Effect of Simvastatin Alone and in Combination With Cytosine Arabinoside on the Proliferation of Myeloid Leukemia Cell Lines

Michael Lishner, Avigdor Bar-Sef, Avishay Elis, and Ina Fabian

ABSTRACT

Background: **Cholesterol biosynthesis is regulated by the activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase. Cholesterol and its derivatives are required in high concentrations by neoplastic proliferating cells for both DNA synthesis and cell growth. Thus, inhibition of HMG-CoA reductase could effect cell cycle progression and proliferation. Therefore, we examined the effect of an HMG-CoA reductase inhibitor (simvastatin) alone and in combination with cytosine arabinoside (ARA-C) on the proliferation of two AML cell lines.**

Methods: **AML blasts derived from two cell lines (HL-60 and AML-2) were incubated with increasing concentrations of either simvastatin alone or simvastatin alone for 24 hours with ARA-C added thereafter. The effect of the drugs on cell proliferation in liquid culture (^{3}H thymidine uptake) and on clonogenic assay was analyzed.**

Results: **We found that the number of proliferating AML blasts (suspension cultures) and colony formations (agar cultures) of both cell lines declined significantly after incubation with simvastatin. Preincubation of both cell lines with simvastatin by the addition of increasing concentrations of ARA-C produced a degree of growth inhibition that was significantly greater than that of the individual compounds. This antigrowth interaction was additive rather than synergistic.**

Conclusions: **We conclude that simvastatin has a major antiproliferative effect on AML blasts in vitro. Also, the combination of simvastatin and ARA-C significantly enhanced the antiproliferative effect of each drug. These findings may open new avenues in both the laboratory and clinical research of the treatment of leukemia. (J Investig Med 2001;49:319–324) Key Words: AML • simvastatin • ARA-C**

INTRODUCTION

Cholesterol is either synthesized intracellularly or obtained from the plasma by receptor-mediated endocytosis of low density lipoprotein.[1] Cholesterol biosynthesis is regulated by the activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase.[1] The product of this enzyme, mevalonic acid, is an essential precursor of several important cellular constituents. These include, among other, sterols (especially cholesterol) that are a major structural component of cell membranes, and the side chains of isopentenyladenosine and ubiquinone that are involved in the progression of cell cycle and cell survival.[2,3] Furthermore, it has been shown recently that membrane association is an important function for mevalonate-derived modifications of several important proteins such as cellular membrane G proteins, proteins encoded by oncogenes (Ras proteins), and nuclear proteins (lamins).[4,5]

Cancer cells seem to require an increased concentration of cholesterol and its precursors. Indeed, proliferating cells posses a high rate of cholesterol production and a high rate of HMG-CoA reductase activity.[6] Cholesterol is required in these neoplastic cells for cell proliferation and for the progression of cell cycle.[7] Also, some proteins (such as p21 ras) that are involved in signal transduction undergo posttranslational modification by the addition of isoprenoid group to the carboxyl terminus.[8]

Because mevalonic acid is the precursor of many metabolites that are involved in both DNA synthesis and cell

From the Department of Medicine, Meir Hospital (M.L., A.B.-S., A.E.), Kfar-Saba, and Department of Cell Biology and Histology, Sackler Faculty of Medicine, Tel-Aviv University (I.F.), Israel.

Address correspondence to: M. Lishner, MD, Department of Medicine, Meir Hospital, Sapir Medical Center, Kfar-Saba 44281, Israel. E-mail lishnm@internet-zahav.net

growth, inhibition of the key enzyme HMG-CoA reductase could affect cell cycle progression and proliferation. Indeed, both in vivo and in vitro studies showed that statins (HMG-CoA reductase inhibitors) inhibit cell proliferation[9,10] and modulate changes in the cell cycle[11] and cell morpholgy.[12] Also, lovastatin slowed growth rate of rat neuroblastoma.[12]

Only a few studies evaluated the effect of HMG-CoA reductase inhibitors on the survival of blasts of acute myeloid leukemia (AML) and AML cell progenitors. Newman et al[13] have shown that simvastatin inhibits the proliferation of human AML progenitors that form colonies in semisolid agar to a greater extent than progenitors from normal bone marrow. The same group of scientists recently reported that simvastatin also inhibited proliferation of AML cells in vivo in severe combined immunodeficient mice.[14] Bansal et al[15] have suggested that HMG-CoA reductase inhibitors may have a therapeutic value in the treatment of leukemia.

