Immunohistochemical and histomorphological analysis of rat mammary tumors after simvastatin treatment

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The results of experimental studies have indicated the pleiotropic effects of statins in organism, e.g. the influence on cell cycle, apoptosis or angiogenesis. In this study, the effects of simvastatin on selected parameters of apoptosis and proliferation in chemocarcinogen-induced mammary tumorigenesis in female rats were determined. Simvastatin was administered dietary at a dose of 18 mg/kg and highly effective dose of 180 mg/kg the entire experiment (18 weeks). At autopsy mammary tumors were removed and prepared for immunohistochemical and histomorphological analysis. In treated animals (simvastatin 180 mg/kg), significant decrease by 12% in Bcl-2 protein expression and non-significant decrease by 27% of Ki67 protein expression in tumor cells compared to tumor cells in control animals were observed after semiquantitative evaluation. Morphometrical analysis has shown significant proapototic shift in Bcl-2/Bax ratio in tumor cells. In high grade control carcinoma cells, the expression of Ki67 increased by 37% (non-significantly) in comparison with control low grade carcinomas. A histomorphological analysis of malignant tumors has revealed a shift from high grade to low grade carcinomas after simvastatin treatment. The noticeable decrease of mammary tumor frequency and incidence in rats after simvastatin treatment was accompanied with antiapoptotic Blc-2 protein decrease and proapoptotic Bax protein increase in this experiment.

Key words: rat, mammary tumors, simvastatin, Bcl-2, Bax, Ki67

Statins are 3-hydroxy-3-methylglutaryl CoA reductase (HMG-CoA) inhibitors with important role as hypolipidemic agents [1]. Within the last ten years, the results of experimental studies have indicated the pleiotropic effects of statins in organism which are mediated by their direct or indirect influence on cell cycle, apoptosis or angiogenesis. These physiological processes play a key role in neoplastic transformation, for this reason statins are seriously discussed by clinical and experimental oncologists.

Statins reduce not only serum cholesterol levels but also mevalonate synthesis. Mevalonate is a precursor of several major products regulating the cell cycle, including dolichol, geranylpyrophosphate (GPP) and farnesylpyrophosphate (FPP) [2]. Another product – geranylgeranylpyrophosphate (GGPP) can be synthesised from FPP. Both FPP and GGPP are substrates essential for the activation of variety protooncogenes, e.g. the inhibition of mevalonate pathway by statins can consequently block the Ras or Rho activation [3]. Statins have been shown to stabilize the cell cycle kinase inhibitors p21 and p27, and to arrest breast cancer cell lines in the G1 phase of the cell cycle [2].

Proposed mechanisms for statin-mediated apoptosis include an upregulation of pro-apoptotic protein expression (e.g., Bax, Bim), combined with decreased anti-apoptotic protein expression (e.g., Bcl-2) [4], or activation of caspase-3, caspase-

8, and caspase-9 [5]. In the study of Agarwal *et al.* [6] it has been shown that addition of geranylgeranylated pyrophosphate prevented lovastatin induced apoptosis in colon cancer cells, whereas cotreatment with farnesyl pyrophosphate had no effect. This study also showed that lovastatin treatment resulted in decreased expression of the antiapoptotic protein Bcl-2 and increased expression of proapoptotic protein Bax in cancer cells. In esophageal adenocarcinoma cells, statin treatment increased mRNA and protein expression of proapoptotic proteins Bax and Bad, but protein levels of antiapoptotic proteins Bcl-2 and Bcl-X-L were unchanged [7]. In another study, the anticarcinogenic potential of fluvastatin was evaluated in three hepatocellular carcinoma cell lines (HepG2, SMMC-7721 and MHCC-97H). After fluvastatin treatment, expression of Bcl-2 and procaspase-9 were downregulated, cytochrome c (cytosolic extract), Bax and cleaved-caspase-3 protein expression increased [8]. In this context, pro-apoptotic shift of ratio in Bax/Bcl-2 mRNA expression in rat mammary tumor cells caused by atorvastatin in our previous experiment was confirmed [9].

