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Cisplatin and doxorubicin repress Vascular Endothelial Growth Factor expression and differentially down-regulate Hypoxia-inducible Factor I activity in human ovarian cancer cells

Monique C.A. Duyndam [a], *Maria P.A. van Berkel* [a], *Josephine C. Dorsman* [b,c], *Davy A.P. Rockx* [b], *Herbert M. Pinedo* [a], *Epie Boven* [a,*]

[a] *Department of Medical Oncology, VU University medical center, De Boelelaan 1117, 1081 HV Amsterdam, The Netherlands*
[b] *Department of Gynaecology and Obstetrics, VU University medical center, Amsterdam, The Netherlands*
[c] *Center of Medical Systems Biology, Leiden, The Netherlands*

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ABSTRACT

Vascular Endothelial Growth Factor (VEGF) and its transcriptional regulator Hypoxia-inducible Factor 1 (HIF-1) play an important role in the process of angiogenesis in many types of cancer, including ovarian cancer. We have examined whether the DNA-damaging drugs cisplatin and doxorubicin and the microtubule inhibitors docetaxel and paclitaxel can affect VEGF expression and HIF-1 activity in three human ovarian cancer cell lines. We demonstrate that cisplatin and doxorubicin abolish hypoxia-induced VEGF mRNA expression in all cell lines, while basal VEGF mRNA expression was also downregulated. Transient transfection with a HIF-1-responsive luciferase construct indicated that cisplatin and doxorubicin inhibited hypoxic activation of HIF-1. Cisplatin repressed HIF-1α protein expression in all cell lines. Stimulation of HIF-1α protein degradation by cisplatin was observed in the only cell line expressing wild-type p53. Cisplatin also inhibited the synthesis of HIF-1α protein for which p53 was dispensable. Interestingly, cisplatin strongly reduced the protein levels of the HIF-1 coactivators p300 and CREB-binding protein (CBP) under hypoxia in all cell lines. Although doxorubicin inhibited hypoxic activation of HIF-1, this drug had no significant effect on the expression levels of HIF-1α and hypoxic expression of p300 and CBP was only weakly reduced. Docetaxel and paclitaxel did neither influence VEGF expression nor hypoxia-induced HIF-1 activity. In total, our findings indicate that cisplatin and doxorubicin can repress hypoxic induction of VEGF expression by inhibiting HIF-1 through different mechanisms. This knowledge may be useful for future treatment schedules including agents that target the HIF-1 signalling pathway.

* *Corresponding author*. Tel.: +31 20 4444336; fax: +31 20 4444079.
E-mail address: E.Boven@vumc.nl (E. Boven).

Abbreviations: VEGF, Vascular Endothelial Growth Factor l; HIF-1, Hypoxia-inducible Factor 1; HRE, hypoxia responsive element; VHL, Von Hippel-Lindau; wt, wildtype; mt, mutant; DFO, desferrioxamine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; CA, carbonic anhydrase; PI3K, phosphatidylinositol 3-kinase; mTOR, mammalian target of rapamycin; MAPK, Mitogen-Activated Protein Kinase

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1. Introduction

Vascular Endothelial Growth Factor (VEGF) is an important regulator of the process of angiogenesis in many types of cancer, including ovarian cancer. High VEGF expression and microvessel density have been correlated with poor survival in ovarian cancer patients [1]. VEGF exists as multiple isoforms that are generated by alternative splicing of a single transcript [2]. The isoforms differ in their molecular weights and in biological properties such as their ability to bind to cell-surface heparan sulfate proteoglycans. The $VEGF_{165}$ isoform is partially secreted and partly retained on the cell surface [2]. We have reported that $VEGF_{165}$ overexpression stimulates angiogenesis in human ovarian cancer xenografts [3].

While VEGF expression can be regulated by many different stimuli, hypoxia is known to be an important activator of VEGF transcription. Hypoxic induction of VEGF is mediated by the transcription factor Hypoxia-inducible Factor 1 (HIF-1), which plays a key role in regulating the adaptation of tumors to hypoxia [4]. Clinical studies support that HIF-1 is also an important regulator of angiogenesis and concomitant tumor growth in ovarian cancer [1,5].

The regulation of HIF-1 has been extensively investigated. HIF-1 is a heterodimeric protein that consists of two subunits, HIF-1α and HIF-1β. The activity of HIF-1 is mainly regulated by the expression level and activity of the HIF-1α subunit. Under normal oxygen tension, the expression of the HIF-1α protein is low due to hydroxylation of proline residues 402 and 564 by prolyl hydroxylases [6]. These modifications allow the binding of HIF-1α to the tumor suppressor protein van Hippel-Lindau, which is associated with an E3 ubiquitin ligase complex that targets HIF-1α protein to the ubiquitin/proteasome degradation pathway [7]. Under normoxia, the ability of HIF-1α to activate transcription is also repressed due to hydroxylation of an asparagine residue in its C-terminal transactivation domain [6]. This latter modification inhibits the interaction of HIF-1α with the highly related coactivators p300 and CBP [6,8]. Apart from oxygen, the activity of the prolyl and asparagine hydroxylases is dependent on the presence of iron. Under hypoxia or upon treatment with iron chelators, HIF-1α protein becomes stabilized and transcriptionally active [9,10]. Following stabilization, HIF-1α is translocated to the nucleus where it dimerizes to HIF-1β and binds to hypoxia-responsive elements (HREs) located within the promoter of target genes [4,11].

Since VEGF and HIF-1 are important regulators of angiogenesis, these are important potential targets for cancer therapy. Efforts are currently undertaken to develop inhibitors of both proteins and to test their efficacy as anticancer agents [12,13], also in combination regimens [13]. In this context, it is of relevance to determine whether conventional anticancer agents can influence VEGF and HIF-1 already themselves, and if so, via which mechanism.

For ovarian cancer, a platinum-based combination including docetaxel or paclitaxel is now the most effective first-line treatment modality [14,15]. Treatment with doxorubicin has also gained renewed interest [16]. Cisplatin and doxorubicin seem to exert their toxic effects primarily by causing DNA damage. Cisplatin covalently binds to DNA and forms inter and intra-strands crosslinks. Doxorubicin damages DNA by intercalation, by generation of free radicals and by inhibition of DNA topoisomerase II. In contrast, the taxanes docetaxel and paclitaxel act by interfering with the microtubule cytoskeleton [17]. These agents bind to microtubule polymers, which results in the inhibition of depolymerization required for a cell to complete mitosis.

In this study, we examined whether cisplatin, doxorubicin and the taxanes docetaxel and paclitaxel can affect VEGF expression and HIF-1 activity under normoxia and hypoxia in three human ovarian cancer cell lines. In addition, drug-mediated effects on the expression of HIF-1α and its coactivators p300 and CBP protein were investigated.

