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Title: METHODS AND COMPOSITIONS FOR STIMULATING BONE REGENERATION

Abstract: The invention provides methods and compositions for stimulating or promoting bone regeneration or repairing bone fracture or for stimulating or increasing differentiation or activation of osteoblasts by administering to a subject a therapeutically effective amount of an adenosine receptor agonist, or an analog, derivative or combination thereof, an adenosine receptor antagonist, or an analog, derivative or combination thereof, or adenosine or a compound that upregulates, increases the amount of or increases the biological activity of adenosine. The invention also extends to pharmaceutical compositions such as those comprising an agent that modulates an adenosine receptor such as an adenosine $A_{2a}$ agonist or $A_1$ antagonist.
Methods and Compositions for Stimulating Bone Regeneration

FIELD OF THE INVENTION

[0001] The present invention relates to methods and compositions for stimulating or promoting bone regeneration, bone fracture healing and osteoblast differentiation and activation.

BACKGROUND OF THE INVENTION

[0002] Adenosine is a nucleoside that occurs naturally in mammals, which acts as a ubiquitous biochemical messenger. The heart, for instance, produces and releases adenosine in order to modulate heart rate and coronary vasodilation. Likewise, adenosine is produced in the kidney to modulate essential physiological responses, including glomerular filtration rate (GFR), electrolyte reabsorption, and renin secretion.

[0003] Adenosine is known to bind to and activate seven-transmembrane spanning G-protein coupled receptors, thereby eliciting a variety of physiological responses. There are 4 known subtypes of adenosine receptors (i.e., A₁, A₂A, A₂B and A₃), which mediate different, and sometimes opposing, effects. For example, activation of the adenosine A₁ receptor, elicits an increase in renal vascular resistance, which leads to a decrease in glomerular filtration rate (GFR), while activation of the adenosine A₂Areceptor elicits a decrease in renal vascular resistance. Conversely, blockade of the A₁ adenosine receptor decreases afferent arteriole pressure, leading to an increase in GFR and urine flow, and sodium excretion. Furthermore, A₂A adenosine receptors modulate coronary vasodilation, whereas A₂B receptors have been implicated in mast cell activation, asthma, vasodilation, regulation of cell growth, intestinal function, and modulation of neurosecretion (See, Adenosine A₂B Receptors as Therapeutic Targets, Drug Dev Res 45:198; Feoktistov et al, Trends Pharmacol Sci 19:148-153 and Ralevic, V and Burnstock, G. (1998), Pharmacological Reviews, Vol. 50: 413-492), and A₃ adenosine receptors modulate cell proliferation processes. Two receptor subtypes (A₁ and A₂A) exhibit affinity for adenosine in the nanomolar range while two other known subtypes A₂Band A₃ are low-affinity receptors, with affinity for adenosine in the low-micromolar range. A₁ and A₃ adenosine receptor activation can lead to an inhibition of adenylate cyclase activity, while A₂A and A₂B activation causes a stimulation of adenylate cyclase.
[0004] It has been shown that adenosine, acting at specific cell surface receptors, has the potential to suppress inflammation and that inflammation itself may increase extracellular adenosine levels (Cronstein, et al., 1986, Journal of Clinical Investigation 78:760-770; Cronstein, et al., 1983, Journal of Experimental Medicine 158:1160-1 177). Further, it has been demonstrated that adenosine mediates the anti-inflammatory effects of low-dose methotrexate therapy for Rheumatoid Arthritis (Reviewed in Cronstein, 2005, Pharmacol Rev 57:163-172). Exploration of the therapeutic and toxic properties of methotrexate in the treatment of RA has led to a number of other potentially important pre-clinical therapeutic developments. Methotrexate increases giant cell formation from peripheral blood monocytes and that this effect is mediated by adenosine acting at A1 receptors (Merrill, et al., Arth.Rheum. 40:1308-1315). In addition, A2A receptor antagonists promote giant cell formation by diminishing the effect of endogenous adenosine although the A1 receptor-mediated promotion of giant cell formation appears to dominate.

[0005] A1 receptor antagonists completely block, in a dose-dependent fashion, osteoclast formation. Similarly, the A1 receptor antagonists block osteoclast function (resorption of dentin). Six-month old A1 KO mice demonstrate increased bone density. Their bones demonstrate diminished resorption, and some evidence indicates that the osteoclasts in the A1 knockout mice do not resorb bone. A murine model of post-menopausal osteoporosis, ovariectomy-induced bone loss, reveals that treatment of mice with an adenosine A1 receptor antagonist completely prevents ovariectomy-induced bone loss. Adenosine A1 receptors may be useful in treating and preventing osteoporosis.

[0006] Osteoblasts are mononucleate cells that are responsible for bone formation. They are specialized fibroblasts that in addition to fibroblastic products, express bone sialoprotein and osteocalcin. Osteoblasts produce a matrix of osteoid, which is composed mainly of Type I collagen. Osteoblasts are also responsible for mineralization of this matrix. Zinc, copper and sodium are some of the minerals required in this process. Bone is a dynamic tissue that is constantly being reshaped by osteoblasts, which are in charge of production of matrix and mineral, and osteoclasts, which break down the tissue. The number of osteoblasts tends to decrease with age, affecting the balance of formation and resorption in the bone tissue, and potentially leading to osteoporosis.
Osteoblasts arise from osteoprogenitor cells located in the deeper layer of periosteum and the bone marrow. Osteoprogenitors are immature progenitor cells that express the master regulatory transcription factor Cbfa1/Runx2. Osteoprogenitors are induced to differentiate under the influence of growth factors, in particular the bone morphogenetic proteins (BMPs). Aside from BMPs, other growth factors including fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) and transforming growth factor beta (TGF-β) may promote the division of osteoprogenitors and potentially increase osteogenesis. Once osteoprogenitors start to differentiate into osteoblasts, they begin to express a range of genetic markers including Osterix, Coll, BSP, M-CSF, ALP, osteocalcin, osteopontin, and osteonectin. Although the term osteoblast implies an immature cell type, osteoblasts are in fact the mature bone cells entirely responsible for generating bone tissue in animals and humans.

Cronstein, U.S. Patent 7,795,427 describes the use of agents that block adenosine A1 receptor antagonists to diminish osteoclast function and thereby prevent the development of osteoporosis. United States Serial No. 12/291,510 describes the activation of adenosine A2A receptors as inhibiting osteoclast formation and function, and use of adenosine A2A receptor agonists to prevent wear particle-induced bone resorption. In all of these actions adenosine receptor blockade or activation was directed solely at preventing bone resorption. Interestingly, these studies do not demonstrate that either adenosine A1 or A2A receptors affect the formation or function of osteoblasts.

The prior art teaches use of adenosine receptor agonists and antagonists or dipyridamole in the regulation of osteoblast differentiation, proliferation and function. The prior art does not teach using adenosine A1 or A2A receptor agonists or antagonists or dipyridamole for the treatment of bone defects following trauma or to promote spinal fusion. Moreover, any proposed use of dipyridamole described in the prior art is to increase adenosine to stimulate adenosine A2B receptors to stimulate osteoblast production of bone matrix and inhibit IL-6 production or increase production of osteoprotegerin. (See, e.g., Kara et al., The FASEB Journal 2010; 24:2325-2333; Kara et al., Arthritis and Rheumatism 2010; 62:534-541; Russell et al., Calcif Tissue Int 2007; 81:316-326; Evans et al., J Bone Miner Res 2006; 21:228-236; Costa et al., Journal of Cellular Physiology 2011; 226: 1353-1366)

All publications, patent applications, patents and other reference material mentioned are incorporated by reference in their entirety. In addition, the materials, methods and examples are
only illustrative and are not intended to be limiting. The citation of references herein is not to be construed as an admission that the references are prior art to the present invention.

SUMMARY OF THE INVENTION

[0011] The invention relates to the use of modulators of an adenosine receptor, including agonists of an adenosine $A_2A$ receptor and antagonists of an $A_1$ receptor, to stimulate bone regeneration and stimulate and promote differentiation and activation of osteoblasts as well as potentially inhibit bone resorption and inhibit differentiation and stimulation of osteoclasts.

[0012] In a first aspect, the invention provides a method for stimulating or promoting bone regeneration by administering to a subject a therapeutically effective amount of an adenosine receptor agonist, or an analog or derivative thereof or by administering to a subject a therapeutically effective amount of an adenosine receptor antagonist, or an analog or derivative thereof. Further, the invention provides a method for stimulating or promoting bone regeneration by administering to a subject a therapeutically effective amount of adenosine or a therapeutically effective amount of an agent that upregulates, increases the amount of or increases the biological activity of adenosine.

[0013] In a second aspect, the invention provides a method for stimulating differentiation and activation of osteoblasts comprising administering to the subject a therapeutically effective amount of an adenosine receptor agonist, or an analog or derivative thereof or by administering to a subject a therapeutically effective amount of an adenosine receptor antagonist, or an analog or derivative thereof. Further, the invention provides a method for stimulating or promoting differentiation and activation of osteoblasts by administering to a subject a therapeutically effective amount of adenosine or a therapeutically effective amount of an agent that upregulates, increases the amount of or the biological activity of adenosine.

