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Apomine Enhances the Antitumor Effects of Lovastatin on Myeloma Cells by Down-Regulating 3-Hydroxy-3-methylglutaryl-Coenzyme A Reductase

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Abstract

Apomine, a 1,1-bisphosphonate-ester with antitumor activity, has previously been reported to strongly down-regulate 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase), the rate-limiting enzyme in the mevalonate pathway responsible for the prenylation of proteins. Here, we show that although apomine down-regulated HMG-CoA reductase protein levels in myeloma cells, it did not inhibit protein prenylation, and apomine-induced apoptosis could not be prevented by mevalonate, indicating that apomine cytotoxicity is independent from its effects on HMG-CoA reductase. Instead, apomine cytotoxicity was prevented by the addition of phosphatidylcholine, which is similar to the previously reported ability of phosphatidylcholine to overcome the cytotoxicity of farnesol, whereas phosphatidylcholine had no effect on down-regulation of HMG-CoA reductase by apomine. These findings raised the possibility that apomine, independent from its own cytotoxic effects, could enhance the antitumor effects of the competitive HMG-CoA reductase inhibitor lovastatin via down-regulating HMG-CoA reductase. Indeed, treatment with apomine in combination with lovastatin resulted in synergistic decreases in viable cell number and induction of apoptosis. At the concentrations used, apomine down-regulated HMG-CoA reductase protein levels without being cytotoxic. Accumulation of unprenylated Rap1A by lovastatin was enhanced in the presence of apomine. Furthermore, synergy was completely prevented by mevalonate, and apomine did not synergize with desoxolovastatin, which does not inhibit HMG-CoA reductase. We conclude that the synergistic drug interaction results from an enhancement by apomine of the effects of lovastatin, mediated by down-regulation of HMG-CoA reductase by apomine. Thus, these findings demonstrate a novel strategy for enhancing the antitumor effects of lovastatin.

Apomine [tetra-iso-propyl 2-(3,5-di-*tert*-butyl-4-hydroxyphenyl)ethyl-1,1-diphosphonate] (Alberts et al., 2001) belongs to the group of 1,1-bisphosphonate-esters. In recent years, a role for apomine as an anticancer drug has been emerging. Apomine inhibits the growth of a variety of cancer cell lines (Flach et al., 2000; Lowe et al., 2005) and primary ovarian tumor

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cells (Alberts et al., 2001). We have previously demonstrated that apomine exerts antitumor effects in the 5T2MM murine myeloma model *in vivo*, as demonstrated by decreased serum paraprotein levels and a reduction in the proportion of 5T2MM cells in the bone marrow (Edwards et al., 2007). However, the antitumor mechanism of action of apomine is poorly understood.

Apomine, and its family member SR-12813, originally attracted attention by their ability to lower plasma cholesterol (Berkhout et al., 1997; Jackson et al., 2000). The cholesterol-lowering mechanism of action of apomine is thought to be similar to that of the statins, involving inhibition of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase) (Roitelman et al., 2004), which catalyzes the rate-limiting step in the mevalonate and cholesterol biosynthesis pathway. However, whereas statins inhibit HMG-CoA reductase by strongly binding to the HMG-CoA binding site of this enzyme, thereby competitively inhibiting its function (Istvan and Deisenhofer, 2001), apomine has been reported to inhibit HMG-CoA reductase by up-regulating degradation of this enzyme by the proteasome, causing a rapid decrease in HMG-CoA reductase protein levels (Roitelman et al., 2004).

The mevalonate pathway, in addition to feeding into the cholesterol biosynthesis pathway, is responsible for the generation of isoprenoid donors required for the prenylation of proteins. Inhibitors of the mevalonate pathway, including statins, and nitrogen-containing bisphosphonates that are widely used in the treatment of multiple myeloma to relieve the tumor-associated bone disease (Kanis and McCloskey, 2000; Conte and Coleman, 2004) block protein prenylation (Sinensky et al., 1990; Luckman et al., 1998). Inhibition of protein geranylgeranylation is believed to be largely responsible for the antitumor effects of these classes of drugs in myeloma cells, at least *in vitro*, because the addition of geranylgeraniol (GGOH) protects cells from their cytotoxic effects (Shipman et al., 1998; van de Donk et al., 2003). The strong down-regulation of HMG-CoA reductase by apomine may also affect the prenylation of small GTPases essential for cell growth and survival and may be responsible for the antitumor effects of apomine on myeloma cells.

