UNIVERSITA' DEGLI STUDI DI VERONA

DIPARTIMENTO DI
PATOLOGIA E DIAGNOSTICA

SCUOLA DI DOTTORATO DI
SCIENZE BIOMEDICHE TRASLAZIONALI

DOTTORATO DI RICERCA IN
PATOLOGIA ONCOLOGICA E FISIOPATOLOGIA RIGENERATIVA TISSUTALE UMANA

CICLO /ANNO XXIV/ 2009

TITOLO DELLA TESI DI DOTTORATO

" STUDY OF THE EFFICACY OF NEW DRUGS IN A MOUSE MODEL OF MULTIPLE MYELOMA"

S.S.D. MED/08

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ABBREVIATIONS LIST

AML = acute myeloid leukemia

Ann-V = annexin-V

BM = bone marrow

CDK = cyclin dependent kinase

CR = complete remission

CVD = cyclophosphamide, bortezomib, and dexamethasone

DKK-1 = dickkopf-1

DMSO = dimethyl sulfoxide

DVD = liposomal doxorubicin, bortezomib, and dexamethasone

ECM = extracellular matrix

EFS = event free survival

FACS = fluorescence-activated cell sorting

FBS = fetal bovine serum

FGF = fibroblast growth factor

FTI = farnesyl-transferase inhibitors

G-CSF = granulocyte colony-stimulating factor

HDAC = histone deacetylase

HDT = High-dose therapy

HGF = hepatocyte growth factor

HHV = human herpesvirus

HL = Hodgkin's lymphoma

HSC = hematopoietic stem cell

HSP = heat-shock protein

IC_{50} = median inhibition concentration

ICAM-1 = intercellular adhesion molecule-1

IFN = interferon

Ig = immunoglobulin

IGF = insulin-like growth factor

IL = interleukin

i.p. = intraperitoneally

i.v. = intravenously

MAPK = mitogen activated protein kinase

MDS = myelodysplastic syndromes

MGUS = monoclonal gammopathy of undetermined significance

MIP-1α = macrophage inflammatory protein-1α

MM = multiple myeloma

MP-1 = MEK partner 1

mTOR = mammalian target of rapamycin

MVD = mean vascular density

NFκB = nuclear factor kappa B

NK = natural killer

NSCLC = non-small-cell lung carcinoma

NTX = amino-terminal cross-linking telopeptide

OAFs = osteoclast activating factors

OPG = osteoprotegerin

OS = overall survival

PBS = phosphate-buffered saline

PDGFR = platelet-derived growth factor receptor

PFS = progression free survival

PI = propidium iodide

p.o. = per os

RANKL = receptor-activating of NF-κB ligand

ROTI = related organ impairment

RVD = lenalidomide, bortezomib and dexamethasone

SCF =stem-cell factor

SDF-1α = stromal cell-derived factor-1α

SMM = smoldering myeloma

SRE = skeletal related event

TGF-β = transforming growth factor-β

TKR = tyrosine-kinase receptors

TNF = tumor necrosis factor

TRAIL = TNF-related apoptosis-inducing ligand

UPR = unfolded protein response

VAD = doxorubicin, vincristine and intermittent high-dose dexamethasone

VCAM-1 = vascular cell adhesion molecule-1

VEGF = vascular endothelial growth factor

VGPR = very good partial response

VTD = bortezomib, thalidomide, and dexamethasone

XTT = 2,3-bis[2-Methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide inner salt

SUMMARY

Multiple myeloma (MM) is a clonal plasma cell disorder accounting for approximately 10% of all haematological malignancies. MM development dramatically depends on plasma cell interactions with bone marrow (BM) microenvironment, which supports plasma cell growth, survival and drug resistance through cell-cell adhesion and release of a large number of growth factors, including interleukin-6 (IL-6) and vascular endothelial growth factor (VEGF). Some crucial pathogenetic events in MM, such as neoangiogenesis and osteolysis, are ignited inside BM by MM cells themselves. Although standard chemotherapy is usually effective in lowering the disease burden, complete remission is achievable only in a minority of patients, and clinical responses are rarely persistent. Recent studies showed that a significantly higher number of patients may achieve clinical major responses when treated with therapeutic schedules based on the use of anti-angiogenic or biological agents, such as thalidomide, lenalidomide and bortezomib, especially when employed in combination with dexamethasone. However, MM still remains an incurable disease. Therefore, the identification of new key targets is crucial for the development of innovative therapeutic strategies, which should have not only direct effects on MM cells, but also interfering effects on MM-supporting BM microenvironment. To this aim, the use of mouse models that may closely resemble human MM development are mostly useful to foresee the clinical effects in patients. Some kinds of murine MM, such as 5TMM, originate from spontaneously developed MM in elderly mice of C57BL/KalwRij strain. The characteristics of these models, including the localization of the MM cells in the BM, the presence of serum M-component, the induction of osteolytic bone disease and neo-angiogenesis in the BM, are similar to human MM. In this study, we examined the effects of several new drugs in different murine and human myeloma cell lines and we tested these molecules *in vivo* in the murine 5T33MM model.

T8 induced a reduction in cell activity only for high concentrations of molecule, with IC_{50} value of 4315 nM after 48 hours of incubation; for this reason it was not used for further studies.

As expected, N80 did not show any cytotoxic activity on treated cells as compared to controls. However, when tested *in vivo* for a preventive and curative treatment, N80 showed immediate toxicity and reduction of the tumor burden. There were not differences in IgG2b levels in serum of mice treated with N80 using the therapeutical schedule as compared to controls, while the preventive schedule induced a statistically significant reduction in IgG2b levels.

L9, N0 and N2 were able to inhibit the activity of MM cells *in vitro*. They appeared the most potent compounds, with nanomolar IC_{50} values, capable of reducing cell viability, in correlation with cell apoptosis *in vitro*. These molecules reduced the M-component and VEGF serum levels *in vivo* and eventually the tumor burden, although they did not modify significantly, with the treatment schedules employed, the overall survival in 5T33vtMM model.

RIASSUNTO

Il mieloma multiplo (MM) è un disordine clonale delle plasmacellule, che rappresenta approssimativamente il 10% di tutte le neoplasie ematologiche. Lo sviluppo del MM dipende dalle interazioni con il microambiente del midollo osseo (BM), che supporta la crescita, la sopravvivenza e la resistenza ai farmaci delle plasmacellule attraverso l'adesione cellula-cellula e il rilascio di un grande numero di fattori di crescita, come l'interleuchina-6 (IL-6) e il fattore di crescita dell'endotelio vascolare (VEGF). Alcuni eventi patogenetici cruciali nel MM, come la neoangiogenesi e l'osteolisi, sono indotti all'interno del BM dalle stesse cellule di MM. Nonostante la chemioterapia standard sia spesso efficace nel ridurre la malattia, la remissione completa è raggiungibile solo in una minoranza di pazienti e le risposte cliniche sono raramente persistenti. Studi recenti mostrano che un numero altamente significativo di pazienti può ottenere buone risposte cliniche con protocolli terapeutici basati sull'utilizzo di agenti anti-angiogenici o biologici, come la talidomide, lenalidomide e il bortezomib, specialmente quando utilizzati in combinazione con il desametasone. Nonostante ciò, il MM rimane ancora una patologia incurabile e perciò l'identificazione di nuovi obiettivi molecolari è cruciale per lo sviluppo di strategie terapeutiche innovative, che dovrebbero non solo avere effetti diretti sulle cellule neoplastiche, ma anche indiretti agendo sul microambiente midollare che supporta il MM. A questo scopo, l'utilizzo di modelli murini con un microambiente di sviluppo della malattia molto simile a quello umano risulta utile per prevedere gli effetti clinici nei pazienti. Tra questi, il 5TMM è di particolare interesse, in quanto origina da un MM sviluppato spontaneamente in topi anziani del ceppo C57BL/KalwRij. Le caratteristiche di tale modello, che comprendono la localizzazione midollare delle cellule di MM, la presenza della componente monoclonale nel siero, l'induzione di osteolisi e la neoangiogenesi nel midollo, lo rendono molto simile al MM umano. In questo studio, sono stati esaminati gli effetti *in vitro* di diversi nuovi farmaci in fase di sviluppo della Ditta Novartis su linee cellulari umane e murine e sono state testate queste molecole *in vivo* nel modello 5T33MM.

La molecola T8 induce una riduzione dell'attività cellulare solo per alte concentrazioni, con un valore di IC_{50} di 4315 nM dopo 48 ore di incubazione; per questa ragione non è stato utilizzato negli studi successivi.

Come atteso, la molecola N80 non mostra alcuna attività citotossica sulle cellule trattate rispetto ai controlli. Comunque, quando è stato testata *in vivo* per un trattamento preventivo e curativo, è stata dimostrata un'immediata tossicità e la riduzione del carico tumorale. Non si evidenziano differenze nei livelli di IgG2b monoclonale nel siero dei topi trattati con N80 utilizzando il protocollo terapeutico rispetto ai controlli, mentre il protocollo preventivo induce una riduzione statisticamente significativa nei livelli di IgG2b.

L9, N0 e N2 sono in grado d'inibire l'attività delle cellule di MM *in vitro*. Si sono dimostrati i composti più potenti, con valori nanomolari di IC_{50} e in grado di indurre una riduzione della vitalità cellulare, correlata con l'apoptosi cellulare *in vitro*. Queste molecole riducono la produzione della componente monoclonale e i livelli sierici di VEGF *in vivo*. Quindi, queste molecole sono in grado di ridurre il carico tumorale, sebbene non modifichino la sopravvivenza dei topi nel modello 5T33vtMM con gli attuali protocolli terapeutici utilizzati.

INTRODUCTION

1. MULTIPLE MYELOMA

1.1 Epidemiology

Multiple myeloma (MM) is a monoclonal plasma cell disorder determined by a clonogenic, neoplastic plasma cells accumulation inside the bone marrow (BM). It is generally characterized by the presence of an entire or fragmented monoclonal immunoglobulin (Ig) in the serum and/or urine and bone osteolytic lesions [1,2].

MM accounts for approximately 1% of all cancers and 10% of hematological malignancies in white populations, with approximately 23,000 new cases diagnosed each year in Europe [1-3]. Male/female ratio is about 2:1. The incidence is higher in black people and lower in Asians than in white people. The incidence increases along age (median age at diagnosis: 67 years) and only about 3% of patients are younger than 40 years [4]. MM incidence has been increasing in many countries because of improved diagnostic accuracy and increasing risk factors, such as obesity [5]. Median survival after conventional treatment is 3-4 years; high-dose treatment followed by autologous stem-cell transplantation can prolong median survival up to 5-7 years. Novel drugs used alone and in combination with existing treatments are currently tested to further improve survival.

MM is characterized by a number of typical features [2,6-8]:

- clonal accumulation of clusters of long-living, poor-proliferating malignant plasma cells inside the bone marrow;
- presence of monoclonal proteins, i.e. paraproteins (usually IgG, IgA and IgD), released by malignant plasma cells in blood and/or urine (Bence Jones proteinuria);
- reduction in the normal levels of Ig (hypogammaglobulinemia).

MM presentation is quite variable, as it may be diagnosed both in asymptomatic patients and in patients with life-threatening clinical events. Clinical features are characterized by multiple symptoms and organ disfunctions [3,6-8]:

- lytic bone disease: BM invasion by MM cells and bone destruction cause bone pain, particularly in weight-bearing bones, pathological fractures secondary to

lytic bone lesions and hypercalcemia, that often dominates the clinical feature. The interactions between plasma cells and BM microenvironment determine the activation and proliferation of osteoclasts and suppress osteoblast activity, thus causing an imbalance between bone resorption and bone formation [9]. Up to 80% of MM patients refer bone pain at diagnosis and more than 70% of patients develop pathological fractures during the disease course [10];

- hypercalcemia: it is due to increased bone resorption;
- renal insufficiency: it may be secondary to light-chain and heavy-chain renal deposition, dehydration, hypercalcemia, hyperuricemia, direct plasma cells infiltration of the kidney. Up to 20% or 30% of MM patients have some form of renal impairment at diagnosis [11,12];
- susceptibility to infections: MM patients are immunosuppressed and particularly susceptible to bacterial infections. This immunodeficiency is mainly caused by abnormal Ig production and impairment of the primary immune response. Pneumonias and urinary tract infections are mostly frequent [13,14];
- anemia: it is a common complication of MM, caused by the abnormal iron utilization, abnormally low serum erythropoietin levels, decreased response to erythropoietin, hemolysis or BM involvement;
- neurological symptoms: the most frequent neurological features include spinal cord compression, radiculopathy, peripheral neuropathy, cerebral disorders and cranial nerve involvement [15];
- alterations in coagulative activity: the direct interaction of monoclonal Ig with hemostatic mechanisms may lead to the development of either hemorrhagic or thrombotic events. Bleeding may be present in 15-30% of the patients, while the baseline thrombotic risk is approximately 3% [16,17]. Rarely, MM can present with hyperviscosity syndrome or cryoglobulinemia causing vascular problems, as well as renal or gastrointestinal disorders [18];
- amyloidosis: amyloid deposition mainly occurs at renal and cardiac level.

1.2 Stages of multiple myeloma

Epidemiologic studies have shown that at least one-third of MM cases is the evolution of a pre-existing monoclonal plasma cell disorder, i.e. monoclonal gammopathy of undetermined significance (MGUS). This suggests that two types of MM exist: MM

secondary to MGUS and primary, *de novo* MM [5]. The malignant transformation of MGUS to MM seems to be a multi-step transformation process, in which genetic changes, BM angiogenesis, various cytokines related to bone disease and possibly infectious agents may be involved [1,19]. (**Figure 1**).

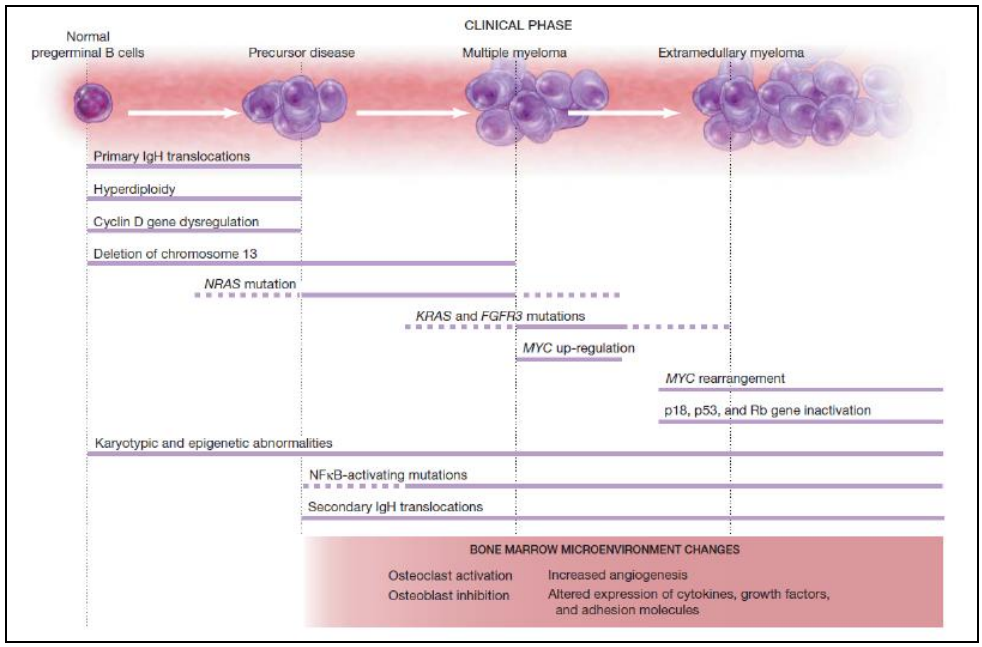

Figure 1: biological events related to MM progression [20]

MGUS is typically asymptomatic and stable. Cells are immortalized but not transformed, and do not accumulate or cause bone destruction. In MGUS there is a lower intramedullary tumor-cell content (tumor cells are no more than 10% of the mononuclear cells in the BM) and lower monoclonal Ig production (≤ 30 g/L IgG or ≤ 20 g/L IgA). MGUS is present in 1% of adults and progresses to malignant MM at 1% per year rate [21]. At the time of diagnosis, the risk of progression to MM cannot be determined, but different parameters are useful, such as the dose and type of serum monoclonal protein (higher risk in presence of IgD or IgA rather than IgG), percentage of BM plasma cells and abnormal serum free light chain (FLC) ratio.

Non-symptomatic smouldering MM (SMM) is a disease variant with stable intramedullary tumor cell content of more than 10%, but no osteolytic lesions or other complications of malignant MM. MGUS and SMM can switch into intramedullary MM accumulating inside BM microenvironment and causing bone destruction *via* cytokine-induced increased osteoclast activity.

Further progression of MM is associated with increasingly severe secondary features (lytic bone lesions, anemia, immunodeficiency, renal impairment) and extramedullary plasma cell invasion, i.e. plasma cell leukemia, which is secondary or primary, depending on whether intramedullary myeloma has been previously recognized or not [3,10,21].

1.3 Etiology

MM etiology remains unknown. Risk factors include chronic immune stimulation, autoimmune disorders, exposure to ionizing radiation and other environmental factors, such as pesticides, herbicides and dioxin. A number of viruses has been implicated in the pathogenesis of MM. Patients with HIV have 4.5 fold increased risk of developing MM, although the precise mechanism still remains unclear. HHV-8 (human herpesvirus-8) infection has been demonstrated in BM stromal cells in MM patients and could be associated with the development and progression of the disease. HHV-8 has also been identified in stromal cells of other plasma cells disorders, such as MGUS and primary systemic amyloidosis, implying a potential casual association [3].

Genetic factors are also important and different studies analyze the genetic background of the malignant MM cell clone. Studies on the chromosome content suggest two molecular pathways of MM pathogenesis: a non-hyperdiploid (or hypodiploid) pathway with high incidence of 5 recurrent IgH translocations and loss of chromosome 13/13q14; and a hyperdiploid pathway with multiple trisomies. Non-hyperdiploid abnormalities are associated with reduced life-span and high-risk translocations of IGHR t(4;14) or t(14;16)), partial or complete loss of chromosome 13 and partial loss of chromosome 17. By contrast, hyperdiploid abnormalities caused by multiple trisomias, and low frequency of monosomy or deletion of chromosome 13 and translocation of IGHR are associated with improved outcome.

Primary reciprocal chromosomal translocations occur most frequently at IGHR on chromosome 14, which is usually juxtaposed to CCND1, CCND3, MAF, FGFR3, WHSC1 and MAFB genes. For example, the t(4;14) translocation generates a constitutive activation of the oncogenic tyrosine-kinases receptor fibroblast growth factor receptor3 (FGFR3), with subsequent phosphorylation of the antiapoptotic signal transducer and activator of transcription 3 (STAT3) signaling pathway, which promotes cell survival.

Secondary translocations and gene mutations are complex karyotypic abnormalities of MYC, activation of NRAS, KRAS and FGFR3 mutations, inactivating mutations or

deletions of TP53, RB1 and PTEN, and inactivation of cyclin-dependent kinase inhibitors CDKN2A and CDKN2C [4,22,23].

Epigenetic activation is another mechanism that may influence the development of MM cell clone. For example, SHPI, a native regulator of the Jak/STAT signaling pathway, is hypermethylated in 80% of MM patients and associated with constitutive STAT3 phosphorylation. Telomeres, specialized nucleoprotein which protect against fusion and degradation of linear chromosomes, represent another potential therapeutic target. In MM cells the mean telomere length is markedly shorter than in peripheral granulocytes and lymphocytes [24], and telomerase activity is increased in MM cells as compared to normal plasma cells [22].

1.4 BM microenvironment in MM

Plasma cells represent <1% of BM mononuclear cells. They derived from activated B cells in the germinal center of the lymph nodes: B cells with a high affinity antigen receptor (Ig) are selected by antigen through mutations of the Ig variable genes and then induced to become either memory B cells or antibody-producing plasmablastic cells. The latter cells migrate rapidly to the BM where they can find additional survival and differentiation factors supporting their long-term survival and differentiation into plasma cells. Hallmarks of mature plasma cells are their large amounts of Ig secretion, high expression of the syndecan-1 proteoglycan that is not expressed on B cells and the lack of most B cell markers, except CD19 [25].

