EUROPEAN JOURNAL OF CANCER 46 (2010) 3022–3036

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journal homepage: www.ejconline.com

Bevacizumab/docetaxel association is more efficient than docetaxel alone in reducing breast and prostate cancer cell growth: A new paradigm for understanding the therapeutic effect of combined treatment

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ARTICLE INFO

Article history:
Received 30 April 2010
Accepted 15 July 2010
Available online 20 August 2010

Keywords:
Bevacizumab
Docetaxel
Angiogenesis
Breast and prostate tumours

ABSTRACT

Bevacizumab (Bvz), a Vascular Endothelial Growth Factor (VEGF)-targeted humanised monoclonal antibody, provides clinical benefit in combination with docetaxel (DXL), a microtubule-stabilising agent, in the treatment of metastatic breast and prostate cancers. Since VEGF and their receptors are expressed by tumour cells, we hypothesised that Bvz, in addition to its impact on neo-vascularisation, could have an impact on tumour cells and enhance the DXL activity. Hence, we studied the effect of DXL and Bvz on metastatic breast (MDA MB-231) and prostate (PC3) cancer cells lines. Bvz alone did not decrease cell proliferation but in combination with DXL, Bvz enhanced the anti-proliferative activity of DXL. Other anti-angiogenic factors Sunitinib, Sorafenib and Gefitinib enhanced the anti-proliferative effect of DXL. qPCR experiments showed that DXL significantly increased the VEGF and VEGF receptor 2 (VEGF-R2) mRNA levels. Activation of VEGF and VEGF-R2 promoters demonstrated that enhanced mRNA levels are partly due to transcriptional activation. ELISA assays showed that DXL induced accumulation of cytoplasmic VEGF but decreased extracellular levels by 39% (MDA) and 48% (PC3). Cell surface localisation of VEGF-R2 was increased by DXL alone, but decreased after combined treatment of DXL plus Bvz. Abnormal expression of VEGF-R2 was also shown on breast and prostate tumour samples reinforcing the results obtained on cellular models. In conclusion, DXL and Bvz in combination decreased extracellular VEGF and VEGF-R2 levels at the plasma membrane thereby blocking an important growth/survival loop. Thus, the combined therapeutic impact of Bvz and DXL observed in clinical trials is associated with enhanced anti-proliferative activity and inhibition of the vascular network.

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0959-8049/$ - see front matter
doi:10.1016/j.ejca.2010.07.021

1. Introduction

Targeting tumour neo-angiogenesis is one of the most burgeoning areas of cancer therapy research. Indeed, tumours growing beyond 2 mm^3 require a vascular supply.[1] The majority of tumour cells express Vascular Endothelial Growth Factor (VEGF), a major regulator of angiogenesis. VEGF induces proliferation of endothelial cells, principally via the VEGF receptor 2 (VEGF-R2/KDR), and then promotes neovascularisation.

The anti-VEGF recombinant humanised monoclonal antibody bevacizumab (Bvz) is one of the best-studied approaches to inhibit neo-angiogenesis. This antibody prevents VEGF/VEGF-R interaction and thereby abrogates VEGF-R-mediated biological effects.[2] Many clinical trials assessing the benefit of Bvz demonstrate that it had minimal activity in several types of cancers when used as a single agent.[3–7] However, some randomised trials report that the combination of Bvz and chemotherapy is more efficient than chemotherapy alone.[5,8,9]

The combination of Bvz and docetaxel (DXL, a taxane class drug) is a promising therapeutic approach for treating breast and hormone refractory prostate cancer (HRPC). Indeed, in these cancers, a high level of VEGF was demonstrated to be a marker of poor prognosis.[10–12]

In metastatic breast cancer: a phase I/II study evaluating the tolerance and the efficacy of Bvz used as a single agent showed a low overall response rate (6.7%).[6] The combination of DXL and Bvz provided a partial response (52%) in another study.[13] Two phase III studies demonstrated that the combination of Bvz and DXL was more efficient in terms of response rate and progression-free survival than DXL alone.[14,15]

In HRPC: a phase II study evaluating the efficacy of Bvz used as a single agent gave disappointing results, with no objective response.[16] Since DXL is the most efficient chemotherapeutic agent for treating HRPC, the combined effect of DXL and Bvz was evaluated in phase II clinical trials. Both studies have reported >80% objective response rate.[17,18] These results matched the effects of DXL plus estramustine alone, in which the objective response rate was 50%.[19] Building on these encouraging results, an ongoing phase III trial is evaluating the benefit of this combined treatment in HRPC.

The results of these breast and prostate cancer clinical trials suggest that the combined effects of Bvz and taxanes are efficacious. Many hypotheses could explain this phenomenon: for example: (i) anti-angiogenic agents could normalise the vasculature and improve the delivery of cytotoxic agents in tumour cells[20,21]; (ii) taxanes alone may also exhibit an anti-angiogenic activity.[22] However, because tumour cells express VEGF receptors, some authors suggest a direct effect of anti-angiogenic agents[23] and we hypothesise that the efficacy of the Bvz and DXL combination could be partly due to a direct effect of Bvz on tumour cells. Since the combination of Bvz and taxanes seem to be promising in breast and prostate cancers, this study focused on the effects of DXL alone and in combination with Bvz on metastatic breast and prostate cancer cell lines.

2. Materials and methods

2.1. Materials

Bvz (Avastin® from Roche/Genentech, San Francisco), DXL (Sanofi Aventis, Bale), Sunitinib (Pfizer, Paris), Sorafenib (Bayer Health Care, Paris) and Gefitinib (AstraZeneca, Paris) were residual materials given to patients. DXL was dissolved in water (83%)/ethanol(13%). Bvz was dissolved in PBS. Sp600-125 was from Calbiochem (Darmstadt, Germany). Gal4, Gal4/Sp1 and Gal4 reporter plasmids were used as already described.[24]

2.2. Cell lines and culture condition

MDA-MB-231 (MDA), hormone-resistant metastatic breast cancer cells, human fibroblasts and PC3, hormone refractory prostate cancer cells, were grown in DMEM, for the two first cell lines, or in RPMI supplemented with 10% inactivated foetal bovine serum (FBS), 2 mM glutamine and 100 U/mL penicillin–streptomycin. Both cell lines were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. HuVEC cells were cultured in SFM (Invitrogen) supplemented with 20% foetal calf serum, 20 ng of FGF-2 and 10 ng of EGF/ml. Hypoxic conditions were induced by incubation of cells in a sealed Bug-Box™ anaerobic workstation (Ruskinn Technology Biotrace International Plc).

2.3. Cell proliferation assay

Cells were plated in 12-well dishes at a density of 150,000 cells/well and allowed to grow for 8 h in standard medium. Cells were exposed to different treatments, trypsinised 48 h after treatment and counted using a Coulter counter. Cell proliferation was assessed by using a colorimetric BrdU immuno-fluorescence assay kit (Roche Applied Science, Indianapolis, United States of America (USA)). Cells were plated in 96-well dishes (3000 cells/well) and allowed to grow for 8 h in standard medium. Cells were exposed to the different treatments for 48 h and incubated for 2 h with BdrU. The proliferative capacities of the cells following the different treatments were also assessed using the 3-[4,5-dimethylthiazol-2yl]-diphenyltetrazolium bromide (MTT) colorimetric assay (Sigma, France) according to the manufacturer's instructions.

2.4. Transient transfection and luciferase assay

VEGF and VEGF-R2 promoter gene constructs were previously described.[24,25] Transfections were carried out using the lipofectamine method for MDA and the calcium phosphate method for PC3 cells. Lipofectamine transfections were performed according to the manufacturer's instructions (Invitrogen™). The calcium phosphate transfection method and the luciferase assays were performed as previously described.[26]

2.5. Western blotting

Cell were incubated with the different effectors for the times indicated in the figure legend and were washed twice with ice-cold PBS and immediately lysed in Laemmli sample buffer. Protein extracts were resolved on a 9% SDS–PAGE gel and transferred onto a PVDF membrane (Immobilon-P; Millipore). The membranes were blocked for 1 h in 5% non-fat dry milk and incubated overnight with the following antibodies: antiphospho-ATF2 (1:1000; 92215, New England Biolabs, Ipswich, MA), antiphospho-JNK (1:1000; 9255, New England Biolabs, Ipswich, MA), antiphospho ERK 1,2 antibody (1:10,000; M8159, Sigma St. Louis, MO), anti-VEGF-R1 (1:1000; SC-317, Santa Cruz Biotechnology, Santa Cruz, CA), anti-VEGF-R2 (1:500; 2479 Cell Signaling, Cambridge, UK), antiphosphoAkt (1:1000; 9271, Cell Signaling, Cambridge, UK), anti-Akt (1:1000; 9272, Cell Signaling, Cambridge, UK), anti-ERKs (RKC16; 1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), anti-EGF-R (1:1000; 2232, Cell Signaling), anti-PDGF-R β (1:1000; 3169, Cell Signaling) and anti-Zyxin (1:1000; SC1-5338 Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were washed three times with PBS and then incubated 1 h with an anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibody. Bound antibody was detected using an enhanced chemiluminescence (ECL) system (Amersham Biosciences).

2.6. Biochemical quantification of cell surface VEGF-R2

Cell surface VEGF-R2 was measured as previously described.[27] Cells were washed three times in PBS and then incubated with 0.15 mg/mL sulfo-NHS-SS-biotin (NHS-SS-biotin; Pierce, Rockford, IL) in PBS for 10 min with rocking. The un-reacted biotinylation reagent was eliminated by washing once with TBA (25 mM Tris, pH 8; 137 mM NaCl; 5 mM KCl; 2.3 mM $CaCl_2$; 0.5 mM $MgCl_2$; and 1 mM Na_2 HPO_4), and with PBS (3×). Cells were then solubilised in lysis buffer (20 mM Tris, pH 7.5; 125 mM NaCl; 10% glycerol; 1% NP40; 1 μg/mL PMSF) containing a protease inhibitor cocktail (Calbiochem), used according to the manufacturer's instructions. Cell lysates were centrifuged at 14000*g* for 10 min at 4 °C and an aliquot (10 μL) was recovered from the supernatant to test for the presence of the total cellular KDR. Streptavidin–agarose beads (Pierce, Rockford, IL) were added to the supernatant (100 μL packed beads per 500 μL lysate) and left to tumble at 4 °C for 2 h. Beads were collected by centrifugation at 14000*g* for 10 s at 4 °C and the supernatant was removed. The supernatant was tested for the intracellular VEGF-R2 pool. The beads were then washed three times in lysis buffer at 4 °C and proteins were extracted from the beads by heating at 95 °C with Laemmli sample buffer.

