Title: USE OF LOW DOSAGE BISPHOSPHONATES TO INHIBIT CARDIAC AND ARTERIAL CALCIFICATION

Abstract: This invention provides methods of inhibiting calcification of a soft tissue (e.g., an artery, a heart valve, an atherosclerotic plaque, a cancer, a kidney, a prostate, skin, muscle, cartilage, viscera, and heart muscle) in a mammal. These methods involve inhibiting osteoclastic bone resorption in said mammal (e.g., a mammal diagnosed as having or at risk for a pathology characterized by calcification of a soft tissue). The inhibition is preferably by administration of a bisphosphonate to the mammal in a concentration sufficient to inhibit bone resorption without inhibiting bone mineralization. The methods of this invention can also be used to mitigate a symptom of atherosclerosis in a mammal. Such methods involve inhibiting osteoclastic bone resorption in the mammal. In a preferred embodiment, the inhibiting is by administration of a bisphosphonate to the mammal in a concentration sufficient to inhibit bone resorption without inhibiting bone mineralization.
USE OF LOW DOSAGE BISPHOSPHONATES TO INHIBIT CARDIAC AND ARTERIAL CALCIFICATION

CROSS-REFERENCE TO RELATED APPLICATIONS
[ Not Applicable ]

STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT
[ Not Applicable ]

FIELD OF THE INVENTION
This invention relates to modulators of soft tissue calcification. In particular, this invention relates to the discovery that inhibition of bone resorption will also result in the inhibition of calcification of soft tissues.

BACKGROUND OF THE INVENTION
The bisphosphonates have been known to chemists since the middle of the 19th century, when the first synthesis occurred in 1865 in Germany (Menschutkin (1865) Ann. Chem. Pharm., 133: 317-320). Bisphosphonates were used in industry, mainly as corrosion inhibitors or as complexing agents in the textile, fertilizer and oil industries. Their ability to inhibit calcium carbonate precipitation, similar to polyphosphates was put to use in the prevention of scaling (Blomen (1995) Pages 111-124 in Bijvoet OLM et al., eds. Bisphosphonate on Bones, Elsevier, Amsterdam).

More recently, bisphosphonates have been developed as drugs for use in various diseases of bone, tooth, and calcium metabolism. The bisphosphonates have two fundamental previously known biological effects: inhibition of calcification when given at high doses and inhibition of bone resorption.

Bisphosphonates have been shown to efficiently inhibit ectopic calcification in vivo. Thus, among others, they prevent experimentally induced calcification of many soft tissues when given both parenterally and orally (Fleisch et al. (1970) Eur. J. Clin. Invest., 1: 12-18; Rosenblum et al. (1977) Calcif. Tissue Res., 23: 151-159). In contrast to pyrophosphate, which acts only when given parenterally, bisphosphonates are active when
administered orally. They have also been shown to have activity when released locally from various matrices (Levy et al. (1985) Science, 228: 190-192; Golomb et al. (1986) J. Contr. Rel., 4: 181-194). In addition, topical administration can lead to a decreased formation of dental calculus (Briner et al. (1971) Int. Dent. J. 21: 61-73). This effect is used to prevent tartar formation in humans by the addition of bisphosphonates to toothpastes. In addition, certain bisphosphonates inhibit ectopic ossification when given systemically (Plasmans et al. (1978) Clin. Orthop., 132: 233-243) or locally (Ahrendt and Lindgren (1986) J. Orthop., Res. 4: 18-26).

Of the bisphosphonates, etidronate has been used in humans to prevent ectopic calcification and ossification. Unfortunately with respect to calcification, the results have been disappointing. In conditions such as scleroderma, dermatomyositis, and calcinosis universalis, the results have proven at best inconclusive (Fleisch (1988) Pages 440-466 in Baker PF (ed) Handbook of Experimental Pharmacology, Springer-Verlag, N.Y.). In urolithiasis, the dose that was believed to potentially be effective was such that normal bone mineralization was inhibited (Baumann et al. (1978) Clin. Sci. Mol. Med., 54: 509-516).


Moreover, while the different bisphosphonates vary greatly in their activity in bone resorption, they do not vary greatly in the inhibition of mineralization. For most bisphosphonates, the effective daily dose was believed to be on the order of 5-20 mg of
compound phosphorus per kg, administered parenterally, suggesting that the bisphosphonates inhibit calcification at high doses via a common mechanism.

Thus, although bisphosphonates have proven successful when administered to humans or other mammals to inhibit bone resorption, the propensity to inhibit the calcification of normal bone when administered at dosages believed high enough to inhibit ectopic calcification, has hampered the therapeutic use of bisphosphonates in the treatment of ectopic calcifications.

**SUMMARY OF THE INVENTION**

This invention provides new approaches to the treatment of ectopic calcifications and various arterioscleroses (e.g., atherosclerosis). The methods of this invention are premised, in part, on the discovery that agents that inhibit bone resorption will also inhibit ectopic calcification and/or plaque formation and related pathologies associated with arteriosclerosis. Without being bound to a particular theory, it is believed that the process of bone resorption, delivers solubilized calcium (e.g. in a calcium phosphate/protein complex) to the blood where it can travel to sites far removed from bone and there act as a nucleation complex for the formation of ectopic calcifications or atherosclerotic plaques and/or contribute to the formation of an existing calcium deposition.

Various agents, in particular bisphosphonates, are often able to inhibit bone resorption at far lower dosages than the dosages at which they have been observed to inhibit bone calcification. It was believed that the effect on bone resorption was mediated via a biological/cellular mechanism and the effect on bone calcification was mediated by a physio-chemical mechanism (e.g. direct binding to hydroxyapatite). Similarly, it was believed that bisphosphonates could inhibit ectopic calcification by the same physio-chemical mechanism as that used to inhibit bone mineralization. Consequently it was believed that although high dosages of bisphosphonates could inhibit ectopic calcification, this approach had little therapeutic value because of the adverse effect on bone mineralization.

The discovery of this invention, that ectopic calcification can be inhibited by inhibition of bone resorption allows the treatment of pathologies associated with undesired calcification at low dosages, e.g. at dosages that do not adversely effect bone mineralization. Thus, in view of the discoveries described herein, a new therapeutic modality is provided for the alleviation of ectopic calcifications and/or arteriosclerotic plaque formation.
Thus, in one embodiment, this invention provides methods of inhibiting calcification of a soft tissue (e.g., an artery, a heart valve, an atherosclerotic plaque, a cancer, a kidney, a prostate, skin, muscle, cartilage, viscera, and heart muscle) in a mammal. These methods involve inhibiting osteoclastic bone resorption in said mammal (e.g., a mammal diagnosed as having or at risk for a pathology characterized by calcification of a soft tissue). The inhibition is preferably by administration of a bisphosphonate to the mammal in a concentration sufficient to inhibit bone resorption without inhibiting bone mineralization. In preferred embodiments, the bisphosphonate effects a significant reduction of bone resorption at a concentration at least 10-fold, more preferably at least 100-fold, and most preferably at least 1000-fold lower than the concentration at which said bisphosphonate effects a significant reduction of bone mineralization (preferably in the same assay and at the same confidence level). The bisphosphonate may be administered at a dosage at least 10-fold, more preferably at least 100-fold, and most preferably at least 1000-fold lower than concentration at which said bisphosphonate effects a significant reduction of bone mineralization (preferably in the same assay and at the same confidence level). Particularly preferred bisphosphonates include, but are not limited to alendronate, ibandronate, zoledronate, incadronate, risedronate, EB-1053, neridronate, olpadronate, pamidronate, YH 529, tiludronate, and clodronate.

In another embodiment this invention provides methods of method of inhibiting calcification of soft tissue (e.g., an artery, a heart valve, an atherosclerotic plaque, a cancer, a kidney, a prostate, skin, muscle, cartilage, viscera, and heart muscle) in a mammal diagnosed as having or at risk for a pathology characterized by calcification of a soft tissue. These methods involve administering to the animal a low dosage of a bisphosphonate, where the low dosage is sufficient to inhibit the calcification, but below the dosage of the bisphosphonate that inhibits normal bone mineralization. Preferred bisphosphonates and dosages include those described above. In one embodiment the bisphosphonate is alendronate administered at a dosage ranging from the minimum dose that produces a detectable inhibition of bone resorption up to 0.5 mg P/kg/day. In another embodiment, the bisphosphonate is alendronate administered to humans at an oral dosage ranging from 5 mg to 40 mg per day. In still another embodiment, the bisphosphonate is ibandronate administered at a dosage ranging from the minimum dose that produces a detectable inhibition of bone resorption up to 0.5 mg P/kg/day, preferably at an intra venous dosage of 1mg per day. In still yet another embodiment, the bisphosphonate is zoledronate,
incadronate, risedronate, EB-1053, neridronate, olpadronate, pamidronate, YH 529,
tiludronate, or clodronate administered at a dosage ranging from the minimum dose that
produces a detectable inhibition of bone resorption up to 0.5 mg P/kg/day. Preferred modes
of administration include, but are not limited to, transdermal patch, orally, intravenous
injection, subcutaneous injection, and intramuscular injection. The bisphosphonate can be
administered as a prophylactic or a therapeutic treatment.

This invention also provides a method of mitigating the symptoms of a
disease in a mammal that involves calcification of a soft tissue (an artery, a heart valve, an
atherosclerotic plaque, a cancer, a kidney, a prostate, skin, muscle, cartilage, viscera, and
heart muscle) The method involves administering to the mammal a low dosage of a
bisphosphonate sufficient to inhibit calcification of the soft tissue without inhibiting bone
calcification. Such diseases include, but are not limited to atherosclerosis, arteriosclerosis,
arteriolosclerosis, hypertensive arteriolosclerosis, Monckeberg's arteriosclerosis, heart valve
stenosis, uremia, diabetes, hyperparathyroidism, blood clot formation, cancer growth, cancer
metastasis, hypertension, vitamin D toxicity, and arthritis. Preferred bisphosphonates and
dosages include, but are not limited to the bisphosphonates and dosages described above.
The mammal may be diagnosed as having or at risk for a pathology characterized by
calcification of a soft tissue.

In still yet another embodiment, this invention provides methods of mitigating
the calcification of an implanted prosthetic device in a mammal. These methods involve
administering to the mammal a low dosage of a bisphosphonate sufficient to inhibit
calcification of the prosthetic device or soft tissue surrounding said prosthetic device without
inhibiting calcification of bone. Such prosthetic devices include, but are not limited to, a
heart valve bioprosthesis, and a heart valve mechanical prosthesis. The prosthetic devices
can also include, but are not limited to, a surgical implant comprising polyetherurethaneuerea,
a surgical implant comprising polyetherurethane; a surgical implant comprising silicon, a
surgical repair material used for the repair of an aneurisms. Preferred bisphosphonates and
dosages include, but are not limited to the bisphosphonates and dosages described above.

The methods of this invention can also be used to mitigate a symptom of
atherosclerosis in a mammal. Such methods involve inhibiting osteoclastic bone resorption
in said mammal. In preferred embodiment, the inhibiting is by administration of a
bisphosphonate to the mammal in a concentration sufficient to inhibit bone resorption
without inhibiting bone mineralization. Preferred mammals include, but are not limited to
mammals diagnosed as having, or at risk for, atherosclerosis. Preferred bisphosphonates and dosages include, but are not limited to the bisphosphonates and dosages described above. The bisphosphonate is administered as a prophylactic or as a therapeutic treatment.

In another embodiment a symptom or progression of atherosclerosis in a mammal is inhibited by inhibiting the removal of mineral by macrophages at sites of calcification. In a preferred embodiment the inhibiting comprises administering a bisphosphonate to the mammal in a concentration sufficient to inhibit calcium removal by said macrophages. The bisphosphonate is preferably administered at a concentration that does not inhibit macrophages at locations other than sites of calcification. Preferred bisphosphonates and dosages include, but are not limited to the bisphosphonates and dosages described above. The method can be prophylactic and/or therapeutic.

Kit are also provided for the mitigation of a pathology associated with calcification of a soft tissue. Preferred kits include a container containing a bisphosphonate that inhibits calcification of a soft tissue at a dosage that does not substantially inhibit calcification of bone and instructional materials teaching the use of said bisphosphonate for treatment of a pathology associated with calcification of a soft tissue or calcification of a prosthetic device. Preferred bisphosphonates and dosages include, but are not limited to the bisphosphonates and dosages described above.

This invention also provides methods of stabilizing the size and/or the crystal structure of calcium or a calcium salt in an aqueous phase. These methods involve contacting the calcium or calcium salt with fetuin.

The stabilized calcium provides a method of delivering a calcification initiator to a preselected site. Such methods involve providing a fetuin-mineral complex attached to a targeting molecule (e.g., antibody, lectin, nucleic acid etc.) where the targeting molecule specifically binds to the preselected site; and contacting the fetuin mineral complex to the preselected site.

Also provided is a method of distributing mineral nuclei within a matrix. This method involves impregnating the matrix with a fetuin-mineral complex and denaturing the fetuin such that the mineral is released from the fetuin mineral complex.

The fetuin can also be used to stabilize the size or crystal structure of a mineral salt in an aqueous phase. This method involves contacting the mineral salt with a fetuin.
This invention also provides substantially isolated mineral salts (e.g. calcium phosphate) stabilized in a complex with fetuin.

Mammals subject to the methods described herein include, but are not limited to humans, non-human primates, canines, felines, equines, bovines, rodents, porcines, and lagomorphs. Thus, veterinary and human medical applications are contemplated.

In particularly preferred embodiments, the bisphosphonates used in the methods of this invention do not include bisphosphonates for which the dosage that inhibits bone mineralization is comparable to or equal to the dosage that inhibits bone resorption. The bisphosphonates used in the methods of this invention preferably do not include etidronate.

DEFINITIONS

Bisphosphonates, previously and erroneously called diphosphonates in the past, are compounds characterized by two C-P bonds. If the two bonds are located on the same carbon atom, the compounds are called geminal bisphosphonates and are analogs of pyrophosphate, containing an oxygen instead of a carbon atom (Formula I).

\[
\begin{align*}
\text{O}^- & \quad \text{R'} \quad \text{O}^- \\
\mid & \quad \mid \\
\text{O} & = \text{P} \quad \text{C} \quad \text{P} & = \text{O} \\
\mid & \quad \mid \\
\text{O}^- & \quad \text{R''} \quad \text{O}^-
\end{align*}
\]

I.

