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Reversal of chemoresistance and enhancement of apoptosis by statins through down-regulation of the NF-κB pathway

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ABSTRACT

We recently found that simvastatin can modulate the nuclear factor-κB (NF-κB) activation pathway, but whether other statins have similar effects to those of simvastatin is unknown. Therefore, we evaluated the effect six different statins on TNF-induced NF-κB activation in human myeloid leukemia cells. We then determined whether the combination of statins and standard chemotherapeutic agents could overcome chemoresistance and augment apoptosis. Of the six statins evaluated, only the natural statins (simvastatin, mevastatin, lovastatin, and pravastatin), not the synthetic statins (fluvastatin and atorvastatin), inhibited TNF-induced NF-κB activation. Simvastatin suppressed the NF-κB activation and potentiated the apoptosis induced by doxorubicin, paclitaxel, and 5-fluorouracil. These results suggest that different statins behave differently from one another and that they may be useful in overcoming chemoresistance.

1. Introduction

Statins may be the most important family of cholesterol-lowering drugs to emerge in the 21st century [1]. Statins primarily inhibit 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which is needed to produce cholesterol through the mevalonate pathway.

Recent evidence suggests that statins have pleiotropic effects and thus may be useful in the treatment of diseases such as cancer [2–6]. Indeed, statins have been found to have anticancer activity in various cancer cell types, including colorectal [7], colon [8], bladder [9], prostate [10], and gastrointestinal [11] cancer, although they do not significantly reduce the risk for breast, prostatic, colorectal, or lung cancer [12]. Browning and Martin [13] suggested that statins are not associated with short-term cancer risk, but longer latency effects are possible.

Recently, we reported that simvastatin can potentiate the TNF-induced apoptosis through down-regulation of nuclear factor-κB (NF-κB) regulated antiapoptotic gene products [14]. NF-κB activation has been associated with tumor cell proliferation, invasion, angiogenesis, and metastasis through its regulation of various gene products [15]. Thus, NF-κB suppression in cancer cells may be useful in the prevention and treatment of cancer [16]. Inducible drug resistance has emerged as a substantial obstacle to effective cancer therapy, and NF-κB activation may play a role in the development of chemoresistance [17]. In fact, chemotherapeutic agents themselves can activate NF-κB, which leads to tumor cells' eventual resistance to therapy [17]. NF-κB activation has been associated with paclitaxel, doxorubicin, and 5-fluorouracil resistance in tumor cells [18–20].

Lovastatin, mevastatin, simvastatin (a methyl derivative of lovastatin), and pravastatin are natural statins, isolated from

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Abbreviations: NF-κB, nuclear factor-κB; TNF, tumor necrosis factor α; EMSA, electrophoretic mobility shift assay.

