

Simvastatin sensitizes human gastric cancer xenograft in nude mice to capecitabine by suppressing nuclear factor-kappa B-regulated gene products

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Abstract

Chemoresistance remains a major problem in the treatment of gastric cancer patients. Hence, novel pharmacological agents that can overcome drug resistance are urgently required. Whether simvastatin can sensitize the gastric cancer to the antitumor effects of capecitabine in vitro and in vivo was investigated. The effect of simvastatin on the proliferation of gastric cancer cells was examined by mitochondrial dye-uptake 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method, apoptosis by esterase staining, NF- κ B activation by DNA binding assay, and protein expression by western blot analysis. The effect of simvastatin on the tumor growth in xenograft mouse model of human gastric cancer was also examined. Simvastatin suppressed the proliferation of gastric cancer cells, enhanced the apoptotic effects of

capecitabine, suppressed the constitutive activation of NF- κ B, and abrogated the expression of cyclooxygenase-2 (COX-2), cyclin D1, Bcl-2, survivin, CXC motif receptor 4, and MMP-9 proteins. In a xenograft mouse model, we observed that the administration of simvastatin alone (5 mg/kg body weight, intraperitoneal thrice/week) significantly suppressed the growth of the tumor and this effect was further potentiated by capecitabine treatment. As compared to the vehicle control, simvastatin also suppressed the expression of NF- κ B-regulated gene products such as cyclin D1, COX-2, ICAM-1, MMP-9, survivin, Bcl-xL, and XIAP in tumor tissues. Overall, our results demonstrate that simvastatin can enhance the effects of capecitabine through suppression of NF- κ B-regulated markers of proliferation, invasion, angiogenesis, and metastasis.

Both KAM and MKS contributed equally to this work.

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Key message

- Simvastatin suppressed the proliferation and augmented apoptotic effects of capecitabine.
- Simvastatin suppressed constitutive activation of NF- κ B and its regulated genes.
- Simvastatin enhanced the tumor growth inhibitory effects of capecitabine.

Keywords Gastric cancer · Chemoresistance · Simvastatin · Apoptosis · NF- κ B

Introduction

Gastric cancer in spite of its decreasing incidence remains one of the most common causes of cancer-related deaths worldwide [1]. In addition to the surgery, various chemotherapeutic agents have been used in the gastric cancer patients, but still the median progression for survival remains

7–10 months [2]. Development of resistance to the chemotherapeutic drugs like capecitabine is frequently encountered in gastric cancer patients and contributes to the poor prognosis and overall median survival rate [3, 4]. Hence, novel treatment modalities to enhance the effects of chemotherapeutic drugs and reduce their resistance are required.

The pro-inflammatory transcription factor, NF- κ B, and its regulated gene products play a critical role in the proliferation, metastasis, and chemoresistance of gastric cancer [4]. In the cells, NF- κ B is composed of homodimers and heterodimers derived from five distinct subunits: RelA (p65), c-Rel, RelB, p50 (NF- κ B1), and p52 (NF- κ B2). All family members share a highly conserved Rel homology domain (~300aa) responsible for DNA binding, dimerization domain, and interaction with I κ Bs, the intracellular inhibitor of NF- κ B [5]. In the resting stage, majority of NF- κ B complexes are localized predominantly in an inactive form in the cytoplasm by binding to a family of inhibitory proteins, the I κ Bs. Phosphorylation of these conserved serine residues in response to stimulator leads to the immediate polyubiquitination of I κ B proteins by the SCF- β -TrCP complex. This modification subsequently targets I κ B proteins for rapid degradation by the 26S proteasome. Activation of the NF- κ B signaling cascade results in complete degradation of I κ B, allowing translocation of NF- κ B to the nucleus, where it induces gene transcription [6]. A number of NF- κ B-regulated gene products, including cyclin D1, CXC motif receptor 4 (CXCR4), and cyclooxygenase-2 (COX-2), have been closely linked with the initiation, progression, and also the development of chemoresistance in gastric cancer [4–6]. Interestingly, Long and coworkers have also reported that NF- κ B is activated in gastric carcinoma tissues and can be considered a prognostic marker of chemotherapy for human stage IV gastric carcinoma [7]. Also, the gastric mucosa of the individuals infected with *Helicobacter pylori*, a major risk factor for gastric cancer show upregulated NF- κ B pathway and Th1-type cytokine responses, which can alter the overall integrity of the gut epithelial barrier [8]. Thus, the identification of novel pharmacological blockers of NF- κ B activation cascade pathway can be a useful therapeutic strategy to circumvent chemoresistance in the gastric cancer patients.

In the present study, we determined whether simvastatin, a cholesterol-lowering drug [9], can sensitize the human gastric cancer to capecitabine in vitro and in a xenograft mouse model. The potential anti-carcinogenic effects of simvastatin alone or in combination with various anticancer therapies have been documented in a number of tumor cell lines and mouse models, including colorectal [10], hepatocellular [11], prostate [12], breast [13], and hematological malignancies [14]. How simvastatin exerts its anticancer effects is not fully understood, but it has been found to modulate various signaling cascades

including mitogen-activated protein kinases [15, 16], PI3-K/Akt [17], NF- κ B [18–20], STAT3 [14], cell cycle-dependent kinases [21], Rho-dependent kinase [22, 23], and Ras-related C3 botulinum toxin substrate 1 [24].

However, no prior reports exist in literature elaborating the potential anticancer effects of simvastatin in the gastric cancer alone or in combination with the chemotherapeutic agents. Hence, we investigated whether simvastatin can potentiate the anticancer effects of capecitabine in gastric cancer and through what molecular mechanism(s). Our findings suggest for the first time that simvastatin can suppress the proliferation of gastric cancer cells, potentiate capecitabine-induced apoptosis, and also the anticancer efficacy of capecitabine in the human xenograft gastric cancer model through the suppression of NF- κ B-regulated gene products.

