Dose-dependent effects of simvastatin, atorvastatin and rosuvastatin on apoptosis and inflammation pathways on cancerous lung cells

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ABSTRACT

The aim of study was to investigate the anti-proliferative and inflammatory effects of atorvastatin, rosuvastatin, and simvastatin in lung cancer. The effects of statins were investigated in Vero, BEAS-2B, and A549 cell lines. In addition to expressions of *BAX, BCL-2, TNFα, IL-10, IL-6*, protein levels of TNFα, IL-10, IL-6 were determined. Cell viability and MDA were also measured. While the cell numbers in groups with low doses of statins were found to be approximately $1x10^6$/mL, proliferation was inhibited at higher rates containing high doses. Simvastatin, rosuvastatin, and high dose atorvastatin upregulated the *BAX*, while high dose of atorvastatin and both doses of rosuvastatin caused downregulation in *BCL-2*. All statin groups had higher MDA. Simvastatin and high dose rosuvastatin upregulated *TNFα*. While low dose simvastatin and atorvastatin and high dose atorvastatin and rosuvastatin upregulated *IL-10*, *IL-6* was upregulated with a low dose of rosuvastatin. TNFα was higher in simvastatin and rosuvastatin groups. IL-10 was highest in rosuvastatin groups. Atorvastatin groups had lower IL-6. Although cell numbers have been reduced by all statins, rosuvastatin is more effective on studied genes.

Introduction

Lung cancer is one of the most common malignant tumors and causes the death of thousands of people (9, 12). Although many new treatment methods are applied, there are still many people who lose their lives due to lung cancer, and its incidence and mortality have increased (18). For this reason, the possibilities of using more than one active substance in the treatment have been constantly investigated (4).

Statins, as HMG-CoA reductase inhibitors, are a group of drugs that decrease plasma cholesterol strongly (13). Statins lead to the production of isoprenoid, which has a vital function in the cell, and this situation affects cell development and differentiation with hypolipidemic effect. As a result of this pleiotropic effect, statins have an effect that prevents the growth of tumor cells (28).

Statins can act by increasing the sensitivity of tumor cells to traditional chemotherapy drugs. In this context, statins have been reported to have an anticancer impact on certain tumor cells (1, 6). Some studies report that statins may show different effects even in the same cell lines and do not cause cancer frequency (14). General knowledge is

that there is an important relationship between statins and cancer (2), but the molecular mechanisms by which this relationship can be controlled have not yet been fully elucidated. While the anticancer effects of statins are reported in the literature (6, 27), it is not known to what extent it affects the natural or synthetic statins on cell proliferation and inflammation in lung cancer.

In this study, the anti-proliferative and inflammatory effects of both synthetic (atorvastatin and rosuvastatin) and natural (simvastatin) statins in lung cancer cells were tested.

Materials and Methods

***Cell culture*:** Vero cell line (African Green Monkey Cells, ATCC CCL-81), human bronchial epithelial cell line (BEAS-2B, ATCC-CRL-9609) and human lung adenocarcinoma cell line (A549, ATCC CCL-185) were used. RPMI 1640 containing 10% fetal calf serum, 10 mM HEPES, 4mM glutamine, and 100 IU mL penicillin/ streptomycin was used as a cell culture medium. Incubation was carried out in an incubator at 37 °C, with 5% CO_2 and 95% air.

***Proliferation Assay*:** Primarily, non-toxic concentrations of statins in healthy cell lines (Vero and BEAS-2B cell lines) were determined. Then, activity studies on BEAS-2B and A549 cell lines were determined by MTT method as described in the literature (19). For this purpose, the effects of different concentrations of Simvastatin, Atorvastatin and Rosuvastatin on the cell proliferation of BEAS-2B and A549 cells were investigated by MTT cell proliferation method. After adding statins to the cells, they were incubated for 96 hours at 37°C in an incubator with 5% carbon dioxide. After the incubation, the culture medium was removed and 10 µl of MTT was added to each well and the plates were incubated under the same conditions for 4 hours to allow the formation of formazan crystals. The crystals formed were dissolved in DMSO added to each well and the optical density was measured spectrophotometrically at a wavelength of 570 nm. Proliferation was expressed as the ratio of cells in statin-treated wells to control cells. In addition, the following experiments were also performed to quantitatively evaluate the effects of statins on cell viability.

