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

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Abstract

1
2 Bisphosphonates, and more specially nitrogen - containing bisphosphonates, which are in
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4 current use for the treatment of bone diseases, demonstrate proapoptotic, antiproliferative,
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6 antiangiogenic and anti-invasive properties on tumor cells. The amino - bisphosphonate
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8 alendronate is considered as a potential anticancer drug. In the case of A431 cells, which
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10 express high levels of VEGF, it had a two-step effect. At 24h, the antitumor properties of
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12 alendronate were counterbalanced by a survival process, which consisted of an enhancement
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14 of VEGF expression (mRNA and protein secretion) and TGF α secretion. It was only at 48h
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16 that alendronate displayed the expected antiproliferative and antiangiogenic properties. The
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18 first step, in which the PI3K pathway was engaged, could be prevented by the use of a VEGF-
19
20 antisense oligonucleotide. The combination of such an antisense with small concentrations of
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22 alendronate ($\sim 2 \mu\text{M}$), which is of the order of clinically used concentrations, was shown to
23
24 have an antiangiogenic effect as soon as 12h.
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Key words:

34 Alendronate, VEGF expression, TGF α , PI3K pathway, VEGF-antisense, proliferation
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1. Introduction

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5 Bisphosphonates (BPs) are analogs of endogenous pyrophosphates in which the P-O-P bond
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7 has been replaced by a nonhydrolyzable P-C-P bond. BPs are widely used to inhibit
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9 osteoclastic activity in benign and malignant bone diseases, such as Paget's disease,
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11 osteoporosis or osteolytic tumor bone metastases [1-3]. The nitrogen containing-BPs (N-BPs)
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13 are more effective and are able to interfere with the metabolism of mevalonate, leading to
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15 inhibition of the prenylation of small GTP-binding proteins (Ras, Rho...) [4, 5]. Those
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17 GTPases are important signaling proteins which regulate a large variety of cellular processes.
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19 It is now clearly established that BPs may exert directly proapoptotic and antiproliferative
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21 effects on tumor cells [3, 6, 7]. Antiangiogenic properties have also been attributed to N-BPs
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23 because of their potency to inhibit the proliferation and the migration of endothelial cells [8,
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25 9]. For instance, the amino - bisphosphonate alendronate (ALN) has been shown to inhibit at
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27 moderate concentrations the endothelial cells proliferation induced by VEGF (Vascular
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29 Endothelial Growth Factor), in vitro as well as in vivo [10]. VEGF is a major angiogenic
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31 factor secreted by tumors [11]; it is produced in highly variable amounts by different tumor
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33 types. High levels of VEGF are of bad prognosis and have been associated with an increased
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35 propensity for metastasis [12]. In the present study, we have investigated the direct action of
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37 ALN on A431 cells (a human squamous cell carcinoma cell line) which produce high amount
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39 of VEGF [13], contrary to MCF7 cells (a human mammary adenocarcinoma cell line) which
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41 were taken as a control cell line. The A431 cells also overexpress epidermal growth factor
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43 receptors (EGFR) and produce its ligand TGF α (Transforming Growth Factor-alpha) [14].
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46 The impact of the important secretion of VEGF by A431 cells on the anticancer properties of
47
48 ALN has been studied at the levels of cell proliferation and apoptosis. We also determined
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50 whether ALN modified the A431 VEGF expression. These questions were investigated by
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1 studying the ALN effect on cells, combined or not with an anti - VEGF antisense
2 oligonucleotide (AS) in order to reduce the high endogenous VEGF level in A431 cells.
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10 **2. Materials and methods**

11 *2.1. Reagents and antisense oligonucleotides*

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18 ALN was synthesized in the lab [15]. LY294002 was purchased from Sigma (Saint-Quentin
19 Fallavier, France). Phosphorothioate oligodeoxynucleotides were synthesized and PAGE
20 purified by Eurogentec (Seraing, Belgium). The antisense AS (5'-
21 TGGCTTGAAGATGTACTCGAT) was designed to target the human VEGF mRNA at the
22 level of the region 257-278 nt relative to start codon [16]. SC was a control scrambled
23 oligodeoxynucleotide.
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32 *2.2. Cell culture and treatments*

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41 The A431 and MCF7 cell lines were received from ATCC (LGC Standards, Molsheim,
42 France). Cells were grown in DMEM medium (A431 cells) or RPMI medium (MCF7 cells)
43 supplemented with 10% decompemented foetal bovine serum (FBS), 50 U/mL penicillin, 50
44 µg/mL streptomycin, 2 mM glutamine, in a 5% CO₂ atmosphere at 37°C. They were
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51 trypsinized and passed twice a week.