Materials and methods

Reagents

Simvastatin with purity >98 % was purchased from Alexis Biochemicals (San Diego, CA, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Tris base, glycine, NaCl, SDS, BSA, β -actin antibody, and corn oil were purchased from Sigma-Aldrich (St. Louis, MO). Simvastatin was dissolved in dimethylsulfoxide as a 50-mM stock solution and stored at 4 °C for in vitro and in corn oil for in vivo experiments, respectively. Further dilution was done in cell culture medium. RPMI-1640 media, fetal bovine serum (FBS), 0.4 % trypan blue vital stain, and antibiotic–antimycotic mixture were obtained from Invitrogen (Carlsbad, CA). Antibodies against p65, MMP-9, Bcl-2, Bcl-xL, XIAP, COX-2, cyclin D1, survivin, and VEGF were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). CXCR4 antibody was obtained from Abcam (Cambridge, MA, USA). CD31 antibody was purchased from and Cell Signaling Technology (Danvers, MA). Ki-67 antibody was purchased from BD PharMingen, Inc. (San Diego, CA). Goat anti-rabbit-horse radish peroxidase (HRP) conjugate and goat anti-mouse HRP were purchased from Invitrogen (Carlsbad, CA). Capecitabine was obtained from Duheng International Trading Company Ltd. Shanghai, China, and dissolved in sterile phosphate buffered saline (PBS) on the day of use.

Cell lines

The gastric cancer cell lines (SNU-5, SNU-16, MKN45 and AGS) were kindly provided by Prof. Patrick Tan from DUKE-NUS, Singapore. The gastric cancer cell lines were cultured in RPMI-1640 media supplemented with 10 % FBS, 100 units/mL penicillin, and 100 μ g/mL streptomycin.

Western blot analysis

For detection of various proteins in gastric cancer cells, 2×10^6 /ml cells were treated with simvastatin for different time intervals and western blot analysis was performed as described previously [25].

Cell proliferation assay

The effect of simvastatin on cell proliferation was determined by the MTT uptake method as described previously [25].

Live and dead assay

To investigate whether simvastatin could enhance the apoptotic effects of capecitabine in gastric cancer cells, we used a LIVE/DEAD cell viability assay kit (Invitrogen), which is used to determine intracellular esterase activity and plasma membrane integrity [4]. Briefly, gastric cancer cells (5,000/well) were incubated in chamber slides, pretreated with simvastatin for 4 h, and treated with capecitabine for 24 h. Cells were then stained with the assay reagents for 30 min at room temperature. Cell viability was determined under a fluorescence microscope by counting live (green) and dead (red) cells.

Flow cytometric analysis

To determine the effect on the cell cycle, cells were exposed to combination of simvastatin for 4 h and treated with capecitabine for 24 h. Thereafter, cells were washed, fixed with 70 % ethanol, and incubated for 30 min at 37 °C with 0.1 % R Nase A in PBS. Cells were then washed again, resuspended, and stained in PBS containing 25 µg/ml propidium iodide for 30 min at room temperature. Cell distribution across the cell cycle was analyzed with a CyAn ADP flow cytometer (Dako Cytomation).

RNA isolation and reverse transcription

Total cellular RNA was extracted from untreated and simvastatin-treated cells using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) as described previously [25].

Real-time polymerase chain reaction

Real-time polymerase chain reaction (PCR) for Bcl-2, Bcl-xl, cyclin D1, VEGF, and Mcl-1 genes was performed as described previously [25].

Xenograft gastric tumor mouse model

All procedures involving animals were reviewed and approved by NUS Institutional Animal Care and Use Committee. Six-week-old athymic nu/nu female mice (Animal Resource Centre, Australia) were implanted subcutaneously in the right flank with SNU-5 cells (3×10^6 cells/100 µl saline). When tumors have reached 0.25 cm in diameter, the mice were randomized into the following treatment groups ($n=5$ /group): (a) untreated control (corn oil, 100 µL daily); (b) simvastatin (5 mg/kg bodyweight, suspended in corn oil, intraperitoneal [i.p.] injection) thrice/week; (c) capecitabine alone (60 mg/kg bodyweight, suspended in corn oil, twice weekly by gavage; and (d) combination (simvastatin, 5 mg/kg bodyweight, suspended in corn oil, i.p. injection thrice/week and capecitabine, 60 mg/kg bodyweight, suspended in corn oil, twice weekly by gavage). Therapy was continued for 4 weeks, and the animals were euthanized 1 week later. Primary tumors were excised and the final tumor volume was measured during the course of experiment and calculated using the formula $V=4/3\pi r^3$: r is the radius of the tumor. Half of the tumor tissue was fixed in formalin and embedded in paraffin for immunohistochemistry analysis. The other half was snap frozen in liquid nitrogen and stored at -80 °C.

