Abstract. Background: Bisphosphonates are used in cancer-related hypercalcaemia, in complications of bone metastasis and in postmenopausal osteoporosis, and have often been associated with adverse complications. Aim: To determine the protective effect of apigenin against growth inhibition of normal epithelial human prostatic (PNT2), transgenic adenocarcinoma of mouse prostate (TRAMP-C1) and metastatic melanoma cells (B16F10) in combined treatments with bisphosphonates and ionizing radiation (IR).

Materials and Methods: The growth inhibition on PNT2, TRAMP-C1 and B16F10 cells in the combined treatments with bisphosphonates (zoledronic acid, ibandronate and pamidronate) and IR in the presence and absence of apigenin was studied using a cell viability test.

Results: Zoledronic acid had a cytotoxic effect on PNT2, TRAMP-C1 and B16F10 cells (p<0.001). However, ibandronate and pamidronate had a cytotoxic effect only on PNT2 cells (p<0.001). The administration of apigenin in combined treatment with bisphosphonates (zoledronic acid, ibandronate and pamidronate) and IR in the presence and absence of apigenin was studied using a cell viability test.

Conclusion: The use of the antioxidant produced a greater decrease in the cytotoxic effect on the non-tumor than in tumor cells when treated with bisphosphonates-alone and could be used in non-tumor pathologies. However, in a combined treatment with IR, it can also provide protection to tumor cells, thus reducing the intended effect of the IR.
to evaluate its influence on combined treatments with bisphosphonates and ionizing radiation (IR) for possible extrapolation to clinical trials.

Materials and Methods

Chemicals and reagents. Zoledronic acid (Zometa®) was obtained from Novartis Pharmaceuticals (Barcelona, Spain). Ibandronate (ibandronate sodium salt) and pamidronate (P:3-amino-1-hydroxypropylydine bisphosphonic acid) were obtained from Sigma-Aldrich Chemicals S.A. (Madrid, Spain). RPMI-1640 medium, Ham’s F10, Phytomagglutinin (PHA), Dimethyl Sulfoxide (DMSO), cytochalasin B, streptomycin, penicillin, phosphate-buffered saline (PBS) and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2h-tetrazolium bromide (MTT) were obtained from Sigma-Aldrich Chemicals S.A. Fetal bovine serum was obtained from Gibco BRL, Louisville, KY (USA) and apigenin was obtained from Sigma Co. (Madrid Spain).

Cell lines and culture conditions. The normal epithelial human prostatic cell line (PNT2) was used was obtained from the European Collection of Cell Cultures (ECACC), Health Protection Agency Culture Collection (catalog n:95012613;UK). The PNT2 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, glutamine (2 mM) and streptomycin plus penicillin (100 μg/ml and 100 IU/ml, respectively). The metastatic melanoma cell line (B16F10) was kindly provided by Dr. Hearing from the National Cancer Institute (Bethesda, MA, USA). The transgenic adenocarcinoma of mouse prostate cell line (TRAMP-C1) used was obtained from the American Type Culture Collection (catalog n:CRl-2730, USA). The TRAMP-C1 and B16F10 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM)/F12K (1:1). Both media contained 10% fetal bovine serum (Gibco BRL) and 5% penicillin/streptomycin. The cell cultures were kept at 37°C and 95% relative humidity, in an atmosphere with 5% CO₂.

Tests were carried out to confirm the absence of Mycoplasma spp. throughout the study.

The substances tested were administered by adding 25 μl to each well. Zoledronic acid was administered at the recommended concentrations for humans (5%); ibandronate, pamidronate and apigenin were administered at a concentration of 20 μM. The cells treated with IR were irradiated immediately after administration of each test substance to the cells.

