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Dose-dependent differential effects of risedronate on gene expression in osteoblasts

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ABSTRACT

Bisphosphonates have multiple effects on bone. Their actions on osteoclasts lead to inhibition of bone resorption, at least partially through apoptosis. Effects on osteoblasts vary, with modifications in the molecule and concentration both resulting in qualitatively different responses. To understand the mechanism of the differential effects of high and low bisphosphonate concentrations on osteoblast activity, we compared the effects of 10^{-8} M and 10^{-4} M risedronate on gene expression in UMR-106 rat osteoblastic cells. Two targeted arrays, an 84-gene signaling array and an 84-gene osteogeneic array were used. Gene expression was measured at 1 and 24 h. Although some genes were regulated similarly by low and high concentrations of the drug, there was also differential regulation. At 1 h, 11 genes (1 signaling and 10 osteogenesis) were solely regulated by the low concentration, and 7 genes (3 signaling, 4 osteogenesis) were solely regulated by the high concentration. At 24 h, 8 genes (3 signaling, 5 osteogenesis) were solely regulated by the low concentration and 30 genes (16 signaling and 14 osteogenesis) were solely regulated by the high concentration. Interestingly, the low, but not the high concentration of risedronate transiently and selectively upregulated several genes associated with cell differentiation. A number of genes related to apoptosis were regulated, and could be involved in effects of bisphosphonates to promote osteoblast apoptosis. Also, observed gene changes associated with decreased angiogenesis and decreased metastasis could, if they occur in other cell types, provide a basis for the effectiveness of bisphosphonates in the prevention of cancer metastases.

1. Introduction

Bisphosphonates are efficacious agents for the prevention and treatment of osteoporosis, for the antagonism of hypercalcemia, and for therapy of cancer metastases to bone. They are effective inhibitors of bone resorption, inhibiting osteoclastogenesis, osteoclast activity and osteoclast survival [1]. Although not all bisphosphonates affect resorption, those that do have essentially unidirectional effects, leading to the suppression of bone breakdown. In contrast, bisphosphonate effects on osteoblasts are more complex. Some bisphosphonates stimulate osteoblast proliferation, differentiation or survival, whereas others have inhibitory effects. Additionally, dose-dependent biphasic effects on proliferation or apoptosis have been documented for several bisphosphonates. Earlier studies showed that bisphosphonates (10^{-4}–10^{-5} M) decrease hFOB cell (fetal osteoblast cell) proliferation, but enhance differentiation and bone formation activities of the osteoblasts [2]. Bisphosphonates, at 10^{-5}–10^{-6} M can inhibit cell proliferation and induce apoptosis in UMR-106 osteoblastic cells [3]. Contrasting with this, other studies showed that low concentrations (10^{-6}–10^{-9} M) of bisphosphonates prevent apoptosis of osteoblasts [4,5]. Recent studies also indicate that bisphosphonates, including risedronate, over a broad concentration range (10^{-5}–10^{-12} M) enhance proliferation and differentiation of osteoblasts [6,7]. However, at a high concentration of 10^{-4} M, bisphosphonates decrease proliferation of MG-63 osteoblasts [7]. Both high and low bisphosphonate concentrations are potentially therapeutically relevant, since bisphosphonates concentrate in bone. High doses or prolonged treatment with bisphosphonates have now been associated with undesirable effects on bone, such as osteonecrosis of the jaw [8] and subtrochanteric fractures [9], which, although rare, can have devastating consequences. Inhibition of angiogenesis, suppressed bone remodeling, bone cell apoptosis, and collagen and mineralization abnormalities have been implicated in these bone side effects of bisphosphonates [10–14]. The more potent bisphosphonates are effective for inhibiting tumor cell metastases to bone [15,16].

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The range of bone effects seen with bisphosphonates leads to the conclusion that there may be qualitative as well as quantitative differences elicited by different concentrations of bisphosphonates on osteoblasts. We have undertaken to investigate that possibility by comparing the effects on gene expression in rat osteosarcoma-derived UMR-106 osteoblastic cells of two widely different risedronate concentrations, a low concentration that is often used to simulate a therapeutic concentration, and the other representing a concentration that could be acquired by bone when exposed to high doses and which has antiproliferative effects on the osteoblastic cells. We have used a targeted osteogenic array to examine responses of the osteoblastic cells at the two concentrations, and also a targeted signaling array that could reveal pathways that lead to different responses. We have also used two time points, a 24 h time point, at which many responses should have been established, and a 1 h time point to identify dose-related differences that could represent possible initiating events.

