Lipophilic Statins But Not Hydrophilic Statins Attenuate Human Atrial Myofibroblast Viability and Induce Apoptosis *In Vitro*.

BY

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A Thesis Submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Department of Physiology

University of Manitoba

Winnipeg

Abstract

Hydroxymethylgluteryl-CoA reductase inhibitors (statins) are commonly used in the treatment of cardiovascular diseases. These drugs have been shown to induce cell death in various cell types. It is unclear if this is a class effect or a phenomenon specific to certain compounds.

We hypothesize that lipophilic statins induce cell death in primary human atrial myofibroblasts (hATMF) whereas hydrophilic statins do not.

hATMF were treated with atorvastatin, simvastatin (lipophilic statins) or pravastatin (hydrophilic statin). Cell viability was assessed using MTT assay. Induction of apoptosis and autophagy were estimated with western blot analysis.

We found that lipophilic statin treatment of hATMF reduced cell viability in a time and dose-dependent manner and increased expression of apoptotic markers. These effects were not observed with the hydrophilic statin.

In conclusion, there are substantial differences between various compounds in the statin family. These differences should be considered when selecting a drug for a particular patient.

Acknowledgements

I would like to thank my supervisors Dr. Darren Freed and Dr. Rakesh Arora for giving me the opportunity to work in their lab, under their guidance and supervision. Dr. Freed, words are cannot describe my gratitude to you for being the best mentor in the world. Your incredible patience, understanding, persistence and positive attitude always inspired me to keep my chin up and work hard. Dr. Arora, thank you for your constant encouragement during our coursework, and ensuring that I was up to date on my cardiovascular research literature. I will forever be grateful to both of you for all the opportunities and experiences during my graduate studies.

To my advisory committee: Dr. Ian Dixon, Dr. Ellisavet Kardami, and Dr. Jeffery Wigle, a very warm thank you for your immense support and guidance throughout my studies. During my three year study, your insight, direction, critique and leadership were all very well taken. Our discussions were always very motivating and inspired my critical way of thinking.

I am extremely grateful for the funding I received from the Institute of Cardiovascular Sciences and St. Boniface General Hospital during my graduate research project.

I would like to thank the all lab members for the making the lab a great place to work. Special mention to Melanie Durston and Yun Li; you tremendously helped me getting started in the lab.

I would also like to acknowledge and say a special thanks to the person who has been a friend, philosopher and guide, from the first day I arrived in Canada, and started my journey as a graduate student: Dr. Jude Uzzona (Faculty of Immunology). Dr. Uzzona, thank you for believing in me and always encouraging me to do my best always. You are a true inspiration, and will always be.

Finally, I would like to thank the people who stayed most close to me. To Meaghan Labine, thanks you for being there for me always. I will forever cherish our friendship. To my sister Bhupinder, thank you for staying by my side through thick and thin. I will always remember our time together in graduate school and forever treasure our memories as friends and sisters. To my little brother Prabhjot, thank you for your constant support and making sure that I was always doing well, not just in school, but life in general. To my parents, thank you for your unconditional love and unfailing support, without which it would not have been possible to achieve this goal. A special thanks to my husband Deepak, thank you for your encouragement and constant support to help me realise this dream and for your unfailing support through some of the most difficult times.

DEDICATED TO MY PARENTS FOR MAKING ME WHO I AM...

AND MY GRANDMOTHER.

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List of Abbreviations

α-SMA	alpha-smooth muscle actin
Ang-II	Angiotensin-II
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CAD	Coronary artery disease
CTGF	Connective tissue growth factor
CVA	Cerebrovascular accident
CVD	Cardiovascular diseases
DMEM	Dulbecco's modified Eagle's medium/Nutrient Mixture F-12
DMSO	Dimethyl Sulfoxide
ECM	Extracellular matrix
EthD-1	Ethidium homodimer-1
EMT	Epithelial-to-mesenchymal transition
ET-1	Endothelin-1
FBS	Fetal bovine serum
FPP	Farnesylpyrophosphate
GGPP	Geranylgeranylpyrophosphate
GI	Gastrointestinal
hATMF	human Atrial Myofibroblasts
HF	Heart failure
HMG-CoA	Hydroxymethylgluteryl-Coenzyme A Reductase

HMGR	Hydroxymethylgluteryl-Coenzyme A Reductase
HRP	Horse Radish Peroxidise
IgG	Immunoglobulin-G
LDL	Low density lipoprotein
LDL-C	Low density lipoprotein-Cholesterol
MI	Myocardial Infarction
MHC-II	Major Histocompatibility Complex-II
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
NCDs	Non Communicable Diseases
NF-κB	Nuclear factor kappa B
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
PBS-T	Phosphate buffer saline (1X) + Tween (0.05%)
PFA	Paraformaldehyde
PVDF	Polyvinylidene difluoride
PMSF	Phenylmethylsulfonyl fluoride
PVDF	Polyvinylidene difluoride
RIPA	Radioimmunoprecipitation assay buffer
SDS	Sodium dodecyl sulphate
SMEM	Suspension Minimum Essential Medium
TGF-β	Tranforming growth factor-β
WHO	World Health Organisation

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I. INTRODUCTION

Cardiovascular diseases (CVD) are the leading cause of mortality and morbidity in the world, and their prevalence continues to rise each year (1-5). CVD collectively can lead to heart failure, and consume a tremendous amount of health care resources (1; 3; 4). Risk factors for CVD include obesity, hypertension, diabetes and altered lipid metabolism, including hypertriglyceridemia and hypercholesterolemia (3; 6-10). Reduction of serum cholesterol levels has been shown to be beneficial in reducing adverse events associated with CVD (6; 11).

The link between serum cholesterol levels, for example, the levels of low density lipoprotein (LDL) and the incidence of coronary artery disease (CAD) is well established (6; 11; 12). The majority of the cholesterol comes from *de novo* synthesis in the body, and a smaller portion comes from diet. The use of lipid lowering drugs has revolutionized the management of risk of CAD/CVD associated with hyperlipidemia. The rate-limiting step in cholesterol biosynthesis is hydroxymethylgluteryl-coenzyme A (HMG-CoA) reductase. There are several compounds that inhibit this enzyme, and their use has been show to provide beneficial effects in both optimizing the lipid profile, and also in reducing adverse cardiovascular events (9). These compounds include pravastatin, simvastatin, rosuvastatin, atorvastatin and lovastatin, among others, and are commonly referred to as "statins" (13). In addition to their effects on the lipid profile, statins also exhibit many other beneficial vascular effects on endothelial function, plaque stability, and angiogenesis, and also have anti-inflammatory, anti-thrombotic, and

immunomodulatory properties (14-17). The positive pleiotropic effects of statins are independent of their cholesterol lowering properties (14). Statins also exhibit direct cellular effects, which have been studied in various cell types. Statins have also been associated with induction of apoptosis, autophagy, and endoplasmic reticulum stress in many cancerous (18; 19) and non-cancerous cell types (20-22). Mevalonate cascade inhibition by simvastatin was shown to induce autophagy and apoptosis in airway mesenchymal cells (22). Statin treatment of human cholangiocarcinoma cells lead to apoptosis induction via suppression of the MAPK pathway (18).

Other pleiotropic effects of statins include reduction of myocardial hypertrophy (23-26) and suppression of phenotypic transformation of rat cardiac fibroblasts to myofibroblasts (27). Myofibroblasts are the mediators of cardiac fibrosis (28). Herein we address the direct cellular effects of statins on human atrial myofibroblasts and examine the differential effects of lipophilic versus hydrophilic statins.

II. LITERATURE REVIEW

1. Epidemiology of the Cardiovascular Diseases

Based on 2012 statistics provided by the World Health Organisation (WHO), 17 million people die of CVD such as myocardial infarction (MI) and stroke each year worldwide, and this number will increase to 25 million in 2030. Furthermore, deaths due to CVD account for the largest proportion (48%) of Non Communicable Deaths (NCDs). In the United States (US, deaths due to CVD accounted for 32% of the total mortality in 2009, which was 1 in every 3 deaths (3). In Canada, 68,342 deaths in 2009 were attributed to CVD, which accounted for approximately 30% deaths [Statistics Canada – 2009; Catalogue no #84F0209X]. The total cost of CVD and stroke (direct and indirect) was estimated to be $312.6 billion in the US in 2009(3); and $21.2 billion in Canada in 2004 (4). Thus CVD represent a major fiscal burden on North American society, and interventions aimed at its prevention and treatments are of significant interest.

There are several risk factors for the development of CVD, including modifiable and non-modifiable factors: age, sex, and inheritance of disease susceptibility are considered to be non-modifiable. Hypertension, dyslipidemia, diabetes, obesity and a sedentary lifestyle are considered to be modifiable risk factors. The association between these factors and CVD is strong, with multiple studies demonstrating that control of these modifiable factors results in reduced cardiovascular event rates and cardiovascular mortality. These risk factors predispose the individual to premature, aggressive atherosclerosis that then

predisposes the individual to stroke, cerebrovascular accident (CVA) and, myocardial infarction through plaque rupture and intravascular thrombosis.

Dyslipidemia is abnormal lipid handling in the body, and is represented by altered levels of cholesterol and triglycerides. It is estimated that 32 million adults in the US, over the age of 20, have total serum cholesterol levels higher than 240 mg/dl (6.216 mM/L), a prevalence rate of 13.8% (3). Medications such as statins are used to modify the lipid profile of these individuals, in an effort to reduce the rate of cardiovascular events such as MI and CVA. Myocardial infarction as a consequence of atherosclerotic plaque rupture leads to scar formation in the heart, with consequent reduction in myocardial function and the onset of heart failure (3).

2. Cardiac Fibrosis

Most CVD leading to heart failure are associated with cardiac fibrosis and varying degrees of myocardial cell loss such as reduced cardiomyocyte number (29). The heart has a limited ability to regenerate functional myocardium, and therefore subsequent myocardial wound healing, following any form of injury, primarily consists of fibrotic response. This process of cardiac remodeling, following MI, involves a cascade of events in response to injury, leading to changes in the organisation of the myocardium (30). This process involves a complex interplay between several different cell types, including, but not limited to, fibroblasts, myofibroblasts endothelial cells, and inflammatory cells (31). Cardiac remodeling is critical and helps the heart to adapt to various changes in mechanical, chemical and electrical signals (30).

3. Pathogenesis of cardiac fibrosis

3.1 Cardiac Remodeling as an adaptive response

Initially, cardiac remodeling is an adaptive process in response to injury. Following acute injury, cardiomyocyte death leads to recruitment of inflammatory cells to the infarct area, which helps remove necrotic myocyte and cell debris by phagocytosis. This is followed by granulation tissue formation consisting of inflammatory cells, newly formed blood vessels to restore the blood supply, and fibroblast like cells (myofibroblasts) that synthesize and secrete matrix proteins including fibrillar collagens (31). Eventually, granulation tissue leads to mature scar formation, which consists of fibrotic tissue, rich in extracellular matrix (ECM), but devoid of inflammatory cells (31). Scar tissue at the infarct site is a passive replacement for lost cardiomyocytes with some biological activity (31). This initial fibrotic response as a reaction to loss of myocardial material is known as reparative fibrosis and it is mainly interstitial (28). This reparative wound healing response is initially critical, but it becomes maladaptive over time and leads to reduced cardiac function (30).

3.2 Progressive fibrosis as a result of adverse cardiac remodeling

The adaptive response of cardiac remodeling after MI often becomes maladaptive and detrimental over a period of time, leading to a net accumulation of collagen, cardiac fibrosis, and loss of cardiac function culminating into heart failure (HF) (28). As a reaction to inflammation, reactive fibrosis is observed, which is primarily perivascular and extends to neighbouring interstitial space (32-34).

The key components in cardiac remodeling are the cardiac fibroblasts, and they tightly regulate ECM synthesis and degradation in a normal heart (30). However, during heart failure, these regulatory pathways are disrupted leading to increased ECM and fibrosis in the infarct area during reparative fibrosis. An increased ECM deposition at non-infarcted areas, i.e., sites unrelated to focal injury, causes reactive fibrosis in the remaining viable myocardium (30). Ultimately, this contributes to increased mechanical stiffness, reduced contractile performance, diastolic dysfunction and ultimately HF (30).

3.3 Cardiac fibrosis has significant consequences on cardiac function

Cardiac fibroblasts play a critical role in maintaining normal cardiac function, including regulation of synthesis, deposition and degradation of ECM; and cell-cell communication with myocytes, other fibroblasts, as well as endothelial cells via direct cell-cell interactions or autocrine and paracrine actions by secretion of growth factors and cytokines (30; 34). Cardiac fibroblasts help maintain and coordinate various signals, and as a result contribute to the structural, mechanical, biochemical, and electric properties of the heart (29; 30). Alterations in these signals and biochemical inputs lead to adaptive and/or deleterious changes in the heart and have significant consequences on cardiac function (29; 30).

Following injury, during wound healing, excess ECM deposition results in increased myocardial stiffness (35). This increased deposition of ECM between the insulating layers of cardiomyocytes, lead to impaired cardiac contractions by disrupting the electrical coupling between the bundles of cardiomyocytes (36). Remodeling of infarcted

and non-infarcted areas in the heart also lead to increased wall stress and reduced ventricular function (37). In total, remodeling results in left ventricular hypertrophy and ventricular dilatation, and heart failure ensues (38). Furthermore, perivascular fibrosis and inflammation impairs the flow of oxygen and nutrient supply and hence it increases the myocyte ischemia (30), (39).

4. Myofibroblasts: Cellular mediator of cardiac fibrosis

4.1 Sources of myofibroblasts

Myofibroblasts, defined as smooth muscle-like fibroblasts, are the key players in cardiac fibrosis (40). Under normal circumstances, myofibroblasts are not found in a healthy heart, but are abundant at the infarct site during wound healing (28; 30; 41-43). Myofibroblasts are thought to have heterogeneous origins, however most myofibroblasts are derived from interstitial and adventitial fibroblasts (30; 44). Additionally, bone-marrow derived cells or epithelial cells via epithelial-mesenchymal transformation, or endothelial cells via endothelial-mesenchymal transition, can differentiate into myofibroblasts (30) (31). Other possible sources could include resident progenitor stem cells in the heart or hematopoietic stem cells from the circulation (30).

4.2 Myofibroblasts after MI

Following injury, fibroblasts are activated and differentiate into contractile and secretory myofibroblasts that subsequently migrate to the infarct area and contribute to scar formation (44). These cells replace the lost cardiomyocytes that cannot be regenerated,

and help maintain the ECM in the scar area, which is subjected to significant mechanical stress in the beating heart (31). Myofibroblasts provide the tensile strength needed for wound healing and help in maintaining a smaller and stronger scar area that prevents further infarct expansion and ventricular dilatation (31; 45).

Myofibroblasts become highly proliferative and invasive in response to various signals such as hormones, cytokines and growth factors in both autocrine and paracrine manner; and actively remodel the cardiac interstitium (40; 46). Myofibroblasts have been shown to produce angiotensin-I and angiotensin-II *de novo*; angiotensin-II is known to promote fibrous tissue contraction, and modulate collagen gene expression in both fibroblasts and myofibroblasts (46; 47). Myofibroblasts are also highly motile in nature, and capable of contracting collagen gels *in vitro.*

In the classical wound healing process, as the scar matures, the myofibroblasts undergo apoptosis and leave behind a healed scar; but these cells often fail to undergo apoptosis and persist for a long durations in the heart after MI (31). This persistence of myofibroblasts in the heart leads to extensive scarring and progressive fibrosis that culminates in heart failure.

4.3 Fibroblast to myofibroblast transition at molecular level

Most myofibroblasts in cardiac remodelling arise from cardiac fibroblasts (30). The cardiac fibroblasts account for approximately 60-70 % of the total cell population in the human heart (28; 30).

Following an injury, cardiac fibroblasts differentiate into proto-myofibroblasts, activated fibroblasts expressing α-smooth muscle actin (α-SMA), which further become myofibroblasts primarily through transforming growth factor-β1 (TGF-β1) signalling. Myofibroblast differentiation and maintenance is influenced by a variety of cytokines, growth factors, and mechanical stimuli. The major pro-remodelling factors comprise of endothelin-1 (ET-1), angiotensin-II (Ang-II) and TGF-β. TGF- β is the key mediator in fibroblast to myofibroblast transformation and ECM deposition; it promotes fibrosis and cardiomyocyte hypertrophy of the infarcted and the non infarcted regions of the myocardium (48). TGF-β m-RNA and protein expression is downstream of Ang-II, a potent stimulator of TGF-β synthesis and expression by cardiac fibroblasts and myofibroblasts (28; 48).

Differentiated myofibroblasts become the major source of these pro-remodelling factors, which then influence the collagen turnover and ECM synthesis in an autocrine manner (46; 49; 50).

5. Statins: Lipid lowering drugs

5.1 Historical Significance

The German pathologist Virchow was the first to provide the link between death from coronary heart disease and cholesterol deposition in the arterial wall (11; 12). However, it was the Framingham study that first prospectively examined the common factors that contribute to cardiovascular diseases, and established the relationship between serum

cholesterol levels, specifically plasma LDL-C (low density lipoprotein-cholesterol) and coronary artery disease (51), (52). The 1960s saw the emergence of cholesterol lowering drugs, such as triparanol (MER-29) and AY-9044, for treatment. These drugs act on the later steps of cholesterol synthesis pathway, and lead to the cellular accumulation of sterols, which were believed to be the cause of many side effects, including ichthysis and posterior lenticular cataracts (53).

