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(ACQUE INTERNE E AGROECOSISTEMI) (XXVII ciclo)

TESI DI DOTTORATO DI RICERCA

# CAP DEPENDENT TRANSLATION CONTRIBUTES TO RESISTANCE OF MYELOMA CELLS TO BORTEZOMIB

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## 1. ABTRACT

Multiple myeloma is the second most predominant blood malignancy. Proteasome inhibitors like bortezomib have increased life expectancy, but eventually patients develop resistance to therapy. It was proposed that bortezomib acts through the induction of the Unfolded Protein Response (UPR), i.e. an accumulation of misfolded proteins that can cause a lethal stress response. By this theory, increasing the proteasome load by the stimulation of translation may worsen the UPR.

We evaluated the crosstalk between translation and bortezomib toxicity in sensitive as well as bortezomib resistant cells. We found that bortezomib toxicity did not correlate with induction of the UPR but caused a late reduction in global translation. The reduction of translation was accompanied by dephosphorylation of the mTORC1 target 4E-BP1. Infection of myeloma cells with mutant forms of 4E-BP, constitutively dephosphorylated, worsened bortezomib induced cell death. Since mTORC1 inhibitors cause pharmacological inhibition of 4E-BP phosphorylation, we tested whether they could act synergistically with bortezomib. We found that rapamycin, a specific mTORC1 blocker, and PP242 a mTOR antagonist induce the arrest of myeloma cells, irrespective of BZ sensitivity. Sensitivity to mTOR inhibitors (rapalogs) has been associated to the levels of eIF4E/4E-BPs. Low eIF4E/4E-BP ratio in malignant cells predicts sensitivity to rapalogs. We found that levels of eIF4E and 4E-BPs are variable among patients, and that 15% of myeloma patients have high levels of 4E-BP1/2 compared to the levels of eIF4E. Primary cells of myeloma retain sensitivity to mTOR inhibition, when plated on stromal cells. We propose that translational load does not contribute to bortezomib-induced death, but rather mTOR targeting may be successful in bortezomib resistant patients, stratified for eIF4E/4EBPs. In conclusion translation load in myeloma cells is not contributing to proteasome-induced cell death. We provide

a rationale for treating patients with multiple myeloma with mTOR inhibitors, independently from their response to bortezomib.

## 2. INTRODUCTION

## 2.1. Multiple Myeloma (MM)

Multiple myeloma (MM) is a B-cell malignancy characterized by the accumulation of malignant plasma cells in the bone marrow in association with the monoclonal immunoglobulin (paraprotein) secreted by tumour cells in serum and/ or urine (Kyle and Rajkumar 2004; Rollig, Knop et al. 2014).

Multiple myeloma (MM) is the second most predominant blood malignancy and accounts for nearly 1% of all cancers. The annual incidence of myeloma in the United States is from 4 to 6 cases per 100,000.

The median age of diagnosis is 66 years old; only 3% of MM patients are younger than 40 years. The occurrence of the disease is more common in men than women, and is twice as high among African–Americans compared with Caucasians (Table 1) (Palumbo and Anderson 2011; Rollig, Knop et al. 2014).

The causes of myeloma are unknown. The different occurrence of myeloma between races suggests the existence of a genetic pre-disposition, however, multiple myeloma is not a hereditary disease. Many environmental factors seem to have an important rule in the pathogenesis of MM. Exposure to herbicides, insecticides, petroleum products, heavy metals, plastics and various dusts including asbestos appear to be risk factors for the disease (Blair 1982; Blair, Malker et al. 1985; Axelson 1987). In addition, people exposed to large amounts of radiation, such as survivors of the atomic bomb explosions, have an increased risk for MM, although this accounts for a very small number of cases (Ichimaru, Ishimaru et al. 1982; Shimizu, Kato et al. 1991).

| | |
|---|---|
| **Incidence (per 100,000 per year in the USA)** | **5,6** |
| **Age of diagnosis** | **37%< 65 years**<br>**26% between 65 and 74 years**<br>**37%> 75 years** |
| **Males:female** | **≈ 3:2** |
| **Incidence between races** | **2 to 3 times higher in African-American compared with Caucasians** |
| **Median survival** | **increased from 3 years to 6 years in the past two decades** |

**Table 1:** Incidence and survival of Multiple Myeloma (Rollig, Knop et al. 2014)

## 2.2. Course of the disease

All cases of myeloma originate from an indolent, asymptomatic condition called monoclonal gammopathy of undetermined significance (MGUS), which progresses to malignant multiple myeloma at a rate of 1% per year (Kyle, Therneau et al. 2002; Kyle and Rajkumar 2006).

MGUS is followed by smouldering multiple myeloma (SMM). This asymptomatic phase is characterized by an intramedullary tumor cell content greater than 10%. The average risk of progression to myeloma is of 10% in the first five years (Kyle, Remstein et al. 2007). The final phase of multiple myeloma is the plasma cell leukemia (PCL). PCL is characterised by the presence of extramedullary clones and rapid progression to death (Ramsingh, Mehan et al. 2009).

The progression of the disease through these phases, occurs via accumulation of genetic lesions and alterations in the bone marrow microenvironment including the induction of angiogenesis, and the development of paracrine signalling loops involving cytokines (Kyle, Therneau et al. 2002; Landgren, Kyle et al. 2009).

The interaction of myeloma cells with the bone marrow stromal cells (BMSCs) triggers adhesion and cytokine-mediated MM cell growth, survival and migration and, after treatment, the development of drug resistance (Roodman 2002).

Multiple myeloma cell binding to bone marrow stromal cells (BMSCs) induces the activation of p24/44 mitogen-activated protein kinase (MAPK) and nuclear factor kB (NF-kB) in BMSCs. The activation of NF-kB upregulates adhesion molecules of both MM cells and BMSCs. The adhesion of tumour cells to BMSCs upregulates the transcription and secretion of cytokines, such as interleukin-6 (IL-6), insulin- like

growth factor 1 (IGF1), vascular endothelial growth factor (VEGF), tumour-necrosis factor-α (TNFα) promoting autocrine and paracrine tumor cell growth and survival (Hideshima and Anderson 2002).

## 2.3. Clinical features and pathogenesis of multiple myeloma

The clinical features of multiple myeloma are generated by the accumulation of MM cells within the bone marrow (BM), the interactions of myeloma cells with the BM stromal cells and the mutations occurring in the genes responsible for IgG production. This last mentioned event, leads to expression of proteins with abnormal aminoacid sequence and protein structure and as result, the normal antibody function. is lost. The reduced levels of normal IgG induce susceptibility to infection (Fahey, Scoggins et al. 1963). Release of "M-protein" (monoclonal protein) into the blood, increases plasma volume and viscosity that can cause renal insufficiency (Sanders 1994).

Bone marrow crowding with malignant plasma cells entails the replacement of hematopoietic tissue with tumour cells inducing anemia. Following homing to the bone marrow, multiple myeloma cells trigger osteoclast activity, including lytic bone lesions. BMSCs and osteoblasts regulate osteoclastogenesis by producing receptor activator of NF-kB ligand (RANKL) and osteoprotegerin (OPG); (Giuliani, Colla et al. 2004). RANKL binds to RANK on osteoclasts, stimulating their differentiation and activity, whereas OPG as a decoy receptor by binding to RANKL and preventing its interaction with RANK, inhibiting its ability to stimulate osteoclastogenesis (Sezer, Heider et al. 2003). The blockade of RANKL binding to RANK receptor by OPG inhibits osteoclast maturation and bone destruction. By contrast, the binding of multiple myeloma cells to BMSCs decreases the secretion of OPG and increases the expression of RANKL promoting osteolysis. Multiple myeloma cells affect the OPG:RANKL ratio in the bone marrow environment promoting lytic bone lesions (Giuliani, Colla et al. 2004). This increase in osteoclastic activity also mediates the hypercalcemia commonly found in multiple myeloma (Oyajobi 2007).

In summary the most common clinical features of MM are extensive skeletal destruction, infections, anemia, hypercalcemia, and renal failure.

## 2.4. Genetic of Multiple myeloma

Aberrant chromosomal translocations is one of the central molecular hallmarks of myeloma. Multiple myeloma, as previously described, is a tumor of antibody-producing plasma cells. Within the germinal centre, B cells that express a functional B cell receptor undergo affinity maturation in response to antigen. This event requires that somatic hypermutation (SHM), within the DNA encoding the hypervariable regions of the immunoglobulin heavy chain (IGH), occurs in order to produce highly specific antibody. The functionality of these antibodies is increased by class switch recombination (CSR) which generates antibodies of different immunoglobulin isotypes. These events, both SHM and CSR are mediated by the generation of double strand DNA breaks (DSB) in the Ig loci (Gonzalez, van der Burg et al. 2007). Although DSB are mostly repaired, DNA rearrangements can malfunction, leading to mutations in crucial oncogenes and tumor suppressor genes.

Myeloma is divided into two broad groups, non hyperdiploidy myeloma and hyperdiploidy myeloma (figure 2.1).

Non-hyperdiploid abnormalities are associated with reduced life-span because of high-risk translocations of IGHR t(4;14) or t(14;16) or complete loss of chromosome 13, and partial loss of chromosome 17 (p13).

Hyperdiploid abnormalities, caused by multiple trisomies, monosomies or deletion of chromosome 13 and translocation of IGHR (t11;14) are, instead associated with improved outcome (Avet-Loiseau, Attal et al. 2007). In the case of chromosomal translocations the transcriptions of various oncogenes (such as cyclin D1 (CCND1), fibroblast growth factor receptor 3 (FGFR3), multiple myeloma SET domain (MMSET,

MAF and MAFB)) are under the control of the enhancers of the Ig loci (Chesi, Nardini et al. 1997).

In general both hyperdiploid and nonhyperdiploid events lead to the deregulation of the G1/S cell cycle transition point through the overexpression of cyclin D genes. This event represents a early molecular abnormality in myeloma (Bergsagel, Kuehl et al. 2005).

Secondary late events including translocations and gene mutations involved in disease progression are: abnormality of MYC (Kyle, Therneau et al. 2002; Fonseca, Barlogie et al. 2004), activation of NRAS, KRAS and FGFR3 mutations, inactivation of mutations or deletions of TP53, RB1 and PTEN; and inactivation of cyclin-dependent kinase inhibitors CDKN2A e CDKN2C (Hideshima, Mitsiades et al. 2007; Bergsagel and Kuehl 2005). All these genes are involved in the NF-KB pathway, indicating that upregulation of NF-KB signalling is important in myeloma. It seems that constitutive activation of NF-KB is central to the ability of malignant B cells to resist apoptosis (Keats, Fonseca et al. 2007).

Moreover, also ERK pathway (NRAS in 24% cases, KRAS in 27% of cases and BRAF in 4% of cases) is frequently mutated in multiple myeloma patients indicating that deregulation of ERK signalling promotes myeloma development (Walker, Leone et al. 2010). Deregulation in the PI3K pathway also occurs in myeloma, but not so frequently. However, phosphorylated AKT, which is indicative of PI3K activity is detected in 50% of cases (Aronson, Davenport et al. 2013).

The complexity of the genetic deregulation of myeloma is further enhanced by the recent identification of recurrent mutations in DIS3, FAM46C and splicing factor 3B1( SF3B1); (Chapman, Lawrence et al. 2011).

## 2.5 Therapy for Multiple Myeloma

Multiple myeloma still remains an incurable disease.

Improvement in the in MM biology knowledge, such as the discovery of the interaction between the plasma cells and the tumor microenvironment, allowed a rapid development in the treatment of multiple myeloma.

The first active classes of drugs used for the treatment of multiple myeloma were: alkylators (e.g., melphalan and cyclophosphamide), corticosteroids (e.g., prednisone and dexamethasone), and anthracyclines (e.g., doxorubicin); (Palumbo and Anderson 2011). Subsequently, the immunomodulatory drugs thalidomide lenalidomide and pomalidomide, were developed (Lacy, Hayman et al. 2009). Lenalidomide has increased the efficacy and the adverse-effects of thalidomide (Carrier, Le Gal et al. 2011). Pomalidomide has demonstrated promising activity in patients refractory to lenalidomide (Lacy, Hayman et al. 2009). The immunomodulatory drugs target both tumor plasma cells and their microenvironment and are able to trigger caspase-8-mediated apoptosis, to decrease binding of tumor cells to bone-marrow stromal cells, to inhibit secretion of cytokines from the bone marrow, and to stimulate immunity against myeloma cells (Palumbo and Anderson 2011). In spite of the introduction of this agents, complete remission (CR) in MM was rare to achieve.

Improvements in progression-free and overall survival have been achieved by the introduction of high-dose drug treatment followed by stem-cell transplantation. However, the introduction of new agents such as the proteasome inhibitor bortezomib (BZ) in the treatment of myeloma patients, has resulted in improved response rates and longer overall survival. Although first approved as a single agent in the relapsed setting, bortezomib is now predominantly used in combination regimens. In conclusion fifty

years ago, with the development of melphalan and prednisone the patient median survival was of 3 years, high-dose therapy followed by stem cell transplantation has prolonged median survival to 4 to 5 years (Anderson 2012). The combination therapy of thalidomide, bortezomib and dexamethasone after autologous hematopoietic stem cell transplantation in patients with newly diagnosed multiple myeloma, has further increased median survival to 7 years (Cavo, Pantani et al. 2012; Munshi and Anderson 2013).

## 2.6 Bortezomib

Bortezomib (previously known as PS-341) is a dipeptide boronic acid derivative, which specifically and reversibly inhibits proteasome activity and triggers apoptosis in MM (Elliott and Ross 2001; Voorhees, Dees et al. 2003).

The function of the ubiquitin-proteasome pathway is to degrade regulatory proteins in eukaryotic cells, such as proteins involved in the control of cell-cycle progression, apoptosis, and DNA repair playing an essential role in maintaining normal cellular homeostasis (Adams 2003; Ciechanover 1994; Ciechanover 1998). The 26S proteasome consists of a barrel- shaped 20S proteolytic core, composed of 2 β-subunit rings and 2 α-subunit rings, plus two 19S regulatory complexes that cap the 20S barrel (Groll, Ditzel et al. 1997). Proteins destined for degradation are first polyubiquitinated; the 19S cap recognizes and binds ubiquitinated proteins and directs them to the 20S core, where proteolytic cleavage is mediated by 3 β-subunits: β1 (caspase-like activity), β2 (trypsin-like activity), and β5 (chymotrypsin-like activity); (Adams 2003).

Disruption of proteasome activity produces rapid accumulation of proteins within the cell resulting in growth arrest and cell death. Treatment of myeloma cells with bortezomib is associated with these events: inhibition of the adhesion of multiple myeloma cells to bone marrow stromal cells, down regulation of growth and antiapoptotic signalling pathway blocks production and intracellular signaling of IL-6 in myeloma cells, stops the production and expression of proangiogenic mediators, and overcomes defects in apoptotic regulators, such as Bcl-2 overexpression and alterations in tumor suppressor p53 (Boccadoro, Morgan et al. 2005; Reddy and Czuczman 2010). These effect are achieved principally, through the inhibition of NF-κB pathway (figure 2.2). The NF-κB pathway is constitutively active in MM. The nuclear factor-kB is

linked to proliferation and drug resistance of cancer B-cells. If NF-κB is inhibited by I-κB, cancer development is prevented. The role of bortezomib in NF-κB pathway is to inhibit NF-κB activation by protecting I-κB from degradation by the 26S proteasome (Chauhan, Hideshima et al. 2005).

