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Bortezomib and zoledronic acid on angiogenic and vasculogenic activities of bone marrow macrophages in patients with multiple myeloma

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ABSTRACT

Bone marrow neovascularisation supports plasma cell tumour progression in patients with multiple myeloma (MM), and is partially sustained by bone marrow macrophages through their angiogenic and vasculogenic activities. As such, macrophages may be a target for antivascular treatment in MM. Here, we show that bortezomib (BZ) and zoledronic acid (ZOL) display distinct and synergistic inhibitory effects on cell proliferation, adhesion, migration and expression of angiogenic cytokines (i.e.: VEGF, bFGF, HGF and PDGF). Similar effects were found on capillarogenic organisation and expression of vascular markers in cells which became vasculogenic. VEGFR2 and ERK1/2 phosphoactivation as well as NF-κB activity were also inhibited. Overall these data provide evidence that the exposure of bone marrow macrophages in MM during the treatment with ZOL and BZ, alone and or in combination, impacts their angiogenic and vasculogenic properties, suggesting that these cells may be considered as a target of both drugs in MM patients.

1. Introduction

Multiple myeloma (MM) is characterised by a clonal expansion of malignant plasma cells in the bone marrow, where they proliferate and acquire resistance to apoptosis and eventually lead to osteolysis, renal dysfunction and anaemia.[1] Since the disease mainly progresses in the bone marrow, signals from its microenvironment play a pivotal role in maintaining plasma cell growth, migration and survival.[2] Macrophages are key cells for these signals[3]: they form capillary-like lumina and branching patterns *in vitro*, thus participating to the *de novo* formation of neovessels, and express and release major

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cytokines, i.e. vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), platelet derived growth factor (PDGF) and metalloproteinases, which promote angiogenesis, hence plasma cell growth and invasion. They form capillary-like structures overlapping morphologically those produced by paired endothelial cells (ECs), and contribute to neovessel development through a vasculogenic pathway,[4] that acts as a plasma cell supporter too. Since bone marrow macrophages function as a permissive cell population for plasma cell growth and invasion, they may be a target in MM.

Bortezomib (BZ) and zoledronic acid (ZOL) are currently and widely used in the management of MM. Bortezomib exerts a direct cytotoxic activity on plasma cells by blocking the activation of nuclear factor κB (NF-κB) an inducer of gene transcription,[5,6] and by preventing the activation of caveolin-1 a promoter of plasma cell growth and migration.[7] ZOL inhibits farnesyl diphosphate (FFP) synthase, a key enzyme in the mevalonate pathway, thus preventing prenylation of several proteins, including the GTP-binding Ras, Rho, Rac and Rab[8] leading to cellular apoptosis, antiangiogenesis and activation of gamma/delta T-cells.

In the MM bone marrow environment, BZ and ZOL impact ECs too: BZ activates their angiogenic genes, including *VEGF*, *Interleukin-6 (IL-6)*, *Angiopoietin-2 (Ang-2)* and *insulin-like growth factor (IGF-1)*,[9] while ZOL down-regulates the VEGF/VEGF receptor 2 (VEGFR2) autocrine system through the inhibition of the farnesyl diphosphate synthase.[10] It is thus plausible that the antitumoural activity of BZ and ZOL may also be due to their antiangiogenic properties.

Here we investigated whether ZOL and BZ alone or in combination inhibit the activities of bone marrow macrophages in MM patients. Data suggest that the combination of both drugs at clinically achievable concentrations acts synergistically and impacts several angiogenic and vasculogenic activities.

2. Patients and methods

2.1. Patients and Macrophages

Twenty-two patients fulfilling the International Myeloma Working Group diagnostic criteria for MM[11] were studied at diagnosis. They were 12 M and 10 F, aged 52–74 (median 64.1) and D&S staged[12] as IIA ($n = 6$), IIIA ($n = 14$) and IIIB ($n = 2$); the M-component was IgG ($n = 16$), IgA ($n = 4$), κ or λ ($n = 2$). The study was approved by the local Ethics Committee and all patients provided their informed consent in accordance with the Declaration of Helsinki.

Bone marrow macrophages were obtained as described by Scavelli et al.[4]: aspirates were subjected to immunoselection with an anti-CD14 mAb (Santa Cruz Biotechnology, Santa Cruz, CA)-coated magnetic microbeads (Dynal, Oslo, Norway) after immunodepletion of ECs, plasma cells and dendritic cells. Beads with bound macrophages were transferred into dishes with DMEM complemented with 10% FCS (complete medium) to allow cell detachment and growth to 90% confluence. Cell purity and viability (more than 95% viable cells) were assessed by fluorescence-activated cell sorting, RT-PCR, and trypan blue viable staining.

2.2. Treatment with ZOL and BZ, and preparation of conditioned media (CM) and RNA

ZOL (Zometa®, Novartis, Basel, Switzerland) was dissolved in distilled water and diluted 1–20 μM in the medium, equivalent to the concentrations at sites of active bone resorption after 4 mg i.v. administration in an adult (70 kg) patient.[13] Bortezomib (Velcade®, Millennium Pharmaceuticals Inc., Cambridge, MA) was dissolved in dimethylsulphoxide (DMSO) and diluted 1–20 nM in the medium.