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53 Cells were plated in 6-well plates and, after reaching 30-50% confluence, incubated in
54 culture medium (10% serum) containing different concentrations of ALN for 24 or 48h or
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58 transfected by AS or SC with OligofectamineTM (Invitrogen, Cergy, France). Transfection
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1 was carried out as directed by the manufacturer, i.e. in OPTI-MEM1 (Invitrogen) without
2 serum or antibiotics. After 4h incubation at 37°C, FBS (10% final concentration), 50 U/ml
3 penicillin and 50 mg/ml streptomycin were added. The final concentration of AS or SC was
4 200 nM. For some experiments, ALN was also added at 4h. For LY294002 treatment, cells
5 were incubated with 50 µM LY294002 for 40 min before addition of ALN [17].
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14 *2.3. Cell proliferation assay*

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19 Cells (10^4 /well) were plated in 96-well plates and allowed to attach for 24h, and then cultured
20 under increasing concentrations of ALN (1-150 µM) in full culture medium for 12, 24 or 48h
21 along, or not, with transfection by AS or SC. Viability was assessed using MTT (3-(4,5-
22 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) at a final concentration of 0.5
23 mg/ml for 2h at 37°C. Optical density of the dissolved formazan product (in DMSO) was read
24 at 570 nm.
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36 *2.4. Annexin V assay*

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41 After 24h treatment with increasing doses of ALN, 10^6 cells were washed with PBS,
42 trypsinized and resuspended in 100 µl of binding buffer. A fluorescein isothiocyanate-
43 conjugated annexin V and propidium iodide were added as directed by the manufacturer
44 (Santa Cruz Biotechnology, (Tebu, Le Perray en Yvelines, France)). Cells were analyzed with
45 a flow cytometer (FACSCalibur, BD Biosciences, Le Pont de Claix, France).
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56 *2.5. VEGF and TGF α Elisa assays*

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

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5 24 and 48h after treatment, total RNA was extracted (Rneasy™ kit, Qiagen, Courtaboeuf,
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7 France) and cDNA was synthesized using Omniscript™ reverse transcription kit (Qiagen).
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9 Reverse transcription was performed using 0.5 μM gene-specific reverse primers of VEGF₁₆₅
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11 and β-actin which was chosen as an internal control. Hot Star Taq™ PCR kit (Qiagen) was
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13 used for DNA amplification, which was kept in its exponential phase. Primers used for
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15 VEGF₁₆₅ amplification (0.5 μM) were: sense, 5'-d(GGAAGTGGTGAAGTTCATGGATG)
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17 and reverse, 5'-d(AGCAAGGCCACAGGGATTT). The amplification consisted of 21 cycles
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19 for A431 cells, 23 for MCF7 cells (denaturation at 94°C for 30 s, annealing at 59°C for 30 s
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21 and extension at 72°C for 1 min). Because of the difference of 4°C between the melting
22
23 temperatures of both primers, a four-cycle touch-down PCR was first carried out with an
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25 annealing temperature decreasing from 63°C to 60°C. Primers for β-actin amplification (0.3
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27 μM) were: sense, 5'-d(ACCAACTGGGACGACATGGA) and reverse, 5'-
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29 d(CTCCTTAATGTCACGCACGA). Amplification consisted of 20 cycles (95°C, 30 s; 56°C,
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31 30 s; 72°C, 1 min). The PCR products were separated on 1.8% agarose gel stained with
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33 ethidium bromide (6 mg/ml). Gene expression level was quantified by densitometric analysis
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35 using Image J software.
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2.7. Statistical analysis of the data

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

3.1. Cell growth inhibition and apoptosis

The ALN-induced growth inhibition of A431 or MCF7 has been evaluated by MTT assays (Figure 1). Whereas a dose-dependent growth decrease was observed as soon as 24h in the case of MCF7 cells ($IC_{50} \sim 60 \mu\text{M}$) (Figure 1a), it is only at 48h that ALN treatment caused a similar dose-dependent decrease in A431 cell number (Figure 1b), with a $\sim 75\%$ decrease at $150 \mu\text{M}$ and a IC_{50} around $50 \mu\text{M}$. (At 72h, the IC_{50} was only $\sim 10 \mu\text{M}$, not shown). During the first 24h, A431 cell number was only reduced of $\sim 20\%$ and this effect was observable as soon as from about $10 \mu\text{M}$ ALN without 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) (Figure 1c). SC had no effect by itself: results were similar to those obtained with ALN alone (Figure 1b). AS had by itself an antineoplastic effect on A431 cells (reduction of 20 % in cell number) (Figure 1c). AS markedly favoured ALN-induced cell growth decrease, which was now efficient as soon as 24h. A reduction in cell number of $\sim 40\%$ was obtained with $150 \mu\text{M}$ ALN. Even if the growth curve tended to a plateau, ALN had now an antiproliferative effect at least up to $\sim 30\text{-}40 \mu\text{M}$ ALN.

