Pamidronate Resistance and Associated Low Ras Levels in Breast Cancer Cells: A Role for Combinatorial Therapy

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Abstract. To identify markers sensitive to inhibitors of the farnesylation pathway, we used 3 breast cancer cell lines (SKBR-3, MDA-175, and MDA-231) to evaluate the in vitro effects of pamidronate, an inhibitor of farnesyl diphosphate synthase. In response to pamidronate, there was significant inhibition of cell proliferation in MDA-231 and SKBR-3 cells, compared to MDA-175 cells. This correlated with their respective basal levels of N-ras and H-ras. N-ras and H-ras protein levels were both reduced in MDA-231 cells, and to lesser extent in SKBR-3 cells, following exposure to pamidronate, whereas these markers were not altered in MDA-175 cells. Combinatorial therapy with pamidronate and Gleevec, an inhibitor of several tyrosine kinases; Velcade, a proteasome inhibitor; or rapamycin, an inhibitor of the mammalian target of rapamycin (m-TOR) all showed additive effects in causing proliferative inhibition in MDA-175 cells. In summary, resistance to pamidronate may result from low levels of GTPase-activating proteins, such as N-ras and H-ras, in tumor cells. Combinatorial therapies directed against other signaling pathways, not dependent upon ras, may be required to overcome such resistance. (received 6 May 2004; accepted 24 May 2004)

Keywords: pamidronate, H-ras, N-ras, apoptosis, breast cancer, Gleevec, Velcade

Introduction

Ras is an important signal transducing protein for growth factor activated pathways. Approximately 20-30% of human tumors contain mutated versions of ras proteins. Because ras is mutated so often in human cancers, much effort has been devoted to devising means to control the activity of renegade ras. Normal ras binds GTP and in the GTP-bound state interacts with numerous effectors including the Raf proto-oncogene kinase and phosphatidylinositol-3-kinase (PI3K). Its intrinsic GTPase activity terminates the signal. Ras function requires lipophilic anchorage to the cell membrane by lipid prenylation and this prenylation reaction is mediated by the enzyme farnesyl protein transferase (FT). Preventing ras protein association with the plasma membrane is a strategy to impair ras transformation. Therefore drugs that target FT and interfere with activated ras formation are good candidates for anti-tumor agents [1].

Farnesyl pyrophosphate synthase in the cholesterol synthesis pathway is the specific molecular target of bisphosphonates, analogues of farnesyl pyrophosphate [2]. Inhibition of farnesyl pyrophosphate synthase by bisphosphonates leads to decreased production of isoprenoid intermediates like farnesyl needed for the prenylation of ras via FT [1]. Among 3 forms of ras proteins (H-ras, N-ras, K-ras), K-ras has been found to be resistant to FT inhibitors [3]. The basis for the resistance may be related to high affinity of K-ras to FT and its capacity to be prenylated by the related enzyme geranylgeranyl transferase (GGT), in the presence of FT. Several bisphosphonates have been approved by the Food and Drug Administration (FDA) to treat malignant lytic lesions (multiple myeloma or
metastatic cancer) in bone [4,5]. But a cellular marker in malignant tumors to predict the effects of bisphosphonates on tumor cells per se has not been defined.

We selected 3 breast cancer cell lines that are negative for hormonal receptors in order to study the cell-growth inhibitory effects of pamidronate and to determine whether the inhibitory effects were correlated with proteins levels of several makers such as N-ras, H-ras, or FTα/GGTα. We found that cancer cells with less expression of N-ras and H-ras had high resistance to pamidronate treatment. This resistance can be overcome by the additive effects of combinatorial drugs, directed at different cellular pathways.