Immunohistochemical analysis of tumor samples

Solid tumors from control and treatment groups were fixed with 10 % phosphate-buffered formalin, processed, and embedded in paraffin. Sections were cut and deparaffinized in xylene, and dehydrated in graded alcohol, and finally hydrated in water. Antigen retrieval was performed by boiling the slide in 10 mM sodium citrate (pH 6.0) for 30 min. Immunohistochemistry was performed following manufacturer instructions (DAKO LSAB kit). Briefly, endogenous peroxidases were quenched with 3 % hydrogen peroxide. Non-specific binding was blocked by incubation in the blocking reagent in the LSAB kit (Dako, Carpinteria, CA) according to the manufacturer's instructions. Sections were incubated overnight with primary antibodies as follows: anti-p65, anti-COX-2, anti-VEGF, anti-MMP-9, anti-Ki-67, and anti-CD31 (each at 1:100 dilutions). Slides were subsequently washed several times in Tris-buffered saline with 0.1 % Tween 20 and were incubated with biotinylated linker for 30 min, followed by incubation with streptavidin conjugate. Immunoreactive species were detected using 3,3'-diaminobenzidine tetrahydrochloride as a substrate. Sections were counterstained with Gill's hematoxylin and mounted under glass cover slips. Images were taken using an Olympus BX51 microscope (magnification, 40×).

Measurement of NF- κ B activation in gastric cancer cells and tumor samples

To determine NF- κ B activation, we performed DNA binding assay using TransAM NF- κ B p65 transcription factor assay kit (Active Motif, Carlsbad, CA, USA) as described previously[4].

Statistical analysis

Statistical analysis was performed by Student's *t* test and one-way analysis of variance. A *p* value of less than 0.05 was considered statistically significant.

Results

Simvastatin suppresses the proliferation and enhances the apoptotic effect of capecitabine in gastric cancer cells The aim of this study was to determine whether simvastatin, a cholesterol-lowering drug (with chemical structure shown in Fig. 1a), has a potential role in the treatment of gastric cancer either alone or in combination with capecitabine and if so, through what mechanism(s). We first analyzed the anticancer effect of simvastatin on the proliferation of four different gastric cancer cell lines. Simvastatin inhibited the growth of all three human gastric cancer cells (SNU-16, MKN-45, AGS, and SNU-5) in a dose- and time-dependent manner (Fig. 1b). Whether simvastatin can also enhance the apoptotic effect of capecitabine against gastric cancer cells was also determined. We use flow cytometric analysis and an esterase staining method (live/dead assay) to establish whether simvastatin can potentiate the apoptosis induced by capecitabine. As shown in Fig. 1c, d, the suboptimal doses of simvastatin (10 μ M) or capecitabine (10 μ M) that had minimum effect on apoptosis alone produced significant increase in apoptosis when used in combination against gastric cancer cells.

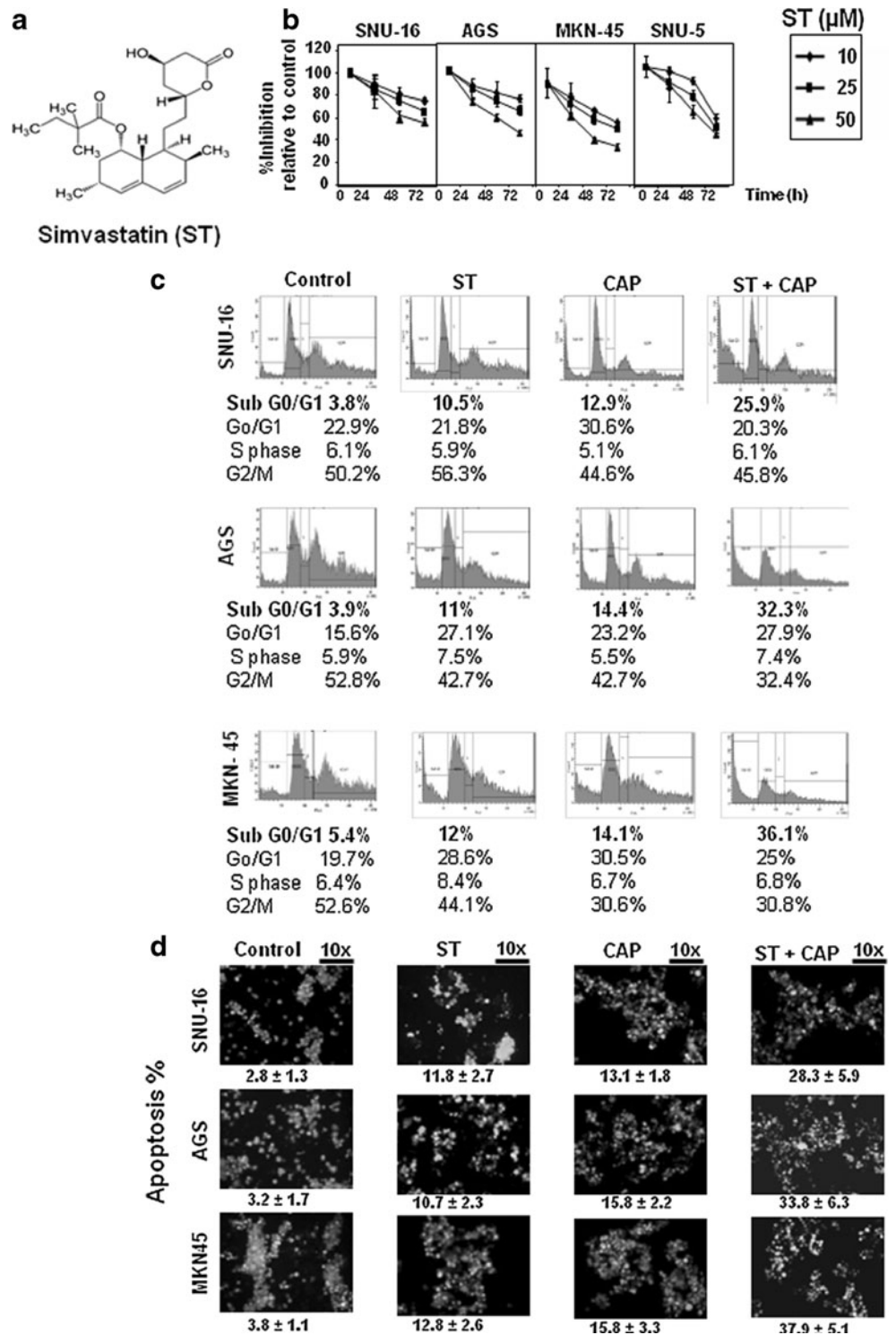
Simvastatin inhibits the constitutive and inducible NF- κ B activation in gastric cancer cells We next examined how simvastatin enhances the apoptotic effects of capecitabine in gastric cancer cells. NF- κ B has been found to be constitutively activated in gastric cancer and mediates resistance to apoptosis [4, 26–28]. Whether simvastatin induces downregulation of constitutive NF- κ B activation in SNU-5 cells was investigated by using an ELISA-based DNA binding assay. The assay results indicate that the treatment with simvastatin inhibited constitutive NF- κ B activation in SNU-5 cells in a dose- and time-dependent manner (Fig. 2a, b). We have previously reported that the chemotherapeutic agent capecitabine can cause NF- κ B activation in a dose-dependent manner in MKN-45 cells, with significant activation observed at

