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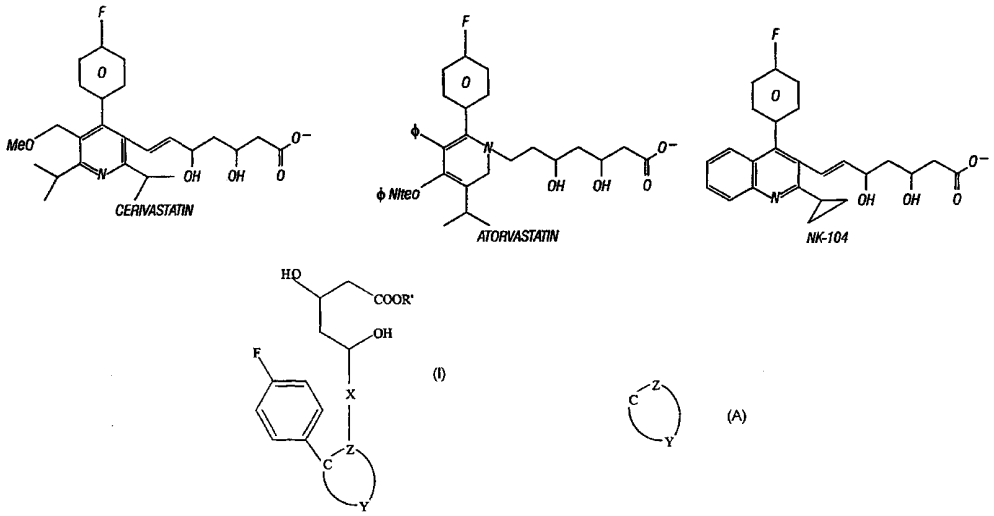
(54) Title: STATIN-TYPE BONE GROWTH STIMULATORS



(57) Abstract: Compounds of formula (I), and the lactone forms thereof, wheren R' is H, a cation, or alkyl (1-6C); is $-CH_2CH_2-$ or -CH=CH-; Z is N or C, and the symbol (a) represents an aromatic or heteroaromatic ring system which may optionally be further substituted in addition to the indicated p-fluorophenyl substituent promote bone formation and are thus useful in treating osteoporosis, bone fracture or deficiency, primary or secondary hyperparathyroidism, periodontal disease or defect, metastatic bone disease, osteolytic bone disease, post-plastic surgery, post-prosthetic joint surgery, and post-dental implantation.

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STATIN-TYPE BONE GROWTH STIMULATORS

Technical Field

The invention relates to treatment of bone disorders in vertebrates including fractures and cartilage disorders. In particular, the invention concerns methods to treat these conditions, and in particular to effect stimulation of osteoblasts and bone formation by use of a preferred group of statin-type compounds.

Background Art

Bone is subject to constant breakdown and resynthesis in a complex process mediated by osteoblasts, which produce new bone, and osteoclasts, which destroy bone. The activities of these cells are regulated by a large number of cytokines and growth factors, many of which have now been identified and cloned.

There is a plethora of conditions which are characterized by the need to enhance bone formation. Perhaps the most obvious is the case of bone fractures, where it would be desirable to stimulate bone growth and to hasten and complete bone repair. Agents that enhance bone formation would also be useful in facial reconstruction procedures. Other bone deficit conditions include bone segmental defects, periodontal disease, metastatic bone disease, osteolytic bone disease and conditions where connective tissue repair would be beneficial, such as healing or regeneration of cartilage defects or injury. Also of great significance is the chronic condition of osteoporosis, including age-related osteoporosis and osteoporosis associated with post-menopausal hormone status. Other conditions characterized by the need for bone growth include primary and secondary hyperparathyroidism, disuse osteoporosis, diabetes-related osteoporosis, and glucocorticoid-related osteoporosis.

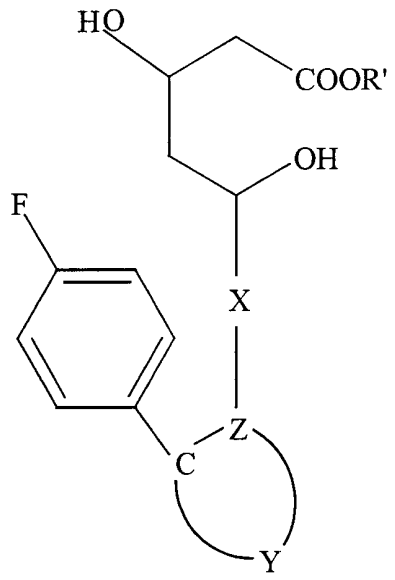
Statins in general, including, for example, mevastatin, lovastatin, simvastatin, and other known statin-type molecules have been shown to enhance directly the production of bone by stimulating osteoblast proliferation and/or differentiation, as described in PCT publication WO 98/25460 published 18 June 1998 and incorporated herein by reference. This published application further describes the use of these members of the statin family in treatment of bone related disorders.

It has now been found that a specific subset of statin-type molecules is particularly useful in treatment of bone conditions, and in particular in enhancing

bone formation by stimulating osteoblasts. The present application is directed to methods to treat bone disorders using this subset of the statin class.

Disclosure of the Invention

In one aspect, the invention provides statin-type compounds that can be administered as ordinary pharmaceuticals and in particular have the metabolic effect of directly enhancing bone growth. These statins can be confirmed in this property using appropriate assays as described below. Thus, the invention is directed to methods and compositions for stimulating the growth of skeletal (bone) tissue, which methods and compositions use, as at least one of the active ingredients, compounds of the formula:

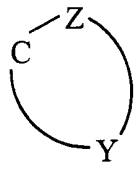


wherein R′ is H, a cation, or alkyl (1-6C);

X is $-CH_2CH_2-$ or -CH=CH-;

Z is N or C, and

the symbol



represents an aromatic or heteroaromatic ring system which may optionally be further substituted in addition to the indicated p-fluorophenyl substituent. The compounds of the invention may also be in the form of their corresponding lactones.

