UNIVERSITA' DEGLI STUDI DI PARMA

Dottorato di Ricerca in Fisiopatologia Renale e Malattie Osteometaboliche

Ciclo XXVII

Convenzione SPINNER 2013

Ciclo XXVII

PROTEASOME INHIBITORS BLUNT MYELOMA INDUCED OSTEOCYTE DEATH TARGETING AUTOPHAGY BOTH IN VITRO AND IN VIVO IN MULTIPLE MYELOMA PATIENTS

Coordinatore:
Chiar.mo Prof. Landino Allegri

Tutor:
Chiar.mo Prof. Nicola Giuliani

Dottoranda: Denise Toscani

PhD researches of Dr. Denise Toscani were supported by a 3 years fellowship of Consorzio SPINNER, Regione Emilia Romagna, and were conducted in the Department of Clinical and Experimental Medicine of the University of Parma.

"A cento anni ho perso un po' la vista, molto l'udito.
Alle conferenze non vedo le proiezioni e non sento bene.
Ma penso più adesso di quando avevo vent'anni.
Il corpo faccia quello che vuole. Io non sono il corpo:
io sono la mente"

Rita Levi-Montalcini

ABSTRACT

Multiple myeloma (MM) is characterized by severe bone destruction. Recently it has been shown that an increased osteocyte death is involved in MM-induced osteolysis. In this study we show that autophagy rather than apoptosis is involved in MM-induced osteocyte death and that proteasome inhibitors (PI)s, a class of drugs known to stimulate osteoblast formation, significantly blunted MM-induced osteocyte and pre-osteocyte death. PIs also reduced osteocyte death induced by high dose of dexamethasone (DEX) and potentiated the anabolic effect of PTH(1-34). PIs, such as Bortezomib (BOR), are able to block the autophagic process in osteocytes and pre-osteocytes, as shown by an increased of the level of the autophagic marker LC3 and a decreased of p62. Moreover, both MM-induced and DEX-induced autophagic cell death were blunted by PIs. Transmission electron microscopy and confocal microscopy evaluation confirmed observations. These data were further expanded in vivo by a histological analysis of osteocyte viability on bone biopsies in MM patients. A significant increase in the number of viable osteocytes was demonstrated in patients treated with Bortezomib (BOR)-based regimen as compared to those treated with other regimen. Our data identify osteocyte autophagy as a potential target in MM bone disease and support the use of PIs to increase osteocyte viability and improve bone integrity in MM patients.

RIASSUNTO

Il Mieloma Multiplo (MM) è caratterizzato da grave distruzione ossea. Recentemente, è stato mostrato il coinvolgimento della morte osteocitaria nell'osteolisi indotta da MM. In questo studio noi mostriamo come l'autofagia, piuttosto che l'apoptosi, sia coinvolta nella morte osteocitaria e che gli inibitori del proteasoma (PI)s, una classe di farmaci nota per stimolare la formazione osteoblastica, riduce la morte osteocitaria e pre-osteocitaria indotta da MM. Gli inibitori del proteasoma, tra cui Bortezomib (BOR), riducono la morte osteocitaria indotta da alte dosi di desametasone (DEX) e potenziano gli effetti anabolici del PTH(1-34). In particolare il Bortezomib, è in grado di bloccare il processo autofagico, come mostrato dagli aumentati livelli del marker autofagico LC3 e dalla riduzione del marker p62. Inoltre, il trattamento con BOR è in grado di bloccare la morte autofagica indotta da MM e da DEX. Questi dati sono stati confermati da valutazioni di microscopia elettronica a trasmissione e microscopia confocale. Questi dati sono stati valutati in vivo con l'analisi istologica della vitalità osteocitaria su biopsie osse dei pazienti MM. E' stato dimostrato un aumento significativo del numero di osteociti vitali nei pazienti trattati con regimi terapeutici basati su BOR rispetto a pazienti trattati con altri regimi terapeutici. I nostri dati mostrano come l'autofagia può essere un potenziale target nella malattia ossea del MM supportando l'uso degli inibitori del proteasoma per migliorare la vitalità osteocitaria e l'integrità ossea nei pazienti con MM.

TABLE OF CONTENTS

1. INTRODUCTION

Multiple myeloma (MM) is a plasma cell malignancy characterized by severely unbalanced and uncoupled bone remodeling in the bone marrow (BM) area of plasma cell infiltration due to increased osteoclast (OCL) formation and activity, and osteoblast (OB) suppression, leading to osteolysis.[1,2] Osteocytes are terminally differentiated cells derived from osteoblasts and involved in bone remodeling through cell death that up-regulates OCL recruitment and by secreting sclerostin (SOST) an inhibitor of bone formation.[3-8] An increased osteocyte cell death is related to pathophysiological conditions such as osteoporosis caused by estrogen deficiency or glucocorticoid administration[9-12] that led to a reduction of bone mineral density with a prolonged lifespan of OCLs.[13-15] On the other hand, intermittent PTH(1-34) administration to mice, or short-term exposure to PTH(1-34), prolonged osteocyte survival and it is also associated with decreased OB apoptosis.[16] Thalidomide and its analogues, bisphosphonates (BPs) and proteasome inhibitors are the principal drugs currently used in the treatment of MM patients.[17] Thalidomide has antiproliferative effects on MM cells, but also affect their interaction with stromal cells, angiogenesis and modulation of immune response.[17] BPs including zoledronic acid (ZOL), are a class of nonhydrolyzable analogs of pyrophosphate used in MM patients to block osteoclast formation.[17-19] The principal mechanism of BPs in reducing bone resorption is the induction of osteoclast apoptosis but, in vitro studies, demonstrated a proapoptotic effect on myeloma cells, by inhibiting the release of growth factor from osteoclast and bone marrow stromal cells.[17] Beside these effects, several works demonstrated their capacity to inhibit osteocyte death and apoptosis.[18-20]. Other than apoptosis, recent data suggest that osteocytes can undergo a tightly regulated process of self-degradation called autophagy.[21] Autophagy is well known as a protective pro-survival mechanism from oxidative stress stimuli or aging[22-23], however under stronger stress, autophagy can be an unregulated self-destructive process leading to cell death independently to apoptosis.[4,21-22] In line with this concept it has been recently reported that dexamethasone (DEX) treatment both in vitro and in vivo

increases the expression of the autophagic marker LC3 in osteocytes[24-25] suggesting that an increased autophagic death is involved in bone loss in patients receiving glucocorticoid therapy as MM patients. Autophagy is controlled by a series of evolutionarily conserved genes (ATG genes) and begins with the formation of vesicle, called autophagosomes, that contains part of cytoplasm and organelles. Subsequently they fuses with the lysosome to form autolysosome and the components are degraded by lysosomal enzymes.[21,22] Different protein are involved in this process such as Light chain3-II (LC3-II) during the formation of autophagosome, and p62/sequestosome 2 (p62/SQTM1) that decreased when autophagy is completed.[21,22] The role of autophagy in programmed cell death are still controversial. Several studies have shown that autophagy can contribute to cell death in the absence of intact apoptosis pathway [26-31]. Indeed, the authors showed that the presence of autophagic inhibitors or reduced ATG gene expression can prevent some type of cell death. [26,28] Moreover, in cells competent for apoptosis, high levels of autophagy can lead to autophagy caspase-independent cell death, such as in cells expressing the short isoform of p19[ARF 32],or in cells exposed to a different environmental stresses and toxic agents.[33] Recently we demonstrated that the number of viable osteocytes was significantly reduced in MM patients in relationship with the presence of bone lesions.[34] These observations were also confirmed by ultra-structural in vitro analysis on osteocytes co-cultured with MM cells showing that MM cells increase osteocyte death.[34] Among the new anti-MM drugs actually used in the treatment of MM the proteasome inhibitors (PIs) including Bortezomib (BOR) affect bone remodeling, stimulating osteoblast differentiation in both mouse and human systems.[35-36] Moreover, recent studies showed a significant level increase of markers of osteoblast activity, such as alkaline phosphatase and osteocalcin, in MM patients treated with BOR.[37-40] The proteasome is a multi-catalytic enzyme complex involved in intracellular proteolysis ensuring the rapid degradation of protein targeted with a chain of ubiquitin.[41,42] Proteasome inhibitors fall into three important class: peptide aldehydes, peptide boronates, and nonpeptide inhibitors.[41] Peptide aldehydes such as MG-132 potently and reversibly, block the chymotrypsin-like activity but also lysosomal cysteine and serine proteases.[41] Bortezomib (BOR), such

as MG 262, is a dipeptide boronic acid analog, more potent than peptide aldehydes, highly selective, and reversible proteasome inhibitor that targets 26S proteasome complex. It binds reversibly to the chymotrypsin-like β5 subunit of the catalytic chamber of the 20S proteasome and inhibits its function.[17,41,42] It was largely estabilished the effects of proteasome inhibition on the stability of different cell cycle–regulatory proteins, including cyclins, cyclin-dependent kinase inhibitors (CDKIs e.g., p21 and p27), tumor suppressors (e.g., p53), and the transcription factor NF-kB.[41,42] Importantly, treatment of multiple myeloma with BOR blocks NF-kB activation, a transcription factor important for proliferation and drug resistance of MM cells, and enhances the downregulation of stromal cell IL-6 secretion, important in the growth and survival of tumor cells.[41,42] Crosstalk between autophagy and the ubiquitine-proteasome system has been reported in different cell types[42-44], to prevent the accumulation of ubiquitinated and aggregated proteins. The potential effects of PIs on osteocytes are not known and were evaluated in two different model of osteocyte death as MM-induced[34] and DEX-induced osteocyte death.[12-13,24-25] Moreover the potential role of autophagy and apoptosis in these models and the effect of PIs were investigated using both a human pre-osteocytic cell line[45] and the murine osteocytic MLO-Y4.[46] Finally to put the results into a clinical perspective we have evaluated osteocyte viability, autophagy and apoptosis in bone biopsies of MM patients underwent to different therapeutic regimen including BOR.

2. METHODS AND SUBJECTS

2.1 Drugs

BOR was purchased from Janssen-Cilag (Milan, Italy). The drug was reconstituted in water at a stock concentration of 2.6mM and diluted in the cell culture medium just before the use. MG 262 and the caspase 3 inhibitor Ac-DEVD-CHO were purchased from Enzo Life Sciences (Florence, Italy). ZOL was supplied as hydrated disodium salt from Novartis Farma (Origgio, VA, Italy). DEX was purchased from Sigma Aldrich (Milan, Italy). Human PTH (1-34), 3-methiladenine (3-MA) and Chloroquine (CQ) were purchased from Tocris Bioscience (Bristol, United Kingdom).

2.2 Cells and cell culture conditions

2.2.1 Cell lines

The human myeloma cell lines (HMCLs) JJN3 were purchased from DSMZ (Braunschweig, Germany) and RPMI 8226 from the American Type Culture Collection (Rockville, MD). The human preosteocytic cells HOB-01 were established from human bone and kindly provided by Julia Billars (Collegeville,PA). The murine osteocytic cells MLO-Y4 were kindly given by Linda Bonewald (Kansas City, MO).

2.2.2 Cell conditions and experimental procedures

Confluent HOB-01 and MLO-Y4 cells were treated in 96-well plates for 12-24-48hrs with BOR (2-5-10nM), or MG 262 (10nM) or vehicle. Confluent HOB-01 and MLO-Y4 cells were incubated in 96-well plates in the presence or the absence of 48-hrs conditioned media (CM) of HMCLs RPMI 8226 and JJN3. Then the culture media was changed and the cells were treated for 12-24-48hrs with BOR (2-5nM), or MG 262 (10nM), or ZOL (10-5-10-11M) or vehicle. Moreover, MLO-Y4 and HOB-01 were treated with DEX or vehicle (ethanol) at 10-6M for 48hrs. Then the culture media was changed and the cells were treated for 12-24-48hrs with BOR (2-5nM), or MG 262 (10nM), or

ZOL (10-5-10-11M) or vehicle. In other series of experiments MLO-Y4 were seeded in 96-well plates and incubated with DEX or vehicle 10-5M for 6 or 24hrs. Then the culture media was changed and the cells treated with PTH (1-34) (10nM) for 1hr or BOR (2nM) for 12hrs or with PTH(1-34) (1hr) followed by BOR (12hrs), or vehicle (water).

2.2.3 Co-cultures

HOB-01 and MLO-Y4 cells were seeded into uncoated and type I collagen–coated Petri dishes, respectively, and co-cultured with the HMCLs RPMI 8226 and JJN3 placed in a transwell insert for 48hrs. Then the culture media was changed and the cells were treated for 12hrs with BOR (2nM) or MG 262 (10nM), or vehicles (water and dimethyl sulfoxide, respectively). The pellets from all experiments were collected for cell viability, caspase 3 activity, western blot, immunofluorescent confocal microscopy and transmission electron microscopy.

2.2.4 Autophagy and apoptosis inhibition

MLO-Y4 were incubated in 96-well plates in the presence or the absence of 48-hrs CM of RPMI 8226, or treated with DEX or vehicle at 10-6M for 48hrs. 3-MA (0.5mM), Chloroquine (25μM), or Ac-DEVD-CHO (50nM) or vehicle (dimethyl sulfoxide and water, respectively) were added 1h before the induction of cell death and maintained for the entire period of culture.

2.2.5 Osteocyte viability

Osteocyte viability was evaluated by means of a cytotoxicity assay in accordance with the manufacturer's protocol (Cell Counting Kit-8; Alexis Biochemicals, Plymouth Meeting, PA). All experiments were performed in culture media supplemented with 5% fetal bovine serum. In the cocultures osteocyte viability was evaluated by Tripan blue

staining (Sigma Aldrich). All results are expressed as the mean value ± standard error. The mean values of the groups were compared by Student's t-test.

2.2.6 Caspase 3 activity assay

To evaluate caspase 3 activity, non-adherent and adherent cells were harvested, combined and assayed using a commercial kit (Caspase-3 Colorimetric Activity Assay kit, Millipore, Billerica, MA) following manufacturer's instruction. The quantification was performed using a spectrophotometer at 405 nm. HOB-01 treated with BMP-2 200ng/mL for 6hrs, and MLO-Y4 treated with Etoposide (ETO) 50uM for 24hrs were used as positive controls. All results are expressed as the mean value ± SE. The mean values of the groups were compared by Student's t-test.

2.3 Western blot analysis

Total and cytosolic extracts were obtained using a commercial kit (Active Motif, Carlsbad, CA). For immunoblotting the following antibodies were used: rabbit polyclonal anti-LC3 antibody (1:1000) (MBL International Corporation, Woburn, MA), mouse anti-p62 antibody (1:250) (R&D System, Minneapolis, MN) mouse monoclonal anti-SOST antibody (1:250) (R&D System, Minneapolis, MN), and mouse anti-β-actin monoclonal antibody (1:5000) (Sigma-Aldrich) as internal control. The secondary antibodies peroxidase conjugated were the anti-rabbit (1:2000) (Chemicon, Temecula, CA,) and anti-mouse (1:10.000) (BD Pharmingen, Franklin Lakes, NJ,). Chemiluminescence was detected by incubating the membranes for 5min with luminol solution (ECL Plus, GE Healthcare Amersham, Milan, Italy). The immunoreactive bands were visualized using an exposure time of minutes (Kodak XOMAT). Protein expression levels were quantified by ImageJ software and normalized by β-actin level. For LC3 we reported, as index of autophagy, the ratio between LC3II and LC3I, normalized by β-actin level. For p62 we reported the level of p62 normalized by β-actin level.