25 μ M [4]. Hence, we next determined whether simvastatin treatment can also modulate capecitabine-induced NF- κ B activation in MKN-45 cells. Moreover, we found that simvastatin was also able to inhibit capecitabine-induced NF- κ B activation in a dose-dependent manner in MKN-45 cells (Fig. 2c), thereby indicating that it is a potent suppressor of both constitutive and inducible NF- κ B activation in gastric cancer cells. The master transcription factor NF- κ B regulates the expression of a wide plethora of genes involved in the proliferation, survival, metastasis, angiogenesis, and chemoresistance in gastric cancer [4]. Whether simvastatin exposure can also modulate the expression of various NF- κ B-regulated gene products was also examined. We found that the simvastatin suppressed the constitutive expression of antiproliferative (cyclin D1, COX-2), anti-apoptotic (Bcl-2, survivin), and invasive/metastatic (MMP-9, CXCR4) protein expression in a time-dependent manner in SNU-5 cells (Fig. 2d). Simvastatin also induced the cleavage of PARP in SNU-5 cells (Fig. 2d). Interestingly, we also noticed that there was a time-dependent decrease in the expression of various genes namely *Bcl-2*, *Bcl-xL*, *survivin*, *VEGF*, and *Mcl-1* upon simvastatin treatment with maximal inhibitory effect observed at 48 h (Fig. 2e). Based on these intriguing findings in vitro, we proceeded to study the effect of simvastatin and capecitabine alone and in combination in a xenograft mouse model.

Simvastatin potentiates the antitumor effects of capecitabine in a xenograft gastric cancer mouse model We examined the therapeutic efficacy of simvastatin and capecitabine either alone or in combination on the growth of subcutaneously implanted human gastric cancer cells in nude mice. The experimental protocol is depicted in Fig. 3a. SNU-5 cells were implanted subcutaneously in the right flank of nude mice. When tumors have reached 0.25 cm in diameter after a week, the mice were randomized into four groups and started the treatment as per the experimental protocol. The treatment was continued for 4 weeks and animals were sacrificed after 5 weeks. We found that simvastatin alone when given at 5 mg/kg body weight significantly inhibited the growth of the tumor ($p < 0.001$ when compared to control) (Fig. 3b, c). Capecitabine alone was also found to be quite effective ($p < 0.001$ when compared to control; $p > 0.05$ when compared to simvastatin alone group), and the combination of the two drugs was significantly more potent in reducing the tumor burden. The tumor volume in the combination of simvastatin and capecitabine group was significantly lower than simvastatin alone group ($p < 0.001$) or capecitabine alone group ($p < 0.001$) on day 35 (Fig. 3c, d).

Simvastatin inhibits the expression of markers of proliferation and angiogenesis in gastric tumor tissues While Ki-67-positive index is used as a marker for cell proliferation, the

Fig. 1 Simvastatin inhibits the proliferation and enhances the apoptotic effects of capecitabine in gastric cancer cells in vitro. **a** The chemical structure of simvastatin. **b** MTT assay results showed dose-dependent suppression of cell proliferation in four different gastric cancer cell lines treated with simvastatin. Points, mean of triplicate; bars, SE. Data are a representative of two independent experiments. **c**, **d** Flow cytometric analysis and Live/Dead assay results indicate that simvastatin (ST, 10 μ M) treatment in combination with capecitabine (CAP, 10 μ M) for 24 h can enhance apoptosis in gastric cancer cells. Data indicated as percentage proportions of apoptotic gastric cancer cells for Live/Dead assay. Values are mean \pm SE of triplicate. Data are a representative of two independent experiments



CD31 level is considered as marker for microvessel density. Whether simvastatin and capecitabine can affect these markers in gastric tumor tissues was also determined. Figure 4a shows that both simvastatin and capecitabine alone downregulated the expression of Ki-67 in tumor tissues and the combination of the two was most effective ($p < 0.001$). Also, when examined for CD31 expression levels, we found

that both agents individually substantially reduced the CD31 expression and the combination of two was most effective ($p < 0.001$) (Fig. 4b).