Particularly preferred in this group are the known statins cerivastatin, atorvastatin, and NK-104.

Brief Description of the Drawings

Figure 1 shows the structures of cerivastatin, atorvastatin and NK-104.

Modes of Carrying Out the Invention

The ultimate goal of the methods and compositions of the invention is to treat or ameliorate bone disorders in vertebrate subjects, particularly mammals, and more particularly humans.

As used herein, "treat" or "treatment" include a postponement of development of bone deficit symptoms and/or a reduction in the severity of such symptoms that will or are expected to develop. The terms further include ameliorating existing bone or cartilage deficit symptoms, preventing additional symptoms, ameliorating or preventing the underlying metabolic causes of symptoms, preventing or reversing bone resorption and/or encouraging bone growth. Thus, the terms denote that a beneficial result has been conferred on a vertebrate subject with a cartilage, bone or skeletal deficit, or with the potential to develop such deficit.

By "bone deficit" is meant an imbalance in the ratio of bone formation to bone resorption, such that, if unmodified, the subject will exhibit less bone than desirable, or the subject's bones will be less intact and coherent than desired. Bone deficit may also result from fracture, from surgical intervention or from dental or periodontal disease. By "cartilage defect" is meant damaged cartilage, less cartilage than desired, or cartilage that is less intact and coherent than desired. "Bone disorders" includes both bone deficits and cartilage defects.

Representative uses of the compounds of the present invention include: repair of bone defects and deficiencies, such as those occurring in closed, open and non-union fractures; prophylactic use in closed and open fracture reduction; promotion of bone healing in plastic surgery; stimulation of bone ingrowth into non-cemented prosthetic joints and dental implants; elevation of peak bone mass in pre-menopausal women; treatment of growth deficiencies; treatment of periodontal disease and defects, and other tooth repair processes; increase in bone formation during distraction osteogenesis; and treatment of other skeletal disorders, such as age-related osteoporosis, post-menopausal osteoporosis, glucocorticoid-induced osteoporosis or disuse osteoporosis and arthritis, or any condition that benefits from stimulation of bone formation. The compounds of the present invention can also be useful in repair of congenital, trauma-induced or surgical resection of bone (for instance, for cancer treatment), and in cosmetic surgery. Further, the compounds of the present invention can be used for limiting or treating cartilage defects or disorders, and may be useful in wound healing or tissue repair.

The compositions useful in the invention may be administered systemically or locally. For systemic use, the compounds herein are formulated for parenteral (*e.g.*, intravenous, subcutaneous, intramuscular, intraperitoneal, intranasal or transdermal) or enteral (*e.g.*, oral or rectal) delivery according to conventional methods. Intravenous administration can be by a series of injections or by continuous infusion over an extended period. Administration by injection or other routes of discretely spaced administration can be performed at intervals ranging from weekly to once to three times daily. Alternatively, the compounds disclosed herein may be administered in a cyclical manner (administration of disclosed compound; followed by no administration; followed by administration of disclosed compound, and the like). Treatment will continue until the desired outcome is achieved. In general, pharmaceutical formulations will include a compound of the present invention in combination with a pharmaceutically acceptable vehicle, such as saline, buffered saline, 5% dextrose in water, borate-buffered saline containing trace metals or the like. Formulations may further include one or more excipients, preservatives, solubilizers, buffering agents, albumin to prevent protein loss on vial surfaces, lubricants, fillers, stabilizers, etc. Methods of formulation are well known in the art and are disclosed, for example, in Remington's Pharmaceutical Sciences, latest

edition, Mack Publishing Co., Easton PA, which is incorporated herein by reference. Pharmaceutical compositions for use within the present invention can be in the form of sterile, non-pyrogenic liquid solutions or suspensions, coated capsules, suppositories, lyophilized powders, transdermal patches or other forms known in the art. Local administration may be by injection at the site of injury or defect, or by insertion or attachment of a solid carrier at the site, or by direct, topical application of a viscous liquid, or the like. For local administration, the delivery vehicle preferably provides a matrix for the growing bone or cartilage, and more preferably is a vehicle that can be absorbed by the subject without adverse effects.

Delivery of compounds herein to wound sites may be enhanced by the use of controlled-release compositions, such as those described in PCT application WO 93/20859, which is incorporated herein by reference. Films of this type are particularly useful as coatings for prosthetic devices and surgical implants. The films may, for example, be wrapped around the outer surfaces of surgical screws, rods, pins, plates and the like. Implantable devices of this type are routinely used in orthopedic surgery. The films can also be used to coat bone filling materials, such as hydroxyapatite blocks, demineralized bone matrix plugs, collagen matrices and the like. In general, a film or device as described herein is applied to the bone at the fracture site. Application is generally by implantation into the bone or attachment to the surface using standard surgical procedures.

In addition to the copolymers and carriers noted above, the biodegradable films and matrices may include other active or inert components. Of particular interest are those agents that promote tissue growth or infiltration, such as growth factors. Exemplary growth factors for this purpose include epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), transforming growth factors (TGFs), parathyroid hormone (PTH), leukemia inhibitory factor (LIF), insulin-like growth factors (IGFs) and the like. Agents that promote bone growth, such as bone morphogenetic proteins (U.S. Patent No. 4,761,471; PCT Publication WO 90/11366), osteogenin (Sampath, *et al., Proc. Natl. Acad. Sci. USA* (1987) 84:7109-13) and NaF (Tencer, *et al., J. Biomed. Mat. Res.* (1989) 23: 571-89) are also contemplated. Biodegradable films or matrices include calcium sulfate, tricalcium phosphate, hydroxyapatite, polylactic acid, polyanhydrides, bone or dermal collagen, pure proteins, extracellular matrix components and the like and

combinations thereof. Such biodegradable materials may be used in combination with non-biodegradable materials, to provide desired mechanical, cosmetic or tissue or matrix interface properties.

