# New treatment strategies for multiple myeloma by targeting Bcl-2 and the mevalonate pathway

**Niels van de Donk**

# New treatment strategies for multiple myeloma by targeting Bcl-2 and the mevalonate pathway

Nieuwe behandelingsstrategieën voor multipel myeloom door remming van Bcl-2 en de mevalonzuur route

(met een samenvatting in het Nederlands)

**Proefschrift**

Ter verkrijging van de graad van doctor aan de Universiteit van Utrecht
op gezag van de rector Magnificus, Prof. Dr. W.H. Gispen,
ingevolge het besluit van het College voor Promoties
in het openbaar te verdedigen op
dinsdag 7 oktober 2003 des ochtends te 10.30 uur

door

**Niels Wilhelmus Christianus Johannes van de Donk**

Geboren op 8 juli 1976 te Nuenen CA

Promotores: Prof. dr. H. C. Clevers[1]
Prof. dr. A. Hagenbeek[2]

Co-promotores: Dr. A. C. Bloem[1]
Dr. H. M. Lokhorst[2]

[1] Department of Immunology
University Medical Center Utrecht
The Netherlands

[2] Department of Haematology
University Medical Center Utrecht
The Netherlands

The research described in this thesis was performed at the Departments of Immunology and Haematology, University Medical Center Utrecht, Utrecht, The Nederlands and was financially supported by a research grant from the Dutch Cancer Society (Nederlandse Kankerbestrijding/Koningin Wilhelmina fonds (KWF)) (UU 99.1941)

ISBN: 90-393-3377-3

Voor mijn ouders

Commissie: Prof. dr. J. G. van den Tweel
Prof. dr. J. L. Bos
Prof. dr. Ph. G. de Groot
Prof. dr. A. J. van Zonneveld

Print: Febodruk B.V., Enschede

Publication of this thesis was financially supported by:
University of Utrecht; Pfizer B.V.; Merck Sharp & Dohme B.V.; Edward Jenner stichting; IQ Products; Sanquin Reagentia; Dr. Ir. Van de Laar Stichting; Genta Incorporated; Roche Nederland B.V.; Novartis Pharma B.V.; Amgen B.V. Breda; Aventis Pharma B.V.; GlaxoSmithKline B.V.

# Contents


| | | |
|---|---|---|
| **Chapter 1:** | General introduction | 9 |
| **Chapter 2:** | Chemosensitization of myeloma plasma cells by an antisense-mediated down-regulation of Bcl-2 protein | 37 |
| **Chapter 3:** | G3139, a Bcl-2 antisense oligodeoxynucleotide, may overcome drug resistance in VAD refractory myeloma | 57 |
| **Chapter 4:** | The cholesterol lowering drug lovastatin induces cell death in myeloma plasma cells | 73 |
| **Chapter 5:** | Inhibition of protein geranylgeranylation induces apoptosis in myeloma plasma cells by reducing Mcl-1 protein levels | 93 |
| **Chapter 6:** | Geranylgeranylated proteins are involved in the regulation of myeloma cell growth | 117 |
| **Chapter 7:** | Protein geranylgeranylation is critical for the regulation of survival and proliferation of lymphoma tumor cells | 143 |
| **Chapter 8:** | General discussion | 169 |
| **Nederlandse samenvatting** | | 183 |
| **Dankwoord** | | 187 |
| **Curriculum Vitae** | | 189 |
| **List of publications** | | 191 |

# Chapter 1

## General Introduction

## Multiple myeloma

Multiple myeloma is characterized by the accumulation of monoclonal plasma cells in the bone marrow (Figure 1). The neoplastic cells are long-lived cells with a low proliferative index, and produce a monoclonal immunoglobulin (M-component, paraprotein). The amount of the M-component in the serum is a reliable measure of tumor burden and can therefore be used as an excellent tumor marker. In about 20% of the myeloma patients only light chains are produced which are secreted in the urine as Bence Jones proteins, and 2% of the patients have nonsecretory myeloma characterized by the absence of detectable monoclonal protein in serum and urine. The tumor cells and the M-component are responsible for a number of organ dysfunctions and symptoms including bone disease, renal failure, increased susceptibility to infection, anemia, and sometimes clotting abnormalities or manifestations of hyperviscosity. Myeloma is an incurable disease and accounts for 1% of all cancers and more than 10% of all malignant hematologic diseases. The incidence of myeloma is 4 per 100,000 per year and the median age of diagnosis is 68 years. Survival of myeloma patients ranges from as short as 2 months to greater than 80 months after diagnosis and depends on a variety of prognostic factors including plasma cell labeling index [1], level of $\beta_2$-microglobulin [2], plasmablastic morphology [3], and cytogenetics [4-7].

## Cytogenetics

Chromosome 14q32 translocations seem to be important in the pathogenesis of myeloma and are detected in the majority of the patients [8,9]. These translocations result in the deregulation of expression of oncogenes by juxtaposing them near one of the IgH enhancers. Primary translocations are early events, occur in germinal center B cells as a result of errors in IgH switch recombination, or less often somatic hypermutation, and are characterized by the absence of clonal heterogeneity. These translocations are also detected in patients with monoclonal gammopathy of undetermined significance (MGUS) [10,11], and therefore it is supposed that they are not sufficient for malignant transformation, but may be initial clone-immortalizing events. Secondary translocations occur during tumor progression, are usually complex, involve other mechanisms than aberrant class switch recombination or somatic hypermutation, are less often IgH associated, and characterized by the presence of intraclonal heterogeneity [12-14]. There are 4 recurrent primary translocations that together account for 40% of all translocations: t(11;14)(q13;q32) (cyclin D1) [15], t(4;14)(p16;q32) (MMSET; FGFR3) [16,17], t(6;14)(p21;q32) (cyclin D3) [18], and t(14;16)(q32;q23) (c-maf) [19]. The t(4;14) translocation is associated with a poor prognosis, whereas the t(11;14) predicts good clinical outcome [19-21]. The biologic consequences regarding apoptosis, drug resistance, and proliferation of these translocations are at the moment poorly understood. Deregulation of Cyclin D1 as a result of the t(11;14) translocation may lead to loss of cell

cycle control [22], and c-Maf can transform fibroblasts [5]. Expression of the fibroblast growth factor receptor-3 (FGFR3) as a result of the t(4;14) translocation leads to activation of the MAPK pathway [23,24] and FGFR3 signaling can substitute for interleukin-6 (IL-6) for the growth and survival of an IL-6-dependent myeloma cell line [25]. In addition, FGFR3 can transform hematopoietic cells [26] and fibroblasts [23,24]. Furthermore, expression of FGFR3 in myeloma cells resulted in dexamethasone-resistance, probably through the up-regulation of Bcl-XL [27]. Disease progression is characterized by an increasing number of chromosomal abnormalities (c-Myc) [28,29], activating mutations of genes such as FGFR3 and Ras [17,30-34], and changes in expression of genes ($p16^{INK4a}$) [35,36], enabling the tumor to survive and proliferate independently of the bone marrow microenvironment.

Monosomy of chromosome 13 or deletion of chromosome 13q14 is observed in about 50% of the patients and is associated with poor response to therapy and a short progression free and overall survival [5,7,37,38]. The gene or genes that are deleted on chromosome 13 and are responsible for the poor prognosis have yet to be defined.

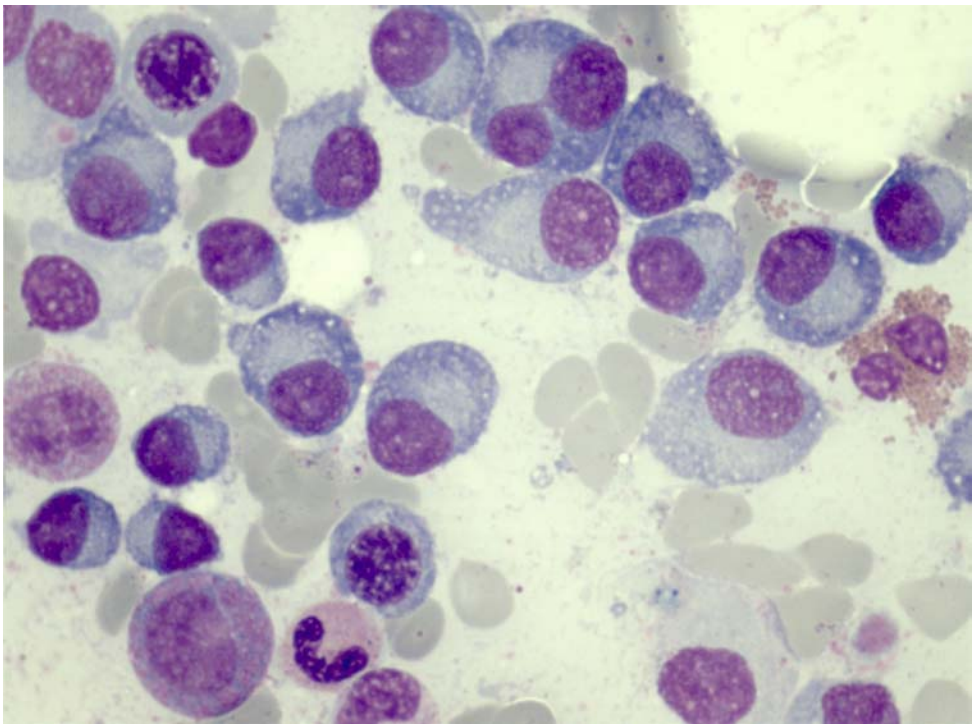

**Figure 1. Bone marrow aspiration of a myeloma patient.** Smear of bone marrow aspirate, with extensive infiltration by polymorphous plasma cells.

## Drug resistance

High-dose therapy followed by autologous stem cell transplantation has improved response rate and survival when compared with conventional chemotherapy [39]. However, in all myeloma patients the disease ultimately relapses and becomes resistant to subsequent drug treatments. In myeloma, many mechanisms of drug resistance exist and insight in these mechanisms is essential to make progress in the therapy of myeloma. These mechanisms include alterations in drug transport by proteins such as P-glycoprotein [40-43] and lung resistance protein (LRP) [44,45]. Inhibition of drug efflux from cancer cells with P-glycoprotein inhibitors has demonstrated variable success in clinical trials [40,46]. Mutations or deletions in the target genes may also be involved, an example of which is expression of a truncated glucocorticoid receptor (GR), which was associated with steroid resistance [47,48]. Furthermore, altered expression of target genes such as DNA Topoisomerase II and the glucocorticoid receptor [47,49,50], and enhanced detoxification of drugs by enzymes such as glutathione S-transferase or by elevated levels of glutathione [51] may contribute to chemoresistance. Increased rates of repair of damage to DNA have also been shown to be a mechanism of clinical resistance to melphalan [52]. However, since cytotoxic agents induce cell death through the triggering of apoptosis [53], inhibition of drug-induced apoptosis has been suggested as a major contributor of drug resistance in myeloma [54,55].

## Deregulation of apoptosis in myeloma

Defects in the regulation of programmed cell death resulting in a survival advantage play an important role in the accumulation of slowly proliferating myeloma cells in the bone marrow. Furthermore, failure of myeloma cells to undergo programmed cell death may result in resistance to chemotherapeutic agents. Several studies have demonstrated the importance of the bone marrow microenvironment in promoting myeloma cell growth, survival, and drug resistance. Growth factors such as IL-6 and insulin-like growth factor I (IGF-I), which are predominantly produced by bone marrow stromal cells result in dysfunction of growth and survival pathways, and may render myeloma cells resistant to a variety of cytotoxic agents. For example, IGF-I and IL-6 protect against drug-induced apoptosis via PI-3K signaling and NF-κB activation. Furthermore, adhesion of myeloma cells to extracellular matrix components protects against a variety of cytotoxic agents.

Various studies have demonstrated constitutive activation of growth and survival signaling pathways in myeloma tumor cells, which may be, at least in part, due to interactions between myeloma cells and the bone marrow microenvironment. However, deregulation of oncogenes and tumor suppressor genes as a result of mutations (Ras, FGFR3) or alterations in expression (PTEN, deletion of chromosome 13q14, Mcl-1) may also be involved in the activation of pathways leading to a variety of cellular processes including drug resistance, survival, and proliferation of myeloma tumor cells.

## Micro-environment

Myeloma cells secrete IL-1β, tumor necrosis factor-α (TNF-α), vascular endothelial growth factor (VEGF), and IL-6 [56-59] and binding of myeloma cells to bone marrow stromal cells triggers the secretion of IL-6, IGF-I, VEGF, and transforming growth factor-β (TGF-β) from bone marrow stromal cells [60,61]. Myeloma cells also produce cytokines (TNF-α, TGF-β, VEGF) that can further upregulate IL-6 secretion in bone marrow stromal cells [56,62,63]. Furthermore, enhanced osteoclastic bone resorption induced by myeloma cells results in the release of diverse cytokines and growth factors from the extracellular matrix. In addition, the osteoclast itself is a potent producer of IL-6 [64]. These findings indicate that there is a symbiotic relationship between myeloma cells and the bone marrow microenvironment. These cytokines not only contribute to the expansion of the myeloma tumor clone through the promotion of survival, proliferation, and angiogenesis, but also contribute to bone destruction and block the apoptotic effects of various cytotoxic agents (Figure 2). IL-6 is one of the most important growth factors for myeloma cells, and is predominantly produced in a paracrine way by bone marrow stromal cells, but also by activated osteoclasts [65-67]. IL-6 induces myeloma cell proliferation and inhibits apoptosis, and overrides the apoptotic signals mediated by cytotoxic drugs [68]. Binding of IL-6 to the IL-6 receptor triggers the activation of at least 3 signaling pathways; the JAK/STAT pathway [69], the Ras/mitogen-activated protein kinase (MAPK) pathway [70,71], and the phosphatidylinositol-3 kinase (PI-3K) pathway [72]. Activation of the JAK/STAT [73] and PI-3K pathways [74] is implicated in protection against apoptosis, whereas activation of the Ras-MAPK [70] and PI-3K pathways [74] induces proliferation. Blocking of the IL-6 receptor can overcome drug resistance of myeloma cells [75]. IGF-I is also an important growth factor for myeloma cells and stimulates proliferation and protects myeloma tumor cells against death induced by dexamethasone [72,76-80]. IGF-I stimulation leads to activation of both the MAPK and the PI-3K pathways, but the PI-3K pathway was demonstrated to be the major regulator of both apoptosis and proliferation, with only minimal contributions to either by the MAPK cascade [76,81]. IGF-I is produced in the liver, and also by osteoblasts in the bone marrow microenvironment [82,83]. Finally, adhesion of myeloma tumor cells to extracellular matrix proteins such as fibronectin via β1 integrins [84,85] or bone marrow stromal cells [86,87] reduces the sensitivity of tumor cells to a variety of chemotherapeutic agents (cell adhesion-mediated drug resistance; CAM-DR).

## Ras

Ras proteins (H-, K-, and N-Ras) have been shown to play a key role in signal transduction pathways leading to survival or growth, and in malignant transformation. Ras proteins cycle between an inactive guanosine 5'-diphosphate (GDP)-bound state and an

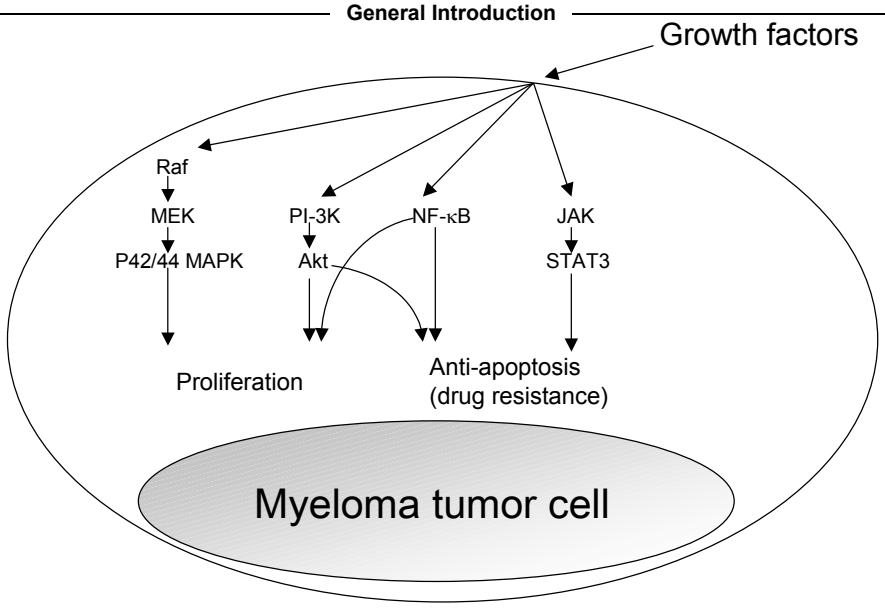


**Figure 2. Growth factors activate signaling pathways leading to proliferation, survival, and resistance to cytotoxic agents.** Myeloma cell proliferation is mediated through activation of MAPK, PI-3K, and NF-κB signaling pathways. Survival is mediated by activation of JAK/STAT, NF-κB, and PI-3K pathways. Inhibition of drug-induced apoptosis is an important mechanism of drug resistance in myeloma.

active guanosine 5'-triphosphate (GTP)-bound state. Regulatory proteins that control the GDP/GTP cycling rate of Ras include GTPase-activating proteins (GAPs), which enhance the rate of GTP hydrolysis to GDP, and guanine nucleotide exchange factors (GEFs), which induce the dissociation of GDP to allow association of GTP [88]. Active GTP-bound Ras modulates the activity of effector proteins including Raf-1 and PI-3K. Point mutations at codons 12, 13, or 61 lead to a constitutive active GTP-bound state.

In multiple myeloma, Ras plays an important role in the control of both proliferation, apoptosis, and drug resistance. In myeloma cells, IL-6 triggers the transition of Ras from the inactive GDP-bound form to the active GTP-bound form and activates the MAPK pathway, leading to proliferation [70,89]. This suggests that Ras proteins transduce proliferation signals through their activation of Raf-MAPK pathway, but they are also involved in the transduction of survival and growth signals through pathways such as PI-3K/Akt and NF-κB [90]. Ras mutations are found in many human cancers [91], including multiple myeloma. The frequency of Ras mutations is 39% in patients with newly diagnosed disease [30], and increases to 64-70% in patients with advanced disease [31,32]. Furthermore, Ras mutations are absent in patients with MGUS [92,93]. This suggests that mutations of Ras represent a progression event and not an initiating event. The presence of Ras mutations at diagnosis is associated with an adverse outcome, a poor response to therapy [31], and a shorter survival [30]. *In vitro* studies showed that Ras mutations are associated with IL-6-independent growth [94,95], and provided resistance against apoptosis

induced by cytotoxic agents such as dexamethasone, doxorubicin, and melphalan [86,95,96] through the constitutive activation of MAPK, PI-3K, and NF-κB signaling pathways [90]. The role of other small GTP-binding proteins including the Rho family members such as Rac-1, RhoA, and Cdc42 in the regulation of myeloma cell growth and drug resistance is currently unknown.

## Nuclear factor-κB

Nuclear factor-κB (NF-κB) is a transcription factor involved in the regulation of a variety of cellular processes including apoptosis, differentiation, and inflammatory responses and is typically a heterodimer of p50 and p65 subunits [97]. In most cell types, NF-κB resides in the cytosol and is inactivated by its association with IκB family inhibitors. Activators of NF-κB such as TNF-α, result in the activation of IκB kinase (IKK), which phosphorylates IκB in a site-specific way. This leads to the ubiquitination-dependent degradation of IκB by the 26S proteasome [98]. The direct consequence is the translocation of NF-κB from the cytoplasm into the nucleus, where it binds to specific DNA sequences in the promoters of target genes leading to stimulation of their transcription.

Various reports have indicated constitutive activity of NF-κB in myeloma patients' samples and in myeloma cell lines [99-101]. Maintenance of constitutive activity of NF-κB was associated with resistance of myeloma cell lines to dexamethasone [99]. Inhibition of NF-κB activity induced apoptosis, inhibited proliferation, potentiated the cytotoxic effects of chemotherapeutic agents, and abrogated the protective effect of IL-6 on dexamethasone- and thalidomide derivatives-induced growth inhibition [100-102]. Furthermore, adhesion of myeloma cells to bone marrow stromal cells and TNF-α secreted by myeloma cells trigger the transcription and secretion of the important growth and survival factor IL-6 through the activation of NF-κB in the stromal cells [103,104]. These results indicate that NF-κB plays an important role in myeloma cell survival, proliferation, and drug resistance. In myeloma cell lines, the proteasome inhibitor PS-341 (Velcade, Bortezomib) has been shown to induce apoptosis, to inhibit proliferation, and to sensitize myeloma cells to conventional chemotherapeutic agents which was, at least in part, due to inhibition of NF-κB activity [102,105-107]. These results provided the framework for clinical trials of PS-341. These clinical studies have shown that PS-341 has activity against refractory multiple myeloma [108,109].

## PI-3K/Akt pathway

Activation of PI-3K results in the generation of phosphatidylinositol (PI) phosphates including $PIP_3$. One target of PI-3K and the phospholipids is Akt (protein kinase B; PKB). The phospholipid molecules bind to the pleckstrin-homology domain of Akt, resulting in its translocation to the cell membrane and phosphorylation at serine and threonine

residues by PDK 1 and 2. This phosphorylation of Akt leads to the efficient activation of Akt kinase activity [110-112]. Activated Akt subsequently phosphorylates several substrates, including BAD, glycogen synthase kinase-3, and the Forkhead family of transcription factors.

Constitutive activation of PI-3K and Akt was found in myeloma cell lines [72] and in myeloma cells from patients, whereas Akt activation was not observed in non-malignant hematopoietic cells from the same patients [72,113]. Various studies have shown that the myeloma growth and survival factors IL-6 and IGF-I can activate the PI-3K/Akt pathway in myeloma cells [72,74,81,114,115]. These growth factors may therefore contribute to the activation of the PI-3K/Akt pathway in myeloma cells *in vivo*. However, loss-of-function mutations in PTEN may also be involved. The tumor suppressor PTEN is an important negative regulator of PI-3K/Akt activation. It dephosphorylates PI-3K products such as $PIP_3$ and leads to a concomitant reduction in Akt activity [116,117]. Loss of PTEN expression was found in myeloma cell lines [115]. Induction of proliferation, survival, and protection against cytotoxic agents mediated by IGF-I and IL-6 were, at least in part, dependent on the activation of a PI-3K-dependent pathway [72,74,81]. Activation of Akt by PI-3K may be responsible for the proliferative and anti-apoptotic effects of PI-3K, since expression of a dominant-negative Akt gene in myeloma cells or pharmacologic inhibition of Akt resulted in a decreased number of cells in S phase and increase in apoptosis [113,118]. In addition, expression of constitutively active Akt protected myeloma cells against both novel and conventional anti-tumor agents [118]. Downstream targets of Akt that are involved in the control of survival and proliferation of myeloma cells include p70S6 kinase and the Forkhead family of proteins [74,81,90,115].

## Bcl-2 family proteins and regulation of apoptosis

The Bcl-2 family consists of anti-apoptotic (Bcl-2, Bcl-XL, Bcl-W, Bfl-1, Mcl-1, A1) and pro-apoptotic (Bax, Bak, Bid, Bcl-XS, Bad, Bik, Bim, Hrk) family members. The ratio of death agonists to death antagonists determines whether a cell will survive or die after an apoptotic signal. Alterations in the highly regulated expression of anti- and pro-apoptotic Bcl-2 family proteins can contribute to the development of cancer. Deregulated overexpression of anti-apoptotic Bcl-2 family members may result in an extended survival. These cells are at risk for the acquisition of additional genetic changes, and subsequent malignant transformation. Moreover, the ratio of anti- and pro-apoptotic Bcl-2 family members determines the susceptibility of cancer cells towards a variety of cytotoxic agents [119,120]. Anti-apoptotic Bcl-2 family proteins do not affect drug transport or drug-induced DNA damage, however, tumor cells with elevated levels of these proteins have a reduced susceptibility to apoptosis induction during drug treatment and can repair drug-induced damage when administration of the drug is stopped.

Bcl-2 family proteins contain at least one of four conserved Bcl-2 homology (BH) domains designated BH1, BH2, BH3, and BH4 [121-123]. Part of the Bcl-2 family proteins also possesses a carboxyl-terminal hydrophobic domain, which is important for membrane docking [124]. Anti-apoptotic Bcl-2 family members are localized in the mitochondrial outer membrane, the endoplasmic reticulum, or nuclear membrane [125-128]. A substantial fraction of the pro-apoptotic Bcl-2 family members, however, reside in the cytosol or localize to the cytoskeleton [129-131]. Following a death signal, these pro-apoptotic proteins undergo a change of conformation and translocate to mitochondria. Moreover, part of the pro-apoptotic proteins can also integrate in the mitochondrial outer membrane. For example, in viable cells Bax is predominantly localized in the cytosol or loosely attached to membranes, but following a death signal Bax translocates to the outer membrane of mitochondria and becomes an integral membrane protein [130,132]. Bid is cleaved at its amino-terminus following TNF-α/Fas signaling by activated caspase-8 and the truncated Bid form moves to mitochondria [133-136]. It is hypothesized that following translocation of pro-apoptotic proteins to mitochondria, the activity of the anti-apoptotic Bcl-2 family members is neutralized through the formation of heterodimers. In addition, the pro-apoptotic members with hydrophobic core α helices such as Bax and Bid can become integral mitochondrial membrane proteins and may form selective channels for cytochrome c release and other factors from the intermembrane space [120,137-139]. Alternatively, these proteins may alter the activity of pre-existing channels such as the permeability transition pore (PTP) [140-142]. Opening of the PTP results in mitochondrial depolarization, uncoupling of oxidative phosphorylation, and mitochondrial swelling leading to the rupture of the mitochondrial outer membrane and release of cytochrome c [143]. The anti-apoptotic Bcl-2 family proteins inhibit apoptosis by neutralizing the function of pro-apoptotic Bcl-2 family members, or by suppression of the activity of components of the PTP, or a combination of these [144]. Cytochrome c in the cytosol binds to Apaf-1 and induces a conformational change in Apaf-1 allowing it to associate with caspase-9, resulting in activation of caspase-9 which, in turn, activates effector caspase-3 [145], resulting in the cleavage of key proteins (Figure 3) [146]. This leads to cellular changes that occur during apoptosis including DNA fragmentation, chromatin condensation, membrane blebbing, cell shrinkage, and disassembly of the cell into membrane-enclosed vesicles (apoptotic bodies). Caspases cleave other caspase family members, signal transduction proteins, cytoskeletal proteins, chromatin modifying proteins (eg, polyADP ribosyl polymerase, PARP), DNA repair proteins, and inhibitory subunits of endonucleases specifically at aspartic acid residues [147]. One group of substrates of the effector caspases includes the anti-apoptotic Bcl-2 family members [148,149]. Cleavage not only inactivates these proteins, but also creates a form that promotes apoptosis, suggesting a positive feed-back loop. Some studies have reported that anti-apoptotic Bcl-2 family proteins can also inhibit the ability of Apaf-1 to activate caspase-9 [150]. The anti-apoptotic family member

Bcl-XL can bind Apaf-1 and some pro-apoptotic Bcl-2 family proteins have been shown to dissociate this complex [151-154]. The inhibitor of apoptosis (IAP) proteins such as survivin and XIAP can inhibit activated caspases and activation of pro-caspases, and thereby prevent apoptosis [155]. Their role in myeloma, however, is unknown. Another apoptogenic protein that is released from mitochondria is Smac/DIABLO. Once released, Smac/DIABLO interacts with and neutralizes XIAP, an inhibitor of caspase-3 and -9, thereby facilitating caspase activation [156,157].

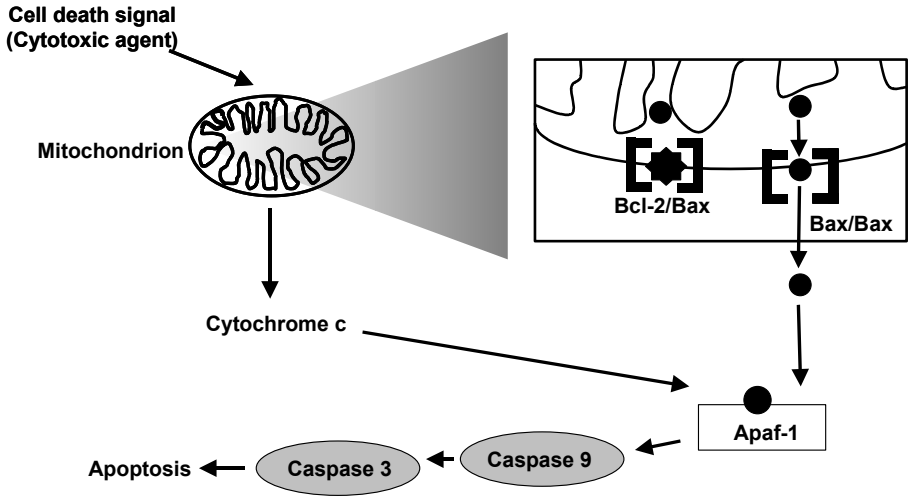


**Figure 3. Cytochrome c-mediated activation of Apaf-1, results in the activation of a downstream caspase program.** The ratio of anti-apoptotic and pro-apoptotic Bcl-2 family proteins determines the susceptibility of cells to a death signal.

## Bcl-2 in myeloma

Bcl-2 was identified at the t(14;18) chromosome translocation breakpoint. This translocation is present in about 90% of follicular lymphomas and in up to 30% in diffuse large B-cell lymphomas [158-160]. This translocation results in the juxtaposition of Bcl-2 normally located on chromosome 18, with transcriptional enhancer regions in 14q32, thus deregulating the expression of Bcl-2 and giving the B cells containing this translocation a survival advantage. Although these B cells have a low growth fraction, the increased life span results in clonal expansion. Several studies have demonstrated significant Bcl-2 expression in malignant cells of patients with myeloma [161-167], however the t(14;18) translocation is not present in myeloma cells. This indicates that other mechanisms such as hypomethylation of the Bcl-2 gene [168], constitutive activity of NF-κB which induces Bcl-2 transcription [169,170], or loss of the p53 tumor suppressor which inhibits Bcl-2 gene expression [171,172], may be involved. Overexpression of Bcl-2 in B cells through use of an

immunoglobulin gene enhancer in transgenic mice prolongs the life span of plasma cells and B cells [173,174], and in combination with abnormal expression of other oncogenes such as c-myc, contributes to the development of immature pre-B cell lymphoma, large cell lymphoma, or plasmacytoma in the mice [175,176]. Altogether these data suggest a role for Bcl-2 in the etiology of myeloma and non-Hodgkin's lymphomas.

There are several lines of evidence that suggest that Bcl-2 plays a role in drug resistance in myeloma. Bcl-2 overexpression using gene transfer approaches rendered myeloma cells more resistant to a variety of cytotoxic drugs [177,178], and protected against IL-6 deprivation-induced apoptosis [179]. In addition, Bcl-2 protein levels increased following exposure of cell lines to doxorubicin, which may account for the relative resistance to a second doxorubicin exposure [178]. Also in leukemia and solid tumor cell lines, Bcl-2 levels correlate with relative sensitivity to various chemotherapeutic agents and γ-radiation [123].

High expression of Bcl-2 in malignant cells of patients with acute myeloid leukemia and high-grade B cell lymphoma is associated with poor response to therapy [180,181]. In myeloma, the prognostic significance of Bcl-2 expression is currently unclear. In 3 studies no correlation between Bcl-2 expression, which was determined by immunohistochemistry or immunofluoresence, and response to chemotherapy or survival was observed [163,164,167]. All these studies, however, suffer from small sample size. Moreover, the expression of other anti- and pro-apoptotic Bcl-2 family proteins was not evaluated. Finally, one study showed that high levels of Bcl-2 expression were associated with resistance to interferon therapy [163]. Larger studies using flow cytometric analysis to objectively quantify Bcl-2 expression are required to further assess the prognostic significance of Bcl-2. In summary, in myeloma the role of Bcl-2 in drug resistance is still unclear and needs further clarification.

## Other Bcl-2 family proteins in myeloma

Bcl-XL is expressed in myeloma cells, and appears to be up-regulated in specimens taken from patients at the time of relapse, when compared to expression levels at initial diagnosis. In addition, Bcl-XL expression correlated with decreased response rates in patient groups treated with either melphalan and prednisone or with vincristine, Adriamycin, and dexamethasone [182]. However, down-regulation of Bcl-XL protein expression in myeloma cell lines did not induce apoptosis or enhance chemosensitivity [183], suggesting that Bcl-XL is not involved in drug resistance in myeloma, but serves as an indicator of chemoresistance. Bcl-XS is a protein that arises through alternate splicing of Bcl-X [184], but is not expressed in myeloma cells [182].

In normal cells Mcl-1 expression is rapidly up-regulated during critical events such as changes in proliferation, differentiation, and cellular stress [185], suggesting that its function is to provide short-term, rapidly inducible enhancement of cell viability [186]. Mcl-

1 is expressed during specific stages of B cell development. Expression is low in naive B cells and memory B cells, while expression is prominent in germinal center B cells [187,188]. The expression of Bcl-2 follows a reciprocal pattern. Germinal center B cells undergo affinity maturation and the majority of the cells die by apoptosis, in contrast to Bcl-2 positive naive and memory B cells, which survive for an extended period of time. Mcl-1 is supposed to promote survival of germinal center B cells for a short period of time, to allow the selection of clones with appropriate antibody affinity. However, deregulated or constitutive expression of Mcl-1, as opposed to the transient expression in normal cells, may result in enhanced survival over the long term and tumorigenic conversion and may attribute to resistance of tumor cells to cytotoxic drugs. In cell lines, Mcl-1 promotes cell viability under a variety of apoptosis-inducing conditions including exposure to cytotoxic agents and the withdrawal of required growth factors [189,190]. Furthermore, overexpression of Mcl-1 has been correlated with failure to achieve complete remission in B-cell chronic lymphocytic leukemia (B-CLL) patients treated with chemotherapy [191]. Overexpression of Mcl-1 in transgenic mice prolongs the life span of myeloid and lymphoid (B and T) cells at both mature and immature stages of differentiation [192] and contributes to the development of follicular B-cell lymphoma and diffuse large-cell lymphoma in approximately 85% of the mice after a follow-up of 2 years [193].

Mcl-1 is overexpressed in myeloma cells, when compared with normal plasma cells [194]. In part of the myeloma cell lines, Mcl-1 and Bcl-XL are coregulated by the important growth and survival factor IL-6 [195]. The JAK-STAT signal transduction pathway seems to be responsible for the up-regulation of Bcl-XL and Mcl-1 by IL-6 [73,196]. In the majority of myeloma cell lines and myeloma patients' samples, however, Mcl-1 regulation is IL-6-independent and not controlled by the JAK-STAT, MAPK, or PI-3K signaling pathways [197], whereas these signaling pathways have been shown to stimulate Mcl-1 transciption in a variety of other cell lines. Down-regulation of Mcl-1 induced apoptosis in myeloma cells [183,198,199] and sensitized myeloma cells towards cytotoxic agents [183]. This indicates that Mcl-1 is an important regulator of survival and chemosensitivity in myeloma cells. The role of the other Bcl-2 family proteins in multiple myeloma remains to be elucidated.

## Mevalonate pathway and prenylation

Deregulated or elevated activity of HMG-CoA reductase, which is the rate-limiting enzyme of the mevalonate pathway, has been demonstrated in a variety of human cancers including leukemia and lymphoma [200,201]. Furthermore, inhibition of the mevalonate pathway induces apoptosis and inhibits proliferation in acute myeloid leukemia cells [202]. The role of the mevalonate pathway in myeloma is unknown.

Cells obtain cholesterol from 2 sources: 1) exogenously from low-density lipoprotein (LDL) which enters the cell via receptor-mediated endocytosis; and 2)

endogenously from synthesis within the cell from acetyl-CoA through the mevalonate pathway (Figure 4). Other non-sterol products of the mevalonate pathway include dolichol, required for glycoprotein synthesis; the polyisoprenoids side chains of heme A and ubiquinone, involved in oxidative respiration; isopentenyladenine, present in some tRNAs; and the isoprenoids farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP), required for protein prenylation [203]. The mevalonate pathway is highly regulated and this is achieved through regulation of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) synthase, HMG-CoA reductase, and LDL receptors. In the absence of LDL, cells maintain high activites of HMG-CoA synthase and HMG-CoA reductase resulting in the increased synthesis of mevalonate for the production of cholesterol and non-sterol products. When LDL is present, the activities of these enzymes decline, and mevalonate is used for the production of non-sterol end-products. Furthermore, the LDL receptor gene is repressed when intracellular pools of sterols increase. When there is both excess of mevalonate and sterols, the residual activity of HMG-CoA reductase is abolished and mevalonate production is stopped [203].

Many proteins such as Ras and Rho family proteins require prenylation, a lipid posttranslational modification, for proper cellular localization. Prenylation is essential for the full biologic activity of the proteins [204-206]. H-, N-, and K-Ras require farnesylation, and Rac-1, Cdc42, and RhoA require geranylgeranylation for their function [207-210]. However, in cells treated with farnesyl transferase inhibitors, K-Ras can be alternatively geranylgeranylated [211,212]. Farnsesyl transferase (FTase) catalyzes the transfer of a farnesyl group from FPP, whereas geranylgeranyl transferases (GGTase) I and II catalyze the transfer of a geranylgeranyl moiety derived from GGPP to cysteines at the carboxy-terminus of the protein. Prenylated proteins share characteristic carboxy-terminal consensus sequences, and can be separated in proteins with a CAAX (C, cysteine; A, aliphatic amino acid; X any amino acid) motif and proteins with a CC or CXC sequence. When the X in the CAAX sequence is methionine, serine, cysteine, or glutamine, the cysteine will be farnesylated; when X is leucine, it will be gerananylgeranylated by GGTase I. GGTase II transfers the geranylgeranyl moieties to both cysteine residues of the CC or CXC motifs [209,210,213,214].

Statins block cholesterol synthesis by inhibition of HMG-CoA reductase, and are therefore widely used for the treatment of hypercholesterolemia [215]. Statins also deplete intracellular pools of FPP and GGPP, thereby preventing the posttranslational prenylation of small GTP-binding proteins involved in diverse cellular processes including the regulation of apoptosis and proliferation [216,217]. In various cancer cell lines and purified tumor cells from acute myeloid leukemia patients statins induce apoptosis, inhibit proliferation, and sensitize tumor cells to a variety of cytotoxic drugs through the inhibition of the mevalonate pathway [216,217]. Furthermore, pravastatin prolonged the survival of patients with advanced hepatocellular carcinoma [218].

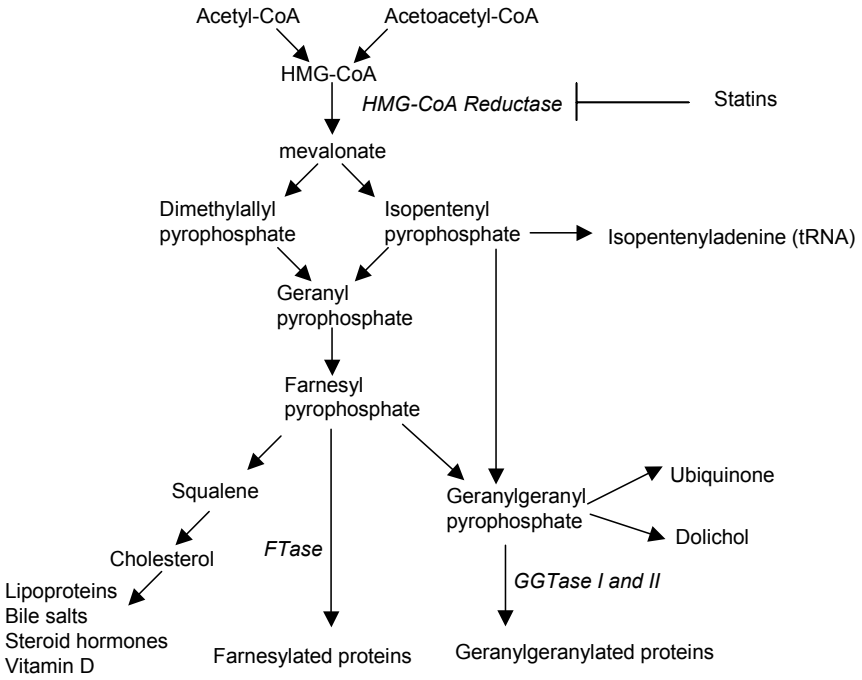


**Figure 4. The mevalonate pathway.** The mevalonate pathway leads to the formation of cholesterol. Other non-sterol products of the mevalonate pathway include dolichol, required for glycoprotein synthesis; the polyisoprenoids side chains of heme A and ubiquinone, involved in oxidative respiration; isopentenyladenine, present in some tRNAs; and the isoprenoids farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP), required for protein prenylation.

## Scope of this thesis

The study of drug resistance and cell survival in myeloma cells has resulted in the identification of a number of potential targets for antimyeloma therapy. Among those are proteins participating in signaling pathways regulating growth and survival. Biologically-based therapies that are currently being explored in myeloma include recombinant osteoprotegerin (OPG), inhibitors of NF-κB such as the proteasome inhibitor PS-341 (Velcade), farnesyl transferase inhibitors to target Ras mutations, and thalidomide and its analogs to target both the myeloma cell and its microenvironment. Development of therapeutic agents that augment the apoptotic effects of conventional chemotherapeutic agents represents a strategy to reverse chemoresistance in patients with multidrug resistant disease.

In this study, we investigated the role of Bcl-2 protein in the regulation of survival and chemoresistance of myeloma cells using an antisense strategy. First, preclinical studies were performed to determine the role of Bcl-2 in the regulation of apoptosis and drug resistance in myeloma tumor cells. Based on the outcome of these *in*

*vitro* studies, a phase 2 trial was designed to evaluate the effect of Bcl-2 protein down-regulation on the therapeutic efficacy of VAD chemotherapy in refractory myeloma patients.

Targeting the mevalonate pathway may be a new therapeutic strategy to overcome resistance to conventional therapies in multiple myeloma. We investigated the importance of the mevalonate pathway for the regulation of growth, survival, and drug resistance in myeloma tumor cells.

## References


1. Greipp, P. R., Lust, J. A., O'Fallon, W. M., Katzmann, J. A., Witzig, T. E., and Kyle, R. A. Plasma cell labeling index and beta 2-microglobulin predict survival independent of thymidine kinase and C-reactive protein in multiple myeloma. Blood, *81*: 3382-3387, 1993.
2. Garewal, H., Durie, B. G., Kyle, R. A., Finley, P., Bower, B., and Serokman, R. Serum beta 2-microglobulin in the initial staging and subsequent monitoring of monoclonal plasma cell disorders. J Clin.Oncol., *2*: 51-57, 1984.
3. Greipp, P. R., Leong, T., Bennett, J. M., Gaillard, J. P., Klein, B., Stewart, J. A., Oken, M. M., Kay, N. E., Van Ness, B., and Kyle, R. A. Plasmablastic morphology--an independent prognostic factor with clinical and laboratory correlates: Eastern Cooperative Oncology Group (ECOG) myeloma trial E9486 report by the ECOG Myeloma Laboratory Group. Blood, *91*: 2501-2507, 1998.
4. Keats, J. J., Reiman, T., Maxwell, C. A., Taylor, B. J., Larratt, L. M., Mant, M. J., Belch, A. R., and Pilarski, L. M. In multiple myeloma, t(4;14)(p16;q32) is an adverse prognostic factor irrespective of FGFR3 expression. Blood, *101*: 1520-1529, 2003.
5. Fonseca, R., Blood, E., Rue, M., Harrington, D., Oken, M. M., Kyle, R. A., Dewald, G. W., Van Ness, B., Van Wier, S. A., Henderson, K. J., Bailey, R. J., and Greipp, P. R. Clinical and biologic implications of recurrent genomic aberrations in myeloma. Blood, *101*: 4569-4575, 2003.
6. Shaughnessy, J., Jacobson, J., Sawyer, J., McCoy, J., Fassas, A., Zhan, F., Bumm, K., Epstein, J., Anaissie, E., Jagannath, S., Vesole, D., Siegel, D., Desikan, R., Munshi, N., Badros, A., Tian, E., Zangari, M., Tricot, G., Crowley, J., and Barlogie, B. Continuous absence of metaphase-defined cytogenetic abnormalities, especially of chromosome 13 and hypodiploidy, ensures long-term survival in multiple myeloma treated with Total Therapy I: interpretation in the context of global gene expression. Blood, *101* : 3849-3856, 2003.
7. Tricot, G., Barlogie, B., Jagannath, S., Bracy, D., Mattox, S., Vesole, D. H., Naucke, S., and Sawyer, J. R. Poor prognosis in multiple myeloma is associated only with partial or complete deletions of chromosome 13 or abnormalities involving 11q and not with other karyotype abnormalities. Blood, *86*: 4250-4256, 1995.
8. Nishida, K., Tamura, A., Nakazawa, N., Ueda, Y., Abe, T., Matsuda, F., Kashima, K., and Taniwaki, M. The Ig heavy chain gene is frequently involved in chromosomal translocations in multiple myeloma and plasma cell leukemia as detected by in situ hybridization. Blood, *90*: 526-534, 1997.
9. Avet-Loiseau, H., Li, J. Y., Facon, T., Brigaudeau, C., Morineau, N., Maloisel, F., Rapp, M. J., Talmant, P., Trimoreau, F., Jaccard, A., Harousseau, J. L., and Bataille, R. High incidence of translocations t(11;14)(q13;q32) and t(4;14)(p16;q32) in patients with plasma cell malignancies. Cancer Res., *58*: 5640-5645, 1998.
10. Avet-Loiseau, H., Facon, T., Daviet, A., Godon, C., Rapp, M. J., Harousseau, J. L., Grosbois, B., and Bataille, R. 14q32 translocations and monosomy 13 observed in monoclonal gammopathy of

undetermined significance delineate a multistep process for the oncogenesis of multiple myeloma. Intergroupe Francophone du Myelome. Cancer Res., *59*: 4546-4550, 1999.

11. Fonseca, R., Bailey, R. J., Ahmann, G. J., Rajkumar, S. V., Hoyer, J. D., Lust, J. A., Kyle, R. A., Gertz, M. A., Greipp, P. R., and Dewald, G. W. Genomic abnormalities in monoclonal gammopathy of undetermined significance. Blood, *100*: 1417-1424, 2002.
12. Bergsagel, P. L. and Kuehl, W. M. Chromosome translocations in multiple myeloma. Oncogene, *20*: 5611-5622, 2001.
13. Kuppers, R. and Dalla-Favera, R. Mechanisms of chromosomal translocations in B cell lymphomas. Oncogene, *20*: 5580-5594, 2001.
14. Korsmeyer, S. J. Chromosomal translocations in lymphoid malignancies reveal novel proto-oncogenes. Annu.Rev.Immunol, *10:785-807.*: 785-807, 1992.
15. Fonseca, R., Witzig, T. E., Gertz, M. A., Kyle, R. A., Hoyer, J. D., Jalal, S. M., and Greipp, P. R. Multiple myeloma and the translocation t(11;14)(q13;q32): a report on 13 cases. Br.J Haematol., *101*: 296-301, 1998.
16. Finelli, P., Fabris, S., Zagano, S., Baldini, L., Intini, D., Nobili, L., Lombardi, L., Maiolo, A. T., and Neri, A. Detection of t(4;14)(p16.3;q32) chromosomal translocation in multiple myeloma by double-color fluorescent in situ hybridization. Blood, *94*: 724-732, 1999.
17. Chesi, M., Nardini, E., Brents, L. A., Schrock, E., Ried, T., Kuehl, W. M., and Bergsagel, P. L. Frequent translocation t(4;14)(p16.3;q32.3) in multiple myeloma is associated with increased expression and activating mutations of fibroblast growth factor receptor 3. Nat.Genet., *16*: 260-264, 1997.
18. Shaughnessy, J., Jr., Gabrea, A., Qi, Y., Brents, L., Zhan, F., Tian, E., Sawyer, J., Barlogie, B., Bergsagel, P. L., and Kuehl, M. Cyclin D3 at 6p21 is dysregulated by recurrent chromosomal translocations to immunoglobulin loci in multiple myeloma. Blood, *98*: 217-223, 2001.
19. Fonseca, R., Blood, E., Rue, M., Harrington, D., Oken, M. M., Kyle, R. A., Dewald, G. W., Van Ness, B., Van Wier, S. A., Henderson, K. J., Bailey, R. J., and Greipp, P. R. Clinical and biologic implications of recurrent genomic aberrations in myeloma. Blood, *101*: 4569-4575, 2003.
20. Avet-Loiseau, H., Facon, T., Grosbois, B., Magrangeas, F., Rapp, M. J., Harousseau, J. L., Minvielle, S., and Bataille, R. Oncogenesis of multiple myeloma: 14q32 and 13q chromosomal abnormalities are not randomly distributed, but correlate with natural history, immunological features, and clinical presentation. Blood, *99*: 2185-2191, 2002.
21. Moreau, P., Facon, T., Leleu, X., Morineau, N., Huyghe, P., Harousseau, J. L., Bataille, R., and Avet-Loiseau, H. Recurrent 14q32 translocations determine the prognosis of multiple myeloma, especially in patients receiving intensive chemotherapy. Blood, *100*: 1579-1583, 2002.
22. Motokura, T. and Arnold, A. Cyclin D and oncogenesis. Curr.Opin.Genet.Dev., *3*: 5-10, 1993.
23. Chesi, M., Brents, L. A., Ely, S. A., Bais, C., Robbiani, D. F., Mesri, E. A., Kuehl, W. M., and Bergsagel, P. L. Activated fibroblast growth factor receptor 3 is an oncogene that contributes to tumor progression in multiple myeloma. Blood, *97*: 729-736, 2001.
24. Ronchetti, D., Greco, A., Compasso, S., Colombo, G., Dell'Era, P., Otsuki, T., Lombardi, L., and Neri, A. Deregulated FGFR3 mutants in multiple myeloma cell lines with t(4;14): comparative analysis of Y373C, K650E and the novel G384D mutations. Oncogene, *20*: 3553-3562, 2001.
25. Plowright, E. E., Li, Z., Bergsagel, P. L., Chesi, M., Barber, D. L., Branch, D. R., Hawley, R. G., and Stewart, A. K. Ectopic expression of fibroblast growth factor receptor 3 promotes myeloma cell proliferation and prevents apoptosis. Blood, *95*: 992-998, 2000.
26. Li, Z., Zhu, Y. X., Plowright, E. E., Bergsagel, P. L., Chesi, M., Patterson, B., Hawley, T. S., Hawley, R. G., and Stewart, A. K. The myeloma-associated oncogene fibroblast growth factor receptor 3 is transforming in hematopoietic cells. Blood, *97*: 2413-2419, 2001.
27. Pollett, J. B., Trudel, S., Stern, D., Li, Z. H., and Stewart, A. K. Overexpression of the myeloma-associated oncogene fibroblast growth factor receptor 3 confers dexamethasone resistance. Blood, *100*: 3819-3821, 2002.

28. Shou, Y., Martelli, M. L., Gabrea, A., Qi, Y., Brents, L. A., Roschke, A., Dewald, G., Kirsch, I. R., Bergsagel, P. L., and Kuehl, W. M. Diverse karyotypic abnormalities of the c-myc locus associated with c-myc dysregulation and tumor progression in multiple myeloma. Proc Natl Acad Sci U S A, *97*: 228-233, 2000.
29. Avet-Loiseau, H., Gerson, F., Magrangeas, F., Minvielle, S., Harousseau, J. L., and Bataille, R. Rearrangements of the c-myc oncogene are present in 15% of primary human multiple myeloma tumors. Blood, *98*: 3082-3086, 2001.
30. Liu, P., Leong, T., Quam, L., Billadeau, D., Kay, N. E., Greipp, P., Kyle, R. A., Oken, M. M., and Van Ness, B. Activating mutations of N- and K-ras in multiple myeloma show different clinical associations: analysis of the Eastern Cooperative Oncology Group Phase III Trial. Blood, *88*: 2699-2706, 1996.
31. Neri, A., Murphy, J. P., Cro, L., Ferrero, D., Tarella, C., Baldini, L., and Dalla-Favera, R. Ras oncogene mutation in multiple myeloma. J.Exp.Med., *170*: 1715-1725, 1989.
32. Portier, M., Moles, J. P., Mazars, G. R., Jeanteur, P., Bataille, R., Klein, B., and Theillet, C. p53 and RAS gene mutations in multiple myeloma. Oncogene, *7*: 2539-2543, 1992.
33. Chesi, M., Brents, L. A., Ely, S. A., Bais, C., Robbiani, D. F., Mesri, E. A., Kuehl, W. M., and Bergsagel, P. L. Activated fibroblast growth factor receptor 3 is an oncogene that contributes to tumor progression in multiple myeloma. Blood, *97*: 729-736, 2001.
34. Intini, D., Baldini, L., Fabris, S., Lombardi, L., Ciceri, G., Maiolo, A. T., and Neri, A. Analysis of FGFR3 gene mutations in multiple myeloma patients with t(4;14). Br.J Haematol., *114*: 362-364, 2001.
35. Tasaka, T., Asou, H., Munker, R., Said, J. W., Berenson, J., Vescio, R. A., Nagai, M., Takahara, J., and Koeffler, H. P. Methylation of the p16INK4A gene in multiple myeloma. Br.J Haematol., *101*: 558-564, 1998.
36. Uchida, T., Kinoshita, T., Ohno, T., Ohashi, H., Nagai, H., and Saito, H. Hypermethylation of p16INK4A gene promoter during the progression of plasma cell dyscrasia. Leukemia, *15*: 157-165, 2001.
37. Zojer, N., Konigsberg, R., Ackermann, J., Fritz, E., Dallinger, S., Kromer, E., Kaufmann, H., Riedl, L., Gisslinger, H., Schreiber, S., Heinz, R., Ludwig, H., Huber, H., and Drach, J. Deletion of 13q14 remains an independent adverse prognostic variable in multiple myeloma despite its frequent detection by interphase fluorescence in situ hybridization. Blood, *95*: 1925-1930, 2000.
38. Facon, T., Avet-Loiseau, H., Guillerm, G., Moreau, P., Genevieve, F., Zandecki, M., Lai, J. L., Leleu, X., Jouet, J. P., Bauters, F., Harousseau, J. L., Bataille, R., and Mary, J. Y. Chromosome 13 abnormalities identified by FISH analysis and serum beta2-microglobulin produce a powerful myeloma staging system for patients receiving high-dose therapy. Blood, *97*: 1566-1571, 2001.
39. Attal, M., Harousseau, J. L., Stoppa, A. M., Sotto, J. J., Fuzibet, J. G., Rossi, J. F., Casassus, P., Maisonneuve, H., Facon, T., Ifrah, N., Payen, C., and Bataille, R. A prospective, randomized trial of autologous bone marrow transplantation and chemotherapy in multiple myeloma. Intergroupe Francais du Myelome. N.Engl.J Med, *335*: 91-97, 1996.
40. Dalton, W. S., Grogan, T. M., Meltzer, P. S., Scheper, R. J., Durie, B. G., Taylor, C. W., Miller, T. P., and Salmon, S. E. Drug-resistance in multiple myeloma and non-Hodgkin's lymphoma: detection of P-glycoprotein and potential circumvention by addition of verapamil to chemotherapy. J Clin.Oncol., *7*: 415-424, 1989.
41. Epstein, J., Xiao, H. Q., and Oba, B. K. P-glycoprotein expression in plasma-cell myeloma is associated with resistance to VAD. Blood, *74*: 913-917, 1989.
42. Sonneveld, P., Durie, B. G., Lokhorst, H. M., Marie, J. P., Solbu, G., Suciu, S., Zittoun, R., Lowenberg, B., and Nooter, K. Modulation of multidrug-resistant multiple myeloma by cyclosporin. The Leukaemia Group of the EORTC and the HOVON. Lancet, *340*: 255-259, 1992.
43. Grogan, T. M., Spier, C. M., Salmon, S. E., Matzner, M., Rybski, J., Weinstein, R. S., Scheper, R. J., and Dalton, W. S. P-glycoprotein expression in human plasma cell myeloma: correlation with prior chemotherapy. Blood, *81*: 490-495, 1993.

44. Raaijmakers, H. G., Izquierdo, M. A., Lokhorst, H. M., de Leeuw, C., Belien, J. A., Bloem, A. C., Dekker, A. W., Scheper, R. J., and Sonneveld, P. Lung-resistance-related protein expression is a negative predictive factor for response to conventional low but not to intensified dose alkylating chemotherapy in multiple myeloma. Blood, *91*: 1029-1036, 1998.
45. Filipits, M., Drach, J., Pohl, G., Schuster, J., Stranzl, T., Ackermann, J., Konigsberg, R., Kaufmann, H., Gisslinger, H., Huber, H., Ludwig, H., and Pirker, R. Expression of the lung resistance protein predicts poor outcome in patients with multiple myeloma. Clin.Cancer Res., *5*: 2426-2430, 1999.
46. Sonneveld, P., Marie, J. P., Huisman, C., Vekhoff, A., Schoester, M., Faussat, A. M., van Kapel, J., Groenewegen, A., Charnick, S., Zittoun, R., and Lowenberg, B. Reversal of multidrug resistance by SDZ PSC 833, combined with VAD (vincristine, doxorubicin, dexamethasone) in refractory multiple myeloma. A phase I study. Leukemia, *10*: 1741-1750, 1996.
47. Moalli, P. A., Pillay, S., Weiner, D., Leikin, R., and Rosen, S. T. A mechanism of resistance to glucocorticoids in multiple myeloma: transient expression of a truncated glucocorticoid receptor mRNA. Blood, *79*: 213-222, 1992.
48. Krett, N. L., Pillay, S., Moalli, P. A., Greipp, P. R., and Rosen, S. T. A variant glucocorticoid receptor messenger RNA is expressed in multiple myeloma patients. Cancer Res., *55*: 2727-2729, 1995.
49. Ishikawa, H., Kawano, M. M., Okada, K., Tanaka, H., Tanabe, O., Sakai, A., Asaoku, H., Iwato, K., Nobuyoshi, M., and Kuramoto, A. Expressions of DNA topoisomerase I and II gene and the genes possibly related to drug resistance in human myeloma cells. Br.J Haematol., *83*: 68-74, 1993.
50. Valkov, N. I. and Sullivan, D. M. Drug resistance to DNA topoisomerase I and II inhibitors in human leukemia, lymphoma, and multiple myeloma. Semin.Hematol, *34*: 48-62, 1997.
51. Linsenmeyer, M. E., Jefferson, S., Wolf, M., Matthews, J. P., Board, P. G., and Woodcock, D. M. Levels of expression of the mdr1 gene and glutathione S-transferase genes 2 and 3 and response to chemotherapy in multiple myeloma. Br.J.Cancer, *65*: 471-475, 1992.
52. Spanswick, V. J., Craddock, C., Sekhar, M., Mahendra, P., Shankaranarayana, P., Hughes, R. G., Hochhauser, D., and Hartley, J. A. Repair of DNA interstrand crosslinks as a mechanism of clinical resistance to melphalan in multiple myeloma. Blood, *100*: 224-229, 2002.
53. Johnstone, R. W., Ruefli, A. A., and Lowe, S. W. Apoptosis: a link between cancer genetics and chemotherapy. Cell, *108*: 153-164, 2002.
54. Solary, E., Droin, N., Bettaieb, A., Corcos, L., Dimanche-Boitrel, M. T., and Garrido, C. Positive and negative regulation of apoptotic pathways by cytotoxic agents in hematological malignancies. Leukemia, *14*: 1833-1849, 2000.
55. O'Gorman, D. M. and Cotter, T. G. Molecular signals in anti-apoptotic survival pathways. Leukemia, *15*: 21-34, 2001.
56. Hideshima, T., Chauhan, D., Schlossman, R., Richardson, P., and Anderson, K. C. The role of tumor necrosis factor alpha in the pathophysiology of human multiple myeloma: therapeutic applications. Oncogene, *20*: 4519-4527, 2001.
57. Dankbar, B., Padro, T., Leo, R., Feldmann, B., Kropff, M., Mesters, R. M., Serve, H., Berdel, W. E., and Kienast, J. Vascular endothelial growth factor and interleukin-6 in paracrine tumor-stromal cell interactions in multiple myeloma. Blood, *95*: 2630-2636, 2000.
58. Urashima, M., Chauhan, D., Uchiyama, H., Freeman, G. J., and Anderson, K. C. CD40 ligand triggered interleukin-6 secretion in multiple myeloma. Blood, *85*: 1903-1912, 1995.
59. Lacy, M. Q., Donovan, K. A., Heimbach, J. K., Ahmann, G. J., and Lust, J. A. Comparison of interleukin-1 beta expression by in situ hybridization in monoclonal gammopathy of undetermined significance and multiple myeloma. Blood, *93*: 300-305, 1999.
60. Uchiyama, H., Barut, B. A., Mohrbacher, A. F., Chauhan, D., and Anderson, K. C. Adhesion of human myeloma-derived cell lines to bone marrow stromal cells stimulates interleukin-6 secretion. Blood, *82*: 3712-3720, 1993.

61. Gimble, J. M., Pietrangeli, C., Henley, A., Dorheim, M. A., Silver, J., Namen, A., Takeichi, M., Goridis, C., and Kincade, P. W. Characterization of murine bone marrow and spleen-derived stromal cells: analysis of leukocyte marker and growth factor mRNA transcript levels. Blood, *74*: 303-311, 1989.
62. Podar, K., Tai, Y. T., Davies, F. E., Lentzsch, S., Sattler, M., Hideshima, T., Lin, B. K., Gupta, D., Shima, Y., Chauhan, D., Mitsiades, C., Raje, N., Richardson, P., and Anderson, K. C. Vascular endothelial growth factor triggers signaling cascades mediating multiple myeloma cell growth and migration. Blood, *98*: 428-435, 2001.
63. Urashima, M., Ogata, A., Chauhan, D., Hatziyanni, M., Vidriales, M. B., Dedera, D. A., Schlossman, R. L., and Anderson, K. C. Transforming growth factor-beta1: differential effects on multiple myeloma versus normal B cells. Blood, *87*: 1928-1938, 1996.
64. Roodman, G. D., Kurihara, N., Ohsaki, Y., Kukita, A., Hosking, D., Demulder, A., Smith, J. F., and Singer, F. R. Interleukin 6. A potential autocrine/paracrine factor in Paget's disease of bone. J Clin.Invest, *89*: 46-52, 1992.
65. Kawano, M., Hirano, T., Matsuda, T., Taga, T., Horii, Y., Iwato, K., Asaoku, H., Tang, B., Tanabe, O., Tanaka, H., and . Autocrine generation and requirement of BSF-2/IL-6 for human multiple myelomas. Nature, *332*: 83-85, 1988.
66. Klein, B., Zhang, X. G., Lu, Z. Y., and Bataille, R. Interleukin-6 in human multiple myeloma. Blood, *85*: 863-872, 1995.
67. Anderson, K. C., Jones, R. M., Morimoto, C., Leavitt, P., and Barut, B. A. Response patterns of purified myeloma cells to hematopoietic growth factors. Blood, *73*: 1915-1924, 1989.
68. Hardin, J., MacLeod, S., Grigorieva, I., Chang, R., Barlogie, B., Xiao, H., and Epstein, J. Interleukin-6 prevents dexamethasone-induced myeloma cell death. Blood, *84*: 3063-3070, 1994.
69. Heinrich, P. C., Behrmann, I., Muller-Newen, G., Schaper, F., and Graeve, L. Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway. Biochem.J, *334*: 297-314, 1998.
70. Ogata, A., Chauhan, D., Teoh, G., Treon, S. P., Urashima, M., Schlossman, R. L., and Anderson, K. C. IL-6 triggers cell growth via the Ras-dependent mitogen-activated protein kinase cascade. J.Immunol., *159*: 2212-2221, 1997.
71. Ogata, A., Chauhan, D., Urashima, M., Teoh, G., Treon, S. P., and Anderson, K. C. Blockade of mitogen-activated protein kinase cascade signaling in interleukin 6-independent multiple myeloma cells. Clin.Cancer Res., *3*: 1017-1022, 1997.
72. Tu, Y., Gardner, A., and Lichtenstein, A. The phosphatidylinositol 3-kinase/AKT kinase pathway in multiple myeloma plasma cells: roles in cytokine-dependent survival and proliferative responses. Cancer Res., *60*: 6763-6770, 2000.
73. Catlett-Falcone, R., Landowski, T. H., Oshiro, M. M., Turkson, J., Levitzki, A., Savino, R., Ciliberto, G., Moscinski, L., Fernandez-Luna, J. L., Nunez, G., Dalton, W. S., and Jove, R. Constitutive activation of Stat3 signaling confers resistance to apoptosis in human U266 myeloma cells. Immunity., *10*: 105-115, 1999.
74. Hideshima, T., Nakamura, N., Chauhan, D., and Anderson, K. C. Biologic sequelae of interleukin-6 induced PI3-K/Akt signaling in multiple myeloma. Oncogene, *%20;20*: 5991-6000, 2001.
75. Honemann, D., Chatterjee, M., Savino, R., Bommert, K., Burger, R., Gramatzki, M., Dorken, B., and Bargou, R. C. The IL-6 receptor antagonist SANT-7 overcomes bone marrow stromal cell-mediated drug resistance of multiple myeloma cells. Int.J Cancer, *93*: 674-680, 2001.
76. Ferlin, M., Noraz, N., Hertogh, C., Brochier, J., Taylor, N., and Klein, B. Insulin-like growth factor induces the survival and proliferation of myeloma cells through an interleukin-6-independent transduction pathway. Br.J Haematol., *111*: 626-634, 2000.
77. Xu, F., Gardner, A., Tu, Y., Michl, P., Prager, D., and Lichtenstein, A. Multiple myeloma cells are protected against dexamethasone-induced apoptosis by insulin-like growth factors. Br.J Haematol., *97*: 429-440, 1997.

78. Freund, G. G., Kulas, D. T., and Mooney, R. A. Insulin and IGF-1 increase mitogenesis and glucose metabolism in the multiple myeloma cell line, RPMI 8226. J Immunol, *151*: 1811-1820, 1993.
79. Georgii-Hemming, P., Wiklund, H. J., Ljunggren, O., and Nilsson, K. Insulin-like growth factor I is a growth and survival factor in human multiple myeloma cell lines. Blood, *88*: 2250-2258, 1996.
80. Jelinek, D. F., Witzig, T. E., and Arendt, B. K. A role for insulin-like growth factor in the regulation of IL-6-responsive human myeloma cell line growth. J Immunol, *159*: 487-496, 1997.
81. Qiang, Y. W., Kopantzev, E., and Rudikoff, S. Insulinlike growth factor-I signaling in multiple myeloma: downstream elements, functional correlates, and pathway cross-talk. Blood, *99*: 4138-4146, 2002.
82. Nilsson, K., Georgii-Hemming, P., Spets, H., and Jernberg-Wiklund, H. The control of proliferation, survival and apoptosis in human multiple myeloma cells in vitro. Curr.Top.Microbiol.Immunol, *246:325-32; discussion 333.*: 325-332, 1999.
83. Middleton, J., Arnott, N., Walsh, S., and Beresford, J. Osteoblasts and osteoclasts in adult human osteophyte tissue express the mRNAs for insulin-like growth factors I and II and the type 1 IGF receptor. Bone, *16*: 287-293, 1995.
84. Damiano, J. S., Cress, A. E., Hazlehurst, L. A., Shtil, A. A., and Dalton, W. S. Cell adhesion mediated drug resistance (CAM-DR): role of integrins and resistance to apoptosis in human myeloma cell lines. Blood, *93*: 1658-1667, 1999.
85. Hazlehurst, L. A., Damiano, J. S., Buyuksal, I., Pledger, W. J., and Dalton, W. S. Adhesion to fibronectin via beta1 integrins regulates p27kip1 levels and contributes to cell adhesion mediated drug resistance (CAM-DR). Oncogene, *19*: 4319-4327, 2000.
86. Cheung, W. C. and Van Ness, B. The bone marrow stromal microenvironment influences myeloma therapeutic response in vitro. Leukemia, *15*: 264-271, 2001.
87. Chatterjee, M., Honemann, D., Lentzsch, S., Bommert, K., Sers, C., Herrmann, P., Mathas, S., Dorken, B., and Bargou, R. C. In the presence of bone marrow stromal cells human multiple myeloma cells become independent of the IL-6/gp130/STAT3 pathway. Blood, *100*: 3311-3318, 2002.
88. Reuter, C. W., Morgan, M. A., and Bergmann, L. Targeting the Ras signaling pathway: a rational, mechanism-based treatment for hematologic malignancies? Blood, *96*: 1655-1669, 2000.
89. Rowley, M. and Van Ness, B. Activation of N-ras and K-ras induced by interleukin-6 in a myeloma cell line: implications for disease progression and therapeutic response. Oncogene, *21*: 8769-8775, 2002.
90. Hu, L., Shi, Y., Hsu, J. H., Gera, J., Van Ness, B., and Lichtenstein, A. Downstream effectors of oncogenic ras in multiple myeloma cells. Blood, *101*: 3126-3135, 2003.
91. Bos, J. L. ras oncogenes in human cancer: a review. Cancer Res., *49*: 4682-4689, 1989.
92. Corradini, P., Ladetto, M., Voena, C., Palumbo, A., Inghirami, G., Knowles, D. M., Boccadoro, M., and Pileri, A. Mutational activation of N- and K-ras oncogenes in plasma cell dyscrasias. Blood, *81*: 2708-2713, 1993.
93. Corradini, P., Ladetto, M., Inghirami, G., Boccadoro, M., and Pileri, A. N- and K-ras oncogenes in plasma cell dyscrasias. Leuk.Lymphoma, *15*: 17-20, 1994.
94. Billadeau, D., Jelinek, D. F., Shah, N., LeBien, T. W., and Van Ness, B. Introduction of an activated N-ras oncogene alters the growth characteristics of the interleukin 6-dependent myeloma cell line ANBL6. Cancer Res., *55*: 3640-3646, 1995.
95. Billadeau, D., Liu, P., Jelinek, D., Shah, N., LeBien, T. W., and Van Ness, B. Activating mutations in the N- and K-ras oncogenes differentially affect the growth properties of the IL-6-dependent myeloma cell line ANBL6. Cancer Res., *57*: 2268-2275, 1997.
96. Rowley, M., Liu, P., and Van Ness, B. Heterogeneity in therapeutic response of genetically altered myeloma cell lines to interleukin 6, dexamethasone, doxorubicin, and melphalan. Blood, *96*: 3175-3180, 2000.
97. Baldwin, A. S., Jr. The NF-kappa B and I kappa B proteins: new discoveries and insights. Annu.Rev.Immunol, *14:649-83.*: 649-683, 1996.