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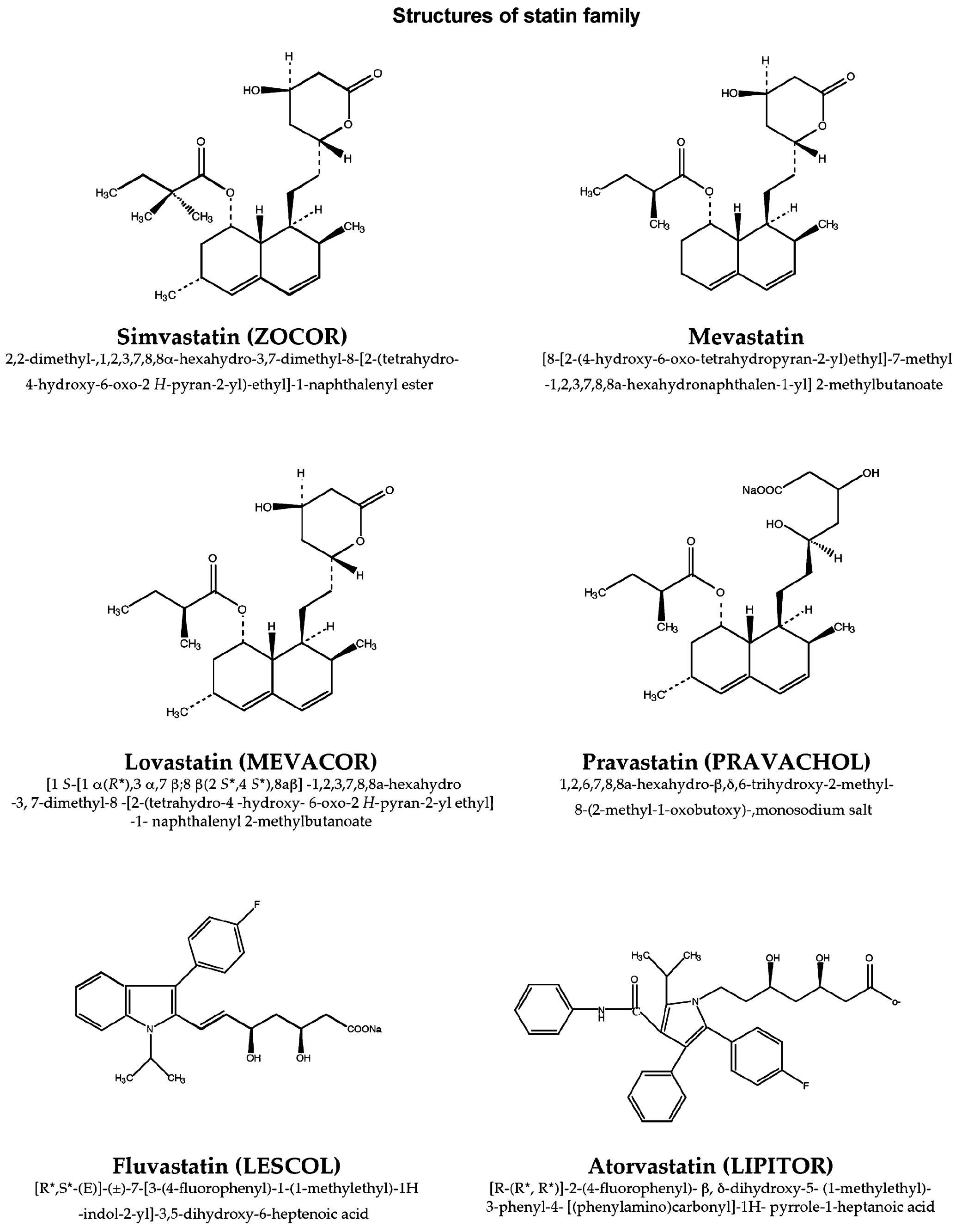

Fig. 1 – Structures of the natural (lovastatin, mevastatin, simvastatin, and pravastatin) and synthetic (fluvastatin, atorvastatin) statins used.

fermented red yeast rice; fluvastatin, atorvastatin, cerivastatin, rosuvastatin, and pitavastatin are synthetic compounds. Natural and synthetic statins have different biologic characteristics (Fig. 1); whether they have similar potency against NF-κB and can potentiate the effects of chemotherapeutic agents is not understood. Therefore, we examined the ability of six statins to suppress TNF-induced NF-κB activation and if this inhibition overcomes chemoresistance and enhances apoptosis in human myeloid leukemia cells. The six statins varied in their ability to suppress NF-κB activation, and simvastatin suppressed chemotherapeutic agent-induced NF-κB activation, leading to potentiation of apoptosis.

2. Materials and methods

2.1. *Materials*

All statins were obtained from LKT Laboratories, Inc. (St. Paul, MN). A 50 mM solution of statins was prepared in 100% dimethyl sulfoxide, stored as small aliquots at −20 °C, and then diluted as needed in cell culture medium. Bacteria-derived recombinant human tumor necrosis factor (TNF), purified to homogeneity with a specific activity of 5×10^7 U/mg, was kindly provided by Genentech (South San Francisco, CA). Penicillin, streptomycin, Iscove's modified Dulbecco's medium, and fetal bovine serum were obtained from Invitrogen (Grand Island, NY). Paclitaxel, doxorubicin, and 5-fluorouracil were obtained from Sigma–Aldrich (St. Louis, MO).

2.2. Cell lines

Human myeloid leukemia KBM-5 cells were obtained from American Type Culture Collection (Manassas, VA). KBM-5 cells were cultured in Iscove's modified Dulbecco's medium supplemented with 15% fetal bovine serum. Media were also supplemented with 100 U/mL of penicillin and 100 μg/mL of streptomycin.