Alternative methods for delivery of compounds of the present invention include use of ALZET osmotic minipumps (Alza Corp., Palo Alto, CA); sustained release matrix materials such as those disclosed in Wang, *et al.* (PCT Publication WO 90/11366); electrically charged dextran beads, as disclosed in Bao, *et al.*, (PCT Publication WO 92/03125); collagen-based delivery systems, for example, as disclosed in Ksander, *et al., Ann. Surg.* (1990) 211(3):288-94; methylcellulose gel systems, as disclosed in Beck, *et al., J. Bone Min. Res.* (1991) 6(11):1257-65; alginate-based systems, as disclosed in Edelman, *et al., Biomaterials* (1991) 12:619-26 and the like. Other methods well known in the art for sustained local delivery in bone include porous coated metal prostheses that can be impregnated and solid plastic rods with therapeutic compositions incorporated within them.

The compounds of the present invention may also be used in conjunction with agents that inhibit bone resorption. Antiresorptive agents, such as estrogen, bisphosphonates and calcitonin, are preferred for this purpose. More specifically, the compounds disclosed herein may be administered for a period of time (for instance, months to years) sufficient to obtain correction of a bone deficit condition. Once the bone deficit condition has been corrected, the vertebrate can be administered an anti-resorptive compound to maintain the corrected bone condition. Alternatively, the compounds disclosed herein may be administered with an anti-resorptive compound in a cyclical manner (administration of disclosed compound, followed by anti-resorptive, followed by disclosed compound, and the like).

In additional formulations, conventional preparations such as those described below may be used.

Aqueous suspensions may contain the active ingredient in admixture with pharmacologically acceptable excipients, comprising suspending agents, such as methyl cellulose; and wetting agents, such as lecithin, lysolecithin or long-chain fatty alcohols. The said aqueous suspensions may also contain preservatives, coloring agents, flavoring agents, sweetening agents and the like in accordance with industry standards.

Preparations for topical and local application comprise aerosol sprays, lotions, gels and ointments in pharmaceutically appropriate vehicles which may comprise lower aliphatic alcohols, polyglycols such as glycerol, polyethylene glycol, esters of fatty acids, oils and fats, and silicones. The preparations may further comprise antioxidants, such as ascorbic acid or tocopherol, and preservatives, such as p-hydroxybenzoic acid esters.

Parenteral preparations comprise particularly sterile or sterilized products. Injectable compositions may be provided containing the active compound and any of the well known injectable carriers. These may contain salts for regulating the osmotic pressure.

If desired, the osteogenic agents can be incorporated into liposomes by any of the reported methods of preparing liposomes for use in treating various pathogenic conditions. The present compositions may utilize the compounds noted above incorporated in liposomes in order to direct these compounds to macrophages, monocytes, as well as other cells and tissues and organs which take up the liposomal composition. The liposome-incorporated compounds of the invention can be utilized by parenteral administration, to allow for the efficacious use of lower doses of the compounds. Ligands may also be incorporated to further focus the specificity of the liposomes.

Suitable conventional methods of liposome preparation include, but are not limited to, those disclosed by Bangham, A.D., *et al., J Mol Biol* (1965) 23:238-252, Olson, F., *et al., Biochim Biophys Acta* (1979) 557:9-23, Szoka, F., *et al., Proc Natl Acad Sci USA* (1978) 75:4194-4198, Kim, S., *et al., Biochim Biophys Acta* (1983) 728:339:348, and Mayer, *et al., Biochim Biophys Acta* (1986) 858:161-168.

The liposomes may be made from the present compounds in combination with any of the conventional synthetic or natural phospholipid liposome materials including phospholipids from natural sources such as egg, plant or animal sources such as phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, sphingomyelin, phosphatidylserine, or phosphatidylinositol and the like. Synthetic phospholipids that may also be used, include, but are not limited to: dimyristoylphosphatidylcholine, dioleoylphosphatidylcholine, dipalmitoylphosphatidylcholine and distearoylphosphatidycholine, and the corresponding synthetic phosphatidylethanolamines and phosphatidylglycerols.

Cholesterol or other sterols, cholesterol hemisuccinate, glycolipids, cerebrosides, fatty acids, gangliosides, sphingolipids, 1,2-bis(oleoyloxy)-3-(trimethyl ammonio) propane (DOTAP), N-[1-(2,3-dioleoyl) propyl-N,N,N-trimethylammonium chloride (DOTMA), and other cationic lipids may be incorporated into the liposomes, as is known to those skilled in the art. The relative amounts of phospholipid and additives used in the liposomes may be varied if desired. The preferred ranges are from about 60 to 90 mole percent of the phospholipid; cholesterol, cholesterol hemisuccinate, fatty acids or cationic lipids may be used in amounts ranging from 0 to 50 mole percent. The amounts of the present compounds incorporated into the lipid layer of liposomes can be varied with the concentration of the lipids ranging from about 0.01 to about 50 mole percent.

The liposomes with the above formulations may be made still more specific for their intended targets with the incorporation of monoclonal antibodies or other ligands specific for a target. For example, monoclonal antibodies to the BMP receptor may be incorporated into the liposome by linkage to phosphatidylethanolamine (PE) incorporated into the liposome by the method of Leserman, L., *et al., Nature* (1980) 288:602-604.

Veterinary uses of the disclosed compounds are also contemplated. Such uses would include treatment of bone or cartilage deficits or defects, *i.e.*, bone disorders, in domestic animals, livestock and thoroughbred horses.

