

Targeting the mevalonate pathway in multiple myeloma

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Targeting the mevalonate pathway in multiple myeloma

Remming van de mevalonzuurroute in multipel myeloom
(met een samenvatting in het Nederlands)

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“Twenty years from now you will be more disappointed by the things you didn’t do than by the ones you did do. So throw off the bowlines. Sail away from the safe harbor. Catch the trade winds in your sails. Explore. Dream. Discover.”

- Mark Twain -

voor mijn vader

Contents

Chapter 1	9
General introduction	
Chapter 2	27
Dose-finding study of high-dose simvastatin combined with standard chemotherapy in patients with relapsed or refractory myeloma and lymphoma	
Chapter 3	37
High-dose simvastatin does not reverse resistance to vincristine, adriamycin, and dexamethasone (VAD) in myeloma	
Chapter 4	43
Inhibition of the mevalonate pathway potentiates the effects of lenalidomide in myeloma	
Chapter 5	59
Genome-wide expression analysis suggests that simvastatin exerts its anti-myeloma effect through upregulation of RhoB and pro-apoptotic genes	
Chapter 6	81
A bioluminescence imaging-based in vivo model for preclinical testing of novel cellular immunotherapy strategies to improve graft versus myeloma	
Chapter 7	101
General discussion	
Chapter 8	113
Samenvatting	
Dankwoord	
Curriculum vitae	

General introduction

chapterone

Introduction

Multiple myeloma is an incurable plasma cell malignancy. Clinical symptoms may include bone pain, infection, renal failure, hypercalcemia, and anemia. It is the second most frequent hematologic malignancy. In the Netherlands, annually approximately 800 new cases of multiple myeloma are diagnosed. Multiple myeloma cells predominantly localize in the bone marrow and display a low proliferation rate. Cyclin D dysregulation appears to occur as an early, nearly universal event in the pathogenesis of myeloma tumors.¹ Accumulation of additional genetic aberrancies contributes to disease progression and drug resistance. Although novel therapies have improved outcome, drug resistance develops in virtually all patients.² Resistance to therapy is mainly due to defective apoptosis, induced by chromosomal abnormalities, extracellular growth signals, and the tumor microenvironment.³

Multiple myeloma and the role of the microenvironment

The bone marrow microenvironment plays a pivotal role in the survival of myeloma cells and development of drug resistance. Interaction of clonal myeloma cells with extracellular matrix proteins (ECM) and bone marrow stromal cells (BMSC) induces release of cytokines and growth factors that bind to their respective receptors on the cell surface. This, in turn, causes activation of signal transduction pathways that lead to sustained growth and survival of malignant cells and drug resistance.^{4,5}

Important cytokines are interleukin-6 (IL-6), tumor-necrosis factor α (TNF- α), vascular endothelial growth factor (VEGF), and insulin-like growth factor I (IGF-I). IL-6 is well studied and plays an important role in growth and survival in myeloma. It is predominantly produced by BMSC⁶ and by the tumor itself.⁷ It inhibits apoptosis⁸ and protects the myeloma cell from dexamethasone-induced apoptosis.^{9,10} Addition of IL-6 to myeloma cells activates the Janus-activated kinase-signal transducers and activators of transcription (JAK/STAT),¹¹ the ras/mitogen-activated protein kinase (ras/MAPK),^{12,13} and the phosphatidylinositol-3' kinase/Akt kinase (PI-3K/Akt) pathways.¹⁴ Resistance of myeloma cells to apoptosis is mediated through JAK/STAT3,^{15,16} nuclear factor- κ B (NF- κ B),^{15,17} and PI-3K/Akt signaling pathways.¹⁸ Proliferation of myeloma cells is primarily mediated by the ras/MAPK pathway,¹³ PI-3K/Akt,¹⁸ and NF- κ B.^{19,20} TNF- α induces secretion of IL-6 in BMSC, NF- κ B activation, and phosphorylation of p42/44 MAPK. Additionally, it induces adhesion molecules on myeloma cells as well as on BMSC.²¹ VEGF is produced by both myeloma cells and BMSC. It stimulates angiogenesis and IL-6

production and triggers migration.^{22,23} Finally, IGF-I is produced by stroma cells, and activates the PI-3K/Akt and Ras/MAPK pathways.²⁴

Myeloma cells home to the bone marrow by selective binding to BMSC (figure 1). Adhesion molecules involved in this process include very-late antigen 4 (VLA-4) and leukocyte function-associated antigen-1 (LFA-1). VLA-4 and LFA-1 respectively bind to vascular cell adhesion molecule (VCAM) and intercellular adhesion molecule 1 (ICAM-1) on BMSC.^{25,26} In addition, VLA-4 binds to fibronectin; a major component of the ECM.²⁷ Binding of the myeloma cell to the BMSC enhances IL-6, IGF-I, and VEGF secretion in BMSC.^{22,28} In multiple myeloma, this is associated with protection from apoptosis and is shown to be an important mechanism for drug resistance.^{29,30} This phenomenon is called cell adhesion mediated-drug resistance (CAM-DR).

All these interactions and signaling pathways underlie the pathogenesis of multiple myeloma and may provide new targets for the treatment of this disease.

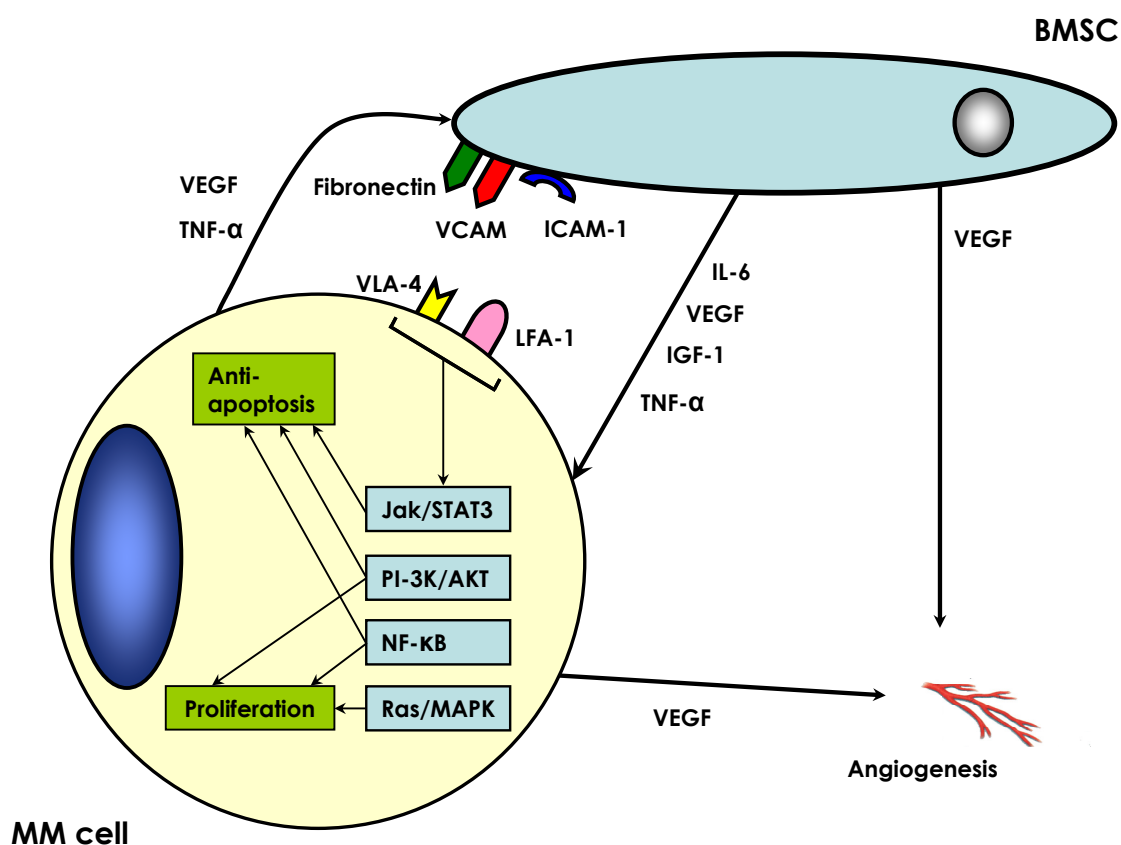


Figure 1. The myeloma plasma cell, its interactions with the bone marrow microenvironment, and the subsequently activated signaling pathways leading to survival and proliferation.

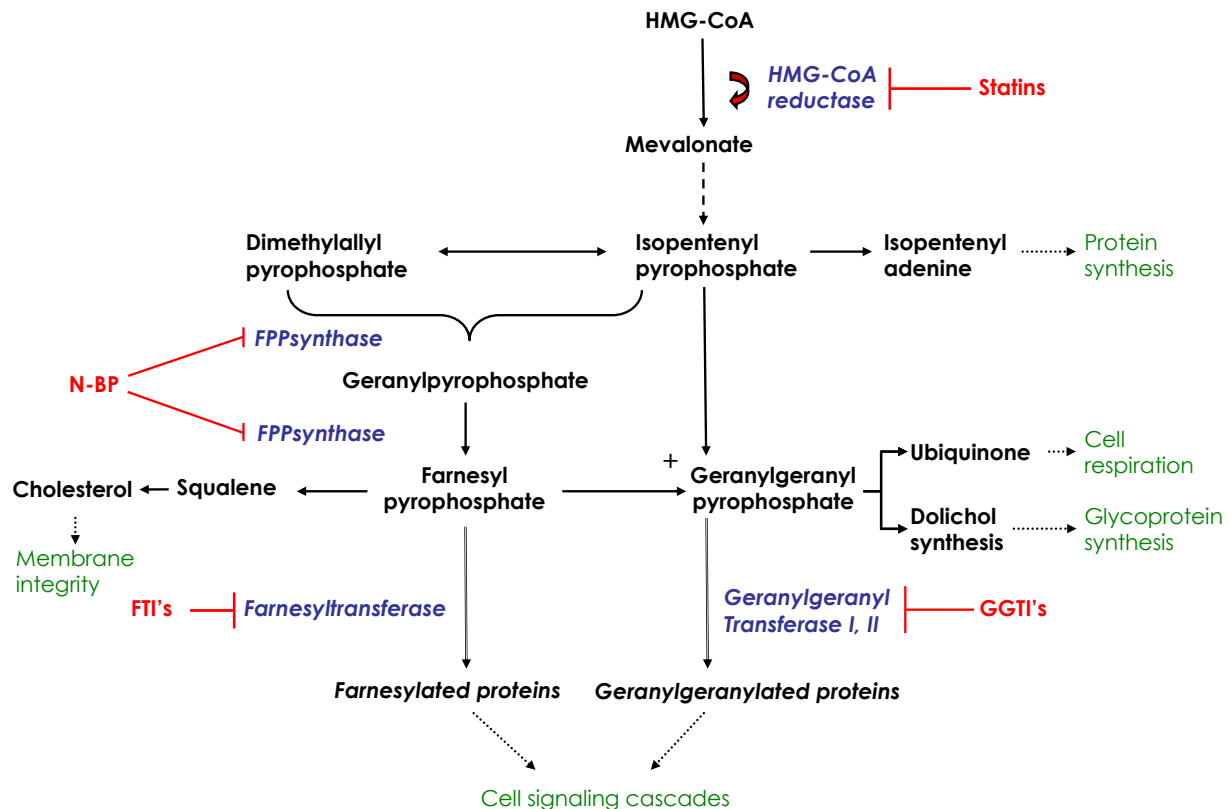


Figure 2. The mevalonate pathway. **Blue** words represent enzymes necessary for translation. **Red** words represent inhibitors of the respective enzymes. **Green** words represent the function of the respective mevalonic acid intermediates.

Novel therapies in myeloma

To improve the clinical outcome in patients with multiple myeloma, there is a continuous search for new therapies. Over the last decade, important changes in the treatment of multiple myeloma patients have occurred. Novel agents like thalidomide, bortezomib, and lenalidomide have changed treatment schedules considerably and have improved the survival of myeloma patients.³¹ These agents either directly interfere with critical signaling pathways in the myeloma cells or, alternatively, affect the microenvironment resulting in the demise of the tumor cells. Despite these new developments, however, multiple myeloma remains incurable and the search for new drugs continues. A new and intriguing possibility in the treatment of myeloma is targeting the mevalonate pathway. Inhibition of 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) reductase, which is the rate-limiting enzyme of the mevalonate pathway, induces apoptosis very effectively *in vitro*, inhibits proliferation of myeloma cells,³² and overcomes CAM-DR.³³ Here, the current knowledge and concepts for the rationale of inhibition of the mevalonate pathway as a treatment modality for multiple myeloma will be discussed.

The mevalonate pathway

Conversion of HMG-CoA into mevalonate by HMG-CoA reductase is the rate limiting step in producing cholesterol and is crucial for membrane and steroid synthesis (figure 2). In addition, mevalonate is the precursor of other non-sterol products, including dolichol required for glycoprotein synthesis, ubiquinone involved in oxidative respiration, and the isoprenoids geranylgeranylpyrophosphate (GGPP) and farnesylpyrophosphate (FPP).³⁴

The isoprenoids GGPP and FPP are essential for prenylation, a lipid posttranslational modification necessary for full biologic activity of small intracellular guanosine triphosphate hydrolases (GTP-ases). GTP-ases, also called G-proteins, serve as molecular switches that cycle between a GTP-bound (active) and a GDP-bound (inactive) state. Examples of G-proteins are ras, rho, rab, rac, and rap, which are involved in proliferation, signal transduction, and apoptosis.³⁵ Prenylation attaches either a farnesylgroup (farnesylation) or a geranylgeranylgroup (geranylgeranylation) to the G-protein. These isoprenoid groups anchor the proteins to the cell membrane at the correct intracellular location. This process is catalyzed by farnesyltransferase (FT-ase) or geranylgeranyltransferase I (GGT-ase-I). Subsequently, the prenylated proteins play a key role in signal transduction, necessary for transcription of genes involved in proliferation and differentiation.

Whether a protein is farnesylated or geranylgeranylated depends on the x in the CAAX-motif in the carboxy-terminal of the protein (where c is a cysteine, A is an aliphatic amino acid and x is any amino acid). If x is serine, methionine, or glutamine, this protein will be preferentially farnesylated, whereas if x is leucine it will be recognized by GGT-ase-I and subsequently geranylgeranylated.³⁶ Inhibition of the mevalonate pathway depletes intracellular pools of FPP and GGPP. This prevents prenylation of G-proteins and interferes with their biologic activity.

Inhibitors of the mevalonate pathway

The mevalonate pathway can be targeted by statins, nitrogen containing bisphosphonates (N-BP), and by direct inhibition of farnesylation and/or geranylgeranylation with farnesyltransferase inhibitors (FTI) or geranylgeranyltransferase inhibitors (GGTI) (figure 2).

Statins

Statins are active competitive inhibitors of the enzyme HMG-CoA reductase, necessary for the production of mevalonate. Interference with the activity of this enzyme reduces the quantity of mevalonate and in this way the production of downstream metabolites. There are eight HMG-CoA reductase inhibitors currently known, three of which are naturally derived from fungal fermentation (lovastatin, simvastatin, and pravastatin). The other five

are synthetically derived (fluvastatin, atorvastatin, cerivastatin, rosuvastatin, and pitavastatin).³⁷ Statins are well known as treatment for hypercholesterolemia and widely used as both primary and secondary prevention of cardiovascular disease.³⁸ Moreover, multiple 'pleiotropic effects' are attributed to statins,³⁹ including improvement of endothelial function, antithrombotic actions, atherosclerotic plaque stabilization, and reduction of the vascular inflammatory process.⁴⁰ Furthermore, use of statins is claimed to be an effective treatment and preventive strategy of osteoporosis,⁴¹ dementia,⁴²⁻⁴⁴ and multiple sclerosis.⁴⁵ Finally, several studies suggest that inhibition of isoprenoid production by statins has an anti-cancer effect. This was substantiated by *in vitro* experiments showing that statins effectively induce apoptosis and inhibit proliferation of several cancer cell lines, including prostate, and breast cancer⁴⁶ and acute myeloid leukemia.⁴⁷ Although case control studies showed a lower prevalence of lung cancer,⁴⁸ colorectal cancer,⁴⁹ kidney cancer,⁵⁰ and a generally lower cancer incidence⁵¹ among statin users, two large meta-analyses did not show a reduction in the overall cancer risk or cancer death of statin use in dosages used to treat hypercholesterolemia.^{52,53}

Bisphosphonates

Bisphosphonates are the treatment of choice for diseases characterized by excessive bone resorption, such as osteoporosis and tumor-associated osteolysis. N-BP inhibit FPP synthase, an enzyme that catalyzes the conversion of dimethylallyl pyrophosphate to geranylpyrophosphate and subsequently to FPP (figure 1). Inhibition of FPP synthase by N-BP leads to inhibition of both farnesylation and geranylgeranylation.⁵⁴ *In vitro*, zoledronic acid induces apoptosis in breast cancer cells and prostate cancer cell lines. Antitumor effects *in vitro* are enhanced by addition of anthracyclines and taxanes.^{55,56}

Farnesyltransferase inhibitors

Farnesyltransferase inhibitors (FTI) inhibit the enzyme farnesyltransferase, which catalyzes the covalent transfer of a lipid farnesyl group from FPP to a cystein residue of the CAAX-motif. Farnesyltransferase inhibitors were developed to inhibit ras proteins.⁵⁷ Ras proto-oncogenes (H-ras, N-ras, and K-ras) play an important role in signal transduction, proliferation, differentiation, and malignant transformation through subsequent activation of downstream signal transduction pathways, like MAPK and PI-3K/Akt pathway.⁵⁸ Many types of malignancies are associated with active ras mutations, including many hematologic malignancies, resulting in constitutive activation of ras and its downstream effectors.⁵⁹ Prenylation of ras is required for (oncogenic) activity. Ras proteins are preferentially farnesylated.⁵⁸ FTI inhibits ras activity, possibly resulting in tumor kill.⁶⁰

Geranylgeranyltransferase inhibitors

Geranylgeranyltransferase 1 catalyzes geranylgeranylation. Geranylgeranyltransferase inhibitors (GGTI) specifically inhibit GGT-ase-1. In this way, the activity of G-proteins requiring geranylgeranylation for their function will be inhibited. Approximately, 0.5 to 1% of cellular proteins need geranylgeranylation.⁶¹ Candidates are rhoA, rac, and CDC42. At this moment, there is no clinical availability of GGTI.

Effect of inhibitors of the mevalonate pathway on multiple myeloma

Statins and multiple myeloma

Several reports have been published in the last decade showing *in vitro* activity of statins against myeloma tumor cells.^{32,62-65} Statins induce apoptosis and inhibit cell proliferation in myeloma cells, overcome the protective effects of IL-6, IGF-I, and fibronectin on myeloma cell survival,⁶⁴ and inhibit cell adhesion mediated-drug resistance (CAM-DR).³³

Statins effectively inhibit proliferation and induce apoptosis in myeloma cell lines and myeloma plasma cells in a dose- and time-dependent fashion. Statin-induced apoptosis is mediated by caspase-9 cleavage, causing mitochondrial damage and the activation of caspase-3 as the downstream effector caspase.^{63,65} The effect of statins is abrogated by addition of mevalonate, indicating that downstream metabolites of mevalonate are vital for myeloma cell survival and proliferation.^{32,62} Although the exact mechanism of cell kill is not clear, geranylgeranylated proteins appear critically involved in these processes.^{63,66,67} Co-administration of GGOH, which is converted into geranylgeranylpyrophosphate,⁶⁸ largely abrogates induction of apoptosis, inhibition of proliferation, and CAM-DR induced by statins. This implies an important role for geranylgeranylated proteins in myeloma cell survival, including CAM-DR and proliferation. Geranylgeranylated GTP-ases like rac1, rhoA, rhoB, rhoC, and CDC42 may be targeted by statin treatment. Statin-induced interference with CAM-DR seems to be mediated by inhibition of rho kinase activity,³³ possibly through inhibition of the LFA-1-ICAM interaction.⁶⁹

The signaling pathways involved in simvastatin-induced myeloma cell death are still unknown. One report showed inactivation of various MAP-kinase pathways, like ERK1/2, MEK1/2, JNK, and p38 in myeloma cell lines after treatment with simvastatin.⁷⁰ In acute myeloid leukemia cells, lovastatin-induced apoptosis by inhibition of ERK.⁶¹ Importantly, statins seem to induce apoptosis and inhibit proliferation to a greater degree in malignant than in non-malignant cells,⁷¹ possibly due to increased expression of HMG-CoA reductase and greater requirement for isoprenoids in tumor cells as opposed to normal cells.⁷²

Statins also synergize with doxorubicin, dexamethasone, zoledronate, FTI, and 7-hydroxystaurosporine (UCN-1) in reducing cell viability in myeloma cells.^{32,73-76} Since

statins are so effective in inducing apoptosis and inhibiting proliferation in myeloma cells, the question arises whether statins show equal *in vivo* activity. Only a small number of studies has been published discussing the role of statins in myeloma as prevention or treatment strategy.

A case-control study in Connecticut women showed an overall reduced risk (odds ratio 0.4; 95% confidence interval, 0.2-0.8) for developing multiple myeloma in statin users.⁷⁷ This suggests that a regular dose of statins might have a preventive effect for the development of myeloma. A small German pilot study showed a positive effect in overcoming drug resistance of adding simvastatin 80 mg to bendamustine or bortezomib to refractory myeloma patients.⁷⁸ A subsequent study is necessary to confirm these interesting results. Another small observational study examined the effect of statin use during autologous stem cell transplantation as treatment for multiple myeloma. The results show a better response rate, but no benefit in progression free or overall survival.⁷⁹ Recently, Sondergaard et al. described the effects of high-dose simvastatin on markers of bone turnover in myeloma patients. Six patients were treated with 15 mg/kg simvastatin for seven days. After treatment, one of five markers of osteoclast activity increased, while other markers remained stable, suggesting a possibly harmful effect on bone turnover for myeloma patients. No response was recorded.⁸⁰

Bisphosphonates and multiple myeloma

N-BP reduce cell viability mainly by inhibition of cell proliferation in several myeloma cell lines.^{81,82} Other studies report induction of apoptosis in myeloma cells after treatment with bisphosphonates.⁸³⁻⁸⁵ Previously, pamidronate was reported to decrease osteolytic bone lesions in multiple myeloma patients, when added to conventional chemotherapy.⁸² However, maintenance therapy with pamidronate in multiple myeloma patients did not reduce skeletal events. Moreover, bisphosphonate therapy failed to improve overall survival.⁸⁶

Farnesyltransferase inhibitors and multiple myeloma

Disease progression of myeloma patients is correlated with an increase in ras mutations. Farnesyltransferase inhibitors inhibit ras activity and can theoretically be effective as treatment for multiple myeloma.^{87,88} *In vitro* results were promising, showing inhibition of cell growth and induction of apoptosis in myeloma cells.⁸⁹ However, cell lines with activated N-Ras were more sensitive for FTI, than cell lines with activated K-Ras or wildtype ras. Compared to GGTI, FTI-induced apoptosis less effectively in myeloma cell lines.⁶³ Furthermore, FTI did not inhibit CAM-DR.³³ Clinical use of FTI only resulted in disease stabilization, but no response was achieved.⁹⁰ A possible explanation for this

disappointing result is evidence that inhibition of FT-ase results in alternative geranylgeranylation of K-Ras, and possibly N-Ras.⁹¹

Geranylgeranyltransferase inhibitors and multiple myeloma

Few studies describe the effects of GGTI on myeloma cells. Treatment of myeloma cells with GGTI-induced apoptosis and inhibited proliferation in myeloma cell lines and primary myeloma cells.^{63,84,92} Treatment with GGTI resulted in similar inhibition of CAM-DR compared to inhibition after statin treatment.³³ These results confirm the importance of geranylgeranylated proteins in myeloma cell survival. Furthermore, combinations of FTI with GGTI resulted in synergistic inhibition of proliferation and induction of apoptosis.^{75,89}

Targeting the mevalonate pathway in multiple myeloma

Inhibition of the mevalonate pathway is an interesting option as new treatment strategy in multiple myeloma. Of all inhibitors of the mevalonate pathway, statins are most effective *in vitro*, directly available and oral drugs. After years of clinical experience, statins show a very good safety profile, and are reasonably priced. However, to reach plasma levels comparable to those that are effective in *in vitro* experiments, high-dosage statins are necessary. To define its clinical role in myeloma, primarily, evaluation of feasibility of high-dose statins is needed to define the maximum tolerated dose of high-dose statins and the dose-limiting toxicity. Subsequently, in a phase II trial, efficacy should be tested. In addition, targets of statins in myeloma cells need to be identified in order to better understand the mechanism of action of statins in myeloma and to develop new targeted therapies. An understanding of cellular and molecular mechanisms regulating myeloma cell growth and survival will derive novel therapies to specifically inhibit tumor cell growth and/or trigger apoptosis and simultaneously target the bone marrow microenvironment. Finally, the development of a myeloma mouse model will create the possibility for *in vivo* monitoring of efficacy of new antimyeloma therapies, like statins.

Outline of this thesis

This thesis investigates the possible role of statins as treatment for multiple myeloma.

Chapter describes the feasibility of high-dose statins combined with chemotherapy in a phase I study in extensively pre-treated myeloma and non-Hodgkin's lymphoma patients.

Chapter discusses the results of a phase II trial, showing response rates of high-dose statins combined with VAD chemotherapy in multiple myeloma patients. **Chapter**

investigates the interaction between simvastatin and lenalidomide in inducing cell kill in myeloma plasma cells. Subsequently, **chapter** describes the results of a microarray experiment performed on a myeloma plasma cell line treated with simvastatin to further

elucidate targets of simvastatin in multiple myeloma. In **chapter** , we present a mouse model for multiple myeloma making use of bioluminescence to assess tumor localization and growth, which can be used for evaluation of *in vivo* novel therapies. Finally, **chapter** summarizes the main findings of the thesis and discusses the implications for patient care and future research.

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Dose-finding study of high-dose simvastatin combined with standard chemotherapy in patients with relapsed or refractory myeloma and lymphoma

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chaptertwo

Abstract

In vitro, statins effectively induce apoptosis in myeloma and lymphoma cells in a dose- and time-dependent way. In combination with dexamethasone and doxorubicin, statins have a chemo-sensitizing effect.