2. Materials and methods

2.1. Reagents

Cisplatin and docetaxel were purchased from Bristol-Myers Squibb (Woerden, The Netherlands) and Sanofi Aventis (Vitry-sur-Seine, France), respectively. Doxorubicin was purchased from Pfizer (Woerden, The Netherlands). Paclitaxel, MG132, cycloheximide (CHX) and desferrioxamine (DFO) were purchased from Sigma–Aldrich Chemie (Zwijndrecht, The Netherlands).

2.2. Cell culture and cell treatment

A2780 (wild-type (wt) p53) and OVCAR-3 (mutant (mt) p53) human ovarian cancer cell lines were selected on the basis of p53 status [18] and have been characterized previously in our laboratory for responses to cisplatin and docetaxel [18,19]. The SKOV-3 human ovarian cancer cell line was added because of p53null status [20]. The lack of p53 expression in our SKOV-3 cells has been confirmed by Western blotting (data not shown). Mean population doubling times for A2780, OVCAR-3 and SKOV-3 cells were 19, 26 and 34 h, respectively.

All cells were cultured in Dulbecco's modified Eagle's medium (Gibco/Life Technologies, Breda, The Netherlands) supplemented with 10% heat-inactivated fetal calf serum (FCS, Sanbio, Uden, The Netherlands), 50 units/ml penicillin (ICN Biochemicals, Zoetermeer, The Netherlands) and 50 μg/ml streptomycin (ICN Biochemicals). Cells were routinely cultured in 95% air and 5% CO_2 at 37 °C. Hypoxic conditions were obtained by incubation of cells in a tightly sealed chamber (Billupus-Rothemberg Inc., Del Mar, USA) maintained at 1% oxygen, 94% N_2 and 5% CO_2 at 37 °C.

Cells were exposed for 6–8 h in the absence and presence of drugs under normoxia or hypoxia. The 6 and 8 h exposure periods to hypoxia were selected to allow a clear detection of HIF-1 activation and VEGF mRNA induction, respectively, while direct toxicity of the drugs on cell viability would be avoided.

2.3. Real-time quantitative PCR

Total RNA was reversed transcribed to cDNA using Moloney Murine Leukemia Virus reverse transcriptase (Invitrogen/Life technologies, Breda, The Netherlands). Quantification of mRNAs encoding HIF-1α, $VEGF_{165}$, Carbonic Anhydrase (CA)

9, p21 and β2-microglobulin was performed using the LightCycler technology (Roche Diagnostics, Almere, The Netherlands). The forward and reversed primers for amplification of $VEGF_{165}$, HIF-1α, CA9, p21 and β2-microglobulin fragments have been described [21–23]. Real-time PCR was performed using the FastStart DNA Master SYBR Green I kit (Roche diagnostics). Amplification was performed in the presence of 4 mM $MgCl_2$. Conditions consisted of an initial denaturation step of 10 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 1 s, annealing at 60 °C for 5 s and extension at 72 °C for 22 s. Fluorescence was measured directly after extension for 5 s at 72 °C. At the end of the PCR cycles, a melting curve was generated to determine the specificity of the PCR product. The relative expression ratio represents the fold induction of the mRNA levels of the gene of interest in drug and/or hypoxia-treated cells over non-treated, normoxic control cells after normalisation to β2-microglobulin. The relative expression ratios were calculated by the equation:

$$\frac{E_{\text{gene of interest}}\,\Delta\text{Cp gene of interest (control} - \text{sample)}}{E_{\beta\text{2-microglobulin}}\,\Delta\text{Cp }\beta 2 - \text{microglobulin(control} - \text{sample)}}$$

Cp represents the crossing point, which is defined as the number of cycles needed to generate a fluorescent signal above a predefined threshold. *E* represents the efficiency of amplification for a particular primer set, which was determined by plotting Cp cycles versus concentrations of a pool of the cDNAs used in the experiments. *E* was calculated according to the equation: $E = 10^{[-1/\text{slope}]}$. $E_{VEGF165}$, $E_{HIF\text{-}1\alpha}$, E_{CA9}, E_{p21} and $E_{\beta\text{-}2\ \text{microglobulin}}$ were 1.86, 1.86, 1.89, 1.72 and 1.85, respectively.

2.4. Elisa

A2780 cells were plated on 5 mm culture dishes in 10-fold at equal densities of 2×10^6 cells per dish and grown overnight. The conditioned medium was collected and divided in 5 equal volumes. Cisplatin and doxorubin was added to individual volumes at a concentration of 50 and 100 μM or 5 and 15 μM, respectively. In a similar experiment, docetaxel and paclitaxel were added at a concentration of 3 and 30 μM. Subsequently, 4 ml of conditioned media with or without drugs was again added to the ten culture dishes with cells. Thus, the amount of VEGF protein in the medium was the same in each culture dish. After an exposure period of 8 h under normoxia or hypoxia in the absence and presence of drugs, 450 μl of conditioned medium was sampled and cells were with lysed in E1A buffer (50 mM Tris–HCl pH 7.5, 250 mM NaCl, 5 mM EDTA and 0.1% NP40), supplemented with 50 mM NaF, 1 mM Na_3VO_4, 1.0 mM phenylmethylsulphonyl fluoride (PMSF), 0.5 mM trypsin inhibitor and 0.5 μg/ml leupeptin. After a 15 min incubation period on ice, the extracts were clarified by centrifugation at 20,000 × *g* for 15 min at 4 °C and stored at −70 °C. Protein concentrations in lysates were determined by the Coomassie Plus Protein assay (Pierce, Omnilabo, Breda, The Netherlands). VEGF concentrations in nondiluted media samples and lysates were determined in duplo by ELISA using the reagents and the protocol supplied with the Quantikine Human VEGF Immunoassay kit (R&D Systems/ITK Diagnostics, Uithoorn, The Netherlands).

2.5. Transient transfection

Transient transfection of OVCAR-3, A2780 and SKOV-3 cells was performed in duplicate wells of 6-well culture plates with 0.5 μg of the pGL3-luciferase reporter construct (Promega, Leiden, The Netherlands) or the 5xHREpGL3 constuct [24] together with 2.0 μg of pUC19 carrier DNA (per well) as described [24]. Luciferase assays (Promega) were performed according to the manufacturer's protocol.

2.6. MTT-viability assay

The effect of drug concentrations on cell viability was evaluated by measurement of metabolic activity in the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay [25]. In short, 1.5×10^4 cells were plated in four replicate wells of 96-well tissue culture plates. The next day, cells were exposed for 8 h to drugs under normoxic or hypoxic conditions. MTT was added directly after exposure of cells and metabolic activity of treated cells was expressed as a percentage of that of control (untreated) cells. In addition, cell morphology was monitored during and at the end of the experiments.

