IDENTIFICATION OF A NOVEL AGENT THAT CAN SUPPRESS PROLIFERATION, INDUCE APOPTOSIS AND OVERCOME CHEMORESISTANCE IN MULTIPLE MYELOMA

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DECLARATION

I hereby declare that this thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.

Ms. RADHAMANI KANNAIYAN

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SUMMARY

Multiple myeloma (MM) is a B cell malignancy characterized by clonal proliferation of plasma cells in the bone marrow. With the advent of novel targeted agents, the median survival rate has increased to 5 -7 years. However, majority of patients with myeloma suffer relapse or develop chemoresistance to existing therapeutic agents. Thus, there is a need to develop alternative therapies for the treatment of myeloma. Activation of pro-inflammatory transcription factors NF-κB and STAT3 is one of the major contributors to both pathogenesis and chemoresistance in MM. These transcription factors regulate diverse genes involved in various hallmark capabilities of MM cells namely proliferation, survival, angiogenesis, migration and invasion and chemoresistance. Natural compounds have been considered a good source of anti-cancer therapies since ancient times. Thus in the present study, we investigated whether celastrol, a triterpenoid derived from Chinese medicinal plant *Tripterygium wilfordii* could suppress the proliferation and induce chemosensitization in myeloma cells and xenograft mouse model by interfering with NF-κB and STAT3 activation pathways. The anti-inflammatory effects of celastrol have been previously investigated in various inflammatory disease models. In fact, the extract derived from the plant *Tripterygium wilfordii* has been tested before clinically in rheumatoid arthritis patients. A number of studies have also described its anticancer efficacy in diverse tumor models in the last few years.

We aimed to investigate the untapped potential of celastrol as an anti-MM agent and found that celastrol can significantly inhibit the proliferation of various myeloma cell lines regardless of their sensitivity to conventional

chemotherapeutic agents. As a single agent, celastrol induced apoptosis, and abrogated migration and invasion of myeloma cells. Celastrol also enhanced the apoptosis induced by novel targeted therapies namely bortezomib and thalidomide in synergistic manner in MM cells. Interestingly, celastrol also reduced the activation of upstream non-receptor tyrsoine kinase JAK2 and thereby abrogated STAT3 phosphorylation, nuclear translocation and DNA binding. Interestingly, we also found that celastrol's thiol reactive function is critical for its observed inhibitory effects on STAT3 activation cascade in MM cells. Celastrol also suppressed the phosphorylation of p65 subunit of the NF-κB transcription factor, its nuclear translocation, DNA binding ability and these effects were found to be mediated via inhibition of activation of IKK-α/β and AKT kinases. Celastrol also potentiated the ability of bortezomib to cause inhibition of NF-κB DNA binding ability in MM cells. Modulation of STAT3 and NF-κB pathways were associated with the downregulation of various oncogenic gene products involved in MM proliferation, survival, invasion and angiogenesis.

Finally, whether celastrol can overcome chemoresistance and enhance the activity of bortezomib was also investigated in MM xenograft model in nude mice. Interestingly, we observed that celastrol significantly potentiated the antitumor effects of bortezomib *in vivo* and this correlated with suppression of various markers survival and angiogenesis. Overall, our cumulative findings suggest that celastrol overcomes chemoresistance and sensitizes myeloma cells to thalidomide and bortezomib by abrogating STAT3/NF-κB signal transduction cascades and thereby highlights the enormous potential of this triterpene as a novel anti-myeloma agent.

LIST OF FIGURES

ABBREVIATIONS

Abl - Abelson murine leukemia viral oncogene homolog

AML - acute myeloid leukemia

ANOVA - analysis of variance

APRIL - a proliferation inducing ligand

AR - androgen receptor

ATF-2 - activating transcription factor-2

ATF-4 - activating transcription factor-4

BAFF - B-cell activating factor

Bax - Bcl-2–associated X protein

Bcl-xL - B-cell lymphoma-extra large

Bcr - breakpoint cluster region

BLIMP-1 - B-lymphocyte induced maturation protein-1

BMME - bone marrow microenvironment

BMSCs - bone marrow stromal cells

Cdk - cyclin-dependent kinase

CHOP - CCAAT/-enhancer-binding protein homologous protein

cIAP - cellular inhibitor of apoptosis

CML - chronic myeloid leukemia

CXCR-4 - C-X-C chemokine receptor type 4

CYLD - cylindromatosis

DAPI - 4',6-diamidino-2- phenylindole, dihydrochloride

DR - death receptors

DTT - dithiothreitol

ECM - extracellular matrix

eEF1A - eukaryotic translation elongation factor 1A

EGFR - endothelial derived growth factor

EM MM - extramedullary multiple myeloma

ER - endoplasmic reticulam

ERAD - ER-associated degradation

ERG - erythroblast transformation- specific related gene

ERK - extracellular signal-regulated kinase

ER-α - estrogen receptor-α

EthD-1 - ethidium monomer dye

FAK - focal adhesion kinase

FDA - food and drug administration

FGF - fibroblast growth factor

FGFR3 - fibroblast growth factor receptor3

FLT3 - fms-like tyrosine kinase 3

GSH- glutathione

GSSG – glutathione oxidized

HCC - hepatocellular carcinoma

HER-2 - human epidermal growth factor receptor-2

HGF - hepatocyte growth factor

HIF-1α - hypoxia inducible factor-1α

HMCLs - human myeloma cell lines

HO-1 - heme oxygenase-1

HSF-1 - heat shock factor-1

HSP - heat shock response protein

HSR - heat shock response

IAP - inhibitors of apoptosis

ICAM - intercellular adhesion molecule

IGF-1 - insulin like growth factor-1

IL-10 - interleukin -10

IL-13 - interleukin-13

IL-6 - interleukin-6

IL-β1 - interleukin- β1

IM MM - intramedullary multiple myeloma

IMWG - international myeloma working group

iNOS - inducible nitric oxide synthase

IRF4- interferon regulatory factor 4

IκB-α - inhibitor of κB

IKK - IκB kinase

JAK - Janus kinase

JNK- c-Jun N-terminal kinase

LFA-1 - lymphocyte function–associated antigen 1

LPS - lipopolysacharide

LT-β - lymphotoxin-β

MAPK - mitogen activated protein kinase

Mcl-1 - induced myeloid leukemia cell differentiation protein -1

MCP-1 - monocyte chemoattractant protein-1

MEK - mitogen activated extracellular kinase

MGUS - monoclonal gammopathy of undetermined significance

MM - multiple myeloma

MMP - matrix metalloproteinase

MMSET - MM set domain gene

mTOR - mammalian target of rapamycin

MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

MVD - micro vessel density

NF-κB - nuclear factor - κB

NIK - NF-κB inducing kinase

Nrf2 - NF-E2- related factor-2

JNK - c-Jun NH2-terminal kinase

OPG - Osteoprotegerin

p70S6-kinase -ribosomal protein S6 kinase

PARP - poly (ADP-ribose) polymerase

PBS - phosphate buffered saline

PCL - plasma cell leukaemia

PCLI - plasma cell labelling index

PDGF - platelet derived growth factor

PI - propidium iodide

PI3K - phosphatidyl inositol-3 kinase

PMA - phorbol 12-myristate 13-acetate

PTEN - phosphatase and tensin homolog

Raf-1 - rapidly accelerated fibrosarcoma -1

RAFTK - related adhesion focal tyrosine kinase

RANK - receptor activator of NF-κB

RANKL - receptor activator of NF-κB ligand

RIP kinase- receptor interacting protein kinase

RIP1-Tag2 - rat insulin promoter- SV40 large T antigen transgene

ROS - reactive oxygen species

RPMI - Roswell park memorial institute

RT-PCR - reverse transcriptase - polymerase chain reaction

SAPK1 - stress activated protein kinase-1

SCID - severe combined immunodeficiency

SDF-1α - stromal derived factor-1α

SDS - sodium dodecyl sulphate

SHP-1 - src homology region 2 domain-containing phosphatase-1

SHP-2 - src homology region 2 domain-containing phosphatase-2

SMM- smouldering multiple myeloma

SOCS1 - suppressor of cytokine signaling1

STAT3 - signal transducer and activator of transcription 3

TACI - tumor necrosis factor receptor superfamily member 13B

TAK1 - TGF-β activated kinase 1

TGF-β - transforming growth factor-β

TMPRSS2 - transmembrane protease, serine 2

TNF-α - tumor necrosis factor-α

TRAF - tumor necrosis factor receptor associated factors

TWEAK - TNF-related weak inducer of apoptosis

UGT - UDP-glucuronosyltransferase

uPAR - urokinase type plasminogen activator receptor

UPR - unfolded protein response

VCAM - vascular cell adhesion molecule

VEGF - vascular endothelial growth factor

VLA-4 - very late antigen

XBP1- X box-binding protein 1

Yap1 - yeast AP-1

LIST OF PUBLICATIONS AND CONFERENCE ABSTRACTS

Publications in Peer Reviewed International Journals

1. **Kannaiyan R**, Hay HS, Rajendran P, Li F, Shanmugam MK, Vali S, Abbasi T, Kapoor S, Sharma A, Kumar AP, Sethi G. Celastrol Inhibits Proliferation and Induces Chemosensitization through downregulation of NF-κB- and STAT3-Regulated Gene Products in Multiple Myeloma Cells. *British Journal of Pharmacology*, 2011 Nov; 164(5):1506-21. (IF= 5.0).

2. **Kannaiyan R,** Manu KA, Chen L, Li F, Rajendran P, Subramaniam A, Lam P, Kumar AP, Sethi G. Celastrol inhibits tumor cell proliferation and promotes apoptosis through the activation of c-Jun N-terminal kinase and suppression of PI3 K/Akt signaling pathways. *Apoptosis,* 2011 Oct; 16(10):1028-41. (IF= 3.95).

3. **Kannaiyan R,** Shanmugam MK, Sethi G. Molecular targets of celastrol derived from Thunder of God Vine: Potential role in the treatment of inflammatory disorders and cancer. *Cancer Letters*, 2011 Apr 1; 303(1):9-20. (IF= 4.86).

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Book Chapter

1. **Kannaiyan R**, Surana S, Shin EM, Ramachandran L, Sethi G, Kumar AP. Targeted Inhibition of Multiple Proinflammatory Signalling Pathways for the Prevention and Treatment of Multiple Myeloma, Multiple Myeloma - An Overview (2012), Dr. Ajay Gupta (Ed.), ISBN: 978-953-307-768-0, InTech, DOI: 10.5772/30444.

Publications in Regional Magazine

1. **Kannaiyan R.** Ancient Medicine with Newer Roles: Potential Role of Celastrol in the Treatment of Multiple Myeloma. *Asia Pacific Biotech News*, August 2012, 16: 51-53.

Oral Presentations in Local Conferences

1.**Kannaiyan R.** Potential novel role of celastrol as an anti-multiple myeloma agent Yong Loo Lin School of Medicine, YLLSoM 1st Annual Graduate Scientific Congress, National University of Singapore, Jan 25th, 2011, NUHS Tower Block, Main Auditorium, Singapore.

Poster Presentations in International Conferences

1. **Kannaiyan R**, Li F, Shanmugam MK, Aryan MK, Siveen KS, Kumar AP, Sethi G. Celastrol potentiates the antitumor effect of Bortezomib in Multiple Myeloma xenograft mice model through the suppression of NF-κB-regulated gene products. YLLSOM 3rd Annual Graduate Scientific Congress, 30th Jan 2013, NUHS Tower Block, Main Auditorium, Singapore.

2. **Kannaiyan R,** Rajendran P, Li F, Shanmugam MK, Goh JN, Wong KF, Wang W, Khin E, Tergaonkar V, Kumar AP, Luk JM, Sethi G. Celastrol, a potent suppressor of hepatocellular carcinoma growth through the inhibition of STAT3/JAK2 signaling cascade: novel mode of action(s) for an old drug". AACR New Horizons in Cancer Research: Biology to Prevention to Therapy, 13-16th. Dec 2011, The Leela Kempinski, Gurgaon, Haryana, India.

3. **Kannaiyan R**, Sethi G. Potential role of celastrol as a novel anti-myeloma drug. National Cancer Research Institute (NCRI) conference, 7-9th November 2010, Liverpool, UK.

4. **Kannaiyan R**, Sethi G. Celastrol, a pentacyclic tritepenoid overcomes chemoresistance through down-regulation of STAT3 and Nuclear Factor-κB-regulated anti-apoptotic gene products in human multiple myeloma cells. The International Conference of Pharmacology-The 3rd Mainland, Hongkong and Singapore Meeting of Pharmacology Sep 24-27th, 2010, Shenyang, China.

Awards & Achievements

1. **Scholar in Training Award** for best abstract submitted for AACR International Conference on New Horizons in Cancer Research: Biology to Prevention to Therapy, December 13-16, 2011 Gurgaon, Haryana, India.

2. **Excellent Youth Paper Reports Award** in The International Conference of Pharmacology-The 3rd Mainland, Hongkong and Singapore Meeting of Pharmacology Sep 24th-27th, 2010, Shenyang, China.

3. **NUS Research Scholarship Award** for graduate studies in Yong Loo Lin School of Medicine, NUS, Singapore.

1. INTRODUCTION

1. 1. Cancer

Cancer is a major public health problem worldwide. According to World Cancer Research Fund International, there were an estimated 12.7 million cancer deaths (13% of all deaths) worldwide in 2008, males accounting for 6.6 million and females accounting for 6 million. According to Singapore Cancer Registry Interim Annual Registry Report detailing the trends in cancer incidence in Singapore 2007-2011, a total of 54,001 cases were diagnosed between 2007 and 2011. Of these, 26,267 (48.6%) were men and (51.4%) cases were women. The understanding of the biology of cancer has increased tremendously in the past few years. Accordingly, the treatment modalities and supportive care have also seen a lot of improvement.

In modern times, it has been established that cancer originates from the normal mammalian cells when they undergo multistep mutagenesis each of which confer a survival advantage to the cancer cells [1]. Weinberg and Hanahan have described the key hallmark principles that control the transformation of a normal cell into a tumor cell. These include 1. Self-sufficiency in growth signals 2. Insensitivity to antigrowth signals 3. Evasion of apoptosis 4. Limitless replicative potential 5. Sustained angiogenesis and 6. Tissue invasion and metastasis. These properties are attained by the cells through 'the oncogenes' resulting from the gain of function mutations and 'tumor suppressor genes' resulting from loss of function mutations [2]. Additional hallmark capabilities described recently include genomic instability and mutations, tumor promoting inflammation, deregulated cellular energetics and avoiding immune destruction [3].

1. 2. Multiple Myeloma (MM)

Multiple myeloma (MM) is a B cell malignancy involving the post germinal center B cells. The disease is characterized by presence of blood and

urinary monoclonal proteins, osteolytic bone lesions and infiltration of bone marrow with malignant plasma cells [4]. Generally, MM is preceded by two premalignant conditions namely monoclonal gammopathy of undetermined significance (MGUS) and smouldering (asymptomatic) multiple myeloma (SMM) [5, 6].

1. 2. 1. Epidemiology

MM is mainly a disease of elderly people. It is twice more common in African Americans than Caucasians [7, 8]. MM is the second most common hematological malignancy next only to non-Hodgkin's lymphoma, contributing 1% of all neoplasias [9]. The advent of existing treatment modalities, has increased the median survival rate to 5-7 years [10]. MGUS is prevalent in 2% of white males of older than 50 years. The risk of progression of SMM to MM or other MM related conditions is 10% over the first 5 years [6]. According to Singapore General Hospital website as dated on October 2011, about 80 new patients with MM are diagnosed every year.

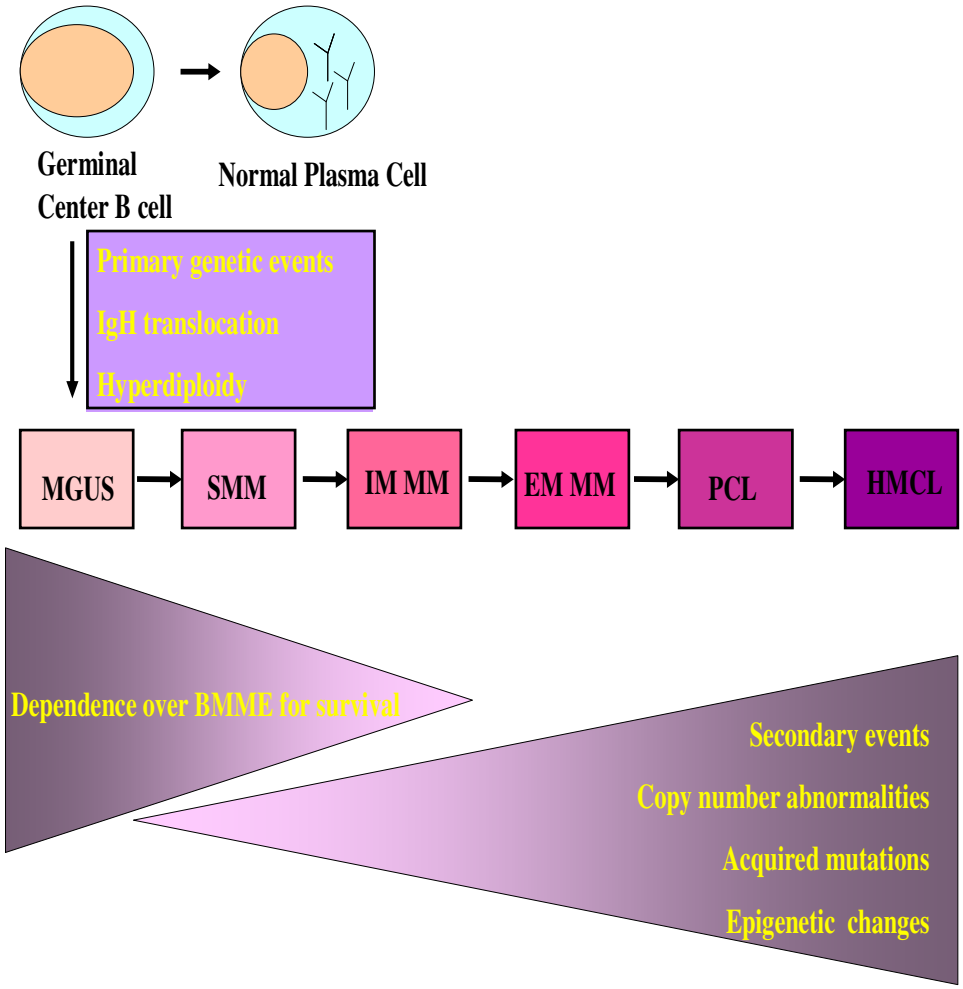
1. 2. 2. Diagnosis

According to International Myeloma Working Group (IMWG) the criteria for the diagnosis of MGUS is presence of less than 10% of bone marrow plasma cells, serum intact immunoglobulin of less than 3g/100 ml with the absence of any clinical symptoms. Diagnosis of SMM requires greater than 10% bone marrow plasma cells, greater 3g/100 ml of serum intact immunoglobulin with the absence of any clinical symptoms. MM is diagnosed when there is greater than 10% of plasma cells in the bone marrow, greater than 3g/100ml of serum intact immunoglobulin with the presence of clinical symptoms. The clinical symptoms defining the diagnosis of MM are hypercalcemia, renal involvement, anemia and bone lesions that can be attributed to the plasma cell disorder and are not related to any other disease or disorder present in the patient [11].

1. 2. 3. Normal B cell development

Understanding of normal B cell development is crucial to study the pathogenesis of MM. Occurrence of programmed mutations during B cell development help in the immune function by increasing diversity of antibodies that can be produced in response to the exposure to any foreign antigen. This interesting phenomenon in the development of B cells (also in T cells) enables body to fight with the diverse environmental antigens. These regulated mutations occur during various steps of B cell development. Naive B cell that resides in the bone marrow expresses IgM (immunoglobulin M) which binds with foreign antigen. They undergo first step of mutation (VDJ recombination) resulting in immunoglobulin genes recombination and move to the lymph node. These naive B cells upon exposure to antigen move to the germinal center of the lymph nodes and undergo somatic hypermutation to increase the affinity with their antigens. Then, these B cells undergo immunoglobulin class switch recombination to produce various isotypes of immunoglobulins. The B cells that enter the bone marrow to produce functional immunoglobulins are called 'plasma cells' or 'post germinal center B cells' [12]. Few plasma cells undergo apoptosis when the immune response wanes. However, remaining plasma cells find their way into the specialised bone marrow niche where they survive as 'long lived plasma cells' to function as memory cells waiting for the next encounter with the same antigens. The differentiation of centroblast residing in the lymph node to the mature plasma cells requires reprogramming to increase their functional efficacy. They undergo cell cycle arrest, condensation of chromatin and silencing of cellular functions which are not necessary for the production of antibody. Simultaneously, processes required for the production of antibody are turned on which are mediated by various transcription factors [13]. The transcription factors required for the plasma cell differentiation include IRF4 (interferon regulatory factor 4), XBP1 (X box-binding protein 1) and BLIMP-1 (transcriptional repressor B lymphocyte-induced maturation protein 1) [14, 15]. Erroneous immunoglobulin translocations and dysregulation in the differentiation of centroblast to the plasma cell leads to of MM [13].

Multi-Step model of MM progression



IM MM - Intramedullary MM EMMM - Extramedullary MM

Figure 1.1. Multi-step molecular model of MM initiation and progression

In the process of B cell development, germinal center B cells develop into long lived plasma cells, which home to bone marrow and survive in the specialized bone marrow niche. Erroneous translocations and other cytogenetic events lead to the development of MGUS. In interaction with the bone marrow microenvironment and with the advantage offered by other genetic events like Ras mutations MGUS progress through SMM to MM. Additional cytogenetic, genetic and epigenetic alterations offer an advantage such that the MM cells no longer require the BMME for their survival, leading to development of EMMM and PCL.

1. 2. 4. Pathogenesis and Classification

The pathogenesis of MM involves characteristic genetic abnormalities and the interaction between the clonal plasma cells and the bone marrow microenvironment [16]. Unlike other hematological malignancies and similar to solid tumors, the genetic abnormalities of MM are heterogeneous and the transformation is not driven by a single gene mutation. The genetic abnormalities include copy number changes of chromosomes, translocations and point mutations which lead to activation of oncogenic pathways or inhibition of tumor suppressor pathways [17].

1. 2. 5. Initiating cytogenetic events

Broadly, based on the initiating cytogenetic events MM can be classified into two groups which are hyperdiploid and non-hyperdiploid. Hyperdiploid MM is characterized by trisomies of odd number chromosomes with low prevalence of IgH (Immunoglobulin heavy chain) translocations. Non-hyperdiploid MM is typified by high prevalence of translocations, hypodiploid, near diploid, pseudodiploid and tetraploid [18]. As a result of these translocations, IgH enhancer loci cause activation of juxtaposed genes leading to their oncogenic activation. The recurrent translocation partners most commonly involved are *cyclin D1* [t(11;14)(q13;q32) translocation] in about 15% of MM patients, *FGFR* (fibroblast growth factor receptor 3) and *MMSET* (MM set domain gene) [(4;14)(p16;q32) translocation] about another 15% of MM patients, *C-Maf* [t(14;16)(q32;q23) translocation] in 5% of MM patients and *cyclin D3* [t(6;14)(p21;q32)] in 4% of MM patients [12]. *Cyclin D* dysregulation is an early perhaps the initiating event in both the groups but it occurs by different mechanisms and leads to variable progression of MM in each group [19].

1. 2. 6. Progression of MGUS to MM

The progression of MGUS to MM has been an interesting area of study. Chromosome 13 abnormalities, especially monosomy 13 or deletion of 13q14 is present in about 50 % of patients with MM [20]. Initially, it was thought that

chromosome 13 abnormalities lead to the progression of MGUS to MM. However, with recent advances in the techniques used in cytogenetics, it has become clear that patients with chromosome 13 abnormality had this chromosomal abnormality even before they had progressed to MM [21-23]. Though in subset of patients, activating mutations of *Ras* [24] and *FGFR3* [25] have been thought to lead the transformation of MGUS to MM, the majority of oncogenomics studies have failed to show any significant difference in the genomic profile of MGUS and MM. So, it is yet to be determined what molecular even is linked to the progression of MGUS to MM. However, the significant difference between MGUS and MM lies in the dysregulated interaction between the MM cells and the bone marrow microenvironment [17, 26]. Though few studies implicate the role of epigenetic events involved in the pathogenesis of MM, their casual role remains yet to be determined. It can be concluded that the progression of normal plasma cell to MM through MGUS involves complex interplay of genetic events, epigenetic events and the bone marrow microenvironment [27].

1. 2. 7. Molecular events in the progression of MM

Thus developed MM, through complex genetic and epigenetic events further progresses and is no longer dependent on bone marrow microenvironment for survival leading to extramedullary MM where in malignant plasma cell seed themselves in various parts of the body and PCL (plasma cell leukemia) wherein MM cells flood the blood stream. Elucidating evolving genetic and epigenetic events have lead us identify dysregulation of few oncogenic and tumor suppressor pathways and their specific role in the further progression of MM. These secondary cytogenetic events rarely involve IgH locus unlike primary initiating events.

1. 2. 7. 1. Mutations involving *Ras*

Mutations affecting *Ras* are consistently less common in MGUS than in newly diagnosed MM and in primary plasma cell leukemia [24]. Activating

Ras mutations are considered to play important role in the transformation of MGUS to MM, at least in the t(11;14) subtype [13]. 40% of newly diagnosed MM patients are found to have *Ras* mutations [28]. Their role in the progression has also been supported by increase in the prevalence of the mutation in treated patients when compared with newly diagnosed patients [29]. Moreover, ectopic expression of *K-ras* or *N-ras* in the interleukin 6 (IL-6)–dependent ANBL6 MM cell line leads to cytokine-independent growth [30]. Ectopic expression of *K-ras* or *N-ras* can lead to constitutive activation of MEK 2 (mitogen activated extracellular kinase 2)/ ERK (extracellular signal-regulated kinase), PI3K (phosphatidylinositol-3 kinase)/Akt, mTOR (mammalian target of rapamycin)/p70S6-kinase (ribosomal protein S6 kinase), and NF-κB (nuclear factor kappa B) pathways in the IL-6-dependent ANBL6 multiple MM cell line [31] and thereby lead to IL-6-independent growth [30].

1. 2. 7. 2. Mutations involving *Myc*:

Translocations involving *Myc* are quite complex and often involve more than two chromosomes. The typical translocation involving immunoglobulin locus found in mouse plasmacytoma [32-34] and Burkit's lymphoma [35] are rarely found in MM. Since, these mutations were found in advanced MM and in even at greater percentage in HMCLs (human myeloma cell lines) they were considered to play role in progression of MM in advanced disease state [36]. However, recent studies support the hypothesis that similar to *Ras* mutations, *Myc* mutations might be co-operatively transform MGUS to MM along with mutations leading dysregulated cell cycle [37].

1. 2. 7. 3. Mutations involving *p53* and *Rb*

Inactivating mutations involving *p53* and *Rb* are not found in MGUS and progressively increase through newly diagnosed MM patients to advanced disease stage indicating that they are not initiating events but are involved in progression of MM [38]. Other inactivating mutations rarely also involve *p15, p18* and *PTEN* (phosphatase and tensin homolog) and their biological consequences are not well determined yet [39].

1. 2. 8. Role of bone marrow microenvironment in the pathogenesis of MM

Normal plasma cells need bone marrow niche for survival. However, genetic events leading to the development of MM alter their interaction with the bone marrow microenvironment [13]. The bone marrow microenvironment can be broadly divided into cellular and non-cellular components. Cellular components include MM cells, BMSCs (bone marrow stromal cells) or bone marrow fibroblasts, hematopoietic precursor cells, osteoclasts, osteoblasts, endothelial cells and immune cells [40, 41]. The BMSCs and osteoclasts provide the MM cells with the ability to grow and survive, either by direct adhesion and/or by secreting growth and survival cytokines [42]. Of these, the supportive role of stromal cells in MM has been studied extensively. The interactions between MM cells and osteoclasts have also been studied to an extent [43].

The non-cellular compartment is comprised of the ECM (extracellular matrix) and the soluble factors. Extracellular matrix consists of various proteins like collagen, fibronectin and laminin. The extracellular matrix not only acts as depots for the growth factors, but also provides the MM cells with the ability to resist cell death induced by chemotherapeutic agents [41]. The survival advantage offered by the bone marrow microenvironment to the MM cells is achieved through the soluble growth factors secreted by various cellular components, insoluble growth factors bound to the extracellular matrix component and adhesion molecules that help MM cells adhere to the extracellular matrix and the cellular compartment [44]. In fact, in a recent study, 22 out of the 51 MM growth factor genes that could be analyzed by affymetrix, were found to be significantly overexpressed by at least one bone marrow environment population in comparison to others [45].

SDF-1α/CXCL12 (Stromal derived factor-1α), secreted by the BMSCs, play an important role in homing of MM cells to the bone marrow, which expresses its receptor CXCR-4 (C-X-C chemokine receptor type 4). Moreover, adhesion of MM cells to stromal cells or fibronectin, induces chemoresistance in MM cells, mediated by integrins [46]. The adhesion molecules namely VLA-4 (very late antigen), VCAM-1 (vascular cell adhesion molecule) and

LFA-1 (lymphocyte function–associated antigen 1), ICAM–1 (intercellular adhesion molecule) mediate integrin-induced chemoresistance [47]. The resistance is mediated partly due to the activation of NF-κB, which upregulates anti-apoptotic gene products [48].

Adhesion of MM cells to the stromal cells induces the latter to secrete IL-6. IL-6 is the main growth factor for the MM cells. In turn IL-6 then induces Janus kinase (JAK)/Signal transducer and activator of transcription3 (STAT3), PI3K/Akt and MAPK (mitogen-activated protein kinase) survival pathways in MM cells [49]. Thus activated STAT3 transcription factor can upregulate the expression of its target genes namely *cyclin D1* and *Mcl-1* (induced myeloid leukemia cell differentiation protein), which promote cell proliferation and anti-apoptosis respectively. In addition to the IL-6-induced activation of STAT3, DNA methylation has also been reported to silence the negative regulators of STAT3 [50]. AKT promotes cell proliferation by phosphorylating GSK3β (glycogen synthase kinase 3β), which regulates cyclin D1 proteolysis [51]. Activated MAPK pathway can further drive MM growth and survival [52, 53]. Numerous novel targeted therapies that act by modulating these diverse players involved in these complex cellular interactions are under investigation.

Diagrammatic representation depicting the interaction between MM cell and the bone marrow microenvironment

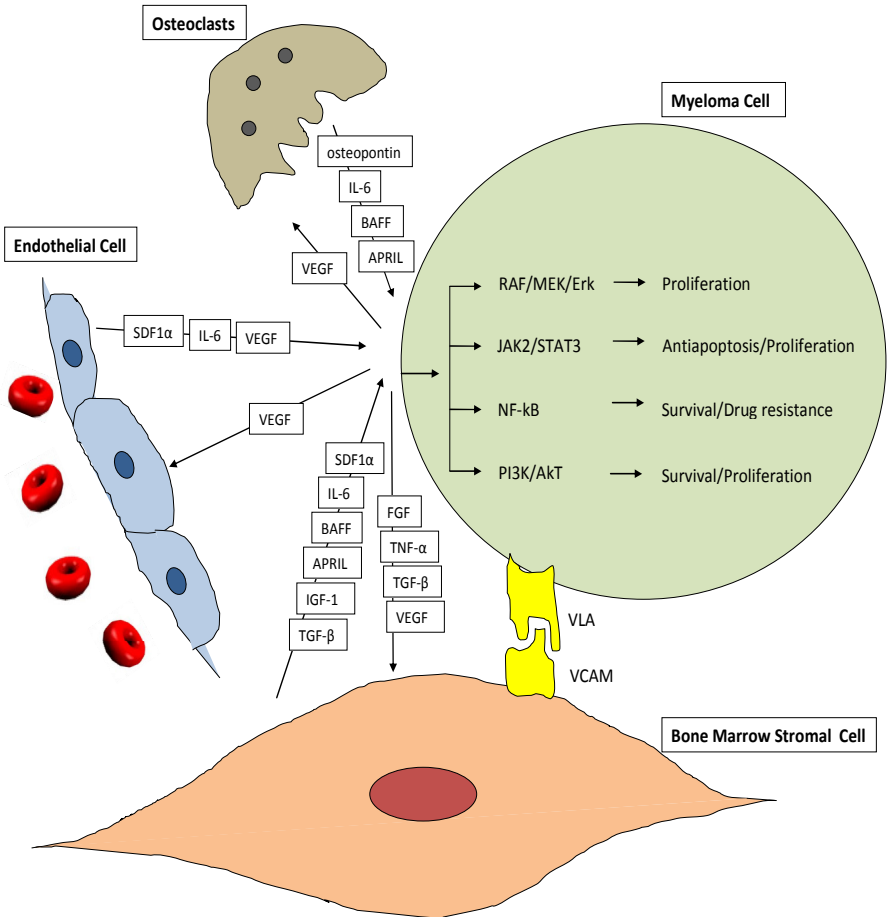


Figure 1.2. Complex interaction between MM cells and BMME

Adhesion of MM cells to the BMSCs induces secretion of various cytokines, chemokines and growth factors mainly by NF-κB-mediated mechanism. Adhesion also confers resistance to MM cells. MM cells further secrete various growth factors which act on BMSCs. Also involved in the reciprocal interaction are osteoclasts and endothelial cells. Adapted from [54].

1.2.8.1. Role of IL-6 in MM pathogenesis

IL-6, a pleiotropic cytokine, is involved in the physiological processes such as haematopoiesis, immunity and inflammation. It was discovered as a factor secreted from mitogen stimulated T cells, which helps mature B cells transform into antibody producing plasma cells [55]. Because of the pleiotropic nature of IL-6, various laboratories have identified its different functions, giving it different names: BSF II (B cell stimulating factor II) for its ability to stimulate B cells to turn into plasma cells and secrete antibodies, interferon-ß2 [56] for it was thought to have the properties of interferon but later it was proven that IL-6 does not have properties of interferon, 26 kDa protein- named after its molecular weight, a hybridoma/plasmacytoma growth factor for it induced plasmacytoma in Balb/c mice injected with mineral oil [57], and a hepatocyte-stimulating factor for it stimulated hepatocytes to produce acute phase proteins [58].

IL-6 binds to its receptor, which is either membrane bound gp130 or in soluble form. It then activates ubiquitously expressed receptor gp130 [59]. Once gp130 gets activated, IL-6 acts by the three of the following signaling pathways: JAK-STAT pathway, MAPK-ERK and PI3K/AKT pathway. Most of the actions of IL-6 are executed by JAK-STAT pathway [60]. IL-6 is also found to be involved in the growth of many solid tumors like prostate cancer and renal cancer [61, 62]. Pathogenesis of Kaposi sarcoma has been proven to be due to dysregulation of the secretion of IL-6 [63].

IL-6 is one of the main growth factors in MM [63]. In fact, it has been found that IL-6 knock out mice fail to develop MM [64]. Moreover, serum level of IL-6 and soluble IL-6 receptor has been proven to be a prognostic marker for tumour load, disease progression and survival [65-69]. Serum levels of IL-6 in patients with smouldering MM and MGUS are comparable with healthy individuals indicating the important role of IL-6 in the disease progression [70].

Initially, it was thought based on the following findings that MM cells secrete and respond to IL-6 in an autocrine manner. Firstly, IL-6 induces *in vitro* growth of freshly isolated MM cells. Secondly, MM cells express the IL-6R (IL-6 receptor). Thirdly, purified MM cells produce IL-6 and lastly, *in vitro*

growth of MM cells is inhibited by anti-IL-6 antibody [71]. But, again controversies prevailed among various scientific groups on the autocrine secretion of IL-6 by MM cells. Because, though all MM derived cell lines and patients cells express IL-6 receptor, only subset of cell lines express IL-6 mRNA [72]. It was also found that BMSCs are the main source of IL-6 [73-75]. Interestingly, when MM cells were co-cultured with BMSCs, they tend to adhere to each other tightly and the IL-6 secretion by these cells reaches the peak. But, when the BMSCs were fixed by paraformaldehyde, there was no increase in the level of IL- 6, confirming that the source of IL-6 was BMSCs and not MM cells.

Moreover, it was found that the stromal cells secrete IL-6 when stimulated by the adhesion of MM cells to the stromal cells. This is evident from experimental set up where these cells were cultured in transwell chambers without any physical contact with the MM cells. As a result, the bone marrow cells failed to secrete IL-6, emphasising the importance of adhesion molecules in the cross talk between group of cells and pathophysiology of MM [76]. The adhesion-mediated secretion of IL-6 was found to be NF-κB-dependent [77]. In addition to BMSCs, adhesion of MM cells to the peripheral blood derived osteoclastic cells protected MM cells from serum deprivation-induced apoptosis and doxorubicin-induced apoptosis. Thus osteoclast-induced survival is also at least partially mediated by IL-6 [78].

IL-6-induced survival of MM cells is mediated by STAT3, which upregulates anti-apoptotic proteins Bcl-xL (B-cell lymphoma-extra large) and Mcl-1 and cell cycle proteins like cyclin D1, c-Myc and proto-oncogene coding for serine/threonine-protein kinase Pim-1. The IL-6-induced proliferation is mediated by MAPK-ERK pathway [53]. PI3K/AKT pathway mediates proliferation and induces survival by phosphorylating Bad and activating cell cycle proteins and NF-κB [79]. Gene expression profiling studies demonstrated that out of 138 genes shown to be regulated by IL-6 in MM cells, 54% regulated cell cycle progression. This finding emphasizes the role of IL-6 in MM cell proliferation [80]. IL-6 was also shown to abrogate Fos-induced apoptosis [81]. IL-6 can inhibit dexamethasone-induced apoptosis of MM cells by gp130 induced activation of SHP-2 (Src homology region 2 domain-containing phosphatase-2) which deactivates RAFTK (RAFTK related

adhesion focal tyrosine kinase) [82, 83] and activates the PI3K/AKT pathway [84]. Partial reduction in the levels of IL-6 can sensitize the MM cells to chemotherapeutic agents [85, 86].

Various strategies including IL-6 antagonist, IL-6 receptor inhibitor (CNTO 328), antisense oligonucleotides against IL-6 and IL-6 super antagonist (SANT7), has been investigated for MM treatment. But, even after effectively blocking IL-6 receptor by the monoclonal antibody, the results were disappointing in clinical trials as a single therapy [87]. Accordingly, in the presence of BMSCs, IL-6 receptor inhibition did not induce apoptosis, indicating the significance of the pleotropism offered by other growth and survival factors present in the bone marrow microenvironment [88, 89].

1.2.8.2. Role of VEGF in MM pathogenesis

VEGF (vascular endothelial growth factor) is a signal protein that stimulates formation of new blood vessels, through vasculogenesis and angiogenesis [90] The activity of VEGF is mediated through three receptor tyrosine kinases: VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1), and VEGFR-3 [91]. VEGFR-1 is expressed on endothelial cells and monocytes and mediates cell motility [92]. The mitogenic activities of VEGF, as well as vascular permeability, are mediated primarily through VEGFR-2 [93]. Lastly, VEGFR-3, is believed to mediate lymphoangiogenesis [94-96]. VEGF isoforms, upon binding to their respective receptors, initiate a tyrosine kinase signaling cascade and initiates the production of distinct growth factors that can stimulate vessel permeability, proliferation/survival, migration and finally differentiation into mature blood vessels [97]. Dysregulation of VEGF has been shown to be a major contributor to tumor angiogenesis as well, promoting tumor growth, invasion and metastasis [98] . Upon stimulation by VEGF, bovine capillary endothelial cells were shown to proliferate and show signs of capillary like tube structures [99]. Significantly elevated levels of VEGF are observed in a variety of hematological malignancies [100-104]. Several studies link VEGF inactivation to observed antitumor effects in various malignancies [105].

Angiogenesis appears to play a key role in hematological malignancies [106]. There is growing evidence that increased bone marrow angiogenesis occurs in MM [107, 108] and is related to disease activity [109, 110] . Angiogenesis in MM also appears to be correlated with the PCLI (plasma cell labelling index) [109]. MVD (micro vessel density) can be measured by histological analysis of tumor samples for markers of angiogenesis and serve as prognostic indicators for various cancers. Interestingly, MVD have been reported to increase five-to-six fold in magnitude during progression from MGUS or SMM to the active MM [111, 112]. Moreover, after chemotherapy, MVD decreases significantly in patients in complete or partial remission [113]. A number of studies implicate dysregulation of VEGF in MM pathogenesis and associated clinical features, including lytic lesions of the bone and immune deficiency. VEGF protein was found in malignant cells from 75% of MM patients studied [114]. Increased serum levels of VEGF have been correlated with a poor prognosis in patients with advanced stages of MM [115]. In fact, Iwasaki *et al.* reports predicted treatment responses and disease progression in MM using serum vascular endothelial growth factor [116]. Another patient study claims that the levels of VEGF, along with FGF, parallel disease activity [117]. VEGF may also affect the immune response in MM patients. Sera from MM patients' bone marrow inhibits antigen presentation by DCs (dendritic cells); conversely, anti-VEGF antibodies neutralized this inhibitory effect, confirming that VEGF can mediate immunosuppression in MM patients [118]. The cytokine is probably involved in the progression of MM to PCL [119]. VEGFR-1 is also widely expressed on both MM cell lines and patient MM cells, confirmed both by RT-PCR (reverse-transcriptase polymerase chain reaction) analyses and immunoprecipitation [120-122].

MM cells release angiogenic factors such as FGF (fibroblast growth factor) and VEGF [112, 117] and are shown to induce angiogenesis *in vivo* in the chick chorioallantoic membrane assay [112]. They secrete MMP-2 and MMP-9 (matrix metalloproteinase-2 and -9) and urokinase-type plasminogen activator [112] and cytokines recruiting inflammatory cells, such as mast cells that then induce angiogenesis through secretion of angiogenic factors in their granules [123]. VEGF is generally present in the bone marrow microenvironment of patients with MM and associated with neovascularization

at sites of MM cell infiltration [124]. The induction of VEGF enhances the microvascular density of bone marrow and accounts for the abnormal structure of MM tumor vessels [125]. VEGF increases both osteoclastic bone-resorbing activity [126] and osteoclast chemotaxis [127] and inhibits maturation of dendritic cells [128]. Marrow neovascularization parallels disease activity in MM and this is postulated to be due to VEGF-induced time- and dose-dependent increase in stromal cell secretion of IL-6, a known MM growth factor [129]. Another cytokine, TNF-α (tumor necrosis factor-α), has been reported to be involved in the control of VEGF production by MM cells [130]. Moreover, VEGF directly, or indirectly through its stimulatory activity on TNF-α and IL-β1 (interleukin- β1), stimulates the activation of osteoclasts and thus contributes to the lytic lesions in MM [126].

Other factors modulating VEGF secretion include Interleukins: IL-1β [131], IL-10 (interleukin -10) and IL-13 (interleukin 13) [132]; secretion of IL-6 [121, 129, 133] or VEGF by both BMSCs and tumor cells (paracrine/autocrine loop); hypoxia and the presence of mutant oncogenes (ie, mutant *Ras* which up-regulate *VEGF* expression); secretion of growth factors such as IGF-1 (insulin like growth factor-1) [134, 135], FGF-4 [136], PDGF (platelet-derived growth factor) [137], TGF-β (transforming growth factor-β) [138], TNF-α [139], and gonadotropins [140]; c-maf–driven expression of tumor integrin β7 [141].

Binding of VEGF to MM cells triggers VEGFR tyrosine phosphorylation, activating several downstream signaling pathways, particularly involving PI3K [142, 143]. PI3K-dependent cascade mediates MM cell migration [142]. This signal transduction pathway is mediated by focal adhesion proteins [144] such as FAK (focal adhesion kinase), paxillin and cortactin, which are responsible for the stabilization of focal adhesion plaques and the reorganization of actin fibers [145]. VEGF also regulates MM cell survival by modulating the expression of Mcl-1 and survivin [146].

MAPK are the final effectors of the signal to the nucleus, thereby activating genes for proliferation, migration and survival [147]. The three well-characterized families of MAPK are ERK1/2, SAPK1 (stress-activated protein kinase-1) and SAPK2/p38 kinases. Treatment of endothelial cells with VEGF

activates both ERK1/2 and SAPK2/p38 MAPK and increases cell migration [148]. This increased migration and cell proliferation is because of the activation of VEGFR-2, since it is totally inhibited by a VEGFR-2 blocking antibody [149]. In fact, MEK-ERK pathway is shown to mediate MM cell proliferation, as evidenced by use of anti-VEGF antibody and PD098059 [120].

Approaches to disrupt the VEGF/VEGF receptor signaling pathways range from small molecule VEGF/ VEGFR inhibitors, anti-VEGF and anti-VEGF receptor antibodies such as bevacizumab [150, 151], and VEGF transcription inhibitors. Of interest are various kinase inhibitors that block the signal transduction mediated by VEGF. The VEGF receptor tyrosine kinase inhibitor PTK787 has been found to be active preclinically and undergoing clinical protocol testing in MM [152, 153]. It acts directly on MM cells to inhibit VEGF-induced MM cell growth and migration and inhibits paracrine IL-6–mediated MM cell growth in the BM milieu. Pazopanib [154], another VEGF receptor tyrosine kinase inhibitor, is also being studied for cancer therapy.

1.2.8.3. Role of TNF-α in MM pathogenesis

In 1894, William Coley, noticed that an injection of bacterial extracts into the tumor, could induce necrosis of tumors [155]. O'Malley et al demonstrated that serum from mice injected with bacterial endotoxins can induce tumor regression [156]. The factor that can induce anticancer activity *in vivo* and *in vitro*, present in the sera of mice treated with endotoxin or LPS (lipopolysacharide) was identified as tumor necrosis factor α [157, 158]. The gene expressing human TNF-α was cloned in 1984 [159]. Thereafter, the recombinant TNF-α was used for experimental and therapeutic purposes. The therapeutic dose of TNF-α induced serious hemodynamic instability and septic shock like symptoms preclinically. TNF-α can induce necrosis of tumor by selective destruction of the blood vessels, only when injected at higher concentrations loco-regionally [160]. Its induction of apoptosis is highly context dependent. Its dysregulation is involved in number of inflammatory and autoimmune conditions including rheumatoid arthritis and Crohn's disease

[161]. Recent research has demonstrated the potent protumorigenic effect of TNF-α [162, 163]. TNF-α KO and TNFα-R1 KO mice do not develop chemical carcinogen-induced skin cancers [164, 165]. TNFα-R1 KO mice do not develop chemical carcinogen-induced liver cancer [166].