[0014] In a third aspect, the invention provides methods for treating, ameliorating or preventing a bone disease or a condition in a subject having such disease or condition, or in a subject at risk for developing such disease or condition, particularly where it is desired to stimulate or increase osteoblast function, differentiation, or activation. The methods feature administering to the subject a therapeutically effective amount of an adenosine receptor agonist, or an analog or derivative thereof or by administering to a subject a therapeutically effective amount of an adenosine receptor antagonist, or an analog or derivative thereof. Also, the methods may feature
administering to a subject a therapeutically effective amount of adenosine or a therapeutically effective amount of an agent that upregulates, increases the amount of or the biological activity of adenosine. The disease or condition may be, for instance, osteoporosis, juvenile osteoporosis, bone loss due to or associated with the onset of menopause, osteoporotic fractures, giant cell tumors of bone, renal osteodystrophy, osteogenesis imperfecta, hypercalcemia, hyperparathyroidism, osteomalacia, osteohalisteresis, osteolytic bone disease, osteonecrosis, Paget's disease of bone, bone loss due to rheumatoid arthritis, inflammatory arthritis, osteoporosis and bone lysis, childhood idiopathic bone loss, periodontal bone loss, age-related loss of bone mass, osteotomy and bone loss associated with prosthetic ingrowth, other forms of osteopenia, and in other conditions where facilitation of bone repair or replacement is desired such as bone fractures, bone defects, plastic surgery, dental and other implantations.

[0015] In a fourth aspect, the invention provides a method for stimulating or promoting bone fracture healing or for repairing bone fractures by administering to a subject a therapeutically effective amount of an adenosine receptor agonist, or an analog or derivative thereof or by administering to a subject a therapeutically effective amount of an adenosine receptor antagonist, or an analog or derivative thereof. Further, the invention provides a method for stimulating or promoting bone fracture healing or for repairing bone fractures by administering to a subject a therapeutically effective amount of adenosine or a therapeutically effective amount of an agent that upregulates, increases the amount of or increases the biological activity of adenosine.

[0016] For each of these aspects, in one particular embodiment, the adenosine receptor of the present invention may be any one of A1, A2A, A2B or A3. In a more particular embodiment, the adenosine receptor is an A2A receptor, and the agonist is an adenosine receptor A2A agonist. In another more particular embodiment, the adenosine receptor is an A2B receptor, and the agonist is an adenosine receptor A2B agonist. In yet another embodiment, the adenosine receptor agonist affects more than one adenosine receptor. In a more particular embodiment, the adenosine receptor is an A1 receptor, and the antagonist is an adenosine receptor A1 antagonist. In yet another embodiment, the adenosine receptor antagonist affects more than one adenosine receptor.

[0017] In another particular embodiment, the adenosine receptor agonist is a selective adenosine receptor agonist. In still other particular embodiments, the adenosine receptor agonist is a non-selective adenosine receptor agonist. In another particular embodiment, the adenosine receptor
antagonist is a selective adenosine receptor agonist. In still other particular embodiments, the adenosine receptor antagonist is a non-selective adenosine receptor antagonist.

[0018] In a more particular embodiment, the agent that agonizes an adenosine receptor is an adenosine A<sub>2A</sub> receptor agonist or an adenosine A<sub>2B</sub> receptor agonist. The adenosine receptor agonist may be, for instance, a small organic molecule, a protein or peptide, a nucleic acid or an antibody. Similarly, in a more particular embodiment, the agent that antagonizes an adenosine receptor is an adenosine A<sub>i</sub> receptor antagonist. The adenosine receptor antagonist may be, for instance, a small organic molecule, a protein or peptide, a nucleic acid or an antibody.

[0019] In yet another more particular embodiment, the adenosine receptor agonist is capable of substantially stimulating the endogenous activity of the adenosine receptor substantially the same as though the adenosine receptor had encountered its natural, endogenous ligand.

[0020] In yet another particular embodiment, the adenosine receptor agonist or antagonist is an adenosine A<sub>2A</sub> receptor agonist or an adenosine A<sub>i</sub> receptor antagonist.

[0021] In some instances, the bone regeneration or differentiation or activation of osteoblasts may be increased by 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, or by two fold, three fold, four fold, five fold, ten fold or more relative to normal. Likewise, in some instances, the speed of bone regeneration or number of differentiated or stimulated osteoblasts may be increased by 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, or by two fold, three fold, four fold, five fold, ten fold or more relative to normal.

[0022] In one particular embodiment, an effective amount of an adenosine receptor agonist or antagonist may be used in combination with one or more drugs useful in inhibiting bone resorption or inhibiting differentiation or stimulation of osteoclasts or in stimulating bone regeneration or growth or stimulating or promoting differentiation of osteoblasts or a combination of any of these agents.

[0023] Adenosine A<sub>2A</sub> receptor agonists are well known in the art. Many are disclosed in, for instance, U.S. Patent Nos. 7,226,913 and 6,326,359 and in United States Patent Publication Nos. 20070225247, 20060100169, 20060034941, 20050261236, 20050182018, 20050171050, 20050020915 and 20040064039, the disclosures of which are herein incorporated by reference in their entireties. In another more particular embodiment, the adenosine A<sub>2A</sub> receptor agonist is
selected from the group consisting of CGS 21680, MRE-0094, IB-MECA and R-PIA, binodenoson, ATL146, for instance. Adenosine A\textsubscript{2B} receptor agonists are also known in the art. Many are disclosed in, for instance, United States Patent Publication Nos. 20070225335 and 20070240433.

Likewise, adenosine A\textsubscript{i} receptor antagonists are well known in the art and include, for instance, DPCPX. Exemplary A\textsubscript{i} receptor antagonists include those disclosed by Cronstein, United States Patent No. 7,795,427 such as DPCPX (8-Cyclopentyl-1,3-dipropylxanthine), N-0861 (N-6-endonorboman-2-yl-9-methyladenine), N-0840 (N-6-cyclopentyl-9-methyladenine), CVT-124, WRC-0342 ([N\textsuperscript{6}-(5'-endohydroxy)-endonorboman-2-yl-9-methyladenine]), CGS-15943, XAC (xanthine carboxylic acid congener), WRC-0571 ([C\textsuperscript{8}-(N-methylisopropyl)-amino-N\textsuperscript{6}(5'-endohydroxy)-endonorboman-2-yl-9-methyladenine],), KW-3902 (8-(noradamantan-3-yl)-1,3-dipropylxanthine), ENX (1,3-Dipropyl-8-[2-(5,6-epoxy)norbornyl]xanthine), KFM 19 (BHIP20, (S)-3,7-dihydro-8-(3-oxocyclopentyl)-1,3-dipropyl-1H-purine-2,6-dione), FK453 ((R)-1-[[(E)-3-(2-phenylpyrazolo[1,5-a]pyridin-3-yl)] acryloyl]-2-piperidine ethanol), FK352 ((R)-1-[(E)-3-(2-phenylpyrazolo[1,5-a]pyridin-3-yl)] acryloyl]-piperidin-2-yl acetic acid), FK838 (6-oxo-3-(2-phenylpyrazolo[1,5-a]pyridin-3-yl)-l(6H)-pyridazinebutyric acid), FR166124 and its analogues, 8-cyclopentyltheophylline, BG9719 and BG9928.

The adenosine receptor agonist or antagonist may be administered alone or in combination with one or more other compounds or agents for inhibiting bone resorption or osteoclast differentiation or for stimulating or increasing bone regeneration or bone growth or stimulating osteoblast differentiation. Such other compounds may be, for instance, anti-inflammatory compounds, bisphosphonates or growth factors. The adenosine receptor agonist may be administered with a second adenosine receptor agonist or with a less selective adenosine receptor agonist, (i.e. one that binds other adenosine receptors in addition to A\textsubscript{2}A or A\textsubscript{2}B for example A\textsubscript{3}B, A\textsubscript{i} or A\textsubscript{3}). The adenosine receptor agonist or antagonist may be administered or provided in a matrix such as, for example a calcium sulfate matrix, a calcium phosphate matrix or bovine collagen. Such a matrix may be directly applied to bone defects to promote bone formation.

In one embodiment, the adenosine receptor agonist may be selective for the receptor, or it may be a non-selective adenosine receptor agonist, which may stimulate or mimic natural ligands of one or more of the following receptors: A\textsubscript{i}, A\textsubscript{2A}, A\textsubscript{2B} or A\textsubscript{3}. In a preferred embodiment, the adenosine receptor agonist is an adenosine A\textsubscript{2A} receptor agonist.
[0027] In another more particular embodiment, the agent that increases endogenous adenosine levels may be an agent that, for instance, diminishes platelet function or induces coronary vasodilation, such as, for instance, dipyridamole or ticagrelor.

[0028] In a further aspect, the present invention provides a pharmaceutical composition comprising an adenosine receptor agonist or an adenosine receptor antagonist, adenosine or an agent that upregulates, increases the amount of or increases the biological activity of adenosine alone or in combination with one or more compounds or agents effective for inhibiting bone resorption, for inhibiting osteoclast differentiation and stimulation or for promoting osteoblast differentiation and activation. The adenosine receptor agonist or antagonist, adenosine or the compound that upregulates, increases the amount of or increases the biological activity of adenosine and the one or more compounds or agents may be formulated and administered alone or together. The pharmaceutical composition(s) comprising the adenosine receptor agonist or antagonist or adenosine or a therapeutically effective amount of a compound that upregulates, increases the amount of or increases the biological activity of adenosine and the one or more compounds or agents may be administered concurrently or sequentially. In another particular embodiment, the one or more compounds or agents effective for inhibiting bone resorption or osteoclast differentiation and stimulation or for stimulating osteoblast differentiation and activation may be selected from the group consisting of those effective for stimulating bone density and those effective for inhibiting or reducing inflammation. The pharmaceutical compositions may be delivered orally or parenterally. They may be delivered via the intravenous route, the intramuscular route, or the subcutaneous route. They may be delivered as an immediate release formulation or as a slow or sustained release formulation. In some particular embodiments, the compositions are delivered on the surface of a prosthetic device or are delivered in the very matrix of a prosthetic device.