The above mentioned observations have led us to hypothesize that statins might inhibit also proliferation and induce apoptosis in rat mammary tumor cells in our preventive-curative model. Ki67 is an excellent marker to determine the growth fraction of a given cell population [10]. The fraction of Ki67 – positive tumor cells (the Ki67 labeling index) is often correlated with the clinical course of cancer. In this study, the immunohistochemical analysis of Ki67 and Bcl-2 and Bax proteins as the apoptotic parameters after simvastatin treatment will be determined. Another aim of this study – a histomorphological analysis of mammary tumors – may have implications for assessment of simvastatin on the differentiation and prognosis of the tumors after long-term treatment and or the efficacy of drug.

Materials and methods

Female rats of Sprague-Dawley strain obtained from AnLab (Prague, Czech Republic) aged 31–35 days were used in the experiment. The animals were adapted to standard vivarium conditions with temperature 23 ± 2 °C, relative humidity 40-60 %, artificial regimen light : dark (12 h : 12 h) (lights on from 6 a.m., light intensity 150 lux per cage). During the experiment the animals were fed by the Ssniff diet (Soest, Germany) and drank tap water ad libitum. Mammary carcinogenesis was induced by N-methyl-N-nitrosourea (NMU) (Sigma, Deisenhofen, Germany) administered intraperitoneally in one dose of 50 mg/kg body weight on average the 41th postnatal day. Car-

cinogen was freshly prepared and dissolved in isotonic saline solution. Simvastatin synthesized by Zentiva (Prague, Czech Republic) was administered in the diet at two concentrations: 18 mg/kg (0.0018 %) and 180 mg/kg (0.018 %). The lower average dose of simvastatin in this experiment was equivalent to the maximal daily clinical dose of Zocor administered to patients with hypercholesterolemia. Based on our previous experience with statins` use in rat mammary carcinogenesis (statins in rats demonstrate different pharmacokinetics and pharmacodynamics than in humans), it was necessary to use high doses of simvastatin (180 mg/kg) to prove its antineoplastic effect in this experiment. Chemoprevention with simvastatin began 1 week before carcinogen administration and lasted until the end of the experiment – 17 weeks after NMU administration. Animals were randomly assigned to one of three experimental groups: 1. control group without chemoprevention; 2. chemoprevention with simvastatin at a concentration of 18 mg/kg in the chow (SIMVA 18); 3. chemoprevention with simvastatin at a concentration of 180 mg/kg in the chow (SIMVA 180). Each group consisted of 20 animals. The animals were weekly weighted and since 6th week post NMU palpated in order to register the presence, number, location and size of each palpable tumor.

In the last - 18th week of the experiment, the animals were quickly decapitated, mammary tumors were excised and tumor size was recorded. Macroscopic changes in selected organs (liver, kidney, stomach, intestine and lung) were evaluated at autopsy. The parameters of mammary carcinogenesis (tumor incidence, frequency, latency and volume) were evaluated in each experimental group. Tissue samples of each mammary tumor were routinely formalin-fixed and paraffin-embedded. The tumors were classified according to the criteria for the classification of rat mammary tumors [11]. The additional parameter – grade of invasive carcinomas was used. Tumor samples were divided into low-grade (LG) and high-grade (HG) carcinomas. The criteria for categorization – solidization, cell atypia, mitotic activity index, and necrosis, were chosen according to the standard diagnostic method of classification. As HG carcinomas were considered tumors with ≥2 positive criteria, LG carcinomas were tumors with ≤ 1 positive criterium. The solidization was considered if >30% of tumor sample displays solid growth, high mitotic activity index if ≥10 mitosis was observed in 10 high power fields and necrosis if the occurrence of comedo (not infarct) was determined.

The paraffin block having the most representative tumor area of each mammary tumor was chosen for immunohistochemical analysis of Ki67, Bcl-2 and Bax protein expression. A Table 1 summarizes the clone, source concentration, and

Table 1. Summary of used antibodies

Antibody against to	Clone	Source	Concentration	Time of incubation
Ki67	mouse monoclonal, MIB-5, M7248	DAKO, Glostrup, DK	1 : 50	30 min
Bcl-2	rabbit polyclonal, N-19, SC492	Santa Cruz Biotechnology, CA, USA	1 : 450	30 min
Bax	rabbit polyclonal, P-19, SC526	Santa Cruz Biotechnology, CA, USA	1 : 450	30 min

Table 2. Histopathological classification of mammary tumors and grade of malignat lessions.