It was also proposed that bortezomib acts through the induction of the Unfolded Protein Response (UPR), i.e., accumulation of misfolded proteins in the Endoplasmic Reticulum (ER) causing a lethal stress response, known as ER stress (Lee, Iwakoshi et al. 2003; Nawrocki, Carew et al. 2005; Obeng, Carlson et al. 2006; Meister, Schubert et al. 2007).

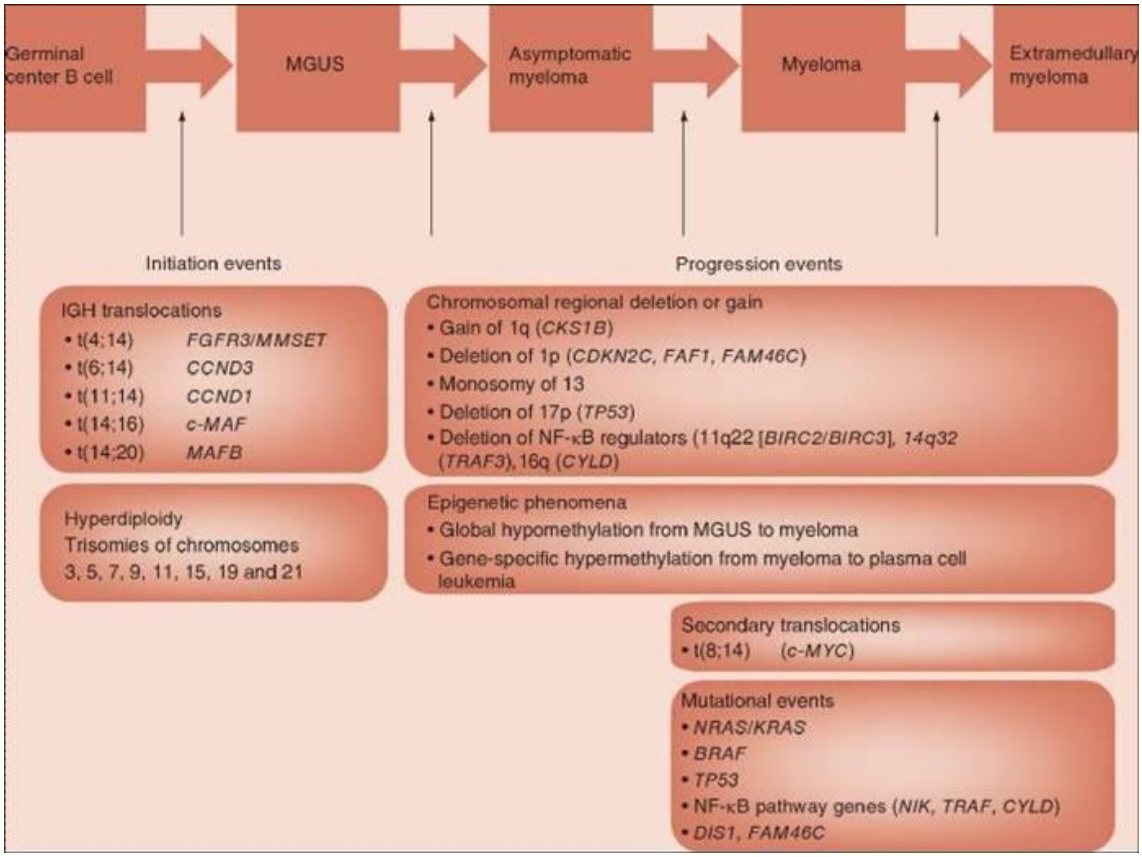


**Figure 2.1:** Genetic events in myeloma pathogenesis (Boyd, Pawlyn et al. 2012).

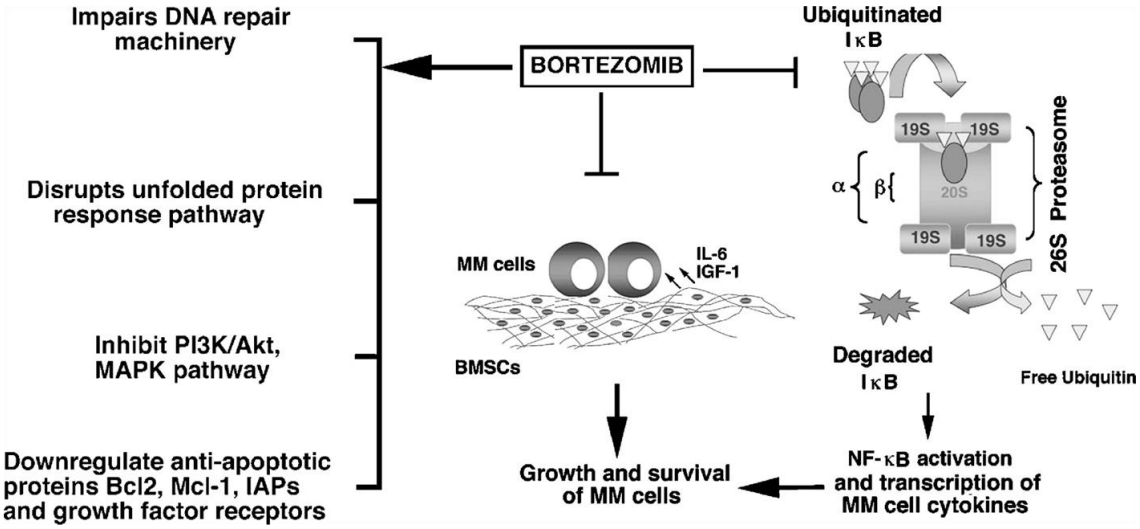


**Figure 2.2:** Effect of bortezomib on growth and survival pathways in MM (Chauhan, Hideshima et al. 2005).

## 2.7. The Unfolded protein response

The endoplasmic reticulum (ER) is the cellular compartment were secretory and membrane proteins are synthesized. In the ER the nascent proteins are folded and posttranslationally modified. Molecular chaperone proteins are involved in the proper folding and modification of newly synthesized proteins.

When the ER lumen is in unfavourable conditions, for instance in the case of high protein load, the accumulation of misfolded proteins occurs in the ER, a condition known as ER stress. The ER stress triggers the unfolded protein response with the aim of restoring protein homeostasis. If the UPR fails, cell death pathways are activated to sacrifice the compromised cells for the sake of the organism.

The UPR consists of three signalling systems controlled by these ER transmembrane proteins: inositol requiring kinase 1 (IRE1), (Sidrauski and Walter 1997; Yoshida, Haze et al. 1998; Chen and Brandizzi 2013) double stranded RNA-activated protein kinase-like ER-kinase (PERK), (Harding, Novoa et al. 2000; Donnelly, Gorman et al. 2013), and the transcription factor activating transcription factor 6 (ATF6);(Yoshida, Okada et al. 2000) (figure 2.3). The three ER-resident transmembrane proteins sense ER stress through GRP78 (glucose regulated protein 78 also know as BIP) chaperone protein binding/release. Under normal conditions these stress sensors bind GRP78. As unfolded proteins accumulate during ER stress, Grp78 dissociates and bind to the misfolded protein, allowing the activation of UPR signalling (Bertolotti, Zhang et al. 2000). The ER stress induces activation of IRE1 by dimerization and phosphorylation (Shamu and Walter 1996) that causes IRE1-mediated splicing of XBP1 mRNA (Sidrauski, Cox et al. 1996). Once XBP1 is translated, it translocates into the nucleus and binds the UPR promoter elements (UPRE). The expression of a variety of genes is promoted. The

proteins encoded are responsible for protein folding, modification and ER-Golgi transport and activation of the ER-associated degradation (ERAD); (Meusser, Hirsch et al. 2005). ERAD retrotraslocates terminally misfolded proteins from the ER for proteasome-dependent degradation (Ruggiano, Foresti et al. 2014).

As for IRE1 the ER stress-sensing mechanism of ATF6 involves the dissociation of Grp78 from its luminal domain during ER stress. GRP78 dissociation reveals two Golgi localization signals allowing to ATF6 to enter into the Golgi compartment (Chen, Shen et al. 2002). In the Golgi, the full length 90-kDa ATF6 is proteolytically processed by site-1 protease (S1P) and site-2 protease (S2P), to release a 50-kD transcription factor (Haze, Yoshida et al. 1999; Chen, Shen et al. 2002; Shen and Prywes 2004; Rawson 2013). This active cytosolic ATF6 domain, translocates to the nucleus and binds the ER stress response element (ERSE) to promote the transcription of ER-resident molecular chaperone and other assistant folding enzymes (Yoshida, Okada et al. 2000; Okada, Yoshida et al. 2002).

Thus, both the IRE1 and the ATF6 branch of the UPR principally activate a transcriptional programme that increases the capacity of the cell to fold and degrade proteins.

In contrast, PERK acts reducing the load of proteins which require folding Activated PERK (EIF2AK3) phosphorylates eukaryotic initiation factor 2a (eIF2α, EIF2A) on Ser51 inhibiting mRNA translation and restoring ER homeostasis (Harding, Novoa et al. 2000) .

eIF2α associates with the initiator Met-tRNA (aminoacylated initiator methionyl-tRNA) and GTP to form the ternary complex recruited on the small 40S ribosomal subunit. In order to allow the joining of the small and large ribosomal subunits, GTP associated with eIF2α, is hydrolyzed to GDP, and eIF2-GDP is released from the translational

machinery. The GDP-bound eIF2 is recycled to the active eIF2-GTP by a reaction catalyzed by the guanine nucleotide-exchange factor eIF2B. When eIF2α is phosphorylated eIF2B can no longer exchange GDP to GTP and this prevents the initiation of translation of almost all mRNAs (Proud 2005) (figure 2.4).

When eIF2α is phosphorylated while the translation of the most proteins is blocked the expression of mRNAs which contain multiple upstream open reading frames in their 5' UTR is increased like for ATF4 mRNA. The ATF4 mRNA contains two short upstream open reading frames (uORF). When eIF2α is not phosphorylated, both uORF are translated. The second uORF is out-of-frame and overlaps with the ATF4 coding sequence, thus the reinitiation at second uORF inhibits the expression of functional ATF4. eIF2α phosphorylation causes a reduction in ternary complex formation and in this condition the translation of second uORF is less likely. As a result, ATF4 is translated (Lu, Harding et al. 2004; Adams 2003) (figure 2.5).

The stress-induced phosphatase growth arrest and DNA damage- inducible gene 34 (GADD34) protein is upregulated by ATF4. GADD34 dephosphorylates eIF2α, promoting translational recovery (Novoa, Zeng et al. 2001; Ron 2002). ATF4 induces the transcription of mRNAs coding for proteins involved in the redox reactions, protein secretions, and transcription of mRNAs coding for molecular chaperones (Harding, Zhang et al. 2003).

This strategy is adaptive and it occurs in short term ER stress conditions. If the ER stress is prolonged PERK signaling involves activation of CHOP, The CHOP promoter includes binding sites for both ATF4 and ATF6, which appear to synergize (Okada, Yoshida et al. 2002).

CHOP is a bZIP-containing transcription factor that was identified as a member of the CCAAT/enhancer binding protein (C/EBP) family (Ron and Habener 1992). CHOP is

also known as growth-arrest and DNA-damage-inducible gene 153 (GADD153). During prolonged ER stress, CHOP is one of the most highly upregulated genes (Harding, Novoa et al. 2000; Okada, Yoshida et al. 2002). CHOP activity results in the downregulation of the anti-apoptotic Bcl2 (B-cell lymphoma 2) and the upregulation of the ER-resident oxidase ERO1-alpha. The final result of CHOP activation is the induction of programmed cell death (Marciniak, Yun et al. 2004).

The role of eIF2α phosphorylation in cancer is context and cell types dependent. In general, prolonged eIF2α phosphorylation may induce cell death. Salubrinal is a specific inhibitor of eIF2α dephosphorylation. The treatment of leukemia and multiple myeloma cells with Salubrinal enhances proteasome inhibitor-induced apoptosis (Drexler 2009; Schewe and Aguirre-Ghiso 2009). In other context, for instance in neural cells, salubrinal prevents ER stress induced apoptosis (Boyce, Bryant et al. 2005). Instead, selective inhibition of eIF2α dephosphorylation causes pancreatic beta-cell dysfunction and apoptosis (Cnop, Ladriere et al. 2007).

