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Impact of alendronate and VEGF-antisense combined treatment on highly VEGF-expressing A431 cells

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ABSTRACT

Bisphosphonates, and more specially nitrogen-containing bisphosphonates, which are in current use for the treatment of bone diseases, demonstrate proapoptotic, antiproliferative, antiangiogenic and anti-invasive properties on tumor cells. The amino-bisphosphonate alendronate is considered as a potential anticancer drug. In the case of A431 cells, which express high levels of VEGF, it had a two-step effect. At 24 h, the antitumor properties of alendronate were counterbalanced by a survival process, which consisted of an enhancement of VEGF expression (mRNA and protein secretion) and TGFα secretion. It was only at 48 h that alendronate displayed the expected antiproliferative and antiangiogenic properties. The first step, in which the PI3K pathway was engaged, could be prevented by the use of a VEGF-antisense oligonucleotide. The combination of such an antisense with small concentrations of alendronate (~2 μM), which is of the order of clinically used concentrations, was shown to have an antiangiogenic effect as soon as 12 h.

1. Introduction

Bisphosphonates (BPs) are analogs of endogenous pyrophosphates in which the P–O–P bond has been replaced by a nonhydrolyzable P–C–P bond. BPs are widely used to inhibit osteoclastic activity in benign and malignant bone diseases, such as Paget's disease, osteoporosis or osteolytic tumor bone metastases [1–3]. The nitrogen-containing BPs (N-BPs) are more effective and are able to interfere with the metabolism of mevalonate, leading to inhibition of the prenylation of small GTP-binding proteins (Ras, Rho, etc.) [4,5]. Those GTPases are important signaling proteins which regulate a large variety of cellular processes. It is now clearly established that BPs may exert directly proapoptotic and antiproliferative effects on tumor cells [3,6,7]. Antiangiogenic properties have also been attributed to N-BPs because of their potency to inhibit the proliferation and the migration of endothelial cells [8,9]. For instance, the amino-bisphosphonate alendronate (ALN) has been shown to inhibit at moderate concentrations the endothelial cells proliferation induced by VEGF (vascular endothelial growth factor), in vitro as well as in vivo [10]. VEGF is a major angiogenic factor secreted by tumors [11]; it is produced in highly variable amounts by different tumor types. High levels of VEGF are of bad prognosis and have been associated with an increased propensity for metastasis [12]. In the present study, we have investigated the direct action of ALN on A431 cells (a human squamous cell carcinoma cell line) which produce high amount of VEGF [13], contrary to MCF7 cells (a human mammary adenocarcinoma cell line) which were taken as a control cell line. The A431 cells also overexpress epidermal growth factor receptors (EGFRs) and produce its ligand TGFα (transforming growth factor-alpha) [14]. The impact of the important secretion of VEGF by A431 cells on the anticancer properties of ALN has been studied at the levels of cell proliferation and apoptosis. We also determined whether ALN modified the A431 VEGF expression. These questions were investigated by studying the ALN effect on cells, combined or not with an anti-VEGF-antisense oligonucleotide (AS) in order to reduce the high endogenous VEGF level in A431 cells.

2. Materials and methods

2.1. Reagents and antisense oligonucleotides

ALN was synthesized in the lab [15]. LY294002 was purchased from Sigma (Saint-Quentin Fallavier, France). Phosphorothioate oligodeoxynucleotides were synthesized and PAGE purified by Eurogentec (Seraing, Belgium). The antisense AS (5′-TGGCTTGAA-GATGTACTCGAT) was designed to target the human VEGF mRNA at

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the level of the region 257–278 nt relative to start codon [16]. SC was a control scrambled oligodeoxynucleotide.

2.2. Cell culture and treatments

The A431 and MCF7 cell lines were received from ATCC (LGC Standards, Molsheim, France). Cells were grown in DMEM medium (A431 cells) or RPMI medium (MCF7 cells) supplemented with 10% decomplemented foetal bovine serum (FBS), 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM glutamine, in a 5% CO_2 atmosphere at 37 °C. They were trypsinized and passed twice a week.

