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Study of the Effect of Statins on a Variety of Null, Wild Type, and Mutant p53 Cancer Cell Lines

Ahmed S. Mehanna*, Ngan Tran

Department of Pharmaceutical Sciences, School of Pharmacy, MCPHS University, USA

***Corresponding author:** Ahmed S. Mehanna, Department of Pharmaceutical Sciences, School of Pharmacy, MCPHS University, 179 Longwood Avenue, Boston Massachusetts 02115, USA. Tel: +1-6177322955; Fax: +1-6177322228; Email: Ahmed.mehanna@mcphs.edu

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Abstract

The aim of the current study is to compare the cytotoxic effects of the most commonly used statins: atorvastatin (Lipitor®) and simvastatin (Zocor®) on a variety of cancer cells with different p53 status. Two breast cancer cell lines: MDA-MB-231 (mutant p53) and 4T1 (null p53), two colon cancer cell lines: HCT-116 (wild-type p53) and CT26.WT (null p53), and two bladder cancer cell lines: 5637 (mutant p53) and RT4 (wild-type p53) were studied. A cell viability assay (MTS) indicated that simvastatin has 15-fold higher activity than atorvastatin in cytotoxicity as reflected by the average IC_{50} values of atorvastatin (30 μM) in comparison to that of simvastatin (2 μM), after 2 days of incubation. Additionally, the study revealed that adding mevalonic acid, as a supplementary rescue agent, to the cells in combination with simvastatin, at different concentrations, only the cell viabilities of MDA-MB-231, 4T1, and CT26.WT were significantly rescued out of the six cell lines used. With the addition of mevalonic acid, the cell viabilities of these cell lines were found to be double of that without adding mevalonic acid. The three cell lines that were not rescued are HCT-116, RT4 and 5637. The mevalonate metabolism is enhanced in mutant p53 and null p53 cells but not in wildtype p53. However, the study showed that not all of mutant p53 cancer cells contain mevalonate metabolism. Mutant p53 MDA-MB-231 cells does go through the mevalonate pathway, but not in mutant p53 5637 cells. Besides, simvastatin showed promising cytotoxic effects on triple negative breast cancer cell lines: MDA-MB-231 and 4T1, the most dangerous type of breast cancer. Simvastatin also showed excellent anti-proliferative effect on resistant cells such as bladder cancer cells (RT4).

Keywords: Atorvastatin; Bladder cancer cells; Breast cancer cells; Colon cancer cells, Cytotoxicity; Simvastatin

Introduction

Statins, also known as 3-Hydroxy-3-Methylglutaryl Coenzyme A (HMG-CoA) reductase inhibitors, are commonly-used drugs for the treatment of hypercholesterolemia since it inhibits the mevalonate pathway that generates cholesterol [1]. Statins mimic the tetrahedral intermediate produced by HMG-CoA reductase, an enzyme which catalyzes the conversion of HMG-CoA into mevalonate, the rate-limiting step in hepatic cholesterol biosynthesis. Statins bind to the active site of enzyme HMG-CoA reductase with ~1,000-fold greater affinity than HMG-CoA itself, hence statins are potent competitive inhibitors [2].

Cholesterol is known to be a major structural component of cell membranes, and the cholesterol biosynthetic pathway is closely related to cell-growth processes [3]. Statins reduce not only serum cholesterol levels but also mevalonate synthesis by inhibiting HMG-CoA reductase. Mevalonate is a precursor of several major products regulating the cell cycle. By inhibiting the mevalonate pathway, the generation of these products including dolichol, Geranylpyrophosphate (GPP) and Farnesylpyrophosphate (FPP) is also inhibited. The mevalonate pathway is not only present in hepatic cells, many studies showed that the mevalonate pathway is up-regulated in several cancers such as leukemia, lymphoma, multiple myeloma; as well as breast, hepatic, pancreatic, esophageal and prostate cancer cells [4]. Therefore, it is believed that statins might be useful for cancer prevention and treatment through their interactions with essential cellular functions, such

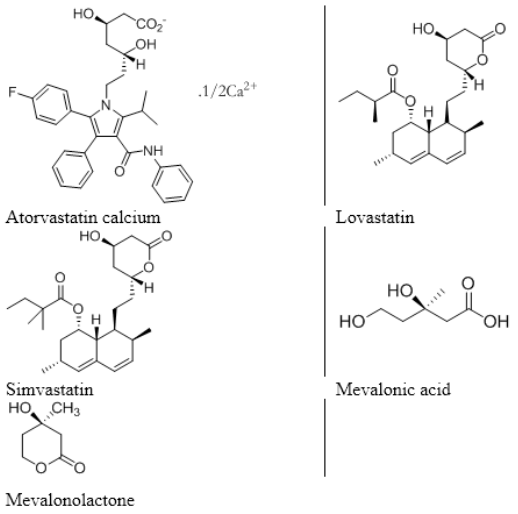
as cell proliferation and differentiation. [5,6] Both *In vitro* and *In vivo* studies have also demonstrated that statins inhibit tumor growth and induce apoptosis in a variety of tumor cells, including melanoma, [7] glioma, [8] neuroblastoma, [9] and leukemia cell lines [10]. Several clinical trials have assessed the antitumor activity of statins [11-16]. The different mechanisms attributed to the anticancer activity of statins in different types of cancer include: stabilization of the cell cycle kinase inhibitors p21 and p27, [17] inhibition of Ras- and Rho-mediated cell proliferation; [18] stimulation of protein kinase B, [19] activation of endothelial nitric oxide synthase at low-dose, [20] inhibition of capillary tube formation, [21] decreased vascular endothelial growth factor release at high-dose in order to prevent angiogenesis; [22] upregulation of proapoptotic proteins such as Bax and Bim, [23] decrease in antiapoptotic proteins such as Bcl-2, [10] activation of Caspase-3, Caspase-7, Caspase-8, and Caspase-9 to induce apoptosis; [24-26] reduction in expression of the endothelial leukocyte adhesion molecule E-selectin, [27] reduction of matrix metalloproteinase 9 expression, [28] and inhibition of epithelial growth factor-induced tumor cell invasion to repress tumor metastases [29].