The compounds of the present invention may be used to stimulate growth of bone-forming cells or their precursors, or to induce differentiation of bone-forming cell precursors, either *in vitro* or *ex vivo*. The compounds described herein may also modify a target tissue or organ environment, so as to attract bone-forming cells to an environment in need of such cells. As used herein, the term "precursor cell" refers to a cell that is committed to a differentiation pathway, but that generally does not express markers or function as a mature, fully differentiated cell. As used herein, the term "mesenchymal cells" or "mesenchymal stem cells" refers to pluripotent progenitor cells that are capable of dividing many times, and whose progeny will give rise to skeletal tissues, including cartilage, bone, tendon, ligament, marrow stroma and connective tissue (see A. Caplan, *J. Orthop. Res.* (1991) 9:641-50). As used herein, the term "osteogenic cells" includes osteoblasts and osteoblast precursor cells. More particularly, the disclosed compounds are useful for stimulating a cell population

containing marrow mesenchymal cells, thereby increasing the number of osteogenic cells in that cell population. In a preferred method, hematopoietic cells are removed from the cell population, either before or after stimulation with the disclosed compounds. Through practice of such methods, osteogenic cells may be expanded. The expanded osteogenic cells can be infused (or reinfused) into a vertebrate subject in need thereof. For instance, a subject's own mesenchymal stem cells can be exposed to compounds of the present invention *ex vivo*, and the resultant osteogenic cells could be infused or directed to a desired site within the subject, where further proliferation and/or differentiation of the osteogenic cells can occur without immunorejection. Alternatively, the cell population exposed to the disclosed compounds may be immortalized human fetal osteoblastic or osteogenic cells. If such cells are infused or implanted in a vertebrate subject, it may be advantageous to "immunoprotect" these non-self cells, or to immunosuppress (preferably locally) the recipient to enhance transplantation and bone or cartilage repair.

Within the present invention, an "effective amount" of a composition is that amount which produces a statistically significant effect. For example, an "effective amount" for therapeutic uses is the amount of the composition comprising an active compound herein required to provide a clinically significant increase in healing rates in fracture repair; reversal of bone loss in osteoporosis; reversal of cartilage defects or disorders; prevention or delay of onset of osteoporosis; stimulation and/or augmentation of bone formation in fracture non-unions and distraction osteogenesis; increase and/or acceleration of bone growth into prosthetic devices; and repair of dental defects. Such effective amounts will be determined using routine optimization techniques and are dependent on the particular condition to be treated, the condition of the patient, the route of administration, the formulation, and the judgment of the practitioner and other factors evident to those skilled in the art. The dosage required for the compounds of the invention (for example, in osteoporosis where an increase in bone formation is desired) is manifested as a statistically significant difference in bone mass between treatment and control groups. This difference in bone mass may be seen, for example, as a 5-20% or more increase in bone mass in the treatment group. Other measurements of clinically significant increases in healing may include, for example, tests for breaking strength and tension, breaking strength and torsion, 4-point bending, increased connectivity in bone biopsies and other biomechanical

tests well known to those skilled in the art. General guidance for treatment regimens is obtained from experiments carried out in animal models of the disease of interest.

The dosage of the compounds of the invention will vary according to the extent and severity of the need for treatment, the activity of the administered compound, the general health of the subject, and other considerations well known to the skilled artisan. Generally, they can be administered to a typical human on a daily basis as an oral dose of about 0.1 mg/kg-1000 mg/kg, and more preferably from about 1 mg/kg to about 200 mg/kg. The parenteral dose will appropriately be 20-100% of the oral dose. While oral administration may be preferable in most instances (for reasons of ease, patient acceptability, and the like), alternative methods of administration may be appropriate for selected compounds and selected defects or diseases. In comparative assays, positive control compounds or other bone-active test compounds may be administered subcutaneously, while statin-type test compounds are administered orally.

Confirmatory Assays

The osteogenic activity of the compounds of formula 1 used in the methods of the invention can be verified in various assay systems.

Neonatal Mouse Calvaria Assay (*In vitro*)

An assay for bone resorption or bone formation is similar to that described by Gowen M. & Mundy G., *J Immunol* (1986) 136:2478-82. Briefly, four days after birth, the front and parietal bones of ICR Swiss white mouse pups are removed by microdissection and split along the sagittal suture. In an assay for resorption, the bones are incubated in BGJb medium (Irvine Scientific, Santa Ana, CA) plus 0.02% (or lower concentration) β-methylcyclodextrin, wherein the medium also contains test or control substances. The medium used when the assay is conducted to assess bone formation is Fitton and Jackson Modified BGJ Medium (Sigma) supplemented with 6 μg/ml insulin, 6 μg/ml transferrin, 6 ng/ml selenous acid, calcium and phosphate concentrations of 1.25 and 3.0 mM, respectively, and ascorbic acid to a concentration of 100 μg/ml is added every two days. The incubation is conducted at 37°C in a humidified atmosphere of 5% CO_2 and 95% air for 96 hours.

Following this, the bones are removed from the incubation media and fixed in 10% buffered formalin for 24-48 hours, decalcified in 14% EDTA for 1 week, processed through graded alcohols; and embedded in paraffin wax. Three μm sections of the calvaria are prepared. Representative sections are selected for histomorphometric assessment of bone formation or bone resorption. Bone changes are measured on sections cut 200 μm apart. Osteoblasts and osteoclasts are identified by their distinctive morphology.

Other auxiliary assays can be used as controls to determine non-BMP promoter-mediated effects of test compounds. For example, mitogenic activity can be measured using screening assays featuring a serum-response element (SRE) as a promoter and a luciferase reporter gene. More specifically, these screening assays can detect signaling through SRE-mediated pathways, such as the protein kinase C pathway. For instance, an osteoblast activator SRE-luciferase screen and an insulin mimetic SRE-luciferase screen are useful for this purpose. Similarly, test compound stimulation of cAMP response element (CRE)-mediated pathways can also be assayed. For instance, cells transfected with receptors for PTH and calcitonin (two bone-active agents) can be used in CRE-luciferase screens to detect elevated cAMP levels. Thus, the BMP promoter specificity of a test compound can be examined through use of these types of auxiliary assays.

