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The Mevalonate/Isoprenoid Pathway Inhibitor Apomine (SR-45023A) Is Antiproliferative and Induces Apoptosis Similar to Farnesol

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Apomine (SR-45023A) is a new antineoplastic compound which is currently in clinical trials and representative of the family of cholesterol synthesis inhibitors 1,1-bisphosphonate esters. Apomine inhibits growth of a wide variety of tumor cell lines with IC_{50} values ranging from 5 to 14 μM. The antiproliferative activity of apomine was studied in comparison with that of other inhibitors of the mevalonate/isoprenoid pathway of cholesterol synthesis, simvastatin, farnesol, and 25-hydroxycholesterol. All these compounds inhibit 3-hydroxy-3-methylglutaryl-coenzyme A reductase activity. Apomine (IC_{50} = 14 μM), simvastatin (IC_{50} = 3 μM), farnesol (IC_{50} = 60 μM), and 25-hydroxycholesterol (IC_{50} = 2 μM) inhibited HL60 cell growth. Growth inhibition due to simvastatin was reverted by mevalonate, whereas the antiproliferative activity of apomine, farnesol, and 25-hydroxycholesterol was not. Apomine triggered apoptosis in HL60 cells in less than 2 h. Apomine and farnesol induced caspase-3 activity at concentrations similar to their IC_{50} values for cell proliferation, whereas a 10-fold excess of simvastatin was necessary to trigger apoptosis compared to its potency on proliferation. Caspase-3 activity was not induced by 25-hydroxycholesterol. The overall similar profile on mevalonate synthesis inhibition, cell growth inhibition, and apoptosis suggests that apomine acts as a synthetic mimetic of farnesol.

The majority of the standard chemotherapeutic drugs are cytotoxic compounds impairing directly DNA replication or cytoskeleton organization, leading to apoptotic cell death in both normal and tumor cells. Apoptosis caused by these drugs is a consequence of the extent of the cellular damage. It is however possible to interfere with cell proliferation by other means. For example, it has been shown that cell proliferation depends on the mevalonate/isoprenoid pathway which is at the origin of farnesol pyrophosphate and geranylgeranyl pyrophosphate (1). These isoprenoid derivatives are essential for the function of proteins involved in cell proliferation such as ras (2) and rho (3). Compounds specifically targeting protein farnesyl transferases and protein geranylgeranyl transferases are also currently under clinical investigation (4, 5).

Interfering with other steps of the mevalonate/isoprenoid pathway could also prove valuable for cancer treatment. So far, drugs targeting the mevalonate pathway were developed with the primary goal of decreasing high level plasma cholesterol which is an established risk factor for the incidence of coronary heart disease (6). The statins, which are competitive inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase), the rate limiting step of the pathway, reduce the rate of isoprenoid synthesis and decrease the activity of ras, rho and other proteins requiring isoprenylation (7, 8). Statins are therefore antiproliferative, in addition to inhibiting cholesterol synthesis. These antiproliferative properties were shown to be mechanistically linked to HMG-CoA reductase inhibition since they could be reverted by the addition of mevalonate (9, 10). Extended cell exposure to statins was reported to trigger apoptosis (11). Although in some cases high doses of statins have been demonstrated to inhibit tumor growth in animal models (12), they have not been successful for cancer treatment. The reason for failure in the clinic might be the fact that the current statins were developed as hypocholesterolemic agents targeting the liver and are in general poorly systemically bioavailable (13). There is then a clear rationale for identifying novel inhibitors of the mevalonate/isoprenoid pathway which provide improved systemic bioavailability and increased tumor exposure.

In addition to the direct competitive inhibition by statins, HMG-CoA reductase activity is regulated at posttranscriptional levels by oxysterols like 25-hydroxycholesterol and by isoprenoid metabolites like farnesol (14, 15). These sterols and non-sterol metabo-

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lites downstream of mevalonate are known to increase the degradation of HMG-CoA reductase (16). Specifically, farnesol has been shown to modulate mevalonate synthesis by this means (17) and to trigger apoptosis (18). Although the mechanism by which farnesol promotes cell death is not yet understood, an interaction with the ceramide pathway was hypothesized (19, 20). Cell growth is also inhibited by 25-hydroxycholesterol (10).

Despite their antiproliferative properties, farnesol and 25-hydroxycholesterol would not be eligible as ideal drug candidates due to their rapid metabolism with poor bioavailability and insufficient exposure of the tumor cells.