In the literature, these compounds are usually called bisphosphonates. This, however, is somewhat misleading, since non-geminal bisphophonates are also bisphophonates. Thus, as used herein bisphosphonates include, both geminal and non-geminal bisphophonates.

The P-C-P structure allows a great number of possible variations, either by changing the two lateral chains on the carbon or by esterifying the phosphate groups. A number of bisphosphonates have been investigated in humans with respect to their effects of bone. A number are commercially available for the treatment of bone disease. These include, but are not limited to, alendronate (4-amino-1-hydroxybutyridene)bis-phosphonate, clodronate (dichloromethylene)-bis-phosphonate, EB-1053 (1-hydroxy-3-(1-pyrrolidinyl)-propyridene)bis-phosphonate, etidronate ((1-hydroxyethylidyridene)-bisphosphonate), ibandronate (1-hydroxy-3-(methylpentylamino)propyridene)bis-phosphonate, incadronate, etc.
(([(cycloheptylamino)-methylene]bis-phosphonate), neridronate ((6-amino-1-
hydroxyhexylidene)bis-phosphonate), olpadronate ((3-dimethylamino)-1-
hydroxypropylylidene)bis-phosphonate), palmidronate (3-amino-1-hydroxypropylylidene)bis-
phosphonate), risedronate (1-hydroxy-2-(3-pyridinyl)-ethylidene)bis-phosphonate),
tildronate (((4-chlorophenyl)thio)-methylene)bis-phosphonate), YH 529 ([1-hydroxy-2-
imidazo-(1,2-a)pyridin-3-ylethylidene)bis-phosphonate), and zoledronate (1-hydroxy-2-(1H-
imidazole-1-y)ethylidene)bis-phosphonate), and the like.

The term "bone resorption" refers to a process by which calcified bone tissue
is removed from the bone, e.g. via the activity of osteoclasts. Elevated bone resorption may
result in decreased bone mass and/or bone density (e.g. osteoporosis).

The terms "calcification" refers to the deposition of calcium in a tissue. The
calcium can be in a number of forms, e.g. calcium phosphate, hydroxyapatite, carbonate
apatite, amorphous calcium phosphate, etc.

The phrase "inhibition of calcification" or "inhibiting calcification" refers to a
decrease in the rate and/or degree of calcification of a soft tissue. The inhibition may be
complete or partial. Any measurable inhibition is viewed as an inhibition. A preferred
inhibition is a statistically significant decrease in the rate and/or degree of calcification (e.g.
at the 90% or better, preferably at the 95% or better, more preferably at the 98% or better,
and most preferably at the 99% or better confidence level).

The phrase "without inhibiting bone mineralization" or "without inhibiting
substantial bone mineralization" refers to the use of an agent in a dosage that it typically has
no substantial effect on bone mineralization. In a preferred embodiment, it typically effects
less than a 10%, more preferably less than a 1%, and most preferably less than a 0.1%
decrease in the rate of bone mineralization. More preferably it has no statistically significant
effect on bone mineralization (e.g. at the 90% or better, preferably at the 95% or better, more
preferably at the 98% or better, and most preferably at the 99% or better confidence level).

In a most preferred embodiment there is no detectable effect on bone mineralization.

**BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1 illustrates a number of commercially available bisphosphonates.

Figure 2 illustrates the effect of alendronate treatment on von Kossa staining
for aorta calcification in rats treated with vitamin D plus warfarin. Twelve 7 week old male
Sprague Dawley rats were given subcutaneous injections of 300,000 IU vitamin D/kg body
-8-
weight at $t = 0$, 24, and 48h. Beginning with the first vitamin D injection, rats were also treated with subcutaneous injections of vitamin K every 24h and warfarin every 12h. Six animals were injected subcutaneously with alendronate at a dose of 0.25 mg P/kg/day beginning 4 days prior to the first vitamin D injection, and the remaining 6 animals received no alendronate. All animals were killed 96h after the first vitamin D injection and the abdominal aorta segment between the renal branch and the femoral bifurcation was immediately removed from each animal and fixed in 10% buffered formalin. Longitudinal sections of each aorta were stained for mineral by von Kossa. The panels illustrate typical sections from the 6 rats treated with alendronate and from the 6 animals which did not receive alendronate.

Figure 3 illustrates the effect of bisphosphonate type and dose on the extent of mineral phosphate accumulation in the thoracic aorta of rats treated with vitamin D plus warfarin. Seventy 7 week old male Sprague Dawley rats were given subcutaneous injections of 300,000 IU vitamin D/kg body weight at $t = 0$, 24, and 48h. Beginning with the first vitamin D injection, all rats were also treated with subcutaneous injections of vitamin K every 24h and warfarin every 12h. Twenty-two rats did not receive a bisphosphonate. The remaining 48 rats were divided among 12 treatment groups and each group was given daily subcutaneous injections of the different bisphosphonates at the doses indicated in the Figure beginning 4 days before the first vitamin D injection. All animals were killed 84h after the first vitamin D injection and the thoracic aorta segment between the renal branch and the heart was immediately removed from each animal. The level of phosphate in the acid demineralization extract of each artery is shown for all 70 animals, and the lines are drawn to connect the mean values of aorta phosphate in each treatment group (○, no bisphosphonate; Δ, ibandronate; ▲, alendronate; and ●, etidronate). The level of phosphate in the thoracic aorta of untreated control rats of this age is 445 +/- 104 (X +/- SD) nmol phosphate per thoracic aorta.

Figure 4 illustrates the effect of bisphosphonate type and dose on the extent of mineral phosphate accumulation in the carotid arteries of rats treated with vitamin D plus warfarin. Both carotid arteries were removed from each of the 70 animals in the experiment described in the legend to figure 3, and the level of phosphate in the acid demineralization extract of the two arteries from each of the 70 animals is shown with lines drawn to connect the mean values of carotid phosphate in each treatment group (○, no bisphosphonate; Δ,
ibandronate; ▲, alendronate; and •, etidronate). The level of phosphate in the carotid artery of untreated control rats of this age is 51 +/- 22 nmol phosphate per carotid artery.

Figure 5 illustrates the effect of timing of alendronate administration on von Kossa staining of artery calcification in rats treated with vitamin D plus warfarin. Twenty six 7 week old male Sprague Dawley rats were given subcutaneous injections of 300,000 IU vitamin D/kg body weight at t = 0, 24, and 48h. Beginning with the first vitamin D injection, rats were also treated with subcutaneous injections of vitamin K every 24h and warfarin every 12h. There were four alendronate treatment groups: 11 animals received no alendronate, 6 received subcutaneous injections of alendronate at 0.25 mg P/kg/day starting four days prior to the first warfarin injection with the eighth and last dose at t = 72h; 6 received alendronate at 0.25 mg P/kg/day starting four days prior to the first warfarin injection with the sixth and last dose at t = 24h; and 9 received alendronate on the last two days only, at t=48 and 72h. All animals were killed 96h after the first vitamin D injection and the abdominal aorta segment between the renal branch and the femoral bifurcation was immediately removed from each animal and fixed in 10% buffered formalin. Longitudinal sections of each aorta were stained for mineral by von Kossa. The panels illustrate typical sections from rats that did not receive alendronate (lower right), rats that received alendronate on the last 2 days only (upper right), rats that received alendronate only on the first 6 days (lower left), and rats that received alendronate for all 8 days (upper left).

Figure 6 illustrates the effect of timing of alendronate administration on the extent of mineral phosphate accumulation in the carotid arteries of rats treated with vitamin D plus warfarin. Both carotid arteries were removed from each of the 26 animals in the experiment described in the legend to Figure 5, and the mean level of phosphate in the acid demineralization extract of the two carotid arteries from each animal in the respective 4 treatment groups are shown. No Alendronate, rats that did not receive alendronate; Late Alendronate, rats that received alendronate only on the last 2 days; Early Alendronate, rats that received alendronate only on the first 6 days; Continuous Alendronate, rats that received alendronate for all 8 days. The level of phosphate in the carotid artery of untreated control rats of this age is 51 +/- 22 nmol phosphate per carotid artery.

Figure 7 illustrates the effect of alendronate treatment on von Kossa staining for aorta calcification in rats treated with warfarin for two weeks. Twelve 42 day old male Sprague Dawley rats were treated with warfarin every 12h and with vitamin K every 24h
for 2 weeks. Starting 4 days prior to the first warfarin injection, 4 rats received alendronate at 0.25 mg P/kg/day, 4 rats received ibandronate at 0.01 mg P/kg/day, and 4 rats received no bisphosphonate. The abdominal aorta segment between the renal branch and the femoral bifurcation was removed immediately after the rats were killed and fixed in 10% buffered formalin, and longitudinal sections of each aorta were stained for mineral by von Kossa. The panels illustrate the typical level of calcification seen in the aorta from two animals in each treatment group. No calcification can be detected in untreated control animals at this age.

Figure 8 illustrates the effect of alendronate treatment on von Kossa staining for aorta calcification in rats treated with warfarin for four weeks. Fourteen 42 day old male Sprague Dawley rats were treated with warfarin every 12h and with vitamin K every 24h for 2 weeks. Starting 4 days prior to the first warfarin injection, 3 rats received alendronate at 0.25 mg P/kg/day, 3 rats received alendronate at 0.025 mg P/kg/day, and 8 rats did not receive alendronate. The abdominal aorta segment between the renal branch and the femoral bifurcation was removed at necropsy and fixed in 10% buffered formalin, and longitudinal sections of each aorta were stained for mineral by von Kossa. The panels illustrate the typical level of calcification seen in the aorta from an animal in each treatment group.

DETAILED DESCRIPTION

I. Bisphosphonates and ectopic calcification.

This invention pertains to the discovery that, contrary to prevailing belief, bisphosphonates can be used to prevent calcification of soft tissues at concentrations sufficiently low that the bisphosphonates will not inhibit bone mineralization. Prior to this discovery it was generally believed that bisphosphonates inhibited calcification of soft tissues and bone mineralization through an identical, physiochemical mechanism (e.g. binding to nucleation sites and blocking crystal growth, etc.). Support for this belief was found, in part, in the observation that the first bisphosphonate to be investigated, etidronate, inhibits bone mineralization and soft tissue calcification at about the same high dosage (parenteral etidronate dose of about 5 mg P/kg/day), a dosage which is comparable to the etidronate dosage needed to inhibit bone resorption.

Because of the interest in the use of specific inhibitors of bone resorption to treat clinical disorders such as osteoporosis, a large number of bisphosphonates were subsequently synthesized and tested for their efficacy in inhibiting bone resorption at doses
which do not inhibit bone mineralization. These investigations revealed that
bisphosphonates differ dramatically in the concentration at which they inhibit bone
resorption, with some bisphosphonates 1000 to 10,000 fold more effective on a dose basis
than etidronate itself. In contrast, the newer bisphosphonates and etidronate were found to
inhibit bone mineralization at comparably high doses (parenteral bisphosphonate doses of
about 5 to about 20 mg P/kg/day). The immense difference in the very low effective dose of
the newer bisphosphonates required to inhibit bone resorption, and the comparably high
doses of the same bisphosphonates required to inhibit bone mineralization, has provided
clinicians with a large therapeutic window of bisphosphonate doses that can be used to
inhibit bone resorption without inhibiting bone mineralization.

It is our understanding that, prior to this invention, investigators believed that
all bisphosphonates inhibit bone mineralization and soft tissue calcification by an identical
physicochemical mechanism, and that both processes consequently were believed to require
comparably high doses of the newer bisphosphonates (parenteral bisphosphonate doses of
about 5 to about 20 mg P/kg/day). Because of this belief, no investigator examined the effect
of low doses of the new bisphosphonates to see if it might be possible to inhibit soft tissue
calcification without inhibiting bone mineralization.

It was a discovery of this invention that bisphosphonates appear to inhibit
calcification of soft tissues by a mechanism fundamentally different from the mechanism by
which they inhibit calcification of bone. It was a discovery of this invention that, as
illustrated in Example 1, soft tissue calcification appears to be coupled to bone resorption.
Thus, inhibition of bone resorption, by any mechanism, will result in an inhibition of soft
tissue calcification. Without being bound to a particular theory, it is believed that the bone
resorption process releases calcium phosphate complexes (e.g. a calcium phosphate/protein
complex) that escape into the blood where they can act as nucleation centers to promote
calcification at some site away from the bone (e.g. in an arterial wall, a heart valve, etc.).

In view of this, it was a discovery of this invention that a dosage of
bisphosphonates (or other agent) sufficient to inhibit bone resorption will also inhibit soft
tissue calcification. As indicated above, bisphosphonates all inhibit bone calcification at
approximately the same dosage, while they differ significantly in the dosages required to
inhibit bone resorption. Where the dosage level of the bisphosphonates required to inhibit
bone resorption (and thereby inhibit soft tissue calcification) is lower than the dosage level
required to inhibit bone calcification there will exist a therapeutic window; that is a dosage
range wherein the bisphosphonate will inhibit soft tissue calcification without substantially inhibiting bone calcification.

Thus, in one embodiment, this invention provides methods of inhibiting calcification of soft tissue in a mammal. The methods involve administering to the animal a low dosage of a bisphosphonate, where the low dosage is sufficient to inhibit said calcification, but below the dosage of said bisphosphonate that inhibits normal bone mineralization. Such bisphosphonates can be used to treat a wide variety of disorders characterized by ectopic calcification as described below.

It was a discovery of this invention that inhibition of macrophages engaged in resorption of dystrophic calcifications can prevent and/or inhibit and/or induce regression in atherosclerosis. Without being bound by a particular theory, it is believed that calcification in the artery intima attracts macrophages which are able to actively resorb calcium deposited at this site. Macrophages in the intima then take up oxidized low density lipoproteins (LDLs) and become foamy cells which eventually die, releasing cholesterol in the intima and creating the cholesterol-rich atheroma. Calcification therefore creates a continuing cycle of macrophage recruitment and cholesterol deposition. This is a cycle which can be broken by specifically inhibiting macrophages at the calcification site.

Thus, in one embodiment, this invention provides methods of treating (prophylactically or therapeutically) atherosclerosis. The methods involve inhibiting macrophage-mediated calcium resorption. In a preferred embodiment, this is accomplished by administering an inhibitor that specifically inhibits macrophages involved in calcium uptake. In a particularly preferred embodiment, this is accomplished using a bisphosphonate at a relatively low dosage (e.g. a dosage comparable to that which inhibits bone resorption).

