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Alendronate Inhibits Proliferation and Invasion of Human Epidermoid Carcinoma Cells *In Vitro*

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Abstract. *There is increasing evidence that bisphosphonates have direct antitumor effects in vivo in addition to their therapeutic antiresorptive properties. Bisphosphonates inhibit proliferation and induce apoptosis of many cancer cell lines. They also exhibit anti-invasive properties in vitro and in vivo. We have previously shown that a novel non-nitrogen-containing bisphosphonate inhibited tumor growth of A431 human epidermoid carcinoma cells. In the present study, we investigated the antitumor properties of three nitrogen-containing bisphosphonates on A431 cells in vitro. We first compared the antiproliferative effects of pamidronate, alendronate and neridronate. Then, by matrigel invasion assay, the effect of alendronate on A431 cell invasiveness was studied. All three bisphosphonates were found to inhibit cell proliferation dose- and time-dependently. The most potent molecule was alendronate. The invasion test demonstrated that alendronate also inhibited cell invasion in a Boyden chamber. These data suggest that alendronate may have beneficial effects in the treatment of carcinomas exhibiting important angiogenesis.*

Bisphosphonates (BPs) are C-substituted pyrophosphate analogs that are potent inhibitors of bone resorption. Initially, BPs were used to control osteolysis and reduce bone loss in Paget's disease and osteoporosis. They are now also widely used in hypercalcemia and in the management of bone pain and osteolytic complications induced by metastatic tumors, like breast and prostate cancers. The beneficial effect of BPs in treatment of such diseases is due to their anti-osteoclastic properties (1, 2). Preclinical and clinical trials, and several *in vitro* reports, have suggested that BPs might also have a specific antitumor action. They act either by inhibiting cell viability and/or inducing apoptosis, or by inhibiting tumor cell adhesion and/or invasiveness. An anti-angiogenic potential of these compounds has also recently been reported *in vitro* and *in vivo* (3-5).

We have previously shown that the new BP, BP7033, exhibited marked anti-angiogenic and antitumor properties in a tumor model of A431 cells xenografted in nude mice (6). In the present study, we investigated the antitumor properties of three second-generation BPs, pamidronate (PAM), alendronate (ALN) and neridronate (NER), on A431 cells *in vitro*. Structurally, these aminobisphosphonates (N-BPs) differ in the length of the aliphatic carbon chain, varying from 2C (PAM) to 5C (NER) (see Figure 1). Antiproliferative and pro-apoptotic activities, as well as the antimigrative and anti-invasive effects of PAM and ALN, have been studied *in vitro* and *in vivo* on several tumor models such as osteosarcoma (7-10), breast (11-14), prostate (15, 16) and melanoma (17, 18) cancer cells. However, to our knowledge, it is the first time that the antitumor effects of N-BPs have been investigated in A431 epidermoid carcinoma cells.

Human squamous cell carcinoma cells like A431 (vulvar epidermoid carcinoma cells) represent a good model of an aggressive, highly angiogenic and metastatic tumor. Indeed, A431 cells display an increase in the expression of epidermal growth factor receptors (EGFR). Increased EGFR expression renders these cells less dependent upon an exogenous source of epidermal growth factor (EGF). It also further enhances the EGF-induced motogenic responses of squamous cell carcinoma cell lines compared with human epidermal keratinocytes, contributing to the invasiveness of malignant cells (19). Moreover, A431 cells produce large amounts of vascular endothelial growth factor (VEGF), promoting neovascularization and neo-angiogenesis (20).

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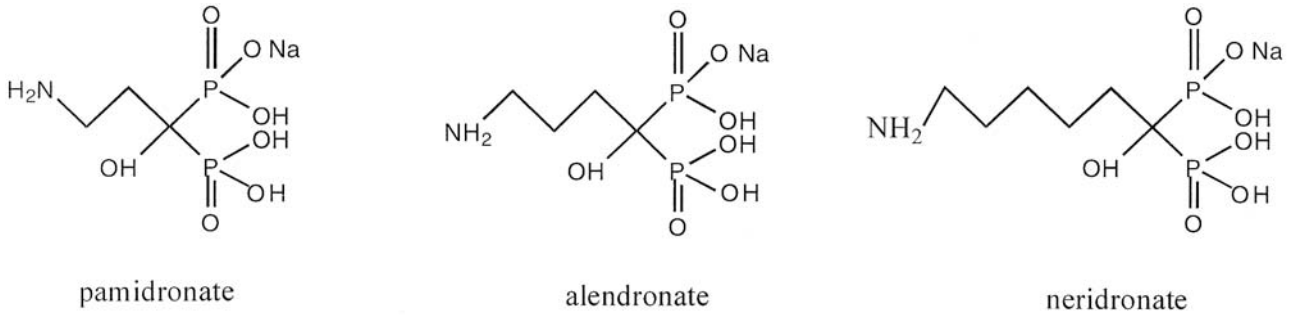