Cell culture studies were performed in 96-well, flat-bottomed sterile microplates. To investigate the activity of statins on BEAS-2B and A549, cell density was adjusted to $1x10^5$/ml cells. For cell adhesion, the plates were incubated for 6 hours under the same conditions as stated. Following the adhesion of the cells to the surface of the plate wells, different concentrations of statins were added to the culture medium. At the end of the 96 hours incubation, the cells in the culture vessel were collected in 0.25% trypsinization solution and transferred to tubes. Cells were centrifuged at +4 °C at 1250 rpm for 10 min. Cell number and viability were determined by hemocytometer.

Two different non-toxic concentrations (simvastatin: 40 and 80 µM, atorvastatin: 65 and 130 µM, rosuvastatin: 40 and 80 µM) of each statin were studied in the experiments. DMSO (Sigma, MI, USA) was chosen as the solvent in order to homogeneously dissolve the statins in the medium. The effects of DMSO on cell growth were evaluated daily with an inverted microscope, both morphologically and cell viability. In order to determine the non-toxic concentration of DMSO, different concentrations of DMSO (8, 4, 2, 1, 0.5%) were treated with cells for 96 hours. Cultures without DMSO (negative control) were left to incubation simultaneously as a control. Samples were collected and the number of viable cells was determined. The 1% concentration of DMSO was chosen as the solvent concentration, which did not show a significant difference in cell viability between the control and negative control groups. All experiments were performed in duplicate in 3 replicates.

***The groups were as follows*:** Control (Con), Atorvastatin Low (Ato-L, 65 µM), Atorvastatin High (Ato-H, 130 µM), Simvastatin Low (Sim-L, 40 µM), Simvastatin High (Sim-H, 80 µM), Rosuvastatin Low (Ros-L, 40 µM), Rosuvastatin High (Ros-H, 80 µM).

Cells were harvested after 48 hours. Half of the samples were homogenized with 1 mL TRIzol (Sigma-Aldrich, USA) and stored at –86 °C until RNA isolation. Other parts of samples were stored at –86 °C within PBS for ELISA.

***Total RNA isolation, cDNA synthesis, and qPCR application*:** After thawing samples at room temperature, total RNA isolation was performed (26). Following the chloroform-isopropyl alcohol and ethyl alcohol steps, the pellets were dried for about 10 min and dissolved with 30-100 µL nuclease free water (NFW). Concentrations, purities, and qualities were checked with a nucleic acid spectrophotometer (Merinton, SMA 1000) and gel electrophoresis (100 V and 30 min). After DNA digestion (DNase I, Thermo Scientific, USA), cDNA was synthesized (High-Capacity cDNA Reverse Transcription kit, Applied Biosystems, USA). Thermal cycler (BioRad T100, USA) protocol was as follows: Following the 10 min at 25 °C, samples were kept at 37 °C for 120 min. Then, the temperature was arranged the 85 °C for 5 min. After the reaction, samples were completed to 150 µL with NFW and stored at – 20 °C. SYBR Green Dye containing kit (Power SYBR® Green PCR Master, ThermoFisher Scientific, USA) was used to analyze the expressions of *TNFα*, *IL-10*, *IL-6*, *BAX*, and *BCL-2* (Table 1). Samples were studied as duplicated and *GAPDH* was the housekeeping gene (8). The protocol in qPCR (Bio-Rad CFX-96) was as follows: Following the 10 min at 95 °C, 95 °C for 10 sec, 60 °C for 60 sec, and 40 cycles.

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Table 1. Forward and reverse sequences of primers studied genes.