Glycolysis in tumor cells generates pyruvate, which is either converted to lactate or introduced into the citrate or Krebs cycle. However, mitochondrial oxidation is incomplete and leads to an enhanced export of Acetyl-CoA in the cytosol. Cytosolic Acetyl-CoA serves as a building block for anabolic reactions that promote cell growth and proliferation. Acetyl-CoA can also be used to form HMG-CoA and thus to initiate mevalonate metabolism pathway, which is enhanced by mutated p53. [30] The mevalonate pathway is believed to help the stabilization of mutant p53 [31]. Statins inhibit the mevalonate pathway, not only inhibits the generation of products involved in cell cycle, cell growth process, but also helps to destabilize mutant p53, hence suppresses cancer progression caused by mutant p53.

Why is it crucial to discover a drug that can stop the actions of mutated p53 cancer cells? Over 50% of human cancers carry loss of function mutations in p53 gene [32]. The p53 oncogene also known as TP53 or tumor protein, is a gene that codes for a protein which regulates the cell cycle and therefore it functions as a tumor suppressor, meaning its activity stops the formation of tumors. Because of the role of the p53 protein in protecting stability by preventing genome mutation, it has been described as "the guardian of the genome" [33]. One of the mechanism of how wildtype-p53 helps to stop the growth of cancers is as follows, p53 protein binds DNA, which in turn stimulates another gene to produce a protein called p21 that interacts with a cell division-stimulating protein (CDK2). When p21 is complexed with CDK2 the cell cannot pass through to the next stage of cell division. Mutant p53 can no longer bind DNA in an effective way, and as a consequence the p21 protein is not made available to act as the 'stop signal' for cell division. Thus cells divide uncontrollably, and form tumors [34]. Therefore, stabilization of mutant p53 in tumours greatly contributes to malignant progression [31].

Without HMG-CoA reductase inhibitors, mevalonic acid after being generated by HMG-CoA will then continue Generate Mevalonate-5-Phosphate (MVP). When MVP present, DNAJA1, a Hsp40 protein family member which is associate with complex assembly, protein folding, and export, [31] binds to mutant p53 and stabilize mutant p53, making cancer progression enhanced. Statins inhibit HMG-CoA reductase, therefore reduce the concentration of MVP, mutant p53 cannot efficiently complex with DNAJA1. Instead, CHIP, a protein that signal ubiquitin degradation, will bind to mutant p53. Hence, mutant p53 gets degraded and stop the growth process of cancer cells. However, is the mevalonate pathway present in all p53 cancer cells, so that when MVP reduced, mutant p53 will be all degraded? What is the correct mechanism if there is a mutant p53 treated by statins? To test this question, atorvastatin calcium, simvastatin, and mevalonic acid are used in this research.

Scheme 1 introduces structure of atorvastatin, simvastatin, lovastatin, mevalonic acid and mevalonolactone. Atorvastatin calcium (Lipitor), which has molecular formula: $C_{33}H_{35}FN_2O_5.1/2Ca$ and molecular weight 578.7 g/mol, is an off-white crystalline powder. It is a synthetic, selective inhibitor of HMG-CoA reductase. Simvastatin (Zocor), which has molecular formula: $C_{25}H_{38}O_5$ and molecular weight: 418.57 g/mol, is a lactone that is readily hydrolyzed *In vivo* to the corresponding β-hydroxy acid, and can be activated prior to use with sodium hydroxide in ethanol treatment. It is a synthetic analog of lovastatin, the first HMG-CoA reductase inhibitor received approval by the U.S. Food and Drug Administration (FDA) for therapeutic use [2]. Mevalonolactone (mevalonic acid), which has molecular formula: $C_6H_{10}O_3$ and molecular weight: 130.14 g/mol, is a dihydroxymethylvalerolactone and a precursor in the biosynthetic pathway known as mevalonate pathway, which produces terpenes and steroids that are vital for diverse cellular functions. Mevalonolactone is chiral and the (3R)-enantiomer is the only one that is biologically active.



Scheme 1: Structure of atorvastatin, simvastatin, lovastatin, mevalonic acid and mevalonolactone.

Materials and Methods

Chemicals

Atorvastatin calcium and simvastatin were purchased from Cayman Chemical (Ann Arbor, MI, USA) Mevalonic acid was obtained from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions (10 mm) of atorvastatin calcium and simvastatin were prepared in DMSO, which was purchased from ATCC (Manassas, VA, USA). Fresh dilutions in complete media were prepared of each of the compounds before each experiment. Mevalonic acid solution was prepared in media before each experiment.