Members of the class of 1,1-bisphosphonate are potent inhibitors of cholesterol synthesis (21), inhibiting HMG-CoA reductase activity by increasing its degradation (22). Contrary to the statins, they were shown to lower plasma cholesterol in animal species including rodents (23) and to have an excellent systemic bioavailability. Here we describe apomine, a bisphosphonate ester also termed SR-45023A and SR-9223i (tetraisopropyl 2-(3,5-di-tert-butyl-4-hydroxyphenyl) ethyl-1,1-diphosphonate) that inhibits cell proliferation and induces apoptosis in a wide variety of tumor cell lines. The antiproliferative activity and proapoptotic properties of apomine were compared with those of simvastatin, farnesol and 25-hydroxycholesterol, other compounds that affect HMGCoA reductase.

METHODS

Cells. HL60 (promyelocytic leukemia), SW480 and SW620 (colorectal adenocarcinoma), HT29 (colon adenocarcinoma), HepG2 (hepatocellular carcinoma) and MesSA (uterine sarcoma) cell lines were obtained from the American Tissue Culture Collection (ATCC). The MCF7 (breast carcinoma) cell line was obtained from the National Cancer Institute (NCI).

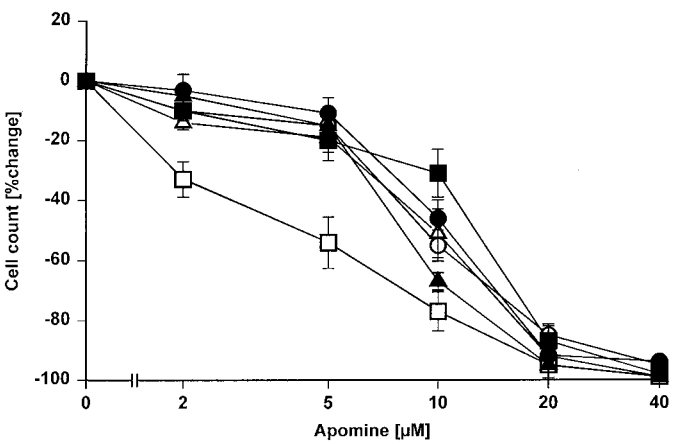

FIG. 1. Antiproliferative activity of apomine on cell lines derived from leukemia (●; HL60), colon (○; SW480; and ■; HT29), liver (□; HepG2), uterus (▲; MesSA), and breast (△; MCF7) tissues.

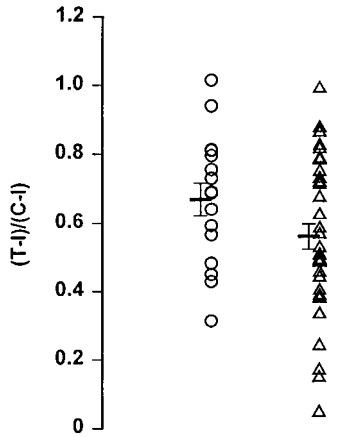

FIG. 2. Sensitivity to 10 μM apomine of wild-type (○) and mutant (△) p53 cell lines from the NCI primary screen panel, expressed as the ratio (T − I)/(C − I) where *T* is the number of cells in the treated culture at the end of the incubation, *C* is the number of cells in the control culture at the end of the incubation, and *I* is the initial number of cells.

Antiproliferative activities on human tumor cell lines. Cells were seeded in 24-well plates, 5×10^5 cells per well, in the medium described by the provider, containing 10% FCS (20% for HL60). They were allowed to install for 48 h prior treatment. The medium was then changed for fresh medium with FCS, containing 0 to 40 μM apomine. The cells were then incubated for 72 h. At the end of the incubation period, the cell were trypsinized (except HL60 grown in suspension) and counted using a Coulter Counter Z1 (Coulter Electronic).

DNA laddering. Colon SW480 cells were plated into 50-cm square plates, 1.5×10^6 cells per plate and allowed to install for 48 h. The medium was then removed and 10 ml of fresh medium without FCS and containing the compounds to be tested was added. The plates were incubated for 16 h at 37°C. The adherent and floating cells were lysed in 5 ml buffer (10 mM Tris, 100 mM NaCl, 10 mM EDTA, 0.5% SDS, pH 8.0). The samples were incubated with 0.1 mg/ml of proteinase K for 4 h at 50°C and were extracted with 1 vol of phenol: chloroform (1:1). The aqueous phase was precipitated with 1/10 volume of sodium acetate 3 M and 2.5 vol of ethanol. The nucleic acids pellet was washed in 70% ice-cold ethanol, resuspended in 5 ml TE buffer (Tris 10 mM, EDTA 1 mM) with 5 μg/ml RNAse A and incubated for 1 h at 37°C. The DNA was precipitated as described above and resuspended in 1 ml TE buffer. Twenty micrograms of DNA were loaded onto 1% agarose gel.

