The HMG-CoA inhibitor, simvastatin, triggers *in vitro* anti-tumour effect and decreases IgM secretion in Waldenstrom macroglobulinaemia

Anne-Sophie Moreau,[1,2] Xiaoying Jia,[1] Christopher J. Patterson,[1] Aldo M. Roccaro,[1] Lian Xu,[1] Antonio Sacco,[1] Kelly O'Connor,[1] Jacob Soumerai,[1] Hai T. Ngo,[1] Evdoxia Hatjiharissi,[1] Zachary R. Hunter,[1] Bryan Ciccarelli,[1] Robert Manning,[1] Irene M. Ghobrial,[1] Xavier Leleu[1,2] and Steven P. Treon[1]

[1]Bing Center for Waldenstrom's Macroglobulinemia, Dana-Farber Cancer Institute, Boston, MA, USA, and [2]Service des maladies du sang, Centre Hospitalier Régional Universitaire, and INSERM U837, Lille, France

Summary

Waldenstrom macroglobulinaemia (WM) is an incurable lymphoplasmacytic lymphoma with secretion of serum monoclonal immunoglobulin M (IgM). We previously showed that patients receiving cholesterol-lowering statins, had the lowest IgM value in a large cohort of patients with WM. Simvastatin, a 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitor, induced inhibition of proliferation, cytotoxic effect and apoptosis in IgM secreting cell lines as well as in primary CD19^{+} WM cells. Interestingly, those effects were reversed by addition of mevalonate and geranylgeranylpyrophosphate, demonstrating that simvastatin inhibited cell growth, survival and IgM secretion on BCWM.1 WM cells by inhibition of geranylgeranylated proteins. Furthermore, simvastatin overcame tumour cell growth induced by co-culture of WM cells with bone-marrow stromal cells. Simvastatin also decreased IgM secretion by BCWM.1 cells at an early time-point that had not affected cell survival. Simvastatin-induced cytotoxicity was preceded by a decrease in Akt (protein kinase B, PKB) and extracellular signal-regulated kinase (ERK) mitogen-activated protein kinase (MAPK) pathways at 18 h. In addition, simvastatin induced an increase in stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) MAPK followed by caspase-8, -9, -3 and poly(ADP-ribose) polymerase (PARP) cleavages at 18 h, leading to apoptosis. Furthermore, simvastatin enhanced the cytotoxicity induced by bortezomib, fludarabine and dexamethasone. Our studies therefore support our earlier observation of statin-mediated anti-WM activity and provide the framework for future clinical trials testing simvastatin in WM.

Keywords: Waldenstrom macroglobulinemia, simvastatin, HMG-CoA reductase inhibitor.

Waldenstrom macroglobulinemia (WM) is an indolent lymphoma characterized by accumulation of lymphoplasmacytic cells in the bone marrow (BM) and production of a monoclonal immunoglobulin M (IgM) (Owen *et al*, 2003). Current treatment options in WM provide an overall response rate of 30–70% in front line and 30–40% in relapse/refractory disease, with less than 10% of patients attaining a complete response (Treon *et al*, 2006; Vijay & Gertz, 2007). Despite advances in the treatment of WM, the disease remains incurable with a median overall survival of 5–6 years. Therefore, there is a need for new drugs.

In a recent study, we observed an inverse relationship between the serum IgM level and the serum low-density lipoprotein cholesterol level on 110 patients with WM. Interestingly, the patients who were treated with statins for hypercholesterolaemia (20–80 mg/d) had the lowest IgM value (Patterson, 2006). These findings led us to investigate the anti-tumour effect of statins in WM disease.

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Statins are a family of 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase inhibitors used for the treatment of hypercholesterolemia, and act by interfering with the mevalonate pathway (Tobert, 2003). Statins inhibit the activity of HMG-CoA reductase, which catalyses the rate-limiting step in mevalonate biosynthesis. Mevalonate is a precursor for cholesterol synthesis as well as for the synthesis of isoprenoids (farnesyl and geranylgeranyl lipid chains), important for the membrane localization and function of small G proteins such as Ras, RhoA, Rac1 and Cdc42 (Seabra, 1998). Those proteins are implicated in many pathways that regulate cell cycle progression, cell growth and survival, such as phosphoinositide-3 kinase (PI3k)/Akt and mitogen-activated protein kinase (MAPK) signalling pathways (Frost *et al*, 1997).

Statin activity has been studied in several malignancies, including breast cancer (Denoyelle *et al*, 2003; Kozar *et al*, 2004), prostate cancer (Moyad *et al*, 2005), multiple myeloma (MM) and lymphoma (van de Donk *et al*, 2003, 2005). At high doses (15 mg/kg/d), statins have been shown to have an anti-tumour effect in MM (Thibault *et al*, 1996; van der Spek *et al*, 2006). In a phase 1 study in patients with cancer, high doses of lovastatin (2–25 mg/kg/d) were shown to be safe (Thibault *et al*, 1996).

The present study sought to determine the effect of statins in WM *in vitro*. We first showed that simvastatin inhibited cell proliferation and triggered a cytotoxic effect in WM cell lines as well as in WM patient samples, and was more efficient than lovastatin and pravastatin. Those effects were mainly due to the inhibition of protein geranylgeranylation of downstream signalling pathways. Finally, this report showed that simvastatin has synergistic activity when combined with several important WM therapeutics.

Material and methods

Cells

The WM cell lines [Bing Center for Waldenstrom's Macroglobulinaemia (BCWM.1) and Wayne State University (WSU)-WM] and IgM-secreting cell lines (MEC-1, RL) were used. BCWM.1 was developed from a patient with untreated WM (Santos *et al*, 2006), WSU-WM (Al-Katib *et al*, 2003) was a gift from Dr. Al Katib (Wayne State University, Detroit, MI, USA) and MEC-1 from Dr. Neil Kay (Mayo Clinic, Rochester, MN, USA). RL was purchased from the American Type Tissue Culture Collection (Manassas, VA, USA). All cell lines were cultured as previously described (Moreau *et al*, 2007). Patient samples were obtained after approval from the Dana-Farber Cancer Institute Institutional Review Board (DFCI-IRB). Informed consent was obtained from all patients according to the declaration of Helsinki. Peripheral blood mononuclear cells (PBMC) from healthy volunteers and primary BM WM cells were obtained as previously described (Moreau *et al*, 2007).