2. Materials and methods

2.1. Cell culture

UMR-106 osteoblastic cells were purchased from American Type Culture Collection (Rockville, MD). The cells were cultured to confluence in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 100 U/ml penicillin G at 37 °C in a 5% CO_2 environment.

2.2. MTT assay

Cells were seeded at 18,000 cells per well in 96-well cell culture dishes in 0.2 ml of medium and allowed to attach overnight. They were then treated for 1 or 24 h with 0.2 ml of medium containing 10^{-4} M or 10^{-8} M risedronate (P&G Pharmaceuticals, Cincinnati, OH) or left untreated. At the end of the incubation period, 20 μl of 5 mg/ml MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) (Sigma, St. Louis, MO) in sterile phosphate-buffered saline was added to the medium in each well. After 1 h (for the 1 h treatment) or 4 h (for the 24 h treatment), the medium was removed and 200 μl dimethyl sulfoxide was added to each well and thoroughly triturated by pipetting up and down five times. Absorbance at 570 nm was measured with a Dynatech MR5000 plate reader.

2.3. ^{3}H-thymidine incorporation

For ^{3}H-thymidine incorporation, cells were seeded at 50,000 cells per well in 24-well cell culture dishes in 1 ml of medium and allowed to attach overnight. They were then treated for 1 or 24 h with 1 ml of medium containing 10^{-4} M or 10^{-8} M risedronate, or left untreated. For the final hour of the incubation, cells were labeled with 1 μCi/ml ^{3}H-thymidine (Amersham, Buckinghamshire, England), which was added in 5 μl of medium. They were then washed with 1 ml of medium. Medium was removed, and the plates placed on ice. The cells were incubated for 10 min with 0.5 ml 10% trichloroacetic acid, then washed 3 times with 0.5 ml 10% trichloroacetic acid and solubilized by incubation for 2 h at room temperature with 0.5 ml 0.5 N KOH. 1 N HCl was added to neutralize, and the samples were counted in Ultima Gold scintillation solution (PerkinElmer, Boston) with a Beckman LS 6500 scintillation counter.

2.4. Gene expression profiling using real-time PCR gene array

Cells were seeded at 500,000 cells per well in six-well cell culture dishes in 2 ml of medium and allowed to attach overnight.