Nucleosome release into the cytoplasm. The cytoplasmic histone-associated DNA assay was carried out in accordance with the instructions of the manufacturer (Cell Death Detection ELISA^{PLUS}, Roche Diagnostics). Briefly, 5×10^4 HL60 cells were incubated for 2 h in 1 ml medium containing the compound. After the incubation, the cells were washed with 1 ml medium and lysed for 30 min at 4°C in the buffer provided by the manufacturer. The lysate was centrifuged and the supernatant containing the nucleosomes released into the cytoplasm was assayed by ELISA. Biotin-conjugated anti-histone antibody was incubated with the sample and peroxidase-conjugated anti-DNA antibody. The resulting complexes were bound to a streptavidin-coated microwell plate and the secondary conjugated antibody was detected by incubation with the peroxidase substrate.

Caspase-3 activity. HL60 cells (2×10^6) were incubated with the compounds for 2 h in FCS-free medium. They were washed in PBS, then lysed in 200 μl 10 mM Tris–HCl (pH 7.5), 10 mM NaH_2PO_4, 10 mM Na_2HPO_4, 130 mM NaCl, 1% Triton X-100 and 10 mM NaPPi.

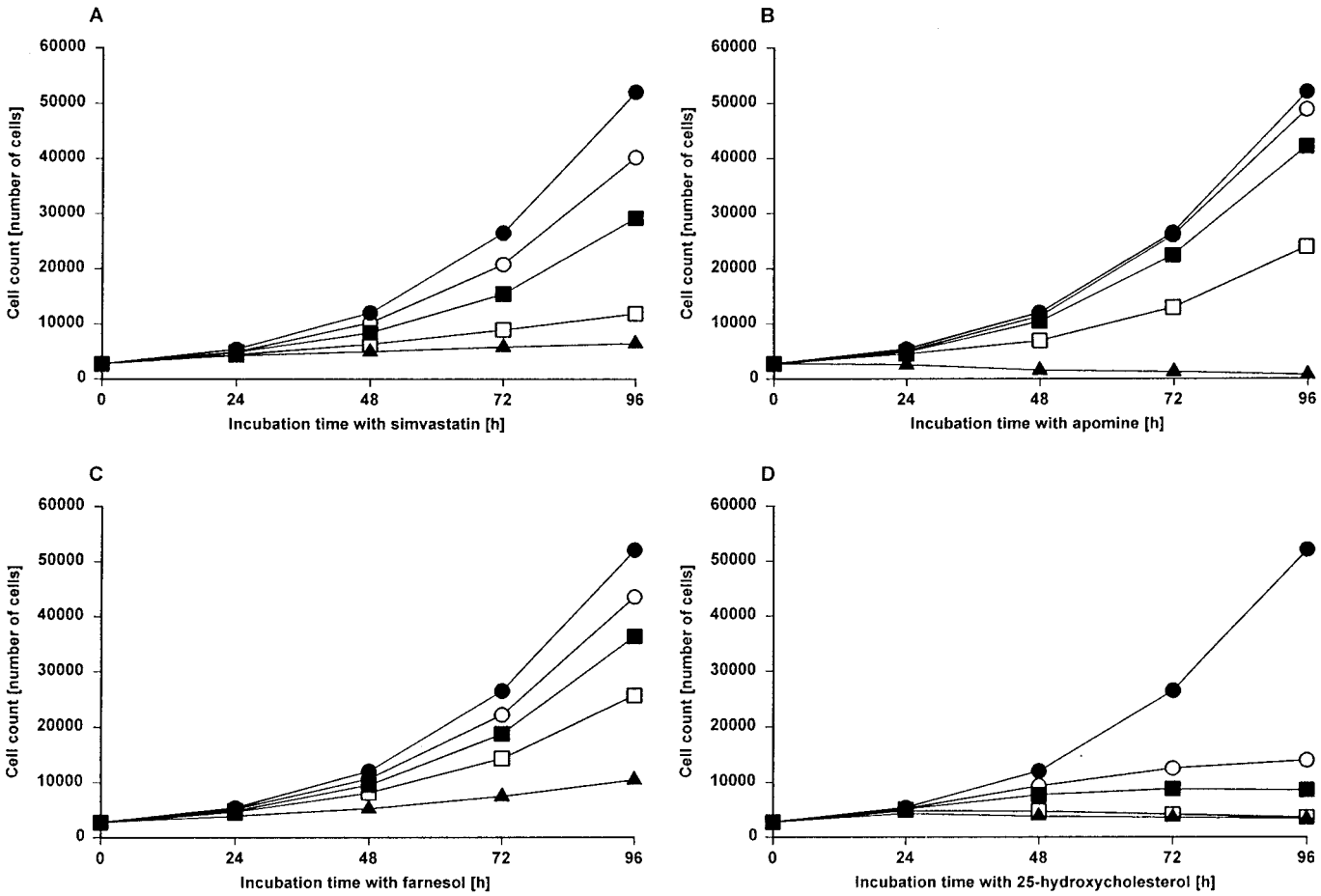

FIG. 3. (A) Time and dose dependent effect of 0 (●), 1 (○), 2 (■), 5 (□), and 10 (▲) μM simvastatin. (B) 0 (●), 2 (○), 5 (■), 10 (□), and 20 (▲) μM apomine. (C) 0 (●), 10 (○), 20 (■), 40 (□), and 100 (▲) μM farnesol. (D) 0 (●), 1 (○), 2 (■), 5 (□), and 10 (▲) μM 25-hydroxycholesterol.

The caspase-3 activity was assayed by adding 500 μl reaction buffer (40 mM Hepes (pH 7.5), 20% glycerol, 4 mM DTT), 2.88 μM substrate (Ac-DEVD-AMC) and adjusting the total volume to 1 ml with H_2O. The samples were incubated at 37°C for 1 h. The AMC liberated was measured using a spectrofluorometer with an excitation wavelength of 380 nm and an emission wavelength of 440 nm.