2.4 Immunofluorescence confocal microscopy

Immunofluorescence analysis was carried out to quantify expression of MAP LC3 (autophagy) and Apaf-1 (apoptosis). The MLO-Y4 cells, cultured in different conditions in multi-well chamber slides, were treated and observed as previously described.[47] The cells were incubated with the following antibodies: rabbit anti-MAP LC3, mouse anti-Apaf-1 (Santa Cruz Biotechnology, Inc. Dallas, TX); goat anti rabbit Cy3 and sheep anti-mouse FITC (Sigma-Aldrich). After washing the samples were counterstained with 1 g/ml DAPI (4',6-diamidin-2-fenilindolo) and then mounted with anti-fading medium (0.21M DABCO (1,4-diazabicyclo[2.2.2]octane) and 90% glycerol in 0.02M Tris (tris(hydroxymethyl)aminomethane), pH 8.0). Negative control samples were not incubated with the primary antibody. The confocal imaging was performed on a Leica TCS SP2 AOBS confocal laser scanning microscope (Leica S.p.A., Milan, Italy). To quantify MAP LC3 positive cells, 5-6 slides were examined at X40 magnification for each condition, in order to count almost 1000 cells as previously described.35 All results are expressed as the mean % ± standard deviation. The mean values of the groups were compared by ANOVA, followed by Bonferroni and Student-Newman-Keuls tests.

2.5 Transmission electron microscopy

After the culture period, monolayers of MLO-Y4 were fixed for 20 min with 4% paraformaldehyde in 0.13 M phosphate buffer (pH 7.4), post-fixed for 20 min with 1% osmium tetroxide in 0.13 M phosphate buffer (pH 7.4), dehydrated in graded ethanol and embedded in epoxy resin (Durcupan ACM -, SPI Supplies, Jeol-Italia, Milan, Italy) before being sectioned with a diamond knife mounted on an Ultracut-Reichert Microtome (Leica, Wetzlar, Germany). Ultra-thin sections (70 -80 nm) were mounted on Formvar- and carbon-coated copper grids, stained with 1% uranyl acetate and lead citrate, and examined by means of a Zeiss EM109 transmission electron microscope (Zeiss AG, Jena, Germany).

2.6 Histological evaluations of osteocytes viability on bone biopsies of MM patients

A histological retrospective analysis on bone biopsies was performed in a cohort of 37 patients (15 females and 22 males) with monoclonal gammopathies, 31 of them with symptomatic MM and 6 smoldering MM (SMM). Bone biopsies were obtained in both symptomatic MM and SMM at diagnosis and after an average time of 12 months of treatment or observation, respectively. The study was carried out in accordance with the principles of the Declaration of Helsinki and informed consent was obtained from the patients. Cylindrical iliac biopsies (3mm in diameter and 10mm in length) were obtained from the patients, fixed in sodium phosphate buffered 4% paraformaldehyde, pH 7.4, dehydrated in a graded series of ethanol, and embedded in paraffin. The bone samples were longitudinally sectioned by means of a microtome cutting system (Leica S.p.A) in order to obtain sections that were 5 micron thick, and then stained with Gomori's trichrome stain (Polysciences, Inc., Eppelheim, Germany). Osteocyte viability was evaluated as previously described.[34] Bone biopsy's sections taken from 22 out of 31 symptomatic MM patients also underwent to in situ end-labeling analysis (TUNEL) in order to evaluate the amount of osteocyte apoptosis using the ApopTag peroxidase in situ apoptosis kit (Millipore, Billerica, MA) according to the manufacturer's recommendations (counterstain Fast Green). Finally in 13 bone biopsy sections out of 31 samples obtained from symptomatic MM patients, MAP LC3 expression was checked by immunofluorescence and evaluated using the confocal microscopy according the above-mentioned procedure. Statistics were performed using non-parametric analysis to evaluate the differences among the median values of viable osteocytes across the different groups of patients. A multiple regression non-parametric analysis was also performed to identify which treatment had a significant impact of the osteocyte viability.

3. RESULTS

3.1 PIs reduced pre-osteocyte and osteocyte death induced by HMCLs

We checked the in vitro effect of PIs on MM-induced osteocyte cell death. Firstly, both HOB-01 and MLO-Y4 were treated with BOR at different concentration and different time and we found that only BOR at 2nM for 12hrs had a significant positive impact on viability of both HOB-01 and MLO-Y4 (Figure 1a) (means % of increase±SE vs control: +9%±1% and +11%±1% respectively, p≤ 0.001). On the contrary at 24 and 48hrs BOR had no effect or a negative effect on osteocytes viability. Similar results were observed with the more potent PIs MG 262 at 10nM.(Figure 1b) Both HOB-01 and MLO-Y4 were co-cultured for 48hrs with or without the CM of RPMI 8226 and JJN3 and then treated with BOR 2nM or MG 262 10nM or vehicle for 12hrs. As expected we found that both CM of RPMI 8226 (Figure 2a) and JJN3 (Figure 2b) significantly reduced HOB-01 and MLO-Y4 viability (means % of reduction±SE vs control: RPMI 8226: -14%±0.8% and -46%±0.9% respectively, p≤ 0.001; JJN3: -25%±2% and -28%±1.1% respectively, p≤ 0.001); thus we found that 12hrs-treatment with both PIs BOR and MG 262 significantly reduced the cytotoxic effect induced by the CM of RPMI 8226 (Figure 2a) and JJN3 (Figure 2b) on HOB-01 and MLO-Y4. A similar effect was observed with ZOL treatment at 10-10 and 10-11M on MLO-Y4 incubated with CM of RPMI 8226 for 48hrs whereas we did not found any significant effect of ZOL on the viability of HOB-01 (Supplementary Figure 1).

3.2 PIs blunts DEX-induced osteocyte death with an enhanced effect in combination with PTH(1-34)

Secondly we evaluated the effect of PIs on DEX-induced osteocyte death. Because HOB-01 did not show any reduction of viability under DEX treatment at a wide range of concentrations (data not shown), we used MLO-Y4 to this purpose. As expected the

treatment with DEX at high dose (10-6M) for 48hrs significantly reduced MLO-Y4 viability whereas the addition of BOR 2nM for 12hrs after DEX treatment, significantly reduced the effect of DEX (Figure 3a). Similar results were obtained with MG 262 (data not shown). On the contrary ZOL at concentrations ranging from 10-5 to 10-11M was not able to reduced DEX-induced MLO-Y4 death (Supplementary Figure 1). Because it is known that PTH(1-34) intermittent administration counteracts the effect of glucocorticoids,[16] we further investigated the potential effect of BOR in combination with PTH(1-34) on DEX-induced osteocyte death. The treatment with BOR (12hrs) or PTH(1-34) (1hr) alone increased the viability of HOB-01 and MLO-Y4 (means % of increase ± SE vs control: BOR: +9%±1% P=0.02; +10%±1 P=0.008 respectively; PTH: +9%±1.2% P=0.02; +7%±1 P≤ 0.001, respectively) (data not shown). The combination of BOR and PTH(1-34) significantly reduced the cytotoxic effect induced either by 6hrs DEX treatment as compared to the treatment with PTH(1-34) alone (Figure 3b) or by 24hrs DEX treatment as compared to BOR alone (Figure 3c).

3.3 Autophagic cell death rather than apoptosis is involved in MM and DEX-induced pre-osteocytes and osteocyte death: effects of PIs

In order to establish the mechanism by which the treatment with PIs blunted both MM and DEX-induced osteocytes death, firstly we checked caspase-3 activation in osteocytes co-cultured with the HMCLs using RPMI 8226 in the presence or the absence of PIs. After 48hrs of co-culture in a transwell system, the presence of RPMI 8226 did not affect caspase-3 activity in both HOB-01 (Figure 4a) and MLO-Y4 (Figure 4b) and consequently PIs did not affect caspase-3 activity in both cell lines (Figure 4a and 4b). Similarly DEX (10-6M) for 48 hrs did not affect caspase-3 activity in MLO-Y4 (Figure 4b). The potential involvement of apoptosis in both MM- and DEX-induced osteocyte death was further investigated by immunofluorescent confocal microscopy checking the expression of the apoptotic marker APAF-1. We found that neither RPMI 8226 nor DEX (10-6M) increased the number of APAF-1 positive cells in MLO-Y4 (data not shown). Because we failed to find a significant effect on osteocyte apoptosis, then

we focused on autophagy. Interestingly we found that BOR (2nM) treatment induced the expression of the autophagic marker LC3 increasing the LC3 II/I ratio as compared to vehicle in both HOB-01 (Figure 5a) and MLO-Y4 (Figure 5b). On the other hand, we found that in the co-culture system RPMI 8226 induced an increase of LC3II/I ratio in HOB-01 (Figure 5c) and in MLO-Y4 (Figure 5d) whereas the 12hrs-treatment with BOR and MG 262 significantly reduced LC3II/I ratio (Figure 5c and 5d). In the same way, MLO-Y4 incubated for 48hrs with DEX (10-6M) showed an increased level of LC3 II/I ratio compared to control blunted by the subsequently 12hrs treatment with both PIs (Figure 5e). The expression of autophagic marker LC3 was also evaluated by confocal microscopy on the monolayers of MLO-Y4. Accordingly, an increase of the % of LC3 positive autophagic osteocytes was observed after 48hrs of co-culture with RPMI 8226 (Figure 6a) and DEX treatment (Figure 6b). The subsequently 12hrs treatment with BOR and MG 262 reduced the % of autophagic cells in both systems (Figure 6a and 6b) (ANOVA: $P<0.001$, Bonferroni: $P<0.05$, Student-Newman-Keuls: $P<0.05$).

3.4 BOR blocked p62 degradation inhibiting the autophagic flux

To better understand the effect of BOR on autophagic flux, we study the level of the protein p62, a useful marker of autophagy that decreased when autophagy is completed.[48,49] Interestingly, we found that, concomitantly with the increased of LC3II/I ratio, the 12hrs- treatment with BOR of MLO-Y4 increased the level of p62, indicating the ability of BOR to inhibit their autophagy-dependent degradation (Figure 7a). In the same way, the presence of RPMI 8226 in co-culture system (Figure 7b) or the treatment of MLO-Y4 with DEX (10-6M) (Figure 7c) for 48 hrs slightly reduced the level of p62 whereas the subsequently 12hrs-treatment with BOR increased their level. Transmission electron microscopy examination confirmed the increase of autophagic osteocytes after treatment with DEX and the lack of apoptotic cells, in MLO-Y4. Furthermore, the subsequently treatment with BOR (2nM) reduced the amount of osteocytes containing autophagic vesicles (Figure 8). Interestingly, TEM evaluation also showed few autophagic osteocytes and mostly cells with a normal ultrastructure and

normal mitochondria after treatment with BOR alone. Moreover, we lack to find evidence of apoptosis (Figure 8). Take together these data showed that BOR block the degradation of p62 inhibiting the autophagic flux in osteocytes.

3.5 Inhibition of both apoptosis and autophagy block the positive effect of BOR on osteocytes viability

To elucidated the effects of BOR on osteocytes viability related to its role in autophagic process, MLO-Y4 were pretreated with 3-MA (0.5mM), CQ (25μM), or vehicle for 1hr and then exposed DEX 10-6M or CM of RPMI 8226 for further 48hrs. At the end cells were then treated with BOR 2nM. Autophagy inhibitors were maintained for the entire period of culture. Interestingly, the presence of autophagy inhibitors block the positive effects of BOR on osteocytes viability with a strongest effect of CQ, maybe due to their different target in the autophagic process (Figure 9a and b). The same set of experiments were performed in the presence or absence of Caspase 3 inhibitor, Ac-DEVD-CHO (50nM); similarly, the inhibition of apoptosis blunted the positive effect of BOR on MLO-Y4 viability (Figure 9a and b).

3.6 Increase of viable osteocytes in MM patients treated with BOR-based regimens

We retrospectively analyzed the bone biopsies obtained from the iliac crests of 37 patients including 31 with symptomatic MM (newly diagnosed: 19 and relapsed: 12; ISS I-III: I=3, II=17, III=11) and 7 with SMM (mean age±SD of all patients 68±10 years; median 68 years; range 35-88 years). 73% of MM patients carried type k and 27% with type λ chains and 65% of symptomatic MM patients had radiographic evidence of osteolytic lesions at the X-rays survey. The patients' characteristics and the drugs used in the treatment regimens are summarized in Table 1. In SMM any significant change was not observed in the number of viable osteocytes in the histological evaluations

carried out after 12 months of observation (median % change: +1.2%, P=0.68, NS). The overall response rate of the MM patients analyzed was 79.3% (nCR/CR: 48.3%; VGPR/PR: 31%). The mean percent change of the osteocyte viability was not correlated with the response rate to treatment (R2 0.01, P=NS). A significant increase of the number of viable osteocytes was found in MM patients treated with BOR-based regimen as compared to those treated without BOR (% median increase: +6% vs +1.30%; P=0.017) (Figure 10a). Patients treated with BOR alone showed the highest increase of osteocyte viability, as compared to either patients treated without BOR (+14% vs +1.3%, P=0.0027) or those treated with BOR plus DEX (+11.6% vs+4.4%, P=0.01); the difference between MM patients treated with BOR alone vs. BOR plus DEX did not reach a statistical significance (Figure 10b). On the other hand any significant difference was not observed in patients treated with Thalidomide (THAL) or Immunomodulatory drugs (IMiDs) than in those untreated with these drugs (P= 0.7). A multiple regression non-parametric analysis showed that BOR had a significant positive impact on osteocyte viability (P=0.042) whereas THAL/IMiDs as well as ZOL treatments had not (P=0.2). In addition BOR counterbalanced the negative effect of DEX treatment (P=0.035). Figure 10c shows Gomori's trichrome staining of sections of the iliac crest biopsies obtained from two representative MM patients treated with or without BOR. In a sub-group of bone biopsies we also performed TUNEL assay to check apoptotic death osteocytes and LC3 expression to identify autophagic osteocytes. A reduction of the percent of apoptotic osteocytes was observed in MM patients treated with BOR-based regimen after 12 months however the reduction did not reach a statistical significance as compared to that observed in MM patients treated without BOR (median % reduction of apoptotic osteocytes: BOR+: -64% vs BOR-: -36%: P=0.3). Similarly when we analyzed the responding MM patients any significant difference was not observed in the reduction of the apoptotic osteocytes between patients treated with BOR as compared with those treated without BOR (median % reduction of apoptotic osteocytes: BOR+: -63% vs BOR-: -39%: P=0.38). Figure 10d shows three representative MM patients. A higher reduction of autophagic osteocytes was recorded in MM patients treated with BOR-based regimen as compared to those

treated without BOR but the difference did not reach a statistical significance (median % reduction of autophagic osteocytes: BOR+: -65% vs BOR-: -52%: P=0.5). On the other hand when we analyzed the responder MM patients we found that patients treated with BOR-based regimen had a reduction of the number of autophagic osteocytes significantly higher as compared to that observed in MM patients treated without BOR (median % reduction of autophagic osteocytes: BOR+: -65% vs BOR-: -4%: P=0.02). Figure 10e shows two representative MM patients.