II. Indications.

In view of the foregoing, the methods of this invention are particularly applicable in two contexts: 1) Where the organism (animal or human) is at risk for or has an ectopic calcification; and 2) Where the organism (animal or human) is at risk for, or has, atherosclerosis or arteriosclerosis.

A) Ectopic calcification.

In one embodiment the methods of this invention are used for the treatment (therapeutic or prophylactic) of an organism having, or at risk for, a calcification of a soft
tissue. As used herein, a "soft tissue" refers to a tissue that is not calcified in a normal healthy mammal. Such ectopic calcifications arise in a wide variety of contexts including, but not limited to calcification of one or more heart valves (e.g. aortic valve), calcifications of lymph nodes, renal calcifications (e.g. nephrocalcinosis), calcifications of muscles and/or tendons, calcifications in the gall bladder, calcifications associated with uremia (e.g. associated with end-stage renal disease), certain cancer growths and/or metastases, calcification associated with blood clot formation, and the like.

The frequency of stenosis (associated with heart valve disease) as a cause of heart valve failure is very high, over 75%, and essentially all stenotic valves fail because of calcification. The number of subjects at risk for stenosis and heart valve replacement is fairly high, since it includes all subjects with some extent of heart valve calcification, which is about 30% of human subjects in their 60s. This high incidence of risk for stenoses suggests that the methods of this invention could be used prophylactically to decrease the risk of heart valve failure in all subjects for which there is evidence of progressive valve calcification.

Other ectopic calcifications are associated with trauma, repetitive stress, surgery, and/or biological implants. In particular, biological implants (e.g. prostheses) are vulnerable to undesired calcification. Bioprosthetic devices in which calcification is a serious problem include, but are not limited to porcine and bovine (i.e., exogenous) aortic, pulmonary, and mitral heart valve bioprotheses (e.g., Carpentier-Edwards Standard and Supraanular porcine bioprosthetic valves and Hancock porcine-heterograft bioprosthetic valves), heart valves and other surgical implants made from bovine, porcine, or human pericardium, and human valve homografts/allografts (human cadaver) and autografts (fabricated from the patient's own pulmonary valve, thigh connective tissue, or pericardium, etc.).

Other prosthetic devices in which calcification is a problem include, but are not limited to mechanical heart valves, particularly those made using polyetherurethane and polyetherurethane; other surgical implants made from polyetherurethane and polyetherurethane; silicone implants (including breast implants); and synthetic materials used for repair of aneurisms and other vascular problems.
B) Atherosclerosis and arteriosclerosis.

As indicated above, the methods of this invention are applicable to mammals (e.g. humans) having, or at risk for, atherosclerosis. Atherosclerosis refers to a progressive narrowing and hardening of the arteries over time. More generally, the methods of this invention are applicable to any arteriosclerosis that involves the deposition of calcium in the vascular intima. Thus, the methods of this invention are applicable to atherosclerotic conditions including, but not limited to, atherosclerotic conditions associated with Diabetes mellitus, hypertension, familial hypercholesterolemia, familial combined hyperlipidemia, familial dysbetalipoproteinemia, familial hypoalphaproteinemia, hypothyroidism, cholesterol ester storage disease, systemic lupus erythematosus, and homocysteinemia. In addition, the methods of this invention are applicable to non-atheromatous arteriosclerotic conditions involving calcium deposition including, but not limited to, Diabetes mellitus, chronic renal insufficiency, chronic vitamin D intoxication, Monckeberg's arteriosclerosis, arteriosclerosis, hypertensive arteriosclerosis, pseudoxanthoma elasticum, idiopathic arterial calcification in infancy, aortic valvular calcification in the elderly, and Werner's syndrome.

Differential diagnosis for these conditions and/or for risk of these conditions are well known to medical personnel.

III. Agents for use in this invention.

A) Preferred agents.

Preferred agents for use in the methods of this invention include, but are not limited to, bisphosphonates, more preferably bisphosphonates for which the dosage that inhibits bone resorption is lower than the dosage that inhibits bone calcification. In particularly preferred bisphosphonates the dosage that inhibits bone resorption is distinguishably lower than the dosage that inhibits bone calcification, more preferably there is a statistically significant difference between the dosage that inhibits bone resorption and the dosage that inhibits bone calcification (e.g. at 90% or better, preferably at 95% or better, more preferably at 98% or better, and most preferably at 99% or better confidence level). Most preferred bisphosphonates inhibit bone resorption at a dosage one or more orders of magnitude, preferably at a dosage two or more orders of magnitude, more preferably at a dosage 3 or more orders of magnitude and most preferably at a dosage four or more orders of magnitude lower than the dosage that inhibits bone calcification.
Particularly preferred bisphosphonates are commercially available and include, but are not limited to, alendronate (4-amino-1-hydroxybutylidene)bis-phosphonate, clodronate (dichloromethylene)bis-phosphonate, EB-1053 (1-hydroxy-3-(1-pyrrolidinyl)propylidene)bis-phosphonate, ibandronate (1-hydroxy-3-(methylpentylamino)propylidene)bis-phosphonate, incadronate ((((cycloheptylamino)methylene)bis-phosphonate), neridronate (6-amino-1-hydroxyhexylidene)bis-phosphonate, olpadronate (3-dimethylamino)-1-hydroxypropylidene)bis-phosphonate, palmidronate (3-amino-1-hydroxypropylidene)bis-phosphonate, risedronate (1-hydroxy-2-(3-pyridinyl)ethylidene)bis-phosphonate, tiludronate ((((4-chlorophenyl)thio)methylene)bis-phosphonate), YH 529 (1-hydroxy-2-imidazo-(1,2-a)pyridin-3-ylethylidene)bis-phosphonate, and zoledronate (1-hydroxy-2-(1H-imidazole-1-yl)ethylidene)bis-phosphonate, and the like.

Other bisphosphonates are also known to those of skill in the art. Thus, for example, U.S. Patent 5,317,015 describing the synthesis and use of azacyclic bisphosphonates, and U.S. Patent 5,103,036 describing the preparation of 3-alkenyldiene-1,1-bisphosphonates. Methods of synthesizing numerous other bisphosphonates are well known to those of skill in the art (see, e.g., U.S. Patents 5,622,973, 5,616,571, 5,616,560, 5,403,829, 5,338,731, 5,196,409, and the like).

Methods of assaying new bisphosphonates for use in the methods of this invention are straightforward. In one embodiment, the bisphosphonates is simply assayed for a dosage that inhibits bone resorption and for the dosage at which it inhibits bone calcification as illustrated in the examples, and as taught in the literature (see, e.g., Muhlbauer et al. (1991) J. Bone and Mineral Res. 6: 1003-1010; Antic et al. (1996) Calcif. Tissue Int. 58: 443-448). If the dosage at which the bisphosphonate in question inhibits bone resorption is lower than the dosage at which the bisphosphonate inhibits bone calcification, and toxicity is acceptable, the bisphosphonates is a good candidate for use in the methods of this invention.

**B) Bisphosphonate dosages.**

It was a discovery of this invention that, in contrast to the prevailing belief, bisphosphonates can be administered at low dosages rather than at high dosages to inhibit ectopic calcification and/or to treat atherosclerosis and related conditions. In particular, suitable low dosages are dosages at which the bisphosphonates inhibit bone resorption
without inhibiting bone mineralization. Thus preferred dosages range from a dosage sufficient to inhibit bone resorption and/or ectopic calcification (a minimum therapeutically effective dose) up to a dosage comparable to that used to inhibit bone calcification. Such a dosage range is often at least 10-fold, preferably at least 100-fold, more preferably at least 1000-fold, and most preferably at least 10000-fold less than the dosage that inhibits bone calcification. Dosages that inhibit bone resorption and that inhibit bone calcification will vary with the formulation and mode of administration and can be determined from the product literature for commercially available bisphosphonates. Suitable dosages for other bisphosphonates can be determined empirically.

In the case of alendronate in one preferred embodiment, the aldendronate is administered to humans at an oral dosage ranging from about 5 mg to about 40 mg per day. In the case of ibandronate, in one preferred embodiment, the ibandronate is preferably administered at an intravenous dosage of 1 mg/day. For zoledronate, incadronate, risedronate, EB-1053, nertidronate, olpadronate, pamidronate, YH 529, tiludronate, or clodronate a preferred dosage ranges from the minimum dose that produces a detectable inhibition of bone resorption up to 0.5 mg P/kg/day.

C) Formulation and administration of bisphosphonates.

Acute, sub-acute, and chronic administration of bisphosphonates has, in general, revealed little toxicity. This is generally explained by their rapid incorporation into calcified tissue and hence their short presence in the circulation. Accordingly, a wide variety of formulations and routes of administration are available.

The compounds of the present invention can be administered to a mammalian host in a variety of forms adapted to the chosen route of administration, i.e., orally, or parentally. Parenteral administration in this respect includes, but is not limited to, administration by the following routes: intravenous, intramuscular, subcutaneous, intraocular, intrasynovial, transepithelially including transdermal, ophthalmic, sublingual and buccal; topically including ophthalmic, dermal, ocular, rectal and nasal inhalation via insufflation and aerosol and rectal systemic.

The active compound may be orally administered, for example, when an inert diluent or with an assimilable edible carrier, or it may be enclosed in hard or soft shell gelatin capsules, or it may be compressed into tablets, or it may be incorporated directly with the food of the diet. For oral therapeutic administration, the active compound may be
incorporated with excipient and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 0.1% of active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 0.1% to about 6% of the weight of the unit. The amount of active compound in such therapeutically useful compositions is such that a suitable dosage, as described above, will be obtained. Preferred compositions or preparations according to the present invention are prepared so that an oral dosage unit form contains between about 0.01 mg and about 300 mg of active compound.

The tablets, troches, pills, capsules and the like may also contain the following: A binder such as gum tragacanth, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, lactose or saccharin may be added or a flavoring agent such as peppermint, oil of wintergreen, or cherry flavoring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active compound, sucrose as a sweetening agent, methyl and propylparabens as a preservative, a dye and flavoring such as cherry or orange flavor. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compound may be incorporated into sustained-release preparations and formulations.

The active compound may also be administered parenterally or intraperitoneally. Solutions of the active compound as an ester, a free base or a pharmacologically acceptable salt can be prepared in water or other aqueous solution (e.g. water suitably mixed with a surfactant such as hydroxypropylcellulose). Dispersion can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to
the extent that easy syringability exists. It may be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent of dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimersal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be obtained by the use of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compound in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, typically followed by filtered sterilization. Generally, dispersions are prepared by incorporating the sterilized active ingredient into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze drying technique which yield a powder of the active ingredient plus any additional desired ingredient from previously sterile-filtered solution thereof.

The therapeutic compounds of this invention may be administered to a mammal alone or in combination with pharmaceutically acceptable carriers, as noted above, the proportion of which is determined by the solubility and chemical nature of the compound, chosen route of administration and standard pharmaceutical practice. Thus, for example, to enhance bioavailability of oral formulations, the therapeutic compound may be formulated with a chelator (e.g. EDTA).

The physician will determine the dosage of the present therapeutic agents which will be most suitable for prophylaxis or treatment in accordance with the advantageous low dosages as taught herein and it will vary with the form of administration and the particular compound chosen, and also, it will vary with the particular patient under
treatment. He will generally wish to initiate treatment with small dosages and increase the dosage by small increments until the optimum effect under the circumstances is reached.

The compounds of the invention may also be employed in combination with one or more other active agents. Thus, for example, the compounds described herein may be administered with an antihyperlipoproteinemic agent such as probucol and/or with one or more serum cholesterol lowering agents such as Lopid (gemfibrozil), bile acid sequestrants such as cholestyramine, colestipol, polidexide (DEAE-Sephadex) as well as clofibrate, nicotinic acid and its derivatives, neomycin, p-aminosalicylic acid, bezafibrate and the like and/or one or more HMG CoA reductase inhibitors such as lovastatin, pravastatin, velastatin or simvastatin, etc.

IV. Other inhibitors of calcification.

The methods described herein are not limited to bisphosphonates. As indicated above, it was a discovery of this invention that essentially any agent that inhibits osteoclastic bone resorption at a dosage that does not also substantially inhibit bone calcification will also inhibit ectopic calcification. Thus, other agents (e.g. non-bisphosphonates) that inhibit osteoclastic bone resorption are good candidates for use in the methods of this invention. Such agents can be identified by routine screening e.g. as illustrated in the Examples.

Similarly, it was a discovery of this invention that agents that inhibit macrophage-mediated removal of mineral will also inhibit the development and/or progression of atherosclerosis. Thus agents that inhibit macrophage activity (e.g. genisteine), more preferably agents that specifically inhibit macrophages involved in the uptake of calcium (e.g. by rapidly associating with calcium so that they are internalized by this subset of macrophages) are good candidates for the methods of this invention.

V. Kits.

In still another embodiment, this invention provides kits for inhibiting ectopic calcification and/or atherosclerosis. The kits include one or more bisphosphonates preferably formulated as pharmaceuticals (e.g. in a pharmacologically acceptable excipient). In addition, the kits preferably include instructional materials containing directions (i.e., protocols) describing the indications for the use of the compositions and recommending dosages as described herein. While the instructional materials typically comprise written or
printed materials they are not limited to such. Any medium capable of storing such instructions and communicating them to an end user is contemplated by this invention. Such media include, but are not limited to electronic storage media (e.g., magnetic discs, tapes, cartridges, chips), optical media (e.g., CD ROM), and the like. Such media may include addresses to internet sites that provide such instructional materials.

VI. Fetuin complexes

It was also a discovery of this invention that the serum protein fetuin forms a stable complex with a calcium phosphate mineral phase and that this complex can under some circumstances be detected in blood. Without being bound to a particular theory it is believed that the fetuin/calcium phosphate complex is a form in which calcium removed during bone resorption is solubilized in plasma and migrates to new sites where it can act as a nucleation site for calcium deposition and thereby contribute to ectopic calcification and to atherosclerotic plaque formation.