The complete elucidation of cholesterol biosynthesis pathway was the crucial discovery that facilitated the development of new therapeutic strategies for lowering serum cholesterol levels (11). After almost two decades, in the 1980s, statins became available for clinical use to lower cholesterol levels in the blood. The first statins to become clinically available were simvastatin and lovastatin (11). The effectiveness and safety of statins have been investigated under large scale studies, which provided strong evidence for the capacity of statins to decrease the risk of CVD and the morbidity and mortality related to CVD (12), (54). Today, statins are among the most widely prescribed drugs to limit cardiovascular events in patients with hypercholesterolemia and cardiovascular diseases, and this effect is mediated by modification of lipid profile (55), (14) (9).

5.2 Pharmacology of statins

Statins competitively inhibit HMG-CoA reductase (HMGR) which is the rate limiting enzyme in the cholesterol production pathway (Figure.1) (53). It is therefore an ideal target for inhibiting cholesterol synthesis without accumulating sterols in cells (14; 55).

Statin induced inhibition of HMGR leads to reduced production of L-mevalonate which is the cholesterol precursor (56).

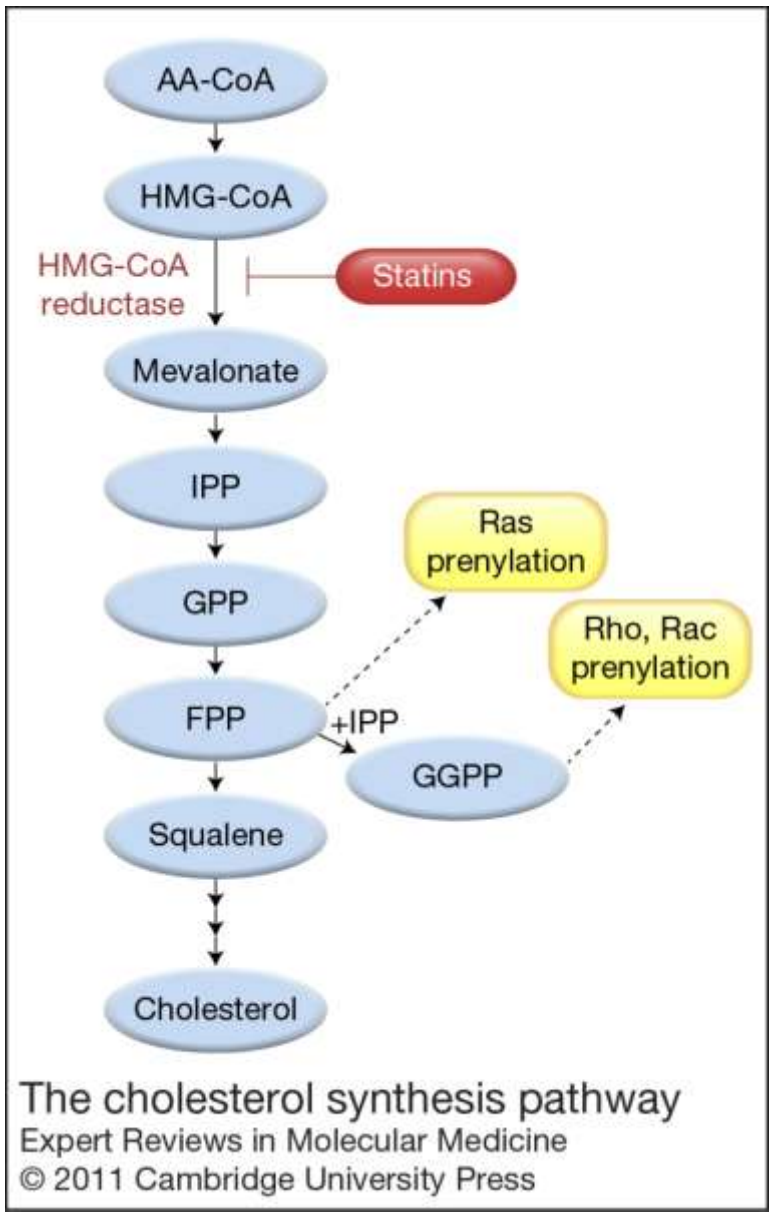


Figure1. The Cholesterol synthesis pathway. Statins inhibit HMGR enzyme in cholesterol production pathway, and hence inhibit the mevalonate production. Mevalonate is not only the cholesterol precursor, but also important for FPP and GGPP synthesis, which are important for post-translational modification for a range of proteins (11) (Used with permission).

There are two pathways for cholesterol production: 1) *De novo* synthesis of cholesterol and, 2) plasma lipoprotein absorption and degradation via LDL receptors (53). HMGR inhibition by statins also increases transcription of LDL-receptor gene, besides blocking cholesterol production (57). The upregulated LDL receptor expression, primarily in the liver, leads to increased cholesterol uptake from circulation, and eventually to reduced plasma LDL- cholesterol levels (12; 53).

Statins also have a profound effect on isoprenoid pathways that mediate many of the pleiotropic effects of statins (58; 59).

5.3 Pleiotropic effects of statins

Pleiotropic effects of a drug refer to the effects exhibited beyond those for which the drug was explicitly designed (11). Statins have many positive pleiotropic effects, independent of effects on lipid metabolism, which include improved endothelial function, enhanced atherosclerotic plaque stability, prevention of thrombus formation, and decreased oxidative stress (14). These effects extend to modulation of inflammation and inhibition of thrombosis (14).

Statins also show direct effects on the myocardium, including reduced cardiac hypertrophy and left ventricular remodeling in patients with heart failure; and lesser number of incidents of atrial fibrillation in patients with CAD (11). Additional beneficial effects of statins on myocardial remodelling could be due to the direct cellular effects of statins.

5.4 Direct Cellular effects of statins

The pleiotropic effects of statins are mediated through mevalonate depletion that leads to the subsequent inhibition of isoprenoid intermediates downstream of mevalonate synthesis, such as farnesylpyrophosphate (FPP) and geranylgeranlypyrophosphate (GGPP) (11; 14). The FPP and GGPP deprivation interferes with the post-translational modification of a variety of proteins, including small GTPases (Ras, Rho, Rac, Rab, Ral and Rap) (11; 14; 56). For example, FPP and GGPP are vital for Ras farnesylation and Rho geranylgeranylation, respectively; both Ras and Rho are essential for many cellular events, including cell adhesion, migration, and actin cytoskeleton (60).

The effect of a lipophilic statin, simvastatin, on cytoskeleton structure in cultured cardiac fibroblast has been studied by Copaja et. al. These studies showed that simvastatin disrupts actin cytoskeleton integrity, affects focal adhesion assembly, and interferes with basic cell signalling pathways and consequently decreases cell migration, adhesion and viability in cultured cardiac fibroblasts. These processes are the key element for cardiac function under physiological and pathological states. These effects were caused by statin mediated inhibition of FPP and GGPP production. Simvastatin has been shown to induce apoptosis in rat neonatal cardiac fibroblasts and myofibroblasts in culture by a Rho-dependent mechanism (58).

5.5 Effects of statins on other conditions

Statins contribute to plaque stability, through a combined effect of lipid reduction, decreased macrophage accumulation, and inhibition of MMPs expression (14). Statins

appear to reduce the number and activity of inflammatory cell in the atherosclerotic plaque (61). This effect appears to be mediated by inhibition of Major Histocompatibility Complex- II (MHC-II) expression on endothelial cells and monocyte-macrophages, which leads to repression of MHC-II mediated T-cell activation (62). All of these cell types are the mediators of atherosclerosis. Atherosclerosis is a chronic inflammatory response to cholesterol accumulation in the wall of arteries that eventually culminate into plaque formation (61; 62). There are other studies, also indicating that statins help reduce atherosclerosis beyond its lipid lowering effects (63; 64).

The vascular benefits of statins are attributed partly to their ability to attenuate chronic vascular inflammation (63; 65). Nuclear factor kappa-B (NF-κB) is a transcription factor, and a key regulator of inflammatory response (66). Upon activation, NF-κB is translocated to the nucleus, where it induces transcription of many target genes, including cytokines and tissue factor (66-68). Statins were shown to prevent NF-κB transactivation in human endothelial cells (65). Statins were also shown to reduce the vascular wall NF-κB activity and aortic wall expression of NF-κB regulated mediators (such as IL-6, and MCP-1), and hence reduce vascular inflammation (69).

Several studies, including the CARE trial and Pravastatin Inflammation/CRP Evaluation, indicate that statins significantly lower plasma levels of high-sensitivity C-Reactive protein, a clinical marker for inflammation (61; 62). These studies signify the effectiveness of statins in decreasing vascular and systemic inflammation (62). Statins

have also been shown to inhibit vascular smooth muscle cell proliferation and limit neointima formation following vascular injury (70) and stent implantation (71).

Statins have been shown to inhibit the epithelial-to-mesenchymal transition (EMT) and decrease the development of renal fibrosis in hypercholesterolemia and renal artery stenosis (72). This effect of statins on EMT modulation and renal fibrosis attenuation is attributable to the inhibition of isoprenylation by statins (73). Statins have also shown to attenuate the development of pulmonary fibrosis (74-76). This effect seems to be mediated by the anti-inflammatory properties of statins (76). Simvastatin was shown to down-regulate the expression of TGF-β1 and connective tissue growth factor (CTGF) in a dose dependent manner, in the bleomycin-induced pulmonary fibrosis mice model (74). A further study utilising a mouse model demonstrated that pravastatin attenuated bleomycin-induced lung injury and pulmonary fibrosis, by reducing TGF-β1, CTGF, RhoA, and cyclin D1 expression. In both of these studies, statins down-regulated the expression of TGF-β1 and CTGF, at both the gene and protein levels (74; 75).

5.6 Adverse side effects of statins

Despite the beneficial effects, statins also have significant adverse side effects. These adverse side effects include statin induced myopathies (77-79), appearance of diabetes (80), effects on gastrointestinal (GI) system (81), elevation of liver enzymes (82), and rarely rhabdomyolysis (79; 80; 82). These side effects can usually be resolved by discontinuation or reduction in statin dose (82). Currently, there is a debate on the other possible side effects of statins such as cognitive decline, cancer, and diabetes induction;

however based on available scientific evidence Jukema et. al conclude that statin therapy does not impose any increased risk of cognitive decline or cancer, but it could be related to the induction of type II diabetes mellitus (82).

III. RATIONALE

Mevalonate depletion has also been shown to induce autophagy, apoptosis, and ER stress in various cells types (18). Simvastatin was shown to induce apoptosis in cultured cardiac fibroblasts in a Rho-dependent mechanism (58). Simvastatin was also shown to alter the cellular response involved in tissue repair in the gingivial fibroblasts in culture (83). Lipophilic statins, such as simvastatin and cerivastatin, induced autophagy even at low concentrations in cultured human rhabdomyosarcoma cells, but the hydrophilic statin pravastatin did not show any effect on these cells, even at a higher concentration (84).

Statins have been shown to exhibit differential cellular effects on various cell types however, whether it is a class effect or a phenomenon specific to various compounds remains to be elucidated. Thus we studied the effect of lipophilic versus hydrophilic statins on hATMF viability with an emphasis on cell death induction mechanisms induced by these compounds.

Hypotheses

1. Lipophilic statins attenuate cell viability of primary human atrial myofibroblasts *in vitro*; whereas hydrophilic statins do not have any effect.
2. Lipophilic statin induced cell death in human atrial myofibroblast is mediated by mevalonate depletion.
3. Lipophilic statins induce apoptosis in human atrial myofibroblasts and may also induce autophagy.

IV. MATERIALS AND METHODS

1. Materials and reagents

Cell culture plates were purchased from BD Falcon VWR (Franklin Lakes, NJ). Collagenase II for digesting atrial tissue was purchased from Worthington Biochemical Corporation (Jackwood, NJ), the SMEM media to dissolve collagenase II, and the cell culture medium- Dulbecco's modified Eagle's medium was purchased from GIBCO (Grand Island, NY). Fetal Bovine Serum was purchased from Hyclone Laboratories Inc. (Logan, UT). Penicillin and streptomycin antibiotics were purchased from LONZA (Walkersville, MD). TryplE was purchased from GIBCO (Grand Island, NY). The coverslips to grow cells for IF and live-dead assay were purchased from BD Falcon VWR (Franklin Lakes, NJ). The live-dead viability/cytotoxicity assay kit was purchased from Molecular Probes (Eugene, OR). BCA kit to estimate protein concentrations was purchased from BIO-RAD (Hercules, CA). Western blot reagent, acrylamide 30% was purchased from Sigma (Saint Louis, MO) and TEMED (electrophoresis grade) was purchased from Fisher Scientific (Fair Lawn, NJ). PVDF membranes were purchased from Millipore (Etobicoke, ON). The pre-stained protein ladder used as molecular weight marker was purchased from BIO-RAD (Hercules, CA). The ECL Plus was purchased from Amersham Biosciences (Buckinghamshire, UK).

Autophagy inhibitor bafilomycin-1 was purchased from Sigma (Saint Louis, MO). The lysotracker used to stain the lysosomes was purchased from Invitrogen (Carlsbad, CA).

Bovine serum albumin was purchased from Fisher Scientific (Fair Lawn, NJ). Mounting medium Vectashield with DAPI was purchased from Cedarlane (Burlingame, CA).

2. Antibodies

Primary antibodies used for western blot analysis, including Bax, Bcl-2, Cleaved-caspase-3, Beclin-1, and Atg5-12 were purchased form Cell Signaling (Danvers, MA). The LC3-β used for western blot analysis and immuno-fluorescence was purchased from Sigma (Saint Louis, MO). The secondary antibody conjugated with horseradish peroxidase: goat anti-rabbit IgG was purchased from Jackson ImmunoResearch Laboratories (Eugene, OR). The loading control for western blot: β-tubulin antibody was purchased from Abcam (Cambridge, MA). The secondary antibody for immuno-fluorescence: Alexa Fluor- 488 was purchased from Invitrogen (Eugene, OR).

3. Isolation and culture of primary human atrial myofibroblasts.

Approval from the University of Manitoba Bannatyne Campus Research Ethics Board was obtained to collect atrial appendage tissue (less than 1 cm x 1 cm size) from patients undergoing cardiac surgery; Ethics approval number H2007:169. These patients provided written informed consent prior to the collection of intraoprative samples.

Atrial tissue was collected, diced into small pieces, approximately 1 mm x 1 mm size, and digested with collagenase II (2 mg/ml) in SMEM media for 2-3 hours at 37 ˚C. The tissue was manually disintergrated by gentle pipetting action and was allowed to settle in a 50 ml tube. The liberated cells remained suspended in the SMEM media while leaving

the tissue chunks to settle at the bottom of the tube (for a second digestion). The media with the cells was carefully transferred to a new 50 ml tube and centrifuged for 7 minutes at 2000 rpm. The supernatant was carefully discarded and the cell pellet was resuspended in standard culture medium: Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 (DMEM), which was supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin, 20 % fetal bovine serum (FBS), and 1ml of 100 mM ascorbic acid. This procedure was repeated for the remaining tissue that was initially incubated with collagenase II.

These cells were then plated and allowed to adhere and grow on a standard 10 cm plastic petri-dishes and incubated at: 37˚C, 95% humidity, and 5% CO_2 (standard culture conditions). The human atrial myofibroblasts (hATMF) were then isolated based on their plastic adherence property. After approximately 16-20 hours of incubation, non-adherent cells, primarily myocytes and debris, were removed by washing the plastic plates twice with 1X-phosphate buffer saline (PBS). The remaining attached myofibroblasts were supplemented with fresh complete media, which was replaced every 3-4 days. These primary cells were sub-cultured within a week upon reaching 70-90% confluency. TrypLE Express was then used to detach the primary cells from the plastic petri plate, and fresh media was added to neutralize the TripLE Express. These cells were then seeded onto fresh plastic petri plates at 5-10% confluency, and allowed to grow in standard culture conditions. The samples were collected for analysis at passage-2 (P2).

4. Effects of statins on cell viability

4.1 MTT assay

The assay was performed to assess cell viability of hATMF treated with simvastatin, atorvastatin, and pravastatin in a dose and time dependent manner. The P2 cells were plated on 96-well plates. Upon reaching 50-60% confluency, the cells were starved for 24 hours using culture media with 1% FBS prior to statin treatment, and then were treated with 0.1, 1, and 10μM concentrations with each of simvastatin, atorvastatin and pravastatin for 24 hour and 48 hour time periods. After completion of the time points, 24 or 48 hours, 20 μl MTT was added and incubated for 2 hours at 37˚C; optical density (OD) readings were then taken using the spectrophotometer at a wavelength of 570 nM. The percentage of cell viability was calculated as follows:

$$\frac{Mean\ OD\ treated\ cells}{Mean\ OD\ control\ cells} * 100$$

The control set included cells treated with media only or, media and Dimethyl Sulfoxide (DMSO). DMSO is the solvent for hydrophobic statins.