Cancer Cell Lines, Culture Conditions and Treatment Protocol

The following human and mouse cancer cell lines Table 1 (with different p53 status) were obtained from American Type Culture Collection -ATCC (Manassas, VA, USA). The p53 statuses of MDA-MB-231, HCT-116, RT4, and 5637 cell lines were obtained from International Agency for Research on Cancer (IARC) TP53 database. The p53 statuses of CT26.WT, and 4T1 cell lines were obtained from Western blot result (Data not shown). 4T1, MDA-MB-231, CT26.WT, and HCT-116 cells were cultured in T-75 flasks with a started number of $1x10^6$ cells in Dulbecco's Modified Eagle Medium (DMEM), RT4 cells were cultured in McCoy's 5a Medium Modified Medium, and 5637 cells were cultured in RPMI-1640 Medium. All media were supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin. 4T1, CT26.WT, and HCT-116 cell lines become 80-90% confluent in 48 hours; MDA-MB-231, RT4, and 5637 cell lines become 80-90% confluent in 72 hours after which they were harvested by 0.25% Trypsin/2.21 mm EDTA and seeded in 96-well plates with a density of 10,000 cells/200 µL/well in triplicates. They were grown for 24 hours after which, they were treated in 1 day or every 24 hours in 2 days with different micromolar concentrations of atorvastatin calcium (100 µM, 50 µM, 20 µM, 10 µM, 5 µM), simvastatin (20 µM, 10 µM, 5 µM, 2 µM, 1 µM, 0.5 µM), mevalonic acid (1 mm, 0.5 mm, 0.2 mm, 0.1 mm), and DMSO (100 µM – 1%, 50 µM – 0.5%, 20 µM – 0.2%, 10 µM – 0.1%, 5 µM – 0.05%, the concentrations showing in DMSO chart correlated to atorvastatin calcium) in complete media. The plates were incubated at 37 ^{0}C and 5% CO_2 for 24 and 48 hours.

Cell line	Type	TP53 status	Protein
4T1	Mouse breast cancer Triple negative (ER-, PR-, HER2-)	Null P53. [35]	N/A
MDA-MB-231	Human breast cancer Triple negative (ER-, PR-, HER2-)	Mutant P53	p. R280K
CT26.WT	Mouse colon cancer	Null P53	N/A
HCT-116	Human colon cancer	Wildtype P53	N/A
RT4	Human bladder cancer	Wildtype P53	N/A
5637	Human bladder cancer	Mutant P53	p. R280T

Table 1: Properties of cancer cell lines.

Cell Viability Assay

The cell viability was determined in terms of assessing the metabolic activity of treated cells using MTS Cell Proliferation Assay (Abcam, Cambridge, MA, USA), which is a colorimetric sensitive quantification of viable cells in proliferation and cytotoxicity assay. The method is based on the reduction of MTS tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) by viable cells to generate a colored formazan product that is soluble in cell culture media as depicted in Figure 1. This conversion is thought to be carried out by NAD(P)H-dependent dehydrogenase enzymes in metabolically active cells. The quantity of formazan (presumably directly proportional to the number of viable cells) is measured by recording changes in absorbance at 490 nm using a plate reading spectrophotometer [36,37].

After treatment of the cells with the compounds every 24 hours in 2 days, MTS reagent will be added to each well in the 96-well plate and incubated for 3 hours. The absorbance of the plates will be read at 490 nm. The percentage of cell growth was

determined as the following formula:

$$\%Cell\ viability = \frac{OD_{drug/DMSO}}{OD_{positive\ control}} \times 100\%$$

Thin Layer Chromatography (TLC)

Thin Layer Chromatography (TLC) is an analytical technique used for determining the purify of materials and also for preliminary identification purposes. In TLC, there are a mobile phase and a stationary phase. The most common stationary phase is silicagel, which is coated on the TLC plate, and is an adsorbent material. The mobile phase is a solvent or solvent mixture. The mixture of compounds of interest is spotted on the silicagel plate and interact with these two phases. The stronger the compound is bound to the adsorbent, the slower it moves up the TLC plate. Non-polar compounds move up the plate more rapidly, whereas polar substances travel up slowly because polar functionality in the analyte molecules can bind to the silicagel in two ways: hydrogen bonds and dipole-dipole interactions, holding the polar compounds back. The spots are visualized by Ultraviolet (UV) lamp [38].

Mevalonic acid solution was prepared in one tube in Dichloromethane (DCM). Simvastatin was prepared in one tube in DMSO. After each preparation, mixture of mevalonic acid and simvastatin was prepared by taking half of each solution and add into one tube. Stationary phase was a silicagel plate. Mobile phase was a mixture of 50% hexane and 50% ethyl acetate. The experiment was run under room temperature. Spots were visualized by an UV lamp.

Statistical Analysis

All data are presented as the mean ± Standard Error of The Mean (SEM). The comparisons between multiple groups were performed with one-way Analysis of Variance (ANOVA) using GraphPad Prism (La Jolla, CA, USA). A p value <0.05 was considered to indicate a statiscially significant difference with * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001. If the ANOVA yielded significant results, a post hoc Dunnett's test or Turkey's test was performed. Three independent experiments were performed for each result.

Results

Cell Viability Assay

The assay was performed to determine the effect of the three compounds individually and in combination on the viability of mutant, null and wildtype p53 cell lines. The assay was also performed to determine the effect of the solvent DMSO on the cells as a vehicle. The percentage of DMSO corresponding to the concentrations of atorvastatin (ATS) as: 0.05% DMSO in 5 µM ATS, 0.1% DMSO in 10 µM ATS, 0.2% DMSO in 20 µM ATS, 0.5% DMSO in 50 µM ATS, 1% DMSO in 100 µM. The highest concentration using for simvastatin (SVS) was 20 µM, therefore the percentage of DMSO in 20 µM SVS was 0.2%, considering DMSO gave insignificantly effect on all cell lines.

According to Figure 1 and Table 2, DMSO caused significant cytotoxicity at 1% (100 µM in drug) in 4T1 and CT26.WT after both 1 and 2 days treament. In MDA-MB-231 cell line, DMSO had no significant changes after 1 day treatment, however, cell growth induced at concentrations 0.1% - 0.5% after 2 days. DMSO had no significant effect on HCT-116 after both 1 and 2 days treament. It also had no significant effect on RT4 and 5637 after 1 day, but caused significant cytotoxicity at 1% in both RT4 and 5637 after 2 days of treatment.

