CYTOTOXICITY OF ETIDRONIC ACID TO HUMAN BREAST CANCER CELLS

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Introduction: Bisphosphonates have been used to treat Paget's disease, osteoporosis, and cancer metastases to the bone. The cancer chemotherapeutic potential of a first-generation bisphosphonate, etidronic acid, was evaluated by using MCF-7 human breast cancer cells.

Methods: *In vitro* cytotoxicity of etidronic acid to MCF-7 cells was estimated on the basis of clonogenicity assays, while cell cycle effects were determined by using flow cytometry. Mutagenicity of etidronic acid was detected by using denaturing high-pressure liquid chromatography analysis of cellular DNA amplified by PCR with primers for exons 5 through 8 of the human p53 gene.

Results: A 24-hour treatment with etidronic acid (10 mM) with or without strontium chloride was cytototoxic to MCF-7 cells. Etidronic acid caused a decrease in the S-phase population and an increase in the G_2/M population. Mutations in the p53 gene were detected in MCF-7 cells treated with etidronic acid. Strontium chloride was not cytotoxic to cells.

Conclusions: Cytotoxicity of etidronic acid to breast cancer cells may complement its inhibitory effects on bone resorption at the site of bone metastasis. Within the cell cycle, late S-phase cells are the most radioresistant, while cells at the G_2/M border are the most sensitive. Therefore the decrease in S-phase population with corresponding increase in G_2/M would make the cells more radiosensitive. This may be useful if etidronic acid were combined with radioactive strontium (^{89}Sr, metastron) or external-beam radiotherapy for treating bone metastases. Tumor cells that survive etidronic acid treatment may acquire drug resistance because of mutations in the p53 tumor-suppressor gene. (*Ethn Dis*. 2008;18[Suppl 2]:S2-87–S2-92)

Key Words: Etidronic Acid, Breast Cancer, Cell Cycle, Cytotoxicity, Mutagenicity

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INTRODUCTION

Breast cancers tend to metastasize to the skeleton, and the risk of fracture increases as a consequence of bone metastases. Chemotherapy and endocrine deprivation therapy for breast cancer also cause bone resorption and decrease bone density. Deterioration of bone health from metastases or endocrine deprivation therapy for breast cancer can diminish quality of life.[1–2] Bisphosphonates are useful to treat postmenopausal osteoporosis and bone metastases.[3–6] Bisphosphonates are nonhydrolysable analogs of pyrophosphates in which the central oxygen atom is substituted by a carbon atom. Since these pyrophosphate analogs have a strong affinity for hydroxyapatite on the bone surface, they are attracted to sites of increased bone formation and resorption. Prolonged treatment with this class of drug can sometimes cause osteonecrosis.[7–8] Both the beneficial and adverse effects of bisphosphonates involve cytotoxic mechanisms. Recent evidence suggests that bisphosphonates may have antitumor effects in addition to their well documented effects on osteoclasts and antiosteoporotic effects.[9] The chemotherapeutic potential of bisphosphonates as single agents or in combination with other anticancer agents have been reported recently.[10–14] In general, later-generation nitrogen-containing bisphosphonates are more potent than first-generation bisphosphonates, such as etidronate.[5,13] The decreased potency of etidronic acid may also be associated with diminished risk of adverse effects. The use of etidronic acid in combination with other drugs for cancer treatment can therefore be contemplated. Investigation of the mode of action of etidronic acid alone and in combination with other agents could be useful for developing safer strategies for cancer treatment.

Strontium is a calcium mimic that stimulates bone formation, and the efficacy of strontium ranelate as an antiosteoporotic agent has been evaluated in clinical trials.[15–16] Moreover, the bone-seeking radioisotope ^{89}Sr has been used for treating bone metastases.[17–19] Therefore, the effects of etidronic acid, a first generation bisphosphonate, were examined in estrogen-dependent human breast cancer cells in culture.

We tested the hypothesis that treatment of MCF-7 cultures with etidronic acid would perturb cell cycle progression and decrease cell viability. We also tested the possibility that etidronic acid could induce mutations in p53, which is one of the cell cycle checkpoint genes.