2.7. Quantification of phosphorylated VEGF-R2

Quantification of phospho-VEGFR-2 was performed using the phospho-VEGFR-2 (Tyr1175) sandwich ELISA Kit (Cell Signaling, Cambridge, UK). Cells were lysed in the following lysis buffer: 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM ethylene diaminetetraacetate, 1 mM ethylene glycolbis(2-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM b-glycerolphosphate, 1 mM Na3VO4 and a cocktail of protease inhibitors (Roche, Mannheim, Germany). Equal amounts of protein were plated in each microwell and ELISA was performed according to the manufacturer's instruction.

2.8. ELISA

Cells (150,000) were plated on 12-well dishes and allowed to grow for 24 h. Cells were washed twice with PBS and incubated for 48 h with various concentrations of DXL. Cell culture supernatants were then collected and the cells were lysed with 100 μL of lysis buffer (Tris, pH 7.40, 4 M, NaCl 1 M, EDTA 50 mM, triton 0.1 M, AEBSF 1 mM). The Pierce Human VEGF ELISA Kit was used according to the manufacturer's instructions (Pierce Biotechnology, Rockford, IL). The intracellular VEGF protein level determined by ELISA was normalised to micrograms of total protein.

2.9. RNA preparation and real-time PCR

Total RNA was extracted with TRIZOL Reagent (Invitrogen). Two micrograms of total RNA were used for reverse transcription, using the Superscript First-Strand Synthesis System (QIAGEN, Hilden, Germany), with oligo (dT) to prime first-strand synthesis. For real-time PCR, primers and dual-labelled probes (5′-FAM, 3′-TAMRA) were from Applied Biosystems (7300 Software System, Europe). Each gene was amplified using the appropriate set of specific primers (Assay ID for VEGF: Hs00900054_m1 for VEGF-R2: Hs00176676_m1 and Hs99999902_m1 for 36B4/RPLP0, Applied Biosystems, Foster City, CA). The relative expression level of transcripts was quantified by real-time RT-PCR using the Taqman PCR Master Mix (Eurogentec, Liege, Belgium) on an ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The relative amount of each gene was determined as already described.[28] The results are representative of at least three independent experiments.

2.10. Statistical analysis

The results for cell count/proliferation, luciferase activity, mRNA and protein levels are given as an average of at least three separate (replicate) experiments for each treatment. Results were presented as means plus or minus the standard deviation. The statistical significance of the differences between the values in the treated and control cells was determined by a non-parametric Mann-Whitney U test. Statistical differences between controls and treated cells were considered significant when p is <0.05. The cell count and cell proliferation were analysed using a two-way ANOVA with Bonferroni *post hoc* analysis.

2.11. Human tissue samples

Samples were collected with the approval of the Local Ethics committee. Five independent breast and prostate tumours were tested. Their characteristics are as follows: *Breast tumours*: the mean patient age was 58 years (range 50–71). Case

1: invasive ductal carcinoma, HER2–, RO30%, RP0% VEGF-R2 negative; Case 2: invasive lobular carcinoma, HER2–, RO10%, RP80%; VEGF-R2 negative; Case 2 bis: node metastasis of case 2; VEGF-R2 negative on the tumour, positive on blood vessels; Case 3: mix carcinoma, HER2–, RO20%, RP0%; VEGFR2 negative on the tumour, positive on blood vessels; Case 3 bis: node metastasis of case 3; VEGFR2 positive on the tumour: nuclear labelling on 5% of tumour cells intensity 3/3; Case 5: cutaneous metastasis of carcinoma; HER2–, RO+, RP–; VEGFR2 negative on the tumour, positive on blood vessels.

Prostate tumours: the mean patient age was 64.2 years (range 63–65). Case 1: Gleason 9/pT3a, VEGF-R2 positive; Case 2: Gleason 9/pT3b, VEGF-R2 positive; Case 3: Gleason 7/pT3a, VEGF-R2 positive; Case 4: Gleason 9/pT3a, VEGF-R2 negative; Case 5: Gleason 9/pT2c, VEGF-R2 positive. Human clear cell renal cell carcinoma was used as a positive control for VEGF-R2 labelling.

2.12. *Immunohistochemistry*

Sections from blocks of formol-fixed and paraffin-embedded tissue were examined for immunostaining for VEGFR2. Briefly, after deparaffinisation, hydration and heat-induced antigen retrieval, the tissue sections were incubated for 20 min at room temperature with a polyclonal rabbit anti-human VEGF-R2 antibody (dilution 1:300; Cell Signaling, Boston, MA, USA). Biotinylated secondary antibody (DAKO) was applied and binding was detected with the substrate diaminobenzidine against a haematoxylin counterstain.

3. Results

3.1. *Combined effects of Bvz and DXL on MDA and PC3 cell accumulation*

We first examined the effect of different concentrations of DXL alone and in combination with Bvz on the number of MDA and PC3 cells. DXL decreased the cell number for both cell lines in a dose-dependent manner. Whilst addition of a saturating concentration of Bvz alone (500 ng/ml)[29] had a negligible effect, the cell numbers were significantly decreased when Bvz was combined with DXL. A major effect was observed at 1 nM DXL in MDA cells and at 10 nM DXL in PC3 cells (Fig. 1A and C). This differential efficiency of DXL in MDA versus PC3 cells is probably due to the taxane resistance of these cells, which is related to a loss of PTEN.[30] BrdU incorporation assays (Fig. 1B and D) showed that such a difference in cell counts is partly due to an anti-proliferative effect of the

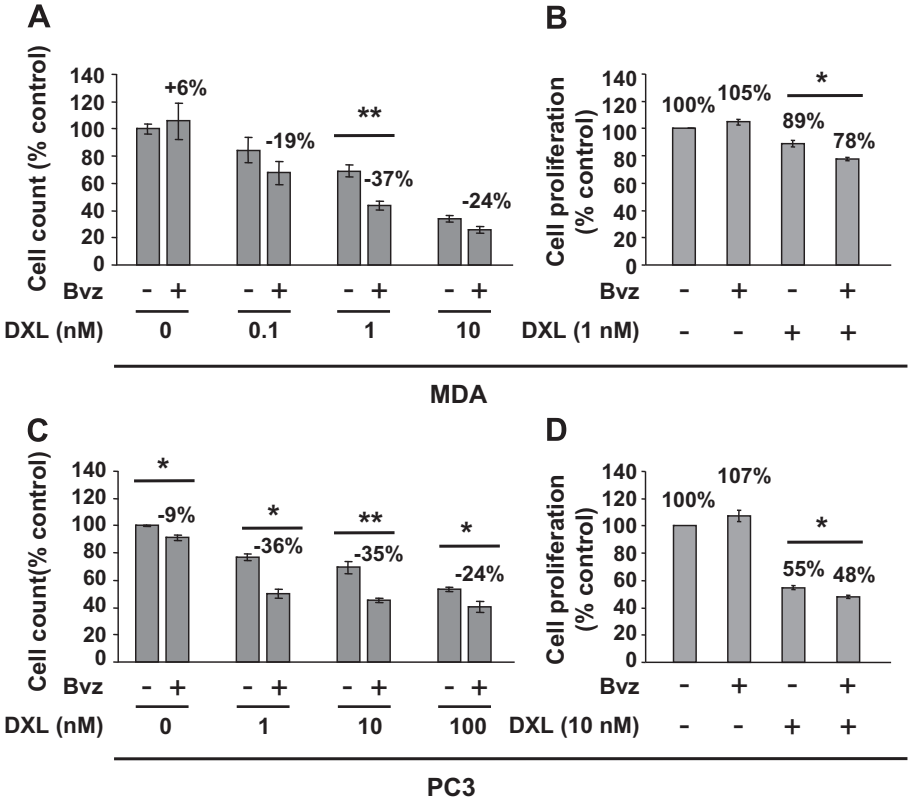

Fig. 1 – Bvz increases the anti-proliferative effect of DXL in MDA and PC3 cells. (A and C) MDA and PC3 cells (150,000 cells/mL) were maintained in 10% serum containing medium for 48 h in the absence or presence of Bvz (500 ng/ml) and/or different concentrations of DXL. The percentage of remaining cells (compared without Bvz) was plotted ± SEM. (B and D) 3000 MDA or PC3 cells were cultured in 10% serum containing medium for 48 h in the absence or presence of Bvz (500 ng/ml) and/or different concentrations of DXL. BrdU incorporation was measured as described in Section 2. Proliferation was expressed as a percentage of baseline ± SEM. These data represent the mean ± SEM of four independent experiments. (*) represents $p < 0.05$ and (**) represents statistical significance according to the Bonferroni *post hoc* test.

combined treatment. To test whether our results with Bvz could be extended to other anti-angiogenic drugs used in the clinic, we combined an optimal dose of DXL (1 nM for MDA or 10 nM for PC3 cells) with different concentrations of Sunitinib, Sorafenib or Gefitinib. Sunitinib and Sorafenib are two anti-angiogenic drugs targeting tyrosine kinase receptors including VEGF-R, PDGF-R, Kit RET, CSF-R and FLT3, which are mainly expressed on endothelial cells or aberrantly expressed on tumour cells. Sorafenib also targets Raf kinases, major regulators of cell growth in all cell types. Gefitinib targets EGF-R which is highly expressed on tumour cells and which is indirectly implicated in angiogenesis through the stimulation of VEGF production.[31,32] Fig. 2A and B shows that the three inhibitors can decrease cell proliferation in a dose-dependent manner, Sunitinib and Sorafenib being more efficient than Gefitinib. An additive effect on cell proliferation was also observed with DXL when sub-optimal concentrations of each inhibitor were used.