The role of BM microenvironment in MM pathogenesis has been thoroughly studied. In MM the balanced homeostasis between cellular, extracellular and liquid compartments within the BM is profoundly disrupted, resulting in lytic bone lesions, immune suppression and cytopenias. These effects are caused not only by the clonal expansion of MM cells in the BM, but also by the impact of tumor cells on the functions of other cells within the BM microenvironment. Direct cell-to-cell/cell-to-extracellular matrix (ECM) interactions or cytokines/growth factors modulate functions of different cells, including endothelial cells, osteoclasts, osteoblasts, fibroblasts and immune effector cells, which in turn promote tumor-cell growth, survival, migration and drug resistance. BM angiogenesis in MM is tightly regulated by pro- and anti-angiogenic factors and BM microvascular density (MVD) is increased and correlates with disease progression and poor prognosis. (**Figure 2**)

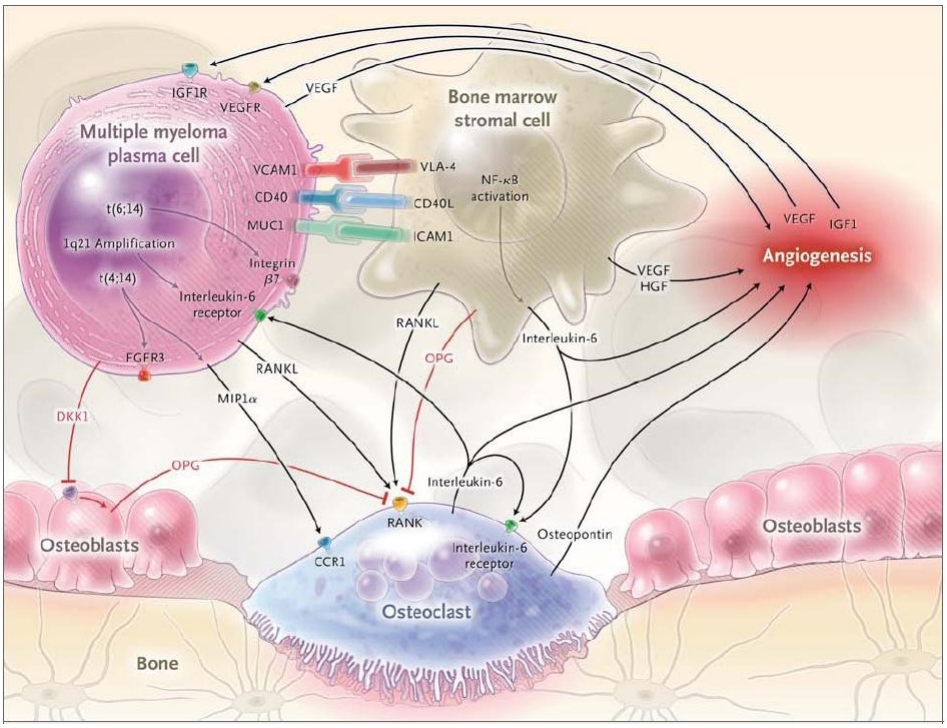

Figure 2: Interaction between plasma cells and bone marrow in multiple myeloma [26]

Increasing knowledge about BM microenvironment in MM can contribute to the design of new biological treatment regimens aimed at targeting not only MM cells, but also MM cell-stromal cell interactions and BM microenviroment (**Figure 3**) [22,27]

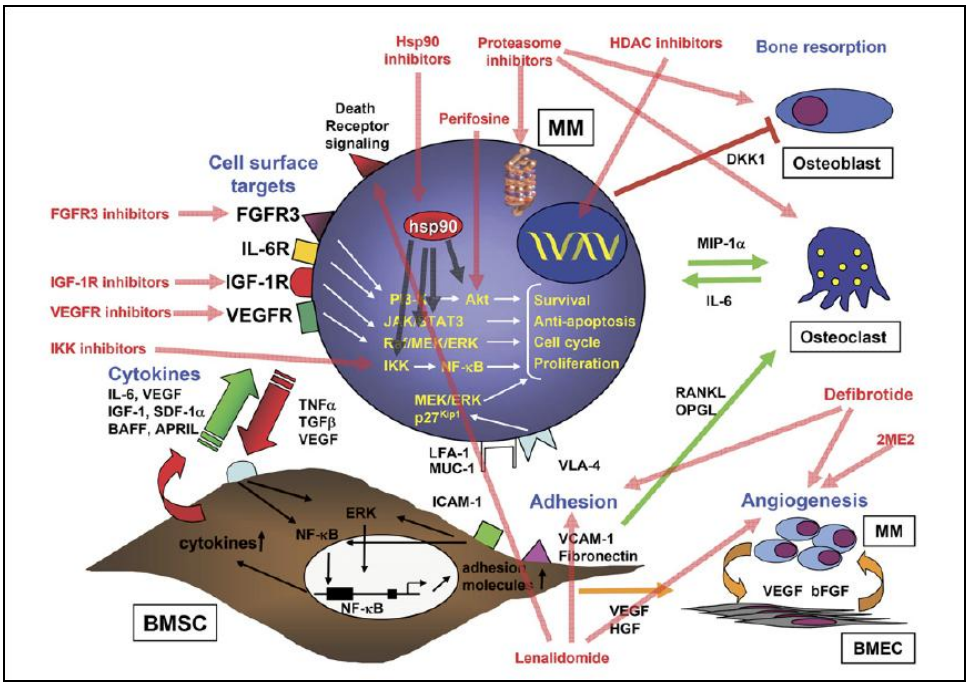

Figure 3: Interactions of neoplastic plasma cell and microenviroment are targets for new drug. [28]

There are different ways of interaction between malignant MM clone and BM microenvironment:

- interactions with the extracellular matrix and other cells within the BM microenvironment: MM plasma cells adhere to the extracellular matrix proteins and BM stromal cells through a number of adhesion molecules, such as β1-integrin family with several very late antigens (VLA, VLA-4, VLA-5 and VLA-6), as well as intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1). The main molecules regulating plasma cell homing into the BM and binding to extracellular matrix are CD44, VLA-4, VLA-5, LFA-1 (CD11a), CD56, CD54 (ICAM-1), syndecan-1 (CD138) and MPC-1 (Monocyte chemotactic protein-1) [29]. This binding induces cell-adhesion-mediated drug resistance and secretion of several cytokines by both MM plasma cells and BM stromal cells.

  In particular, MM cells express β1 integrins, including VLA-4 and VLA-5, that mediate MM cell adhesion to endothelial cells, fibroblasts and ECM proteins, such as fibronectin and laminin. Adhesion enhances MM cell growth and survival and confers protection against drug-induced apoptosis. For example, MM cell adhesion to fibronectin protects tumor cells from DNA-damaging drugs (such as anthracyclines and alkylating agents) by inducing cell-adhesion-mediated drug resistance, with reversible G1-arrest and increased p27kip1 levels. Moreover, MM cells express also αvβ3-integrins that mediate the binding to vitronectin and fibronectin, stimulating the production and release of metalloproteinase 2 and 9, both promoting tumor-cell invasion and spread. Ongoing studies are testing novel molecules, that are able to alter adhesive interaction among MM cells, accessory cells and ECM to increase anti-MM activity and reduce drug-resistance [22];
- cytokines and growth factors: tumor cells activate the stroma, that triggers the paracrine and autocrine production and secretion of a variety of cytokines and growth factors into the BM-microenvironment, including IL-6, IGF-1 (insulin-like growth factor), VEGF, TNF-α (tumor necrosis factor-α), SDF-1α (stromal cell-derived factor-1α), TGF-β (transforming growth factor-β), bFGF (basic fibroblast growth factor), MIP-1 α (macrophage inflammatory protein-1α), SCF (stem-cell factor), HGF (hepatocyte growth factor), MP-1 (MEK partner 1), osteoprotegerin

(OPG), RANKL (receptor-activating nuclear factor kappa-beta ligand), IL-1β, IL-3, IL-10, IL-15, IL-21, Ang-1 and matrix metalloproteinases (e.g. MMP-2 and MMP-9). These cytokines trigger signaling pathways (NF-kB, RAF/MEK/MAPK; PI3K/AKT and JAK/STAT pathways), thus promoting cell proliferation and preventing apoptosis (IL-6, TNF-α, IGF-I, IL-1β, VEGF), inducing angiogenesis (VEGF, FGF, HGF), osteoclast activity (IL-1β, RANKL, HGF) and immunodeficiency (TGF-β, VEGF) [23].

In particular:

- IL-6: it is the major growth and survival factor for MM cells. It has pleiotropic effects on hematopoietic and non-hematopoietic cells, induces purified B cells to differentiate into Ig-secreting plasma cells, and acts as a growth factor for MM. Thus, IL-6 mediates the expansion of plasmablastic cells and their malignant counterparts [19,25]. The evidence that IL-6 is involved in MM pathogenesis was established by the following experimental and clinical findings:
    - serum levels of IL-6 and soluble IL-6R are increased in patients with MM in association with poor prognosis [30];
    - antibodies against IL-6 block MM cell proliferation [31];
    - injection of anti-IL-6 monoclonal antibody inhibits MM cell proliferation in patients with terminal disease if the antibody is injected at concentrations blocking completely the large amount of IL-6 produced *in vivo* [32];
    - BM microenvironment of MM patients, consisting mainly of monocytes, myeloid cells and stromal cells, overproduces IL-6 [33,34]. IL-6 production is mediated by soluble factors, cytokines and by intercellular interactions;
    - survival of cell lines, obtained from patients with extramedullary proliferation, depends on the addition of exogenous IL-6 [35,36].

  IL-6 is predominantly produced and secreted by BM stromal cells and osteoblasts, and correlates with MM tumor cell mass, disease stage and prognosis [37,38]. MM cells also shed the soluble form of IL-6 receptor (sIL-6R, gp80), which can amplify their response to IL-6. [3]. In fact, about 50% of serum IL-6 is bound to gp80. The complex of IL-6/sIL-6R is much

more powerful than IL-6 alone in stimulating malignant plasma cells through the gp130 receptor signal-transduction pathway [37]. IL-6 triggers activation of MEK/MAPK-, JAK/STAT3- and PI3K/AKT- signaling pathways [30,39]. IL-6 triggered JAK/STAT3 pathway induces upregulation/activation of antiapoptotic proteins Mcl-1 and Bcl_{XL}, Pim1 and c-Myc. Besides MM cell growth and survival, IL-6 also triggers drug resistance. However, many cell lines grow independently of IL-6 and different therapeutic approaches targeting IL-6 have not induced responses in phase I clinical trials. Taken together, these data show that IL-6 is crucial, but not the only factor in MM pathogenesis [27];

- IGF-1: it is a survival and proliferation factor for most myeloma cell lines [40]. In MM, IGF-1 induces tumor cell growth, survival and migration. It is mostly produced by the liver, but also by osteoblasts in the bone matrix, where myeloma cells survive and proliferate *in vivo*. IGF-1 serum levels are predictive of poor survival in patients with MM, although they are not increased [25]. IGF-1 induces the activation of the PI-3 kinase (PI3K) pathway, that can induce the phosphorylation of the anti-apoptotic AKT protein and MAPK [41,42]. Different substrates of PI3K/AKT pathway are involved [43], such as: Bad (pro-apoptotic protein: after the phosphorylation by AKT, Bad sequestration by the 14-3-3 protein prevents its migration to the mitochondrial membrane); P70S6-kinase (its phosphorylation by AKT induces its kinesic activity to ribosomial subunit 40S, with a consequent increase of protein synthesis); Forkhead proteins; Glycogen synthase kinase-3β (GSK-3β, that is inactivated) and NF-κB;
- members of the TNF superfamily: it includes SDF-1α, CD40, BAFF and APRIL. SDF-1α and its cognate receptor CXCR4 are expressed in the BM of MM patients. SDF-1α is primarly produced by BM stromal cells, but also by MM cells; it upregulates rapidly and transiently VLA-4-mediated MM cell adhesion to both fibronectin and VCAM-1. Furthermore, SDF-1α promotes proliferation, induces migration and protects against drug-induced apoptosis in MM cells through MAPK, NF-κB and AKT-mediated pathways. In addition, SDF-1α upregulates secretion of IL-6 and

VEGF in BM stromal cells and is a critical regulator of MM cell migration and homing.

CD40 is expressed by antigen-presenting cells, T cells and B cell malignancies, including MM. Functionally, it mediates p53-dependent increases in MM cell growth, PI3K/AKT/NF-κB- dependent MM cell migration and triggers VEGF secretion. Moreover, CD40-activated MM cells adhere to fibronectin and are protected against apoptosis induced by irradiation and doxorubicin.

TNF-α, secreted mainly by macrophages, poorly triggers MM cell proliferation, survival and drug resistance. However, it markedly upregulates secretion of IL-6 by BM stromal cells and induced NF-κB-dependent expression of different molecules (e.g. CD49d, CD54), thus resulting in increased specific binding of MM cells to BM stromal cells and consequent induction of IL-6 transcription and secretion, as well as drug-resistance [22].

BAFF or B lymphocyte stimulator is normally expressed by monocytes, macrophages, dendritic cells (DCs), T cells and BM stromal cells, and exists as both membrane-bound and cleaved soluble protein. In MM, both tumor cells and BM stromal cells express high levels of BAFF and APRIL, as well as their receptors. Functionally, BAFF and APRIL protect MM cells from apoptosis induced by IL-6 deprivation and promote MM cell growth and adhesion to BM stromal cells. These processes are mediated by NF-κB-, PI3K/AKT- and MAPK- pathways [27];

- FGF: although serum levels of FGF2 are increased in MM, there is no direct evidence so far that its expression plays a role in the survival or proliferation of human myeloma cells. FGFs likely play an important role in myeloma biology: in fact, FGFs bind syndecan-1 and activation of FGFR3 may induce the PI3-K/AKT pathway that is critical for myeloma cell survival and proliferation [44];
- VEGF: various growth factors belong to the VEGF family: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and placenta growth factor (PIGF). VEGF is secreted by several MM cell lines and is present in patient plasma. VEGFR-1 is highly expressed by MM cells, consistently

with autocrine signaling [45,46]. Specifically, VEGF triggers Flt-1 phosphorylation and activation of MEK/ERK and PI-3K/PKC-α signaling cascades in MM cell lines and patient cells, thus promoting modest proliferation and pronounced migration, respectively [29]. VEGF also influences also osteoblasts, NK cells, monocytes and endothelial progenitors. In fact, it stimulates BM angiogenesis and inhibits antigen-presenting functions of DCs, thus contributing to immune suppression in MM [2];

- IL-10: it enhances proliferation of freshly isolated MM cells in short-term BM culture. Moreover, the growth of MM lines is supported by IL-10 [19,25];

- neoangiogenesis: the importance of tumor angiogenesis in the evolution of solid tumors and hematological malignancies is well established: the increase in MVD within BM microenvironment is considered an important prognostic factor for median overall survival (OS) and event-free survival (EFS) at the time of initial diagnosis, and correlates with disease progression and poor prognosis [47,48]. The main angiogenic factor involved is VEGF that is secreted by several MM cell lines and is present in MM BM aspirates. [15]. There is a hypothetical paracrine loop for angiogenesis and tumor growth in the BM of MM patients: Plasma cells secrete VEGF-A, which induces proliferation and chemotaxis in endothelial cells through VEGFR-2, a VEGF-A receptor expressed by those cells. VEGF-A acts also through its receptor VEGFR-1, expressed by stromal cells, inducing their proliferation. Activation of these cells results in VEGF-C and VEGF-D production that stimulates malignant plasma cell growth *via* VEGFR3. Finally, the VEGF–VEGFR-2 interaction by endothelial cells increases IL-6 levels, creating another paracrine loop for plasma cell growth [49];
- myeloma bone disease and osteoblastic niche (**Figure 3**) one of the earlier symptom in MM is the excessive bone resorption. Histomorphometric analysis of bone biopsies from MM patients shows that osteolytic bone lesions are due to the unbalanced bone remodeling that increases osteoclastic resorption and lowers bone formation. As a result, MM bone lesions are highly destructive and appear as areas of extensive demineralization at x-ray scan [50]. In particular, there is a significant increase in both the recruitment of new osteoclasts and osteoclast

activity in close contact with myeloma cells. This suggests that bone disease results from local production of osteoclast activating factors (OAFs), such as RANKL/OPG system and the chemokine MIP-1, secreted by either myeloma cells or BM stromal cells. RANKL is expressed by osteoblasts and binds to its receptor (RANK) present on osteoclasts, thus triggering differentiation and activation signals in osteoclast precursors and promoting bone resorption. OPG (osteoprotegerin) is the natural antagonist of RANKL and its expression regulates osteoclast activity and bone resorption, thus preserving bone integrity. Different studies have shown that myeloma cells are able to induce increased RANKL expression and decreased OPG production in the BM environment. In BM biopsies from MM patients, RANKL is overexpressed in stromal cells in the interface between MM and normal BM elements, and also in myeloma cells. Several other OAFs, including IL-1β, IL-6 and TNF-α, are overproduced by BM stroma in response to MM. However, the addition of blocking antibodies against these molecules in co-cultures does not prevent RANKL upregulation.

In addition, in BM of MM patients decreased production of OPG occurs through two mechanisms: i. a decrease in OPG production by stromal cells is directly induced by MM cells; ii. myeloma cells internalize and degrade OPG within the lysosomal compartment [*10*].

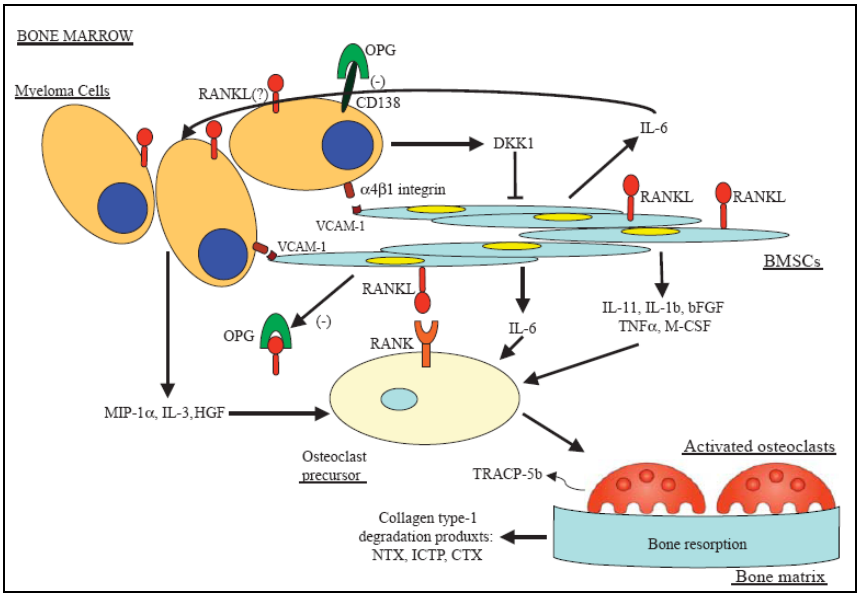

Figure 3: myeloma bone disease. [9]

Denosumab is a fully human monoclonal antibody against RANKL. In a recent study, 1776 adult patients with solid tumors or MM (10% of the total patients), not previously treated with intravenous bisphosphonates, were randomized to receive either subcutaneous denosumab 120 mg or intravenous zoledronic acid every 4 weeks. Denosumab produced similar results regarding the delay of the first onset or subsequent of skeletal related event (SRE) as compared to zoledronic acid; in addition, Denosumab reduced rapidly and significantly (>80% in the first month) urinary amino-terminal cross-linking telopeptide of type I collagen (NTX) levels, a marker of bone resorption. [*51*].

The other system involved in myeloma-induced bone disease is the chemokine MIP-1. MIP-1α and β are overexpressed and secreted by myeloma cells. MIP-1 belongs to the RANTES family and acts as chemoattractant and activator of monocytes. Both osteoclast precursors and stromal cells express the chemokine receptor for MIP-1α and β (CCR5) and MIP-1/CCR5 interaction induces expression of RANKL in stromal cells. Furthermore, CCR5 is expressed by MM cells; therefore, MIP-1α and β may act on MM cells in both autocrine and paracrine fashion. In fact, MIP-1α triggers migration and signaling cascades mediating survival and proliferation in MM cells. MIP-1α and β are potent modulator of hematopoiesis: MIP-1α inhibits early erythropoiesis and MIP-1β increases apoptosis in pre-B cells. Therefore, MIP-1α and MIP-1β are pleiotropic chemokines that play important roles in the pathogenesis of several clinical features of MM, including not only destructive bone lesions, but also suppression of erythropoiesis, B lymphopoiesis and immunoglobulin production. RANKL/OPG and MIP-1 pathways enhance osteoclast formation and activity through osteoblasts, although studies in mice demonstrate the importance also of the myeloma-osteoclast loop for sustaining the disease progression through a still unclear mechanism. [*10*].

Recent research has identified other factors that regulate osteoblast function and help maintaining skeletal homeostasis. Specifically, the dickkopf-1 (DKK-1) protein plays an important role in regulating bone homeostasis; DKK-1 overexpression by myeloma cells is involved in bone loss, leading to osteolytic bone lesions in MM patient. In fact, DKK-1 inhibits the differentiation and

maturation of mesenchymal stem cells into bone-producing osteoblasts, thus promoting osteoclast activation. Treatment with DKK-1 neutralizing antibody prevents bone loss, increases the number of osteoblasts and inhibits MM cell proliferation in a mouse model of MM. Clinical evidence for the role of DKK-1 in MM is emerging. In a study by Tian E. *et al.* in 173 patients with newly diagnosed MM, elevated DKK-1 levels in blood and BM correlated with the presence of bone lesions. In another study by Kaiser M. *et al.* in 184 patients with MM, high DKK-1 levels were associated with the presence and number of bone lesions, as well as with Durie–Salmon disease stage. Therefore, the available data support the concept of targeting DKK-1 to develop new therapeutic strategies for MM [50].

Over the past two decades, the isolation and characterization of cellular and extracellular components of the skeletal matrix led to the development of biochemical markers that reflect either bone formation or resorption. Markers of bone resorption and formation are depicted in **Tables 1 and 2**. Measurement of bone turnover markers is non-invasive, cheap and, when applied and interpreted correctly, helpful in assessing bone disorders.

However, factors that affect bone turnover and marker levels, including circadian rhythm, diet, age, gender, renal function and drugs, should be clearly defined and appropriately adjusted, whenever possible. It is also important to recognize that these biochemical measurements reflect whole-body bone turnover and give little information about the function of local changes in skeletal homeostasis [*51*].

Table 1: Markers of bone resorption

Marker	*Abbreviation*[a]	*Tissue of origin*	*Analytical method*	*Analytical specimen*
Hydroxyproline	Hyp	All tissues and all genetic types of collagen	Colorimetric, assay, HPLC	Urine
Hydroxylysine	Hyl	All tissues and all genetic types of collagen	Reversed-phase HPLC	Urine
Galactosyl-hydroxylysine	Gal-Hyl	Both Gal-Hyl and Glc-Gal-Hyl appears to be specific for bone collagen degradation	Reversed-phase HPLC	Urine
Glucosyl- galactosyl-hydroxylysine	Glc-Gal-Hyl	Bone, cartilage, tendon, blood vessels	Reversed-phase HPLC	Urine
Pyridinoline	PYD	Bone, dentin	HPLC, ELISA	Urine
Deoxypyridinoline	DPD	All tissues containing type-I collagen	RIA	Urine (free DPD can be also measured in serum or plasma)
N-terminal cross-linking telopeptide of type-I collagen	NTX	All tissues containing type-I collagen	ELISA, RIA	Urine, serum
C-terminal cross-linking telopeptide of type-I collagen	CTX	All tissues containing type-I collagen	ELISA, RIA	Urine, serum (β-form only)
C-terminal cross-linking telopeptide of type-I collagen generated by MMPs	CTX-MMP or ICTP	All tissues containing type-I collagen	RIA	Serum
Tartrate-resistant acid phosphatase isoform 5b	TRACP-5b	Bone (osteoclasts)	Colorimetric RIA, ELISA	Serum, plasma
Bone sialoprotein	BSP	Bone, dentin, hypertrophic cartilage, cancer cells	RIA, ELISA	Serum

Abbreviations: ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography; MMP, matrix metalloproteinase; RIA, radioimmunoassay.