Gene	Primers	Product size (bp)	Reference
GAPDH	**F:** 5'-TGCACCACCAACTGCTTAGC-3' **R:** 5'-GGCATGGACTGTGGTCATGAG-3'	87	(7)
TNFα	**F:** 5'-AGAACTCACTGGGGCCTACA-3' **R:** 5'-GCTCCGTGTCTCAAGGAAGT-3'	177	*
IL-6	**F:** 5'-GGTACATCCTCGACGGCATCT-3' **R:** 5'-GTGCCTCTTTGCTGCTTTCAC-3'	81	(11)
IL-10	**F:** 5'-GGAGGTGATGCCCCAAGCTGA-3' **R:** 5'-AATCGATGACAGCGCCGTAGC-3'	111	(28)
BAX	**F:** 5'-TGGCAGCTGACATGTTTTCTGAC-3' **R:** 5'-TCACCCAACCACCCTGGTCTT-3'	195	(10)
BCL-2	**F:** 5'-CATGTGTGTGGAGAGCGTCAA-3' **R:** 5'-GCCGGTTCAGGTACTCAGTCA-3'	83	(20)

*: Designed by the current study.

***MDA analyzes and ELISA application*:** The levels of MDA were determined according to the Esterbauer ve Cheeseman method (5). TNFα, IL-10, IL-6 levels were determined with ELISA kits according to the manufacturer instructions (Bioassay Technology Laboratory, CHINA) via ELISA Reader (Thermo Multiskan GO) at 450 nm. Also, the total protein contents of samples were determined (16).

***Statistical analyses*:** Two-way analysis of variance was performed in order to identify the effects of cell line, and statin on dependent variables (protein levels of TNFα, IL-10, IL-6 and MDA, and cell counts):

$$Y_{ij} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + e_{ij}$$

Where, Y_{ij}, dependent variable (TNFα, IL-10, IL-6, MDA or cell counts); μ, overall mean; α_i, effect of cell line (i = cancer and healthy); β_j, effect of statin (j = control, Ato-L, Ato-H, Sim-L, Sim-H, Ros-L, Ros-H); $(\alpha\beta)_{ij}$, two-way interaction term of cell line and statin; and e_{ij}, residual error. In case any interaction term was found statistically significant, simple effect analysis with Bonferroni correction was performed to find out the differences among the statin groups in each cell line and among the cell lines in each statin group. The normality of the data was checked using Shapiro Wilk Test in each level of the statin and cell line variables. In addition, the residuals of each model were controlled in terms of the normality assumption. Levene's Test of Equality of Error Variances was used to assess the assumption of homogeneity. For expression analysis $2^{-\Delta\Delta Ct}$ method was used (15). Groups which used different statins with different dose were compared with control in each cell line. All statistical analyses were conducted using IBM SPSS Statistics for Windows, Version 23.0. All descriptive statistics were given as mean ± SE and $P<0.05$ was considered as significant.

Results

The determination of DMSO concentrations that did not affect cell viability and growth were performed in three different cell lines. The 1% concentration of DMSO selected as a solvent was not toxic to cells in both the healthy cell lines (Vero and BEAS-2B cell lines) and the cancer cell line (A-549 cell line), (Figures 1-3).

The effects of statins on cell viability in Vero cells at different concentrations were given. At a concentration of 140 µM, atorvastatin was beginning to be toxic to cells. Therefore, a lower dose (130 µM) than 140 µM concentration was studied in the expression experiments. Concentrations of 100 µM of both Simvastatin and Rosuvastatin induced toxicity on cells. However, these two statins were not toxic at concentrations of 80 µM (Figure 4).

Concentrations of statins that did not affect cell viability were similar in BEAS cells as in Vero cells. Concentrations<140 µM for Atovastatin and 80 µM for Simvastatin and Rosuvastatin were non-toxic (Figure 5).

The cell numbers in statin groups were found to be lower than the control in BEAS-2B and A549 ($P<0.01$; $P<0.05$, respectively). Moreover, low-dose simvastatin and rosuvastatin had a similar effect on A549 as well as BEAS-2B. However, cell proliferation was inhibited at higher rates at most of the groups containing high doses of statins on A549 ($P<0.01$). In terms of MDA, the highest was Ato-H in BEAS-2B, while it was Sim-L in A549. While the high dose of rosuvastatin had similar MDA levels in both cell lines, it was determined that there were differences in other statin-treated groups ($P<0.001$) (Table 2).