Treatment and irradiation. The samples were exposed to X-rays using an Andrex SMART 200E instrument (Yxlon International, Hamburg, Germany) operating at 200 kV, 4.5 mA, 36 cm (FOD), at room temperature. The radiation doses were monitored by a UNIDOS® Universal Dosimeter with PTW Farme® ionization chambers TW30010 (PTW-Freiburg, Freiburg, Germany) in the radiation cabin and the dose of radiation of X-rays was confirmed by means of thermoluminescent dosimeters (TLDs) (GR-200®, Conqueror Electronics Technology Co Ltd, P.R. China). The Ministry of Industry and Energy (Spain) supplied the TLDs and also measured their absorbed doses after the experiments. Different doses of X-rays (2, 4, 6, 8, 10 Gy and 0 Gy as control) were used in the MTT cell viability assay.

MTT test. To analyze for the effects of zoledronic acid, ibandronate, pamidronate, apigenin and IR on PNT2, TRAMP-C1, B16F10 cell viability and survival, we used the MTT assay. Briefly, the cultures were incubated in 200 μl growth medium and allowed to adhere for 24 hours. After treatment, supplemented growth medium and 50 μl of MTT (5 mg/ml) were added to each well and microplates were further incubated in a 5% CO₂ atmosphere at 37°C for 4 h. After centrifugation to carefully remove the medium and non-metabolized MTT, 100 μl of DMSO was added to each well to solubilize the MTT formazan produced by the cultured cells. After shaking for 30 min at room temperature, the plates were read with a Multiskan MCC/340P spectrophotometer using 570 nm for the test reading and 690 nm as the reference wavelength. The negative control well was used for the baseline zero. Each experiment was repeated on three occasions.

Statistical analysis. In the cytotoxicity assay, an analysis of variance (ANOVA) of repeated means was carried out to compare the percentages of surviving cells in the cultures with different concentrations of the various compounds, complemented by least significant difference analyses to contrast pairs and means. The analyses were carried out by logarithmically transforming the data to comply with ANOVA conditions.

Results

The treatment with zoledronic acid for 48 h caused a decrease in PNT2, TRAMP-C1 and B16F10 cell survival (p<0.001) (Figure 1). The highest degree of cytotoxicity was shown in the TRAMP-C1 cell line (Figure 1) followed by the B16F10 cell line (Figure 1). Treatment with apigenin showed no significant differences in cell growth compared to control cells showing no cellular toxicity at the doses tested. However, combined treatment with zoledronic acid and apigenin showed a significant increase in cell survival compared with the administration of zoledronic acid alone in all treated cell lines but manifested significant differences only in the PNT2 cells after 48 h of incubation (Figure 1). Thus, in the PNT2 cells, the administration of zoledronic acid + apigenin led to greater survival than when the cells were treated with zoledronic acid alone (p<0.001) (Figure 1), and illustrates a reduced cytotoxic effect of zoledronic acid when administered together with apigenin.

The treatment of cells with ibandronate only led to a significant decrease in the survival of PNT2 cells (p<0.001) (Figure 2). The treatment with apigenin led to no significant differences in cell growth compared with control cells in any of the cell lines tested. The effect of combined administration of apigenin plus ibandronate did not differ significantly from when ibandronate was administered alone in any of the cell lines tested (Figure 2).

Treatment with pamidronate only caused a significant decrease in the survival of the PNT2 cell line (p<0.001) (Figure 2). The treatment with apigenin caused no significant differences in cell growth compared with control cells in any of the cell lines tested. The effects of combined administration of apigenin plus pamidronate did not significantly differ when compared to the control cells in any of the cell lines tested (Figure 2).
Irradiation with 10 Gy of X-rays produced a significant reduction in cell survival in all cell lines studied (p<0.001). The irradiated cells treated with zoledronic acid +apigenin exhibited increased cell survival in all cell lines tested showing statistical significance in the PNT2 and B16F10 cells (p<0.001) (Figure 3). This may be a demonstration of an anti-cytotoxic effect of apigenin against damage induced by combined treatment with zoledronic acid plus IR.

