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Apomine enhances the anti-tumor effects of lovastatin on myeloma cells by downregulating 3-hydroxy-3-methylglutaryl-coenzyme A reductase

Anke J Roelofs, Claire M Edwards, R Graham G Russell, F Hal Ebetino, Michael J Rogers, Philippa A Hulley

Botnar Research Centre, Nuffield Department of Orthopaedic Surgery, University of Oxford, Oxford, UK [AJR, PAH, RGGR]; Bone Research Group, Department of Medicine and Therapeutics, University of Aberdeen, Aberdeen, UK [AJR, MJR]; Department of Cancer Biology, Vanderbilt University Medical Center, Nashville, TN, USA [CME]; Procter & Gamble Pharmaceuticals, Cincinnati, OH, USA [FHE]

Running title: Mechanism of action of apomine and synergy with lovastatin

Address correspondence to: Anke J Roelofs, Bone Research Group, Institute of Medical Sciences, Foresterhill, Aberdeen AB25 2ZD, UK. Phone: +441224554593; Fax: +441224559533; Email: a.roelofs@abdn.ac.uk

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Abstract

Apomine, a 1,1-bisphosphonate-ester with anti-tumor activity, has previously been reported to strongly downregulate 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 downregulated 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 addition of phosphatidylcholine, which is similar to the previously reported ability of phosphatidylcholine to overcome the cytotoxicity of farnesol, while phosphatidylcholine had no effect on downregulation of HMG-CoA reductase by apomine. These findings raised the possibility that apomine, independent from its own cytotoxic effects, could enhance the anti-tumor effects of the competitive HMG-CoA reductase inhibitor lovastatin via downregulating 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 downregulated 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 synergise with des*oxo*lovastatin, 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 downregulation of HMG-CoA reductase by apomine. Thus, these findings demonstrate a novel strategy for enhancing the anti-tumor effects of lovastatin.

Introduction

Apomine (tetraisopropyl 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 wide variety of cancer cell lines (Flach et al., 2000; Lowe et al., 2005), and primary ovarian tumor cells (Alberts et al., 2001). We have previously demonstrated that apomine exerts anti-tumor 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 anti-tumor 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 (Jackson et al., 2000; Berkhout et al., 1997). 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 catalyses the rate-limiting step in the mevalonate and cholesterol biosynthesis pathway. However, while 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 upregulating 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 tumour-associated bone disease (Kanis and McCloskey, 2000; Conte and Coleman, 2004) block protein prenylation (Sinenski et al., 1990; Luckman et al., 1998). Inhibition of protein geranylgeranylation is believed to be largely responsible for the anti-tumour effects of these classes of drugs in myeloma cells, at least *in vitro*, since addition of geranylgeraniol (GGOH) protects cells from their cytotoxic effects (Shipman et al., 1998; Van de Donk et al., 2003). The strong downregulation 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 anti-tumour effects of apomine on myeloma cells.

Alternatively, Flach et al. suggested that apomine may act as a synthetic mimetic of farnesol, since apomine induces apoptosis in HL60 leukaemia cells with characteristics similar to the cytotoxicity induced by farnesol (Flach et al., 2000). 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 downregulation of HMG-CoA reductase by apomine, competitive inhibition of this enzyme by statins triggers compensatory feedback mechanisms, which cause a strong upregulation 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 anti-tumor agents, as 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 anti-tumor potential of statins may prove valuable.

We hypothesized that apomine, by downregulating HMG-CoA reductase protein levels, is able to enhance the effects of lovastatin. The aims of this study were to gain insight into the anti-tumor mechanism of action of apomine on myeloma cells, and to investigate whether apomine, via downregulation of HMG-CoA reductase, could enhance the anti-tumor effects of lovastatin on myeloma cells *in vitro*.

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Materials and methods

Chemicals

Apomine (tetra-iso-propyl 2-(3,5-*di*-*tert*-butyl-4-hydroxyphenyl)ethyl-1,1-diphosphonate; Alberts et al., 2001) was synthesised as previously described (Nguyen et al., 1991). Des*oxo*lovastatin (Weitz-Schmidt et al., 2001) was a kind gift of Dr G. Weitz-Schmidt (Novartis Pharma AG, Basle, Switzerland). Chemicals were from Sigma Chemical Co. (Poole, UK), unless stated otherwise. Stock solutions of apomine, mevalonate, geranylgeraniol (GGOH), and phosphatidylcholine (PC) were prepared in 100% ethanol. Stock solutions of lovastatin were prepared in 100% dimethylsulfoxide.

Cell culture

JJN-3 human myeloma cells were kindly provided by Professor Ian Franklin (University of 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 (Life Technologies, Paisley, UK), supplemented with 10% fetal calf serum (FCS), 1 mM glutamine, 1 mM sodium pyruvate, 1xMEM non-essential amino acids, 100 units/ml penicillin, 100 µg/ml streptomycin, and 50 µM 2-mercaptoethanol. For all experiments, cells were plated out in multiwell-plates (Costar, Corning, NY, USA) at a density of 5 x 10^5 cells/ml.