Alternatively, Flach et al. (2000) suggested that apomine may act as a synthetic mimetic of farnesol, because apomine induces apoptosis in HL60 leukemia cells with characteristics similar to the cytotoxicity induced by farnesol. Although the mechanism of farnesol cytotoxicity is not completely understood, farnesol has been demonstrated to inhibit the conversion of CDP-choline into phosphatidylcholine (PC) (Voziyan et al., 1993; Miquel et al., 1998; Anthony et al., 1999), and exogenous administration of PC has been shown to prevent induction of apoptosis by farnesol in CEM-C1 and HL60 leukemia cells (Melnykovich et al., 1992; Haug et al., 1994; Anthony et al., 1999). If apomine indeed acts similarly to farnesol, its cytotoxic effects should also be prevented by PC.

In contrast to the reported down-regulation of HMG-CoA reductase by apomine, competitive inhibition of this enzyme by statins triggers compensatory feedback mechanisms, which cause a strong up-regulation of HMG-CoA reductase protein levels (Brown et al., 1978; Nakanishi et al., 1988). Therefore, although statins are very potent inhibitors of HMG-CoA reductase, the effectiveness of these drugs in cellular systems is limited. This is especially relevant in terms of their potential as antitumor agents, because concentrations required to significantly affect the proliferation and/or survival of the vast majority of tumor cells *in vitro* may not be achieved *in vivo* (Dimitroulakos et al., 2001). Thus, novel approaches to increase the antitumor potential of statins may prove valuable.

We hypothesized that apomine, by down-regulating HMG-CoA reductase protein levels, is able to enhance the effects of lovastatin. The aims of this study were to gain insight into the

antitumor mechanism of action of apomine on myeloma cells, and to investigate whether apomine, via down-regulation of HMG-CoA reductase, could enhance the antitumor effects of lovastatin on myeloma cells in vitro.

Materials and Methods

Chemicals

Apomine was synthesized as described previously (Nguyen et al., 1991). Des α lovastatin (Weitz-Schmidt et al., 2001) was a kind gift of Dr. G. Weitz-Schmidt (Novartis Pharma AG, Basel, Switzerland). Chemicals were from Sigma Chemical Co. (Poole, UK) unless stated otherwise. Stock solutions of apomine, mevalonate, GGOH, and PC were prepared in 100% ethanol. Stock solutions of lovastatin were prepared in 100% dimethyl sulfoxide.

Cell Culture

JJN-3 human myeloma cells were kindly provided by Professor Ian Franklin (University of Glasgow, Glasgow, UK). The human myeloma cell line RPMI 8226 was obtained from the European Collection of Animal Cell Cultures (Salisbury, UK). Cells were cultured in RPMI 1640 medium (Invitrogen, Paisley, UK), supplemented with 10% fetal calf serum, 1 mM glutamine, 1 mM sodium pyruvate, 1 $\times$ minimal essential medium nonessential amino acids, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 50 μ M 2-mercaptoethanol. For all experiments, cells were plated out in multiwell plates (Corning Life Sciences, Acton, MA) at a density of 5×10^5 cells/ml.

Preparation of Cell Lysates and Western Blotting

Cells were lysed in radioimmunoprecipitation assay buffer [phosphate-buffered saline containing 1% (v/v) Nonidet P40, 0.1% (w/v) sodium dodecyl sulfate, 0.5% (w/v) sodium deoxycholate, and 1:100 Sigma protease inhibitor cocktail] or in Tris-buffered saline containing 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1% (v/v) Triton X-100, and 2% (v/v) dimethyl sulfoxide. Protein content was determined using a bicinchoninic acid assay (Pierce Biotechnology, Rockford, IL). Lysates for detection of Rap1A and Rab6 were heated for 5 min at 95°C to optimize the denaturation of protein. This procedure was not carried out for HMG-CoA reductase samples, since this has been reported to cause dimerization of HMG-CoA reductase (Roitelman et al., 2004). Equal protein amounts were electrophoresed on 12% (Rap1A and Rab6) or either 7.5 or 10% (HMG-CoA reductase) polyacrylamide-SDS gels and transferred onto polyvinylidene difluoride membranes. Blots were probed with a polyclonal goat anti-Rap1A antibody (Santa Cruz Biotechnology, Santa Cruz, CA) that specifically recognizes the unprenylated form of this small GTPase (Coxon et al., 2001; Roelofs et al., 2006) and a rabbit polyclonal anti-Rab6 antibody (Santa Cruz Biotechnology) that detects both the unprenylated and the prenylated form (Coxon et al., 2005; Roelofs et al., 2006). HMG-CoA reductase was detected with a polyclonal rabbit anti-HMG-CoA reductase antibody (Upstate, Dundee, UK). As loading control, blots were probed with either a mouse monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Abcam Ltd., Cambridge, UK) or rabbit polyclonal anti- β -actin (Sigma).

