

The Amino Bisphosphonate Ibandronate Prevents Vitamin D Toxicity and Inhibits Vitamin D-Induced Calcification of Arteries, Cartilage, Lungs and Kidneys in Rats¹

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ABSTRACT Experiments were carried out to determine whether the doses of the amino bisphosphonate ibandronate that inhibit bone resorption inhibit soft tissue calcification and death in rats treated with a toxic dose of vitamin D. These studies were prompted by the recent discovery that ibandronate doses that inhibit bone resorption potently inhibit artery calcification induced by treatment with the vitamin K antagonist warfarin. All 16 rats treated with the toxic dose of vitamin D ($12.5 \text{ mg cholecalciferol} \cdot \text{kg}^{-1}$) died by d 6 after the first vitamin D injection (median survival: 4.5 d), whereas the 12 rats treated with vitamin D plus ibandronate ($0.25 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$) were alive and in good health at d 10. Rats treated with vitamin D alone and examined at d 4 had extensive Alizarin red staining for calcification in the aorta, the carotid, hepatic, mesenteric, renal and femoral arteries, kidneys and lungs, whereas rats treated with vitamin D plus ibandronate had no evidence for calcification at any of these tissues when examined at d 7 and 10. Ibandronate treatment also inhibited the dramatic increase in the levels of calcium and phosphate seen in the abdominal aorta, kidneys, lungs and trachea of the vitamin D-treated rats ($P < 0.001$). Serum calcium levels were, however, not different in rats treated with vitamin D alone ($3.4 \pm 0.2 \text{ mmol} \cdot \text{L}^{-1}$) and in rats treated with vitamin D plus ibandronate ($3.5 \pm 0.2 \text{ mmol} \cdot \text{L}^{-1}$). Treatment with vitamin D alone increased levels of matrix Gla protein, an inhibitor of soft tissue calcification, in the arteries, kidneys, lungs and trachea by 10- to 100-fold, and ibandronate treatment prevented this increase. The importance of these studies in the rat model is that they identify a class of drugs in current clinical use that can be used to treat patients with vitamin D toxicity and that they identify the dose of the drug that is predicted to be effective, namely the dose that inhibits bone resorption. Because there is no other known treatment for vitamin D toxicity, there would seem to be good reason to try bisphosphonates such as ibandronate in future studies aimed at treating patients who have been exposed to toxic levels of vitamin D. *J. Nutr.* 131: 2910–2915, 2001.

KEY WORDS: • rats • warfarin • vitamin K • vitamin D • calcification • matrix Gla protein

High doses of vitamin D have been known for many years to be toxic to humans, rats and other animals (1–3). In humans, manifestations of vitamin D toxicity include hypercalcemia, hypercalciuria, nausea, anorexia, lethargy, mental disturbances, ectopic soft tissue calcification, including vascular calcification and nephrocalcinosis, and renal failure (1–3). In the years immediately after the introduction of vitamin D into clinical use, vitamin D was used to treat diseases that are not associated with hypocalcemia, including arthritis, gout and various disorders in children, and treatment often led to vitamin D intoxication (2). Despite the current appreciation of the potential toxicity of vitamin D, however, it is still used unnecessarily and cases of vitamin D intoxication still occur (4). Vitamin D intoxication can also occur even when it is used to treat hypocalcemic disorders (1). In rats, vitamin D intoxication causes hypercalcemia, artery and renal calcification, anorexia, lethargy and death (5–7). Although the exact cause

of death has not been established in rats given toxic doses of vitamin D, it is possible that the ectopic calcification of arteries, kidneys or other soft tissue structures may play a critical role in morbidity.

In a previous study we found that the amino bisphosphonates alendronate and ibandronate inhibit warfarin-induced artery calcification in rats (8). The present experiments were carried out to determine whether the more potent of these bisphosphonates, ibandronate, would inhibit calcification of soft tissues and death in rats that have been given toxic doses of vitamin D. A second goal of these studies was to investigate the effect of vitamin D-induced soft tissue calcification on the level of the vitamin K-dependent matrix Gla protein (MGP)³, a proven inhibitor of soft tissue calcifications. A deficiency in the activity of MGP has been shown to cause calcification of arteries and cartilage in rats treated with the vitamin K antagonist warfarin (9,10), in the MGP gene knockout mouse (11) and in Keutel syndrome in humans (12).

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³ Abbreviation used: MGP, matrix Gla protein.

