SAPIENZA
UNIVERSITÀ DI ROMA

PhD in Immunological Sciences
CYCLE XXVI
Department of Molecular Medicine

"The effects of cannabidiol and its synergism with bortezomib in multiple myeloma cell lines. A role for transient receptor potential vanilloid type-2"

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CHAPTER 1: INTRODUTION

1.1 Multiple Myeloma (MM)

1.1.1 Pathogenesis and epidemiology

MM, also known as plasma cell myeloma or Kahler's disease, is a B cell malignancy that accounts for 10% of all hematologic malignancies and 1% of all cancers [1].
MM is characterised by clonal proliferation of plasmacells (PCs), which accumulate in the bone marrow (BM), leading to impairment of normal immune functions and to destruction and invasion of bones surrounding the BM cavity [1].
The pathogenesis of MM is only partially understood but the main cause of PCs transformation seems associated to recurrent genetic alterations occurring during the terminal differentiation of B lymphocytes [2].
As a matter of fact, myeloma PCs posses the phenotypic features of post-germinal center (GC) cells and are present in different sites of BM. Moreover the main genetic variations associated to cell transformation involve translocations of the immunoglobulin heavy chain (IgH) locus during one of the three B cells DNA modification mechanisms, confirming that tumor-initiating cells are pre-B cells or naive B cells migrating from BM to the lymph node [3]. The homogeneous mutated immunoglobulin (Ig) sequences indicate no continuative exposure to somatic hypermutation mechanism [4].
In addition transformed cells posses surface proteins typical of different hematopoietic lineages, supporting the theory that malignant clones evolve from early stage of differentiation [2].
Myeloma cells produce monoclonal Igs, also called paraproteins, M-proteins, or protein spike, due to the way they appear on protein electrophoresis. These identical Igs have an abnormal amino acid sequence and protein structure, and lose their normal functions due to the presence of genetic mutations on genes responsible of their production [5].
Because their enormous quantity, kidneys are not able to completely recycle these abnormal proteins that, after secretion in bloodstream by myeloma cells, accumulate in the body causing tissue and organs damages [5].
Also the light chains of these monoclonal proteins, known as Bence Jones proteins, are produced in excess respect the amount needed for the formation of whole Igs, and increase blood viscosity when secreted or can deposit in tissues causing amyloidosis or Light Chain Deposition Disease (LCDD) [6].
Plasmacytoma are localized tumor plasma cells that can growth inside (intramedullary) or outside (extramedullary or soft tissue) BM, and the condition in which multiple plasmacytomas are present is defined MM.
Epidemiological studies suggest that this incurable pathology can evolve in most cases, if not all, from other gammophaties such as monoclonal gammopathy of undetermined significance (MGUS) (Figure 1.1.1) [7].
MGUS is considered a premalignant state of MM and patients could have had this disease for over 10 year before the clinical diagnosis, in fact the stage from the occurrence of the first clonal plasma cell to development of MGUS results asyntomatic. To date no treatments are proposed for this disease, however yearly monitoring is recommended because about 1% per year of MGUS patient can progress to myeloma [8]. This benign disorder is characterized by the presence of low monoclonal PCs in BM (< 10%) and low amount of monoclonal proteins (< 30 g/L) in blood or urine [8].

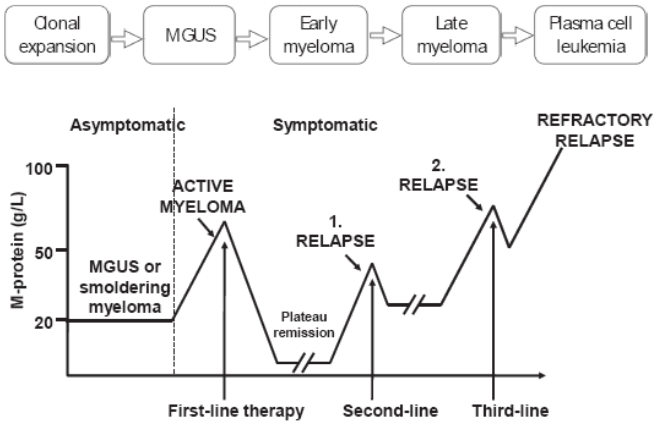

Figure 1.1.1. History of multiple myeloma [8]

Another premalignant condition that can evolve to MM is the smoldering multiple myeloma (SMM) and exhibits an higher amount of PCs (≥ 10%) and paraprotein (≥ 30 g/L) concentration in blood than MGUS. Also in this disease, clinical manifestations are absent but SMM patients have to be constantly monitored because have a 10% of change per year to develop myeloma and no interventions are provided to delay or prevent this progression [9].
On regard to symptomatic MM, its development results highly influenced by BM microenvironment that is constituted by different cell types, like stromal cells, osteoclasts, osteoblasts, immune cells, precursor cells and extracellular matrix [10].
MM cells express adhesion molecules that allow their interaction with BM stromal cells (BMSCs), but are also subjected to cytokines effects, important events in the progression of this malignancy [10]. Adhesion molecules mediate moreover the MM cells homing after class switching from BM to the lymph nodes, and influence several pathways of MM pathogenesis. An example is given by Syndecan-1, also known as CD138, that is able to induce apoptosis inhibiting MM growth and controlling osteoclast and osteoblast differentiation [11].
A rare variant of MM is the non-secretory multiple myeloma (NSMM) that presents the same clinical symptoms but show no gammophaty, so is more difficult to diagnosed [12].
Myeloma cells can also extend outside the BM to extramedullary location, such as upper respiratory tract, spleen and liver, giving rise to a more aggressive form of this pathology known as extramedullary MM (EMM) [10].
About epidemiology of symptomatic MM, each year over 20,000 new cases are diagnosed in the United States, with median age of patients around 65 years [2]. In all racial groups the incidence is 50% higher in men than in woman and this pathology results twice common in Afro-Carribeans as in white people [13].
Among factors contributing to MM development contamination of food by heavy or chemicals and particular medical conditions, including immune system disorders and infections, seems to have relevant importance [14]. Moreover MM was associated to several work cathegories, like agricultural worker exposed to pesticides, miners, sheet-metal workers, farmer, paper producer, furniture manufacturers and wood-worker, suggesting a relationship between environmental/occupational risk factors and development of MM [15].
Nevertheless tumor-initiating events are not completely identified and to date it is difficult to predict if and when a premalignant condition will progress to symptomatic MM.

1.1.2 Diagnostic criteria and standard laboratory tests

Active MM can be distinguished from precursor states based on identification of clinical manifestations and end-organ impairment.
Key criteria have been established for MM diagnosis and they consist in: detection of ≥ 10% monoclonal plasma cells in the BM, of high levels of monoclonal proteins in serum and urine and in evidence of organ or tissue damages, usually identified with the acronym "CRAB" (hypercalcemia, renal insufficiency, anemia and bone lesion) (Figure 1.1.2) [16].

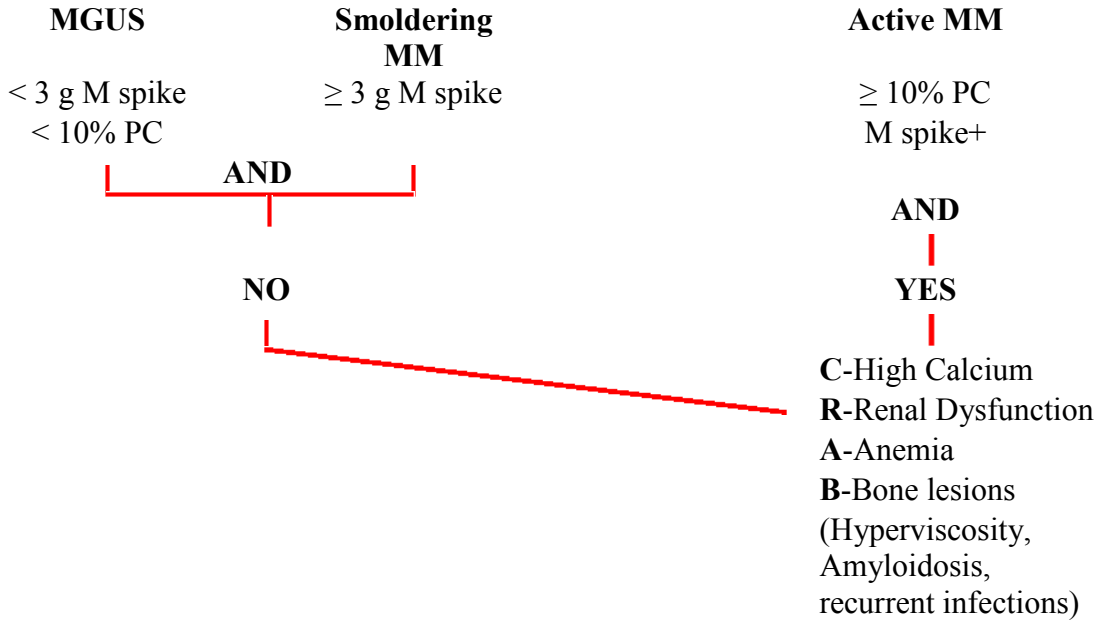

Figure 1.1.2. Criteria for MM identification

Among the CRAB symptoms, bone disease is considered the most common and was evidenced in 70% of patients at diagnosis. Manifestation of bone disorders is characterized by painful lytic lesions, typically back pain due to myeloma cells in the bone, but also vertebral crush and long bone fractures, that usually lead to spinal cord compression or extramedullary soft tissue plasmacytomas [17]. The origin of bone lesions has been attributed to over-expression of osteoclast activating factors (OAFs) by BMSCs, such as cytokine Receptor Activator for Nuclear Factor κ B Ligand (RANKL) or chemokine macrophage inflammatory protein-1α (MIP-1 α) [17].
These molecules stimulate osteoclast formation, proliferation and activation, giving rise to bone resorption, and reduce, in the mean time, osteoblasts differentiation and functions [17].
Uncontrolled osteolysis in myeloma patients is associated to another common CRAB symptom, hypercalcemia, that is connected to fatigue, weakness and confusion and characterized by increase in blood calcium levels [18].
Kidney damages are caused by high amount of monoclonal Igs (usually IgG or IgA) and free light chains (FLCs) in blood and urine, in fact these proteins exceed the renal re-absorptive capacity and lead to their precipitation out the distal tubule causing inflammation [19].
Finally exposure of renal cells to FLCs results in inhibition of cell proliferation, damage of actin cytoskeletal molecules and chromatin condensation, events that lead to cell apoptosis and acute kidney injury (AKI).
Accumulation of abnormal Igs in different tissues can produce a broad range of potential complications. For example these proteins can adhere to each other or to blood components

reducing blood flow and circulation, and leading to hyperviscosity syndrome usually accompanied to headaches, epistaxis, blurred vision, and confusion [18].
However, despite the enormous amount of antibodies produced by myeloma plasma cells, the majority of them result ineffective and this condition causes immune deficiency, exposing myeloma patient to an higher infection risk [18].
Anemia, leukopenia and thrombocytopenia are other common disease associated to MM, and are caused by impairment of BM capacity to produce red blood cells, white blood cells and platelets, that result replaced by malignant myeloma cells [18].
So, the presence of CRAB symptoms at diagnosis suggest that patients could be affected by MM, however this first evaluation has to be confirmed by specific screening tests.
Standard laboratory tests performed to better identify MM include [16]:
Complete blood count (CBC), that measures the amount of each blood cell type in blood and urine.
Igs quantization and electrophoresis (SPEP), that evaluates level of each type of antibodies and the presence of some abnormal proteins in blood.
Free light chains (FLCs) assay, that measures the amount of light chain in blood no detected by the SPEP.
Moreover a blood chemistry profile including calcium, creatinine, lactate dehydrogenase (LDH), and albumin assay is required and permit to determine tumor stage and prognosis.
Beta-2-microglobulin, a protein produced by myeloma cells, and serum albumin are in fact considered important prognostic factors used by the International Staging System (ISS) for MM classification (Table 1.1.1).

Stage	Criteria	Median Survival (Months)
I	Serum β-2 microglobulin < 3.5 mg/L and serum albumin > 3.5 mg/dl	62
II	Not stage I or III*	44
III	Serum β-2 microglobulin > 5.5 mg/L	29

*There are two cathegories for Stage II: serum β-2 microglobulin < 3.5 mg/L, but serum albumin < 3.5 g/dl; or serum β-2 microglobulin 3.5 to < 5.5 mg/L irrespective of the serum albumin level.

Table 1.1.1. International Staging System

Evaluation of plasma cells amount in BM aspirates or in biopsy, conventional cytogenetics, and fluorescence *in situ* hybridization (FISH) analysis are further useful diagnostic tests, and the presence of more than 10% of plasma cells in the examined samples is associated to MM [16].
Moreover, examinations under the microscope are used to verify the presence and the cell division rate of myeloma cells in the analyzed samples.
Bone erosion in vertebrae, ribs, pelvic bones and bone of the arms can be established by X-ray, but also magnetic resonance imaging (MRI) or computerized tomography (CT) scan may be needed to evaluate symptomatic bony sites [16].
Finally, gene expression profiling (GEP) could be performed to determine the presence of recurrent chromosomal alterations in myeloma plasma cells and this test could provide prognostic markers to find specific MM treatment [20].

1.1.3 Genetic Abnormalities In Multiple Myeloma

MM is an heterogeneous disease that displays enormous genomic instability, leading to marked variation in clinical characteristics and patient survival.
Several alterations seems to be recurrent in this pathology and function as useful markers for identification of personalized or combined target therapy. Mutations may involve changes in chromosome number or in chromosome structure and sometimes transformed plasma cells can undergo clonal expansion generating further subclones with secondary alterations that enhances heterogenicity and disease progression (Figure 1.1.3) [21].

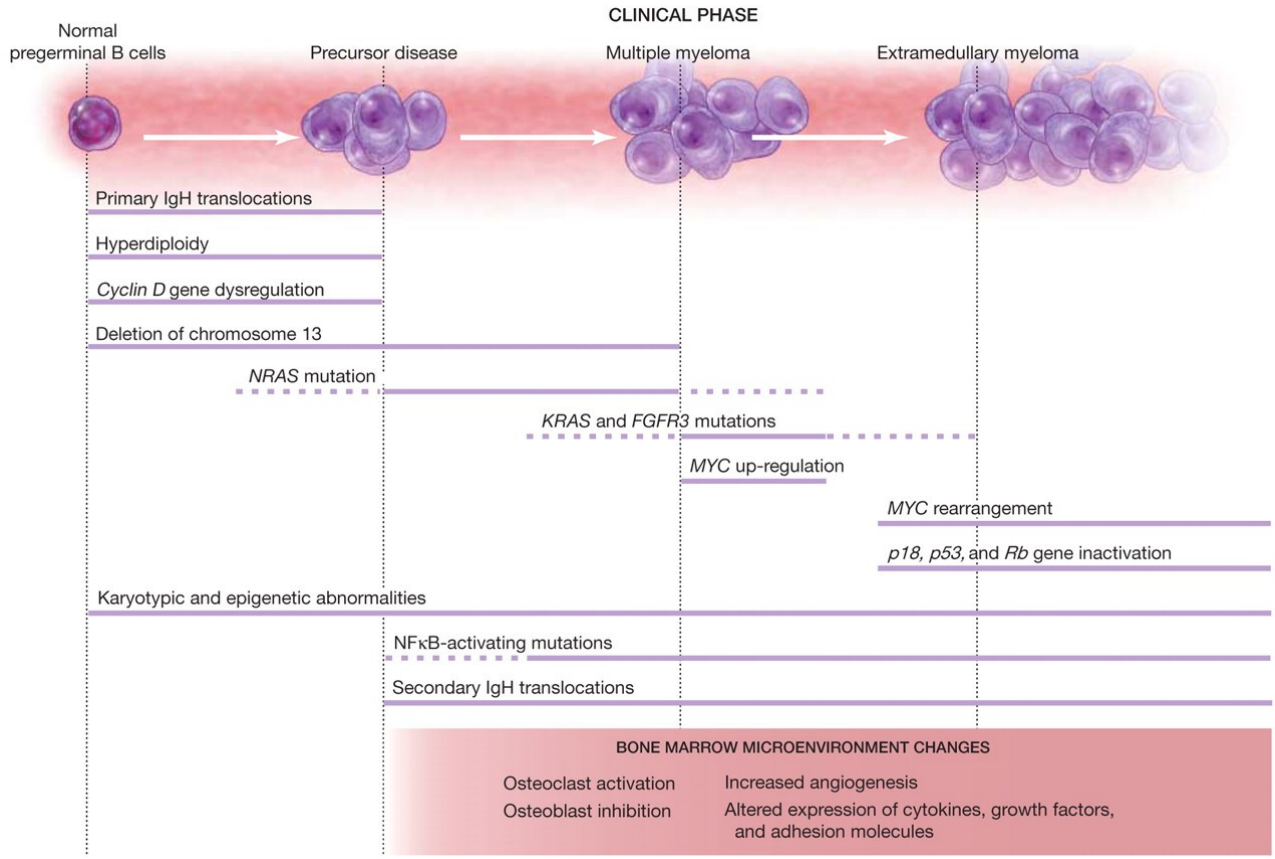

Figure 1.1.3. Events related to progression of multiple myeloma [22]

Translocations of the IgH locus are defined as early genetic alterations in MM pathogenesis but are also highly recurrent events that have been evidenced in 50% of MGUS cases and in 90% of human myeloma cell lines (HMCL).
These mutations consist in the juxtaposition of dysregulated oncogenes to an Ig enhancer and occur in one of the three B cell DNA modification mechanisms (IgH switch recombination, somatic hypermutation and, rarely, VDJ recombination) [23].