98. Zandi, E. and Karin, M. Bridging the gap: composition, regulation, and physiological function of the IkappaB kinase complex. Mol.Cell Biol., *19*: 4547-4551, 1999.
99. Feinman, R., Koury, J., Thames, M., Barlogie, B., Epstein, J., and Siegel, D. S. Role of NF-kappaB in the rescue of multiple myeloma cells from glucocorticoid-induced apoptosis by bcl-2. Blood, *93*: 3044-3052, 1999.
100. Ni, H., Ergin, M., Huang, Q., Qin, J. Z., Amin, H. M., Martinez, R. L., Saeed, S., Barton, K., and Alkan, S. Analysis of expression of nuclear factor kappa B (NF-kappa B) in multiple myeloma: downregulation of NF-kappa B induces apoptosis. Br.J Haematol., *115*: 279-286, 2001.
101. Bharti, A. C., Donato, N., Singh, S., and Aggarwal, B. B. Curcumin (diferuloylmethane) down-regulates the constitutive activation of nuclear factor-kappa B and IkappaBalpha kinase in human multiple myeloma cells, leading to suppression of proliferation and induction of apoptosis. Blood, *101*: 1053-1062, 2003.
102. Hideshima, T., Chauhan, D., Richardson, P., Mitsiades, C., Mitsiades, N., Hayashi, T., Munshi, N., Dang, L., Castro, A., Palombella, V., Adams, J., and Anderson, K. C. NF-kappa B as a therapeutic target in multiple myeloma. J Biol.Chem., *277*: 16639-16647, 2002.
103. Chauhan, D., Uchiyama, H., Akbarali, Y., Urashima, M., Yamamoto, K., Libermann, T. A., and Anderson, K. C. Multiple myeloma cell adhesion-induced interleukin-6 expression in bone marrow stromal cells involves activation of NF-kappa B. Blood, *87*: 1104-1112, 1996.
104. Hideshima, T., Chauhan, D., Schlossman, R., Richardson, P., and Anderson, K. C. The role of tumor necrosis factor alpha in the pathophysiology of human multiple myeloma: therapeutic applications. Oncogene, *20*: 4519-4527, 2001.
105. Mitsiades, N., Mitsiades, C. S., Poulaki, V., Chauhan, D., Fanourakis, G., Gu, X., Bailey, C., Joseph, M., Libermann, T. A., Treon, S. P., Munshi, N. C., Richardson, P. G., Hideshima, T., and Anderson, K. C. Molecular sequelae of proteasome inhibition in human multiple myeloma cells. Proc Natl Acad Sci U S A, *99*: 14374-14379, 2002.
106. Hideshima, T., Mitsiades, C., Akiyama, M., Hayashi, T., Chauhan, D., Richardson, P., Schlossman, R., Podar, K., Munshi, N. C., Mitsiades, N., and Anderson, K. C. Molecular mechanisms mediating antimyeloma activity of proteasome inhibitor PS-341. Blood, *101*: 1530-1534, 2003.
107. Mitsiades, N., Mitsiades, C. S., Richardson, P. G., Poulaki, V., Tai, Y. T., Chauhan, D., Fanourakis, G., Gu, X., Bailey, C., Joseph, M., Libermann, T. A., Schlossman, R., Munshi, N. C., Hideshima, T., and Anderson, K. C. The proteasome inhibitor PS-341 potentiates sensitivity of multiple myeloma cells to conventional chemotherapeutic agents: therapeutic applications. Blood, *101*: 2377-2380, 2003.
108. Orlowski, R. Z., Stinchcombe, T. E., Mitchell, B. S., Shea, T. C., Baldwin, A. S., Stahl, S., Adams, J., Esseltine, D. L., Elliott, P. J., Pien, C. S., Guerciolini, R., Anderson, J. K., Depcik-Smith, N. D., Bhagat, R., Lehman, M. J., Novick, S. C., O'Connor, O. A., and Soignet, S. L. Phase I trial of the proteasome inhibitor PS-341 in patients with refractory hematologic malignancies. J Clin.Oncol., *20*: 4420-4427, 2002.
109. Richardson, P. G., Barlogie, B., Berenson, J., Singhal, S., Jagannath, S., Irwin, D., Rajkumar, S. V., Srkalovic, G., Alsina, M., Alexanian, R., Siegel, D., Orlowski, R. Z., Kuter, D., Limentani, S. A., Lee, S., Hideshima, T., Esseltine, D. L., Kauffman, M., Adams, J., Schenkein, D. P., and Anderson, K. C. A phase 2 study of bortezomib in relapsed, refractory myeloma. N.Engl.J Med, *348*: 2609-2617, 2003.
110. Andjelkovic, M., Alessi, D. R., Meier, R., Fernandez, A., Lamb, N. J., Frech, M., Cron, P., Cohen, P., Lucocq, J. M., and Hemmings, B. A. Role of translocation in the activation and function of protein kinase B. J Biol.Chem., *272*: 31515-31524, 1997.
111. Franke, T. F., Yang, S. I., Chan, T. O., Datta, K., Kazlauskas, A., Morrison, D. K., Kaplan, D. R., and Tsichlis, P. N. The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. Cell, *81*: 727-736, 1995.
112. Burgering, B. M. and Coffer, P. J. Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. Nature, *376*: 599-602, 1995.

113. Hsu, J., Shi, Y., Krajewski, S., Renner, S., Fisher, M., Reed, J. C., Franke, T. F., and Lichtenstein, A. The AKT kinase is activated in multiple myeloma tumor cells. Blood, *98*: 2853-2855, 2001.
114. Ge, N. L. and Rudikoff, S. Insulin-like growth factor I is a dual effector of multiple myeloma cell growth. Blood, *96*: 2856-2861, 2000.
115. Hyun, T., Yam, A., Pece, S., Xie, X., Zhang, J., Miki, T., Gutkind, J. S., and Li, W. Loss of PTEN expression leading to high Akt activation in human multiple myelomas. Blood, *96*: 3560-3568, 2000.
116. Maehama, T. and Dixon, J. E. The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. J Biol.Chem., *273*: 13375-13378, 1998.
117. Cantley, L. C. and Neel, B. G. New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. Proc Natl Acad Sci U S A, *96*: 4240-4245, 1999.
118. Mitsiades, C. S., Mitsiades, N., Poulaki, V., Schlossman, R., Akiyama, M., Chauhan, D., Hideshima, T., Treon, S. P., Munshi, N. C., Richardson, P. G., and Anderson, K. C. Activation of NF-kappaB and upregulation of intracellular anti-apoptotic proteins via the IGF-1/Akt signaling in human multiple myeloma cells: therapeutic implications. Oncogene, *21*: 5673-5683, 2002.
119. Kroemer, G. and Reed, J. C. Mitochondrial control of cell death. Nat.Med, *6*: 513-519, 2000.
120. Gross, A., McDonnell, J. M., and Korsmeyer, S. J. BCL-2 family members and the mitochondria in apoptosis. Genes Dev., *13*: 1899-1911, 1999.
121. Adams, J. M. and Cory, S. The Bcl-2 protein family: arbiters of cell survival. Science, *281*: 1322-1326, 1998.
122. Kelekar, A. and Thompson, C. B. Bcl-2-family proteins: the role of the BH3 domain in apoptosis. Trends Cell Biol., *8*: 324-330, 1998.
123. Reed, J. C. Bcl-2 family proteins. Oncogene, *17*: 3225-3236, 1998.
124. Nguyen, M., Millar, D. G., Yong, V. W., Korsmeyer, S. J., and Shore, G. C. Targeting of Bcl-2 to the mitochondrial outer membrane by a COOH-terminal signal anchor sequence. J Biol.Chem., *268*: 25265-25268, 1993.
125. Hockenbery, D., Nunez, G., Milliman, C., Schreiber, R. D., and Korsmeyer, S. J. Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. Nature, *348*: 334-336, 1990.
126. Krajewski, S., Tanaka, S., Takayama, S., Schibler, M. J., Fenton, W., and Reed, J. C. Investigation of the subcellular distribution of the bcl-2 oncoprotein: residence in the nuclear envelope, endoplasmic reticulum, and outer mitochondrial membranes. Cancer Res., *53*: 4701-4714, 1993.
127. de Jong, D., Prins, F. A., Mason, D. Y., Reed, J. C., van Ommen, G. B., and Kluin, P. M. Subcellular localization of the bcl-2 protein in malignant and normal lymphoid cells. Cancer Res., *54*: 256-260, 1994.
128. Zhu, W., Cowie, A., Wasfy, G. W., Penn, L. Z., Leber, B., and Andrews, D. W. Bcl-2 mutants with restricted subcellular location reveal spatially distinct pathways for apoptosis in different cell types. EMBO J, *15*: 4130-4141, 1996.
129. Hsu, Y. T., Wolter, K. G., and Youle, R. J. Cytosol-to-membrane redistribution of Bax and Bcl-X(L) during apoptosis. Proc Natl Acad Sci U S A, *94*: 3668-3672, 1997.
130. Gross, A., Jockel, J., Wei, M. C., and Korsmeyer, S. J. Enforced dimerization of BAX results in its translocation, mitochondrial dysfunction and apoptosis. EMBO J, *17*: 3878-3885, 1998.
131. Puthalakath, H., Huang, D. C., O'Reilly, L. A., King, S. M., and Strasser, A. The proapoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex. Mol.Cell, *3*: 287-296, 1999.
132. Wolter, K. G., Hsu, Y. T., Smith, C. L., Nechushtan, A., Xi, X. G., and Youle, R. J. Movement of Bax from the cytosol to mitochondria during apoptosis. J Cell Biol., *139*: 1281-1292, 1997.
133. Li, H., Zhu, H., Xu, C. J., and Yuan, J. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell, *94*: 491-501, 1998.

134. Luo, X., Budihardjo, I., Zou, H., Slaughter, C., and Wang, X. Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. Cell, *94*: 481-490, 1998.
135. Gross, A., Yin, X. M., Wang, K., Wei, M. C., Jockel, J., Milliman, C., Erdjument-Bromage, H., Tempst, P., and Korsmeyer, S. J. Caspase cleaved BID targets mitochondria and is required for cytochrome c release, while BCL-XL prevents this release but not tumor necrosis factor-R1/Fas death. J Biol.Chem., *274*: 1156-1163, 1999.
136. Han, Z., Bhalla, K., Pantazis, P., Hendrickson, E. A., and Wyche, J. H. Cif (Cytochrome c efflux-inducing factor) activity is regulated by Bcl-2 and caspases and correlates with the activation of Bid. Mol.Cell Biol., *19*: 1381-1389, 1999.
137. Manon, S., Chaudhuri, B., and Guerin, M. Release of cytochrome c and decrease of cytochrome c oxidase in Bax-expressing yeast cells, and prevention of these effects by coexpression of Bcl-xL. FEBS Lett., *415*: 29-32, 1997.
138. Reed, J. C. Cytochrome c: can't live with it--can't live without it. Cell, *91*: 559-562, 1997.
139. Green, D. R. and Reed, J. C. Mitochondria and apoptosis. Science, *281*: 1309-1312, 1998.
140. Marzo, I., Brenner, C., Zamzami, N., Jurgensmeier, J. M., Susin, S. A., Vieira, H. L., Prevost, M. C., Xie, Z., Matsuyama, S., Reed, J. C., and Kroemer, G. Bax and adenine nucleotide translocator cooperate in the mitochondrial control of apoptosis. Science, *281*: 2027-2031, 1998.
141. Shimizu, S., Eguchi, Y., Kamiike, W., Funahashi, Y., Mignon, A., Lacronique, V., Matsuda, H., and Tsujimoto, Y. Bcl-2 prevents apoptotic mitochondrial dysfunction by regulating proton flux. Proc Natl Acad Sci U S A, *95*: 1455-1459, 1998.
142. Narita, M., Shimizu, S., Ito, T., Chittenden, T., Lutz, R. J., Matsuda, H., and Tsujimoto, Y. Bax interacts with the permeability transition pore to induce permeability transition and cytochrome c release in isolated mitochondria. Proc Natl Acad Sci U S A, *95*: 14681-14686, 1998.
143. Vander Heiden, M. G., Chandel, N. S., Williamson, E. K., Schumacker, P. T., and Thompson, C. B. Bcl-xL regulates the membrane potential and volume homeostasis of mitochondria. Cell, *91*: 627-637, 1997.
144. Debatin, K. M., Poncet, D., and Kroemer, G. Chemotherapy: targeting the mitochondrial cell death pathway. Oncogene, *21*: 8786-8803, 2002.
145. Thornberry, N. A. and Lazebnik, Y. Caspases: enemies within. Science, *281*: 1312-1316, 1998.
146. Cryns, V. and Yuan, J. Proteases to die for. Genes Dev., *12*: 1551-1570, 1998.
147. Thornberry, N. A. Caspases: a decade of death research. Cell Death.Differ., *6*: 1023-1027, 1999.
148. Cheng, E. H., Kirsch, D. G., Clem, R. J., Ravi, R., Kastan, M. B., Bedi, A., Ueno, K., and Hardwick, J. M. Conversion of Bcl-2 to a Bax-like death effector by caspases. Science, *278*: 1966-1968, 1997.
149. Kirsch, D. G., Doseff, A., Chau, B. N., Lim, D. S., Souza-Pinto, N. C., Hansford, R., Kastan, M. B., Lazebnik, Y. A., and Hardwick, J. M. Caspase-3-dependent cleavage of Bcl-2 promotes release of cytochrome c. J Biol.Chem., *274*: 21155-21161, 1999.
150. Huang, D. C., Adams, J. M., and Cory, S. The conserved N-terminal BH4 domain of Bcl-2 homologues is essential for inhibition of apoptosis and interaction with CED-4. EMBO J, *17*: 1029-1039, 1998.
151. Hu, Y., Benedict, M. A., Wu, D., Inohara, N., and Nunez, G. Bcl-XL interacts with Apaf-1 and inhibits Apaf-1-dependent caspase-9 activation. Proc Natl Acad Sci U S A, *95*: 4386-4391, 1998.
152. Pan, G., O'Rourke, K., and Dixit, V. M. Caspase-9, Bcl-XL, and Apaf-1 form a ternary complex. J Biol.Chem., *273*: 5841-5845, 1998.
153. Inohara, N., Gourley, T. S., Carrio, R., Muniz, M., Merino, J., Garcia, I., Koseki, T., Hu, Y., Chen, S., and Nunez, G. Diva, a Bcl-2 homologue that binds directly to Apaf-1 and induces BH3-independent cell death. J Biol.Chem., *273*: 32479-32486, 1998.
154. Song, Q., Kuang, Y., Dixit, V. M., and Vincenz, C. Boo, a novel negative regulator of cell death, interacts with Apaf-1. EMBO J, *18*: 167-178, 1999.
155. Deveraux, Q. L., Takahashi, R., Salvesen, G. S., and Reed, J. C. X-linked IAP is a direct inhibitor of cell-death proteases. Nature, *388*: 300-304, 1997.

156. Du, C., Fang, M., Li, Y., Li, L., and Wang, X. Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. Cell, *102*: 33-42, 2000.
157. Verhagen, A. M., Ekert, P. G., Pakusch, M., Silke, J., Connolly, L. M., Reid, G. E., Moritz, R. L., Simpson, R. J., and Vaux, D. L. Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. Cell, *102*: 43-53, 2000.
158. Tsujimoto, Y. and Croce, C. M. Analysis of the structure, transcripts, and protein products of bcl-2, the gene involved in human follicular lymphoma. Proc Natl Acad Sci U S A, *83*: 5214-5218, 1986.
159. Weiss, L. M., Warnke, R. A., Sklar, J., and Cleary, M. L. Molecular analysis of the t(14;18) chromosomal translocation in malignant lymphomas. N.Engl.J Med, *317*: 1185-1189, 1987.
160. Jacobson, J. O., Wilkes, B. M., Kwaiatkowski, D. J., Medeiros, L. J., Aisenberg, A. C., and Harris, N. L. bcl-2 rearrangements in de novo diffuse large cell lymphoma. Association with distinctive clinical features. Cancer, *72*: 231-236, 1993.
161. Hamilton, M. S., Barker, H. F., Ball, J., Drew, M., Abbot, S. D., and Franklin, I. M. Normal and neoplastic human plasma cells express bcl-2 antigen. Leukemia, *5*: 768-771, 1991.
162. Pettersson, M., Jernberg-Wiklund, H., Larsson, L. G., Sundstrom, C., Givol, I., Tsujimoto, Y., and Nilsson, K. Expression of the bcl-2 gene in human multiple myeloma cell lines and normal plasma cells. Blood, *79*: 495-502, 1992.
163. Sangfelt, O., Osterborg, A., Grander, D., Anderbring, E., Ost, A., Mellstedt, H., and Einhorn, S. Response to interferon therapy in patients with multiple myeloma correlates with expression of the Bcl-2 oncoprotein. Int.J.Cancer, *63*: 190-192, 1995.
164. Ong, F., van Nieuwkoop, J. A., Groot-Swings, G. M., Hermans, J., Harvey, M. S., Kluin, P. M., and Kluin-Nelemans, J. C. Bcl-2 protein expression is not related to short survival in multiple myeloma. Leukemia, *9*: 1282-1284, 1995.
165. Harada, N., Hata, H., Yoshida, M., Soniki, T., Nagasaki, A., Kuribayashi, N., Kimura, T., Matsuzaki, H., and Mitsuya, H. Expression of Bcl-2 family of proteins in fresh myeloma cells. Leukemia, *12*: 1817-1820, 1998.
166. Puthier, D., Pellat-Deceunynck, C., Barille, S., Robillard, N., Rapp, M. J., Juge-Morineau, N., Harousseau, J. L., Bataille, R., and Amiot, M. Differential expression of Bcl-2 in human plasma cell disorders according to proliferation status and malignancy. Leukemia, *13* : 289-294, 1999.
167. Renner, S., Weisz, J., Krajewski, S., Krajewska, M., Reed, J. C., and Lichtenstein, A. Expression of BAX in plasma cell dyscrasias. Clin.Cancer Res., *6*: 2371-2380, 2000.
168. Hanada, M., Delia, D., Aiello, A., Stadtmauer, E., and Reed, J. C. bcl-2 gene hypomethylation and high-level expression in B-cell chronic lymphocytic leukemia. Blood, *82*: 1820-1828, 1993.
169. Pahl, H. L. Activators and target genes of Rel/NF-kappaB transcription factors. Oncogene, *18*: 6853-6866, 1999.
170. Bharti, A. C., Donato, N., Singh, S., and Aggarwal, B. B. Curcumin (diferuloylmethane) down-regulates the constitutive activation of nuclear factor-kappa B and IkappaBalpha kinase in human multiple myeloma cells, leading to suppression of proliferation and induction of apoptosis. Blood, *101*: 1053-1062, 2003.
171. Miyashita, T., Krajewski, S., Krajewska, M., Wang, H. G., Lin, H. K., Liebermann, D. A., Hoffman, B., and Reed, J. C. Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression in vitro and in vivo. Oncogene, *9*: 1799-1805, 1994.
172. Miyashita, T., Harigai, M., Hanada, M., and Reed, J. C. Identification of a p53-dependent negative response element in the bcl-2 gene. Cancer Res., *54*: 3131-3135, 1994.
173. Strasser, A., Whittingham, S., Vaux, D. L., Bath, M. L., Adams, J. M., Cory, S., and Harris, A. W. Enforced BCL2 expression in B-lymphoid cells prolongs antibody responses and elicits autoimmune disease. Proc.Natl.Acad.Sci.U.S.A, *88*: 8661-8665, 1991.

174. McDonnell, T. J., Deane, N., Platt, F. M., Nunez, G., Jaeger, U., McKearn, J. P., and Korsmeyer, S. J. bcl-2-immunoglobulin transgenic mice demonstrate extended B cell survival and follicular lymphoproliferation. Cell, *57*: 79-88, 1989.
175. McDonnell, T. J. and Korsmeyer, S. J. Progression from lymphoid hyperplasia to high-grade malignant lymphoma in mice transgenic for the t(14; 18). Nature, *349*: 254-256, 1991.
176. Strasser, A., Harris, A. W., and Cory, S. E mu-bcl-2 transgene facilitates spontaneous transformation of early pre-B and immunoglobulin-secreting cells but not T cells. Oncogene, *8*: 1-9, 1993.
177. Gazitt, Y., Fey, V., Thomas, C., and Alvarez, R. Bcl-2 overexpression is associated with resistance to dexamethasone, but not melphalan, in multiple myeloma cells. Int.J Oncol., *13*: 397-405, 1998.
178. Tu, Y., Xu, F. H., Liu, J., Vescio, R., Berenson, J., Fady, C., and Lichtenstein, A. Upregulated expression of BCL-2 in multiple myeloma cells induced by exposure to doxorubicin, etoposide, and hydrogen peroxide. Blood, *88*: 1805-1812, 1996.
179. Schwarze, M. M. and Hawley, R. G. Prevention of myeloma cell apoptosis by ectopic bcl-2 expression or interleukin 6-mediated up-regulation of bcl-xL. Cancer Res., *55*: 2262-2265, 1995.
180. Campos, L., Rouault, J. P., Sabido, O., Oriol, P., Roubi, N., Vasselon, C., Archimbaud, E., Magaud, J. P., and Guyotat, D. High expression of bcl-2 protein in acute myeloid leukemia cells is associated with poor response to chemotherapy. Blood, *81*: 3091-3096, 1993.
181. Piris, M. A., Pezzella, F., Martinez-Montero, J. C., Orradre, J. L., Villuendas, R., Sanchez-Beato, M., Cuena, R., Cruz, M. A., Martinez, B., Pezella, F., and . p53 and bcl-2 expression in high-grade B-cell lymphomas: correlation with survival time. Br.J Cancer, *69*: 337-341, 1994.
182. Tu, Y., Renner, S., Xu, F., Fleishman, A., Taylor, J., Weisz, J., Vescio, R., Rettig, M., Berenson, J., Krajewski, S., Reed, J. C., and Lichtenstein, A. BCL-X expression in multiple myeloma: possible indicator of chemoresistance. Cancer Res., *58*: 256-262, 1998.
183. Derenne, S., Monia, B., Dean, N. M., Taylor, J. K., Rapp, M. J., Harousseau, J. L., Bataille, R., and Amiot, M. Antisense strategy shows that Mcl-1 rather than Bcl-2 or Bcl-x(L) is an essential survival protein of human myeloma cells. Blood, *100*: 194-199, 2002.
184. Boise, L. H., Gonzalez-Garcia, M., Postema, C. E., Ding, L., Lindsten, T., Turka, L. A., Mao, X., Nunez, G., and Thompson, C. B. bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. Cell, *74*: 597-608, 1993.
185. Kozopas, K. M., Yang, T., Buchan, H. L., Zhou, P., and Craig, R. W. MCL1, a gene expressed in programmed myeloid cell differentiation, has sequence similarity to BCL2. Proc Natl Acad Sci U S A, *90*: 3516-3520, 1993.
186. Craig, R. W. MCL1 provides a window on the role of the BCL2 family in cell proliferation, differentiation and tumorigenesis. Leukemia, *16*: 444-454, 2002.
187. Krajewski, S., Bodrug, S., Gascoyne, R., Berean, K., Krajewska, M., and Reed, J. C. Immunohistochemical analysis of Mcl-1 and Bcl-2 proteins in normal and neoplastic lymph nodes. Am J Pathol., *145*: 515-525, 1994.
188. Krajewski, S., Bodrug, S., Krajewska, M., Shabaik, A., Gascoyne, R., Berean, K., and Reed, J. C. Immunohistochemical analysis of Mcl-1 protein in human tissues. Differential regulation of Mcl-1 and Bcl-2 protein production suggests a unique role for Mcl-1 in control of programmed cell death in vivo. Am J Pathol., *146*: 1309-1319, 1995.
189. Zhou, P., Qian, L., Kozopas, K. M., and Craig, R. W. Mcl-1, a Bcl-2 family member, delays the death of hematopoietic cells under a variety of apoptosis-inducing conditions. Blood, *89*: 630-643, 1997.
190. Vrana, J. A., Bieszczad, C. K., Cleaveland, E. S., Ma, Y., Park, J. P., Mohandas, T. K., and Craig, R. W. An MCL1-overexpressing Burkitt lymphoma subline exhibits enhanced survival on exposure to serum deprivation, topoisomerase inhibitors, or staurosporine but remains sensitive to 1-beta-D-arabinofuranosylcytosine. Cancer Res., *62*: 892-900, 2002.
191. Kitada, S., Andersen, J., Akar, S., Zapata, J. M., Takayama, S., Krajewski, S., Wang, H. G., Zhang, X., Bullrich, F., Croce, C. M., Rai, K., Hines, J., and Reed, J. C. Expression of apoptosis-regulating proteins

in chronic lymphocytic leukemia: correlations with In vitro and In vivo chemoresponses. Blood, *91*: 3379-3389, 1998.

192. Zhou, P., Qian, L., Bieszczad, C. K., Noelle, R., Binder, M., Levy, N. B., and Craig, R. W. Mcl-1 in transgenic mice promotes survival in a spectrum of hematopoietic cell types and immortalization in the myeloid lineage. Blood, *92*: 3226-3239, 1998.
193. Zhou, P., Levy, N. B., Xie, H., Qian, L., Lee, C. Y., Gascoyne, R. D., and Craig, R. W. MCL1 transgenic mice exhibit a high incidence of B-cell lymphoma manifested as a spectrum of histologic subtypes. Blood, *97*: 3902-3909, 2001.
194. Spets, H., Stromberg, T., Georgii-Hemming, P., Siljason, J., Nilsson, K., and Jernberg-Wiklund, H. Expression of the bcl-2 family of pro- and anti-apoptotic genes in multiple myeloma and normal plasma cells: regulation during interleukin-6(IL-6)-induced growth and survival. Eur.J Haematol., *69*: 76-89, 2002.
195. Puthier, D., Derenne, S., Barille, S., Moreau, P., Harousseau, J. L., Bataille, R., and Amiot, M. Mcl-1 and Bcl-xL are co-regulated by IL-6 in human myeloma cells. Br.J Haematol., *107*: 392-395, 1999.
196. Puthier, D., Bataille, R., and Amiot, M. IL-6 up-regulates mcl-1 in human myeloma cells through JAK / STAT rather than ras / MAP kinase pathway. Eur.J Immunol, *29*: 3945-3950, 1999.
197. Zhang, B., Potyagaylo, V., and Fenton, R. G. IL-6-independent expression of Mcl-1 in human multiple myeloma. Oncogene, *22*: 1848-1859, 2003.
198. Gojo, I., Zhang, B., and Fenton, R. G. The Cyclin-dependent Kinase Inhibitor Flavopiridol Induces Apoptosis in Multiple Myeloma Cells through Transcriptional Repression and Down-Regulation of Mcl-1. Clin.Cancer Res., *8*: 3527-3538, 2002.
199. Zhang, B., Gojo, I., and Fenton, R. G. Myeloid cell factor-1 is a critical survival factor for multiple myeloma. Blood, *99*: 1885-1893, 2002.
200. Vitols, S., Norgren, S., Juliusson, G., Tatidis, L., and Luthman, H. Multilevel regulation of low-density lipoprotein receptor and 3-hydroxy-3-methylglutaryl coenzyme A reductase gene expression in normal and leukemic cells. Blood, *84*: 2689-2698, 1994.
201. Harwood, H. J., Jr., Alvarez, I. M., Noyes, W. D., and Stacpoole, P. W. In vivo regulation of human leukocyte 3-hydroxy-3-methylglutaryl coenzyme A reductase: increased enzyme protein concentration and catalytic efficiency in human leukemia and lymphoma. J Lipid Res., *32*: 1237-1252, 1991.
202. Dimitroulakos, J., Nohynek, D., Backway, K. L., Hedley, D. W., Yeger, H., Freedman, M. H., Minden, M. D., and Penn, L. Z. Increased sensitivity of acute myeloid leukemias to lovastatin-induced apoptosis: A potential therapeutic approach. Blood, *93*: 1308-1318, 1999.
203. Goldstein, J. L. and Brown, M. S. Regulation of the mevalonate pathway. Nature, *343*: 425-430, 1990.
204. Magee, T. and Marshall, C. New insights into the interaction of Ras with the plasma membrane. Cell, *98*: 9-12, 1999.
205. Lerner, E. C., Qian, Y., Blaskovich, M. A., Fossum, R. D., Vogt, A., Sun, J., Cox, A. D., Der, C. J., Hamilton, A. D., and Sebti, S. M. Ras CAAX peptidomimetic FTI-277 selectively blocks oncogenic Ras signaling by inducing cytoplasmic accumulation of inactive Ras-Raf complexes. J Biol.Chem., *270*: 26802-26806, 1995.
206. Lerner, E. C., Qian, Y., Hamilton, A. D., and Sebti, S. M. Disruption of oncogenic K-Ras4B processing and signaling by a potent geranylgeranyltransferase I inhibitor. J Biol.Chem., *270*: 26770-26773, 1995.
207. Sebti, S. M. and Hamilton, A. D. Farnesyltransferase and geranylgeranyltransferase I inhibitors and cancer therapy: lessons from mechanism and bench-to-bedside translational studies. Oncogene, *19*: 6584-6593, 2000.
208. Zohn, I. M., Campbell, S. L., Khosravi-Far, R., Rossman, K. L., and Der, C. J. Rho family proteins and Ras transformation: the RHOad less traveled gets congested. Oncogene, *17*: 1415-1438, 1998.
209. Reiss, Y., Goldstein, J. L., Seabra, M. C., Casey, P. J., and Brown, M. S. Inhibition of purified p21ras farnesyl:protein transferase by Cys-AAX tetrapeptides. Cell, *62*: 81-88, 1990.
210. Casey, P. J. Biochemistry of protein prenylation. J Lipid Res., *33*: 1731-1740, 1992.

211. James, G. L., Goldstein, J. L., and Brown, M. S. Polylysine and CVIM sequences of K-RasB dictate specificity of prenylation and confer resistance to benzodiazepine peptidomimetic in vitro. J Biol.Chem., *270*: 6221-6226, 1995.
212. Rowell, C. A., Kowalczyk, J. J., Lewis, M. D., and Garcia, A. M. Direct demonstration of geranylgeranylation and farnesylation of Ki-Ras in vivo. J Biol.Chem., *272*: 14093-14097, 1997.
213. Reiss, Y., Stradley, S. J., Gierasch, L. M., Brown, M. S., and Goldstein, J. L. Sequence requirement for peptide recognition by rat brain p21ras protein farnesyltransferase. Proc Natl Acad Sci U S A, *88*: 732-736, 1991.
214. Yokoyama, K., Goodwin, G. W., Ghomashchi, F., Glomset, J. A., and Gelb, M. H. A protein geranylgeranyltransferase from bovine brain: implications for protein prenylation specificity. Proc Natl Acad Sci U S A, *88*: 5302-5306, 1991.
215. Downs, J. R., Clearfield, M., Weis, S., Whitney, E., Shapiro, D. R., Beere, P. A., Langendorfer, A., Stein, E. A., Kruyer, W., and Gotto, A. M. Primary prevention of acute coronary events with lovastatin in men and women with average cholesterol levels: results of AFCAPS/TexCAPS. Air Force/Texas Coronary Atherosclerosis Prevention Study. JAMA, *279* : 1615-1622, 1998.
216. Xia, Z., Tan, M. M., Wong, W. W., Dimitroulakos, J., Minden, M. D., and Penn, L. Z. Blocking protein geranylgeranylation is essential for lovastatin-induced apoptosis of human acute myeloid leukemia cells. Leukemia, *15*: 1398-1407, 2001.
217. Miquel, K., Pradines, A., Sun, J., Qian, Y., Hamilton, A. D., Sebti, S. M., and Favre, G. GGTI-298 induces G0-G1 block and apoptosis whereas FTI-277 causes G2-M enrichment in A549 cells. Cancer Res., *57*: 1846-1850, 1997.
218. Kawata, S., Yamasaki, E., Nagase, T., Inui, Y., Ito, N., Matsuda, Y., Inada, M., Tamura, S., Noda, S., Imai, Y., and Matsuzawa, Y. Effect of pravastatin on survival in patients with advanced hepatocellular carcinoma. A randomized controlled trial. Br.J Cancer, *84*: 886-891, 2001.

# Chapter 2

## Chemosensitization of myeloma plasma cells by an antisense-mediated down-regulation of Bcl-2 protein

Niels W.C.J. van de Donk, Marloes M.J. Kamphuis, Mirjam van Dijk, H.P. Eric Borst, Andries C. Bloem, and Henk M. Lokhorst

Leukemia 2003; 17: 211-219

## Abstract

An antisense oligodeoxynucleotide (ODN) complementary to the first 6 codons of the Bcl-2 mRNA, G3139 (oblimersen sodium; Genasense), has been shown to down-regulate Bcl-2 and produce responses in a variety of malignancies including drug-resistant lymphoma. Incubation of *ex vivo* purified plasma cells from patients with multiple myeloma (MM) with carboxyfluorescein (FAM)-labeled antisense ODNs resulted in a time- and dose-dependent uptake in the cytoplasm and nucleus. No major differences in uptake of Bcl-2 antisense ODNs were observed among patients' samples. Incubation of purified myeloma plasma cells with G3139, but not solvent or reverse polarity control ODNs, resulted in a reduction (>75%) of Bcl-2 mRNA levels after 2 and 4 days, as measured by Real-Time PCR. Treatment with G3139 led to a sequence-specific reduction of Bcl-2 protein levels within 4 days of exposure in 10 out of 11 clinical samples from patients with chemosensitive and multidrug resistant disease, without significant reduction of α-actin, Bax, Bcl-XL, or Mcl-1 proteins. This resulted in a significantly enhanced sensitivity of the myeloma tumor cells to dexamethasone or doxorubicin-induced apoptosis. G3139 can consistently enter myeloma cells, down-regulate the expression of Bcl-2, and enhance the efficacy of myeloma therapy. These data support further clinical evaluation of G3139 therapy in multiple myeloma.

## Keywords

Bcl-2, antisense oligodeoxynucleotide, multiple myeloma, chemosensitization, drug resistance

## Introduction

Although chemotherapy is initially effective in the majority of patients with multiple myeloma (MM), they all eventually develop multidrug resistant disease. Different mechanisms contribute to the drug resistant phenotype of myeloma tumor cells [1]. These mechanisms include decreased accumulation of the cytotoxic drug in cells due to increased efflux [2-6], altered drug metabolism [7], and altered intracellular targets of the drug [8-11]. However, there is increasing evidence that cytotoxic drugs exert their effects through the triggering of apoptotic pathways and that prevention of drug-induced apoptosis is associated with multidrug resistant disease [12,13]. Several studies have demonstrated that interleukin-6 (IL-6) [14-16], Ras mutations [16-18], adhesion of myeloma plasma cells to bone marrow stromal cells [19,20] or extracellular matrix components [21], and the anti-apoptotic proteins Bcl-XL [22,23], Mcl-1 [24,25], and Bcl-2 [26,27], inhibit apoptosis of myeloma plasma cells.

Bcl-2 overexpression was first identified in the translocation t(14;18) observed in follicular lymphoma. Despite the low incidence (0-15%) of this translocation in MM, overexpression of Bcl-2 is found in the majority of myeloma patients and plasma cell lines [28-34]. Bcl-2 protects against apoptosis by forming heterodimers with pro-apoptotic Bcl-2 family members such as Bax and Bad, and by preventing both cytochrome c release from mitochondria and activation of caspases [35,36]. Overexpression of Bcl-2 protein in myeloma cell lines confers protection from apoptosis induced by IL-6 deprivation [37], dexamethasone [27], etoposide, or doxorubicin [26] and may therefore contribute to tumor cell survival and a multidrug resistant phenotype. For these reasons, abrogating the anti-apoptotic effect of Bcl-2 protein may increase chemosensitivity and reverse chemoresistance in myeloma tumor cells.

Antisense oligodeoxynucleotides (ODNs) are short single-stranded DNA molecules that are complementary to a known target mRNA. This results in a sequence-specific hybridization of the antisense ODNs with their target mRNAs, which prevents translation or recruits endogenous RNase H to cleave the mRNA backbone [38]. G3139 (oblimersen sodium; Genasense) is an 18-mer phosphorothioate antisense ODN, directed against the first 6 codons of Bcl-2 mRNA. Several *in vitro* studies and studies using tumor xenograft models in mice demonstrate that antisense-mediated Bcl-2 down-regulation induces apoptosis [39-45] and reverses chemoresistance [46-51]. In a Phase I study in relapsed non-Hodgkin's lymphoma patients [52,53] and in a Phase I-II study in patients with advanced melanoma [54], objective antitumor responses without significant toxicity were reported for G3139 as a single agent or G3139 combined with dacarbazine, respectively.

In this study, we show that G3139 decreases Bcl-2 mRNA and protein levels in myeloma plasma cells resulting in an enhanced susceptibility towards dexamethasone and doxorubicin-induced apoptosis.

## Materials and methods

### Reagents

Dexamethasone was purchased from Sigma (St Louis, MO, USA) and doxorubicin from Pharmacia & Upjohn (Woerden, The Netherlands).

### ODNs

Phosphorothioate antisense oligodeoxynucleotides used in this study, were provided by Genta Inc. (Berkeley Heights, NJ, USA). The sequence of Bcl-2 antisense ODN (Genasense$^{TM}$; oblimersen sodium; G3139), corresponding to the first 6 codons of the open reading frame of Bcl-2 mRNA was 5'-TCT CCC AGC GTG CGC CAT-3'. Reverse polarity ODNs (G3622; sequence, 5'-TAC CGC GTG CGA CCC TCT-3') were used as a control. The ODNs were dissolved in Iscove's Modified Dulbecco's Medium (IMDM) and stored at –80$^{o}$C until use. ODNs were administered to cells in free form in the absence of cationic lipids.

### Patients

Myeloma plasma cells were obtained from bone marrow aspirates taken from the posterior iliac crest in 14 patients and from peripheral blood in one patient (patient 3) with plasma cell leukemia after obtaining informed consent. The clinical characteristics of these patients are shown in Table 1. The plasma cell percentage in the patient samples varied from 6-54% of the mononuclear cells as determined by co-expression of CD38 (anti-CD38-FITC, Immunotech, Marseille, France) and CD138 (anti-CD138-PE, Immunotech) by flow cytometric analysis (FACSCalibur, Becton Dickinson, Erembodegem, Belgium (BDIS)). Myeloma plasma cells were purified *ex vivo* from mononuclear cells obtained by Ficoll-Paque (Amersham; Pharmacia BiotechAB, Uppsala, Sweden) density centrifugation, by magnetic cell sorting (MACS) based on CD138 expression [55]. To this end, mononuclear cells were subsequently labeled with anti-CD138 (Immunotech) and rat anti-mouse IgG1 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and then separated using a high gradient magnetic separation column placed in a strong magnetic field (Miltenyi), exactly following the instructions of the manufacturer. Samples obtained in this way contained >95% plasma cells as determined by analysis of CD38/CD138 co-expression. For experiments, myeloma cells were resuspended in RPMI-1640 (GIBCO, Breda, The Netherlands) supplemented with 10% fetal calf serum (FCS) (Integro, Zaandam, The Netherlands), 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10 μM β-mercaptoethanol.

**Table 1.** Clinical characteristics of multiple myeloma patients

| Patient | Age/Sex | Stage | M-component type | Disease status |
|---|---|---|---|---|
| 1 | 46/F | III-A | κ-LC | Untreated |
| 2 | 50/M | III-A | IgG-λ | Untreated |
| 3 | 76/F | III-B | non-secretory | Relapse after MP |
| 4 | 58/M | III-A | IgG-λ | Relapse after autoPBSCT |
| 5 | 49/F | II-A | IgG-κ | Untreated |
| 6 | 73/M | I-A | IgG-λ | MP refractory |
| 7 | 69/M | II-A | IgA-κ | VAD refractory |
| 8 | 52/M | III-A | IgG-κ | VAD/Thalidomide sensitive |
| 9 | 58/F | III-A | IgG-κ | MP refractory |
| 10 | 54/F | II-A | IgG-κ | VAD sensitive |
| 11 | 59/M | II-B | IgG-κ | MP-Dexamethasone-Thalidomide refractory |
| 12 | 63/M | III-B | IgA-κ | VAD refractory |
| 13 | 63/M | III-A | κ-LC | VAD refractory |
| 14 | 61/M | III-A | IgG-κ | VAD sensitive |
| 15 | 69/M | III-B | IgG-λ | VAD refractory |

The patients were classified according to the Durie-Salmon staging system [64]. The M-component type was determined by immunofixation. Bone marrow plasma cell percentage was determined by simultaneous detection of CD38 and CD138 in bone marrow mononuclear cells by flow cytometric analysis. Plasma cell monoclonality was established by means of immunofluorescence microscopy based on heavy- and light-chain expression.

M, male; F, female; Ig, immunoglobulin; LC, light chain; κ, kappa; λ, lambda; MP, melphalan and prednisone; autoPBSCT, autologous peripheral blood stem cell transplantation; VAD, vincristine, doxorubicin, and dexamethasone.

### Flow cytometric analysis of ODN-uptake

For the time-course studies, cells (1 X $10^6$ cells/ml) were incubated with 5 μM FAM-labeled antisense or unlabeled antisense ODNs for various periods of time. For the dose-response study, cells were incubated with 0, 0.05, 0.1, 0.5, 1, 5, or 10 μM FAM-labeled or unlabeled antisense ODNs for 24 hours. After incubation, cells were harvested, washed 3 times in ice-cold PBS, and incubated with an ice-cold solution of 0.2 M acetic acid and 0.5 M NaCl (pH 2.5) at 4°C for 10 minutes to remove membrane-bound ODN as described by Haigler et al [56]. After acid-salt elution, the cells were washed in PBS and incubated with propidium iodide (Calbiochem, La Jolla, CA, USA; 5 μg/ml). Green fluorescence of propidium iodide negative cells was determined by flow cytometric analysis (BDIS). The relative amount of intracellular ODNs was expressed as the ratio of the mean fluorescence intensities of FAM-labeled antisense and unlabeled antisense treated cells.

### Viability assay

Viability of cells was examined by means of the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay as described previously [57]. Purified myeloma plasma cells were seeded in a concentration of 1 X $10^6$/ml in a 96-well flat bottom plate (100 μl/well) (Nunc, Roskilde, Denmark) and treated with 10 μM ODNs. After 2, 4, or 7

days, 25 µl of MTT (5 mg/mL) was added to each well. After an incubation of 2 hours at 37°C the reaction was stopped by the addition of 100 µl 20% sodium dodecyl sulphate (SDS; Boehringer Mannheim, Mannheim, Germany) / 0.025 M HCl / 0.35 M HAc in a mixture of (1:1; v/v) N,N-dimethylformamide (Merck, Darmstadt, Germany) and distilled water. After an overnight incubation at 37°C the optical density of the samples was determined at 570 nm.

**Apoptosis assessment by Annexin V staining**

Purified myeloma plasma cells (5 X $10^5$ in 0.5 ml) were treated with 10 µM ODNs in a 48-well plate (Nunc). After 2, 4, or 7 days, cells were harvested, washed in ice cold PBS and directly stained with Annexin V-FITC (Nexins Research, Kattendijke, The Netherlands) and propidium iodide (PI). After an incubation for 10 minutes at 4°C, the cells were subsequently analyzed by flow cytometry as described previously [58]. Apoptotic cells were defined as early apoptotic cells (Annexin V positive and PI negative) and late apoptotic cells (Annexin V positive and PI positive).

**Real-Time PCR**

Total RNA was isolated from cells (2 X $10^6$ in 2 ml), which were incubated with solvent control or 10 µM ODNs for 2 or 4 days, using RNAzol (Campro, Veenendaal, The Netherlands) according to the manufacturer's instructions. For cDNA synthesis 1 µg of RNA was incubated with 750 U/ml AMV Reverse Transcriptase (Promega, Madison, WI, USA), 100 µg/ml Hexamer primer (Roche, Almere, The Netherlands), 1 mM of dATP, dTTP, dGTP and dCTP (Amersham Pharmacia Biotech Inc, Little Chalfont, UK), and 1350 U/ml RNAguard (Amersham) in a total volume of 20 µl for 90 minutes at 42°C.

Bcl-2 and porphobilinogen deaminase (PBGD) cDNAs were amplified using primers and probes that were selected with the Primer Express Applications-Based Primer Design Software (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). For Bcl-2, the sequences of the primers were 5'-CGC CCT GTG GAT GAC TGA GT-3' and 5'-GGG CCG TAC AGT TCC ACA A-3' and for PBGD 5'-GGC AAT GCG GCT GCA A-3' and 5'-GGG TAC CCA CGC GAA TCA C-3'. The sequences of the Bcl-2 and PBGD TaqMan probes (Applied Biosystems) were 5'-CTG AAC CGG CAC CTG CAC ACC TG-3' and 5'-CAT CTT TGG GCT GTT TTC TTC CGC C-3', respectively. The probes were labeled at the 5' end with the reporter dye molecule (FAM and VIC for the Bcl-2 and PBGD probes, respectively) and at the 3' end with the quencher dye molecule (TAMRA). The PCR reactions (50 µl total volume; 5 µl 10 X ROX buffer (Applied Biosystems), 3 mM $MgCl_2$ for Bcl-2 or 5 mM for PBGD, 1 µl cDNA, 12.5 pmol of each of the Bcl-2 or 15 pmol of each of the PBGD primers, 10 pmol Bcl-2 or PBGD probe, 25 U/ml AmpliTaq gold polymerase (Applied Biosystems), 0.8 mM of dATP, dTTP, dGTP and dCTP (Amersham)) were heated to 95°C for 10 minutes and then amplified for 40

cycles at 95°C for 15 seconds and 60 seconds at 60°C by using the ABI Prism 7700 Sequence Detection system. Relative quantitation of gene expression was performed as described in the manual using the comparative $C_T$ (treshold cycle) method. Relative Bcl-2 mRNA levels were normalized to PBGD mRNA levels and presented as a percentage of solvent control treated cells.

**Western blot analysis**

Cells (1.5 X $10^6$ cells in 1.5 ml) were incubated for 2 or 4 days in growth medium with ODNs (10 μM). After harvesting, whole cell lysates were made by washing cells twice in ice-cold PBS and then resuspending them in lysis buffer (10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% Triton X-100, and a cocktail of protease inhibitors (Boehringer Mannheim)) at 4°C for 20 minutes. Insoluble material was removed by centrifugation at 14 000 r.p.m. for 6 minutes at 4°C. Protein concentrations were determined by the BCA assay (Pierce, Rockford, IL, USA). Samples containing equal amounts of protein were mixed with 2 X Laemmli sample buffer (0.125 M Tris (pH 6.9) with 4% SDS, 20% Glycerol, and 10% β-mercaptoethanol) and boiled for 5 minutes. Proteins were subsequently fractionated in 12% SDS-PAGE at room temperature and electrically transferred from the gel to a PVDF-membrane (Biorad, Hercules, CA, USA). After blocking in 0.1% Tween-20, 5% skimmed powder milk, 2% BSA in 10 mM Tris and 150 mM NaCl, the membranes were incubated with anti-Bcl-2 (Dako, Glostrup, Denmark), anti-Bax (Immunotech), anti-Mcl-1 (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA), or anti-Bcl-XL/S (Santa Cruz). Antibody binding was visualized with enhanced chemoluminescence (Amersham) detection with hyperfilm ECL (Amersham) after incubation with a horseradish peroxidase (HRP)-conjugated secondary antibody (Dako). Membranes incubated with anti-Bcl-2, anti-Bax, anti-Mcl-1, or anti-Bcl-XL/S were extensively washed in PBS and reprobed with anti-α-actin (Sigma). Relative amounts of protein expression were determined by densitometry and normalized to α-actin expression.

**Statistical analysis**

Data analysis was performed using the SPSS statistical software package (SPSS Inc, Chicago, IL, USA). A two-sided Student's t-test or a Welch's t-test in case of unequal variances, was used to determine differences between groups. Differences were considered statistically significant when $P < 0.05$. Data are plotted as means ± standard error of the mean (s.e.m.).

## Results

### Uptake of antisense ODNs

Uptake of Bcl-2 antisense ODNs was studied in purified myeloma plasma cells from patients 1, 2, 3, and 4. Myeloma cells were purified from mononuclear cells from bone marrow aspirates of patients 1, 2, and 4, and from peripheral blood of patient 3 with secondary plasma cell leukemia by MACS based on CD138 expression. Plasma cell percentage in all cases was >95% after purification (data not shown). Myeloma cells were incubated with 5 μM FAM-labeled or unlabeled Bcl-2 antisense ODNs for various periods of time. Figure 1A shows a time-dependent increase in ODN-uptake. ODN-uptake was higher for myeloma cells from patients 1 and 3 compared to cells from patients 2 and 4. For all 4 patients' samples ODN-uptake was highest during the first 48 hours of incubation. During the following 48 hours ODNs accumulated at a significantly lower rate. The concentration-dependent uptake of antisense ODNs was studied by incubating myeloma cells from patient 4 with 0, 0.05, 0.1, 0.5, 1, 5, or 10 μM FAM-labeled or unlabeled antisense ODNs for 24 hours. Figure 1B demonstrates that uptake was more efficient at lower concentrations of ODN in medium.

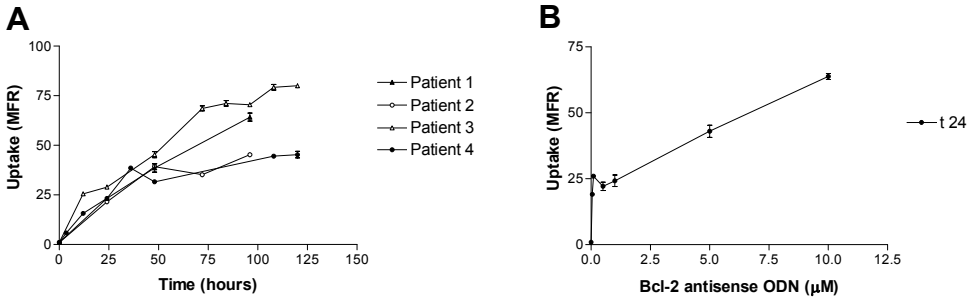


**Figure 1. Dose- and time-dependent uptake of Bcl-2 antisense ODNs.** Plasma cells from myeloma patients 1, 2, and 4 were purified from bone marrow mononuclear cells and from patient 3 with secondary plasma cell leukemia from peripheral blood mononuclear cells by MACS based on CD138 expression. Plasma cell percentage was >95% after purification. Myeloma plasma cells were (A) incubated with 5 μM unlabeled or FAM-labeled antisense ODNs for various periods of time or (B) with various concentrations of unlabeled or FAM-labeled Bcl-2 antisense ODNs for 24 hours. The relative amount of intracellular ODNs was studied by flow cytometry and expressed as the ratio of the mean fluorescence intensities of FAM-labeled antisense and unlabeled antisense treated cells (MFR). Experiments were performed in triplicate. Data are presented as mean ± s.e.m.

### Effect of Bcl-2 antisense ODNs on Bcl-2 mRNA levels in *ex vivo* purified myeloma plasma cells

Real-Time PCR was performed to examine Bcl-2 mRNA levels in purified myeloma plasma cells from patients 8 and 12. In myeloma cells from patient 8, Bcl-2 mRNA levels normalized to PBGD mRNA levels, were reduced by 10 μM (60.6 μg/mL) antisense

ODNs by 92.5%, when compared to the solvent control after 4 days of treatment. Reverse polarity ODNs did not significantly decrease Bcl-2 mRNA levels in cells from patient 8 (4.8%; $P < 0.01$). Treatment of myeloma cells from patient 12 with 10 μM Bcl-2 antisense for 2 and 4 days reduced Bcl-2 mRNA levels normalized to PBGD mRNA levels by 89.4 and 77.2%, compared to 2.2% ($P < 0.01$) down-regulation and 14.1% ($P < 0.01$) induction in reverse polarity-treated cells, respectively (Figure 2A). This indicates that there is a sequence-specific inhibition of Bcl-2 mRNA by Bcl-2 antisense ODNs.

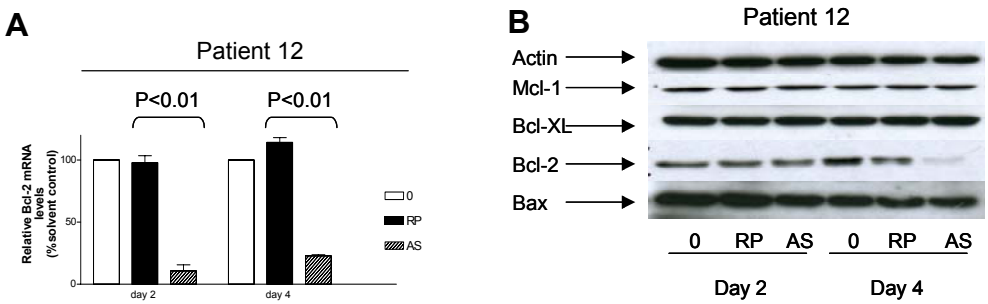


**Figure 2. Sequence-specific reduction of Bcl-2 mRNA and protein levels by Bcl-2 antisense ODNs in purified myeloma tumor cells from patient 12.** Plasma cells were purified from bone marrow mononuclear cells by MACS based on CD138 expression (purity >95%). Cells from patients 12 were treated for 2 or 4 days with solvent control (0), 10 μM antisense (AS), or 10 μM reverse polarity (RP) ODNs. (A) Bcl-2 and PBGD mRNA levels were determined by Real-Time PCR. Bcl-2 mRNA levels normalized to PBGD, were expressed as a percentage of the solvent control values. The experiment was performed once in triplicate. Shown are the mean ± s.e.m. and the significance (*P* values). (B) After protein isolation, Bcl-2, Mcl-1, Bcl-XL, and Bax were determined by Western blot analysis. Blots were reprobed with anti-α-actin to confirm the presence of equal amounts of protein in each lane.

### Effect of Bcl-2 antisense ODNs on Bcl-2 protein levels in *ex vivo* purified myeloma plasma cells

Western blot analysis was performed to determine the effect of the ODNs on Bcl-2 protein expression. Myeloma cells from 11 myeloma patients (patients 5-15) were purified from bone marrow mononuclear cells by MACS based on CD138 expression. The *ex vivo* purified plasma cells were incubated with solvent control, 10 μM antisense, or 10 μM reverse polarity ODNs. Myeloma cells were treated for 2 and 4 days in all cases with the exception of cells from patients 7, 9, 10, and 13 which were treated for 4 days only because of insufficient myeloma cells. Expression of Bcl-2 and Bax was found in all

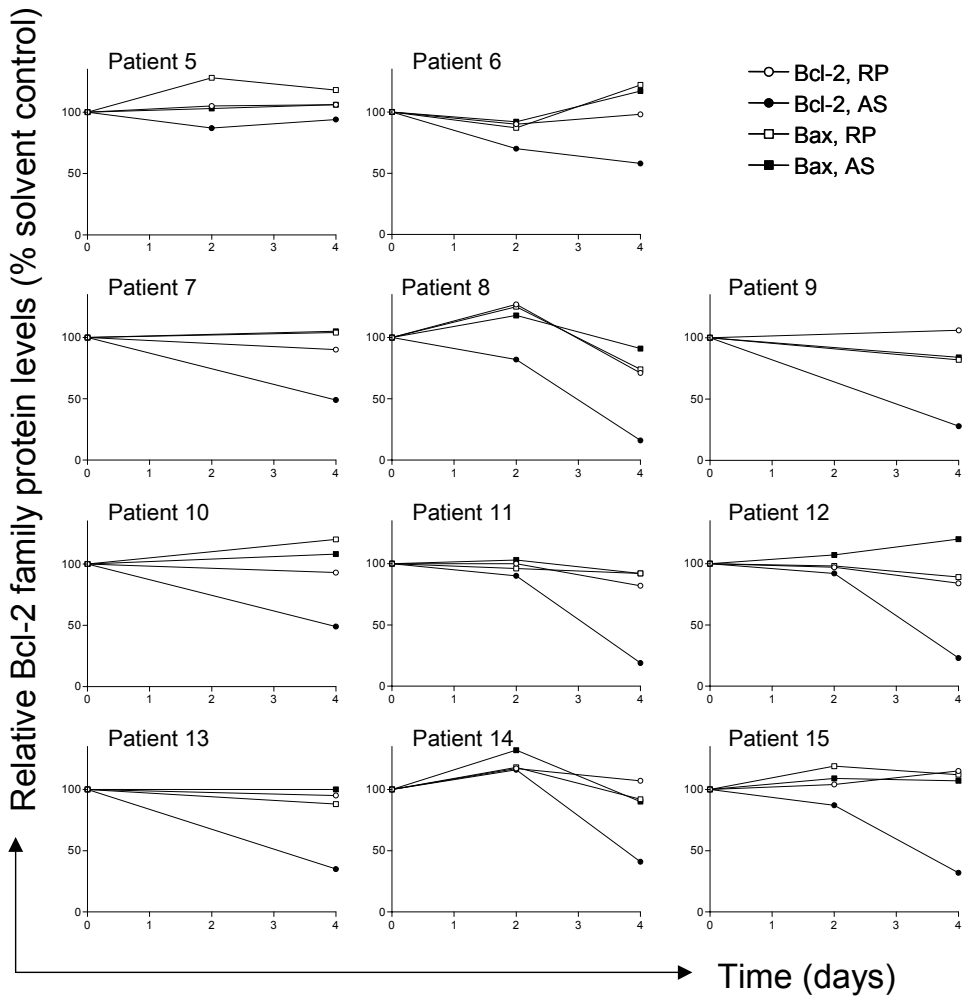


**Figure 3. Sequence-specific and time-dependent down-regulation of Bcl-2 protein levels by Bcl-2 antisense ODNs in purified myeloma cells from patients, whereas protein levels of Bax were not affected**. The reduction of Bcl-2 protein levels ranged from 8-18% and 42-84% in the 6 out of 7 and 10 out of 11 responding patients after 2 and 4 days of treatment, respectively. Plasma cells from myeloma patients 5-15 were purified from bone marrow mononuclear cells by MACS based on CD138 expression. Plasma cell percentage was >95% after purification. *Ex vivo* purified myeloma plasma cells were incubated with solvent control, 10 μM Bcl-2 antisense (AS), or 10 μM reverse polarity (RP) ODNs for 2 or 4 days. After protein isolation, Bcl-2, Bax, and α-actin were determined by Western blot analysis. In 8 patients' samples, Western blot analysis was performed once; in 3 patients' samples 2 times, with identical results. Bcl-2 and Bax protein levels were quantitated by densitometry analysis of the protein bands and normalized to α-actin. Bcl-2 and Bax protein levels are expressed as a percentage of the solvent control values.

cases. After 2 days of treatment Bcl-2 protein levels were slightly decreased in antisense-treated cells in 6 out of 7 patients. However, after 4 days of incubation Bcl-2 protein expression was significantly down-regulated by antisense ODNs in all 11 patients' samples, except in myeloma cells from patient 5. On day 2 Bcl-2 levels were not changed in myeloma cells derived from patient 14. In contrast, on day 4 Bcl-2 protein levels were significantly diminished in Bcl-2 antisense-treated cells from patient 14, when compared to solvent or reverse polarity control-treated cells.

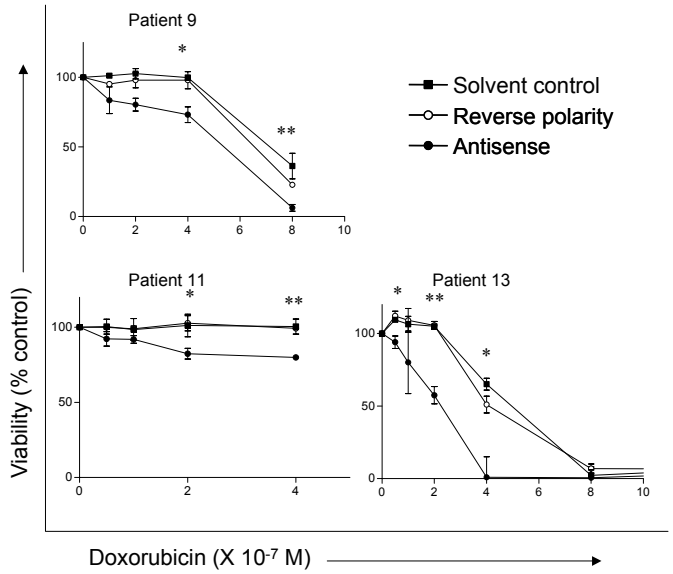


**Figure 4. Bcl-2 antisense enhances sensitivity of purified myeloma tumor cells from patients to doxorubicin-induced cell death**. Plasma cells from myeloma patients 9, 11, and 13 were purified from bone marrow mononuclear cells by MACS based on CD138 expression (purity >95%). The myeloma tumor cells were incubated for 4 days with solvent control, 10 μM Bcl-2 antisense, or 10 μM reverse polarity ODNs. Then cells were resuspended in RPMI-1640 with 10% FCS and incubated for 2 days with doxorubicin (0.5 to 20 X $10^{-7}$ M). The percentage of viable cells, relative to the untreated cells, was measured by using the MTT assay. The experiment was performed once in triplicate. Data are presented as mean ± s.e.m. *: $P < 0.05$; **: $P < 0.005$ (Bcl-2 antisense treatment *vs* reverse polarity control treatment).

Densitometry analysis after normalization for α-actin, demonstrated a reduction of Bcl-2 protein levels that ranged from 8 to 18% and 42 to 84% in the 6 out of 7 and 10 out of 11 responding patients after 2 and 4 days of treatment, respectively (Figure 3). This was in contrast to reverse polarity ODNs and solvent control, which did not affect Bcl-2 protein expression indicating a sequence-specific effect (Figure 3). For example, treatment of myeloma cells from patient 9 with Bcl-2 antisense for 4 days reduced Bcl-2 protein levels by 72%, compared to an increase of 6% in reverse polarity treated cells. Antisense treatment did not result in a significant change in the levels of α-actin or the pro-apoptotic

Bcl-2 family protein Bax (Figure 3). In addition, protein levels of the anti-apoptotic Bcl-2 family members Mcl-1 and Bcl-XL were not affected by Bcl-2 antisense treatment, as determined in myeloma patients 7, 8, 12, 14, and 15. Bcl-XS expression was not detected in the different patients' samples. Figure 2B shows a representative example of an antisense-mediated Bcl-2 protein down-regulation in myeloma plasma cells derived from patient 12, while Bcl-XL, Bax, Mcl-1, and α-actin protein levels remained unchanged.

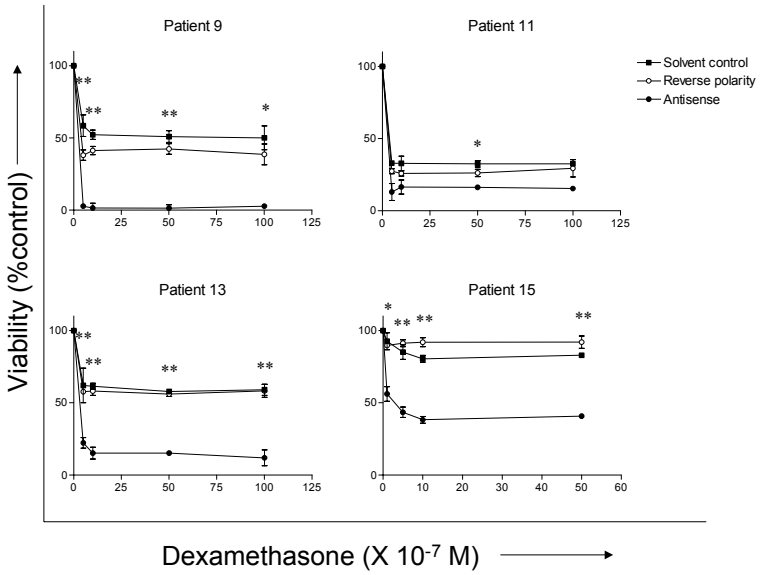


**Figure 5. Bcl-2 antisense enhances sensitivity of purified myeloma tumor cells from patients to dexamethasone-induced cell death.** Plasma cells from myeloma patients 9, 11, 13, and 15 were purified from bone marrow mononuclear cells by MACS based on CD138 expression (purity >95%). The myeloma tumor cells were incubated for 4 days with solvent control, 10 μM Bcl-2 antisense, or reverse polarity ODNs. Then cells were resuspended in RPMI-1640 with 10% FCS and incubated for 2 days with dexamethasone (0.5 to 100 X $10^{-7}$ M). The percentage of viable cells, relative to the untreated cells, was measured by using the MTT assay. The experiment was performed once in triplicate. Data are presented as mean ± s.e.m. *: $P < 0.05$; **: $P < 0.005$ (Bcl-2 antisense treatment *vs* reverse polarity control treatment).

### Bcl-2 antisense ODNs chemosensitize *ex vivo* purified myeloma plasma cells

MTT assay was used to determine the effect of Bcl-2 antisense ODNs on cell viability of *ex vivo* purified plasma cells derived from myeloma patients 1-15. Although treatment with 10 μM Bcl-2 antisense resulted in down-regulation of Bcl-2 mRNA and protein levels, it did not reduce cell viability after 2, 4, and 7 days when compared to solvent, or reverse polarity control-treated cells (data not shown).

We also tested whether the Bcl-2 protein down-regulation resulted in an increased susceptibility of myeloma plasma cells to dexamethasone or doxorubicin-

induced apoptosis. *Ex vivo* purified myeloma cells from myeloma patients were treated for 4 days with solvent control, 10 μM antisense, or reverse polarity control ODNs. This was followed by a 2 day incubation with various concentrations of dexamethasone (0.5 to 100 X $10^{-7}$ M) (patients 9, 11, 13, and 15) or doxorubicin (0.5 to 20 X $10^{-7}$ M) (patients 9, 11, and 13). In all 4 patients' samples a significant reduction of Bcl-2 protein expression was detected after 4 days of treatment with Bcl-2 antisense (Figure 3). Reduction of cell viability by doxorubicin or dexamethasone was significantly higher in myeloma cells which were pre-treated with antisense ODNs when compared to solvent or reverse polarity control pre-treated cells (Figure 4 and 5). For instance, doxorubicin (4 X $10^{-7}$ M) reduced cell viability of plasma cells from patient 13, relative to untreated controls with 99% in antisense pre-treated cells, whereas the reduction was 35% ($P = 0.012$) in solvent control and 49% ($P = 0.03$) in reverse polarity control pre-treated cells (Figure 4). The reduction in cell viability of myeloma plasma cells from patient 13 after addition of 10 X $10^{-7}$ M dexamethasone was 85% in antisense pre-treated cells, compared to 39% ($P = 0.001$) and 42% ($P = 0.001$) in solvent and reverse polarity control pre-treated cells, respectively (Figure 5). The results obtained by MTT assay were confirmed by flow cytometric detection of apoptotic cells by using the Annexin V assay as shown in figure 6 for patient 12.

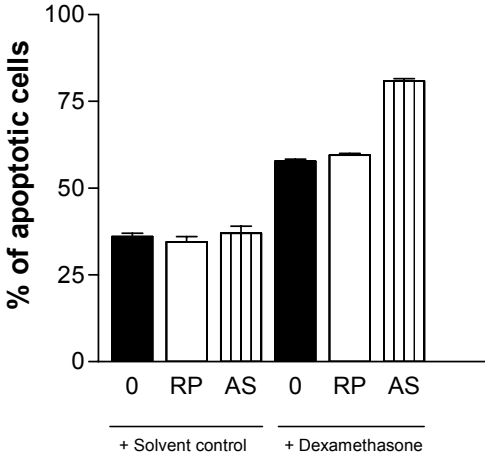


**Figure 6. Bcl-2 antisense enhances sensitivity of purified myeloma tumor cells from patient 12 to dexamethasone-induced apoptosis.** Plasma cells from myeloma patient 12 were purified from bone marrow mononuclear cells by MACS based on CD138 expression (purity >95%). The myeloma tumor cells were incubated for 4 days with solvent control (0), 10 μM Bcl-2 antisense (AS), or reverse polarity ODNs (RP). Then cells were resuspended in RPMI-1640 with 10% FCS and incubated for 2 days with dexamethasone (50 X $10^{-7}$ M) or solvent control. The percentage of apoptotic cells was determined by flow cytometric analysis using the Annexin V assay. The experiment was performed once in triplicate. Data are presented as mean ± s.e.m.

## Discussion

Bcl-2 is expressed in the majority of myeloma patients and myeloma cell lines [28-34]. Gene transfection experiments show that overexpression of Bcl-2 renders myeloma cell lines

resistant to the induction of apoptosis by various cytotoxic drugs [26,27]. In addition, exposure of plasma cell lines to doxorubicin increases Bcl-2 protein levels, which may account for the relative resistance to a second doxorubicin exposure [26]. This suggests that Bcl-2 overexpression protects myeloma tumor cells from apoptosis induced by cytotoxic drugs, and that modulation of Bcl-2 protein levels may enhance the susceptibility to antimyeloma agents and reverse chemoresistance.

Bcl-2 gene expression can be specifically manipulated by using antisense ODNs targeting the Bcl-2 mRNA. G3139 is a phosphorothioate antisense ODN rendering it resistant to nuclease digestion, directed against the first 6 codons of the Bcl-2 mRNA. In this study, we show that treatment with G3139 reduced Bcl-2 protein in a time-dependent and sequence-specific manner in purified myeloma cells from patients, while α-actin, Bcl-XL, Mcl-1, and Bax levels remained unchanged. G3139 treatment decreased Bcl-2 protein levels in myeloma cells both in patients with chemosensitive and refractory disease. The reduction of Bcl-2 protein by G3139 was mediated by a sequence-specific reduction of Bcl-2 mRNA, as the control, PBGD, mRNA levels remained unchanged. This suggests that the decrease of Bcl-2 mRNA levels is a sequence-specific effect, possibly due to degradation of the target mRNA by RNase H, an enzyme that hydrolyzes the RNA moiety of RNA-DNA complexes [38]. The phosphorothioate DNA oligonucleotide is resistant to RNase H cleavage and can therefore hybridize with other Bcl-2 mRNA molecules. However, it can not be excluded that other mechanisms, such as inhibition of transcription initiation or elongation, inhibition of RNA transport, interference with binding to ribosomes, and the inability of the ribosomal complex to scan the message may also contribute to the inhibition of Bcl-2 protein expression by Bcl-2 antisense ODNs [38].