According to the bone marker nomenclature by the Committee of Scientific Advisors of the International Osteoporosis Foundation.

Table 2: Markers of bone formation

Marker	*Abbreviation*[a]	*Tissue of origin*	*Analytical method*	*Analytical specimen*
Osteocalcin (or bone gla-protein)	OC	Bone, platelets	RIA, ELISA, IRMA	Serum
Bone-specific alkaline phosphatase	Bone ALP	Bone	ELISA, IRMA, colorimetric assay	Serum
Procollagen type-I N-propeptide	PINP	Bone, soft tissue, skin	RIA, ELISA	Serum
Procollagen type I C-propeptide	PICP	Bone, soft tissue, skin	RIA, ELISA	Serum

Abbreviations: ELISA, enzyme-linked immunosorbent assay; RIA, radioimmunoassay; IRMA, immunoradiometric assay.

According to the bone marker nomenclature by the Committee of Scientific Advisors of the International Osteoporosis Foundation.

1.5 Disease management

MM remains an incurable disease and, therefore, new therapeutical approaches are needed to improve the patient outcome (**Figure 4**). Increasing knowledge on the biology of MM is already contributing to the design of new drugs targeting cellular proteins or pathways involved in the pathophysiology of the disease.

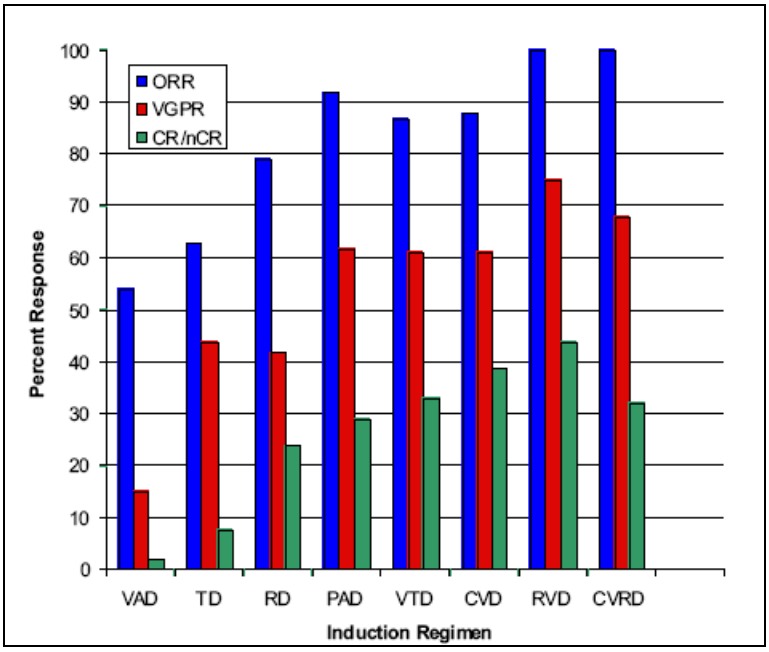

Figure 4. A continuous improvement in response is seen with the combination of newer agents. [52]

<u>When to treat</u>

Although novel agents has been employed for the early treatment of smouldering myeloma, there is no evidence yet that such approach improves survival in patients with no symptoms and stable disease. Up to 25% of smoldering myeloma patients does not require active treatment for 10 to 15 years, although the majority undergoes disease progression during that time. Thus, the employment of the maximum efficacy of the drugs only in symptomatic patients still remain preferable, as some data suggest that early intervention might select more aggressive subclones of myeloma.

Smoldering myeloma patients require frequent monitoring to identify the need of treatment before organ damage is evident, although the use of certain supportive therapies, such as bisphosphonates for osteopenia, is justified in selected patients [52].

Recommendations

• Chemotherapy is only indicated in patients with symptomatic MM on the basis of the presence of related organ impairment - ROTI (Grade A recommendation; level of evidence Ib);

• patients with asymptomatic myeloma should be monitored under the supervision of a Consultant Hematologist. These patients should entry into clinical trials if available (Grade C recommendation; level of evidence IV);

• monitoring of patients with asymptomatic myeloma should include regular (every 3 months) clinical assessment of the emergence of ROTI and measurement of serum and urinary M-protein (and SFLC, when indicated). Repeated BM examination and skeletal imaging should be considered prior to start the treatment (Grade C recommendation; level of evidence IV) [53].

Therapeutic targets

Novel therapeutic cellular targets are MM cells, BM stromal cells, endothelial cells, genes (e.g. FGFR3), cytokines, growth factors and specific signaling pathways (KRAS, RAF1, MAPK2K1, PI3K and AKT, JAK and STAT3, PRKC, NF-κB and WNT). For example, thalidomide, lenalidomide and bortezomib have been shown to overcome the supportive effects of BM microenvironment. Other preclinical drugs have been designed to inhibit either transport of ubiquitinated proteins to the aggresome (tubacin) or histone deacetylase (LBH589 - vorinostat). These drugs are capable of inducing synergistic cytotoxic effects against MM cells in BM. Additional drugs with increased potency and reduced adverse effects (e.g. antiangiogenic and endothelial-targeting drugs) are more active when combined with conventional or novel agents for MM.

Newly-diagnosed disease

High-dose therapy (HDT) plus autologous stem cell transplantation is considered the standard of care for front-line treatment of < 65 year old MM patients. New drugs have been investigated in various combinations as induction therapy. In particular:

- thalidomide has been used for MM treatment because of its anti-angiogenic, direct pro-apoptotic and G1 growth arrest-inducing effects, even in drug-resistant myeloma cells. It abrogates the increased secretion of IL-6 and VEGF and

stimulates autologous NK cell-mediated anti-myeloma immunity. Common side effects of this drug in patients with all stages of disease are deep vein thrombosis, constipation, peripheral neuropathy and fatigue, which often restrict dose and treatment duration to the detriment of drug effectiveness. Different trials have shown better responses by combining thalidomide and dexamethasone or thalidomide, dexamethasone and doxorubicin, as compared to conventional chemotherapy (e.g. high-dose dexamethasone or VAD – doxorubicin, vincristine and intermittent high-dose dexamethasone);

- lenalidomide is an immunomodulatory drug, more powerful than thalidomide and without sedative and neurotoxic adverse effects. The most common side-effect is myelosuppression, which is usually reversible with dose reduction and growth factor support. Clinical trials have suggested that lenalidomide, in combination with low-dose dexamethasone, leads to better progression free survival (PFS) and OS rates. Moreover, combined lenalidomide and dexamethasone improve response as compared to conventional high-dose dexamethasone alone. Risk of deep vein thrombosis is an important side effect and prophylaxis have to be used;
- bortezomib is a dipeptide boronic acid that inhibits the function of the 26S proteasome complex. It determines the accumulation of misfolded/damaged proteins and cell apoptosis mediated by caspase 8 and 9, inhibits MM cell growth, survival and migration and targets MM microenvironment. Inhibition of NF-κB through accumulation of IκB plays a key role in this process. Bortezomib induces apoptosis in drug-resistant MM cells, and inhibits both secretion of cytokines (such as IL-6) and binding of MM cells to the BM microenvironment. The anti-angiogenic effect of Bortezomib is another potential mechanism of its anti-MM activity. Most common side-effects are peripheral neuropathy, transient thrombocytopenia, fatigue and gastrointestinal disorders [2,4,5,27].

Choice of initial drug therapy

Although successful and long-term remission has been achieved in many transplantation-eligible patients by using limited treatment regimens (thalidomide + dexamethasone, bortezomib + dexamethasone, and lenalidomide + dexamethasone), complete and very good partial response (VGPR) rates can be substantially increased by combining 3 or 4 different drugs. Preliminary results from ongoing phase 3 randomized trials show

improved initial response rates and increased frequency of complete remission (CR) after induction therapy in patients randomized to receive bortezomib and dexamethasone *versus* VAD chemotherapy, and in patients randomized to receive bortezomib, thalidomide and dexamethasone *versus* thalidomide and dexamethasone alone. These higher response rate was followed by higher frequency of CR after transplantation and, at least in preliminary reports, improved PFS. As durable CR and PFS appear to be valuable surrogates for long-term outcome, multiple drug combination therapies have been applied to younger patients suitable for high-dose therapy approaches.

The earliest reports of 3 drug-therapeutical schedules came from the combination of bortezomib, thalidomide, and dexamethasone (VTD) and the encouraging results have been confirmed by using either lenalidomide, bortezomib and dexamethasone (RVD), or liposomal doxorubicin, bortezomib, and dexamethasone (DVD), or cyclophosphamide, bortezomib, and dexamethasone (CVD).

Although response rates are clearly improved with new drug combinations, it is difficult to prove OS advantage considering the large numbers of patients and the long duration of follow-up required. Clinical studies with OS as endpoint are further complicated by the availability of a large number of effective salvage therapies. Nevertheless, RVD, CVD, or VTD are the most commonly used clinical trials so far. Consequently, VAD-based regimens or single-agent dexamethasone are less and less used, and even the simple combination of thalidomide and dexamethasone may be considered suboptimal therapies. Refractory or progressive disease is now uncommon when using multidrug combinations with overall response rates exceeding 90% in almost all recent studies. However, high-dose melphalan should be considered for truly refractory patients, as this group of patients may benefit from this approach [52].

Summary of treatment recommendations

General (all Grade C recommendations; level IV evidence)

• Chemotherapy prescribing should be undertaken by an experienced clinician with input from a chemotherapy-trained pharmacist;

• SPC recommendations for dose adjustments of chemotherapeutic drugs and use of G-CSF support should be followed wherever possible;

• doses should be appropriately calculated to preserve renal and liver functions;

• patients with cytopenias at baseline, due to limited BM hematopoiesis, require more frequent monitoring and dose adjustment;
• all patients should be considered for enrolment into clinical trials;
• therapy choice should consider patient's preference, co-morbidities and toxicity.

Specific treatment recommendations for induction therapy prior to HDT
• VAD or single agent dexamethasone should no longer be used routinely as induction therapy;
• induction regimens should contain at least one novel agent;
• induction regimens that are superior to VAD in terms of response rates include CTD, TAD, bortezomib/dexamethasone and PAD;
• decisions regarding the most appropriate induction schedule require the assessment of a number of factors, such as renal function, thrombotic risk and pre-existing neuropathy, although some agents are not routinely funded as initial therapy in some countries (i.e. U.K. - the most clinical experience in the UK is based on the use of the CTD combination).

Specific treatment recommendations for older and/or less fit patients in whom HDT is not planned as initial therapy
Induction therapy should consist of either thalidomide-containing regimens in combination with alkylating agent and steroid, such as MPT or CTDa (Grade A recommendation; level Ib evidence), or bortezomib in combination with melphalan and prednisolone (Grade A recommendation; level Ib evidence).

Specific treatment recommendations for patients with plasma cell leukemia and rare myeloma subtypes (all are Grade C based on level IV evidence)
• The use of initial treatment with bortezomib and autologous stem cell transplantation should be considered in responding patients with plasma cell leukemia;
• IgD, IgE and IgM myeloma are associated with a poor outcome, but there are insufficient data to support specific alternative treatment strategies at this time [53].

How much treatment before stem cell transplantation?

For the patient eligible for transplantation, autologous stem cell collection and transplantation after 4 to 6 cycles of induction therapy is recommended. However, as the stated goal of therapy is to maximize the depth and duration of remission, induction therapy can be continued in some patients in presence of evident response and tolerability. High-dose melphalan and stem cell transplantation are consolidation schedules after obtaining the best possible response to frontline treatment.

What to do if the patient achieves CR before transplantation is still controversial (continuing chemotherapy *versus* transplantation). So far, transplantation is carried out in most cases, but the option has to be discussed with the patient. CR assessment is a poorly sensitive parameter, as minimal residual disease is present in most patients. If the patient does not achieve complete CR after transplantation, additional consolidation/maintenance therapy, including a second autologous transplantation, should be considered.

Consolidation and maintenance therapy after transplantation

Three separate phase 3 studies showed that thalidomide maintenance improves OS. Despite these findings, thalidomide is not routinely used for maintenance in many centers in consideration of its cumulative toxicity. Lenalidomide may offer the same advantages with less toxicity, and large randomized trials are now addressing its role in the post-transplantation setting. Maintenance therapy is currently used when patients do not achieve CR after stem cell transplantation or in presence of genetic risk markers associated with very high risk of early relapse: in these cases, thalidomide and lenalidomide proved suitable. There are still insufficient data on bortezomib-based maintenance. It is unknown how long maintenance should be, but it is normally tailored on patient's tolerance. Anticoagulation does not seem to be required in the maintenance setting [*52*].

Recommendations (Grade C recommendation, level IV evidence unless stated)

• Interferon (IFN)-α or single-agent corticosteroids cannot be routinely recommended as maintenance therapy (grade A, level Ib recommendation). In the allograft setting, IFN-α may be useful for patients who did not achieve a CR (grade B, level IIa);

• maintenance with thalidomide may improve EFS and OS in patients who did not achieve VGPR post high-dose chemotherapy and maintenance therapy could be considered (grade A, level Ia). Patients with deletion 13q may not benefit from it;
• the dose of thalidomide should not exceed 150 mg (grade B, level IIa recommendation) and no recommendation can be made concerning the duration of thalidomide maintenance;
• in the maintenance setting, routine anticoagulant prophylaxis is not required;
• at present, there is no evidence of benefit for the use of thalidomide maintenance in elderly patients who did not undergo autologous transplantation;
• the combination of steroids and thalidomide is not recommended in the maintenance setting due to increase toxicity and unclear benefit over thalidomide alone;
• although promising data are emerging about the use of bortezomib or lenalidomide in the maintenance setting, long term are needed to recommend their use outside clinical trials [53].

Patients unwilling or unsuitable for high-dose melphalan and transplantation

In these patients, induction therapy (such as VTD, RVD, or CVD) can be prolonged to maximal response as an alternative to transplantation, followed by maintenance in those patients not achieving CR or at high risk for early relapse.

Alternative regimens in younger patients, who do not undergo transplantation, could include alkylating agents in combinations, such as melphalan, prednisone, and bortezomib or cyclophosphamide, thalidomide, and dexamethasone, on the basis of drug availability. Less intensive treatment with lenalidomide and low-dose dexamethasone may be appropriate for patients not requiring a very rapid response to initial therapy and with low risk of early relapse on the basis of clinical features (e.g., low β-$_2$ microglobulin, low LDH, absence of high-risk genetic features).

In patients with significant co-morbidities precluding transplantation, also combination therapies could not be easily administered: in such patients less toxic approaches based on lenalidomide and low-dose dexamethasone could be employed, with the aim to obtain the best possible response with acceptable toxicity. Therapy efficacy in these conditions is unknown [52].

Relapsed and refractory disease

Relapsed/refractory disease is defined in patients who achieve minor response or responses followed by relapse requiring salvage therapy, or in patients who experience progression within 60 days of their last chemotherapy. Subsequent treatment regimens result in progressively shorter response duration, which reflects the emerging drug resistance. The treatment choice depends on the previous schedules, the duration of the response obtained, and the potential side-effects that patient experienced. In general, patients with relapsed/refractory disease are preferentially treated with bortezomib, thalidomide, or lenalidomide. If these agents are no longer effective or unavailable, other treatment options (such as combination chemotherapy or high-dose dexamethasone) can be explored. Future trials will test combinations of novel drugs to increase effectiveness, overcome resistance, and reduce toxic effects, thus further improving patient outcome [5].

Bone disease

The treatment for osteolytic lesions consists in the use of bisphosphonates, radiotherapy, analgesics for bone pain and, rarely, vertebroplasty or surgical procedure for pathological fractures [9]. Bisphosphonates (e.g. clodronate, pamidronate, zoledronate) are inhibitors of osteoclastic activity and may reduce tumor burden by changing BM microenvironment and promoting directly tumor cell apoptosis. They reduce the incidence of skeletal events, prevente hypercalcemia, relieve bone pain, and improve patient's quality of life. Adverse effects include renal toxicity and jaw osteonecrosis in presence of scarse oral hygiene. Bone disease can also be treated with bortezomib, which inhibits osteoclasts and stimulates osteoblasts, thus increasing bone formation [3,9,10].

1.6 New therapeutic strategy in MM

MM is still considered an incurable disease. Consequently, there are different new therapeutic strategies that are currently tested in preclinical and clinical studies in patients with MM. These agents have been classified according to the cellular mechanisms they specifically target: agents acting through cell-surface receptors (e.g, activators of cell-death receptors, inhibitors of tyrosine-kinase receptors (TKR), and monoclonal antibodies against plasma-cell antigens); inhibitors of signalling pathways (e.g., nuclear factor kappa B (NFκB) inhibitors, farnesyl-transferase inhibitors (FTI), mitogen activated protein kinase (MAPK) inhibitors, mammalian target of rapamycin (mTOR) inhibitors, and AKT inhibitors); drugs interfering with the cell cycle (e.g., cyclin dependent kinase (CDK) inhibitors and aurorakinase inhibitors); inhibitors of the unfolded protein response (UPR) (e.g., heat-shock protein (HSP) inhibitors, proteasome inhibitors, and aggressome-formation inhibitors) and epigenetic agents (e.g., hypomethylating compounds and deacetylase inhibitors).

Agents acting on cell-surface receptors

Direct activators of the extrinsic pathway of apoptosis

Two members of the TNF-receptor superfamily have been studied as potential anti-myeloma targets: the TNF-related apoptosis-inducing ligand (TRAIL) receptors (i.e. TRAIL-R1 and TRAIL-R2) and FAS (CD95). Several reports have shown the *in vitro* activity of the ligand TRAIL/APO2L against MM cell lines, *ex vivo* freshly isolated MM cells and *in vivo* human plasmocytomas xenografted into mice [54].

Small-molecule inhibitors that target receptor tyrosine kinases

A second interesting target for anticancer therapy is the group of TKRs. These receptors are very frequently overexpressed in cancer, including MM; consequently, several drugs have been designed to block their proliferative and antiapoptotic effects. Two examples are imatinib and dasatinib. The rationale for using these c-KIT or platelet-derived growth factor receptor (PDGFR) inhibitors is based on the evidence that about a third of patients with MM express c-KIT on plasma cell surface.

Another group of these drugs are the FGF-3 inhibitors. The t(4;14) translocation, involving the FGF-3 gene, is present in 15% of MM patients and confers adverse prognosis. Studies, using the small-molecule inhibitor PD173074,44 or the inhibitor of

class III, IV, and V TKR (dovitinib), or another FGFR3 inhibitor (AB1010) have shown the capacity to inhibit FGFR3 autophosphorylation in cells carrying the translocation, resulting in plasma cell growth arrest and apoptosis. Different clinical studies has been started. In this study, we have tested a new FGFR3 inhibitor, as reported below.

Another interesting target is VEGF because of its role in MM pathogenesis, as reported above. Several tyrosine-kinase inhibitors that target the three VEGFR are already available and have shown *in vitro* activity; in addition, a humanized monoclonal antibody against VEGF (bevacizumab) has already been tested in combination with lenalidomide and dexamethasone in 10 patients. Unfortunately, the expectations raised by the use of these agents have not been confirmed in the clinic so far, as the small-molecule antagonist pazopanib was ineffective in a phase II trial. Nevertheless, these agents might be used in combination with other drugs.

IGF-1 is a relevant cytokine in the pathogenesis of MM. This receptor has been identified on most MM cell lines and *ex vivo* plasma cells, and its activation stimulates proliferation of MM cells and protects them from dexamethasone- or TRAIL-induced apoptosis. Some studies with monoclonal antibodies that block ligand binding are in progress for MM treatment [*23*].

Monoclonal antibodies against surface antigens

The crucial role for IL-6 receptor in plasma cells has been previously discussed. Nevertheless, clinical results with monoclonal antibodies against this receptor, used as single agents, are poor. Preliminary data with the combination of the monoclonal antibody CNTO 328 with bortezomib in 6 patients have shown five partial responses (two of them unconfirmed) [*55*].

CD56 (neural cell adhesion molecule) is involved in the adhesion of MM plasma cells to BM stromal cells: the lack of this marker is associated with extramedullary spreading and tendency to poor prognosis. Around 75% of patients express this antigen on the surface of plasma cells. A new monoclonal antibody, HuN901-DM1, which covalently binds to CD56 and, once internalized, releases the novel cytotoxic maytansinoid DM1, has been developed with promising *in vitro* and *in vivo* preclinical activity. A phase I study is exploring its clinical activity in patients with $CD56^{+}$ refractory MM [*56*]. CD56 is also

expressed by NK cells, and, therefore, this molecule might theoretically hamper the anti-myeloma immune response, but there are not sufficient data so far.

CS1 (cell surface 1 antigen) is a glycoprotein expressed at high levels in MM plasma cells, $CD8^+$ lymphocytes, and NK cells, and is important for the adhesion of plasma cells to stroma. A humanized monoclonal antibody (HuLuc 63) has been generated against this antigen with interesting preclinical activity; nevertheless, no responses have been observed when used as single-agent in the first 7 treated patients [57].

CD40 is expressed on cells with high proliferative activity and in all antigen-presenting cells. In MM cell lines, the monoclonal antibody anti-CD40 (SGN-40) induced apoptosis by growth-inhibitory signaling, antibody-dependent cellular cytotoxicity and antibody-dependent cellular phagocytosis. In a phase I trial that included 32 MM patients, only transient drops in the M-component or stable disease were obtained [58].