Macrophages were cultured in the medium alone or supplemented with daily addition for 7 days of human recombinant $VEGF_{165}$ (50 ng/ml) and bFGF (10 ng/ml), both from PeproTech (Rocky Hill, NJ). Exposure to VEGF + bFGF (which are major cytokines secreted by plasma cells in the MM microenvironment[2]) induces vasculogenic activities in MM macrophages resulting in the formation of a vascular network and acquirement of EC markers, including factor VIII-related antigen (FVIII-RA), VEGFR2, Tie2/Tek and VE-cadherin.[4] Unexposed ('resting') and exposed ('vasculogenic') macrophages were then treated with ZOL, BZ or their combination for 24 h, with or without pre-treatment with ZOL for 18 h. CM were collected and stored as described in Ref.[14] after a 24 h-culture in serum-free medium (SFM) alone or supplemented with BZ, ZOL or their combination.

Total RNA was extracted with the Trizol reagent (Invitrogen, Life Technologies, Carlsbad, CA), purified using the RNeasy total RNA Isolation Kit (Qiagen, Valencia, CA) and verified for integrity with an Agilent Bioanalyzer (Agilent Technologies, Waldbronn, Germany).

2.3. Functional studies

2.3.1. Proliferation assay

This was performed with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test[15]: cells were plated in 96-well plates (4×10^3/100 μl/well in triplicate) and exposed to ZOL, BZ or their combination, then treated with MTT (10 μl) on the last 4 h of 48-h and 72-h cultures, and absorbance measured at 570/690 nm.

2.3.2. 'Wound' assay

Macrophages grown in triplicate on fibronectin (10 μg/ml)-coated 10 cm^2 dishes (1×10^6/dish) were scraped as a 'wound' with a P200 pipette tip in the middle of the monolayer, and exposed for 24 h to a complete medium alone (positive control) or to the medium containing ZOL, BZ or their combination, fixed (Diff-Quik staining, Dade Behring, Dudingen, Switzerland), and quantified for their motility rate by counting migrated cells into the wound area in at least three randomly-chosen 10× fields across the wound length as described in Ref.[15]

2.3.3. Adhesion assay

Macrophages were plated (4×10^3 cells/well in triplicate) in 96-well fibronectin (10 μg/ml)-coated plates in SFM alone (positive control) or medium containing ZOL, BZ, or the combination for 90 min at 37 °C in 5% CO_2 humidified atmosphere, then fixed at 90 min with 2.5% glutaraldehyde, and counted as in the proliferation assay.[15]

2.3.4. Capillarogenesis assay

Vasculogenic macrophages were plated in Matrigel® (300 μl/well, Becton Dickinson, San Jose, CA)-coated 24-well plates (2×10^5 cells/well in duplicate) in 1 ml complete medium. Cells were exposed to ZOL, BZ or their combination, and after 24 h the skeletonisation of the mesh was followed by measurement of its topological parameters, i.e. 'mesh areas', 'length' and 'branching points' with a computed image analysis as described in Ref.[4]

2.4. Reverse transcriptase-polymerase chain reaction (RT-PCR) and real-time RT-PCR

Two micrograms of total RNA were reverse transcribed by Moloney murine leukaemia virus-reverse transcriptase (MMLV-RT, Invitrogen Corp., Carlsbad, CA), and 1 μg of cDNA was subjected to PCR with primers (Invitrogen Corp.) shown in Table 1. The PCR products were separated and stained, and bands were measured as numbers of pixels as described in Ref.[15]

Real-time RT-PCR was performed using the StepOne™ Real-Time PCR System (Applied Biosystems, TaqMan® Gene Expression Assays, Foster City, CA) added with the SYBR Green PCR master mix (Applied Biosystems), and the following primers (Invitrogen Corp., forward/reverse): *VEGF*, 5′-AAGGAGGAGGGCAGAATCAT-3′/5′-CCAGGCCCTCGTCATTG-3′; *bFGF*, 5′-CCCGACGGCCGAGTTGAC-3′/5′-CACATTTAGAAGCCAGTAATCT-3′; *HGF*, 5′-AGAAATGCAGCCAGCATCATC-3′/5′-CACATGGTCCTGATCCAATCTTT-3′; *PDGF-BB*, 5′-CTTTAAGAAGGCCACGGTGA-3′/5′-TCCAAGGGTCTCCTTCAGTG-3′; and control housekeeping gene *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), 5′-CGACACCCACTCCTCCACCT-3′/5′-GAGGTCCACCACCCTGTTGC-3′. The PCR profile was: 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Data were analysed with the Sequence Detection System software (Applied Biosystems). The average threshold cycle (Ct) of *GAPDH* was subtracted from the average Ct of each gene to yield the ΔCt. The ΔCt of the basal (medium) macrophages was then subtracted from the ΔCt of cells exposed to ZOL and BZ, alone and together, to obtain the ΔΔCt as described in Ref.[10]

2.5. Western blot and enzyme-linked immunosorbent assay (ELISA)

Western blot[15] evaluated the content of phosphorylated (p) VEGFR2 and pERK1/2 in cell extracts without (medium) and with exposure to ZOL, BZ alone or together in vasculogenic macrophages. Proteins (40 μg) subjected to 8% SDS–polyacrylamide gel electrophoresis, electrotransferred to a polyvinylidene difluoride membrane, incubated with primary and secondary antibodies (AbCam, Cambridge, UK and Santa Cruz Biotechnology Inc., Santa Cruz, CA) were then visualised by enhanced chemiluminescence with Kodak Biomax

Table 1 – RT-PCR primers, amplification and products.