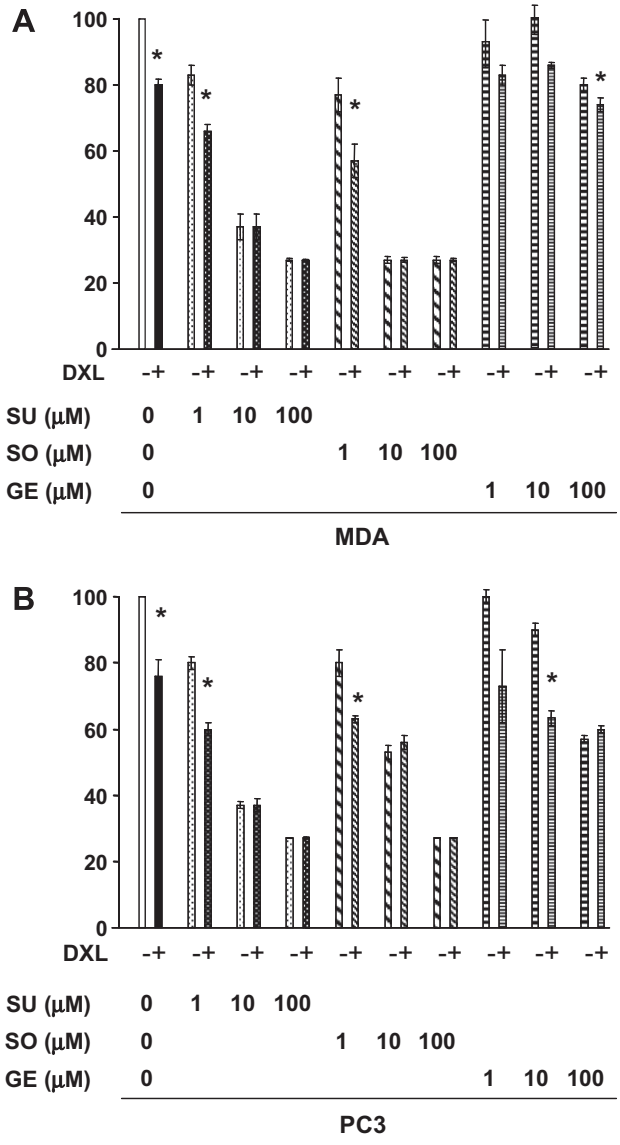

Fig. 2 – Sunitinib, Sorafenib and Gefitinib increase the anti-proliferative effect of DXL in MDA and PC3 cells. MDA (A) and PC3 (B) cells were maintained in 10% serum containing medium for 48 h in the absence or presence of suboptimal concentrations of DXL (1 nM for MDA and 10 nM for PC3 cells) and/or increasing concentrations of Sunitinib (Su), Sorafenib (So) or Gefitinib (Ge). The percentage of remaining cells was evaluated with an MTT assay and the results were plotted ± SEM. The condition without any treatment is considered as the reference value (100%). These data represent the mean ± SEM of four independent experiments. (*) represents a p value <0.05.

3.2. *DXL stimulates transcription of the VEGF gene through GC-rich sequences and the transcription factor Sp1*

In order to elucidate the molecular mechanisms implicated in the cytostatic effects of Bvz in combination with DXL, we first analysed the effect of DXL on the expression of VEGF. In both cell lines, DXL increased the level of VEGF mRNA in a dose-dependent manner (Fig. 3A). Hypoxia, a classical inducer of VEGF mRNA expression, resulted in a comparable increase (Fig. 3A). Since these results suggest a transcription-dependent mechanism, we then investigated the effect of DXL on the VEGF promoter activity using a DXL concentration that induces optimal mRNA expression. For this purpose, the entire VEGF promoter (–1176, +54) coupled to the luciferase reporter gene was used, as previously reported.[24,26] DXL increased the VEGF promoter activity thereby supporting the hypothesis that DXL increases VEGF mRNA levels by transcriptional activation (Fig. 3B). To more precisely define the region of the VEGF promoter targeted by DXL, successive deletions of the VEGF promoter were analysed and tested for their response to DXL treatment (Fig. 3C). Mutation or deletion of the GC-rich sequences targeted by Sp transcription factors strongly decreased the DXL-mediated activation of the promoter. These results demonstrated that GC-rich domains of the VEGF promoter are strongly implicated in the response to DXL. Deletion of the HIF-1-targeted domain (–888) did not affect induction of the luciferase activity by DXL and HIF-1α expression is not modified by DXL treatment (not shown) suggesting that this important regulatory domain of the VEGF promoter is not implicated in the effect of DXL. Previous studies demonstrated that the Sp1 DNA-binding activity and trans-activation capacity could be mediated by several pathways, including the ERK 1, 2 and the stress kinase p38/JNK pathways. In both cell lines, DXL activated phosphorylation of p38/JNK stress kinase pathway targets (ATF2/JUN) whereas it had no effect on the ERK pathway (Fig. 4A). Inhibition of the stress kinase pathways by Sp 600-125 reversed the DXL-mediated activation of the full-length and the minimal region of the VEGF promoter containing GC-rich sequences (Fig. 4B). To test the effect of DXL on the trans-activation capacity of Sp1, one of the major Sp proteins implicated in VEGF regulation, we used constructs in which Sp1 was fused to the DNA-binding domain of the Gal4 transcription factor. The effect of DXL was evaluated after co-transfection with a construct encoding a Gal4-dependent luciferase reporter gene. DXL stimulated the transcriptional activity of the Sp1/Gal4 fusion protein, whilst Sp 600-125 inhibited the trans-activation capacity (Fig. 4C). Since DXL did not change the level of Sp1 (Fig. 4A), our results suggest that DXL stimulates the

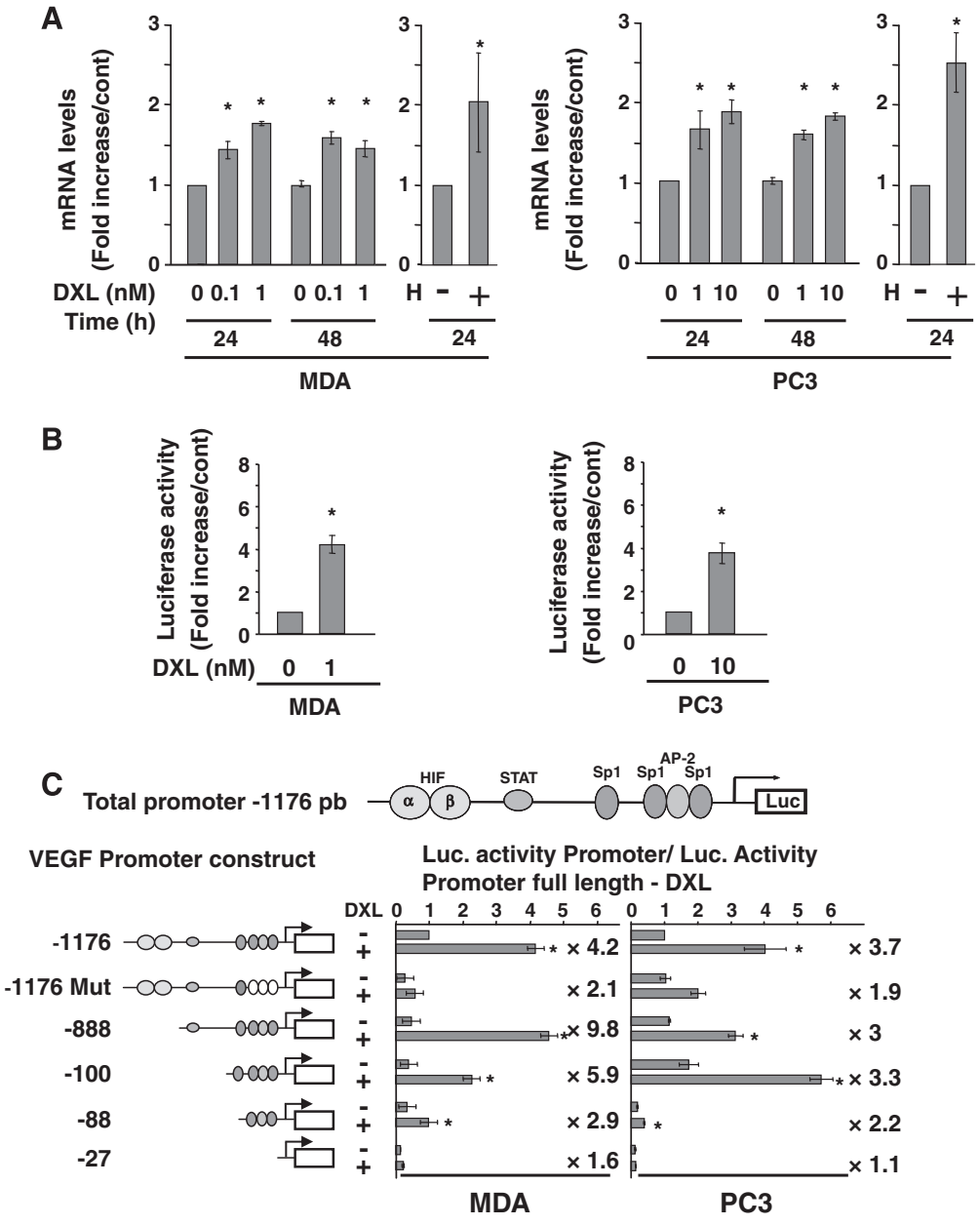

Fig. 3 – DXL stimulates transcription of the VEGF gene through GC-rich sequences and the transcription factor Sp1. (A) The relative level of VEGF mRNA in DXL-treated MDA and PC3 cells was evaluated by qPCR and normalised to 36B4. Cells were maintained in normal medium supplemented with 10% serum for 24 and 48 h in the absence or presence of different concentrations of DXL. Fold induction of VEGF mRNA is relative to the untreated condition after normalisation to 36B4. As a positive control, cells were cultured 24 h under hypoxic conditions (1% oxygen, H).These results are representative of three independent experiments performed in duplicate ± SEM. Statistically significant differences between controls and DXL-treated cells are indicated by an asterisk (*). (B) VEGF promoter activity in the presence or absence of DXL (1 nM for MDA and 10 nM for PC3). The promoter activity in transient transfection assays is shown. The relative light units normalised to the protein concentration were measured after transfection of 500 ng of the reporter gene in the absence or presence of DXL for 24 h. These results (fold-increase compared to the untreated condition) are representative of three independent experiments performed in triplicate ± SEM. Statistically significant differences between controls and DXL-treated cells are indicated by an asterisk (*). (C) The activity of different VEGF promoter constructs was tested in MDA and PC3 cells in the absence or presence of DXL in transient transfection assays. The relative light units normalised to the protein concentration were measured after transfection of 500 ng of the reporter gene in the absence or presence of DXL for 24 h. Results are expressed as a ratio between the luciferase activity obtained for each construct in the absence or presence of DXL and the luciferase activity obtained for the full-length promoter (–1176) in the absence of DXL, which was taken as the reference value (1). For each construct, the fold-increase between control and DXL treated cells is indicated. These results are representative of three independent experiments performed in duplicate ± SEM. Statistically significant differences between control and DXL-treated cells are indicated by an asterisk (*).