Twenty-eight relapsed myeloma and lymphoma patients were treated with a dose-escalating regimen of simvastatin for seven days followed by vincristine, adriamycin, dexamethasone (VAD) in myeloma patients and cyclophosphamide, doxorubicin, vincristine, and prednisolone (CHOP) in lymphoma patients.

The maximum tolerated dose was 15 mg/kg/day simvastatin. The most reported side effects were fatigue, gastrointestinal (both grade 1-2), and neutropenic fever. The dose-limiting toxicity was neutropenic sepsis and grade 3 gastrointestinal side effects. High-dose simvastatin sequentially given with chemotherapy is safe and tolerable up to 15 mg/kg/day.

Introduction

Inhibitors of HMG-CoA reductase, such as lovastatin and simvastatin, are widely used for the treatment of hypercholesterolemia.¹ HMG-CoA reductase is the rate-limiting enzyme of the mevalonate pathway and catalyses the reduction of HMG-CoA to mevalonate, which is an essential product for the synthesis of various compounds, including cholesterol and isoprenoids like farnesylpyrophosphate and geranylgeranylpyrophosphate.² Isoprenoids are essential for prenylation of proteins, like ras and rho, which are important in myeloma and lymphoma.³ Prenylation is necessary for membrane localization and participation of these proteins in various signaling pathways.⁴

In vitro, HMG-CoA reductase inhibitors induce apoptosis^{5,6} and inhibit proliferation in myeloma and lymphoma cells in a dose- and time-dependent way.^{7,8} Statins sensitize myeloma and lymphoma tumor cells to various chemotherapeutic agents^{5,8,9} and succeed in overcoming cell adhesion-mediated drug resistance in myeloma cells, probably due to the inhibition of geranylgeranylation of rho.¹⁰ Incubation of myeloma tumor cells with lovastatin resulted in the downregulation of Mcl-1, an important anti-apoptotic protein in myeloma.^{7,11} Phase I and II studies on high-dose statins as single agent for anticancer therapy have been performed on solid tumors¹² and separately on astrocytoma¹³ and gastric cancer.¹⁴ Lovastatin could be given safely at a dose up to 25 mg/kg/day.

The objectives of this study were to determine the maximum tolerated dose (MTD) of simvastatin, which is twice as potent as lovastatin, administered prior to standard chemotherapy for myeloma and lymphoma and to establish whether an *in vivo* blockade of the mevalonate pathway was achieved by the MTD.

Patients and methods

Patient eligibility

Patients under the age of 75 years old with multiple myeloma or non-Hodgkin's lymphoma who had been treated with at least two lines of chemotherapy, including treatment with anthracyclines and steroids, and World Health Organization (WHO) performance 0-2 were eligible for enrolment. Adequate hepatic and renal function (creatinine clearance of ≥ 40 ml/min) was required. The study was conducted according to the Declaration of Helsinki and was approved by the University Medical Center Utrecht institutional review board (01/051-E). All patients gave written informed consent.

Study design and treatment schedule

The study was an open phase I dose-escalation study. The initial dosage was 5 mg simvastatin/kg per day orally for seven consecutive days, divided in two doses. The last

dose of simvastatin was given on the morning of day 7, before start of chemotherapy. Patients with myeloma received a shortened, full dose of vincristine 1.6 mg/m², adriamycin 36 mg/m², over two days on days 7 and 8 and dexamethasone 40 mg on days 7-10 (VAD). The lymphoma patients were treated with CHOP (cyclophosphamide 750 mg/m², vincristine 2 mg, adriamycin 50 mg/m² on day 7, and prednisone 100 mg on day 7-11). Patients who responded or whose disease was stable received a maximum of three cycles, each lasting 28 days.

The dose of simvastatin was escalated by 2.5 mg/kg, unless grade 3 or 4 non-hematologic toxicity was observed, excluding neutropenic fever grade 3. Three patients were studied at each dose level. If one out of three patients developed dose-limiting toxicity (DLT), three more patients were enrolled at the same dose level. If two patients at one dose level developed DLT, the subsequent cohort was enrolled at the previous dose level. If no grade 3 or 4 toxicity was detected, this level was considered MTD. Once the maximum tolerated dose (MTD) had been determined, additional patients were included at this dose level to confirm feasibility and to determine the *in vivo* effects of simvastatin on the mevalonate pathway and the anti-apoptotic protein Mcl-1.

Pretreatment and follow-up studies

Patients underwent standard pretreatment evaluation. Cardiac function was evaluated by MUGA scan. Blood chemistry tests and peripheral blood counts were repeated on days 7 and 21 to check for toxicity, especially rhabdomyolysis. In five patients, bone marrow aspiration was repeated on day 7 to evaluate the *in vivo* effects of simvastatin. In six patients cholesterol levels were measured before and seven days after treatment.

Evaluation of adverse effects

Adverse events were evaluated according to Common Toxicity Criteria Version 3.0.

Assessment and definitions of response

Responses were evaluated using the EBMT myeloma response criteria and according to the recommendations of the Non-Hodgkin's Lymphoma International Working Group.^{15,16}

Laboratory studies

Plasma cells were purified by magnetic cell sorting (MACS) on the basis of CD138 expression.⁵ Mononuclear cells were analyzed by flow cytometry as described previously.¹⁷ Western blots were performed as described elsewhere.⁵

Statistical analysis

The two-tailed paired Student's t-test and the Mann-Whitney U test were used for statistical analysis. P-values <0.05 were considered statistically significant.

Table 1. Patient characteristics (n=28)

	Number (%) [*]
Sex	
female	12 (43)
male	16 (57)
Age (years)	
median	56
range	27-72
Disease	
multiple myeloma	19 (68)
non-Hodgkin's lymphoma	9 (32)
Previous lines of chemotherapy	
median	4
range	2-7
Previous treatment multiple myeloma	
anthracyclines	19 (100)
thalidomide	18 (95)
bortezomib	4 (21)
stem cell transplantation	13 (68)
Previous treatment non-Hodgkin's lymphoma	
anthracyclines	9 (100)
autologous stem cell transplantation	5 (56)
Refractory or relapse patients	
relapse	15 (54)
refractory	13 (46)
Hematologic values at start	
leucocytes less than $3.0 \times 10^9/l$	3 (11)
hemoglobin at least grade 2	6 (21)
platelet count less than $75 \times 10^9/l$	4 (14)

* values in this column are number of patients and values in parentheses are percentages, except when indicated otherwise in the row headings.

Results

Patients

Twenty-eight patients were enrolled in the study between August 2002 and February 2005. The baseline characteristics of these patients are shown in table 1. All patients had been heavily pretreated and had received a median of four previous chemotherapy regimens (range 2-7). Each patient had previously received anthracyclines and steroids. All patients could be assessed for toxicity.

Table 2A. Non-hematologic toxicity

No. of patients	Simvastatin dose level (mg/kg)													
	5		7.5		10		12.5		15		17.5		total (28)	
	3	3	3	3	3	3	3	3	13	3	3	3		
Grade	1-2	≥3	1-2	≥3	1-2	≥3	1-2	≥3	1-2	≥3	1-2	≥3	1-2	≥3
Fatigue	1	-	3	-	1	-	1	-	7	-	3	-	16	-
Nausea	-	-	-	-	2	-	1	-	6	-	2	1	10	1
Diarrhea	-	-	2	-	2	-	-	-	3	1	1	1	8	2
Anorexia	-	-	1	-	2	-	-	-	5	-	1	-	9	-
Fever	1	-	-	-	-	-	1	-	-	-	1	-	3	-
Febrile neutropenia	na	-	na	2	na	1	na	-	na	2	na	1	na	6
Renal	-	-	-	-	-	-	-	-	-	1	-	-	-	1
Mucositis	-	-	-	-	-	-	-	-	1	-	-	-	1	-
Myalgia	-	-	-	-	-	-	1	-	1	-	-	-	2	-
Depression	-	-	-	-	-	-	-	-	2	1	-	-	2	1
Muscle weakness	-	-	-	-	-	-	1	-	1	-	-	-	2	-

na: not applicable

Dose escalation

The initial dosage was 5 mg/kg followed by chemotherapy. At the dose level of 17.5 mg/kg two patients experienced DLT. We subsequently treated ten more patients at dose level 15 mg/kg to confirm DLT and MTD.

Toxicity

Non-hematologic toxic effects are shown in table 2A. The most reported side effect was fatigue (57%), but this did not exceed grade 2 toxicity. Treatment with a dosage of 12.5 mg/kg and under mainly resulted in gastrointestinal complaints and fatigue. Three patients developed neutropenic fever, necessitating hospitalization in two cases.

At the dose level 15 mg/kg one patient with a history of depression developed severe depression and attempted suicide. One patient who received 17.5 mg/kg simvastatin died two days after chemotherapy due to overwhelming neutropenic sepsis. Another patient receiving 17.5 mg/kg developed grade 2 diarrhea and stopped taking simvastatin at day 4, the other patient had grade 3 nausea and grade 3 diarrhea and simvastatin stopped on day 5. According to the dose-finding protocol, ten patients were then treated with 15 mg/kg to confirm the MTD. One more patient developed neutropenic fever and had to be admitted to hospital. Another patient was hospitalized after four days of simvastatin therapy with neutropenic fever and severe dehydration. This patient already had diarrhea before start of therapy, which severely worsened with simvastatin. She developed septic shock, complicated by acute tubular necrosis. She died three months later as a consequence of the

Table 2B. Hematologic toxicity

Cycles	Simvastatin dose level, mg/kg													
	5		7.5		10		12.5		15		17.5		total (52)	
	9	7	7	7	8	8	5	5	22	22	1	1		
Grade	1-2	≥3	1-2	≥3	1-2	≥3	0-2	≥3	0-2	≥3	0-2	≥3	0-2	≥3
Anemia	7	-	6	-	4	-	2	-	16	1	1	-	36	1
Thrombocytopenia	5	-	3	3	2	1	1	1	7	4	-	-	18	9
Leucopenia	3	2	-	4	1	2	3	-	12	7	-	1	19	15
Neutropenia	3	-	-	4	1	2	1	1	1	12	-	1	6	20

progression of multiple myeloma. The other patients at dose level 15 mg/kg mainly experienced fatigue and gastrointestinal problems.

Liver enzymes did not increase in any of the patients, except in the woman with the septic shock. No rhabdomyolysis occurred. There was no clear cumulative toxicity: the first cycle was a good predictor of how the next cycles would be experienced.

Hematologic toxicity is shown in table 2B. Neutropenia grade ≥ 3 occurred in 38% of cycles. The nadir white cell count occurred a mean of 11.5 days after chemotherapy. Altogether six out of 28 patients developed neutropenic fever. Other grade ≥ 3 hematologic toxic effects occurred less frequently (leucopenia 29%, thrombocytopenia 17%, and anemia 2% in all cycles at all dose levels).

Efficacy

Twenty-three patients were evaluated after completing at least one cycle. The total response rate was 30%. One patient with a diffuse large B cell lymphoma treated with 5 mg/kg achieved a complete response, which is still ongoing following subsequent non-myeloablative allogeneic stem-cell transplantation. Three patients (two with multiple myeloma and one with non-Hodgkin's Lymphoma) had a partial response. Three patients had a minimal response. Five had stable disease. The mean time to progression was two months. There was no correlation between dose level and response rate ($p=0.52$).

The effect of high-dose simvastatin on cholesterol levels

As a surrogate pharmacodynamic end-point of *in vivo* simvastatin activity, cholesterol levels were measured in six patients who received simvastatin 15 mg/kg. The mean cholesterol level at the start of treatment was 4.7 mmol/l (range 2.7-6.3). After seven days of simvastatin therapy, the mean cholesterol level was 2.5 mmol/l (range 1.3-3.4; $p=0.003$). Cholesterol levels normalized in the 21 days after simvastatin therapy had been stopped. The same decrease in cholesterol levels was seen after the second and third cycles of therapy.

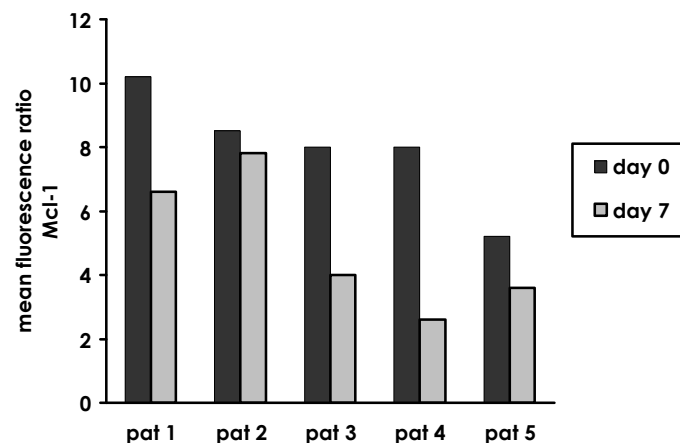


Figure 1. Mean fluorescence ratio of Mcl-1 measured in CD38+/138+ cells before and after therapy using 15 mg/kg simvastatin for seven consecutive days

In vivo mcl- downregulation in myeloma plasma cells

The effect of *in vivo* simvastatin treatment on mcl-1 protein expression in myeloma plasma cells was analysed using flow-cytometry of bone marrow nuclear cells collected at the start and after seven days of simvastatin, before the administration of chemotherapy. In all patients investigated by flow cytometry for this parameter (n=5), simvastatin induced a significant downregulation of mcl-1 in bone marrow myeloma plasma cells ($p=0.021$) (figure 1). This downregulation was confirmed by western blot analysis in the purified plasma cells of two patients. There was no significant effect on mcl-1 expression in monocytes, T cells, and B cells as determined by flow cytometry (data not shown).

Inhibition of prenylation in vivo

Western blot analysis demonstrated inhibition of prenylation in two patients. In one patient partial inhibition of farnesylation of DNA-J was seen in purified plasma cells and in the second patient partial inhibition of geranylgeranylation of rap-1A was observed in bone marrow mononuclear cells (data not shown).

Discussion

In vitro data show that inhibition of the mevalonate pathway by HMG-CoA inhibitors results in apoptosis and the sensitization of tumor cells to chemotherapeutic agents by inhibition of prenylation, leading to downregulation of anti-apoptotic proteins.⁴ This prompted us to evaluate the safety and tolerability of statins followed by conventional chemotherapy in end-stage myeloma and lymphoma. The rationale for performing a dose-escalating study was the dose- and time-dependent effects of statins demonstrated *in vitro*. The rationale for the sequential administration of chemotherapy was based on

previous preclinical and phase I studies. Preclinical studies had shown that the chemosensitization of myeloma plasma cells by statins requires several days of pre-incubation.^{5,8} A phase I study showed that the expected maximum peak levels of serum simvastatin could synergize effectively with doxorubicin and dexamethasone but were not sufficient to induce spontaneous apoptosis in myeloma and lymphoma tumor cells.¹² No dexamethasone pulse was given in between since simvastatin has a short half-life, such that the effect of simvastatin on anti-apoptotic proteins is transitory.

We determined that the MTD of simvastatin was 15 mg/kg/day for seven days in association with systemic chemotherapy. The most frequently reported non-hematologic side effects were fatigue, nausea, and diarrhea. The dose limiting toxicities were gastrointestinal complaints and neutropenic sepsis. Although the high incidence of neutropenia (38% grade ≥ 3) in this heavily pretreated group of patients may not be unusual after chemotherapy, it cannot be excluded that simvastatin followed immediately by chemotherapy enhances bone marrow suppression. The last cohort of patients received prophylactic antibiotics. We did not find any correlation between cholesterol levels and the development of toxicity.

A significant downregulation of Mcl-1 in myeloma tumor cells was seen. In addition, inhibition of farnesylation and geranylgeranylation was observed.

Seven out of the 23 patients (30%) who could be evaluated responded. It cannot be concluded from this study, however, that simvastatin pre-treatment contributed to the response and disease stabilization. Patients previously treated with anthracyclines and prednisone/dexamethasone may respond again to regimens containing these agents. It is nevertheless worth noting that three out of the seven patients who responded were refractory to CHOP or VAD.

We conclude that simvastatin can be administered safely with acceptable toxicity at a dose of 15 mg/kg for seven days followed by standard chemotherapy and that at this dose level *in vivo* downregulation of Mcl-1 and inhibition of prenylation is achieved.

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High-dose simvastatin does not reverse resistance to vincristine, adriamycin, and dexamethasone (VAD) in myeloma

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chapterthree

Abstract

In a prospective phase II study, we evaluated the combination of high-dose simvastatin and VAD chemotherapy in patients with refractory or relapsed multiple myeloma. Although treatment was feasible with mild side effects, we found that after treatment of 12 patients, only one patient achieved a partial response. According to our predefined criteria, this was insufficient to continue the study.

Introduction

Simvastatin is a HMG-CoA reductase inhibitor widely used for the treatment of hypercholesterolemia. *In vitro*, statins are cytotoxic against myeloma cells by inducing apoptosis and inhibition of proliferation. Synergistic activity of simvastatin with doxorubicin and dexamethasone was shown *in vitro*.¹ We recently performed a phase I study to determine the maximum tolerated dose (MTD) of high-dose simvastatin combined with chemotherapy in relapsed or refractory myeloma and lymphoma patients.² The MTD was 15 mg/kg simvastatin for seven days and dose limiting toxicities were gastrointestinal complaints and neutropenic fever. The response rate, in this study defined as minimal response (MR) + partial response (PR) + complete response (CR), in myeloma and lymphoma was 30%.

To further explore its potential anti-myeloma activity, and to confirm the feasibility of the phase I study, we subsequently conducted a phase II study of high-dose simvastatin combined with chemotherapy in patients with relapsed or refractory multiple myeloma.

Material and methods

Patients under the age of 75 years were eligible if they had received at least two lines of chemotherapy, which included anthracyclines and dexamethasone, and had acceptable organ function (total bilirubin and transaminases ≤ 2 times the upper limit of normal, creatinine clearance ≥ 40 ml/min, and no severe cardiac dysfunction).

Simvastatin 15 mg/kg/day was prescribed orally on day 1-7 of a 28 day cycle, administered twice daily, followed by rapid intravenous infusion of vincristine (1.6 mg), adriamycin (36 mg/m²) on day 7 and dexamethasone 40 mg (VAD) orally on day 7 to 10. Response according to EBMT criteria was determined after two cycles. In case of stable disease or at least minimal response, two additional courses were given. In case of progressive disease during therapy, the treatment was discontinued. All patients received prophylactic treatment with co-trimoxazole. The medical ethical board approved the protocol and all patients signed informed consent before start of treatment.

Based on literature,³ we defined that for this category of patients a response rate (defined as CR + PR) below 10% and a non-hematologic toxicity rate WHO grade 3-4 larger than 30% would be unacceptable. The aim of the study was to include 40 patients, with a planned interim analysis after 12 patients. According to predefined stopping rules, at least two patients required a partial or complete response to continue the study.

Table 1. Patient characteristics (n=12)

	N (%) [*]
Sex	
Female	4 (33)
Male	8 (67)
Age (years)	
median	64
range	38-71
Previous lines of chemotherapy	
median	4
range	2-6
Previous treatment	
anthracyclines	12 (100)
thalidomide	12 (100)
bortezomib	10 (83)
stem cell transplantation	9 (75)
Type of myeloma	
IgG	5 (42)
IgA	5 (42)
light chain	2 (17)
International staging system (ISS) stage	
I	5 (42)
II	6 (50)
III	1 (8)

* Values are number of patients with percentage of patients in parentheses, unless indicated otherwise in the row headings. The sum of percentages may be less than 100 because of rounding.

Results

Median age of the 12 patients was 64 years (range 38-71). Previously, they received a median of 4 (range 2-6) anti-myeloma treatments (table 1). Seven out of 12 patients were VAD resistant. Evaluation after 12 patients, who received a median of three cycles (range 1-4), showed a PR in one patient, stable disease in six patients -five of whom had progressive disease before starting treatment- and progressive disease in five patients. In the group with stable disease, two patients experienced a reduction of pain on simvastatin alone. One patient had plasma cell leukemia, which was stable during treatment. After the end of the study, he had a rapid deterioration and died 45 days afterwards. Patients in stable disease remained stable during a median of 128 days (range 94-258 days). The patient with the partial response relapsed after 230 days from start of treatment. According to our predefined criteria, one partial response was insufficient to continue the study.

Table 2A. Hematologic toxicity

	Grade			
	1	2	3	4
Anemia	4	7	-	-
Thrombocytopenia	4	2	1	1
Leucopenia	1	6	5	-
Neutropenia	-	1	4	4

Values in the table denote the number of patients affected.

Table 2B. Non-hematologic toxicity

	Grade			
	1	2	3	4
Fatigue	5	-	-	-
Nausea	2	-	1	-
Anorexia	3	-	-	-
Febrile neutropenia	na	na	-	-
Muscle weakness	-	1	-	-

Values in the table denote the number of patients affected; na: not applicable

The main adverse event was hematologic toxicity: WHO grade 3-4 neutropenia occurred in eight patients and grade 3-4 thrombocytopenia in two patients (table 2A). This effect was more prominent in the second course. No neutropenic fever occurred. One patient experienced grade 3 gastro-intestinal toxicity with dehydration due to nausea and vomiting after his first cycle of chemotherapy. The subsequent three cycles were without side effects. No other \geq grade 3 toxicity was observed, especially no rhabdomyolysis (table 2B).

Discussion

To our knowledge, this is the first clinical study with high-dose statins in myeloma. Although statins are very effective *in vitro*, high-dose simvastatin *in vivo* did not reverse clinical resistance to VAD chemotherapy. An explanation for this may be that although dose levels of statins that are effective *in vitro* can be reached *in vivo*,⁴ these levels are minimally required and should probably be maintained for a prolonged period. This may not be the case *in vivo* in patients, due to the short half-life of simvastatin. Higher bioavailability may be obtained by simultaneous, rather than subsequent treatment with anti-myeloma agents. It may also be that in our heavily pretreated patients - almost all were pretreated with novel agents bortezomib and thalidomide - multiple anti-apoptotic pathways were active that are not influenced by statins. In conclusion, our design of high-dose statins followed by VAD chemotherapy in refractory myeloma cannot be

recommended for further exploration. Other strategies are required to determine if statins deserve a place in the treatment of myeloma. These strategies may include prolonged and/or simultaneous administration with other myeloma agents in less pre-treated patients.

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Inhibition of the mevalonate pathway potentiates the effects of lenalidomide in myeloma

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chapterfour

Abstract

The effects of the combination of simvastatin and lenalidomide were analyzed in myeloma. Myeloma cell lines and patient myeloma cells were incubated with different concentrations of lenalidomide, simvastatin, or the combination. Co-exposure to simvastatin and lenalidomide resulted in a synergistic reduction of cell viability in myeloma cells. This effect was due to induction of apoptosis and inhibition of proliferation. The combination augmented induction of caspase-8 cleavage and enhanced downregulation of pSTAT3. Mevalonate and GGOH abrogated the synergy between lenalidomide and simvastatin. These data provide a rationale for the clinical evaluation of lenalidomide and simvastatin in patients with myeloma.

Introduction

Important progress has been made in the treatment of multiple myeloma by melphalan-based high-dose therapy and, more recently, by the introduction of novel agents like thalidomide and bortezomib. Nevertheless, myeloma remains an incurable disease.¹ Thalidomide is an effective agent in the treatment of myeloma, but is associated with important side effects. Lenalidomide (CC-5013, Revlimid®) is an analogue of thalidomide that shows higher anti-myeloma activity *in vitro* compared to thalidomide.² Lenalidomide has a response rate of 25% in relapsed and refractory myeloma patients, with manageable side effects.³ In newly diagnosed patients, the combination of lenalidomide and dexamethasone has a response rate of 91%.⁴

Modes of action of lenalidomide include inhibition of anti-angiogenesis,⁵ immunomodulation by inhibiting tumor necrosis factor- α production, stimulation of T cell proliferation,⁶ and augmentation of NK cell activity.⁷ In addition, lenalidomide directly inhibits proliferation of myeloma cells, sensitizes myeloma cell lines to conventional anti-myeloma agents like doxorubicin and dexamethasone,² and acts synergistically with bortezomib⁸ and rapamycin, an mTOR inhibitor,⁹ in reducing myeloma cell viability.

Mevalonate is an intermediate in the production of various compounds such as cholesterol and the isoprenoids geranylgeranylpyrophosphate (GGPP) and farnesylpyrophosphate (FPP). Both GGPP and FPP are essential for geranylgeranylation and farnesylation, respectively. These posttranslational modifications are necessary for membrane localization and activation of small G-proteins, such as Ras and Rho.¹⁰ The rate-limiting step of the mevalonate pathway is the conversion of HMG-CoA to mevalonate. Simvastatin is a HMG-CoA reductase inhibitor, which reduces cardiovascular risk,¹¹ induces apoptosis, and inhibits proliferation in myeloma plasma cells.^{12,13} Co-treatment of myeloma cells with mevalonate and GGPP, but not FPP, abrogates statin-induced apoptosis and inhibition of proliferation. This implies an important role for protein geranylgeranylation in myeloma cell survival and proliferation.¹⁴ Simvastatin overcomes the protective effects of IL-6, IGF-I, and fibronectin on myeloma cell survival,¹⁵ and inhibits cell adhesion mediated drug resistance via inhibition of geranylgeranylation.¹⁶ Furthermore, statins act synergistically with doxorubicin, dexamethasone, and 7-hydroxystaurosporine in reducing cell viability in myeloma cells.^{12,17,18}

The combination of agents that target different pathways is important to avoid drug resistance and to achieve long-term remissions in myeloma. In this report, we show that the combination of lenalidomide and simvastatin results in reduction of cell-viability and induction of apoptosis in a synergistic fashion.

Material and methods

Cell lines

Plasma cell lines RPMI-8226 and U266 were obtained from the American Tissue Culture Collection. L-363 and OPM-2 were obtained from the German Collection of Micro-organisms and Cell Cultures (DSMZ, Braunschweig, Germany). The IL-6 dependent cell line XG-1 was a kind gift from dr. B. Klein (Institute for molecular genetics, Montpellier, France). The cell lines UM-6 and UM-8 were both obtained after prolonged *in vitro* culture of bone marrow aspirates of myeloma patients. UM-6 was described before.¹² Both cell lines are IL-6 dependent. Cell lines were maintained in RPMI-1640 (GIBCO, Breda, the Netherlands) supplemented with 10% fetal calf serum (FCS; Integro, Zaandam, the Netherlands) and antibiotics. In XG-1, 1.25 ng/ml rhIL-6 (Roche, Almere, the Netherlands) was added. Experiments with XG-1, UM-6, and UM-8 were performed in presence of IL-6.