In vivo Assay of Effects of Compounds on Murine Calvarial Bone Growth

Male ICR Swiss white mice, aged 4-6 weeks and weighing 13-26 gm, are employed, using 4-5 mice per group. The calvarial bone growth assay is performed as described in PCT application WO 95/24211, incorporated by reference. Briefly, the test compound or appropriate control vehicle is injected into the subcutaneous tissue over the right calvaria of normal mice. Typically, the control vehicle is the vehicle in which the compound was solubilized, and is PBS containing 5% DMSO or is PBS containing Tween (2 μl/10 ml). The animals are sacrificed on day 14 and bone growth measured by histomorphometry. Bone samples for quantitation are cleaned from adjacent tissues and fixed in 10% buffered formalin for 24-48 hours, decalcified in 14% EDTA for 1-3 weeks, processed through graded alcohols; and embedded in paraffin wax. Three to five μm sections of the calvaria are prepared, and representative sections are selected for histomorphometric assessment of the effects

on bone formation and bone resorption. Sections are measured by using a camera lucida attachment to trace directly the microscopic image onto a digitizing plate. Bone changes are measured on sections cut 200 μm apart, over 4 adjacent 1x1 mm fields on both the injected and noninjected sides of the calvaria. New bone is identified by its characteristic woven structure, and osteoclasts and osteoblasts are identified by their distinctive morphology. Histomorphometry software (OsteoMeasure, Osteometrix, Inc., Atlanta) is used to process digitizer input to determine cell counts and measure areas or perimeters.

Additional *In Vivo* Assays

Compounds can be further confirmed to have the desired activity in intact animals using an *in vivo,* dosing assay. Prototypical dosing may be accomplished by subcutaneous, intraperitoneal or oral administration, and may be performed by injection, sustained release or other delivery techniques. The time period for administration of test compound may vary (for instance, 28 days as well as 35 days may be appropriate). An exemplary, *in vivo* oral or subcutaneous dosing assay may be conducted as follows:

In a typical study, 70 three-month-old female Sprague-Dawley rats are weight-matched and divided into seven groups, with ten animals in each group. This includes a baseline control group of animals sacrificed at the initiation of the study; a control group administered vehicle only; a PBS-treated control group; and a positive control group administered a compound (non-protein or protein) known to promote bone growth. Three dosage levels of the compound to be tested are administered to the remaining three groups.

Briefly, test compound, positive control compound, PBS, or vehicle alone is administered subcutaneously once per day for 35 days. All animals are injected with calcein nine days and two days before sacrifice (two injections of calcein administered each designated day). Weekly body weights are determined. At the end of the 35-day cycle, the animals are weighed and bled by orbital or cardiac puncture. Serum calcium, phosphate, osteocalcin, and CBCs are determined. Both leg bones (femur and tibia) and lumbar vertebrae are removed, cleaned of adhering soft tissue, and stored in 70% ethanol for evaluation, as performed by peripheral quantitative computed tomography (pQCT; Ferretti, J., *Bone* (1995) 17:353S-64S), dual energy

X-ray absorptiometry (DEXA; Laval-Jeantet A., *et al., Calcif Tissue Intl* (1995) 56:14-18; J. Casez, *et al., Bone and Mineral* (1994) 26:61-68) and/or histomorphometry. The effect of test compounds on bone remodeling can thus be evaluated.

Lead compounds can also be tested in acute ovariectomized animals (prevention model) using an *in vivo* dosing assay. Such assays may also include an estrogen-treated group as a control. An exemplary subcutaneous dosing assay is performed as follows:

In a typical study, 80 three-month-old female Sprague-Dawley rats are weight-matched and divided into eight groups, with ten animals in each group. This includes a baseline control group of animals sacrificed at the initiation of the study; three control groups (sham ovariectomized (sham OVX) + vehicle only; ovariectomized (OVX) + vehicle only; PBS-treated OVX); and a control OVX group that is administered a compound known to promote bone growth. Three dosage levels of the compound to be tested are administered to the remaining three groups of OVX animals.

Since ovariectomy (OVX) induces hyperphagia, all OVX animals are pair-fed with sham OVX animals throughout the 35 day study. Briefly, test compound, positive control compound, PBS, or vehicle alone is administered orally or subcutaneously once per day for 35 days. Alternatively, test compound can be formulated in implantable pellets that are implanted for 35 days, or may be administered orally, such as by gastric gavage. All animals, including sham OVX/vehicle and OVX/vehicle groups, are injected intraperitoneally with calcein nine days and two days before sacrifice (two injections of calcein administered each designated day, to ensure proper labeling of newly formed bone). Weekly body weights are determined. At the end of the 35-day cycle, the animals' blood and tissues are processed as described above.

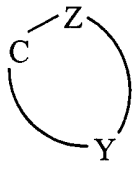
Compounds may also be tested in chronic OVX animals (treatment model). An exemplary protocol for treatment of established bone loss in ovariectomized animals that can be used to assess efficacy of anabolic agents may be performed as follows. Briefly, 80 to 100 six month old female, Sprague-Dawley rats are subjected to sham surgery (sham OVX) or ovariectomy (OVX) at time 0, and 10 rats are sacrificed to serve as baseline controls. Body weights are recorded weekly during the

experiment. After approximately 6 weeks (42 days) or more of bone depletion, 10 sham OVX and 10 OVX rats are randomly selected for sacrifice as depletion period controls. Of the remaining animals, 10 sham OVX and 10 OVX rats are used as placebo-treated controls. The remaining OVX animals are treated with 3 to 5 doses of test drug for a period of 5 weeks (35 days). As a positive control, a group of OVX rats can be treated with an agent such as PTH, a known anabolic agent in this model (Kimmel, *et al., Endocrinology* (1993) 132:1577-84). To determine effects on bone formation, the following procedure can be followed. The femurs, tibiae and lumbar vertebrae 1 to 4 are excised and collected. The proximal left and right tibiae are used for pQCT measurements, cancellous bone mineral density (BMD) (gravimetric determination), and histology, while the midshaft of each tibiae is subjected to cortical BMD or histology. The femurs are prepared for pQCT scanning of the midshaft prior to biomechanical testing. With respect to lumbar vertebrae (LV), LV2 are processed for BMD (pQCT may also be performed); LV3 are prepared for undecalcified bone histology; and LV4 are processed for mechanical testing.