Chemicals. Apomine (SR-45023A) (tetraisopropyl 2-(3,5-di-tert-butyl-4-hydroxyphenyl) ethyl-1,1-diphosphonate) was synthesized by the Division of Chemical Sciences, Symphar, Switzerland. Other chemicals were purchased from Fluka and Sigma and cell culture media from GibcoBRL and Life Technologies.

RESULTS

Antiproliferative activity of apomine in human cancer cell lines. HL60 (promyelocytic leukemia), SW480, HT29 (colon carcinoma), HepG2 (hepatocellular carcinoma), MesSA (uterine sarcoma) and MCF7 (breast carcinoma) cells were challenged with concentrations up to 40 μM apomine (Fig. 1). In all the tested cell lines, except HepG2, concentrations of less than 5 μM had little or no effect on proliferation. However above 5 μM the cell numbers dropped quickly with increasing dose levels, generally growth being inhibited between 80 and 100% at 20 μM. The IC_{50} values were 5 μM (HepG2), 8 μM (MesSA), 10 μM (HL60, SW620, and MCF7) and 14 μM (HT29).

Apomine was subsequently tested in the primary screen for anticancer drug discovery by the National Cancer Institute (NCI) (24). Ten micromolar of the compound significantly inhibited growth of the majority of the cell lines. Of 53 cell lines, only 16 were inhibited by less than 25%, 16 scored between 25 and 50%, 17 scored between 50 and 75% and 4 showed more than 75% growth inhibition. The most sensitive cell lines were HL60 (leukemia), HOP-92 (NSCLC), HT29 (colon carcinoma) and PC3 (prostate cancer). No specificity toward a particular set of cell lines with similar tissue origin could be observed *in vitro*, although leukemia cells may be slightly more sensitive. O'Connor *et al.* analyzed the p53 status of the cell lines used in the NCI primary screen (25). Sixteen cell lines were reported to be wild type and 36 were reported as having p53 mutation. Figure 2 shows the individual and mean sensitivity of these cell lines to 10 μM apomine. On average, mutant cells were slightly more sensitive to the compound than those that had functional p53. This means that the antiproliferative activity of apomine