Atorvastatin caused no significant effect on 4T1, MDA-MB-231, CT26.WT, HCT-116, and RT4 after 1 day treatment, respectively. However, at the same condition, atorvastatin caused significant effect on 5637 cell line. After treating the cells daily for 2 days with atorvastin, significant cytotoxicity was seen on 4T1, MDA-MB-231, CT26.WT, HCT-116, and 5637 cell lines. Even though atorvastatin and simvastatin have similar structures and IC_{50} values for HMG-CoA reductase enzyme (10 nM and 9 nM, respectively), simvastatin showed much more potent effect on cancer cells compared to atorvastatin. After 1 day treatment, simvastatin caused significant cytotoxicity on all cell lines at low concentration. Specifically, 5 µM in 4T1; 2 µM in MDA-MB-231and RT4; 1 µM in CT26.WT, HCT-116, and 5637 cell lines. This was also observed after treating all cells with simvastatin daily for 2 days.

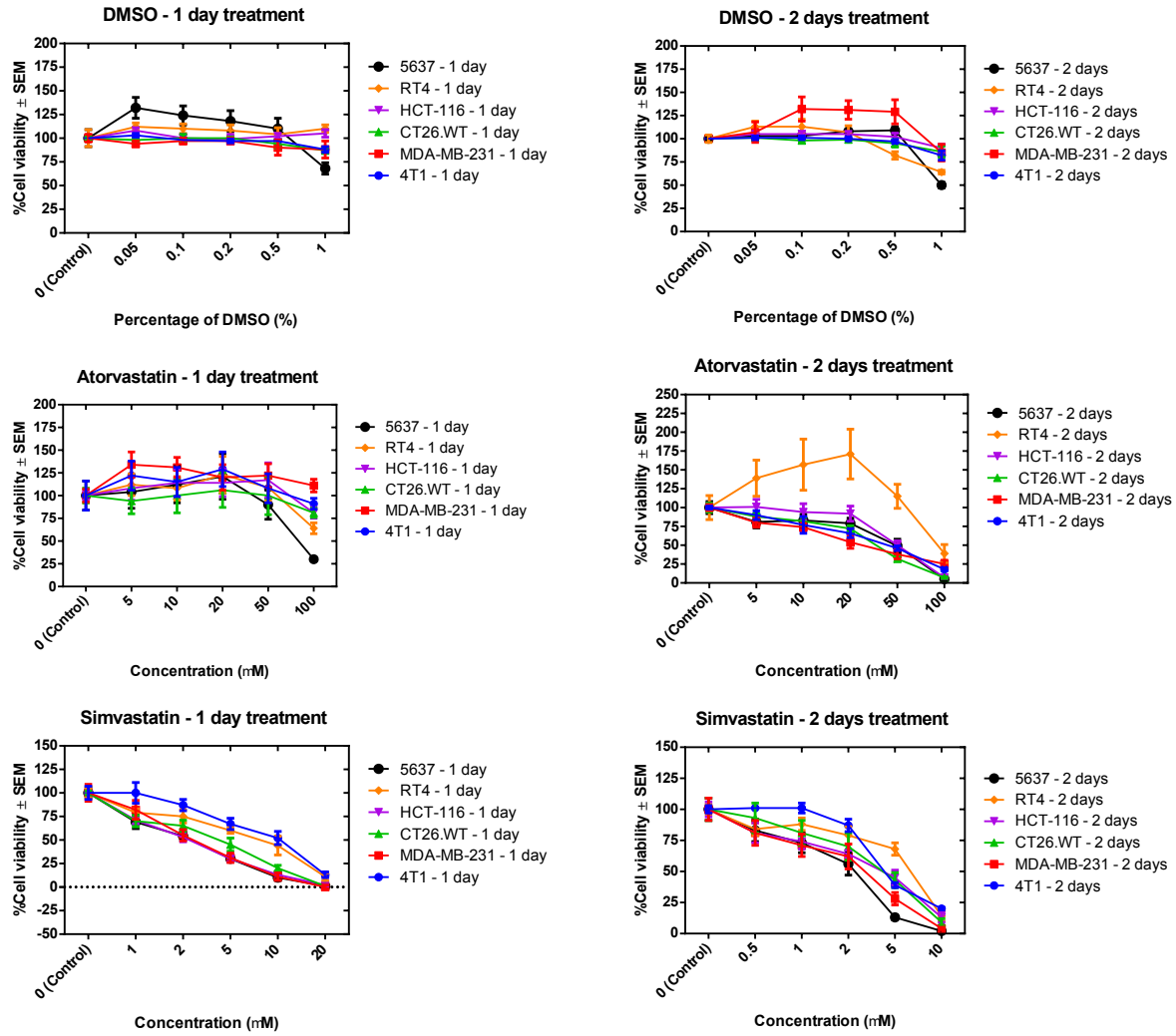


Figure 1: Effect of DMSO, atorvastatin, and simvastatin on 5637, RT4, HCT-116, CT26.WT, MDA-MB-231, and 4T1 cancer cell lines after 1 and 2-day treatment.