In this study, we investigated and characterized the in vitro effects of simvastatin, an HMG-CoA reductase inhibitor, on the proliferation and colony formation of two AML cell lines (HL60 and AML-2). We also tested the hypothesis that simvastatin would potentiate the inhibitory effect of cytosine arabinoside (ARA-C) on the growth of AML blasts.

MATERIALS AND METHODS

Drugs

Simvastatin (a gift from Merck Sharp and Dohme Ltd, Petah Tikva, Israel) was dissolved in dimethyl sulfoxide (4.0 mg/mL) followed by stepwise dilution with Iscove's Modified Dulbecco's Medium (IMDM) (Gibco, Grand Island, NY). ARA-C (a gift from Upjohn Co, Kalamazoo, Mich) was dissolved in IMDM.

Cell Culture

Cells from two continuous AML cell lines were studied: HL-60[16] and OCI/AML-2 (a gift from Dr Minden, Ontario Cancer Institute).[17] The cell lines were maintained in IMDM, supplemented with fetal bovine serum (growth medium; Hyclone Laboratories, Logan, Utah).[16] Cells were harvested in their logarithmic growth phase for experiments.

Incubation of Cells With Drugs in Liquid Culture

HL-60 cells or AML-2 cells were seeded at 1×10^5/mL growth medium (in 0.8-mL portions) in 24-well tissue culture dishes. Simvastatin was added to each well (0.5–10 μg/mL) and cell cultures were incubated at 37°C in an atmosphere of 5% CO_2/air for 5 days. In other experiments, 24 hours after the addition of simvastatin, ARA-C ($10^{-9}-10^{-11}$ M) was added to the wells and cells were incubated for 4 additional days. In the controls in all the experiments, we added dimethyl sulfoxide at the same concentrations as for the corresponding experimental groups in which simvastatin was used. After incubation, cultures were assayed for surviving, proliferating, and colony-forming cells.

Cell Proliferation and ^{3}H-Thymidine Incorporation

Cell viability and proliferation were determined by trypan blue exclusion and by ^{3}H-thymidine incorporation. On the fifth day after incubation of the cells with the drug(s), 180 μL of cell suspension was transferred to each well in 96-well plates in quadruplicates. Cultures were pulsed with 1 μCi/mL of ^{3}H-thymidine (1 mCi/mol/L; Nuclear Research Center, Negev, Israel; final volume, 200 μL) and harvested 4 hours later onto glass filter with an automated cell harvester under hypotonic conditions. Filter discs were dried and counted in a Packard Tri-carb liquid scintillation counter (Downers Grove, Ill).

Clonogenic Assay

HL-60 cells or AML-2 cells, grown in liquid culture in the presence of the drug(s), were harvested on day 5, cell viability was determined, and 5×10^3 viable cells were plated in 35-mm Petri dishes containing IMDM, 15% fetal bovine serum, and 0.3% agar. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2/air. On day 8, cell aggregates containing 40 cells or more were enumerated as colonies.

Statistical Analysis

Statistical analyses were performed using the analysis of variance with repeated measures test.

RESULTS

Effect of Exposure to Simvastatin

The number of proliferating AML blasts (suspension cultures) and colony formation (agar cultures) of both cell lines declined significantly after incubation with simvastatin. On the fifth day of culture, the number of HL-60 cells and AML-2 cells not treated with drugs (control cells) was $19\pm1.9\times10^5$/mL (n=6) and $10.9\pm0.6\times10^5$/mL (n=4), respectively. The proliferation of HL-60 cells or AML-2 cells in liquid culture in the presence of 1 μg/mL of simvastatin for 5 days was significantly inhibited, as determined by trypan blue exclusion (by 66% and 42%, respectively; $P<0.05$) (Figure 1A), and DNA synthesis was significantly inhibited by 90%, as determined by ^{3}H-thymidine incorporation ($P<0.05$) (Figure 1B). When