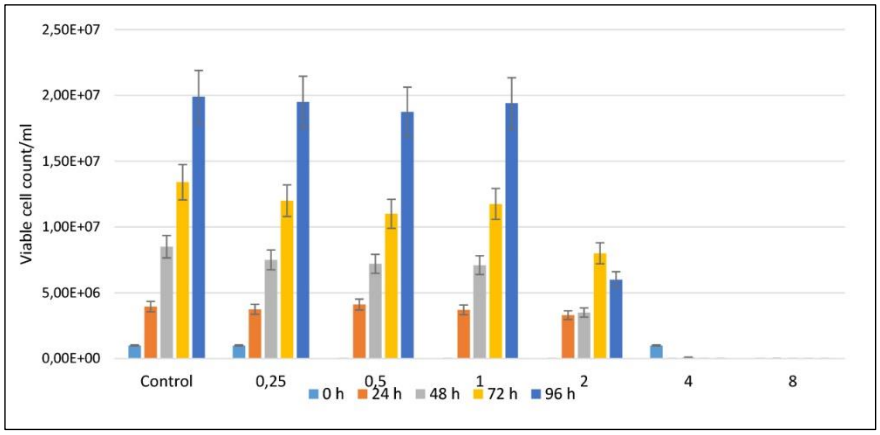

Figure 1. Determination of non-toxic concentrations of DMSO on Vero cells.

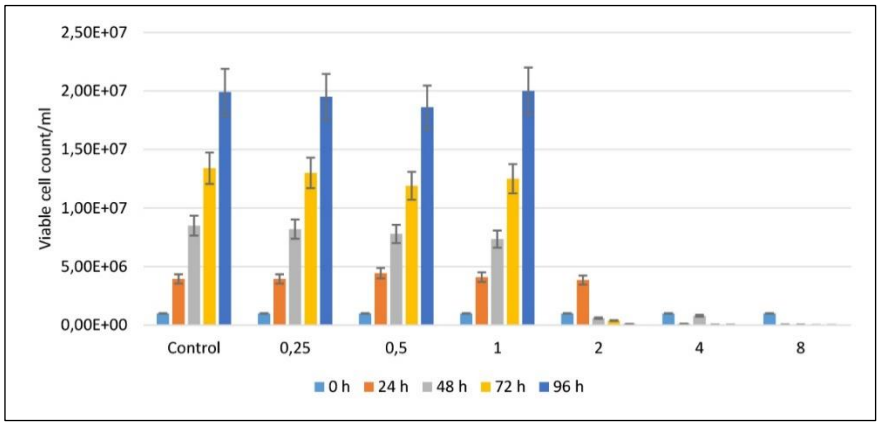

Figure 2. Determination of non-toxic concentrations of DMSO on BEAS-2B cells.

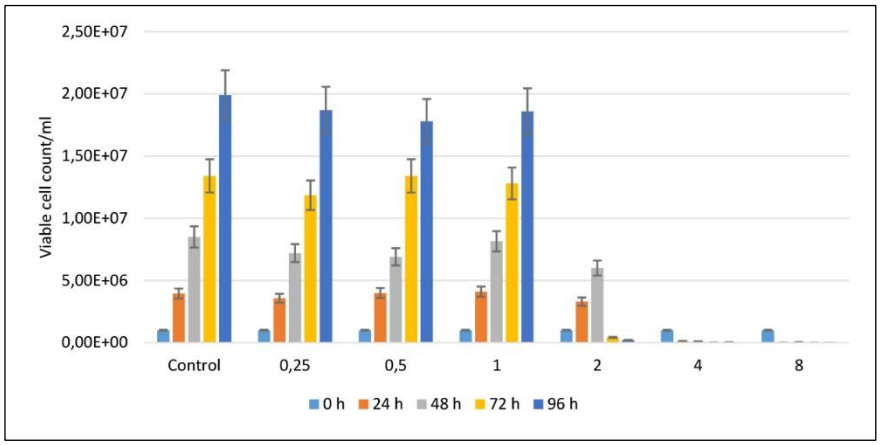

Figure 3. Determination of non-toxic concentrations of DMSO on A549 cells.