In MM, TNF-α also acts as an important growth factor although not as potent as IL-6 [167]. But, it is an important factor secreted from MM cells to act on BMSCs to stimulate the secretion of IL-6. TNF-α induces the expression of adhesion molecules on both MM cells and BMSCs. TNF-α secreted by MM cells acts both directly, and by increasing the adhesion between MM cells and the BMSCs to secrete IL-6 by an NF-κB-mediated mechanism in BMSCs [76, 77, 168]. TNF-α is very potent when compared to other growth factors [169]. TNF-α also participates in transendothelial migration of MM cells by acting via TNF-R2 and upregulating the secretion of MCP-1 (monocyte chemoattractant protein-1) in MM cells [170]. Clinically, the agents which are known to inhibit TNF-α such as thalidomide and other immunomodulatory agents have exhibited significant anti-myeloma activity [171].

1.2.8.4. Role of chemokines in MM pathogenesis

Chemokines are a family of cytokines originally found to be responsible for chemotaxis of inflammatory cells, thus deriving their name 'chemotactic cytokines – chemokines'. They are expressed by injured tissue and inflammatory cells. Thus the resulting chemokine gradient helps in the recruitment of the inflammatory cells towards the site of high concentration of chemokines [172]. They are involved in various biological processes like wound healing, lymphoid recruitment etc. However, their role in angiogenesis, metastasis and cancer progression has also been unraveled [173-176].

In MM, chemokines mainly help homing the MM cells to the bone marrow microenvironment [177]. Their role in proliferation and survival of MM cells can be either direct, or mediated indirectly by inducing the secretion of IL-6, VEGF or any other growth factor involved in the growth and survival of MM cells [178]. The potential role of chemokines, especially that of MIP (macrophage inflammatory proteins), in osteolytic bone lesions is well

established [179]. Homing is defined by transendothelial migration of cells from the blood stream towards the chemokine gradient. This involves adhesion of cells to the endothelial layer, transendothelial migration and eventually residing in the microenvironment. This process is also aided by the chemokine gradient in the bone marrow microenvironment because certain chemokines are said to be present in higher concentrations in bone marrow microenvironment than in bone marrow endothelial cells which ensures that the cells are confined to the bone marrow microenvironment [180].

CXC12 is a member of CXC family of cytokines and its cognate receptor is CXCR4. It has been implicated in progression, migration, invasion and metastasis of various cancers [181]. The role of CXC12/CXCR4 has been well established in the homing of hematopoietic progenitor cells [182-184]. BMSCs secrete this chemokine, with the MM cells from the patient sample and MM derived cell lines expressing the cognate receptors [177].

This chemokine mediates the secretion of IL-6 and VEGF and induces proliferation, migration and inhibits dexamethasone-induced cell death [178]. In the 5TMM mouse model, BMSCs and endothelial cells secrete CXC12 and MM cells express the receptor. *In vitro*, CXC12 induces moderate proliferation of MM cells, which was abrogated by blocking antibody. 5T MM cells migrated towards stromal cell-conditioned medium which was partially inhibited by CXCR4 inhibitor. CXC12 also stimulated MM cells to secrete MMP9. Accordingly, CXC12 induces invasion and the CXCR4 inhibitor inhibits CXC12-induced invasion. *In vivo*, CXCR4 inhibitor decreased the tumor burden and the immediate homing to about 40% [179].

Adhesion molecules and CXCL12/CXCR4 signaling play an important role in the homing and mobilization of hematopoietic stem cells. Among the various adhesion molecules, very late antigen 4 is critical for homing and mobilisation of myeloma cells. Moreover, when the MM cells are mobilized, the CXCL12/CXCR4 axis is generally downregulated. Interestingly, there is also a downregulation in the expression of adhesion molecule very late antigen 4 in the peripheral blood MM cells after mobilization. This results in a suppression of the adhesion of MM cells to the BMSCs, which can be rescued by induction with IL-6 [185]. Moreover, bone marrow endothelial cells are also shown to secrete CXCL12 and induce migration of MM cells towards the bone

marrow endothelial cells. Thus, angiogenesis-induced migration of MM cells is also mediated by CXCL12 chemokine [186]. The expression of CXCR4 was higher in bone marrow plasma cells of patients with MM than in patients with MGUS. Moreover, the bone marrow plasma of MM patients has higher CXC12 levels than that of peripheral blood of MM cells and bone marrow plasma of healthy individuals [187]. Consistent with its effect on migration, invasion, homing, proliferation and survival, CXCL12/CXCR4 axis can also induce activation of MAPK/ERK, AKT, PKC (protein kinase C) and NF-κB pathway [178, 187].

1.2.8.5. Role of MMPs in MM pathogenesis

MMPs belong to a family of proteases, capable of degrading all kinds of extracellular matrix proteins [188]. In 1962, Gross and colleagues discovered MMP, when they found collagenase activity in the tail of a tadpole during metamorphogenesis [189]. These proteins function not only to remodel the extracellular matrix, but also are involved in the cleavage and thereby activation and inactivation of various biologically significant proteins like chemokines and growth factors [188]. In the context of cancer, both the cancer cells and stromal cells secrete MMPs. Their involvement in invasion and metastasis was examined in various clinical models. Recent evidences suggest the role of MMPs in various hall marks of cancer progression [190].

Culture supernatants of bone marrow derived stromal cells from MM patients were found to have higher levels of MMP-1 and MMP-2 compared to the control samples [191]. Moreover, endothelial cells secrete hepatocyte growth factor, which acts on MM cells to stimulate the secretion of MMP-9 [192]. In addition, 5T mouse model of MM bone marrow expresses various distinct MMPs such as MMP2, MMP8, MMP9 and MMP13. Adequate inhibition of these MMPs by a broad spectrum MMP inhibitor SC-964 can suppress angiogenesis, reduce tumor load and osteolytic lesions [193].

1.2.8.6. Role of adhesion Molecules in MM pathogenesis

Adhesion molecules mediate cell-cell and cell-ECM interactions [194] and are also involved in intracellular signaling after engagement with their receptors. Broadly, there are five groups of adhesion molecules. They are 1) the integrins-mediating cell-ECM and cell-cell adhesion 2) the cadherin family- mediating homotypic cell-cell adhesion 3) the selectin family-mediating heterotypic cell-cell adhesion 4) the immunoglobulin superfamily-mediating cell-cell adhesion and 5) other transmembrane proteoglycans such as CD44 adhesion molecules and syndecan that mediate cell-extracellular matrix adhesion [47]. Dysregulated expression or function of adhesion molecules are involved in various steps of cancer progression [195].

In MM, adhesion molecules mediate homing of MM cells to the bone marrow, secretion of cytokines and growth factors and development of chemoresistance [46, 196]. Out of all the adhesion molecules, VLA-4 and VLA-5 expressed by the MM cells play a crucial role in the MM pathogenesis [197]. VCAM-1 and fibronectin are the receptors for VLA. VLA adheres to the BMSCs by binding to VCAM, CS-1 fragment and H1 region of fibronectin [198]. Inhibition of VLA using blocking antibodies can abrogate the adhesion of MM cells to the BMSCs and fibronectin [199]. VLA-dependent adhesion to the bone marrow, is regulated by the CXCL12/CXCR4 axis [200]. This is further supported by the finding that disruption of CXCL12/CXCR4 axis results in downregulation of VLA-4 and decreased adhesive capacity in the mobilised MM cells when compared to pre-mobilization bone marrow MM cells [185].

VLA-dependent adhesion of MM cells to the BMSCs induces secretion of IL-6 by an NF-κB-mediated mechanism [76, 77]. Drug-sensitive 8226 human MM cells, expressing both VLA-4 and VLA-5 receptors, are relatively resistant to the apoptotic effects of doxorubicin and melphalan, when pre-adhered to fibronectin and compared with cells grown in suspension. Upon exposure to chemotherapeutic agents, MM cells expressing high level of VLA-4 have survival advantage than those that express them at low level [168]. It has shown that adhesion of MM cells to fibronectin activates NF-κB and its regulated gene products, leading to drug resistance [48]. It is also possible that fibronectin augments IL-6-induced STAT3 activation [201].

1.2.8.7. Role of JAK/STAT3 pathway in MM pathogenesis

STAT3 is a member of the STAT family of transcription factors. STAT family proteins were first discovered in the context of the specificity of the IFN signaling [202]. STAT3 was first described as a DNA-binding factor, in IL-6 stimulated hepatocytes, capable of selectively interacting with an enhancer element in the promoter region of acute-phase genes [203]. Later, it was found that STAT3 can be activated by the entire IL-6 family of cytokines and some other growth factors. STAT3 has diverse functions in various biological processes [204, 205]. STAT3 is involved in the induction of an acute-phase response in hepatoma cells, stimulation of proliferation in B lymphocytes, activation of terminal differentiation, growth arrest in monocytes and maintenance of the pluripotency of embryonic stem cells [206].

Soon after its discovery, the oncogenic role of STAT3 has been established by the presence of constitutively activated STAT3 in cancer cell lines. STAT3 is constitutively phosphorylated in v-Src–transformed cells and has been found to be necessary for the v-Src-induced carcinogenesis [207, 208]. Expression of a constitutively active version of STAT3 on its own, can lead to fibroblast transformation, showing that STAT3 is an oncogene [209]. In addition to its role in cellular transformation, its role in various stages of cancer progression has been established [210]. Genes regulated by STAT3 that are involved in proliferation and growth, include *c-myc, p21, cyclin D1, Bcl-xL* [211]. STAT3-mediated angiogenesis is mediated by VEGF; STAT3 also regulates MMP family members MMP2 and MMP9 [212]. STAT3 is vital for development, as evidenced from STAT3 knock out mice which succumb to embryonic lethality [213]. This observation is critical for the development of therapeutic strategies with higher therapeutic index.

Unlike NF-κB pathway there are no activating mutations involving JAK/STAT3 pathway. However, around 48% of MM patient samples have constitutively activated STAT3 [214]. Dysregulated STAT3 activation in MM is mainly thought to be due to the aberrant secretion of cytokines in the bone marrow microenvironment by both MM cells and BMSCs [215]. SOCS1 (suppressor of cytokine signaling1) is important for the differentiation of normal lymphocytes. It negatively regulates JAK/STAT3 pathway induced by

various cytokines including IL-6 and 4, leukaemia inhibitory factor, oncostatin M, and interferon. In a study, almost 60% of MM patients had hypermethylation of *SOCS1* gene. However, in normal peripheral blood lymphocytes and in bone marrow cells there were no epigenetic silencing [216]. Moreover, epigenetic silencing of SOCS1 was also strongly associated with disease progression and poor survival in MM patients [217]. Overexpression of SOCS1 in IL-6-dependent MM cells induced apoptosis, implicating the importance of negative role played by SOCS in MM cell survival [218]. In another study, almost 79% of MM samples had hypermethylation of SHP-1 (Src homology region 2 domain-containing phosphatase-1). SHP-1 is a phosphatase that negatively regulates JAK/STAT pathway by dephosphorylating STAT3 [50]. Although there is no strong evidence for the presence of activating mutations involving JAK/STAT3 pathway to date in MM, it is conceivable from the above studies that presence of constitutively activated STAT3 in MM samples can at least be partially explained by the epigenetic silencing involving the negative regulators of JAK/STAT3 pathway.

In MM, STAT3 plays an important role in survival. It can upregulate antiapoptotic proteins like Bcl2, Bcl-xL and Mcl-1 [219-221]. Constitutive expression of STAT3 confers MM cells resistance to apoptosis [222]. Out of all the antiapoptotic proteins regulated by STAT3, Mcl-1 seems to be more important. While antisense inhibition of Bcl-xL did not inhibit survival, knock down of Mcl-1 was sufficient to inhibit survival in MM cells. Overexpression of Mcl-1 was able to promote proliferation of MM cells lines even in the absence of IL-6 [223]. Knock down of Bcl-2 can augment dexamethasone-induced apoptosis [224]. But again, the importance of STAT3 in regulating the anti-apoptotic proteins and thereby the survival of MM cells remains controversial in the light of a lack of correlation between the constitutive expression of STAT3 and the anti-apoptotic proteins [214]. However, it is clear that STAT3 is not the only factor which regulates the survival of MM cells because MM cells become independent of IL-6-gp130-STAT3 pathway in the presence of BMSCs [89]. There are lot of therapeutic strategies that are being developed to target JAK-STAT3 pathway in MM. In fact, the novel agents that are being used nowadays namely; thalidomide and its derivatives and bortezomib, act partially to disrupt the NF-κB-induced activation of IL-6 and

thereby STAT3 activation. Numerous drugs that inhibit IL-6-JAK-STAT3 pathway at various levels induce apoptosis both *in vitro* and *in vivo* [225-257].

1.2.8.8. Role of NF-κB in MM pathogenesis

NF-κB is a Rel family of transcription factors consisting of p50, p52, c-Rel, p65/RelA, and RelB subunits [258, 259]. It was discovered by Dr. Baltimore and colleagues in 1986 as a DNA binding protein, recognising specific sequences in the immunoglobulin kappa light chain joining (J) segment gene region in B cells [260]. NF-κB family of transcription factors consists of Rel (c-Rel), RelA (p65), RelB, NF-κB1 (p50 and its precursor p105), and NF-κB2 (p52 and its precursor p100) [261]. There are two pathways that lead to the activation of NF-κB. In canonical pathway, IKK-β (inhibitor of IkappaB kinase-β) activates the pathway by phosphorylating the negative regulator of p50/p65 subunits namely IκB-α (inhibitor of kappa B-α), thereby leading to the proteasomal-dependent degradation. In non-canonical or alternative pathway, NF-κB inducing kinase (NIK) activates this pathway by phosphorylating IKK-α (inhibitor of IkappaB kinase-α) which in turn phosphorylates p100. P100 is then processed to active p52 by proteasome-dependent degradation of its inhibitory C-terminal inhibitory protein domain IκB-γ [262]. Canonical pathway is activated through TRAF 5, TRAF2 (TNFR-associated factors), RIP (receptor-interacting protein) kinase and TAK1 (TGF-beta activated kinase 1) and this pathway is involved in immune response and inflammation. Canonical pathway is negatively regulated by CYLD (cylindromatosis) and A20 proteins. The alternative pathway is involved in B cell maturation. Accordingly, it is activated by cytokines, including CD40L, LT-aß (lymphotoxin-aß), BAFF, RANKL (receptor activator of NF-κB ligand), and TWEAK (TNF-related weak inducer of apoptosis). This pathway is mainly regulated by stability of NIK, in which TRAF 3, TRAF 2 and cIAP1/cIAP2 (cellular inhibitor of apoptosis 1 and 2) are critically involved [263]. However, both pathways often overlap and interplay [264]. Physiologically NF-κB functions to regulate immunity and inflammation [265]. NF-κB transcription factor regulates various players involved in inflammation including the expression of adhesion molecules both in leucocytes and endothelial cells [266,

267], recruitment of neutrophils [268], and the production of inflammatory mediators such as iNOS (inducible nitric oxide synthase) [269], prostaglandins [270-272], MMPs [273-275].

Schematic diagram of NF-κB activation pathway

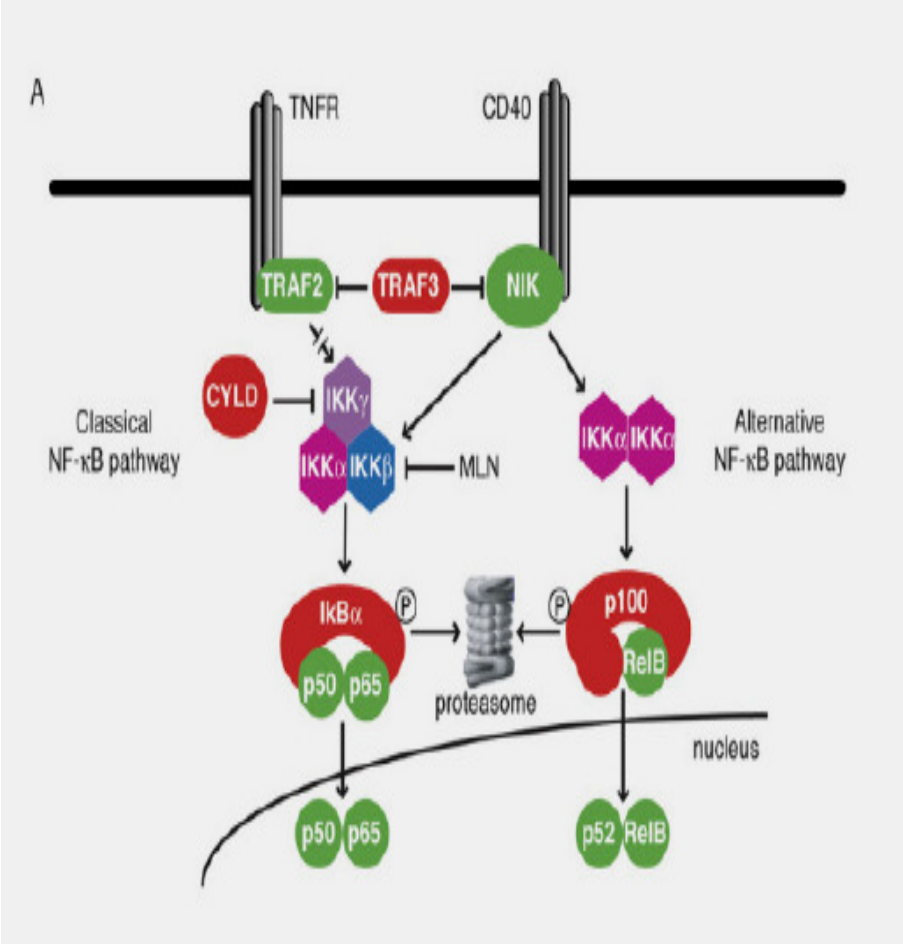
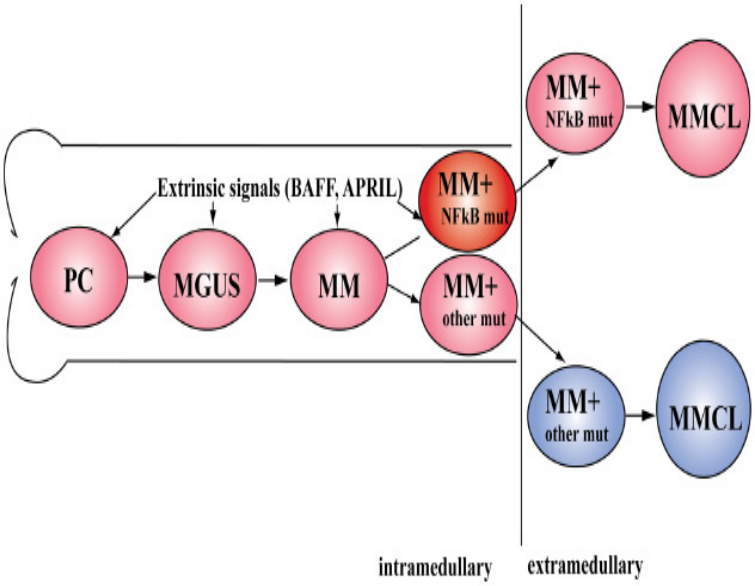


Figure 1.3. Schematic diagram of NF-κB activation pathway

Classical pathway is mediated by IKK-β and results in activation of p50/p65 subunits followed by proteasome-dependent degradation of IκB-α. Conversely, alternative pathway is mediated by IKK-α and results in activation of P52/RelB subunits followed by proteasome mediated-degradation of C-terminal inhibitory domain IκB-γ. Adapted from [276].

Model of NF-κB activation in normal and malignant plasma cells



The colors correspond to the different levels of the NF-κB activity

low activity-blue
high activity due to extrinsic or mutational activation - pink
highest activity due to extrinsic plus mutational activation -red

PC- plasma cell
MMCL - multiple MM cell line

Figure 1.4. Model of NF-κB activation in normal plasma cells and malignant plasma cells

In normal plasma cells NF-κB pathway is activated by extrinsic signals from the bone marrow microenvironment. However, in malignant plasma cells this

pathway is activated by extrinsic signals and/or by mutations. Adapted from [263].

NF-κB pathway is constitutively activated in many cancers either due to the mutations or dysregulation of cytokines involved in the activation of NF-κB pathway [277]. Likewise, the role of NF-κB in various stages of tumor progression has been identified [278, 279]. NF-κB has been casually implicated in progression of various types of tumors [280]. Selective deletion of NF-κB in hepatocytes, or inhibition of TNF-α production by neighbouring parenchymal cells, induced programmed cell death of transformed hepatocytes and reduced the incidence of liver cancer [281]. In colitis-associated colon cancer model, NF-κB affects both tumor cells and inflammatory stromal cells to induce and promote cancer. In enteroctyes, NF-κB inhibits apoptosis and in inflammatory cells stimulates the secretion of various mediators of inflammation, which in turn act on enteroctyes to initiate cancer [282]. However, in some tissues, NF-κB prevents cancer. For example, inhibition of NF-κB in keratinocytes leads to squamous cell carcinoma of skin [283]. Many studies have shown that the NF-κB signaling pathway plays an important role in anti-apoptosis and the drug resistance of tumor cells during chemotherapy. First, many chemotherapeutic drugs and radiotherapy induce NF-κB expression *in vitro* and *in vivo* [284, 285]. Second, activation of NF-κB rescues cells from chemotherapy-induced apoptosis [286]. Third, induction of chemoresistance and radioresistance is mediated *via* genes regulated by NF-κB [287]. Fourth, inhibition of NF-κB and NF-κB-regulated gene products increases the sensitivity of cancer cells to apoptosis induced by chemotherapeutic agents and to radiation exposure [288, 289].

NF-κB pathway is constitutively activated in normal plasma cells. Indeed, normal plasma cells shows the highest NF-κB among the all other B cells, mainly due to BAFF-, APRIL- mediated signaling from the bone marrow [290]. However, MGUS MM cells show even higher activation and in these cells it is due to the combined effect of extrinsic signaling stimuli from cytokines present in the BMME and also caused by activation due to mutations. Eventually, activating mutations of NF-κB pathway helps the MM cells become independent of the bone marrow, as they overcome the need for

external cytokines to activate the pathway [291]. MM patient samples show a constitutive activation of NF-κB to variable degree. Soluble cytokines belonging to TNF-α super family including TNF-α, BAFF (B-cell activating factor), APRIL (a proliferation inducing ligand), LTβ (lymphotoxin β) are known to activate NF-κB and are present in the bone marrow microenvironment. Adhesion of MM cells to the BMSCs and osteoclasts also activates NF-κB pathway in MM cells and osteoclasts and BMSCs [292]. These cytokines are said to activate NF-κB mainly by the canonical pathway. 15-20% of MM samples and 40% of the cell lines show mutations that lead to constitutive activation of NF-κB pathway [276, 291, 293]. There could also be some unidentified genetic mutations or epigenetic modifications that might explain the constitutive activation. Gain of function mutations involve receptors known to activate NF-κB namely *CD40, LTβR, TACI* (tumor necrosis factor receptor superfamily member 13B) or direct regulator of the non-canonical pathway *NIK* (NF-κB-inducing kinase), and also direct mutations involving *NF-κB1 p50/p105, and NF-κB2 p52/ p100*. Loss of function mutations involve negative regulators of NF-κB activation namely *TRAF3, TRAF2, CYLD and cIAP1/cIAP2,* inactivation of *TRAF3* being the most common. Also, *CD40, LTβR, TAC1* receptor overexpression may be sufficient to activate the NF-κB pathway or might enhance the sensitivity of MM cells to factors in the tumor microenvironment to activate NF-κB. Overexpression of *NIK or NF-κB1 p105* directly leads to constitutive activation of NF-κB. Deletion of sequences in the *p100 IκB*-like domain of *NF-κB2*, promotes processing of p100 to p52 and activation of the alternative NF-κB pathway [276, 293]. Though mutations mainly involve the alternative pathway, the canonical pathway is also activated. Mutations that selectively involve either the canonical or the non-canonical actually lead to activation of both the pathways and gene expression profiles induced by these pathways are quite similar if not the same. This could be because once dysregulated the canonical or the alternative pathways may no longer relay the signal in orderly fashion [264]. Though, conventionally NIK was thought to activate the alternative pathway, NIK activation due to mutations mainly activate the classical pathway suggesting that IKK-β is a good target to inhibit consequences of deregulated NF-κB pathway activation [276]. However, total NF-κB activity is

the combined activity of both the canonical and the alternative pathways. Also proving the role of the alternative pathway in the pathogenesis of MM is the development of plasma cell tumors in the *NF-κB2* mutant *p80HT* transgenic mice model [294]. Thus dual inhibition of both the canonical and the alternative NF-κB pathway may offer greater anti-myeloma activity [295].

Activation of NF-κB in MM cells induces proliferation, survival and chemoresistance. When compared to chemosensitive MM cell lines chemoresistant MM cells express higher levels of NF-κB, suggesting a link between NF-κB and development of chemoresistance [286, 296]. Moreover, dexamethasone-induced apoptosis is associated with a decrease in the NF-κB DNA binding activity. Interestingly, NF-κB can also serve as a prognostic indicator for response to dexamethasone. Only patients who responded to dexamethasone, demonstrated decreased NF-κB DNA binding activity in their samples. Enforced ectopic expression of Bcl-2 in MM cells conferred resistance to dexamethasone-induced apoptosis, and this was also associated with enhanced NF-κB DNA binding [286]. Certain cancer cells when in contact with cell or extracellular matrix display substantial multi-drug resistance. This phenotype is called cell adhesion-mediated drug resistance (CAM-DR). The resistance is conferred by diverse mechanisms including alteration of intracellular target, upregulation of antiapoptotic proteins and increased p27 levels. In MM cells, NF-κB pathway activation plays a major role in cell adhesion-mediated drug resistance [48]. Alkylating agents used in the treatment of MM namely melphalan and doxorubicin can also induce the NF-κB activation in HMCLs. Interestingly, NF-κB activation was inhibited when bortezomib was added, suggesting that bortezomib sensitizes MM cells to alkylating agents by inhibiting NF-κB activation [297]. Inhibition of NF-κB by either proteasome inhibitor or inhibitors of IκB phosphorylation induces apoptosis in HMCLs. IKK inhibitor can abrogate the protective effect of IL-6 on dexamethasone- induced apoptosis. It also potentiated TNF-α-induced apoptosis in MM cells. NF-κB inhibition abrogated the TNF-α-induced upregulation of ICAM-1 both in MM cells and in BMSCs. It also inhibited the MM cell adhesion-induced IL-6 secretion by BMSCs and resulting proliferation of MM cells. These findings indicate that pro-survival functions of bone marrow microenvironment is abrogated upon NF-κB inhibition [298].

The novel therapeutic agents namely bortezomib, a proteasome inhibitor and thalidomide and its immunomodulatory derivatives also act at least partially by inhibiting NF-κB [290].

1. 2. 9. MM associated bone disease

Most of the cancers that metastasize to bone form osteolytic lesions, which are followed by new bone formation. However, in MM there is increased osteolysis and decreased new bone formation resulting in pure osteolytic lesions [299]. Consequently, patients with MM often present with bone fractures and is in fact an important cause of increased morbidity in MM [300]. MM cells increase the osteoclast formation and activation leading to osteolytic lesions. Conversely, osteoclast cells secrete many cytokines which provide survival advantage to MM cells. Thus, there is a symbiotic relationship between osteolysis and MM. Thus, MM bone disease is not only a complication of MM but also a contributing factor to the progression of MM [301]. There are many factors involved in MM bone disease pathogenesis. The TNF receptor super family members RANK (receptor activator of NF-κB)/RANKL/ OPG (oteoprotegerin) system plays an important role in the osteoclast activation. RANK is secreted by BMSCs and osteoblastic cells which act on its cognate receptor RANKL which is present on the osteoclastic cells to stimulate bone resorption. The soluble decoy molecule osteoprotegerin secreted by BMSCs and osteoblasts bind to RANK to inhibit its interaction with RANKL [302]. In addition to the RANK; VEGF, CXCL12/CXCR4 and MIP-1α also plays roles in osteoclastic activation. Factors leading to osteoblastic inhibition are inhibition of Wnt signaling, TGF-β, Runx2, IL-3 and HGF (hepatocyte growth factor) [303]. Apart from surgical intervention and radiation, there have been many improvements in the pharmacotherapy of MM bone disease including the advent of bisphosphonates which inhibit osteoclastic activity . Recent improvement in the understanding of MM bone disease has lead to the development of novel targeted therapies which are in various phase of clinical trials [304]. Interestingly, novel anti-myeloma agents including thalidomide and bortezomib also inhibit bone resorption by

inhibiting interaction of various players in the bone marrow microenvironment [305].

1. 2. 10. Treatment options

Treatment of MM is based fairly on whether the patient is eligible for bone marrow transplantation. Generally patients below 65 yrs old with no comorbidities are treated with non-melphalan based high dose chemotherapy followed by autologous stem cell transplantation. Those who do not qualify for stem cell transplant are treated with combination chemotherapy [306]. Common front line agents used in the induction therapy of MM are either two drug or three drug combinations of melphalan, dexamethasone, thalidomide, lenalidomide and bortezomib [307]. The treatment of relapsed and refractory MM has been updated with the approval of carfilzomib a new proteasome inhibitor by FDA (Food and Drug Administrative Agency). The regimens available for the treatment of relapsed and refractory MM are carfilzomib, bortezomib-thalidomide-dexamethasone combination, lenalidomide-bendamustine-dexamethasone combination [308]. Pomalidomide, a third generation immunomodulatory agent has also been recently approved by the FDA for the treatment of relapsed and refractory MM [309].

1. 2. 11. Drugs in the clinical trials

A number of pharmacological agents are being currently investigated for MM treatment. For example, histone deacetylase inhibitors vorinostat and panobinostat are in the phase III clinical trial in combination with bortezomib. Perifosine, a PI3K/Akt pathway inhibitor is in phase III clinical trial in combination with bortezomib. Elotuzumab, a monoclonal antibody against the surface-expressed glycoprotein CS-1 is in phase III clinical trial in combination with lenalidomide. As a survival mechanism, bortezomib-treated cells are found to activate heat shock response proteins; therefore, HSP 90 inhibitors are rational drugs to be tried in combination with bortezomib. These drugs are likely to be approved in the near future and expected to expand the armamentarium of anti-myeloma agents [307]. Apart from the above

mentioned drugs, there are several other drugs in clinical trials including but not limited to those that target the signaling cascades involved in the pathogenesis of MM, monoclonal antibodies against the receptors that are expressed over the cell membrane of plasma cells and various players involved in the MM bone disease [310]. However, there remains an urgent need to develop novel anti-myeloma agents because despite the availability of various anti-myeloma agents MM still remains incurable. Most of the patients relapse after initial response or develop chemoresistance. Moreover, most of the available drugs have severe dose-limiting toxicity including but not limited to bone marrow suppression, peripheral neuropathy, and reactivation of herpes zoster infection [310].

1. 2. 12. MM animal models

Biologically relevant animal models are crucial in understanding the biology, evaluating potential targets and novel therapies of any disease. These preclinical models enable us to quickly translate the results obtained to clinical studies [311]. Many types of animal models of MM have been used so far, each having its own advantages and disadvantages. Subcutaneous xenograft of MM in immunocompromised mice is the most commonly used model. They are relatively easy to establish and allow easy monitoring of the tumor volume. However, since the tumor in this model grow in subcutaneous microenvironment, this model does not account the role of bone marrow microenvironment in MM cell survival, proliferation and drug resistance. In addition, they do not form the typical MM bone disease. So, drugs evaluating MM bone disease cannot be effectively studied using this model [312]. Inspite of these limitations, it is interesting that many agents that have been approved recently by FDA for the treatment of MM have been tested in these model before they were brought into clinical trials [313]. SCID (severe combined immunodeficiency)-human model overcomes the disadvantages of the subcutaneous xenograft model. In this model, human foetal bone is transplanted bilaterally into the SCID mice and MM cells are injected into the human foetal bone marrow of one side. The MM cells infiltrate the human bone marrow and eventually metastasize to the foetal bone of the other side.

This model offers the advantage of addressing the role of bone marrow microenvironment in disease biology [314]. Interestingly, the effect of thalidomide on the MM cell-bone marrow microenvironment has been studied using the SCID-Human model [315]. SCID-rabbit model resembles the SCID-Human model, except that rabbit bone tissue is used to avoid the ethical issues of using human foetal bone tissue [316]. To mimic the characteristic of MM in inducing multifocal osteolytic lesions in the skeletal system, MM cells were injected intravenously into the immunocompromised mice and these cells lodged into the mouse bone marrow and produced characteristic lesions in the bones [317]. Many anti-myeloma agents have been evaluated using this model including humanized-anti-IL-6 receptor antibody [317]. When fresh core bone marrow biopsies of patients were engrafted en bloc into the hind limb muscles of the mice, 3 of the 33 such patients' samples developed palpable tumors. These three tumors was further passaged in mice by intramuscular injections. One of these was derived from cells of an IgGλ-1producing patient who had MM, was used for further studies. When these cells were injected into the mice subcutaneously or intramuscularly they formed plasmacytomas and this animal model is described as LAGλ-1 model [318]. Aged C57BL/KaLwRij mice spontaneously develop MGUS and MM like features. When the these murine MM cells were injected into the young syngenic mice, the young mouse develop same features. This model is known as 5T MM model. This model differs from other mice models from its immunocompetent state. Moreover, this model has shed light into the involvement of cytokine, adhesion molecules in MM biology and offered better understanding of MM bone disease. In fact, the effects of bisphosphonates were evaluated using this model [312]. Transgenic mouse models of MM include AID (activation-induced deaminase) to a conditional *Myc* transgene leading to the development of plasma cell tumor resembling human MM [319] and *p80HT* transgenic mice model induced by *NF-κB2* mutant *p80HT* [294], which lead to the development of plasma cell tumor resembling human MM. Thus, transgenic models are invaluable in helping us dissect how each molecule is involved in the pathogenesis of MM.

1. 3. Identification of celastrol as an anti-cancer agent

Interest in medicinal plants has been gaining momentum in the last few decades. Various native medicinal systems like Ayurveda (India) and Chinese natural medicine are based on plant derived medicinal products [320]. These plant derived medicinal compounds are good source of alternative therapy because of their effectiveness in various diseases and better understanding of their mechanisms of action nowadays [321]. *Trypterigyum wilfordii* commonly known as 'Thunder of God Vine' is a perennial creeper plant seen in China. The extract of this plant has been used in Chinese natural medicine for inflammatory conditions like arthritis, fever and edema. Numerous studies have been conducted with this extract using various *in vitro* and *in vivo* models. The beneficial effects of the this extract were tested in clinical trials conducted in patients with rheumatoid arthritis [322]. The extract has been found to contain numerous active compounds including triterpenoids, diterpenoids, alkaloids etc. Celastrol, a quinone methide triterpenoid, is one among these compounds, derived from the root bark extracts of *T. wilfordii*. Celastrol initially attracted attention because of its anti-inflammatory effects, though it was also found to have antimicrobial activities [323-325]. The effects of celastrol have been investigated in various models of inflammation. Interestingly, in the last few years, celastrol has been extensively studied for its potential anticancer effects in various *in vitr*o and *in vivo* cancer models [326].

Chemical structure of celastrol

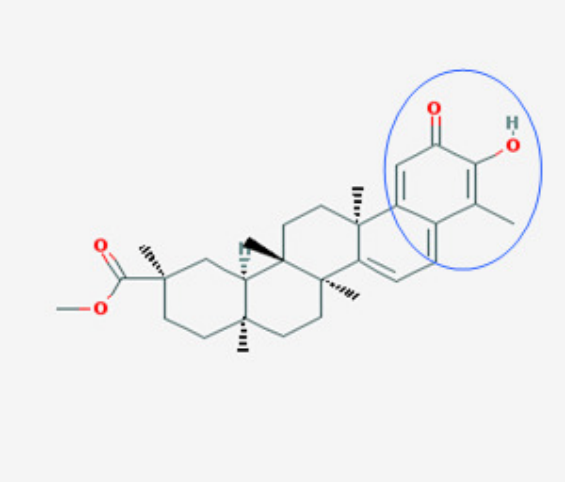


Figure 1.5. Chemical structure of celastrol

The thiol reacting quinone methide ring is circled in blue.

Sources of Celastrol

Celastrus orbiculatus

***Tripterygium wilfordii (*Thunder of God Vine)**

Figure 1.6. Biological sources of celastrol

1.3.1. Identified key molecular targets of celastrol

1.3.1.1. Proteasome

The ubiquitin-proteasome pathway functions to degrade cellular proteins. The 26S proteasome is made of two 19S regulatory particles and a 20S coreparticle. The 20S core particle is a multicatalytic threonine protease with at least three distinct catalytic activities which are chymotrypsin-like, trypsin like and PGPH (peptidylglutamyl peptide-hydrolyzing)-like activities. By acting as a tag, a chain of ubiquitin can target a substrate protein to the 26S proteasome for destruction [327]. The ubiquitin/proteasome-dependent pathway is known to degrade many endogenous proteins including transcription factors, cyclins, tumor suppressor proteins and signaling proteins involved in tumorigenesis and also misfolded and damaged proteins. Several studies have shown that inhibition of the proteasomal activity resulted in accumulation of several target proteins like IκB-α, Bax (Bcl-2–associated X protein), and p27 [328-331] and induction of apoptosis in various types of cells

[332, 333]. Proteasome is considered to be an attractive target for the treatment of cancer. Indeed, small molecule inhibitor bortezomib or velcade, a proteasome inhibitor has already been approved by US FDA for the treatment of MM [334]. Additionally, more potent, less toxic proteasome inhibitors are being investigated [335].

Celastrol has been shown to inhibit proteasome in *Xenopus laevis* A6 cells [336], human prostate cancer cells [337] RAW264.7 macrophage cells [331] and rat glioma cells [338]. Celastol-induced inhibition of proteasome was associated with upregulated expression of heat shock protein genes *hsp30 and hsp70* [336]. Celastrol did not seem to inhibit the binding of ubiquitin to protein chains by binding to ubiquitin moieties [331]. However, celastrol has been found to inhibit chymotrypsin like activity of proteasome in RAW264.7 cells [331] prostate cancer cells [337] and rat glioma cells [338]. This inhibiton of chymotrypsin activity was associated with accumulation of proteasome targets like Bax, IκB-α and p27 and was accompanied by apoptosis [337].

1.3.1.2. HSP (heat shock response protein) 90

HSR (heat shock response) is one of the conserved molecular pathways which is induced in response to various cellular stresses and rescues cells from subsequent and more lethal stresses. In response to heat, the heat inducible transcription factor HSF-1 (heat shock factor-1) is activated. Upon activation, HSF-1 moves to nucleus, binds to DNA and gets hyperphosphorylated to modulate the expression of various genes involved in HSR including HSP90 [339, 340]. Most of Hsp90 client proteins including HER-2 (human epidermal growth factor receptor-2), Akt, Cdk 4 (cyclin-dependent kinase 4), Bcr-Abl (Breakpoint cluster region-Abelson murine leukemia viral oncogene homolog 1), p53, and Raf-1 (rapidly accelerated fibrosarcoma -1) play crucial role in the pathogenesis of various cancers. Targeting molecular chaperone Hsp90 might offer many advantages for cancer therapy by simultaneously modulating various key players involved in the pathogenesis of cancer [341].

Celastrol has been shown to inhibit HSP90, its co-chaparones and their interactions [342-345]. Celastrol differed from conventional HSP90 inhibitors in the fact that celastrol did not act by binding to ATP binding site of HSP90.

In fact, celastrol did not compete with geldanamycin which is known to inhibit HSP90 by binding to ATP binding pocket. Consequently, celastrol can synergize with conventional HSP90 inhibitors in inhibiting HSP90 because of different mechanisms of action [343]. Celastrol's ability to inhibit HSP90 client kinases are found to be mediated by the inhibition of interaction between HSP90 and the co-chaparone cdc37 [342, 346]. However, Sreeramulu and coworkers have demonstrated using NMR spectroscopy and chemical mutational analysis that celastrol can directly bind to cdc37 but not to N-terminal domain of HSP90 to inhibit HSP90-cdc37 interaction [347]. Moreover, Zhang and coworkers have demonstrated that celastrol can inhibit HSP90's client transcription factors by inhibiting the interaction between HSP90/Hop and HSP90/transcription factors, indicating that celastrol's ability to inhibit HSP90 clients is not only mediated by the inhibition the co-chaparone cdc37 but mediated by other cochaperones as well [348]. Along this line, celastrol has also been demonstrated to inhibit the interaction of HSP90 and its co-chaperone p23 in breast cancer SKBR-3 cells [343] and in HeLa cells [344] though in pancreatic cancer cell lines HSP90-P23 interaction was found to be unaffected [342]. Celastrol-induced inhibition of HSP90 and/or its cochaperones lead to cell cycle arrest in monocytic leukemia U937 cell line [346], induced paraptosis and apoptosis in HeLa cells [349] and caused radiosensitization in lung cancer NCI-H460 cell line [350].

Celastrol has been shown to activate heat shock response in human breast cancer MCF7 and BT474 cell lines, human non-small cell lung carcinoma H157 cell line, human neuroblastoma SH-SY5Y cell line and HeLa cell line, *Saccharomyces cerevisiae* yeast and in the amphibian *Xenopus laevis* A6 cells [336, 351-353]. Furthermore, celastrol synergistically induced heat shock response, in combination with mild heat and proteasomal inhibitor MG132 [336, 351]. Celastrol's ability to induce heat shock response has been shown to offer cytoprotection in various other model systems [351, 354-356]. Celastrol's ability to induce heat shock proteins is regulated at transcriptional level because, celastrol upregulated HSF-1 in L444P fibroblasts [357] and celastrol has also been found to induce DNA binding and hyperphosphorylation of HSF-1 in yeast and mammalian cells [351, 352]. Moreover, celastrol was unable to upregulate HSP70 accumulation in *HSF1–/–*

mouse embryo fibroblast cells [358] and celastrol-induced upregulation of HSP 30 and HSP 70 were abrogated by the HSF-1 activation inhibitor KNK437 [336].

UPR is a stress pathway induced by cells when cells are loaded with unfolded proteins by various stimuli. This pathway serves by increasing protein folding capacity and enhanced degradation of misfolded proteins within the ER (endoplasmic reticulum) through a process called ERAD (ER-associated degradation) [359]. Celastrol has been shown to induce unfolded protein response (UPR) in L444P GC Gaucher fibroblasts [357], HCC (human hepatocellular carcinoma) Bel-7402 and HepG2 cell lines. In HCC cell lines Bel-7402 and HepG2, celastrol-induced activation of UPR lead to upregulation of Noxa, cytochrome C release, caspase activation cascade and apoptosis [353]. However in Gaucher GC fibroblasts, celastrol-induced UPR offered cytoprotection [357]. How celastrol induced UPR leads to cell protection in Gaucher fibroblasts and apoptosis in hepatocellular carcinoma cell line has not been studied yet.

By modulating various pathways involved in protein homeostasis, celastrol has been found to be one of the hit compouds found during the screening for small molecule compounds as proteostasis regulator, suggesting its potential role in diseases involving dysregulated protein homeostasis [360]. Interestingly, Wang and coworkers found that celastrol treatment in HeLa cells induced features of apoptosis, paraptosis and autophagy at the same dose. This is explained by the fact that celastrol modulates various pathways involved in protein homeostasis including ubiquitin-proteasome pathway and heat shock response [349].

Antioxidant defense is an important stress pathway activated by cells in response to oxidative stress [361]. Celastrol has been shown to not only upregulate HSR and UPR, but also, one other important cell stress pathway, antioxidant defense pathway [326]. Trott and coworkers have found that celastrol can induce an array of genes involved not only in heat shock response that are controlled by HSF-1, but also genes involved in antioxidant defense. In human colon carcinoma RKO cells, celastrol induced the transcription factors Nrf2 (NF-E2-related factor-2) and ATF-4 (activating transcription factor-4) which are involved in the regulation of antioxidant defense [352].

How celastrol induces cell stress pathways like HSR, UPR and antioxidant response is not well understood. Whether these are the primary effect caused by celastrol or induced by cellular stress caused by celastrol-induced inhibition of proteasome, HSP90 and celastrol-induced ROS (reactive oxygen species) remains to be elucidated. It is also possible that the dose and cellular context also might play a role in deciding the outcome of celastrol treatment. It can be conceived that initially at low dose and short burst celastrol might prove to be a cytoprotective agent by inducing cell stress pathways. However, when the cells are already under physiological stress, as in case of cancer cells, addition of celastrol might induce cell death [362].

1.3.1.3. NF-κB

Celastrol has been reported to inhibit NF-κB transcription factor in various inflammatory and tumor models. In fact, celastrol's NF-κB inhibiting ability is considered to be the main mechanism of its anti-inflammatory property [326]. The role of NF-κB in cancer initiation and cancer progression is well established [278, 279]. Celastrol has been shown to inhibit NF-κB in various cancer cells. Concurrent with our findings on NF-κB inhibitory effects of celastrol [363], Tozawa and coworkers have also shown that inhibition of NF-κB nuclear translocation is associated with apoptosis in U266 MM cells [364]. Celastrol-induced inhibition of nuclear translocation of NF-κB was associated with induction of cell cycle arrest and apoptosis in human leukemia U937 cells [365]. Inhibition of constitutive NF-κB pathway and NF-κB-regulated gene products involved in proliferation, survival, migration and invasion by celastrol was associated with induction of apoptosis, inhibition of migration and invasion in androgen-independent prostate cancer cell lines [366]. Celastrol inhibited NF-κB-mediated expression of MMP-9 and thereby inhibited PMA (phorbol 12-myristate 13-acetate) induced invasion and migration of MCF-7 breast cancer cell lines [367]. Osteolytic metastatic lesions to bones are common occurrence in the advanced stages of various malignancies [368]. Aberrant NF-κB activity has been implicated in osteolytic lesions induced by bone metastasis. Celastrol-induced inhibition of NF-κB

activity was associated with induction of apoptosis, inhibition of migration and downregulation of MMP9 and urinary plasminogen activator in rat W256 cells. Interestingly, celastrol also inhibited the W256 cells-induced osteolysis in rats [369]. TMPRSS2/ERG (transmembrane protease, serine2/ Erythroblast transformation-specific related gene) fusion gene has been sought as an attractive target in prostate cancer. NF-κB pathway has been shown to act downstream to this fusion gene product. Celastrol inhibited NF-κB activation in fusion gene expressing VCaP prostate cancer cells and inhibited cell growth in both *in vitro* and in xenograft mouse model [370].

Celastrol has been reported to inhibit TNF-α-induced activation of NF-κB in HT-1080 human fibrosarcoma cell line, leading to induction of apoptosis [371]. Celastrol can also potentiate TNF-α-, paclitaxel- and doxorubicin-induced apoptosis and inhibited TNF-α-induced NF-κB activity [372]. Celastrol can also augment the apoptosis induced by temozolamide in melanoma cell line SK-MEL-173 by inhibiting TNF-α-induced NF-κB activity [373]. Celastrol sensitized oral squamous cell carcinoma Tca8113 cells to gambogic acid [374]. In mouse xenograft retinoblastoma model celastrol nanoparticles decreased the expression of NF-κB, phospho-NF-κB which was associated with significant decrease in tumor growth [375].

