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Inhibiting Translation as a Novel Strategy to Target Multiple Myeloma

William Roman,

Division of Experimental Medicine,

McGill University, Montreal

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Abstract

Multiple Myeloma (MM) cell survival has been shown to depend on precise control of protein production and degradation. Disruption of protein catabolism through proteosomal or aggresomal blockade results in MM cell death. We hypothesized that inhibiting protein production would have a similarly toxic effect in MM. We explored the consequences of inhibiting mRNA translation in MM using silvestrol, a powerful inhibitor of ribosomal recruitment, which preferentially disrupts the production of certain cell regulatory and survival proteins. A panel of silvestrol-treated MM cell lines showed profound inhibition of growth and a rapid induction of apoptosis, as seen by MTT viability and AnnexinV flow cytometric assays respectively. The average IC50 in MM cells was determined to be 20nM while it was considerably higher in primary cultures of senescent fibroblasts. We show that silvestrol inhibits protein translation by inhibiting ribosome binding, decreasing polysome content and increasing 80S ribosomes. Western blot analyses show that silvestrol rapidly decreases the expression of c-Myc and non-canonical NF-KB signaling. Expression of anti-apoptotic proteins such as Mcl-1 and Bcl-2 decreased while pro-apoptotic proteins, such as BAX, increased with silvestrol treatment. In a novel transgenic mouse model of MM (vk*myc), which has been shown to behave clinically like human MM, silvestrol does not appear to be toxic and is therapeutically effective. Our results warrant clinical evaluation of silvestrol.

Abstracte

Il a été démontré que la survie des cellules cancéreuses chez les patients atteints du Myélome Multiple (MM), dépend de la régulation de la production et de la dégradation des protéines. Une perturbation du système de catabolisme des protéines, à travers un blocage proteosomale, engendre la mort de ces dernières. Nous avons donc formulé l'hypothèse que l'inhibition de la production de protéines aurait des effets toxiques similaires. Grâce à un puissant inhibiteur, le silvestrol, nous avons déterminé les conséquences liées à l'inhibition de la translation de l'ARN messager. Le silvestrol est un inhibiteur de recrutement ribosomale qui affecte de manière préférentielle certaines protéines impliquées dans la division et la survie cellulaire. Une panoplie de lignées cellulaire spécifiques au Myélome ont été susceptibles au silvestrol, ce qui s'est exprimé par une inhibition de la croissance cellulaire et une amorce rapide de l'apoptose. Cela a été observé grâce au test de viabilité MTT et par l'analyse de cytométrie en flux avec les marqueurs Annexin V et 7-AAD. La moyenne du ci50 des cellules du myélome testées a été établie à 20 nM, tandis qu'une concentration considérablement plus élevé est nécessaire pour obtenir les mêmes résultats chez les cultures primaires de fibroblaste sénescents. Nous démontrons que le silvestrol inhibe la translation des protéines en bloquant le ribosome 80S au départ du codon d'initiation de la translation. Ceci engendre une accumulation des ribosomes 80S et une décroissance du nombre de polysome par ARN messager. Nos analyses de western blot montrent que le silvestrol provoque une décroissance de

l'expression de l'oncogène c-Myc et de NF-KB. De plus, l'expression de protéine anti-apoptotiques tel que Mcl-1, Bcl-Xl et Bcl-2 décroit avec un traitement de silvestrol, alors que l'expression de la protéine pro-apoptotique Bax augmente. Nos résultats montrent que le silvestrol est efficace et non toxique dans le nouveau model transgénique du MM (la souris Vk*Myc) qui est réputé pour sa similarité de caractéristiques avec le MM humain. Nous prônons une évaluation clinique du silvestrol.

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I. Introduction

A. Multiple Myeloma overview

In September 1844, Thomas Alexander MacBean, a 45 years of age tradesman felt as if something had snapped within his chest during his hiking vacation in a cavern. Despite the use of leeches, steel and quinine that temporarily alleviated the symptoms of his bone pain, Mr. MacBean died in 1846 revealing, after autopsy, brittle bones and a 'gelatineform substance of a blood red and uncruous feel' in the bone marrow. Inspection of the latter unveiled multinucleated round or oval cells larger and brighter than an average erythrocyte [1]. These observations, alongside those of Dr. Solly, were the first documented cases of multiple myeloma [2]. It was however not before 1900 that Wright hypothesized Multiple Myeloma to be caused by plasma cell tumors [3] after their histological description by Marschalko in 1895 [4]. Multiple Myeloma's recognition dramatically increased after the introduction of bone marrow aspirates in medical practice by Arinkin in 1929 [5].

Multiple Myeloma is a haematological disorder of white blood cells named plasma cells. These cells are differentiated/activated B lymphocytes and are essential components of the adaptive immune system as they are responsible for the production of antibodies. MM accounts for 0.8% of all cancers worldwide with incidence rates increasing with age. Although the rarity of the disease has rendered the study of potential etiological factors inconsistent, a genetic predisposition seems to be of importance since the

disease is most prevalent in the African American population [6]. It remains the second most common hematological malignancy in adults, and despite tremendous advances in our understanding and treatment of this disease, it remains incurable with median life expectancy of 7 years with novel treatments [7].

B. Signs and Symptoms

As stated in the case study of Thomas Alexander Macbean, the main symptom of MM is frequent fractures due to low bone density. The bone phenotype in MM is more severe than that of other major metastatic cancers such as breast or prostate cancer [8]. Patients suffer dramatically of frequent fractures and spinal cord compressions leading to neurological pathologies [9]. These osteolytic lesions are mostly due to an imbalance between increased bone resorption and decreased production [10].

Although the greatest discomfort of MM is the associated bone disease, the cause of death is often the result of infection [11]. MM patients regularly develop immune deficiencies due to a combination of old age, treatments and the disease itself. These immune impairments render patients susceptible to infection by encapsulated bacteria like Streptococcus pneumoniae [12] and Haemophilus influenzae [13]. It is believed the immune disorder is caused by hypogammaglobulinemia, the lack of competent immunoclobulin production by B cells (thought to be the most important), as

well as possibly dendritic cell and natural killer cell dysfunction [14, 15], lack of T cell diversity [16], abnormal Th1/Th2 ratios [17] and bone marrow failure [18].

The excessive production of antibodies by myeloma cells provokes renal failure in about 40 percent of newly diagnosed MM patients. In normal individuals, the light chains of monoclonal antibodies are freely filtered and are catabolized by the proximal tubule through an endocytosis/lysosomal pathway [19]. In MM, the excess of light chains renders the re-absorption of these latter impossible creating an obstruction of the distal tubule and thick ascending loop of Henle [20]. This blockade augments the intraluminal pressure thus reducing glomerular filtration rate and interstitial blood flow [21]. Hypercalcemia, due to bone resorption, has also been implicated in renal failure [22].

Anemia is another symptom of MM. One of the causes has recently been established to be due to increased levels of bone morphogenetic protein 2 (BMP-2) in MM patients. The study showed that BMP-2 was over expressed in the serum of MM patients, which in turn stimulates hepcidin, an iron regulatory hormone that inhibits iron availability. High levels of hepcidin inversely correlate with hemoglobin and are believed to be the major mechanism explaining the anemia found in MM[23].

Finally, peripheral neuropathy (PN) can be observed in MM patients for a variety of reasons such as spinal cord compression [24], light chain deposits (amyloidosis) [25] or autoimmunity against myelin sheath [26]. PN in

MM patients can vary from fatigue or loss of bladder control to paraplegia [27]. Therapies for MM such as bortezomib and thalidomide have also been accounted for generating PN and may be the primary source of PN induction[28].

C. Pathogenesis

1. Chromosome abnormalities

MM stems from tumor cells that have the capacity to evade normal growth regulatory control. Cancers can originate from a variety of different factors (viral, carcinogens, UV, etc…); in the case of MM, chromosome abnormalities appear to be the initial mutation that renders the cell tumorigenic. MM patients have a wide variety of different aberrations ranging from chromosomal addition or deletion to important translocations. The type of chromosome abnormality is a key factor to establish prognosis and therapeutic approaches [29].

Analysis of myeloma cells with conventional cytogenetics (CC) or fluorescence in situ hybridization (FISH) can reveal chromosomal abnormalities in almost all cases. Alongside the gain or loss of certain chromosomes, the most common anomalies involve the translocation (~50 – 70% of patients) of chromosome 14 on which is located the immunoglobulin heavy-chain gene (IgH) (14q32) [30]. This translocation juxtaposes strong immunoglobulin enhancers to a variety of genes leading to their overexpression [31]. Amongst these are the cyclin D family genes [32] and

oncogenes such as MAFB [33] and Myc [34] that alter cell proliferation, survival and DNA repair mechanism.

2. Molecular pathways

Secondary events can include mutations that lead to alterations of key proteins involved in molecular pathways such as PI3K/Akt, JAK/Stat-, Ras/MEK/MAPK-, NF-$_K$B and Wnt.

Before exploring these pathways in detail, it is important to mention the crucial role that the bone marrow microenvironment plays in cell signaling. The bone marrow niche is composed of extracellular matrix (ECM), itself constituted of a variety of signaling proteins, including fibronectin, collagen, and laminin many of which derive from resident stromal cells [35]. These provide an architectural and cellular meshwork that hosts diverse cellular components that can interact with one another. The interaction of myeloma cells with this milieu results in the induction of autocrine/paracrine release of cytokines and growth factors and triggers growth and survival pathways mentioned above [36]. The main cytokines/growth factors present are interlukin-6 (IL-6; involved in signaling and oscteoclastogenesis [37]), vascular endothelial growth factor (VEGF; involved in angiogenesis [38]) and insuline growth factor 1 (IGF-1; also involved in cell signaling [39]).

The Ras/MEK/MAPK-pathway is important in the pathogenesis of multiple myeloma. The pathway is triggered by the signaling molecules

discussed above (IL-6, VEGF and IGF-1) and leads to the cell's uncontrolled growth and resistance to drug [40]. Inhibition of this pathway using farnesyltransferase inhibitors (FTI or Tipifarnib) demonstrated stabilization of the disease in a clinical phase II trial of advanced MM patients [41]. These results are concordant with Ras' role for cell proliferation. Mutations in the Ras pathway are also an indicator of poor prognosis but can be found in approximately 35% of patient samples in a recent analysis of whole genomic sequencing in MM[41].

Another important pathway involves the PI3K/Akt proteins. Similarly to Ras, the pathway is activated by the same cytokines and growth factors but the pathway is more specialized in keeping the cell alive through the inhibition of the intrinsic pathway of apoptosis (Mcl-1, Bcl-x and Bcl-2) [42]. The PI3K/Akt pathway also has the ability to inhibit anti-proliferative proteins such as p21 and p27 that are cyclin inhibitors. Perifosine, an Akt phosphorylation inhibitor, has shown promising results and clinical trials are ongoing [43].

The JAK/Stat pathway is found constitutively active in primary MM samples through the Stat3 protein [44]. Stat3 is mostly involved in gene expression of anti-apoptotic proteins (bcl-xl and bcl-2) as well as oncogenes (c-myc) and a few cyclin D proteins. Inhibition of stat3 activity sensitizes MM cells to apoptosis induced by conventional chemotherapy [45].

Finally, the NF_KB pathway is the most implicated in the pathogenesis of MM. Recent studies demonstrate that a high percentage of MM cells possess constitutive NF-$_K$B activation of the non-canonical pathway [46]. NF-$_K$B is involved in survival, proliferation, metastasis, angiogenesis and also inflammation; which creates a positive feedback loop by triggering the release of IL-6 from surrounding stromal cells [47]. Many NF_KB and IKKβ inhibitors have been identified and have demonstrated a decrease in both growth and survival of MM cells [48].

3. Bone pathogenesis

The molecular pathways above explain the capacity of the tumor cells to proliferate and survive but do not account for the symptoms. The osteoporosis witnessed in MM is due to alterations to the RANK/RANKL/OPG system.

Bone marrow stromal cells and osteoblasts express RANKL, a protein that binds to the receptor RANK present on the surface of oscteoclast progenitors [49]. The RANK/RANKL interaction stimulates osteoclastogenesis and bone resorption. Since RANKL is produced by the osteoblast, the RANK/RANKL interaction creates a balanced system in which variations in osteoblastic activity are matched by the stimulation of osteoclasts [50]. It was shown that this equilibrium was ruptured in MM because tumor cells stimulate surrounding osteoblasts, stromal cells and themselves to overexpress RANKL [51, 52, 53]. The importance of the RANK/RANKL

relationship in myeloma bone disease can be best appreciated when the MM murine and the SCID-hu models were treated with RANK.Fc: a RANKL antagonist. RANK.Fc almost completely prevented the development of the bone phenotype and reduced tumor burden [54, 55].