Materials and Methods

Materials. Pamidronate (BC Cancer Agency, Vancouver, BC, Canada) and Velcade (Millenium Pharmaceuticals, Inc, Cambridge, MA) were obtained from the pharmacy of Geisinger Medical Center. Gleevec was a gift from Novartis Pharma AG (Basel, Switzerland). Ramamycin was purchased from Calbiochem (San Diego, CA). N-ras, FTα/GGTα, and actin antibodies were purchased from Santa Cruz Technology, Inc. (Santa Cruz, CA). H-ras (p21ras) was purchased from DAKO Cytomation (Carpinteria, CA). p-ERK1/2 and cleaved caspase-3 antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA).

Cells and cell culture. SKBR-3, MDA-175, and MDA-231 breast cancer cell lines (American Type Culture Collection, Manassas, VA) were incubated in Dulbecco’s Modified Eagle’s Medium (DMEM, Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (Gibco/Invitrogen, Grand Island, NY) at 37°C in a humidified atmosphere of 5% CO₂ in air.

Cell proliferation assay. The 3 lines of breast cancer cells were grown in 96-well plates, and treated with pamidronate at specified concentrations for 4 days. Viable cells in each respective well were determined colorimetrically (CellTiter 96 one solution proliferation assay, Promega, Madison, WI). The cells were washed once with Hank’s Balanced Salt Solution (HBSS, Gibco/Invitrogen), and then with 100 µl of HBSS. One Step Assay Solution (20 µl, Promega) containing a tetrazolium compound was added to each well. The tetrazolium compound was bioreduced by viable cells to a colored formazan product. After a 30-min incubation, absorbance measurements at 490 nm were made using a plate reader. The proliferation rates of the control cells and inhibitor-treated cells were compared.

Separation of particulate and soluble proteins. To determine if pamidronate diminished the ras activity by dislocating ras from cell membranes into the cytoplasmic compartment, cells with or without pamidronate treatments were processed to separate particulate (membrane) and soluble (cytosolic) proteins, as described previously [3]. In brief, cells were suspended in ice-cold hypotonic buffer and sonicated for 5 sec. The cell debris was pelleted at 1500 x g for 4 min at 4°C and discarded. The supernatant was transferred to Beckman polyallomer tubes and spun at 46,000 rpm (100,000 x g) for 45 min. The pellet was washed once with ice-cold hypotonic buffer with protease inhibitors, re-pelleted at 46,000 rpm for 15 min, and resuspended in 200 µl of ice-cold hypotonic buffer with protease inhibitors to prepare the particulate sample. Using 10K NMWL concentrators (Millipore, Bedford, MA), the supernatant was concentrated to prepare the soluble sample. The resulting particulate and soluble samples were analyzed by Western blotting.

Western blots. Control and pamidronate-treated breast cancer cells were harvested and sonicated. Cell homogenates (30 µg total protein per lane) were electrophoresed on 6-12% SDS PAGE. Fractionated proteins were transferred onto PVDF membranes. For immunostaining of N-ras, monoclonal mouse anti-N-ras antibody (1:500 dilution) was used. The second antibody was horseradish peroxidase-linked anti-mouse whole antibody (from donkey; 1:1,000 dilution). Immunoreactive proteins were visualized by an enhanced chemiluminescence-Western blotting system (Amersham Biotech, Piscataway, NJ). Western blots for other antibodies were performed as described above, using primary and
secondary antibodies as recommended by the respective producers.

**Statistics**  In vitro inhibitory rates were expressed as mean ± SE. One-way ANOVA was used to compare the inhibitory rates among the 3 types of breast cancer cells; p <0.05 was considered statistically significant.

**Results**

**Ras protein levels are correlated with the inhibitory effects of pamidronate.** In Fig. 1, N-ras in the particular part (membranous protein) of MDA-231 and SKBR-3 cells showed a reduction in protein expression after pamidronate treatment. In response to pamidronate, dislocation from the membranous to the cytoplasmic compartment was observed only in N-ras of MDA-231 cells, since soluble protein showed a mild increase in N-ras expression post-treatment in MDA-231. Based on densitometric analysis of the corresponding band, the ratio of particulate N-ras to soluble N-ras was reduced from 8.5 to 1.7 in MDA-231 cells after pamidronate treatment. SKBR-3 and MDA-175 cells revealed minimal ratio changes of N-ras before and after the 48-hr pamidronate challenge. In contrast, H-ras remained largely in the particulate component in all 3 types of cells. Membranous FTα/GGTα (particulate component) was slightly reduced after pamidronate treatment in the MDA-231 cells. Otherwise, soluble FTα/GGTα remained stable after pamidronate treatment in all 3 types of cells.