Reagents

Lenalidomide was a kind gift from Celgene (Summit, NJ, USA). It was dissolved in dimethyl sulfoxide (DMSO, Sigma, St. Louis, USA) and stored at -20°C until use. The final concentration of DMSO in culture medium was less than 0.01%. Simvastatin was a kind gift from Merck (Rahway, NJ, USA). It was chemically activated by alkaline hydrolysis prior to use, as described previously.¹⁹ 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.²⁰

Cell viability

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described.¹² In short, cells were seeded in a concentration of 0.2×10^6 /ml in a 96-well flat bottom plate (100 µl/well) (Nunc, Roskilde, Denmark) and treated with lenalidomide, simvastatin, or the combination of lenalidomide with simvastatin in a fixed ratio (lenalidomide/simvastatin 2:1) at day 0. After two and four days, 25 µl of MTT (5 mg/ml) was added to each well. After an incubation period of two 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) and distilled water. After an overnight incubation at 37°C the optical density of the samples was determined at 570 nm.

Cell proliferation

Cell proliferation was measured by tritiated thymidine uptake (³H-TdR). A total of 0.2×10^6 cells/ml were seeded in a 96-flat bottom plate (Nunc) as described previously.¹² After two and four days ³H-TdR (Amersham, Little Chalfont, UK) (1 µCi/well) was added for

the remaining eight hours of the assay. ^3H -Tdr incorporation was analyzed by liquid scintillation counting.

Apoptosis assay

Apoptosis was assessed by using the Annexin v–fluorescein isothiocyanate (FITC) apoptosis detection kit II (BD Biosciences, Heidelberg, Germany) as described previously.¹² Flow cytometric analysis was done with a FACS calibur flow cytometer with the use of CellQuest software (BD Biosciences). Apoptotic cells were defined as Annexin positive and PI negative.

Apoptosis detection in patient myeloma cells

After informed consent, bone marrow was obtained during routine diagnostic procedures. Mononuclear cells were harvested by standard Ficoll/Paque density gradient centrifugation (Amersham). Cells ($1 \times 10^6/\text{ml}$ in 0.5 ml) were incubated with different concentrations of lenalidomide, simvastatin, or the combination during four days. Myeloma cell apoptosis was determined by Annexin v/PI assay. Myeloma cells were identified by CD38 positivity, forward scatter, and side scatter. The percentage of apoptotic cells (AV+/PI-) was determined in at least 5,000 myeloma cells.

Western blot

Western blot analysis was performed as described before.¹² In short, cells ($0.2 \times 10^6/\text{ml}$) were treated with lenalidomide ($12 \mu\text{M}$), simvastatin ($6 \mu\text{M}$), or the combination. After two and four days, 3×10^6 viable cells were directly lysed and diluted in sample buffer, after protein concentrations were determined. Equal amounts of protein were then fractionated in 12% SDS-PAGE and blotted. Equal loading was confirmed by anti-actin expression. The following primary antibodies were used: caspase-8, caspase-9, STAT3, phospho-STAT3 (Tyr705), Akt, phospho-Akt (ser473), p42/44 MAPK, phospho-p42/44 MAPK (Thr202/Tyr204) (Cell Signaling Technology, Beverly, MA, USA). Primary antibodies were detected by horseradish peroxidase (HRP)-conjugated secondary antibodies (Dako, Glostrup, Denmark), followed by detection using enhanced chemiluminescence (Roche).

Statistics

The combination effect analysis of Chou and Talalay was used to identify synergy between lenalidomide and simvastatin.²¹ CalcuSyn (Biosoft, Cambridge, UK) software version 2.0 was used to calculate the combination index (CI). CI values of <1 , $=1$, and >1 indicate synergy, additivity, and antagonism, respectively. CI values at ED₅₀ and ED₉₀ indicate CI at which 50% and 90% reduction of cell viability is achieved, respectively. CI values can only be calculated if both drugs show a dose-response relationship. A Wilcoxon test was used to test the differences between groups. A p-value <0.05 was considered significant.

Results

Simvastatin synergizes with lenalidomide in reducing cell viability

The effect of lenalidomide and simvastatin on cell viability in myeloma plasma cell lines L-363, OPM-2, RPMI-8226, UM-6, UM-8, U266, and XG-1 was determined by using the MTT assay. Incubation with lenalidomide alone showed a time-dependent reduction of cell viability ranging from 0% to 35% in the myeloma cell lines tested. The cell lines OPM-2, UM-6, UM-8, and XG-1, showed a dose-dependent effect of lenalidomide. As reported previously, simvastatin as a single agent reduced cell viability in all myeloma cell lines tested in a time- and dose-dependent way.¹² The combination of lenalidomide and simvastatin showed no synergy in RPMI-8226, U266, and L-363 cells (data not shown). However, in OPM-2, UM-6, UM-8, and XG-1 myeloma cell lines, the cytotoxic effect of simvastatin was clearly potentiated by lenalidomide after two days (data not shown) and even more evidently after four days of *in vitro* culture. Representative examples are given in figure 1A for XG-1 and OPM-2 plasma cell lines. To determine whether the cytotoxic effect of the combination of lenalidomide and simvastatin was synergistic, we generated an isobologram and calculated the CI at four days. CI values of <1 indicate synergy. CI at ED₅₀ and ED₉₀ were 0.42 and 0.73 in the OPM-2 cell line, 0.55 and 0.86 in the UM-6 cell line, 0.72 and 0.38 in the UM-8 cell line, and 0.39 and 0.54 in the XG-1 cell line, respectively. This implies a significant synergistic effect between lenalidomide and simvastatin in the reduction of myeloma cell viability. For the other cell lines L263, RPMI, and U266, CI could not be calculated because there was no dose-response relationship between lenalidomide and reduction of cell viability, which is obligatory for the calculation of CI.

Lenalidomide potentiates the inhibitory effect of simvastatin on proliferation of OPM- and XG- plasma cell lines

Reduction of the number of viable cells is mediated by inhibition of proliferation, induction of apoptosis, or both. To investigate the effect of lenalidomide on simvastatin-induced inhibition of myeloma cell proliferation, plasma cell lines XG-1 and OPM-2 were cultured with different concentrations of lenalidomide, simvastatin, or the combination. ³H-TdR incorporation was measured after two days (data not shown) and after four days. Lenalidomide alone inhibited proliferation of both cell lines by 20-30% in a dose-independent way. As reported earlier, simvastatin reduced myeloma cell proliferation in a time- and dose-dependent fashion.¹² Lenalidomide potentiated the effect of simvastatin in reduction of cell proliferation in both cell lines. This effect was most pronounced at low concentrations of both drugs after four days of treatment (figure 1B).

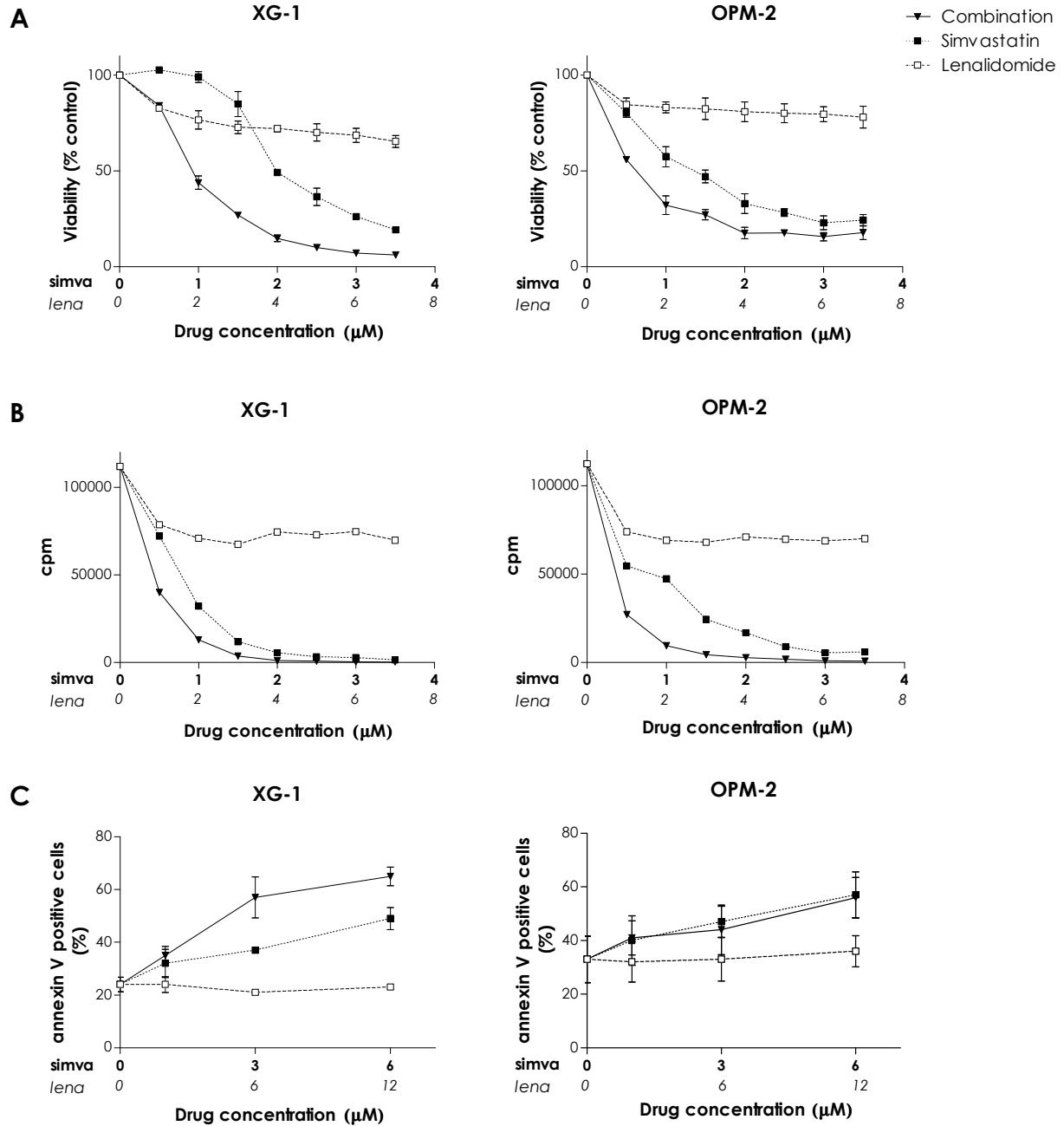


Figure 1. Reduction of the number of viable cells through inhibition of proliferation and, in XG-1, induction of apoptosis by the combination of lenalidomide with simvastatin. **(A)** XG-1 and OPM-2 cells were incubated with solvent control, lenalidomide (0-7 μM), simvastatin (0-3.5 μM), or the combination of lenalidomide and simvastatin in a 2:1 ratio for four days. The percentage of viable cells, relative to solvent control-treated cells, was determined by using MTT assay. Results are representative of at least three experiments performed in triplicate. Data are presented as mean \pm S.E.M. **(B)** XG-1 and OPM-2 were incubated with solvent control, lenalidomide (0-7 μM), simvastatin (0-3.5 μM), or the combination of lenalidomide and simvastatin in a 2:1 ratio for four days. Proliferation was determined by ^3H -thymidine incorporation during the last eight hours of culture. Experiments were performed three times in triplicate. A representative example is shown. **(C)** XG-1 and OPM-2 were incubated with solvent control, lenalidomide (0, 2, 6, and 12 μM), simvastatin (0, 1, 3, and 6 μM), or the combination of lenalidomide and simvastatin in a 2:1 ratio, for two days. Apoptosis was determined by Annexin V assay. Experiments were performed three times. Data are presented as mean \pm S.E.M.

Lenalidomide potentiates simvastatin-induced apoptosis in the XG-1 plasma cell line

Treatment of myeloma cells with simvastatin results in induction of apoptosis via activation of the intrinsic cell-death pathway.²² The XG-1 and OPM-2 cells were treated with simvastatin, lenalidomide, or the combination in a fixed ratio. The percentage of apoptotic cells was assessed using the Annexin V-assay. Lenalidomide alone did not induce apoptosis in OPM-2 and XG-1 cells (figure 1C). Treatment with simvastatin, however, increased the percentage of apoptotic cells compared with solvent control. The combination of lenalidomide and simvastatin resulted in an increase in apoptotic cells in the XG-1 plasma cell line compared to single agent simvastatin. This synergistic effect was not observed in OPM-2 cells.

Lenalidomide enhances induction of apoptosis by simvastatin in bone marrow myeloma plasma cells

Freshly obtained bone marrow mononuclear cells derived from seven myeloma patients containing >5% monoclonal plasma cells were incubated with lenalidomide, simvastatin, or the combination. After an *in vitro* culture period of four days, induction of apoptosis was measured by Annexin V-assay. Lenalidomide had no or only a small effect, whereas simvastatin increased the percentage of apoptotic cells in all patients' samples. Importantly, lenalidomide potentiated simvastatin-induced apoptosis in six out of seven patients. For example, in patient 7, 3 μ M simvastatin increased the percentage of apoptotic cells from 4% to 30%, 6 μ M lenalidomide increased the percentage from 4% to 10% while the combination of simvastatin and lenalidomide resulted in 46% apoptotic cells. No synergy was observed in patient 6. Figure 2 shows the results of all samples tested.

The combination of lenalidomide with simvastatin induces caspase-8 cleavage

Caspase-8 and caspase-9 are well known initiators of apoptosis. Activation of these caspases leads to the subsequent activation of effector caspases-3, -6, and -7, resulting in cell death. We detected the cleavage products of both caspase-8 and -9 after treatment of XG-1 cells with simvastatin (figure 3). Lenalidomide as a single agent had hardly any effect on caspase cleavage. The combination of simvastatin and lenalidomide resulted in enhanced cleavage of caspase-8 on day 2. This effect was even more pronounced on day 4. Caspase-9 cleavage was not increased by the combined incubation with simvastatin and lenalidomide, when compared to simvastatin alone.

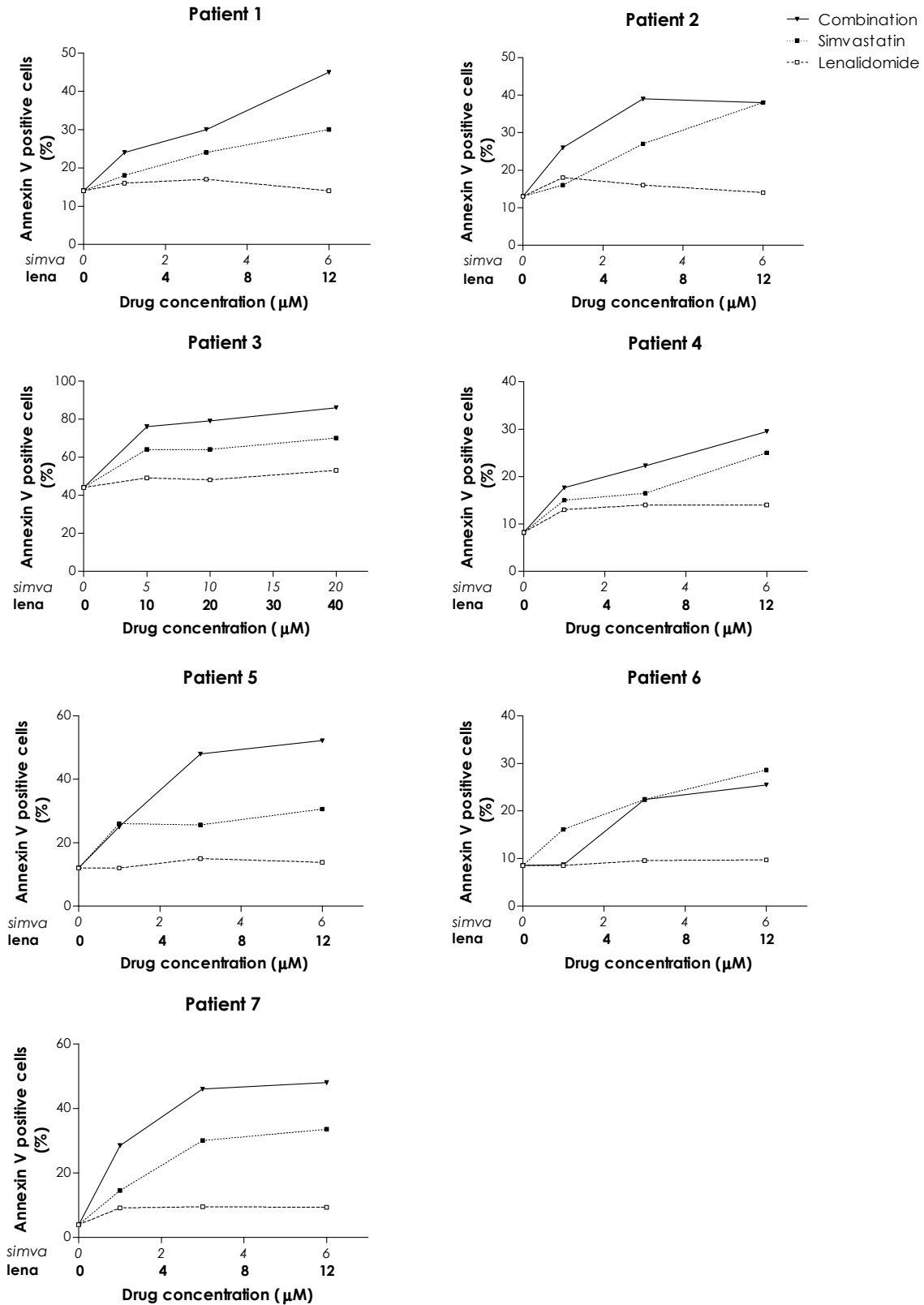


Figure 2. Lenalidomide combined with simvastatin increases apoptosis induction in myeloma plasma cells derived from patients. Bone marrow mononuclear cells of seven myeloma patients with >5% plasma cells were incubated with different concentrations of lenalidomide (0, 2, 6, and 12 μM), simvastatin (0, 1, 3, and 6 μM), or the combination of lenalidomide and simvastatin in a 2:1 ratio for four days. However, for patient 3 other concentrations were used (lenalidomide: 0, 10, 20, and 40 μM; simvastatin: 0, 5, 10, and 20 μM). Apoptosis of plasma cells was determined by Annexin V assay.

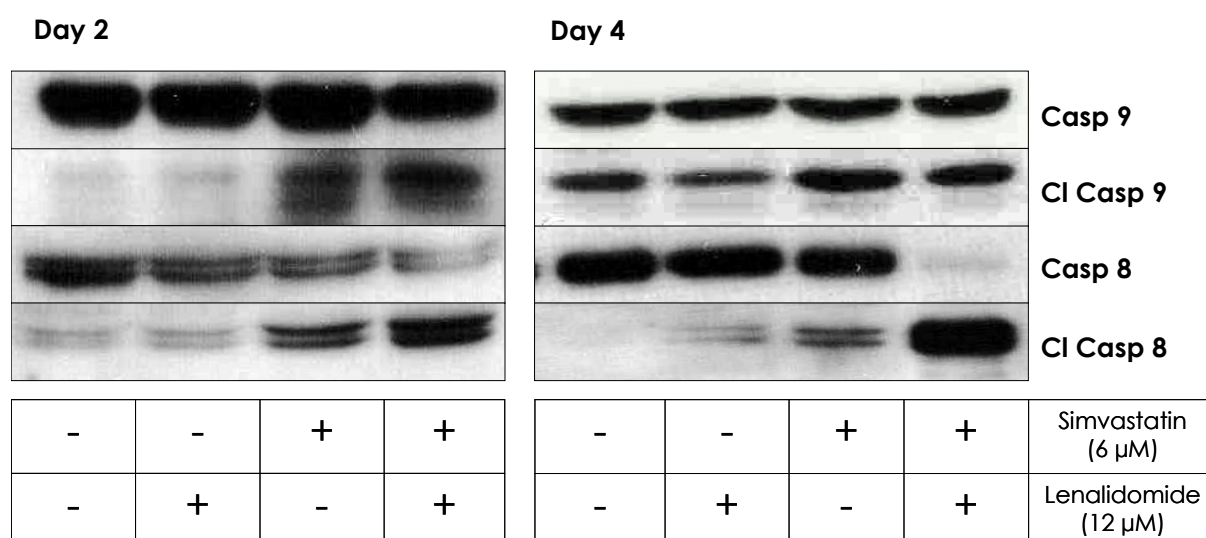


Figure 3. Co-exposure of XG-1 plasma cells to lenalidomide and simvastatin induces caspase-8 cleavage. XG-1 cells were treated for two and four days with solvent control, lenalidomide 12 μ M, simvastatin 6 μ M, or the combination of lenalidomide and simvastatin. After protein isolation, caspase-8 and -9 were determined by western blot analysis. The data shown are representative of at least three independent experiments.

Inactivation of the STAT pathway by lenalidomide in combination with simvastatin

Several signaling pathways, like Jak/STAT₃,²³ PI-3K/Akt,²⁴ and Ras/MAPK,²⁵ play an important role in myeloma cell survival and proliferation. We investigated the effect of simvastatin and lenalidomide on different signaling cascades in XG-1 cells to explain the observed synergy in the induction of apoptosis by the combination of both reagents. The PI-3K/Akt signaling pathway was inactive in XG-1 cells as determined by the analysis of phosphorylated Akt (data not shown). Phosphorylated p42/44 MAPK and STAT₃ were present in XG-1 cells, suggesting that both the MAPK and the Jak/STAT₃ pathways were constitutively active in XG-1 cells. Simvastatin, lenalidomide, and the combination of both agents did not affect the phosphorylation status of p42/44 MAPK (figure 4). However, the combination resulted in a marked inhibition of phosphorylation of STAT₃ in a time- and dose-dependent fashion, while both simvastatin alone and lenalidomide alone had a less pronounced effect on phosphorylation of STAT₃ (figure 4A and 4B).

Inhibition of prenylation abrogates the synergistic effect of simvastatin with lenalidomide on induction of apoptosis

Simvastatin inhibits the conversion of HMG-CoA to mevalonate by inhibiting HMG-CoA reductase. As a result, it reduces the synthesis of FPP and GGPP leading to inhibition of protein prenylation. Furthermore, simvastatin induces apoptosis and inhibits proliferation in myeloma cells. These effects are abrogated by the addition of mevalonate or GGOH,¹⁴ indicating that protein geranylgeranylation is important in the regulation of myeloma cell

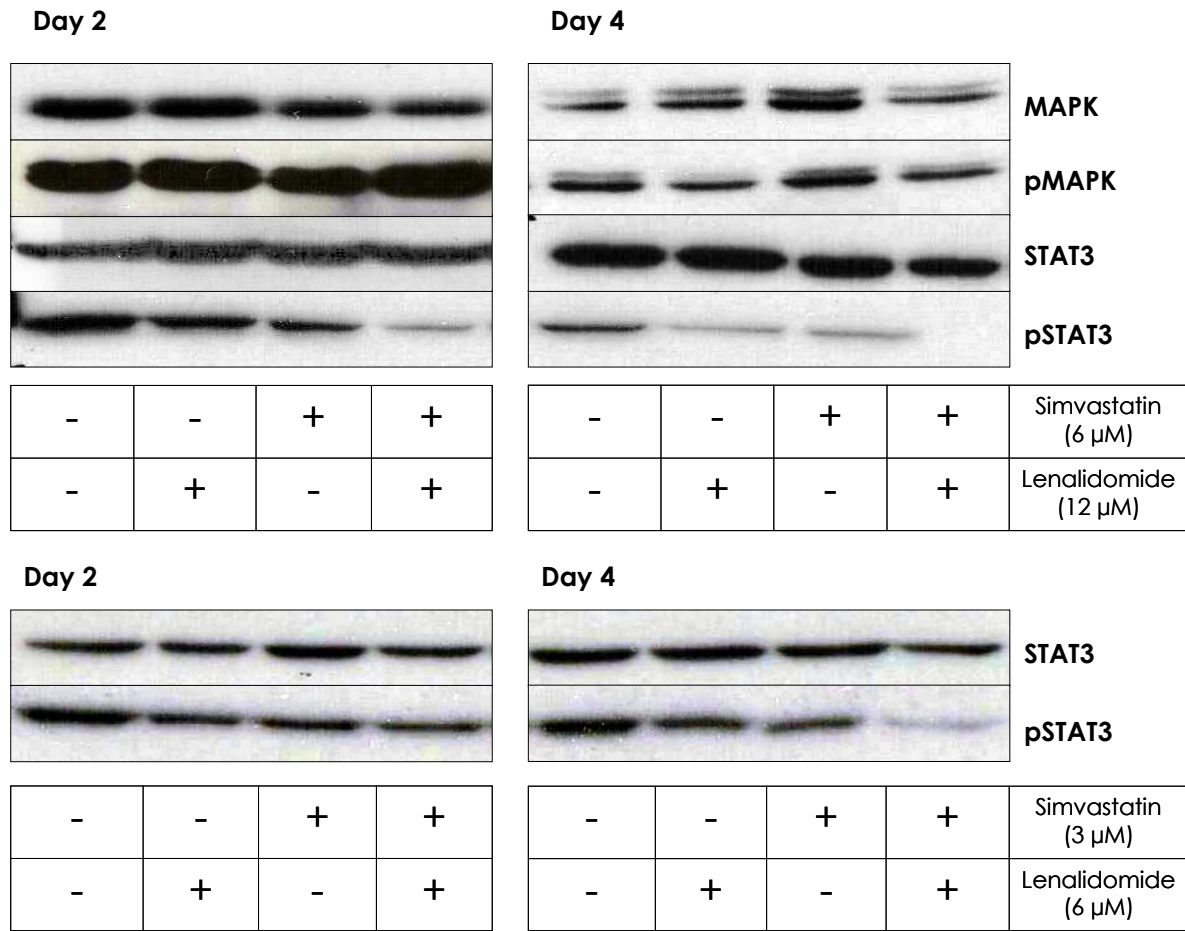


Figure 4. Synergy between lenalidomide and simvastatin in the reduction of phosphorylation of STAT3. XG-1 cells were treated for two and four days with solvent control, lenalidomide, simvastatin, or the combination of lenalidomide and simvastatin. After protein isolation, p42/44 MAPK, STAT3, phosphorylated p42/44 MAPK, and phosphorylated STAT3 were determined by western blot analysis. **(A)** shows the effect of the combination of 12 μ M lenalidomide and 6 μ M simvastatin. In **(B)** the effect on pSTAT3 is shown of 6 μ M lenalidomide and 3 μ M simvastatin. The data shown are representative of at least three independent experiments.

survival and proliferation. As shown in figure 5, treatment of XG-1 myeloma cells with 12 μ M lenalidomide for two days increased the percentage of apoptotic cells by 2%, as compared to solvent control. Six μ M simvastatin increased the percentage of apoptotic cells by 32%, as compared to solvent control. However, the combination of lenalidomide and simvastatin led to a significant increase of apoptotic cells of 46%, as compared to solvent control. Addition of mevalonate (100 μ M), GGOH (10 μ M), and to a lesser extent FOH (10 μ M) to XG-1 plasma cells treated with the combination of lenalidomide and simvastatin for two days abrogated the synergistic effect of lenalidomide on simvastatin-induced apoptosis (figure 5). Thus, the mevalonate pathway, and more specifically geranylgeranylated proteins, appears to be involved in the potentiation of simvastatin-induced apoptosis by lenalidomide.