4. DISCUSSION

Among the new drugs used in the treatment of MM patients, the PIs including BOR have been demonstrated to stimulate bone formation with a positive effect on bone disease.[35-40] MM patients treated with BOR show a significant increase in bone architectural parameters such as bone volume and trabecular thickness[50] as well as those responding to BOR show an increase of osteoblastic serum markers as alkaline phosphatase.[50] Our previously data and those of others indicate that PIs are able to induce the osteogenic differentiation process of mesenchymal stem cells and to stimulate osteoblasts activity.[36,39,51] In this study we found that PIs affect osteocyte and pre-osteocyte viability and potentiated the effect of PTH(1-34) showing a new mechanism of action of this class of drugs. In order to check the potential effects of PIs on osteocyte survival, two different in vitro models were used (i) MM cell–induced osteocyte death and (ii) drug-induced osteocyte death with high dose of DEX. In both systems we demonstrated that PIs reduced the rate of pre-osteocyte and osteocyte death. The in vitro effects of PIs on osteocyte survival were observed at low concentrations comparable to those able to stimulate osteogenic differentiation of mesenchymal cells.[36] BOR was also able to potentiate the stimulatory effect of PTH(1-34) on osteocytes viability after DEX treatment. The PTH receptor 1 has been detected in osteocytes and it is known that PTH has opposite effects on bone[52]: exogenous and intermittent administration of PTH in vivo stimulates the bone formation, while continuous PTH elevation decreases bone mass.[53] Interestingly in MM mouse models it has been reported that PTH administration increases osteoblast formation and bone mass and consequently reduces tumoral burden. [54] Our data show that the positive effect of BOR on DEX-induced osteocytes death was enhanced by the combination with a PTH(1-34) short-term treatment as compared to the treatment with BOR and PTH(1-34) alone, in turns it has been recently reported that PTH receptor signaling mediates the anti myeloma effect of PIs including BOR.[55] The mechanism involved in MM-induced osteocyte death and in the protective effect on osteocyte survival by PIs was further investigated. Because evidences suggest that both apoptosis

and autophagy are involved in the regulation of bone remodeling by osteocytes[7,10-11] and in the pathophysiological of bone loss in several conditions such as glucocorticoid administration[12-14] both mechanisms were evaluated in our in vitro systems. Firstly we found that autophagic death rather than apoptosis is involved in the increased osteocyte death induced by MM cells either in human pre-osteocytes or in murine osteocytes as shown by the increased expression of the autophagic marker LC3 by western blot and confocal microscopy and the lack of effect on caspase-3 activation and APAF-1 expression. Several work established that DEX treatment induced apoptosis in MLO-Y4 but after 6hrs-treatment[12,18,56,57] and preceded by cell detachment, in a process called anoikis.[58] In our system, with a longer DEX-treatment, apoptosis can be followed by an excessive of autophagy leading to death. These observation were further confirmed by TEM analysis that shows the increase of autophagic osteocytes and the lack of apoptotic cells after treatment with DEX. Moreover, both treatment with DEX and the presence of MM cells reduce the protein level of p62 a key player in the selective autophagic degradation of many proteins; this protein is commonly used to study the autophagic flux since it interacts with LC3 during the formation of autophagolysosomes and is then degraded as autophagy completed. [48,49] Take together this observation clarifies which mechanism is involved in the previously reported MM-induced osteocyte death[34] nevertheless further studies will be necessary to identified which factors produced by MM cells may activate autophagic osteocyte death. TNFa was reported to induce apoptosis but not autophagy in osteocyte.[24] Accordingly we have previously reported that anti-TNFa antibody lack to block MM cell-induced osteocyte death.[34] Recently data reported that direct cell-to cell interactions between osteocyte and MM cells up-regulate SOST in osteocytes[59] however in our system SOST is likely to be not involved because we perform co-culture system with a transwell system failing to find an upregulation of SOST at protein level (data not shown) and there are not evidences that SOST induces autophagy in osteocytes. Autophagy has a critical role in the regulation of cell survival and cell death in response to environmental stress stimuli.[60-61] Growing evidences underline the role of autophagy in the regulation of osteocytes survival and

consequently in the control of skeletal homeostasis and bone remodeling.[62] A suppression of the basal level of autophagy was reported in relationship with aging related bone changes[23], on the other hand an increase of both osteocyte and osteoblast autophagic death was reported in relationship with glucocorticoid treatment characterized by a rapid bone loss.[12,14] Similarly it was found that treatment with high dose of DEX triggered autophagic markers in osteocyte.[24] Here we report the involvement of autophagy in MM-induced osteocyte death and related bone remodeling alterations. Similarly it has been recently reported that impaired osteoblastogenesis in osteogenesis imperfecta is related to an upregulation of autophagy.[63] These evidences suggest that autophagy of bone cells could be a potential target in the bone microenvironment. In line with this new hypothesis we find that PIs stimulate osteocyte and preosteocyte survival blocking both MM cell and DEX-induced autophagic death. The role of BOR in autophagic pathway has been extensively examined in different cellular types,[64-70] as well as a cross talk and integration between autophagy and proteasomal degradation.[71-72] The capacity of BOR to induce autophagy has been recently reported in pancreatic and colon cancer cells[64] as well as in melanoma[65], hepatocarcinoma cells[66], myeloid leukemic[69] and lymphoma cells.[70] On the other hand, recent data indicate that BOR can inhibit autophagy via multiple mechanism.[43-44] In particular, it has been showed that BOR can inhibit the autophagic flux by increasing the level of LC3II but blocking p62 degradation stimulating ERK phosphorylation in multiple ovarian cancer cell lines[43] as well as in breast cancer cells via a cathepsin-dependent mechanism.[44] In line with these data, we find that BOR is able to increase the level of LC3II/I ratio and to block the degradation of p62 in MLO-Y4, indicating the ability to target the autophagic pathway. The same effect was found both after DEX-treatment or after co-cultivation with MM cells. Indeed, the capacity of BOR to inhibit autophagy was also demonstrated by transmission electron microscopy examinations. Interestingly, we found few autophagic osteocytes after treatment of MLO-Y4 with BOR alone and the lack of apoptotic cells. Autophagy is known to be inhibited by both phosphoratidylinositol-3-kinase (PI3K) inhibitors such as 3-MA and lysosomal lumen alkalizers such as

chloroquine, that block the early and the late stage of autophagy, respectively. [73-75] We found that blocking autophagy by both inhibitors blunted the positive effects of BOR on MLO-Y4 viability, both after DEX and CM of RPMI 8226 treatment. Similarly the positive effect of BOR was blunted by Caspase 3 inhibitor, Ac-DEVD. These data indicated that there is a strong connection between autophagy and apoptosis, as confirmed by several works showing that there are many physical and functional between autophagy and apoptosis.[75,76] In particular, it is known that p62 is needed for the activation of polyubiquitinated caspase-8 [77] and caspase 8, in turn, cleaves p62 in response to death receptors activation.[78] Moreover, caspase-8 colocalizes also with Atg8/LC3 and Atg5, and the assembly of the death-inducing signaling complex requires the autophagosomal membrane.[79] On the other hand, inhibition of autophagy may enhanced apoptosis because of the lack of degradation of p62-complexed apoptosis proteins[80], while the inhibition of autophagy in cancer cells, caspase-8 dependent cell death was associated with the concomitantly elevated p62 level.[81] The in vitro effects of PIs are likely to occur in vivo in MM patients because we show that MM patients treated with BOR-based regiments have significant increased of osteocyte viability as compared to those treated without BOR. In addition although the difference between MM patients treated with BOR alone vs BOR plus DEX did not reach a statistical significance, by a multiple regression analysis we found that BOR counterbalanced the negative effect of DEX treatment. Together to a direct effect of PIs on osteocytes an indirect one mediated by PTH, could occur in vivo in MM patients treated with BOR. Other authors have demonstrated that the treatment with BOR determines a significant and reproducible pulsatile increase of serum PTH in responding MM patients36 supporting the hypothesis that the anabolic effect of BOR may be mediated at least in part by PTH. In our cohort of MM patients the effect of BOR on osteocyte viability was independent to the response to the treatment suggesting that the direct effect is likely to be predominant compared to the indirect one, however, we cannot exclude that both effects may coexist in vivo. The analysis of both osteocyte apoptosis and autophagy in MM patients indicate that both processes are reduced after the treatment although we lack to find a statistical significant difference between MM

patients treated with BOR as compared to those treated without BOR. However in responding MM patients we show that autophagy but not apoptosis were significantly inhibited in patients treated with BOR-based regimens. These evidences indicate that it is likely that in vivo both apoptosis and autophagy are involved in the reduction of osteocyte viability and are affected by PIs with a predominance of the effect on autophagy. In conclusion our data identify osteocyte autophagy as a potential target in MM bone disease and suggest that PIs, increasing osteocyte viability through the modulation of the autophagy, improve bone integrity in MM patients. In Figure 11 we summarized the interplay between MM cells and OBs/osteocytes/OCLs and the positive effects of PIs on osteocytes viability.

5. REFERENCES

1. Galson DL, Silbermann R, Roodman GD. Mechanisms of multiple myeloma bone disease. *Bonekey Rep*. 2012;1:135.
2. Giuliani N, Rizzoli V, Roodman GD. Multiple myeloma bone disease: Pathophysiology of osteoblast inhibition. *Blood*. 2006;108(13):3992-3996.
3. Bonewald LF. The amazing osteocyte. J Bone Miner Res. 2011;26(2):229-238.
4. Heino TJ, Hentunen TA. Differentiation of osteoblasts and osteocytes from mesenchymal stem cells. *Curr Stem Cell Res Ther*. 2008;3(2):131-145.
5. Noble BS, Reeve J. Osteocyte function, osteocyte death and bone fracture resistance. *Mol Cell Endocrinol*. 2000; 159(1-2):7-13.
6. Palumbo C, Palazzini S, Marotti G. Morphological study of intercellular junctions during osteocytes differentiation. *Bone*. 1990;11(6):401-406.
7. Teti A, Zallone A. Do osteocytes contribute to bone mineral homeostasis? Osteocytic osteolysis revisited. *Bone*. 2009;44(1):11-16.
8. van Bezooijen RL, ten Dijke P, Papapoulos SE, Löwik CW. SOST/-sclerostin, an osteocyte-derived negative regulator of bone formation. *Cytokine Growth Factor Rev*. 2005;16(3):319-327.
9. Weinstein RS, Nicholas RW, Manolagas SC. Apoptosis of osteocytes in glucocorticoid-induced osteonecrosis of the hip. *J Clin Endocrinol Metab*. 2000; 85(8):2907-2912.
10. Tomkinson A, Reeve J, Shaw RW, Noble BS. The death of osteocytes via apoptosis accompanies estrogen withdrawal in human bone. *J Clin Endocrinol Metab*. 1997;82(9):3128-3135.
11. Emerton KB, Hu B, Woo AA, et al. Osteocyte apoptosis and control of bone resorption following ovariectomy in mice. *Bone*. 2010;46(3):577-583.
12. O'Brien CA, Jia D, Plotkin LI, et al. Glucocorticoids act directly on osteoblasts and osteocytes to induce their apoptosis and reduce bone formation and strength. *Endocrinology*. 2004;145(4):1835-1841.
13. Canalis E, Delany AM. Mechanisms of glucocorticoid action in bone. *Ann N Y Acad Sci.* 2002;966:73-81.

14. Lane NE, Yao W, Balooch M, et al. Glucocorticoid-treated mice have localized changes in trabecular bone material properties and osteocytes lacunar size that are not observed in placebo-treated or estrogen-deficient mice. *J Bone Miner Res*. 2006;21(3):466-476.
15. Tatsumi S, Ishii K, Amizuka N, et al. Targeted ablation of osteocytes induces osteoporosis with defective mechanotransduction. *Cell Metab*. 2007;5(6):464-475.
16. Weinstein RS, Jilka RL, Almeida M, Roberson PK, Manolagas SC. Intermittent parathyroid hormone administration counteracts the adverse effects of glucocorticoids on osteoblast and osteocytes viability, bone formation, and strength in mice. *Endocrinology.* 2010;151:2641-9.
17. Raje N, Roodman GD. Advances in the biology and treatment of bone disease in multiple myeloma. Clin Cancer Res. 2011;17(6):1278-86.
18. Plotkin LI, Weinstein RS, Parfitt AM, Roberson PK, Manolagas SC, Bellido T. Prevention of osteocytes and osteoblast apoptosis by bisphosphonates and calcitonin. *J Clin Invest*. 1999;104(10):1363-74.
19. Plotkin LI, Manolagas SC, Bellido T. Dissociation of the pro-apoptotic effects of bisphosphonates on osteoclasts from their anti-apoptotic effects on osteoblasts/osteocytes with novel analogs. *Bone*. 2006;39(3):443-52.
20. Bellido T, Plotkin LI. Novel actions of bisphosphonates in bone: preservation of osteoblast and osteocyte viability. *Bone.* 2011;49(1):50-5.
21. Srinivas V, Bohensky J, Zahm AM, Shapiro IM. Autophagy in mineralizing tissues: microenvironmental perspectives. *Cell cycle*. 2009;8(3):391-393.
22. Tsujimoto Y, Shimizu S. Another way to die: autophagic programmed cell death. *Cell Death Differ*. 2005;12:1528-1534.
23. Onal M, Piemontese M, Xiong J, et al. Suppression of autophagy in osteocytes mimics skeletal aging. *J Biol Chem*. 2013;288(24):17432-17440.
24. Xia X, Kar R, Gluhak-Heinrich J, et al. Glucocorticoid-induced autophagy in osteocytes. *J Bone Miner Res*. 2010;25(11):2479-2488.
25. Yao W, Dai W, Jiang JX, Lane NE. Glucocorticoids and osteocyte autophagy. *Bone*. 2013;54(2):279-284.
26. Shimizu S, Kanaseki T, Mizushima N, Mizuta T, Arakawa-Kobayashi S, Thompson CB et al. Role of Bcl-2 family proteins in a non-apoptotic programmed cell death dependent on autophagy genes. *Nat Cell Biol* 2004; 6: 1221–1228.