The fetuin-mineral complex can be synthesized using pure fetuin, calcium, and phosphate (see, Example 2). In brief, the procedure allows the synthesis of small mineral particles of uniform size which can be seen by transmission electron microscopy. Because the size of the fetuin mineral complex is very small, a solution containing very high concentrations of the fetuin mineral complex is quite clear and the complex does not settle. The particles are stable, with no apparent changes over 7 days of observation. We believe that the mineral phase trapped by this complex is the first phase formed in calcification of bone and teeth. Prior to this invention, there was no method for preparing this phase in a stable form, and no method for preparing a solution containing this phase at uniform concentration.

The formation of such complexes is readily demonstrated. When concentrated solutions of calcium and phosphate are mixed to create a final mixture containing e.g., 40 mM Calcium and 40 mM phosphate, a dense white precipitate forms within a fraction of a second which slowly sinks to the bottom the test tube. If fetuin is added prior to mixing, the dense white precipitate fails to form and the solution remains quite clear for days. If one looks at the solution by electron microscopy, numerous small mineral nuclei are present which have remarkably uniform size and shape. The nuclei, which are coated with fetuin, account for over 95 % of the calcium and phosphate in the mixture. This
experiment illustrates the power of the fetuin molecule to direct the course of a mineralization process.

This discovery can be exploited in a number of contexts. For example, this discovery provides a general method for the preparation of any unstable mineral phase by using a protein which binds to this mineral phase selectively in order to trap the unstable phase and prevent its transformation to more stable phases. A fetuin mineral complex can be used to distribute mineral nuclei within a suitable matrix so that subsequent inactivation of fetuin (e.g. by heat, acid, addition of a chaotropic agent, etc.) would cause rapid and uniform calcification of this matrix. This method could be used, for example, to prepare a calcified structure for use in a bio prosthetic device or other device.

Because the fetuin mineral complex is stable in blood, it can be used as a transport vehicle to deliver calcification initiators to desired sites in the body. For example, the fetuin in the complex could be modified so that it binds to a site where calcification is desired (e.g. teeth, bone, etc.) and so that fetuin can be inactivated at this site to allow mineralization to proceed. Typically such a modification would involve coupling a targeting molecule (e.g., an antibody, antibody fragment, single chain antibody, a lectin, a lipid, a carbohydrate, a sugar, etc.) to the fetuin-mineral complex. The targeting molecule is selected to specifically bind to the target (e.g. cell receptor, ligand, etc.) whereby the mineral complex is delivered to the desired target.

It is noted that fetuin is a glycoprotein and methods of attaching molecules to glycoproteins (directly or through a linker) are well known to those of skill in the art. The attachment is preferably by way of a linker. A "linker" as used herein, is a molecule that is used to join the targeting molecule to the fetuin-mineral complex. The linker is capable of forming covalent bonds to both the fetuin and to the targeting molecule. Suitable linkers are well known to those of skill in the art and include, but are not limited to, straight or branched-chain carbon linkers, heterocyclic carbon linkers, or peptide connectors. The linkers may be joined to the constituent amino acids through their side groups (e.g., through a disulfide linkage to cysteine) or joined to the alpha carbon amino and carboxyl groups of the terminal amino acids.

Many procedures and linkers molecules for attachment of various polypeptides are known (see, e.g., European Patent Application No. 188,256; U.S. Patent Nos. 4,545,985 and 4,894,443, 4,671,958, 4,659,839, 4,414,148, 4,699,784; 4,680,338;

The fetuin mineral complex can also be used as a reagent to develop fetuin-mineral specific assays which, in turn, can be used to determine the levels of a fetuin mineral complex in human blood. This would provide a method to measure bone metabolic processes relevant to the management of patients with bone disease.

Without being bound to a particular theory, it is believed that a surface of the fetuin molecule binds strongly and specifically to the target mineral phase. This binding exposes surfaces on fetuin which have a high affinity for other bound fetuin molecules, forming strong lateral associations that arrest crystal growth. The oligosaccharide moieties in fetuin, which account for about half of its mass, project away from mineral and form a hydrated shell which keeps the fetuin mineral complex from aggregating or settling from solution. This model suggests that engineered modifications in the mineral interaction surface of fetuin could direct the protein to any desired mineral phase, thereby enabling the protein to control the synthesis of this mineral phase.

EXAMPLES

The following examples are offered to illustrate, but not to limit the claimed invention.

**Example 1**

**Bisphosphonates prevent artery calcification at doses that inhibit bone resorption, but not bone mineralization.**

The present experiments were carried out to test the hypothesis that bisphosphonates inhibit artery calcification by virtue of their ability to inhibit bone resorption. The hypothesis that bone resorption is linked with artery calcification originated in experiments carried out to understand the factors which enhance artery calcification in rats treated with high doses of warfarin, a vitamin K antagonist which inhibits the γ-carboxylation of matrix Gla protein and thereby causes arteries and other soft tissues to calcify. In the course of these studies we observed that warfarin treatment induces artery calcification to the greatest extent in young, rapidly growing rats, and that adult rats are completely resistant to warfarin induced artery calcification. The susceptibility of young rats to warfarin induced artery calcification is related to growth and not age per se, since warfarin
treatment fails to induce artery calcification in young rats fed a restricted diet with a caloric content adequate to maintain body weight without permitting bone growth or weight gain. These experiments showed that growth processes promote artery calcification, and were consistent with the hypothesis that bone metabolism could in fact be the critical determinant for susceptibility to warfarin-induced artery calcification. In a second series of experiments we observed that high doses of vitamin D accentuate artery calcification in rats treated with warfarin. Since vitamin D is known to potently stimulate bone resorption, one explanation for the increased susceptibility of vitamin D treated rats to warfarin-induced artery calcification could be a link between bone resorption and artery calcification.

**Background for these experiments.**

Bisphosphonates have been previously shown to inhibit bone resorption, normal bone mineralization, and experimentally induced artery calcification (see Fleisch (1998) Endocrine Rev.19: 80-100 for a recent review). The first bisphosphonate to be studied thoroughly, etidronate, inhibits bone resorption, normal bone mineralization, and artery calcification at comparably high doses. Because of the interest in the use of specific inhibitors of bone resorption to treat clinical disorders such as osteoporosis, a large number of bisphosphonates have been synthesized and tested for their efficacy in inhibiting bone resorption at doses which do not inhibit bone mineralization. Among the bisphosphonates currently in clinical use, two of the most potent bone resorption inhibitors are alendronate and ibandronate, which are 1000 and 10000 fold more effective resorption inhibitors than etidronate, respectively. Since all bisphosphonates inhibit bone mineralization at comparably high doses (Fleisch (1998) Endocrine Rev.19: 80-100; Fleisch (1997) Ann. Med. 29: 55-62), the discovery that alendronate and ibandronate are far more potent bone resorption inhibitors allows the inhibition of bone resorption in patients using doses of these drugs which do not affect normal mineralization processes.

The mechanisms by which bisphosphonates inhibit mineralization processes *in vivo* are poorly understood. Bisphosphonates bind strongly to hydroxyapatite, the mineral phase of bone, and are cleared rapidly from blood by virtue of their ability to bind to bone mineral (*Ibid.*). *In vitro*, all bisphosphonates potently inhibit formation of calcium phosphate mineral phases from supersaturated solutions of calcium and phosphate, and the concentrations of different bisphosphonates that are required to inhibit calcification *in vitro* are comparable (*Ibid.*). *In vivo*, all bisphosphonates potently inhibit normal mineralization of
bone and other structures, and the concentrations of different bisphosphonates that are required to inhibit normal mineralization are comparable and quite high (parenteral bisphosphonate doses of 5 to 20 mg P/kg/day) (Fleisch (1998) Endocrine Rev.19: 80-100). Because all bisphosphonates inhibit normal bone mineralization in vivo at comparably high doses, and all bisphosphonates inhibit formation of hydroxyapatite from supersaturated solutions in vitro at comparable concentrations, it has been suggested that both actions reflect the same basic ability of these compounds to bind to hydroxyapatite crystals and inhibit crystal growth by a physicochemical mechanism.

Investigators have held that the inhibition of experimentally induced artery calcification by bisphosphonates is also related to the ability of these compounds to bind to hydroxyapatite crystals and inhibit crystal growth in vitro, and to the ability of these compounds to inhibit normal bone mineralization in vivo, and have indeed stated that it would be impossible to use such drugs to inhibit calcification of arteries and other soft tissues without also inhibiting normal mineralization process (Fleisch (1998) Endocrine Rev.19: 80-100; Fleisch (1997) Ann. Med. 29: 55-62). In the present study we have for the first time demonstrated that bisphosphonates inhibit artery calcification by a different mechanism than the mechanism by which they inhibit normal bone mineralization, namely by inhibiting bone resorption. It is therefore possible to use the newer class of more potent bone resorption inhibitors, such as alendronate and ibandronate, to inhibit calcification of arteries and heart valves without affecting normal mineralization processes.

**Experimental procedures.**

**Materials.**

Vitamin K₁ (phyloquinone), vitamin D₃ (cholecalciferol), and warfarin were purchased from Sigma (St. Louis, Mo). Etidronate (Didronel, Proctor and Gamble Pharmaceuticals) and alendronate (Fosamax, Merck and Co., Inc.) were purchased from University City Pharmacy, San Diego, Ca., and Ibandronate (Bondronat, Boehringer Mannheim) was purchased from Idis World Medicines, Surrey, United Kingdom. Stock solutions of alendronate and etidronate were prepared in 0.15 M NaCl, titrated to pH7.4 with NaOH, and stored at 4° C. Ibandronate was diluted with 0.15M NaCl and stored at 4° C.

All bisphosphonate doses are stated in mg P so that the molar effectiveness of the drugs can be compared directly, a method which has been employed in earlier studies (Muhlbauer et al.
(1991) *J. Bone and Mineral Res.* 6: 1003-1010; Antic *et al.* (1996) *Calcif. Tissue Int.* 58: 443-448). The following values were used to convert from actual measured weight of bisphosphonate to mg P for each drug used: Alendronate (Na)(H$_2$O)$_3$ = 62 mg P per 325 mg drug, etidronate (Na)$_2$ = 62 mg P per 250 mg drug, and ibandronate (Na)(H$_2$O) = 62 mg P per 357 mg drug. Stock solutions of vitamin K$_1$ were prepared at 10 mg per ml and stored in sterile, foil wrapped containers at 4°C. Stock solutions of sodium warfarin were prepared at 50 mg per ml in 0.15M NaCl and stored at 4°C. Finally, stock solutions of vitamin D were prepared fresh for each 3 day subcutaneous injection cycle at a concentration of 1.65 mg/ml in 7% emulphor (alkamuls EL-620, Rhone-Poulenc) and then wrapped in foil and stored at 4°C. Simonsen albino rats (Sprague-Dawley derived) were purchased from Simonsen labs (Gilroy, Ca).

**Methods.**

For measurement of mineral accumulation in arteries, each tissue was removed within 30 minutes of death and immediately frozen. Tissues were subsequently washed extensively with buffer and extracted with 1 ml of 10% formic acid for 24h at room temperature, as described. Calcium levels in serum were determined colorimetrically using cresolphthalein complexone (Sigma) and phosphate levels in serum and in acid tissue extracts were determined colorimetrically as described (Zhu *et al.* (1994) *Cardiology*, 85: 370-377). Tissue sectioning and staining were carried out by Biomedical Testing Services, Inc., (San Diego, Ca).

Male Sprague Dawley rats were fed ad libitum with rodent diet 5001 (Purina Mills Inc., St. Louis, Mo), a diet that is 0.67% phosphorus and 0.95% calcium by weight. This diet contains 500µg per kg of phylloquinone and has no added menadione. In all experiments, animals were killed by exsanguination while under metofane anesthetic, and selected tissues were removed immediately and fixed in 10% buffered formalin or frozen at -20°C for later studies. All animal experiments were approved by the UCSD animal subjects committee.

The effect of bisphosphonates on artery calcification was first examined in rats in which artery calcification was induced by treatment with warfarin plus high doses of vitamin D. In brief, 49 day old male rats received subcutaneous doses of 300,000 IU vitamin D per kg at t = 0, 24, and 48h. Starting at t = 0, each animal also received injections of warfarin every 12h and of vitamin K every 24h. All animals were killed by
exsanguination at 96h. In the initial experiment (Figure 2), 6 rats were treated with alendronate at a dose of 0.25 mg P/kg/day starting four days prior to the first vitamin D injection, and 6 rats received no alendronate. All animals were killed by exsanguination at 96h after the first vitamin D dose. In the dose dependence experiments, animals were given identical treatment with warfarin, vitamin K, and vitamin D together with the desired dose and type of bisphosphonate (4 rats per dose) starting 4 days prior to the first vitamin D injection; all animals in the dose dependence experiments (Figures 3 and 4, and Table III) were killed by exsanguination at 84h after the first vitamin D dose. In the experiments on the effect of the timing of alendronate administration on artery calcification (Figures 5 and 6), animals were again given identical treatment with warfarin, vitamin K, and vitamin D together with the following treatment with alendronate at a dose of 0.25 mg P/kg/day: Group A received no alendronate (11 rats); Group B received alendronate continuously for 8 days, starting 4 days prior to the first vitamin D injection (6 rats); Group C received alendronate for 6 days, starting 4 days prior to the first vitamin D treatment and ending with the final dose on the second day of vitamin D treatment (at t = 24h) (6 rats); and Group D received alendronate only for the last two days of the 8 day experiment (at t = 48 and 72h) (9 rats). All animals in the experiments on the timing of alendronate dose were killed by exsanguination 96h after the first vitamin D injection. In the final experiment, 49 day old male rats received subcutaneous doses of 300,000 IU vitamin D per kg at t = 0, 24, and 48h but did not receive warfarin; 4 rats received alendronate at a daily dose of 0.25 mg P/kg/day starting 4 days prior to the first vitamin D injection, 4 rats received ibandronate at dose of 0.01 mg P/kg/day starting 4 days prior to the first vitamin D injection, and 4 rats did not receive bisphosphonate. All animals were killed by exsanguination 96h after the first vitamin D injection.