How celastrol modulates NF-κB is not yet clear. However, celastrol has been demonstrated to act by inhibiting NF-κB by directly targeting cysteine 179 of IKK by reactive quinine moiety [371]. Moreover, proteasome inhibition also leads to NF-κB pathway inhibition by accumulating IκB [337]. Inhibition of TNF-α-induced activation of NF-κB has been shown to be mediated by celastrol-induced abrogation of TAK1 activation [372].

1.3.1.4. Other important molecular targets modulated by celastrol

Apart from the above discussed targets, celastrol has been shown to affect various other players involved in the pathogenesis of cancer. In human lung cancer 95-D and mouse melanoma cells, celastrol-induced activation of p38 MAPK partially inhibited β-1 integrin activity, inhibited the phosphorylation of focal adhesion kinase and the suppressed the adhesion of β-

1 integrin to fibronectin [376]. How celastrol inhibits MAPKs is not known yet. However, molecular docking analysis shows that celastrol can directly bind to the large pocket ATP binding site to inhibit its kinase activity [377]. ATF-2 (activating transcription factor-2) acts downstream to the MAPK signaling. Celastrol has been shown to modify ATF-2 activity to induce apoptosis in melanoma cells [378].

Topoisomerase inhibitors have been used in the cancer chemotherapy since long time. They are known for their ability to induce apoptosis in cancer cells by inhibiting topoisomerase II. Nagase *et al.,* have reported that in human promyelocytic leukemia HL-60 cells, the rank order of potency of celastrol and its derivatives in causing apoptosis correlated well with their potency of inhibiting the enzyme topoisomerase II [379]. Dysregulation of Wnt-β-catenin pathway is involved in the pathogenesis of various cancers. In HeLa cells, celastrol-induced apoptosis was found to be associated with the increased nuclear translocation of β-catenin [380]. Sp (Specificity protein) transcription factors are involved in the pathogenesis of myriad cancers including bladder carcinoma [381]. In human bladder cancer cells celastrol downregulated Sp transcription factors by both proteasome-dependent and proteasome-independent mechanisms. ROS induction by celastrol mediated the proteasome-independent downregulation of Sp proteins by celastrol [382].

HIF-1α (hypoxia inducible factor-1α) is upregulated in hypoxic tumor environment and induces angiogenesis by upregulating angiogenic factors thereby leading to the development of more aggressive phenotypic cancer cells. Celastrol inhibited hypoxia-induced proliferation and migration of human HCC HepG2 cells which was associated with the downregulation of HIF-1α and decrease in its transcriptional activity [383].

Whether celastrol-induced modulation of these targets are secondary to its effect on proteasome and/or HSP90 or independent of proteasome and/or HSP90 inhibition has yet to elucidated. However, celastrol has been shown to directly bind to tubulin and affects its polymerization leading to mitotic arrest and cell death [384, 385]. In addition to tubulin, celastrol was also shown to directly bind to annexin II and eEF1A (eukaryotic translation elongation factor 1A) [385].

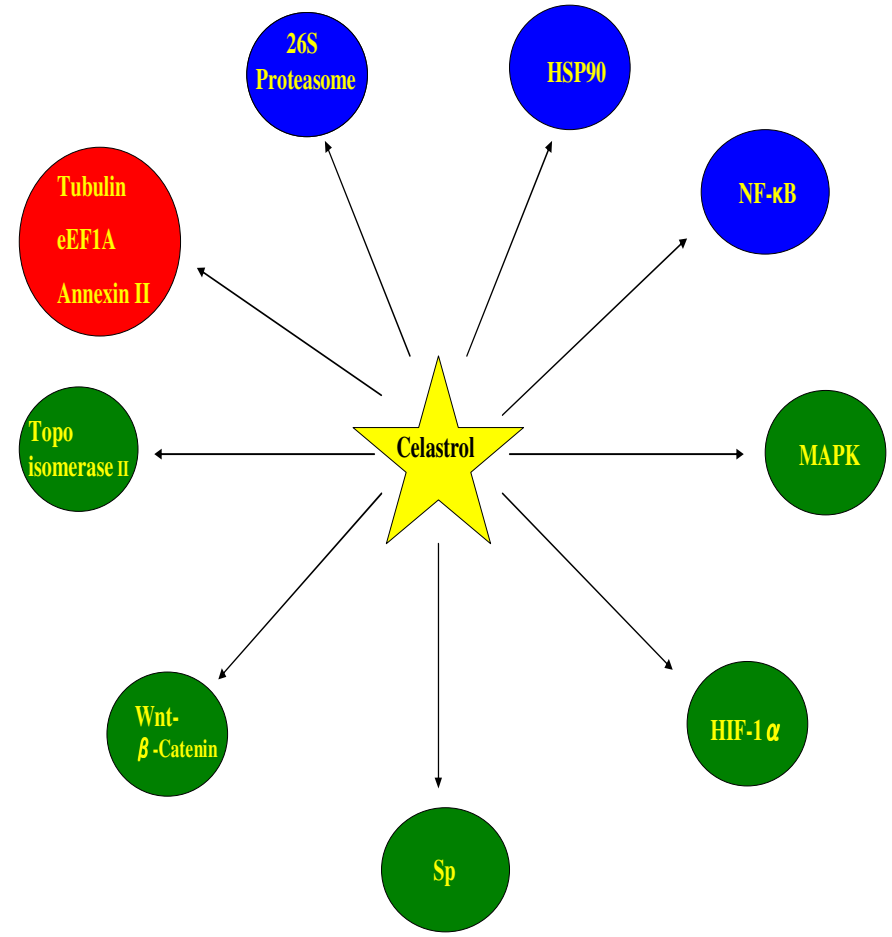


Figure 1.7. Reported molecular targets of celastrol

1.3.2. Anti-cancer effects of celastrol *in vitro*

1.3.2.1. Celastrol induces cell cycle arrest

Most of the anti-cancer drugs eventually inhibit tumor growth by inducing cell cycle arrest and apoptosis. In recent years, the anti-cancer effects of celastrol has been studied using various cancer models [326]. In the various tumor cell lines tested, celastrol induced cell cycle arrest and cell death mainly by apoptosis. Agents that could arrest cell cycle in G_1 to S phase (G_1/G_0 arrest) or G_2 to M phase (G_2 M arrest) or mitosis inhibitors are of potential interest in anti-cancer therapy [386]. Celastrol has been found to induce cell cycle arrest in various cell lines tested. For instance, celastrol has been shown to induce G_1/G_0 arrest in human monocytic leukemia U937 cells by modulating proteins invovled in cell cycle progression [346]. Celastrol has also been shown to induce G2/M cell cycle arrest in glioma C6 cells and this was found to be associated with the inhibition of cyclin B and its kinase cdk2 which are necessary for the progression of cell cycle from G2 to mitosis and upregulation of p21 and p27 which are known to induce cell cycle arrest [338]. In agreement with the study showing that celastrol binds directly to tubulin [385], celastrol was also found to inhibit tubulin polymerization in an *in vitro* tubulin polymerization assay, suggesting its potential role of celastrol as mitosis inhibitor [387]. On a similar note, celastrol has been shown to suppress tubulin-mediated processes, resulting in mitotic arrest and mitotic arrest-mediated cell death [384].

1.3.2.2. Celastrol induces apoptosis in cancer cells

Many drugs induce apoptosis by inducing ROS. Celastrol has also been found to induce ROS in various cancer cells and thereby leading to apoptosis.

Celastrol-induced ROS lead to suppression of breast cancer MCF-7 cell viability [388] and apoptosis of melanoma B16 cells [389], bladder carcinoma 253JB-V cells [382] and Erb2 overexpressing breast cancer cell lines [390]. Celastrol in combination with ionizing radiation induced ROS and augmented ionizing radiation-induced cell death in human non-small cell lung cancer NCI-H460 cells [391]. Celastrol induced accumulation of ROS-induced

phosphorylation of JNK (c-Jun NH2-terminal kinase), which in turn induced mitochondrial-mediated apoptosis in H1299 and HepG2 cells in a dose-dependent manner [392].

Anti-cancer drugs induce apoptosis either by death receptor-mediated extrinsic apoptotic pathway or by mitochondria-mediated intrinsic apoptotic pathway or by both [393]. Celastrol induces apoptosis by engaging both the pathways. In human breast adenocarcinoma MCF-7 cells, celastrol induced both caspase-8-dependent extrinsic apoptotic pathway and mitochondria-dependent intrinsic apoptotic pathway [394]. The apoptotic pathways engaged in celastrol-induced apoptosis has been thoroughly examined in human non-small-cell lung cancer A549 cells. In this *in vitro* model, celastrol-induced apoptosis was associated with upregulation of Fas expression and loss of mitochondrial membrane potential resulting in activation of both extrinsic and intrinsic pathway of apoptosis [395]. Celastrol-induced ROS also has been shown to activate both intrinsic and extrinsic apoptotic pathways [390].

1.3.2.3. Celastrol potentiates the cyokine-mediated apoptosis

TNF-α is a cytokine which belongs to the tumor necrosis factor superfamily. The response of cells to the activation of TNF-α is context dependent [396]. While identifying the effects of celastrol on TNF-α-mediated cell signaling, Sethi, G., *et al.,* repoted that celastrol can potentiate apoptosis induced by TNF-α [372]. Celastrol also potentiated TNF-α-induced cytotoxicity in HT-1080 fibrosarcoma cell line [371].

DR4 and DR5 death receptors provoke extrinsic apoptotic pathway upon binding of TRAIL, preferentially in transformed malignant cells without causing any significant side effects to normal cells. The agonistic antibodies that mimic the ability of TRAIL in activating death receptors and engaging apoptotic pathways are already in clinical trials for the treatment of various cancers [397]. However, cancer cells develop resistance to TRAIL-induced cell death by various mechanisms [398]. Hong Zhu and coworkers demonstrated the synergistic effect of celastrol with TRAIL in human ovarian carcinoma cell line OVCAR-8, human lung cancer cell line 95-D, and human colorectal

carcinoma SW620 cell line in inducing apoptosis. They have further shown that this combination engages both intrinsic and extrinsic pathways [399]. The same authors in a different study have demonstrated that augmentation of apoptosis induced by celastrol was due to the upregulation of death receptors DR4 and DR5 [400]. Sung and co-workers have also reported that celastrol can indeed enhance TRAIL-induced inhibition of tumor cell proliferation and induction of apoptosis. This was associated with upregulation of DR4, DR5, pro-apoptotic protein Bax and downregulation of anti-apoptotic proteins [401].

1.3.2.4. Celastrol potentiates the apoptotic effects of chemotherapeutic agents

Combination strategies are often used in cancer treatment to increase the efficacy, decrease the possible development of chemoresistance and to decrease the side effects. Celastrol has enhanced the apoptotic effect of multiple chemotherapeutic agents in various cell lines tested. For instance, celastrol enhanced the apoptotic effect of gambogic acid in human oral carcinoma cell line Tca8113 [374], sensitized SK-MEL-173 melanoma cells to temozolomide [373], potentiated conventional chemotherapeutic agents daunorubicin- and cytarabine-mediated apoptosis in K-562 and Jurkat T human leukemia cells [402]. It also synergistically induced apoptosis in ErbB2-targeted therapeutics trastuzumab and lapatinib in ErbB2 overexpressing breast cancer cells [390] and sensitized taxol-resistant HeLa cells to paclitaxel [384].

1.3.2.5. Celastrol potentiates the effects of radiotherapy

Development of acquired radioresistance remains a major therapeutic obstacle in hormone resistant prostate cancer and is associated with poor prognosis. Celastrol sensitized PC-3 prostate cancer cells to ionizing radiation in a dose- and schedule-dependent manner [403]. Celastrol sensitized NCI-H460 lung cancer cells to radiation and this effect was associated with the inhibition of HSP90 and upregulation of p53 tumor suppressor gene [350]. Among the many quinone methide triterpenoids that were tested, celastrol had

the most potent radio-sensitizing ability in various human non-small cell lung cancer cell lines namely NCI-H460, NCI-H1299 and A549 [391].

1.3.2.6. Celastrol suppresses tumor angiogenesis

Tumor angiogenesis is another target that is being exploited in cancer therapy. Indeed, bevacizumab, a humanized monoclonal antibody has been approved by FDA for the treatment of colorectal cancer, non-small cell lung cancer, breast cancer, metastatic renal cell cancer and glioblastoma multiformae. Celastrol has been shown to inhibit TNF-α-induced upregulation of vasculogenic factor VEGF [372], to inhibit the proliferation, migration, and tube formation of ECV-304 endothelial cells [404]. It also suppressed the hypoxia-induced proliferation, migration and tube formation of endothelial cells by modulating HIF-1α [383]. In bone marrow endothelial progenitor cells, celastrol abrogated VEGF-induced proliferation, cell-cell adhesion, cell-ECM adhesion, migration response and vascular tube formation [405]. Celastrol inhibited VEGF-induced proliferation, migration, invasion, and capillary-like structure formation of cultured HUVEC (human umbilical vascular endothelial cells) by suppressing VEGF-induced activation of AKT/mTOR/P70S6K signaling [406]. Inhibition of β1-integrin-mediated HUVEC-fibronectin cell adhesion and migration is also demonstrated to be a mechanism by which celastrol can modulate angiogenesis [407].

1.3.2.7. Celastrol abrogates tumor cell invasion and migration

In human lung cancer 95-D and mouse melanoma B16F10 cells celastrol inhibited focal adhesion-dependent cell migration and invasion by modulating β1-integrin ligand affinity, focal adhesion formation, which are accompanied by reduced phosphorylation of FAK (focal adhesion kinase) [376]. Celastrol has been shown to inhibit TNF-α-induced cell migration and invasion [372], suppress invasion and migration in breast cancer cell lines [408]; both of which were accompanied by the downregulation of NF-κB-mediated MMP9 expression. Celastrol inhibited the invasion and migration of colon and pancreatic cancer cells by modulating CXCL12/CXCR4 axis [409].

Celastrol suppressed the migration and invasion of prostate cancer cells [366] and hypoxia-induced invasion of HCC cells [383]. In W256 cells, celastrol-induced inhibition of migration was associated with downreguation of MMP9 and uPAR (urokinase type plasminogen activator receptor) [410].

1.3.2.8. Celastrol exploits oncogene addiction of cancer cells

Certain cancer cells are addicted to one particular molecule or one particular pathway for their proliferation and survival, which is termed as 'oncogene addiction'. Oncogenes act as 'Achilles heel' which can be exploited for the treatment of cancers by using targeted therapies. Interestingly, in certain cancers tested, celastrol preferentially induced apoptosis in the oncogene-addicted cancer cell lines. This finding is not surprising considering many dysregulated oncogenes are regualated by proteasome and Hsp90 in cancer cells which are known to be inhibited by celastrol.

CML (chronic myeloid leukemia) is addicted to the fusion gene *Bcr-Abl*, which is targeted by tyrosine kinase inhibitor imatinib. Interestingly, in CML cells bearing *Bcr-Abl*, celastrol can downregulate Bcr-Abl and induce apoptosis regardless of their sensitivity to imatinib [402, 411]. Moreover, celastrol has also been shown to downregulate other mutant proteins which drive oncogenesis like FLT3 (Fms-like tyrosine kinase 3), EGFR (epidermal derived growth factor receptor) in Ba/F3 cell [343]. Certain prostate cancer cells are dependent on AR (androgen receptor)-mediated cell growth. Celastrol can also downregulate AR expression in human prostate cancer LNCap cells to induce apoptosis [337, 343]. In breast cancer, celastrol modulated the expression of ER-α (estrogen receptor-α) and induced apoptosis in MCF7 and T47D human breast cancer cells [412] and also decreased the stability of ErbB2 , in ErbB2 overexpressing breast cancer cells and induced apoptosis [390].

1.3.3. Anticancer effects of celastrol *in vivo*

1.3.3.1. Celastrol inhibits tumor growth *in vivo*

Animal models are indispensable for the evaluation of chemotherapeutic/chemopreventive agents to bring these agents safely into the clinical trials. Celastrol has been tested in various animal models of inflammation and cancer [326]. Inhibition of tumor growth is an important end point in using of animal models to evaluate their efficacy. Celastrol inhibited tumor growth in human prostate cancer nude mice xenograft model [337, 406], human breast cancer xenograft model [371], human and mouse melanoma tumors and reduced the number of lung metastases in syngeneic and xenograft mouse models [378], human pancreatic cancer [342], glioma [413] and CML xenograft mice models [411].

1.3.3.2. Celastrol potentiates the tumor growth inhibitory effects of TRAIL chemokine and radiotherapy *in vivo*

Xenograft mice models are often used to evaluate the combination therapy of cancer. Celastrol showed enhanced anti-tumor activity with radiotherapy in prostate cancer xenograft mice model [403] and human lung cancer nude mice xenograft model [391]. Celastrol also potentiated the anti-tumor activity of the cytokine TRAIL in 95-D xenograft mice [399] and SW620 xenograft mice model [400].

1.3.3.3. Celastrol inhibits angiogenesis *in vivo*

Celastrol downregulated VEGFR1 and VEGFR2 and decreased the MVD of human xenograft glioma model [413], inhibited VEGF-induced angiogenesis in matrigel plug assay, aortic ring assay [406], and inhibited angiogenesis in chick chorioallantoic membrane assay and *in vivo* matrigel plug assay [404]. Celastrol decreased the MVD in intracranial glioma in rats in combination with adeno-associated virus-mediated recombinant angiostatin [414] and in combination with X-ray radiation it caused significant reduction of angiogenesis in PC-3 xenograft mice model [403].

1.3.3.4. Celastrol inhibits metastasis in *vivo*

RIP1-Tag2 (rat insulin promoter- SV40 Large T antigen transgene) is a mouse model of pancreatic islet carcinoma metastasis. Celastrol caused reduction of metastatic tumors in the mesenterium and inhibition of the tumors in the pancreas resulting in prolongation of longevity [342]. In the rat W256 model of osteolytic bone metastases, celastrol decreased the size of osteolytic bone leisons, reduced the trabacular bone loss, increased the trabacular separation and decreased the number of osteoclasts [410]. In xenograft and syngenic mice models of human melanoma, celastrol decreased the lung metastasis [378]. Celastrol was also found to reduce the lung metastases in mouse melanoma model [376].

1.3.4. Pharmacokinetic studies with celastrol

In a pharmacokinetic study done using Sprague-Dawley rats, it was revealed that the oral bioavailability of celastrol was poor. However, when the whole extract of *Tripterygium wilfordii* was given as a tablet, bioavailability of celastrol increased dramatically, suggesting that other components of the plant extract somehow increase the bioavailability of celastrol. Interestingly, female rats exhibited better oral bioavailability profile when compared to male rats significantly [415]. It is customary to modify the drug formulation to improve the pharmacokinetic profile of a drug without modulating the bioactive component of the drug. Accordingly, liposomal formulations of celastrol had better bioavailability, distribution profile, greater efficacy and fewer side effects in nude mice xenograft model of glioma [416]. Celastrol-loaded polymeric nanomicelles were prepared and it was demonstrated using *in vitro* experiments that these polymeric nanomicelle formulations showed gradual release over 24 hrs. However, this formulation remains to be evaluated in animal models [417]. The efficacy of celastrol nanoparticles have been tested in human retinoblastoma xenograft model of mice [375] and in suture-induced corneal neovascularisation model of rats [418].

Interaction of any therapeutic agent with liver enzymes represents an important pharmacokinetic study analyzing the metabolism of the drug and its potential interactions with other drugs. Celastrol has been shown to inhibit the

liver enzyme UDP-glucuronosyltransferase (UGT) 1A7 [419], UGT1A6 and UGT2B7 [420], UGT 1A3 [421]. In addition, celastrol has also been shown to inhibit intestinal UGT1A8 and UGT1A10, which will lead to increase in the plasma concentrations of co-administered drugs which are metabolized by these enzymes [422]. So, it is prudent that the possibilities of celastrol-mediated drug-drug interactions are considered while conducting combination studies in animal models and while planning future clinical trials.

1.3.5. Toxicological analysis of celastrol

Systematic toxicological analyses of celastrol are yet to be conducted. However, in the various animal models that have been tested so far, celsatrol was not found to cause any obvious side effects, except in one study where authors noticed about 10% weight loss of celastrol-treated RIP1-Tag2 transgenic mice [342]. Zebra fish embryo model is commonly used to analyze the teratogenic potential of therapeutic agents. Celastrol was found to cause tail malformation [423] and cardiotoxicity [424] in zebra fish embryo. The above findings warrant a systematic study of side effects, toxic effects and teratogenic effects of celastrol.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1. Cell lines

The human MM cell lines U266 and RPMI 8226 were bought from ATCC. RPMI-8226-Dox-6 (a doxorubicin-resistant clone), RPMI-8226-LR-5 (a melphalan-resistant clone) and RPMI-8226 Vel-R (a bortezomib resistant clone) were kindly provided by Prof. Chng Wee Joo, Department of Medicine, Yong Loo Lin School of Medicine, National University of Singapore. All the MM cells were cultured in RPMI 1640 (Rosewell Park Memorial Institute) medium containing 1X antibiotic-antimycotic with 10% FBS. All cell lines used were grown and passaged in a humidified incubator at 37°C with 5% CO_2.

2.1.2. Cell culture reagents

RPMI 1640 - Life Technologies (Carlsbad, CA)

0.4% trypan blue vital stain - Life Technologies (Carlsbad, CA)

Antibiotic-antimycotic mixture - Life Technologies (Carlsbad, CA)

FBS (fetal bovine serum) - BioWest (Miami, FL, USA)

ThinCert 8-µm pore size tissue culture insert - Greiner Bio-one ThinCerts™, NC, USA.

2.1.3. Test compounds

Celastrol - Alexis Biochemicals (San Diego, CA)

50 mM stock of celastrol with purity greater than 98% was prepared and stored in -20° C.

Bortezomib (Velcade, PS341) - LC Laboratories (Woburn, MA, USA)

Thalidomide - Sigma-Aldrich Chemical Co. (St. Louis, MO)

DTT (Dithiothreitol) - Sigma-Aldrich Chemical Co. (St. Louis, MO)

GSH (Glutathione reduced) - Sigma-Aldrich Chemical Co. (St. Louis, MO)

GSSG (Glutathione oxidized) - Sigma-Aldrich Chemical Co. (St. Louis, MO)

Vit C (Ascorbic acid) - Sigma-Aldrich Chemical Co. (St. Louis, MO)

SP600125 - Sigma-Aldrich Chemical Co. (St. Louis, MO)

2.1.4. Reagents and chemicals

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] reagent - Life Technologies (Carlsbad, CA, USA)

SDS (sodium dodecyl sulfate) - Life Technologies (Carlsbad, CA, USA)

Dimethylformamide - Life Technologies (Carlsbad, CA, USA)

CXCL12 - Pro-Spec-Tany TechnoGene Ltd. (Rehovot, Israel)

Human recombinant IL-6 - ProSpec-TanyTechnoGene Ltd (Rehovot, Israel)

The Live/Dead Viability/Cytotoxicity Assay Kit - Life Technologies (Carlsbad, CA)

Nuclear extraction kit - Active Motif (Carlsbad, CA)

NF-κB p65 DNA binding kit - Active Motif (Carlsbad, CA)

Annexin V-FITC assay kit - Santa Cruz Biotechnology (Santa Cruz, CA)

Propidium iodide (PI) - Sigma-Aldrich Chemical Co. (St. Louis, MO)

RNAase - Roche (U.S.A)

DAPI (4',6-Diamidino-2- Phenylindole, Dihydrochloride) - Santa Cruz Biotechnology (Santa Cruz, CA)

Hoechst stain - Life Technologies (Carlsbad, CA, USA)

Calcein-AM - Becton Dickinson (Bedford, MA, USA)

Matrigel - Becton Dickinson, (Bedford, MA, USA)

Chemiluminescence ECL- GE Healthcare**, (**Little Chalfont, Buckinghamshire, UK)

Trizol reagent - Life Technologies (Carlsbad, CA, USA)

Immunohistochemistry kit - DAKO LSAB kit, Dako Corporation, (Carpinteria, California, USA)

ELISA kits for TNF-α and IL-6 - R&D systems, (USA).

2.1.5. Antibodies

Antibodies purchased from Santa Cruz Biotechnology (Santa Cruz, CA) are as follows:

sc-81523- anti-phospho-STAT3 (Tyr 705)

sc-8001-R-anti-phospho-STAT3 (Ser 727)

sc-836 - anti-STAT5

sc-482 - anti-STAT-3

sc-56960 - anti-PTP1B

sc-280 - anti-SHPTP2

sc-101629- anti-phospho-AKT (Ser 473)

sc-56878 - anti-AKT-

sc-137179 - anti-GAPDH

sc-20682 - anti-Lamin B

sc-371 - anti-IκBα

sc-23470-R -anti-phospho-IKKα/β (Ser 180/Ser 181)

sc-493 - anti-Bax

sc-832 - anti-Bak

sc-509 - anti-Bcl-2

sc-8392 - anti-Bcl-xL

sc-20679 - anti-Mcl-1

sc-11423 - anti-Bid

sc-47750 - anti-Survivin

sc-753 - anti-Cyclin D1

sc-481 - anti-Cyclin E

sc-817 - anti-p21

sc-528 - anti-p27

sc-57496 - anti-VEGF

sc-40 - anti-c-Myc

sc-19999 - anti-COX2

sc-10737 - anti-MMP 9

sc-7150 - anti-PARP

sc-47778 - anti-β-actin

sc-2005 - HRP conjugated goat anti-mouse

sc-2004 - HRP conjugated goat anti-rabbit

sc-2354 - HRP conjugated mouse anti-goat

Antibodies purchased from Cell Signaling Technology (Beverly, MA) are as follows:

#4322 - anti-phospho-STAT5 (Tyr 694)

#2101S - anti-phospho-Src (Tyr 416)

#2108S - anti-Src

#3771S - anti-phospho-JAK2 (Tyr 1007/ 1008)

#3230S - anti-JAK2

#2859 - anti-phospho-IκBα (Ser 32/36)

#2684 - anti-IKKβ

#3033 - anti-phospho-p65 (Ser 536)

#3034 - anti-p65

#9251S - anti-phospho-SAPK/JNK (Thr183/Tyr185)

#9252 - anti-SAPK/JNK

#2042 - anti-XIAP

#9662 - anti-Caspase 3

#9746 - anti-Caspase 8

#9502 - anti-Caspase 9

Antibody purchased from Abcam (Cambridge, MA, USA) is as follows:

ab2074 -anti-CXCR4

Antibody purchased from Life Technologies (Carlsbad, CA, USA) is as follows:

A-11012 - Goat anti-rabbit IgG-Alexa 594.

2.1.6. Primers and probes

Please refer to the appendix I.

2.2. Methods

2.2.1. *In Silico* Analysis

Please refer to the appendix II.

2.2.2. Cell viability assay

The cytotoxic effect of celastrol on various myeloma cells was measured using MTT dye uptake method. This assay is primarily based on the principle that live and metabolically active cells readily take up MTT and reduce it to insoluble purple formazan crystals. The soluble formazan product can be quantified colorimetrically by measuring the absorbance at 570 nm. Thus this assay is useful for quantitative determination of cellular proliferation and viability, as it occurs only in metabolically intact cells. Briefly, MM cells ($5x10^3$/ml) were incubated in triplicate in a 96-well plate in the presence or absence of different concentrations of celastrol in a final volume of 0.2 ml for different time intervals at 37°C with 5% CO_2 atmosphere. Thereafter, 20 µl MTT solution was added to each well. After a 4 hours (hrs) incubation at 37°C, lysis buffer was added, cells were incubated at 37°C for 4 hrs, and the optical density was then measured at 570 nm using a Tecan microplate reader. The percentage cell viability was calculated using the following formula.

Percentage cell viability = average OD of treated cells / average OD of control cells x 100.

MTT reagent was prepared at the concentration of 5mg/ml in PBS and stored at 4°C in the dark. Lysis buffer consisting of 20% SDS and 50% dimethylformamide was prepared in autoclaved water and stored at room temperature.

2.2.3. DNA fragmentation analysis

DNA fragmentation analysis was carried out to detect apoptosis in test compounds-treated cells. Briefly, MM cells ($1x10^6$) cells were seeded and treated with the test compounds for the indicated time duration. Following treatment with the test compounds, the cells were collected, washed and resuspended in 1 ml of 1X PBS (phosphate buffered saline) and immediately fixed with 70% ice cold ethanol while vortexing the cells. The vortexing was done to prevent the cells from clumping together. The cells were incubated at

4°C for 30 minutes (min) and then were washed with 1X PBS and resuspended in 500 µl PI (10µg/ml)-RNAase A (1µg/ml) solution. The cells were incubated for another 30 min before DNA content analysis. At least 1000 events were analyzed with CyAn ADP flow cytometer (Dako Cytomation) with the excitation set at 488 nm and emission at 610 nm, after which they were analyzed with WINMDI software. The histogram data shows percentage of cells containing hypodiploid, diploid and hyperdiploid DNA content. The cells containing hypodiploid DNA content are considered to be apoptotic. PI (Propidium Iodide) was dissolved in PBS to a stock concentration of 5 mg/ml stored in 4°C in the dark. RNAase was dissolved in PBS to a stock concentration of 1 mg/ml and kept at -20°C.

2.2.4. Annexin V-FITC staining

One of the early indicators of apoptosis is the rapid translocation of the membrane phospholipid phosphatidylserine from the cell's cytoplasmic interface to the extracellular surface and its accumulation there, and thereby causing loss of membrane asymmetry that can be detected using annexin V. Annexin V is a phospholipid binding protein that preferentially binds to negatively charged phospholipids including phosphatidylserine. PI a red flourescent dye, stains the chromosomal components of the nucleus and does not permeate live cells. It is used to identify the dead cells in a given cell population. So, cells progressing through apoptosis can be monitored according to their Annexin V and PI staining pattern. Early apoptotic cells will bind Annexin V, but will not take up PI. As cells progress through apoptosis the integrity of the plasma membrane is lost, thereby allowing PI to penetrate and label the cells with a strong yellow-red fluorescence. Briefly, MM cells (1 x 10^6) were treated with test compounds for indicated time points. Cells were collected, washed with PBS and resuspended with 1 ml of assay buffer. Then, 50 µl of Annexin V FITC and 100 µl of PI were added into the cells and incubated in dark at room temperature for 15 min. Cells were analyzed with a flow cytometer (BD FACS Calibur, BD Biosciences, US) and the data obtained was analyzed using WINMDI software.

2.2.5. Live and dead assay

The Live/Dead Viability/Cytotoxicity Assay Kit provides a two-color fluorescence cell viability assay that is based on the simultaneous determination of live and dead cells with two probes that measure recognized parameters of cell viability which are intracellular esterase activity and plasma membrane integrity. Live cells are distinguished by the presence of ubiquitous intracellular esterase activity, determined by the enzymatic conversion of the virtually non-fluorescent cell-permeant calcein-AM to the intensely fluorescent calcein. The polyanionic dye calcein is well retained within live cells, producing an intense uniform green fluorescence in live cells (excitation/emission ~495 nm/~515 nm). EthD-1 (Ethidium monomer dye) enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, thereby producing a bright red fluorescence in dead cells (excitation/emission ~495 nm/~635 nm). EthD-1 is excluded by the intact plasma membrane of live cells. Briefly, MM cells (1 x 10^6) were incubated with test compounds for various times at 37°C. Cells were collected and washed with 1X PBS. Cells were resuspended using the working solution consisting of EthD-1 (4µM) and Calcein-AM (2µM) and incubated for 45 min in the dark. After the incubation, cells were collected by centrifugation and the working solution was discarded. Cells are resuspended using 20 µl 1XPBS and put on a glass slide, covered with a cover slip and cells were observed and captured under a fluorescence microscope (Olympus DP 70, Japan).

2.2.6. Migration assay

U266 cells (50 x10^4/well) were plated in 300-µl cell culture media with and without 1µM Celastrol in the top chambers of 24-well transwell inserts with 8-µm pores. Cell culture medium (600 µl) containing the recombinant human B-cell chemoattractant CXCL12 (100 ng/ml) was added to the bottom chamber and incubated for 12 hrs. After incubation, the insert was removed and calcein-AM (5µM) was added to the wells. The cells were incubated at 37°C for 1 hr to allow the cells to internalize Calcein-AM. The fluorescence

was measured (485 nm excitation, 520 nm emission). A standard curve was generated with cells ranging from 50000-1000 cells using Calcein-AM under same condition and cells number calculated from relative fluorescence of treated samples.

2.2.7. Invasion assay

An invasion assay was performed with U266 cells in 24-well plates with polycarbonate membranes (ThinCert 8-µm pore size tissue culture insert). Briefly the upper chambers were coated with 50-µl matrigel in advance. 50 $x10^4$ U266 cells in suspension were starved in serum-free RPMI-1640 for 3 hrs, and then loaded onto the matrigel-coated inserts in the upper chambers. The wells of the plate were filled with 600-µl of 10% FBS-containing cell culture media with 100 ng/ml CXCL12. Celastrol was added with the cells to the upper chamber. Plates were then incubated at 37°C for 12 hrs. At the end of the incubation period, Calcein-AM (5µM) was added to the wells containing invasive cells. The cells were incubated at 37°C for 1 hr to allow the cells to internalize Calcein-AM. The fluorescence was measured (485 nm excitation, 520 nm emission). A standard curve was generated with cells ranging from 50000-1000 cells using Calcein-AM under same condition and cells number calculated from relative fluorescence of treated samples.

2.2.8. Preparation of cytosolic and nuclear extracts

Briefly, MM cells (3 $x10^6$/flask) were seeded and treated with the test compounds for the indicated duration. After that cells were spun at 1000 rpm at 4 °C for 5 min and the media was removed. The cell pellet was washed with 10 ml of ice cold PBS. Then the cells were incubated in hypotonic buffer with protease and phosphatase inhibitors for 15 min, when 25µl of DTT was added and vortexed for 10 seconds at highest speed. Then the cell suspension was centrifuged for 30 seconds at 14,000 rpm in a microcentrifuge pre-cooled at 4ºC. The supernatant (cytoplasmic fraction) was transferred into a pre-chilled microcentrifuge tube and stored at –80ºC until it was used. The

remaining nuclear pellet was resuspended in 50 µl of complete lysis buffer by pipetting up and down and vortexing for 10 seconds at the highest setting and incubating on ice over a rocking platform for about 30 min before, it was spun at 14,000 rpm for 5 min. The supernatant (nuclear extract) was collected, transferred to a pre-chilled microcentrifuge tube and stored at –80ºC until being used.

2.2.9. Western blot analysis

For detection of various proteins, test compound-treated whole cell extracts were lysed in lysis buffer. Thereafter, the cells in the lysis buffer were incubated on ice for 30 min with intermittent vortexing and were subsequently spun at 14,000 rpm for 10 min and resolved on a SDS gel. After electrophoresis, the proteins were electrotransferred to a nitrocellulose membrane, blocked with 5% non-fat milk, and probed with various primary antibodies (1:1000) overnight at 4°C. The blot was washed, exposed to HRP-conjugated secondary antibodies for 1 hr, and finally examined by chemiluminescence. The densitometric analysis of the scanned blots was done using Image J software and the results are expressed as fold change relative to the control.

Lysis buffer consisting of 20 mM Tris (pH 7.4), 250 mM NaCl, 2 mM EDTA (pH 8.0), 0.1% Triton X-100 was prepared and stored at -20°C. Protease inhibitors namely 0.01 mg/ml aprotinin, 0.005 mg/ml leupeptin, 0.4 mM PMSF, and 4 mM $NaVO_4$ were added into the lysis buffer immediately before being used.

2.2.10. RNA extraction and Real-time PCR analysis:

Please refer to the appendix II.

2.2.11. Immunocytochemistry

Immunocytochemistry analysis was done to study the nuclear translocation of STAT3 and NF-κB. Briefly, MM cells (1 x10^6) were seeded and incubated overnight. Then the MM cells were treated with the celastrol for various time points. Cells were plated on a glass slide by centrifugation with the use of a cytospin. The glass slide was air dried for 1hr at room temperature. Cells were fixed with pre-chilled acetone at -20°C for 15 min. Then the slides were washed with 1XPBS and incubated with 0.2% Triton-X 100 in PBS for 15 min at room temperature. After washing with 1X PBS, cells were blocked with 5% normal goat serum in PBS for 1 hr at room temperature. After washing, the cells were incubated in primary antibody (1:100 in normal goat serum) at 4°C. After overnight incubation and washing with 1XPBS, cells were incubated with goat anti-rabbit IgG-Alexa 594 (1/100) for 1 hr and counterstained with Hoechst (50 ng/mL) for 5 min. Then, the slides were mounted using the mounting medium and analyzed under a fluorescence microscope (DP 70, Olympus, Tokyo, Japan). Positive cells (brown) were quantitated using the Image-Pro plus 6.0 software package (Media Cybernetics, Inc.).

2.2.12. NF-κB DNA binding assay

To determine NF-κB activation, we performed DNA binding assay. Briefly, the binding of NF-κB to DNA was measured in nuclear extracts with a fast, user-friendly, ELISA based TransAM NF-κB p65 assay kit. This assay uses multiwell plates coated with an unlabeled oligonucleotide containing the consensus binding site for NF-κB (5'-GGGACTTTCC-3'). MM cells (2x 10^6) were treated with the indicated concentrations of test compounds for indicated time intervals after which, nuclear extracts were prepared using the nuclear extraction kit. Nuclear proteins (20 μg) were added to each well and incubated for 1 hr to allow NF-κB DNA binding. Subsequently, by using an antibody that is directed against NF-κB p65 subunit, the NF-κB complex bound to the oligonucleotide is detected. A horseradish peroxidase-conjugated secondary antibody provided the basis for the colorimetric quantification.

2.2.13. Xenograft MM mouse model

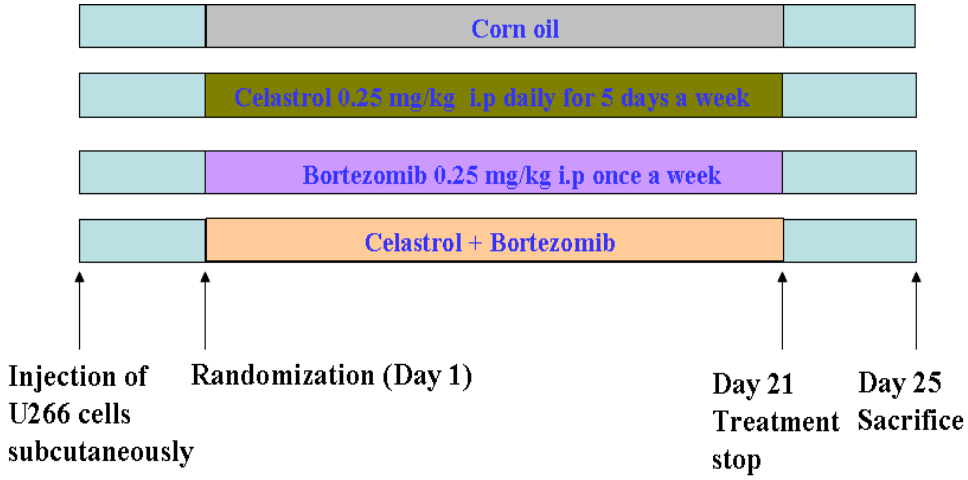


Figure 2.1. Schematic representation of *in vivo* experimental protocol

One week after the tumor cell injection mice were randomized into four treatment groups and treatment was started. On day twenty one, the treatment was stopped and the mice were observed for another four days after which they were sacrificed.

Male athymic balb/c nude mice (BRC, Biopolis, Singapore) were implanted with U266 cells ($2x10^7$) subcutaneously. One week after the tumor cell implantation, the mice were randomized into four groups (n=5) based on the tumor volume. Group I (control) received corn oil 100 µl i.p. for five days a week, group II received 0.25 mg/kg celastrol in 100 µl corn oil for five days a week, group III received 0.25 mg/kg bortezomib in 100 µl corn oil i.p. weekly and group IV received 0.25mg/kg celastrol in 100 µl corn oil i.p. 5 days a week and 0. 25 mg/kg bortezomib in 100 µl corn oil administered i.p. weekly for 3 consecutive weeks. The tumor diameters and body weight of the mice were monitored and measured on every third day during the duration of the experiment. The tumor volume was calculated using the formula [L x W2]/2, where W and L are the width and the length of the tumor On completion of the treatment period, mice were euthanized by i.p. phentobarbital (40 mg/kg b.w) followed by cervical dislocation and blood was collected. Then tumors were

dissected and subjected to histological examination.

2.2.14. Immunohistochemical analysis of tumor tissues

Solid tumors from control and celastrol-treated groups were fixed with 10% phosphate-buffered formalin, processed, and embedded in paraffin. The sections were cut and deparafinized in xylene, dehydrated in graded alcohol, and finally hydrated in water. Antigen retrieval was performed by boiling the slide in 10 mM sodium citrate (pH 6.0) for 30 min. Briefly, endogenous peroxidases were quenched with 3% hydrogen peroxide. Non-specific binding was blocked by incubating in the blocking reagent in the LSAB kit (Dako). Sections were incubated overnight with primary antibodies as follows: anti-p65, anti-phospho-STAT3, anti-Bcl-2, and anti-VEGF (each at 1:100 dilutions). The slides were subsequently washed several times in Tris-buffered saline with 0.1% Tween 20 and were incubated with biotinylated linker for 30 min, followed by incubation with streptavidin conjugate provided in LSAB kit according to the manufacturer's instructions. Immunoreactive species were detected using 3, 3'-diaminobenzidine tetrahydrochloride as a substrate. The sections were counterstained with Gill's hematoxylin and mounted under glass cover slips. Images were taken using an Olympus BX51 microscope (magnification, X 40).

2.2.15. ELISA assay

Blood was collected as the mice were sacrificed and sera was separated and stored in -80°C. Serum level of mouse TNF-α and IL-6 was measured using ELISA kit (R&D systems, USA) according to manufacturer's instructions. Briefly, 50 µl of assay diluent was added to each well and 50 µl standard and samples was added in the wells and incubated for 2 hrs at room temperature. At the end of two hrs, the contents of the wells were aspirated. The wells were washed with the wash buffer for five times. After that 100 µl of mouse TNF-α and IL-6 conjugate were added to the wells respectively and incubated for 2 hours at room temperature. At the end of two hrs contents of the wells were aspirated and the wells were washed with wash buffer. Then

100 µl of substrate solution was added to each well and incubated in the room temperature for 30 min in dark. After that 100 µl of stop solution was added to each well and was mixed with the substrate solution by gentle tapping. The optical density was measured using a microplate reader at 450 nm. The wavelength correction was done at 540 nm.

2.3. Statistical analysis

Statistical analysis was performed by student's unpaired t test. The probability (p) value less than 0.05 was considered statistically significant. When multiple comparisons were done one way ANOVA test was used and post-test comparisons among the groups were done using Bonferroni method. The probability (p) value less than 0.05 were considered statistically significant.

3. RESULTS

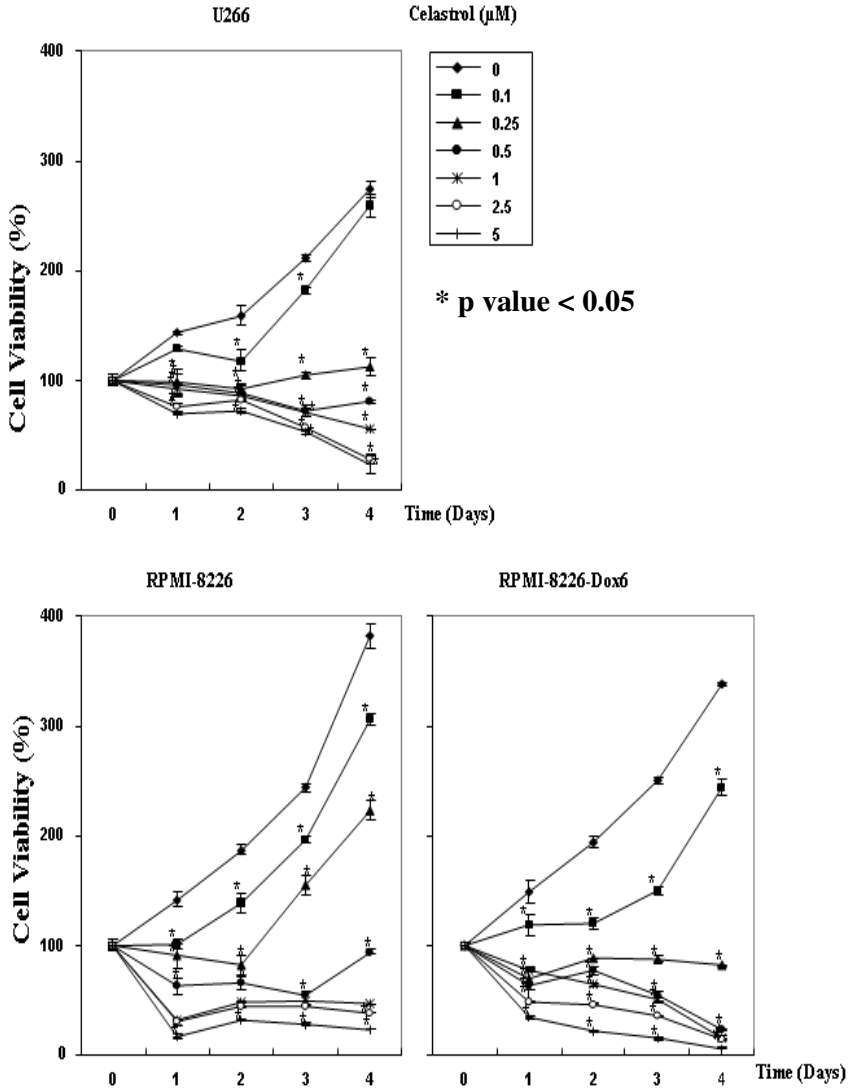
3. 1. *In silico* analysis of anti-myeloma effects of celastrol

Please refer to the appendix I.

3.2. Anti-myeloma effects of celastrol *in vitro*

3.2.1. Celastrol suppresses the viability of various drug resistant MM cells

Anti-myeloma effect of celastrol was evaluated by assessing the cell viability using MTT assay. MTT assay is a colorimetric assay that is generally used to determine the cytotoxicity of potential pharmacological agents [425]. Different MM cell lines including U266, RPMI-8226-Dox6 (resistant to doxorubicin), RPMI 8226-LR5 (resistant to melphalan), and RPMI-8226-BR (bortezomib resistant) were used for these experiments. The cells were treated with 0, 0.1, 0.25, 0.5, 1, 2.5 and 5 μM of celastrol for 0, 1, 2, 3 and 4 days and subjected to MTT assay. The data obtained indicated that celastrol suppressed the proliferation of all MM cell types examined in a dose- and time-dependent manner.


