologically active (unbound) drug is low. Statins hepato-selectivity reduces their widespread tissue distribution however their amphiphilic nature allows them to exert pleiotropic effects at extrahepatic sites [37, 62].

The unique lipophilicity of each statin affects its distribution within the phospholipid bilayer of the cellular membranes (figure 10). Hydrophilic statins (pravastatin and rosuvastatin) are bound to the polar surface of the membrane, whereas the lipophilic statins (atorvastatin and simvastatin) partition more deeply into the membrane, where they interact with the surrounding acyl chains [63]. The lactone form of statins also has higher lipophilicity than the acid form which enables their penetration into the cells and consequently induces high local drug concentrations [61].

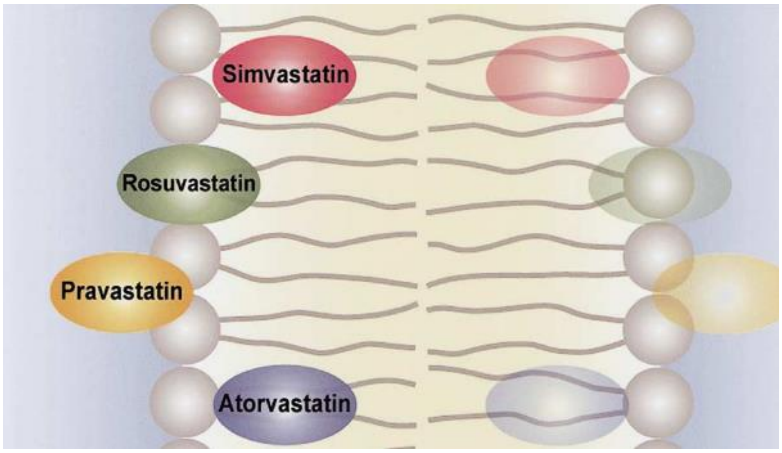



**Figure 10. Location of statins in biological membranes [63].**

Statins were shown to aggregate in the membrane [47]. Statins are amphiphilic drugs possessing hydrophilic and hydrophobic regions. Amphiphilic drugs are soluble in aqueous biological fluids and can diffuse through the body, and partition into membranes that may further impact lipid bilayer parameters like permeability and thickness [47]. In fact, statins were observed to alter lipid bilayer properties by increasing membrane elasticity, with fluvastatin being the most effective membrane disruptor [64].

iv. Metabolism

The cytochrome P450 are a superfamily (CYP) of enzymes containing heme as a cofactor that function as monooxygenases catalyzing the oxidative biotransformation of most drugs and other organic substances [65]. All statins undergo extensive microsomal metabolism by oxidation, reduction, and/or hydrolysis via CYP450 isoenzyme systems such as CYP3A4 and CYP2C9 [52]. On the other hand, pravastatin is minimally metabolized by CYP450 enzymes and undergoes sulfation in the liver cytosol. The metabolizing enzymes and corresponding metabolites of statins are represented in figure 11 [66].

Whereas the lactone form of simvastatin and lovastatin are inactive, their acid metabolites are active. Metabolism of simvastatin and lovastatin into their respective active product, such as 3′α,5′β-dihydrodiol simvastatin, 3′α-hydroxy simvastatin, 6′β-hydroxy simvastatin, and 6′β-hydroxy lovastatin, exerts an inhibitory effect on HMG-CoAR (figure 11). These metabolites can contribute to adverse side effects such as muscle and liver toxicity. The CYP3A4 isoenzyme is responsible for the metabolism of lovastatin, simvastatin, and atorvastatin [66].

On the other hand, the metabolites of the other statins show minimal activity. Fluvastatin is metabolized primarily by the CYP2C9 enzyme, with CYP3A4 and CYP2C8 contributing to a lesser extent. Rosuvastatin, pravastatin, and pitavastatin are not extensively metabolized but have some interaction with the CYP2C9 enzyme yielding inactive metabolites [67].

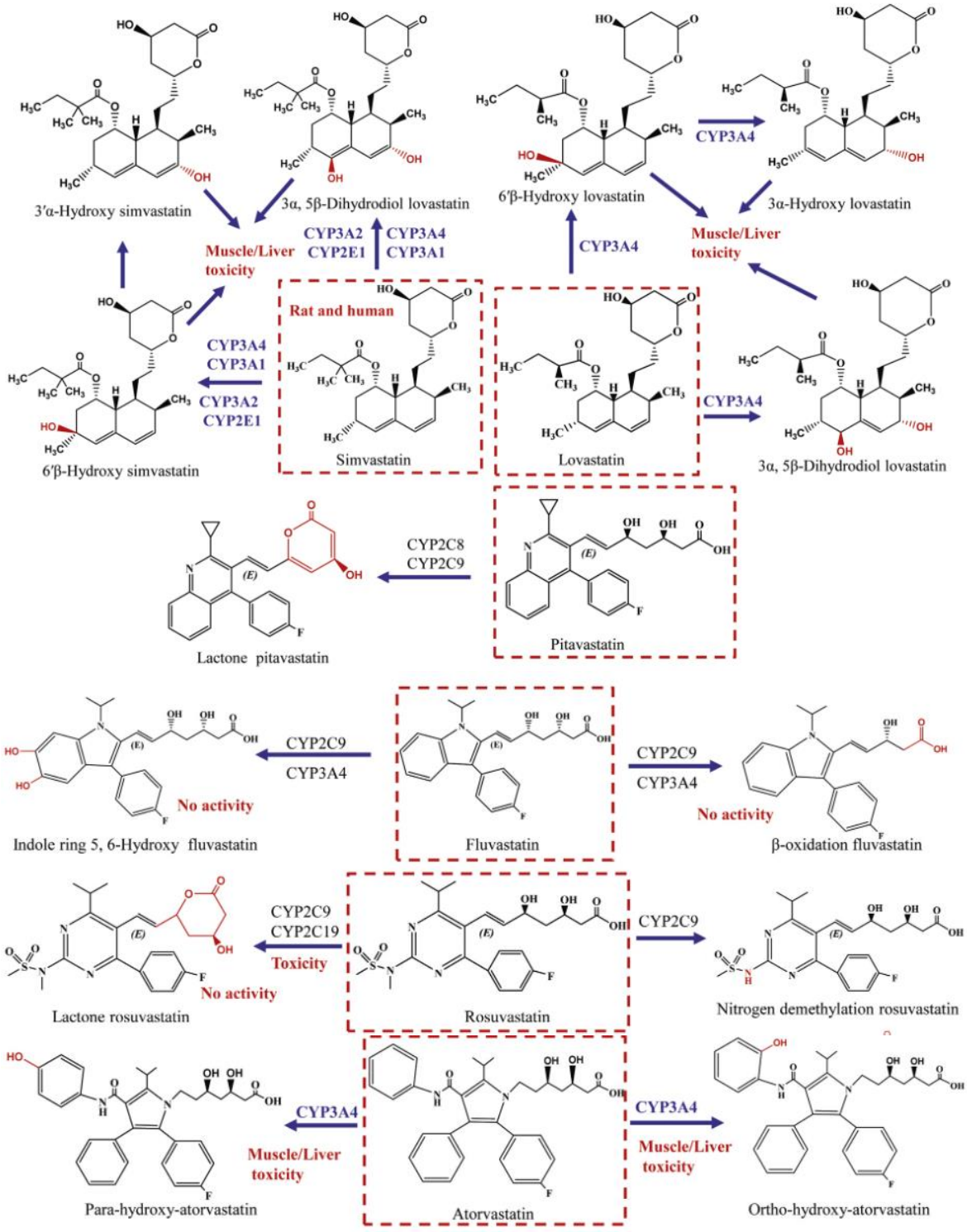



**Figure 11. Metabolic pathways of statins, metabolizing enzymes and their associated metabolites [66]**

v. Excretion

Following their metabolism and biotransformation, statins are excreted by bile except for hydrophilic statins that are eliminated by the kidneys. The half-life of fluvastatin, lovastatin, pravastatin, and simvastatin are short (<5 hours) compared to rosuvastatin, atorvastatin, and pitavastatin which relates to their greater efficacy in lowering LDL cholesterol levels [68]. The longer the half-life of the statin, the longer the inhibition of reductase hence the greater reduction in LDL cholesterol. Clinical studies confirmed rosuvastatin to be the most effective for reducing low-density lipoprotein cholesterol, followed by atorvastatin, simvastatin, and pravastatin [37, 48, 55, 65, 69].

Efflux transporters eliminate statins. They include the superfamily of plasma membrane proteins, the ATP-binding cassettes (ABC), transport statins across the cell membrane that are expressed on hepatocytes and enterocytes [70]. ABCs that are primarily involved in the removal of statins by active transport are multidrug-resistant-associated protein (MRP2/ABCC2), breast cancer resistance protein (BCRP/ABCG2), and P-glycoprotein (Pg-P/MDR1/ABCB1) (figure 9) [71].

## D. Statin toxicity

Although statin therapy is the first choice of treatment of hyperlipidemias, it is not well-tolerated by a significant proportion of developing side effects as well as they do not achieve the targeted hypocholesterolemic effect [19, 72, 73]. Mechanistically, the toxicity of statin is thought to arise as a consequence of HMG-CoAR inhibition yet other non-lipid-related effects are induced by the downstream inhibition of important biomolecules such as

prenylated proteins (GPP and FPP), dolichols, and ubiquinone that are required for a variety of important cellular functions (figure 12) [73]. Among the side effects known as statins intolerance or statins, toxicity includes renal, liver [74], muscle[59], and brain toxicity [69]. Different effects had been reported such as new-onset type 2 diabetes mellitus[75], mitochondrial dysfunction [76], as well as neurological and neurocognitive effects [37, 77].

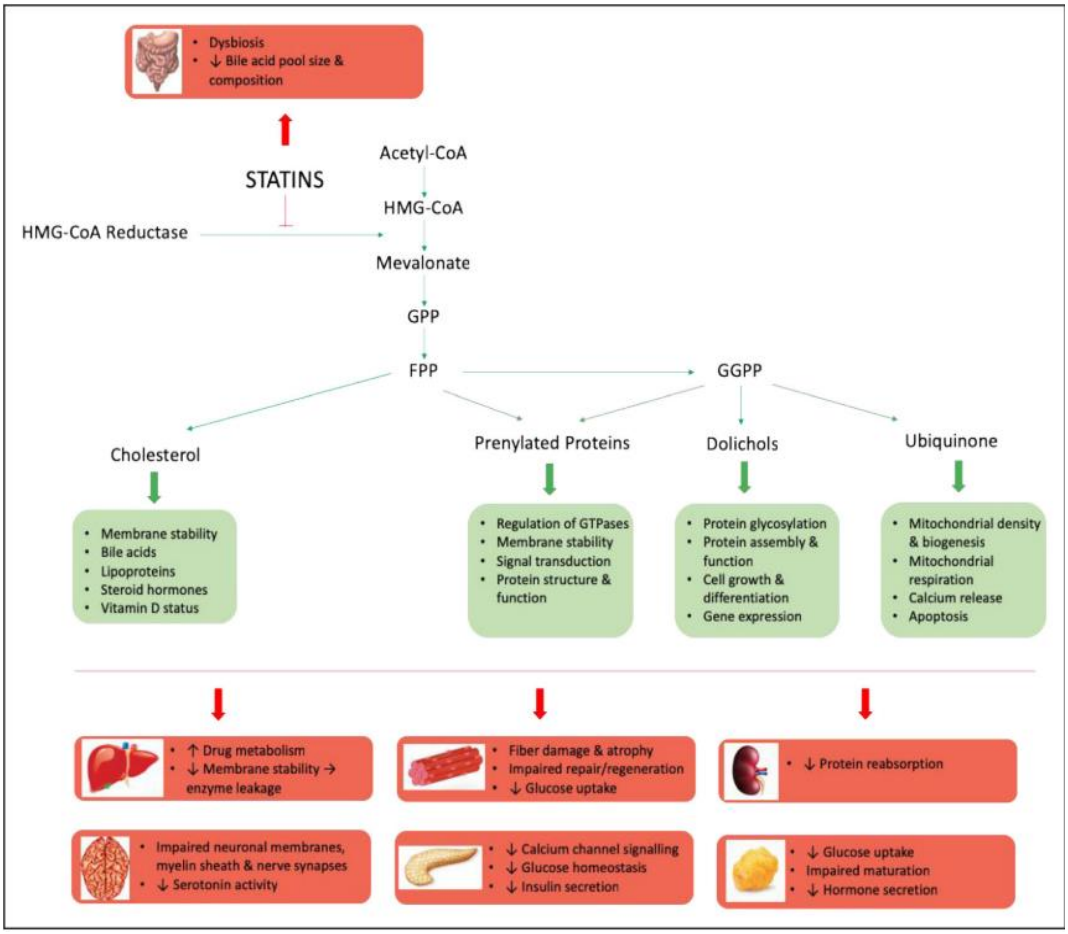



**Figure 12. Potential mechanisms for the development of statin toxicity [37].**

### *1. New-onset type 2 diabetes mellitus*

Chronic treatment of statins (hydrophilic, hydrophobic) may contribute to the predisposition of many diseases as new-onset type 2 diabetes mellitus [78, 79]. This effect may be enhanced in a dose-dependent manner when combined with preexisting risk factors: high body mass index or lack of exercise, impaired fasting glucose, and in aged people (>60) [80].