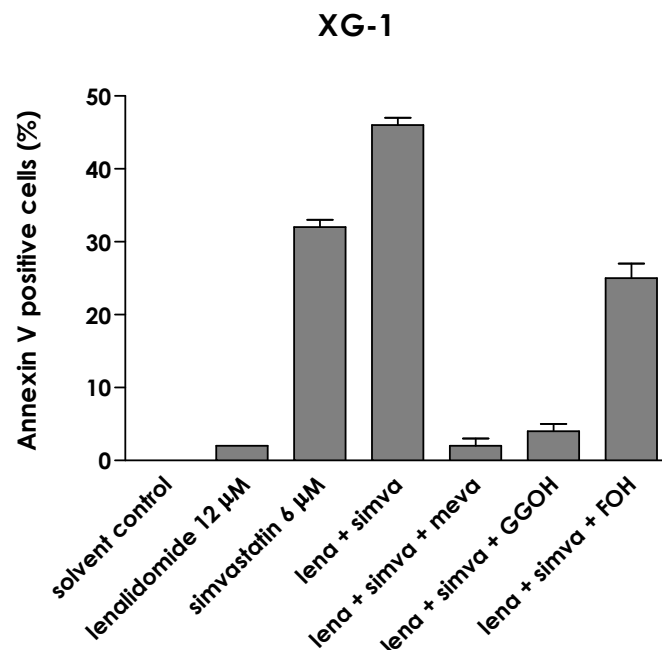


Figure 5. Mevalonate, GGOH, and partly FOH, abrogate the synergistic effect between lenalidomide and simvastatin in XG-1 cells. XG-1 cells were incubated with solvent control, lenalidomide (12 μ M), simvastatin (6 μ M), or the combination of lenalidomide and simvastatin. In addition, mevalonate (100 μ M), GGOH (10 μ M) or FOH (10 μ M) were added to XG-1 cells treated with the combination of lenalidomide and simvastatin. After two days the percentage of apoptotic cells was determined by Annexin V assay. Experiments were performed three times. The percentage of apoptotic cells in solvent control-treated cells was subtracted from the percentage of apoptotic cells in the different treatment groups. Data are presented as mean \pm S.E.M.

Discussion

New combination regimens incorporating novel agents offer the opportunity to treat myeloma patients more effectively and with less toxicity. In the present study, we investigated whether inhibition of the mevalonate pathway in combination with lenalidomide augments anti-myeloma activity. We showed that the HMG-CoA reductase inhibitor simvastatin induced apoptosis and inhibited proliferation of myeloma cells, whereas lenalidomide alone showed only modest *in vitro* anti-myeloma activity. However, lenalidomide significantly potentiated simvastatin-induced cytotoxicity in myeloma cell lines and in six out of seven patient bone marrow samples, through induction of apoptosis and inhibition of proliferation.

In patients, a high-dose of statins is needed to achieve the concentrations used in the experiments described in this study. Previously, we and others demonstrated this to be feasible, with only mild side effects.^{26,27} The limited effect of lenalidomide on myeloma cells *in vitro* is in contrast to the *in vivo* effect of lenalidomide in myeloma.^{3,28} This finding can be explained by the observation that lenalidomide *in vivo* also targets the myeloma environment and modulates the immune response.^{6,7}

It has previously been shown that in the MM.1s plasma cell line lenalidomide activates caspase-8, leading to induction of apoptosis.⁸ We showed that in XG-1 cells lenalidomide alone did not induce apoptosis and had no or only a limited effect on caspase-8 or -9 activation. Simvastatin resulted in cleavage of caspase-8 and -9, as described previously.²² However, the combination of lenalidomide and simvastatin resulted in enhanced activation of caspase-8, which may be responsible for the synergistic induction of apoptosis.

We also examined which signaling cascades may be involved in the potentiation of apoptosis induced by co-exposure to simvastatin and lenalidomide. The PI-3K/Akt signaling pathway was not active in XG-1 cells and activation of the p42/44 MAPK signaling pathway was not altered by lenalidomide, simvastatin, or the combination of both agents. However, the combination of lenalidomide and simvastatin resulted in a strongly augmented and time-dependent down-regulation of STAT3 phosphorylation. Three out of four cell lines showing synergy between lenalidomide and simvastatin are IL-6-dependent. IL-6 is a well-known activator of the STAT3 pathway and is implicated in resistance of myeloma cells to apoptosis.²³ STAT3 has been shown to be constitutively active in part of myeloma cell lines and in the majority of myeloma cells from patients, whereas plasma cells from healthy individuals were not.^{23,29} STAT3 plays an important role in myeloma cell proliferation, survival, and chemotherapy resistance.^{23,30} Therefore, inhibition of STAT3 activation may be involved in the synergistic effects between lenalidomide and simvastatin. Bcl-XL is one of the downstream effector proteins of the STAT3-signaling pathway in myeloma.²³ However, we could not demonstrate a change in Bcl-XL protein expression or in expression levels of other members of the bcl-2 family, using the combination of simvastatin and lenalidomide (data not shown). Additional studies including microarray experiments are required to further unravel the target genes of simvastatin.

Addition of mevalonate abrogated the potentiation of apoptosis induced by the combination of lenalidomide and simvastatin and reduced apoptosis levels to those induced with lenalidomide alone. This implies an important role of mevalonate pathway metabolites, such as FPP and GGPP, in the synergistic effects between lenalidomide and simvastatin. We demonstrated that addition of GGOH, which is converted to GGPP, and to a lesser extent FOH (which is converted to FPP), abrogated the potentiation of apoptosis induced by the combination of simvastatin and lenalidomide. This suggests that geranylgeranylated proteins are involved in the synergistic induction of apoptosis by lenalidomide and simvastatin. At present it is unclear which geranylgeranylated proteins are involved in this process. A possible candidate is RAC1, a member of the rho family of small GTP-ases. It has been implicated in regulation of apoptosis and cell cycle

progression, including myeloma cell growth. Statins affect rac1 function *in vitro* by inhibition of geranylgeranylation.³¹ Interestingly, rac1 mediates STAT3 activation.^{32,33} Further studies are needed to elucidate the role of rac1 in myeloma cell survival and chemo resistance.

In conclusion, the combination of simvastatin and lenalidomide is synergistic, possibly through combined inhibition of STAT3 activity and cleavage of caspase-8. Both drugs are orally available and relatively non-toxic. Inhibition of the mevalonate pathway by statins in combination with lenalidomide may represent a new treatment strategy in refractory myeloma.

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Genome-wide expression analysis suggests that simvastatin exerts its anti-myeloma effect through upregulation of RhoB and pro-apoptotic genes

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chapterfive

Abstract

Inhibition of HMG-CoA reductase with statins is a possible new therapy for multiple myeloma, since statins exhibit a strong anti-myeloma effect *in vitro*, by inhibition of proliferation and induction of apoptosis. The antimyeloma effect of statins has been shown to act through inhibition of the mevalonate pathway and more specifically through inhibition of geranylgeranylation. However, the downstream targets of statins remain elusive. Analysis of alterations in gene expression can help to identify targets of statins in myeloma and is the focus of the here reported investigation.

The myeloma plasma cell line XG-1 was treated with simvastatin alone and with combinations of simvastatin and agents that (partly) rescue the myeloma cell from simvastatin-induced cell kill (mevalonate, GGOH, and FOH). Subsequently, relative gene expression profiles were assessed using Affymetrix microarray chips. Simvastatin treatment resulted in a twofold differential expression of 535 genes compared with control cells. Co-culture with GGOH or mevalonate resulted in reduction of the simvastatin-induced effect in 479 of these genes. The ten most upregulated and downregulated genes by simvastatin included *RHOB* (up), *EGR-1*, *CCR2*, *CDC25A*, and *CDK6* (down). The microarray results were confirmed using western blot. Analysis of these genes using Ingenuity pathway software showed that most of the differentially-expressed genes are related to cancer (135 genes) and are associated with cell death (109 genes) and growth and proliferation, including cell cycle (157 genes). The most affected canonical pathway was the G₁/S checkpoint regulation.

Microarray analysis of statin treated XG-1 myeloma cells resulted in the identification of genes that may be involved in the regulation of myeloma survival and proliferation. Among these 479 genes, expression of *RHOB*, *CCR2*, and *CDK6* were distinctly altered. Further targets include apoptotic genes and genes involved in cellular proliferation. This analysis helps to further understand how simvastatin exerts its anti-myeloma effects.

Introduction

Multiple myeloma is a plasma cell malignancy, which is characterized by the accumulation of slowly proliferating monoclonal plasma cells in the bone marrow. Although recently novel agents like bortezomib and lenalidomide improved response rates, the disease is still incurable.¹

HMG-CoA reductase inhibitors, like lovastatin and simvastatin, inhibit the conversion of HMG-CoA into mevalonate.² Statins have been shown to effectively induce apoptosis in myeloma cell lines and primary myeloma cells *in vitro*.^{3,4} Furthermore, statins synergize with doxorubicin and lenalidomide to induce apoptosis in myeloma cells.^{3,5} In addition, simvastatin inhibits cell adhesion mediated drug-resistance (CAM-DR) - an important survival mechanism of myeloma cells. These effects are achieved by inhibition of the mevalonate pathway, and more specifically by inhibition of geranylgeranylation.^{6-8,9} The geranylgeranylated proteins, which are involved in statin-induced apoptosis and inhibition of proliferation, and their downstream effector proteins, remain elusive until now. A clinical study in myeloma showed feasibility of high-dose simvastatin combined with chemotherapy.¹⁰ However, clinical efficacy in extensively pre-treated myeloma patients was disappointing.¹¹ Unraveling the targets of statins can lead to better application of statins in clinical studies, and can define new therapeutic targets in multiple myeloma.

We employed RNA expression profiling to identify genes that are targeted by simvastatin in myeloma cells. Since geranylgeranylated proteins are of key importance in myeloma cell growth and survival and seem to be the major targets of statin-induced apoptosis, we analyzed differentially-expressed genes in simvastatin-treated myeloma cells that were rescued both by mevalonate and GGOH.

Material and methods

Reagents

Simvastatin was a kind gift of Merck & Co (Rahway, NJ, USA) and was chemically activated by alkaline hydrolysis prior to use as described previously.¹² Mevalonate and farnesol (FOH) were purchased from Sigma (St. Louis, MO, USA) and geranylgeraniol (GGOH) was obtained from ICN Biomedicals BV (Zoetermeer, the Netherlands). In the cells, FOH and GGOH are metabolized to FPP and GGPP, respectively.¹³

Cell lines

The IL-6-dependent plasma cell line XG-1 was a kind gift from dr. B. Klein (Institute for Molecular Genetics, Montpellier, France). The RPMI-8226 plasma cell line was obtained from the American Tissue Culture Collection (ATCC) and the L3-63 from the German

Collection of Microorganisms and Cell Cultures (GCMC). The cell lines were maintained in RPMI-1640 (GIBCO, Breda, the Netherlands) supplemented with 10% foetal calf serum (FCS; Integro, Zaandam, the Netherlands), and antibiotics. All the experiments with XG-1 were performed in the presence of 1.25 ng/ml rhIL-6 (Roche, Almere, the Netherlands).

Cell viability

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described.³ In short, cells were seeded in a concentration of 0.3×10^6 /ml in a 96-well flat bottom plate (100 μ l/well) (Nunc, Roskilde, Denmark) and treated with simvastatin (for concentrations see legends) alone or in the presence of mevalonate (100 μ M), FOH (10 μ M) or GGOH (10 μ M). After 24, 48, 72, and 96 hours, 25 μ l of MTT (5 mg/ml) was added to each well. After an incubation period of two hours at 37°C, the reaction was stopped by the addition of 100 μ l 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) and distilled water. After an overnight incubation at 37°C, the optical density of the samples was determined at 570 nm.

Apoptosis detection

XG-1 myeloma cells were incubated with solvent control and simvastatin (see legends for concentrations). Cells were seeded in a concentration of 0.3×10^6 /ml in a 48-well flat bottom plate (Nunc, Roskilde, Denmark). After one and two days, cells were harvested, washed in ice-cold PBS and directly stained with Annexin v FITC and propidium iodide (PI). After ten minutes of incubation at room temperature in the dark, cells were analyzed by flow cytometry (FACS calibur, BDIS) as described previously.³ Viable cells were defined as Annexin v negative/PI positive. Apoptotic cells were defined as Annexin v positive cells.

Total RNA isolation

Total RNA of XG-1 cells treated with solvent control, simvastatin (7.5 μ M) alone, or the combination of simvastatin and mevalonate (100 μ M), FOH (10 μ M), or GGOH (10 μ M) was isolated with the RN-easy Mini Kit (Qiagen, Valencia, CA), according to the manufacturer's instruction. In brief, cells were pelleted, lysed in RLT buffer containing 1% β -mercaptoethanol, and passed through a QiaShredder (Qiagen) to prevent cell clumps. RNA was quantified by spectrophotometric analysis at 260 nm and 280 nm, and the quality of all RNA samples was assessed by electrophoresis on a 1.2% agarose gel containing formaldehyde/formamide.

Sample preparation and processing for microarrays

cDNA was prepared, amplified, and biotinylated from 1 μ g of total RNA, using the 3' amplification One-Cycle Target labeling (Affymetrix). The amplified cDNA samples were

fragmented and hybridized onto HG-U133 plus 2.0 GeneChips. After sample hybridization, arrays were washed, stained, and scanned according to the standard Affymetrix protocol.

Microarray data analysis

Data reported here are extracted from five Affymetrix HG-U133 plus 2.0 GeneChips (54,675 probe sets per chip) used under the various conditions described. Affymetrix microarray data were quantile normalised¹⁴ and background was removed using robust multichip analysis (RMA).¹⁵ Arrays or array groups were compared based on the perfect match (PM) probe intensity levels only,¹⁵ by performing a per-probeset two-way analysis of variance (ANOVA, with factors “probe” and “stage”). This results in average expression levels for each probeset in each stage, as well as p-values for the significance of the difference between the stages. The latter were adjusted for multiple testing using Šidák step-up adjustment¹⁶ and all differences with adjusted p-values <0.05 were considered significant. For the purpose of clustering, expression data were transformed to z-scores by subtracting the mean over all five arrays from each probeset value and dividing by its standard deviation. Hierarchical clustering was then performed based on Pearson correlation, using complete linkage. We subsequently analyzed genes that were induced or repressed at least twofold after treatment with simvastatin compared to solvent control. As statins exert their anti-myeloma effect by inhibition of the mevalonate pathway and, more specifically, by inhibition of geranylgeranylation, we selected genes in which the twofold differential expression was at least partly reversed by adding both mevalonate and GGOH.

Ten highest up- and downregulated genes

The function and biologic processes of the ten highest up- and downregulated genes were retrieved from EntrezGene (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene>).

Western blot

Western blot analysis was performed as described before.³ In short, cells (0.3×10^6 /ml) were treated with solvent control, simvastatin (7.5 μ M), or the combination of simvastatin with mevalonate (100 μ M), GGOH (10 μ M) or FOH (10 μ M). After two days, 3×10^6 viable cells were directly lysed and diluted in sample buffer. Afterwards, protein concentrations were determined. Equal amounts of protein were subsequently fractionated in 12% SDS-PAGE and were blotted. Equal loading was confirmed by anti- α -actin expression (Sigma). The following primary antibodies were used: rhob, rhoc (both Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA), and CDC25A (Cell Signaling Technology, Beverly, MA, SA). Primary antibodies were detected by horseradish peroxidase (HRP)-conjugated secondary antibodies (Dako, Glostrup, Denmark), followed by detection with enhanced chemiluminescence (Amersham).

Ingenuity pathways analysis

Ingenuity Pathways Analysis (IPA) (Ingenuity Systems, Mountain View, CA, USA) is a database containing up-to-date information on genes and proteins. This information is integrated with other relevant databases such as EntrezGene and Gene Ontology. IPA computes a score for each network/disease/canonical pathway according to the fit of the set of supplied focus genes. A network describes a functional relationship between gene products based on known interactions in the literature. A network does not have directionality. There is a maximum network size of 35 molecules. A canonical pathway is a core pathway in the cell, which molecular interactions occur in a linear and stepwise manner. It may represent both metabolic and signaling pathways. The significance is expressed as a p-value, which is calculated using the right-tailed Fisher's Exact Test. In this method, the p-value is calculated by comparing the number of user-specified genes of interest that participate in a given function or pathway, relative to the total number of occurrences of these genes in all functional/pathway annotations stored in the Ingenuity Pathways Knowledge Base. The score is not an indication of the quality or significance of the network; it rather calculates the approximate fit between the supplied genes and each network.

Results

Effects of simvastatin on cell viability in a myeloma plasma cell line

As previously described,^{3,9} statins reduce cell viability in myeloma plasma cells. Here, we examined the effect of simvastatin on the XG-1 plasma myeloma cell line. As expected, simvastatin effectively reduced cell viability in a time- and dose- dependent manner. The effect of simvastatin was abrogated by the addition of mevalonate or GGOH, especially at earlier time points, while FOH only marginally prevented simvastatin-induced reduction of cell viability (figure 1A). These results indicate an important role for geranylgeranylated proteins in myeloma cell survival and proliferation. To study the targets of simvastatin in myeloma, gene expression profiles of untreated cells were compared with expression profiles of XG-1 cells treated with simvastatin alone or simvastatin combined with mevalonate, GGOH or FOH. We argued that gene expression profiles were best measured prior to the onset of overt apoptosis triggered by simvastatin. Simvastatin induced apoptosis in a time- and dose-dependent way in the XG-1 plasma cell line (figure 1B). Consequently, RNA of cells treated with simvastatin 7.5 μ M was collected after a 24 hour incubation.

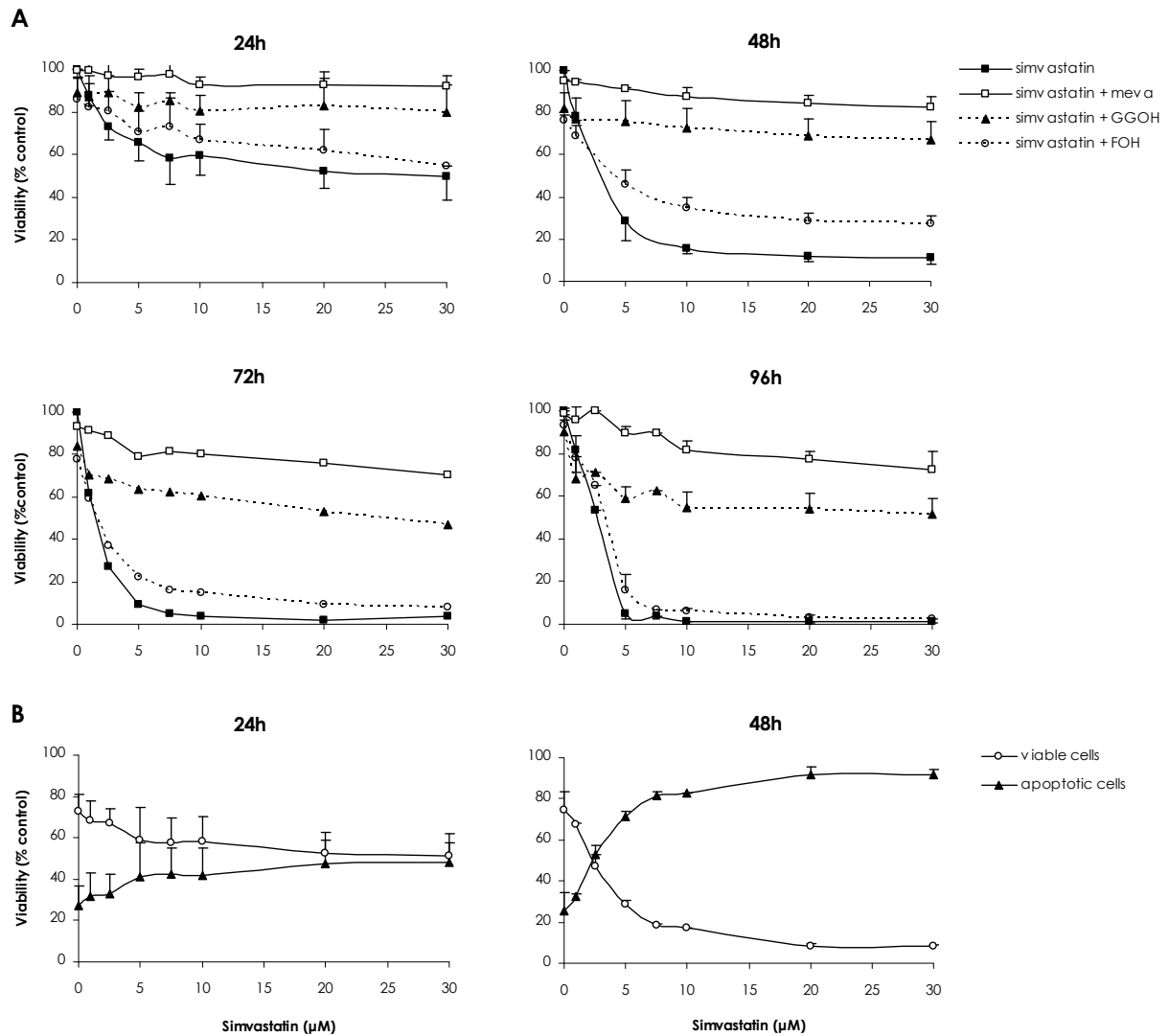


Figure 1 A. XG-1 myeloma cells were treated for 24 hour, 48 hour, 72 hour, and 96 hour with solvent control, different concentrations of simvastatin (1, 2.5, 5, 7.5, 10, 20, and 30 μM) alone or in the presence of mevalonate (meva; 100 μM), GGOH (10 μM) or FOH (10 μM). The percentage of viable cells, 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 \pm SD. **B.** XG-1 myeloma cells were treated for 24 and 48 hour with solvent control and different concentrations of simvastatin (1, 2.5, 5, 7.5, 10, 20, and 30 μM). The percentage apoptotic cells was examined by using the Annexin V assay. The percentage of viable plasma cells (Annexin V-/PI-), and apoptotic cells (Annexin V+) are shown. Experiments were performed three times in triplicate. Data are presented as mean \pm SD.

Table 1A. The ten most upregulated genes by simvastatin

Gene symbol	Gene name	Function	Biologic process	Fold
RHOB	ras homolog gene B	GTP binding; GTPase activity; nucleotide binding; protein binding	Rho protein signal transduction; cell adhesion; cell differentiation; negative regulation of cell cycle; positive regulation of angiogenesis; small GTPase mediated signal transduction; transformed cell apoptosis	19.1
TSC22D3	TSC22, domain 3	transcription factor activity	regulation of transcription, DNA-dependent	15.8
ANXA1	annexin 1	calcium ion binding; calcium-dependent phospholipid binding; phospholipase A2 inhibitor activity; protein binding, bridging; receptor binding;	anti-apoptosis; cell cycle; cell motility; cell surface receptor linked signal transduction; inflammatory response; lipid metabolic process; regulation of cell proliferation	9.6
ANXA3	annexin 3	calcium ion binding; calcium-dependent phospholipid binding; phospholipase A2 inhibitor activity	signal transduction	8.0
KLF2	kruppel like factor 2	metal ion binding; protein binding; transcription activator activity; transcription factor activity;	regulation of transcription, DNA-dependent; positive regulation of transcription ;	5.5
S100A10	S100 calcium binding protein A10	calcium ion binding; receptor binding	signal transduction	5.4
ATF3	activating transcription factor 3	DNA binding; Identical protein binding; protein dimerization activity; transcription factor activity	regulation of transcription, DNA dependent	5.3
NCF2	neutrophil cytosolic factor 2	binding; electron carrier activity;	cellular defense response	5.1
SESN2	sestrin 2	-	cell cycle arrest	4.8
INHBE	inhibin E	-	growth factor activity; hormone activity	4.6

Table 1B. The ten most downregulated genes by simvastatin

Gene symbol	Gene name	Function	Biologic process	Fold
EGR1	early growth response 1	DNA binding, metal ion binding, nucleic acid binding, transcription factor activity	T cell differentiation, regulation of transcription, DNA dependent; negative regulation of transcription from RNA polymerase II promoter	5.5
MMRN1	multimerin 1	-	blood coagulation, cell adhesion	4.6
CCR2	chemokine (C-C motif) receptor 2	C-C chemokine receptor activity; CCR2 chemokine receptor binding; receptor activity;	G-protein coupled receptor protein signaling pathway; JAK-STAT cascade cellular defense response; chemotaxis; cytokine and chemokine mediated signalling pathway; immune response; inflammatory response; signal transduction	4.3
HBD	hemoglobin delta	heme binding; iron ion binding; metal ion binding; oxygen binding;	oxygen transport; transport	4.2
HELLS	helicase, lymphoid specific	ATP binding; DNA binding; helicase activity; hydrolase activity; nucleotide binding	cell cycle; cell division; centromeric heterochromatin formation; lymphocyte proliferation; maintenance of DNA methylation; mitosis; regulation of transcription; transcription	4.0
SUV39H2	suppressor of variegation 3-9, homolog 2	chromatin binding; histone lysine N-methyl transferase activity (H3-K9 specific); methyltransferase activity; transferase activity; zinc ion binding	cell differentiation; multicellular organismal development	3.9
HEMGN	hemogen	-	cell differentiation; multicellular organismal development	3.8
CDC25A	cell division cycle 25, homolog A	hydrolase activity; protein binding; protein tyrosine phosphatase activity	DNA replication; cell cycle; cell division; cell proliferation; mitosis; regulation of cyclin dependent protein kinase activity	3.6
CDK6	cyclin-dependent kinase 6	ATP binding; cyclin binding; cyclin -dependent protein kinase activity; nucleotide binding; transferase activity	G1 phase of mitotic cell cycle; cell cycle; cell dedifferentiation; cell division; negative regulation of osteoblast differentiation; positive regulation of cell-matrix adhesion; regulation of gene expression	3.6
DUSP6	dual specificity phosphatase	MAP kinase thyrrosine/serine/threonine phosphotase activity; hydrolase activity; protein serine/threonine phosphatase activity; protein tyrosine activity	inactivation of MAPK activity; protein amino acid dephosphorylation	3.3

Microarray results

Using microarray analysis, relative expression data of 54,675 probesets were generated of five microarrays (1. XG-1, solvent control; 2. XG-1 treated with simvastatin (7.5 μM); 3. XG-1 treated with simvastatin (7.5 μM) and with mevalonate (100 μM); 4. XG-1 treated with simvastatin (7.5 μM) and GGOH (10 μM), 5. XG-1 treated with simvastatin (7.5 μM) and (FOH 10 μM)). Differential gene expression between samples 1 and 2, 2 and 3, 2 and 4, and 2 and 5 was calculated using the method described by Dik et al.¹⁷ ANOVA analysis on all samples resulted in 5,776 probesets significantly differentially-expressed between at least two of the five samples, as shown in a clustered heat-map in figure 2A. ANOVA analysis on sample 1 vs sample 2 (733), 2 vs 3 (579), 2 vs 4 (506), 2 vs 5 (351) gives a total of 1,392 unique significantly differentially-expressed probesets (shown clustered in figure 2B).