DMSO (%)	% Cell viability ± SEM											
	4T1		MDA-MB-231		CT26.WT		HCT-116		RT4		5637	
	1-day	2-day	1-day	2-day	1-day	2-day	1-day	2-day	1-day	2-day	1-day	2-day
0 (Control)	100±0	100±0	100±4	100±0	100±2	100±0	100±0	100±0	100±9	100±4	100±9	100±2
0.05	103±2	101±2	94±3	107±11	98±2	101±0	108±3	105±3	112±4	113±6	132±11	103±3
0.1	98±0	101±2	97±3	132±13	100±4	98±2	100±4	105±3	110±5	113±8	124±10	103±1
0.2	97±2	100±2	97±3	131±10	100±1	99±1	99±5	105±3	108±6	107±7	118±11	108±1
0.5	97±0	97±2	90±8	129±13	94±4	95±4	102±3	102±4	104±7	82±4	110±11	109±4
1	88±3	82±5	88±9	85±9	87±3	86±6	105±4	90±4	110±4	64±2	68±6	50±3

ATS (μM)	4T1		MDA-MB-231		CT26.WT		HCT-116		RT4		5637	
	1-day	2-day	1-day	2-day	1-day	2-day	1-day	2-day	1-day	2-day	1-day	2-day
0 (Control)	100±16	100±2	100±7	100±4	100±8	100±6	100±5	100±6	100±8	100±16	100±16	100±8
5	122±16	90±6	134±14	80±6	94±14	88±4	108±13	101±10	112±12	139±24	104±18	81±8
10	115±16	77±11	131±11	74±5	100±19	82±5	114±15	94±11	108±13	157±34	112±20	83±8
20	129±19	66±6	120±14	54±8	106±19	72±4	114±15	92±10	123±20	171±33	121±25	79±7
50	108±16	46±3	122±13	38±6	100±21	32±3	117±19	50±6	109±15	115±16	90±16	49±9
100	91±6	18±2	111±7	25±5	81±5	7±1	81±6	7±1	64±6	39±12	30±2	5±2
SVS (μM)	4T1		MDA-MB-231		CT26.WT		HCT-116		RT4		5637	
	1-day treatment											
0 (Control)	100±7		100±9		100±2		100±2		100±5		100±6	
1	100±11		82±10		70±6		71±4		79±4		69±7	
2	87±6		55±6		65±6		53±5		75±7		54±4	
5	67±6		31±5		45±7		30±3		60±3		30±3	
10	52±7		11±4		20±3		13±1		44±10		10±3	
20	13±3		0±2		1±0		2±1		11±4		1±0	
SVS (μM)	4T1		MDA-MB-231		CT26.WT		HCT-116		RT4		5637	
	2-day treatment											
0 (Control)	100±3		100±9		100±9		100±6		100±4		100±9	
0.5	101±0		81±10		93±12		81±8		84±5		83±9	
1	101±4		71±9		81±10		74±6		88±5		73±8	
2	87±5		62±10		70±11		64±3		79±7		56±9	
5	39±2		28±5		43±6		45±6		68±5		13±2	
10	20±1		4±1		9±3		13±4		14±4		2±0	

Table 2: Summary data of effect of DMSO, atorvastatin, and simvastatin on 5637, RT4, HCT-116, CT26.WT, MDA-MB-231, and 4T1 cancer cell lines after 1 and 2-days treatment.

In order to make sure mevalonic acid does not have any cytotoxicity effect. We treated all cell lines with the concentration of mevalonic acid range from 0.1 mm to 1 mm as depicted in Figure 2 and Table 3, mevalonic acid had no significant effect on all of the cell lines at the concentrations from 0.1 mm to 1 mm.

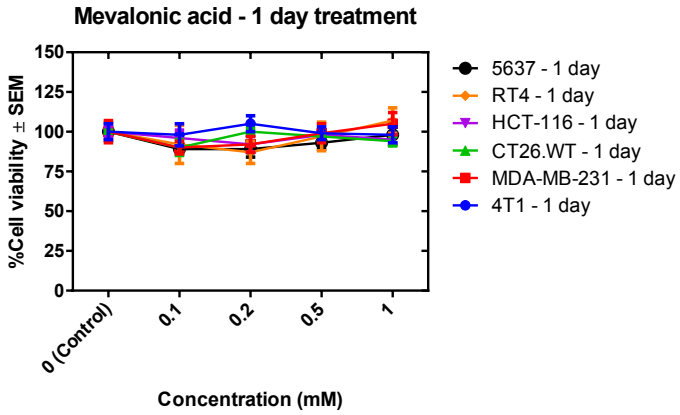


Figure 2: Effect of mevalonic acid on 5637, RT4, HCT-116, CT26.WT, MDA-MB-231, and 4T1 cancer cell lines after 1-day treatment.

MVA (mm)	4T1	MDA-MB-231	CT26.WT	HCT-116	RT4	5637
0 (Control)	100±5	100±7	100±1	100±3	100±6	100±5
0.1	98±7	90±4	90±5	96±5	92±12	89±3
0.2	105±5	92±5	100±2	92±5	87±7	89±5
0.5	99±4	99±6	97±1	98±0	97±9	93±4
1	98±5	105±7	94±3	95±3	107±8	98±5

Table 3: Summary data of effect of mevalonic acid on 5637, RT4, HCT-116, CT26.WT, MDA-MB-231, and 4T1 cancer cell lines after 1-day treatment.

As depicted in Figure 3 and Table 4, mevalonic acid had no significant effect on 4T1 cells at the concentrations of 0.5 mm and 1 mm. Simvastatin started to cause significant decrease in cell viabilities at the concentration of 5 µM. When added mevalonic acid and simvastatin together, cells are able to recover their viabilities. Significantly increased in cell viabilities was seen when adding mevalonic acid 1 mm into the wells contained cells treated with simvastatin 10 µM. Without mevalonic acid, the cell viability was only 42%. However, when adding 1 mm mevalonic acid, the cell viability went up to 80%, almost two times higher. This result demonstrated that in 4T1 cell line, there is mevalonate pathway present. Also taken into account that the higher concentration of mevalonic acid, the more cell viability was recovered in 4T1 cell line.