G3139 treatment alone did not reduce cell viability of myeloma plasma cells from patients despite the significant reduction of Bcl-2 protein levels. It is possible that other anti-apoptotic proteins such as Bcl-XL [22,23] or Mcl-1, which is an important survival protein in myeloma [24,25], substitute for Bcl-2 after its down-regulation resulting in protection against apoptosis. In *ex vivo* purified myeloma tumor cells, however, we show that an antisense-mediated down-regulation of Bcl-2 protein resulted in a significantly enhanced susceptibility to dexamethasone or doxorubicin-induced apoptosis. Importantly, chemosensitization was observed in myeloma plasma cells from both patients with refractory and chemosensitive disease, and in samples with low *in vitro* sensitivity to dexamethasone or doxorubicin. Interestingly, Bcl-2 antisense ODNs sensitized myeloma cells more efficiently to dexamethasone, in comparison to doxorubicin. This may suggest that Bcl-2 is a more important regulator of response to dexamethasone. Some reports indicate different nonantisense effects of antisense ODNs. For instance, products of enzymatic degradation of ODNs can influence cell proliferation [59]. Moreover, G3139 contains 2 unmethylated CpG motifs within the ODN sequence which are known to activate NK cells and macrophages [60], and to stimulate B cells [61]. Phosphorothioate ODNs

have also been shown to competitively inhibit a variety of nucleases and polymerases [62]. However, in contrast to G3139, the phosphorothioate reverse polarity control ODN (G3622) which contains the same nucleotides and also 2 CpG motifs did not enhance the sensitivity of myeloma cells to dexamethasone or doxorubicin-induced apoptosis. These data support an antisense mechanism for the chemosensitizing activity of G3139. Chemosensitization by an antisense-mediated Bcl-2 protein reduction was also detected in myeloma cells which were purified from bone marrow mononuclear cells by negative selection based on absence of CD45 expression on myeloma cells (data not shown), which excludes the possibilty that chemosensitization is a phenomenon that can only be observed in positively selected myeloma cells. Determination of apoptosis by flow cytometric detection of Annexin V staining and exclusion of propidium iodide by cells confirmed the results obtained from MTT assay, concerning chemosensitization and the absence of a direct cytotoxic effect of G3139. These results indicate that manipulation of Bcl-2 protein levels by treating patients with G3139 may enhance sensitivity to dexamethasone or doxorubicin-containing regimens, and induce more durable responses.

Antisense ODNs must cross cell membranes in order to hybridize with their target mRNA. Uptake of Bcl-2 antisense was studied in plasma cells from 4 myeloma patients by using flow cytometry. Although phosphorothioate modified Bcl-2 antisense ODNs have a high polyanionic charge and molecular weight, they were rapidly taken up by cells. No major differences in uptake of Bcl-2 antisense ODNs were observed among patients. A recent study reported that ODN-uptake at low concentrations is predominantly via receptor-mediated endocytosis while at higher concentrations, a fluid phase endocytosis mechanism appears to predominate [38,63]. This may explain the dose-dependent uptake of fluorochrome-labeled G3139 observed in myeloma cells.

In summary, uptake of G3139 resulted in a sequence-specific reduction of Bcl-2 mRNA and protein levels in *ex vivo* purified myeloma plasma cells. In clinical samples from myeloma patients, Bcl-2 down-regulation alone did not induce apoptosis, but it did enhance the sensitivity of myeloma plasma cells to dexamethasone and doxorubicin. Our findings suggest that Bcl-2 protein plays a significant role in multidrug resistance, and that treatment with cytotoxic antimyeloma agents following down-regulation of Bcl-2 by G3139 may reduce the induction of or reverse the effect of multidrug resistance. This approach is currently undergoing evaluation in clinical trials.

## References

1. Dalton, W. S. and Jove, R. Drug resistance in multiple myeloma: approaches to circumvention. Semin.Oncol., *26*: 23-27, 1999.
2. Epstein, J., Xiao, H. Q., and Oba, B. K. P-glycoprotein expression in plasma-cell myeloma is associated with resistance to VAD. Blood, *74*: 913-917, 1989.

3. Sonneveld, P., Durie, B. G., Lokhorst, H. M., Marie, J. P., Solbu, G., Suciu, S., Zittoun, R., Lowenberg, B., and Nooter, K. Modulation of multidrug-resistant multiple myeloma by cyclosporin. The Leukaemia Group of the EORTC and the HOVON. Lancet, *340*: 255-259, 1992.
4. Grogan, T. M., Spier, C. M., Salmon, S. E., Matzner, M., Rybski, J., Weinstein, R. S., Scheper, R. J., and Dalton, W. S. P-glycoprotein expression in human plasma cell myeloma: correlation with prior chemotherapy. Blood, *81*: 490-495, 1993.
5. Raaijmakers, H. G., Izquierdo, M. A., Lokhorst, H. M., de Leeuw, C., Belien, J. A., Bloem, A. C., Dekker, A. W., Scheper, R. J., and Sonneveld, P. Lung-resistance-related protein expression is a negative predictive factor for response to conventional low but not to intensified dose alkylating chemotherapy in multiple myeloma. Blood, *91*: 1029-1036, 1998.
6. Filipits, M., Drach, J., Pohl, G., Schuster, J., Stranzl, T., Ackermann, J., Konigsberg, R., Kaufmann, H., Gisslinger, H., Huber, H., Ludwig, H., and Pirker, R. Expression of the lung resistance protein predicts poor outcome in patients with multiple myeloma. Clin.Cancer Res., *5*: 2426-2430, 1999.
7. Linsenmeyer, M. E., Jefferson, S., Wolf, M., Matthews, J. P., Board, P. G., and Woodcock, D. M. Levels of expression of the mdr1 gene and glutathione S-transferase genes 2 and 3 and response to chemotherapy in multiple myeloma. Br.J.Cancer, *65*: 471-475, 1992.
8. Moalli, P. A., Pillay, S., Weiner, D., Leikin, R., and Rosen, S. T. A mechanism of resistance to glucocorticoids in multiple myeloma: transient expression of a truncated glucocorticoid receptor mRNA. Blood, *79*: 213-222, 1992.
9. Ishikawa, H., Kawano, M. M., Okada, K., Tanaka, H., Tanabe, O., Sakai, A., Asaoku, H., Iwato, K., Nobuyoshi, M., and Kuramoto, A. Expressions of DNA topoisomerase I and II gene and the genes possibly related to drug resistance in human myeloma cells. Br.J.Haematol., *83*: 68-74, 1993.
10. Krett, N. L., Pillay, S., Moalli, P. A., Greipp, P. R., and Rosen, S. T. A variant glucocorticoid receptor messenger RNA is expressed in multiple myeloma patients. Cancer Res., *55*: 2727-2729, 1995.
11. Futscher, B. W., Foley, N. E., Gleason-Guzman, M. C., Meltzer, P. S., Sullivan, D. M., and Dalton, W. S. Verapamil suppresses the emergence of P-glycoprotein-mediated multi-drug resistance. Int.J.Cancer, *66*: 520-525, 1996.
12. Solary, E., Droin, N., Bettaieb, A., Corcos, L., Dimanche-Boitrel, M. T., and Garrido, C. Positive and negative regulation of apoptotic pathways by cytotoxic agents in hematological malignancies. Leukemia, *14*: 1833-1849, 2000.
13. O'Gorman, D. M. and Cotter, T. G. Molecular signals in anti-apoptotic survival pathways. Leukemia, *15*: 21-34, 2001.
14. Hardin, J., MacLeod, S., Grigorieva, I., Chang, R., Barlogie, B., Xiao, H., and Epstein, J. Interleukin-6 prevents dexamethasone-induced myeloma cell death. Blood, *84*: 3063-3070, 1994.
15. Lichtenstein, A., Tu, Y., Fady, C., Vescio, R., and Berenson, J. Interleukin-6 inhibits apoptosis of malignant plasma cells. Cell Immunol., *162*: 248-255, 1995.
16. Rowley, M., Liu, P., and Van Ness, B. Heterogeneity in therapeutic response of genetically altered myeloma cell lines to interleukin 6, dexamethasone, doxorubicin, and melphalan. Blood, *96*: 3175-3180, 2000.
17. Neri, A., Murphy, J. P., Cro, L., Ferrero, D., Tarella, C., Baldini, L., and Dalla-Favera, R. Ras oncogene mutation in multiple myeloma. J.Exp.Med., *170*: 1715-1725, 1989.
18. Billadeau, D., Liu, P., Jelinek, D., Shah, N., LeBien, T. W., and Van Ness, B. Activating mutations in the N- and K-ras oncogenes differentially affect the growth properties of the IL-6-dependent myeloma cell line ANBL6. Cancer Res., *57*: 2268-2275, 1997.
19. Grigorieva, I., Thomas, X., and Epstein, J. The bone marrow stromal environment is a major factor in myeloma cell resistance to dexamethasone. Exp.Hematol., *26*: 597-603, 1998.
20. Cheung, W. C. and Van Ness, B. The bone marrow stromal microenvironment influences myeloma therapeutic response in vitro. Leukemia, *15*: 264-271, 2001.

21. Damiano, J. S., Cress, A. E., Hazlehurst, L. A., Shtil, A. A., and Dalton, W. S. Cell adhesion mediated drug resistance (CAM-DR): role of integrins and resistance to apoptosis in human myeloma cell lines. Blood, *93*: 1658-1667, 1999.
22. Gauthier, E. R., Piche, L., Lemieux, G., and Lemieux, R. Role of bcl-X(L) in the control of apoptosis in murine myeloma cells. Cancer Res., *56*: 1451-1456, 1996.
23. Tu, Y., Renner, S., Xu, F., Fleishman, A., Taylor, J., Weisz, J., Vescio, R., Rettig, M., Berenson, J., Krajewski, S., Reed, J. C., and Lichtenstein, A. BCL-X expression in multiple myeloma: possible indicator of chemoresistance. Cancer Res., *58*: 256-262, 1998.
24. Zhang, B., Gojo, I., and Fenton, R. G. Myeloid cell factor-1 is a critical survival factor for multiple myeloma. Blood, *99*: 1885-1893, 2002.
25. Derenne, S., Monia, B., Dean, N. M., Taylor, J. K., Rapp, M. J., Harousseau, J. L., Bataille, R., and Amiot, M. Antisense strategy shows that Mcl-1 rather than Bcl-2 or Bcl-x(L) is an essential survival protein of human myeloma cells. Blood, *100*: 194-199, 2002.
26. Tu, Y., Xu, F. H., Liu, J., Vescio, R., Berenson, J., Fady, C., and Lichtenstein, A. Upregulated expression of BCL-2 in multiple myeloma cells induced by exposure to doxorubicin, etoposide, and hydrogen peroxide. Blood, *88*: 1805-1812, 1996.
27. Gazitt, Y., Fey, V., Thomas, C., and Alvarez, R. Bcl-2 overexpression is associated with resistance to dexamethasone, but not melphalan, in multiple myeloma cells. Int.J.Oncol., *13*: 397-405, 1998.
28. Hamilton, M. S., Barker, H. F., Ball, J., Drew, M., Abbot, S. D., and Franklin, I. M. Normal and neoplastic human plasma cells express bcl-2 antigen. Leukemia, *5*: 768-771, 1991.
29. Pettersson, M., Jernberg-Wiklund, H., Larsson, L. G., Sundstrom, C., Givol, I., Tsujimoto, Y., and Nilsson, K. Expression of the bcl-2 gene in human multiple myeloma cell lines and normal plasma cells. Blood, *79*: 495-502, 1992.
30. Sangfelt, O., Osterborg, A., Grander, D., Anderbring, E., Ost, A., Mellstedt, H., and Einhorn, S. Response to interferon therapy in patients with multiple myeloma correlates with expression of the Bcl-2 oncoprotein. Int.J.Cancer, *63*: 190-192, 1995.
31. Ong, F., van Nieuwkoop, J. A., Groot-Swings, G. M., Hermans, J., Harvey, M. S., Kluin, P. M., and Kluin-Nelemans, J. C. Bcl-2 protein expression is not related to short survival in multiple myeloma. Leukemia, *9*: 1282-1284, 1995.
32. Harada, N., Hata, H., Yoshida, M., Soniki, T., Nagasaki, A., Kuribayashi, N., Kimura, T., Matsuzaki, H., and Mitsuya, H. Expression of Bcl-2 family of proteins in fresh myeloma cells. Leukemia, *12*: 1817-1820, 1998.
33. Puthier, D., Pellat-Deceunynck, C., Barille, S., Robillard, N., Rapp, M. J., Juge-Morineau, N., Harousseau, J. L., Bataille, R., and Amiot, M. Differential expression of Bcl-2 in human plasma cell disorders according to proliferation status and malignancy. Leukemia, *13*: 289-294, 1999.
34. Renner, S., Weisz, J., Krajewski, S., Krajewska, M., Reed, J. C., and Lichtenstein, A. Expression of BAX in plasma cell dyscrasias. Clin.Cancer Res., *6*: 2371-2380, 2000.
35. Gross, A., McDonnell, J. M., and Korsmeyer, S. J. BCL-2 family members and the mitochondria in apoptosis. Genes Dev., *13*: 1899-1911, 1999.
36. Kroemer, G. and Reed, J. C. Mitochondrial control of cell death. Nat.Med., *6*: 513-519, 2000.
37. Schwarze, M. M. and Hawley, R. G. Prevention of myeloma cell apoptosis by ectopic bcl-2 expression or interleukin 6-mediated up-regulation of bcl-xL. Cancer Res., *55*: 2262-2265, 1995.
38. Gewirtz, A. M., Sokol, D. L., and Ratajczak, M. Z. Nucleic acid therapeutics: state of the art and future prospects. Blood, *92*: 712-736, 1998.
39. Campos, L., Sabido, O., Rouault, J. P., and Guyotat, D. Effects of BCL-2 antisense oligodeoxynucleotides on in vitro proliferation and survival of normal marrow progenitors and leukemic cells. Blood, *84*: 595-600, 1994.

40. Cotter, F. E., Johnson, P., Hall, P., Pocock, C., al Mahdi, N., Cowell, J. K., and Morgan, G. Antisense oligonucleotides suppress B-cell lymphoma growth in a SCID-hu mouse model. Oncogene, *9* : 3049-3055, 1994.
41. Pepper, C., Thomas, A., Hoy, T., Cotter, F., and Bentley, P. Antisense-mediated suppression of Bcl-2 highlights its pivotal role in failed apoptosis in B-cell chronic lymphocytic leukaemia. Br.J.Haematol., *107*: 611-615, 1999.
42. Miyake, H., Tolcher, A., and Gleave, M. E. Antisense Bcl-2 oligodeoxynucleotides inhibit progression to androgen-independence after castration in the Shionogi tumor model. Cancer Res., *59*: 4030-4034, 1999.
43. Schlagbauer-Wadl, H., Klosner, G., Heere-Ress, E., Waltering, S., Moll, I., Wolff, K., Pehamberger, H., and Jansen, B. Bcl-2 antisense oligonucleotides (G3139) inhibit Merkel cell carcinoma growth in SCID mice. J.Invest Dermatol., *114*: 725-730, 2000.
44. Guinness, M. E., Kenney, J. L., Reiss, M., and Lacy, J. Bcl-2 antisense oligodeoxynucleotide therapy of Epstein-Barr virus-associated lymphoproliferative disease in severe combined immunodeficient mice. Cancer Res., *60*: 5354-5358, 2000.
45. Tortora, G., Caputo, R., Damiano, V., Bianco, R., Fontanini, G., Cuccato, S., De Placido, S., Bianco, A. R., and Ciardiello, F. Combined blockade of protein kinase A and bcl-2 by antisense strategy induces apoptosis and inhibits tumor growth and angiogenesis. Clin.Cancer Res., *7*: 2537-2544, 2001.
46. Zangemeister-Wittke, U., Schenker, T., Luedke, G. H., and Stahel, R. A. Synergistic cytotoxicity of bcl-2 antisense oligodeoxynucleotides and etoposide, doxorubicin and cisplatin on small-cell lung cancer cell lines. Br.J.Cancer, *78*: 1035-1042, 1998.
47. Jansen, B., Schlagbauer-Wadl, H., Brown, B. D., Bryan, R. N., van Elsas, A., Muller, M., Wolff, K., Eichler, H. G., and Pehamberger, H. bcl-2 antisense therapy chemosensitizes human melanoma in SCID mice. Nat.Med., *4*: 232-234, 1998.
48. Gleave, M. E., Miayake, H., Goldie, J., Nelson, C., and Tolcher, A. Targeting bcl-2 gene to delay androgen-independent progression and enhance chemosensitivity in prostate cancer using antisense bcl-2 oligodeoxynucleotides. Urology, *54*: 36-46, 1999.
49. Konopleva, M., Tari, A. M., Estrov, Z., Harris, D., Xie, Z., Zhao, S., Lopez-Berestein, G., and Andreeff, M. Liposomal Bcl-2 antisense oligonucleotides enhance proliferation, sensitize acute myeloid leukemia to cytosine-arabinoside, and induce apoptosis independent of other antiapoptotic proteins. Blood, *95*: 3929-3938, 2000.
50. Klasa, R. J., Bally, M. B., Ng, R., Goldie, J. H., Gascoyne, R. D., and Wong, F. M. Eradication of human non-Hodgkin's lymphoma in SCID mice by BCL-2 antisense oligonucleotides combined with low-dose cyclophosphamide. Clin.Cancer Res., *6*: 2492-2500, 2000.
51. Lopes de Menezes, D. E., Hudon, N., McIntosh, N., and Mayer, L. D. Molecular and pharmacokinetic properties associated with the therapeutics of bcl-2 antisense oligonucleotide G3139 combined with free and liposomal doxorubicin. Clin.Cancer Res., *6*: 2891-2902, 2000.
52. Webb, A., Cunningham, D., Cotter, F., Clarke, P. A., di Stefano, F., Ross, P., Corbo, M., and Dziewanowska, Z. BCL-2 antisense therapy in patients with non-Hodgkin lymphoma. Lancet, *349*: 1137-1141, 1997.
53. Waters, J. S., Webb, A., Cunningham, D., Clarke, P. A., Raynaud, F., di Stefano, F., and Cotter, F. E. Phase I clinical and pharmacokinetic study of bcl-2 antisense oligonucleotide therapy in patients with non-Hodgkin's lymphoma. J.Clin.Oncol., *18*: 1812-1823, 2000.
54. Jansen, B., Wacheck, V., Heere-Ress, E., Schlagbauer-Wadl, H., Hoeller, C., Lucas, T., Hoermann, M., Hollenstein, U., Wolff, K., and Pehamberger, H. Chemosensitisation of malignant melanoma by BCL2 antisense therapy. Lancet, *356*: 1728-1733, 2000.
55. Jego, G., Robillard, N., Puthier, D., Amiot, M., Accard, F., Pineau, D., Harousseau, J. L., Bataille, R., and Pellat-Deceunynck, C. Reactive plasmacytoses are expansions of plasmablasts retaining the capacity to differentiate into plasma cells. Blood, *94*: 701-712, 1999.

56. Haigler, H. T., Maxfield, F. R., Willingham, M. C., and Pastan, I. Dansylcadaverine inhibits internalization of 125I-epidermal growth factor in BALB 3T3 cells. J.Biol.Chem., *255*: 1239-1241, 1980.
57. Roovers, D. J., van Vliet, M., Bloem, A. C., and Lokhorst, H. M. Idarubicin overcomes P-glycoprotein-related multidrug resistance: comparison with doxorubicin and daunorubicin in human multiple myeloma cell lines. Leuk.Res., *23*: 539-548, 1999.
58. van de Donk, N. W., Kamphuis, M. M., Lokhorst, H. M., and Bloem, A. C. The cholesterol lowering drug lovastatin induces cell death in myeloma plasma cells. Leukemia, *16*: 1362-1371, 2002.
59. Koziolkiewicz, M., Gendaszewska, E., Maszewska, M., Stein, C. A., and Stec, W. J. The mononucleotide-dependent, nonantisense mechanism of action of phosphodiester and phosphorothioate oligonucleotides depends upon the activity of an ecto-5'-nucleotidase. Blood, *98*: 995-1002, 2001.
60. Ballas, Z. K., Rasmussen, W. L., and Krieg, A. M. Induction of NK activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA. J.Immunol., *157*: 1840-1845, 1996.
61. Yi, A. K., Chang, M., Peckham, D. W., Krieg, A. M., and Ashman, R. F. CpG oligodeoxyribonucleotides rescue mature spleen B cells from spontaneous apoptosis and promote cell cycle entry. J.Immunol., *160*: 5898-5906, 1998.
62. Stein, C. A. Does antisense exist? Nat.Med., *1*: 1119-1121, 1995.
63. Beltinger, C., Saragovi, H. U., Smith, R. M., LeSauteur, L., Shah, N., DeDionisio, L., Christensen, L., Raible, A., Jarett, L., and Gewirtz, A. M. Binding, uptake, and intracellular trafficking of phosphorothioate-modified oligodeoxynucleotides. J.Clin.Invest, *95*: 1814-1823, 1995.
64. Durie, B. G. Staging and kinetics of multiple myeloma. Semin.Oncol., *13*: 300-309, 1986.

# Chapter 3

## G3139, a Bcl-2 antisense oligodeoxynucleotide, may overcome drug resistance in VAD refractory myeloma

Niels W.C.J. van de Donk, Okke de Weerdt, Gerda Veth, Marijke Eurelings, Esther van Stralen, Stanley R. Frankel, Anton Hagenbeek, Andries C. Bloem, and Henk M. Lokhorst

Submitted

## Abstract

Expression of Bcl-2 in multiple myeloma is associated with resistance to chemotherapeutic drugs. Conversely, suppression of Bcl-2 enhanced the chemosensitivity of myeloma cells *in vitro*. G3139 is an antisense oligodeoxynucleotide targeted to the first 6 codons of the Bcl-2 mRNA. In this study, G3139 was delivered as a continous intravenous infusion for 7 days at a fixed dose of 7 mg/kg/day in combination with VAD chemotherapy. Ten patients with refractory myeloma participated in this trial, including 8 patients with VAD refractory disease, 1 patient with thalidomide refractory disease, and 6 patients with disease refractory to thalidomide/dexamethasone. The combination of G3139 and VAD was feasible and well tolerated. Seven patients (70%) responded including 4 patients (40%) with a partial response and 3 patients (30%) with a minor response. Baseline Bcl-2 protein levels in bone marrow myeloma cells, Ki-67 growth fraction, and presence of deletion of chromosome 13 were not predictive of response. Median progression free survival was 6 months (range, 2 to 7+ months) and median overall survival has not been reached. G3139 down-regulated Bcl-2 protein levels in peripheral blood circulating myeloma cells, B cells, T cells, and monocytes. There was a trend towards correlation between decrease in circulating B and T cells and reduction of Bcl-2 protein levels in these cells. These results indicate that G3139 may overcome classical resistance and restore sensitivity of myeloma tumor cells to VAD chemotherapy.

## Introduction

Multiple myeloma is characterized by the accumulation of monoclonal plasma cells in the bone marrow. The emergence of drug-resistant disease is the primary cause of treatment failure in myeloma. Well established mechanisms of drug resistance for myeloma tumor cells *in vitro* are mutations or altered expression of target genes [1-4], increased drug metabolism [5], changes in drug accumulation [6-11], and increased repair of drug-induced damage [12]. Recent studies have demonstrated that defects in apoptotic pathways contribute significantly to resistance of cancer cells to chemotherapeutic drugs [13-15]. Tumor cells, which are unable to undergo programmed cell death are therefore resistant to chemotherapeutic agents.

Bcl-2 is a potent enhancer of cell viability through the inhibition of apoptotic death in various cell lines. Bcl-2 is expressed in myeloma cell lines and in myeloma patients' samples despite the absence of molecular evidence of Bcl-2 rearrangements [16-22]. Bcl-2 inhibits apoptosis by forming inactivating heterodimers with pro-apoptotic Bcl-2 family proteins, and by preventing the collapse of the mitochondrial transmembrane potential, cytochrome c release from mitochondria into the cytosol, and caspase activation [23-25]. Overexpression of Bcl-2 in transgenic mice prolongs the life span of plasma cells and in combination with abnormal expression of other oncogenes such as c-myc, contributes to the development of immature pre-B cell lymphoma, large cell lymphoma or plasmacytoma in 40-60% of the mice [26,27]. This suggests a role for Bcl-2 in the etiology of myeloma. Gene transfer-mediated overexpression of Bcl-2 in myeloma cells has been reported to block the process of chemotherapy-induced apoptosis [28,29]. In addition, Bcl-2 protein levels increased following exposure of cell lines to doxorubicin, which may account for the relative resistance to a second doxorubicin exposure [28]. We and others have shown that specific down-regulation of Bcl-2 protein by Bcl-2 antisense oligodeoxynucleotides (ODNs) restored chemosensitivity in chemoresistant myeloma cell lines and in primary myeloma cells from patients [30-32].

G3139 is a 18-mer fully phosphorothioated antisense ODN targeted to the first 6 codons of the Bcl-2 mRNA. Preclinical studies showed that G3139 was effectively taken up by myeloma cells, decreased Bcl-2 mRNA levels and protein levels, and sensitized myeloma cells to chemotherapeutic agents including dexamethasone and doxorubicin [31,32]. Furthermore, G3139 as a single agent has resulted in tumor regression in patients with relapsed non-Hodgkin's lymphoma (NHL) [33,34], and studies in patients with metastatic melanoma [35], chemorefractory small-cell lung cancer [36], metastatic hormone-refractory prostate cancer [37], and in refractory/relapsed acute leukemia [38] showed that G3139 can be administered safely in combination with chemotherapy. Based upon these data we initiated a phase 2 study of G3139 in combination with vincristine, adriamycin, and

dexamethasone (VAD) in patients with heavily pretreated multiple myeloma including patients with disease resistant to VAD, thalidomide, and other chemotherapy regimens.

## Patients, materials, and methods

### Criteria for enrollment

Patients up to 75 years of age with multiple myeloma refractory to or relapsing after completion of at least 2 lines of chemotherapy which included VAD chemotherapy and with satisfactory venous access for 7-day infusion were eligible for the study. Criteria for exclusion were World Health Organization (WHO) performance status 4; severe cardiac, pulmonary, neurologic, metabolic, or psychiatric disease; absolute neutrophil count $<1000/mm^3$ and platelets $<50000/mm^3$; need for transfusion support; inadequate liver function; creatinine clearance <40 ml/min; prior malignant disease except nonmelanoma skin tumors or stage 0 cervical carcinoma; therapy for myeloma within 3 weeks prior to start G3139 in combination with VAD; active uncontrolled infections; known hypersensitivity to phophorothiate-containing oligodeoxynucleotides, dexamethasone, or doxorubicin; and use of any investigational therapy within 4 weeks prior to registration. Approval was obtained from the University Medical Center Utrecht institutional review board for these studies (01/227-E). Written informed consent was obtained from all patients before inclusion. The study was performed according to the Helsinki agreement.

### Study protocol

G3139 (Genasense, oblimersen sodium; Genta, Inc., Berkeley Heights, NJ, USA; 5'-TCT CCC AGC GTG CGC CAT-3') was delivered as a continous intravenous infusion through a separate line using a portable pump on days 1 to 7 at a dose of 7 mg/kg/day. Infusion sites were changed when early signs of inflammation were observed. VAD was started at day 4 of the G3139 infusion. The VAD regimen consisted of daily doses of 0.4 mg vincristine and 9 $mg/m^2$ doxorubicin on days 4 to 7 and were administered by rapid intravenous infusion as described previously [39]. Dexamethasone (40 mg) was given orally on days 4 to 7 on even cycles and on days 4 to 7, 12 to 15, and 20 to 23 on odd cycles of VAD. The treatment cycles were repeated at 4-week intervals. Three courses of treatment were planned per patient, but additional courses could be administered in the case of response. Antibacterial and anti-fungal prophylaxis was given according to local guidelines. Adverse events were graded according to the National Cancer Institute (NCI) Common Toxicity Criteria (CTC) version 2.0 (http://ctep.info.nih.gov).

**Evaluation of response**

Response evaluation was performed according to the Blade criteria [40]. Partial response (PR) was defined as 50% or more reduction of monoclonal immunoglobulins (M-protein) in serum and/or urine or more than 50% reduction of bone marrow infiltration in nonsecretory myeloma. Minor response (MR) was defined as 25 to 50% reduction of M-protein in serum and/or urine or 25 to 50% reduction of bone marrow infiltration in nonsecretory myeloma. Complete response (CR) was defined as no M-protein measurable in serum and/or 10 times concentrated urine by immunofixation analysis and less than 5% plasma cells which had to be polyclonal by immunofluorescence staining. Patients with a reduction of less than 25% of M-protein in serum and/or urine or of bone marrow infiltration were considered to be refractory. Relapse from CR was defined as recurrence of M-protein in serum and/or urine measured by immunofixation on at least 2 occasions. Progression from PR or MR was defined as an increase in serum and/or urine M-protein levels or bone marrow infiltration by more than 25% on 2 consecutive measurements or any increase of M-protein in the presence of clinical evidence of disease progression.

**Immuno-FISH analysis**

To detect a (partial) deletion of chromosome 13, FISH analysis using the commercially available SpectrumOrange-conjugated probe LSI13 (Rb1) (Vysis, Downers Grove, IL, USA) was performed according to the manufacturer's guidelines with minor modifications and combined with immunological staining of the cytoplasmatic Ig light chains to positively identify plasma cells as described previously [41]. Signals of the probe were detected by standard procedures. We intended to analyze a total of 100 plasma cells in each patient. A patient was considered as having deletion of chromosome 13 when the percentage of plasma cells with a deletion exceeded 20%.

**Quantification of Bcl-2 by flow cytometry**

Bcl-2 and Bax protein levels were determined in myeloma plasma cells ($CD38^+$ $CD138^+$ $CD45^{\pm}$) in bone marrow prior to start of G3139 treatment and in peripheral blood circulating myeloma cells ($CD38^+$, $CD138^+$, $CD45^{\pm}$), $CD3^+$ cells, $CD19^+$ cells, and $CD14^+$ cells at day 0, 4, and 7 of the treatment cycle by flow cytometry. Peripheral blood or bone marrow mononuclear cells, which were obtained by Ficoll-Paque (Amersham; Pharmacia BiotechAB, Uppsala, Sweden) density centrifugation, ($1 \times 10^6$) were fixed and permeabilized using FACS Lysing Solution (FLS, 10% in aqua destillata; Becton Dickinson, Erembodegem, Belgium (BDIS)) and subsequently incubated with anti-Bcl-2-FITC (IgG1, Dako, Glostrup, Denmark) antibody or the isotype and subclass matched control antibody. All incubations were performed at room temperature for 30 minutes. Cells were then incubated with mouse serum for 15 minutes at room temperature, and subsequently incubated with CD3-PE (BDIS), CD14-APC (BDIS), and CD19-Percp Cy5.5 (BDIS) or with CD38-APC (BDIS), CD138-PE (Immunotech, Marseille , France),

and CD45-PERCP (BDIS) for 15 minutes at room temperature, and analyzed on a FACSCalibur (BDIS). Between different steps the cells were washed with PBS. The mean fluorescence ratio (MFR), defined as the ratio of the mean fluorescence intensities (MFI) of primary antibody and isotype control stained cells was used as a measure for Bcl-2 expression.

**Flow cytometric analysis of lymphocyte subsets**

Lymphocyte populations were analyzed using four-colour flow cytometry at day 0, 4, and 7 of each treatment cycle. Peripheral blood, collected in sodium heparine, was stained at room temperature with a panel of antibodies, lysed using FACS Lysing Solution (BDIS), washed with PBS, and analyzed on a FACSCalibur (BDIS). The following antibody combinations were used to analyze the lymphocyte populations: CD3, CD16+56, CD45, CD19, and CD8, CD4, CD45, CD3. CD3, CD4, CD8, CD19, and CD45 were obtained from BDIS and CD16+56 was derived from Immunotech. B cells were defined as $CD3^-$ $CD19^+$, natural killer cells as $CD3^-$ $CD16^+/CD56^+$, CD8 cells as $CD3^+$ $CD8^+$, and CD4 cells as $CD3^+$ $CD4^+$. Absolute counts of a certain lymphocyte population were calculated by multiplying the total white blood cell (WBC) count by the frequency of that population in the lymphocyte gate and by the fraction of total WBCs included in the lymphocyte gate.

**Statistical analysis**

Data analysis was performed using the SPSS statistical software package (SPSS, Chicago, IL, USA). Correlations were calculated by using the Spearmann test. The Mann-Whitney U-test was used to determine differences in case of continuous variables. The Fisher's exact test for two by two tables was used to determine differences in case of categorial variables. Correlations were considered significant when $P < 0.05$. Progression free and overall survival were computed by Kaplan Meier survival analysis.

## Results

**Patient characteristics**

A total of 10 patients were enrolled in this study. Clinical characteristics are shown in Table 1. There were 5 males and 5 females. Median age was 56 years, with a range of 39 to 64 years. All patients were heavily pretreated and had received a median of 4 previous chemotherapy regimens (range, 2 to 6). Eight patients (80%) had VAD refractory disease, 6 (60%) had thalidomide/dexamethasone refractory disease, and 1 patient (10%) had thalidomide refractory disease. Five patients had received high-dose chemotherapy with autologous hematopoietic stem-cell support, and one of these patients received a second cycle of high-dose chemotherapy for relapse. Two patients had received an allograft. The

median number of cycles of G3139 and VAD was 2.5. Five patients received 2 cycles of treatment, and went off study because of progression during treatment or because of infection (patient 10). Three cycles were administered to 3 patients. Two patients received 4 or 6 treatment cycles. A total of 29 cycles of G3139 and VAD have been administered.

**Table 1.** Clinical characteristics

| **Characteristic** | |
|---|---|
| Total number of patients enrolled | 10 |
| Gender | |
| Male | 5 |
| Female | 5 |
| Age (years) | |
| Median | 56 |
| Range | 39-64 |
| Deletion of chromosome 13 (%) | 77.8 |
| Ki-67 (%) | |
| Median | 2 |
| Range | 0-70 |
| Number of prior chemotherapy regimens | |
| 2 | 1 |
| 3 | 1 |
| 4 | 4 |
| 5 | 2 |
| 6 | 2 |
| Number of cycles completed | |
| 2 | 5 |
| 3 | 3 |
| 4 | 1 |
| 6 | 1 |

**Toxicity**

At the start of treatment, anemia was present in 8 patients (6 grade 1, and 2 grade 2), leucopenia was present in 4 (1 grade 1, 3 grade 2), neutropenia in 4 (1 grade 1, 2 grade 2, 1 grade 3), lymphopenia in 2 (1 grade 1, 1 grade 2), and grade 1 thrombocytopenia in 2 patients. Hematological toxicity during the first cycle and during all cycles is shown in Table 2.

**Table 2.** Hematological toxicity in patients treated with G3139 and VAD

| No. of patients with event of common toxicity criteria during first cycle (all cycles; n=29) | | | | |
|---|---|---|---|---|
| Toxicity | Grade 1 | Grade 2 | Grade 3 | Grade 4 |
| Leucopenia | 0 (6) | 1 (4) | 1 (2) | 1 (3) |
| Neutropenia | 0 (3) | 1 (1) | 1 (3) | 1 (3) |
| Lymphopenia | 1 (2) | 1 (1) | 3 (8) | 1 (2) |
| Thrombocytopenia | 3 (7) | 1 (1) | 0 (4) | 1 (2) |
| Anemia | 7 (17) | 2 (6) | 0 (1) | 0 |

Non-hematological adverse events included fatigue in 5 patients (grade 1 in 3 patients, and grade 2 in 2 patients) (Table 3). Nine patients had a grade 1 local inflammatory reaction around the infusion site. One patient (patient 5) developed skin and subcutaneous tissue necrosis (grade 3) during administration of G3139 before start of chemotherapy. This was likely the consequence of extravasation of G3139 at cycle 1. The local skin inflammation around the infusion site required change of the infusion site every 2 or 3 days in all patients. Furthermore, 3 patients (5, 9, 10) underwent placement of a central venous catheter. One of these patients (patient 5) developed a catheter-related venous thrombosis and patient 10 had a catheter-related infection. These events were likely related to G3139 and VAD treatment. Patients 4 and 10 developed a grade 3 infection.

**Table 3.** Non-hematological toxicity in patients treated with G3139 and VAD

| No. of patients with event of common toxicity criteria during first cycle (all cycles; n=29) | | | | |
|---|---|---|---|---|
| Toxicity | Grade 1 | Grade 2 | Grade 3 | Grade 4 |
| Fatigue | 3 (13) | 2 (4) | 0 | 0 |
| Thrombosis | 0 | 0 | 0 (1) | 0 |
| Catheter-related infection | 0 | 0 | 0 (1) | 0 |
| Infection | 0 | 0 | 0 (2) | 0 |
| Renal | 2 (5) | 0 | 0 | 0 |
| Injection site reaction | 9 (23) | 0 | 1 (1) | 0 |

**Disease response**

Four patients (40%) achieved a partial response and 3 patients (30%) achieved a minor response, indicating that 70% of the patients responded to the combination of G3139 and VAD (Table 4). Of the 7 responders, 5 had VAD refractory disease (Table 4). This group of 5 included 3 patients who sequentially received VAD alone followed by the combination of G3139 and VAD (patients 5, 9, and 10). In addition, 3 of the 7 responding patients had thalidomide/dexamethasone refractory disease and 1 had thalidomide refractory disease. Patient 2 had VAD and thalidomide/dexamethasone refractory disease and obtained only a minor response after allogeneic stem cell transplantation, but achieved a partial response on the current protocol (Table 4). Three patients did not respond. Deletion of chromosome 13 (del13) was present in 7 out 9 evaluable patients (77.8%). Del13 was present in 5 out 6 responding and in 2 out 3 non-responding patients. The Ki-67 growth fraction was not predictive of response. Among the responding patients, 5 had anemia at base line. Hemoglobin levels increased in 3 patients, decreased in 1 patient, and remained unchanged in 1 patient. Responses were also associated with increases in Karnofsky performance-status scores in 4 (57%) patients and improvement of pain in 7 (100%) patients. Four out 7 responding patients relapsed, whereas response persists in 3 patients. Median progression free survival of these patients was 6 months (range, 2 to more than 7 months) without any further therapy before relapse occurred.

Three patients had died of progressive disease. The median overall survival for all patients to date exceeds 12 months (range, 3 to more than 16 months). Although not significant, achievement of response was associated with longer overall survival when compared to non-responding patients ($P = 0.17$).

**Table 4.** Previous treatment and outcome of G3139 combined with VAD

| Patient | Cycles completed | Previous therapy and outcome of G3139+VAD therapy |
|---|---|---|
| 1 | 2 | VAD *PR*---IDM *refr*---VAD *refr*---CVP *refr*---Thal+dexa *refr*---G3139+VAD *refr* |
| 2 | 3 | VAD *refr*---Thal+dexa *refr*—alloSCT *MR*---G3139+VAD *PR* |
| 3 | 6 | VAD *refr*---IDM *refr*---autoSCT *PR*---Thal+dexa *refr*---G3139+VAD *PR* |
| 4 | 2 | VAD *PR*---IDM *PR*---autoSCT *PR*---Thal+dexa *refr*---G3139+VAD *MR* |
| 5 | 3 | VAD *refr*---IDM *PR*---autoSCT *CR*---VAD *refr*---G3139+VAD *PR* |
| 6 | 2 | VAD *refr*---IDM *PR*---TAD *PR*---autoSCT *PR*---Thal+dexa *refr*---G3139+VAD *refr* |
| 7 | 4 | VAD *PR*---IDM *PR*---autoSCT *CR*---VAD *PR*---autoSCT *PR*---Thal *refr*---G3139+VAD *PR* |
| 8 | 2 | VAD *PR*---IDM *CR*---VAD *refr*---Thal+dexa *refr*---G3139+VAD *refr* |
| 9 | 3 | VAD *PR*---IDM *PR*---alloSCT *PR*---TAD *PR*---DLI *refr*---VAD *refr*---G3139+VAD *MR* |
| 10 | 2 | MP *refr*---VAD *refr*---G3139+VAD *MR* |

VAD, vincristine, doxorubicin, and dexamethasone; IDM, intermediate-dose melphalan; CVP, cyclophosphamide, vincristine, and prednisone; Thal, thalidomide; dexa, dexamethasone; alloSCT, allogeneic stem cell transplantation; autoSCT, autologous stem cell transplantation; TAD, thalidomide, doxorubicin, and dexamethasone; DLI, donor lymphocyte infusion; MP, melphalan and prednisone; MR, minor response; PR, partial response; CR, complete response; refr, refractory.

**Bcl-2 protein levels in bone marrow myeloma cells**

In 9 patients, Bcl-2 was determined in bone marrow myeloma cells by flow cytometry prior to start of therapy. No material for analysis was available from patient 2. Bcl-2 protein levels, expressed as mean fluorescence ratio, varied among patients (median 12.2; range: 2.4 to 27.4). No association between Bcl-2 protein levels and response was observed.

**Effect of treatment on Bcl-2 protein levels in peripheral blood circulating myeloma cells, B cells, T cells, and monocytes**

In peripheral blood from patients 3, 4, 5, 6, 9, and 10 more than 0.01% circulating myeloma cells were detected (range: 0.01 to 4.0%), enabling analysis of Bcl-2 protein levels in these cells. In the other patients the level of peripheral blood infiltration was too low, to reliably quantify Bcl-2 protein expression. Bcl-2 protein expression was determined by flow cytometry at day 0 before treatment and at day 4 of G3139 infusion prior to initiation of VAD. After 4 days of G3139 treatment Bcl-2 protein levels in circulating myeloma cells were reduced in 4 out 6 patients (median change: -13.3%) (Table 5). Bcl-2 protein expression was also determined by flow cytometry in peripheral blood T cells ($CD3^+$), B cells ($CD19^+$), and monocytes ($CD14^+$). This analysis was performed during cycle 1 (patients 1, 2, 6, 7, 9, and 10) or cycle 2 (patients 3, 5, and 8). Treatment for 4 days with G3139 resulted in a reduction of Bcl-2 protein levels in B cells in 9 out 9 patients when compared with baseline values (median change: -25.0%). In monocytes, Bcl-2 protein levels were reduced in 8 out 9 patients on day 4 (median

change: -21.8%). Bcl-2 protein levels were reduced in T cells in 6 out 9 patients on day 4 (median change: -16.4%) (Table 5). There was no significant association between Bcl-2 protein down-regulation in peripheral blood circulating myeloma cells, B cells, T cells, or monocytes and response to G3139 in combination with VAD chemotherapy.

**Table 5.** Effect of G3139 on Bcl-2 protein levels at day 4 (presented as the change from baseline (day 0) in %)

| Patient | B cells | T cells | Monocytes | Myeloma cells |
|---|---|---|---|---|
| 1 | -16.3 | 13.8 | -21.8 | |
| 2 | -3.6 | -5.1 | -11.1 | |
| 3 | -62.8 | -19.3 | -8.3 | 5.5 |
| 4 | | | | 1.9 |
| 5 | -27.6 | -16.4 | -22.2 | -19.7 |
| 6 | -25.0 | -25.1 | -25.0 | -25.6 |
| 7 | -5.3 | 2.0 | -19.5 | |
| 8 | -14.8 | 15.5 | 15.7 | |
| 9 | -33.6 | -18.0 | -27.7 | -26.4 |
| 10 | -37.4 | -19.3 | -47.3 | -6.9 |

**Effect of treatment on circulating leucocytes and thrombocytes**

In 8 patients, the effect of treatment on numbers of circulating lymphocytes, monocytes, granulocytes, and thrombocytes in peripheral blood was determined on day 0, 4, 7, and 28 of cycle 1 (patients 2, 4, 6, 7, 9, and 10) or cycle 2 (patients 3 and 8) (Table 6). The largest effect was observed in B cells, which showed mean reductions of 50.9% on day 4 and 36.5% on day 7, when compared with baseline numbers. There was a minor reduction of T cells on day 4 and a more profound reduction on day 7. This was due to a decrease of both $CD4^+$ and $CD8^+$ T cells. NK cells either increased (n=5) or decreased (n=3) after 4 days of G3139 treatment. However, on day 7 natural killer (NK) cells were reduced in all patients. Both on day 4 and 7, a reduction in monocytes was observed. G3139 did not affect granulocytes on day 4, but on day 7 there was a significant increase in the number of granulocytes, which was probably related to administration of dexamethasone. Thrombocytes were reduced on day 4 and day 7. T cells (CD4 and CD8) and monocytes returned to baseline or near baseline values on day 28 prior to start of the following course of G3139 and VAD. However, B cells and thrombocytes did not fully recover, and NK cells and granulocytes were increased on day 28. Although not significant, there was a trend for correlation between Bcl-2 protein reduction in B or T cells and reduction of B cells (day 4: R=0.643; $P = 0.119$, day 7: R=0.657; $P = 0.156$) or T cells (day 4: R=0.739; $P = 0.058$, day 7: R=0.771; $P = 0.072$). No association was observed between Bcl-2 down-regulation in monocytes and reduction of monocytes.

**Table 6.** Effect of G3139 combined with VAD on numbers of circulating leucocytes and thrombocytes (presented as the change from baseline in %)

| | Day 4 | | Day 7 | | Day 28 | |
|---|---|---|---|---|---|---|
| **Cell type** | **Mean (%)** | **Range (%)** | **Mean (%)** | **Range (%)** | **Mean (%)** | **Range (%)** |
| **B cells** | -50.9 | -90 to -1.1 | -36.5 | -95.8 to 3.4 | -32.8 | -88.8 to 60.0 |
| **T cells** | -20.4 | -78.5 to 54.1 | -43.4 | -78.9 to 36.8 | -7.5 | -55.3 to 63.2 |
| **$CD4^+$ T cells** | -11.6 | -65.3 to 66.1 | -45.4 | -84.2 to 31.3 | -1.1 | -59.8 to 107.1 |
| **$CD8^+$ T cells** | -21.5 | -95.9 to 49.6 | -37.4 | -71.6 to 47.6 | 5.4 | -54.2 to 71.2 |
| **NK cells** | 3.3 | -51.3 to 54.4 | -40.3 | -62.5 to -13.2 | 22.8 | -65.0 to 100.0 |
| **Granulocytes** | -5.3 | -50.0 to 26.4 | 172.5 | 4.1 to 293.1 | 55.7 | 0.0 to 122.4 |
| **Monocytes** | -27.1 | -67.7 to 72.2 | -28.9 | -45.2 to -12.3 | 6.3 | -20.9 to 43.1 |
| **Thrombocytes** | -14.9 | -26.8 to 2.6 | -20.5 | -38.9 to 3.6 | -19.3 | -75.9 to 1.6 |

## Discussion

In this study, we evaluated the effect of Bcl-2 antisense in combination with VAD chemotherapy in heavily pretreated multiple myeloma patients who failed previous therapy. G3139 was administered as a continuous intravenous infusion on days 1 to 7 at a dose of 7 mg/kg/day. VAD was started at day 4 of the G3139 infusion. The sequential order was based on preclinical data indicating that in purified myeloma cells the maximum decrease in Bcl-2 protein expression was typically observed after 4 days of treatment with G3139 [31]. In this study, 7 of the 10 patients (70%) responded including 4 patients (40%) with a partial response and 3 (30%) with a minor response. Of the 7 responders 5 had VAD refractory disease. This group of 5 included 3 patients who sequentially received VAD followed by the combination of G3139 and VAD. Responses were associated with increased hemoglobin levels and performance status, and improvements in disease symptoms including pain. These data indicate that Bcl-2 antisense may overcome classical drug resistance and restore the sensitivity of myeloma tumor cells to VAD chemotherapy. Median progression free survival was 6 months, which is usual for this category of patients [42,43], and the median overall survival has not been reached. Response did not appear to be correlated with Ki-67 growth fraction or Bcl-2 protein levels in bone marrow myeloma tumor cells at baseline. Furthermore, it is remarkable that responsiveness to G3139 in combination with VAD chemotherapy did not correlate with the presence of deletion of chromosome 13, which is a strong unfavourable prognostic factor in myeloma.

This study showed that G3139 and VAD could be administered safely in heavily pretreated myeloma patients. Also in patients with refractory/relapsed acute leukemia [38], metastatic melanoma [35], chemorefractory small-cell lung cancer [36], or metastatic hormone-

refractory prostate cancer [37], the combination of G3139 with chemotherapy was feasible. In the current study, the clinically most significant adverse event of G3139 was a local inflammatory reaction around the infusion site. This was also observed in NHL patients who were treated with a continuous subcutaneous infusion of G3139 [33,34]. Hematological toxicity may be primarily attributable to VAD chemotherapy and was predominantly observed in patients with cytopenias at baseline. Patient 8 developed grade 2 anemia, grade 3 leucopenia, neutropenia, and thrombopenia, and grade 4 lymphopenia, which were probably due to advanced infiltration of bone marrow. Thrombocytopenia and fatigue were observed during G3139 infusion that precedes VAD chemotherapy and are adverse events that were also reported in trials evaluating other phosphorothioate oligodeoxynucleotides [44,45], suggesting that these effects resulted from non-sequence specific effects and can be attributed to the phosphorothioate backbone of the oligodeoxynucleotide molecule.

G3139 treatment for 4 days moderately reduced Bcl-2 protein levels in peripheral blood circulating myeloma cells, B cells, T cells, and monocytes in the majority of the patients. Similar moderate reductions of Bcl-2 were found in other studies evaluating G3139 [34,35,38]. No significant association was found between Bcl-2 down-regulation in peripheral blood myeloma cells, B cells, T cells, or monocytes and response to G3139 in combination with VAD. G3139 administration alone preceding chemotherapy resulted in a transient reduction of absolute numbers of circulating lymphocytes, monocytes, and thrombocytes, but did not alter the number of circulating neutrophils or NK cells. We further show for the first time that the decline in circulating lymphocytes is due to reduction of B cells and to a lesser extent of T cells (CD4 and CD8). After completion of the treatment course there was a full recovery of the number of circulating T cells and monocytes and a partial recovery of platelets and B cells. This is in agreement with studies indicating that Bcl-2 is not essential for survival of pluripotent hematopoietic stem cells [46], and therefore Bcl-2 down-regulation by G3139 treatment will not result in a loss of renewal potential for normal hematopoietic cells. The transient reduction of B cells and T cells by G3139 is likely related to a sequence-specific Bcl-2 down-regulation in these cells. First, survival of mature B and T (CD4 and CD8) cells has been shown to be critically dependent on Bcl-2 protein expression [46]. Furthermore, there was a trend towards a correlation between down-regulation of Bcl-2 protein in B and T cells and decline of circulating lymphocytes. Moreover, although lymphopenia was also reported in other studies evaluating G3139 [34,35,37], there have been no reports of lymphopenia in clinical trials of other phosphorothioate antisense molecules developed to inhibit other targets [44,45].

In conclusion, G3139 induced significant clinical responses when combined with VAD chemotherapy in patients with (VAD) refractory disease. The combination of Bcl-2 antisense and VAD chemotherapy was feasible and well tolerated. The results of this trial warrant exploration of Bcl-2 antisense in earlier-stage disease.

## References


1. Moalli, P. A., Pillay, S., Weiner, D., Leikin, R., and Rosen, S. T. A mechanism of resistance to glucocorticoids in multiple myeloma: transient expression of a truncated glucocorticoid receptor mRNA. Blood, *79*: 213-222, 1992.
2. Ishikawa, H., Kawano, M. M., Okada, K., Tanaka, H., Tanabe, O., Sakai, A., Asaoku, H., Iwato, K., Nobuyoshi, M., and Kuramoto, A. Expressions of DNA topoisomerase I and II gene and the genes possibly related to drug resistance in human myeloma cells. Br.J Haematol., *83*: 68-74, 1993.
3. Krett, N. L., Pillay, S., Moalli, P. A., Greipp, P. R., and Rosen, S. T. A variant glucocorticoid receptor messenger RNA is expressed in multiple myeloma patients. Cancer Res., *55*: 2727-2729, 1995.
4. Valkov, N. I. and Sullivan, D. M. Drug resistance to DNA topoisomerase I and II inhibitors in human leukemia, lymphoma, and multiple myeloma. Semin.Hematol, *34*: 48-62, 1997.
5. Linsenmeyer, M. E., Jefferson, S., Wolf, M., Matthews, J. P., Board, P. G., and Woodcock, D. M. Levels of expression of the mdr1 gene and glutathione S-transferase genes 2 and 3 and response to chemotherapy in multiple myeloma. Br.J.Cancer, *65*: 471-475, 1992.
6. Dalton, W. S., Grogan, T. M., Meltzer, P. S., Scheper, R. J., Durie, B. G., Taylor, C. W., Miller, T. P., and Salmon, S. E. Drug-resistance in multiple myeloma and non-Hodgkin's lymphoma: detection of P-glycoprotein and potential circumvention by addition of verapamil to chemotherapy. J Clin.Oncol., *7*: 415-424, 1989.
7. Epstein, J., Xiao, H. Q., and Oba, B. K. P-glycoprotein expression in plasma-cell myeloma is associated with resistance to VAD. Blood, *74*: 913-917, 1989.
8. Sonneveld, P., Durie, B. G., Lokhorst, H. M., Marie, J. P., Solbu, G., Suciu, S., Zittoun, R., Lowenberg, B., and Nooter, K. Modulation of multidrug-resistant multiple myeloma by cyclosporin. The Leukaemia Group of the EORTC and the HOVON. Lancet, *340*: 255-259, 1992.
9. Grogan, T. M., Spier, C. M., Salmon, S. E., Matzner, M., Rybski, J., Weinstein, R. S., Scheper, R. J., and Dalton, W. S. P-glycoprotein expression in human plasma cell myeloma: correlation with prior chemotherapy. Blood, *81*: 490-495, 1993.
10. Raaijmakers, H. G., Izquierdo, M. A., Lokhorst, H. M., de Leeuw, C., Belien, J. A., Bloem, A. C., Dekker, A. W., Scheper, R. J., and Sonneveld, P. Lung-resistance-related protein expression is a negative predictive factor for response to conventional low but not to intensified dose alkylating chemotherapy in multiple myeloma. Blood, *91*: 1029-1036, 1998.
11. Filipits, M., Drach, J., Pohl, G., Schuster, J., Stranzl, T., Ackermann, J., Konigsberg, R., Kaufmann, H., Gisslinger, H., Huber, H., Ludwig, H., and Pirker, R. Expression of the lung resistance protein predicts poor outcome in patients with multiple myeloma. Clin.Cancer Res., *5*: 2426-2430, 1999.
12. Spanswick, V. J., Craddock, C., Sekhar, M., Mahendra, P., Shankaranarayana, P., Hughes, R. G., Hochhauser, D., and Hartley, J. A. Repair of DNA interstrand crosslinks as a mechanism of clinical resistance to melphalan in multiple myeloma. Blood, *100*: 224-229, 2002.
13. Solary, E., Droin, N., Bettaieb, A., Corcos, L., Dimanche-Boitrel, M. T., and Garrido, C. Positive and negative regulation of apoptotic pathways by cytotoxic agents in hematological malignancies. Leukemia, *14*: 1833-1849, 2000.
14. O'Gorman, D. M. and Cotter, T. G. Molecular signals in anti-apoptotic survival pathways. Leukemia, *15*: 21-34, 2001.
15. Johnstone, R. W., Ruefli, A. A., and Lowe, S. W. Apoptosis: a link between cancer genetics and chemotherapy. Cell, *108*: 153-164, 2002.
16. Hamilton, M. S., Barker, H. F., Ball, J., Drew, M., Abbot, S. D., and Franklin, I. M. Normal and neoplastic human plasma cells express bcl-2 antigen. Leukemia, *5*: 768-771, 1991.

17. Pettersson, M., Jernberg-Wiklund, H., Larsson, L. G., Sundstrom, C., Givol, I., Tsujimoto, Y., and Nilsson, K. Expression of the bcl-2 gene in human multiple myeloma cell lines and normal plasma cells. Blood, *79*: 495-502, 1992.
18. Sangfelt, O., Osterborg, A., Grander, D., Anderbring, E., Ost, A., Mellstedt, H., and Einhorn, S. Response to interferon therapy in patients with multiple myeloma correlates with expression of the Bcl-2 oncoprotein. Int.J.Cancer, *63*: 190-192, 1995.
19. Ong, F., van Nieuwkoop, J. A., Groot-Swings, G. M., Hermans, J., Harvey, M. S., Kluin, P. M., and Kluin-Nelemans, J. C. Bcl-2 protein expression is not related to short survival in multiple myeloma. Leukemia, *9*: 1282-1284, 1995.
20. Harada, N., Hata, H., Yoshida, M., Soniki, T., Nagasaki, A., Kuribayashi, N., Kimura, T., Matsuzaki, H., and Mitsuya, H. Expression of Bcl-2 family of proteins in fresh myeloma cells. Leukemia, *12*: 1817-1820, 1998.
21. Puthier, D., Pellat-Deceunynck, C., Barille, S., Robillard, N., Rapp, M. J., Juge-Morineau, N., Harousseau, J. L., Bataille, R., and Amiot, M. Differential expression of Bcl-2 in human plasma cell disorders according to proliferation status and malignancy. Leukemia, *13*: 289-294, 1999.
22. Renner, S., Weisz, J., Krajewski, S., Krajewska, M., Reed, J. C., and Lichtenstein, A. Expression of BAX in plasma cell dyscrasias. Clin.Cancer Res., *6*: 2371-2380, 2000.
23. Gross, A., McDonnell, J. M., and Korsmeyer, S. J. BCL-2 family members and the mitochondria in apoptosis. Genes Dev., *13*: 1899-1911, 1999.
24. Kroemer, G. and Reed, J. C. Mitochondrial control of cell death. Nat.Med, *6*: 513-519, 2000.
25. Debatin, K. M., Poncet, D., and Kroemer, G. Chemotherapy: targeting the mitochondrial cell death pathway. Oncogene, *21*: 8786-8803, 2002.
26. McDonnell, T. J. and Korsmeyer, S. J. Progression from lymphoid hyperplasia to high-grade malignant lymphoma in mice transgenic for the t(14; 18). Nature, *349*: 254-256, 1991.
27. Strasser, A., Harris, A. W., and Cory, S. E mu-bcl-2 transgene facilitates spontaneous transformation of early pre-B and immunoglobulin-secreting cells but not T cells. Oncogene, *8*: 1-9, 1993.
28. Tu, Y., Xu, F. H., Liu, J., Vescio, R., Berenson, J., Fady, C., and Lichtenstein, A. Upregulated expression of BCL-2 in multiple myeloma cells induced by exposure to doxorubicin, etoposide, and hydrogen peroxide. Blood, *88*: 1805-1812, 1996.
29. Gazitt, Y., Fey, V., Thomas, C., and Alvarez, R. Bcl-2 overexpression is associated with resistance to dexamethasone, but not melphalan, in multiple myeloma cells. Int.J Oncol., *13*: 397-405, 1998.
30. Derenne, S., Monia, B., Dean, N. M., Taylor, J. K., Rapp, M. J., Harousseau, J. L., Bataille, R., and Amiot, M. Antisense strategy shows that Mcl-1 rather than Bcl-2 or Bcl-x(L) is an essential survival protein of human myeloma cells. Blood, *100*: 194-199, 2002.
31. van de Donk, N. W., Kamphuis, M. M., Van Dijk, M., Borst, H. P., Bloem, A. C., and Lokhorst, H. M. Chemosensitization of myeloma plasma cells by an antisense-mediated downregulation of Bcl-2 protein. Leukemia, *17*: 211-219, 2003.
32. Liu, Q. and Gazitt, Y. Potentiation of dexamethasone-, paclitaxel-, and Ad-p53-induced apoptosis by Bcl-2 antisense oligodeoxynucleotides in drug-resistant multiple myeloma cells. Blood, *101* : 4105-4114, 2003.
33. Webb, A., Cunningham, D., Cotter, F., Clarke, P. A., di Stefano, F., Ross, P., Corbo, M., and Dziewanowska, Z. BCL-2 antisense therapy in patients with non-Hodgkin lymphoma. Lancet, *%19;349*: 1137-1141, 1997.
34. Waters, J. S., Webb, A., Cunningham, D., Clarke, P. A., Raynaud, F., di Stefano, F., and Cotter, F. E. Phase I clinical and pharmacokinetic study of bcl-2 antisense oligonucleotide therapy in patients with non-Hodgkin's lymphoma. J Clin.Oncol., *18*: 1812-1823, 2000.
35. Jansen, B., Wacheck, V., Heere-Ress, E., Schlagbauer-Wadl, H., Hoeller, C., Lucas, T., Hoermann, M., Hollenstein, U., Wolff, K., and Pehamberger, H. Chemosensitisation of malignant melanoma by BCL2 antisense therapy. Lancet, *356*: 1728-1733, 2000.

36. Rudin, C. M., Otterson, G. A., Mauer, A. M., Villalona-Calero, M. A., Tomek, R., Prange, B., George, C. M., Szeto, L., and Vokes, E. E. A pilot trial of G3139, a bcl-2 antisense oligonucleotide, and paclitaxel in patients with chemorefractory small-cell lung cancer. Ann.Oncol., *13*: 539-545, 2002.
37. Chi, K. N., Gleave, M. E., Klasa, R., Murray, N., Bryce, C., Lopes de Menezes, D. E., D'Aloisio, S., and Tolcher, A. W. A phase I dose-finding study of combined treatment with an antisense Bcl-2 oligonucleotide (Genasense) and mitoxantrone in patients with metastatic hormone-refractory prostate cancer. Clin.Cancer Res., *7*: 3920-3927, 2001.
38. Marcucci, G., Byrd, J. C., Dai, G., Klisovic, M. I., Kourlas, P. J., Young, D. C., Cataland, S. R., Fisher, D. B., Lucas, D., Chan, K. K., Porcu, P., Lin, Z. P., Farag, S. F., Frankel, S. R., Zwiebel, J. A., Kraut, E. H., Balcerzak, S. P., Bloomfield, C. D., Grever, M. R., and Caligiuri, M. A. Phase 1 and pharmacodynamic studies of G3139, a Bcl-2 antisense oligonucleotide, in combination with chemotherapy in refractory or relapsed acute leukemia. Blood, *101*: 425-432, 2003.
39. Segeren, C. M., Sonneveld, P., van der, H. B., Baars, J. W., Biesma, D. H., Cornellissen, J. J., Croockewit, A. J., Dekker, A. W., Fibbe, W. E., Lowenberg, B., van Marwijk, K. M., van Oers, M. H., Richel, D. J., Schouten, H. C., Vellenga, E., Verhoef, G. E., Wijermans, P. W., Wittebol, S., and Lokhorst, H. M. Vincristine, doxorubicin and dexamethasone (VAD) administered as rapid intravenous infusion for first-line treatment in untreated multiple myeloma. Br.J Haematol., *105*: 127-130, 1999.
40. Blade, J., Samson, D., Reece, D., Apperley, J., Bjorkstrand, B., Gahrton, G., Gertz, M., Giralt, S., Jagannath, S., and Vesole, D. Criteria for evaluating disease response and progression in patients with multiple myeloma treated by high-dose therapy and haemopoietic stem cell transplantation. Myeloma Subcommittee of the EBMT. European Group for Blood and Marrow Transplant. Br.J Haematol., *102*: 1115-1123, 1998.
41. Boersma-Vreugdenhil, G. R., Peeters, T., and Bast, B. J. Translocation of the IgH locus is nearly ubiquitous in multiple myeloma as detected by immuno-FISH. Blood, *101*: 1653, 2003.
42. Blade, J. and Esteve, J. Treatment approaches for relapsing and refractory multiple myeloma. Acta Oncol., *39*: 843-847, 2000.
43. Richardson, P. G., Schlossman, R. L., Weller, E., Hideshima, T., Mitsiades, C., Davies, F., LeBlanc, R., Catley, L. P., Doss, D., Kelly, K., McKenney, M., Mechlowicz, J., Freeman, A., Deocampo, R., Rich, R., Ryoo, J. J., Chauhan, D., Balinski, K., Zeldis, J., and Anderson, K. C. Immunomodulatory drug CC-5013 overcomes drug resistance and is well tolerated in patients with relapsed multiple myeloma. Blood, *100*: 3063-3067, 2002.
44. Bishop, M. R., Iversen, P. L., Bayever, E., Sharp, J. G., Greiner, T. C., Copple, B. L., Ruddon, R., Zon, G., Spinolo, J., Arneson, M., Armitage, J. O., and Kessinger, A. Phase I trial of an antisense oligonucleotide OL(1)p53 in hematologic malignancies. J Clin.Oncol., *14*: 1320-1326, 1996.
45. Glover, J. M., Leeds, J. M., Mant, T. G., Amin, D., Kisner, D. L., Zuckerman, J. E., Geary, R. S., Levin, A. A., and Shanahan, W. R., Jr. Phase I safety and pharmacokinetic profile of an intercellular adhesion molecule-1 antisense oligodeoxynucleotide (ISIS 2302). J Pharmacol.Exp Ther, *282*: 1173-1180, 1997.
46. Veis, D. J., Sorenson, C. M., Shutter, J. R., and Korsmeyer, S. J. Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. Cell, *75*: 229-240, 1993.

# Chapter 4

## The cholesterol lowering drug lovastatin induces cell death in myeloma plasma cells

Niels W.C.J. van de Donk, Marloes M.J. Kamphuis, Henk M. Lokhorst,
and Andries C. Bloem

Leukemia 2002; 16: 1362-1371

## Abstract

Lovastatin is an irreversible inhibitor of HMG-CoA reductase and blocks the production of mevalonate, a critical compound in the production of cholesterol and isoprenoids. Isoprenylation of target proteins, like the GTP-binding protein Ras, is essential for their membrane localization and subsequent participation in intracellular signaling cascades. Lovastatin effectively decreased the viability of plasma cells from cell lines (n=10) and myeloma patients' samples (n=8) in a dose- and time-dependent way. Importantly, co-incubation of lovastatin with dexamethasone had a synergistic effect in inducing plasma cell cytotoxity. This effect was not the consequence of a change in the protein expression levels of Bcl-2 or Bax induced by lovastatin. The decrease in plasma cell viability was the result of induction of apoptosis and inhibition of proliferation. Mevalonate effectively reversed the cytotoxic and cytostatic effects of lovastatin in plasma cells. The cytotoxic activity of lovastatin was higher in Pgp expressing cell lines, but did not correlate with the multidrug resistance (MDR)-related proteins LRP, Bcl-2 and Bax. Lovastatin treatment resulted in a shift of Ras localization from the membrane to the cytosol that was reversed by mevalonate. The data presented in this paper warrant study of lovastatin alone or in combination with therapeutic drugs, in the treatment of myeloma patients.

**Keywords:** lovastatin, HMG-CoA reductase, multiple myeloma, multidrug resistance, apoptosis, isoprenylation

## Introduction

The enzyme 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase catalyzes the reduction of HMG-CoA to mevalonate, which is an intermediate in the synthesis of cholesterol, essential for membrane integrity; of dolichol, required for glycoprotein synthesis; of polyisoprenoids side chains of heme A and ubiquinone, involved in oxidative respiration; and of isopentyl-adenine, present in some tRNAs. Furthermore mevalonate is also a precursor of the isoprenoids farnesylpyrophosphate and geranylgeranylpyrophosphate [1]. These isoprenoids are used for posttranslational modification of a variety of proteins, including Ras and Ras-related GTP binding proteins. Isoprenylation is essential for membrane attachment [2] and participation of the modified proteins in signal transduction processes [3-7].

Lovastatin is a potent competitive inhibitor of HMG-CoA reductase and is widely used for the treatment of hypercholesterolemia [8]. Lovastatin blocks the enzymatic conversion of HMG-CoA to mevalonate and subsequently the synthesis of downstream mevalonate products. In this way lovastatin interferes with isoprenylation and the subsequent membrane localization of G proteins like Ras and Ras-related proteins [9,10]. Lovastatin inhibits the proliferation of several tumor cells by inducing a growth arrest at the G1/S boundary [10,11]. Furthermore, lovastatin has a chemosensitizing capacity in cancer cells, rendering tumor cells susceptible to chemotherapeutic drugs [12-18] and can directly induce apoptosis as has been documented for several tumor cell lines [10,15,19,20] and tumor cells from patients with acute myeloid leukemia [19], possibly via down-regulation of the anti-apoptotic protein Bcl-2 [15]. The observation that long-term treatment of hypercholesterolemia patients with statins was associated with fewer cancer deaths [21], suggests that lovastatin interferes with processes involved in tumorigenesis.

Multiple myeloma is a plasma cell tumor localized in the bone marrow. Although the majority of the patients respond to initial treatment, almost all patients relapse and die from multidrug resistant disease. Interleukin-6 (IL-6) is the most important growth and survival factor in myeloma [22-24]. Through binding to the IL-6 receptor, IL-6 activates the JAK-STAT [25] and Ras-MAPK [23] pathways, resulting in proliferation and protection from apoptosis of plasma cells. Activating Ras mutations are associated with IL-6-independent growth [26] and are associated with a dexamethasone and doxorubicin-resistant phenotype [27].

Since lovastatin effectively interferes with the membrane localization of Ras and its subsequent involvement in signaling processes [20,28], and knowing the importance of Ras signaling cascades in myeloma, we investigated the ability of lovastatin to induce apoptosis in myeloma cell lines and purified myeloma plasma cells. The results imply that lovastatin alone or in combination with chemotherapeutic drugs might be an effective therapy in multiple myeloma.

## Materials and methods

### Reagents

Lovastatin was a generous gift from Merck, Sharp & Dohme Research Laboratories (Rahway, NJ, USA) and prepared as described previously [29]. Dexamethasone and mevalonate were purchased from Sigma (St Louis, MO, USA).

### Cell lines and culture conditions

Plasma cell lines RPMI-8226 and U266 were obtained from the American Tissue Culture Collection (ATCC) and L363 from the German Collection of Microorganisms and Cell Cultures (GCMC). Plasma cell lines UM-1 and UM-3 were obtained after prolonged *in vitro* culture of bone marrow aspirates of myeloma patients and have been described before [30]. The plasma cell line UM-6 was obtained under identical conditions as described above. UM-6 cells express high levels of plasma cell associated antigens CD38, CD138 and cytoplasmic IgAkappa, as determined by flow cytometric analysis and immunofluorescence microscopy, respectively. Analysis using ASO-PCR established the clonal relationship between UM-6 and the original myeloma tumor (data not shown). The doxorubicin-resistant cell lines RPMI-DOX40 (DOX40) and RPMI-DOX6 (DOX6) were kind gifts of Dr W. Dalton (University of South Florida, Tampa, FL, USA) and Dr R. Scheper (Free University, Amsterdam, The Netherlands), respectively. The IL-6-dependent plasma cell line XG-1 (XG-1+) was a kind gift of Dr B. Klein (Institut for Molecular Genetics, Montpellier, France) [31]. An IL-6-independent variant (XG-1-) was selected by limiting dilution of XG-1+ cells in the absence of IL-6. Cell lines were cultured in RPMI-1640 (GIBCO, Breda, The Netherlands) supplemented with 10% fetal calf serum (FCS) (Integro, Zaandam, The Netherlands), 100 IU/ml penicillin, 100 μg/ml streptomycin and 10 μM β-mercaptoethanol (growth medium). The IL-6-dependent cell lines XG-1+ and UM-6 were cultured in the continuous presence of exogenous IL-6 (1.25 ng/ml rhIL-6 (Roche, Almere, The Netherlands)) and the RPMI-DOX6 cell line was cultured in the continuous presence of 6 X $10^{-8}$ M doxorubicin.