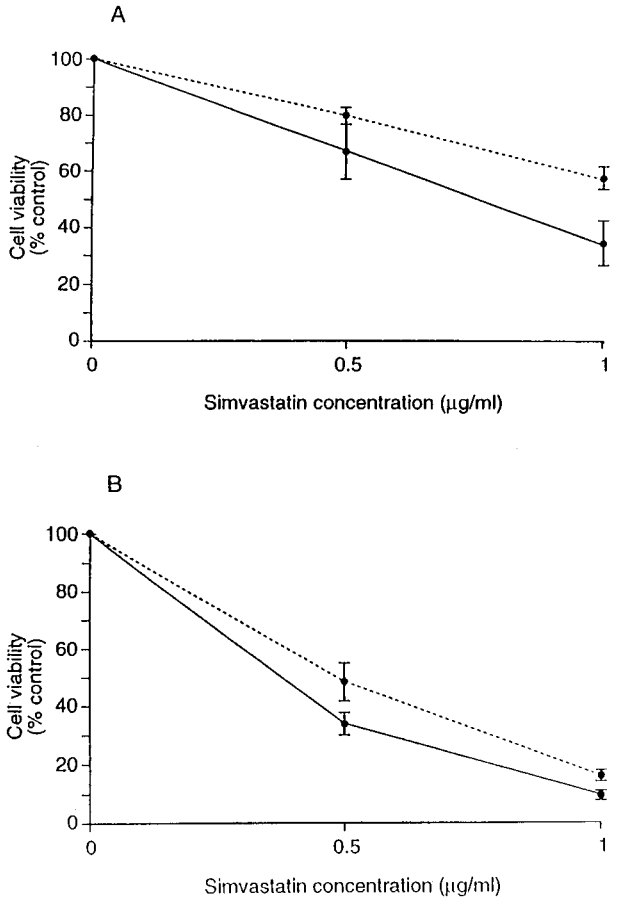

Figure 1. *Effect of simvastatin on the growth of AML blasts in liquid culture. HL-60 cells (solid line) and AML-2 cells (dotted line) were grown in liquid culture in the presence of increasing concentrations of simvastatin. (A) On the fifth day, cells were counted in a hemocytometer chamber. Viability was determined by trypan blue exclusion. (B) The cells were grown as described in (A), and ^{3}H-thymidine incorporation studies were performed as described in Materials and Methods. Results are the mean±SE of 4 experiments performed in duplicates for trypan blue exclusion studies and in quadruplicates for ^{3}H-thymidine incorporation studies.*

higher concentrations of simvastatin (5 μg/mL) were used, viability was reduced to less than 10% (data not shown), and therefore, the rest of the experiments were performed at concentrations of 0.5 and 1 μg/mL.

The effect of simvastatin on clonogenic potential of both cell lines was evaluated in the next set of experiments. Figure 2 demonstrates that, at a concentration of 1 μg/mL, a significant decrease in clonogenic cells was observed (up to 30% of the controls; $P<0.05$). A similar reduction in clonogenic potential was found in both cell lines.

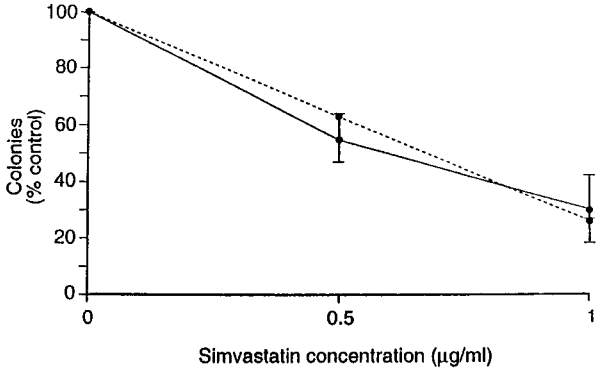

Figure 2. *Effect of simvastatin on the clonogenic potential of AML blasts. HL-60 cells (solid line) and AML-2 cells (dotted line) were grown in liquid culture in the presence of increasing concentrations of simvastatin. On the fifth day 5×10^3 viable cells were plated in semisolid culture, as described in Materials and Methods. On day 8, colonies (containing>40 cells) were enumerated. Results are the mean±SE of 3 experiments performed in duplicates.*