Another actor in the RANK/RANKL relationship is OPG, a soluble decoy produced by stromal bone marrow cells and osteoblasts in order to bind RANKL and prevent its interaction with RANK [56]. OPG's role as a bone resorption inhibitor is suppressed in MM patients who possess low levels of the protein in the serum [57]. Studies have shown that MM cells interact with osteoblasts and stromal cells to reduce their secretion of OPG thus leading to an overall increased RANKL:OPG ratio [58].

Resolving the Myeloma bone disease is of primordial importance as it strongly contributes to the patient's well being and survival. It would also prevent osteoclast stimulation that enhances the myeloma cell's tumorigenicity through osteoclast's production of bone-derived tumor growth factors [59].

D. Diagnosis of MM

Multiple myeloma diagnosis usually occurs after the symptoms of the disease kick in. To confirm the diagnosis, the International Myeloma Working Group has elaborated three measurable criteria. The first involves witnessing an accumulation of plasma cells exceeding 10 percent of the total bone marrow. The second is evidence that the patient is suffering from the usual

MM symptoms that are referred to by the acronym "CRAB": hyper**C**alcemia, **R**enal insuffiency, **A**nemia and **B**one lesions. Finally, the third criterion is the dramatic increase of paraprotein in the serum seen on a serum protein electrophoresis gel (SPEP) [60]. The International Myeloma Working Group also characterized the disease in three stages according to the levels of Serum beta$_2$ –microglobulin (Sβ_2M), albumin, creatinine and platelet count which emerged as powerful prognosis factors. Data was gathered from 10 750 patients in 15 clinical centers around the world and analyzed to consolidate these three stages. Increasing levels of serum β_2M and decreasing levels of albumin arose as the main predicting factors as they correlated with poor prognosis[61].

E. Treatment

MM remains incurable despite researchers' relentless quest to find novel treatments. Alkylating agents like melphalan, the corticosteroid prednisone and conventional chemotherapy were the first treatments discovered that had a significant clinical effect [62, 63, and 64]. Drugs were tested in combinations like melphalan and prednisone (MP) and showed greater efficacy than the agents alone. This treatment remained the MM therapy for decades, however, targeting cell growth proved to be limited due to the slow turnover rate of the tumor [65]. Autologous stem cell/bone marrow transplantation (ASCT) was the second wave of treatment and offered a means to use higher, lethal doses of chemotherapy that could more

effectively destroy the myeloma involved bone marrow[66]. A French and English study demonstrated that high dose therapy followed by ASCT significantly improved the overall survival and prolonged the event-free period to an average of 5 years. However, the benefits witnessed with this treatment were not sustained and patients ultimately relapsed [67, 68].

The past decade has seen a tremendous evolution in MM treatments. With the growing understanding of cellular and molecular mechanisms, scientists found new approaches to target the cancerous cells. Thalidomide, an anti-angiogenic agent, was first tested in MM for its potency in inhibiting bone marrow vascularization and endothelial growth [69]. This proved to be effective and combinational therapy with the two existing treatments MP and dexamethasone showed even greater efficacy than the two treatments alone [70, 71]. It is now understood that the potency of the drug is mostly due to its capacity to disrupt adhesion between MM and surrounding stromal cells as well as inducing caspase-8 mediated apoptosis. The use of thalidomide exposed the drugs' toxicity and therefore led to the creation of lenalidomide, an analogue of thalidomide that shares the same biological properties but without the toxic side effects of the parent compound. Lenalidomide is currently being evaluated in a number of clinical studies alone or in combination with other agents [72, 73].

Adams in 1998 developed bortezomib (velcade), the first agent that blocks the proteaosome from degrading ubiquitinated proteins [74]. Our

understanding of bortezomib's mechanism of action is still not clear, but it is hypothesized that it blocks the degradation of cell cycle-regulated proteins forcing the cell into cycle arrest thus leading to its destruction by caspase activation [75]. Bortezomib is also believed to inhibit NF-kB activation, through stabilization of its inhibitor, IkkB [76]. Clinical trials of the drug as a single agent demonstrated an equal or better response in 27% of patients in contrast to their previous treatments [77]. Bortezomib's combination with existing drugs showed great efficacy in many clinical trials [78] and novel proteaosome inhibitors such as Carfilzomib or salinosporamide are currently being tested and developed [79, 80]. Other strategies are also being explored including Akt inhibitors (perifosine [81], Heat-shock-protein Inhibitors (geldanamycin [82]) and Histone Deacetylase Inhibitors (vorinostat [83]). The need for innovation is constant in MM. Finding alternative drugs that kill the myeloma cells or re-sensitizes them to existing treatments like bortezomib may extensively prolong the life of patients or cure them completely.

F. Apoptosis

Most MM drugs are designed to induce the myeloma cells to apoptose. Two signaling pathways exist that lead to programmed cell death: the extrinsic and intrinsic pathways of apoptosis.

The extrinsic pathway operates via death receptors on the cell surface such as tumor necrosis factor family (TNF). When ligands bind these transmembrane proteins, their intracellular domains activate and bind

adaptor proteins Fas-associated death domain (FADD) or TRADD (TNFR1-associated death domain protein) to form the death inducing complex (DISC). Disc recruits and catalytically activates procaspase 8. If sufficient activated caspase 8 is present, it serves as an activator of other downstream caspases like procaspase 3, 7 and 9, which inevitably induce apoptosis [84].

The intrinsic pathway involves the mitochondria and is usually activated in response to a loss of growth signals or stimuli from inside the cell. The mitochondrion possesses two different compartments: the matrix and the intermembrane space (IMS). The IMS contains many proteins involved in cell death induction such as cytochrome c or apoptosis-inducing factors (AIF). These proteins are, in normal cells, isolated from the cytoplasm but can be released resulting in cell death. The intrinsic pathway is controlled by interactions between pro-apoptotic (Bax, Bak, PUMA, Bad) and anti-apoptotic (Bcl-2, Mcl-1, Bcl-w, Bcl-xL) members of the Bcl-2 protein family. Anti-apoptotic proteins preserve the integrity of the mitochondrial membrane by inhibiting proteins like PUMA and Bad that initiate the cell death signaling. PUMA and Bad become activated in response to signals from inside the cell and initiate cell death signaling by activating the mitochondrial transmembrane proteins Bax and Bak. These latter promote the formation of proteolipid pores known as mitochondrial apoptosis-induced channels (MACs) that create openings between the cytosol and the IMS. This leads to the release of cytochrome c or AIF into the cytoplasm, which results in the cleavage of PARP and ultimately, apoptosis [85, 86].

G. Rationale of Silvestrol

The unique sensitivity of MM to proteaosomal blockers underlines the reliance of MM cells on protein metabolic pathways for their survival. These cells are antibody (protein) producing factories and have been shown to apoptose when the processes of protein production are altered. Targeting these pathways is therefore sensible. In our study we explore the effects of silvestrol, an inhibitor of protein translation initiation, in multiple myeloma.

1. Translation initiation overview

The rate-limiting step of mRNA translation is that of initiation: the formation of a 43S pre-initiation complex. This complex consists of the 40S small ribosomal subunit, the initiating methionyl tRNA (Met-tRNAi) and a group of eukaryotic initiation factors (eIFs). Next, the 43S ribosome complex is recruited to the 5′ end of the mRNA, identified by an inverted m7GpppN cap. Association of the 43S ribosome complex with the cap is mediated by the cap-binding (initiation) complex, eIF4F. Also involved in initiation are several other initiation factors, including the multisubunit complex eIF3, and mRNA binding proteins such as the polyA tail binding proteins (PABP). eIF4F is a heterotrimeric complex composed of eIF4E (a cap binding protein), eIF4A (a RNA helicase), and eIF4G (a RNA binding protein that also interacts with PABP). eIF4F assembly depends on the availability of eIF4E and eIF4A to interact with the scaffolding protein eIF4G. eIF4E and 4A are freed from inhibitory binding proteins after mTOR mediated phosphorylation. mTOR

itself can phosphorylate the scaffolding protein eIF4G directly promoting its association with the eIF3 translation complex, thereby bringing together eIF4F with the 40S ribosome leading to increased small ribosome positioning on the mRNA [87].

2. eIF4F in Cancer

Increased eIF4F complexes lead to enhanced translation of all cap-dependent mRNAs and increase total protein synthesis. The relative differences in the rate of mRNA translation depends upon specific sequence elements such as the length and structure of the 5'UTRs and the presence of discrete hairpin structures in that region [88]. Most mRNAs have short simple 5'UTRs (such as 'housekeeping' genes ('strong' mRNAs) like ß-actin and GAPDH) that can be easily read by the eIF4F complex and form a ribosome landing pad. These mRNAs are translated well even when the eIF4F is limited. Other groups of mRNAs are exquisitely sensitive to and may even depend upon eIF4F for translation, their structures are complex, they are G+C rich and harbour many hairpin structures [89]. Examples of eIF4F dependent mRNAs ('weak' mRNAs) include c-myc, survivin and cyclin D [90]; these appear to be preferentially and disproportionately affected by the activity of eIF4F. The enhanced translation of these oncogenic proteins is thought to contribute to the oncogenicity of eIF4F. Overexpression of eIF4F is oncogenenic in transgenic mouse models [91].

Several strategies have been devised to disrupt the activity or prevent the formation of the eIF4F complex (eIF4A, eIF4E and eIF4G). The small

molecule 4EGI-1 was identified as an inhibitor of eIF4E:eIF4G interaction and leads to decreased expression of c-myc and cyclin D1 [92]. Rapamycin and its analogues are allosteric inhibitors of mTORC1, which can inhibit the release of eIF4E and eIF4A from binding proteins, but these have shown limited useful clinical activity [93]. Another, potentially more successful strategy to inhibit the formation of eIF4F would be to target its subunits (such as eIF4E and eIF4A), that also play a role in carcinogenesis.

3. eIF4A in Cancer

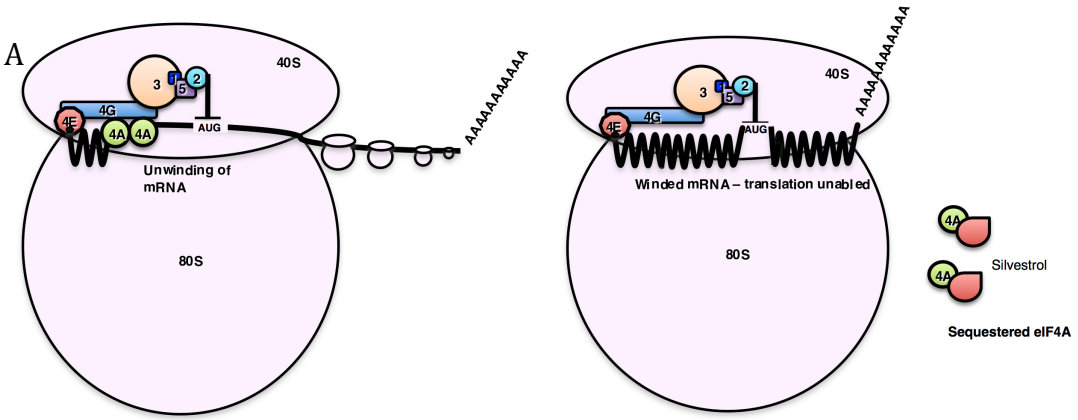
In contrast to eIF4E inhibitors, there has been little exploration into the role of eIF4A in cancer. However, it remains a tantalizing target for drug development, as it is a crucial mediator of the other components of the eIF4F complex. BC1 a small non-coding RNA expressed predominantly in the CNS, can sequester eIF4A and thereby repress translation [94]. Pateamine, purified from a marine sponge, has been shown to inhibit protein synthesis by inducing conformational changes in eIF4A and forcing its engagement to mRNA thereby decreasing its availability to join the eIF4F complex [95]. Hippuristanol, another marine derived molecule (this one a steroid), is a potent inhibitor of protein synthesis that appears to work by inhibiting the helicase activity of eIF4A and decreasing the RNA binding of eIF4A [96].

