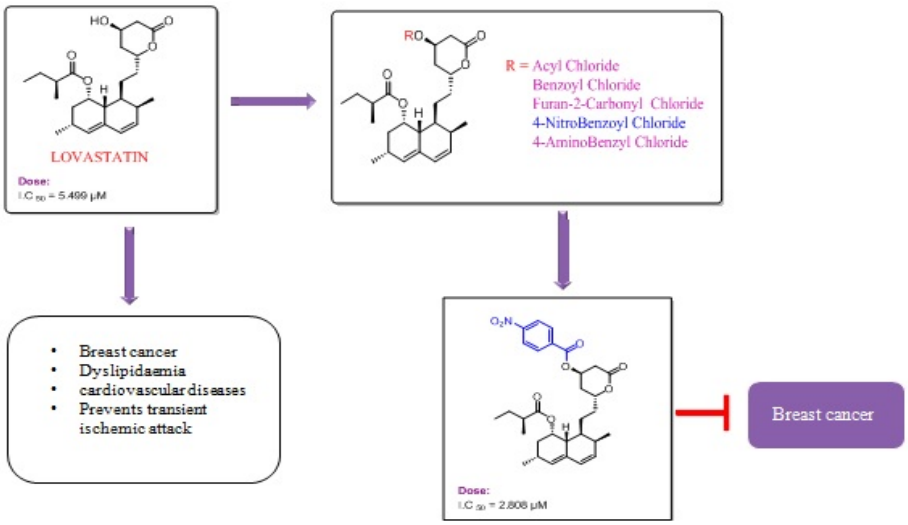
Swapna Vadlamani et al /J. Pharm. Sci. & Res. Vol. 11(9), 2019, 3331-3338

ISSN:0975-1459
Journal of Pharmaceutical Sciences and Research
www.jpsr.pharmainfo.in

Anti cancer Potential of Cholestrol Lowering drug Lovastatin and its New Analogues

Swapna Vadlamani,[*a] Siva Kumar,[b] Venkatesh Pooladanda,[c] Chandraih godugu,[d].
** Assistant Professor, Process chemistry, NIPER Hyderabad*

Graphical Abstract



Abstract

The Paper portrays the recent and brief update of statin therapy. Our work implicated on the synthesis of new analogues of natural product Lovastatin and their characterization using mass and NMR spectra. Preliminary screening of best analogues were done using molecular modelling and further from cell proliferation activity studies best activity analogue was chosen for further cell cycle analysis and DAPI staining. The studies signify that paranitro analogue of lovastatin showed a promising activity towards breast cancer.

Key Words: Statins, HDL Cholesterol Lowering Drugs, Lovastatin

1 INTRODUCTION

Cholesterol is a complex molecule and it is one of the major causes for coronary heart diseases (CHD). There are two different cholesterol good (HDL) high density lipoprotein cholesterol and low density-lipoprotein cholesterol (LDL) bad cholesterol.LDL bad cholesterol is actually leading cause for CHD. In the 1950s and 1960s, it became apparent that elevated concentrations of plasma cholesterol were a major risk factor for the development of coronary heart disease, which led to the search for drugs that could reduce plasma cholesterol. One possibility was to reduce cholesterol biosynthesis, and the rate-limiting enzyme in the cholesterol biosynthetic pathway, 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase, was a natural target1. Recent research in medicine has demonstrated that high levels of cholesterol in blood can lead to atherosclerosis, heart attacks and strokes. Statins are a group of drugs primarily used in lowering blood cholesterol by 20 to 60 percent [2].The inhibition of HMG-CoA (3-hydroxy-3- methyl-glutaryl-coenzyme A) reductase, the essential enzyme in cholesterol biosynthesis by statins was breakthrough in the prevention of hypercholesterolemia and related diseases

Lovastatin was shown to be dramatically effective for lowering LDL cholesterol in healthy volunteers, with no obvious adverse effects [5-6]. Lovastatin, pravastatin are natural statins of fungal origin. All natural statins have a common molecular structure, a Hexahydronaphthalene ring and a hydroxylactone, but they differ from each other due to side chains and a methyl group around the ring. The statins differ with respect to their ring structure and substituent's. These differences in structure affect the pharmacological properties of the statins. Inhibition of HMG-CoA reductase reduces levels of mevalonate [8-9], which leads to a reduction in the regulatory sterol pool, which in turn causes up regulation of HMG-CoA reductase [10], other enzymes of cholesterol biosynthesis[11- 12], and most importantly the LDL receptor[13- 14].

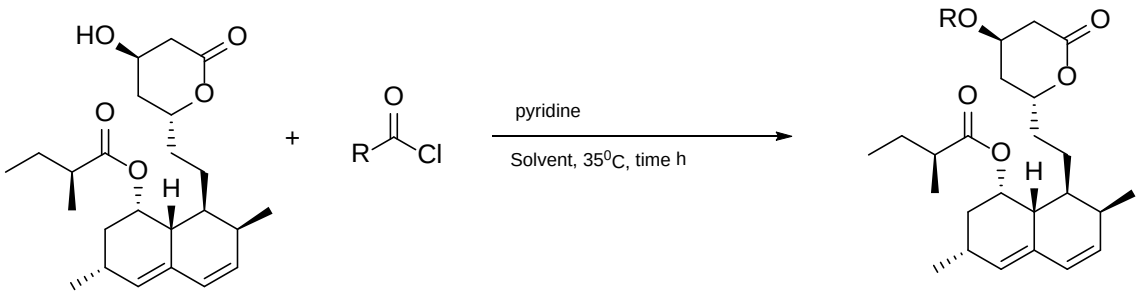
1.1 Statin Therapy for Different Diseases

The Statin therapy mainly targets HMG-R which regulates multi step biosynthetic pathway where almost 30 enzymes are involved. Targeting this enzymes open new avenues for statins in treating various diseases as described in the table 1.