Combined Effect of Simvastatin and ARA-C

The effect of simvastatin in combination with increasing concentrations of ARA-C was explored in each cell line. ARA-C alone inhibited cell proliferation and colony formation in a concentration-dependent fashion in the range of concentrations used. Preincubation of AML-2 cells with simvastatin (0.5 or 1 μg/mL) followed by the addition of $10^{-11}-10^{-9}$ M ARA-C resulted in a marked and significant inhibition of proliferation (compared with ARA-C alone), as determined by the trypan blue exclusion test (Figure 3A), [^{3}H] thymidine incorporation assay (Figure 3B), and clonogenic assay (Figure 3C). Similar statistically significant inhibition was also demonstrated in HL60 cells (Figure 4 A–C). Thus, combination of simvastatin with ARA-C produced an antigrowth interaction that was more active than the individual compounds in inhibiting cell growth. To determine whether the combined effect of simvastatin and ARA-C was additive or synergistic, we used analysis of variance. We found the combination to be additive.

DISCUSSION

The main finding of our study is that simvastatin significantly reduced cell proliferation and clonogenic potential of two AML cell lines, HL-60 and AML-2. In addition, preincubation of the cells with simvastatin before exposure to ARA-C increased the cytotoxic effect of ARA-C significantly in an additive fashion and decreased DNA synthesis as manifested by [^{3}H] thymidine uptake. These results lend further support to the concept of antiprolifera-

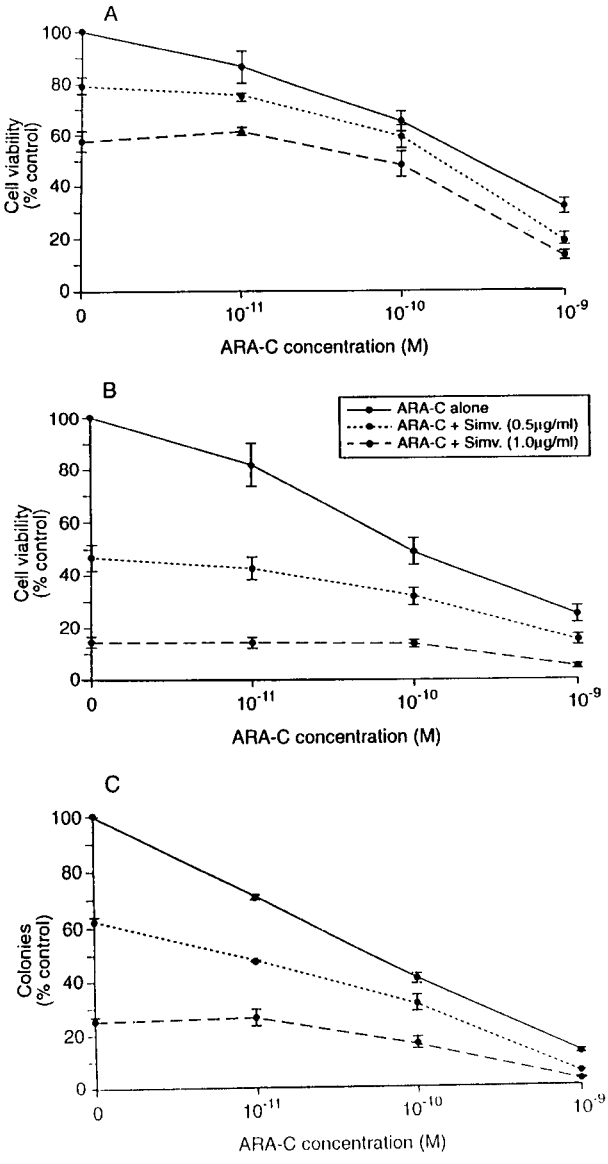

Figure 3. *Effect of ARA-C and a combination of ARA-C and simvastatin on the growth of AML-2 cells. AML-2 cells (1×10^5/mL) were grown in liquid culture in the presence of 0–1 μg/mL simvastatin for 5 days. After 24 hours of incubation of simvastatin, ARA-C was added to the cultures and cells were incubated for 4 additional days. After incubation, cells were assayed for survival. (A) Trypan blue exclusion test. (B) ^{3}H thymidine incorporation assay and (C) clonogenic assay: aliquots of 5×10^3 viable cells were plated in agar, as described in Materials and Methods. Colony growth was scored after incubation for 8 days. Results are expressed as mean±SE of experiments performed in duplicates for trypan blue and clonogenic assay and in quadruplicates for ^{3}H thymidine assay.*

tive effects of simvastatin and may open new directions in the clinical management of AML.