4. Targeting eIF4A with Silvestrol

Silvestrol is a plant derived cyclopenta (b) benzofuran (CBF) that has been identified as a powerful inhibitor of eIEF4A. CBFs have been reported to inhibit mdm2, NF-AT and NF-κB activity, likely through the reduction of mRNA translation [97,98]. Silvestrol appears to act as a chemical inducer of dimerization between mRNA and eIF4A in a cap-dependent manner [99], likely preventing its availability to properly join the eIF4F complex (see Figure 1) [100]. In a variety of studies, it appears to be toxic in vitro to myeloid leukemia cells, low grade B cell leukemia/lymphoma and more aggressive lymphoma [101]. When given to transgenic Eμ-TC-1 mice, it severely reduces their B cell numbers and also prolongs the survival in a xenographic model of B cell leukemia [101]. Silvestrol was shown to reduce the viability of patient derived CLL cells *ex vivo* [101] and was able to synergize with an anthracyclin in a breast cancer animal model [99]. Silvestrol's greater potency on B cells over T cells presumably relies on its mechanism of action, B cells produce much more proteins.

Silvestrol has not yet been tested in MM; but when one considers the biology of MM and the mechanism of action of silvestrol, these would appear to be an ideal match.

A



B

Cell line	IC50 (nM)	
AML2 (AML cell line)	3.8 at 48 hours	MTS assay
AML3 (AML cell line)	4.46 at 48 hours	
HL-60 (PML cell line)	7.7 at 48 hours	
NB4 (PML cell line)	10 at 48 hours	
CLL tumor cells (patients)	6.9 at 72 hours	Apoptosis assay
Ramos (B-lymphoma cell line)	< 5 at 72 hours	
Mino (B-lymphoma cell line)	< 5 at 72 hours	
JeKo-1 (B-lymphoma cell line)	< 5 at 72 hours	
ALL tumor cells (patients)	7 at 72 hours	

Figure 1A. Silvestrol's inhibitory effect on translation initiation by eIF4A sequestration. 1B. IC50 of silvestrol-treated cells from previous studies [101, 106].

II. Objective

The objective of our project was to study the cytotoxic effect of silvestrol, a novel inhibitor of translation, in Multiple Myeloma.

III. Materials and methods

Cells and reagents

Human MM cells (U266, KMS11, KMS28, JJN3, OPM1, MM.1S and MM.1R see table) were cultured using RPMI 1640 (Wisent Inc.) and supplemented with 10% Fetal Bovine Serum (FBS). Mouse Embryonic Fibroblasts (MEFs) were generated by isolating and dismembering embryos harvested at 13.5 days to which Trypsin and EDTA were added. The cells were passed 10 times every 3 – 4 days in DMEM supplemented with 10% FBS. The following table describes the characteristics of the different cell lines used.

Name	Sex	Ethnic Origin	Heavy Chain	Light Chain	Tissue Source of Origin	Chromosomal Translocation	Translocation protein	NF-κB Abnormality
JJN-3	Female	-	IgA	Kappa	BM, PCL	16q23	MAF, MYC	yes
KMS-11	Female	Japan	IgG	Kappa	Pleural Effusion	4p16	MMSET/FGFR3, MAF, MYC	yes
KMS-28BM	Female	Japan	IgG	Lambda	BM	4p16	MMSET/FGFR3	yes
MM.1R	-	-	-	-	-	16q23	MAF, MYC	yes
MM.1S	-	-	-	-	-	16q23	MAF, MYC	yes
OPM-1	Female	Japan	IgG	Lamda	PB, PCL	4p16	MMSET/FGFR3, MYC	no
U-266	Male	Caucasian	IgE	Lambda	PB	11q13	CCND1	yes

Silvestrol was synthesized by Dr. Rob Porco at Boston University and supplied courtesy of Dr. Jerry Pelletier, McGill Univerisity Deparment of

Biochemistry. Silvestrol was re-suspended in DMSO and stored at −80 ◦C. Velcade (bortezomib)(Ortho Jannsen, Toronto, ON) was diluted in sterile saline and kept at -4 °C.

Animal studies

The transgenic and immunocompetent Vk*MYC mouse model (courtesy of Dr. Leif Bergsagel, Mayo Clinic, AZ) of MM was used for its biologic and therapeutic fidelity to human MM. The mice were engineered by introducing a vector containing the human Myc gene controlled by the V-kappa promoter. The vector was constructed to harbor a TAG stop codon after the second exon that prematurely terminates translation once the mRNA is synthesized exclusively in B-lymphocytes. This stop codon also resides in a sequence known to undergo regional somatic hypermutation during the germinal center phase of late B cell development. It was hypothesized that this premature stop codon would get mutated in B cells undergoing somatic hypermutation (SHM) thus resuming Myc translation in fully mature B cells (plasma cells)(figure 2A). Myc was shown to be over-expressed in PCs once these develop MM. It was reported that these mice demonstrate the same clinical characteristics as human MM. The monoclonal PC expansion, confirmed by $CD138^{+}$ (membrane marker on plasma cells) flow analysis, lead to the characteristic monoclonal band seen on SPEP (M-Peak) (figure 2B), elevated IgG levels, anemia, protein deposition in the glomeruli, and to bone destruction and/or lesions. Most importantly, the mice were shown to respond to the current MM treatments such as

melphalan, dexamethasone and bortezomib in a similar fashion to humans, while they did not respond to treatments that do not work in humans [102]. This therapeutic fidelity makes this animal model unique and highly predictive of drugs that will work in the clinic against MM.

Animals were housed in the vivarium of the McGill University Health Centre at the Royal Victoria Hospital in cages with access to food and water ad libitum. All animal experiments were conducted according to the guidelines of the McGill University Animal Care Committee and approved by this committee (Protocol #5608).

Vk*MYC mice were genotyped using polymerase chain reaction (PCR) with primers and a technique described elsewhere[102].

Mice were treated with silvestrol after dilution in a 100 µl PBS solution with 5% PEG 400/Tween 80 and IP Injection.

Bortezomib resistant mice were generated by treating vk*myc mice with low bortezomib doses (0.15 mg/kg) two times a week for a month. The mice were then treated with silvestrol twice a day with a 0.25mg/kg dose and with bortezomib (0.5 mg/kg) (n=3) for 3 weeks [99].

Treatment efficacy was determined by serum protein electrophoresis as detailed below.

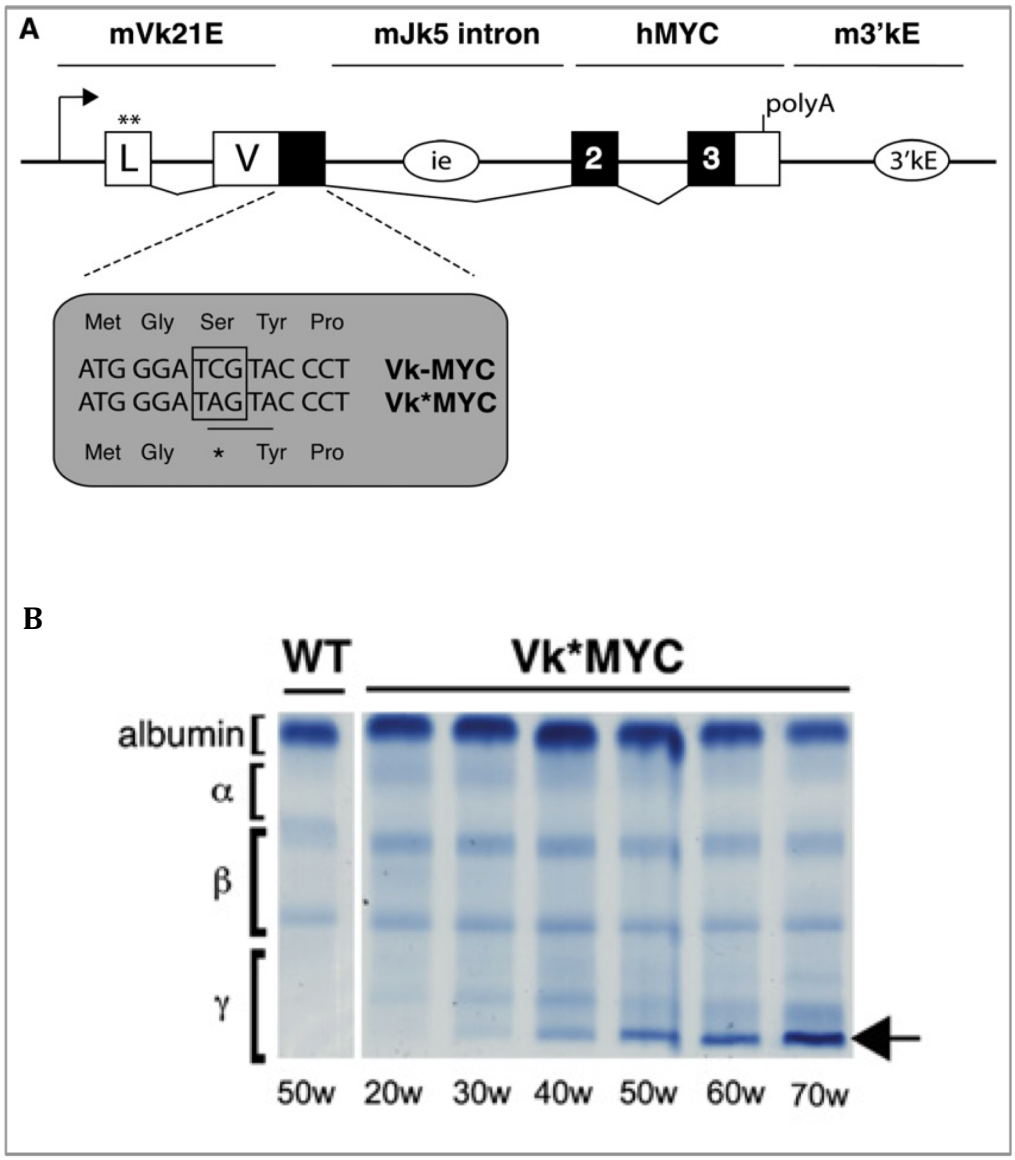


Figure 2A and B are from Chesi M, Robbiani DF, Sebag M, Chng WJ, Affer M, Tiedemann R, Valdez R, Palmer SE, Haas SS, Stewart AK, Fonseca R, Kremer R, Cattoretti G, Bergsagel PL.
AID-dependent activation of a MYC transgene induces multiple myeloma in a conditional mouse model of post-germinal center malignancies.
Cancer Cell. 2008 Feb;13(2):167-80.

MTT and Apoptosis assays

Cell proliferation was determined using MTT assays. Cell lines were seeded in 96-well plates, each well containing 100 μl of medium (RPMI, 10% FCS) with $5x10^4$ cells. Cells were then treated with increasing concentrations of silvestrol for 24, 48 and 72 hours. A CellTiter 96 Cell Proliferation Assay (Promega, Madison, WI) was used according to the manufacturer's instruction. Plates were read on a SpectramaxPlus384 (Molecular Devices) after a 3-hour incubation. Values obtained were standardized against vehicle (DMSO) control, which was set at 1; each value represents the mean of at least 3 replicates. Statistical analysis for standard deviation and statistical significance was performed using Microsoft Excel software, (student t-test).

Cell apoptosis following silvestrol administration was determined using 7-Aminoactinomycin D (7-AAD) and the Annexin V-PE protein (BD Pharmingen™) binding for detection of early apoptosis. 7-AAD only binds to DNA of apoptotic cells that possess disrupted membranes. Annexin V is a phosphatidylserine, a peptide only present on the surface of apoptotic cells. MM cell lines were seeded at 10^6 cells in 2 ml of medium and treated with increasing concentrations of silvestrol (2.5, 5, 10, 25, 50 and 100 nM) for 24 or 48 hours. Cells were collected and washed twice in PBS (300 x g for 10 minutes) and re-suspended in 100 μl PBS. 5 μl of both 7-AAD and Annexin V were added to cells resuspended in buffer then analyzed using a FACScaliber flow cytometer (BD Biosciences, Mississauga, ON) and CellQuestPro and FlowJo software (BD Biosciences and Tree Star Software, Ashland OR).

In vivo metabolic labeling

To measure the rate of ^{35}S-Met incorporation into proteins, 120 000 cells/well were seeded in a 24-well plate in the presence of increasing concentrations of silvestrol. For the last 15 min, cells were labeled with [^{35}S]-methionine. Cells were harvested by centrifugation for 10 min at 2000xg at 4 ^{o}C. Cell pellets were washed in PBS and lyzed in RIPA buffer (20 mM Tris [pH 7.5], 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1% NP-40, 0.5% sodium desoxycholate, 0.1% SDS, 20 mM ß-glycerophosphate, 10 mM NaF, 1 mM PMSF, 4 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml pepstatin). The protein was TCA precipitated and radioactivity quantitated by scintillation counting. Protein content in the cell lysates was measured using the Bio-Rad D_C ProteinAssay (Bio-Rad Laboratories) and used to standardize the counts obtained by TCA precipitation.