U266
Celastrol (μM)
0
0.1
0.25
0.5
1
2.5
5
* p value < 0.05
Cell Viability (%)
Time (Days)
RPMI-8226
RPMI-8226-Dox6
Cell Viability (%)
Time (Days)

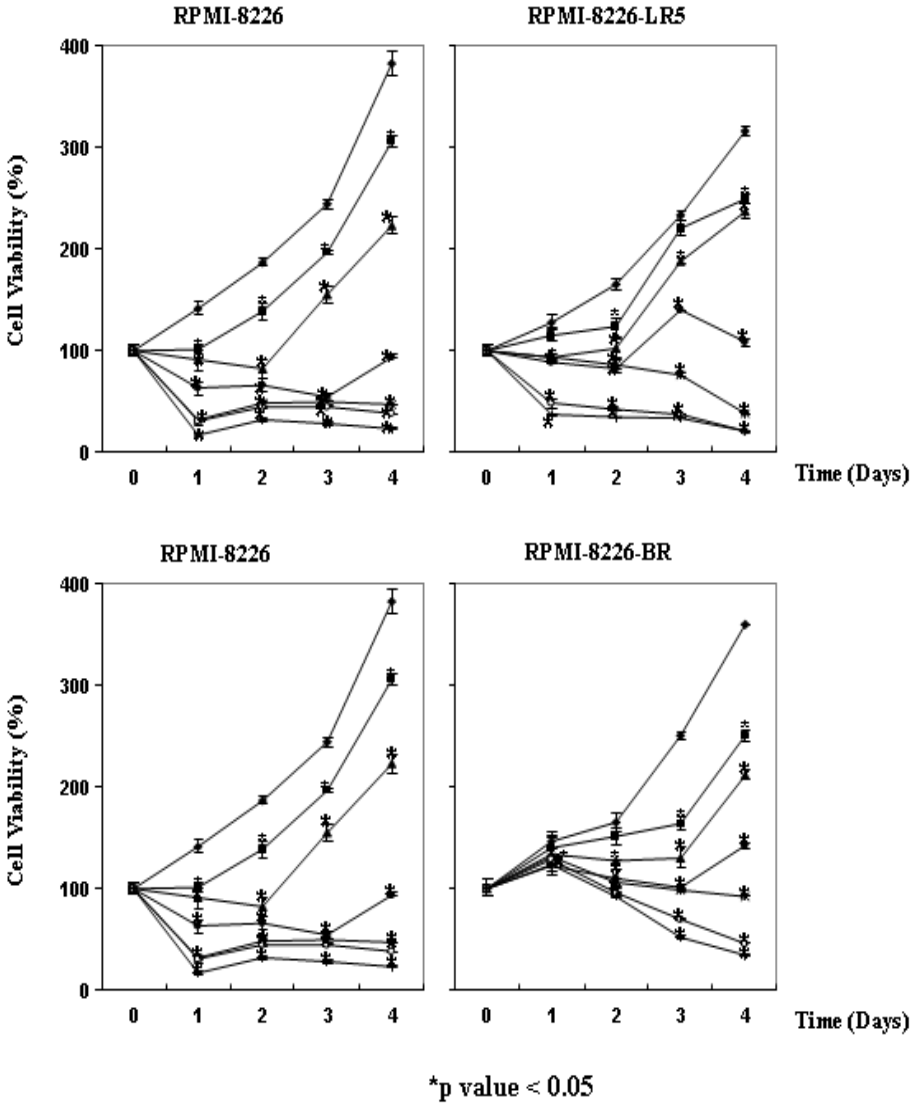


Figure 3.2.1. Celastrol suppresses the viability of various drug resistant MM cells

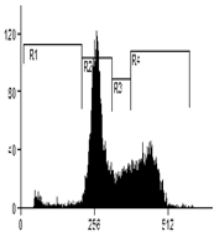
U266, RPMI-8226-Dox6, RPMI-8226-LR5, and RPMI-8226-BR cells were treated with 0, 0.1, 0.25, 0.5, 1, 2.5 and 5 μM of celastrol for 0, 1, 2, 3 and 4 days and subjected to MTT assay. Cell viability in celastrol-treated group decreased to statistically significant levels as compared to control group ($p < 0.05$) after treatment for indicated time points. Data expressed as mean $\pm$ standard deviation (SD).

3.2.2. Celastrol induces apoptosis in MM cells

3.2.2.1. Celastrol causes increased accumulation of MM cells in sub G_1 phase

To analyze, if the decrease in the cell viability of MM cells upon treatment with celastrol was due to induction of apoptosis, DNA fragmentation, an important hallmark of apoptosis was analyzed. Fragmentation of DNA which occurs during apoptosis will result in accumulation cells in subG_1 (sub-diploid) population when analyzed by FACS after PI staining. Different MM cells were seeded in triplicate and treated with 1 μM celastrol for indicated time points. Cells were then washed, fixed and stained with PI before analyzed by FACS. Cells with fragmented DNA will appear in the subG_1 fraction of cell cycle profile. Our data clearly reveals that celastrol treatment caused substantial accumulation of cells in subG_1 phase in a time-dependent manner in U266, RPMI-8266, and RPMI-8226-BR cell lines.

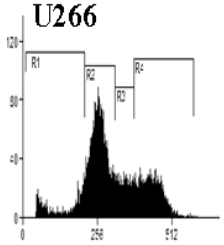
A.



G0/G1	51%
S	14%
G2/M	32%
SubG1	3%

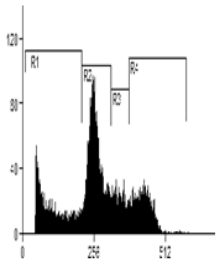
Untreated

U266



G0/G1	49%
S	14%
G2/M	26%
SubG1	11%

12 hrs

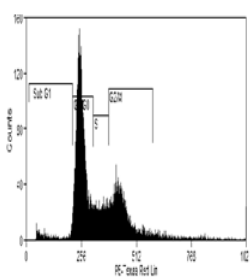


G0/G1	44%
S	13%
G2/M	20%
SubG1	23%

24 hrs

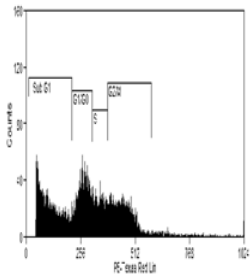
B.

RPMI-8226



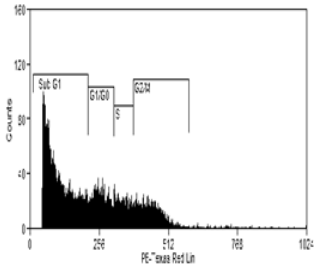
G0/G1	57%
S	13%
G2/M	27%
SubG1	3%

Untreated



G0/G1	29%
S	15%
G2/M	26%
SubG1	30%

12 hrs

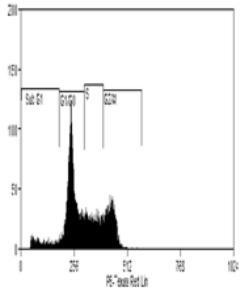


G0/G1	19%
S	11%
G2/M	17%
SubG1	53%

24 hrs

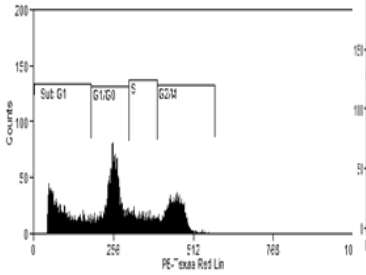
C.

RPMI-8226-BR



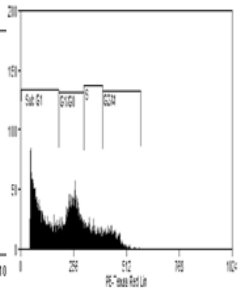
G0/G1	48.91%
S	22.51%
G2/M	21.97%
SubG1	7%

Untreated



G0/G1	37.86%
S	13.53%
G2/M	23.05%
SubG1	26.01%

24 hrs



G0/G1	34.94%
S	13.50%
G2/M	11.13%
SubG1	40.83%

48 hrs

Figure 3.2.2.1. Celastrol causes accumulation of MM cells in sub G_1 phase

A. U266 cells were treated with 1 μM celastrol for 0, 12, and 24 hrs, after which the cells were washed, fixed, stained with PI, and analyzed for DNA content by flow cytometry.

B. RPMI-8266 cells were treated with 1 μM celastrol for 0, 12, and 24 hrs, after which the cells were washed, fixed, stained with PI, and analyzed for DNA content by flow cytometry.

C. RPMI-8226-BR cells were treated with 1 μM celastrol for 0, 24, and 48 hrs, after which the cells were washed, fixed, stained with PI, and analyzed for DNA content by flow cytometry.

Representative histogram pictures with the percentage of cells in hypodiploid (subG_1) diploid G_1/G_0 and hyperdiploid (S and G_2M) phases for each cell line has been shown.

3.2.2.2. Celastrol causes loss of membrane integrity in MM cells

Loss of membrane integrity is one of the earliest features of apoptosis. To measure effect of celastrol on membrane integrity, we used the Live/Dead cell viability assay kit which determines intracellular esterase activity and plasma membrane integrity. MM cells were treated with 1 μM celastrol for 0, 6, 12, and 24 hrs and stained with EthD-1 and calcein-AM and processed as discussed under Materials and Methods. Live cells will exclude EthD-1 and allow calcein-AM which is converted by metabolically active cells to a green fluorescent dye when analyzed under fluorescent microscope. Dead cells will allow EthD-1 because of loss of membrane integrity and will appear as red cells when analyzed under fluorescent microscope. The green cells indicate live cells and the red cells indicate dead cells. As evident from our results shown below, the percentage of ethidium monomer dye (red fluorescence) stained cells significantly increased in a time-dependent manner upon celastrol treatment which indicated that this triterpene can induce substantial apoptosis in MM cells.

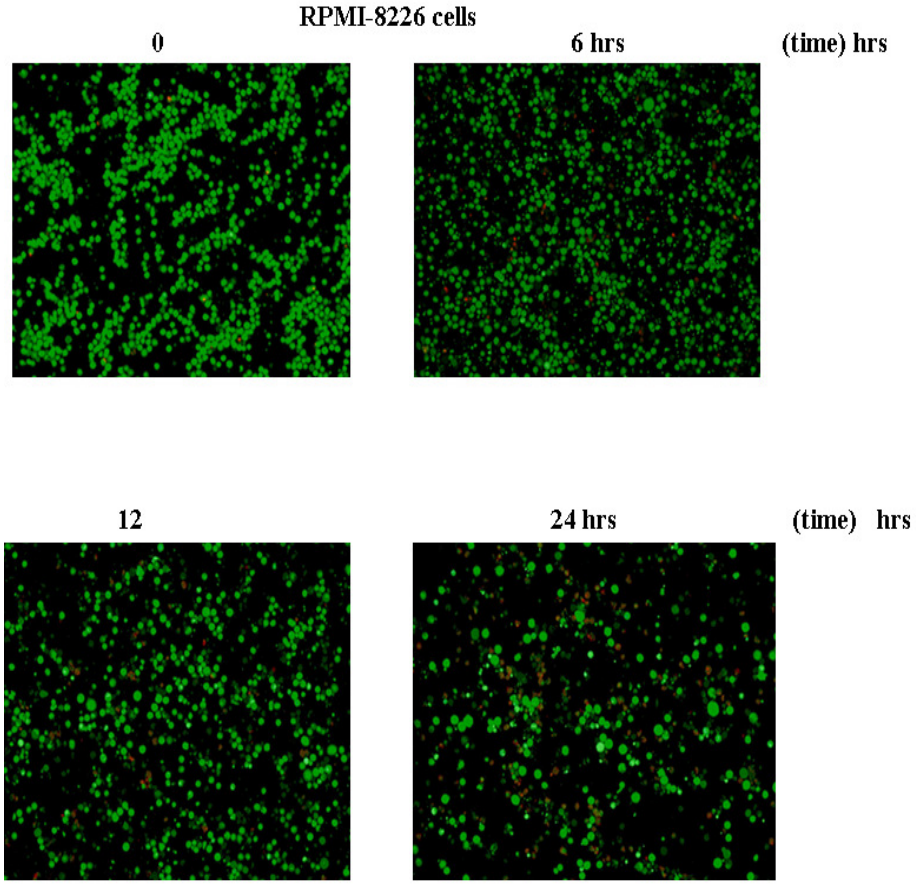


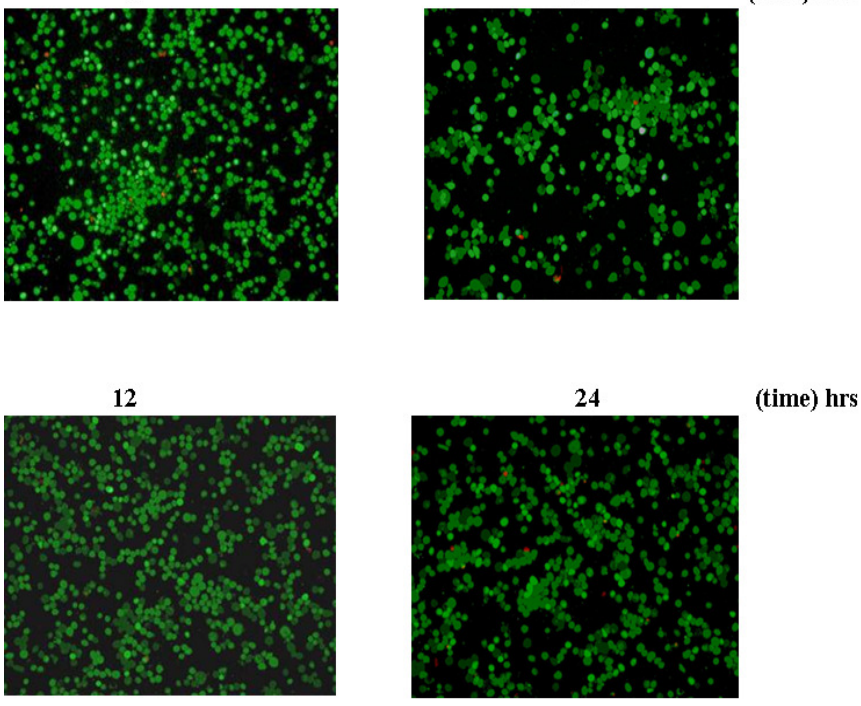
Figure 3.2.2.2. Celastrol causes loss of membrane integrity in a time-dependent manner in MM cells

RPMI-8266 cells were treated with 1 μM celastrol for 0, 6, 12, and 24 hrs. Cells were stained with Live/Dead assay reagent for 30 mins and then analyzed under a fluorescence microscope as described under Materials and Methods. A representative image for each group is shown.

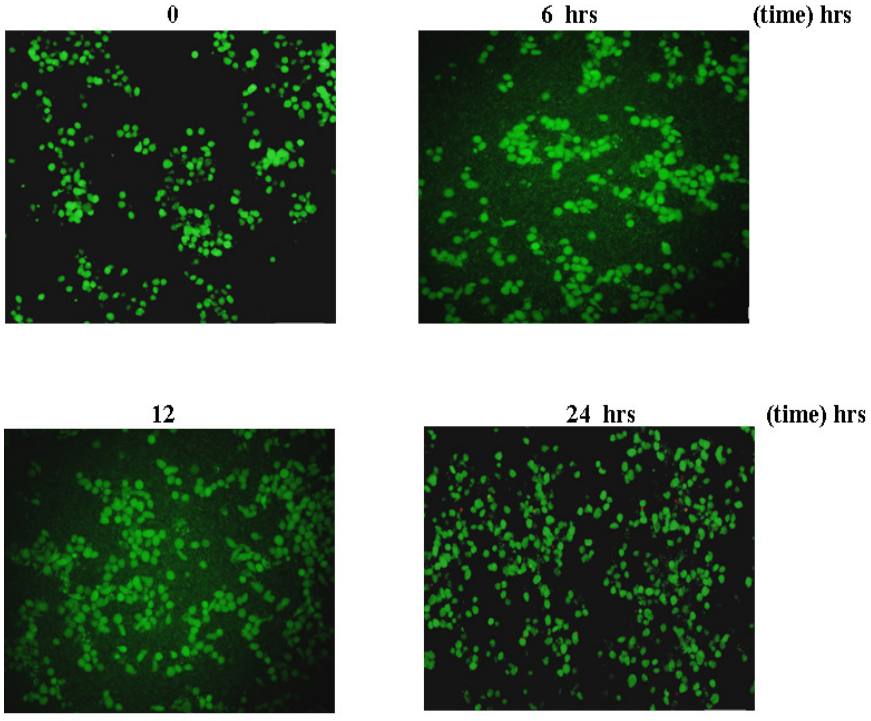
3.2.2.3. Celastrol causes relatively less cell death in normal mouse embryonic fibroblasts

Our above mentioned results clearly show that celastrol induces apoptosis in MM cells. Next we went on to investigate the apoptotic effects of celastrol on normal cells to rule out its potential toxicity. We used mouse embryonic fibroblasts (MEF) and human normal breast epithelial cell line MCF-10A to represent non-cancer cells. To analyze the apoptotic effect of celastrol on these cells, we performed Live and Dead assay which detects intracellular esterase activity and plasma membrane integrity. MEF cells and MCF-10A cells were treated with 1 μM celastrol for 0, 6, 12 and 24 hrs. Live and Dead assay was performed as described under Materials and Methods. The results shown below clearly indicate that celastrol induces relatively less cell death in normal cells when compared to MM cells.

A. Mouse Embryonic Fibroblast Cells



B. MCF-10A



C.

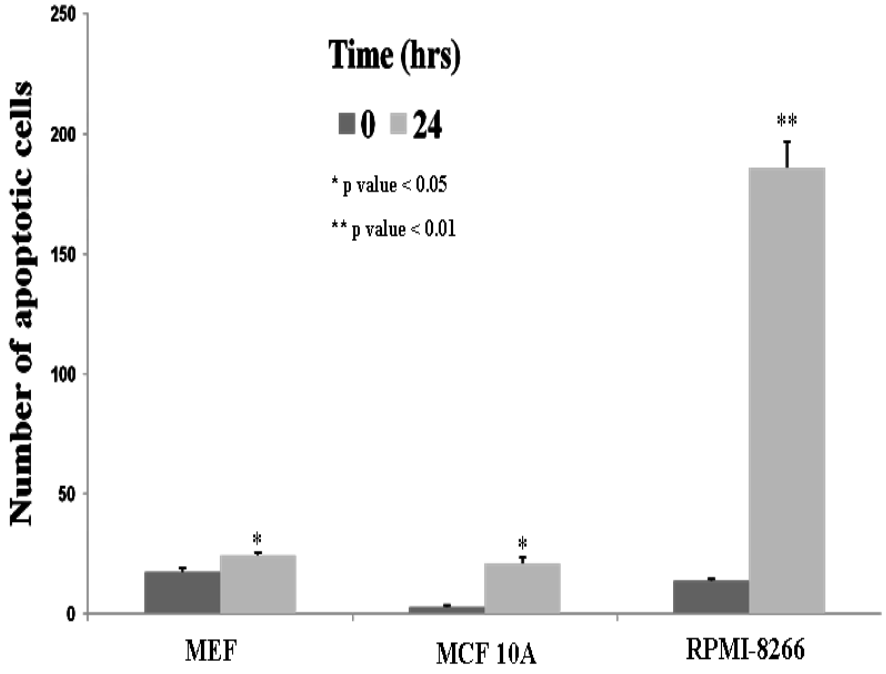


Figure 3.2.2.3. Celastrol causes relatively less cell death in normal mouse embryonic fibroblasts

A. Mouse embryonic fibroblasts were treated with 1 μM celastrol for 0, 6, 12, and 24 hrs. Cells were stained with Live/Dead assay reagent for 30 mins and then analyzed under a fluorescence microscope as described under Materials and Methods. A representative image for each group is shown.

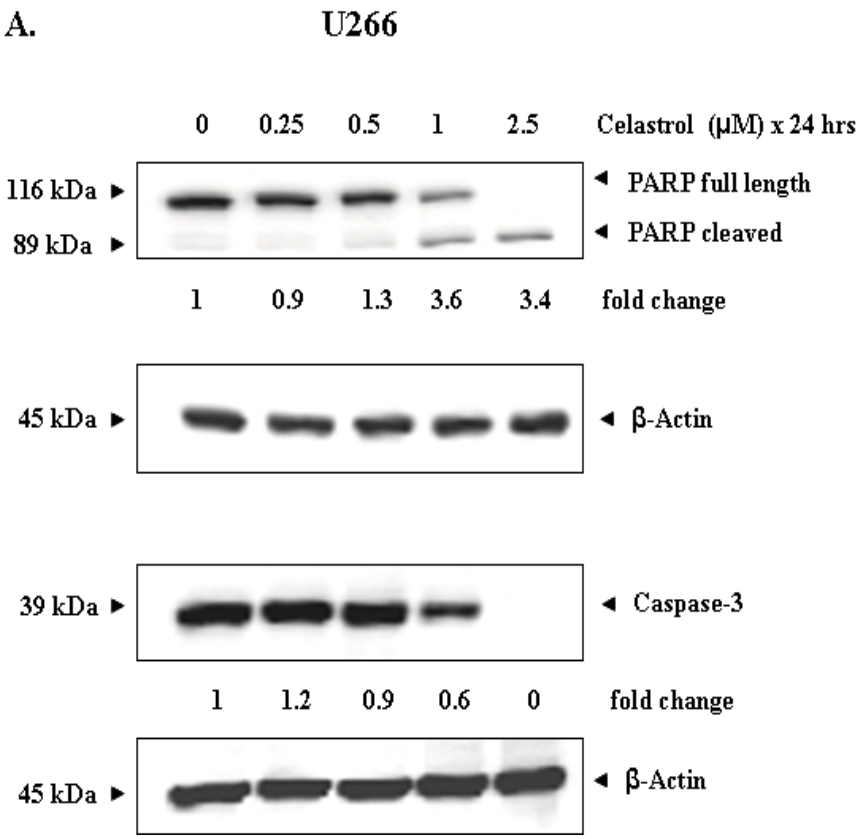
B. MCF-10A cells were treated with 1 μM celastrol for 0, 6, 12, and 24 hrs. Cells were stained with Live/Dead assay reagent for 30 mins and then analyzed under a fluorescence microscope as described under Materials and Methods. A representative image for each group is shown.

C. Live and dead assay was performed using RPMI-8226 cells, mouse embryonic fibroblast cells and MCF-10A cells as mentioned above. Number of apoptotic cells of RPMI-8226 cells, mouse embryonic fibroblast cells and MCF-10A cells in 10 microscopic fields were counted. Data is represented as average number of apoptotic cells $\pm$ SD (n=10).

3.2.2.4. Celastrol causes activation of caspase-3 to induce apoptosis in MM cells

Activation of the executioner caspases such as caspase-3 is the final step in the apoptotic pathway [426]. Thus activated caspase-3 can cleave its various substrates including PARP which will appear in its cleaved form during western blot analysis. MM cells were treated with 0, 0.25, 0.5, 1 and 2.5 uM celastrol for 24 hrs and 1 μM celastrol for 0, 3, 6, 12 and 24 hrs respectively and western blot assay was done to detect the full length caspase-3 and PARP. Our results indicate that there is a dose-dependent decrease in full length caspase-3, full length PARP and dose-dependent increase in cleaved PARP in both the cell lines. There was also a time-dependent decrease in full length caspase-3, full length PARP and a time-dependent increase in cleaved PARP in both the cell lines thereby indicating that celastrol induces apoptosis in both dose and time-dependent manner in MM cells.

A.


U266
0 0.25 0.5 1 2.5 Celastrol (μM) x 24 hrs
116 kDa
89 kDa
PARP full length
PARP cleaved
1 0.9 1.3 3.6 3.4 fold change
45 kDa
β-Actin
39 kDa
Caspase-3
1 1.2 0.9 0.6 0 fold change
45 kDa
β-Actin

B.

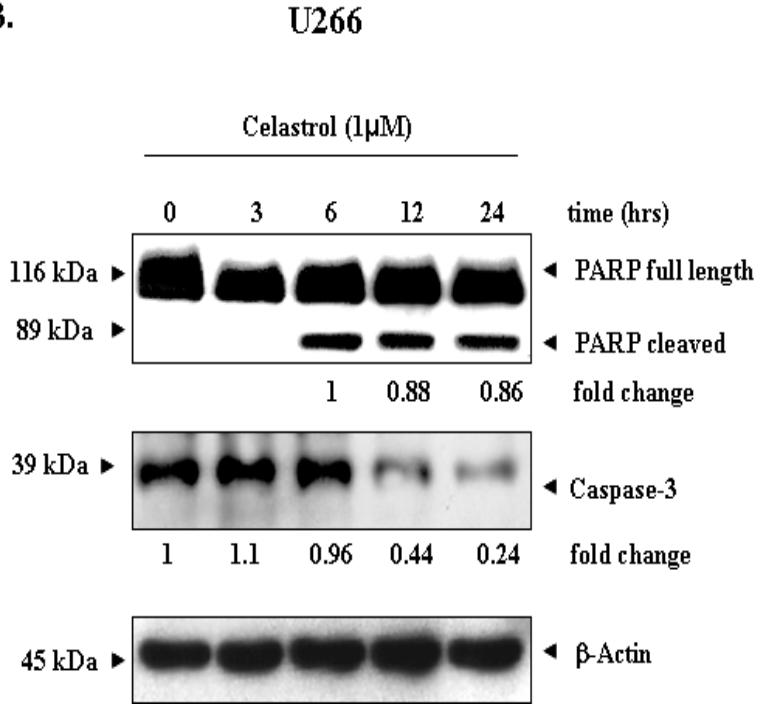

U266
Celastrol (1μM)
0 3 6 12 24 time (hrs)
116 kDa
89 kDa
PARP full length
PARP cleaved
1 0.88 0.86 fold change
39 kDa
Caspase-3
1 1.1 0.96 0.44 0.24 fold change
45 kDa
β-Actin

Figure 3.2.2.4. Celastrol activates caspase-3 and causes PARP cleavage

A. After treating U266 cells with 1 μM celastrol for the indicated duration, western blot assay was done by preparing whole-cell extracts. 30 μg protein was loaded and run on SDS-PAGE and transferred to nitrocellulose membrane. The blots were probed with anti-caspase-3 and anti-PARP antibodies. As a loading control, β-actin was probed after stripping the same blots. The blot shown is representative of at least two independent experiments. Densitometric analysis was done to determine fold change in intensity between treated and control groups.

B. After treating U266 cells with 0, 0.25, 0.5, 1 and 2.5 μM concentrations of celastrol for indicated duration, western blot assay was done using whole cell extracts. 30μg protein was loaded and run on SDS-PAGE and transferred to nitrocellulose membrane. The blots were probed with anti-caspase-3 and anti-PARP antibodies. As a loading control, β-actin was probed after stripping the same blots. The blot shown is representative of at least two independent experiments. Densitometric analysis was done to determine fold change in intensity between treated and control groups.

3.2.2.5. Celastrol activates both extrinsic and intrinsic caspase activation cascade to induce apoptosis in MM cells

Apoptosis can be the result of activation of extrinsic caspase cascade or intrinsic caspase cascade or by both. To analyze if celastrol-induced apoptosis involves extrinsic pathway and or intrinsic pathway, RPMI-8226 cells were treated with 1 μM celastrol for 0, 3, 6, 12 and 24 hrs and western blot assay was done to detect the expression of both caspase-8 and caspase-9. Bid which is activated by caspase-8-induced cleavage can also activate caspase-9. Bid, a BH3 containing protein is involved in relaying caspase mediated extrinsic and mitochondria mediated intrinsic pathway. Upon cleavage by activated caspase-8, the cleaved Bid causes the release of cytochrome c which in turn activates caspase- 9. The activity of Bid is opposed by Bcl-2 [427]. To determine if celastrol-induced apoptosis involves activation of Bid, western blot analysis of Bid was also done using specific antibodies under the same conditions used to detect caspase-8 and caspase-9. There was a time-dependent decrease in full length caspase-8 and caspase-9 and a corresponding increase in the cleaved band of caspase-8 and caspase-9, indicating that activation of both extrinsic and intrinsic apoptotic cascade is involved in the observed apoptotic effects of celastrol in MM cells. Also, a time-dependent decrease in the level of full length Bid was noted upon celastrol treatment in MM cells.

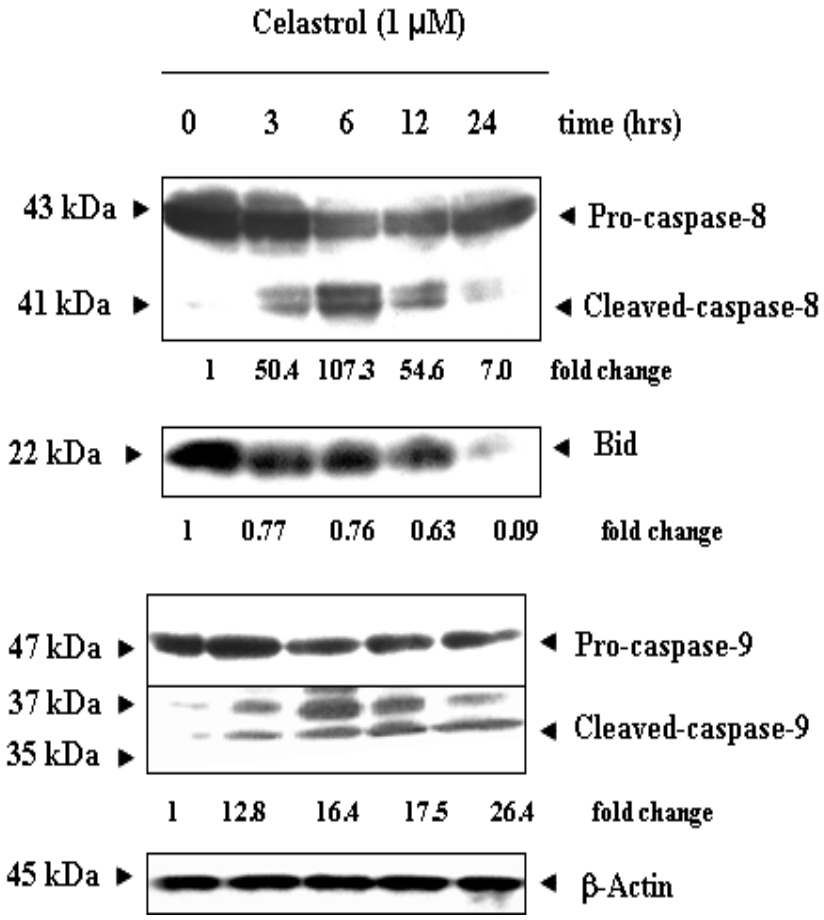


Figure 3.2.2.5. Celastrol causes the activation of both extrinsic and intrinsic caspase cascades in MM cells

After treating RPMI-8226 with 1 μM celastrol for various time points, western blot assay was done using whole-cell extracts. 50 μg protein was loaded and run on SDS-PAGE and transferred to nitrocellulose membrane. The blots were probed with anti-caspase-8, anti-caspase-9 and anti-Bid antibodies. As a loading control, β-actin was probed after stripping the same blots. The blot shown is representative of at least two independent experiments. Densitometric analysis was done to determine fold change in intensity between treated and control groups.

3.2.2.6. Celastrol causes downregulation of expression of various anti-apoptotic proteins involved in survival of MM cells

The anti-apoptotic and pro-apoptotic proteins maintain a balance which determines whether a cell is going to survive or undergo apoptosis [428]. To analyze if various anti-apoptotic proteins are downregulated during celastrol-induced apoptosis in MM cells, western blot analysis was performed. MM cells were treated with 1 μM celastrol for 0, 3, 6, 12 and 24 hrs in western blot analysis. Cells were harvested and western blot assay was done using specific antibodies to detect Bcl-2, Bcl-xL, Mcl-1, survivin and XIAP. It was found that celastrol can downregulate the above indicated anti-apoptotic proteins in a time-dependent manner in both the cell lines. It is evident from the result 3.2.2.5 that celastrol treated cells show a decrease in full length Bid level, which can be explained by cleavage of caspase-8. It is interesting to note that celastrol treated cells also show a substantial downregulation in the expression of Bcl-2 family of anti-apoptotic proteins including Bcl-2, Bcl-xL, Mcl-1, as well as survivin and XIAP in MM cells.

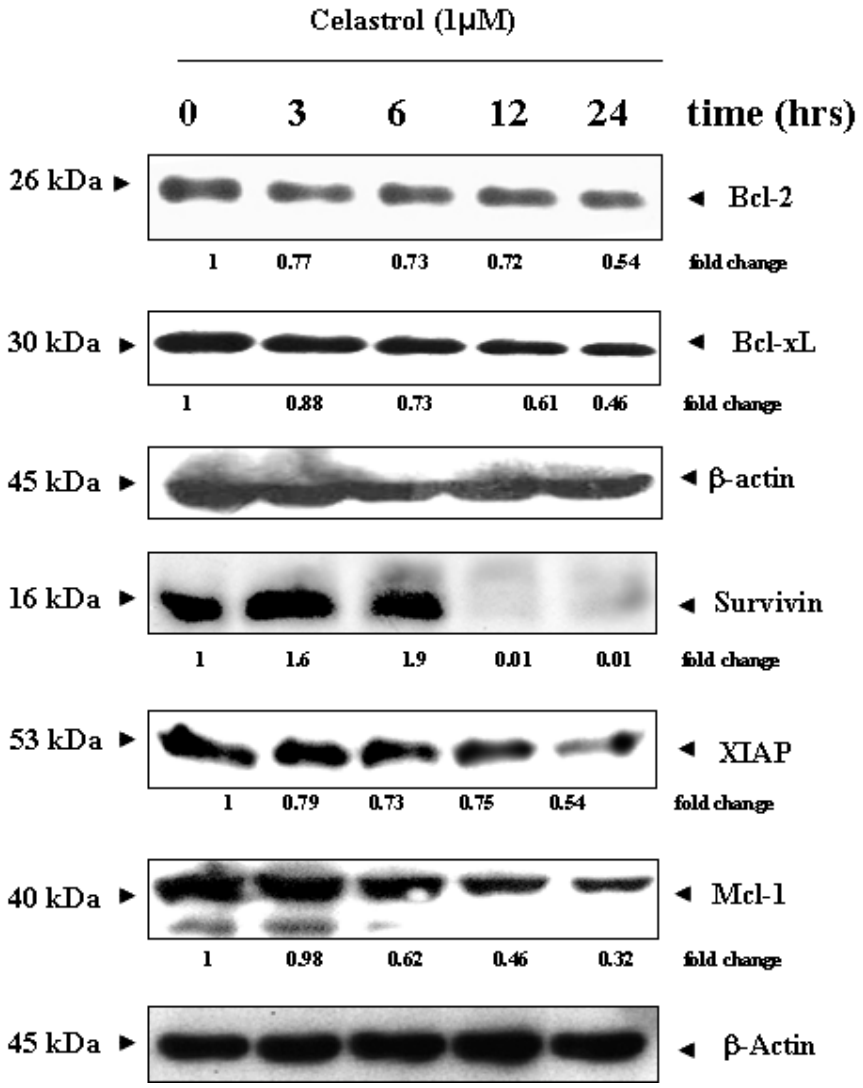


Figure 3.2.2.6. Celastrol downregulates expression of various anti-apoptotic proteins involved in survival of MM cells

After treating U266 cells with 1 μM celastrol for the indicated duration, western blot assay was done by preparing whole-cell extracts. 50 μg protein was loaded and run on SDS-PAGE and transferred to nitrocellulose membrane. The blots were probed for shown proteins using specific antibodies. As a loading control, β-actin was probed after stripping the same blots. The blot shown is representative of at least two independent experiments. Densitometric analysis was done to determine fold change in intensity between treated and control groups.

3.2.2.7. Celastrol causes downregulation of expression of various anti-apoptotic proteins involved in survival of MM cells at transcriptional level

Please refer to the appendix II.

3.2.2.8. Celastrol increases the expression of various pro-apoptotic proteins in MM cells

The balance between proapoptotic and anti-apoptotic members of the Bcl-2 family of proteins is a crucial factor which determines whether a cell is going to survive or undergo apoptosis [428]**.** As observed from the above mentioned results, a down-regulation of various Bcl-2 families of anti-apoptotic proteins namely Bcl-2, Bcl-xL and Mcl-1 was noticed in celastrol-treated MM cells. To analyze whether pro-apoptotic members of Bcl-2 family proteins are also upregulated upon celastrol-induced apoptosis in MM cells, U266 and RPMI-8226 cells were treated with 1 μM celastrol for 0, 3, 6, 12 and 24 hrs. Cells were harvested and western blot assay was done using specific antibodies to detect the expression level of Bax and Bak proteins. Our results reveal that celastrol upregulates the expression of pro-apoptotic proteins Bax and Bak in a time-dependent manner in both the cell lines.

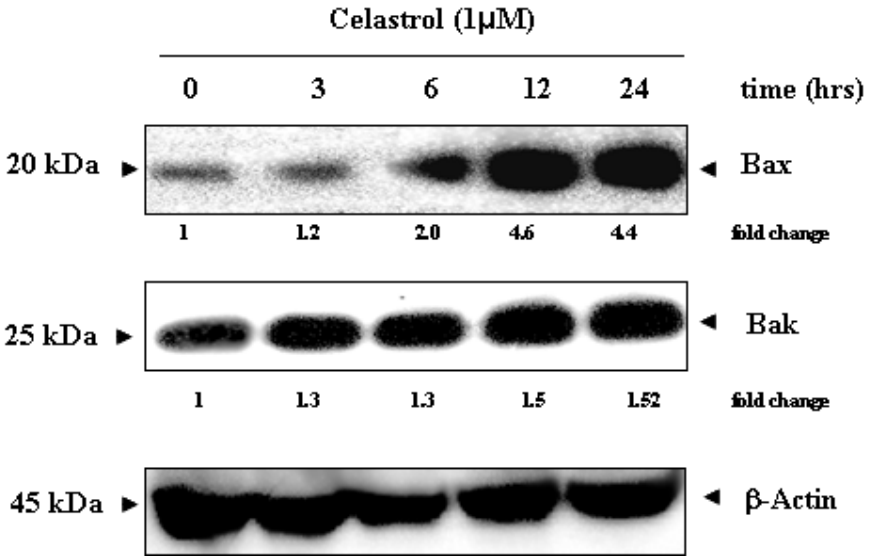


Figure 3.2.2.8. Celastrol causes upregulation of pro-apoptotic members of Bcl-2 family proteins in MM cells.

After treating U266 cells with 1 μM celastrol for the indicated duration, western blot assay was done by preparing whole-cell extracts. 50 μg protein was loaded and run on SDS-PAGE and transferred to nitrocellulose membrane. The blots were probed for shown proteins using specific antibodies. As a loading control, β-actin was probed after stripping the same blots. The blot shown is representative of at least two independent experiments. Densitometric analysis was done to determine fold change in intensity between treated and control groups.

3.2.2.9. Celastrol causes JNK kinase activation in MM cells

JNK kinase is a stress kinase that is often activated when cancer cells are exposed to chemotherapeutic agents [429, 430]. Activation of JNK kinase has been found to mediate apoptosis induced by various stress stimuli. Since celastrol caused significant apoptosis in MM cells, we aimed to determine if activation of JNK MAP kinase can mediate the observed apoptotic effects of celastrol. To analyze this possibility, western blot assay was performed to detect p-JNK. RPMI-8226 cells were incubated with 1 μM celastrol for the indicated duration of time. It is evident from the result shown below that celastrol induced JNK activation as early as 15 minutes after treatment without altering the levels of JNK protein expression (Figure A). It is also evident that the specific JNK inhibitor, SP600125 completely inhibited celastrol-induced JNK activation (Figure B). However, we found that celastrol had a minimal effect on the activation of p38 and p42/44MAPkinases in MM cells (data not shown), thereby suggesting that JNK MAP Kinase may play a pivotal role in the observed apoptotic effects of celastrol.

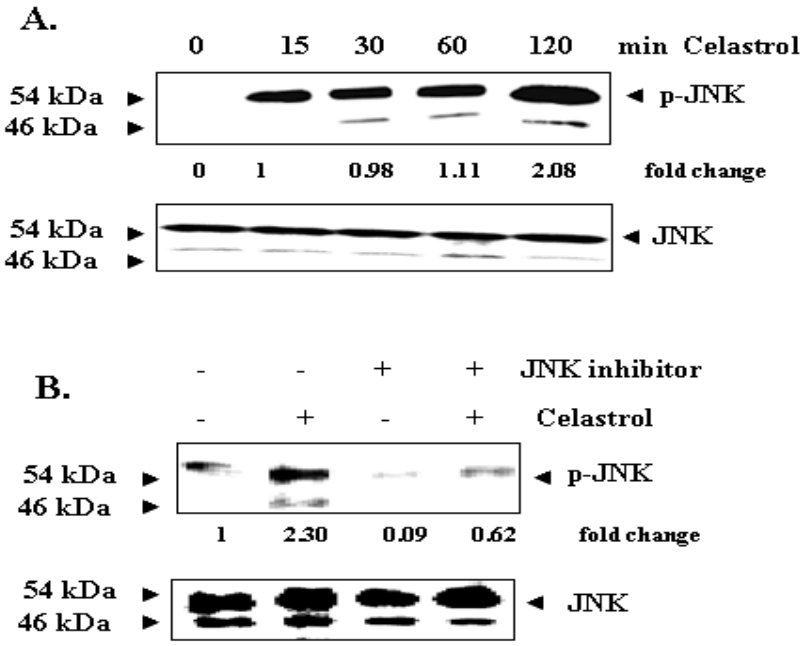


Figure 3.2.2.9. Celastrol induces JNK activation and celastrol-induced JNK activation is inhibited by JNK inhibitor (SP600125)

A. After treating RPMI-8226 cells with 1 μM celastrol for the indicated duration, western blot assay was done by preparing whole-cell extracts. 50μg protein was loaded and run on SDS-PAGE and transferred to nitrocellulose membrane. The blot was probed for p-JNK using anti-p-JNK antibody. As a loading control, total JNK protein was probed after stripping the same blot. The blot shown is representative of at least two independent experiments. Densitometric analysis was done to determine fold change in intensity between treated and control groups.

B. RPMI-8226 cells were pretreated with 10 μM JNK inhibitor (SP600125) for 1 h and then treated with 1 μM celastrol for 2 hrs, western blot assay was done by preparing whole-cell extracts. 50 μg protein was loaded and run on SDS-PAGE and transferred to nitrocellulose membrane. The blot was probed for p-JNK using anti-p-JNK antibody. As a loading control, total JNK was probed using anti-JNK antibody after stripping the same blot. The blot shown is representative of at least two independent experiments. Densitometric analysis was done to determine fold change in intensity between treated and control groups.

3.2.2.10. Inhibition of JNK activation abrogates celastrol-induced apoptosis in MM cells

From our previous experimental result, we understand that celastrol induces JNK activation. We next sought to determine whether suppression of JNK by a specific inhibitor of JNK blocks cell death induced by celastrol. To analyze this possibility $SubG_1$ analysis was done. Cells were pre-treated with SP600125 and then treated with indicated concentrations of celastrol for 24 hrs. We next examined the effect of SP600125 on celastrol-induced apoptosis. The results shown below clearly suggest that inhibition of JNK activation can suppress apoptosis induced by celastrol. To further confirm if celastrol induced apoptosis is mediated by JNK, cells were pretreated with SP600125 and then treated with 1 μM of celastrol for indicated time duration. Western blot assay was done to detect the PARP level. The results show that cleaved PARP level increase in time dependent manner in cells treated with celastrol alone. Whereas, in cells treated with celastrol and SP600125 there is no cleavage of PAPR indicating that SP600125 abrogated celastrol induced apoptosis. These observations suggest that celastrol-induced apoptosis at least partially mediated through the activation of JNK.

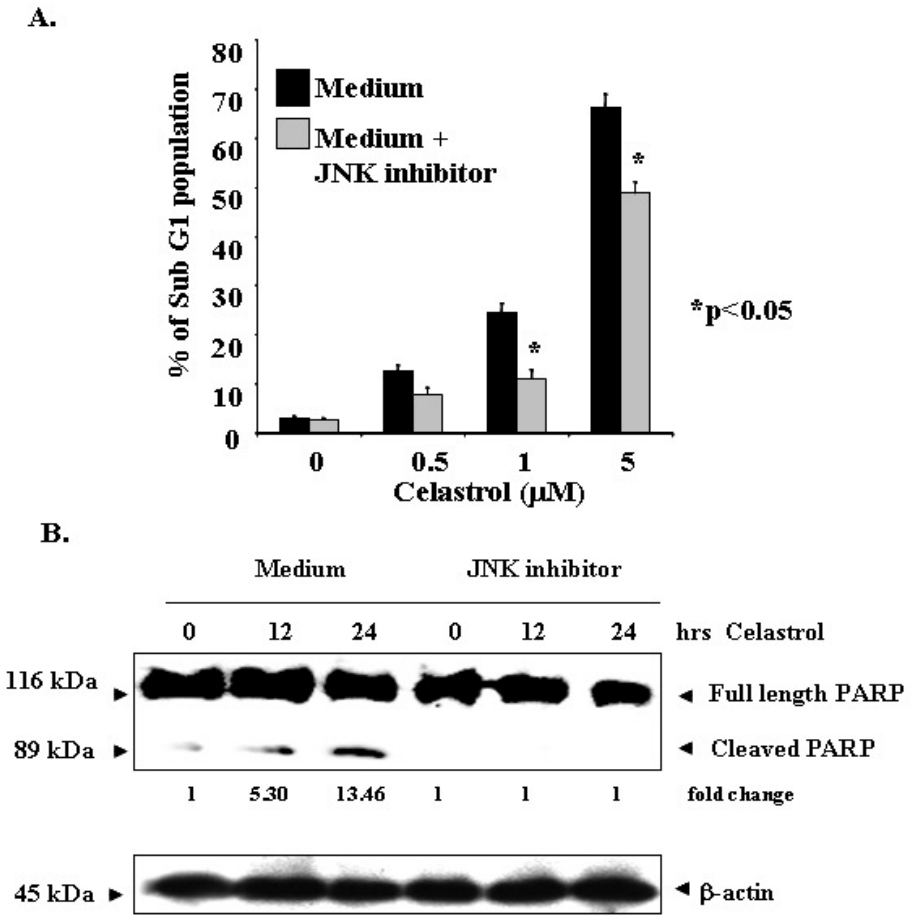


Figure 3.2.2.10. JNK inhibition blocks celastrol-induced apoptosis

A. After treating RPMI-8226 cells with indicated concentrations of celastrol alone and or in combination with JNK inhibitor for 24 hrs, sub G_1 analysis was done using flow cytometry to assess the apoptotic cells.