4.2 Live-Dead assay

This assay determines the cell viability based on physical and biochemical properties of the cell, by simultaneous determination of live cells and dead cells by using two different probes that measure parameters of cell viability: the plasma membrane integrity and the intracellular esterase activity. The principle behind the live-dead assay is that the live

cells stay attached to the cover slip, whereas the dead cells detach; live cells stain fluorescent green using the Calsein dye and dead cells stain red using the Ethidium homodimer-1 stain.

The cells were grown on cover slips and treated with each of simvastatin, atorvastatin or pravastatin at 0.1, 1, or 10 μM concentrations. After 24 hours and 48 hours of treatment, the LIVE/DEAD Viability/Cytotoxicity kit was used to determine the live and dead cells. The kit comprised of two dyes: Ethidium homodimer-1 (EthD- 1) 2mM solution in 1:4 DMSO/water (v/v), and Calsein AM 4 mM solution in anhydrous DMSO. The principle behind live-dead assay in brief is: the intracellular esterase enzyme present in live cells, converts non-fluorescent cell-permeant calcein AM into highly fluorescent calcein, which is retained within the cells, and produces an intense fluorescent green color in the live cells. EthD-1 goes into the dead cells through their damaged membranes, then binds to the nucleic acids, thereby producing a bright red fluorescent color in the dead cells. EthD-1 is unable to enter the live cells because of their intact plasma membrane.

The assay was performed based on the manufacturer's instructions provided with the kit, as follows: Cells were washed with 1X-PBS once, and then 300 μl live-dead solution was added on top of each cover slip. Then the cells were protected from light by covering in aluminum foil, and then incubated for 30 minutes at 37˚C. The live-dead solution consisted of: 2 μl calcein and 3 μl EthD-1 in 10 ml serum free DMEM media. Photomicrographs were taken with a fluorescent microscope. Each treatment group was done in duplicates.

5. Apoptosis and autophagy analysis by western blot technique

5.1 Protein isolation by cell lysis

The cells were grown on up to P2 on 10 cm plates, and the treatment was started when they reached 70% confluency. They were starved for a period of 24 hours and then treated with simvastatin, atorvastatin, or pravastatin, for 0.1 μM, 1 μM, or 10 μM concentrations for 48 hours and 96 hours for analyzing the apoptotic and autophagic markers.

The protein isolation was done on ice as follows: cells were washed twice with cold 1X-PBS, and then lysed with RIPA lysis buffer containing a protease inhibitor cocktail. The new RIPA lysis buffer was made up of 50 mM Tris base (pH 8.0), 1mM EDTA, 1mM EGTA, 150 mM NaCl, 0.5% Sodium deoxycholate, 0.1% Sodium dodecyl sulphate (SDS), and 1% Triton X-100; and the protease inhibitors comprised of 0.1 M phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml leupepetin, 2 μg/ml aprotinin, and 1 μg/ml pepstatin. 100 μl lysis buffer was used per 10 cm plate and the cells were scraped using a plastic scraper after lysis (incubated with lysis buffer for 5 minutes). This mixture of cells and lysis buffer was collected in an eppendorf tube (1.5 ml tube), and allowed to sit on ice for 1 hour. At this point, the cells were either stored at -20 ˚C, or directly sonicated to perform protein assay. Cells were sonicated for 10 -15 seconds, three times and then centrifuged for 5 minutes at 14000 rpm at 4 ˚C. Then, the supernatant was carefully transferred to a new 1.5 ml eppendorf tube, without disturbing the pellet.

5.2 Estimation of protein concentration

Protein concentration of whole cell lysate was determined by using the Bio-Rad BCA Protein assay kit, based on the bicinchoninic acid (BCA) method. Standards of 0.25, 0.50, 0.75, 1.0, 2.0, and 3.0 µg /µl were used. This assay was done in a 96-well plate, in dulicates. Working reagents were prepared as per the manufacturer's instructions, as follows: to make reagent A', "20 µl of reagent S was added to 1 ml of reagent A" and 25 µl of this solution was added to each well, then 5 µl of sample/ standard was added. Finally, 200 µl of reagent B was added. After 15 minutes of incubation at room temperature, the absorbance readings were taken at 750 nm using the microplate reader with the help of revelation mex software.

5.3 Protein samples preparation

Protein samples for western blot were prepared by diluting the protein stock with new RIPA, to get the same amount of 20 µg protein in all the samples, for loading onto the gel. Equal amount of sample buffer was added to each of these samples. Sample buffer consisted of Laemmli buffer (125 mM Tris-HCl) pH 6.8, 2.5% SDS, 5% glycerol, 0.125% bromophenol-blue, and 5% 2-mercaptoethanol. Then, these samples were boiled for 5 minutes to denature the proteins.

5.4 Western blot analysis

Protein samples were separated using polyacrylamide gel electrophoresis (PAGE). 20 µg of protein sample was loaded on a 10% SDS-polyacrylamide gel, along with a prestained protein ladder (BIO-RAD). Proteins were then separated at 130 Volts, and transferred on

to a 0.45 μM polyvinylidene difluoride (PVDF) membrane at 300 milli Ampere for 2 hours. Membranes were then blocked with 5% non-fat skim milk in 1X-PBS for 1-2 hours at room temperature, and incubated with primary antibody at a recommended concentration (as per the antibody's datasheet) overnight at 4˚C. The membranes were then washed three times with 1X-PBS + 0.05% Tween (PBS-T) for 10 minutes each, and incubated with secondary antibody Horse Radish Peroxidase (HRP) conjugated anti-mouse Immunoglobulin-G (IgG) or HRP labeled anti-rabbit IgG prepared in 1X-PBS, for 1 hour at room-temperature, followed by three 10-minute washes with PBS-T. Protein bands were then visualized with ECL-Plus based on manufacturer's instructions, and developed on a film. β-tubulin was used as a loading control. These films were then scanned using the densitometer, and the protein band densities were measured using the Quantity One software, and the band density (OD/mm2) was normalized to that of the β-tubulin.

Primary antibodies used include: Bax, Bcl-2, Beclin-1, Atg5-12, cleaved caspase-3, and LC3-β.

5.5 Bafilomycin treatment

The role of autophagy in statin-induced cells death was assessed by inhibiting autophagy with bafilomycin-1. Bafilomycin inhibits autophagy by preventing the maturation of autophagic vacuoles, and this effect of Bafilomycin is mediated by inhibiting the fusion of autophagosome with lysosomes (85). Cells were treated with bafilomycin-1 for 4 hours to inhibit the basal autophagy. Then, they were co-treated with statins and

bafilomycin-1 for both immuno-fluorescence and western blot analysis. The cells were harvested to isolate protein at 6, 12, 24, and 48 hour time points after the treatment, and western blot experiments were performed on these samples for analyzing the protein markers for apoptosis and autophagy: cleaved caspase-3, LC3-β, and Atg5-12. For immuno-cytochemistry analysis, these cells were grown on coverslips and immuno-fluorescence was performed.

6. Immunocytochemistry

Induction of autophagy was analyzed through identification of co-localization of LC3-β and lysosomes using the lysotracker. Cells were grown on cover slips in 24 well plates at passage-2, and treated with simvastatin, atorvastatin, or pravastatin at 1 μM concentration with and without Bafilomycin-1. After 24 hours of treatment, these cells were washed with 1X-PBS thrice. Then they were fixed using 4% paraformaldehyde (PFA) pH 7.4, for 15 minutes at 37˚C, and then washed again and permeabelized with 0.2% triton-X 100 prepared in 1X-PBS, for 15minutes at room temperature. The cells were again washed to rinse off any permeabilizing reagent that is left, followed by blocking with 5% bovine serum albumin (BSA) prepared in 1X-PBS, for 30 minutes at room temperature. After fixing, the cells were incubated with 90 μl primary antibody, LC3-β overnight at 4˚C. Cells were then washed with cold 1X-PBS, and incubated with secondary antibody (Alexa Fluor-488) for 1 hour at room temperature, followed by washes as above. The coverslips were mounted using mounting medium Vectashield with DAPI. The cells were then visualized and pictures were taken under the fluorescent microscope.

7. Statistical analysis

All the data in the current study are expressed as the mean ± SEM. GraphPad Prism-5 was used to process the data. Two-tailed student's t-test was used to compare the means between two groups. One-way ANOVA was used for multiple group comparisons. P value < 0.05 was considered to be statistically significant, unless otherwise indicated.

V. RESULTS

1. Differential effects of lipophilic versus hydrophilic statins on cell viability of human atrial myofibroblasts

1.1 Lipophilic statins reduce the human atrial myofibroblasts viability *in vitro*

To determine the effect of lipophilic and hydrophilic statins on human atrial myofibroblasts *in vitro,* we employed the MTT assay, an established assay to assess cell viability *in vitro*. Lipophilic statins, simvastatin and atorvastatin significantly reduced hATMF viability in a dose and time dependent manner, whereas the hydrophilic statin, pravastatin did not affect hATMF viability. After 48 hours of treatment with atorvastatin or simvastatin, there was over 20% decline in cell viability at 1 μM dose and 50% decline at 10 μM dose as compared to the control (Figure. 2A).

After 96 hours of treatment, 20% decline with 1 μM atorvastatin and approximately 50% decline with simvastatin 1 μM in hATMF viability was observed. Further an 80% reduction in cell viability at 10 μM dose of atorvastatin or simvastatin after 96 hours of treatment of hATMF was observed (Figure. 2B). Similar results were obtained in separate experiments (n=5). In contrast, the hydrophilic statin, pravastatin did not affect cell viability of hATMF, even at the higher dose of 10 μM.

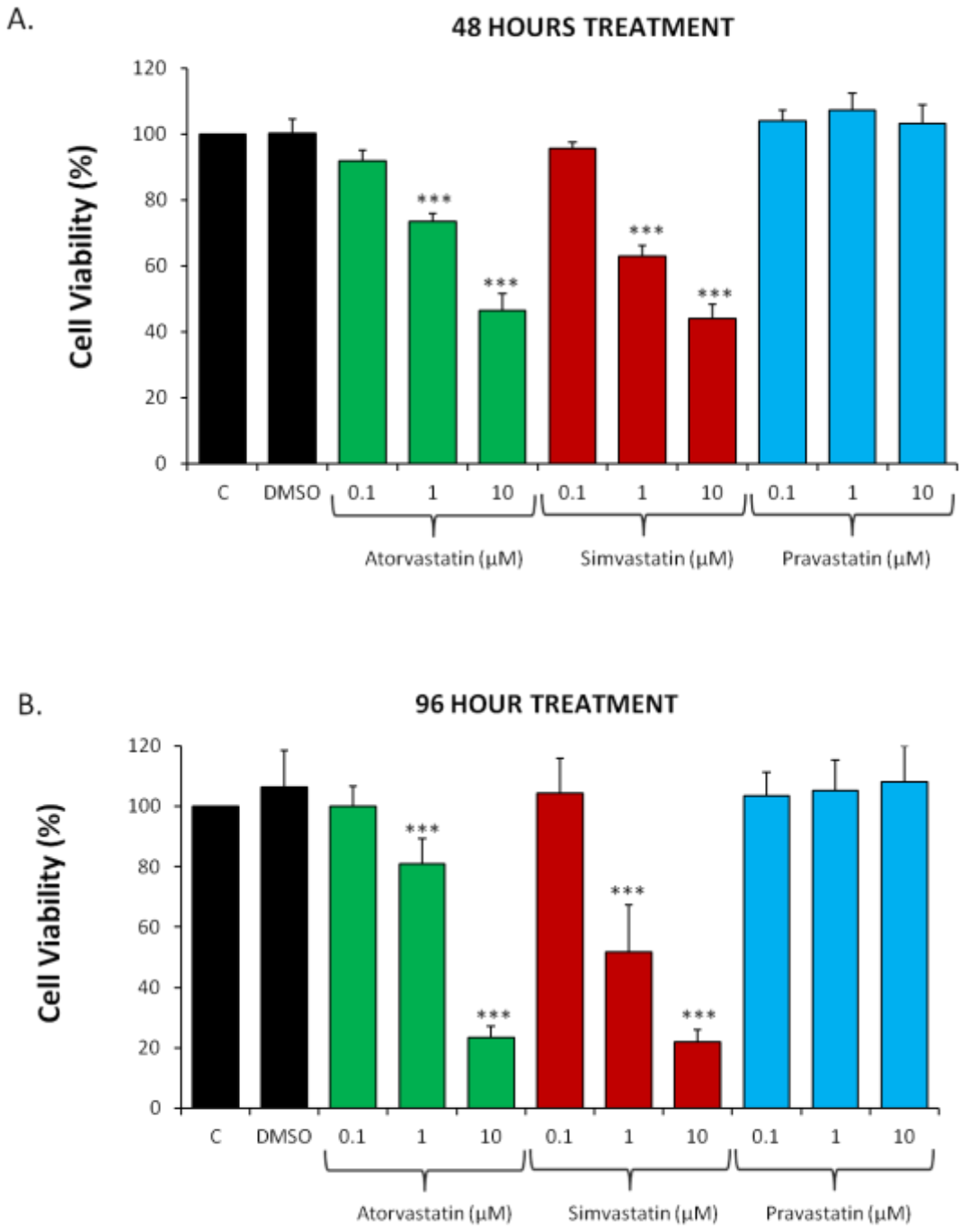


Figure 2. Lipophilic statins reduced hATMF viability. After 48 hour treatment, hATMF treated with atorvastatin or pravastatin showed significant decline in cell viability as indicated by the MTT assay; over 20% decline at 1µM and over 50% decline

at 10μM doses was observed (figure A). Pravastatin did not affect cell viability of hATMF when compared with the control group. *** P Value < 0.001 Vs Control, n=5. After 96 hour treatment, significant decline in cell viability of hATMF treated with 1μM or 10μM dose of atorvastatin or simvastatin was observed (Figure B). Approximately 80% decline in hATMF viability was observed at 10μM dose of atorvastatin or simvastatin. Pravastatin did not affect cell viability of hATMF when compared with the control group. Similar results were obtained in separate MTT experiments. C represents Control. *** P Value < 0.001 Vs Control, n=5.

1.2 Immunofluorescence confirms differential effects of statins on hATMF viability

The live-dead assay was used to determine the effects of lipophilic versus hydrophilic statins on the morphology of hATMF and to support the MTT data showing the differential effects of statins on hATMF. The hATMF were grown on cover slips at passage-2, and then treated with all three statins separately (atorvastatin, simvastatin, or pravastatin) at doses of 0.1 μM, 1.0 μM, or 10 μM for 48 and 96 hours, and followed by live-dead assay. At the end of the 48 hour treatment, similar to the morphology of the control cells, the pravastatin treated cells appeared healthy and remained attached to the cover slip; however, the cells treated with atorvastatin and simvastatin showed increased cell death in a dose dependent manner (Figure 3A). At 10 μM atorvastatin or simvastatin dose, the cells had lifted from the cover slip.

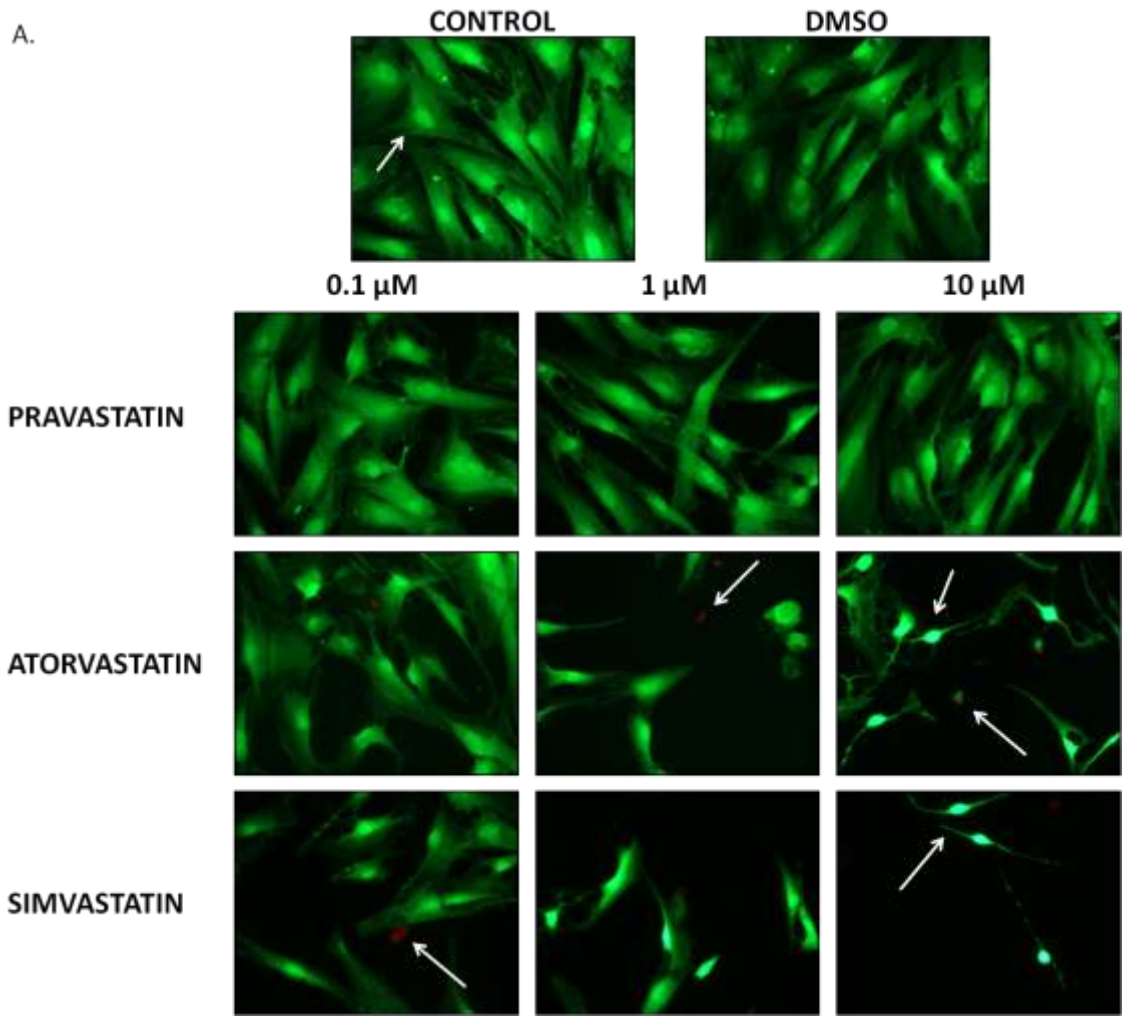


Figure 3A. Differential effects of lipophilic versus hydrophilic statins after 48 hour treatment on hATMF morphology and viability. Atorvastatin and simvastatin altered the morphology of hATMF and reduced the number of viable hATMF; whereas pravastatin did not affect the morphology or viability of hATMF. The live cells are stained green and the dead cells are stained red and are indicated in the figure. The difference in cell morphology within each treatment group can be appreciated. Images were taken on the fluorescent microscope, and are representative of n=3.