Polysome profiling analyses

To perform polysome-profiling analysis, 10 million cells were seeded in the presence of 0, 25, 50, 100, 250, 500 nM silvestrol for 1 hour. Cells were harvested by addition of 100 μg/ml cycloheximide followed by centrifugation for 10 min at 2000xg at 4 ^{o}C. After washing with cold PBS containing 100 μg/ml cycloheximide and an additional spin as before, cell pellets were resuspended in 425 μl hypotonic lysis buffer (5 mM Tris $_{7.5}$, 2.5 mM $MgCl_2$, 1.5 mM KCl). Five μl 10μg/ml cycloheximide, 1 μM DTT, 0.5% Triton X-100 and 0.5% sodium deoxycholate were added and samples loaded on 10-50%

sucrose gradients. Following a spin in an SW40 rotor at 35 000 rpm for 2 h at 4 °C, gradients were analyzed by piercing the bottom of the tube with a Brandel tube piercer and passing a 60% sucrose solution through the bottom. Recording of the data was performed using InstaCal Version 5.70 and TracerDaq Version 1.9.0.0 (Measurement Computing Corporation, Norton, MA).

CD138 cell isolation

Bone marrow samples from sacrificed Vk*MYC mice were harvested following treatment with silvestrol or vehicle alone for two days with two 0.25 mg/kg dose per day. Bone marrows from tibiae were flushed and washed with PBS. Approximately 10^8 cells were collected per mice and subsequently washed two times (300 x g for 10 minutes) with buffer (PBS, 0.5% BSA and 2mM EDTA). 100 µl of anti-CD138 micro-beads (Miltenyi Biotec) were added and incubated 15 minutes at 4°C. A Miltenyi Automacs automated magnetic cell separator (Miltenyi Biotech, Auburn, CA) was used to separate the CD138 labeled cells. Cells were counted and $5x10^5$ cells were used for purity analysis using flow cytometry and an antibody that recognizes a different CD138 epitope than used for separation (mouseCD138-PE (Stemcell Technologies)). Intracelullar IgG staining was done using the intracellular staining anti-IgG-FITC kit (Miltenyi Biotech).

Cellular Extracts and Western Blotting

All cells were spun down by centrifugation (300 x g for 10 minutes), re-suspended in lysis CAT buffer and transferred to eppendorf tubes. Cell debris was pulled down by centrifugation (8000 rpm for 10 minutes) and the supernatant was collected into fresh eppendorf tubes. Protein extracts were stored at -30 °C. For western blotting, the protein extracts were mixed with loading buffer and denaturing solution and then heated for 5 minutes before loading on 10% SDS-polyacrylamide gels. After protein migration and transfer onto a PDVF membrane, the proteins were blocked with 5% milk solutions then hybridized with primary and or secondary antibodies. Primary antibodies employed were anti-Mcl1 (human and mice), anti-Bcl-Xl, anti-caspase 3, anti-caspase 9, anti-caspase 8 (Cell Signaling), anti-NF-κB (R&D Systems), anti-c-Myc, anti-PARP, anti-actin, anti-tubulin, anti-Gapdh, anti-Bax and anti-Bcl2 (Santa Cruz biotechnology, Inc.). All antibodies were diluted according to the manufacturer's specifications.

Serum Protein Electrophoresis (SPEP)

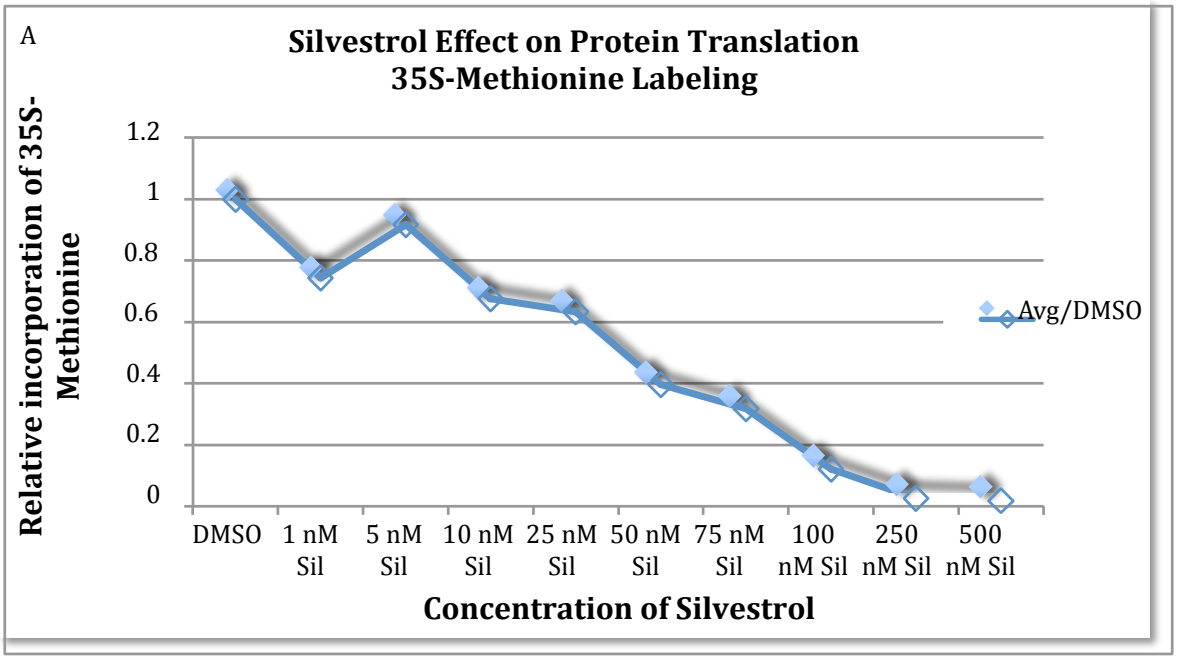
Blood was collected by tail or leg puncture and was centrifuged for 10 minutes at 8000 rpm. Red blood cells were discarded and plasma was run on non-denaturing agarose gels and stained for protein content according to the manufacturer's indications (Sebia-USA, Norcross, GA).

IV. Results

Silvestrol mechanism

To determine the mechanism of protein translation inhibition we looked at [^{35}S]-methionine labeling to observe the cell's capacity to produce proteins when exposed to silvestrol. The methionine amino acid is encoded by the AUG start codon and therefore correlates with overall protein production. MM cells demonstrated a decrease of ^{35}S-methionine uptake that was inversely proportional to increasing concentrations of silvestrol. At 100 nM, translation was almost completely abrogated (figure 3.A), suggesting that silvestrol inhibits protein production in MM cells.

To have a better understanding of silvestrol's effect on protein translation we performed polysome profiling of MM cells exposed to silvestrol. Polysome profiles were raised after treating the JJn3 cell line for 1 hour with different concentrations of silvestrol. We found that the polysome to monosome ratio greatly decreased in correlation with increasing silvestrol concentrations (figure 3.B and C). The first four peaks (left to right) represent the cell debris, 40S, 60S and 80S ribosome respectively whereas the later smaller peaks represent nascent polysomes. The height of the 80S peak indicates the accumulation of monosomes thus showing that silvestrol does not affect ribosome loading but rather blocks the first step of elongation. The absence of nascent polysomes in the silvestrol treated samples confirms this compound inhibits the steps of translation elongation.


A
Silvestrol Effect on Protein Translation
35S-Methionine Labeling
Relative incorporation of 35S-Methionine
1.2
1
0.8
0.6
0.4
0.2
0
DMSO
1 nM Sil
5 nM Sil
10 nM Sil
25 nM Sil
50 nM Sil
75 nM Sil
100 nM Sil
250 nM Sil
500 nM Sil
Concentration of Silvestrol
Avg/DMSO

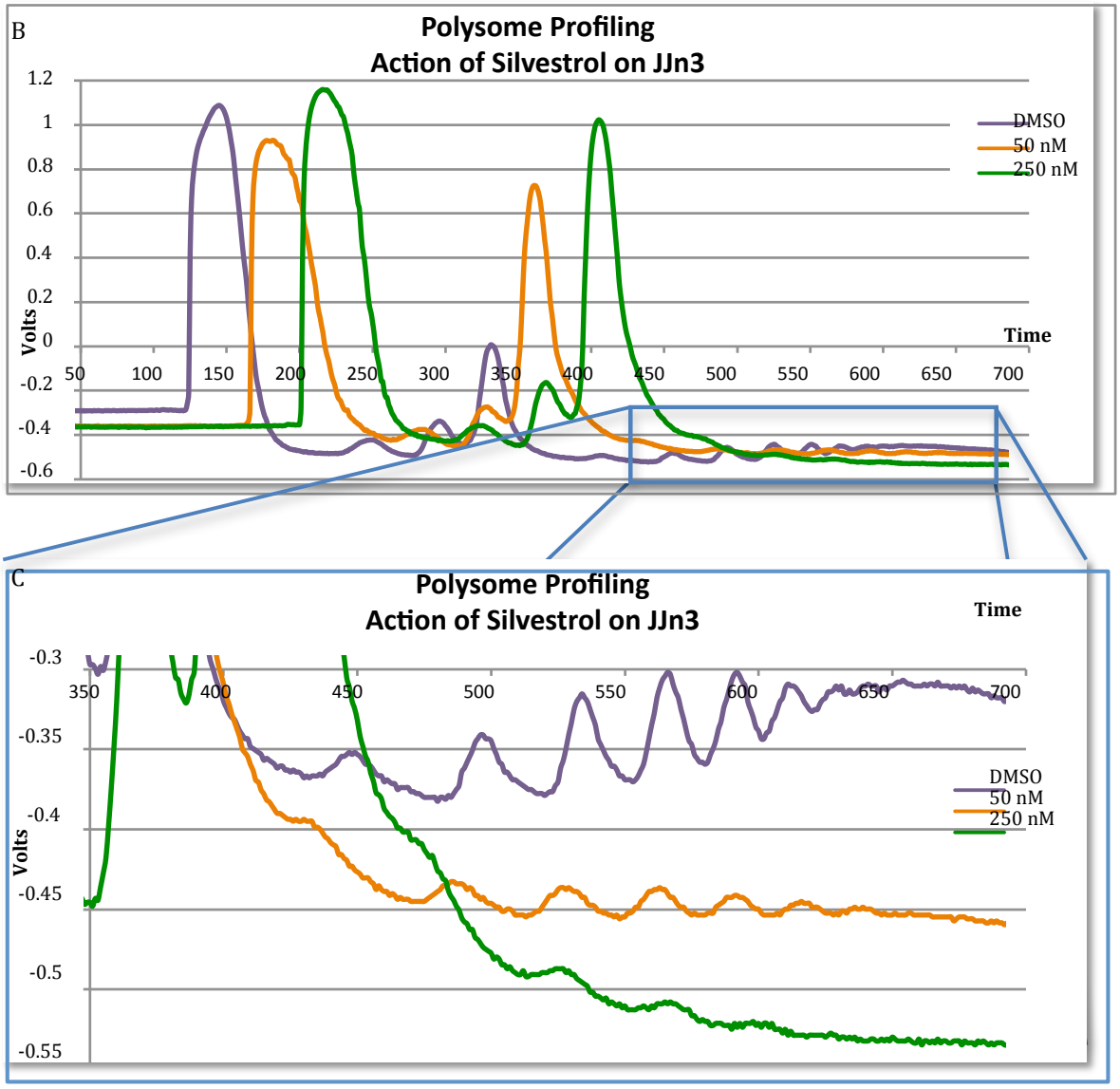
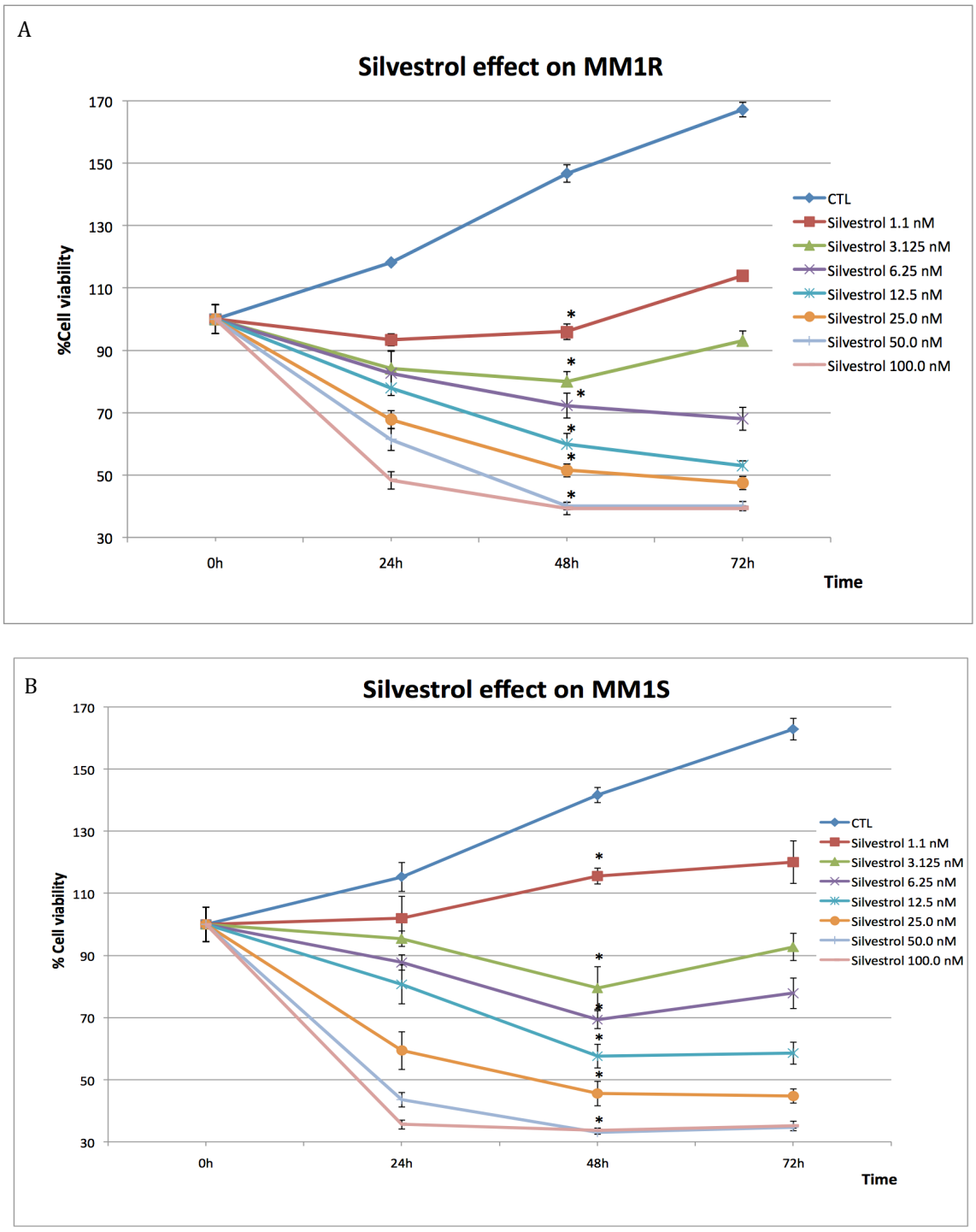