Animal (number)	Type	Grade	SOLID	ATYP	MAI	NECR
CONTROL GROUP, 36 lesions, 12 tumor bearing animals						
3	C	HG	-	+	+	+/-
3	TA					
4	P-C	HG	+	-	-	+
4	P	LG	-	-	-	-
4	C	LG	-	-	-	-
5	P-C	HG	+	+	+	-
5	P-C	LG	-	-	-	-
7	PAP					
8	P	LG	-	-	-	-
8	C	LG	-	-	-	-
8	P	LG	-	-	-	-
8	P-C	HG	-/+	+	+	-
9	P-C	HG	-/+	+	+	-
10	C-P-CO	HG	-	+	-	+
11	C-P	LG	-	-	-	-
14	C	HG	+	+	+	-
14	C	LG	-	-	-	-
14	C	LG	-	-	-	-
14	C-P	LG	-	-	-	-
14	P-C	HG	-	+	+	-
16	C-P	LG	-	-	-	-
16	C-CO	LG	-	-	-	+
16	C-P	LG	-	-	-	-
16	C-CO	HG	+	+	+	+
16	C-P	LG	-	-	-	-
16	C-P	LG	-	-	-	-
16	PAP					
16	C-P	HG	+	+	+	+
16	C-P	HG	-	+	-	+
18	P-C	LG	-	-	-	-
20	C-P	HG	-	+	+	-/+
20	C-P	HG	+	+	-	+
20	C-P-CO	HG	+	+	+	+
20	P-C	HG	+	+	+	-
20	C-CO	HG	+	+	+	+
20	C-P	LG	-	-	-	-
SIMVA 18 GROUP, 35 lesions, 15 tumor bearing animals						
22	C-P	LG	-	-	-	-
22	C-P	HG	+	+	-	-
22	C-P	LG	-	-	-	-
23	PAP					
24	P-C	LG	+	-	-	-
24	C-P	LG	-	-	-	+
24	P-C-CO	HG	+	+	+	+

Animal (number)	Type	Grade	SOLID	ATYP	MAI	NECR
24	C-P	HG	+	+	+	+
25	C-P	LG	+	-	-	-
25	PAP					
27	C-P	HG	+	+	-	-
27	C-P	LG	-	-	-	-
27	C-P	HG	+	+	+	+
28	C-P	LG	-	-	-	-
28	C-P	HG	+	+	+	+
28	C-P	LG	-	-	-	-
29	P-C	HG	-	+	+	-
30	P-C	LG	-	-	-	-
30	C-P	LG	-	-	-	-
30	C-P	LG	-	-	-	-
31	C	LG	-	-	-	-
31	C	HG	+	+	+	+
31	P-C	LG	-	-	-	-
33	C-CO	HG	+	+	+	+
34	C-P-CO	HG	+	+	+	+
36	P	LG	-	-	-	-
36	C	LG	-	-	-	-
37	C	LG	-	-	-	-
37	DCIS					
38	P	LG	-	-	-	-
38	P	LG	-	-	-	-
38	C-CO	HG	-	+	+	+
39	C	LG	-	+	-	-
39	PAP-LN					
39	C-P	LG	-	+	-	-
SIMVA 180 GROUP – 7 lesions, 5 tumor bearing animals						
50	PAP					
51	C	HG	+	+	+	+
51	PAP					
55	C	LG	-	-	-	-
57	PAP					
57	P-C	LG	-	-	-	-
58	PAP					

Type: invasive carcinoma (C- cribriform, P – papillary, CO – comedo), DCIS – ductal carcinoma in situ,
PAP – papilloma, TA – tubular adenoma, LN – lymph node. Dominant type in mixed tumors is the first in order.
Staging was evaluated only in invasive carcinomas, LG (low-grade) - well differentiated carcinomas,
HG (high-grade) – poorly differentiated carcinomas; SOLID – solidization; ATYP – cell atypia;
MAI – mitotic activity index, NECR – necrosis.
Explanatory note: SOLID – if >30% of tumor sample displays solid growth;
MAI – if ≥10 mitosis is observed in 10 high power fields; NECR – occurrence of comedo (not infarct) necrosis.
HG – tumor with ≥2 positive criteria, LG – tumor with ≤ 1 positive criteria.