Similarly, at the end of the 96 hour treatment, like the morphology of the control cells, pravastatin treated cells appeared healthy and remained attached to the cover slip. However, the cells treated with 10µM atorvastatin or simvastatin showed increased cell (Figure 3B).

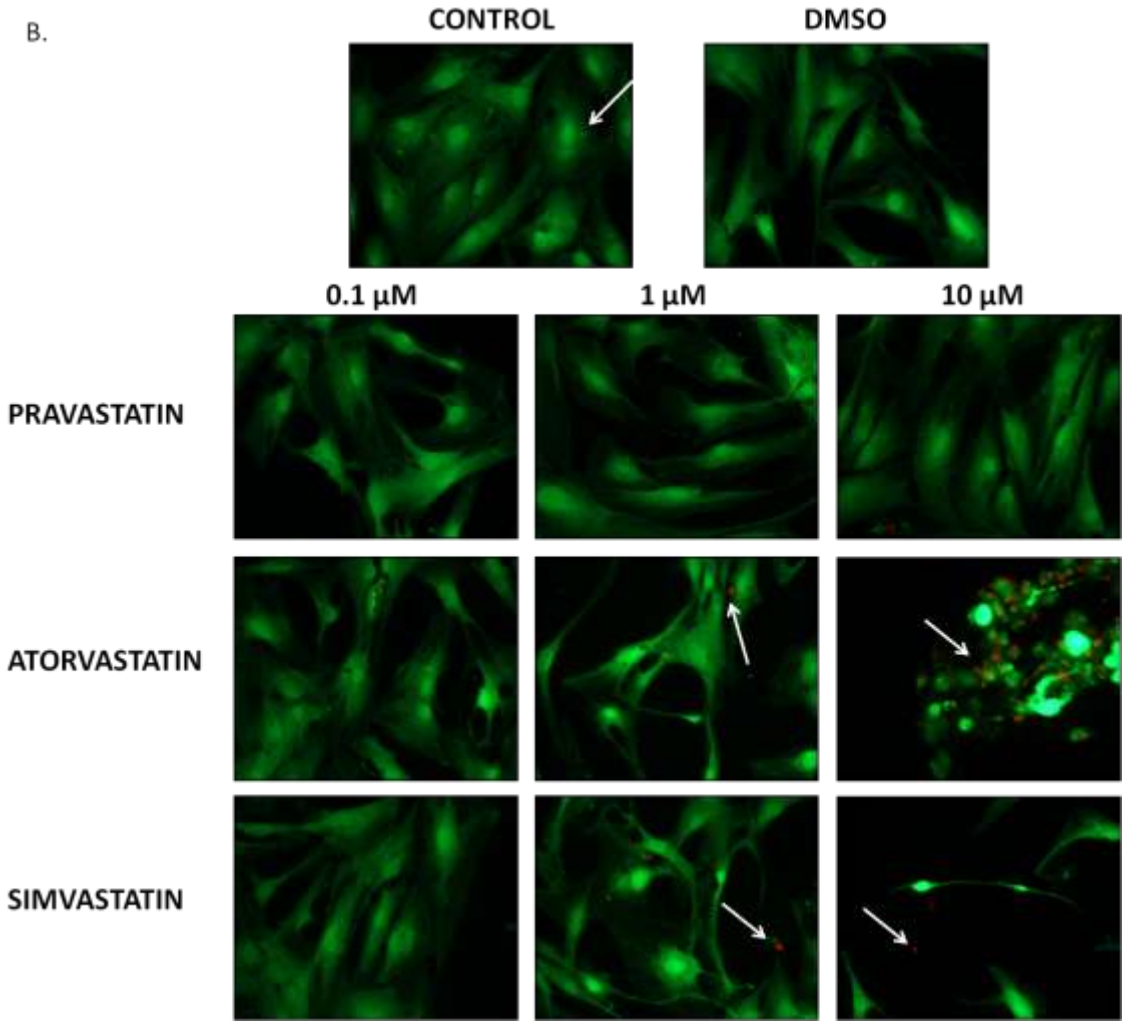


Figure 3B. Differential effects of lipophilic versus hydrophilic statins on hATMF morphology and viability after 96 hour treatment. After 96 hour treatment 1 µM or 10 µM atorvastatin or simvastatin altered the morphology of hATMF. Reduced numbers of viable hATMF were observed with atorvastatin or simvastatin treatment; whereas pravastatin treated hATMF appeared to be similar to morphology of control cells. The

live cells are stained green and the dead cells are stained red. The difference in cell morphology within each treatment group can be appreciated. Images were taken on the fluorescent microscope, and are representative of n=3.

2. Statin induced cell death in primary hATMF *in vitro* is mediated by mevalonate depletion by statins

2.1 Mevalonate rescues viability of statin treated human atrial myofibroblasts *in vitro*

HMGR inhibition by statins results in mevalonate depletion. We examined the effect of mevalonate co-incubation with statins on the viability of human atrial myofibroblasts *in vitro*. MTT assay was used to determine the cell viability of hATMF, after co-incubating with 10 μM of simvastatin or atorvastatin with 0.1 μM or 0.3 μM mevalonate for 48 and 96 hours. After 48 hours of co-treatment of hATMF with mevalonate and statins, the effect of atorvastatin (Figure 4A) and simvastatin (Figure 4B) on cell viability was reversed by mevalonate. The treated groups (statin + mevalonate) showed no significant difference in cell viability of hATMF when compared to the control group. This result suggests that statin induced hATMF cell death is mediated through mevalonate depletion.

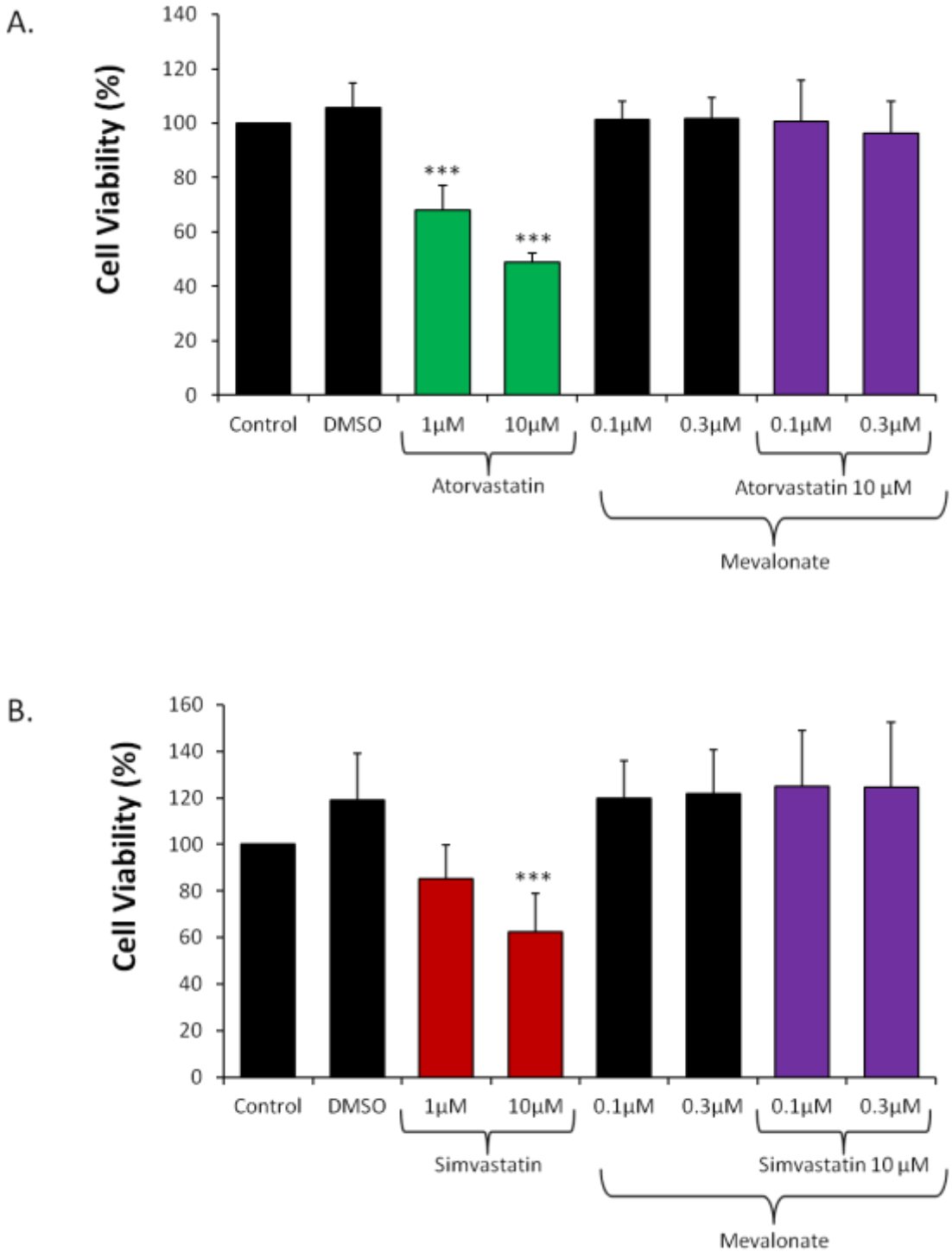
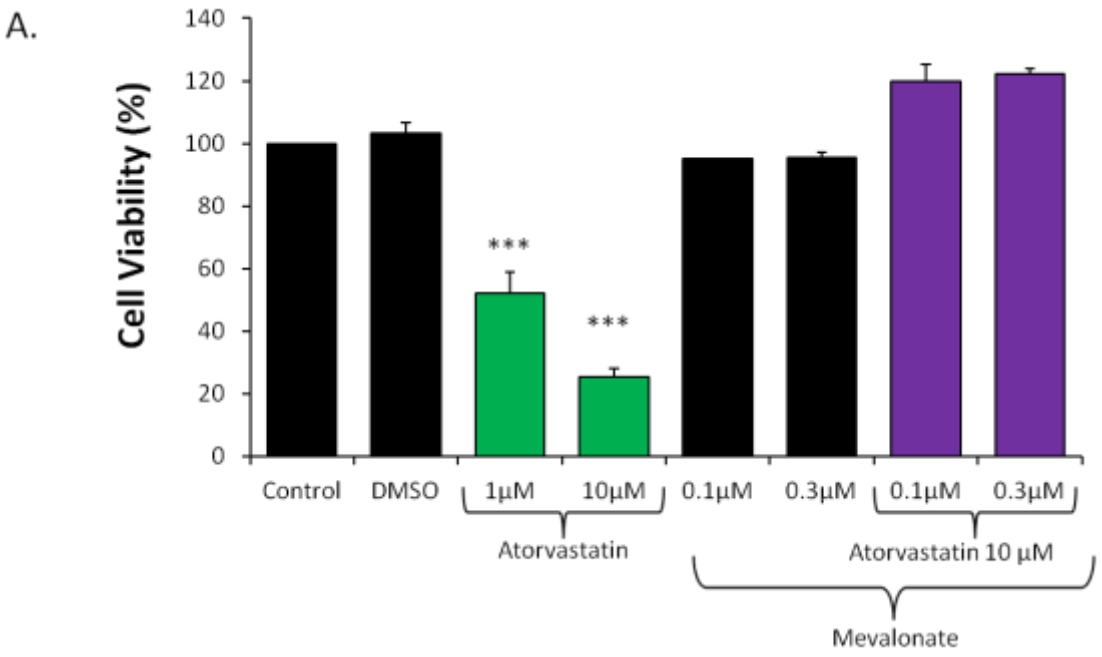


Figure 4. Mevalonate rescues viability of hATMF treated with lipophilic statins for 48 hour treatment. MTT assay after 48 hours indicated significant decline in hATMF viability with 1μM and 10 μM atorvastatin, and this effect was completely reversed when

hATMF were co-treated with 10 µM atorvastatin and 0.1µM or 0.3 µM mevalonate (figure A). Similarly, 10 µM simvastatin treatment also showed a significant decline in cell viability after 48 hour treatment, but this effect was reversed when hATMF were co-treated with 10 µM simvastatin and 0.1 µM or 0.3 µM mevalonate (figure B). These results confirmed that statin mediated decreased in cell viability is mediated through mevalonate depletion. *** P Value < 0.001 Vs Control, n=5.

Similar results were obtained after 96 hours of treatment. Treatment with 0.1 µM or 10 µM atorvastatin or simvastatin showed a significant decrease in hATMF viability, which could be restored by co-incubating with mevalonate (Figure 5A and 5B).