Table 1 Applications of statins		
S.No	Diseases	Statins therapeutic role
1	Auto immune and inflammatory diseases	They suppress vascular and myocardial inflammation.
2	Cancer	They cause pro–apoptosis and 17 patents were reported for treating different cancers like breast, prostate, lymphoma etc.
3	Diabetes	Acetyl L-carnitine mixed with anti hypertensive drug and statins reduce risk of developing diabetes
4	Asthma	Decrease oxidative stress and reduce airway inflammation
5	Bone regeneration	Important pleiotropic effect
6	Neurodegenerative disorders	Dementia, Alzheimer's and antidepressant effect
7	Others	Migrane,dental caries, biological tissue repair etc.

In spite of certain side effects the statins therapy is extensively used. Recent studies on cardiovascular risk factors claim dosage induced variation of diabetogenic potential and also type of the statins used. The pleiotropic effects and long term benefits of statins subside the side effects and also if administered little cautiously and selectively for the patient. The appropriate combination of Statin with other drugs selectively will be a great potential drug with no side effects. With this motivation we synthesized few analogues of natural lovastatin and in our study anti cancer potential of lovastatin and some new analogues of lovastatin were explored and one of the new analogues showed good activity towards breast cancer.

2 SYNTHETIC SCHEME FOR THE ANALOGUES:



1. Acyl Chloride
2.Benzoyl Chloride
3.Furan-2-CarbonylChloride
4.4-NiroBenzoyl Chloride
5.4-AminoBenzyl Chloride

3 MOLECULAR MODELLING STUDIES

Molecular modelling studies were carried out for all the designed analogues .The lymphocyte function-associated antigen (LFA-1) belongs to the family of beta2-integrins and plays an important role in T-cell activation inactivation of Rac-1 which in turn leads to apoptosis, through different signalling mechanisms. The Docking score for the inhibition of this protein by lovastatin and its analogue PNBL LOVA is good in comparison with others. This PNBL Lova also showed good docking score with other apoptosis related proteins. The good docking score and also interactions of the analogue motivated us to further investigate this analogue for cell culture studies.

3.1 MOLECULAR DOCKING STUDIES RESULTS

PDB ID:- 1CQP (Crystal structure analysis of the complex LFA-1 (CD11A) I-domain / lovastatin at 2.6 Å resolution) Bound ligand:- Lovastatin.

Table 2 GLIDE docking results for lovastatin analogs at inhibitor binding site				
S.No	Ligand Name	Glide Score	Interactions	
			H- bonds	π-Cation
1	Lovastatin	-8.171	Tyr257, Ile309, Lys287	-
2	PNBL LOVA	-8.148	Tyr166, Tyr 257, Lys 287	Tyr307

4 RESULTS AND DISCUSSION

4.1 Cell culture studies

The Human cancer cell lines Cal-27(Head and Neck cancer), HCC827 (Lung cancer), MDAMB-231(Breast cancer) were maintained in complete media and supplemented with 1%pencillin/streptomycin and 10%FBS. Cell Viability was assessed using cell proliferation kit (MTT Roche) contain MTT labelling reagent and solubilisation solution.

Cells were grown in 96well micro plates in a final volume of 100µlculture medium per well at 37^0C, 0.5% CO2 overnight. The overnight culture cells are treated with Lovastatin and Its analogues in a concentration dependent manner ranging from 0.1nM to 10000nm (From stock using drug 10mM dissolved in 100% DMSO) and 0.1% DMSO as control. Cells were incubated for 24-96 Hrs. After incubation time 15µl of MTT labelling reagent (final concentration of 0.5mg/ml) was added to each well and incubated for 4 Hours at 37^0C, 0.5% CO2. Then 100µl of the solubilisation solution was added to each well and incubated overnight at 37^0C, 0.5% CO2. After overnight incubation absorbance was measured spectrophotometrically using (ELISA) reader at 550-600 nm. Cell viability was calculated in percent of control. IC50 was calculated for each molecule. The best analogue for breast cancer PNBL LOVA based on IC50 value was further analysed. IC50 values of Lovastatin analogues for different cell lines

4.2 Acridine orange/ethidium bromide (AO/EB) staining

Acridine orange/ethidium bromide (AO/EB) staining assay was carried out to differentiate between live, apoptotic and necrotic cells. AO can permeate the intact cell membrane and stain the nuclei green, whereas EB can only stain the nucleus of cells that have lost membrane integrity in red. It can be inferred from Fig. 1 that the control cells display normal morphology and appeared green in colour. Fluorescence microscopic images of MDA-MB-231 cells treated with 5 and 10 µM of both compounds Lovastatin and PNBL LOVA, the compounds clearly showed morphological changes such as cell shrinkage, membrane blebbing, chromatin condensation and apoptotic body formation, suggesting that it induced apoptosis in breast cancer cells. The results from Fig. 1 indicate that the control cells showed normal morphology and appeared green in colour, whereas, PNBL LOVA has induced increased apoptosis as compared to Lovastatin in breast cancer cells at both the 5 and 10 µM concentration after 24 h of incubation.