time of incubation of antibodies used in experiment. Immunohistochemical staining was evaluated by a semiquantitative method based on percentage representation of positively responded elements. Positivity of staining was assessed as percentage of positive cells. The only criterion of positivity in semiquantitative method was the difference against negative stromal component. In the Bcl-2/Bax ratio evaluation, a morphometrical analysis as a method for precise analysis of protein expression in tumor cells was used. In morphometry, the expression of markers (positivity) was evaluated from the set limit. Moreover in this method not only carcinoma cells were evaluated but also stromal elements ("all the cells of tumor tissue"). Possible differences in results between semiquantitative and morphometrical analysis arise from different threshold positivity setting and from different spectrum of evaluated elements. Morphometric analysis of the digital images was done by software QuickPhoto Micro version 2.3 (Promicra, Prague, Czech Republic). Phase analysis function was used for the quantitative determination of Bcl-2 and Bax immunohistochemically positive cells. The area of labeled cells was percentually evaluated with standard thresholds for weak, mild and strong intensity /for three degrees of intensity/ of immunoreactivity in standard fields. Protein expression of Bcl-2 and Bax was analysed in the cytoplasm of tumor cells separately. During interphase, Ki67 can be exclusively detected within the nucleus, whereas in mitosis most of the protein is relocated to the surface of the chromosomes. The Ki67 protein is present during all active phases of the cell cycle, but is absent in resting cells, therefore it is an excellent marker for determining the growth fraction of cell population. Photographs were taken by an Olympus BX41, digital camera model No. SP 350. Mann-Whitney –U test, Kruskal-Wallis and one-way analysis of variance were statistical methods used in data evaluation in this experiment.

Results

This experiment is a continuation to our previous study where the significant tumor-suppressive effects of simvastatin (SIMVA 180) in the chemoprevention of rat mammary carcinogenesis were observed [12]. Simvastatin administered at a lower dose (SIMVA 18) did not change parameters of mammary carcinogenesis; therefore immunohistochemical

analysis was realized only in rat mammary tumor cells from the control and the SIMVA 180 group.

A histopathological classification of mammary tumors has revealed a shift in the rate of poorly differentiated (high grade, HG) and well differentiated (low grade, LG) invasive tumors to higher representation LG invasive tumors after treatment with simvastatin [control group: 16/17 (HG/LG); SIMVA 18: 11/20; SIMVA 180: 1/2]. The treatment with simvastatin in higher dose apparently changed the rate from malignant to benign lesions (control group – 33/3, SIMVA 180 – 3/4) (Table 2).

The expression of Ki67, Bcl-2 and Bax proteins as percentage of positive cells in treated and control tumor cells and in control low grade and high grade invasive tumors were evaluated by semiquantitative method (Table 3). The decrease by 27% (12.93±3.01 vs 17.70±1.80) of Ki67 protein expression in treated tumor cells compared to tumor cells in control animals was observed. The median Ki67 percentage positivity in the control tumor cells was 20.0 (range 1 – 40) and in the treated tumor cells was 15.0 (0.5 – 20). Using non-parametric Mann-Whitney test, the difference was non-significant (P=0.273). Figure 1a shows immunohistochemical detection of Ki67 in control and treated tumor cells. Only slight correlation was found between Ki67 and Bcl-2 expression decrease in tumor cells in treated rats (r=0.454; P=0.084). In high grade control tumor cells, the expression of Ki67 protein increased by 37% (P=0.127) in comparison with low grade control group (Figure 2, Table 3).