[0029] In another more particular embodiment, the pharmaceutical composition comprising the adenosine receptor agonist or antagonist may also contain one or more drugs selected from the group consisting of anti-inflammatory agents, growth factors, bone morphogenetic protein, soluble RANK. In some instances, the pharmaceutical composition may be administered or provided in a matrix such as, for example a calcium sulfate matrix. Such a matrix may be directly applied to bone defects to promote bone formation.
Other objects and advantages will become apparent to those skilled in the art from a review of the following description which proceeds with reference to the following illustrative drawings.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 demonstrates that agents that block adenosine A1 receptors, stimulate A2A receptors or block adenosine uptake promote bone growth. Under general anesthesia a 3mm in diameter defect is drilled into the skull of C57B6 mice. A collagen matrix soaked in PBS or PBS containing the A1 antagonist DPCPX (1µM), the A2A agonist CGS21680 (CGS, 1µM), the adenosine uptake inhibitor dipyridamole (1µM) or the A3 receptor agonist IB-MECA is placed in the defect. The matrix is then reinjected with 100 µl of these agents daily for 8 weeks before harvest. Figure 1A shows microCT images of representative calvaria, for comparison a calvarium with a fresh defect is included (Day 0). Figure 1B represents the percentage bone regeneration calculated as the percent of the original defect that was now filled with bone. Shown are the means (±SD) of results obtained in 3 animals for each condition. Results were analyzed by ANOVA. ***p<0.001

Figure 2 demonstrates that the adenosine A1 receptor antagonist DPCPX, the A2A agonist CGS21680 and the A2B agonist BAY606583 all stimulate bone regeneration. Similarly the adenosine uptake inhibitor dipyridamole, which increases local adenosine concentration, promotes bone regeneration as well. In contrast, the adenosine A3 receptor agonist has no effect on bone regeneration. Thus blockade of adenosine A1 receptors and stimulation of both adenosine A2A and A2B receptors promotes bone regeneration. As shown in Figure 2B, the effects are first detectable after as little as 2-4 weeks for the A2A agonist, the A1 antagonist and dipyridamole. Figure 2A shows microCT images of representative calvaria, for comparison a calvarium with a fresh defect is included (Day 0). In Figure 2B, the percentage bone regeneration is calculated as the percent of the original defect that was now filled with bone. Shown are the means (+SD) of results obtained in 10 animals for each condition. Results were analyzed by ANOVA. ***p<0.001 Figure 2B represents the percent bone formation for each agent 8 weeks post trephination, and Figure 2C represents the percent bone formation for each agent at 2, 4, and 8 week intervals post trephination.
[0033] Figure 3 demonstrates that the effects of all four of these agents, the adenosine A\textsubscript{i} receptor antagonist DPCPX, the A\textsubscript{2A} agonist CGS21680, and dipyridamole are dose-dependent and appear to achieve maximal effect at 1\textmu M. For each Figure 3A, 3B and 3C, the top line shows microCT images of representative calvaria, for comparison a calvarium with a fresh defect is included (Day 0). The second line shows the percentage bone formation calculated as the percent of the original defect that was now filled with bone. Shown are the means (\pm SD) of results obtained in 10 animals for each condition. Results were analyzed by ANOVA. ***p<0.001 The third line represents bone volume in mm\textsuperscript{3} regenerated 8 weeks post trephination.

[0034] Figure 4 demonstrates that the adenosine A\textsubscript{2A} receptor antagonist ZM241385 blocks the effect of both the A\textsubscript{2A} agonist CGS21680 and dipyridamole indicating that the A\textsubscript{2A} receptor plays a role in promoting bone regeneration. Moreover, the A\textsubscript{2B} receptor antagonist MRS 1764 blocks the effect of dipyridamole as well indicating that the A\textsubscript{2B} receptor is also involved in promoting bone regeneration by extracellular adenosine. Figure 4A shows microCT images of representative calvaria, for comparison a calvarium with a fresh defect is included (Day 0). In Figure 4B, the percentage bone formation is calculated as the percent of the original defect that was now filled with bone. Shown are the means (\pm SD) of results obtained in 10 animals for each condition. Results were analyzed by ANOVA. ***p<0.001 Figure 4B represents the bone volume for each agent 8 weeks post trephination, and Figure 4C represents the bone volume as measured in mm\textsuperscript{3} regenerated 8 weeks post trephination.

[0035] Figure 5 provides further evidence for the involvement of the A\textsubscript{i} and A\textsubscript{2B} receptors. Deletion of the A\textsubscript{i} receptor (AiKO), like blockade of that receptor, increases bone regeneration. Moreover, deletion of the A\textsubscript{2B} receptor (A\textsubscript{2B}KO) reverses the effect of the A\textsubscript{2A} agonist CGS21680 and dipyridamole as well as the A\textsubscript{i} receptor antagonist DPCPX further supporting the role of the A\textsubscript{2B} receptor. These results further support the effect of A\textsubscript{i} and A\textsubscript{2B} receptors in bone regeneration and do not rule out a role for the A\textsubscript{2A} receptor as well. Figure 5A shows microCT images of representative calvaria, for comparison a calvarium with a fresh defect is included (Day 0). In Figure 5B, the percentage bone formation is calculated as the percent of the original defect that was now filled with bone. Shown are the means (+SD) of results obtained in 10 animals for each condition. Results were analyzed by ANOVA. ***p<0.001 Figure 5C represents the percent bone formation for each agent 8 weeks post trephination.
Figure 6 directly demonstrates the effect of A1 receptor blockade and A2A receptor stimulation on bone formation using a fluorescent indicator of bone formation. Figure 6A shows representative IVIS images of skulls of mice. The yellow represents increased bone formation, red is least bone formation. Shown in Figure 6B is the bone formation over time measured as a % of control bone formation (fluorescence) measured at that time point. Each point represents the mean (+/-SEM) of determinations carried out in 6 animals.

Figure 7 provides further evidence for the effect of the A1 antagonist DPCPX, the A2A receptor agonist CGS21680 and dipyridamole on bone formation in which all three of these agents increase alkaline phosphatase positive osteoblasts and reduce TRAP stained osteoclasts in the regenerating bone. Shown in Figure 7A are representative medium powered fields of calvarial sections stained immunohistochemically for alkaline phosphatase (left hand column, a marker for osteoblasts). Shown in the next column of figures are sections stained histochemically for tartrate resistant acid phosphatase (a marker for osteoclasts). Arrows in these figures point to osteoblasts (first column) or osteoclasts (second column). Figure 7B shows the mean (+/-SEM) number of osteoblasts (alkaline phosphatase positive cells)/hpf over time in groups of 6 mice. Shown in Figure 7C are the mean (+/-SEM) number of osteoclasts (TRAP+ cells)/hpf. ***p<0.001 vs control, 2-way ANOVA.

Figure 8 demonstrates a reduction of both RANK+ osteoblasts, RANKL+ osteoclasts and an increase in osteoprotegerin+ cells in the regenerating bone of mice treated with the A1 antagonist DPCPX, the A2A receptor agonist CGS21680 and the adenosine uptake inhibitor dipyridamole. Paraffin embedded sections are stained immunohistochemically for RANK, RANKL or osteoprotegerin. Slides are imaged and positive cells (brown) are enumerated in three fields. Shown on the left are representative medium power sections of immunohistochemically stained sections. Figure 8B shows the mean (+/-SEM) number of RANK positive cells/hpf in tissue sections of 6 mice. There are more RANK positive osteoblasts in bone from control mice than treated mice. Figure 8C shows the mean (+/-SEM) number of RANKL positive cells/hpf in tissue sections of 6 mice. There are more RANKL positive osteoblasts in bone from control mice than treated mice. Figure 8D shows the mean (+/-SEM) number of osteoprotegerin positive cells/hpf in tissue sections of 6 mice. There are fewer osteoprotegerin positive osteoblasts in bone from control mice than treated mice. *p<0.05, **p<0.01, ***p<0.001 vs control (2-way ANOVA, Bonferroni post-hoc test).
[0039] Figure 9 provides the histology of calvaria from representative mice immediately after formation of the lesion, in control mice or mice treated with the A1 antagonist DPCPX, the A2A agonist (CGS), the A3 agonist (IB-MECA) or dipyridamole. This demonstrates histologically the bone formation that occurs associated with treatment with the A1 antagonist DPCPX, the A2A agonist CGS21680 and the adenosine uptake inhibitor dipyridamole.

DETAILED DESCRIPTION OF THE INVENTION

[0040] Before the present methods and treatment methodology are described, it is to be understood that this invention is not limited to particular methods and experimental conditions described, as such methods and conditions may vary. It is also to be understood that the terminology used herein is for purposes of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims.

[0041] A common problem encountered clinically is poor bone healing after trauma or fracture. In addition promotion of new bone growth at the site of surgery is a common maneuver during such procedures as spinal fusion. Agents used topically or during surgery to promote bone growth are generally limited to BMPs.

