# Antineoplastic Effects of Simvastatin in Experimental Breast Cancer

## Antineoplastické účinky simvastatínu u experimentálnej rakoviny prsníka

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### Summary

***Backgrounds:*** Statins (3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors) have proven therapeutic and preventive effects on cardiovascular diseases. Preclinical evidence demonstrates tumor-suppressive effects of statins in several human neoplasias, including breast cancer. ***Materials and Methods:*** In this study, antineoplastic effects of simvastatin in chemoprevention of N-methyl-N-nitrosourea-induced mammary carcinogenesis in female rats were evaluated. The drug was dietary administered at two concentrations – 18 mg/kg (SIMVA 18) and 180 mg/kg (SIMVA 180). ***Results:*** Basic parameters of experimental carcinogenesis after long-term simvastatin treatment in animals were assessed. In the SIMVA 180 group, simvastatin significantly suppressed tumour frequency by 80.5% and tumour incidence by 58.5% in comparison to the controls. Higher dose simvastatin non-significantly decreased the mean tumor volume by 23.5%, as well as non-significantly lengthened the latency period by 14.5 days compared to the control animals. Simvastatin, administered at a lower dose did not change parameters of mammary carcinogenesis in comparison to the control group. Simvastatin in both treated groups significantly decreased serum levels of triacylglycerols and VLDL-cholesterol in comparison to the control animals. Compared to the controls, a significant increase in food intake by the animals was recorded in the SIMVA 18 and SIMVA 180 groups. No significant differences in the final body weight gain between the simvastatin-administered and the control group were found. ***Conclusion:*** This study represents the first report of simvastatin use in experimental mammary carcinogenesis *in vivo*.

### Key words

mammary carcinogenesis – rat – chemoprevention – simvastatin

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## Súhrn

***Východiská:*** Statíny (inhibítory 3-hydroxy-3-metylglutaryl koenzým A reduktázy) predstavujú látky s dobre dokumentovanými terapeutickými a preventívnymi účinkami u kardiovaskulárnych ochorení. V predklinických štúdiách statíny preukázali tumor-supresívne účinky u viacerých typov neoplázií vrátane rakoviny prsníka. ***Materiál a metódy:*** V tejto štúdii sme hodnotili antineoplastický účinok simvastatínu v chemoprevencii N-metyl-N-nitrozoureou – indukovanej mamárnej karcinogenézy u samíc potkanov. Farmakum bolo aplikované v potrave vo dvoch koncentráciách – 18 mg/ kg (SIMVA 18) a 180 mg/ kg (SIMVA 180). ***Výsledky:*** Po dlhodobej aplikácii simvastatínu sme na konci pokusu vyhodnotili základné parametre experimentálnej karcinogenézy. Simvastatín v skupine SIMVA 180 signifikantne znížil frekvenciu nádorov o 80,5 % a incidenciu nádorov o 58,5 % v porovnaní s kontrolou. Simvastatín v tej istej skupine zvierat nesignifikantne znížil priemerný objem nádorov o 23,5 % a nesignifikantne predĺžil latenciu o 14,5 dňa v porovnaní s kontrolnými zvieratami. U simvastatínu podávaného v nižšej dávke sme nepozorovali antineoplastické účinky. Simvastatín v oboch liečených skupinách signifikantne znížil sérové hladiny triacylglycerolov a VLDL-cholesterolu v porovnaní s kontrolnými zvieratami. V porovnaní s kontrolami sme v skupinách SIMVA 18 a SIMVA 180 pozorovali signifikantný nárast príjmu potravy u zvierat. Signifikantné zmeny prírastku telesnej hmotnosti medzi skupinami so simvastatínom a kontrolnou skupinou neboli zistené. ***Záver:*** Táto štúdia je prvou zmienkou o simvastatíne použitom v experimentálnej mamárnej karcinogenéze *in vivo.*



## Kľúčové slová

mamárna karcinogenéza – potkan – chemoprevencia – simvastatín


## Introduction

Except for non-melanoma skin cancers, breast cancer is the neoplasia with highest incidence in females all over the world. Chemoprevention is assumed to become an effective way to combat the above neoplasia. The aim of the chemopreventive trials is to find an efficient substance that can be administered for a long period with minimum adverse effects. The statins are highly effective drugs in lowering cholesterol by inhibiting 3-hydroxy-3-methylglutaryl coenzyme A reductase. The statins have been shown to decrease the incidence of adverse cardiovascular events, including death, myocardial infarction, stroke, atrial fibrillation and renal dysfunction. However, increasing evidence suggests that statins exert pleiotropic effects in organism, independent of cholesterol reduction.