Figure 1. *Chemical structures of pamidronate, alendronate and neridronate.*

We first compared the antiproliferative effects of the three BPs on A431 cells. It was found that these compounds caused an irreversible loss of A431 cell viability, inhibiting proliferation in a time- and dose-dependent manner. The anti-invasive effect of ALN, the most potent BP of the three tested, was subsequently investigated. In an *in vitro* matrigel assay, we demonstrated that ALN exhibited a significant anti-invasive activity, without inhibiting either cell adhesion or cell migration.

Materials and Methods

Drugs. Pamidronate (3-amino-1-hydroxy-propylidene-1,1-bisphosphonate) (PAM), alendronate (4-amino-1-hydroxy-butylidiene-1,1-bisphosphonate) (ALN) and neridronate (6-amino-1-hydroxy-hexylidene-1,1-bisphosphonate) (NER) were synthesized by the Structural Chemistry and Biomolecular Spectroscopy Laboratory (CNRS UMR 7033, Université Paris 13, Bobigny, France). Stock solutions of the compounds (10 or 20 mM) were prepared in distilled water (pH adjusted to 7.4) and filter-sterilized using a 0.2 μm filter.

Taxol® (paclitaxel) was purchased from Sigma-Aldrich (L'Isles d'Abeau, France).

Cell culture. The human epidermoid carcinoma cell line A431 was purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were grown in DMEM-medium (4,500 mg/ml of glucose, glutaMAX and sodium pyruvate) (Life Technologies, Gaithersburg, MD, USA) supplemented with 10% fetal calf serum (FCS), 50 U/ml penicillin and 50 μg/ml streptomycin (all from Life Technologies), at 37˚C in a 5% CO_2-humidified atmosphere.

Cell viability experiments. The effects of PAM, ALN and NER on viability of the A431 cells were evaluated using the MTT microculture tetrazolium assay. Cells were seeded at a density of $5x10^3$ cells/well in 96-well flat-bottom plates (Falcon, Strasbourg, France) and incubated in complete culture medium for 24 h. The medium was then removed and replaced by 2% FCS-medium containing increasing concentrations of BP (10^{-7} to 10^{-3}M). After 24-, 48- and 72-h incubation, the cells were washed with phosphate-buffered saline (PBS, Life Technologies) and incubated with 0.1ml of MTT (2 mg/ml, Sigma-Aldrich) for an additional 4 h at 37˚C. The insoluble product was then dissolved by addition of DMSO (Sigma-Aldrich). The optical density was measured at 570 nm using a Labsystems Multiskan MS microplate reader.

The effect of short-term exposure to BP was then evaluated on A431 cells. Cells were seeded at a density of $5x10^3$ cells/well in 96-well flat-bottom plates and incubated in complete culture medium for 24 h. The cells were then treated with 2.5, 5 or 10 μM BP for only 8, 16 or 24 h. After each period of treatment, all media was removed and replaced by 2% FCS-medium. A MTT assay was assessed after a total of 72-h incubation from the start of BP treatment, as described above. The effects of short-term exposure were compared to the effects of complete 72-h drug exposure.

The cell viability experiments were performed in triplicate.

Matrigel invasion assay. Cell invasion experiments were performed using Bio-coat cell migration chambers (Becton Dickinson, Le Pont de Claix, France), which consist of a 24-well companion plate with cell culture inserts containing 8 μM pore size filters. The filters were coated with matrigel (100 μl/insert, Becton Dickinson) overnight. Untreated and ALN-pretreated cells (1-50 μM for 24 h) were added to each insert (upper chamber, $3x10^4$ cells/250 μL) in medium containing 0.1% bovine serum albumin (BSA, Sigma-Aldrich). Taxol®-pretreated cells (1 μM for 1 h) were used as positive controls. The chemoattractant (10% FCS-medium) was placed in each well of the 24-well companion plate (lower chamber, 500 μL/well). Serum-free medium was placed in the lower chamber for negative controls. After 48-h incubation at 37˚C in a 5% CO_2-incubator, the upper surface of the filters was wiped with a cotton-tipped applicator to remove non-invading cells. Cells that had invaded the matrigel and migrated through the filter pores were fixed with 4% paraformaldehyde and stained with Mayer's hematoxylin and eosin. The membranes were mounted on glass slides, and cells of the entire membranes were counted using an optical microscope (Zeiss Axiophot, Germany, magnification X200). In a parallel experiment, the viability of the untreated and drug-treated cells used in this assay was ensured by performing a MTT assay after 24, 48 and 72 h, as described above. The experiments were run in duplicate.