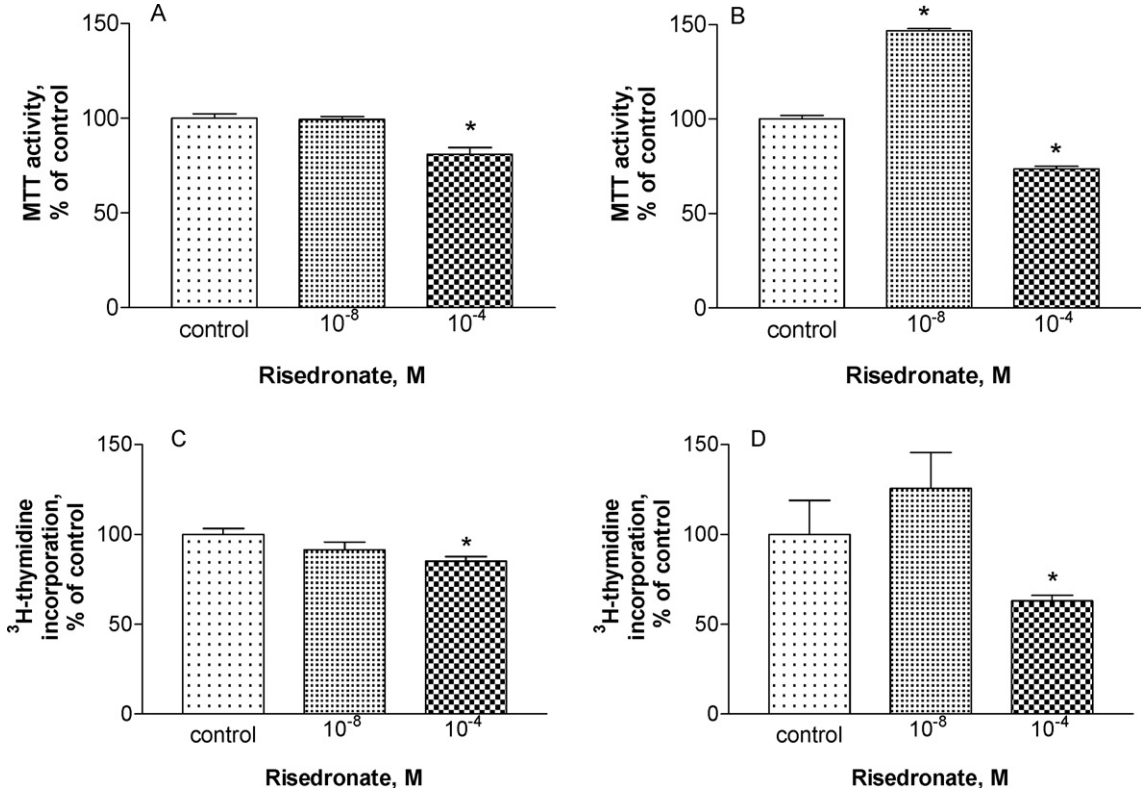

Fig. 1. MTT activity (A and B) and ^{3}H-thymidine incorporation (C and D) elicited by treatment of UMR-106 cells for 1 h (A and C) or 24 h (B and D) with 10^{-8} or 10^{-4} M risedronate. For MTT assays, $N = 6$, for ^{3}H-thymidine incorporation, $N = 3$. Data were analyzed by analysis of variance and significance determined by and Dunnett's test. *$P < 0.05$ vs. untreated control.

Table 1
Genes regulated by both low dose and high dose risedronate with 1 h treatment.

Symbol	Description	10^{-8} M		10^{-4} M	
		Fold	*P* value	Fold	*P* value
Signaling array					
Pparg	Peroxisome proliferator activated receptor gamma	10.1	0.0006	6.1	0.0009
Hk2	Hexokinase 2	10.1	0.003	6.5	0.004
Faslg	Fas ligand (TNF superfamily, member 6)	6.0	0.0001	4.8	0.0002
Cdh1	Cadherin 1	4.4	0.001	2.6	0.003
Odc1	Ornithine decarboxylase 1	3.1	0.002	4.9	0.001
Ptch1	Patched homolog 1 (Drosophila)	2.5	0.004	2.5	0.004
Il4ra	Interleukin 4 receptor, alpha	2.1	0.04	2.7	0.02
Birc1b	Baculoviral IAP repeat-containing 1b	0.4	0.007	0.2	0.002
Osteogenesis array					
Col4a1	Procollagen, type IV, alpha 1	14.8	0.05	56.1	0.02
Comp	Cartilage oligomeric matrix protein	0.07	0.000007	0.1	0.00001
Bmpr1a	Bone morphogenetic protein receptor, type 1A	0.03	0.005	0.03	0.005

Cells were then treated with risedronate at 10^{-4} M or 10^{-8} M for 1 h or 24 h in DMEM containing 20 mM HEPES, 0.1% BSA, and 1% absolute ethanol. The cells were trypsinized and collected for total RNA extraction. Total RNA was extracted from the cells using an RNeasy kit (Qiagen, Maryland). Genomic DNA contamination was eliminated and the first strand cDNA was synthesized from 1 μg total RNA using an RT^2 first strand kit (SABiosciences, Frederick, MD). Gene expression profiles in the cells were studied using signal transduction pathwayFinder PCR arrays and osteogenesis PCR arrays (SABiosciences), which contained 84 key genes representative of bone formation and 18 different signal transduction pathways, as well as 5 different housekeeping genes as loading controls for normalization. The cDNA samples mixed with qPCR master mix (containing SYBR green) were loaded in 96-well PCR arrays and qPCR reactions were performed on a BioRad iQ real-time PCR detection system. After denaturing the template and activating the HotStart DNA polymerase at 95 °C for 10 min, the two-step cycling program was run for 40 cycles at 95 °C for 15 s, 60 °C for 60 s. The PCR array data were analyzed using data analysis web portal and software, performing $\Delta\Delta C_t$ based fold-change calculations from raw threshold cycle data. Pair-wise comparison between test samples and control sample were performed. Genes whose expression was increased by greater than 2-fold or decreased to less than 0.5-fold with a *P* value less than 0.05 were considered to be significantly modified.