Reagents

Simvastatin, lovastatin and pravastatin were purchased from Calbiochem (San Diego, CA, USA). Mevalonate, squalene, farnesylpyrophosphate (FPP), geranylgeranylpyrophosphate (GGPP), the selective farnesyl-transferase inhibitor FTI-277 and the geranylgeranyl-transferase inhibitor GGTI-298 as well as fludarabine and dexamethasone were purchased from Sigma (St. Louis, MO, USA). Bortezomib was provided by Millenium Pharmaceuticals (Cambridge, MA, USA). Recombinant human interleukin 6 (rhIL-6) and recombinant B lymphocyte stimulator (BLyS) were purchased from R&D systems (Minneapolis, MN, USA).

Cytotoxicity assay

The inhibitory effect of simvastatin on WM cell survival was assessed by measuring 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT assay; Chemicon International, Temecula, CA, USA) dye absorbance as previously described (Hideshima *et al*, 2000).

DNA synthesis

The WM cells proliferation was measured by the uptake of the [^{3}H]-thymidine ([^{3}H]-TdR; Perkin Elmer, Boston, MA, USA), as previously described (Hideshima *et al*, 2000).

Effect of simvastatin on paracrine WM cell growth in the BM

To evaluate growth stimulation and signalling in WM cells adherent to BM stromal cells (BMSC), BCWM.1 cells were cultured in BMSC-coated 96-well plates for 48 h, and DNA synthesis was measured as previously described (Hideshima *et al*, 2000).

Effect of the statins on IgM secretion by WM tumour cells

The BCWM.1 cells were cultured with simvastatin (10–25 μmol/l) or media control (RPMI medium with 1% fetal bovine serum [FBS]), in the presence or absence of mevalonate, squalene, GGPP or FPP. Culture supernatant was harvested at 24 h and IgM concentration in the supernatant was determined by enzyme-linked immunosorbent assay (ELISA; Bethyl, Montgomery, TX, USA) as previously described (Tassone *et al*, 2005).

Colony forming units assay

Colony forming units (CFU) assay was performed as previously described (Moreau *et al*, 2007). The following colonies: erythroid burst-forming units (BFU-E)-, granulocyte CFU, macrophage CFU, and granulocyte-macrophage-erythroid-

megakaryocyte CFU were counted at day 14–16 with an inverted microscope.

Cell cycle and apoptosis analysis

Cell-cycle analysis was performed using flow cytometry with propidium iodide (PI) staining as previously described (Hideshima *et al*, 2000). Apo2.7-PE staining was used to detect and quantify apoptosis by flow cytometry. Cells were processed with a cytomics FC500 analyser.

Immunoblotting

Immunoblotting was performed as previously described (Moreau *et al*, 2007). The antibodies included: anti-phospho (p)-Akt (Ser473), -Akt, -p-ERK (Thr202/Tyr204), -ERK1/2, -p-S6 ribosomal (Ser240/244), -p-p38MAPK, -p-stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK), -caspase-3,-caspase-8,-caspase-9, -PARP, -Mcl1, -Cdc42, -RhoA, -Rac1/2/3, -Ras (p21), -p21$^{waf-1/cip-1}$, -p27^{kip-1}, -p-cycline-dependent kinase (CDK)2 (Thr160), -CDK2, -p-Rb (Ser807/811) (Cell Signaling Technology, MA), -p53 and -α-tubulin (Santa Cruz Biotechnology, CA) antibodies.

Statistical analysis

The interaction between simvastatin and either bortezomib, fludarabine and dexamethasone was determined by isobol-ogram analysis using the CalcuSyn software program (Biosoft, Ferguson, MO, USA). The Chou-Talalay method calculates a combination index (CI) to indicate additive or synergistic effects. When CI < 0·8, effects are synergistic. Results from viability assays, using the MTT assays, were expressed as fraction of cells killed by the individual drugs or the combination in drug-treated *versus* untreated cells.

Results

Simvastatin inhibits proliferation and induces cytotoxicity in WM cells

The WM (BCWM.1 and WSU-WM) and IgM secreting cell lines (RL and MEC-1) were cultured for 72 h in the presence of simvastatin (1·25–25 μmol/l) or media alone. Simvastatin inhibited BCWM.1 cells proliferation at 72 h as measured by the ^{3}H-thymidine uptake assay with 50% inhibitory concentration (IC$_{50}$) between 2·5 and 5 μmol/l (Fig 1A). A similar degree of inhibition was observed on all other cell lines using ^{3}H-thymidine uptake assay (data not shown). Simvastatin also triggered cytotoxic activity on all cell lines tested, including BCWM.1, at 72 h by the MTT assay, with an IC$_{50}$ between 2·5 and 10 μmol/l (Fig 1B). Similar results were obtained with lovastatin (IC$_{50}$ = 5 μϖ mol/l), whereas the lipophobic agent pravastatin showed no

efficacy (Fig 1C). Because simvastatin is a derivative of lovastatin, simvastatin was chosen for further experiments in this study.

Simvastatin-induced cytotoxic effect was also observed on CD19$^+$ WM patient cells (IC$_{50}$ = 10–50 μmol/l) using the MTT assay at 5 d (*n* = 3, Fig 1D). In contrast, simvastatin (5–50 μmol/l) had no cytotoxic effect on PBMCs from three healthy volunteers at 72 h (data not shown). However, we observed a cytotoxic effect of simvastatin (10 μmol/l) on erythroid progenitors (BFU-E) using the CFU assay. At 15 d, a decrease in BFU-E formation of 80% was observed but not of other hematopoietic progenitors upon simvastatin treatment (Fig 1E). These results demonstrated that simvastatin triggers significant and selective cytotoxicity of WM cells.