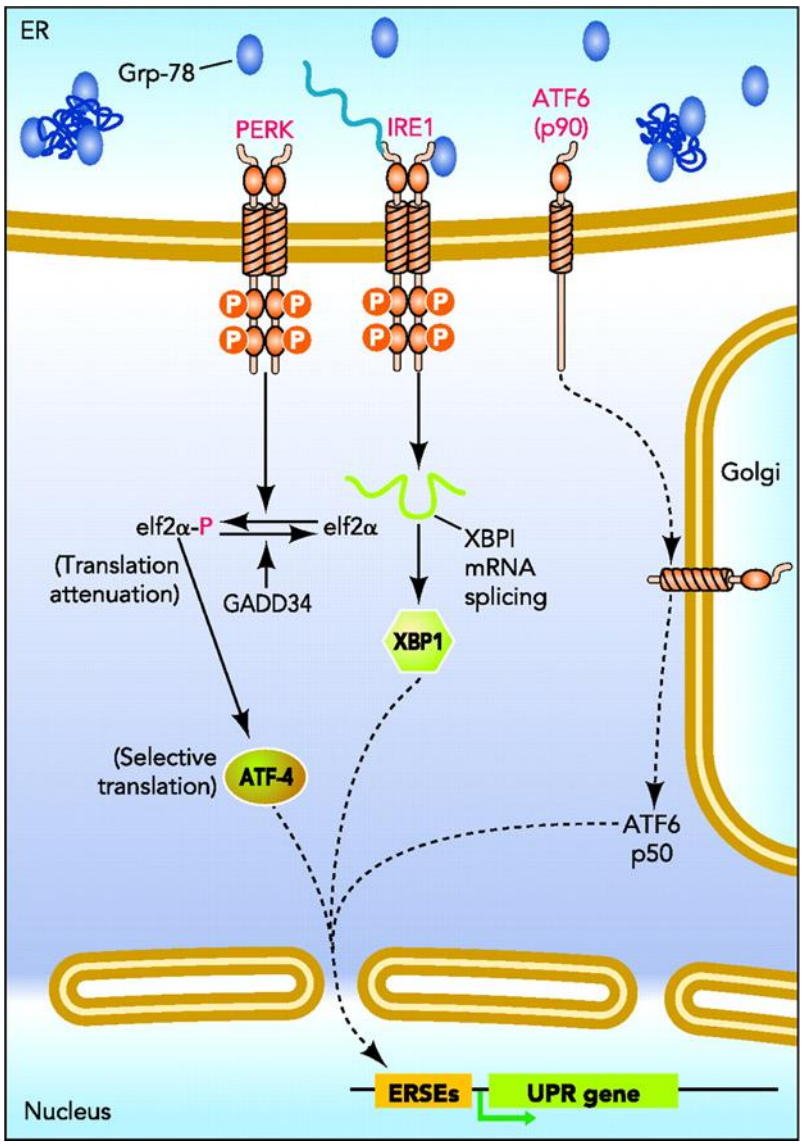


**Figure 2.3:**UPR signaling pathways in mammalian cells (Lai, Teodoro et al. 2007)

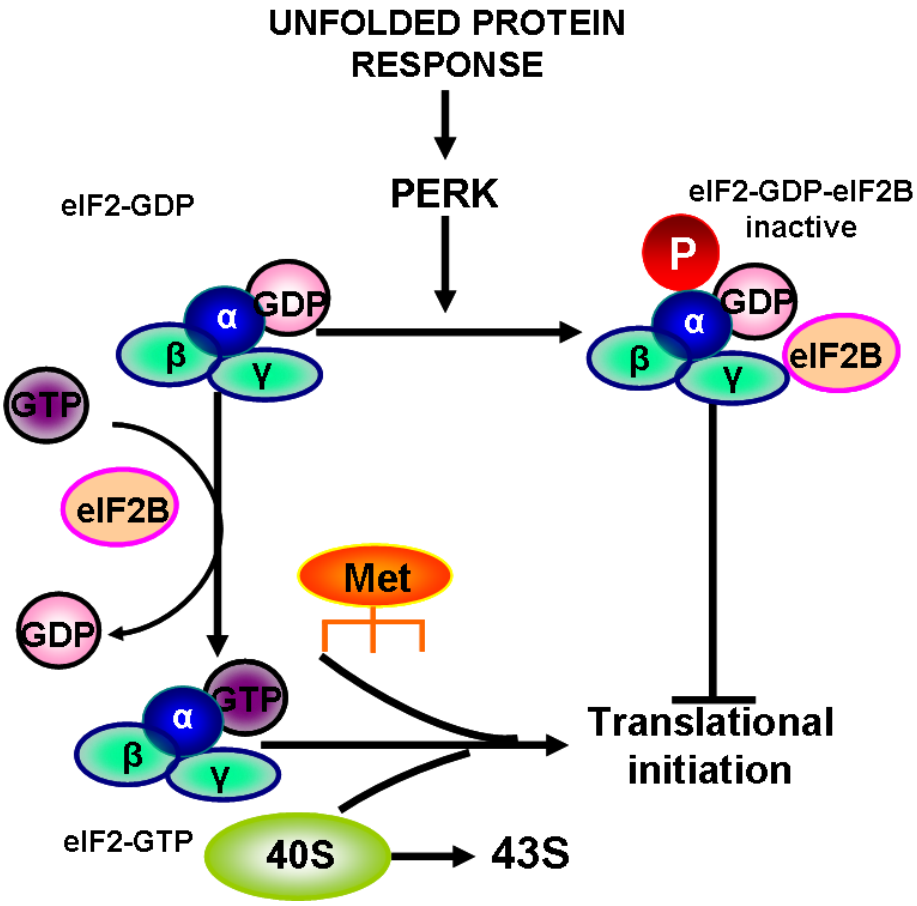


**Figure 2.4:** eIF2α phosphorylation leads to inhibition of translation.

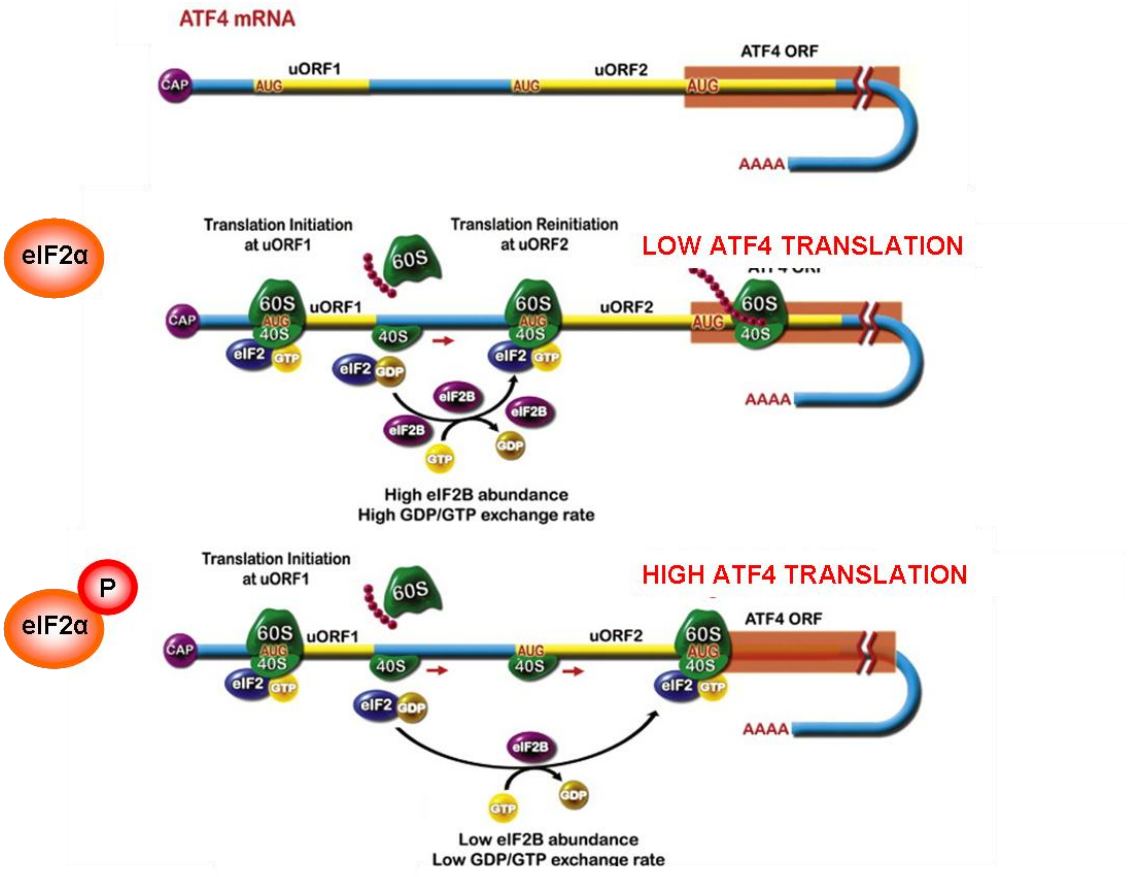


**Figure 2.5:** Effect of eIF2α phosphorylation on ATF4 mRNA translation, (adapted from (Kilberg, Shan et al. 2009)).

## 2.8. Initiation of protein synthesis

Protein synthesis, or translation, is the fundamental process in which mRNA previously transcribed from DNA is decoded by specialized cellular structures, called ribosomes, to make proteins.

Ribosomes are ribozimes, constituted by ribosomal RNA (rRNA) and structural proteins. They are formed, in eukaryotes, by a small subunit (40S), and by a large subunit (60S). The joining between the two subunits generates a translational competent ribosome (80S).

Protein synthesis comprises three stages: initiation, elongation and termination and ribosomal recycling. Each of these steps is facilitated by protein factors, which transiently associate with the ribosome and/or the mRNA. They are called eukaryotic initiation factors (eIFs), eukaryotic elongation factors (eEFs), and eukaryotic termination factors (eRFs).

The limiting step of protein synthesis is translation initiation, during which the small ribosome subunit is recruited to the 5' end of mRNA and scan toward the start codon, where the complete ribosome is subsequently assembled to begin polypeptide formation (Pestova, Kolupaeva et al. 2001; Gebauer and Hentze 2004; Sonenberg and Hinnebusch 2009). Initiation of translation can be subdivided into four steps: first, binding of the specific initiator Met-tRNAi to the 40S subunit; second, binding of the formed complex to the cap structure at the 5’ end of the mRNA; third “scanning” of the 5’ untranslated region (UTR) of the mRNA and start codon recognition; fourth, joining of 60S ribosomal subunit to generate the 80S complex.

The first step requires the help of the initiation factor eIF2. As mention before, eIF2 is an essential component of the ternary complex which comprises eIF2, Met-tRNA and GTP.

The ternary complex then associates to the 40S ribosomal subunit, eIF3, eIF1, eIF1A and eIF5, to form the 43S pre-initiation complex. Both AUG recognition and joining of the 60S subunit trigger GTP hydrolysis on eIF2.

The second step is the recruitment of the 43S pre-initiation complex to mRNA to form the 48S pre-initiation complex. This requires the assembly of the eukaryotic translation initiation factor 4F (eIF4F) complex on the 5' m7 GpppN cap structure of mRNA. eIF4F is composed of the cap binding protein eIF4E , the scaffold protein eIF4G and the ATP-dependent helicase eIF4A which, assisted by eIF4B, unwinds secondary structures in the 5'UTR of the mRNA. The exact molecular mechanism by which 43S pre- initiation complex is loaded on the mRNA is not completely understood. It involves interactions of the eIF4F with the eIF3 and the poly(A) binging protein (PABP), which is bound to the poly(A) tail at the 3' end of the transcript. Several studies have provided evidence indicating that the specific interaction between PABP and the eIF4F component eIF4G stimulates 43S recruitment to the mRNA (Tarun and Sachs 1995; Preiss and Hentze 1998; Kahvejian, Svitkin et al. 2005). The PABP-eIF4G interaction is thought to promote a circularization of the mRNA molecule forming a closed loop.

The 48S after the binding of mRNA, complexed to eIF4F, initiates the scanning to the AUG start codon.

Some mRNA species contain inhibitory secondary structures in the 5' untranslated region (UTR). The structured 5' UTR prevents efficient scanning of the small ribosome subunit to the start codon. A group of initiation factors, such as eIF4A, are RNA helycase that can "unwind" mRNA secondary structures and have a crucial role in

translating these mRNAs (Pestova and Kolupaeva 2002). The helicase activity of eIF4A is significantly stimulated by the presence of the translation initiation factors, eIF4B or eIF4H (Rogers, Richter et al. 2001). The helicase activity of eIF4A is enhanced when its regulatory factor eIF4B associated with eIF4A. Growth factor-mediated phosphorylation of eIF4B can increase its association eIF4A (Holz, Ballif et al. 2005; Shahbazian, Roux et al. 2006). eIF4H is an RNA binding factor, eIF4H can significantly enhance the helicase activity of eIF4A by binding both to loop structures within the RNA transcript and to eIF4A (Parsyan, Svitkin et al. 2011; Sun, Atas et al. 2012).

Joining of the 60S ribosomal subunit to the 48S complex requires hydrolysis of two GTP molecules. First eIF5 triggers GTP hydrolysis on eIF2, which leaves the complex thereafter in the GDP bound state together with eIF5 (Unbehaun, Borukhov et al. 2004). eIF1 and eIF3 remain associated with the complex until a second GTPase, eIF5B binds to the 43S pre-initiation complex and allows the 60S subunit to join (Pestova, Lomakin et al. 2000).

Finally, GTP hydrolysis on eIF5B, triggered by 60S subunit joining, results in the dissociation of eIF5B in the GTP bound form and the formation of an elongation competent 80S ribosome (Pestova, Lomakin et al. 2000; Lee, Pestova et al. 2002; Jackson, Hellen et al. 2010).

## 2.9. Regulation of cap complex formation (mTOR pathway)

Most cap-dependent translation is regulated by the pathway, which involves mTOR (mammalian Target of Rapamycin, also Known as FRAP1). mTOR is a highly conserved serine/threonine chinase. Mammalian TOR complex 1 (mTORC1), which contains mTOR in complex with raptor (regulatory associated protein of mTOR) and LST8 (also known as GβL), directly regulates protein synthesis in mammals (Ma and Blenis 2009; Kim, Buel et al. 2013).

A second kinase complex, mTORC2, elicits distinct functions to mTORC1. The mTORC2 complex contains mTOR, LST8, rector (rapamycin-independent companion of mTOR) and SIN1 (stress-activated protein kinase- interacting protein 1). mTORC2 phosphorylates AKT on Ser 473, RAC1 and protein kinase (PKCα) and is involved in the regulation of cytoskeletal organization (Guertin and Sabatini 2007; Yang and Guan 2007; Oh and Jacinto 2011).

Main downstream targets of mTOR kinase are eIF4E-binding proteins 4E-BPs) and p70 rpS6 kinase (S6K) (figure 2.6). As it was previously described, eIF4E binds to the 5' cap structure of eukaryotic mRNAs and, together with eIF4G, eIF4A, eIF4B, and PAPB, forms the cap complex. The so-called eIF4E binding protein (4E-BPs) comprise low molecular mass proteins that blocks the interaction of eIF4E with eIF4G. In mammals three eIF4E binding proteins are known, 4E-BP1, 4E-BP2, 4E-BP3. When hypophosphorylated, 4E-BPs are able to bind a small domain required for the interactions between eIF4E and EIF4G, and thereby displace eIF4G. Without eIF4G binding, cap complex and 48S pre-initiation complex cannot form (Haghighat, Mader et al. 1995; Mader, Lee et al. 1995; Marcotrigiano, Gingras et al. 1999).

Active mTORC1 phosphorylates 4E-BPs at multiple sites causing their release from eIF4E and allowing 48S pre-initiation complex formation (Lawrence and Abraham 1997; Lawrence, Fadden et al. 1997). mTORC1 also activates S6 Kinase 1and 2 (S6K1/2) which in turn phosphorylates 40S ribosomal protein S6 and eIF4B. The meaning of S6 phosphorylation is still unknown. The phosphorylation of eIF4B, as described before, enhances the helicase activity of eIF4A (Hay and Sonenberg 2004; Laplante and Sabatini 2012).

mTORC1 phosphotransferase activity is stimulated by the small G protein *Rheb* (Ras homologue enriched in brain) bound to GTP. In turn, *Rheb* is regulated by a tumour suppressor heterodimer that is composed of TSC1 and TSC2 (Tuberous Sclerosis 1 and 2, also known as hamartin and tuberin, respectively). TCS2 exhibits GTPase- activating protein (GAP) activity towards *Rheb*, converging it to the inactive GDP-bound form(Manning and Cantley 2003; Kwiatkowski and Manning 2005). Thus, TSC1-TSC2 complex is a negative regulator of mTORC1.

Multiple upstream signalling inputs from PI3K-AKT, RAS-ERK-RSK, TNFα-IKKβ, AMPK-GSK3β pathways either positively ore negatively regulate mTORC1 signalling (figure 2.7). For instance, PI3K -AKT pathway and RAS-ERK pathway (Shaw and Cantley 2006; Mendoza, Er et al. 2011) stimulate mTORC1 signalling by inhibiting the tumor suppressor complex TSC1-TSC2, thus allowing *Rheb* activity. Insulin stimulation results in PI3K (PhosphoInositide 3Kinase) activation. PI3K converts $PIP_2$ (Phosphotidy1Inositol 3,4-bisPhosphate) in $PIP_3$ (Phosphotidy1Inositol 3,4,5-trisPhosphate), which recruits AKT to the membrane. Akt is then phosphorylated by PDK1 (Phosphoinositide Dependent protein Kinase 1) and thereby activated. Active Akt directly phosphorylates TSC2, inhibiting its GAP activity.