27. Shimizu S, Konishi A, Nishida Y, Mizuta T, Nishina H, Yamamoto A et al. Involvement of JNK in the regulation of autophagic cell death. *Oncogene* 2010; 29: 2070–2082.
28. Yu L, Alva A, Su H, Dutt P, Freundt E, Welsh S et al. Regulation of an ATG7-beclin 1 program of autophagic cell death by caspase-8. *Science* 2004; 304: 1500–1502.
29. Yu L, Wan F, Dutta S, Welsh S, Liu Z, Freundt E et al. Autophagic programmed cell death by selective catalase degradation. *Proc Natl Acad Sci USA* 2006; 103:4952–4957.
30. Pattingre S, Tassa A, Qu X, Garuti R, Liang XH, Mizushima N et al. Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. *Cell* 2005; 122: 927–939.
31. Lamy L, Ngo VN, Emre NC, Shaffer AL 3rd, Yang Y, Tian E et al. Control of autophagic cell death by caspase-10 in multiple myeloma. *Cancer Cell* 2013; 23: 435–449.
32. Reef S, Zalckvar E, Shifman O, Bialik S, Sabanay H, Oren M et al. A short mitochondrial form of p19ARF induces autophagy and caspase-independent cell death. *Mol Cell* 2006; 22:463–475.
33. Gewirtz DA. When cytoprotective autophagy isn't... and even when it is. *Autophagy* 2014; 10:391-392
34. Giuliani N, Ferretti M, Bolzoni M, et al. Increased osteocyte death in multiple myeloma patients: role in myeloma-induced osteoclast formation. *Leukemia.* 2012;26(6):1391-1401.
35. Garrett IR, Chen D, Gutierrez G, et al. Selective inhibitors of the osteoblast proteasome stimulate bone formation in vivo and in vitro. *J Clin Invest*. 2003;111(11):1771-1782.
36. Giuliani N,Morandi F, Tagliaferri S, et al. The proteasome inhibitor bortezomib affects osteoblast differentiation in vitro and in vivo in multiple myeloma patients. *Blood.* 2007;110(1): 334-338.
37. Zangari M, Esseltine D, Lee CK, et al. Response to bortezomib is associated to osteoblastic activation in patients with multiple myeloma. *Br J Haematol*. 2005;131(1):71-73.
38. Shimazaki C, Uchida R, Nakano S, et al. High serum bone-specific alkaline phosphatase level after bortezomib-combined therapy in refractory multiple myeloma: possible role of bortezomib on osteoblast differentiation. *Leukemia*. 2005;19(6):1102-1103.
39. Heider U, Kaiser M, Müller C, et al. Bortezomib increases osteoblast activity in myeloma patients irrespective of response to treatment. *Eur J Haematol*. 2006;77(3):233-238.

40. Zangari M, Yaccoby S, Cavallo F, Esseltine D, Tricot G. Response to bortezomib and activation of osteoblasts in multiple myeloma. *Clin Lymphoma Myeloma*. 2006;7(2):109-114.
41. Terpos, Christoulas. Effects of proteasome inhibitors on bone cancer. *Bonekey Rep*. 2013; 2:395
42. Crawford LJ, Walker B and Irvine AE. Proteasome inhibitors in cancer therapy. J Cell Commun Signal 2011; 5: 101–110.
43. Kao C, Chao A, Tsai CL. Bortezomib enhances cancer cell death by blocking the autophagic flux through stimulating ERK phosphorylation. *Cell Death Dis*. 2014;5:e1510.
44. Periyasamy-Thandavan S, Jackson WH, Samaddar JS. Bortezomib blocks the catabolic process of autophagy via a cathepsin-dependent mechanism, affects endoplasmic reticulum stress and induces caspase-dependent cell death in antiestrogen-sensitive and resistant ER+ breast cancer cells. *Autophagy*. 2010; 6(1):19-35.
45. Bodine PV, Vernon SK, Komm BS. Establishment and hormonal regulation of a conditionally transformed preosteocytic cell line from adult human bone. *Endocrinology.* 1996;137:4592-604
46. Bonewald LF. Establishment and characterization of an osteocyte-like cell line, MLO-Y4. *J Bone Miner Metab.* 1999;17:61-5.
47. Sena P, Manfredini G, Benincasa M, et al. Up-regulation of the chemo-attractive receptor ChemR23 and occurrence of apoptosis in human chondrocytes isolated from fractured calcaneal osteochondral fragments. *J Anat*. 2014;224(6):659-68
48. Jiang P, Mizushima N. LC3- and p62-based biochemical methods for the analysis of autophagy progression in mammalian cells. *Methods*. 2014 S1046-2023(14)00384-3.
49. Pankiv S, Clausen TH, Lamark T J. p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. *Biol Chem*. 2007;282(33):24131-45.
50. Zangari M, Yaccoby S, Pappas L, et al. A prospective evaluation of the biochemical, metabolic, hormonal and structural bone changes associated with bortezomib response in multiple myeloma patients. *Haematologica*. 2011; 96(2):333-6
51. Mukherjee S, Raje N, Schoonmaker JA, et al. Pharmacologic targeting of a stem/progenitor population in vivo is associated with enhanced bone regeneration in mice. *J Clin Invest*. 2008; 118(2):491-504

52. Bringhurst FR. PTH receptors and apoptosis in osteocytes. *J Musculoskelet Neuronal Interact*.2002; 2: 245-251.

53. Qin L, Raggatt LJ, Partridge NC. Parathyroid hormone: a double-edged sword for bone metabolism. *Trends Endocrinol Metab*. 2005; 15:60-65.

54. Pennisi A, Ling W, Li X, et al. Consequences of daily administered parathyroid hormone on myeloma growth, bone disease, and molecular profiling of whole myelomatous bone. *PLoS One*. 2010; 5(12):e15233.

55. Zangari M, Berno T, Yang Y, et al. Parathyroid hormone receptor mediates the anti-myeloma effect of proteasome inhibitors. *Bone.* 2014; 61:39-43.

56. Plotkin LI, Manolagas SC, Bellido T. Transduction of cell survival signals by connexin-43 hemichannels. *Biol Chem*. 2002; 277(10):8648-57.

57. Ahuja SS, Zhao S, Bellido T, Plotkin LI, Jimenez F, Bonewald LF. CD40 ligand blocks apoptosis induced by tumor necrosis factor alpha, glucocorticoids, and etoposide in osteoblasts and the osteocyte-like cell line murine long bone osteocyte-Y4. *Endocrinology*. 2003;144(5):1761-9.

58. Plotkin LI, Manolagas SC, Bellido T. Glucocorticoids induce osteocyte apoptosis by blocking focal adhesion kinase-mediated survival. Evidence for inside-out signaling leading to anoikis. *J Biol Chem*. 2007; 282(33):24120-30.

59. Calle JD, Bellido T, Roodman GD. Direct Cell-To-Cell Interactions Between Osteocytes and Multiple Myeloma (MM) Cells Upregulate Sost and Downregulate OPG Expression In Osteocytes: Evidence For Osteocytic Contributions To MM-Induced Bone Disease [abstract]. *Blood*. 2013; 122 (21). Abstract 3140.

60. Codogno P, Meijer AJ. Autophagy and signaling: their role in cell survival and cell death. *Cell Death Differ*. 2005;12 Suppl 2:1509-18.

61. Nikoletopoulou V, Markaki M, Palikaras K, Tavernarakis N. Crosstalk between apoptosis, necrosis and autophagy. *Biochim Biophys Acta*. 2013;1833(12):3448-59.

62. Hocking LJ, Whitehouse C, Helfrich MH. Autophagy: a new player in skeletal maintenance? *J Bone Miner Res*. 2012;27(7):1439-47.

63. Gioia R, Panaroni C, Besio R et al. Impaired osteoblastogenesis in a murine model of dominant osteogenesis imperfecta: a new target for osteogenesis imperfecta pharmacological therapy. *Stem Cells*. 2012;30(7):1465-76.

64. Min H, Xu M, Chen ZR, et al. Bortezomib induces protective autophagy through AMP-activated protein kinase activation in cultured pancreatic and colorectal cancer cells. *Cancer Chemother Pharmacol*. 2014;74(1):167-76.

65. Selimovic D, Porzig BB, El-Khattouti A et al. Bortezomib/proteasome inhibitor triggers both apoptosis and autophagy-dependent pathways inmelanoma cells. *Cell Signal*. 2013; 25(1):308-18.

66. Hui B, Shi YH, Ding ZB et al. Proteasome inhibitor interacts synergistically with autophagy inhibitor to suppress proliferation and induce apoptosis in hepatocellular carcinoma. *Cancer*. 2012; 118(22):5560-71.

67. Kim SY, Song X, Zhang L, Bartlett DL, Lee YJ. Role of Bcl-xL/Beclin-1 in interplay between apoptosis and autophagy in oxaliplatin andbortezomib-induced cell death. *Biochem Pharmacol*. 2014; 88(2):178-88.

68. Armstrong JL, Corazzari M, Martin S et al. Oncogenic B-RAF signaling in melanoma impairs the therapeutic advantage of autophagy inhibition. *Clin Cancer Res.* 2011 ;17(8):2216-26.

69. Fang J, Rhyasen G, Bolanos L et al. Cytotoxic effects of bortezomib in myelodysplastic syndrome/acute myeloid leukemia depend on autophagy-mediated lysosomal degradation of TRAF6 and repression of PSMA1. *Blood*. 2012;120(4):858-67.

70. Granato M, Santarelli R, Lotti LV et al. JNK and macroautophagy activation by bortezomib has a pro-survival effect in primary effusion lymphoma cells. *PLoS One.* 2013;8(9):e75965.

71. Wojcik S. Crosstalk between autophagy and proteasome protein degradation systems: possible implications for cancer therapy. *Folia Histochem Cytobiol*. 2013;51(4):249-64.

72. Benbrook DM, Long A. Integration of autophagy, proteasomal degradation, unfolded protein response and apoptosis. *Exp Oncol.* 2012; 34(3):286-97.

73. Klionsky DJ, Abdalla FC, Abeliovich H et al. Guidelines for the use and interpretation of assays for monitoring autophagy. *Autophagy*. 2012; 8:4,445-544.

74. Yang YP, Hu LF, Zheng HF Application and interpretation of current autophagy inhibitors and activators. *Acta Pharmacol Sin*. 2013;34(5):625-35.

75. Denton D, Xu T, Kumar S. Autophagy as a pro-death pathway. *Immunol Cell Biol.* 2015; 93(1):35-42.

76. Gump JM, Thorburn A. Autophagy and apoptosis- what's the connection? *Trends Cell Biol*. 2011 July ; 21(7): 387–392.

77. Jin Z, Li Y, Pitti R, et al. Cullin3-based polyubiquitination and p62-dependent aggregation of caspase-8 mediate extrinsic apoptosis signaling. *Cell*. 2009;137(4):721-35

78. Norman JM, Cohen GM, Bampton ET. The in vitro cleavage of the hAtg proteins by cell death proteases. *Autophagy*. 2010;6(8):1042-56.

79. Young MM, Takahashi Y, Khan O et al. Autophagosomal membrane serves as platform for intracellular death-inducing signaling complex (iDISC)-mediated caspase-8 activation and apoptosis. *J Biol Chem*. 2012;287(15):12455-68.

80. Kovacs JR, Li C, Yang Q et al. Autophagy promotes T-cell survival through degradation of proteins of the cell death machinery. *Cell Death Differ*. 2012;19(1):144-52.

81. Huang S, Okamoto K, Yu C, Sinicrope FA. p62/sequestosome-1 up-regulation promotes ABT-263-induced caspase-8 aggregation/activation on the autophagosome. *J Biol Chem.* 2013;288(47):33654-66.

6. TABLE

Table 1: Main characteristics of the cohort of MM patients

	SEX	AGE	STAGE	ISS	OSTEOLYSIS	% PC PRE	% PC POST	BOR	DEX	THAL/IMIDs	ZOL
SMM1	F	69			NO	20%	40%	-	-	-	-
SMM2	M	76			NO	15%	30%	-	-	-	-
SMM3	F	60			NO	20%	40%	-	-	-	-
SMM4	M	61			NO	15%	25%	-	-	-	-
SMM5	F	62			NO	12%	12%	-	-	-	-
SMM6	F	68			NO	10%	30%	-	-	-	+
MM1	F	60	D	II	YES	60%	NEG.	+	+	+	+
MM2	F	76	D	II	YES	30%	90%	-	-	+	+
MM3	M	68	D	II	NO	40%	NEG.	+	+	-	-
MM4	F	61	D	III	YES	60%	NEG.	-	+	-	+
MM5	F	71	D	III	YES	40%	NEG.	+	+	+	+
MM6	M	77	R	II	YES	15%	40%	+	-	-	-
MM7	M	65	D	III	NO	50%	15%	+	+	+	+
MM8	M	65	R	III	N.D.	40%	15%	+	+	+	+
MM9	M	73	R	III	YES	60%	2%	+	-	-	-
MM10	F	71	D	III	YES	90%	NEG.	+	+	-	+
MM11	M	62	R	I	YES	15%	10%	+	-	-	+
MM12	F	75	R	III	NO	80%	60%	+	+	-	-
MM13	M	49	D	II	N.D.	30%	NEG.	-	+	+	+
MM14	M	67	D	II	N.D.	90%	80%	+	+	-	-
MM15	F	75	D	I	YES	40%	15%	-	-	+	-
MM16	F	82	D	II	YES	70%	30%	-	-	-	+
MM17	M	58	D	III	YES	70%	NEG.	+	+	+	+
MM18	M	61	D	III	N.D.	30%	NEG.	-	+	+	-
MM19	M	73	R	II	YES	20%	15%	+	-	-	-
MM20	M	88	R	II	YES	70%	NEG.	+	-	-	-
MM21	M	85	D	III	YES	30%	NEG.	+	-	+	+
MM22	F	78	R	I	YES	10%	NEG.	+	+	-	-
MM23	M	66	D	III	YES	40%	NEG.	-	+	+	+
MM24	M	35	D	II	NO	30%	NEG.	-	+	+	-
MM25	M	65	D	II	NO	25%	5%	+	+	-	+
MM26	M	50	D	II	NO	60%	NEG.	+	+	+	+
MM27	M	80	R	II	NO	15%	40%	+	-	+	-
MM28	F	73	R	II	NO	20%	30%	-	-	+	-
MM29	M	63	R	II	YES	60%	25%	+	-	-	-
MM30	F	63	D	II	N.D.	90%	NEG.	+	+	+	+
MM31	M	73	R	II	NO	50%	20%	-	-	+	+

Abbreviations: F, female; M, male; D, diagnosis; R, relapsed; N.D., not determined; ISS, International Staging System; PC, plasma cell; NEG., absence of PCs infiltration; BOR, Bortezomib; DEX, Dexamathasone; THAL/IMIDs, Thalidomide/Immunomodulatory drugs; ZOL, Zoledronic Acid.