DAPI (4',6-diamidino-2-phenylindole) is a fluorescent dye capable of strong binding to A–T rich sequences of DNA and aids in the visualization of chromatin condensation or nuclear damage. It distinguishes live cells from apoptotic cells by staining the characteristic condensed nuclei of the latter bright blue. Therefore, this staining technique was considered of interest to detect the induction of apoptosis by both the compounds **Lovastatin** and **PNBL LOVA** in MDA-MB-231 cells, respectively. The results from **Fig.1** demonstrated that the nuclear structure of untreated control cells was intact whereas breast cancer cells treated with **Lovastatin** and **PNBL LOVA,** respectively displayed condensed, horse-shoe shaped or fragmented nuclei in cells. Here, **PNBL LOVA** has shown increased apoptosis as compared to standard derivative **Lovastatin** at 5 and 10 µM concentrations at 24 h.

4.3 Mitochondrial membrane potential

The apoptotic effect of **Lovastatin** and its derivative treatment in breast cancer cells was explored. Mitochondria are known to play a pivotal role to apoptosis in response to many stresses and the loss of mitochondrial membrane potential (MMP) is a sensitive marker of early mitochondrial damage during apoptosis. We therefore used JC1 staining assay to investigate the MMP of Lovastatin and its derivative. **Lovastatin** treatment of MDA-MB-231 cells for 24 h results in 68.54% increased in mitochondrial membrane potential at 10 µM. Whereas, **PNBL LOVA** had shown 97.25% increased membrane potential as compared to **Lovastatin** observed at same concentration and incubation time. Thus, mitochondrial membrane potential was significantly increased after **PNBL LOVA** treatment in breast cancer cells as compared to **Lovastatin**, which indicated that **PNBL LOVA** is more potent than standard derivative.

Table 3 IC50 values of Lovastatin analogues for different cell lines

S.No	Name of the drug/Molecule	IC 50 value on various cell lines		
		Cal-27	HCC-827	MDA-MB-231
1	Lovastatin standard	1.539±0.0265	1.33±0.0127	5.499±0.014
2	ACL LOVA	12.58±0.2347	11.73±0.0.253	10.53±0.006
3	BZL LOVA	13.23±0.134	12.72±0.0304	11.76±0.043
4	FURO LOVA	12.63±0.0321	11.47±0.1203	11.12±0.013
5	PABL LOVA	6.78±0.001	8.92±0.001	5.24±0.003
6	PNBL LOVA	1.789±0.0043	7.38±0.001	2.88±0.102