Detection of Apoptotic Cells

Apoptotic cells were identified by either annexin V labeling or a fluorescence in situ nick translation assay. For annexin V labeling, cells were washed once in ice-cold phosphate-buffered saline and resuspended in annexin-binding buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM CaCl₂, pH 7.4). Cells were incubated with annexin V conjugated to either Alexa Fluor 488 or Alexa Fluor 647 (Invitrogen) for 15 min at room temperature, placed

back on ice, and immediately analyzed by flow cytometry on a FACSCalibur (BD BioSciences, Oxford, UK). The nick translation assay was performed as described previously (Roelofs et al., 2006). Results were confirmed by staining with 4'-6-diamidino-2-phenyl-indole and investigating changes in nuclear morphology using a fluorescence microscope. Apoptotic cells were identified by their characteristic nuclear morphology, including chromatin condensation and DNA fragmentation (Ramachandra and Studzinsky, 1995).

Determination of Viable Cell Number

Viable cell number was determined using the alamarBlue assay (BioSource International, Camarillo, CA). Three hours before the end of the culture period, alamarBlue was added [1:10 (v/v)] to the cell cultures. After 3 h, fluorescence was detected using a Bio-Tek fluorescence plate-reader (Winooski, VT) at an excitation wavelength of 530 nm and emission wavelength of 590 nm. Results are corrected for background fluorescence and expressed as a percentage of the control.

Statistical Analysis

Experiments determining the percentage of apoptotic cells were performed in triplicates (annexin V) or quadruplicates (nick translation), and experiments determining viable cell number were carried out with six replicates. All results shown are representative, or combined data, of at least three independent experiments. IC_{50} concentrations were calculated using GraphPad Prism software (San Diego, CA) by nonlinear regression (curve-fit) analysis, using the sigmoidal dose-response equation with variable slope (R^2 values were 0.98 for all curves). Two-way analysis of variance was used to analyze the interaction between apomine and lovastatin on viable cell number, and changes in IC_{50} concentrations were tested for statistically significant differences using one-way analysis of variance and the Dunnett's post hoc test. All other experiments were analyzed using one-way analysis of variance and Tukey's post hoc test.

Results

Apomine Decreases HMG-CoA Reductase Protein Levels in Myeloma Cells

It has previously been shown that apomine decreases HMG-CoA reductase protein levels in HeLa, LP-90, and Chinese hamster ovary cells (Roitelman et al., 2004). To investigate whether apomine causes a decrease in HMG-CoA reductase protein levels in myeloma cells, JJN-3 cells were treated with 2–12 μ M apomine for 24 h, and HMG-CoA reductase was detected by immunoblotting. Apomine dose-dependently decreased the amount of HMG-CoA reductase enzyme present in the cells (Fig. 1). HMG-CoA reductase was strongly down-regulated in response to 2 and 4 μ M apomine and was barely detectable after treatment with 12 μ M apomine. Similar results were obtained in the RPMI 8226 myeloma cell line (data not shown). It is unclear what the band of higher molecular weight in the 12 μ M apomine sample is, but this could represent ubiquitinated HMG-CoA reductase, since HMGCoA reductase has been reported to be down-regulated by increased ubiquitinylation and proteasomal degradation (Roitelman et al., 2004).

Apomine Does Not Inhibit Protein Prenylation

To investigate whether the down-regulation of HMG-CoA reductase protein levels by apomine causes inhibition of protein prenylation, the prenylation status of two small GTPases, Rap1A and Rab6, was assessed. As a positive control, cells were treated with lovastatin. Whereas incubation with 5 μ M lovastatin for 24 h clearly caused accumulation of unprenylated Rap1A and Rab6, treatment with 10 μ M apomine (a concentration that down-

regulates HMG-CoA reductase and induces apoptosis; see Figs. 1 and 2B) had no detectable effect on the prenylation status of these two proteins in JJN-3 cells (Fig. 2A). Treatment of RPMI 8226 cells with 10 μ M apomine did not cause the appearance of unprenylated Rap1A or Rab6 either, nor did longer treatment times (up to 72 h) with apomine affect prenylation (not shown). Treatment with different concentrations of apomine did not result in accumulation of unprenylated Rap1A either (Fig. 5B).