Dot-plots of FITC-annexin V (x-axis)/propidium iodide (y-axis) fluorescence and corresponding histograms for FITC-annexin V fluorescence are presented on Figure 2. ALN induced apoptosis as soon as 24h 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 $50 \mu\text{M}$ ALN, evidenced by the shift of annexin V fluorescence on the

1 histogram. By contrast, in the case of A431 cells, apoptosis increased with ALN treatment up
2 to 10 μM for which it became very significant (24% with only $\sim 70\%$ viable cells), then
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4 decreased back with 50 μM ALN. It is worth noticing that, after a 50 μM ALN treatment, the
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6 percentage of viable cells was comparable to that of untreated cells ($\sim 90\%$) whereas the
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8 percentage of dead cells had doubled ($\sim 10\%$ instead of 5%). 30 nM EGF has been taken as a
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10 positive control of apoptosis in the case of A431 cells [19].
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17 3.2. VEGF and TGF α expression

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22 The amount of secreted VEGF into the culture medium, determined by Elisa test, was largely
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24 superior in A431 cells compared to MCF7 cells. (Figure 3a). When cells were treated by
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26 increasing amounts of ALN, a consistent increase ($\sim 30\%$) in secreted VEGF was evidenced
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28 in A431 cells at 24h, followed by a decrease at 48h ($\sim 40\%$). The overall increase in VEGF
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30 secretion along the time (48h compared to 24h) is a usual observation on cells in culture [20].
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32 Correlation between VEGF secretion and VEGF mRNA level in A431 cells has been
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34 established by RT-PCR (Figure 3b). Results concerning the VEGF₁₆₅ isoform are presented
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36 since VEGF₁₆₅ is considered as the most mitogenic isoform but similar results have been
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38 obtained for VEGF₁₂₁ (not shown). After 24h treatment with ALN (0 to 15 μM), the mRNA
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40 expression of VEGF in A431 cells increased of $\sim 60\%$. This was no longer observed at 48h.
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42 Figure 3c presents the amount of TGF α secreted by A431 cells after ALN treatment. In a very
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44 similar way to what was observed for VEGF, increasing doses of ALN induced enhancement
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46 in TGF α secretion at 24h, followed by a decrease at 48h.
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54 VEGF secretion was lowered after treatment with AS (Figure 4a and 4b), compared to
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56 SC. When combined with AS, ALN treatment of A431 cells did not induce any more increase
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58 in VEGF secretion at 24h, like it was still the case after combination with SC (Figure 4a) or in
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1 the case of treatment by ALN alone (Figure 3a). The VEGF level was kept constant for small
2 ALN concentrations, up to 10 μ M ALN for which it increased. After only 12h treatment, the
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4 combination of AS with ALN even induced decrease in VEGF secretion (Figure 4b). It was
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6 only with 20 μ M ALN that VEGF secretion increased again. More surprisingly, AS also led
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8 to a decrease in TGF α secretion at 24h, compared to SC (Figure 4c) and, up to 5 μ M, opposed
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10 ALN-induced TGF α secretion, which had been observed at 24h (Figure 3c). At 12h, the level
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12 of TGF α was under the sensitivity of the assay. After pretreatment with 50 μ M LY294002, an
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14 inhibitor of the phosphatidyl inositol 3-kinase (PI3K) pathway, a global decrease in VEGF
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16 secretion was observed (Figure 4d) and ALN did not induce any more increase in VEGF
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18 secretion by A431 cells at 24h.
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27 **4. Discussion**

28 *4.1. Cell growth inhibition and apoptosis*

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37 The antiproliferative and proapoptotic properties of BPs have generally been described after
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39 48h treatment or more [21]. The slight modifications observed for earlier times were usually
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41 considered as resulting from a metabolic shift in the cell line stressed by the treatment or from
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43 a cytostatic drug effect. In the present work, we specifically focused on the effects observed
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45 after only 24h ALN treatment. Whereas a dose-dependent cell growth decrease was observed
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47 in the case of MCF7 cells, increasing doses of ALN only led to a plateau with 20 % cell
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49 growth inhibition (Figure 1) in the case of A431 cells. It is only at 48h that ALN treatment
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51 caused a dose-dependent decrease in cell number. This delay in cell growth inhibition is not
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53 attributed to a cytostatic effect since there is a clear correlation between (i) the efficient A431
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55 cell apoptosis (24 %) measured at 24h with 10 μ M ALN (Figure 2) and (ii) the dose-
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1 dependent decrease in cell number, which was observed at 24h (Fig 1b) up to 10 μ M ALN.
2 With 50 μ M ALN, whereas the percentage of apoptotic cells increased in the case of MCF7
3 cells (Figure 2), apoptosis was no longer observable in A431 cells. The percentage of dead
4 cells has doubled but that of viable cells has returned to its initial value, suggesting new
5 proliferation. Therefore we assume that, in the first 24h of treatment, there is superimposition
6 of two different processes in the case of A431 cells: an ALN-induced reduction in cell
7 viability evidenced by apoptosis and some mechanism stimulating cell proliferation and
8 leading to new daughter cells. This phenomenon was not observed for MCF7 cells. A
9 decrease in VEGF expression by using a VEGF-antisense (AS) allowed an improvement of
10 the antiproliferative effect of ALN at 24h to be obtained: it was now observed for ALN
11 concentrations up to \sim 30-40 μ M (Figure 1c). High VEGF expression in A431 cells may be
12 therefore involved in the de novo cell proliferation observed after 24h ALN treatment.
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32 *4.2. VEGF and TGF α expression*