7. FIGURE LEGENDS

Figure 1: PIs had positive effect on osteocyte and pre-osteocyte viability

Confluent HOB-01 and MLO-Y4 were incubated with BOR (2-5-10 nM) (a) and with MG 262 (10nM) (b) for 12-24-48hrs. At the end of the culture period, osteocyte viability was assessed by means of colorimetry using tetrazolium salt as described in Methods and Subjects. The graphs show the mean % change of cell viability values compared to control ± S.E in five independent experiments performed in triplicate. Data were analyzed by Student's t-test . (Control= HOB-01 and MLO-Y4 incubated with vehicle; * and **= HOB-01 or MLO-Y4 incubated with BOR or MG262 plus vs control; *P=0.001, **P<0.001)

Figure 2: PIs reduce MM-induced osteocyte and pre-osteocyte death

Confluent HOB-01 and MLO-Y4 were incubated with or without the 48hrs-CM of the HMCLs RPMI 8226 (a) or JJN3 (b) for 48hrs. Then the culture media was changed and osteocytes were treated with BOR (2nM) or MG 262 (10nM) for 12hrs. At the end of the culture period, osteocyte viability was assessed by means of colorimetry using tetrazolium salt as described in Methods and Subjects. The graphs show the mean % change of cell viability values compared to control ± S.E in eight independent experiments performed in triplicate. Data were analyzed by Student's t-test (Control= HOB-01 or MLO-Y4 incubated without CM of HMCLs in the presence of vehicle; * and **= HOB-01 or MLO-Y4 incubated with RPMI 8226 or JJN3 plus vehicle vs control; *P=0.001, **P<0.001).

Figure 3: PIs reduce DEX-induced osteocyte death and potentiate the effect of PTH(1-34)

Confluent MLO-Y4 were treated with DEX (10-6M) or vehicle for 48hrs, thereafter the culture media was changed and osteocytes exposed to BOR (2nM) or vehicle for further 12hrs (Control=MLO-Y4 treated with vehicle) (a). In another series of experiments MLO-Y4 were incubated with vehicle or high dose of DEX (10- 5M) for 6hrs (b) or 24hrs (c) respectively to induce cell death. Then the culture media was changed and MLO-Y4 treated with PTH(1-34) (10nM) for 1hr or BOR (2nM) for 12hrs or with PTH(1-34) (1hr) followed by BOR (12hrs). At the end of the culture period, osteocyte viability was assessed by means of colorimetry using tetrazolium salt as described in Methods and Subjects. The graphs show the mean % change of cell viability values compared to control ± SE in three independent experiments performed in triplicate. Data were analyzed by Student's t-test (Control = MLO-Y4 treated with vehicle).

Figure 4: Lack of effect on the activity of the apoptotic marker Caspase 3 in osteocytes by HMCLs and proteasome inhibitors

HOB-01 were co-cultured with HMCL RPMI 8226 in the presence or absence of BOR (2nM) or vehicle (a). MLO-Y4 were co-cultured with HMCL RPMI 8226 or treated with DEX 10-6M for 48hrs in the presence or absence of BOR (2nM), MG 262 (10nM), or vehicle (b). Both non-adherent and adherent cells were harvested, combined and analyzed for Caspase 3 activity. The graphs show the concentration of chromophore p-nitroaniline releated to caspase 3 activity, as described in Materials and Subjects. The data represent the mean value ± SD of two independent experiments. BMP2 and Etoposide treatment were used as positive control for caspase 3 activation, in HOB-01 and MLO-Y4, respectively. Data were analyzed by Student's t-test.

Figure 5: PIs increase the expression of the autophagic marker LC3 by pre-osteocytes and osteocytes but blunted its up-regulation induced by HMCL and DEX treatment

Both HOB-01 (a) and MLO-Y4 (b) were treated with BOR (2nM) or vehicle for 12hrs. Both HOB-01 (c) and MLO-Y4 (d) were co-cultured with RPMI 8226 for 48hrs and then treated with BOR (2nM) or MG 262 (10nM) or vehicle for 12hrs. MLO-Y4 were incubated with DEX 10-6M or vehicle for 48hrs and then treated with BOR (2nM) or MG 262 (10nM) or vehicle for 12hrs (e). LC3 protein level was evaluated by means of western blot. Graphs show the LC3II/LC3I ratio normalized for the internal control Actin level relative to vehicle treatment.

Figure 6: Confocal microscopy reveals that PIs blunted HMCL and DEX-induced autophagic death in MLOY4

Osteocytes autophagy was assessed by immunofluorescent confocal microscopy as described in the Methods and Subjects. Confluent MLO-Y4 were co-cultured for 48hrs with or without RPMI 8226 in the presence of transwell insert and then treated for 12hrs with BOR (2nM), MG 262(10nM) or vehicle (a). Confluent MLO-Y4 were also incubated with DEX 10-6M for 48hrs and then treated with BOR (2nM) or vehicle for 12hrs (b). The graphs show the mean % ± SD of MAP LC3 positive osteocytes for each condition. Original magnification X40. DAPI (blue) for nuclei; MAP-LC3 (red) for autophagy. Data were analyzed by ANOVA followed by Bonferroni and Student-Newman-Keuls tests (**= MLO-Y4 incubated with RPMI 8226 or treated with DEX plus vehicle vs vehicle; ANOVA: $P<0.001$, Student-Newman-Keuls: $P<0.05$, Bonferroni: $P<0.05$).

Figure 7: BOR blocked p62 degradation during autophagic death in MLO-Y4

MLO-Y4 was treated with BOR (2nM) or vehicle for 12hrs (a), or co-cultured with RPMI 8226 (b) or incubated with DEX 10-6M or vehicle (c) for 48hrs and then treated with BOR (2nM) or vehicle for 12hrs. p62 protein level was evaluated by means of western blot. Graphs show the p62 level normalized for the internal control Actin level relative to vehicle treatment.

Figure 8: Trasmission electron microscopy shows that BOR reduced the autophagic osteocytes.

Osteocytes autophagy was assessed by transmission electron microscopy as described in the Methods and Subjects. Confluent MLO-Y4 were treated for 48hrs with or without DEX 10-6M or vehicle and then treated for 12hrs with BOR (2nM), or vehicle (a). TEM micrographs showing cell ultrastructure. The arrows indicate autophagic cells. In b selected region of the micrographs of MLO-Y4 treated with DEX and enlarged section showing a typical autophagic vesicle.

Figure 9: Blocking autophagy and apoptosis blunted the positive effect of BOR on MLO-Y4 viability

Confluent MLO-Y4 were incubated with 3-MA (0.5 mM) or CQ (25 µM) or Ac-DEVD-CHO (50nM) or vehicle for 1hr before addition of 48h-CM of the HMCL RPMI8226 (a) or DEX (10^{-6}M) (b) or vehicle for 48hrs**.** Then the culture media was changed and osteocytes were treated with BOR (2 nM) or vehicle for 12 hrs. At the end of the culture period, osteocyte viability was assessed by means of colorimetry using tetrazolium salt as described in Methods and Subjects. The graphs show the mean % change of cell viability values compared to control ± S.E in three independent

experiments performed in triplicate. Data were analyzed by Student's t-test. (Control= MLO-Y4 incubated with DEX or CM of RPMI8226 in the presence of vehicle; **= MLO-Y4 incubated with DEX or CM of RPMI 8226 plus vehicle versus control, P<0.001).

Figure 10: BOR-based regimen increases the number of viable osteocytes in MM patients

The number of viable osteocytes was evaluated on bone biopsies of both MM patients treated with BOR-based regimen as compared to those treated without BOR (a), and MM patients treated with BOR alone as compared to either patients treated without BOR or those treated with BOR plus DEX (b). The graphs and bars show the median increase of viable osteocytes evaluated by Gomori's trichrome staining. Gomori's trichrome staining of sections of the iliac crest biopsy samples obtained from two representative MM patients treated with (MM 29) or without (MM 27) BOR, pre (top) and post (bottom) treatment (c). MM 29 and MM 27 are responder and non responder patients, respectively. Note the higher amount of well-stained viable osteocytes after treatment.

In situ end-labelling (TUNEL) of longitudinal sections of iliac crest biopsies taken from three representative MM patients treated without BOR-based regiment (MM 2) or with BOR (MM 27 and MM 30). pre (top): baseline; post (bottom): after treatment (d).

Figure 11: PIs role in MM BM microenvironment

Osteocyte viability is influenced by both positive and negative signals. Intermittent PTH simulates bone formation. Indeed, mechanical stimulus and glucocorticoids lead to osteocytes death. In MM patients, the presence of MM cells modifies BM microenvironment in different manners, such as increasing osteocytes death. PIs treatment improves osteocyte viability, blocking MM- and glucocorticoid-mediated osteocyte death. Moreover PIs potentiate the positive effect of PTH.

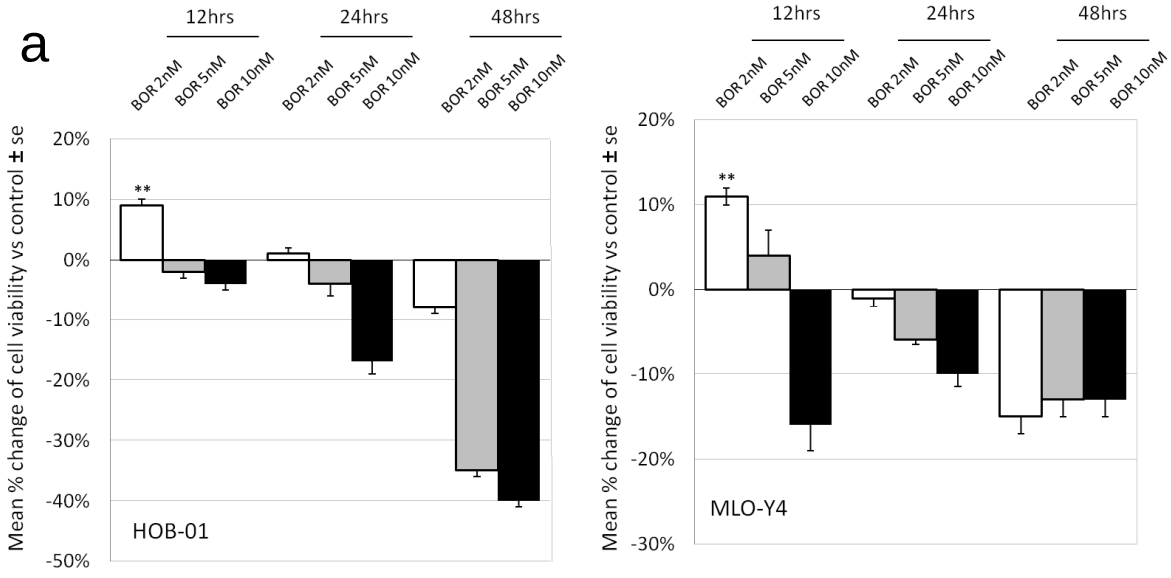
Supplementary Figure 1:

Confluent HOB-01 and MLO-Y4 were incubated with or without the 48h-CM of the HMCL RPMI-8226 for 48hrs. Then the culture media was changed and osteocytes were treated with BOR (2nM) or ZOL (10-10-10- 11M), or vehicle, for 12 h (a). Confluent MLO-Y4 were incubated with DEX 10-6M or vehicle for 48h and then treated with BOR (2nM) or ZOL (10-10-10-11 M), or vehicle for 12h (b). At the end of the culture period, osteocyte viability was assessed by means of colorimetry using tetrazolium salt as described in Methods and Subjects. The graphs show the mean % change of cell viability values compared to control ± S.E in two independent experiments performed in triplicate. Data were analyzed by Student's t-test (Control= HOB-01 or MLO-Y4 incubated without HMCL or DEX in the presence of vehicle; **= HOB-01 incubated with RPMI 8226 plus vehicle versus control, $P<0.001$; *MLO-Y4 incubated with RPMI 8226 plus vehicle versus control, $P<0.01$).