Gene sequence of primers (to)	Amplification conditions	Product length (base pairs)
VEGF-A Forward: CAGAACAGTCCTTAATCCAG Reverse: CATGCCAGAGTCTCTCATCT	35 Cycles at 1 min and 30 s, 1 min, 30 s, 1 min	443
VEGFR-2 Forward: CCGTCAAGGGAAAGACTACG Reverse: CTTTACCCCAGGATATGGAG	35 Cycles at 1 min and 30 s, 30 s, 30 s, 1 min	496
bFGF Forward: CTCACGTGGCACCAGTGGAT Reverse: CACAGAGGATGAATAGTAGC	35 Cycles at 1 min and 30 s, 1 min, 45 s, 1 min	918
FVIII-RA Forward: GTTCGTCCTGGAAGGATCGG Reverse: CACTGACACCTGAGTGAGAC	35 Cycles at 1 min and 30 s, 45 s, 45 s, 1 min	696
Tie-2/Tek Forward: TTACGGGCCAGATTGTAAGC Reverse: CATCCCCAAAAGTAAGGCTCA	35 Cycles at 1 min and 30 s, 1 min, 45 s, 1 min	502
VE-cadherin Forward: ACGGGATGACCAAGTACAGC Reverse: ACACACTTTGGGCTGGTAGG	35 Cycles at 1 min and 30 s, 1 min, 45 s, 1 min	592
PDGF-BB Forward: GATCCGCTCCTTTGATGATC Reverse: GTCTCACACTTGCATGCCAG	35 Cycles at 3 min, 1 min, 1 min, 1 min	435
HGF Forward: CTCAGATCAGTATCTAATTG Reverse: GACATGACTCTACCCTGTTC	38 Cycles at 1 min and 30 s, 1 min, 45 s, 1 min	1375
GAPDH Forward: CCCTCCAAAATCAAGTGGGG Reverse: CGCCACAGTTTCCCGGAGGG	22 Cycles at 1 min and 30 s, 45 s, 45 s, 30 s	347

film (Eastman Kodak Co., Rochester, NY), and their band intensity expressed as fold expression of the medium value by arbitrary optical density (OD) units using the Kodak Molecular Imaging Software.

CM (50 μl) were tested in triplicate with an ELISA (Human Angiogenesis Array 2, SearchLight, Pierce, Rockford, IL) for quantification of VEGF, bFGF, HGF and PDGF.

2.6. Measurement of NF-κB activation

Confluent vasculogenic macrophages were treated with ZOL, BZ and their combination for 4 h, then stimulated with tumour necrosis factor-α (TNF-α, 10 ng/ml) on the last 20 min. NF-κB activation was measured in nuclear extracts with an ELISA system (TransAM NF-κB p65, Active motif, Belgium): cells washed with ice-cold PBS were lysed with dithiothreitol and a protease inhibitor cocktail, centrifuged (14,000 rpm at 4 °C for 20 min), and supernatant (containing the nuclear extract) measured for protein concentration (Bradford assay). Next an oligonucleotide of the NF-κB consensus binding site (5′-GGGACTTTCC-3′) specific for the NF-κB active form was immobilised to a 96-well plate, and duplicate wells were filled with 1 μg of the nuclear extracts for 1 h, then incubated 1-h with a primary antibody to an epitope of the p65 subunit accessible only in activated NF-κB and bound to its target DNA. A horseradish peroxidase-conjugated secondary antibody was added, and reading, expressed as percentage of control, made by a spectrophotometer at 450 nm.

2.7. Statistical analysis

Statistical significance of differences in drug-treated versus control cultures was determined using the Wilcoxon signed-rank test. The minimal level of significance was $P < 0.05$. The synergism ZOL-BZ was analysed by isobologram analysis using the CalcuSyn software program (Biosoft, Ferguson, MO), according to the Chou–Talalay method[16] based on the equation: combination index (CI) = (D)1/(Dx)1 + (D)2/(Dx)2 + (D)1 (D)2/(Dx)1(Dx)2, where (D)1 and (D)2 are the doses of drug1 and drug2 that have x effect when used in combination, and (Dx)1 and (Dx)2 are the doses of drug1 and drug2 that have x effect when used alone. A CI < 1 indicates synergism, whereas >1 indicates additive effects.

3. Results

3.1. Inhibition of resting macrophages

3.1.1. Proliferation

MM macrophages were treated with ZOL (1, 2.5, 5, 10, 20 μM) and BZ (1, 2.5, 5, 10, 20 nM) both separately and in combination (Fig. 1). Proliferation was reduced by both drugs in a dose-dependent fashion with BZ exerting greater inhibition (panel A). The combination of BZ (20 nM) and ZOL (20 μM) in the 1:1000 ratio after a 18-h pre-treatment with ZOL produced the highest significant synergistic inhibition ($P < 0.005$ or better, Wilcoxon signed-rank test; CI: 0.77 or lower). The most effective dose–response relationship was found in a range between the BZ:ZOL 5 nM:5 μM (93% of control for ZOL; 79% for BZ; 46% for the combination; $P < 0.001$, CI: 0.45) and 10 nM:10 μM (65% for ZOL; 47% for BZ; 28% for combination $P < 0.005$, CI: 0.67). A synergistic effect was also evident at lower doses: BZ:ZOL 2.5nM:2.5 μM (96% for ZA; 91% for BZ; 48% for combination; CI: 0.55) and 1 nM:1 μM (98% for ZOL; 93% for BZ; 64% for the combination; CI: 0.58).