Cells were plated in six-well plates and, after reaching 30–50% confluence, incubated in culture medium (10% serum) containing different concentrations of ALN for 24 or 48 h or transfected by AS or SC with Oligofectamine™ (Invitrogen, Cergy, France). Transfection was carried out as directed by the manufacturer, i.e. in OPTI-MEM1 (Invitrogen) without serum or antibiotics. After 4 h incubation at 37 °C, FBS (10% final concentration), 50 U/ml penicillin and 50 mg/ml streptomycin were added. The final concentration of AS or SC was 200 nM. For some experiments, ALN was also added at 4 h. For LY294002 treatment, cells were incubated with 50 μM LY294002 for 40 min before addition of ALN [17].

2.3. Cell proliferation assay

Cells (10^4/well) were plated in 96-well plates and allowed to attach for 24 h, and then cultured under increasing concentrations of ALN (1–150 μM) in full culture medium for 12, 24 or 48 h along, or not, with transfection by AS or SC. Viability was assessed using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) at a final concentration of 0.5 mg/ml for 2 h at 37 °C. Optical density of the dissolved formazan product (in DMSO) was read at 570 nm.

2.4. Annexin V assay

After 24 h treatment with increasing doses of ALN, 10^6 cells were washed with PBS, trypsinized and resuspended in 100 μl of binding buffer. A fluorescein isothiocyanate-conjugated annexin V and propidium iodide were added as directed by the manufacturer (Santa Cruz Biotechnology (Tebu, Le Perray en Yvelines, France)). Cells were analyzed with a flow cytometer (FACSCalibur, BD Biosciences, Le Pont de Claix, France).

2.5. VEGF and TGFα ELISA assays

Secretion of VEGF or TGFα into the cell culture supernatant after the different treatments was determined using Ray Bio Human VEGF ELISA kits (Tebu, Le Perray en Yvelines, France), according to the manufacturer's instructions. In the case of VEGF, supernatant samples were diluted 50-fold (A431 cells) or 30-fold (MCF7 cells).

2.6. RT-PCR

24 and 48 h after treatment, total RNA was extracted (Rneasy™ kit, Qiagen, Courtaboeuf, France) and cDNA was synthesized using Omniscript™ reverse transcription kit (Qiagen). Reverse transcription was performed using 0.5 μM gene-specific reverse primers of $VEGF_{165}$ and β-actin which was chosen as an internal control. Hot Star Taq™ PCR kit (Qiagen) was used for DNA amplification, which was kept in its exponential phase. Primers used for $VEGF_{165}$ amplification (0.5 μM) were: sense, 5′-d(GGAAGTGGTGAAGTTCATGGATG) and reverse, 5′-d(AGCAAGGCCCACAGGGATTT). The amplification consisted of 21 cycles for A431 cells, 23 for MCF7 cells (denaturation at 94 °C for 30 s, annealing at 59 °C for 30 s and extension at 72 °C for 1 min). Because of the difference of 4 °C between the melting temperatures of both primers, a four-cycle touch-down PCR was first carried out with an annealing temperature decreasing from 63 to 60 °C. Primers for β-actin amplification (0.3 μM) were: sense, 5′-d(ACCAACTGGGACGACATGGA) and reverse, 5′-d(CTCCTTAATGTCACGCACGA). Amplification consisted of 20 cycles (95 °C, 30 s; 56 °C, 30 s; 72 °C, 1 min). The PCR products were separated on 1.8% agarose gel stained with ethidium bromide (6 mg/ml). Gene expression level was quantified by densitometric analysis using Image J software.

2.7. Statistical analysis of the data

The data were subjected to one-way analysis of variance (ANOVA), followed by Dunnett's test. $P < 0.05$ was considered a significant difference.