Treatment with ibandronate and IR caused an increment in the cell survival of TRAMP-C1 and B16F10 cells (p<0.001) (Figure 4) compared with irradiated control cells, showing the characteristics of a radioprotective agent only for these tumor cells (TRAMP-C1 and B16F10). Administration of apigenin to the irradiated TRAMP-C1 and B16F10 cells led to an increase in cellular survival compared with the irradiated control cells (p<0.001). Co-administration of ibandronate + apigenin had a protective effect only in the tumor cells (TRAMP-C1 and B16F10) (p<0.001) (Figure 4).

In the cells treated with pamidronate and IR showed no significant differences with respect to irradiated control cells; treatment with pamidronate only led to a decrease in cell survival in the B16F10 melanoma cells (p<0.001) (Figure 4). The administration of apigenin to the irradiated PNT2 and B16F10 cells led to an increased cell survival compared with the irradiated control cells (p<0.001). Co-administration of pamidronate plus IR plus apigenin had a protective effect only in the PNT2 cells (p<0.001) (Figure 4).

**Discussion**

Our study shows the cytotoxic effects of zoledronic acid on PNT2, TRAMP-C1 and B16F10 melanoma cells in vitro. These results are consistent with earlier reports that zoledronic acid can inhibit cellular proliferation in different tumor cells (10-13). However, ibandronate and pamidronate had such a cytotoxic effect only on normal prostate epithelial cells, but not in transgenic prostate cancer or metastatic melanoma cells.

There is dearth of information on the in vitro cytotoxic effects of ibandronate and pamidronate on cell viability (14). A cytotoxic effect induced by ibandronate through the inhibition of cell growth has been described in PC-3 human prostate cancer cell line (15) and in breast cancer (14, 16, 17), which shows a significant increase in cell apoptosis (14, 15). Some authors have reported that in in vitro rat models, ibandronate led to a reduced tumor burden by inhibiting growth of cancer cells (18) and significantly reducing the infiltration of cancer cells to metastatic sites (19).

Several studies have suggested that the administration of antioxidants in combination with chemotherapy (zoledronic acid and/or ibandronate) may reduce chemotherapy-induced toxicity and that it may even be possible to increase the dose
of the drug with a consequent decrease in its adverse toxic effects (10). Some authors propose that the administration of zoledronic acid induces oxidative stress and reduces endogenous antioxidant levels. These authors suggested that the administration of zoledronic acid could be clinically useful and that as a further investigation, it should be combined with antioxidant supplements (20). In our study, administration of zoledronic acid in combination with an antioxidant (apigenin) reduced the toxic effect of zoledronic acid on normal prostate cells, had no protective effect towards TRAMP-C1 tumor and melanoma cells, but also had no noticeable lower effect on their cellular survival.

A different situation prevailed with ibandronate. Recently it has been described that ibandronate reduces endogenous levels of reactive oxygen species in cultured prostate cancer and endothelial cells (14). It has also been hypothesized that
it exerts its antioxidant effects through a reduction in the prenylation of RAC proteins and a disruption of NADPH oxidase complex (14). This described in vivo antioxidant capability of ibandronate could help in the elimination of free radicals induced by phenytoin used in the treatment of epilepsy (21). In our study, the administration of the apigenin did not reduce the toxic effect of ibandronate on human prostate epithelial cells and prostate tumoral cells, but did protect melanoma cells. In short, the administration of an antioxidant in combination with zoledronic acid can reduce oxidative stress produced by zoledronic acid (20), or reduce the adverse effects of ibandronate (14). This protective effect is more intense against the cytotoxic effect on human non-tumor epithelial cells than on prostate tumor or metastatic melanoma cells.