2.7. Western blot

Cells were lysed in luciferase lysis reagent (25 mM Tris-phosphate pH 7.8, 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid, 10% glycerol, 1% Triton X-100) or in FosRipa buffer (10 mM Tris/HCl pH 7.5, 250 mM NaCl, 0.1% SDS, 1% Nonidet P40, 1% sodium deoxycholate). Insoluble pellets were solubilized in Laemmli samples buffer (62.5 mM Tris–HCl pH 6.8, 2.4% SDS, 100 mM DDT, 10% glycerol, 1 mM EDTA, 0.001% bromphenol blue), heated for 5 min at 96 °C and centrifuged briefly before loading. Determination of protein concentrations and Western blotting was performed according to standard procedures [21,26]. Equal amounts of protein (50 μg) were loaded for separation via 7.5% SDS-PAGE. Mouse monoclonal antisera directed against HIF-1α (BD Transduction Laboratories, Alphen a/d Rijn, The Netherlands), HIF-1β (BD Transduction Laboratories), p53 (DO-7; Dako, Glostrup, Denmark), β-Actin (Sigma–Aldrich Chemie) and rabbit polyclonal antisera against p300 (N15; Santa Cruz MP Biochemicals, Amsterdam, The Netherlands) and CBP (A22; Santa Cruz MP Biochemicals) were used as primary antibodies. Horse-radish peroxidase-coupled anti-mouse and anti-rabbit sera (Dako) were used as a secondary antibodies.

3. Results

3.1. Cisplatin and doxorubicin inhibit both basal and hypoxia-induced expression of VEGF and CA9

When A2780 human ovarian cancer cells were exposed for 8 h to hypoxia, $VEGF_{165}$ mRNA levels were induced 4.1-fold (Fig. 1A). Hypoxic induction of $VEGF_{165}$ mRNA was strongly inhibited by cisplatin (50 and 100 μM) and even completely by doxorubicin (5 and 15 μM). VEGF protein levels were regulated in a similar manner. Exposure to hypoxia increased VEGF

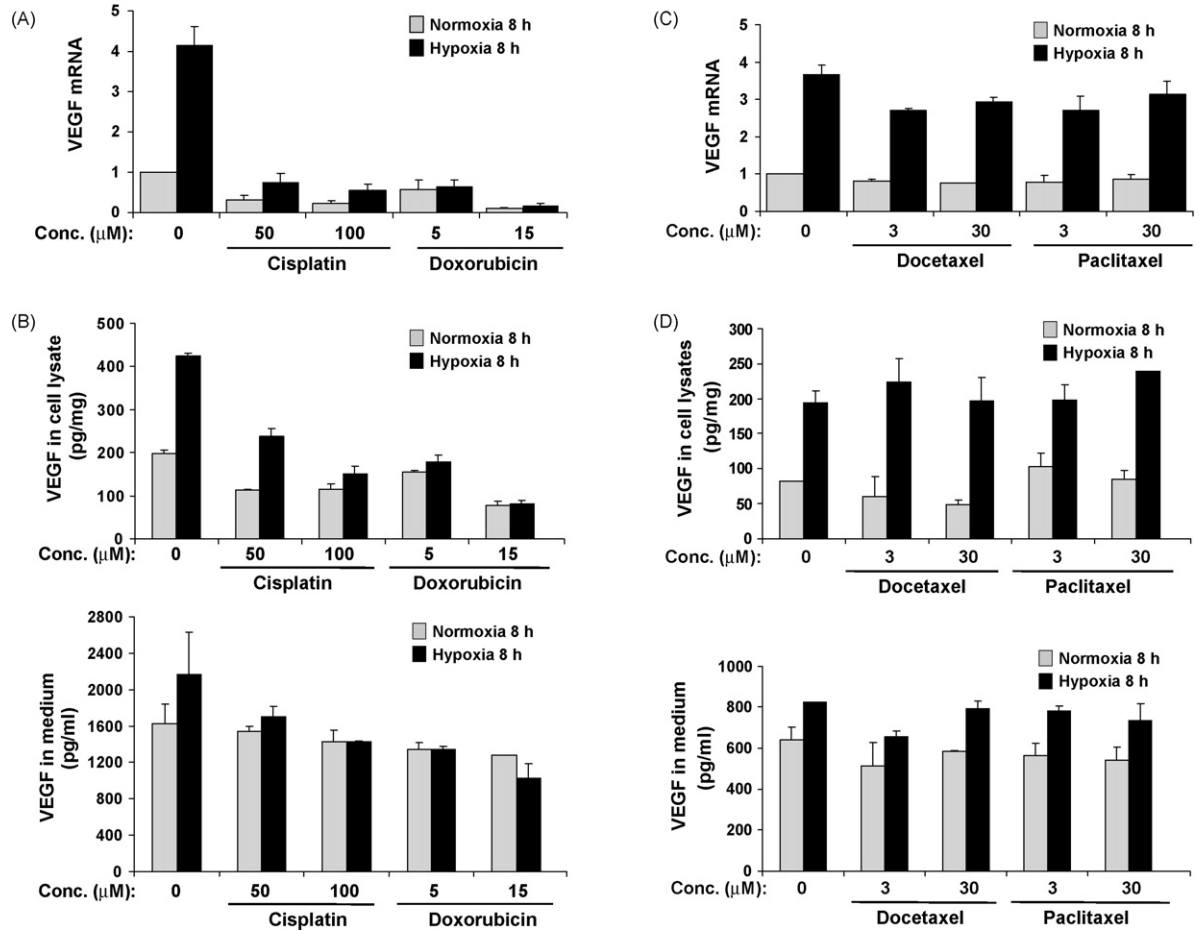

Fig. 1 – Cisplatin and doxorubicin, but not docetaxel and paclitaxel, inhibit hypoxia-induced and basal expression of VEGF mRNA and VEGF protein in A2780 cells. A2780 cells were left untreated or exposed to the indicated concentrations of cisplatin, doxorubicin (A and B), docetaxel or paclitaxel (C and D) under normoxia or hypoxia for 8 h. (A and C) $VEGF_{165}$ mRNA levels were assessed by real-time PCR and normalized to β2-microglobulin mRNA levels. Mean relative expression ratios of $VEGF_{165}$/β2-microglobulin are shown. Non-treated A2780 cells under normoxia were set at 1. The bars represent the mean ± range of 2 independent runs of cDNA samples of one experiment. An independent experiment gave comparable results. (B and D) VEGF protein in cell lysates and conditioned media was measured by ELISA as described in Section 2. Results are given in pg of VEGF per mg of total cell protein or per ml of conditioned medium. The bars represent the mean ± S.D. of duplicate samples in a representative experiment of three independent experiments that gave comparable results.

protein concentrations in lysate and in conditioned medium of A2780 cells and this increase was abolished by cisplatin and doxorubicin (Fig. 1B). Under normoxia, the expression of $VEGF_{165}$ mRNA and protein was also reduced by cisplatin and doxorubicin. In contrast, the taxanes docetaxel and paclitaxel (10, 30 μM) only weakly reduced basal and hypoxia-induced $VEGF_{165}$ mRNA expression, which did not result in a reduction in VEGF protein expression (Fig. 1C and D). As assessed by the MTT assay, 8-h exposures to cisplatin and doxorubicin under normoxia and hypoxia did not affect the viability of the cells (data not shown). Eight-hour exposures to the highest concentrations of docetaxel and paclitaxel slightly reduced (<10%) the viability of A2780 cells (data not shown). Under the various conditions, the morphology of the cells was not changed and no cell detachment was observed.