B. After treating RPMI-8226 cells with indicated concentrations of celastrol alone and or in combination with JNK inhibitor for 24 hrs and the cells, western blot assay was done by preparing whole-cell extracts. 5μg protein was loaded and run on SDS-PAGE and transferred to nitrocellulose membrane. The blot was probed for PARP using anti-PARP antibody. As a loading control, β-actin was probed after stripping the same blots. The blot shown is representative of at least two independent experiments. Densitometric analysis was done to determine fold change in intensity between treated and control groups.

3.2.3. Celastrol modulates several proteins involved in cell cycle progression in MM cells

Most of the anti-cancer drugs exert their anti-cancer activity either by inducing apoptosis or by arresting cell cycle progression or exert both these activities [431]. To analyze, if celastrol can modulate the expression of various proteins involved in cell cycle progression, western blot analysis to detect levels of cyclin D1, cyclin E, p21 and p27 was performed. Interestingly, it was found in our experimental results that celastrol downregulated the expression of several proteins involved in cell cycle progression namely cyclin D and cyclin E and upregulated proteins involved in cell cycle arrest namely p21 and p27 in a time-dependent manner.

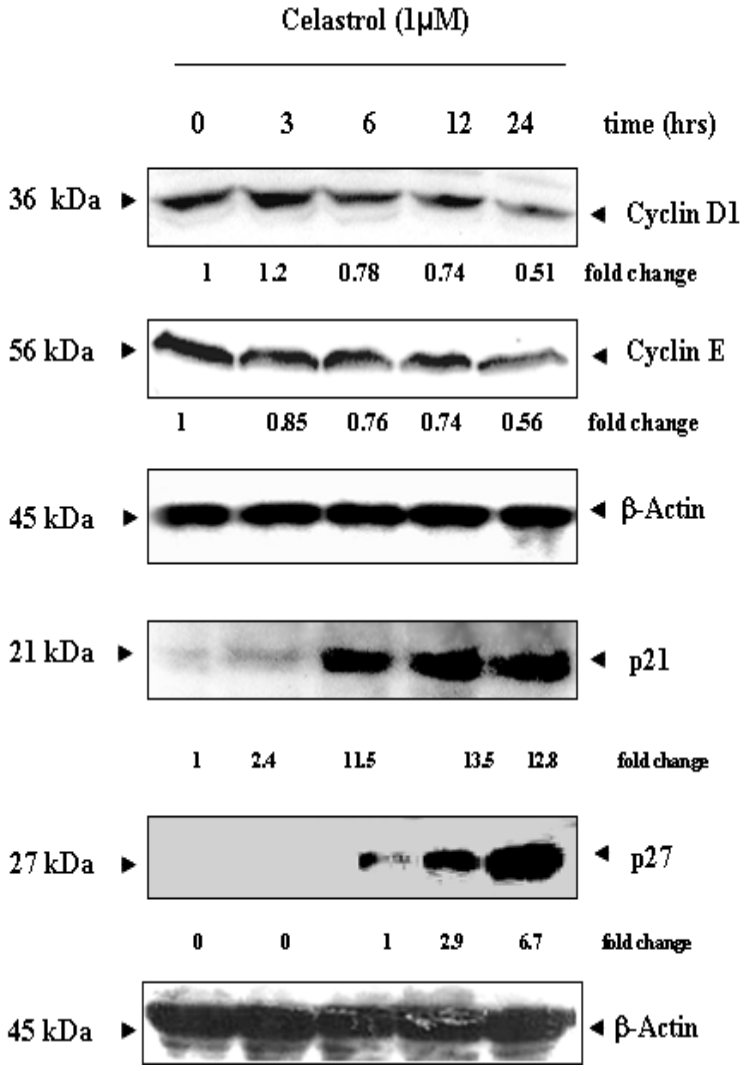


Figure 3.2.3. Celastrol modulates the expression of proteins involved in cell cycle progression

After treating RPMI-8266 cells with 1 μM celastrol for the indicated duration, western blot assay was done by preparing whole-cell extracts. 50 μg protein was loaded and run on SDS-PAGE and transferred to nitrocellulose membrane. The blots were probed for shown proteins using specific antibodies. As a loading control, β-actin was probed after stripping the same blots. The blot shown is representative of at least two independent experiments. Densitometric analysis was done to determine fold change in intensity between treated and control groups.

3.2.4. Celastrol downregulates the expression of proteins involved in myeloma angiogenesis/aggressiveness

Angiogenesis is another crucial hallmark characteristic feature of cancer cells, thereby increasing the aggressiveness of the cancer. [2, 3]. VEGF and c-Myc not only play an important role in myeloma angiogenesis but also in cell survival. To investigate if celastrol can modulate the expression of VEGF and c-Myc in MM cells, RPMI-8226 cells were treated with 1 μM celastrol for 0, 3, 6, 12 and 24 hrs. Thereafter, western blot analysis was done using VEGF-specific and c-Myc specific antibodies and β-actin as loading control. Our experimental results clearly show that celastrol at 1 μM concentration can downregulate the expression of both VEGF and c-Myc proteins in a time-dependent manner in RPMI-8226 cells.

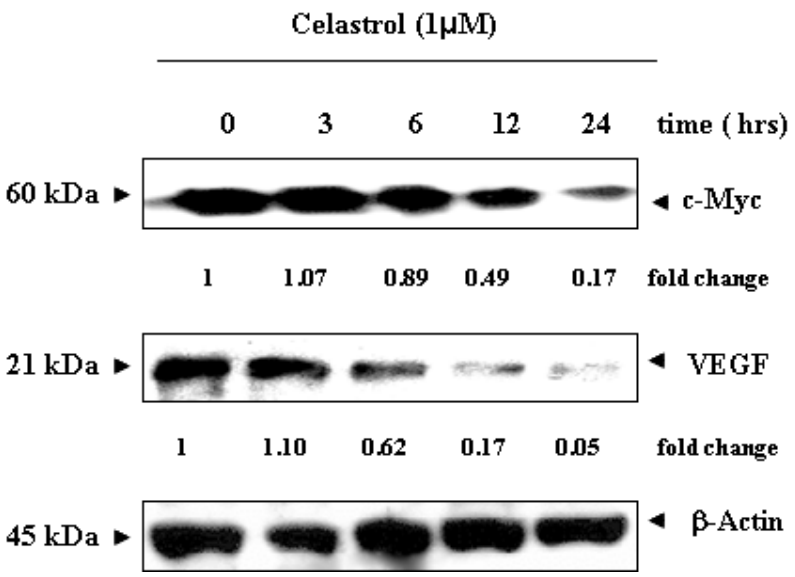


Figure 3.2.4. Celastrol downregulates the expression of proteins involved in myeloma angiogenesis/aggressiveness

After treating RPMI-8266 cells with 1 μM celastrol for the indicated duration, western blot assay was done by preparing whole-cell extracts. 50 μg protein was loaded and run on SDS-PAGE and transferred to nitrocellulose membrane. The blots were probed for shown proteins using specific antibodies. As a loading control, β-actin was probed after stripping the same blots. The blot shown is representative of at least two independent experiments. Densitometric analysis was done to determine fold change in intensity between treated and control groups.

3.2.5. Celastrol inhibits MM cell migration and invasion

3.2.5.1. Celastrol inhibits CXCL12-induced MM cell migration

Migration of tumor cell is an important hallmark characteristic feature which is crucial to the processes of angiogenesis and to metastasis [2, 3]. We next analyzed if celastrol treatment can also abrogate migration potential of MM cells. To explore this possibility, a transwell migration assay was performed. In a transwell migration assay, tumor cells migrate through the transwell inserts with 8-um pores towards chemokine (CXCL12) containing outer chamber. To analyze whether, celastrol can inhibit this chemo-attractant stimulated migration of MM cells, U266 cells were treated with 1 μM celastrol and or 100 ng/ml CXCL12 for 12 hrs using transwell migration chambers. Migrated cells were stained with calcein-AM and the fluorescence was measured. Using the standard curve, number of migrated cells was calculated from relative fluorescence of treated samples. There was 40.9% inhibition in migration in the absence of CXCL12 ($p < 0.05$) and 28.37 % inhibition in migration in the presence of CXCL12 ($p < 0.05$). Results of our experiments clearly show that celastrol can indeed inhibit CXCL12-induced myeloma cell transwell migration in statistically significant manner.

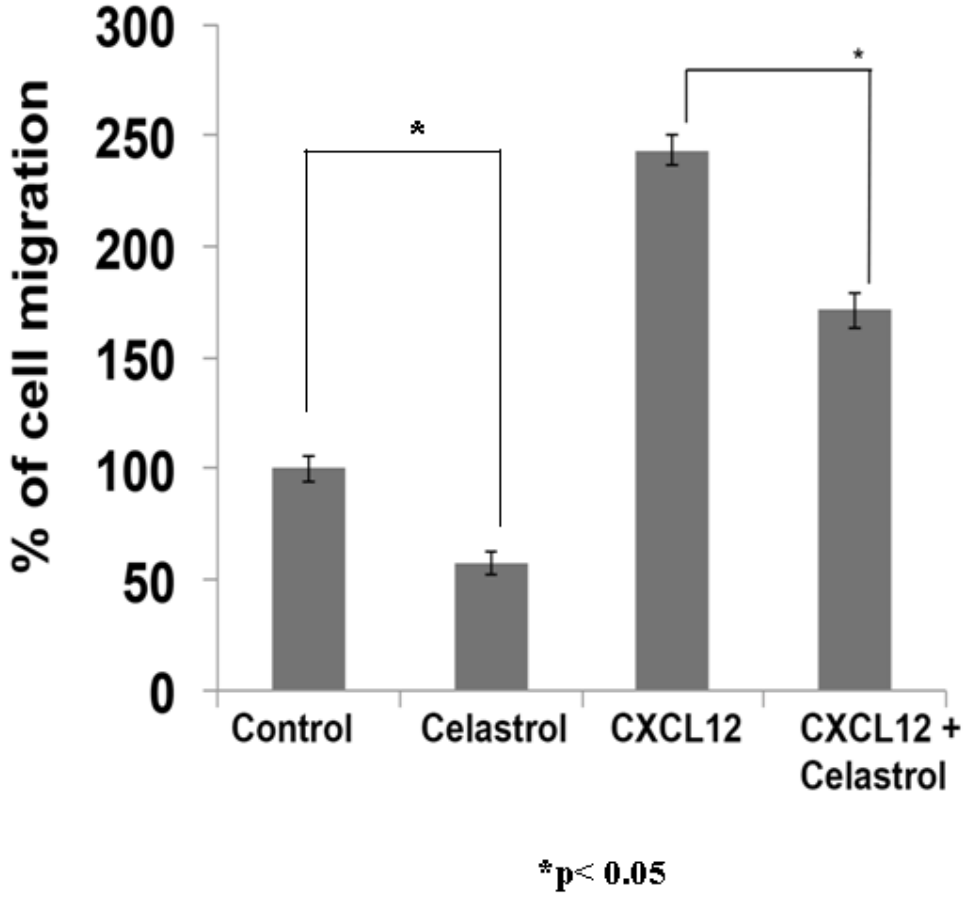


Figure 3.2.5.1. Celastrol inhibits CXCL12-induced MM cell migration

U266 cells were seeded in triplicate and treated with 1 μM celastrol and or 100 ng/ml CXCL12 for12 hrs using transwell migration chambers. Transwell migration towards CXCL12 was measured using calcein-AM staining and measuring the fluorescence intensity. Data are expressed as percentage of mean cell migration ± SD relative to the control group.

3.2.5.2. Celastrol inhibits CXCL12-induced MM cell invasion

Invasive capability of tumor cells not only enables them to get into the circulatory system but also to spread to various parts of the body and finally to get distributed into various potential sites of metastasis [2, 3]. To analyze if celastrol can affect invasive potential of MM cells, a matrigel invasion assay was performed. Matrigel chamber used in migration assay has inner and outer chambers. The base of the inner chamber is made up of polycarbonate membrane containing 8-μm pores. The outer chamber contains the chemo-attractant (CXCL12) and the cells with greater migratory potential move towards the chemoattractive gradient through the pores. However, in invasion assay, the inner chamber is often coated with basement membrane proteins. Cancer cells with invasive potential move towards the chemoattractant gradient by invading the basement membrane.

U266 cells were treated with 1 μM celastrol and or CXCL12 (100 ng/ml) for 12 hrs in matrigel invasion chambers. Invaded myeloma cells were stained with calcein-AM and the fluorescence was measured. Using the standard curve, number of invaded cells was calculated from relative fluorescence of treated samples. There was 35.53% inhibition in the invasive potential in the absence of CXCL12 ($p < 0.05$) and 37.82 % reduction in the invasive potential in the presence of CXCL12 ($p < 0.05$). It was found that celastrol can significantly inhibit CXCL12-induced myeloma cell invasion in a statistically significant manner.

The migration assay just measures the movement of cells across a polycarbonate membrane with pores, while the invasion assay analyzes movement across the polycarbonate membrane coated with matrigel. So in the invasion assay, the degradation of matrigel by the cells is also taken into consideration. The cells with migration potential will not be counted in invasion assay if the cells do not have invasion potential. So even if the inhibition percentage for migration and invasion looks similar, the migration is not the only factor contributing to the invasive potential of tumor cells.

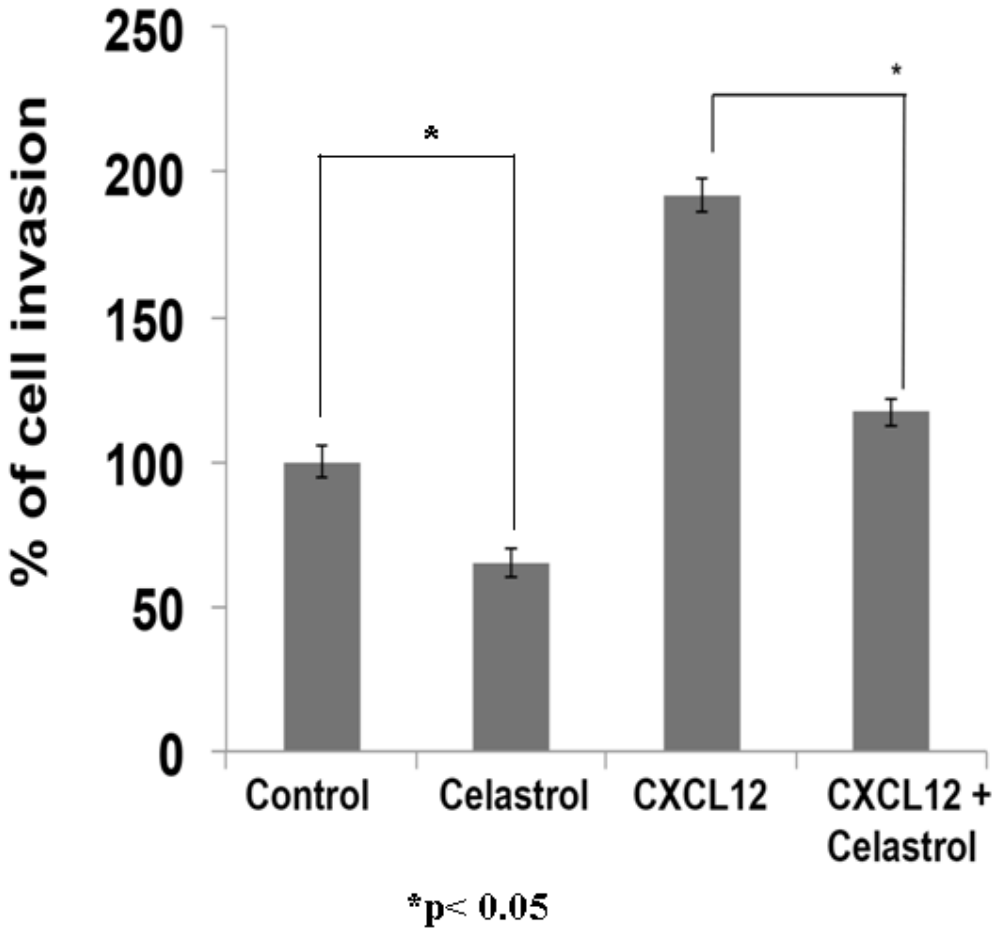


Figure 3.2.5.2. Celastrol inhibits CXCL12-induced MM cell invasion

U266 cells were seeded in triplicate and treated with 1 μM celastrol and or 100 ng/ml CXCL12 for 12 hrs in matrigel invasion chambers. Matrigel invasion towards CXCL12 was measured by staining the cells with calcein-AM and measuring the fluorescence intensity. Data are expressed as percentage of mean cell invasion $\pm$ SD relative to the control group.

3.2.5.3. Celastrol downregulates the expression of various proteins involved in MM cell migration and invasion

Several proteins have been reported to be involved in the orchestration of migration and invasion processes in tumor cells [432]**.** Among these, CXCR4 and MMP-9 are the two key molecules, which play crucial role in migration and invasion of various tumor cells, including MM [433, 434]. To analyze if celastrol-induced inhibition of migration and invasion also involves modulation of CXCR4 and MMP-9 expression, U266 cells were treated with 1 μM celastrol for 0, 3, 6 and 12 hrs. Whole cell extract was prepared, separated using SDS-page and probed for CXCR4 and MMP-9 using specific antibodies. It was found that celastrol substantially downregulated CXCR4 and MMP-9 in a time-dependent manner. These results clearly establish the anti-migratory/anti-invasive potential of celastrol through the suppression of CXCR4 and MMP-9 proteins in MM cells.

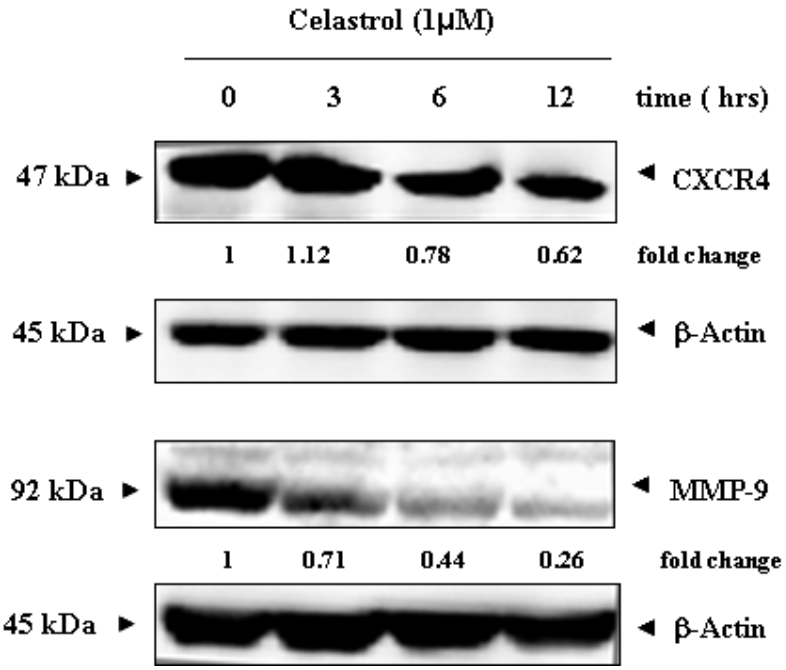


Figure 3.2.5.3. Celastrol downregulates the expression of proteins involved in MM cell migration and invasion

U266 cells were treated with 1 μM celastrol for the indicated time points, western blot assay was done by preparing whole-cell extracts. 50 μg protein was loaded and run on SDS-PAGE and transferred to nitrocellulose membrane. The blots were probed for shown proteins using specific antibodies. As a loading control, β-actin was probed after stripping the same blots. The blot shown is representative of at least two independent experiments. Densitometric analysis was done to determine fold change in intensity between treated and control groups.

3.3. Identification of celastrol as a novel inhibitor of STAT3 activation pathway

3.3.1. U266 cells express constitutively active STAT3

As discussed in the introduction, constitutive activation of transcription factor, STAT3 plays an important role in progression of many cancers including MM. To analyze the expression and activation profile of STAT3 in human MM cell lines, various MM cells including U266, RPMI-8226, RPMI-8226-Dox6, RPMI-8226-LR5, and RPMI-8226-BR were used and western blot analysis was done to detect the basal expression level of p-STAT3 (Tyr-705) and STAT3. (Hereafter in this thesis, p-STAT3 indicates tyrosine phosphorylation of STAT3 at 705 residue unless stated otherwise). Our experimental results clearly confirm that U266 cells express substantial levels of constitutively phosphorylated STAT3. On the contrary, although RPMI-8226 cells express STAT3, these cell lines do not display phosphorylated levels of STAT3 under non-stimulated conditions.

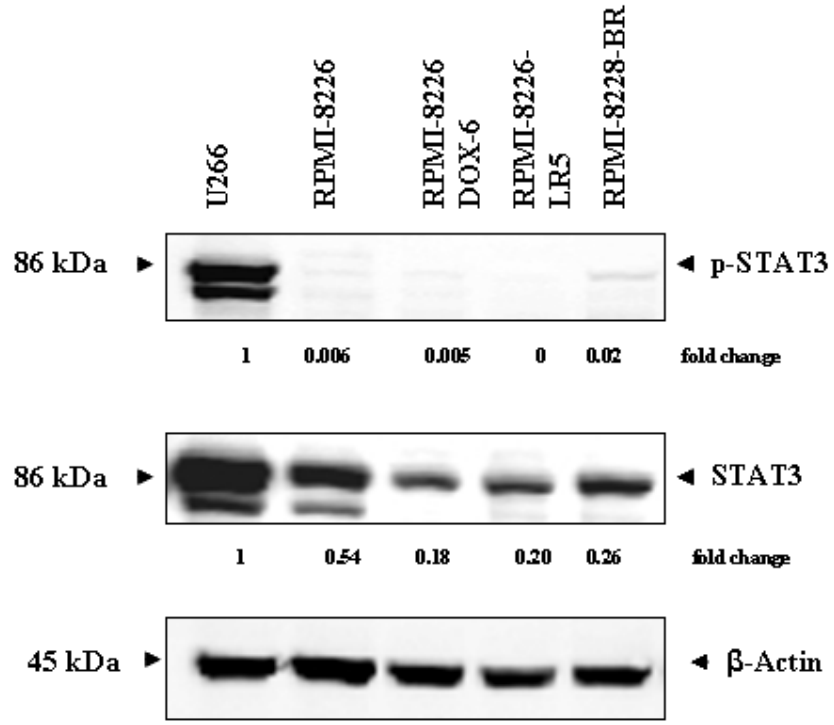


Figure 3.3.1. Basal level of p-STAT3 and STAT3 expression in MM cell lines

Whole cell extracts of different human MM cells including U266, RPMI-8226, RPMI-8226-Dox6 cells, RPMI-8226-LR5, and RPMI-8226-BR were prepared. 50μg protein was loaded and run on SDS-PAGE and transferred to nitrocellulose membrane. The blot was probed for p-STAT3 using anti-p-STAT3 antibody. As a loading control, total STAT3 and β-actin proteins were probed after stripping the same blot. The blot shown is representative of at least two independent experiments. Densitometric analysis was done to determine fold change in intensity between proteins obtained from U266 and other MM cell lines.

3.3.2. Treatment with cytokine IL-6 induces STAT3 phosphorylation in RPMI-8226 cells and its resistant clones

As observed in the above indicated experimental results, RPMI-8226 clones do not constitutively phosphorylate STAT3 under basal conditions. However, it has been reported that these cells can phosphorylate STAT3 upon induction with cytokines such as, IL-6. To confirm this, RPMI-8226 cells were treated with indicated concentrations of IL-6 for 15 mins and in another set of experiments RPMI-8226 cells were treated with 10 ng/ml of IL-6 for indicated time points. Thereafter, western blot assay was done to detect IL-6-induced phosphorylation of STAT3. Our experimental findings clearly show that IL-6 can induce phosphorylation of STAT3 in a time and dose-dependent manner.

To analyze the phosphorylation profile of STAT3 in resistant clones of RPMI- 8226 cells western blot assay was performed. RPMI-8226 cells, RPMI-8226-Dox6 cells (resistant to doxorubicin), RPMI 8226-LR5 cells (resistant to melphalan), and RPMI-8226-BR (resistant to bortezomib) were treated with 10 ng/ml of IL-6 for 15 mins and western blot assay was performed using p-STAT3 specific antibodies. Our experimental results clearly reveal that analogous to RPMI-8226 cells, the resistant clones of RPMI-8226 cells also phosphorylate STAT3 upon induction with IL-6.

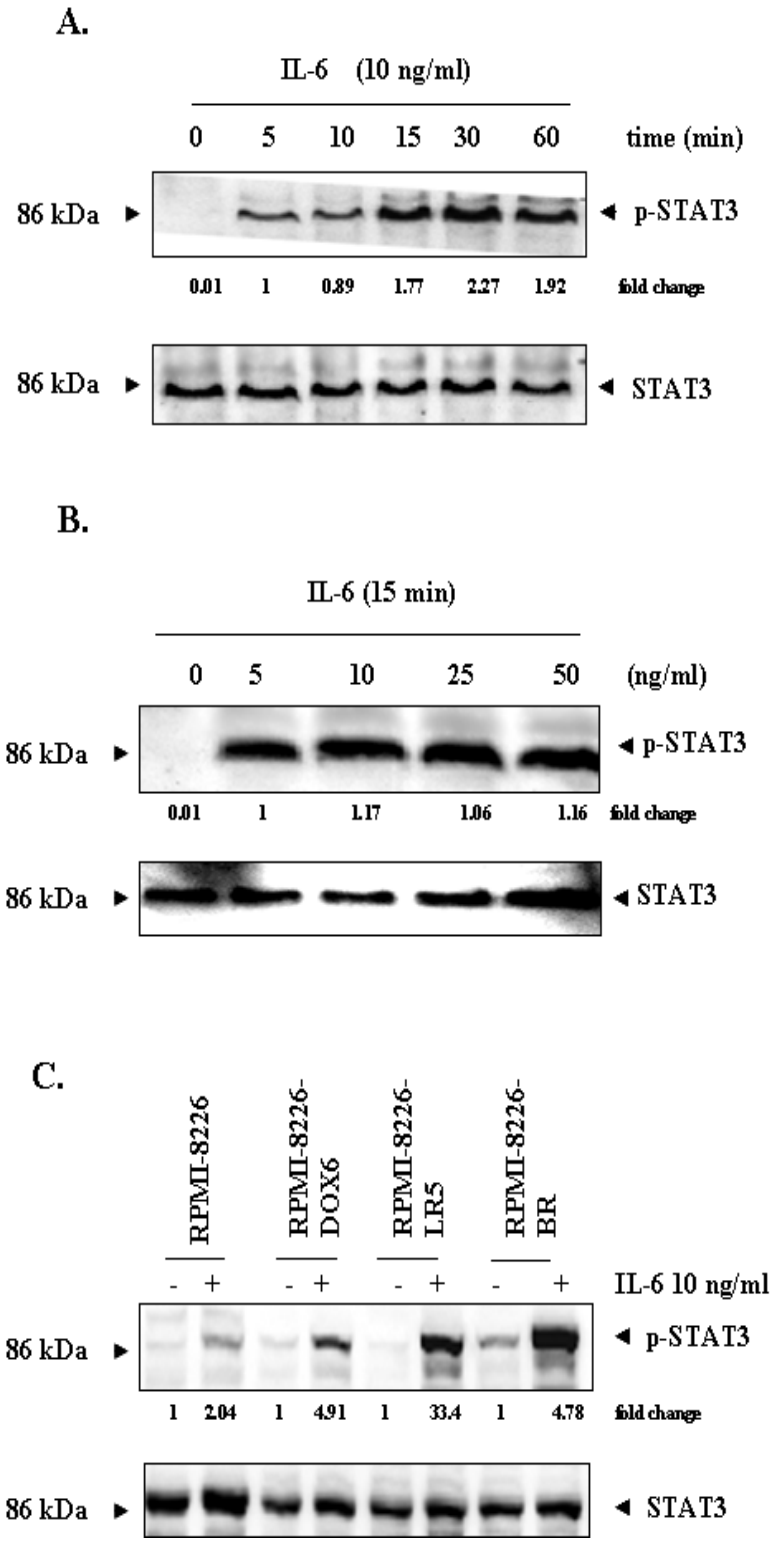

A.
IL-6 (10 ng/ml)
0 5 10 15 30 60 time (min)
86 kDa
p-STAT3
0.01 1 0.89 1.77 2.27 1.92 fold change
86 kDa
STAT3
B.
IL-6 (15 min)
0 5 10 25 50 (ng/ml)
86 kDa
p-STAT3
0.01 1 1.17 1.06 1.16 fold change
86 kDa
STAT3
C.
RPMI-8226
RPMI-8226-DOX6
RPMI-8226-LR5
RPMI-8226-BR
- + - + - + - + IL-6 10 ng/ml
86 kDa
p-STAT3
1 2.04 1 4.91 1 33.4 1 4.78 fold change
86 kDa
STAT3

Figure 3.3.2. RPMI-8226 cells and its resistant clones phosphorylate STAT3 upon induction with IL-6

A. RPMI-8226 cells were treated with 10 ng/ml of IL-6 for 0, 5, 10, 15, 30 and 60 mins and whole cell extract was prepared. 50 μg protein was loaded and run on SDS-PAGE and transferred to nitrocellulose membrane. The blot was probed for p-STAT3 using anti-p-STAT3 antibody. As a loading control, total STAT3 protein was probed using anti-STAT3 antibody after stripping the same blot. The blot shown is representative of at least two independent experiments. Densitometric analysis was done to determine fold change in intensity between treated and control groups.

B. RPMI 8226 cells were treated with 0, 5, 10, 25 and 50 ng/ml of IL-6 for 15 mins and whole cell extract was prepared. 50 μg protein was loaded and run on SDS-PAGE and transferred to nitrocellulose membrane. The blot was probed for p-STAT3 using anti-p-STAT3 antibody. As a loading control, total STAT3 was probed using anti-STAT3 antibody after stripping the same blot. The blot shown is representative of at least two independent experiments. Densitometric analysis was done to determine fold change in intensity between treated and control groups.

C. RPMI 8226 cells, RPMI 8226-Dox6 cells, RPMI 8226-LR5 cells, and RPMI-8226-BR were treated with 10 ng/ml of IL-6 for 15 mins and whole cell extracts were prepared. 50 μg protein was loaded and run on SDS-PAGE and transferred to nitrocellulose membrane. The blot was probed for p-STAT3 using anti-p-STAT3 antibody. As a loading control, total STAT3 was probed using anti-STAT3 antibody after stripping the same blot. The blot shown is representative of at least two independent experiments. Densitometric analysis was done to determine fold change in intensity between treated and control groups.

3.3.3. Celastrol inhibits the constitutive phosphorylation of STAT3 in a dose- and time-dependent manner in MM cells

To analyze whether above observed anti-myeloma effects of celastrol are associated with inhibition of constitutive STAT3 activation, U266 cells were treated with 0, 0.5, 1, 2.5, 5 μM celastrol for 4 hrs and western blot analysis was done to detect the level of p-STAT3 and STAT3. Our experimental results demonstrate that celastrol can inhibit constitutive phosphorylation of STAT3 in a dose-dependent manner in U266 cells with minimal effect on total STAT3 levels.

For time kinetics study, U266 cells were treated with 2.5 μM celastrol for 0, 30, 60, 120 and 240 mins and western blot assay was done to detect p-STAT3 and STAT3 expression levels. Total STAT3 served as loading control. Our experimental findings reveal that celastrol inhibits constitutive phosphorylation of STAT3 in a time-dependent manner with minimal effect on total STAT3 levels.

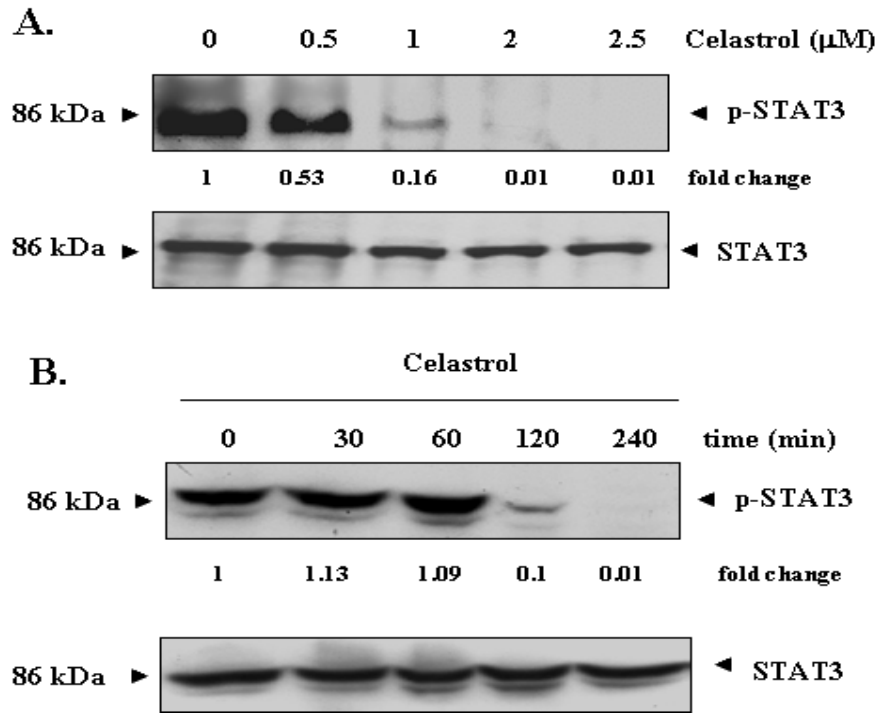


Figure 3.3.3. Celastrol inhibits the constitutively activated STAT3 in a dose- and time-dependent manner

A. U266 cells were treated with 0, 0.5, 1, 2.5 and 5 μM celastrol for 4 hrs and whole cell extract was prepared. 50 μg protein was loaded and run on SDS-PAGE and transferred to nitrocellulose membrane. The blot was probed for p-STAT3 using anti-p-STAT3 antibody. As a loading control, total STAT3 was probed using anti-STAT3 antibody after stripping the same blot. The blot shown is representative of at least two independent experiments. Densitometric analysis was done to determine fold change in intensity between treated and control groups.

B. U266 cells were treated with 2.5 μM celastrol for 0, 30, 60, 120 and 240 mins and whole cell extract was prepared. 50 μg protein was loaded and run on SDS-PAGE and transferred to nitrocellulose membrane. The blot was probed for p-STAT3 using anti-p-STAT3 antibody. As a loading control, total STAT3 was probed using anti-STAT3 antibody after stripping the same blot. The blot shown is representative of at least two independent experiments. Densitometric analysis was done to determine fold change in intensity between treated and control groups.

3.3.4. Celastrol inhibits IL-6-induced phosphorylation of STAT3 in a time-dependent manner

As demonstrated from the above experiments, RPMI-8266 cells display phosphorylated STAT3 expression upon stimulation with IL-6. Also, we have shown that celastrol can inhibit constitutive phosphorylation of STAT3 in U266 cells. On a similar note, we next aimed to determine whether celastrol has any potential effect on the IL-6-induced phosphorylation of STAT3 in MM cells. To analyze this possibility, RPMI-8266 cells were pretreated with 2.5 μM celastrol for 0, 30, 60, 120 and 240 mins and then the cells were induced with 10 ng/ml of IL-6 for 15 mins. Western blot assay was done to detect p-STAT3 levels. It was found that celastrol indeed inhibited the IL-6-induced phosphorylation of STAT3 in a time-dependent manner with minimal effect on total STAT3 levels.

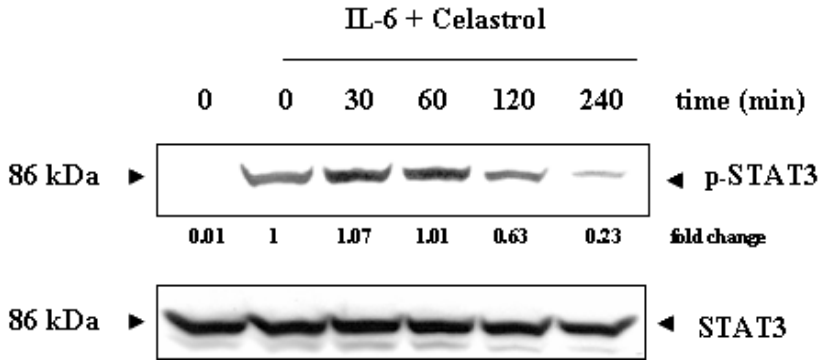


Figure 3.3.4. Celastrol inhibits IL-6-induced phosphorylation of STAT3 in a time-dependent manner

RPMI-8266 cells were pretreated with 2.5 μM celastrol for 0, 30, 60, 120 and 240 mins and then the cells were induced by 10 ng/ml of IL-6 for 15 mins and whole cell extract was prepared. 50 μg protein was loaded and run on SDS-PAGE and transferred to nitrocellulose membrane. The blot was probed for p-STAT3 using anti-p-STAT3 antibody. As a loading control, total STAT3 was probed using anti-STAT3 antibody after stripping the same blot. The blot shown is representative of at least two independent experiments. Densitometric analysis was done to determine fold change in intensity between treated and control groups.

3.3.5. Celastrol does not affect serine phosphorylation of STAT3 in MM cells

In U266 cells, STAT3 can be phosphorylated at both tyrosine residue (Tyr-705) and serine (Ser 727) residues. From our above results, it was observed that celastrol can inhibit phosphorylation of STAT3 at tyrosine 705 residue. We next proceeded to investigate whether celastrol can also modulate phosphorylation of STAT3 at serine 727 residue as well. To analyze this possibility, western blot assay was performed using p-STAT3 antibody specific to STAT3 serine 727 residue. Interestingly, it was found from our experimental results that celastrol did not substantially affect the phosphorylation of STAT3 at Ser 727 residue in U266 cells.

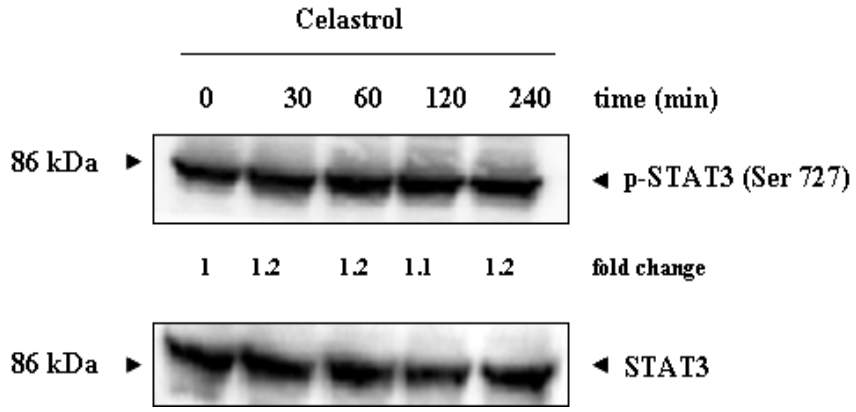


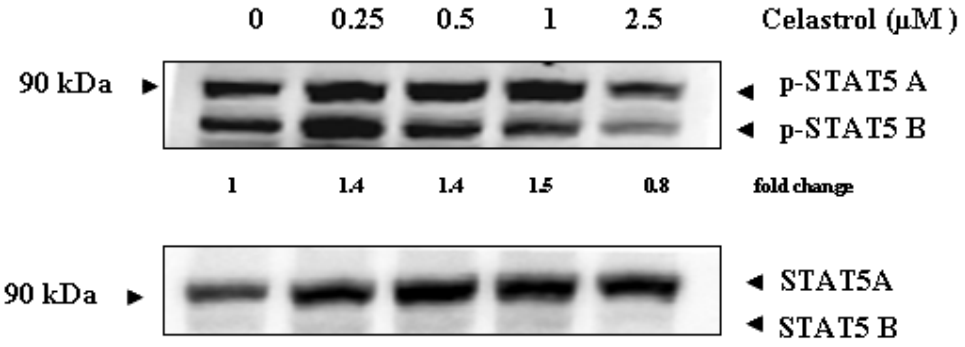
Figure 3.3.5. Celastrol did not affect serine phosphorylation of STAT3 in MM cells

U266 cells were treated with 2.5 μM celastrol for 0, 30, 60, 120 and 240 mins and whole cell extract was prepared. 50 μg protein was loaded and run on SDS-PAGE and transferred to nitrocellulose membrane. The blot was probed for p-STAT3 (Ser 727) using anti-p-STAT3 antibody. As a loading control, total STAT3 was probed using anti-STAT3 antibody after stripping the same blot. The blot shown is representative of at least two independent experiments. Densitometric analysis was done to determine fold change in intensity between treated and control groups.

3.3.6. Celastrol does not affect the phosphorylation status of STAT5 in MM cells

STAT5 is another member of STAT family of transcription factors that has been implicated in pathogenesis of various cancers including MM. Since, it has been demonstrated from our above results, that celastrol can substantially inhibit phosphorylation of STAT3 in MM cells, we next determined whether celastrol also has an effect on the phosphorylation status of STAT5. MM cells were treated with various concentrations of celastrol for 4 hrs and western blot assay was performed to detect p-STAT5 levels. Total STAT5 protein served as loading control. Interestingly, it is found from our experimental results that celastrol did not affect the phosphorylation of STAT5 transcription factor in MM cells.

A. U266



B. RPMI-8226

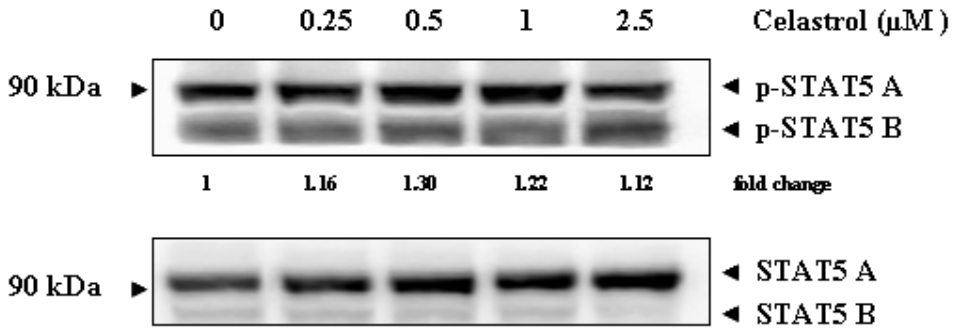


Figure 3.3.6. Celastrol did not affect phosphorylation of STAT5 in MM cells

A. U266 cells treated with 0, 0.5, 1, 2.5 and 5 μM celastrol for 4 hrs and whole cell extract was prepared. 50 μg protein was loaded and run on SDS-PAGE and transferred to nitrocellulose membrane. The blot was probed for p-STAT5 using anti-p-STAT5 antibody. As a loading control, total STAT5 was probed using anti-STAT5 antibody after stripping the same blot. The blot shown is representative of at least two independent experiments. Densitometric analysis was done to determine fold change in intensity between treated and control groups.

B. RPMI-8226 cells treated with 0, 0.5, 1, 2.5 and 5 μM celastrol for 4 hrs and whole cell extract was prepared. 50 μg protein was loaded and run on SDS-PAGE and transferred to nitrocellulose membrane. The blot was probed for p-STAT5 using anti-p-STAT5 antibody. As a loading control, total STAT5 was probed using anti-STAT5 antibody after stripping the same blot. The blot shown is representative of at least two independent experiments. Densitometric analysis was done to determine fold change in intensity between treated and control groups.

3.3.7. Celastrol-induced inhibition of STAT3 is not majorly mediated via the induction of phosphatases in MM cells

STAT3 activation is tightly regulated by a family of phosphatases which can dephosphorylate STAT3 to inactivate it. Since celastrol inhibited the phosphorylation of STAT3 in MM cells, we next determined if celastrol-induced inhibition of STAT3 phosphorylation is mediated via phosphatases. To analyze this possibility, U266 cells were pretreated with indicated concentrations of broad spectrum phosphatase inhibitor pervanadate for 2 hrs. Thereafter, the cells were treated with 2.5 μM celastrol for 4 hrs and western blot assay was done to detect the levels of p-STAT3. Our experimental results indicated that treatment with pervanadate could not substantially reverse STAT3 inhibitory effects of celastrol in MM cells thereby, indicating that phosphatases are not majorly involved in the celastrol-induced inhibition of STAT3 activation.

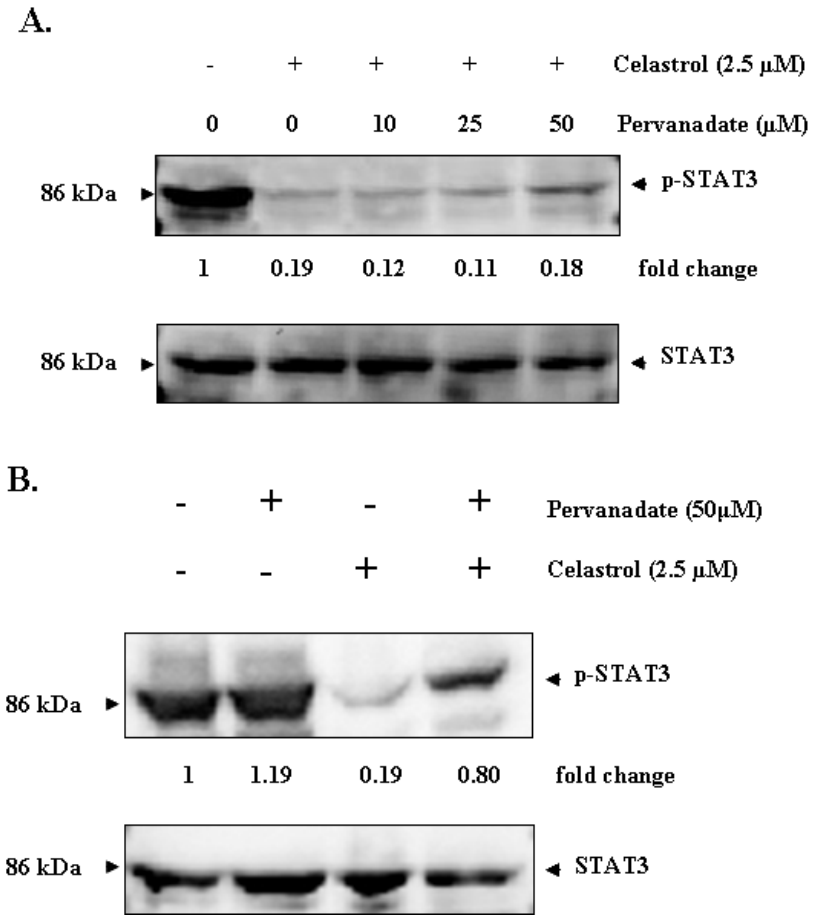

A.
- + + + + Celastrol (2.5 μM)
0 0 10 25 50 Pervanadate (μM)
86 kDa
p-STAT3
1 0.19 0.12 0.11 0.18 fold change
86 kDa
STAT3
B.
- + - + Pervanadate (50μM)
- - + + Celastrol (2.5 μM)
86 kDa
p-STAT3
1 1.19 0.19 0.80 fold change
86 kDa
STAT3

Figure 3.3.7. Celastrol-induced inhibition of STAT3 is not mediated via the induction of phosphatases in MM cells

A. U266 cells were pretreated with 10, 25 and 50 μM of pervanadate for 2 hrs. Thereafter, the cells were treated with 2.5 μM of celastrol for 4 hrs and whole cell extract was prepared. 50 μg protein was loaded and run on SDS-PAGE and transferred to nitrocellulose membrane. The blot was probed for p-STAT3 using anti-p-STAT3 antibody. As a loading control, total STAT3 was probed using anti-STAT3 antibody after stripping the same blot. The blot shown is representative of at least two independent experiments. Densitometric analysis was done to determine fold change in intensity between treated and control groups.