Simvastatin targets Akt and MEK/ERK MAPK pathways

As the PI3K/Akt and mitogen-activated protein kinase kinase (MEK)/ERK MAPK pathways are known to promote growth and survival of tumour B cells (Okkenhaug & Vanhaesebroeck, 2003), we studied Akt and ERK1/2 activity by immunoblot upon simvastatin treatment. Simvastatin partially inhibited phosphorylation of Akt (Ser473) and of downstream ribo-somal protein S6 in a time- (Fig 2D) and dose-dependent fashion at 18 h (Fig 2A). Simvastatin also inhibited phosphor-ylation of ERK1/2 (Thr202/Tyr204) and of p38MAPK at higher doses at 18 h (Fig 2B), whereas SAPK/JNK was activated at low doses of simvastatin. The activation of SAPK/JNK has previ-ously been reported to precede induction of apoptosis in statin-treated cells (Liang *et al*, 2006) and MM tumour cells (Hideshima *et al*, 2006).

Nuclear factor (NF) κB is one of the major pathways implicated in plasma cell dyscrasia cell growth and survival (Hideshima *et al*, 2002). Recently, NFκB activity has also been shown to be downregulated upon statin treatment (Ahn *et al*, 2007). We therefore investigated the effect of simvastatin on the NFκB canonical pathway in BCWM.1 cells. Tumour necrosis factor α (10 ng/ml)-induced IκBα phosphorylation at 10 min and subsequent degradation were inhibited by sim-vastatin 25 μmol/l in a time- (Fig 2D) and dose-dependent manner (Fig 2C), leading to retention of IκBα and NFκBp50 in the cytoplasm at 18 h.

Simvastatin induces apoptosis in WM cells

We next examined the mechanisms whereby simvastatin induces cytotoxicity in WM cells. Simvastatin induced significant dose-dependent apoptosis at 72 h in BCWM.1 cells, with 10 μmol/l of simvastatin inducing 45% apoptosis (Fig 3A). Similar results were observed in other IgM-secreting cell lines, RL, MEC1 and WM-WSU (data not shown). To determine the mechanism of simvastatin-induced apoptosis, we investigated the effect of simvastatin on BCWM.1 cells using immunoblotting. Simvastatin (25 μmol/l) induced activation of both the extrinsic and intrinsic pathways of apoptosis with caspases-8, -9, -3 and
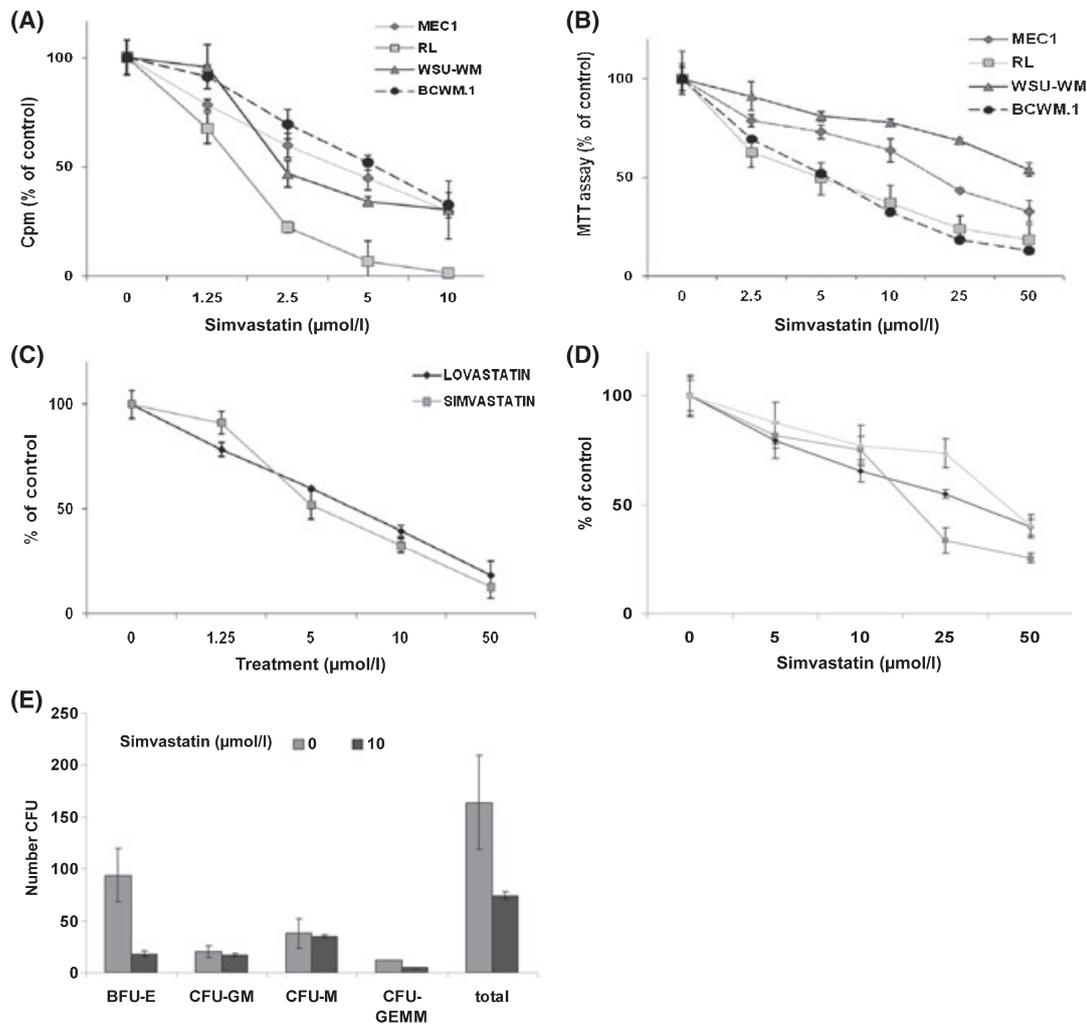
Fig 1. Simvastatin induced a decrease in proliferation and triggered cytotoxicity. Several immunoglobulin M (IgM)-secreting cell lines, RL (□), MEC-1 (◇), WM-WSU (Δ) and BCWM.1 (•) were cultured with simvastatin (2·5–25 μmol/l) or solvent control for 72 h. (A)Thymidine uptake assay. (B) MTT assay. (C) MTT assay. Simvastatin triggers significant cytotoxicity in BCWM.1 WM cells similar to lovastatin, in favour of a class effect on WM cells. (D) Freshly isolated bone marrow CD19+ tumour cells from three patients with WM were cultured with simvastatin (5–50 μmol/l). Cytotoxicity was assessed by the MTT assay. (E) Colony forming-unit Assay. Negative fraction after CD19+ selection of bone marrow mononuclear cells from patients with WM was cultured using methylcellulose semi-solid technique in absence and presence of simvastatin (10 μmol/l) for 14–16 d. BFU-E, erythroid burst-forming units; CFU-GM, granulocyte-macrophage colony-forming units; CFU-M, macrophage colony-forming units; CFU-GEMM, granulocyte/erythroid/macrophage/megakaryocyte colony-forming units. All results represent mean values (±SD) of triplicate experiments (A–C), D was done in duplicate.

poly(ADP-ribose) polymerase (PARP) cleavages and a decrease of the anti-apoptotic protein Mcl-1 in a dose-dependent fashion at 18 h (Fig 3B).