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37 The expected antiproliferative but also antiangiogenic properties of ALN were observed in
38 A431 cells only after 48h. Direct expression of VEGF by tumor cells has rarely been
39 considered. In one study, ALN did not alter VEGF expression (protein or mRNA) in human
40 ovarian Caov-3 cells [10], leading the authors to conclude that antiangiogenic effect of ALN
41 might not be derived from inhibition of the production of VEGF by the tumor cells. But in
42 this study we have shown that ALN treatment led to a two-step effect in the case of A431
43 cells: enhancement of VEGF expression at 24h (mRNA and protein secretion) followed by a
44 decrease of VEGF expression at 48h (protein secretion) (Figure 3a,b). This two-step effect of
45 ALN on VEGF has also been observed in the case of TGF α : a dose-dependent increase in
46 secretion at 24h followed by a decrease at 48h (Figure 3c). TGF α is a ligand of EGFR and has
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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]. TGF α 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 TGF α secretion induced by ALN during the first 24h 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 24h (Figure 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 TGF α secretion. Above $\sim 10\mu\text{M}$ ALN, the amount of secreted TGF α becomes sufficient to counterbalance the antiproliferative effect of ALN. This transitory increase in TGF α induces up-regulation of VEGF secretion and consequently masks at 24h the expected antiangiogenic effect of ALN. (ii) the antiproliferative and antiangiogenic properties of ALN occur only at 48h.

AS lowered VEGF secretion. It allowed an antiangiogenic effect of ALN to be observed at 12h up to $10\mu\text{M}$ ALN and prevented the ALN-dose dependent increase in VEGF secretion at 24h, at least up to $5\mu\text{M}$ ALN. In an unexpected way, AS had a comparable effect on TGF α secretion, which decreased at least for the smallest concentration. If regulation of VEGF by TGF α is well established [23], it is the first time, to our knowledge, that influence of VEGF on TGF α expression has been described: reduction in TGF α 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 TGF α and since (i) TGF α is directly involved in cell proliferation and (ii) A431 cells express a high VEGF growth dependence

1 [18] through an autocrine pathway [25], it is possible that both effects of AS treatment (direct
2 on VEGF and indirect on TGF α) contributed to the resulting improvement of the
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4 antiproliferative effect of ALN, now observable at least up to $\sim 30\text{-}40\ \mu\text{M}$ ALN, instead of
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7 $10\ \mu\text{M}$.
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12 In conclusion, our results agree with the general idea that the combination of N-BPs
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14 with chemotherapeutic or other molecularly targeted anticancer agent should lead to an
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16 enhanced antitumor activity [26]. In the present case, the combination of ALN with AS would
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18 lead to the suppression of the proangiogenic step preceding the expected antiangiogenic
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20 action of ALN in A431 cells. Of particular interest is the fact that the combination of AS with
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22 small concentrations of ALN ($\sim 2\ \mu\text{M}$) induced either the reversion (at 12h) or the inhibition
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24 (at 24h) of the proangiogenic step. Indeed, $2\ \mu\text{M}$ ALN is of the order of a clinically relevant
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26 ALN concentration [27]. It has been estimated that, after rapid clearance of BPs by uptake in
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28 the skeleton or by the kidney where they are excreted, BP concentration in the circulation
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30 peaks may vary between $0,01\ \mu\text{M}$ and $1\ \mu\text{M}$, depending on the administration route [27].
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37 When patients were treated by zoledronate, the more efficient of the N-BPs, the peak serum
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39 concentration has been estimated in the range of $1\text{-}3\ \mu\text{M}$ and was maintained for only a few
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41 hours [28].
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44 In this study, the early action (proliferative and proangiogenic) of ALN has been described in
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46 cells which express high levels of VEGF. Therefore, the presented results cannot be
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48 generalized. However, hypoxia is a strong inducer of VEGF gene expression, and that more
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50 particularly in cells with low basal abundance of VEGF mRNA [29]. Since low levels of
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52 oxygenation have been demonstrated in malignant solid tumors, It is therefore conceivable
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54 that, in vivo, the two-step effect of ALN could frequently occur.
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Figure legends