Recent pre-clinical *in vitro* studies have proven direct or indirect effects of statins on regulation mechanisms of the cell e. g. proliferation, differentiation and apoptosis. These physiological processes play a key role in neoplastic transformation; therefore statins is being seriously discussed in oncology. Statins, through mevalonate, inhibit dolichol-, farnesyl- and geranylgeranyl pyrophosphate production and block tumor cell proliferation [1,2]. Lovastatin has been demonstrated to stabilize the cell cycle kinase inhibitors p21 and p27 and to arrest breast cancer cell lines in G1 phase of the cell cycle [1]. Cerivastatin has been shown to inhibit Ras- and Rho-mediated cell growth [2]. Proposed mechanisms for statin-mediated apoptosis include an upregulation of proapoptotic protein expression (e. g., Bax, Bim), combined with decreased antiapoptotic protein expression (e. g., Bcl-2) [3], or activation of caspase-3, caspase-8, and caspase-9 [4]. Angiogenesis play an important role in the growth of primary tumors and metastasis. High-dose of cerivastatin decreased tumor vascularisation by 51% in a murine Lewis lung cancer model [5]. Statins have been shown to decrease vascular endothelial growth factor production and to inhibit capillary tube formation [6]. Several lines of evidence suggest that statins impair the metastatic potential of tumor cells. Statins have been demonstrated to reduce endothelial leukocyte adhesion molecule E-selectin [7] and matrix metalloproteinase-9 expression [8]. Fluvastatin and lovastatin reduced liver tumorigenesis and liver metastases in pancreatic cancer cells [9]; atorvastatin decreased melanoma cell metastases [10].

Also data from experimental studies *in vivo* indicated antineoplastic effects of statins in rodent colon [11] and hepatal [12] carcinogenesis. Actual results of our group demonstrated an apparent antineoplastic effect of dietary administered atorvastatin in the chemoprevention of rat mammary carcinogenesis [Kubatka et al., unpublished results]. Epidemiologic studies [13–17] and several human clinical trials have reported beneficial effects of statins in certain neoplasias [18–20].

Antitumor properties of statins in human breast cancer have not been tested so far. Original experimental studies are necessary, which should answer the question about expected tumor suppressive effects of statins in mammary carcinogenesis. The aim of this study is to evaluate the chemopreventive potential of simvastatin in rat mammary carcinogenesis. The adverse effects of the drug after long-term treatment will be assessed.

## 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 60–70%, artificial regimen light : dark (12 h : 12 h) (lights on from 6 a. m., light intensity 150 lux per cage). During the experiment animals drank tap water *ad libitum*. The chow containing simvastatin synthesized by Zentiva (Prague, Czech Republic) was prepared at SSNIFF Spezialdiäten GmbH (Soest, Germany). Simvastatin was administered in the chow at two concentrations – 18 mg/ kg (0.0018%) and 180 mg/ kg (0.018%). Mammary carcinogenesis was induced by N-methyl-N-nitrosourea (Sigma, Deisenhofen, Germany) administered intraperitoneally in one dose of 50 mg/ kg body weight on average the 41$^{th}$ post-

**Tab. 1. Effects of simvastatin in NMU-induced mammary carcinogenesis in female Sprague-Dawley rats at the end of experiment.**

| Group | CONT | SIMVA 18 | SIMVA 180 |
|---|---|---|---|
| tumor incidence (%) | 63.16 | 78.95 (+ 25%) | 26.32a,b (–58.5%) |
| tumor frequency* | 1.89 ± 0.57 | 1.84 ± 0.30 (–2.5%) | 0.37 ± 0.16a,b (–80.5%) |
| tumor latency* (days) | 97.42 ± 5.94 | 95.20 ± 3.73 (–2 days) | 112.00 ± 5.42c (+14.5 days) |
| tumor volume* ($cm^3$) | 0.93 ± 0.28 | 0.74 ± 0.29 (–20.5%) | 0.71 ± 0.43 (–23.5%) |