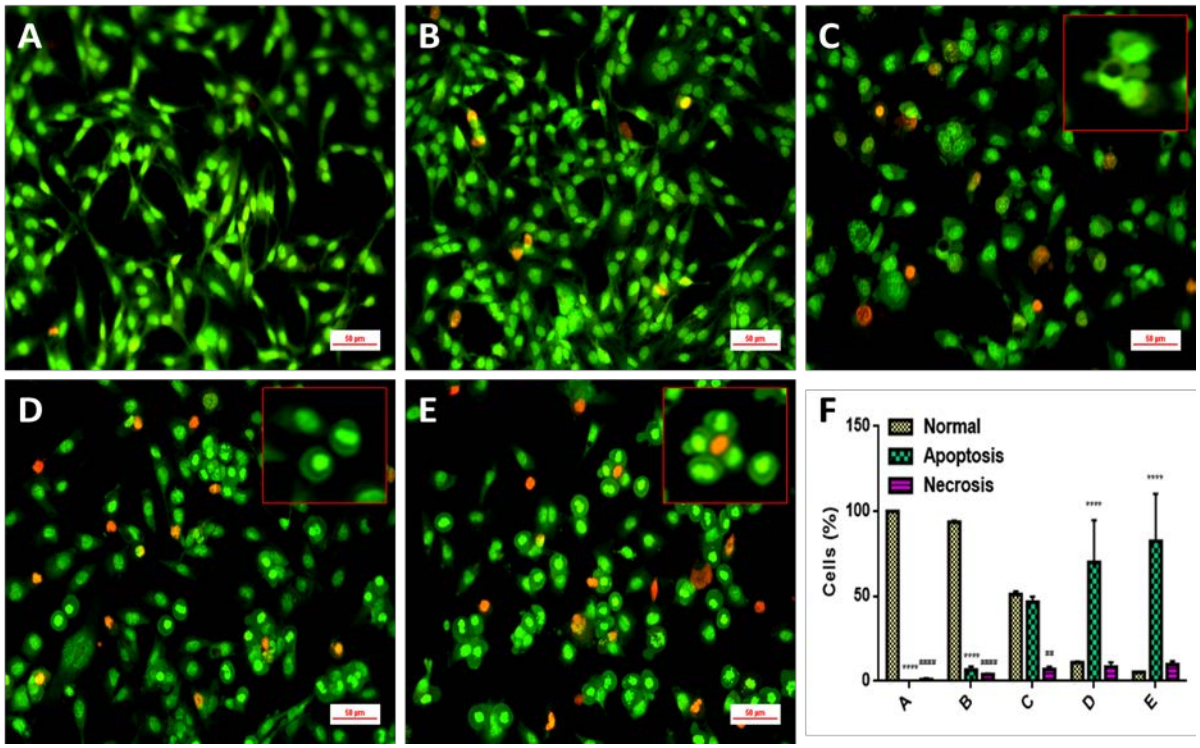


Figure 1 AO/EB staining of Lovastatin and PNBL LOVA compounds

MDA-MB-231 cells were grown in culture plates for 24 h and then treated with 5 and 10 µM of Lovastatin and PNBL LOVA. Representative photomicrographs are shown of breast cancer cells (A) control (B) 5 µM Lovastatin (C) 10 µM Lovastatin (D) 5 µM PNBL LOVA and (E) 10 µM PNBL LOVA. The normal, apoptotic and necrotic cells were counted randomly from AO/EB images and represented as bar chart. Data presented as mean ± SEM (n = 3). ****p < 0.0001 is significantly different from the normal verses apoptotic cells; ##p < 0.01 and ####p < 0.0001 are significantly different from the normal verses necrotic cells.

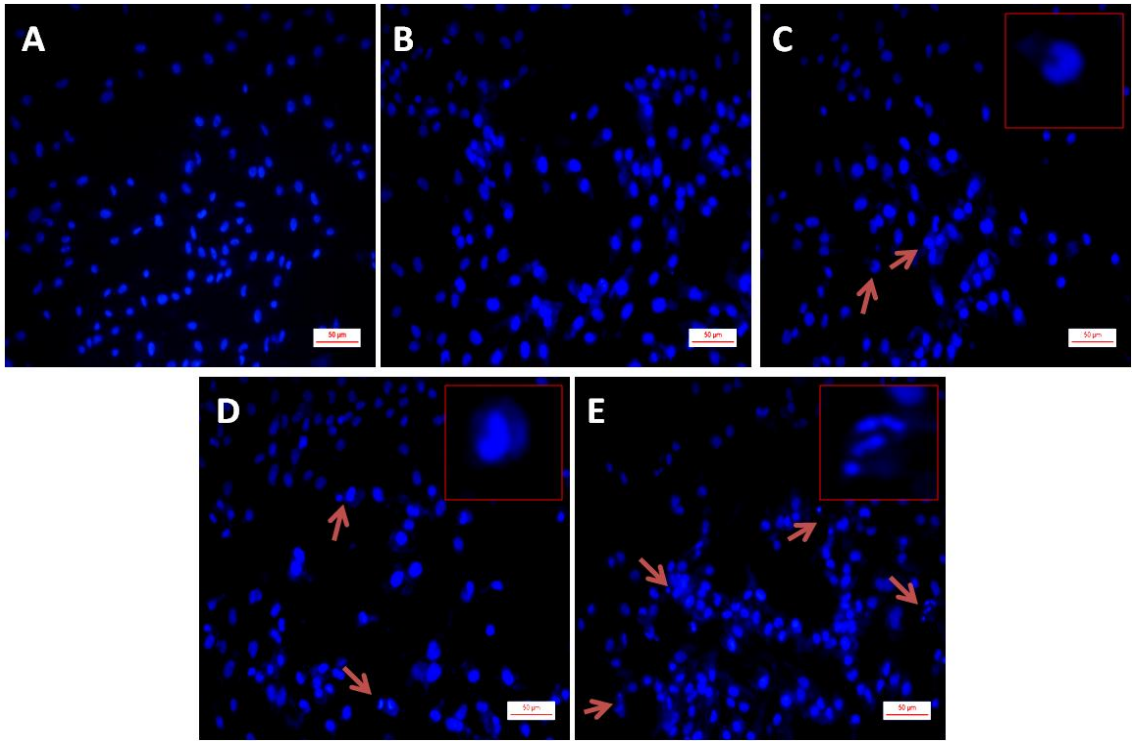


Figure 2 Nuclear morphology of cancer cells after DAPI staining

MDA-MB-231 cells were treated with 5 and 10 µM concentrations of Lovastatin and PNBL LOVA for 24 h and stained with DAPI. The images were captured with fluorescence microscope at 200X. Here, the photomicrographs are represented as (A) control (B) 5 µM Lovastatin (C) 10 µM Lovastatin (D) 5 µM PNBL LOVA and (E) 10 µM PNBL LOVA.