3. Results

3.1. Effects of risedronate treatment on MTT activity and ^{3}H-thymidine incorporation

To determine whether the risedronate concentrations selected for the microarray analyses had significantly different responses in assays of cell viability (MTT activity) and DNA synthesis (^{3}H-thymidine incorporation), these parameters were measured. Both parameters were decreased by 10^{-4} M risedronate, with greater effects seen at 24 h than at 1 h (Fig. 1A–D). At 24 h, 10^{-8} M risedronate significantly stimulated MTT activity (Fig. 1B).

3.2. Effects of risedronate on gene responses in gene arrays

Twenty-seven genes from the two arrays were found to be affected by 1 h risedronate treatment. Eleven genes were affected similarly by the two risedronate concentrations. Eight genes from the signaling array and 3 genes from the osteogenesis array were similarly regulated by both the low dose and high dose of risedronate with 1 h treatment. These genes were Pparg, Hk2, Faslg, Cdh1, Odc1, Ptch1, Il4ra and Col4a1, which were upregulated, and Birc1b, Comp and Bmpr1a, which were downregulated (Table 1). Of the similarly regulated genes, Col4a1 was increased more markedly by the high risedronate concentration. In contrast to the genes that were similarly regulated, 16 genes were differentially regulated by the low or high risedronate concentration. Nine genes from the osteogenesis array were modified solely by the lower concentration of risedronate, these being Vdr, Fgf2, Alpl, Ambn, Enam, Col2a1 and NfkB1, which were upregulated, and Bmp7 and Flt1, which were downregulated (Table 2A). Two genes from the signaling array and 3 genes from the osteogenesis array were modified solely by the higher concentration of risendronate, these genes being Vegfa, which was upregulated, and Bmp2, Mmp2, Smad3 and Fgfr1, which were downregulated (Table 2B). Interestingly, 2 genes, one from the signaling array and one from the osteogenesis array were conversely regulated by the two risedronate concentrations. Specifically, Tank was downregulated by 10^{-8} M risedronate but upregulated by 10^{-4} M risedronate. Mmp10 was upregulated by 10^{-8} M risedronate but downregulated by 10^{-4} M risedronate.

Fifty genes were affected by 24 h treatment with risedronate. After 24 h treatment, 14 genes, 6 from the signaling array and 8 from the osteogenesis array were similarly regulated by the low and high concentrations of risedronate. These were Hk2, Fos, Fas, Cebpb, Pparg, Mmp8 and Mmp10, which were upregulated, and Birc1b, Col7a1, Col6a1, Tfip11, Comp, Flt1 and Bmpr1a, which were downregulated (Table 3). As at 1 h, 36 other genes were differentially regulated by the low or high risedronate concentration. Two genes from the signaling array and 4 genes from the osteogenesis array were modified solely by the low concentration of risendronate. Selp, Odc1 and Bmp7 were upregulated solely by the low concentration of risedronate, and Smad2, Cdh11 and Enam were downregulated solely by the low risedronate concentration (Table 4A). The largest number of genes responding to risedronate treatment was those that was affected solely by 24 h treatment with the high concentration of risedronate. Fifteen genes from the signaling array and 13 genes from the osteogenesis array were modified solely by the high concentration of risendronate (Table 4B). At 24 h, Egr1, Faslg, Ptgs2, Fasn, Cdkn1a, Prkce, Brca1, Jun, Vegfa, Hoxa1, Cdk2, Bcl2l1, Hspb1, Tcf7, Gadd45a, Col4a1, Bmp2, Icam1, Dmp1, Scarb1, Tgfb3, Ctsk, Twist1, Smad3, Bmp5 and Col14a1 were upregulated solely by the high risedronate concentration, whereas Sox9 and Bmpr1b were downregulated solely by the high risedronate concentration. Again, two genes were conversely regulated by the high and low concentrations of risedronate. Specifically, Cdh1 was upregulated by 10^{-8} M risedronate but downregulated by 10^{-4} M risedronate. Mmp2 was downregulated by 10^{-8} M risedronate but upregulated by 10^{-4} M risedronate.

Table 2
Genes specifically or differentially regulated by (A) low dose or (B) high dose risedronate with 1 h treatment.