3. Results

3.1. Cell growth inhibition and apoptosis

The ALN-induced growth inhibition of A431 or MCF7 cells has been evaluated by MTT assays (Fig. 1). Whereas a dose-dependent growth decrease was observed as soon as 24 h in the case of MCF7 cells (IC_{50} ~60 μM) (Fig. 1a), it is only at 48 h that ALN treatment caused a similar dose-dependent decrease in A431 cell number (Fig. 1b), with a ~75% decrease at 150 μM and a IC_{50} around 50 μM. (At 72 h, the IC_{50} was only ~10 μM, not shown.) During the first 24 h, A431 cell number was only reduced of ~20% and this effect was observable as soon as from about 10 μM ALN without

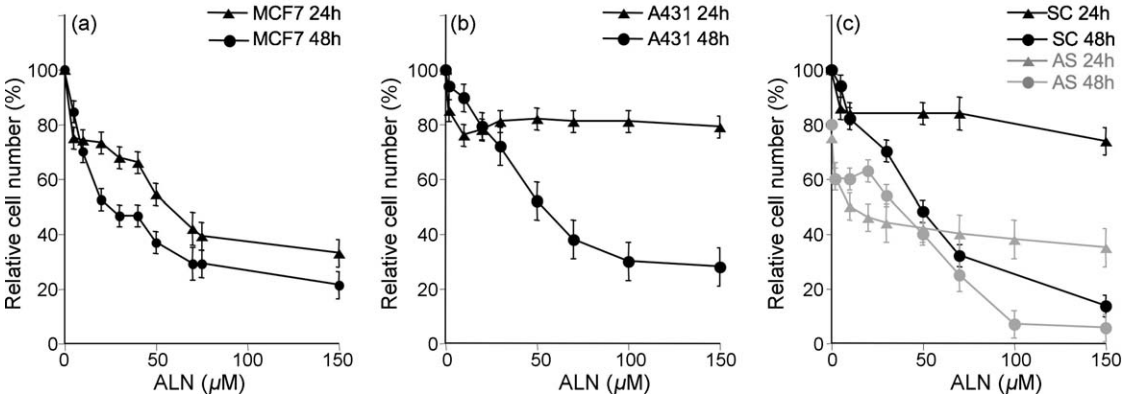

Fig. 1. MTT cell proliferation tests after 24 or 48 h treatment with increasing amounts of ALN. Results are the mean of three separate experiments. (a) MCF7 cells, (b) A431 cells and (c) A431 cells with ALN combined to 0.2 μM VEGF-antisense (AS) or control scrambled (SC) oligonucleotides.

further decrease for higher ALN concentrations. Because of the high growth dependence of A431 cells on VEGF [18], antiproliferative properties of ALN were also checked in combination with an anti-VEGF-antisense oligonucleotide (AS), compared to a scrambled control (SC) (Fig. 1c). SC had no effect by itself: results were similar to those obtained with ALN alone (Fig. 1b). AS had by itself an antineoplastic effect on A431 cells (reduction of 20% in cell number) (Fig. 1c). AS markedly favoured ALN-induced cell growth decrease, which was now efficient as soon as 24 h. A reduction in cell number of ~40% was obtained with 150 μM ALN. Even if the growth curve tended to a plateau, ALN had now an antiproliferative effect at least up to ~30–40 μM ALN.

Dot-plots of FITC-annexin V (*x*-axis)/propidium iodide (*y*-axis) fluorescence and corresponding histograms for FITC-annexin V fluorescence are presented in Fig. 2. ALN induced apoptosis as soon as 24 h in MCF7 cells and, more surprisingly, in A431 cells. In the case of MCF7 cells, there was a dose-dependent moderate increase in FITC-annexin V staining up to 50 μM ALN, evidenced by the shift of annexin V fluorescence on the histogram. By contrast, in the case of A431 cells, apoptosis increased with ALN treatment up to 10 μM for which it became very significant (24% with only ~70% viable cells), then decreased back with 50 μM ALN. It is worth noticing that, after a 50 μM ALN treatment, the percentage of viable cells was comparable to that of untreated cells (~90%) whereas the percentage of dead cells had doubled (~10% instead of 5%). 30 nM EGF has been taken as a positive control of apoptosis in the case of A431 cells [19].