Growth factors are able to stimulate Ras activity towards Raf, which in turn activates MAP kinase pathway by phosphorylating MEK (MAP/Erk Kinase). MEK activates Erk (Extracellular signal-Regulated Kinase), which acts on TSC2 inhibiting the GAP function of TSC2 towards *Rheb*.

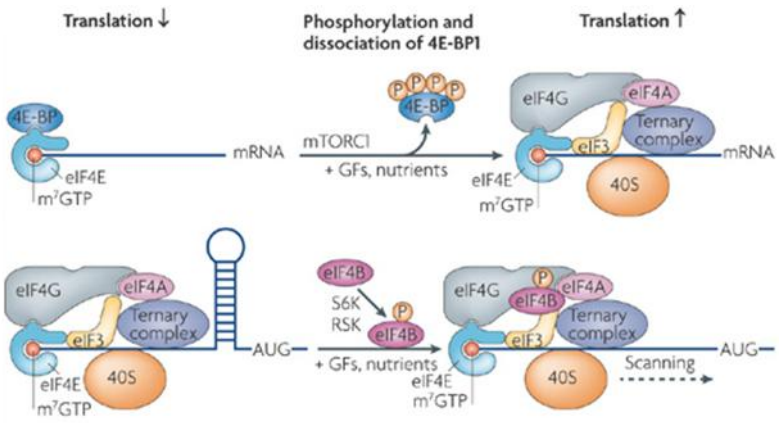


**Figure 2.6:** Regulation of cap dependent translation (Ma and Blenis 2009)

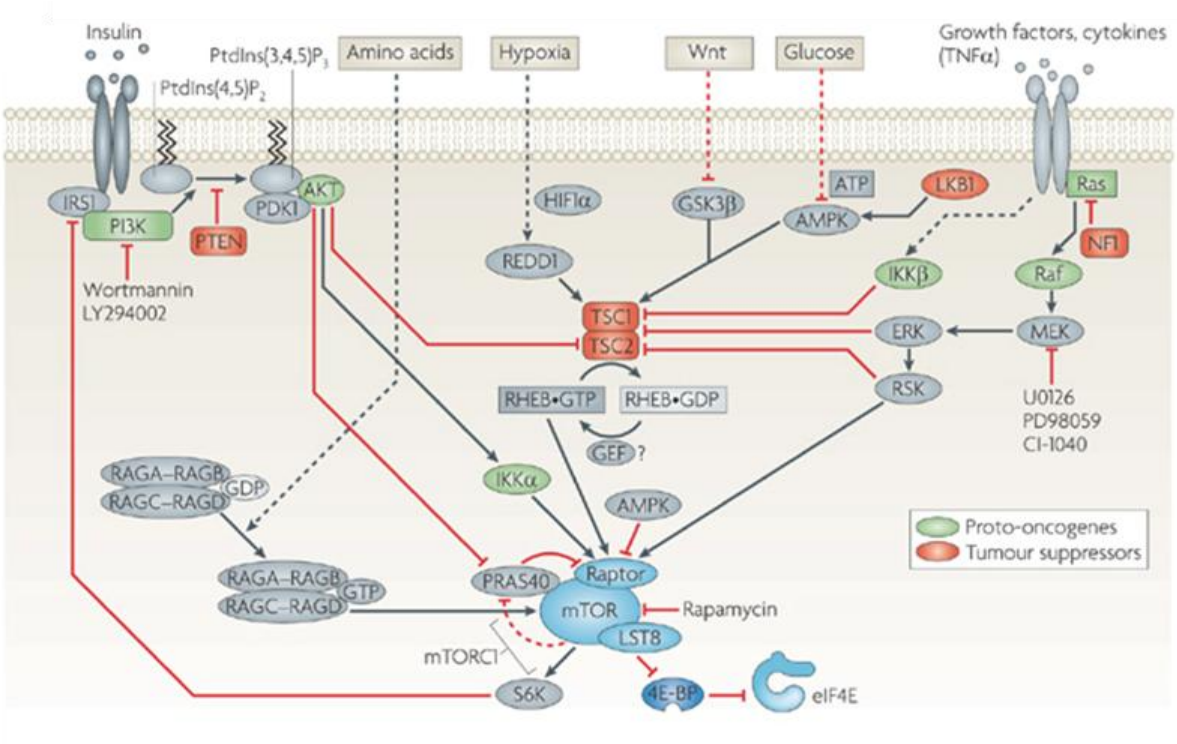


**Figure 2.7:** Regulation of mTOR pathway (Ma and Blenis 2009).

## 2.10. Rapamycin and mTOR inhibitors

The activation of mTOR results in the production of proteins involved in cell metabolism, growth, proliferation and angiogenesis.

Aberrant activation of mTOR signalling has been identified in a number of cancers (Hanahan and Weinberg 2011). Therefore, inhibition of translation through the silencing mTOR signalling pathway is emerging as a promising therapeutic strategy. Rapamycin was the first agent found to interfere with mTOR pathway. Rapamycin binds to the immunophilin FKBP12 and generates a specific inhibitor of mTORC1-dependent signalling through direct binding to the FRB domain. Rapamycin-FKBP12 is not able to bind mTORC2, although long-term rapamycin treatment impairs mTORC2 activity in some cancer cell lines through an unknown mechanism (Sarbassov, Ali et al. 2006). In preclinical cancer models, rapamycin exhibits antitumor properties, such as inhibiting cell proliferation and cell survival and anti-angiogenesis (Guba, von Breitenbuch et al. 2002).

Rapamycin derivates, temsirolimus and everolimus, have shown clinical efficacy in a variety of tumors such as advanced renal cell carcinoma, giant cell astrocytoma, breast cancer and progressive neuroendocrine tumors (Ramirez-Fort, Case et al. 2014). However accumulating evidence suggest that the antitumor properties of rapamycin vary significantly among different cell lines. For example, rapamycin can be pro-apoptotic (Beuvink, Boulay et al. 2005; Teachey, Obzut et al. 2006) or pro-survival (Fumarola, La Monica et al. 2005), depending on the cancer cell line tested. The pro-survival or cytotoxic effects of rapamycin can be explained by the inhibition of the S6K1-dependent feedback loops. When mTORC1 is activated, the resulting activation of S6 kinase 1, results in the phosphorylation and inhibition of insulin receptor substrate

1 (IRS1)-dependent activation of phosphoinositide 3-kinase (PI3K), thus leading to inhibition of AKT and MEK-ERK signalling cascade. mTORC1 inhibition by rapamycin suppresses this negative feedback loop and leads to the upregulation of PI3K signalling pathway providing pro-survival and proliferative signals (O'Reilly, Rojo et al. 2006; Carracedo, Ma et al. 2008) (figure 2.8).

Furthemore, rapamycin affects 4E-BP phosphorylation only transiently and partially (Feldman, Apsel et al. 2009; Thoreen and Sabatini 2009).

For these reasons, active-site mTOR inhibitors (asTORi) were generated. PP242, the first of a series of mTOR inhibitors has been used in a preclinical trial for AKT-driven lymphomagenesis. (Hseieh et al 2010). INK128, a more potent derivative of PP242, as well as a several other ATP-site inhibitors, are currently in phase I/II trials in patients with advanced solid tumours and haematological malignancies (Benjiamin et al. 2011; Hsieh et al 2012). These compounds differ from rapalogs for their ability to bind the ATP-binding pocket directly on mTOR. mTOR ATP-competitive inhibitors are able to inhibit both mTORC1 and mTORC2. The mTOR inhibitors completely repress 4E-BP1 phosphorylation and reduce phosphorylation of TORC2 substrates, e.g. AKT. (Feldman, Apsel et al. 2009). Moreover, asTORi, as well as rapamycin, cause the inhibition of the negative feedback loop driven by S6K (indirectly upregulating phosphatidylinositol 3 kinase (PI3K)). However, since ATP-site inhibitors are able to block both mTORC1 and mTORC2 activities (while rapamycin can not) they result in AKT not being hyperactivated. (figure 2.8). In general, mTOR inhibitors have more dramatic effects on cell growth and proliferation with their ability to inhibit both mTORC1 and mTORC2 (Benjamin, Colombi et al. 2011).

However, several cancer cells and patients do not respond to rapamycin and to dual mTORC1/mTORC2 inhibitors. One mechanism that accounts for resistance is the ratio

between 4E-BPs and eIF4E. Overexpression of eIF4E and variations in 4E-BP levels and phosphorylation, are frequently observed in tumors, suggesting that eIF4E/4E-BP stoichiometry may differ among patients. Two different works demonstrated that malignant cells with high eIF4E/4E-BP ratio are resistant to rapamacyn and asTOR1(Grosso, Pesce et al. 2011; Alain, Morita et al. 2012). In fact, the downregulation of 4E-BPs or overexpression of eIF4E make neoplastic growth and translation of tumor-promoting mRNAs refractory to mTOR inhibition. Conversely, moderate depletion of eIF4E increases the anti-neoplastic effects of active-site mTOR inhibitors (Alain, Morita et al. 2012). In summary, the levels of eIF4E and 4E-BP, should be predictive biomarkers, that would allow the stratification of patients that most likely, may respond to these drugs.

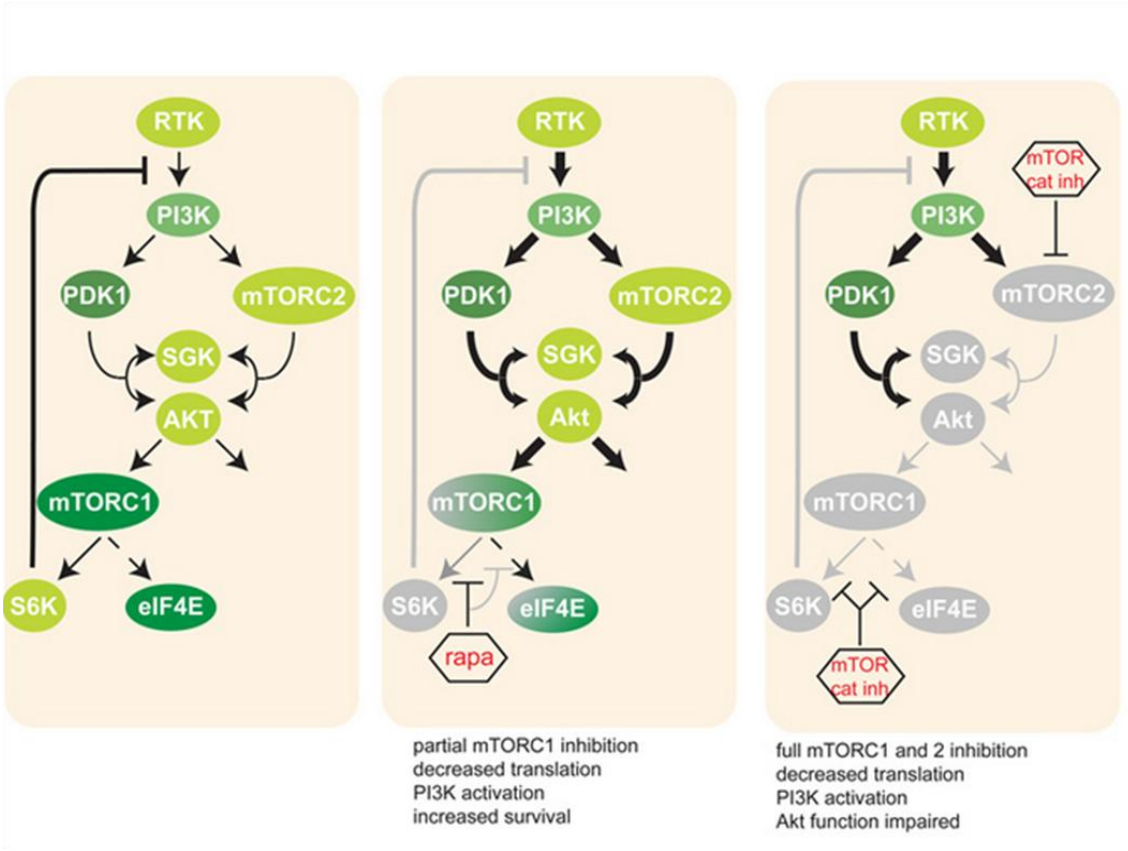


**Figure 2.8:** Activity of rapamacyn and mTOR inhibitors on mTORC1 and mTORC2 pathways (Zoncu, Efeyan et al. 2011).

## 2.11. Hypothesis of the work

Multiple myeloma still remains an incurable disease, even if the introduction of bortezomib in the myeloma therapy has improved the patient's outlook, patients developed resistance to bortezomib. Many attempts have been done in order to discover the causes of BZ resistance.

Recently, it has been proposed that BZ is able to induce ER stress, activating the unfolded protein response. The induction of the unfolded protein response impairs the initiation of translation, through eIF2α phosphorylation. As consequence, the inhibition of protein synthesis reduces the proteasome load.

It has been suggested that multiple myeloma cells are extremely sensitive to BZ induced ER stress for their peculiar characteristic to produce high amount of immunoglobulins. Accordingly to this theory, by reducing the proteasome workload, multiple myeloma should acquire resistance to proteasome inhibitors.

In this work we investigated the hypothesis that the translational load contribute to BZ toxicity or survival and whether the inhibition of protein synthesis protects or prevents BZ induced toxicity in multiple myeloma.

# 3. MATERIALS and METHODS

## 3.1. Cell culture and proliferation assay

The MM cell lines MM.1S and U266 cells were kindly provided by Dr Tonon (San Raffaele Scientific Institute, Milan, Italy). Cells were cultured in RPM1 1640 (Euroclone) supplemented with 10% FBS (fetal bovine serum; Gibco) 1% glutamine and a commercial antibiotic mix (Gibco). Cell proliferation was measured by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide]. Cells were plated in 96 well plate at a density of 20.000 cells per well. After treatment, MTT solution was added and incubated for 3 hours. The reaction product was quantified reading the absorbance at 570 nm using a microplate reader (Biorad) (Hideshima, Catley et al. 2006).

### 3.2. Primary myeloma specimens and proliferation assay

Bone marrow (BM) samples for molecular studies were obtained during standard diagnostic procedures. Written informed consent was obtained from each patient. Plasma cells were purified from mononuclear BM cells obtained by Ficoll-Hypaque density gradient centrifugation using anti-CD138 micro beads on an AutoMacs Magnetic Cell Separator (MACS system, Miltenyi Biotec). The purity of positively selected plasma cells was assessed by flow cytometry and was ≥ 90% in all cases. For co-colture experiment HS-5 stromal cells were plated O/N in 96 well plates at a density of 500 cells per well. Primary myeloma cells were plated at density of 10.000 cells/well on a layer of HS-5 stromal cells. Drugs were added at the indicated concentrations and compared with DMSO treated controls. Cultures were then incubated for 24, 48 and 72 h in a 37°C incubator with 5% of $CO_2$. The MTT assay was performed. The experiment was done in triplicate measurement.