Apomine-Induced Apoptosis Is Not Prevented by Intermediates of the Mevalonate Pathway

The lack of effect of apomine on protein prenylation suggests that apomine may induce apoptosis via mechanisms distinct from the inhibition of the mevalonate pathway. To confirm this, JJN-3 cells were treated with vehicle only, 10 μ M apomine, or 10 μ M lovastatin (positive control) in the presence or absence of 500 μ M mevalonate or 10 μ M GGOH for 72 h, and apoptosis was detected using annexin V labeling (Fig. 2B). Mevalonate is the product of the reaction catalyzed by HMG-CoA reductase. GGOH is intracellularly metabolized to geranylgeranyl pyrophosphate (Crick et al., 1997), the isoprenoid donor for the geranylgeranylation of proteins such as Rap1A and Rab6. Apomine-induced apoptosis was not prevented by the addition of either of these two intermediates of the mevalonate pathway downstream from HMG-CoA reductase. In contrast, apoptosis in response to lovastatin was completely blocked by either mevalonate ($p < 0.001$) or GGOH ($p < 0.001$). Similar results were obtained using the nick translation assay, which detects endonuclease-mediated DNA fragmentation characteristic of apoptosis (data not shown).

Phosphatidylcholine Protects against the Cytotoxic Effects of Apomine on Myeloma Cells

Apomine has been suggested to mimic the actions of farnesol (Flach et al., 2000), and because various cell types can be rescued from farnesol-induced apoptosis by the exogenous addition of PC (Melnykovich et al., 1992; Haug et al., 1994; Anthony et al., 1999), we investigated whether PC was able to rescue JJN-3 and RPMI 8226 myeloma cells from the cytotoxic effects of apomine. PC completely prevented the decrease in viable cell number in response to 10 μ M apomine in JJN-3 cells ($p < 0.001$; Fig. 3A). In addition, 100 μ g/ml PC was able to prevent induction of apoptosis by apomine in these cells ($p < 0.001$; Fig. 3B). PC also dose-dependently protected RPMI 8226 cells from the decrease in viable cell number by apomine, with 100 μ g/ml PC conferring partial protection ($p < 0.01$) and 200 μ g/ml PC almost completely preventing the decrease in viable cell number by apomine ($p < 0.001$; Fig. 3A). Interestingly, a trend was observed for a partially protective effect of PC against the decrease in viable cell number (Fig. 3A) and the increase in apoptotic cell death (Fig. 3B) induced by lovastatin in JJN-3 cells, although this was not significant at these concentrations. However, the protective effect of PC was more pronounced at lower concentrations of lovastatin, with 100 μ g/ml PC significantly rescuing JJN-3 cells from induction of apoptosis by 1 μ M lovastatin ($p < 0.01$; Fig. 6A). In contrast, however, PC had no effect on the decrease in viable cell number induced by treatment with 10 μ M lovastatin in RPMI 8226 cells, even at the higher concentration of 200 μ g/ml PC (Fig. 3A), suggesting that this is a cell line-specific effect.

Apomine Counteracts Up-Regulation of HMG-CoA Reductase by Lovastatin, and This Is Not Prevented by Phosphatidylcholine

Inhibition of HMG-CoA reductase by statins typically triggers compensatory feedback mechanisms, which cause a strong up-regulation of HMG-CoA reductase protein levels (Brown et al., 1978; Nakanishi et al., 1988). A strong increase in HMG-CoA reductase enzyme levels was also observed in JJN-3 cells treated with increasing concentrations of lovastatin for 24 h (Fig. 4A). Because apomine alone down-regulated HMG-CoA reductase, we investigated the effect of combined treatment with these two compounds on HMG-CoA reductase protein levels. Simultaneous treatment of JJN-3 cells with apomine and lovastatin

for 24 h caused a decrease in HMG-CoA reductase protein levels compared with the increase observed with lovastatin treatment alone (Fig. 4B). HMG-CoA reductase protein was similar to control levels after coincubation with 1 μ M lovastatin and 12 μ M apomine (data not shown). Importantly, PC had no effect on the down-regulation of either basal or lovastatin-up-regulated HMG-CoA reductase protein levels by apomine (Fig. 4C), indicating that the down-regulation of HMG-CoA reductase by apomine is not secondary to its cytotoxic effects. Similar results were obtained using RPMI 8226 cells (data not shown).

Lovastatin and Apomine Synergistically Decrease Viable Cell Number and Increase Apoptosis