We next determined the effects of cisplatin and doxorubicin on VEGF mRNA expression in two other human ovarian cancer cell lines OVCAR-3 and SKOV-3. Also in these cell lines, hypoxia-induced and basal expression of VEGF mRNA was inhibited by cisplatin and doxorubicin (Fig. 2A and B). As VEGF induction by hypoxia involves the activation of the transcription factor HIF-1, we further examined the expression of another HIF-1-responsive gene, CA9, in SKOV-3 cells. As can be seen in Fig. 2C, hypoxic induction of CA9 mRNA expression was inhibited by cisplatin and doxorubicin. Both drugs also reduced CA9 expression in normoxic SKOV-3 cells.

These data demonstrate that cisplatin and doxorubicin can inhibit basal and hypoxia-induced VEGF and CA9 expression in human ovarian cancer cell lines.

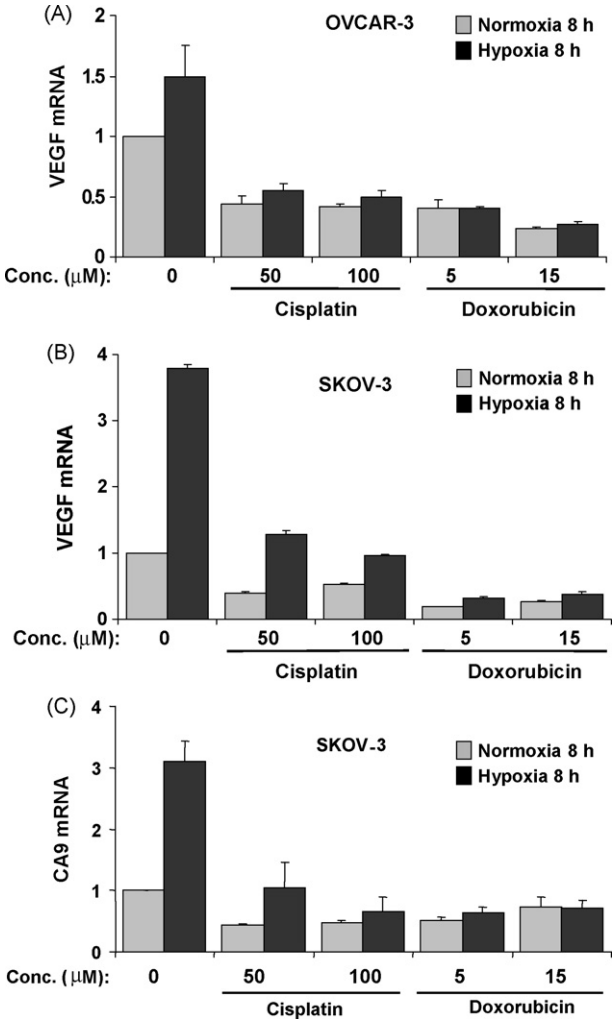

Fig. 2 – Cisplatin and doxorubicin inhibit hypoxia-induced and basal expression of VEGF and CA9 mRNA in OVCAR-3 and SKOV-3 cells. OVCAR-3 (A) and SKOV-3 cells (B and C) were left untreated or exposed to the indicated concentrations of cisplatin and doxorubicin under normoxia or hypoxia for 8 h. $VEGF_{165}$ and/or CA9 mRNA levels were assessed by real-time PCR and normalized to β2-microglobulin mRNA levels. Mean relative expression ratio of $VEGF_{165}$/β2-microglobulin or CA9/β2-microglobulin mRNA are shown. Non-treated OVCAR-3 and SKOV-3 cells under normoxia were set at 1. The bars represent the mean ± range of 2 independent runs of cDNA samples of one experiment. An independent experiment gave comparable results.

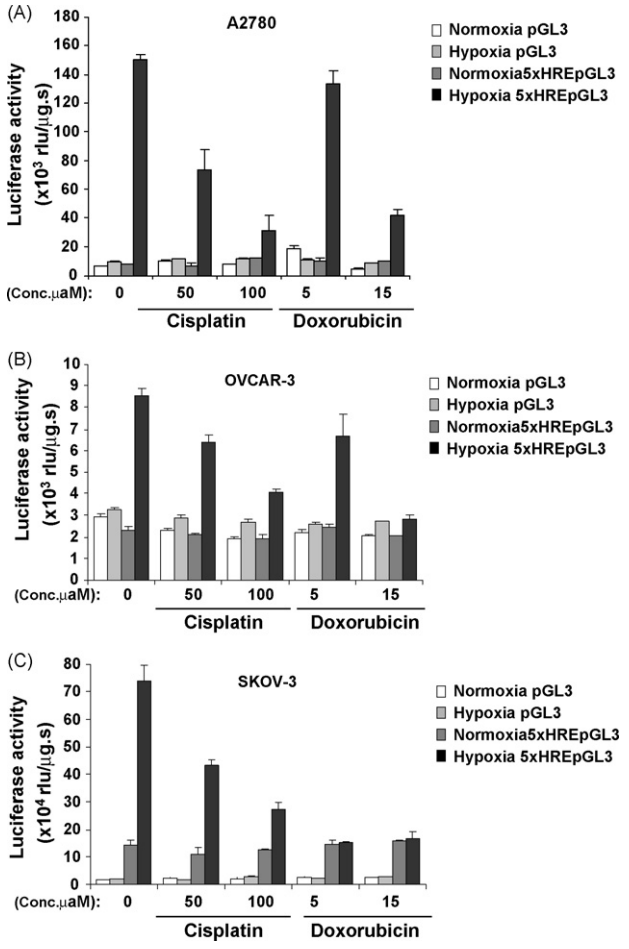

Fig. 3 – Cisplatin and doxorubicin inhibit hypoxia-induced HIF-1 activity. A2780 (A), OVCAR-3 (B) and SKOV-3 (C) cells were transiently transfected with the indicated pGL3-luciferase reporter constructs. After 40 h, cells were left untreated or exposed to the indicated concentrations of cisplatin or doxorubicin under normoxia or hypoxia for a period of 6 h. The relative luciferase activity in the histogram represents the mean luciferase (RUL) per μg total protein per 10 s ± range of duplicate extracts. Three independent experiments were performed with similar outcome. Results of one of these are shown.