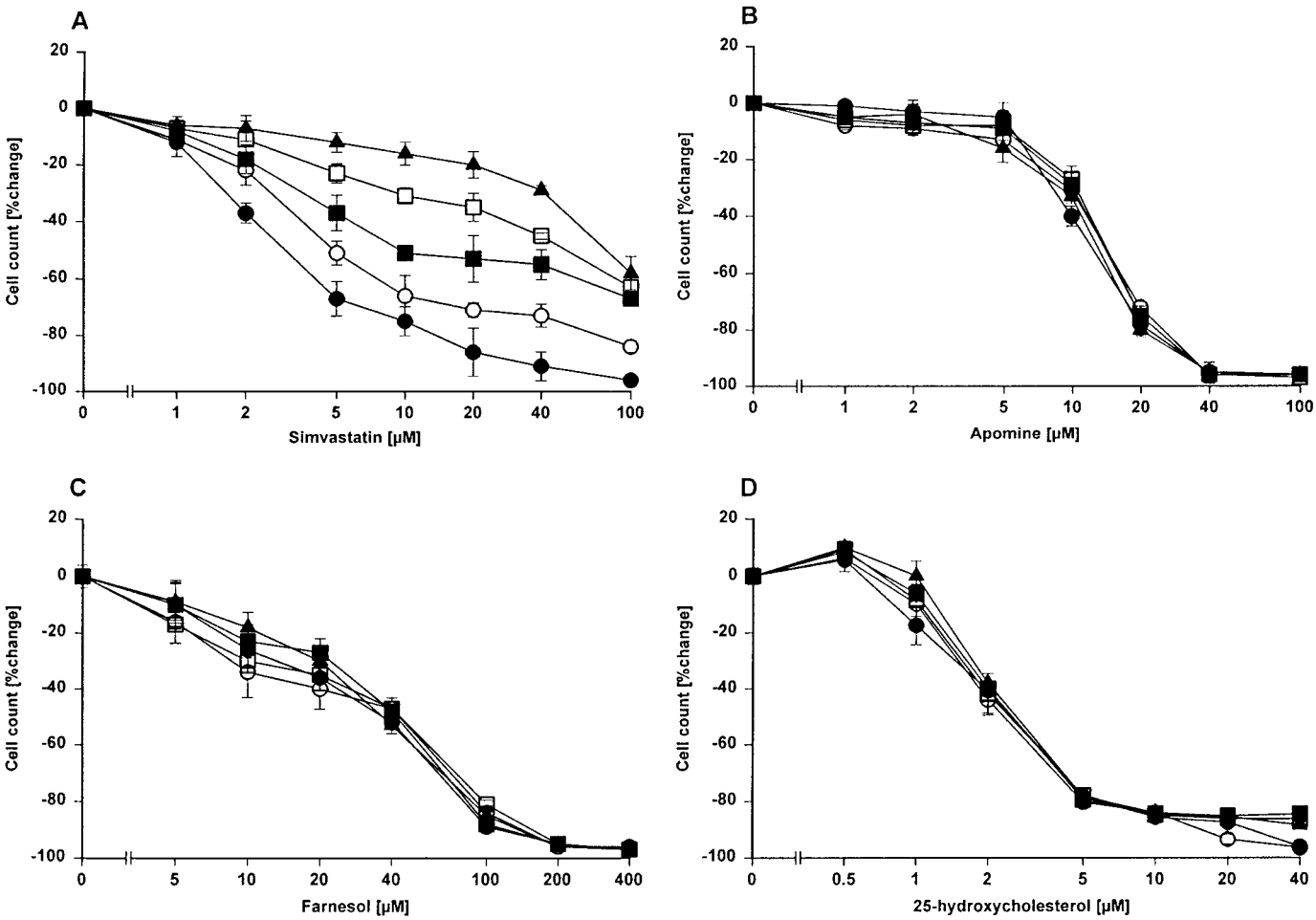

FIG. 4. Reversion by mevalonate of simvastatin (A) induced growth inhibition and lack of reversion by mevalonate of apomine (B), farnesol (C) and 25-hydroxycholesterol (D) induced growth inhibition in HL60. Cells are treated with 0 (●), 50 (○), 100 (■), 200 (□), and 400 (▲) μM mevalonate.

appears to be independent of the p53 status of the cell lines.

Addition of mevalonate does not prevent apomine-induced growth inhibition. Apomine, farnesol, 25-hydroxycholesterol and simvastatin are four inhibitors of the mevalonate pathway. All of them inhibited HL60 growth in a dose and time dependent manner (Figs. 3A–3D). Mevalonate reverted the effect of simvastatin on cell growth (Fig. 4A). The reversion was proportional to the dose of mevalonate between 50 and 400 μM. With the highest concentration of mevalonate, the growth was similar to that of the control despite the presence of 10 μM simvastatin. In contrast, mevalonate did not revert growth inhibition due to apomine (Fig. 4B), farnesol (Fig. 4C) and 25-hydroxycholesterol (Fig. 4D).