MATERIALS AND METHODS

Materials. Cholecalciferol (vitamin D) was purchased from Sigma (St. Louis, MO) and ibandronate (Bondronat; Boehringer Mannheim, Indianapolis, IN) was purchased from Idis World Medicines (Surrey, United Kingdom). Ibandronate was diluted with $0.15 \text{ mol} \cdot \text{L}^{-1}$ NaCl and stored at 4°C . Stock solutions of vitamin D were prepared fresh for each 3-d subcutaneous injection cycle at a concentration of 4.3 mmol/L in 7% emulphor (alkamuls EL-620; Rodia, Crabury, NJ) and then placed in foil wrapped containers and stored at 4°C , as described previously (10). Simonsen albino rats (Sprague-Dawley derived) were purchased from Simonsen Laboratories (Gilroy, CA).

Maintenance of rats. Male Sprague-Dawley rats consumed ad libitum rodent diet 5001 (Purina Mills, St. Louis, MO), a diet that is 0.67% phosphorus and 0.95% calcium by weight. This diet contains $500 \mu\text{g/kg}$ of phyloquinone and has no added menadione. In all experiments, rats were killed by exsanguination while under ether anesthesia. All animal experiments were approved by the University of California, San Diego Animal Subjects Committee.

Treatment of rats. In the survival study (Fig. 1), 28 seven-week-old male rats were given subcutaneous injections of 12.5 mg of vitamin $\text{D} \cdot \text{kg body}^{-1}$ at $t = 0, 24,$ and 48 h . Twelve of these rats also received subcutaneous injections of ibandronate at a dose of $0.25 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ beginning 4 d before the first vitamin D injection and continuing until the rats were killed. The number of rats surviving was determined at 12-h intervals; as noted in Figure 1, two of the vitamin D-treated rats were judged to be close to death at 120 h and, therefore, were anesthetized and killed. For histological analysis of tissues, rats were given the same treatments of vitamin D and of vitamin D plus ibandronate and were killed at 96 h (vitamin D only) and at 96 or 168 h (vitamin D plus ibandronate). For chemical analysis of tissue levels of calcium, phosphate and MGP, rats were given the same treatments of vitamin D and of vitamin D plus ibandronate and were killed at 96 h.

Methods. For measurement of mineral and MGP accumulation, the appropriate tissues were removed within 30 min of death and immediately frozen at -20°C until analysis. For each animal, the abdominal aorta section beginning 1 cm above the renal branch and ending at the femoral bifurcation, and the segment of trachea obtained by cutting between tracheal rings 12 and 13 (counting from the larynx) and rings 22 and 23, were placed into different 2-mL epitubes and 1 mL of $150 \text{ mmol} \cdot \text{L}^{-1}$ HCl was added to each tube.

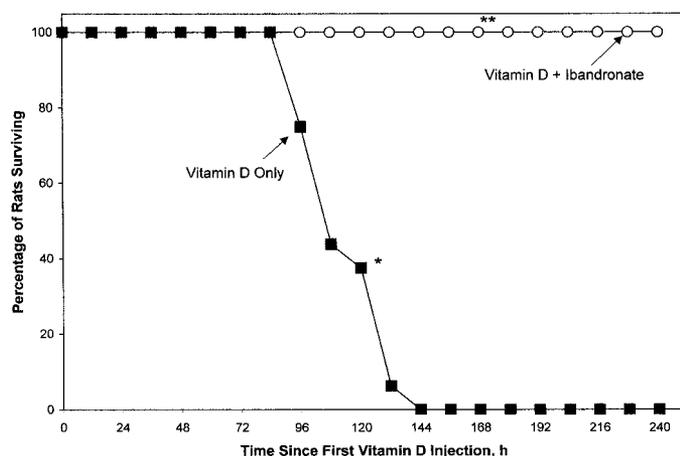


FIGURE 1 Effect of ibandronate on the survival of rats treated with a toxic dose of vitamin D. Twenty-eight 7-wk-old male Sprague-Dawley rats were given subcutaneous injections of 12.5 mg of vitamin $\text{D} \cdot \text{kg body}^{-1}$ at $t = 0, 24$ and 48 h . Twelve of these rats were also injected subcutaneously with ibandronate at a dose of $0.25 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ beginning 4 d before the first vitamin D injection, and the remaining 16 rats were given no ibandronate (see Materials and Methods). *Two rats treated with vitamin D alone were killed at this time-point due to poor health. **Six rats from the vitamin D plus ibandronate group were killed at 168 h for use in histological analyses.