[0042] The present invention demonstrates that a matrix containing either an adenosine A1 receptor antagonist or an A2A receptor stimulus at the site of a bone defect dramatically increases repair of the defect with new bone. Moreover, increase of endogenous adenosine levels at the site of the bone defect by application of dipyridamole, an agent that has been in clinical use for nearly 50 years to diminish platelet function and to induce coronary vasodilation by increasing endogenous adenosine levels, similarly promotes new bone growth at the site of a bone defect.

[0043] Promotion of local bone growth is critical for rapid healing of bone defects following trauma or invention. Similarly, agents that promote bone growth are commonly applied in a gel at the site of spinal fusion and other similar procedures.Currently BMPs are the principal stimuli for bone growth during spinal fusion or at sites of trauma. However, recent studies indicate that use of BMPs to promote bone growth during spinal fusion is associated with a significant increase in the risk for developing cancer. This understanding presents a novel opportunity for developing new agents useful for promoting bone growth to repair bone defects or stimulate formation of new bone during such procedures as spinal fusion.
[0044] The methods and compositions described promote bone repair following trauma or bone growth after spinal fusion or similar surgeries. Topical application in a gel or matrix or more frequent applications in solution to bony defects of either A1 receptor antagonists or A2A agonists or similar topical applications of dipyridamole to bone defects, such as, for instance, after spinal fusion surgery are provided herein.

[0045] As used in this specification and the appended claims, the singular forms "a", "an", and "the" include plural references unless the context clearly dictates otherwise. Thus, for example, references to "the method" includes one or more methods, and/or steps of the type described herein and/or which will become apparent to those persons skilled in the art upon reading this disclosure and so forth in their entirety.

[0046] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the invention, the preferred methods and materials are now described. All publications mentioned herein are incorporated herein by reference in their entireties.


Definitions

[0048] The terms used herein have the meanings recognized and known to those of skill in the art, however, for convenience and completeness, particular terms and their meanings are set forth
below.

[0049] "Agent" refers to all materials that may be used to prepare pharmaceutical and diagnostic compositions, or that may be compounds such as small synthetic or naturally derived organic compounds, nucleic acids, polypeptides, antibodies, fragments, isoforms, variants, or other materials that may be used independently for such purposes, all in accordance with the present invention.

[0050] By "agonist" is meant a substance that binds to a specific receptor and triggers a response in a cell. It mimics the action of an endogenous ligand (such as hormone or neurotransmitter) that binds to the same receptor. A "full agonist" binds (has affinity for) and activates a receptor, displaying full efficacy at that receptor. One example of a drug that acts as a full agonist is isoproterenol which mimics the action of acetylcholine at β adrenoreceptors. A "partial agonist" (such as buspirone, aripiprazole, buprenorphine, or norclozapine) also binds and activates a given receptor, but has only partial efficacy at the receptor relative to a full agonist. A "partial agonist" may also be considered a ligand that displays both agonistic and antagonistic effects - when both a full agonist and partial agonist are present, the partial agonist actually acts as a competitive antagonist, competing with the full agonist for receptor occupancy and producing a net decrease in the receptor activation observed with the full agonist alone. A "co-agonist" works with other co-agonists to produce the desired effect together. An antagonist blocks a receptor from activation by agonists. Receptors can be activated or inactivated either by endogenous (such as hormones and neurotransmitters) or exogenous (such as drugs) agonists and antagonists, resulting in stimulating or inhibiting a biological response. A ligand can concurrently behave as agonist and antagonist at the same receptor, depending on effector pathways.

[0051] The potency of an agonist is usually defined by its EC50 value. This can be calculated for a given agonist by determining the concentration of agonist needed to elicit half of the maximum biological response of the agonist. Elucidating an EC50 value is useful for comparing the potency of drugs with similar efficacies producing physiologically similar effects. The lower the EC50, the greater the potency of the agonist and the lower the concentration of drug that is required to elicit a maximum biological response.

[0052] "Antagonist" refers to an agent that down-regulates (e.g., suppresses or inhibits) at least one bioactivity of a protein. An "antagonist" or an agent that "antagonizes" may be a compound
which inhibits or decreases the interaction between a protein and another molecule, e.g., a target peptide or enzyme substrate. An antagonist may also be a compound that down-regulates expression of a gene or which reduces the amount of expressed protein present. Methods for assessing the ability of an agent to "antagonize" or "inhibit" an adenosine receptor are known to those skilled in the art.

[0053] "Analog" as used herein, refers to a chemical compound, a nucleotide, a protein, or a polypeptide that possesses similar or identical activity or function(s) as the chemical compounds, nucleotides, proteins or polypeptides having the desired activity and therapeutic effect of the present invention (e.g. to treat or prevent bone disease, or to modulate osteoclast differentiation), but need not necessarily comprise a compound that is similar or identical to those compounds of the preferred embodiment, or possess a structure that is similar or identical to the agents of the present invention.

[0054] "Derivative" refers to the chemical modification of molecules, either synthetic organic molecules or proteins, nucleic acids, or any class of small molecules such as fatty acids, or other small molecules that are prepared either synthetically or isolated from a natural source, such as a plant, that retain at least one function of the active parent molecule, but may be structurally different. Chemical modifications may include, for example, replacement of hydrogen by an alkyl, acyl, or amino group. It may also refer to chemically similar compounds which have been chemically altered to increase bioavailability, absorption, or to decrease toxicity. A derivative polypeptide is one modified by glycosylation, pegylation, or any similar process that retains at least one biological or immunological function of the polypeptide from which it was derived.

[0055] By "medical prosthetic device" or "prosthesis" is meant an artificial component, device or extension that replaces a portion or all of a body part whether the body part is entirely or partially missing. The term includes artificial limbs, breast prosthesis such as those implanted post-mastectomy, cochlear implants, corrective lenses, craniofacial prosthesis, dental/maxillofacial prosthetics such as those implanted to correct a cleft palate, dentures, dental restoration, facial prosthetics, hair prosthesis, neuroprosthetics, ocular prosthetics, ostomies such as colostomy, ileostomy and urostomy, penile prosthetics, replacement joints such as hips, knees and shoulders, simato prosthetics, prosthetic testis and transtibial prosthesis.

[0056] A "small molecule" refers to a molecule that has a molecular weight of less than 3
kilodaltons (kDa), preferably less than about 1.5 kilodaltons, more preferably less than about 1 kilodalton. Small molecules may be nucleic acids, peptides, polypeptides, peptidomimetics, carbohydrates, lipids or other organic (carbon-containing) or inorganic molecules. As those skilled in the art will appreciate, based on the present description, extensive libraries of chemical and/or biological mixtures, often fungal, bacterial, or algal extracts, may be screened with any of the assays of the invention to identify compounds that modulate a bioactivity. A "small organic molecule" is normally an organic compound (or organic compound complexed with an inorganic compound (e.g., metal)) that has a molecular weight of less than 3 kilodaltons, and preferably less than 1.5 kilodaltons, and more preferably less than about 1 kDa.

[0057] "Diagnosis" or "screening" refers to diagnosis, prognosis, monitoring, characterizing, selecting patients, including participants in clinical trials, and identifying patients at risk for or having a particular disorder or clinical event or those most likely to respond to a particular therapeutic treatment, or for assessing or monitoring a patient's response to a particular therapeutic treatment.

[0058] The concept of "combination therapy" is well exploited in current medical practice. Treatment of a pathology by combining two or more agents that target the same pathogen or biochemical pathway sometimes results in greater efficacy and diminished side effects relative to the use of the therapeutically relevant dose of each agent alone. In some cases, the efficacy of the drug combination is additive (the efficacy of the combination is approximately equal to the sum of the effects of each drug alone), but in other cases the effect can be synergistic (the efficacy of the combination is greater than the sum of the effects of each drug given alone). As used herein, the term "combination therapy" means the two compounds can be delivered in a simultaneous manner, e.g. concurrently, or one of the compounds may be administered first, followed by the second agent, e.g sequentially. The desired result can be either a subjective relief of one or more symptoms or an objectively identifiable improvement in the recipient of the dosage.

[0059] "Differentiate" or "differentiation" as used herein, generally refers to the process by which precursor or progenitor cells differentiate into specific cell types. In the present invention, the term refers to the process by which pre-osteoblasts become osteoblasts or pre-osteoclasts become osteoclasts. Differentiated cells can be identified by their patterns of gene expression and cell surface protein expression. As used herein, the term "differentiate" refers to having a different character or function from the original type of tissues or cells. Thus, "differentiation" is the process or act of differentiating. The term "Osteoclast Differentiation" refers to the process
whereby osteoclast precursors in the bone marrow become functional osteoclasts, and the term
"Osteoblast Differentiation" refers to the process whereby osteoblast precursors in the bone
marrow become functional osteoblasts.

[0060] "Modulation" or "modulates" or "modulating" refers to up regulation (i.e., activation or
stimulation), down regulation (i.e., inhibition or suppression) of a response, or the two in
combination or apart. As used herein, an adenosine receptor "modulator" or "modulating"
compound or agent is a compound or agent that modulates at least one biological marker or
biological activity characteristic of osteoclasts and bone formation. The term "modulating" as
related to osteoclast differentiation, refers to the ability of a compound or agent to exert an effect
on precursors to osteoclasts, or to alter the expression of at least one gene related to
osteoclastogenesis. For example, expression of the following genes is modulated during
osteoclastogenesis: DC-Stamp, tartrate resistant alkaline phosphatase (TRAP), cathepsin K,
calcitonin receptor, and integrin.