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2 Figure 1: MTT cell proliferation tests after 24h or 48h treatment with increasing amounts of
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4 ALN. Results are the mean of three separate experiments. (a) MCF7 cells; (b) A431 cells; (c)
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6 A431 cells with ALN combined to 0.2 μ M VEGF - antisense (AS) or control scrambled (SC)
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8 oligonucleotides.
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14 Figure 2: Flow cytometry analysis of apoptosis in A431 or MCF7 cells after 24h treatment
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16 with 0, 2, 10 and 50 μ M ALN. Above: dot plots of FITC-annexin V / propidium iodide.
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18 Percentage of viable cells (lower left part), early apoptotic cells (lower right), late apoptotic
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20 cells (upper right) and dead cells (upper left) are indicated in each graph. 30 nM EGF was a
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22 positive control of apoptosis for A431 cells. Below: corresponding histograms for FITC-
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24 annexin V fluorescence. The experiment was done in triplicate and is representative of three
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26 independent experiments.
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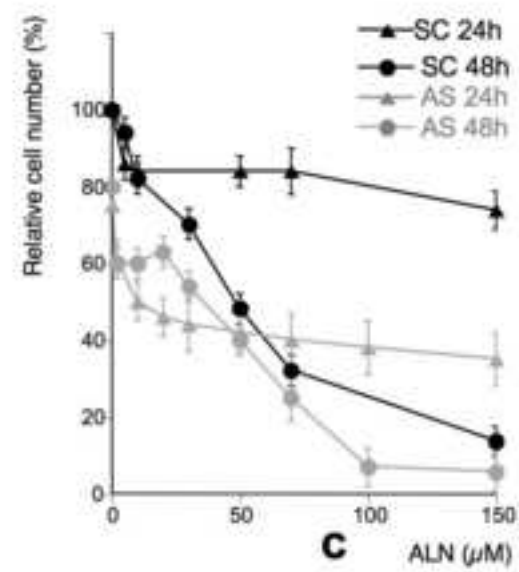
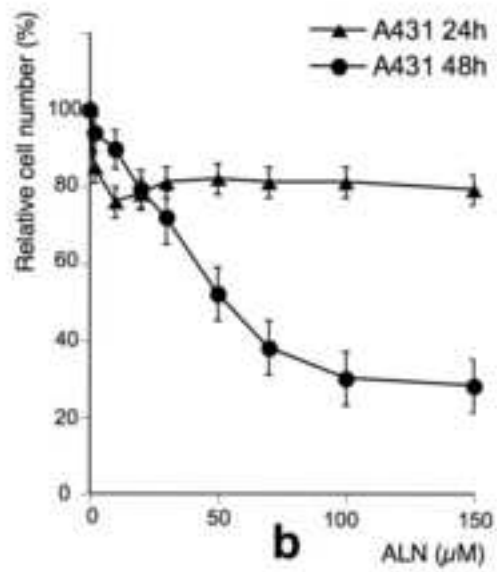
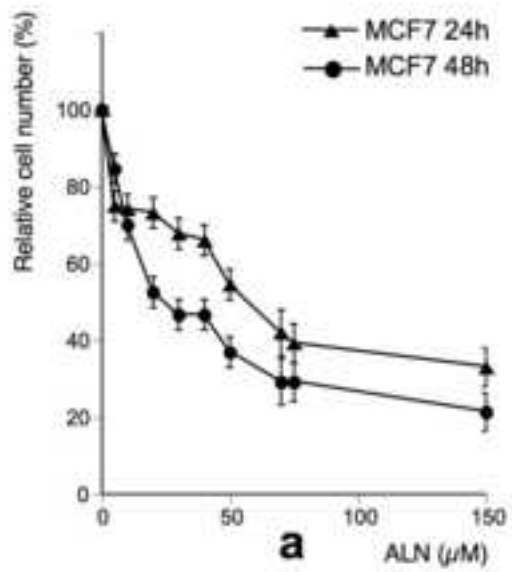
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34 Figure 3: (a): Elisa detection of secreted VEGF at 24h and 48h after treatment with increasing
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36 concentrations of ALN (A431 and MCF7 cells). The histograms (mean \pm S.E.M.) are
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38 representative of the average of three different cell treatments, each of them followed by Elisa
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40 test. Values indicated by an * ($p < 0.05$, ANOVA) are significantly different from control
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42 value, i.e. 0 μ M ALN at 24 or 48h. (b) Modulation of the expression of VEGF mRNA by
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44 ALN in A431 cells (24h and 48h). β -actin was used as an internal control. The histogram is
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46 representative of the average of four different cell treatments, each of them followed by PCR
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48 in duplicate. *: $p < 0.05$ compared to 0 μ M ALN. (c) the same as (a) for TGF α .
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56 Figure 4: (a) Elisa detection of secreted VEGF after 24h treatment with AS or SC, combined
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58 with increasing concentrations of ALN (A431 cells). The histogram (mean \pm S.E.M.) is
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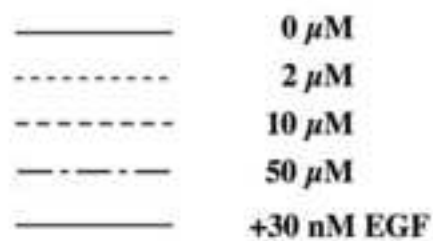
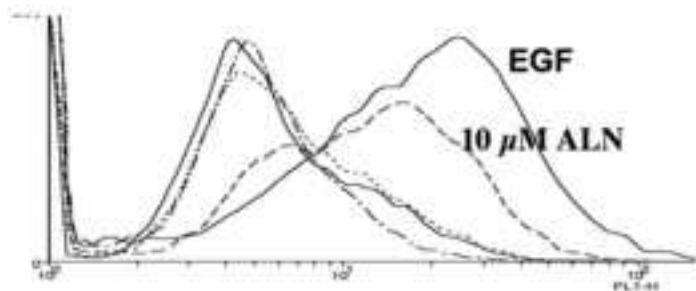
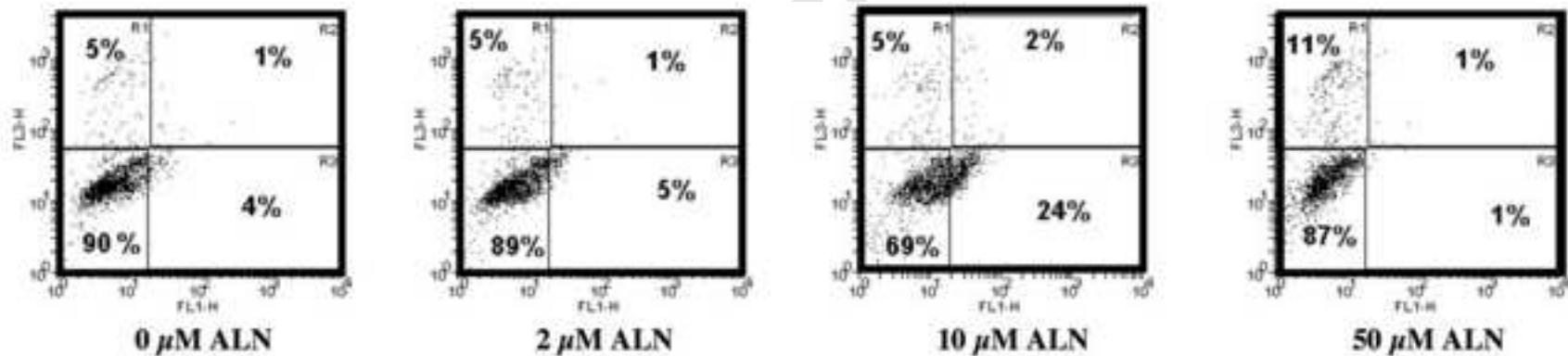
1 representative of the average of three different cell treatments, each of them followed by Elisa
2 test. (b) the same at 12h. (c) the same for TGF α at 24h. (d) Elisa detection of VEGF secretion
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4 after pretreatment with 50 μ M LY294002 and 24h treatment with increasing concentrations of
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6 ALN (A431 cells).
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Figure 1