A total of 535 genes were differentially-expressed at least twofold after treatment with simvastatin compared to solvent control. Of these, 210 genes were downregulated and 325 were upregulated. Mevalonate reversed the effect of simvastatin in 481 genes (325 upregulated genes and 156 downregulated genes). Addition of GGOH reversed the effect of simvastatin in 479 of these 481 affected genes (figure 3). In 392 genes, FOH showed equal inversion of simvastatin-induced gene expression compared to GGOH, while in 27 genes this effect was more pronounced when compared to GGOH. In 87 genes, addition of FOH showed an opposite effect compared with GGOH.

The ten most up- and downregulated genes are shown in table 1. Ras homolog gene B (RHOB) was the highest upregulated gene. Early growth response 1 (EGR-1), chemokine receptor 2 (CCR2), cell division cycle 25, homolog A (CDC25A), and cyclin-dependent kinase 6 (CDK 6) were all among the highest downregulated genes. These genes are further discussed below.

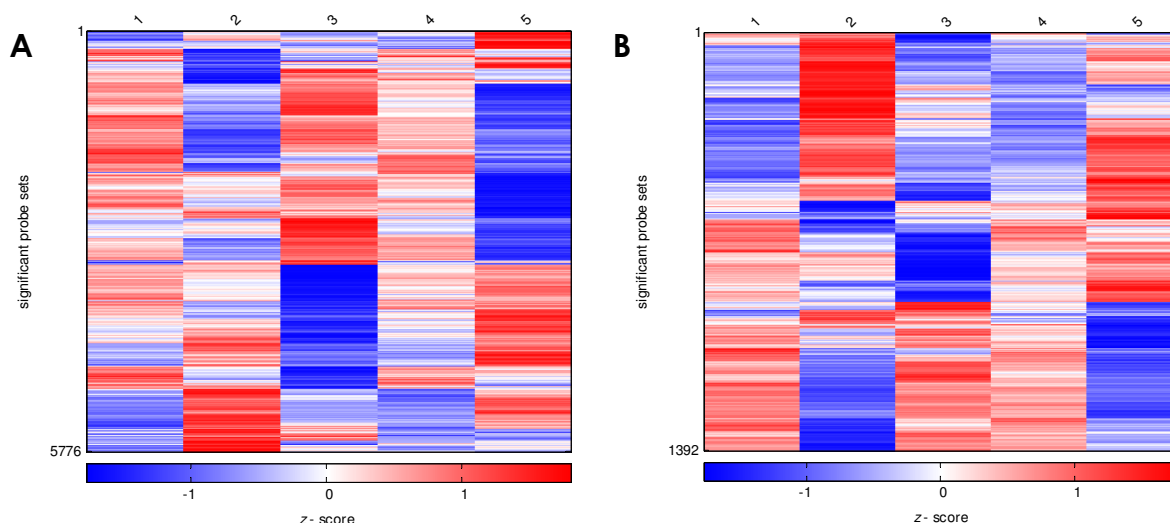


Figure 2 **A.** Heatplot of 5,776 probe sets (ANOVA on all samples) **B.** Heatplot of 1,392 probe sets (superset of individual comparisons)

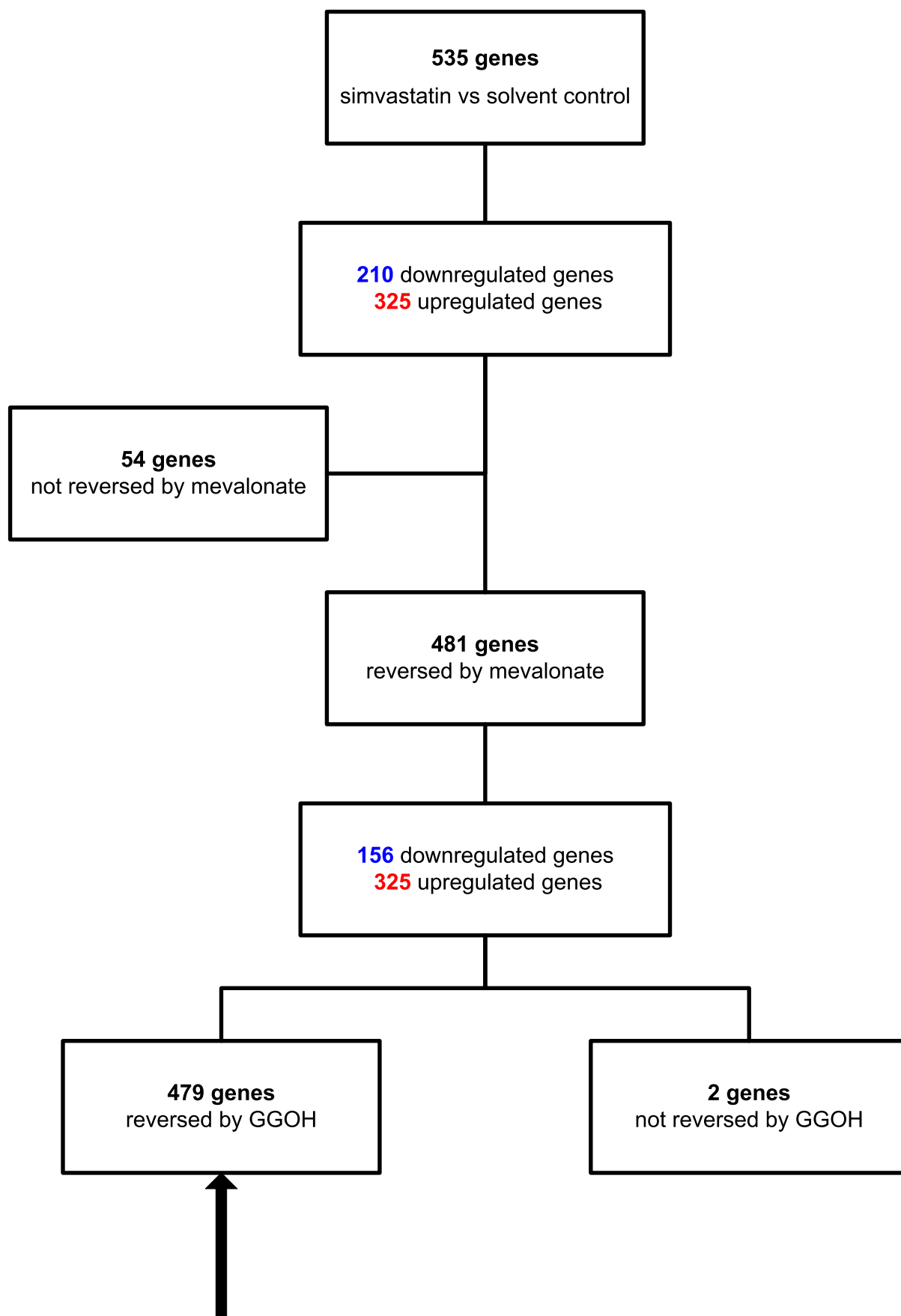


Figure 3. Flow chart of gene sorting preceding the analysis in Ingenuity. The box with the arrow indicates the genes that are analyzed in Ingenuity.

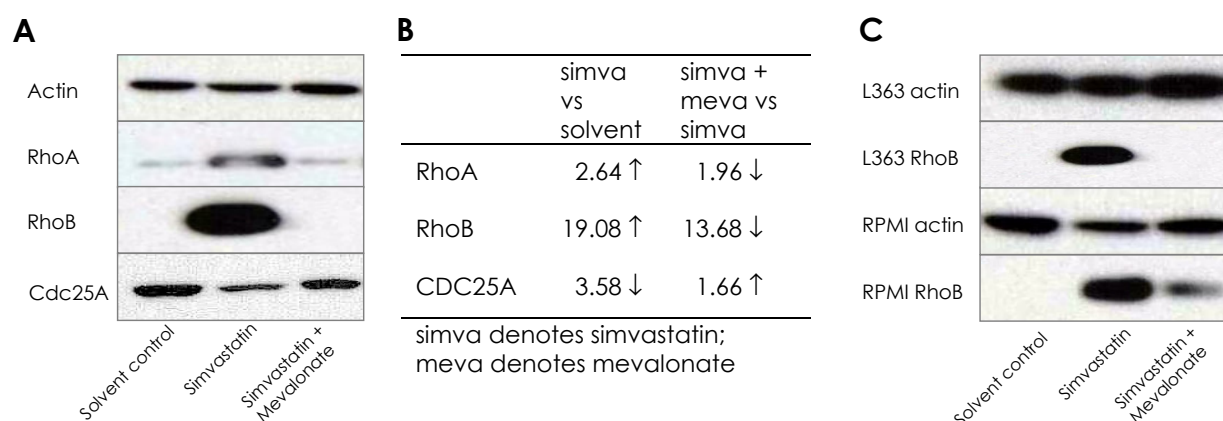


Figure 4A. XG-1 cells were treated for two days with solvent control, or simvastatin (7.5 μ M) alone or in the presence of mevalonate (100 μ M). After protein isolation, actin, RhoA, RhoB, and CDC25A were identified by western blot analysis. **B.** Fold change as determined by microarray. ↑: upregulation; ↓: downregulation; e.g. treatment of XG-1 cells with simvastatin combined with mevalonate results in a 1.96 fold downregulation when compared to simvastatin treatment alone. **C.** L-363 cells and RPMI-8226 cells were treated for two days with solvent control, or simvastatin (7.5 μ M) alone or in the presence of mevalonate (100 μ M). After protein isolation, actin, and RhoB were identified by western blot analysis. The data are representative of three independent experiments.

Confirmation of microarray results by western blot analysis

Microarray data were validated by using western blot analysis. We confirmed our microarray data for three differentially-expressed genes, by using lysates of XG-1 cells treated for 48 hours with solvent control, simvastatin only or the combination of simvastatin and mevalonate. Protein levels confirmed the differential expression of genes as determined in the microarray (figure 4A). For example, western blot analysis showed that *rhoB* was highly upregulated after simvastatin treatment and addition of mevalonate abrogated this effect. This finding was in accordance with the microarray data (figure 4B). To determine whether the same effects of simvastatin could be demonstrated in other plasma cell lines, we confirmed differential expression of *rhoB* in L-363 plasma cells and RPMI-8226 cells (figure 4C).

Ingenuity pathways analysis

The 479 GGOH responsive genes after simvastatin treatment were uploaded in IPA. Of the uploaded genes, 449 genes were eligible for analysis; the other 30 could not be mapped to a molecule in Ingenuity's knowledge base. Two highly significant networks with a score of ≥ 26 (out of 35) were identified from the imported data set. These affected networks were both associated with cell cycle. Furthermore, network A was associated with cancer, cellular assembly, and organization (figure 5A), while network B correlated with cell death and neurologic disease (figure 5B) (both at $p=10^{-44}$).

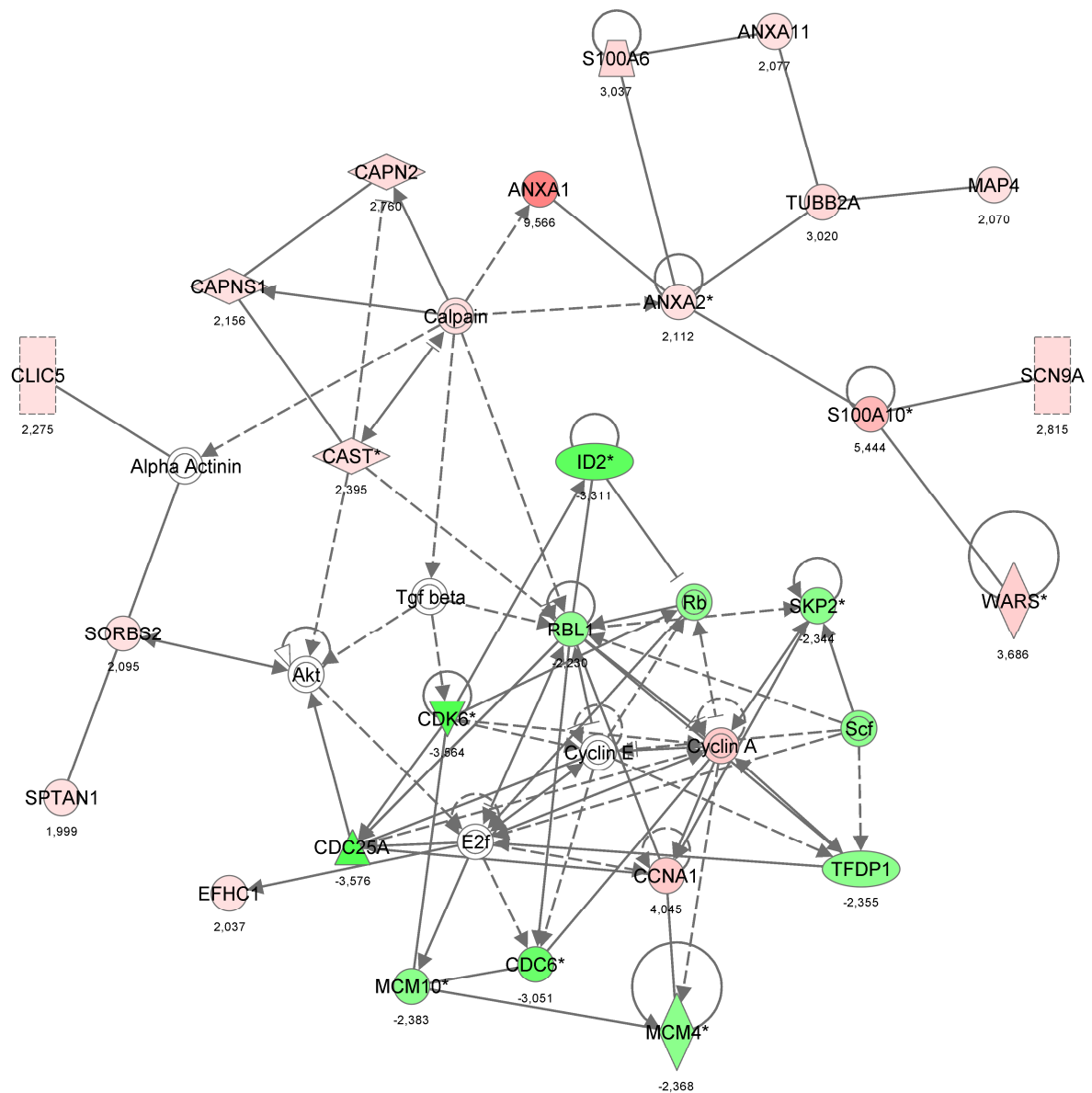
In both networks, 26 out of 35 molecules were affected by statin treatment. Network A presents CDK6 and Retinoblastoma-like 1 (RBL1) in central position and overlays with the G₁/S signaling pathway. CDC6, CDC25A, and PRB were all downregulated members of this pathway. Network B presents p21(CDKN1A) and PI3K in central position and overlays especially with the p53 canonical pathway. BBC3, p21, and TP53INP were found upregulated. The analysis of biologic functions (table 2A and 2B) shows that most of the affected genes (135 genes) are involved in processes related with cancer (table 2A) and are associated with the regulation of cell death (109 genes) or cell cycle (60 genes) and growth and proliferation (97 genes). These results fit with our previously published functional findings that blocking of the mevalonate pathway induces apoptosis and inhibits cell proliferation. Finally, table 2C shows the canonical pathways associated with the analyzed dataset of genes.

Discussion

Simvastatin targets important genes known to be involved in the regulation of growth and survival in multiple myeloma. Using microarray analysis in XG-1 myeloma cells, we were able to identify 535 genes that were at least twofold up- or downregulated after treatment with simvastatin compared to solvent control. The effects of simvastatin were abolished in 479 genes by both addition of mevalonate and GGOH, indicating their contribution in simvastatin-induced effects. These 479 genes were the subject of our further analyses as they represent *bona fide* direct targets of the transcriptional response to simvastatin in myeloma cells. Western blot analysis confirmed the array results. Among the ten most up- and downregulated genes were RHOB (up), EGR-1, CDC25A, and CCR2 (down). Ingenuity pathway analysis showed that a significant part of these genes is related to cancer, cell death, and cell cycle processes. Thus, we identified the molecular response of simvastatin at a genome-wide level, explaining the observed anti-myeloma effects.

Previously, microarray studies investigated the effect of fluvastatin in combination with zoledronate in myeloma.⁹ In agreement with our data, these other mevalonate pathway inhibitors also affected genes related to cell cycle and apoptosis, including RHOB. Exact comparison with our study is difficult however, since the authors use another type of microarray chip and analyzer, only report data of gene expression changes using the combination of zoledronate with fluvastatin, and do not specify up- or downregulation of genes. Wong et al. studied expression profiling in several lovastatin sensitive and insensitive myeloma cell lines after lovastatin treatment. They showed differential expression of RHOB mRNAs in two sensitive cell lines analyzed upon treatment with lovastatin.

A



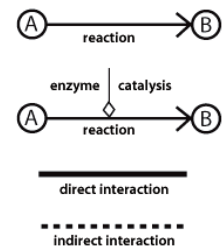
Network Shapes

- Chemical or Drug
- Cytokine
- Enzyme
- G-protein Coupled Receptor
- Group or Complex
- Growth Factor
- Ion Channel
- Kinase

- Ligand-dependent Nuclear Receptor
- Peptidase
- Phosphatase
- Transcription Regulator
- Translation Regulator
- Transmembrane Receptor
- Transporter
- Other

Relationships

- binding only
- inhibits
- acts on
- inhibits AND acts on
- leads to
- translocates to



Note: "Acts on" and "inhibits" edges may also include a binding event.

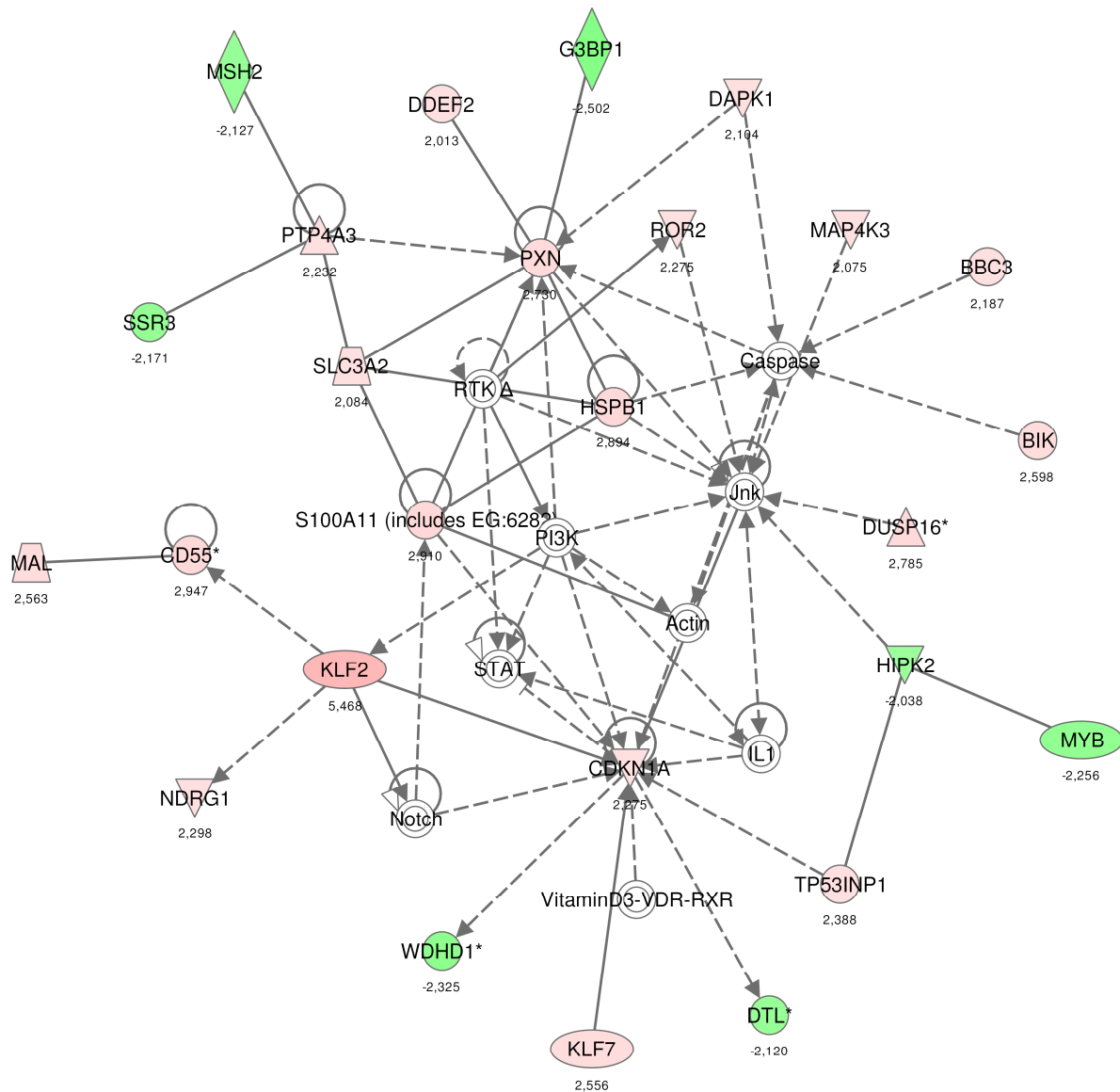
B

Figure 5 A. Network A is associated with cell cycle, cancer, and cellular assembly and organization. **B.** Network B is associated with cell death, neurologic disease, and cell cycle. Genes were uploaded in Ingenuity Pathways Analysis (IPA) for a core analysis, which put the datasets in the context of biologic processes, pathways, and molecular networks. The intensity of the node color indicate the degree of down (green) or up (red) regulation. Genes in uncolored nodes were not identified as differentially-expressed in our uploaded dataset.

Table 2A. Diseases and disorders by Ingenuity knowledge based analysis associated with differentially-expressed genes in simvastatin-treated XG-1 cells

Diseases and disorders	P-value	Number of genes
Cancer	$6.02 \times 10^{-13} - 3.53 \times 10^{-3}$	135
Connective tissue disorders	$1.55 \times 10^{-09} - 2.97 \times 10^{-3}$	63
Gastrointestinal disease	$5.53 \times 10^{-09} - 1.36 \times 10^{-3}$	59
Immunologic disease	$2.79 \times 10^{-06} - 3.04 \times 10^{-3}$	34
Neurologic disease	$3.52 \times 10^{-06} - 2.90 \times 10^{-3}$	28

Table 2B. Molecular and cellular functions by Ingenuity knowledge based analysis associated with differentially-expressed genes in simvastatin-treated XG-1 cells

Molecular and cellular functions	P-value	Number of genes
Cell death	$4.64 \times 10^{-15} - 3.66 \times 10^{-3}$	109
Cell cycle	$4.04 \times 10^{-11} - 3.53 \times 10^{-3}$	60
Cell morphology	$6.37 \times 10^{-08} - 2.11 \times 10^{-3}$	53
Cellular growth and proliferation	$1.69 \times 10^{-07} - 3.53 \times 10^{-3}$	97
Gene expression	$2.05 \times 10^{-06} - 3.34 \times 10^{-3}$	62

Table 2C. Top canonical pathways identified by Ingenuity knowledge based analysis in simvastatin-treated XG-1 cells

Canonical pathway	P-value	Differentially-expressed genes associated with this pathway
Cell cycle: G ₁ /S checkpoint regulation	1.66×10^{-05}	CDC25A, CDK6, CDKN1A, HDAC5, RBL1, SKP2, SMAD3, TDFP1
Aryl Hydrocarbon receptor signaling	6.37×10^{-03}	ALDH1L2, CCNA1, CDK6, CDKN1A, HSPB1, JUN, MGST3, TDFP1,
Aminoacyl-t-RNA biosynthesis	8.21×10^{-03}	CARS, GARS, SARS, WARS
P53 signaling	8.47×10^{-03}	BBC3, CDKN1A, HIPK2, JMY, JUN, TP53INP1
Methionine metabolism	1.18×10^{-2}	CBS, CTH, MTAP

Ectopic expression of constitutively activated rhoA, rhoB, rac1, CDC42, and N- and K-Ras proteins, however, was insufficient to change sensitivity to lovastatin.⁸ These results suggest that rho and ras are not directly involved in lovastatin-induced responses in myeloma. However, it is unclear if the levels of expression reached were sufficient to overcome the dysregulated effects. Moreover, rhoB can have pro-apoptotic effects, which may inadvertently have been induced.