Cell migration assay. Cell migration experiments were performed using Bio-coat cell migration chambers (Becton Dickinson), as described above. The undersides of the inserts were coated with 100 μL of fibronectine (100 μg/ml, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and were allowed to stand overnight at 4˚C. The filters were then washed twice with PBS and blocked with

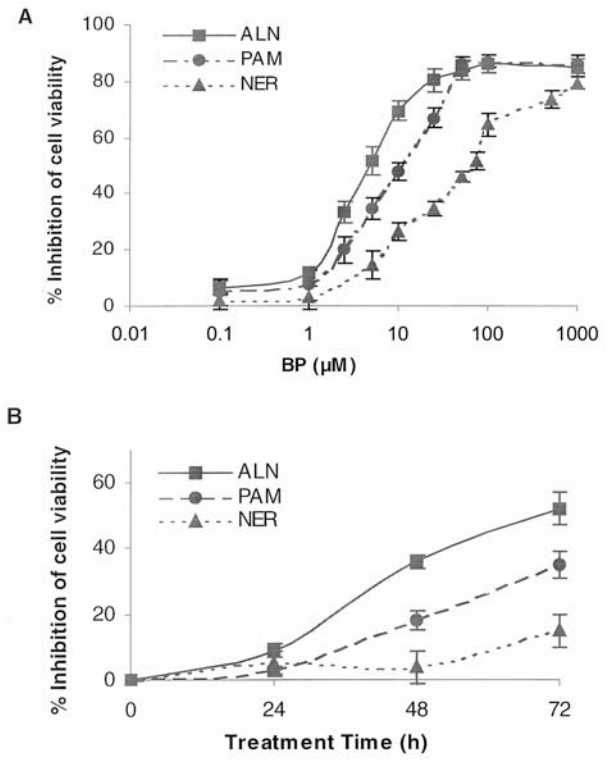

Figure 2. *Dose- and time-dependent effects of PAM, ALN and NER on A431 cell viability. A: Cells were treated with increasing concentrations (10^{-7}-10^{-3}M) of BPs for 72 h. At this time, cell viability was evaluated using the MTT assay. B: Cells were treated with 5 μM of BPs for 24, 48 and 72 h. After each incubation period, cell viability was evaluated using the MTT assay, as described in Materials and Methods. Results are expressed as the mean±SD from three independent experiments.*

1 mg/ml BSA for 1 h. As described for the invasion assay, untreated and drug-pretreated cells were added to each insert (upper chamber). After 6-h incubation at 37˚C in a 5% CO_2-incubator, non-migrated cells were removed by scraping and migrated cells were fixed, stained and counted. The experiments were run in duplicate.

Cell adhesion assay. The adhesion ability of untreated and ALN-pretreated A431 cells (1-50 μM for 24 h) was assessed. Cells were seeded in 96-well plates (10^5 cells/well). After 1.5-h incubation in 10% BSA medium, the proportion of attached cells was evaluated using the MTT assay described above. The experiments were run in duplicate.

Statistical analysis. All data are presented as means±SD. Statistical significance was determined by the Student's *t*-test. A *p*-value <0.05 was considered to be statistically significant.