Molecules interfering with different signaling pathways

NFκB inhibitors

NFκB is a transcription factor involved in the regulation of genes that participate in cell cycle and apoptosis control. Interaction of myeloma cells with BM microenvironment triggers NFκB-dependent transcription of cytokines, such as IL-6. Studies have shown specific mutations in genes that regulate the NFκB pathway in up to 80% of MM patients. Drugs that inhibit NFκB activation, such as inhibitors of the upstream kinase IKKβ, have shown anti-myeloma activity in preclinical models. Also bortezomib targets this pathway, and a correlation has been noted between the clinical response to this agent and TNF-receptor-associated factor 3 (TRAF3) function. The latter is a protein that negatively regulates NFκB activity. (**Figure 5**)

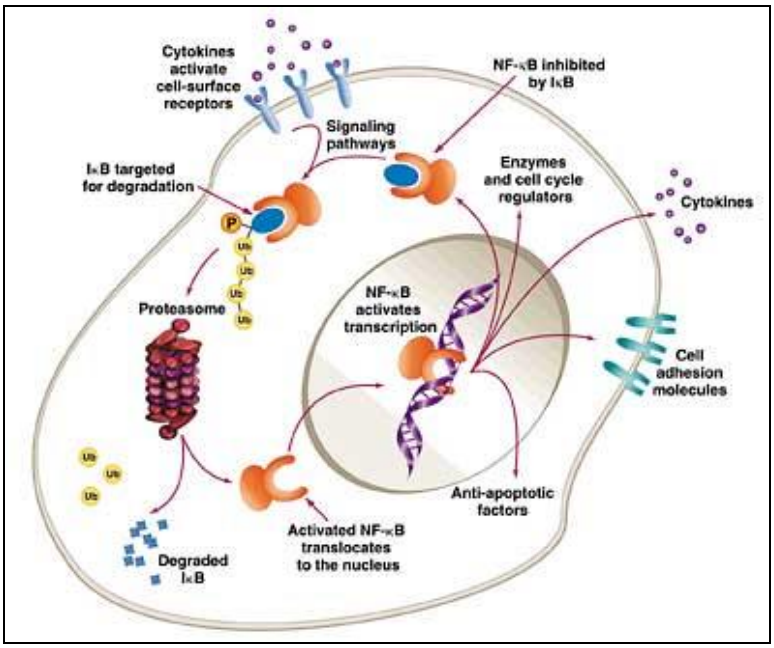

Figure 5. Ubiquitine-proteasome pathway and NF-kB.

Farnesyl-transferase inhibitors

N-RAS and K-RAS mutations are frequent in advanced MM and are associated with adverse prognosis. The use of drugs to inhibit RAS farnesylation in MM patients seems to be an attractive approach, especially in patients with RAS mutations. The preliminary findings of a phase II trial with the FTI inhibitor tipifarnib are not encouraging, because stable disease was achieved only in few patients. It has *in vitro* synergy with other agents, such as bortezomib, but no clinical data have been reported so far.

MAPK inhibitors

The use of P38 MAPK inhibitors (e.g., SCIOS-469) resulted in the inhibition of MM cell proliferation and decrease of IL-6 and VEGF secretion; these inhibitors show synergy with bortezomib. A phase II trial with SCIOS-469 in 62 patients with relapsed MM showed stable disease in 24% of the 28 patients receiving the drug in monotherapy. When combined with bortezomib, 9 of the 34 patients achieved partial response, 2 showed minor response and 3 stable disease. Nevertheless, these responses might be mainly due to bortezomib and not to the P38 inhibitor or the combination. Another drug acting on this pathway is the MAPK-kinase inhibitor AZD 6244, which is effective against MM tumor cells and improves myeloma bone disease in preclinical models.

mTOR inhibitors

mTOR is a downstream target of PI3K/AKT and mediates phosphorylation of proteins responsible for the regulation of protein synthesis and expression of multiple proteins, including D-type cyclins and c-MYC. On the basis of the *in vitro* findings, mTOR inhibitors, such as rapamycin and its analogue temsirolimus, are currently under clinical assessment in MM, both alone and in combination with lenalidomide.

PI3K/AKT inhibitors.

Perifosine is an oral bioactive new alkylphospholipid that inhibits AKT and induces *in vitro* and *in vivo* cytotoxicity in human MM cells; it is synergistic with other agents, such as dexamethasone, doxorubicin, melphalan, and bortezomib. These data provided the rationale for the ongoing clinical trial with perifosine alone and with dexamethasone, bortezomib and/or lenalidomide [*23*].

Agents interfering with the cell cycle

In MM, the control of cell-cycle deregulation is of particular relevance due to the nearly generalized over-expression of cyclins in MM plasma cells. Two families of agents have been employed:

CDK inhibitors

CDK activity is regulated by cyclins; as mentioned above, cyclins D1, D2, or D3 are deregulated in virtually all MM patients. Moreover, gene expression profiling and clinical follow-up have shown differences between the distinct cyclin-D subgroups. As this deregulation seems to be a unifying molecular abnormality in this disease, attempts to inhibit cyclin-D activity by targeting its CDK partners have been carried out with MM cells *in vitro*. Several of these CDK inhibitors resulted effective, by inducing not only cell cycle arrest, but also apoptosis. Clinical trials with these inhibitors will start soon.

Aurora-kinase inhibitors

Aurora kinases are proteins involved in mitotic process. Preclinical studies have shown that aurora-kinase inhibitors determine hyperdiploidy and myeloma cell death [*59,60*].

Inhibitors of the UPR

The UPR is responsible for the detection of misfolded proteins and the coordination of their disposal. UPR proper activity is necessary to maintain cell homoeostasis, especially

in case of neoplastic cells, due to their increased metabolism and protein turnover. MM cells secrete large amounts of Ig, which need to be correctly folded by the chaperone system. If this process fails, the misfolded proteins have to be eliminated by the two main garbage-disposal systems of the cell: the proteasome and the aggresome. Their blockade results in the accumulation of Ig and other toxic proteins in the cytoplasm, and cell death.

HSP inhibitors

The proper activity of HSPs is crucial for the survival of MM cells, not only because they require appropriate chaperon machinery to manage the large amounts of proteins synthesized, but also because they need this system to stabilize survival signaling key-proteins, such as AKT, MEK and NFκB. In fact, HSP27, HSP70 and HSP90 are overexpressed in MM. The use of HSP inhibitors leads to cytotoxic intracellular accumulation of misfolded proteins with subsequent cell death. *In vitro* studies have shown anti-myeloma activity of HSP inhibitors and a synergistic effect with bortezomib. After phase I studies that confirmed their activity, a phase II trial of tanespimycin plus bortezomib was started, with preliminary good responses [*61*].

In these work we used two new HSP90 inhibitors NO and N2, by Novartis Oncology, as reported below.

New proteasome inhibitors

As previously mentioned, the proteasome inhibitor bortezomib, together with the immunomodulatory drugs, has changed the clinical course of MM. Other proteasome inhibitors have been tested with the aims to increase the potency and bioavailability, lower toxicity profile and, eventually, facilitate the administration route. Carfilzomib is one of this new group of proteasome inhibitors with potent *in vitro* activity by inhibiting the chymotrypsin-like activity.

Other proteasome inhibitors have been developed, such as NPI-0052, an oral inhibitor that blocks *in vitro* the three catalytic activities of the proteasome (trypsinlike, chymotrypsin-like, and caspase-like); phase I studies with this drug are ongoing. Finally, other agents are in the first stages of development, such as the oral inhibitor CEP-18770[*62,63*].

Inhibitors of aggresome formation

The simultaneous inhibition of the two cellular garbage-disposal systems, the proteasome and the aggresome, by the histone deacetylase [HDAC]-6 inhibitor tubacin resulted in a

synergistic increase in cytotoxicity of MM cell lines and *ex vivo* isolated fresh cells; however, no clinical data has been reported so far [23].

Epigenetic agents

HDAC inhibitors

Many tumors, including MM cells, show enhanced HDAC activity resulting in the decrease in DNA transcription, especially of tumor-suppressor genes, thus leading to pro-oncogenic conditions. Treatment with HDAC inhibitors can revert this deacetylated pattern, leadingto the final differentiation or apoptosis of MM cells through different mechanisms. Pan-DACi have been shown to have multiple effects in tumor cell lines, such as decreased oncoprotein expression (e.g., Bcr-Abl, HER-2), angiogenesis and tumor cell motility and invasion, as well as induction of apoptosis and cell cycle arrest. A present, several clinically relevant HDAC inhibitors are available and clinical trials with HDAC alone and in combination with other anti-myeloma agents are in progress [23].

In this study we have tested the effect of a histone deacetylase inhibitors, as reported below.

Hypomethylating agents

5-Azacytidine is active against MM cells and its clinical efficacy is currently under assessment in a phase II trial. Combinations of hypo-methylating agents and HDAC inhibitors will be explored to verify whether the simultaneous targeting of both epigenetic pathways may result in antitumoral synergism [23].

Other agents

Plitidepsin (aplidin)

Aplidin is an antitumor agent derived from the marine tunicate *Aplidium albicans*. Although its target has not been defined yet, the involvement of Jun N-terminal kinase and P38 pathways has been shown in aplidin-induced apoptosis in MM. Moreover, this compound is synergistic *in vitro* with several anti-myeloma agents.

Arsenic trioxide

The rationale for using arsenic trioxide in MM is based on its multifaceted effects on MM cell lines and *ex vivo* fresh MM cells. A phase II trial in heavily pretreated patients with relapsed or refractory MM showed minor responses or stabilization of the M-component.

Glutathione has been shown as inhibitor of arsenic trioxide-induced cell death. As ascorbic acid can decrease glutathione concentrations, its combination with arsenic trioxide as well as with dexamethasone would seem appropriate, because they use different proapoptotic pathways. The activity of this drug should be further investigated, because it is active in patients resistant to bortezomib, melphalan or both [23].

On the basis of a written consensus agreement with Novartis-Oncology company, we tested in this study the efficacy and toxicity *in vitro* (on MM cell lines) and *in vivo* (in a mouse MM model) of 5 new molecules:

Hyston deacetilasi (HiDAC) inhibitor (L9): it is a pan-DACi that has been studied in many hematological and solid malignancies, including Hodgkin's lymphoma, MM, acute myeloid leukemia (AML), and myelodysplastic syndromes (MDS). In preclinical studies, L9 has shown nanomolar activity in different hematological malignancies and synergy with chemotherapeutic drugs, demethylators, proteasome inhibitors, and other agents. Phase Ib/II studies of L9, as monotherapy and in combination with chemotherapy and/or targeted therapy, are ongoing in relapsed/refractory Hodgkin's lymphoma (HL), MM, AML/MDS and other hematological malignancies. Phase III trials are ongoing with global enrollment in relapsed MM (PANORAMA-1) and post–transplant HL maintenance (PATH).

N0 and N2: they are novel, non-geldanamycin derivative HSP90 inhibitors. N2 is orally bioavailable. Both *in vitro* and *in vivo* models indicate that they have significant antitumor activity in a wide range of mutated and wild-type cancer cell lines, primary tumor cells and animal models of cancer, including MM, gastric cancer, non-small-cell lung carcinoma (NSCLC), hepatocellular cancer, sarcoma, and breast cancer. Phase I and II studies in hematological malignancies and in solid tumors, including breast cancer and MM, are ongoing with N0. N2 is being investigated in Phase I clinical trials in patients with advanced solid tumors.

FGFR3 inhibitor (T8): it is orally bioavailable and inhibits VEGFR and FGFRs. Phase III trial in renal cell carcinoma, and phase II trial in advanced breast cancer, relapsed MM and urothelial cancer are ongoing with this molecule.

Human neutralizing antibody, anti-DKK1 (N80): Preclinical studies support the hypothesis that N80 promotes bone formation and inhibits tumor-induced osteolytic disease. N80 is currently in Phase I/II clinical trials in MM.
[http://www.novartisoncology.com]

2. 5T MOUSE MODEL

In our study we have used a mouse cell line of MM called 5T33vtMM, which derives from a murine myeloma developing in BM of C57BL/KaLwRij mice.

In 1974 Jiri Radl and Carel Hollander examined by agar gel electrophoresis 800 sera from BALB/c, C57BL/Ka, CBA and C3H mice ranging in age from 1 to 30 months. They found that only in C57BL/Ka 50% of mice by 24 months of age had homogeneous Ig (H-Ig) bands in the β2-γ region. The incidence of these H-Igs increased with age and could be enhanced by neonatal thymectomy or immunization with various antigens given without adjuvants. In BALB/c mice they found only a scarce incidence of spontaneously H-Igs, while in athymic BALB/c/nu/nu mice they were found in 63% of animals by 12 months of age. Dr. Radl transplanted BM from affected old mice into young irradiated recipients ($2x10^6$ bone marrow or $2.5x10^6$ spleen cells were injected intravenously) and obtained the propagation of H-Ig clones.

During these studies, the occasional onset of progressive plasma cell tumors was observed: they produced large amounts of monoclonal Ig and grew serially in a high percentage of recipients, following intravenous transplantation. These mice also developed osteolytic bone lesions. It was estimated that 0.5% of ageing C57BL/KaLwRij mice developed aggressive plasma cell tumors in BM, defined as 5T myelomas. This model is now considered a cellular surrogate of human MM.

Of 5T tumors, 5T33 and 5T2 are mostly used. 5T2 tumor is morphologically heterogeneous and contains lymphoid cells as well as plasma cells; its growth is more restricted to BM and displays less progressive evolution. 5T33 tumor cell line were derived, growing *in vitro* with (5T33vvMM) or without (5T33vtMM) adherent stromal cell layer. If injected intravenously, 5T33 tumor variants induce paralysis at 35-40 days after injection, as well as infiltration of BM, liver, spleen and lymph nodes, and osteolytic lesions. Unlike the induced peritoneal plasmocytomas (such as Sp6), 5T tumors do not have either c-myc activating t(12;15), as shown by the absence of rearranged myc bands, or t(6;15). 5T plasma cells express isoforms of CD44-adhesion molecules V6, V7 and V10, which are critical for the adhesion of 5T cells to BM endothelium.

In conclusion, 5T transplantable MM model resembles human MM in several aspects: myeloma occurred spontaneously, the frequency of development of the disease is age-

related, tumor load can be assessed by paraproteinemia and the concentration of normal Ig is low in the serum (**Table 3**) [*37, 64, 65*].

Table 3. Characteristics of models of plasma cell tumor development (Adapted by Potter et al.)

Model system	Species	Genetics	c-myc activation by CT	Microenvironment of origin	IL-6	Anti-apoptotic factors
Peritoneal PTCs induced by pristane*	Mouse	++, BALB/c is hypersusceptible	Yes	Chronic inflammatory oil granuloma	Inflammation cells and macrophage	IL-6
IL-6 transgenic	Mouse	Suspected, not yet defined	Yes	Lymphonodes and medullary cords	Trangene	IL-6
Eμ-v-abl-transgenic	Mouse	Not yet defined	Yes	Lymphonodes and bone marrow	?	v-abl-transgenic
ABL/MYC retrovirus	Mouse	+	No	LP-GI tract, lymphonodes and spleen	?	v-abl
Bcl_2/Bcl_{XL}	Mouse	?	Yes	Peritoneal cavity	?	transgene
5T	Mouse	C57BL/KaLwRij	No	Bone marrow	?	?
MGUS, Multiple Myelom	Human	?	No	Bone marrow	Stromal cells	?

CT: chromosomal translocation; GI: gastrointestinal; IL: interleukin; *Other poorly metabolized materials are effective as well

AIMS OF STUDY

In this study, we examined the therapeutic potential of five new drugs in MM treatment. We analyzed their effect *in vitro* on different human and mouse myeloma cell lines. In particular, we performed cytotoxic assay and, in case of reduction in cell viability, we assessed whether this effect was associated with the induction of apoptosis.

We also explored the effects of these new potential drugs *in vitro* and *in vivo* in a mouse preclinical model of myeloma, 5T33vtMM, cell line, tumorigenic in the syngenic C57BL/KaLwRij mouse. Thus, we could evaluate at different time points the changes of tumor burden in treated mice, as compared to untreated, myeloma-bearing control animals.

MATERIALS AND METHODS

1. Cell lines

In this study, we have used different myeloma cell lines: human cell lines were RPMI-8226, U-266, KMS-11; mouse cell line was 5T33vtMM. RPMI-8226, KMS-11, 5T33vtMM were maintained in RPMI 1640 medium (Gibco Invitrogen Corporation, San Diego, CA, USA) supplied with glutamine 1mM (Biochrom AG, Berlin, Germany) and 10% heat-inactivated fetal bovine serum (FBS; Euroclone, Pavia, Italy). U-266 was supplied with 15% of FBS. All cell lines were incubated at 37°C and 5% CO_2.

2. Drugs solution and formulation

All the molecules were supplied by Novartis Pharmaceuticals (Basel, Switzerland). For *in vitro* studies, N0, N2 and T8 were dissolved in dimethyl sulfoxide (DMSO) at stock concentration of 10 mM, while L9 was dissolved at stock concentration of 30 mM. The stock solutions were aliquoted and stored at -20°C. N80 was freshly dissolved before use. For *in vivo* studies, all the drugs were freshly dissolved before use, according to drug company's instructions.

3. Cell viability assay

In the cytotoxic assays cells were seeded in 90 µl of RPMI medium (Sigma-Aldrich, Milan, Italy) without red fenol, with 10% FBS at a density of $0.2x10^5$ cells/well in flat-bottomed 96-well plates (Falcon, Becton Dickinson, Lincoln Park, NJ, USA). The molecules were added to wells at serial dilutions. In details: N80: 4, 2, 1, 0.5 and 0.25 ng/ml; N0, N2 and L9: 1000, 333, 111, 55, 27, 13.8, 4.6, 1.5 nM; T8: 5000, 2500, 1250, 750, 375, 187.5, 93.7, 46.8 nM. Total volume of each well was 100 µl and all drug concentrations were added in triplicate for each experiment. Plates were incubated for 24 and 48 hours at 37°C and 5% CO_2. Cytotoxicity assays were performed by using the vital standard 2,3-bis[2-Methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide

inner salt (XTT) colorimetric assay. 10 µl of a 1.5-mg phenazin (Sigma-Aldrich, Milan, Italy)/ml and phosphate-buffered saline (PBS) were added to 10 ml of 5-mg XTT (Sigma-Aldrich, Milan, Italy)/ml RPMI medium stock solution. This freshly prepared mixture was sterilely filtered, and 40 µl were added to each well containing the cells to be tested. Absorption was measured at 450 nm after 2 hours of incubation at 37°C with 5% CO_2. Three independent experiments were performed for each condition.

4. Evaluation of apoptosis *in vitro*

Cells were treated with different concentrations (12.5, 20, 200 nM) of N0, N2 and L9 for 48 hours. Annexin V (Ann-V)/propidium iodide (PI) apoptosis assay was performed according to manufacturer's protocol (Roche diagnostics, Milan, Italy). Cells were gently mixed and the fractions of apoptotic (Ann-V^+/PI^-), dead (Ann-V^+/PI^+) and alive (Ann-V^-/PI^-) cells were measured by flow cytometry (BD-Pharmigen, Milan, Italy). Active Caspase-3 was quantified by using homogenous caspase assay kit, according to manufacturer's protocol (BD-Pharmingen, Milan, Italy) and measured by using FACSCalibur flow cytometer (BD-Pharmigen, Milan, Italy) and FlowJo analysis software. Three independent experiments were performed for each condition.

5. *In vivo* experiments

C57BL/KaLwRij mice (Harlan Laboratories, Belgium) were bred in the animal facility of the University of Verona. Two month-old male and/or female mice were injected intravenously (i.v.) through the tail vein with 0.5×10^6 5T33vtMM tumor cells resuspended in 0.1 ml PBS. The dose of 0.5×10^6 cells is tumorigenic *in vivo* in 100% of syngeneic animals and gave rise to paraplegia in mice in 45-50 days. Tumors grew in BM, spleen and liver.

Tumor bearing mice were divided into different groups to receive different treatments, starting at day +10 from tumor injection. In details: mice in group 1 (n=6) were treated with vehicle (PBS) intraperitoneally (i.p.); group 2 (n=8) were treated with N0 50 mg/Kg i.p- once a week; group 3 (n=8) were treated with N0 50 mg/Kg i.p. twice a

week for 2 week with a week of resting; group 4 (n=8) received N2 10 mg/Kg per os (p.o.) once a week; group 5 (n=8) received N2 10 mg/Kg p.o. twice a week for 2 week with a week of resting; group 6 (n=8) were treated with L9 10 mg/Kg i.p. 5 days/week and group 7 (n=8) were treated with L9 20 mg/Kg i.p. 5 days/week for 2 week with a week of resting. Two different treatment schedule were used for N80: both the groups were treated with N80 10 mg/kg i.p. twice/week, but group 8 (n=8) started treatment on day +0 (preventive schedule), while group 9 (n=8) started on day +10 (curative schedule).

Mice were sacrificed when they developed paraplegia. If signs of pain and fatigue became evident in the animals earlier, they were sacrificed immediately. All *in vivo* experiments were part of Research Protocols authorized by the Italian Ministry of Health: Decreto n. 199/2008-B and 14/2012-B,.

6. Evaluation of tumor burden

Tumor burden was measured in mice by determining the concentration of paraprotein and VEGF in serum, which were collected at specific time point (+31 and +45 days after tumor cell injection). ELISA assay was used to quantify the levels of murine IgG2b (BETHYL Laboratories.Inc, Montgomery, TX, USA) and VEGF (R&D System, Minneapolis, MN, USA). Serum samples were diluted prior to test (dilution 1:10.000) for ELISA IgG2b, and required 5-fold dilution for ELISA VEGF. The range of sensitivity of the ELISA IgG2b assay was 0.34-250 ng/ml.