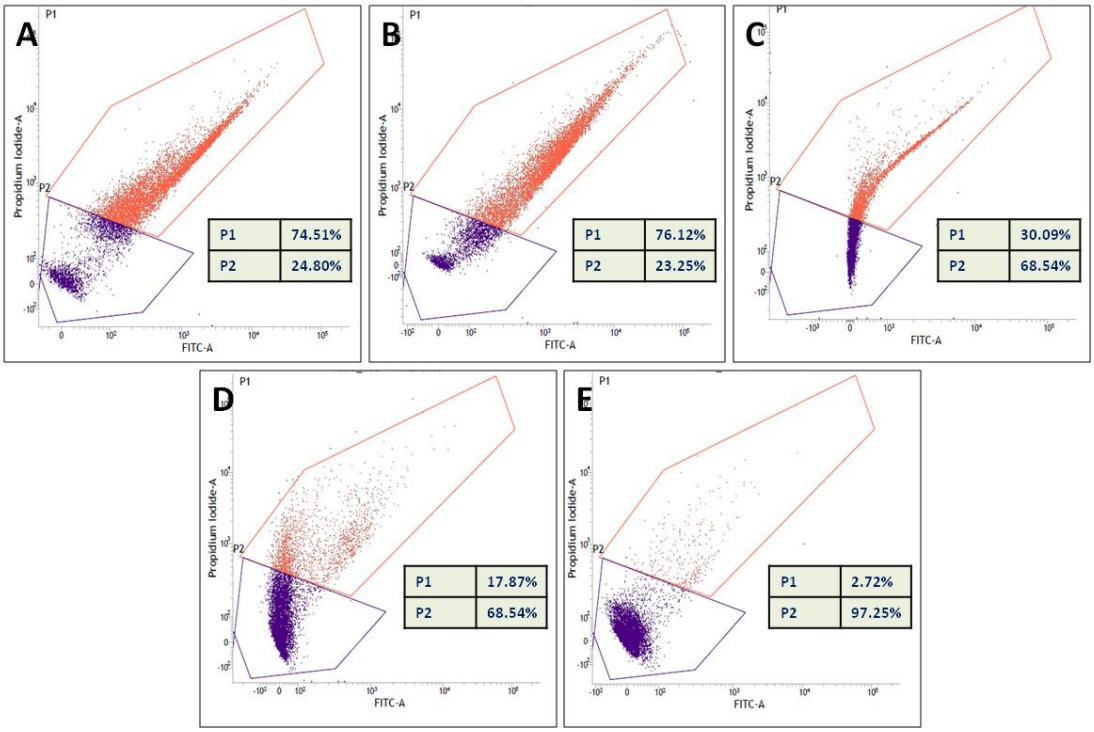


Figure 3 Increased mitochondrial membrane potential (MMP) induced by Lovastatin derivatives

Cells were incubated with JC-1 and subjected to flow cytometry for measuring JC-1 aggregates and monomers. Representative data were shown in (A) control (B) 5 µM Lovastatin (C) 10 µM Lovastatin (D) 5 µM PNBL LOVA and (E) 10 µM PNBL LOVA. Here, P1=indicates live cells and P2=indicates dead cells.

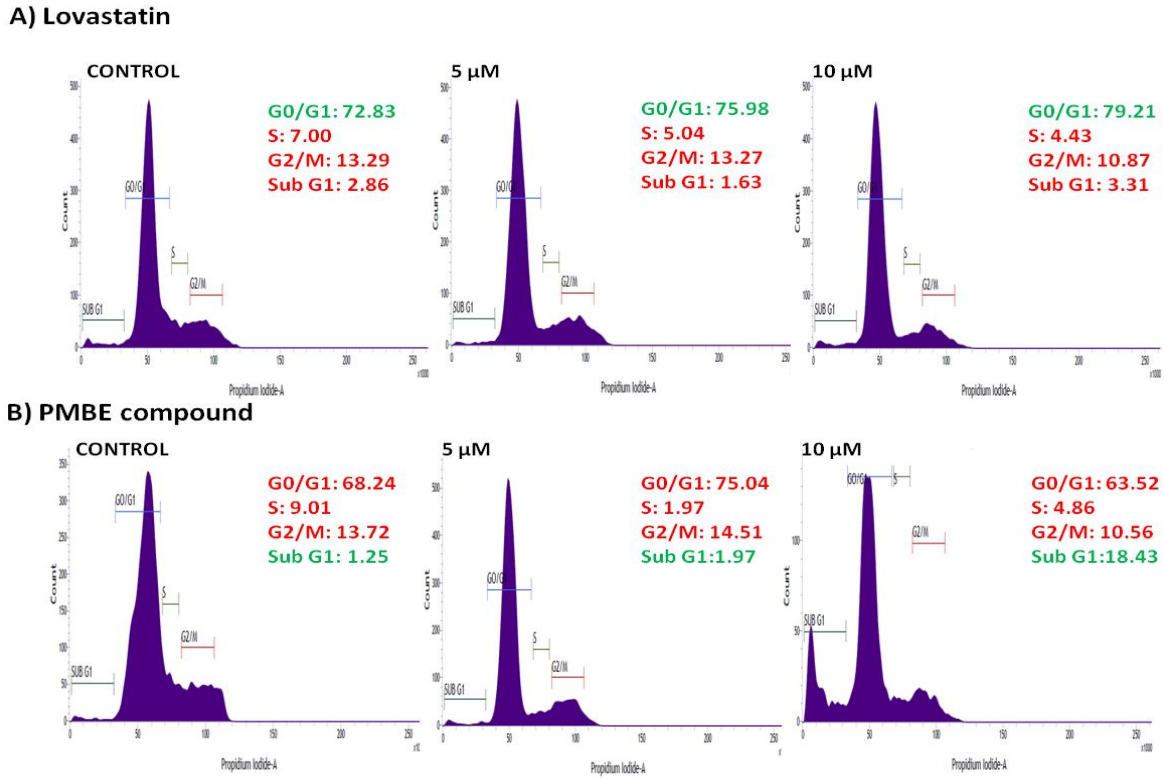


Figure 4 Effect of Lovastatin and PNBL LOVA on cell cycle progression of MDA-MB-231 cells

(A) MDA-MB-231 cells were treated with Lovastatin and (B) PNBL LOVA cell cycle analysis was performed after 24 h of incubation by using 5 and 10 µM concentration. The analysis of cell cycle distribution was performed by using propidium iodide staining method.