Whole cell lysates showed basal protein expressions of N-ras and H-ras to be high in MDA-231 cells, moderate in SKBR-3 cells, and low in MDA-175 cells (Fig. 2). Subjected to pamidronate treatment, the whole N-ras protein levels were not altered in any of the 3 cell lines. The whole H-ras level was prominently reduced in the MDA-231 cells after pamidronate treatment, but was essentially unchanged in the SKBK-3 and MDA-175 cells. Basal levels of FTα/GGTα were slightly higher in SKBK-3 cells, when compared to either MDA-175 cells or MDA-231 cells. No alterations in the whole FTα/GGTα expression was seen in any of 3 cell types following pamidronate treatment.

![Fig. 1. Particulate (P) and soluble (S) protein expression of N-ras, H-ras, and FTα/GGTα in SKBR-3, MDA-175, and MDA-231 cells before and after 24 or 48 hr incubation with 90 µM pamidronate.](image)
The proliferation assay showed markedly enhanced inhibitory rates in the pamidronate-treated MDA-231 cells in a dose-dependent manner (Table 1). This dose-dependent pattern was also seen in pamidronate-treated SKBR-3 cells. However, the inhibitory rates in the pamidronate-treated MDA-175 cells were significantly lower than in the corresponding MDA-231 or SKBR-3 cells. As shown in Figs. 1 and 2 and Table 1, there was general correlation between the basal ras expression and the diminution of inhibitory rate following pamidronate treatment.

Pamidronate inhibits cell proliferation by blocking the Erk pathway and promoting apoptosis. To illustrate the alteration of downstream signals along the ras pathway responsible for the inhibitory effects of pamidronate, Western blotting was performed using a series of antibodies in the 3 types of cells incubated with pamidronate for 1 and 2 days. The p-Erk expression was markedly reduced at 48 hr in both SKBR-3 and MDA-231 cells, but not in MDA-175 cells (Fig. 2). The expression of p-JNK was also reduced after 24 hr incubation with pamidronate in MDA-231 and SKBR-3 cells, but not in MDA-175 cells.

Fig. 2. Protein expression of N-ras, H-ras, FTα/GGTα, p-Erk, p-JNK, cleaved (C) caspase-3, and actin in SKBR-3, MDA-175, and MDA-231 cells before and after 24 or 48 hr incubation with 90 µM pamidronate.
not in MDA-175 cells. This reduced expression of p-JNK was seen following 48 hr of incubation with pamidronate in all 3 cell lines. To examine the effects of pamidronate on the apoptotic pathway, cleaved caspase-3 antibody was used to detect this protein expression. Subjected to pamidronate treatment, there was a sequential increase in cleaved caspase-3 from the MDA-175 cells to the SKBR-3 cells, and finally to the MDA-231 cells, indicating a graduated enhancement in apoptotic activity in the 3 cell lines. Actin protein was expressed equally in each cell line before and after pamidronate treatment for either 24 or 48 hr.

Additive effects of drugs at varying cellular pathways overcame drug resistance to pamidronate. To demonstrate the “additive” effects of pamidronate with Gleevec, an inhibitor of farnesylation-independent platelet-derived growth factor receptor and c-kit signaling, pamidronate at 30 µM and Gleevec at 20 µM were both added to MDA-175 cells to result in 75.2 ± 1.9% inhibition; pamidronate alone caused 39.5 ± 2.2% inhibition, whereas Gleevec alone caused 50.2 ± 3.7% inhibition in MDA-175 cells (p <0.05 vs combinatorial effects) (Fig. 3).