3.2. VEGF and TGFα expression

The amount of secreted VEGF into the culture medium, determined by ELISA test, was largely superior in A431 cells compared to MCF7 cells (Fig. 3a). When cells were treated by increasing amounts of ALN, a consistent increase (~30%) in secreted VEGF was evidenced in A431 cells at 24 h, followed by a decrease at 48 h (~40%). The overall increase in VEGF secretion along the time (48 h compared to 24 h) is a usual observation on cells in culture [20]. Correlation between VEGF secretion and VEGF mRNA level in A431 cells has been established by RT-PCR (Fig. 3b). Results concerning the $VEGF_{165}$ isoform are presented since $VEGF_{165}$ is considered as the most mitogenic isoform but similar results have been obtained for $VEGF_{121}$ (not shown). After 24 h treatment with ALN (0–15 μM), the mRNA expression of VEGF in A431 cells increased of ~60%. This was no longer observed at 48 h. Fig. 3c presents the amount of TGFα secreted by A431 cells after ALN treatment. In a very similar way to what was observed for VEGF, increasing doses of ALN induced enhancement in TGFα secretion at 24 h, followed by a decrease at 48 h.

VEGF secretion was lowered after treatment with AS (Fig. 4a and b), compared to SC. When combined with AS, ALN treatment of