Statin Compounds Useful in the Invention

The compounds of the invention may be in the open chain form, as shown, or may be in the form of a lactone. The compounds contain chiral carbons; all stereoisomeric forms of these compounds and mixtures thereof are included.

The statin compounds useful in the methods of the invention contain an aromatic or heteroaromatic system represented by:



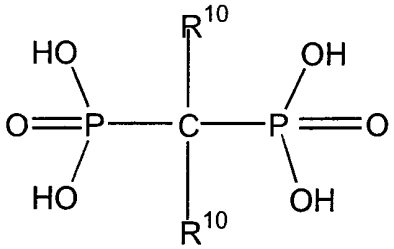
By aromatic or heteroaromatic system is meant a monocyclic or fused bicyclic system which is aromatic in nature and which contains 5-12 ring members. Typical aromatic systems are, of course, benzene and naphthalene. For heteroaromatic embodiments, the carbon in one or more positions may be replaced by nitrogen, sulfur, or oxygen. Replacement by nitrogen is preferred. Thus, the heteroaromatic system may preferably be pyrrole, pyrazole, imidazole, pyridine, pyrazine, pyrimidine, indole,

purine, benzimidazole, quinoline, isoquinoline, quinazoline, and the like. Particularly preferred are pyrrole, quinoline, and pyridine. When the heteroaromatic system is pyrrole, it is preferred that Z occupies position 1; when the heteroaromatic system is quinoline, it is preferred that Z is at position 3, and when the heteroaromatic system is pyridine, position 3 for Z is also preferred.

The heteroaromatic system is required to be substituted at the position adjacent Z by p-fluorophenyl. Additional substituents may also be present. These substituents may contain additional aromatic groups, and may be alkyl, including cycloalkyl, OR, SR, NR_2, and the like wherein R is H or alkyl (1-6C), halo, and/or phenyl. Substituents also may include those of the formula R″COO, R″CONH, R″CO, R″HNCO, and R″OOC wherein R″ is H, alkyl (1-6C) or phenyl. Particularly preferred substituents are phenyl, lower alkyl, methoxy, and cycloalkyl.

The compounds of the invention which are useful in treating bone disorders by enhancing bone growth are generally classified as "statins." Particularly preferred for use in the invention are cerivastatin, marketed under the name Baycol® by Bayer (See U.S. patents 5,006,530 and 5,177,080), atorvastatin, marketed under the name Lipotor® by Warner-Lambert (See U.S. patent 5,273,995), and NK-104 developed by NEGMA. (See Akiba, T et al., *J Toxicol Sci* (1998) 23V:713-720.) All the above-cited documents are incorporated herein by reference.

The compositions of the invention may also include the bisphosphonates and their analogs. Typically, and preferably, the bisphosphonates are of the formula



and the pharmaceutically acceptable salts, esters and amides thereof. Typical salts are those of the inorganic ions, such as sodium ion, potassium ion, calcium ion, magnesium ion and the like; any pharmaceutically acceptable cation may be used. Typical esters are the ethyl, methyl, isobutyl, ethylene glycol, and other typical pharmaceutically acceptable esters; typical amides are the unsubstituted -NH_2 amides as well as the alkyl and dialkyl amides.

Embodiments of R^{10} include halo, OR, SR, NR_2, where R is H or alkyl (1-6C) or alkyl or arylalkyl with optional substitutions. Particularly preferred are the amino-substituted alkyl embodiments. Typically, both R^{10} are not identical, although in some embodiments, such as clodronate, both R^{10} are halo. Particularly preferred compounds among the bisphosphonates are risedronate, alandronate, pamidronate, clodronate and in particular ibandronate. These compounds are particularly useful in combination with the statins.

In addition to the statins or other isoprenoid inhibiting compounds of the invention, the compositions may also include other agents, including those which inhibit bond resorption such as estrogens or their analogs and compounds of the formula Ar-L-Ar wherein Ar represents an aryl substituent and L represents a linker.

The following examples are intended to illustrate but not to limit the invention.

Example 1

In vivo Calvarial Bone Growth Assay

Cerivastatin, atorvastatin and NK-104 are assayed *in vivo* according to the procedure described previously (see "*In vivo* Assay of Effects of Compounds on Murine Calvarial Bone Growth," *supra*). Vehicle control, bFGF and varying doses of test compound are tested.

Example 2

In vitro Bone Formation

Cerivastatin, atorvastatin and NK-104 and appropriate controls are assayed *in vitro* (*ex vivo*) for bone formation activity (described above in "Neonatal Mouse Calvaria Assay (*in vitro*)"). Histomorphometrical assessments of *ex vivo* calvaria are carried out using an OsteoMetrics bone morphometry measurement program, according to the manufacturer's instructions. Measurements are determined using either a 10- or 20-fold objective with a standard point counting eyepiece graticule.

New bone formation is determined (using a 10X objective) by measuring the new bone area formed in one field in 3 representative sections of each bone (4 bones per group). Each measurement is carried out ½ field distance from the end of the

suture. Both total bone and old bone area were measured. Data are expressed as new bone area in mm^2.

Osteoblast numbers are determined by point counting. The number of osteoblast cells lining the bone surface on both sides of the bone are counted in one field using a 20X objective. Data are expressed as osteoblast numbers/mm of bone surface.