In contrast to these studies, we focused on those simvastatin-induced genes whose expression was reversed after addition of both mevalonate and GGOH. We chose this approach because the effects of simvastatin on apoptosis and proliferation in myeloma cells can be inverted by mevalonate or GGOH.^{6,18} Therefore, including up- or downregulated genes whose effects are not abrogated by mevalonate or GGOH can contaminate the analysis. Consequently, we optimized our analysis by excluding such genes. This method helped to identify target genes more distinctively. Ten percent of twofold expressed genes were thus eliminated as candidate genes and these genes are likely to represent indirect targets.

The ten most up- and downregulated genes point to important targets of simvastatin in myeloma. The highest upregulated gene is *RHOB*. *RHOB* is a member of the rho GTP-ase family involved in cell adhesion, motility, proliferation, and survival.^{19,20} It differs in many aspects from other members of the same family, *rhoA* and *rhoC*. Both *rhoA* and *rhoC* are associated with malignant transformation and proliferation and are exclusively geranylgeranylated. Conversely, *rhoB* is associated with anticancer properties and is a negative regulator of cell proliferation. *RHOB* was shown to repress NF- κ B signalling,^{21,22} which is an important transcription factor in myeloma.^{23,24} To be active, *rhoB* can both be geranylgeranylated and farnesylated. As statins inhibit geranylgeranylation and farnesylation, the upregulated *rhoB* may not be functional. However, one study showed activity of unprenylated *rhoA*, *rhoB*, and *rhoC* after inhibition of prenylation.²⁵ Possibly, a negative feedback mechanism upregulates *rhoB* to counteract the reduced prenylation caused by statins. This may cause an anti-myeloma effect of statins, or, alternatively, upregulated *rhoB* is an innocent bystander. Further studies are needed to explore the exact role of *rhoB* in myeloma.

We identified other interesting targets that were all downregulated, including *EGR-1*, *CDC25A*, *CCR2*, and *CDK6*. *EGR-1* is a transcription factor that activates transcription by binding to DNA. Although no association has been reported between *EGR-1* and multiple myeloma, *EGR-1* is extensively studied in other malignancies. It has opposite roles in cancer: it upregulates tumor suppressors like *PTEN*, and *p53*.²⁶ Conversely, *EGR-1* deficiency impairs transition from carcinoma in situ to invasive cancer in prostate carcinoma.²⁷ Furthermore, *EGR-1* induces transforming growth factor (TGF)- β ₁,²⁸ which is associated with IL-6 induction and important in myeloma cell survival.²⁹ Fibronectin is strongly upregulated by *EGR-1*.³⁰ In multiple myeloma, fibronectin is an important regulator of bone marrow stroma-tumor interaction and is crucial for cell survival. *EGR-1* downregulation may thus play an important role in the inhibition of CAM-DR by simvastatin in myeloma. Inhibition of CAM-DR by simvastatin was shown to be regulated

through inhibition of rho proteins.⁷ Interestingly, EGR-I activation by a mutated G protein was shown to be mediated by rho proteins.³¹

Another compelling target is CDC25A. This phosphatase controls both the G₁/S transition and G₂/M transition. In addition, it acts as a checkpoint, making it an essential component for all phases of the cell cycle. CDC25A is overexpressed in a significant number of cancers and inhibition may limit tumorigenesis.^{32,33}

Another downregulated target of simvastatin is CCR2. This chemokine receptor is expressed on peripheral monocytes and activated T cells, B cells, and immature dendritic cells. In myeloma cells, CCR2 is overexpressed compared to autologous B lymphoblastoid cell lines,^{34,35} and may be involved in homing of plasma cells to the bone marrow and thus play a role in the pathogenesis of multiple myeloma.³⁶

The final target that is discussed here is CDK6, a cyclin-dependent kinase. It regulates progression and re-entry through the G₁ phase of the cell cycle in cooperation with cyclin D.³⁷ Cyclin D expression is increased or dysregulated in virtually all multiple myeloma tumors and thought to be an early and perhaps initiating event in the pathogenesis of multiple myeloma.³⁸ Downregulation of CDK6 can possibly inhibit cell cycle progression. Interestingly, p21, one of the CDK inhibitors that inhibit the CDK4/6/cyclin D complex, is upregulated in our array 2.28 times by simvastatin.

In summary, these data strongly suggest that RHOB, EGR-I, CDC25A, CCR2, and CDK6 are candidate targets of statin treatment in myeloma. Evaluation of the micro-array data employing IPA showed that differentially-expressed genes were involved in cellular and molecular pathways related to cell death, cell cycle, cellular growth and proliferation, cell morphology, and gene expression. Network analysis showed involvement of statins in networks related to cell death and cell cycle, with overlaying canonical pathways G₁/S regulation and p53 signaling. Possible targets in the G₁/S regulation pathway are CDC6 and CDC25A. Like CDC25A, CDC6 is a checkpoint regulator of the cell cycle, and shows proto-oncogenic activity.³⁹ Remarkably, retinoblastoma-like 1, similar to the retinoblastoma gene, well known for its tumor suppressor activity, and inhibitor of IL-6⁴⁰ is downregulated by statins. Activation of the p53 pathway induces apoptosis in myeloma cells.⁴¹ IPA shows upregulation of several molecules that belong to the p53 pathway. This could explain induction of apoptosis in myeloma cells. Interesting candidates are p21(CDKN1A), BBC3, a strongly pro-apoptotic protein,⁴² and TP53INP1, all related to p53 signaling. To understand the exact meaning of these pathways, a closer look at affected genes and their downstream metabolites associated with these pathways is warranted.

Treatment of myeloma cells with simvastatin affects genes related to cancer, apoptosis, and proliferation. To further determine the exact significance of these results, additional tests are needed to explore the exact role of these differentially-expressed genes in simvastatin-induced effects in myeloma. An interesting technique in this context is RNA interference, which we currently use to test whether *rhoB* has a role in inducing apoptosis in myeloma cells after treatment with simvastatin. In conclusion, this study defines a number of important targets for novel therapies in multiple myeloma.

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A bioluminescence imaging-based in vivo model for preclinical testing of novel cellular immunotherapy strategies to improve graft versus myeloma

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chapter six

Abstract

Development and preclinical testing of novel immuno-therapy strategies for multiple myeloma can substantially benefit from a humanized animal model that permits quantitative real-time monitoring of tumor progression. Here we have explored the feasibility of establishing such a model in immuno-deficient $RAG2^{-/-}\gamma C^{-/-}$ mice, by utilizing non-invasive bioluminescent imaging (BLI) for real-time monitoring of multiple myeloma cell growth.

Seven MM cell lines, marked with a GFP-firefly luciferase fusion gene, were intravenously injected in $RAG2^{-/-}\gamma C^{-/-}$ mice. Tumor localization and outgrowth was monitored by BLI. The sensitivity of BLI was compared to that of free Ig light chain (FLC)-based myeloma monitoring. Established tumors were treated with radiotherapy or with allogeneic PBMC infusions to evaluate the application areas of the model.

Five out of seven tested myeloma cell lines progressed as myeloma-like tumors growing mainly in the bone marrow; the two other lines showed additional growth in soft tissues. Bioluminescence imaging appeared superior to FLC-based monitoring and allowed semi-quantitative monitoring of individual myeloma foci. While tumors treated with radiotherapy showed temporary regression, infusion of allogeneic PBMC was associated with the development of xenogeneic GVHD and a powerful cell dose-dependent graft versus myeloma effect, resulting in complete eradication of tumors, depending on the *in vitro* immunogenicity of inoculated myeloma cells.

Our results indicate that this new model allows convenient and sensitive real-time monitoring of cellular approaches for immunotherapy of myeloma-like tumors with different immunogenicities. Thus, this model allows comprehensive preclinical evaluation of novel combination therapies for multiple myeloma.

Introduction

Multiple myeloma is a neoplastic disease, characterized by the outgrowth of monoclonal plasma cells in the bone marrow. Over the past decade, treatment of multiple myeloma has been changed significantly by the introduction of several novel agents such as bortezomib, thalidomide, and lenalidomide.¹ Furthermore, with the success of non-myeloablative conditioning in reducing transplant-related mortality, allogeneic stem cell transplantation may become a realistic and attractive therapeutic option for patients with chemotherapy-resistant myeloma.² Despite these promising achievements, myeloma still remains incurable, indicating the need for novel combination therapies. Unfortunately, clinical testing of several potentially promising drugs in combination or not with allogeneic stem cell transplantation is cumbersome and requires large controlled studies, once again highlighting the need for useful animal models that enable pre-clinical testing of novel immunologic and pharmacologic combination therapy strategies against multiple myeloma.

To date, three xenograft models of human myeloma have been developed: SCID-Hu,³⁻⁵ NOD/SCID,⁶⁻¹⁰ and SCID-Rab.¹¹ While these models offer several opportunities to test therapeutics *in vivo* against human myeloma, each of them has also reported limitations, in particular for reproducible, convenient, and sensitive monitoring of cellular immunologic therapies in combination with immunomodulatory agents.¹²

To develop a model that offers an optimal platform for preclinical evaluation of cellular immunotherapies, we here tested the feasibility of using immuno-deficient $RAG2^{-/-}\gamma C^{-/-}$ mice for this purpose because these mice completely lack B, T, and NK cells,¹³ and are more suitable than NOD/SCID mice for reproducible engraftment of human T and B cells.^{14,15}

To this end, we transduced seven different human myeloma cell lines with a retroviral vector encoding a green fluorescent protein (GFP)-luciferase fusion protein and injected them in the $RAG2^{-/-}\gamma C^{-/-}$ mice. Bioluminescence imaging (BLI) was used to monitor the *in vivo* engraftment, outgrowth, and distribution of the myeloma cell lines semi-quantitatively. Subsequently, radiotherapy and allogeneic lymphocyte infusions were used to eradicate established tumors.

Design and methods

Animals

The animals used in this study are the $RAG2^{-/-}\gamma C^{-/-}$ mice that were bred and housed in the specified pathogen-free breeding unit of the Central Animal Facility of the University of Utrecht.¹³ The animals were supplied with autoclaved sterilized food pellets and distilled

water *ad libitum*. All animal experiments were conducted according to Institutional Guidelines after acquiring permission from the local Ethical Committee for Animal Experimentation and in accordance with current Dutch laws on Animal Experiments.

Cell lines and cell culturing

The human myeloma cell lines that we studied were U266, RPMI-8226/s (both obtained from the American Tissue Culture Collection (ATCC), UM-9 (generated in our institute),¹⁶ LME-I,¹⁷ XG-I,¹⁸ OPM-I,¹⁹ and L-363.²⁰ All myeloma cell lines were cultured in RPMI-1640 (Gibco, Breda, the Netherlands) supplemented with 10% fetal calf serum (Integro, Zaandam, the Netherlands), 100 U/ml penicillin (Gibco), 100 µg/ml streptomycin (Gibco), and 10 µM β-mercaptoethanol (Merck, Darmstadt, Germany). The amphotropic packaging cell line Phoenix (a kind gift from dr. G. Nolan), PG13 cell line (ATCC), and NIH-3T3 fibroblasts (ATCC) were cultured in DMEM (Gibco) supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin. The cultures were maintained at 37°C and 5% CO₂ in a humidified atmosphere.

Retroviral vector production

The GFP-luciferase retroviral vector encoding a fusion gene of GFP and luciferase was generated by inserting the luciferase gene from pBSSKGFP-Luc²¹ (kindly provided by dr. R. Day) into the XhoI-NotI sites of LZRSBPMN-IRES-EGFP (S-001-AB provided by dr. G. Nolan). The amphotropic Phoenix packaging cell line and the gibbon ape leukemia virus (GALV) pseudotyped cell line PG13 were transfected with the GFP-luciferase plasmid using calcium phosphate precipitation. The viral titer was 10⁵ infectious virus particles per milliliter, as determined on 3T3 cells by flow cytometry (FACS) analyses of GFP expression.

Retroviral transduction of the myeloma cell line

For retroviral transduction of myeloma cell lines, we used Retronectin-coated tissue culture plates (CH-296, Takara Shuzo, Otsu, Japan) as described previously.²² Transduction efficiencies, as assessed by FACS analysis of GFP expression, were 11-17% with GALV pseudotyped viruses (RPMI-8226/s) or amphotropic pseudotyped viruses (rest of myeloma cell lines). The percentage of GFP-luciferase expressing cells was increased to 85% by FACS sorting of the transduced cells. Luciferase activity was confirmed according to manufacturer's protocol (Promega, Madison, WI, USA). FACS sorted myeloma cells were expanded to produce the amounts necessary for the assays. Before *in vivo* transfer, the cells were analyzed for the expression of CD45, CD38, CD86, and CD138 by labeling with phycoerythrin-conjugated antibodies (Becton, Dickinson Biosciences, San Jose, CA, USA).

Transplantation of myeloma cells into the $RAG2^{-/-}\gamma C^{-/-}$ mice

$RAG2^{-/-}\gamma C^{-/-}$ mice (age 9–14 weeks) were used in the experiments. Twenty-four hours before the injection of the freshly cultured myeloma cells, mice received total body irradiation (TBI; 3.0 Gy x-rays). Between $5\text{--}20 \times 10^6$ myeloma cells, suspended in 200 μ l phosphate-buffered saline containing 0.1% bovine serum albumin (Gibco) were injected intravenously (i.v.) via the lateral tail vein. The tumor load in the mice was determined by weekly BLI measurements as described. In case of paralysis of the hind limbs or when the mice became moribund, they were sacrificed by cervical dislocation. Cell isolates from various skeletal parts and soft tissues were analyzed using FACS for the presence of GFP⁺ myeloma cells in the total nucleated cell fraction. Bone marrow was obtained by flushing the bones with RPMI-1640. Single cell suspensions from the soft tissues were produced by passage through 70 μ m cell strainers (Becton Dickinson). The cell concentration was determined by adding a fixed number of 4 μ m beads to the single cell suspensions just prior to flow cytometry. Ten thousand events were analyzed for blood and bone marrow samples, whereas 100,000 events from the soft tissue cell suspensions were analyzed. Organs were isolated from some of the mice and fixed in 4% formalin and used for further immuno-histochemical analyses.

Bioluminescent imaging

A few minutes before BLI, the mice were anesthetized by intramuscular injection of 50 μ l of ketamine-xylazine-atropine. One minute before imaging, the mice received an intraperitoneal injection of 100 μ l 7.5 mM D-luciferine (i.e. 125 mg/kg) (Synchem Chemie, Kassel, Germany) and were placed in a light-tight chamber. Bioluminescence images were taken from both the ventral and the dorsal side of the mice using a cooled charge-coupled device (CCCD) camera (Roper Scientific, Princeton Instrument, Trenton, NJ, USA), fitted to a light-tight chamber and mounted with a 50 mm FL2 Nikon lens, controlled by the Metavue Software package software (Universal Imaging Corporation, Downingtown, PA, USA). The instrument is especially designed for photon counting. The integrated light intensity of a stack of ten sequential one-minute exposures was used to quantify the amount of light emitted by the myeloma cells. A low intensity visible light image was made and used to produce overlay images. The images were analyzed with Metamorph Imaging System software (Universal Imaging Corporation).

Histopathologic examination

Femora were fixed and decalcified in saturated EDTA for seven days and embedded in paraffin. Five-micron thick sections were stained with hematoxylin and eosin for histologic examination. The sections were labeled with anti-CD138 (Labvision, Fremont,

USA) for detection of human myeloma cells. PowerVision Poly HRP-anti-Rabbit IgG (ImmunoLogic/Klinpath, Duiven, the Netherlands) was used as secondary antibody followed by the peroxidase enzymatic reaction.

Determination of free immunoglobulin light chain levels

The levels of human free immunoglobulin light chains (FLC) were determined in undiluted cell culture supernatants and in urine samples using automated immunoassays as described previously.²³

Tumor load reduction by radiotherapy

To induce a temporary tumor load reduction, mice bearing myeloma tumors were subjected to TBI of 6.0 Gy x-rays, in week 5 after i.v. injection of 5×10^6 u266 cells. To rescue the irradiated mice from bone marrow failure, 5×10^6 syngeneic bone marrow cells were injected i.v. The effect of the TBI on the total tumor load per mouse as well as on individual foci of myeloma growth was monitored with BLI.

Human peripheral blood mononuclear cells and mixed lymphocyte reaction

Human peripheral blood mononuclear cells (PBMC) isolated from buffy-coats of healthy blood donors were thawed. The percentages of CD3⁺ cells were determined by FACS analysis and the cells were then suspended in phosphate-buffered saline/0.1% human serum albumin at $5-40 \times 10^6$ T cells, which were injected in a volume of 0.2 ml as effector cells into the mice. To determine the reactivity against the myeloma cell lines, irradiated u266 and RPMI-8226/s cells were used as target cells in a ratio of 1:1. Briefly, 40,000 effector and 40,000 target cells were cultured in round-bottom 96-well plates for six days and pulsed with [³H] thymidine for the last 18 hours.

Tumor load reduction by immunotherapy (the graft-versus-myeloma effect)

In order to induce a graft-versus-myeloma (GVM) effect, mice bearing myeloma tumors were injected with various doses of human PBMC at week 5, in which the BLI signal was clearly visible in the image analysis (approximately twofold above the lower BLI baseline). To facilitate the engraftment of human cells, resident murine macrophage function was suppressed by i.v. injection of 2-chloromethyl biphosphonate liposomes, one day prior to the PBMC injection. The effect of the PBMC infusions on the myeloma tumor load was monitored by BLI. Mice were also monitored for the development of xenogeneic graft-versus-host disease (GVHD) as described previously.¹⁴ The diagnosis of xenogeneic GVHD diagnosis was based on a combination of poor physical condition of the mice, hunched posture, ruffled fur, a body weight loss above 20%, and high human T cell engraftment of >50%.

Table 1. Outgrowth of different myeloma cell lines after i.v. injection in RAG2^{-/-}γC^{-/-} mice

Cell line	# Cells (i.v.)	Location	Experiment termination*	Time of death mean (wks)
U266	5 x 10 ⁶	skeleton	paralysis	13
UM-9	20 x 10 ⁶	skeleton	paralysis	12
RPMI-8226/S	5 x 10 ⁶	skeleton	paralysis	8
XG-1	15 x 10 ⁶	skeleton	paralysis	8
L-363	5 x 10 ⁶	skeleton	paralysis	8
OPM-1	5 x 10 ⁶	skeleton, liver	weight loss	4
LME-1	5 x 10 ⁶	skeleton, intestinal tract	weight loss	5

Seven cell lines were transduced with the GFP-LUC gene, selected for GFP+ cells, and injected intravenously in RAG2^{-/-}γC^{-/-} mice. The location of myeloma growth was determined by BLI during the terminal stage of myeloma development.

* reason to sacrifice the animals; hind leg paralysis or loss of >20% of body weight

Results

Engraftment and tumor outgrowth of multiple myeloma cell lines in RAG2^{-/-}γC^{-/-} mice

To test the suitability of RAG2^{-/-}γC^{-/-} mice for generating a useful myeloma murine model, seven different myeloma cell lines (U266, RPMI-8226/S, XG-1, L-363, UM-9, LME-1, and OPM-1) were transduced with GFP-luciferase and inoculated in the mice. The U266, RPMI-8226/S, XG-1, L-363, and UM-9 cell lines revealed similar results in terms of selective growth (survival time 7-12 weeks) in the bone marrow compartment only. The LME-1 and OPM-1 lines also showed growth in soft tissues, such as the liver and intestines (table 1).

Detailed results for the two cell lines, U266 and RPMI-8226/S are depicted in figures 1 and 2. After i.v. injection of the mice with 5 x 10⁶ transduced myeloma cells the first signs of tumor growth, i.e. areas of luciferase activity, in the mice were detectable in the femur and/or tibia after two weeks, followed by additional foci of luciferase activity in the pelvic region, skull, limbs, sternum, ribs, and the spinal vertebrae (figure 1A and 1B). On average 5-15 foci of myeloma growth were detected per mouse, although the pattern of distribution varied; it was different for each mouse (figure 1A, 1B, and 1C). Myeloma growth in the vertebrae resulted in the development of hind leg paralysis in late-stage disease in about 70% of the animals and was a reason to sacrifice the mice. The non-paralyzed mice also became moribund and were sacrificed when they lost 20% of their body weight. BLI revealed no myeloma lesion in soft tissues such as spleen, lung, liver, and kidneys (figure 1C).

In the terminal stage of U266 myeloma growth, mice were sacrificed and from several bone and soft tissue samples cell suspensions were prepared and analyzed by FACS (10,000-100,000 cells per sample) for the presence of myeloma cells. All bone marrow specimens

that were positive for BLI *in vivo*, contained large numbers of myeloma cells. Very few or no myeloma cells were found in specimens that did not reveal BLI signals. The expression pattern of GFP, CD45, CD38, CD138, and CD86 was not changed after the *in vivo* passage (data not shown). For the U266-derived tumors, we examined the presence of myeloma cells in soft tissues (table 2). Using flow cytometry as a read-out system with a lower threshold of 0.005%, we did not detect any GFP positive cell (table 2).

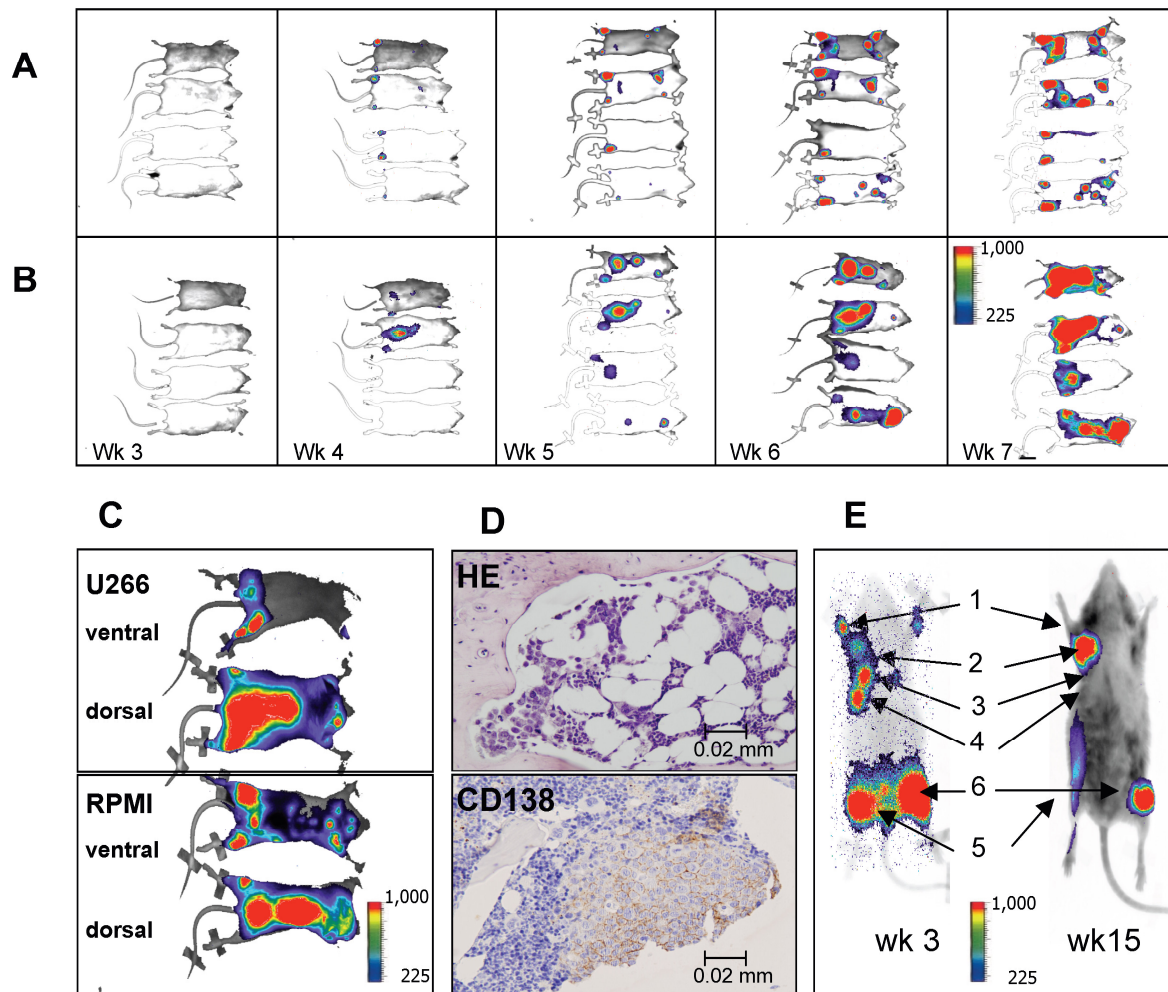


Figure 1. In vivo growth dynamics of luciferase gene marked RPMI-8226/S and U266 Multiple Myeloma Bioluminescence images (BLI) taken from the ventral side **(A)** or from the dorsal side **(B)** of the mice at five sequential time points from week 3 to 7, with one week interval, after i.v. injection with GFP-LUC transduced RPMI-8226/S myeloma cells. **(C)** Higher magnification images taken from the dorsal and ventral side showing distinct foci of myeloma growth at various sites in the BM compartment of the mice inoculated with U266 or with RPMI-8226/S. **(D)** Presence of myeloma cells in the bone marrow of a mouse injected with U266 shown by hematoxylin-eosin (HE) staining and by immuno-staining for expression of CD138. **(E)** Location of various foci of U266 myeloma growth before total body irradiation (week 3) and after myeloma re-growth measured at week 15.