Figure 3.A: Silvestrol effect on protein translation ^{35}S-methionine labeling. JJN3 cells were treated with silvestrol for one hour in which ^{35}S-methionine was added at the 45th minute. Values are standardized against total protein content and plotted relative to DMSO controls. **B. Polysome profile - Effect of silvestrol on ribosomal activity.** JJN3 cells were treated with varying concentrations of silvestrol for one hour. After treatment, the cells were lysed and their translation machinery was frozen with

cycloheximide. Cell extracts were deposited on a 10-50% sucrose gradient and centrifuged for 2 hours at 35, 000 rpm at 4^{o}C. 80S ribosome peak is greater with increasing concentrations of Silvestrol demonstrating an arrest of translation machinery after only one mRNA is bound to the complex. **C.** Inversely, the polysome peaks decrease in amplitude with increasing levels of silvestrol in contrast to control

Silvestrol cytotoxicity

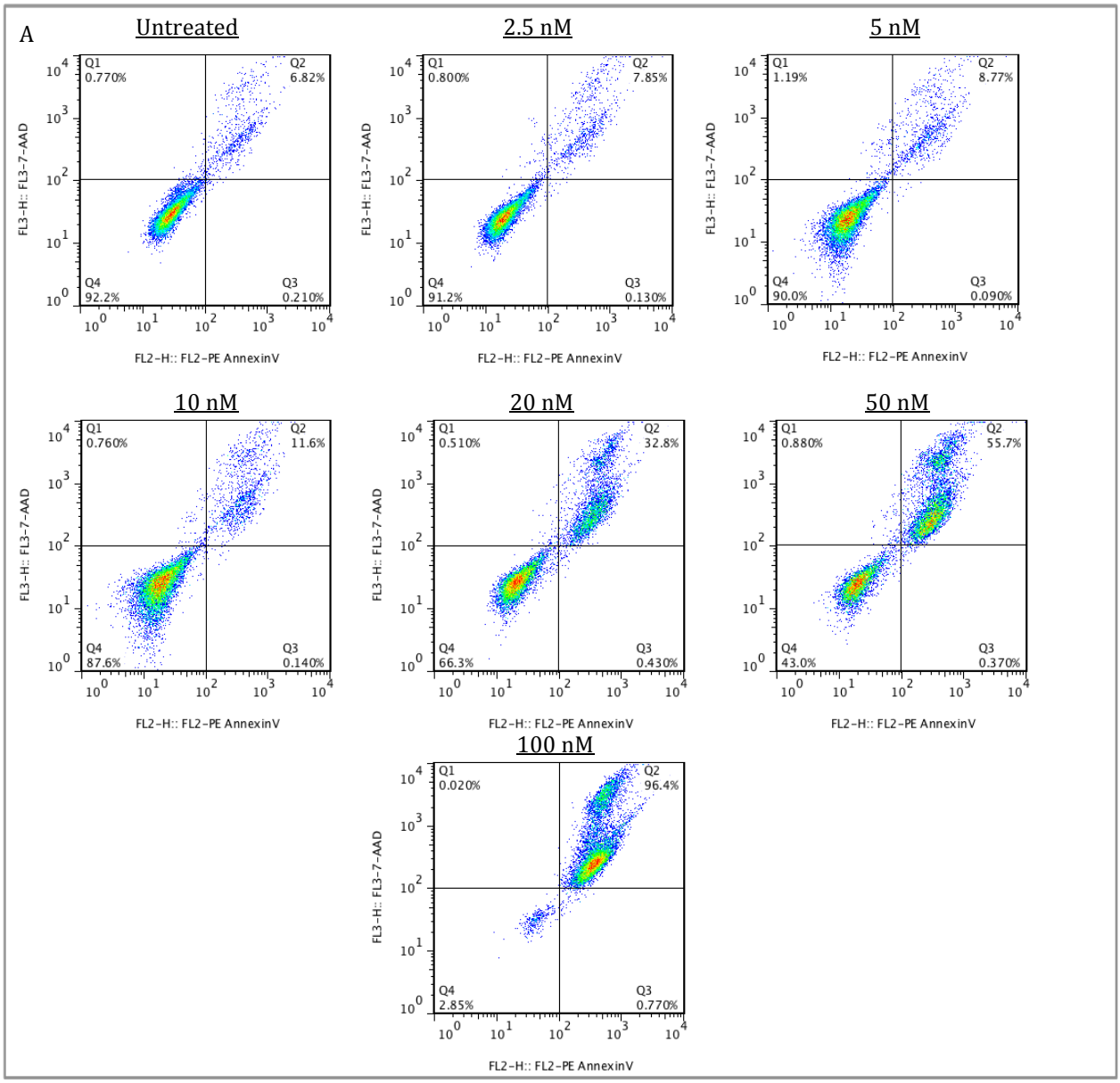
To determine the cytotoxic potential of silvestrol, we performed MTT assays to assess cell viability and proliferation after exposure to increasing doses of silvestrol. We noted a powerful inhibition of cell growth at very low silvestrol concentrations (nM range) in all tested MM cell lines. KMS11, MM1.S, MM1.R cell lines were found to be the most sensitive showing little viability after 48h of silvestrol exposure ($p<0.001$). (figure 4A and 4B).

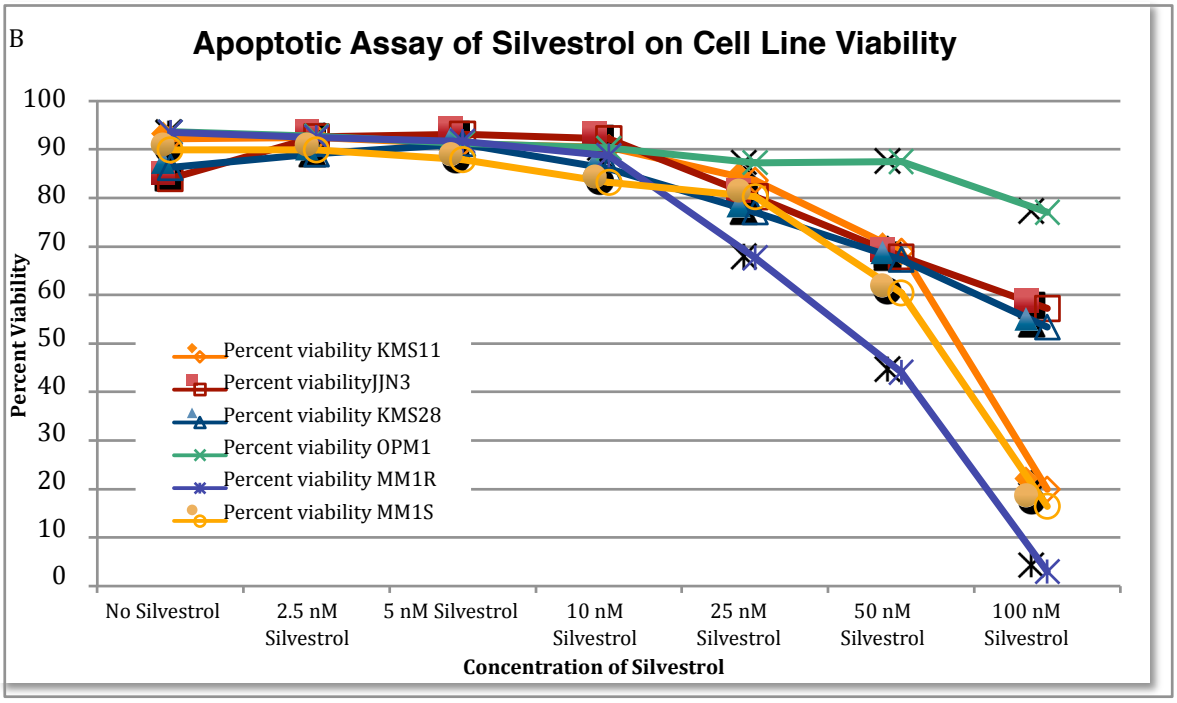


Mean results are plotted +\- standard error (SEM). *indicates statistical difference from control p<0.01 (at 48 hours)

Figure 4A + B. Cytotoxicity of silvestrol on MM1S and OPM1 myeloma cell line. Myeloma cell lines were treated with increasing concentrations of Silvestrol at 24, 48 and 72 hours.

To determine the cytotoxic potential and mechanism of silvestrol on MM cells, we looked at silvestrol's ability to induce cellular apoptosis. Flow cytometry results revealed a rapid decrease in cell viability as demonstrated by the expression of early apoptotic markers, Annexin V and 7-AAD (double positive) (figure 5A). We noted that silvestrol was able to induce most cell lines to undergo apoptosis when exposed to as little as 50nM.