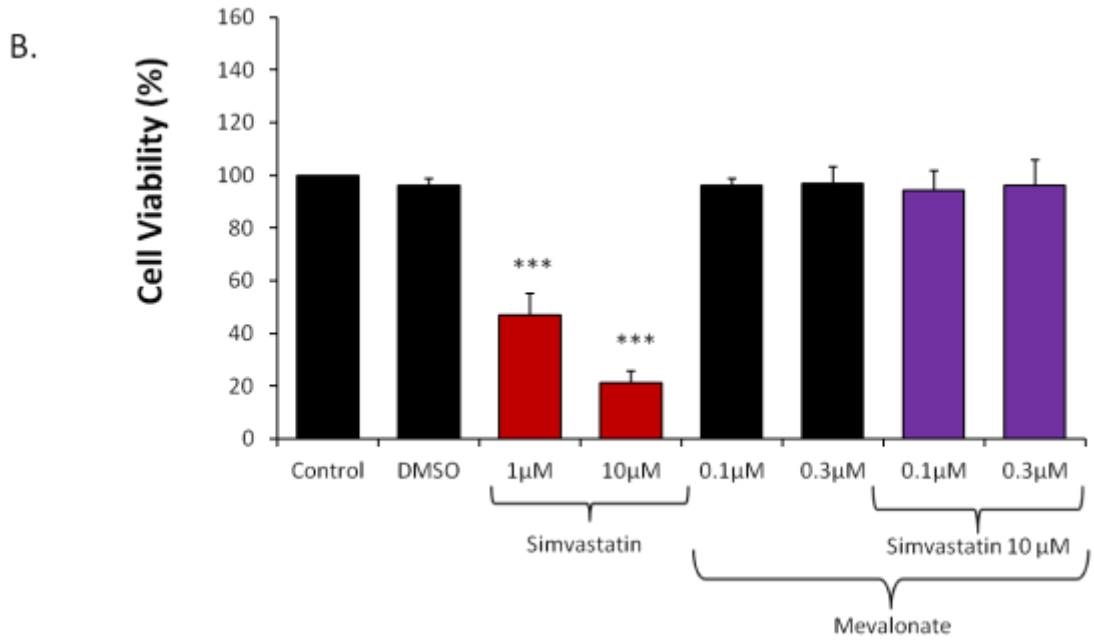


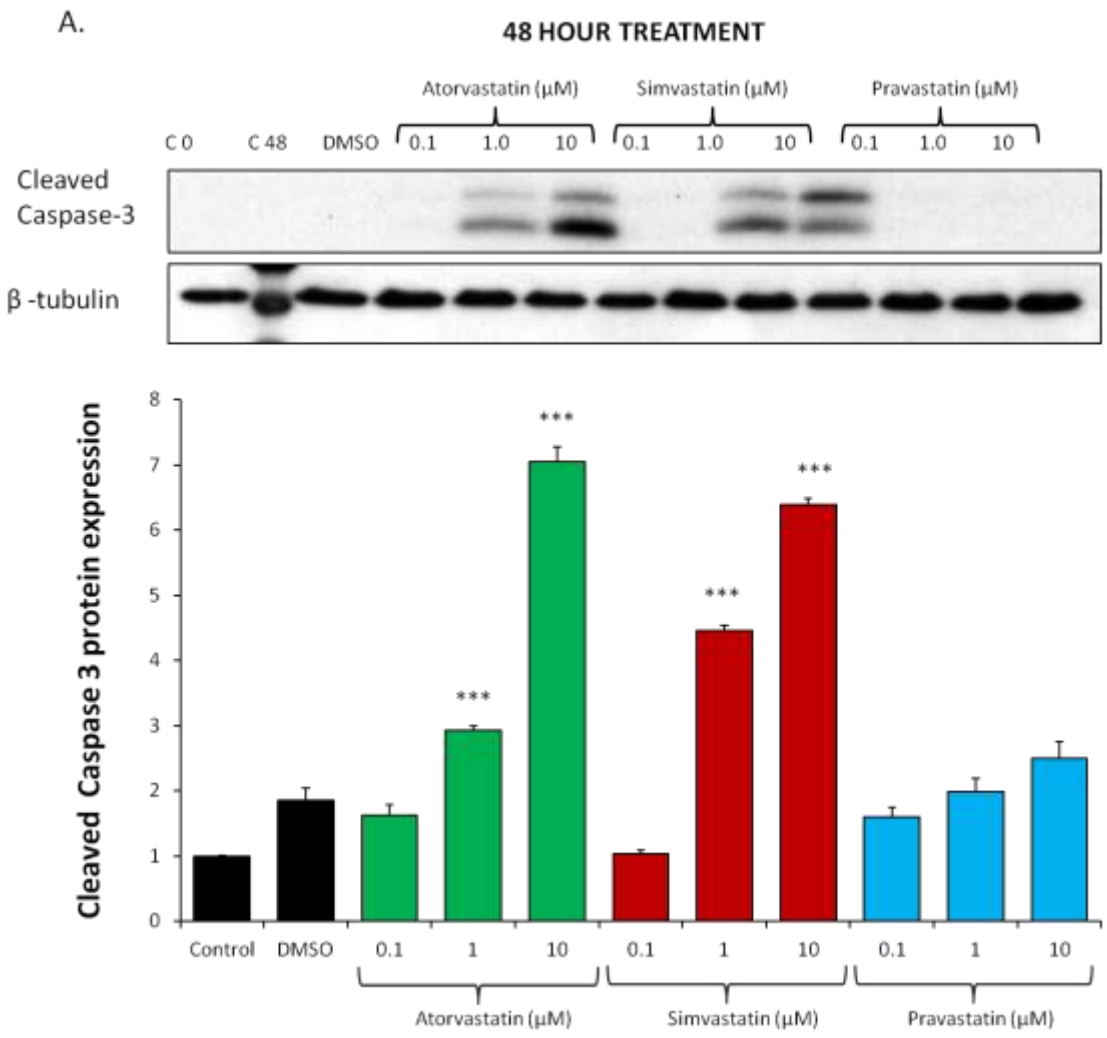
Figure 5. Mevalonate rescues viability of hATMF treated with lipophilic statins after 96 hour treatment. MTT assay after 96 hours indicated significant decline in hATMF viability with 1μM or 10 μM atorvastatin (figure A); and 1μM or 10 μM simvastatin (figure B). This effect was completely reversed when hATMF were co-treated with 10 μM atorvastatin (figure A) or simvastatin (figure B) and 0.1μM or 0.3 μM mevalonate. These results confirmed that statin mediated cell death of hATMF is mediated through mevalonate depletion.

*** P Value < 0.001 Vs Control, n=5.

3. Lipophilic statins, but not the hydrophilic statin induce programmed cell death in hATMFs *in vitro*

3.1 Apoptosis induction in human atrial myofibroblasts *in vitro* by lipophilic statins

Cleaved Caspase 3 is a marker used to assess the induction of the programmed cell death pathway, apoptosis. Caspase 3 is a critical marker of apoptosis and is responsible for proteolytic cleavage of many key proteins in the cell. Total protein was collected after 24, 48, 72, and 96 hour treatment of hATMF with atorvastatin, simvastatin, and pravastatin at 0.1 μM, 1 μM, or 10 μM concentrations. Western blot analysis showed that there was a significant increase in expression of the cleaved caspase-3 in hATMF treated with 1 μM or 10 μM of atorvastatin or simvastatin in a dose dependent manner, whereas hATMF treated with pravastatin did not induce expression of the cleaved form of caspase 3. This result indicates a clear induction of intrinsic apoptotic pathway with lipophilic statin treatment (Figure 6). Similar results were obtained with repeated experiments (n = 3). β-tubulin was used as a loading control.


A.
48 HOUR TREATMENT
Atorvastatin (μM)
Simvastatin (μM)
Pravastatin (μM)
C 0
C 48
DMSO
0.1
1.0
10
Cleaved Caspase-3
β -tubulin
Cleaved Caspase 3 protein expression
***
Control
DMSO
0.1
1
10

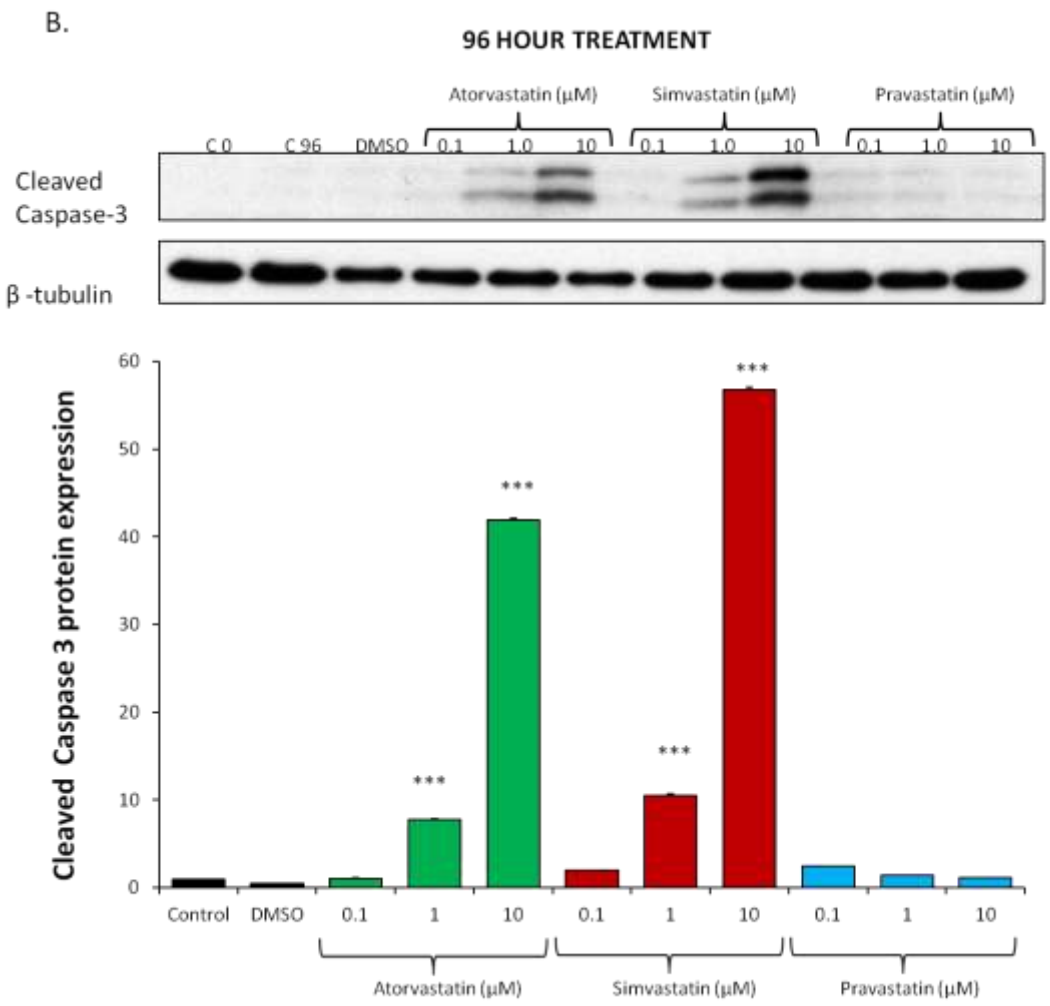
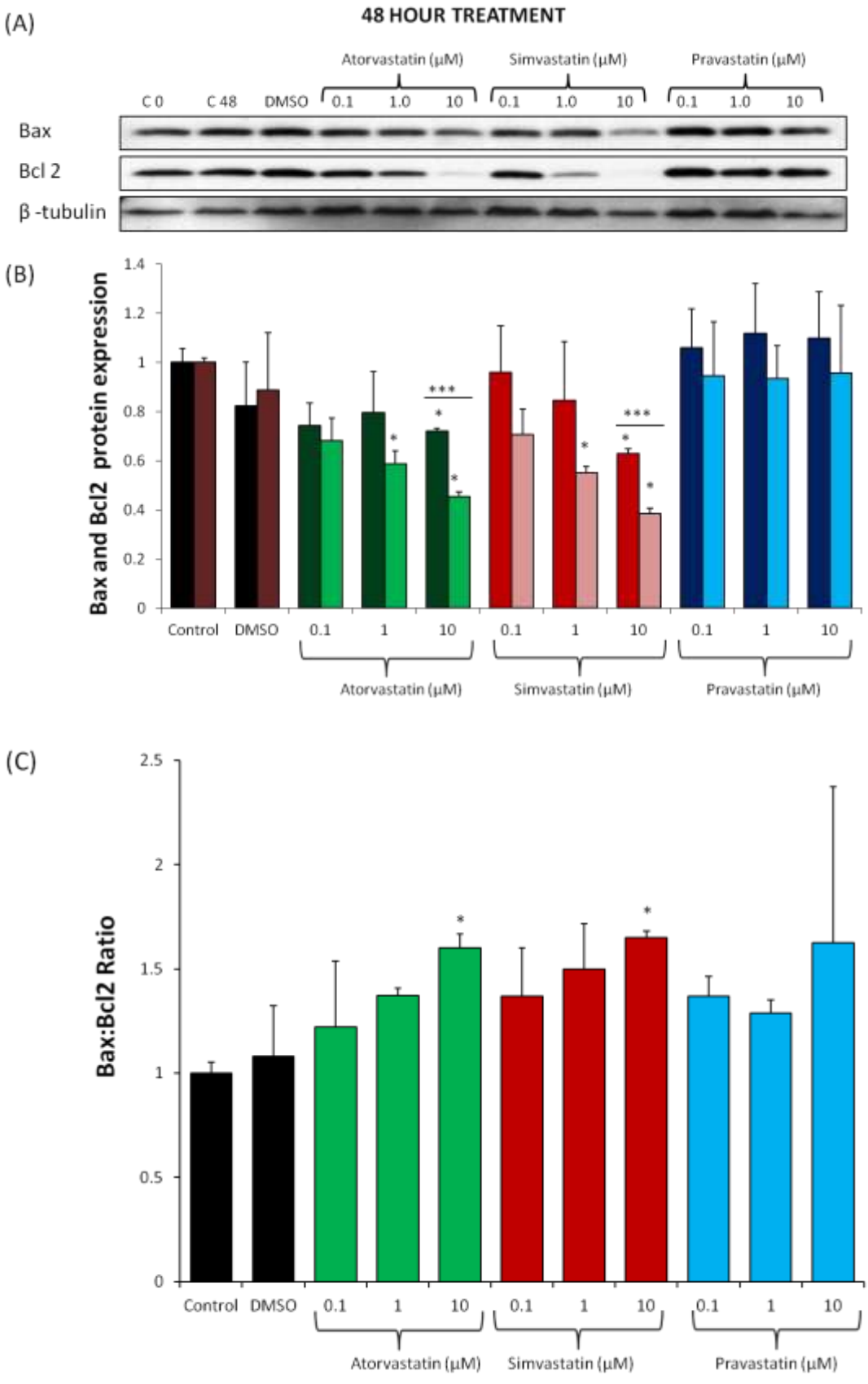


Figure 6. Increased cleaved caspase 3 protein expression in lipophilic statin treated hATMFs. Representative western blots for hATMFs treated with atorvastatin, simvastatin or pravastatin at 0.1μM, 1 μM or 10μM concentrations, probed with cleaved caspase 3 at 48 hour treatment (figure A), and 96 hour treatment (figure B). hATMFs treated with atorvastatin or simvastatin at 1μM or 10μM concentration showed an increase in the expression of cleaved caspase 3. However pravastatin treated hATMF did not show any expression of caspase 3. The bar graph shows the fold change expression of cleaved caspase 3 compared to the control group at 48 hour or 96 hour treatment

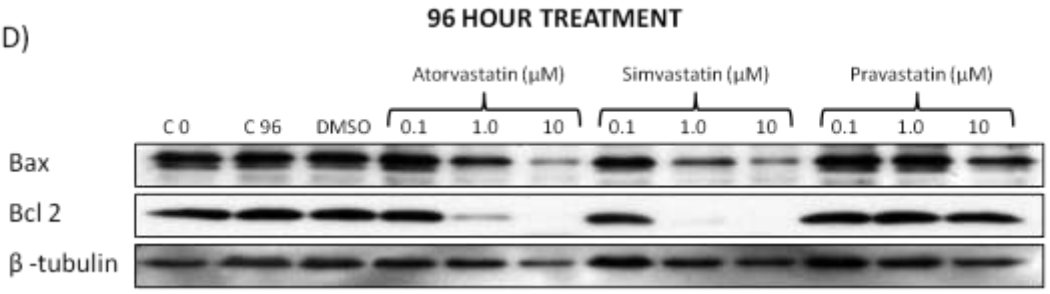
indicating a clear induction of apoptosis. β-tubulin was used as a loading control. C0, C48, and C96 represent control at 0, 48, and 96 hours respectively. This figure is representative of western blot analysis with n = 3. *** P value < 0.05 Vs control.

3.2 Lipophilic statins induce pro-apoptotic response in hATMFs *in vitro*, whereas hydrophilic statins do not

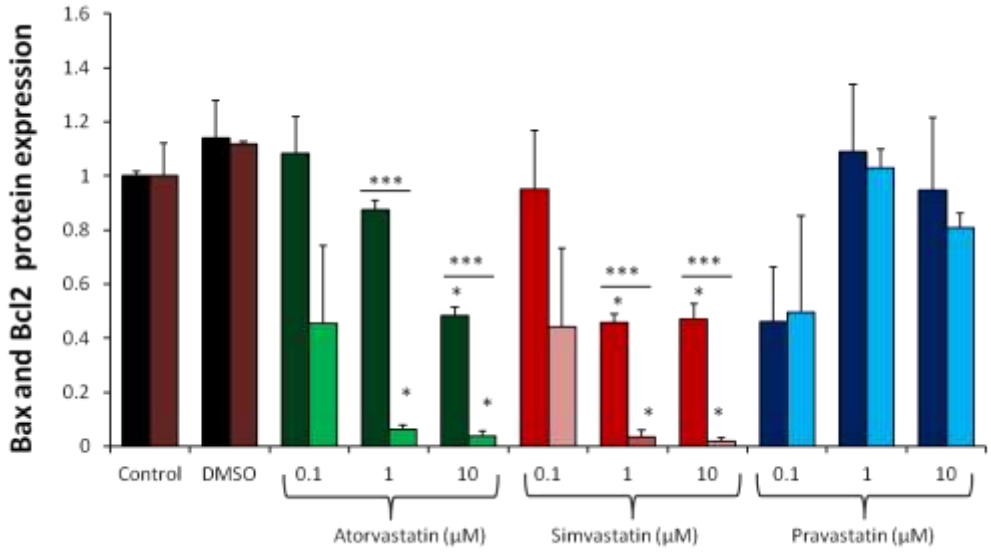
To further investigate the apoptosis induction, we looked at the protein expression of both the pro-apoptotic marker (Bax) and the anti-apoptotic marker (Bcl2) in our treatment groups. Western blot analysis of Bax and Bcl2 protein markers showed a clear induction of a pro-apoptotic signal at 48 hour treatment (Figure 7A) or 96 hour treatment (Figure 7D) with simvastatin or atorvastatin, while pravastatin did not show any significant difference from the control group at both the time points. Although the expression of both Bax and Bcl2 appeared to decrease in dose dependent manner, but the bar graph for Bax and Bcl2 protein expression at 48 hour treatment (Figure 7B) or 96 hour treatment (Figure 7E) shows a significantly higher expression for Bax than Bcl2, indicating a pro-apoptotic response in lipophilic statin treated hATMFs. The Bax protein expression was significantly higher than its corresponding Bcl2 protein expression at 10 µM atorvatstain or simvastatin treatment after 48 hours (Figure 7B). After 96 hours of treatment, there was a significantly higher expression of Bax than Bcl2 at 1 µM or 10 µM dose of atorvastatin or simvastatin (Figure 7E). Also, the ratio of Bax: Bcl2 was compared with control at individual treatment conditions, and a significant increase in ratio compared with control group was observed at 48 hour (Figure 7C) and 96 hour (Figure 7F) treatment. Similar results were obtained in repeated experiments (n = 3).