In treated animals (SIMVA 180), significant decrease by 12% (82.82±6.06 vs 94.41±1.47) (P=0.015) in Bcl-2 protein expression in tumor cells compared to tumor cells in control animals was observed. The median Bcl-2 percentage positivity in tumor cells in treated animals was 90.0 (50 – 100) and in control animals was 100.0 (70 – 100). Compared to control cells, Bax protein expression was decreased in treated tumor cells by 13% (67.14±11.69 vs 77.06±3.57) but the difference was not significant compared to control tumors (P=0.584). The median Bax percentage positivity in treated tumor cells was 70.0 (30 – 100) respectively 80.0 (20 – 100) in control tumor cells. Compared to semiquantitative method, the morphometrical analysis has shown more evident proapoptotic shift in the evaluation of Bcl-2/Bax protein expression (Table 4). Figure 1 b,c shows immunohistochemical detection of Bcl-2 and Bax proteins in control and treated tumor cells. Protein

Table 3. Semiquantitative analysis of Ki67, Bcl-2 and Bax protein expression in the rat mammary tumor cells after simvastatin treatment and the expression of Ki67, Bcl-2 and Bax in the control carcinoma cells of low or high grade, respectively.

Antibody against to	Control group	Simvastatin group	Control low grade cells	Control high grade cells
Ki67	17.70±1.80	12.93±3.01 (- 27 %)	15.17±2.40	20.73±2.58 [+ 37 %]
Bcl-2	94.41±1.47	82.82±6.06 (- 12 %) [a]	92.63±2.00	96.67±2.11 [+ 4 %]
Bax	77.06±3.57	67.14±11.69 (- 13 %)	73.16±5.25	82.00±4.49 [+ 12 %]

Data are expressed as means±SEM. Values in brackets are calculated as %-ual deviation from the 100% of non-influenced control group (round brackets) or low grade cells (square brackets).
Significantly different, [a] P<0.05 vs CONT.

a) Ki67 detection has shown higher proliferation activity in the cells of control group compared to simvastatin group (magnification 400x).
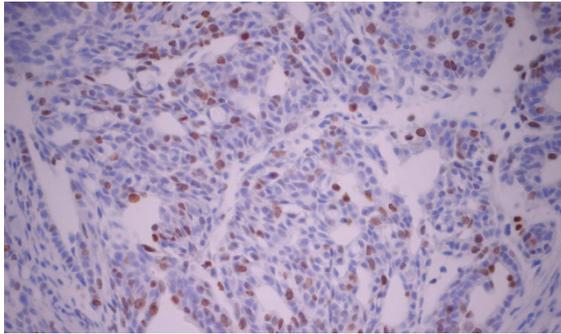
control group
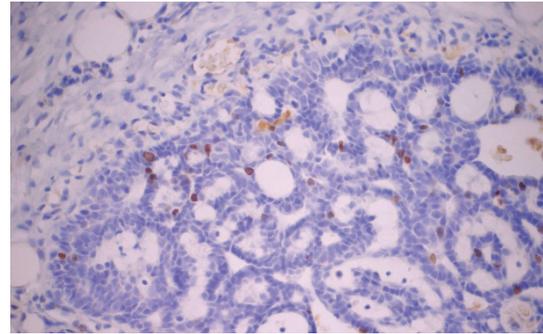
simvastatin group

b) Bcl-2 detection has shown significantly higher expression in tumor cells of control group (400x).
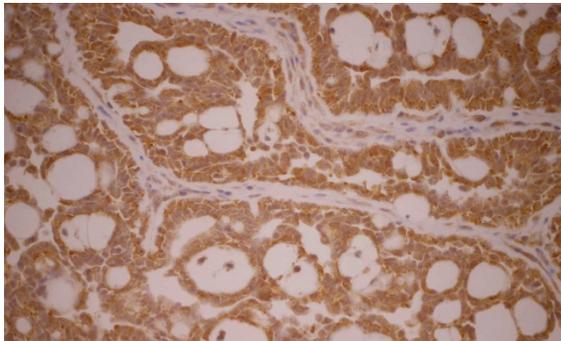
control group
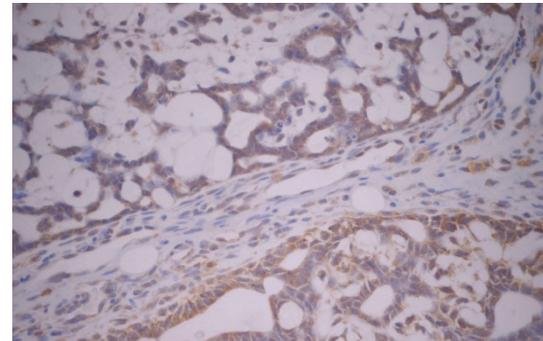
simvastatin group