8. FIGURES

Figure 1

a



b

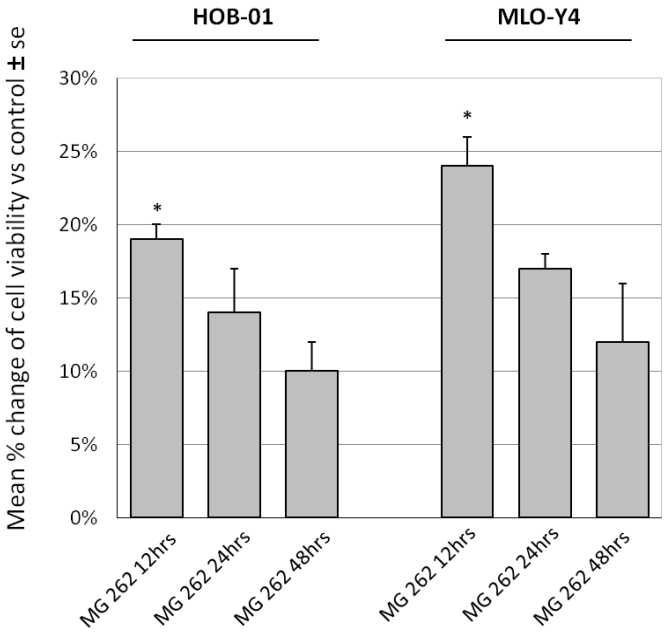


Figure 2

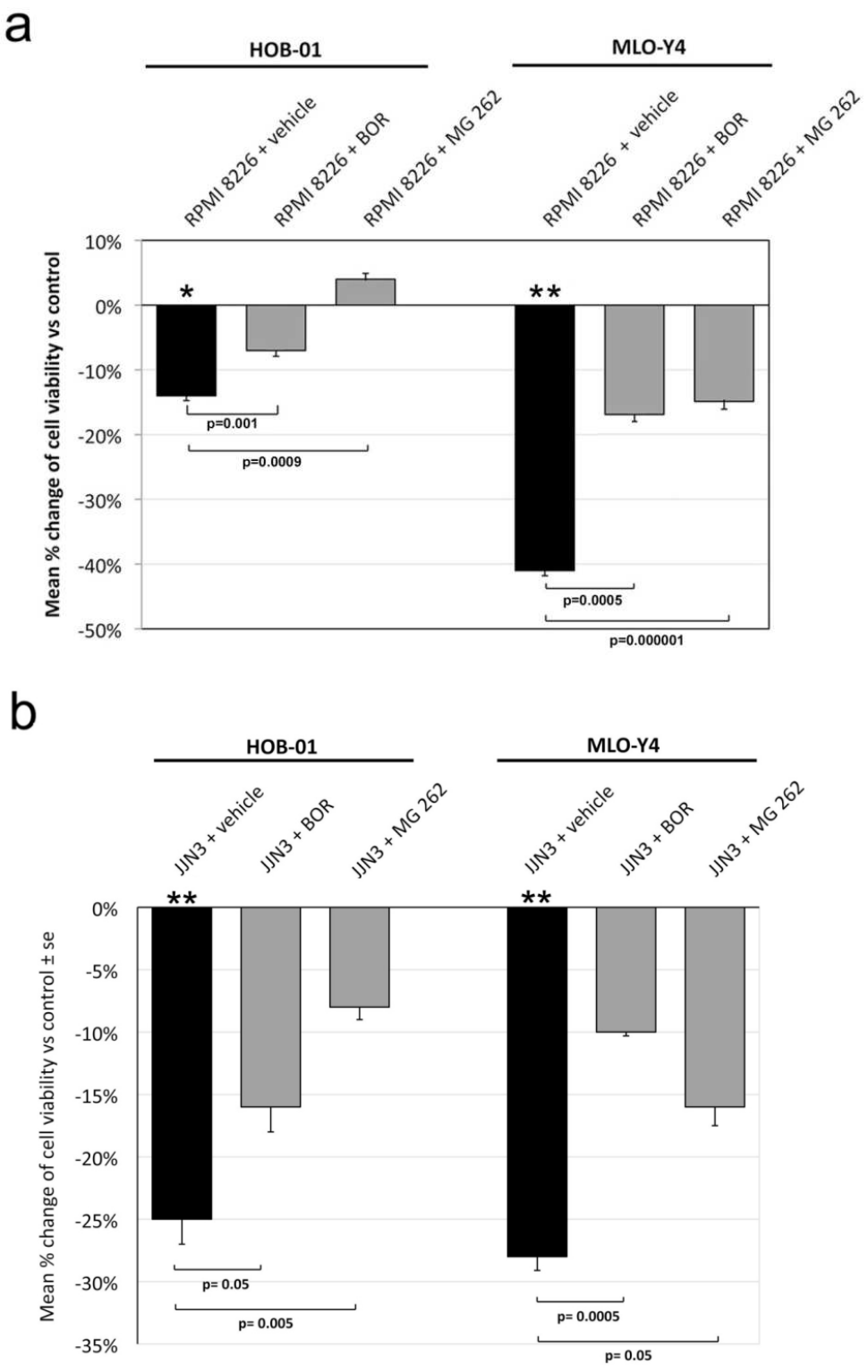

a
HOB-01
MLO-Y4
RPMI 8226 + vehicle
RPMI 8226 + BOR
RPMI 8226 + MG 262
RPMI 8226 + vehicle
RPMI 8226 + BOR
RPMI 8226 + MG 262
Mean % change of cell viability vs control
10%
0%
-10%
-20%
-30%
-40%
-50%
*
**
p=0.001
p=0.0009
p=0.0005
p=0.000001
b
HOB-01
MLO-Y4
JJN3 + vehicle
JJN3 + BOR
JJN3 + MG 262
JJN3 + vehicle
JJN3 + BOR
JJN3 + MG 262
Mean % change of cell viability vs control ± se
0%
-5%
-10%
-15%
-20%
-25%
-30%
-35%
**
**
p= 0.05
p= 0.005
p= 0.0005
p= 0.05

Figure 3

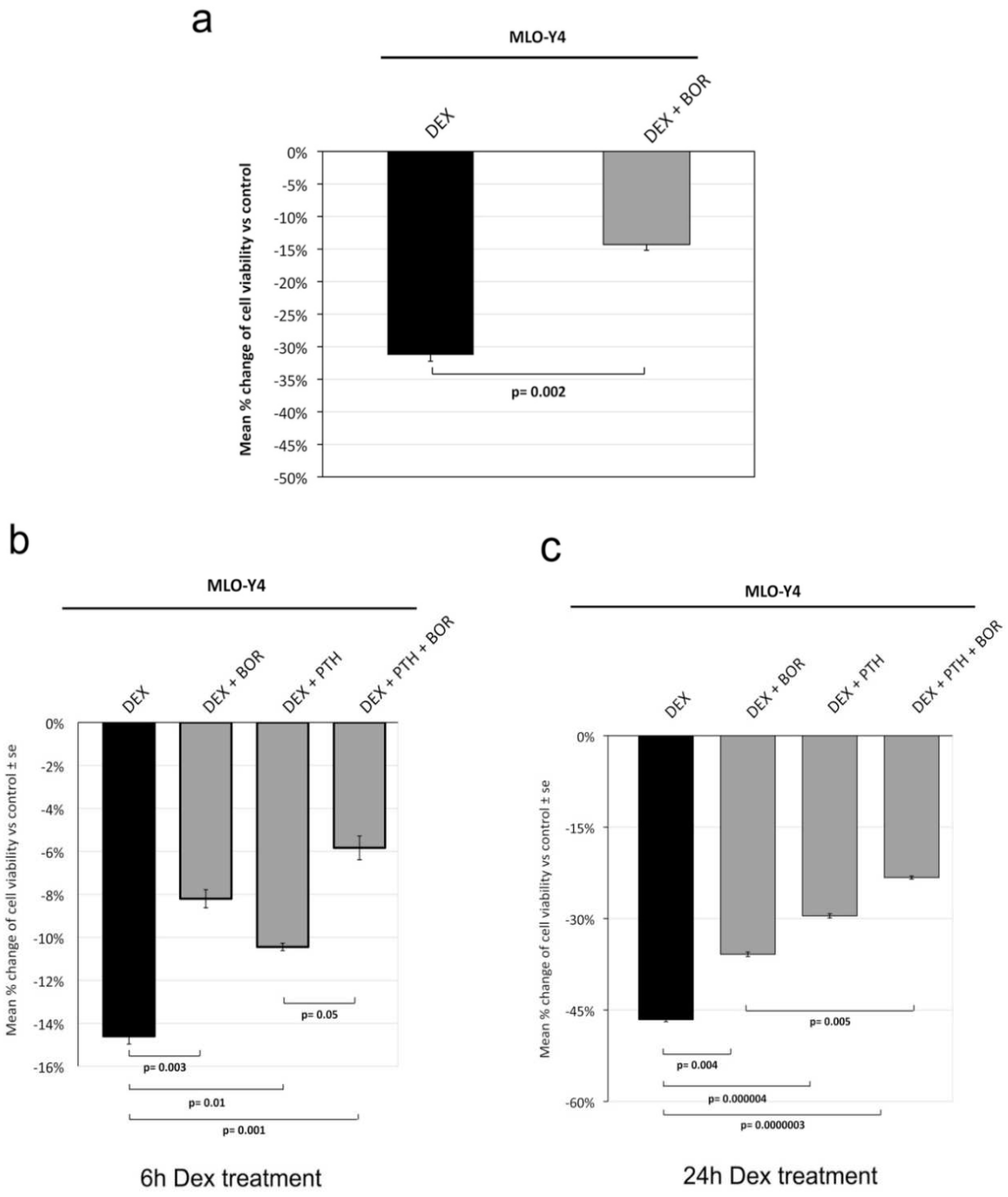
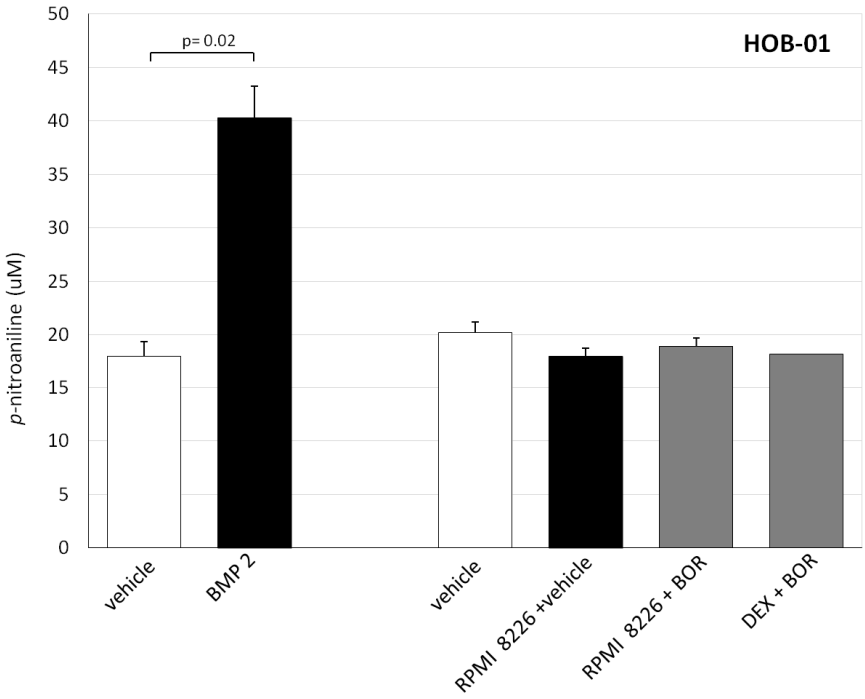

a
MLO-Y4
DEX
DEX + BOR
Mean % change of cell viability vs control
0%
-5%
-10%
-15%
-20%
-25%
-30%
-35%
-40%
-45%
-50%
p= 0.002
b
MLO-Y4
DEX
DEX + BOR
DEX + PTH
DEX + PTH + BOR
Mean % change of cell viability vs control ± se
0%
-2%
-4%
-6%
-8%
-10%
-12%
-14%
-16%
p= 0.003
p= 0.01
p= 0.001
p= 0.05
6h Dex treatment
c
MLO-Y4
DEX
DEX + BOR
DEX + PTH
DEX + PTH + BOR
Mean % change of cell viability vs control ± se
0%
-15%
-30%
-45%
-60%
p= 0.004
p= 0.000004
p= 0.0000003
p= 0.005
24h Dex treatment

Figure 4

a


HOB-01
p= 0.02
p-nitroaniline (uM)
0
5
10
15
20
25
30
35
40
45
50
vehicle
BMP 2
vehicle
RPMI 8226 +vehicle
RPMI 8226 + BOR
DEX + BOR

b

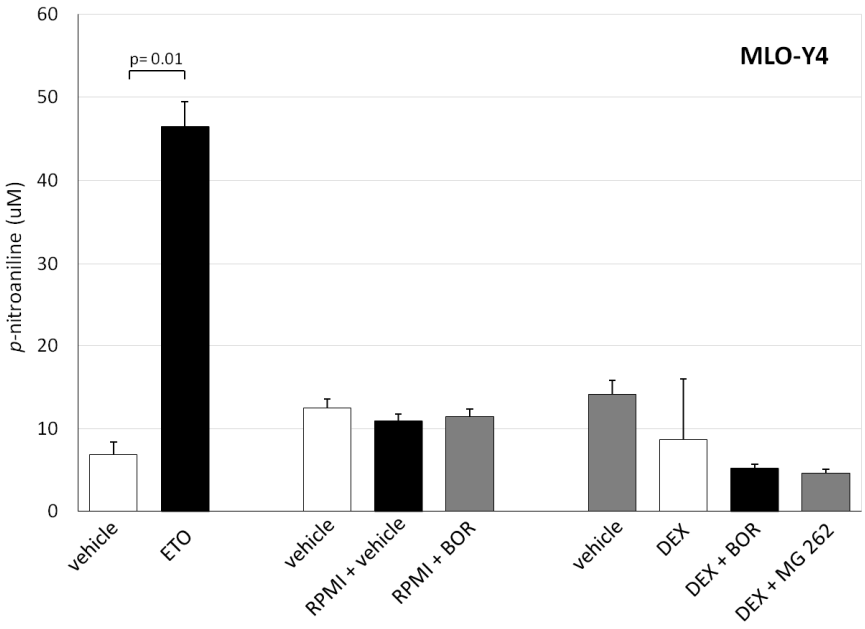

MLO-Y4
p= 0.01
p-nitroaniline (uM)
0
10
20
30
40
50
60
vehicle
ETO
vehicle
RPMI + vehicle
RPMI + BOR
vehicle
DEX
DEX + BOR
DEX + MG 262

Figure 5

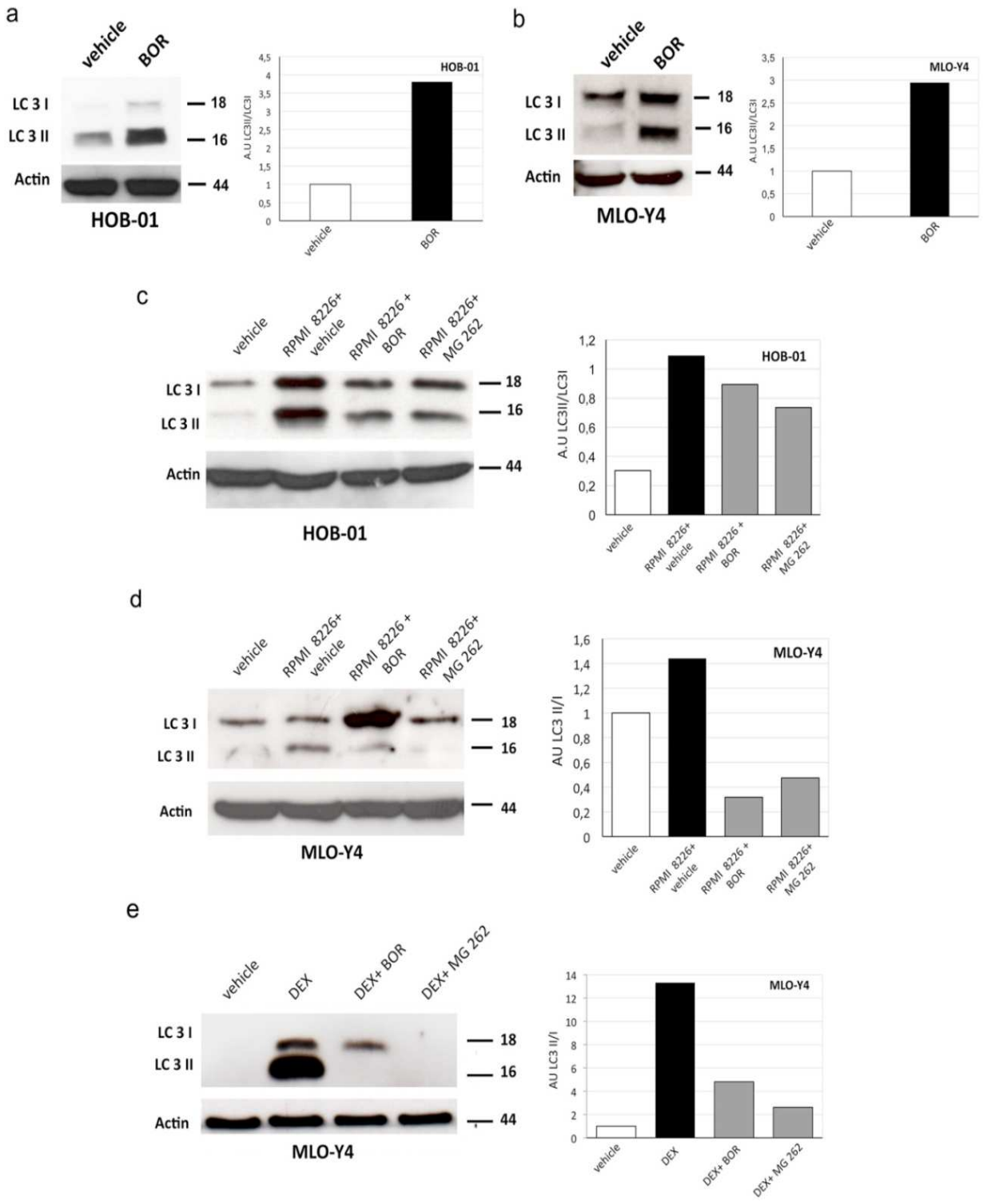
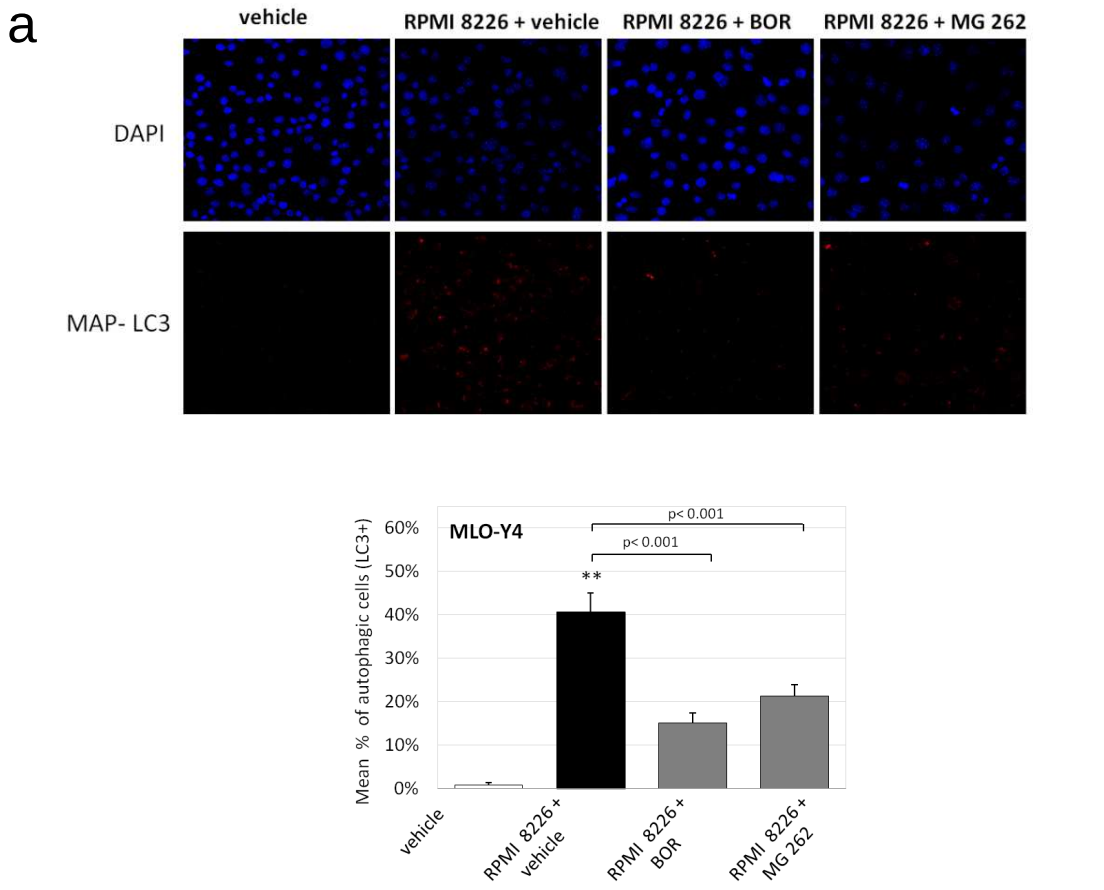