To test whether the Akt pathway (associated with the mammalian target of rapamycin [mTOR] and nuclear factor-kappaB [NF-κB]) may be an alternative farnesylation-independent growth pathway associated with cell proliferation in MDA-175 cells, we combined rapamycin at 1.0 µM (an inhibitor of mTOR) and Velcade at 30 nM (an inhibitor of proteasome and in turn NF-κB). These additive effects resulted in 76.9 ± 0.9 % inhibition, which was significantly more than the solo effect of rapamycin (28.1 ± 4.3% inhibition) or Velcade (64.3 ± 1.3% inhibition) (Fig. 3).

Table 1. Inhibitory effects of pamidronate in 3 cell lines

<table>
<thead>
<tr>
<th>Pamidronate exposure</th>
<th>Inhibitory rates in 3 cell lines (%)</th>
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<tbody>
<tr>
<td>SKBR-3</td>
<td>MDA-175</td>
</tr>
<tr>
<td>10 µM, 4 days</td>
<td>7.0±1.8</td>
</tr>
<tr>
<td>30 µM, 4 days</td>
<td>47.4±1.1</td>
</tr>
<tr>
<td>90 µM, 4 days</td>
<td>74.1±5.0</td>
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*a p <0.05 vs SKBR-3 cells; b p <0.05 vs MDA-175 cells.
Inhibitory rates = (controls-treated)/controls.

Fig. 3. Upper panel: inhibitory effects of pamidronate at 30 µM, Gleevec at 20 µM, or both in MDA-175 cells. Lower panel: inhibitory effects of rapamycin at 1 µM, Velcade at 30 nM, or both in MDA-175 cells. *p <0.05 vs drug 1; #p <0.05 vs drug 2.
Discussion

We found that in response to pamidronate, the higher the basal level of N-ras and H-ras, the deeper the reduction of related protein levels in the 3 breast cancer cells. The reduction appears correlated with the inhibitory extent of pamidronate. Previous studies showed that there was a reduction in membrane H-ras after several types of treatment (SCH56582, an inhibitor of FT, and zoledronic acid, a bisphosphonate) [3,6,7]. Whyte et al [3] reported diminished N-ras, but not K-ras, following SCH56582 treatment. In this study, we also observed that pamidronate treatment resulted in a decreased level of membranous N-ras expression in MDA-231 cells, which correlated with inhibited proliferation in this breast carcinoma line. In summary, H-ras and N-ras appear to be good markers for predicting the inhibitory effects of pamidronate in the breast cancer cells; a low expression level of the markers may be related to a relatively high resistance to pamidronate treatment.

Using the same method as Whyte et al [3], we could not demonstrate that pamidronate caused dislocation of membranous H-ras into the cytosolic compartment. The membranous expression of FT\(\alpha\)/GGT\(\alpha\) was diminished after pamidronate treatment, while abundant cytosolic FT\(\alpha\)/GGT\(\alpha\) was not altered in MDA-231 cells (Fig. 1). The finding indicates that pamidronate affected the enzyme level FT\(\alpha\)/GGT\(\alpha\), thus diminishing production of farnesylated N-ras and H-ras at the level of the cell membrane. Only a mild increase in cytosolic N-ras was seen in MDA-231 cells after pamidronate treatment, indicating a weak dislocating effect of pamidronate on membrane-bound N-ras. This discrepancy between the current study and the previous study [3] may reflect the difference in cell lines and different types of inhibitors affecting ras.

Activation of p-Erk, a marker for cell proliferation, is triggered by activation of H-ras (p21ras) [8]. Since we observed reduced expression of p-Erk following pamidronate treatment in MDA-231 cells and to a lesser extent in SKBR-3 cells, this effect on p-Erk appeared to be associated with reduction in H-ras and N-ras signaling and partially contributed to the inhibition of cell proliferation. Because the expression of p-JNK, a marker for apoptosis, was also reduced when cells were subjected to pamidronate treatment, it is likely that pamidronate treatment results in inhibition of ras-associated enzymes for the activation of both p-Erk and p-JNK [9]. But p-Akt, a marker of another pathway of ras, was not altered (data not shown) after pamidronate treatment, as was observed using another bisphosphonate, YM529, in a previous study [10].