Mevalonic acid caused no significant effect on MDA-MB-231 cells at the concentrations of 0.5 mm and 1 mm. Simvastatin inhibited about 55% and 70% at the concentrations of 5 µM and 10 µM, respectively. Similar to what observed on 4T1 cell line, when added mevalonic acid and simvastatin together, MDA-MB-231 cells are able to recover their viabilities. With simvastatin 5 µM, cells viability was only 44%. In the cells that contained both mevalonic acid 1 mm and simvastatin 5 µM, cells viability was significantly increased to 84%, higher than the simvastatin alone twice. A significant increase in cell viabilities was also observed when adding mevalonic acid 1 mm into the wells contained cells treated with simvastatin 10 µM. Without mevalonic acid, the cell viability was only 29%. However, when adding 1 mm mevalonic acid, the cell viability went up to 66%, more than two times higher. Additionally, the result also showed that the higher concentration of mevalonic acid, the more cell viability was recovered in this cell line. Therefore, it demonstrated that in MDA-MB-231 cell line, mevalonate pathway involved.

Mevalonic acid had no significant effect on CT26.WT cells at the concentrations of 0.5 mm and 1 mm. Simvastatin started to cause significantly decreased in cell viabilities at the concentration of 5 µM, it inhibited almost 60% cell growth. In the wells which contained both simvastatin 5 µM and mevalonic acid 0.5 mm, the cell viability was significantly recovered up to 72%. Hence, statins worked through the mevalonate pathway mechanism in this cell line. Interestingly, there was no increase when the concentration of mevalonic acid increased, showing some differences with those two breast cancer cell lines, 4T1 and MDA-MB-231.

The results for HCT-116 cell line was unexpected, the results showed above were different with what the first three cell lines demonstrated. At the concentrations of 5 μM and 10 μM, mevalonic acid caused no significant effect on HCT-116 cells. Furthermore, there was no significant increase in cell viability in the wells that contained both mevalonic acid and simvastatin as what observed in the other three cell lines (4T1, MDA-MB-231, and CT26. WT). Simvastatin 5 μM showed 51% cell viability, when adding mevalonic acid at both concentrations 0.5 mm and 1 mm, the cell viabilities were recovered about 10%. Better than simvastatin 5 μM, at 10 μM and 20 μM when adding back mevalonic acid, cells were able to recover about 20% viabilities. According to the results, HCT-116 cells may not have mevalonate pathway presented or may have but not a major metabolism pathway in this cell line.

Mevalonic acid had no significant effect on RT4 cells at the two concentrations, 0.5 mm and 1 mm. Simvastatin showed about 40% inhibition at the concentration of 5 μM. When adding mevalonic acid together, the cell viability was not recovered, it was still only 56% at MVA 0.5 mm and 64% at MVA 1 mm. Increase in cell viability was seen when the concentration of mevalonic acid increased from 0.5 mm to 1 mm, however this increase was insignificant. Similarly, at the concentration of 10 μM and 20 μM, simvastatin inhibited about 60% and 85% of cell growth. In the wells which contained both mevalonic acid and simvastatin at those two concentrations, the cell viabilities were slightly recovered for about 10%. At the concentration of 1 mm, mevalonic acid also showed a slight increase in the cell viability for about 10% but not significantly. This cell line showed the result pretty much similar to what observed in the human colon HCT-116 cell line, showing that RT4 cells may not contain mevalonate pathway in their biological metabolism, that was why when adding back mevalonic acid, the cell viability was not recovered in both cell lines. Note that both HCT-116 and RT4 cell lines are wildtype p53 cell lines. Mevalonate metabolism was known to be enhanced by mutant p53 but not on wildtype p53 cells.

The results were similar to HCT-116 and RT4. There was no significant increase in this cell line when adding back mevalonic acid to the wells which contained cells and simvastatin. At the concentration of 5 μM, simvastatin showed 26% cell viability. When adding mevalonic together, the cell viabilities were 26% and 33% at the concentration of 0.5 mm and 1 mm, respectively. Surprisingly, at the concentration of 20 μM simvastatin, adding mevalonic acid was completely not be able to recover the cell viabilities at the both concentrations 0.5 mm and 1 mm. Both RT4 and 5637 are bladder cancer cells, showing that bladder cancer cells may not contain the mevalonate metabolism or statins' actions are not through this mechanism.