2.3. NF-κB activation

We performed an electrophoretic mobility shift assay (EMSA), essentially as previously described [21]. In brief, nuclear extracts prepared from TNF-treated cells ($1 \times 10^6\ mL^{-1}$) were incubated with ^{32}P-end-labeled 45-mer double-stranded NF-κB oligonucleotides (15 μg of protein with 16 fmol of DNA) from the human immunodeficiency virus long terminal repeat 5′-TTGTTACAA **GGGACTTTC** CGCTG **GGGACTTTC** CAGGGAGGCGTGG-3′ (boldface indicates NF-κB-binding sites) for 30 min at 37 °C, and the DNA-protein complex that formed was separated from free oligonucleotides on 6.6% native polyacrylamide gels. A double-stranded mutated oligonucleotide, 5′-TTGTTACAA **CTCACTTTC** CGCTG **CTCACTTTC** CAGGGAGGCGTGG-3′, was used to determine the specificity of NF-κB binding to DNA. The specificity of binding was also evaluated by competition with the unlabeled oligonucleotide. The dried gels were visualized with a Storm 820 phosporimager, and radioactive bands were quantified using Imagequant software (Amersham, Piscataway, NJ).

2.4. Cytotoxicity assay

The effect of simvastatin on the cytotoxic effects of chemotherapeutic reagents was determined by the MTT uptake method, as previously described [22]. In brief, 5000 cells were incubated with simvastatin for 12 h in triplicate on a 96-well plate and then treated with various concentrations of reagents for 24 h at 37 °C. Thereafter, an MTT solution was added to each well. After a 2 h incubation at 37 °C, extraction buffer (20% SDS and 50% dimethylformamide) was added, the cells were incubated overnight at 37 °C, and the optical density was measured at 570 nm using a 96-well multiscanner (MRX Revelation; Dynex Technologies, Chantilly, VA).

2.5. Live/dead assay

To measure apoptosis, we used the live/dead assay (Molecular Probes, OR), which determines intracellular esterase activity and plasma membrane integrity. This assay uses calcein, a polyanionic dye, which is retained in live cells and provides green fluorescence [14]. It also uses the ethidium monomer dye (red fluorescence), which can enter cells only through damaged membranes and bind to nucleic acids but is excluded by the intact plasma membrane of live cells.

In brief, 1×10^6 cells were incubated with 5 μM simvastatin for 12 h and then treated with 100 nM paclitaxel, 100 nM doxorubicin, and 5 μM 5-fluorouracil for 24 h at 37 °C. Cells were stained with the live/dead reagent (5 μM ethidium homodimer and 5 μM calcein-AM) and then incubated at 37 °C for 30 min. Cells were analyzed under a fluorescence microscope (Labophot-2; Nikon, Tokyo, Japan). The % values were derived by counting of the red and green cell numbers manually.

3. Results

The structures of the six statins (Fig. 1) suggest that natural statin molecules are more similar to each other than composed to synthetic molecules. The conditions used to investigate their effects on the NF-κB pathway had no effect on cell viability (data not shown).

3.1. Only natural statins suppressed TNF-induced NF-κB activation

KBM-5 cells were pretreated with different doses of statins for 12 h and then treated with 0.1 nM TNF for 30 min to activate NF-κB. Natural statins (Fig. 2, panels A–D) inhibited TNF-induced NF-κB activation; synthetic statins (panels E and F) did not. Among the natural statins, simvastatin was the most active, and pravastatin was the least active. Therefore, in all subsequent studies, only simvastatin was used.

3.2. Simvastatin suppressed NF-κB activation induced by chemotherapeutic agents

Whether chemotherapeutic agent-induced NF-κB activation could be modulated by simvastatin. KBM-5 cells were pretreated with simvastatin for 12 h and then with doxorubicin, paclitaxel, or 5-fluorouracil. As shown in Fig. 3, all three chemotherapeutic agents activated NF-κB, and simvastatin treatment suppressed this activation.

3.3. Simvastatin enhanced the cytotoxic effects of doxorubicin, paclitaxel, and 5-fluorouracil

Because NF-κB activation has been shown to suppress apoptosis [23], we determined whether simvastatin modulated chemotherapeutic agent-induced apoptosis in KBM-5 cells using the MTT assay. Simvastatin synergistically enhanced the cytotoxic effects of paclitaxel, doxorubicin, and 5-fluorouracil (Fig. 4A–C).