Results

N-BPs inhibit A431 cell proliferation in a dose- and time-dependent manner. The effects of PAM, ALN and NER were studied, at doses ranging from 10^{-7} to 10^{-3}M, on A431 cell viability. All three BPs induced a concentration-related and time-dependent reduction in cell viability (Figure 2). ALN was the most potent BP with maximum inhibition (80%) obtained with 25 μM at 72 h ($IC_{50} \approx 5$ μM). PAM was the second most efficient BP with a maximum inhibition rate (86%) obtained after 72-h treatment with 50 μM of PAM ($IC_{50} \approx 10$ μM). NER exhibited the weakest inhibitory effect with maximum inhibition (79%) detected after 72-h treatment with concentrations in excess of 500 μM ($IC_{50} \approx 75$ μM) (Figure 2A). The decrease in cell viability induced by BP treatment was time-dependent (Figure 2B). At 24 h, 5 μM ALN induced 9% reduction in cell viability. A greater inhibition was observed after 48-h (36%) and 72-h (52%) treatment at the same concentration. A time-dependent effect was also seen after 5 μM PAM treatment: at 24 h, PAM had no effect, whereas 18% inhibition was observed at 48 h and 35% at 72 h. Finally, neither 24-h nor 48-h treatment with 5 μM NER affected A431 cells, while 72-h treatment of cells with this concentration induced 15% inhibition.

According to these results, the most potent BP was clearly ALN. Further experiments were thus carried out with this BP.

Short-time exposure to ALN causes irreversible loss of A431 cell viability. Treatment of A431 cells for 72 h with ALN (2.5 μM, 5 μM, 10 μM) resulted in, respectively, 33%, 52% and 69% inhibition of cell viability. To estimate the minimum time of exposure to ALN required to observe these effects at 72 h, A431 cells were exposed to 2.5, 5 and 10 μM ALN for 8, 16 or 24 h. The BP was then replaced by fresh medium until the end of the experiment. Cell growth inhibition was evaluated 72 h after starting BP treatment and was compared to inhibition of 72-h-exposed cells (Figure 3). A minimum of 16 h exposure to ALN resulted in a similar inhibition of cell viability compared to 72-h continuous exposure, whatever the dose tested. Incubation of A431 cells for 8 h with ALN at 2.5 μM resulted in 15% inhibition (compared to 33% for 72-h exposure). The inhibition induced by 8-h incubation with 5 μM ALN was 37% (compared to 52% for 72-h exposure). Eight-hour cell exposure to 10 μM ALN induced 63% inhibition (compared to 69% for 72-h exposure). Our results suggest that cell exposure to ALN for a minimum duration of 8 h could induce a significant irreversible loss of cell viability.

ALN inhibits A431 cell invasion. A matrigel invasion assay was performed to study the effect of ALN (1, 10, 25 and 50 μM) on the invasive ability of A431 cells. As shown in Figure 4, ALN inhibited A431 cell invasion in a dose-dependent manner. Cell pretreatment with 1 μM ALN did not affect cell invasion. Pretreatment with 10 μM ALN reduced the invasiveness of A431 cells to 60% of controls

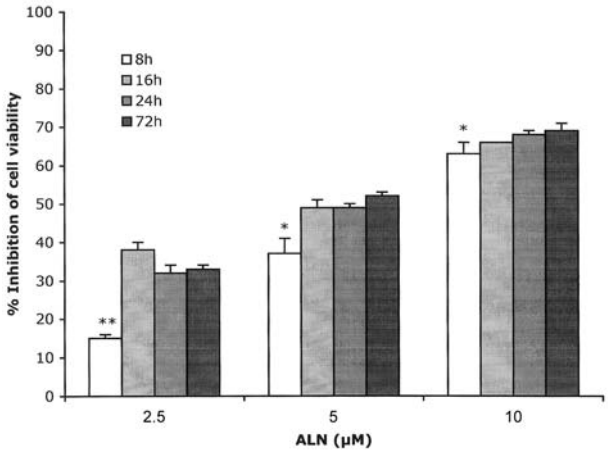

Figure 3. *Effects of short-term exposure to ALN on A431 cell viability. Cells were incubated with different doses of ALN for 8, 16, 24 or 72 h. At 72 h, cell viability was assessed using the MTT assay, as described in Materials and Methods. Results are expressed as the mean±SD from one representative of three independent experiments (*p<0.05; **p<0.01).*