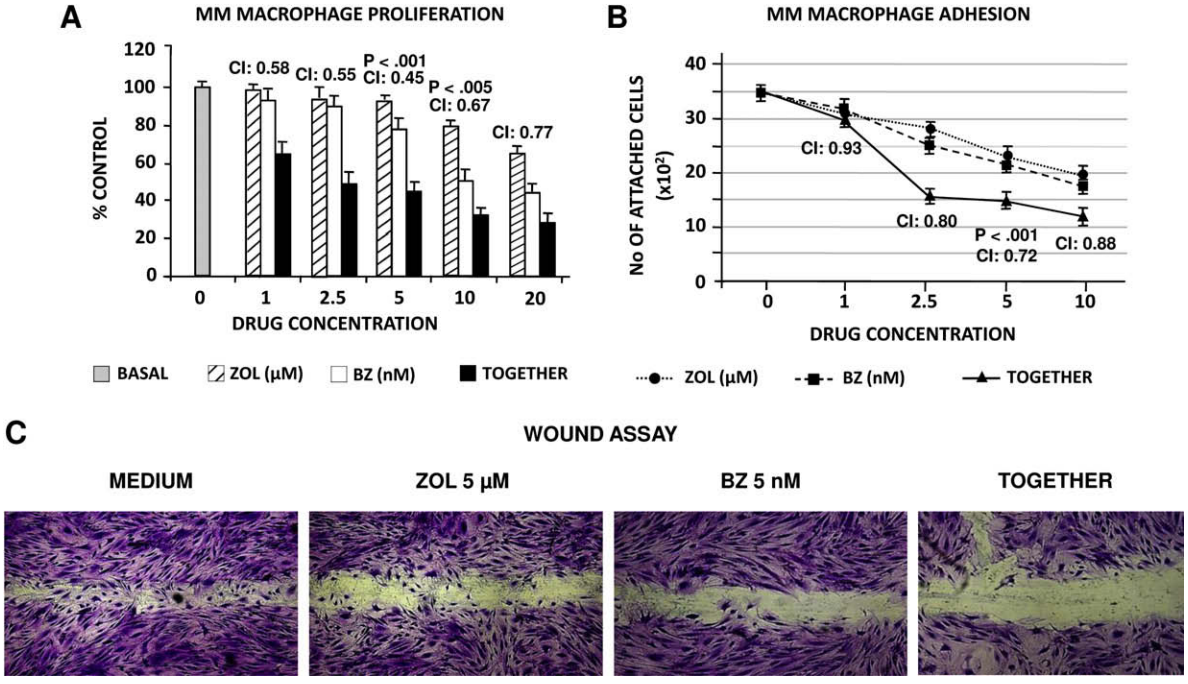

Fig. 1 – Inhibitory effect of different doses of ZOL and BZ, alone and together, on MM bone marrow macrophages. Inhibition of (A) proliferation, (B) adhesion and (C) migration in the 'wound'. Cell counts are presented as mean ± 1 SD for the group of patients. Synergistic inhibition is indicated by CI < 1. Significance of changes was assessed by the Wilcoxon signed-rank test.

3.1.2. *Adhesion*

Both drugs inhibited adhesion in a dose-dependent fashion both singularly and together in a synergistic manner (panel B). The most effective dose-response relationship was found in the same ranges as in the proliferation test. The combined BZ:ZOL 5 nM:5 μM gave an average of 1500 attached macrophages (–58% of the control, CI: 0.72) while ZOL and BZ alone gave 2300 (–35% of the control) and 2100 (–40%) cells, respectively ($P < 0.01$).

3.1.3. *Wound assay*

MM macrophage migration was influenced by the drugs (panel C). Doses were in the middle range of the best results obtained from the previous tests, i.e. 5 μM ZOL and 5 nM BZ. A significant and synergistic lowering of cell migration was produced by the combined drugs: ZOL 37 ± 6 cells (–40% of control), BZ 25 ± 9 cells (–57%, $P < 0.005$), together 11 ± 4 cells (–77%, $P < 0.001$, CI: 0.45).

3.1.4. *Angiogenic cytokine modulation*

Fig. 2 (panel A) shows that MM macrophages treated with ZOL 5 μM and BZ 5 nM down-regulated VEGF (–10% of control RT-PCR OD, on average, for ZOL, –46% for BZ), bFGF (–33% for ZOL, –71% for BZ), HGF (+36% for ZOL, –43% for BZ) and PDGF (+23% for ZOL, –21% for BZ). The combined treatment, again, gave the strongest effect: –79% for VEGF, –96% for bFGF, –60% for HGF ($P < 0.005$ or better), –35% for PDGF. Real-time RT-PCR (panel B) showed significant inhibition by both drugs and the highest, synergistic inhibition with the combination. The most prominent effects were seen with bFGF and HGF (–70% and –75%, respectively, $P < 0.001$; CI: 0.56 and 0.78).

3.1.5. *ELISA*

The drugs alone and especially together significantly lowered the cytokine content in the CM of MM macrophages, with VEGF and bFGF being maximally affected (panel C, –76% and –72% of control, respectively; $P < 0.001$).