3.2. *Cisplatin and doxorubicin inhibit induction of HIF-1 activity by hypoxia in human ovarian cancer cell lines*

We subsequently focused on the effects of cisplatin and doxorubicin on hypoxia-induced gene expression and on the transcriptional activity of HIF-1. To that end, cells were transiently transfected with the 5xHREpGL3 and pGL3 luciferase reporter gene constructs (Fig. 3). The 5xHREpGL3 construct contains 5 copies of a HIF-1 consensus sequence of the human VEGF promoter. As activation of HIF-1 is expected to precede induction of VEGF mRNA expression, transfected cells were exposed for a shorter period of 6 h to hypoxia in the absence and presence of drugs. Exposure to hypoxia alone induced the activity of 5xHREpGL3 15-, 4- and 5-fold in A2780, OVCAR-3 and SKOV-3 cells, respectively, indicating activation of HIF-1. Exposure to cisplatin (50, 100 μM) or doxorubicin (5, 15 μM) did not affect the basal activity of 5xHREpGL3 and pGL3 in all cell lines. Hypoxic induction of 5xHREpGL3 activity, however, could be inhibited by both cisplatin and doxorubicin in a dose-dependent manner. These effects were mediated by the HREs, as expression of the pGL3 construct was hardly affected upon drug treatment. The taxanes docetaxel (3–30 μM) and paclitaxel (3–30 μM) did neither affect basal expression of pGL3 and 5xHREpGL3 constructs, nor alter hypoxic induction of

5xHREpGL3 expression, as tested after a 6 h period in A2780 and OVCAR-3 cells (Supplementary Fig. 1 online).

Together, these results indicate that cisplatin and doxorubicin can inhibit hypoxic activation of HIF-1 in human ovarian cancer cells. In contrast, the taxanes docetaxel and paclitaxel did neither influence HIF-1 activity under normoxia nor under hypoxia.

3.3. *Cisplatin, but not doxorubicin, strongly down-regulates the expression of HIF-1α protein*

The levels of HIF-1α and HIF-1β protein in normoxic and hypoxic A2780, OVCAR-3 and SKOV-3 cells upon drug treatment were investigated in lysates by Western blotting (Fig. 4A and B). In all cell lines, HIF-1α protein expression was highly increased after 6 h of exposure to hypoxia. Cisplatin strongly inhibited HIF-1α protein expression under hypoxia and normoxia in the OVCAR-3 and SKOV-3 cells and to a limited extent in normoxic and hypoxic A2780 cells. The observed down-regulation of HIF-1α protein expression by cisplatin was specific, since no alterations were detected in the levels of the HIF-1β protein. In contrast, doxorubicin did not influence HIF-1α protein expression under hypoxia (Fig. 4A). The taxanes docetaxel and paclitaxel did neither exert a specific effect on the expression of HIF-1α protein under normoxia nor under hypoxia in A2780 and OVCAR-3 cells (not shown), in keeping with their lack of an effect on HIF-1 activity.

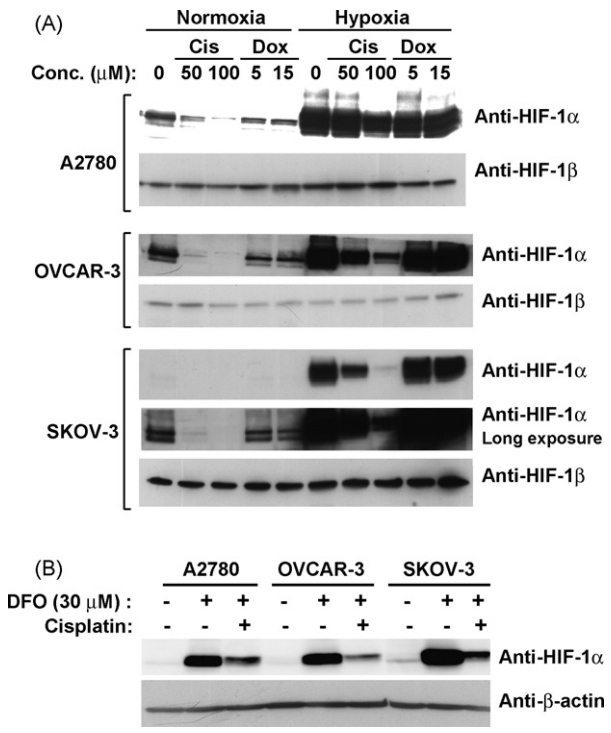

Fig. 4 – Cisplatin, but not doxorubicin, down-regulates the expression of HIF-1α protein under normoxia and hypoxia. The levels of HIF-1α, HIF-1β and/or β-Actin proteins in lysates were assessed by Western blot. (A) A2780, OVCAR-3 and SKOV-3 cells were left untreated or exposed to the indicated concentrations of cisplatin (Cis) and doxorubicin (Dox) under normoxia and hypoxia for 6 h. A longer exposure of the Western blot of SKOV-3 cells is shown to visualize alterations in HIF-1α protein expression under normoxia. (B) A2780, OVCAR-3 and SKOV-3 cells were treated for 6 h with DFO (30 μM) under normoxia in the absence or presence of cisplatin (100 μM).

We further examined the effect of cisplatin on the induction of HIF-1α protein by a 6-h treatment with the iron chelator DFO (30 μM). This agent induces the expression and the transactivating activity of HIF-1α under normoxia by inhibiting the hydroxylation reactions for which Fe(II) is an important cofactor [27]. Fig. 4B shows that cisplatin (100 μM) also strongly inhibited the accumulation of HIF-1α protein in DFO-treated A2780, OVCAR-3 and SKOV-3 cells.

One possible mechanism by which cisplatin could reduce the HIF-1α protein levels is the inhibition of HIF-1α gene transcription. Therefore, we examined the effect of cisplatin (50 and 100 μM) on the levels of HIF-1α mRNA relative to that of the control β2-microglobulin under hypoxia. Upon treatment with cisplatin, HIF-1α mRNA expression was only weakly reduced in hypoxic A2780 and OVCAR-3 cells (25% and 20% reduction, respectively), while a slight elevation of HIF-1α mRNA levels was observed in hypoxic SKOV-3 cell (Supplementary Fig. 2A online). Similar effects of cisplatin on HIF-1α mRNA expression were observed in DFO-treated A2780, OVCAR-3 and SKOV-3 cells (Supplementary Fig. 2B online). Evidently, inhibition of HIF-1α protein expression by cisplatin is not due to a decreased transcription of the HIF-1α gene in SKOV-3 cells. In addition, the weak reduction of the HIF-1α mRNA levels observed in OVCAR-3 cells can only contribute to a minor extent to the potent downregulation of HIF-1α protein expression by cisplatin. In OVCAR-3 and in A2780 cells, HIF-1α mRNA expression was reduced to a similar extent by doxorubicin (15 μM; Supplementary Fig. 2A and B online), while this agent showed no effect on the level of HIF-1α protein In total, these results indicate that cisplatin inhibits hypoxia-induced HIF-1 activity at least in part by down-regulation of HIF-1α protein expression at posttranscriptional levels.