It has been shown that statins can impair insulin sensitivity and secretion and increase insulin resistance in peripheral tissues. The mechanisms involved in these processes include impaired $Ca^{2+}$ signaling in pancreatic β-cells, down-regulation of GLUT4 in adipocytes, and compromised insulin signaling [81].

a. Pancreatic insulin secretion is triggered by glucose-induced $Ca^{2+}$ entry via calcium channels. Previous studies have reported that *in vivo* lipophilic statins (e.g. simvastatin) block the L-type calcium channels in mice pancreatic islet-β cells leading to the inhibition of glucose-induced calcium signaling [82].
b. The inhibition of isoprenoid production down-regulates GLUT4 expression. Isoprenoids stimulate upregulation of GLUT4 in adipocytes leading to an increase in glucose uptake in skeletal muscles and adipocytes. Hence inhibiting isoprenoid synthesis may downregulate GLUT4 as reported for lovastatin that induced insulin resistance via decreasing the expression of GLUT4 and caveolin-1 in adipocytes [83].
c. Decrease in insulin receptor substrate (IRS-1) activation *in vitro* as has been reported for simvastatin and atorvastatin [84, 85].

### *2. Hepatotoxicity*

Clinical trials with statins revealed elevated levels of transaminases in 0.5-2% of patients. No significant increase in bilirubin levels was reported indicating no drug-induced liver injury [86]. This adverse effect has been noted in all statins in a dose-dependent manner [87] however atorvastatin was predominant in inducing cholestatic/mixed liver injury and drug-induced autoimmune hepatitis. Among the postulated mechanisms involved in hepatotoxicity:

Membrane fluidity: cholesterol regulates membrane permeability. By inhibiting cholesterol synthesis, statins alter lipid membrane composition that leads to increased permeability hence leaking of the liver enzymes and elevate transaminases [88].

### *3. Renal toxicity*

Albumin uptake in proximal tubular cells is maintained by receptor-mediated endocytosis (RME) that requires the presence of prenylated GTP binding proteins [89]. Statins inhibit the mevalonate pathway hence the generation of isoprenoid pyrophosphates that are required for the prenylation of GTP binding proteins and can impair RME. Mild transient proteinuria has been reported in some cases on high-dose statin treatment, this was not associated with any clinical deterioration in renal function [90]. On the other hand, high potency statins (rosuvastatin >10 mg, atorvastatin>20 mg, or simvastatin >40 mg) have been associated with a 34% higher rate of hospitalization for acute kidney injury within 120 days of drug initiation than less potent statin doses [91]. This might be caused by the

inhibition of protein prenylation (of GTP binding proteins) that leads to the repression of the tubular reabsorption of albumin [89, 92].

### *4. Statin-associated muscle symptoms*

Statin-associated muscle symptoms (SAMS) is the most commonly reported statin adverse event in 5-10% of the statin consumers [93]. Symptoms involve muscular pain, stiffness, and cramps with or without weakness. The phenotypic presentation of SAMS is heterogeneous and might include the relatively more common myalgias, myopathies, rhabdomyolysis, and the very rare immune-mediated necrotizing myositis[94]. The pathogenesis of SAMS depends on the dose and duration of exposure to the statins. The median time of onset of muscular symptoms was 1 month following statin administration. In the PRIMO study (Prediction of Muscular Risk in Observational conditions), hyperlipidemic patients treated with high doses of statins (40 or 80 mg fluvastatin, atorvastatin, and simvastatin) represented mild to moderate muscular symptoms [95]. The frequency however was highest with type 1 simvastatin followed by type 2 atorvastatin then fluvastatin. The risk of muscular side effects increased with drug-drug interactions that interfere with statin elimination, more specifically CYP3A4 enzyme inhibitors (e.g. clarithromycin, erythromycin, etc.). The low incidence of SAMS with fluvastatin may be attributed to the fact that it is primarily metabolized by CYP2C9 and to a lesser extent by CYP3A4 [95].

Statin uptake by myocytes is facilitated by several transporters including OATP2B1, multidrug resistance-associated protein: MRP 1, MRP 4, MRP 5, and

monocarboxylated transporter-4 (MCT4) [96]. It is noteworthy that the hydrophobic statins (atorvastatin, simvastatin…) compared to hydrophilic statins are more likely to accumulate in skeletal [96]. Statins in the lactone form (lipophilic) can permeate cell membranes easily while acid forms (hydrophilic) inhibit this passage [97]. Acidic media maintain the statins in their lactone form facilitating their uptake by muscle cells which may explain the greater myotoxicity of lipophilic statins [98]. This may result from the:

a. Reduction of cholesterol content in skeletal muscle membranes lowering membrane fluidity [99]. This may affect several ion channels (sodium, potassium, and chloride) thus modifying muscle membrane excitability. For example, simvastatin but not the hydrophilic pravastatin induced a dose-dependent decrease of membrane chloride conductance in rat muscle fibers that leads to myofiber hyperexcitability and spontaneous myofiber action potentials [100].
b. Reduction of protein prenylation downstream the mevalonate pathway is implicated in the *in vitro* statin myotoxicity. The statin mediated decreased in geranyl-geranyl pyro-phosphate (GGPP): reduced myotube ATP levels [101], blocked protein prenylation of small GTPases including Rab and RhoA [101, 102], induced atrogin-1 expression (gene required for muscle atrophy) [103] hence inducing muscle damage and apoptosis. Among the possible mechanisms explaining cellular death include The mislocalization of RhoA (membrane to cytoplasm), loss of Rab1 activity due to ATP depletion leading to the activation of caspase-3 activation [102].

c. Reduction in Coenzyme $Q_{10}$ level. Ubiquinone plays a central role in the electron transport chain mediating electron transfer from complexes I and II to cytochromes. The inhibition of HMG-CoAR leads to the decrease in ubiquinone formation consequently a decrease in ATP [104] that is required for the skeletal myocyte contraction-relaxation cycle hence causing SAMS [59]. A decrease in coenzyme $Q_{10}$ was reported in the muscle biopsy of patients [105] and circulation [106].

d. Impaired mitochondrial function. Statins may cause mitochondrial impairment in patients with myotoxicity [107]. Histochemical staining of skeletal muscle biopsies from statin-treated patients consistently demonstrated: increased intramuscular lipids indicating abnormal aerobic metabolism, reduced cytochrome oxidase staining, and ragged red fibers (clumps of diseased mitochondria) indicating mitochondrial dysfunction [108]. Simvastatin (80 mg) treatment for 8 weeks reported a significant decrease in mitochondrial DNA levels [109]. Other studies tested for mitochondrial dysfunction by targeting the activity of co-enzyme $Q_{10}$ reductase within complex III that contains two binding sites for ubiquinone. Statins (lactone more potent than acid form) inhibited the activity of this enzyme up to 84% in vitro and 18% in vivo [96]. On the other hand, the effect of statins on myocytes was different from cardiac muscles (figure 13). Statins triggered the transcriptional activation of mitochondrial biogenesis by enhancing antioxidant capacity in atrial cells whereas in skeletal cells statins induced a much higher oxidative stress that inhibited the transcription of mitochondrial biogenesis leading to mitochondrial dysfunction (figure 13) [110].

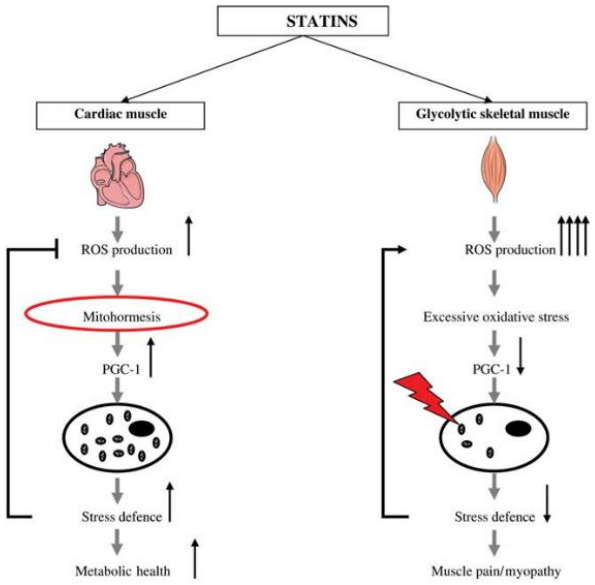



**Figure 13. Statin differential effect according to muscular phenotype [110].**

ROS: reactive oxygen species; PGC-1: peroxisome proliferator-activated receptor- γ co-activator

### *5. Mitochondrial toxicity*

Mitochondria have been proposed as one of the targets underlying statin-induced toxicity [76]. Mitochondrial impairment associated with statins' treatment is demonstrated by abnormal morphology, a decrease in the oxidative phosphorylation capacity hence an increase in ROS, and intrinsic apoptotic pathway activation [111]. Mechanisms are not fully elucidated however proposed causes are the following: $CoQ_{10}$ deficiency, inhibition of respiratory chain complexes, inhibitory effect on protein prenylation, and induction of mitochondrial apoptosis pathway [112].

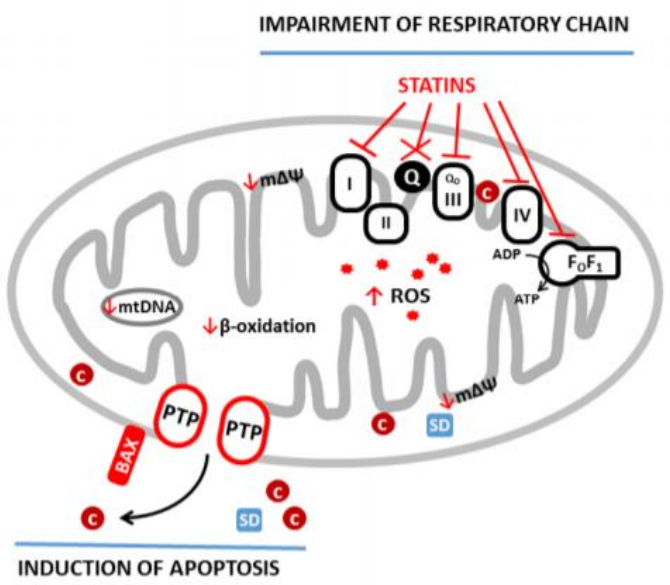



**Figure 14. Scheme illustrating the possible effects of statins on mitochondria**

A review by Broniarek et. al summarized (figure 14) the possible effects of statins on mitochondria. Statins especially the lipophilic ones inhibit respiratory chain complexes I, III, IV, and V function, uncouple oxidative phosphorylation, and dissipates the membrane potential [113, 114]. These effects result in an increase in reactive oxygen species levels (ROS) increasing the permeability of the inner mitochondrial membrane causing mitochondrial swelling and cytochrome c release from the intermembrane space [113]. The release of cytochrome c from mitochondria will 1) activate caspase-3 that triggers the activation of a cascade of caspases inducing apoptosis; 2) increase the level of the pro-apoptotic BAX protein and suppresses that of anti-apoptotic Bcl-2 [114, 115]; 3) Activate mitochondrial mega-channel PTP (permeability transition pore). Consequently, ions and

small molecules escape from the mitochondria, resulting in the final collapse of the membrane potential [113, 116].

### *6. Neurological and neurocognitive conditions*

Due to reported behavioral effects associated with statin (mainly lipophilic) administration, the FDA changed the security label to indicate that memory loss and confusion may occur during medication yet reversible once the drug is no longer administered [117-120]. Of the neurological conditions associated with statin use: hemorrhagic stroke, cognitive decline, peripheral neuropathy, depression, confusion/memory loss and aggression, and personality changes [121]. However, it is unclear whether these are because of the direct action of statins given the blood-brain barrier's selective permeability to substrates and the brain's self-sufficiency of cholesterol [122]. Several mechanisms have been proposed most of which focus on the importance of lipids in proper brain function. Reducing serum lipids may affect the formation of the neuronal cell membrane, myelin sheath, and nerve synapses. The reduction of cholesterol availability for neurons can result in lowering the expression level of serotonin receptors contributing to the behavioral and adverse psychiatric effects [123-125].

## E. Cholesterol in the central nervous system

### *1. The complex role of cholesterol in the brain*

Cholesterol is highly abundant in the brain, estimated to contain 25% of the body's non-esterified cholesterol content. Cholesterol is essential for normal brain development

and a major component of the myelin sheath and the plasma membrane of neurons and astrocytes [126, 127]. In addition, cholesterol participates in signaling, axonal guidance, and synaptogenesis which influence synapse plasticity and neurotransmission [6, 128, 129]. Changes in brain cholesterol levels contribute to the development of neurodegenerative (Alzheimer's disease, autism spectrum disorder), and metabolic diseases (Niemann-Pick, Smith-Lemli-Opitz syndrome) [32].