3.4. Simvastatin potentiated the apoptotic effects of doxorubicin, paclitaxel, and 5-fluorouracil

We next performed the live/dead assay and found that simvastatin up-regulated paclitaxel-induced apoptosis (from 12% to 55%), doxorubicin-induced apoptosis (10–45%), and 5-fluorouracil-induced apoptosis (3–37%) (Fig. 5A). The results of these assays suggest that simvastatin enhances the apoptotic effects of chemotherapeutic agents. We also investigated whether atorvastatin enhances paclitaxel-induced apoptosis. In agreement with NF-κB activity, atorvastatin did not significantly enhance the apoptotic effect of paclitaxel (Fig. 5B).

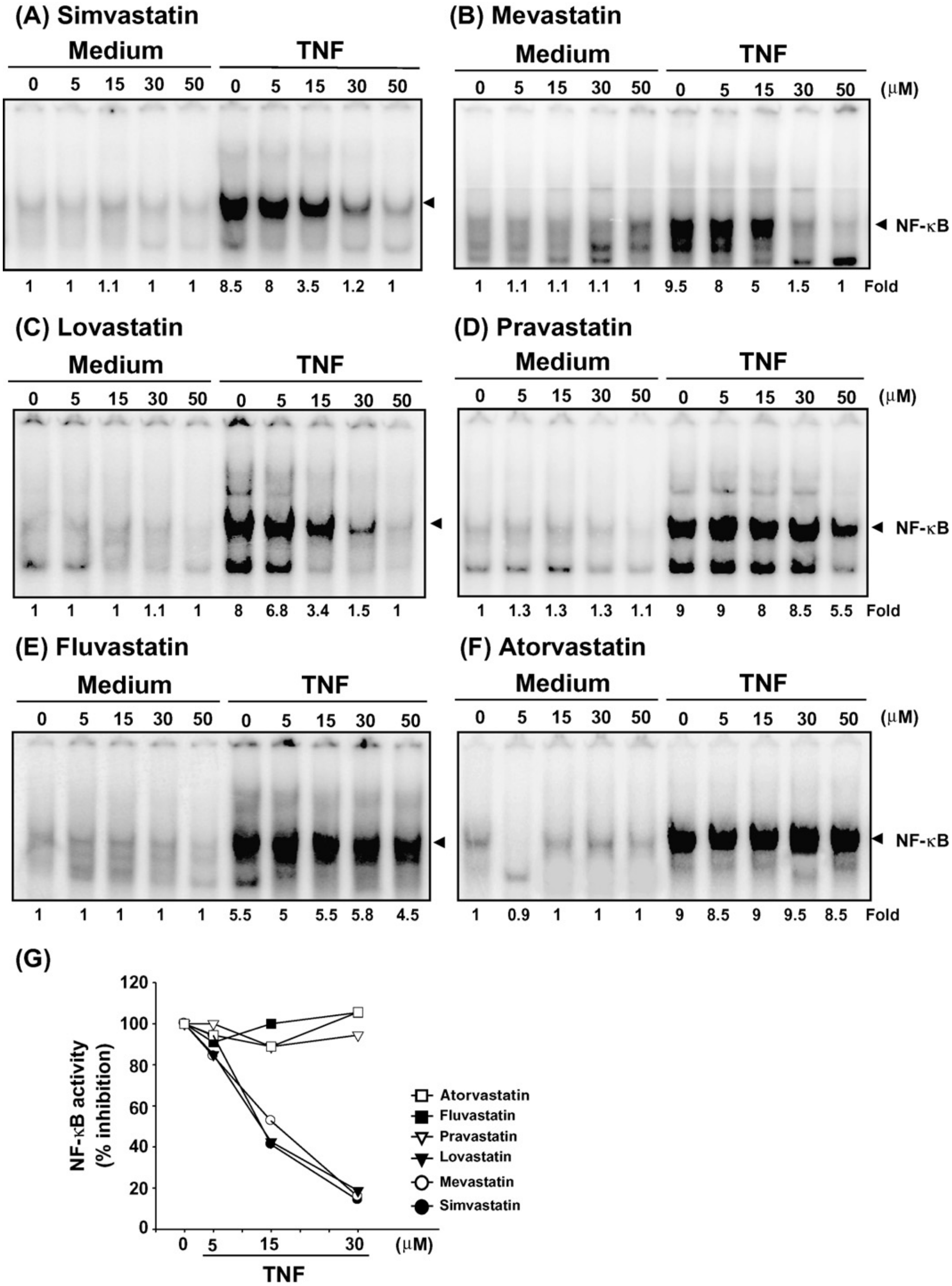

Fig. 2 – (A–F) Statins differ in their ability to suppress NF-κB activation in KBM-5 cells. KBM-5 cells ($1 \times 10^6\ mL^{-1}$) were preincubated with the indicated concentrations of statins for 12 h at 37 °C and then treated with 0.1 nM TNF for 30 min. Nuclear extracts were prepared and tested for NF-κB activation as described in Section 2. (G) Quantitative analysis of NF-κB inhibitory effect of different statins.

4. Discussion