IgH translocations increase with the disease stage and three main recurrent groups have been evidenced [24]:

- CYCLIN D translocation group (11q13-CYCLIN D1; 12p13-CYCLIN D2; 6p21-CYCLIN D3), where *CYCLIN D* gene results dysregulated, but do not cause increase in cell

proliferation. These are the main common translocations, and are associated to a favourable outcome [25].

- MAF translocation group (16q23-c-MAF; 20q12-MAFB; 8q24.3-MAFA) where the MAF transcription factors is altered leading to increased expression of many other genes, including *CYCLIN D2* and adhesion molecules, that allow interaction between tumor cells and BM environment.

- MMSET/FGFR3 translocation group -4p16-(MMSET and usually FGFR3), where the histone methyltransferase MMSET associated to t(4;14) tumors, when over-expressed, function as initiation and progression factor in MM. The fibroblast growth factor receptor 3 (FGFR3) deregulation is instead not always associated to t(4;14) translocation.

During tumor progression, other genetic mutations can contribute to the transformation of MGUS cells into MM cells, such as :
c-MYC transcription factor up-regulation, responsible of MM progression. In addition c-MYC gene can be subjected to translocation, a secondary event occurring when tumor become more proliferative and less stromal-dependent [26].
Chromosome 13 alteration (85% monosomy and 15% delation), an early event associated to an aggressive clinical course, and necessary for clonal expansion of myeloma cells [27,28].
Loss of chromosome 17p and abnormalities in *p53* gene, occurring in 10% of MM patients and, mutation of *p53* gene is found in 37% of patients presenting del17p, which is usually associated to a more aggressive phenotype and to a shorter survival prognosis [29,30]. Recently it has been also demonstrated that p53 activity could be lost for the presence of micro-RNAs that increase its inhibitor MDM2 in MM development [31].
Chromosome 1q alterations (gain or loss), usually occur in MM and determine a poor prognosis [32].
Activating mutations of RAS and BRAF proteins, lead to NRAS and KRAS activation in MM tumors, mainly in those tumor expressing *CYCLIN D1* gene [32,33,34].
Finally activating mutations of NF-κB pathway, caused by continuous stimulation of receptors through ligands produced by BM stromal cells, such as APRIL and BAFF, that activate NF-κB and promote survival of long-lived PCs. These continuous stimulations are justified by the higher expression level of NF-κB target genes observed in MM tumors [35,36]. In addition positive regulation of NF-κB activating factor or negative regulation of NF-κB inactivating factors are frequent in MM patients and rend cells less dependent to ligand-activation (Figure 1.1.4) [34].

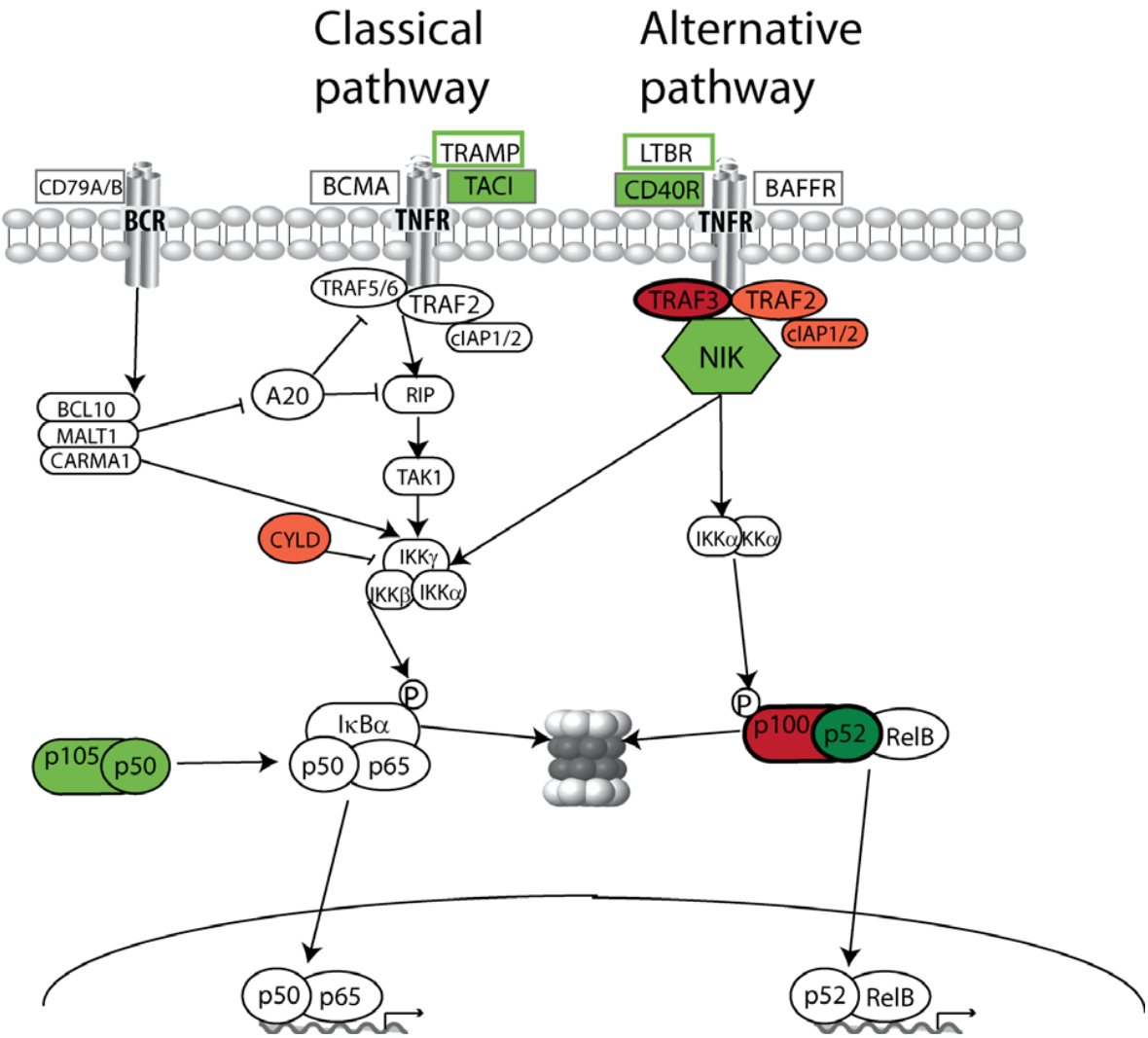

Figure 1.1.4. Mutations activate the classical and alternative NFκB pathways in MM [37]
Frequent mutations in positive (green) and negative (red) regulators of NFkB pathway in multiple myeloma.

MM tumors could be classified in two categories based on variation in chromosome number that correspond to different pathways of pathogenesis :

- Hyperdiploid (HRD) tumors, that account for the 50% of all MM neoplasms and are characterized by the presence of chromosomal trisomies, so extra number of three or more chromosome 3, 5, 7, 9, 11, 15, 19 and 21. They are associated with a good prognosis and around 10% show IgH translocations.

- Non hyperdiploid (NHRD) tumors, which are the other half of the tumors and have a poor prognosis respect the HRDs. 70% of them present IgH traslocation at 14q32, even if tumors with t(11;14) translocations are diploid or pseudodiploid and constituted another category.

Secondary IgH translocations, including IgK and IgL translocations, occur as progression events in MM [31]. Genomic rearrangement are caused also by mutations of genes related to homeostasis,

RNA metabolism, protein translation and histone-modifying enzymes, even if further studies are necessary to clarify their importance [34].
In conclusion, heterogenicity could be considered an high-risk factor in MM development and progression, and diverse malignant subclones can contribute independently to the tumour progression and confer different chemoresistance to myeloma cells [38].

1.1.4 Therapies for multiple myeloma

In recent years strategies for treatment of MM changed and increased, giving an encouraging life expectancy to patients (Figure 1.1.5).

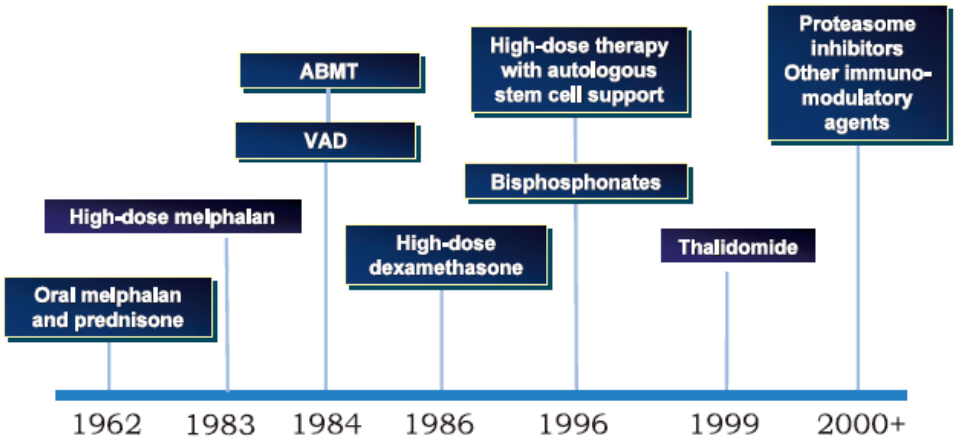

Figure 1.1.5. History of multiple myeloma treatment

Since 1960 it has been reported that the alkaloid L-phenylalanine mustard, (melphalan), induces remission in approximately one-third of patients with MM [39], but also high doses of glucocorticoids could have good effects in patients with refractory or relapsing MM [40].
Later, a 50% response was obtained from combination therapy with alkylators and corticosteroids but complete remission (CR) was rare [41]. Not even drugs such as vincristine, BCNU, melphalan, cyclophosphamide, and prednisone used in this combination gave better results.
For relapsed or primary refractory diseases, the vincristine plus doxorubicin plus dexamethasone (VAD) combination was used in order to reduce the exposure of stem cell to cytotoxic drug and produced a response rate from 50 to 30% [42].
However the MM heterogenicity confers a native resistance to the tumor, so a high-dose therapy combined to single autologous stem cell transplantation was used in patients with refractory disease [43]. Autologous BM transplantation (ABMT) consists in subcutaneous injection of BM cells previously collected from patient in remission with the aim to significantly reduce morbidity and treatment-related mortality caused by drug-induced myelosuppression [44].
This new approach improved patient survival and was extended to newly diagnosed symptomatic myeloma patients who are young (less than 65 years) and can undergo ABMT.
Results obtained from randomized trials showed that second transplant was beneficial for patient that do not obtain a complete remission by the first, and sequential autologous tandem trasplantation was used in order to increase drug dose and remission time in myeloma patients.
Bisphosphonates were introduced in MM therapy in order to reduce bone resorption by inhibition of osteoclast activity. This drug attach calcium and calcium containing molecules, usually exposed within the lytic lesion, and accumulates in this space avoiding further osteoclast activity [45].

To date these conventional therapies are not excluded, but in the last years new potent agents have been discovered improving MM treatment.
Immunomodulatory drugs (iMID), proteasome inhibitors and other specific therapies have been developed to target myeloma cells and/or the BM microenvironment, increasing rate and durability of the response [46]. In addition, a number of new inhibitory agents targeting farnesyl-transferase (FT), mitogen-activated protein kinases (MAPKs), AKT and cell cycle proteins (*e.g.*, cyclin D1, D2) are currently under investigation for the treatment of relapsed/refractory MM in preclinical and clinical studies [47].
Thalidomide (Thalomid) and several thalidomide analogs (lenalidomide and pomalidomide), are among the new iMIDs and have shown effectiveness in relapsed/refractory MM patients [48].
Their mechanism of action is still unknown but they seem to cause angiogenesis inhibition, down-regulating vascular endothelial growth factor (VEGF), and stimulate natural killer cells activity, interleukin-2, gamma interferon and apoptotic factors. A better rate response is given when thalidomide is used in combination with the old standard melphalan and prednisone (MPT) in elderly patients, increasing the survival rate of 32 months respect the MP alone [49].
Lenalidomide (Revlimid) is considered the most advanced iMIDs and exhibits only occasionally neurotoxic side effects, so is well tolerated by patients. However the combination with melphalan and prednisone (RMP) is currently being evaluated as a treatment option for patients newly diagnosed with MM [50].
Another new agent for MM treatment is the proteasome inhibitor Bortezomib (BORT), also known as Velcade, that was approved in 2003 by the US FDA for treatment of relapsed or refractory MM. Although the molecular mechanisms responsible for the favourable outcome of this treatment remain unclear [51,52], it seems to block the 26S proteasome, an essential complex of eukaryotic cells responsible of cellular protein degradation, causing protein accumulation and impairment of cell metabolism and regulation.
Moreover MM cells exhibit mutations in the NF-κB pathway, and this family of transcription factors, that include NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB and c-Rel, is involved in the canonical and alternative pathways regulating MM proliferation, survival and chemoresistance [35,36]. The use of BORT in the treatment of MM is further rationalised by the fact that this drug can inhibit IκBα, a known regulator of the canonical RelA/p50 pathway. However, it is not clear whether BORT-induced cytotoxicity is entirely due to its inhibition of the canonical NF-kB pathway [53,54-55]. The initial overall rate of response to BORT is promising, although the vast majority of patients who respond to this therapy develop resistance over time [1,58].
The choice of initial treatment after MM diagnosis depends on individual risk profile, such as chance of disease progression, patient age and general health, that also help to determine whether a patient is eligible of stem cell transplantation [56].
Therapy for patients that admit stem cell transplantation, excludes the use of melphalan, that have toxic effect on stem cells and impede their collection, so initial treatment is typically with iMID drugs combined with low-dose dexamethasone. After three to four months of treatment with these initial agents, stem cells are collected and their transplantation can be done soon or delayed until a relapse occurs. In high-risk patients, after this step, a new course of treatment with a drug combination that includes BORT and melphalan can be started (Figure 1.1.6) [56].
If patients can be not considered for stem cell transplantation, initial therapy correspond to a combination of melphalan, prednisone and thalidomide (MPT) or melphalan, prednisone and bortezomib (MPV). If side effects are intolerable, melphalan plus prednisone (MP) or lenalidomide plus low-dose dexamethasone could be additional options that can be given for about 12 to 18 months (Figure 1.1.7) [56].

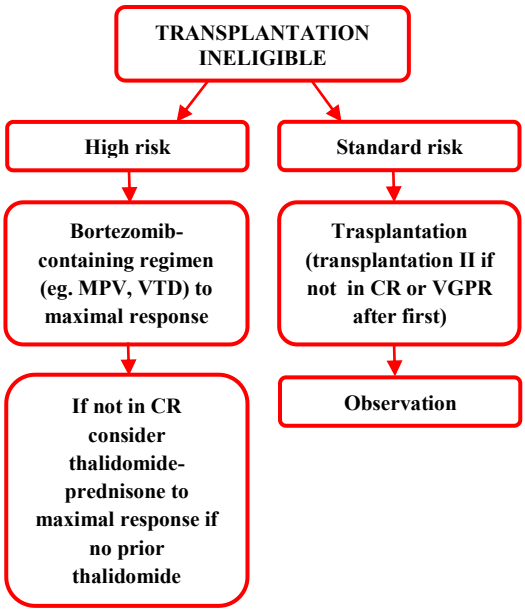

Figure 1.1.6. Algoritm for transplant elegible patients

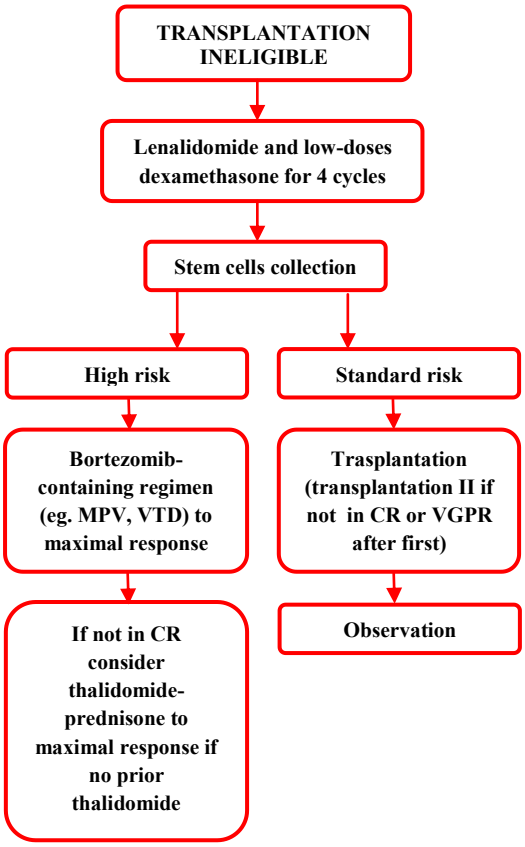

Figure 1.1.7. Algoritm for transplant inelegible patients

In conclusion, the limited clinical activity of a single agent can be enhanced by combining a single drug with conventional agents and/or steroids or with ASCT [57].