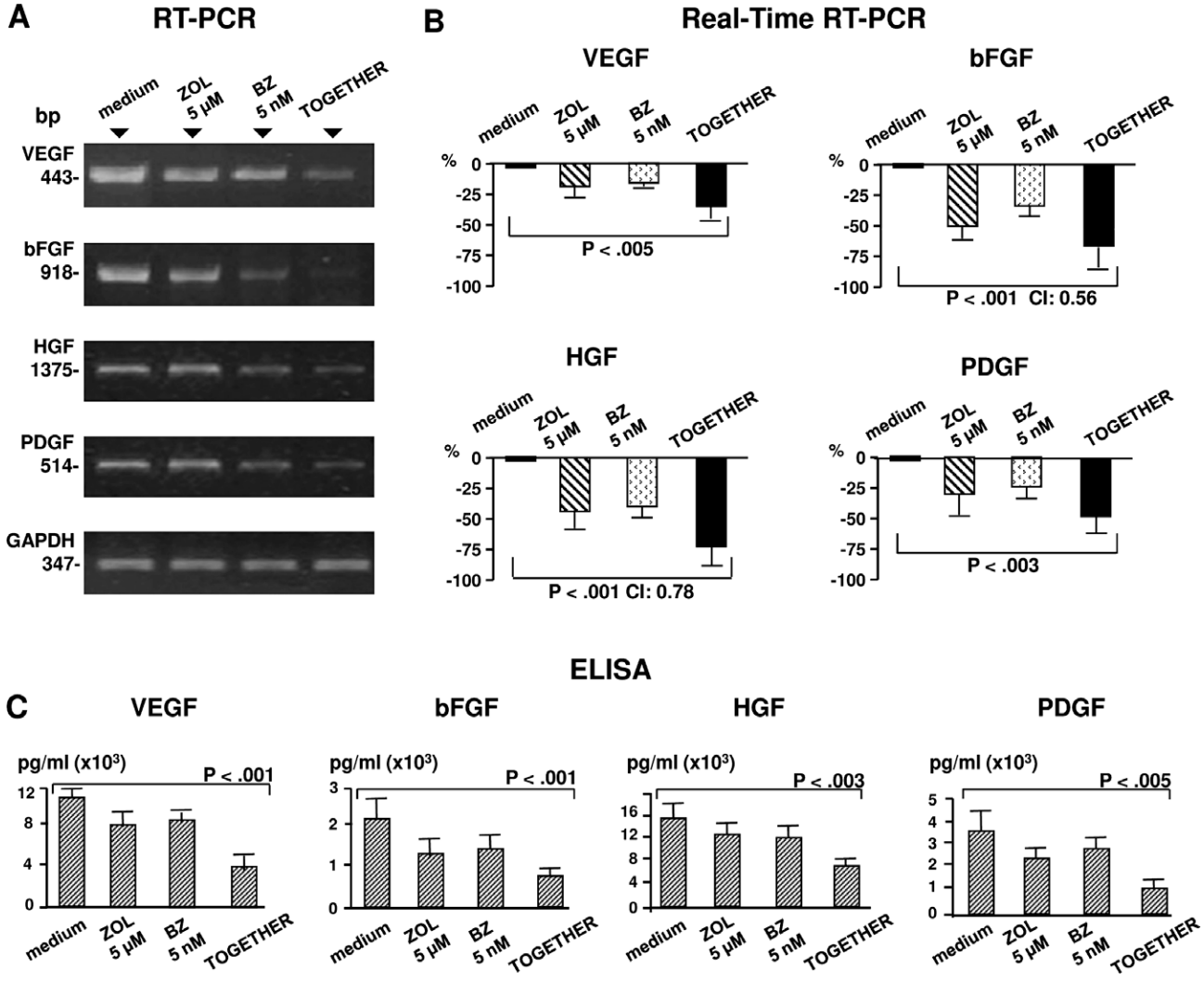

Fig. 2 – Expression levels of *VEGF*, *bFGF*, *HGF* and *PDGF* genes in MM macrophages of a representative patient without (medium) and with exposure to ZOL and BZ, as evaluated by the (A) RT-PCR and (B) Real-Time RT-PCR. Data are expressed as mean ± 1 SD for the group of patients of percentage of inhibition compared to the medium value. Significance of changes was assessed by the Wilcoxon signed-rank test. bp = Base pairs; *GAPDH* = glyceraldehyde-3-phosphate dehydrogenase. (C) ELISA analysis of the indicated cytokines secreted into the cell conditioned medium: synergistic inhibition is indicated by CI < 1. Significance of changes was assessed by the Wilcoxon signed-rank test.

Overall, the results provide direct evidence that ZOL and BZ, especially in combination, inhibit key functions of MM macrophages needed for their angiogenic and vasculogenic activity.

3.2. *Inhibition of vasculogenic macrophages*

3.2.1. *In vitro* neovascularisation on matrigel

Vasculogenic MM macrophages produce a closely knit capillary network on Matrigel® with thin, branching and anastomosing tubes linked through numerous junctions.[4] Capillarogenesis of this type was slightly inhibited by ZOL 5 μM and BZ 5 nM, while the drugs together gave the highest inhibitory effect leading to a poorly organised mesh with straight few tubes and junctions (Fig. 3). The drugs together inhibited the mesh topological parameters by –72% to –87% versus –35% to –61% of the drugs alone ($P < 0.005$ or better), and the branching activity synergistically (CI: 0.69).

3.2.2. *Vasculogenic markers*

Vasculogenic MM macrophages express the endothelial cell (EC) markers FVIII-RA, VEGFR2, Tie2/Tek and VE-cadherin, which implies their transdifferentiation into EC-like cells.[4] We found significant down-regulation of all these markers (especially FVIII-RA and VEGFR2) by the combined treatment (Fig. 4).