Preparation of cell lysates and western blotting

Cells were lysed in RIPA buffer [PBS containing 1% (v/v) NP-40, 0.1% (w/v) sodium dodecyl sulfate, 0.5% (w/v) sodium deoxycholate, 1:100 Sigma protease inhibitor cocktail], or in TBS containing 1 mM EDTA, 1 mM PMSF, 1% (v/v) Triton-X100,

and 2% (v/v) dimethylsulfoxide. Protein content was determined using a bicinchoninic acid (BCA) assay (Pierce Biotechnology, Rockford, IL, USA). Lysates for detection of Rap1A and Rab6 were heated for 5 min at 95˚C to optimise denaturation of protein. This procedure was not carried out for HMG-CoA reductase samples, as 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 polyvinyl 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-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 PBS, and resuspended in annexin-binding buffer [10 mM HEPES, 140 mM NaCl, and 2.5 mM $CaCl_2$, pH 7.4]. Cells were incubated with annexin V conjugated to either alexafluor 488 or alexafluor 647 (Molecular Probes, Eugene, OR) for 15 min at RT, placed back on ice, and immediately analysed by flow cytometry on a FACS Calibur (BD BioSciences, Oxford, UK). The nick translation assay was performed as

described previously (Roelofs et al., 2006). Results were confirmed by staining with DAPI (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, Camarillo, CA, USA). 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 Biotek fluorescence plate-reader (Winooski, VT), at excitation wavelength 530 nm, and emission wavelength 590 nm. Results are corrected for background fluorescence and expressed as 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 3 independent experiments. IC_{50} concentrations were calculated using GraphPad Prism software by non-linear 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 analyse 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 analysed using one-way analysis of variance, and Tukey's post-hoc test.

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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 CHO 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 μM 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 downregulated in response to 2 and 4 μM apomine, and was barely detectable following treatment with 12 μM apomine. Similar results were obtained in the RPMI 8226 myeloma cell line (not shown). It is unclear what the band of higher molecular weight in the 12 μM apomine sample is, but this could represent ubiquitinylated HMG-CoA reductase, since HMG-CoA reductase has been reported to be downregulated by increased ubiquitinylation and proteasomal degradation (Roitelman et al., 2004).

Apomine does not inhibit protein prenylation

To investigate whether the downregulation 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, or with the bisphosphonate risedronate. Whereas incubation with 5 μM lovastatin for 24 h clearly caused accumulation of unprenylated Rap1A and Rab6, treatment with 10 μM apomine (a concentration that downregulates HMG-CoA reductase and induces apoptosis, see Fig. 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 μM 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 inhibition of the mevalonate pathway. To confirm this, JJN-3 cells were treated with vehicle only, 10 μM apomine, or 10 μM lovastatin (positive control) in the presence or absence of 500 μM mevalonate or 10 μM GGOH for 72 h, and apoptosis was detected using annexin V labeling (Fig. 2B). Mevalonate is the product of the reaction catalysed 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 (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 since various cell types can be rescued from farnesol-induced apoptosis by 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 μM apomine in JJN-3 cells ($p<0.001$; Fig. 3A). In addition, 100 μg/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 μg/ml PC conferring partial protection ($p<0.01$), and 200 μg/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 μg/ml PC significantly rescuing JJN-3 cells from induction of apoptosis by 1 μM 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 μM lovastatin in RPMI 8226 cells, even at the higher concentration of 200 μg/ml PC (Fig. 3A), suggesting this is a cell line specific effect.

Apomine counteracts upregulation 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 upregulation 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). Since apomine alone downregulated 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 as compared to the increase observed with lovastatin treatment alone (Fig. 4B). HMG-CoA reductase protein was similar to control levels after co-incubation with 1 μM lovastatin and 12 μM apomine (data not shown). Importantly, PC had no effect on the downregulation of either basal or lovastatin-upregulated HMG-CoA reductase protein levels by apomine (Fig 4C), indicating that the downregulation of HMG-CoA reductase by apomine is not secondary to its cytotoxic effects. Similar results were obtained using RPMI 8226 cells (not shown).

Lovastatin and apomine synergistically decrease viable cell number and increase apoptosis

The strong upregulation 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. Since apomine was able to counteract this strong lovastatin -induced upregulation of HMG-CoA reductase enzyme levels (Fig. 4), we investigated whether apomine enhances the anti-tumor 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 ± SD 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 ± 0.4 μM (lovastatin alone) to 2.6 ± 0.3 μM ($p<0.001$), 1.9 ± 0.1 μM ($p<0.001$), and 0.9 ± 0.0 μM ($p<0.001$) in the presence of 1, 2, and 4 μM 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 ± 1.4 μM in JJN-3 cells, and 8.7 ± 1.5 μM in RPMI 8226 cells (data from 3 independent experiments; not shown). lovastatin and apomine also caused a synergistic increase in the percentage of apoptotic cells (Fig. 6A). Treatment with 1 μM lovastatin alone caused a modest increase in apoptosis from 26.6 ± 3.6% in the control, to 43.2 ± 5.1% in response to 1 μM lovastatin (mean ± S.E.M. of 3 independent experiments; $p<0.05$). However, when combined with 4 μM apomine, which on its own did not affect the percentage of apoptotic cells, apoptosis was strongly increased to 87.0 ± 2.1% (mean ± S.E.M. of 3 independent experiments; $p<0.001$ as compared to 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 μM lovastatin (a

concentration which 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 ($p<0.01$; mean ± S.D. of 4 independent experiments) in response to 0.1 μM lovastatin in the presence of 8 or 12 μM apomine, as compared to lovastatin treatment alone. The apparent decrease in amount of unprenylated Rap1A at 12 μM apomine (in the presence of lovastatin), as compared to 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 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 μM mevalonate ($p<0.001$; Fig. 6A). Addition of mevalonate also completely blocked the synergistic increase in unprenylated Rap1A by combination treatment with 1 μM lovastatin and 4 μM apomine (Fig. 6B). In contrast, PC, which protects against the cytotoxic effects of apomine, only had a partially protective effect

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against the synergistic induction of apoptosis (Fig. 6A), similar to lovastatin treatment alone (Fig. 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 (not shown). Furthermore, PC did not prevent the enhanced accumulation of unprenylated Rap1A (Fig. 6B).