CONT – control group, SIMVA 18 – group with administered simvastatin at a concentration of 18 mg/kg in food, SIMVA 180 – group with administered simvastatin at a concentration of 180 mg/kg in food. *Data are expressed as means ±SEM. Values in brackets are calculated as percentual deviation from the 100% of non-influenced control group (with exception of latency). Significantly different, [a] $P < 0.05$ vs CONT, [b] $P < 0.01$ vs SIMVA 18, [c] $P < 0.05$ vs SIMVA 18

natal day. Carcinogen was freshly prepared and dissolved in isotonic saline solution.

Chemoprevention with simvastatin began 8 days before carcinogen administration and lasted until the end of the experiment – 17 weeks after N-methyl-N-nitrosourea (NMU) application. 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 – 17th 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. Tissue samples of each mammary tumor were fixed in 10% formol and prepared for histological analysis. The tumors were classified according to the criteria for the classification of rat mammary tumors [21]. At sacrifice, the blood was collected from each animal. The selected parameters of serum lipid metabolism were assessed. The following basic parameters of mammary carcinogenesis were evaluated in each group: tumor incidence as the percentage representation of tumor-bearing animals, tumor frequency as the number of tumors per group, latency period determined by the period from carcinogen administration to the appearance of first tumor in an animal and average tumor volume. The effect of simvastatin on food, water intake and final body weight gain was observed. Food and water intake of animals during 24 hours in 7th and 14th week after carcinogen administration were found out, overall in 4 measurements (twice in a mentioned week). The simvastatin doses were calculated in accordance with the amount of chow consumed.

Tumor incidence was evaluated by Mann-Whitney test, other parameters by one-way analysis of variance or Kruskal-Wallis test. Tumor volume was calculated according to: $V = \pi . (S_1)^2 . S_2 / 12$; $S_1$ and $S_2$ are tumor diameters ($S_1 < S_2$).

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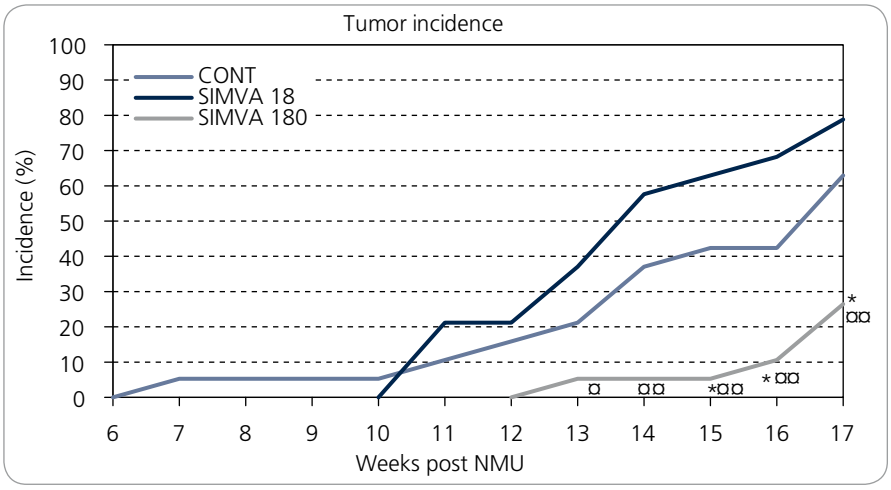
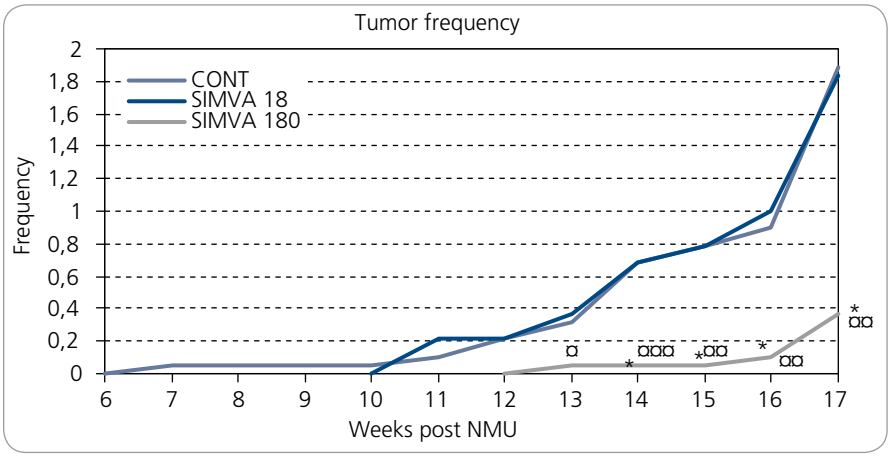
## Results