Myeloma plasma cells were purified from mononuclear cells obtained by Ficoll-Paque (Amersham; Pharmacia BiotechAB, Uppsala, Sweden) density centrifugation from bone marrow aspirates of 7 myeloma patients and peripheral blood of one patient with primary plasma cell leukemia after obtaining informed consent. The plasma cell percentage in the patient samples varied from 4 - 46% of the mononuclear cells (see Table 2) as determined by simultaneous detection of CD38 (anti-CD38-FITC, Immunotech, Marseille, France) and CD138 (anti-CD138-PE, Immunotech) by flow cytometric analysis (FACSCalibur, Becton and Dickinson, Erembodegem, Belgium (BDIS)). Myeloma plasma cells were purified *ex vivo* by magnetic cell sorting (MACS) based on CD138 expression [32]. To this end, mononuclear cells were subsequently labeled with anti-CD138

(Immunotech) and rat anti-mouse IgG1 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and then separated using a high gradient magnetic separation column placed in a strong magnetic field (Miltenyi), exactly following the instructions of the manufacturer. Samples obtained in this way contained >95% plasma cells as determined by analysis of CD38/CD138 co-expression. For experiments myeloma cells were resuspended in growth medium (see above).

***In vitro* cell cultures**

*Viability assay* Viability of cells was examined by means of the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay as described previously [33]. In short, cells were seeded in a concentration of 0.3 X $10^6$/ml for the plasma cell lines or 1 X $10^6$/ml for the purified myeloma cells and NBM mononuclear cells in a 96-well flat bottom plate (100 µl/well) (Nunc, Roskilde, Denmark) and treated with different concentrations lovastatin (0, 1, 5, 10, 20, 40, 60, 80, 100 and 150 µM). After 2 or 4 days, 25 µl of MTT (5 mg/ml) was added to each well. After an incubation of 2 hours at 37°C the reaction was stopped by the addition of 100 µl 20% sodium dodecyl sulphate (SDS; Boehringer Mannheim, Mannheim, Germany) / 0.025 M HCl / 0.35 M HAc in a mixture of (1:1; v/v) N,N-dimethylformamide (Merck, Darmstadt, Germany) and aqua destillata. After an overnight incubation at 37°C the optical density of the samples was determined at 570 nm.

*Cell proliferation assay* Cells (3 X $10^4$) were seeded in 96-well flat bottom plates (Nunc) in 100 µl growth medium with different concentrations lovastatin (0, 1, 5, 10, 20, 40, 60, 80, 100 and 150 µM). After 32 and 80 hours, $^3$H-thymidine (Amersham, Little Chalfont, UK) (1 µCi/well) was added for the remaining 16 hours of the assay. $^3$H-thymidine incorporation was analyzed by liquid scintillation counting as described previously [34].

*Apoptosis assessment by Annexin V staining* Mononuclear cells (3 X $10^5$ in 0.5 ml) were incubated with different concentrations lovastatin (0, 5, 10, 40, 100 and 150 µM) in a 48-well plate (Nunc). After 2 or 4 days, cells were harvested, washed in ice cold PBS and directly stained with Annexin V-FITC (Nexins Research, Kattendijke, The Netherlands) and propidium iodide (PI). After an incubation for 10 minutes at 4°C, the cells were subsequently analyzed by flow cytometry as described previously [30]. Apoptotic cells were defined as early apoptotic cells (Annexin V positive and PI negative) and late apoptotic cells (Annexin V positive and PI positive).

*Apoptosis assessment by the TUNEL assay* Cells were incubated with lovastatin as described above. DNA strand breaks in apoptotic cells were detected by incorporation of FITC-labeled deoxyuridine triphosphate (UTP-FITC) into fragmented DNA by terminal deoxynucleotidyl transferase (TdT) (Boehringer Mannheim). Briefly, cells were subsequently washed in 1% bovine serum albumin (BSA) in PBS at 4°C, fixated in 4% paraformaldehyde solution for 1 hour at room temperature, permeabilized in 0.1% Triton

X-100 in 0.1% sodiumcitrate solution for 2 minutes at 4°C and incubated with UTP-FITC and TdT for 1 hour at 37°C in the dark. Cells were analyzed by flow cytometry.

**Expression of MDR-related proteins**

For flow cytometric detection of intracellular Bcl-2, Bax and LRP, cells (0.5 - 1 X $10^6$) were fixed and permeabilized using FACS Lysing Solution (FLS, 10% in aqua destillata; BDIS) and subsequently incubated with anti-Bcl-2-FITC (IgG1, Dako, Glostrup, Denmark), anti-Bax (IgG2b, Immunotech) or anti-LRP (IgG2b, a kind gift of Dr R. Scheper) antibodies or their isotype and subclass matched control antibodies, followed by incubation with goat anti-mouse IgG F(ab')$_2$-FITC (Dako) for detection of Bax and LRP. All incubations were performed at room temperature for 30 minutes except staining for LRP that was performed at 4°C for 60 minutes. Between different steps the cells were washed with PBS. Membrane Pgp expression was determined by using the MRK16 antibody (kindly provided by Dr R. Scheeper) as described before [33]. The mean fluorescence ratio (MFR), defined as the ratio of the mean fluorescence intensities (MFI) of primary antibody and isotype control stained cells was used as a measure for the Bcl-2, Bax, LRP or Pgp protein expression.

**Western blotting**

Cells (1 X $10^6$ cells in 1.5 ml) were incubated for varying lengths of time in growth medium with lovastatin (0, 10 or 30 μM) in the presence or absence of 100 μM mevalonate. After harvesting, whole cell lysates were made by washing cells twice in ice-cold PBS and then resuspending them in lysis buffer (10 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% Triton X-100 and a cocktail of protease inhibitors (Boehringer Mannheim)) at 4°C for 20 minutes. Insoluble material was removed by centrifugation at 14 000 r.p.m. for 6 minutes at 4°C. When membrane and cytosolic protein fractions were analyzed, cells were suspended in extraction solution (10% glycerol, 20 mM Hepes, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT and a cocktail of protease inhibitors (Boehringer Mannheim)). The lysate was sonificated twice for 5 seconds and centrifuged at 4°C for 30 minutes at 48 000 *g*. The supernatant contained the proteins of the cytosolic fraction. The insoluble material was subsequently resuspended in solubilization buffer (1% NP-40, 20 mM Hepes, 20 mM MgAc, 5 mM NaF, 0.2 mM EDTA, 0.8 mM EGTA, 1 mM DTT and a cocktail of protease inhibitors). After 2 rounds of sonification for 10 seconds, an incubation for 30 minutes at 4°C and a centrifugation step at 4°C for 30 minutes at 48 000 *g*, proteins of the membrane fraction were present in the supernatant.

Protein concentrations were determined by the BCA assay (Pierce, Rockford, IL, USA). Samples containing equal amounts of protein were mixed with 2 X Laemmli sample buffer (0.125 M Tris pH 6.9 with 4% SDS, 20% Glycerol and 10% β-mercaptoethanol) and boiled for 5 minutes. Proteins were subsequently fractionated in

10% SDS-PAGE at room temperature and electrically transferred from the gel to PVDF-membrane (Biorad, Hercules, CA, USA). After blocking in 0.1% Tween-20, 5% skimmed powder milk, 2% BSA in 10 mM Tris and 150 mM NaCl, the membranes were incubated with anti-Bcl-2 (Dako) or anti-Ras (Transduction Laboratories, BDIS). Antibody binding was visualized with enhanced chemoluminescence (Amersham) detection with hyperfilm ECL (Amersham) after incubation with a horseradish peroxidase (HRP)-conjugated secondary antibody (Dako). Membranes incubated with anti-Bcl-2 or anti-Ras were extensively washed in PBS and reprobed with anti-Bax (Dako) and anti-α-actin (Sigma) or anti-γ-adaptin (Sigma) and anti-ICAM-1 (clone F7.11 [35]), respectively. The level of protein expression was determined by densitometry following normalization for α-actin expression.

**Statistics**

Data analysis was performed using the SPSS statistical software package (SPSS Inc, Chicago, IL, USA). A two-sided Student's t-test or a Welch's t-test in case of unequal variances, was used to determine differences between groups. The correlation between lovastatin sensitivity and MDR-related proteins was calculated by using the Spearmann test. Differences and correlations were considered statistically significant when $P < 0.05$. Data are plotted as means ± standard error of the mean (s.e.m.).

## Results

**Lovastatin reduces cell viability in plasma cell lines and in *ex vivo* purified myeloma plasma cells by inhibition of HMG-CoA reductase**

The effect of lovastatin on cell viability was examined on 10 plasma cell lines by using the MTT assay. *In vitro* incubation with lovastatin decreased cell viability in all plasma cell lines tested in a time- (data not shown) and dose-dependent way, as shown in Figure 1A for 3 representative cell lines. The concentration of lovastatin that reduced cell viability of the plasma cell lines with 50% (MTT50) or 75% (MTT25) after 4 days of *in vitro* culture ranged from 0.8 – 13.3 μM (MTT50) and 1.8 – 18.8 μM (MTT25) (Table 1). Lovastatin inhibits the enzyme HMG-CoA reductase that catalyzes the conversion of 3-hydroxy-3-methylglutaryl CoA to mevalonate. To examine whether the reduction in myeloma cell viability was mediated by an inhibition of HMG-CoA reductase activity, we investigated whether mevalonate rescued plasma cells from lovastatin-induced cell death. Co-incubation with mevalonate significantly diminished the effect of lovastatin on plasma cell lines, as shown in Figure 1B for U266 cells.

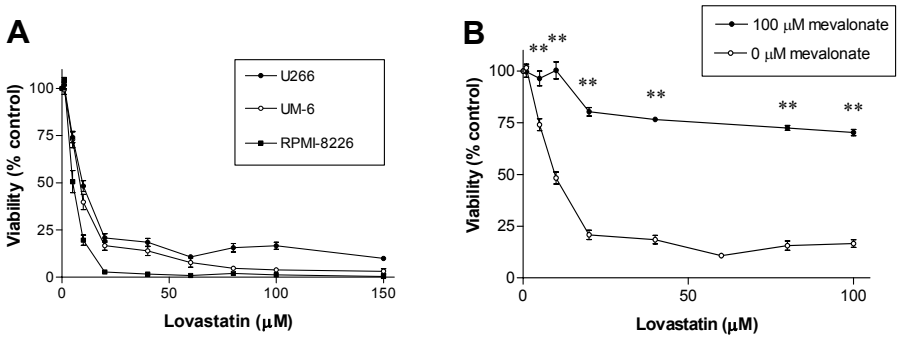


**Figure 1. Effect of lovastatin alone or in combination with mevalonate on viability of plasma cell lines.** (A) Three representative plasma cell lines were incubated for 4 days with different concentrations of lovastatin or solvent control. (B) U266 cells were incubated with lovastatin for 4 days in the presence or absence of 100 μM mevalonate. Cell viability was examined by using the MTT assay as compared with control cells treated with solvent control. Experiments were performed at least 3 times in triplo. Data are presented as mean ± s.e.m. **: $P < 0.005$.

Myeloma plasma cells were purified from bone marrow aspirates from 7 myeloma patients and peripheral blood from 1 patient with primary plasma cell leukemia (for clinical data of these patients see Table 2) based on CD138 expression using magnetic beads. This isolation method resulted in cell samples that contained >95% plasma cells (data not shown). Lovastatin also effectively induced tumor cell death in these *ex vivo* purified myeloma plasma cells in a dose- and time-dependent fashion (Figure 2A). Lovastatin sensitivity differed among the myeloma patients' samples. MTT50 and MTT25 were in the range of 6.0 to 63.4 and 8.9 to >150 μM, respectively (Table 2). The reduction of viability of myeloma tumor cells from patients by lovastatin was significantly reversed by co-treatment with mevalonate (shown for cells from patient 5 in Figure 2B).

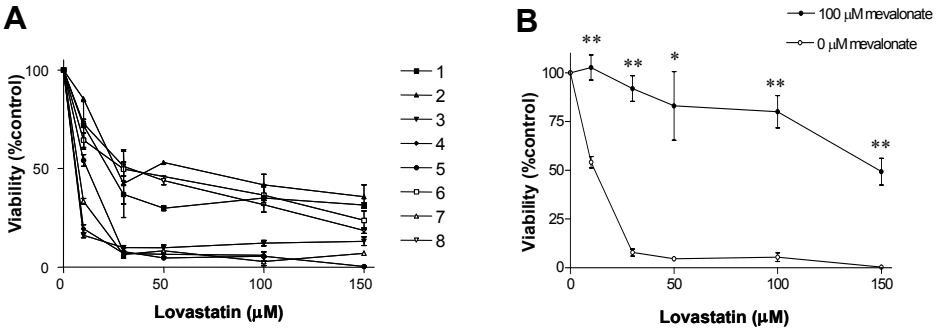


**Figure 2. Effect of lovastatin alone or in combination with mevalonate on viability of *ex vivo* purified plasma cells.** (A) *Ex vivo* purified myeloma plasma cells from myeloma bone marrow samples (n=7) and peripheral blood from one patient with primary plasma cell leukemia were incubated for 4 days with different concentrations of lovastatin or solvent control. (B) Purified myeloma tumor cells from patient 5 were incubated with lovastatin for 4 days in the presence or absence of 100 μM mevalonate. Cell viability was examined by using the MTT assay as compared with control cells treated with solvent control. Experiments were performed once in triplo. Data are presented as mean ± s.e.m. *: $P < 0.05$ ; **: $P < 0.005$.

**Table 1.** Expression of MDR-related proteins and effect of lovastatin on cell viability in 10 plasma cell lines

| Cell line | Pgp | LRP | Bcl-2 | Bax | Bcl-2/Bax | MTT50 | MTT25 |
|---|---|---|---|---|---|---|---|
| RPMI-8226 | 1.62 | 4.77 | 19.20 | 18.06 | 1.06 | 5.11 | 9.13 |
| DOX6 | 22.30 | 6.61 | 11.05 | 17.49 | 0.63 | 1.65 | 4.94 |
| DOX40 | 67.30 | 4.20 | 25.30 | 43.39 | 0.58 | 0.84 | 1.80 |
| UM-1 | 2.15 | 9.92 | 18.53 | 33.18 | 0.56 | 4.57 | 8.67 |
| UM-3 | 1.59 | 2.62 | 17.38 | 19.42 | 0.89 | 13.34 | 17.38 |
| UM-6 | 1.48 | 0.99 | 9.87 | 8.45 | 1.17 | 8.46 | 16.42 |
| XG-1- | 1.53 | 2.44 | 12.20 | 41.63 | 0.29 | 3.36 | 4.56 |
| XG-1+ | 1.71 | 3.00 | 8.51 | 20.64 | 0.41 | 2.79 | 4.10 |
| U266 | 1.55 | 6.07 | 19.87 | 23.89 | 0.83 | 9.68 | 18.48 |
| L363 | 0.97 | 7.33 | 17.52 | 35.29 | 0.50 | 10.37 | 18.78 |

Cells of 10 different plasma cell lines were incubated with different concentrations of lovastatin for 4 days. MTT50 and MTT25 are the concentrations of lovastatin that decreased cell viability with 50 or 75%, compared to solvent control-treated cells, respectively. The expression of Pgp, Bcl-2, Bax, and LRP was determined by using flow cytometry. The ratio of the mean fluorescence intensity of the primary antibody and the isotype control (MFR) is shown. The Bcl-2/Bax ratio was calculated by dividing the MFR of Bcl-2 and Bax. UM-6, XG-1+, and U266 plasma cell lines are dependent on IL-6 for their growth.

**Table 2.** Clinical data and effect of lovastatin on cell viability of purified myeloma cells

| Patient | Disease | MTT50 (μM) | MTT25 (μM) | Age/Sex | Stage diagnosis | M-protein | Plasma cell (%) | Treatments | Disease status |
|---|---|---|---|---|---|---|---|---|---|
| 1 | MM | 22.48 | >150 | 49/M | III-B | IgG-κ | 4 | alloBMT | plateau |
| 2 | MM | 63.40 | >150 | 57/M | II-B | IgG-κ | 11 | MP-dexa-Thal | relapse |
| 3 | MM | 5.96 | 8.94 | 59/M | III-B | IgA-κ | 28 | VAD-MP | relapse |
| 4 | MM | 6.20 | 9.30 | 60/M | III-A | κ-LC | 16 | autoPBSCT-Thal | relapse |
| 5 | MM | 11.78 | 22.58 | 57/M | III-A | IgG-λ | 39 | none | untreated/active |
| 6 | MM | 29.66 | 144.8 | 65/F | III-A | IgG-κ | 13 | autoPBSCT | relapse |
| 7 | MM | 7.51 | 16.23 | 66/F | III-A | IgG-λ | 17 | MP | relapse |
| 8 | PCL | 33.20 | 125.2 | 55/F | III-A | IgG-κ | 46 | none | untreated/active |

MTT50 and MTT25 indicate the concentrations of lovastatin, which after a 4 day incubation, reduced cell viability of *ex vivo* purified myeloma cells with 50 and 75%, compared to solvent control-treated cells, respectively. The patients were classified according to the Durie-Salmon staging system[54]. The M-protein type was determined by immunofixation. Bone marrow plasma cell percentage was determined by simultaneous detection of CD38 and CD138 in bone marrow mononuclear cells by flow cytometric analysis. Plasma cell monoclonality was established by means of immunofluorescence microscopy based on heavy and light chain expression. MM, multiple myeloma; PCL, plasma cell leukemia; M, male; F, female; Ig, immunoglobulin; κ, kappa; λ, lambda; LC, light chain; autoPBSCT, autologous peripheral blood stem cell transplantation; alloBMT, allogeneic bone marrow transplantation; MP, melphalan and prednisone; dexa, dexamethasone; Thal, thalidomide; VAD, vincristine, doxorubicin, and dexamethasone.

### Lovastatin induces apoptosis and inhibits proliferation

The effect of lovastatin on plasma cell proliferation and induction of apoptosis was investigated in three plasma cell lines that differed in their lovastatin sensitivity (RPMI-

8226, UM-6, and U266). *In vitro* incubation with lovastatin induced apoptosis in the plasma cell lines in a dose- and time-dependent way, as measured in the Annexin V assay (data not shown). The percentage of apoptotic cells was 19.2, 31.9 and 58.2% after 2 days and 32.4, 65.7, and 97.2% after 4 days of incubation with 40 μM lovastatin for U266, UM-6, and RPMI-8226 cells, respectively. Mevalonate significantly blocked induction of apoptosis by lovastatin in all 3 cell lines. Data from a representative experiment performed with UM-6 cells, are shown in Figure 3A. The Annexin V data were confirmed in an independent flow cytometric apoptosis assay, the Tunel assay (Figure 3B for a representative experiment performed with the RPMI-8226 cell line). The effect of lovastatin on proliferation of RPMI-8226, U266, and UM-6 plasma cell lines was evaluated by measuring $^{3}$H-thymidine incorporation. Figure 4A shows a concentration-dependent decrease of plasma cell proliferation at day 4 in all 3 cell lines. As with the induction of apoptosis, inhibition of proliferation by lovastatin was significantly abrogated by addition of 100 μM mevalonate, as shown in Figure 4B for U266 cells. Thus, these results imply that the effect of lovastatin on plasma cell viability is the consequence of induction of apoptosis and inhibition of proliferation.

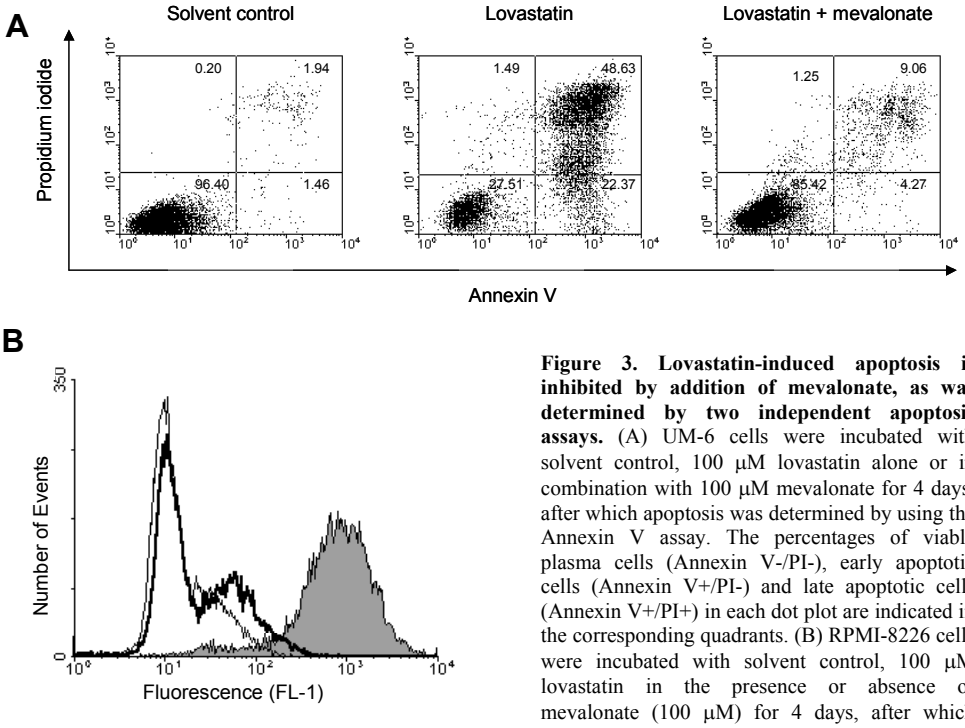


**Figure 3. Lovastatin-induced apoptosis is inhibited by addition of mevalonate, as was determined by two independent apoptosis assays.** (A) UM-6 cells were incubated with solvent control, 100 μM lovastatin alone or in combination with 100 μM mevalonate for 4 days, after which apoptosis was determined by using the Annexin V assay. The percentages of viable plasma cells (Annexin V-/PI-), early apoptotic cells (Annexin V+/PI-) and late apoptotic cells (Annexin V+/PI+) in each dot plot are indicated in the corresponding quadrants. (B) RPMI-8226 cells were incubated with solvent control, 100 μM lovastatin in the presence or absence of mevalonate (100 μM) for 4 days, after which DNA strand breaks were detected by using the TUNEL assay. In the overlay diagram, the filled histogram represents the cells treated with lovastatin alone, the histogram with the bold line the cells treated with mevalonate and lovastatin and the histogram with the thin line cells treated with solvent control.

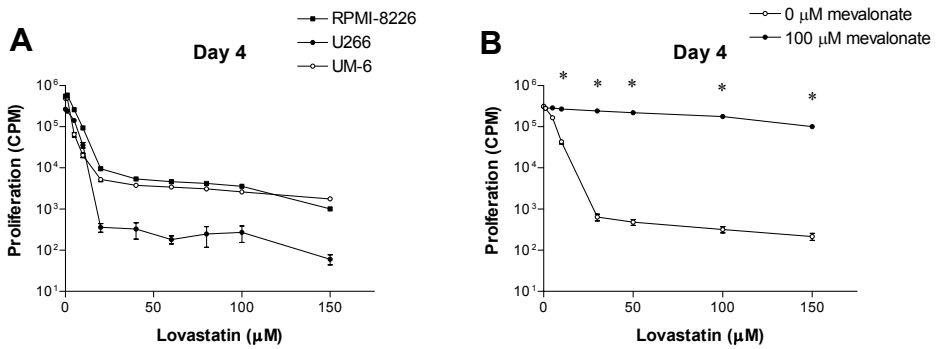


**Figure 4. Lovastatin inhibits proliferation of plasma cell lines, which was abrogated by addition of mevalonate.** (A) RPMI-8226, U266, and UM-6 cells were incubated with various concentrations of lovastatin or solvent control for 4 days, and proliferation was determined by $^{3}$H-thymidine incorporation during the last 16 hours of culture. Data were from 3 experiments performed in triplo. Data are presented as mean ± s.e.m. (B) Inhibition of proliferation of U266 cells by lovastatin was abrogated by addition of 100 μM mevalonate. Data were from 3 experiments performed in triplo. Data are presented as mean ± s.e.m. *: $P < 0.001$.

### Correlation between expression of MDR-related proteins and lovastatin sensitivity

The plasma cell lines differed in their sensitivity to lovastatin-induced cell death. Therefore, we investigated a possible correlation between lovastatin sensitivity and IL-6-dependent growth and expression of the anti-apoptotic protein Bcl-2, the pro-apoptotic protein Bax, the membrane efflux pump Pgp, and the human major vault protein LRP (lung resistance-related protein) in 10 different plasma cell lines. No difference in lovastatin sensitivity was found between IL-6-dependent (XG-1+, UM-6, and U266) and -independent plasma cell lines (Table 1). Moreover addition of exogenous IL-6 (5 ng/ml) did not protect against lovastatin-induced cell death (data not shown). All cell lines expressed different levels of intracellular Bcl-2 and Bax, and with the exception of the doxorubicin-resistant cell lines RPMI-DOX40 and RPMI-DOX6, the plasma cell lines hardly expressed Pgp and had low or undetectable intracellular LRP expression (Table 1). There was no significant correlation between Bcl-2, Bax, Bcl-2/Bax-ratio, or LRP expression and lovastatin sensitivity. However, lovastatin sensitivity significantly correlated with Pgp expression (R= –0.733 and –0.697; $P = 0.016$ and 0.025 for MTT50 and MTT25, respectively). DOX6 and DOX40 are doxorubicin-resistant variants of the RPMI-8226 plasma cell line. In these lines doxorubicin resistance positively correlated with the expression of Pgp. The most doxorubicin resistant cell line (DOX40) was most sensitive for lovastatin treatment, followed by DOX6 and the RPMI-8226.

### Dexamethasone synergizes with lovastatin in reducing plasma cell viability

The combined effect of lovastatin and dexamethasone on cell viability was examined by using the MTT assay in 3 dexamethasone-sensitive plasma cell lines (U266, L363, and XG-1+). In all cell lines, lovastatin had a synergistic effect with dexamethasone in reducing cell viability. A representative experiment with XG-1+ cells is shown in Figure

5. Dexamethasone (5 μM) alone reduced cell viability by 27.5%, lovastatin (1 μM) alone by 11.4%, while the combination of both decreased the percentage of viable cells by 54.5%. In the other 2 cell lines, U266 and L363, a synergistic effect between dexamethasone (0.5 and 5 μM) and lovastatin (1-10 μM) was found (data not shown).

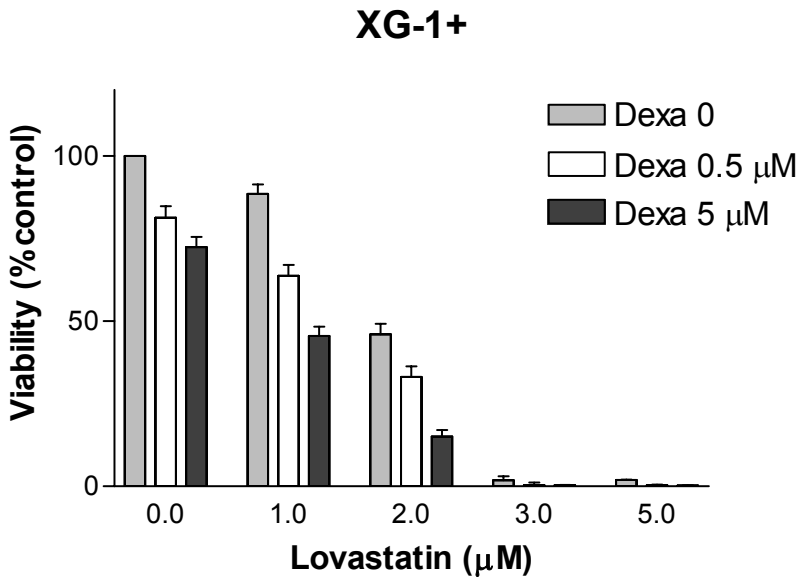


**Figure 5. Dexamethasone synergizes with lovastatin in reducing plasma cell viability.** Cells from the XG-1+ plasma cell line were incubated with different lovastatin concentrations (0-5 μM) in combination with dexamethasone (0.5 or 5 μM) or solvent control for 4 days. Cell viability was determined by using the MTT assay as compared to cells treated without lovastatin and dexamethasone. Data were from 3 experiments performed in triplo. Data are presented as mean ± s.e.m.

**Lovastatin down-regulates Bcl-2 expression only in the U266 cell line**

High levels of Bcl-2 protein expression are associated with *in vitro* resistance to dexamethasone [36]. In colon carcinoma cell lines, lovastatin reduced expression of the anti-apoptotic protein Bcl-2 and increased expression of the pro-apoptotic protein Bax [15]. To determine whether the synergism between dexamethasone and lovastatin in inducing plasma cell cytotoxicity was related to changes in the protein expression of Bcl-2 and Bax by lovastatin, Western blotting was performed. Cells from plasma cell lines (XG-1+, U266, and L363) were incubated for 2 or 4 days with different concentrations of lovastatin in the presence or absence of mevalonate. Lovastatin reduced Bcl-2 protein expression only in U266 cells (shown for day 4 in Figure 6A), but not in the other two cell lines tested (shown for XG-1+ in Figure 6B). Bax and α-actin levels remained unchanged. Mevalonate prevented lovastatin-induced Bcl-2 down-regulation in U266 cells. In 2 out of

8 patients lovastatin treatment resulted in a decreased Bcl-2 protein expression, without changing Bax or α-actin expression levels (data not shown).

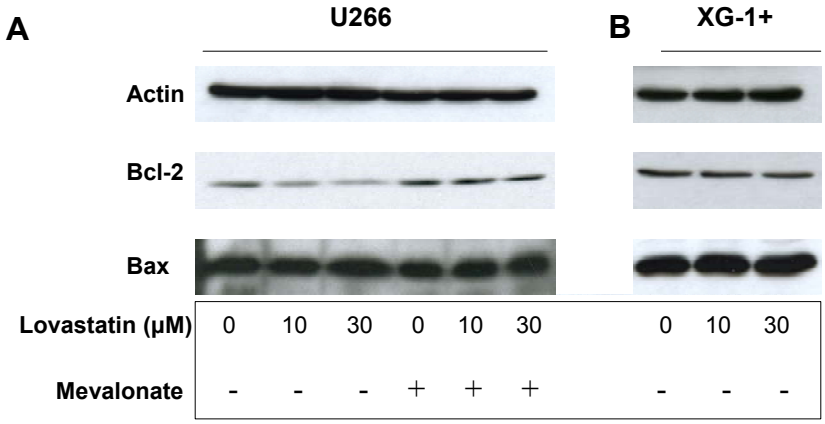


**Figure 6. Down-regulation of Bcl-2, but not Bax protein levels in the U266 cell line, which was abrogated by mevalonate.** U266 cells were incubated for 4 days with solvent control or 10 or 30 μM lovastatin in the presence or absence of 100 μM mevalonate. XG-1+ cells were incubated for 4 days with solvent control or the same doses lovastatin. After protein isolation, Bcl-2 and Bax were determined by Western blot analysis. Blots were reprobed with anti-actin to confirm the presence of equal amounts of protein in each lane. The data presented are representative of 3 independent experiments.

**Lovastatin and protein isoprenylation**

Lovastatin depletes the cellular pool of mevalonate by inhibition of HMG-CoA reductase and potentially impairs isoprenylation and a subsequent membrane localization of small GTPases like Ras. The effect of Lovastatin on Ras localization was evaluated by incubating U266 cells with different doses of lovastatin in the absence or presence of mevalonate for 2 days. Membrane and cytosol protein fractions were isolated and analyzed by Western blot. In the untreated cells, Ras was predominantly membrane associated (Figure 7). Lovastatin induced a dose-dependent shift of Ras from the membrane to the cytosolic protein fraction. Co-incubation with mevalonate prevented this effect of lovastatin on Ras localization. The localization of non-isoprenylated control proteins, ICAM-1 (membrane protein) and γ-adaptin (cytosolic protein) was not affected by lovastatin treatment (Figure 7). Identical results were obtained with the RPMI-8226 and UM-6 plasma cell lines (data not shown). These results show that Lovastatin treatment of plasma cells impairs membrane localization of Ras, probably via inhibition of isoprenylation.

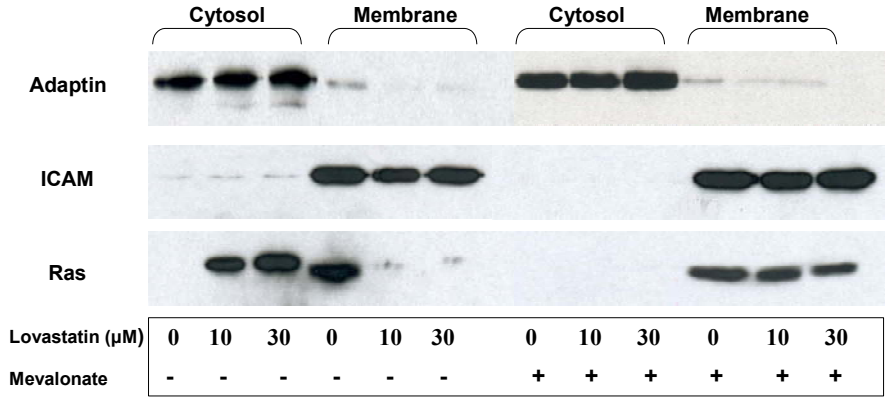


**Figure 7. Lovastatin induces a shift of membrane-associated Ras to cytosol localized Ras, which is abrogated by the addition of mevalonate.** U266 cells were incubated with solvent control, 10, or 30 μM lovastatin in the presence or absence of 100 μM mevalonate. After a 2 day incubation, protein was separated in membrane and cytosolic fractions. Ras was identified by Western blot analysis. The blots were reprobed with anti-ICAM-1 and anti-γ-adaptin, to confirm a well-performed separation and the presence of equal amounts of protein in each lane. The data are representative of 3 independent experiments.

## Discussion

Lovastatin is a HMG-CoA reductase inhibitor, which, because of its inhibition of hepatic cholesterol synthesis, is prescribed as a cholesterol-lowering drug. In this report we demonstrated for the first time that lovastatin induced cell death in *ex vivo* purified plasma cells from myeloma patients and cell lines at concentrations that can be achieved *in vivo* [37], which was mediated by induction of apoptosis and by inhibition of proliferation. Furthermore, lovastatin in combination with dexamethasone displayed a synergistic effect in inducing cell death.

Lovastatin sensitivity differed among the plasma cell lines and the purified myeloma samples. In plasma cell lines we examined possible correlations between lovastatin sensitivity and IL-6-dependent growth and expression of well-known multiple myeloma multidrug resistance-related proteins [38] such as P-glycoprotein (Pgp) [39-41], lung resistance protein (LRP) [42,43], and Bcl-2 family proteins [36,44,45]. There was no obvious relationship between IL-6-independent or -dependent plasma cell growth and lovastatin sensitivity. Moreover, addition of exogenous IL-6 did not protect against lovastatin-induced cell death. No correlation was found between lovastatin sensitivity and LRP, Bax, or Bcl-2 expression, but cell lines with a high Pgp expression were more sensitive to lovastatin than cell lines, which did not express Pgp. This is in agreement with previously published *in vitro* studies performed in neuroblastoma, myeloid leukemia, and two

myeloma cell lines [46-48]. The correlation between lovastatin sensitivity and Pgp expression indicates that lovastatin can be used for the treatment of myeloma patients with multidrug-resistant disease related to Pgp overexpression.

Induction of apoptosis and inhibition of proliferation of myeloma plasma cells by lovastatin was reversed by addition of mevalonate. Mevalonate is an intermediate in the synthesis of cholesterol, but also of the isoprenoids farnesylpyrophosphate and geranylgeranylpyrophosphate [1]. Ras and Ras-related proteins depend on the covalent attachment of these isoprenoids to their C-terminus for their proper localization to the cell membrane [2]. Plasma membrane localization of Ras is necessary for the conversion of its inactive GDP- to its active GTP-bound state [49], following activation of transmembrane receptors by growth factors such as the myeloma growth and survival factor IL-6 [22-24]. GTP-bound Ras subsequently activates downstream signaling proteins, which regulate growth and survival [3-7]. Activating Ras mutations are described in 39% of newly diagnosed myeloma patients [50] and in 64 - 70% of patients with terminal disease [24,51,52]. The presence of Ras mutations at diagnosis is also associated with a poor response to chemotherapy [51] and a shorter survival [50]. *In vitro*, Ras mutations are associated with IL-6-independent growth and protection from apoptosis induced by IL-6 withdrawal [26] or treatment with dexamethasone and doxorubicin [27]. Importantly, lovastatin inhibits cell proliferation both in cell lines with wild type and mutant Ras [53]. Our results demonstrate that treatment of plasma cells with lovastatin resulted in a depletion of membrane-localized Ras and an accumulation of cytosolic Ras, probably by inhibition of isoprenylation. This effect was abrogated by mevalonate. The cytotoxic effect of lovastatin on plasma cells may be the consequence of interference with the isoprenylation of Ras and its subsequent involvement in signaling cascades and thereby abrogating proliferation and cell survival signals. This is consistent with experiments, which show that lovastatin disrupted oncogenic Ras signaling through MAPK, causing apoptosis and inhibiting proliferation [20,28]. Current experiments focus on the characterization of isoprenylated target proteins that regulate plasma cell survival and proliferation.

In multiple myeloma high levels of the anti-apoptotic protein Bcl-2 are associated *in vitro* with protection against IL-6-deprived apoptosis [44] and resistance to cytotoxic drugs and dexamethasone [36]. In colon cancer cells lovastatin treatment resulted in a decreased Bcl-2 and an increased Bax protein expression [15]. The results presented in this paper showed that lovastatin and dexamethasone had a synergistic effect in inducing cell death in plasma cell lines. However, lovastatin treatment reduced Bcl-2 protein levels only in the U266 cell line. In L363 and XG-1+ cells, Bcl-2 levels remained unchanged. This implies that although down-regulation of Bcl-2 in U266 cells may contribute to the observed synergistic effect between lovastatin and dexamethasone in the induction of plasma cell cytotoxicity, other mechanisms may account for the synergistic response in the other two cell lines. The same observation was made in *ex vivo* purified plasma cells;

only in 2 out of 8 patients did lovastatin treatment reduce Bcl-2 protein expression, without changing Bax and α-actin expression levels.

Serum peak levels in the range of 0.1 to 4 μM were achieved in individuals, who received high-doses of lovastatin (30 mg/kg/day). These dosages were well tolerated, but as a mono-therapy had little effect in reducing tumor load in patients with solid tumors [37]. However, tumor cells from patients with acute myeloid leukemia (AML) were more sensitive to lovastatin compared to solid tumors. Dosages of lovastatin that are easily reached *in vivo*, induced cell death of freshly isolated AML cells and decreased the colony forming potential of AML primary cultures, with minimal effects on normal bone marrow and normal bone marrow progenitors [19]. In this paper we demonstrate that concentrations that can be achieved *in vivo*, also effectively killed plasma cell lines and *ex vivo* purified myeloma tumor cells from patients *in vitro*. Furthermore, we show that lovastatin at low concentrations (0-10 μM) displayed a synergistic effect with dexamethasone in inducing cell death. These results agree with earlier published findings, which show that lovastatin enhanced antitumor activity of several chemotherapeutical agents in solid tumors [12-18].

In conclusion, lovastatin reduced cell viability in plasma cell lines and *ex vivo* purified plasma cells from myeloma patients. Dexamethasone synergized with low concentrations of lovastatin in reducing plasma cell viability. Induction of apoptosis in myeloma cell lines by lovastatin was independent of expression of MDR-related proteins like Bcl-2, Bax, and LRP, but cells expressing Pgp were more sensitive to lovastatin. Together, our data indicate that lovastatin as single agent or combined with dexamethasone or dexamethasone-containing regimens, should be evaluated in multiple myeloma.

## References


1. Goldstein, J. L. and Brown, M. S. Regulation of the mevalonate pathway. Nature, *343*: 425-430, 1990.
2. Magee, T. and Marshall, C. New insights into the interaction of Ras with the plasma membrane. Cell, *98*: 9-12, 1999.
3. Kato, K., Cox, A. D., Hisaka, M. M., Graham, S. M., Buss, J. E., and Der, C. J. Isoprenoid addition to Ras protein is the critical modification for its membrane association and transforming activity. Proc.Natl.Acad.Sci.U.S.A, *89*: 6403-6407, 1992.
4. Gelb, M. H. Protein prenylation, et cetera: signal transduction in two dimensions. Science, *275*: 1750-1751, 1997.
5. Rebollo, A. and Martinez, A. Ras proteins: recent advances and new functions. Blood, *94*: 2971-2980, 1999.
6. Reuter, C. W., Morgan, M. A., and Bergmann, L. Targeting the Ras signaling pathway: a rational, mechanism-based treatment for hematologic malignancies? Blood, *96*: 1655-1669, 2000.
7. O'Gorman, D. M. and Cotter, T. G. Molecular signals in anti-apoptotic survival pathways. Leukemia, *15*: 21-34, 2001.

8. Downs, J. R., Clearfield, M., Weis, S., Whitney, E., Shapiro, D. R., Beere, P. A., Langendorfer, A., Stein, E. A., Kruyer, W., and Gotto, A. M. Primary prevention of acute coronary events with lovastatin in men and women with average cholesterol levels: results of AFCAPS/TexCAPS. Air Force/Texas Coronary Atherosclerosis Prevention Study. JAMA, *279*: 1615-1622, 1998.
9. Cuthbert, J. A. and Lipsky, P. E. Regulation of proliferation and Ras localization in transformed cells by products of mevalonate metabolism. Cancer Res., *57*: 3498-3505, 1997.
10. Ghosh, P. M., Ghosh-Choudhury, N., Moyer, M. L., Mott, G. E., Thomas, C. A., Foster, B. A., Greenberg, N. M., and Kreisberg, J. I. Role of RhoA activation in the growth and morphology of a murine prostate tumor cell line. Oncogene, *18*: 4120-4130, 1999.
11. Jakobisiak, M., Bruno, S., Skierski, J. S., and Darzynkiewicz, Z. Cell cycle-specific effects of lovastatin. Proc.Natl.Acad.Sci.U.S.A, *88*: 3628-3632, 1991.
12. Soma, M. R., Pagliarini, P., Butti, G., Paoletti, R., Paoletti, P., and Fumagalli, R. Simvastatin, an inhibitor of cholesterol biosynthesis, shows a synergistic effect with N,N'-bis(2-chloroethyl)-N-nitrosourea and beta-interferon on human glioma cells. Cancer Res., *52*: 4348-4355, 1992.
13. Soma, M. R., Baetta, R., De Renzis, M. R., Mazzini, G., Davegna, C., Magrassi, L., Butti, G., Pezzotta, S., Paoletti, R., and Fumagalli, R. In vivo enhanced antitumor activity of carmustine [N,N'-bis(2-chloroethyl)-N-nitrosourea] by simvastatin. Cancer Res., *55*: 597-602, 1995.
14. Feleszko, W., Zagozdzon, R., Golab, J., and Jakobisiak, M. Potentiated antitumour effects of cisplatin and lovastatin against MmB16 melanoma in mice. Eur.J.Cancer, *34*: 406-411, 1998.
15. Agarwal, B., Bhendwal, S., Halmos, B., Moss, S. F., Ramey, W. G., and Holt, P. R. Lovastatin augments apoptosis induced by chemotherapeutic agents in colon cancer cells. Clin.Cancer Res., *5*: 2223-2229, 1999.
16. Feleszko, W., Balkowiec, E. Z., Sieberth, E., Marczak, M., Dabrowska, A., Giermasz, A., Czajka, A., and Jakobisiak, M. Lovastatin and tumor necrosis factor-alpha exhibit potentiated antitumor effects against Ha-ras-transformed murine tumor via inhibition of tumor-induced angiogenesis. Int.J.Cancer, *81*: 560-567, 1999.
17. Agarwal, B., Rao, C. V., Bhendwal, S., Ramey, W. R., Shirin, H., Reddy, B. S., and Holt, P. R. Lovastatin augments sulindac-induced apoptosis in colon cancer cells and potentiates chemopreventive effects of sulindac. Gastroenterology, *117*: 838-847, 1999.
18. Feleszko, W., Mlynarczuk, I., Balkowiec-Iskra, E. Z., Czajka, A., Switaj, T., Stoklosa, T., Giermasz, A., and Jakobisiak, M. Lovastatin potentiates antitumor activity and attenuates cardiotoxicity of doxorubicin in three tumor models in mice. Clin.Cancer Res., *6*: 2044-2052, 2000.
19. Dimitroulakos, J., Nohynek, D., Backway, K. L., Hedley, D. W., Yeger, H., Freedman, M. H., Minden, M. D., and Penn, L. Z. Increased sensitivity of acute myeloid leukemias to lovastatin-induced apoptosis: A potential therapeutic approach. Blood, *93*: 1308-1318, 1999.
20. Bouterfa, H. L., Sattelmeyer, V., Czub, S., Vordermark, D., Roosen, K., and Tonn, J. C. Inhibition of Ras farnesylation by lovastatin leads to downregulation of proliferation and migration in primary cultured human glioblastoma cells. Anticancer Res., *20*: 2761-2771, 2000.
21. Pedersen, T. R., Wilhelmsen, L., Faergeman, O., Strandberg, T. E., Thorgeirsson, G., Troedsson, L., Kristianson, J., Berg, K., Cook, T. J., Haghfelt, T., Kjekshus, J., Miettinen, T., Olsson, A. G., Pyorala, K., and Wedel, H. Follow-up study of patients randomized in the Scandinavian simvastatin survival study (4S) of cholesterol lowering. Am.J.Cardiol., *86*: 257-262, 2000.
22. Klein, B., Zhang, X. G., Lu, Z. Y., and Bataille, R. Interleukin-6 in human multiple myeloma. Blood, *85*: 863-872, 1995.
23. Ogata, A., Chauhan, D., Teoh, G., Treon, S. P., Urashima, M., Schlossman, R. L., and Anderson, K. C. IL-6 triggers cell growth via the Ras-dependent mitogen-activated protein kinase cascade. J.Immunol., *159*: 2212-2221, 1997.
24. Hallek, M., Bergsagel, P. L., and Anderson, K. C. Multiple myeloma: increasing evidence for a multistep transformation process. Blood, *91*: 3-21, 1998.

25. Hirano, T., Ishihara, K., and Hibi, M. Roles of STAT3 in mediating the cell growth, differentiation and survival signals relayed through the IL-6 family of cytokine receptors. Oncogene, *19*: 2548-2556, 2000.
26. Billadeau, D., Liu, P., Jelinek, D., Shah, N., LeBien, T. W., and Van Ness, B. Activating mutations in the N- and K-ras oncogenes differentially affect the growth properties of the IL-6-dependent myeloma cell line ANBL6. Cancer Res., *57*: 2268-2275, 1997.
27. Rowley, M., Liu, P., and Van Ness, B. Heterogeneity in therapeutic response of genetically altered myeloma cell lines to interleukin 6, dexamethasone, doxorubicin, and melphalan. Blood, *96*: 3175-3180, 2000.
28. Bassa, B. V., Roh, D. D., Vaziri, N. D., Kirschenbaum, M. A., and Kamanna, V. S. Effect of inhibition of cholesterol synthetic pathway on the activation of Ras and MAP kinase in mesangial cells. Biochim.Biophys.Acta, *1449*: 137-149, 1999.
29. Keyomarsi, K., Sandoval, L., Band, V., and Pardee, A. B. Synchronization of tumor and normal cells from G1 to multiple cell cycles by lovastatin. Cancer Res., *51*: 3602-3609, 1991.
30. Bloem, A. C., Lamme, T., de Smet, M., Kok, H., Vooijs, W., Wijdenes, J., Boom, S. E., and Lokhorst, H. M. Long-term bone marrow cultured stromal cells regulate myeloma tumour growth in vitro: studies with primary tumour cells and LTBMC-dependent cell lines. Br.J.Haematol., *100*: 166-175, 1998.
31. Zhang, X. G., Gaillard, J. P., Robillard, N., Lu, Z. Y., Gu, Z. J., Jourdan, M., Boiron, J. M., Bataille, R., and Klein, B. Reproducible obtaining of human myeloma cell lines as a model for tumor stem cell study in human multiple myeloma. Blood, *83*: 3654-3663, 1994.
32. Jego, G., Robillard, N., Puthier, D., Amiot, M., Accard, F., Pineau, D., Harousseau, J. L., Bataille, R., and Pellat-Deceunynck, C. Reactive plasmacytoses are expansions of plasmablasts retaining the capacity to differentiate into plasma cells. Blood, *94*: 701-712, 1999.
33. Roovers, D. J., van Vliet, M., Bloem, A. C., and Lokhorst, H. M. Idarubicin overcomes P-glycoprotein-related multidrug resistance: comparison with doxorubicin and daunorubicin in human multiple myeloma cell lines. Leuk.Res., *23*: 539-548, 1999.
34. Daha, M. R., Bloem, A. C., and Ballieux, R. E. Immunoglobulin production by human peripheral lymphocytes induced by anti-C3 receptor antibodies. J.Immunol., *132*: 1197-1201, 1984.
35. van Horssen, M., Loman, S., Rijkers, G. T., Boom, S. E., and Bloem, A. C. Co-ligation of ICAM-1 (CD54) and membrane IgM negatively affects B cell receptor signaling. Eur.J.Immunol., *25*: 154-158, 1995.
36. Gazitt, Y., Fey, V., Thomas, C., and Alvarez, R. Bcl-2 overexpression is associated with resistance to dexamethasone, but not melphalan, in multiple myeloma cells. Int.J.Oncol., *13*: 397-405, 1998.
37. Thibault, A., Samid, D., Tompkins, A. C., Figg, W. D., Cooper, M. R., Hohl, R. J., Trepel, J., Liang, B., Patronas, N., Venzon, D. J., Reed, E., and Myers, C. E. Phase I study of lovastatin, an inhibitor of the mevalonate pathway, in patients with cancer. Clin.Cancer Res., *2*: 483-491, 1996.
38. Dalton, W. S. and Jove, R. Drug resistance in multiple myeloma: approaches to circumvention. Semin.Oncol., *26*: 23-27, 1999.
39. Epstein, J., Xiao, H. Q., and Oba, B. K. P-glycoprotein expression in plasma-cell myeloma is associated with resistance to VAD. Blood, *74*: 913-917, 1989.
40. Sonneveld, P., Durie, B. G., Lokhorst, H. M., Marie, J. P., Solbu, G., Suciu, S., Zittoun, R., Lowenberg, B., and Nooter, K. Modulation of multidrug-resistant multiple myeloma by cyclosporin. The Leukaemia Group of the EORTC and the HOVON. Lancet, *340*: 255-259, 1992.
41. Grogan, T. M., Spier, C. M., Salmon, S. E., Matzner, M., Rybski, J., Weinstein, R. S., Scheper, R. J., and Dalton, W. S. P-glycoprotein expression in human plasma cell myeloma: correlation with prior chemotherapy. Blood, *81*: 490-495, 1993.
42. Raaijmakers, H. G., Izquierdo, M. A., Lokhorst, H. M., de Leeuw, C., Belien, J. A., Bloem, A. C., Dekker, A. W., Scheper, R. J., and Sonneveld, P. Lung-resistance-related protein expression is a negative predictive factor for response to conventional low but not to intensified dose alkylating chemotherapy in multiple myeloma. Blood, *91*: 1029-1036, 1998.

43. Filipits, M., Drach, J., Pohl, G., Schuster, J., Stranzl, T., Ackermann, J., Konigsberg, R., Kaufmann, H., Gisslinger, H., Huber, H., Ludwig, H., and Pirker, R. Expression of the lung resistance protein predicts poor outcome in patients with multiple myeloma. Clin.Cancer Res., *5*: 2426-2430, 1999.
44. Schwarze, M. M. and Hawley, R. G. Prevention of myeloma cell apoptosis by ectopic bcl-2 expression or interleukin 6-mediated up-regulation of bcl-xL. Cancer Res., *55*: 2262-2265, 1995.
45. Renner, S., Weisz, J., Krajewski, S., Krajewska, M., Reed, J. C., and Lichtenstein, A. Expression of BAX in plasma cell dyscrasias. Clin.Cancer Res., *6*: 2371-2380, 2000.
46. Holmberg, M., Sandberg, C., Nygren, P., and Larsson, R. Effects of lovastatin on a human myeloma cell line: increased sensitivity of a multidrug-resistant subline that expresses the 170 kDa P-glycoprotein. Anticancer Drugs, *5*: 598-600, 1994.
47. Dimitroulakos, J. and Yeger, H. HMG-CoA reductase mediates the biological effects of retinoic acid on human neuroblastoma cells: lovastatin specifically targets P-glycoprotein-expressing cells. Nat.Med., *2*: 326-333, 1996.
48. Maksumova, L., Ohnishi, K., Muratkhodjaev, F., Zhang, W., Pan, L., Takeshita, A., and Ohno, R. Increased sensitivity of multidrug-resistant myeloid leukemia cell lines to lovastatin. Leukemia, *14*: 1444-1450, 2000.
49. Nakafuku, M., Satoh, T., and Kaziro, Y. Differentiation factors, including nerve growth factor, fibroblast growth factor, and interleukin-6, induce an accumulation of an active Ras.GTP complex in rat pheochromocytoma PC12 cells. J.Biol.Chem., *267*: 19448-19454, 1992.
50. Liu, P., Leong, T., Quam, L., Billadeau, D., Kay, N. E., Greipp, P., Kyle, R. A., Oken, M. M., and Van Ness, B. Activating mutations of N- and K-ras in multiple myeloma show different clinical associations: analysis of the Eastern Cooperative Oncology Group Phase III Trial. Blood, *88*: 2699-2706, 1996.
51. Neri, A., Murphy, J. P., Cro, L., Ferrero, D., Tarella, C., Baldini, L., and Dalla-Favera, R. Ras oncogene mutation in multiple myeloma. J.Exp.Med., *170*: 1715-1725, 1989.
52. Portier, M., Moles, J. P., Mazars, G. R., Jeanteur, P., Bataille, R., Klein, B., and Theillet, C. p53 and RAS gene mutations in multiple myeloma. Oncogene, *7*: 2539-2543, 1992.
53. Muller, C., Bockhorn, A. G., Klusmeier, S., Kiehl, M., Roeder, C., Kalthoff, H., and Koch, O. M. Lovastatin inhibits proliferation of pancreatic cancer cell lines with mutant as well as with wild-type K-ras oncogene but has different effects on protein phosphorylation and induction of apoptosis. Int.J.Oncol., *12*: 717-723, 1998.
54. Durie, B. G. Staging and kinetics of multiple myeloma. Semin.Oncol., *13*: 300-309, 1986.

# Chapter 5

## Inhibition of protein geranylgeranylation induces apoptosis in myeloma plasma cells by reducing Mcl-1 protein levels

Niels W.C.J. van de Donk, Marloes M.J. Kamphuis, Berris van Kessel, Henk M. Lokhorst, and Andries C. Bloem

Blood, in press for publication in November 2003

## Abstract

HMG-CoA reductase is the rate-limiting enzyme of the mevalonate pathway leading to the formation of cholesterol and isoprenoids like farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP). Inhibition of HMG-CoA reductase by lovastatin induced apoptosis in plasma cell lines and tumor cells from patients with multiple myeloma. Here we show that co-treatment with mevalonate or geranylgeranyl moieties, but not farnesyl groups, rescued myeloma cells from lovastatin-induced apoptosis. Also inhibition of geranylgeranylation by specific inhibition of geranylgeranyl transferase I (GGTase I) resulted in induction of apoptosis of myeloma cells. Apoptosis triggered by inhibition of geranylgeranylation was associated with reduction of Mcl-1 protein expression, collapse of the mitochondrial transmembrane potential, expression of the mitochondrial membrane protein 7A6, cytochrome c release from mitochondria into the cytosol, and stimulation of caspase-3 activity. These results imply that protein geranylgeranylation is critical for the regulation of myeloma tumor cell survival, possibly through the regulation of Mcl-1 expression. Our results show that pharmacological agents such as lovastatin or GGTase inhibitors may be useful in the treatment of multiple myeloma.

## Introduction

Prenylation is a class of lipid modification involving the covalent attachment of hydrophobic isoprenoid molecules to target proteins [1]. The enzymes farnesyl transferase (FTase) and geranylgeranyl transferase (GGTase) catalyze the transfer and subsequent binding of farnesyl and geranylgeranyl isoprenoid moieties from farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP), respectively, to a cysteine-containing motif located at or near the C-terminus of a target protein. Prenylation is essential for membrane attachment [1] and the subsequent participation of prenylated proteins in diverse signaling pathways regulating cell growth and survival [2-5]. Proteins that require geranylgeranylation or farnesylation for their function include GTP-binding proteins such as the Rho family members Rac-1, RhoA, and Cdc42 (geranylgeranylation) [6] and Ras (farnesylation) [1,5]. Oncogenic Ras mutations have been found in several human cancers including multiple myeloma [7,8]. Based on *in vitro* studies indicating that inhibition of farnesylation has antimyeloma activity [9-12], clinical studies have been initiated to evaluate the efficacy of FTase inhibitors in myeloma treatment [13].

Multiple myeloma is a so far incurable neoplastic disease of the B-cell lineage, characterized by the presence of monoclonal plasma cells in the bone marrow. Although chemotherapy is initially effective in most patients with myeloma, they all eventually develop multidrug-resistant disease. Recently, we described that lovastatin effectively decreases the viability of myeloma cells from cell lines and patients' samples, including patients with drug-resistant disease [14]. In addition, low concentrations of lovastatin synergized with dexamethasone in inducing plasma cell cytotoxicity [14]. Lovastatin is a potent competitive inhibitor of the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase and is widely used for the treatment of hypercholesterolemia [15]. HMG-CoA reductase is the rate-limiting enzyme of the mevalonate pathway and catalyzes the reduction of HMG-CoA to mevalonate, which is an intermediate in the synthesis of FPP and GGPP [16]. The apoptotic effect of lovastatin on myeloma plasma cells was reversed by the addition of mevalonate [14]. This suggests that downstream metabolites of mevalonate play a role in the regulation of lovastatin-induced apoptosis.

Recent reports imply that anti-apoptotic and pro-apoptotic Bcl-2 family proteins are important regulators of cell survival and resistance to cytotoxic drugs. The anti-apoptotic members of the Bcl-2 family, such as Bcl-2, Bcl-XL and Mcl-1 protect against apoptosis by the formation of heterodimers with pro-apoptotic Bcl-2 family members, such as Bax and Bad [17,18]. This prevents the collapse of the mitochondrial transmembrane potential and the release of cytochrome c from mitochondria, which may facilitate a change in Apaf-1 structure to allow procaspase-9 recruitment, processing and activation. Activated caspase-9 then activates effector caspases such as caspase-3 [17,18]. Our work and that of others has shown that Bcl-2 protects myeloma cells against cytotoxic drugs and

contributes to chemoresistance [19-22]. Bcl-XL is upregulated in myeloma cells at the time of relapse and correlates with a decreased response rate to subsequent chemotherapy [23]. Recently it was reported that a threshold level of Mcl-1 expression is required to prevent apoptosis and maintain viability of myeloma cells, marking Mcl-1 as a critical survival factor for myeloma cells [24-26].

In this study, we examined the importance of protein farnesylation and geranylgeranylation for the regulation of survival of myeloma tumor cells. We demonstrate that geranylgeranylation is essential for myeloma tumor cell survival through the regulation of Mcl-1 protein expression.

## Materials and methods

### Reagents

Lovastatin and simvastatin were obtained from Merck & Co., Inc (Rahway, NJ, USA) and were chemically activated by alkaline hydrolysis prior to use as described previously [27]. Pravastatin Sodium was purchased from Bristol-Meyers Squibb (New Brunswick, NJ, USA) and dissolved in PBS (20 mM). Atorvastatin Calcium was obtained from Pfizer GmbH (Freiburg, Germany) and dissolved in ethanol containing 3% DMSO (Riedel-de Haen, Seelze, Germany) (10 mM). Mevalonate and farnesol (FOH) were purchased from Sigma (St Louis, MO, USA) and geranylgeraniol (GGOH) was obtained from ICN Biomedicals, BV (Zoetermeer, The Netherlands). FOH and GGOH are metabolized to FPP and GGPP in the cells, respectively [28]. FTI-277 and GGTI-298 were obtained from Calbiochem (Schwallbach, Germany).

### Cell lines and patients

Plasma cell lines RPMI-8226 and U266 were obtained from the American Tissue Culture Collection (ATCC) and L363 from the German Collection of Microorganisms and Cell Cultures (GCMC). These cell lines were cultured in the absence of exogenous IL-6 [29]. The IL-6-dependent plasma cell line XG-1 was a kind gift of Dr B. Klein (Institute for Molecular Genetics, Montpellier, France) [30]. Cell lines were cultured in RPMI-1640 (GIBCO, Breda, The Netherlands) supplemented with 10% fetal calf serum (FCS) (Integro, Zaandam, The Netherlands), 100 IU/ml penicillin, 100 μg/ml streptomycin and 10 μM β-mercaptoethanol (growth medium). The IL-6-dependent cell line XG-1 was cultured in the continuous presence of exogenous IL-6 (1.25 ng/ml rhIL-6 (Roche, Almere, The Netherlands).

Myeloma plasma cells were obtained from bone marrow aspirates taken from the posterior iliac crest in 4 patients and from peripheral blood in one patient with plasma cell leukemia after obtaining informed consent. The plasma cell percentage in the patient

samples varied from 12-96% of the mononuclear cells as determined by co-expression of CD38 (anti-CD38-FITC, Immunotech, Marseille, France) and CD138 (anti-CD138-PE, Immunotech) by flow cytometric analysis (FACSCalibur, Becton Dickinson, Erembodegem, Belgium (BDIS)). Except for patient 2, who had 96% myeloma cells in her bone marrow, tumor cells were purified *ex vivo* from mononuclear cells obtained by Ficoll-Paque (Amersham; Pharmacia BiotechAB, Uppsala, Sweden) density centrifugation, by magnetic cell sorting (MACS) based on CD138 expression. To this end, mononuclear cells were subsequently labeled with anti-CD138 (Immunotech) and rat anti-mouse IgG1 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and then separated using a high gradient magnetic separation column placed in a strong magnetic field (Miltenyi), exactly following the instructions of the manufacturer. Samples obtained in this way contained >95% myeloma plasma cells as determined by analysis of CD38/CD138 co-expression. For experiments, myeloma cells were resuspended in RPMI-1640 (GIBCO) supplemented with 10% fetal calf serum (FCS) (Integro), 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10 μM β-mercaptoethanol.

**Cell viability**

Viability of cells was examined by means of the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay as described previously [14]. In short, cells were seeded in a concentration of 0.3 x $10^6$/ml for the myeloma cell lines or 1 X $10^6$/ml for the tumor cells of patients in a 96-well flat bottom plate (100 μl/well) (Nunc, Roskilde, Denmark) and treated with lovastatin (for concentrations see legends) alone or in the presence of mevalonate, FOH or GGOH. Fixed concentrations of mevalonate (100 μM), FOH (10 μM), or GGOH (10 μM) were used. These concentrations proved to be optimal in rescuing myeloma cells from lovastatin-induced apoptosis (data not shown). Inhibition of FTase and GGTase I was accomplished by treating cells with FTI-277 and GGTI-298, respectively (for concentrations see legends). After 2 or 4 days, 25 μl of MTT (5 mg/ml) was added to each well. After an incubation of 2 hours at 37°C the reaction was stopped by the addition of 100 μl 20% sodium dodecyl sulphate (SDS; Boehringer Mannheim, Mannheim, Germany) / 0.025 M HCl / 0.35 M HAc in a mixture of (1:1; v/v) N,N-dimethylformamide (Merck, Darmstadt, Germany) and distilled water. After an overnight incubation at 37°C the optical density of the samples was determined at 570 nm.

**Apoptosis detection by Annexin V staining**

Myeloma cells (1.5 X $10^5$ in 0.5 ml) were incubated with lovastatin (for concentrations see legends) alone or in the presence of mevalonate (100 μM), FOH (10 μM) or GGOH (10 μM) in a 48-well plate (Nunc). Inhibition of FTase and GGTase I was accomplished by treating cells with FTI-277 and GGTI-298 (for concentrations see legends), respectively. After 2 or 4 days, cells were harvested, washed in ice-cold PBS and directly

stained with Annexin V-FITC (Nexins Research, Kattendijke, The Netherlands) and propidium iodide (PI). After 10 minutes of incubation at room temperature in the dark, cells were analyzed by flow cytometry (FACSCalibur, BDIS) as described previously [14]. Apoptotic cells were defined as early apoptotic cells (Annexin V positive and PI negative) and late apoptotic cells (Annexin V positive and PI positive).

**Apoptosis detection by APO2.7 staining**

Myeloma cells (1.5 X $10^5$ in 0.5 ml) were incubated with lovastatin (for concentrations see legends) alone or in the presence of mevalonate (100 μM), FOH (10 μM) or GGOH (10 μM) in a 48-well plate (Nunc). Cells were washed in PBS with 2.5% FCS and permeabilized in 100 μg/ml Digitonin (Sigma) in PBS, and incubated for 20 minutes on ice. Cells were washed and resuspended in 80 μl PBS with 2.5% FCS and 20 μl APO2.7-PE (Immunotech), and incubated for 15 minutes at room temperature in the dark. After a washing step, cells were analyzed by flow cytometry (FACSCalibur, BDIS).

**Caspase-3 activity**

The caspase-3 activity assay (Roche) was used to determine caspase-3 activity. Briefly, cells were washed in ice-cold PBS and then resuspended in lysis buffer (1 X DTT) and incubated for 1 minute on ice. Supernatants were obtained after centrifugation at 14 000 r.p.m. for 1 minute at room temperature. Supernatant was added to anti-caspase-3 coated wells and incubated at 37$^o$C for 1 hour. After 3 washing steps, substrate solution (Ac-DEVD-AFC) was added and the wells were incubated for 2 hours at 37$^o$C. Fluorescence was measured with an excitation filter 400 nm and an emission filter 505 nm.

**Cytochrome c ELISA**

A cytochrome c ELISA kit (MBL, Watertown, MA, USA) was used to quantitate cytochrome c that was released from mitochondria into the cytosol. Briefly, cells were washed three times with ice-cold PBS and resuspended at the concentration of 5 X $10^6$ cells/ml in 10 mM Tris-HCl (pH 7.5), 0.3 M sucrose, and a cocktail of protease inhibitors (Boehringer Mannheim). Cells were homogenized by douncing 10 times in a Dounce homogenizer with a sandpaper-polished pestle. After a centrifugation step at 4$^o$C for 60 minutes at 14 000 r.p.m. the cytosolic fraction was present in the supernatant. Sixty μl of the 1:5 diluted cytosolic fraction was mixed with 60 μl conjugate reagent. Hundred μl of the mixture was added to anti-cytochrome c coated wells and incubated for 60 minutes at room temperature. After 4 washing steps, substrate solution was added and the wells were incubated for 15 minutes at room temperature. After addition of stop solution, the absorbance of each well was read at 450 nm.

**Measurement of mitochondrial transmembrane potential**

Changes in mitochondrial transmembrane potential ($\Delta\psi_m$) were evaluated by staining with 40 nM 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6[3]$; Molecular Probes, Leiden, The Netherlands). Cells were incubated with $DiOC_6[3]$ in PBS for 15 minutes at 37°C, washed and resuspended in PBS. The cells were then analyzed on a flow cytometer (FACSCalibur, BDIS).

**Western blotting**

Cells (1 X $10^6$ cells in 1.5 ml) were incubated for 2 or 4 days with lovastatin (for concentrations see legends) in the presence or absence of mevalonate (100 μM), FOH (10 μM) or GGOH (10 μM). Inhibition of FTase and GGTase I was accomplished by treating cells with FTI-277 and GGTI-298 (for concentrations see legends), respectively. After harvesting, whole cell lysates were made by washing cells twice in ice-cold PBS and then resuspending them in lysis buffer (10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% Triton X-100 and a cocktail of protease inhibitors (Boehringer Mannheim)) at 4°C for 20 minutes. Insoluble material was removed by centrifugation at 14 000 r.p.m. for 6 minutes at 4°C. When membrane and cytosolic protein fractions were analyzed, cells were suspended in extraction solution (10% glycerol, 20 mM Hepes, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT and a cocktail of protease inhibitors (Boehringer Mannheim)). The lysate was sonificated twice for 5 seconds and centrifuged at 4°C for 30 minutes at 48 000 *g*. The supernatant contained the proteins of the cytosolic fraction. The insoluble material was subsequently resuspended in solubilization buffer (1% NP-40, 20 mM Hepes, 20 mM MgAc, 5 mM NaF, 0.2 mM EDTA, 0.8 mM EGTA, 1 mM DTT and a cocktail of protease inhibitors). After 2 rounds of sonification for 10 seconds, an incubation for 30 minutes at 4°C and a centrifugation step at 4 °C for 30 minutes at 48 000 *g*, proteins of the membrane fraction were present in the supernatant.

Protein concentrations were determined by the BCA assay (Pierce, Rockford, IL, USA). Samples containing equal amounts of protein were mixed with 2 X Laemmli sample buffer (0.125 M Tris pH 6.9 with 4% SDS, 20% Glycerol and 10% β-mercaptoethanol) and boiled for 5 minutes. Proteins were subsequently fractionated in 10% SDS-PAGE at room temperature and electrically transferred from the gel to PVDF-membrane (Biorad). After blocking in 0.1% Tween-20, 5% skimmed powder milk, 2% BSA in 10 mM Tris and 150 mM NaCl, the membranes were incubated with anti-Bcl-2 (Dako, Glostrup, Denmark), anti-Mcl-1 (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA), anti-Bcl-XL (Santa Cruz), anti-Bax (Immunotech), anti-Rap1a (Santa Cruz), or anti-HDJ-2 (DnaJ) (Neomarkers, Lab Vision, Fremont, CA, USA). Antibody binding was visualized with enhanced chemoluminescence (Amersham) detection with hyperfilm ECL (Amersham) after incubation with a horseradish peroxidase (HRP)-conjugated secondary

antibody (Dako). Finally, the membranes were extensively washed in PBS and reprobed with anti-α-actin (Sigma) as a control for equal loading of protein. Relative amounts of protein were determined by densitometry and expressed as a percentage of the solvent control.