The strong up-regulation of HMG-CoA reductase protein levels in response to lovastatin treatment (Fig. 4A) serves to protect cells from the competitive inhibitory actions of lovastatin on HMG-CoA reductase and results in higher concentrations of the drug being required to block the synthesis of mevalonate, prevent downstream protein prenylation, and ultimately induce apoptosis. Because apomine was able to counteract this strong lovastatin-induced up-regulation of HMG-CoA reductase enzyme levels (Fig. 4), we investigated whether apomine enhances the antitumor effects of lovastatin on myeloma cells. We established dose-response curves illustrating the effect of lovastatin treatment for 72 h on viable cell number, either on its own or in the presence of increasing concentrations of apomine within the same experiment. Apomine caused a concentration-dependent shift in the lovastatin cell viability curve, characteristic of a synergistic interaction (Fig. 5A). This shift was constantly observed between repeat experiments, which is reflected in the small error bars (results shown are mean $\pm$ S.D. of three experiments). Two-way analysis of variance revealed that this interaction between lovastatin and apomine was statistically significant ($p < 0.001$). The IC_{50} of lovastatin decreased from $5.4 \pm 0.4 \mu$ M (lovastatin alone) to $2.6 \pm 0.3 \mu$ M ($p < 0.001$), $1.9 \pm 0.1 \mu$ M ($p < 0.001$), and $0.9 \pm 0.0 \mu$ M ($p < 0.001$) in the presence of 1, 2, and 4 μ M apomine, respectively. Importantly, at these concentrations, apomine had no effect on the number of viable cells on its own. Dose-response curves of apomine revealed an IC_{50} for decrease in viable cell number by apomine on its own of $10.8 \pm 1.4 \mu$ M in JJN-3 cells and $8.7 \pm 1.5 \mu$ M in RPMI 8226 cells (data from three independent experiments; not shown). Lovastatin and apomine also caused a synergistic increase in the percentage of apoptotic cells (Fig. 6A). Treatment with 1 μ M lovastatin alone caused a modest increase in apoptosis from $26.6 \pm 3.6\%$ in the control to $43.2 \pm 5.1\%$ in response to 1 μ M lovastatin (mean $\pm$ S.E.M. of three independent experiments; $p < 0.05$). However, when combined with 4 μ M apomine, which on its own did not affect the percentage of apoptotic cells, apoptosis was strongly increased to $87.0 \pm 2.1\%$ (mean $\pm$ S.E.M. of three independent experiments; $p < 0.001$ compared with the control and either drug alone).

Apomine Augments the Inhibition of Protein Prenylation by Lovastatin

To investigate whether the interaction between lovastatin and apomine could be observed at the level of inhibition of protein prenylation, the prenylation status of Rap1A was investigated. Treatment of JJN-3 cells with 0.1 μ M lovastatin (a concentration that had very little effect on the prenylation of Rap1A on its own) in the presence of increasing concentrations of apomine for 24 h resulted in a concentration-dependent accumulation of unprenylated Rap1A (Fig. 5B). The amount of unprenylated Rap1A was significantly increased ($p < 0.01$; of four independent experiments) in response to 0.1 μ M lovastatin in the presence of 8 or 12 μ M apomine, compared with lovastatin treatment alone. The apparent decrease in amount of unprenylated Rap1A at 12 μ M apomine (in the presence of lovastatin), compared with lower concentrations of apomine, was associated with a decrease in β -actin levels and is most likely the result of the potent cytotoxic effects of apomine at

this concentration. Apomine did not induce accumulation of unprenylated Rap1A on its own at any of the concentrations used, consistent with the results presented in Fig. 2A.

Synergy between Apomine and Lovastatin Is Completely Prevented by Mevalonate, but Not or Only Partly by Phosphatidylcholine

The increase in unprenylated Rap1A, which served as a marker for inhibition of protein prenylation, suggests that the synergistic interaction between lovastatin and apomine is mediated via enhanced inhibition of HMG-CoA reductase. Therefore, we investigated whether the addition of mevalonate to the culture medium was able to prevent the drug interaction. Indeed, the strong increase in apoptosis induced by combination treatment with lovastatin and apomine was completely prevented by the addition of 500 μ M mevalonate ($p < 0.001$; Fig. 6A). The addition of mevalonate also completely blocked the synergistic increase in unprenylated Rap1A by combination treatment with 1 μ M lovastatin and 4 μ M apomine (Fig. 6B). In contrast, PC, which protects against the cytotoxic effects of apomine, only had a partially protective effect against the synergistic induction of apoptosis (Fig. 6A), similar to lovastatin treatment alone (Figs. 3A and 6A), in JJN-3 cells. PC did not confer any protection against the synergistic decrease in viable cell number by apomine and lovastatin in RPMI 8226 cells (data not shown). Furthermore, PC did not prevent the enhanced accumulation of unprenylated Rap1A (Fig. 6B).

Apomine Does Not Act in Synergy with Desoxolovastatin

The complete rescue from the synergistic interaction between apomine and lovastatin by the addition of mevalonate strongly suggests that synergy between apomine and lovastatin is the result of an enhanced inhibition of mevalonate synthesis. To further confirm this, we investigated whether apomine synergizes with desoxolovastatin, an analog of lovastatin that is unable to inhibit HMG-CoA reductase. Treatment with 10 μ M desoxolovastatin on its own did not affect viable cell number, consistent with a lack of effect on HMG-CoA reductase (Fig. 6C). Combination treatment with 10 μ M desoxolovastatin and 2 or 4 μ M apomine had no effect on viable cell number either. In contrast, treatment with 3 μ M lovastatin in combination with these concentrations of apomine resulted in a clear synergistic effect ($p < 0.001$), consistent with the results presented in Fig. 5A.