### 3.3. Drugs and reagents

The following antibodies were used: rabbit polyclonal anti-rpS6, anti-phospho-rpS6 (Ser235/236), anti-phospho-rpS6 (Ser 240/244), anti-4E-BP1, anti-p44/42 MAPK (ERK1/2), anti-phospho-p44/42 MAPK-ERK (Thr202/Tyr204), anti-eIF2α, anti-phospho-eIF2α (Ser51), anti-mTOR, (Cell signalling); mouse monoclonals anti-β actin (Sigma), anti caspase -3 (Alexis), anti-PARP-1 (Millipore), anti-HA (Covance). Retroviruses pBABE-puro and 4E-BP1 4Ala were a gift of Dr. N. Sonenberg (McGill University, Montréal, Quebec, Canada). The proteasome inhibitor bortezomib (BZ) was from Millennium Pharmaceuticals. mTOR inhibitor PP242, rapamycin and cycloheximide were from Sigma.

### 3.4. Western blot

MM cells were lysed with buffer containing 10 mM NaCl, 10 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.3), 1% Triton X-100, 1% sodium deoxycholate, 1 mM DTT, 5 mM NaF, 2 mM $Na_3VO_4$, 40 units/ml RNasin® (Promega) and protease inhibitor cocktail. The whole cell extract was clarified at 4 °C at 15.000 g for 10 min. The amount of recovered protein was quantified by the bicinchoninic acid (BCA) protein assay (Smith, Krohn et al. 1985). Equal amount of proteins were added with Laemmli buffer (2x Laemmli buffer: 2gr glycerol, 0,4gr SDS, 0,5 ml 2-mercaptoethanol, 250mM Tris HCL pH 6.8, a bit of bromophenol blue), resolved on SDS-PAGE (Laemmli 1970), transferred to Immobilon-P membranes (Millipore), and probed with appropriate antibodies (Renart, Reiser et al. 1979).

### 3.5. Kinase assay

Proteins from MM.1S cells were extracted in lysis buffer (50 mM Tris-HCL pH 7.4, 150 mM NaCl, 1 mM EDTA, 5 mM EGTA, 20 mM β-glycerolphosphate and protease inhibitor cocktail) by freezing and thawing and clarified by centrifugation. Protein

concentration was quantified by BCA. One mg of total extract protein was incubated with anti-mTOR antibody for 2 hours at 4$^{o}$C in constant rotation. Immunoprecipitation was performed with protein A for 30 min. Beads were washed one time with high-salt wash buffer (100 mM TRIS-HCl pH 7.4, 500 mM LiCl) and three times with kinase buffer (10 mM Hepes pH 7.4, 50 mM β-glycerolphosphate, 150 mM NaCl). The beads were resuspended in kinase buffer. The kinase assay was performed by adding 10 μg GST-4E-BP1 recombinant protein or GST alone, 10 mM $MnCl_2$ and 4 μCi of γ-$^{32}$P-ATP (10 Ci/mmol). The reaction was run at 30$^{o}$C for 1 hour and terminated by adding one volume of sample buffer. Samples were boiled 5 min, separated by SDS PAGE, transferred to Immobilon-P membranes (Millipore), and probed with anti-GST, anti-mTOR. Autoradiography was performed for one hour at room temperature.

### 3.6. m$^{7}$GTP Cap Column Pull-Down

Cells were collected by scraping, washed three times with cold PBS and pelleted by centrifugation. Cell pellets were resuspended in lysis buffer (50 mM Tris-HCl at pH 7.4, 100 mM KCl, 1 mM EDTA, 1 mM EGTA, 20 mM NaF, 0,5 mM Na3VO4, 1% Triton X-100 , and protease inhibitors), and incubated for 30 min at 4°C. Cell debris was removed by centrifugation at 10.000 g for 10 min at 4°C, and protein concentration was determined using the BCA protein assay (Thermo scientific). The extract (300 μg) was incubated with 30 μL of m$^{7}$GTP-agarose resin (GE Healthcare) for 1 h at 4°C (Pyronnet, Dostie et al. 2001). The resin was washed three times with 1 mL of lysis buffer, boiled for 6 min in Laemmli buffer, and proteins were resolved by SDS-PAGE.

### 3.7. Polysomal Profiles

Cells ($40\times10^{6}$) were pre-treated 10 minutes with 100 μg/ml cycloheximide, collected and washed with cold PBS (phosphate saline buffer) with 10 μg/ml cycloheximide. Cells were resuspended in lysis buffer composed by 50 mM Tris–HCl pH 7.5, 100 mM

NaCl, 30 mM $MgCl_2$, 0.1% NP-40, 100 μg/ml cycloheximide, 40 U/ml RNasin® (Promega), protease inhibitor cocktail (Sigma). Lysed cells were left for 20 min at 4°C, and lysates were then clarified by centrifugating at 18.000g for 5 min at 4°C. Supernatants were collected, and RNA concentration was quantified by reading $OD_{254}$.The equivalent of 10 absorbance units at 254 nm were layered on to a 15–50% sucrose gradient in 50 mM Tris/acetate (pH 7.5), 50 mM $NH_4Cl$, 12 mM $MgCl_2$ and 1 mM DTT and centrifuged at 4 °C in a SW41Ti Beckman rotor for 3:30 h at 39,000 rpm (170.000 g). Samples were analyzed with BioLogic LP (BioRad) by reading $OD_{254}$.

**3.8. Methionine Labeling**

One million cells were incubated at 37 $^oC$ with methionine-free medium and pulsed with 33 μCi of Promix $^{35}S$-labelled methionine (PerkinElmer Life Sciences) for 1 hour. Cells were lysed in 50μl of RIPA buffer without SDS (10mM Tris-HCL, pH 7.5, 1% Na-deoxycholate, 1% Triton X-100, 150 mM NaCl, 1mM EDTA, protease inhibitor cocktail from Sigma). The lysate was cleared by centrifugation. Extracts of 10 μl were TCA (TriChloroacetic Acid) precipitated on glass microfibre filters (Whatman) and counted. Obtained values were normalized by sample protein content, quantified using the BCA protein assay (Thermo scientific). Each sample was analysed in triplicate and results are expressed as a means ± sd.

**3.9. Retroviral infections**

Retroviral constructs, pBABE empty vector and pBABE -HA-4E-BP1 (4Ala) were transfected into amphotropic phoenix 293T packaging cells (Rong, Livingstone et al. 2008). After 48 hr, the virus containing medium was collected. $10^6$ cells were plated in 24-well plates and incubated with virus supernatant and 8mg/ml polybrene. Cells were spun at 1100 g for 2 hours and then incubated for 3 hours at 37$^oC$, 5% $CO_2$. Transduced

cells were selected with puromycin 3mg/ml, for one week and further analyzed by immunoblot.

### 3.10. Gene expression profiling

Total RNA from CD138 positive cells was obtained from each sample by the RNeasy® kit (Qiagen) extraction procedure. To measure concentration and purity of RNA, a NanoDrop ND-1000 spectrophotometer was used (NanoDrop Technologies), purity of the extracted RNA was based on the 260/280 and the 260/230 O.D. ratios, as calculated and displayed by the NanoDrop spectrophotometer. Moreover, disposable RNA chips (Agilent RNA 6000 Nano LabChip kit) were used to determine the concentration and purity/integrity of RNA samples using Agilent 2100 Bioanalyzer. Samples with at least 30 ng/uL RNA were labelled for gene expression profiling, using the Affymetrix Two-cycles Gene Chip microarray system (Affymetrix). cDNA synthesis, biotin-labelled target synthesis, HG U133 Plus 2.0 GeneChip arrays hybridization, staining, and scanning were performed according to the standard protocol supplied by Affymetrix. Microarray data were used to identify gene expression profile of 4E-BP1, 4E-BP2 and eIF4E in BM samples obtained from 122 newly diagnosed MM.

# 4. RESULTS

## 4.1. Bortezomib toxicity is not associated with proapoptotic eIF2α Phosphorylation.

It has been proposed that bortezomib can induce the unfolded protein response in multiple myeloma and that this contributes to its pro-apoptotic activity (Lee, Iwakoshi et al. 2003; Obeng, Carlson et al. 2006; Ling, Lau et al. 2012). Here we examined whether, the induction of unfolded protein response, can explain the BZ resistance and or sensitivity of multiple myeloma to BZ treatment. To this aim, we compared the BZ effect on MM.1S and U266 cell lines, defined respectively sensitive and resistant to BZ.

We first validated the BZ effect on the inhibition of growth, proteasome activities, and on the induction of apoptosis. We performed MTT analysis on MM.1S and U266 treated with different concentrations of BZ for 24 and 48 hours (figure 4.1.1). Calculated $EC_{50}$ at 48 h were 11,93 ± 1,68 nM for MM.1S cells and 16,15 ± 1,81 nM for U266. Calculated $EC_{50}$ at 24 hours were 18,26 nM ± 1,68 for MM.1S, whereas U266 were resistant to BZ at the concentrations up to 50 nM. These data, in agreement with previous works, show that MM.1S cells are more sensitive than U266 to BZ-induced toxicity. The inhibition of proteasome activity leads to polyubiquitinated proteins accumulation. Consistently, 8h BZ treatment caused, in both MM1.S and U266, accumulation of polyubiquitinated proteins. Further accumulation of polyubiquitinated proteins at 24 hours was observed only in MM.1S cells (figure 4.1.2).We also analyzed the induction of apoptosis by analyzing cleaved Parp and cleaved caspase 3 in both MM.1S and U266. In agreement with MTT data, cleaved caspase 3 and PARP were observed only in MM.1S at 25 and 50 nM of BZ treatment (figure 4.1.3). Taken together, these data indicate that long term proteasome inhibition has more dramatic

effects on MM.1S, (compared to U266) in terms of accumulation of poly-Ubiquitinated proteins, inhibition of cell proliferation and induction of apoptosis.

In order to investigate whether the activity of BZ on the induction of unfolded protein response was correlated to the sensitivity and/or resistance to the drug, we evaluated the phosphorylation status of eIF2α upon BZ treatment in MM.1S and U266. As positive control of eIF2α phosphorylation we administrated thapsigargin to NIH-3T3 fibroblasts. Thapsigargin blocks the ER calcium ATPase pump leading to the depletion of ER calcium stores (Thastrup, Dawson et al. 1994). This in turn leads to the induction of ER stress that robustly induces eIF2α phosphorylation.

As shown by Western blot analysis, myeloma cells have high basal level of eIF2α phosphorylation compared to fibroblast. eIF2α phosphorylation is induced by thapsigargin in fibroblast cells. Instead, the induction of eIF2α phosphorylation by BZ treatment was minimal, and present both in BZ-sensitive MM1.S cells and in BZ-insensitive U266 cells (figure 4.1.4).This result indicates that the timing and the extent of induction of eIF2α phosphorylation does not associate with BZ induced-death.

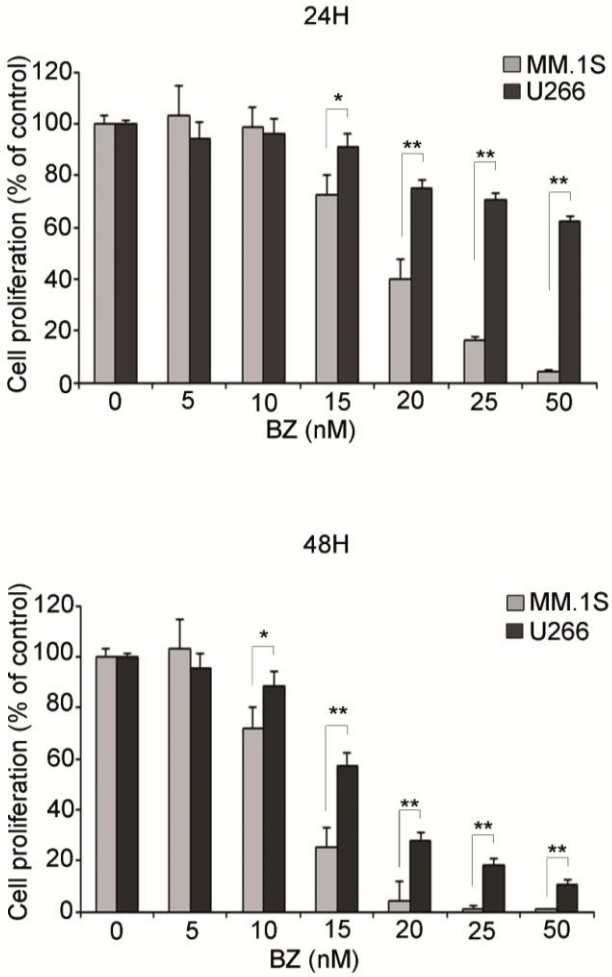


**Figure 4.1.1**

MM.1S cells are more sensitive to BZ than U266 cells. Cells were treated with BZ (5 nM to 100 nM) for 24 and 48 hours. MTT assay was performed. Data are presented as percentage of untreated control. Statistical significance was assessed by Student's t-test **P < 0,01.

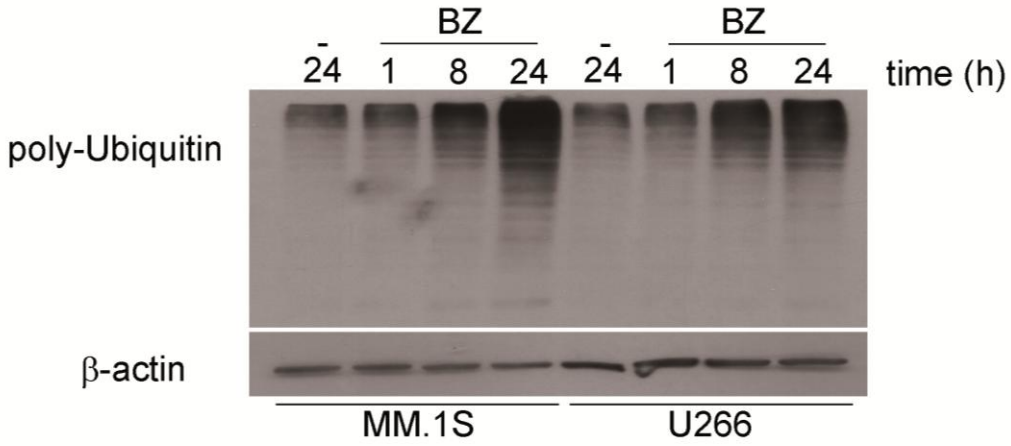


**Figure 4.1.2**

BZ induces polyubiquitin accumulation in both sensitive and insensitive cells. MM cells were treated with 20 nM BZ for 1, 8 and 24 hours. Total protein extracts were analyzed in WB to test for polyubiquitin accumulation. Data were normalized with anti-β-actin.