## Results

**Inhibition of prenylation by lovastatin, FTI-277, and GGTI-298 in myeloma cell lines**

The effect of lovastatin, FTase inhibitor FTI-277, and GGTase I inhibitor GGTI-298 on prenylation in myeloma cell lines was determined by analysis of the migratory behaviour during electrophoresis of DnaJ, a protein prenylated exclusively by FTase [31,32], and of Rap1a, a protein prenylated exclusively by GGTase I [33]. Inhibition of prenylation of these proteins can be monitored by immunoblotting, because the unprenylated forms of these proteins display reduced mobility in SDS-PAGE relative to their prenylated versions. All experiments were performed with the myeloma cell lines RPMI-8226, L363, U266, and XG-1. For brevity, only representative results from the cell line RPMI-8226 are shown in Figure 1A. In solvent control-treated myeloma cell lines DnaJ and Rap1a were in the processed, prenylated, forms. Treatment with lovastatin inhibited the processing of DnaJ and of Rap1a in a dose-dependent way, resulting in unprenylated protein forms with reduced electrophoretic mobility (Figure 1A). The effect of lovastatin on prenylation was reversed by the addition of mevalonate (shown for 30 μM lovastatin and 100 μM mevalonate). Treatment with lovastatin (30 μM) in the presence of GGOH (10 μM), which is metabolized to GGPP in the cells [28], restored geranylgeranylation of Rap1a, but had no effect on the inhibition of the farnesylation of DnaJ. In contrast, FOH (10 μM), which is metabolized to FPP [28], restored DnaJ farnesylation, but not Rap1a geranylgeranylation. Inhibition of FTase by FTI-277 disrupted the processing of DnaJ in a dose-dependent way. Similarly, a dose-dependent mobility shift of Rap1a was observed on exposure to GGTI-298. The specificity of these prenylation inhibitors is illustrated by the lack of inhibition of Rap1a prenylation by FTI-277, and, likewise, the lack of inhibition of DnaJ prenylation by GGTI-298 (Figure 1B).

Prenylation is crucial for the membrane attachment of proteins. This is shown in Figure 1C for RhoA, which is geranylgeranylated by GGTase I. Lovastatin reduced the amount of RhoA present in the membrane protein fraction and increased the amount present in the cytosolic protein fraction when compared with the solvent control. Co-treatment of cells with mevalonate or GGOH, but not FOH, prevented the cytosolic accumulation and restored the membrane localization of RhoA.

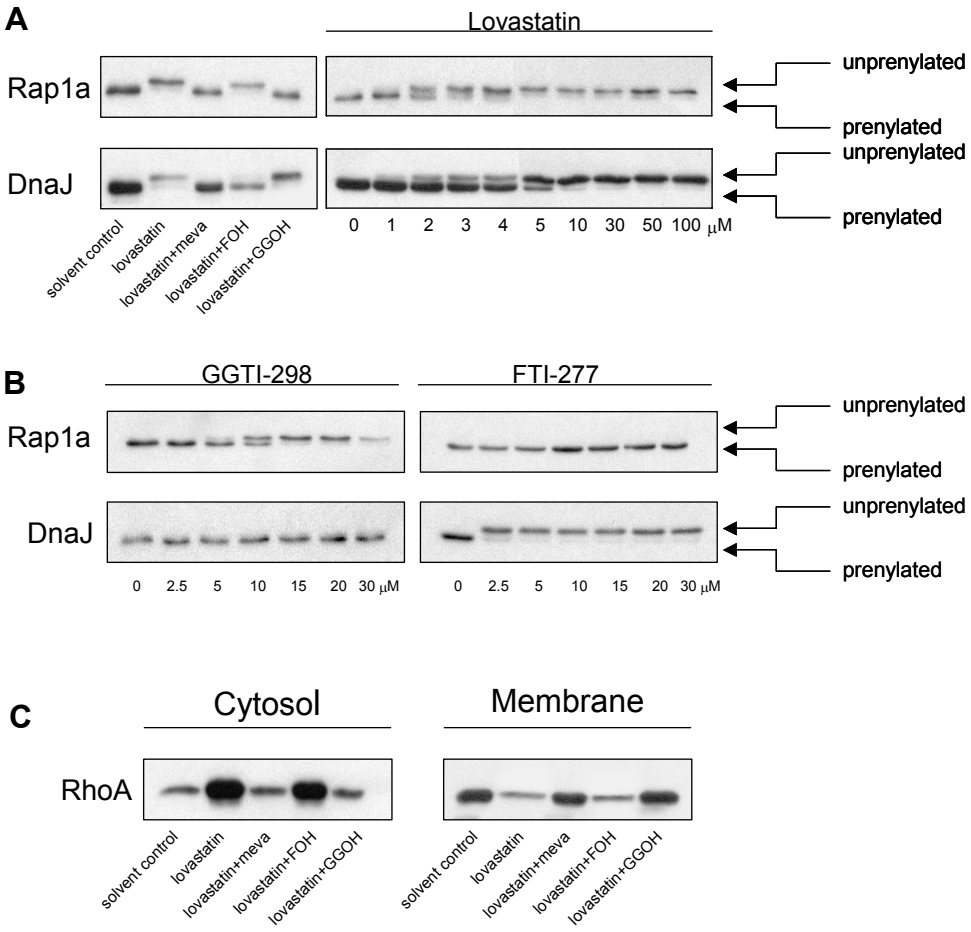


**Figure 1. Effect of lovastatin, FTI-277, and GGTI-298 on farnesylation and geranylgeranylation.** (A) RPMI-8226 cells were treated for 2 days with solvent control, or lovastatin (30 μM) alone or in the presence of mevalonate (meva; 100 μM), GGOH (10 μM), or FOH (10 μM). For the dose-response, RPMI-8226 cells were treated with solvent control or lovastatin (1, 2, 3, 4, 5, 10, 30, 50, 100 μM). After protein isolation, prenylation status of DnaJ and Rap1a was determined by Western blot analysis. The faster-migrating band represents prenylated protein, the slower band represents unprenylated protein. The data shown are representative of at least three independent experiments. (B) RPMI-8226 cells were treated for 2 days with solvent control, FTI-277 (2.5, 5, 10, 15, 20, or 30 μM), or GGTI-298 (2.5, 5, 10, 15, 20, or 30 μM). After protein isolation, prenylation status of DnaJ and Rap1a was determined by Western blot analysis. The data shown are representative of at least three independent experiments. (C) RPMI-8226 cells were exposed to solvent control, or lovastatin (30 μM) alone or in the presence of mevalonate (100 μM), GGOH (10 μM), or FOH (10 μM). After a 2 day incubation, protein was separated in membrane and cytosolic fractions. RhoA was identified by Western blot analysis. The data are representative of three independent experiments.

**Depletion of intracellular pools of geranylgeranylpyrophosphate induces apoptosis in myeloma cell lines**

Previously we have shown that lovastatin reduces cell viability in myeloma cell lines and in *ex vivo* purified myeloma tumor cells [14]. Reduction of cell viability was abrogated by the addition of mevalonate. This suggests that the effect of lovastatin resulted from inhibition of mevalonate formation and not from non-specific cell toxicity. Here we examined the effect of the simultaneous exposure of cells to lovastatin and the isoprenoids GGOH or FOH, which are metabolites downstream of mevalonate. A dose-response analysis revealed a reduction of cell viability by lovastatin beginning at a concentration of 1 μM for XG-1 cells, 2 μM for RPMI-8226 and L363 cells, and 3 μM for U266 cells. Lovastatin sensitivity varied among the myeloma cell lines, with MTT50 (concentration that reduces cell viability with 50%) ranging from 1.86 μM (XG-1) to 10.17 μM (U266) at day 4 (Figure 2). GGOH (10 μM), but not FOH (10 μM), prevented lovastatin-induced reduction of myeloma cell viability (Figure 2). This demonstrates that inhibition of protein geranylgeranylation by lovastatin results in reduction of cell viability.

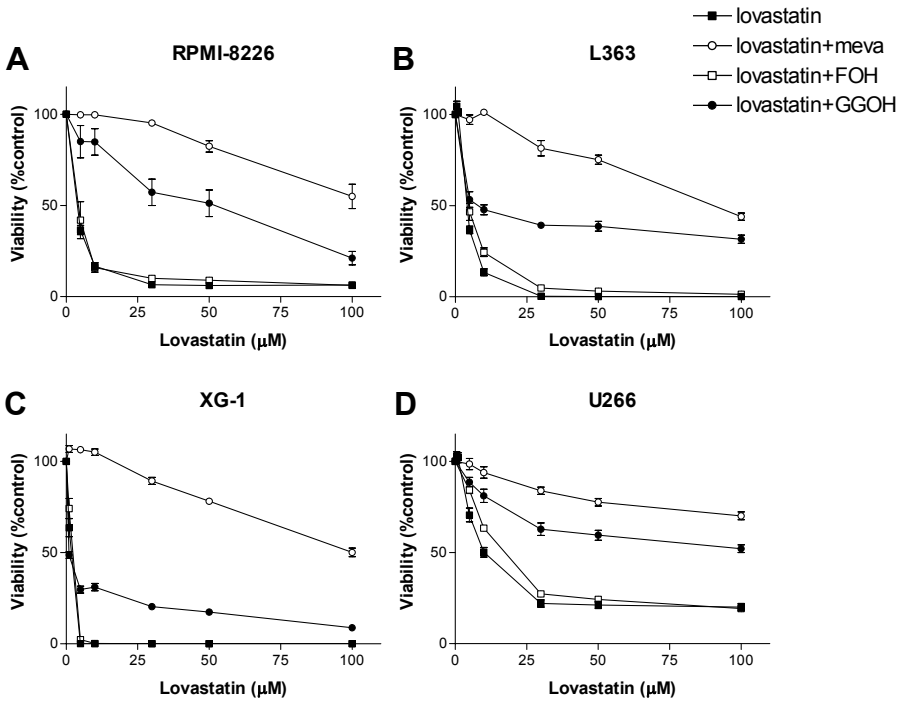


**Figure 2. Lovastatin reduces cell viability in plasma cell lines, which is restored by GGOH.** Plasma cell lines (A) RPMI-8226, (B) L363, (C) XG-1, and (D) U266 were treated for 4 days with solvent control, or different concentrations of lovastatin (5, 10, 30, 50, 100 μM) alone or in the presence of mevalonate (meva; 100 μM), GGOH (10 μM), or FOH (10 μM). The percentage of viable cells, relative to the solvent control-treated cells, was measured by using MTT assay. Experiments were performed three times in triplicate. Data are presented as mean ± s.e.m.

Then it was investigated whether the reduction of cell viability was mediated by induction of apoptosis by using the Annexin V assay. Lovastatin induced apoptosis in a dose-dependent way in the U266, L363, RPMI-8226, and XG-1 cell lines. Mevalonate and GGOH prevented lovastatin-induced apoptosis. FOH was without effect (RPMI-8226 and XG-1) or had only partial protective effects (L363 and U266) (Figure 3 A and B). These data were confirmed by the TUNEL (terminal deoxynucleotidyl transferase [TdT]-mediated deoxyuracil triphosphate [dUTP] nick-end labeling) assay (data not shown).

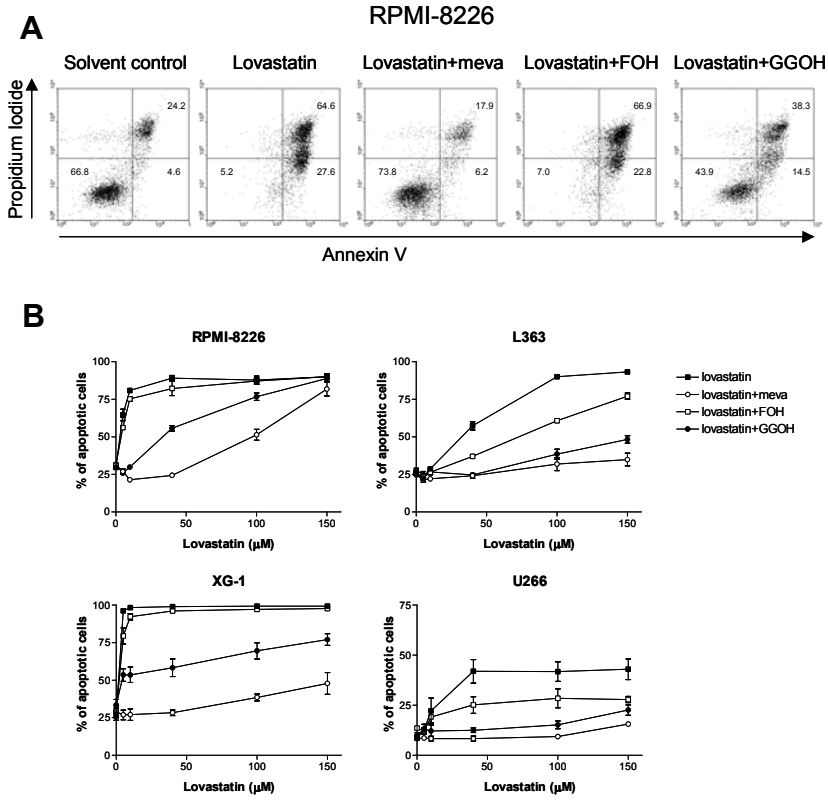


**Figure 3. Lovastatin induces apoptosis in plasma cell lines by depletion of intracellular pools of GGPP.** (A) RPMI-8226 cells were treated for 4 days with solvent control, or lovastatin (30 μM) alone or in combination with mevalonate (meva; 100 μM), GGOH (10 μM), or FOH (10 μM). The percentage of apoptotic cells was examined by using the Annexin V assay. The percentages of viable plasma cells (Annexin V-/PI-), early apoptotic cells (Annexin V+/PI-), and late apoptotic cells (Annexin V+/PI+) in each dot plot are indicated in the corresponding quadrants. Results are representative of three experiments performed in triplicate. (B) RPMI-8226, L363, XG-1, and U266 cells were treated for 4 days with solvent control, or different concentrations of lovastatin (5,10, 40, 100, 150 μM) in combination with mevalonate (meva; 100 μM), GGOH (10 μM), or FOH (10 μM), after which apoptosis was determined by the Annexin V assay. Shown is the sum of the percentages of early and late apoptotic cells. Experiments were performed three times in triplicate. Data are presented as mean ± s.e.m.

Identical results were obtained with other inhibitors of HMG-CoA reductase including simvastatin and atorvastatin. Cells from the different myeloma cell lines were most sensitive to simvastatin, followed by lovastatin and atorvastatin. However, the hydrophilic HMG-CoA reductase inhibitor pravastatin had no effect (data not shown).

**Inhibition of geranylgeranyl transferase I induces apoptosis in myeloma cell lines**

The importance of protein geranylgeranylation versus farnesylation for the regulation of survival of myeloma cells was further evaluated by treating cell lines with different concentrations of FTI-277 or GGTI-298 for 2 or 4 days. Cell viability was examined by MTT assay. GGTI-298 and FTI-277 reduced cell viability in a dose- and time-dependent way in the 4 plasma cell lines analyzed (Figure 4A). However, GGTI-298 had, by far, the most pronounced effect on cell viability when compared to FTI-277. The reduction of cell viability at day 2 and 4 by GGTI-298 (20 μM) in the cell lines varied from 52.9 to 86.2% and 40.4 to 98.7%, whereas the reduction by FTI-277 (20 μM) varied from 22.1 to 43.7% and 0 to 57.0%, respectively. The reduction of cell viability by GGTI-298 was mediated by induction of apoptosis (Figure 4B). In all cell lines, treatment with GGTI-298 (20 μM) increased the percentage of apoptotic cells when compared with the solvent control. In contrast, FTI-277 (20 μM) induced low to moderate apoptosis in XG-1 and L363 cells, and did not induce apoptosis in U266 and RPMI-8226 cells.

**Inhibition of geranylgeranylation reduces Mcl-1 protein levels**

Inhibition of geranylgeranylation either by depletion of intracellular pools of GGPP or by inhibition of GGTase I, induces apoptosis in myeloma cell lines. To investigate how inhibition of geranylgeranylation results in apoptosis, the expression levels of several Bcl-2 family proteins were determined by Western blot analysis in cells treated with lovastatin in combination with mevalonate, FOH, or GGOH. Lovastatin treatment resulted in a down-regulation of Mcl-1 protein levels at both day 2 and 4 in RPMI-8226, L363, U266, and XG-1 cells when compared with solvent control-treated cells (Figure 5). The Mcl-1 down-regulation by lovastatin preceded (U266 cells) or was concomitant (RPMI-8226, L363, and XG-1 cells) with apoptosis induction. Co-treatment of cells with mevalonate or GGOH restored Mcl-1 protein expression. Co-inbubation of myeloma cell lines with FOH was without effect. Lovastatin treatment also resulted in a reduction of Bcl-XL protein levels in U266, L363, and XG-1 cells and an increase in Bax protein levels in RPMI-8226 and XG-1 cells. However, the altered expression levels of Bcl-XL and Bax were relatively late effects and observed only after 4 days of treatment. Bcl-XL was detected as a doublet of mass 29-31 kD, which represent two conformations of Bcl-XL that migrate differently in SDS-page [23]. Lovastatin did not alter the expression levels of Bcl-2. However, there was a time-dependent up-regulation of the pro-apoptotic 23 kD Bcl-2 form, which could be visualized after long exposure of the film when compared with Bcl-2. Bcl-XS expression was not detected in the different plasma cell lines. Mevalonate and GGOH

prevented lovastatin-mediated reduction of Bcl-XL, and the increase of Bax and the pro-apoptotic Bcl-2 form. In contrast, FOH was without effect. Similar results were obtained with simvastatin and atorvastatin, whereas pravastatin had no effect (data not shown).

Similar to lovastatin, GGTI-298 also reduced Mcl-1 protein levels after 2 days in all 4 plasma cell lines tested (Figure 6). Furthermore, GGTI-298 treatment resulted in an increase of the pro-apoptotic 23 kD Bcl-2 form and reduced Bcl-XL expression levels in L363 and XG-1 cell lines. FTI-277 did not alter Mcl-1, Bcl-XL, Bax, or Bcl-2 protein expression and did not consistently change expression of the pro-apoptotic 23 kD Bcl-2 form.

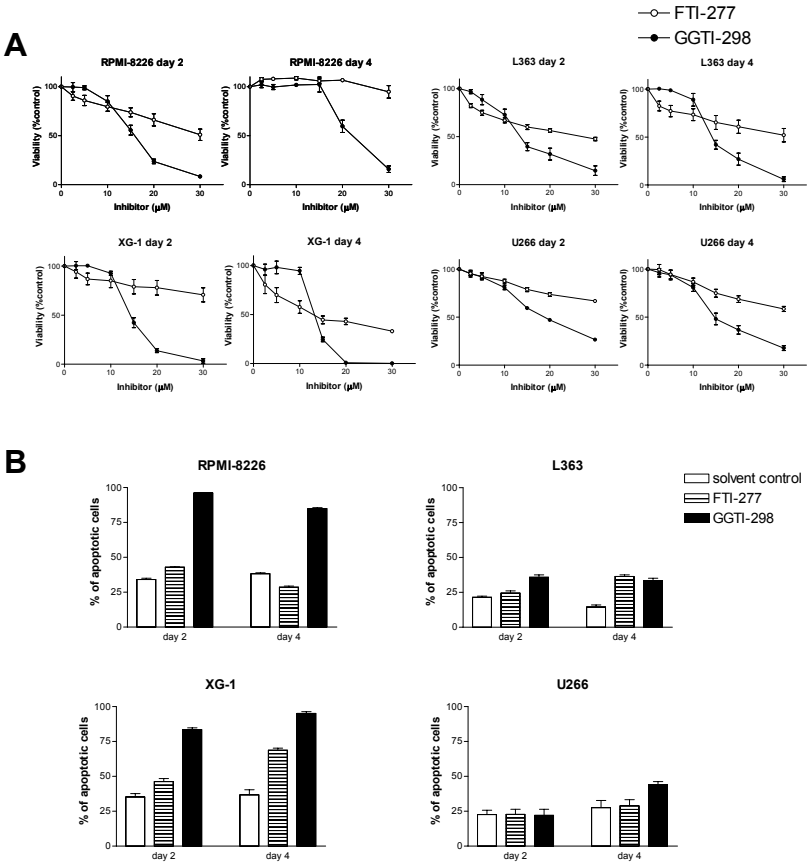


**Figure 4. Inhibition of GGTase I by GGTI-298 reduces cell viability and induces apoptosis**. (A) Plasma cell lines RPMI-8226, L363, XG-1, and U266 were treated for 2 or 4 days with solvent control, different concentrations of the farnesyl transferase inhibitor FTI-277 (2.5, 5, 10, 15, 20, 30 μM), or with different concentrations of the geranylgeranyl transferase I inhibitor GGTI-298 (2.5, 5, 10, 15, 20, 30 μM). The percentage of viable cells, relative to the solvent control-treated cells, was measured by using MTT assay. Experiments were performed three times in triplicate. Data are presented as mean ± s.e.m. (B) Plasma cell lines RPMI-8226, L363, XG-1, and U266 were treated for 2 or 4 days with solvent control, FTI-277 (20 μM), or with GGTI-298 (20 μM). The percentage of apoptotic cells was determined by Annexin V assay. Shown is the sum of the percentages of early and late apoptotic cells. Experiments were performed three times in triplicate. Data are presented as mean ± s.e.m.

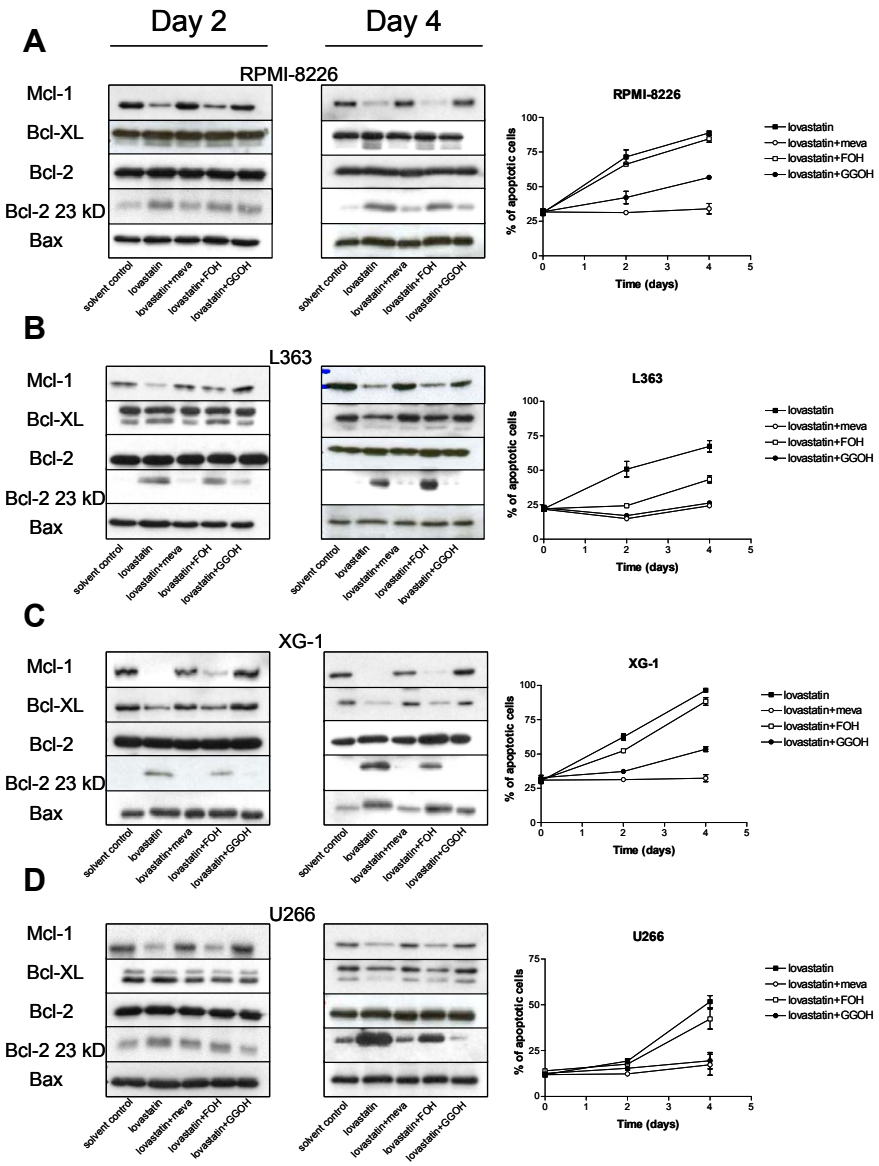


**Figure 5. Lovastatin reduces Mcl-1 protein levels by depletion of intacellular pools of GGPP.** Plasma cell lines (A) RPMI-8226, (B) L363, (C) XG-1, and (D) U266 were treated for 2 or 4 days with solvent control, or lovastatin (5 μM for XG-1 and 30 μM for RPMI-8226, U266, and L363) alone or in combination with mevalonate (meva; 100 μM), FOH (10 μM), or GGOH (10 μM). After protein isolation, Mcl-1, Bcl-XL, Bcl-2, and Bax were determined by Western blot analysis. Furthermore, the pro-apoptotic 23 kD Bcl-2 form was detected after long exposure of the film. The data shown are representative of at least three independent experiments. The percentage of apoptotic cells was determined by Annexin V assay. Shown is the sum of the percentages of early and late apoptotic cells. Experiments were performed three times in triplicate. Data are presented as mean ± s.e.m.

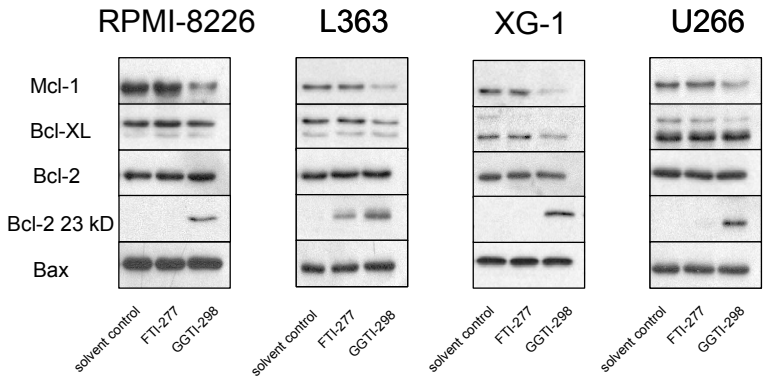


**Figure 6. GGTI-298 reduces Mcl-1 protein levels.** Plasma cell lines RPMI-8226, L363, XG-1, and U266 were treated for 2 days with solvent control, FTI-277 (20 μM), or GGTI-298 (20 μM). After protein isolation, Mcl-1, Bcl-XL, Bcl-2, and Bax expression levels were determined by Western blot analysis. Furthermore, the pro-apoptotic 23 kD Bcl-2 form was detected after long exposure of the film. The data shown are representative of at least three independent experiments.

**Inhibition of geranylgeranylation results in reduction of the mitochondrial transmembrane potential, APO2.7 staining, release of cytochrome c, and caspase activation**

Members of the Bcl-2 family are involved in the regulation of the mitochondrial transmembrane potential, release of cytochrome c, and caspase-3 activation [17,18]. Treatment of RPMI-8226, L363, U266, and XG-1 cells with lovastatin resulted in loss of the mitochondrial transmembrane potential as shown in Figure 7A for RPMI-8226 cells. The collapse was time- (data not shown) and dose-dependent (Figure 7B). APO2.7 reacts with a mitochondrial membrane protein (7A6 antigen) that is exposed in cells undergoing apoptosis. Lovastatin increased the percentage of APO2.7-positive cells in a time- (data not shown) and dose-dependent way (Figure 7 A and B). This together with the loss of the mitochondrial transmembrane potential suggests that mitochondrial changes were induced by lovastatin.

Collapse of the mitochondrial transmembrane potential results in the release of several pro-apoptogenic factors such as cytochrome c from the mitochondria into the cytosol [17,18]. Treatment of myeloma cell lines with lovastatin resulted in an increase of cytosolic cytochrome c as shown for RPMI-8226 cells in Figure 7C.

Cytosolic cytochrome c promotes activation of caspases through the cytochrome c/Apaf-1/caspase-9 pathway [17,18]. To determine whether lovastatin-induced cytochrome c release resulted in activation of caspase-3, cell lysates were analyzed for caspase-3 activity. Treatment of plasma cell lines with lovastatin resulted in activation of caspase-3 as shown for RPMI-8226 cells in Figure 7D. Bcl-2 is a known substrate of caspase-3 [34,35]. Exposure of cell lines to lovastatin stimulates caspase-3 activity, which, in turn, results in the generation of the Bcl-2 cleavage product that promotes apoptosis. The time-dependent

increase of the pro-apoptotic 23 kD Bcl-2 form was demonstrated by Western blot analysis (Figure 5).

Importantly, incubation of the plasma cell lines with lovastatin in the presence of mevalonate or GGOH, markedly prevented the collapse of the mitochondrial transmembrane potential, APO2.7 staining, the release of cytochrome c from mitochondria, and the activation of caspase-3 (as shown for myeloma cell line RPMI-8226 in Figure 7A-D). In contrast, co-treatment of cells with FOH had no effect when compared to cells treated with lovastatin alone.

**Inhibition of geranylgeranylation induces apoptosis in *ex vivo* purified tumor cells from myeloma patients**

Studies were then conducted to determine the effect of lovastatin in purified tumor cells from multiple myeloma patients (n=5). Except for patient 2 who had 96% myeloma cells in her bone marrow, tumor cells were purified from mononuclear cells by MACS based on the expression of CD138. The percentage of tumor cells was >95%. Treatment of *ex vivo* purified myeloma cells with 30 μM lovastatin resulted in the reduction of myeloma cell viability in all patients (Table 1). Moreover, as in cell lines treatment of *ex vivo* purified myeloma tumor cells with lovastatin reduced Mcl-1 protein levels (Table 1). Similar to the effects in plasma cell lines, addition of mevalonate or GGOH resulted in complete inhibition of lovastatin-induced reduction of cell viability and lovastatin-induced apoptosis. However, co-incubation of cells with FOH had no effect. Co-treatment of tumor cells with mevalonate or GGOH restored Mcl-1 protein expression, whereas FOH was without effect. Figure 8 shows representative examples from 2 myeloma patients. Only in tumor cells from patient 2, lovastatin treatment additionally reduced Bcl-XL protein expression (>50% reduction when compared to the solvent control) and resulted in the increase of the pro-apoptotic Bcl-2 cleavage product.

**Table 1.** Effect of lovastatin on expression of Bcl-2 family proteins and viability in purified tumor cells derived from myeloma patients

| Patient | Mcl-1 | Bcl-XL | Bcl-2 | Bax | Viability |
|---|---|---|---|---|---|
| 1 | 3.7 | 86.8 | 103.1 | 76.7 | 18.4 |
| 2 | 4.8 | 33.0 | 144.9 | 181.5 | 18.9 |
| 3 | 21.8 | 69.7 | 103.7 | 92.5 | 60 |
| 4 | 2.3 | 112.0 | 95.0 | 98.7 | 23.7 |
| 5 | 3.3 | 82.5 | 101.5 | 76.9 | 4.6 |

Purified myeloma cells were treated with solvent control or 30 μM lovastatin for 4 days. After protein isolation, Mcl-1, Bcl-XL, Bcl-2, and Bax were detected by Western blot analysis. Protein levels were quantitated by densitometry analysis of the protein bands and expressed as a percentage of the solvent control values. The percentage of viable cells, relative to the solvent control-treated cells, was measured by using MTT assay.

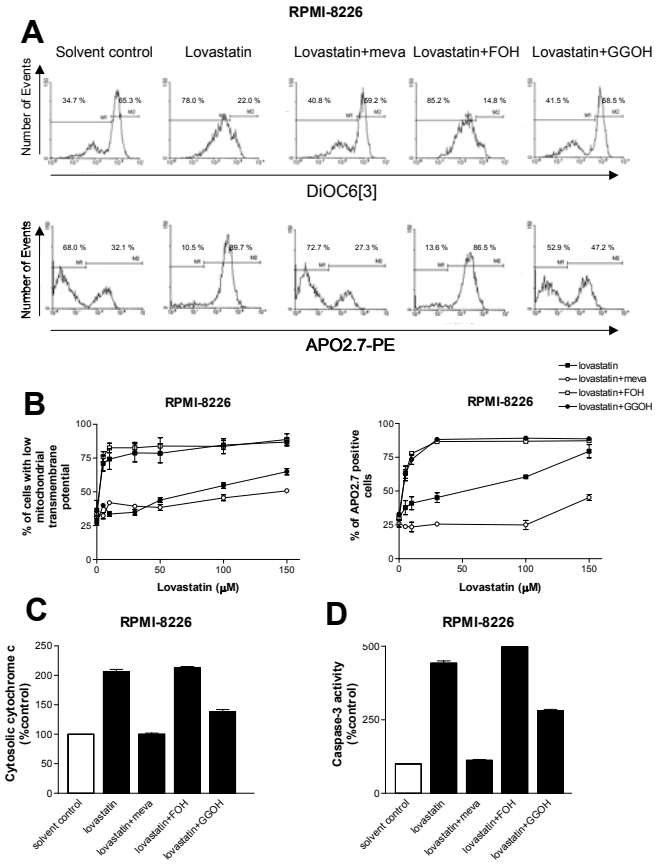


**Figure 7. Lovastatin treatment results in collapse of the mitochondrial transmembrane potential, APO2.7 staining, release of cytochrome c into the cytosol, and caspase-3 activation by depletion of GGPP.** (A) RPMI-8226 cells were treated for 4 days with solvent control, or lovastatin (30 μM) alone or in combination with mevalonate (meva; 100 μM), FOH (10 μM), or GGOH (10 μM). Collapse of the mitochondrial transmembrane potential was determined by staining the cells with $DiOC_6[3]$. The fraction of cells with low ($DiOC_6[3]^{low}$) and the fraction of cells with intact ($DiOC_6[3]^{high}$) mitochondrial transmembrane potential are indicated in gate M1 and M2, respectively. APO2.7 staining was determined by flow cytometry in Digitonin-permeabilized cells. The fraction of cells staining with APO2.7-PE (M2) and the fraction of cells not staining with APO2.7-PE (M1) are indicated. Results are representative of 3 experiments performed in triplicate (B) RPMI-8226 cells were treated for 4 days with solvent control, or different concentrations of lovastatin (5, 10, 30, 50, 100, 150 μM) alone or in combination with mevalonate (meva; 100 μM), FOH (10 μM), or GGOH (10 μM). Collapse of the mitochondrial transmembrane potential was determined by staining the cells with $DiOC_6[3]$. Shown is the percentage of cells with low mitochondrial transmembrane potential (gate M1). APO2.7 staining was determined by flow cytometry in Digitonin-permeabilized cells. Shown is the percentage of cells staining with APO2.7-PE (gate M2). Experiments were performed three times in triplicate. Data are presented as mean ± s.e.m. (C) RPMI-8226 cells were exposed to solvent control, or lovastatin (30 μM) alone or in combination with mevalonate (meva; 100 μM), FOH (10 μM), or GGOH (10 μM) for 2 days, at which time the cells were harvested and cytochrome c present in the cytosolic fraction was determined by ELISA. Experiments were performed three times in duplicate. Data are presented as mean ± s.e.m. (D) RPMI-8226 cells were exposed to solvent control, or lovastatin (30 μM) alone or in combination with mevalonate (meva; 100 μM), FOH (10 μM), or GGOH (10 μM) for 2 days, at which time the cells were harvested and caspase-3 activation was assessed as described in "Materials and Methods." Experiments were performed three times in duplicate. Data are presented as mean ± s.e.m.

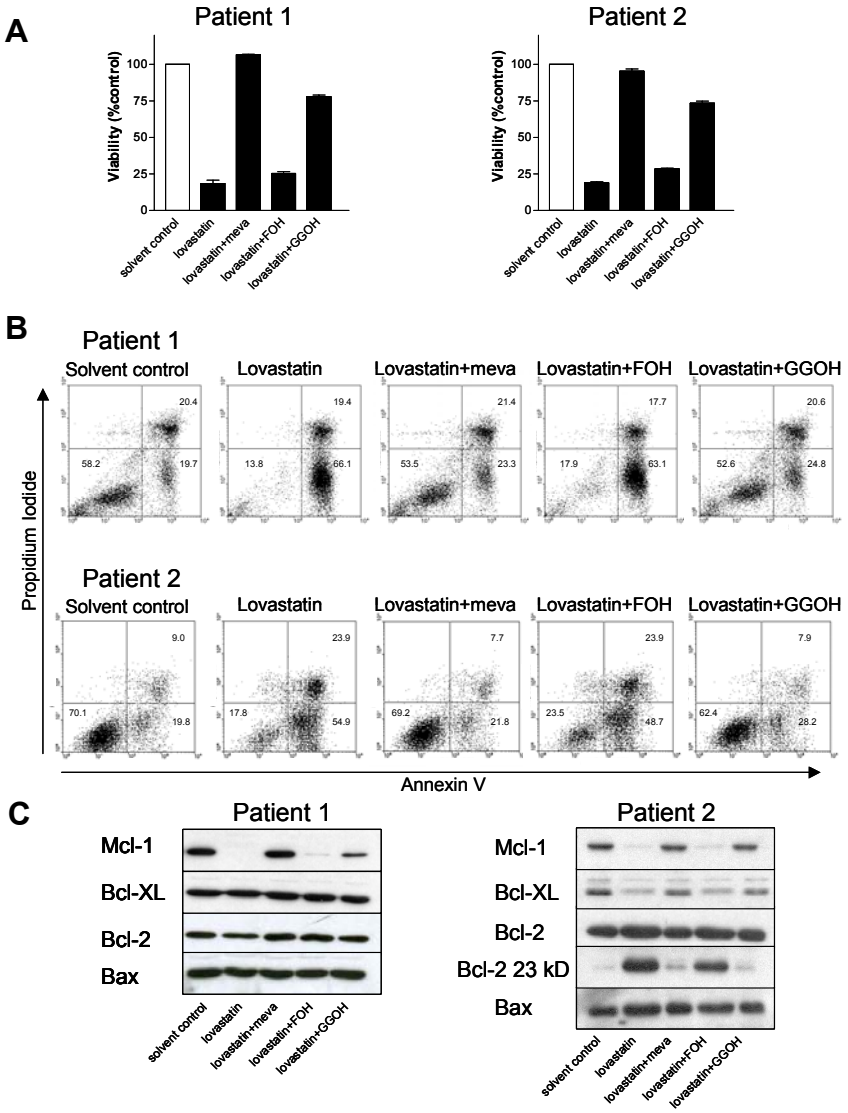


**Figure 8. Treatment of *ex vivo* purified myeloma cells with lovastatin results in reduction of cell viability and induction of apoptosis by reducing Mcl-1 protein levels.** Plasma cells from myeloma patient 1 were purified from bone marrow mononuclear cells by MACS based on CD138 expression. Plasma cell percentage was 97% after purification. Patient 2 had 96% myeloma cells in her bone marrow mononuclear cells and therefore purification was not necessary. Tumor cells derived from patients 1 and 2 were treated for 4 days with solvent control, or lovastatin (30 μM) alone or in the presence of mevalonate (meva; 100 μM), FOH (10 μM), or GGOH (10 μM). (A) The percentage of viable cells, relative to the solvent control-treated cells, was measured by using MTT assay. Experiments were performed once in triplicate. Data are presented as mean ± s.e.m. (B) The percentage of apoptotic cells was determined by the Annexin V assay. The percentages of viable myeloma cells (Annexin V-/PI-), early apoptotic cells (Annexin V+/PI-), and late apoptotic cells (Annexin V+/PI+) in each dot plot are indicated in the corresponding quadrants. (C) After protein isolation, Mcl-1, Bcl-XL, Bcl-2, and Bax expression levels were determined by Western blot analysis. Furthermore, the pro-apoptotic 23 kD Bcl-2 form was detected after long exposure of the film.

## Discussion

In this study we demonstrated that inhibition of protein geranylgeranylation induced apoptosis in myeloma plasma cells. Inhibition of geranylgeranylation was established in two independent ways, by depletion of intracellular pools of GGPP by lovastatin and by specific inhibition of GGTase I activity. Incubation of myeloma cells with lovastatin effectively depleted pools of FPP and GGPP resulting in a dose-dependent inhibition of protein farnesylation and geranylgeranylation. This was demonstrated by analysis of the migratory behaviour of DnaJ and Rap1a during electrophoresis. Addition of FOH, which is converted to FPP in cells [28], to lovastatin-treated myeloma cells restored farnesylation of DnaJ and addition of GGOH, which is converted to GGPP [28], restored geranylgeranylation of Rap1a. In addition, geranylgeranylation and farnesylation were specifically blocked in a dose-dependent way by GGTase I and FTase inhibitors GGTI-298 and FTI-277, respectively. In functional assays, addition of GGOH, but not FOH, rescued myeloma cells from lovastatin-induced apoptosis. However, GGOH was less effective than mevalonate in the rescue of myeloma cell survival. This suggests that depletion of other metabolites downstream of mevalonate may also contribute partly to the effects of lovastatin. The GGTase I inhibitor GGTI-298 also induced apoptosis in myeloma cells in a time- and dose-dependent way. Both inhibition of geranylgeranylation by lovastatin and GGTI-298 preceded the reduction of cell viability. Rescue of farnesylation by addition of FOH to lovastatin-treated cells was without effect. Furthermore, although the FTase inhibitor FTI-277 completely blocked farnesylation, it was less effective in the induction of apoptosis, when compared with GGTI-298. These results point to an important role for geranylgeranylated proteins in the regulation of apoptosis in myeloma plasma cells.

The induction of apoptosis by inhibition of geranylgeranylation either by lovastatin or GGTI-298 was preceded or was concomitant with a consistent reduction of Mcl-1 protein expression in all myeloma cell lines and patients' samples. Down-regulation of Mcl-1 protein expression at day two was accompanied by disruption of the mitochondrial transmembrane potential, increased APO2.7 staining, cytochrome c release, and activation of caspase-3; suggesting that apoptosis was carried out via the intrinsic cell-death pathway. In some cell lines and patients' samples a reduction of Bcl-XL expression levels or an induction of Bax or the pro-apoptotic 23 kD Bcl-2 form was found. However, this was not a consistent finding or only observed 4 days after inhibition of geranylgeranylation. The observed temporal sequence in the regulation of expression of apoptosis regulating proteins, suggests for the first time that down-regulation of Mcl-1 is the key event in the induction of apoptosis by inhibition of geranylgeranylation.

In multiple myeloma, Mcl-1 is one of the key-regulators of apoptosis [24-26]. Specific depletion of Mcl-1 by antisense oligodeoxynucleotides resulted in rapid cell

death, whereas overexpression of Mcl-1 in myeloma cells delayed the activation of caspases in a manner consistent with the hypothesis that a threshold level of Mcl-1 is required for myeloma cell survival [24-26]. In addition, Mcl-1 promotes cell viability under a variety of apoptosis-inducing conditions including exposure to cytotoxic agents and the withdrawal of required growth factors [36]. Similarly, high levels of Mcl-1 have been correlated with failure to achieve complete remission to chemotherapy in CLL [37]. At present, it is unclear which pathway(s) are involved in the down-regulation of Mcl-1 protein expression in myeloma cells after lovastatin treatment or GGTase I inhibition. Studies using a variety of cell types imply that multiple pathways, including PI-3K, JAK/STAT3, and MEK/ERK, are involved in the regulation of Mcl-1 transcription [38]. However, recent studies performed by Zhang et al [39] suggest that Mcl-1 regulation in myeloma is more complex and may be independent from the above mentioned signaling pathways.

Statins have been shown to induce apoptosis in other tumor cell lines and *ex vivo* purified tumor cells [14,40-42]. Similar to myeloma, in acute myeloid leukemia (AML) [40], lung adenocarcinoma [41], and in pulmonary smooth muscle cells [42], the effect of lovastatin could be attributed to inhibition of geranylgeranylation. Identical results as presented here for myeloma, were obtained with lymphoma cell lines and purified tumor cells from patients with B-cell non-Hodgkin's lymphoma (manuscript in preparation). In addition to the induction of apoptosis, recent studies have shown that statins also enhance new bone formation by stimulating osteoblast differentiation and activity [43] and inhibit bone resorption by inhibition of osteoclast formation and function [44]. This action may be beneficial in myeloma patients with bone disease. Furthermore, in various cell types HMG-CoA reductase inhibitors inhibit expression of cytokines including the important myeloma growth factor IL-6 [45]. This indicates that in addition to induction of apoptosis by Mcl-1 down-regulation, these pleiotropic properties of statins may have important additional clinical implications in the treatment of multiple myeloma.

At present it is unclear which geranylgeranylated target protein(s) are involved in the regulation of myeloma tumor cell apoptosis. Possible geranylgeranylated target proteins include RhoA, Cdc42, and Rac-1. These small GTP-binding proteins are involved in important cellular functions, such as organization of the cytoskeleton [46]. In addition, they are also involved in regulation of apoptosis and proliferation [6,47-49] through the activation of various signaling pathways including PI-3K [50,51], several serine/threonine kinases [52], NF-kappaB [53,54], and SAPK/JNK [55,56]. Furthermore, Rac-1, RhoA, and Cdc42 have been demonstrated to have transforming and oncogenic potential in cell lines [57-60]. Whether one of the above mentioned GTP-binding proteins is involved in the regulation of myeloma cell survival or apoptosis is currently under investigation.

In summary, we demonstrated that inhibition of geranylgeranylation rather than inhibition of farnesylation reduces cell viability of myeloma plasma cells by induction of apoptosis. Apoptosis triggered by inhibition of geranylgeranylation was associated with

reduction of Mcl-1 protein expression, which, in turn, resulted in the collapse of the mitochondrial transmembrane potential, cytochrome c release from mitochondria into the cytosol, and stimulation of caspase-3 activity. These results show that geranylgeranylation of proteins is a key event in the regulation of myeloma tumor cell survival. Furthermore, our results suggest that pharmacologic agents such as lovastatin or GGTase inhibitors may be useful in the treatment of multiple myeloma.

## References


1. Magee, T. and Marshall, C. New insights into the interaction of Ras with the plasma membrane. Cell, *98*: 9-12, 1999.
2. Kato, K., Cox, A. D., Hisaka, M. M., Graham, S. M., Buss, J. E., and Der, C. J. Isoprenoid addition to Ras protein is the critical modification for its membrane association and transforming activity. Proc.Natl.Acad.Sci.U.S.A, *89*: 6403-6407, 1992.
3. Gelb, M. H. Protein prenylation, et cetera: signal transduction in two dimensions. Science, *275*: 1750-1751, 1997.
4. Rebollo, A. and Martinez, A. Ras proteins: recent advances and new functions. Blood, *94*: 2971-2980, 1999.
5. Reuter, C. W., Morgan, M. A., and Bergmann, L. Targeting the Ras signaling pathway: a rational, mechanism-based treatment for hematologic malignancies? Blood, *96*: 1655-1669, 2000.
6. Bishop, A. L. and Hall, A. Rho GTPases and their effector proteins. Biochem.J, *348 Pt 2:241-55.*: 241-255, 2000.
7. Neri, A., Murphy, J. P., Cro, L., Ferrero, D., Tarella, C., Baldini, L., and Dalla-Favera, R. Ras oncogene mutation in multiple myeloma. J.Exp.Med., *170*: 1715-1725, 1989.
8. Liu, P., Leong, T., Quam, L., Billadeau, D., Kay, N. E., Greipp, P., Kyle, R. A., Oken, M. M., and Van Ness, B. Activating mutations of N- and K-ras in multiple myeloma show different clinical associations: analysis of the Eastern Cooperative Oncology Group Phase III Trial. Blood, *88*: 2699-2706, 1996.
9. Le Gouill, S., Pellat-Deceunynck, C., Harousseau, J. L., Rapp, M. J., Robillard, N., Bataille, R., and Amiot, M. Farnesyl transferase inhibitor R115777 induces apoptosis of human myeloma cells. Leukemia, *16*: 1664-1667, 2002.
10. Chatterjee, M., Honemann, D., Lentzsch, S., Bommert, K., Sers, C., Herrmann, P., Mathas, S., Dorken, B., and Bargou, R. C. In the presence of bone marrow stromal cells human multiple myeloma cells become independent of the IL-6/gp130/STAT3 pathway. Blood, *100*: 3311-3318, 2002.
11. Frassanito, M. A., Cusmai, A., Piccoli, C., and Dammacco, F. Manumycin inhibits farnesyltransferase and induces apoptosis of drug-resistant interleukin 6-producing myeloma cells. Br.J Haematol., *118*: 157-165, 2002.
12. Bolick, S. C., Landowski, T. H., Boulware, D., Oshiro, M. M., Ohkanda, J., Hamilton, A. D., Sebti, S. M., and Dalton, W. S. The farnesyl transferase inhibitor, FTI-277, inhibits growth and induces apoptosis in drug-resistant myeloma tumor cells. Leukemia, *17*: 451-457, 2003.
13. Cortes, J., Albitar, M., Thomas, D., Giles, F., Kurzrock, R., Thibault, A., Rackoff, W., Koller, C., O'Brien, S., Garcia-Manero, G., Talpaz, M., and Kantarjian, H. Efficacy of the farnesyl transferase inhibitor R115777 in chronic myeloid leukemia and other hematologic malignancies. Blood, *101*: 1692-1697, 2003.
14. van de Donk, N. W., Kamphuis, M. M., Lokhorst, H. M., and Bloem, A. C. The cholesterol lowering drug lovastatin induces cell death in myeloma plasma cells. Leukemia, *16*: 1362-1371, 2002.

15. Downs, J. R., Clearfield, M., Weis, S., Whitney, E., Shapiro, D. R., Beere, P. A., Langendorfer, A., Stein, E. A., Kruyer, W., and Gotto, A. M. Primary prevention of acute coronary events with lovastatin in men and women with average cholesterol levels: results of AFCAPS/TexCAPS. Air Force/Texas Coronary Atherosclerosis Prevention Study. JAMA, *279*: 1615-1622, 1998.
16. Goldstein, J. L. and Brown, M. S. Regulation of the mevalonate pathway. Nature, *343*: 425-430, 1990.
17. Gross, A., McDonnell, J. M., and Korsmeyer, S. J. BCL-2 family members and the mitochondria in apoptosis. Genes Dev., *13*: 1899-1911, 1999.
18. Kroemer, G. and Reed, J. C. Mitochondrial control of cell death. Nat.Med, *6*: 513-519, 2000.
19. Schwarze, M. M. and Hawley, R. G. Prevention of myeloma cell apoptosis by ectopic bcl-2 expression or interleukin 6-mediated up-regulation of bcl-xL. Cancer Res., *55*: 2262-2265, 1995.
20. Tu, Y., Xu, F. H., Liu, J., Vescio, R., Berenson, J., Fady, C., and Lichtenstein, A. Upregulated expression of BCL-2 in multiple myeloma cells induced by exposure to doxorubicin, etoposide, and hydrogen peroxide. Blood, *88*: 1805-1812, 1996.
21. Gazitt, Y., Fey, V., Thomas, C., and Alvarez, R. Bcl-2 overexpression is associated with resistance to dexamethasone, but not melphalan, in multiple myeloma cells. Int.J Oncol., *13*: 397-405, 1998.
22. van de Donk, N. W., Kamphuis, M. M., Van Dijk, M., Borst, H. P., Bloem, A. C., and Lokhorst, H. M. Chemosensitization of myeloma plasma cells by an antisense-mediated downregulation of Bcl-2 protein. Leukemia, *17*: 211-219, 2003.
23. Tu, Y., Renner, S., Xu, F., Fleishman, A., Taylor, J., Weisz, J., Vescio, R., Rettig, M., Berenson, J., Krajewski, S., Reed, J. C., and Lichtenstein, A. BCL-X expression in multiple myeloma: possible indicator of chemoresistance. Cancer Res., *58*: 256-262, 1998.
24. Gojo, I., Zhang, B., and Fenton, R. G. The Cyclin-dependent Kinase Inhibitor Flavopiridol Induces Apoptosis in Multiple Myeloma Cells through Transcriptional Repression and Down-Regulation of Mcl-1. Clin.Cancer Res., *8*: 3527-3538, 2002.
25. Derenne, S., Monia, B., Dean, N. M., Taylor, J. K., Rapp, M. J., Harousseau, J. L., Bataille, R., and Amiot, M. Antisense strategy shows that Mcl-1 rather than Bcl-2 or Bcl-x(L) is an essential survival protein of human myeloma cells. Blood, *100*: 194-199, 2002.
26. Zhang, B., Gojo, I., and Fenton, R. G. Myeloid cell factor-1 is a critical survival factor for multiple myeloma. Blood, *99*: 1885-1893, 2002.
27. Keyomarsi, K., Sandoval, L., Band, V., and Pardee, A. B. Synchronization of tumor and normal cells from G1 to multiple cell cycles by lovastatin. Cancer Res., *51*: 3602-3609, 1991.
28. Crick, D. C., Andres, D. A., and Waechter, C. J. Novel salvage pathway utilizing farnesol and geranylgeraniol for protein isoprenylation. Biochem.Biophys.Res.Commun., *237*: 483-487, 1997.
29. Barut, B. A., Zon, L. I., Cochran, M. K., Paul, S. R., Chauhan, D., Mohrbacher, A., Fingeroth, J., and Anderson, K. C. Role of interleukin 6 in the growth of myeloma-derived cell lines. Leuk.Res., *16*: 951-959, 1992.
30. Zhang, X. G., Gaillard, J. P., Robillard, N., Lu, Z. Y., Gu, Z. J., Jourdan, M., Boiron, J. M., Bataille, R., and Klein, B. Reproducible obtaining of human myeloma cell lines as a model for tumor stem cell study in human multiple myeloma. Blood, *83*: 3654-3663, 1994.
31. Davis, A. R., Alevy, Y. G., Chellaiah, A., Quinn, M. T., and Mohanakumar, T. Characterization of HDJ-2, a human 40 kD heat shock protein. Int.J Biochem.Cell Biol., *30*: 1203-1221, 1998.
32. Omer, C. A., Chen, Z., Diehl, R. E., Conner, M. W., Chen, H. Y., Trumbauer, M. E., Gopal-Truter, S., Seeburger, G., Bhimnathwala, H., Abrams, M. T., Davide, J. P., Ellis, M. S., Gibbs, J. B., Greenberg, I., Koblan, K. S., Kral, A. M., Liu, D., Lobell, R. B., Miller, P. J., Mosser, S. D., O'Neill, T. J., Rands, E., Schaber, M. D., Senderak, E. T., Oliff, A., and Kohl, N. E. Mouse mammary tumor virus-Ki-rasB transgenic mice develop mammary carcinomas that can be growth-inhibited by a farnesyl:protein transferase inhibitor. Cancer Res., *60*: 2680-2688, 2000.

33. Vogt, A., Qian, Y., McGuire, T. F., Hamilton, A. D., and Sebti, S. M. Protein geranylgeranylation, not farnesylation, is required for the G1 to S phase transition in mouse fibroblasts. Oncogene, *13*: 1991-1999, 1996.
34. Cheng, E. H., Kirsch, D. G., Clem, R. J., Ravi, R., Kastan, M. B., Bedi, A., Ueno, K., and Hardwick, J. M. Conversion of Bcl-2 to a Bax-like death effector by caspases. Science, *278*: 1966-1968, 1997.
35. Kirsch, D. G., Doseff, A., Chau, B. N., Lim, D. S., Souza-Pinto, N. C., Hansford, R., Kastan, M. B., Lazebnik, Y. A., and Hardwick, J. M. Caspase-3-dependent cleavage of Bcl-2 promotes release of cytochrome c. J Biol.Chem., *274*: 21155-21161, 1999.
36. Zhou, P., Qian, L., Kozopas, K. M., and Craig, R. W. Mcl-1, a Bcl-2 family member, delays the death of hematopoietic cells under a variety of apoptosis-inducing conditions. Blood, *89*: 630-643, 1997.
37. Kitada, S., Andersen, J., Akar, S., Zapata, J. M., Takayama, S., Krajewski, S., Wang, H. G., Zhang, X., Bullrich, F., Croce, C. M., Rai, K., Hines, J., and Reed, J. C. Expression of apoptosis-regulating proteins in chronic lymphocytic leukemia: correlations with In vitro and In vivo chemoresponses. Blood, *91*: 3379-3389, 1998.
38. Craig, R. W. MCL1 provides a window on the role of the BCL2 family in cell proliferation, differentiation and tumorigenesis. Leukemia, *16*: 444-454, 2002.
39. Zhang, B., Potyagaylo, V., and Fenton, R. G. IL-6-independent expression of Mcl-1 in human multiple myeloma. Oncogene, *22*: 1848-1859, 2003.
40. Xia, Z., Tan, M. M., Wong, W. W., Dimitroulakos, J., Minden, M. D., and Penn, L. Z. Blocking protein geranylgeranylation is essential for lovastatin-induced apoptosis of human acute myeloid leukemia cells. Leukemia, *15*: 1398-1407, 2001.
41. Miquel, K., Pradines, A., Sun, J., Qian, Y., Hamilton, A. D., Sebti, S. M., and Favre, G. GGTI-298 induces G0-G1 block and apoptosis whereas FTI-277 causes G2-M enrichment in A549 cells. Cancer Res., *57*: 1846-1850, 1997.
42. Stark, W. W., Jr., Blaskovich, M. A., Johnson, B. A., Qian, Y., Vasudevan, A., Pitt, B., Hamilton, A. D., Sebti, S. M., and Davies, P. Inhibiting geranylgeranylation blocks growth and promotes apoptosis in pulmonary vascular smooth muscle cells. Am J Physiol, *275*: L55-L63, 1998.
43. Mundy, G., Garrett, R., Harris, S., Chan, J., Chen, D., Rossini, G., Boyce, B., Zhao, M., and Gutierrez, G. Stimulation of bone formation in vitro and in rodents by statins. Science, *286*: 1946-1949, 1999.
44. Staal, A., Frith, J. C., French, M. H., Swartz, J., Gungor, T., Harrity, T. W., Tamasi, J., Rogers, M. J., and Feyen, J. H. The ability of statins to inhibit bone resorption is directly related to their inhibitory effect on HMG-CoA reductase activity. J Bone Miner.Res., *18*: 88-96, 2003.
45. Pahan, K., Sheikh, F. G., Namboodiri, A. M., and Singh, I. Lovastatin and phenylacetate inhibit the induction of nitric oxide synthase and cytokines in rat primary astrocytes, microglia, and macrophages. J Clin.Invest, *100*: 2671-2679, 1997.
46. Hall, A. Rho GTPases and the actin cytoskeleton. Science, *279*: 509-514, 1998.
47. Van Aelst, L. and D'Souza-Schorey, C. Rho GTPases and signaling networks. Genes Dev., *11*: 2295-2322, 1997.
48. Zohn, I. M., Campbell, S. L., Khosravi-Far, R., Rossman, K. L., and Der, C. J. Rho family proteins and Ras transformation: the RHOad less traveled gets congested. Oncogene, *17*: 1415-1438, 1998.
49. Bar-Sagi, D. and Hall, A. Ras and Rho GTPases: a family reunion. Cell, *103*: 227-238, 2000.
50. Kumagai, N., Morii, N., Fujisawa, K., Nemoto, Y., and Narumiya, S. ADP-ribosylation of rho p21 inhibits lysophosphatidic acid-induced protein tyrosine phosphorylation and phosphatidylinositol 3-kinase activation in cultured Swiss 3T3 cells. J Biol.Chem., *268*: 24535-24538, 1993.
51. Zheng, Y., Bagrodia, S., and Cerione, R. A. Activation of phosphoinositide 3-kinase activity by Cdc42Hs binding to p85. J Biol.Chem., *269*: 18727-18730, 1994.
52. Amano, M., Mukai, H., Ono, Y., Chihara, K., Matsui, T., Hamajima, Y., Okawa, K., Iwamatsu, A., and Kaibuchi, K. Identification of a putative target for Rho as the serine-threonine kinase protein kinase N. Science, *271*: 648-650, 1996.

53. Sulciner, D. J., Irani, K., Yu, Z. X., Ferrans, V. J., Goldschmidt-Clermont, P., and Finkel, T. rac1 regulates a cytokine-stimulated, redox-dependent pathway necessary for NF-kappaB activation. Mol.Cell Biol., *16*: 7115-7121, 1996.
54. Perona, R., Montaner, S., Saniger, L., Sanchez-Perez, I., Bravo, R., and Lacal, J. C. Activation of the nuclear factor-kappaB by Rho, CDC42, and Rac-1 proteins. Genes Dev., *11*: 463-475, 1997.
55. Coso, O. A., Chiariello, M., Yu, J. C., Teramoto, H., Crespo, P., Xu, N., Miki, T., and Gutkind, J. S. The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway. Cell, *81*: 1137-1146, 1995.
56. Teramoto, H., Crespo, P., Coso, O. A., Igishi, T., Xu, N., and Gutkind, J. S. The small GTP-binding protein rho activates c-Jun N-terminal kinases/stress-activated protein kinases in human kidney 293T cells. Evidence for a Pak-independent signaling pathway. J Biol.Chem., *271*: 25731-25734, 1996.
57. Khosravi-Far, R., Solski, P. A., Clark, G. J., Kinch, M. S., and Der, C. J. Activation of Rac1, RhoA, and mitogen-activated protein kinases is required for Ras transformation. Mol.Cell Biol., *15*: 6443-6453, 1995.
58. Qiu, R. G., Chen, J., McCormick, F., and Symons, M. A role for Rho in Ras transformation. Proc Natl Acad Sci U S A, *92*: 11781-11785, 1995.
59. Qiu, R. G., Abo, A., McCormick, F., and Symons, M. Cdc42 regulates anchorage-independent growth and is necessary for Ras transformation. Mol.Cell Biol., *17*: 3449-3458, 1997.
60. Lin, R., Bagrodia, S., Cerione, R., and Manor, D. A novel Cdc42Hs mutant induces cellular transformation. Curr.Biol., *7*: 794-797, 1997.

# Chapter 6

## Geranylgeranylated proteins are involved in the regulation of myeloma cell growth

Niels W.C.J. van de Donk, Marloes M.J. Kamphuis, Evert H.J. Nijhuis, Berris van Kessel, Henk M. Lokhorst, and Andries C. Bloem

In preparation

## Abstract

Lovastatin inhibits HMG-CoA reductase resulting in the depletion of mevalonate and the isoprenoid molecules farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP). Lovastatin blocked the transition from G1 to S phase of the cell cycle by the specific depletion of GGPP in myeloma cell lines and *ex vivo* purified myeloma cells. Furthermore, inhibition of geranylgeranyl transferase I activity was more effective in the inhibition of myeloma cell growth when compared to inhibition of farnesyl transferase activity. This indicates that protein geranylgeranylation is important for myeloma cell proliferation and cell cycle progression through G1. Geranylgeranylated target proteins involved in the control of proliferation include Rac-1, Cdc42, and RhoA. Inhibition of Rho, Rac, and Cdc42 GTPases by toxin B reduced proliferation, without affecting cell viability, but specific inhibition of Rho GTPases by C3 exoenzyme had no effect. This suggests a role for Rac and/or Cdc42 GTPases in myeloma cell growth. Rac-1 activity was found in all myeloma cell lines and was suppressed by lovastatin through the depletion of intracellular pools of GGPP. IL-6 rapidly stimulated Rac-1 activity, indicating a role for Rac-1 in the signal transduction cascade of IL-6 leading to myeloma cell proliferation. Specific reduction of Rac protein levels by antisense oligodeoxynucleotides reduced spontaneous proliferation of primary myeloma cells. Furthermore, dominant-negative Tat-Rac-1 reduced myeloma cell proliferation, while constitutively active Tat-Rac-1 enhanced proliferation. These data imply that Rac-1 is involved in the regulation of myeloma cell growth.

## Introduction

Multiple myeloma is characterized by the proliferation and accumulation of monoclonal plasma cells in the bone marrow. The proteins interleukin-6 (IL-6) and insulin-like growth factor I (IGF-I) [1-8] function as growth factors for myeloma cells and promote cell survival. Binding of IL-6 to the IL-6 receptor triggers the activation of at least 3 signaling pathways; the JAK-STAT pathway [9,10], the phosphatidylinositol-3 kinase (PI-3K) pathway [8,11], and the mitogen-activated protein kinase (MAPK) pathway [12]. Activation of the JAK-STAT [10] and PI-3K [11] pathways has been implicated in the protection against apoptosis, whereas activation of the PI-3K [8,11] and MAPK [12] pathways induces proliferation in myeloma cell lines. IGF-I stimulation leads to activation of both the MAPK and the PI-3K pathways in myeloma cells, however the PI-3K pathway was demonstrated to be the major regulator of both apoptosis and proliferation, with only minimal contributions to either by the MAPK cascade [6-8,13]. Activation of Akt by PI-3K may be responsible for the proliferative and anti-apoptotic effects of PI-3K in myeloma, since expression of a dominant-negative Akt gene in myeloma cells or pharmacologic inhibition of Akt resulted in a decreased number of cells in S phase and an increase in apoptosis [14,15]. Various elements downstream of Akt have been implicated in the control of myeloma cell growth and survival including the Forkhead family of transcription factors [11,13] and p70S6 kinase [13]. Furthermore, NF-κB, which is constitutively active in myeloma cells promotes survival and proliferation through the induction of progression of cells from the G1 phase of the cell cycle to S phase [16-18].

GTPases of the Ras and Rho families cycle between an inactive GDP-bound form, and a GTP-bound form with affinity for various effector proteins that control signal transduction cascades regulating multiple cellular processes including migration, cytoskeletal reorganization, stimulation of cell proliferation, and survival. Activating Ras mutations are frequently detected in myeloma [19-21] and contribute to reduced apoptosis [22-24], increased cell proliferation [22,23], and an adverse clinical outcome [19,20]. Rac-1, Cdc42, and RhoA have been implicated in the regulation of cell cycle progression through G1 phase of the cell cycle. Constitutively activated mutants of Rac-1, Cdc42, and RhoA caused G1 progression and stimulation of DNA synthesis in fibroblasts [25-27]. Furthermore, expression of active forms of Rac-1, Cdc42, and RhoA can transform fibroblasts [28] and activation of Rac-1, RhoA, and Cdc42 is required for full Ras transforming activity [29-33]. The role of Rac, Cdc42, and RhoA in multiple myeloma is currently unknown.

Participation of Ras and Rho family proteins in signaling pathways depends on the proper subcellular localization to the plasma membrane, which is facilitated by a series of posttranslational modifications of the carboxyl-terminus [34-36]. This includes the addition of a farnesyl lipid derived from farnesylpyrophosphate (FPP) or a geranylgeranyl lipid derived from geranylgeranylpyrophosphate (GGPP) to a conserved cysteine residue at or

near the carboxyl-terminus of target proteins including Ras (farnesylation) and Rho family proteins such as Rac-1, Cdc42, and RhoA (geranylgeranylation). These reactions are catalyzed by farnesyl transferase (FTase) and geranylgeranyl transferase I (GGTase I). The mevalonate pathway produces many critical substances in cells, including cholesterol and the isoprenoid molecules FPP and GGPP. The rate-limiting step of this pathway is the conversion of HMG-CoA to mevalonate and is catalyzed by HMG-CoA reductase [37]. HMG-CoA reductase inhibitors such as lovastatin are widely used to treat patients with hypercholesterolemia [38].

In this report we show that inhibition of protein geranylgeranylation either by depletion of GGPP by lovastatin or by specific inhibition of GGTase I activity inhibits proliferation of myeloma cells. Furthermore, our data suggest that the geranylgeranylated GTP-binding protein Rac-1 is involved in the control of myeloma cell growth.

## Materials and methods

### Reagents

Lovastatin and simvastatin were obtained from Merck & Co., Inc (Rahway, NJ, USA) and were chemically activated by alkaline hydrolysis prior to use as described previously [39]. Mevalonate and farnesol (FOH) were purchased from Sigma (St Louis, MO, USA) and geranylgeraniol (GGOH) was obtained from ICN Biomedicals, BV (Zoetermeer, The Netherlands). FOH and GGOH are metabolized to FPP and GGPP in the cells, respectively [40]. FTI-277 and GGTI-298 were obtained from Calbiochem (Schwallbach, Germany). Tat-Rac-1 Q61L and Tat-Rac-1 N17 vectors were a kind gift of Dr S. Dowdy (Howard Hughes Medical Institute, Dept. of Pathology, Washington University School of Medicine, St Louis, Missouri 63110, USA). The *Clostridium botulinum* C3 exoenzyme was purchased from List Biological Laboratories, Inc. (Campbell, CA) and *Clostridium difficile* toxin B was obtained from Sigma. A rabbit polyclonal antiserum against Cdk2 was purchased from Santa Cruz (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA). Mouse monoclonal antibodies recognizing RhoA and Rac-1 were obtained from Santa Cruz and Pierce (Rockford, IL, USA), repectively. Mouse monoclonal antibodies recognizing $p21^{Cip1}$ and $p27^{Kip1}$ were from Pharmingen (Becton Dickinson, Erembodegem, Belgium (BDIS)).

### Cell lines

Plasma cell lines RPMI-8226 and U266 were obtained from the American Tissue Culture Collection (ATCC) and L363 from the German Collection of Microorganisms and Cell Cultures (GCMC). The IL-6 dependent plasma cell line XG-1 was a kind gift of Dr B. Klein (Institute for Molecular Genetics, Montpellier, France) [41]. Cell lines were cultured

in RPMI-1640 (GIBCO, Breda, The Netherlands) supplemented with 10% fetal calf serum (FCS) (Integro, Zaandam, The Netherlands), 100 IU/ml penicillin, 100 μg/ml streptomycin and 10 μM β-mercaptoethanol (growth medium). The IL-6-dependent cell line XG-1 was cultured in the continuous presence of exogenous IL-6 (1.25 ng/ml rhIL-6 (Roche, Almere, The Netherlands).

**Isolation of myeloma tumor cells**

Myeloma plasma cells were obtained from bone marrow aspirates taken from the posterior iliac crest in 8 patients and from peripheral blood in one patient with plasma cell leukemia (patient 4) after obtaining informed consent. The plasma cell percentage in the patient samples varied from 15-96% of the mononuclear cells as determined by co-expression of CD38 (anti-CD38-FITC, Immunotech, Marseille, France) and CD138 (anti-CD138-PE, Immunotech) by flow cytometric analysis (FACSCalibur, BDIS). Except for patient 3, who had 96% myeloma cells in her bone marrow, tumor cells were purified *ex vivo* from mononuclear cells obtained by Ficoll-Paque (Amersham; Pharmacia BiotechAB, Uppsala, Sweden) density centrifugation, by magnetic cell sorting (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany) based on CD138 expression as described previously [42]. Samples obtained in this way contained >95% myeloma plasma cells as determined by analysis of CD38/CD138 co-expression. For experiments, myeloma cells were resuspended in RPMI-1640 (GIBCO) supplemented with 10% fetal calf serum (FCS) (Integro), 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10 μM β-mercaptoethanol.

**Cell viability**

Viability of cells was examined by means of the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay as described previously [43]. In short, cells were seeded in a concentration of 0.3 X $10^6$/ml for the myeloma cell lines or 1 X $10^6$/ml for the tumor cells of patients in a 96-well flat bottom plate (100 μl/well) (Nunc, Roskilde, Denmark) and treated with different concentrations of lovastatin (for concentrations see legends) alone or in the presence of mevalonate (100 μM), FOH (10 μM), or GGOH (10 μM). Fixed concentrations of mevalonate (100 μM), FOH (10 μM), or GGOH (10 μM) were used. These concentrations proved to be optimal in rescuing myeloma cells from lovastatin-induced inhibition of proliferation. Inhibition of FTase and GGTase I was accomplished by treating cells with FTI-277 and GGTI-298, respectively (for concentrations see legends). After 2 or 4 days, 25 μl of MTT (5 mg/ml) was added to each well. After an incubation of 2 hours at 37°C the reaction was stopped by the addition of 100 μl 20% sodium dodecyl sulphate (SDS; Boehringer Mannheim, Mannheim, Germany) / 0.025 M HCl / 0.35 M HAc in a mixture of (1:1; v/v) N,N-dimethylformamide (Merck, Darmstadt, Germany) and distilled water. After an overnight incubation at 37°C the optical density of the samples was determined at 570 nm.