### *2. Cholesterol homeostasis*

Unlike cholesterol in peripheral organs, brain cholesterol is primarily derived by the *de novo* synthesis in astrocytes, oligodendrocytes, microglia, and to a lesser extent in neurons [127, 130]. Peripheral cells meet their cholesterol need by the uptake of LDL as well as by the de novo synthesis. However, in the CNS the brain is separated from the plasma cholesterol pools by two barriers: the blood-brain barrier (BBB) and the blood-cerebrospinal fluid (CSF). This restricts plasma lipids, including cholesterol and plasma lipoproteins, from entering or leaving the CNS [126, 131].

In the CNS, cholesterol level is tightly maintained by a transport mechanism involving neurons and glia (figure 15). Neurons depend on glial-derived cholesterol, mainly astrocytes, to form numerous and efficient synapses [132-134]. The rate of cholesterol level and synthesis in different brain regions vary with the stage of brain development [135]. During the embryonic stage or myelination, neurons can meet their own cholesterol requirements by de novo synthesis (kandutsch-Russell pathway). Postnatally, neuron's ability to synthesize cholesterol is reduced favoring cholesterol import via expressing LDLR or LDL receptor-related protein (LRP) [136]. Astrocytes synthesize cholesterol by

the Bloch-pathway providing it to neurons. Cholesterol is secreted via ATP-binding cassette transporter (ABCA1) into HDL-like particles containing apoE which is delivered to neurons [130, 137].

To maintain homeostasis, excess cholesterol can cross the BBB into the circulation after its conversion to oxysterols that regulate cholesterol synthesis and reuptake between the neurons and glial cells. Cholesterol hydroxylation to 24-hydroxycholesterol (24S-OHC) by cholesterol 24-hydroxylase (CYP46A1) occurs selectively where it is expressed in neurons, but not in astrocytes [127, 137]. CYP27A1 expressed by neurons and glial cells converts cholesterol to 27S-OHC. The 24S-/27S-OHC regulate cholesterol synthesis and transport from glia to neurons by acting on the nuclear liver X receptors (LXR) that regulate the expression and synthesis of apoE and ABC transporters. The oxysterols can be transported

into circulation, esterified with fatty acids on lipoproteins (LDL or HDL), that get converted in the liver into bile acids to be eliminated.

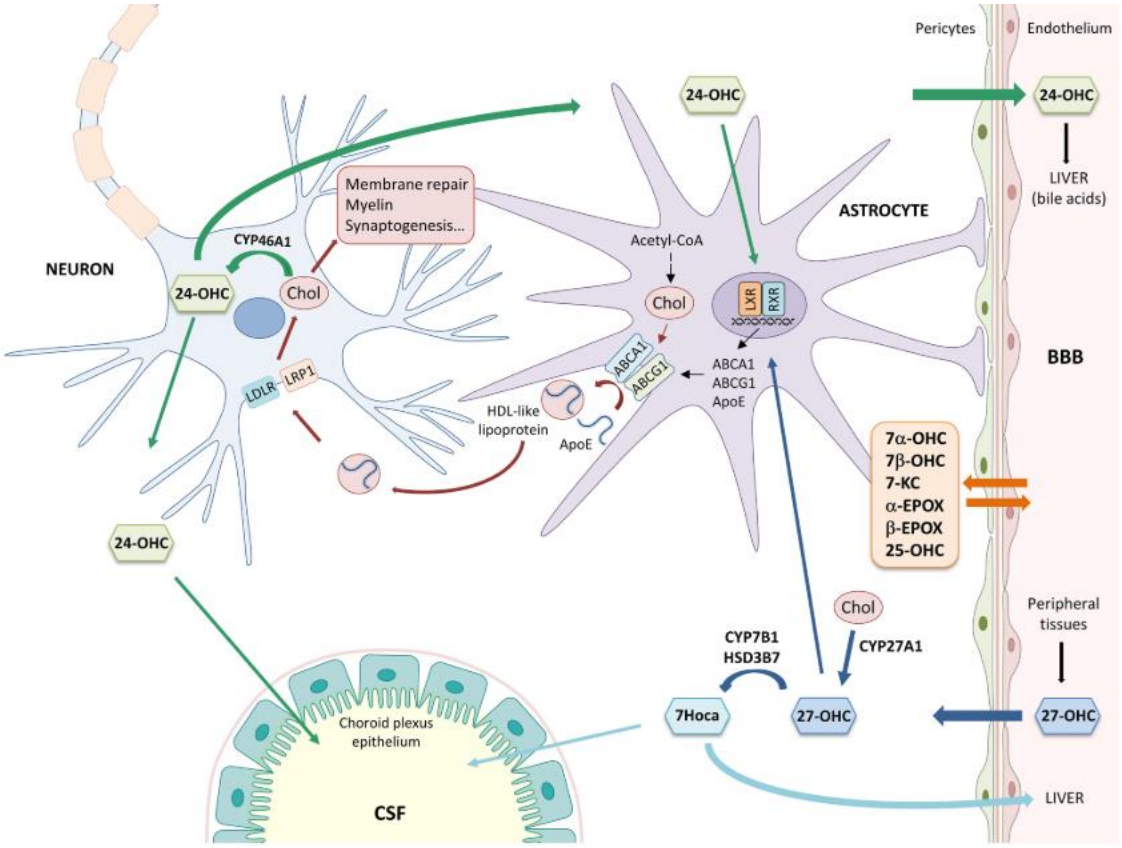



**Figure 15. Cholesterol homeostasis in the brain [130]**

### *3. Regulation of brain cholesterol*

Cholesterol homeostasis is critical for maintaining brain structure and function that is equally controlled by astrocytes and neurons [131]. Disturbances in its synthesis or transport cause either deficiency or accumulation of brain cholesterol [32, 126, 138]. Oxysterols influence lipid synthesis by regulating SREBP as they interact with Insig promoting it to bind SCAP thus preventing its maturation [139]. As a consequence, the

transcription of genes encoding for enzymes involved in cholesterol synthesis and transport is attenuated (figure 16).

When cholesterol level is high, or the mevalonate biosynthetic enzyme levels are high, excess cholesterol is converted to the 24S–OHC. In astrocytes, 24OH-C decreases the expression of HMGCR and increase LXR regulated ApoE expression by activating the nuclear transcription factor LXR that dimerizes with the retinoid X receptor (RXR). The dimerization induces the expression of apoE and its lipid transporters ABCA1 and ABCG1 facilitating cholesterol export from astrocyte to neurons [133, 140].

In contrast, a decrease in cholesterol level or enzymes involved in its synthesis as well as a decrease in cholesterol transport and uptake by neurons induces SREBP-2 cleavage. This in turn 1) activates the transcription of genes encoding enzymes involved in cholesterol synthesis in astrocytes [141] 2) increases the expression of LDLR 3) blocks ABCA1 and ABCG1 expression thus reducing cholesterol efflux [137].

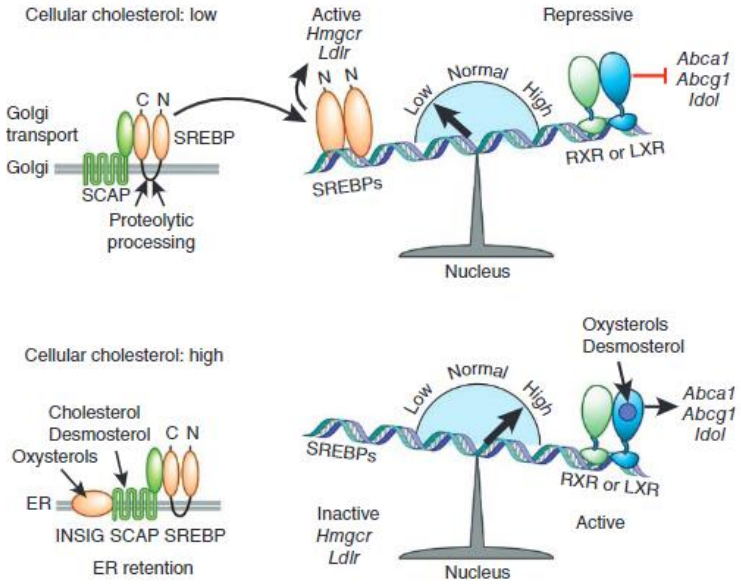


**Figure 16. Regulation of cellular cholesterol homeostasis by SREBPs and LXR [142]**

## F. Statins in the central nervous system

The cholesterol-lowering effect of statins in peripheral tissue is less likely to affect the level of cholesterol in the CNS due to the brain's inherited capacity of synthesizing its own cholesterol. Unlike cholesterol in plasma which has a half-life of only a few days, brain cholesterol has a half-life from 6 months to 5 years with a turnover rate of 0.02% [126]. Thus chronic statin administration is needed to alter brain cholesterol levels [143].

The neurological effects of statins rely on whether these compounds differ in their ability to permeate the CNS through the BBB via passive diffusion or active transporters. While lipophilic statins can pass the BBB passively, hydrophilic statins cannot. Active transporters including the OATP1A2 and OATP1C1 as well as monocarboxylic acid transporters are expressed in the brain that facilitate the entry of statins [46, 143].

Regardless of specific transporters, statins accumulate at different rates and concentrations within the CNS depending on their lipid solubilities as well as the size of these molecules [46, 144, 145]. Table 2 shows the various statins parameters related to their ability to penetrate through the BBB as well as their efficiency to inhibit HMG-CoAR.

**Table 2. Statins' parameters related to BBB penetration and inhibition of HMG-CoAR (modified) [145]**

| Statin | CLOGP | PAMPA assay (% of crossing) | Pe (x $10^{-6}$ cm/s) | Mw |
|---|---|---|---|---|

| | | | | |
|---|---|---|---|---|
| Mevastatin | 4.09 | 10.08 ± 3.5 | 1.3 ±0.6 | 422 |
| Atorvastatin | 5.55 | 4.9 ± 2.0 | 0.5 ± 0.2 | 558 |
| Fluvastatin | 4.18 | 28.0 ± 2.5 | 3.2 ± 0.3 | 411 |
| Pitavastatin | 4.58 | 12.7 ± 2.6 | 1.4 ± 0.2 | 421 |
| Rosuvastatin | 2.29 | 0.1 ± 0.2 | 0.0 ± 0.0 | 482 |

**Table legend: Parallel Artificial Membrane Permeation Assay (PAMPA); Effective permeability (Pe) of the compounds; Mw molecular weight; CLOGP the logarithm of the partition coefficient between n-octanol and water**

Supporting evidence claim that statins' effect on brain cells is controversial [146]. In some cases, statins were shown to have a neuroprotective effect by decreasing the risk of dementia [147, 148], Alzheimer's disease, and improving cognitive impairment [149]. Discordant were the results of cognitive impaired effects associated with statin use [118, 121]. Furthermore, randomized controlled trials however failed to find a significant beneficial nor impairing effect [150, 151].

### *4. Neuroprotective effect*

Epidemiological studies alluded to hypercholesterolemia as one of the main risk factors for Alzheimer's disease (AD) [152]. AD is a chronic neurodegenerative disorder and the most common cause of dementia in the elderly characterized by the appearance of brain senile plaques composed of β-amyloid proteins, and neurofibrillary tangles of hyper-phosphorylated tau protein [153]. Amyloid plaque formation has been shown to increase with a cholesterol-rich diet. Regression however was observed when dietary cholesterol

intake was diminished [154]. Furthermore, an increase in the level of isoprenoid (FPP, GGPP) was observed in the postmortem tissue from the frontal cortex [155] being more prevalent in non-statin treated than statin-treated patients [156].

Statins were useful in treating or preventing AD [157] as well as in reducing the incidence of cognitive impairment with or without dementia in patients [158]. Statins neuroprotective activity is based on the inhibition of the biosynthesis of cholesterol and isoprenoid by-products. Statins had a positive effect on memory and cognitive level *in vitro* and *in* patients by inhibiting amyloid production via modification of GTPase prenylation or reduction of APP in lipid raft and APP phosphorylation [159-161]. Furthermore, statins had a direct *in vitro* neuroprotective effect against glutamate-induced toxicity [162].

- Dose-dependent effects

Toxicity or beneficial effects of statin depends on the dosage. Low-dose of statins (nM) attenuated the entry of aberrant neuronal into mitosis [163], impaired inflammation [164], and activated anti-apoptotic pathways *in vivo* [165]. High dose administration of atorvastatin significantly reduced lipoperoxidation, protein oxidation, and nitration and increased the glutathione (GSH) levels in the parietal cortex of aged beagles [166]. Although high levels of statins (μM) decreased Aβ production, the significant decrease of cellular cholesterol and isoprenoid levels induced cellular death [146]. Moderate doses (μM) of statins induced a small reduction of cholesterol level more specifically in lipid rafts facilitating the interaction of β-secretase and APP [146].