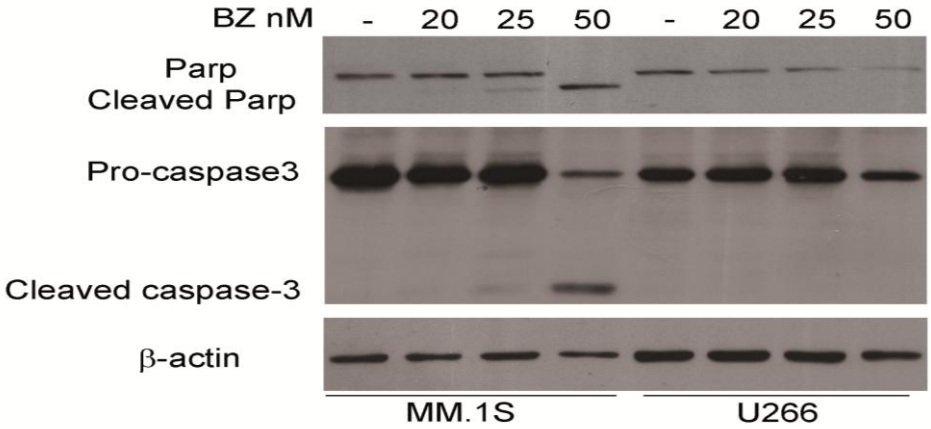


**Figure 4.1.3**

Apoptosis is activated only in MM.1S cells. MM cells were treated as indicated for 24 hours. Total protein extracts were analyzed by WB for anti-caspase 3 and anti PARP antibodies.

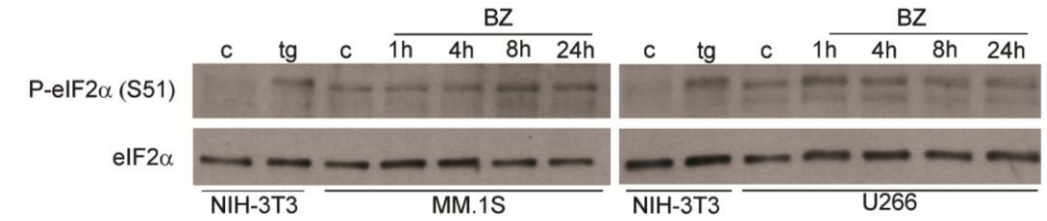


**Figure 4.1.4**

Constitutive eIF2α phosphorylation is only transiently affected by BZ treatment both in sensitive MM.1S cells and resistant U266 cells. MM cells were treated with 20 nM BZ for indicated times. Thapsigargin (tg) treatment in NIH-3T3 cells was used as a positive control for eIF2α phosphorylation. Data were normalized for the total amount of eIF2α.

## 4.2. Bortezomib treatment induces 4E-BP1 dephosphorylation.

The mTOR signalling pathway plays a key role in regulating mRNA translation, cell growth and cell cycle progression. In order to assess whether the proteasome inhibitor bortezomib was able to affect this pathway, we assessed its effect on two well-characterized downstream components of mTOR signalling, p70 S6 kinase and 4E-BP1. The p70 ribosomal S6 kinases, S6K1 and S6K2, induce rpS6 C terminus phosphorylation in response to insulin, serum, and amino acids stimulation S6K1 and S6K2 phosphorylate Ser-240 and Ser-244 (Meyuhas 2008). rpS6 phosphorylation is also regulated by the RAS/ERK pathway through the activation of p90 ribosomal S6K kinases. RSK1 and RSK2, phosphorylate rpS6 on Ser-235 and Ser-236 in response to phorbol ester, serum, and oncogenic RAS (Roux, Shahbazian et al. 2007).

We analyzed the BZ effect on the downstream targets of mTORC1 (4E-BP1 and rpS6) in both MM.1S and U266 cell lines. We analyzed the phosphorylation status of 4E-BP1 and rpS6. In untreated MM.1S and U266, S6 and 4E-BP1 were phosphorylated. The BZ treatment caused, only in MM.1S cells, 4E-BP1 dephosphorylation (figure 4.2.1), while the phosphorylation of S6 on Ser (240/244) and Ser (235/236) was not affected by BZ in both cell lines (figure 4.2.1). Our data indicate that while 24h BZ treatment affects 4E-BP1 phosphorylation, S6 phosphorylation is not compromised by BZ. We investigated whether 4E-BP1 dephosphorylation was due to mTORC1 activity impairment. We performed mTORC1 kinase assay (figure 4.2.2). We immunoprecipitated mTORC1 complex from untreated and BZ treated MM.1S and analyzed mTORC1 capability to phosphorylate 4E-BP1 synthetic substrate. As negative control of mTOR activity PP242 drug was used in order to inhibit mTORC compex 1

and 2 activity. We found that BZ does not reduce in vitro mTORC1 activity, at least in vitro.

Since 4E-BP is dephosphorylated upon BZ treatment in MM.1S, we analyzed whether BZ treatment causes an enrichment of 4E-BP1 bound to eIF4E. We evaluated cap complex assembly of initiation factors in both MM.1S and U266 treated with BZ. Data in figure 4.2.3 show that BZ treatment causes an enrichment of 4E-BP1 bound to eIF4E at 24 hours treatment only in MM.1S. In agreement with the observed absence of effects of BZ treatment on 4E-BP1 phosphorylation in U266 cells, BZ did not affect 4E-BP1 binding to eIF4E in the same context (figure 4.2.3).

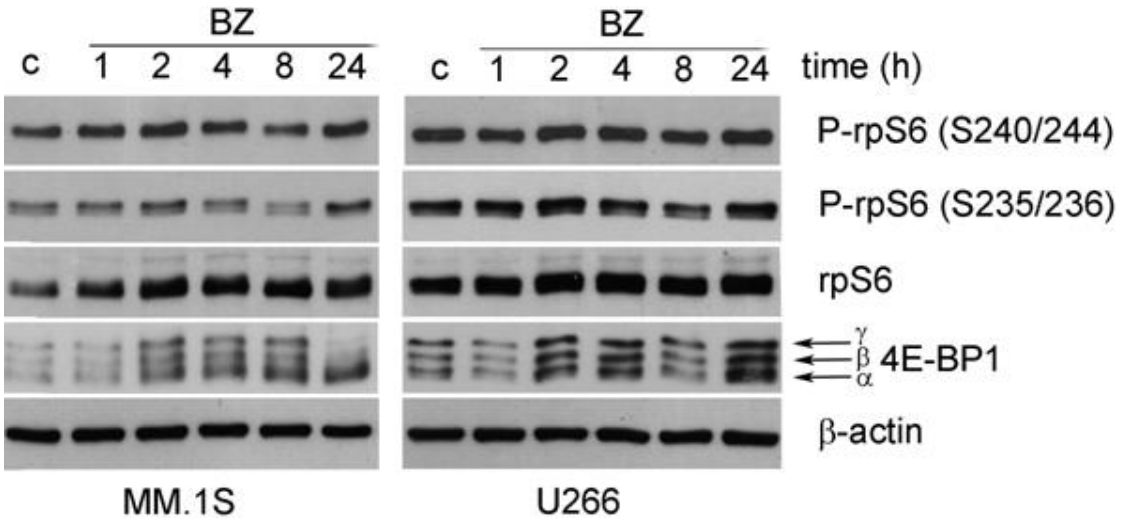


**Figure 4.2.1**

MM cell lines were treated with 20 nM of BZ for indicated times. The samples were subjected to SDS-PAGE and Western blotting to analyze 4E-BP1 isoforms and S6 phosphorylation.

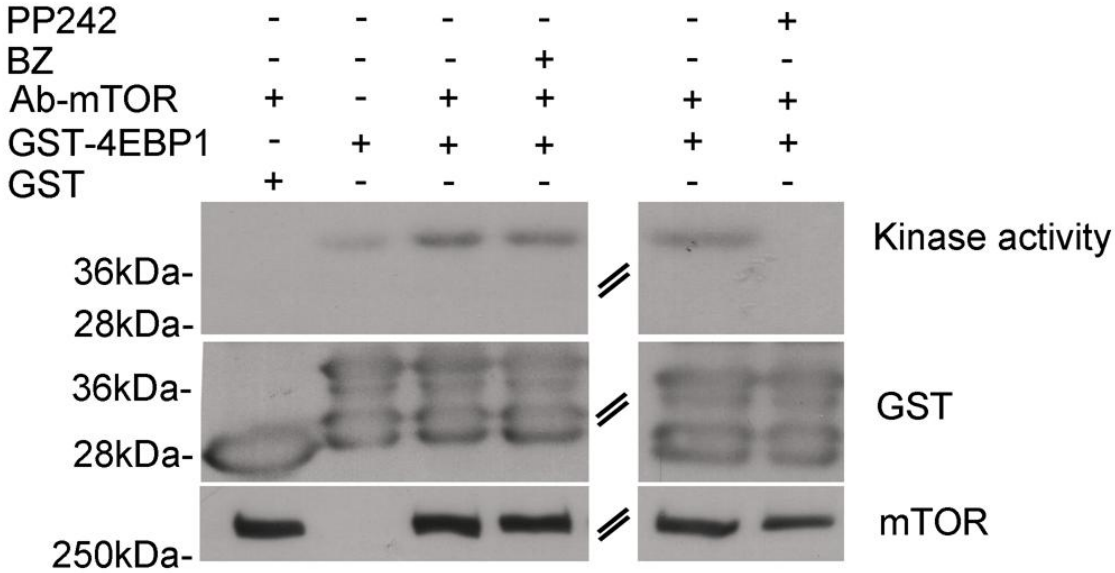


**Figure 4.2.2**

MM.1S cells were exposed to 20 nM BZ for 24 hours. mTOR,immunoprecipitated with anti-mTOR antibody, was analyzed for kinase activity with γ-32P ATP. GST-4E-BP1 was used as substrate and GST as negative control of kinase assay *(left panel)* Immunoprecipitated mTOR from MM.1S cellular extracts was preincubated with 1,5 μM PP242 for 30 min prior to the kinase reaction. PP242 is a negatve control of mTOR Kinase activity *(right panel).*

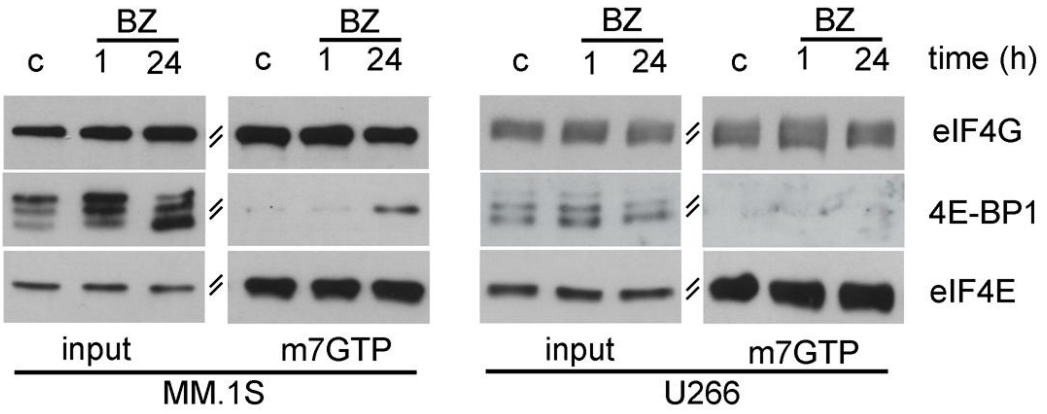


**Figure 4.2.3**

BZ treatment induces 4E-BP1 binding to eIF4E in MM1S treated with BZ for 24 hours. MM cells were treated as indicated and total proteins were incubated with 7-Methyl GTP-Sepharose beads. Input is 10% of the purification. Cap binding proteins were analyzed by WB with anti 4E-BP1 and eIF4G. eIF4E shows equal amount of purified proteins.

## 4.3. Bortezomib treatment induces attenuation of cap dependent translation.

The experiments described in figure 4.2.1, showed that BZ treatment inhibited 4E-BP1 phosphorylation in MM.1S and that this correlated with an impairment of cap complex formation. A readout of the inhibition of cap complex assembly is the reduction of protein synthesis. We measured whether BZ treatment was able to induce a reduction of translational rate. Global protein synthesis was measured by methionine incorporation in MM.1S and U266 cells, treated with BZ. Short term, BZ treatment did not affect global translation. In contrast, long term BZ treatment induced only in MM.1S cells a reduction of translation rate (figure 4.3.1).

To better address the effect of BZ on translation, polysomal profile were performed in both cell lines. This technique allows to separate mRNAs bound to polyribosomes from mRNA bound to monosome (40S, 60S and 80S) using sucrose gradient density centrifugation. BZ treatment caused 80S ribosome subunit accumulation and polysome reduction only in MM.1S BZ treated cells. Polysome distribution in U266 was not affected by BZ (figure 4.3.2). These data suggest that translational load does not increase BZ toxicity, but rather that attenuation of cap dependent translation exacerbates BZ toxicity.

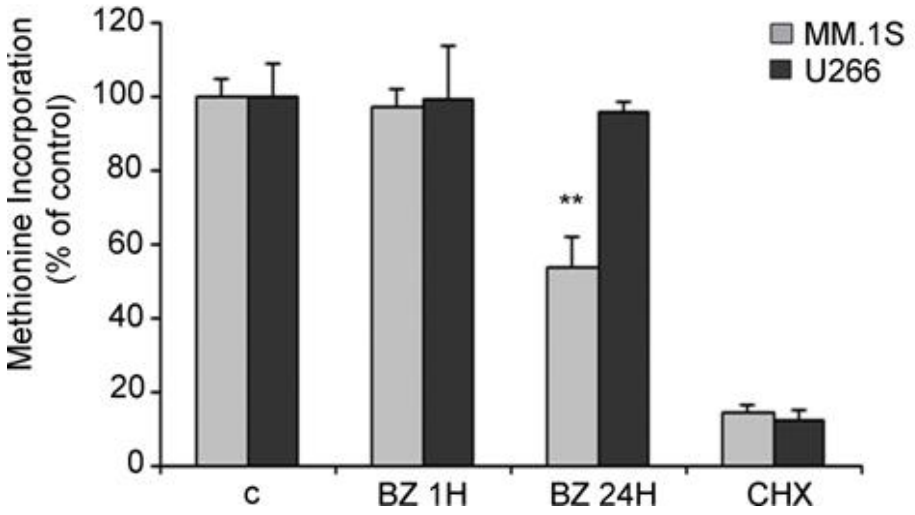


**Figure 4.3.1**

MM cells were treated with 20 nM BZ for indicated times and pulsed with $^{35}$S Methionine. Methionine incorporation was normalized on proteins. Cycloheximide (CHX) is a negative control of translational elongation. Statistical significance was assessed by Student's t-test **P < 0,01.