Nonetheless, MM is a malignancy that shows high chemoresistance, so new therapeutic approaches are needed in order to improve the activity and of recent discovered drugs, like the proteasome inhibitor BORT.

1.2 Transient receptor potential vanilloid-2 (TRPV2)

1.2.1 TRPV2 structure and physiological role

The human transient receptor potential vanilloid-2 (TRPV2) belongs to the family of transient receptor potential (TRP) cation channels [58], an ion channel superfamily represented across the phylogenetic tree from yeast to human and considered the second largest family of voltage-gated-like ion channels after the potassium channel family [59].
TRPs are classified based on their amino acid sequence identity and six subfamilies have been identified, showing significant sequence similarity within the transmembrane domains (TMDs) but very low similarity in their N- and C-terminal cytoplasmic regions.
The name of each subfamilies depend on founding members: TRPA (ANKTM1); TRPC (canonical); TRPM (melastatin); TRPML (mucolipin); TRPP (polycystin); and TRPV (vanilloid) [60].
In particular, TRPV2 channel is a member of the TRPV subfamily and its gene is located on chromosome 17p11.2 encoding a non-selective Ca^{2+} channel, that differ from other TRPV members for its divalent/monovalent cation permeability [62].
This receptor results highly conserved among the mammalian orthologs and it is structurally composed of an N-terminal cytoplasmic domain with three ankyrin-repeat domains, a 6 transmembrane domains containing a putative pore-loop region between S5 and S6 loops and a C-terminal cytoplasmic domain (Figure 1.2.1).

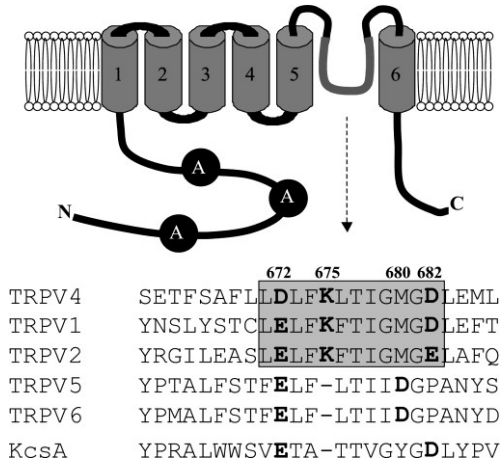

Figure 1.2.1. Structure of TRPV2 cation channel

About the receptor pharmacology, TRPV2 does not respond to vanilloids or acidic pH as TRPV1, and to date no selective agonists have been identified, however it seems to be responsive to several exogenous chemical ligands, like probenecid, cannabidiol (CBD) and tetrahydrocannabinol (THC) [61]. TRPV2 could be also regulated by growth factors, such as IGF-1 that, via phosphatidylinositol 3-kinase (PI3K), induce receptor translocation from the intracellular compartment to the plasma membrane, a mechanism results very important for receptor activation [61].
TRPV2 has been proposed as a potential pain target for its sequence similarity to TRPV1 receptor (50% identical) but also as a thermoreceptor because it results activated by noxious high temperatures (□52°C) [58].
Nevertheless, recently it has been demonstrated that TRPV2 is not essential for nociception, as shown by knockout mice displaying normal thermal and mechanical nociception responses, or by analysis of sensory ganglia development, no showing significant differences between wild-type and

knockout tissues. However knockout mice show a reduced perinatal viability and a decrease in embryo and adult body weight [62].
In addition there are several lines of evidence suggesting that TRPV2 function is important in various immune cell types, for example studies on cultured TRPV2 knock-out mice cells, indicate an impairment in macrophage function [63].
TRPV2 is also important in various osmosensory mechanisms, including osmotic balance, autonomous regulation, somatosensation, food and fluid intake, and cardiovascular functions, and these evidences suggest a potential implication of this receptor in several disorders [64].

1.2.2 TRPV2 in cancer

Several works have demonstrated that TRPV2 is involved in the regulation of tumour growth, progression, invasion and angiogenesis [65,66], however the role of this receptor in cancer progression is poorly understood.
In liver cancer for example, TRPV2 results highly expressed in human hepatocarcinoma cells (HepG2) [67], and high TRPV2 levels has been found in cirrhotic liver and well-differentiated hepatic tumors, compared with poorly differentiated tumors, suggesting a potential role for TRPV2 as a prognostic marker [68].
TRPV2 is also correlated to bladder cancer where receptor expression increases with grade and stage of UC specimens and UC cell lines respect normal samples. In addition, in non-tumor patients a short splice variant (s-TRPV2) was detected, but this variant is less abundant in late-stage tumors respect the full-length TRPV2 [69]. Similarly, a second study observed higher TRPV2 expression in a poorly differentiated bladder cancer cell line, compared with a well-differentiated line, which underwent apoptosis when exposed to the TRPV2 agonist CBD [70].
In prostate cancer TRPV2 levels are higher in metastatic cancers compared with solid tumors [71], and lysophospholipids mediated the receptor translocation to the plasma membrane, stimulating prostate cancer cell migration but not cell growth [72].
The role of TRPV2 has been investigated also in glioma cancer cells, where results down-regulated in primary glioma cells, compared with benign astrocyte control tissues, and a progressive reduction was observed in high grade gliomas [73]. TRPV2 over-expression was correlated with a decrease in proliferation via a mechanism involving the inhibition of the Ras/Raf/MEK/ERK pathway [73]. Moreover, functional studies on the TRPV2 channels further revealed that agonists, such as CBD, trigger TRPV2 activation increasing drug uptake and cytotoxicity in glioma cells [74].
TRPV2 also promote brain cancer stem-like cell differentiation [75], and altogether these results suggest a negative regulatory role for TRPV2 in glioma survival and proliferation.
Mutations of TRPV2 gene (gain or loss of function) have been reported also in haematological cancers. Aberrant TRPV2 expression has been found in B lymphocytes from Mantle Cell Lymphoma (MCL) cell lines and tissues [76], and this receptor has been evidenced among the transmembrane proteins expressed in MCL cells plasma membrane [77].
Moreover TRPV2 down-regulation has been demonstrated in acute myeloid leukemia and myelodisplastic syndrome (AML/MDS) patients [78].
A short TRPV2 splice variant, similar to that observed in bladder cells [69], has been identified in human leukemic cell lines [79] and seems to act as dominant-negative mutant, forming heteromers with full-length TRPV2 and preventing its traffic to the plasma membrane [79]. This in part explain the role of TRPV2 in tumoral chemoresistance.
Finally the involvement of TRPV2 has been confirmed also in MM, where a SNP analysis on KMS-26 myeloma cells detected a 5-Mb 17p11.2-p12 amplified region, and 12 significantly over-expressed genes have been identified in this region, including TRPV2 [80].
However the large involvement of TRPV2 in several cancer, to date, no functional role has been ascribed to this receptor in MM.

1.3. Cannabidiol (CBD)

1.3.1 Cannabidiol structure and therapeutic effects

CBD is a component of *Cannabis sativa* isolated from the first time 1940 that represents up to 40% of the plant extracts [81]. No works has been reported until the 1963, when its chemical structure was elucidated (Figure 1.3.1) opening a new research field on the pharmacological activity of cannabis constituents [82].

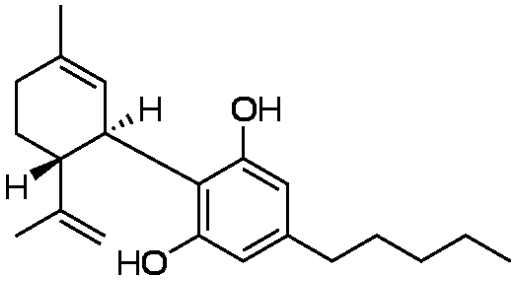

Figure 1.3.1. Cannabidiol chemical structure

In 1990, the description and cloning of specific cannabinoids receptors in the central nervous system (CNS) increase the interest about cannabis extracts, especially after and the subsequent isolation of anandamide, an endogenous cannabinoid [83].
CBD, in particular, demonstrates affinity for cannabinoid (CB1 and CB2) receptors, but also for vanilloid receptors (*e.g.*, TRPV1, TRPV2) [84] and peroxisome proliferator-activated receptor gamma (PPAR-γ) [85].
In the last years many works about its pharmacological roles have been produced and a plethora of therapeutic effects have been associated to CBD, making this compound an ideal candidate for chronic administration [86]. The use of this plant extract in therapy has shown several advantages, in fact it is a stable compound with low-toxicity profiles, well tolerated by animals and humans during chronic administration compared to other cannabis components, such as THC, which does not show psychotropic activity [86].
CBD could be considered a neuroprotective agent; in fact the first property attributed to this substance is the anticonvulsive capacity, as shown by the reduction or blockage of convulsions produced in experimental animals and by the enhancement of other anticonvulsant drugs action when administered in combination [87].
CBD showed also sedative [88] and anti-anxiety properties [89] both in animal and in human, even if higher doses of this substance are required respect the clinically used drugs. This plant extract is also used for treatment of sleep disorders, in fact is able to induce sleep [90].
It could function as a future therapeutic option for psychosis, and in particular for schizophrenia [91], and in CNS CBD shows anti-inflammatory and immunosuppressive action [92] useful in neuro-inflammatory disorders, like multiple sclerosis (MS). On this regard, CBD has been recently combined with THC (Sativex®), for the treatment of spasticity and pain in MS [93].
Finally, nausea and vomiting are significant side effects of cancer chemotherapy and preclinical research indicates that cannabinioids, including CBD, may be effective in treatment of these disturbs [94].
CBD show a wide range of clinical application and although many discoveries have been made about the medicinal benefits of this compound, there is much left to be revealed.

1.3.2 Cannabidiol and cancer

Among the various beneficial roles attributed to CBD, *in vitro* and *in vivo* studies have demonstrated also anti-tumoral property for this compound. In fact it is able to increase cell death and reduce proliferation, invasion, and migration of different human cancer cells in a receptor-dependent and -independent manner [70,74,75,84].
Its anti-neoplastic activity has been evidenced for the first time in breast cancer, where CBD inhibits cancer cell growth inducing oxidative stress, with low potency for non-cancer cells [95]. This effect was confirmed *in vivo* where CBD injection reduced growth of xenografted breast cancer cells and prevented lung metastasis formation [95]. Also cancer cell migration resulted reduced after CBD treatment [96] in fact this compound is able to regulate expression of key genes involved in cell proliferation and invasion, like extracellular signal-regulated kinase phosphorylation (pERK) [97].
Moreover CBD is able to induce programmed cell death in breast cancer through the coordination of apoptotic and autophagic processes [98]: it activates the intrinsic apoptotic pathway, reducing mitochondrial membrane potential through the translocation of the Beclin2 interacting protein (Bid) and the release of cytochrome C to the cytosol [98-100], but, in the same time, it inhibits autophagy blocking the Akt/mTOR/4EBP1 signaling pathway.
The CBD antitumoral activity has been demonstrated also in U87-MG and U373 human glioma cells, where reduces cell proliferation and induces apoptosis *in vitro* and *in vivo*, increasing ROS production and GSH-associated enzymatic activity and depleting intracellular glutathione [101] without affecting non-transformed primary glial cells [102].
CBD moreover favor the release of cytochrome C and the activation of effector caspases, suggesting the involvement of both the intrinsic and extrinsic apoptotic pathways in glioma cancer cell death [102].
Combined treatment with CBD and THC enhance both autophagic and apoptotic processes, triggering to caspase-3 activation *in vitro* and *in vivo* and reducing glioma cell viability [103].
This is in line with the discovery that CBD is able to potentiate the THC inhibitory effect on glioblastoma cell growth through sustained activation of caspases and specific modulation of the extracellular signal-regulated kinase, ERK [104].
CBD *per se* strongly down-regulates two crucial molecules in tumour cell proliferation, ERK and PI3K/Akt, and inhibits the hypoxia-inducible transcription factor HIF-1a, one of the most critical stimuli for cell survival, motility and tumor angiogenesis [105] and at low concentrations CBD reduced glioma cell migration [106] and invasion [104] in a receptor-independent manner.
In lung cancer CBD impairs tumor cell invasion *in vitro* and metastasis formation *in vivo* [107], modulating expression of factors involved in cell spreading, such as the plasminogen activator inhibitor PAI-1, and in cell invasion, like p38 and ERK phosphorylation [108].
Among other beneficial effects, it has been shown that in rat thyroid KiMol cells CBD protects DNA from oxidative damage [109] and in colon cancer it reduces polyps and tumor formation *in vivo* [110].
The use of CBD in cancer therapy is encouraged by its anti-angiogenic effects, in fact it affects endothelial cell differentiation *in vitro* and down-regulates several molecules associated to angiogenesis [111].
Finally CBD is used also in lymphoblastic diseases, as demonstrated in human acute myeloid leukemia (AML) HL-60 cell line where induces apoptosis through caspase-3 activation without affecting monocytes from normal individuals [112].
Human leukemias and lymphomas expressed significantly higher levels of CB2 receptors compared with other tumor cell lines, suggesting that tumors of immune origin may be highly sensitive to the CB2-mediated effects of CBD [113].

In murine EL-4 lymphoma cell line and in human Jurkat and Molt-4 leukemia cell lines, CBD exposure led to a significant reduction of cell viability with a possible cross-talk between the intrinsic and extrinsic apoptotic pathways, *in vitro* and *in vivo* [114].
The observation that CBD induces oxidative stress in thymocytes, EL-4 cells and splenocytes [115] stress the notion that both primary and immortalized T cells are sensitive and respond similarly to CBD.
In conclusion CBD might be worthy of clinical consideration for cancer therapy as demonstated by the reported finding that shed new light on CBD ability to target multiple cellular pathways controlling tumourigenesis through the modulation of different intracellular signaling, and on its selectivity for cancer cells, at least *in vitro*. Moreover its low toxicity and the non-psychoactive properties are good factors that make this drug useful for chronic administration.

CHAPTER 2: AIM OF THE WORK

As earlier reported, MM is characterized by complex cytogenetic and molecular aberrations, some of which are recurrent among MM patients, allowing the identification of MM groups with different prognostic significance [21].
Among these genetic alterations, chromosome 17p deletion occurs as secondary event, when the disease becomes more proliferative, and has been associated with poor prognosis [21].
The non-selective Ca^{2+} channel TRPV2 gene is located on the short arm of this chromosome (17p11.2), and this receptor is involved in several physiological processes [64], showing a relevant role in tumour growth, progression, invasion and angiogenesis [65,66].
To date no selective agonists have been identified for TRPV2 channel, however it seems to be responsive to CBD [69], an exogenous chemical ligand that posses many therapeutic effects and reduces proliferation of several cancer cell types [70,74,75,84]
MM remains an incurable disease, and although the promising initial rate of response to new developed agents, such as BORT, the vast majority of patients who respond to this therapy develop resistance over time [1,56].
To date only an altered expression of TRPV2 gene has been observed in MM [80], but no functional role has been ascribed to this receptor or to its ligands in this disorder.
On this regard, my research work has been focused on the study of TRPV2 receptor expression in MM cell lines and PCs obtained from newly diagnosed MM patients, in order to verify the involvement of TRPV2 in MM development.
Moreover the TRPV2-dependent and -independent effects of CBD alone and in combination with BORT has been evaluated in MM cell lines in order to define the CBD effect in this plasma cell malignancy and so rationalise the putative use of CBD in combination therapy with BORT.

CHAPTER 3: MATERIALS AND METHODS

Cells

RPMI8226 (RPMI) and U266 MM cell lines were purchased from ATCC (LGC Standards, Milan, IT). Cell authentication was performed by IST (Genova, Italy). Briefly, short tandem repeat [STR] profile has been carried out on RPMI and U266 cell lines. Nine highly polymorphic STR loci plus amelogenin (Cell IDTM System, Promega, WI) were used. Detection of amplified fragments was obtained by ABI PRISM 3100
Genetic Analyser (Paisley, UK). Data analysis was performer by GeneMapper software, version 4.0. Cell lines were cultured in RPMI medium (Lonza, Milan, IT) supplemented with 10% foetal bovine serum (FBS), 2 mM L-glutamine, 100 IU/ml penicillin, 100 lg streptomycin and 1 mM sodium pyruvate. Cell lines were maintained at 37°C with 5% CO2 and 95% humidity. Freshly isolated $CD138^+$ PCs from BM aspirates were obtained from newly diagnosed MM patients. Informed consent was provided for a protocol approved by the Polytechnic University of the Marche (study n CLEM 01-09), in accordance with the Declaration of Helsinki. $CD138^+$ PCs were isolated using the EasySep Human CD138 Positive Selection kit (Stem Cell Technologies, Vancouver, Canada). Whole blood from healthy donors was used to isolate $CD34^+$ haematopoietic progenitors using the CD34 MicroBead kit (StemCell Technologies). $CD138^+$ and $CD34^+$ purity was determined by flow cytometry analysis.