Apparent tumor-suppressive effects of simvastatin in the chemoprevention of rat mammary carcinogenesis are summarized in Tab. 1. The continuous development of tumor incidence and frequency is presented in Graph 1 and Graph 2, respectively. In experimental group SIMVA 180, simvastatin decreased the incidence by 58.5 % (P = 0.023), frequency by 80.5 % (P = 0.013) and average tumor volume by 23.5 % (P = 0.738), and lengthened the latency by 14.5 days (P = 0.163) in




**Graph 1. Percentage of animals with mammary tumors in NMU-induced tumorigenesis during simvastatin treatment. Values are expressed as means. Significant difference: * $P < 0.05$ vs CONT, ¤ $P < 0.05$ vs SIMVA 18, ¤¤ $P < 0.01$ vs SIMVA 18.**




**Graph 2. Frequency of mammary tumors per group in NMU-induced tumorigenesis during simvastatin treatment. Values are expressed as means. Significant difference: * P < 0.05 vs CONT, ¤ P < 0.05 vs SIMVA 18, ¤¤ P < 0.01 vs SIMVA 18, ¤¤¤ P < 0.001 vs SIMVA 18.**

comparison with the control animals. Chemoprevention with simvastatin beneficially shifted the rate of malignant to benign lesions in the group SIMVA 180 (43% : 57%) in comparison with untreated control group (92% : 8%). In comparison with the control group, simvastatin administered at a lower dose in experimental group SIMVA 18 did not significantly change the monitored parameters of experimental rat mammary carcinogenesis.

No macroscopic changes due to simvastatin administration in the selected organs – liver, kidney, stomach, intestine and lung were observed. With regard to plasma lipid metabolism, simvastatin in both treated groups significantly decreased the levels of triacylglycerols (P = 0.041, resp. P < 0.0001 in SIMVA 18, resp. SIMVA 180) and VLDL-cholesterol (P = 0.035, resp. P < 0.0001 in SIMVA 18, resp. SIMVA 180) in comparison with the controls. The total cholesterol, HDL- and LDL-cholesterol serum levels were not changed in animals. The evaluation of final body weight gain did not reveal significant changes in animals with administered simvastatin compared to control animals. Average daily food intake per rat in all experimental groups was between 17.7–19.0 g of the chow. Compared to controls, a significant increase in food intake of animals in the group SIMVA 18 (P = 0.025) and SIMVA 180 (P = 0.013) were found. Daily average dose of simvastatin per rat was 0.34 mg in the group SIMVA 18, and 3.42 mg in group SIMVA 180.

## Discussion

This study is the first report about simvastatin – a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, used in experimental rat mammary carcinogenesis. A substantial chemopreventive effect of simvastatin administered in the concentration of 180 mg/kg of the diet was recorded in all evaluated parameters in rat mammary carcinogenesis. In order to choose the optimal simvastatin doses in this experiment we took into consideration daily doses of the drug in clinical practice. The lower concentration of simvastatin – 18 mg/mg in our experiment was equivalent to daily dose of the drug (40 mg/day) administered to patients with hypercholesterolemia. On our previous experience with atorvastatin [Kubatka et al., unpublished results] we have used also the 10 times higher concentration of simvastatin in the diet (180 mg/kg) and this dose has been shown to be very effective in this experiment.