The effect of bisphosphonates on artery calcification was also examined in rats treated with warfarin alone using procedures which have been described elsewhere (Price et al. (1998) Arterioscler. Thromb. Vasc. Biol. 18: 1400-1407). This procedure induces artery calcification within 2 weeks without the presence of hypercalcemia. In the first series of experiments, 42 day old male rats were treated with warfarin for two weeks and with bisphosphonates beginning 4 days prior to the first warfarin dose according to the following doses: 8 rats received no bisphosphonate; 4 rats received alendronate at 0.25 mg P/kg/day; and 4 rats received ibandronate at 0.01 mg P/kg/day. In the second series of experiments, 42 day old male rats were treated with warfarin for 4 weeks and with
bisphosphonates beginning 4 days prior to the first warfarin dose according to the following doses: 8 rats received no bisphosphonate; 3 rats received alendronate at 0.25 mg P/kg/day; 3 rats received alendronate at 0.025 mgP/kg/day; and 4 rats received etidronate at 6.25 mg P/kg/day.

5 Results

Effect of bisphosphonates on artery calcification in rats treated with high doses of vitamin D.

To evaluate the possible relationship between bone resorption and artery calcification we initially examined the effect of alendronate, a bisphosphonate which potently inhibits bone resorption, on artery calcification in rats treated with high doses of vitamin D. In most of these experiments we also treated the animals with the vitamin K antagonist warfarin in order to suppress the γ-carboxylation of matrix Gla protein, a vitamin K-dependent inhibitor of artery calcification, and thereby enhance the extent of artery calcification compared to that seen in animals treated with high doses of vitamin D alone. In previous studies we have shown that treatment with vitamin D plus warfarin causes rapid and massive calcification of the elastic lamellae of the aorta and other arteries, and that significant calcification is apparent 72h after the first vitamin D injection.

In the initial experiments, we injected 49 day old male rats subcutaneously with alendronate at a dose of 0.25 mg P/kg/day. This dose is comparable to the 0.3 mg P/kg/day subcutaneous dose of alendronate which that has been demonstrated to completely inhibit the increase in bone resorption induced by a calcium deficient diet in 58 day old male rats (Antic et al. (1996) Calcif. Tissue Int. 58: 443-448). The daily alendronate treatment was initiated four days prior to the first vitamin D injection because previous studies have shown that it takes about 4 days of alendronate treatment to maximally inhibit bone resorption (Figure 3 in Antic et al. (1996) Calcif. Tissue Int. 58: 443-448). When the animals were examined 96h after the first vitamin D injection there was no detectable von Kossa staining for mineral in the abdominal aorta of any of the 6 animals treated with vitamin D plus alendronate, while there was massive von Kossa staining for mineral in the elastic lamella of the abdominal aorta media in all 6 of the animals treated with vitamin D plus vehicle (Figure 2). Alendronate treatment also completely eliminated von Kossa staining in the elastic lamella of aortic heart valves and in the elastic lamella of the media in
all other arteries examined, which included the renal, pulmonary, and carotid arteries (Figures not shown). In addition, alendronate treatment eliminated the von Kossa staining of the kidney, a calcification which we have observed in rats treated with high doses of vitamin D plus warfarin that is not associated with arteries. Quantitative analysis of the extent of mineral accumulation revealed that the acid demineralization extracts of the thoracic aorta and carotid arteries of the vitamin D treated animals had calcium and phosphate levels which were at least 40 times higher than found in the corresponding tissues from control rats, while the acid demineralization extracts of the thoracic aorta and carotid artery of animals treated with vitamin D plus alendronate had calcium and phosphate levels which were not significantly elevated compared to levels in control tissues (data not shown).

Previous studies have shown that treatment with high doses of vitamin D alone is highly toxic to animals (Takeo et al. (1989) Atherosclerosis, 77: 175-181; Takeo et al. (1991) Molec. Cell. Biochem. 107: 169-183), and that concurrent treatment with warfarin augments the lethal nature of vitamin D administration. To examine the relationship between alendronate treatment and mortality, animals were treated with vitamin D plus warfarin and given either alendronate at a dose of 0.25 mg P/kg/day or vehicle starting 4 days prior to the first vitamin D injection. All 4 of the animals treated with vehicle plus vitamin D were dead within 6 days of the first vitamin D administration, while all 4 of the animals treated with alendronate plus vitamin D were alive and healthy at day 12, the end of the period of observation. We conclude that mortality in animals treated with high doses of vitamin D is prevented by treatment with alendronate, and that the probable cause of death in animals treated with high doses of vitamin D is the calcification of some as yet unidentified structure which is critical for life.

In the next series of experiments, we established the dependence of artery calcification in vitamin D treated rats on the dose of three bisphosphonate inhibitors of bone resorption, etidronate, alendronate, and ibandronate. In these experiments we examined the extent of artery calcification at 84h after the first vitamin D dose rather than at 96h because of the significant mortality observed in rats treated with vitamin D plus warfarin at the 96h time point. All three bisphosphonates dramatically inhibited artery calcification, and the dose of bisphosphonate required for inhibition correlated with the known differences in the potency of these drugs as inhibitors of bone resorption. Ibandronate and alendronate completely eliminated von Kossa staining for mineral in the abdominal aorta at doses of ibandronate of 0.0018 mg P/kg/day and above and at an alendronate dose of 0.25 mg
P/kg/day, and etidronate significantly reduced the extent of von Kossa staining in the abdominal aorta at the highest dose tested, 6.25 mg P/kg/day (Table I). Quantitative analysis of the accumulation of mineral phosphate in the acid demineralization extracts of the thoracic aorta and of the two carotid arteries revealed that the dose of bisphosphonate required to reduce the extent of mineralization by half in the thoracic aorta is 0.0002 mg P/kg/day for ibandronate, 0.005 for alendronate, and 2 for etidronate (Figure 3) and that the dose required to reduce the extent of mineralization by half in the carotid artery is 0.00018 mg P/kg/day for ibandronate, 0.005 for alendronate, and 2 for etidronate (Figure 4). The level of mineral phosphate in the acid demineralization extracts of the thoracic aorta and of the two carotid arteries at the two highest doses of alendronate and ibandronate were not significantly above control values, which were 445 +/- 104 (X +/- SD) nmol phosphate per thoracic aorta and 51 +/- 22 nmol phosphate per carotid artery.

Table I. Effect of bisphosphonate dose on the extent of von Kossa staining for calcification in the thoracic aorta of rats treated with vitamin D plus warfarin (see legends to Figures 3 and 4) Seventy 7 week old male Sprague Dawley rats were given subcutaneous injections of 300,000 IU vitamin D/kg body weight at t = 0, 24, and 48h. Beginning with the first vitamin D injection, all rats were also treated with subcutaneous injections of vitamin K every 24h and warfarin every 12h. Twenty two rats did not receive a bisphosphonate. The remaining 48 rats were divided among 12 treatment groups and each group was given bisphosphonates at the doses indicated in the Table beginning 4 days before the first vitamin D injection. All animals were killed 84h after the first vitamin D injection and the abdominal aorta segment between the renal branch and the femoral bifurcation was immediately removed from each animal and fixed in 10% buffered formalin. Longitudinal sections of each abdominal aorta were stained for mineral by von Kossa, and all sections from each of the 70 animals were examined blindly by two observers, and the extent of calcification was scored with 5 as most calcified and with 0 as no calcification. (n) = number of rats in the indicated treatment group.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>Observer #1</th>
<th>Observer #2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>x ± S.D.</td>
<td>x ± S.D.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Range</td>
<td>Range</td>
</tr>
<tr>
<td>Bisphosphonate</td>
<td>Dosage (mg P/kg/day)</td>
<td>Calcium (%)</td>
<td>Bone Loss (%)</td>
</tr>
<tr>
<td>---------------</td>
<td>---------------------</td>
<td>-------------</td>
<td>---------------</td>
</tr>
<tr>
<td>No Bisphosphonate</td>
<td>22</td>
<td>3.2 ± 1.5</td>
<td>0.0-5</td>
</tr>
<tr>
<td>Ibandronate:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.00036</td>
<td>4</td>
<td>1.0 ± 0.8</td>
<td>0-2</td>
</tr>
<tr>
<td>0.0018</td>
<td>4</td>
<td>0.0 ± 0.0</td>
<td>0-0</td>
</tr>
<tr>
<td>0.009</td>
<td>4</td>
<td>0.0 ± 0.0</td>
<td>0-0</td>
</tr>
<tr>
<td>0.045</td>
<td>4</td>
<td>0.0 ± 0.0</td>
<td>0-0</td>
</tr>
<tr>
<td>Alendronate:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.0004</td>
<td>4</td>
<td>2.8 ± 1.0</td>
<td>2-4</td>
</tr>
<tr>
<td>0.002</td>
<td>4</td>
<td>2.5 ± 0.6</td>
<td>2-3</td>
</tr>
<tr>
<td>0.010</td>
<td>4</td>
<td>2.0 ± 1.8</td>
<td>1-5</td>
</tr>
<tr>
<td>0.050</td>
<td>4</td>
<td>0.8 ± 0.5</td>
<td>0-1</td>
</tr>
<tr>
<td>0.25</td>
<td>4</td>
<td>0.0 ± 0.0</td>
<td>0-0</td>
</tr>
<tr>
<td>Etidronate:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.25</td>
<td>4</td>
<td>3.5 ± 1.3</td>
<td>2-5</td>
</tr>
<tr>
<td>1.25</td>
<td>4</td>
<td>1.8 ± 2.4</td>
<td>0-5</td>
</tr>
<tr>
<td>6.25</td>
<td>4</td>
<td>0.3 ± 0.5</td>
<td>0-1</td>
</tr>
</tbody>
</table>

The dose of bisphosphonate required to inhibit vitamin D-induced artery calcification is compared with the dose of bisphosphonate required to inhibit arthrotinoid-induced bone resorption in Table II. As seen, the relative potency of etidronate, alendronate, and ibandronate as inhibitors of artery calcification parallels the relative potency of these drugs as inhibitors of bone resorption. The absolute parenteral dose of alendronate and ibandronate needed to inhibit artery calcification by half is actually about 2- to 5-fold lower than the dose required to inhibit arthrotinoid-induced bone resorption by half. Taken together, these comparisons strongly suggest that the actions of bisphosphonates on bone resorption and artery calcification are linked. The most reasonable hypothesis is that artery calcification is in fact linked to bone resorption.
Table II. A comparison of the dose dependence of the effects of bisphosphonates on artery calcification and on bone resorption. The data for the effect of daily subcutaneous dose of bisphosphonate on vitamin D-induced artery calcification is taken from figures 2 and 3. The data for the effect of daily subcutaneous dose of alendronate and ibandronate on arotinoid-induced bone resorption are from Figure 3 in (Muhlbauer et al. (1991) J. Bone and Mineral Res. 6: 1003-1010), and the relative antiresorption potency of bisphosphonates in the rat is from Table I in (Muhlbauer et al. (1991) J. Bone and Mineral Res. 6: 1003-1010). Note that the bone resorption studies were carried out in male rats initially 200-230g, and the vitamin D induced artery calcification studies presented here were carried out in male rats initially 200g.

<table>
<thead>
<tr>
<th>Bisphosphonate</th>
<th>Artery Calcification</th>
<th>Bone Resorption</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dose for 50% inhibition (mg P/kg/day)</td>
<td>Relative potency</td>
</tr>
<tr>
<td>Etidronate</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Alendronate</td>
<td>0.005</td>
<td>400</td>
</tr>
<tr>
<td>Ibandronate</td>
<td>0.00019</td>
<td>10500</td>
</tr>
</tbody>
</table>

A potentially trivial explanation for the correlation between the bisphosphonate doses required to inhibit artery calcification and bone resorption could be that the hypercalcemia induced by high doses of vitamin D, a potent stimulator of bone resorption, might be reduced by bisphosphonate treatment. As seen in Table III, however, none of the bisphosphonates tested here significantly reduced the increased level of serum calcium caused by vitamin D treatment, which remained at 40% above normal serum calcium levels at all bisphosphonate doses tested. We therefore conclude that the effectiveness of bisphosphonates as inhibitors of artery calcification in the vitamin D-treated rat is not due to a simple reduction in the extent of hypercalcemia induced by treatment with vitamin D. Bisphosphonate treatment did significantly reduce the level of serum phosphate, but the magnitude of the reduction was only about 16% for the two highest doses of alendronate and 11% for the two highest doses of ibandronate (Table III). In repeat experiments using the 0.25 mg P/kg/day dose of alendronate, no significant reduction in serum calcium or phosphate could be demonstrated at 48h, 60h, and 72h after the first
vitamin D injection, and so the reduction in serum phosphate levels in the bisphosphonate treated animals occurs relatively late in the time course of vitamin D induced artery calcification.

5 **Table III.** Effect of bisphosphonate type and dose on serum levels of calcium and phosphate in rats treated with vitamin D plus warfarin. See legend to Table I for a more detailed description of this experiment. Serum calcium and phosphate levels were determined using blood removed from all 70 animals at the end of the experiment, 84h after the first vitamin D injection. The values given are the mean and standard deviation for serum calcium and phosphate levels in the animals in each bisphosphonate treatment group. *, p < 0.05 when compared with the W/D control; **, p < 0.025 when compared with the W/D control; ***, p < 0.001 when compared with the W/D control.

<table>
<thead>
<tr>
<th>Bisphosphonate</th>
<th>Dose (mg P/kg/day)</th>
<th>Serum Ca (mg/dL)</th>
<th>Serum P (mg/dl)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Etidronate</td>
<td>6.25</td>
<td>15.0 ± 0.5</td>
<td>8.2 ± 0.4 ****</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>1.25</td>
<td>13.5 ± 0.7</td>
<td>9.5 ± 0.3 **</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>13.7 ± 1.2</td>
<td>10.8 ± 0.5</td>
<td>4</td>
</tr>
<tr>
<td>Alendronate</td>
<td>0.25</td>
<td>14.5 ± 0.1</td>
<td>9.5 ± 0.7 **</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>15.5 ± 0.4</td>
<td>9.9 ± 0.5 *</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>15.9 ± 0.4</td>
<td>10.0 ± 0.9</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>0.002</td>
<td>13.6 ± 0.2</td>
<td>10.2 ± 0.6</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>0.0004</td>
<td>13.3 ± 0.7</td>
<td>10.8 ± 1.2</td>
<td>4</td>
</tr>
<tr>
<td>Iblandronate</td>
<td>0.045</td>
<td>15.4 ± 0.3</td>
<td>9.9 ± 0.4 *</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>0.009</td>
<td>14.9 ± 0.6</td>
<td>10.4 ± 0.7</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>0.0018</td>
<td>15.5 ± 0.9</td>
<td>10.3 ± 0.6</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>0.00036</td>
<td>13.8 ± 0.6</td>
<td>9.9 ± 0.5 *</td>
<td>4</td>
</tr>
<tr>
<td>W/D Control</td>
<td>0</td>
<td>14.5 ± 1.1</td>
<td>11.4 ± 1.4</td>
<td>22</td>
</tr>
</tbody>
</table>

We employed concurrent treatment with the vitamin K antagonist warfarin in all of the experiments discussed above in order to enhance the extent of artery calcification compared to that seen in animals treated with vitamin D alone. In order to establish that the effectiveness of bisphosphonates as inhibitors of artery calcification is not due to a possible interaction between the bisphosphonate and warfarin, we also examined the effectiveness of bisphosphonates as inhibitors of artery calcification in animals treated with vitamin D that were not also treated with warfarin. These experiments demonstrated that 0.01 mg P/kg/day of iblandronate and 0.25 mg P/kg/day of alendronate completely eliminated all von Kossa
staining for mineral in the aorta and carotid arteries of the 4 animals in each group when examined at 96h after the first vitamin D injection, while the 4 animals which did not receive bisphosphonate all had extensive artery calcification (figure not shown). We therefore conclude that the effectiveness of bisphosphonates as inhibitors of artery calcification is equivalent in animals treated with vitamin D alone and in animals treated with vitamin D plus warfarin.