In addition, simvastatin (1·25–10 μmol/l) induced a G0/G1 arrest on cell cycle analysis at 24 h (Fig 3C). Immunoblotting was used to study the molecular pathways of cell cycle G0/G1 arrest on BCWM.1 cells. Simvastatin induced an increased expression of p53 tumour suppressor protein as well as an increase of the expression of the CDK inhibitors p21[waf−1/cip−1] and p27[kip−1], in a dose-dependent fashion at 24 h (Fig 3D). Those proteins are known to regulate CDK2 (Poon *et al*, 1996), important for the G1 to S transition of cell cycle through inactivation of retinoblastoma tumour suppressor

protein (Rb) (Lundberg & Weinberg, 1998). Simvastatin induced a decrease in CDK2 expression and phosphorylation on Thr160 on BCWM.1 cells as well as an activation of Rb protein as shown by a decrease in Rb phosphorylation on Ser807/811 (Fig 3D) and inhibition of cell cycle progression (Lundberg & Weinberg, 1998).

GGPP reverses simvastatin-induced cytotoxicity

The HMG-CoA reductase inhibitors inhibit the whole mevalonate pathway, including cholesterol synthesis, protein farnesylation and protein geranylgeranylation. We therefore investigated which of these lipid forms was important in WM
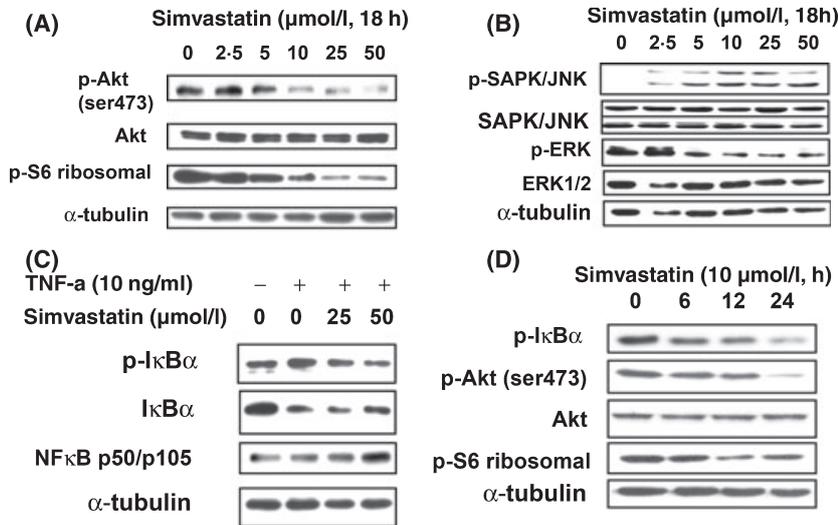
Fig 2. Simvastatin targets Akt and MEK/ERK MAPK pathways. (A) BCWM.1 cells were cultured with control media, simvastatin (2·5–50 μmol/l) for 18 h. Whole cell lysates were subjected to Western blotting using anti-p-Akt (Ser473), -Akt, -p-S6 ribosomal and -α-tubulin antibodies. (B) BCWM.1 cells were cultured with simvastatin (2·5–50 μmol/l) for 18 h. Whole-cell lysates were subjected to Western blotting using anti-p-SAPK/JNK, -SAPK/JNK, -p-ERK (Thr202/Tyr204), -ERK and -α-tubulin antibodies. (C) BCWM.1 cells were cultured with simvastatin (25–50 μmol/l) for 18 h in presence or absence of TNF-α (10 ng/ml) for 10 min. Whole-cell lysates were subjected to Western blotting using anti-p-IκBα, -IκBα, -NFκBp50/105 and -α-tubulin antibodies. (D) BCWM.1 cells were cultured with simvastatin (10 μmol/l) for 6, 12 and 24 h. Whole-cell lysates were subjected to Western blotting using anti-p-IκBα, -p-Akt, -Akt, -p-S6R and -α-tubulin antibodies.
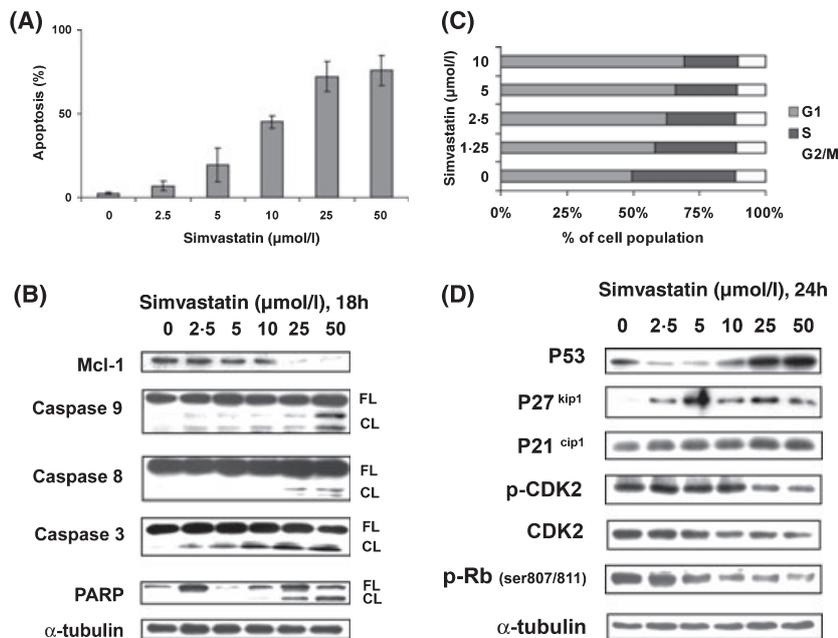