(A)
48 HOUR TREATMENT
Atorvastatin (μM)
Simvastatin (μM)
Pravastatin (μM)
C 0
C 48
DMSO
0.1
1.0
10
Bax
Bcl 2
β -tubulin
(B)
Bax and Bcl2 protein expression
Control
DMSO
0.1
1
10
Atorvastatin (μM)
Simvastatin (μM)
Pravastatin (μM)
(C)
Bax:Bcl2 Ratio

(D)


96 HOUR TREATMENT
Atorvastatin (μM)
Simvastatin (μM)
Pravastatin (μM)
C 0
C 96
DMSO
0.1
1.0
10
0.1
1.0
10
0.1
1.0
10
Bax
Bcl 2
β -tubulin

(E)


Bax and Bcl2 protein expression
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
***
***
***
*
*
*
*
*
*
*
Control
DMSO
0.1
1
10
0.1
1
10
0.1
1
10
Atorvastatin (μM)
Simvastatin (μM)
Pravastatin (μM)

(F)

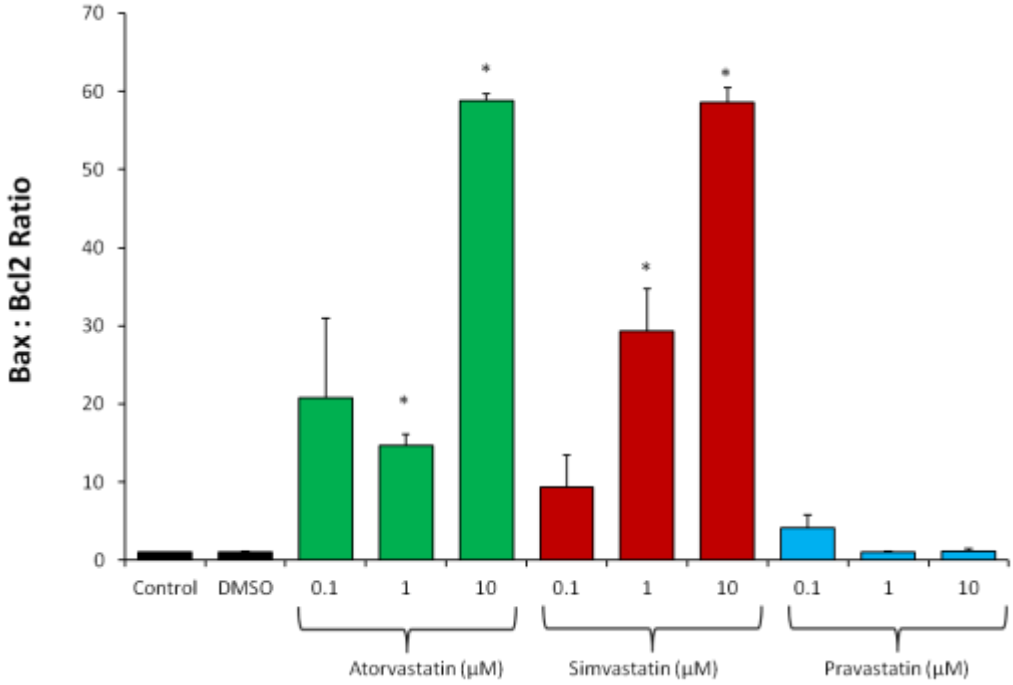

Bax : Bcl2 Ratio
70
60
50
40
30
20
10
0
*
*
*
*
*
Control
DMSO
0.1
1
10
0.1
1
10
0.1
1
10
Atorvastatin (μM)
Simvastatin (μM)
Pravastatin (μM)

Figure 7. Lipophilic statins induced pro-apoptotic response in hATMF. Western blot analysis of hATMFs treated with atorvastatin, simvastatin or pravastatin at 0.1μM, 1μM, or 10μM probed with Bax and Bcl2 markers after 48 hour treatment (figure. A) and 96 hour treatment (figure D). hATMFs treated with atorvastatin or simvastatin showed a pro-apoptotic expression indicated by a comparatively higher Bax expression than Bcl2 expression. However, pravastatin did not show any significant difference in expression of Bax and Bcl2. β-tubulin was used as a loading control.

Higher Bax expression than Bcl2 expression in hATMF treated with lipophilic statins. Bar graph representing the western blot analysis of hATMFs treated with atorvastatin, simvastatin or pravastatin after 48 hour (figure. B) and 96 hour (figure E), probed with Bax and Bcl2 proteins. Within each group, the darker colour (the left side bar) represents Bax expression and the lighter colour (the right side bar) represents Bcl2 expression. After 48 hour treatment, the hATMF treated with 10 μM atorvastatin or simvastatin showed a higher expression of Bax than Bcl2 (figure7B); After 96 hours of treatment, there was a significantly higher expression of Bax than Bcl2 both at 1 μM or 10 μM dose of atorvastatin or simvastatin (Figure 7E). Pravastatin showed a consistent Bax and Bcl2 expression with no significant difference with control at both the time points. * P value < 0.05 Vs control, n = 3. *** P value < 0.005 for Bax Vs Bcl2, n = 3.

Increase in Bax: Bcl2 ratio in lipophilic statin treated hATMF. Significant increase in Bax: Bcl2 ratio was observed after 48 hour treatment at 10 μM dose of atorvastatin or simvastatin (figure 7C). After 96 hour treatment, there was a significant increase in Bax: Bcl2 ratio at 1μM or 10 μM dose of atorvastatin or simvastatin (figure 7F). This indicates an increase in pro-apoptotic signal with increase in dose of lipophilic statins.

However, pravastatin did not show any significant change in ratio. * P value < 0.05 Vs control, n = 3.

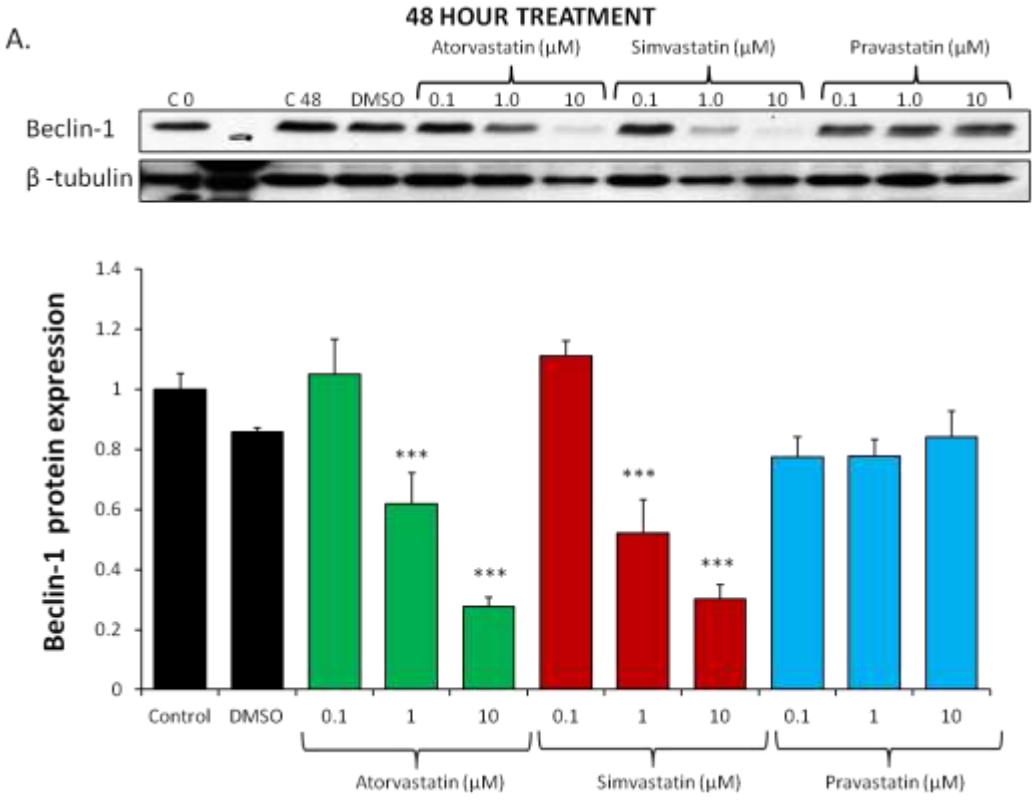
4. Autophagic protein marker expression varies in lipophilic versus hydrophilic statins treated hATMF *in vitro*

4.1 Lipophilic statins lead to a decreased Beclin-1 expression in treated hATMF

To further examine the differential effect of statins in cell death induction, we analysed the expression of some of the key autophagic markers to determine if there was an induction of autophagy in the treated groups. Autophagy is mainly a pro-survival mechanism and ensures cellular survival, but in some cases excess autophagy can lead to cell death due to high turnover of various proteins and cellular organelles. Autophagy and apoptosis are both linked to each other and there is extensive crosstalk at different levels between these pathways.

Beclin-1 is a mammalian ortholog of yeast Atg-6, and is necessary for the induction of autophagy. Western blot analysis was done with protein collected at 24, 48, 72, and 96 hour treatments, and probed with Beclin-1 antibody. There was a significant decline in Beclin-1 expression in a dose dependent manner observed at 1µM or 10 µM atorvastatin or simvastatin after 48 hour (Figure 8A) or 96 hour (Figure 8B) treatment. Pravastatin treated hATMF did not show any increase or decrease in Beclin-1 expression. Because Beclin-1 is expressed upstream of autophagosome formation, and is one of the

components for class III PI3K complex, this decrease in Beclin-1 expression by lipophilic statins indicates a decreased autophagic process with increase in dose and time.



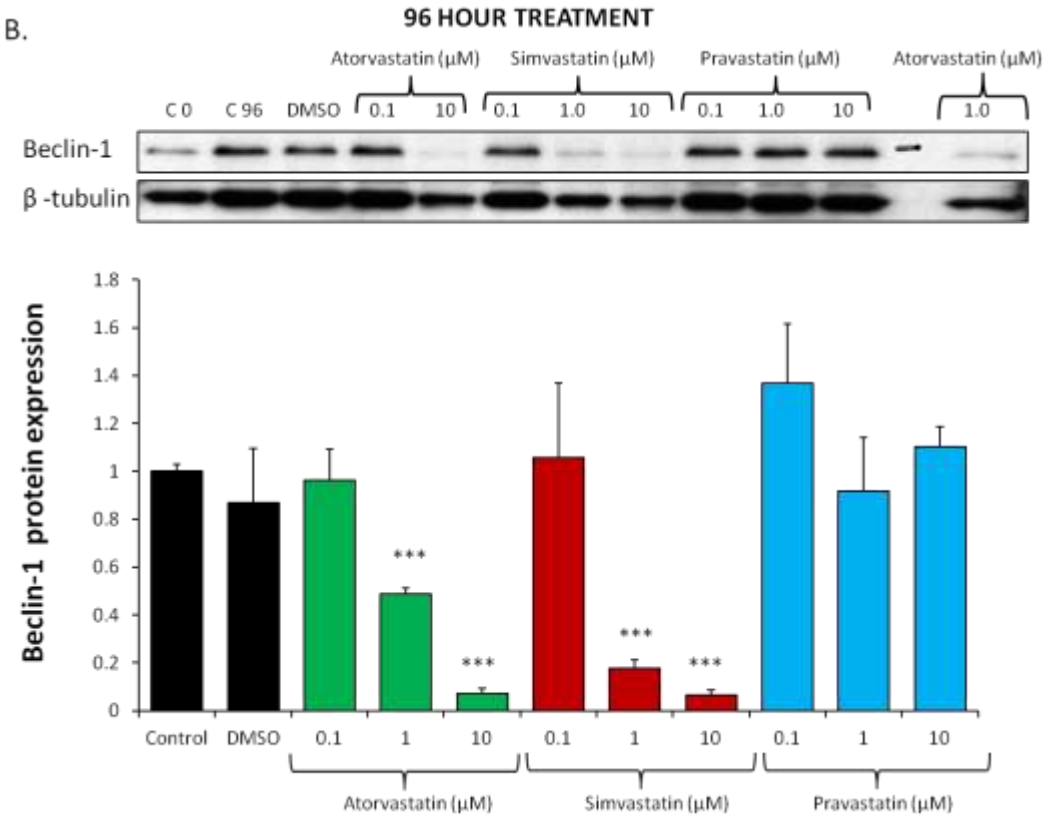
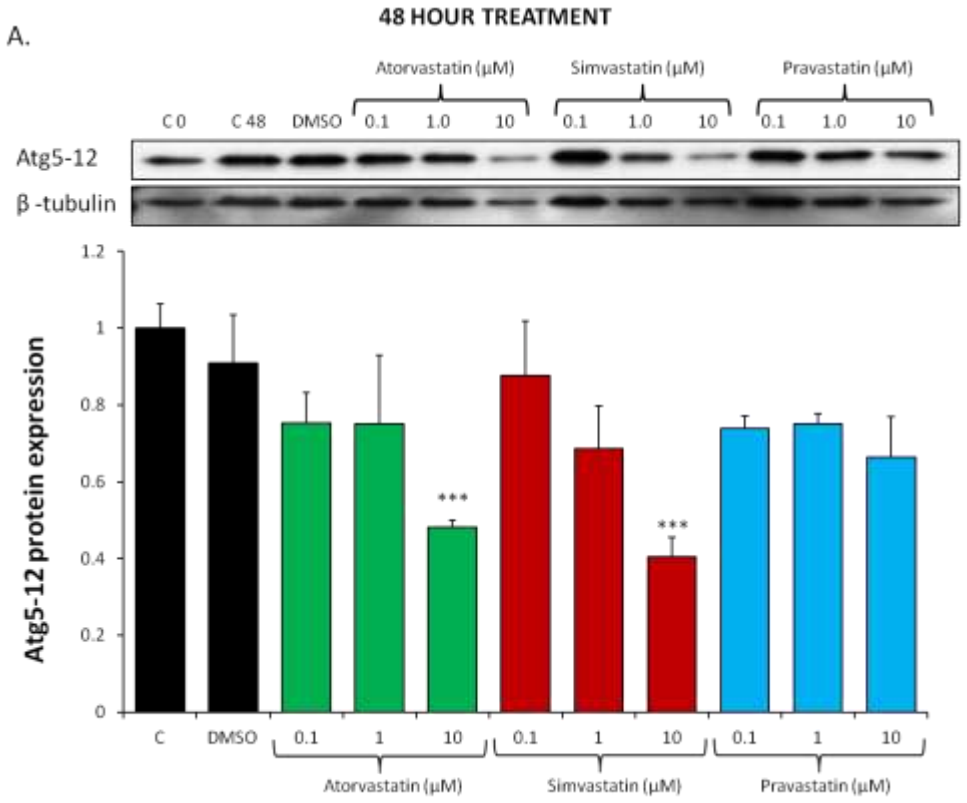


Figure 8. Decline in Beclin-1 expression in lipophilic statin treated hATMF.

Western blot analysis of hATMF treated with atorvastatin, simvastatin or pravastatin after 48 hour (figure A) or 96 hour (figure B), probed with Beclin-1 protein marker. The hATMF treated with 1 µM or 10 µM atorvastatin or simvastatin showed a significant decrease in Beclin-1 expression. Pravastatin did not show any significant change in Beclin-1 expression even at a higher dose. C0, C48, and C96 represent control at 0, 48, and 96 hours respectively. β-tubulin was used as a loading control. *** P value < 0.05 Vs control, n = 3.

4.2 Lipophilic statins down-regulate the expression of Atg5-12 complex indicating a decrease in autophagy

To further investigate the effect of statins on autophagy in hATMF, we analysed the protein expression of Atg 5-12 in statin treated hATMF. Atg genes regulate and control the formation of autophagosome. Western blot analysis for proteins collected at 24, 48, 72, and 96 hours was done for Atg5-12 expression. The hATMF treated with 10µM atorvastatin or simvastatin showed a significant decline in the Atg5-12 expression after 48 hours (Figure 9A) or 96 hour (Figure 9B) treatment. Similar results were obtained in separate experiments (n = 3). This data is consistent with the Beclin-1 data, indicative of down regulation of autophagy with induction of apoptosis as shown above. β-tubulin was used as the loading control.