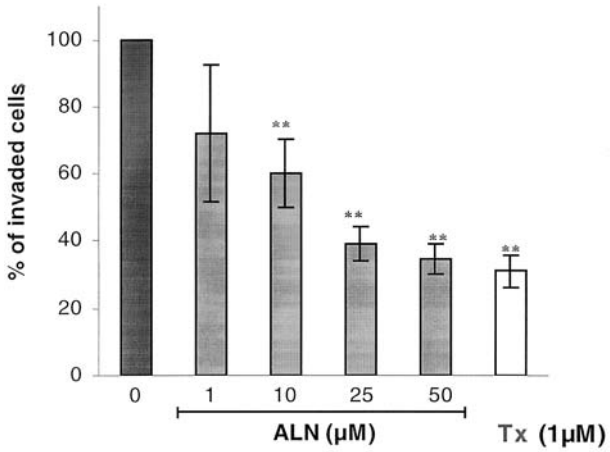

Figure 4. *Effects of increasing concentrations of ALN on A431 cell invasiveness through matrigel. A431 cells were pretreated with the indicated concentrations of ALN for 24 h. Positive control cells were treated for 1 h with 1 μM of Taxol® (Tx). The cells were subsequently added to Bio-coat chambers coated with matrigel. After 48-h incubation at 37˚C, the invaded cells were fixed, stained and counted, as described in Materials and Methods. The percentage of untreated cells invading matrigel was set to 100%. Data are expressed as the mean±SD of two independent experiments (**p<0.01).*

(=untreated cells, set to 100%); at 25 μM, cell invasion was reduced to 39% of controls. Maximum reduction was observed after treatment with 50 μM ALN (35%). This effect was comparable to that caused by Taxol® pretreatment (31.5%, positive control). In our experimental procedure, pretreatment of cells with higher doses of ALN (100 μM) affected cell viability and, thus such high concentrations were disregarded for further study.

ALN does not inhibit A431 cell migration or adhesion. We next assessed the effect of ALN on the adhesion and migration abilities of A431 cells. ALN, at all doses tested (from 1 to 50 μM), did not significantly affect either cell migration (compared to Taxol, ≈ 60% inhibition), or cell adhesion (data not shown).

Discussion

BPs, potent inhibitors of bone resorption, are currently used in cancer-induced bone diseases. The beneficial effect of BPs in the treatment of such diseases is due to their anti-osteoclastic properties. There is increasing evidence that BPs may also have direct inhibitory effects on cancer cells, acting either on tumor cell proliferation/survival or on cell invasiveness. In the present study, the antitumor potency of three second-generation BPs (PAM, ALN and NER) was examined on A431 cells, a model of highly angiogenic and aggressive epidermoid carcinoma. We first investigated the effects of the three BPs on cell viability and then analyzed the effect of ALN on the invasiveness of A431 cells.

Several reports of *in vitro* studies have cited the antiproliferative effects of PAM or ALN on different cancer cells, such as osteosarcoma (7, 8, 10), breast (11-13), prostate (15), myeloma (21), melanoma (22) and, recently, neuroblastoma (23) cancer cells. The antiproliferative effects of NER on cancer cells have still, to our knowledge, not been investigated. In the present study, we demonstrated that PAM, ALN and NER all inhibited A431 cell proliferation, in a dose- and time-dependent manner. ALN was the most potent BP (maximum inhibition ≈ 80%, observed after 72-h treatment with 25 μM ALN), followed by PAM, which was also very efficient (maximum inhibition ≈ 86%, observed after 72-h treatment with 50 μM PAM). NER exhibited the weakest inhibitory effect (maximum inhibition ≈ 80%, observed after 72-h treatment with 1 mM NER). Vortonjak *et al.* (23) have reported the same order of potency of ALN and PAM on neuroblastoma cell lines (ALN>PAM), with similar doses of BP. The concentrations of ALN and PAM that were effective on A431 cells are also comparable to those reported in many other studies (7, 8, 10-13, 15). However, Takahashi *et al.* (21) and Sawada *et al.* (24) reported that only high concentrations (≥100 μM) of, respectively, PAM and ALN significantly inhibited tumor cell proliferation after 48-h and 72-h treatment.

Our results indicated that the three BPs inhibit proliferation of A431 cells *in vitro* in a dose- and time-dependent manner. However, *in vivo*, cancer cells are exposed to micromolar concentrations of BP for only a few