3.4. *Cisplatin can stimulate the degradation and inhibit the synthesis of HIF-1α protein*

To investigate whether cisplatin can stimulate the degradation of HIF-1α protein, we mimicked hypoxia by exposure of the cells to DFO (100 μM). HIF-1α protein expression was induced by a 2-h treatment with DFO in the absence and presence of cisplatin (100 μM). Thereafter, ($t = 0$), the synthesis of HIF-1α protein was inhibited by the addition of cycloheximide (40 μg/ml) and the degradation of DFO-stabilized HIF-1α was monitored for a further period of 180 min. Fig. 5A and B shows that cisplatin was able to stimulate the degradation of DFO-stabilized HIF-1α in A2780 cells. The stimulatory effect of cisplatin was observed at 60 min after cycloheximide addition and degradation gradually increased after 120 and 180 min. The stability of HIF-1α protein was not influenced by cisplatin in DFO-treated OVCAR-3 and SKOV-3 cells, indicating that cisplatin does not promote the degradation of HIF-1α protein in these cells.

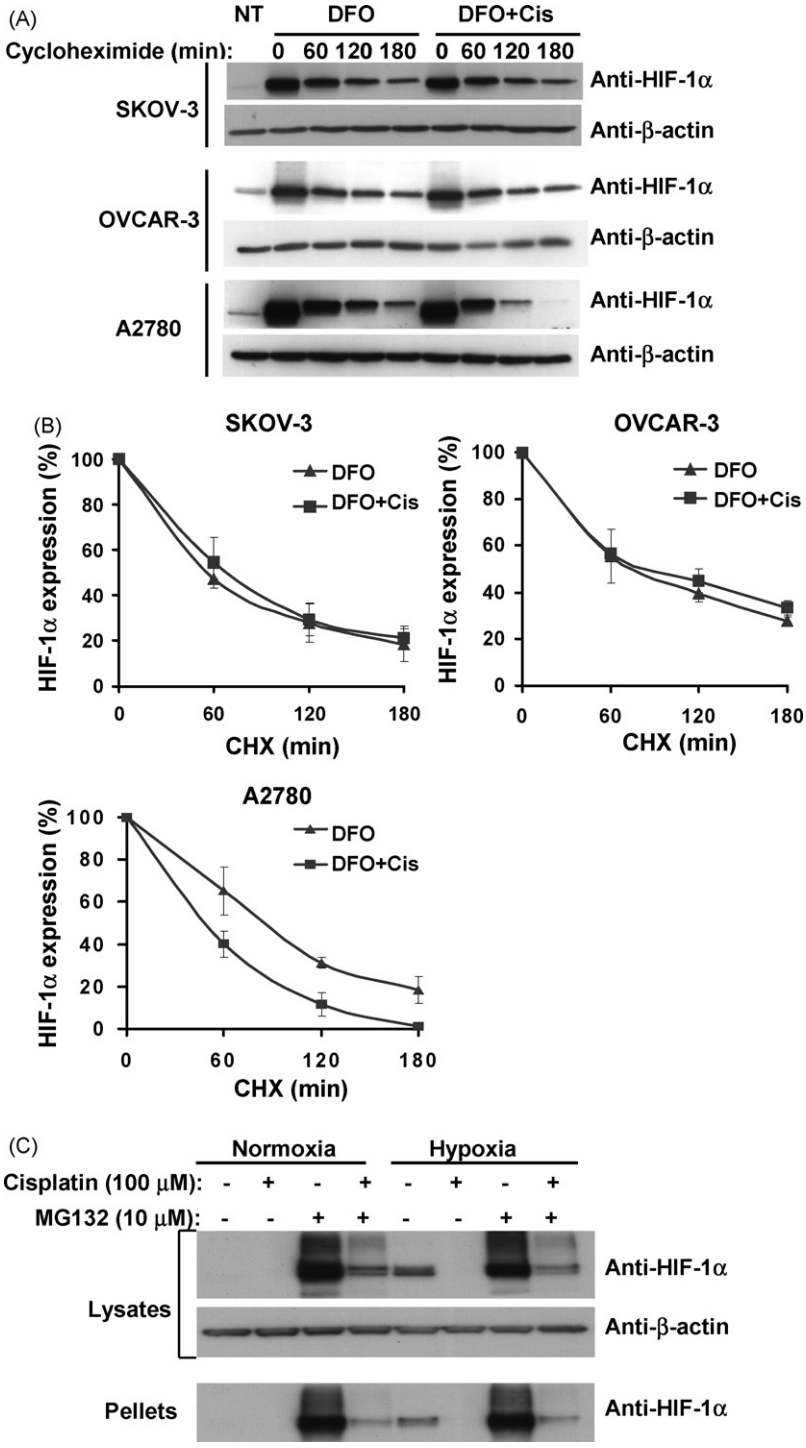

Fig. 5 – Cisplatin can stimulate the degradation and can inhibit the synthesis of HIF-1α protein. (A) Cells were not treated (NT) or treated for 2 h with DFO (100 μM) in the absence and presence of cisplatin (100 μM). Subsequently, cycloheximide (40 μg/ml, t = 0) was added and cells were lysed after the indicated time periods. (B) The graphs represent the quantification of the autoradiographic HIF-1α signal from the Western blots in (A) by densitometry. Values were normalized to the expression of β-actin and expressed as a percentage relative to t = 0, which was set at 100%. The values in the graphs indicate the mean ± range of two different experiments. (C) SKOV-3 cells were treated with MG132 (10 μM) alone or in combination with cisplatin under normoxia and hypoxia for 6 h. The HIF-1α and β-actin protein levels in lysates and HIF-1α protein levels in solubilized pellets of lysates were assessed by Western blot.

To examine whether cisplatin could also inhibit the synthesis of the HIF-1α protein, we performed experiments with the proteasome inhibitor MG132. Because rapid proteasomal destruction of HIF-1α occurs in normoxic cells, the degree of accumulation of HIF-1α after proteasomal inhibition indirectly reflects the synthesis rate of the protein. The experiments were performed in SKOV-3 cells, because cisplatin did not reduce HIF-1α mRNA levels in these cells (Supplementary Fig. 2A online). As demonstrated in cell lysates in Fig. 5C, a high amount of HIF-1α protein accumulated after treatment with MG132 (10 μM) under normoxia. Accumulation of HIF-1α protein under hypoxia was also more pronounced in the presence of MG132. Treatment with cisplatin in the presence of MG132 strongly reduced the accumulation of HIF-1α protein under normoxia and hypoxia. This suggests that cisplatin can indeed inhibit the synthesis of the HIF-1α protein in normoxic and hypoxic SKOV-3 cells.