Antibodies

The following mouse monoclonal antibodies (Abs) were used: phycoerythrin [PE]-conjugated anti-human CD138 (20 µl/sample), PE-conjugated IgG1 κ isotype control (20 µl/sample), PE conjugated anti-human CD34 (20 µl/sample) (all purchased from BD Biosciences, San Jose, CA); anti-phospho extracellular signal-related kinase [pERK] (1:2000) (Cell Signalling Technology, Danvers, MA) and anti-GAPDH-peroxidase (1:5000) (Sigma-Aldrich, St. Louis, MO). The following polyclonal Abs were used: goat anti-TRPV2 (1:50 for fluorescence activated cell sorting [FACS] analysis, 1:200 for Western blot analysis), normal goat IgG (1:200), FITC-conjugated donkey anti-goat (1:20), rabbit anti-cyclin D1 (1:200), horseradish peroxidase (HRP)-conjugated donkey anti-goat IgG (1:1000) (all purchased from Santa Cruz Biotechnology, Santa Cruz, CA); rabbit anti-phospho protein kinase B (pAKT; 1:1000) and rabbit anti-ERK (1:1000) (Cell Signalling Technology); rabbit anti-AKT (1:1000) (Signalway Antibody, Pearland, TX) and HRP-conjugated donkey antirabbit IgG (1:2000) (GE Healthcare, Munich, Germany).

Compounds

CBD, Ruthenium Red (RR), AM251, AM630 and GW9662 were purchased from Tocris Bioscience (Bristol, UK), and 25mM aliquots were prepared (CBD, AM251, AM630 and GW9662 in dimethyl sulphoxide [DMSO], RR in water). NAcetyl cysteine (NAC) was purchased from Sigma-Aldrich and 0.5 M aliquots were prepared. BORT was provided by Janssen-Cilag International N.V. (Beerse, Belgium), and 1 µg/ml aliquots were prepared in water.

FISH analysis

FISH analysis using Vysis Locus Specific Identifier probes was performed as recommended by the manufacturer (Vysis, Inc., Downers Grove, IL). The following probes were used to analyse the structure of chromosome 17p: LIS1 in 17p13.3, p53 in 17p13.1 and proteasome Magenis syndrome

(SMS) in 17p11.2. The control probes were D17Z1 in 17p10q10 (centromere 17) and RAR-A in 17q22. Slides were examined
using a Zeiss Axioplan 2 (Carl Zeiss AG, G€ottingen, Germany) epifluorescent microscope with single and dual bandpass filter sets for the visualization of spectrum green, orange and 4',6-diamidino-2-phenylindole [DAPI] fluorescence. Images were captured and enhanced using a photometrics image point CCD camera coupled with MacProbe 4.0 software. In each sample, the number of locus-specific signals was evaluated for interphase nuclei.

FACS analysis

The expression of CD138, CD34 and TRPV2 was determined using PE-conjugated anti-CD138, PE-conjugated anti-CD34, anti-TRPV2 and FITC-conjugated anti-goat Abs. Briefly, cells were fixed with 4% paraformaldehyde and incubated with the primary or isotype-specific Abs. The cells were then washed with staining buffer (phosphate-buffered saline [PBS], 1% FBS and 0.1% NaN_3) and permeabilisation buffer (PBS, 1% FBS, 0.1% NaN_3 and 1% saponin) and incubated with anti-TRPV2 or normal goat IgG (negative control). The pellets were then incubated with FITC-conjugated anti-goat Abs and analysed using a FACScan cytofluorimeter (BD Bioscience) with CellQuest software (BD Pharmingen, Milan, IT).

Cell transfection

RPMI and U266 cells were plated at a density of 3 X 10^4 per cm^2, and after overnight incubation, 1 μg/ml of $pCMV_{TRPV2}$ or $pCMV_{empty}$ (empty vector) were added to the wells according to the METAFECTENE EASY protocol (Biontex Laboratories, San Diego, CA). The cells were harvested at day 3 post-transfection for analysis. The efficiency of transfection was evaluated by Western blot and flow cytometry analysis.
Only transfected cells (*transient receptor potential vanilloid type 2*-transfected RPMI cells [$RPMI_{TRPV2}$] and *transient receptor potential vanilloid type 2*-transfected U266 cells [$U266_{TRPV2}$]) with 70% or more $TRPV2^+$ cells were used for the experiments.

Colony forming assay

Purified $CD34^+$ cells were resuspended in Iscoves Modified Dulbecco's medium [MDM] (StemCell Technologies) supplemented with 2% FBS and treated with CBD and BORT alone or in combination. The cells were then diluted in MethoCult H4034 Optimum (StemCell Technologies) and dispensed in duplicate 35-mm culture plates, with 5 X 10^3 cells in each plate. Colonies were scored on day 12.

Gene expression analysis

Total RNA was isolated using the RNeasy Mini kit (Qiagen, Milan, IT). Complementary DNA (cDNA) was synthesized using the high-capacity cDNA archive kit (Life Technology, Milan, IT). Polymerase chain reaction (PCR) for TRPV1, TRPV2, TRPV3 and p53 was performed using specific primers and the following thermal cycling conditions: 5*'* at 95°C; 35 cycles of 10*''* at 95°C, 20*''* at 60°C, 30*''* at 72°C; and finally 5*'* at 72°C (MyCycler instrument, Bio-Rad, Hercules, CA). PCR products were run on 1.5% agarose gels, and bands were detected using Sybr Green staining and Chemi-Doc (Bio-Rad) detection.

Western blot analysis

MM cells were lysed as described previously [77]. Twenty micrograms of the lysate was separated on a SDS-polyacrylamide gel, transferred onto Hybond-C extra membranes (GE Healthcare), blocked with 5% low-fat dry milk in PBS-Tween 20, immunoblotted with TRPV2, pERK, ERK, pAKT, AKT, cyclin D1 and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) Abs overnight and then incubated with HRP-conjugated Ab for 1 hr. Immunostaining was revealed using an enhanced chemiluminescence Western blotting analysis system (GE Healthcare). Densitometric analysis was performed using ChemiDoc with Quantity One software (Bio-Rad).

3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide [MTT] assay

Three thousand cells per well were seeded in 96-well plates. After 1 day of incubation, compounds or vehicles were added. Four replicates were used for each treatment. At the indicated time point, cell viability was assessed by adding 0.8 mg/ml of MTT (Sigma-Aldrich) to the media. After 3 hr, the plates were
centrifuged, the supernatant was discharged, and the pellet was solubilized with 100 µl/well DMSO. The absorbance of the samples against a background control (medium alone) was measured at 570 nm using an ELISA reader microliter plate (BioTek Instruments, Winooski, VT). In some experiments, preincubation (3 hr) with 10 mM NAC was performed. Synergistic activity of the CBD/BORT combination was determined by the isobologram and combination index (CI) methods (CompuSyn Software, ComboSyn, Inc. Paramus, NJ 2007). The CI was used to express synergism (CI < 1), additivity (CI = 1) or antagonism (CI > 1) and was calculated according to the standard isobologram equation [120].

BrdU cell proliferation assay

The incorporation of 5-bromo-2-deoxyuridine (BrdU) was assessed using the BrdU Cell Proliferation Assay (Millipore, Billerica, MA). Incorporated BrdU was detected by adding the peroxidase substrate. Spectrophotometric detection was performed at a dual wavelength of 450/550 nm using an ELISA reader microliter plate.

Cell cycle analysis

For this analysis, 3 X 10^5 cells/ml were incubated with the appropriate drugs for up to 72 hr. Cells were fixed for 1 hr by adding ice-cold 70% ethanol and then washed with staining buffer (PBS, 2% FBS and 0.01% NaN_3). Next, the cells were treated with 100 µg/ml ribonuclease A solution (Sigma- Aldrich), incubated for 30 min at 37°C, stained for 30 min at room temperature with PI 20 ug/ml (Sigma-Aldrich) and finally analysed by flow cytometry using linear amplification.

Apoptosis assays

The exposure of phosphatidylserine on MM cells was detected by Annexin V staining and cytofluorimetric analysis. Briefly, 2 X 10^4 cells were treated with different doses of the appropriate drugs for a maximum of 72 hr. Four replicates were used for each treatment. After treatment, the cells were stained with 5 µl of
Annexin V FITC (Vinci Biochem, Vinci, Italy) for 10 min at room temperature, washed once with binding buffer (10 mM N-(2-Hydroxyethyl)piperazine-N'-2-ethanesulfonic acid [HEPES]/sodium hydroxide, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) and analysed on a FACScan flow cytometer using CellQuest software.

PI staining

After treatment with the appropriate drugs for a maximum of 72 hr, 2 X 10^4 MM cells were incubated in a binding buffer containing 20 μg/ml PI for 10 min at room temperature. The cells were then analysed by flow cytometry using CellQuest software.

Mitochondrial transmembrane potential

Mitochondrial transmembrane potential (Dwm) was evaluated by 5,5',6,6'-tetrachloro-1,1',3,3' tetraehylbenzimidazolylcarbocyanineiodide (JC-1) staining. Briefly, 2 X 10^4 cells were treated with the appropriate drugs for different times (up to 6 hr) and then incubated for 10 min at room temperature with 10 μg/ml of JC-1. JC-1 was excited by an argon laser (488 nm), and the green (530 nm)/red (570 nm) emission fluorescence was collected simultaneously. Carbonyl cyanide chlorophenylhydrazone protonophore, a mitochondrial uncoupler that collapses ($\Delta\psi_m$), was used as a positive control (data not shown). Samples were analysed using a FACScan cytofluorimeter with CellQuest software.

ROS production

The fluorescent probe dichlorodihydrofluorescein diacetate (DCFDA) was used to assess oxidative stress levels. Briefly, 2 X 10^4 cells treated with the appropriate compounds were incubated with 20 μM DCFDA (Life Technologies Italia, Italy) 20 min prior to the harvest time point. In some experiments, cells were preincubated for 3 hr with 10 mM NAC. The cells were then washed, and the intensity of the fluorescence
was assayed using flow cytometry and CellQuest software.

DNA fragmentation assay

Electrophoresis of DNA extracts was performed to assess DNA fragmentation as a criterion for necrosis and apoptosis. Briefly, 1.5 X 10^6 cells were treated with the appropriate compounds for a maximum of 3 days, and genomic DNA was extracted using a DNA extraction kit (Qiagen). The purified samples were then subjected to electrophoresis on a 1.25% agarose gel, and DNA was stained with ethidium bromide. Ultraviolet spectroscopy at 302 nm was used to obtain the results.

NF-κB DNA-binding activity

Activated p50, p65, p52 and RelB NF-κB subunit proteins were quantified using the TransAm Flexi NF-jB family transcription factor ELISA assay (Active Motif, La Hulpe, Belgium).

Statistical analysis

The data presented represent the mean and standard deviation (SD) of at least 3 independent experiments. The statistical significance was determined by analysis of variance or Student's t-test; $^{*,\#,\S,c}$,$p < 0.01$. The statistical analysis of IC50 levels was performed using Prism 5.0a (Graph Pad). Data from $pCMV_{empty}$-transfected RPMI and U266 cells were omitted because no effects were observed when compared with untransfected cells. No differences were found between drug treatments in the untransfected *versus* $pCMV_{empty}$-transfected cells.

CHAPTER 4: RESULTS

Expression of TRPV2 and FISH analysis in CD138$^+$ PCs from MM patients and in MM cell lines.

The percentage of pathological cells was determined according to the PC counts of BM slides from 13 newly diagnosed patients. As shown, the percentages varied from approximately 3–100%. None of the samples analysed by FISH demonstrated the presence of del17p (Table 4.1).

In all samples, flow cytometry analysis of TRPV2 expression on CD138$^+$ enriched PCs demonstrated the presence of two distinct CD138$^+$ PC subpopulations based on the expression of TRPV2 (CD138$^+$ TRPV2$^+$ and CD138$^+$ TRPV2$^-$; Table 4.1; Figs. 4.1a and 4.1b).

Then, we performed cytogenetic analysis to investigate TRPV2 in the RPMI and U266 MM cell lines. By FISH analysis, we found tetraploid RPMI cells with a short deletion of two 17*p* arms and diploid U266 cells showing a large deletion of one 17*p* phenotype (Table 4.2).

By flow cytometry and Western blot analysis, we found that both MM cell lines were CD138$^+$ TRPV2$^-$ (Fig. 4.2a; Figs. 4.1c and 4.1d), resembling the CD138$^+$ TRPV2$^-$ subpopulation identified in MM patients. PCR analysis of total RNA extracted from RPMI and U266 cells showed that genes such as *TRPV1, TRPV2, TRPV3* and *p53* that were localized in the 17*p* region were not expressed (Fig. 4.3). Furthermore, transfection of RPMI and U266 cells with a plasmid expressing h-TRPV2 gave rise to MM cells with a CD138$^+$ TRPV2$^+$ phenotype, as evaluated by flow cytometry and Western blot analyses (Fig. 4.2a; Figs. 4.1c and 4.1d).

P	FISH	% Pathological cells	del17p	% CD138$^+$/TRPV2$^+$	% CD138$^+$/TRPV2$^-$
1	Hyperdiploid	25	-	44	56
2	t(11;14)	40	-	91	9
3	del 13	100	-	93	7
4	Hyperdiploid	20	-	70	30
5	Hyperdiploid	40	-	68	32
6	Hyperdiploid	35	-	40	60
7	t(11;14) Hypodiploid	50	-	56	44
8	Diploid	90	-	90	10
9	del 13	40	-	33	67
10	Diploid	3	-	33	67
11	t(11; 14)	90	-	9	91
12	Diploid	70	-	45	55
13	t(11; 14)	15	-	70	30

Table 4.1. Expression of TRPV2 in CD138$^+$ PCs derived from MM patients

BM aspirates were collected from MM patients (P) (n = 13), and the percentage of pathological cells in aspirates was determined. All samples were also karyotyped by FISH to detect the 17p deletion (del17p). All samples were subjected to an enrichment procedure to increase CD138$^+$

tumour cells by CD138$^+$ cell sorting. The percentage of TRPV2$^+$ and TRPV2$^-$ cells among CD138$^+$ purified PCs was evaluated using flow cytometry.

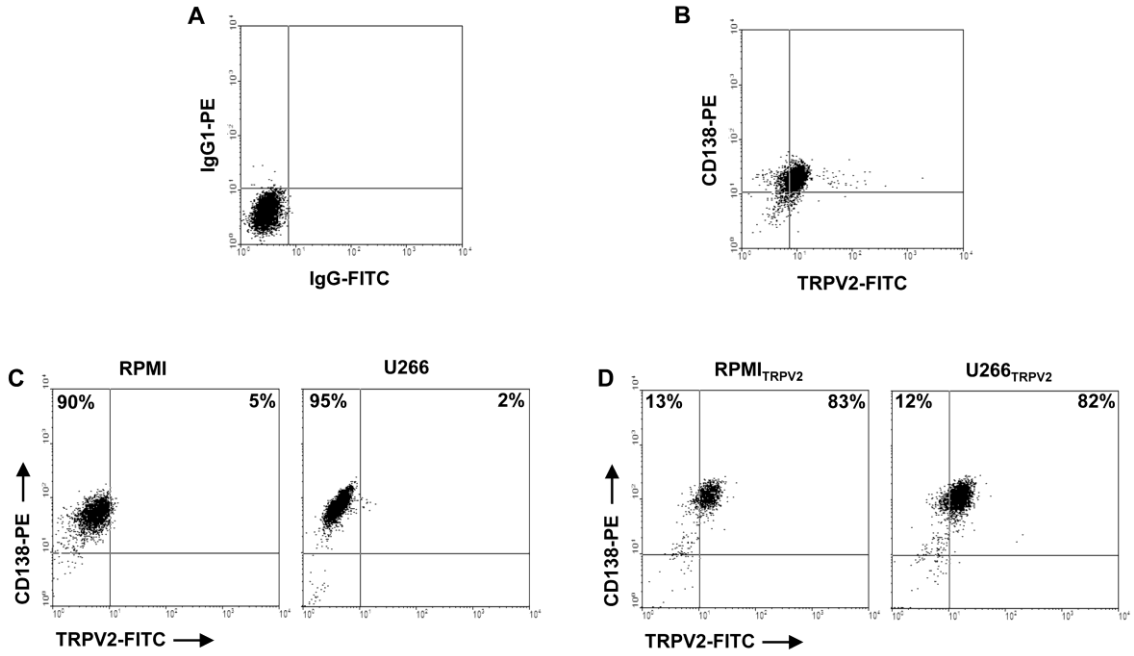

Fig. 4.1. TRPV2 and CD138 expression in PCs derived from MM patients
(A,B) Representative dot plots showing TRPV2 expression in purified PCs from CD138$^+$ patients. (C,D) Representative dot plots showing TRPV2 expression in RPMI, U266 cells and TRPV2-transfected RPMI (RPMI$_{TRPV2}$) and U266 (U266$_{TRPV2}$) cells. FACS analysis was performed using PE anti-mouse IgG1, FITC anti-goat IgG, PE anti-CD138 and FITC anti-TRPV2 Abs.