3.2.3. *Inhibition of VEGFR2 and ERK1/2 phosphorylation*

VEGFR2, a key receptor for vasculogenic activities of macrophages,[4] and the downstream extracellular signal-regulated

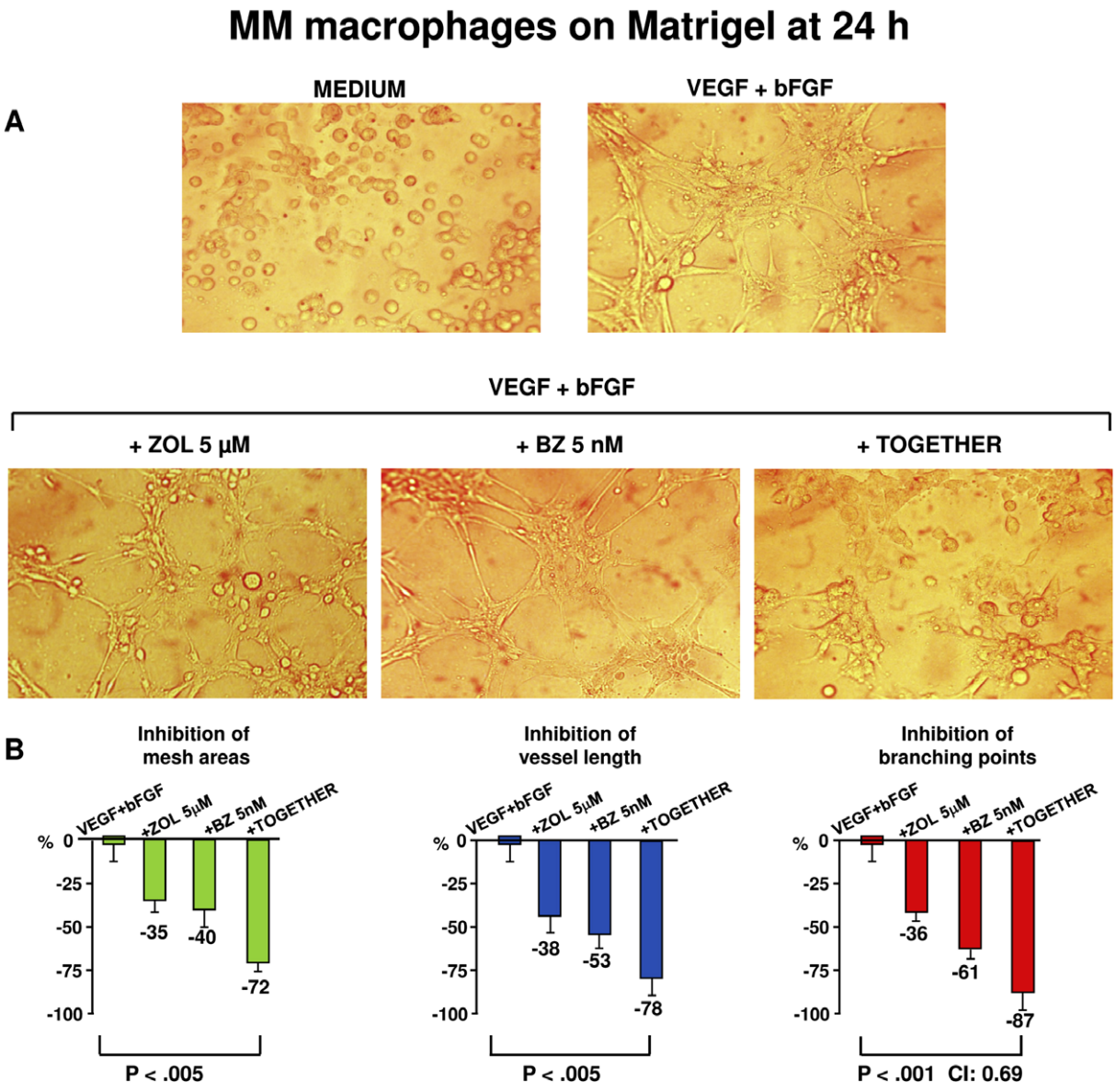

Fig. 3 – Capillarogenesis on Matrigel. (A) Bone marrow vasculogenic macrophages of a representative patient were seeded on Matrigel and exposed to ZOL and BZ, alone and together. After a 24-h incubation, their 3-D organisation was examined planimetrically by computer image analysis. (B) Bars represent the mean ± 1 SD of percentage of inhibition of the indicated topological parameters. Synergistic inhibition is indicated by CI < 1. Significance of changes was assessed by the Wilcoxon signed-rank test.

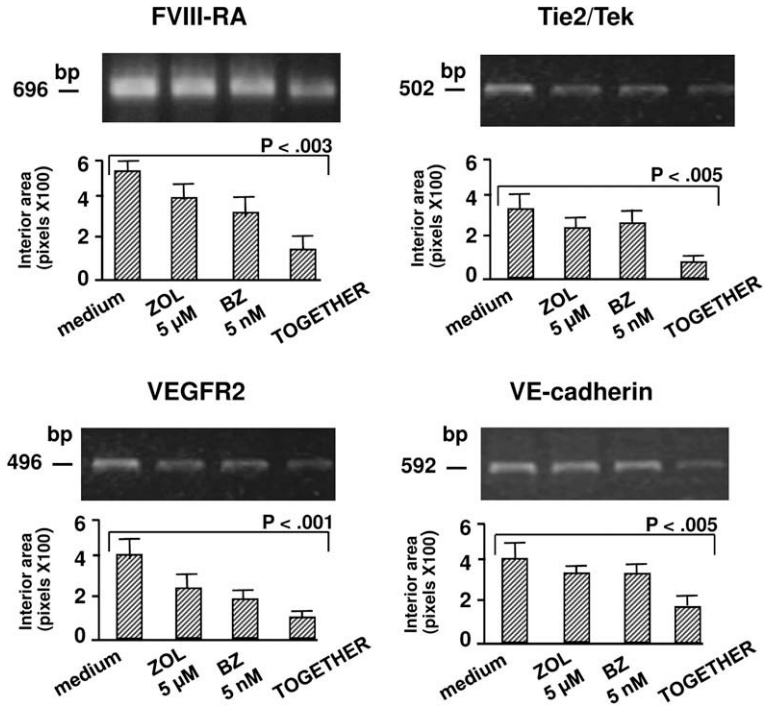

Fig. 4 – Expression levels of the indicated vascular markers in bone marrow macrophages of a representative MM patients after exposure to ZOL and BZ, as evaluated by the RT-PCR. Bars represent mean ± 1 SD for the group of patients of percentage of inhibition compared to the medium value. Significance of changes was assessed by the Wilcoxon signed-rank test. bp = Base pairs.