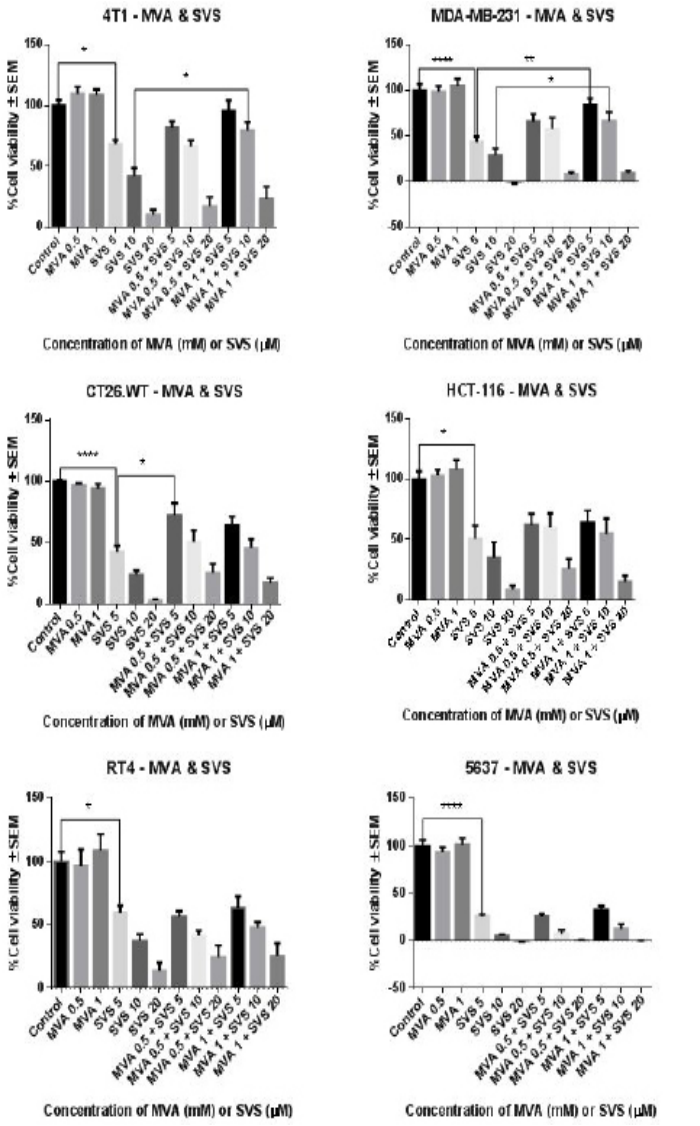


Figure 3: Effect of mevalonic acid (MVA) and simvastatin (SVS) on 4T1, MDA-MB-231, CT26.WT, HCT-116, RT4, and 5637 cell lines after 1 day treatment. The concentration of MVA is in mm. The concentration of SVS is in μM.

4T1					
Concentration of Mevalonic acid (MVA)	**%Cell viability ± SEM**	**Concentration of Simvastatin (SVS)**	**%Cell viability ±SEM**		
			No MVA	**+ MVA 0.5 mm**	**+ MVA 1 mm**
0 (Control)	100±4	SVS 5 µM	69 ± 3	82 ± 5	96 ± 9
MVA 0.5 mm	109±6	SVS 10 µM	42 ± 7	66 ± 5	80 ± 7
MVA 1 mm	108±5	SVS 20 µM	10 ± 4	17 ± 7	23 ± 10
MDA-MB-231					
Concentration of Mevalonic acid (MVA)	**%Cell viability ± SEM**	**Concentration of Simvastatin (SVS)**	**%Cell viability ±SEM**		
			No MVA	**+ MVA 0.5 mm**	**+ MVA 1 mm**
0 (Control)	100±7	SVS 5 µM	44 ± 6	66 ± 8	84 ± 8
MVA 0.5 mm	99±6	SVS 10 µM	29 ± 7	56 ± 12	66 ± 10
MVA 1 mm	105±7	SVS 20 µM	0 ± 2	8 ± 2	9 ± 3
CT26.WT					
Concentration of Mevalonic acid (MVA)	**%Cell viability ± SEM**	**Concentration of Simvastatin (SVS)**	**%Cell viability ±SEM**		
			No MVA	**+ MVA 0.5 mm**	**+ MVA 1 mm**
0 (Control)	100±1	SVS 5 µM	43 ± 5	72 ± 10	64 ± 7
MVA 0.5 mm	97±1	SVS 10 µM	24 ± 4	51 ± 9	46 ± 7
MVA 1 mm	94±4	SVS 20 µM	3 ± 1	25 ± 7	17 ± 4
HCT-116					
Concentration of Mevalonic acid (MVA)	**%Cell viability ± SEM**	**Concentration of Simvastatin (SVS)**	**%Cell viability ±SEM**		
			No MVA	**+ MVA 0.5 mm**	**+ MVA 1 mm**
0 (Control)	100±6	SVS 5 µM	51 ± 11	62 ± 9	64 ± 10
MVA 0.5 mm	103±5	SVS 10 µM	35 ± 13	60 ± 12	55 ± 13
MVA 1 mm	108±8	SVS 20 µM	8 ± 3	26 ± 8	15 ± 5
RT4					
Concentration of Mevalonic acid (MVA)	**%Cell viability ± SEM**	**Concentration of Simvastatin (SVS)**	**%Cell viability ±SEM**		
			No MVA	**+ MVA 0.5 mm**	**+ MVA 1 mm**
0 (Control)	100±8	SVS 5 µM	60 ± 6	56 ± 5	64 ± 9

MVA 0.5 mm	97±13	SVS 10 µM	37 ± 5	42 ± 4	48 ± 4
MVA 1 mm	109±12	SVS 20 µM	14 ± 6	24 ± 9	26 ± 10
5637					
Concentration of Mevalonic acid (MVA)	**%Cell viability ± SEM**	**Concentration of Simvastatin (SVS)**	**%Cell viability ±SEM**		
			No MVA	**+ MVA 0.5 mm**	**+ MVA 1 mm**
0 (Control)	100±6	SVS 5 µM	26 ± 2	26 ± 3	33 ± 4
MVA 0.5 mm	93±5	SVS 10 µM	6 ± 1	8 ± 4	13 ± 5
MVA 1 mm	101±6	SVS 20 µM	0 ± 0	1 ± 0	0 ± 0

Table 4: Summary data of effect of Mevalonic Acid (MVA) and Simvastatin (SVS) on 4T1, MDA-MB-231, CT26.WT, HCT-116, RT4, and 5637 cell lines after 1 day treatment.

Thin Layer Chromatography (TLC)

Thin layer chromatography was performed to make sure there is no interaction between simvastatin and mevalonic acid. The result showed that, there is no interaction between these two compounds (Figure 4). Mevalonic acid is a very polar compound, bound to the absorbent which is silica gel stronger, hence it traveled up the TLC plate slowly. Simvastatin is a non-polar compound, therefore it moved up the plate more rapid than mevalonic acid. Based on those differences in the structures, separation of the two compounds were able to be conducted. Spots A and B were clearly seen separated from each other.