[0061] As used herein, the term "candidate compound" or "test compound" or "agent" or "test
agent" refers to any compound or molecule that is to be tested. As used herein, the terms, which
are used interchangeably, refer to biological or chemical compounds such as simple or complex
organic or inorganic molecules, peptides, proteins, oligonucleotides, polynucleotides,
carbohydrates, or lipoproteins. A vast array of compounds can be synthesized, for example
oligomers, such as oligopeptides and oligonucleotides, and synthetic organic compounds based
on various core structures, and these are also included in the terms noted above. In addition,
various natural sources can provide compounds for screening, such as plant or animal extracts,
and the like. Compounds can be tested singly or in combination with one another. Agents or
candidate compounds can be randomly selected or rationally selected or designed. As used
herein, an agent or candidate compound is said to be "randomly selected" when the agent is
chosen randomly without considering the specific interaction between the agent and the target
compound or site. As used herein, an agent is said to be "rationally selected or designed", when
the agent is chosen on a nonrandom basis which takes into account the specific interaction
between the agent and the target site and/or the conformation in connection with the agent's
action.

[0062] "Treatment" or "treating" refers to therapy, prevention and prophylaxis and particularly
refers to administering medicine or performing medical procedures on a patient, for either
prophylaxis (prevention) or to cure or reduce the extent of or likelihood of occurrence of the
infirmity or malady or condition or event. In the present invention, the treatments using the agents described may be provided to stimulate or promote bone regeneration, to slow or halt bone loss, or to increase the amount or quality of bone density. Most preferably, the treating is for the purpose of stimulating or promoting bone regeneration or reducing or diminishing bone resorption. Treating as used herein also means administering the compounds for increasing bone density or for modulating osteoblastogenesis or osteoclastogenesis in individuals. Furthermore, in treating a subject, the compounds of the invention may be administered to a subject already suffering from loss of bone mass or other bone disease as provided herein or to prevent or inhibit the occurrence of such condition.

[0063] "Subject" or "patient" refers to a mammal, preferably a human, in need of treatment for a condition, disorder or disease.


[0065] "Osteoclast precursor" refers to a cell or cell structure, such as a pre-osteoclast, which is any cellular entity on the pathway of differentiation between a macrophage and a differentiated and functional osteoclast. The term osteoclast includes any osteoclast-like cell or cell structure which has differentiated fully or partially from a macrophage, and which has osteoclast character, including but not limited to positive staining for tartrate-resistant acid phosphatase (TRAP), but which is not a fully differentiated or functional osteoclast, including particularly aberrantly differentiated or non functional osteoclasts or pre-osteoclasts.

[0066] "Osteoclast culture" refers to any in vitro or ex vivo culture or system for the growth,
differentiation and/or functional assessment of osteoclasts or osteoclast precursors, whether in the absence or presence of other cells or cell types, for instance, but not limited to, osteoblasts, macrophages, hematopoietic or stromal cells.

[0067] "Osteoclast function", as used herein, refers to bone resorption and the processes required for bone resorption.

[0068] An "amount sufficient to inhibit osteoclast differentiation, formation or function" refers to the amount of the adenosine receptor agonist sufficient to block either the differentiation, the formation or the function of osteoclasts, more particularly, an amount ranging from about 0.1 nM to about 10 μM, or more preferentially from about 0.1 nM to about 5 μM, and most preferentially from about 0.1 nM to about 1 μM in vitro. In vivo amounts of an adenosine receptor agonist such as an adenosine A1 receptor agonist sufficient to block either the differentiation, the formation or the function of osteoclasts may range from about 0.1 mg/Kg of body weight per day to about 200 mg/Kg of body weight per day in vivo, or more preferentially from about 1 mg/Kg to about 100 mg/Kg, and most preferentially from about 25 mg/Kg to about 50 mg/Kg of body weight per day in vivo. It is understood that the dose, when administered in vivo, may vary depending on the clinical circumstances, such as route of administration, age, weight and clinical status of the subject in which inhibition of osteoclast differentiation, formation or function is desired.

[0069] In a specific embodiment, the term "about" means within 20%, preferably within 10%, and more preferably within 5% or even within 1%.

[0070] An "effective amount" or a "therapeutically effective amount" is an amount sufficient to stimulate or promote bone regeneration or decrease or prevent the symptoms associated with the conditions disclosed herein, including bone loss or in a decrease in bone mass or density, such as that which occurs with medical prosthetic devices or other related conditions contemplated for therapy with the compositions of the present invention. For example, an "effective amount" for therapeutic uses is the amount of the composition comprising an active compound herein required to provide reversal or inhibition of bone loss or delay the onset of prosthetic device loosening, increase and/or accelerate bone growth into prosthetic devices, etc. Such effective amounts may be determined using routine optimization techniques and are dependent on the particular condition to be treated, the condition of the subject, 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 is that which induces a statistically
significant difference in bone mass between treatment and control groups. This difference in bone mass or bone loss may be seen, for example, as at least 1-2%, or any clinically significant increase in bone mass or reduction in bone loss in the treatment group. Other measurements of clinically significant increases in healing may include, for example, an assay for the N-terminal propeptide of Type I collagen, 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 may be obtained from experiments carried out in vitro or in animal models of the disease of interest. The "effective amount" or "therapeutically effective amount" may range from about 1 mg/Kg to about 200 mg/Kg in vivo, or more preferentially from about 10 mg/Kg to about 100 mg/Kg, and most preferentially from about 25 mg/Kg to about 50 mg/Kg in vivo.

[0071] The phrase "pharmacologically acceptable" refers to molecular entities and compositions that are physiologically tolerable and do not typically produce an allergic or similar untoward reaction, such as gastric upset, dizziness and the like, when administered to a human. Preferably, as used herein, the term "pharmacologically acceptable" means approved by a regulatory agency of the federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeias for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the compound is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water or aqueous solution saline solutions and aqueous dextrose and glycerol solutions are preferably employed as carriers, particularly for injectable solutions. Suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E. W. Martin.

[0072] Binding compounds can also be characterized by their effect on the activity of the target molecule. Thus, a "low activity" compound has an inhibitory concentration (IC50) (for inhibitors or antagonists) or effective concentration (EC50) (applicable to agonists) of greater than 1 μM under standard conditions. By "very low activity" is meant an IC50 or EC50 of above 100 μM under standard conditions. By "extremely low activity" is meant an IC50 or EC50 of above 1 mM under standard conditions. By "moderate activity" is meant an IC50 or EC50 of 200 nM to 1 μM under standard conditions. By "moderately high activity" is meant an IC50 or EC50 of 1 nM to 200 nM. By "high activity" is meant an IC50 or EC50 of below 1 nM under standard conditions. The IC50 (or EC50) is defined as the concentration of compound at which 50% of the activity of the
target molecule (e.g., enzyme or other protein) activity being measured is lost (or gained) relative to activity when no compound is present. Activity can be measured using methods known to those of ordinary skill in the art, e.g., by measuring any detectable product or signal produced by occurrence of an enzymatic reaction, or other activity by a protein being measured.

[0073] An individual "at risk" may or may not have detectable disease, and may or may not have displayed detectable disease prior to the treatment methods described herein. "At risk" denotes that an individual who is determined to be more likely to develop a symptom based on conventional risk assessment methods or has one or more risk factors that correlate with development of a bone disease or low bone mass or density or enhanced susceptibility to bone resorption. An individual having one or more of these risk factors has a higher probability of developing bone resorption than an individual without these risk factors.

[0074] "Prophylactic" or "therapeutic" treatment refers to administration to the host of one or more of the subject compositions. If it is administered prior to clinical manifestation of the unwanted condition (e.g., disease or other unwanted state of the host animal) then the treatment is prophylactic, i.e., it protects the host against developing the unwanted condition, whereas if administered after manifestation of the unwanted condition, the treatment is therapeutic (i.e., it is intended to diminish, ameliorate or maintain the existing unwanted condition or side effects thereof).

General Description

[0075] Adenosine, a potent endogenous physiological mediator, regulates a wide variety of physiological processes via interaction with one or more of four G protein-coupled receptors (A₁, A₂A, A₂B and A₃), expressed on many cell types, including neutrophils, macrophages, fibroblasts, and endothelial cells. Because adenosine A₂A receptors inhibit the formation of giant cells from peripheral blood monocytes in vitro it was determined that adenosine, acting through one or another of these receptors, regulated the formation of osteoclasts.

[0076] In one embodiment, agents that interact with (e.g., bind to) and block, agonize or stimulate an adenosine receptor, in particular, A₂A (e.g., a functionally active fragment), are identified in a cell-based assay system. In accordance with this embodiment, cells expressing an adenosine receptor, a fragment of an adenosine receptor, an adenosine receptor related polypeptide, or a binding fragment thereof, are contacted with a candidate compound or a control compound and the ability of the candidate compound to interact with the receptor or fragment
thereof is determined. Alternatively, the ability of a candidate compound to compete for binding
with a known ligand or compound known to bind the receptor is measured. If desired, this assay
may be used to screen a plurality (e.g. a library) of candidate compounds. The cell, for example,
can be of prokaryotic origin (e.g., E. coli) or eukaryotic origin (e.g., yeast, insect or mammalian).
Further, the cells can express the receptor endogenously or be genetically engineered to express
the receptor, a binding fragment or a receptor fusion protein. In some embodiments, the receptor
or fragment thereof, or the candidate compound is labeled, for example with a radioactive label
(such as $^{32}$P, $^{35}$S or $^{125}$I) or a fluorescent label (such as fluorescein isothiocyanate, rhodamine,
phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde or fluorescamine) to enable
detecting an interaction between the $A_2A$ receptor and a candidate compound. The ability of the
candidate compound to interact directly or indirectly with a receptor or binding fragment thereof
or a fusion protein or to modulate the activity of the receptor can be determined by methods
known to those of skill in the art. For example, the interaction or modulation by a candidate
compound can be determined by flow cytometry, a scintillation assay, immunoprecipitation or
western blot analysis, based on the present description, or by a competitive radioreceptor assay.