The lung and one kidney from each rat were placed into separate 50-mL test tubes and 20 mL of 150 mmol/L HCl was added to each tube. Each tube was closed securely and mixed end over end for 24 h at room temperature. Calcium levels in the serum and in the acid extract of tissues were determined colorimetrically, using cresolphthalein complexone (Sigma) and phosphate levels in serum and in the acid extract of tissues were determined colorimetrically as described (13). The MGP levels in serum and in the acid extract of tissues were determined by radioimmunoassay as described previously (14). Serum samples were analyzed to determine the level of cross-linked N-telopeptides (OSTEOMARK NTx) by Ostex (Seattle, WA) using a specific enzyme-linked immunosorbent assay (15).

For histological analysis of mineral accumulation, the appropriate tissues were removed within 30 min of death and fixed in formalin for at least 24 h at room temperature. Sectioning and histological staining (hematoxylin and eosin and von Kossa) of formalin fixed tissues were carried out by Biomedical Testing Services (San Diego, CA). Alizarin red staining of formalin fixed tissues was carried out as described (16,17).

Statistical analysis. All data are presented as means \pm SD. Differences between groups were analyzed by the Student's t test. Differences with $P < 0.05$ were accepted as significant.

RESULTS

The first experiment was carried out to determine the effects of ibandronate on the mortality of rats given $12.5 \text{ mg} \cdot \text{kg}^{-1}$ of cholecalciferol (vitamin D) for 3 consecutive days, a dose that has been previously shown to be toxic (5–7). All 16 of the rats treated with vitamin D alone died by 144 h (Fig. 1). The symptoms before death included: anorexia and lethargy beginning by 48 h and a dramatic weight loss between 48 h and death, with a 21% weight loss between 48 and 96 h alone. In contrast, the 12 rats treated with vitamin D plus ibandronate at a dose of $0.25 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ were healthy at 168 h with no signs of anorexia, lethargy or weight loss. Six of the rats treated with vitamin D plus ibandronate were killed at this time and the remaining 6 rats continued to receive ibandronate until 240 h, when they were killed. These rats were judged to be healthy at 240 h by the criteria of normal intake of food and water and normal levels of activity. The serum chemistry values measured at 240 h were also normal except for serum calcium, which remained $\sim 40\%$ above normal levels.

Additional experiments were conducted to identify the tissues that calcify in rats treated with toxic doses of vitamin D and to determine the effects of ibandronate on each calcification. Figure 2 shows a typical example of the level of Alizarin red staining seen in the arteries from the 15 rats treated for 4 d with vitamin D alone, and an example of the absence of Alizarin red staining seen in the arteries from the 7 rats treated for 4 or 7 d with vitamin D plus ibandronate. Calcification in the vitamin D-treated rats was more pronounced in the smaller branch arteries, such as the mesenteric, hepatic and renal arteries, than it was in the aorta itself. Microscopic examination of von Kossa stained sections revealed massive calcification of the elastic lamellae in the media of arteries from the vitamin D-treated rats and the absence of staining in the arteries from rats treated with vitamin D plus ibandronate (not shown).

Figure 3 shows the typical Alizarin red staining seen in the lungs of rats treated for 4 d with vitamin D alone and an example of the absence of Alizarin red staining seen in the lungs from rats treated for 4 or 7 d with vitamin D plus ibandronate⁴. Microscopic examination of von Kossa stained

⁴ There was a red-appearing region at the center of the lungs from the control rats and the rats treated with vitamin D plus ibandronate. This color was an artifact of the photography and no alizarin red stain can be seen on visual inspection of either lung.

FIGURE 2 Effect of ibandronate on artery calcification in rats treated with a toxic dose of vitamin D. Seven-week-old male Sprague-Dawley rats were given subcutaneous injections of 12.5 mg of vitamin D · kg body⁻¹ at *t* = 0, 1, and 2 d. A subset of these rats was also injected subcutaneously with ibandronate at a dose of 0.25 mg · kg⁻¹ · d⁻¹ beginning 4 d before the first vitamin D injection. Rats were killed 96 h after the first vitamin D injection and the carotid arteries, aorta and portions of the pulmonary, mesenteric, hepatic, renal and femoral arteries were dissected as a unit, fixed in formalin and stained with Alizarin red (see Materials and Methods). Typical artery from a rat treated with vitamin D alone (carotid arteries on left; *upper panel*); typical artery from a rat treated with vitamin D plus ibandronate (*middle panel*); and artery from an age-matched control rat (*lower panel*).



sections showed that calcification is associated with the alveolar wall and pulmonary arteries (not shown). As can also be seen in Figure 3, vitamin D treatment increased calcification of tracheal ring cartilage, and ibandronate treatment reduced this increase. Figure 4 shows the typical level of von Kossa staining seen in the elastic lamellae of aortic heart valves from rats treated with vitamin D alone and an example of the absence of von Kossa staining seen in the aortic heart valves of rats treated with vitamin D plus ibandronate.