Apomine does not act in synergy with des*oxo*lovastatin

The complete rescue from the synergistic interaction between apomine and lovastatin by 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 synergises with des*oxo*lovastatin, and analogue of lovastatin that is unable to inhibit HMG-CoA reductase. Treatment with 10 μM des*oxo*lovastatin 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 μM des*oxo*lovastatin and 2 or 4 μM apomine had no effect on viable cell number either. In contrast, treatment with 3 μM 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 non-sterol 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 downregulate HMG-CoA reductase by causing this enzyme to be ubiquitinylated 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 downregulating the target enzyme HMG-CoA reductase, may enhance the effects of lovastatin.

Apomine is a potent anti-neoplastic agent, inducing apoptosis in a wide variety of tumor cells (Flach et al., 2000), and we have recently shown that apomine alone has anti-myeloma 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 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 anticancer drugs. However, its anti-tumor 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 anti-tumor effects of apomine involve downregulation of HMG-CoA reductase and/or inhibition of protein

prenylation. Our findings show that although apomine strongly downregulates 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 localisation in breast cancer cells (Lowe et al., 2005). A possible explanation for the lack of effect of apomine on protein prenylation, despite the strong downregulation 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 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.* suggested that apomine acts as a synthetic mimetic of farnesol (Flach et al., 2000; Niesor et al., 2001). 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 synergises with sterols to downregulate HMG-CoA reductase (Sever et al., 2003).

Since 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 downregulation 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 downregulation 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 upregulation of HMG-CoA reductase protein levels in these cells. A recent study showed that the simvastatin upregulated expression of HMG-CoA reductase protein in the liver of rats *in vivo* was decreased following 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 as compared to either drug alone, determined by decreases in viable cell number and induction of apoptosis. Importantly, this was observed at concentrations at which

apomine downregulated 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 i) accumulation of unprenylated Rap1A (an indicator of inhibition of protein prenylation) was increased in response to combination treatment with lovastatin and apomine, as compared to lovastatin alone, ii) 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, iii) 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 iv) apomine did not synergise with des*oxo*lovastatin, an analogue of lovastatin that lacks the ability to inhibit HMG-CoA reductase.

In recent years, there has been increasing interest in statins as potential anti-tumor agents (Graaf et al., 2004), especially in combination regimens with other anti-cancer agents to enhance their anti-tumor potential *in vivo* (Sleijfer et al., 2005). These findings suggest a novel approach to increasing the anti-tumor activity of statins, by combining treatment with an agent that downregulates 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 utilise 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 and co-workers 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/day for 7 consecutive days) resulted in a maximum serum concentration of 3.9 μM lovastatin

(Thibault et al., 1996). 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 tumours 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 anti-tumor 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* appeared to be due to enhanced transcription of BMP-2 in (pre-)osteoblasts, this effect could be blocked by mevalonate, suggesting that stimulation of the BMP-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 anti-tumor 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 synergy between these two drugs is the direct result of increased inhibition of HMG-CoA reductase, most likely due to downregulation of HMG-CoA reductase enzyme levels by apomine.

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Footnotes

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Requests for reprints should be addressed to:

Anke Roelofs, Bone Research Group, Institute of Medical Sciences, Foresterhill, Aberdeen AB25 2ZD, UK. Email: a.roelofs@abdn.ac.uk

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Legends for Figures

Figure 1

Apomine downregulates HMG-CoA reductase protein levels in myeloma cells. JJN-3 cells were treated for 24 h with vehicle only, 2, 4, or 12 μM apomine, and HMG-CoA reductase (HMGR) was detected by SDS-PAGE and immunoblotting. Detection of GAPDH served as loading control.

Figure 2

Apomine cytotoxicity is not due to inhibition of the mevalonate pathway. (A) JJN-3 cells were treated with vehicle only, 10 μM apomine, or 5 μM 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 μM apomine or 10 μM lovastatin (LOV) (positive control) in the presence or absence of 500 μM mevalonate (MVA) or 10 μM geranylgeraniol (GGOH) for 72 h. Apoptosis was detected using annexin V labeling. Results are given as mean ± SD (triplicates). *** indicates $p<0.001$.

Figure 3

Phosphatidylcholine protects against the cytotoxic effects of apomine. (A) JJN-3 (left) and RPMI 8226 cells (right) were treated with 10 μM apomine or 10 μM lovastatin (LOV) in the presence or absence of 100 μg/ml phosphatidylcholine (PC) (both cell lines) or 200 μg/ml phosphatidylcholine (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 ± S.E.M. of 4 (JJN-3) or 3 (RPMI 8226) independent experiments. ** indicates $p<0.01$; *** indicates $p<0.001$; ††† indicates $p<0.001$ compared with PC alone. (B) JJN-3 cells were treated with 10 μM apomine or 10 μM lovastatin (LOV) in the presence or absence of 100 μg/ml phosphatidylcholine (PC) for 72 h. The percentage of apoptotic cells was determined by annexin V labeling. Results are given as mean ± S.E.M. of 3 independent experiments. *** indicates $p<0.001$.