7. Histomorphometry

Three mice for each group, at day +45 from tumor injection, were sacrificed for histomorphometric analysis. They were injected with demeclocycline 25 mg/Kg subcutanuosly (s.c.) 14 and 13 days before sacrifice, and with calcein 10 mg/Kg s.c. 4 and 3 days before sacrifice.

The right tibiae were removed, dissected to remove soft tissue, and fixed in 70% reagent alcohol. The samples were embedded undecalcified in methyl-methacrylate resin (Merck 800590, Darmstadt, Germany). Bone sections were cut by using a microtome

(Polycut S, Leica Microsystems, Wetzlar, Germany) equipped with a carbide-tungsten blade, stained with Goldner's stain and toluidine blue, and mounted on microscope slides for histomorphometric measurements. Some sections were left unstained for the measurement of fluorescent labeling. The sections were obtained from three different levels of the methyl-methacrylate block, each separated by a thickness of 250 µm. Histomorphometric results were calculated as the mean of the values obtained from the three different levels as an approximation of a 3-D evaluation. This also avoids replicating the sampling of any single bone remodeling unit.

Measurements were performed by means of an image analysis system consisting of epifluorescent microscope (Leica DM2500, Leica Microsystems, Wetzlar, Germany) connected to a digital camera (Leica DFC420 C) and a computer equipped with a specific software for histomorphometric analyses (Bone 3.5, Explora Nova, La Rochelle, France).

The area analyzed was restricted to the trabecular bone of the secondary spongiosa area between 2 and 4 mm distal to the growth plate-metaphyseal junction[66]. Histomorphometric parameters are reported in accordance with the ASBMR Committee nomenclature [67].

Thickness results were adjusted for the obliquity of sections by multiplying by $\pi/4$ [67]. As to the assessment of trabecular microarchitecture, trabecular number (Tb.N), trabecular separation (Tb.Sp; µm), and trabecular thickness (Tb.Th; µm) were measured. After skeletonization, the trabecular network was evaluated by measuring the connections between nodes (points at which three or more trabeculae joined), connection branches (struts), terminal or free-ends branches (termini) and by calculating Total Skeletonized Structure Length (TSL), number of nodes (N.Nd/TV), and nodes/termini ratio (Nd/Tm), as previously described [68].

In addition, indirect parameters of microarchitecture were assessed: Marrow Star Volume (MSV; N/mm^3), that is the mean volume of all the parts of an object that can be unobscured in all the directions from a point inside the object [69]**;** Fractal Dimension (D), which describes how an object fills space with relation to its structure. This parameter permits to evaluate bone structural anisotropy **[70].**

8. Statistical analysis

When applicable, results are presented as mean ± standard deviation. Annexin-V, Caspase-3 and ELISA tests were analyzed by analysis of variance (ANOVA) with the *post hoc* Tukey test for intra-group comparison. Survival analysis has been carried out using the method developed by Kaplan and Meiers, and the log-rank test to compare survival. Differences were considered statistically significant for P values ≤ 0.05. Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA).

RESULTS

1. Effect of N80, T8, L9, N0 and N2 on MM cells *in vitro*

Concentration-dependent anti-survival activity of N80, T8, L9, N0, and N2 was assessed *in vitro* on a panel of human (RPMI-8226, U-266, KMS-11) and mouse (5T33vtMM) myeloma cell lines by using the XTT assay (see Materials and Methods) at 24 and 48 hours.

N80 was tested on all cell lines at different concentrations (4, 2, 1, 0.5 and 0.25 ng/mL) for 24 and 48 hours of incubation. As expected, no effect of N80 on cell survival was observed on treated cells as compared to controls, either after 24 (data not shown) or 48 hours (**Figure 6 and Table 4A**).

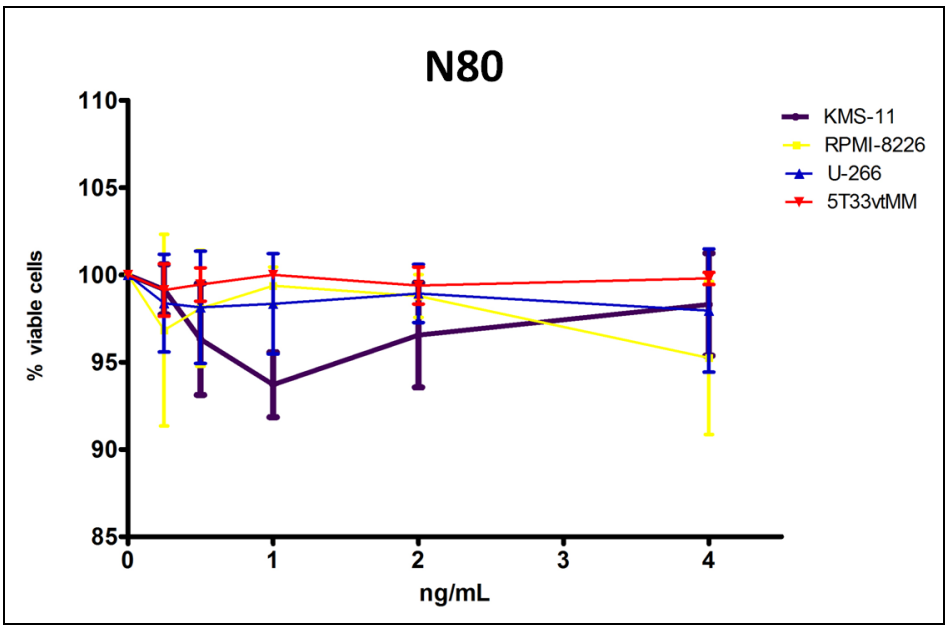

Figure 6. N80 has no cytotoxic effect on MM cells *in vitro*: XTT test at different concentrations of N80 in medium culture after 48 hours of incubation. The percentage of viable cells is on y axis, while the different concentrations of N80 (ng/mL) are on x axis. Mean value ± standard deviation (n=3).

T8 effect was assessed on 5T33vtMM cell line at concentrations ranging from 5,000 nM to 20 nM; we found a reduction in cell survival only for high concentrations of the molecule, with median inhibition concentration (IC_{50}) value of 4,315 nM after 48 hours

of incubation (**Figure 7A, B and Table 4B**). No inhibition was found after 24 hours of treatment (data not shown).

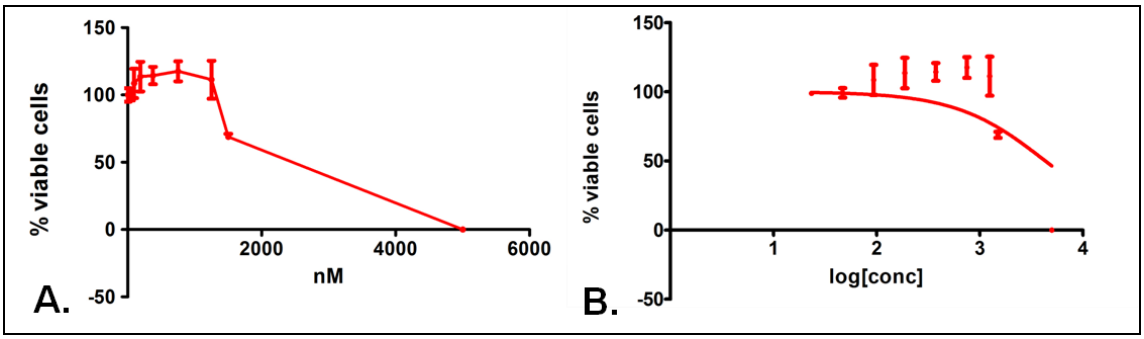

Figure 7. T8 has cytotoxic effect on 5T33vtMM cells only at high doses *in vitro*: XTT test on 5T33vtMM at different concentrations of T8 in medium culture after 48 hours of incubation. The percentage of viable cells is on y axis, while the different concentrations of T8 (nM) are on x axis. Normalized data in linear graph **(A)** and transformed in logarithmic graph **(B)**. Mean value ± standard deviation (n=3).

The human myeloma cell lines and the mouse 5T33vtMM cell line were exposed to **N0** at concentrations ranging from 0.15 nM up to 1,000 nM for 24 hours (data not shown) and 48 hours (**Figure 8**). After 48 hours IC_{50} values raging from 2.15 to 7.88 nM were observed in all myeloma cell lines (**Table 4C**). The maximum decrease in myeloma cell viability varied from 100% in the sensitive cell lines 5T33vtMM to 23% in RPMI-8226 (**Table 4C**).

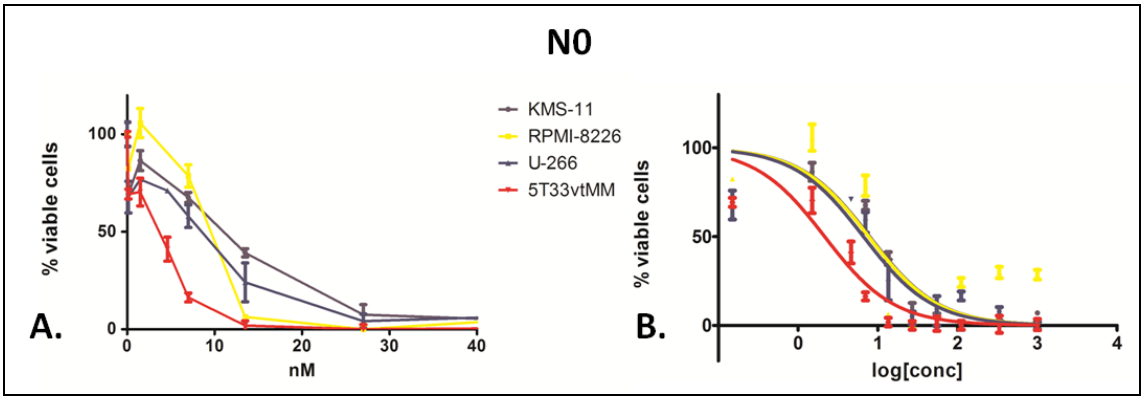

Figure 8. A linear dose-response effect is observed treating MM cells with increasing amounts of N0: XTT test at different concentrations of N0 in medium culture after 48 hours. The percentage of viable cells is on y axis, while the different concentrations of N0 (nM) are on x axis. Normalized data in linear graph **(A)** and transformed in logarithmic **(B)**. Mean value ± standard deviation (n=3).

N2 activity on human and mouse myeloma cell lines was tested for concentrations ranging from 0.15 nM up to 1,000 nM for 24 (data not shown) and 48 hours (**Figure 9**). After 48 hours IC_{50} values ranging from 3.63 to 26.72 nM were observed in all myeloma cell lines (**Table 4D**). The maximum decrease in myeloma cell viability was very similar for all cell lines, reaching 1% viability in RPMI-8226 and the total abrogation in the sensitive cell lines 5T33vtMM, KMS-11 and U-266 (**Table 4D**).

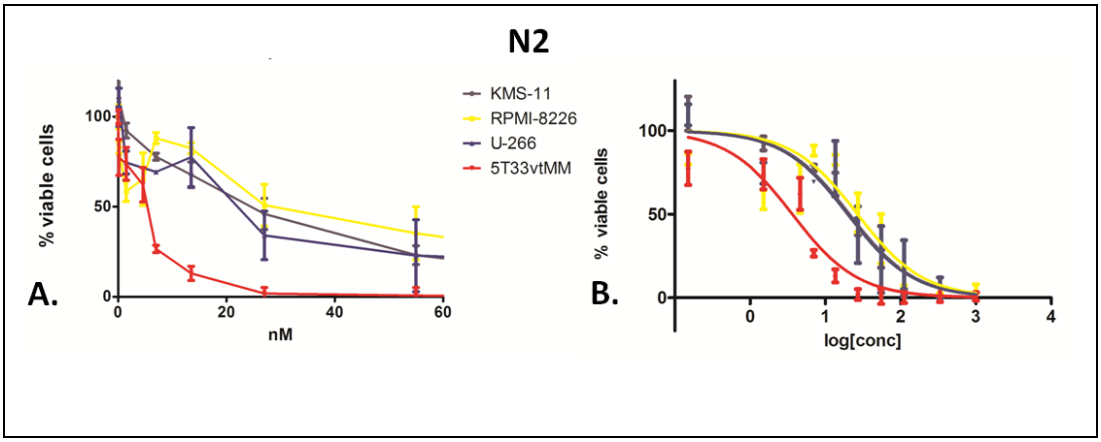

Figure 9. N2 is effective in reducing the viability of MM cells *in vitro* after 48 hours: XTT test at different concentrations of N2 in medium culture at 48 hours. The percentage of viable cells is on y axis, while the different concentrations of N2 (nM) are on x axis. Normalized data in linear graph **(A)** and transformed in logarithmic **(B)**. Mean value ± standard deviation (n=3).

Finally, we tested **L9** on 5T33vtMM, RPMI-8226, U-266 and KMS-11 at concentrations ranging from 1,000 nM to 0.15 nM for 24 (data not shown) and 48 hours. We found a reduction in cell activity for different concentrations of L9 in different cell lines, with IC_{50} values ranging from 3.63 nM for 5T33vtMM to 25.72 nM for RPMI-8226 after 48 hours of incubation (**Figure 10A and B, Table 4E**). The maximum decrease in myeloma cell viability varied from total abrogation in the sensitive cell lines 5T33vtMM, RPMI-8226 and U-266, to 27% viability in KMS-11 (**Table 4E**).

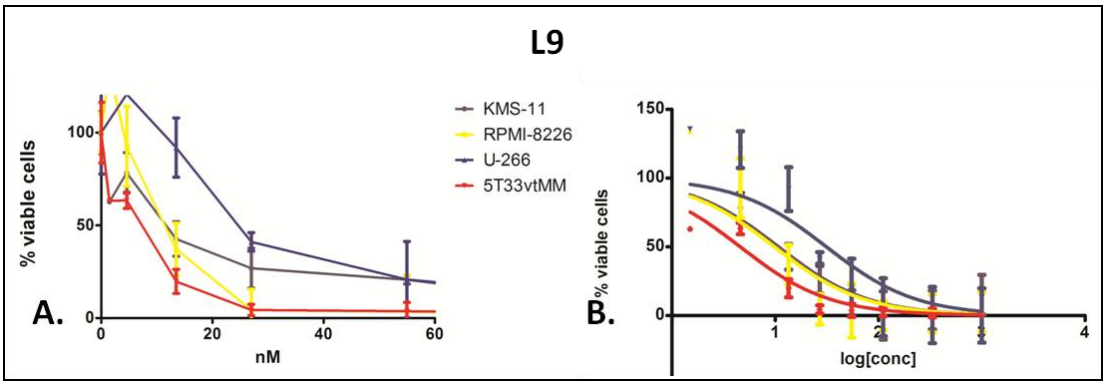

Figure 10. L9 reduces viability of MM cells in different manner *in vitro*: XTT test at different concentrations of L9 in medium culture at 48 hours. The percentage of viable cells is on y axis, while the different concentrations of L9 (nM) are on x axis. Normalized data in linear graph **(A)** and transformed in logarithmic **(B)**. Mean value ± standard deviation (n=3).

Table 4: IC_{50} and minimum viability values for N80 (A), T8 (B), N0 (C), N2 (D), L9 (E) in MM cell lines. Cells were cultured in the presence of 0.25-4 ng/mL of N80, 20-5,000 nM of T8, 0.15-1,000 nM of N0, N2 and L9 for 48 hours and viability was determinated using XTT assay.

Cell line	IC_{50} (nM)	Min. cell viability (% of control)
A) N80		
KMS-11	Not reached	70%
RPMI-8226	Not reached	65%
U-266	Not reached	100%
5T33vtMM	Not reached	75%
B) T8		
5T33vtMM	4315	1%
C) N0		
KMS-11	7.88	7%
RPMI-8226	7.68	23%
U-266	6.6	3%
5T33vtMM	2.15	0%
D) N2		
KMS-11	20.79	0%
RPMI-8226	26.72	1%
U-266	19.76	0%
5T33vtMM	3.63	0%
E) L9		
KMS-11	10.73	27%
RPMI-8226	9.797	0%
U-266	31.27	0%
5T33vtMM	4.585	0%

N2, N0 and L9 were the most potent compounds with nanomolar IC_{50} values and for this reason they were used for further studies.

2. N0, N2 and L9 induce cell apoptosis *in vitro*

In XTT test, N0, N2 and L9 reduced the viability of MM cells in all cell lines. To assess whether their effect was either cytostatic or cytotoxic, these molecules were analyzed for apoptosis induction by using Ann-V/PI analysis and Caspase-3 protein expression after 48 hour culture with drugs at different concentrations (12.5, 20 and 200 nM).

N0 (**Figure 11, Table5 and B**): N0 was capable of exerting a statistically significant (*P*=0.05) pro-apoptotic effect already at 12.5 nM dose in all cell lines, as compared to untreated RPMI-8226, KMS-11, U-266 and 5T33vtMM (n=3). The effect was not dose-dependent, as there were not statistically significant differences with the dose of 25 nM (n=3) and 200 nM (n=3), as compared to 12.5 nM.

Table 5: Studies of apoptosis after culture with different doses of N0 for 48 hours. A) percentage of alive cells (Annexin-V negative and PI negative); **B)** percentage of Caspase-3 positive cells. Mean value ± standard deviation (n=3).

	A) N0: % Ann-V$^-$/PI$^-$ cells (alive)				B) N0: % caspase-3$^+$ cells			
nM	**RPMI-8226**	**KMS-11**	**U-266**	**5T33vtMM**	**RPMI-8226**	**KMS-11**	**U-266**	**5T33vtMM**
NT	72.23±2.3	70.87±0.25	94.67±1.16	83.2± 1.65	17.84 ± 1.23	16.33±1.78	10.44±0.55	5.61±0.53
12.5	15.97±1.01	29.53±2.03	69.17±1.35	1.35 ± 0.11	60.47± 5.25	58.33 ±2.77	57.03±2	80.43±3.51
25	18.4±0.51	15.6±1.04	68.8±2.1	0.6 ± 0.31	64.63±1.4	55.17 ±3.38	66.37±5.72	84.8±3.27
200	17.9±1.76	16.23±1.45	67.63±0.6	1.04 ± 0.32	70.2±1.3	53.74±1.3	66.27±6.57	82.5±5.42

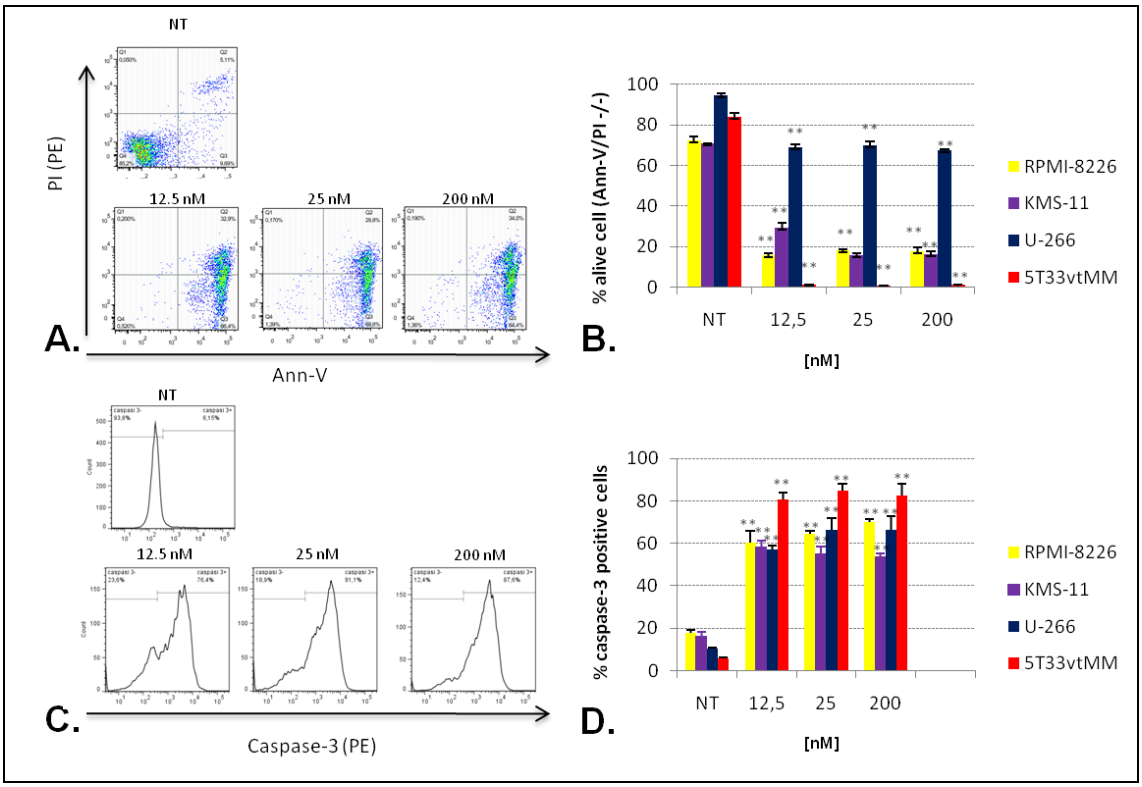

Figure 11. N0 inhibits the survival of MM cells *in vitro*, activating apoptosis. (A): Annexin-V/PI test: a representative experiment on 5T33vtMM cells. After 3 days, the presence of N0 at all concentrations strongly inhibited the growth of 5T33vtMM *in vitro*, as shown by the increase of double-positive (Ann-V^+/PI^+) cells. **(B):** Mean percentage of viable MM cells (Ann-V^-/PI^-) at 48 hours (n=3). The percentage of viable cells is on y axis, while the different concentrations of N0 (nM) are on x axis. Mean value ± standard deviation. As compared to MM cell lines cultured alone, the presence of N0 at different concentrations exerted a statistically significant pro-apoptotic effect. This effect was appreciable in all 4 cell lines (human and mouse). **(C):** Caspase-3 test: a representative experiment on 5T33vtMM cells. The presence of N0 at all concentrations induced the expression of Caspase-3. **(D):** Mean percentage of Caspase-3^+cells at 48 hours (n=3). The percentage of Caspase-3^+ cells is on y axis, while the different concentrations of N0 (nM) are on x axis. Mean value ± standard deviation. Reduction of cell survival was due to activation of apoptotic pathway through the expression of Caspase-3 (*= P<0.05; **= P<0.01; ***= P<0.001).