Sections of arteries, kidneys and lungs were also stained with hematoxylin and eosin and examined. There was no evidence of cell necrosis or degeneration in any tissue from the rats treated with vitamin D plus ibandronate, and the microscopic appearance of these tissues was indistinguishable from the appearance of corresponding tissues from age-matched, untreated control rats.

Tissues were analyzed for calcium and phosphate to obtain a quantitative measure of the effects of treatment with vitamin D and with vitamin D plus ibandronate on the accumulation of calcium and phosphate in the tissues. Treatment with

vitamin D alone significantly increased tissue levels of calcium and phosphate in the aorta, lung, kidney and trachea, whereas tissue levels of calcium and phosphate in the corresponding tissues from the rats treated with vitamin D plus ibandronate were at control levels (Table 1). The average molar ratio of the increase in calcium to the increase in phosphate found in the aorta, lung, kidney and trachea of rats treated with vitamin D alone was 1.54 ± 0.12 . Vitamin D treatment significantly increased serum calcium levels at d 4 compared with control rats (Table 2), in agreement with earlier studies (10). Ibandronate treatment did not reduce the levels of serum calcium at d 4 compared with rats treated with vitamin D alone (Table 2), but did produce a significant 23% reduction in the level of serum phosphate. In the 6 rats treated with vitamin D plus ibandronate for 10 d, serum calcium remained high (3.5 ± 0.1 mmol · L⁻¹) and serum phosphate remained low (2.3 ± 0.2 mmol · L⁻¹). Treatment with toxic levels of vitamin D increased serum levels of cross-linked N-telopeptides, a marker for bone resorption activity, by 110% (Table 2). Serum levels of cross-linked N-telopeptides were at control values in the

FIGURE 3 Effect of ibandronate on the calcification of lungs and trachea in rats treated with a toxic dose of vitamin D. In the experiment described in the legend of Figure 2, lungs and attached trachea were dissected as a unit, fixed in formalin and stained with Alizarin red (see Materials and Methods). Typical lung from a rat treated with vitamin D alone (left); typical lung from a rat treated with vitamin D plus ibandronate (middle); and lung from an age-matched control rat (right).