In present study, we first investigated the effect of various statins on TNF and chemotherapeutic agents induced the NF-κB activation and apoptosis in myeloid leukemia cells. Only natural statins (simvastatin, mevastatin, lovastatin, and pravastatin) blocked TNF-induced NF-κB activation; synthetic statins did not. Simvastatin also suppressed doxorubicin-, paclitaxel-, and 5-fluorouracil-induced NF-κB activation in human myeloid leukemia cells. Simvastatin also potentiated the apoptosis induced by TNF and these chemotherapeutic agents.

Our results indicate that only statins derived from fungal fermentation inhibited NF-κB activation. Synthetic statins, fluvastatin and atorvastatin did not. Why lovastatin, simvastatin, mevastatin, and pravastatin suppressed NF-κB activa-

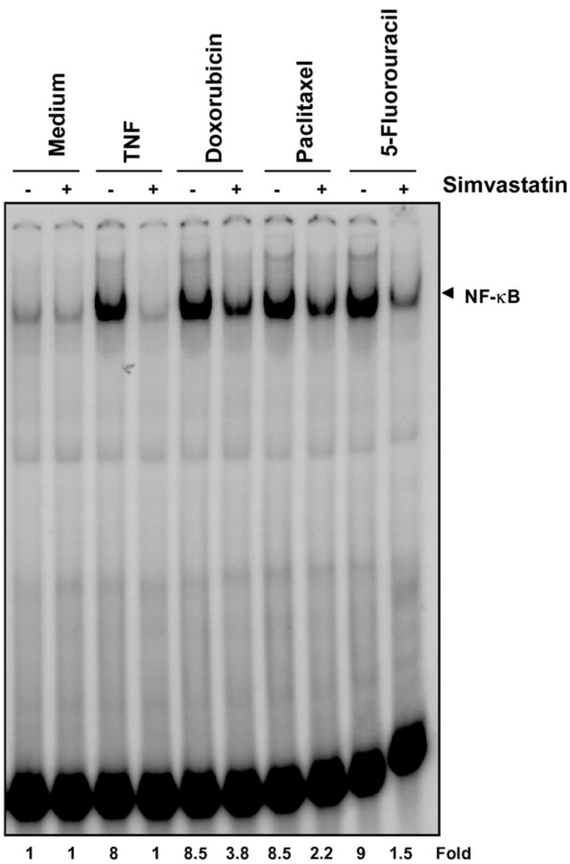

Fig. 3 – Simvastatin suppresses chemotherapeutic agent-induced NF-κB activation in KBM-5 cells. KBM-5 cells (1×10^6 mL^{-1}) were preincubated with 50 μM simvastatin for 12 h at 37 °C and then treated with TNF (0.1 nM) for 30 min, doxorubicin (2 μM) for 6 h, paclitaxel (100 μM) for 8 h, and 5-fluorouracil (100 μM) for 6 h. Nuclear extracts were prepared and tested for NF-κB activation as described in Section 2.