[0077] Selecting the compounds that interact with or bind to an adenosine receptor or otherwise
agonize or stimulate or antagonize or inhibit the receptor may be performed in multiple ways.
The compounds may first be chosen based on their structural and functional characteristics, using
one of a number of approaches known in the art. For instance, homology modeling can be used to
screen small molecule libraries in order to determine which molecules are candidates to interact
with the receptor thereby selecting plausible targets. The compounds to be screened can include
both natural and synthetic ligands. Furthermore, any desired compound may be examined for its
ability to interact with or bind to the receptor.

[0078] Binding to or interaction with adenosine receptors may be determined by performing an
assay such as, for example, a binding assay between a desired compound and an adenosine
receptor. In one aspect, this is done by contacting said compound to an adenosine receptor and
determining its dissociation rate. Numerous possibilities for performing binding assays are well
known in the art. The indication of a compound's ability to bind to an adenosine receptor is
determined, e.g., by a dissociation rate, and the correlation of binding activity and dissociation
rates is well established in the art. For example, the assay may be performed by radio-labeling a
reference compound, or other suitable radioactive marker, and incubating it with the cell bearing
an adenosine receptor, in particular, an $A_1$ or $A_2A$. Test compounds are then added to these
reactions in increasing concentrations. After optimal incubation, the reference compound and
receptor complexes are separated, e.g., with chromatography columns, and evaluated for bound 
\(^{125}\)I-labeled peptide with a gamma (\(\gamma\)) counter. The amount of the test compound necessary to 
inhibit 50% of the reference compound's binding is determined. These values are then normalized 
to the concentration of unlabeled reference compound's binding (relative inhibitory concentration 
(RIC) \(= \text{concentration}_{\text{test}} / \text{concentration}_{\text{reference}}\)). A small RIC\(^{-1}\) value indicates strong relative 
binding, whereas a large RIC\(^{-1}\) value indicates weak relative binding. See, for example, Latek 
agonist mimic may be computationally evaluated and designed by means of a series of steps in 
which chemical groups or fragments are screened and selected for their ability to associate with 
the individual binding pockets or interface surfaces of the protein (e.g., the A\(_{2}\)A receptor). One 
skilled in the art may employ one of several methods to screen chemical groups or fragments for 
their ability to associate with the adenosine receptor. This process may begin by visual inspection 
of, for example, the protein/protein interfaces or the binding site on a computer screen based on 
the available crystal complex coordinates of the receptor, including a protein known to interact 
with selected fragments or chemical groups may then be positioned in a variety of orientations, or 
docked, at an individual surface of the receptor that participates in a protein/protein interface or 
in the binding pocket. Docking may be accomplished using software such as QUANTA and 
SYBYL, followed by energy minimization and molecular dynamics with standard molecular 
mechanics forcefields, such as CHARMM and AMBER (AMBER, version 4.0 (Kollman, 
University of California at San Francisco, copyright, 1994); QUANTA/CHARMM (Molecular 
Simulations, Inc., Burlington, Mass., copyright, 1994)). Specialized computer programs may also 
assist in the process of selecting fragments or chemical groups. These include: GRID (Goodford, 
(Miranker & Karplus, 1991, Proteins: Structure, Function and Genetics 11:29-34), available from 
Molecular Simulations, Burlington, Mass.; AUTODOCK (Goodsell & Olsen, 1990, Proteins: 
Structure, Function, and Genetics 8:195-202), available from Scripps Research Institute, La Jolla, 
Calif; and DOCK (Kuntz et al., 1982, J. Mol. Biol. 161:269-288), available from University of 
California, San Francisco, Calif. Once suitable chemical groups or fragments that bind to an 
adenosine receptor have been selected, they can be assembled into a single compound or agonist. 
Assembly may proceed by visual inspection of the relationship of the fragments to each other in 
the three-dimensional image displayed on a computer screen in relation to the structure 
coordinates thereof. This would be followed by manual model building using software such as 
QUANTA or SYBYL. Useful programs to aid one of skill in the art in connecting the individual 
chemical groups or fragments include: CAVEAT (Bartlett et al, 1989, ‘CAVEAT: A Program to 
Facilitate the Structure-Derived Design of Biologically Active Molecules’. In Molecular
Recognition in Chemical and Biological Problems’, Special Pub., Royal Chem. Soc. 78:182-196), available from the University of California, Berkeley, Calif; 3D Database systems such as MACCS-3D (MDL Information Systems, San Leandro, Calif). This area is reviewed in Martin, 1992, J. Med. Chem. 35:2145-2154); and HOOK (available from Molecular Simulations, Burlington, Mass.). Instead of proceeding to build an adenosine receptor agonist mimic, in a step-wise fashion one fragment or chemical group at a time, as described above, such compounds may be designed as a whole or ‘de novo’ using either an empty binding site or the surface of a protein that participates in protein/protein interactions or optionally including some portion(s) of a known activator(s). These methods include: LUDI (Bohm, 1992, J. Comp. Aid. Molec. Design 6:61-78), available from Molecular Simulations, Inc., San Diego, Calif; LEGEND (Nishibata et al, 1991, Tetrahedron 47:8985), available from Molecular Simulations, Burlington, Mass.; and LeapFrog (available from Tripos, Inc., St. Louis, Mo.). Other molecular modeling techniques may also be employed in accordance with this invention. See, e.g., Cohen et al, 1990, J. Med. Chem. 33:883-894. See also, Navia & Murcko, 1992, Current Opinions in Structural Biology 2:202-210.

[0079] Once a compound has been designed by the above methods, the efficiency with which that compound may bind to or interact with the adenosine receptor protein may be tested and optimized by computational evaluation. Agonists may interact with the receptor in more than one conformation that is similar in overall binding energy. In those cases, the deformation energy of binding is taken to be the difference between the energy of the free compound and the average energy of the conformations observed when the inhibitor binds to the receptor protein.

[0080] A compound selected for binding to the adenosine receptor may be further computationally optimized so that in its bound state it would preferably lack repulsive electrostatic interaction with the target protein. Such non-complementary electrostatic interactions include repulsive charge-charge, dipole-dipole and charge-dipole interactions. Specifically, the sum of all electrostatic interactions between the compound and the receptor protein when the mimic is bound to it preferably make a neutral or favorable contribution to the enthalpy of binding. Specific computer software is available in the art to evaluate compound deformation energy and electrostatic interaction. Examples of programs designed for such uses include: Gaussian 92, revision C (Frisch, Gaussian, Inc., Pittsburgh, Pa. copyright 1992); AMBER, version 4.0 (Kollman, University of California at San Francisco, copyright 1994); QUANTA/CHARMM (Molecular Simulations, Inc., Burlington, Mass., copyright 1994); and Insight II/Discover (Biosym Technologies Inc., San Diego, Calif, copyright 1994).
programs may be implemented, for instance, using a computer workstation, as are well-known in the art. Other hardware systems and software packages will be known to those skilled in the art.

[0081] Once an adenosine receptor modulating compound, such as an agonist, has been optimally designed, for example as described above, substitutions may then be made in some of its atoms or chemical groups in order to improve or modify its binding properties, or its pharmaceutical properties such as stability or toxicity. Generally, initial substitutions are conservative, i.e., the replacement group will have approximately the same size, shape, hydrophobicity and charge as the original group. Substitutions known in the art to alter conformation should be avoided. Such altered chemical compounds may then be analyzed for efficiency of binding to the receptor by the same computer methods described in detail above.

Candidate Compounds and Agents

[0082] Examples of agents, candidate compounds or test compounds include, but are not limited to, nucleic acids (e.g., DNA and RNA), carbohydrates, lipids, proteins, peptides, peptidomimetics, small molecules and other drugs. In one preferred aspect, agents can be obtained using any of the numerous suitable approaches in combinatorial library methods known in the art, including: biological libraries; spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the "one-bead one-compound" library method; and synthetic library methods using affinity chromatography selection. The biological library approach is limited to peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds (Lam, 1997, Anticancer Drug Des. 12:145; U.S. Patent No. 5,738,996 and U.S. Patent No. 5,807,683).

[0083] Phage display libraries may be used to screen potential ligands or adenosine receptor modulators. Their usefulness lies in the ability to screen, for example, a library displaying a large number of different compounds. For use of phage display libraries in a screening process, see, for instance, Kay et al, Methods, 240-246, 2001. An exemplary scheme for using phage display libraries to identify compounds that bind or interact with an adenosine receptor may be described as follows: initially, an aliquot of the library is introduced into microtiter plate wells that have previously been coated with target protein, e.g. A1 or A2 receptor. After incubation (e.g., 2 hours), the nonbinding phage are washed away, and the bound phage are recovered by denaturing or destroying the target with exposure to harsh conditions such as, for instance pH 2, but leaving the phage intact. After transferring the phage to another tube, the conditions are neutralized, followed by infection of bacteria with the phage and production of more phage particles. The
amplified phage are then rescreened to complete one cycle of affinity selection. After three or more rounds of screening, the phage are plated out such that there are individual plaques that can be further analyzed. For example, the conformation of binding activity of affinity-purified phage for the adenosine A_{2A} receptor may be obtained by performing ELISAs. One skilled in the art can easily perform these experiments. In one aspect, an A_{1} or A_{2A} receptor molecule used for any of the assays may be a recombinant A_{1} or A_{2A} receptor protein, or an A_{1} or A_{2A} fusion protein, an analog, derivative, or mimic thereof.