N2 (**Figure 12, Table 6A and B**): culture with N2 was capable of exerting a statistically significant pro-apoptotic effect already at dose of 12.5 nM for U-266 and 5T33vtMM (P=0.01), but not for KMS-11 and RPMI-8226. An increase of apoptotic cells, as compared to untreated cells, was obtained at 20 nM dose for KMS-11 (P=0.05) and only at 200 nM dose for RPMI-8226 (P=0.01). The effect was dose-dependent, with the statistically significant linear reduction of alive cells along with increasing N2 dose in culture medium (P=0.01 for all conditions) (**Table 3A**). We could observe the same effect on the induction of Caspase-3 expression (**Table 6B and Figure 12**).

Table 6: Studies of apoptosis after culture with different doses of N2 for 48 hours. A) percentage of alive cells (Annexin-V negative and PI negative); **B)** percentage of Caspase-3 positive cells Mean value ± standard deviation (n=3).

	A) N2: % Ann-V$^-$/PI$^-$ cells (alive)				B) N2: % caspase-3$^+$ cells			
nM	**RPMI-8226**	**KMS-11**	**U-266**	**5T33vtMM**	**RPMI-8226**	**KMS-11**	**U-266**	**5T33vtMM**
NT	74.23±0.23	67.7±3.74	92.87±0.06	83.17± 1.50	22.4±4.6	10.8±3.2	8.14±0.6	6±0.6
12.5	62.27±28.66	61.87±1.39	78.87±1.55	73.4 ± 2.08	31.6±7.3	9.7±1.5	28.5±2.3	17.8±4.3
25	64.07±2.13	57.37±2.81	74.33±0.67	60.97 ± 1.01	51.3±3.6	15.4±3.8	36.2±4.9	32.7±3.9
200	28.67±3.28	14.87±4.82	55.73±1.16	4.64 ± 0.56	59.2±3.4	62.9±0.7	47.7±2.1	80.5±5.3

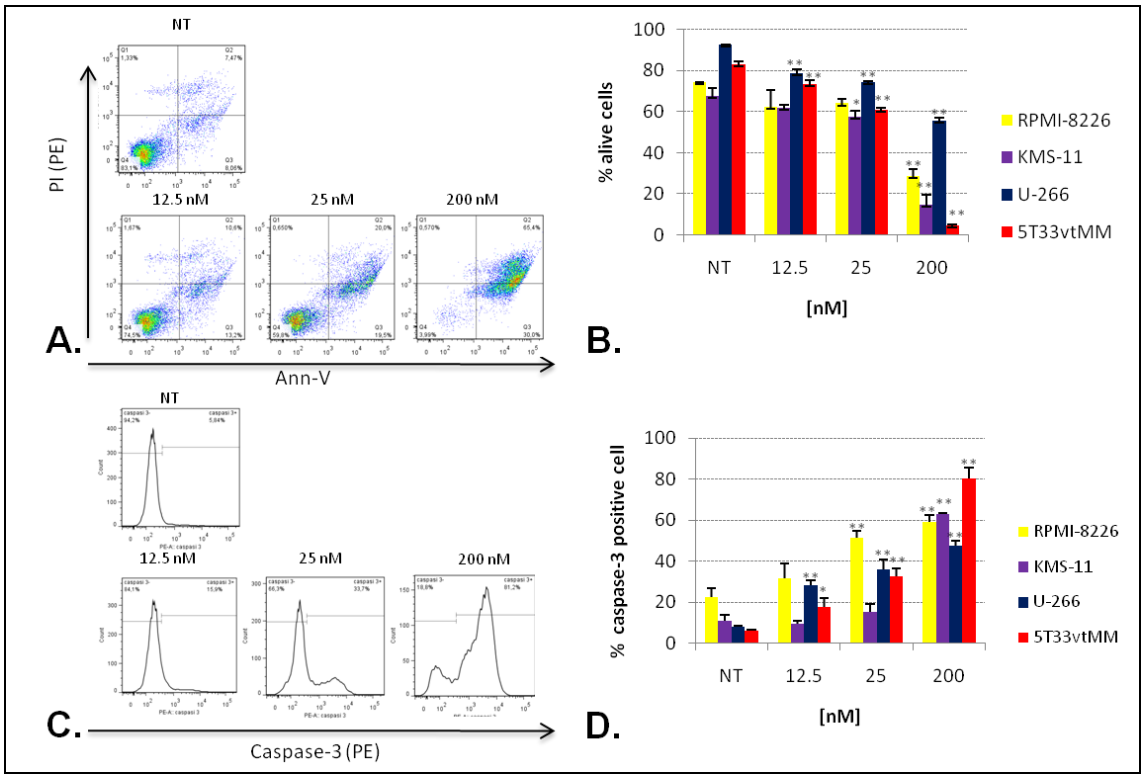

Figure 12. N2 inhibits the survival of MM cells *in vitro*, activating apoptosis. (A): Annexin-V/PI test: a representative experiment on 5T33vtMM cells. After 3 days, the presence of N2 at different concentrations strongly inhibited the growth of 5T33vtMM *in vitro*, as shown by the increase of double-positive (Ann-V$^+$/PI$^+$) cells. **(B):** Mean percentage of viable MM cells (Ann-V$^-$/PI$^-$) at 48 hours (n=3). The percentage of viable cells is on y axis, while the different concentrations of N2 (nM) are on x axis. Mean value ± standard deviation. As compared to MM cell lines cultured alone, the presence of N2 at different concentrations exerted a statistically significant pro-apoptotic effect. This effect was appreciable in all 4 cell lines, for concentration of 200 nM, but only in 5T33vtMM and U-266 for 12.5 nM and 25 nM. **(C):** Caspase-3 test: representative experiment on 5T33vtMM cells. The presence of N2 at all concentrations induced the expression of Caspase-3. **(D):** Mean percentage of Caspase-3$^+$ cells at 48 hours (n=3). The percentage of Caspase-3$^+$ cells is on y axis, while the different concentrations of N2 (nM) are on x axis. Mean value ±

standard deviation. Reduction of cell survival was due to activation of apoptotic pathway, by the expression of Caspase-3 (*= $P<0.05$; **= $P<0.01$; ***= $P<0.001$).

L9 (**Figure 13, Table 7A and B**): culture with L9 was capable of exerting a statistically significant pro-apoptotic effect already at dose of 12.5 nM for all cell lines ($P=0.01$). The effect was dose-dependent for all cell lines: we could observe a statistically significant linear reduction of alive cell along with increasing L9 dose in culture medium for all conditions with U-266 and 5T33vtMM ($P=0.01$), while there were no differences between 12.5 and 25 nM for RPMI-8226 and KMS-11. A significant difference was observed for 200 nM as compared to 12.5 nM ($P=0.05$ for KMS-11 and $P=0.01$ for RPMI-8226) and 25 nM ($P=0.05$ for both cell lines) (**Table 7A**). We could observe the same effects in the induction of Caspase-3 expression (**Table 7B and Figure 13**).

Table 7: Studies of apoptosis after culture with different doses of L9 for 48 hours. A) percentage of alive cells (Annexin-V negative and PI negative); **B)** percentage of Caspase-3 positive cells. Mean value ± standard deviation (n=3).

	A) L9: % Ann-V$^-$/PI$^-$ cells (alive)				B) L9: % caspase-3$^+$ cells			
nM	**RPMI-8226**	**KMS-11**	**U-266**	**5T33vtMM**	**RPMI-8226**	**KMS-11**	**U-266**	**5T33vtMM**
NT	68.47±0.47	73.2±0.95	90.07±0.11	71.63±0.46	14.13±0.59	24.06±0.69	8.95±0.69	14.,44±1.12
12.5	6.72±1.47	19.97±1.42	64.13±3.11	39.03±1.15	54.03±7.34	65.3±3.22	44,7±0.53	55,5±3.35
25	4.76±0.83	18.67±1.8	51.4±0.86	3.23±0.24	73.97±0.80	58.3±2.15	60.27±2.98	78.37±6.31
200	2-21±0.29	14.87±4.82	55.73±1.16	4.64 ±0.56	71.4±2.74	55.33±4.20	77.67±4.08	84.03±0.83

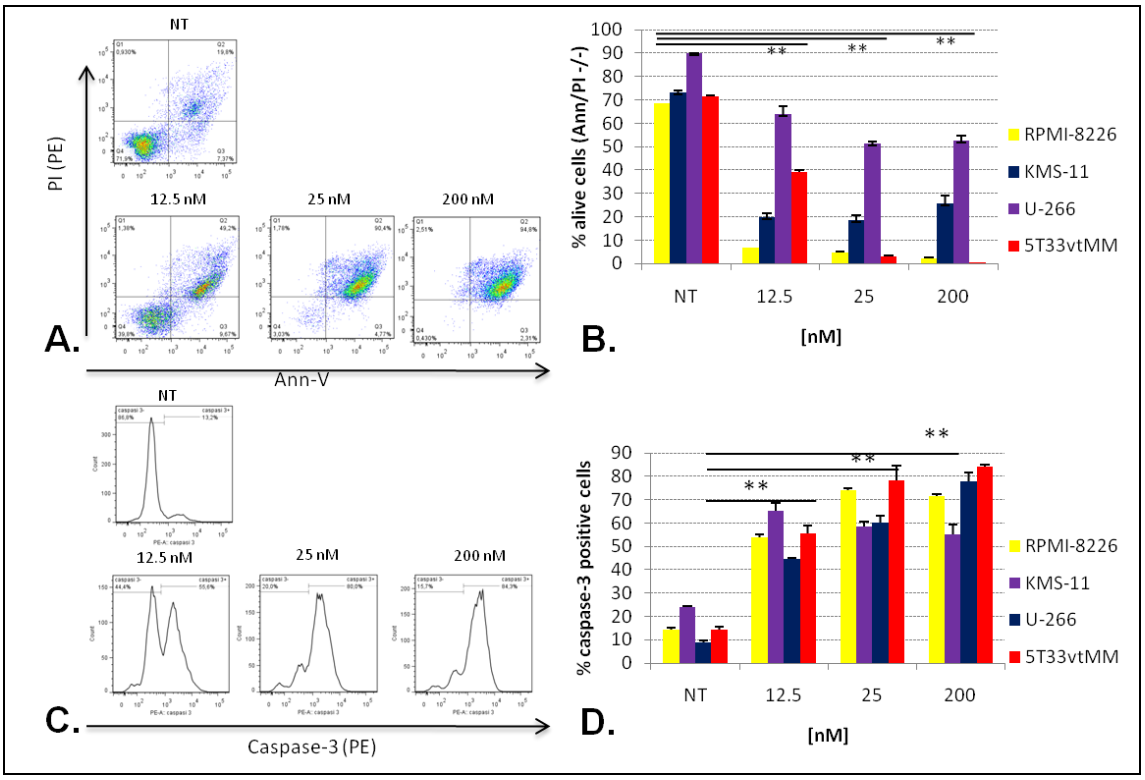

Figure 13. L9 inhibits the survival of MM cells *in vitro*, activating apoptosis. (A): Annexin-V/PI test: a representative experiment on 5T33vtMM cells. After 3 days, the presence of L9 at different concentrations strongly inhibited the growth of 5T33vtMM *in vitro*, as shown by the increase of double-positive (Ann-V^+/PI^+) cells. **(B):** Mean percentage of viable MM cells (Ann-V^-/PI^-) at 48 hours (n=3). The percentage of viable cells is on y axis, while the different concentrations of L9 (nM) are on x axis. Mean value ± standard deviation. As compared to MM cell lines cultured alone, the presence of L9 at different concentrations exerted a statistically significant pro-apoptotic effect. This effect was appreciable in all 4 cell lines (human and mouse) **(C):** Caspase-3 test: a representative experiment on 5T33vtMM cells. The presence of L9 at all concentrations induced the expression of Caspase-3. **(D):** Mean percentage of Caspase-3+ cells at 48 hours (n=3). The percentage of Caspase-3^+ cells is on y axis, while the different concentrations of L9 (nM) are on x axis. Mean value ± standard deviation. Reduction of cell survival was due to apoptotic pathway activation through the expression of Caspase-3 (*= P<0.05; **= P<0.01; ***= P<0.001).

In summary, studies on 5T33vtMM have shown the cytotoxic activity of N0, N2 and L9. XTT test has detected the almost complete reduction of viable cells with high doses of drugs, and IC_{50} values of 2.15 nM for N0, 3.63 nM for N2, and 4.585 nM for L9 (**Table 4 and Figure 14A**). This effect is correlated with the induction of apoptosis, as demonstrated by Annexin-V/PI analysis. As compared to 5T33vtMM cultured alone (79.67 ± 6.15% viable cells, n=9), the presence of 12.5 nM of drug was capable of exerting a statistically significant pro-apoptotic effect: 1.35 ± 0.11% alive cells for N0 (n=3; P=0.01), 73.4 ± 2.08% for N2 (n=3; P=0.05) and 39.03±1.15%, (n=3; P=0.01) for

L9. N0 is significantly more effective in inducing apoptosis than N2 (*P*=0.01) and L9 (*P*=0.01); and 5T33vtMM cells are more sensible to L9 than N2 (*P*=0.01) (**Table 5, 6 and 7, and Figure 14B**). Further analysis have shown that cell death occurred through caspase-3 activation (**Figure 14C**).

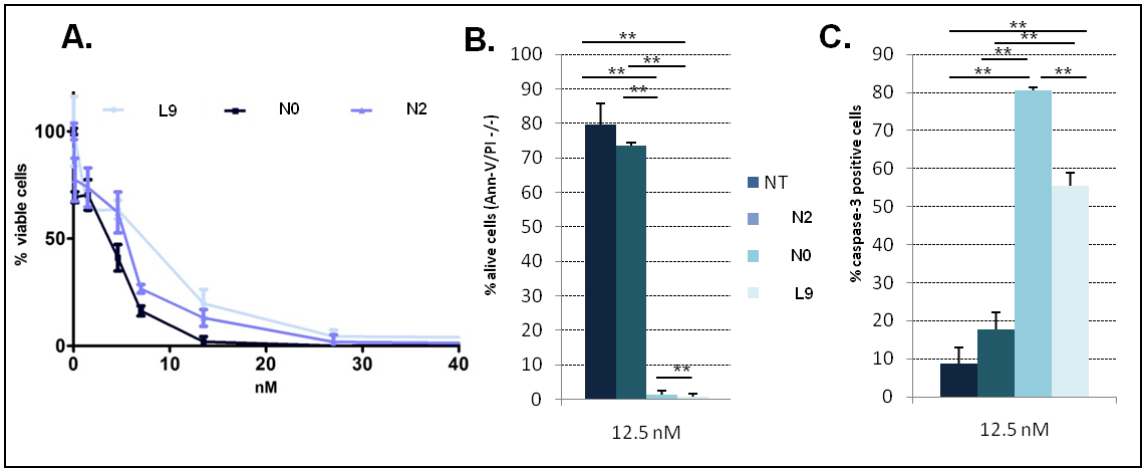

Figure 14. 5T33vtMM is sensible to N0, N2 and L9 *in vitro*. (A): XTT test, linear data. The percentage of viable cells is on y axis, while the different concentrations of the drugs (nM) are on x axis. Mean value ± standard deviation. **(B):** Annexin-V/PI test: mean percentage of viable MM cells (Ann-V^-/PI^-) at 48 hours (mean value ± standard deviation) at 12.5 nM of N0, N2 and L9. **(C):** Caspase-3 test: mean percentage of Caspase-3^+ cells at 48 hours (mean value ± standard deviation) with 12.5 nM of N0, N2 and L9. N0 and L9 induced a significant reduction of viable cells as compared to untreated samples, activating apoptosis. 5T33vtMM is more susceptible to N0 and L9 as compared to N2 at low concentration (*= *P*<0.05; **= *P*<0.01; ***= *P*<0.001).

3. Treatment with N0, N2, L9 and N80 reduces M-component production *in vivo*

To evaluate tumor development from 5T33vtMM injected in C57BL/KaLwRij, we measured IgG2b level, the paraprotein produced by neoplastic cells, by using ELISA assay on serum samples collected at different time points. At day +31 from cell injection no differences were found in mice treated with all drugs, as compared to untreated mice (data not shown). In details, the levels of serum IgG2b was 5.34 ± 9.97 g/L in untreated mice (n=6), 5.07± 1.78 g/L in mice treated with L9 20 mg/Kg/day for 5 days/week (n=7), 4.09± 0.98 g/L in mice treated with N0 50 mg/Kg twice/week (n=4), and 5.30± 0.64 g/L in mice treated with N2 10 mg/Kg twice/week (n=7).

At day +45, we found a reduction of IgG2b serum levels in mice treated with N80, N0, N2 and L9, as compared to untreated mice (n=3) (36.03± 12.88 g/L). In particular, N0 was tested at two different doses (50 mg/kg i.p. once a week and 50 mg/kg i.p. twice a

week): both treatments started at day +10 from tumor cells injection and led to a significant reduction in IgG2b serum levels of mice treated with N0 (once a week schedule: 6.78± 1.80 g/L; *P*<0.01, n=3; double week schedule: 9.58± 8.84 g/L; *P*<0.05, n=3), without differences between the two schedules (**Figure 15**).

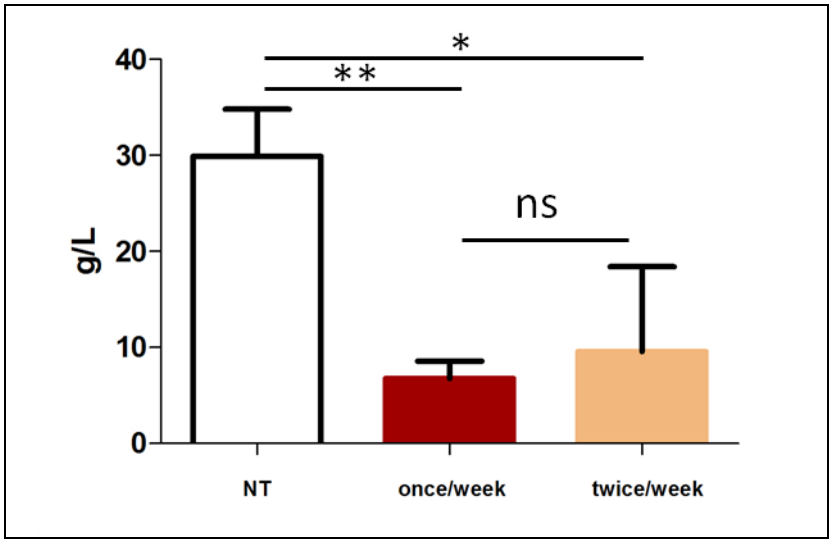

Figure 15: treatment with N0 reduces tumor burden at day +45: ELISA assay of IgG2b (g/L) in serum of mice untreated and treated with N0 50 mg/Kg i.p. once a week or twice a week. Mean value ± standard deviation (n=3). (*= *P*<0.05; **= *P*<0.01; ***= *P*<0.001).

Similarly, N2 was tested at two different schedules of treatment: 10 mg/kg *orally* once a week and 10 mg/kg *orally* twice a week. Both schedules started at day +10 from tumor cells injection. We found a significant reduction in IgG2b serum levels in mice treated with N2 (once a week schedule: 5.24± 4.10 g/L; *P*<0.001, n=3; double week schedule: 4.08± 0.98 g/L; *P*<0.001, n=3), without differences between the two schedules (**Figure 16**).

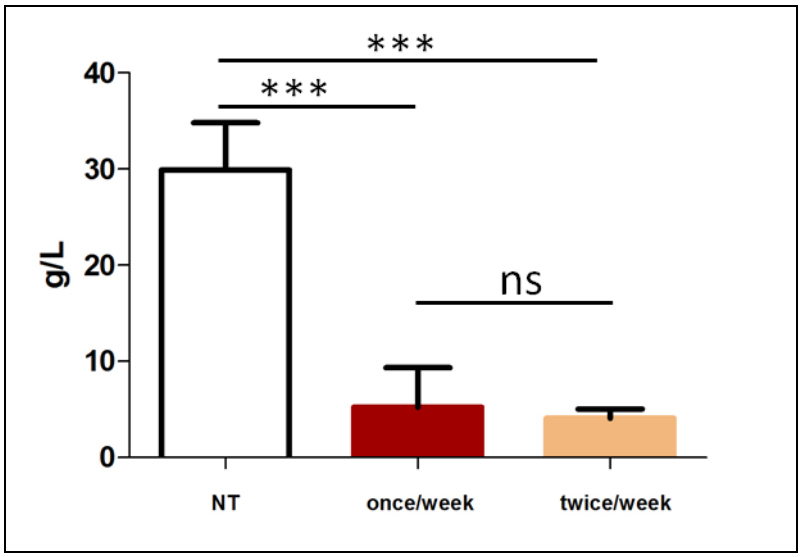

Figure 16: treatment with N2 reduces tumor burden at day +45: ELISA assay of IgG2b (g/L) in serum of mice untreated, treated with N2 10 mg/Kg *orally* once a week or twice a week. Mean value ± standard deviation (n=3). (*= $P<0.05$; **= $P<0.01$; ***= $P<0.001$).

L9 was tested at two different schedules of treatment: 10 mg/kg i.p. 5 days a week and 20 mg/kg i.p. 5 days a week. Both schedules started at day +10 from tumor cells injection. No differences were found in IgG2b serum levels of mice treated with L9 at low dose (27.63± 5.48 g/L, n=3), while the highest dose was effective (5.34± 0.58 g/L n=3) as compared to control group ($P<0.001$) and low dose schedule ($P<0.01$) (**Figure 17**).