### *5. Neurotoxic effect*

Numerous studies in different cellular models confirmed the neurotoxic effect of statins. Statins decreased the viability of rat brain neuroblasts [167] and primary neurons [168], as well as of human and rat malignant glioma cell lines [169, 170]. Atorvastatin and simvastatin triggered differentiation and cell death in neurons and astroglia [171]. In addition, mevastatin but not pravastatin induced the degeneration of differentiated neuroblastoma cells (NBP2) following 72 hours (50% cell death at 10 µM) [172]. Another study, on the human neuroblastoma line SH-SY5Y, demonstrated the time and dose-dependent apoptosis induced by lovastatin (0-100 µM) and had no effect on cell viability at 24 hours. However, at 48 and 72 hours, significant cytotoxicity was observed at relatively low doses of less than 10 µM which induced a greater than 50% reduction in MTT activity [173].

Multiple mechanisms were proposed as contributors to statins neurotoxic effects:

a. Decrease in neurotransmission: cholesterol is an element of the exocytosis apparatus where it plays a role in the biogenesis and transport of synaptic vesicles. Low cholesterol levels may reduce synaptic activity due to lower amounts of released neurotransmitters in general [174]. Statins impact neuronal and glial cells, decreasing neurotransmitter synaptic levels (serotonin) [175].
b. Deficiency of nonsterol isoprenoids attenuated cell growth and induced apoptosis [176]. In the CNS, Rho GTPases participate in cytoskeleton remodeling, and in neuronal development, migration, plasticity, and protection [177-180]. The decrease in FPP and GGPP levels in both, cultured neurons [181] and mouse

brain [155] decreased signal transduction pathways are crucial for neuronal growth and survival.

c. Oxidative stress: the release of caspases [182] or an increase in mitochondrial ROS levels [77] triggered cellular death. The oxidative stress induced by statins can cause neuronal damage thereby leading to the pathogenesis of an array of neurodegenerative diseases (figure 17).

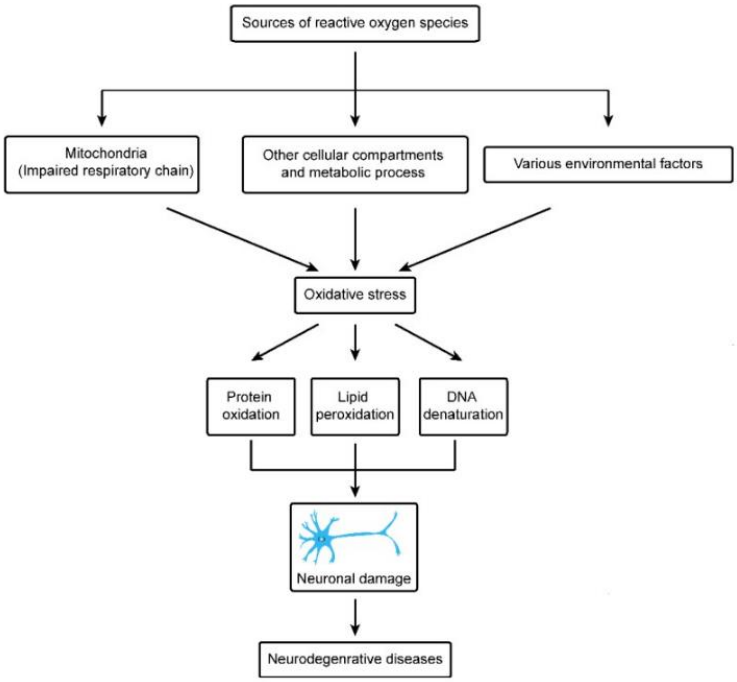



**Figure 17. The causes of oxidative stress in neurodegenerative diseases [183].**

## G. Objective of the study

Statins are cholesterol-lowering drugs. They are competitive inhibitors of the HMG-CoA reductase enzyme that catalyzes the conversion of HMG-CoA into mevalonate that ultimately is metabolized into cholesterol and ubiquinone. Mevalonate diverges in 2

different pathways leading to cholesterol and ubiquinone which mediates electron transfer between complexes I and II and the cytochromes. Side effects of statins treatment include myopathies, renal, and liver toxicity neurological. In addition, statins' neurotherapeutic effects were controversial being neuroprotective in some and neurodegenerative in others.

1. ***Hypothesis:***

Inhibition of HMG-CoA reductase by statins will decrease levels of cholesterol and ubiquinone. Statins' cytotoxicity, therefore, may be attributed to a decrease in ubiquinone level consequently to a decrease in energy production. We aim to investigate the in vitro effects of statins on rat and human neuronal cells. Five-different statins belonging to type 1 (mevalonate) and type 2 fluvastatin, pitavastatin, rosuvastatin, and atorvastatin) will be used addressing the following

1. How toxic are statins to neuronal cells?

The cytotoxicity of the 5 different statins on PC12 and SH-SY5Y cells in culture using MTT assay and trypan blue exclusion assay: concentration effect and time effect (24, 48 hours) at sub-$IC_{50}$ will be determined

2. What is the effect of statin-treated neuronal cells on ATP level?
3. What is the effect of different statins on the level of reactive oxygen species on neuronal cells?
4. Will pre- or co-treatment with MitoQ prevent statin-induced cytotoxicity in neuronal cells?

# CHAPTER II

# MATERIALS AND METHODS

## A. Materials:

### *1. Cell lines:*

The two different cell lines used in this study were a generous gift of Dr. F. kobaissy originally purchased from the American Type Culture Collection, Manassas, VA, USA. PC12: Rat pheochromocytoma and SH-SY5Y cells: Human neuroblastoma.

### *2. Disposable LabWare:*

- Cell culture plastic wares – Corning
- MTP black plate (Costar, Cat# 266)

### *3. Cell Culture Reagents were supplied as follows by:*

- **Sigma**: Heat inactivated Fetal Bovine Serum (FBS) (SF9665); Trypsin -1X (Cat#: T3924); Phosphate buffered saline (PBS) without calcium and magnesium (Cat#: D8537); Dulbecco's Modified Eagle's medium (DMEM, Cat#: D5796); DMEM F12 (Cat#:D8437); Heat Inactivated Horse Serum Donor Herd, (Cat#: H1138); Sodium pyruvate (Cat#: S83636),
- **Biowest**: Penicillin-Streptomycin Solution 100x (Cat#: L0022-100)

### *4. In Vitro Kits*

- **Sigma**: HMG-CoA Reductase Assay Kit (Cat#: CS1090); Cholesterol Quantitation Kit (Cat#: MAK043); ATP Bioluminescence Assay Kit (Cat#: MAK190)

### *5. Chemicals and Reagents*

- **Sigma-Aldrich**

  Dimethyl sulfoxide (DMSO; Cat#: 41640); Bovine Serum Albumin (BSA; Cat#: A2153); Thiazolyl Blue Tetrazolium (MTT; Cat#: M5655); IGEPAL (Cat#: CA-630); Mevastatin (Cat#: M2537); Catalase (Cat#: C-3515)
- **FOCUS BIOMOLECULES:** Mitoquinone (MitoQ, Cat#: 10-1363)
- **ACROS Organics**

  Nitro Blue Tetrazolium Chloride (NBT; Cat#: A0317685)
- **MERCK**

  Potassium Hydroxide (KOH; Cat#: C140532)
- **VWR-Chemicals**

  Methanol; Ethanol Absolute; Hydrogen Peroxide
- **Cayman Chemicals**: Atorvastatin calcium salt (Cat#: 10493); Rosuvastatin calcium salt (Cat#: 18813); Pitavastatin calcium salt (Cat#: 15414);
- **Calbiochem**

  Fluvastatin sodium salt (Cat#: 344095)

### *6. Drug and Reagent Preparation*

- **Statins:**

  Statins used in this study include mevastatin purchased from Sigma and from which were a generous gift of Dr. A. Habib: atorvastatin, rosuvastatin, pitavastatin, and fluvastatin; originally, they were purchased from Cayman chemicals Co, Ann Arbor, MI, USA. Stocks were prepared in DMSO at 12 mM concentration for atorvastatin, rosuvastatin, and pitavastatin; 4 mM for mevastatin; and 5 mM for fluvastatin.

- **MitoQ:**

  A stock of MitoQ solution (376 mM) was prepared by dissolving the MitoQ powder (12.5 mg) in DMSO (50 µl). To treat cells, a final concentration of 50 nM was used by diluting the stock in DMSO/Media with DMSO not exceeding 1%.

### *7. Equipment*

- Centrifuge 5416 (eppendorf)
- Centrifuge 5810 (eppendorf)
- Mini spin centrifuge (Thermo)
- Hematocytometer (Fisher scientific) (0267110)
- AES-2010 Speed Vac System (Thermo Savant)
- Multiskan EX (ELISA reader) (Thermo).
- Fluoroskan Ascent FL (Thermo).

## B. Methods

### *1. Cell Culture and Treatment*

PC12 and SH-SY5Y cells were cultured in their respective media and incubated in a humidified 5% $CO_2$ incubator at 37ºC for the indicated time. PC12 cells were cultured in Dulbecco's Modified Eagle's medium DMEM (500 mL) supplemented with FBS (10%), PS (1%), Horse Serum (5%), and Sodium Pyruvate (1%). SH-SY5Y cells were cultured in DMEM (500 mL) supplemented with FBS (10%) and PS (1%).

The following statins were initially examined at the indicated final concentrations: 1, 5, 10, and 15 µM for each of atorvastatin, rosuvastatin, mevastatin, and pitavastatin and 1, 3, and 5 µM for fluvastatin. The % concentration of DMSO was constant in all and did not exceed 1%.

### *2. Viability Assays*

a. MTT viability assay: Viability Assays

PC12 and SH-SY5Y cells were seeded for 24 hours in a 96-well plate ($10^4$ cells/100 µl media) then treated (24 and 48 hours) with each of the different statins at concentrations ranging between 1-15 µM. Using MTT assay, viability was assessed in treated and control (DMSO treated) cells. In brief, 10 µl MTT (5 mg/mL dissolved in PBS) was added to freshly introduced media and incubated for 3 hours ( 37ºC). The media was then removed and followed by the addition of DMSO (50 µl) to dissolve the formazan crystals. The

intensity of the purple color formed was quantified by measuring the absorbance (λ = 595 nm) using an ELISA reader.

Viability was calculated and expressed as follows

$$\% \text{ Viability} = \frac{\text{Abs (Treated cells) x 100}}{\text{Abs (Control)}}$$

b. Trypan blue exclusion assay

PC12 and SH-SY5Y cells were seeded for 24 hours in a 24 well plate ($5 \times 10^4$ cells/ 0.5 ml media/ well). Following treatment with statins (24 and 48 hours), media was aspirated, and cells were washed with 1X PBS, trypsinized for 3 minutes, collected, mixed with trypan blue at a ratio of 1:1 (µl), and counted using a hemocytometer under a light microscope. Dead cells are stained blue due to the diffusion of the dye whereas viable cells remained unstained and appeared opaque. Viability was calculated as follows:

$$\% \text{ Viability/well} = \frac{\text{Number of Viable unstained cells x 100}}{\text{Number of stained + unstained cells}}$$

$$\% \text{ Viability} = \frac{\text{Viability of treated x 100}}{\text{Viability of control}}$$

### *3. Determination of ROS level: NBT Assay*

Using p-nitro-blue-tetrazolium (NBT) reduction assay, intracellular ROS level was determined in SH-SY5Y cells. Cells were seeded for 24 hours in a 96-well plate ($10^4$ cells/100 µl media/well) then treated (24 and 48 hours) with increasing statin

concentrations (1-10 µM). In brief, media was aspired followed by NBT (1 mg/ml $ddH_2O$, 100 µl) addition and incubation for one-hour at 37° C. Cells were then washed with methanol (100 µl) and allowed to air dry. The formed formazan crystals were solubilized by the consecutive addition of potassium hydroxide (2M, 120 µl) and DMSO (140 µl). The intensity of the blue color was quantified by measuring the absorbance ( λ= 630 nm) using an ELISA reader. ROS level was calculated as follows:

$$\% \text{ NBT reduced} = \frac{\text{Abs (Treated cells) x 100}}{\text{Abs (Control)}}$$

% ROS production = 100% - % NBT reduced

### *4. Effect of the antioxidant enzyme catalase*

Catalase is an enzyme responsible for the detoxification of oxidizing molecule hydrogen peroxide. Cells were seeded for 24 hours in a 96-well plate ($10^4$ cells/100 µl media), pre-treated for 2 hours with catalase (33 U/well) followed by statin treatment for 24 and 48 hours. Using MTT assay, the viability of catalase-statin-treated SH-SY5Y cells was assessed and compared to statin-treated cells. Hydrogen peroxide (200 µM final concentration) was used as a positive inhibitory control of catalase activity.