Similarly, significant antitumor effects of atorvastatin administered in the chemoprevention of NMU-induced rat mammary carcinogenesis in our previous study were observed [Kubatka et al., unpublished results]. Dietary administered atorvastatin in the dose of 100 mg/kg (concentration of 0.01%) significantly decreased tumor frequency by 80.5% and tumor incidence by 49.5%, and lengthened latency by 14 days in comparison with control animals. Our study pointed to fact that antineoplastic effect of atorvastatin in rat mammary carcinogenesis is independent from its effects on plasma lipid metabolism: atorvastatin in both concentrations in the diet did not change the serum levels of triacylglycerols, total cholesterol, and LDL-cholesterol. Narisawa et al [11] 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%. Simvastatin and pravastatin (with exception of pravastatin concentration of 0.001%) significantly reduced tumor frequency; the tumor incidence was reduced non-significantly by both agents. 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 [12] and to reduce N-methyl-N-nitrosourea induced F344 rat colon carcinogenesis [22]. On the other hand, an actual paper of Lubet et al [23] reported about dietary administered atorvastatin and lovastatin either as single agents or in combination with suboptimal doses of tamoxifen or rexinoid bexarotene in the prevention of NMU – induced rat mammary carcinogenesis. Atorvastatin alone in this experiment in high doses of 125 and 500 mg/kg of chow did not significantly alter incidence and frequency of mammary tumors. Combining atorvastatin (500 mg/kg diet) with either of tamoxifen and bexarotene minimally altered their efficacy. Lovastatin in the doses of 100 and 400 mg/kg diet yielded similar results as atorvastatin with limited oncostatic effects administered alone, without obvious synergy with tamoxifen or bexarotene [23]. The results of both above mentioned experiments with atorvastatin and lovastatin of Lu-

bet`s group are in strong contrary with the apparent antineoplastic effects of atrovastatin or simvastatin observed in our experiments.

In this experiment, a significant antineoplastic effect of simvastatin in rat mammary carcinogenesis could be explained by several mechanisms. Above cited results from preclinical research suggested that statins have antiproliferative, antiangiogenic and antimetastatic properties. In addition, data from experimental studies *in vitro* demonstrated the link between statin application and apoptosis induction in various human cells [3,4,24]. In order to prove proapoptotic effects of atorvastatin in our previous study with atorvastatin [Kubatka et al., unpublished results], the specimens of each mammary tumor from all experimental groups were evaluated for the mRNA expression of anti-apoptotic Bcl-2 and pro-apoptotic Bax genes. In this regard, a significant pro-apoptotic shift of ratio in Bax/Bcl-2 mRNA expression in mammary tumors after atorvastatin treatment (concentration of 0.01% in the diet) in our experiment was confirmed.

Although the favourable effects of statins in the prevention of cardiovascular diseases resulting from hypercholesterolemia are well established, the increasing evidence suggests, that these drugs exert pleiotropic effect independent of cholesterol reduction. Based on favourable results from oncological research, statins may thus represent a novel clinical approach for cancer risk reduction or maybe treatment. Several questions are unanswered about the role of statins in cancer patients. It is unknown, which types of tumors are responsive to statin therapy. Actual experimental data suggested that statins may be potentially effective in the treatment of melanoma, leukaemia, brain cancer, hepatocellular cancer and squamous cell cancer of the head and neck [25]. Further, it is not known, which statins are most effective in carcinogenesis – hydrophilic statins (pravastatin, rosuvastatin) or lipophilic statins (atorvastatin, simvastatin, fluvastatin, lovastatin). Finally, the optimal statin regimens were not defined yet. Statins administered in combination with other oncostatic substances may enhance tumor suppressive effects. In order to reduce statin adverse effects (myopathy, hepatotoxicity, rhabdomyolysis), it is favoured continuous low-dose drug clinical regimens.

## Conclusion

Pleiotropic properties of statins with proven anticarcinogenic effects in human cells can open a new era in clinical medicine. The results of this study clearly pointed to simvastatin favourable effects in experimental rat mammary carcinogenesis and gave the drug a chance to become a substance with chemopreventive efficacy in various neoplasias including breast cancer. Our experiment provided a rationale for the use of the 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor simvastatin in women who require the treatment of hypercholesterolemia and moreover are high-risk for breast cancer.

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