**Effect of timing of alendronate administration on the inhibition of artery calcification in vitamin D- treated rats.**

To further address the mechanism by which bisphosphonates inhibit artery calcification, we examined the relationship between the timing of alendronate administration and the extent to which alendronate inhibits artery calcification. The strategy of this experiment was to adjust the timing of alendronate administration so that one group would receive alendronate only during the period prior to artery calcification and another group would receive alendronate only during the period in which artery calcification actually occurs. Animals were given the same doses of vitamin D and warfarin and were divided into four treatment groups based on the timing of the 0.25 mg P/kg/day dose of alendronate: Group A received no alendronate; Group B received alendronate continuously for 8 days, starting 4 days prior to the first vitamin D injection; Group C received alendronate for 6 days, starting 4 days prior to the first vitamin D treatment and ending with the final dose on the second day of vitamin D treatment (at t = 24h); and Group D received alendronate only for the last two days of the 8 day experiment (at t = 48 and 72h).

Histochemical examination of mineralization in the abdominal aorta using the von Kossa stain revealed massive calcification in the 11 animals treated with vitamin D alone (group A), reduced levels of calcification in the 9 animals treated with alendronate for the last 2 days of the 8 day experiment (group D), and no evidence of calcification in the 6 animals treated with alendronate for the first 6 days only (group C) and in the 6 animals treated with alendronate for the entire 8 days (group B) (Figure 5). Quantitative analysis of the accumulation of mineral phosphate in the acid demineralization extracts of the carotid arteries revealed essentially identical results, with very high levels of mineral in the carotid arteries of animals which received vitamin D and no alendronate (Figure 6), intermediate levels of mineral in the carotid arteries of rats treated with alendronate for the last 2 days of the 8 day experiment (group D), and control levels of mineral in the carotid arteries of rats.
treated continuously for 8 days with alendronate (group B) and in rats treated with
alendronate for the first 6 days only (group C).

The absence of artery calcification in the animals in group C may be revealing as to the mechanism by which bisphosphonates inhibit artery calcification. Since alendronate is cleared rapidly from serum and so would not be expected to be present in the blood of animals in group C during the actual period in which mineralization occurs, the group C results indicate that alendronate need not be present during the actual progression of artery calcification in order to inhibit the calcification process. It is also worth noting that alendronate need not be present during the period of vitamin D-induced hypercalcemia, since serum calcium levels are normal at the time of the last alendronate administration to group C, which is 24h after the first vitamin D injection, and subsequently rise to 21% above normal at 48h and to 40% above normal at 72 and 96h. We believe that the absence of artery calcification in the animals in group C is consistent with the hypothesis that alendronate inhibits artery calcification by virtue of its ability to inhibit bone resorption, since treatment of animals in group C with alendronate for the first 6 days should inhibit resorption through the last two days of the experiment due to the long term action of the drug on osteoclasts (Figure 6 in Antic et al. (1996) Calcif. Tissue Int. 58: 443-448).

It should be noted that treatment with alendronate during the actual period in which mineralization occurred, the last two days of the experiment, did not completely inhibit artery calcification (Figures 5 and 6). This result is also consistent with the hypothesis that alendronate acts by inhibiting bone resorption, since resorption will be only partially inhibited by two days of treatment with this drug (Antic et al. (1996) Calcif. Tissue Int. 58: 443-448). This result is not, however, consistent with the hypothesis that alendronate inhibits artery calcification by virtue of its ability to interact directly with mineral surfaces and so inhibit crystal growth by a direct physicochemical mechanism, since the animals in group D received alendronate throughout the entire period in which artery calcification actually occurred (that is, 48 to 96h after the first vitamin D injection) and nevertheless had significant artery calcification.

**Effect of bisphosphonates on artery calcification in rats treated with warfarin.**

In order to examine the relationship between bone resorption and artery calcification in rats treated with warfarin, rats were treated with warfarin for 2 weeks, which is the minimum treatment period required for warfarin-induced artery calcification, together
with bisphosphonates at doses which proved to be effective in inhibiting artery calcification in the vitamin D treated rat. In each case, the daily bisphosphonate treatment was begun 4 days prior to the start of warfarin treatment because previous studies have shown that it takes about 4 days for bisphosphonates to completely inhibit bone resorption (Antic et al. (1996) *Calcif. Tissue Int.* 58: 443-448). All 8 of the rats treated with warfarin alone had extensive calcification of the abdominal aorta, in agreement with earlier studies (Price et al. (1998) *Arterioscler. Thromb. Vasc. Biol.* 18: 1400-1407), while no calcification could be detected in the abdominal aorta of any of the 4 animals treated with warfarin together with alendronate at 0.25 mg P/kg/day. Two of the four animals treated with warfarin together with ibandronate at 0.01 mg P/kg/day had no evidence of calcification in their abdominal aorta, while one had a single small calcification foci and the other had two small calcification foci. These foci were much less intensely stained than the typical calcification foci found in rats treated with warfarin alone. Representative histological sections of the abdominal aorta from each group are shown in Figure 7. The effect of the 0.25 mg P/kg/day dose of alendronate was examined in a repeat two week warfarin treatment experiment, and again no calcification could be detected in the abdominal aorta of any of the 4 alendronate treated rats.

The effect of alendronate and etidronate on warfarin-induced artery calcification was also examined in rats treated for four weeks with warfarin because a longer period of warfarin treatment is necessary in order to achieve calcification levels which can be measured accurately by quantitative analysis of acid demineralization extracts (Price et al. (1998) *Arterioscler. Thromb. Vasc. Biol.* 18: 1400-1407). Rats were again pretreated for 4 days with the bisphosphonate alone, and then treated with the bisphosphonate plus warfarin for a total warfarin treatment time of four weeks. As shown in Table IV and Figure 8, alendronate treatment produced a dose dependent decrease in the level of mineral phosphate in the carotid arteries without affecting bone growth or weight gain. Etidronate also inhibited calcification of the carotid artery, but the dose needed for this effect is sufficiently high that it inhibits weight gain (Table IV). The final length of the tibia in the 4 animals treated with etidronate plus warfarin (3.59±0.03 cm) was also significantly lower than the length of tibia in the 8 treated with warfarin alone (3.80±0.04 cm, p < 0.001).

**Table IV.** Effect of alendronate and etidronate on the accumulation of mineral phosphate in the carotid arteries of rats treated for 4 weeks with warfarin. Beginning at 42 days of age,
18 male rats were given subcutaneous injections of vitamin K every 24h and warfarin every 12h for 4 weeks and 11 control rats received no warfarin. Beginning 4 days prior to the first warfarin injection, three warfarin treated rats received alendronate at a dose of 0.25 mg P/kg/day, 3 received alendronate at a dose of 0.025 mg P/kg/day, and 4 received etidronate at a dose of 6.25 mg P/kg/day. All animals were exsanguinated at 4 weeks and both carotid arteries were removed and demineralized with acid. Phosphate levels were determined in all acid extracts, and the mean and SD for phosphate levels in both carotid arteries are shown for each treatment group. Also shown in the table are average serum calcium and phosphate levels for each group determined on the blood obtained from animals at the end of the experiment, and the average beginning and final weights of the animals.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of Rats</th>
<th>Starting wt (gm)</th>
<th>Ending wt (gm)</th>
<th>nmol PO₄ Carotid</th>
<th>mg Ca dl serum</th>
<th>mg P dl serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>11</td>
<td>N.D.</td>
<td>N.D.</td>
<td>49.4 ± 28.4</td>
<td>10.1 ± 0.6</td>
<td>9.8 ± 1.0</td>
</tr>
<tr>
<td>Warfarin Only</td>
<td>8</td>
<td>149 ± 6</td>
<td>301 ± 10</td>
<td>735.2 ± 476.1</td>
<td>11.1 ± 0.6</td>
<td>11.0 ± 1.4</td>
</tr>
<tr>
<td>Warfarin + Alendronate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.025 mg P/kg/day</td>
<td>3</td>
<td>134 ± 6</td>
<td>301 ± 8</td>
<td>374.0 ± 217.4</td>
<td>10.0 ± 0.1</td>
<td>10.0 ± 0.1</td>
</tr>
<tr>
<td>0.25 mg P/kg/day</td>
<td>3</td>
<td>151 ± 3</td>
<td>300 ± 5</td>
<td>63.7 ± 43.1</td>
<td>10.8 ± 0.1</td>
<td>10.9 ± 0.7</td>
</tr>
<tr>
<td>Warfarin + Etidronate</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.25 mg P/kg/day</td>
<td>4</td>
<td>154 ± 4</td>
<td>238 ± 11</td>
<td>55.2 ± 14.5</td>
<td>10.9 ± 0.8</td>
<td>8.1 ± 0.6</td>
</tr>
</tbody>
</table>

Calcium and phosphate levels were determined in serum obtained at exsanguination for all animals in the 2 and 4 week warfarin treatment experiments, and in no instance were serum calcium or phosphate levels significantly affected by treatment either with warfarin alone or with warfarin in conjunction with a bisphosphonate (data not shown). This result indicates that the effectiveness of bisphosphonates as inhibitors of warfarin-induced calcification of arteries is not due to an effect of bisphosphonates on serum levels of calcium and phosphate.

**Discussion.**

A major conclusion of the present study is that bisphosphonates inhibit the calcification of arteries, heart valves, and kidneys by virtue of their ability to inhibit bone resorption in three artery calcification models, treatment with vitamin D, treatment with vitamin D plus warfarin, and treatment with warfarin alone. The previous hypothesis to
explain the ability of high etidronate doses to inhibit artery calcification is probably incorrect, and alendronate and ibandronate, which are members of the newer generation of bisphosphonates developed to more potently inhibit bone resorption, clearly do not inhibit artery calcification by virtue of their ability to directly interact with mineralization sites and thereby inhibit artery calcification by a physicochemical mechanism. This new conclusion is supported by several lines of evidence: 1. The actual daily subcutaneous doses of alendronate and ibandronate which are required to inhibit artery calcification are in good agreement with the daily subcutaneous doses of these drugs which are required to inhibit bone resorption in male rats of this age (Table II). In contrast, the dose of these drugs required to inhibit normal bone mineralization in vivo, 5 mg P/kg/day, is over 1000 fold greater than the dose required to inhibit artery calcification. 2. The 10,500 fold difference in the doses of the etidronate and ibandronate that are required to inhibit artery calcification is in excellent agreement with the 10,000 fold difference in the doses of the same bisphosphonates that are required to inhibit bone resorption (Table II). This is in marked contrast to the essentially identical doses of etidronate and ibandronate that are required to inhibit normal bone mineralization in vivo (Fleisch (1998) Endocrine Rev.19: 80-100; Fleisch (1997) Ann. Med. 29: 55-62.). 3. Studies on the timing of alendronate administration that is required to inhibit artery calcification (Figures 5 and 6) demonstrate that the drug is completely effective in inhibiting artery calcification even when administered prior to the first appearance of mineral in the artery and prior to the appearance of vitamin D - induced hypercalcemia. This result is difficult to reconcile with the hypothesis that alendronate inhibits artery calcification by a direct physicochemical mechanism analogous to the mechanism by which it inhibits mineralization in vitro and is thought to inhibit normal bone mineralization in vivo. Because the inhibition of bone resorption by alendronate is known to persist for at least 10 days after the daily administration of the drug is discontinued in male rats of this age (Figure 6 in Antic et al. (1996) Calcif. Tissue Int. 58: 443-448), however, this result is entirely consistent with the conclusion that alendronate inhibits artery calcification by virtue of its ability to inhibit bone resorption.

The discovery that bisphosphonates inhibit artery calcification by virtue of their ability to inhibit bone resorption may well be clinically significant. The previous hypothesis, that the mechanism by which bisphosphonates inhibit artery calcification is related to the mechanism by which they inhibit bone mineralization, led investigators to conclude that bisphosphonates could not be employed as inhibitors of soft tissue calcification
without inhibiting normal bone mineralization. To quote from a 1998 review (Fleisch (1998) Endocrine Rev.19: 80-100): "Unfortunately, however, when administered in doses approximating those that inhibit soft tissue calcification, bisphosphonates can impair the mineralization of normal calcified tissues such as bone and cartilage and, when given in higher amounts, also dentine, enamel, and cementum." and "The propensity to inhibit the calcification of normal bone has hampered the therapeutic use of bisphosphonates in ectopic calcification." Our data support the conclusion that etidronate doses which inhibit artery calcification and other ectopic calcifications also inhibit bone mineralization (Table IV), but show for the first time that the newer class of bisphosphonates, such as alendronate and ibandronate, inhibit artery calcification at doses far below the doses which inhibit normal mineralization. The doses of these drugs which inhibit artery calcification are in fact the doses which inhibit bone resorption, and so it seems possible that the inhibition of the calcification of arteries and soft tissues could be an unanticipated and beneficial side effect of doses of these drugs already used in clinical practice to inhibit bone resorption. In this context it is worth noting that the intravenous dose of ibandronate used to inhibit bone resorption in human subjects, 1 mg ibandronate over a 24h period, translates to an ibandronate dose of 10 µg P/kg/day for a 50kg human. This is well above the 0.2 µg P/kg/day subcutaneous ibandronate dose required for 50% inhibition of artery calcification in the rat (Figures 3 and 4).