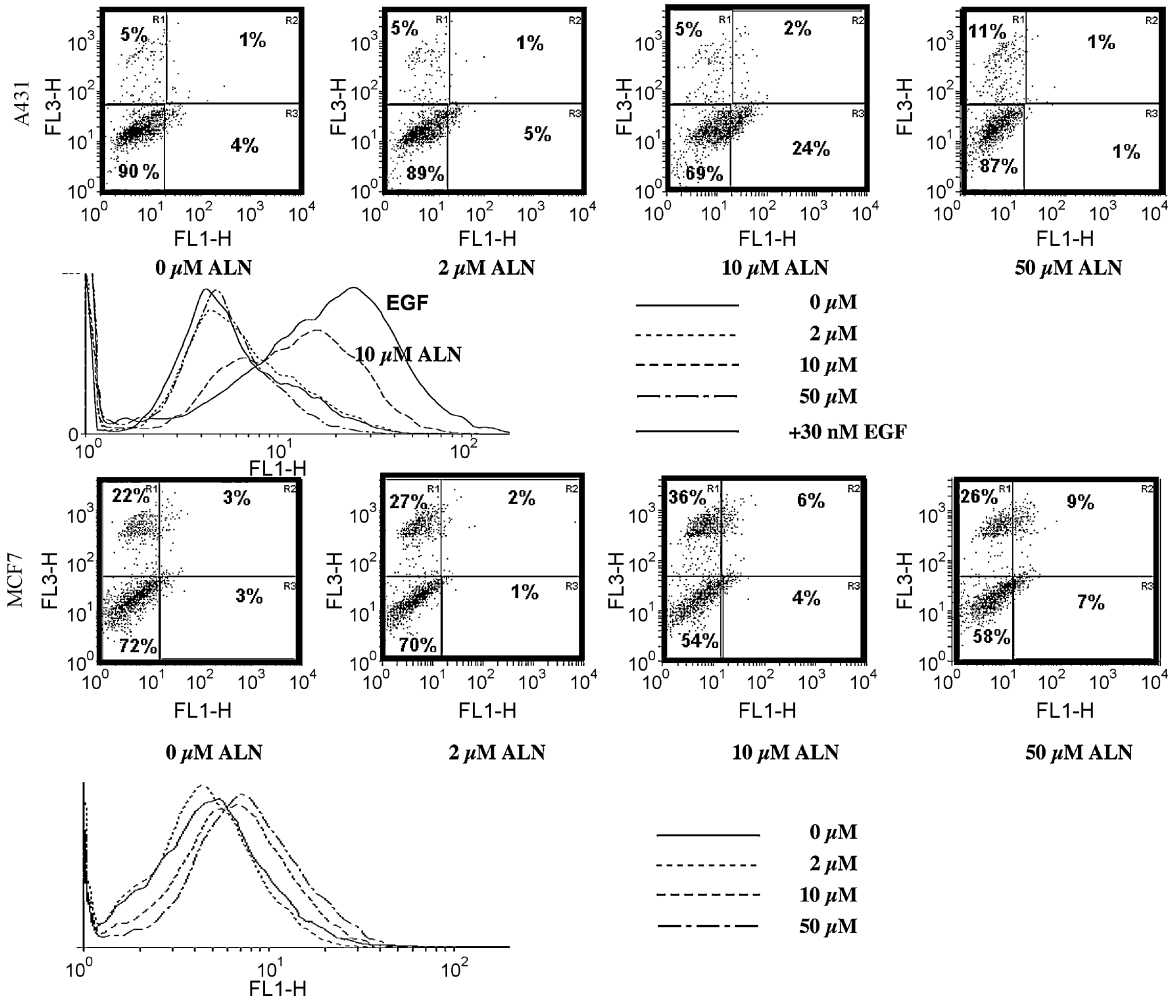

Fig. 2. Flow cytometry analysis of apoptosis in A431 or MCF7 cells after 24 h treatment with 0, 2, 10 and 50 μM ALN. Above: dot-plots of FITC-annexin V/propidium iodide. Percentage of viable cells (lower left part), early apoptotic cells (lower right), late apoptotic cells (upper right) and dead cells (upper left) are indicated in each graph. 30 nM EGF was a positive control of apoptosis for A431 cells. Below: corresponding histograms for FITC-annexin V fluorescence. The experiment was done in triplicate and is representative of three independent experiments.