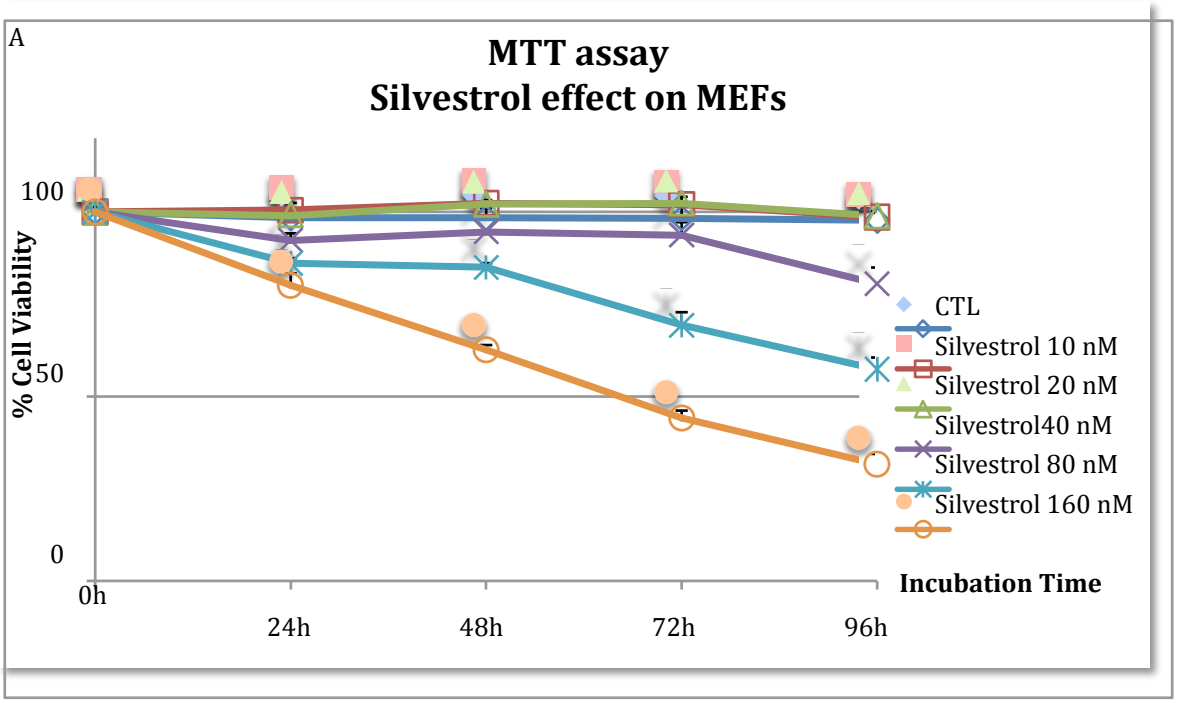


C

Cell line	IC50 (nM)
KMS11	50
JJN3	100
KMS28	100
OPM1	-
MM1R	50
MM1S	50

Figure 5A. Flow cytometry analysis of the MM1R cell line. Cells were exposed to increasing concentrations of silvestrol and analyzed using Annexin V and 7 AAD after 24 and 48 hours. **B. Silvestrol's apoptotic potency on MM cell lines.** Results from the flow cytometry were generated by quadrant statistical analysis. **C. IC50s of myeloma cell line tested.**

As virtually all cells require protein translation for maintenance and possibly survival, we explored the cytotoxicity of silvestrol in normal non-dividing tissues. If a higher concentration of silvestrol was required to be cytotoxic to non-dividing cell, it would demonstrate the possibility of a useable therapeutic window. We used mouse embryonic fibroblasts that were passaged sufficiently to demonstrate viability and senescence. Treatment with increasing concentrations of silvestrol demonstrated cytotoxicity as seen in MTT assays (figure 6A), but at significantly higher concentrations then that seen in even the slowest growing MM cells lines (MM1.S and MM1.R) (Figure 6B). We concluded that tumor cells (even slow growing ones) were still more sensitive to inhibition of protein translation than normal slow or non-replicating cells.



B Cell line	IC50 (nM)
KMS11	50
JJN3	50
OPM1	50
MM1R	25
MM1S	25
MEF	160

Figure 6A. MTT assay depicting silvestrol's effect on MEFs. MEFs were treated for 96 hours with different concentrations of silvestrol. **B. Comparaison between IC50s of myeloma cell lines and MEFs at 72 hours.**

Bortezomib remains one of the most powerful treatment options in MM. Its anti-myeloma effects have been known to be enhanced when used in combination with other agents. We hypothesized that blocking protein degradation with bortezomib while simultaneously blocking protein synthesis with silvestrol would be a powerful combination. We set out to see if we could demonstrate synergy between these two agents. Cell lines were treated with increasing concentrations of both silvestrol and bortezomib. Viability was then assessed with an MTT assay. To determine synergy the

Berenbaum's equation [105] was applied to the result and silvestrol and bortezomib were found to be synergistic (Figure 7).

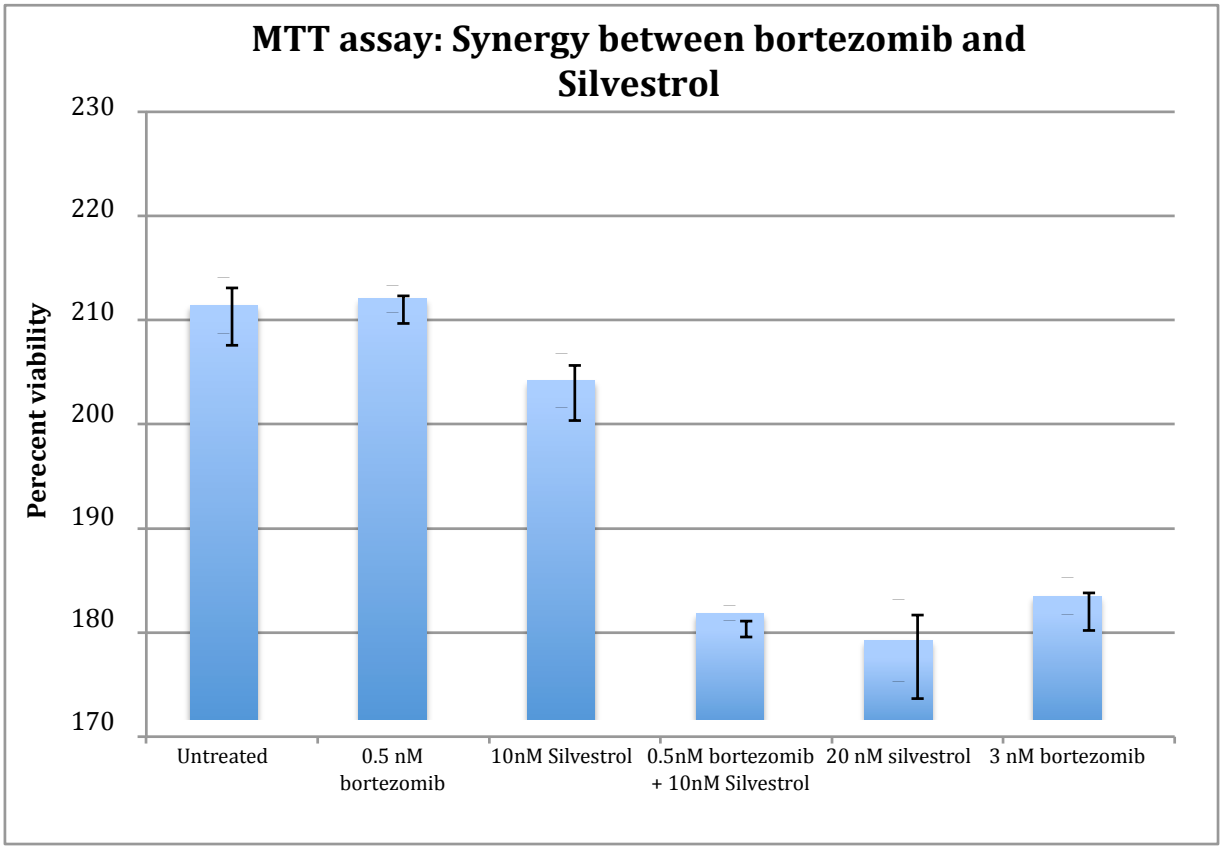


Figure 7. Synergy between silvestrol and bortezomib witnessed in a MTT assay at 48 hours.

Berenbaum formula (1989): $d_a/D_a + d_b/D_b < 1$ synergy effect.

Where d_a and d_b are the concentrations of drugs A and B used in combination and D_a and D_b are their single concentrations that were isoeffective with the combination ($d_a + d_b$) at any specified level of effect.

Here at 48 hours: $0.5/3 + 10/20 = 0.66$ (values < 1, are synergistic)

da: 0.5 nM bortezomib

db: 10 nM silvestrol

Da: 3 nM bortezomib

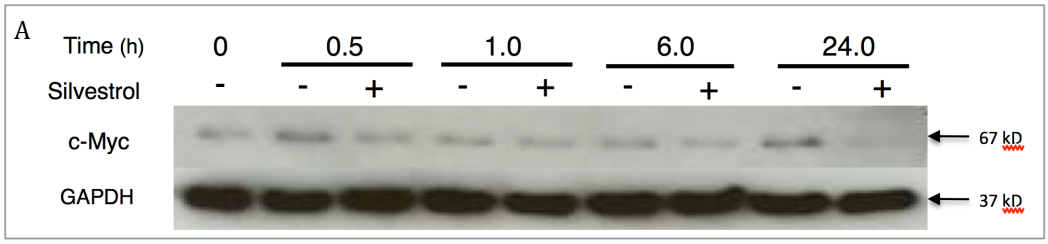
Db: 20 nM silvestrol

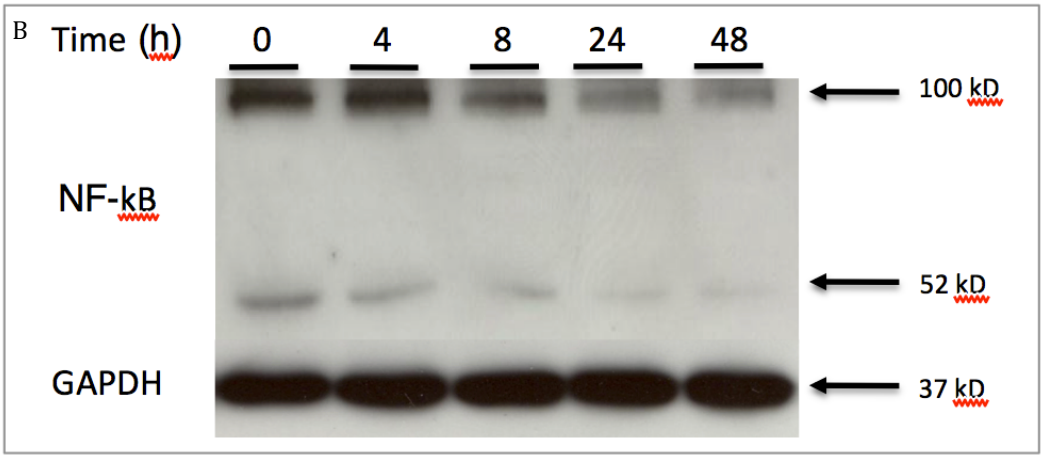
Cellular mechanism of silvestrol cytotoxicity

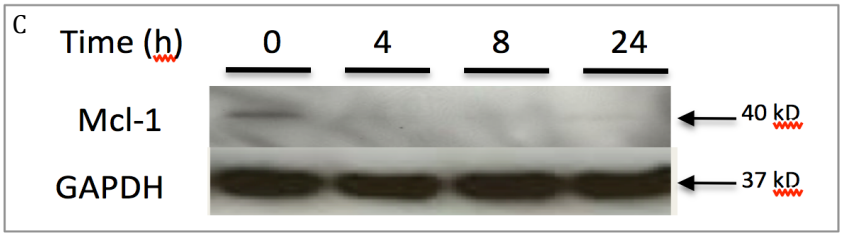
To further elucidate the mechanism of cell cytoxicity, we explored silvestrol's effect on various key regulatory pathways. We first looked at two pathways that are known to play a role in MM cell survival and growth: c-Myc and Nf-κb. Both MM1R and JJn3 cell lines (known to overexpress c-Myc) exhibited a decrease in c-Myc expression especially notable at 24 hours (figure 8A). The KMS11 cell line, which is known to demonstrate constitutive Nf-κb signaling, was used to observe silvestrol's effect on the non-canonical pathway of Nf-κb. Endogenous p100 Nf-κb as well as its processed activated subunit p52 were found to decrease gradually with increase time of silvestrol exposure (figure 8B).