Example 3

Effect on Resorption

Cerivastatin, atorvastatin and NK-104 and controls are tested in an antiresorptive assay. Briefly, 15 day timed pregnant CD-1 female mice are injected with ^{45}Ca (25 μCi/mouse). The calvaria from the 4 day old pups are dissected out and cut in half. The excised half calvaria are placed on metal grids (at the surface) in 1 ml of BGJ medium (Sigma) containing 0.1% BSA with glutamine and Pen/Strep added. The bones are incubated at 37°C in a 5% humidified incubator for a period of 24 h, and then transferred to wells containing 1 ml medium with factors added (IL-1, PTH, and/or test compounds). The treated bones are incubated under the above conditions for a further 72 h. After this incubation period, the bones are removed and placed into 20% TCA in a scintillation vial for 1.5 h, and then counted with scintillation fluid. An aliquot of medium (0.4 ml) is also counted. The results are expressed as % ^{45}Ca release.

This assay may be modified by including test compounds/factors or control compounds/factors in the preincubation medium (*i.e.*, during the first 24 h). Since most of the osteoclasts are formed in the calvaria following the preincubation period, compounds or factors that affect osteoclast formation may have a greater effect during the preincubation period.

In this assay, compound toxicity is indicated by obvious death of the cells in the periosteal region and within the marrow cavity of the bone organ cultures. These cells are characterized by pyknotic nuclei and vacuolated cytoplasm, characteristic of cell necrosis and distinct from apoptosis.

Example 4

Systemic Administration of Statins in OVX Models

Cerivastatin, atorvastatin and NK-104 are also analyzed *in vivo* using an acute OVX (prevention model) and/or chronic (treatment model) OVX model system, as described above under "Additional *In Vivo* Assays".

Example 5

Statin-Mediated Fracture Repair Effects of Test Compounds

Cerivastatin, atorvastatin and NK-104 are examined for effects on surgical defects in the rabbit radius. Healing of these defects may be assessed by X-ray, histology and biomechanical strength.

The test compound is weighed out in a microcentrifuge tube, and 50 µl of 1.5% sodium alginate solution is added as a carrier. This test sample is vortexed to wet all of the powder. The sample is sonicated for 20-30 min, and then vortexed again. Disks are created in the top of the microcentrifuge tube (by placing the tube lid or stopper-side down). The indent in the top of the stopper (*i.e.*, lid) is used to form the disk (7.5 mm diameter). $CaCl_2$ solution (100 µl of 100 mM) is added to the sodium alginate/drug solution. The samples are allowed to sit for 5-10 min, and then the calcium-alginate disks are carefully removed. The disks are rinsed in a beaker filled with water to rinse off the excess calcium solution, and are saved in tubes using water as vehicle. All solutions and containers are sterile, and all procedures for preparations of disks are performed under a laminar flow hood under sterile conditions.

Bone healing is examined as follows. Briefly, six-month old male rabbits are obtained, and are divided into 4 treatment groups (n=3 animals/group). The treatment groups received either: 1) placebo; 2) test compound (5 mg/disk); 3) test compound (10 mg/disk); or 4) an autologous bone graft. Animals are anesthetized with rabbit cocktail (1 ml/1.5 kg intramuscularly), and the right forelimb is clipped, prepped and draped for aseptic surgery. Anesthesia is maintained using isofluorane delivered with a face mask. To create a 20 mm gap defect in the right mid-radius, an incision is made over the lateral aspect of the forearm, and an osteotomy is performed with an oscillating bone saw. The cerivastatin, atorvastatin and NK-104 or vehicle is applied

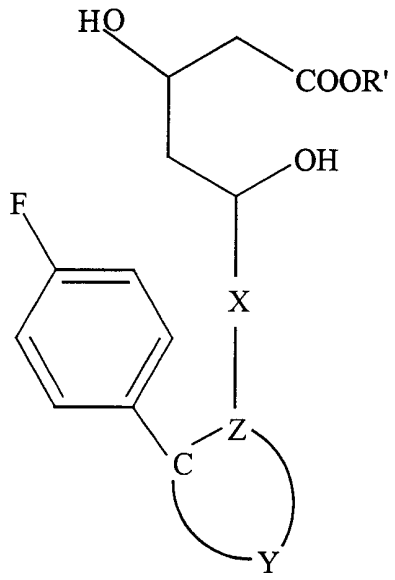
to the defect and the defect is closed in layers. No external splinting is needed, as the radius is paired with the ulna, which functions to allow normal ambulation in the rabbit. Disks are cut on strips and inserted in the fracture to cover all the defect. Radiologic evaluation is performed at zero time and at 4 weeks.

Because the vehicle (placebo treatment group) prevented full healing in the control group, only X-ray results are obtained and analyzed. Accordingly, X-ray analysis 4 weeks after initiation of treatment showed callus formation at the bone treatment site in the treated (both doses), but not the placebo (vehicle or autologous bone graft) groups.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

Claims

1. A method to treat bone deficiency conditions in a vertebrate animal which method comprises administering to a vertebrate subject in need of such treatment an amount of compound of the formula:



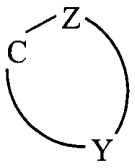
or the lactone form thereof,

wherein R' is H, a cation, or alkyl (1-6C);

X is $-CH_2CH_2-$ or -CH=CH-;

Z is N or C, and

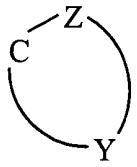
the symbol



represents an aromatic or heteroaromatic ring system which may optionally be further substituted in addition to the indicated p-fluorophenyl substituent,

effective to treat said bone deficiency.

2. The method of claim 1 wherein



is a heteroaromatic system.

3. The method of claim 2 wherein the heteroaromatic system is pyrrole, pyrazole, imidazole, pyridine, pyrazine, pyrimidine, indole, purine, benzimidazole, quinoline, isoquinoline, or quinazoline.