Figure 4

Apomine counteracts the upregulation of HMG-CoA reductase by lovastatin, and this is not prevented by phosphatidylcholine (PC). JJN-3 cells were treated for 24 h with 0-5 μM lovastatin (LOV) (A), or 1 μM lovastatin (LOV) in the presence or absence of 2 or 4 μM apomine (B), or combinations of 1 μM lovastatin (LOV), 4 μM apomine, and 100 μg/ml phosphatidylcholine (PC) (C). HMG-CoA reductase (HMGR) was detected by SDS-PAGE and immunoblotting. GAPDH served as loading control.

Figure 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 lovastatin (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 ± S.E.M. of 3 independent experiments. (B) JJN-3 cells were treated for 24 h with 0.1 μM lovastatin (LOV) or vehicle, in the presence of 0-12 μM apomine, and unprenylated Rap1A (uRap1A) was detected by immunoblotting. Detection of β-actin served as loading control.

Figure 6

Synergy between apomine and lovastatin is completely prevented by mevalonate, and apomine does not synergise with the inactive des*oxo*lovastatin. (A) JJN-3 cells were treated with combinations of 1 μM lovastatin (LOV), 4 μM apomine, 500 μM mevalonate (MVA), or 100 μg/ml phosphatidylcholine (PC) for 72 h, and the percentage of apoptotic cells was determined using annexin V labeling. Results are given as mean ± S.E.M. of 3 independent experiments. ** indicates $p<0.01$; *** indicates $p<0.001$; † indicates $p<0.05$ compared to the vehicle only control; ††† indicates $p<0.001$ compared to the respective control (vehicle or PC only). (B) JJN-3 cells were treated for 24 h with vehicle only, 1 μM lovastatin (LOV), 4 μM apomine, or a combination of both drugs, in the presence or absence of 500 μM mevalonate (MVA) or 100 μg/ml phosphatidylcholine (PC), and unprenylated Rap1A (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 μM lovastatin (LOV), or 10 μM des*oxo*lovastatin (des*oxo*LOV) in the presence or absence of 2 or 4 μM 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 ± S.E.M. of 3 independent experiments. *** indicates $p<0.001$.