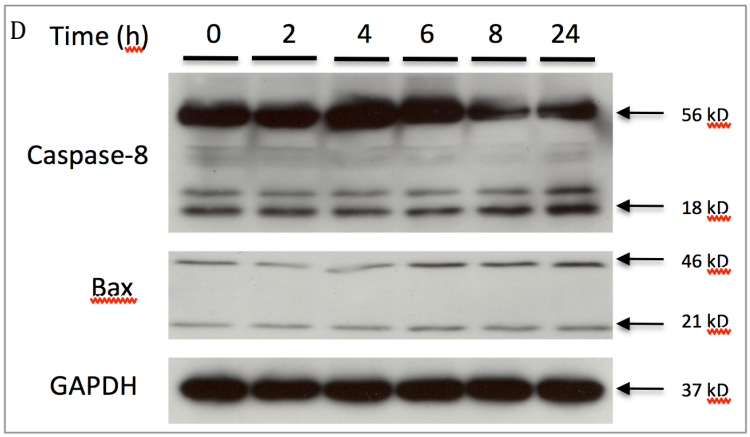
We then verified the protein levels of cytosolic and mitochondrial proteins involved in apoptosis. These include the various caspases and the Bcl-2 family proteins such as Mcl-1, bcl-2, blc-xl and bax that are involved in the intrinsic pathways of apoptosis. Upon silvestrol exposure, the anti-apoptotic proteins Mcl-1 and Bcl-Xl were seen to decrease rapidly whereas changes in bcl-2 content were not significant even at 48 hours (figure 8C, D, E). The pro-apoptotic bax protein was slightly upregulated in its monomeric form and clearly upregulated in its dimeric form after 6 hours. Increased

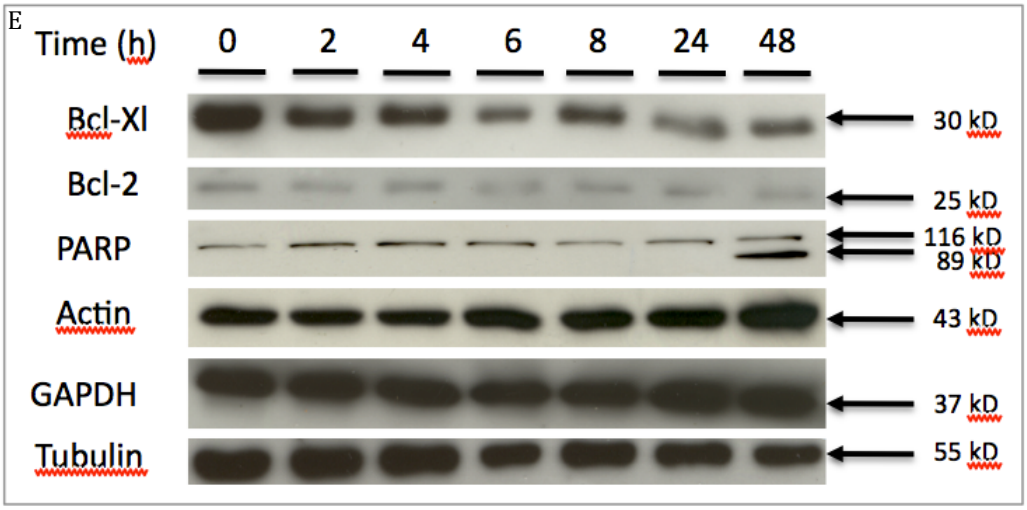
Caspase 8 cleavage is also observed at 8 hours (figure 8D). These western blots demonstrate the activation of the intrinsic apoptotic pathway that ultimately leads to PARP cleavage (the penultimate step before apoptosis) between 24 and 48 hours (Figure 8E). Analysis of caspase 3 and 9 cleavage revealed that silvestrol does not appear to activate the extrinsic pathway of apoptosis as their expression levels were unchanged compared to untreated cells (figure 8F).











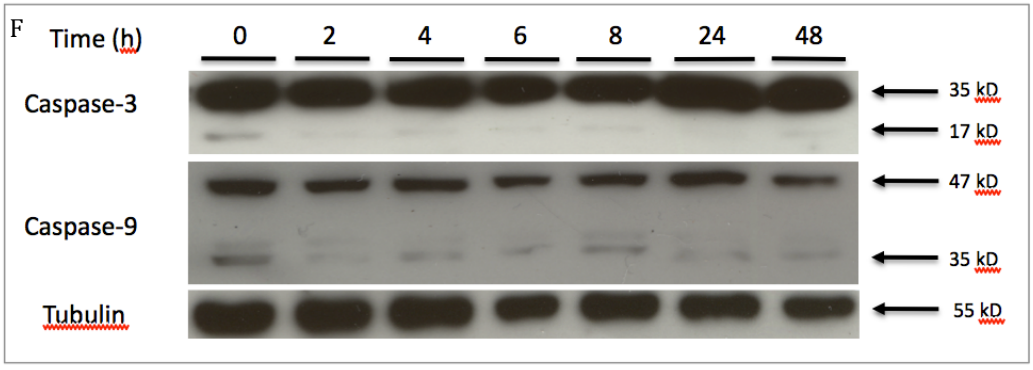


Figure 8A and B. Westernblot analysis assessing the expression of Myc and Nf-κb when exposed to silvestrol. Both these proteins are believed to be instrumental in the development of the MM tumor. **8C, D, E and F.**

Expression of proteins involved in the intrinsic (C, D and E) and extrinsic (F) pathways of apoptosis. The decreased expression of Mcl-1, Bcl-Xl and Bcl-2 associated with the increase in dimeric Bax and cleavage of caspase-8 and PARP demonstrate silvestrol's ability to trigger the intrinsic pathway of apoptosis. Caspase-3 and 9 were unchanged thus showing that silvestrol does not affect the extrinsic apoptotic pathway.

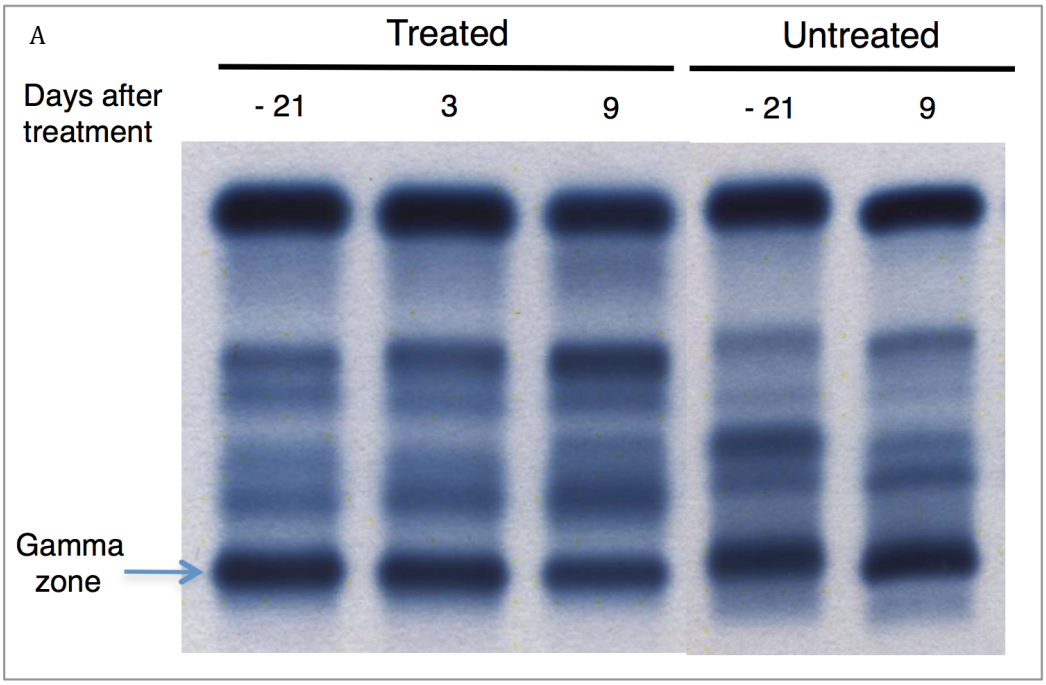
Anti-MM actions of Silvestrol in an animal model of MM

The fidelity of the vk*myc mice to human MM treatment responses was utilized to pre-clinically test silvestrol in an *in vivo* setting. Silvestrol was previously shown not to be toxic to mice given at concentrations high enough to effectuate tumoral cytotoxicity in xenographic models of other hematologic diseases [99]. In human disease, a decrease in M-protein on serum protein electrophoresis (SPEP) correlates with a decrease in MM disease burden.

Mice were treated with silvestrol at either 0.5mg/kg per day or at 0.25mg/kg twice a day by IP injection. In all cases, we observed a decrease in the pathologic M-protein indicating therapeutic efficacy (figure 9A)

To ensure that the decrease in serum M-protein levels seen in silvestrol treated mice is not simply due to silvestrol's ability to decrease total protein production, we looked at *in vivo* evidence of cellular apoptosis. Bone marrow extracts of mice treated with silvestrol were harvested and both the CD138 positive and negative fractions were analyzed for Mcl-1

content. We observed that the CD138+ cells overexpress Mcl-1 (as compared to the CD138- cells) in keeping with MM known overexpression of Mcl-1 [103]. In addition, treatment with silvestrol dramatically decreased Mcl-1 production in the CD138+ cells (Figure 9B, C).



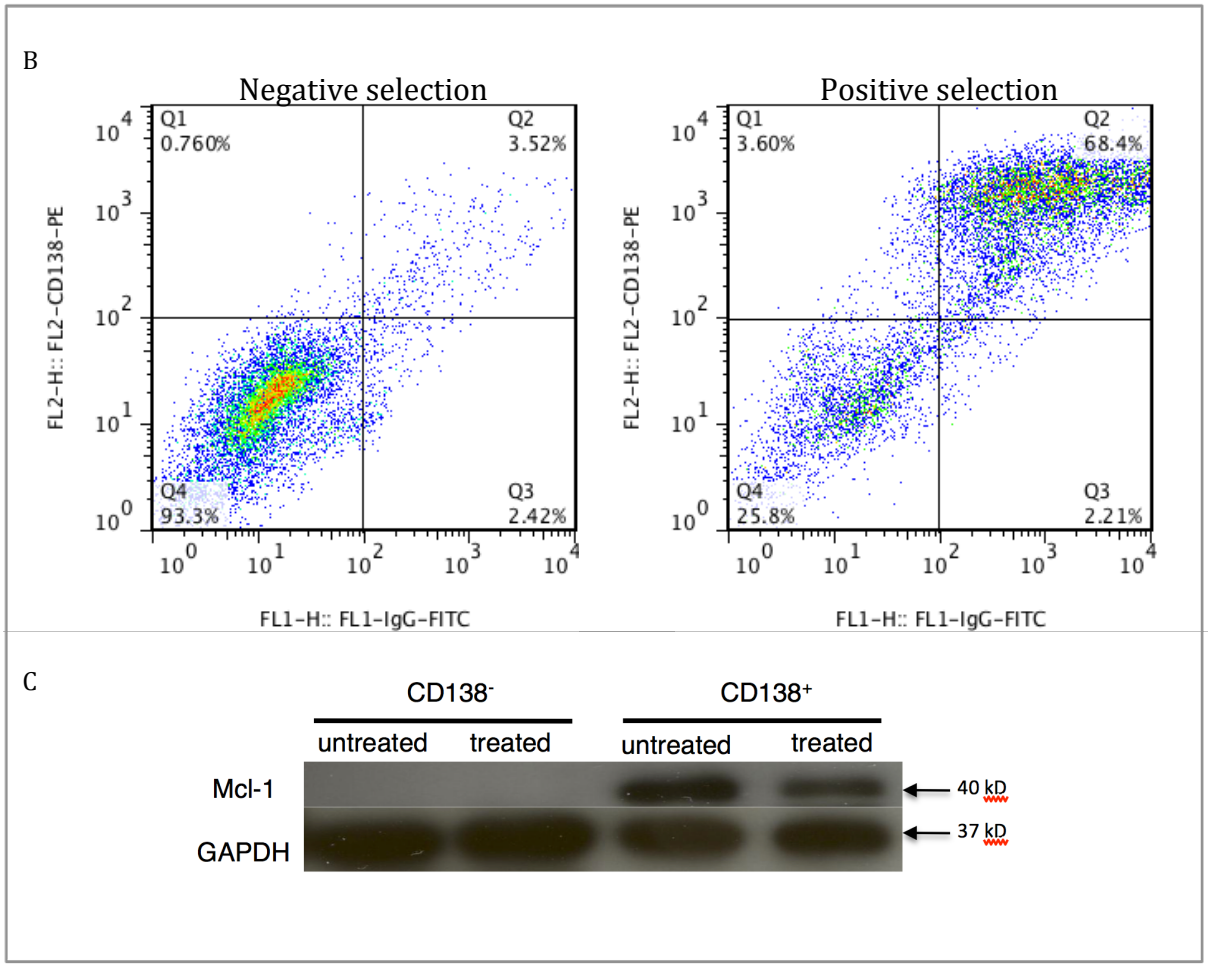
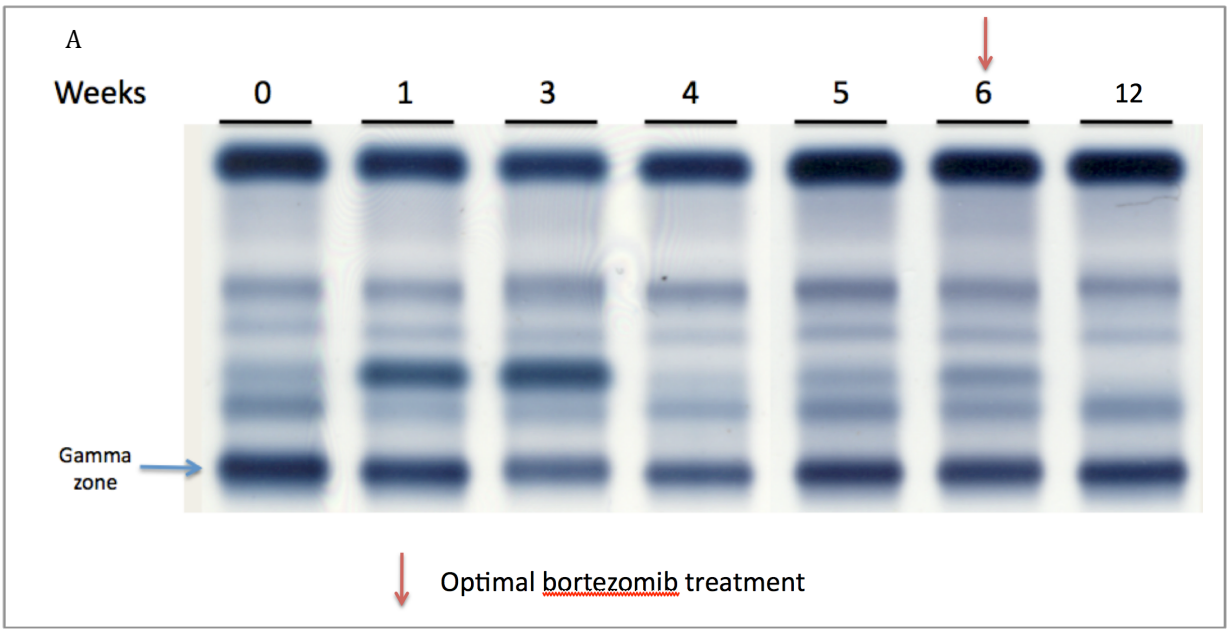