B. U266 cells were pretreated with 50 μM of pervanadate for 2 hrs. Thereafter, the cells were treated with 2.5 μM of celastrol for 4 hrs and whole cell extract was prepared. 50 μg protein was loaded and run on SDS-PAGE and transferred to nitrocellulose membrane. The blot was probed for p-STAT3 using anti-p-STAT3 antibody. As a loading control, total STAT3 was probed using anti-STAT3 antibody after stripping the same blot. The blot shown is representative of at least two independent experiments. Densitometric analysis was done to determine fold change in intensity between treated and control groups.

3.3.8. Celastrol inhibits phosphorylation of Janus Kinase and Src kinase in MM cells

As discussed in the introduction section, non-receptor tyrosine kinases like Janus kinase and Src kinase play a critical role in the tyrosine phosphorylation of STAT3. As our earlier experimental findings showed that celastrol can substantially inhibit the tyrosine phosphorylation of STAT3 in MM cells, we next determined if celastrol does it so by modulating the activation of upstream kinases namely JAK2 and Src involved in STAT3 activation cascade. To analyze this possibility, western blot assay was performed with p-JAK2 and p-Src specific antibodies. U266 cells were treated with 2.5 μM celastrol for 0, 30, 60, 120 and 240 mins and western blot assay was done to detect the levels of p-JAK2 and p-Src. interestingly, it was found that celastrol inhibited the phosphorylation of JAK2 and Src in a time-dependent manner, with minimal effect on the level of non-phosphorylated JAK2 and Src proteins.

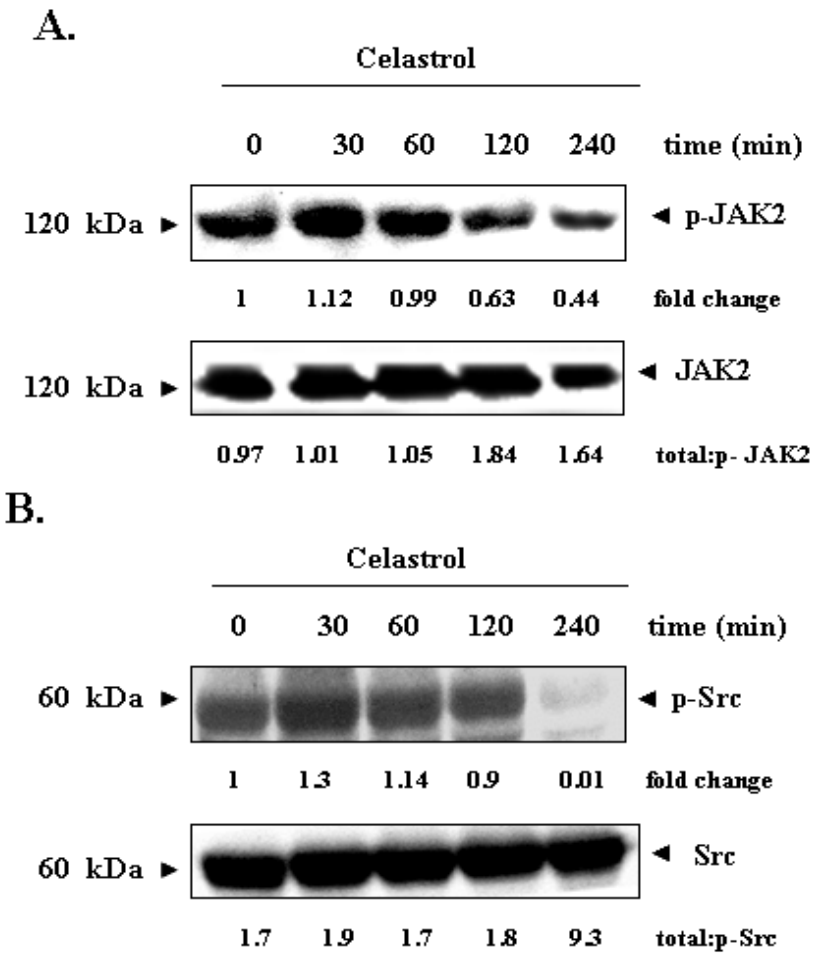

A.
Celastrol
0 30 60 120 240 time (min)
120 kDa
p-JAK2
1 1.12 0.99 0.63 0.44 fold change
120 kDa
JAK2
0.97 1.01 1.05 1.84 1.64 total:p- JAK2
B.
Celastrol
0 30 60 120 240 time (min)
60 kDa
p-Src
1 1.3 1.14 0.9 0.01 fold change
60 kDa
Src
1.7 1.9 1.7 1.8 9.3 total:p-Src

Figure 3.3.8. Celastrol inhibits phosphorylation of Janus Kinase and Src kinase in MM cells

A. U266 cells were treated with 2.5 μM celastrol for 0, 30, 60, 120 and 240 mins and whole cell extract was prepared. 80 μg protein was loaded and run on SDS-PAGE and transferred to nitrocellulose membrane. The blot was probed for p-JAK2 protein using anti-p-JAK2 antibody. As a loading control, total JAK2 was probed using anti-JAK2 antibody after stripping the same blot. As a loading control, β-actin was probed after stripping the same blots. The blot shown is representative of at least two independent experiments. Densitometric analysis was done to determine fold change in intensity between the ratios of treated and control groups.

B. U266 cells were treated with 2.5 μM celastrol for 0, 30, 60, 120 and 240 mins and whole cell extract was prepared. 80 μg protein was loaded and run on SDS-PAGE and transferred to nitrocellulose membrane. The blot was probed for p-Src protein using anti-p-Src antibody. As a loading control, total Src was probed using anti-Src antibody after stripping the same blot. The blot shown is representative of at least two independent experiments. Densitometric analysis was done to determine fold change in intensity between the ratios of treated and control groups.

3.3.9. Celastrol inhibits the nuclear translocation of STAT3 in MM cells

As discussed previously in the introduction section, once STAT3 gets phosphorylated, STAT3 monomers form dimers and translocate to the nucleus where they bind to DNA to regulate transcriptional activation. Since, celastrol inhibited the phosphorylation of STAT3, we next analyzed using immunocytochemistry analysis whether celastrol can also affect nuclear translocation of STAT3 in MM cells. U266 cells were treated with 2.5 μM celastrol for 4 hrs. Immunocytochemistry was performed as described under Materials and Methods section using STAT3 specific antibodies. It is clearly evident from our experimental findings that in the control group, STAT3 is mainly found distributed in the nuclei, whereas in celastrol treated group, it was observed to be localized predominantly in the cytoplasm. The above data clearly indicates that celastrol can substantially inhibit the nuclear translocation of STAT3 in MM cells.

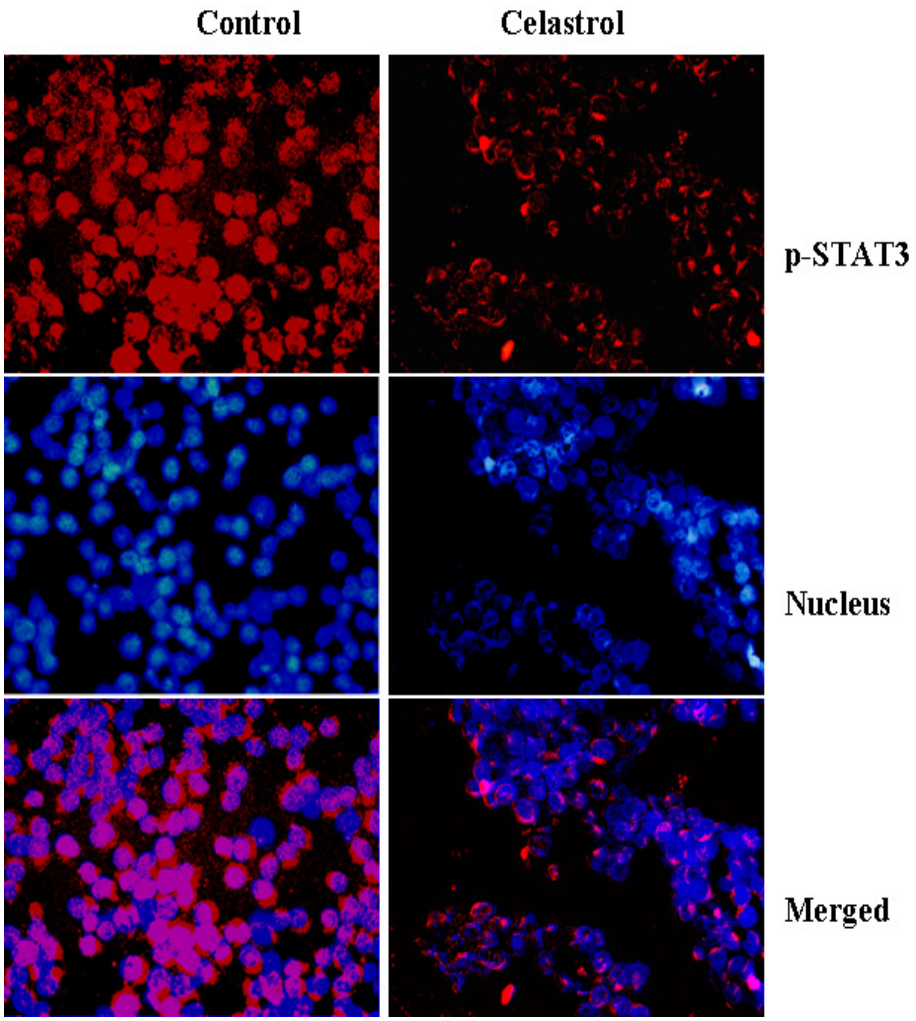
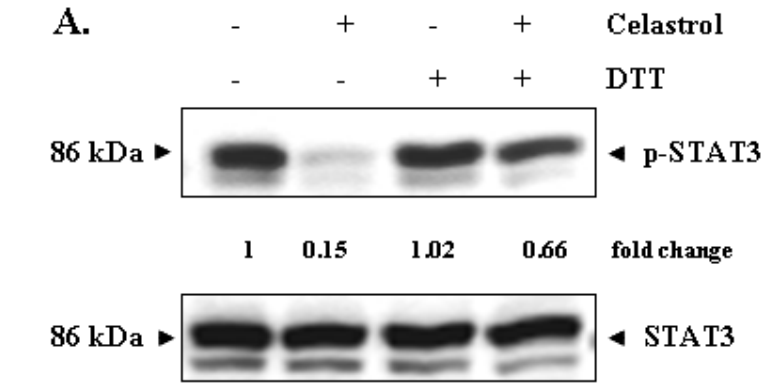


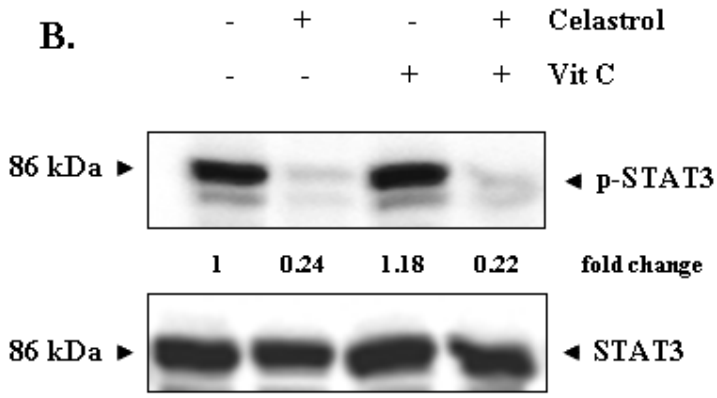
Figure 3.3.9. Celastrol inhibits the nuclear translocation of STAT3 in MM cells

U266 cells were incubated with 2.5 μM celastrol for 4 hrs and then analyzed for the intracellular distribution of STAT3 by immunocytochemistry. Red fluorescence indicates STAT3, and blue fluorescence indicates nucleus (original magnification × 20). Representative image for each treatment group has been shown. The images were merged using Image J software.

3.3.10. Free thiol containing agents prevented the STAT3 inhibitory effects of celastrol in MM cells

Since, thiol reactive moiety of celastrol is considered to be an important functional group for its previously reported anticancer effects, we next investigated, if the same moiety is also involved in the celastrol's ability to inhibit STAT3 phosphorylation in MM cells. To explore this possibility, we used various thiol containing agents including DTT (dithiothreitol) and GSH (glutathione reduced) which are known to react with the thiol reactive groups on proteins to form thioester coupled products. U266 cells were treated with 2.5 μM celastrol and the 250 μM DTT and 2 mM GSH for 4 hrs. Non-thiol containing agents such as Vitamin C, (ascorbic acid, a non-thiol reducing agent) and GSSG (glutathione oxidized, a non-thiol analogue of GSH) were used as negative controls. Interestingly, we found that treatment with thiol containing agents at least partially reverse the celastrol-induced inhibition of STAT3 phosphorylation. Conversely, the non-thiol containing compounds did not do so, indicating that thiol reactive moiety of celastrol is at least partially responsible for its ability to inhibit STAT3 phosphorylation in MM cells.


A.
- + - + Celastrol
- - + + DTT
86 kDa
p-STAT3
1 0.15 1.02 0.66 fold change
86 kDa
STAT3


B.
- + - + Celastrol
- - + + Vit C
86 kDa
p-STAT3
1 0.24 1.18 0.22 fold change
86 kDa
STAT3

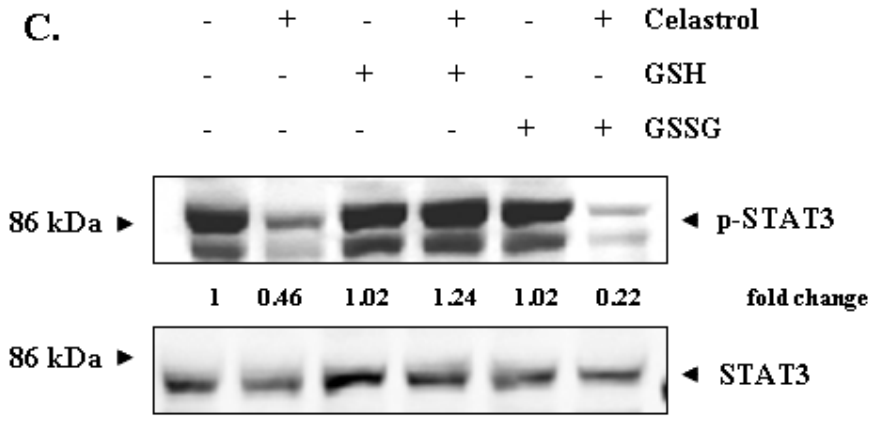

C.
- + - + - + Celastrol
- - + + - - GSH
- - - - + + GSSG
86 kDa
p-STAT3
1 0.46 1.02 1.24 1.02 0.22 fold change
86 kDa
STAT3

Figure 3.3.10. Free thiol containing agents reversed the STAT3 inhibitory effects of celastrol in MM cells

A. U266 cells were treated with 250 μM DTT and 2.5 μM celastrol for 4 hrs and whole cell extract was prepared. 50 μg protein was loaded and run on SDS-PAGE and transferred to nitrocellulose membrane. The blot was probed for p-STAT3 using anti-p-STAT3 antibody. As a loading control, total STAT3 was probed using anti-STAT3 antibody after stripping the same blot. The blot shown is representative of at least two independent experiments. Densitometric analysis was done to determine fold change in intensity between treated and control groups.

B. U266 cells were treated with 0.1 mM Vit C and 2.5 μM celastrol for 4 hrs and whole cell extract was prepared. 50 μg protein was loaded and run on SDS-PAGE and transferred to nitrocellulose membrane. The blot was probed for p-STAT3 using anti-p-STAT3 antibody. As a loading control, total STAT3 was probed using anti-STAT3 antibody after stripping the same blot. The blot shown is representative of at least two independent experiments. Densitometric analysis was done to determine fold change in intensity between treated and control groups.

C. U266 cells were treated with 2 mM GSH and 2.5 μM celastrol for 4 hrs and whole cell extract was prepared. 50 μg protein was loaded and run on SDS-PAGE and transferred to nitrocellulose membrane. The blot was probed for p-STAT3 using anti-p-STAT3 antibody. As a loading control, total STAT3 was probed using anti-STAT3 antibody after stripping the same blot. The blot shown is representative of at least two independent experiments. Densitometric analysis was done to determine fold change in intensity between treated and control groups.

3.4. Effect of celastrol on NF-κB signaling cascade in MM cells

3.4.1. Celastrol inhibits NF-κB activation pathway in MM cells

3.4.1.1. Myeloma cells express constitutively activated p65

As discussed in the introduction section, constitutive NF-κB activation plays a major role in the pathogenesis of various cancers including MM. Human myeloma cells have been reported to express constitutively activated NF-κB. To confirm this finding, various MM cells including U226, RPMI-8226 cells, RPMI-8226-Dox6 cells (resistant to doxorubicin), RPMI-8226-LR5 cells (resistant to melphalan) and RPMI 8226-BR (resistant to bortezomib) cell lines were used. Nuclear extract was prepared as discussed under Materials and Methods section and western blot assay was done using p-p65 and p65 specific antibodies. Lamin B1 was used as a loading control. As shown in the figure, our experimental findings confirm the fact that the all the above analyzed human MM cells express constitutively activate NF-κB p65 levels.

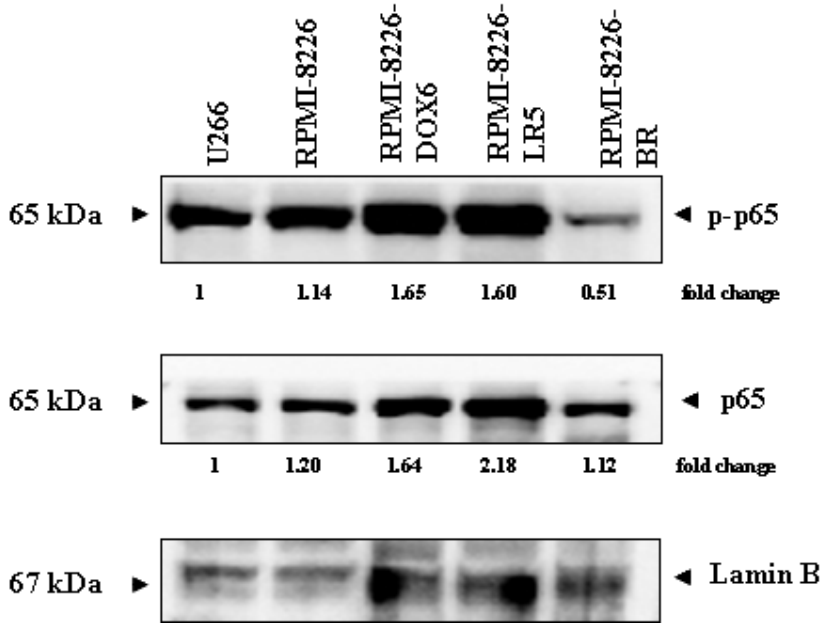


Figure 3.4.1.1. Myeloma cells express constitutively activated p65

Nuclear extracts of human MM cells including U226, RPMI-8226, RPMI 8226-Dox6 cells, RPMI-8226-LR5, and RPMI-8226-BR cells were prepared. 50μg protein was loaded and run on SDS-PAGE and transferred to nitrocellulose membrane. The blot was probed for p-p65 and p65 using specific antibodies. As a loading control, Lamin B was probed using anti-Lamin B antibody after stripping the same blot. The blot shown is representative of at least two independent experiments. Densitometric analysis was done to determine fold change in intensity between proteins obtained from U266 and other MM cell lines.

3.4.1.2. Celastrol inhibits the phosphorylation of IκBα and p65 proteins in MM cells

As discussed in the introduction section, NF-κB p65 subunits remain bound with IκBα protein in the cytoplasm. Upon activation by various stimuli, phosphorylation of IκBα protein leads to its ubiquitination and proteasome-dependent degradation and thus released p65 enters the nucleus to bind with the DNA and transcriptionally modulate its target genes. Since celastrol has been reported to abrogate NF-κB activation in tumor cells, we next analyzed whether celastrol can also inhibit NF-κB p65 activation in MM cell lines. To analyze this aspect, a western blot assay was performed.

U266 cells were treated with 2.5 μM celastrol for 0, 30, 60, 120 and 240 mins. Cytoplasmic and nuclear extracts were prepared as described under Materials and Methods. The extracts were separated on SDS-PAGE and subjected to western blot analysis. Cytoplasmic extract was used to detect p-IκB levels. GAPDH was used as a loading control for cytoplasmic extract. It is evident from our experimental finding that celastrol inhibits the phosphorylation of IκB in a time-dependent manner. Nuclear extracts were used to detect p-p65. Lamin B1 was used as a loading control for nuclear extract. It is clearly evident from our experimental results that celastrol can indeed inhibit the phoshorylation of p65 in a time-dependent manner.

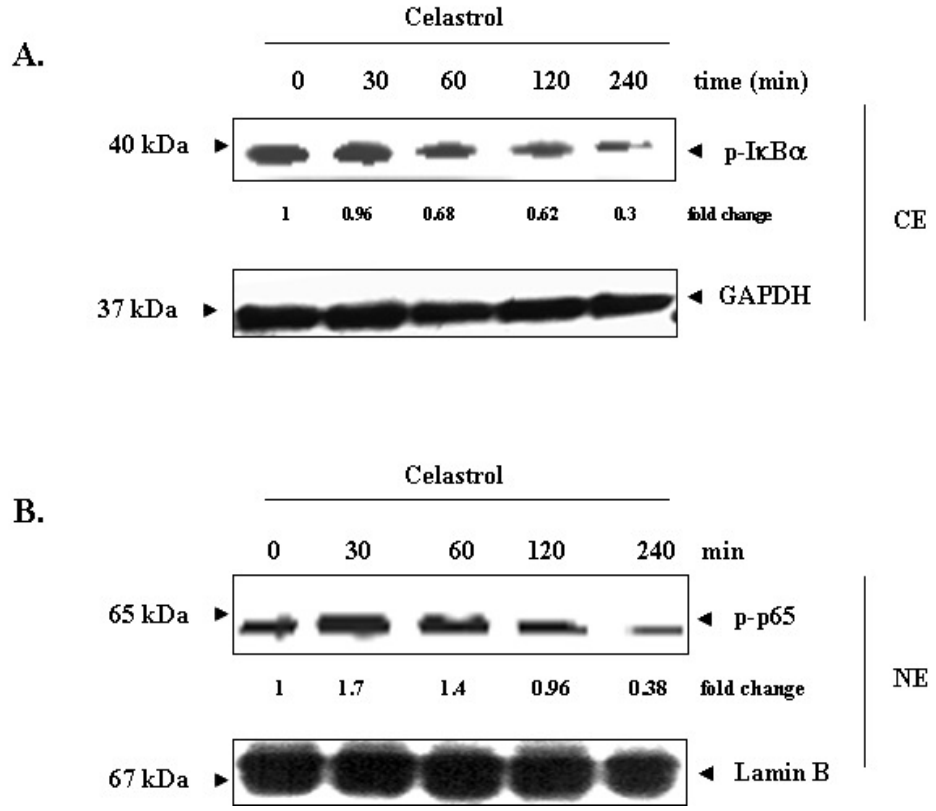


Figure 3.4.1.2. Celastrol inhibits the phosphorylation of IκB and p65 proteins in MM cells

A. U266 cells were treated with 2.5 μM celastrol for 0, 30, 60, 120 and 240 mins and cytoplasmic extract was prepared. 50 μg protein was loaded and run on SDS-PAGE and transferred to nitrocellulose membrane. The blot was probed for p-IκBα using anti-p-IκBα antibody. As a loading control, GAPDH was probed using anti-GAPDH antibody after stripping the same blot. The blot shown is representative of at least two independent experiments. Densitometric analysis was done to determine fold change in intensity between treated and control groups.

B. U266 cells were treated with 2.5 μM celastrol for 0, 30, 60, 120 and 240 mins and nuclear extract was prepared. 50 μg protein was loaded and run on SDS-PAGE and transferred to nitrocellulose membrane. The blot was probed for p-p65 using anti-p-p65 antibody. As a loading control, Lamin B was probed using anti-Lamin B antibody after stripping the same blot. The blot shown is representative of at least two independent experiments. Densitometric analysis

was done to determine fold change in intensity between treated and control groups.

3.4.1.3. Celastrol inhibits the activation of upstream kinases involved in the NF-κB signaling cascade

As discussed previously in the introduction, IKK is involved in the phosphorylation of IκBα protein and thus regulate activation of p65 upon stimulation by external stimuli. Since, it is clearly evident from the above data that celastrol inhibits the phoshorylation of IκBα and p65 proteins, we next determined whether celastrol has any effect on the phosphorylation status and thus activation of IKK in MM cells. U266 cells were treated with 2.5 μM celastrol for 0, 30, 60, 120 and 240 mins and western blot assay was performed using p-IKK-α/β specific antibodies. IKK-α protein served as a loading control. It is clearly demonstrated from our experimental results that celastrol can indeed suppress the phosphorylation of IKK-α/β in a time-dependent manner in MM cells.

It has been reported that AKT may also act upstream of IKK-α/β to activate the NF-κB signaling pathway. Moreover, as discussed in the introduction section, AKT has also been found to be involved in myeloma progression by mediating cell proliferation, anti-apoptosis and survival [435]. So, we next aimed to investigate, if celastrol can also modulate the activation of AKT in MM cells. To analyze this possibility, U266 cells were treated with 2.5 μM celastrol for 0, 30, 60, 120 and 240 mins and western blot assay was performed using p-AKT specific antibodies, AKT serving as loading control. It is evident from our experimental results that celastrol can indeed suppress the phosphorylation of AKT in a time-dependent manner in MM cells without significantly affecting the basal level of AKT.

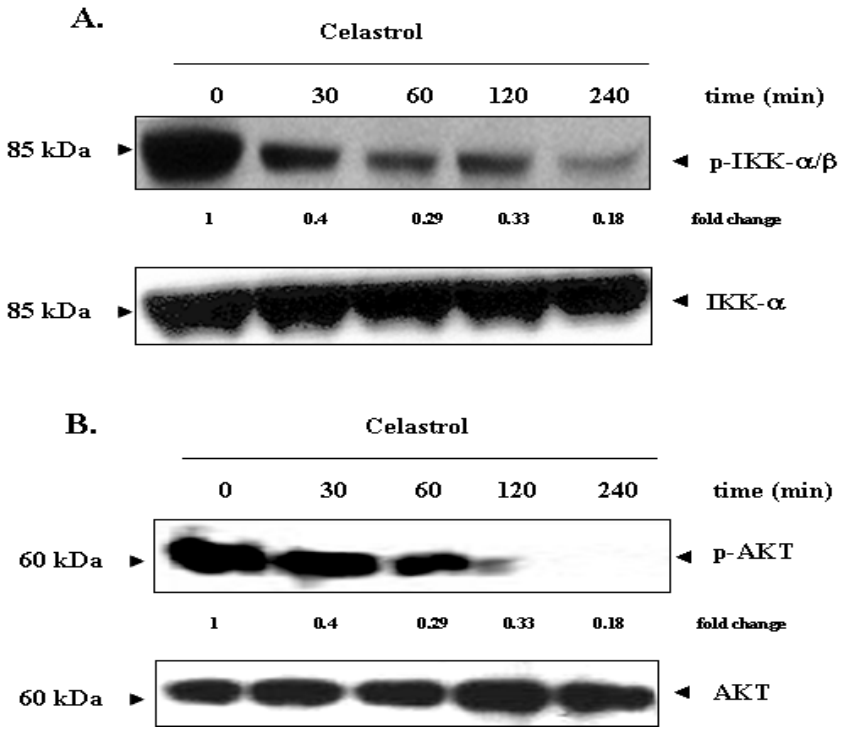


Figure 3.4.1.3. Celastrol inhibits the activation of upstream kinsaes involved in the NF-κB signaling cascade

A. U266 cells were treated with 2.5 μM celastrol for 0, 30, 60, 120 and 240 mins and whole cell extract was prepared. 50 μg protein was loaded and run on SDS-PAGE and transferred to nitrocellulose membrane. The blot was probed for p-IKK-α/β using anti-p-IKK-α/β antibody. As a loading control, IKK-α was probed using anti-IKK-α antibody after stripping the same blot. The blot shown is representative of at least two independent experiments. Densitometric analysis was done to determine fold change in intensity between treated and control groups.

B. U266 cells were treated with 2.5 μM celastrol for 0, 30, 60, 120 and 240 mins and whole cell extract was prepared. 50 μg protein was loaded and run on SDS-PAGE and transferred to nitrocellulose membrane. The blot was probed for p-AKT using anti-p-AKT antibody. As a loading control, AKT was probed using anti-AKT antibody after stripping the same blot. The blot shown is representative of at least two independent experiments. Densitometric analysis was done to determine fold change in intensity between treated and control groups.

3.4.1.4. Celastrol treatment abrogates the nuclear translocation of p65 in MM cells

As discussed previously in the introduction section, once IκBα protein gets phosphorylated and degraded by ubiquitin-proteasome system, p65 subunit enter the nucleus to bind with the DNA and to cause transactivation. Since, we know that celastrol inhibits the phosphorylation of IκBα, we next determined using immunohistochemistry analysis whether celastrol can also cause failure of nuclear translocation of p65 subunit in MM cells. U266 cells were treated with 2.5 μM celastrol for 4 hrs. Immunocytochemistry was performed as discussed under the Materials and Methods section using p65 specific antibodies. It is clearly evident from our experimental findings that in the control group, p65 is mainly found distributed in the nuclei, whereas in celastrol treated group, it was observed to be localized predominantly in the cytoplasm. The above data clearly indicates that celastrol suppresses the nuclear translocation of p65 subunits in MM cells.

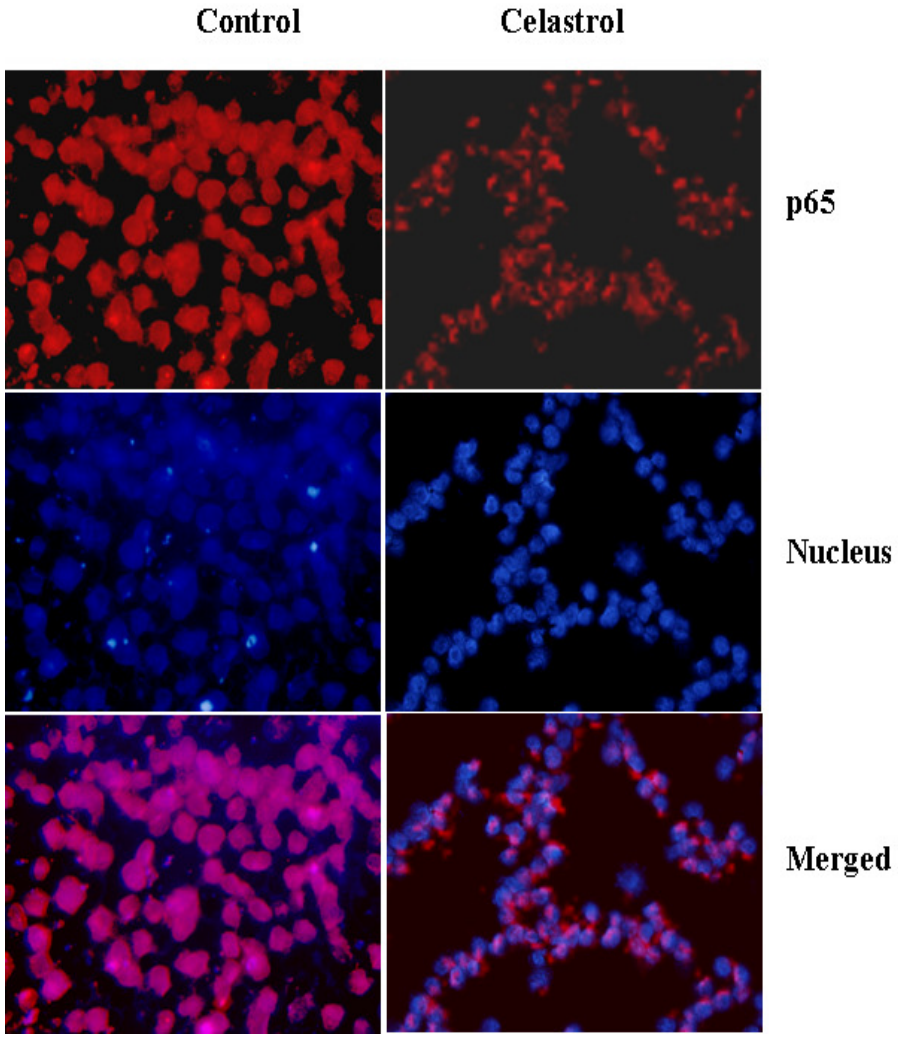


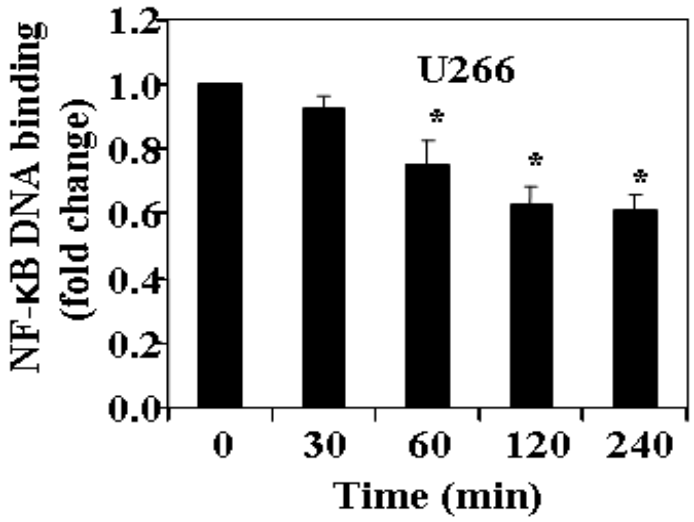
Figure 3.4.1.4. Celastrol treatment abrogates the nuclear translocation of p65 in MM cells

U266 cells were incubated with 2.5 μM celastrol for 4 hrs and then analyzed for the intracelullar distribution of p65 by immunocytochemistry. Red fluorescence indicates p65, and blue fluorescence indicates nucleus (original magnification × 20). Representative image for each group has been shown. The images were merged using Image J software.

3.4.1.5. Celastrol inhibits DNA binding ability of NF-κB in MM cells

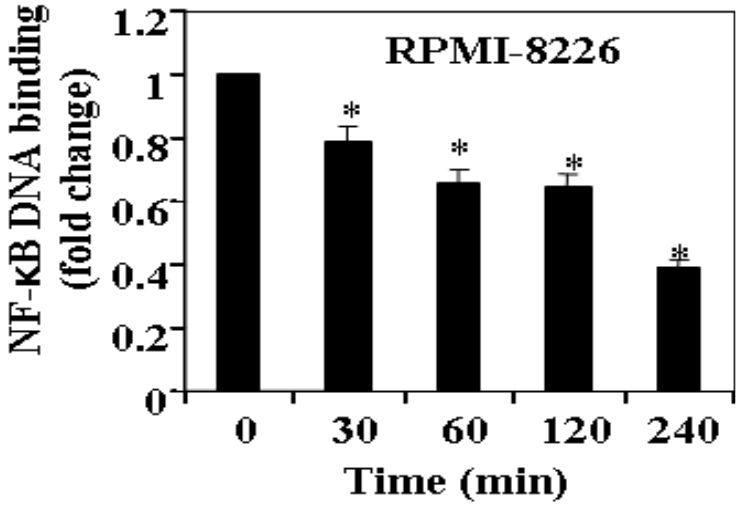
Since celastrol inhibited the nuclear translocation of p65 protein which is evident from our earlier experiments and any transcription factor has to bind with DNA to transactivate it, we aimed to analyze if celastrol also inhibited the DNA binding ability of p65 subunits. To analyze this, U266 and RPMI-8226 cells were treated with 2.5 μM celastrol for 0, 30, 60, 120 and 240 mins. Nuclear extract was prepared and DNA binding assay was performed using ELISA based NF-κB p65 DNA binding assay kit as described under Materials and Methods section. It is evident from our experimental findings that celastrol significantly inhibited the DNA binding ability of NF-κB in a time-dependent manner in MM cells.

A.


U266
NF-κB DNA binding (fold change)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
30
60
120
240
Time (min)
*
*
*

*p<0.05

B.


RPMI-8226
NF-κB DNA binding (fold change)
1.2
1
0.8
0.6
0.4
0.2
0
0
30
60
120
240
Time (min)
*
*
*
*

*p<0.05

Figure 3.4.1.5. Celastrol inhibits DNA binding ability of NF-κB in MM cells

A. U266 cells were treated with 2.5 μM celastrol for 0, 30, 60, 120 and 240 mins. Nuclear extracts were prepared, and 20 μg of the nuclear extract protein was used for ELISA based DNA binding assay as described under Materials and Methods. Data are presented as relative DNA binding as compared to the control $\pm$ SD. *Significant difference when compared to the control group.

B. RPMI-8226 cells were treated with 2.5 μM celastrol for 0, 30, 60, 120 and 240 mins. Nuclear extracts were prepared, and 20 μg of the nuclear extract protein was used for ELISA based DNA binding assay as described under Materials and Methods. Data are presented as relative DNA binding as compared to the control $\pm$ SD. *Significant difference when compared to the control group.

3.4.2. Celastrol augments the NF-κB inhibitory effects of bortezomib in MM cells

Bortezomib, a well characterized pharmacological proteasome inhibitor can also inhibit NF-κB pathway by increasing the availability of IκBα to bind with p65 subunits [435]. Since bortezomib is a standard of care for MM patients and reported previously to inhibit NF-κB activation in MM cells, we next determined whether sub-optimal concentrations of celastrol can enhance NF-κB inhibiting ability of bortezomib. To explore this possibility, U266 cells were treated with 0.5 μM celastrol and or 10 nM bortezomib for 4 hrs. Nuclear extract was prepared and DNA binding assay was performed using ELISA based NF-κB p65 DNA binding assay kit as described under Materials and Methods section. While the sub-optimal concentrations of celastrol and bortezomib alone did not affect the DNA binding ability of NF-κB, the combination of the two drugs significantly decreased NF-κB activity to 0.7 fold (*$P<0.05$). Thus, it is evident from our experimental findings that celastrol can indeed potentiate the NF-κB inhibitory effect of bortezomib in a statistically significant manner in MM cells.

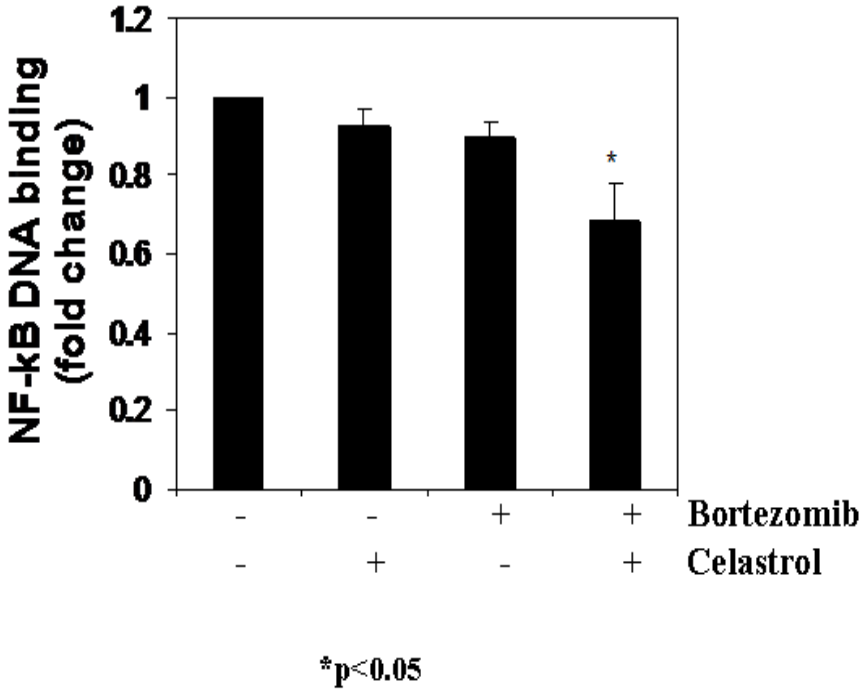


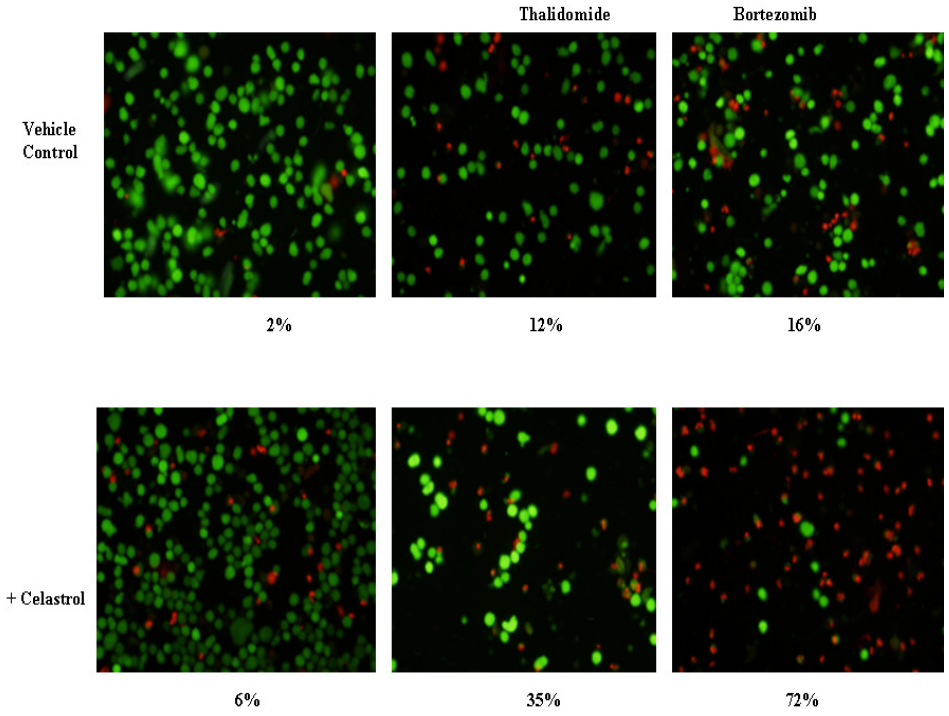
Figure 3.4.2. Celastrol augments the NF-κB inhibitory effects of bortezomib in MM cells

U266 cells were treated with 0.5 μM celastrol and or 10 nM bortezomib for 4 hrs. Nuclear extracts were prepared and 20 μg of the nuclear extract protein was used for ELISA based DNA binding assay as described under Materials and Methods. Data are represented as relative DNA binding as compared to the control $\pm$ SD. *Significant difference when compared to the control group.

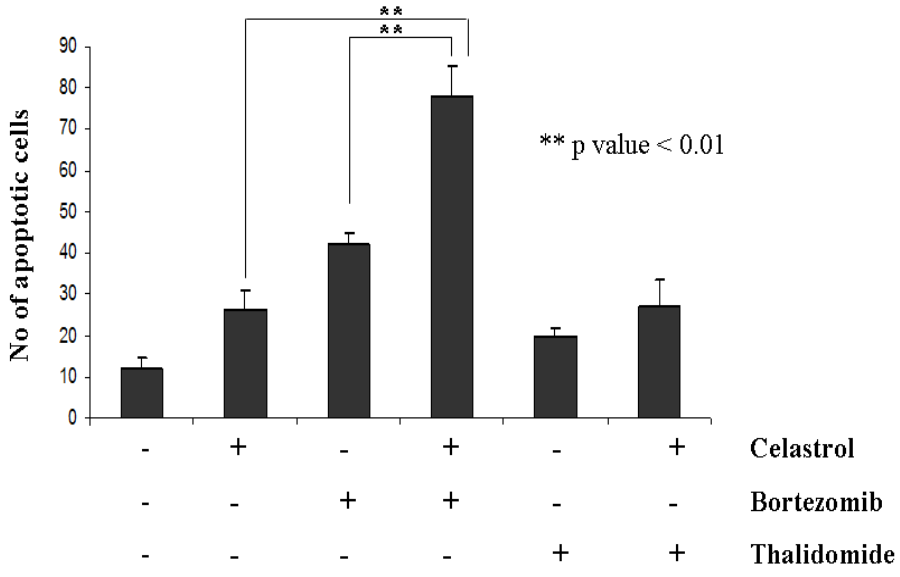
3.4.3. Celastrol enhances apoptosis induced by novel anti-myeloma agents in MM cells

As discussed in the introduction section, thalidomide (an inhibitor of TNF-α expression), and bortezomib (a proteasome inhibitor) have been approved by FDA for the treatment of MM patients. Also, as evident from our previous experimental results celastrol can indeed potentiate the NF-κB inhibitory effects of bortezomib in MM cells, though reported NF-κB inhibition by bortezomib is considered to be only one of its proposed mechanism(s) of action. We next investigated whether celastrol when employed at suboptimal concentration can potentiate the apoptotic effect of thalidomide and bortezomib in MM cells. To explore this possibility, U266 cells were treated with 0.5 μM celastrol together with different concentrations of either thalidomide or bortezomib; and then examined for apoptosis using live and dead assays, annexin V staining, and cell cycle analysis. The results of live and dead (figure A), annexin V (figure B) and cell cycle analysis (figure C) clearly indicate that celastrol can substantially potentiate the apoptotic effects of both thalidomide and bortezomib in MM cells. Based on the cell cycle analysis isobologram-illustrated results, we found that 0.5 μM celastrol synergistically induced the accumulation of MM cells in sub-G1 phase when used in combination with 10 μg/ml thalidomide or 10 nM bortezomib for 24 hrs when analyzed by Chou-Talalay multiple drug effect equation.

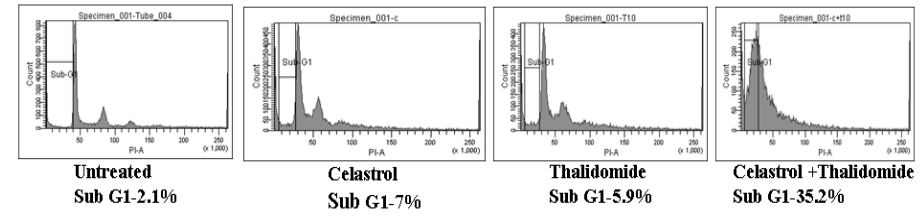
A


Thalidomide
Bortezomib
Vehicle Control
2%
12%
16%
+ Celastrol
6%
35%
72%

B.