4. The method of claim 3 wherein the heteroaromatic system is pyrrole, quinoline, or pyridine.

5. The method of claim 2 wherein the heteroaromatic system is further substituted.

6. The method of claim 5 wherein the substitution is by at least one group selected from the group consisting of alkyl, including cycloalkyl, OR, SR, NR_2, R″COO, R″CONH, R″CO, R″HNCO, and R″OOC wherein R″ is H, alkyl (1-6C) or phenyl.

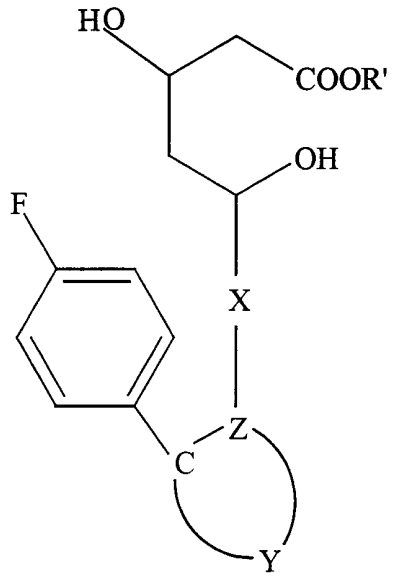
7. The method of claim 2 wherein said compound is atorvastatin, cerivastatin, or NK-104 in hydrolyzed or unhydrolyzed form.

8. The method of claim 1 wherein said subject is characterized by a condition selected from the group consisting of osteoporosis, bone fracture or deficiency, primary or secondary hyperparathyroidism, periodontal disease or defect, metastatic bone disease, osteolytic bone disease, post-plastic surgery, post-prosthetic joint surgery, and post-dental implantation.

9. The method of claim 1 which further comprises administering to said subject one or more agents that promote bone growth or that inhibit bone resorption.

10. The method of claim 1 wherein said treating enhances bone formation.

11. A pharmaceutical composition in unit dosage form to enhance bone formation in a vertebrate animal which composition comprises a pharmaceutically acceptable excipient and an amount, effective to promote bone formation, of a compound of the formula:



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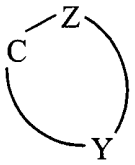
including the lactone form thereof,

wherein R′ is H, a cation, or alkyl (1-6C);

X is $-CH_2CH_2-$ or -CH=CH-;

Z is N or C, and

10 the symbol



represents an aromatic or heteroaromatic ring system which may optionally be further substituted in addition to the indicated p-fluorophenyl substituent.

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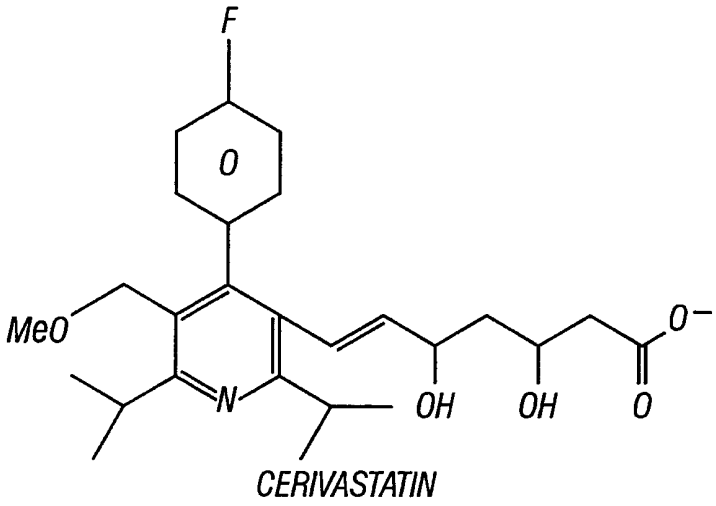
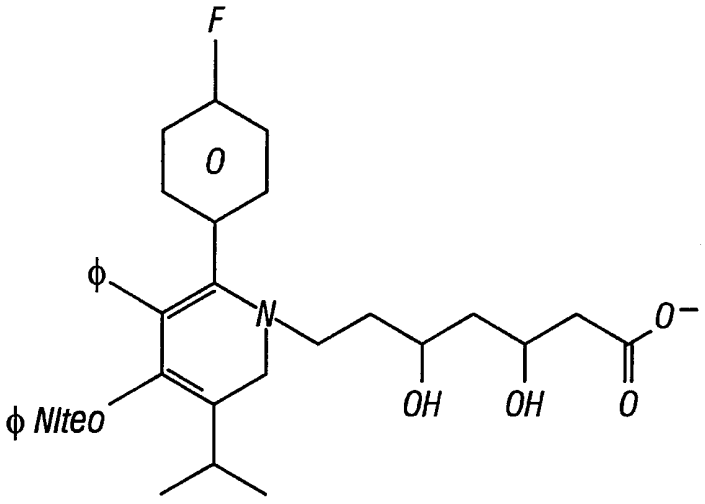
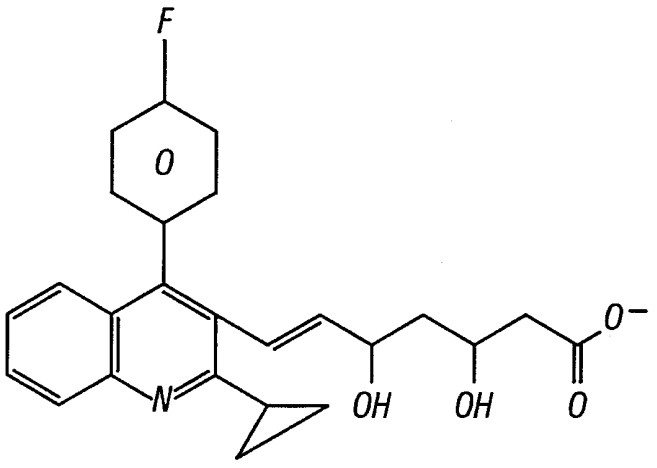


CERIVASTATIN



ATORVASTATIN



NK-104

FIG. 1