### *5. Determination of intracellular ATP level Determination*

ATP level was quantified using an ATP Bioluminescence Assay Kit HS II. SH-SY5Y cells were seeded for 24 hours in 24 well plate (5 x $10^4$ cells/ 0.5 ml media/ well) and

treated with statins for 24, 36, and 48 hours. Cells were trypsinized, collected by centrifugation (1200 rpm, 10 mins), washed with 1X PBS, and lysed using the kit Lysis reagent (100 µl, 5 min). Sample of the fresh lysate (50 µl) was then transferred over luciferase enzyme (50 µl) introduced in black MTP plate. The intensity of bioluminescence was quantified using fluoroscan. The percentage of ATP was calculated as follows:

$$\% \text{ ATP} = \frac{\text{Bioluminescence (treated) x 100}}{\text{Bioluminescence control}}$$

### *6. Effect of MitoQ*

Following seeding for 24 hours in a 96-well plate ($10^4$ cells/100 µl media), the effect of MitoQ on the viability of statin-treated SH-SY5Y cells (24 and 48 hours) was assessed using MTT assay. Two sets of experiments were designed: in the first set cells were co-treated with MitoQ (50 nM) and statins; in the second set cells were pre-treated for 2 hours with MitoQ (50 nM) followed by the addition of statins for the indicated time. Treated cells were compared to statin-treated cells as a control to examine if MitoQ would protect against the statin cytotoxicity.

### *7. HMG-CoA Reductase activity*

The direct effect of statins on the purified catalytic subunit of the enzyme (HMG-CoA Reductase) was assessed using HMG-CoA Reductase Assay Kit following manual instructions. Briefly the reaction assay (96 well plate) of final volume 200 µl/ well contained: 1 µl of 100 µM statin (0.5 µM final concentration), 4 µl NADPH (400 µM), 12

µl HMG-CoA substrate (0.3 mg/ml) and 181 µl of 1X assay buffer. The reaction was initiated by the addition of 2 µl of the enzyme HMGR (0.50–0.70 mg/ml) to each well.

Oxidation of NADPH by HMG-CoAR was kinetically monitored at 37°C every 10 seconds over 20 minutes by measuring absorbance (λ=340 nm) using a microplate spectrophotometer reader. Results were expressed as % of the control specific activity of the enzyme in the absence of statin. Pravastatin (1 µl, 100 µM) was used as a positive inhibitory control of HMGR activity.

### *8. Determination of intracellular cholesterol level*

a. Cholesterol extraction

SH-SY5Y cells were seeded for 24 hours in 100 mm Petri dishes ($10^6$ cells/10 ml). Statin treated cells (24 hours) were scraped, washed with 1X PBS, and centrifuged then resuspended in PBS (1 ml). The cell suspension (200 µl) was removed to determine protein concentration using Bradford assay (described below) while the remaining 800 µls cell suspension was used for cholesterol extraction. In brief, pelleted cells were extracted by adding 200 µl chloroform:isopropanol:IGEPAL (7:11:0.1 ml/ml/ml) which were vortexed at room temperature, homogenized then centrifuged (13,000 g, 10 min). The organic phase was then transferred to a new tube, dried using a heat block (50 °C) initially, followed by drying under vacuum for 30 min using a SpeedVac. The dried lipid extract was finally dissolved in cholesterol buffer (200 µl) provided by the kit.

b. Cholesterol assay

In a 96-well plate, samples (50 µl) of the cholesterol extract were added to the reaction mixture kit (50 µl) and incubated for one hour at 37 °C. The intensity of the formed purple color reflecting total cholesterol content was measured (λ=570 nm) using a microplate spectrophotometer reader. Cholesterol level was determined compared relative to a cholesterol standard curve as described in the kit instruction manual.

**Bradford assay:** Protein content was determined according to Bradford assay using bovine serum albumin as a standard (0, 2, 4, 6, 8, and 10 µg/ml final concentration). Bradford reagent (200 µl) was added to appropriately diluted samples, vortexed, and incubated (10 mins). The absorbance of the blue color was measured (λ=595 nm) using an ELISA reader.

### *9. Statistical Analysis*

Statistical analysis was determined using Excel Microsoft independent student t-test. For multiple comparisons, all analysis was done on graph pad prism (version 7) based on Ordinary One Way Anova analysis and compared by Dunnett's and Tukey's multiple comparisons test. P-value $<0.05$ was considered significant. For each parameter tested, a set of at least three experiments were done where a triplicate of three determinants was applied. Also, for each parameter, both inter-categorical statistical significance and significance relative to control were analyzed.

# CHAPTER III

# RESULTS

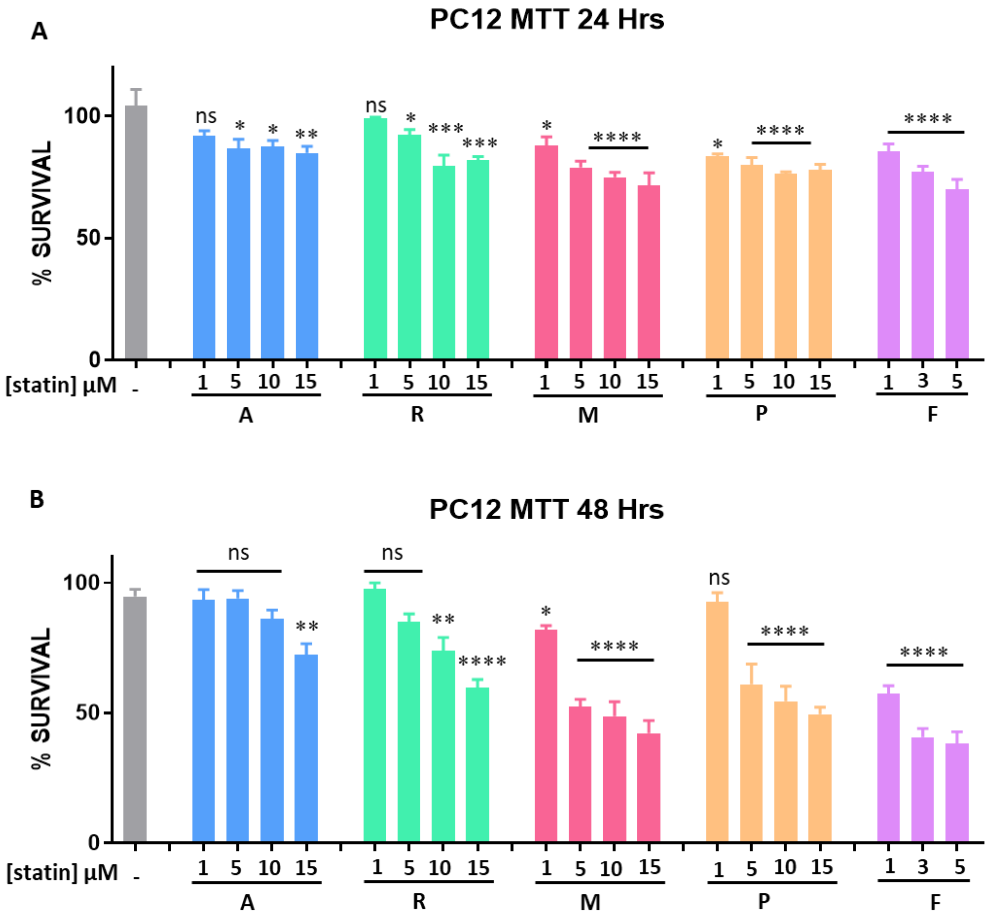
## A. Cytotoxicity of statins

We have initially screened for the cytotoxicity of the 5 statins on PC12 and SH-SY5Y cells. The viability of the treated cells for 24 and 48 hours was determined using MTT assay and was further confirmed by trypan blue exclusion assay. The final concentration used for each of atorvastatin, rosuvastatin, mevastatin, and pitavastatin ranged between 1 and 15 µM, while fluvastatin concentration ranged between 1 and 5 µM. Our findings show:

### *1. Statins decreased the viability of PC12 cells*

Using the MTT assay, the decrease in the viability of PC12 treated with the different statins was more significant at 48 hours than 24 hours. Treating PC12, for 24 hours at the maximal concentration of all the statins caused a 30% decrease in the viability. Fluvastatin at 5 µM exhibited almost the same effect as 15 µM of the other statins. On the other hand, treating PC12 with statins for 48 hours, more significant cell death was obtained; with atorvastatin being the least effective (30% decrease at 15 µM) and fluvastatin the most effective causing a 60% decrease in viability at 3 µM. Similarly, treating with 15 µM each rosuvastatin, mevastatin, and pitavastatin, resulted in cell death ranging between 40%, 50%, and 60% respectively (figure 18B).

We next verified the statin-induced effects by examining the viability of PC12 using trypan blue exclusion assay. We opted to use the maximal concentration examined for the different statins that caused approximately 50% cell death. The decrease in viability was more significant after 48 hours of treatment with statins compared to 24 hours (figure 18C). The percentage decrease in viability varied between 55% and 60%. While atorvastatin showed an insignificant effect with MTT assay, it caused significant cell death of 25% and 50% at 24 and 48 hours respectively using trypan blue. These findings suggest that long-time exposure of PC12 to statins disturb the plasma membrane integrity leading to an increase in cell death.



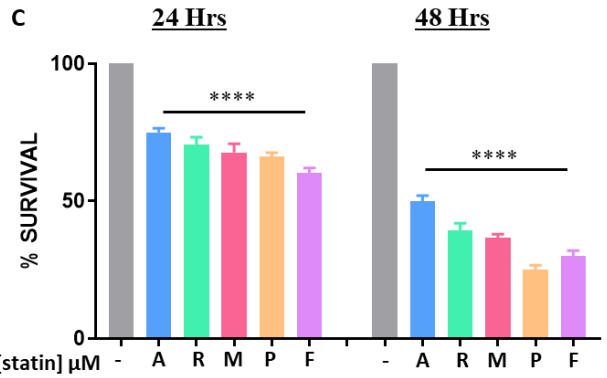



**Figure 18. Statins decreased the viability of PC12 cells.**

Cells were treated with indicated statins A: atorvastatin, R: rosuvastatin, M: mevastatin, P: pitavastatin, and F: fluvastatin. Viability was then determined using MTT assay following A) 24 hours and B) 48-hour treatment. Using trypan blue assay (C), the viability of cells, treated with statins (15 µM for A, R, M, and P, and 5 µM for F), was also determined. Data presented are mean ± SEM of 9 determinations from 3 different experiments. Asterisks on bars represent significance relative to the control (*), (**), (***), and (****) correspond to P<0.05, 0.01, 0.001, and 0.0001 respectively, ns: not significant.

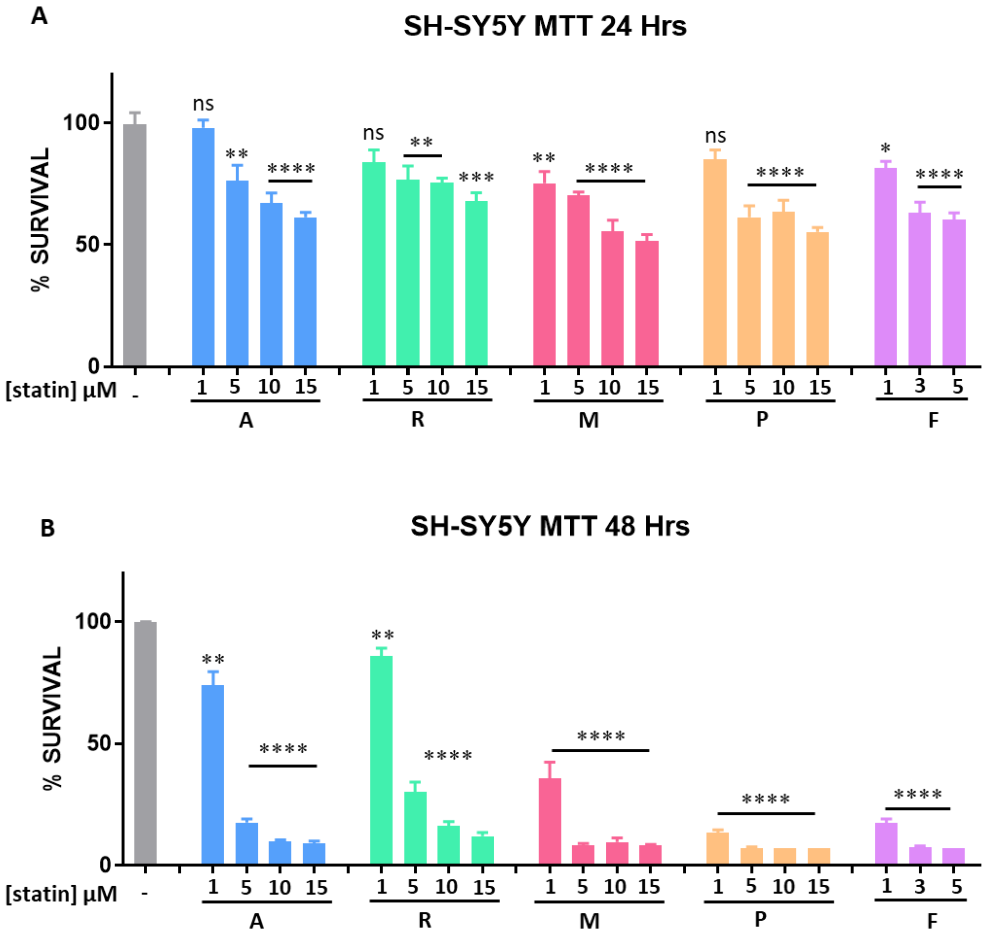
### *2. Statins decreased the viability of SH-SY5Y cells*

The effect of statins on the viability of human neuronal cells was assessed using the MTT assay. Compared to 24 hours of treatment, the viability of SH-SY5Y cells significantly decreased following 48 hours of treatment.