Simvastatin suppressed the expression of NF- κ B-regulated gene products in gastric tumor tissues We also investigated the effect of simvastatin and capecitabine on NF- κ B levels in

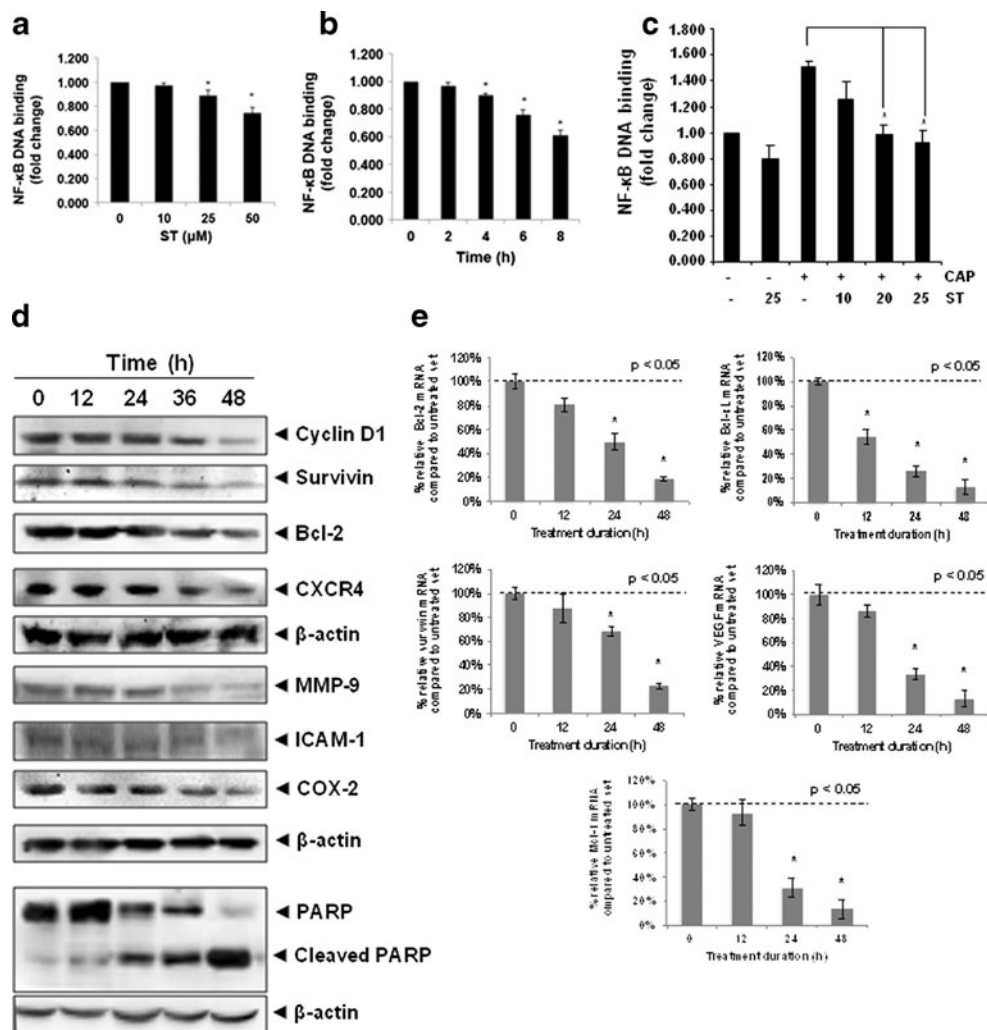


Fig. 2 DNA binding assay results showing that simvastatin suppresses the constitutive activation of NF-κB in SNU-5 cells in a dose- and time-dependent manner. **a** SNU-5 (1×10^6) cells were treated with indicated concentrations of simvastatin for 8 h and nuclear extracts were prepared and assayed for NF-κB activation by ELISA-linked DNA binding assay. **b** SNU-5 (1×10^6) cells were treated with 50 μM simvastatin for 0, 2, 4, 6, and 8 h, and nuclear extracts were prepared and assayed for NF-κB activation by ELISA-linked DNA binding assay. **c** MKN-45 (1×10^6) cells were pretreated with simvastatin (10, 20, and 25 μM) for 12 h, stimulated with capecitabine 25 μM for 4 h, and then the nuclear extracts were prepared and assayed for NF-κB activation by ELISA-linked DNA

binding assay. $*p < 0.05$. **d** Simvastatin suppressed the constitutive expression of gene products involved in proliferation, metastasis, and anti-apoptosis in gastric cancer cells. SNU-5 (1×10^6) cells were treated with 50 μM simvastatin for the indicated time points and western blot was performed as described under “Materials and methods” section. $*p < 0.05$. **e** Simvastatin suppressed the expression of various genes involved in tumor progression in gastric cancer cells. SNU-5 (1×10^6) cells were treated with 50 μM simvastatin for the indicated time points and real-time PCR analyses was performed as described under “Materials and methods” section. $*p < 0.05$