All images are depicted in false colour, blue is at least 225 a.u./pixel and red is a maximal light intensity of 1,000 a.u./pixel.

Table 2. Localization of myeloma lesions in RAG2^{-/-}γC^{-/-} mice detected by bioluminescence imaging confirmed by FACS analysis

Location	BLI (a.u.) range	FACS GFP ⁺ cells	Myeloma cells detected	Correlation between FACS and BLI
skeletal parts				
femur	1.4 x 10 ⁵ – 1.5 x 10 ⁷	1.0 – 2.3	14/14	yes
tibia	5.0 x 10 ⁴ – 1.2 x 10 ⁷	0.8 – 21.8	14/14	yes
humerus	1.2 x 10 ⁵ – 9.0 x 10 ⁶	0.9 – 10.2	14/14	yes
sternum	BDL	<0.01	6/7*	yes
soft tissues				
spleen	BDL	<0.005	0/7	yes
lungs	BDL	<0.005	0/7	yes
liver	BDL	<0.005	0/7	yes
kidney	BDL	<0.005	0/7	yes
brain	BDL	<0.005	0/7	yes
blood		<0.01	-	-

The overall distribution of myeloma cells was assessed by bioluminescence imaging (BLI) in seven mice, nine weeks after inoculation of 5 x 10⁶ U266 myeloma cells i.v. Cell isolates from various skeletal parts and soft tissues were analyzed by flow cytometry (FACS) for the presence of GFP⁺ cells in the total nucleated cell fraction; 100,000 cells were analyzed per sample (10,000 for the blood).

BLI is expressed as arbitrary light units (a.u.).

BDL: below detection limit (in region not covered by skeletal parts)

* One sternum showed a small BLI signal not confirmed by FACS analysis.

Although these results do not completely exclude the presence of myeloma in soft tissues, they indicate that soft tissues were not the primary site for myeloma growth for five out of the seven cell lines that we studied. This predominant bone marrow outgrowth was also observed for RPMI-8226/s, XG-I, L-363, and UM-9 (data not shown), indicating that in this model myeloma cell homing and growth strongly resembles that occurring in human myeloma. Two other lines LME-I and OPM-I appeared to show not only medullary, but also extramedullary growth (data not shown).

Based on sequential BLI measurements, the growth curves of U266 and RPMI-8226/s show an exponential increase of the myeloma tumor cell load over time, for the whole mouse (figure 2A and 2B) as well as for individual foci of myeloma scattered throughout the bone marrow compartment (figure 2C and 2D). The myeloma tumor growth could be monitored over a range of 4 log (a factor of 10,000). The mice inoculated with U266-tumors survived 13.6 ± 2.4 weeks. The RPMI-8226/s tumors appeared to develop a little more rapidly such that animals with these tumors had a mean survival time of 7.9 ± 0.8 weeks. The medullary

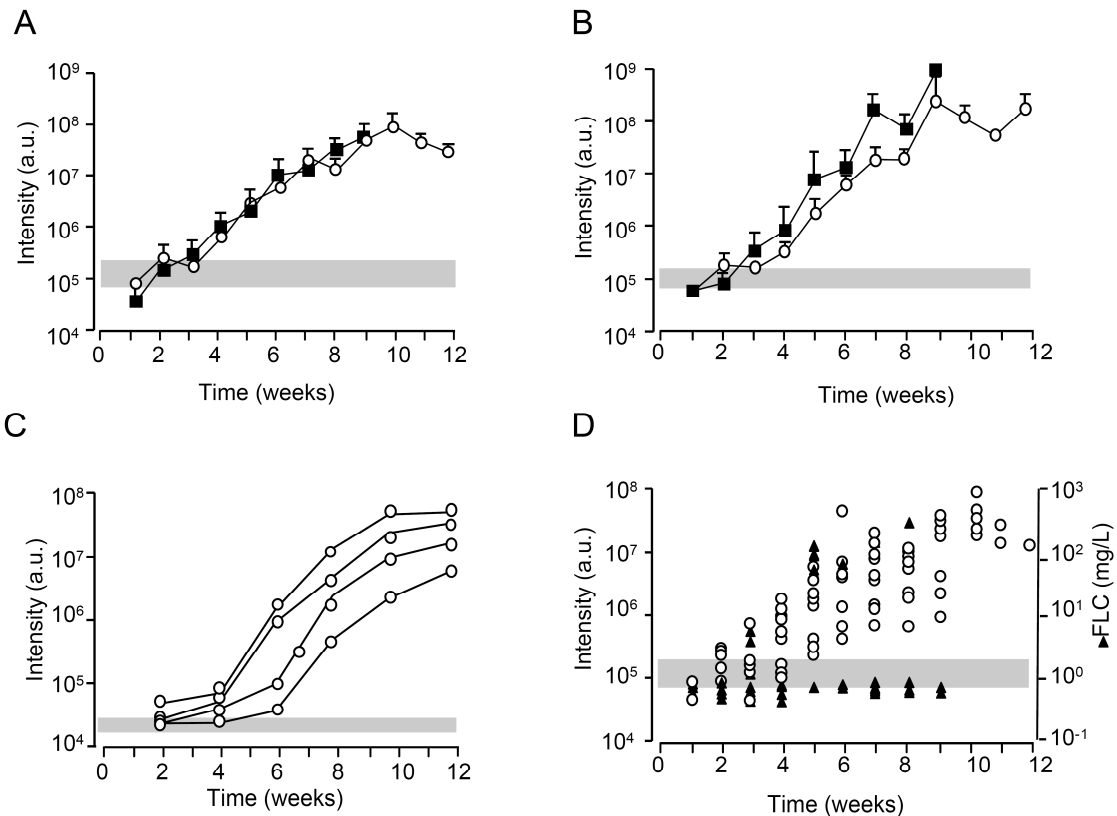


Figure 2. BLI based growth kinetics of RPMI-8226/S and U266 myeloma in RAG2- γ c-/- mice. Myeloma growth curves based on bioluminescence photon emission measurements and shown as mean \pm SD (n=8). The curves reflect the increase in photon emission measured for the whole mouse inoculated with RPMI-8226/S (■) or U266 myeloma cells (O) and imaged from the ventral side (A) as well as from the dorsal side (B) at various time intervals after inoculation of the myeloma cells. (C) Growth curves for several individual foci of bioluminescence from RPMI-8226/S in a single mouse imaged from the ventral side. (D) Comparison between levels of bioluminescence (O) and Free-light-chain (FLC) (▲) concentrations that are recorded on several time points during Multiple Myeloma (U266) growth FLC is measured in the urine. The grey area at the bottom of the graphs represents the mean photon emission count from an identical sized control region in the same image over the 15 weeks.

and extramedullary growing cell lines LME-I and OPM-I behaved as more aggressive tumors and animals inoculated with these cells survived approximately four to five weeks. In these mice the initial signs of tumor growth were in the femoral and tibial areas; soon thereafter fast growing myeloma tumors were observed in the liver (OPM-I) or in the abdominal (intestinal tract) region (LME-I) and in the rest of the skeleton.

Comparison of BLI- and free immunoglobulin light chain-based tumor monitoring

A sensitive parameter that has been recognized as an indicator of myeloma burden is the concentration of FLC in urine or plasma. *In vitro*, U266 cells produce high concentrations of FLC in culture medium. We therefore compared the sensitivity of tumor monitoring either with BLI or urine FLC measurements in 12 mice that were inoculated with U266 cells.

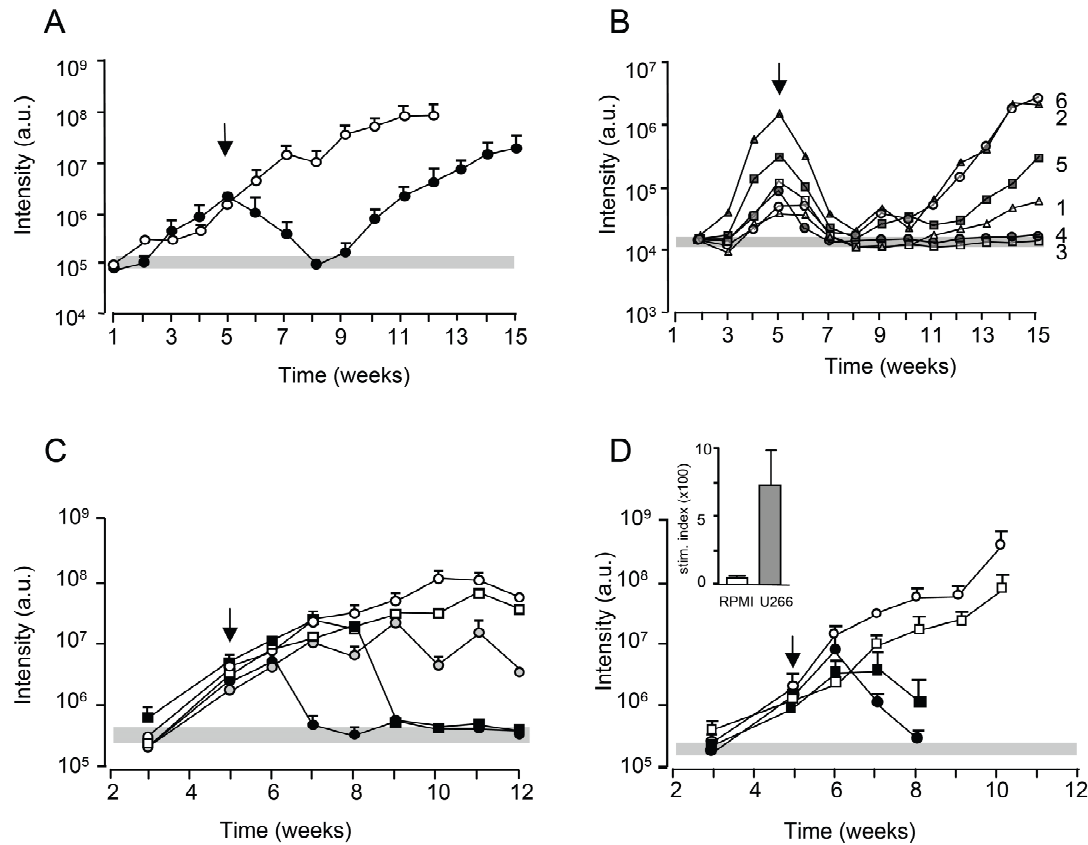


Figure 3. BLI based monitoring of myeloma tumor reduction after radiotherapy (total body irradiation, TBI) or the graft-versus-myeloma effect by cellular immunotherapy with human PBMC **(A)** RAG2-/- γ c-/- mice were inoculated i.v. with U266 cells and subjected to TBI in week 5 (indicated by the arrow). The myeloma tumor load in treated mice (●) and in non-irradiated control mice (○) was monitored weekly with BLI. The curves show the bioluminescence photon emission in arbitrary units (a.u.) for the whole mouse \pm SD (n=4). **(B)** Individual foci of myeloma growth (1 through 6) in one representative mouse. The BLI images of this mouse are shown in Figure 1E and the corresponding foci of myeloma 1 through 6 are indicated. **(C)** GVM effect in mice with U266 myeloma cells after inoculation of 40 x 10^6 T cells (●) and 10 x 10^6 T cells (○) in week 5 (indicated by the arrow) compared to a non-treated group of mice (○). The GVM effect was also evident after inoculation of 10 x 10^6 T cells in CL2MDP liposome pretreated mice (■) and is compared with only liposome treated animals (□). **(D)** RAG2-/- γ c-/- mice were inoculated with 5 x 10^6 RPMI-8226/S or 5 x 10^6 U266 myeloma cells. At week 5 all mice were pretreated with CL2MDP liposomes followed by inoculation of 10 x 10^6 T cells the next day (indicated by the arrow). The GVM effect is shown in the RPMI-8226/S (■) and the U266 mice (●) compared with the non-treated RPMI-8226/S (□) and U266 mice (○). The bar graph inset shows the results of the in vitro mixed lymphocyte reaction analysis to RPMI-8226/S and U266 myeloma cells respectively. The grey area at the bottom of the graphs represents the mean photon emission count from an identical sized control region in the same image over the 12 weeks.

While in all mice clearly increasing BLI-signals were observed starting from week 2 and onwards, in six out of nine mice the FLC measurements remained negative until week 8 (figure 2D), indicating that in our model BLI is a more sensitive method for monitoring tumor load. At all time points the plasma creatinine levels remained normal (data not shown), indicating that the measured FLC concentrations were not confounded by renal dysfunction.

Suitability of BLI for tumor monitoring after therapeutic intervention

To evaluate whether the newly developed myeloma model was suitable for sensitive tumor monitoring after therapeutic intervention, we first subjected mice bearing U266-tumors to a dose of 6.0 Gy x-rays TBI, which is the maximum tolerated dose for this strain of mouse.²² At the time of TBI (week 5), the BLI signals were approximately 10-20 times higher than baseline level. As depicted in figure 3A, the BLI signals in treated mice were significantly reduced to baseline levels within two weeks. In the meantime, the controls showed a 10-fold increase in signal. Thus, at the nadir there was a 100-fold (2 log) difference in tumor load between the treated mice and the control mice. However, BLI measurements beyond the second week after treatment revealed that, similar to the human situation, TBI was not sufficient to eradicate the myeloma tumors. This was evident from the steady increase of BLI signals with kinetics similar to that of the non-treated control group. These experiments demonstrated that the intervention in tumor growth in the new model was possible and that BLI could easily and sensitively monitor tumor regression and progression. In addition, BLI appeared suitable to monitor individual tumor foci. A representative mouse depicted in figure 3B and figure 1E illustrates that, before TBI, two of the total six myeloma foci detected in this mouse displayed weak BLI signals. Following treatment, these two foci disappeared completely, indicating eradication of myeloma from the affected bones. The foci with high BLI signals, however, showed only temporary regression by TBI treatment and eventually showed progression (figure 1E and 3B).

Table 3. Development of xenogeneic GVHD and GVM effects in PBMC-treated mice with U266-tumors

Group	N	TRM	X-GVHD symptoms	Cause of death		
				X-GVHD ^a	Myeloma ^b	Myeloma-free ^c
Untreated myeloma controls	35	0	0	0	3	50
40 x 10 ⁶ T cells	7	0	7	7	0	7
10 x 10 ⁶ T cells	6	1	5	0	5	0
Liposomes only controls	16	6	0	0	10	0
Liposomes + 10 x 10 ⁶ PBMC	31	5	26	26*	0	20
Liposomes + 5 x 10 ⁶ PBMC	6	1	5	4	1	0

Data are pooled from four independent experiments with PBMC of four different individuals.

Treatment: day 0: 5 x 10⁶ myeloma cells; day 35: Cl2MDP-liposomes (i.v.); day 36 PBMC i.v. (based on % CD3+) as indicated.

TRM treatment related mortality (death <2 weeks after treatment, not GvH or myeloma related)

A GVHD: graft-versus-host disease, i.e. severe weight loss, ruffled fur, and high huCD45 chimerism in blood.

B myeloma: high myeloma- associated luciferase signal plus hind leg paralysis

C myeloma free: no myeloma- associated luciferase signal (BLI)

* based on BLI, 20 animals were myeloma-free and six had substantially reduced BLI signal at time of death.

Induction of a GVM effect in the $RAG2^{-/-}\gamma C^{-/-}$ based human myeloma model

One of our most important goals was to develop a myeloma model suitable for evaluation of cellular immunotherapy approaches, such as donor lymphocyte infusions (DLI). To this end, we first explored whether infusion of human PBMC in U266-bearing mice would induce a GVM effect. Human PBMC containing either 10×10^6 or 40×10^6 T cells were injected in mice at week 5, when the U266-tumors were clearly detectable by BLI (figure 3c). Another group received PBMC containing 10×10^6 T cells, one day after the mice had been preconditioned with clodronate-containing liposomes. The preconditioning depletes murine macrophages and in our experience this improves the engraftment of human cells in the $RAG2^{-/-}\gamma C^{-/-}$ mice.^{14,22} In a control group, liposomes had no anti-tumor effect (figure 3c). As expected, all animals treated with human PBMC developed, from week 7 onwards, a cell-dose-dependent xenogeneic GVHD (data not shown). In mice treated with 40×10^6 T cells, the BLI signals started to drop for all tumor locations one week after the onset of xenogeneic GVHD. BLI signals decreased below the lower threshold levels within two weeks (figure 3c). All mice were free of myeloma, but eventually died from lethal xenogeneic GVHD between weeks 8-12 (table 3). At the T cell dose of 10×10^6 (without liposome pretreatment) the xenogeneic GVHD and GVM effects were merely sufficient to stabilize the tumor progression, but did not lead to tumor clearance. Only one out of six mice developed a clear xenogeneic graft-versus-host reaction. However, when combined with liposome pretreatment, 10×10^6 T cells mediated strong xenogeneic graft-versus-host and GVM effects and similar results as for the 40×10^6 T cell group were observed.

Thirty-one mice were treated with PBMC from three different donors. Five animals died early from treatment-related toxicity. The remaining 26 mice all developed GVHD, although less rapidly than the group of mice that received 40×10^6 T cells; six mice died within three weeks, however, already with significantly reduced tumor load. The remaining 20 mice survived longer, but all mice ultimately died from xenogeneic GVHD between week 8-12. All these mice, however, were free of myeloma (figure 3c and table 3).

In our *in vitro* mixed lymphocyte reactions, the RPMI-8226/s cell line consistently failed to induce significant T cell proliferation from PBMC of five different donors, while the U266 cell line was highly immunogenic (figure 3D inset). The stimulation index is 697 ± 581 for the U266 versus 30 ± 48 for the RPMI. This suggested that RPMI-8226/s-derived tumors could represent, less immunogenic myeloma tumors in our new model. To test this we compared the development of GVM effects against U266- or RPMI-8226/s-derived tumors in a separate experiment. Mice bearing either U266- or RPMI-8226/s- tumors received PBMC containing 10×10^6 T cells one day after the liposome conditioning at week 5 after inoculation of the myeloma cells. All mice developed severe xenogeneic GVHD and died

between week 8 and 9. BLI measurements performed within this short period revealed that the GVM effect was more pronounced for the U266-tumors than for the RPMI-8226/s ones (figure 3D). Tumor load reduction started in the U266 group at week 7, one week earlier than in the RPMI-8226/s group. At week 9, when the experiments were terminated due to severe xenogeneic GVHD, tumor load in the U266 group had almost returned to baseline levels ($10 \pm 9\%$ of starting signal), whereas the tumor load in the RPMI-8226/s group was greater ($91 \pm 53\%$), with the difference being statistically significantly ($p < 0.05$). These results suggest that RPMI-8226/s-tumors might be used as a model for non-immunogenic or DLI-resistant tumors when testing the efficacy of new combination therapies.

Discussion

In this study we present a convenient and reproducible *in vivo* model for human myeloma that can facilitate pre-clinical testing of cellular therapy strategies in combination with immunomodulatory agents to improve GVM effects after allogeneic stem cell transplantation. Since we aimed at convenient and sensitive immunomonitoring of tumor load, the model was established by intravenous transfer of GFP-luciferase transduced myeloma cell lines in $RAG2^{-/-}\gamma C^{-/-}$ mice. Despite the use of myeloma cell lines, five out of seven tested myeloma cell lines developed tumors with phenotypic dissemination, and growth patterns highly similar to those of human myeloma. In particular, the multifocal growth predominantly in the bone marrow, production of FLC, and expression of myeloma-associated molecule CD138 by the established tumors, are the most striking similarities with human myeloma. Furthermore, the differences in the *in vivo* growth kinetics and immunogenicity of the myeloma cell lines used enables us a comprehensive evaluation of novel strategies on various predefined conditions.

Previously, other investigators developed myeloma models by injecting myeloma cell lines into nod/scid mice.³⁻¹¹ A recently developed model also exploited the advantages of fluorescence-based optical imaging, which has a high resolution for GFP imaging and also allows frequent and serial non-invasive monitoring.^{24,25} While highly useful and convenient, an important drawback of this recent model was the extramedullary growth of tumors, which is not a typical feature of myeloma.

The mechanisms controlling the homing behavior of myeloma cells to the bone marrow have not yet been elucidated but it is believed that chemokines and their respective receptor counterparts involved in lymphocyte trafficking and homing also play a role in myeloma homing. Differential expression of chemokine receptors has been reported for primary myeloma²⁶ as well as for myeloma cell lines.²⁷ It has been shown that SDF-1/CXCL12 is a critical regulator of myeloma homing.²⁸ Murine SDF-1, expressed by the

stromal elements in mouse bone marrow, which can interact with human hematopoietic cells,²⁹ may play an important role in this process. Whatever the reason, extramedullary growth may introduce a significant bias if the model is aimed at testing the efficacy of cellular immunotherapy strategies. Furthermore, outgrowth of human τ cells in the NOD/SCID mice is far less reproducible than that in $RAG2^{-/-}\gamma C^{-/-}$ mice.¹⁴ Thus, in our view, our model offers several advantages over the NOD/SCID model and may therefore be better suited for preclinical testing of cellular immunotherapeutic approaches. Furthermore, the availability of other cell lines with different growth kinetics and with medullary or extramedullary outgrowth that we developed, gives us the possibility of comparing the efficacy of combination therapies in more detail. Nevertheless, it should be noted that our model, which uses cell lines rather than primary tumor cells, may be less suitable for studying a number of the biologic aspects of myeloma such as stroma-myeloma interactions or natural growth kinetics. Thus far we have not tested whether $RAG2^{-/-}\gamma C^{-/-}$ immune deficient mice facilitate the outgrowth of primary human myeloma cells after intravenous transfer in a similar way as has been described by other investigators who showed that primary myeloma cells can only engraft when injected in combination with previously implanted human fetal bone^{3,10} or rabbit bone fragments.¹¹

In our study, an important reason why myeloma cell lines were preferred to primary myeloma cells was to exploit the advantages of BLI as a sensitive and non-invasive technology for tumor monitoring. In our model, BLI appeared superior to FLC-based monitoring and allowed semi-quantitative monitoring of individual myeloma foci. Indeed, BLI appeared not only suitable for quantitative detection of the tumor load in the whole body, but it was also highly useful for visualization and quantitative analysis of individual foci of myeloma growth throughout the marrow compartment. Bioluminescence is very sensitive for the detection of low levels of marker gene expression,³⁰ allowing the detection of even small tumors, an aspect that becomes relevant when monitoring small tumors after therapy. Obviously, the location of the tumor (depth inside the animals) influences the lower detection limits of BLI. This is clearly seen in panel 2c for the individual foci of growth. However, once the light signals from a tumor at a given location reach levels beyond the baseline, the doubling times are similar indicating that the growth kinetics are not effected by the location. A positive correlation between tumor burden and BLI signal intensity has been shown in a variety of *in vivo* studies of animal tumor models.³⁰⁻³⁴ We show here that this correlation also applies to the human myeloma cell lines that we studied and confirms reports with human myeloma by others.³⁵⁻³⁷ This unique advantage enables us to evaluate the effects of therapeutic interventions on individual tumor foci, which often differ in size.

To evaluate the application areas of our model we treated established full-blown tumors with radiotherapy as well as with allogeneic cellular immunotherapy. Radiotherapy resulted in a slow decay in tumor load, initially diminishing the tumor load to below the detection limits of the BLI. Based on the whole body BLI measurements we calculated that an approximate 2 log tumor reduction was achieved by radiotherapy, which is in agreement with the radiosensitivity of the clonogenic myeloma cells.³⁸ While for small individual lesions radiotherapy seemed to be sufficient for tumor eradication (there was no reappearance of the tumor), this was clearly not the case for the larger tumor foci. Tumors in these latter foci eventually progressed at the same pace, with the radiotherapy providing only a six-week prolongation in overall survival. These results, therefore, once again emphasize the need for additional therapies after radiotherapy. Most importantly, however, the radiotherapy experiments illustrated the feasibility of using BLI as a sensitive technology for tumor monitoring.

Finally, the results of allo-immune therapy experiments demonstrated that the myeloma model developed in $RAG2^{-/-}\gamma C^{-/-}$ mice is very useful for preclinical testing of cellular immune therapies, as the mice easily permit the engraftment and outgrowth of human T cells. The potent GVM effect obtained by infusion of allogeneic, thus MHC-mismatched, human T cells reveals first of all the proof of principle that it may be possible to eradicate full-blown myeloma by potent allo-immune cellular therapies. Secondly, the results obtained so far suggest that the cell dose and immunogenicity of myeloma cells are important parameters determining the chances of cure. While the U266-derived tumors could be completely eradicated by allo-immune treatment containing 10×10^6 T cells, the RPMI-8226/s-based tumors appeared less susceptible to the same treatment dose. Furthermore, in *in vitro* assays, the RPMI-8226/s cell line evoked little or poor responses from several HLA-mismatched PBMC. Taken together these results suggest that RPMI-8226/s-based tumors are less immunogenic than U266-derived tumors, providing us a unique opportunity to compare and optimize the efficacy of cellular immunotherapy approaches.

While at this stage we used HLA-mismatched PBMC to develop the model, it would obviously be quite interesting to evaluate the GVM effects of three locus HLA-matched PBMC, which can be selected among HLA-typed blood-bank donations. Such a model will obviously be more comparable to HLA-identical matched unrelated donor transplantation. As mice also develop xenogeneic GVHD after infusion of human PBMC, effects on xenogeneic GVHD can be used as a control for all therapeutic interventions in combination with infusion of allogeneic lymphocytes. In this respect, we think that one of the interesting application areas of our current model will be the evaluation of the impact of

regulatory T cells on the GVM effect, since we have recently shown in this model that co-infusion of human PBMC with autologous regulatory T cells effectively downregulates the lethal xenogeneic GVHD.¹⁵

In conclusion, we present here a model of human myeloma growth in an immunodeficient mouse, strongly resembling human myeloma. This model can be used to monitor non-invasively and quantitatively the outgrowth of myeloma cells. It offers the possibility for preclinical evaluation of several therapeutic strategies and may therefore facilitate the design of optimal protocols for multiple myeloma treatment.