METHODS

Cells

MCF-7 human breast cancer cells were cultured and maintained as exponential monolayers in a humidified 5% carbon dioxide air atmosphere in a 37°C incubator. RPMI 1640 medium fortified with 10% fetal bovine serum, glutamine (2 mM), sodium pyruvate (1 mM), 100 U/mL penicillin, and 100 mg/mL streptomycin was used for culturing MCF-7 cells.

Clonogenicity Assay

Cells were seeded at densities of 1500 and 4500 cells per 100 mm diameter tissue culture dish, and the cells were allowed to attach overnight. Control cultures were treated with same volume of medium without drug. After 24-hour exposure to etidronic acid (0, 1.0, and 10.0 mM) with or without

Table 1. PCR primers and PCR conditions

Exon	Forward Primer Sequence	Reverse Primer Sequence	Amplicon Length (bp)	Annealing Temperature (°C)	
				Predicted	Recommended
5	ATC TGT TCA CTT GTG CCC TA	AAC CAG CCC TGT CGT CTC TC	239	63	60
6	AGG GTC CCC AGG CCT CTG AT	CAC CCT TAA CCC CTC CTC CC	197	61	62
7	CCA AGG CGC ACT GGC CTC ATC	CAG AGG CTG GGG CAC AGC AGG	205	62	56
8	TTC CTT ACT GCC TCT TGC TT	TGT CCT GCT TGC TTA CCT CG	194	60	56

strontium chloride (0, 3.5, and 7.0 mM), the medium was removed from each culture dish, and the cells were washed with Dulbecco phosphate buffered saline (PBS), and fresh, drug-free culture medium (15 mL) was added. The cultures were then returned to the incubator for colony formation to progress for 10 days. Any colony containing >50 cells was considered to represent a viable clonogenic cell. The colonies in the different dishes were counted after staining with methylene blue. Survival was calculated relative to a 100% value for untreated controls.

Cell Cycle Analysis

The effect of etidronate treatment with or without strontium chloride on cell cycle was analyzed by using a flow cytometry assay. The cells were trypsinzed and washed twice with PBS after treatment. The suspended cells were fixed overnight with ice-cold 80% ethanol and then centrifuged for five minutes at 1500 rpm. The fixed cells were washed again with PBS two times. The cells were stained at 37°C in the dark with 1 mL propidium iodide (PI) and RNase solution. The cell cycle distribution was analyzed by FACScaliber flow cytometry (Becton Dickson, San Jose, Calif) using ModFit LT software (Verity Software House, Topsham, Maine). Ten thousand cells were analyzed per sample. PI solution contained 50 μg/mL RNase and 50 μg/mL PI in PBS.

DNA Extraction and PCR Amplification of p53 Gene

After treating MCF-7 cell cultures with etidronic acid, the mutagenic effect of etidronic acid on p53 was studied by using denaturing high pressure liquid chromatography (DHPLC) analysis to detect changes (if any) on the highly conserved exon 5 to exon 8 regions of the p53 gene.

Exons 5 through 8 constitute the highly conserved region of the human p53 genome. Mutations are recognizable when heteroduplex DHPLC profiles are obtained. First, the genomic DNA from control and treated cultures were extracted according to the QIAmp DNA Mini Kit procedure (Qiagen, Valencia, Calif). The primers listed in Table 1 were used to amplify the highly conserved exons 5 to 8 of the p53 gene, including the intron/exon boundaries by using PCR. The quality and correct size of the PCR products were checked on 2% agarose gel. The temperature for optimal resolution of heteroduplex and homoduplex DNA detection was determined by using the predicted melting temperature from the DHPLC wave analysis system and the temperature recommended by Quintanilla-Martinez et al.[20]

The PCR products were denatured for 5 minutes at 95°C and cooled to 65°C within three minutes. Ten to 15 μL of PCR product was applied to a

Table 2. Cytotoxicity and cell cycle effects in MCF-7 cells treated with etidronic acid alone, strontium chloride alone, and etidronic acid in combination with strontium chloride*