Figure 1

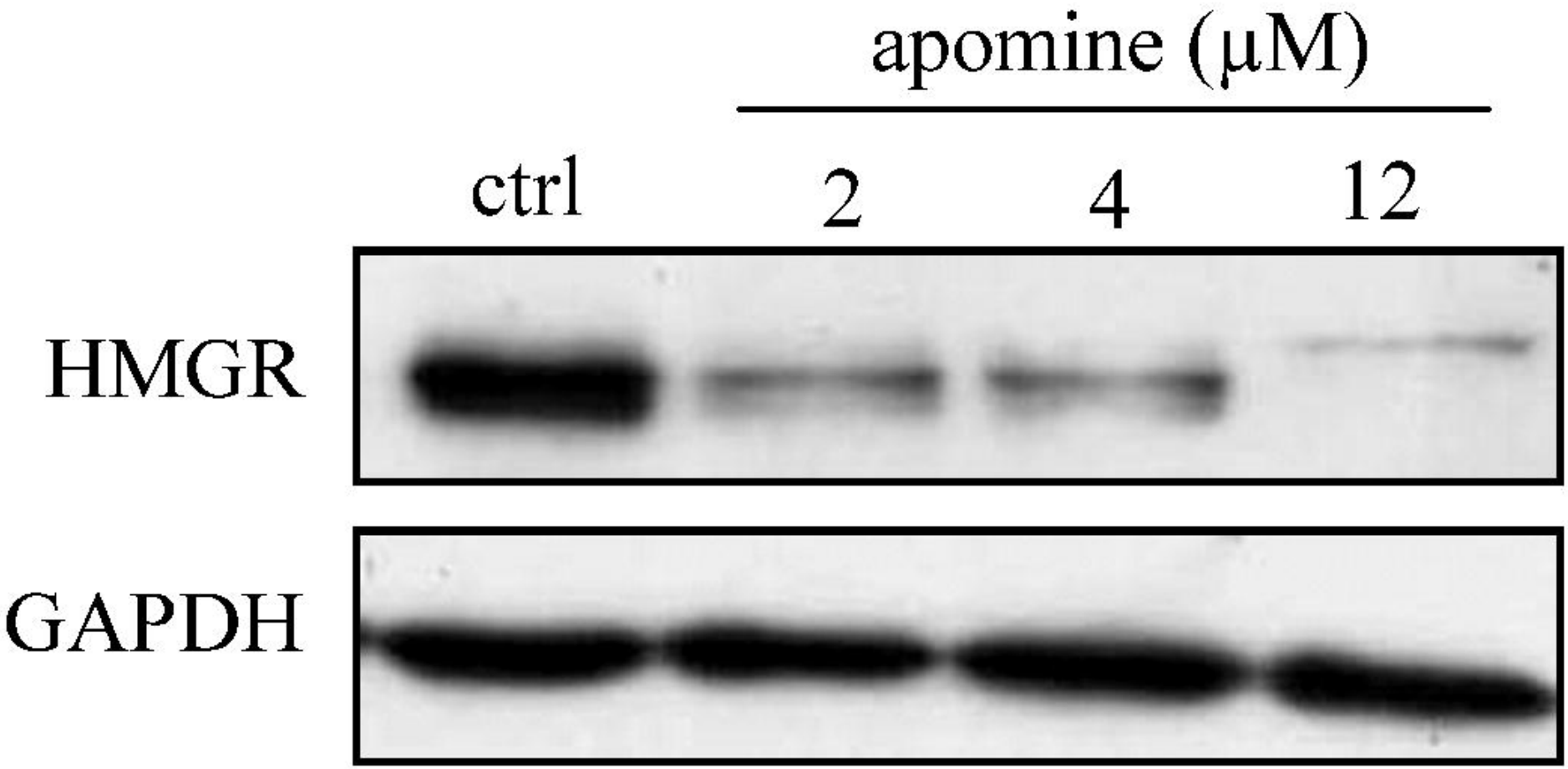

Figure 2

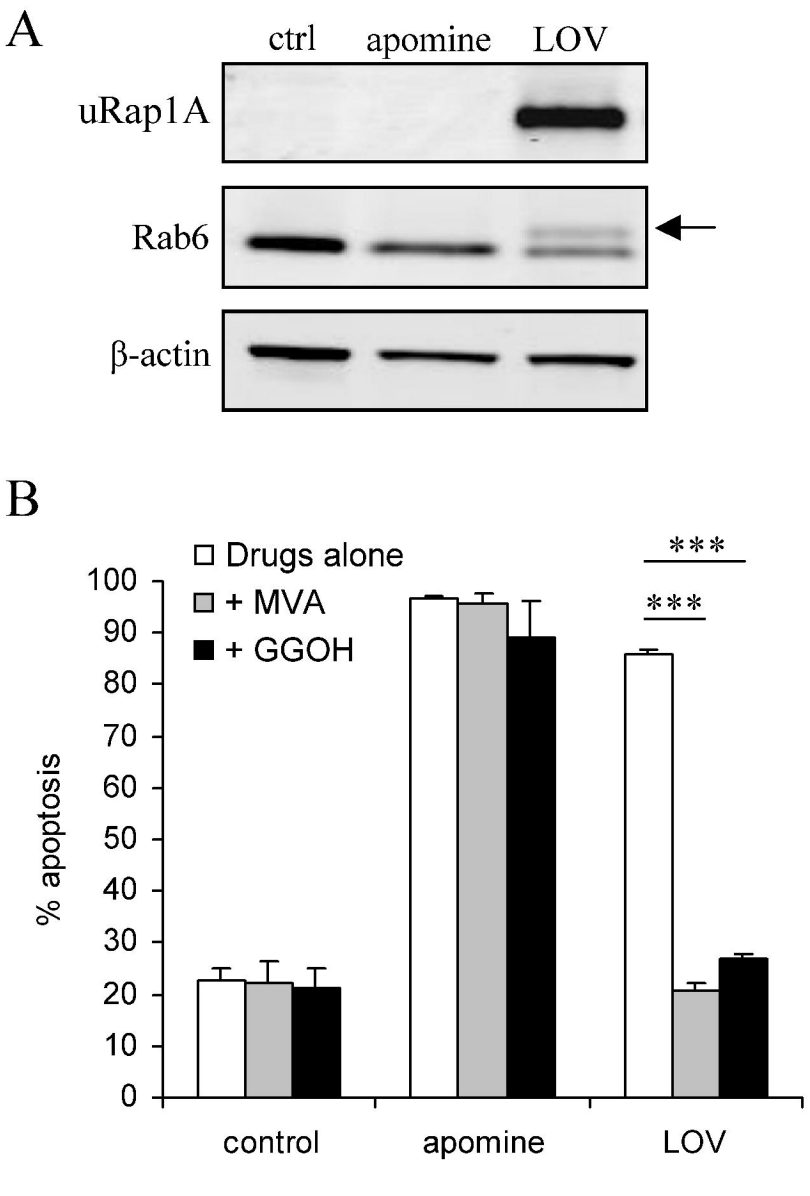
A
ctrl
apomine
LOV
uRap1A
Rab6
β-actin
B
Drugs alone
+ MVA
+ GGOH
***
***
% apoptosis
100
90
80
70
60
50
40
30
20
10
0
control
apomine
LOV