hours, since BPs are rapidly removed from the circulation following their administration and subsequently accumulate in the bone (1, 2). In order to examine those conditions that may more accurately reflect the *in vivo* situation, cells were exposed to BP for shorter periods of time (8, 16 and 24 h). We observed that cell exposure to ALN at doses ≥5 μM (IC_{50} value) for only 8 h was sufficient to induce 71 to 100% of the inhibition observed with full 72-h exposure. This result suggests that acute exposure to relatively low doses of ALN is sufficient to induce an antitumor effect in A431 cells. Moreover, removal of ALN after 16 and 24 h did not allow recovery of A431 cell proliferation, demonstrating an irreversible loss of cell viability. Comparable results were obtained with PAM and NER short-term exposure experiments (data not shown). Our results support previous data reported by Lee *et al.* (15), who observed that treatment of prostate cancer cells with high doses of PAM or zoledronate (100 μM) for 6 h inhibited cell growth. However, in this study, the authors showed that 12- to 24-h treatment with BP at lower doses (25-50 μM) was the minimum time required to observe statistically relevant inhibition of cancer cell growth. Similar results were obtained by Fromigue *et al.* (11) on breast cancer cells with PAM, zoledronate, clodronate and ibandronate. These authors reported that removal of 1 μM BP after 24 h did not allow return to the basal proliferation rate (observed 48 h later). Senaratne *et al.* (12) also showed that the presence of 100 μM PAM for only 24 h led to irreversible loss of cell viability at 4 days.

Metastasis, a multistep process leading to the formation of secondary tumors from the original tumor, is a complication of most cancers. It is also the main failure of cancer treatment. One important step in this process consists of the invasion of a secondary tissue by metastatic cells. Several reports from animal and clinical studies demonstrated that BPs affect the invasive behavior of metastatic cells *in vivo* (25, 26). The anti-invasive effects of BPs were also described in several tumor *in vitro* models (3, 4), and the anti-invasive effects of ALN in matrigel assays were reported for prostate and breast cancer cells (16), osteosarcoma (9), and fibrosarcoma and melanoma cells (17). In the present study, we showed that ALN displayed, *in vitro,* a significant anti-invasive effect on A431 cells (61-65% inhibition with 25-50 μM). These results are in agreement with those observed with ALN on osteosarcoma cells by Cheng *et al.* (9), who observed 50-70% inhibition after 48-h invasion of cells pretreated with 50 μM ALN, and Heikkila *et al.* (17), who reported an ALN IC_{50}=40-70 μM. Similar inhibition was found in prostate cancer cells, but for pico-nanomolar doses of ALN (16). In contrast, in the study of Andela *et al.* (27), ALN failed to inhibit invasion of lung cancer cells. However, the invasion assay was only performed for 24 h, which may explain the ineffectiveness of ALN in their study.

Tumor cell invasion requires both cell adhesion and cell migration. BPs have been reported to inhibit these two functions. ALN (1-30 μM) significantly suppressed the lysophosphatidic acid-induced migration and adhesion of human ovarian carcinoma cells *in vitro* (24). Similar data were reported for breast and prostate cancer cells (16) and fibrosarcoma and melanoma cells (17). An ALN-induced inhibition of breast cancer cell adhesion to bone matrices was also reported *in vitro* (28). In order to characterize the specific phenomenon inhibited by ALN in A431 cells, we also performed migration and adhesion assays. Our results suggest that, at similar doses (1-50 μM), ALN was not able to inhibit either adhesion or migration of A431 cells *in vitro*. However, our results are in agreement with those obtained by Boissier *et al.* (29) in similar migration and invasion assays. Indeed, these authors demonstrated that ALN doses that inhibited breast cancer cell invasion did not inhibit cell migration. Magnetto *et al.* (30) also reported that the N-BP ibandronate is able to partially inhibit breast cancer cell invasion without affecting cell motility.

The anti-invasive effect of ALN that we observed on A431 cells was not due to either an inhibition of cell adhesion or of cell migration. The anti-invasive effects of many BPs, including ALN, have been correlated to inhibition of production and/or activity of some matrix metalloproteinases (MMPs), proteases implicated in the required degradation of extracellular matrix during invasion (31). Thus, the anti-invasive effect of ALN observed in our study may be caused by dysregulation of secretion or activity from one or more MMPs. Further investigations must be carried out to answer this question.

In conclusion, the present work supports observations that N-BPs can act as direct anticancer drugs by inducing irreversible cell death of A431 tumor cells. One possible mechanism for N-BPs to induce tumor cell death is by induction of apoptosis (3, 4). The hypothesis that PAM, ALN and NER induce apoptosis in A431 cells should be considered in further investigations. We have also demonstrated that ALN can exhibit antimetastatic properties *in vitro* by inhibiting the invasion of cancer cells. Our results suggest that a BP, such as ALN, may be very efficient in the treatment of aggressive tumors. However, further *in vivo* experiments are required to determine whether this BP is clinically relevant in the treatment of aggressive and highly angiogenic squamous cell carcinomas.

Acknowledgements

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