A431



MCF7

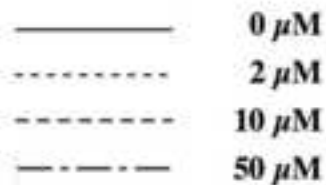
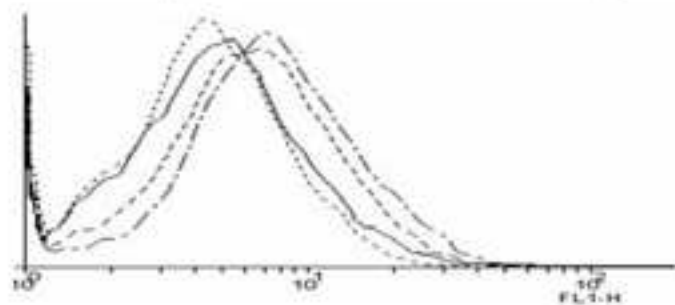
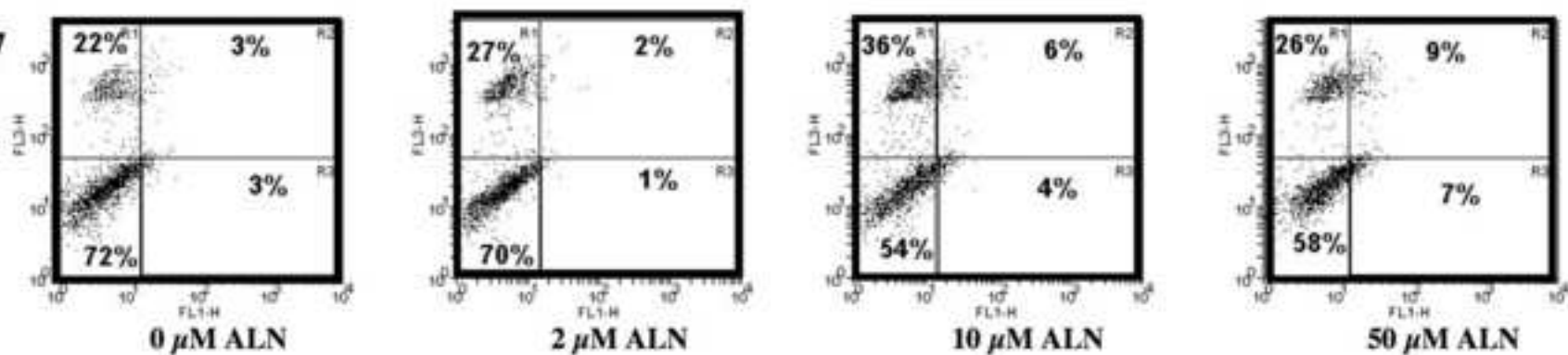