c) Bax protein expression in tumor cells from control group and tumor cells treated by simvastatin (400x).
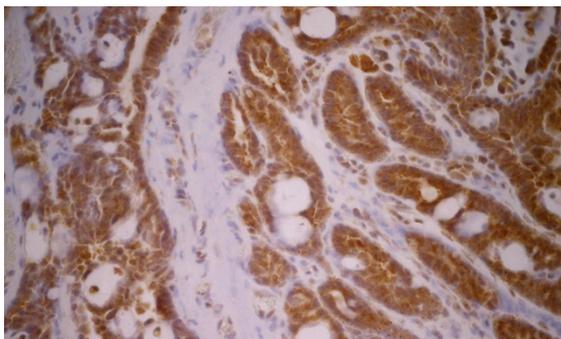
control group
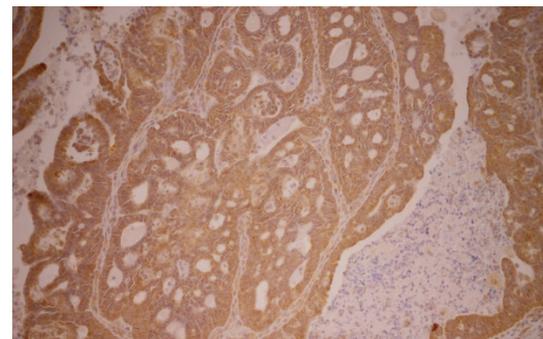
simvastatin group

Figure 1. The immunohistochemical detection of Ki67, Bcl-2 and Bax protein expression in mammary tumor cells after treatment with simvastatin (180 mg/kg).

expression of Bcl-2 and Bax did not significantly differ between control LG and control HG invasive tumor cell groups. In addition to the above mentioned statistical analysis, in the tumor cells from treated group (compared to control tumor cells), more frequent occurrence of some histomorphological signs of apoptosis were observed – shrinking of the cells, fragmentation of the nucleus, dense cytoplasm, karyorhexis, apoptotic bodies.

Discussion

This experiment is the first report about simvastatin used in mammary carcinogenesis in female rats. A significant chemopreventive effect of simvastatin administered in the concentration of 180 mg/kg of the diet was recorded in all parameters of mammary carcinogenesis. In addition, simvastatin in this experimental group was also effective in the
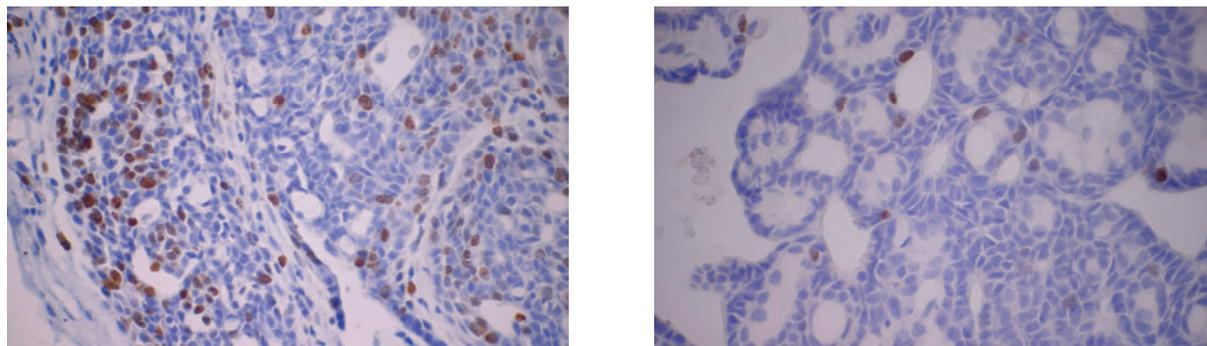
Figure 2. The nuclear positivity of Ki67 protein in high grade (on the left) and low grade (on the right) control carcinoma cells (magnification 600x).