4.4 Cell cycle analysis

Usually, anticancer therapeutics prevents the proliferation of cancer cells by blockade of cell cycle at a specific checkpoint. From the in vitro screening results, it was evident that test compound PNBL LOVA showed significant activity against MDA-MB-231 cells. As a result, it was our interest to figure out whether this cytotoxicity was due to cell cycle arrest through cell cycle analysis. MDA-MB-231 cells were treated with PNBL LOVA and Lovastatin compounds respectively, at concentrations of 5 and 10 µM for 24 h, and stained with propidium iodide and further analyzed by using BD FACSVerseTM flow analyzer. The results from Fig. 4A showed that the control untreated cells showed 72.83% cells in G0/G1 phase, whereas cells treated with Lovastatin showed 79.21% increase in G0/G1 population at 10 µM concentration at 24 h. On the other hand, the results from Fig. 4B indicated that the MDA-MB-231 untreated control cells exposed to DMSO showed 1.25% cells in sub-G1 phase, whereas compound PNBL LOVA treatment resulted in increased sub-G1 population to 18.43% in 24 h at 10 µM concentration as compared to control where 1.25% phase arrest was observed. These results clearly indicated that treatment of MDA-MB-231 cells with compound PNBL LOVA resulted in sub-G1 phase arrest. These results indicated that there was an increased sub-G1 and G0/G1 phase arrest in Lovastatin and PNBL LOVA treated breast cancer cells respectively and they were showing phase arrest dissimilar due changes in structural modification.

5 CONCLUSION

The natural product lovastatin itself is having a very good antipoliferating activity compared to the analogues. Para amino analogue of lovastatin is having better activity compared to other analogues and showing good activity towards breast cancer even better than lovastatin.From molecualr modelling studies siminar activity for para nitro anolgue as lovastatin was obseved. From the cell poliferation assay this analogue showed better activity towards breast cancer than lovastatin with IC50 value 2.88 was observed. This paranitro analogue from cell cycle analysis arresting sub G1 phase and high Mitochondrion membrane potential is observed. This again proves the potential of statins towards treating multiple deseases and scope for discovering new anaologues in the pipeline.

6 BIBILOGRAPHY

1. Tobert JA. Lovastatin and beyond: the history of the HMG-CoA reductase inhibitors. Nature reviews Drug discovery. 2003 Jul;2(7):517.
2. Liu J, Zhang J, Shi Y, Grimsgaard S, Alraek T, Fønnebø V. Chinese red yeast rice (Monascus purpureus) for primary hyperlipidemia: a meta-analysis of randomized controlled trials. Chinese medicine. 2006 Dec;1(1):4.
3. Endo A, Kuroda M, Tsujita Y. ML-236A, ML-236B, and ML-236C, new inhibitors of cholesterogenesis produced by Penicillium citrinum. The Journal of antibiotics. 1976;29(12):1346-8.
4. Tobert JA, Hitzenberger G, Kukovetz WR, Holmes IB, Jones KH. Rapid and substantial lowering of human serum cholesterol by mevinolin (MK-803), an inhibitor of hydroxymethylglutaryl-coenzyme A reductase. Atherosclerosis. 1982 Jan 1;41(1):61-5.
5. Tobert JA, Bell GD, Birtwell J, James I, Kukovetz WR, Pryor JS, Buntinx A, Holmes IB, Chao YS, Bolognese JA. Cholesterol-lowering effect of mevinolin, an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme a reductase, in healthy volunteers. The Journal of clinical investigation. 1982 Apr 1;69(4):913-9.
6. Brown MS, Goldstein JL. A receptor-mediated pathway for cholesterol homeostasis. Science. 1986 Apr 4;232(4746):34-47
7. Pappu AS, Illingworth DR. Contrasting effects of lovastatin and cholestyramine on low-density lipoprotein cholesterol and 24-hour urinary mevalonate excretion in patients with heterozygous familial hypercholesterolemia. The Journal of laboratory and clinical medicine. 1989 Nov;114(5):554-62.
8. Naoumova RP, Marais AD, Mountney J, Firth JC, Rendell NB, Taylor GW, Thompson GR. Plasma mevalonic acid, an index of cholesterol synthesis in vivo, and responsiveness to HMG-CoA reductase inhibitors in familial hypercholesterolaemia. Atherosclerosis. 1996 Jan 26;119(2):203-13.
9. Brown MS, Goldstein JL. Multivalent feedback regulation of HMG CoA reductase, a control mechanism coordinating isoprenoid synthesis and cell growth. Journal of lipid research. 1980 Jul 1;21(5):505-17.
10. Balasubramaniam S, Goldstein JL, Brown MS. Regulation of cholesterol synthesis in rat adrenal gland through coordinate control of 3-hydroxy-3-methylglutaryl coenzyme A synthase and reductase activities. Proceedings of the National Academy of Sciences. 1977 Apr 1;74(4):1421-5.
11. Bergstrom JD, Wong GA, Edwards PA, Edmond J. The regulation of acetoacetyl-CoA synthetase activity by modulators of cholesterol synthesis in vivo and the utilization of acetoacetate for cholesterogenesis. Journal of Biological Chemistry. 1984 Dec 10;259(23):14548-53.
12. Kovanen PT, Bilheimer DW, Goldstein JL, Jaramillo JJ, Brown MS. Regulatory role for hepatic low density lipoprotein receptors in vivo in the dog. Proceedings of the National Academy of Sciences. 1981 Feb 1;78(2):1194-8.
13. Ma PT, Gil G, Südhof TC, Bilheimer DW, Goldstein JL, Brown MS. Mevinolin, an inhibitor of cholesterol synthesis, induces mRNA for low density lipoprotein receptor in livers of hamsters and rabbits. Proceedings of the National Academy of Sciences. 1986 Nov 1;83(21):8370-4.
14. Alberts AW, Chen J, Kuron G, Hunt V, Huff J, Hoffman C, Rothrock J, Lopez M, Joshua H, Harris E, Patchett A. Mevinolin: a highly potent competitive inhibitor of hydroxymethylglutaryl-coenzyme A reductase and a cholesterol-lowering agent. Proceedings of the National Academy of Sciences. 1980 Jul 1;77(7):3957-61.
15. Thurnher M, Nussbaumer O, Gruenbacher G. Novel aspects of mevalonate pathway inhibitors as antitumor agents. Clinical Cancer Research. 2012 Apr 23:clincanres-0489.