HIF-1α protein has been reported to aggregate into detergent-insoluble complexes upon proteasome inhibition and treatment with additional agents can further decrease its solubility [23]. To examine the possibility that cisplatin decreased the solubility of HIF-1α protein, we measured the amount of HIF-1α in the pellets of the lysates after solubilization in the stringent Laemmli sample buffer (Fig. 5C). A high amount of HIF-1α protein was indeed present in the pellets of cells treated with MG132 alone. This amount, however, was also strongly reduced after co-treatment with cisplatin. Thus, co-treatment with cisplatin did not significantly decrease the solubility of the HIF-1α protein, but rather down-regulated its synthesis.

In summary, these data indicate that cisplatin can stimulate HIF-1α degradation exclusively in A2780 cells. In addition, our findings in SKOV-3 cells indicate that cisplatin can also inhibit the synthesis of HIF-1α protein through an oxygen-independent pathway.

3.5. *Cisplatin down-regulates the expression of p300 and CBP proteins under hypoxia*

Since the transcriptional coactivators p300 and CBP are important for the transactivating activity of HIF-1α under hypoxia, we examined the effect of cisplatin and doxorubicin on the levels of these proteins (Fig. 6). Cisplatin (50 and 100 μM) strongly reduced the expression of p300 and CBP proteins in hypoxic OVCAR-3 and SKOV-3 cells. This also occurred in hypoxic A2780 cells, although to a lesser extent. Doxorubicin (5 and 15 μM) decreased p300 and CBP protein levels as well in A2780 and SKOV-3 cells, whereas only p300 expression was slightly reduced by 15 μM of doxorubicin in OVCAR-3 cells. In SKOV-3 cells, the inhibitory effect of doxorubicin was clearly less potent than that of cisplatin. These findings suggest that cisplatin, and perhaps also doxorubicin, may also inhibit the transcriptional activity of HIF-1 under hypoxia by reducing the expression of important coactivators.

4. Discussion

This study was undertaken to examine whether the conventional anticancer agents, cisplatin, doxorubicin, docetaxel and paclitaxel, can influence two important mediators of angiogenesis, VEGF and its transcriptional regulator HIF-1, in normoxic and hypoxic A2780, OVCAR-3 and SKOV-3 human ovarian cancer cells. Our data indicate that the DNA-damaging drugs cisplatin and doxorubicin can inhibit hypoxia-induced VEGF expression through the inhibition of HIF-1. Our data

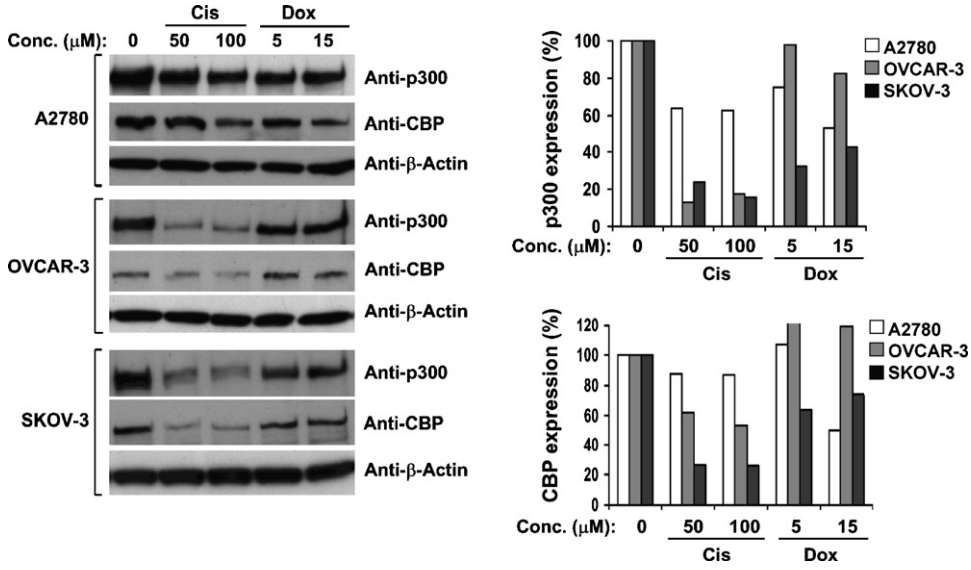

Fig. 6 – Cisplatin and doxorubicin reduce the expression of p300 and CBP proteins under hypoxia. A2780, OVCAR-3 and SKOV-3 cells were left untreated or exposed to the indicated concentrations of cisplatin (Cis) or doxorubicin (Dox) under normoxia and hypoxia for 6 h. Expression of p300, CBP and β-actin proteins was assessed by Western blot. The histograms represent the quantification of the autoradiographic p300 and CBP signals by densitometry. Values were normalized to the expression of β-actin and expressed as percentage relative to non-treated, hypoxic cells that were set at 100%. For optimal visualization of inhibitory effects of doxorubicin on CBP expression, the maximal value of the Y-axis was set at 120%. In OVCAR-3 cells, the values for CBP expression after exposure to 5 and 15 μM doxorubicin were 212% and 119%, respectively.

further indicate that repression of HIF-1 activity by cisplatin and doxorubicin under hypoxia occurred through different mechanisms. Cisplatin strongly down-regulated the expression of the HIF-1α protein and of its transcriptional coactivators p300 and CBP in all cell lines, whereas doxorubicin did not. Both drugs also down-regulated the basal expression of VEGF. In contrast to cisplatin and doxorubicin, the taxanes docetaxel and paclitaxel did neither influence HIF-1 activity nor VEGF expression in normoxic and hypoxic cells under the employed experimental conditions.

Our data indicate that inhibition of HIF-1α protein expression by cisplatin mainly occurs at the post-transcriptional level and that it involves different mechanisms. Experiments with the proteasome inhibitor MG132 strongly suggest that cisplatin can inhibit *de novo* HIF-1α protein synthesis through a pathway independent of oxygen. It has already been demonstrated that HIF-1α is regulated at the translational level by the phosphatidylinositol 3-kinase (PI3K)/AKT-1/mammalian target of rapamycin (mTOR) pathway [28]. As assessed by monitoring AKT-1 phosphorylation on Western blots, cisplatin did not inhibit PI3K activity in normoxic and hypoxic SKOV-3 (and OVCAR-3) cells (not shown). Since cisplatin can cause inactivation of translational regulators that are linked to mTOR signaling, this drug might exert its inhibitory effect on translation by the latter mechanism [29].