kinase (ERK)1/2, which is involved in the Ras-pathway and needed for VEGFR2 signalling transduction,[17] showed on the Western blot a significant reduction of their phosphorylation (p) upon the ZOL and BZ treatment, maximally when combined. BZ was more effective, while the drugs together were additive for pVEGFR2 and synergistic for pERK1/2 (Fig. 5, panel A).

3.2.4. *NF-κB activity*

NF-κB pathway plays a pivotal role in regulating cell growth and survival.[18] By studying the effects on NF-κB p65 DNA-binding activity in nuclear extracts, we found (panel B) that drugs alone minimally affected the NF-κB p65 expression, while together they gave significant synergistic inhibition (–4% ZOL versus the medium, –16% BZ, and –45% together; $P < 0.001$, CI: 0.76).

4. Discussion

Most human malignancies result from multiple aberrant pathways that are not easily reversed by a single agent. Bortezomib (BZ) induces, respectively, a 7% and 31% complete/partial remission in patients with MM[19] while its combination with Doxil,[20] thalidomide[21] or melphalan[22] improves the response rates, suggesting that in addition to its direct pro-apoptotic activity on plasma cells, it also enhances cell responses to other agents.[23] Zoledronic acid (ZOL) is used for the management of bone disease in MM[24]; its direct/indirect clinical antitumour activities leading to improved disease-free survival (DFS) and reduced recurrences have been well described in recent publications in adjuvant breast cancer,[25] advanced disease[26] and MM.[27] Here, we tested their effect on the MM patients' bone marrow macrophages, focusing on the cell angiogenic[2] and vasculogenic properties[4] which favour disease progression. MM macrophages induce angiogenesis via secretion of VEGF, bFGF, HGF and PDGF,[2] and build neovessels themselves via vasculogenic mimicry in which proliferation, adhesion, migration and VEGFR2/VE-cadherin expression play a key role.[4] Also, VEGF, bFGF and HGF support plasma cell growth, migration and survival,[28] while high local VEGF levels suppress the antiproliferative effect of chemotherapeutic agents and promote multidrug resistance.[29]

We demonstrated that ZOL and BZ synergistically impact MM macrophage proliferation, adhesion and migration, as well as VEGF, bFGF, HGF and PDGF secretion. The drugs synergistically inhibit macrophage vasculogenesis on Matrigel, and the expression the FVIII-RA, Tie2/Tek and VEGFR2/VE-cadherin, indicative of cell transdifferentiation into EC-like cells.

To better understand the molecular mechanisms of this synergistic activity, we analysed the phosphoactivation of VEGFR2 and ERK1/2, and the NF-κB activity. Interaction between VEGF and VEGFR2 rapidly phosphorylates this receptor and the downstream ERK1/2.[30] Here we show that both drugs reduce phosphoactivation of VEGFR2 and ERK1/2 and NF-κB activity, all these actions being more effective with the combined treatment.

The more relevant effects of the combined drugs are probably due to their separate mechanisms of action. Only nitrogen-containing bisphosphonates, such as ZOL, act by inhibiting farnesyl pyrophosphate (FPP)-synthase, a key