Discussion

Statins are very potent competitive inhibitors of HMG-CoA reductase. However, their effectiveness is diminished by the strong (up to 200-fold) cellular increase in HMG-CoA reductase protein levels as a result of feedback mechanisms activated by the depletion of cellular sterols and nonsterol mevalonate pathway intermediates (Nakanishi et al., 1988). This reduces the efficacy of these drugs to inhibit the synthesis of mevalonate by HMG-CoA reductase. The 1,1-bisphosphonate-ester apomine has previously been shown to down-regulate HMG-CoA reductase by causing this enzyme to be ubiquitinated and targeted to the proteasome for degradation, resulting in a rapid decrease in HMG-CoA reductase protein levels (Roitelman et al., 2004). This raised the possibility that apomine, via down-regulating the target enzyme HMG-CoA reductase, may enhance the effects of lovastatin.

Apomine is a potent antineoplastic agent, inducing apoptosis in a wide variety of tumor cells (Flach et al., 2000), and we have recently shown that apomine alone has antimyeloma effects both in vitro and in vivo in a murine model of multiple myeloma (Edwards et al., 2007). Furthermore, results from a pharmacokinetic trial indicated that the concentrations needed to induce tumor cell apoptosis in vitro can be achieved in vivo with minimal side effects (Alberts et al., 2001). This compound therefore represents a potential new class of anti-cancer drugs. However, its antitumor mechanism of action is poorly understood.

HMG-CoA reductase is the rate-limiting enzyme of the mevalonate pathway ultimately responsible for the synthesis of cholesterol and for the production of farnesyl and geranylgeranyl diphosphates that are required as substrates for the prenylation of proteins. This led us to investigate whether the antitumor effects of apomine involve down-regulation of HMG-CoA reductase and/or inhibition of protein prenylation. Our findings show that although apomine strongly down-regulates HMG-CoA reductase in myeloma cells in vitro, prenylation of the representative small GTPases Rap1A or Rab6 was not affected by apomine treatment. In addition, neither mevalonate nor GGOH was able to prevent induction of apoptosis in response to apomine, demonstrating that inhibition of mevalonate synthesis or of geranylgeranylation of proteins does not play a role in the cytotoxic effects of apomine on myeloma cells. This supports previous findings that mevalonate was unable to prevent the growth inhibitory effect of apomine on HL60 cells (Flach et al., 2000) or rescue breast cancer cells from apomine-induced cell death (Lowe et al., 2005), and that apomine treatment did not affect Ras membrane localization in breast cancer cells (Lowe et al., 2005). A possible explanation for the lack of effect of apomine on protein prenylation, despite the strong down-regulation of HMG-CoA reductase protein levels, and the previously reported resulting inhibition of intracellular cholesterol biosynthesis (Roitelman et al., 2004), is that the protein prenyltransferases that mediate the addition of isoprenyl groups to proteins have a much higher affinity for their substrates than the enzymes of the cholesterol biosynthesis pathway (Brown and Goldstein, 1980).

Flach et al. (2000) suggested that apomine acts as a synthetic mimetic of farnesol. In support of this, apomine has been shown to be an agonist of the farnesoid nuclear receptor, for which farnesol is a natural ligand (Niesor et al., 2001). Furthermore, farnesol has been reported to mediate the negative feedback regulation of the mevalonate and cholesterol biosynthesis pathway on HMG-CoA reductase by increasing the degradation rate of this enzyme (Correll et al., 1994), similar to the effects of apomine on HMG-CoA reductase (Roitelman et al., 2004), although a more recent study suggested that GGOH and not farnesol synergizes with sterols to down-regulate HMG-CoA reductase (Sever et al., 2003).

Because the cytotoxicity of farnesol has been shown to be due to inhibition of PC synthesis (Voziyan et al., 1993; Miquel et al., 1998; Anthony et al., 1999), and can be prevented by the addition of PC (Melnykovich et al., 1992; Haug et al., 1994; Anthony et al., 1999), we investigated whether PC was able to prevent apomine cytotoxicity on myeloma cells. Both the decrease in viable cell number and the induction of apoptosis by apomine could be prevented by the addition of PC. These findings support the suggestion that apomine acts in a similar way to farnesol.