Symbol	Description	Fold	*P* value
A			
Signaling array			
Tank	TRAF family member-associated Nf-kappa B activator	0.4	0.02
Osteogenesis array			
Vdr	Vitamin D receptor	57.3	0.02
Fgf2	Fibroblast growth factor 2	3.7	0.003
Alpl	Alkaline phosphatase, tissue-nonspecific	3.7	0.04
Ambn	Ameloblastin	3.6	0.04
Enam	Enamelin	3.2	0.05
Mmp10	Matrix metallopeptidase 10	2.6	0.02
Col2a1	Procollagen, type II, alpha 1	2.1	0.04
Nfkb1	Nuclear factor of kappa light chain gene enhancer in B-cells 1, p105	2.1	0.03
Bmp7	Bone morphogenetic protein 7	0.3	0.04
Flt1	FMS-like tyrosine kinase 1, VEGFR1	0.2	0.003
B			
Signaling array			
Tank	TRAF family member-associated Nf-kappa B activator	64.0	0.001
Vegfa	Vascular endothelial growth factor A	3.4	0.03
Bmp2	Bone morphogenetic protein 2	0.2	0.04
Osteogenesis array			
Smad3	MAD homolog 3 (Drosophila)	0.5	0.007
Mmp10	Matrix metallopeptidase 10	0.5	0.04
Fgfr1	Fibroblast growth factor receptor 1	0.4	0.02
Mmp2	Matrix metallopeptidase 2	0.1	0.01

Table 3
Genes regulated by both low dose and high dose risedronate with 24 h treatment.

Symbol	Description	10^{-8} M		10^{-4} M	
		Fold	*P* value	Fold	*P* value
Signaling array					
Hk2	Hexokinase 2	10.1	0.003	2.3	0.02
Fos	FBJ murine osteosarcoma viral oncogene homolog	10.1	0.003	12.3	0.002
Fas	Fas (TNF receptor superfamily, member 6)	2.9	0.003	2.5	0.004
Cebpb	CCAAT/enhancer binding protein (C/EBP), beta	2.4	0.0003	3.1	0.0002
Pparg	Peroxisome proliferator activated receptor gamma	2.1	0.006	5.4	0.001
Birc1b	Baculoviral IAP repeat-containing 1b	0.4	0.009	0.2	0.003
Osteogenesis array					
Mmp8	Matrix metallopeptidase 8	5.4	0.02	6.5	0.02
Mmp10	Matrix metallopeptidase 10	2.9	0.02	5.2	0.008
Col7a1	Procollagen, type VII, alpha 1 (predicted)	0.4	0.00007	0.3	0.00003
Col6a1	Procollagen, type VI, alpha 1 (predicted)	0.4	0.0008	0.4	0.0009
Tfip11	Tuftelin interacting protein 11	0.3	0.0005	0.5	0.001
Comp	Cartilage oligomeric matrix protein	0.2	0.00002	0.1	0.00001
Flt1	FMS-like tyrosine kinase 1, VEGFR1	0.08	0.001	0.09	0.002
Bmpr1a	Bone morphogenetic protein receptor, type 1A	0.04	0.006	0.05	0.006

Five genes were affected similarly, and to a relatively similar extent, by both risedronate concentrations at both time points. These were Hk2 and Pparg, which were upregulated, and Birc1b, Bmpr1a, and Comp, which were downregulated.

4. Discussion

The current studies indicate that treatment with a bisphosphonate can affect factors involved in both signaling and osteogenesis in UMR-106 rat osteosarcoma-derived osteoblastic cells. They also provide a basis for the observations that the bisphosphonates have not only quantitative, but also qualitative differences in their dose-dependent effects on bone. This was even apparent in the measurement of metabolic activity in the MTT assay, in which the lower concentration of risedronate increased MTT activity at 24 h, whereas it was decreased by the high concentration at both 1 and 24 h.