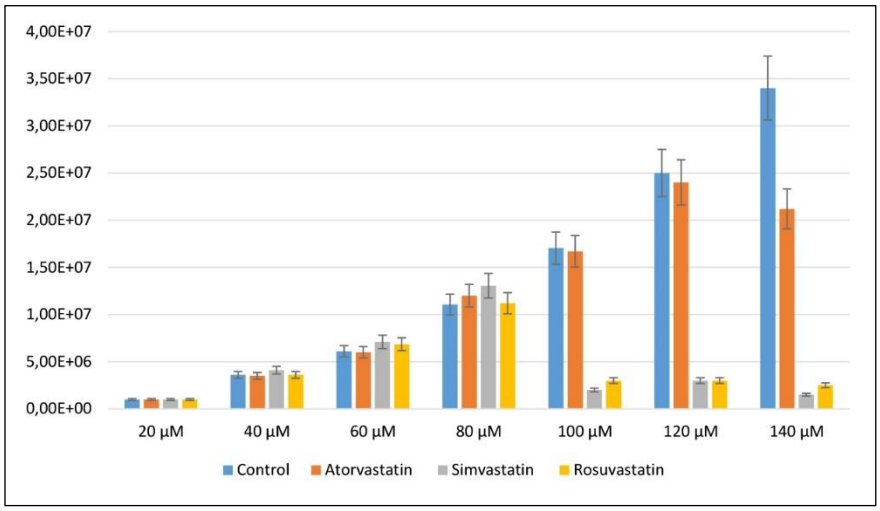

Figure 4. Effects of the three Statins on cell viability in Vero cells compared to the control group.

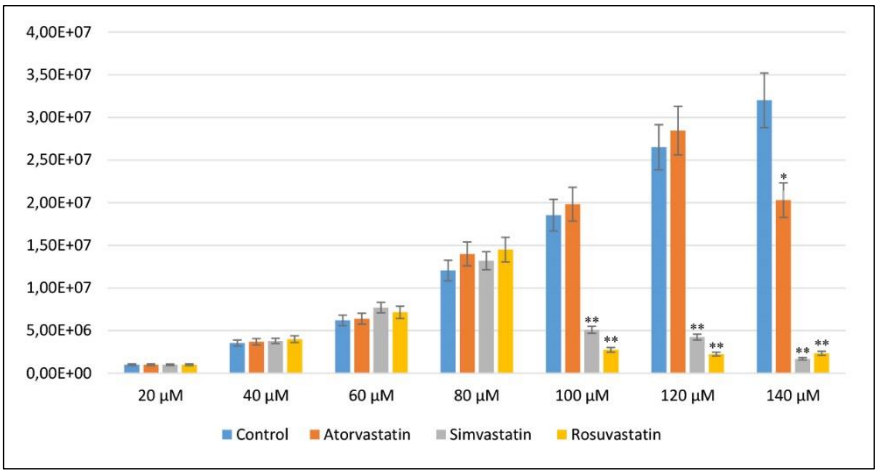

Figure 5. Effects of the three Statins on cell viability in BEAS-2B cells compared to the control group.
*: P<0.05, **: P<0.01.

Table 2. Cell counts and MDA levels in BEAS-2B and A549 cell lines.

Cell Line	Group	Cell Counts (x10^3/mL)	MDA (nmol/mg protein)
BEAS-2B	**Con**	1394.00±93.56^{a}	80.08±0.74^{e,B}
	Sim-L	476.67±40.55^{bc}	277.90±3.67^{b,B}
	Sim-H	318.33±22.42^{c}	272.60±3.16^{b,A}
	Ato-L	639.33±34.80^{b}	232.48±1.14^{c,A}
	Ato-H	500.00±34.64^{bc}	463.40±1.99^{a,B}
	Ros-L	743.33±53.64^{b}	114.88±0.22^{d,B}
	Ros-H	410.00±35.12^{bc}	219.63±2.67^{c}
A549	**Con**	1316.33±139.56^{a}	96.13±1.43^{e,A}
	Sim-L	870.00±64.29^{b}	289.56±5.02^{a,A}
	Sim-H	336.67±63.60^{c}	92.68±4.47^{e,B}
	Ato-L	510.00±94.52^{c}	150.14±0.75^{d,B}
	Ato-H	400.00±80.83^{c}	162.29±6.59^{d,B}
	Ros-L	943.33±74.46^{b}	248.73±7.24^{b,A}
	Ros-H	380.00±87.18^{c}	216.11±2.19^{c}
P	**Cell Line**	0.423	<0.001
	Statin	<0.001	<0.001
	Cell Line*Statin	0.007	<0.001

Con: Control; **Sim-L**: Low dose of simvastatin; **Sim-H**: High dose of simvastatin; **Ato-L**: Low dose of atorvastatin: **Ato-H**: High dose of atorvastatin; **Ros-L**: Low dose of rosuvastatin; **Ros-H**: High dose of rosuvastatin.
a,b: Different lower-case superscript letters indicate significant difference among Statin groups. A,B: Different upper-case superscript letters indicate significant difference among Cell line groups.