There are several human diseases in which calcification plays a role, and in which there could be a therapeutic advantage to treating patients with the newer bisphosphonates at doses which are sufficient to inhibit bone resorption but are far below the doses which inhibit normal bone mineralization. From the viewpoint of human health, the most important of these are diseases which affect arteries and heart valves. Since these are the two tissues in which we have here demonstrated the profound ability of low doses of bisphosphonates to inhibit calcification, it seems appropriate to briefly review the human diseases in which artery or heart valve calcification plays a role.

Artery calcification is associated with arteriosclerosis, a term which is derived in part from the Greek word for hardness, sklerosis. Arteriosclerosis refers to hardening of arteries, and the types of arteriosclerosis include atherosclerosis, Monckeberg's arteriosclerosis, hypertensive arteriosclerosis, and arteriolar sclerosis. Atherosclerosis is the most prevalent arteriosclerosis, and calcification is typically associated with the

It is also worth noting that, in rabbit (Rosenblum et al. (1975) Atherosclerosis. 22:411-424) and monkey (Kramschi et al. (1981) Science 213:1511-1512) models of atherosclerosis, high doses of the bisphosphonate etidronate have been shown not only to inhibit artery calcification, but also to inhibit the accumulation of cholesterol in the artery. High etidronate doses have also been shown to cause the regression of pre-established atherosclerosis in the cholesterol fed New Zealand white rabbit model (Hollander et al. (1979) Atherosclerosis 33: 111-123; Zhu et al. (1994) Cardiology 85:370-377). As noted above, etidronate is a first generation bisphosphonate and inhibits bone resorption, artery calcification, and normal bone mineralization at comparably high doses. In the studies of etidronate and atherosclerosis cited above, the high doses of etidronate needed to inhibit artery calcification and atherosclerosis do indeed affect normal bone mineralization (Zhu et al. (1994) Cardiology 85:370-377). We have here shown for the first time that the newer bisphosphonates can be used to inhibit artery calcification at doses which inhibit bone resorption, but which are at least 1000 times lower than the doses which inhibit normal bone mineralization.

Arteriosclerosis is also frequently associated with uremia and, in dialysis patients, the frequency of artery calcification increases with the duration of dialysis to an incidence of 92% at 16 years (Goldsmith et al. (1997) Nephron. 77:37-43). There are two patterns of vascular calcification in uremic patients, calcification of axial arteries (aorta, femoral, iliac) and calcification of peripheral arteries. The latter calcification, referred to as arteriolosclerosis, can lead to cutaneous necrosis and ulceration and is associated with high

While the mechanism by which the serum calcium X phosphate product affects mortality in uremic patients has not yet been established, the rate of calcification is known to be exponentially dependent on the calcium X phosphate product. It is therefore tempting to speculate that dystrophic calcification does indeed account for increased mortality in uremic patients.

Heart valve calcification is frequently associated with valvular dysfunction. In a recent study of 236 aortic heart valves excised at the Mayo Clinic in 1990 (Dare et al. (1993) Human Pathology. 24:1330-1338), stenosis related to calcification was found in 64% of the excised valves and pure insufficiency without calcification was found in 25%. Ten percent of the heart valves had both stenosis and insufficiency, in these valves insufficiency was typically secondary to degenerative calcification. The prevalence of aortic heart valve calcification increases with age, and in a recent study was detected incidentally on CT scans in 30% of the subjects examined (Lippert et al. (1995) Am. J. Roentgenology. 164:73-77). Fifteen percent of the subjects with incidental heart valve calcification were found to have abnormal aortic valve gradients at echocardiography, while none of the subjects without aortic valve calcification had abnormal aortic valve gradients. Calcification is also the major cause of structural valve degeneration in aortic valve bioprostheses (Jamieson et al. (1995) Ann. Thorac. Surg. 60:S241-S247; Schoen et al. (1988) Cardiovasc. Clin. 18:289-317; Cohn et al. (1989) Ann. Surg. 210:435-443) and it is possible that bisphosphonates could inhibit such calcification and thereby reduce the frequency of bioprosthetic valve failure.

We believe that the probable mechanism by which osteoclastic bone resorption promotes artery calcification is by the generation of calcium phosphate crystal nuclei. Some of these nuclei escape to blood and are subsequently deposited in the elastic lamella of arteries and at other soft tissue sites. These nuclei are then able to grow at the deposition sites, due to the fact that serum is supersaturated with respect to calcium phosphate mineral phases such as hydroxyapatite. Our hypothesis is supported in part by evidence that, under some circumstances, a protein mineral complex is released from bone and can be detected in blood.
Example 2

Synthesis and Use of a Fetuin-Mineral Complex

Background.

We discovered the existence of a complex between a calcium phosphate mineral phase and the serum protein fetuin in the course of investigating the effects of high etidronate doses on the chemical composition of serum in rats. To confirm the chemical composition and nature of this complex, we developed the methods for creating the complex in vitro which are described below.

In a preferred embodiment, the creation of a fetuin mineral complex involves the creation of a solution which is supersaturated with respect to the calcium phosphate mineral phase. This is done in the presence of fetuin at physiological pH (that is, pH values found in serum). In the two procedures outlined below, we have generated the supersaturated conditions by the rapid mixing of calcium and phosphate solutions in order to generate mineral nuclei by a homogeneous nucleation process. It was one of the discoveries of this research that the presence of fetuin arrests the growth and aggregation of the mineral phase so that many small crystallites are formed. Since the size of these crystallites is small, the solution itself remains clear for many days at room temperature in spite of the presence of rather large amounts of the fetuin mineral complex.

Procedures.

Procedure for the preparation of fetuin mineral complex using fetal calf serum, calcium, and phosphate.

A first approach to preparing a fetuin-mineral complex uses fetal calf serum. The fetal calf serum is brought and about 2 mL is aliquoted into a test tube. Then 0.5mL of 1M HEPES (pH 7.4) is added to the fetal calf serum to give a final concentration of 0.2M HEPES. (The buffer is added at this step in order to prevent a drop in the pH of the solution due to the formation of the mineral phase. If buffer is not present the pH decreases and rather large crystals of brushite form and precipitate.) Then 160μL of 0.5M phosphate buffer* into a 12x75mm tube.

About 80μL of 1M CaCl₂ is placed into a separate 12x75mm tube. Then 1mL of the fetal calf serum-HEPES buffer solution prepared above is added to both tubes. The
calcium containing tube is covered with parafilm and a hole is poked through the parafilm with a pipette tip. Using a Pasteur pipette and a rubber bulb, the contents of the PO₄ tube is rapidly added to that of the Ca tube. (It is critical that mixing be as close to instantaneous as possible at this step in order to create conditions which favor homogeneous nucleation of the mineral phase. Leisurably mixing of the two solutions will form large crystals of calcium phosphate mineral, which are evident as a cloudy precipitate which sinks to the bottom of the tube.) (The order of mixing can be reversed, and the calcium containing solution can be added to the phosphate containing solution.)

The mixture is then re-covered with parafilm and left at room temperature. The size of the crystallites which form are so small that they can only be seen by transmission electron microscopy. Within a few minutes, small spherical crystallites form. These grow and change in structure over the next 3h to generate numerous crystallites of fairly uniform size. Once the final size is obtained, it remains stable over a period of many days.

**Procedure for the preparation of the fetuin mineral complex using purified bovine fetuin, calcium, and phosphate.**

A second approach to preparing a fetuin-mineral complex uses purified bovine fetuin, calcium, and phosphate fetal calf serum. First, 50mg of purified bovine fetuin are dissolved in 2.5mL of 0.2M HEPES pH 7.4. The mixture is spun at top speed for 30 minutes in an epifuge to clarify the solution. (The Sigma fetuin we use in these experiments contains a small portion of protein which does not dissolve in this buffer.) About 160µl of 0.5M Phosphate buffer* is placed into a 12x75 tube. In a separate 12x75 tube is placed 80µl of 1M CaCl₂. 1mL of the fetuin-HEPES buffer solution prepared in step 2 is rapidly added to both tubes.

The tube containing calcium is covered with parafilm and a hole is poked in the parafilm with a pipette tip. Using a Pasteur pipette and a rubber bulb, rapidlyadd the contents of the PO₄ tube is rapidly added to that of the Ca tube. (It is critical that mixing be as close to instantaneous as possible at this step in order to create conditions which favor homogeneous nucleation of the mineral phase. Leisurably mixing of the two solutions will form large crystals of calcium phosphate mineral, which are evident as a cloudy precipitate which sinks to the bottom of the tube.) (The order of mixing can be reversed, and the calcium containing solution can be added to the phosphate containing solution.)
The mixture is re-covered with parafilm and left at room temperature. The size of the crystallites which form are so small that they can only be seen by transmission electron microscopy. Within a few minutes, small spherical crystallites form. These grow and change in structure over the next 3h to generate numerous crystallites of fairly uniform size. Once the final size distribution is obtained, it remains stable over a period of many days.

The phosphate buffer used above is prepared by preparing 50mL of 0.5M Na₂HPO₄ (Dibasic) and 25mL of 0.5M NaH₂PO₄ (Monobasic). The dibasic is titrated to pH 7.4 with the monobasic solution. Then sodium azide is added to a to a final concentration of 0.02% as a preservative. (This step is optional.)

**Modifications of procedures:**

The initial concentrations of calcium and phosphate can be varied considerably. In the above experiments the final ion composition is slightly less than 40mM in calcium and phosphate. We have formed the complex using final ion compositions as low as 5mM in calcium and phosphate; the major difference is that the complex forms slowly over a period of several days under these conditions. The crystallites which form are still too small to be visualized as cloudiness in the solution, which remains clear, and no crystals sink to the bottom of the tube. The crystallites can be seen by transmission electron microscopy, and are similar in size and structure to those formed after 3h at room temperature in the experiments outlined above. We have also formed the fetuin mineral complex using initial molar ratios of calcium to phosphate ranging from 2:1 to 0.5:1, and find that the final crystallites formed are identical in properties and structure to those formed under the 1:1 molar ratio conditions.

The fetuin mineral complexes formed by the above procedures can be sedimented by centrifugation for 5 to 30 minutes at high speed in an epifuge. The pellet which forms is translucent and glassy in appearance, and contains fetuin, calcium, and phosphate. The molar ratio of calcium to phosphate in this complex is about 1.25 and the weight ratio of fetuin to calcium in this complex is about 3.

The temperature of the calcium phosphate mixture can be varied. We have also prepared the complex at 7 and 37° C. The complex forms more rapidly at the higher temperature and more slowly at the lower, but the final crystallites formed are identical in properties and structure to those formed at room temperature.
The initial concentration of purified bovine fetuin can be varied. We have successfully formed the fetuin mineral complex using fetuin at 5 mg/ml and an initial ion composition of 10mM calcium and phosphate, and using fetuin at 1mg/ml and an initial ion composition of 5mM calcium and phosphate. In general, less fetuin is required to form a stable complex of uniform size and structure at lower initial concentrations of calcium and phosphate.

The species source of fetuin can be varied. While we have not investigated complex formation using purified fetuin from other species, we have successfully formed the fetuin mineral complex using rat and human serum starting with initial calcium and phosphate concentrations of 10mM. (Human fetuin is also called α2-HS Glycoprotein.)

Because the rate of homogeneous crystal nucleation is strongly dependent on the initial ion composition, the importance of mixing rapidly is greatest at the higher calcium phosphate concentrations. If time is not a factor, it is easier to mix rapidly enough to create homogeneous nucleation conditions using a low initial ion concentrations, and it is therefore these conditions which favor the formation of fetuin mineral complexes which are the most uniform in structure.

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.
What is claimed is:


2. The method of claim 1, wherein said mammal is a mammal diagnosed as having or at risk for a pathology characterized by calcification of a soft tissue.

3. The method of claim 3, wherein said inhibiting is by administration of a bisphosphonate to said mammal in a concentration sufficient to inhibit bone resorption without inhibiting bone mineralization.

4. The method of claim 3, wherein said bisphosphonate effects a significant reduction of bone resorption at a concentration at least 10-fold lower than the concentration at which said bisphosphonate effects a significant reduction of bone mineralization.

5. The method of claim 4, wherein said bisphosphonate is administered at a dosage at least 10-fold lower than concentration at which said bisphosphonate effects a significant reduction of bone mineralization.

6. The method of claim 3, wherein said bisphosphonate effects a significant reduction of bone resorption at a concentration at least 1000-fold lower than the concentration at which said bisphosphonate effects a significant reduction of bone mineralization.

7. The method of claim 6, wherein said bisphosphonate is administered at a dosage at least 1000-fold lower than concentration at which said bisphosphonate effects a significant reduction of bone mineralization.

8. The method of claim 3, wherein said bisphosphonate is selected from the group consisting of alendronate, ibandronate, zoledronate, incadronate, risedronate, EB-1053, neridronate, olpadronate, pamidronate, YH 529, tiludronate, and clodronate.
9. The method of claim 3, wherein said soft tissue is selected from the group consisting of an artery, a heart valve, an atherosclerotic plaque, a cancer, a kidney, a prostate, skin, muscle, cartilage, viscera, and heart muscle.

10. A method of inhibiting calcification of soft tissue in a mammal diagnosed as having or at risk for a pathology characterized by calcification of a soft tissue, said method comprising administering to said animal a low dosage of a bisphosphonate, where said low dosage is sufficient to inhibit said calcification, but below the dosage of said bisphosphonate that inhibits normal bone mineralization.

11. The method of claim 10, wherein said bisphosphonate effects a significant reduction of bone resorption at a concentration at least 10-fold lower than the concentration at which said bisphosphonate effects a significant reduction of bone mineralization.

12. The method of claim 10, wherein said bisphosphonate is administered at a dosage at least 10-fold lower than concentration at which said bisphosphonate effects a significant reduction of bone mineralization.

13. The method of claim 10, wherein said bisphosphonate is selected from the group consisting of alendronate, ibandronate, zoledronate, incadronate, risedronate, EB-1053, neridronate, olpadronate, pamidronate, YH 529, tiludronate, and clodronate.