No of apoptotic cells
90
80
70
60
50
40
30
20
10
0
** p value < 0.01
- + - + - +
Celastrol
- - + + - -
Bortezomib
- - - - + +
Thalidomide

C

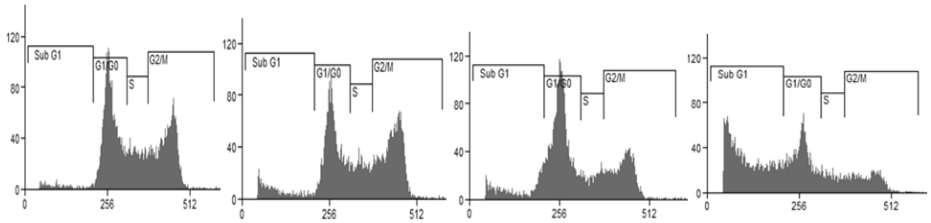


Untreated
Sub G1-2.1%

Celastrol
Sub G1-7%

Thalidomide
Sub G1-5.9%

Celastrol +Thalidomide
Sub G1-35.2%



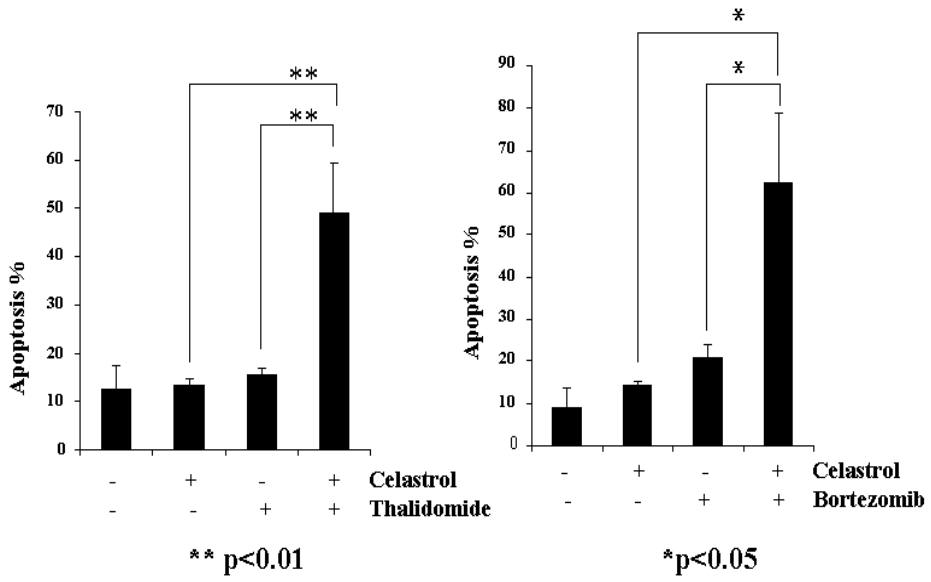
Untreated
Sub G1-2%

Celastrol
Sub G1-8%

Bortezomib
Sub G1-5.9%

Celastrol +Bortezomib
Sub G1- 47%

D.



** p<0.01

*p<0.05

E

Cell line	Drugs	Dose	CI value	Relationship
U266	Celastrol	0.5 μM	n/a	n/a
	Thalidomide	10 μg/ml	n/a	n/a
	Bortezomib	10 nM	n/a	n/a
	Celastrol + Thalidomide	0.5 μM + 10 μg/ml	4.54E-6	Highly Synergistic
	Celastrol + Thalidomide	0.5 μM + 20 μg/ml	0.068	Synergistic
	Celastrol + Bortezomib	0.5 μM + 10 nM	1.17E-5	Highly Synergistic
	Celastrol + Bortezomib	0.5 μM + 20 nM	0.0058	Synergistic

Figure 3.4.3. Celastrol augments apoptosis induced by anti-myeloma agents in MM cells

A. U266 cells were treated with 0.5 μM celastrol, and 10 μg/ml thalidomide or 10 nM bortezomib alone or in combination for 24 hrs at 37°C. Cells were stained with a Live/Dead assay reagent for 30 mins and then analyzed under a fluorescence microscope as described under Materials and Methods. A representative image for each group is shown.

B. No of apoptotic cells in three microscopic fields for each group in the above experiment was counted. Data is represented as mean $\pm$ SD (n=3). p value <0.05 was considered statistically significant.

C. U266 cells were treated with 0.5 μM celastrol, 10 μg/ml and 20 μg/ml thalidomide or 10 nM, and 20nM bortezomib alone or in combination for 24 hrs at 37°C after which the cells were washed, fixed, stained with PI, and analyzed for DNA content by flow cytometry. The representative histograms are shown only for thalidomide (10 μg/ml), bortezomib (10 nM) alone or in combination with celastrol (0.5 μM). The percentage of sub-G1 population cells were taken for combination index (CI) calculation.

D. U266 cells were treated with 0.5 μM celastrol, and 10 μg/ml thalidomide (left panel) or 10 nM bortezomib (right panel) alone or in combination for 24 hrs at 37°C. Cells were incubated with anti-annexin V antibody conjugated with FITC and then analyzed with a flow cytometer. Data are represented as percentage of apoptotic cells in each group.

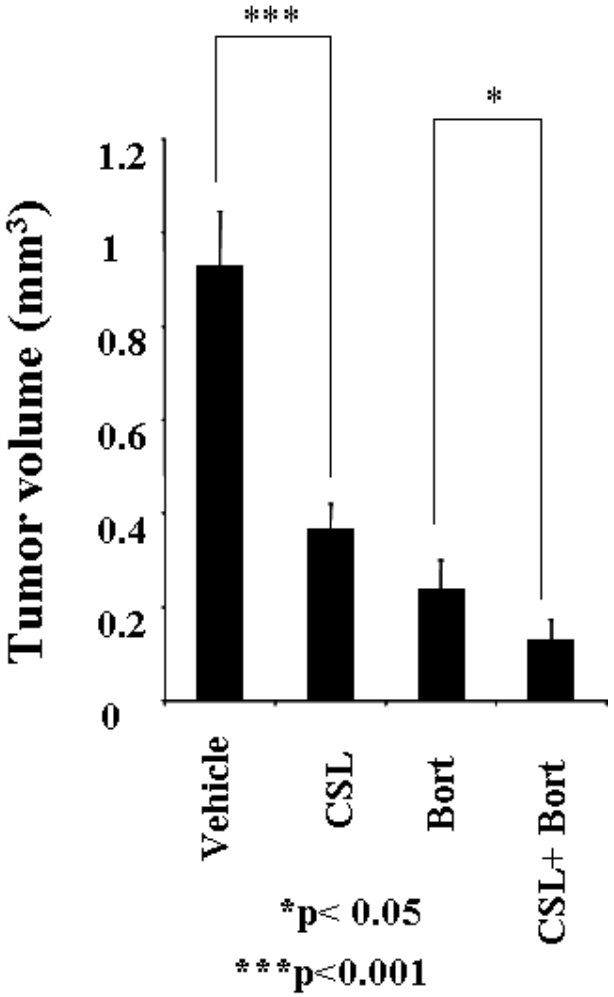
E. Assessment on the type of combination relationship was done using the method developed by Chou-Talalay multiple drug effect equation. The degree of synergy was assessed based on CI values, where CI values of >1.0 implies antagonism, 1.0 implies additive and <1.0 implies synergistic effect relationships.

3.5. Celastrol potentiates the anti-myeloma effects of bortezomib in a xenograft MM mouse model

3.5.1. Celastrol enhances the inhibition of tumor growth induced by bortezomib *in vivo*

To determine whether celastrol enhances the antitumor effects of bortezomib against MM, we developed a human MM xenograft in nude mice using U266 cells. A week after implantation, the animals were randomized into four treatment groups based on tumor volume. Treatment was started 1 week after tumor cell implantation and was continued up to 20 days, in accordance with the experimental protocol under Materials and Methods. Tumor diameters and body weight were measured on every third day. Animals were killed 32 days after tumor cell injection and 25 days after the treatment start date, and the tumors were excised. We found that the tumor volume increased rapidly in the control group compared with the other treatment groups. The tumor volume in the celastrol alone group was significantly lower than that in the control group ($p< 0.001$, vehicle versus celastrol; Fig.A). When we analyzed if celastrol has augmented the tumor inhibition caused by bortezomib, we found that celastrol significantly potentiated the tumor inhibition induced by bortezomib ($p < 0.05$, bortezomib versus celastrol + bortezomib; Fig.A). When examined for tumor volume on different days, we found that tumor volume started to decrease in all the three treatment groups when compared to control group from day 9 onwards and the trend became even more statistically significant as the treatment was continued (Fig. B).

A.


***
*
Tumor volume (mm3)
1.2
1
0.8
0.6
0.4
0.2
0
Vehicle
CSL
Bort
CSL+ Bort
*p< 0.05
***p<0.001

B.

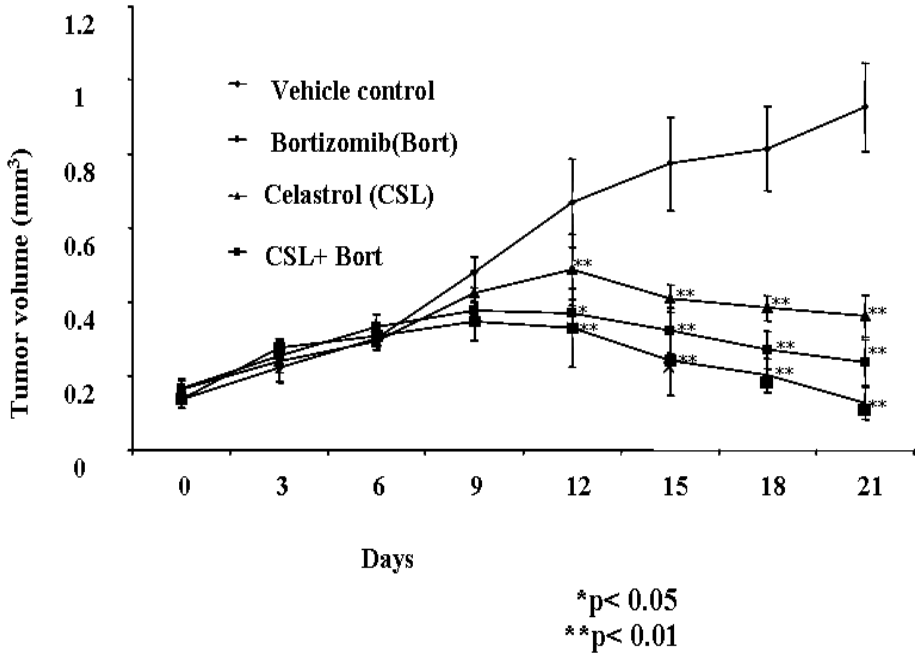


Figure 3.5.1. Celastrol enhances the inhibition of tumor growth induced by bortezomib *in vivo*

A. On the last day of the experimental protocol, tumor diameters were measured and tumor volume was calculated using the formula [L x W2]/2, where W and L are the width. Data is represented as mean $\pm$ SD (n=5).

B. Tumor diameters were measured on every third day and tumor volume was calculated using the formula [L x W2]/2, where W and L are the width. Data are represented as mean $\pm$ SD (n=5).

3.5.2. Celastrol exhibits no obvious side effects *in vivo*

To determine if celastrol caused any serious side effects *in vivo*, mice were closely observed throughout the treatment duration. Interestingly, we did not notice any obvious side effects throughout our experiment. Body weight was measured on every third day as a parameter of side effects and the results are shown in the figure below. As can be inferred from our findings, celastrol did not cause any significant weight loss in mice upon administration, thereby indicating that it can be developed as a non-toxic treatment regimen for MM patients.

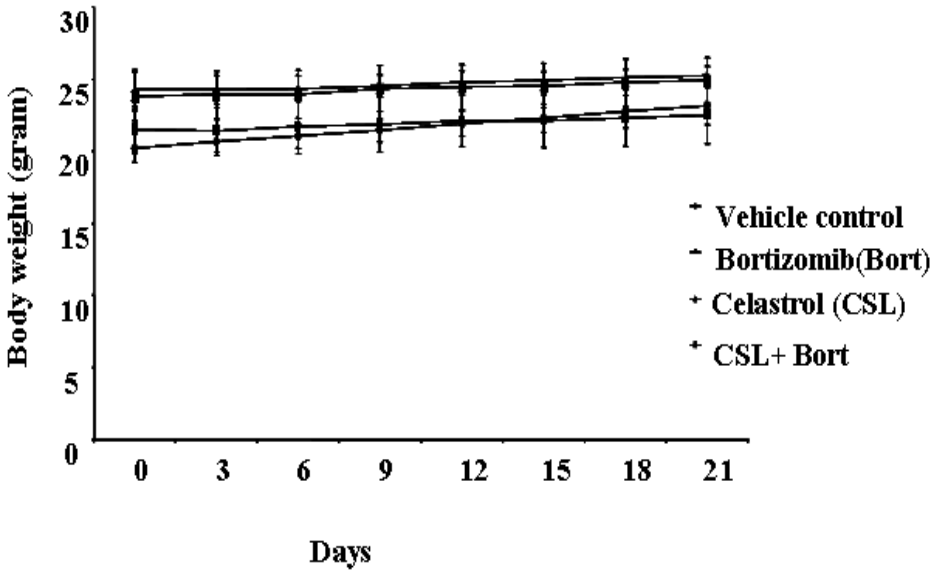


Figure 3.5.2. Celastrol exhibits no obvious side effects *in vivo*

Body weight was measured on every third day during the experimental protocol. Data is represented as mean $\pm$ SD (n=5).

3.5.3. Celastrol downregulates the expression of p-STAT3 and p65 *in vivo* in a MM xenograft mouse model

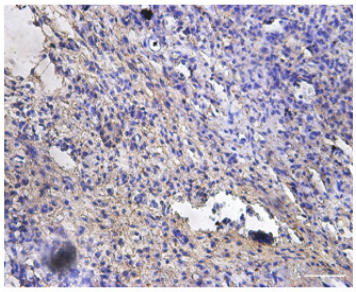
It is clearly evident from our *in vitro* experiments that celastrol inhibits STAT3 and p65 activation in MM cells. Since celastrol alone and in combination with bortezomib significantly decreased the tumor volume in MM xenograft mouse model, we next determined whether celastrol alone and in combination with bortezomib can modulate STAT3 and p65 activation under *in vivo* conditions as well. To explore this possibility, immunohistochemistry assay was performed using tumor tissues from all four treatment groups excised as described under Materials and Methods. As evident from our experimental results shown below, celastrol alone and also in combination with bortezomib significantly decreased the p-STAT3 and p65 expression in tumor tissues.

A.

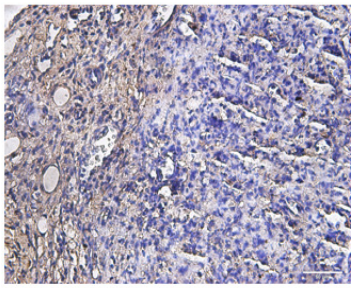
Vehicle

Celastrol

p-STAT3



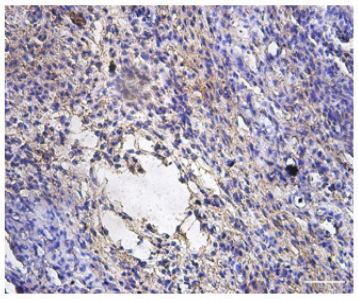
79 ± 2%



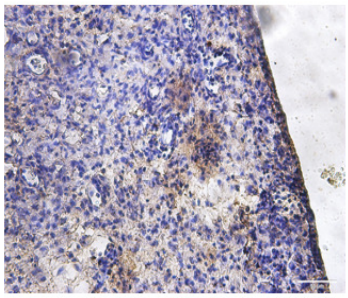
39 ± 4%

Bortezomib

Celastrol + Bortezomib



44 ± 3%



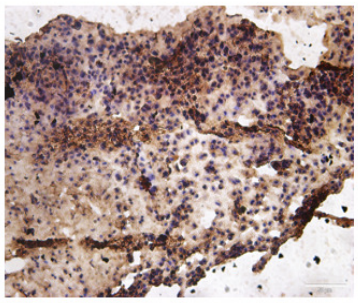
24 ± 2%

B.

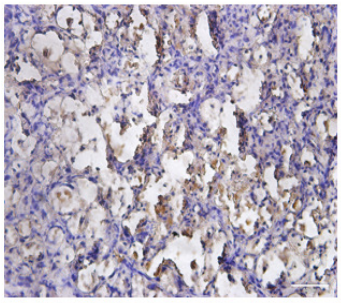
Vehicle

Celastrol

p65



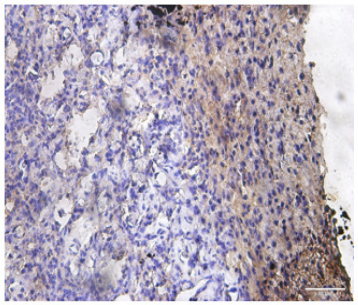
98 ± 1%



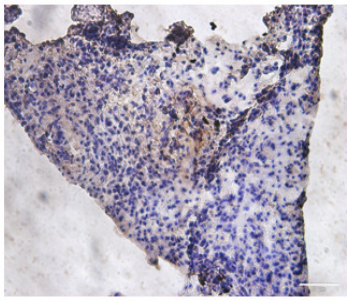
41 ± 3%

Bortezomib

Celastrol + Bortezomib



49 ± 5%



21 ± 3%

Figure 3.5.3. Celastrol donwregulates p-STAT3 and p65 expression *in vivo* in a MM xenograft mouse model

A. Tumor tissues obtained were subjected to immunohistochemistry using p-STAT3 specific antibodies as described under Materials and Methods section. Images were taken using an Olympus BX51 microscope (magnification ×40). Representative image for each group is shown above. Positive cells (brown) were quantitated using the Image-Pro plus 6.0 software package (Media Cybernetics, Inc.).

B. Tumor tissue obtained was subjected to immunohistochemistry using p65 specific antibodies as described under Materials and Methods section. Images were taken using an Olympus BX51 microscope (magnification ×40). Representative image for each group is shown above. Positive cells (brown) were quantitated using the Image-Pro plus 6.0 software package (Media Cybernetics, Inc.).

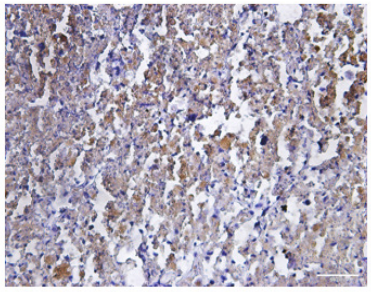
3.5.4. Celastrol modulates the expression of various proteins involved in myeloma progression *in vivo* in a MM xenograft mouse model

It was observed in our *in vitro* experiments that celastrol can downregulate the expression of various proteins involved in MM survival, metastasis and angiogenesis. Since celastrol alone and in combination with bortezomib decreased significantly decreased the tumor volume in MM xenograft mouse model, we next determined whether celastrol alone and in combination with bortezomib can affect Bcl-2 and VEGF protein expression *in vivo*. To explore this possibility, immunohistochemistry assay was performed using tumor tissues excised from all four treatment groups under Materials and Methods. As evidenced from our experimental results, celastrol alone and also in combination with bortezomib significantly downregulated the expression of Bcl-2 and VEGF expression in tumor tissues. This data clearly indicates the proapoptotic and antiangiogenic potential of celastrol *in vivo*.

A.

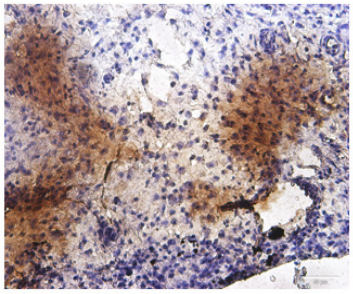
Bcl-2

Vehicle



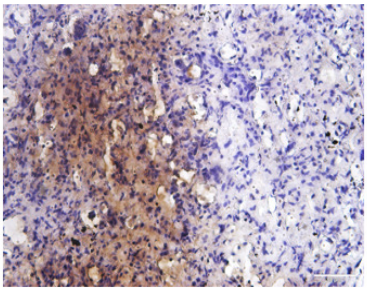
75 ± 2%

Celastrol



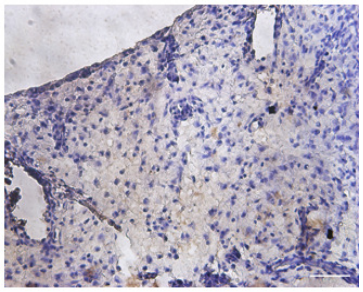
46 ± 3%

Bortezomib



48 ± 4%

Celastrol + Bortezomib

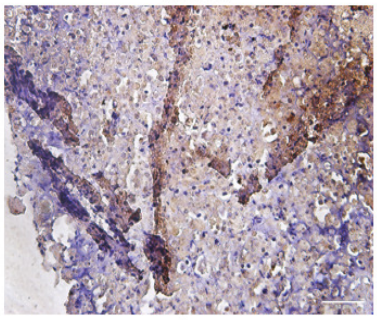


22 ± 3%

B.

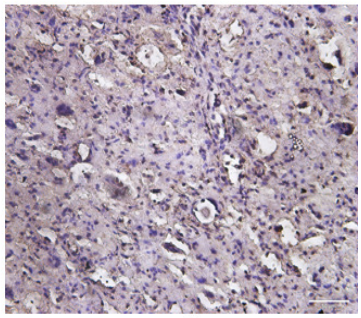
VEGF

Vehicle



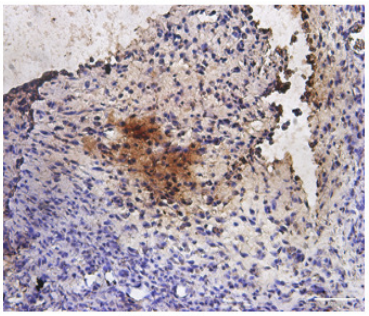
91 ± 2%

Celastrol



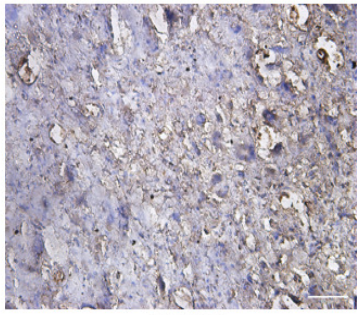
45 ± 3%

Bortezomib



56 ± 4%

Celastrol + Bortezomib



19 ± 2%

Figure 3.5.4. Celastrol modulates the expression of proteins involved in myeloma progression *in vivo* in a MM xenograft mouse model

A. Tumor tissues obtained were subjected to immunohistochemistry using Bcl-2 specific antibody as described under Materials and Methods section. Images were taken using an Olympus BX51 microscope (magnification ×40). Representative image for each group is shown above. Positive cells (brown) were quantitated using the Image-Pro plus 6.0 software package (Media Cybernetics, Inc.).

B. Tumor tissues obtained were subjected to immunohistochemistry using VEGF specific antibody as described under Materials and Methods section. Images were taken using an Olympus BX51 microscope (magnification ×40). Representative image for each group is shown above. Positive cells (brown) were quantitated using the Image-Pro plus 6.0 software package (Media Cybernetics, Inc.).

3.5.5. Celastrol alters the cytokine profile *in vivo* in a MM xenograft mouse model

As discussed in the introduction, pro-inflammatory cytokines such as, IL-6 and TNF-α play a crucial role in myeloma progression by not only acting on myeloma cells but also on various cells present in the bone marrow microenvironment. Novel therapies including thalidomide modulate myeloma progression at least partially by altering the cytokine profile. Since, so far our experimental findings clearly suggest the potential of celastrol as a promising anti-myeloma agent, we next sought to determine if celastrol has an effect on the pro-inflammatory cytokine profile both as a single agent and also in combination with bortezomib. Blood was collected as the mice were sacrificed and sera were separated. ELISA assay was done to measure the serum IL-6 and TNF-α level as discussed under Materials and Methods. As shown from our experimental findings below, celastrol alone and also in combination with bortezomib decreased the serum IL-6 and TNF-α level significantly, which clearly establishes its anticancer and anti-inflammatory potential.

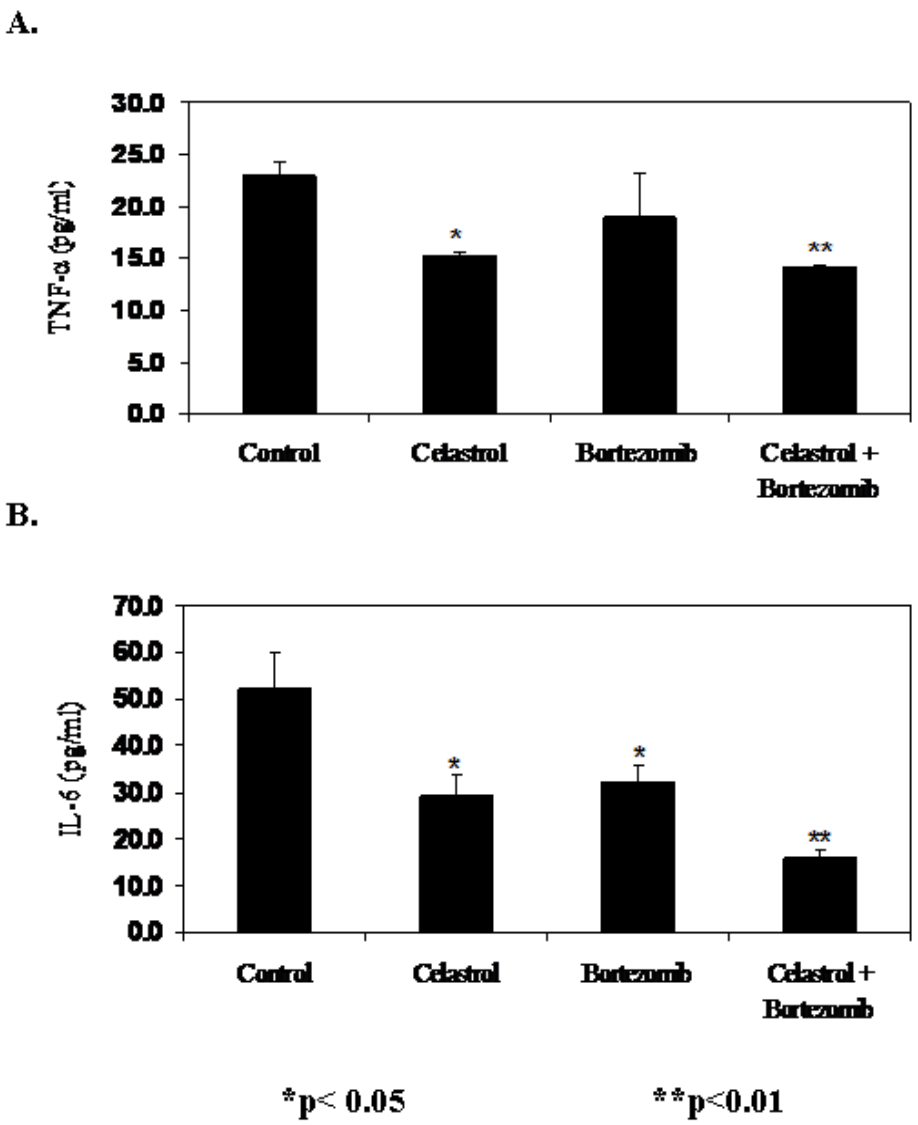


Figure 3.5.5. Celastrol alters the cytokine profile *in vivo* in MM xenograft mouse model

A. TNF-α level was measured using the ELISA kit as described under Materials and Methods section. Data is presented as mean ± SD. * Significant difference as compared to the control, $p<0.05$.

B. IL-6 level was measured using the ELISA kit as described under Materials and Methods section. Data is presented as mean ± SD. * Significant difference as compared to the control, $p<0.05$.

4. DISCUSSION

4.1. Anti-myeloma effects of celastrol *in vitro*

MM is a B cell malignancy characterised by abnormal proliferation of plasma cells with low proliferative index. It is often preceded by the two premalignant conditions MGUS and SMM [4]. MM is common among elderly male [7]. MM is the second most common hematological malignancy next only to non-Hodgkin's lymphoma, contributing 1% of all neoplasias [9]. According to Singapore General Hospital website as dated on October 2011, about 80 new patients with MM are diagnosed every year.

Treatment of MM involves bone marrow transplantation for those who are eligible and those who are not eligible for the bone marrow transplantation are treated with chemotherapy [306]. The common front line agents used are alkylating agent melphalan, corticosteroid dexamethasone, immunomodulatory agent thalidomide and second generation immunomodulatory agent lenalidomide and the proteasome inhibitor bortezomib [307]. The relapsed and refractory MM are treated with bortezomib-thalidomide-dexamethasone combination, lenalidomide-bendamustine-dexamethasone combination, carfilzomib and pamolidomide [308, 309]. However, there remains an urgent need to develop novel anti-myeloma agents because despite the availability of various targeted therapies, MM still remains an incurable disease. Most of the patients relapse after initial response or develop chemoresistance. Moreover, most of the available drugs have severe dose-limiting toxicity [310]. Consequently, there are many drugs that are currently being investigated in preclinical and clinical studies to improve the disease outcome [307, 310].

Plant derived medicinal compounds are good source of alternative therapy. Interest in medicinal plants is gaining momentum in the last few decades. Various native medicinal systems like Ayurveda (India) and Chinese natural medicine are based on plant derived medicinal products [436]. The extract of the medicinal plant *T. wilfordii* (Thunder of God Vine) has been used in Chinese natural medicine for inflammatory conditions like arthritis, fever and edema. Various *in vitro* and *in vivo* preclinical and clinical studies

prove the effectiveness of this plant extract in various inflammatory conditions [322]. Celastrol is one of the active compounds found in this plant extract that has attracted great interest among the scientific community over the last decade because of its diverse anti-inflammatory and anti-cancer properties [326].

4.1.1. Celastrol induces cell death in MM cells

Resisting cell death is considered to be one of important hallmark capabilities of cancer cells. Most of the anti-cancer drugs eventually inhibit tumor growth by inducing cell cycle arrest and or apoptosis [2, 3]. We observed that celastrol decreased the viability of a wide variety of MM cells, including ones resistant to doxorubicin, melphalan and bortezomib. Celastrol not only induced apoptosis in U266 and RPMI-8226 cells but also in bortezomib resistant clone of RPMI-8226. Celastrol-induced cell death in RPMI-8226 cell line resistant to bortezomib that was comparable to its observed effect on drug-sensitive counterpart. Celastrol increased the number of apoptotic cells as demonstrated by annexin V staining, TUNEL, live-dead assay, and DNA fragmentation assays. In accordance with the previous studies which investigated the apoptotic effect of celastrol in numerous cancers, our study shows that celastrol induced cell death unequivocally in MM cells [326]. Concurrent with our study [437], another study using MM cell model also reported that celastrol indeed induces programmed cell death in MM cells [364]. Interestingly, we found that celastrol induced relatively less death in mouse embryonic fibroblasts and human breast epithelial cell line MCF-10A which were used as a representative of non-tumor cells, thereby indicating that it can preferentially targets cancer cells.

Exposure to ionizing radiation, conventional chemotherapy, TRAIL ligand and thalidomide and its other immunomodulatory agents induce apoptosis by caspase-8-mediated extrinsic apoptotic pathway, while dexamethasone induces apoptosis by caspase-9-mediated intrinsic apoptotic pathway. However, proteasome inhibitor bortezomib can induce apoptosis by both intrinsic and extrinsic pathways [437]. Along this line, we further investigated the apoptotic pathways involved in celastrol-induced apoptosis. In accordance with other studies done in breast cancer and lung cancer cell lines

[394, 395], celastrol-induced apoptosis was found to involve both caspase-8-mediated extrinsic apoptotic pathway and caspase-9-mediated intrinsic apoptotic pathway.

4.1.2. Celastrol activates JNK MAP kinase to induce apoptosis in MM cells

JNK is one of the MAPKs activated in cells in response to cytokines and exposure to environmental stresses resulting in cellular proliferation, differentiation and apoptosis [429]. Various environmental stresses including ionising radiation and chemotherapeutic agents induce JNK. c-Jun and c-Fos are proteins which are activated after being phosphorylated by JNK. These two proteins form dimers with each other to activate AP-1 transcription factor which than mediates the downstream effects of JNK. Whether activated JNK induces cellular proliferation or induces apoptosis is context dependent. When inducing apoptosis JNK can induce apoptosis through both caspase-8-dependent extrinsic pathway or caspase-9-dependent intrinsic pathway [430]. JNK activation is also needed for apoptosis induced by chemotherapeutic agents [438]. Since celastrol acted as a cytotoxic agent in MM cells, we further investigated if celastrol-induced apoptosis is also mediated through JNK activation. We found that celastrol induced JNK phosphorylation as early as 15 minutes after treatment without altering the levels of JNK protein expression. Further, we investigated if celastrol-induced activation of JNK is involved in celastrol- induced apoptosis. It was found that suppression of JNK activation by the specific JNK inhibitor SP600125 partially blocked celastrol- induced apoptosis, indicating that activation of JNK is at least partially necessary for celastrol-induced apoptosis. In melanoma cells, celastrol was found to activate JNK and increase the transcriptional activity of c-Jun to inhibit the melanoma cell proliferation. Moreover, in the presence of JNK inhibitor celastrol failed to inhibit the melanoma cell growth [378]. However, we did not investigate the downstream effects of celastrol on c-jun, c-Fos expression in our study.

Numerous studies have shown that celastrol induces ROS [389, 390, 392, 401]. JNK has been found to be an important mediator of ROS-induced apoptosis [439]. Along this line, while our manuscript was under review [440], it was

reported by another group that celastrol induced ROS and celastrol-induced ROS lead to activation of JNK which in turn induced apoptosis [392]. However, whether celastrol-induced activation of JNK in MM cells is mediated by induction of ROS in MM cells is yet to be investigated.

4.1.3. Celastrol alters the balance between pro-apoptotic and anti-apoptotic proteins to favour apoptosis

Evasion of apoptosis or development of acquired resistance to apoptosis is a hallmark capability of cancer cells which is almost universal to all human cancers [2]. U226 cells display upregulated expression of anti-apoptotic proteins which leads to their inherent resistance to apoptosis [222]. Interestingly, celastrol downregulated the expression of Bcl-2 family of anti-apoptotic proteins including Bcl-2, Bcl-xL and Mcl-1 and IAP (Inhibitors of apoptotic protein), including survivin and XIAP in both U266 and RPMI-8226 cell lines. Moreover, celastrol was found to upregulate the expression of pro-apoptotic members Bcl-2 family of proteins including Bax and Bak. This alteration in anti-apoptotic and pro-apoptotic proteins might lead to celastrol-induced apoptosis. These observations are in agreement with a recent report in which celastrol was shown to downregulate the expression of Bcl-2 and induce Bax expression in breast cancer cell lines [394].

4.1.4. Celastrol modulates the expression of proteins involved in cell cycle progression

Insensitivity to anti-growth signal hallmark capability of cancer cells enable these to evade the anti-growth signals present in the environment. Most of the anti-growth signals control the regulation of cell cycle in order to prevent the cells from multiplying in an uncontrolled manner [2]. Cyclin D1 and cyclin E are required for the progression of cells from the G1 phase to the S-phase of the cell cycle [441]. Cyclin D is dysregulated in almost all types of MM and is considered to be one of the early initiating event in MM pathogenesis [19]. Interestingly, in our study celastrol suppressed the expression of both cyclin D1 and cyclin E in a time-dependent manner in

RPMI-8226 cells. The expression of the cyclin-dependent kinase inhibitors p21 $^{WAF1/CIP1}$ and p27, which block cell cycle progression by inhibiting the activity of cyclin/Cdk2 complexes, was also upregulated by celastrol. These observations are in agreement with the studies done with human monocytic leukemia cells and glioma cells in which celastrol was observed to downregulate cyclin D1 expression to cause G0/G1 arrest and upregulate the expression of cell cycle progression inhibitors p21 and p27 to cause G2/M arrest respectively [338, 346].

4.1.5. Celastrol modulates the expression of proteins involved MM angiogenesis/aggressiveness

Angiogenesis hallmark capability of cancer cells enable the tumors to outgrow the 100 μm diameter limit, within which cells survive by diffusion of oxygen from nearby capillaries. This process is initiated by angiogenic switch in which the balance between proangiogenic and antiangiogenic factors is tipped towards angiogenesis [2]. In MM, there is dysregulated bone marrow angiogenesis [107, 108]. Moreover, increase in bone marrow angiogenesis is correlated with disease progression [111, 112] and disease activity [109, 110]. Dysregulation of c-Myc is considered to play an important role both in myeloma initiation and progression [36, 37]. It is considered that dysregulated c-Myc exerts its role in myeloma pathogenesis by inducing bone marrow angiogenesis [442]. VEGF is secreted both by MM and bone marrow stromal cells which in turn stimulate IL-6 secretion by stromal cells, thus stimulating paracrine growth of MM cells. c-Myc mediates anigogenesis by inducing autocrine secretion of VEGF by MM cells [443]. So, we sought to assess the anti-angiogenic potential of celastrol if any, by its effect on the expression of mediators of MM angiogenesis namely c-Myc and VEGF in RPMI-8226 cells. Interestingly, we found that celastrol downregulated the expression of these two proteins in a time-dependent manner, indicating its potential role in inhibiting myeloma angiogenesis, thereby MM progression.

4.1.6. Celastrol inhibits MM cell migration, invasion and modulates the expression of proteins involved in these cellular processes

CXCL12/CXCR4 axis mediates myeloma cell migration and thus, homing to the bone marrow [178]. In 5TMM model, inhibition of CXCR4 results in 20% reduction in tumor load [179]. Moreover, CXCR4 expression is correlative with disease progression [187]. Along this line, we sought to investigate whether celastrol has any effect on MM cell migration. We found using transwell migration chamber assay that celastrol can inhibit CXCL12-induced migration of myeloma cell. CXC12/CXCR4 axis is also involved in invasion of myeloma cell which is mediated by MMP-9 protein [179]. So, we also investigated if celastrol can inhibit myeloma cell invasion using matrigel invasion assay. We further observed that celastrol inhibits CXCL12-induced myeloma cell invasion. Interestingly, celastrol-induced inhibition of myeloma cell migration and invasion are associated with time-dependent downregulation of both CXCR4 and MMP-9 expression.

4.2. Celastrol suppresses STAT3 signaling casacde in MM cells

Resistant to apoptosis hallmark capability is crucial in the pathogenesis of myeloma, especially since it is a cancer with low proliferative index [444]. IL-6/JAK/STAT3 pathway is considered to enable this hallmark capability to myeloma cells by upregulating anti-apoptotic proteins [219, 222]. Since, celastrol downregulated anti-apoptotic proteins and induced myeloma cell apoptosis, we next aimed to investigate if celastrol modulated IL-6/JAK/STAT3 pathway. We found out celastrol inhibited both constitutive and IL-6 inducible tyrosine phosphorylation of STAT3 and nuclear translocation of STAT3. Involvement of negative regulators of STAT3, especially SHP-1 protein tyrosine phosphatases has also been implicated in the constitutive activation of STAT3 [50]. Many natural agents have been found to modulate IL-6/JAK/STAT3 pathways by upregulating SHP-1 [235, 247, 445, 446]. On a similar note, we aimed to explore the involvement of phosphatases in celastrol-induced inhibition of tyrosine phosphorylation of STAT3, if any. Our

experimental findings proved that celastrol does not involve protein tyrosine phosphatases in its ability to inhibit tyrosine phosphorylation of STAT3.

STAT3 is phoshporylated at both tyrosine and serine residues [447]. Tyrosine phosphorylation of STAT3 is executed by receptor tyrosine kinases and non-receptor tyrosine kinases like JAK and Src kinase, where as serine phosporylation of STAT3 is carried out by MAPK kinases including JNK and ERK. Most of the growth factors regulate serine phosphorylation of STAT3 by ERK signaling, though IL-6 is also reported to serine phosphorylate STAT3 via an ERK independent mechanism. JNK is involved in stress induced serine phosphorylation of STAT3 [448]. There are conflicting reports about the role of serine phosphorylated STAT3 in the transcriptional activity of STAT3. Wen *et al.,* had previously indicated that either the presence or absence of serine phosphorylation does not affect the DNA binding ability of STAT3 [449]. However, earlier reports suggest that serine phosphorylation is essential for maximum transcriptional activity of STAT3 [450]. Chung *et al.,* have previously reported that serine phosphorylation of STAT3 can negatively regulate its tyrosine phosphorylation, thus abrogating the DNA binding ability of STAT3 [448]. The role of serine phosphorylation in the oncogenic potential of STAT3 is established by employing diethylnitrosamine-induced mouse hepatocarcinogenesis model. So, we aimed to investigate if celastrol also inhibited the serine phosphorylation of STAT3 using serine phosphorylation specific antibodies. We found out that celastrol specifically inhibited tyrosine phosphorylation of STAT3 with minimal effect on serine phosphorylation. JNK is also known to negatively regulate tyrosine phosphorylation of STAT3 through both serine phosphorylation-dependent and serine phosphorylation-independent mechanisms.

STAT5, an another member of STAT family of transcription factors, has also been implicated in myeloma pathogenesis and is mainly activated by IGF [451]. So, we aimed to investigate if celastrol can also modulate STAT5 activation in MM cells. Interestingly, we found that celastrol does not have any effect on phosphorylation of STAT5. These findings indicate that celastrol is a specific blocker of STAT3 tyrosine phosphorylation. So, we next proceeded to

investigate whether celastrol affects any upstream kinases involved in the tyrosine phosphorylation of STAT3. We found out that celastrol can inhibit activation of both JAK2 and Src kinases involved in the tyrosine phosphorylation of STAT3. Our group has also confirmed the STAT3 inhibiting ability of celastrol in HCC cell lines and in human HCC xenograft mouse model. In HCC cell lines, celastrol not only inhibited constitutive and IL-6-induced phosphorylation of STAT3 but also blocked its transcriptional activity as evidenced from the luciferase reporter assay [452].

Quinone methide moiety of celastrol is considered to be an important functional group involved in its observed pharmacological activities. Quinone methide functional group is also known to react with thiols [346, 352]. So, we sought to investigate if celastrol-induced inhibition of tyrosine phosphorylation of STAT3 is mediated through the quinone methide moiety, using various cellular and non-cellular thiols. Our experimental findings clearly indicate that thiol reactive functionality of celastrol is necessary for its observed inhibitory effects on STAT3 activation.

In sum, we report for the first time that celastrol could suppress both constitutive and inducible STAT3 activation in MM cells, concomitant with inhibition of upstream Src and JAK2 kinases. Previous studies have indicated that JAK2 and Src kinases cooperate to mediate constitutive activation of STAT3 [453, 454]. Our observations also suggest that celastrol may block cooperation of Src and JAK2 involved in tyrosine phosphorylation of STAT3. This study further analyzes and proves that thiol reactive functionality of celastrol is necessary for its observed inhibitory effects on STAT3 tyrosine phosphorylation. Overall, it was found that this triterpene could suppress both constitutive and inducible STAT3 activation leading to the other downstream effects as confirmed through the corroboration between the experimental and predictive data.

4.3. Celastrol inhibits NF-κB activation pathway in MM cells

NF-κB plays myriad role in myeloma pathogenesis. NF-κB enables sustained proliferation of myeloma cells by stimulating bone marrow stromal cells to secrete IL-6, thereby promoting paracrine growth and proliferation of

MM cells [77]. NF-κB confers MM cells resistance to apoptosis by upregulating members of IAP including XIAP and survivin [455]. NF-κB also aids in evasion of growth suppressors by upregulating cyclin D1 and there by leading to cell cycle progression [435]. Moreover, constitutive activation of NF-κB is proved to be essential for myeloma survival [298]. NF-κB is considered to be involved in bone marrow angiogenesis [456, 457]. NF-κB has also been found to be involved in myeloma cell migration and invasion [458].

In agreement with previous reports [286, 293, 459], we found that MM cell lines expressed constitutively activated NF-κB. Since, in our study celastrol downregulated the expression of IAP members of anti-apoptotic proteins including survivin and XIAP, induced apoptosis, downregulated the expression of proteins involved in angiogenesis, inhibited myeloma cell migration/invasion and downregulated the expression of proteins involved in myeloma cell migration and invasion, we hypothesized that celastrol may affect the master transcription factor NF-κB, that plays a key role in proliferation, survival, invasion and angiogenesis in MM cells. Our experimental findings indeed show that celastrol inhibits phosphorylation of IκB, thereby preventing nuclear translocation of NF-κB p65 subunit and NF-κB DNA binding activity in a time-dependent manner. These observations are in agreement with an another study published [364] concurrent with our study [363] showing that celastrol can indeed inhibit nuclear translocation of p65 subunit in MM cells. Though, total NF-κB activity is the combination of canonical and alternative pathway activity [295] and both the pathways are dysregulated in MM [291], but interestingly dysregulated alternative pathway can also transduce signals through IKK-β in MM, thereby indicating the importance of targeting IKK-β activation [276]. Along this line, we sought to investigate if celastrol can also target phosphorylation of IKK-β, an upstream kinase involved in NF-κB activation. Interestingly, our study showed that celastrol inhibited IKK-α/β activation in time-dependent manner. Although there are previous studies which indicate celastrol inhibits IKK-α/β, those studies analyzed the effect of celastrol on either TNF-α-induced IKK-α/β in human myeloid KBM-5 cells [372] or IL-1β and TNF-α-induced IKK-α/β in W256 mammary sarcoma cells [369], Therefore, this is the first study to show inhibition of constitutive IKK-α/β activation by celastrol. However, whether

celastrol modulates constitutive IKK-α/β activation directly or indirectly via suppression of other important upstream kinases such as TAK1, MEKK1, NIK etc needs additional investigation.

AKT is constitutively activated in MM cells [460] and since it mediates various processes involved in MM progression AKT pathway is considered to be an important therapeutic target in the treatment of MM [79]. AKT has been found to be involved in MM progression by mediating cell proliferation [84, 461], anti-apoptosis [84, 462] and cell migration [463]. Interestingly, our study also shows that celastrol can inhibit the phosphorylation of AKT. It has been established that AKT can act upstream of NF-κB signalling cascade to positively regulate its activation [455]. Thus, it is feasible that celastrol-induced inhibition of both IKK-α/β and AKT activation could be involved in the suppression of NF-κB activation pathway.