[0086] The methods of screening compounds may also include the specific identification or characterization of such compounds, whose effect on bone resorption is determined by the methods described above. If the identity of the compound is known from the start of the experiment, no additional assays are needed to determine its identity. However, if the screening for compounds that modulate the adenosine A_{2A} receptor is done with a library of compounds, it may be necessary to perform additional tests to positively identify a compound that satisfies all required conditions of the screening process. There are multiple ways to determine the identity of the compound. One process involves mass spectrometry, for which various methods are available and known to the skilled artisan (e.g. the neogenesis website). Neogenesis' ALIS (automated ligand identification system) spectral search engine and data analysis software allow for a highly specific identification of a ligand structure based on the exact mass of the ligand. One skilled in the art can also readily perform mass spectrometry experiments to determine the identity of the compound.
[0087] Antibodies, including polyclonal and monoclonal antibodies, particularly anti-A2A receptor antibodies and neutralizing antibodies may be useful as compounds to modulate osteoclast differentiation and/or function. These antibodies are available from such vendors as Upstate Biologicals, Santa Cruz, or they made be prepared using standard procedures for preparation of polyclonal or monoclonal antibodies known to those skilled in the art. Also, antibodies including both polyclonal and monoclonal antibodies, and drugs that modulate the activity of the adenosine receptor and/or its subunits may possess certain diagnostic applications and may for example, be utilized for the purpose of detecting and/or measuring conditions such as bone diseases, bone loss, or osteoclast differentiation and/or function. The adenosine receptor or its subunits may be used to produce both polyclonal and monoclonal antibodies to themselves in a variety of cellular media, by known techniques such as the hybridoma technique utilizing, for example, fused mouse spleen lymphocytes and myeloma cells. Likewise, small molecules that mimic or act as agonists for the activities of the A2A receptor may be discovered or synthesized, and may be used in diagnostic and/or therapeutic protocols.

**Therapeutic and Prophylactic Compositions and Their Use**

[0088] Candidates for therapy with the agents identified by the methods described herein are patients suffering from bone diseases, procedures involving bone injury, bone resorption or patients who have a medical prosthesis implanted or who contemplate receiving an implant medical prothetic device.

[0089] The invention provides methods of treatment featuring administering to a subject an effective amount of an agent of the invention. The compound is preferably substantially purified (e.g., substantially free from substances that limit its effect or produce undesired side-effects). The subject is preferably an animal, including but not limited to animals such as monkeys, cows, pigs, horses, chickens, cats, dogs, etc., and is preferably a mammal, and most preferably human. In one specific embodiment, a non-human mammal is the subject. In another specific embodiment, a human mammal is the subject. Accordingly, the agents identified by the methods described herein may be formulated as pharmaceutical compositions to be used for prophylaxis or therapeutic use to treat these patients.

[0090] Various delivery systems are known and can be used to administer a compound of the invention, e.g., encapsulation in liposomes, microparticles, or microcapsules. Methods of introduction can be enteral or parenteral and include but are not limited to intradermal,
intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, topical and oral routes. The compounds may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (e.g., oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically active agents. Administration can be systemic or local. In addition, it may be desirable to introduce the pharmaceutical compositions of the invention into the central nervous system by any suitable route, including intraventricular and intrathecal injection; intraventricular injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir, such as an Ommaya reservoir. Pulmonary administration can also be employed, e.g., by use of an inhaler or nebulizer, and formulation with an aerosolizing agent. In a specific embodiment, it may be desirable to administer the pharmaceutical compositions of the invention locally to the area in need of treatment.

[0091] Such compositions comprise a therapeutically effective amount of an agent, and a pharmaceutically acceptable carrier. In a particular embodiment, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopoeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E. W. Martin. Such compositions will contain a therapeutically effective amount of the compound, preferably in purified form, together with a suitable amount of carrier so as to provide the form
for proper administration to the subject. The formulation should suit the mode of administration.

[0092] In a preferred embodiment, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic such as lidocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

[0093] In another embodiment, the compound can be delivered in a vesicle, in particular a liposome (Langer (1990) Science 249:1527-1533; Treat et al., in Liposomes in the Therapy of Infectious Disease and Cancer, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353-365 (1989); Lopez-Berestein, ibid., pp. 317-327)


[0095] The present invention further contemplates therapeutic compositions useful in practicing
the therapeutic methods of this invention. A subject therapeutic composition includes, in admixture, a pharmaceutically acceptable excipient (carrier) and one or more of an adenosine receptor modulator, such as an adenosine A_1 or A_2A receptor agonist or antagonist, as described herein as an active ingredient. In a preferred embodiment, the composition comprises one or more compounds or agents capable of mimicking or serving as an agonist for the adenosine A_2A receptor or as an antagonist for the adenosine A_1 receptor.

[0096] Effects of the compounds or agents of the invention can first be tested for their ability to stimulate or mimic the adenosine receptor using standard techniques known in the art. More particularly, the selectivity of the compounds for the receptor can be assessed using radioligand binding assays whereby a test or candidate compound can be assayed for its ability to bind to a cell having or expressing the receptor (including any of the known adenosine receptors, A_1, A_2A, A_2B or A_3). Cells can be transfected with the nucleic acid encoding the various adenosine receptors and competitive binding assays with radiolabeled ligands run to evaluate the specificity of the particular candidate compounds. The cDNAs for human A_1 (see GenBank accession number BC026340), A_2A (see GenBank accession number NM000675), A_2B (see GenBank accession number NM000676) or A_3 (see GenBank accession number AY136749 or L22607 or NM_000777) can be used to prepare the nucleic acid constructs for use in these methods.

[0097] The present compounds or agents that modulate the adenosine receptor, in particular, the agonists of the A_2A receptor and antagonists of the A_1 receptor, themselves can be used as the sole active agents, or can be used in combination with one or more other active ingredients. In particular, combination therapy using the adenosine receptor agonists with one or more other agents is contemplated. These agents are known in the art, and can be selected from anti-inflammatory compounds, bisphosphonates, soluble RANK, and bone morphogenetic proteins, for instance.

[0098] When contemplating combination therapy with an adenosine receptor agonist and one or more of the above-noted agents, it is important to assess clinical safety by methods known to those skilled in the art. Appropriate dose titration may be necessary when certain groups of compounds are contemplated for use together.

[0099] The compounds or compositions of the invention may be combined for administration with or embedded in polymeric carrier(s), biodegradable or biomimetic matrices or in a scaffold. The carrier, matrix or scaffold may be of any material that will allow composition to be
incorporated and expressed and will be compatible with the addition of cells or in the presence of cells. Preferably, the carrier matrix or scaffold is predominantly non-immunogenic and is biodegradable. Examples of biodegradable materials include, but are not limited to, polyglycolic acid (PGA), polylactic acid (PLA), hyaluronic acid, catgut suture material, gelatin, cellulose, nitrocellulose, collagen, albumin, fibrin, alginate, cotton, or other naturally-occurring biodegradable materials. It may be preferable to sterilize the matrix or scaffold material prior to administration or implantation, e.g., by treating it with ethylene oxide or by gamma irradiation or irradiation with an electron beam. In addition, a number of other materials may be used to form the scaffold or framework structure, including but not limited to: nylon (polyamides), dacron (polyesters), polystyrene, polypropylene, polyacrylates, polyvinyl compounds (e.g., polyvinylchloride), polycarbonate (PVC), polytetrafluorethylene (PTFE, teflon), thermox (TPX), polymers of hydroxy acids such as polylactic acid (PLA), polyglycolic acid (PGA), and polylactic acid-glycolic acid (PLGA), polyorthoesters, polyanhydrides, polyphosphazenes, and a variety of polyhydroxyalkanoates, and combinations thereof. Matrices suitable include a polymeric mesh or sponge and a polymeric hydrogel. In the preferred embodiment, the matrix is biodegradable over a time period of less than a year, more preferably less than six months, most preferably over two to ten weeks. The polymer composition, as well as method of manufacture, can be used to determine the rate of degradation. For example, mixing increasing amounts of polylactic acid with polyglycolic acid decreases the degradation time. Meshes of polyglycolic acid that can be used can be obtained commercially, for instance, from surgical supply companies (e.g., Ethicon, N.J.). A hydrogel is defined as a substance formed when an organic polymer (natural or synthetic) is cross-linked via covalent, ionic, or hydrogen bonds to create a three-dimensional open-lattice structure which entraps water molecules to form a gel. In general, these polymers are at least partially soluble in aqueous solutions, such as water, buffered salt solutions, or aqueous alcohol solutions, that have charged side groups, or a monovalent ionic salt thereof.

[0100] For use in treating animal subjects, the compositions of the invention can be formulated as pharmaceutical or veterinary compositions. Depending on the subject to be treated, the mode of administration, and the type of treatment desired, e.g., prevention, prophylaxis, therapy; the compositions are formulated in ways consonant with these parameters. A summary of such techniques is found in Remington's Pharmaceutical Sciences, latest edition, Mack Publishing Co., Easton, Pa.