Figure 8A. Serum protein electrophoresis showing a decrease in the paraprotein gamma zone. Mice were treated with 15 doses of 0.5 mg/kg silvestrol over a three week period (IP injections). **B. Flow Cytometry analysis of mice bone marrow sample selected for CD138⁺.** Mice were treated with 0.25mg/kg silvestrol twice a day for two days. Bone marrow was isolated and CD138⁺ cells were separated using magnetically labeled antibodies. Anti-CD138 and intracellular anti-mouse IgG were used for purity analysis. **C. Mcl-1 Western blot analysis.**

Rescuing bortezomib resistance

Treatment of MM patients with bortezomib inevitably leads to drug resistance. To see if we could overcome bortezomib resistance we first generated bortezomib-resistant mice by treating them with chronic low, suboptimal, concentration of bortezomib (0.15mg/kg) Figure 10A. Resistance was demonstrated by a lack of response following challenge with a higher (optimal) concentration of bortezomib (0.5mg/kg) Figure 10A.

Resistant mice were then treated with either silvestrol or the combination of silvestrol and bortezomib. As demonstrated by SPEP analysis of secreted M-protein levels, bortezomib resistant mice showed no decrease in M-protein production when exposed to the higher 0.5mg/kg dose of bortezomib. However, they did show a significant response to silvestrol treatment alone. Promisingly, they showed an even greater response when bortezomib was added to silvestrol (figure 10B)



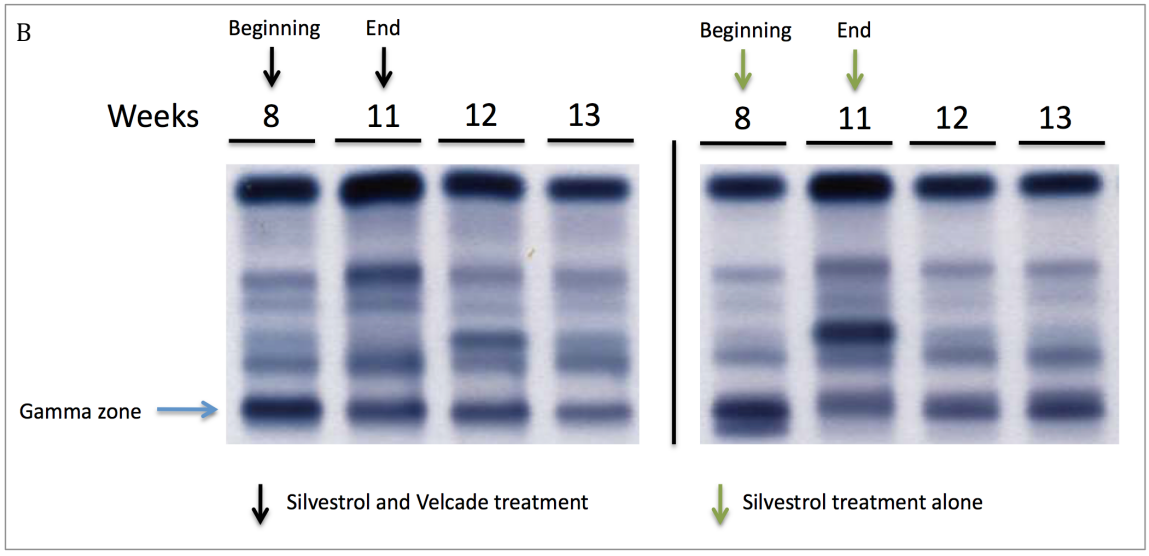


Figure 10A. Serum Protein Electrophoresis (SPEP) signature of a bortezomib resistant mouse. Mice were treated for 6 weeks with a low dose of 0.15 mg/kg bortezomib and 3 weeks with 0.5mg/kg. **B. SPEP of bortezomib resistant mice to silvestrol, silvestrol and bortezomib.**

V. Discussion

In our studies we have shown that silvestrol inhibits protein translation by blocking ribosome from reading complex mRNAs. Inhibiting protein translation led to a decreased in cell growth and increase apoptosis. Induction of apoptosis appears to be mediated by the intrinsic pathway of apoptosis while crucial cell cycle proteins such as c-myc and NF-κB that depend on post-transcriptional control are decreased by silvestrol in MM cells. Normal tissues may be affected by silvestrol, but truly non-dividing cells that are non-tumorigenic require much higher concentrations of silvestrol to exhibit toxicity. In addition, silvestrol at doses which inhibit tumor growth in xenographic and in our transgenic mouse models do not cause significant mouse toxicity. The mice survive, but their tumors appear not to. We show that the cytotoxic effects due to the inhibition of protein translation with silvestrol can synergize with protesomal blockers like bortezomib. We also demonstrate that bortezomib resistance can be overcome by treatment with silvestrol with or without bortezomib *in vivo*.

Proteaosomal blockade, in which agents like bortezomib block the proteolytic degradation of ubiquitinated targets, has been shown to be lethal to MM cells both *in vitro* and in the clinical setting. The unique sensitivity of MM to this therapeutic mechanism underlines the reliance of these cells on

protein metabolic pathways for their survival. MM cells are antibody (protein) producing factories, therefore targeting these pathways is sensible.

We explored the use of silvestrol, a plant derived cyclopenta (b) benzofuran that has been shown to bind and sequester eIF4A, inhibiting it from properly joining the eIF4F complex and initiating mRNA translation. Decreasing eIF4F complex formation reduces translation of cap-dependent, CG rich and complex structured mRNAs [99]. These include mRNAs that code for proteins regulating the cell cycle or that are involved in cell survival pathways, but not those that perform basic 'housekeeping' functions. Silvestrol inhibits translation initiation by sequestering eIF4A and forcing cap-dependant mRNAs to bind to it. However, comprehending why complex mRNAs do not get translated with silvestrol is still uncertain and two hypotheses arise. 1. Selective inhibition of complex mRNAs can be due to the lack of eIF4A. This would block the ribosome from reading the complex mRNA since it would remain un-wound. 2. Silvestrol forces cap-dependant mRNAs to bind the sequestered eIF4A making these latter unreachable to the ribosomal complexes.

Our understanding of silvestrol's mechanism is still limited but its effects *in vitro* and *in vivo* demonstrate promising results. Silvestrol was cytotoxic in all of the cell lines tested, and in *in vivo* experiments. It was even toxic in very slowly dividing myeloma cell lines and, at much higher concentrations, in non-dividing cells. While senescent mouse embryonic

fibroblasts were sensitive to silvestrol, these required much higher doses, perhaps due to the fact that, unlike myeloma cells, do not rely so much on protein metabolism. As myeloma is not a highly proliferative tumor, solely targeting cell division is not necessarily the best therapeutic choice. Silvestrol does not appear to principally target cell division. It is compelling to believe that cells that rely on protein metabolism would be more sensitive to its disruption by silvestrol.

We believe that silvestrol is lethal to myeloma cells by two complementary mechanisms. It was shown that one third of the mutations found in myeloma were related to the protein translation pathway including the eIF4 co-factor system [104]. Blocking this pathway with silvestrol may engender a stress response from the cell that renders it more susceptible to apoptosis. Simultaneously, silvestrol inhibits translation of key oncogenes (c-myc), transcription factors (NF-κB) and proteins (Bcl-2 family), which disturbs the cell's reliance on its anti-apoptotic pathways. These hypotheses are confirmed by our western blots and cell line data. However, cell lines were not equally sensitive to silvestrol. These observations were comparable in MTT and apoptosis assays and we believe they may be due to the different tumorigenic pathways the cell lines rely on, with some pathways dependant on oncogenes with more or less complex mRNA structures.

Our experimental data show that silvestrol induces cell death by principally activating the intrinsic pathway of apoptosis. The expression of

proteins belonging to the bcl-2 family decreased, thus demonstrating a pro-apoptotic response. Mcl-1 has been shown to be upregulated in MM and may be a principle factor preventing MM cell death in normal circumstances [103]. We have shown that Mcl-1 levels are profoundly decreased by silvestrol. The upregulation of monomeric and dimeric bax depicts the ongoing apoptosis but it is also evidence that silvestrol does not inhibit translation completely and indiscriminately. Non-activation of caspases 3 and 9 demonstrates that the extrinsic pathway is not triggered by silvestrol. The intrinsic pathway yields the cleavage of PARP after 24 hours and ultimately confirms programmed cell death.

The onset of Multiple myeloma is typically quite late in life (average 70 years) and new therapies have prolonged life expectancy to 7 – 10 years but eventually relapse occurs due to bortezomib resistance. Increasing the potency of bortezomib and vanquishing the resistance is a crucial and realistic strategy to extend life expectancy even further. Our MTT assays reveal that silvestrol and bortezomib act in a synergistic manner. In combination, these drugs not only kill myeloma cells more efficiently, but our *in vivo* data with bortezomib resistant mice suggests that silvestrol helps overcome the resistance for a more durable bortezomib treatment. It is currently difficult to determine the mechanism underlying the powerful effect of these two drugs in combination, as we are still unsure of how bortezomib and silvestrol exert their lethality individually.

VI. Summary and Conclusion

Multiple Myeloma remains incurable despite our greater understanding of the processes that promote this cancer. Exploiting myeloma cell physiology with new drugs is a sensible approach to gain further knowledge about MM and may generate novel therapeutic approaches.

Silvestrol exhibits cytotoxic effects *in vitro* (cell lines and primary bone marrow) and *in vivo* in the vk*myc mouse model. It may also exert a greater effect on MM as compared to other cell types and tumors (not shown) because of myeloma's reliance on protein metabolism. Furthermore, its potency in a low concentration range may limit the adverse effects of the drug.

Altogether, silvestrol warrants further investigation and may prove to be a good therapeutic approach for myeloma.

VII. Future Perspectives

Silvestrol's efficacy in this study has shown that translation inhibition in MM may play an important role towards curing the disease. However, many questions and logistics remain to be solved. Clearly understanding silvestrol's mechanism of action is necessary and would permit us to create new experimental plans that would exploit silvestrol's mechanism to a greater extent. We also wish to test silvestrol on more evolved species in the hopes of one day reaching clinical trials. Targeting eIF4A is one strategy to inhibit protein translation; but there are other viable targets within the protein translation apparatuses. In addition, other cellular processes that are involved in protein metabolism like the ER or Golgi apparatus (blocking protein trafficking, folding or packaging) could be explored in this diseaae.

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