Treatment	G_0/G_1	S	G_2/M	Cytotoxicity (%) ± SD
Control	68.3	25.3	6.4	100±11.9†
Sr 3.5 mM only	70.1	23.7	6.1	101±13.9
Sr 7.0 mM only	70.7	21.8	7.6	101±2.8
Eti 1.0 mM only	69.5	22.6	7.9	88±9.0
Sr 3.5 mM + Eti 1.0 mM	72.1	21.4	6.5	84±7.7
Sr 7.0 mM + Eti 1.0 mM	70.8	21.3	7.9	89±8.1
Eti 10.0 mM only	63.8	16.9	19.3	52±3.4†
Sr 3.5 mM + Eti 10.0 mM	59.9	17.4	22.7	52±7.8†
Sr 7.0 mM + Eti 10.0 mM	67.1	14.7	18.2	45±6.1†

SD = standard deviation, Sr = strontium chloride, Eti = etidronic acid.

* Cell cycle parameters (percentage of cells in G_0/G_1, S, and G_2/M phase) are from one representative series of flow cytometry experiments. Duplicate flow cytometry experiments gave similar results. Cytotoxicity was determined on the basis of clonogenicity assays. Cytotoxicity data are presented as mean ± SD from four separate experiments.

† $P<.001$ (Student *t* test).

preheated reverse-phase column (DNA-Sep Transgenomic, Omaha, Neb). Mutational screening was performed according to the method described by Giardano et al[21] on an automated DHPLC analysis system (WAVE Transgenomic, Omaha, Neb). The overall sensitivity of this method for the detection of p53 mutations in exons 5 to 8 is reported to be in the 95% range.

The forward and reverse primers listed in Table 1 correspond to the reported sequences for the highly conserved region (exons 5 to 8) of the human p53 gene as shown below

Exon 5 of the human p53 gene:
ATCTGTTCACTTGTGCCCTACTCCCC-TGCCCTCAACAAGATGTTTTGC-CAACTGGCCAAGACCTGCCCTG-TGCAGCTGTGGGTTGATTCCA-CACCCCCGCCCGGCACCCGCGTC-CGCGCCATGGCCATCTACAAG-CAGTCACAGCACATGACG-GAGGTTGTGAGGCGCTGCCCC-CACCATGAGCGCTGCTCAGA-TAGCGATG**GTGAGCAGCTGGGG-CTGGAGAGACGACAGGGCTG-GTT**

Exon 6 of the human p53 gene:
AGGGTCCCCAGGCCTCT-GATTCCTCACTGATTGCTCT-TAGGTCTGGCCCCTCCTCAG-CATCTTATCCGAGTGGAAG-GAAATTTGCGTGTGGAG-TATTTGGATGACAGAAA-CACTTTTCGACATAGTGTGGTG-GTGCCCTATGAGCCGCCT-GAG**GTCTGGTTTGCAACTGGG-GTCTCTGGGAGGAGGGGT-TAAGGGTG**

Exon 7 of the human p53 gene:
CCAAGGCGCACTGGCCT-CATCTTGGGCCTGTGT-TATCTCCTAGGTTGGCTCT-GACTGTACCACCATCCACTA-CAACTACATGTGTAA-CAGTTCCTGCATGGGCGGCAT-GAACCGGAGGCCCATCCTCAC-CATCATCACACTGGAAGACTC-CAG**GTCAGGAGCCACTTGC-CACCCTGCACACTGGCCTGCTG-TGCCCCAGCCTCTG**