Importantly, the down-regulation of HMG-CoA reductase by apomine still occurred in the presence of PC, indicating that PC selectively prevents the cytotoxic effects of this drug and suggesting that down-regulation of HMG-CoA reductase and induction of apoptosis by apomine may occur via distinct mechanisms. This indicated that apomine may enhance the effects of lovastatin independent from its own cytotoxic effects, resulting in a novel synergistic interaction.

Treatment of JJN-3 myeloma cells with lovastatin resulted in a strong increase in the amount of HMG-CoA reductase, and we show here that apomine is able to counteract this compensatory up-regulation of HMG-CoA reductase protein levels in these cells. A recent study showed that the simvastatin up-regulated expression of HMG-CoA reductase protein in the liver of rats in vivo was decreased after a single injection with apomine (Roitelman et al., 2004), demonstrating that a similar effect occurs in vivo, at least in the liver of rats. Further experiments showed that combination treatment with apomine and lovastatin caused enhanced cytotoxic effects compared with either drug alone, determined by decreases in

viable cell number and induction of apoptosis. Importantly, this was observed at concentrations at which apomine down-regulated HMG-CoA reductase protein levels, without inducing any cytotoxic effects on its own. That synergy is indeed the result of an enhancement of the effects of lovastatin by apomine is supported by our findings that 1) accumulation of unprenylated Rap1A (an indicator of inhibition of protein prenylation) was increased in response to combination treatment with lovastatin and apomine, compared with lovastatin alone; 2) the synergistic interaction between lovastatin and apomine, both the increase in unprenylated Rap1A and the strong increase in the percentage of apoptotic cells, was completely prevented by mevalonate; 3) PC did not protect against the enhanced accumulation of unprenylated Rap1A and only partially protected against the synergistic increase in apoptosis in JJN-3 cells, similar to the effects of lovastatin on its own; and 4) apomine did not synergize with desoxolovastatin, an analog of lovastatin that lacks the ability to inhibit HMG-CoA reductase.

In recent years, there has been increasing interest in statins as potential antitumor agents (Graaf et al., 2004), especially in combination regimens with other anticancer agents to enhance their antitumor potential in vivo (Sleijfer et al., 2005). These findings suggest a novel approach to increasing the antitumor activity of statins, by combining treatment with an agent that down-regulates the target protein HMG-CoA reductase, thus effectively increasing the ratio between the drug and the target enzyme, and preventing the compensatory mechanism that cells use to overcome HMG-CoA reductase inhibition. The concentrations of both apomine and lovastatin used in this study are within the range of concentrations that may be achieved in vivo. Thibault et al. (1996) demonstrated in a phase I study of lovastatin in patients with cancer that the highest dose of lovastatin that was well tolerated (25 mg/kg per day for 7 consecutive days) resulted in a maximum serum concentration of 3.9 μM lovastatin. Pharmacokinetic trials with apomine showed that this drug has a good systemic bioavailability in humans (Alberts et al., 2001; Bonate et al., 2004), and the peak plasma concentration achieved in a phase I clinical trial of patients with solid tumors was 29.1 μM , which was not associated with significant signs of toxicity (Alberts et al., 2001).

In addition to the ability of apomine to enhance the antitumor effects of lovastatin, at least in vitro, these findings of synergy between apomine and lovastatin could have important implications for a variety of other drug applications. Statins are commonly used as cholesterol-lowering drugs. Apomine may enhance their cholesterol-lowering properties, in addition to its own beneficial effects on plasma cholesterol (Jackson et al., 2000). Another potential application is in the treatment of metabolic bone diseases. Statins have been shown to stimulate bone formation in vitro and in rats (Mundy et al., 1999; Gutierrez et al., 2006). Although the increase in bone formation in vitro seemed to be due to enhanced transcription of bone morphogenetic protein-2 in (pre-)osteoblasts, this effect could be blocked by mevalonate, suggesting that the stimulation of the bone morphogenetic protein-2 promoter is a downstream event from inhibition of HMG-CoA reductase (Mundy et al., 1999; Ohnaka et al., 2001). Our findings therefore raise the possibility that combination treatment with apomine could increase the therapeutic potential of statins as bone anabolic agents.

Taken together, this study provides new insights into the antitumor mechanism of action of apomine and demonstrates a novel synergistic interaction between apomine and lovastatin, at least in vitro. Furthermore, our findings strongly suggest that the synergy between these two drugs is the direct result of increased inhibition of HMG-CoA reductase, most likely due to down-regulation of HMG-CoA reductase enzyme levels by apomine.

ABBREVIATIONS

SR-12813	tetra-ethyl 2-(3,5-di- <i>tert</i> -butyl-4-hydroxyphenyl)ethenyl-1,1-bisphosphonate
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
GGOH	geranylgeraniol
PC	phosphatidylcholine
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
LOV	lovastatin
MVA	mevalonate
uRap1A	unprenylated Rap1A

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Fig. 1.