Cell line	Locus signals	Interpretation
RPMI	**RAR-A x 3 D17Z1 x 4 SMS x 4 P53 x 2 LIS1 x 2**	**4 chromosomes 17 with small deletion of two short arms and a deletion of one long arm.**
U266	**RAR-A x 2 D17Z1 x 2 SMS x 1 P53 x 1 LIS1 x 1**	**2 chromosomes 17 with large deletion of one short arm.**

Table 4.2. Chromosome 17 abnormalities in RPMI and U266 cell lines
FISH analysis of RPMI and U266 interphase nuclei used to map the rearrangement breakpoints. FISH was performed with LIS1, P53 and SMS probes. Two probes were used as control: D17Z1 in 17p10q10 (centromere 17) and RAR-A in 17q22. Slides were examined using a Zeiss Axioplan 2 epifluorescent microscope for visualization of Spectrum Green, Spectrum Orange and DAPI fluorescence.

A

Target gene	Map position	Forward	Reverse	Amplicon (bp)
β-ACTIN	7p22	GACATCCGCAAAGACCTGTACG	CGGCCACATTGTTGTTGAACTTTG	478
TRPV1	17p13.2	GGTGGGGTCTAAGGACAAGC	CTGGGACAGCAGCCTGG	394
TRPV2	17p11.2	CTCATGAGCGAGACCGTCAA	CTCGAGAGTTCGAGGGACAC	276
TRPV3	17p13.2	GCCTGTAAGACGAACAGCAGA	CCCTCAGTTCAGACACCCAC	278
p53	17p13.1	CTGGATTGGCAGCCAGACT	CTGGCATTCTGGGAGCTTCA	239

B

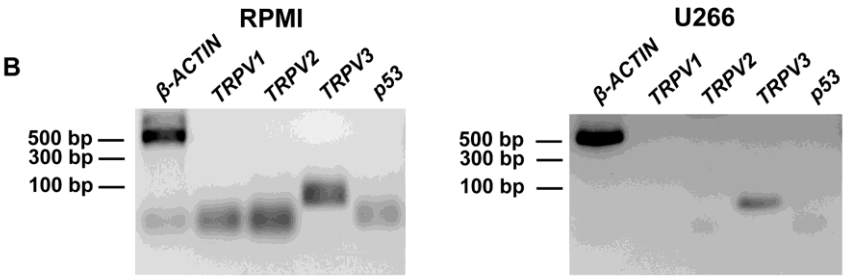

Figure 4.3 PCR analysis of the chromosomal 17p region in RPMI and U266 cell lines
(A) Description of the target genes, map position, primers used and amplicon size in base pairs (bp). Primers were designed to span an exon-exon junction. (B) Agarose gel electrophoresis of the PCR products obtained from RPMI and U266 cDNA samples. Reference markers are indicated.

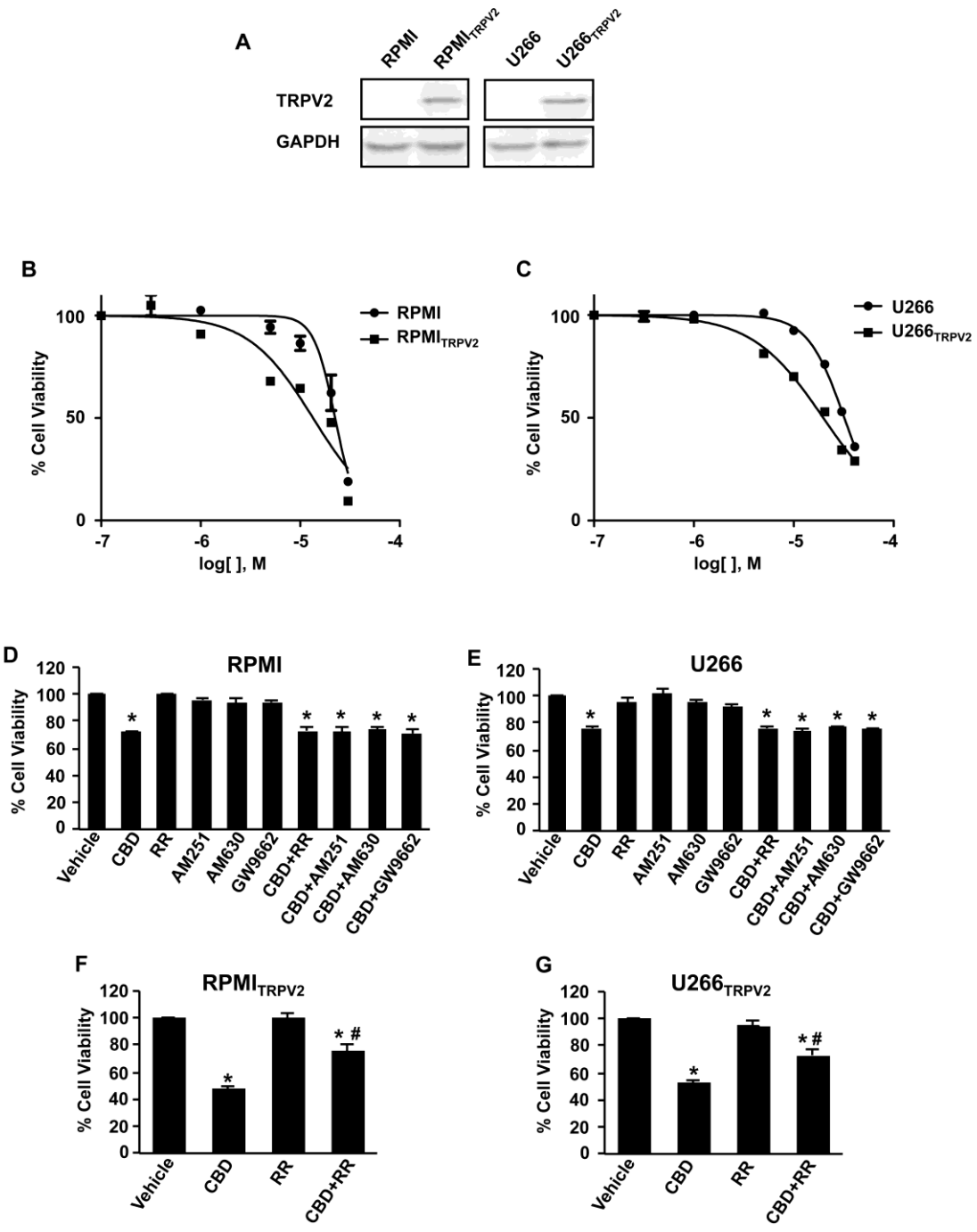

Figure 4.2. Expression of TRPV2 and TRPV2 dependent and independent induced CBD cytotoxicity in RPMI and U266 MM cell lines.

(A) Representative analysis of TRPV2 protein expression (MW 86 kDa) in RPMI, RPMI$_{TRPV2}$, U266 and U266$_{TRPV2}$ cells evaluated by Western blot analysis. Lysates were immunoblotted with anti-TRPV2 Ab. GAPDH protein levels were used as loading control. Blots are representative of one of three separate experiments. (B,C) Transfected and untransfected MM cell lines were cultured for 3 days with different doses of CBD. Cell viability was determined by MTT assay. Data shown are expressed as mean ± standard error of three separate experiments. (D,G) RPMI, U266, RPMI$_{TRPV2}$ and U266$_{TRPV2}$ cells were treated for 3 days with CBD (20 μM) alone and in combination with RR (10 μM), AM251 (20 μM), AM630 (20 μM) or GW9662 (12.5 μM). The percentage of viable cells

*was determined by MTT assay. Data shown are the mean ± SD of at least three separate experiments. *p < 0.01 vs. vehicle; #p < 0.01 CBD 1 RR vs. CBD or RR.*

TRPV2 expression increases CBD-induced cytotoxicity in MM cell lines

The effects of CBD were evaluated in TRPV2-transfected and untransfected cells. We found that the $RPMI_{TRPV2}$ and $U266_{TRPV2}$ cell lines were more susceptible to the effects of CBD in a time- and dose-dependent manner compared with untransfected MM cells (IC_{50} at day 3: RPMI 22.4 μM, $RPMI_{TRPV2}$ 13.5 μM, U266 32.2 μM, $U266_{TRPV2}$ 19.8 μM), demonstrating that TRPV2 expression reduced the IC50 in CBD-treated transfected-MM cells by approximately 40% (Figs. 4.2b and 4.2c; Fig. 4.4).

To further evaluate the selectivity of CBD for TRPV2, TRPV2- transfected and untransfected MM cell lines were treated with 20 μM of AM251 (CB1 selective antagonist), 20 μM AM630 (CB2 selective antagonist), 12.5 μM GW9662 (selective PPARγ antagonist) or 10 μM RR (TRP channel blocker); these drugs were administered alone or in combination with the lowest effective dose of CBD (20 μM), which demonstrated an inhibition rate of 25% in RPMI cells and 24% in U266 cells at day 3 post-treatment.

As shown by MTT assay, the AM251, AM630, GW9662 and RR treatments did not revert the effect of CBD on cell viability in RPMI and U266 cells (Figs. 4.2d and 4.2e), whereas RR markedly reverted the TRPV2-dependent CBD-induced effects in $RPMI_{TRPV2}$ and $U266_{TRPV2}$ cells (Figs. 4.2f and 4.2g).

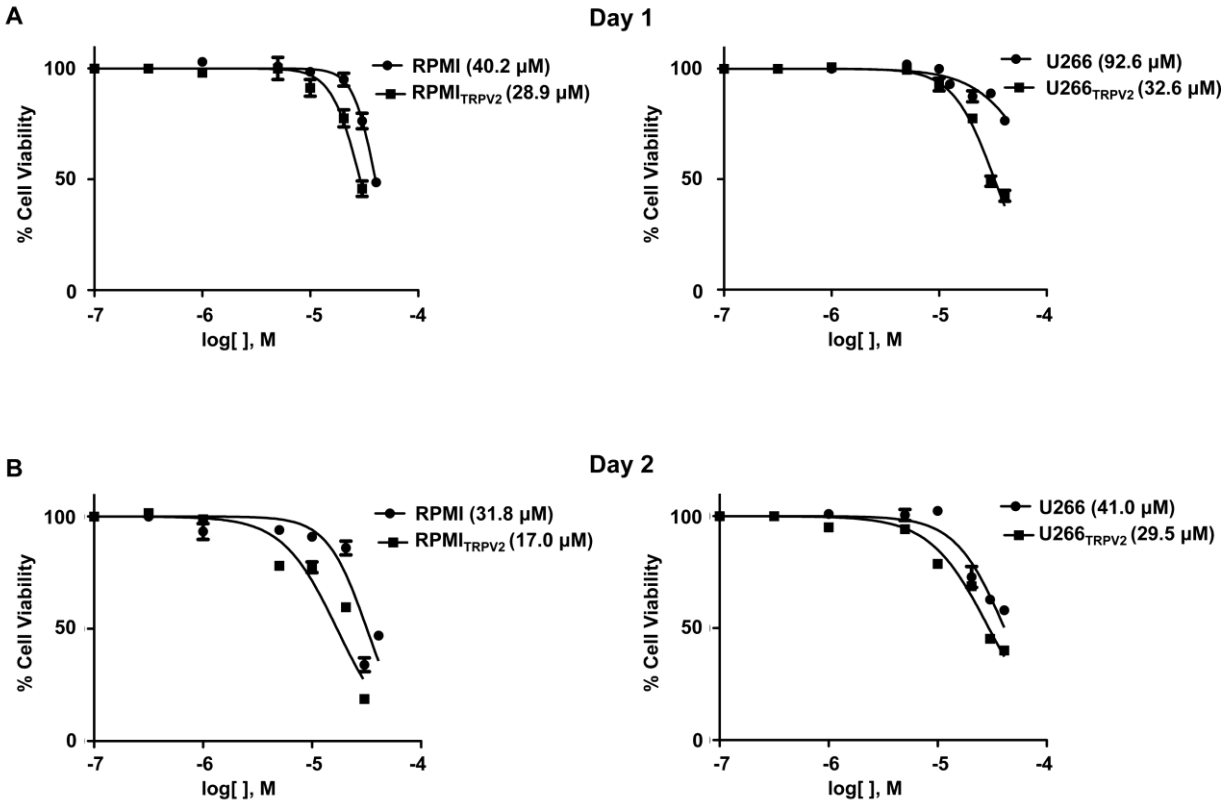

Figure 4.4. Dose-response curves of the CBD effects in MM cells.

(A,B) Transfected and untransfected MM cell lines were cultured for 1 and 2 days with different doses of CBD. Cell viability was determined by MTT assay. Data shown are expressed as mean ± SE of three separate experiments. IC_{50} values were shown.

BORT and CBD synergise to increase cytotoxicity in MM cell lines

We performed time-course (up to 3 incubation days) and dose–response (BORT 1 to 25 ng/ml) experiments in RPMI and U266 cell lines (IC_{50} at day 3: RPMI = 3.24 ng/ml, $RPMI_{TRPV2}$ = 2.98 ng/ml, U266 = 4.36 ng/ml; $U266_{TRPV2}$ = 4.07 ng/ml; $p < 0.01$). We found that treatment with 3 ng/ml of BORT was the lowest effective dose that reduced the viability of both transfected and untransfected MM cell lines (percentage of inhibition: RPMI = 44, $RPMI_{TRPV2}$ = 48, U266 = 38, $U266_{TRPV2}$ = 37) at day 3 of treatment (Figs.4.5a–4.5d). Furthermore, the co-administration of 20 μM CBD and 3 ng/ml of BORT acted synergistically to inhibit the viability of MM cells (CI < 1; Figs. 4.5e and 4.5f; Table 4.3 and Fig. 4.6). So, CBD added in BORT treatment strongly enhances BORT effect, observing a response comparable to that obtained by the use of higher BORT doses, in both cell lines. In addition, the cytotoxic effect of CBD alone or in combination with BORT was evaluated in $CD34^+$ cells isolated from healthy blood donors expressing low TRPV2 levels (Fig. 4.7a). Colony forming assays (CFU-GM) demonstrated that the growth of $CD34^+$ cells was unaffected by this combination of drugs (Fig. 4.7b).

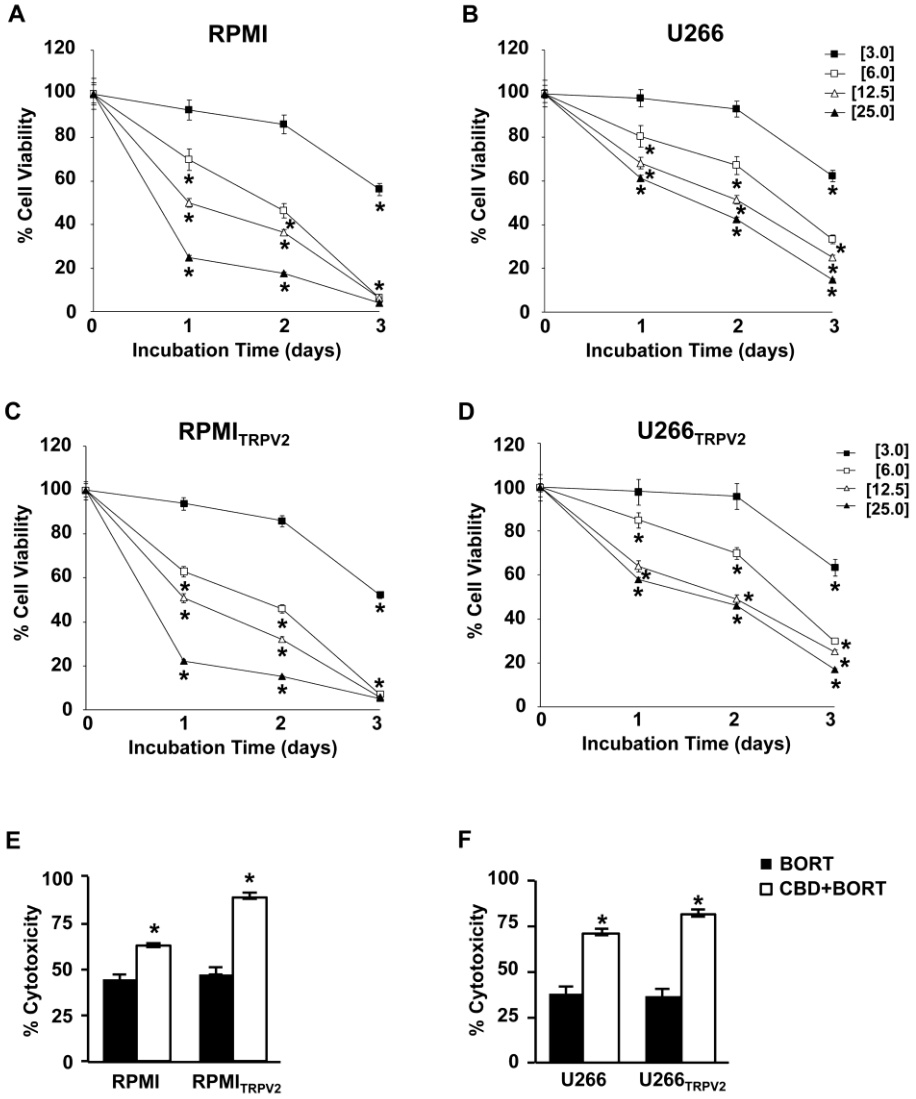

Figure 4.5. TRPV2 triggers the synergistic cytotoxic effect of CBD and BORT on MM cell lines
(A-D) RPMI, U266, $RPMI_{TRPV2}$ and $U266_{TRPV2}$ cell lines were cultured for a maximum of 3 days in the presence of BORT (3–25 ng/ml). The percentage of viable cells was determined by MTT assay.