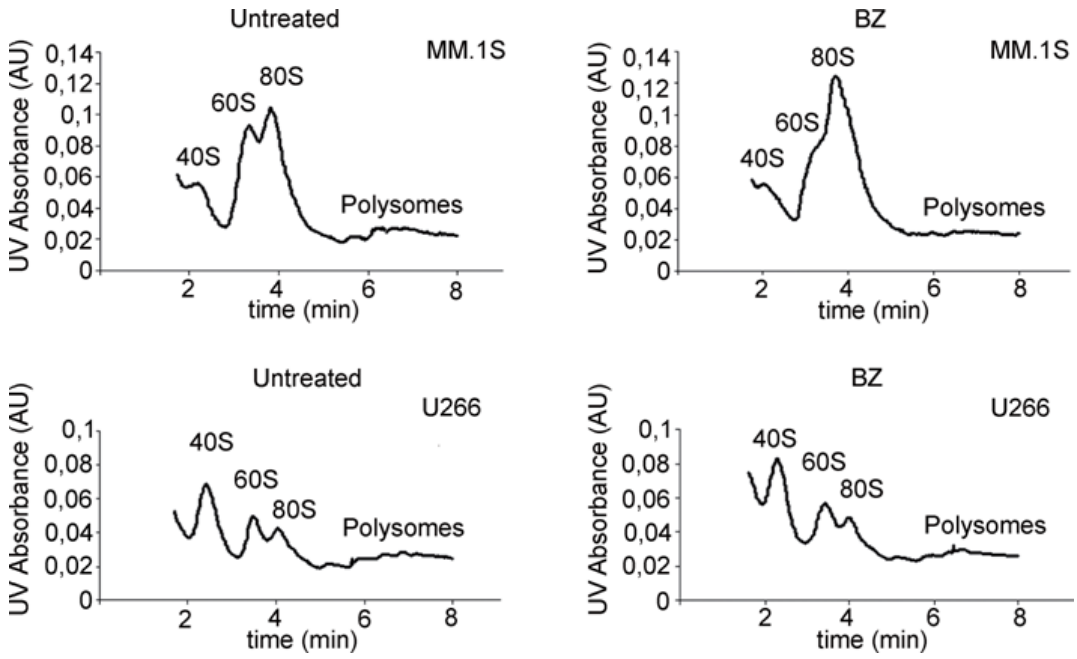


**Figure 4.3.2**

Polysome profile of MM.1S and U266 treated with BZ 20 nM for 24 hours. BZ treated MM.1S cells have reduced polysomal peaks and augmented 80S. BZ does not affect polysome distribution in U266 cells.

.

## 4.4. Dephosphorylated 4E-BP1 enhances bortezomib toxicity

The data obtained, demonstrated that MM.1S cells have dephosphorylated 4E-BP1 upon BZ treatment, while 4E-BP1 remains phosphorylated in BZ insensitive U266. We infected U266 cells with retrovirus expressing non-phosphorylable 4E-BP1 (4Ala) or empty control. We evaluated the efficiency of infection by Western blotting (figure 4.4.1). Next, we observed the viability of infected cells with the MTT assay. We found that 4E-BP1-4Ala infected cells are, significantly, more sensitive to 48h BZ treatment (figure 4.4.1).

Taken together these data demonstrate that dephosphorylated 4E-BP1 worsen BZ toxicity and 4E-BP1 targeting can block myeloma growth.

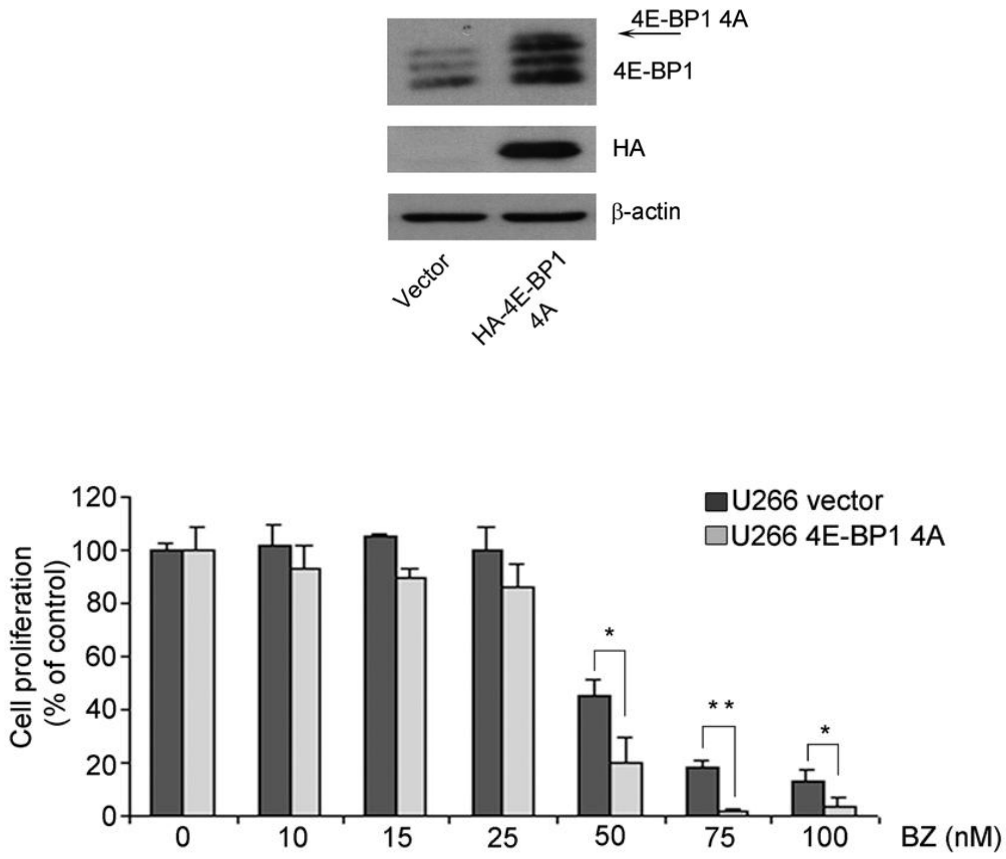


**Figure 4.4.1**

U266 cells expressing empty vector and HA-4E-BP1(4Ala) were immunoblotted with anti-HA and anti-4E-BP1 antibodies. The HA-4E-BP1(4Ala) construct is over-expressed *(top panel)*. The transduced cells were treated with increasing concentrations of BZ for 48 hours. MTT assay was performed *(bottom panel)*. The viability of untreated cells was set as 100%. Statistical significance was assessed by Student's t-test * $P < 0,05$ , **$P < 0,01$.

## 4.5. mTOR inhibition delays MM growth independently from bortezomib resistance

The data obtained suggest that the pharmacological inhibition of 4E-BP1 may act synergistically to BZ and/or be of value in treatment of MM. 4E-BP phosphorylation is inhibited by mTORC1 inhibitors including rapamycin and related rapalogs, such as temsirolimus and everolimus (Fingar and Blenis 2004). Moreover a new generation of mTOR inhibitors (such as PP242) have been developed, these compounds specifically inhibit the mTOR kinase domain and suppress both TORC2 as well as TORC1 activity, preventing AKT phosphorylation (Feldman, Apsel et al. 2009).We analyzed the effect of either rapamacyn or PP242 on myeloma growth (figure 4.5.1).

MM.1S and U266 were treated with growing concentrations of the PP242 and rapamycin for 24 and 48 hours. MTT analysis was performed. PP242 was more effective than rapamycin in achieving cytoreduction in multiple myeloma cell lines. In addition the rapamicyn cytotoxicity dose response was rather flat, reaching an early plateau in efficacy at 40% of inhibition. These data suggest the superior anti-tumor effect of PP242 versus rapamycin.

By inhibiting mTORC1 and mTORC2 a pro-survival feedback loop is activated by MAPK signalling (Wan, Harkavy et al. 2007; Carracedo, Ma et al. 2008). The ERK activation may provide some protection against the cytotoxicity induced by rapamycin and PP242. We investigated whether, BZ was able to stimulate ERK phosphorylation. Next we investigated the effect of ERK inhibition on BZ treated cells. We found that BZ treatment induces ERK phosphorylation in MM.1S. Importantly, ERK inhibition, in conditions of BZ treatment, does not worsen BZ-mediated toxicity (figure 4.5.2). These results provide a rational for further analysis of mTOR inhibitors in myeloma cells.

Next, we analyzed the effect of either mTORC1 inactivation by rapamycin or mTOR pharmacological blockade by PP242, singly or in combination with BZ. We found that the effects of PP242 and rapamycin were independent from the one of BZ (figure 4.5.3). Early trials with rapalogues have shown a limited response in myeloma cells (Farag, Zhang et al. 2009; Ghobrial, Weller et al. 2011). Thus, we examined the effects of PP242 on the growth of primary myeloma cells derived from patients (figure 4.5.4). Since the interaction between stromal cells and myeloma cells is critical to their survival, the effect of mTORC blockers was examined on cocultured tumor and stromal cells. Of five patients cells tested, one was found to partly respond to PP242, in conditions of BZ resistance. All together, these data suggest that a subset of patients may respond to mTORC inhibition.

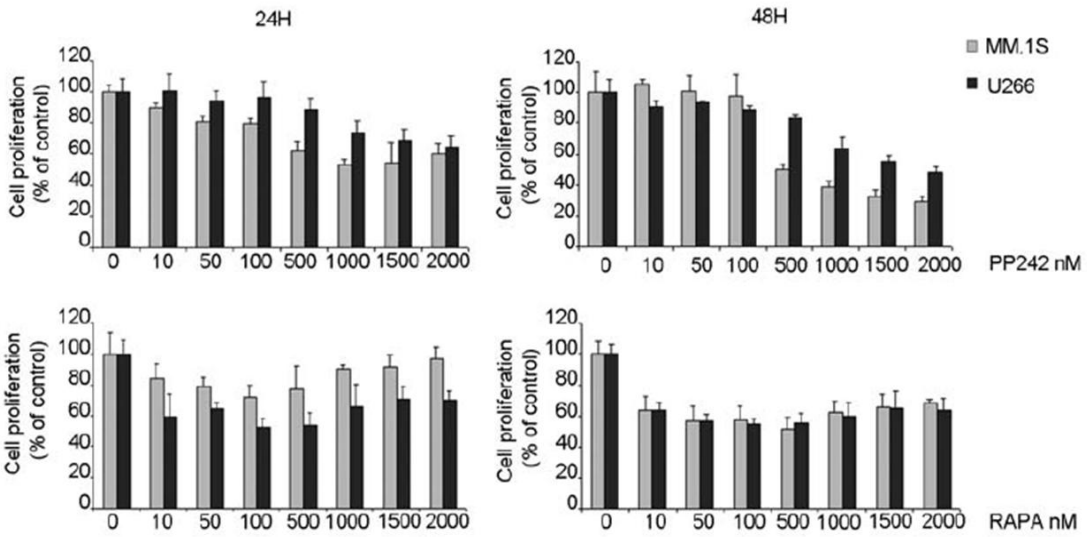


**Figure 4.5.1**

MTT assay was performed on MM.1S and U266 treated with growing concentrations of PP242 and Rapamycin for 24 and 48 hours. Data are presented as a percentage of the control, in which cells were treated with 0,02% (v/v) DMSO.

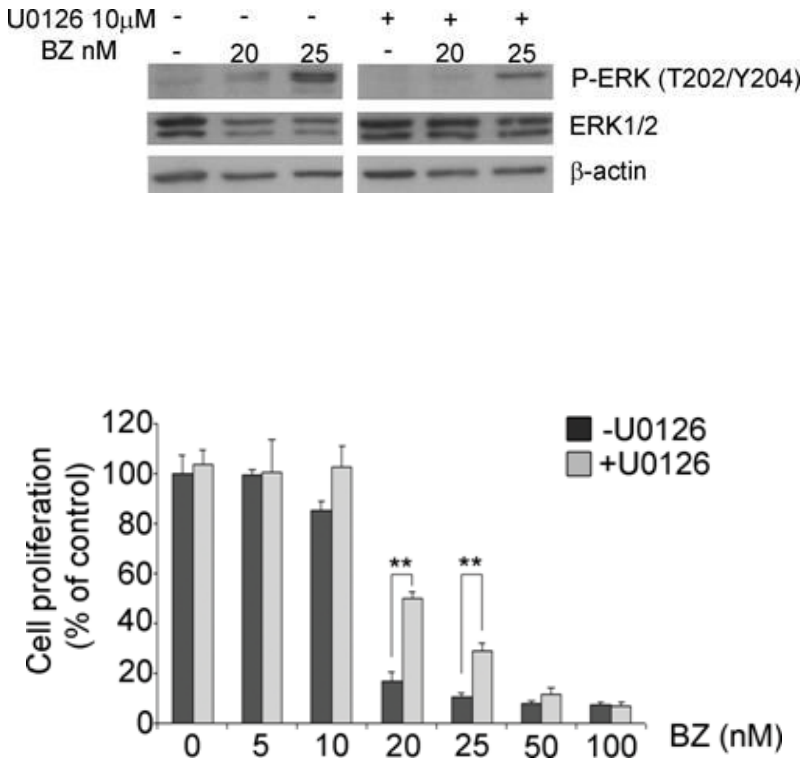


**Figure 4.5.2**

MM.1S cells were treated with BZ and ERK inhibitor, U0126, alone and in combination for 24 hours. The U0126 effect on ERK dephosphorylation was verified by Western blot for anti-P-ERK antibody. MTT assay on MM.1S treated with U0126 10 μM and with increasing concentration of BZ for 24 hours. Data are presented as a percentage of the control. Statistical significance was assessed by Student's t-test **P < 0,01.

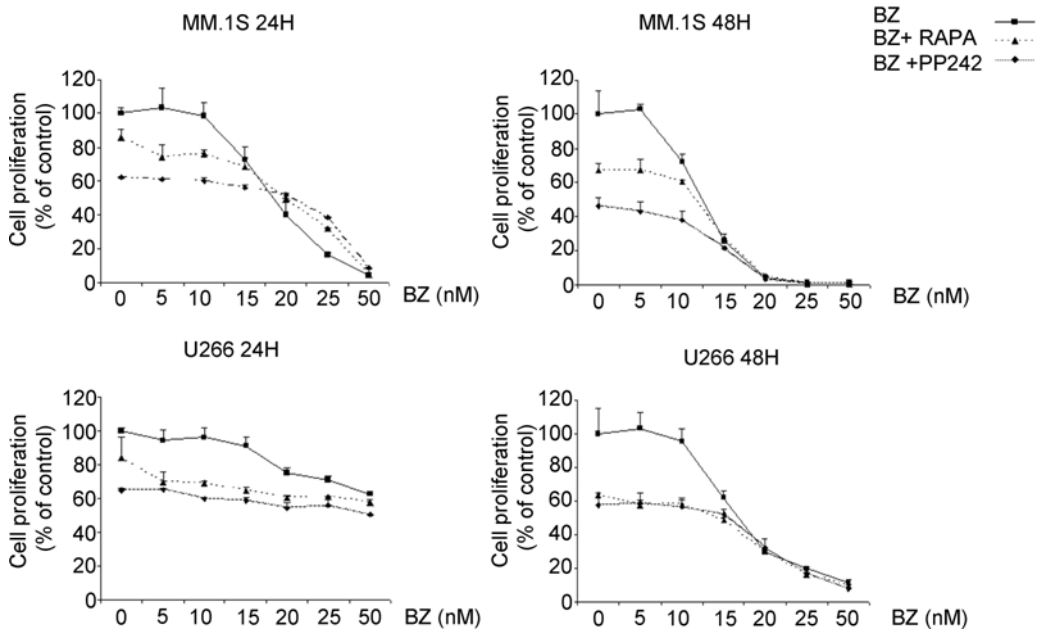


**Figure 4.5.3**

Growth inhibition induced by BZ alone and in combination with mTOR inhibitors. MM.1S and U266 were treated for 24 and 48 hours with increasing concentrations of BZ alone and in the presence of Rapamycin (10 nM) or PP242 (500 nM). MTT assay was subsequently performed. Data are presented as a percentage of the control, in which cells were treated with 0,02% (v/v) DMSO.