However, the entire observed trend seems to change when treatment is combined with X-ray irradiation. Our study shows an in vitro dose-dependent cytotoxic effect of IR on PNT2, TRAMP-C1 and B16F10 melanoma cells and is consistent with findings described by others (10, 11, 22). The combined administration of zoledronic acid plus IR significantly reduced cell survival in all cell lines. Previously, we identified a synergic cytotoxic effect of zoledronic acid with IR which portrayed the characteristics of a powerful chemical radiosensitizing agent (8). Others have described a synergic effect of zoledronic acid in combination with IR in several tumor lines (11-13) and described a sensitizing effect of zoledronic acid and have therefore suggested that reduced doses could be used in oncological RT. In contrast, in our study, ibandronate had a direct radioprotective effect on tumor cells (TRAMP-C1 and melanoma) with no radioprotective effect on normal cells (PNT2). Pamidronate had no significant radioprotective effect on either of the three cell lines studied (PNT2, TRAMP-C1 and B16F10).

Currently, the ability of different substances to prevent cytotoxic damage is measured in terms of their capacity to inhibit the activity of ROS. Antitoxic effects are directly proportional to the antioxidant capacity, although it is also dependent on their bioavailability characteristics in the medium assayed (22, 23). Apigenin is a known radioprotector whose mechanism of action is thought to proceed via the elimination of free radicals induced by IR (22-24).

It has been suggested that the ability of zoledronic acid to arrest cells in the G2M phase or to prolong cell-cycle progression raises the possibility of it functioning as a potential cell-cycle chemosensitizer (12, 25). However, all the above are compatible with the cellular mechanism of radiosensitization produced by zoledronic acid in normal cells. Karabulut et al. (20) described how the in vivo administration of zoledronic acid caused a significant reduction in glutathione (GSH) and the antioxidant γ-glutamate-cysteine; an increase in nitrites and nitrates, which are the end products of nitric oxide metabolism; and a significant increase in malondialdehyde. Concluding, the authors explained that the administration of zoledronic acid induces oxidative stress and reduces endogenous antioxidant levels. The diminution in the intracellular levels of GSH,
which increases sensitivity to subsequent radiation effects, explained the radiosensitizing effect of cisplatin (22), could act in a similar way in our combined treatments using zoledronic acid.

The mechanisms of action described above, involving the induction of oxidative stress by the formation of free radicals and a fall in the endogenous levels of antioxidants, are similar to the mechanisms of IR, both in regards to cell death and cytotoxic capacity, and have been previously described for exposure to X-rays (9, 13). The effect of apigenin as a free radical-scavenging antioxidant acts to supplement exogenous antioxidant levels and reduce oxidative stress.
Our study showed that in tumor and prostate non-tumor cells, the administration of apigenin in combined treatment with IR plus zoledronic acid or pamidronate provides a higher level of protection to normal cells than TRAMP-C1 tumor cells, but when ibandronate is used, it offers more protection to TRAMP-C1 cells than it does to non-tumor cells. In contrast, administration of apigenin to melanoma cells protected against the detrimental effects of zoledronic acid and ibandronate along with IR, whereas the administration of pamidronate did not show any effect.

Our results suggest that if the mechanism of chemotherapy and/or RT induced cell damage is through the production of oxidative stress with an increase in free radicals, co-administration of antioxidants to minimize the harmful effect is obvious, but this treatment may also result in the reduction of the detrimental effect of these ROS on tumor cells. Perhaps for this reason, at present, many are against an indiscriminate supplementation of antioxidants to oncology patients undergoing RT or chemotherapy and have identified increase tumor survival, prolonged disease duration, or increase of disease recurrence in these patients (8). Possibly, knowledge of the different mechanisms of cells damage produced through different routes of antioxidant action can help achieve selective protection of non-tumor cells without reducing the damaging effect on tumoral cells.

In conclusion, the administration of an antioxidant substance with a bisphosphonates produces a more profound decrease in the detrimental effect on non-tumor cells than on tumor cells and could be used in the treatment of non-tumor pathologies. However, in a combined treatment with IR, the antioxidant supplement can protect both tumoral (TRAMP-C1 and B16F10) as well as non-tumor cells (PNT2), thereby diminishing the intended effect of treatment with IR.

Conflicts of Interest

None.

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