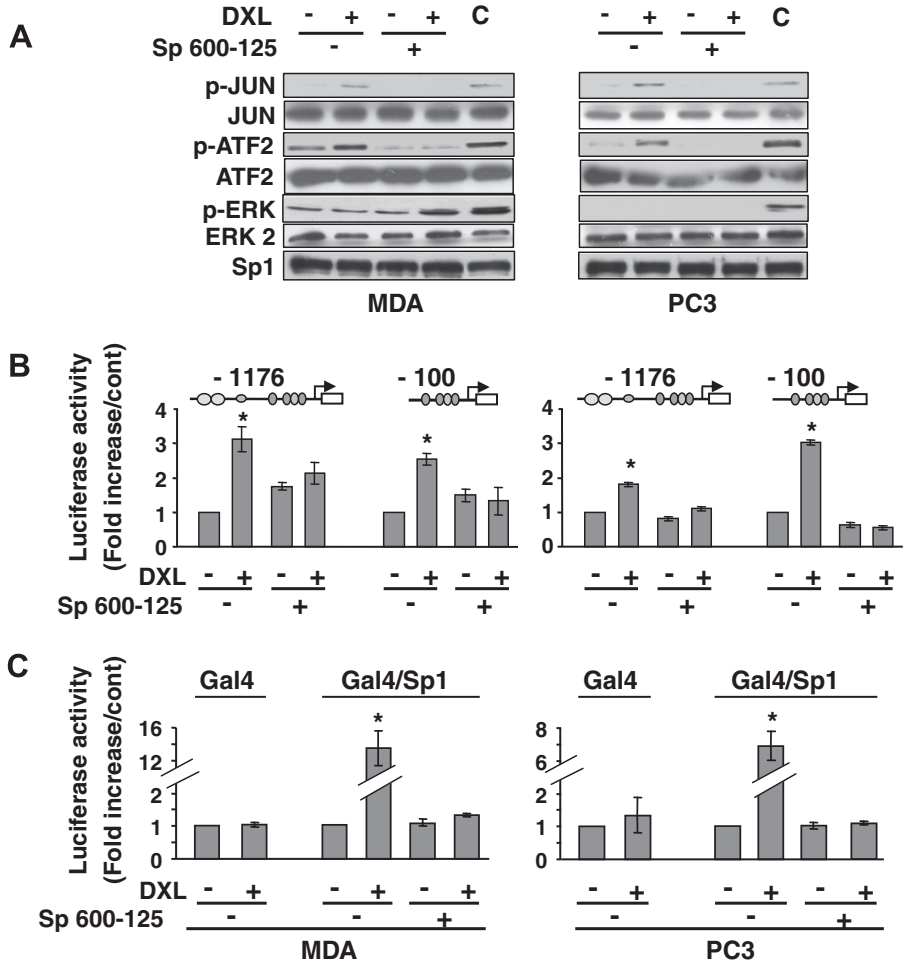

Fig. 4 – DXL stimulates transcription of the VEGF gene through the transcription factor Sp1 and stress kinase pathways. (A) Both stress kinase pathways (p38 and JNK) are activated by DXL in MDA and PC3 cells. MDA and PC3 cells were pre-treated or not with 40 mM of Sp 600-125, then exposed for 16 h to DXL. Extracts were tested for the presence of p-p38, p-JNK and p-ERK. Cells stimulated with 100 mM of anisomycin for 30 min were used as a positive control (C) for JNK and p38 activation. Stimulation with 10% of serum for 10 min following 24 h of serum starvation was used as a positive control for p-ERK (C). These results are representative of two independent experiments. (B) Sp 600-125 inhibits DXL-dependent induction of VEGF promoter activity. The activity of the full-length and the minimal region of the VEGF promoter (containing GC-rich sequences) was tested upon DXL treatment in the absence or presence of Sp 600-125 (40 nM, pre-treatment for one hour before stimulation with DXL for 16 h) in MDA and PC3 cells. The luciferase counts obtained for the untreated situation (- DXL, - Sp600-125) were considered as the reference value for each tested promoter construct. These results are representative of two independent experiments performed in triplicate ± SEM. Statistically significant differences between controls and DXL-treated cells are indicated by an asterisk (*). (C) Stimulation of the Sp1 trans-activation capacity by DXL is inhibited by Sp600-125. Gal4 and Gal4/Sp1 trans-activation in transient transfection assays is shown. The relative luciferase activity was measured after transfection of a 5X Gal4 reporter vector (500 ng) in the presence of a control Gal4 vector or Gal4/Sp1 (500 ng). Cells were stimulated or not with DXL in the presence or absence of Sp600-125 (40 nM) for 16 h. The luciferase counts obtained for the untreated situation (- DXL, - Sp600-125) for Gal4 or Gal4/Sp1 were considered as the reference value (1). These results are representative of two independent experiments performed in triplicate ± SEM. Statistically significant differences between controls and DXL-treated cells are indicated by an asterisk (*).

activation of VEGF by enhancing the Sp1-dependent trans-activation capacity through p38/JNK stress kinase pathways.

3.3. *DXL treatment results in VEGF accumulation in the cytoplasm*

To examine a possible correlation between DXL-mediated induction of VEGF mRNA and increased VEGF production, we measured intracellular and secreted VEGF protein levels in cell lysates and cell supernatants (Fig. 5). DXL treatment resulted in significant intracellular accumulation of VEGF, whilst extracellular VEGF levels were decreased compared to controls (Fig. 5). Although the intracellular increase of VEGF is modest, such a modification is physiologically relevant since mutation of only one VEGF allele or overexpression of VEGF in transgenic mice results in embryonic lethality.[33,34]

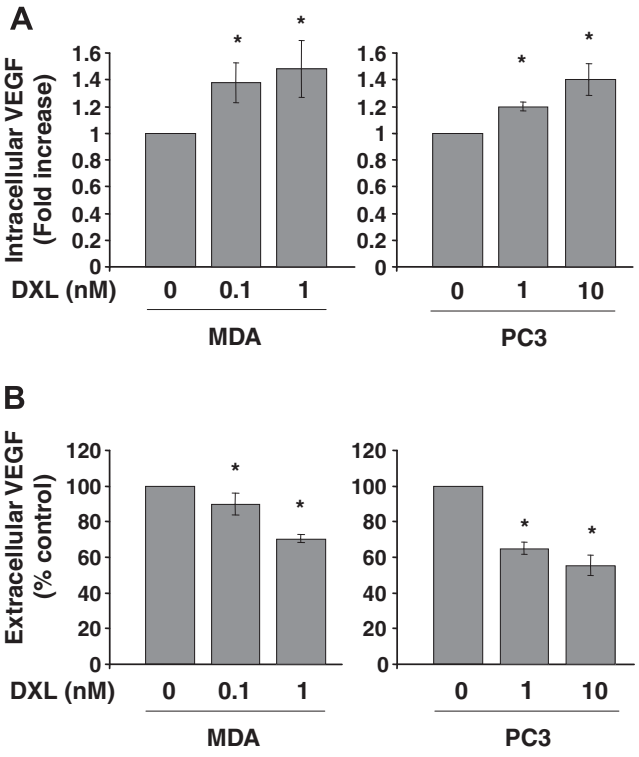

Fig. 5 – VEGF production in MDA and PC3 cells in response to DXL. Cells were maintained in normal medium supplemented with 10% serum for 48 h in the absence or in the presence of different concentrations of DXL. The VEGF concentration was tested with an ELISA. Intracellular amounts of VEGF were normalised to the total intracellular protein concentration. These results are representative of three independent experiments performed in duplicate ± SEM. Statistically significant differences between control and DXL-treated cells are indicated by an asterisk (*). (A) The increase in the intracellular amount of VEGF in MDA and PC3 cells treated with different concentrations of DXL during 48 h is presented as the fold-increase compared to the control condition without DXL. (B) The decrease in the amount of VEGF in conditioned medium of MDA and PC3 cells treated with different concentrations of DXL during 48 h is presented as the percentage of the control condition without DXL.

Hence, any up- or down-regulation of VEGF above or below its steady state level could be physiologically relevant. To extend our results to others members of the VEGF family implicated in lymphangiogenesis and in metastatic dissemination,[35] we also tested the expression of VEGF-C and VEGF-D in response to DXL. The results were similar to those observed for VEGF (increased intracellular and decreased extracellular levels of VEGF-C were observed in both cell lines, data not shown). VEGF-D expression was minimal to undetectable in both cell lines.

3.4. *DXL stimulates production of VEGF-R2*

Western blot analysis showed that both cell lines express VEGF-R2, a major cellular target for VEGF. This receptor is also regulated by Sp proteins in some cancer cell lines[25] and VEGF-R2 mediates the mitogenic activity of VEGF.[36] Therefore, the effect of DXL on VEGF-R2 expression in MDA and PC3 cells was also investigated. DXL increased VEGF-R2 mRNA levels in both cell lines (Fig. 6A). Since we observed activation of a VEGF-R2 promoter/luciferase reporter gene (–716 to +268) the increase in VEGF-R2 mRNA is partly attributable to a transcriptional mechanism (Fig. 6B). DXL also increased VEGF-R2 protein expression in MDA and PC3 cells (Fig. 6C and D), which is consistent with Sp-dependent activation of VEGF and the reported co-regulation of both VEGF/VEGF-R2 by Sp transcription factors. In contrast, Bvz did not affect VEGF-R2 expression (data not shown). Although the expression of VEGF-R2 is low compared to that detected in endothelial cells HUVEC, it is significantly increased compared to that obtained in fibroblasts (Fig. 6E). These data confirm results of previous studies showing that tumour cells of different origin have established an autocrine proliferation/survival pathway by co-expressing VEGF and VEGF-R2.[37–42] However, none of these articles describe the effect of a chemotherapeutic agent on VEGF-R2 expression.