NF-κB inhibition is considered to be at least one of the mechanisms of action of the novel proteasome inhibitor bortezomib that is approved for MM treatment [290]. Since celastrol was observed to inhibit NF-κB pathway activation in our study, we further investigated if celastrol potentiated bortezomib-induced inhibition of NF-κB activation. Our experimental results demonstrate that celastrol indeed potentiates the bortezomib-induced NF-κB inhibition in MM cells.

Whether celastrol-induced inhibition of STAT3 and NF-κB activation as observed in MM cell lines are linked with each other is not clear as of yet. However, there is an evidence to suggest that STAT3 activation might positively regulate NF-κB activity by preventing its nuclear export [464]. However, activation of STAT3 and NF-κB pathway are mediated by different cytokines. STAT3 pathway is activated by IL-6 cytokine; whereas, NF-κB pathway is primarily activated by TNF-α and B cell growth factors. Interestingly, erythropoientin-activated JAK2 kinase has been shown to positively regulate NF-κB activity. Hence, it is conceivable that celastrol-mediated inhibition of these two master transcription factors might be related through its ability to abrogate JAK2 kinase activation [465]. Our study also demonstrates that thiol reactive quinone methide functional group of celastrol is involved in the inhibition of STAT3. Thus, it would be interesting to analyze if the same functional group also mediates the inhibition of NF-κB as observed

upon celastrol treatment.

Based on our predictive analysis, inhibition of NF-κB and STAT3 can be attributed as the potential downstream effects of HSP90 inhibition and HO-1 induction by celastrol in MM cells. Interestingly, a study by Malara *et al.,* showed that simultaneous inhibition of both STAT3 and NF-κB is necessary and sufficient to induce apoptosis in U266 cells, which exhibit constitutive activation of both STAT3 and NF-κB pathway [242]. Our study propose and prove that celastrol at micromolar concentrations effectively block these two pathways to induce MM cell apoptosis.

4.4. Celastrol potentiates the apoptosis induced by novel targeted therapies in MM cells

Celastrol has been recently reported to enhance TRAIL-induced cell death [399, 401] and TNF-α-induced apoptosis [371, 372]. Celastrol has also been found to potentiate the apoptotic effects of gambogic acid in human oral carcinoma cell line Tca8113 [374], temozolomide in melanoma [373], and conventional chemotherapeutic agents (daunorubicin and cytarabine) in K-562 and Jurkat T human leukemia cells [402]. It also synergistically induced apoptosis in combination with ErbB2-targeted therapeutics (trastuzumab and lapatinib) in ErbB2 overexpressing breast cancer cells [390] and sensitized taxol-resistant HeLa cells to paclitaxel [384]. However, so far there are no reports to analyze the apoptotic effects of celastrol in combination with targeted anti-MM drugs. Interestingly, our studies show for the first time that celastrol synergistically induces apoptosis in combination with either the bortezomib or thalidomide as deducted from Chou-Talalay multiple drug effect equation. The plausible explanation for this synergistic effect could be myriad. As evidenced from our studies and other reports published previously, celastrol can inhibit NF-κB pathway, although via distinct mechanisms in diverse tumor cell types. Both bortezomib [466] and thalidomide [467] are known to inhibit NF-κB activation cascade. Therefore, it is conceivable that celastrol-bortezomib and celastrol-thalidomide combination might cooperatively inhibit

NF-κB pathway that may lead to observed synergistic effect on apoptosis. Moreover, both bortezomib and celastrol are known to act as proteasome inhibitors. Thus, these two agents might cooperatively modulate various proteins that are degraded by ubiquitin-proteasome system and are involved in the survival and progression of myeloma. However, there exists one interesting difference between pharmacological actions of bortezomib and celastrol; Bortezomib treatment upregulate HSP90 and this upregulated HSP90 is considered to be a stress response by which tumor cells manage to recover from the cytotoxic effects of bortezomib [435]. Inhibition of HSP90 by HSP09 inhibitors enhances bortezomib-induced apoptosis [468]. Consequently, many HSP90 inhibitors are currently being tested in combination with bortezomib to achieve greater clinical efficacy [469]. Interestingly, celastrol is a bonafide HSP90 inhibitor [342, 344, 345, 350, 353]. Therefore, celastrol induced inhibition of HSP90 that is upregulated by bortezomib treatment can be another plausible mechanism for the synergism exhibited by these two agents. Inhibition of IL-6-mediated signaling in combination with bortezomib has also been shown to exhibit synergistic apoptosis [470]. Hence, celastrol-induced inhibition of STAT3 activation pathway can also account for the observed synergy with celastrol-bortezomib combination in MM cells.

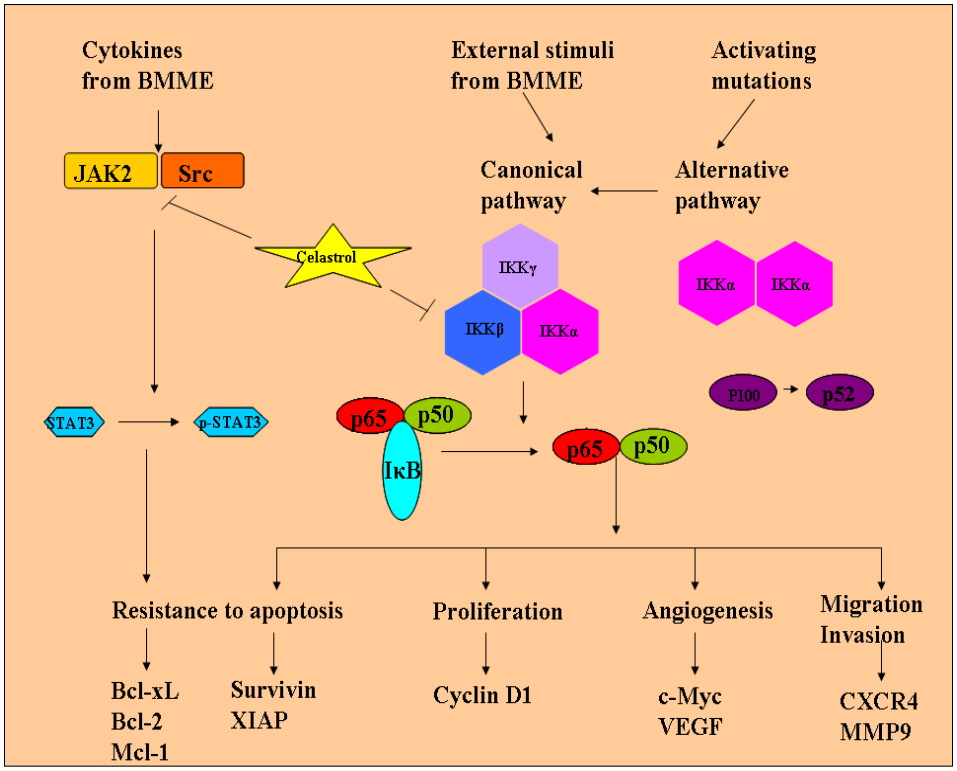


Figure 4.1. Schematic diagram depicting inhibition of STAT3 and NF-κB pathways by celastrol in MM cells

STAT3 plays crucial role in myeloma pathogenesis by upregulating Bcl-2 members of anti-apoptotic proteins which confer myeloma cells resistance to apoptosis. In MM, STAT3 pathways are mainly activated by cytokines present in the bone marrow microenvironment. These cytokines activate the non-receptor tyrosine kinases JAK2 and Src which in turn can phosphorylate STAT3 to induce its nuclear translocation and DNA binding. In MM, NF-κB pathway is dysregulated by both activating mutations and cytokines present in the BMME. Thus dysregulated NF-κB pathway mainly channels through canonical pathway which is mediated by IKK-β. NF-κB governs many processes involved in myeloma progression including resistance to apoptosis, proliferation, angiogenesis, migration and invasion by regulating the indicated players. Celastrol suppresses STAT3 activation pathway by inhibiting upstream JAK2 and Src kinases. Inhibition of NF-κB pathway by celastrol is found to be mediated by abrogation of IKK-α/β and AKT activation. Overall, inhibition of these two important pro-inflammatory transcription factors by

celastrol can account for its observed anti-proliferative, apoptotic, anti-invasive and antiangiogenic effects in MM cells.

4.5. Anti-myeloma effects of celastrol *in vivo*

4.5.1. Celastrol potentiates the inhibition of tumor growth induced by bortezomib in a xenograft nude mouse model

To analyze the anti-myeloma effects of celastrol *in vivo*, we established human MM xenograft nude mouse model by subcutaneously injecting U226 cells. Xenograft mouse model is the most commonly used animal model to test the efficacy of anticancer drugs. This model is relatively easy to establish and allow easy monitoring of the tumor volume [312]. Several anti-myeloma agents that have been approved recently by FDA for the treatment of MM has been tested in xenograft model before being tested in clinical trials [313]. Though, various studies have been done to analyze the *in vivo* effects of celastrol in various tumor models, this is the first study to analyze the *in vivo* effects of celastrol alone and also in combination with bortezomib in MM. In our study, celastrol alone inhibited MM tumor growth when compared to control group. In accordance with our *in vitro* studies, celastrol also potentiated the tumor growth inhibition induced by bortezomib in statistically significant manner. These observations are in agreement with studies conducted in human prostate cancer [337, 406], breast cancer [371], melanoma [378], pancreatic cancer [342], glioma [413], and CML xenograft mouse models [411], where celastrol significantly inhibited tumor growth *in vivo* as single agent. Though, there have been previous studies to analyze the effect of celastrol in combination with TRAIL cytokine in lung cancer [399], and colon cancer xenograft mouse models [400] as well as with radiotherapy in prostate [403] and lung cancers [391], this is the first study to analyze the effect of celastrol in combination with an approved targeted therapy (bortezomib) *in vivo* in a tumor model of haematological malignancy.

4.5.2. Celastrol in combination with bortezomib inhibits the expression of STAT3 and NF-κB p65 in tumor tissues

As mentioned previously, combined and simultaneous inhibition of STAT3 and NF-κB is sufficient and necessary for induction of apoptosis in myeloma cells [242]. Since, celastrol as a single agent induced apoptosis *in vitro* and inhibited tumor growth *in vivo*, we also aimed to investigate if celastrol-induced abrogation of tumor growth correlated with the modulation of STAT3 and NF-κB activation. Interestingly, we found that celastrol inhibited the expression of both p-STAT3 and NF-κB in mouse xenograft model. Also, though the effect of bortezomib on STAT3 activation cascade is not well characterized as it is on NF-κB, our study demonstrates that celastrol in combination with bortezomib can inhibit expression of both p-STAT3 and NF-κB, which is associated with greater inhibition of tumor growth. These observations are in agreement with the studies showing that inhibition of IL-6-mediated pathway in MM can indeed potentiate the apoptosis induced by bortezomib [470].

4.5.3. Celastrol in combination with bortezomib downregulates the expression of proteins involved in myeloma progression *in vivo*

Since celastrol in combination with bortezomib inhibited MM tumor growth and was associated with the inhibition of expression of p-STAT3 and NF-κB, we aimed to investigate if celastrol-bortezomib combination was associated with downregulation of survival/angiogenic proteins involved in myeloma progression and regulated by these two transcription factors. Interestingly, we found that both celastrol alone and bortezomib alone downregulated the expression of Bcl-2 and VEGF proteins in tumor tissues. However, the celastrol-bortezomib combination exhibited substantially better inhibition than when both the compounds were used as a single agent at suboptimal doses. This observation is in accordance with the greater inhibition of tumor growth and in the expression of STAT3 and NF-κB transcription factors exhibited by the combination treatment group.

4.5.4. Celastrol in combination with bortezomib modulates cytokine profile *in vivo*

IL-6 cytokine drives myeloma progression by simultaneously activating JAK/STAT3, MAPK/ERK and PI3K/AKT pathways [435]. Moreover, serum levels of IL-6 and soluble IL-6 receptor has been proven to be a prognostic marker for tumour load, disease progression and survival [65-69]. Likewise, TNF-α is also involved in myeloma cell survival and progression [435]. Moreover, TNF-α secreted by MM cells act on the bone marrow stromal cells to stimulate the latter to secrete IL-6 which in turn aids in paracrine growth of MM cells [76, 77, 168]. Bortezomib has been shown to modulate cytokine profile favoring MM cell death [435]. On a similar note, we aimed to investigate if celastrol and bortezomib combination can cause modulation of cytokines in the sera derived from MM xenograft mouse model. Interestingly, we noted that celastrol alone decreased the serum level of both IL-6 and TNF-α when compared to the control group. However, celastrol-bortezomib combination treatment group showed a greater decrease in these cytokine levels. Thus, these findings suggest that by modulating cytokine levels celastrol might alter the interaction between MM cells and BMME thereby favouring cell death, as observed similarly with bortezomib [471].

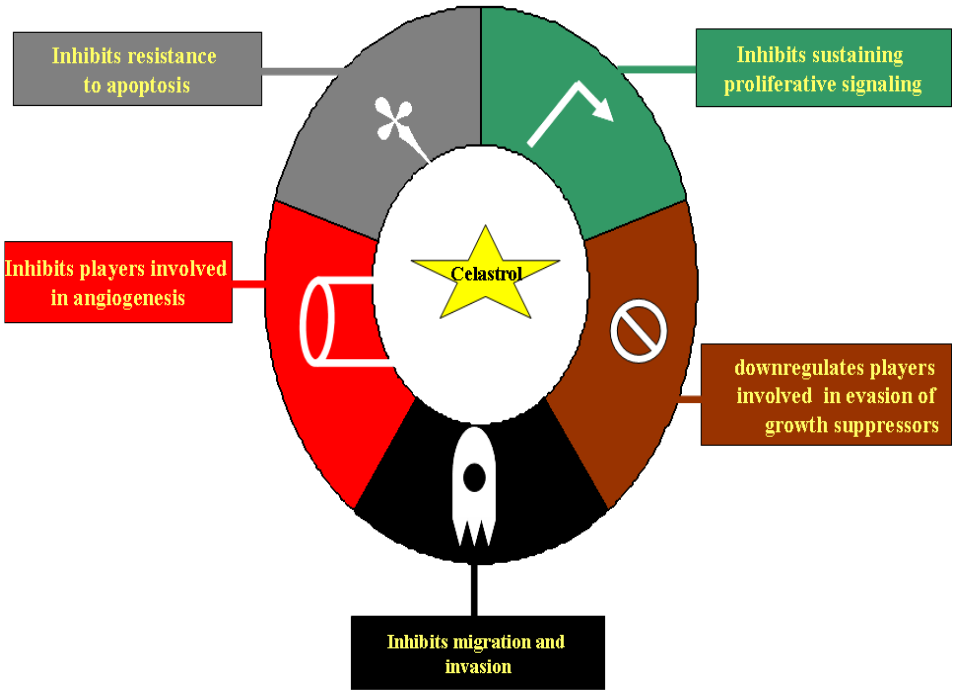


Figure 4.2. Schematic diagram showing celastrol-induced inhibition of various characteristics hallmark features of MM

Celastrol inhibits resistance to apoptosis by downregulating anti-apoptotic proteins and upregulating pro-apoptotic proteins, suppresses sustaining proliferative signaling by inhibiting STAT3/NF-κB activation pathways, inhibits evasion of growth suppressors by downregulating cyclin D1 and cyclin E, and upregulating p21 and p27 expressions, modulates angiogenic signal by downregulating the expression of VEGF /c-Myc, and abrogates CXCL12-induced migration/invasion by downregulating the expression of CXCR4 and MMP-9. However, it is possible that celastrol may still modulate several other important proteins that play an important role in MM initiation and progression.

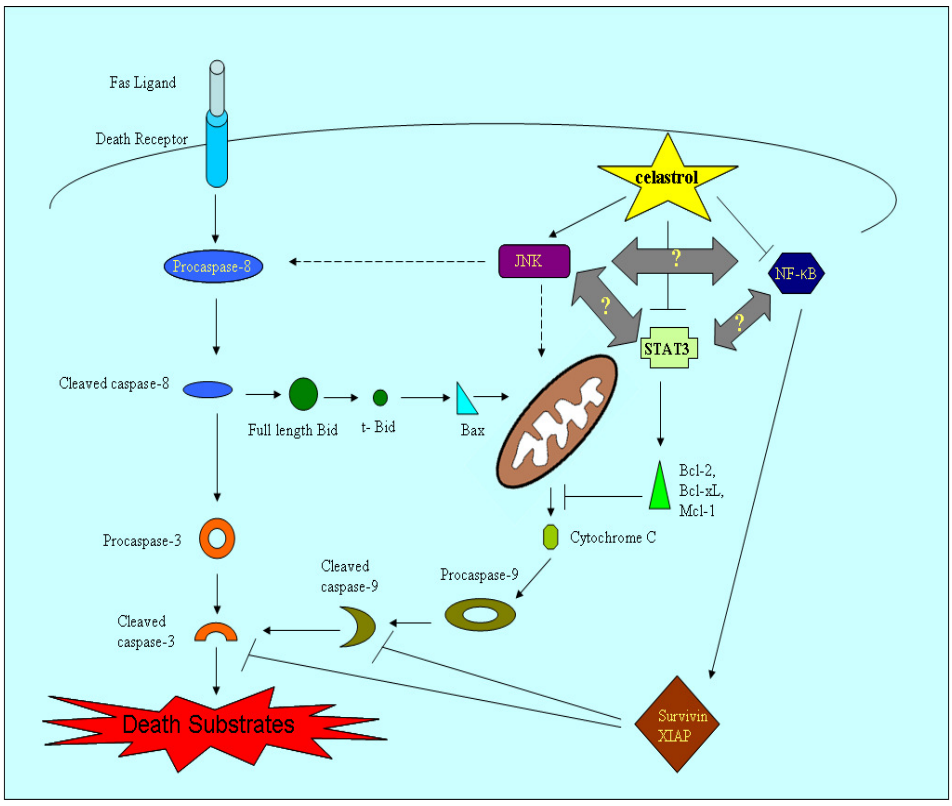


Figure 4.3. Schematic diagram representing the molecular mechanism(s) of celastrol-induced apoptosis in MM cells

MM cells have upregulated expression of anti-apoptotic members of Bcl-2 family of proteins namely Bcl-2, Bcl-xL and Mcl-1 by virtue of the activated STAT3 pathway. Constitutively active NF-κB pathway also contributes to MM cell survival by upregulating members of IAP family of proteins namely survivin and XIAP. Celastrol activates both caspase-8-mediated extrinsic apoptotic pathway and caspase-9-mediated intrinsic apoptotic pathway. Celastrol treatment induces JNK activation which can mediate its apoptotic effects in MM cells. The dashed lines indicate how JNK can possibly mediate celastrol-induced apoptosis. Celastrol also aids in apoptosis by inhibiting both STAT3 and NF-κB activation pathways. However, whether STAT3, NF-κB and JNK proteins can directly interact with each other needs additional investigation.

5. CONCLUSIONS

In summary, our study shows for the first time that celastrol exhibits potent anti-myeloma effects including induction of apoptosis, inhibition of expression of various proteins involved in cell cycle progression, suppression of expression of proteins involved in angiogenesis, as well as abrogation of chemokine-induced migration and invasion. We also provide mechanistic evidences, namely induction of JNK by which celastrol can induce apoptosis in MM cells. This study also establishes celastrol as a novel inhibitor of STAT3 transcription factor, an unexplored pharmacological property of this triterpene. We also identified the key functional groups of celastrol responsible for its observed STAT3 inhibitory effects. Moreover, we also validate that celastrol can indeed inhibit NF-κB cascade through the modulation of constitutive IKK-α/β and AKT activation in MM cells. These findings were found to be in agreement with the *in silico* analysis data provided by Cell works Inc. We also demonstrate that celastrol can significantly potentiate NF-κB inhibitory potential of bortezomib in MM cells. In addition, we provide evidence showing that celastrol can synergistically induce apoptosis in combination with novel anti-myeloma agents. Finally, we demonstrate the significant potentiation of anti-myeloma effects of bortezomib by celastrol in a xenograft MM mouse model without any substantial side effects. Our *in vivo* data also confirms inhibition of p-STAT3, NF-κB and downregulation of survival/angiogenic/inflammatory biomarkers by celastrol alone and also in combination with bortezomib mediates its observed anti-myeloma effects. Also, when examined in $CD138^+$ plasma cells isolated from patients with MM, we found that celastrol suppressed the viability in a dose-dependent manner, suggesting its enormous clinical potential for MM treatment.

6. FUTURE DIRECTIONS

Analysis of effects of celastrol on MM cells co-cultured with bone marrow stromal cells will help to elucidate its effect on the interaction between MM cells and BMME. Elucidation of detailed molecular mechanisms of action by which celastrol can modulate multiple cell signal transduction cascades will provide novel insights into its potential therapeutic uses. Moreover, these proposed experiments will also help to analyze the potential effect of celastrol on cell adhesion mediated drug resistance. *SCID-Rb* MM xenograft model can be employed to analyze the effect of celastrol on the interaction between MM cells and BMME *in vivo*. Cultured MM cells do not exactly resemble human MM pathological process. So, it would be prudent to confirm the anti-myeloma effects of celastrol in plasma cells isolated from MM patients. Additionally the effect of this triterpene should also be investigated in peripheral blood mononuclear cells to rule out potential toxicities. Also, detailed pharmacokinetic and toxicological studies in suitable mouse models should be performed to facilitate the rapid use of celastrol from bench to bedside research.

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8. APPENDIX-I

2.2. Methods

2.2.1. *In Silico* Analysis

The Cellworks (Cellworks, USA) tumor cell platform provides a dynamic and transparent view of human cellular physiology at the proteomics abstraction level. The virtual tumor cell platform consists of a dynamic and kinetic representation of the signaling pathways underlying tumor physiology at the bio-molecular level. The platform has been co-related against an extensive set of pre-defined *in vitro* and *in vivo* studies [1].

2.2.2. Platform Description

The virtual tumor cell platform consists of a dynamic and kinetic representation of the signaling pathways underlying tumor physiology at the bio-molecular level. All the key relevant protein players and associated gene and mRNA species with regard to tumor related signaling are comprehensively included in the system with their relationship quantitatively represented. Pathways and signaling for different cancer phenotypes comprise 75 major signaling networks with more than 3900 intracellular molecules. The platform comprises of crucial oncogenic proteins including EGFR, PDGFRA, FGFR, c-MET, VEGFR and IGF-1R, cell cycle regulators, mTOR signaling, p53 signaling cascade, cytokine pathways like IL1, IL4, IL6, IL12, TNF, lipid mediators, and tumor metabolism. The modeling of the time-dependent changes in the fluxes of the constituent pathways has been done utilizing modified ordinary differential equations (ODE) and mass action kinetics. The platform has been correlated against an extensive set of pre-defined *in vitro* and *in vivo* studies.

2.2.3. Study Details

The base line used for the study was a BRAF-over expressed, RB1 and P53 mutant, aligned to U266 cell line [472, 473]. The following studies were

conducted in disease state and the biomarker trends evaluated as percentage change from disease values.

- HSP90 activity was inhibited by 80%
- NF-κB activation was inhibited by 80%
- HO-1 expression was induced by 2 folds
- A combination of the HSP90 and NF-κB inhibition, NF-κB inhibition and HO-1 induction, HSP90 inhibition with HO-1 induction and all the three in combination was also tested along with the above three individual experiments

The results for the above studies individually or in combination were analyzed across known biomarker trends for celastrol and the trends were compared with known literature.

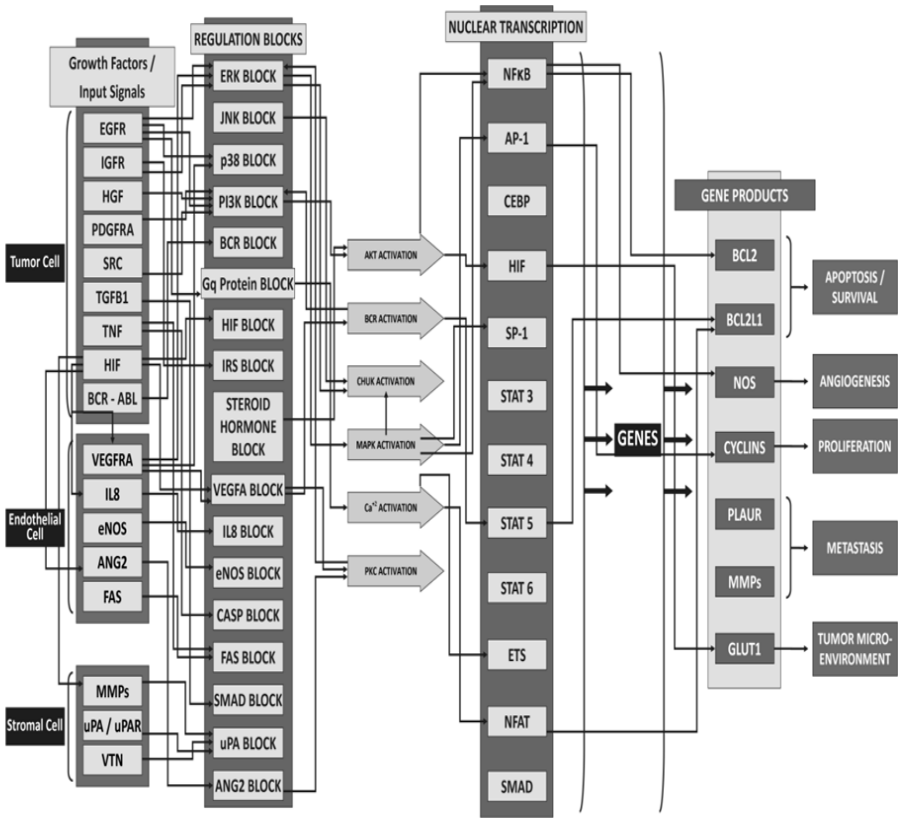
3. RESULTS

3. 1. *In silico* analysis of anti-myeloma effects of celastrol

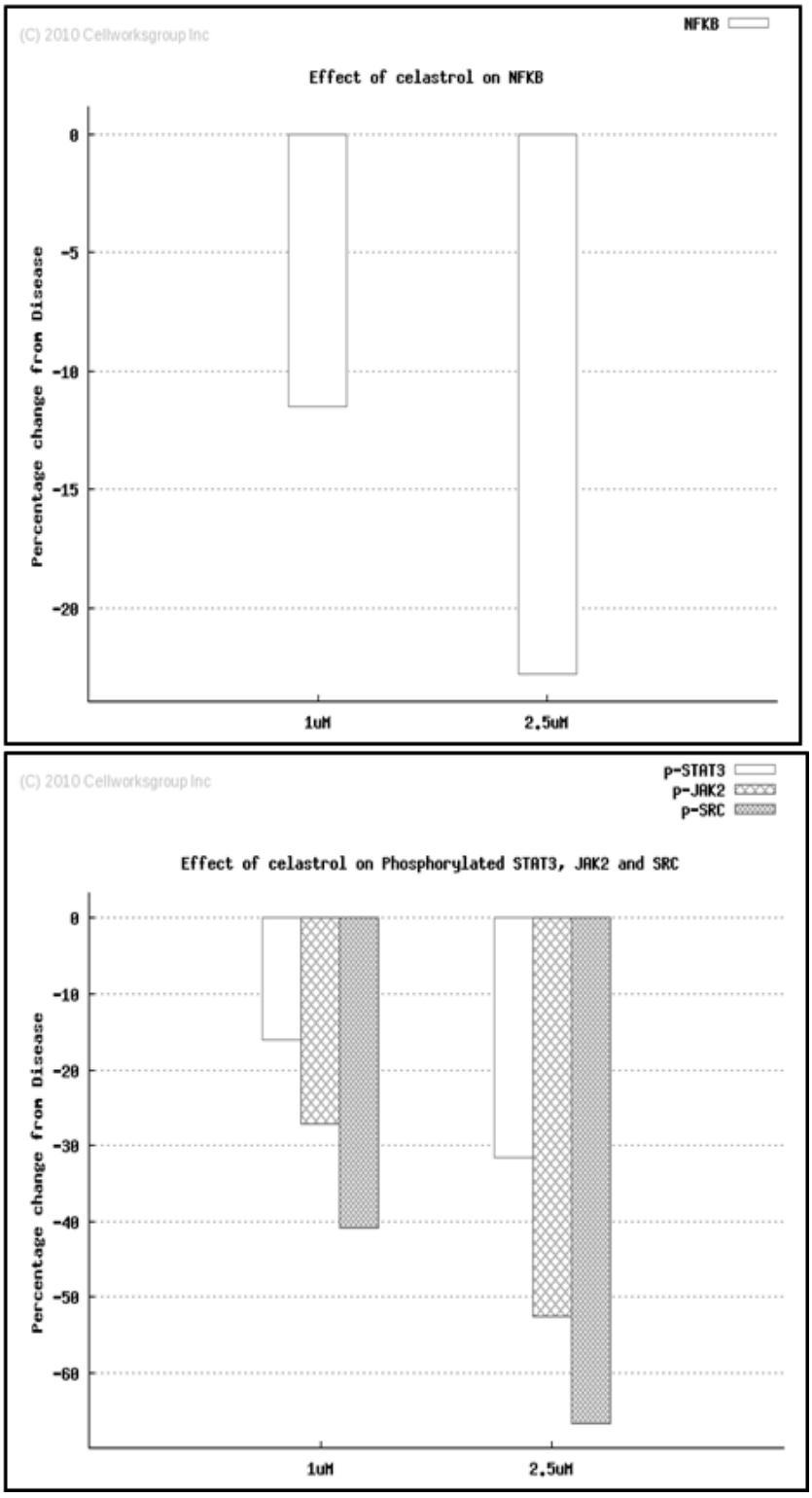
3.1.1. Predictive analysis of anti-myeloma effects of celastrol upon HSP90 activity knock-down along with HO-1 induction:

Predictive analysis data related to the potential anti-myeloma effects of celastrol under *in silico* conditions was kindly provided by Cellworks Group Inc. California, USA. For predictive analysis, the HSP90 activity was inhibited by 55% and 92% along with the induction of HO-1 expression by 2 folds, in a *BRAF* (gene that codes for B-Raf) over-expressed, *RB1* (retinoblastoma1 gene) and *p53* mutant virtual tumor cell aligned to U266 cell. **Fig. 3.1.1 A**, illustrates a high-level view of the virtual tumor cell platform. Fig. B, upper panel clearly shows that knocking down HSP90 activity by 55% and 92% along with HO-1 induction causes a reduction in NF-κB activity. Active phosphorylated STAT3, JAK2 and Src all show a reduction (Fig B, lower panel), and also all the key survival markers including Bcl-2, Bcl-xL, XIAP and survivin (BIRC5) showed a decrease with HSP90 inhibition (Fig C, upper panel). Also, increased predictive trends were observed for the apoptotic markers cleaved caspase-3 and cleaved-PARP1 (Fig. C, lower panel). Only HO-1 induction and HSP90 inhibition resulted in abrogation of STAT3 and NF-κB activity. Moreover, the predicted modulation for various oncogenic proteins by celastrol co-related with our experimental results. Thus, findings of this *in silico* model supports our central hypothesis that celastrol might modulate diverse oncogenic proteins through the negative regulation of these two master transcription factors.

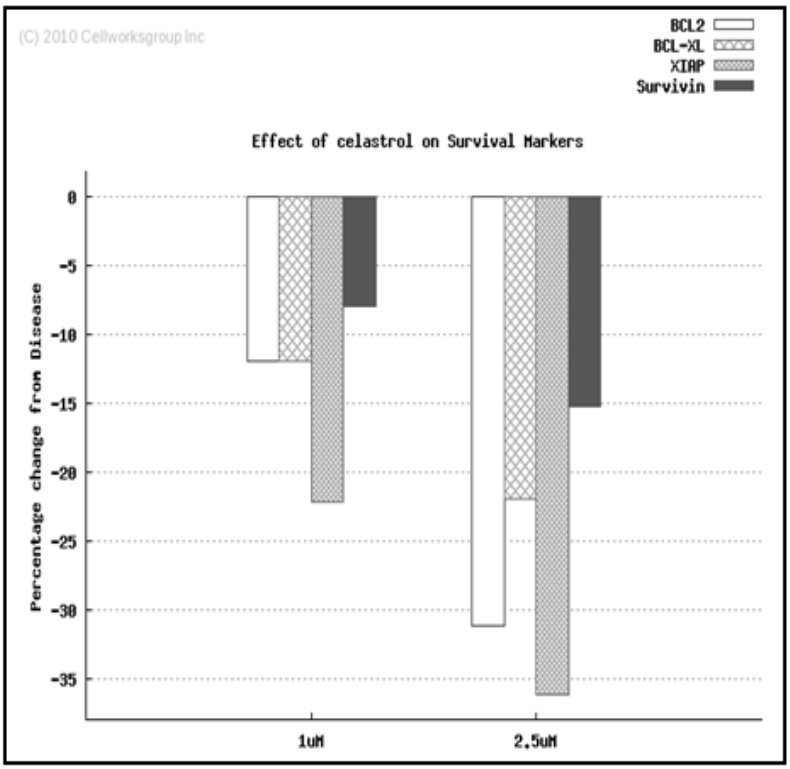
A.


Growth Factors / Input Signals
EGFR
IGFR
HGF
PDGFRA
SRC
TGFB1
TNF
HIF
BCR - ABL
Tumor Cell
VEGFRA
IL8
eNOS
ANG2
FAS
Endothelial Cell
MMPs
uPA / uPAR
VTN
Stromal Cell
REGULATION BLOCKS
ERK BLOCK
JNK BLOCK
p38 BLOCK
PI3K BLOCK
BCR BLOCK
Gq Protein BLOCK
HIF BLOCK
IRS BLOCK
STEROID HORMONE BLOCK
VEGFA BLOCK
IL8 BLOCK
eNOS BLOCK
CASP BLOCK
FAS BLOCK
SMAD BLOCK
uPA BLOCK
ANG2 BLOCK
AKT ACTIVATION
BCR ACTIVATION
CHUK ACTIVATION
MAPK ACTIVATION
Ca+2 ACTIVATION
PKC ACTIVATION
NUCLEAR TRANSCRIPTION
NFκB
AP-1
CEBP
HIF
SP-1
STAT 3
STAT 4
STAT 5
STAT 6
ETS
NFAT
SMAD
GENES
GENE PRODUCTS
BCL2
BCL2L1
NOS
CYCLINS
PLAUR
MMPs
GLUT1
APOPTOSIS / SURVIVAL
ANGIOGENESIS
PROLIFERATION
METASTASIS
TUMOR MICRO-ENVIRONMENT

B.


(C) 2010 Cellworksgroup Inc
NFKB
Effect of celastrol on NFKB
Percentage change from Disease
0
-5
-10
-15
-20
1uM
2.5uM
(C) 2010 Cellworksgroup Inc
p-STAT3
p-JAK2
p-SRC
Effect of celastrol on Phosphorylated STAT3, JAK2 and SRC
Percentage change from Disease
0
-10
-20
-30
-40
-50
-60
1uM
2.5uM

C.


(C) 2010 Cellworksgroup Inc
BCL2
BCL-XL
XIAP
Survivin
Effect of celastrol on Survival Markers
Percentage change from Disease
0
-5
-10
-15
-20
-25
-30
-35
1uM
2.5uM

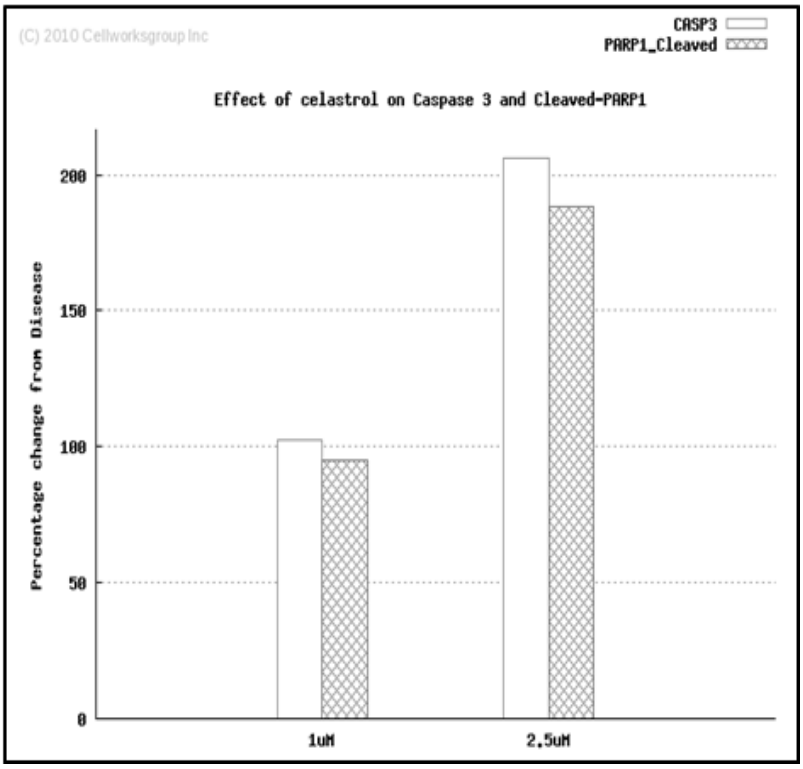

(C) 2010 Cellworksgroup Inc
CASP3
PARP1_Cleaved
Effect of celastrol on Caspase 3 and Cleaved-PARP1
Percentage change from Disease
200
150
100
50
0
1uM
2.5uM

Figure 3.1.1. Predictive analysis of anti-myeloma effects of celastrol upon HSP90 activity knock-down along with HO-1 induction

A. The figure illustrates a high-level view of the Cellworks group Inc. virtual tumor cell platform.

B. The figure in the upper panel illustrates the percentage reduction in NF-κB activity following HSP90 inhibition and HO-1 induction. The figure in the lower panel illustrates the percentage reduction in phosphorylated STAT3, JAK2 and Src kinases.

C. The figure in the upper panel, illustrates the percentage reduction in the various survival markers- Bcl-2, Bcl-xL, XIAP and survivin. The figure in the lower panel illustrates the percentage increase in caspase-3 and cleaved-PARP1 and the increasing trend of these markers supports the increase in apoptotic endpoint seen experimentally.

REFERENCES

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9. APPENDIX-II

2.1. Materials

2.1.6. Primers and probes

Primers and probes used in this study were obtained from Applied Biosystems (Foster City, CA, USA) and are as follows:

Human Bcl-2

Human Bcl-xL

Human Survivin

Human Mcl-1

Human cyclin D1

2.2. Methods

2.2.10. RNA extraction and Real-time PCR analysis

Trizol reagent was used for RNA extraction. Briefly, for a 50µl reaction, 10µl of RT product was mixed with 1x TaqMan® Universal PCR Master mix, 2.5µl of 20x TaqMan probes for Bcl-2, Bcl-xl, survivin, and Mcl-1 respectively, 2.5µl of 20x 18S RNA TaqMan probe as the endogenous control for each targeting gene, and topped up to 50 µl with sterile water. A negative control for RT, in which sterile water replaced the RNA template, was included. Another control, where RT mix was replaced with sterile water, was included to check for DNA contamination. Real-time PCR was done using 7500 Fast Real-Time PCR System (ABI PRISM 7500, Applied Biosystems, Foster City, CA, USA) with a protocol that consists of 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of denaturing at 95°C for 15 seconds and extension at 60°C for 1 min. Results were analyzed using Sequence Detection Software version 1.3 provided by Applied Biosystems. Relative gene expression was obtained after normalization with endogenous human GAPDH and determination of the difference in threshold cycle (Ct) between treated and untreated cells using 2-ΔΔCt method.

3. RESULTS

3.2.2.7. Celastrol causes downregulation of expression of various anti-apoptotic proteins involved in survival of MM cells at the transcriptional level

It is evident from our western blot results that celastrol causes downregulation of the expression of various prosurvival proteins. To analyze if the celsatrol-induced downregulation of the prosurvival proteins, occurs also at transcriptional level real time PCR analysis was also done. U266 cells were treated with 1 μM celastrol for 0, 2, 4 and 6 hrs and real time PCR assay was done as described under Materials and Methods. The results clearly reveal that celastrol down-regulates the expression of various indicated proteins at the transcriptional level in statistically significant manner.

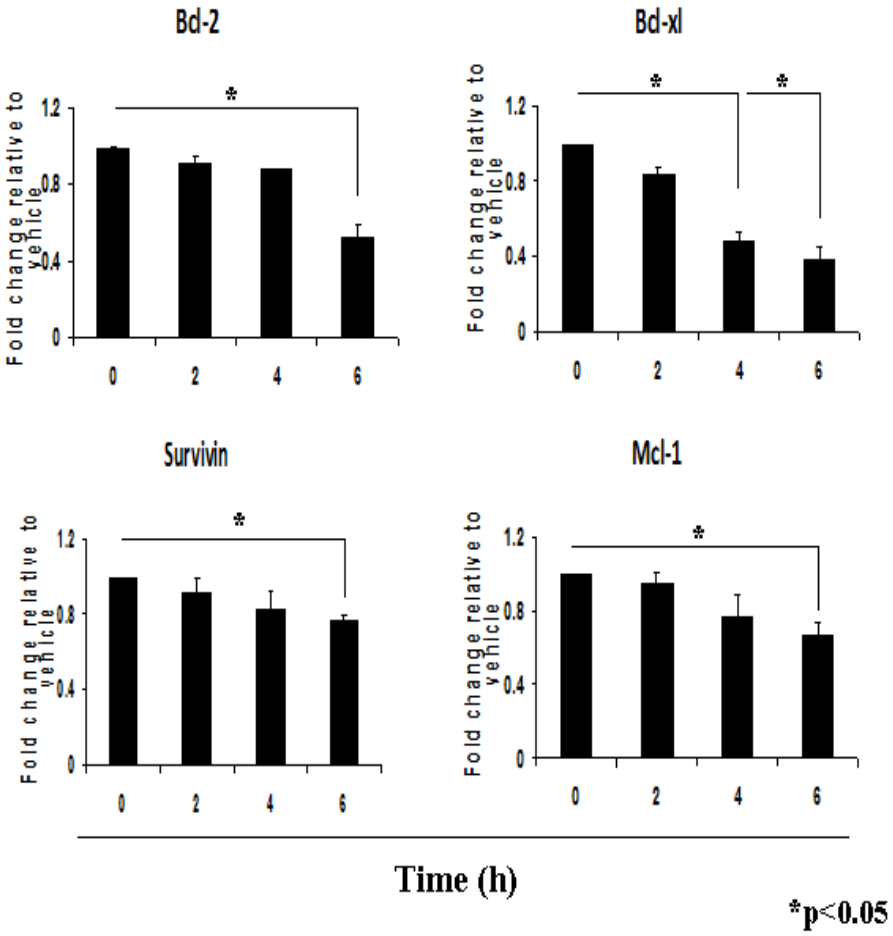

Figure 3.2.2.7. Celastrol causes downregulation of expression of various anti-apoptotic proteins involved in survival of MM cells at transcriptional level

U266 cells were treated with 1 μM celastrol for the indicated time intervals, after which RNA samples were extracted and used for real time PCR with 18S RNA as endogenous control. Results were analyzed using Sequence Detection Software version 1.3 provided by Applied Biosystems. Relative gene expression was obtained after normalization with endogenous 18S RNA and determination of the difference in threshold cycle (Ct) between treated and untreated cells using 2-ΔΔCt method. The results shown are representative of two independent experiments.

10. APPENDIX-III

Methods

Isolation and culture of primary MM plasma cells

Primary cells were obtained from bone marrow aspirates of MM patients after informed consent and with ethical approval from the NUS IRB. Peripheral blood mononuclear cells were separated with RBC lysis buffer and subsequently $CD138^+$ plasma cells were isolated using magnetic cell sorting with $CD138^+$ EasySep magnetic nanoparticles (Stemcells Technologies, Singapore) according to manufacturer's instructions. Purified $CD138^+$ patient cells were grown in IMDM, Glutamax (Gibco, Life Technologies), supplemented with 20% fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin, 10ng/mL of IL-6 (Militenyi Biotech, Surrey UK) and 100 ng/mL of rhIGF-1(R&D Systems, Abingdon, Oxford,UK). All the cells were grown at 37°C in a humidified atmosphere with 5% CO_2.

RESULTS

Celastrol suppresses the viability of $CD138^+$ primary MM plasma cells isolated from patients in a dose-dependent manner

Since celastrol suppresses the viability of a variety of human MM cell lines and tumor growth in xenograft MM model, we next aimed to investigate if celastrol can also affect the viability of $CD138^+$ cells isolated from the patients. $CD138^+$ cells isolated from MM patients were treated with 0, 1, 2.5 and 5 µM of celastrol for 72 hrs and thereafter MTT assay was performed to analyze the viability as described in Materials and Methods section in the main body of this thesis. It is evident from the results shown below that celastrol can substantially suppress the viability of $CD138^+$ cells in a dose-dependent manner in all the five tested patients samples.

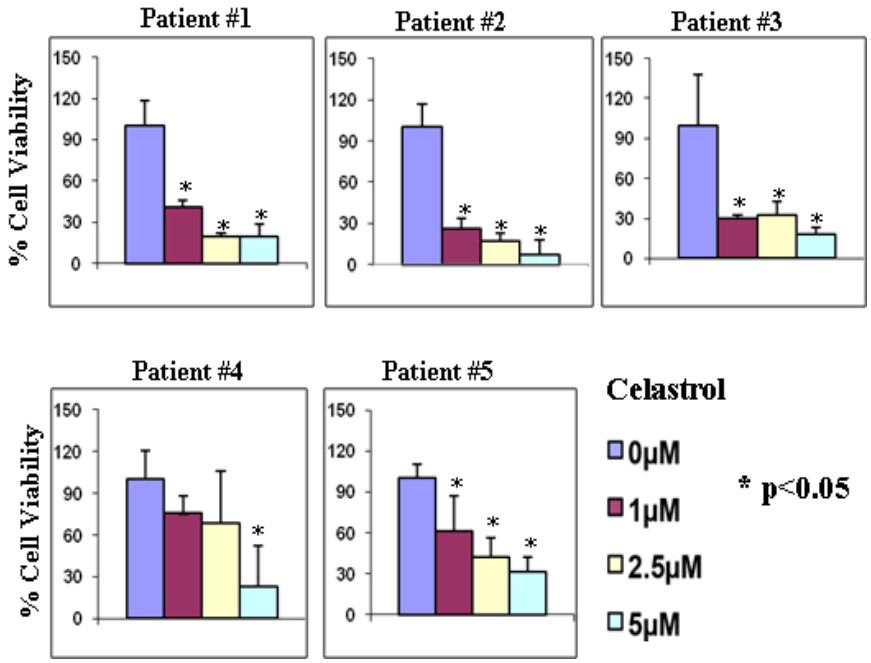

Figure 1: Celastrol suppresses the viability of CD138$^+$ MM plasma cells in a dose-dependent manner

CD138$^+$ cells were isolated from MM patients as described above in the Methods section. Cells were thereafter treated with the indicated concentrations of celastrol for 72 hrs and MTT assay was performed. Data is represented as the relative viability of each treatment group as compared to the control for each patient sample.