The percentage of cell death occurring after 24 hours of treatment with the 5 statins, at maximal concentration, ranged between 30% and 50% (figure 19A) with rosuvastatin being the least effective. However, treating cells with the various statins for 48 hours caused a pronounced cell death including atorvastatin and rosuvastatin. Interestingly, at 5 µM, all statins reduced significantly ( >70%) the viability of SH-SY5Y. However, at lower concentrations (1 µM), atorvastatin and rosuvastatin were the least effective causing cell

death of 25% and 15% respectively while pitavastatin and fluvastatin were the most potent causing cell death of 80% (Figure 19B).

Using trypan blue exclusion assay we further examined the viability of statin-treated cells at a final concentration that caused less than 50% cell death at 24 hours by the MTT assay. Trypan blue findings were concordant with those of MTT. Cell death ranged between 25% and 40% at 24 hours, but increased drastically, following 48 hours, ranging between 90% and 70% (figure 19C).



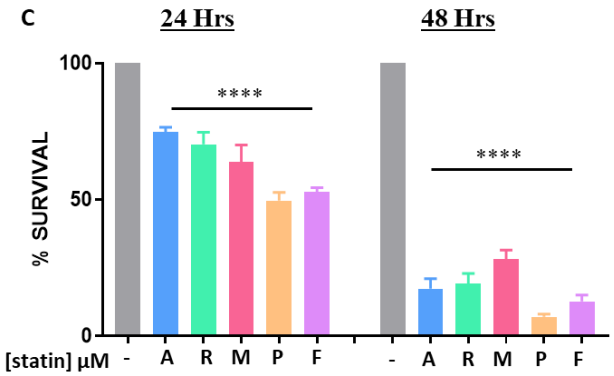



**Figure 19. Statins decreased the viability of SH-SY5Y cells.**

Cells were treated with indicated statins A: atorvastatin, R: rosuvastatin, M: mevastatin, P: pitavastatin, and F: fluvastatin at concentrations ranging between 1 and 15 µM. Viability was then determined using MTT assay following A) 24 hours and B) 48-hour treatment. Using trypan blue assay (C) the viability of cells treated with statins (5 µM for A, R, and P, 1 µM for M, and 3 µM for F) was also determined. Data presented are Mean ± SEM of 9 determinations from 3 different experiments. Asterisks on bars represent significance relative to the control (*), (**), (***), and (****) correspond to $P<0.05$, 0.01, 0.001, and 0.0001 respectively, ns: not significant.

To sum up, cell death in statin-treated PC12 or SH-SY5Y cells was more significant following 48 hours than 24 hours with the human SH-SY5Y cell line being more sensitive than the rat PC12 cell line. Hence, we opted in all subsequent experiments to continue with SH-SY5Y cells treating them at concentrations that caused less than 50% death as estimated by the MTT 24 hours.

## B. Statins induced ROS production

We next examined using NBT assay, whether the pronounced cell death occurring at 48 hours in statin-treated SH-SY5Y cells is due to an increase in reactive oxygen species (ROS). The ability of cellular dehydrogenase to reduce NBT will decrease with an increase in oxidative stress level which correlates with ROS levels.

Treatment of SH-SY5Y cells for 24 hours with various statins (1-10 μM) showed a dose-dependent increase in ROS level (Figure 20A) ranging between 8% and 35%. However extended exposure to statins (48 hours), caused a dose-dependent increase in ROS production with each atorvastatin, rosuvastatin, and mevastatin reaching 65% at the highest tested concentration (10 μM). Regarding pitavastatin and fluvastatin, the maximal obtained increase occurred at concentrations as low as 1 μM with no further increase with concentration. Therefore, the increase in cell death induced following 48 hours of treatment with statins is due to the significant increase in oxidative stress levels.

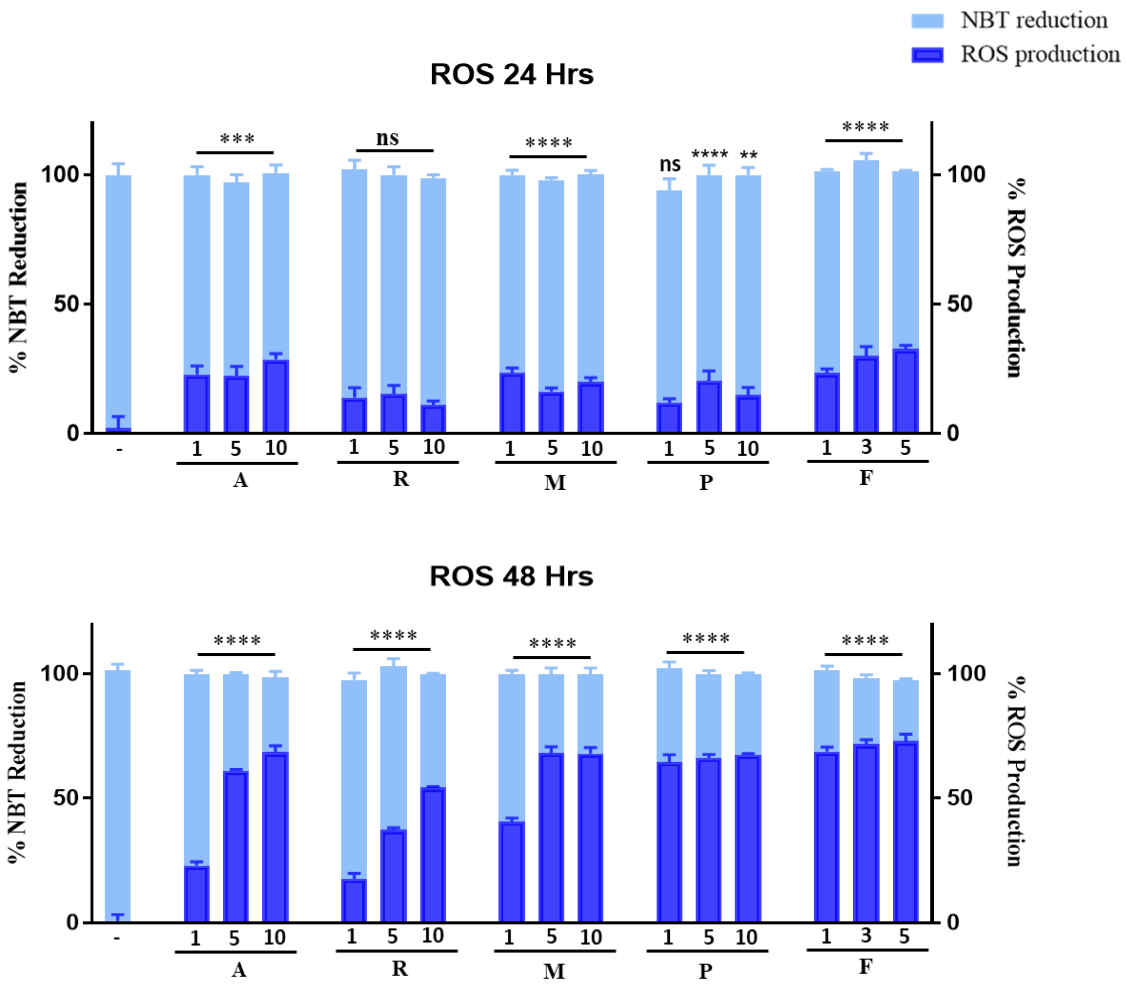
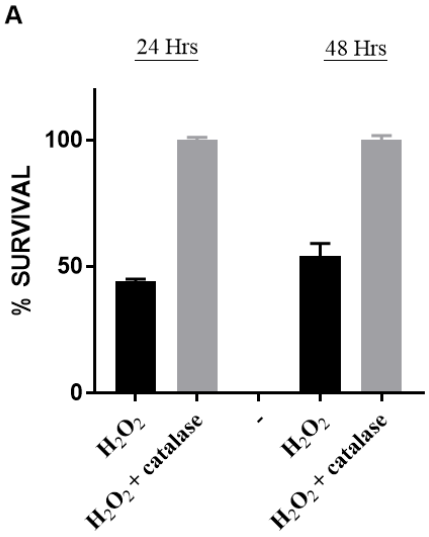



**Figure 20. Stains induced time-dependent ROS production.**

Cells were treated with the indicated statins: A: atorvastatin, R: rosuvastatin, M: mevastatin, P: pitavastatin, and F: fluvastatin, with concentrations ranging between 1 and 10 µM for 24 and 48 hours. ROS production was then assessed using the NBT assay. Data presented are Mean ± SEM of 16 determination from 4 different experiments. Asterisks on bars represent significance relative to the control. (*), (**), (***), and (****) correspond to P<0.05, 0.01, 0.001, and 0.0001 respectively.

### C. Catalase exhibited no protective effect on statin-treated SH-SY5Y cells

We next investigated if statin-induced oxidative stress is due to increased production of $H_2O_2$. The protective effect of the catalase enzyme responsible for the reduction of hydrogen peroxide ($H_2O_2$) into water and oxygen was examined. Hydrogen peroxide $H_2O_2$ (200 µM) treated SH-SY5Y cells were protected when treated with catalase. Viability was completely restored at both 24 and 48 hours (figure 21 A). However, catalase pretreatment exhibited no protective effect on statin-induced death. No restoration in viability was obtained in catalase pretreated cells compared to statin-treated at both time points (figure 21 B & C) indicating the cytotoxicity of statins in neuronal cells is independent of $H_2O_2$ production.



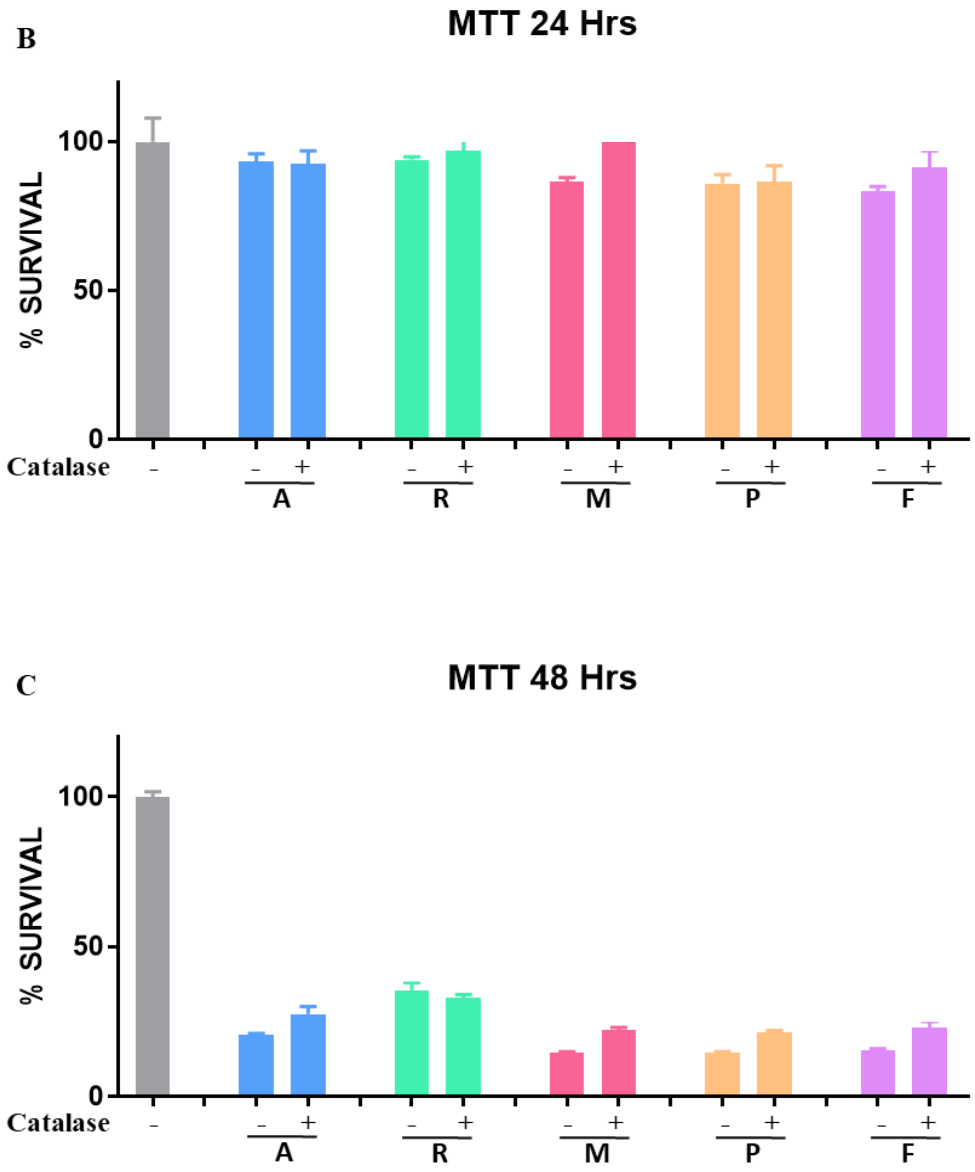



**Figure 21. Catalase exhibited no protective effect on statin-treated SH-SY5Y cells**.

Following treatment with catalase (2 hours), cells were treated for 24 and 48 hours with A) 200 µM $H_2O_2$ and (B & C) with the indicated statins A: atorvastatin, R: rosuvastatin, M: mevastatin, P: pitavastatin, and F: fluvastatin at a concentration of 5 µM for each of A, R, and P; 1 µM for M; and 3 µM for F. Viability was then determined using MTT assay. Data presented are the Mean ± SEM of 6 determinations from 2 different experiments compared to control.