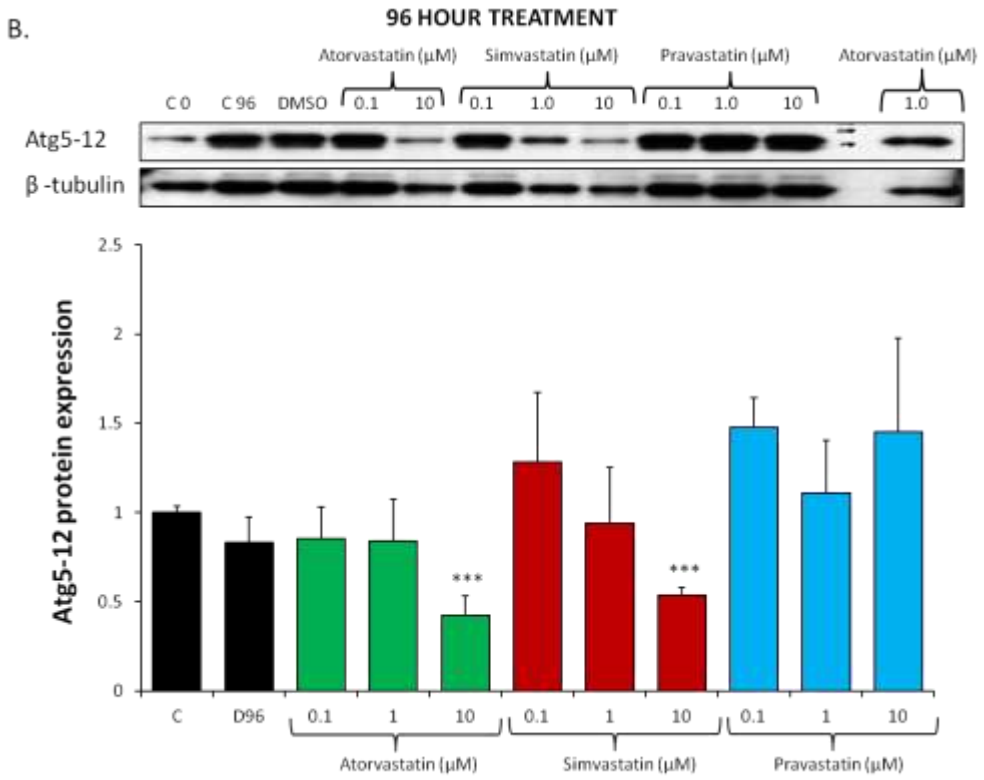
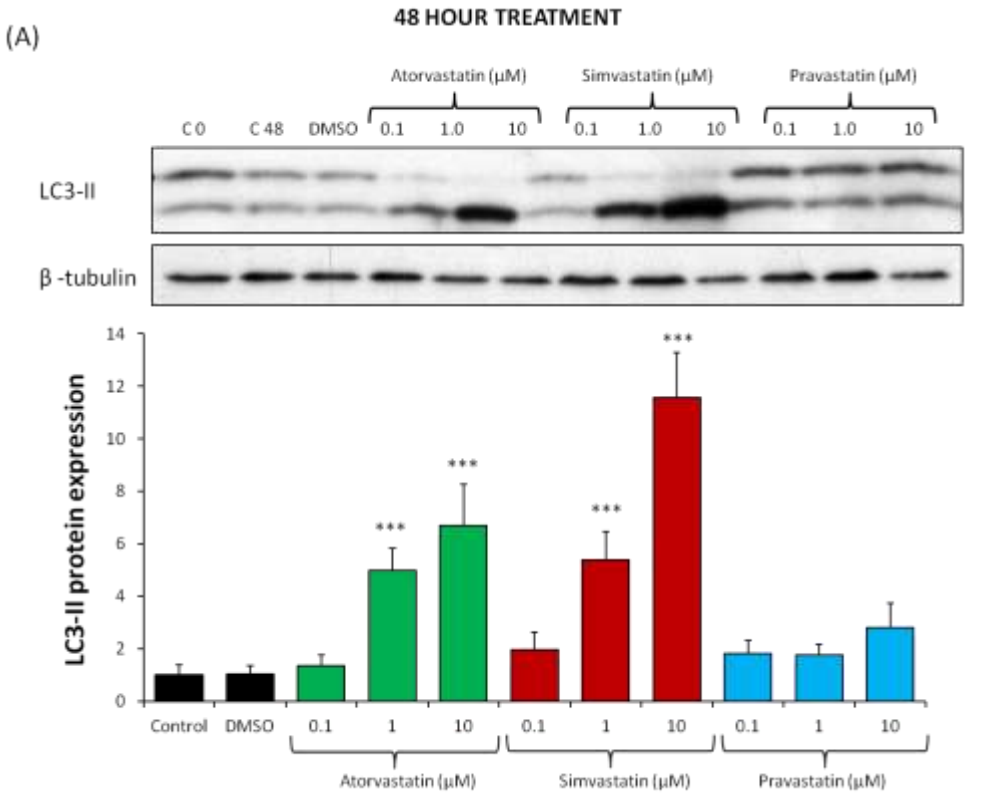


Figure 9. Decline in Atg5-12 expression in lipophilic statin treated hATMF

Western blot analysis of hATMF treated with atorvastatin, simvastatin, or pravastatin after 48 hours (figure. A) or 96 hours (figure. B) probed with Atg5-12 marker. 10 µM atorvastatin or simvastatin treated hATMF showed a significant decline in Atg5-12 expression, while pravastatin treated hATMF showed no change. C0, C48, and C96 represent control at 0, 48, and 96 hours respectively. β-tubulin was used as a loading control. *** P value < 0.05 Vs control, n = 3.

4.3 Increased LC3-II expression may indicate an early onset of autophagic induction in hATMF treated with lipophilic statins

To further investigate the effect of atorvastatin, simvastatin and pravastatin on the autophagic mechanism in treated hATMF, we analysed the expression of LC3-II in these statin treatment groups. LC3-II is expressed during the autophagosome formation and is a lipidated form of LC3, which is expressed and attached inside the autophagosome membrane. LC3-II expression indicates that the autophagosome formation is complete, which is one of the critical steps in autophagy before the fusion of autophagosome with the lysosome. Unlike Beclin-1 and Atg5-12 expressions, there was a significant increase in a dose dependent manner in LC3-II expression at 1 μM or 10 μM atorvastatin or simvastatin treatment of hATMF at 48 hour (Figure 10A) and 96 hour (Figure 10B) treatment. This could be due to the fact that LC3-II accumulates after autophagosome formation and is expressed later in the autophagy pathway. However, the pravastatin treated hATMF did not have LC3-II expression that was different from the control at both 48 and 96 hour time points of treatment.


(A)
48 HOUR TREATMENT
Atorvastatin (μM)
Simvastatin (μM)
Pravastatin (μM)
C 0
C 48
DMSO
0.1
1.0
10
LC3-II
β -tubulin
LC3-II protein expression
Control
DMSO
Atorvastatin (μM)
Simvastatin (μM)
Pravastatin (μM)

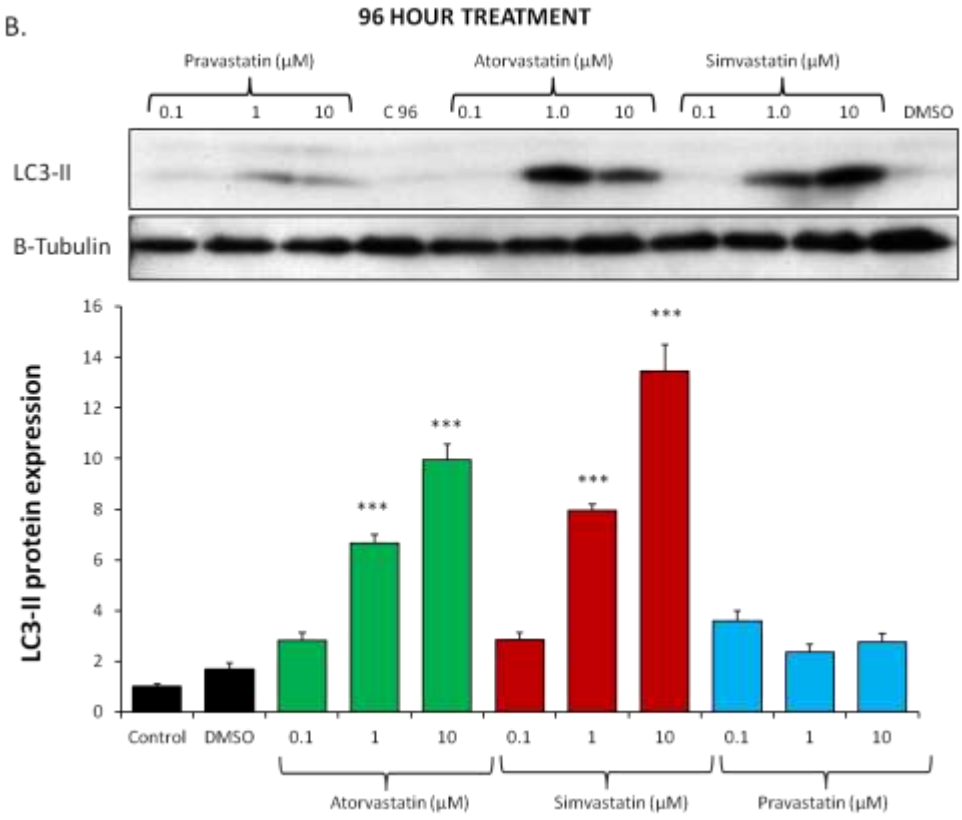

B.
96 HOUR TREATMENT
Pravastatin (μM)
Atorvastatin (μM)
Simvastatin (μM)
C 96
DMSO
LC3-II
B-Tubulin
LC3-II protein expression
Control
DMSO
Atorvastatin (μM)
Simvastatin (μM)
Pravastatin (μM)

Figure 10. Increased LC3-II expression in lipophilic statin treated hATMF

Western blot analysis of hATMF treated with atorvastatin, simvastatin, or pravastatin after 48 hours (figure. A) or 96 hours (figure. B) probed with LC3 marker. hATMF treated with 1μM or 10 μM atorvastatin or simvastatin showed a significant increase in LC3-II expression after 48 hour or 96 hour treatment, while pravastatin treated hATMF showed no change. This may indicate an early on induction of autophagy. C0, C48 and C96 represent control at 0 and 48 hours respectively. β-tubulin was used as a loading control. *** P value < 0.05 Vs control, n = 3.

4.4 Differential effects of lipophilic versus hydrophilic statins on autophagy induction in hATMF

To confirm autophagy induction, immunofluorescence was used to identify co-localisation of autophagosomes and lysosomes. LC3-II was used to identify the autophagosome formation, and lysotracker was used to visualise the lysosomes. hATMF were treated with 1μM of atorvastatin, simvastatin or pravastatin for 24 hours, and then immunofluorescence was performed to assess and compare the differential effect of statins in autophagy induction in hATMF.

Atorvastatin or simvastatin treated cells contained a higher number of autophagosomes visualised as green spots (LC3-II), and lysosomes visualised as red spots. The co-localisation of autophagosome and lysosome can be appreciated in the overlay image (Figure 11). Simvastatin treated hATMF showed a very high number of autophagosomes

confirming autophagy induction. Pravastatin treated hATMF appeared to similar to control group.

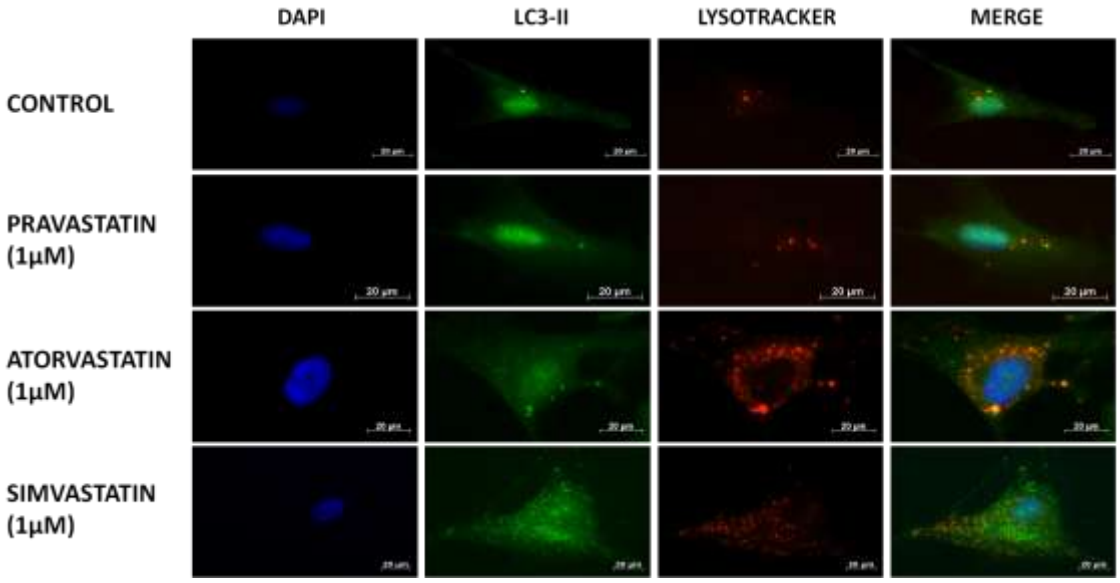


Figure 11. IF indicates autophagy induction in lipophilic statin treated hATMF. Immuno fluorescence pictures of hATMF treated with 1 μM atorvastatin, simvastatin or pravastatin. Nuclei are stained blue with DAPI. LC3-II (green stain) and lysotracker (red stain) indicates autophagosome formation and lysosomes respectively in the hATMF treated with 1μM of atorvastatin, simvastatin or pravastatin for 24 hours. There was an increase in the autophagosome number in atorvastatin or simvastatin treated hATMF. Images were taken on the fluorescent microscope, and are representative of n=3

5. The effect of autophagy inhibition in statin treated hATMF

5.1 Autophagy inhibition lead to decline cell viability in lipophilic statin treated hATMF

To investigate the role of autophagy in statin treated hATMF, autophagy inhibitor Bafilomycin was used. hATMF were co-treated with Bafilomycin (2.5nM or 5nM) and statins (1μM): atorvastatin, simvastatin or pravastatin, and then an MTT assay was performed after 6, 12, 24, and 48 hours of treatment. These time points were chosen based on the observation that there might be an early onset of autophagy induction by statins. The hATMF were treated with bafilomycin for 4 hours to inhibit basal autophagy, prior to the co-treatment with bafilomycin and statins. There was no change in viability after 6 hours and 12 hours of treatment with statins (data not shown). After 24 hour treatment, there was a significant decline in cell viability of hATMF co-treated with 1 μM atorvastatin or simvastatin, and Bafilomycin (2.5nM or 5nM), when compared to the control at the 24 hour treatment (Figure 12A), but the pravastatin did not show any significant difference from the control group. Also, there was a significant decrease in hATMF viability when co-treated with 1 μM atorvastatin or simvastatin and 2.5nM Bafilomycin after 48 hours (Figure12 A).

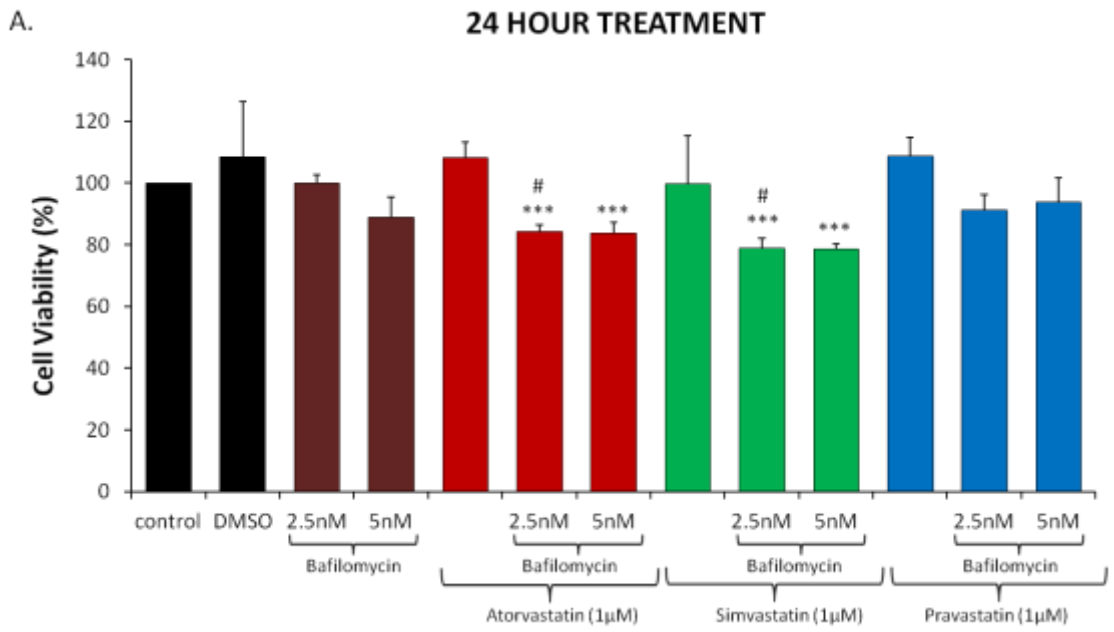


Figure 12A. Decrease in hATMF viability, when co-treated with lipophilic statin and Bafilomycin for 24 hours. MTT assay result for hATMF treated with 1μM of atorvastatin, simvastatin or pravastatin at 24 hours (with Bafilomycin 2.5nM or 5nM, or without Bafilomycin). Atorvastatin or simvastatin treated hATMF showed a significant decline in cell viability as compared with Bafilomycin 2.5 nM control. This indicates a further reduction in cell viability of hATMF when autophagy was inhibited. Pravastatin treated hATMF did not show any difference from the control group.