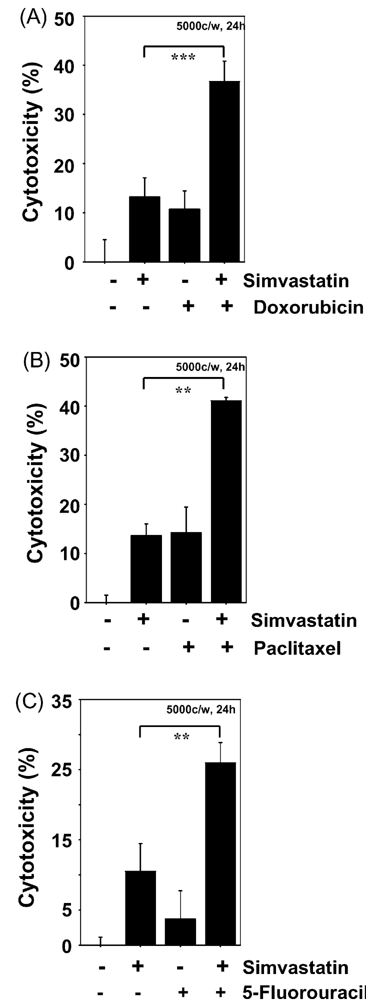

Fig. 4 – Simvastatin enhances cytotoxicity induced by chemotherapeutic agents. KBM-5 cells (5000 cells/0.1 mL) were incubated at 37 °C with (A) 100 nM doxorubicin; (B) 100 nM paclitaxel; (C) 5 μM 5-fluorouracil in the presence and absence of 5 μM simvastatin as indicated for 24 h, and viable cells were assayed using the MTT reagent. The results are expressed as mean cytotoxicity ± standard deviation from triplicate cultures. Determinations were made in triplicate. Data represent the mean of three measurements ± S.D. $^{***}p < 0.001$, $^{**}p < 0.01$.

tion and fluvastatin and atorvastatin did not is unclear; lovastatin, mevastatin, and simvastatin contain a lactone ring (Fig. 1), which may be essential for suppressing NF-κB activation. In contrast, fluvastatin and atorvastatin are acidic in nature. The K_i for 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase of lovastatin, simvastatin, and pravastatin are 0.6, 0.12, and 2.3 nM, respectively [24], which may also explain the difference in their activities.

Lovastatin in combination with TNF has been reported to inhibit proliferation of both murine melanoma and leukemia cells [25]. That mevastatin was found to suppress TNF-induced NF-κB activation is in agreement with the results of an earlier report study in endothelial cells [26]. Hilgendorff et al. [27] determined the effects of different statins on lipopolysaccharide-induced NF-κB activation in human monocytes; and reported that atorvastatin was the most effective in at blocking NF-κB activation, followed by simvastatin, pravastatin, lovastatin, and fluvastatin. Why these results differ so substantially from ours is not clear, it could relate to the inducer and cells used. Hilgendorff et al. [27] found that fluvastatin inhibited NF-κB activation by only 5%; this result is similar to ours.

Chemotherapeutic agents have been shown to induce NF-κB activation through the up-regulation of anti-apoptotic gene products that leads to chemoresistance [17]. In this study, we found that doxorubicin, paclitaxel, and 5-fluorourcil were potent inducers of NF-κB. This NF-κB activation, however, was abrogated by simvastatin. The combination of simvastatin and standard chemotherapeutic agents resulted in enhanced cytotoxic effects and potentiated apoptosis in tumor cells. Thus, simvastatin by the virtue of its NF-κB inhibitory effect can sensitize the cells to these drugs and hence overcome chemoresistance. Under identical conditions, atorvastatin did not have any substantial effect on NF-κB activation and apoptosis. We previously demonstrated that, concurrent with down-