Effects on gene expression were consistent with the possibility that lower concentrations of bisphosphonates could be beneficial to bone formation. At 1 h, a number of genes associated with growth and differentiation were increased in the osteoblastic cells by the lower concentration of risedronate. Of the genes whose expression was increased by the low concentration of risedronate at 1 h, the Vdr gene, encoding the vitamin D receptor, which is a transcriptional regulator of osteocalcin and an activator of alkaline phosphatase [17], had the largest magnitude response, a 57.3-fold increase. A number of genes associated with differentiation of mineralized tissues were increased 2.1–3.7-fold by the low concentration of risedronate at 1 h including fibroblast growth factor 2 (Fgf2), alkaline phosphatase (Alp1), collagen IIa1 (Col2a1), ameloblastin (Ambn), and enamelin (Enam). Fibroblast growth factor 2 is an activator of MAP kinase and a regulator of osteogenesis [18]. Alkaline phosphatase is an established marker of early osteoblast differentiation [19]. Collagen IIa1 encodes the alpha 1 chain of type II collagen a major component of the cartilage

Table 4
Genes specifically or differentially regulated by (A) low dose or (B) high dose risedronate with 24 h treatment.

Symbol	Description	Fold	*P* value
A			
Signaling array			
Selp	Selectin, platelet	4.7	0.003
Cdh1	Cadherin 1	3.1	0.002
Odc1	Ornithine decarboxylase 1	2.1	0.006
Osteogenesis array			
Bmp7	Bone morphogenetic protein 7	4.5	0.02
Smad2	MAD homolog 2 (Drosophila)	0.5	0.04
Mmp2	Matrix metallopeptidase 2	0.2	0.02
Cdh11	Cadherin 11	0.2	0.02
Enam	Enamelin	0.1	0.02
B			
Signaling array			
Egr1	Early growth response 1	27.3	0.01
Faslg	Fas ligand (TNF superfamily, member 6)	9.0	0.00009
Ptgs2	Prostaglandin-endoperoxide synthase 2	5.0	0.003
Fasn	Fatty acid synthase	4.1	0.02
Cdkn1a	Cyclin-dependent kinase inhibitor 1A	4.1	0.03
Prkce	Protein kinase C, epsilon	3.9	0.04
Brca1	Breast cancer 1	3.9	0.01
Jun	Jun oncogene	3.5	0.02
Vegfa	Vascular endothelial growth factor A	3.0	0.04
Hoxa1	Homeo box A1	2.8	0.05
Cdk2	Cyclin dependent kinase 2	2.8	0.05
Bcl2l1	Bcl2-like 1	2.7	0.04
Hspb1	Heat shock 27kDa protein 1	2.7	0.02
Tcf7	Transcription factor 7, T-cell specific (predicted)	2.3	0.05
Gadd45a	Growth arrest and DNA-damage-inducible 45 alpha	2.3	0.01
Cdh1	Cadherin 1	0.4	0.003
Osteogenesis array			
Mmp2	Matrix metallopeptidase 2	22.5	0.005
Col4a1	Procollagen, type IV, alpha 1	15.9	0.05
Bmp2	Bone morphogenetic protein 2	6.9	0.03
Icam1	Intercellular adhesion molecule 1	5.6	0.02
Dmp1	Dentin matrix protein 1	4.1	0.04
Scarb1	Scavenger receptor class B, member 1	3.7	0.02
Tgfb3	Transforming growth factor, beta 3	3.2	0.05
Ctsk	Cathepsin K	2.9	0.05
Twist1	Twist gene homolog 1 (Drosophila)	2.8	0.04
Smad3	MAD homolog 3 (Drosophila)	2.6	0.004
Bmp5	Bone morphogenetic protein 5 (predicted)	2.6	0.05
Col14a1	Procollagen, type XIV, alpha 1 (predicted)	2.1	0.02
Sox9	SRY-box containing gene 9	0.3	0.05
Bmpr1b	Bone morphogenetic protein receptor, type 1B	0.2	0.02

matrix [20] Although ameloblastin has been mainly associated with dentin differentiation, recent studies reveal that it can promote osteogenic differentiation [21]. Enamelin effects on mineralized tissue have focused on its actions on enamel matrix organization and mineralization [22]. None of these genes was increased by 1 h treatment with the high concentration of risedronate, and Enam was decreased at 24 h by the low concentration of risedronate. The expression of Fgfr1, encoding a member of the fibroblast growth factor receptor family, and the gene for the bone growth factor Bmp2 were unaffected by 1 h treatment with the low concentration of risedronate, but was decreased by 1 h treatment with the high concentration of the drug. Ptch1, which encodes the receptor for sonic hedgehog, a factor crucial for osteogenic differentiation [23], was increased by both high and low risedronate, suggesting that even at higher concentrations, the bisphosphonate has effects that promote bone formation. The effects of this factor are complex, however, as activation of hedgehog signaling in marrow led to loss and osteoblast precursors [24] and Patched 1 haploinsufficiency increased adult bone mass [25]. It is also possible that different anabolic factors promoting osteoblast differentiation are elicited by higher concentrations of risedronate. The gene for the growth factor, vascular endothelial growth factor α (Vegfa), was increased by the high concentration of risedronate only. Genes encoding the osteogenic factors bone morphogenic protein 2 (Bmp2) [26], bone morphogenic protein 5 (Bmp5) [27] and dentin morphogenic protein 1 (Dmp1) [28], transforming growth factor beta 3 (Tgfb3) [29,30] and the wnt pathway effector Tcf7 [31] required 24 h treatment with the high risedronate concentration to elicit increases in their expression. Egr1, which has mixed effects on collagen gene expression [32–35] was increased by the high risedronate concentration at 24 h.