High doses of simvastatin and atorvastatin caused upregulation of the *BAX*, while high dose of rosuvastatin caused downregulation in BEAS-2B (P<0.05). *BAX* upregulated in all statin groups in A549. *BCL-2* was downregulated in BEAS-2B with low doses of all statins. However, *BCL-2* downregulated in Ato-H, Ros-L, and Ros-H groups in A549 (P<0.05). In addition, *TNFα* upregulated in all groups except for Ato-L and Ros-H in BEAS-2B (P < 0.05). While this gene was downregulated in Ato-L, it was upregulated in most of the groups in A549. *IL-10* was similar in all groups with control except for Ato-H in BEAS-2B. In A549, it was upregulated in Ato-L, Ato-H, Sim-L, Ros-H (P<0.05). In BEAS-2B, IL-6 was only upregulated in Sim-L (P<0.001). However, it was lowest in Ros-L (P<0.05). But, *IL-6* in Ros-L was upregulated almost 2 folds in A549 (P<0.05) (Figure 6).

TNFα protein levels increased approximately twice as much at both doses of simvastatin while it decreased in Ato-H in BEAS-2B (P<0.05). Also, TNFα increased in Ato-L and Ros-L. In A549, all groups treated with simvastatin and rosuvastatin had higher TNFα protein levels. However, Ato-L had lower TNFα levels (P<0.01). IL-10 levels were increased in the groups using statins except for Ato-H in BEAS-2B (P<0.05). However, it had the highest levels in Ros-L and Ros-H in A549 (P<0.001). IL-6 was found the highest in Sim-L in BEAS-2B (P<0.05).