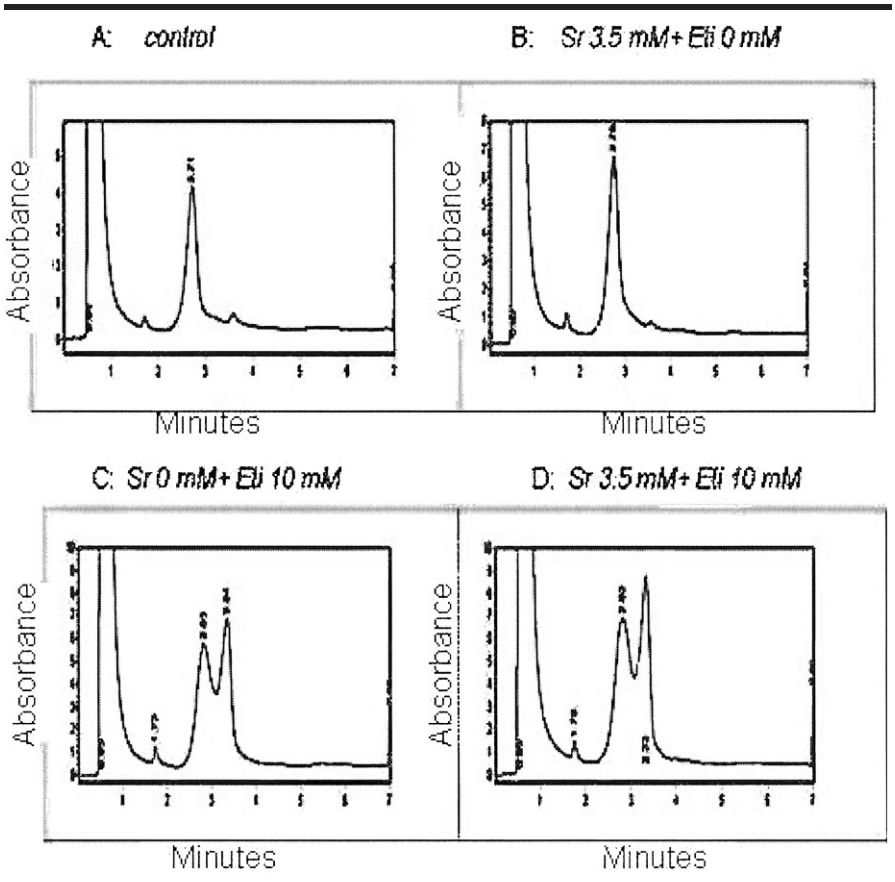

Fig 1. Denaturing high-pressure liquid chromatography profiles of exon 6 of p53 from untreated control (A), treated with strontium chloride (3.5 mM) (B), treated with etidronic acid (10 mM) (C), or treated with etidronic acid (10 mM) in combination with strontium chloride (3.5 mM) (D). The chromatograms show the UV absorbance due to DNA (vertical axis) as a function of elution time (horizontal axis).

Exon 8 of the human p53 gene:
TTCCTTACTGCCTCTTGCTTCT-CTTTTCCTATCCTGAGTAGTGG-TAACTACTGGGACGGAA-CAGCTTTGAGGTGCGTGTTTGT-GCCTGTCCTGGGAGA-GACCGGCGCACAGAGGAAGA-GAATCTCCGCAAGAAAGGG-GAGCCTCACCACGAGCTGCCCC-CAGGGAGCACTAAGCGAG**G-TAAGCAAGCAGGACA**

RESULTS

Cytotoxicity

Clonogenicity assays revealed that a 24-hour exposure to etidronic acid (10.0 mM) was toxic to MCF-7 cells, while the addition of strontium chloride (3.5 mM and 7.0 mM) had no effect. A 24-hour treatment with etidronic acid (1.0 mM) caused a statistically insignificant decrease in clonogenicity, whereas exposure to etidronic acid (10.0 mM) with or without strontium chloride caused ≈50% decrease in clonogenicity of MCF-7 cells (Table 2). In addition to its cytotoxicity, etidronic acid caused significant changes in the cell cycle distribution.

Cell Cycle

Flow cytometry studies revealed that etidronic acid caused a decrease in the S-phase population with a concomitant increase in G_2/M-phase population