**Apoptosis detection by Annexin V staining**

Myeloma cells (1.5 X $10^5$ in 0.5 ml) were incubated with different concentrations of lovastatin (for concentrations see legends) alone or in the presence of mevalonate (100 μM), FOH (10 μM), or GGOH (10 μM) in a 48-well plate (Nunc). Inhibition of FTase and GGTase I was accomplished by treating cells with FTI-277 and GGTI-298, respectively (for concentrations see legends). After 2 or 4 days, cells were harvested and apoptosis was determined by using the Annexin V assay as described previously [43]. Apoptotic cells were defined as early apoptotic cells (Annexin V positive and PI negative) and late apoptotic cells (Annexin V positive and PI positive).

**Cell proliferation**

Cells (3 X $10^4$) were seeded in 96-well flat bottom plates (Nunc) in 100 μl growth medium with different concentrations of lovastatin (for concentrations see legends) alone or in the presence of mevalonate (100 μM), FOH (10 μM), or GGOH (10 μM). Inhibition of FTase and GGTase I was accomplished by treating cells with different concentrations of FTI-277 (0-30 μM) and GGTI-298 (0-30 μM), respectively. After 32 and 80 hours, $^3$H-thymidine (Amersham, Little Chalfont, UK) (1 μCi/well) was added for the remaining 16 hours of the assay. $^3$H-thymidine incorporation was analyzed by liquid scintillation counting as described previously [43].

**Cell cycle analysis**

Myeloma cells (1 X $10^6$ in 1.5 ml) were incubated with different concentrations of lovastatin (for concentrations see legends) in the presence or absence of mevalonate (100 μM), FOH (10 μM), or GGOH (10 μM) in a 48-well plate (Nunc) for 2 days. Inhibition of FTase and GGTase I was accomplished by treating cells for 2 days with FTI-277 (20 μM) and GGTI-298 (20 μM), respectively (for concentrations see legends). Cells were then pulsed with 10 μM bromodeoxyuridine (BrdU) for 30 minutes at 37$^o$C, after which the cells were washed twice with ice-cold PBS containing 1% bovine serum albumin (BSA). Cells were subsequently fixed with 70% ethanol for 30 minutes at 4$^o$C. After the ethanol was washed away, the cells were treated with 2 M HCl containing 0.5% Triton-X-100 for 30 minutes at room temperature, followed by neutralization with 1M natrium tetraborate (pH 8.5). Following washing, cells were resuspended in PBS containing 0.5% Tween-20 and 1% BSA and then incubated with anti-BrdU-FITC (Becton and Dickinson) for 30 minutes at room temperature. After washing with PBS containing 0.5% Tween-20 and 1% BSA, cells were stained with propidium iodide in PBS (5 μg/ml) and analyzed by flow cytometry.

**Cdk2 kinase assay**

Cells (1.67 X $10^6$ cells in 2.5 ml) were incubated for 2 days with lovastatin (30 μM) alone or in the presence of mevalonate (100 μM), FOH (10 μM), or GGOH (10 μM). After harvesting, whole cell lysates were made by washing cells in ice-cold PBS and then resuspending them in ELB buffer (150 mM NaCl, 50 mM HEPES pH 7.5, 5 mM EDTA, 0.1% NP-40 with a cocktail of protease inhibitors (Boehringer Mannheim)) for 30 minutes at 4°C. Insoluble material was removed by centrifugation at 14 000 r.p.m. for 15 minutes at 4°C. Supernatants were then precleared with protein A-Sepharose CL-4B beads (Amersham, Little Chalfont, UK) for 30 minutes at 4°C. Following a short 14 000 r.p.m. centrifugation, protein A-Sepharose CL-4B beads (Amersham) and 0.4 μg of anti-Cdk2 antibody (Santa Cruz) were added to the precleared supernatants and Cdk2 was immunoprecipitated overnight at 4°C. The precipitates were washed three times with ELB buffer and twice with kinase buffer (20 mM HEPES pH 7.5 with 5 mM $MgCl_2$ and 2.5 mM $MnCl_2$). The precipitates were then dried with an insulin syringe and incubated in kinase buffer containing 2.5 μCi ($\gamma$-$^{32}$P) ATP (Amersham), 10 μg histone H1 (Roche, Basel, Switzerland) and 50 μM ATP (Sigma) for 30 minutes at 30°C. Samples were then mixed with 2 X Laemmli sample buffer (0.125 M Tris pH 6.9 with 4% SDS, 20% Glycerol and 10% β-mercaptoethanol) and boiled for 5 minutes. Proteins were separated on a 10% SDS-PAGE gel and the gel was dried in a gel dryer (Biorad, Hercules, CA, USA) for 60 minutes at 80°C. The phosphorylated histone H1 was visualized using hyperfilm ECL (Amersham).

**Treatment of cells with *C. botulinum* C3 exoenzyme and *C. difficile* toxin B**

Cells were washed in PBS and resuspended in buffer (114 mM KCl, 15 mM NaCl, 5.5 mM $MgCl_2$, 10 mM Tris) in the presence of solvent control or 50 μg/ml *C.botulinum* C3 exoenzyme for 1 hour at room temperature as described previously [44,45]. Cells were washed and resuspended in medium. *C.difficile* toxin B (50 ng/ml) was directly administered to the cells. Toxin B and C3 exoenzyme were used at concentrations, which inhibited proliferation of various cell lines as shown previously [44-46].

**Incubation of cells with oligodeoxynucleotides**

A 16-mer phosphorothioated antisense Rac oligodeoxynucleotide (5'-ACT TGA TGG CCT GCA T-3'), complementary to a sequence shared by Rac-1 and Rac-2 genes and starting at the initiation codon, was synthesized by Isogen (Maarssen, The Netherlands) [47,48]. Reverse polarity ODNs (sequence; 5'-ATG CAG GCC ATC AAG T-3' were used as a control. The ODNs were dissolved in Iscove's Modified Dulbecco's medium (IMDM) and stored at –80°C until use. ODNs were administered to cells in free form in the absence of cationic lipids.

**Tat-Rac-1 protein isolation**

Polyhistidine-tagged Tat-Rac-1 Q61L and Tat-Rac-1 N17 constructs were expressed in BL21 bacteria (Novagen, Madison, WI, USA). Expression and isolation of Tat-Rac-1 Q61L and Tat-Rac-1 N17 were performed similarly as has been described by Hall et al [49], except that the isolation of the proteins was performed under non-reducing conditions. Briefly, expression of Tat-Rac-1 Q61L and Tat-Rac-1 N17 in bacteria was induced by IPTG for 3-4 hours at 37°C, before the bacteria pellet was solubilized in lysis buffer (50 mM $NaH_2PO_4$, 300 mM NaCl, 10 mM imidazole, 10% glycerol, 1 mM PMSF, pH 8.0). Lysozyme (1 mg/ml) and DNase (5 μg/ml) were added to the bacteria solution and incubated for 30 minutes on ice. The solution was sonicated 6-8 times for 10 seconds each, and centrifuged twice at 15 000 r.p.m. for 20 minutes. The supernatant containing the fusion protein was added to Ni-NTA beads and rotated for 30 minutes at 4°C. The beads were incubated with lysis buffer, twice with wash buffer 1 (50 mM $NaH_2PO_4$, 300 mM NaCl, 20 mM imidazole, 0.5% Tween-20, 10% glycerol, pH 8.0), and 3-4 times with wash buffer 2 (50 mM $NaH_2PO_4$, 300 mM NaCl, 20 mM imidazole, 10% glycerol, pH 8.0). Protein was eluted from the beads with elution buffer (50 mM $NaH_2PO_4$, 300 mM NaCl, 500 mM imidazole, 10% glycerol, pH 8.0). LPS was removed from the protein elution by a method described previously [50]. In short, 0.1 volume of LPS removal buffer (50 mM $NaH_2PO_4$, 300 mM NaCl, 10% glycerol, 10% Triton-X114, pH 7.5) was added and rotated at 4°C for 10 minutes. After transferring the solution to 37°C for 5 minutes, it was centrifuged at 3 000 r.p.m. for 5 minutes. Upper layer was transferred to a new tube and the procedure was repeated 4 times and 2 times with the LPS removal buffer without Triton-X114. Imidazole, and last traces of Triton-X114, were removed from the resultant protein solution by dialyzing two times against PBS (pH 7.4) containing 10% glycerol for at least 4 hours at 4°C each time, using Slide-A-Lyzer dialysis cassette (extra strength; 3-12 ml; MW cut off 10 kD; Pierce). Protein concentration was measured and purity of Tat-Rac-1 Q61L and Tat-Rac-1 N17 was approximately 95%, as determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent Coomassie blue staining. Protein expression of Tat-Rac-1 Q61L and Tat-Rac-1 N17 was confirmed by SDS-PAGE and immunoblotting using anti-HA and anti-Rac-1 antibodies. Protein solution was aliquoted and frozen at –80°C.

**Detection of Rac-1 activity**

Rac-1 activity was determined by using the EZ-Detect Rac-1 activation kit (Pierce). Cells were treated for 2 days with lovastatin (for concentrations see legends) alone or in the presence of mevalonate (100 μM), FOH (10 μM), or GGOH (10 μM). Prior to stimulation of cell lines with IL-6, cells were cultured overnight in serum-free RPMI-1640 medium. After purification of myeloma cells from patients, cells were washed 2 times in serum-free medium, resuspended in serum-free medium, and incubated for 1 hour at 37°C. Cell lines

and purified tumor cells from myeloma patients were stimulated with 10 ng/ml IL-6. Equal numbers of cells (10-15 X $10^6$) were washed in ice-cold PBS and then resuspended in 1 ml lysis buffer (25 mM Tris HCl, pH 7.5, 150 mM NaCl, 5mM $MgCl_2$, 1% NP-40, 1 mM DTT and 5% glycerol) at 4°C for 5 minutes. The supernatant, which was obtained after a centrifugation step at 16 000 *g* for 15 minutes at 4°C, was added to a spin column containing an immobilized glutathione disc and 20 µg GST-Pak1-PBD. After an incubation of the reaction mixture at 4°C for 60 minutes with gentle rocking, the columns were centrifuged at 7 200 *g* for 2 minutes. The resin was washed four times with lysis buffer and then 50 µl 2 x SDS sample buffer containing 5% β-mercaptoethanol was added to the resin. The samples were boiled at 100°C for 5 minutes. After a centrifugation step at 7 200 *g* for 2 minutes, the samples were electrophoresed on a gel.

**Western blotting**

Cells (1 X $10^6$ cells in 1.5 ml) were incubated for 2 or 4 days with lovastatin (for concentrations see legends) in the presence or absence of mevalonate, FOH, or GGOH. Inhibition of FTase and GGTase I was accomplished by treating cells with FTI-277 and GGTI-298, respectively (for concentrations see legends). Cell lysates were made as described previously [51] and protein concentrations were determined by the BCA assay (Pierce). Samples containing equal amounts of protein were mixed with 2 X Laemmli sample buffer (0.125 M Tris pH 6.9 with 4% SDS, 20% Glycerol and 10% β-mercaptoethanol) and boiled for 5 minutes. Proteins were subsequently fractionated in 10% SDS-PAGE at room temperature and electrically transferred from the gel to PVDF-membrane (Biorad). After blocking in 0.1% Tween-20, 5% skimmed powder milk, 2% BSA in 10 mM Tris and 150 mM NaCl, the membranes were incubated with anti-$p27^{Kip1}$, $p21^{Cip1}$, Cdk2, RhoA, or Rac-1. Antibody binding was visualized with enhanced chemoluminescence (Amersham) detection with hyperfilm ECL (Amersham) after incubation with a horseradish peroxidase (HRP)-conjugated secondary antibody (Dako). Finally, the membranes were extensively washed in PBS and reprobed with anti-α-actin (Sigma) as a control for equal loading of protein. Relative amounts of protein were determined by densitometry.

## Results

**Depletion of GGPP inhibits proliferation by inducing G1 arrest in myeloma cell lines**

Lovastatin inhibits the conversion of HMG-CoA to mevalonate, and thereby the synthesis of the isoprenoids FPP and GGPP. We have previously shown that the depletion of FPP and GGPP in myeloma cells results in inhibition of protein farnesylation and geranylgeranylation, respectively [52], and that geranylgeranylated proteins play an

important role in the regulation of myeloma cell survival [52]. To investigate the effect of depletion of FPP and GGPP on myeloma cell proliferation, myeloma cell lines were incubated for 2 or 4 days with different concentrations of lovastatin alone or in the presence of mevalonate (100 μM), FOH (10 μM), or GGOH (10 μM). Lovastatin inhibited proliferation of RPMI-8226 and L363 cells, and the IL-6-dependent U266 and XG-1 cell lines in a dose- and time-dependent way as determined by detection of $^{3}$H-thymidine incorporation (Figure 1A). Inhibition of proliferation by 30 μM lovastatin varied from 64.1-98.2% at day 2, while proliferation was reduced by more than 95% in all 4 cell lines tested at day 4. Addition of mevalonate to lovastatin-treated cell lines restored cell proliferation. This indicates that lovastatin inhibits proliferation through the decrease of mevalonate production as a consequence of the specific inhibition of HMG-CoA reductase and not through non-specific cell toxicity. FPP and GGPP are metabolites of mevalonate and are donors of farnesyl and geranylgeranyl molecules, which are attached to a variety of proteins including small GTPases. FOH and GGOH are metabolized to FPP and GGPP in the cells [40]. GGOH restored proliferation in lovastatin-treated myeloma cells. In contrast, FOH had no effect or only partial protective effects (Figure 1A). Cell cycle analysis showed that lovastatin treatment for 2 days caused accumulation of cells in G1 phase of the cell cycle and a loss of cells in S phase in a dose-dependent way as shown for U266 cells in Figure 1B. At day 2, there was no significant increase in apoptosis in U266 cells, as determined by the absence of a sub-G1 population. Addition of mevalonate or GGOH, but not FOH prevented the G1 to S phase cell cycle arrest (Figure 1C). Addition of mevalonate, GGOH, or FOH to myeloma cells in the absence of lovastatin had no effect on cell cycle distribution. Lovastatin-induced G1/S phase growth arrest was accompanied by the up-regulation of the cyclin dependent kinase (Cdk) inhibitors $p21^{Cip1}$ and/or $p27^{Kip1}$. Lovastatin increased $p21^{Cip1}$ protein levels in U266 (shown in Figure 1D), XG-1, and RPMI-8226 cells and $p27^{Kip1}$ levels in RPMI-8226 and L363 cells. To evaluate whether the induction of the Cdk inhibitors resulted in the inhibition of kinase activity, we measured the phosphorylation of histone H1 in anti-Cdk2 immunoprecipitates. In all 4 myeloma cell lines, Cdk2 activity was significantly reduced after treatment with lovastatin for 2 days, whereas Cdk2 protein levels did not change. Mevalonate and GGOH, but not FOH prevented the up-regulation of the Cdk inhibitors and the decrease in Cdk2 activity as shown for U266 cells in Figure 1D.

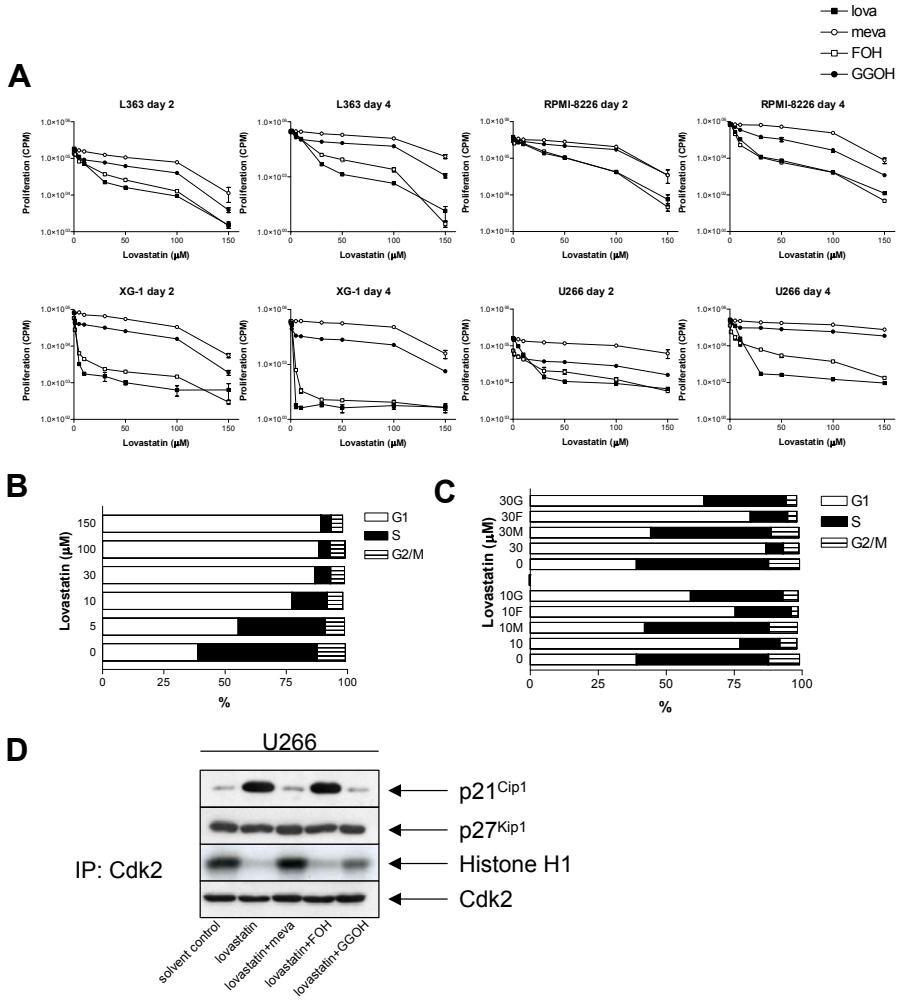


**Figure 1. Lovastatin reduces proliferation of myeloma cell lines through the depletion of GGPP.** (A) L363, RPMI-8226, XG-1, and U266 cells were treated for 2 or 4 days with solvent control, or different concentrations of lovastatin (1, 5, 10, 30, 50, 100, 150 μM) alone or in combination with mevalonate (meva; 100 μM), FOH (10 μM), or GGOH (10 μM). Proliferation was determined by $^{3}$H -thymidine incorporation during the last 16 hours of culture. Data were from three experiments performed in triplicate. Data are presented as mean ± s.e.m. (B and C) U266 cells were treated with solvent control (0), or lovastatin (5, 10, 30, 100, or 150 μM) alone or in combination with mevalonate (M; 100 μM), FOH (F; 10 μM), or GGOH (G; 10 μM) for 2 days, at which time the cells were harvested and cell cycle distribution was determined by BrdU assay. The percentages of cells in G1 phase, G2/M phase, and S phase of the cell cycle are shown. Results are representative of three experiments performed in triplicate. (D) U266 cells were treated for 2 days with solvent control or lovastatin (30 μM) alone or in the presence of mevalonate (100 μM), FOH (10 μM), or GGOH (10 μM). After protein isolation p21$^{Cip1}$, p27$^{Kip1}$, and Cdk2 were determined by Western blot analysis. In addition, cell lysates were immunoprecipitated (IP) with anti-Cdk2, and kinase activity was determined using histone H1 as substrate. The data shown are representative of at least three independent experiments.

**Depletion of GGPP inhibits proliferation of purified tumor cells derived from myeloma patients**

Similar to myeloma cell lines, lovastatin inhibited both spontaneous and IL-6-induced proliferation of purified myeloma tumor cells (n=7) in a dose- and time- (data not shown) dependent way as determined by detection of $^{3}$H-thymidine incorporation (Figure 2A-C). Reduction of spontaneous proliferation induced by 30 μM lovastatin varied between 61.9-99.8% and 73.0-99.9% at day 2 and 4, respectively. Addition of exogenous IL-6 enhanced myeloma cell proliferation, as shown in Figure 2A for two myeloma patients' samples. Lovastatin reduced myeloma cell proliferation both in the presence and in the absence of exogenous IL-6 (n=2). Proliferation was restored by addition of mevalonate or GGOH to lovastatin-treated myeloma tumor cells, whereas FOH had no effect (n=4), as shown in Figure 2B for two representative myeloma patients' samples. Also in primary myeloma cells, Cdk2 activity was reduced by lovastatin and activity was restored by addition of mevalonate to lovastatin-treated cells (n=3), as shown in Figure 2C for a representative myeloma patient sample. The inhibition of Cdk2 activity was accompanied by up-regulation of $p21^{Cip1}$ protein levels in myeloma cells (Figure 2C). These data clearly suggest that lovastatin-reduced myeloma cell proliferation is the result of depletion of intracellular pools of GGPP leading to inhibition of protein geranylgeranylation.

**Inhibition of GGTase I activity reduces proliferation of myeloma cells**

We have previously shown that in myeloma cells GGTI-298 is a specific inhibitor of GGTase I, whereas FTase activity is specifically inhibited by FTI-277 [52]. To confirm that geranylgeranylation is critical for the regulation of myeloma cell proliferation, we incubated myeloma cell lines with FTI-277 and GGTI-298 for 2 or 4 days. GGTI-298 inhibited proliferation in a dose- and time-dependent way (Figure 3A). FTI-277 had no effect or inhibited proliferation only to a small extent when compared to GGTI-298. Cell cycle analysis showed that GGTI-298 treatment for 2 days caused myeloma cells to arrest at the G1 phase of the cell cycle and reduced the number of cells in S phase (Figure 3B).

**Rho family members are involved in the regulation of myeloma cell proliferation**

These data support the involvement of geranylgeranylated proteins in myeloma cell growth. Potential geranylgeranylated target proteins are the Rho family members RhoA, Rac-1, and Cdc42, which are involved in a host of cellular processes including cell proliferation. To discriminate between these proteins, we studied the effect of toxin B and C3 exoenzyme. *C. difficile* toxin B specifically glucosylates and inactivates Rho, Rac, and Cdc42 GTPases [46,53]. Treatment of myeloma cell lines with toxin B for 2 or 4 days reduced the number of viable cells in a dose- (data not shown) and time-dependent way (Table 1).

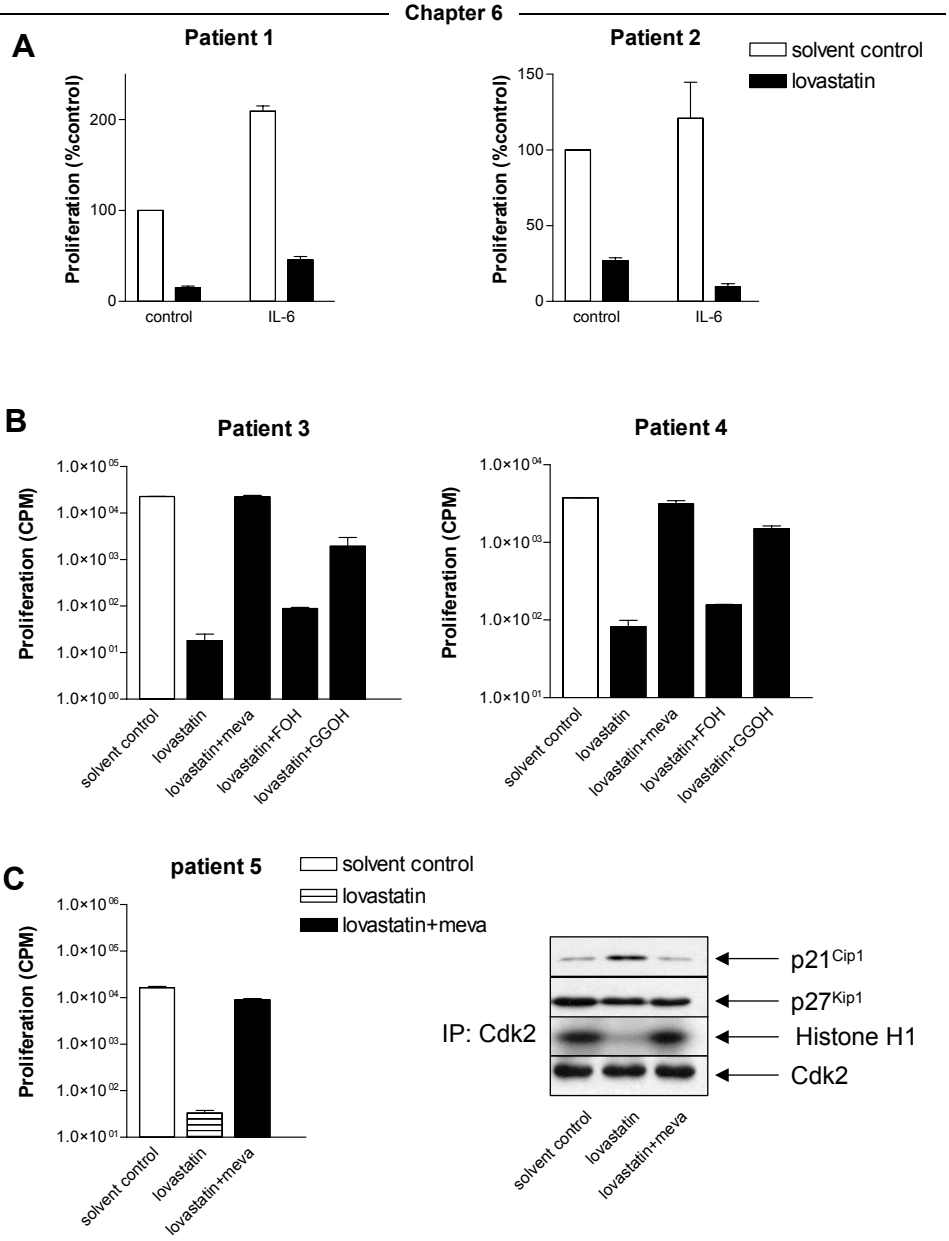


**Figure 2. Lovastatin reduces proliferation of purified myeloma cells from patients through depletion of GGPP.** Plasma cells from myeloma patients were purified from bone marrow mononuclear cells by MACS based on CD138 expression. Plasma cell percentage was >95% after purification. (A) Myeloma tumor cells were treated for 4 days with lovastatin (30 μM) or solvent control in the presence or absence of IL-6 (10 ng/ml). (B) Myeloma cells were treated for 4 days with solvent control, or lovastatin (30 μM) alone or in combination with mevalonate (meva; 100 μM), FOH (10 μM), or GGOH (10 μM). (C) Myeloma cells were treated for 2 days with solvent control, or lovastatin (30 μM) alone or in combination with mevalonate (meva; 100 μM). Proliferation was determined by $^{3}$H-thymidine incorporation during the last 16 hours of culture. Experiments were performed once in triplicate. Data are presented as mean ± s.e.m. After protein isolation $p21^{Cip1}$, $p27^{Kip1}$, and Cdk2 were determined by Western blot analysis. In addition, cell lysates were immunoprecipitated (IP) with anti-Cdk2, and kinase activity was determined using histone H1 as substrate.

This was caused by a dose- (data not shown) and time-dependent inhibition of myeloma cell proliferation, since the percentage of apoptotic cells was not affected by toxin B (Table 1) as shown for XG-1 cells in Figure 4A. *C. botulinum* C3 exoenzyme selectively inactivates Rho GTPases by ADP-ribosylating asparagine 41 [54-56]. Treatment of myeloma cells with C3 exoenzyme (50 μg/ml) for 2 or 4 days had no effect on the number of viable cells, and did not inhibit proliferation or induce apoptosis as shown for the XG-1 cell line, which was the most sensitive cell line to toxin B, in Figure 4B. In addition, introduction of C3 exoenzyme into cells using reversible permeabilization of myeloma cells with streptolysin-O had also no effect (data not shown). These data suggest that in myeloma cells, Rac and/or Cdc42 small GTPases rather than Rho proteins are involved in the regulation of myeloma cell proliferation.

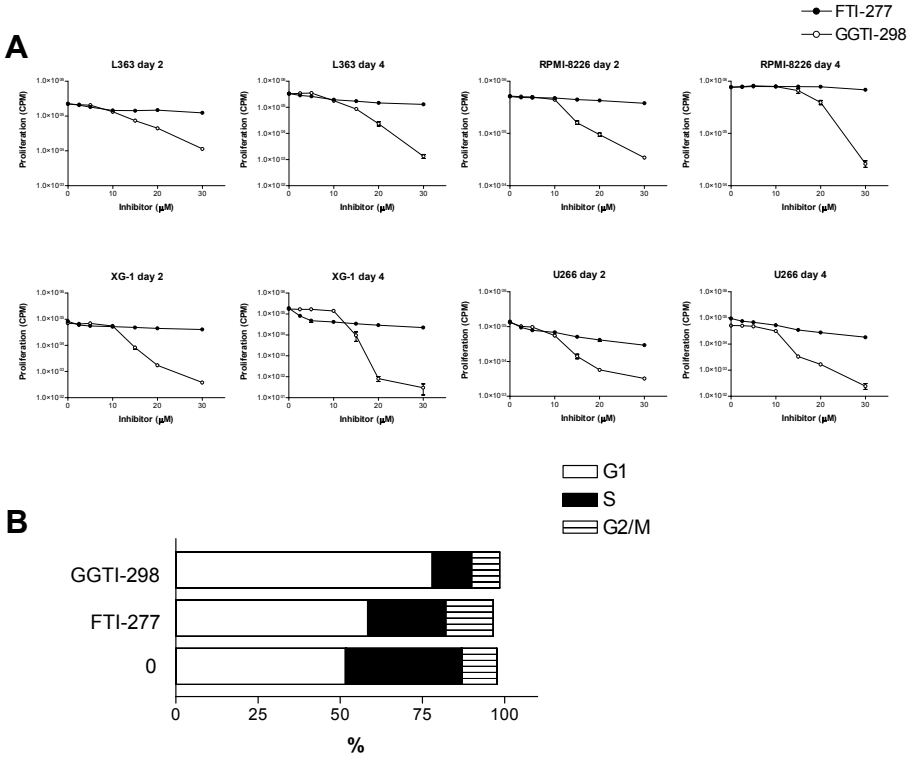


**Figure 3. Inhibition of geranylgeranyl transferase I activity inhibits proliferation of myeloma cell lines.** (A) L363, RPMI-8226, XG-1, and U266 cells were treated for 2 or 4 days with solvent control, or different concentrations of FTI-277 (2.5, 5, 10, 15, 20, 30 μM) or GGTI-298 (2.5, 5, 10, 15, 20, 30 μM) for 2 or 4 days. Proliferation was determined by $^{3}$H-thymidine incorporation during the last 16 hours of culture. Data were from three experiments performed in triplicate. Data are presented as mean ± s.e.m. (B) U266 cells were treated with solvent control, FTI-277 (20 μM), or GGTI-298 (20 μM) for 2 days, at which time the cells were harvested and cell cycle distribution was determined by BrdU assay. The percentages of cells in G1 phase, G2/M phase, and S phase of the cell cycle are shown. Results are representative of three experiments performed in triplicate.

**Table 1.** Effect of *C. difficile* toxin B on viability, proliferation, and apoptosis of myeloma cell lines

| | L363 | | RPMI-8226 | | XG-1 | | U266 | |
|---|---|---|---|---|---|---|---|---|
| | Day 2 | Day 4 | Day 2 | Day 4 | Day 2 | Day 4 | Day 2 | Day 4 |
| Number of viable cells (%control) | 53.1 | 39.6 | 93.1 | 79.8 | 42.9 | 13.7 | 85.8 | 59.6 |
| Proliferation (%control) | 61.5 | 65.2 | 93.8 | 83.7 | 39.8 | 22.7 | 39.6 | 36.3 |
| % of apoptotic cells | 31.9/36.8 | 42.8/41.6 | 30.2/33.4 | 48.1/50.5 | 30.2/38.1 | 41.5/48.8 | 10.9/12.7 | 11.3/16.7 |

Myeloma cell lines were treated for 2 or 4 days with solvent control or toxin B (50 ng/ml). The number of viable cells was determined by MTT assay, the percentage of apoptotic cells was evaluated by Annexin V/PI assay, and proliferation was determined by $^{3}$H-thymidine incorporation. The number of viable cells and proliferation are shown as a percentage of solvent control-treated cells. The percentage of apoptotic cells is shown for solvent control-(left) and toxin B-treated cells (right).

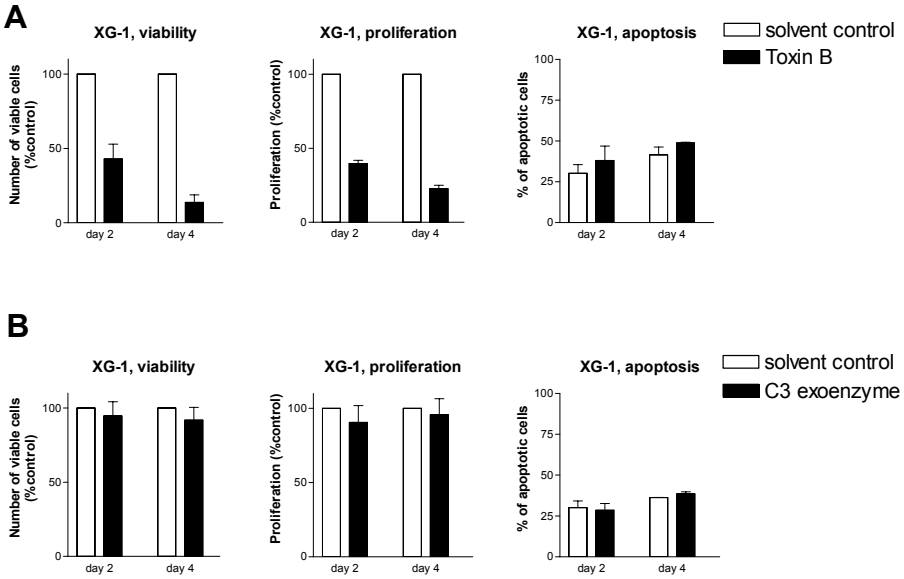


**Figure 4. Toxin B inhibits proliferation, but has no effect on the survival of myeloma cell lines.** (A) XG-1 cells were treated for 2 or 4 days with solvent control or *Clostridium difficile* toxin B (50 ng/ml). (B) XG-1 cells were treated for 2 or 4 days with solvent control or *Clostridium botulinum* C3 exoenzyme (50 µg/ml). The percentage of viable cells, relative to the solvent control, was measured by using MTT assay. Proliferation was determined by $^{3}$H-thymidine incorporation during the last 16 hours of culture. The percentage of apoptotic cells was determined by Annexin V assay. Shown is the percentage of early and late apoptotic cells. Experiments were performed three times in triplicate. Data are presented as mean ± s.e.m.

## Lovastatin reduces Rac-1 activity in myeloma cell lines

We analyzed in myeloma cell lines whether lovastatin, through inhibition of geranylgeranylation, reduced Rac-1 activity. The activation of Rac-1 was measured by specifically coprecipitating the GTP-bound form of Rac-1 using a recombinant fusion

protein of GST and amino acid residues 59-145 of PAK-1β, including the Rac binding domain (GST-PAK). Rac-1 was activated in all myeloma cell lines tested as shown for XG-1 cells in Figure 5A. Lovastatin reduced Rac-1 activity to near background levels. Rac-1 activity was restored by addition of mevalonate or GGOH to lovastatin-treated cells, whereas FOH had no effect.

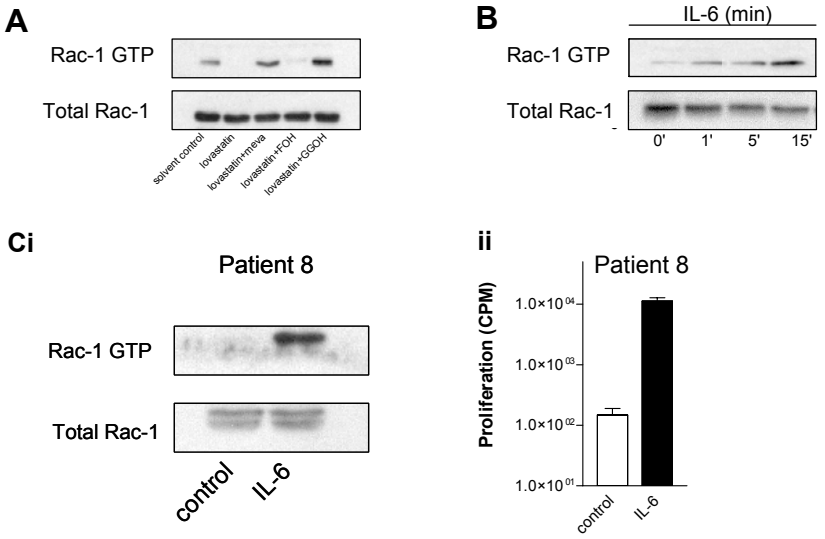


**Figure 5. Lovastatin inhibits Rac-1 activity, and IL-6 induces activation of Rac-1 in myeloma cell lines and purified tumor cells from patients.** (A) XG-1 cells were treated for 2 days with solvent control, or lovastatin (5 μM) alone or in combination with mevalonate (meva; 100 μM), FOH (10 μM), or GGOH (10 μM). Rac-1 activation state was examined using GST-PAK pull-down assays. Results are representative of three experiments performed in triplicate. (B) XG-1 cells were stimulated with IL-6 for 1, 5, and 15 minutes. Prior to stimulation with IL-6, XG-1 cells were cultured overnight in serum-free RPMI-1640 medium. Rac-1 activation state was examined using GST-PAK pull-down assays. Results are representative of three experiments performed in triplicate. (Ci) Plasma cells from myeloma patient 8 were purified from bone marrow mononuclear cells by MACS based on CD138 expression. Plasma cell percentage was >95% after purification. Purified myeloma tumor cells were incubated for 1 hour at 37°C in serum-free RPMI-1640 medium, and then incubated with 10 ng/ml IL-6 for 15 minutes. Rac-1 activation state was examined using GST-PAK pull-down assays. (Cii) Purified myeloma cells from patient 8 were incubated for 2 days in growth medium in the presence or absence of IL-6. Proliferation was determined by $^{3}$H-thymidine incorporation during the last 16 hours of culture. Experiments were performed once in triplicate. Data are presented as mean ± s.e.m.

## IL-6 induces Rac-1 activity in myeloma cell lines and tumor cells derived from patients

IL-6 is an important growth and survival factor for myeloma tumor cells. Rac-1 activation state was examined in IL-6-stimulated myeloma cell lines, using GST-PAK pull-down assays. We observed that IL-6 rapidly stimulated Rac-1 activity in serum-starved myeloma cell lines. This activation was already observed after 1 minute and was maintained for at least 15 minutes as shown in Figure 5B. Myeloma tumor cells derived from patients were also used to confirm that Rac-1 activity was induced by IL-6 in

primary cells. Similar to cell lines, IL-6-induced proliferation coincided with a significant increase in Rac-1-GTP levels (Figure 5C).

**Antisense Rac suppresses myeloma cell proliferation**

The role of Rac in the regulation of proliferation was investigated in purified tumor cells from a myeloma patient using antisense oligodeoxynucleotides (ODNs) complementary to a sequence shared by Rac-1 and Rac-2 genes. Myeloma cells were incubated for 4 days with solvent control, Rac antisense ODNs, or reverse polarity control ODNs prior to detection of Rac protein levels and measurement of proliferation. Rac protein levels were not affected by reverse polarity ODNs, whereas these were markedly decreased by Rac antisense ODNs when compared with the solvent control-treated cells. The down-regulation of Rac was sequence-specific since Rac antisense ODNs did not alter expression levels of RhoA (Figure 6A). Antisense-mediated Rac down-regulation significantly reduced the spontaneous proliferation of *ex vivo* purified myeloma cells (Figure 6B). In contrast, reverse polarity control ODNs had no effect when compared with solvent control-treated cells. Myeloma cell survival was not altered by Rac antisense ODNs (data not shown).

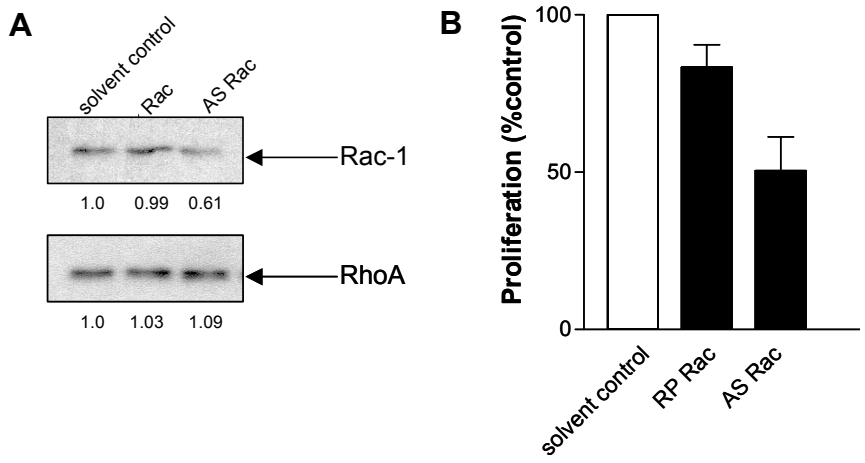


**Figure 6. Rac antisense ODNs reduce myeloma cell proliferation.** Plasma cells from myeloma patient 9, were purified from bone marrow mononuclear cells by MACS based on CD138 expression. Plasma cell percentage was >95% after purification. Purified myeloma tumor cells were incubated for 4 days with solvent control, reverse polarity Rac ODNs (RP Rac), or antisense Rac ODNs (AS Rac). (A) After protein isolation, Rac-1 and RhoA were determined by Western blot analysis. Numbers below blots represent the ratios of the optical density of a band relative to the optical density of the band from solvent control-treated cells. (B) Proliferation was determined by $^{3}$H-thymidine incorporation during the last 16 hours of culture. Experiments were performed once in triplicate. Data are presented as mean ± s.e.m.

**Dominant-negative Tat-Rac-1 reduces proliferation, whereas constitutively active Tat-Rac-1 enhances proliferation of myeloma cells**

Previous studies have shown that Tat fusion proteins rapidly enter cells following their addition to cell culture medium [49,57], and therefore Tat fusion proteins offer a novel method for transduction of proteins into cells. In this study, Tat-HA was fused with dominant-negative or constitutively active Rac-1. The effect of dominant-negative Tat-Rac-1 N17 or constitutively active Tat-Rac-1 Q61L on myeloma cell proliferation was analyzed in U266 and XG-1 cells. Myeloma cells were incubated with the Tat-Rac-1 proteins for 2 or 4 days after which proliferation was determined by detection of $^{3}$H-incorporation. Transduction of myeloma cells with dominant-negative Tat-Rac-1 N17 decreased proliferation when compared with solvent control-treated cells. In contrast, cells transduced with constitutively active Tat-Rac-1 Q61L showed increased proliferation (Figure 7A). However, both dominant-negative and constitutively active Tat-Rac-1 protein had no effect on viability of myeloma cells as determined by the Annexin V/PI assay (Figure 7B).

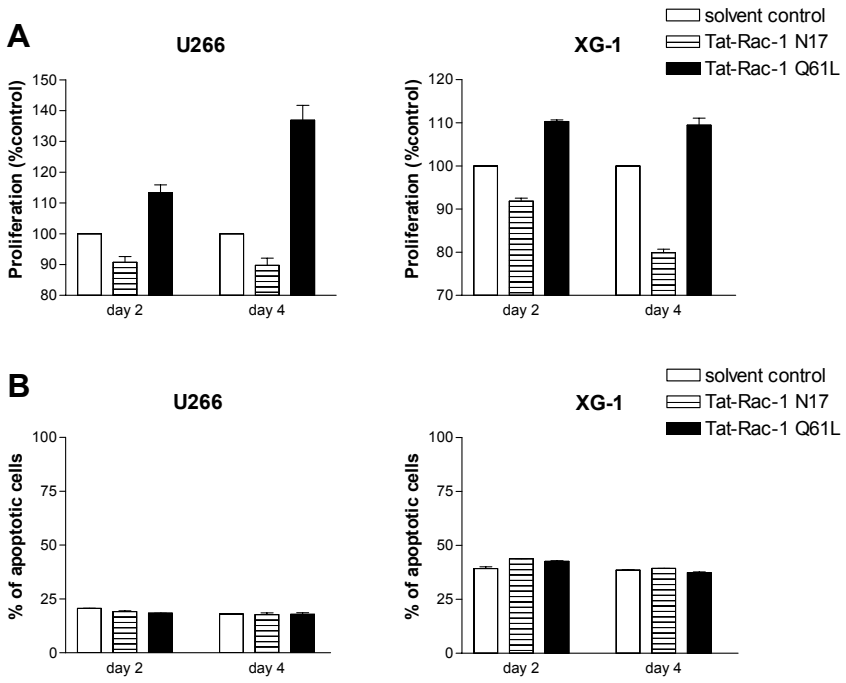


**Figure 7. Dominant-negative Tat-Rac-1 inhibits proliferation and constitutively active Tat-Rac-1 induces proliferation of myeloma cells.** U266 and XG-1 cells were treated for 2 or 4 days with solvent control, dominant-negative Tat-Rac-1 N17 (18.7 μg/ml), or constitutively active Tat-Rac-1 Q61L (18.7 μg/ml). (A) Proliferation was determined by $^{3}$H-thymidine incorporation during the last 16 hours of culture. Data were from three experiments performed in triplicate. Data are presented as mean ± s.e.m. (B) The percentage of apoptotic cells was determined by Annexin V assay. Shown is the percentage of early and late apoptotic cells. Experiments were performed three times in triplicate. Data are presented as mean ± s.e.m.

## Discussion

In this study we investigated the importance of protein prenylation in the control of myeloma cell growth. In a previous study we showed that inhibition of HMG-CoA reductase by statins induced apoptosis in myeloma cells via down-regulation of the anti-apoptotic protein Mcl-1 [52]. In these experiments addition of geranylgeranyl isoprenoids, but not farnesyl isoprenoids, rescued myeloma cells from statin-induced apoptosis. Furthermore, specific inhibition of GGTase I activity, but not FTase activity, also resulted in myeloma cell death. These results implied that geranylgeranylated proteins are involved in statin-induced apoptosis. In this paper we show for the first time that geranylgeranylated proteins regulate myeloma cell growth. Analysis of potential candidate geranylgeranylated proteins suggests that the GTPase Rac-1 is involved in the control of myeloma proliferation without affecting apoptosis.

We have previously shown that inhibition of HMG-CoA reductase in myeloma cells inhibited both farnesylation and geranylgeranylation of target proteins by depletion of the isoprenoids FPP and GGPP in a time- and dose-dependent fashion. Inhibition of geranylgeranylation coincided with induction of apoptosis and these effects could be prevented by addition of mevalonate or GGOH, which is metabolized to GGPP in cells [40]. Studies performed with lung adenocarcinoma [58] and in mouse fibroblasts [59] implicated that inhibition of geranylgeranylation resulted in inhibition of proliferation by arrest in the G1 phase of the cell cycle. Here we show that lovastatin blocks Cdk2 activity, in association with enhanced expression of Cdk inhibitors $p21^{Cip1}$ and/or $p27^{Kip1}$, and inhibits myeloma cell proliferation in a time- and dose-dependent way through the accumulation of cells in the G1 phase of the cell cycle. Addition of GGOH restored Cdk2 activity, the G1 to S phase cell cycle progression, and proliferation. FOH, which is metabolized to FPP in cells [40], completely restored farnesylation [52], but had no or only minor effects on myeloma cell proliferation. Furthermore, specific inhibition of GGTase I activity was more effective in reducing myeloma cell proliferation when compared with inhibition of FTase. This indicates that farnesylated proteins are not involved in the regulation of myeloma proliferation or are unable to support proliferation in the absence of geranylgeranylated proteins. These data support a role for geranylgeranylated proteins in the progression of myeloma cells from G1 to the S phase of the cell cycle.

Geranylgeranylated target proteins that regulate proliferation include Rac-1, RhoA, and Cdc42. *Clostridium difficile* toxin B glycolysates and inactivates Rho, Cdc42 and Rac GTPases [46,53]. Toxin B did not induce apoptosis, but inhibited the proliferation of myeloma cells. *Clostridium botulinum* C3 exoenzyme which ADP-ribolysates and inactivates Rho GTPases [54-56], had no effect on myeloma cell growth or survival. This suggests that reduced cell growth by inhibition of geranylgeranylation is likely due to, at least in part, inhibition of Rac and/or Cdc42 protein activity. Furthermore, these data

indicate that the regulation of proliferation by these geranylgeranylated proteins is independent of effects on survival. Since Rac-1 is required for proliferation, but not survival of BCR/ABL-expressing myeloid precursor cells [60] and plays a role in the regulation of invasion and metastasis of lymphoma tumor cells [61], we investigated the role of Rac-1 in myeloma in this study. Rac-1 is a regulator of diverse cellular processes including the control of cytoskeleton organization, membrane trafficking, cellular adhesion, and gene expression. In addition, Rac-1 plays an essential role in cell cycle progression through G1 [25,62]. Studies in fibroblasts with activated Rac-1 mutants showed that activation of Rac-1 alone was sufficient to initiate cell cycle progression [25,26], while dominant-negative versions of Rac-1 blocked serum-induced DNA synthesis [25].

Rac-1 activity was observed in all myeloma cell lines tested. Depletion of GGPP by lovastatin reduced Rac-1 activity in myeloma cell lines. We also investigated whether the myeloma growth factor IL-6 stimulated Rac-1 activity. Both in myeloma cell lines and in purified myeloma cells from patients Rac-1 activity was induced by IL-6. The role of Rac-1 in the regulation of myeloma cell survival and proliferation was investigated by using both Tat-Rac-1 mutants and Rac antisense ODNs. Specific reduction of Rac protein levels by antisense ODNs reduced spontaneous proliferation of myeloma cells. Furthermore, we demonstrated that dominant-negative Tat-Rac-1 reduced proliferation, whereas constitutively active Tat-Rac-1 stimulated proliferation of myeloma cell lines including IL-6-dependent XG-1 cells. These data suggest that in myeloma cells IL-6 induces proliferation, at least in part, through Rac-1-dependent pathways. Several studies have indicated that the Ras-Raf-MEK-ERK [12,63,64] and PI-3K/Akt [8,11,14,64] pathways are involved in IL-6-induced proliferation. Rac-1 may be a downstream effector of Ras and/or PI-3K signaling pathways in myeloma cells, since both Ras [29,31,65,66] and PI-3K [66-71] mediate Rac-1 activation. In addition, in myeloma cells IL-6 induces tyrosine phosphorylation of Vav [72], which is a GDP/GTP exchange factor (GEF) for members of the Rho family of GTPases, including Rac-1 [73-75]. Phosphorylation of Vav and products of PI-3K activation have been proposed to contribute to the GEF activation of Vav. We are currently investigating which IL-6-triggered signaling pathways lead to the activation of Rac-1 in myeloma. Interestingly, dominant-negative Tat-Rac-1, constitutively active Tat-Rac-1, and Rac antisense ODNs did not affect myeloma cell survival. This suggests that Rac-1-dependent pathways play an important role in the control of myeloma cell growth, but not in myeloma cell viability.

In conclusion, our findings indicate that protein geranylgeranylation is essential for myeloma cell proliferation through the induction of G1/S progression. We have identified the geranylgeranylated GTP-binding protein Rac-1 as an important regulator of myeloma tumor cell proliferation. Furthermore, we provide evidence that IL-6-mediated Rac-1 activation is necessary for IL-6-dependent myeloma cell growth. However, Rac-1 activity was not required for myeloma cell survival. These results suggest that inhibition of protein geranylgeranylation may be a new treatment strategy in multiple myeloma.

## Acknowledgements

We thank Dr S. Bhakdi (Institute of Medical Microbiology and Hygiene, Johannes Gutenberg University, Mainz, Germany) for the kind gift of streptolysin-O.

## References

1. Freund, G. G., Kulas, D. T., and Mooney, R. A. Insulin and IGF-1 increase mitogenesis and glucose metabolism in the multiple myeloma cell line, RPMI 8226. J Immunol, *151*: 1811-1820, 1993.
2. Klein, B., Zhang, X. G., Lu, Z. Y., and Bataille, R. Interleukin-6 in human multiple myeloma. Blood, *85*: 863-872, 1995.
3. Georgii-Hemming, P., Wiklund, H. J., Ljunggren, O., and Nilsson, K. Insulin-like growth factor I is a growth and survival factor in human multiple myeloma cell lines. Blood, *88*: 2250-2258, 1996.
4. Jelinek, D. F., Witzig, T. E., and Arendt, B. K. A role for insulin-like growth factor in the regulation of IL-6-responsive human myeloma cell line growth. J Immunol, *159*: 487-496, 1997.
5. Xu, F., Gardner, A., Tu, Y., Michl, P., Prager, D., and Lichtenstein, A. Multiple myeloma cells are protected against dexamethasone-induced apoptosis by insulin-like growth factors. Br.J Haematol., *97*: 429-440, 1997.
6. Ge, N. L. and Rudikoff, S. Insulin-like growth factor I is a dual effector of multiple myeloma cell growth. Blood, *96*: 2856-2861, 2000.
7. Ferlin, M., Noraz, N., Hertogh, C., Brochier, J., Taylor, N., and Klein, B. Insulin-like growth factor induces the survival and proliferation of myeloma cells through an interleukin-6-independent transduction pathway. Br.J Haematol., *111*: 626-634, 2000.
8. Tu, Y., Gardner, A., and Lichtenstein, A. The phosphatidylinositol 3-kinase/AKT kinase pathway in multiple myeloma plasma cells: roles in cytokine-dependent survival and proliferative responses. Cancer Res., *60*: 6763-6770, 2000.
9. Heinrich, P. C., Behrmann, I., Muller-Newen, G., Schaper, F., and Graeve, L. Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway. Biochem.J, *334*: 297-314, 1998.
10. Catlett-Falcone, R., Landowski, T. H., Oshiro, M. M., Turkson, J., Levitzki, A., Savino, R., Ciliberto, G., Moscinski, L., Fernandez-Luna, J. L., Nunez, G., Dalton, W. S., and Jove, R. Constitutive activation of Stat3 signaling confers resistance to apoptosis in human U266 myeloma cells. Immunity., *10*: 105-115, 1999.
11. Hideshima, T., Nakamura, N., Chauhan, D., and Anderson, K. C. Biologic sequelae of interleukin-6 induced PI3-K/Akt signaling in multiple myeloma. Oncogene, *%20;20*: 5991-6000, 2001.
12. Ogata, A., Chauhan, D., Teoh, G., Treon, S. P., Urashima, M., Schlossman, R. L., and Anderson, K. C. IL-6 triggers cell growth via the Ras-dependent mitogen-activated protein kinase cascade. J.Immunol., *159*: 2212-2221, 1997.
13. Qiang, Y. W., Kopantzev, E., and Rudikoff, S. Insulinlike growth factor-I signaling in multiple myeloma: downstream elements, functional correlates, and pathway cross-talk. Blood, *99*: 4138-4146, 2002.
14. Hsu, J., Shi, Y., Krajewski, S., Renner, S., Fisher, M., Reed, J. C., Franke, T. F., and Lichtenstein, A. The AKT kinase is activated in multiple myeloma tumor cells. Blood, *98*: 2853-2855, 2001.
15. Mitsiades, C. S., Mitsiades, N., Poulaki, V., Schlossman, R., Akiyama, M., Chauhan, D., Hideshima, T., Treon, S. P., Munshi, N. C., Richardson, P. G., and Anderson, K. C. Activation of NF-kappaB and upregulation of intracellular anti-apoptotic proteins via the IGF-1/Akt signaling in human multiple myeloma cells: therapeutic implications. Oncogene, *21*: 5673-5683, 2002.

16. Ni, H., Ergin, M., Huang, Q., Qin, J. Z., Amin, H. M., Martinez, R. L., Saeed, S., Barton, K., and Alkan, S. Analysis of expression of nuclear factor kappa B (NF-kappa B) in multiple myeloma: downregulation of NF-kappa B induces apoptosis. Br.J Haematol., *115*: 279-286, 2001.
17. Hideshima, T., Chauhan, D., Richardson, P., Mitsiades, C., Mitsiades, N., Hayashi, T., Munshi, N., Dang, L., Castro, A., Palombella, V., Adams, J., and Anderson, K. C. NF-kappa B as a therapeutic target in multiple myeloma. J Biol.Chem., *277*: 16639-16647, 2002.
18. Bharti, A. C., Donato, N., Singh, S., and Aggarwal, B. B. Curcumin (diferuloylmethane) down-regulates the constitutive activation of nuclear factor-kappa B and IkappaBalpha kinase in human multiple myeloma cells, leading to suppression of proliferation and induction of apoptosis. Blood, *101*: 1053-1062, 2003.
19. Liu, P., Leong, T., Quam, L., Billadeau, D., Kay, N. E., Greipp, P., Kyle, R. A., Oken, M. M., and Van Ness, B. Activating mutations of N- and K-ras in multiple myeloma show different clinical associations: analysis of the Eastern Cooperative Oncology Group Phase III Trial. Blood, *88*: 2699-2706, 1996.
20. Neri, A., Murphy, J. P., Cro, L., Ferrero, D., Tarella, C., Baldini, L., and Dalla-Favera, R. Ras oncogene mutation in multiple myeloma. J.Exp.Med., *170*: 1715-1725, 1989.
21. Portier, M., Moles, J. P., Mazars, G. R., Jeanteur, P., Bataille, R., Klein, B., and Theillet, C. p53 and RAS gene mutations in multiple myeloma. Oncogene, *7*: 2539-2543, 1992.
22. Billadeau, D., Jelinek, D. F., Shah, N., LeBien, T. W., and Van Ness, B. Introduction of an activated N-ras oncogene alters the growth characteristics of the interleukin 6-dependent myeloma cell line ANBL6. Cancer Res., *55*: 3640-3646, 1995.
23. Billadeau, D., Liu, P., Jelinek, D., Shah, N., LeBien, T. W., and Van Ness, B. Activating mutations in the N- and K-ras oncogenes differentially affect the growth properties of the IL-6-dependent myeloma cell line ANBL6. Cancer Res., *57*: 2268-2275, 1997.
24. Rowley, M., Liu, P., and Van Ness, B. Heterogeneity in therapeutic response of genetically altered myeloma cell lines to interleukin 6, dexamethasone, doxorubicin, and melphalan. Blood, *96*: 3175-3180, 2000.
25. Olson, M. F., Ashworth, A., and Hall, A. An essential role for Rho, Rac, and Cdc42 GTPases in cell cycle progression through G1. Science, *269*: 1270-1272, 1995.
26. Lamarche, N., Tapon, N., Stowers, L., Burbelo, P. D., Aspenstrom, P., Bridges, T., Chant, J., and Hall, A. Rac and Cdc42 induce actin polymerization and G1 cell cycle progression independently of p65PAK and the JNK/SAPK MAP kinase cascade. Cell, *87*: 519-529, 1996.
27. Philips, A., Roux, P., Coulon, V., Bellanger, J. M., Vie, A., Vignais, M. L., and Blanchard, J. M. Differential effect of Rac and Cdc42 on p38 kinase activity and cell cycle progression of nonadherent primary mouse fibroblasts. J Biol.Chem., *275*: 5911-5917, 2000.
28. Westwick, J. K., Lambert, Q. T., Clark, G. J., Symons, M., Van Aelst, L., Pestell, R. G., and Der, C. J. Rac regulation of transformation, gene expression, and actin organization by multiple, PAK-independent pathways. Mol.Cell Biol., *17*: 1324-1335, 1997.
29. Khosravi-Far, R., Solski, P. A., Clark, G. J., Kinch, M. S., and Der, C. J. Activation of Rac1, RhoA, and mitogen-activated protein kinases is required for Ras transformation. Mol.Cell Biol., *15*: 6443-6453, 1995.
30. Prendergast, G. C., Khosravi-Far, R., Solski, P. A., Kurzawa, H., Lebowitz, P. F., and Der, C. J. Critical role of Rho in cell transformation by oncogenic Ras. Oncogene, *10*: 2289-2296, 1995.
31. Qiu, R. G., Chen, J., Kirn, D., McCormick, F., and Symons, M. An essential role for Rac in Ras transformation. Nature, *374*: 457-459, 1995.
32. Qiu, R. G., Chen, J., McCormick, F., and Symons, M. A role for Rho in Ras transformation. Proc Natl Acad Sci U S A, *92*: 11781-11785, 1995.
33. Qiu, R. G., Abo, A., McCormick, F., and Symons, M. Cdc42 regulates anchorage-independent growth and is necessary for Ras transformation. Mol.Cell Biol., *17*: 3449-3458, 1997.

34. Gelb, M. H. Protein prenylation, et cetera: signal transduction in two dimensions. Science, *275*: 1750-1751, 1997.
35. Magee, T. and Marshall, C. New insights into the interaction of Ras with the plasma membrane. Cell, *98*: 9-12, 1999.
36. Bishop, A. L. and Hall, A. Rho GTPases and their effector proteins. Biochem.J, *348 Pt 2:241-55.*: 241-255, 2000.
37. Goldstein, J. L. and Brown, M. S. Regulation of the mevalonate pathway. Nature, *343*: 425-430, 1990.
38. Downs, J. R., Clearfield, M., Weis, S., Whitney, E., Shapiro, D. R., Beere, P. A., Langendorfer, A., Stein, E. A., Kruyer, W., and Gotto, A. M. Primary prevention of acute coronary events with lovastatin in men and women with average cholesterol levels: results of AFCAPS/TexCAPS. Air Force/Texas Coronary Atherosclerosis Prevention Study. JAMA, *279*: 1615-1622, 1998.
39. Keyomarsi, K., Sandoval, L., Band, V., and Pardee, A. B. Synchronization of tumor and normal cells from G1 to multiple cell cycles by lovastatin. Cancer Res., *51*: 3602-3609, 1991.
40. Crick, D. C., Andres, D. A., and Waechter, C. J. Novel salvage pathway utilizing farnesol and geranylgeraniol for protein isoprenylation. Biochem.Biophys.Res.Commun., *237*: 483-487, 1997.
41. Zhang, X. G., Gaillard, J. P., Robillard, N., Lu, Z. Y., Gu, Z. J., Jourdan, M., Boiron, J. M., Bataille, R., and Klein, B. Reproducible obtaining of human myeloma cell lines as a model for tumor stem cell study in human multiple myeloma. Blood, *83*: 3654-3663, 1994.
42. Jego, G., Robillard, N., Puthier, D., Amiot, M., Accard, F., Pineau, D., Harousseau, J. L., Bataille, R., and Pellat-Deceunynck, C. Reactive plasmacytoses are expansions of plasmablasts retaining the capacity to differentiate into plasma cells. Blood, *94*: 701-712, 1999.
43. van de Donk, N. W., Kamphuis, M. M., Lokhorst, H. M., and Bloem, A. C. The cholesterol lowering drug lovastatin induces cell death in myeloma plasma cells. Leukemia, *16*: 1362-1371, 2002.
44. Malcolm, K. C., Elliott, C. M., and Exton, J. H. Evidence for Rho-mediated agonist stimulation of phospholipase D in rat1 fibroblasts. Effects of Clostridium botulinum C3 exoenzyme. J Biol.Chem., *271*: 13135-13139, 1996.
45. Weiss, R. H., Ramirez, A., and Joo, A. Short-term pravastatin mediates growth inhibition and apoptosis, independently of Ras, via the signaling proteins p27Kip1 and P13 kinase. J Am Soc.Nephrol., *10*: 1880-1890, 1999.
46. Linseman, D. A., Laessig, T., Meintzer, M. K., McClure, M., Barth, H., Aktories, K., and Heidenreich, K. A. An essential role for Rac/Cdc42 GTPases in cerebellar granule neuron survival. J Biol.Chem., *%19;276*: 39123-39131, 2001.
47. Dorseuil, O., Vazquez, A., Lang, P., Bertoglio, J., Gacon, G., and Leca, G. Inhibition of superoxide production in B lymphocytes by rac antisense oligonucleotides. J Biol.Chem., *267*: 20540-20542, 1992.
48. Dorseuil, O., Leca, G., Vazquez, A., and Gacon, G. Inhibition of Rac function using antisense oligonucleotides. Methods Enzymol., *256:358-66.*: 358-366, 1995.
49. Hall, D. J., Cui, J., Bates, M. E., Stout, B. A., Koenderman, L., Coffer, P. J., and Bertics, P. J. Transduction of a dominant-negative H-Ras into human eosinophils attenuates extracellular signal-regulated kinase activation and interleukin-5-mediated cell viability. Blood, *98*: 2014-2021, 2001.
50. Aida, Y. and Pabst, M. J. Removal of endotoxin from protein solutions by phase separation using Triton X-114. J Immunol Methods, *132*: 191-195, 1990.
51. van de Donk, N. W., Kamphuis, M. M., Van Dijk, M., Borst, H. P., Bloem, A. C., and Lokhorst, H. M. Chemosensitization of myeloma plasma cells by an antisense-mediated downregulation of Bcl-2 protein. Leukemia, *17*: 211-219, 2003.
52. van de Donk, N. W., Kamphuis, M. M., Van Kessel, B., Lokhorst, H. M., and Bloem, A. C. Inhibition of protein geranylgeranylation induces apoptosis in myeloma plasma cells by reducing Mcl-1 protein levels. Blood, .: 2003.
53. Just, I., Selzer, J., Wilm, M., Eichel-Streiber, C., Mann, M., and Aktories, K. Glucosylation of Rho proteins by Clostridium difficile toxin B. Nature, *375*: 500-503, 1995.

54. Nemoto, Y., Namba, T., Kozaki, S., and Narumiya, S. Clostridium botulinum C3 ADP-ribosyltransferase gene. Cloning, sequencing, and expression of a functional protein in Escherichia coli. J Biol.Chem., *266*: 19312-19319, 1991.
55. Aktories, K. Bacterial toxins that target Rho proteins. J Clin.Invest, *99*: 827-829, 1997.
56. Han, S., Arvai, A. S., Clancy, S. B., and Tainer, J. A. Crystal structure and novel recognition motif of rho ADP-ribosylating C3 exoenzyme from Clostridium botulinum: structural insights for recognition specificity and catalysis. J Mol.Biol., *305*: 95-107, 2001.
57. Nagahara, H., Vocero-Akbani, A. M., Snyder, E. L., Ho, A., Latham, D. G., Lissy, N. A., Becker-Hapak, M., Ezhevsky, S. A., and Dowdy, S. F. Transduction of full-length TAT fusion proteins into mammalian cells: TAT-p27Kip1 induces cell migration. Nat.Med, *4*: 1449-1452, 1998.
58. Miquel, K., Pradines, A., Sun, J., Qian, Y., Hamilton, A. D., Sebti, S. M., and Favre, G. GGTI-298 induces G0-G1 block and apoptosis whereas FTI-277 causes G2-M enrichment in A549 cells. Cancer Res., *57*: 1846-1850, 1997.
59. Vogt, A., Qian, Y., McGuire, T. F., Hamilton, A. D., and Sebti, S. M. Protein geranylgeranylation, not farnesylation, is required for the G1 to S phase transition in mouse fibroblasts. Oncogene, *13*: 1991-1999, 1996.
60. Skorski, T., Wlodarski, P., Daheron, L., Salomoni, P., Nieborowska-Skorska, M., Majewski, M., Wasik, M., and Calabretta, B. BCR/ABL-mediated leukemogenesis requires the activity of the small GTP-binding protein Rac. Proc Natl Acad Sci U S A, *95*: 11858-11862, 1998.
61. Michiels, F., Habets, G. G., Stam, J. C., van der Kammen, R. A., and Collard, J. G. A role for Rac in Tiam1-induced membrane ruffling and invasion. Nature, *375*: 338-340, 1995.
62. Joneson, T., McDonough, M., Bar-Sagi, D., and Van Aelst, L. RAC regulation of actin polymerization and proliferation by a pathway distinct from Jun kinase. Science, *274*: 1374-1376, 1996.
63. Rowley, M. and Van Ness, B. Activation of N-ras and K-ras induced by interleukin-6 in a myeloma cell line: implications for disease progression and therapeutic response. Oncogene, *21*: 8769-8775, 2002.
64. Hu, L., Shi, Y., Hsu, J. H., Gera, J., Van Ness, B., and Lichtenstein, A. Downstream effectors of oncogenic ras in multiple myeloma cells. Blood, *101*: 3126-3135, 2003.
65. Rodriguez-Viciana, P., Warne, P. H., Khwaja, A., Marte, B. M., Pappin, D., Das, P., Waterfield, M. D., Ridley, A., and Downward, J. Role of phosphoinositide 3-OH kinase in cell transformation and control of the actin cytoskeleton by Ras. Cell, *89*: 457-467, 1997.
66. Grill, B. and Schrader, J. W. Activation of Rac-1, Rac-2, and Cdc42 by hemopoietic growth factors or cross-linking of the B-lymphocyte receptor for antigen. Blood, *100*: 3183-3192, 2002.
67. Nobes, C. D., Hawkins, P., Stephens, L., and Hall, A. Activation of the small GTP-binding proteins rho and rac by growth factor receptors. J Cell Sci, *108*: 225-233, 1995.
68. Arrieumerlou, C., Donnadieu, E., Brennan, P., Keryer, G., Bismuth, G., Cantrell, D., and Trautmann, A. Involvement of phosphoinositide 3-kinase and Rac in membrane ruffling induced by IL-2 in T cells. Eur.J Immunol, *28*: 1877-1885, 1998.
69. Cheng, H. L., Steinway, M. L., Russell, J. W., and Feldman, E. L. GTPases and phosphatidylinositol 3-kinase are critical for insulin-like growth factor-I-mediated Schwann cell motility. J Biol.Chem., *275*: 27197-27204, 2000.
70. Jiang, K., Zhong, B., Ritchey, C., Gilvary, D. L., Hong-Geller, E., Wei, S., and Djeu, J. Y. Regulation of Akt-dependent cell survival by Syk and Rac. Blood, *101*: 236-244, 2003.
71. Van Leeuwen, F. N., Olivo, C., Grivell, S., Giepmans, B. N., Collard, J. G., and Moolenaar, W. H. Rac activation by lysophosphatidic acid LPA1 receptors through the guanine nucleotide exchange factor Tiam1. J Biol.Chem., *278*: 400-406, 2003.
72. Lee, I. S., Liu, Y., Narazaki, M., Hibi, M., Kishimoto, T., and Taga, T. Vav is associated with signal transducing molecules gp130, Grb2 and Erk2, and is tyrosine phosphorylated in response to interleukin-6. FEBS Lett., *%20;401*: 133-137, 1997.