3.5. *Combination of DXL and Bvz inhibits cell surface localisation of VEGF-R2*

In endothelial cells, a significant pool of VEGF-R2 is stored in intracellular endocytic compartments and VEGF mobilises intracellular VEGF-R2 resulting in increased cell surface VEGF-R2 expression.[27] In non-endothelial cells, the mechanisms of VEGF-R2 trafficking are unknown. The effect of Bvz alone or in combination with DXL on membrane VEGF-R2 expression has been determined by biotinylation of the surface VEGF-R2, as previously reported.[27] Membrane-associated biotinylated VEGF-R2 was collected by binding to streptavidin–agarose. Total cellular and surface extracts were analysed by Western blotting with an anti-VEGF-R2 antibody (Fig. 7A). In control conditions, a substantial proportion of VEGF-R2 was expressed at the cell surface. This result suggests that VEGF has the capacity to establish an autocrine loop on cellular models of breast and prostate cancers. Addition of Bvz moderately increased the total cellular expression of VEGF-R2 in PC3 cells. We demonstrated that DXL decreased VEGF secretion (Fig. 5B) and induced VEGF-R2 expression (Fig. 6C and D). However, DXL alone moderately increased surface localisation of VEGF-R2 (Fig. 7A). Nonetheless, in cells treated with Bvz plus DXL, surface VEGF-R2 localisation was significantly decreased (30 ± 5% and 80 ± 7% reduction compared to control in, respectively, MDA and PC3 cells). Fig. 7B and supplementary Fig. 1 show that VEGF-R2 is constitutively phosphorylated on tyrosine 1175, a residue shown to be phosphorylated upon VEGF stimulation of the receptor in endothelial cells ([43] and Fig. 7 B, right). This result suggests that VEGF-R2 is activated in both cell lines. Surprisingly, the rate of phosphorylation of VEGF-R2 remained constant in all conditions in MDA and PC3 cells whereas VEGF-R2 phosphorylation could be stimulated by VEGF in quiescent HuVEC cells (Fig. 7B). The consequences of combined treatment on the ERK and PI3-kinase/Akt signalling pathways downstream of VEGF-R2 were also investigated. DXL treatment alone had no effect on ERK activity in MDA cells but significantly

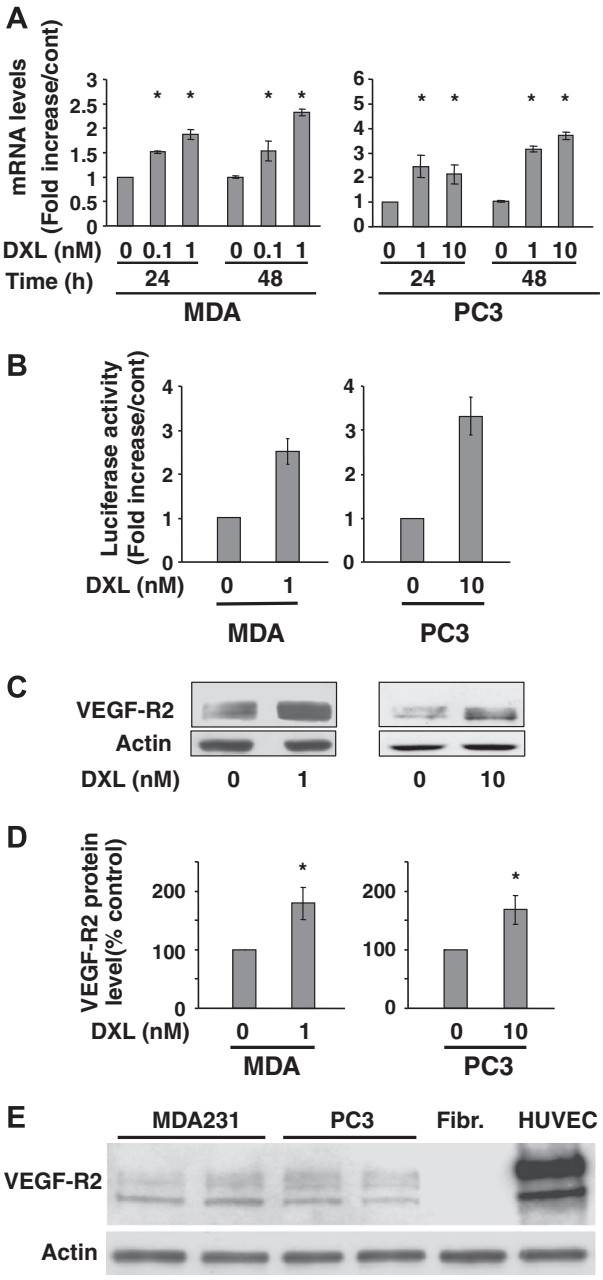

Fig. 6 – DXL stimulates transcriptional activation of VEGF receptor 2 (VEGF-R2). (A) Relative levels of VEGF-R2 mRNA in MDA and PC3 cells upon DXL treatment were evaluated by qPCR and normalised to 36B4. Cells were maintained in normal medium supplemented with 10% serum for 24 and 48 h in the absence or presence of different concentrations of DXL. Fold induction of VEGF-R2 mRNA is relative to the untreated condition after normalisation to 36B4. As a positive control, cells were cultured 24 h under hypoxic conditions (1% oxygen, H). These results are representative of three independent experiments performed in duplicate ± SEM. Statistically significant differences between control and DXL-treated cells are indicated by an asterisk (*). (B) VEGF-R2 promoter activity in the presence of DXL. The relative light units normalised to protein concentration were measured after transfection of 500 ng of the reporter gene in the absence or presence of DXL for 24 h. The luciferase activity for the untreated cells was considered as the reference value (1). These results (fold-increase compared to the untreated condition) are representative of three independent experiments performed in triplicate ± SEM. (C) Cells were maintained in normal medium supplemented with 10% serum for 48 h in the absence or presence DXL. Extracts were analysed by Western Blotting with an anti-VEGF-R2 antibody. Actin is shown as a loading control. These results are representative of three independent experiments. (D) Quantification of the results shown in (C). Statistically significant differences between control and DXL-treated cells are indicated by an asterisk (*). (E) MDA, PC3, human fibroblasts (Fibr) and endothelial cells (HUVEC) were maintained in normal medium supplemented with 10% serum for 48 h in the absence or presence DXL. Extracts were analysed by Western Blotting with an anti-VEGF-R2 antibody. Actin is shown as a loading control. These results are representative of three independent experiments.

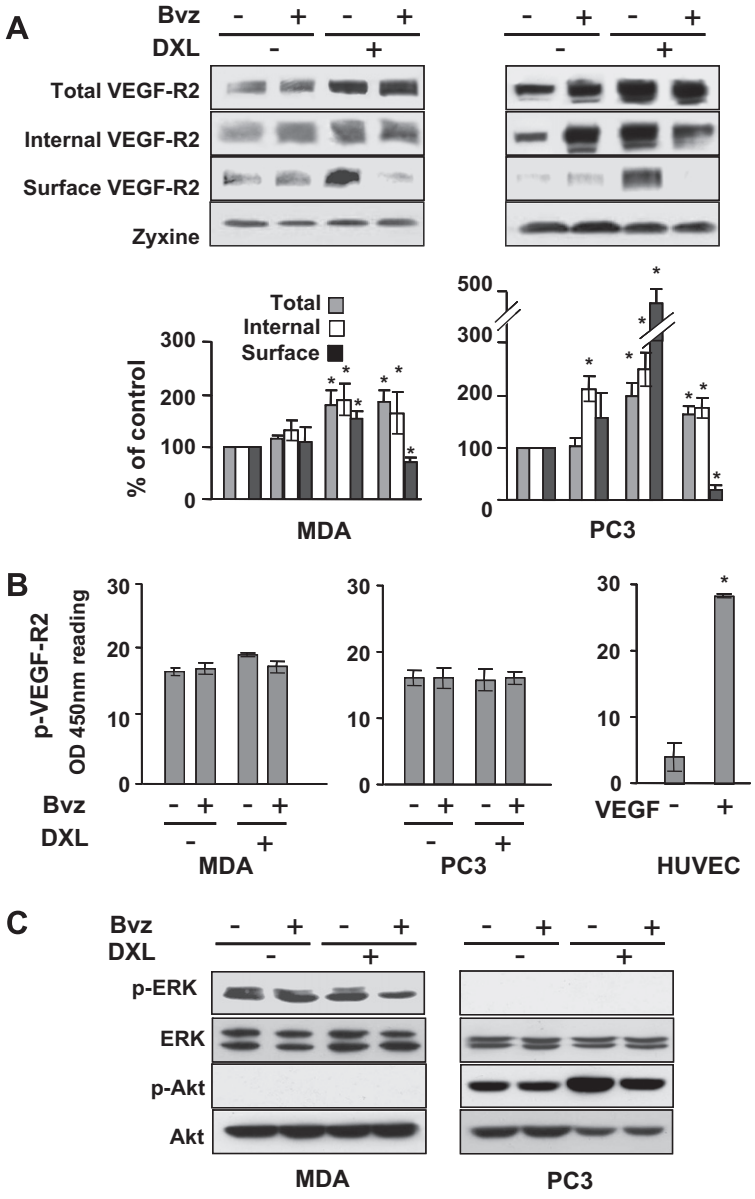

Fig. 7 – VEGF-R2 production in MDA and PC3 cells in response to DXL and/or Bvz. Cells were maintained in normal medium supplemented with 10% serum for 48 h in the absence or presence of 1 nM (MDA) or 10 nM (PC3) of DXL and/or 500 ng/ml of Bvz. (A) Extracts were tested for the presence of total and surface VEGF-R2. Surface VEGR-R2 was labelled with the membrane-impermeant biotinylation reagent sulfo-NHS-SS biotin. Biotinylated surface VEGF-R2 was collected by binding to streptavidin–agarose. Extracts were analysed by Western Blotting with an anti VEGF-R2 antibody for total and membrane VEGF-R2. Graphs represent quantification of three independent experiments. Zyxin is shown as a loading control. (B) Total extracts were analysed for the presence of phosphorylated forms of VEGF-R2 with an ELISA kit (see Section 2). Extracts from HuVEC cells, deprived of growth factors for 24 h and stimulated (+) or not (–) with 100 ng/ml of VEGF for 5 min, were analysed in parallel and serve as positive and negative controls, respectively. Values represent the mean and the standard deviation of three independent experiments. (C) Cell lysates were analysed with antibodies directed against ERKs (total and phosphorylated forms) and Akt (total and phosphorylated forms).

increased phosphorylation of Akt in PC3 cells. Bvz alone did not modify the activity of these kinases. Combined treatment with DXL plus Bvz decreased the ERK activity below basal levels in MDA cells whereas, in PC3 cells, combined treatment reduced the DXL-induced Akt activity (Fig. 7C). Phosphorylated Akt and phosphorylated ERK were not detected in MDA and PC3 cells.