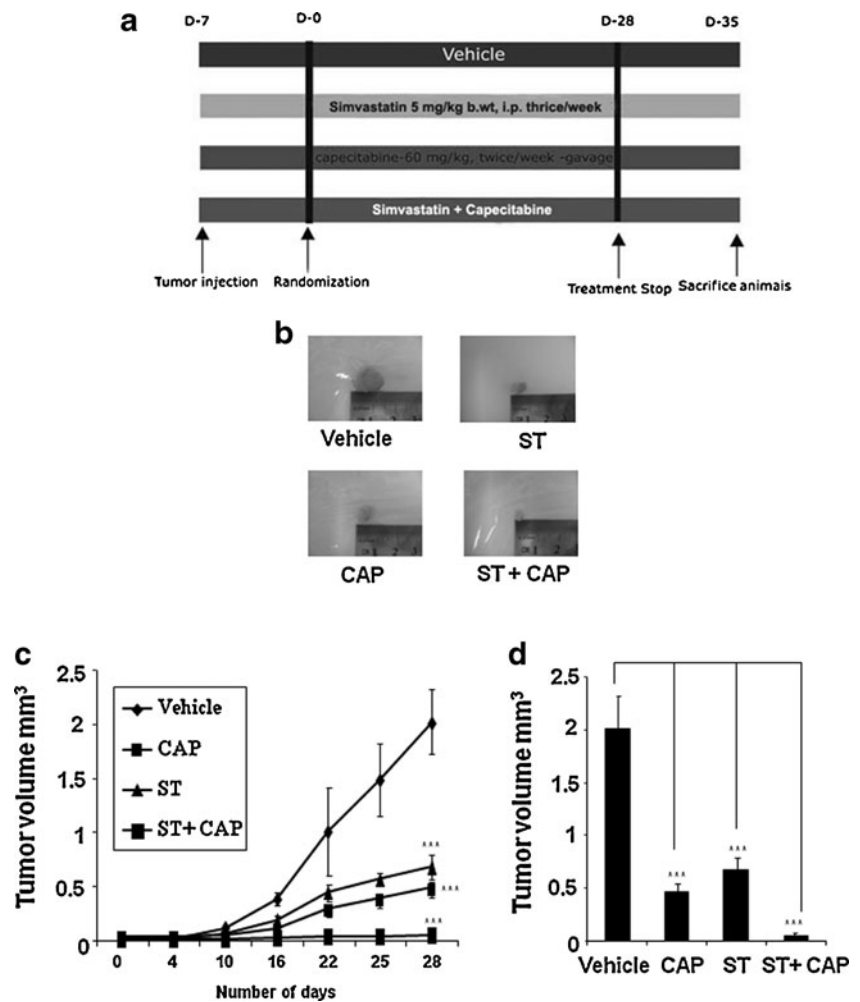
gastric tumor tissue. Figure 5a shows that simvastatin either alone or in combination was quite effective in suppressing the constitutive activation of NF-κB in gastric cancer tissues, whereas capecitabine treatment alone had no significant effect on constitutive NF-κB activation in gastric tissue as evident by DNA binding analysis (Fig. 5a).

NF-κB is known to regulate the expression of number of proteins, including those involved in proliferation (cyclin D1, COX-2), invasion/metastasis (ICAM-1, MMP-9, CXCR4) and survival (Bcl-2, Bcl-xL, survivin, XIAP) [6]. Whether simvastatin can affect the expression of these diverse

NF-κB-regulated gene products in tumor tissues was examined by western blot analysis. We found that treatment with combination of simvastatin and capecitabine was quite effective in substantially downregulating the expression of various gene products involved in the gastric cancer growth, survival, invasion, and metastasis (Fig. 5b, c).

The observed suppression of NF-κB, COX2, VEGF, and MMP-9 by western blot analysis was further confirmed by immunohistochemical methods. As shown in Fig. 6, these gene products were significantly downregulated in gastric tumor samples treated with simvastatin in combination with

Fig. 3 Simvastatin potentiates the anticancer effect of capecitabine to inhibit growth of gastric cancer in xenograft mouse model. **a** Schematic representation of experimental protocol described in “Materials and methods.” Group I was given corn oil (100 µl, p.o., daily), group II was given simvastatin (5 mg/kg body weight, i.p. thrice/week), group III was given capecitabine (60 mg/kg body weight, twice weekly by gavage), and group IV was given simvastatin (5 mg/kg body weight, i.p. thrice/week) and capecitabine (60 mg/kg body weight, twice weekly by gavage). **b** Necropsy photographs of mice bearing subcutaneously implanted SNU-5 cells; **c** Tumor volumes in mice measured during the course of experiment and calculated using the formula $V=4/3\pi r^3$. **d** Tumor volumes in mice measured on the last day of the experiment at autopsy using Vernier calipers and calculated using the formula $V=4/3\pi r^3$ ($n=5$). Columns, mean; bars, SE. *** $p < 0.001$



capecitabine. Overall, the data indicates that simvastatin can suppress the expression of various NF-κB-regulated gene products involved in the proliferation, survival, invasion, and angiogenesis, thereby causing inhibition of tumor growth in gastric cancer xenograft mouse model.

Discussion

Despite significant improvements in the available treatment regimens following the introduction of targeted therapies, gastric cancer remains the second most lethal cancers, with only less than 20 % of patients surviving up to 5 years due to the existing problems of chemoresistance and tumor recurrence. Thus, novel pharmacological agents that are efficacious and can significantly enhance the effects of existing drugs are urgently needed. The goal of the present study was to investigate whether simvastatin, a cholesterol-lowering drug, can enhance the antitumor efficacy of capecitabine against human gastric cancer. We observed that

simvastatin inhibited the proliferation of gastric cancer cells, enhanced capecitabine-induced apoptosis, modulated both constitutive as well the inducible NF-κB activation, and attenuated the expression of various NF-κB-regulated gene products. We also found that in a xenograft mouse model, simvastatin effectively suppressed the growth of gastric cancer alone and in combination with capecitabine.