Fig 3. Simvastatin induced apoptosis in WM cell line BCWM1. (A) BCWM.1 cells were cultured with simvastatin (2·5–50 μmol/l) for 72 h and the percentage of cells undergoing apoptosis was studied by Apo2.7 staining at 72 h. (B) BCWM.1 cells were cultured with simvastatin (2·5–50 μmol/l) for 18 h. Whole-cell lysates were subjected to Western blotting using anti-Mcl1, -caspase 9, 8, 3, -PARP and -α-tubulin antibodies. (C) Cell cycle was studied using PI staining by flow cytometry. BCWM.1 cells were cultured for 24 h in 10% RPMI medium. Percentages indicate cells in G1 phase, S phase and G2 phase. (D) BCWM.1 cells were cultured with simvastatin (2·5–50 μmol/l) for 24 h. Whole-cell lysates were subjected to Western blotting using anti-phospho (p)-CDK2 (Thr160), -CDK2, -P53, -P27[kip1], -P21[cip1], -p-Rb (Thr807/811) and -α-tubulin antibodies. All data represent mean (±SD) of triplicate experiments (A and C).

cell survival by adding mevalonate, squalene, FPP or GGPP to simvastatin-treated BCWM.1 cells. As shown in Fig 4A, addition of mevalonate (100 μmol/l) and GGPP (10 μmol/l)

completely reversed simvastatin (1·25–25 μmol/l)-induced cytotoxic effects at 72 h, whereas the addition of squalene (200 μmol/l) or FPP (10 μmol/l) had no effect. These results
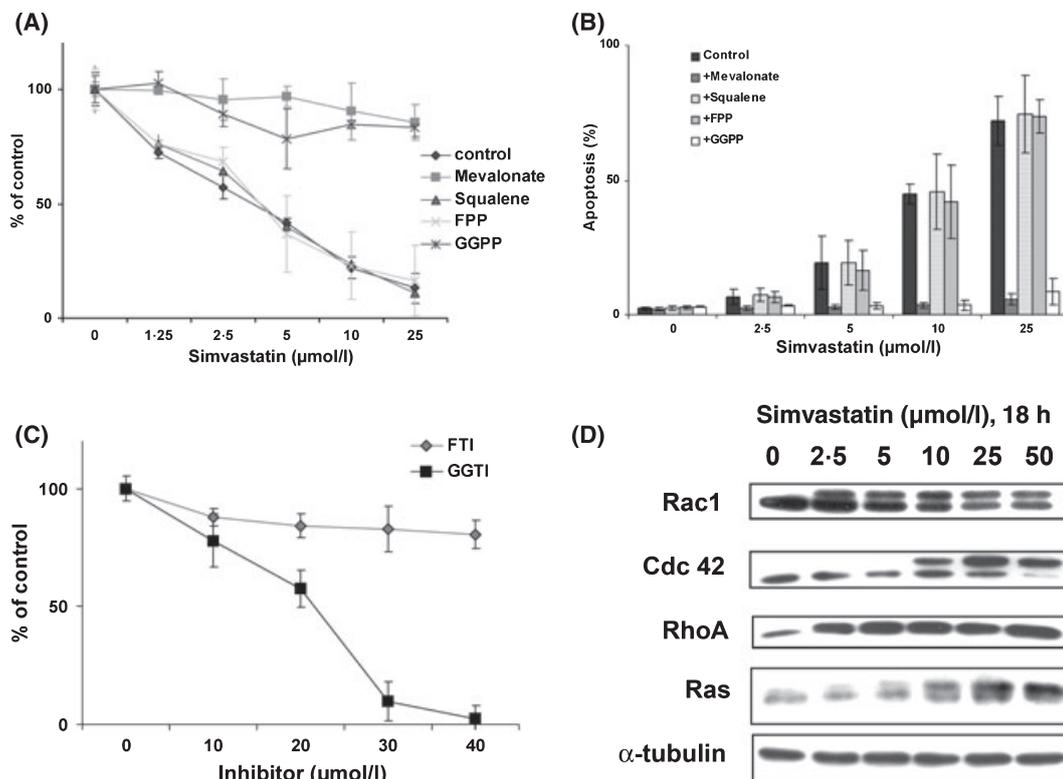
Fig 4. Geranylgeranylated proteins are essential for WM cell growth and survival. (A) BCWM.1 cells were treated with simvastatin (1·25–25 µmol/l) for 72 h and cultured with control media in the absence and presence of mevalonate (100 µmol/l), squalene (200 µmol/l), FPP (10 µmol/l) or GGPP (10 µmol/l). (B) BCWM.1 cells were cultured with control media in the absence and presence of mevalonate (100 µmol/l), squalene (200 µmol/l), FPP (10 µmol/l) or GGPP (10 µmol/l) and treated with simvastatin (1·25–25 µmol/l) for 72 h. Apoptosis was assessed by apo2.7 staining and flow cytometry analysis. (C) BCWM.1 was cultured with FTI or GGTI (10–40 µmol/l) or media control for 72 h. (D) BCWM.1 cells were cultured with simvastatin (2·5–50 µmol/l) for 24 h. Whole-cell lysates were subjected to Western blotting using anti-RhoA, -Cdc42, -Rac1/2/3, -Ras and α-tubulin antibodies. Cell viability was assessed using the MTT assay (A and C). All data represent mean (±SD) of triplicate experiments (A–C).

suggest that simvastatin inhibited cell growth and survival in BCWM.1 WM cells by inhibition of geranylgeranylated proteins. Simvastatin-induced apoptosis was also reversed by the addition of mevalonate (100 µmol/l) and GGPP (10 µmol/l) at 72 h (Fig 4B). Therefore, the geranylgeranylated proteins appear to be important for WM cell growth and survival.

To confirm the above results, we used the specific farnesyl-transferase inhibitor FTI-277 (10–40 µmol/l) and geranylger-anyl-transferases inhibitor GGTI-298 (10–40 µmol/l) on BCWM.1 cells. GGTI-298 induced 90% cell death at 20 µmol/l using the MTT assay, whereas FTI-277 (40 µmol/l) induced only a slight cytotoxic effect with 20% cell death at 48 h (Fig 4C). These results confirmed that simvastatin inhibited cell growth and survival in BCWM.1 WM cells by inhibition of geranylgeranylated proteins, but not farnesylated proteins, as was observed with the addition of GGPP and FPP to the simvastatin-treated cells.