*The data are represented as the mean ± SD of at least three separate experiments. *p < 0.01 BORT vs. vehicle. (E-F) MM cell lines were cultured for 3 days in the presence of CBD (20 μM) and BORT (3 ng/ml) alone or in combination. Cell viability was determined by MTT assay. The data are represented as the mean ± SD of at least three separate experiments. *p < 0.01 CBD-BORT vs. BORT.*

RPMI			
BORT (ng/ml)	**CBD (μM)**	**BORT+CBD effect**	**CI**
3.0	1.0	0.48	1.40
3.0	5.0	0.48	1.58
3.0	10.0	0.46	1.94
3.0	20.0	0.73	0.90
3.0	30.0	0.98	0.14

$RPMI_{TRPV2}$			
BORT (ng/ml)	**CBD (μM)**	**BORT+CBD effect**	**CI**
3.0	1.0	0.49	1.58
3.0	5.0	0.52	1.63
3.0	10.0	0.58	1.57
3.0	20.0	0.85	0.76
3.0	30.0	0.98	0.22

U266			
BORT (ng/ml)	**CBD (μM)**	**BORT+CBD effect**	**CI**
3.0	1.0	0.38	1.00
3.0	5.0	0.41	1.04
3.0	10.0	0.44	1.09
3.0	20.0	0.74	0.72
3.0	30.0	0.98	0.13

$U266_{TRPV2}$			
BORT (ng/ml)	**CBD (μM)**	**BORT+CBD effect**	**CI**
3.0	1.0	0.49	1.71
3.0	5.0	0.52	1.93
3.0	10.0	0.58	1.97
3.0	20.0	0.88	0.62
3.0	30.0	0.98	0.14

Table 4.3. CI analysis of the CBD/BORT combination in MM cell lines.
Synergistic activity of CBD/BORT was assessed by CompuSyn software. The range of concentration varies from 1 to 30 μM for CBD. The BORT concentration used, instead, was 3 to 25 ng/ml. 3 ng/ml, that was the lowest effective dose that reduced the viability of both transfected and untransfected MM cell lines at day 3 post treatments, was shown. CI, combination index; CBD, cannabidiol; BORT, bortezomib.

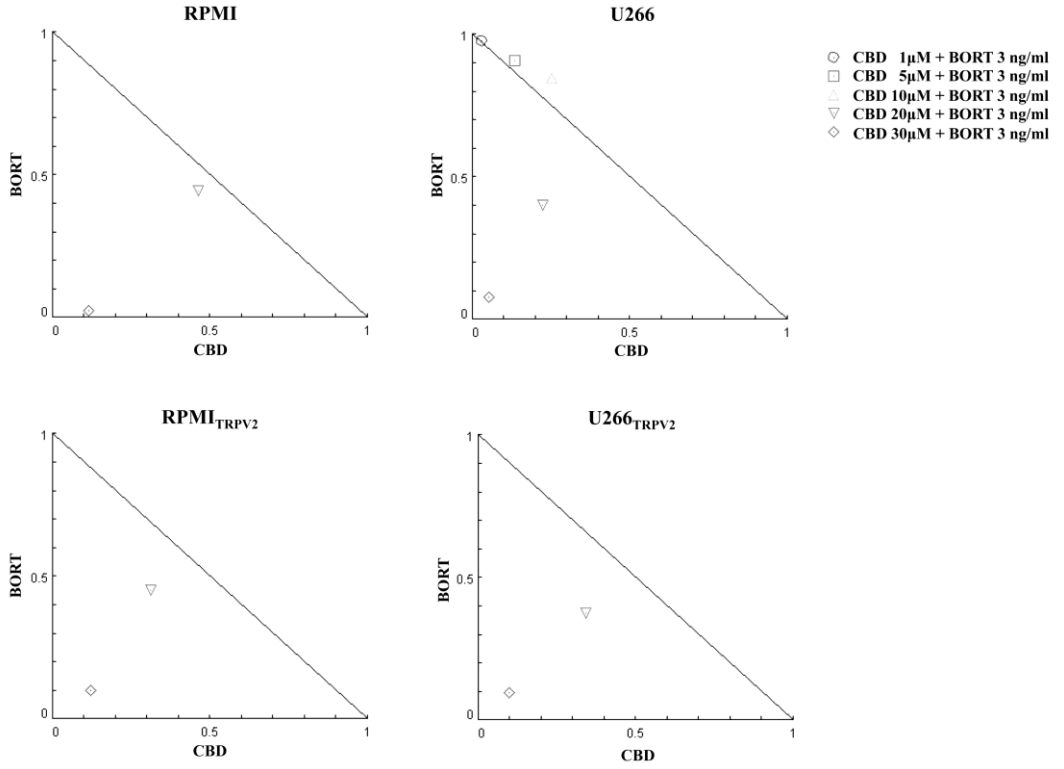

Figure 4.6. Isobologram analysis of the CBD/BORT combination in MM cell lines.
Normalized isobolograms. Symbols represent different concentration of CBD and BORT used in combination at day 3. The range of concentration varies from 1 to 30 μM for CBD. The BORT concentration used, instead, was 3 ng/ml, that is the lowest effective dose that reduced the viability of both transfected and untransfected MM cell lines at day 3 of treatment. Values above 1.2 were not shown.

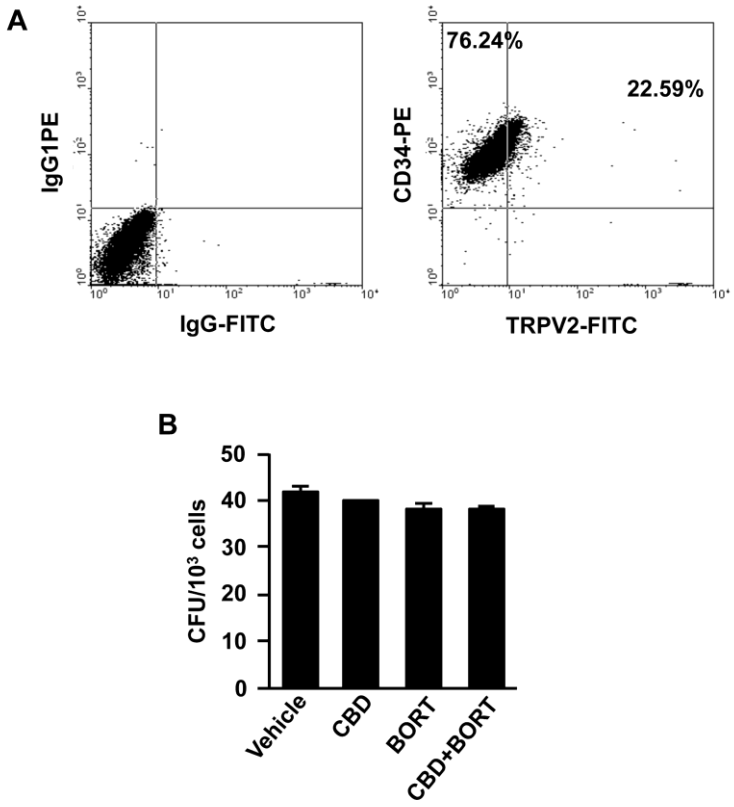

Figure 4.7. Expression of TRPV2 in peripheral blood CD34+ cells and differential response to CBD and/or BORT treatments.

(A) Percentage of TRPV2^{+} and TRPV2^{-} cells in peripheral blood CD34^{+} purified cells was evaluated by FACS analysis. Representative dot plot of TRPV2 expression on CD34^{+} purified cells performed using PE anti-mouse IgG1, FITC anti-goat IgG, PE anti-mouse CD34 and FITC anti-goat TRPV2 Abs. (B) The effect of CBD and/or BORT treatments on the number of clonogenic progenitors was analysed in methylcellulose culture following 12 days of culture. The mean frequency of progenitor colonies per 1000 cells plated is shown. The indicated values represent the mean ± SD of at least 3 separate experiments.

BORT and CBD inhibit proliferation by inducing cell cycle arrest in MM cell lines.
By performing a BrdU incorporation assay, the effects of CBD and BORT alone and in combination were evaluated at day 3 after treatment in RPMI, U266, $RPMI_{TRPV2}$ and $U266_{TRPV2}$ cells. CBD or BORT treatment reduced the proliferation in both MM cell lines, with the major effects observed in TRPV2-transfected compared with untransfected cells (Figs. 4.8a and 4.8b; Figs. 4.9a and 4.9b). Moreover, CBD and BORT coadministration strongly reduced proliferation, mainly in TRPV2-transfected cells compared with untransfected cells (Figs. 4.8a and 4.8b; Figs. 4.9a and 4.9b). In addition, the effects of CBD and BORT on cell cycle progression were analysed. The results showed that CBD and BORT arrested the cell cycle at the G1 phase and induced cell accumulation in the subdiploid (sub-G1) phase, with stronger effects observed in BORT plus CBD-treated cells compared with vehicle-treated cells (Figs. 4.8c and 4.8d; Figs. 4.9c and 4.9d). In TRPV2-transfected cells, CBD or BORT increased the percentage of cells in the sub-G1 and G1 phases compared with untransfected cells (Figs. 4.8c and 4.8d; Figs. 4.9c and 4.9d). Moreover, CBD and BORT strongly increased frequency of TRPV2-transfected cells in the sub-G1 phase as compared with untransfected cells (70% in $RPMI_{TRPV2}$ vs. 40% in RPMI; 70% in $U266_{TRPV2}$ vs. 38% in U266; $p < 0.01$; Figs. 4.8c and 4.8d; Fig. 4.9c and 4.9d).

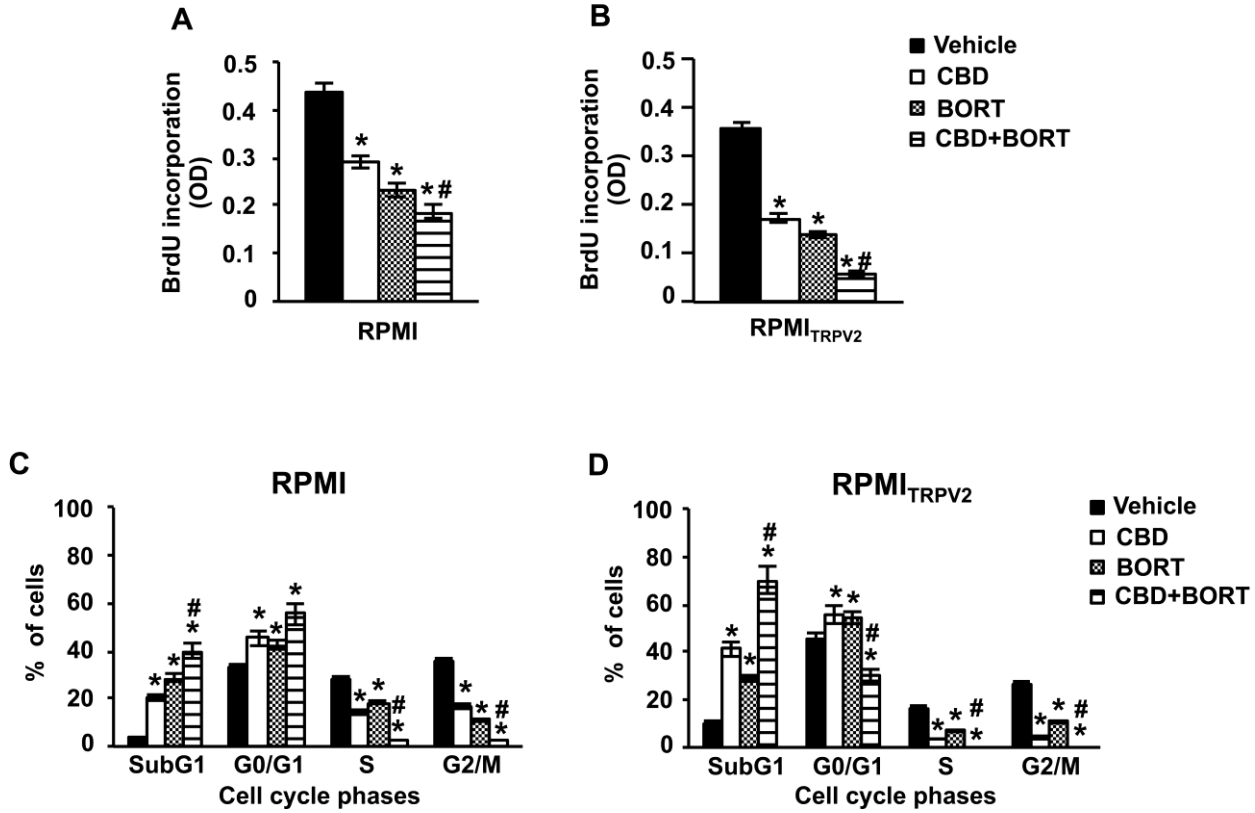

Figure 4.8. TRPV2 triggers a CBD-mediated reduction in proliferation and cell cycle regulation in RPMI and $RPMI_{TRPV2}$ cells.
*RPMI and $RPMI_{TRPV2}$ cells were cultured for 3 days in the presence of CBD (20 μM) and BORT (3 ng/ml) alone or in combination. (A,B) Proliferation was assessed with the BrdU incorporation assay. The values of BrdU incorporation were reported in terms of OD. (C,D) MM cells were stained with PI solution to assess the cell cycle distribution pattern. The values were expressed as the percentage of cells in each phase. The data are represented as the mean ± SD of at least three separate experiments. *p < 0.01 vs. vehicle; #p < 0.01 CBD-BORT vs. CBD or BORT alone.*

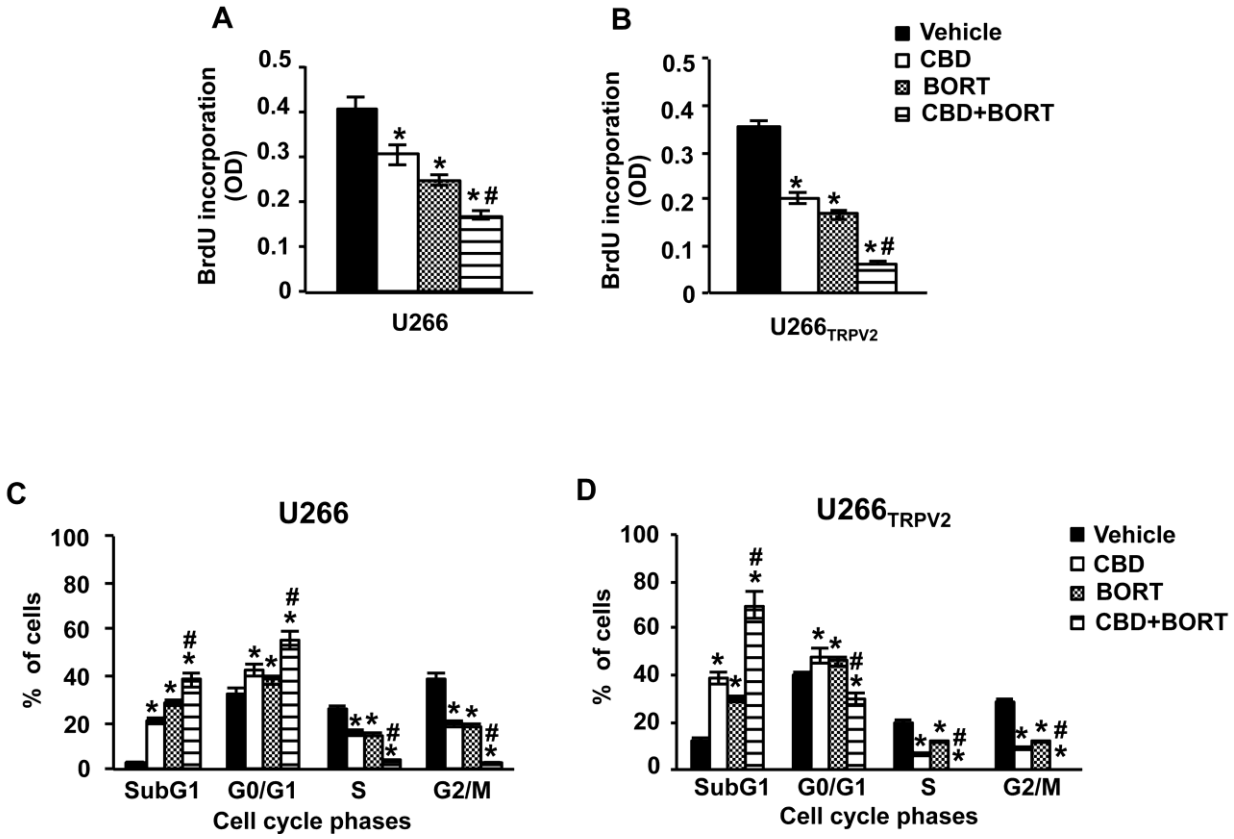

Figure 4.9. TRPV2 triggers a CBD-mediated reduction in proliferation and cell cycle regulation in U266 and U266$_{TRPV2}$ cells.