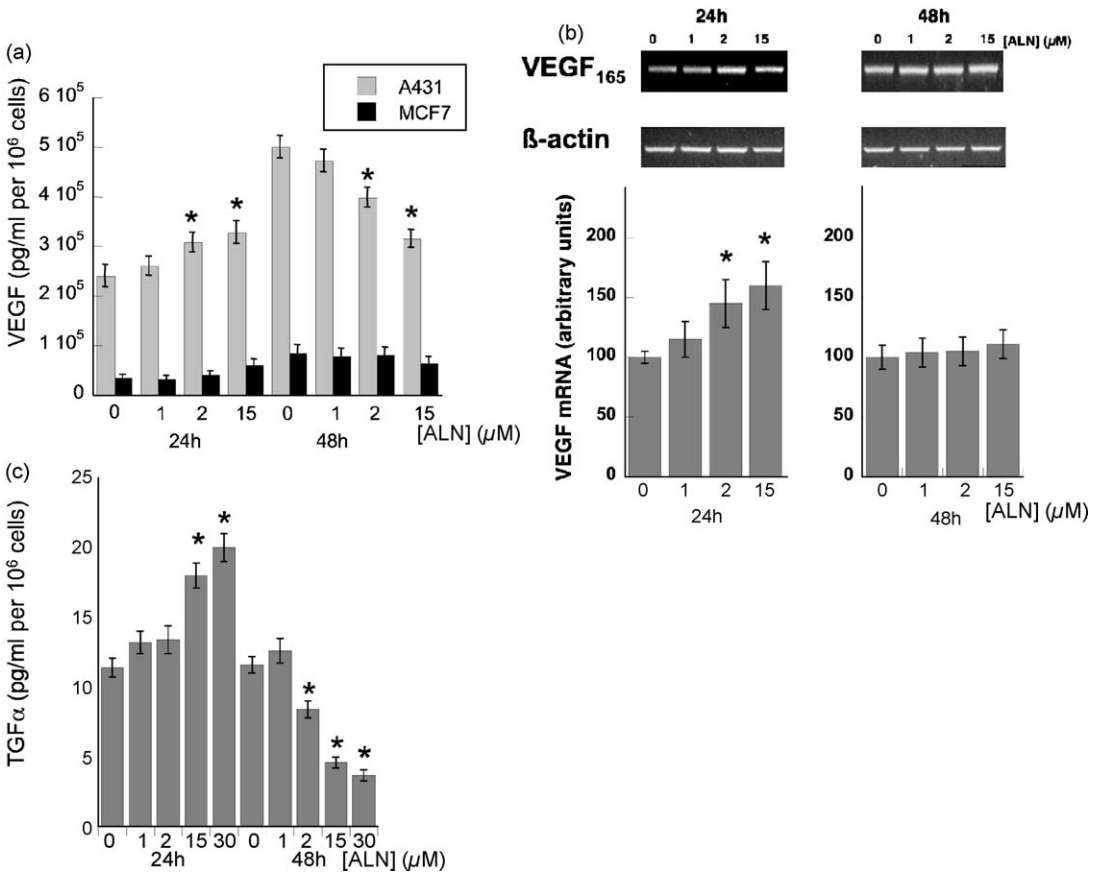

Fig. 3. (a) ELISA detection of secreted VEGF at 24 and 48 h after treatment with increasing concentrations of ALN (A431 and MCF7 cells). The histograms (mean ± S.E.M.) are representative of the average of three different cell treatments, each of them followed by ELISA test. Values indicated by an asterisk ($p < 0.05$, ANOVA) are significantly different from control value, i.e. 0 μM ALN at 24 or 48 h. (b) Modulation of the expression of VEGF mRNA by ALN in A431 cells (24 and 48 h). β-Actin was used as an internal control. The histogram is representative of the average of four different cell treatments, each of them followed by PCR in duplicate. Asterisk: $p < 0.05$ compared to 0 μM ALN. (c) The same as (a) for TGFα.

A431 cells did not induce any more increase in VEGF secretion at 24 h, like it was still the case after combination with SC (Fig. 4a) or in the case of treatment by ALN alone (Fig. 3a). The VEGF level was kept constant for small ALN concentrations, up to 10 μM ALN for which it increased. After only 12 h treatment, the combination of AS with ALN even induced decrease in VEGF secretion (Fig. 4b). It was only with 20 μM ALN that VEGF secretion increased again. More surprisingly, AS also led to a decrease in TGFα secretion at 24 h, compared to SC (Fig. 4c) and, up to 5 μM, opposed ALN-induced TGFα secretion, which had been observed at 24 h (Fig. 3c). At 12 h, the level of TGFα was under the sensitivity of the assay. After pretreatment with 50 μM LY294002, an inhibitor of the phosphatidyl inositol 3-kinase (PI3K) pathway, a global decrease in VEGF secretion was observed (Fig. 4d) and ALN did not induce any more increase in VEGF secretion by A431 cells at 24 h.

4. Discussion

4.1. Cell growth inhibition and apoptosis

The antiproliferative and proapoptotic properties of BPs have generally been described after 48 h treatment or more [21]. The slight modifications observed for earlier times were usually considered as resulting from a metabolic shift in the cell line stressed by the treatment or from a cytostatic drug effect. In the present work, we specifically focused on the effects observed after only 24 h ALN treatment. Whereas a dose-dependent cell growth decrease was observed in the case of MCF7 cells, increasing doses of ALN only led to a plateau with 20% cell growth inhibition (Fig. 1) in the case of A431 cells. It is only at 48 h that ALN treatment caused a dose-dependent decrease in cell number. This delay in cell growth inhibition is not attributed to a cytostatic effect since there is a clear correlation between (i) the efficient A431 cell apoptosis (24%) measured at 24 h with 10 μM ALN (Fig. 2) and (ii) the dose-dependent decrease in cell number, which was observed at 24 h (Fig. 1b) up to 10 μM ALN. With 50 μM ALN, whereas the percentage of apoptotic cells increased in the case of MCF7 cells (Fig. 2), apoptosis was no longer observable in A431 cells. The percentage of dead cells has doubled but that of viable cells has returned to its initial value, suggesting new proliferation. Therefore we assume that, in the first 24 h of treatment, there is superimposition of two different processes in the case of A431 cells: an ALN-induced reduction in cell viability evidenced by apoptosis and some mechanisms stimulating cell proliferation and leading to new daughter cells. This phenomenon was not observed for MCF7 cells. A decrease in VEGF expression by using a VEGF-antisense (AS) allowed an improvement of the antiproliferative effect of ALN at 24 h to be obtained: it was now observed for ALN concentrations up to ~30–40 μM (Fig. 1c). High VEGF expression in A431 cells may be therefore involved in the de novo cell proliferation observed after 24 h ALN treatment.