Apomine down-regulates HMG-CoA reductase protein levels in myeloma cells. JJN-3 cells were treated for 24 h with vehicle only (ctrl), 2, 4, or 12 μ M apomine, and HMG-CoA reductase (HMGR) was detected by SDS-polyacrylamide gel electrophoresis and immunoblotting. Detection of GAPDH served as loading control.

Fig. 2.

Apomine cytotoxicity is not due to inhibition of the mevalonate pathway. A, JJN-3 cells were treated with vehicle only, 10 μ M apomine, or 5 μ M lovastatin (LOV) (positive control) for 24 h. Unprenylated Rap1A (uRap1A) and total Rab6 were detected by immunoblotting (arrow indicates unprenylated Rab6). Detection of β -actin served as loading control. B, JJN-3 cells were cultured with 10 μ M apomine or 10 μ M LOV (positive control) in the presence or absence of 500 μ M mevalonate (MVA) or 10 μ M GGOH for 72 h. Apoptosis was detected using annexin V labeling. Results are given as mean $\pm$ S.D. (triplicates). ***, $p < 0.001$.

Fig. 3.

Phosphatidylcholine protects against the cytotoxic effects of apomine. A, JJN-3 (left) and RPMI 8226 cells (right) were treated with 10 μ M apomine or 10 μ M LOV in the presence or absence of 100 μ g/ml PC (both cell lines) or 200 μ g/ml PC (RPMI 8226). Viable cell number relative to the control was determined after 72 h of treatment using the alamarBlue assay and expressed as percentage of the vehicle-only control. Results are presented as mean $\pm$ S.E.M. of four (JJN-3) or three (RPMI 8226) independent experiments. **, $p < 0.01$; ***, $p < 0.001$. B, JJN-3 cells were treated with 10 μ M apomine or 10 μ M LOV in the presence or absence of 100 μ g/ml PC for 72 h. The percentage of apoptotic cells was determined by annexin V labeling. Results are given as mean $\pm$ S.E.M. of three independent experiments. ***, $p < 0.001$.

Fig. 4.

Apomine counteracts the up-regulation of HMG-CoA reductase by lovastatin, and this is not prevented by PC. JJN-3 cells were treated for 24 h with 0–5 μM LOV (A) or 1 μM LOV in the presence or absence of 2 or 4 μM apomine (B) or combinations of 1 μM LOV, 4 μM apomine, and 100 $\mu\text{g/ml}$ PC (C). HMG-CoA reductase (HMGR) was detected by SDS-polyacrylamide gel electrophoresis and immunoblotting. GAPDH served as loading control.

Fig. 5.

Apomine augments the lovastatin-induced decrease in viable cell number and inhibition of Rap1A prenylation. A, JJN-3 cells were treated for 72 h with combinations of 0–10 μM LOV and 0–4 μM apomine within single experiments, and viable cell number relative to the control was assessed using an alamarBlue assay. Results are expressed as percentage of the vehicle-only control and given as mean $\pm$ S.E.M. of three independent experiments. B, JJN-3 cells were treated for 24 h with 0.1 μM LOV or vehicle in the presence of 0–12 μM apomine, and uRap1A was detected by immunoblotting. Detection of β -actin served as loading control.

Fig. 6.

Synergy between apomine and lovastatin is completely prevented by mevalonate, and apomine does not synergize with the inactive desoxolovastatin. A, JJN-3 cells were treated with combinations of 1 μ M LOV, 4 μ M apomine, 500 μ M MVA, or 100 μ g/ml PC for 72 h, and the percentage of apoptotic cells was determined using annexin V labeling. Results are given as mean $\pm$ S.E.M. of three independent experiments. **, $p < 0.01$; ***, $p < 0.001$; †, $p < 0.05$ compared with the vehicle only control; †††, $p < 0.001$ compared with the respective control (vehicle or PC only). B, JJN-3 cells were treated for 24 h with vehicle only, 1 μ M LOV, 4 μ M apomine, or a combination of both drugs, in the presence or absence of 500 μ M MVA or 100 μ g/ml PC, and uRap1A was detected by immunoblotting. Detection of β -actin served as loading control. C, JJN-3 cells were treated for 72 h with vehicle only, 3 μ M LOV, or 10 μ M desoxolovastatin (desoxo-LOV) in the presence or absence of 2 or 4 μ M apomine, and viable cell number relative to the control was determined using the alamarBlue assay. Results are expressed as percentage of the vehicle-only control and given as mean $\pm$ S.E.M. of three independent experiments. ***, $p < 0.001$.