We first noticed that simvastatin treatment can inhibit the proliferation of various gastric cancer cell lines in a dose- and time-dependent manner. Interestingly, the antiproliferative effects of simvastatin have not been evaluated previously in gastric cancer cells, although it has been found to suppress the proliferation of other gastrointestinal cancers. For example, in a recent study, Kodach and coworkers reported that simvastatin can reduce the proliferation and augment the chemosensitivity of colorectal cancer cells via the bone morphogenetic protein pathway [29]. Also, simvastatin has been found to attenuate the growth and malignant potential of human esophageal adenocarcinoma [30] and hepatic cancer cells [31], but most of these studies were restricted only to cell lines with no in vivo evidence. We observed that simvastatin

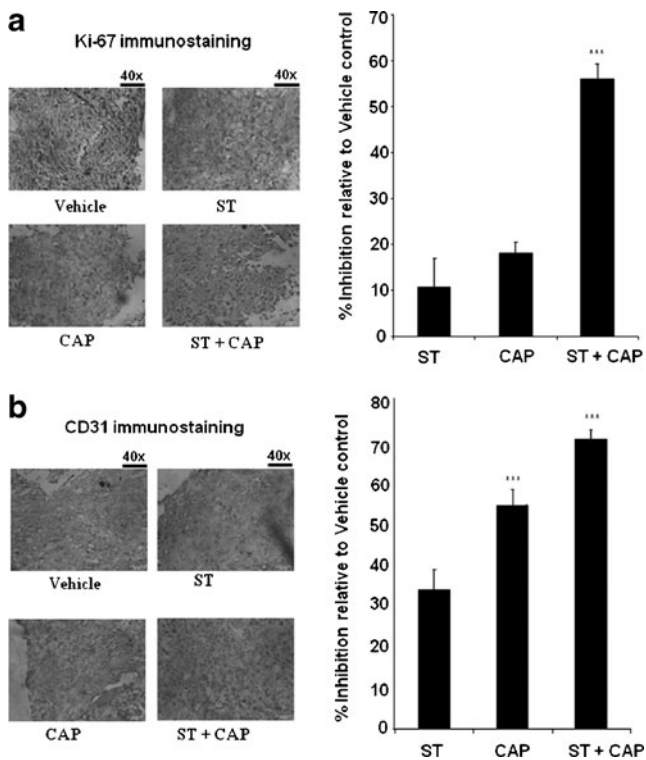


Fig. 4 Simvastatin enhances the effect of capecitabine against tumor cell proliferation and angiogenesis in gastric cancer. **a** *Left panel*, immunohistochemical analysis of proliferation marker Ki-67 indicates the inhibition of gastric cancer cell proliferation following simvastatin exposure either alone or in combination with capecitabine-treated groups of animals. *A right panel*, quantification of Ki-67+ cells as described in “Materials and methods.” *Columns*, mean of triplicate; *bars*, SE. *** $p < 0.001$. **b** *Left panel*, immunohistochemical analysis of CD31 for microvessel density in gastric cancer tumors indicates the inhibition of angiogenesis by either simvastatin alone and in combination with capecitabine; *b right panel*, quantification of CD31+ microvessel density as described in “Materials and methods.” *Columns*, mean of triplicate; *bars*, SE. *** $p < 0.001$

caused the downregulation of cell proliferative gene products such as cyclin D1, which may explain its potent antiproliferative effects in gastric cancer.

We further observed that simvastatin when used in combination with capecitabine can cause increased apoptosis in three different gastric cancer cells. This is very intriguing because simvastatin has never been previously reported to induce apoptosis in gastric cancer cells either alone or in combination with the chemotherapeutic agents. We also noticed that this effect may be mediated due to the downregulation of cell survival proteins such as Bcl-2 and survivin in gastric cancer cells. Moreover, we also found that both the constitutive and capecitabine-induced NF- κ B activation was suppressed by simvastatin in gastric cancer cells. We had previously reported that both constitutive and chemotherapy-induced NF- κ B activation in chronic myeloid leukemia cells can be abrogated by simvastatin [18, 19], but its potential effect on NF- κ B signaling cascade in gastric

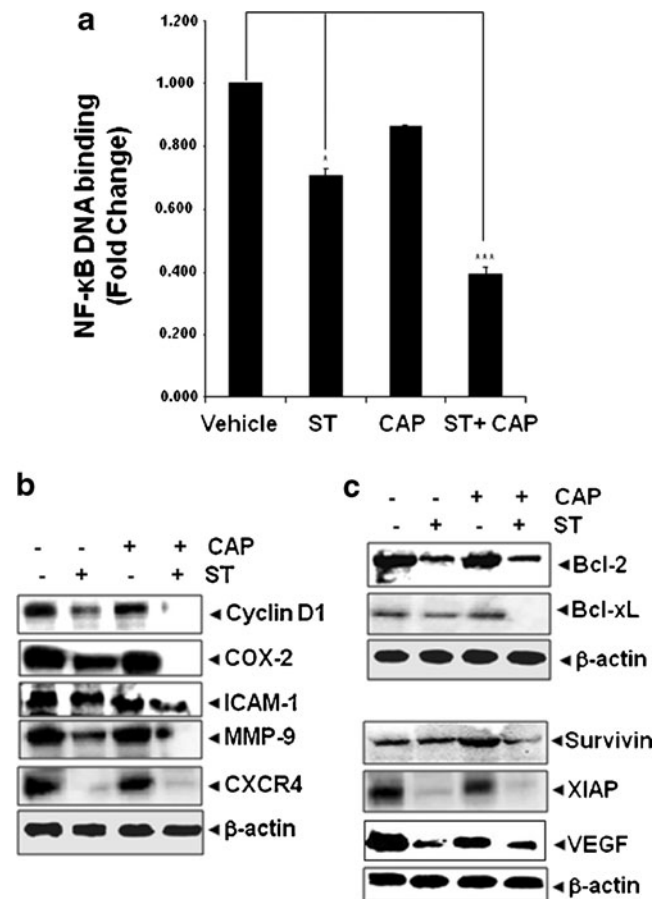
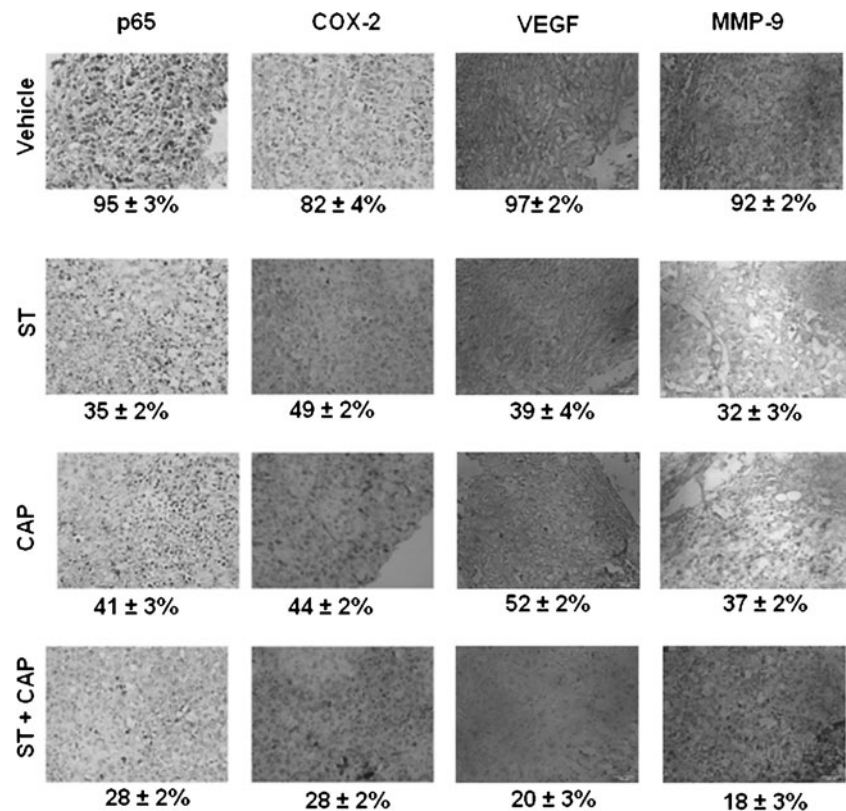