Apomine induces apoptosis with characteristics differing from those observed with simvastatin and 25-hydroxycholesterol, however similar to that of farnesol. The release of DNA fragments associated with histones from the nucleus into the cytoplasm and the induction of caspase-3 activity are hallmarks of apoptosis. Figure 5A shows on an arbitrary scale the accumulation of histone-associated DNA fragment into the cytoplasm during the first few hours of HL60 incubation with 20 μM apomine, while Fig. 5B shows the DNA degradation in SW480 cells. A signal was already observed after a 2-h period. Caspase-3 was activated during apomine (Fig. 6B) and farnesol (Fig. 6C) induced apoptosis, and its level correlated with growth inhibition. In contrast, simvastatin (Fig. 6A) and 25-hydroxycholesterol (Fig. 6D) induced growth inhibition was observed at concentrations that did not elicit caspase-3 activity. Higher concentrations (above 10 μM) of simvastatin did induce caspase-3 activity but this activity was associated with a mechanism of growth inhibition that cannot be prevented by mevalonate (Fig. 7). The effect of simvastatin on cell growth was subdivided in its mevalonate dependent and mevalonate independent components. The mevalonate independent growth inhibition matched the caspase-3 activation suggesting

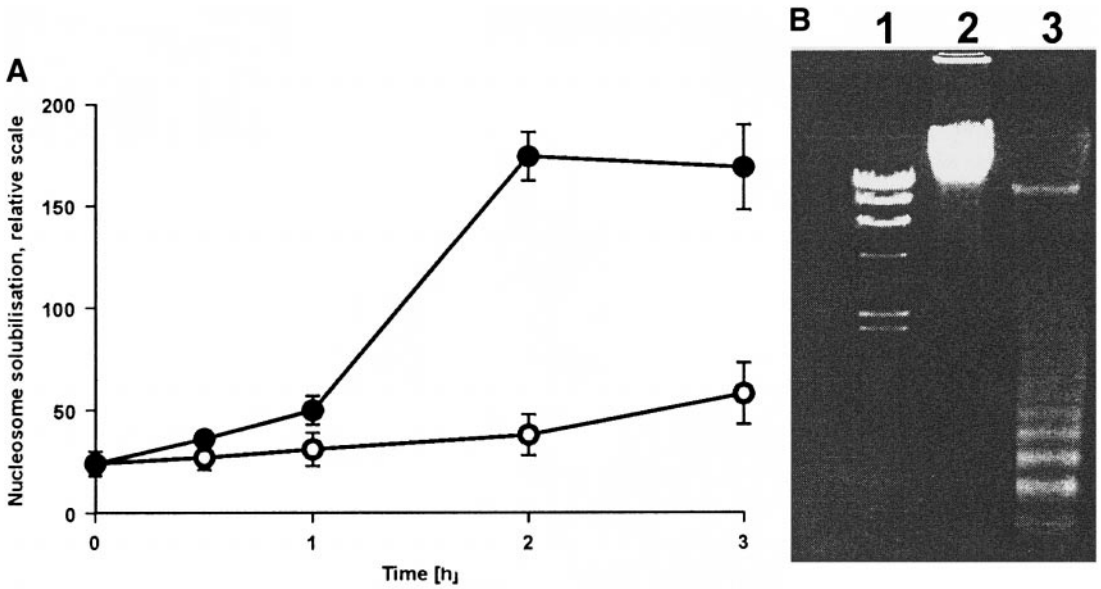

FIG. 5. (A) Time course release of cytoplasmic histone-associated DNA in control (○) and 20 μM apomine (●) treated HL60 cells. (B) Degradation of DNA from SW480 cells treated with 20 μM apomine for 16 h. Lane 1, lambda/*Hin*dIII DNA. Lane 2, control. Lane 3, 20 μM apomine.

distinct mechanisms between simvastatin-induced apoptosis and isoprenoid synthesis inhibition.

DISCUSSION

Apomine represents a family of structurally related 1,1-bisphosphonate esters with multiple biological activities. These compounds are inhibitors of cholesterol synthesis by decreasing the activity of HMG-CoA reductase through a mechanism which differs from that of the statins. While statins are competitive inhibitors of the enzyme HMG-CoA reductase, farnesol (17), 25-hydroxycholesterol (also acting at the transcription level) (14, 15) and bisphosphonate esters (22) increase the rate of HMG-CoA reductase enzyme degradation thus decreasing mevalonate synthesis.