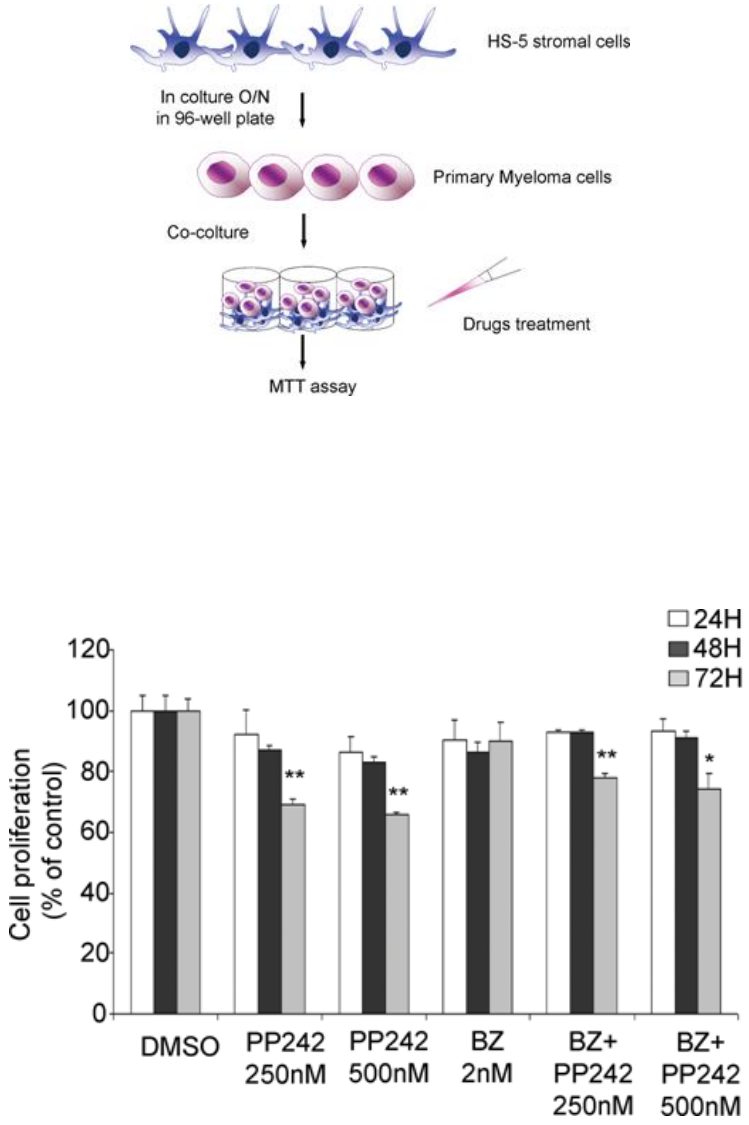


**Figure 4.5.4**

HS-5 stromal cells were plated and incubated O/N. Isolated primary MM cells from a patient were cultured on HS-5 stromal cells layer. The co-culture was treated with 2 nM BZ, 250 nM, 500 nM of PP242. 2 nM BZ was combined with 250 nM PP242 and 500 nM PP242. The co-culture was incubated for 24, 48 and 72 hours. MTT assay was performed. Data are presented as percentage of control treated with 0,02% (v/v) DMSO. Statistical significance was assessed by Student's t-test * $P < 0,05$ , **$P < 0,01$.

## 4.6. Variable levels of eIF4E and 4E-BP1/2 in myeloma patients

The data obtained indicate that a subset of patients resistant to BZ may respond to pharmacological inhibition of mTORC. It has been demonstrated that determinants of sensitivity to mTORC1 inhibition are mutation in PI3K-RAS pathway; in particular PTEN mutations are associated with sensitivity to rapamicyn, while RAS mutation are associated to rapamycin resistance (Di Nicolantonio, Arena et al. 2010).

It has also been demonstrated that higher levels of 4E-BP1 and low levels of eIF4E are related to rapamycin sensitivity (Grosso, Pesce et al. 2011). The ratio of the translational regulatory factors eIF4E and 4E-BP, rather than their individual levels may therefore serve as a marker to predict the clinical therapeutic response to mTOR inhibitors (Alain, Morita et al. 2012). Since, we found that 4E-BP inhibition increases BZ toxicity rather than decrease it (figure 4.4.1) and that 1/5 of patient cells are sensitive to rapalogs (figure 4.5.4), we analyzed in 122 myeloma patients the transcriptional levels of 4E-BP1, 4E-BP2 and EIF4E. The 4E-BP1/EIF4E, 4E-BP2/EIF4E ratio were calculated for each patient. Data show that approximately 15% of patients have higher relative levels of 4E-BP1 and 4E-BP2 (figure 4.6.1). We suggest that these patients should have benefit of pharmacologically mTOR inhibition.

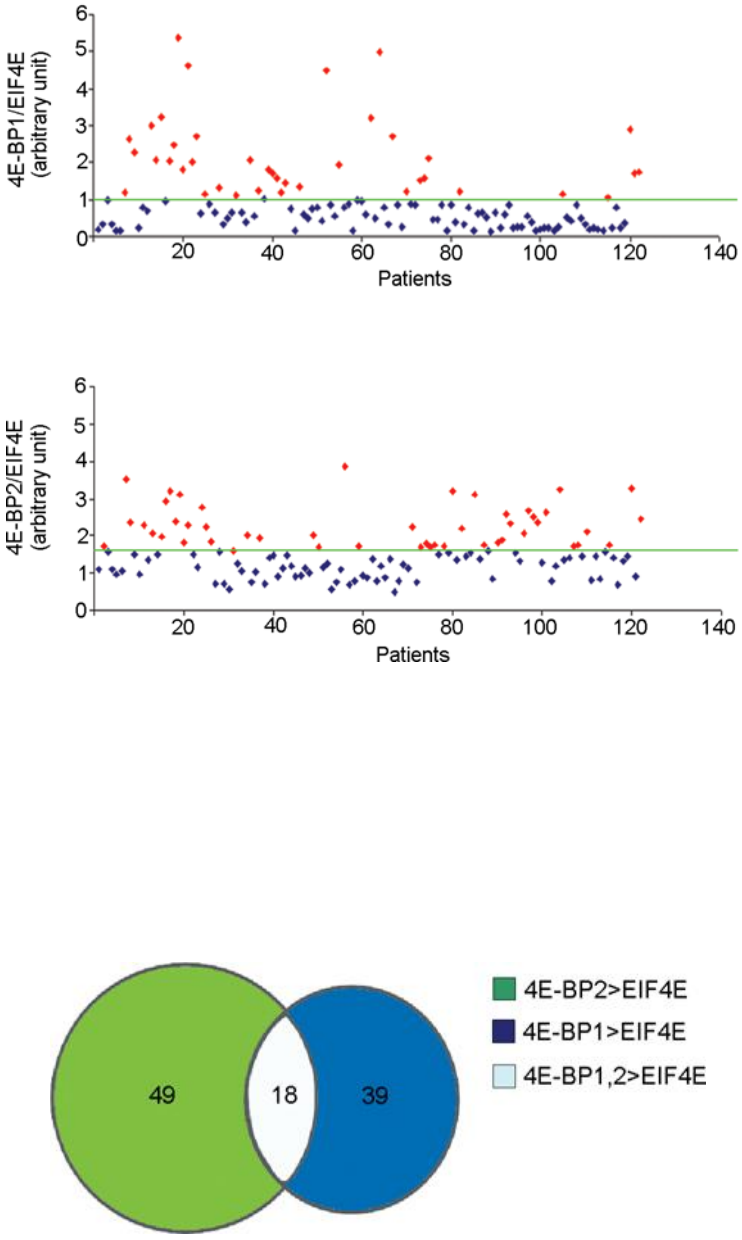


**Figure 4.6.1**

Transcriptional levels of 4E-BP1, 4E-BP2 and eIF4E were evaluated in 122 Multiple Myeloma patients at the onset of disease. The 4E-BP1/EIF4E, 4E-BP2/EIF4E ratio were calculated for each patient. The average ratio was imposed as threshold. In both graphs the red dots represent the patients who have 4E-BP1/EIF4E or 4E-BP2/EIF4E ratio higher than the threshold *(top panel)*. The chart indicates that 39 patients have 4E-BP1 levels higher than eIF4E, 49 patients have 4E-BP2 levels higher than EIF4E and 18 patients have both 4E-BP1, 4E-BP2 mRNA levels higher than EIF4E *(bottom panel)*.

# 5. DISCUSSION

On the basis of available literature, we hypothesized that BZ induced an UPR response in myeloma cells, allowing us to identify eIF2α phosphorylation and uORF mRNAs critical for survival and resistance of cells (Obeng, Carlson et al. 2006; Ling, Lau et al. 2012; Vincenz, Jager et al. 2013). To address the problem, we set up conditions that allowed us to discriminate between BZ-induced toxicity and survival. In these conditions, we did not find evidence for eIF2α phosphorylation in BZ-induced lethality. This result is in apparent contrast with previous reports that analyzed the UPR in MM cells (Schewe and Aguirre-Ghiso 2009; Ling, Lau et al. 2012). Of note, here we focused on low BZ concentrations able to induce cell death, thus ruling out a direct relationship between eIF2α phosphorylation and toxicity.

Several groups have tried to explain the molecular bases of different individual responsiveness to bortezomib, exploiting human MM lines characterized by differential sensitivity (Bianchi, Oliva et al. 2009; Campanella, Santambrogio et al. 2013). Nevertheless, the molecular basis of sensitivity or resistance to BZ among patients, remain largely unknown. We note that, MM1S cells have only 2 fold higher sensitivity than U266 to BZ. In the case of other drugs, such as for instance rapamacyn, sensitive cells show about 100 fold difference versus resistant cells in term of response to the drug (Di Nicolantonio, Arena et al. 2010). Sensitivity/ resistance to BZ seems more a clinical concept than a genetically driven clear-cut difference. This said, U266 cells are considered by all means a good paradigm of BZ-resistant myeloma cells, whereas MM1.S are considered sensitive.

We found that, as in other tumor models, myeloma cells exhibit a sensitivity to translational inhibition, and a prosurvival activity of the mTORC1-eIF4E axis. Similarly to other tumor cell types, inhibition of mTORC1 may be beneficial to therapy (Ramirez-Fort, Case et al. 2014). Mechanistic evidence demonstrates that the cytostatic effects of

rapamycin, an highly specific mTORC1 inhibitor, is due to inhibition of eIF4F formation through dephosphorylation of the eIF4E repressor 4E-BPs (Sonenberg and Hinnebusch 2009). Indeed, either decrease of eIF4E or increase of 4E-BP1 can bypass mTORC1 inhibition in vivo. Conversely, eIF4E increase or 4E-BP1 downregulation overcome rapamycin inhibition (Grosso, Pesce et al. 2011). We found that when BZ represses 4E-BP phosphorylation, in vivo, overexpression of not phosphorylable 4E-BP1 worsens BZ toxicity. We also found an unexpected effect of ERK inhibition on BZ-treated cells. Rapamycin induces a feedback loop that activates ERK and can lead to resistance in other cancer cell types (Carracedo, Ma et al. 2008). However, in MM ERK inhibition does not increase BZ-induced toxicity, but reduces it.

One unexpected observation is that BZ induces 4E-BP1 dephoshorylation, but not rpS6 dephoshorylation as rapamycin does. However, it is intriguing that kinase activity assays suggest that mTOR activity is not affected directly by BZ treatment. Thus, the dephosphorylation of 4E-BP1 may be due to specific phosphatase activities stimulated by BZ or other mechanisms such as mTOR and 4E-BP1 delocalization. Alternatively, BZ may induce variations in steady state levels of adaptors regulating mTORC1 specificity, in vivo. Future work is needed.

We show that both mTORC1 and mTOR inhibition are effective in myeloma cell lines. Moreover, as shown in our and another study, the mTOR inhibitor PP242 has demonstrated efficacy against primary MM cells (Hoang, Frost et al. 2010). The major effect of PP242 on tumour cells is the inhibition of cell proliferation (Feldman, Apsel et al. 2009). Primary myeloma cells grow poorly outside their bone marrow microenvironment. In spite of different conditions of culture (various cytokine combinations, various stroma substrates), primary MM cells cultured in vitro display a decline in growth and proliferation within three days of culture (Zlei, Egert et al. 2007).

This aspect limits the measurement of drug sensitivity in primary myeloma cells, especially for cytostatic agents like for PP242. Therefore, the inhibitory effect of PP242, in vitro in our conditions, is underestimated.

Which would be the best way to employ mTORC1 inhibitors? In this sense, in agreement with a recent study, we found that blockade of mTOR is not synergistic with BZ treatment (Maiso, Liu et al. 2011). Thus, mTOR inhibition may be useful in patients resistant to BZ. Data from this and other work would however suggest that patients that would benefit most from mTOR inhibition are without RAS mutations, (Di Nicolantonio, Arena et al. 2010; Steinbrunn, Stuhmer et al. 2011) and have high levels of 4E-BP, low eIF4E (Grosso, Pesce et al. 2011; Alain, Morita et al. 2012). These patients represents a subset of the whole MM population: it might be thus mandatory to identify them, before treatment.

As a final remark, recent genome sequencing has unveiled new somatic mutations in myeloma cancer cells. Among them, it is curious to note that several of them are on factors associated with translational control, and that at least 50% patients have one mutation in one gene involved in protein synthesis (Chapman, Lawrence et al. 2011). Specifically, two genes FAM46C and DIS3, were found recurrently mutated in multiple myeloma patients (Chapman, Lawrence et al. 2011; Lohr, Stojanov et al. 2014). These mutations were accompanied by loss of heterozygosity and correlated to poor prognosis (Boyd, Ross et al. 2011). The high rate of inactivating mutations accompanied by loss of heterozygosity indicates that FAM46C and DIS3 are a *bona fide* tumor suppressor genes. DIS3 is a ribonuclease involved in RNA processing. DIS3 is a component of the exosome complex which regulates the mRNAs degradation and in turn regulates the pool of mRNAs available for translation (Dziembowski, Lorentzen et al. 2007). Although, the function of FAM46C is largely unknown, it has been demonstrated that

the expression of FAM46C is highly correlated to the expression of the set of ribosomal proteins, eukaryotic initiation and elongation factors, involved in protein translation (Chapman, Lawrence et al. 2011). This finding suggests that FAM46C should be functionally related in some way to the regulation of translation. Sequence analyses of FAM46C protein indicates that FAM46C sequence contains conserved active site residues present in all members of the large family of nucleotidyltransferase. This indicates that FAM46C may retain a nucleotidyl transferase activity (Kuchta, Knizewski et al. 2009). It has also been proposed that FAM46C can function as mRNA stability factor. Although the molecular mechanism, and targets of DIS3 and FAM46C are still unknown, it seems clear their involvement in the regulation of protein synthesis.

These data, together with our observations, may suggest that the translational machinery will be an attractive target for therapy in myeloma cells.

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