a
vehicle
BOR
LC 3 I
LC 3 II
Actin
18
16
44
HOB-01
A.U LC3II/LC3I
HOB-01
vehicle
BOR
b
vehicle
BOR
LC 3 I
LC 3 II
Actin
18
16
44
MLO-Y4
A.U LC3II/LC3I
MLO-Y4
vehicle
BOR
c
vehicle
RPMI 8226+ vehicle
RPMI 8226 + BOR
RPMI 8226+ MG 262
LC 3 I
LC 3 II
Actin
18
16
44
HOB-01
A.U LC3II/LC3I
HOB-01
vehicle
RPMI 8226+ vehicle
RPMI 8226 + BOR
RPMI 8226+ MG 262
d
vehicle
RPMI 8226+ vehicle
RPMI 8226 + BOR
RPMI 8226+ MG 262
LC 3 I
LC 3 II
Actin
18
16
44
MLO-Y4
AU LC3 II/I
MLO-Y4
vehicle
RPMI 8226+ vehicle
RPMI 8226 + BOR
RPMI 8226+ MG 262
e
vehicle
DEX
DEX+ BOR
DEX+ MG 262
LC 3 I
LC 3 II
Actin
18
16
44
MLO-Y4
AU LC3 II/I
MLO-Y4
vehicle
DEX
DEX+ BOR
DEX+ MG 262

Figure 6


a
vehicle
RPMI 8226 + vehicle
RPMI 8226 + BOR
RPMI 8226 + MG 262
DAPI
MAP- LC3
MLO-Y4
p< 0.001
p< 0.001
**
Mean % of autophagic cells (LC3+)
60%
50%
40%
30%
20%
10%
0%
vehicle
RPMI 8226 + vehicle
RPMI 8226 + BOR
RPMI 8226 + MG 262

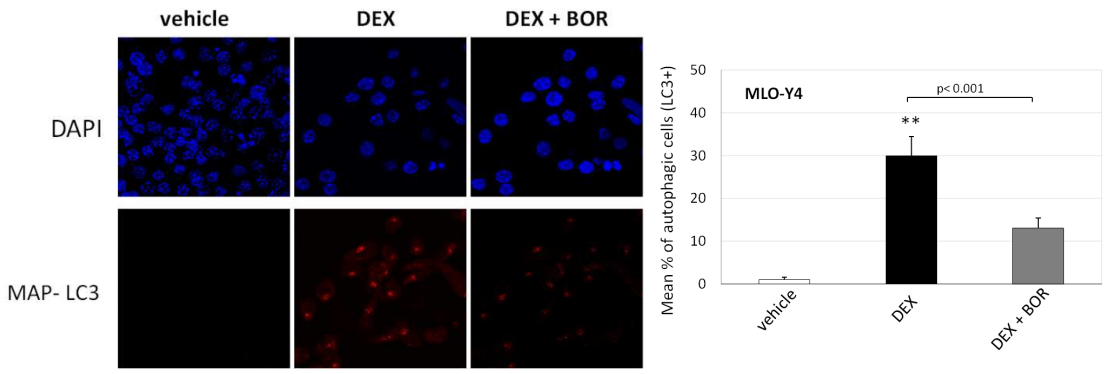
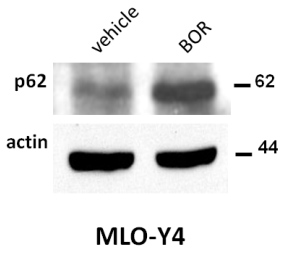
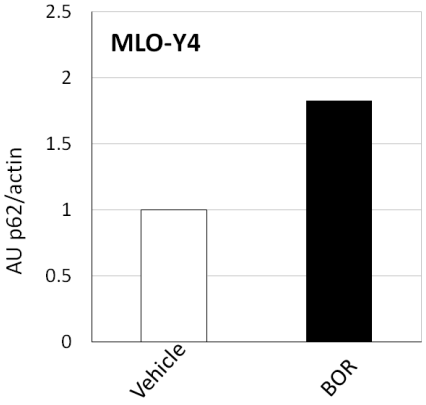

b
vehicle
DEX
DEX + BOR
DAPI
MAP- LC3
MLO-Y4
p< 0.001
**
Mean % of autophagic cells (LC3+)
50
40
30
20
10
0
vehicle
DEX
DEX + BOR

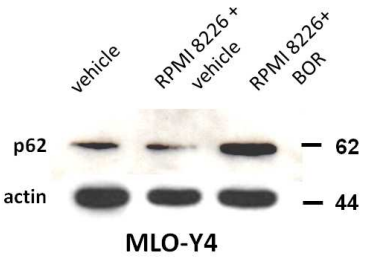
Figure 7

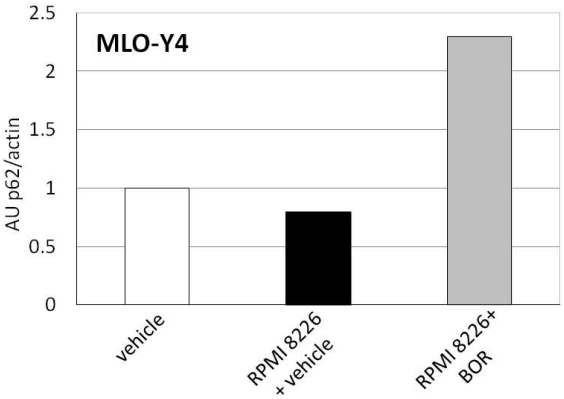
a


vehicle
BOR
p62
62
actin
44
MLO-Y4

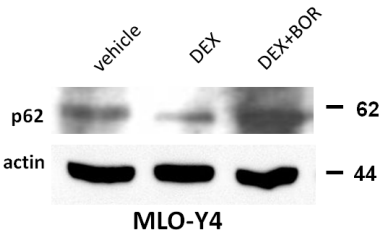

MLO-Y4
AU p62/actin
2.5
2
1.5
1
0.5
0
Vehicle
BOR

b


vehicle
RPMI 8226 + vehicle
RPMI 8226+ BOR
p62
62
actin
44
MLO-Y4


MLO-Y4
AU p62/actin
2.5
2
1.5
1
0.5
0
vehicle
RPMI 8226 + vehicle
RPMI 8226+ BOR

c


vehicle
DEX
DEX+BOR
p62
62
actin
44
MLO-Y4

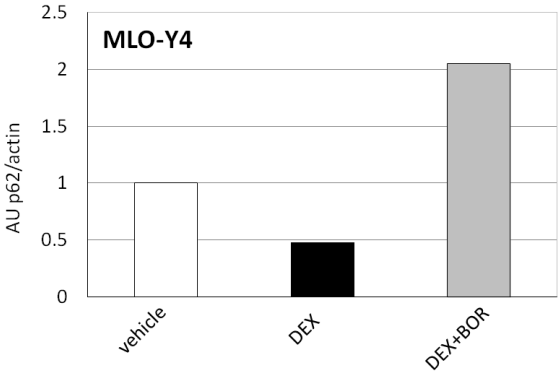
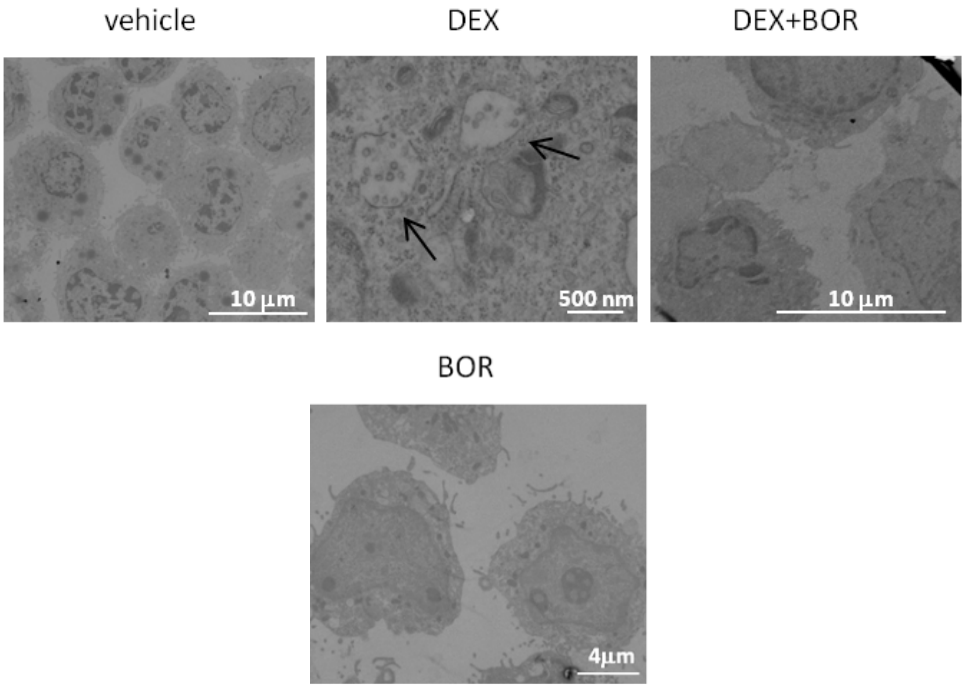

MLO-Y4
AU p62/actin
2.5
2
1.5
1
0.5
0
vehicle
DEX
DEX+BOR

Figure 8

a



b

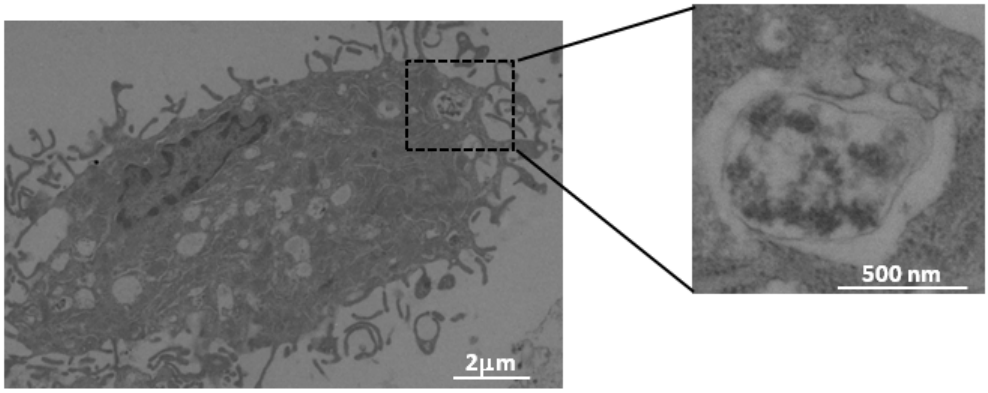
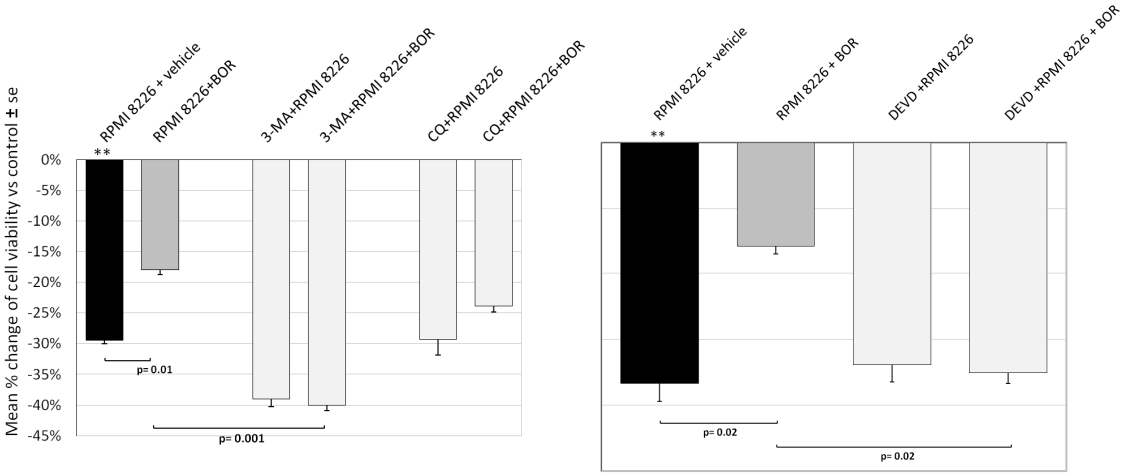