Apomine inhibits the growth of the majority of tested tumor cell lines derived from leukemia, colon, liver, ovary and breast cancer with IC_{50} values ranging between 5 and 14 μM. Initially this effect could be considered secondary to the deprivation of mevalonate which impacts not only on cholesterol synthesis but also on other mevalonate derived metabolites such as isoprenoids. This leads to the loss of the activity of a number of proteins involved in cell proliferation which require isoprenylation, i.e., farnesylation or geranylgeranylation for their proper function (1). Among these proteins are key signal transduction proteins such as the small G proteins ras and rho. Inhibition of farnesylation of these proteins could account for growth inhibition. This is considered the antiproliferative mechanism of the statins (26). Further evidence of growth inhibition being secondary to inhibition of HMG-CoA reductase and of protein prenylation is that it can be reversed by mevalonate supplementation, allowing the synthesis of prenyl pyrophosphates. Indeed, mevalonate allows growth to resume in simvastatin treated cells but not for apomine, farnesol and 25-hydroxycholesterol treated cells. Apomine is not an inhibitor of protein prenyl transferases (data not shown) and the fact that mevalonate does not prevent the growth inhibition due to apomine leads to the conclusion that isoprenoid deprivation with subsequent inhibition of protein prenylation is not the only cause of the antiproliferative activity of apomine.

As demonstrated by several methods, apomine triggers cell death very rapidly. The release of nucleosomes into the cytoplasm is observed as early as 2 h after treatment and DNA laddering can be demonstrated by agarose gel after 16 h. Growth inhibition is well correlated with the rapid occurrence of apoptosis for apomine as shown by the concentration required for inducing caspase-3 activity that is similar to the IC_{50} values for cell proliferation. Farnesol also induces caspase-3 activity at similar doses required for growth inhibition. In contrast simvastatin and 25-hydroxycholesterol block cell proliferation at concentrations that do not elicit caspase-3 activity.

In this work, apomine is compared to three other inhibitors of HMG-CoA reductase for their capability to inhibit cell growth and trigger apoptosis. Apomine is found to differ from simvastatin and 25-hydroxycholesterol in that they do not share the ability to rapidly induce apoptosis. Farnesol is considered as the natu-