We next studied geranylgeranylated proteins by immuno-blot analysis. BCMW.1 cells were treated with simvastatin (2·5–50 µmol/l), and blots stained with specific antibodies for Cdc42, Rac1/2/3 RhoA and Ras. We observed a single band of 21 kD in untreated cells, while cells treated with simvas-

tatin demonstrated a mobility-shifted isoform corresponding to unprenylated proteins (Fig 4D). As previously shown, the electrophoretic mobility of unmodified proteins is slower than geranylgeranylated forms in sodium dodecyl sulphate (SDS)-containing gels (Detter *et al*, 2000). Similarly, the study of farnesylated proteins, such as Ras, showed an additional band on Western blots (15% SDS-polyacrylamide gel elctrophoresis), which confirmed that simvastatin was also efficient in inhibiting protein farnesylation, although it might have only a slight impact on cell survival upon simvastatin treatment.

Simvastatin decreases IgM secretion by WM cells

As serum IgM secretion is a hallmark of WM and serves as a biomarker for studying response to therapy, we next sought to determine whether or not simvastatin could inhibit IgM secretion by tumour cells. Simvastatin (25 µmol/l) inhibited IgM secretion by BCWM.1 cells by 60% at 24 h, whereas simvastatin-induced cytotoxicity was only 20% at 25 µmol/l using the MTT assay (Fig 5A). We previously showed that GGTI-298 significantly inhibited BCWM.1 growth and
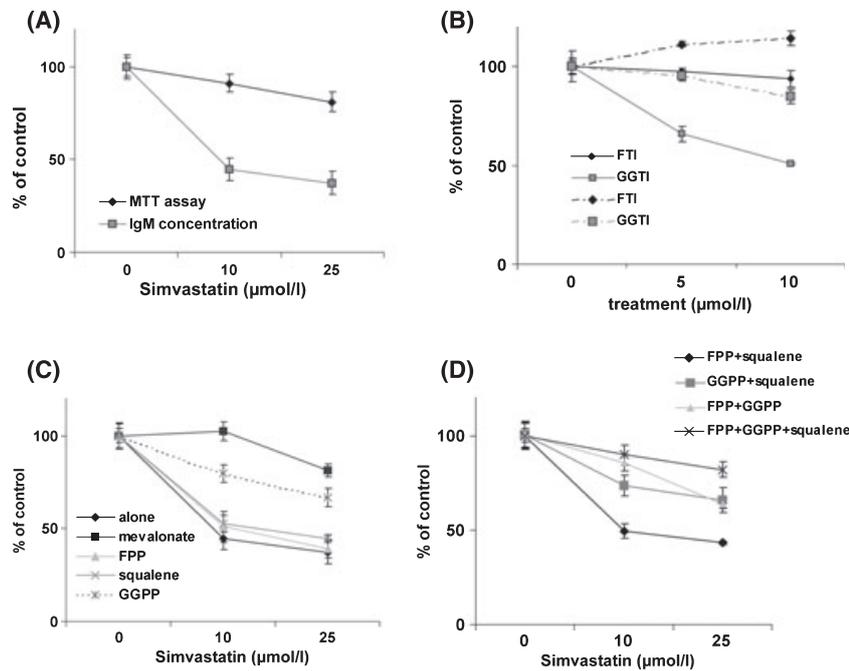
Fig 5. Simvastatin decreases IgM secretion by tumour cells. (A) BCWM.1 cells were cultured with control media (RPMI medium with 1% FBS) or simvastatin (10–25 μmol/l) for 24 h. Cell viability at 24 h was assessed by MTT assay and IgM secretion assessed by ELISA. (B) BCWM.1 cells were cultured with control media, FTI or GGTI (5–10 μmol/l) for 6 h then cell viability was assessed by the MTT assay (dotted line) and IgM concentration (full line) in the supernatant was determined by ELISA assay. (C and D) BCWM.1 cells were cultured with control media, simvastatin (10–25 μmol/l) for 24 h in presence or absence of mevalonate (100 μmol/l), squalene (200 μmol/l), FPP (10 μmol/l) or GGPP (10 μmol/l), alone or in combination. IgM concentration in the supernatant was determined by ELISA assay. All data represent mean (±SD) of triplicate experiments.

survival compared to FTI-277. We then sought to determine whether inhibition of geranylgeranylated proteins and downstream pathway also inhibited IgM secretion by BCWM.1 cells greater than inhibition of farnesylated proteins. GGTI-298 (10 μmol/l) induced 50% decrease of IgM secretion by BCWM.1 cells compared to FTI-277 (10 μmol/l) at 6 h (Fig 5B – full lines). This effect was not related to a significant decrease of survival and number of cells, as shown in Fig 5B (dotted lines). These results suggest that simvastatin inhibited IgM secretion in BCWM.1 WM cells by inhibition of geranylgeranylated proteins and, to lesser extent, farnesylated proteins.

To confirm those results, we next investigated whether mevalonate, squalene, FPP and GGPP metabolites could rescue IgM secretion of simvastatin-treated BCWM.1 cells. As shown in Fig 5C, mevalonate (100 μmol/l) completely rescued IgM secretion of BCWM.1 cells treated with 10 μmol/l of simvastatin, whereas GGPP (10 μmol/l) only partially rescued IgM secretion up to 80%. In contrast, squalene (200 μmol/l) and FPP (10 μmol/l) did not significantly rescue IgM secretion. Interestingly, only the combination of squalene, FPP and GGPP could completely rescue IgM secretion by BCWM.1 cells (Fig 5D), mimicking the effect observed with mevalonate. This suggested that not only GGPP, but also squalene and FPP to a lesser extent, and the pathways downstream of farnesylated and

geranygeranylated proteins are important for simvastatin inhibition of IgM secretion in BCWM.1 WM cells.