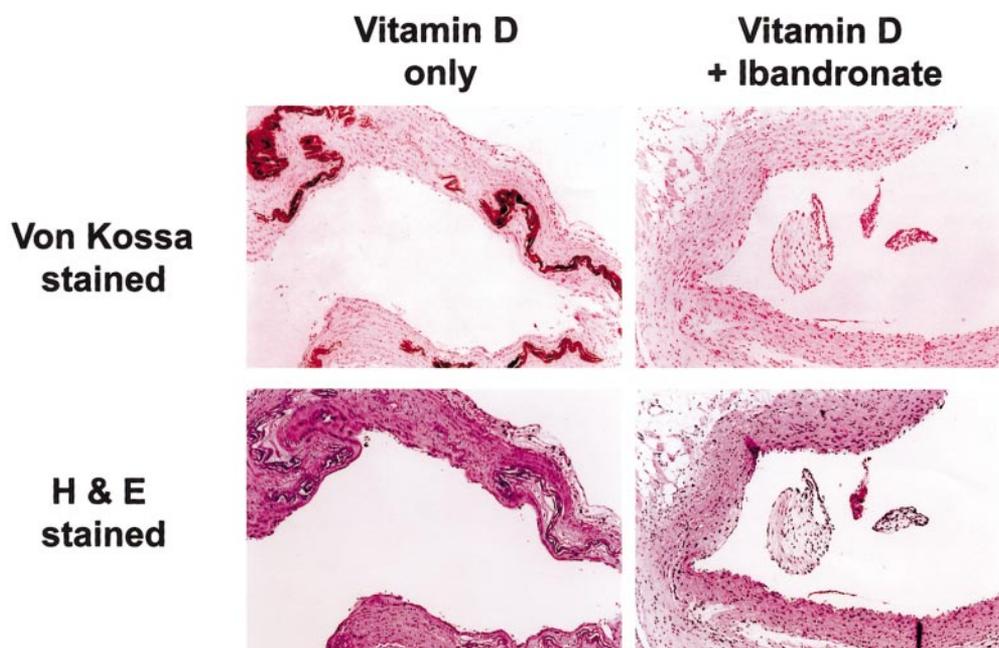


FIGURE 4 Effect of ibandronate on the calcification of aortic heart valves in rats treated with a toxic dose of vitamin D. In the experiment described in the legend of Figure 2, the hearts were removed and fixed in formalin. Sections of each heart were obtained that included the aortic heart valve, and each section was stained by the von Kossa procedure, which stains calcifications brown, and by the hematoxylin & eosin procedure.

rats treated with vitamin D plus ibandronate, which shows that ibandronate treatment completely inhibited the increased level of bone resorption activity produced by vitamin D treatment. Because ibandronate did not reduce the hypercalcemia due to vitamin D intoxication, the vitamin D-induced hypercalcemia observed in these rats cannot be due to accelerated bone resorption.

Additional analyses were carried out to determine whether the soft tissue calcifications induced by toxic doses of vitamin

D are associated with increased levels of MGP, a potent vitamin K-dependent inhibitor of soft tissue calcifications. Treatment with toxic doses of vitamin D increased serum levels of MGP by twofold and increased tissue levels of MGP from sixfold (cartilage) to over 100-fold (lung; Table 3). Treatment with ibandronate reduced serum levels of MGP to control levels and reduced tissue levels of MGP from 64% (cartilage) to 93% (lung) below the levels found in rats treated with vitamin D alone. Lung levels of MGP in the ibandronate-treated rats remained significantly above the MGP levels in control rats, however.

TABLE 1

Effect of ibandronate treatment on soft tissue calcification in rats given a toxic dose of vitamin D^{1,2}

	<i>n</i>	Vitamin D only	Vitamin D + ibandronate	Age-matched control
		<i>μmol</i>		
Abdominal aorta	3			
Calcium		4.01 ± 0.59	0.31 ± 0.03 ³	0.29 ± 0.01 ³
Phosphate		2.43 ± 0.59	0.03 ± 0.00 ³	0.03 ± 0.00 ³
Lung	3			
Calcium		91.0 ± 12.7	8.2 ± 1.1 ³	5.2 ± 2.3 ³
Phosphate		73.8 ± 7.7	13.4 ± 0.5 ³	13.9 ± 0.6 ³
Kidney	4			
Calcium		51.0 ± 10.1	7.0 ± 1.5 ³	5.9 ± 2.3 ³
Phosphate		49.8 ± 5.8	23.6 ± 1.0 ³	23.3 ± 1.5 ³
Trachea	3			
Calcium		4.12 ± 0.38	1.62 ± 0.54 ³	1.04 ± 0.54 ³
Phosphate		2.86 ± 0.12	1.16 ± 0.33 ³	0.79 ± 0.41 ³

¹ Results are means ± SD.

² Seven-week-old male rats received subcutaneous injections of 12.5 mg of vitamin D · kg body⁻¹ at *t* = 0, 24 and 48 h. A subset of these rats were also injected with ibandronate at a dose of 0.25 mg · kg⁻¹ · d⁻¹ beginning 4 d before the first vitamin D injection. Rats were killed 96 h after the first vitamin D injection, and the indicated tissues were extracted with acid to dissolve mineral. Each acid extract was then analyzed for calcium and phosphate.

³ *P* < 0.001 when compared with vitamin D only.

DISCUSSION

To our knowledge, this is the first study to identify a drug that prevents soft tissue calcification and death in experimental animals given a toxic dose of vitamin D. This discovery raises the possibility that the drug identified in this study,

TABLE 2

Effect of treatment with vitamin D and ibandronate on serum levels of calcium, phosphate and cross-linked N-telopeptides in rats^{1,2}

	Vitamin D only	Vitamin D + ibandronate	Age-matched control
Calcium, <i>mmol</i> · <i>L</i> ⁻¹	3.4 ± 0.2 ^a (6)	3.5 ± 0.2 ^a (6)	2.6 ± 0.1 (4)
Phosphate, <i>mmol</i> · <i>L</i> ⁻¹	3.1 ± 0.6 (6)	2.3 ± 0.1 ^a (6)	3.0 ± 0.2 (4)
Cross-linked N-telopeptides, <i>μg/L</i>	119 ± 11 ^a (4)	54 ± 8 (4)	57 ± 3 (4)

¹ Results are means ± SD (*n*). ^a Significantly different from control, *P* < 0.001.