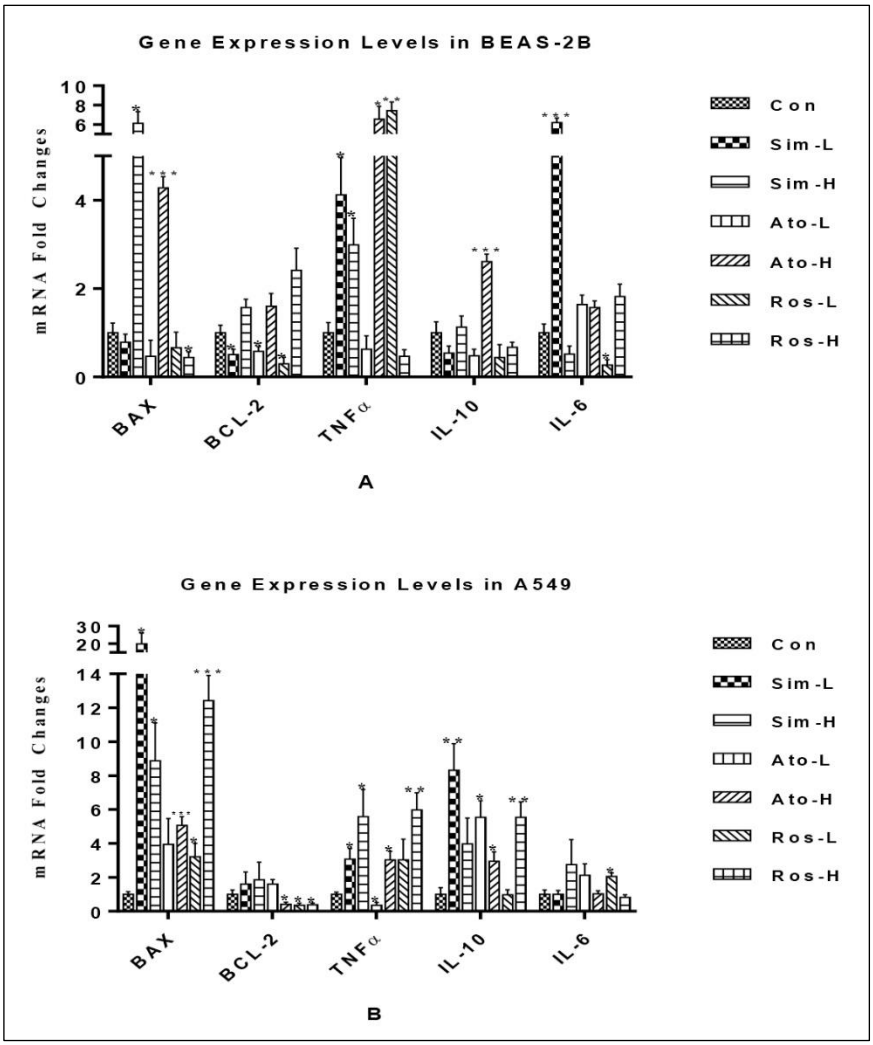

Figure 6. Gene expression levels in BEAS-2B and A549 cell lines.
A: Gene expression levels in BEAS-2B cell line; **B:** Gene expression levels in A-549cell line; *: P<0.05, **: P<0.01, ***: P<0.001; **Con**: Control; **Sim-L**: Low dose of simvastatin; **Sim-H**: High dose of simvastatin; **Ato-L**: Low dose of atorvastatin: **Ato-H**: High dose of atorvastatin; **Ros-L**: Low dose of rosuvastatin; **Ros-H**: High dose of rosuvastatin.

Table 3. Protein levels (ng/mg protein) in BEAS-2B and A549 cell lines.

Cell Line	Group	TNFα	IL-10	IL-6
BEAS-2B	Con	15.34±0,51^{c,A}	8.99±0.35^{d,A}	7.40±0.04^{de,A}
	Sim-L	27.40±1,02^{a,A}	31.25±0.47^{a,A}	16.70±0.25^{a,A}
	Sim-H	26.59±0,26^{a,A}	23.11±0.60^{b,A}	13.05±0.25^{b,A}
	Ato-L	21.44±0,90^{b,A}	23.25±0.37^{b,A}	7.04±0.20^{e,A}
	Ato-H	11.63±0,48^{d,B}	8.17±0.54^{d}	4.32±0.15^{f,A}
	Ros-L	20.54±0,59^{b,B}	13.71±0.50^{c,B}	9.39±0.16^{c}
	Ros-H	16.36±0,59^{c,B}	11.97±0.10^{c,B}	8.41±0.10^{cd,A}
A549	Con	12.39±0,75^{c,B}	5.36±0.30^{d,B}	5.74±0.33^{c,B}
	Sim-L	22.05±0,59^{b,B}	12.23±0.52^{b,B}	5.89±0.09^{c,B}
	Sim-H	20.22±1,25^{b,B}	16.83±1.03^{a,B}	7.66±0.47^{b,B}
	Ato-L	6.21±0,15^{d,B}	5.31±0.21^{d,B}	1.68±0.14^{e,B}
	Ato-H	14.81±0,77^{c,A}	8.39±0.67^{c}	2.76±0.21^{d,B}
	Ros-L	25.62±0,36^{a,A}	18.12±0.39^{a,A}	9.84±0.13^{a}
	Ros-H	27.41±0,43^{a,A}	18.73±0.44^{a,A}	6.75±0.24b^{c,B}
P	Cell Line	<0.001	<0.001	<0.001
	Statin	<0.001	<0.001	<0.001
	Cell Line*Statin	<0.001	<0.001	<0.001

Con: Control; **Sim-L**: Low dose of simvastatin; **Sim-H**: High dose of simvastatin; **Ato-L**: Low dose of atorvastatin: **Ato-H**: High dose of atorvastatin; **Ros-L**: Low dose of rosuvastatin; **Ros-H**: High dose of rosuvastatin.
a,b: Different lower-case superscript letters indicate significant difference among Statin groups. A,B: Different upper-case superscript letters indicate significant difference among Cell line groups.