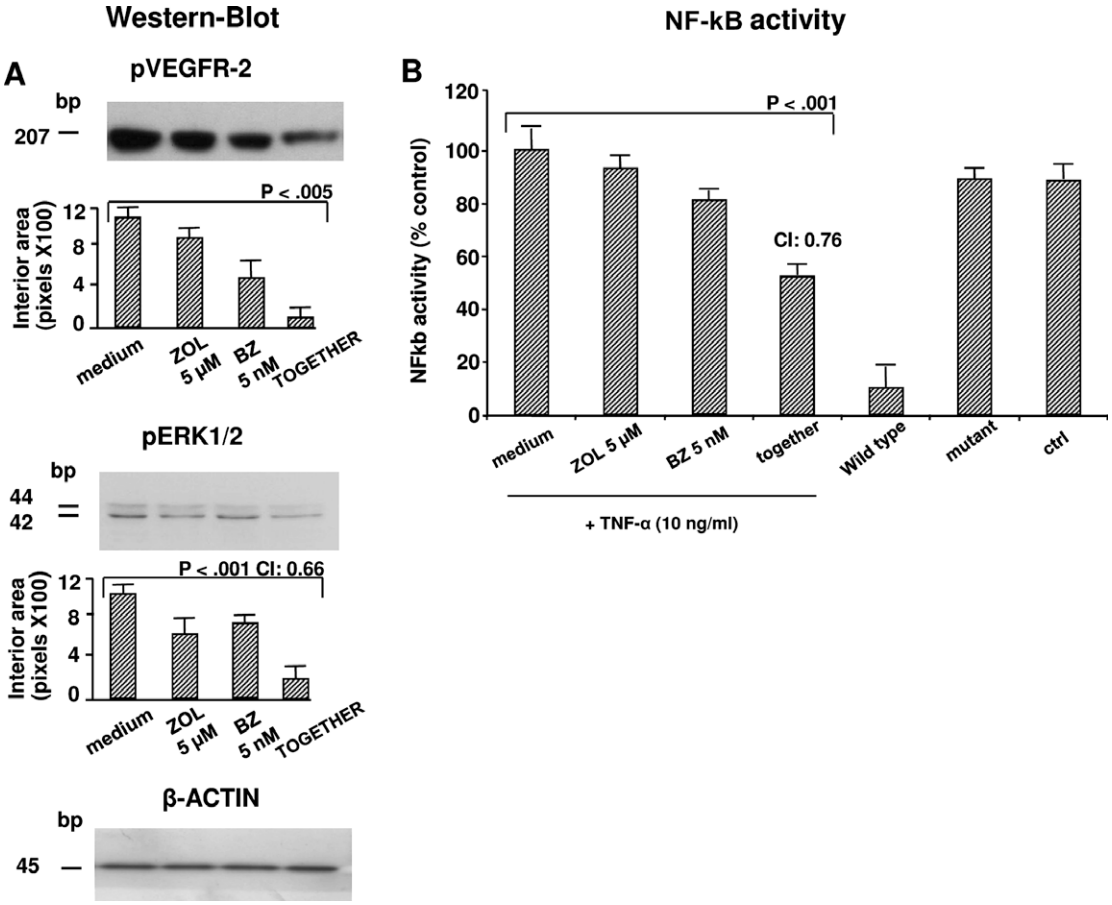

Fig. 5 – (A) Inhibition of VEGFR2 and ERK1/2 phosphorylation (p). Western blotting shows that single drug and combination treatments down-regulate the expression of pVEGFR2 and pERK1/2, the combination treatment being more effective. The band intensity was expressed as fold expression of the medium value by arbitrary absorbance. (B) ZOL and BZ inhibit NF-κB activity in MM macrophages. Macrophages were cultured with either ZOL 5 μM, BZ 5 nM or the combination. Data are expressed as mean ± 1 SD for the group of patients. Synergistic inhibition is indicated by CI < 1. Significance of changes was assessed by the Wilcoxon signed-rank test.

enzyme of the mevalonate pathway.[31,32] As a consequence, ZOL prevents the synthesis of farnesyl pyrophosphate (FPP) and its downstream metabolite geranylgeranyl diphosphate. These isoprenoid lipids are required for post-translational modification (i.e. prenylation) of proteins, including small GTPases, such as those of the Ras, Rho, and Rab families, which are important signalling proteins for regulation of a variety of cell functions. The inhibition of these proteins could explain why ZOL reduces the survival, proliferation, adhesion and migration of MM macrophages as happens in tumour cells.[33,34] Here phosphoactivation of ERK1/2 is reduced by both drugs with ZOL exerting a major effect probably through inhibition of prenylation of Ras and other GTPase proteins. Ras inhibition leads to disruption of Ras/Raf/Mek/Erk MAP kinase signalling and finally of NF-κB, with ZOL and BZ acting synergistically. In fact, BZ blocks the activation of NF-κB directly by preventing proteasome degradation of its inhibitor IκBα.[35,36] It may be well that ZOL (via Ras/Raf/Mek/Erk MAP kinase signalling) and BZ (directly) simultaneously inhibit NF-κB activity in a synergistic way. The activity exhibited by both drugs on NF-κB could also explain the greater efficacy of ZOL pre-treatment before the combination treatment. In fact, the partial inhibition of NF-κB, due to the reduced prenylation of signalling proteins such as Ras and Rho caused by ZOL, could make NF-κB more vulnerable to BZ.

NF-κB is important for MM plasma cell survival and it is normally activated in response to cell stress, including that induced by cytotoxic agents, radiation or DNA damage.[37–39] It is overexpressed in several tumours and regulates the expression of genes involved in apoptosis (including Bcl-2 and Bcl-xL), cell cycle progression, inflammation and angiogenesis (including interleukin IL-6, IL-8 and VEGF).[38,39]

Also, the therapeutic action of BZ-induced inhibition of the proteasome in MM probably results from a direct cytotoxicity on the bone marrow milieu.[40] The BZ antiangiogenic ability is another potential mechanism of its anti-MM activity.[9] Moreover, BZ down-regulates caveolin-1 tyrosine phosphorylation, which is required for VEGF-mediated MM cell migration, and also blocks the caveolin-1 phosphorylation induced by VEGF (transcriptional target of NF-κB) in ECs, thereby inhibiting ERK-dependent cell proliferation.[18] It inhibits the transcription of important adhesion molecules such as ICAM-1, VCAM1 and E-selectin.[41] It down-regulates IL-6 secretion in

bone marrow stromal cells, hence the related plasma cell growth.[39] Inhibition of IL-6 secretion in MM bone marrow leads to a reduction of VEGF secretion. Together, these findings imply important mechanisms by which BZ alone may inhibit migration of MM macrophages similarly to what happens in plasma cells.

In conclusion, this study adds new information on the antivascular activity of ZOL and BZ through their effect on macrophage angiogenic and vasculogenic properties when applied both separately and in combination. This study suggests that dose levels, which were much lower in combination than separately *in vitro*, would allow less toxic dose levels *in vivo*. A new administration schedule such as pretreatment with ZOL followed by a combination of both drugs could be validated in a larger clinical trial program.

Conflict of interest statement

None declared.

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