Figure 3

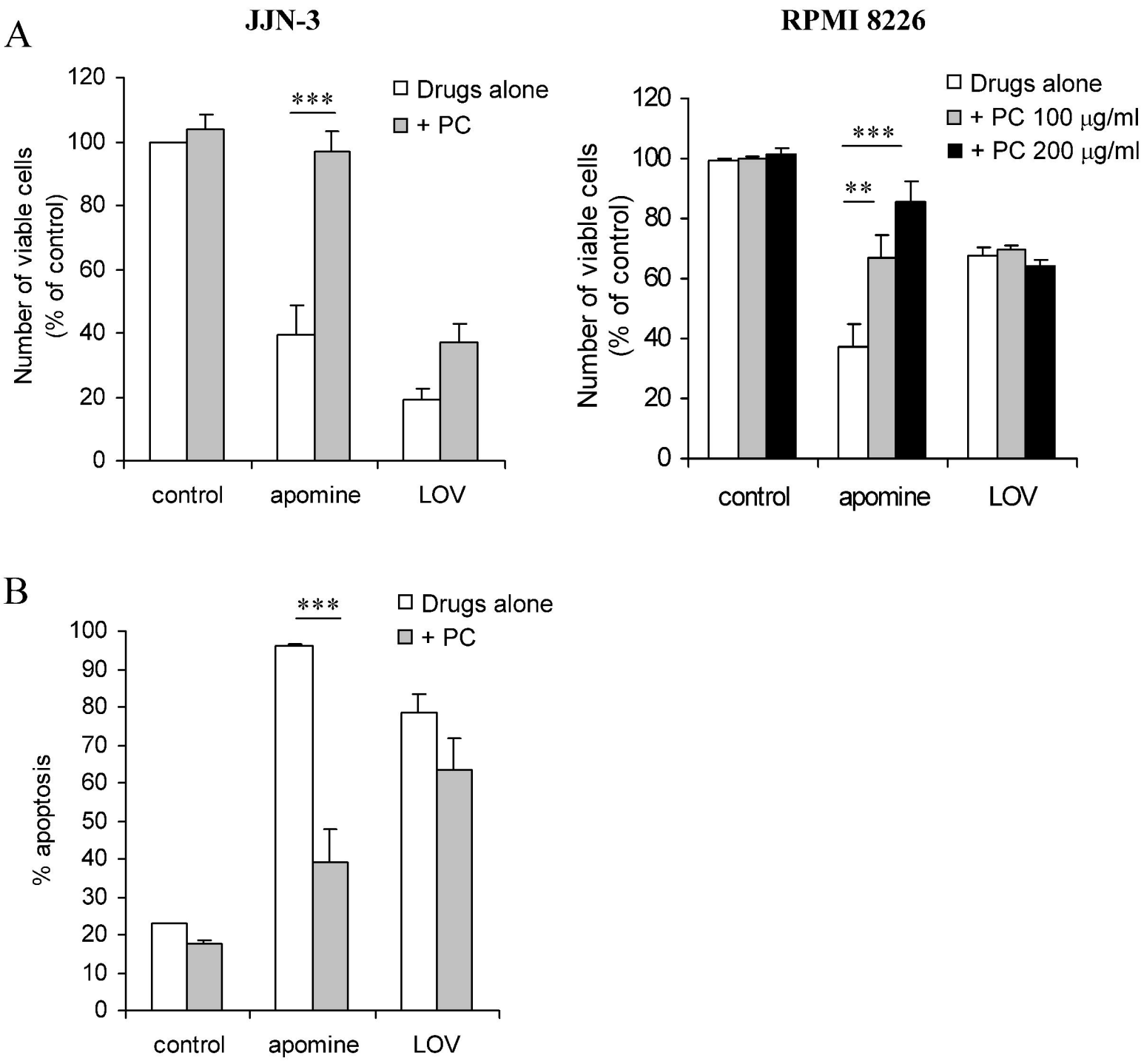
A
JJN-3
RPMI 8226
Number of viable cells (% of control)
Drugs alone
+ PC
+ PC 100 μg/ml
+ PC 200 μg/ml
***
**
control
apomine
LOV
B
% apoptosis