3.6. *VEGF-R2 is present on breast and prostate tumour samples*

To validate our observations in model cell lines, we tested the presence of VEGF-R2 on five independent breast and prostate tumour samples (Fig. 8). Whereas analysis of VEGF-R2 in breast samples has already been performed, no published

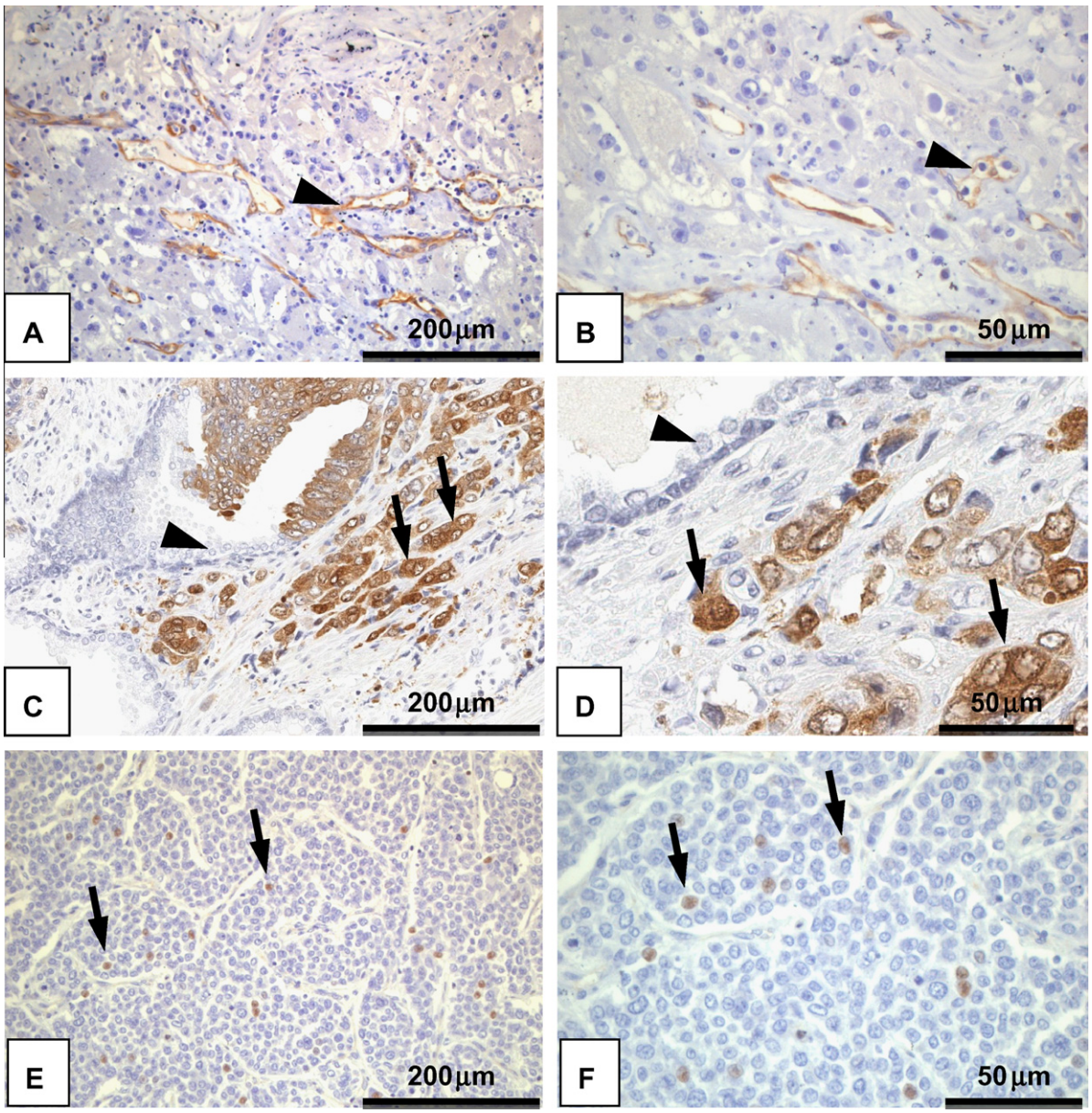

Fig. 8 – VEGF-R2 is expressed in breast and prostate tumours. (A and B) In clear cell renal cell carcinoma. IHC control: immunohistochemical stain shows strong endothelial reactivity (arrowhead) – (Magnification (A):20× and (B):40×). (C and D) In prostatic carcinoma: immunohistochemical staining for VEGFR2 shows tumour cell positivity (arrow) and normal prostatic gland negativity (arrowhead) (Magnification (C/E):20× and (D/F):40×). (E and F) In breast carcinoma: immunohistochemical staining for VEGFR2 shows focal tumour nuclear cell positivity (arrow) (Magnification (G):20× (H):40×).

data are available for prostate tumours. Four out of five prostate tumours were positive for VEGF-R2 whereas only one out of five breast tumours was positive. The results for breast tumours are representative of the already published data showing a high variability of VEGF-R2 staining with negative tumours, low staining, intermediate staining and intense staining.[44] A positive signal was detected on metastases of a tumour that was originally negative. Analysis of prostate tumours also showed aberrant expression of VEGF-R2 on the majority of the tumours tested. These results strongly suggest that the expression of VEGF-R2 observed in our model cell lines is not an aberration linked to the culture conditions of the tumour cells.

3.7. *DXL inhibits other growth/survival pathways*

Under basal conditions, the relative importance of the VEGF signalling pathway is modest, as suggested by the results of Fig. 1, probably because tumour cells express many other growth factor receptors and secrete their cognate ligands. Since VEGF secretion is inhibited by DXL, we examined the effect of DXL on secretion of EGF and PDGF, two major growth

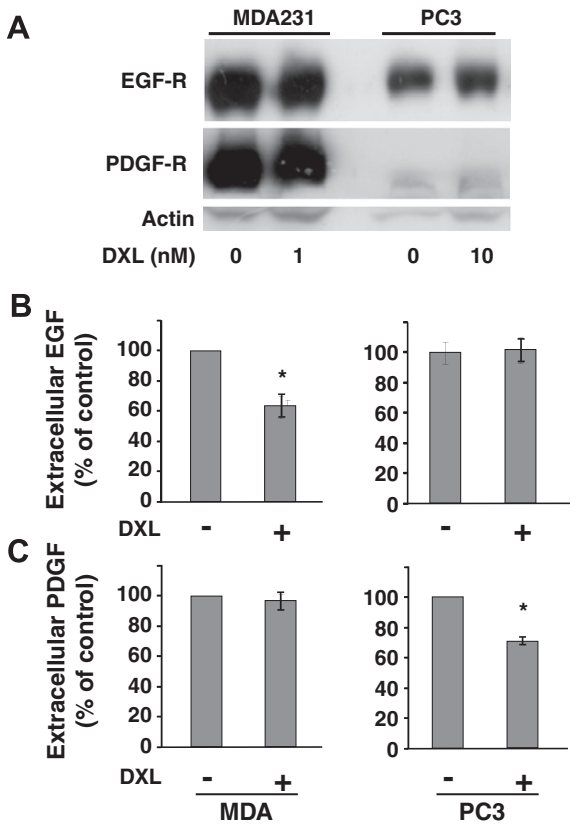

Fig. 9 – Expression of EGF/EGF-R and PDGF/PDGF-R β in MDA and PC3 cells treated with DXL. Cells were maintained in normal medium supplemented with 10% serum for 48 h in the absence or presence of different concentrations of DXL (1 nM (MDA) or 10 nM (PC3)). The EGF and PDGF concentrations were determined by ELISA. These results are representative of two independent experiments performed in triplicate ± SEM. Statistically significant differences between controls and DXL-treated cells are indicated by an asterisk (*). (A) Extracts were tested for the presence of EGF-R and PDGF-R β by Western blotting. Actin is shown as a loading control. This result is representative of two independent experiments. (B) The decrease in the amount of EGF in conditioned medium of MDA and PC3 cells treated with DXL (1 and 10 nM, respectively) during 48 h is presented as a percentage of the control condition without DXL. (C) The decrease in the amount of PDGF in conditioned medium of MDA and PC3 cells treated with DXL (1 and 10 nM, respectively) during 48 h is presented as a percentage of the control condition without DXL.

factors implicated in autocrine growth/survival pathways. Fig. 9A clearly shows that both EGF and PDGF receptors are expressed on both cell lines, as already described, although PDGF-R is expressed in PC3 cells to a lesser extent.[45–48] DXL significantly decreases extracellular levels of EGF and PDGF in MDA and PC3 cells, respectively (Fig. 9B and C), whereas Bvz had no effect on growth factor secretion in either cell line (data not shown). In summary, our experiments show that DXL has the potential to down-regulate production of major autocrine growth factors. Such general inhibition could make the VEGF/VEGF-R2 autocrine loop a limiting growth/survival pathway.

4. Discussion

The anti-cancer activity of taxane plus Bvz combination has been documented in clinical trials. DXL exerts its cytotoxic activity on both tumour and endothelial cells whereas Bvz primarily targets the vascular network. However, we have observed a significant effect of DXL plus Bvz on tumour cell proliferation. Our results show that DXL induced transcriptional activation of VEGF through stress kinase pathways that are hypoxia independent (Fig. 4). However, there is no strict correlation between the fold inductions observed by quantitative PCR and by luciferase assays. Epigenetic regulation of the endogenous VEGF promoter may explain the discrepancy between the results observed with reporter genes and the level of VEGF mRNA. However, these results raised an interesting hypothesis that suggests that DXL could stimulate transcription and at the same time stimulate mRNA degradation. We do not favour this hypothesis since p38 or JNK activation results in VEGF mRNA stabilisation through a domain of the VEGF 3'UTR that forms a hairpin loop.[49] Nevertheless, we have tested this hypothesis with another reporter gene in which the domain corresponding to the VEGF mRNA 3' untranslated region, the domain mainly implicated in VEGF mRNA stability, was placed downstream of the luciferase reporter gene.[28] Using this reporter assay, DXL also increased the luciferase activity suggesting that DXL may increase the VEGF mRNA half-life (see supplementary Fig. 2). Such an activation is compatible with previous results showing that stimulation of the p38 or JNK activity results in VEGF mRNA stabilisation through a domain of the VEGF 3'UTR that forms a hairpin loop.[49] Quantitative PCR experiments have been performed with oligonucleotides that can detect all VEGF mRNA, potentially including pro- and anti-angiogenic isoforms.[50] However, the anti-angiogenic isoforms of VEGF mRNA were not detected in both cell lines and were not affected by the different treatments (supplementary Fig. 3).