Figure 3

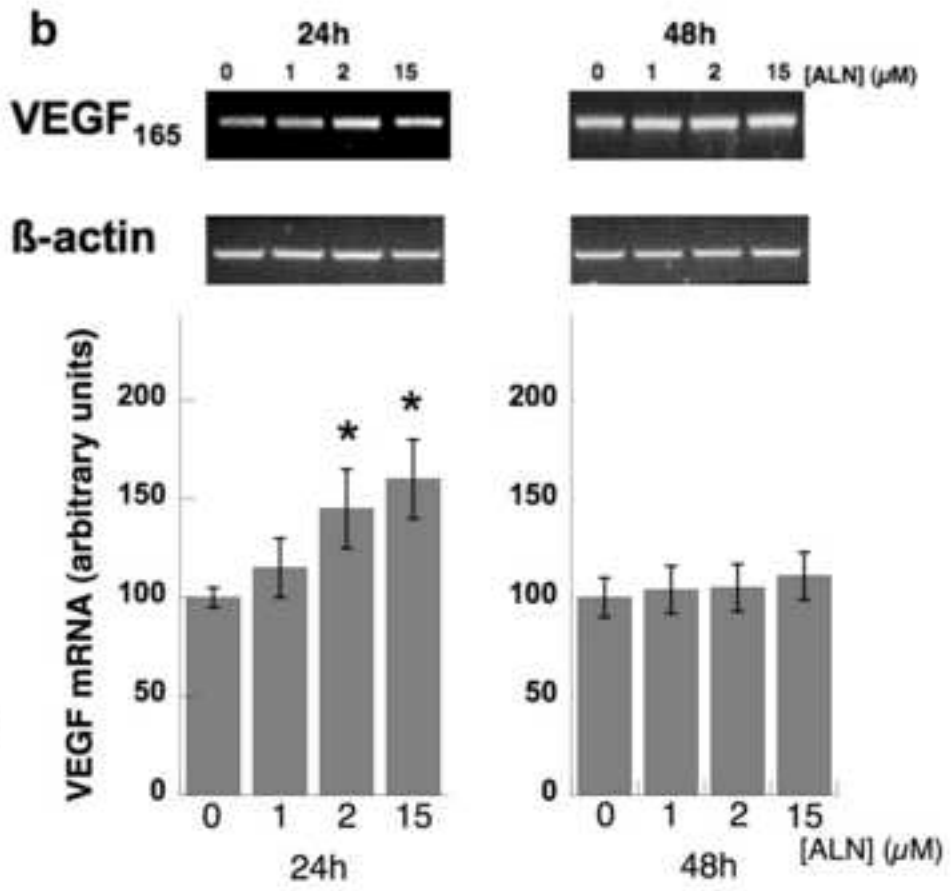
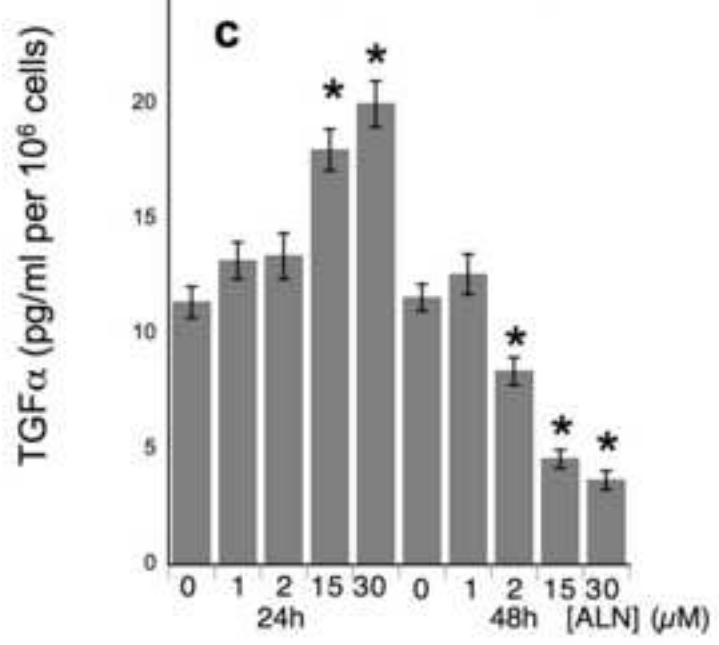
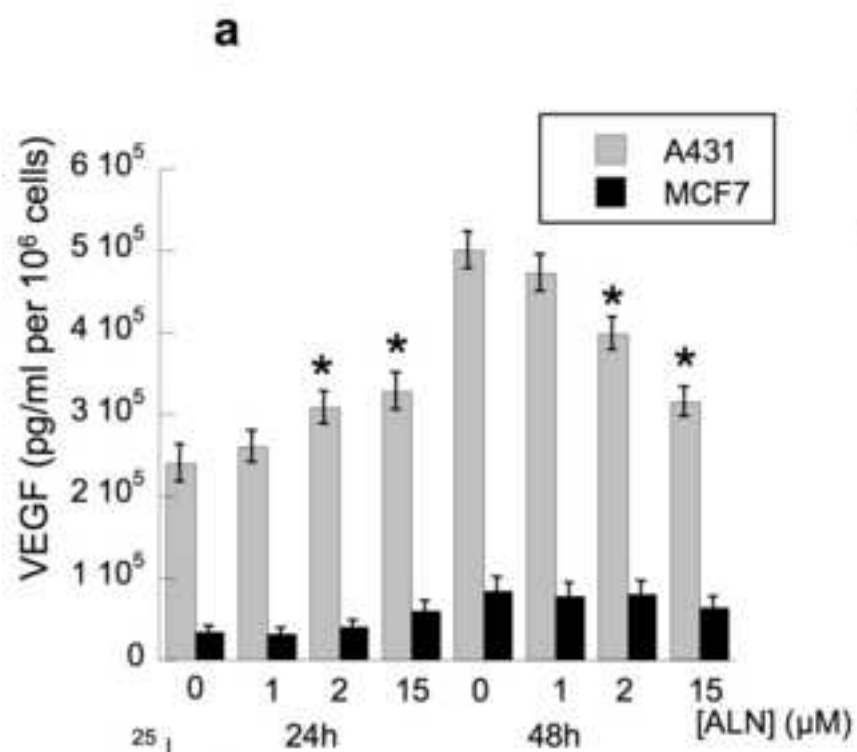
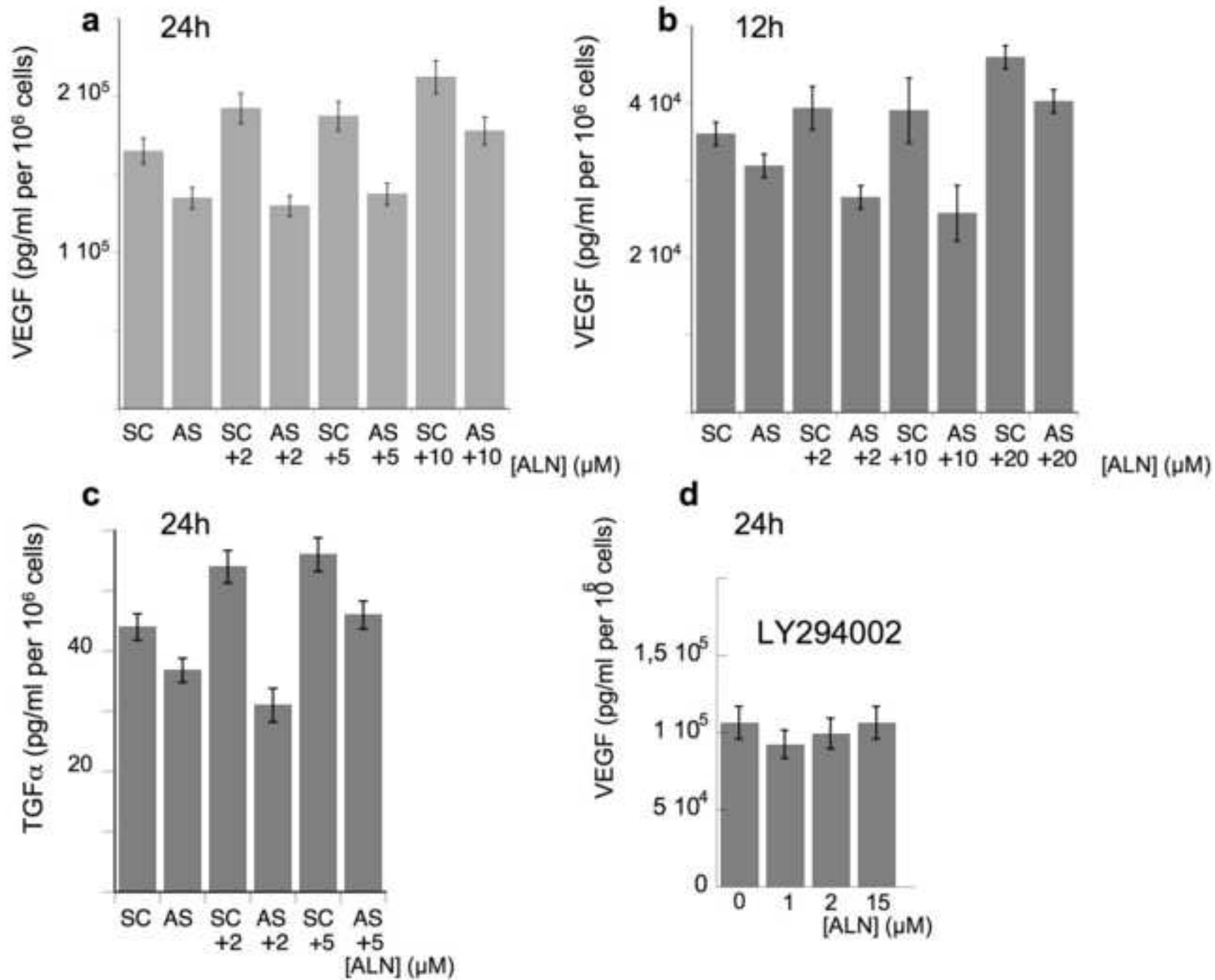


Figure 4





The early proliferative and proangiogenic response induced by alendronate in highly VEGF-expressing A431 cells is suppressed by combination with a VEGF- antisense oligonucleotide.