The precise mechanism of action of statins on the proliferation of malignant cells is unknown. Farnesyl residues derived from the conversion of mevalonate to cholesterol have a major regulatory role in cell growth.[18] It was repeatedly shown that posttranslational fernesylation of ras protein (P21 ras protein) and the nuclear membrane protein lamin B is required for their attachment to the cell membrane and is essential for oncogenic transformation.[5,19,20] The inhibition of mevalonate synthesis would limit the availability of farnesyl pyrophosphate and thereby limit the cell growth promotion of the ras oncoprotein.[21] However, Clutterbuck et al[14] have shown recently that ketoconazole, which inhibits cholesterol biosynthesis after farnesyl pyrophosphate synthesis, had a similar effect to simvastatin on HL-60 colony development. Furthermore, the clonogenicity of N-ras mutated primary AML cells was not more sensitive to simvastatin than those of a population without the mutation. It was concluded that inhibition of AML cell proliferation by simvastatin may be independent of the ras signaling pathway. Because statins could interfere with the function of more than 40 other proteins that are normally isoprenylated, it is possible that the inhibition of proliferation by simvastatin may be related to the interference of function of other proteins.[22]

ARA-C is the most important agent in the treatment of AML. In this study, the combination of simvastatin and ARA-C enhanced the antiproliferative effect of the drugs. In previous in vitro studies, HMG-CoA reductase inhibitors had a synergistic or additive effect when used in combination with various antineoplastic agents (BCNU, interferon-β, mitomycin-C) in different malignant cell lines (hepatoma cell culture-4, human glioma cells).[23,24] Simvastatin was used in our study at concentrations that are usually achieved in the plasma of patients treated for hypercholesterolemia (0.1–10 μm). At these concentrations, simvastatin alone inhibited cell proliferation significantly. The combination of the drugs induced a major increase in the inhibition of cell proliferation over each drug alone. This may allow the combination of lower concentrations of antineoplastic agents with therapeutic doses of simvastatin to achieve the same inhibition of cell growth. It also may allow the increase of neoplastic cell kill over what is achieved with chemotherapy alone, because it was already shown that AML progenitors have greater sensitivity to the antiproliferative properties of simvastatin compared with normal bone marrow progenitors.[13]

The possible effects of HMG-CoA reductase inhibitors on tumor growth have drawn much attention. An inhibitory effect was demonstrated in rats,[25] mice,[26] or severe combined immunodeficient mice implanted with human tumors.[27] A phase I study in patients with high-grade