treatment of tumors – the drug distinctly reduced the number of malignant lesions in favour of benign tumors. Moreover, a histopathological analysis of mammary tumors has revealed a shift from poorly to well differentiated invasive tumors after simvastatin administration (180 mg/kg). On the other hand, the lower concentration of simvastatin – 18 mg/kg in our experiment (equivalent to daily clinical doses) was ineffective in animals. Similarly, significant antitumor effects of atorvastatin (100 mg/kg of diet) administered in the chemoprevention of NMU-induced rat mammary carcinogenesis in our previous study were observed [9]. In our last experiment, hydrophilic rosuvastatin (250 mg/kg of diet) has shown lower chemopreventive activity than lipophilic statins in this model of experimental breast cancer [13]. Narisawa *et al.* [14] in 1,2-dimethylhydrazine-induced colon carcinogenesis in ICR mice, used as a chemopreventive agent dietary administered simvastatin at concentrations of 0.01% (100 mg/kg) and 0.002% (20 mg/kg) and pravastatin administered in drinking water at concentrations of 0.01%, 0.001% and 0.005%. Both agents significantly reduced tumor frequency. Anticarcinogenic effects of statins were proved also in other *in vivo* experiments. Pravastatin administered in drinking water has been shown to reduce the incidence and volume of N-nitrosomorpholine-induced hepatic neoplastic nodules in Sprague-Dawley rats [15] and to reduce N-methyl-N-nitrosourea induced F344 rat colon carcinogenesis [16].

Recent in vitro studies have proven direct or indirect effects of statins on regulation mechanisms of the cell e.g. proliferation or apoptosis. Antiproliferative effects of statins include the inhibition of dolichol, geranylpyrophosphate and farnesylpyrophosphate, the stabilization of the cell cycle kinase inhibitors p21 and p27 and inhibition of Ras- and Rho-mediated cell proliferation [2,3]. Emerging data indicate that statins demonstrate antiproliferative effects in several types of proliferating tumor cells. In vitro studies on various cell lines have shown that statins stimulated inhibitory potential, either by induction of G1-arrest or G2/M-arrest [17,18]. Zhang *et al.* [8] have observed that fluvastatin inhibited proliferation of hepatocellular carcinoma cell lines (HepG2, SMMC-7721 and

Table 4. Morphometrical analysis of Bcl-2 and Bax protein expression in mammary tumor cells after treatment with simvastatin.

Group	Percentage of all labelled cells	Percentage of cells with weak and mild/strong immunoreactivity	
		+	++/+++
Bcl-2 protein			
Control group	7.39±0.66	6.13±0.61	1.26±0.55
Simvastatin group	3.42±1.29*	3.23±1.27*	0.19±0.05
Bax protein			
Control group	1.85±0.29	1.33±0.22	0.51±0.22
Simvastatin group	3.08±0.84	2.25±0.56	0.82±0.34

Data are expressed as means±SEM. Intensity of immunoreactivity: weak (+), mild (++), strong (+++).
Significantly different, * P<0.05 vs CONT.

MHCC-97H) by inducing apoptosis and G2/M phase arrest in a dose-dependent manner. In another experiment, acquired data demonstrated that lovastatin inhibits the proliferation of MART-1-specific human cytolytic T lymphocytes without affecting cytolytic capacity [19]. In the study of Bessler *et al.* [20] malignant cell lines – HuCC, EHEB, K562, and Raji were incubated with three lipophilic statins (atorvastatin, lovastatin, and simvastatin) and one hydrophilic statin (pravastatin). All used statins inhibited HuCC cell proliferation. EHEB and K562 cell proliferation was inhibited by the lipophilic statins, but not by pravastatin. Raji cell proliferation was not affected by any of the used statins. The results of this experiment pointed out that the antiproliferative effect of statins on tumor cells depends on their dosage, physiochemical property, and the type of the malignant cells. Additionally, cerivastatin induced G1-arrest in breast cancer cells, but signs of apoptosis were not observed [3]. These results suggest that statin-induced effects on proliferation and apoptosis are independent. Ki67 is a cancer antigen that is found in growing, dividing cells but is absent in the resting phase of cell growth. This characteristic makes Ki67 a good tumor marker, e.g. meta-analysis of De Azambuja et al. [21] suggested that Ki67/MIB-1 positivity confers a higher risk of relapse and a worse survival in patients