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General discussion

chapterseven

General discussion

Multiple myeloma is a plasma cell malignancy that runs a progressive clinical course. Due to the introduction of novel drugs, like bortezomib, thalidomide, and lenalidomide, median overall survival for newly diagnosed myeloma patients has been improved in the last decade from 29.9 to 44.8 months.¹ However, the disease is still incurable, resulting in an ongoing need for drug development. Statins effectively induce programmed cell death and inhibition of proliferation in myeloma cells *in vitro*, by inhibition of the mevalonate pathway,² and more specifically by inhibition of geranylgeranylation.³⁻⁵ In addition, cell adhesion-mediated drug resistance (CAM-DR) is inhibited by statins like simvastatin and in this way the protective effects of fibronectin may be overcome.⁶ Furthermore, statins synergize with dexamethasone, doxorubicin, zoledronate, FTI, and UCN-01 resulting in improved *in vitro* cytotoxicity.^{2,7-9} Since statins are orally available, have few side effects, and are relatively inexpensive, they are attractive drugs to deploy in multiple myeloma. Furthermore, there is extensive clinical experience with statins.¹⁰ This thesis discusses the rationale, efficacy and future perspectives of statin use as a treatment strategy in multiple myeloma.

The first focus of this thesis was to assess the clinical utility of statins in myeloma patients. In **chapter two** results of the first clinical experience with high-dose simvastatin in myeloma are reported. It was a phase I dose-escalating study using simvastatin in a dosage ranging from 5 - 17.5 mg/kg during seven days combined with chemotherapy in myeloma and lymphoma patients.¹¹ The subsequent phase II study was exclusively performed in refractory myeloma patients, and is described in **chapter three**. High-dose simvastatin was combined with VAD and showed only one partial response in 12 patients, which was insufficient to continue the study according to predefined criteria.¹² In **chapter four** a synergistic cytotoxic activity *in vitro* of simvastatin and lenalidomide is shown in myeloma plasma cells, possibly through downregulation of pSTAT3- and cleavage of caspase-8. Subsequently, in **chapter five** microarray technology helped to define targets in statin-induced cell death, which may facilitate the discovery of new drugs in myeloma. Finally, **chapter six** described a new mouse model using bioluminescence to monitor myeloma cell growth *in vivo*. This model can efficiently monitor the efficacy of new medications.

Feasibility of high-dose statins

In the dose-escalating study, we show the feasibility of high-dose statins combined with chemotherapy in extensively pretreated myeloma- and lymphoma patients.¹¹ The maximum tolerated dose was 15 mg/kg/day simvastatin during seven days, combined with

chemotherapy. Dose-limiting toxicities were gastrointestinal complaints and neutropenic fever. One patient died from neutropenic sepsis after treatment with dose level 17.5 mg/kg/day simvastatin. The subsequent cohort of patients was treated with prophylactic antibiotics, and hereafter no neutropenic fever was observed. There was a relatively high incidence of neutropenia (38% grade ≥ 3) after treatment with simvastatin followed by chemotherapy. This might indicate that simvastatin enhances bone marrow suppression, although such incidence of bone marrow toxicity by chemotherapy alone may not be unusual in extensively pretreated patients.

HMG-CoA reductase inhibitors are widely used throughout the world, and, in regular dosage, they exhibit a mild safety profile; the most serious adverse effect is rhabdomyolysis, but this is relatively uncommon.^{10,13} Using high dosage simvastatin during seven days, we neither saw rhabdomyolysis, nor liver toxicity. Previously, one dose-escalating study using lovastatin was published showing the feasibility of high-dose lovastatin in cancer patients. Twenty-five mg/kg/day lovastatin for seven consecutive days was well tolerated.¹⁴ This study described myopathy as dose-limiting toxicity, conspicuously a side effect that we hardly observed in our study. In a recently published study of the effects of 15 mg/kg simvastatin during seven days in six myeloma patients on markers of bone turnover, all patients experienced grade 1-2 gastrointestinal side effects, which is slightly more than in our study. Myalgia grade 1-2 was reported in five patients without an increase in creatinine kinase. Two patients died in week 3 of pneumonia without neutropenia. There was no response reported.

In our dose escalating study, the response rate was 30% (CR + PR + MR), which is remarkable considering the fact that all patients were heavily pre-treated. However, it cannot be concluded from this study that simvastatin pre-treatment contributed to the response and disease stabilisation. Patients previously treated with anthracyclines and prednisone/dexamethasone may respond again to regimens containing these agents. It is, however, worth noting that three out of the seven patients who responded were refractory to CHOP or VAD chemotherapy.

Clinical utility of high-dose statins in myeloma

Our phase II study showed only one partial response in 12 myeloma patients using 15 mg/kg/day simvastatin followed by VAD chemotherapy. However, all patients were heavily pretreated with a median of 4 (range 2-6) lines of chemotherapy; all patients had received thalidomide and the majority (83%) had received bortezomib.¹² Non-hematologic side effects were mild; only one patient experienced grade 3 nausea. Grade 3/4 neutropenia was the most observed hematologic side effect.

The question is why statins are very effective in inducing apoptosis, inhibiting proliferation, and overcoming CAM-DR in myeloma *in vitro*, but not *in vivo*. Two explanations are conceivable. First, administration of 15 mg/kg simvastatin is insufficient to reach plasma levels similar to dosages that are effective in *in vitro* experiments. Second, in myeloma patients additional mechanisms exist, like multi-drug resistance, which inactivate the effect of simvastatin on the plasma cell.

Insufficient plasma levels in vivo

Simvastatin 40 mg, a dosage used to treat hypercholesterolemia, has a peak concentration (C_{max}) of 10–34 ng/ml, which is equivalent to 23.9 – 81.2 nM.¹⁵ Effective dosages used in *in vitro* studies that induce apoptosis and inhibit proliferation in myeloma cells range from 1 μ M to >100 μ M, which is 10 – 1000 fold higher than reached by ‘antihypercholesterolemic dosages’. High statin plasma levels are hard to reach due to an intestinal first-pass effect. Actually, fluvastatin is the only statin able to achieve peak plasma concentration in the micromolar range, when used in dosages used to treat hypercholesterolemia.¹⁶ Unfortunately, due to the shortest half-life of all available statins, this exposure is only short-lived. In addition, in humans, peak concentration of simvastatin is reached only for a brief moment because of the short $t_{1/2}$ of two hours,¹⁷ in contrast to *in vitro* tests, where ‘plasma levels’ are stable. In the study from Thibault, dosages of 2 to 45 mg/kg of lovastatin were tested in cancer patients. Peak levels ranged from 0.10–3.92 μ M (mean \pm SD, 2.32 ± 1.27 μ M). Trough levels at the 25 mg/kg dose and higher averaged 0.28 ± 0.09 μ M. More importantly, plasma levels showed marked interpatient variability and there was no direct relationship to the dose administered.¹⁴ In our phase II study, we measured simvastatin peak and trough plasma levels and reached similar levels.

Both lovastatin and simvastatin are both CYP3A4 substrates. As a consequence, this interpatient variability can likely be explained through interindividual differences in CYP3A4 metabolism, caused by genetic and environmental differences and, possibly, age.¹⁸ As a consequence, simvastatin plasma levels reached *in vivo* are unpredictable. However, even the highest peak plasma levels reached are lower than the concentrations that are effective *in vitro*.

Additional mechanisms

Whether additional mechanisms are important in *in vivo* resistance is uncertain. Heavily pretreated patients, like our study population, have usually developed multi-drug resistance by activation of anti-apoptotic pathways and expression of multidrug resistance (MDR)-related proteins.¹⁹ A possible explanation for statin-resistance may be enhanced expression of P-glycoprotein (PGP). Pgp-expression is increased in patients treated with

VAD.²⁰ Previously, it was demonstrated that lovastatin sensitivity is reduced with enhanced PGP expression.² However, to demonstrate whether this evokes the lack of effect of statins, however, adequate statin plasma levels should be reached first.

Strategies to improve the clinical anti-myeloma efficacy of statins

Statins are remarkably effective *in vitro* and are relatively non-toxic, easily available and inexpensive. This urges the further exploration of strategies to improve its clinical efficacy. Four strategies can be considered: First, seeking possibilities to raise the plasma levels of statins. Second, considering application of other statins to reach greater bioavailability. Third, testing combinations of statins with anti-myeloma agents that show more synergy. Fourth, identifying specific target genes of simvastatin that are involved in the *in vitro* myeloma cell kill.

1. Raising plasma levels

Intravenous administration of statins avoids the intestinal first pass effect, enabling higher plasma levels. Unfortunately, intravenous formulations are currently not available. Recent endeavors by neurologists for intravenous statin formulations is encouraging and increases their chance of development by pharmaceutical companies.¹⁹ Another, perhaps more controversial option to reach higher simvastatin or lovastatin plasma levels, is combining these HMG-CoA reductase inhibitors with drugs that inhibit CYP3A4. Previous studies showed increase of AUC and peak plasma drug concentration (C_{max}) of both simvastatin and lovastatin in combination with grapefruit juice^{20,21} and verapamil.²² High intra- and interpatient variability hampers this approach in individual patients.²³ This option will therefore only be conceivable with extensive measurement of statin levels and monitoring CK levels to detect rhabdomyolysis.

2. Statins with greater bioavailability

Since all statins have different characteristics, including half life, bioavailability, and potency,²⁴ it is reasonable to assume a difference in potency against myeloma. Lipophilic statins, like simvastatin and atorvastatin, are favored over hydrophilic statins, like rosuvastatin and pravastatin, as treatment for multiple myeloma, since lipophilic statins will readily enter extrahepatic cells,¹⁷ including malignant cells. Conversely, hydrophilic statins, show greater hepatoselectivity,^{25,26} and will theoretically be less effective. One of the disadvantages of simvastatin is its short half life of two hours. Most others statins have similar short half-lives of three hours or less, except for rosuvastatin (19 hours) and atorvastatin (14 hours).¹⁷ Based on its favourable half-life atorvastatin could be considered as a possible alternative, although *in vitro* myeloma cell lines were less sensitive for atorvastatin, compared to simvastatin.²⁷ Instead of statins, direct inhibition of

geranylgeranylation can be considered, but unfortunately, at this moment, inhibitors of geranylgeranylation (GGTIs) are not clinically available.

3. Search for combinations with anti-myeloma agents that show more synergy

Since plasma levels needed for tumor cell kill are hard to reach *in vivo* and several studies showed synergy between statins and other compounds in levels that are reached *in vivo*,^{2,7-9,28} combining statins with these synergistic conventional or novel anti-myeloma agents is a logical step. An additional advantage of combining drugs is the possibility to overcome drug resistance by simultaneous targeting different mechanisms of resistance, for instance agents that target the two main apoptosis pathways (the death receptor pathway and the intrinsic mitochondrial pathway).²⁹ One of the possible combinations to test *in vivo* is statins combined with lenalidomide. *In vitro*, the combination is synergistic, possibly through downregulation of pSTAT3 and enhanced caspase-8 cleavage.³⁰ Addition of GGOH and to a lesser extent FOH, abrogates the synergistic induction of apoptosis by the combination of simvastatin and lenalidomide. This indicates a role for geranylgeranylated proteins in the synergistic induction of apoptosis by lenalidomide and simvastatin. A potential target is RAC1. This small GTP-ase is involved in regulation of apoptosis and cell cycle progression, including myeloma cell growth. Statins affect RAC1 function *in vitro* by inhibition of geranylgeranylation.²⁷ Interestingly, RAC1 mediates STAT3 activation.^{31,32} Both are orally available and have relatively few side effects (**chapter four**). Patients who are refractory to lenalidomide treatment form an interesting population to test this combination. Since the combination of lenalidomide with simvastatin is synergistic, addition of simvastatin may restore sensitivity to lenalidomide. In order for the combination to have optimal synergistic effect, both agents should have maximum simultaneous bioavailability, suggesting simultaneous administration to be preferable. In our phase I and II studies, however, we used sequential rather than simultaneous administration, which may have negatively influenced the outcome. The short half-life of statins further adds to this effect.

4. Identification of the specific genes targeted by statins in the myeloma cell

Inhibition of the mevalonate pathway influences many downstream targets. The exact mechanism of statin-induced effects in myeloma, however, is still elusive. The knowledge of specific targets necessary for myeloma cell survival creates possibilities to develop more targeted and possibly less toxic drugs. Furthermore, it opens possibilities to identify rational based drug combinations with statins. In **chapter five**, an effort is made to define new targets of simvastatin in myeloma. Our genome wide microarray study shows interesting targets including apoptotic genes and genes involved in cellular proliferation.

The ten highest upregulated and downregulated genes by simvastatin included *rhob* (up), *EGR-1*, *CCR2*, *CDC25A*, and *CDK6* (down).

An interesting target is *rhob*, which in previous microarray studies ^{4,5} already appeared as highly differentially-expressed. *Rhob* is associated with anticancer properties and is a negative regulator of cell proliferation and is both geranylgeranylated and farnesylated.^{33,34} Furthermore, targets in the *G1/s* canonical pathway, like *CDK6* and the *p53* pathway, like *BBC3* and *p21* have been discovered using Ingenuity pathways analysis. Further studies have to indicate if these differentially-expressed genes play a functional role in myeloma cell kill by simvastatin. We are currently using RNA interference to investigate the role of *rhob* in simvastatin-induced effects.

Downregulation of *Mcl-1* was previously reported to be an important target of lovastatin in myeloma cell kill,³ and in our phase I study, we showed *Mcl-1* downregulation in myeloma plasma cells after high-dose simvastatin treatment using flow cytometry.¹¹ Unfortunately, we could not confirm our previous results in repeat studies, and cell lines and patient plasma cells treated with simvastatin no longer showed *Mcl-1* downregulation. In contrast, our microarray analysis revealed *Mcl-1* upregulation by simvastatin treatment. All six probesets representing *Mcl-1* show upregulation (fold change range 1.04-1.73), although only one probe set is significantly upregulated (1.38 fold). There is no good explanation for this caprice.

A possible expedient to test the clinical utility of statins is the use of a *RAG2*^{-/-}*γC*^{-/-} myeloma mouse model as described in **chapter six**.³⁵ Mouse models provide a rapid testing ground, and can excellently be used for preliminary work. In the article this mouse model is used to test cellular immunotherapy, but it can also perfectly be deployed to test *in vivo* activity of statins, possibly in combination with other agents. The advantage of this specific model compared with other mouse models is the possibility to monitor tumor-growth non-invasively and semi-quantitatively using bioluminescence. Bioluminescence is a very sensitive method for detection of even small tumors that can individually be monitored.³⁶ Furthermore, the localization of tumor growth is limited to the bone, resembling human myeloma, which is in contrast to many other myeloma mouse models showing extramedullary growth. This model can easily be used to test whether low dosage statins prevail over pulsatile high-dosage, or which drug combination with statins is most effective. When efficacy is proven, subsequently the effective regimens can be tested on myeloma patients. Feasibility of high-dose statins in SCID mice was previously shown.³⁷

Conclusion

Targeting the mevalonate pathway is an intriguing new treatment option in multiple myeloma. *In vitro*, statins very effectively induce apoptosis and inhibit proliferation in multiple myeloma plasma cells by inhibition of geranylgeranylation. However, current clinical strategies combining high-dose simvastatin with chemotherapy show that this regimen lacks satisfactory efficacy, probably due to inadequate plasma levels of statins. Statins are an attractive drug to use as treatment for multiple myeloma since long-term experience shows mild side effects, high-dosage statins is feasible, and prices of statins are low, certainly compared to the price of novel agents. New strategies need to be considered, exploring options to make use of the *in vitro* efficacy.

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Samenvatting
Dankwoord
Curriculum vitae

chaptereight

Samenvatting

Multipel myeloom, ook wel de ziekte van Kahler genoemd, is een vorm van beenmergkanker die vooral voorkomt bij oudere mensen. Bij multipel myeloom is er sprake van een kwaadaardige woekering van plasmacellen in het beenmerg. Plasmacellen behoren tot de witte bloedcellen, en spelen een rol in de afweer door antistoffen te produceren tegen virussen en bacteriën. Symptomen die passen bij multipel myeloom zijn onder andere botpijnen, bloedarmoede en verhoogde vatbaarheid voor infecties. Per jaar wordt in Nederland bij ongeveer 800 mensen de diagnose multipel myeloom gesteld. Hoewel er de laatste jaren een aantal effectieve nieuwe middelen bij zijn gekomen in de behandeling van myeloom is de ziekte nog steeds ongeneeslijk, en komt de aandoening uiteindelijk altijd terug. De helft van de patiënten leeft op dit moment na de diagnose langer dan 3,5 jaar. Jongere patiënten die met intensieve therapie worden behandeld leven nu gemiddeld vijf jaar.

Cholesterol verlagende middelen (ook wel statines genoemd) worden op grote schaal ingezet in de preventie van hart- en vaatziekten. Deze statines remmen de mevalonzuur route door de omzetting van HMG-CoA in mevalonzuur te onderdrukken. Eén van de producten die in deze mevalonzuur route geproduceerd wordt is cholesterol; op deze manier verlagen statines het cholesterol. Maar cholesterol is niet de enige stof die wordt geproduceerd in de mevalonzuur route; ook producten als farnesylpyrofosfaat en geranylgeranylpyrofosfaat worden gevormd in deze route. Deze twee stoffen zijn nodig voor de prenylering van bepaalde eiwitten. Prenylering zorgt ervoor dat deze eiwitten in de cel aan de binnenkant van de celmembranen worden 'verankerd'. Deze 'verankering' hebben die eiwitten nodig om actief te zijn. Belangrijke eiwitten die dit nodig hebben spelen een grote rol in celoverleving en celdeling.

Remming van de mevalonzuur route door statines, zoals simvastatine of lovastatine induceert bij myeloomcellen geprogrammeerde celdood, het remt de deling van myeloomcellen en het maakt myeloomcellen gevoeliger voor chemotherapie. Hierbij speelt vooral geranylgeranylpyrofosfaat een belangrijke rol.

Om te kijken of statines ingezet kunnen worden als middel om multipel myeloom te behandelen hebben we een studie ontworpen, welke beschreven is in **hoofdstuk 2**. Om spiegels te bereiken die effectief zijn tegen myeloomcellen moeten veel hogere doseringen gegeven worden aan patiënten, dan voor de behandeling van hypercholesterolemie gebruikelijk is. Deze studie kijkt naar de haalbaarheid van hoge doseringen simvastatine en de daaraan gerelateerde bijwerkingen. Om de effectiviteit te verhogen wilden we statines combineren met chemotherapie. We hebben in totaal 28 uitbehandelde myeloom

en lymfoom (lymfklierkanker) patiënten behandeld met steeds hogere doseringen statines gedurende zeven dagen, in combinatie met chemotherapie. Bij de dosering 17,5 mg/kg simvastatine overleed één patiënt aan een neutropene sepsis, en één patiënt had ernstige last van misselijkheid en braken. Eén dosering lager, namelijk 15 mg/kg, bleek wel haalbaar. Patiënten konden dit goed verdragen. Dertig procent van de behandelde patiënten die ten minste één kuur had af kunnen maken repondeerde.

In **hoofdstuk** zijn de resultaten van een zogeheten fase II studie beschreven, waarin wordt bekeken of de hoge dosering simvastatine in combinatie met chemotherapie ook daadwerkelijk effectief is in patiënten met multipel myeloom. Hoewel de patiënten de behandeling goed verdroegen, bleek de behandeling onvoldoende effectief. Van te voren wilden we 40 patiënten behandelen, maar na 12 patiënten was er een interim-analyse gepland. Op dat moment bleek dat slechts één van de 12 patiënten gerespondeerd had. Dit was onvoldoende om de studie voort te zetten.

Op dat moment is het duidelijk dat simvastatine bij patiënten met multipel myeloom in de huidige combinatie met chemotherapie onvoldoende effectief is. Nu is het van belang op zoek te gaan naar effectievere combinaties. Ook is het onvoldoende duidelijk waarom statines *in vitro* wel zo effectief de myeloomcel doden. Remming van prenylering, en voornamelijk remming van geranylgeranylering, speelt een belangrijke rol. We weten alleen niet welke eiwitten er daarna voor zorgen dat de myeloomcel geprogrammeerde celdood ondergaat of stopt met delen.

In **hoofdstuk** tonen we dat de combinatie simvastatine met lenalidomide synergistisch is in multipel myeloom. Lenalidomide is een nieuw en effectief middel bij multipel myeloom, dat ook als tablet ingenomen wordt. De combinatie geeft effectievere geprogrammeerde celdood en remming van celdeling dan de (optelsom van) de middelen alleen. Dit blijkt wederom te komen door remming van de mevalonzuur route en onder invloed van remming van gegeranylgeranyleerde eiwitten. Ook blijkt een bepaalde signaalroute, waarmee in een cel signalen doorgegeven worden, namelijk de JAK/STAT route, geremd te worden door de combinatie.

In **hoofdstuk** gaan we op zoek naar genen en op die manier naar eiwitten die verantwoordelijk zijn voor de effecten van statines op de myeloomcel. Dit hebben we bekeken met een zogeheten microarray, waarbij meer dan 50.000 ‘stukjes’ genen tegelijk onderzocht kunnen worden. Door een myeloomcellijn te behandelen met en zonder simvastatine kan er gekeken worden welke genen opgereguleerd worden en welke genen afnemen in expressie na behandeling met simvastatine. Daarnaast hebben we gekeken bij welke veranderde genen toevoeging van mevalonzuur en geranylgeranylpyrofosfaat de

effecten van simvastatine weer opheft. Op dat moment hebben we 479 genen over, die ten minste tweemaal verhoogd of verlaagd zijn. Het sterkst opgereguleerde gen is *rhob*, wat een rol speelt in een remming van kanker. Ook andere genen die bij de 'hoogste tien' zitten zijn interessant, waarvan een paar genen belangrijk zijn bij de celdeling. Daarnaast hebben we de 479 genen geanalyseerd in Ingenuity, een programma wat grote groepen genen analyseert, en bekijkt wat voor functies al die genen hebben en of er connecties bekend zijn. De aangedane genen blijken een rol te spelen in kanker en in celdeling. Verder onderzoek is nodig om de precieze rol van deze genen in de effecten van statines op de myeloomcel beter te begrijpen, en te ontdekken welke genen een functionele rol hebben.

Hoofdstuk beschrijft een muizenmodel om multipel myeloom te bestuderen. De myeloomcellen die bij de muizen worden ingespoten zijn genetisch zodanig veranderd dat ze licht uitzenden, en op deze manier kan de myeloomgroei onder een specifieke camera gevolgd worden. Op deze manier kan je volgen hoe snel en waar myeloomhaarden op komen en of ze reageren op oude en nieuwe behandelingen. Behandeling met bestraling zorgde voor afname van de hoeveelheid tumorcellen, maar de tumoren kwamen wel weer terug. Daarna hebben we gekeken of het model ook kon dienen ter evaluatie van graft versus myeloma door inspuiten van humane perifere bloed monocyten. Er bleek een krachtig graft versus myeloom effect, echter ten koste van een ernstig graft versus host effect.

Op dit moment zijn hoge doseringen statines onvoldoende werkzaam als nieuwe behandeling voor multipel myeloom. Een mogelijke verklaring hiervoor die we beschrijven in **hoofdstuk** is dat simvastatinespiegels die in mensen bereikt worden, ondanks de hoge doseringen die gebruikt worden, onvoldoende zijn om werkzaam te zijn. Er kunnen een aantal strategieën bedacht worden om toch gebruik te maken van de effectiviteit die statines in het laboratorium hebben tegen myeloom. Eén mogelijkheid is om te proberen de statinespiegels te verhogen. Dat kan onder andere door intraveneuze toediening. Een andere optie is de keuze voor een andere statine dan simvastatine, die wellicht betere spiegels haalt. Daarnaast kan er gekeken worden of een andere combinatie van statines dan die met chemotherapie effectiever is, zoals een combinatie met lenalidomide. Als er beter begrepen wordt hoe statines myeloomcellen remmen in hun celdeling en aanzetten tot geprogrammeerde celdood kan dit leiden tot nieuwe medicatie die hier specifiek op aangrijpt, of het kan zorgen voor betere medicijncombinaties door te snappen wat statines precies doen. Als laatste kunnen met behulp van het muizenmodel snel verschillende soorten statines en combinaties met statines getest worden, zodat bij patiënten direct een veelbelovende combinatie kan worden uitgetest.

Het cliché bleek ook deze keer waar, promoveren doe je niet alleen...

Allereerst wil ik alle patiënten bedanken die mee wilden doen aan het onderzoek. Zonder hen was dit boekje in deze vorm nooit mogelijk geweest.

Dr. H.M. Lokhorst, beste Henk,

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En nu is het weer tijd voor gezelligheid, kunst, cultuur, culinaire hoogstandjes, sport, vakantie en... meer wetenschap!

Curriculum vitae

Ellen van der Spek werd geboren op 2 januari 1976 te 's-Gravenhage. In 1993 werd zij verkozen tot burgemeester van Madurodam. Zij behaalde haar vwo diploma in 1994 op het christelijk gymnasium Sorghvliet, te 's-Gravenhage. Na uitgeloot te zijn voor de studie geneeskunde, vertrok zij naar de Katholieke Universiteit Leuven, te België, waar zij slaagde voor de 1^e kandidatuur. Na wederom uitgeloot te zijn kon zij door dit diploma instromen in het 2^e jaar geneeskunde aan de Universiteit Maastricht in 1995. In 1996 volgde zij een stage infectieziekten aan de Universiteit van Linköping, Zweden. Zij behaalde het artsexamen in 2001. Daarna werkte zij als arts-assistent niet in opleiding op de afdeling interne geneeskunde van het Groene Hart Ziekenhuis te Gouda en het Diaconessenhuis te Utrecht. In 2003 begon zij de opleiding interne geneeskunde in het Universitair Medisch Centrum Utrecht (umcu), onder leiding van prof. dr. D.W. Erkelens en vervolgens prof. dr. E. van der Wall. In 2005 startte zij met fulltime onderzoek onder begeleiding van dr. A. Bloem en dr. H.M. Lokhorst, gefinancierd door een onderzoeksjaar voor arts-assistenten van het Koningin Wilhelmina Fonds. In 2007 vervolgde zij de opleiding tot internist in het Diaconessenhuis Utrecht met als opleider dr. W.M.N. Hustinx. In september 2008 is zij gestart met haar aandachtsgebied hematologie in het umcu onder leiding van prof. dr. D.H. Biesma en dr. H.K. Nieuwenhuis. In 2010 hoopt zij haar opleiding tot internist-hematoloog af te ronden.