Inhibition of HIF-1α protein accumulation by cisplatin was clearly less pronounced in hypoxic A2780 cells than in hypoxic OVCAR-3 and SKOV-3 cells. We found that cisplatin reduced the half-life of DFO-stabilized HIF-1α protein in A2780 cells only. It is interesting to speculate that stimulation of HIF-1α degradation by cisplatin in A2780 cells may be related their wild-type p53 status [18]. OVCAR-3 express mutant p53 [18], while our SKOV-3 cells lack expression of p53. It has been demonstrated that wtp53 can stimulate the degradation of HIF-1α protein both under conditions of 0.5% hypoxia after its activation by the DNA-damaging agent mitomycin C [23,30,31]. In our system, cisplatin also potently induced the expression of wtp53 and of the p53-responsive gene p21 in normoxic and hypoxic A2780 cells (see Supplementary Fig. 3 online). These findings and the findings of Kalusova et al. [23], would suggest that wtp53 is a potential candidate to mediate cisplatin-induced degradation of HIF-1α under true hypoxia in A2780 cells.

The binding of the related co-activators p300 and CBP to the C-terminal activation domain of HIF-1α is crucial for the transcriptional activity of HIF-1 under hypoxia [32]. CBP also appears to coordinate the co-localization and complex formation of HIF-1α, HIF-1β, CBP and another transcriptional co-activator, SRC-1, in nuclear accumulation foci [33]. Therefore, repression of transcription via HRE elements could also be caused by a reduction in the levels of p300 and CBP. We demonstrated that cisplatin can indeed down-regulate the protein levels of p300 and CBP under hypoxia in all three ovarian cancer cell lines. As p300 and CBP are limiting factors, because of competition among many factors for binding to overlapping interaction domains, it is an interesting hypothesis that their down-regulation may contribute importantly to the cisplatin-mediated inhibition of HIF-1 under hypoxia.

Our data indicate that doxorubicin inhibits hypoxic activation of HIF-1 at levels that are downstream of HIF-1α protein induction in the HIF-1 activation cascade in the three ovarian cancer cell lines. Our findings in SKOV-3 cells provide unquestionable evidence that this can occur in the absence of p53. Of note, doxorubicin also induced the accumulation of wtp53 in A2780 cells, but obviously this did not result in the degradation of HIF-1α protein. Doxorubicin did not up-regulate p21 mRNA expression after 8 h of exposure (see Supplementary Fig. 3 online), which may be explained by a slower kinetics of p53 activation or p53-mediated gene activation.

Doxorubicin was able to reduce CBP and p300 protein levels under hypoxia, but this inhibitory effect was not observed in hypoxic OVCAR-3 cells and was much less pronounced than the inhibitory effect of cisplatin in hypoxic SKOV-3. Recently, doxorubicin has been shown to induce phosphorylation-dependent proteasomal degradation of p300 by activating p38 Mitogen-Activated Protein Kinase (MAPK) in rat cardiac cells [34]. It should be examined whether p38 also mediates down-regulation of p300/CBP by doxorubicin (and cisplatin) in our hypoxic ovarian cancer cells. Although it is possible that the weak reduction of the expression of limiting p300/CBP proteins by doxorubicin is of negative consequence for HIF-1 function, it is likely that additional mechanisms are involved in the inhibition of HIF-1. In addition to p300 and CBP, p44/p42 MAPK and PI3K have been suggested to increase the transactivating activity of HIF-1α [35,36]. Western blots of phosphorylated p44/p42 MAPK and AKT-1 indicated, however, that doxorubicin did not inhibit p44/42MAPK activity and PI3K activity in our cell lines under hypoxia (data not shown).

Cisplatin and doxorubicin did not only suppress VEGF and CA9 expression under hypoxia, but also under normoxia. By introducing DNA damage, both drugs might exert an inhibitory effect on transcription in general. It cannot be excluded that this non-specific effect may contribute to suppression of the basal and, to a minor extent, to hypoxia-induced expression of VEGF and CA9. Cisplatin and doxorubicin, however, did not influence the expression of endogenous β2-microglobulin mRNA or that derived from the pGL3 luciferase construct in transfected cells. Moreover, the protein levels of HIF-1β, β-actin, p44/p42MAPK (not shown) and AKT (not shown) remained unaltered upon treatment with cisplatin and doxorubicin. In principle, a direct role for HIF-1 in drug-mediated repression of VEGF and CA9 expression under normoxia could also not be excluded. The lack of HRE-dependent transcription suggests, however, that HIF-1 is not active under normoxia and indicate that other transcription factors are involved.

Whereas cisplatin and doxorubicin displayed a clear repression of HIF-1 and VEGF, neither inhibition of HIF-1 activity nor repression of VEGF expression was observed when cells were treated with the microtubule-inhibiting drugs docetaxel and paclitaxel. Recent evidence indicates that HIF-1 regulation is linked to the microtubule cytoskeleton. Microtubule-stabilizing agents (including docetaxel and paclitaxel) and microtubule-destabilizing agents have recently been shown to down-regulate HIF-1α protein accumulation and HIF-1 activity in hypoxic cells [37,38]. The underlying mechanisms are not well explored, but may involve the inhibition of *de novo* HIF-1α protein synthesis [38]. The discrepancy between these findings and our data may be

due to differences in the cell types or to differences in experimental design. In the previous studies of Mabjeesh et al. [37] and Escuin et al. [38], cells were incubated overnight with lower concentrations of docetaxel and paclitaxel before exposure to hypoxia for 4 or 6 h, whereas we introduced simultaneous exposure. We have shown earlier that A2780 and OVCAR-3 cells already accumulate in the G2/M phase within 8 h of exposure to concentrations of docetaxel below those used in this study [18]. Our selected exposure times are thus sufficient to affect the organization of the microtubule skeleton.

In summary, our results demonstrate that cisplatin and doxorubicin, but not taxanes, can negatively influence HIF-1 activity with a concomitant reduction of VEGF expression in human ovarian cancer cell lines and that this can occur in the absence of p53. Our experiments were carried out with relatively high concentrations of the four anticancer agents to obtain a clear manifestation of potential inhibitory effects on HIF-1 and VEGF. In all our experiments, we assessed the toxicity of the drugs within the short time frame of the exposure of 6–8 h and confirmed that treated cells had retained their viability. Although overestimated in this study, the inhibitory effects of cisplatin and doxorubicin on VEGF production in ovarian cancer cells may contribute to tumor response in patients. Therefore, our observations may be of relevance to decide whether a treatment regimen of HIF-1 inhibitors with conventional anticancer agents is warranted.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2007.04.003.

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