Neither growth factors nor adherence to BMSCs protect against simvastatin-induced cytotoxicity

Previous studies using gene expression analysis in WM have demonstrated an upregulation in IL-6 signaling (Hatzimichael et al, 2001; Chng et al, 2006). IL-6 also promotes tumour cell growth in WM, and serum IL-6 levels reflect tumour burden and disease severity (Hatzimichael et al, 2001). We therefore tested the effect of rhIL-6 (50 ng/ml) on WM cells and determined whether simvastatin could overcome its protective effects. As shown in Fig 6A, simvastatin (1·25–25 μmol/l) was able to overcome survival induced by rhIL-6 at 72 h using the MTT assay. Similarly, BLyS (200 ng/ml), a known B-cell maturation and survival factor (Elsawa et al, 2006), was tested on BCWM.1 cells, using the MTT assay. BLyS-induced survival was overcome by simvastatin (1·25–25 μmol/l) at 72 h (Fig 6B).

We recently demonstrated that the BM microenvironment conferred growth and drug resistance to WM cells (Moreau et al, 2007). We therefore studied whether simvastatin could overcome the protective effect conferred by BMCSs on BCWM.1 cells. BCWM.1 cells were cultured for 48 h with
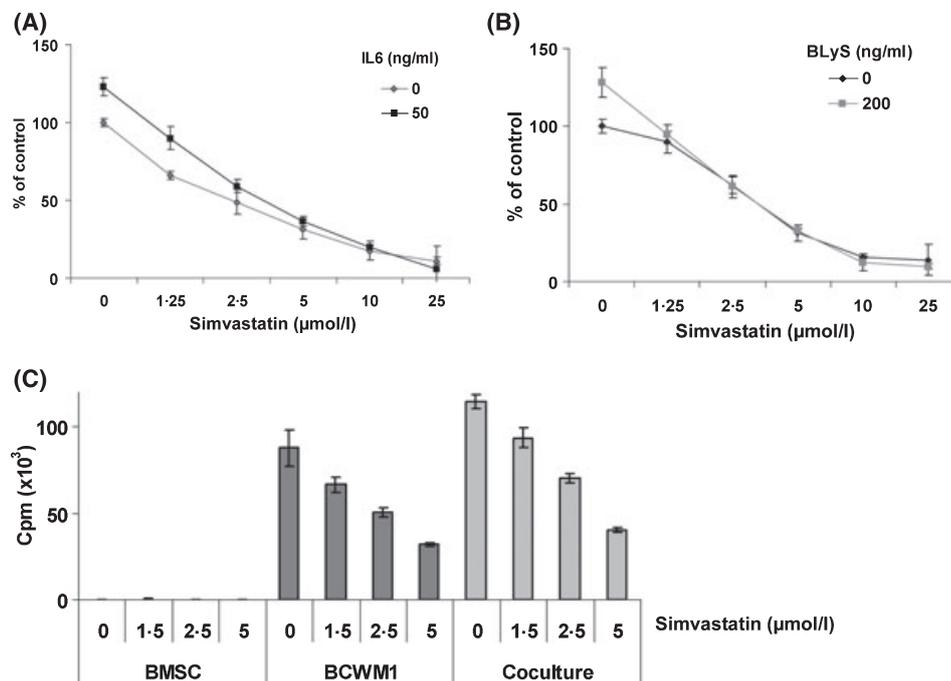
Fig 6. Growth factors and coculture with BMSC do not protect against simvastatin-induced WM cell cytotoxicity. (A) BCWM.1 cells were cultured with control media in the absence and presence of IL-6 (50 ng/ml) and treated with simvastatin (1·25–25 µmol/l) for 72 h. (B) BCWM.1 cells were cultured with control media in the absence and presence of BLyS (200 ng/ml) and treated with simvastatin (1·25–25 µmol/l) for 72 h. Cell survival was assessed by the MTT assay (A and B). (C) BCWM.1 cells were cultured with control media, and with 1·25, 2·5 and 5 µmol/l of simvastatin for 48 h, in the presence or in the absence of BMSCs. Cell proliferation was assessed using the [^{3}H]-thymidin uptake assay. All data represent mean (±SD) of triplicate experiments (A–C).

simvastatin (1·25–5 µmol/l), in the presence or absence of BMSCs. Using the thymidine uptake assay, adherence of BCWM.1 cells to BMSC conferred increased cell proliferation (30%), which was inhibited by simvastatin in a dose-dependent fashion ($P = 0·02$) (Fig 6C). These data indicated that simvastatin can trigger significant anti-tumour activity in the bone marrow milieu.

Simvastatin enhances cytotoxicity of other therapeutics in WM

Steroids, nucleoside analogues (Treon *et al*, 2006) and most recently bortezomib (Chen *et al*, 2007) have become commonly employed drugs in the treatment of WM (Table I). However, there is a need to better define the best combinations including those drugs, as response rates and complete response rates are limited when those agents are used alone.

We therefore determined the effects of simvastatin in combination with bortezomib, fludarabine and dexamethasone on BCWM.1 cells using the MTT assay. BCWM.1 cells were cultured for 48 h with bortezomib (5–10 nmol/l) and simvastatin (1·25–5 µmol/l). Bortezomib-induced cytotoxicity was significantly increased by simvastatin in a dose-dependent fashion. Bortezomib (5 nmol/l) induced 28% cytotoxicity, which was augmented to 53% (CI = 0·83) and 63% (CI = 0·76) by 2·5 and 5 µmol/l simvastatin, respectively, indicating a moderate synergistic effect. We also studied the

Table I. Fractions affected (FA) and combination index (CI) of the combinations of simvastatin with bortezomib, fludarabine or dexamethasone.