7 SUPPORTING INFORMATION

7.1 Experimental Section

General all reactions are carried out under an inert atmosphere of nitrogen and with glassware dried in oven. The following solvents and reagents were dried over molecular sieves prior to use THF, Toluene, DMF. T.L.C Plates pre coated with silica gel. Spots are visualized any of the following methods U.V., I2, Anisaldehyde,KmnO4, Ninhydrin. MS spectrum were obtained from LC/MS system.

7.2 Preparation of acetylated product of lovastatin

To a solution of lovastatin (1g, 2.47 mmol) in dry toluene (10ml) were added pyridine (0.42g, 5.43 mmol) and then slowly add acetyl chloride (0.38g, 4.94 mmol)at room temperature. The mixture was stirred 19 hours and then diluted with toluene (10ml). The resulting solution was washed with 1N HCl solution (2x10ml),10% $NaHCO_3$(10ml) and water (10ml).The organic phase was separated and dried with Na_2SO_4.Na_2SO_4 was filtered off, and the filtrate was concentrate in *vacuo.* The product precipitated up on addition of hexane.

LCMS RESULTS: m/z ratio ([$C_{26}H_{38}O_6]^+H)^+$
Mass peak at 447.2757

7.3 Preparation of benzoylated product of lovastatin

To a solution of lovastatin (1g, 2.47 mmol) in dry toluene (10ml) were added pyridine (0.42g, 5.43 mmol) and then slowly add benzoyl chloride (0.64g, 4.94 mmol) at room temperature. The mixture was stirred 19 hours and then diluted with toluene (10ml). The resulting solution was washed with 1N HCl solution (2x10ml), 10% $NaHCO_3$ (10ml) and water (10ml).The organic phase was separated and dried with Na_2SO_4.Na_2SO_4 was filtered off, and the filtrate was concentrate in *vacuo.* The product precipitated up on addition of hexane.

LCMS RESULTS : m/z ratio ([$C_{31}H_{40}O_6]^+H)^+$
Mass peak at 509.2911

7.4 Preparation of furan-2-carbonyl chloride from furan-2-carboxylic acid

To a solution of furan-2- carboxylic acid (0.5g, 4.46 mmol) in 9ml of DCM was added oxalyl chloride (1.5ml 34.4 mmol) and add two drops of DMF and the mixture was stirred at room temperature for 30 min. the excess oxalyl chloride and DCM removed in *vacuo* to provide furan-2-carbonyl chloride

7.5 Preparation of furan-2- carbonyl lovastatin

To a solution of lovastatin (200mg, 0.49 mmol) in dry toluene (10ml) were added pyridine (0.085g, 1.07 mmol) and then slowly added furan-2-carbonyl chloride (0.12g, 0.98 mmol)at room temperature. The mixture was stirred 19 hours and then diluted with toluene (10ml). The resulting solution was washed with 1N HCl solution (2x10ml), 10% $NaHCO_3$ (10ml) and water (10ml). The organic phase was separated and dried with Na_2SO_4.Na_2SO_4 was filtered off, and the filtrate was concentrate in *vacuo.* The product precipitated up on addition of hexane.

LCMS RESULTS : m/z ratio ([$C_{29}H_{38}O_7]^+H)^+$
Mass peak at 499.2693

7.6 Preparation of P-nitro benzoyl chloride from P-nitro benzoic acid

To solution of P-nitro benzoic acid (0.5mg,2.99 mmol) in thionyl chloride (1.068g, 8.98mmol) was refluxed about 1h, the excess of thionyl chloride was removed by repeated evaporation with dry toluene in *vacuo.*

7.7 Preparation of P-nitro benzoyl lovastatin

To a solution of lovastatin (1g, 2.47 mmol) in dry toluene (10ml) were added pyridine (0.42g, 5.43 mmol) and then slowly added P-nitro benzoyl chloride (0.91g, 4.94 mmol) at room temperature. The mixture was stirred 19 hours and then diluted with toluene (10ml). The resulting solution was washed with 1N HCl solution (2x10ml), 10% $NaHCO_3$ (10ml) and water (10ml). The organic phase was separated and dried with Na_2SO_4.Na_2SO_4 was filtered off, and the filtrate was concentrate in *vacuo.* The product precipitated up on addition of hexane.