Figure 4

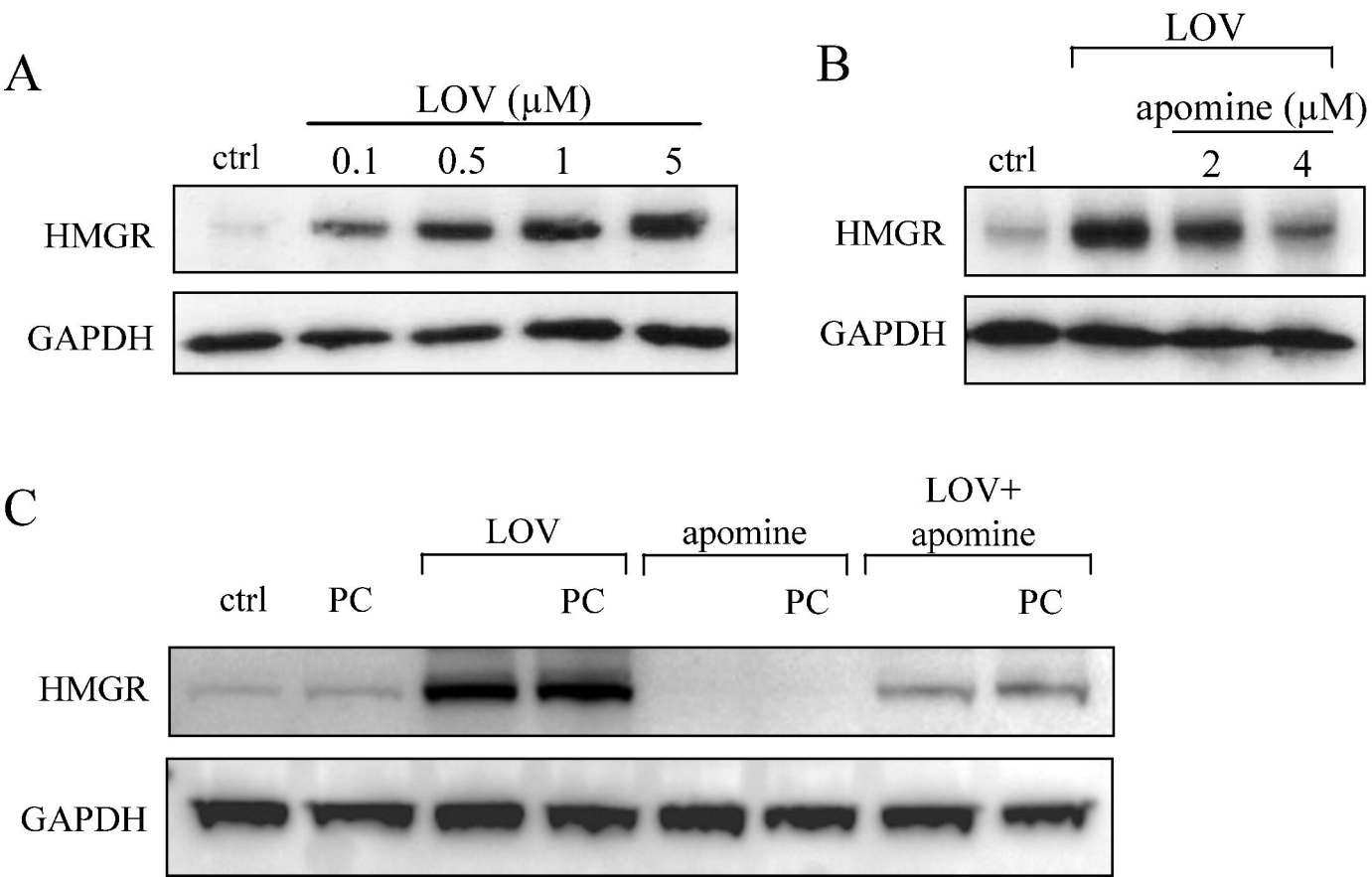
A
LOV (μM)
ctrl
0.1
0.5
1
5
HMGR
GAPDH
B
LOV
apomine (μM)
ctrl
2
4
HMGR
GAPDH
C
LOV
apomine
LOV+
apomine
ctrl
PC
PC
PC
PC
HMGR
GAPDH

Figure 5

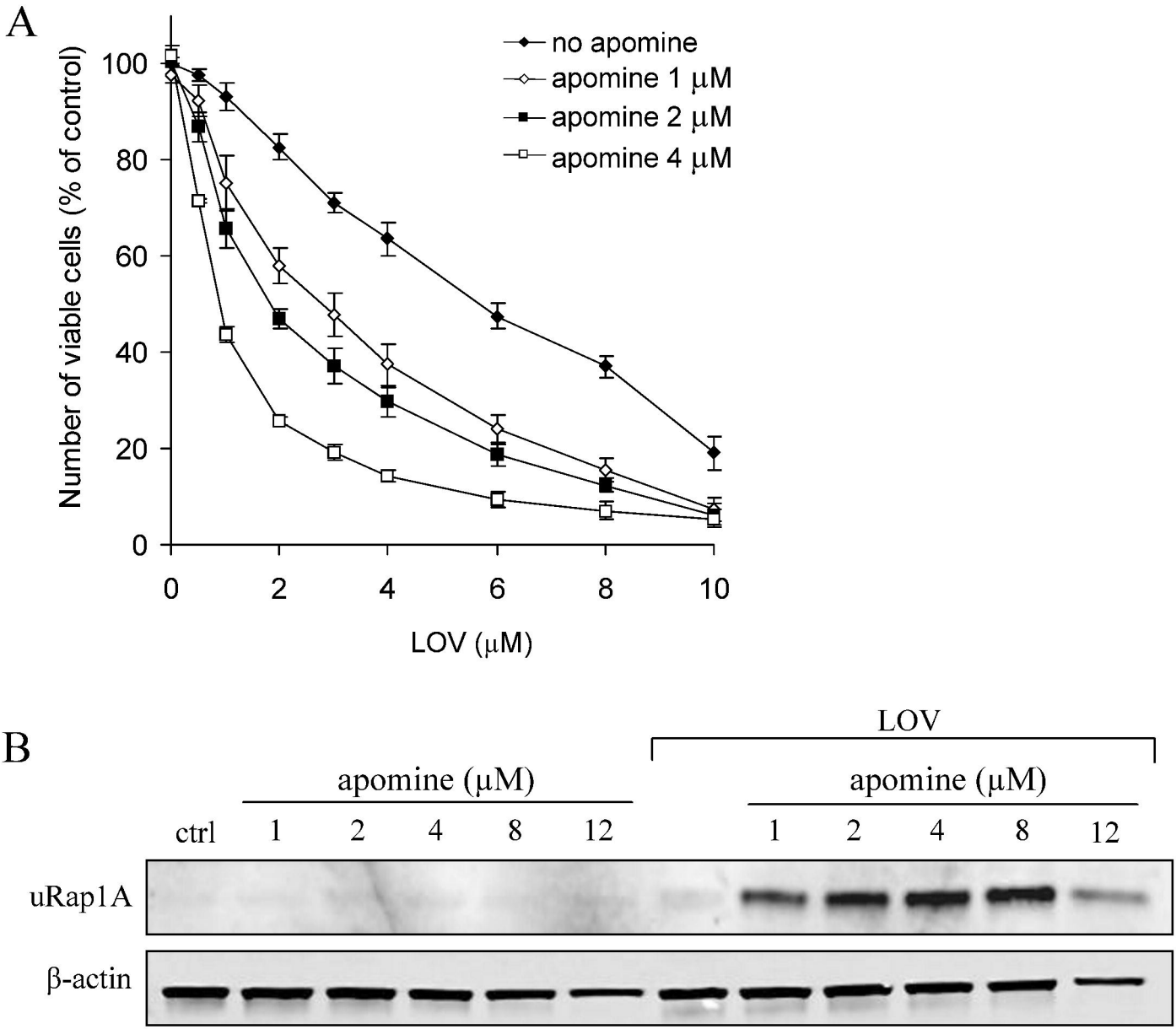
A
no apomine
apomine 1 μM
apomine 2 μM
apomine 4 μM
Number of viable cells (% of control)
100
80
60
40
20
0
0
2
4
6
8
10
LOV (μM)
B
LOV
apomine (μM)
apomine (μM)
ctrl
1
2
4
8
12
1
2
4
8
12
uRap1A
β-actin