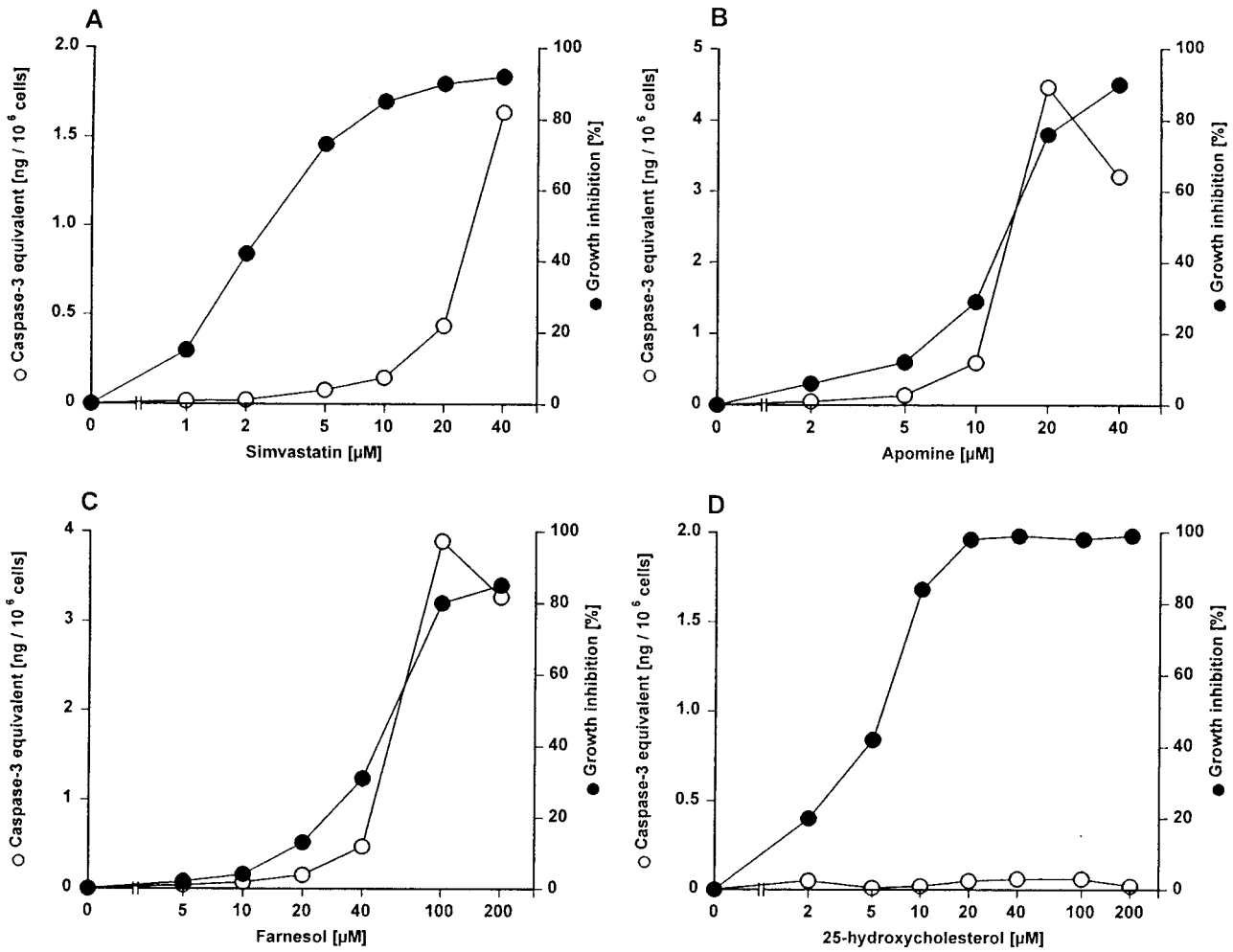

FIG. 6. Relationship between concentrations of simvastatin (A), apomine (B), farnesol (C) and 25-hydroxycholesterol required for cell growth inhibition (●) and induction of caspase-3 activity (○) in HL60.

rally occurring non-sterol regulator of cholesterol synthesis (27). It was previously shown that it regulates HMG-CoA reductase by increasing the degradation rate of the protein (28), and also triggers apoptosis (29). Mechanisms of apoptosis induction by farnesol involving inhibition of choline phosphotransferase (19, 20) were proposed. The antiproliferative and apoptotic activities of apomine could be partly counterbalanced by diacylglycerol and phosphatidylcholine, but no inhibition of phophatidylcholine biosynthesis was observed (data not shown). Thus, apomine and farnesol present a similar profile for apoptosis and cholesterol synthesis.

Recently 1,1-bisphosphonate esters, including apomine, have been discovered to be potent synthetic activators of the farnesoid X activated receptor FXR (30), a nuclear receptor previously reported to be activated by the isoprenoids farnesol, juvenile hormone (31) and bile acids (32). A clear correlation was demonstrated between compounds that activate FXR, interfere with

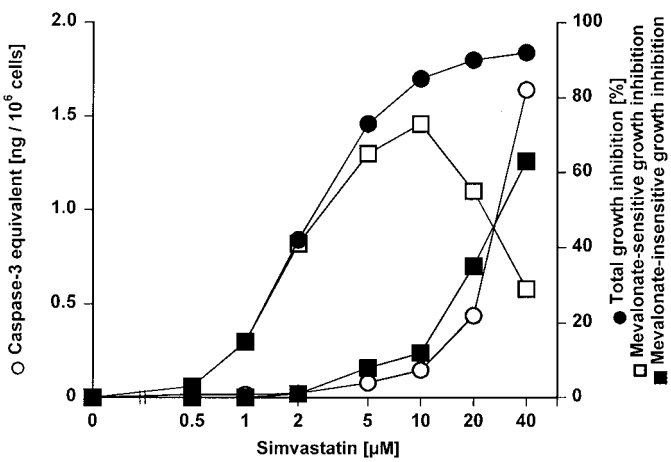

FIG. 7. Simvastatin-induced growth inhibition (●) could be subdivided in mevalonate sensitive (□) and mevalonate insensitive (■) growth inhibition. The mevalonate-insensitive growth inhibition matched the induction of caspase-3 activity (○).

the mevalonate pathway and induce apoptosis. However a direct mechanism of apoptosis implicating FXR is still to be demonstrated.

The overall profile on mevalonate synthesis inhibition, cell growth inhibition, and apoptosis suggests that apomine acts as a synthetic mimetic of farnesol.

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