² Rats were treated as described in the legend to Table 1, and serum was obtained from blood collected at 96 h after the first vitamin D injection.

TABLE 3

Effect of treatment with vitamin D and ibandronate on the levels of matrix Gla protein (MGP) in serum and in soft tissues of rats^{1,2}

	n	Vitamin D only	Vitamin D + ibandronate	Age-matched control
Serum, nmol · L ⁻¹	4	85.0 ± 10.0 ^{a,b}	29.6 ± 2.5	27.6 ± 1.7
Abdominal aorta, pmol	3	109.4 ± 6.4 ^{a,b}	10.5 ± 0.7	6.1 ± 1.6
Lung, pmol	3	1650.0 ± 33.3 ^{a,b}	110.0 ± 2.7 ^a	15.3 ± 1.8
Kidney, pmol	4	1350 ± 550	<80	<80
Trachea, pmol	3	306.7 ± 44.4 ^{a,c}	112.0 ± 78.7	43.0 ± 8.0

¹ Results are means ± SD. ^a *P* < 0.001 compared with age-matched control; ^b *P* < 0.001 compared with D + ibandronate; ^c *P* < 0.025 compared with D + ibandronate.

² MGP levels were determined by the radioimmunoassay of the serum and of the same acid extracts used for measurements of tissue levels of calcium and phosphate.

ibandronate, could be useful in the treatment of vitamin D toxicity in humans.

Because ibandronate prevented both soft tissue calcification and death in rats given a toxic dose of vitamin D, it seems likely that death is caused by soft tissue calcification. This possibility is supported by the observation that warfarin and a lower 7.5 mg · kg⁻¹ dose of vitamin D act synergistically in causing increased calcification of soft tissues and death (10), and by the observation that in rats treated with the same doses of warfarin and vitamin D, the amino bisphosphonate alendronate inhibits both the calcification of soft tissues (8) and death (personal observations). To better understand how soft tissue calcification could affect normal physiological processes, we investigated the possible calcification of a variety of tissues in rats given toxic doses of vitamin D. In agreement with earlier studies (6,18,19), we found that toxic doses of vitamin D induced extensive calcification of arteries and kidneys, and for the first time, we report that toxic doses of vitamin D also caused extensive calcification of lungs, heart valves and tracheal ring cartilage. In each of these tissues, calcification was reduced markedly or prevented by treatment with ibandronate. These results document the diversity of tissues that calcify in rats given toxic doses of vitamin D as well as the ability of ibandronate to inhibit each calcification process. The number of tissues that calcify and the extensive degree of each calcification are consistent with the hypothesis that the cumulative physiological effect of soft tissue calcification causes death and with the hypothesis that ibandronate prevents death by preventing soft tissue calcification.

The fact that ibandronate inhibited calcification of arteries, heart valves, lungs, kidneys and cartilage is intriguing because it suggests that vitamin D-induced calcification of these diverse tissues may proceed by a common biochemical mechanism. We previously hypothesized that artery calcification is linked to bone resorption to account for the effects of vitamin D and growth status on warfarin-induced artery calcification (10). This hypothesis is supported by studies that show that deficiency in osteoprotegerin, a secreted protein that normally inhibits osteoclast activity, causes both the expected rapid loss of bone calcium and the calcification of arteries (20). Our recent discovery that the bisphosphonates alendronate and ibandronate inhibit warfarin-induced artery calcification at doses that inhibit bone resorption also supports this hypothesis

(8). We speculate that the demonstrated ability of toxic doses of vitamin D to strongly stimulate bone resorption (Table 2) accounts for the calcification of the diverse set of soft tissues observed in the present studies, and that bone resorption, therefore, is linked to the calcification of a wide variety of tissues in vitamin D-treated rats. The ability of ibandronate to inhibit each of these soft tissue calcifications supports this hypothesis, because ibandronate completely inhibited the increased level of bone resorption activity produced by vitamin D treatment (Table 2).