Unexpectedly, despite DXL-mediated VEGF induction, extracellular concentrations of VEGF were decreased in MDA and PC3 cells and similar results were observed in other cell lines treated with taxanes. For example, treatment of wild-type and drug-resistant human leukaemia cells with 0.1 μM or 10 μM of DXL for 24 h strongly inhibited VEGF secretion[51] and paclitaxel and other chemotherapeutic agents decreased VEGF secretion in lung cancer cell lines.[52] Our findings are also consistent with results of a clinical study showing that paclitaxel alone significantly decreased serum VEGF levels in metastatic breast cancer in patients with a partial tumour response or stable disease.[53] A relevant hypothesis is that VEGF, produced in response to DXL, is sequestrated in the cytoplasm and not released into the medium and this is consistent with studies showing that anti-microtubule drugs affect sub-cellular protein localisation by modulating their transit and secretion.[54–56] Increased intracellular levels of VEGF in MDA and PC3 cells treated with DXL suggest that DXL blocks cellular excretion of this protein and intracellular accumulation of VEGF may be a protective mechanism since VEGF could have an intracrine activity.[57]

VEGF-R2 is mainly expressed by endothelial cells, but it is also moderately expressed by tumour cells.[37–42] Regulation of both the total pool and cell surface expression of VEGF-R2 is complex. In endothelial cells, a significant proportion of VEGF-R2 is held in an endosomal storage pool and VEGF-R2 recycling to the cell surface is stimulated by VEGF.[27] However, regulation of VEGF-R2 in tumour cells is relatively unknown. In our study, Bvz moderately increased the rate of the VEGF-R2 cell surface expression in MDA and PC3 cells (Fig. 7). A clinical study showed a related phenomenon: patients with a breast cancer underwent neoadjuvant treatment with one cycle of Bvz, used as a single agent, and the serum levels of soluble VEGF-R2 significantly increased after treatment with Bvz.[58] Little is known about the effect of chemotherapy on VEGF-R2 expression. We demonstrated that DXL increased VEGF-R2 transcription, mRNA and protein expression in MDA and PC3 cells (Fig. 6) and enhanced cell surface expression of VEGF-R2 (Fig. 7). Furthermore, the combination of both Bvz plus DXL strongly inhibited the cell surface levels of VEGF-R2 in MDA and PC3 cells (Fig. 7). In growth factor-deprived endothelial cells, the addition of VEGF resulted in mobilisation of intracellular VEGF-R2 and subsequent delivery to the cell membrane. In contrast, DXL treatment results in a reduction of extracellular VEGF in MDA and PC3 cells, which is accompanied by increased total (Fig. 6C) and membrane-associated (Fig. 7A) VEGF-R2. An interpretation of these findings could be the following: tumour cells, by producing high amount of VEGF, aberrantly expressed VEGF-R2 at the plasma membrane creating a pathological growth/survival pathway. Bvz traps extracellular VEGF but this may not be sufficient to prevent long-term VEGF accumulation since VEGF is still produced and Bvz could be degraded/inactivated in culture medium. VEGF, even in low amounts, is probably necessary and sufficient to mobilise intracellular VEGF-R2 pools to the membrane in tumour cells, as it does in endothelial cells.[27] Hence, tumour cells react by moderately increasing VEGF-R2. DXL treatment is much more aggressive since it could affect the production of different growth/survival factors (VEGF, EGF and PDGF). Hence, the potential down-regulation of the VEGF/VEGF-R2 pathway through decreased accessibility of VEGF to VEGF-R2 could be compensated by an increase in VEGF-R2 at the plasma membrane. When Bvz and DXL were combined, VEGF was stored in the cell and the remaining extracellular VEGF was trapped by Bvz. Hence, the amount of VEGF could be insufficient to mobilise VEGF-R2 pools resulting in a dramatic decrease in membrane-associated VEGF-R2. Surprisingly, we showed that the rate of phosphorylation of VEGF-R2 remained constant and was independent of the VEGF in the medium and the expression of membrane VEGF-R2. VEGF-R2 phosphorylation could be stimulated by VEGF sequestrated in the cytoplasm with DXL treatment, as suggested by Lee and co-workers who showed an intracrine activity of VEGF[57] and by Lee and colleagues who have shown that VEGF-R2 could be phosphorylated intracellularly.[59] However, internalisation of VEGF-R2 upon Bvz/DXL treatment, although VEGF-R2 is phosphorylated (Fig. 7B), may not correctly recruit signalling intermediates such as GRB2 and PI3 Kinase, hence altering downstream ERK or Akt signalling pathways.

The VEGF/VEGF-R2 loop has been characterised as autocrine signalling in breast and prostate cancer cell lines.[60,61] In MDA and PC3 cells our results show that Bvz had no effect on cell growth, suggesting that this autocrine signalling loop has a minor effect in control conditions. However, we have observed that the suppression of the VEGF pathway by Bvz plus DXL partly inhibited cell growth. The differences in the cell count and the measures for proliferation can be attributed to the induced apoptosis mediated by the combined treatment since VEGF is also a survival factor. Moreover Lee and co-workers have shown that inhibition of VEGF by antisense oligonucleotides or siRNA induces apoptosis in MDA cells.[57] However, we did not observe any cleavage of PARP, caspase 3 or caspase 9 with DXL treatment alone or in combination with Bvz suggesting that the difference in cell number is not due to apoptosis (data no shown). A possible explanation is that DXL induced multinucleation because of defects in cytokinesis.[62] A recent article addressed the role of different concentrations of DXL on breast cancer cells.[63] Hernandez-Vargas et al. described aberrant mitosis at low concentrations whereas high concentrations resulted in apoptosis. We have obtained similar observations with multi-nucleated cells suggestive of cytokinesis.

In addition, we have shown that extracellular levels of other pro-growth/survival factors are also decreased by the treatment. Hence, we hypothesise that tumour cells require a threshold of autocrine factors and the corresponding receptors for their optimal growth and survival and that DXL probably decreases this threshold below its optimal value. Hence, any further down-regulation of one of the growth/survival pathways, even if it is minor in control conditions, would result in a decreased cell number.

In summary, our results suggest a new paradigm for understanding the combined effects of Bvz and DXL observed in clinical trials.

Conflict of interest statement

All authors have no conflict of interest. Roche supported financially this study. However, Roche did not participate in the study design, analysis and interpretation of data; writing the manuscript and the decision to submit the manuscript for publication.

Acknowledgements

This work was supported by the ‘Centre National pour la Recherche Scientifique’, the University of Nice Sophia Antipolis, the ‘Ligue Nationale Contre le Cancer’ Equipe Labellisée, the Association for International Cancer Research (AICR), the National Institute of Cancer (INCA), the Association for Cancer Research (ARC, contract 4932) and the Cancéropole PACA. We thank Roche-France for financial support. C.O., J.D., A.V., C.B. and G.P. performed the experiments, S.S. provided plasmids, J.M.H.L. has initiated the work, C.O. and G.P. wrote the manuscript. We thank Dr. M.C. Brahimi-Horn for editorial correction of the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2010.07.021.

REFERENCES

1. Folkman J. Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat Med* 1995;**1**(1):27–31.
2. Ferrara N, Alitalo K. Clinical applications of angiogenic growth factors and their inhibitors. *Nat Med* 1999;**5**(12):1359–64.
3. Burger RA. Experience with bevacizumab in the management of epithelial ovarian cancer. *J Clin Oncol* 2007;**25**(20):2902–8.
4. Cannistra SA, Matulonis UA, Penson RT, et al. Phase II study of bevacizumab in patients with platinum-resistant ovarian cancer or peritoneal serous cancer. *J Clin Oncol* 2007;**25**(33):5180–6.
5. Giantonio BJ, Catalano PJ, Meropol NJ, et al. Bevacizumab in combination with oxaliplatin, fluorouracil, and leucovorin (FOLFOX4) for previously treated metastatic colorectal cancer: results from the Eastern Cooperative Oncology Group Study E3200. *J Clin Oncol* 2007;**25**(12):1539–44.
6. Cobleigh MA, Langmuir VK, Sledge GW, et al. A phase I/II dose-escalation trial of bevacizumab in previously treated metastatic breast cancer. *Semin Oncol* 2003;**30**(5 Suppl. 16): 117–24.
7. Yang JC, Haworth L, Sherry RM, et al. A randomized trial of bevacizumab, an anti-vascular endothelial growth factor antibody, for metastatic renal cancer. *N Engl J Med* 2003;**349**(5):427–34.
8. Sandler A, Gray R, Perry MC, et al. Paclitaxel-carboplatin alone or with bevacizumab for non-small-cell lung cancer. *N Engl J Med* 2006;**355**(24):2542–50.
9. Hurwitz H, Fehrenbacher L, Novotny W, et al. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. *N Engl J Med* 2004;**350**(23):2335–42.
10. Foekens JA, Peters HA, Grebenchtchikov N, et al. High tumor levels of vascular endothelial growth factor predict poor response to systemic therapy in advanced breast cancer. *Cancer Res* 2001;**61**(14):5407–14.
11. Bok RA, Halabi S, Fei DT, et al. Vascular endothelial growth factor and basic fibroblast growth factor urine levels as predictors of outcome in hormone-refractory prostate cancer patients: a cancer and leukemia group B study. *Cancer Res* 2001;**61**(6):2533–6.
12. George DJ, Halabi S, Shepard TF, et al. Prognostic significance of plasma vascular endothelial growth factor levels in patients with hormone-refractory prostate cancer treated on Cancer and Leukemia Group B 9480. *Clin Cancer Res* 2001;**7**(7):1932–6.
13. Ramaswamy B, Elias AD, Kelbick NT, et al. Phase II trial of bevacizumab in combination with weekly docetaxel in metastatic breast cancer patients. *Clin Cancer Res* 2006;**12**(10):3124–9.
14. Miller K, Wang M, Gralow J, et al. Paclitaxel plus bevacizumab versus paclitaxel alone for metastatic breast cancer. *N Engl J Med* 2007;**357**(26):2666–76.
15. Miles D, Chan A, Romieu G, et al. Randomized, double-blind, placebo-controlled, phase III study of bevacizumab with docetaxel or docetaxel with placebo as first-line therapy for patients with locally recurrent or metastatic breast cancer (mBC): AVADO. *J Clin Oncol* 2008;**26**(May 20). Abstract LBA 1011.
16. Reese D, Fratesi P, Corry M, et al. A phase II trial of humanized anti-vascular endothelial growth factor antibody for the treatment of androgen-independent prostate cancer. *The Prostate J* 2001;**3**(2):65–70.
17. Picus J, Halabi S, Rini B, et al. The use of bevacizumab (B) with docetaxel (D) and estramustine (E) in hormone refractory prostate cancer (HRPC): initial results of CALGB 90006. Proc Am Soc Clin Oncol 22;2003 [2003:Abstract No: 1578].
18. Di Lorenzo G, Figg WD, Fossa SD, et al. Combination of bevacizumab and docetaxel in docetaxel-pretreated hormone-refractory prostate cancer: a phase 2 study. Eur Urol 2008.
19. Petrylak DP, Tangen CM, Hussain MH, et al. Docetaxel and estramustine compared with mitoxantrone and prednisone for advanced refractory prostate cancer. *N Engl J Med* 2004;**351**(15):1513–20.
20. Jain RK. Antiangiogenic therapy for cancer: current and emerging concepts. *Oncology (Williston Park)* 2005;**19**(4 Suppl 3): 7–16.
21. Yang AD, Bauer TW, Camp ER, et al. Improving delivery of antineoplastic agents with anti-vascular endothelial growth factor therapy. *Cancer* 2005;**103**(8):1561–70.
22. Grant DS, Williams TL, Zahaczewsky M, Dicker AP. Comparison of antiangiogenic activities using paclitaxel (taxol) and docetaxel (taxotere). *Int J Cancer* 2003;**104**(1): 121–9.
23. Moser C, Lang SA, Stoeltzing O. The direct effects of anti-vascular endothelial growth factor therapy on tumor cells. *Clin Colorectal Cancer* 2007;**6**(8):564–71.
24. Legros L, Bourcier C, Jacquel A, et al. Imatinib mesylate (STI571) decreases the vascular endothelial growth factor plasma concentration in patients with chronic myeloid leukemia. *Blood* 2004;**104**(2):495–501.
25. Higgins KJ, Abdelrahim M, Liu S, Yoon K, Safe S. Regulation of vascular endothelial growth factor receptor-2 expression in pancreatic cancer cells by Sp proteins. *Biochem Biophys Res Commun* 2006;**345**(1):292–301.
26. Milanini J, Vinals F, Pouysségur J, Pagès G. p42/p44 MAP Kinase module plays a key role in the transcriptional regulation of vascular endothelial growth factor gene in fibroblasts. *J Biol Chem* 1998;**273**:18165–72.
27. Gampel A, Moss L, Jones MC, et al. VEGF regulates the mobilization of VEGFR2/KDR from an intracellular endothelial storage compartment. *Blood* 2006;**108**(8):2624–31.
28. Essafi-Benkhadir K, Onesto C, Stebe E, Moroni C, Pages G. Tristetraprolin inhibits Ras-dependent tumor vascularization by inducing vascular endothelial growth factor mRNA degradation. *Mol Biol Cell* 2007;**18**(11):4648–58.
29. Wang Y, Fei D, Vanderlaan M, Song A. Biological activity of bevacizumab, a humanized anti-VEGF antibody in vitro. *Angiogenesis* 2004;**7**(4):335–45.
30. Lee Jr JT, Steelman LS, McCubrey JA. Phosphatidylinositol 3'-kinase activation leads to multidrug resistance protein-1 expression and subsequent chemoresistance in advanced prostate cancer cells. *Cancer Res* 2004;**64**(22):8397–404.
31. Hirata A, Ogawa S, Kometani T, et al. ZD1839 (Iressa) induces antiangiogenic effects through inhibition of epidermal growth factor receptor tyrosine kinase. *Cancer Res* 2002;**62**(9):2554–60.
32. Goldman CK, Kim J, Wong WL, et al. Epidermal growth factor stimulates vascular endothelial growth factor production by human malignant glioma cells: a model of glioblastoma multiforme pathophysiology. *Mol Biol Cell* 1993;**4**(1):121–33.
33. Ferrara N, Carver-Moore K, Chen H, et al. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature* 1996;**380**(6573):439–42.