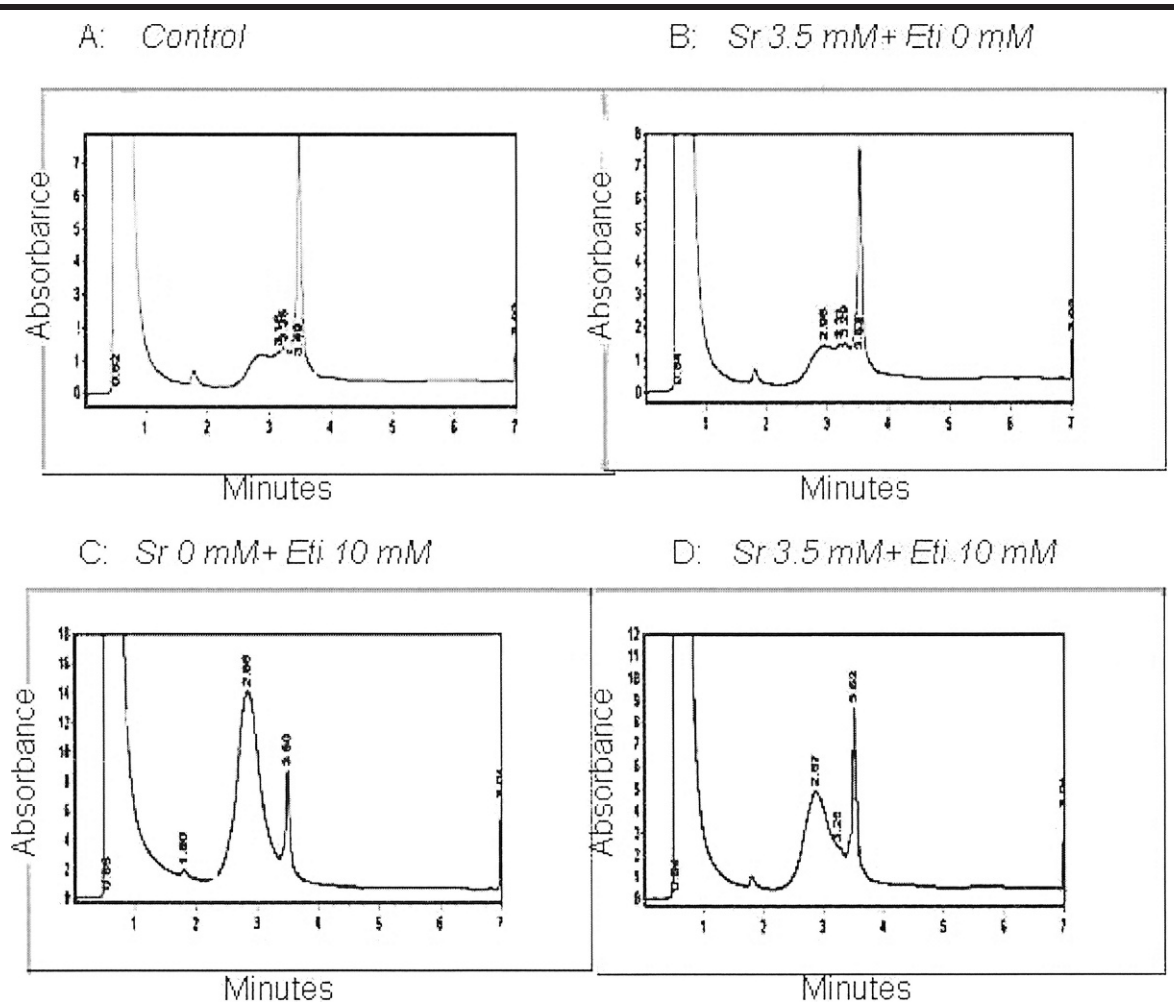

Fig 2. Denaturing high-pressure liquid chromatography profiles of exon 8 of p53 from untreated control (A), treated with strontium chloride (3.5 mM) (B), treated with etidronic acid (10 mM) (C) or treated with etidronic acid (10 mM) in combination with strontium chloride (3.5 mM) (D). The chromatograms show the UV absorbance due to DNA (vertical axis) as a function of elution time (horizontal axis).

(Table 2). Again, strontium had no effect on cell cycle distribution. A 24-hour exposure of MCF-7 cultures to etidronic acid (10 mM) with or without strontium caused ≈30% decrease in the S-phase population, while the proportion of cells in the G_2/M border increased more than threefold.

Mutagenicity of Etidronic Acid

Treatment of MCF-7 human breast cancer cells with etidronic acid (10 mM) for six hours caused mutations in exons 6 and 8 of the p53 gene in MCF-7 cells. The exons 5 through 8 constitute the highly conserved region of the human p53 genome. Mutations are recognizable in the DHPLC profiles (Figure 1 and 2). There were no appreciable changes in the DHPLC profiles of exons 7 and 8 of control and treated cells (DHPLC profiles not shown).