73. Matsuguchi, T., Inhorn, R. C., Carlesso, N., Xu, G., Druker, B., and Griffin, J. D. Tyrosine phosphorylation of p95Vav in myeloid cells is regulated by GM-CSF, IL-3 and steel factor and is constitutively increased by p210BCR/ABL. EMBO J, *14*: 257-265, 1995.
74. Crespo, P., Schuebel, K. E., Ostrom, A. A., Gutkind, J. S., and Bustelo, X. R. Phosphotyrosine-dependent activation of Rac-1 GDP/GTP exchange by the vav proto-oncogene product. Nature, *385*: 169-172, 1997.
75. Han, J., Das, B., Wei, W., Van Aelst, L., Mosteller, R. D., Khosravi-Far, R., Westwick, J. K., Der, C. J., and Broek, D. Lck regulates Vav activation of members of the Rho family of GTPases. Mol.Cell Biol., *17*: 1346-1353, 1997.

# Chapter 7

## Protein geranylgeranylation is critical for the regulation of survival and proliferation of lymphoma tumor cells

Niels W.C.J. van de Donk, Diana Schotte, Marloes M.J. Kamphuis, Ariënne M.W. van Marion, Berris van Kessel, Andries C. Bloem, and Henk M. Lokhorst

Clinical Cancer Research, in press

## Abstract

***Purpose:*** Prenylation is essential for membrane localization and participation of proteins in various signaling pathways. The following study was conducted to examine the importance of protein farnesylation and geranylgeranylation for the regulation of lymphoma cell survival and proliferation.

***Experimental design:*** Lymphoma cells were treated with the HMG-CoA reductase inhibitor lovastatin, which inhibits protein farnesylation and geranylgeranylation by the depletion of intracellular pools of farnesylpyrophosphate and geranylgeranylpyrophosphate. In addition, farnesyl transferase and geranylgeranyl transferase (GGTase I) activity were specifically inhibited by FTI-277 and GGTI-298, respectively.

***Results:*** Only inhibition of geranylgeranylation by lovastatin resulted in the reduction of cell viability in lymphoma cell lines and purified tumor cells from lymphoma patients in a time- and dose-dependent way. The reduction in the number of viable cells was mediated by both induction of apoptosis and inhibition of proliferation. In addition, GGTI-298 was more effective in the induction of apoptosis and inhibition of proliferation, when compared with FTI-277. Apoptosis induced by inhibition of protein geranylgeranylation was associated with a reduction of Mcl-1 protein levels, collapse of the mitochondrial transmembrane potential, and caspase-3 activation. Inhibition of proliferation resulted from the induction of a G1 arrest. Furthermore, lovastatin at low concentrations sensitized lymphoma cells to dexamethasone, including cells resistant to this drug.

***Conclusion:*** These results indicate that protein geranylgeranylation is critical for the regulation of lymphoma tumor cell survival and proliferation, and that pharmacological agents such as lovastatin or GGTase inhibitors, alone or in combination with other drugs, may be useful in the treatment of lymphoma.


## Abbreviations

The abbreviations used are: FPP, farnesylpyrophosphate; GGPP, geranylgeranylpyrophosphate; GGTase I, geranylgeranyl transferase I; FTase, farnesyl transferase; AML, acute myeloid leukemia; FOH, farnesol; GGOH, geranylgeraniol; FCS, fetal calf serum; NHL, non-Hodgkin's lymphoma; B-CLL, B cell chronic lymphocytic leukemia; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; SDS, sodium dodecyl sulphate; BrdU, bromodeoxyuridine; BSA, bovine serum albumin; PI, propidium iodide; $DiOC_6$[3], 3,3'-dihexyloxacarbocyanine iodide; s.e.m., standard error of the mean.

## Introduction

The rate-limiting step of the mevalonate pathway is the conversion of HMG-CoA to mevalonate and is catalyzed by the enzyme HMG-CoA reductase [1]. Mevalonate is an intermediate in the synthesis of cholesterol, essential for membrane integrity; of dolichol, required for glycoprotein synthesis; of polyisoprenoids side chains of heme A and ubiquinone, involved in oxidative respiration; and of isopentyl-adenine, present in some tRNAs. Furthermore, mevalonate is also a precursor of the isoprenoids FPP and GGPP [1]. These isoprenoids are used for the post-translational modification of proteins including Ras (farnesylation) [2,3] and the Rho familiy members Rac-1, RhoA, and Cdc42 (geranylgeranylation) [4]. FTase and GGTase transfer farnesyl and geranylgeranyl moieties from FPP and GGPP, respectively, to the thiol group of conserved cysteine residues at or near the C-terminus of target proteins. Prenylation is essential for membrane association [2] and participation of proteins in various signaling pathways regulating growth and survival [3,5-7]. Lovastatin is a potent competitive inhibitor of HMG-CoA reductase and thereby prevents the conversion of HMG-CoA to mevalonate and the synthesis of the other products of the mevalonate pathway. We and others have previously shown that lovastatin induces apoptosis and inhibits proliferation in various cancer cell lines [8-12] and in purified tumor cells derived from multiple myeloma [12] and AML patients [11].

NHL and B-CLL are characterized by initial sensitivity to cytotoxic drugs in the majority of the patients. However, multidrug-resistant disease will ultimately develop in many of these patients. Recent studies have shown that defects in apoptotic pathways contribute significantly to resistance of cancer cells to chemotherapeutic agents [13]. Anti-apoptotic signaling pathways such as PI-3K/Akt [14-20] or NF-kappaB [21], and expression of anti-apoptotic Bcl-2 familiy members including Bcl-2 [22-27], Mcl-1 [17,28-30], and Bcl-XL [31], are involved in the regulation of lymphoma tumor cell survival and protection against cytotoxic drugs. Therefore, interference with such pathways may induce apoptosis in lymphoma tumor cells or restore chemosensitivity in chemoresistant tumor cells.

This report shows that inhibition of protein geranylgeranylation by depletion of GGPP by lovastatin or by inhibition of GGTase I activity, induces apoptosis and inhibits proliferation in lymphoma cell lines and tumor cells from patients. In addition, we found that lovastatin enhanced the sensitivity of lymphoma cells to dexamethasone. Altogether our results indicate that interference with the geranylgeranylation of proteins involved in survival and proliferation, may be a new treatment strategy in lymphoma.

## Materials and methods

### Reagents

Lovastatin and simvastatin were obtained from Merck & Co., Inc (Rahway, NJ, USA) and were chemically activated by alkaline hydrolysis prior to use as described previously [32]. Pravastatin Sodium was purchased from Bristol-Meyers Squibb (New Brunswick, NJ, USA) and dissolved in PBS (20 mM). Atorvastatin Calcium was obtained from Pfizer GmbH (Freiburg, Germany) and dissolved in ethanol containing 3% DMSO (Riedel-de Haen, Seelze, Germany) (10 mM). Mevalonate and FOH were purchased from Sigma (St Louis, MO, USA) and GGOH was obtained from ICN Biomedicals, BV (Zoetermeer, The Netherlands). FOH and GGOH were metabolized in cells to FPP and GGPP, respectively [33]. FTI-277 and GGTI-298 were obtained from Calbiochem (Schwallbach, Germany).

### Cell lines

The follicular lymphoma cell line DoHH2 was obtained from the German Collection of Microorganisms and Cell Cultures (GCMC) and the follicular lymphoma cell line SU-DHL-6 [34], was kindly provided by Dr J. Jansen (University Medical Center Nijmegen, The Netherlands). The Burkitt's lymphoma cell lines Raji, Ramos, and Daudi were purchased from the American Tissue Culture Collection. Cell lines were cultured in RPMI-1640 (GIBCO, Breda, The Netherlands) supplemented with 10% FCS (Integro, Zaandam, The Netherlands), 100 IU/ml penicillin, 100 μg/ml streptomycin and 10 μM β-mercaptoethanol (growth medium).

### Patients

After obtaining informed consent, mononuclear cells were obtained by Ficoll-Paque (Amersham; Pharmacia BiotechAB, Uppsala, Sweden) density centrifugation of peripheral blood from 13 patients suffering from leukemic NHL, B-CLL or hairy cell leukemia, of bone marrow from one patient with follicular lymphoma, and of pleural fluid from one patient with mantle cell lymphoma. Clinical characteristics of the patients are shown in Table 1. Tumor cells from patients 3 and 9 were purified from bone marrow mononuclear cells (patient 3) or from peripheral blood mononuclear cells (patient 9) by MACS, based on CD19 expression. To this end, mononuclear cells were subsequently labeled with anti-CD19 (Becton Dickinson, Erembodegem, Belgium (BDIS)) and rat anti-mouse IgG1 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and then separated using a high gradient magnetic separation column placed in a strong magnetic field (Miltenyi), following the instructions of the manufacturer. Purity after MACS selection of these samples was >95%.

Tumor cells from patient 7 were purified from an axillary lymph node. The organ was cut into small pieces with a scalpel blade and incubated with 1 mg/ml collagenase IV

(Sigma) and 0.1 mg/ml DNase I (Sigma) for one hour at 37°C, 5% $CO_2$. The cell suspension was subsequently filtered through an open-filter chamber (NPBI, Emmer-Copascuum, The Netherlands) and mononuclear cells were obtained by Ficoll-Paque density centrifugation.

Percentage and monoclonality of the malignant cells was established by flow cytometry (FACSCalibur, BDIS) by analysis of the light chain distribution within the surface bound immunoglobulins on CD19 positive cells or the CD4/CD8 ratio within CD3 positive T cells in case of patient 11. Percentages of clonal cells present in the samples after density centrifugation or MACS selection (patients 3 and 9) ranged from 75 to 99.4% of the nucleated cells (Table 3). For experiments, tumor cells were resuspended in growth medium (see above).

**Table 1.** Clinical characteristics

| Patient | Age/Sex | Diagnosis | Stage | Disease status |
|---|---|---|---|---|
| 1 | 64/M | FL grade I | IVA | untreated |
| 2 | 43/M | FL grade I | IVA | refractory |
| 3 | 70/M | FL grade I | IVA | refractory |
| 4 | 71/F | FL grade I | IVA | refractory |
| 5 | 59/M | FL grade I | IVA | responsive disease |
| 6 | 45/M | FL grade III | IIIA | refractory |
| 7 | 66/F | MCL | IVA | refractory |
| 8 | 67/M | MCL | IVA | refractory |
| 9 | 47/F | MCL | IVB | refractory |
| 10 | 49/M | MZL | IVA | responsive disease |
| 11 | 67/F | PTL | IVA | untreated |
| 12 | 59/M | Richter | IVA | refractory |
| 13 | 59/M | B-CLL | Rai II | untreated |
| 14 | 59/M | B-CLL | Rai III | untreated |
| 15 | 62/F | B-CLL | Rai IV | refractory |
| 16 | 73/M | HCL | | untreated |

The lymphoproliferative disorders were diagnosed according to the REAL classification. The NHL and CLL patients were classified according to the Ann Arbor and Rai classification. F, female; M, male; FL, Follicular lymphoma; MCL, Mantle cell lymphoma; MZL, Marginal zone lymphoma; PTL, Peripheral T-cell lymphoma; Richter, Richter's syndrome; B-CLL, B-cell chronic lymphocytic leukemia; HCL, Hairy cell leukemia.

**Cell viability**

Viability of cells was examined by means of the MTT assay as described previously [12]. In short, cells were seeded in a concentration of 0.3 X $10^6$/ml for the lymphoma cell lines or 1 X $10^6$/ml for the tumor cells of patients in a 96-well flat bottom plate (100 μl/well) (Nunc, Roskilde, Denmark) and treated with different concentrations of lovastatin (for concentrations see legends) alone or in the presence of mevalonate, FOH, or GGOH. Fixed concentrations of mevalonate (100 μM), FOH (10 μM), or GGOH (10 μM) were used. These concentrations proved to be optimal in rescuing myeloma cells from

lovastatin-induced apoptosis (data not shown). After 2 or 4 days, 25 µl of MTT (5 mg/ml) was added to each well. After an incubation of 2 hours at 37°C the reaction was stopped by the addition of 100 µL 20% SDS (Boehringer Mannheim, Mannheim, Germany) / 0.025 M HCl / 0.35 M HAc in a mixture of (1:1; v/v) N,N-dimethylformamide (Merck, Darmstadt, Germany) and distilled water. After an overnight incubation at 37°C the optical density of the samples was determined at 570 nm.

**Cell proliferation**

Cells (3 X $10^4$) were seeded in 96-well flat bottom plates (Nunc) in 100 µl growth medium with lovastatin (for concentrations see legends) alone or in the presence of mevalonate (100 µM), FOH (10 µM), or GGOH (10 µM). Inhibition of FTase and GGTase I was accomplished by treating cells with FTI-277 and GGTI-298, respectively (for concentrations see legends). After 32 and 80 hours, $^3$H-thymidine (Amersham, Little Chalfont, UK) (1 µCi/well) was added for the remaining 16 hours of the assay. $^3$H-thymidine incorporation was analyzed by liquid scintillation counting as described previously [12].

**Cell cycle analysis**

Lymphoma cells (1 X $10^6$ in 1.5 ml) were incubated with different concentrations of lovastatin (for concentrations see legends) in the presence or absence of mevalonate (100 µM), FOH (10 µM), or GGOH (10 µM) in a 48-well plate (Nunc) for 2 days. Cells were then pulsed with 10 µM BrdU for 30 minutes at 37°C, after which the cells were washed twice with ice-cold PBS containing 1% BSA. Cells were subsequently fixed with 70% ethanol for 30 minutes at 4°C. After the ethanol was washed away, the cells were treated with 2 M HCl containing 0.5% Triton-X-100 for 30 minutes at room temperature, followed by neutralization with 1M natrium tetraborate (pH 8.5). Following washing, cells were resuspended in PBS containing 0.5% Tween-20 and 1% BSA and incubated with anti-BrdU-FITC (BDIS) for 30 minutes at room temperature. After washing with PBS containing 0.5% Tween-20 and 1% BSA, cells were stained with propidium iodide in PBS (5 µg/ml) and analyzed by flow cytometry.

**Apoptosis detection by Annexin V staining**

Lymphoma cells (1.5 X $10^5$ in 0.5 ml) were incubated with different concentrations of lovastatin (for concentrations see legends) alone or in the presence of mevalonate (100 µM), FOH (10 µM), or GGOH (10 µM) in a 48-well plate (Nunc). Inhibition of FTase and GGTase I was accomplished by FTI-277 and GGTI-298, respectively (for concentrations see legends). After 2 or 4 days, cells were harvested, washed in ice-cold PBS and directly stained with Annexin V-FITC (Nexins Research, Kattendijke, The Netherlands) and PI. After 10 minutes of incubation at room temperature in the dark, cells were analyzed by

flow cytometry as described previously [12]. Apoptotic cells were defined as early apoptotic cells (Annexin V positive and PI negative) and late apoptotic cells (Annexin V positive and PI positive).

**Caspase-3 activity**

The caspase-3 activity assay (Roche) was used to determine caspase-3 activity. Briefly, cells were washed in ice-cold PBS and then resuspended in lysis buffer (1X DTT) and incubated for 1 minute on ice. Supernatants were obtained after centrifugation at 14 000 r.p.m. for 1 minute at room temperature. Supernatant was added to anti-caspase-3 coated wells and incubated at 37°C for 1 hour. After 3 washing steps, substrate solution (Ac-DEVD-AFC) was added and the wells were incubated for 2 hours at 37°C. Fluorescence was measured with an excitation filter 400 nm and an emission filter 505 nm.

**Measurement of mitochondrial transmembrane potential**

Changes in mitochondrial transmembrane potential ($\Delta\psi_m$) were evaluated by staining with 40 nM $DiOC_6[3]$ (Molecular Probes, Leiden, The Netherlands). Cells were incubated with $DiOC_6[3]$ in PBS for 15 minutes at 37°C, washed and resuspended in PBS. The cells were then analyzed on a flow cytometer (FACSCalibur, BDIS).

**Western blotting**

Cells (1 X $10^6$ cells in 1.5 ml) were incubated for 2 days with lovastatin (for concentrations see legends) in the presence or absence of mevalonate (100 μM), FOH (10 μM), or GGOH (10 μM). Inhibition of FTase and GGTase I was accomplished by FTI-277 and GGTI-298, respectively (for concentrations see legends). After harvesting, whole cell lysates were made by washing cells twice in ice-cold PBS and then resuspending them in lysis buffer (10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% Triton X-100 and a cocktail of protease inhibitors) at 4°C for 20 minutes. Insoluble material was removed by centrifugation at 14 000 r.p.m. for 6 minutes at 4°C. Protein concentrations were determined by the BCA assay (Pierce, Rockford, IL, USA). Samples containing equal amounts of protein were mixed with 2 X Laemmli sample buffer (0.125 M Tris pH 6.9 with 4% SDS, 20% Glycerol and 10% β-mercaptoethanol) and boiled for 5 minutes. Proteins were subsequently fractionated in 10% SDS-PAGE at room temperature and electrically transferred from the gel to PVDF-membrane (Biorad). After blocking in 0.1% Tween-20, 5% skimmed powder milk, 2% BSA in 10 mM Tris and 150 mM NaCl, the membranes were incubated with anti-Bcl-2 (Dako, Glostrup, Denmark), anti-Mcl-1 (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA), anti-Bcl-XL (Santa Cruz) or anti-Bax (Dako), anti-Rap1a (Santa Cruz), or anti-HDJ-2 (DnaJ) (Neomarkers, Lab Vision, Fremont, CA, USA). Antibody binding was visualized with enhanced chemoluminescence (Amersham) detection with hyperfilm ECL (Amersham) after incubation with a

horseradish peroxidase-conjugated secondary antibody (Dako). Finally, the membranes were extensively washed in PBS and reprobed with anti-α-actin (Sigma) as a control for equal loading of protein. Relative amounts of protein were determined by densitometry and expressed as a percentage of the solvent control.

### Statistics

Data analysis was performed using the SPSS statistical software package (SPSS Inc, Chicago, IL, USA). A two-sided Student's t-test or a Welch's t-test in case of unequal variances was used to determine differences between groups. Differences were considered statistically significant when $P < 0.05$. Data are plotted as means ± s.e.m.

## Results

### Lovastatin reduces cell viability of lymphoma cell lines

The lymphoma cell lines (DoHH2, Raji, Daudi, Ramos, and SU-DHL-6) were incubated with different concentrations of lovastatin for 2 or 4 days. Lovastatin reduced viability in a dose- and time-dependent way. Representative examples from the DoHH2 and Raji cell lines are shown in Figure 1A and B. DoHH2 was the most sensitive cell line to lovastatin, whereas SU-DHL-6 was most resistant to lovastatin. The concentration of lovastatin that reduced viability with 50% (MTT50) and 75% (MTT25) ranged from 4.7 to 45.7 and 9.7 to 118 μM at day 2, respectively. At day 4, MTT50 varied between 4.4 and 29.3 and MTT25 between 7.9 and 46.6 μM (Table 2).

**Table 2.** Effect of lovastatin on cell viability in 5 lymphoma cell lines

| Cell line | MTT50 t2 (μM) | MTT25 t2 (μM) | MTT50 t4 (μM) | MTT25 t4 (μM) |
|---|---|---|---|---|
| DoHH2 | 4.7 | 9.7 | 4.4 | 7.9 |
| Raji | 20.6 | 110.9 | 14.0 | 25.1 |
| Daudi | 29.2 | 118.4 | 18.5 | 27.4 |
| Ramos | 27.4 | 60.1 | 19.5 | 27.2 |
| SU-DHL-6 | 45.7 | 87.5 | 29.3 | 46.6 |

MTT50 and MTT25 represent the concentrations of lovastatin, which after a 2 (t2) or 4 (t4) day incubation reduced cell viability of purified lymphoma tumor cells with 50% and 75%, compared to the solvent control-treated cells, respectively.

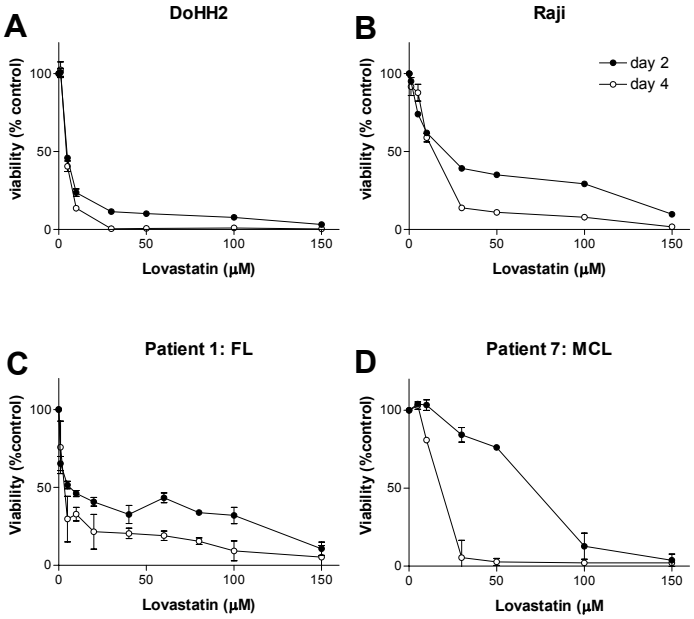


**Figure 1. Lovastatin reduces cell viability in cell lines and in purified tumor cells from lymphoma patients in a time- and dose-dependent way.** Cell lines (A) DoHH2, (B) Raji, (C) purified tumor cells from patient 1 (FL, follicular lymphoma), and (D) purified tumor cells from patient 7 (MCL, mantle cell lymphoma) were treated for 2 or 4 days with solvent control or with different concentrations of lovastatin (1-150 μM). The percentage of viable cells, relative to the solvent control-treated cells, was measured by using MTT assay. Experiments were performed three times in triplicate in case of cell lines and one time in triplicate in case of purified tumor cells from patients. Data are presented as mean ± s.e.m. In some cases the s.e.m. was smaller than the symbol.

### Lovastatin reduces cell viability of tumor cells from NHL patients

Studies were then conducted to determine the effect of lovastatin in purified tumor cells from lymhoma patients (n=16). Tumor cells were purified from peripheral blood (n=13), bone marrow (n=1), pleural fluid (n=1), or from an axillary lymph node (n=1). Tumor cell percentage was >75% after purification (Table 3). The purified tumor cells were incubated with different concentrations of lovastatin for 2 or 4 days. Similar to cell lines, lovastatin reduced cell viability in a dose- and time-dependent way in tumor cells from all patients. Figure 1C and D show representative examples of dose-response data from 2 patients. Lovastatin sensitivity differed among the lymphoma patients' samples. MTT50 and MTT25 were in the range of 1.9 to >150 and 3.1 to >150 at day 2, respectively. At day 4 MTT50 varied from 0.72 to 118 and MTT25 from 1.8 to 142 μM (Table 3). No obvious correlations were observed between lymphoma type or prior therapy and the effects of lovastatin on cell viability. Patients 1, 11, 13, 14, and 16 had not received treatment at the moment that the *in vitro* experiments were performed. Patients 1, 13, and 16 responded to subsequent chemotherapy, whereas patients 11 and 14 were lost to follow-up. There was

no difference between patients with chemosensitive or chemoresistant disease and lovastatin sensitivity.

**Table 3.** Effect of lovastatin on cell viability of purified tumor cells from patients with lymphoproliferative disorders

| Patient | Source | Purity (%) | MTT50 t2 (μM) | MTT25 t2 (μM) | MTT50 t4 (μM) | MTT25 t4 (μM) |
|---|---|---|---|---|---|---|
| 1 | PB | 90.2 | 6.4 | 116 | 3.2 | 16.3 |
| 2 | PB | 87.2 | | | 4.4 | 8.8 |
| 3 | BM | 99.4* | n.r. | n.r. | 16.9 | 57.0 |
| 4 | PB | 89.2 | 144 | n.r. | 118 | 142 |
| 5 | PB | 88.5 | 146 | n.r. | | |
| 6 | PF | 82.0 | 42.3 | 124 | 8.4 | 19.1 |
| 7 | LN | 93.4 | 70.6 | 90.4 | 18.2 | 24.4 |
| 8 | PB | 94.5 | 101 | 148 | 17.5 | 27.6 |
| 9 | PB | 97.8* | 14.7 | 18.8 | | |
| 10 | PB | 75.0 | 92.5 | n.r. | 10.3 | 129 |
| 11 | PB | 96.0 | n.r. | n.r. | 14.3 | 77.6 |
| 12 | PB | 84.0 | | | 7.0 | 14.7 |
| 13 | PB | 94.6 | 3.3 | 49.6 | 4.3 | 71.9 |
| 14 | PB | 77.5 | | | 4.4 | 15.0 |
| 15 | PB | 94.2 | 1.9 | 3.1 | 0.72 | 1.8 |
| 16 | PB | 75.0 | 108 | n.r. | 41.5 | 91.2 |

Purity indicates the percentage of tumor cells in patients' samples and was determined by detection of CD19 and membrane immunoglobulin light chain (sκ/sλ) detection in case of a B-cell malignancy or by detection of CD3/CD4/CD8 in case of a T-cell malignancy by flow cytometric analysis. * indicates that selection was performed by MACS based on CD19 expression. MTT50 and MTT25 represent the concentrations of lovastatin, which after a 2 (t2) or 4 (t4) day incubation reduced cell viability of purified lymphoma tumor cells with 50% and 75%, compared to the solvent control-treated cells, respectively. n.r., indicates that MTT50 or MTT25 values could not be calculated, because the concentration response curves reached a plateau. PB, peripheral blood; BM, bone marrow; PF, pleural fluid; LN, axillary lymph node.

**Lovastatin inhibits protein prenylation**

The effect of lovastatin on prenylation in DoHH2 and Raji cell lines was determined by analysis of the migratory behaviour during electrophoresis of DnaJ, a protein prenylated exclusively by FTase [35,36], and of Rap1a, a protein prenylated exclusively by GGTase I [37] (Figure 2A). Inhibition of prenylation of these proteins can be monitored by immunoblotting, because the unprenylated forms of these proteins display reduced mobility in SDS-PAGE relative to their prenylated versions. In solvent control-treated cells DnaJ and Rap1a were in the processed, prenylated forms. Lovastatin inhibited the farnesylation of DnaJ and geranylgeranylation of Rap1a. Addition of mevalonate restored the processing of DnaJ and Rap1a. Treatment with lovastatin in the presence of GGOH (10 μM), which is metabolized to GGPP in the cells [33], restored geranylgeranylation of Rap1a, but had no effect on the inhibition of the farnesylation of DnaJ. In contrast, FOH (10 μM), which is metabolized to FPP [33], restored DnaJ farnesylation, but not Rap1a geranylgeranylation.

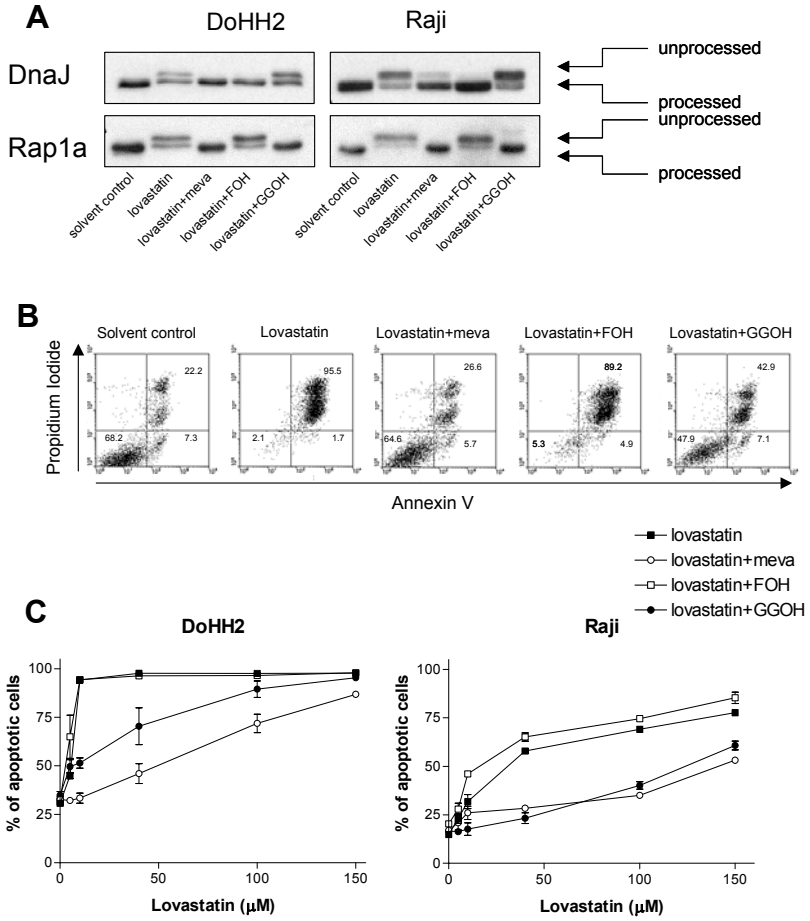


**Figure 2. Lovastatin inhibits protein farnesylation and geranylgeranylation, and induces apoptosis by depletion of intracellular pools of GGPP.** (A) DoHH2 and Raji cells were treated for 2 days with solvent control, or lovastatin (30 μM) alone or in the presence of mevalonate (meva; 100 μM), GGOH (10 μM), or FOH (10 μM). After protein isolation, processing of DnaJ and Rap1a was determined by Western blot analysis. The faster-migrating band represents mature and processed protein, the slower band represents unprenylated, unprocessed protein. The data shown are representative of at least three independent experiments. (B) DoHH2 cells were treated for 4 days with solvent control, or lovastatin (30 μM) alone or in combination with mevalonate (meva; 100 μM), GGOH (10 μM), or FOH (10 μM). The percentage of apoptotic cells was examined by using the Annexin V assay. The percentages of viable plasma cells (Annexin V-/PI-), early apoptotic cells (Annexin V+/PI-), and late apoptotic cells (Annexin V+/PI+) in each dot plot are indicated in the corresponding quadrants. Results are representative of three experiments performed in triplicate. (C) DoHH2 and Raji cells were treated for 4 days with solvent control, or lovastatin (5, 10, 40, 100, 150 μM) in combination with mevalonate (meva; 100 μM), GGOH (10 μM), or FOH (10 μM), after which apoptosis was determined by Annexin V assay. Shown is the sum of the percentages of early and late apoptotic cells. Experiments were performed three times in triplicate. Data are presented as mean ± s.e.m. In some cases the s.e.m. was smaller than the symbol.

### Lovastatin induces apoptosis by depletion of geranylgeranylpyrophosphate

DoHH2, Raji, Daudi, Ramos, and SU-DHL-6 cells were treated with lovastatin alone or in combination with mevalonate (100 μM), FOH (10 μM), or GGOH (10 μM) and apoptosis

was assessed by using the Annexin V assay. Lovastatin treatment resulted in an increase of the percentage of apoptotic cells in a time- and dose-dependent way as shown for DoHH2 and Raji in Figure 2 B and C. Treatment of cells with mevalonate or GGOH prevented lovastatin-induced apoptosis. However, addition of FOH had no effect. Similar to lovastatin, other hydrophobic HMG-CoA reductase inhibitors including simvastatin and atorvastatin also induced apoptosis, which could be abrogated by addition of mevalonate or GGOH, but not FOH. However, the hydrophilic inhibitor pravastatin was without effect (data not shown). This indicates that inhibition of geranylgeranylation by depletion of intracellular pools of GGPP by inhibition of HMG-CoA reductase induces apoptosis in lymphoma cell lines.

**Apoptosis induction by lovastatin is associated with Mcl-1 protein reduction, collapse of the mitochondrial transmembrane potential, and caspase-3 activation**

Expression levels of Bcl-2 family proteins were determined in DoHH2 and Raji cells treated with lovastatin (30 μM) alone or in combination with mevalonate (100 μM), FOH (10 μM), or GGOH (10 μM) by Western blot analysis. Lovastatin treatment resulted in a significant reduction of Mcl-1 expression levels (Figure 3A). Only in DoHH2 cells, lovastatin reduced Bcl-XL and increased Bax protein expression. Although Bcl-2 protein levels remained unaltered, there was an increase of the pro-apoptotic 23 kD Bcl-2 form, which could be visualized after long exposure of the film when compared with Bcl-2. Importantly, mevalonate and GGOH prevented the reduction of Mcl-1 and Bcl-XL expression, and the increase of Bax and the pro-apoptotic Bcl-2 form. In contrast, FOH was without effect.

Members of the Bcl-2 family are involved in the regulation of the mitochondrial transmembrane potential, release of cytochrome c, and caspase-3 activation [38,39]. Treatment of DoHH2 and Raji cells with lovastatin resulted in loss of the mitochondrial transmembrane potential as shown in Figure 3 B and C for DoHH2 cells. The collapse was time- (data not shown) and dose-dependent (Figure 3C). To determine whether lovastatin treatment resulted in activation of caspase-3, cell lysates were analyzed for caspase-3 activity. Treatment of DoHH2 and Raji cells with lovastatin resulted in activation of caspase-3 as shown for DoHH2 cells in Figure 3D. Bcl-2 is a known substrate of caspase-3 [40,41]. Exposure of cell lines to lovastatin stimulates caspase-3 activity, which, in turn, results in the generation of the Bcl-2 cleavage product that promotes apoptosis. The increase of the pro-apoptotic 23 kD Bcl-2 form was demonstrated by Western blot analysis (Figure 3A).

Treatment of DoHH2 and Raji cells with lovastatin in the presence of mevalonate or GGOH prevented the collapse of the mitochondrial transmembrane potential and the activation of caspase-3. However, incubation of cells with lovastatin in combination with FOH had no effect, when compared with lovastatin alone (Figure 3B-D).

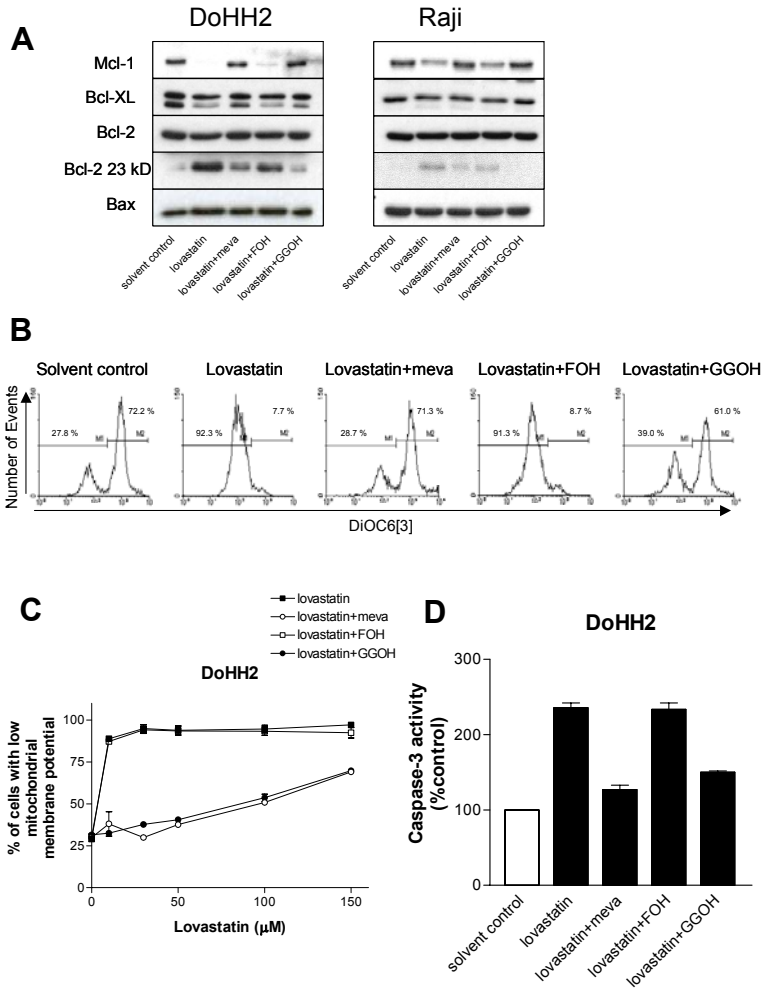


**Figure 3. Depletion of GGPP by lovastatin is associated with Mcl-1 protein reduction, collapse of the mitochondrial transmembrane potential, and caspase-3 activation.** (A) Lymphoma cell lines DoHH2 and Raji were treated for 2 days with solvent control, or lovastatin (30 μM) alone or in combination with mevalonate (meva; 100 μM), FOH (10 μM), or GGOH (10 μM). After protein isolation, Mcl-1, Bcl-XL, Bcl-2, and Bax were determined by Western blot analysis. Furthermore, the pro-apoptotic 23 kD Bcl-2 form was detected after long exposure of the film. The data shown are representative of at least three independent experiments. (B) DoHH2 cells were treated for 4 days with solvent control, or lovastatin (30 μM) alone or in combination with mevalonate (meva; 10 μM), FOH (10 μM), or GGOH (10 μM). Collapse of the mitochondrial transmembrane potential was determined by staining the cells with $DiOC_6[3]$. Results are representative of 3 experiments performed in triplicate. The fraction of cells with low ($DiOC_6[3]^{low}$) and the fraction of cells with intact ($DiOC_6[3]^{high}$) mitochondrial transmembrane potential are indicated in gate M1 and M2, respectively. (C) DoHH2 cells were treated for 4 days with solvent control, or different concentrations of lovastatin (10, 30, 50, 100, 150 μM) alone or in combination with mevalonate (meva; 100 μM), FOH (10 μM), or GGOH (10 μM). Collapse of the mitochondrial transmembrane potential was determined by staining the cells with $DiOC_6[3]$. Shown is the percentage of cells with low mitochondrial transmembrane potential. Experiments were performed three times in triplicate. Data are presented as mean ± s.e.m. In some cases the s.e.m. was smaller than the symbol. (D) DoHH2 cells were exposed to solvent control, or lovastatin (30 μM) alone or in combination with mevalonate (meva; 100 μM), FOH (10 μM), or GGOH (10 μM) for 2 days, at which time the cells were harvested and caspase-3 activation was assessed as described in "Materials and Methods." Experiments were performed three times in duplicate. Data are presented as mean ± s.e.m.

**Synergism between lovastatin and dexamethasone in the induction of lymphoma cell death**

Cell lines were treated with dexamethasone (0, 5, 10, 50, or 100 μM) or doxorubicin (0, 10, 25, 50, 100, 200, or 500 nM) alone or in combination with lovastatin (0-30 μM). The combination of lovastatin with doxorubicin produced a significant additive effect (data not shown). Furthermore, low concentrations of lovastatin potently sensitized both dexamethasone-sensitive (DoHH2, SU-DHL-6, Ramos) and dexamethasone-resistant (Raji, Daudi) lymphoma cells to dexamethasone in a synergistic fashion (Figure 4).

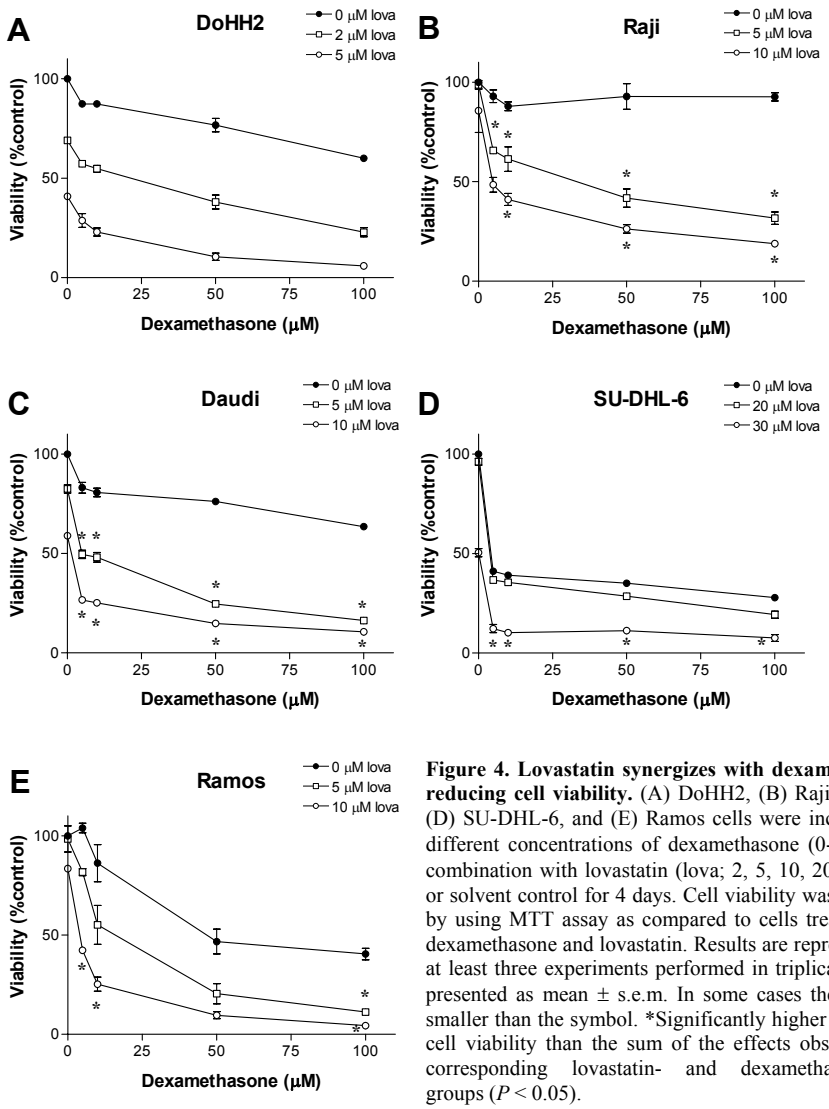


**Figure 4. Lovastatin synergizes with dexamethasone in reducing cell viability.** (A) DoHH2, (B) Raji, (C) Daudi, (D) SU-DHL-6, and (E) Ramos cells were incubated with different concentrations of dexamethasone (0-100 μM) in combination with lovastatin (lova; 2, 5, 10, 20, or 30 μM) or solvent control for 4 days. Cell viability was determined by using MTT assay as compared to cells treated without dexamethasone and lovastatin. Results are representative of at least three experiments performed in triplicate. Data are presented as mean ± s.e.m. In some cases the s.e.m. was smaller than the symbol. *Significantly higher reduction of cell viability than the sum of the effects observed in the corresponding lovastatin- and dexamethasone-treated groups ($P < 0.05$).

**Depletion of GGPP inhibits proliferation by induction of a G1 arrest**

DoHH2, Raji, Daudi, Ramos, and SU-DHL-6 were treated with lovastatin (0-150 μM) alone or in combination with mevalonate (100 μM), FOH (10 μM), or GGOH (10 μM). Proliferation was examined by measuring $^{3}$H-thymidine incorporation. Lovastatin inhibited proliferation in a time- (data not shown) and dose-dependent way as shown for DoHH2 and Raji at day 4 in Figure 5A. Mevalonate and GGOH, but not FOH restored proliferation in lovastatin-treated lymphoma cell lines. Cell cycle analysis revealed that lovastatin-treatment increased the number of cells in the G1 phase of the cell cycle, whereas the number of cells in the S phase decreased (Figure 5B). The blockade of cell cycle progression responsible for the inhibition of proliferation of lymphoma cells was abrogated by addition of mevalonate or GGOH, whereas FOH was without effect (Figure 5B).

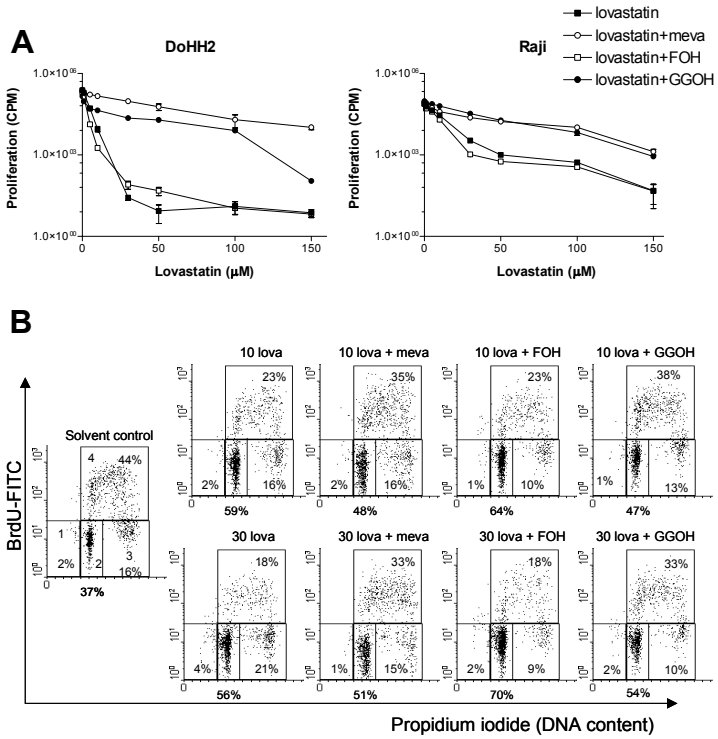


**Figure 5. Depletion of GGPP by lovastatin inhibits proliferation by the induction of a G1 block.** (A) DoHH2 and Raji cells were treated for 4 days with solvent control, or different concentrations of lovastatin (1, 5, 10, 30, 50, 100, 150 μM) alone or in combination with mevalonate (meva; 100 μM), FOH (10 μM), or GGOH (10 μM). Proliferation was determined by $^{3}$H-thymidine incorporation during the last 16 hours of culture. Data were from three experiments performed in triplicate. Data are presented as mean ± s.e.m. In some cases the s.e.m. was smaller than the symbol. (B) DoHH2 cells were treated with solvent control, or lovastatin (lova; 10 or 30 μM) alone or in combination with mevalonate (meva; 100 μM), FOH (10 μM), or GGOH (10 μM) for 2 days, at which time the cells were harvested and cell cycle distribution was determined by BrdU assay. The percentage of apoptotic cells (1) and the percentages of cells in G1 phase (2), G2/M phase (3), and S phase (4) of the cell cycle in each dot plot are indicated in the corresponding quadrants. Results are representative of three experiments performed in triplicate.

**Depletion of GGPP reduces cell viability, down-regulates Mcl-1 expression levels, and inhibits proliferation in purified tumor cells from lymphoma patients**

Purified tumor cells from lymphoma patients were incubated with lovastatin alone or in combination with mevalonate (100 μM), FOH (10 μM), or GGOH (10 μM). Similar to the effects in cell lines, addition of mevalonate and GGOH to purified tumor cells from patients (n=4) abrogated lovastatin-induced reduction of cell viability, whereas FOH had no effect. This is shown for a representative patient (patient 15) in Figure 6A. Mcl-1 levels were determined in cells from 3 patients. In all cases, Mcl-1 protein levels were decreased when the cells were incubated with lovastatin. The reduction was 54.3% for patient 3, 35.1% for patient 7, and 98.4% for patient 15. Bcl-XL, Bcl-2, and Bax protein levels remained unchanged. Mcl-1 protein expression was recovered when mevalonate or GGOH were added to lovastatin-treated cells. FOH had no effect, as shown for patient 15 in Figure 6B. In addition, proliferation of purified tumor cells (n=2) was restored by addition of mevalonate or GGOH, but not FOH. A representative example from patient 7 is shown in Figure 6C.

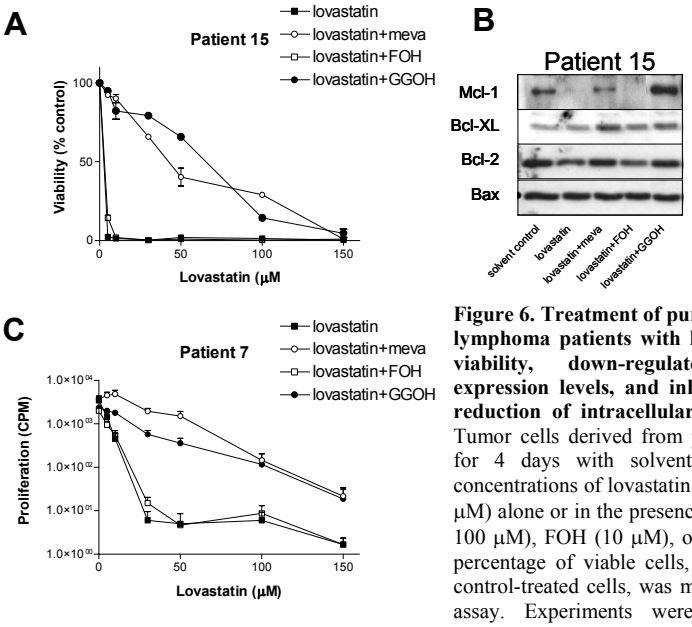


**Figure 6. Treatment of purified tumor cells from lymphoma patients with lovastatin reduces cell viability, down-regulates Mcl-1 protein expression levels, and inhibits proliferation by reduction of intracellular pools of GGPP.** (A) Tumor cells derived from patient 15 were treated for 4 days with solvent control, or different concentrations of lovastatin (5, 10, 30, 50, 100, 150 μM) alone or in the presence of mevalonate (meva; 100 μM), FOH (10 μM), or GGOH (10 μM). The percentage of viable cells, relative to the solvent control-treated cells, was measured by using MTT assay. Experiments were performed once in triplicate. Data are presented as mean ± s.e.m. In some cases the s.e.m. was smaller than the symbol. (B) Tumor cells derived from patient 15 were treated for 4 days with solvent control, or lovastatin (30 μM) alone or in the presence of mevalonate (meva; 100 μM), FOH (10 μM), or GGOH (10 μM). After protein isolation, Mcl-1, Bcl-XL, Bcl-2, and Bax were determined by Western blot analysis. (C) Tumor cells derived from patient 7 were treated for 4 days with solvent control, or different concentrations of lovastatin (5, 10, 30, 50, 100, 150 μM) alone or in the presence of mevalonate (meva; 100 μM), FOH (10 μM), or GGOH (10 μM). Proliferation was determined by $^{3}$H-thymidine incorporation during the last 16 hours of culture. Experiments were performed once in triplicate. Data are presented as mean ± s.e.m. In some cases the s.e.m. was smaller than the symbol.

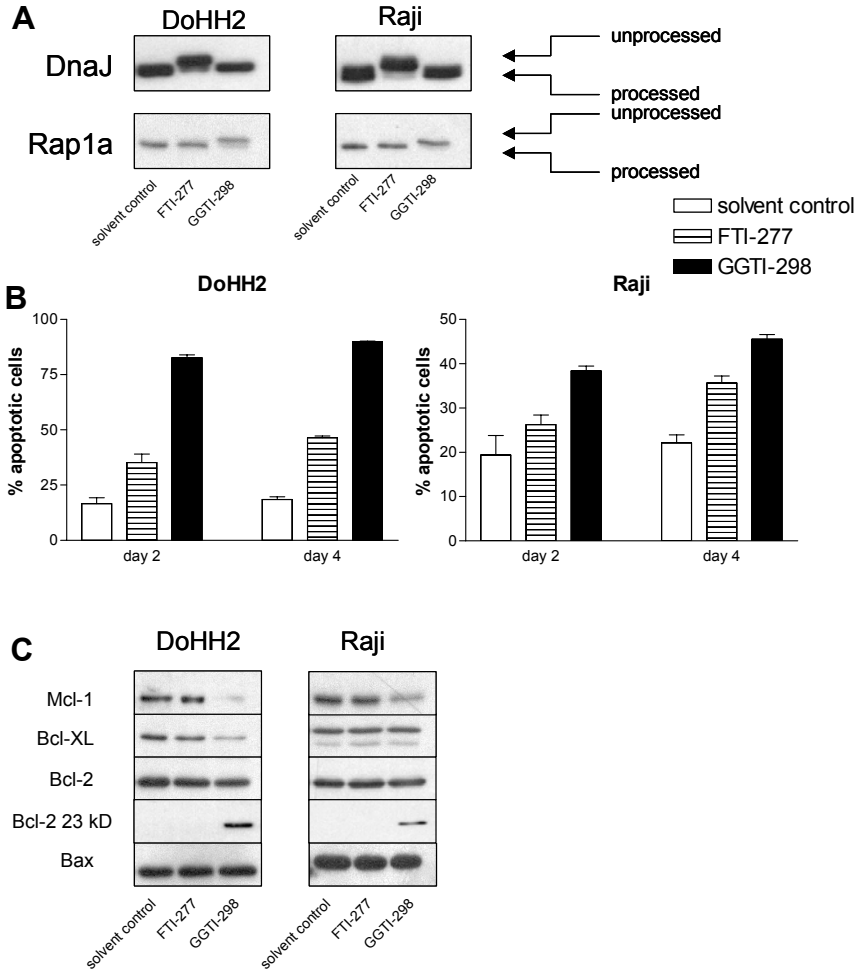


**Figure 7. Inhibition of GGTase I by GGTI-298 inhibits protein geranylgeranylation resulting in Mcl-1 down-regulation and induction of apoptosis.** DoHH2 and Raji cells were treated for 2 or 4 days with solvent control, the farnesyl transferase inhibitor FTI-277 (20 μM), or with the geranylgeranyl transferase I inhibitor GGTI-298 (20 μM). (A) After protein isolation at day 2, processing of DnaJ and Rap1a was determined by Western blot analysis. The faster-migrating band represents prenylated protein, the slower band represents unprenylated, unprocessed protein. The data shown are representative of at least three independent experiments. (B) The percentage of apoptotic cells was determined by Annexin V assay. Shown is the sum of the percentages of early and late apoptotic cells. Experiments were performed three times in triplicate. Data are presented as mean ± s.e.m. (C) After protein isolation at day 2, Mcl-1, Bcl-XL, Bcl-2, and Bax were determined by Western blot analysis. Furthermore, the pro-apoptotic 23 kD Bcl-2 form was detected after long exposure of the film. The data shown are representative of at least three independent experiments.

### Inhibition of GGTase I reduces Mcl-1 protein expression and induces apoptosis

The importance of geranylgeranylation for the regulation of apoptosis and proliferation was further evaluated by using specific inhibitors of FTase (FTI-277) and GGTase I (GGTI-298). FTI-277 (20 μM) inhibited the farnesylation of DnaJ and GGTI-298 (20 μM) inhibited the geranylgeranylation of Rap1a in DoHH2 and Raji cells. The specificity of

these prenylation inhibitors is illustrated by the lack of inhibition of Rap1a prenylation by FTI-277, and, likewise, the lack of inhibition of DnaJ prenylation by GGTI-298 (Figure 7A). Treatment of DoHH2 and Raji cells with GGTI-298 (20 μM) resulted in the induction of apoptosis in a time-dependent way (Figure 7B). The effect of FTI-277 (20 μM) on induction of apoptosis was significantly less pronounced when compared with GGTI-298. Apoptosis induced by GGTI-298 treatment was associated with reduction of Mcl-1 protein expression (Figure 7C). Furthermore, in DoHH2 cells, Bcl-XL expression levels were reduced. Bax and Bcl-2 levels remained unchanged, however, GGTI-298 treatment led to an increase of the 23 kD pro-apoptotic Bcl-2 form. In contrast, inhibition of FTase activity by FTI-277 had no effect on expression of Mcl-1, Bcl-XL, Bax, Bcl-2, and the pro-apoptotic Bcl-2 form (Figure 7C).

**Inhibition of GGTase I inhibits proliferation**

The effect of FTI-277 (0-30 μM) and GGTI-298 (0-30 μM) on proliferation of DoHH2 and Raji cells was examined by measuring $^{3}$H-thymidine incorporation. Inhibition of GGTase I activity by GGTI-298 reduced proliferation in a time- and dose-dependent way (Figure 8 A and B). Inhibition of FTase activity by FTI-277 did not result in inhibition of proliferation or inhibited proliferation only to a small extent, when compared with GGTI-298 (Figure 8 A and B).

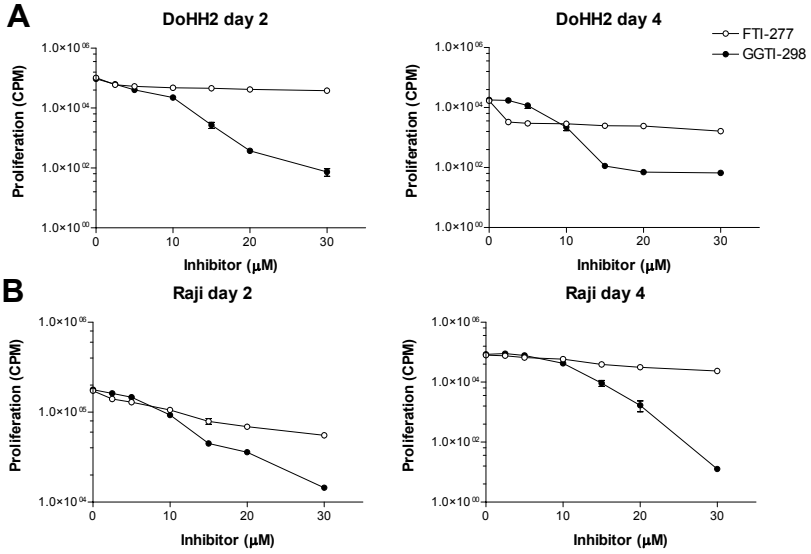


**Figure 8. Inhibition of GGTase I by GGTI-298 inhibits proliferation.** (A) DoHH2 and (B) Raji cells were treated for 2 or 4 days with solvent control, different concentrations of FTI-277 (2.5, 5, 10, 15, 20, 30 μM), or with different concentrations of GGTI-298 (2.5, 5, 10, 15, 20, 30 μM). Proliferation was determined by $^{3}$H-thymidine incorporation during the last 16 hours of culture. Data were from three experiments performed in triplicate. Data are presented as mean ± s.e.m. In some cases the s.e.m. was smaller than the symbol.

## Discussion

Treatment of cell lines (n=6) and purified tumor cells from lymphoma patients (n=16) with the HMG-CoA reductase inhibitor lovastatin reduced the number of viable cells. The reduction in the number of viable cells was mediated by both induction of apoptosis and inhibition of proliferation. Addition of mevalonate to lovastatin-treated cells prevented apoptosis induction and restored proliferation. This indicates that the effects of lovastatin resulted from the inhibition of mevalonate formation, and not from non-specific cell toxicity. There was no significant difference in lovastatin sensitivity between tumor cells from pre-treated and untreated patients or from patients with chemosensitive or chemoresistant disease.

Mevalonate is the precursor of various molecules including the isoprenoids FPP and GGPP [1]. We found that geranylgeranylated protein(s) are important regulators of survival and proliferation in lymphoma tumor cells. Although lovastatin depletes intracellular pools of both GGPP and FPP resulting in inhibition of geranylgeranylation of Rap1a and farnesylation of DnaJ, only addition of GGOH, which is converted to GGPP in cells [33], abrogated the inhibition of geranylgeranylation by lovastatin and restored both cell viability and proliferation. In contrast, although addition of FOH, which is converted to FPP [33], completely restored farnesylation of DnaJ, it had no effect on viability or proliferation. However, in some cases GGOH was less effective than mevalonate in rescuing lymphoma tumor cells from apoptosis and growth arrest. This suggests that depletion of other metabolites downstream of mevalonate may also contribute partly to the effects of lovastatin. The importance of geranylgeranylation for the regulation of growth and survival of lymphoma cells was further confirmed by specific inhibition of FTase by FTI-277 and GGTase I by GGTI-298. GGTI-298 inhibited protein geranylgeranylation and resulted in the induction of apoptosis and inhibition of proliferation, while inhibition of farnesylation by FTI-277 had no or only small effects.

The Bcl-2 family consists of pro-apoptotic proteins such as Bax, Bad, and Bak, and anti-apoptotic family members including Mcl-1, Bcl-XL, and Bcl-2. The balance of anti- and pro-apoptotic Bcl-2 family proteins determines survival or death of cells [38,39]. The anti-apoptotic Bcl-2 family members inhibit apoptosis by forming inactivating heterodimers with pro-apoptotic Bcl-2 family proteins, and by preventing the collapse of the mitochondrial transmembrane potential and cytochrome c release from mitochondria into the cytosol [38,39]. In the cytosol, cytochrome c and Apaf-1 can activate pro-caspase-9, which subsequently activates caspase-3 [38,39]. Mcl-1 is expressed in various types of human leukemia and lymphoma including CLL [28], follicular lymphoma [31,42], and anaplastic large cell lymphoma [43]. There is increasing evidence that Mcl-1 plays a prominent role in lymphomagenesis [44], and in the survival [17,30,45] and chemoresistance [28,29] of lymphoma tumor cells. We showed that apoptosis induced by inhibition of geranylgeranylation was

associated with a reduction of Mcl-1 protein expression levels in lymphoma cell lines and patient cells, which in turn, resulted in the collapse of the mitochondrial transmembrane potential, and caspase-3 activation.

Lovastatin treatment also resulted in the increase of the 23 kD pro-apoptotic Bcl-2 form. Bcl-2 is a known substrate of caspase-3 [40,41] and it is, therefore, likely that lovastatin-mediated stimulation of caspase-3 activity resulted in the increase of the pro-apoptotic Bcl-2 form. The pro-apoptotic 23 kD Bcl-2 form is able to localize to mitochondria and to stimulate the release of cytochrome c into the cytosol [41]. Therefore, caspase-3 dependent cleavage of Bcl-2 appears to promote further caspase activation as part of a positive feed-back loop. Only in DoHH2 cells, expression of the anti-apoptotic protein Bcl-XL was decreased and the pro-apoptotic protein Bax was increased. Although the alterations in expression levels of these proteins were relatively small, when compared to the change in Mcl-1 expression, they may contribute partly to the induction of apoptosis induced by inhibition of geranylgeranylation in this cell line.

The geranylgeranylated target protein(s) of lovastatin and GGTI-298 that mediate the protection against cell death and regulate the proliferation of lymphoma tumor cells remain to be identified. Candidates, however, include RhoA, Rac-1, R-Ras, and Cdc42, which are involved in important cellular functions including the regulation of apoptosis [4,46-50]. Several pathways, including PI-3K, JAK/STAT3, and MEK/ERK, can stimulate Mcl-1 transcription [51]. Since RhoA [52,53], Rac-1 [54], R-Ras [49,55], and Cdc42 [56] have been shown to activate the PI-3K pathway, inhibition of these proteins by lovastatin or GGTI-298 may be involved in Mcl-1 down-regulation and induction of apoptosis. Furthermore, RhoA, Rac-1, R-Ras, and Cdc42 have also been implicated as regulators of proliferation [4,47,48,50,57]. We showed that inhibition of geranylgeranylation resulted in a reduction of proliferation due to arrest in G1 phase of the cell cycle. This indicates that geranylgeranylated proteins are critical for G1-S transition in lymphoma tumor cells. Also in lung adenocarcinoma [58] and in mouse fibroblasts [37] inhibition of geranylgeranylation arrested cells in the G1 phase of their cycle. Rac-1 [59,60], Cdc42 [60-62], and RhoA [59,60] play an essential role in G1-S phase transition, and inhibition of processing of these proteins by lovastatin or GGTI-298 may, therefore, be responsible for the G1 block. Ongoing studies are directed at identifying the geranylgeranylated target proteins.

Lovastatin augmented the sensitivity of both drug-sensitive and drug-resistant lymphoma cells to dexamethasone in a synergistic way. Moreover, additive cytotoxicity was observed between lovastatin and doxorubicin. Our findings are consistent with the ability of lovastatin to sensitize tumor cells to cytotoxic agents in other models [10,12,63-65]. Since Mcl-1 is an important regulator of response to cytotoxic agents [28,29,66], the synergism between lovastatin and dexamethasone may be due, at least in part, to the lovastatin-mediated Mcl-1 down-regulation. Importantly, cell death and chemosensitization induced by lovastatin were observed at concentrations that could be achieved *in vivo* without significant tissue toxicity [67].

In summary, we have shown that geranylgeranylation of proteins is critical for the survival and proliferation of lymphoma tumor cells. Inhibition of geranylgeranylation either by depletion of intracellular pools of GGPP through the inhibition of HMG-CoA reductase by lovastatin or by the specific inhibition of GGTase I activity by GGTI-298 resulted in the induction of apoptosis and reduction of proliferation. Apoptosis induced by inhibition of geranylgeranylation was probably due to reduction of the anti-apoptotic Bcl-2 familiy member Mcl-1, which, in turn, resulted in the collapse of the mitochondrial transmembrane potential and activation of caspase-3. The Mcl-1 down-regulation may also explain the chemosensitizing activity of lovastatin. Furthermore, inhibition of proliferation was associated with a G1 block. These data suggest that inhibition of protein geranylgeranylation either alone or in combination with chemotherapy warrants further investigation as a new therapeutic strategy in lymphoma and CLL.

## References


1. Goldstein, J. L. and Brown, M. S. Regulation of the mevalonate pathway. Nature, *343*: 425-430, 1990.
2. Magee, T. and Marshall, C. New insights into the interaction of Ras with the plasma membrane. Cell, *98*: 9-12, 1999.
3. Reuter, C. W., Morgan, M. A., and Bergmann, L. Targeting the Ras signaling pathway: a rational, mechanism-based treatment for hematologic malignancies? Blood, *96*: 1655-1669, 2000.
4. Bishop, A. L. and Hall, A. Rho GTPases and their effector proteins. Biochem.J, *348 Pt 2:241-55*.: 241-255, 2000.
5. Kato, K., Cox, A. D., Hisaka, M. M., Graham, S. M., Buss, J. E., and Der, C. J. Isoprenoid addition to Ras protein is the critical modification for its membrane association and transforming activity. Proc.Natl.Acad.Sci.U.S.A, *89*: 6403-6407, 1992.
6. Gelb, M. H. Protein prenylation, et cetera: signal transduction in two dimensions. Science, *275*: 1750-1751, 1997.
7. Rebollo, A. and Martinez, A. Ras proteins: recent advances and new functions. Blood, *94*: 2971-2980, 1999.
8. Dimitroulakos, J. and Yeger, H. HMG-CoA reductase mediates the biological effects of retinoic acid on human neuroblastoma cells: lovastatin specifically targets P-glycoprotein-expressing cells. Nat.Med., *2*: 326-333, 1996.
9. Ghosh, P. M., Ghosh-Choudhury, N., Moyer, M. L., Mott, G. E., Thomas, C. A., Foster, B. A., Greenberg, N. M., and Kreisberg, J. I. Role of RhoA activation in the growth and morphology of a murine prostate tumor cell line. Oncogene, *18*: 4120-4130, 1999.
10. Agarwal, B., Bhendwal, S., Halmos, B., Moss, S. F., Ramey, W. G., and Holt, P. R. Lovastatin augments apoptosis induced by chemotherapeutic agents in colon cancer cells. Clin.Cancer Res., *5*: 2223-2229, 1999.
11. Dimitroulakos, J., Nohynek, D., Backway, K. L., Hedley, D. W., Yeger, H., Freedman, M. H., Minden, M. D., and Penn, L. Z. Increased sensitivity of acute myeloid leukemias to lovastatin-induced apoptosis: A potential therapeutic approach. Blood, *93*: 1308-1318, 1999.
12. van de Donk, N. W., Kamphuis, M. M., Lokhorst, H. M., and Bloem, A. C. The cholesterol lowering drug lovastatin induces cell death in myeloma plasma cells. Leukemia, *16*: 1362-1371, 2002.

13. O'Gorman, D. M. and Cotter, T. G. Molecular signals in anti-apoptotic survival pathways. Leukemia, *15*: 21-34, 2001.
14. Borlado, L. R., Redondo, C., Alvarez, B., Jimenez, C., Criado, L. M., Flores, J., Marcos, M. A., Martinez, A., Balomenos, D., and Carrera, A. C. Increased phosphoinositide 3-kinase activity induces a lymphoproliferative disorder and contributes to tumor generation in vivo. FASEB J, *14*: 895-903, 2000.
15. Slupianek, A., Nieborowska-Skorska, M., Hoser, G., Morrione, A., Majewski, M., Xue, L., Morris, S. W., Wasik, M. A., and Skorski, T. Role of phosphatidylinositol 3-kinase-Akt pathway in nucleophosmin/anaplastic lymphoma kinase-mediated lymphomagenesis. Cancer Res., *61*: 2194-2199, 2001.
16. Wickremasinghe, R. G., Ganeshaguru, K., Jones, D. T., Lindsay, C., Spanswick, V. J., Hartley, J. A., Wadhwa, M., Thorpe, R., Hoffbrand, A. V., Prentice, H. G., and Mehta, A. B. Autologous plasma activates Akt/protein kinase B and enhances basal survival and resistance to DNA damage-induced apoptosis in B-chronic lymphocytic leukaemia cells. Br.J Haematol., *114*: 608-615, 2001.
17. Bernal, A., Pastore, R. D., Asgary, Z., Keller, S. A., Cesarman, E., Liou, H. C., and Schattner, E. J. Survival of leukemic B cells promoted by engagement of the antigen receptor. Blood, *98*: 3050-3057, 2001.
18. Brennan, P., Mehl, A. M., Jones, M., and Rowe, M. Phosphatidylinositol 3-kinase is essential for the proliferation of lymphoblastoid cells. Oncogene, *21*: 1263-1271, 2002.
19. Ringshausen, I., Schneller, F., Bogner, C., Hipp, S., Duyster, J., Peschel, C., and Decker, T. Constitutively activated phosphatidylinositol-3 kinase (PI-3K) is involved in the defect of apoptosis in B-CLL: association with protein kinase Cdelta. Blood, *100*: 3741-3748, 2002.
20. Jones, D. T., Ganeshaguru, K., Anderson, R. J., Jackson, T. R., Bruckdorfer, K. R., Low, S. Y., Palmqvist, L., Prentice, H. G., Hoffbrand, A. V., Mehta, A. B., and Wickremasinghe, R. G. Albumin activates the AKT signaling pathway and protects B-chronic lymphocytic leukemia cells from chlorambucil- and radiation-induced apoptosis. Blood, *101*: 3174-3180, 2003.
21. Furman, R. R., Asgary, Z., Mascarenhas, J. O., Liou, H. C., and Schattner, E. J. Modulation of NF-kappa B activity and apoptosis in chronic lymphocytic leukemia B cells. J Immunol, *164*: 2200-2206, 2000.
22. Cotter, F. E., Johnson, P., Hall, P., Pocock, C., al Mahdi, N., Cowell, J. K., and Morgan, G. Antisense oligonucleotides suppress B-cell lymphoma growth in a SCID-hu mouse model. Oncogene, *9* : 3049-3055, 1994.
23. McConkey, D. J., Chandra, J., Wright, S., Plunkett, W., McDonnell, T. J., Reed, J. C., and Keating, M. Apoptosis sensitivity in chronic lymphocytic leukemia is determined by endogenous endonuclease content and relative expression of BCL-2 and BAX. J Immunol, *156*: 2624-2630, 1996.
24. Thomas, A., El Rouby, S., Reed, J. C., Krajewski, S., Silber, R., Potmesil, M., and Newcomb, E. W. Drug-induced apoptosis in B-cell chronic lymphocytic leukemia: relationship between p53 gene mutation and bcl-2/bax proteins in drug resistance. Oncogene, *12*: 1055-1062, 1996.
25. Pepper, C., Hoy, T., and Bentley, D. P. Bcl-2/Bax ratios in chronic lymphocytic leukaemia and their correlation with in vitro apoptosis and clinical resistance. Br.J Cancer, *76*: 935-938, 1997.
26. Pepper, C., Thomas, A., Hoy, T., Cotter, F., and Bentley, P. Antisense-mediated suppression of Bcl-2 highlights its pivotal role in failed apoptosis in B-cell chronic lymphocytic leukaemia. Br.J Haematol., *107*: 611-615, 1999.
27. Guinness, M. E., Kenney, J. L., Reiss, M., and Lacy, J. Bcl-2 antisense oligodeoxynucleotide therapy of Epstein-Barr virus-associated lymphoproliferative disease in severe combined immunodeficient mice. Cancer Res., *60*: 5354-5358, 2000.
28. Kitada, S., Andersen, J., Akar, S., Zapata, J. M., Takayama, S., Krajewski, S., Wang, H. G., Zhang, X., Bullrich, F., Croce, C. M., Rai, K., Hines, J., and Reed, J. C. Expression of apoptosis-regulating proteins in chronic lymphocytic leukemia: correlations with In vitro and In vivo chemoresponses. Blood, *91*: 3379-3389, 1998.