No.	Simvastatin (µmol/l)	Bortezomib (nmol/l)	FA	CI
1	1·25	5	0·46	0·857
2	1·25	10	0·81	0·605
3	2·5	5	0·53	0·835
4	2·5	10	0·84	0·556
5	5	5	0·63	0·758
6	5	10	0·86	0·534
No.	Simvastatin (µmol/l)	Fludarabine (µg/mol/l)	FA	CI
1	2·5	5	0·56	0·852
2	2·5	10	0·65	0·873
3	5	5	0·74	0·839
4	5	10	0·84	0·657
No.	Simvastatin (µmol/l)	Dexamethasone (nmol/l)	FA	CI
1	1·25	50	0·63	0·301
2	2·5	50	0·78	0·428
3	5	50	0·9	0·555

All experiments were repeated in triplicate.

combination of simvastatin with fludarabine. BCWM.1 cells were cultured for 48 h with fludarabine (5−10 µg/ml) and simvastatin (1·25–5 µmol/l). Fludarabine (5 µg/ml) induced

25% cytotoxicity, which increased to 56% with 2·5 μmol/l of simvastatin (CI = 0·85) and to 74% with 5 μmol/l of simvastatin (CI = 0·84), indicating an, at least, additive effect. Finally, we studied the effect of dexamethasone (50 nmol/l) with simvastatin (1·25–5 μmol/l). At 72 h, simvastatin (1·25 μmol/l) induced 10% cytotoxicity, which was increased to 63% in combination with dexamethasone (50 ng/ml) (CI = 0·30) indicating a strong synergistic effect.

Discussion

The molecular pathways dysregulated in WM have not been well defined, and most of the therapeutic agents used in WM have been applied based on their activity in other related lymphoproliferative disorders, such as MM and chronic lymphocytic leukaemia. Therefore, there is a need to better understand the pathogenesis of WM to develop targeted therapies.

We studied the effect of simvastatin on WM cell growth and signalling *in vitro*. Simvastatin is an HMG-CoA reductase inhibitor that inhibits cholesterol synthesis at the mevalonate pathway and also disrupts cholesterol synthesis as well as protein farnesylation and geranylgeranylation. Simvastatin was found to inhibit proliferation of WM tumour cells at the clinically achievable dose of 5 μmol/l, induced a G0/G1 cell cycle arrest and targeted the PI3K/Akt and MEK/ERK MAPK pathways and downstream NFκB pathway, as previously described (Roudier *et al*, 2006),(Ahn *et al*, 2007). Interestingly, simvastatin induced apoptosis in WM cell lines through the phosphorylation of SAPK/JNK MAPK and activation of both the intrinsic and extrinsic apoptotic pathways resulting in caspases-3 and PARP cleavage.

Statins have been tested *in vitro* in MM cell lines and patient samples, with an IC_{50} of 2–60 μmol/l at day 4 (van de Donk *et al*, 2002), in accordance with our IC_{50} of 5 μmol/l on WM cell lines and 10 μmol/l on patient samples. Lovastatin has been tested at doses of 2–45 mg/kg/d in a phase 1 study in patients with cancer, and appeared to be safe at higher doses than those commonly used for the treatment of hypercholesterolemia (20–80 mg/d) (Thibault *et al*, 1996). Simvastatin has been tested in an open phase 1 dose escalation study for relapse/refractory patients with MM or lymphoma, in association with vincristine, adriamycin and dexamethasone (VAD) or cyclophosphamide, doxorubicin, prednisone, vincristine (CHOP) (van der Spek *et al*, 2006). The dose-limiting toxicity, in combination with those regimens, was 15 mg/kg/d and overall response was 30%. Our experimental results are therefore encouraging and clinically achievable. However, due to the slow mode of action of statins, clinical trials using statins should be designed for low progressing or smouldering WM patients, to delay the progression of the disease.

Simvastatin inhibited IgM secretion by tumour cells *in vitro*, independently of a cytotoxic effect on tumour cells, in accordance with the lower serum IgM levels observed in patients with WM receiving statins. This effect was completely rescued by addition of mevalonate but only partially by the addition of squalene, FPP or GGPP. Interestingly, mevalonate and GGPP completely rescued cells from simvastatin-induced cytotoxicity whereas squalene or FPP had no effect. This suggests that pathways downstream of farnesylated or geranylgeranylated proteins are altogether part of the process of simvastatin-induced decrease in IgM secretion, while only geranylgeranylated proteins and their downstream pathways are important for simvastatin-induced WM cell cytotoxicity, as observed in MM (Thibault *et al*, 1996; van de Donk *et al*, 2005). This result might provide new insights in the understanding of WM cell survival and IgM secretion.

Given its use in future clinical trials, we studied simvastatin effects on PBMC and haematopoietic progenitors from patients. Although we did not find any toxicity of simvastatin on PBMCs, on the CFU assay, simvastatin inhibited BFU-E formation, suggesting a possible induction of anaemia in patients receiving high doses of simvastatin. These data are in concordance with the occurrence of temporary anaemia in clinical trials in relapse/refractory MM patients receiving high dose simvastatin (>5 mg/kg/d) (van der Spek *et al*, 2006).

The role of the BM microenvironment in regulation of growth and drug resistance of malignant cells in WM is not well defined. Previous studies in other B-cell malignancies have demonstrated that cytokines, such as IL-6 (Hatzimichael *et al*, 2001) and BLyS (Elsawa *et al*, 2006), as well as binding of tumour cells to BM stromal cells, are critical regulators of WM tumour cell growth. The present study showed that adherence to BM stromal cells and cytokines induced proliferation in WM cells and, importantly, that simvastatin induces cytotoxicity even in the BM milieu.

The regulation of signalling pathways in malignant cells is complex, and therefore rationally designed combinations of novel agents that target specific dysregulated pathways in WM are essential to overcome resistance and induce apoptosis. We thus tested the effect of simvastatin combined to other agents that are active against WM (Vijay & Gertz, 2007), such as the nucleoside analogue fludarabine (Treon *et al*, 2006), the proteasome inhibitor bortezomib (Chen *et al*, 2007), and dexamethasone (Treon *et al*, 2006). We demonstrated that simvastatin enhanced bortezomib, fludarabine and dexamethasone anti-tumour activity, suggesting that combining these agents may be useful in future clinical trials. Like other reports, we also observed that simvastatin strongly synergized with dexamethasone, although the biological rational of this synergism is not fully understood (van de Donk *et al*, 2003).

In summary, this study demonstrated that the HMG-CoA reductase inhibitor, simvastatin, induced apoptosis and growth inhibition *in vitro* in WM cells even in presence of the BM microenvironment and cytokines that promote tumour cell growth. Most importantly, the combination of simvastatin with other novel therapeutic agents mediates synergistic WM cytotoxicity. Together, these studies provide the framework for clinical studies of simvastatin, alone or in combination, to improve patient outcome in WM.

Conflict of interest

The authors declare no competing financial interests.

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