with early breast cancer. The effect of statin on Ki67 expression in tumor cells was evaluated in a recent experimental study [22]. The nude mouse xenograft assay showed that combination of epigallocatechin-3-gallate and lovastatin suppressed esophageal cancer cell growth and reduced the expression of Ki67. In our study, the expression of Ki67 protein in mammary tumor cells was also evaluated. The decrease of Ki67 protein expression by 27% observed in simvastatin treated cells compared to control cells points out to milder antiproliferative effects of the drug by above mentioned mechanisms of action. Additional immunohistochemical analysis of Ki67 confirmed higher protein expression in control high grade invasive tumor cells (by 37%) compared to control low grade group – this result indicate that Ki67 as a reliable marker of proliferation may have a reasonable prognostic and predictive value in invasive breast cancer cells.

It is known that statins differs in their proapoptotic effects in variety of cancers. Lipophilic statins have been shown to induce apoptosis in various cell types, including vascular smooth muscle cells [23] cardiac myocytes [24], hepatocytes [25] and glioma cells [26], whereas hydrophilic statins (rosuvastatin and pravastatin) have not [27]. In the recent study of Kato et al. [28], the apoptotic potential of two lipophilic statins – lovastatin and simvastatin and one hydrophilic statin – pravastatin was assessed in ovarian, endometrial and cervical cancer cell lines. Lovastatin and simvastatin, but not pravastatin induced cell death through activation of extrinsic and intrinsic apoptotic cascades (caspase-8 and -9; BID cleavage, cytochrome C release and PARP cleavage) in cancerous cells, which expressed high levels of HMG-CoA reductase. In our study, after highly effective treatment of rat mammary tumors by simvastatin, we have evaluated the possible role of Bcl-2 and Bax proteins in the drug`s mechanism of action. Using semiquantitative and morphometrical methods, simvastatin has indicated proapoptotic shift in Bax/Bcl-2 ratio. The drug significantly decreased antiapoptotic Bcl-2 protein expression in treated tumor cells compared to tumor cells in control animals. Non-significant, but evident increase of proapoptotic Bax protein expression in the group of treated tumor cells was observed after morphometrical analysis (despite the fact that semiquantitative analysis has shown non-significant decrease of Bax expression in treated cells). The results of this study indicate an important role of Bcl-2 apoptosis-regulatory protein and probably not relevant role for Bax protein in the pathogenesis of rat mammary tumors treated by simvastatin. Some experimental evidence suggests that in breast cancer, development, progression and an increased proliferation rate can be linked to apoptosis-related factors. Similarly than in our study, Sirvent et al. [29] studied 186 cases of infiltrating ductal breast carcinoma and concluded that Bax expression did not provide a new prognostic role, although it contrasted to the Bcl-2 action. Another study summarized that a high Bcl-2 expression had prognostic value in breast cancer patients treated with neoadjuvant anthracycline based chemotherapy [30].

The results of this study clearly pointed to simvastatin`s antineoplastic effects in experimental rat mammary carcinogenesis. Our study is the first report about Ki67, Bcl-2 and Bax protein immunohistochemical analysis in mammary tumor cells in vivo after treatment with statin. Our results pointed out to proapoptotic and antiproliferative effects of simvastatin in rat mammary carcinogenesis. Histomorphological analysis of tumors has shown apparent shift from malignant to benign lesions and shift from high grade to low grade carcinomas after simvastatin administration; in terms of clinical use this is an interesting result because long-term applied simvastatin may improve the disease prognosis and thus lifespan in oncological patients. Anticipated proapoptotic and antiproliferative effects or antiangiogenetic and antimetastatic mechanism of action of statins along with the favourable results from clinical oncological research have provided a rationale for future use of statins as potential anticancer reagents in the growth control of breast cancer.

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