4.2. VEGF and TGFα expression

The expected antiproliferative but also antiangiogenic properties of ALN were observed in A431 cells only after 48 h. Direct expression of VEGF by tumor cells has rarely been considered. In one study, ALN did not alter VEGF expression (protein or mRNA) in

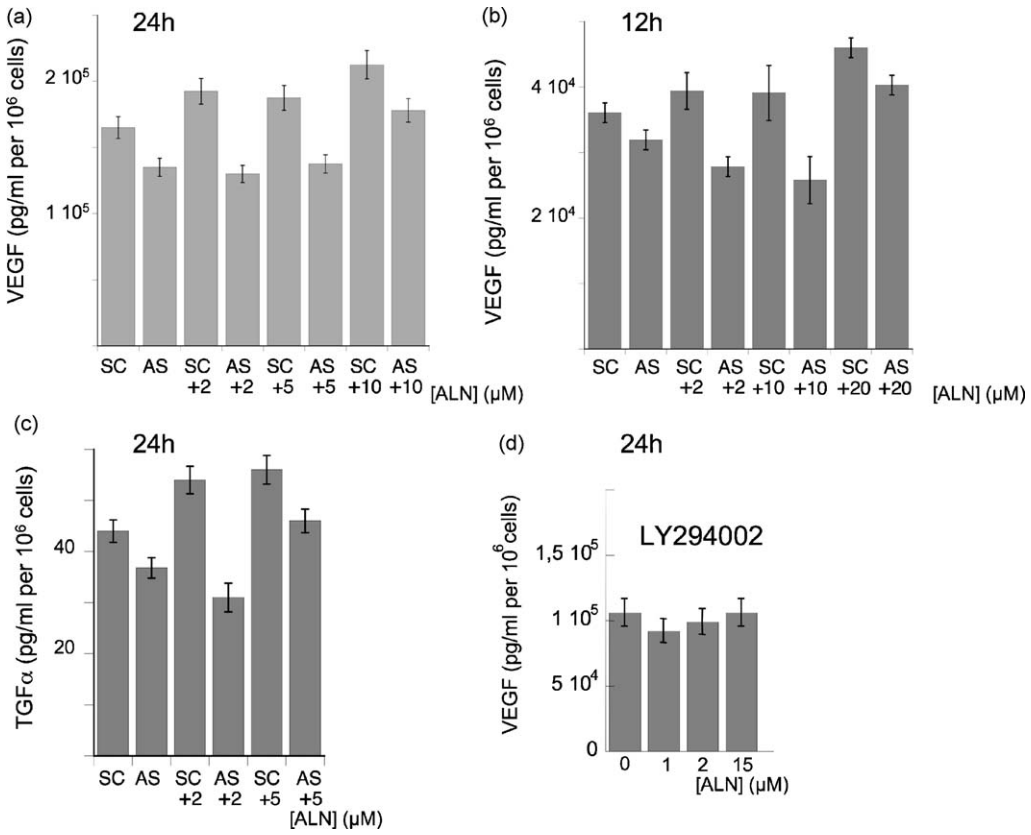

Fig. 4. (a) ELISA detection of secreted VEGF after 24 h treatment with AS or SC, combined with increasing concentrations of ALN (A431 cells). The histogram (mean ± S.E.M.) is representative of the average of three different cell treatments, each of them followed by ELISA test. (b) The same at 12 h. (c) The same for TGFα at 24 h. (d) ELISA detection of VEGF secretion after pretreatment with 50 μM LY294002 and 24 h treatment with increasing concentrations of ALN (A431 cells).