34. Dor Y, Djonov V, Abramovitch R, et al. Conditional switching of VEGF provides new insights into adult neovascularization and pro-angiogenic therapy. *Embo J* 2002;**21**(8):1939–47.
35. Alitalo K, Carmeliet P. Molecular mechanisms of lymphangiogenesis in health and disease. *Cancer Cell* 2002;**1**(3):219–27.
36. Shibuya M. Vascular endothelial growth factor (VEGF)-Receptor2: its biological functions, major signaling pathway, and specific ligand VEGF-E. *Endothelium* 2006;**13**(2):63–9.
37. Duff SE, Jeziorska M, Rosa DD, et al. Vascular endothelial growth factors and receptors in colorectal cancer: implications for anti-angiogenic therapy. *Eur J Cancer* 2006;**42**(1):112–7.
38. Giannelli G, Azzariti A, Sgarra C, et al. ZD6474 inhibits proliferation and invasion of human hepatocellular carcinoma cells. *Biochem Pharmacol* 2006;**71**(4):479–85.
39. Yang AD, Camp ER, Fan F, et al. Vascular endothelial growth factor receptor-1 activation mediates epithelial to mesenchymal transition in human pancreatic carcinoma cells. *Cancer Res* 2006;**66**(1):46–51.
40. Hiramatsu A, Miwa H, Shikami M, et al. Disease-specific expression of VEGF and its receptors in AML cells: possible autocrine pathway of VEGF/type1 receptor of VEGF in t(15;17) AML and VEGF/type2 receptor of VEGF in t(8;21) AML. *Leuk Lymphoma* 2006;**47**(1):89–95.
41. Seto T, Higashiyama M, Funai H, et al. Prognostic value of expression of vascular endothelial growth factor and its flt-1 and KDR receptors in stage I non-small-cell lung cancer. *Lung Cancer* 2006;**53**(1):91–6.
42. Lacal PM, Ruffini F, Pagani E, D'Atri S. An autocrine loop directed by the vascular endothelial growth factor promotes invasiveness of human melanoma cells. *Int J Oncol* 2005;**27**(6):1625–32.
43. Claesson-Welsh L. Signal transduction by vascular endothelial growth factor receptors. *Biochem Soc Trans* 2003;**31**(Pt. 1):20–4.
44. Ryden L, Linderholm B, Nielsen NH, et al. Tumor specific VEGF-A and VEGFR2/KDR protein are co-expressed in breast cancer. *Breast Cancer Res Treat* 2003;**82**(3):147–54.
45. Gril B, Palmieri D, Bronder JL, et al. Effect of lapatinib on the outgrowth of metastatic breast cancer cells to the brain. *J Natl Cancer Inst* 2008;**100**(15):1092–103.
46. Uckun FM, Narla RK, Jun X, et al. Cytotoxic activity of epidermal growth factor-genistein against breast cancer cells. *Clin Cancer Res* 1998;**4**(4):901–12.
47. Timke C, Zieher H, Roth A, et al. Combination of vascular endothelial growth factor receptor/platelet-derived growth factor receptor inhibition markedly improves radiation tumor therapy. *Clin Cancer Res* 2008;**14**(7):2210–9.
48. Kong D, Banerjee S, Huang W, et al. Mammalian target of rapamycin repression by 3,3'-diindolylmethane inhibits invasion and angiogenesis in platelet-derived growth factor-D-overexpressing PC3 cells. *Cancer Res* 2008;**68**(6):1927–34.
49. Pagès G, Berra E, Milanini J, Levy AP, Pouysségur J. Stress-activated protein kinases (JNK and p38/HOG) are essential for vascular endothelial growth factor mRNA stability. *J Biol Chem* 2000;**275**(34):26484–91.
50. Bates DO, Cui TG, Doughty JM, et al. VEGF165b, an inhibitory splice variant of vascular endothelial growth factor, is down-regulated in renal cell carcinoma. *Cancer Res* 2002;**62**(14):4123–31.
51. Avramis IA, Kwock R, Avramis VI. Taxotere and vincristine inhibit the secretion of the angiogenesis inducing vascular endothelial growth factor (VEGF) by wild-type and drug-resistant human leukemia T-cell lines. *Anticancer Res* 2001;**21**(4A):2281–6.
52. Keyes K, Cox K, Treadway P, et al. An in vitro tumor model: analysis of angiogenic factor expression after chemotherapy. *Cancer Res* 2002;**62**(19):5597–602.
53. Lissoni P, Fugamalli E, Malugani F, et al. Chemotherapy and angiogenesis in advanced cancer: vascular endothelial growth factor (VEGF) decline as predictor of disease control during taxol therapy in metastatic breast cancer. *Int J Biol Markers* 2000;**15**(4):308–11.
54. Sonee M, Barron E, Yarber FA, Hamm-Alvarez SF. Taxol inhibits endosomal–lysosomal membrane trafficking at two distinct steps in CV-1 cells. *Am J Physiol* 1998;**275**(6 Pt. 1): C1630–9.
55. Robin P, Rossignol B, Raymond MN. Effect of microtubule network disturbance by nocodazole and docetaxel (Taxotere) on protein secretion in rat extraorbital lacrimal and parotid glands. *Eur J Cell Biol* 1995;**67**(3):227–37.
56. Van De Moortele S, Picart R, Tixier-Vidal A, Tougard C. Nocodazole and taxol affect subcellular compartments but not secretory activity of GH3B6 prolactin cells. *Eur J Cell Biol* 1993;**60**(2):217–27.
57. Lee TH, Seng S, Sekine M, et al. Vascular endothelial growth factor mediates intracrine survival in human breast carcinoma cells through internally expressed VEGFR1/FLT1. *PLoS Med* 2007;**4**(6):e186.
58. Denduluri N, Yang SX, Berman AW, et al. Circulating biomarkers of bevacizumab activity in patients with breast cancer. *Cancer Biol Ther* 2007;**7**(1):1.
59. Lee S, Chen TT, Barber CL, et al. Autocrine VEGF signaling is required for vascular homeostasis. *Cell* 2007;**130**(4): 691–703.
60. Weigand M, Hantel P, Kreienberg R, Waltenberger J. Autocrine vascular endothelial growth factor signalling in breast cancer. Evidence from cell lines and primary breast cancer cultures in vitro. *Angiogenesis* 2005;**8**(3):197–204.
61. Jackson MW, Roberts JS, Heckford SE, et al. A potential autocrine role for vascular endothelial growth factor in prostate cancer. *Cancer Res* 2002;**62**(3):854–9.
62. Zhu J, Beattie EC, Yang Y, et al. Centrosome impairments and consequent cytokinesis defects are possible mechanisms of taxane drugs. *Anticancer Res* 2005;**25**(3B):1919–25.
63. Hernandez-Vargas H, Palacios J, Moreno-Bueno G. Molecular profiling of docetaxel cytotoxicity in breast cancer cells: uncoupling of aberrant mitosis and apoptosis. *Oncogene* 2007;**26**(20):2902–13.