### D. Statins reduce intracellular ATP level in human neuronal SH-SY5Y cells

Statins inhibit HMG-CoAR, the rate-determining step in the cholesterol pathway, hence it decreases cholesterol level and expectedly UQ level consequently influencing ATP level. We assessed the impact on ATP levels in statin-treated SH-SY5Y cells. Our findings show a decrease in ATP level with time. Following the 24 hour treatment, atorvastatin and rosuvastatin decreased ATP levels by 17% while mevastatin and pitavastatin by ~ 35%. However, fluvastatin caused a drastic decrease that dropped the ATP levels to 25%.

Further investigation showed that the drop in ATP levels was time-dependent (figure 22). Atorvastatin and mevastatin induced a gradual decrease in ATP that reached 45% and 22% at 36 hours and further dropped to 10% and 15% following 48 hours of treatment respectively. The decrease induced by other statins however plateaued at 36 hours of treatment. Fluvastatin and pitavastatin decreased ATP to non-detectable levels whereas rosuvastatin decreased ATP levels to 40%.

These results conclude that each of the statins distinctively alters ATP production, in a time-dependent manner, concordant with the viability results.

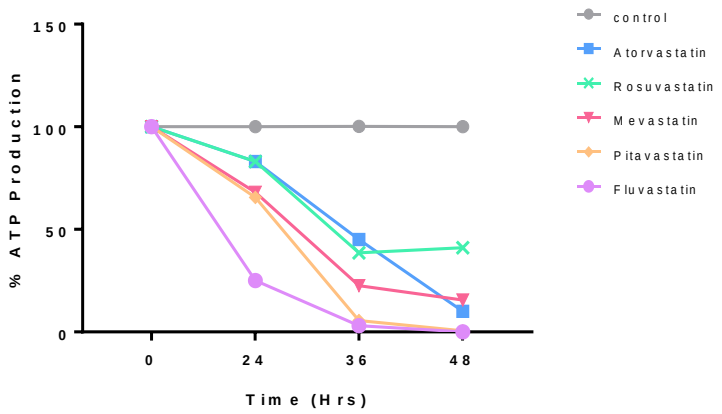


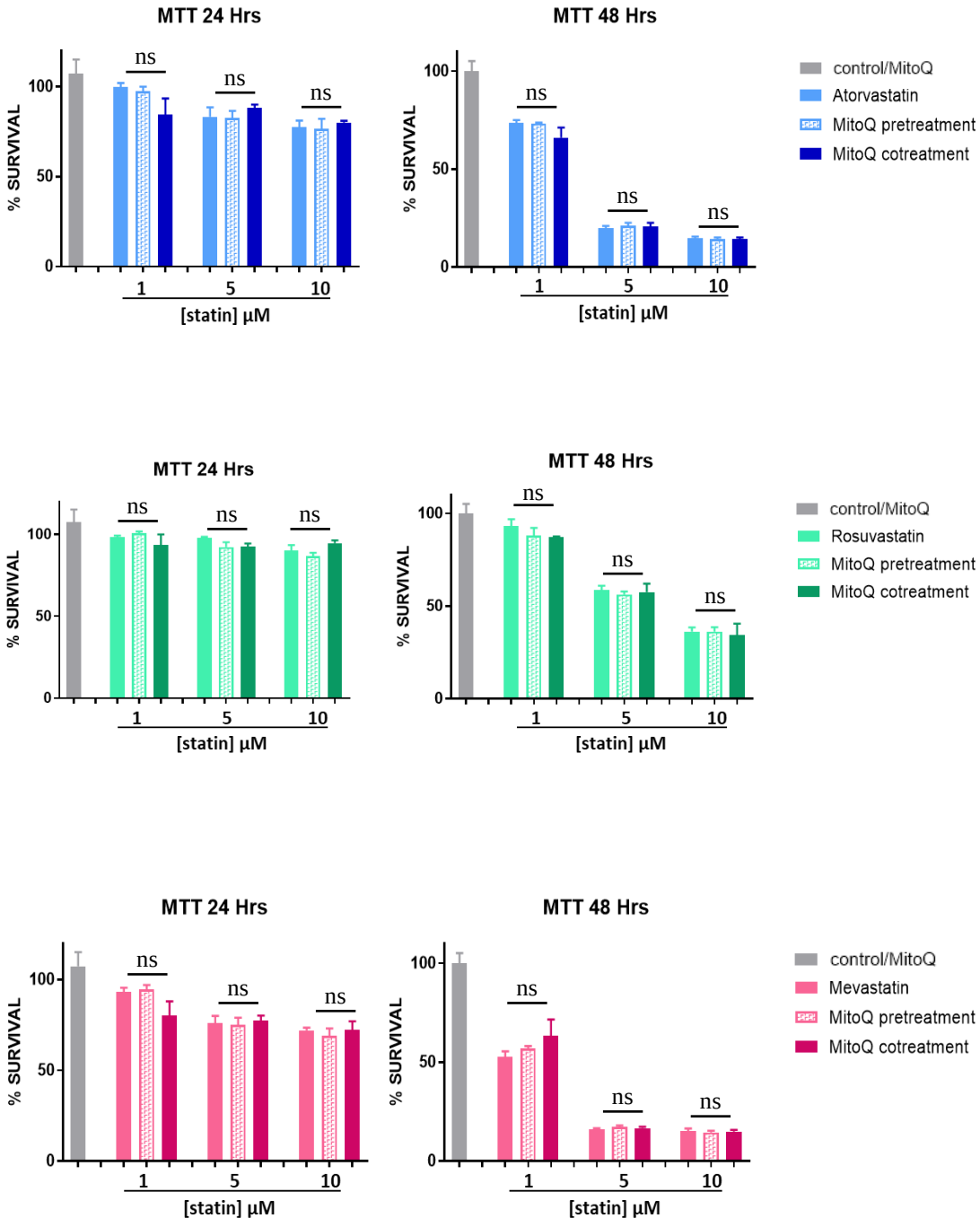

**Figure 22. Statins reduced intracellular ATP level.**

Cells were treated for 24, 36, and 48 hours with the indicated statins: A: atorvastatin, R: rosuvastatin, M: mevastatin, P: pitavastatin, and F: fluvastatin at a concentration of 5 µM for each of A, R, and P; 1 µM for M and 3 µM for F. ATP level was determined using ATP assay kit. Data presented are the Mean of 4 determinations from 2 different experiments compared to control.

### E. MitoQ has no protective effect against statin-induced cell death

Statins inhibit the mevalonate pathway which diverges into pathways that leads to various important biomolecules namely: cholesterol and ubiquinone. The latter mediates the transfer of electrons to cytochrome complexes from complexes I and/ or II of the electron transport chain (ETC). Hence statin treatment would influence ubiquinone (UQ), impairing mitochondrial function, and energy level. We, therefore, tested if the administration of MitoQ, the permeable analog of UQ, would prevent the cytotoxicity of statin. Our findings show (Figure 23) that neither pre-treatment with MitoQ (2 hours before statin addition) nor co-treatment (MitoQ-statin) protected against the statin-induced cell death. MitoQ treatment showed similar viability when compared to control. The decrease in

the viability in statin-treated SH-SY5Y cells remained the same with no restoration in viability with MitoQ pretreatment and cotreatment.



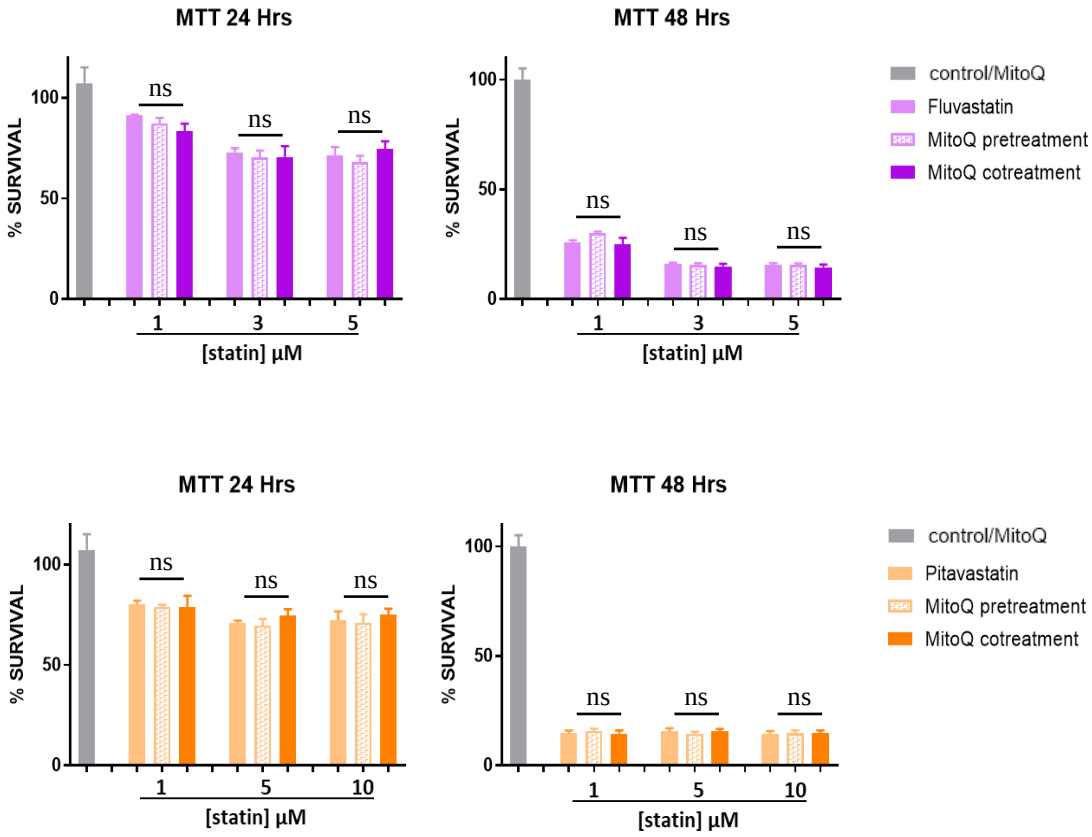



**Figure 23. MitoQ does not protect SH-SY5Y against statin-induced cell death.**

Cells were pre/co-treated with MitoQ (50 nM) along with the indicated statins: A: atorvastatin, R: rosuvastatin, M: mevastatin, P: pitavastatin, and F: fluvastatin with concentrations ranging between 1 and 10 µM for 24 and 48 hours. Viability was then determined using the MTT assay. Data presented are Mean ± SEM of 16 determinations from 4 different experiments. ns refers to statistically not significant (P>0.005) compared to the control/ MitoQ-treated cells.

## F. HMG-CoAR activity: Direct effect of statins

It is well known that statins are competitive inhibitors of HMG-CoAR. Using the HMG-CoA Reductase assay kit, we compared the direct inhibitory effect of the various statins used in this study with respect to the positive control (inhibitor provided by the kit).

All statins were tested at a final concentration of 0.5 µM similar to that of pravastatin (the Kit provided inhibitor).

Compared to pravastatin, all examined statins except for mevalonate showed a similar effect (figure 24). The activity of HMG-CoAR was significantly inhibited (>70% ) with each of atorvastatin, rosuvastatin, pitavastatin, and fluvastatin similar to that of pravastatin (75%). No inhibition was obtained by mevastatin, which is attributed to the structural state of the compound being a lactone. This requires initial activation by hydrolysis which occurs in vivo by intracellular esterases, following which the inhibition of HMG-CoAR.