In this study, caspase-3 was cleaved in MDA-231 cells and to lesser extent in SKBR-3 cells, but not in MDA-175 cells. This finding is consistent with enhanced apoptosis after bisphosphonates, as previously reported [6,7,11-13]. The present study showed diminished p-JNK and unaltered p53 (data not shown) following pamidronate treatment, ruling out activation of apoptosis via these two pathways. Increased cytochrome c release and diminished bcl-2 expression in other studies indicate that apoptosis is, at least in part, associated with the activated apoptotic cascades through mitochondrial factors by bisphosphonates [6,7]. Another study found that bisphosphonates induced apoptosis and caused a simultaneous elevation in glucose-regulated protein 78, a major chaperone protein in the endoplasmic reticulum (ER), indicating the involvement of the ER pathway in the apoptosis [12].

Ras can be activated by stimulating several types of growth factor receptors. Platelet-derived growth factor receptors (PDGFR) are among those. In recent years, Gleevec, an inhibitor of tyrosine kinases for PDGFR and c-kit (CD117) [14], has been approved by the FDA for treating chronic myelogenous leukemia and c-kit-positive gastrointestinal stromal tumor and found to be effective in treating other types of malignancy as well [15]. In the current study, we found an additive effect of pamidronate and Gleevec in pamidronate-resistant MDA-175 cells. The additive effects contributed by Gleevec may be carried out through ras-independent pathways [16], since total N-ras and H-ras are low in MDA-175 cells. Similarly, both the phosphatidylinositol-3-kinase (PI3K)/Akt pathway and NF-\(\kappa\)B activation can involve ras-dependent and independent pathways [17,18]. In this regard, the additive effects of Velcade and rapamycin in MDA-175 cells are not surprising.
In recent years, the proteasome inhibitor, Velcade, has been found to have potent inhibitory effects on tumor growth, at least partially resulting from its inhibitory effects on NF-κB binding activity to DNA for proliferation [19,20]. Rapamycin is a macroclide fungicide that binds intracellularly to the immunophilins FKBP12, and the resultant complex inhibits the activity of a 290-kDa kinase known as mammalian target of rapamycin (mTOR), a downstream signal of Akt. Velcade has been approved by the FDA to treat malignant myeloma and CCI-779, an ester of rapamycin, has been tested in clinical trials for human cancer. Combination of the 2 drugs showed more profound inhibition on cell growth than either drug alone. The additive inhibition most likely represents simultaneous effects on ras-dependent and ras-independent pathways in pamidronate-resistant MDA-175 cells.

In summary, at both 30 and 90 µM of pamidronate, MDA-175 cells showed higher resistance than SKBR-3 and MDA-231 cells, which may be related to the higher levels of N-ras and H-ras in the latter two cell lines compared to MDA-175 cells. N-ras and H-ras may be useful protein markers to predict effects of pamidronate in breast cancer cells. Inhibitory effects of pamidronate on breast cancer cells were, at least partially, mediated by a p-Erk mediated pathway and by promoting apoptosis. In MDA-175 cells, combinatorial use of pamidronate and Gleevec resulted in an additive effect on proliferative inhibition. To test the Akt pathway as an alternative farnesylation-independent growth pathway associated with cell proliferation in MDA-175 cells, combinatorial therapy with rapamycin (an inhibitor of m-TOR) and Velcade (an inhibitor of proteasome and NF-κB) also led to additive inhibition of proliferation in MDA-175 cells. Resistance to pamidronate in breast cancer cells may not be simply determined by the level of FT. Combinatorial therapy with inhibition of other signal pathways, which can be ras-independent, may provide additive effects and overcome the drug resistance.

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