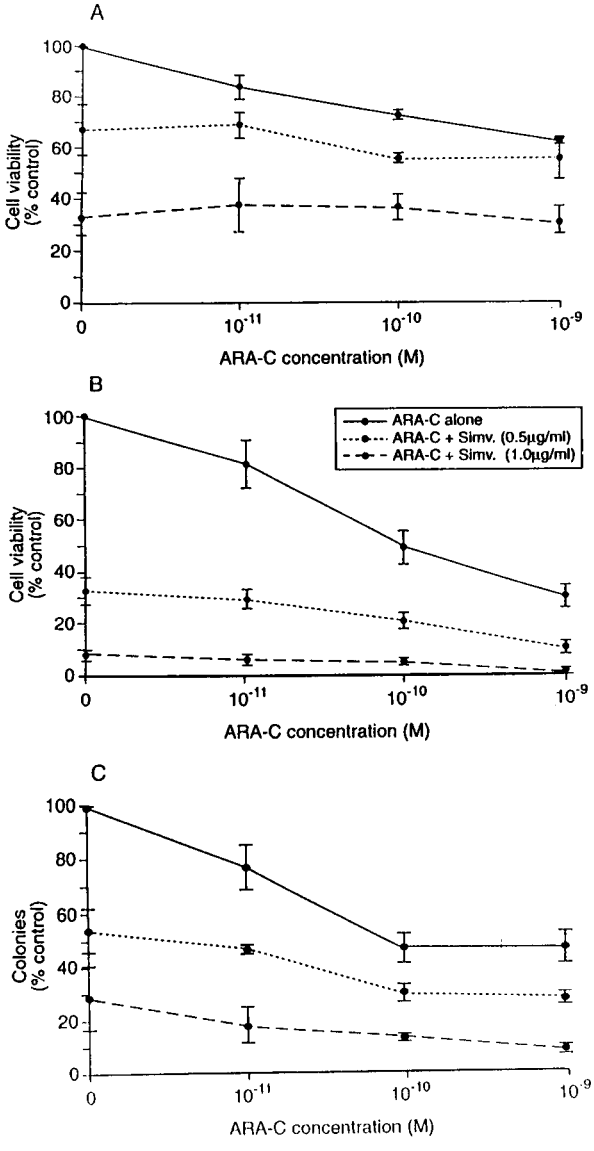

Figure 4. *Effect of ARA-C and a combination of ARA-C and simvastatin on the growth of HL-60 cells. HL-60 cells (1×10^5/mL) were grown in liquid culture in the presence of 0–1 μg/mL simvastatin for 5 days. After 24 hours of incubation with simvastatin, ARA-C was added to the cultures and cells were incubated for 4 additional days. After incubation, cells were assayed for survival. (A) Trypan blue exclusion test. (B) ^3H thymidine incorporation assay and (C) clonogenic assay: aliquots of 5×10^3 viable cells were plated in agar, as described in Materials and Methods. Colony growth was scored after incubation for 8 days. Results are expressed as mean±SE of 4 experiments performed in duplicates for trypan blue and clonogenic assay and in quadruplicates for ^3H thymidine assay.*

glioma was disappointing.[28] We suggest that the model we describe be used in future clinical studies in patients with leukemia resistant to chemotherapy. The possible therapeutic options include either simvastatin alone or in combination with chemotherapeutic medications.

REFERENCES

1. Goldstein JL, Brown MS. Regulation of mevalonate pathway. Nature 1990;343:425–430.
2. Nakanishi M, Goldstein JL, Brown MS. Multivalent control of HMG-CoA reductase. Mevalonate-derived product inhibits translation of mRNA and accelerates degradation of enzyme. J Biol Chem 1988;263: 8929–8937.
3. Maltese WA, Sheridan KM. Isoprenoid synthesis during the cell cycle. J Biol Chem 1988;263:10104–10110.
4. Maltese WA, Sheridan KM, Repko EM, Erdman RA. Post translational modification of low molecular mass GPT-binding proteins by isoprenoid. J Biol Chem 1990;265:2148–2155.
5. Wolda SL, Glomset JA. Evidence for modification of Lamin B by a product of mevalonic acid. J Biol Chem 1988;263:5997–6000.
6. Ho YK, Smith G, Brown MS, Goldstein JL. Low density lipoprotein (LDL) receptor activity in human acute myelogenous leukemia cells. Blood 1978;52:1099–1113.
7. Quesney-Huneeus V, Galick HA, Siperstein MD, Erickson SK, Spencer TA, Nelson IA. The dual role of mevalonate in the cell cycle. J Biol Chem 1983;258:378–385.
8. Hoffman M. Playing tag with membrane proteins. Science 1991; 254:650–651.
9. Habenicht AJR, Glomset JA, Ross R. Relation of cholesterol and mevalonic acid to the cell cycle in smooth muscle and Swiss 3T3 growth factor. J Biol Chem 1980;255:5134–5140.
10. Morris TJ, Palm SL, Pena J, Furcht LT, Buchwald H. Inhibition of hepatoma tissue culture growth by lovastatin: A novel chemotherapeutic strategy. Surg Forum 1991;42:952–954.
11. Takobisiak M, Bruno S, Skierski JS, Darzynkiewicz Z. Cell cycle specific effects of lovastatin. Proc Natl Acad Sci U S A 1980;77: 3957–3961.
12. Maltese WA, Sheridan KN. Differentiation of neuroblastoma cells induced by an inhibitor of mevalonate. Relation of neuritic outgrowth and acetylcholinesterase activity to changes in cell proliferation and blocked isoprenoid synthesis. J Cell Physiol 1985;125: 540–558.
13. Newman A, Clutterbuck RD, Powels RL, Millar JL. Selective inhibition of primary acute myeloid leukemia cell growth by simvastatin. Leukemia 1994;8:2023–2029.
14. Clutterbuck RD, Millar BC, Pawels RL, Newman A, Catovsky D, Jarman M, Millar JL. Inhibitory effect of simvastatin on the proliferation of human myeloid leukemia cells in severe combined immunodeficient (SCID) mice. Br J Haematol 1998;102:522–527.
15. Bansal N, Houle AG, Melnykovych G. Comparison of dexamethazone and lovastatin (mevinolin) as growth inhibitors in cultures of T-cell derived human acute leukemia lines (CEM). Leuk Res 1989;13: 875–882.
16. Lishner M, Shur I, Bleiberg I, Rudi A, Kashman Y, Fabian I. Sensitivity of hematopoietic progenitors of acute myeloblastic leukemia to new compounds derived from marine organisms. Leukemia 1995;9: 1543–1548.
17. Hu ZB, Yang GS, Li M, Miyamoto N, Minden MD, McCulloch EA. Mechanism of cytosine arabinoside toxicity to the blast cells of acute myeloblastic leukemia. Leukemia 1995;9:789–798.
18. Buchwald H. Cholesterol inhibition, cancer, and chemotherapy. Lancet 1992;339:1154–1156.