LCMS RESULTS: m/z ratio ([$C_{31}H_{39}NO8]^+H)^+$
Mass peak at 554.2761

7.8 Preparation of P-amino benzoyl chloride from P-amino benzoic acid

P-amino benzoic acid (1g, 7.5 mmol) is dissolved in thionyl chloride (5.8ml, 72.9 mmol) under nitrogen atmosphere and the reaction is heated under weak reflux (T= 50-60^{0}C) for about 2-hours until the acid is completely converted in to the corresponding acyl chloride. The excess thionyl chloride is evaporated under reduced pressure. The pale yellow oil is obtained that at 0^{0}C becomes yellow crystalize powder. The product is used in the next step without purification.

LCMS RESULTS: m/z ratio ([$C_{31}H_{41}NO6]^+H)^+$
Mass peak at 524.3008

7.9 Preparation of P-amino benzoyl lovastatin

To a solution of lovastatin (0.05g, 0.12mmol) in dry toluene (5ml) were added pyridine (0.021g, 0.27 mmol) and then slowly added P-amino benzoyl chloride (0.03g, 0.24 mmol) at room temperature. The mixture was stirred 19 hours and then diluted with toluene (10ml). The resulting solution was washed with 1N HCl solution (2x10ml),10% $NaHCO_3$ (10ml) and water (10ml). The organic phase was separated and dried with Na_2SO_4.Na_2SO_4 was filtered off, and the filtrate was concentrate in *vacuo.* The product precipitated up on addition of hexane

7.10 In-vitro anti Proliferative activity of Lovastatin analogues on various cancer cell lines.

The Human cancer cell lines (Cal-27(Head and Neck cancer), HCC827 (Lung cancer), MDAMB-231(Breast cancer) were maintained in complete media and supplemented with 1%pencillin/streptomycin and 10%FBS. Cell Viability was asses using cell proliferation kit (MTT)(Roche) contain MTT labelling reagent and solubilisation solution.

Cells were grown in 96well micro plates in a final volume of 100µlculture medium per well at 37^{0}C, 0.5% CO2 overnight. The overnight culture cells are

treated with Lovastatin and Its analogues in a dose dependent manner ranging from 0.1nM to 10000nm (From stock using drug 10mM dissolved in 100% DMSO) and 0.1% DMSO as control. Cells were incubated for 24-96 Hrs. After incubation time 15µl of MTT labelling reagent(final concentration of 0.5mg/ml) was added to each well and incubated for 4 Hours at 37^0C, 0.5% CO2. Then 100µl of the solubilisation solution was added to each well and incubated overnight at 37^0C, 0.5% CO2. After overnight incubation absorbance was measured spectrophotometrically using (ELISA) reader at 550-600 nm. Cell viability was calculated in percent of control. IC50 was calculated for each molecule.

7.11 Cell culture

Breast cancer (MDA-MB-231) cells were procured from National Centre for Cell Science (NCCS) Pune, India and the cells were grown in appropriate L-15 (Leibovitz's L-15) medium. Medium was supplemented with 10% fetal bovine serum stabilized with 1% antibiotic-antimycotic solution (Sigma) in an incubator with 0% CO_2 and 98% relative humidity at 37^o C. When the cells reached up to 80-90% of confluence, they were sub-cultured using 0.25% trypsin/1 mM EDTA solution for further passages.

7.12 Acridine orange and ethidium bromide (AO-EB) staining

MDA-MB-231 cells were treated with 5 and 10 µM of **Lovastatin** and **PMBE** compounds. Plates were incubated in an atmosphere of 0% CO_2 at 37^o C for 24 h, followed by addition of fluorescent dyes containing acridine orange-ethidium bromide (AO-EB; 10 mg/mL) into each well in equal volumes and within 10 min, cells were visualized under fluorescence microscope (Nikon, Inc. Japan) with excitation (488 nm) and emission (550 nm) at 200X magnification.

7.13 DAPI staining

MDA-MB-231 cells (1 X 10^6 cells/well) were cultured in six well plates and treated with 5 and 10 µM of compounds **Lovastatin and PMBE** or without compounds (Control-DMSO). Then the cells were washed twice with PBS, followed by 4% paraformaldehyde fixation and staining with 10 µg/mL of DAPI. The cells were observed for apoptotic characteristics under fluorescence microscope with excitation at 359 nm and emission at 461 nm using DAPI filter at 200X magnification.

7.14 Assay of mitochondrial membrane potential ($\Delta\psi_m$)

The mitochondrial- specific cationic dye JC-1 (Invitrogen, Carlsbad, CA), which undergoes potential-dependent accumulation in the mitochondria was used to detect effect of Lovastatin and its derivatives on mitochondrial membrane potential. Briefly, cells were plated at a seeding density of 2×105 cells/well in a 12-well plate. After 24 h of treatment with Lovastatin and PMBE (5 and 10 µM), cells were incubated with 2 µM JC-1 for 30 min at room temperature in the dark followed by flow cytometry for the quantitative analysis of $\Delta\psi$m. At least 10,000 events per sample were recorded.

7.15 Cell cycle analysis

MDA-MB-231 cells (1 X 10^6 cells/well) in 6 well plates were treated with 5 and 10 µM of Lovastatin and PMBE compounds. Cells were collected by trypsinisation, washed with PBS and fixed with 70% ethanol for 30 min at 4^o C. After fixing, cells were again washed with PBS and stained with 400 µL of propidium iodide staining buffer [PI (200 mg), Triton X (100 µL), DNAse-free RNAse A (2 mg) in 10 mL of PBS] for 15 min in dark condition at room temperature. The samples were then analyzed for propidium iodide fluorescence from 15,000 events by flow cytometry using BD Accuri C6 flow cytometer.