The nature of the biochemical mechanism that is responsible for the putative linkage between bone resorption and soft tissue calcification in vitamin D-treated rats is presently unclear. One possibility is that soft tissue calcification could be a direct physicochemical consequence of the effect of the observed vitamin D-induced hypercalcemia on the nucleation and growth of the mineral phase in soft tissues. This hypothesis is not, however, supported by the failure of ibandronate to normalize serum calcium levels in vitamin D-treated rats (Table 2). Another possibility is that soft tissue calcification is promoted by crystal nuclei generated at sites of bone resorption, which travel in blood and occasionally lodge in soft tissue structures. This hypothesis is supported by the observation that, under some circumstances, a complex of calcium, phosphate and MGP is released from bone and can be detected in blood, and by the observation that the release of this complex from bone is inhibited by inhibitors of bone resorption (personal observations).

The present studies show that soft tissue calcification dramatically increases tissue levels of MGP. We believe that the MGP that accumulates in these tissues is bound directly to the tissue calcifications and that the accumulation of MGP is, therefore, a direct reflection of the amount of calcification within the tissue. This hypothesis is supported by the observation that ibandronate treatment reduces both the calcification of soft tissues and the accumulation of MGP in these tissues. It is probable that the MGP that accumulates at sites of ectopic calcifications in vitamin D-treated rats actually plays a direct role in retarding the further growth of the soft tissue calcification, because previous studies have shown that the rate of ectopic calcification is accelerated when vitamin D-treated rats are treated concurrently with warfarin (10). It should be noted that previous studies have also shown that the direct association of MGP with calcium phosphate crystallites is required for MGP to inhibit the *in vitro* calcification of human aortic elastin in human plasma (21). Because the sites of prominent soft tissue calcification in vitamin D-treated rats are also the tissues with the highest rate of MGP expression in rats (22,23), it seems likely that the increased levels of MGP found at sites of ectopic calcification arise from local synthesis of the protein within the tissue.

There are clinical implications of the present findings that should be noted. Bisphosphonates are currently used to inhibit bone resorption in patients with osteoporosis (24,25). We previously showed that the same bisphosphonate doses that inhibit bone resorption have the unexpected ability to inhibit warfarin-induced artery calcification (8). In the present study we have further shown that doses of the bisphosphonate ibandronate, which in previous studies inhibited bone resorption and warfarin-induced artery calcification, also inhibit the soft tissue calcification induced by toxic doses of vitamin D and prevent death due to vitamin D intoxication. This discovery in the rat model, therefore, identifies a class of drugs in current clinical use that can be used to treat patients with vitamin D toxicity and identifies the dose of the drug that is predicted to be effective, namely the dose that inhibits bone resorption.

Because there is no other known treatment for vitamin D toxicity, there would seem to be little reason not to try bisphosphonates such as ibandronate in future studies aimed at treating patients who have been exposed to toxic levels of vitamin D.