Figure 6

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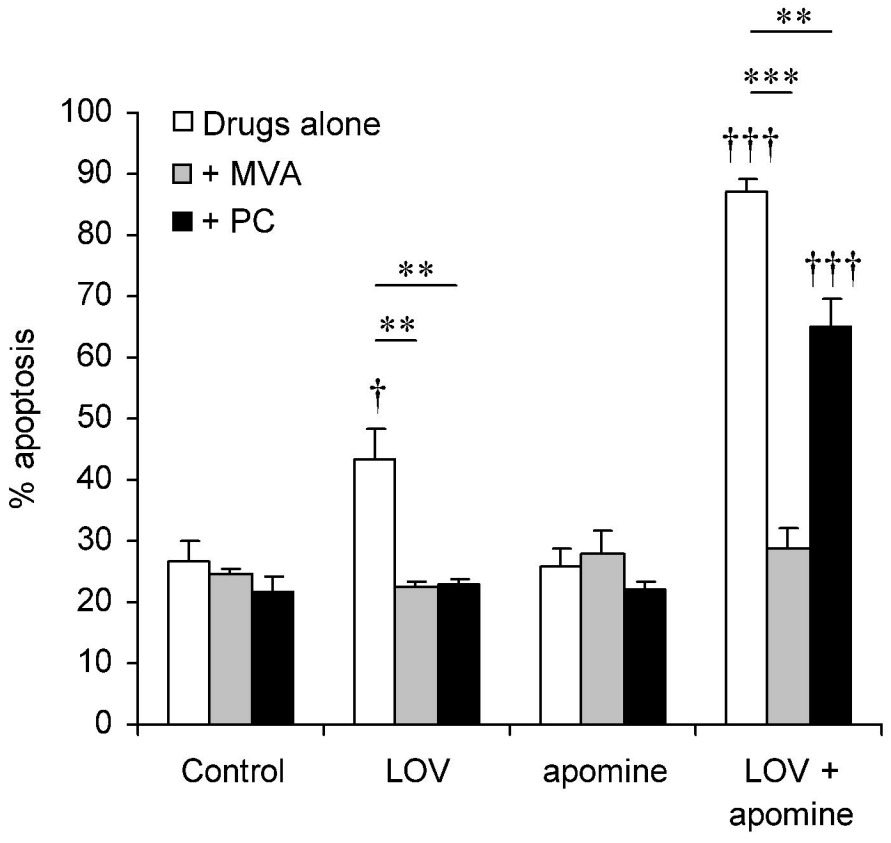
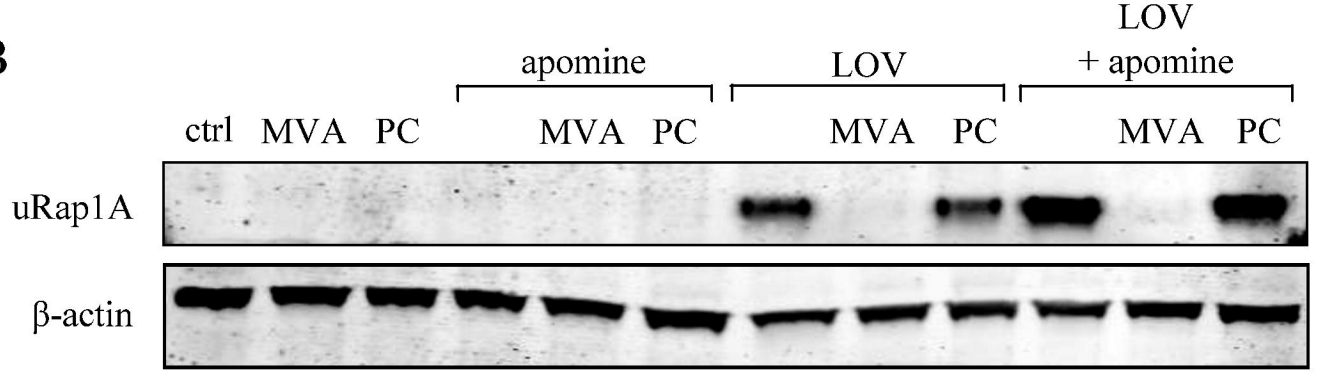
A



B



C