*U266 and U266$_{TRPV2}$ cells were cultured for 3 days in the presence of CBD (20 μM) and BORT (3 ng/ml) alone or in combination. (A,B) Proliferation was assessed with the BrdU incorporation assay. The values of BrdU incorporation were reported in terms of optical density (OD). (C,D) MM cells were stained with PI solution to assess the cell cycle distribution pattern. The values were expressed as the percentage of cells in each phase. The data are represented as the mean ± SD of at least 3 separate experiments. *p<0.01 vs. vehicle; $^{\#}$p<0.01 CBD-BORT vs. CBD or BORT alone.*

BORT and CBD induce mitochondrial and ROS-dependent necrosis in MM cell lines

CBD and BORT markedly increased the percentage of PI^+/ Annexin V^- necrotic MM cells and the intensity of DNA fragmentation in TRPV2-transfected cell lines compared with untransfected cells (Figs. 4.10a and 4.10b; Figs. 4.11a and 4.11b). In addition, a slight increase in PI^+ necrotic cells and DNA fragmentation was found both in un-transfected and transfected MM cells treated with CBD (RPMI = 52, $RPMI_{TRPV2}$ = 65, U266 = 66, $U266_{TRPV2}$ = 88; $p < 0.01$) or BORT (RPMI = 72, $RPMI_{TRPV2}$ = 71, U266 = 57, $U266_{TRPV2}$ = 66; $p < 0.01$) alone, whereas this effect was significantly increased upon co-administration of CBD and BORT (RPMI = 104, $RPMI_{TRPV2}$ = 199, U266 = 133, $U266_{TRPV2}$ = 192; $p < 0.01$; Figs. 4.10a and 4.10b; Figs. 4.11a and 4.11b). In addition, CBD, but not BORT, significantly reduced $\Delta\psi_m$, mainly in TRPV2-transfected MM cells (RPMI = 85, $RPMI_{TRPV2}$ = 35, U266 = 80, $U266_{TRPV2}$ = 45; $p < 0.01$), and this value further increased following CBD and BORT coadministration (RPMI = 74, $RPMI_{TRPV2}$ = 28, U266 = 64, $U266_{TRPV2}$ = 37; $p < 0.01$; Fig. 4.10c; Fig. 4.11c). Mitochondrial depolarisation was accompanied by the generation of CBD-induced reactive oxygen species (ROS) mainly in TRPV2-transfected MM cells (RPMI = 58, $RPMI_{TRPV2}$ = 154 U266 = 76, $U266_{TRPV2}$ = 206; $p < 0.01$), and this level was further increased in MM cells cotreated with CBD and BORT (RPMI = 156, $RPMI_{TRPV2}$ = 253, U266 = 143, $U266_{TRPV2}$ = 279; $p < 0.01$; Fig. 4.10d; Fig. 4.11d), inducing a reduction in cell viability, mainly in TRPV2-transfected cell lines (Fig. 4.10e; Fig. 4.11e). To further examine the effects of BORT and CBD, the free radical scavenger NAC was used. The pretreatment with NAC 10 mM abrogated the increase in ROS generation (Fig. 4.10d; Fig. 4.11d) and the reduction in cell viability (Fig. 4.10e; Fig. 4.11e), mainly in CBD and BORT-treated cells.

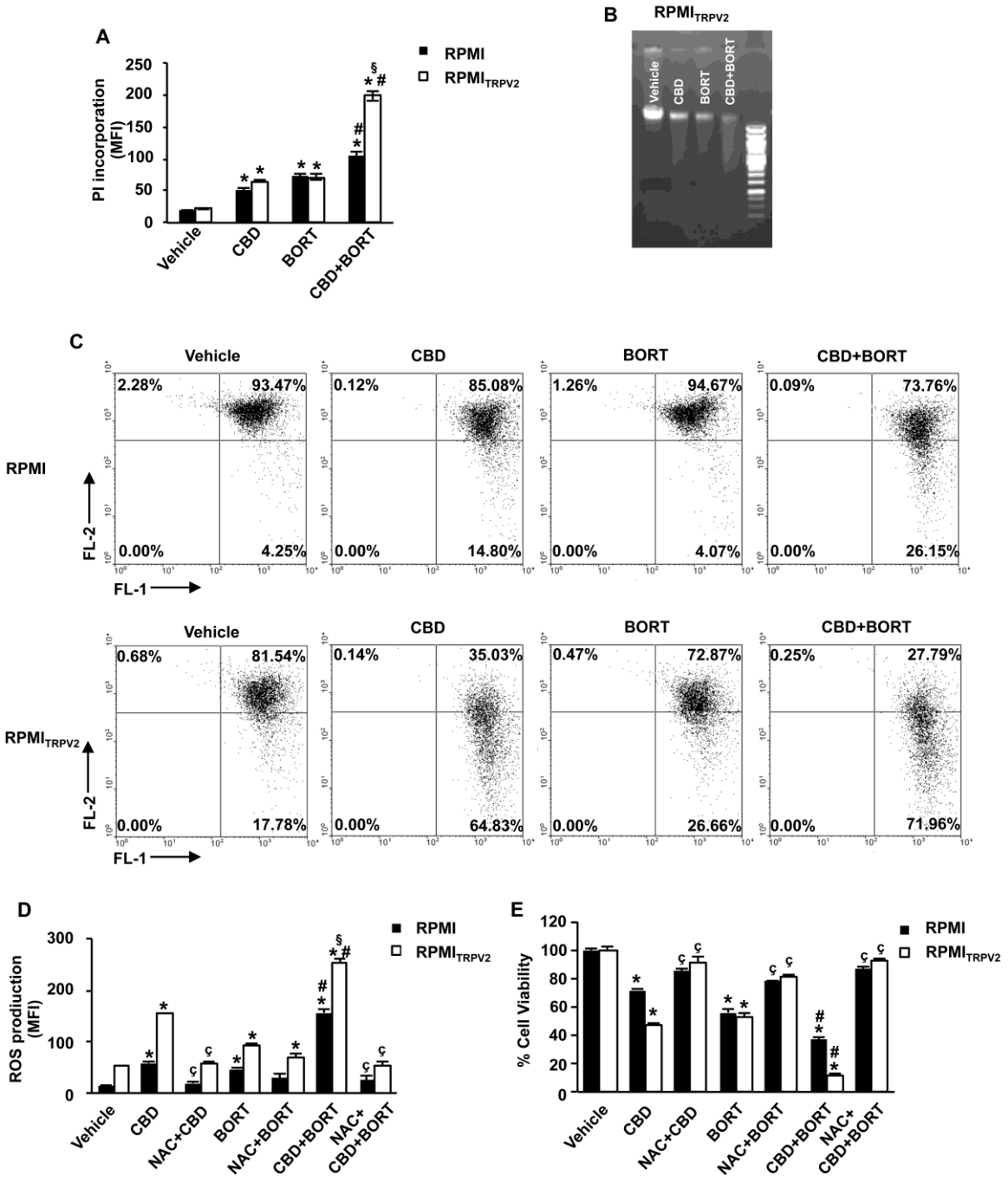

Figure 4.10. TRPV2 increases CBD and BORT combination therapy-induced necrosis in RPMI and RPMI$_{TRPV2}$ cells.

RPMI and RPMI$_{TRPV2}$ cells were cultured in the presence of CBD (20 μM) and BORT (3 ng/ml) alone or in combination.

*(A) Cells treated for 3 days were permeabilised, stained with PI and then assessed for fluorescence by flow cytometry. The data are represented as the mean fluorescence intensity ± SD of at least three separate experiments. *p < 0.01 vs vehicle; #p < 0.01 CBD-BORT vs CBD or BORT alone; §p < 0.01 TRPV2-transfected vs. untransfected cell lines. (B) Representative agarose gel electrophoresis of DNA extracts obtained from RPMI$_{TRPV2}$-treated cells at day 3 for the assessment of DNA fragmentation. (C) RPMI and RPMI$_{TRPV2}$ cells were treated with CBD (20 μM) and BORT (3 ng/ml) alone or in combination for 1 hr. Changes in Δψm were evaluated by JC-1 staining and biparametric FL1(green)/FL2(red) flow cytometric analysis. Numbers indicate the percentage of*

*cells showing a drop in the Dwm-related red fluorescence intensity. The data are representative of at least three independent experiments. (D) RPMI and RPMI$_{TRPV2}$ cell lines were pretreated or not with NAC (10 mM) for 3 hr and then treated with CBD (20 μM) and BORT (3 ng/ml) alone or in combination for 2 hr. ROS production was determined by cytofluorimetric analysis. The data are represented as the mean ± SD of at least three independent experiments. *p < 0.01 vs. vehicle; #p < 0.01 CBD-BORT vs. CBD or BORT alone; §p < 0.01 TRPV2-transfected vs. untransfected cell lines; c¸p < 0.01 NAC-treated vs. NAC untreated. (E) MM cells treated as above described were cultured for 3 days. Cell viability was determined by MTT assay. The data are represented as the mean ± SD of at least three separate experiments. *p < 0.01 vs. vehicle; #p < 0.01 CBD-BORT vs. CBD or BORT alone; c¸p < 0.01 NAC-treated vs. NAC untreated. (d–e) NAC alone treatments data were omitted, since no differences were observed compared with vehicle.*

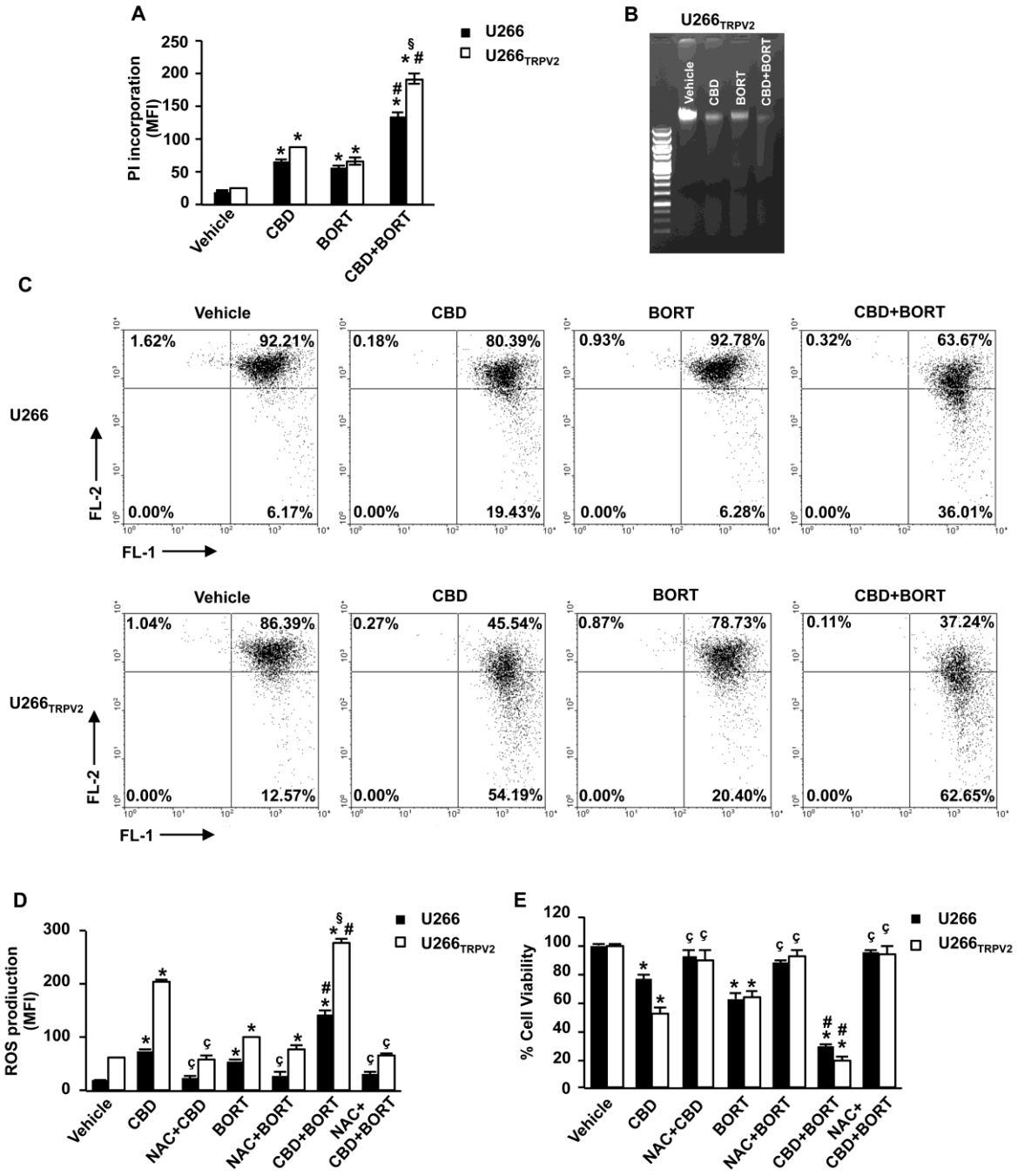

Figure 4.11. TRPV2 increases CBD and BORT combination therapy-induced necrosis in U266 and U266$_{TRPV2}$ cells.

*U266 and U266$_{TRPV2}$ cells were cultured in presence of CBD (20 μM) and BORT (3 ng/ml) alone or in combination. A, The cells treated for 3 days were permeabilised, stained with PI and analysed for fluorescence by flow cytometry. The data shown represent the MFI mean ± SD of at least 3 independent experiments. *p<0.01 vs. vehicle; $^{\#}$p<0.01 CBD-BORT vs. CBD or BORT alone; §p<0.01 TRPV2-transfected vs. untransfected cell lines. B, A representative agarose gel electrophoresis for DNA extracts from U266$_{TRPV2}$-treated cells at day 3 to assess DNA fragmentation is shown. C, U266 and U266$_{TRPV2}$ cells were treated with CBD (20 μM) and BORT (3 ng/ml) alone or in combination for 1 h. Changes in $\Delta\psi_m$ were evaluated by JC-1 staining and biparametric FL1(green)/FL2(red) flow cytometric analysis. Numbers indicate the percentage of cells showing a drop in the Δψm-related red fluorescence intensity. Data are representative of at*

*least 3 separate experiments. D, U266 and U266$_{TRPV2}$ cells were pretreated or not with NAC (10mM) for 3 h and then treated with CBD (20 μM) and BORT (3 ng/ml) alone or in combination for 2 h. ROS production was determined by cytofluorimetric analysis. The data are represented as the mean ± SD of at least 3 independent experiments. *p<0.01 vs. vehicle; $^{\#}$p<0.01 CBD-BORT vs. CBD or BORT alone; §p<0.01 TRPV2-transfected vs. untransfected cell lines; $^{\varsigma}$p<0.01 NAC-treated vs. NAC untreated. E, MM cells treated as above described were cultured for 3 days. Cell viability was determined by MTT assay. The data are represented as the mean ± SD of at least 3 separate experiments. *p<0.01 vs. vehicle; $^{\#}$p<0.01 CBD-BORT vs. CBD or BORT alone; $^{\varsigma}$p<0.01 NAC-treated vs. NAC untreated.*

BORT and CBD abrogate activation of the AKT and ERK pathways in MM cell lines

We found that both ERK and AKT proteins were basally phosphorylated in untransfected and TRPV2-transfected MM cells (Fig. 4.12a; Fig. 4.13a), and CBD and BORT strongly synergised to decrease pERK levels in all MM cell lines. Similarly, CBD or BORT alone inhibited ERK activation in TRPV2-transfected and untransfected MM cells, albeit at lower levels. In addition, CBD and BORT strongly

abrogated AKT phosphorylation (approximately 80% inhibition in TRPV2-transfected MM cells). Finally, the effect of CBD and BORT in regulating cyclin D1 levels was evaluated.