LITERATURE CITED

1. Coburn, J. W. & Barbour, G. L. (1984) Vitamin D intoxication and sarcoidosis. In: *Hypercalciuric States: Pathogenesis, Consequences and Treatment* (Coe, F. L., ed.), pp. 379–406. Grune and Stratton, Orlando, FL.
2. Stern, P. H. & Bell, N. H. (1989) Disorders of vitamin D metabolism: toxicity and hypersensitivity. In: *Metabolic Bone Disease: Cellular and Tissue Mechanisms* (Tam, C. S., Heersche, J.N.M. & Murray, T. M., eds.), pp. 203–213. CRC Press, Boca Raton, FL.
3. Pettifor, J. M., Bikle, D. D., Cavaleros, M. T., Zachen, M. T., Kamdar, M. C. & Ross, F. P. (1995) Serum levels of free 1,25-dihydroxyvitamin D in vitamin D toxicity. *Ann. Intern. Med.* 122: 511–513.
4. Schwartzman, M. S. & Franck, W. A. (1987) Vitamin D toxicity complicating the treatment of senile, postmenopausal, and glucocorticoid-induced osteoporosis. *Am. J. Med.* 82: 224–230.
5. Kitagawa, S., Yamaguchi, Y., Kunitomo, M., Imaizumi, N. & Fujiwara, M. (1992) Impairment of endothelium-dependent relaxation in aorta from rats with arteriosclerosis induced by excess vitamin D and a high-cholesterol diet. *Japan. J. Pharmacol.* 59: 339–347.
6. Takeo, S., Anan, M., Fujioka, K., Kajihara, T., Hiraga, S., Miyake, K., Tanonaka, K., Minematsu, R., Mori, H. & Taniguchi, Y. (1989) Functional changes of aorta with massive accumulation of calcium. *Atherosclerosis* 77: 175–181.
7. Takeo, S., Tanonaka, R., Tanonaka, K., Miyake, K., Hisayama, H., Ueda, N., Kawakami, K., Tsumura, H., Katsushika, S. & Taniguchi, Y. (1991) Alterations in cardiac function and subcellular membrane activities after hypervitaminosis D₃. *Mol. Cell. Biochem.* 107: 169–183.
8. Price, P. A., Faus, S. A. & Williamson, M. K. (2001) The bisphosphonates alendronate and ibandronate inhibit artery calcification at doses comparable to those which inhibit bone resorption. *Arterioscler. Thromb. Vasc. Biol.* 21: 817–824.
9. Price, P. A., Faus, S. A. & Williamson, M. K. (1998) Warfarin causes rapid calcification of the elastic lamellae in rat arteries and heart valves. *Arterioscler. Thromb. Vasc. Biol.* 18: 1400–1407.
10. Price, P. A., Faus, S. A. & Williamson, M. K. (2000) Warfarin induced artery calcification is accelerated by growth and by vitamin D. *Arterioscler. Thromb. Vasc. Biol.* 20: 317–327.
11. Luo, G., Ducy, P., McKee, M. D., Pinero, G. J., Loyer, E., Behringer, R. R. & Karsenty, G. (1997) Spontaneous calcification of arteries and cartilage in mice lacking matrix Gla protein. *Nature* 386: 78–81.
12. Munroe, P. B., Olgunturk, R. O., Fryns, J. P., Maldergem, L. V., Ziereisen, F., Yuksel, B., Gardiner, R. M. & Chung, E. (1999) Mutations in the gene encoding the human matrix Gla protein cause Keutel syndrome. *Nat. Genet.* 21: 142–144.
13. Chen, P. S., Toribara, T. Y. & Warner, H. (1956) Microdetermination of phosphorus. *Anal. Chem.* 28: 1756–1758.
14. Otawara, Y. & Price, P. A. (1986) Developmental appearance of matrix Gla protein during calcification in the rat. *J. Biol. Chem.* 261: 10828–10832.
15. Gorski, J. P., Apone, S., Shaffer, K. A., Batchelder, A., Williams, J. A., Shacter, E. & Eyre, D. R. (2000) Hypercalcemia during the osteogenic phase after rat marrow ablation coincides with increased bone resorption assessed by the NTx marker. *Bone* 27: 103–110.
16. Hanken, J. & Wassersug, R. (1981) A new double-stain technique reveals the nature of the hard tissues. *Funct. Photog.* 16: 22–26.
17. Rosa-Molinar, E., Proskocil, B. J., Ettl, M. & Fritsch, B. (1999) Whole-mount procedures for simultaneous visualization of nerves, neurons, cartilage, and bone. *Brain Res. Protocols* 4: 115–123.
18. Kingma, J. G. & Roy, P. E. (1988) Ultrastructural study of hypervitaminosis D induced arterial calcification in wistar rats. *Artery* 16: 51–61.
19. Sanderson, P. H. (1959) Functional aspects of renal calcification in rats. *Clin. Sci.* 18: 67–79.
20. Bucay, N., Sarosi, I., Dunstan, C. R., Morony, S., Tarpley, J., Capparelli, C., Scully, S., Tan, H. L., Xu, W., Lacey, D. L., Boyle, W. & Simonset, W. S. (1998) Osteoprotegerin-deficient mice develop early onset osteoporosis and arterial calcification. *Genes Dev.* 12: 1260–1268.
21. Alagao, F. C., Patel, R. & Price, P. A. (2000) Matrix Gla protein specifically inhibits calcification of human aortic elastin in vitro. *J. Bone Miner. Res.* 15(suppl 1): S208.
22. Fraser, J. D. & Price, P. A. (1988) Lung, heart, and kidney express high levels of mRNA for the vitamin K-dependent matrix Gla protein: implications for the possible functions of matrix Gla protein and for the tissue distribution of the γ -carboxylase. *J. Biol. Chem.* 263: 11033–11036.
23. Hale, J. E., Fraser, J. D. & Price, P. A. (1988) The identification of matrix Gla protein in cartilage. *J. Biol. Chem.* 263: 5820–5824.
24. Fleisch, H. A. (1997) Bisphosphonates: preclinical aspects and use in osteoporosis. *Ann. Med.* 29: 55–62.
25. Fleisch, H. (1998) Bisphosphonates: mechanisms of action. *Endocr. Rev.* 19: 80–100.