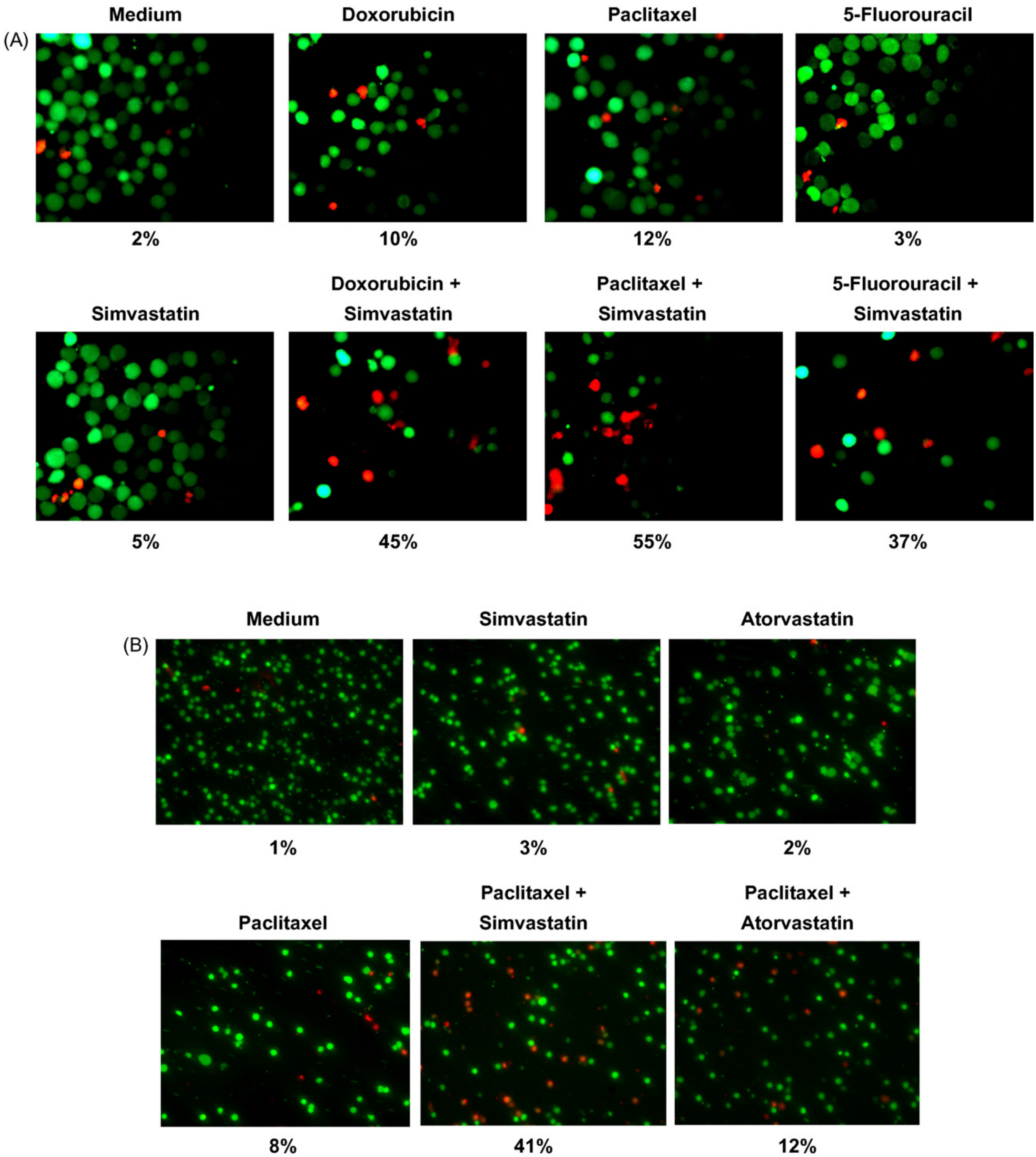

Fig. 5 – (A) Simvastatin potentiates apoptotic effect induced by chemotherapeutic agents. KBM-5 cells ($1 \times 10^6\ mL^{-1}$) were incubated with chemotherapeutic agents, alone or in combination with simvastatin, as indicated above, for 24 h. Cell death was determined by the calcein-AM-based live/dead assay, as described in Section 2. (B) Atorvastain has no effect on paclitaxel induced apoptosis. KBM-5 cells ($1 \times 10^6\ mL^{-1}$) were incubated with paclitaxel, alone or in combination with atorvastatin, as indicated above, for 24 h. Cell death was determined by the calcein-AM-based live/dead assay, as described in Section 2. Experiments were performed in triplicate. Data are from one representative experiment. The percentage of apoptosis is indicated below of each figure.

regulation of gene expression, apoptosis induced by TNF is potentiated by simvastatin [14]. This provides a novel opportunity to exploit statins, not only in the prevention but also the treatment of cancer.

Overall, our results provide novel insights into statins' role in overcoming chemoresistance through the modulation of NF-κB. Considering an extensive experience on the safety of statins in human subjects, statins may be a novel approach for the treatment of various cancers.

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