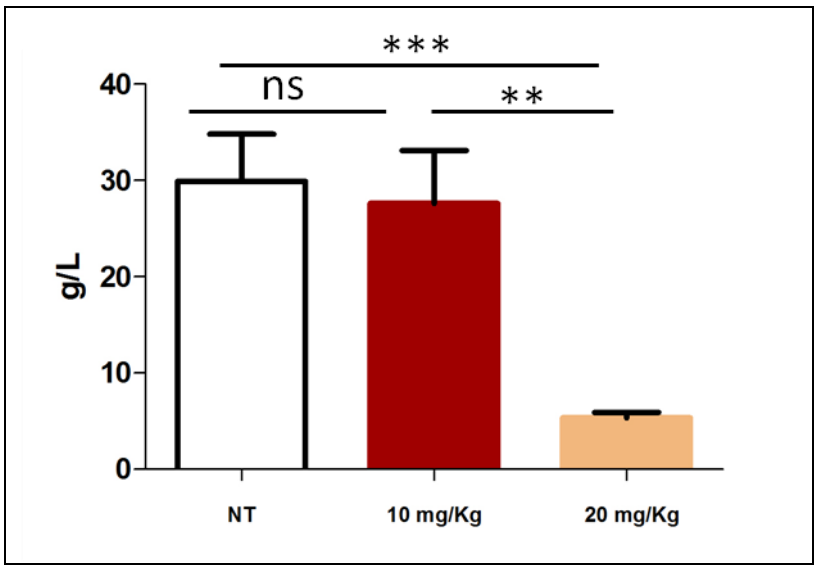

Figure 17: treatment with L9 reduces tumor burden at day +45 at a dose of 20 mg/kg for 5 days a week: ELISA assay of IgG2b (g/L) in serum of mice untreated, treated with L9 10 mg/Kg or 20 mg/kg i.p. 5 days/week Mean value ± standard deviation (n=3). (*= *P*<0.05; **= *P*<0.01; ***= *P*<0.001).

N80 was tested at two different schedules of treatment: 10 mg/kg i.p. twice a week for preventive treatment (started at day +0 from tumor cells injection) and therapeutical treatment (started at day +10). No differences were found in IgG2b serum levels of mice treated with N80 using the therapeutical schedule (24.79± 6.69 g/L, n=3), while the preventive schedule was effective (7.05± 1.40 g/L n=3) as compared to control group (*P*<0.01) and therapeutical schedule (*P*<0.05) (**Figure 18**).

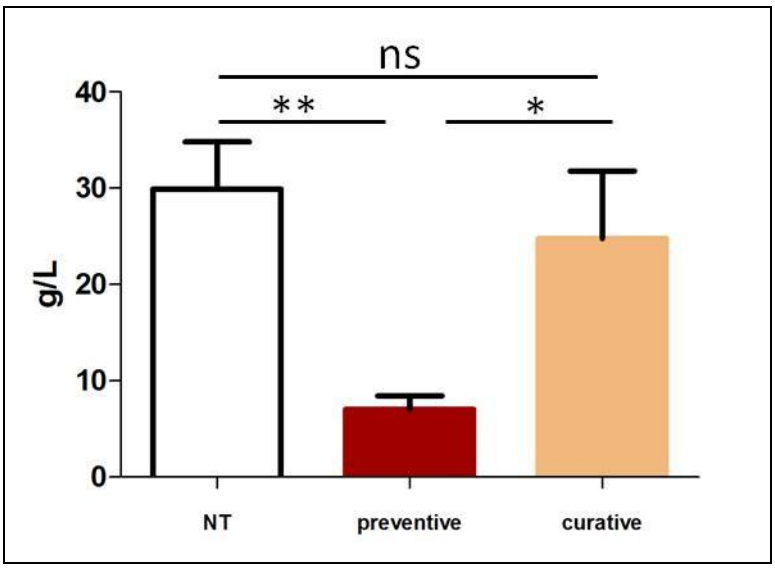

Figure 18: treatment with N80 reduces tumor burden at day +45 in a preventive schedule: ELISA assay of IgG2b (g/L) in serum of mice untreated, and treated with N80 10 mg/Kg i.p twice a week according to preventive and therapeutical schedules. Mean value ± standard deviation (n=3). (*=*P*<0.05; **= *P*<0.01; ***= *P*<0.001).

In summary, treatment with N0 and N2 was effective in lowering IgG2b serum levels at low doses, while L9 required high doses. Once a week schedule of N0 and N2 has similar effects, while is significantly more effective than L9 at low dose (*P*<0.01 for N0 and *P*<0.001 for N2). All drugs reduced M-component levels when used at high dose, with no differences among the various schedules.

4. Treatments with N0, N2, L9 and N80 reduce serum VEGF *in vivo*

The level of VEGF was measured in mice serum collected at day +45 by using ELISA test. We found a statistically significant reduction in VEGF levels in mice treated with all drugs as compared to controls. In details, the levels of VEGF were: 76.35± 6.69 pg/ml (n=3) in tumor bearing mice not receiving treatment; 43.22± 14.43 pg/ml in mice treated with N0 50 mg/Kg i.p. once a week (P<0.01, n=3); 47.06± 12.47 pg/ml in mice treated with N2 10 mg/Kg *orally* once a week (P<0.05, n=3); 37.22± 4.08 pg/ml in mice treated with L9 10 mg/Kg i.p. 5 days at week (P<0.01, n=3); 26.79± 3.15 pg/ml in mice treated with N80 10 mg/Kg i.p. twice a week in a preventive schedule (P<0.01, n=3) and 38.49± 8.93 pg/mlin mice treated with N80 10 mg/Kg i.p. twice a week in a therapeutical schedule (P<0.001, n=3) (**Figure 19**).

No significant differences were observed among the various schedules.

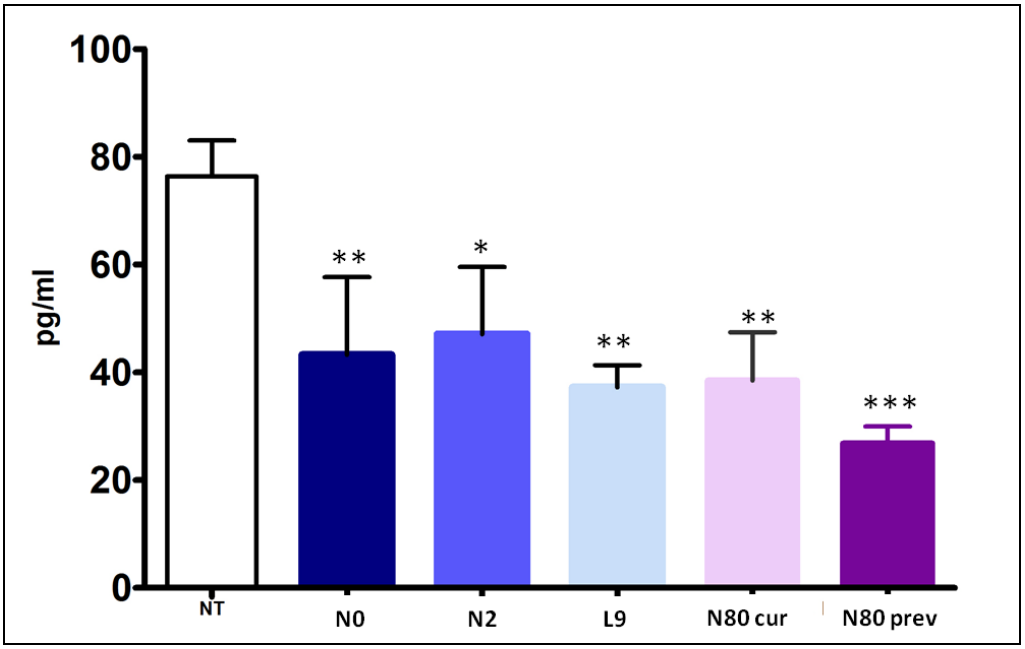

Figure 19: all the drugs reduce VEGF levels at day +45: ELISA assay of VEGF (pg/mL) in serum of mice untreated, treated with N0 50 mg/Kg i.p. once a week, N2 10 mg/Kg *orally* once a week, L9 10 mg/Kg i.p. 5 days at week, N80 10 mg/Kg i.p. twice a week in a preventive and therapeutical schedule. Mean value ± standard deviation (n=3). (*= P<0.05; **= P<0.01; ***= P<0.001).

5. Treatments with L9 improved bone architecture of mice injected *in vivo* with 5T33vtMM cells

Tumor bearing mice showed significant reduction in trabecular bone volume, thickness and number of trabeculae. Moreover, we could observed an increased osteoclast activity expressed as the extent and depth of erosion surfaces and number of active osteoclasts. There was also reduction of osteoblastic activity, of osteoid parameters (data not shown) and we could detect an abnormal microarchitecture.

The administrations of N80, N0, N2 were not able to prevent bone damage (trabeculae thin, non-interconnected, large gaps bone marrow and localization of disease, -data not shown). Otherwise, the administration of L9, already at low dose, showed the ability to preserve the trabecular microarchitecture and a partially osteoblastic activity. This effect was significantly increased when mice were treated with L9 higher dose, showing an organization trabecular most represented, with trabeculae more closed to each other and with a thickness significantly higher compared to untreated mice (**Figure 20**).

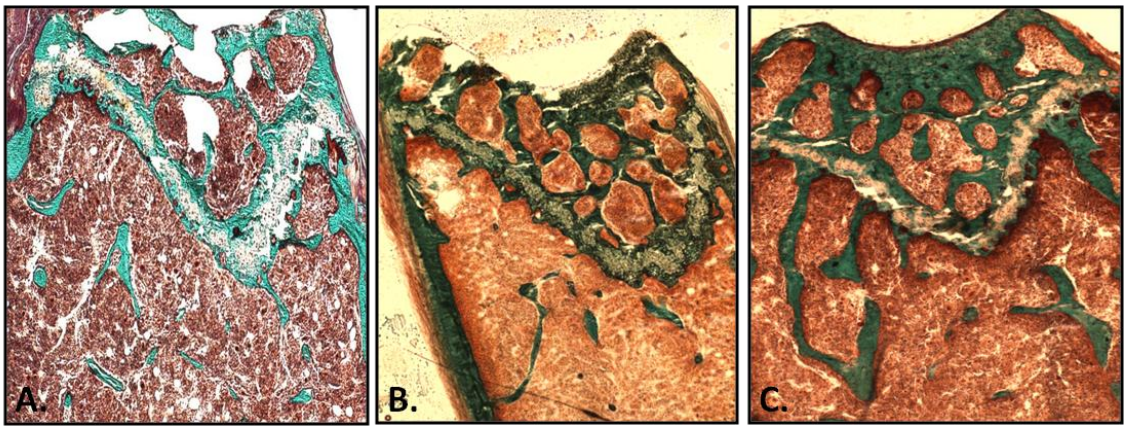

Figure 20: L9 influence bone architecture *in vivo* in 5T33MM model. Histomorphometric section of mice injected i.v. with 0.5×10^6 5T33vtMM cells at day 0 and treated with: L9 10 mg/Kg i.p. 5 days a week **(A)** or 20 mg/kg i.p. 5 days a week **(B)**.

6. Treatments with N0, N2, L9 and N80 do not modify the overall survival of mice injected *in vivo* with 5T33vtMM cells

Distinct Groups of C57BL/KaLwRij mice were injected i.v. with 5T33vtMM cells. On day +10, all animals started treatment with the different drugs. Median OS was not significantly improved by the administration of the different treatments. In details: median OS was 44.5 days in control animals (range: 38-53 days, n=6), 45 days in mice treated with N0 50 mg/Kg i.p. once a week (range 37-52 days, n=8) and 22.5 days twice a week (range 11-31 days, n=8) (**Figure 21A**). We observed increasing toxicity in mice treated with high dose schedule, with necrosis at the injection site (**Figure 22**).

Treatment with N2 induced a median OS of 39 days (range 31-47 days, n=8) for mice receiving 10 mg/Kg *orally* once a week, and 54 days (range 17-69 days, n=8) for mice receiving a twice a week schedule (**Figure 21B**).

Treatment with L9 induced a median OS of 42 days (range 31-47 days, n=6) for mice receiving 10 mg/Kg i.p. 5 days a week and 45 days (range 38-56 days, n=6) for mice receiving 20 mg/Kg i.p. 5 days a week (**Figure 21C**).

Treatment with N80 induced a median OS of 46 days (range 42-55 days, n=6) for mice receiving 10 mg/Kg i.p. twice a week in a preventive schedule, and 41 days (range 38-56 days, n=6) for mice receiving the therapeutical schedule (**Figure 21D**).

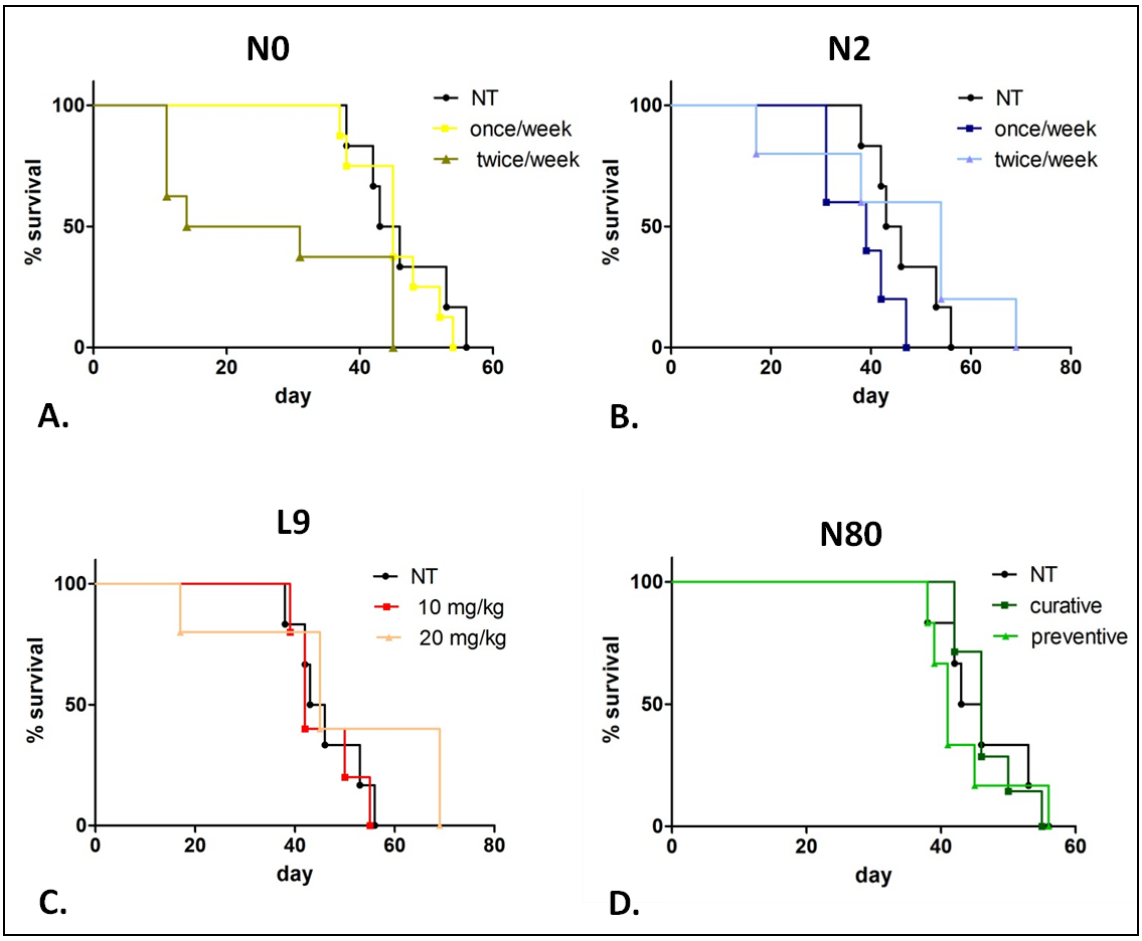

Figure 20: None of the drugs influence OS *in vivo* in 5T33MM model. OS of mice injected i.v. with 0.5×10^6 5T33vtMM cells at day 0 and treated with: N0 50 mg/Kg i.p. once a week or twice a week **(A)**, N2 10 mg/Kg *orally* once a week or twice a week **(B)**, L9 10 mg/Kg i.p. 5 days a week or 20 mg/kg i.p. 5 days a week **(C)**, N80 10 mg/Kg i.p. twice a week in a preventive and therapeutical schedule **(D)**.

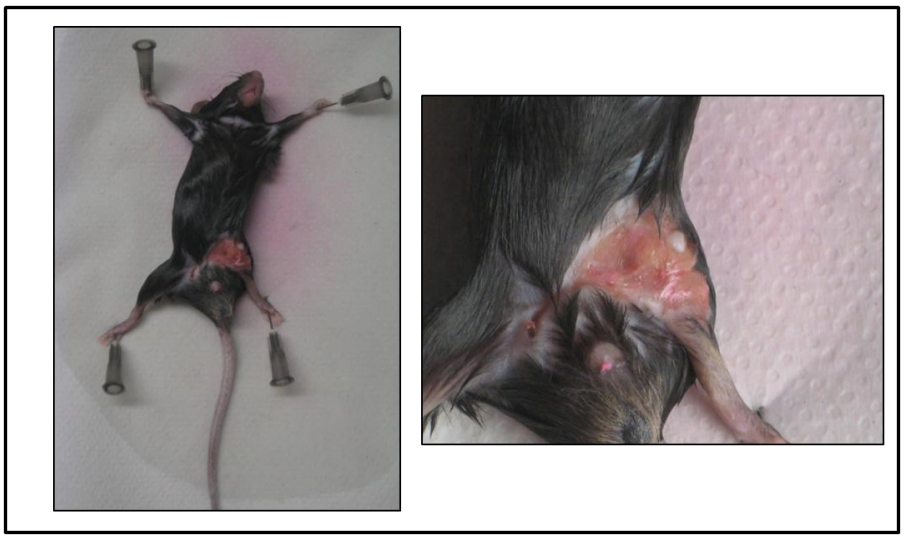

Figure 21: N0 high dose causes necrosis at the injection site. Mice injected i.v. with 0.5×10^6 5T33vtMM cells at day 0 and treated with N0 50 mg/Kg i.p. twice a week developed necrosis at the site of injection, already after 2 doses.

DISCUSSION

Multiple myeloma is still an incurable disease, although new therapeutical strategies have become available in the last years. New treatments based on either biologic drugs, such as thalidomide and lenalidomide, or NFkB inhibitors, such as bortezomib, have improved patients' quality of life by reducing bone pain, anemia, fatigue and complication related to the presence of M-component. In addition, they have increased the event free survival, although often without improving the overall survival, especially when used as monotherapy. Thus, novel therapeutic targets and synergistic combinations with appropriate anti-myeloma agents are urgently needed.

In particular, the large amount of studies on MM microenvironment, biology of neoplastic plasma cell and multistep progression from MGUS to MM facilitates the development of new compounds with either direct cytotoxic effect on myeloma cells, or specific interference with tumor microenvironment.

In this study, in collaborations with a drug company (Novartis Farma S.p.A.), we tested five new molecules with different action mechanisms: nuclear inhibitors (**L9**); growth factor receptor inhibitors (**T8**); intracellular inhibitors (**N0, N2**); and monoclonal antibodies with potential anti-cancer effects (**N80**). The results of the experiments can be shown only partially until the end of the project, because of the confidentiality agreement with Novartis Farma S.p.A.

The *in vitro* experiments have permitted to demonstrate that the mouse cell line 5T33vtMM and human cell lines (RPMI-8226, U-266, KMS-11) are sensible to drugs in a similar manner. This finding is quite relevant, as it shows that these drugs have a general effect on plasma cell biology, regardless the species of origin.

L9 is a HiDAC inhibitor with well known effects on MM cell lines. Deacetylase (DACs) are enzymes specialized in removing acetyl groups from their target proteins, which can be histone- and non-histone proteins. The use of this kind of drugs in MM is supported by the evidence that there is a general pattern of deacetylation in neoplastic cells that could be reverted by the use of HiDAC inhibitors. So far, phase III randomized trials are in progress to assess whether the combination of HiDAC inhibitors with either bortezomib or lenalidomide plus dexametasone is superior to standard protocols currently used for

relapsed/refractory MM patients [71]. By adding L9 to culture medium, we confirmed the reduction of cell viability in all human ad mouse cell lines *in vitro*. This effect was related to the induction of apoptosis, as shown by Annexin-V test and the expression of Caspase-3 in all death cells. For *in vivo* studies, we divided mice in two groups, one treated with 10 mg/kg/five days week and one with 20 mg/kg/5 days week. We collected serum of mice at days +45 after injection for the assays. We observed a decrease in serum M-component (IgG2b) levels for high doses, but not for the 10 mg/Kg dose, while serum VEGF level was already reduced with lower dose of the agent. Thus, a better definition of the therapeutical window of L9 has to be done.

T8 blocks multiple signalling pathways activated by FGFRs, inhibits proliferation and causes a strong reduction in mammary tumor outgrowth and lung metastasis formation. In addition, it inhibits VEGFR) and PDGFR [72]. Theoretically, T8 may have a specific application in MM, as activating mutations of FGFR3 occurs in about 10% of patients with t(4;14) MM and play an important role in the progression of t(4;14) (p16;q32) MM. This chromosome translocation identifies MM with poor prognosis, in particular when treated with melphalan and thalidomide/lenalidomide, while bortezomib appears to abrogate the early mortality, even if the poor prognosis is not completely overcome. However, the absence of FGFR3 expression in about 25% of patients with t(4;14) MM cases suggests that the persistent expression of FGFR3 is important, at least in some patients [73]. In our study we did not find any cytotoxic activity of T8 on 5T33vtMM cells, probably because this mouse cell line has not the mutation enhancing FGFR3 activity. Consequently, we decided not to proceed with other experiments with this molecule.