human ovarian Caov-3 cells [10], leading the authors to conclude that antiangiogenic effect of ALN might not be derived from inhibition of the production of VEGF by the tumor cells. But in this study we have shown that ALN treatment led to a two-step effect in the case of A431 cells: enhancement of VEGF expression at 24 h (mRNA and protein secretion) followed by a decrease of VEGF expression at 48 h (protein secretion) (Fig. 3a and b). This two-step effect of ALN on VEGF has also been observed in the case of TGFα: a dose-dependent increase in secretion at 24 h followed by a decrease at 48 h (Fig. 3c). TGFα is a ligand of EGFR and has been shown to activate this receptor through an autocrine pathway in A431 cells [14]. Link between EGFR kinase activation and VEGF up-regulation (VEGF secretion as well as mRNA expression) has been established, in particular in the case of A431 cells [22]. TGFα is therefore a potent inducer of VEGF expression in these cells and leads to up-regulation of VEGF mRNA level in a concentration-dependent fashion [23]. The increase in TGFα secretion induced by ALN during the first 24 h could lead to a survival mechanism of A431 cells, via the EGFR pathway. The PI3K pathway could be involved in this process. Its inhibition by LY294002 suppressed the increase in VEGF secretion induced by ALN treatment at 24 h (Fig. 4d). As a matter of fact, EGFR-mediated activation of the PI3K pathway has been shown to be directly involved in the survival of tumor cells [24]. The two-step effect of ALN on proliferation and angiogenesis in A431 cells is therefore explained in the following way: (i) the first step is characterized by an increase in TGFα secretion. Above ∼10 μM ALN, the amount of secreted TGFα becomes sufficient to counterbalance the antiproliferative effect of ALN. This transitory increase in TGFα induces up-regulation of VEGF secretion and consequently masks at 24 h the expected antiangiogenic effect of ALN. (ii) The antiproliferative and antiangiogenic properties of ALN occur only at 48 h.

AS lowered VEGF secretion. It allowed an antiangiogenic effect of ALN to be observed at 12 h up to 10 μM ALN and prevented the ALN dose-dependent increase in VEGF secretion at 24 h, at least up to 5 μM ALN. In an unexpected way, AS had a comparable effect on TGFα secretion, which decreased at least for the smallest concentration. If regulation of VEGF by TGFα is well established [23], it is the first time, to our knowledge, that influence of VEGF on TGFα expression has been described: reduction in TGFα secretion after anti-VEGF AS treatment. The potential role of VEGF on the EGFR pathway should be explored. Since A431 cells have both receptors to VEGF and TGFα and since (i) TGFα is directly involved in cell proliferation and (ii) A431 cells express a high VEGF growth dependence [18] through an autocrine pathway [25], it is possible that both effects of AS treatment (direct on VEGF and indirect on TGFα) contributed to the resulting improvement of the anti-proliferative effect of ALN, now observable at least up to ∼30–40 μM ALN, instead of 10 μM.

In conclusion, our results agree with the general idea that the combination of N-BPs with chemotherapeutic or other molecularly targeted anticancer agent should lead to an enhanced antitumor activity [26]. In the present case, the combination of ALN with AS would lead to the suppression of the proangiogenic step preceding the expected antiangiogenic action of ALN in A431 cells. Of particular interest is the fact that the combination of AS with small concentrations of ALN (∼2 μM) induced either the reversion (at 12 h) or the inhibition (at 24 h) of the proangiogenic step. Indeed, 2 μM ALN is of the order of a clinically relevant ALN concentration [27]. It has been estimated that, after rapid clearance of BPs by uptake in the skeleton or by the kidney where they are excreted, BP concentration in the circulation peaks may vary between 0.01 and 1 μM, depending on the administration route [27]. When patients were treated by zoledronate, the more efficient of the N-BPs, the

peak serum concentration has been estimated in the range of 1–3 μM and was maintained for only a few hours [28].

In this study, the early action (proliferative and proangiogenic) of ALN has been described in cells which express high levels of VEGF. Therefore, the presented results cannot be generalized. However, hypoxia is a strong inducer of VEGF gene expression, and that more particularly in cells with low basal abundance of VEGF mRNA [29]. Since low levels of oxygenation have been demonstrated in malignant solid tumors, it is therefore conceivable that, in vivo, the two-step effect of ALN could frequently occur.

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