Figure 9

a


RPMI 8226 + vehicle
RPMI 8226+BOR
3-MA+RPMI 8226
3-MA+RPMI 8226+BOR
CQ+RPMI 8226
CQ+RPMI 8226+BOR
Mean % change of cell viability vs control ± se
0%
-5%
-10%
-15%
-20%
-25%
-30%
-35%
-40%
-45%
**
p= 0.01
p= 0.001
RPMI 8226 + vehicle
RPMI 8226 + BOR
DEVD +RPMI 8226
DEVD +RPMI 8226 + BOR
**
p= 0.02
p= 0.02

b

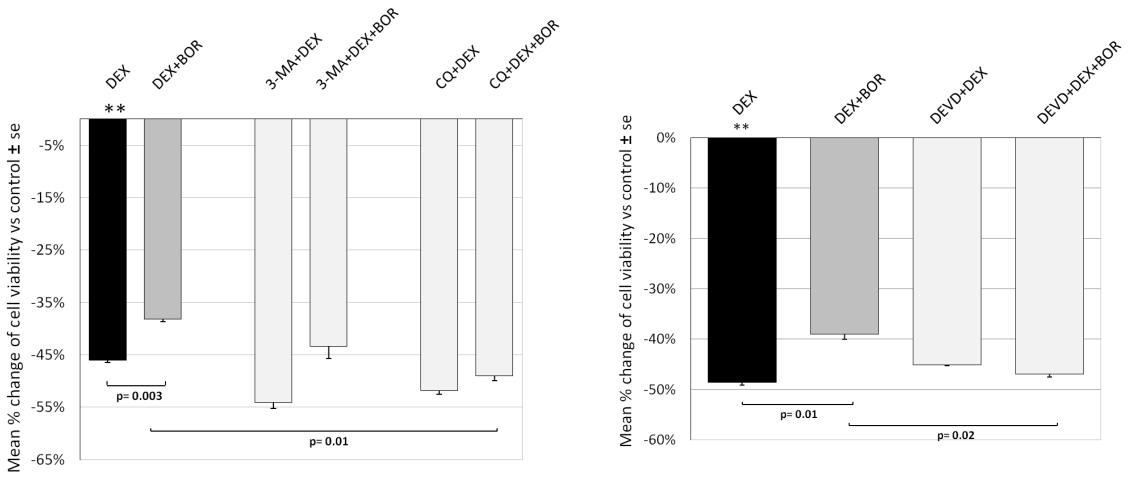

DEX
DEX+BOR
3-MA+DEX
3-MA+DEX+BOR
CQ+DEX
CQ+DEX+BOR
Mean % change of cell viability vs control ± se
-5%
-15%
-25%
-35%
-45%
-55%
-65%
**
p= 0.003
p= 0.01
DEX
DEX+BOR
DEVD+DEX
DEVD+DEX+BOR
0%
-10%
-20%
-30%
-40%
-50%
-60%
**
p= 0.01
p= 0.02

Figure 10

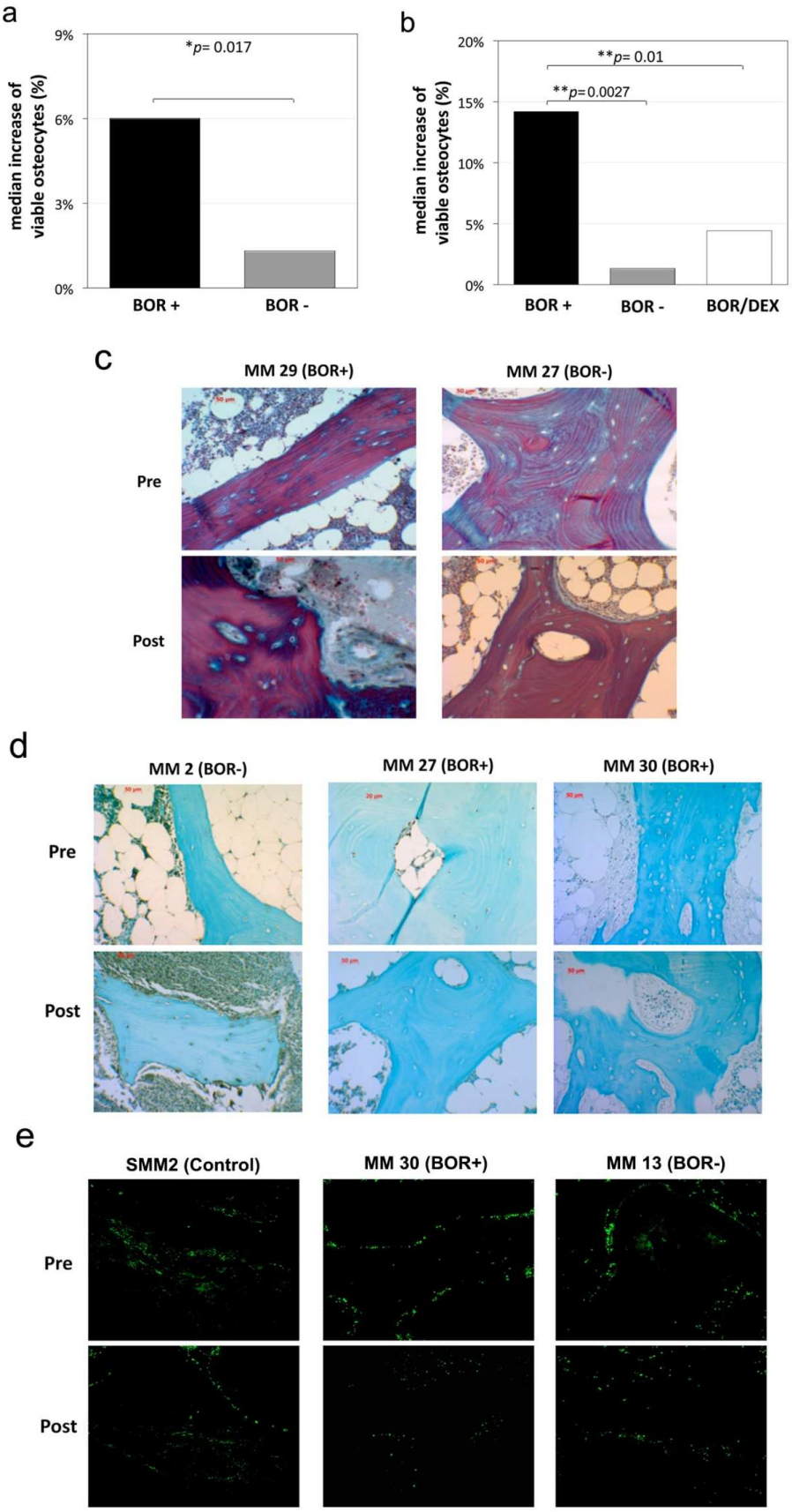
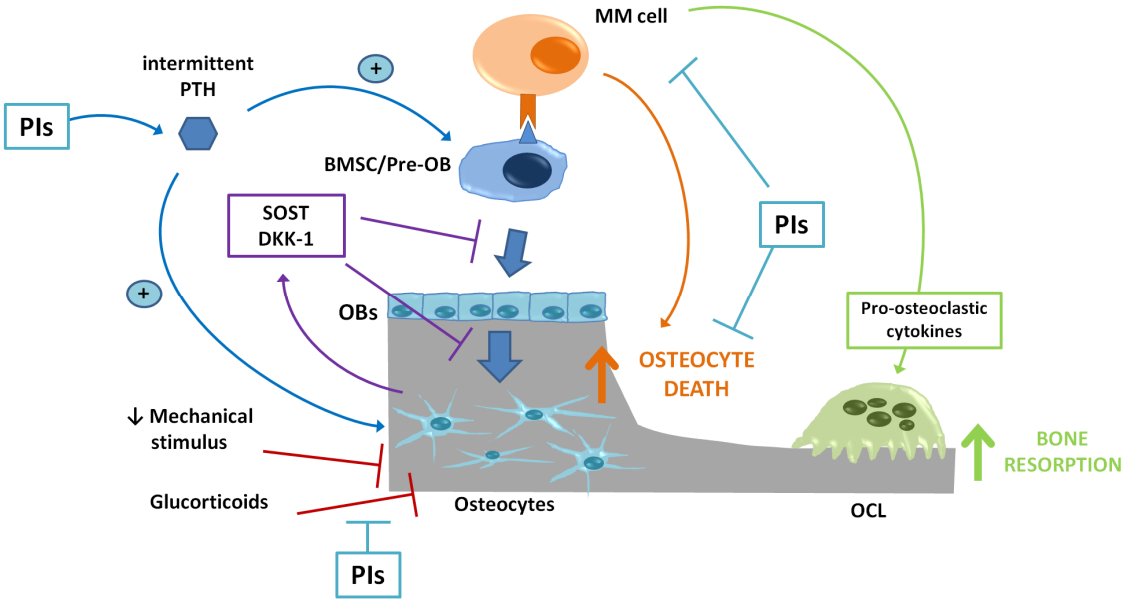
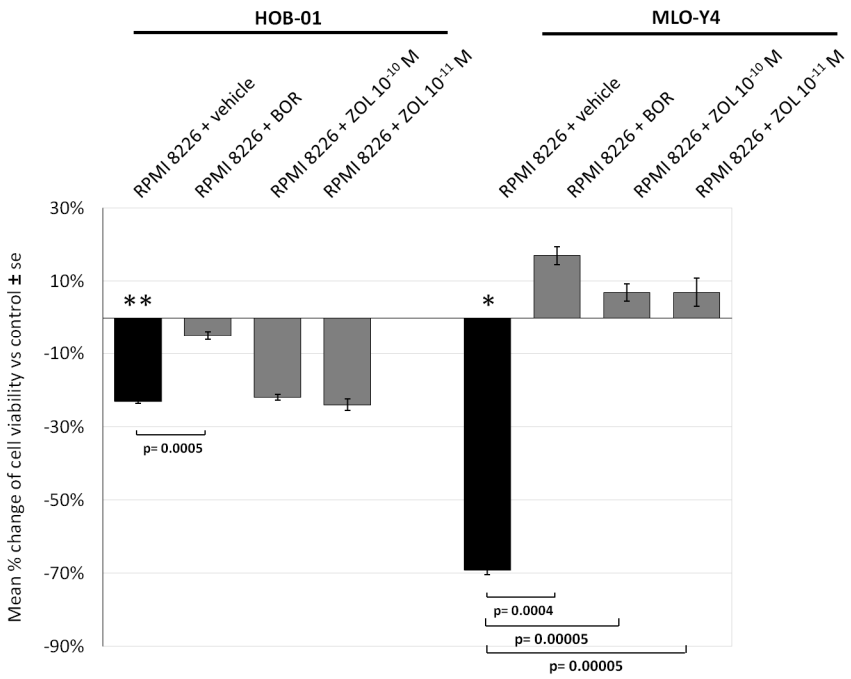

a
9%
6%
3%
0%
*p= 0.017
median increase of viable osteocytes (%)
BOR +
BOR -
b
20%
15%
10%
5%
0%
**p= 0.01
**p=0.0027
median increase of viable osteocytes (%)
BOR +
BOR -
BOR/DEX
c
MM 29 (BOR+)
MM 27 (BOR-)
Pre
Post
d
MM 2 (BOR-)
MM 27 (BOR+)
MM 30 (BOR+)
Pre
Post
e
SMM2 (Control)
MM 30 (BOR+)
MM 13 (BOR-)
Pre
Post

Figure 11

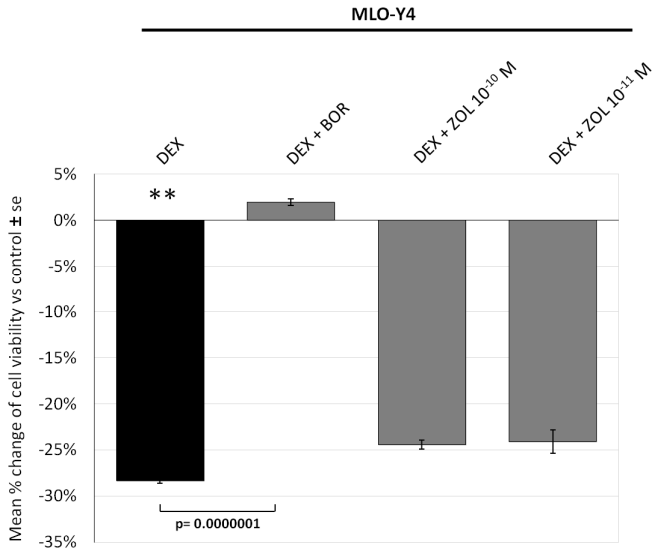

MM cell
intermittent
PTH
+
PIs
BMSC/Pre-OB
SOST
DKK-1
PIs
+
OBs
OSTEOCYTE
DEATH
Pro-osteoclastic
cytokines
↓ Mechanical
stimulus
BONE
RESORPTION
Glucorticoids
Osteocytes
OCL
PIs

Supplementary Figure 1

a



b



Grazie…

Al Professor Giuliani, che mi ha dato fiducia fin dall'inizio, mi ha sgridato quanto lo meritavo, mi ha incoraggiato quando ne avevo bisogno. Non sempre sono stata all'altezza ma ce l'ho messa tutta. Lo ringrazio per avermi dato la possibilità di crescere e imparare.

Al Professor Aversa, direttore della Sezione di Ematologia e Centro Trapianti Midollo Osseo (CTMO), Dip. Medicina Clinica e Sperimentale.

Alla mia amica e compagna di laboratorio Marina, che mi ha "cresciuta" e aiutata ogni giorno, nonostante le difficoltà e il mio brutto carattere. Grazie per quello che mi hai dato dentro e fuori.

A Paola, Daniela, Federica, Valentina M e Valentina F per le risate, l'affetto, il rispetto e l'amicizia che ci lega.

A mamma, papà, Sabrina e Fabio che, nonostante non abbiano ancora capito cosa faccio in laboratorio, mi sono stati vicini anche solo con il pensiero. Sabrina ora è il tuo turno! Metticela tutta!

Al mio Amore, che con tanta pazienza ha preso il brutto di me e lo ha reso bello. E' stato tutto più facile insieme e ora sempre di più. Sei tu la mia gioia.
Finally, as I promised, sorry english.

«Nel momento in cui uno si impegna a fondo, anche la provvidenza allora si muove. Infinite cose accadono per aiutarlo, cose che altrimenti non sarebbero mai avvenute. Qualunque cosa tu possa fare o sognare di fare, cominciala! L'audacia ha in sé genio, potere e magia». (W. Goethe)