However, it was significantly lower in Ato-H. In A549, both doses of atorvastatin had lower IL-6 ($P<0.05$). On the other hand, both doses of simvastatin and a high dose of rosuvastatin had the same effect on IL-6 in A549. The highest IL-6 was in Ros-L ($P<0.001$) (Table 3).

Discussion and Conclusion

Recent studies have focused the effectiveness of statins in the treatment of diseases develop due to inflammation and cancer (24). Studies on the effects of statins on the organism are mostly conducted on cell lines as well as on human and experimental animals. There are some studies controversial results on the effects of statins (4, 7, 30, 31). However, in cell line studies, mostly healthy cell lines are tested (31). However, it has been reported in a study that statins do not affect the growth of normal human embryonic stem cells, but inhibit the growth of cancer cells (6). In another study, the statin family of drugs have been reported as triggers of tumor-specific apoptosis (30). This suggests that effects of statins on cells proliferative activity change depending on cell type and distinctive conditions such as cancer (30, 31).

Oxidative damage is mostly determined by the detection of MDA level and is considered a significant marker of inflammation in the cell (29). Atorvastatin and simvastatin were stated to increase MDA levels in tissues in mice and rats (20, 22). In this study, it was determined that both doses of rosuvastatin had a similar effect on MDA, which is indicator of oxidative stress and inflammation in cell, in BEAS-2B and A549, however, the effects of simvastatin and atorvastatin were varied in different cell lines (20).

The apoptotic activities of statins were reported to be dose and type dependent (31). In a study in which 5 µM of simvastatin was applied to breast cancer cells, it was reported that *BCL-2* decreased, while *BAX* was unchanged, although apoptosis was induced (10, 21). This suggests that apoptosis might be regulated independently of the activity *BAX* with statin treatment. It was reported that simvastatin administration caused inconsistent activation of *BCL-2* and *BAX* (7). It was stated that apoptosis and *BAX* increased in MCF7 cells, while *BCL-2* decreased with 20 µM simvastatin treatment. On the other hand, no effect as stated above was observed in healthy cells (27). It was reported in a study that low and high doses of atorvastatin and simvastatin in ViBo cells showed similar effects on cell proliferation, but high doses in CaSki cell line showed anti-proliferative effect (2). It was understood that the effects of statins on cell proliferation and apoptosis are dose-dependent as well as tissue and cancer type.

It was reported a positive relationship between TNFα levels and the rate of apoptosis (3). In the related study, TNFα increased apoptosis by suppressing cell proliferation with its activities at both mRNA and protein levels. In our study, the suppression in cell numbers might have been caused by TNFα-induced apoptosis as reported (3). Metastatic melanoma cells were reported to be sensitive in apoptosis induced by simvastatin, and IL-6 was reported to act as a growth inhibitor in the early melanoma stage (17). In this study, it was determined that simvastatin increased IL-6 gene and protein expression in healthy cell lines, while high dose simvastatin increased IL-6 protein levels in both cell lines. However, IL-6 in the cancer cell line was found significantly higher in Sim-H, Ros-L, and Ros-H. Both doses of rosuvastatin were more effective than other statins in terms of IL-6 activity. Rosuvastatin groups were the highest groups in terms of TNFα, IL-10, and IL-6 levels in the cancer cell line. Possible reasons for the difference between protein and gene expression levels might be due to some post-transcriptional factors such as miRNAs (25). The activity of cytokines changes in macrophages and monocytes with statin activity and the cholesterol biosynthesis pathway regulates the IL-10 activity (23). Statins regulate IL-10 as well as TNFα and IL-6 in cancer cell lines. In a study, it has been reported that IL-10 levels increased with higher dose of atorvastatin (23).

In conclusion, although all statins have been shown to reduce cell numbers in cancerous cell lines, it may be said both doses of rosuvastatin are more effective on genes in both apoptosis and inflammation pathways. The findings of this study give important insights about drugs and target receptors to be used with and without statins in cancer treatment. More studies are needed regarding the dose-dependent activity of rosuvastatin.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

NDi and AY planned the experiments and took the lead in writing the manuscript. HÖ and BÇ practiced the gene expression analyses. NDu, EA and FÇA carried out cell culture steps. PA made statistic analyses. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

This study does not present any ethical concerns.

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