Fig. 5 Simvastatin enhances the effect of capecitabine against the activation of NF- κ B and expression of NF- κ B-regulated gene products in gastric cancer tissue samples. **a** Detection of NF- κ B by DNA binding assay in tumor tissue samples showed the significant inhibition of NF- κ B by combination, * $p < 0.05$; *** $p < 0.001$. **b** Western blot showing that combination of simvastatin and capecitabine inhibit the expression of NF- κ B-dependent gene products involved in the proliferation (cyclin D1, COX-2, ICAM-1), invasion (MMP-9), and metastasis (CXCR4) in gastric tumor tissues. **c** Western blot showing that combination of simvastatin and capecitabine inhibits the expression of NF- κ B-dependent gene products involved in the survival (Bcl-2, Bcl-xL, survivin, XIAP) and angiogenesis (VEGF) in gastric tumor tissues. Samples from three mice in each group were analyzed and representative data are shown

cancer cells has never been evaluated before. Also, simvastatin was observed to downregulate the expression of various invasive, metastatic, and angiogenic gene products (ICAM-1, MMP-9, CXCR4, and VEGF) which further support its application for gastric cancer treatment.

We also noticed for the first time that the intraperitoneal administration of simvastatin alone inhibited the growth of human gastric tumors when examined in vivo in a xenograft mouse model. Tumor growth was inhibited by more than 50 % on treatment with simvastatin and capecitabine alone, respectively. Also, when the two drugs were used in combination, they were found to be much more effective in modulating tumor growth. When investigated for the molecular mechanism(s) by which simvastatin exerts its

Fig. 6 Immunohistochemical analysis of nuclear p65, COX-2, MMP-9, and VEGF showed the inhibition of these diverse biomarkers by either simvastatin alone or in combination with capecitabine in gastric tumor tissues. *Percentage*, positive staining for the given biomarker



effects in xenograft model, we noticed that the expression of proliferation marker Ki67 as well as microvessel density indicator CD31 was attenuated upon simvastatin treatment. Further analyses revealed that simvastatin caused the downregulation of NF- κ B and various proteins (Cyclin D1, COX-2, survivin, Bcl-xL, XIAP, ICAM-1, MMP-9, and VEGF) regulated by NF- κ B which play a pivotal role in the proliferation, survival, angiogenesis, and metastasis of gastric cancer. All of these observed effects were further enhanced upon capecitabine treatment. These findings are also consistent with a previous study from our group in which vitamin E-gamma tocotrienol was found to potentiate the effect of chemotherapy in gastric cancer through the modulation of NF- κ B signaling cascade [4].

Simvastatin has been used in combination therapy with several chemotherapeutic agents/targeted therapies such as farnesyl transferase inhibitors, EGFR inhibitors gefitinib and cetuximab, doxorubicin, non-steroidal anti-inflammatory drugs, and vitamin-E gamma tocotrienol in diverse tumor cell lines and in vivo cancer models [10, 13, 16, 32–34] but so far its effects either alone or in combination with anticancer therapies in gastric cancer mouse models has never been studied before. Recently conducted couple of population-based case–control studies clearly indicate that statins uptake can reduce the risk of gastric cancer [35], and our findings provide further scientific evidence that simvastatin has significant potential for the treatment of gastric cancer and

its effects can be further enhanced by capecitabine. Finally, a phase III clinical study of capecitabine/CDDP chemotherapy plus a low dose of simvastatin is already in progress in Korea (<http://www.clinicaltrials.gov>) and may further help to substantiate our preclinical findings as reported here.

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Conflict of interest The authors declare no conflict of interest.

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