29. Vrana, J. A., Bieszczad, C. K., Cleaveland, E. S., Ma, Y., Park, J. P., Mohandas, T. K., and Craig, R. W. An MCL1-overexpressing Burkitt lymphoma subline exhibits enhanced survival on exposure to serum deprivation, topoisomerase inhibitors, or staurosporine but remains sensitive to 1-beta-D-arabinofuranosylcytosine. Cancer Res., *62*: 892-900, 2002.
30. Pedersen, I. M., Kitada, S., Leoni, L. M., Zapata, J. M., Karras, J. G., Tsukada, N., Kipps, T. J., Choi, Y. S., Bennett, F., and Reed, J. C. Protection of CLL B cells by a follicular dendritic cell line is dependent on induction of Mcl-1. Blood, *100*: 1795-1801, 2002.
31. Ghia, P., Boussiotis, V. A., Schultze, J. L., Cardoso, A. A., Dorfman, D. M., Gribben, J. G., Freedman, A. S., and Nadler, L. M. Unbalanced expression of bcl-2 family proteins in follicular lymphoma: contribution of CD40 signaling in promoting survival. Blood, *91*: 244-251, 1998.
32. Keyomarsi, K., Sandoval, L., Band, V., and Pardee, A. B. Synchronization of tumor and normal cells from G1 to multiple cell cycles by lovastatin. Cancer Res., *51*: 3602-3609, 1991.
33. Crick, D. C., Andres, D. A., and Waechter, C. J. Novel salvage pathway utilizing farnesol and geranylgeraniol for protein isoprenylation. Biochem.Biophys.Res.Commun., *237*: 483-487, 1997.
34. Siminovitch, K. A., Jensen, J. P., Epstein, A. L., and Korsmeyer, S. J. Immunoglobulin gene rearrangements and expression in diffuse histiocytic lymphomas reveal cellular lineage, molecular defects, and sites of chromosomal translocation. Blood, *67*: 391-397, 1986.
35. Davis, A. R., Alevy, Y. G., Chellaiah, A., Quinn, M. T., and Mohanakumar, T. Characterization of HDJ-2, a human 40 kD heat shock protein. Int.J Biochem.Cell Biol., *30*: 1203-1221, 1998.
36. Omer, C. A., Chen, Z., Diehl, R. E., Conner, M. W., Chen, H. Y., Trumbauer, M. E., Gopal-Truter, S., Seeburger, G., Bhimnathwala, H., Abrams, M. T., Davide, J. P., Ellis, M. S., Gibbs, J. B., Greenberg, I., Koblan, K. S., Kral, A. M., Liu, D., Lobell, R. B., Miller, P. J., Mosser, S. D., O'Neill, T. J., Rands, E., Schaber, M. D., Senderak, E. T., Oliff, A., and Kohl, N. E. Mouse mammary tumor virus-Ki-rasB transgenic mice develop mammary carcinomas that can be growth-inhibited by a farnesyl:protein transferase inhibitor. Cancer Res., *60*: 2680-2688, 2000.
37. Vogt, A., Qian, Y., McGuire, T. F., Hamilton, A. D., and Sebti, S. M. Protein geranylgeranylation, not farnesylation, is required for the G1 to S phase transition in mouse fibroblasts. Oncogene, *13*: 1991-1999, 1996.
38. Gross, A., McDonnell, J. M., and Korsmeyer, S. J. BCL-2 family members and the mitochondria in apoptosis. Genes Dev., *13*: 1899-1911, 1999.
39. Kroemer, G. and Reed, J. C. Mitochondrial control of cell death. Nat.Med, *6*: 513-519, 2000.
40. Cheng, E. H., Kirsch, D. G., Clem, R. J., Ravi, R., Kastan, M. B., Bedi, A., Ueno, K., and Hardwick, J. M. Conversion of Bcl-2 to a Bax-like death effector by caspases. Science, *278*: 1966-1968, 1997.
41. Kirsch, D. G., Doseff, A., Chau, B. N., Lim, D. S., Souza-Pinto, N. C., Hansford, R., Kastan, M. B., Lazebnik, Y. A., and Hardwick, J. M. Caspase-3-dependent cleavage of Bcl-2 promotes release of cytochrome c. J Biol.Chem., *274*: 21155-21161, 1999.
42. Krajewski, S., Bodrug, S., Gascoyne, R., Berean, K., Krajewska, M., and Reed, J. C. Immunohistochemical analysis of Mcl-1 and Bcl-2 proteins in normal and neoplastic lymph nodes. Am J Pathol., *145*: 515-525, 1994.
43. Rassidakis, G. Z., Lai, R., McDonnell, T. J., Cabanillas, F., Sarris, A. H., and Medeiros, L. J. Overexpression of Mcl-1 in anaplastic large cell lymphoma cell lines and tumors. Am J Pathol., *160*: 2309-2310, 2002.
44. Zhou, P., Levy, N. B., Xie, H., Qian, L., Lee, C. Y., Gascoyne, R. D., and Craig, R. W. MCL1 transgenic mice exhibit a high incidence of B-cell lymphoma manifested as a spectrum of histologic subtypes. Blood, *97*: 3902-3909, 2001.
45. Zhou, P., Qian, L., Bieszczad, C. K., Noelle, R., Binder, M., Levy, N. B., and Craig, R. W. Mcl-1 in transgenic mice promotes survival in a spectrum of hematopoietic cell types and immortalization in the myeloid lineage. Blood, *92*: 3226-3239, 1998.

46. Suzuki, J., Kaziro, Y., and Koide, H. An activated mutant of R-Ras inhibits cell death caused by cytokine deprivation in BaF3 cells in the presence of IGF-I. Oncogene, *15*: 1689-1697, 1997.
47. Van Aelst, L. and D'Souza-Schorey, C. Rho GTPases and signaling networks. Genes Dev., *11*: 2295-2322, 1997.
48. Zohn, I. M., Campbell, S. L., Khosravi-Far, R., Rossman, K. L., and Der, C. J. Rho family proteins and Ras transformation: the RHOad less traveled gets congested. Oncogene, *17*: 1415-1438, 1998.
49. Osada, M., Tolkacheva, T., Li, W., Chan, T. O., Tsichlis, P. N., Saez, R., Kimmelman, A. C., and Chan, A. M. Differential roles of Akt, Rac, and Ral in R-Ras-mediated cellular transformation, adhesion, and survival. Mol.Cell Biol., *19*: 6333-6344, 1999.
50. Bar-Sagi, D. and Hall, A. Ras and Rho GTPases: a family reunion. Cell, *103*: 227-238, 2000.
51. Craig, R. W. MCL1 provides a window on the role of the BCL2 family in cell proliferation, differentiation and tumorigenesis. Leukemia, *16*: 444-454, 2002.
52. Kumagai, N., Morii, N., Fujisawa, K., Nemoto, Y., and Narumiya, S. ADP-ribosylation of rho p21 inhibits lysophosphatidic acid-induced protein tyrosine phosphorylation and phosphatidylinositol 3-kinase activation in cultured Swiss 3T3 cells. J Biol.Chem., *268*: 24535-24538, 1993.
53. Tolias, K. F., Cantley, L. C., and Carpenter, C. L. Rho family GTPases bind to phosphoinositide kinases. J Biol.Chem., *270*: 17656-17659, 1995.
54. Bokoch, G. M., Vlahos, C. J., Wang, Y., Knaus, U. G., and Traynor-Kaplan, A. E. Rac GTPase interacts specifically with phosphatidylinositol 3-kinase. Biochem.J, *315*: 775-779, 1996.
55. Marte, B. M., Rodriguez-Viciana, P., Wennstrom, S., Warne, P. H., and Downward, J. R-Ras can activate the phosphoinositide 3-kinase but not the MAP kinase arm of the Ras effector pathways. Curr.Biol., *7*: 63-70, 1997.
56. Zheng, Y., Bagrodia, S., and Cerione, R. A. Activation of phosphoinositide 3-kinase activity by Cdc42Hs binding to p85. J Biol.Chem., *269*: 18727-18730, 1994.
57. Yu, Y. and Feig, L. A. Involvement of R-Ras and Ral GTPases in estrogen-independent proliferation of breast cancer cells. Oncogene, *21*: 7557-7568, 2002.
58. Miquel, K., Pradines, A., Sun, J., Qian, Y., Hamilton, A. D., Sebti, S. M., and Favre, G. GGTI-298 induces G0-G1 block and apoptosis whereas FTI-277 causes G2-M enrichment in A549 cells. Cancer Res., *57*: 1846-1850, 1997.
59. Khosravi-Far, R., Solski, P. A., Clark, G. J., Kinch, M. S., and Der, C. J. Activation of Rac1, RhoA, and mitogen-activated protein kinases is required for Ras transformation. Mol.Cell Biol., *15*: 6443-6453, 1995.
60. Olson, M. F., Ashworth, A., and Hall, A. An essential role for Rho, Rac, and Cdc42 GTPases in cell cycle progression through G1. Science, *269*: 1270-1272, 1995.
61. Qiu, R. G., Abo, A., McCormick, F., and Symons, M. Cdc42 regulates anchorage-independent growth and is necessary for Ras transformation. Mol.Cell Biol., *17*: 3449-3458, 1997.
62. Lin, R., Bagrodia, S., Cerione, R., and Manor, D. A novel Cdc42Hs mutant induces cellular transformation. Curr.Biol., *7*: 794-797, 1997.
63. Soma, M. R., Pagliarini, P., Butti, G., Paoletti, R., Paoletti, P., and Fumagalli, R. Simvastatin, an inhibitor of cholesterol biosynthesis, shows a synergistic effect with N,N'-bis(2-chloroethyl)-N-nitrosourea and beta-interferon on human glioma cells. Cancer Res., *52*: 4348-4355, 1992.
64. Soma, M. R., Baetta, R., De Renzis, M. R., Mazzini, G., Davegna, C., Magrassi, L., Butti, G., Pezzotta, S., Paoletti, R., and Fumagalli, R. In vivo enhanced antitumor activity of carmustine [N,N'-bis(2-chloroethyl)-N-nitrosourea] by simvastatin. Cancer Res., *55*: 597-602, 1995.
65. Feleszko, W., Mlynarczuk, I., Balkowiec-Iskra, E. Z., Czajka, A., Switaj, T., Stoklosa, T., Giermasz, A., and Jakobisiak, M. Lovastatin potentiates antitumor activity and attenuates cardiotoxicity of doxorubicin in three tumor models in mice. Clin.Cancer Res., *6*: 2044-2052, 2000.
66. Zhou, P., Qian, L., Kozopas, K. M., and Craig, R. W. Mcl-1, a Bcl-2 family member, delays the death of hematopoietic cells under a variety of apoptosis-inducing conditions. Blood, *89*: 630-643, 1997.

67. Thibault, A., Samid, D., Tompkins, A. C., Figg, W. D., Cooper, M. R., Hohl, R. J., Trepel, J., Liang, B., Patronas, N., Venzon, D. J., Reed, E., and Myers, C. E. Phase I study of lovastatin, an inhibitor of the mevalonate pathway, in patients with cancer. Clin.Cancer Res., *2*: 483-491, 1996.

# Chapter 8

## General Discussion

## Multiple myeloma

Multiple myeloma is a B-lineage neoplasia and is characterized by the accumulation of malignant plasma cells in the bone marrow. Enhanced proliferation and defects in the regulation of programmed cell death account for the expansion of the malignant clone. Emergence of drug resistance is the primary cause of treatment failure in myeloma. Various studies have indicated that inhibition of drug-induced apoptosis is an important mechanism of drug resistance in various human cancers including myeloma. Understanding of mechanisms that regulate apoptosis and growth will allow for the development of new biologically-based therapies that overcome drug resistance and reduce myeloma tumor growth.

This thesis has focused on the role of Bcl-2 and the mevalonate pathway in the maintenance of myeloma tumor cell survival and resistance to drug-induced apoptosis.

## Bcl-2 in myeloma

The anti-apoptotic protein Bcl-2 is an important regulator of survival and drug resistance in various cancers including non-Hodgkin's lymphoma (NHL) and acute myeloid leukemia (AML) [1]. In myeloma, the role of Bcl-2 is unclear. Some studies suggest that in myeloma Bcl-2 contributes to malignant cell expansion by preventing programmed cell death and mediates resistance to cytotoxic drugs [2-5], whereas others claim that there is no correlation between Bcl-2 expression and response to treatment *in vivo* [6-8]. In chapter 2, we investigated the importance of Bcl-2 in drug-induced apoptosis in myeloma using an antisense approach (Figure 1). G3139 (Genasense, oblimersen sodium; 5'-TCT CCC AGC GTG CGC CAT-3') is a phosphorothioate 18-mer oligodeoxynucleotide (ODN) complementary to the first 6 codons of the Bcl-2 open reading frame. Treatment of *ex vivo* purified myeloma tumor cells with Bcl-2 antisense ODNs resulted in a sequence-specific reduction of Bcl-2 mRNA, followed by a sequence-specific decrease in Bcl-2 protein levels [9]. Down-regulation of Bcl-2 resulted in enhanced sensitivity of myeloma tumor cells to dexamethasone or doxorubicin-induced apoptosis [9]. This chemosensitizing effect was observed in both chemosensitive and chemoresistant myeloma cells. Treatment of myeloma cells with Bcl-2 antisense alone did not induce apoptosis, indicating that in primary myeloma tumor cells other anti-apoptotic proteins or signaling cascades confer protection against Bcl-2 down-regulation. These results agree with those obtained by two other groups and imply that Bcl-2 down-regulation by antisense ODNs decreases the apoptotic threshold and potentiates drug-induced apoptosis or reverses resistance to cytotoxic agents, indicating that Bcl-2 plays a role in drug resistance in myeloma [9-11] (Figure 2).

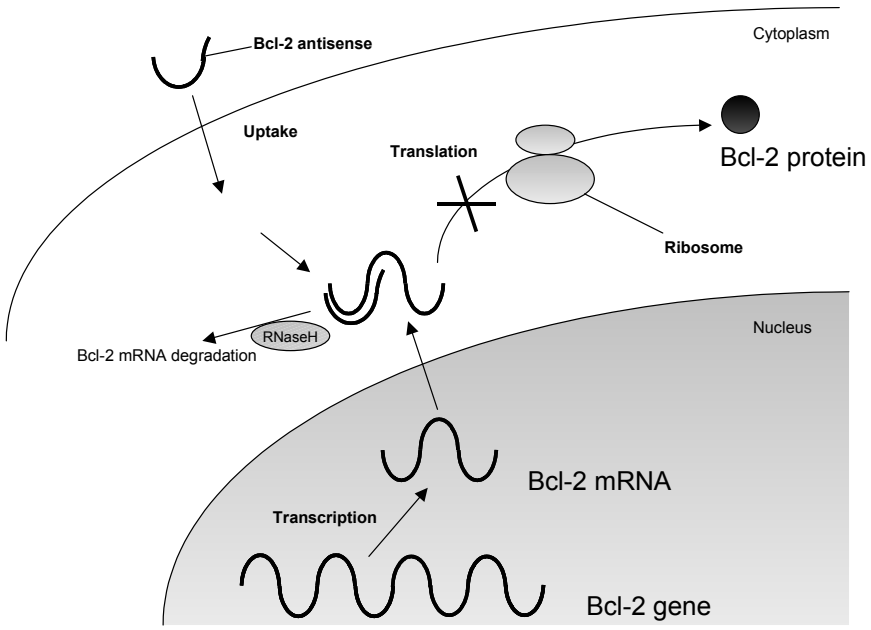


**Figure 1. Mechanism of action of Bcl-2 antisense oligodeoxynucleotides.** Uptake of antisense ODNs by cells is through receptor-mediated endocytosis and/or fluid phase endocytosis. Bcl-2 antisense (G3139) is complementary to the first 6 codons of the Bcl-2 mRNA. This results in a sequence-specific hybridization of the Bcl-2 antisense with the target mRNAs, which prevents translation or recruits endogenous RNase H to cleave the mRNA backbone. The phosphorothioate DNA ODN is resistant to RNase H cleavage and can therefore hybridize with other Bcl-2 mRNA molecules. Altogether, this results in Bcl-2 protein down-regulation.

Bcl-2 protein is expressed in almost all myeloma patients, and along with its role in the regulation of chemosensitivity (Chapter 2) makes this anti-apoptotic protein an attractive target for therapy in multiple myeloma. In a phase 2 clinical trial, we studied the effect of Bcl-2 antisense in 10 myeloma patients that were heavily pretreated, with a median of 4 chemotherapy regimens prior to entering the study (chapter 3). G3139 was administered for 7 days as a continuous intravenous infusion of 7 mg/kg/day and VAD chemotherapy was started at day 4. This sequential order was chosen, since our preclinical studies showed that maximum inhibition of Bcl-2 protein was observed after 4 days of treatment of purified myeloma tumor cells with G3139 [9]. This may be due to the relatively long half-life of the Bcl-2 protein. Bcl-2 mRNA levels, however, were already reduced after 2 days of treatment [9]. Administration of Bcl-2 antisense resulted in a moderate reduction of Bcl-2 protein levels in peripheral blood circulating myeloma cells, monocytes, B cells, and T cells. Four (40%) achieved a partial response and 3 (30%) patients a minor response, indicating that 70% of the patients responded to the combination of G3139 and VAD. Five out 7 responding patients were refractory to VAD chemotherapy alone, which indicates that the administration of Bcl-2 antisense can

overcome classical drug resistance in myeloma. The combination of Bcl-2 antisense and VAD chemotherapy was well tolerated, and toxicity was primarily attributable to VAD. The toxicity associated with administration of Bcl-2 antisense alone prior to start of VAD chemotherapy included inflammation at the infusion site, fatigue, and a transient reduction of absolute numbers of circulating thrombocytes, monocytes, B cells, and T cells. Since Bcl-2 expression is critical for survival of mature B and T cells [12,13], down-regulation of Bcl-2 may be responsible for the reduction of circulating B and T cells. Antisense-mediated Bcl-2 down-regulation did not result in loss of the renewal potential of normal hematopoietic cells, allowing for the repopulation of depleted cells. This is in accordance with the observation that hematopoiesis was not affected in Bcl-2 knock-out mice [12,13] suggesting that Bcl-2 is not essential for survival of pluripotent hematopoietic stem cells. Thrombocytopenia and fatigue have also been described in clinical studies that evaluated the effect of other phosphorothiate oligodeoxynucleotides, indicating that these effects probably resulted from non-sequence specific effects and can be attributed to the phosphorothioate backbone of the oligodeoxynucleotide molecule [14,15].

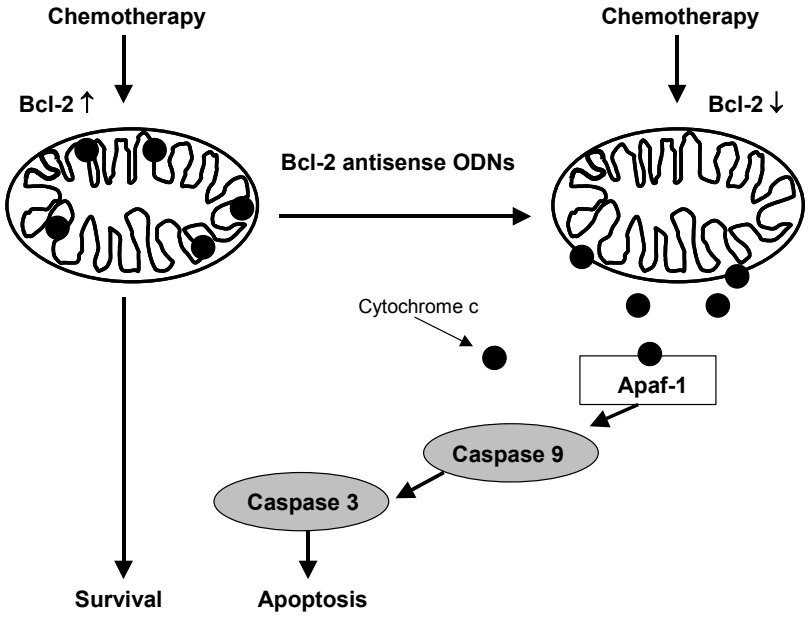


**Figure 2.** Bcl-2 antisense-mediated down-regulation of Bcl-2 protein levels overcomes drug resistance and restores sensitivity to cytotoxic agents in myeloma cells.

Since Bcl-2 protein is expressed in a variety of tissues, it is remarkable that Bcl-2 down-regulation was relatively non-toxic to normal cells. One of the questions at the start of this study, was indeed whether Bcl-2 antisense combined with chemotherapy would

selectively modulate apoptosis in the tumor cells without severe adverse effects in normal tissues. Pro-apoptotic Bcl-2 family proteins such as Bax are expressed in myeloma tumor cells. Moreover, myeloma cells express oncoproteins, like c-Myc, that deregulate the cell cycle, but also have pro-apoptotic effects [16]. Deregulated expression of c-Myc is common in multiple myeloma [17,18], and although c-Myc stimulates proliferation, c-Myc also has an apoptosis-inducing effect [19]. The oncogenic potential of c-Myc is reduced by its induction of apoptosis, as demonstrated in c-Myc transgenic mice [20]. Lymphomagenesis was dramatically increased in these mice by Bcl-2, as c-Myc-induced apoptosis was blocked by Bcl-2 [19]. Furthermore, overexpression of c-Myc in serum-deprived CHO cells or fibroblasts induces proliferation accompanied by apoptosis, however coexpression of Bcl-2 abrogates this c-Myc-induced programmed cell death [19,21]. Thus the balance between pro-apoptotic proliferation effects and anti-apoptotic survival signals provided by various Bcl-2 family proteins seems to be critical for tumor cell viability and tumor cell growth. Reduction of one anti-apoptotic Bcl-2 family protein may then tip the balance directly towards apoptosis or sensitize tumor cells to a second apoptotic-trigger such as a cytotoxic drug, while sparing normal cells without deregulated oncogenes. This may explain the observation that, although Bcl-2 is expressed in many tissues, there was no excess tissue toxicity observed in our clinical study over and above what would be expected from VAD alone except for lymphopenia, fatigue, and inflammation around the infusion site.

One of the disadvantages of this antisense approach includes the need of a continuous intravenous infusion for 7 days, since the plasma half-life of G3139 is in the order of 2 hours. Current research is directed at examining the feasibility of a slow-release subcutaneous formulation of Bcl-2 antisense ODNs. Another strategy to target the Bcl-2 protein includes the inhibition of Bcl-2 by small molecules that disrupt the binding of Bcl-2 to pro-apoptotic Bcl-2 family members and thus abrogate at least part of the anti-apoptotic function of Bcl-2 [22-24]. Other approaches include manipulation of signaling pathways that are critical for the expression of Bcl-2 protein or manipulation of post-translational modifications to alter its activity [25-28].

The responses observed in our trial provide the proof of principle that Bcl-2 protein is involved in drug resistance *in vivo* and that inhibition of Bcl-2 expression increases the *in vivo* therapeutic efficacy of cytotoxic agents in myeloma (Figure 2). A randomized phase 3 clinical study has been initiated to evaluate the clinical efficacy of dexamethasone alone compared with Bcl-2 antisense combined with dexamethasone in relapsed or refractory multiple myeloma patients.

## Mevalonate pathway in myeloma

The mevalonate pathway produces a variety of products including farnesyl and geranylgeranyl isoprenoids, dolichol, ubiquinone, and cholesterol, which may be essential for cell survival and growth [29]. Deregulated or elevated activity of HMG-CoA reductase

has been demonstrated in a variety of tumors including leukemia and lymphoma [30,31]. In some cancer cell lines and purified tumor cells from AML patients, suppression of the mevalonate pathway by statins resulted in inhibition of growth and survival, and significantly enhanced sensitivity of tumor cells to novel drugs or conventional chemotherapeutic agents. Importantly, lovastatin had minimal effects on normal bone marrow progenitor cells [32]. The role of the mevalonate pathway and its metabolites in myeloma, however, was unknown. In the second part of this thesis, we focused on the importance of the mevalonate pathway in myeloma. Blocking of the mevalonate pathway through the inhibition of HMG-CoA reductase by lovastatin induced apoptosis and inhibited proliferation even in resistant myeloma cell lines and primary myeloma cells. Lovastatin also sensitized myeloma cell lines to cytotoxic agents (chapter 4) [33].

These effects were due to specific inhibition of HMG-CoA reductase since addition of mevalonate restored survival and proliferation in lovastatin-treated cells [33]. This also indicates that products downstream of mevalonate were involved in apoptosis induction and inhibition of proliferation. We found that depletion of geranylgeranylpyrophosphate (GGPP) by lovastatin resulted in inhibition of protein geranylgeranylation, induction of apoptosis, and suppression of proliferation, whereas depletion of farnesylpyrophosphate (FPP) leading to inhibition of farnesylation had no or only minor effects. The role of protein geranylgeranylation in the regulation of myeloma tumor cell growth and survival was confirmed in experiments with specific inhibitors of geranylgeranyl transferase I (GGTase I) and farnesyl transferase (FTase) [34]. Inhibition of GGTase I activity reduced proliferation and induced apoptosis, with only small effects of inhibition of FTase (chapter 5 and 6). Inhibition of geranylgeranylation by both lovastatin and the GGTase I inhibitor preceded the reduction of cell viability. These results regarding FTase inhibition agree with several recently published *in vitro* studies that showed that inhibition of farnesylation by FTase inhibitors had only small effects in myeloma [35-39]. Furthermore, two clinical studies have evaluated the therapeutic efficacy of the FTase inhibitor Zarnestra (R115777) in patients with relapsed or refractory myeloma. Although treatment with Zarnestra suppressed FTase activity and inhibited protein farnesylation in bone marrow and peripheral blood mononuclear cells of myeloma patients, it had only modest antimyeloma activity [40,41].

Addition of mevalonate to lovastatin-treated cells was more effective in rescuing cells from apoptosis and growth arrest than geranylgeranyl isoprenoids. This suggests that in addition to inhibition of geranylgeranylation, depletion of other metabolites downstream of mevalonate may also contribute to the effects of lovastatin. For instance, isopentenyladenine is an essential substrate for the modification of some tRNAs [29]. Furthermore, the GGPP metabolite dolichol-phosphate is involved in N-linked glycosylation and plays a role in the translocation of survival receptors including insulin-like growth factor-I (IGF-I) receptors from the intracellular compartment to the cell membrane. Depletion of intracellular pools of dolichol-phosphate by statins in various

cancer cell lines resulted in decreased trafficking of the IGF-I receptor to the cell membrane [42,43]. The IGF-I signaling pathways have been shown to play an important role in the regulation of myeloma growth and survival. This suggests that in addition to inhibition of geranylgeranylation, depletion of dolichol-phosphate or isopentenyladenine by lovastatin may also contribute to the induction of apoptosis and inhibition of proliferation in myeloma cells. However, in add-back experiments isopentenyladenine or dolichol-phosphate did not abrogate lovastatin-induced apoptosis in AML cell lines [44].

Our data indicate an important role for geranylgeranylated proteins in the regulation of myeloma cell survival [34]. The mechanisms of apoptosis induction through the inhibition of geranylgeranylation were investigated in chapter 5. In all myeloma cell lines and in all primary myeloma cells, it was demonstrated that inhibition of geranylgeranylation either by depletion of GGPP or inhibition of GGTase I activity reduced expression of Mcl-1 protein levels [34]. This reduction preceded or was concomitant with induction of apoptosis, collapse of the mitochondrial transmembrane potential, and caspase activation suggesting that Mcl-1 down-regulation is involved in lovastatin- or GGTase I inhibitor-induced programmed cell death. Recently, several studies have demonstrated that the anti-apoptotic Bcl-2 family member Mcl-1 is a key regulator of survival and chemosensitivity in myeloma [10,45,46]. Reduction of Mcl-1 protein induced apoptosis and restored sensitivity to dexamethasone [10]. Furthermore, high expression of Mcl-1 was associated with a poor response to therapy in B-cell chronic lymphocytic leukemia (B-CLL) [47], AML, and acute lymphoblastic leukemia (ALL) patients [48]. This suggests that the synergistic effect between lovastatin and dexamethasone in myeloma cells may be due to lovastatin-mediated reduction of Mcl-1 expression.

Myeloma tumor cell growth and survival is regulated by various signaling pathways including MAPK [49], PI-3K [50,51], JAK-STAT [52], NF-κB [53-56] signaling cascades. The MAPK [49], PI-3K [50,51,57], and NF-κB [55,56] signaling pathways stimulate myeloma cell proliferation and activation of JAK-STAT [52], PI-3K [50,51,57], and NF-κB [54,56] pathways promotes myeloma cell survival. Inhibition of geranylgeranylation either by depletion of geranylgeranyl isoprenoids through inhibition of HMG-CoA reductase or by inhibition of GGTase I activity suppressed myeloma proliferation due to G1 arrest (chapter 6). Approximately 0.5 to 1% of cellular proteins are geranylgeranylated, however only a small number of proteins have been identified [58], of these the geranylgeranylated proteins Rac-1, RhoA, and Cdc42 are implicated in the regulation of various cellular processes including survival and proliferation [59-63]. Studies using specific inhibitors of these proteins suggested that Rac-1 and/or Cdc42 GTPases play a role in myeloma cell growth, but not survival. Although, Rho activity seems to be important for the rearrangement of the actin cytoskeleton in myeloma cells and for morphological changes and attachment to culture dishes [64], inhibition of Rho proteins did not affect myeloma cell survival or proliferation. This suggests that Rho may play a role in the motility and metastatic potential of myeloma

cells [64]. Rac-1 was activated in all myeloma cell lines tested, and the important growth and survival factor IL-6 induced Rac-1 activity. Introduction of dominant-negative Tat-Rac-1 protein and reduction of Rac protein levels by antisense oligodeoxynucleotides reduced proliferation, whereas constitutively active Tat-Rac-1 stimulated proliferation of myeloma cells. Both dominant-negative and constitutively active Rac-1 had no effect on myeloma cell survival. These data suggest that Rac-1 plays a role in myeloma cell proliferation, and that the suppression of proliferation by lovastatin and GGTase I inhibitors may be, at least in part, due to inhibition of geranylgeranylation of Rac-1. Since IL-6 stimulates Rac-1 activity and dominant-negative Rac-1 blocks IL-6-induced growth, Rac-1 may be a downstream effector in IL-6-triggered signaling pathways. The effect of expression of constitutively active and dominant-negative Tat-Rac-1 mutants on proliferation is currently being evaluated in primary myeloma cells.

An important question to address is how Rac-1 is activated in myeloma cells. Various studies have shown that PI-3K stimulates Rac-1 activity probably through the activation of Rac GDP/GTP exchange factors (GEFs) [65-67]. Alternatively, Rac-1 can be activated by Ras in a PI-3K-dependent or -independent way [66,68]. Both PI-3K [50,51,57] and Ras [39] play an important role in myeloma cell growth. Activation of Akt by PI-3K in myeloma cells has been shown to contribute to proliferation and survival [69,70]. Several pathways that contribute to growth are activated downstream of Ras in myeloma cells including PI-3K, NF-κB, and MAPK signaling cascades [39]. Our data suggest that Rac-1 may be a downstream effector of Ras and/or PI-3K signaling pathways leading to myeloma tumor cell proliferation.

Induction of apoptosis and suppression of proliferation through inhibition of protein geranylgeranylation were not restricted to myeloma tumor cells. Also in lymphoma cell lines and in purified tumor cells from patients with NHL and B-CLL inhibition of protein geranylgeranylation either by inhibition of the mevalonate pathway or by specific suppression of GGTase I activity induced apoptosis even in resistant cell lines and patient cells, blocked proliferation, and reversed resistance to dexamethasone (chapter 7).

These preclinical studies indicate that modulation of the mevalonate pathway through the inhibition of HMG-CoA reductase by statins represents a novel therapeutic approach in the treatment of myeloma and lymphoma, and provided the basis for the evaluation of simvastatin in combination with chemotherapy in a phase 1 clinical trial. In this study refractory NHL, B-CLL, and myeloma patients are treated with simvastatin for 7 days, followed by VAD chemotherapy in myeloma or CHOP in NHL or CLL. The primary objectives of this study are to determine the safety and tolerability of simvastatin administered in combination with chemotherapy, and to define the maximum-tolerated dose of simvastatin.

## Concluding remarks

The identification and increased understanding of mechanisms that regulate survival, drug resistance, and proliferation will contribute to the development of new specific and effective therapeutic strategies to modulate apoptosis, growth, and drug resistance in multiple myeloma [71,72]. With rare exceptions, malignant cell growth is the result of multiple genetic alterations [73]. The combination of novel agents that simultaneously target a single pathway or that target different pathways will therefore be critical to avoid drug resistance and to achieve long-term remissions [74]. The introduction of novel agents together with technologies such as gene expression profiling to identify those patients who are most likely to respond, will result in a more selective and potent molecular-based myeloma therapy that will improve patient outcome.

## References


1. Reed, J. C. Bcl-2 family proteins. Oncogene, *17*: 3225-3236, 1998.
2. McDonnell, T. J. and Korsmeyer, S. J. Progression from lymphoid hyperplasia to high-grade malignant lymphoma in mice transgenic for the t(14; 18). Nature, *349*: 254-256, 1991.
3. Strasser, A., Harris, A. W., and Cory, S. E mu-bcl-2 transgene facilitates spontaneous transformation of early pre-B and immunoglobulin-secreting cells but not T cells. Oncogene, *8*: 1-9, 1993.
4. Gazitt, Y., Fey, V., Thomas, C., and Alvarez, R. Bcl-2 overexpression is associated with resistance to dexamethasone, but not melphalan, in multiple myeloma cells. Int.J Oncol., *13*: 397-405, 1998.
5. Tu, Y., Xu, F. H., Liu, J., Vescio, R., Berenson, J., Fady, C., and Lichtenstein, A. Upregulated expression of BCL-2 in multiple myeloma cells induced by exposure to doxorubicin, etoposide, and hydrogen peroxide. Blood, *88*: 1805-1812, 1996.
6. Sangfelt, O., Osterborg, A., Grander, D., Anderbring, E., Ost, A., Mellstedt, H., and Einhorn, S. Response to interferon therapy in patients with multiple myeloma correlates with expression of the Bcl-2 oncoprotein. Int.J.Cancer, *63*: 190-192, 1995.
7. Ong, F., van Nieuwkoop, J. A., Groot-Swings, G. M., Hermans, J., Harvey, M. S., Kluin, P. M., and Kluin-Nelemans, J. C. Bcl-2 protein expression is not related to short survival in multiple myeloma. Leukemia, *9*: 1282-1284, 1995.
8. Renner, S., Weisz, J., Krajewski, S., Krajewska, M., Reed, J. C., and Lichtenstein, A. Expression of BAX in plasma cell dyscrasias. Clin.Cancer Res., *6*: 2371-2380, 2000.
9. van de Donk, N. W., Kamphuis, M. M., Van Dijk, M., Borst, H. P., Bloem, A. C., and Lokhorst, H. M. Chemosensitization of myeloma plasma cells by an antisense-mediated downregulation of Bcl-2 protein. Leukemia, *17*: 211-219, 2003.
10. Derenne, S., Monia, B., Dean, N. M., Taylor, J. K., Rapp, M. J., Harousseau, J. L., Bataille, R., and Amiot, M. Antisense strategy shows that Mcl-1 rather than Bcl-2 or Bcl-x(L) is an essential survival protein of human myeloma cells. Blood, *100*: 194-199, 2002.
11. Liu, Q. and Gazitt, Y. Potentiation of dexamethasone-, paclitaxel-, and Ad-p53-induced apoptosis by Bcl-2 antisense oligodeoxynucleotides in drug-resistant multiple myeloma cells. Blood, *101* : 4105-4114, 2003.
12. Veis, D. J., Sorenson, C. M., Shutter, J. R., and Korsmeyer, S. J. Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. Cell, *75*: 229-240, 1993.

13. Kamada, S., Shimono, A., Shinto, Y., Tsujimura, T., Takahashi, T., Noda, T., Kitamura, Y., Kondoh, H., and Tsujimoto, Y. bcl-2 deficiency in mice leads to pleiotropic abnormalities: accelerated lymphoid cell death in thymus and spleen, polycystic kidney, hair hypopigmentation, and distorted small intestine. Cancer Res., *55*: 354-359, 1995.
14. Glover, J. M., Leeds, J. M., Mant, T. G., Amin, D., Kisner, D. L., Zuckerman, J. E., Geary, R. S., Levin, A. A., and Shanahan, W. R., Jr. Phase I safety and pharmacokinetic profile of an intercellular adhesion molecule-1 antisense oligodeoxynucleotide (ISIS 2302). J Pharmacol.Exp Ther, *282*: 1173-1180, 1997.
15. Bishop, M. R., Iversen, P. L., Bayever, E., Sharp, J. G., Greiner, T. C., Copple, B. L., Ruddon, R., Zon, G., Spinolo, J., Arneson, M., Armitage, J. O., and Kessinger, A. Phase I trial of an antisense oligonucleotide OL(1)p53 in hematologic malignancies. J Clin.Oncol., *14*: 1320-1326, 1996.
16. Evan, G. and Littlewood, T. A matter of life and cell death. Science, *281*: 1317-1322, 1998.
17. Shou, Y., Martelli, M. L., Gabrea, A., Qi, Y., Brents, L. A., Roschke, A., Dewald, G., Kirsch, I. R., Bergsagel, P. L., and Kuehl, W. M. Diverse karyotypic abnormalities of the c-myc locus associated with c-myc dysregulation and tumor progression in multiple myeloma. Proc Natl Acad Sci U S A, *97*: 228-233, 2000.
18. Avet-Loiseau, H., Gerson, F., Magrangeas, F., Minvielle, S., Harousseau, J. L., and Bataille, R. Rearrangements of the c-myc oncogene are present in 15% of primary human multiple myeloma tumors. Blood, *98*: 3082-3086, 2001.
19. Bissonnette, R. P., Echeverri, F., Mahboubi, A., and Green, D. R. Apoptotic cell death induced by c-myc is inhibited by bcl-2. Nature, *359*: 552-554, 1992.
20. Strasser, A., Harris, A. W., Bath, M. L., and Cory, S. Novel primitive lymphoid tumours induced in transgenic mice by cooperation between myc and bcl-2. Nature, *348*: 331-333, 1990.
21. Fanidi, A., Harrington, E. A., and Evan, G. I. Cooperative interaction between c-myc and bcl-2 proto-oncogenes. Nature, *359*: 554-556, 1992.
22. Huang, Z. Bcl-2 family proteins as targets for anticancer drug design. Oncogene, *19*: 6627-6631, 2000.
23. Wang, J. L., Zhang, Z. J., Choksi, S., Shan, S., Lu, Z., Croce, C. M., Alnemri, E. S., Korngold, R., and Huang, Z. Cell permeable Bcl-2 binding peptides: a chemical approach to apoptosis induction in tumor cells. Cancer Res., *60*: 1498-1502, 2000.
24. Wang, J. L., Liu, D., Zhang, Z. J., Shan, S., Han, X., Srinivasula, S. M., Croce, C. M., Alnemri, E. S., and Huang, Z. Structure-based discovery of an organic compound that binds Bcl-2 protein and induces apoptosis of tumor cells. Proc Natl Acad Sci U S A, *%20;97*: 7124-7129, 2000.
25. Haldar, S., Jena, N., and Croce, C. M. Inactivation of Bcl-2 by phosphorylation. Proc Natl Acad Sci U S A, *92*: 4507-4511, 1995.
26. Haldar, S., Chintapalli, J., and Croce, C. M. Taxol induces bcl-2 phosphorylation and death of prostate cancer cells. Cancer Res., *56*: 1253-1255, 1996.
27. Ito, T., Deng, X., Carr, B., and May, W. S. Bcl-2 phosphorylation required for anti-apoptosis function. J Biol.Chem., *272*: 11671-11673, 1997.
28. Deng, X., Ruvolo, P., Carr, B., and May, W. S., Jr. Survival function of ERK1/2 as IL-3-activated, staurosporine-resistant Bcl2 kinases. Proc Natl Acad Sci U S A, *97*: 1578-1583, 2000.
29. Goldstein, J. L. and Brown, M. S. Regulation of the mevalonate pathway. Nature, *343*: 425-430, 1990.
30. Vitols, S., Norgren, S., Juliusson, G., Tatidis, L., and Luthman, H. Multilevel regulation of low-density lipoprotein receptor and 3-hydroxy-3-methylglutaryl coenzyme A reductase gene expression in normal and leukemic cells. Blood, *84*: 2689-2698, 1994.
31. Harwood, H. J., Jr., Alvarez, I. M., Noyes, W. D., and Stacpoole, P. W. In vivo regulation of human leukocyte 3-hydroxy-3-methylglutaryl coenzyme A reductase: increased enzyme protein concentration and catalytic efficiency in human leukemia and lymphoma. J Lipid Res., *32*: 1237-1252, 1991.
32. Dimitroulakos, J., Nohynek, D., Backway, K. L., Hedley, D. W., Yeger, H., Freedman, M. H., Minden, M. D., and Penn, L. Z. Increased sensitivity of acute myeloid leukemias to lovastatin-induced apoptosis: A potential therapeutic approach. Blood, *93*: 1308-1318, 1999.

33. van de Donk, N. W., Kamphuis, M. M., Lokhorst, H. M., and Bloem, A. C. The cholesterol lowering drug lovastatin induces cell death in myeloma plasma cells. Leukemia, *16*: 1362-1371, 2002.
34. van de Donk, N. W., Kamphuis, M. M., Van Kessel, B., Lokhorst, H. M., and Bloem, A. C. Inhibition of protein geranylgeranylation induces apoptosis in myeloma plasma cells by reducing Mcl-1 protein levels. Blood, .: 2003.
35. Le Gouill, S., Pellat-Deceunynck, C., Harousseau, J. L., Rapp, M. J., Robillard, N., Bataille, R., and Amiot, M. Farnesyl transferase inhibitor R115777 induces apoptosis of human myeloma cells. Leukemia, *16*: 1664-1667, 2002.
36. Chatterjee, M., Honemann, D., Lentzsch, S., Bommert, K., Sers, C., Herrmann, P., Mathas, S., Dorken, B., and Bargou, R. C. In the presence of bone marrow stromal cells human multiple myeloma cells become independent of the IL-6/gp130/STAT3 pathway. Blood, *100*: 3311-3318, 2002.
37. Frassanito, M. A., Cusmai, A., Piccoli, C., and Dammacco, F. Manumycin inhibits farnesyltransferase and induces apoptosis of drug-resistant interleukin 6-producing myeloma cells. Br.J Haematol., *118*: 157-165, 2002.
38. Bolick, S. C., Landowski, T. H., Boulware, D., Oshiro, M. M., Ohkanda, J., Hamilton, A. D., Sebti, S. M., and Dalton, W. S. The farnesyl transferase inhibitor, FTI-277, inhibits growth and induces apoptosis in drug-resistant myeloma tumor cells. Leukemia, *17*: 451-457, 2003.
39. Hu, L., Shi, Y., Hsu, J. H., Gera, J., Van Ness, B., and Lichtenstein, A. Downstream effectors of oncogenic ras in multiple myeloma cells. Blood, *101*: 3126-3135, 2003.
40. Cortes, J., Albitar, M., Thomas, D., Giles, F., Kurzrock, R., Thibault, A., Rackoff, W., Koller, C., O'Brien, S., Garcia-Manero, G., Talpaz, M., and Kantarjian, H. Efficacy of the farnesyl transferase inhibitor R115777 in chronic myeloid leukemia and other hematologic malignancies. Blood, *101*: 1692-1697, 2003.
41. Mackley, P. A., Shain, K. H., Dalton, W. S., and Alsina, M. Farnesyl transferase inhibitor-R11577-decreases Akt phosphorylation in multiple myeloma cell lines and its apoptotic effects correlate with phosphoAkt expression levels. Blood, 3198, 2002.
42. Dricu, A., Wang, M., Hjertman, M., Malec, M., Blegen, H., Wejde, J., Carlberg, M., and Larsson, O. Mevalonate-regulated mechanisms in cell growth control: role of dolichyl phosphate in expression of the insulin-like growth factor-1 receptor (IGF-1R) in comparison to Ras prenylation and expression of c-myc. Glycobiology, *7*: 625-633, 1997.
43. Carlberg, M., Dricu, A., Blegen, H., Wang, M., Hjertman, M., Zickert, P., Hoog, A., and Larsson, O. Mevalonic acid is limiting for N-linked glycosylation and translocation of the insulin-like growth factor-1 receptor to the cell surface. Evidence for a new link between 3-hydroxy-3-methylglutaryl-coenzyme a reductase and cell growth. J Biol.Chem., *%19;271*: 17453-17462, 1996.
44. Xia, Z., Tan, M. M., Wong, W. W., Dimitroulakos, J., Minden, M. D., and Penn, L. Z. Blocking protein geranylgeranylation is essential for lovastatin-induced apoptosis of human acute myeloid leukemia cells. Leukemia, *15*: 1398-1407, 2001.
45. Gojo, I., Zhang, B., and Fenton, R. G. The Cyclin-dependent Kinase Inhibitor Flavopiridol Induces Apoptosis in Multiple Myeloma Cells through Transcriptional Repression and Down-Regulation of Mcl-1. Clin.Cancer Res., *8*: 3527-3538, 2002.
46. Zhang, B., Gojo, I., and Fenton, R. G. Myeloid cell factor-1 is a critical survival factor for multiple myeloma. Blood, *99*: 1885-1893, 2002.
47. Kitada, S., Andersen, J., Akar, S., Zapata, J. M., Takayama, S., Krajewski, S., Wang, H. G., Zhang, X., Bullrich, F., Croce, C. M., Rai, K., Hines, J., and Reed, J. C. Expression of apoptosis-regulating proteins in chronic lymphocytic leukemia: correlations with In vitro and In vivo chemoresponses. Blood, *91*: 3379-3389, 1998.
48. Kaufmann, S. H., Karp, J. E., Svingen, P. A., Krajewski, S., Burke, P. J., Gore, S. D., and Reed, J. C. Elevated expression of the apoptotic regulator Mcl-1 at the time of leukemic relapse. Blood, *91*: 991-1000, 1998.

49. Ogata, A., Chauhan, D., Teoh, G., Treon, S. P., Urashima, M., Schlossman, R. L., and Anderson, K. C. IL-6 triggers cell growth via the Ras-dependent mitogen-activated protein kinase cascade. J.Immunol., *159*: 2212-2221, 1997.
50. Tu, Y., Gardner, A., and Lichtenstein, A. The phosphatidylinositol 3-kinase/AKT kinase pathway in multiple myeloma plasma cells: roles in cytokine-dependent survival and proliferative responses. Cancer Res., *60*: 6763-6770, 2000.
51. Hideshima, T., Nakamura, N., Chauhan, D., and Anderson, K. C. Biologic sequelae of interleukin-6 induced PI3-K/Akt signaling in multiple myeloma. Oncogene, *%20;20*: 5991-6000, 2001.
52. Catlett-Falcone, R., Landowski, T. H., Oshiro, M. M., Turkson, J., Levitzki, A., Savino, R., Ciliberto, G., Moscinski, L., Fernandez-Luna, J. L., Nunez, G., Dalton, W. S., and Jove, R. Constitutive activation of Stat3 signaling confers resistance to apoptosis in human U266 myeloma cells. Immunity., *10*: 105-115, 1999.
53. Feinman, R., Koury, J., Thames, M., Barlogie, B., Epstein, J., and Siegel, D. S. Role of NF-kappaB in the rescue of multiple myeloma cells from glucocorticoid-induced apoptosis by bcl-2. Blood, *93*: 3044-3052, 1999.
54. Ni, H., Ergin, M., Huang, Q., Qin, J. Z., Amin, H. M., Martinez, R. L., Saeed, S., Barton, K., and Alkan, S. Analysis of expression of nuclear factor kappa B (NF-kappa B) in multiple myeloma: downregulation of NF-kappa B induces apoptosis. Br.J Haematol., *115*: 279-286, 2001.
55. Bharti, A. C., Donato, N., Singh, S., and Aggarwal, B. B. Curcumin (diferuloylmethane) down-regulates the constitutive activation of nuclear factor-kappa B and IkappaBalpha kinase in human multiple myeloma cells, leading to suppression of proliferation and induction of apoptosis. Blood, *101*: 1053-1062, 2003.
56. Hideshima, T., Chauhan, D., Richardson, P., Mitsiades, C., Mitsiades, N., Hayashi, T., Munshi, N., Dang, L., Castro, A., Palombella, V., Adams, J., and Anderson, K. C. NF-kappa B as a therapeutic target in multiple myeloma. J Biol.Chem., *277*: 16639-16647, 2002.
57. Qiang, Y. W., Kopantzev, E., and Rudikoff, S. Insulinlike growth factor-I signaling in multiple myeloma: downstream elements, functional correlates, and pathway cross-talk. Blood, *99*: 4138-4146, 2002.
58. Wong, W. W., Dimitroulakos, J., Minden, M. D., and Penn, L. Z. HMG-CoA reductase inhibitors and the malignant cell: the statin family of drugs as triggers of tumor-specific apoptosis. Leukemia, *16*: 508-519, 2002.
59. Bishop, A. L. and Hall, A. Rho GTPases and their effector proteins. Biochem.J, *348 Pt 2:241-55.*: 241-255, 2000.
60. Van Aelst, L. and D'Souza-Schorey, C. Rho GTPases and signaling networks. Genes Dev., *11*: 2295-2322, 1997.
61. Zohn, I. M., Campbell, S. L., Khosravi-Far, R., Rossman, K. L., and Der, C. J. Rho family proteins and Ras transformation: the RHOad less traveled gets congested. Oncogene, *17*: 1415-1438, 1998.
62. Bar-Sagi, D. and Hall, A. Ras and Rho GTPases: a family reunion. Cell, *103*: 227-238, 2000.
63. Hall, A. Rho GTPases and the actin cytoskeleton. Science, *279*: 509-514, 1998.
64. Qiang, Y. W., Endo, Y., Rubin, J. S., and Rudikoff, S. WNT signaling in multiple myeloma. The Hematology Journal, *4*: S36, 2003.
65. Han, J., Luby-Phelps, K., Das, B., Shu, X., Xia, Y., Mosteller, R. D., Krishna, U. M., Falck, J. R., White, M. A., and Broek, D. Role of substrates and products of PI 3-kinase in regulating activation of Rac-related guanosine triphosphatases by Vav. Science, *279*: 558-560, 1998.
66. Grill, B. and Schrader, J. W. Activation of Rac-1, Rac-2, and Cdc42 by hemopoietic growth factors or cross-linking of the B-lymphocyte receptor for antigen. Blood, *100*: 3183-3192, 2002.
67. Van Leeuwen, F. N., Olivo, C., Grivell, S., Giepmans, B. N., Collard, J. G., and Moolenaar, W. H. Rac activation by lysophosphatidic acid LPA1 receptors through the guanine nucleotide exchange factor Tiam1. J Biol.Chem., *278*: 400-406, 2003.

68. Rodriguez-Viciana, P., Warne, P. H., Khwaja, A., Marte, B. M., Pappin, D., Das, P., Waterfield, M. D., Ridley, A., and Downward, J. Role of phosphoinositide 3-OH kinase in cell transformation and control of the actin cytoskeleton by Ras. Cell, *89*: 457-467, 1997.
69. Hsu, J., Shi, Y., Krajewski, S., Renner, S., Fisher, M., Reed, J. C., Franke, T. F., and Lichtenstein, A. The AKT kinase is activated in multiple myeloma tumor cells. Blood, *98*: 2853-2855, 2001.
70. Mitsiades, C. S., Mitsiades, N., Poulaki, V., Schlossman, R., Akiyama, M., Chauhan, D., Hideshima, T., Treon, S. P., Munshi, N. C., Richardson, P. G., and Anderson, K. C. Activation of NF-kappaB and upregulation of intracellular anti-apoptotic proteins via the IGF-1/Akt signaling in human multiple myeloma cells: therapeutic implications. Oncogene, *21*: 5673-5683, 2002.
71. Hideshima, T. and Anderson, K. C. Molecular mechanisms of novel therapeutic approaches for multiple myeloma. Nat.Rev.Cancer, *2*: 927-937, 2002.
72. Rabbitts, T. H. and Stocks, M. R. Chromosomal translocation products engender new intracellular therapeutic technologies. Nat.Med, *9*: 383-386, 2003.
73. Hunter, T. Oncoprotein networks. Cell, *88*: 333-346, 1997.
74. Golub, T. R. Mining the genome for combination therapies. Nat.Med, *9*: 510-511, 2003.

# Nederlandse samenvatting

Multipel myeloom, ook wel ziekte van Kahler genoemd, behoort samen met de verschillende soorten leukemieën en lymfomen tot de hematologische maligniteiten. Bij multipel myeloom is er sprake van een kwaadaardige woekering van plasmacellen in het beenmerg. Plasmacellen behoren tot de witte bloedcellen en zijn verantwoordelijk voor een deel van de afweer tegen bacteriën en virussen door de productie van antistoffen. De myeloom tumorcellen en de producten van de tumorcellen zijn verantwoordelijk voor symptomen zoals infecties, botpijn, vermoeidheid (onder andere ten gevolge van bloedarmoede) en nierfunctie stoornissen. Multipel myeloom is een tot nu toe ongeneeslijke vorm van kanker met een overleving die varieert van minder dan 2 tot meer dan 80 maanden na diagnose. In Nederland wordt jaarlijks bij ongeveer 800 mensen de diagnose multipel myeloom gesteld en overlijdt een ongeveer even groot aantal aan deze ziekte. Multipel myeloom wordt over het algemeen behandeld met chemotherapie. Ondanks een vaak goede initiële respons op chemotherapie, reageert de meerderheid van de patiënten uiteindelijk niet meer op chemotherapie en is er sprake van chemotherapieresistente ziekte. Een beter inzicht in het ontstaan van chemotherapieresistentie en kennis van de regulatie van celdeling en overleving van myeloom tumorcellen kan bijdragen aan de ontwikkeling van nieuwe therapieën. In dit proefschrift werd gekeken naar de rol van Bcl-2 en de mevalonzuur route bij de regulatie van dit soort processen in multipel myeloom.

Bcl-2 is een eiwit dat geprogrammeerde celdood (apoptose) tegen gaat en dat ook celdood geïnduceerd door chemotherapeutica kan remmen. De rol van Bcl-2 in multipel myeloom was nog grotendeels onbekend. De functie van Bcl-2 bij multipel myeloom werd onderzocht door middel van antisense oligodeoxynucleotiden (ODNs), dit zijn korte stukken enkelstrengs DNA, die in dit geval een sequentie hebben die complementair is aan de sequentie van het Bcl-2 messengerRNA (mRNA). Bcl-2 mRNA is een kopie van het Bcl-2 gen en bevat de code die nodig is voor de productie van het Bcl-2 eiwit in de cel. Bcl-2 antisense ODNs binden specifiek aan Bcl-2 mRNA moleculen, omdat ze een complementaire sequentie hebben. Dit leidt tot de vorming van een Bcl-2 antisense/Bcl-2 mRNA duplex. De vorming van deze duplex remt de vertaling van het Bcl-2 mRNA tot eiwit en bovendien wordt het Bcl-2 mRNA in de duplex afgebroken door het enzym RNase H. In hoofdstuk 2 is beschreven dat behandeling van opgezuiverde myeloom tumorcellen van patiënten met Bcl-2 antisense ODNs resulteerde in een specifieke daling van de hoeveelheid Bcl-2 mRNA en Bcl-2 eiwit. De reductie van Bcl-2 eiwit alleen induceerde geen celdood, maar maakte de myeloom tumorcellen wel significant gevoeliger voor verschillende soorten chemotherapeutica.

Dit laboratorium onderzoek vormde de basis voor een klinische studie (hoofdstuk 3). Het doel van dit onderzoek was om te bestuderen of een daling van de hoeveelheid

Bcl-2 eiwit de effectiviteit van chemotherapie in myeloom patiënten zou vergroten. In dit onderzoek werd aan uitvoerig voorbehandelde en niet meer op chemotherapie reagerende myeloom patiënten gedurende 7 dagen Bcl-2 antisense ODNs toegediend. Op dag 4 van de antisense infusie werd gestart met VAD-chemotherapiekuren. De combinatie van Bcl-2 antisense en VAD-chemotherapie werd goed verdragen. Behandeling van patiënten met Bcl-2 antisense ODNs gecombineerd met VAD-kuren resulteerde in een reductie van de hoeveelheid tumor bij 7 van de 10 (70%) patiënten, wat zich vertaalde in een vermindering van de pijn en verbetering van de lichamelijke conditie. Vijf van deze 7 responderende patiënten reageerden niet meer op VAD-kuren alleen. Dit geeft aan dat daling van de hoeveelheid Bcl-2 eiwit in de tumor met behulp van Bcl-2 antisense ODNs de gevoeligheid van de tumor voor chemotherapie kan herstellen.

Het tweede deel van dit proefschrift gaat over de rol van de mevalonzuur route in multipel myeloom. De mevalonzuur route produceert een diversiteit aan producten waaronder farnesylpyrofosfaat, geranylgeranylpyrofosfaat, dolichol en ubiquinon, maar ook cholesterol. De snelheidsbepalende stap van de mevalonzuur route is de omzetting van HMG-CoA naar mevalonzuur en wordt geremd door statines zoals simvastatine en lovastatine. Remming van de mevalonzuur route door lovastatine in myeloom tumorcellen verminderde de celdeling, induceerde geprogrammeerde celdood en verhoogde de effectiviteit van chemotherapie (hoofdstuk 4). Vervolgens werd in hoofdstuk 5 en 6 onderzocht op welke wijze remming van de mevalonzuur route resulteerde in celdood en vermindering van celdeling. Afname van de hoeveelheid geranylgeranylpyrofosfaat in de myeloom tumorcel bleek verantwoordelijk te zijn voor zowel de inductie van celdood als de remming van celdeling. Geranylgeranylpyrofosfaat moleculen worden door het enzym geranylgeranyl transferase gehecht aan bepaalde eiwitten. Dit proces, dat ook wel geranylgeranylering wordt genoemd, is een vereiste voor de bevestiging van deze eiwitten aan membranen, wat noodzakelijk is voor het kunnen uitoefenen van hun functie in de cel. Ook remming van geranylgeranylering door inhibitie van geranylgeranyl transferase induceerde celdood en verminderde de celdeling van myeloom tumor cellen.

Deze gegevens wijzen op een belangrijke rol van ge-geranylgeranyleerde eiwitten in de regulatie van deling en overleving van myeloom tumorcellen. Remming van de geranylgeranylering in myeloom tumorcellen resulteerde waarschijnlijk in celdood, doordat de expressie van het Bcl-2 familie eiwit Mcl-1 verlaagd werd. Mcl-1 beschermt net als Bcl-2 tegen geprogrammeerde celdood en een afname van dit eiwit kan direct leiden tot het dood gaan van myeloom tumorcellen. De toename van de effectiviteit van chemotherapie door lovastatine kan ook verklaard worden door de afname van de hoeveelheid Mcl-1 eiwit, aangezien Mcl-1 myeloom tumorcellen kan beschermen tegen de effecten van chemotherapie.

Door gebruik te maken van verschillende remmers van ge-geranylgeranyleerde eiwitten werd gevonden dat het eiwit Rac-1 waarschijnlijk een rol speelt bij de deling van

myeloom tumorcellen, maar niet bij de overleving van deze cellen. Rac-1 was geactiveerd in myeloom tumorcellen en de belangrijkste myeloom groeifactor interleukine-6 (IL-6) verhoogde de activiteit van Rac-1. Specifieke verlaging van de hoeveelheid Rac eiwit en behandeling van de cellen met een dominant-negatief Tat-Rac-1 eiwit leidde tot remming van de celdeling. Dit geeft dus aan dat het ge-geranylgeranyleerde eiwit Rac-1 betrokken is bij de regulatie van multipel myeloom celdeling.

In hoofdstuk 7 is beschreven dat geranylgeranylering van eiwitten ook belangrijk is voor de overleving en celdeling van tumorcellen die afkomstig zijn van patiënten met verschillende soorten non-Hodgkin lymfomen en chronische lymfatische leukemie. Ook bij deze vormen van kanker leidde remming van de mevalonzuur route en inhibitie van geranylgeranyl transferase tot celdood, remming van de celdeling en toename van de effectiviteit van cytostatica.

De preklinische studies zoals beschreven in de hoofdstukken 4 tot en met 7 geven aan dat remming van de mevalonzuur route door statines een nieuwe strategie is voor de behandeling van patiënten met multipel myeloom, non-Hodgkin lymfoom en chronische lymfatische leukemie. Dit onderzoek heeft geleid tot de initiatie van een klinische studie waarin de effectiviteit van simvastatine in combinatie met chemotherapie momenteel onderzocht wordt bij deze patiënten.

Een beter begrip van mechanismen, die van belang zijn voor de regulatie van overleving, deling en chemotherapieresistentie van myeloom tumorcellen, kan bijdragen aan de ontwikkeling van nieuwe specifieke en effectieve therapieën voor de behandeling van multipel myeloom. In dit proefschrift werd de functie van het eiwit Bcl-2 en de mevalonzuur route in multipel myeloom beschreven. Deze kennis leidde tot de initiatie van twee klinische studies. Het uiteindelijke doel van dit soort laboratorium onderzoek en klinische studies is het verbeteren van de behandeling van multipel myeloom patiënten.

# Dankwoord

Dit proefschrift kwam tot stand met de steun en medewerking van veel mensen, die ik hier graag wil bedanken.
Allereerst wil ik Henk Lokhorst en Andries Bloem bedanken voor de mogelijkheid die ik als geneeskunde student kreeg om in het multipel myeloom laboratorium te promoveren. Jullie vriendschappelijke, motiverende, enthousiaste en kritische begeleiding heb ik al die 4 jaren erg gewaardeerd. Ik heb ontzettend veel van jullie geleerd.

Hans Clevers en Ton Hagenbeek, bedankt dat jullie mijn promotores wilden zijn en voor jullie adviezen en ideeën.

Marloes, samen met jou ben ik 4 jaar geleden aan dit project begonnen. Mede dankzij jouw enorme inspanning en ideeën is dit boekje tot stand gekomen. Jammer dat je terug ging naar het Verre Oo(o)sten, maar wel ontzettend leuk dat je nu, samen met Maarten, mijn paranimf wilt zijn. Berris, door jouw inzet en vele blots werden de laatste artikelen snel en succesvol afgerond. Diana, mijn studente, wil ik bedanken voor je harde werk dat onder andere geresulteerd heeft in hoofdstuk 7. Je was een goede, maar ook gezellige studente.

Mijn andere collega's en oud-collega's van het Jordan I laboratorium wil ik ook bedanken voor de gezellige samenwerking, hulp en discussie gedurende de afgelopen 4 jaar. Penny (succes met jouw laatste periode, collega AIO), Mirjam en Henriëtte (door jullie stoomcursus kon ik snel en doelgericht met mijn onderzoek aan de slag), Niels K. (altijd kalm), Kristin (bedankt voor het helpen maken van de voorkant van het boekje), Peter N(ukki)g, Okke (mede door jou enthousiasme ben ik in het myeloom onderzoek terecht gekomen), Esther (bedankt voor het FISH'en), Marijke (bedankt voor het opsporen van de deleties 13 en voor de prettige MGUS samenwerking), Arnold, Lucie, Jessica, Nadine, Alienke, Ton, Gienke, Peter V. (FITC-gelabelde…), Jeroen, Wim, Gerrit (bedankt voor alle computer en FACS problemen die je uit de wereld hielp), en Ariënne (succes, gezellige buurvrouw), bedankt!!

De cytomorfologie dames (dus: Elsbeth, Mariëlle, Anja, Hanneke, Ingrid, Dorine, Jeanette en Joke) dank ik voor bloed en beenmerg, welke ik gebruikt heb bij ontzettend veel experimenten, maar ook voor de bepaling van lymfocyten subsets bij patiënten die deelnamen aan de klinische studies. Bepaling van Bcl-2 mRNA, door middel van Real-Time PCR, was onmogelijk zonder de hulp van Eric (Taqman) Borst. Bedankt.

De dames van de prik-poli, van de dagbehandeling en van het secretariaat van D2Oost wil ik bedanken voor het afnemen van bloed, wat de basis vormde voor vele proeven.
Dames van het secretariaat immunologie (Yvonne en Annette) en hematologie (Joukje en Monique), bedankt voor het papierwerk.
De collega's en oud-collega's van de andere hematologie- en immunologie-laboratoria (inclusief Crucell) wil ik bedanken voor discussies, hulp en adviezen, maar ook voor gezelligheid tijdens of na het werk. Evert Nijhuis, Prof. dr. L. Koenderman en Dr. P. Coffer van de afdeling longziekten wil ik bedanken voor de hulp bij en het mogelijk maken van de Tat-Rac experimenten. Evert en Mirjam ook bedankt voor de hulp bij het het lay-outen van mijn proefschrift.
Ook wil ik de leden van de promotiecommissie bedanken (Prof. dr. J. G. van den Tweel, Prof. dr. J. L. Bos, Prof. dr. Ph. G. de Groot en Prof. dr. A. J. van Zonneveld) voor de tijd die ze aan dit proefschrift hebben besteed.
Tenslotte, vrienden en familie bedankt voor alle leuke dingen buiten het UMCU (etentjes, waterpolo [toch nog gepromoveerd], vakanties enzovoorts). Als ik nu te laat kom is het (voorlopig) in ieder geval zonder het excuus dat ik nog een proef af moest maken.

Niels

# Curriculum Vitae

De schrijver van dit proefschrift werd geboren op 8 juli 1976 te Nuenen. In 1994 behaalde hij het VWO diploma aan het Eckartcollege te Eindhoven. In dat zelfde jaar werd begonnen met de studie Farmacie aan de Universiteit Utrecht. Na het behalen van de propedeuse Farmacie, startte hij in 1995 met de studie Geneeskunde aan de Universiteit Utrecht. Tijdens de studie deed hij onderzoek bij het Rudolf Magnus instituut (Dr. G. J. Biessels, Prof. dr. W. H. Gispen) en bij de afdeling Haematologie van het Universitair Medisch Centrum Utrecht (drs. O. de Weerdt, Dr. H. M. Lokhorst). In september 1999 haalde hij het doctoraal examen Geneeskunde (cum laude). Van augustus 1999 tot oktober 2003 was de schrijver werkzaam als assistent in opleiding bij de afdelingen Immunologie en Haematologie van het Universitair Medisch Centrum Utrecht. Onder begeleiding van Dr. A. C. Bloem, Dr. H. M. Lokhorst, Prof. dr. H. C. Clevers en Prof. dr. A. Hagenbeek heeft hij gewerkt aan een project gefinancierd door het Koningin Wilhelmina fonds (KWF). Het onderzoek verrricht in deze periode, is beschreven in dit proefschrift.

# List of Publications

Lokhorst, H. M., Schattenberg, A., Cornelissen, J. J., van Oers, M. H., Fibbe, W., Russell, I., van de Donk, N. W., and Verdonck, L. F. Donor lymphocyte infusions for relapsed multiple myeloma after allogeneic stem-cell transplantation: predictive factors for response and long-term outcome. J Clin.Oncol., *18*: 3031-3037, 2000.

de Weerdt, O., van de Donk, N. W., Veth, G., Bloem, A. C., Hagenbeek, A., and Lokhorst, H. M. Continuous low-dose cyclophosphamide-prednisone is effective and well tolerated in patients with advanced multiple myeloma. Neth.J Med, *59*: 50-56, 2001.

Eurelings, M., Notermans, N. C., van de Donk, N. W., and Lokhorst, H. M. Risk factors for hematological malignancy in polyneuropathy associated with monoclonal gammopathy. Muscle Nerve, *24*: 1295-1302, 2001.

van de Donk, N. W., de Weerdt, O., Eurelings, M., Bloem, A., and Lokhorst, H. Malignant transformation of monoclonal gammopathy of undetermined significance: cumulative incidence and prognostic factors. Leuk.Lymphoma, *42*: 609-618, 2001.

van de Donk, N. W., Kamphuis, M. M., Lokhorst, H. M., and Bloem, A. C. The cholesterol lowering drug lovastatin induces cell death in myeloma plasma cells. Leukemia, *16*: 1362-1371, 2002.

van Marion, A. M., Lokhorst, H. M., van de Donk, N. W., and van den Tweel, J. G. The significance of plasma cells in bone marrow biopsies of patients with multiple myeloma following autologous and allogeneic stem cell transplantation. Histopathology, *41 suppl 2*: 79-83, 2002.

van de Donk, N. W., Kamphuis, M. M., van Dijk, M., Borst, H. P., Bloem, A. C., and Lokhorst, H. M. Chemosensitization of myeloma plasma cells by an antisense-mediated downregulation of Bcl-2 protein. Leukemia, *17*: 211-219, 2003.

van de Donk, N. W., Kamphuis, M. M., van Kessel, B., Lokhorst, H. M., and Bloem, A. C. Inhibition of protein geranylgeranylation induces apoptosis in myeloma plasma cells by reducing Mcl-1 protein levels. Blood, in press.

van de Donk, N. W., Schotte, D., Kamphuis, M. M., van Marion , A. M., van Kessel, B., Bloem, A. C., and Lokhorst, H. M. Protein geranylgeranylation is critical for the regulation of survival and proliferation of lymphoma tumor cells. Clin. Cancer Res., in press.

van de Donk, N. W., de Weerdt, O., Veth, G., Eurelings, M., van Stralen, E., Frankel, S. R., Hagenbeek, A., Bloem, A. C., and Lokhorst, H. M. G3139, a Bcl-2 antisense oligodeoxynucleotide, may overcome drug resistance in VAD refractory myeloma. Submitted.