Several genes encoding cytokine factors were affected by 1 h risedronate treatment in the osteoblastic cells. TANK, a negative regulator of tumor necrosis factor signaling [36], was downregulated by the low concentration of risedronate but upregulated by the high concentration, which could contribute to the antiresorptive effect of the risedronate. IL4Ra, which encodes the alpha chain of the interleukin-4 receptor, was upregulated by both the high and low concentrations of the drug. IL-4 inhibits osteoblast proliferation, and stimulates IL-6 and alkaline phosphatase activity in osteoblasts [37]. IL-4 also supppressed Cox-2 dependent prostaglandin synthesis in osteoblasts, which was proposed to play a role in inhibition of bone resorption by IL-4 [38].

Cell-cell interaction is likely to be important in bone responses to treatments. Thus, it is interesting that that genes encoding a protein involved in cell attachment, cadherin 1 (Cdh1) [39], was differentially affected by high and low concentrations of risedronate. Cdh1 was upregulated at both 1 and 24 h by the low concentration of risedronate, and was downregulated at 24 h by the high concentration. However, another adhesion molecule, Icam, was upregulated by the high concentration of risedronate at 24 h.

Other responses observed in the current studies identify potential mechanisms for established effects of bisphosphonates in osteoblasts and suggest genes that could mediate the effects of bisphosphonates in other cell types. Bisphosphonates can cause apoptosis of both osteoclasts [40] and osteoblasts [3], effects that may be involved in the deleterious effects of the bisphosphonates on bone. A number of genes involved in the apoptotic process were affected by the risedronate treatments. Perhaps most interesting was that the apoptosis inhibitor Birc1b [41] was decreased by both risedronate concentrations at both time points, suggesting that the potential for apopotosis was present at a range of risedronate treatment conditions but that other factors can override this effect. Other genes associated with apoptosis were affected. Faslg, which is important in triggering apoptosis in many cell types [42], was increased by risedronate at both time points. Fas was increased at 24 h by both concentrations. Egr1, which was increased by the high risedronate concentration at 24 h, can play a role in apoptosis [43,44].

It is interesting that two genes noted to be important for angiogenesis, Comp [45], and Flt1 [46], were decreased in the osteoblastic cells by the bisphosphonate treatments. Flt 1 was affected by both concentrations at 24 h, and Comp was affected by both concentrations at both time points. This suggests that the drug has antiangiogenic potential, which is borne out by functional studies [47,48]. Inhibition of angiogenesis has also been proposed to be a mechanism for deleterious effects of bisphosphonates on bone [48], and it is possible that these genes may also be affected in the cells of the vasculature, and contribute to the antiangiogenic effects.

Finally, bisphosphonates have recently been found to have antimetastasis properties apparent clinically in the treatment of

both breast cancer [49] and prostate cancer [50]. The decreases in Cdh11 with 24 h treatment are consistent with antimetastatic effects [51,52]. The increase in Cdk2, which can antagonize prostate cancer progression [53,54], could also contribute to antitumor effects.

In conclusion, the studies provide potential mechanisms for the differential effects of low and high concentrations of risedronate on osteoblasts. The most marked pattern was the effect of the low risedronate concentration to increase expression of genes associated with mineralized tissue differentiation. The studies also identify genes that could mediate established effects of the bisphosphonates to promote apoptosis and antagonize angiogenesis and metastasis. The bisphosphonate also affected a number of additional genes whose importance and functional role in the actions of bisphosphonates are yet to be determined.

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