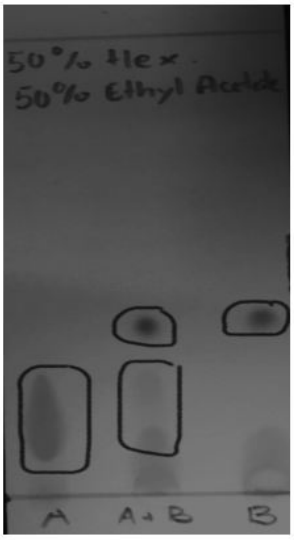


Figure 4: Thin Layer Chromatography (TLC) of mevalonic acid (A), mevalonic acid and simvastatin (A+B), and simvastatin (B). Mobile phase is 50% hexane and 50% ethyl acetate. Stationary phase is a silica gel plate.

Discussion

Simvastatin showed stronger anti-proliferative effects on cancer cell lines than atorvastatin at the same conditions. Based on the characteristics of atorvastatin calcium and simvastatin, we thought that atorvastatin would work more effectively than simvastatin since atorvastatin is more lipophilic (logP = 5.7) than simvastatin (logP = 4.68). Interestingly, according to the results, simvastatin gave much higher activity than atorvastatin since simvastatin started to show significant decrease in viability after 1 day of treatment at the lowest concentrations (1-20 µM) compared to atorvastatin (5-100 µM) causing no significant effect on the cells. After 2 days of treatment, atorvastin caused significant cytotoxicity. These results could be due to the lipophilicity of atorvastatin, it is highly lipophilic therefore it keeps staying inside the membrane of the cells, cannot get out as fast as simvastatin to enter inside the cells and start its action.

Statins showed that they worked independent of p53 status. With atorvastatin, after 2 days of incubation, the cell viabilities of 4T1, MDA-MB-231, CT26.WT, and 5637 were all reduced about 50% at the average concentration of 25 µM. 4T1 and CT26.WT are null p53 cell lines, MDA-MB-231 and CT26.WT are mutant p53 cell lines. Interestingly, the cell viabilities of HCT-116 and RT4 cell lines were decreased about 50% at the average concentration of 50 µM, both cell lines are wildtype p53. Showing that atorvastatin selectively works better on null and mutant p53 cancer cell lines than wildtype p53 cell lines. These results confirmed from previous studies that wildtype p53 suppresses the mevalonate pathway in cancer cells since it is the pathway which helps the proliferation of cancer cells. With simvastatin, it works well on all the cell lines regardless p53 statuses of the cells. After 2 days of incubation, the IC_{50} value for all of the cells is around 2 µM and there is not much different between null, mutant, or wildtype p53 cell lines.

The results after treating all cells with simvastatin and mevalonic acid for 1 day showed that the mevalonate metabolism not presented in all of the cells. It has been demonstrated that the mevalonate pathway presented in breast cancer cells but not yet mentioned about colon cancer and bladder cancer cells. Based on the results, it seems that mevalonate pathway exists in colon cancer but not in bladder cancer. CT26.WT is a null p53 colon cancer cell line, when adding back mevalonic acid, the cell viability is able to recover. However, in another colon cancer which is HCT-116 is a wiltype p53 cell line, the cell viability was not significantly recovered, showing the fact that the mevalonate pathway is not supported with wildtype p53, and this is true because wildtype p53 is supposed to stop cell proliferation as mentioned above. With the two bladder cancer cell lines, RT4 and 5637, the cell viabilities of these two cell lines were not recovered at all. Even 5637 is a mutant p53 cancer cell line, if in the cells do not contain the mevalonate pathway, adding mevalonate back to the cells have no use.

Resistance to chemotherapy and radiotherapy is a major problem in the treatment of urothelial bladder cancer. RT4 is a bladder cancer cell line which has this kind of resistance, hence it is hard to look for a drug that be able to treat this cell line. An example for that is atorvastatin. When using atorvastatin, even though at the highest concentration which is 100 μM, atorvastatin still could not suppress the cell growth. However, with simvastatin at the concentration of 2 μM, it already works on this cell line and significantly decreases the cell viability. Showing that simvastatin would be a promising treatment for this kind of resistant cancer cells. Besides, triple negative breast cancer is the most dangerous type of breast cancer and tends to recur early because it lacks of necessary receptors (estrogen, progesterone, and HER-2), making hormone therapy ineffective. Both atorvastatin and simvastatin showed excellent anti-proliferative effects for the two triple negative breast cancer cell lines (MDA-MB-231 and 4T1), therefore statins can become a promising treatment for this kind of cancer.

Conclusion

Mevalonate pathway is one of the most important metabolism pathways involved in cell proliferation. Increasing evidence stated that inhibition of the mevalonate pathway contributed to destabilization of mutant p53 cancer cells, which is appeared about 50% in human cancers. However, the question here is whether all cancer cells have mevalonate metabolism pathway? To examine this question, statins were used in this thesis as mevalonate pathway inhibitors. Interestingly, based on the results, statins demonstrated that they worked regardless p53 statuses of cancer cells. Furthermore, simvastatin is much more potent than atorvastatin based on their IC_{50} values. The average IC_{50} value of atorvastatin after 2 days of incubation was 30 μM, compared to that of simvastatin was 2 μM. Also adding supplementary mevalonic acid to confirm whether cell viability rescued when the mevalonate pathway in cells recovered. As expected, the results showed that not all of the cancer cells have the mevalonate metabolism, and not all of the mutant p53 cells go through mevalonate metabolism. Wildtype p53 cells suppressed the mevalonate metabolism in cancers therefore adding mevalonic acid back had no use for these cells (HCT-116 and RT4). Null and mutant p53 enhanced the mevalonate metabolism (CT26,WT, 4T1 and MDA-MB-231) but not all of mutant p53 cells expressed mevalonate pathway (5637). Another assumtion to be noticed that bladder cancers do not contain mevalonate metabolism (RT4 and 5637). So next step would be the determination of the right mechanism of how simvastatin works on bladder cancers.

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