19. Hancock JT, Magee AI, Childs JE, Marshall CJ. All ras proteins are polyisoprenylated but only some are palmitoylated. Cell 1989;57: 1167–1177.

20. Schafer WR, Kim R, Sterne R, Thorner J, Kim SH, Rine J. Common modifications of trimeric G proteins and ras protein: Involvement of polyisoprenylation. Science 1989;245:379–384.

21. Kung HF, Smith MR, Bekesi E, Maune V, Stacey DW. Reversal of transformed phenotype by monoclonal antibodies against Ha-ras P21 proteins. Exp Cell Res 1986;162:363–371.

22. Mikulski SM, Viera A, Darzynkiewicz Z, Shogen K. Synergism between a novel amphibian oocyte ribonuclease and lovastatin in inducing cytostatic and cytotoxic effects in human lung and pancreatic carcinoma cell lines. Br J Cancer 1992;66:304–310.

23. Morris PJ, Palm S, Frucht L, Buchwald H. The effect of lovastatin as an adjuvant to mitomycin C on HPC-4 cell growth. Presented at a conference of the Society of University Surgeons, February 15, 1992.

24. Soma RS, Pagliarini P, Butti G, Paoletti R, Paoletti P, Fumagali R. Simvastatin, an inhibitor of cholesterol biosynthesis, shows a synergistic effect with N, N'-Bis (2-chloroethyl)–N-Nitrosurea and β-interferon on human glioma cells. Cancer Res 1992;52:4348–4355.

25. Kawata S, Sakimoto LH, Ishiguro H, Yamusaki E, Inui Y, Matsuzawa Y. Effect of pravastatin, a potent 3-hydroxy-3-methylglutaryl–coenzyme A reductase inhibitor, on survival of AH 30 hepatoma bearing rats. Jpn J Cancer Res 1992;83:1120–1123.

26. Jani JP, Specht S, Stemmler N, Blanock K, Singh SV, Gupta V, Katoh A. Metastasis of B16F10 mouse melanoma inhibited by lovastatin, an inhibitor of cholesterol biosynthesis. Invasion Metastasis 1993;13: 314–324.

27. Sebti SM, Tkalcevic GT, Jani JP. Lovastatin, a cholesterol biosynthetic inhibitor, inhibits the growth of human H-ras transformed cells in nude mice. Cancer Commun 1991;3:141–147.

28. Thaibault A, Samid D, Tompkins AC, Figg WD, Cooper MR, Hohl RJ, Trepel J, Liang B, Patronas N, Venzon DJ, Reed E, Myers CE. Phase I study of lovastatin, an inhibitor of the mevalonate pathway, in patients with cancer. Clin Cancer Res 1996;2: 483–493.