14. The method of claim 10, wherein said soft tissue is selected from the group consisting of an artery, a heart valve, an atherosclerotic plaque, a cancer, a kidney, a prostate, skin, muscle, cartilage, viscera, and heart muscle.

15. The method of claim 13, wherein said bisphosphonate is alendronate administered at a dosage ranging from the minimum dose that produces a detectable inhibition of bone resorption up to 0.5 mg P/kg/day.

16. The method of claim 13, wherein said bisphosphonate is alendronate administered to humans at an oral dosage ranging from 5 mg to 40 mg per day.
17. The method of claim 13, wherein said bisphosphonate is ibandronate administered at a dosage ranging from the minimum dose that produces a detectable inhibition of bone resorption up to 0.5 mg P/kg/day.

18. The method of claim 13, where said bisphosphonate is ibandronate administered to humans at an intravenous dosage of 1mg per day.

19. The method of claim 13, where in said bisphosphonate is zoledronate, incadronate, risedronate, EB-1053, neridronate, olpadronate, pamidronate, YH 529, tiludronate, or clodronate administered at a dosage ranging from the minimum dose that produces a detectable inhibition of bone resorption up to 0.5 mg P/kg/day.

20. The method of claim 10, wherein said bisphosphonate is administered according to a method selected from the group consisting of orally, intravenous injection, subcutaneous injection, and intramuscular injection.

21. The method of claim 10, where in said bisphosphonate is administered as a prophylactic treatment.

22. The method of claim 10, where in said bisphosphonate is administered as a therapeutic treatment.

23. The method of claim 10, wherein said mammal is selected from the group consisting of a human, a non-human primate, a canine, a feline, an equine, a bovine, a rodent, a porcine, and a lagomorph.

24. A method of mitigating the symptoms of a disease in a mammal that involves calcification of a soft tissue, said method comprising administering to said mammal a low dosage of a bisphosphonate sufficient to inhibit calcification of said soft tissue without inhibiting bone calcification.

25. The method of claim 24, wherein said disease is selected from the group consisting of atherosclerosis, arteriosclerosis, arteriolosclerosis, hypertensive arteriolosclerosis, Monckeberg's arteriosclerosis, heart valve stenosis, uremia, diabetes, hyperparathyroidism, blood clot formation, cancer growth, cancer metastasis, hypertension, vitamin D toxicity, and arthritis.
26. The method of claim 24, wherein said bisphosphonate effects a significant reduction of bone resorption at a concentration at least 10-fold lower than the concentration at which said bisphosphonate effects a significant reduction of bone mineralization.

27. The method of claim 24, wherein said bisphosphonate is administered at a dosage at least 10-fold lower than concentration at which said bisphosphonate effects a significant reduction of bone mineralization.

28. The method of claim 24, wherein said bisphosphonate is selected from the group consisting of alendronate, ibandronate, zoledronate, incadronate, risedronate, EB-1053, neridronate, olpadronate, pamidronate, YH 529, tiludronate, and clodronate.

29. The method of claim 24, wherein mammal is diagnosed as having or at risk for a pathology characterized by calcification of a soft tissue.

30. The method of claim 24, wherein said soft tissue is selected from the group consisting of an artery, a heart valve, an atherosclerotic plaque, a cancer, a kidney, a prostate, skin, muscle, cartilage, viscera, and heart muscle.

31. The method of claim 28, wherein said bisphosphonate is alendronate administered at a dosage ranging from the minimum dose that produces a detectable inhibition of bone resorption up to 0.5 mg P/kg/day.

32. The method of claim 28, where said bisphosphonate is alendronate administered to humans at an oral dosage ranging from 5 mg to 40 mg per day.

33. The method of claim 28, wherein said bisphosphonate is ibandronate administered at a dosage ranging from the minimum dose that produces a detectable inhibition of bone resorption up to 0.5 mg P/kg/day.

34. The method of claim 28, wherein said bisphosphonate is ibandronate administered to humans at an intravenous dosage of 1 mg per day.

35. The method of claim 28, wherein said bisphosphonate is zoledronate, incadronate, risedronate, EB-1053, neridronate, olpadronate, pamidronate, YH 529,
tiludronate, or clodronate administered at a dosage ranging from the minimum dose that produces a detectable inhibition of bone resorption up to 0.5 mg P/kg/day.

36. The method of claim 24, wherein said bisphosphonate is administered according to a method selected from the group consisting of orally, intravenous injection, subcutaneous injection, and intramuscular injection.

37. The method of claim 24, wherein said mammal is selected from the group consisting of a human, a non-human primate, a canine, a feline, an equine, a bovine, a rodent, a porcine, and a lagomorph.

38. A method of mitigating the calcification of an implanted prosthetic device in a mammal, said method comprising administering to said mammal a low dosage of a bisphosphonate sufficient to inhibit calcification of said prosthetic device or soft tissue surrounding said prosthetic device without inhibiting calcification of bone.

39. The method of claim 38, wherein said prosthetic device is selected from the group consisting of a heart valve bioprosthesis, and a heart valve mechanical prosthesis.

40. The method of claim 38, wherein said prosthetic device is selected from the group consisting of a surgical implant comprising polyetherurethaneurea, a surgical implant comprising polyetherurethane; a surgical implant comprising silicon, a surgical repair material used for the repair of an aneurisms.

41. The method of claim 38, wherein said bisphosphonate effects a significant reduction of bone resorption at a concentration at least 10-fold lower than the concentration at which said bisphosphonate effects a significant reduction of bone mineralization.

42. The method of claim 38, wherein said bisphosphonate is administered at a dosage at least 10-fold lower than concentration at which said bisphosphonate effects a significant reduction of bone mineralization.
43. The method of claim 38, wherein said bisphosphonate is selected from the group consisting of alendronate, ibandronate, zoledronate, incadronate, EB-1053, neridronate, olpadronate, pamidronate, YH 529, risedronate, tiludronate, and clodronate.

44. The method of claim 43, wherein said bisphosphonate is alendronate administered at a dosage ranging from the minimum dose that produces a detectable inhibition of bone resorption up to 0.5 mg P/kg/day.

45. The method of claim 43, where said bisphosphonate is alendronate administered to humans at an oral dosage ranging from 5 mg to 40 mg per day.

46. The method of claim 43, wherein said bisphosphonate is ibandronate administered at a dosage ranging from the minimum dose that produces a detectable inhibition of bone resorption up to 0.5 mg P/kg/day.

47. The method of claim 43, where said bisphosphonate is ibandronate administered to humans at an intravenous dosage of 1 mg per day.

48. The method of claim 43, wherein said bisphosphonate is zoledronate, incadronate, risedronate, EB-1053, neridronate, olpadronate, pamidronate, YH 529, tiludronate, or clodronate administered at a dosage ranging from the minimum dose that produces a detectable inhibition of bone resorption up to 0.5 mg P/kg/day.

49. The method of claim 38, wherein said bisphosphonate is administered according to a method selected from the group consisting of orally, intravenous injection, subcutaneous injection, and intramuscular injection.

50. The method of claim 38, where in said bisphosphonate is administered as a prophylactic treatment.

51. The method of claim 38, where in said bisphosphonate administered as a therapeutic treatment.

52. The method of claim 38, wherein said mammal is selected from the group consisting of a human, a non-human primate, a canine, a feline, an equine, a bovine, a rodent, a porcine, and a lagomorph.

54. The method of claim 53, wherein said inhibiting is by administration of a bisphosphonate to said mammal in a concentration sufficient to inhibit bone resorption without inhibiting bone mineralization.

55. The method of claim 53, wherein said mammal is a mammal diagnosed as having or at risk for atherosclerosis.

56. The method of claim 54, wherein said bisphosphonate effects a significant reduction of bone resorption at a concentration at least 10-fold lower than the concentration at which said bisphosphonate effects a significant reduction of bone mineralization.

57. The method of claim 56, wherein said bisphosphonate is administered at a dosage at least 10-fold lower than concentration at which said bisphosphonate effects a significant reduction of bone mineralization.

58. The method of claim 54, wherein said bisphosphonate effects a significant reduction of bone resorption at a concentration at least 1000-fold lower than the concentration at which said bisphosphonate effects a significant reduction of bone mineralization.

59. The method of claim 58, wherein said bisphosphonate is administered at a dosage at least 1000-fold lower than concentration at which said bisphosphonate effects a significant reduction of bone mineralization.

60. The method of claim 56, wherein said bisphosphonate is selected from the group consisting of alendronate, ibandronate, zoledronate, incadronate, risedronate, EB-1053, neridronate, olpadronate, pamidronate, YH 529, tiludronate, and clodronate.

61. The method of claim 60, wherein said bisphosphonate is alendronate administered at a dosage ranging from the minimum dose that produces a detectable inhibition of bone resorption up to 0.5 mg P/kg/day.
62. The method of claim 60, where said bisphosphonate is alendronate administered to humans at an oral dosage ranging from 5 mg to 40 mg per day.

63. The method of claim 60, wherein said bisphosphonate is ibandronate administered at a dosage ranging from the minimum dose that produces a detectable inhibition of bone resorption up to 0.5 mg P/kg/day.

64. The method of claim 60, wherein said bisphosphonate is ibandronate administered to humans at an intra venous dosage of 1 mg per day.

65. The method of claim 60, wherein said bisphosphonate is zoledronate, incadronate, risedronate, EB-1053, neridronate, olpadronate, pamidronate, YH 529, tiludronate, or clodronate administered at a dosage ranging from the minimum dose that produces a detectable inhibition of bone resorption up to 0.5 mg P/kg/day.

66. The method of claim 53, wherein said bisphosphonate is administered as a prophylactic treatment.

67. The method of claim 53, wherein said bisphosphonate is administered as a therapeutic treatment.

68. A method of mitigating a symptom of, or progression of, atherosclerosis in a mammal, said method comprising inhibiting the removal of mineral by macrophages at sites of calcification.

69. The method of claim 68, wherein said inhibiting comprises administering a bisphosphonate to said mammal in a concentration sufficient to inhibit mineral removal by said macrophages.

70. The method of claim 69, wherein said bisphosphonate is administered at a concentration that does not inhibit macrophages at locations other than sites of calcification.

71. The method of claim 70, wherein said bisphosphonate is selected from the group consisting of alendronate, ibandronate, zoledronate, incadronate, risedronate, EB-1053, neridronate, olpadronate, pamidronate, YH 529, tiludronate, and clodronate.
72. The method of claim 70, wherein said bisphosphonate is alendronate administered at a dosage ranging from the minimum dose that produces a detectable inhibition of bone resorption up to 0.5 mg P/kg/day.

73. The method of claim 70, where said bisphosphonate is alendronate administered to humans at an oral dosage ranging from 5 mg to 40 mg per day.

74. The method of claim 70, wherein said bisphosphonate is ibandronate administered at a dosage ranging from the minimum dose that produces a detectable inhibition of bone resorption up to 0.5 mg P/kg/day.

75. The method of claim 70, wherein said bisphosphonate is ibandronate administered to humans at an intra venous dosage of 1mg per day.

76. The method of claim 70, wherein said bisphosphonate is zoledronate, incadronate, risedronate, EB-1053, neridronate, olpadronate, pamidronate, YH 529, tiludronate, or clodronate administered at a dosage ranging from the minimum dose that produces a detectable inhibition of bone resorption up to 0.5 mg P/kg/day.

77. The method of claim 70, wherein said bisphosphonate is administered as a prophylactic treatment.

78. The method of claim 70, wherein said bisphosphonate is administered as a therapeutic treatment.

79. A kit for the mitigation of a pathology associated with calcification of a soft tissue, said kit comprising a container containing a bisphosphonate that inhibits calcification of a soft tissue at a dosage that does not substantially inhibit calcification of bone and instructional materials teaching the use of said bisphosphonate for treatment of a pathology associated with calcification of a soft tissue or calcification of a prosthetic device.

80. The kit of claim 79, wherein said bisphosphonate is selected from the group consisting of alendronate, ibandronate, zoledronate, incadronate, risedronate, EB-1053, neridronate, olpadronate, pamidronate, YH 529, tiludronate, and clodronate.
81. A kit for mitigating a symptom of, or progression of, atherosclerosis in a mammal, said kit comprising a container containing a bisphosphonate that inhibits the removal of mineral by macrophages at sites of calcification and instructional materials teaching the use of said bisphosphonate for treatment of a symptom of, or progression of atherosclerosis in a mammal.

82. The kit of claim 81, wherein said bisphosphonate is selected from the group consisting of alendronate, ibandronate, zoledronate, incadronate, risedronate, EB-1053, neridronate, olpadronate, pamidronate, YH 529, tiludronate, and clodronate.

83. A method of stabilizing the size or the crystal structure of a calcium salt in an aqueous phase, said method comprising contacting said calcium or calcium salt with fetuin.

84. A method of delivering a calcification initiator to a preselected site, said method comprising:

   providing a fetuin-mineral complex attached to a targeting molecule

   wherein said targeting molecule specifically binds to said preselected site; and

   contacting said fetuin mineral complex to said preselected site.

85. A method of distributing mineral nuclei within a matrix, said method comprising impregnating said matrix with a fetuin-mineral complex and denaturing said fetuin such that the mineral is released from said fetuin mineral complex.

86. A method of stabilizing a size or crystal structure of a mineral salt in an aqueous phase, said method comprising contacting said mineral salt with a fetuin.

87. An mineral or mineral salt stabilized in an aqueous solution, said mineral or mineral salt being contacted with an isolated fetuin.
Fig. 1
No Alendronate

Alendronate at 0.25 mg P/kg/day

Fig 2
Figure 3
Fig 8

No Alendronate

Alendronate at 0.025 mg P/kg/day

Alendronate at 0.25 mg P/kg/day
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A61K 31/66

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)


Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN
search terms: bisphosphonates and artery and calcification

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y</td>
<td>US 5,403,829 A (LEHTINEN et al.) 04 April 1995, col. 1, line 15 to col. 2, line 18.</td>
<td>1-87</td>
</tr>
<tr>
<td>Y</td>
<td>US 5,733,564 A (LEHTINEN) 31 March 1998, col. 1, line 12 to col. 2, line 24.</td>
<td>1-87</td>
</tr>
</tbody>
</table>

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
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Date of the actual completion of the international search

12 MARCH 2001

Date of mailing of the international search report

26 APR 2001

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