CBD alone or in combination with BORT reduced the cyclin D1 levels mainly in TRPV2-transfected compared with untransfected MM cells (Fig. 4.12a; Fig. 4.13a).

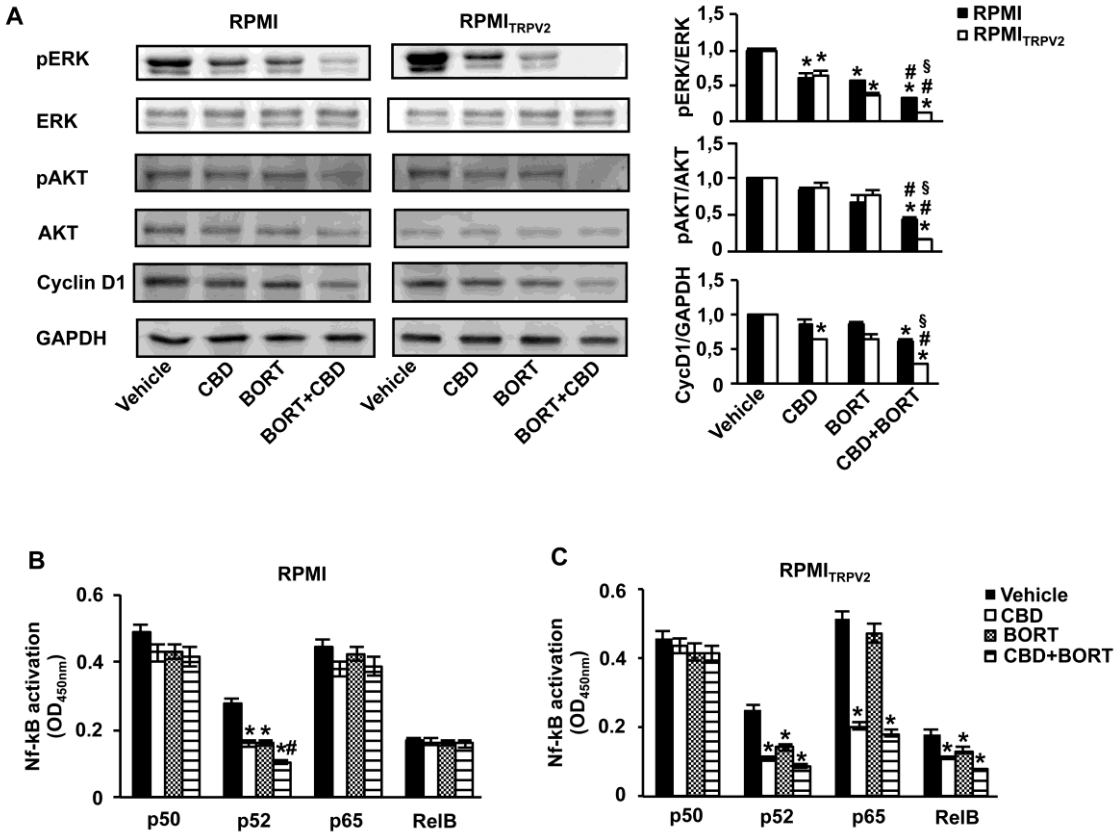

Figure 4.12. TRPV2 triggers the suppression of AKT and ERK activation in MM cells cotreated with CBD and BORT and increases CBD-mediated inhibition of NF-κB canonical and alternative pathways in RPMI and RPMI$_{TRPV2}$ cells.

*(A) RPMI and RPMI$_{TRPV2}$ cells were cultured for 3 days in the presence of CBD (20 μM) and BORT (3 ng/ml) alone or in combination. pERK, ERK, pAKT, AKT and cyclin D1 levels were analysed by Western blot. The relative protein levels were determined using GAPDH as a loading control. Blots are representative of one of three separate experiments. Bar graphs represent the mean 6 SD of at least three separate experiments. *p < 0.01 vs. vehicle; #p < 0.01 CBD-BORT vs. CBD or BORT alone; §p < 0.01 TRPV2-transfected vs. untransfected cell lines. (B,C) RPMI and RPMI$_{TRPV2}$ cells were cultured for 3 days in the presence of CBD (20 μM) and BORT (3 ng/ml) alone or in combination, and nuclear NF-κB subunits were analysed by enzyme-linked immunosorbent assay. The data shown represent the mean OD ± SD of at least three separate experiments. *p < 0.01 vs. vehicle; #p < 0.01 CBD-BORT vs. CBD or BORT alone.*

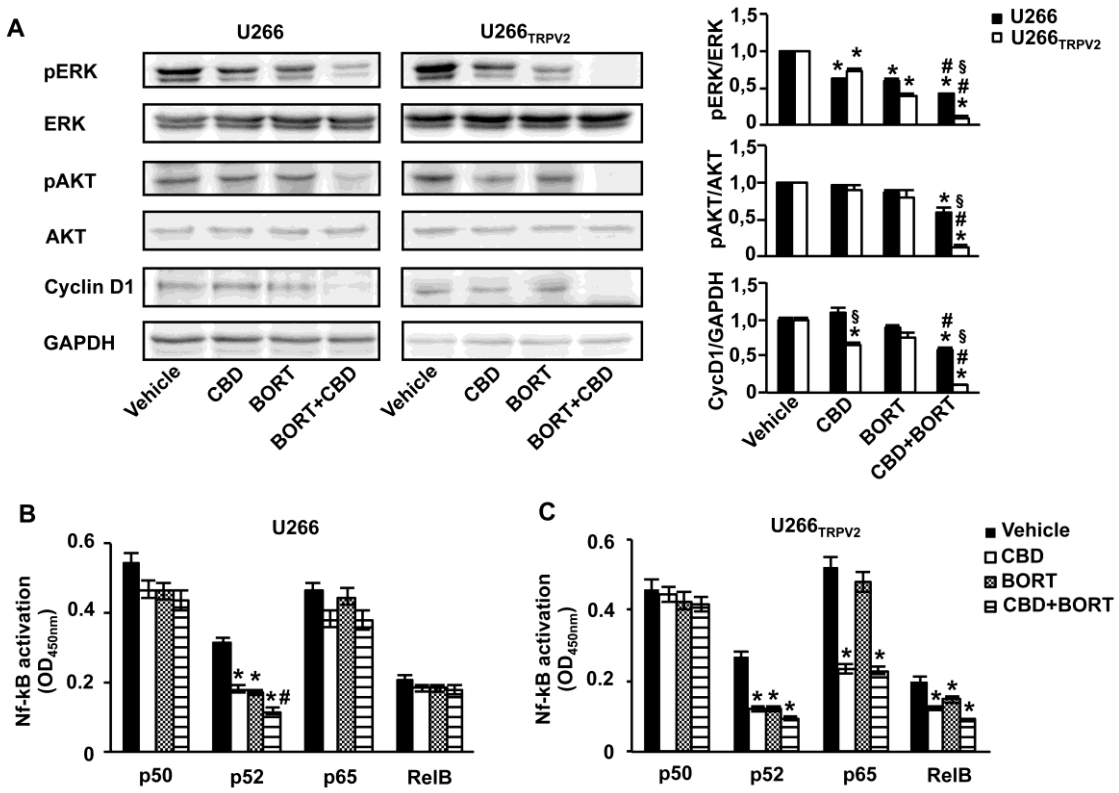

Figure 4.13. TRPV2 triggers the suppression of AKT and ERK activation in MM cells co-treated with CBD and BORT and increases CBD-mediated inhibition of NF-κB canonical and alternative pathways in U266 and U266$_{TRPV2}$ cells

*(A) U266 and U266$_{TRPV2}$ cells were cultured for 3 days in the presence of CBD (20 μM) and BORT (3 ng/ml) alone or in combination. pERK, ERK, pAKT, AKT and cyclin D1 levels were analysed by western blot. The relative protein levels were determined using GAPDH as a loading control. Blots are representative of 1 of 3 separate experiments. Bar graphs represent the mean ± SD of at least 3 separate experiments. *p<0.01 vs. vehicle; #p<0.01 CBD-BORT vs. CBD or BORT alone; §p<0.01 TRPV2-transfected vs. untransfected cell lines. (B,C) U266 and U266TRPV2 cells were cultured for 3 days in the presence of CBD (20 μM) and BORT (3 ng/ml) alone or in combination, and nuclear NF-κB subunits were analysed by enzyme-linked immunosorbent assay. The data shown represent the mean OD ± SD of at least 3 separate experiments. *p<0.01 vs. vehicle; #p<0.01 CBD-BORT vs. CBD or BORT alone.*

TRPV2 expression increases CBD-mediated inhibition of classical and alternative NF-κB pathways in MM cells

We next evaluated the DNA binding activation of the p50, p65, p52 and RelB NF-κB subunits in nuclear extracts from TRPV2-transfected and un-transfected MM cell lines, which had been treated for 3 days with CBD or BORT alone and in combination.

A reduction in p52 DNA binding activation in TRPV2-transfected and un-transfected MM cells treated with CBD (optical density [OD]: RPMI = 0.16, $RPMI_{TRPV2}$ = 0.11, U266 = 0.17, $U266_{TRPV2}$ = 0.12; $p < 0.01$) and/or BORT (OD: RPMI = 0.16, $RPMI_{TRPV2}$ = 0.14, U266 = 0.17, $U266_{TRPV2}$ = 0.12; $p < 0.01$) was observed (Figs. 4.12b and 4.12c; Figs. 4.13b and 4.13c).

In addition, CBD alone or in combination with BORT strongly reduced the DNA binding activation of p65 (CBD-treated OD: $RPMI_{TRPV2}$ = 0.20, $U266_{TRPV2}$ = 0.23; CBD plus BORT-treated OD: $RPMI_{TRPV2}$ = 0.18, $U266_{TRPV2}$ = 0.22; $p < 0.01$) and RelB (CBD-treated OD: $RPMI_{TRPV2}$ = 0.11, $U266_{TRPV2}$ = 0.12; CBD plus BORT-treated OD: $RPMI_{TRPV2}$ = 0.07, $U266_{TRPV2}$ = 0.09; $p < 0.01$) in TRPV2-trasfected MM cells (Figs. 4.12b and 4.12c; Figs. 4.13b and 4.13c), whereas BORT alone slightly reduced only RelB (OD: $RPMI_{TRPV2}$ = 0.13, $U266_{TRPV2}$ = 0.14; $p < 0.01$) activation in TRPV2-transfected MM cells (Figs. 4.12b and 4.12c; Figs. 4.13b and 4.13c).

CHAPTER 5: DISCUSSION

To better define the molecular basis of MM, patient outcomes and new therapeutic targets, FISH analysis and gene expression profiling of highly purified $CD138^+$ PCs have been used [117,52,118].
TRPV2, which is located on 17p11.2, has been shown to regulate malignant transformation processes such as cell proliferation, survival and chemoresistance in different cancer cells and tissues [119,74,75,70], TRPV2 gene mutations have also been identified in MM [77, 120], although the role of TRPV2 has not been investigated thus far.
We first evaluated the expression profile of TRPV2 in $CD138^+$ cells derived from newly diagnosed MM patients as well as RPMI and U266 MM cell lines. By flow cytometric analysis, we demonstrated the presence of two distinct $CD138^+$ subpopulations according to the expression of TRPV2 ($CD138^+TRPV2^+$ and $CD138^+TRPV2^-$) in MM samples, although deletion of 17*p* was not detected in the MM patients analysed.
This finding strengthens the modern concept of the coexistence of genetic heterogeneity in MM cells from early stages [129] when only the $CD138^+TRPV2^-$ population exists, as observed in the RPMI and U266 MM cell lines.
Thus, to mimic the *in vivo* MM phenotypes, we transiently transfected MM cell lines with TRPV2 cDNA to obtain $TRPV2^+$ MM cell lines ($RPMI_{TRPV2}$ and $U266_{TRPV2}$ cells).
We recently reported the ability of CBD, via its role as a TRPV2 agonist, to inhibit the proliferation and increase the cytotoxicity of glioma cells [74]. In addition, CBD was shown to suppress proliferation and induce apoptosis in Jurkat and MOLT-4 leukaemia cells [114].
However, the effect of CBD in myeloma cells remained unclear. Our results are the first to show that CBD induces cytotoxicity in MM cells and that this effect was amplified in TRPV2-positive cells.
BORT, a highly selective and reversible inhibitor of 26S proteasomes and ubiquitin-dependent proteolysis, is currently used as single agent to treat front-line and relapsed, refractory MM. In addition, the combination of various anticancer therapies has demonstrated several advantages over single-agent based strategies, particularly in overcoming drug resistance. In fact, the administration of CBD together with anticancer drugs has been shown to increase the susceptibility of cancer cells to the cytotoxic effects of drugs [74,70,103].
We found that in MM cell lines, the co-administration of CBD and BORT (using the lowest effective dose for each drug) synergistically reduced the viability of TRPV2-transfected and untransfected MM cell lines. Previous findings demonstrated that both CBD than BORT acted inducing cell death in haematological cancer cells, at least in part, by generation of ROS and oxidative stress [114,122]. Herein, we found that CBD and BORT synergistically inhibited cell growth, cell cycle arrest at the G1 phase and accumulation of cells in the subG1 phase and induced mitochondrial and ROS-dependent necrosis, mainly in $TRPV2^+CD138^+$ MM cells. The action of CBD is mediated by different cellular receptors, including the CB1 and CB2 receptors, TRPV1 and TRPV2 and PPARγ [123,70,84,124]. We found, as evaluated by the use of specific antagonists, that the effect of CBD on $TRPV2^-$ MM cells was independent of the CB1 and CB2 receptors, TRPs and PPARγ. Finally, our colony forming assays established that CBD and BORT do not target $CD34^+$ cells, providing an ideal strategy for developing combination therapies to eliminate MM cell populations without cytotoxic effects on haematopoietic progenitor cells.
In the BM, the mechanisms contributing to pathogenesis and chemoresistance of MM include the Ras/Raf/MEK/ERK, phosphatidylinositol 3-kinase (PI3K)-AKT and NF-κB signaling pathways [125,126].
In MM cells, complete blockade of pERK modulates the cell cycle by reducing cyclin D1 expression, increasing subG1 and decreasing S phase cells; moreover, ERK inhibitors showed cytotoxicity against the majority of tumour cells obtained from relapsed and refractory MM patients [127]. Additionally, inactivation of ERK signalling was shown to result in markedly increased DNA

damage and massive cell death in MM cells [128]. Cyclin D1 mRNA is frequently over-expressed in myeloma due to an IgH/cyclin D1 translocation [129]. In addition, translation of cyclin D1 is regulated by ERK and AKT in MM cell lines [130,131]. The PI3K/AKT pathway is constitutively active in MM and shows pleiotropic effects ranging from functional inactivation of pro-apoptotic BAD and cell cycle regulatory molecules, such as mammalian target of rapamycin [mTOR] and NF-κB [132]. However, inhibition of AKT phosphorylation was less effective in MM cells where ERK phosphorylation was still evident, suggesting that combination therapy with ERK and AKT inhibitors may be more effective for MM therapy [133]. BORT induces cell cycle arrest and death in MM cells via a plethora of mechanisms, including interference with AKT phosphorylation [134,135] and synergism with ERK inhibitors [135].

Herein, we found that CBD-BORT treatment reduced ERK and AKT signals and switched off of ERK, AKT phosphorylation and cyclinD1 levels in CBD-BORT-treated MM cell lines, particularly TRPV2-positive cells.

Therefore, given the role of the MEK/ERK and AKT pathways in MM, the combination of CBD-BORT may constitute a rational strategy for MM treatment.

The NF-κB pathway is constitutively active in $CD138^{+}$ cells derived from MM patients, and its suppression has been shown to induce MM cell death [136]. NF-κB is activated by the classical (p50/p65) and alternative (p52/RelB) pathways. In MM patients, both pathways are activated, suggesting that classical and alternative NF-κB signalling may both play a role in MM pathogenesis [55,136,137]. We found that BORT inhibited p52 DNA binding activity and therefore activation of alternative pathway in MM cells expressing p52, [137, 138] as reported previously. Furthermore, the expression of TRPV2 in MM cells not only increased the effects of CBD alone or in combination with BORT but also affected the canonical NF-κB pathway by strongly reducing the nuclear binding activation of p65, which suggests that TRPV2 expression in MM cells specifically sensitises these cells to the effects of CBD.

In conclusion, this study demonstrated that freshly isolated PCs are heterogeneous in terms of TRPV2 expression and that TRPV2 activation may represent be a promising target to deregulate MM signalling pathways. Moreover, CBD used alone or in combination with BORT-induced necrotic death both in $CD138^{+}TRPV2^{+}$ and $CD138^{+}TRPV2^{-}$ MM cells by regulating ERK, AKT and the canonical and alternative NF-κB pathways. Thus, these findings rationalize the putative use of CBD in combination therapy with BORT, for preclinical studies to evaluated the potential use of CBD and BORT combination as MM therapy. Moreover suggesting the possibility to use reduced BORT dose and consequently reducing adverse effects in MM patients.

CHAPTER 6: REFERENCES

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