N0 and **N2** are Hsp90 inhibitors. These molecules have shown potent anti-cancer activity in preclinical studies of leukemia, lymphoma and selected solid tumors, where they showed synergistic effects with either conventional cytotoxic drugs, such as etoposide, taxol and cytarabine, or target therapies such as imatinib and rituximab. [74]. In particular, the promising preclinical data obtained with N0 supported the start of clinical phase I trials in patients with solid tumors. [75]. By using N0 and N2, we observed a different cytotoxic effect on the cells lines, depending on drug concentration and cell type, with IC_{50} values in the low nanomolar range. Annexin-V assay demonstrated that significant apoptosis may be induced with N0 in all cell lines low doses (12.5 nM), in absence of dose-effect curve. By contrast, N2 addition led to significant late and early apoptosis at

12.5 nM dose only using U-266 and 5T33vtMM cerll lines, but not KMS-11 and RPMI-8226, which required at least 25 nM concentration to observe significant cell death, as shown by Caspase-3 expression. In addition, N2 pro-apoptotic effect was dose-dependent, differently from N0. Thus, N0 and N2 seem to act differently, regardless their common inhibitory effect on Hsp90.

I*n vivo* studies with N0 and N2 were based on two different administration protocols, once or twice a week, to test the kinetics of action of both molecules. At day +45 after tumor injection, we found a significant reduction in tumor burden in all treated mice as compared to controls, as measured by serum M-component (IgG2b) and VEGF levels, and with both the molecules and treatment schedules. However, we did not find any advantage from using higher doses of both drugs, rather signs of toxicity (necrosis in the side injection) in mice treated with N0 twice/week. N2 was administered orally, with no apparent toxicity along dose increase. In summary, N0 and N2 seem to be promising in terms of efficacy and their use has to be further investigated.

N80 is a fully humanized, anti-DKK1 monoclonal antibody, with high affinity and neutralizing effect on both human and mouse DKK1. In MM, the overexpression of DKK1 by plasma cells inversely correlates with lytic bone disease, because it inhibits osteoclasts. N80-based clinical trials are in progress both in relapsed and high-risk asymptomatic MM [76]. As expected, we did not find any direct *in vitro* cytotoxic effect of N80 on 5T33vtMM cells after both 24 and 48 hours of incubation; however, in consideration of its mechanism of action on osteoclasts, we tested its clinical effectiveness *in vivo*. We performed experiments with two treatment schedules: early treatment, administered the same days of neoplastic cell injection (preventive schedule), and late treatment, at days +10 from tumor injection (therapeutical schedule), thus mimicking N80 use in clinical trials. We found a significant reduction in IgG2b serum levels only by using preventive schedule, while VEGF reduction occurred following both treatments, although less evident, in a non statistically manner, with the preventive schedule. Thus, our data confirm other studies [77] and show that the anti-DKK1 antibody does not act directly on MM cells, but may interfere with bone microenvironment leading to bone formation and indirect inhibition of tumor growth.

Although all the treatment schedules tested in this study did not modify significantly the overall survival of tumor bearing mice, the reduction in tumor burden observed with some of these new molecules (L9, N2, N0 and N80) has to be anyway considered a good result for myeloma treatments based on single agents. In fact, most of the new molecules tested so far were not effective when used alone. Consequently, our results give support to plan new therapeutic strategies by using different doses, administration schedules and combinations of L9, N2, N0 and N80. Different therapeutical approaches might eventually influence OS, such as increasing the dose of single drugs, combining new molecules with high-dose steroids, or melphalan, or bortezomib and thalidomide/lenalidomide. Moreover, different MM mouse models could be used: for instance, 5T33vtMM cells could be injected into SCID Rag2-/- γ chain -/- mice, which are profoundly immunodeficient mice capable of accepting human cells without any sign of rejection. In this case, developing MM would have the same growth kinetics than using C57BL/KalwRij mice (personal observation), with the advantage to evaluate indirectly the role in the response to anti-myeloma treatment of recipient immune system, which completely lacks in SCID Rag2-/- γ chain -/- mice.

Another experimental approach will be to assess the efficacy of different drugs on fresh plasma cells, once collected *ex vivo* from BM samples of MM patients and positively selected by immunosorting for CD38+. This way, the sensitivity to drugs could be tested *in vitro* in a personalized manner for each single patient with MM at diagnosis.

REFERENCES

1. Kyle RA, Rajkumar SV. Monoclonal gammopathy of undetermined significance. Br J Haematol 2006; 134:573-89.

2. Sirohi B, Powles R. Multiple Myeloma. Lancet 2004; 363(9412):875-87.

3. Zaidi AA, Vesole DH. Multiple myeloma: an old disease with new hope for the future. CA Cancer J Clin 2001; 51, 273-85.

4. Raab MS, Podar K, Breitkreutz I, Richardson PG, Anderson K. Multiple myeloma. Lancet 2009; 374: 324-39.

5. Caers J, Vande Broek I, De Raeve H, Michaux L, Trullemans F, Schots R, Van Camp B, Vanderkerken K. Multiple myeloma - an update on diagnosis and treatment. Eur J Haematol. 2008; 81: 329-43.

6. Greenlee RT, Hill-Harmon MB, Murray T, Thun M. Cancer statistics, 2001. Cancer J Clin 2001; 51: 15-36.

7. Morgan GJ, Davies FE, Linet M. Myeloma aetiology and epidemiology. Biomed Pharmacother 2002; 56: 223-34.

8. Ludwig H. Advances in biology and treatment of multiple myeloma. Ann Oncol 2005; 16: 106-12.

9. Terpos E, Dimopoulos MA. Myeloma bone disease: pathophysiology and management. Ann Oncol 2005; 16(8):1223-31.

10. Barillé-Nion S, Bartologie B, Bataille R, Bergsagel PL, Epstein J, Fenton RG, Jacobson J, Kuehl WM, Shsughnessy J, Tricot G. Advances in biology and therapy of multiple myeloma. Hematology Am Soc Hematol Educ Program 2003; 248-78.

11. Alexanian R, Barlogie B, Dixon D. Renal failure in multiple myeloma. Pathogenesis and prognostic implications. Arch Intern Med 1990; 150(8):1693-95.

12. Chow CC, Mo KL, Chan CK, Lo HK, Wong KS, Chan JC. Renal impairment in patients with multiple myeloma. Hong Kong Med J 2003; 9(2):78-82.

13. Pruzanski W, Gidon MS, Roy A. Suppression of polyclonal immunoglobulins in multiple myeloma: relationship to the staging and other manifestations at diagnosis. Clin Immunol Immunopathol 1980; 17(2):280-86.

14. Barosi G, Boccadorio M, Cavio M, Corradini P, Marchetti M, Massaia M, Merlini G, Tosi P, Tura S. Management of multiple myeloma and related-disorders: guidelines from the Italian Society of Hematology (SIE), Italian Society of Experimental Hematology (SIES) and Italian Group for Bone Marrow Transplantation (GITMO). Haematologica 2004; 89:717-41.

15. Dispenzieri A, Kyle RA. Neurological aspects of multiple myeloma and related disorders. Best Pract Res Clin Haematol 2005; 18(4):673-88.

16. Dispenzieri A, Kyle RA. Multiple myeloma: clinical features and indications for therapy. Best Pract Res Clin Haematol 2005; 18(4):553-68.

17. Zangari M, Saghafifar F, Mehta P, Barlogie B, Fink L, Tricot G. The blood coagulation mechanism in multiple myeloma. Semin Thromb Hemost 2003; 29(3):275-82.

18. Tedeschi A, Baratè C, Minola E, Morra E. Cryoglobulinemia Blood Rev. 2007 Jul;21(4):183-200.

19. Hallek M, Bergsagel PL, Anderson KC. Multiple myeloma: increasing evidence for a multistep transformation process. Blood 1998; 91:3-21.

20. Korde N, Kristinsson SY, Landgren O Monoclonal gammopathy of undetermined significance (MGUS) and smoldering multiple myeloma (SMM): novel biological insights and development of early treatment strategies. Blood. 2011 May 26;117(21):5573-81.

21. Kuehl WM, Bergsagel PL. Multiple myeloma: evolving genetic events and host interactions. Nat Rev Cancer 2002; 2:175-87.

22. Podar K, Richardson PG, Hideshima T, Chauhan D, Anderson KC. The malignant clone and the bone-marrow environment. Best Pract Res Clin Haematol 2007; 20(4):597-612.

23. Ocio EM, Mateos MV, Maiso P, Pandiella A, San-Miguel JF. New drugs in multiple myeloma: mechanisms of action and phase I/II clinical findings. Lancet Oncol 2008; 9:1157-65.

24. Wu KD, Orme LM, Shaughnessy J Jr, Jacobson J, Barlogie B, Moore MA "Telomerase and telomere length in multiple myeloma: correlations with disease heterogeneity, cytogenetic status, and overall survival. Blood. 2003 Jun 15;101(12):4982-9.

25. Klein B, Tarte K, Jourdan M, Mathouk K, Moreaux J, Jourdan E, Legouffe E, De Vos J, Rossi JF. Survival and proliferation factors of normal and malignant plasma cells. Int J Hematol 2003; 78:106-13.

26. Palumbo A, Anderson K, Multiple Myeloma N Engl J Med. 2011 Mar 17;364(11):1046-60.

27. Podar K, Chauhan D, Anderson KC. Bone marrow microenvironment and the identification of new targets for myeloma therapy. Leukemia 2009; 23:10-24.

28. Hayden PJ, Mitsiades CS, Anderson KC, Richardson PG Novel therapies in myeloma. Curr Opin Hematol. 2007 Nov;14(6):609-15. Review.

29. Hideshima T, Bergsagel PL, Kuehl WM, Anderson KC Advances in biology of multiple myeloma: clinical applications Blood. 2004 Aug 1;104(3):607-18. Review.

30. Bataille R, Jourdan M, Zhang XG, Klein B. Serum levels of interleukin 6, a potent myeloma cell growth factor, as a reflect of disease severity in plasma cell dyscrasias J Clin Invest. 1989 Dec;84(6):2008-11.

31. Kawano M, Hirano T, Matsuda T, Taga T, Horii Y, Iwato K, Asaoku H, Tang B, Tanabe O, Tanaka H, et al.. Autocrine generation and requirement of BSF-2/IL-6 for human multiple myelomas Nature. 1988 Mar 3;332(6159):83-5.

32. Lu ZY, Brailly H, Wijdenes J, Bataille R, Rossi JF, Klein B. Measurement of whole body interleukin-6 (IL-6) production: prediction of the efficacy of anti-IL-6 treatments Blood. 1995 Oct 15;86(8):3123-31.

33. Klein B, Zhang XG, Jourdan M, Content J, Houssiau F, Aarden L, Piechaczyk M, Bataille R Paracrine rather than autocrine regulation of myeloma-cell growth and differentiation by interleukin-6 Blood. 1989 Feb;73(2):517-26.

34. Portier M, Rajzbaum G, Zhang XG, Attal M, Rusalen C, Wijdenes J, Mannoni P, Maraninchi D, Piechaczyk M, Bataille R, et al. In vivo interleukin 6 gene expression in the tumoral environment in multiple myeloma Eur J Immunol. 1991 Jul;21(7):1759-62.

35. Zhang XG, Gaillard JP, Robillard N, Lu ZY, Gu ZJ, Jourdan M, Boiron JM, Bataille R, Klein B.. Reproducible obtaining of human myeloma cell lines as a model for tumor stem cell study in human multiple myeloma Blood. 1994 Jun 15;83(12):3654-63.

36. Dalton WS, Bergsagel PL, Kuehl WM, Anderson KC, Harousseau JL.Multiple Myeloma Hematology Am Soc Hematol Educ Program. 2001:157-77. Review.

37. Potter M. Neoplastic development in plasma cells. Immunological Reviews 2003; 194:177-95.

38. Giuliani N, Rizzoli V, Roodman GD. Multiple myeloma bone disease: pathophysiology of osteoblast inhibition Blood. 2006 Dec 15;108(13):3992-6. Review.

39. Hideshima T, Richardson P, Anderson KC. Novel therapeutic approaches for multiple myeloma Immunol Rev. 2003 Aug;194:164-76.

40. Georgii-Hemming P, Wiklund HJ, Ljunggren O, Nilsson K. Insulin-like growth factor I is a growth and survival factor in human multiple myeloma cell lines. Blood 1996; 88:2250-58.

41. Ferlin M, Noraz N, Hertogh C, Brochier J, Taylor N, Klein B.. Insulin-like growth factor induces the survival and proliferation of myeloma cells through an interleukin-6-independent transduction pathway Br J Haematol. 2000 Nov;111(2):626-34

42. Ge NL, Rudikoff S. Insulin-like growth factor I is a dual effector of multiple myeloma cell growth Blood. 2000 Oct 15;96(8):2856-61.

43. Hsu JH, Shi Y, Hu L, Fisher M, Franke TF, Lichtenstein A.. Role of the AKT kinase in expansion of multiple myeloma clones: effects on cytokine-dependent proliferative and survival responses Oncogene. 2002 Feb 21;21(9):1391-400.

44. Sato N, Hattori Y, Wenlin D, Yamada T, Kamata T, Kakimoto T, Okamoto S, Kawamura C, Kizaki M, Shimada N, Ote Y, Hata J, Ikeda Y. Elevated level of plasma basic fibroblast growth factor in multiple myeloma correlates with increased disease activity Jpn J Cancer Res. 2002 Apr;93(4):459-66.

45. Podar K, Tai YT, Davies FE, Lentzsch S, Sattler M, Hideshima T, Lin BK, Gupta D, Shima Y, Chauhan D, Mitsiades C, Raje N, Richardson P, Anderson KC.

Vascular endothelial growth factor triggers signaling cascades mediating multiple myeloma cell growth and migration Blood. 2001 Jul 15;98(2):428-35.

46. Cohen T, Nahari D, Cerem LW, Neufeld G, Levi BZ.. Interleukin 6 induces the expression of vascular endothelial growth factor J Biol Chem. 1996 Jan 12;271(2):736-41.

47. Rajkumar SV, Mesa RA, Fonseca R, Schroeder G, Plevak MF, Dispenzieri A, Lacy MQ, Lust JA, Witzig TE, Gertz MA, Kyle RA, Russell SJ, Greipp PR. Bone marrow angiogenesis in 400 patients with monoclonal gammopathy of undetermined significance, multiple myeloma, and primary amyloidosis. Clin Cancer Res 2002; 8:2210-16.

48. Zangari M, Saghafifar F, Mehta P, Barlogie B, Fink L, Tricot G The blood coagulation mechanism in multiple myeloma. Semin Thromb Hemost. 2003 Jun;29(3):275-82. Review.

49. Vacca A, Ria R, Ribatti D, Semeraro F, Djonov V, Di Raimondo F, Dammacco F. A paracrine loop in the vascular endothelial growth factor pathway triggers tumor angiogenesis and growth in multiple myeloma. Haematologica 2003; 88(2):176-85.

50. Terpos E, Dimopoulos MA, Berenson J. Established role of bisphosphonate therapy for prevention of skeletal complications from myeloma bone disease Crit Rev Oncol Hematol. 2011 Feb;77 Suppl 1:S13-23.

51. Terpos E, Dimopoulos MA, Sezer O, Roodman D, Abildgaard N, Vescio R, Tosi P, Garcia-Sanz R, Davies F, Chanan-Khan A, Palumbo A, Sonneveld P, Drake MT, Harousseau JL, Anderson KC, Durie BG; International Myeloma Working Group The use of biochemical markers of bone remodeling in multiple myeloma:

a report of the International Myeloma Working Group. Leukemia. 2010 Oct;24(10):1700-12. Review.

52. Stewart AK, Richardson PG, San-Miguel JF. How I teat multiple myeloma in younger patients Blood.2009 Dec 24;114(27):5436-43.

53. Bird JM, Owen RG, D'Sa S, Snowden JA, Pratt G, Ashcroft J, Yong K, Cook G, Feyler S, Davies F, Morgan G, Cavenagh J, Low E, Behrens J; Haemato-oncology Task Force of British Committee for Standards in Haematology (BCSH) and UK Myeloma Forum. Guidelines for the diagnosis and management of multiple myeloma 2011.11, Br J Haematol. 2011 Jul;154(1):32-75.

54. Mitsiades CS, Treon SP, Mitsiades N, et al. TRAIL/Apo2L ligand selectively induces apoptosis and overcomes drug resistance in multiple myeloma: therapeutic applications. Blood 2001;98: 795–804.

55. Van Zaanen HC, Lokhorst HM, Aarden LA, et al. Chimaeric antiinterleukin 6 monoclonal antibodies in the treatment of advanced multiple myeloma: a phase I dose-escalating study. Br J Haematol 1998; 102: 783–90.

56. Chanan-Khan AA, Jagannath S, Munshi NC, et al. Phase I study of huN901-DM1 (BB-10901) in patients with relapsed and relapsed/ refractory CD56-positive multiple myeloma. Blood 2007; 110: abstr 1174.

57. Bensinger W, Zonder J, Singhal S, et al. Phase I trial of HuLuc63 in multiple myeloma. Blood 2007; 110: abstr 1180.

58. Hussein MA, Berenson JR, Niesvizky R, et al. Results of a phase I trial of SGN-40 (anti-huCD40 mAb) in patients with relapsed multiple myeloma. Blood 2006; 108: abstr 3576.

59. Baughn LB, Di LM, Wu K, et al. A novel orally active small molecule potently induces G1 arrest in primary myeloma cells and prevents tumor growth by specifi c inhibition of cyclin-dependent kinase 4/6. Cancer Res 2006; 66: 7661–67.

60. Raje N, Kumar S, Hideshima T, et al. Seliciclib (CYC202 or Rroscovitine), a small-molecule cyclin-dependent kinase inhibitor, mediates activity via down-regulation of Mcl-1 in multiple myeloma. Blood 2005; 106: 1042–47.

61. Richardson PG, Chanan-khan AA, Lonial S, et al. Tanespimycin (T) + bortezomib (BZ) in multiple myeloma (MM): confirmation of the recommended dose using a novel formulation. Blood 2007; 110: abstr 1165.

62. Chauhan D, Catley L, Li G, et al. A novel orally active proteasome inhibitor induces apoptosis in multiple myeloma cells with mechanisms distinct from Bortezomib. Cancer Cell 2005; 8: 407–19.

63. Dorsey BD, Iqbal M, Chatterjee S, et al. Discovery of a potent, selective, and orally active proteasome inhibitor for the treatment of cancer. J Med Chem 2008; 51: 1068–72.

64. Vanderkerken K, De Raeve H, Goes E, Van Meirvenne S, Radl J, Van Riet I, Thielemans K, Van Camp B, Organ involvement and phenotypic adhesion profile of 5T2 and 5T33 myeloma cells in C57BL/KaLwRij mouse", Br J Cancer. 1997;76(4):451-60.

65. ManningLS, Berger JD, O'Donoghue HL, Sheridan GN, Claringbold PG, Turner JH A model of multiple myeloma: culture of 5T33 murine myeloma cells and evaluation of tumorigenicity in the C57BL/KaLwRij mouse. Br J Cancer. 1992 Dec;66(6):1088-93.

66. Kimmel DB. Quantitative histologic changes in the proximal tibial growth cartilage of aged female rats. Scanning Microsc 1991, suppl 1:11-18.

67. Parfitt AM, Drezner MK, Glorieux FH, et al. Bone histomorphometry: standardization of nomenclature, symbols, and units. J Bone Mineral Res 1987, 6:595-610.

68. Mellish RW, Ferguson-Pell MW, Cochran GV, Lindsay R, Dempster DW. A new manual method for assessing two-dimensional cancellous bone structure: comparison between iliac crest and lumbar vertebra. J Bone Miner Res 1991,6:689-696.

69. Vesterby A, Gundersen HJG, Melsen F. Star volume of marrow space and trabeculae of the first lumbar vertebra: sampling efficiency and biological variation. Bone 1989,10:7-13.

70. Weinstein RS, Majumdar S. Fractal geometry and vertebral compression fractures. J Bone Miner Res 1994,9:1797-1802.

71. E.M Ocio, J.F San Miguel, Deacetylase inhibitors in Multiple Myeloma, Haematologica.2011 May;96(S1):22.

72. Dey JH, Bianchi F, Voshol J, Bonenfant D, Oakeley EJ, Hynes NE., Targeting fibroblast growth factor receptors blocks PI3K/AKT signaling, induces apoptosis, and impairs mammary tumor outgrowth and metastasis. Cancer Res. 2010 May 15;70(10):4151-62.

73. P.L. Bergsagel, M. Chesi, T(4;14) and genomic instability in high risk multiple myeloma, Haematologica.2011 May;96(S1):10.

74. Kaiser M, Lamottke B, Mieth M, Jensen MR, Quadt C, Garcia-Echeverria C, Atadja P, Heider U, von Metzler I, Türkmen S, Sezer O Synergistic action of the novel HSP90 inhibitor NVP-AUY922 with histone deacetylase inhibitors,

melphalan, or doxorubicin in multiple myeloma. Eur J Haematol. 2010 Apr;84(4):337-44.

75. Tauchi T, Okabe S, Ashihara E, Kimura S, Maekawa T, Ohyashiki K Combined effects of novel heat shock protein 90 inhibitor NVP-AUY922 and nilotinib in a random mutagenesis screen. Oncogene. 2011 Jun 16;30(24):2789-97.

76. S. Lional, S. Jagannath. Monoclonal antibodies in the treatment of multiple myeloma, Haematologica.2011 May;96(S1):22.

77. Fulciniti M, Tassone P, Hideshima T, Vallet S, Nanjappa P, Ettenberg SA, Shen Z, Patel N, Tai YT, Chauhan D, Mitsiades C, Prabhala R, Raje N, Anderson KC, Stover DR, Munshi NC. Anti-DKK1 mAb (BHQ880) as a potential therapeutic agent for multiple myeloma. Blood. 2009 Jul 9;114(2):371-9.