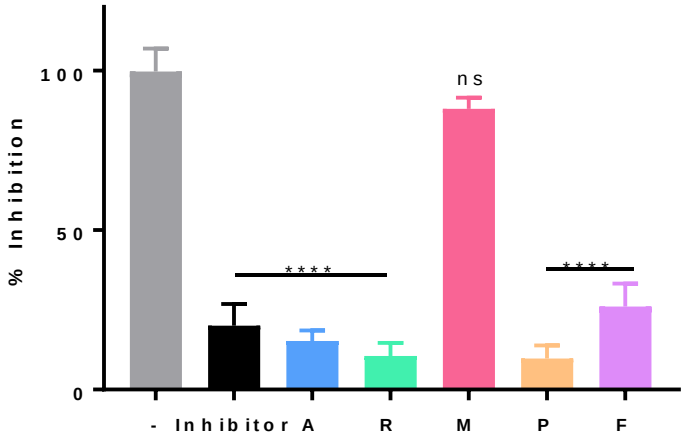



**Figure 24. Statins inhibit the activity of HMG-CoAR**.

The inhibitory effect on the purified catalytic subunit of HMGR of the indicated statins (0.5 µM) for each inhibitor, A: atorvastatin, R: rosuvastatin, M: mevastatin, P: pitavastatin, and F: fluvastatin was determined using HMG-CoA Reductase assay kit. Data presented are the Mean ± SEM of 3 determinations from 3 different experiments Asterisks on bars represent significance relative to the control. (****) correspond to P<0.0001, ns: not significant P>0.05.

## G. Statins alter intracellular cholesterol content

Finally, we assessed the impact of statins on intracellular cholesterol levels. SH-SY5Y cells treated with different statins for 24 hours were extracted and quantified for cholesterol level (free and esterified) using the cholesterol assay kit.

Compared to control, our preliminary findings show that all statins except for pitavastatin increased intracellular cholesterol levels. This may be attributed to statins inhibitory effect on HMG-CoAR that induces a reduction in intracellular cholesterol and a subsequent increase in LDL receptors leading to an increase in cholesterol uptake from culture media.

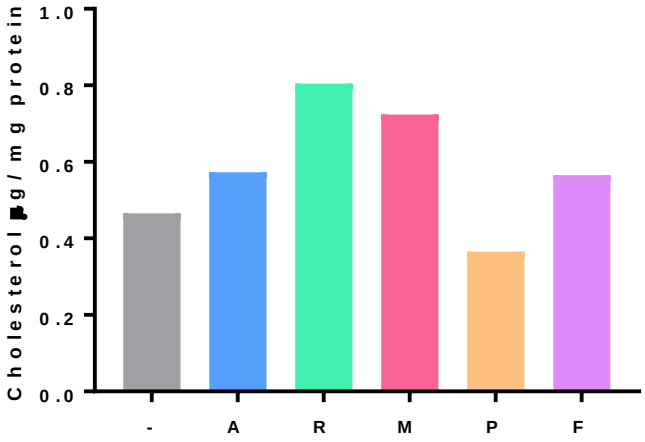



**Figure 25. Statins inhibit intracellular cholesterol content**.

Cells were treated for 24 hours with the indicated statins: A: atorvastatin, R: rosuvastatin, M: mevastatin, P: pitavastatin, and F: fluvastatin at a concentration of 5 µM for each of A, R, and P; 1 µM for M and 3 µM for F. Intracellular cholesterol was determined using cholesterol quantification kit. Data presented are the Mean of 2 determinations from 1 experiment.

# CHAPTER IV

# DISCUSSION

The anti-atherosclerotic effect of statins, labeled them among the most commonly prescribed drugs, to patients with cardiovascular events or diseases [16, 184]. They are competitive inhibitors of the HMG-CoA reductase, the enzyme catalyzing the rate-limiting step in the mevalonate pathway leading to cholesterol [63]. In addition to their hypocholesterolemic effect, they exhibit a pleiotropic role in different tissues and body organs [37]. However, the decrease in cholesterol level by statins is usually accompanied by a decrease in the level of important biomolecules such as isoprenoids and ubiquinone [36] essential for the regulation of many cellular activities including those in the brain and central nervous system [185]. While the effects of statins on the cholesterol pathway in the liver have been extensively characterized, their effects on CNS remain relatively limited [8, 143].

In this *in-vitro* study, we have examined the direct effect of 5 different statins, on rat PC12 and human SH-SY5Y cells including the natural product, mevastatin; and 4-synthetic products (fluvastatin, atorvastatin, rosuvastatin, and pitavastatin) belonging to type1 and type 2 respectively. We hereby report, the sensitivity of human over rat cell lines to statins. Being undifferentiated, the PC12 are insensitive to statins that inhibit Rho-associated coiled-coil forming protein serine/threonine kinase (ROCK) [186, 187]. In all subsequent experiments, we opted to proceed with SH-SY5Y the human neuronal cell line.

All tested statins caused dose- and time-dependent cell death (20%-50%) but was more pronounced (80%-95%) following 24- and 48- hour treatment respectively. Comparing statin-treated SH-SY5Y cells, a significant time-dependent increase in ROS level with a concomitant decrease in ATP level was obtained in the following order from the most potent F>P>A>R (least) for type 2. Mevastatin (type 1) exhibited an effect similar to that of pitavastatin. Neither pre-treatment with catalase nor the co- or pre-treatment with MitoQ, the structural analog of ubiquinone, were protective; No restoration in viability was obtained. Our preliminary findings suggest that statin cytotoxicity may not be limited to the inhibition of HMG-CoAR activity (decrease cholesterol) or lowered ubiquinone (UQ), but to their plausible effect on the plasma membrane as well as the decrease in products of the mevalonate pathway.

Although inhibition of cholesterol biosynthesis by statins alters membrane composition leading to increased membrane permeability [28], statins aggregation in the membranes change the bilayer nanomechanical stability of the bilayers increasing their elasticity [47]. The viability of statin-treated cells varied between MTT and trypan blue exclusion assay. The decrease in viability was more pronounced using trypan blue exclusion assay than MTT assay. While the MTT assay depends on the activity of a mitochondrial dehydrogenase, which involves the transport of statins through the plasma membranes and possibly mitochondrial membranes [188]; Trypan blue assay however reflects the derangements in the plasma membrane integrity [189]. The difference in SH-SY5Y sensitivity to statins may be attributed to their physiochemical properties, lipophilicity, hydrophilicity, and size (molecular weight). Therefore, statins' level and

consequently their effects depend on statin association with the plasma membrane and/or transport intracellularly. Fluvastatin and pitavastatin were the most potent in our study, being lipophilic and small in size, they may favorably permeate the plasma membrane. Whereas the hydrophilic rosuvastatin, with polar ionizable negatively charged sulfate group, and the bulky side groups of atorvastatin, attenuate their diffusion through the cell membrane hence were the least potent.

Hence disturbances in membrane integrity, leading to cell death, may not be ignored, even at sub-$IC_{50}$ statin-treated SH-SY5Y cells (48hrs). In line with our findings, fluvastatin, the most potent in our study, was previously reported as the most effective membrane disruptor [64]. Other mechanisms explaining the decrease in neuronal viability by statin proposed the disruption of cytoskeleton integrity, involving actin that plays a role in maintaining neuronal polarity and dendrites plasticity [190-193].

The toxicity of statins is not limited to the decrease in cholesterol level, but may also result from the downstream decrease in the level of biomolecules such as prenylated intermediates, dolichols, and ubiquinone that serve important cellular functions including cell signaling, growth, and differentiation as well as mitochondrial respiration [37, 107]. Some statins were reported to target the mitochondria, inhibiting different complexes in the electron transport chain that may result in deleterious consequences on neuronal cells [76]. The effect of statins on brain cells and mitochondria remains controversial [76, 194, 195]. Whereas some studies reported on the neuroprotective effects of statins, others demonstrated neurodegenerative effects [150, 151]. Furthermore, there is no consensus concerning alterations in the ubiquinone level, with both *in-vitro* and *in-vivo* statin-treated

studies [196, 197]. Ubiquinone is an electron carrier in the electron transport chain that mediates the transfer of electrons from complexes I and II to cytochromes [198].

Previous *in-vivo* and *in-vitro* studies on isolated mitochondria, reported the occurrence of mitochondrial dysfunction following high dose or chronic statin treatment [109, 199, 200]. In addition to their role in cellular respiration and the site for many metabolic pathways (fatty-acid oxidation, TCA), mitochondria are involved in: $Ca^{2+}$ homeostasis [201], controlling antioxidant activity (e.g. glutathione peroxidase, catalase) [202], and reactive oxygen species generation [203]. Depending on the level of the latter, ROS can either act as second messengers or promoters of cellular damage [110]. Many mechanisms were proposed regarding statin-induced cytotoxicity includes: 1) A decrease in coenzyme Q10 levels [204, 205]; 2) Down-regulation of antioxidant enzymes [74, 206]; 3) inhibition of respiratory chain complexes [96, 205, 207]; and 4) inducing the mitochondrial apoptosis pathway [208, 209].

Increased ROS is considered an early event in the progression of neurological diseases such as Alzheimer's and Parkinson's disease [210, 211]. In our study, we demonstrated that the pronounced statin-induced cell death following 48 hours treatment was accompanied by a significant: a) increase in intracellular ROS that was not counteracted by catalase treatment, indicating that $H_2O_2$ if generated, is not released, and b) a decrease in ATP levels. These findings are in line with *in-vivo* studies relating ROS production liver and renal tubular damage following atorvastatin administration [39]. Similarly, skeletal muscle biopsies from statin-treated patients [115] and animals [212] showed altered mitochondrial function caused by diminished production of ATP, excess

production of ROS, and apoptosis. Treating breast cancer cells (MCF-7) with statins increased ROS production causing cell cycle arrest, inhibition of proliferation leading to cell death [213]. Surprisingly discordant to our findings, a previous study on cardiomyocytes, Jones et al. reported the protective effect of nM concentration simvastatin on mitochondrial oxidative stress [110]. Additionally, simvastatin reversed Amyloid β-induced mitochondrial dysfunction by increasing intracellular ATP production, oxygen consumption, and mitochondrial number [214].

UQ deficiency has been suggested to be involved in the pathogenesis of statin-induced myopathy [105, 106]. The decrease in the level of UQ reduces cell energy, promotes oxidation, impair mitochondrial function, and promotes apoptosis [76, 215]. A recent clinical meta-analysis showed that UQ supplementation ameliorated statin-associated muscle symptoms [105]. However, the uniform effectiveness of UQ supplementation, both *in-vivo or in-vitro* studies, remained controversial with no consensus [196, 197, 216].

Mitoquinone (MitoQ), a mitochondrial-targeted antioxidant, is a ubiquinone analog. It consists of a ubiquinone moiety linked to a triphenylphosphonium ($TPP^+$) molecule. The positively charged lipophilic $TPP^+$ moiety allows MitoQ to pass through the phospholipid bilayers and to accumulate within the mitochondrial inner membrane driven by the mitochondrial membrane potential [217]. The active antioxidative form of MitoQ (ubiquinol), is oxidized by ROS and has been used as an antioxidant in a range of *in-vivo* studies in rats and mice and phase II human trials [218]. As an antioxidant, MitoQ has been effective against lipid peroxidation, peroxy-nitrite, the hydroperoxyl radical, and superoxide but not $H_2O_2$ [219]. We examined whether MitoQ treatment (50 nM) would

prevent the cytotoxicity of statin. Regardless of the mode of combined treatment (pre/co) with statins, neither the pre-treatment with MitoQ nor co-treatment (MitoQ-statin) protected against the statin-induced cell death. While other studies have used twice the concentration we did, the protective role of MitoQ was not sustained with time which explains our findings. MitoQ (200 nM) protected the human-derived desmoplastic cerebellar medulloblastoma cell line (DAOY) from the lovastatin induced toxicity at 12 hours but not at 48 hours [220].

To sum up, in this study the direct exposure of SH-SY5Y cells to a sub-$IC_{50}$ concentration of statins exhibited a neurotoxic effect. Cell death induced may involve perturbations occurring at the plasma membrane, an increase in ROS, and a decrease in ATP production. The order of toxicity fluvastatin> pitavastatin> atorvastatin> rosuvastatin is proportional to lipophilicity. The structural differences of the various statins support the difference in the pharmacokinetic response of these drugs in the central nervous system. It is thus important to emphasize the mechanistic difference between statins to achieve the best therapeutic decision.

**Future studies**

- Compare the effect of statins on differentiated PC12.
- Determine the protective effect of antioxidants: NAC, GSH, and activity or expression of antioxidant enzymes.
- Determine the RNS level in statin-treated cells.
- Investigate the effect of statins on cytoskeletal proteins (F-actin).

- Assess mitochondrial dysfunction.
- Measure the effect of statins on cellular HMG-CoA reductase enzymatic activities.
- Monitor changes in cholesterol level in the plasma membrane (Flippin III staining).
- Monitor changes in LDL receptor levels (immune-staining).
- Monitor changes in isoprenoids and ubiquinone level (HPLC).
- Determine the effect of statin on brain injury markers (e.g. Tau, S100 Calcium Binding Protein B (S100B), myelin basic protein).

**Limitations**

- In this study, neuronal cells were directly exposed to statins.
- The use of undifferentiated neuronal cells.
- The use of a cell-free HMG-CoAR assay.

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