***P-Value < 0.005 Vs Control, n = 5.

P-Value < 0.005 Vs Bafilomycin 2.5nM, n = 5

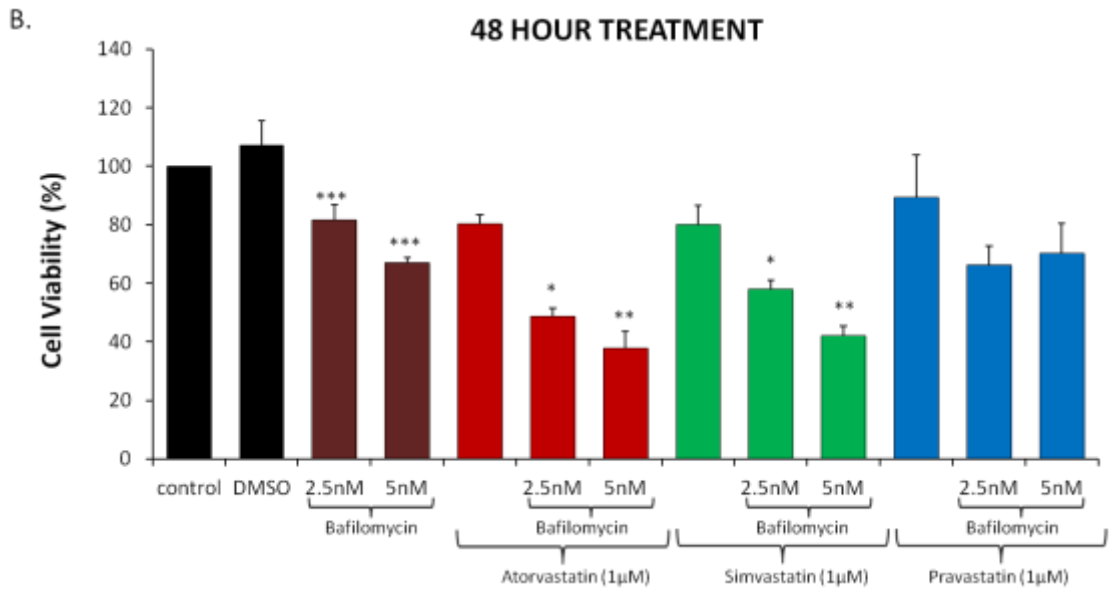


Figure 12B. Decrease in cell viability of lipophilic statin treated hATMF, when autophagy was inhibited for 48 hours. MTT assay result for hATMF treated with 1μM of atorvastatin, simvastatin or pravastatin at 48 hours (with 2.5nM or 5nM of Bafilomycin, or without Bafilomycin). Atorvastatin or simvastatin treated hATMF showed a significant decline in cell viability as compared with Bafilomycin group when autophagy was inhibited with either 2.5 nM or 5 nM Bafilomycin treatment. Pravastatin treated hATMF did not show any difference from any of the control groups.

* P value < 0.05 Vs 2.5nM Bafilomycin Control, n = 5

**P value < 0.05 Vs 5nM Bafilomycin Control, n = 5

***P value < 0.05 Vs Control, n = 5

5.2 Immunoflorescence confirms that autophagy inhibition in statin treated hATMF results in increased autophagic flux

To analyse and support the increased effect of statins when co-incubated with Bafilomycin as seen with the MTT viability assay, the immunofluorescence technique was used to identify co-localisation of autophagosomes and lysosomes in the sttains and Bafilomycin co-treated groups. Autophagosomes were identifyied by staining with LC3-II, and lysosomes were stained with lysotracker. The hATMF were co-treated with 1μM of atorvastatin, simvastatin or pravastatin with 2.5 nM Bafilomycin for 24 hours, and then immunofluorescence was performed.

Atorvastatin and simvastatin treated hATMF showed higher number of autophagosomes visualised as green spots (LC3-II), and lysosomes visualised as red spots (Lysotracker). The co-localisation of autophagosome and lysosome can be appreciated in the overlay image (Figure 13). Simvastatin treated hATMF showed a very high number of autophagosomes confirming autophagy induction. Pravastatin treated hATMF appeared to similar to control group.

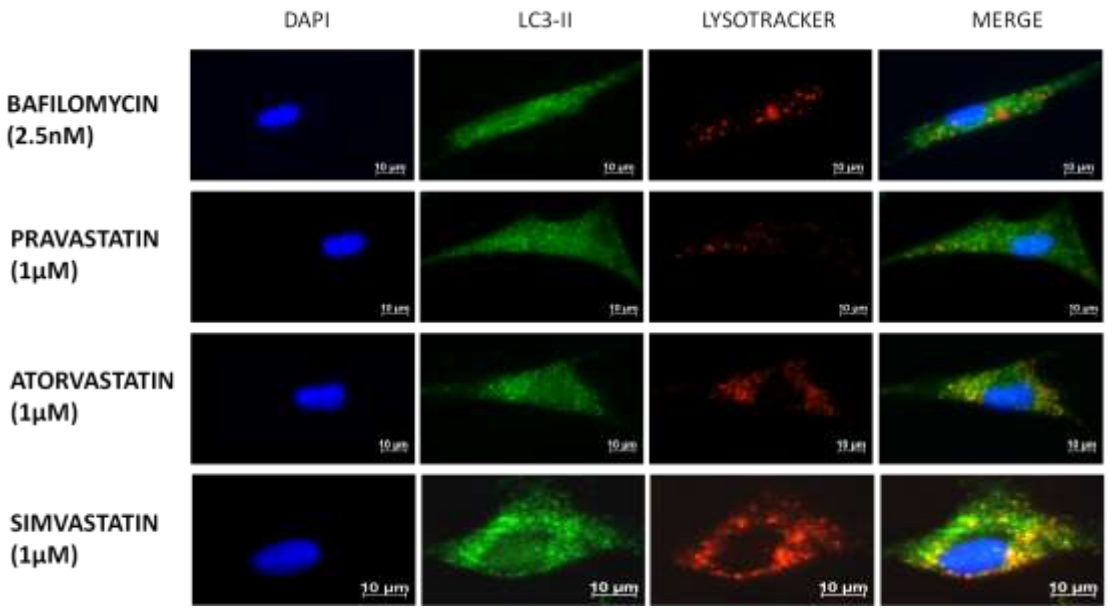


Figure 13. IF data showed increased autophagy induction in lipophilic statin and Bafilomycin co-treated hATMF. Immuno fluorescence pictures of hATMF treated with 1 μM atorvastatin, simvastatin or pravastatin co-treated with 2.5 nM Bafilomycin. Nuclei are stained blue with DAPI. LC3-II (green stain) and lysotracker (red stain) indicates autophagosome formation and lysosomes respectively. There was an increase in the autophagosome number in hATMF co-treated with atorvastatin or simvastatin and Bafilomycin. Images were taken on the fluorescent microscope, and are representative of n=3.

VI. DISCUSSION

Statins are shown to have many pleiotropic effects. *In-vitro*, statins have shown to have varying effects on different cell types (11; 58; 84; 86). The effects of statins on cells from different origin *in vitro* and *in vivo* are still under investigation. To our knowledge, the current study is the first of its kind to demonstrate the differential effects of lipophilic versus hydrophilic statins on human atrial myofibroblasts *in vitro*. Also, it is the first study to demonstrate the differential effects of lipophilic versus hydrophilic statins in the cell death induction mechanisms in human atrial myofibroblasts *in vitro*. Another major strength is that our study used primary human cardiac myofibroblasts, as opposed to fibroblasts/myofibroblasts derived from an animal model as previously investigated by others (58; 60; 86). Although studies done with fibroblasts or myofibroblasts from animals (neonatal rat or rabbit) generate useful information, we cannot assume that human cardiac myofibroblasts will behave in the same manner. As reported by Agocha et al, there are significant differences observed between adult human cardiac fibroblasts and rabbit cardiac fibroblasts in growth and proliferation of these cells in culture (87). Our study focused on the human atrial myofibroblasts, which are the key mediators in myocardial remodeling associated with fibrosis.

Various clinical trials and research on statins have shown that statins exhibit differential side effects (88-91). The current study also sought to understand how lipophilic statins versus hydrophilic statins differ in their effects on cell death induction in human atrial myofibroblasts *in vitro.* This information can provide further insight into why some

statins differ in their severity of side effects than others, based on their lipophilic or hydrophilic nature.

There have been numerous studies undertaken to address the future therapeutic options for fibrosis, however no effective treatments are currently available (92-94). Our study explored the direct cellular effects of statins on human atrial myofibroblasts *in vitro*, with particular focus on the contribution of myofibroblasts to cardiac fibrosis. Porter et. al showed that simvastatin reduced cell proliferation of human atrial fibroblasts *in vitro* (95). Copaja et al have reported induction of apoptosis by simvastatin in cultured cardiac fibroblasts and myofibroblasts from neonatal rats (58). Most of these studies used simvastatin (58; 60; 95; 96) which is lipophilic in nature. We investigated the effects of both lipophilic and hydrophilic statins on hATMF. Our data indicate that lipophilic statins attenuate hATMF viability after 48 hours or 96 hours of treatment *in vitro*, whereas hydrophilic statins have no effect on cell viability.

There are other studies looking at the effects of statins on various other cell types as well, such as induction of apoptosis in vascular smooth muscle cells and renal tubular epithelial cells (97; 98). Studies also indicate that statins stimulate apoptosis, autophagy, and endoplasmic reticulum stress in many cancerous and non-cancerous cell types (18-21). Saied et al showed that simvastatin induces cell death in human atrial fibroblasts *in vitro* (96). We compared the effect of the two groups, lipophilic versus hydrophilic statins in the cell death induction mechanism in human atrial myofibroblasts *in vitro*. We found that after 48 hours of treatment, 0.1, 1 or 10 μM of lipophilic statins (atorvastatin

and simvastatin) induced the programmed cell death "apoptosis" in human atrial myofibroblasts, as indicated by increased expression of key apoptotic marker cleaved caspase-3 and an increased Bax: Bcl2 ratio. However, hydrophilic statins did not show any change in the expression of these markers. This indicates that lipophilic statins induce programmed cell death via apoptosis in human atrial myofibroblasts, in a time and dose dependent manner. Porter et al demonstrated that simvastatin inhibits myofibroblast proliferation (95). In the future, this could be a potential therapeutic alternative to limit myocardial remodeling associated with fibrosis.

Fibrosis in the atria has largely been ignored in the past, as compared to the studies of ventricular fibrosis. Recently it has become clear that atrial remodeling may play an important role in atrial fibrillation and may also be responsible for its origin (99-101). Therefore we suggest that regulation of myofibroblast proliferation in the heart is important in controlling atrial as well as ventricular remodeling and associated fibrosis. The results from our study provide some insight into the effects of statins on human atrial myofibroblasts, and could also be used to better understand the effects of statins on cardiac myofibroblasts in general.

We also looked at the induction of autophagy by statins in human atrial myofibroblasts. We noted that lipophilic statins such as atorvastatin and simvastatin induce autophagy, but it may be an early response to the drug. The induction of autophagy was examined by analyzing the expression of various autophagic markers, such as Beclin-1, Atg5-12 complex, and LC3-II. Although there was a decline in Beclin1 and ATg5-12 expression,

but there was significant increase in the LC3-II expression after 48 hour and 96 hour treatment with lipophilic statins. Autophagy induction was further confirmed by testing LC3-II levels and the autophagosome formation in the human atrial myofibroblasts following statin treatment, using immunofluorescence. We noted that lipophilic statins induced significant amounts of autophagy in the human atrial myofibroblasts whereas; hydrophilic statins appeared to have similar effect as the control group. Autophagy induction could be the initial response of the cells to the lipophilic statins. Initially, the autophagy may contribute as a protective mechanism, but it seems inadequate to sustain cell survival. Statins have also been shown to induce autophagy in other cell types, such as autophagy induction by lipophilic statins in human rhabdomyosarcoma cells (84). We also investigated the effect of autophagy inhibition with Bafilomycin in statin treated human atrial myofibroblasts. When autophagy was inhibited, a further significant decline in cell viability was observed. Autophagy is a complex cellular mechanism, which involves degradation of cellular components or segregated proteins enclosed in the autophagosomes by the lysosomes, after autophagosome-lysosome fusion. Autophagy in the dying cells may be indicative of a different type of cell death than apoptosis or may pinpoint an initial protective adaptive response of the cells in reaction to stress.

Both apoptosis and autophagy play important roles in regulating the homeostasis of the cells in various conditions of cellular stress. Therefore, these results should be carefully interpreted. Whether autophagy is playing a cell protective role or assisting as a cell death mechanism still needs further investigation.

Statins inhibit HMGR enzyme and interfere with the cholesterol production pathway, known as the mevalonate pathway (11). We investigated the role of mevalonate pathway in statin mediated cell death of human atrial myofibroblasts *in vitro*. When co-incubated with statins and mevalonate, the hATMF viability could be rescued even after 48 or 96 hours of statin and mevalonate co-treatment. This result suggests that statin induced cell death in hATMF is mediated by mevalonate depletion by statins. Similar results have been shown by Copaja et al, with 10 μM simvastatin treatment of rat cardiac fibroblasts and myofibroblasts (58). Other investigators have also shown that the statin induced effects on myofibroblasts proliferation could be restored by co-incubating mevalonate or GGPP with simvastatin, but not with FPP (95). Simvastatin induced apoptosis was shown to be prevented by mevalonate, GGPP and FPP (58). These isoprenoid intermediates play an important role as lipid anchoring molecules. Many effects of statins are mediated by the inhibition of these isoprenoid intermediates by statins. This indicates the involvement of Rho-family proteins in maintaining cell viability (58; 60; 95).

Copaja et al showed that simvastatin at 10 μM dose reduces cell adhesion, migration, and viability, as well as disrupts cytoskeleton of rat cardiac fibroblasts *in vitro* (60). Many clinical trials have demonstrated the varying side effects of different statins (88-91; 102). The most common side effects induced by statins include myalgias, effects on gastrointestinal (GI) tract such as upset GI, and elevated CK activity in serum; rhabdomyolysis (rapid muscle breakdown). It is for this reason that, in 2001, cerivastatin was pulled from the market primarily due to patients exhibiting severe rhabdomyolysis as

a side effect (84; 103). Reijneveld et al showed the differential effect of statins on the development of myopathy in young rats. They showed that lipophilic statins (simvastatin and lovastatin) caused stunted growth and severe myopathy, while the hydrophilic statin (pravastatin) did not cause any of these symptoms (86). Our study showed the differential effects of statins in cell death induction mechanism, which could explain some of these potential side effects of statins. Also, this may be indicative of the difference in toxicity amongst statins. This may suggest why some statins have more potent side effects than others. This effect could be due to the hydrophilic versus lipophilic nature of the compound, which may determine how it is absorbed into the human atrial myofibroblast cells.

Also, some studies have indicated a cell type specific effect of statins in induction of autophagy. A study by Makoto et al showed that, lipophilic statins induce autophagy in a cell type specific manner, inducing autophagy in rhabdomyosarcoma cells, but none in HEK293 or HepG2 cells (84). Our study established that lipophilic statins induce apoptosis and autophagy in human atrial myofibroblasts also.

VII. CONCLUSIONS

We have shown that lipophilic statins significantly attenuate the viability of human atrial myofibroblasts *in vitro*, whereas hydrophilic statins do not affect hATMF viability. We also conclude that statin induced cell death in hATMF is mediated through mevalonate depletion by statins through HMGR inhibition. Moreover, the induction of intrinsic apoptotic pathways in human atrial myofibroblasts occurs in association with lipophilic statins only with no effect by hydrophilic statins. This may explain the difference in toxicity of lipophilic versus hydrophilic statins. Lipophilic statins showed an onset autophagy as an initial response, which could be an initial autophagic response as a cell survival mechanism.

VIII. SIGNIFICANCE AND FUTURE DIRECTIONS

These results may represent a novel therapeutic option for the prevention and treatment of cardiac fibrosis, not just ventricular fibrosis, but atrial fibrosis as well. Thus, a novel pleiotropic effect of statins is revealed in this study. A further *in vivo* study with animal models to investigate the effect of statins in controlling the adverse cardiac remodeling associated with fibrosis is worthy of further investigation.

The differential effects of statins in the induction of apoptotic pathways could explain the difference is toxicity between lipophilic versus hydrophilic statins. Many clinical trials have indicated the difference in severity of side effects caused by different statins. Therefore, our study showing the direct cellular effects of lipophilic versus hydrophilic statins on human atrial myofibroblasts, may explain why some statins have more severe side effects than others.

IX. LIMITATIONS

This study is purely an *in vitro* research study. Although it provides an important insight into the differential effects of statins on cardiac myofibroblasts, but a similar *in vivo* study could be helpful in translating the effect of statins in controlling fibrosis.

Current study utilizes primary human cells which could be the source of variability. The current ethics approval for the study did not allow us to collect patient information, such as whether the patient was already on statins or not, or whether they were put on statins after the cardiac surgery; and if they were on statins, which class of statin drug was prescribed and whether there was a difference in the effectiveness of the drug or the side effects experienced. This could possibly explain the reason behind some of the variability observed among samples.

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