DISCUSSION

Nitrogen-containing bisphosphonates such as zoledronic acid, pamidronate, and risedronate are more potent inhibitors of bone resorption than are compounds that lack nitrogen, such as clodronate and etidronate.[5] Bisphosphonates are extensively used to treat bone metastases and osteoporosis. Prolonged use of bisphosphonates may cause osteonecrosis of the jaw, and other adverse effects of bisphosphonates include gastrointestinal disturbance, fever, myalgia, and flu-like syndrome.[5,22] The molecular mechanisms associated with these adverse effects involve release of the cytokines tumor necrosis factor α, interleukin-6 and the inhibition of farnesyl pyrophosphate synthase.[22,23] Recently there have been reports of direct cytotoxicity of bisphosphonates in cancer cells.[9] The ability of zoledronic acid to potentiate the cytotoxic effects of paclitaxel and doxorubicin have been documented in recent literature.[11,13] This

may suggest an added dimension to the clinical usefulness of bisphosphonates. Our results clearly demonstrate the cytotoxicity of etidronic acid towards human breast cancer cells in culture. In addition, this less potent bisphosphonate causes a G_2/M block in the cell cycle progression with a modest decrease in the S-phase population. Within the cell cycle, late S-phase cells are the most radioresistant, while G_2/M cells are the most radiosensitive.[24] Therefore the decrease in the S-phase population with a corresponding increase in G_2/M would position the cells in a relatively more radiosensitive setting. Such a shift in cell cycle distribution may be useful if etidronic acid were combined with radioactive strontium (^{89}Sr), which is a beta emitter used in the treatment of bone metastases from breast cancer.

Many studies have shown an association between p53 alterations/mutations and clinical outcome in breast cancer.[25] The overall frequency of p53 mutation in breast cancer is ≈20%. This demonstrates the direct cytotoxic effect of etidronic acid on MCF-7 human breast cancer cells. Etidronate may have some radiosensitizing properties because of its effects on the cell cycle progression of MCF-7 cells. The antiosteoporotic element strontium does not alter the cytotoxicity of etidronic acid to MCF-7 cells. The exons 5 through 8 constitute the highly conserved region of the human p53 genome. DHPLC analysis of the conserved region of p53 showed clear alterations in exons 6 and 8 with 10 mM etidronic acid alone and in combination with strontium chloride (3.5 mM). The results suggest that tumor cells surviving etidronic treatment may harbor p53 mutations. These mutations may protect these cells from apoptosis and render them refractory to therapy.[26,27] The development of treatment resistance due to mutated p53 gene could increase the probability of tumor recurrence in patients receiving etidronic acid or other bisphosphonates. However, a recent report suggests that zoledronic acid kills breast cancer cells by mechanisms independent of p53 status.[14]

CONCLUSIONS

Etidronic acid is cytotoxic to MCF-7 breast cancer cells. The antiosteoporotic element strontium does not alter the cytotoxicity of etidronic acid to MCF-7 cells. Flow cytometry showed inhibition of cell proliferation by etidronic acid. The S-phase population decreased while the G_2/M population increased slightly. Etidronate may have some radiosensitive properties because of its effects on the cell cycle progression of MCF-7 cells. Etidronic acid treatment may induce p53 mutation and select for cells that may be resistant to apoptosis. Mutation of the p53 gene can sometimes lead to drug resistance. Therefore, the induction of p53 mutations by etidronate and other bisphosphonates can select for drug-resistant tumor cells. This possibility is worth investigating in preclinical studies and clinical trials. Racial disparity in the incidence of aggressive breast cancer is well documented. African Americans are at an increased risk of breast cancer-related mortality compared with White Americans. Bisphosphonates are likely to be used to manage bone metastases in women with aggressive breast cancer. There is some concern about the safety of prolonged use of bisphosphonates. Therefore, it is important to assess the safety of bisphosphonates in the management of bone metastases in clinical trials that include African Americans and other ethnic groups.

ACKNOWLEDGMENTS

This research was partially supported by grant 2 G12 RR003048 from the RCMI program, Division of Research Infrastructure, National Center for Research Resources, and grant DAMD17-03-1-0759, and DAMD17-03-1-0123, from US Army Medical Research Materiel Command.

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