[0101] The preparation of therapeutic compositions containing small organic molecules polypeptides, analogs or active fragments as active ingredients is well understood in the art. The
compositions of the present invention may be administered parenterally, orally, by inhalation spray, topically, rectally, nasally, buccally, vaginally or via an implanted reservoir. The term "parenteral" as used herein includes subcutaneous, intravenous, intramuscular, intra-articular, intra-synovial, intrasternal, intrathecal, intrahepatic, intralesional and intracranial injection or infusion techniques. Formulations may be prepared in a manner suitable for systemic administration or for topical or local administration. Systemic formulations include, but are not limited to those designed for injection (e.g., intramuscular, intravenous or subcutaneous injection) or may be prepared for transdermal, transmucosal, nasal, or oral administration. Such compositions may be prepared as injectables, either as liquid solutions or suspensions, however, solid forms suitable for solution in, or suspension in, liquid prior to injection can also be prepared. The preparation can also be emulsified. The active therapeutic ingredient is often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof. The formulation will generally include a diluent as well as, in some cases, adjuvants, buffers, preservatives and the like. In addition, if desired, the composition can contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents which enhance the effectiveness of the active ingredient.

[0102] A small organic molecule/compound, a polypeptide, an analog or active fragment thereof can be formulated into the therapeutic composition as neutralized pharmaceutically acceptable salt forms. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the polypeptide or antibody molecule) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed from the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like. For oral administration, the compositions can be administered also in liposomal compositions or as microemulsions. Suitable forms include syrups, capsules, tablets, as is understood in the art.

[0103] The compositions of the present invention may also be administered locally to sites in subjects, both human and other vertebrates, such as domestic animals, rodents and livestock, using a variety of techniques known to those skilled in the art. For example, these may include sprays, lotions, gels or other vehicles such as alcohols, polyglycols, esters, oils and silicones.
[0104] The administration of the compositions of the present invention may be pharmacokinetically and pharmacodynamically controlled by calibrating various parameters of administration, including the frequency, dosage, duration mode and route of administration. Variations in the dosage, duration and mode of administration may also be manipulated to produce the activity required.

[0105] The therapeutic adenosine receptor modulator (e.g. agonist or antagonist) compositions are conventionally administered in the form of a unit dose, for instance intravenously, as by injection of a unit dose, for example. The term "unit dose" when used in reference to a therapeutic composition of the present invention refers to physically discrete units suitable as unitary dosage for humans, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required diluent; i.e., carrier, or vehicle.

[0106] The compositions are administered in a manner compatible with the agent selected for treating the subject, the dosage formulation, and in a therapeutically effective amount. If one desires to achieve the desired effect in vitro, the effective amounts may range from about 0.1 nM to about 10 µM, more preferably about 0.1 nM to about 5 µM, and most preferably from about 0.1 nM to about 1 nM. The desired effect refers to the effect of the agent on reducing or inhibiting osteoclast differentiation or stimulation, reducing or inhibiting bone resorption and reducing or inhibiting loosening of a medical prosthesis. Moreover, the quantity of the adenosine receptor agonist to be administered depends on the subject to be treated, and degree of stimulation or mimicry of the adenosine receptor desired or the extent or severity of bone resorption. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner and are peculiar to each individual. However, suitable dosages to achieve the desired therapeutic effect in vivo may range from about 0.1 mg/kg body weight per day to about 200 mg/kg body weight per day, or from about 1.0 mg/kg body weight per day to about 100 mg/kg body weight per day, preferably about 25 mg/kg body weight per day to about 50 mg/kg body weight per day. In a particular embodiment, the term "about" means within 20%, preferably within 10%, and more preferably within 5%. The preferred dose will depend on the route of administration. However, dosage levels are highly dependent on the nature of the disease or situation, the condition of the subject, the judgment of the practitioner, and the frequency and mode of administration. If the oral route is employed, the absorption of the substance will be a factor effecting bioavailability. A low absorption will have the effect that in the gastro-intestinal
tract higher concentrations, and thus higher dosages, will be necessary. Suitable regimes for initial administration and further administration are also variable, but are typified by an initial administration followed by repeated doses at one or more hour intervals by a subsequent injection or other administration. Alternatively, continuous intravenous infusion sufficient to maintain desired concentrations, e.g. in the blood, are contemplated. The composition may be administered as a single dose multiple doses or over an established period of time in an infusion.

[0107] It will be understood that the appropriate dosage of the substance should suitably be assessed by performing animal model tests, where the effective dose level (e.g., ED50) and the toxic dose level (e.g. TD50) as well as the lethal dose level (e.g. LD50 or LD10) are established in suitable and acceptable animal models. Further, if a substance has proven efficient in such animal tests, controlled clinical trials should be performed.

[0108] The compounds or compositions of the present invention may be modified or formulated for administration at the site of pathology. Such modification may include, for instance, formulation which facilitate or prolong the half-life of the compound or composition, particularly in the environment. Additionally, such modification may include the formulation of a compound or composition to include a targeting protein or sequence which facilitates or enhances the uptake of the compound/composition to bone or bone precursor cells. In a particular embodiment, such modification results in the preferential targeting of the compound to bone or bone precursor cells versus other locations or cells. In one embodiment, a tetracycline, tetracycline family or bisphosphonate may be utilized to target the compound or composition of the present invention to bone or bone cells, including osteoclasts and osteoclast precursors. Novel heterocycles as bone targeting compounds are disclosed in U.S. Patent Publication No. 2002/0103 161 A1, which is incorporated herein by reference in its entirety.

[0109] Pharmaceutically acceptable carriers useful in these pharmaceutical compositions include, e.g., ion exchangers, alumina, aluminum stearate, lecithin, serum proteins, such as human serum albumin, buffer substances such as phosphates, glycine, sorbic acid, potassium sorbate, partial glyceride mixtures of saturated vegetable fatty acids, water, salts or electrolytes, such as protamine sulfate, disodium hydrogen phosphate, potassium hydrogen phosphate, sodium chloride, zinc salts, colloidal silica, magnesium trisilicate, polyvinyl pyrrolidone, cellulose-based substances, polyethylene glycol, sodium carboxymethylcellulose, polyacrylates, waxes, polyethylene-polyoxypropylene-block polymers, polyethylene glycol and wool fat.
[0110] Sterile injectable forms of the compositions may be aqueous or oleaginous suspensions. The suspensions may be formulated according to techniques known in the art using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation may also be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose, any bland fixed oil may be employed including synthetic mono- or di-glycerides. Fatty acids, such as oleic acid and its glyceride derivatives are useful in the preparation of injectables, as are natural pharmaceutically-acceptable oils, such as olive oil or castor oil, especially in their polyoxylethylated versions. These oil solutions or suspensions may also contain a long-chain alcohol diluent or dispersant, such as carboxymethyl cellulose or similar dispersing agents which are commonly used in the formulation of pharmaceutically acceptable dosage forms including emulsions and suspensions. Other commonly used surfactants, such as Tweens, Spans and other emulsifying agents or bioavailability enhancers which are commonly used in the manufacture of pharmaceutically acceptable solid, liquid, or other dosage forms may also be used for the purposes of formulation.

[0111] Parenteral formulations may be a single bolus dose, an infusion or a loading bolus dose followed with a maintenance dose. These compositions may be administered once a day or on an "as needed" basis.

[0112] The pharmaceutical compositions may be orally administered in any orally acceptable dosage form including, capsules, tablets, aqueous suspensions or solutions. In the case of tablets for oral use, carriers commonly used include lactose and corn starch. Lubricating agents, such as magnesium stearate, are also typically added. For oral administration in a capsule form, useful diluents include lactose and dried cornstarch. When aqueous suspensions are required for oral use, the active ingredient is combined with emulsifying and suspending agents. If desired, certain sweetening, flavoring or coloring agents may also be added.

[0113] Alternatively, the pharmaceutical compositions may be administered in the form of suppositories for rectal administration. These can be prepared by mixing the agent with a suitable non-irritating excipient which is solid at room temperature but liquid at rectal temperature and therefore will melt in the rectum to release the drug. Such materials include cocoa butter, beeswax and polyethylene glycols.
The pharmaceutical compositions of this invention may also be administered topically. Topical application can be effected in a rectal suppository formulation (see above) or in a suitable enema formulation. Topically-transdermal patches may also be used.

For topical applications, the pharmaceutical compositions may be formulated in a suitable ointment containing the active component suspended or dissolved in one or more carriers. Carriers for topical administration of the compounds of this invention include, mineral oil, liquid petrolatum, white petrolatum, propylene glycol, polyoxyethylene, polyoxypropylene compound, emulsifying wax and water. Alternatively, the pharmaceutical compositions can be formulated in a suitable lotion or cream containing the active components suspended or dissolved in one or more pharmaceutically acceptable carriers. Suitable carriers include, but are not limited to, mineral oil, sorbitan monostearate, polysorbate 60, cetyl esters wax, cetearyl alcohol, 2-octyldodecanol, benzyl alcohol and water.

For ophthalmic use, the pharmaceutical compositions may be formulated as micronized suspensions in isotonic, pH adjusted sterile saline, or, preferably, as solutions in isotonic, pH adjusted sterile saline, either with or without a preservative such as benzylalkonium chloride. Alternatively, for ophthalmic uses, the pharmaceutical compositions may be formulated in an ointment such as petrolatum.

The pharmaceutical compositions of this invention may also be administered by nasal aerosol or inhalation. Such compositions are prepared according to techniques well-known in the art of pharmaceutical formulation and may be prepared as solutions in saline, employing benzyl alcohol or other suitable preservatives, absorption promoters to enhance bioavailability, fluorocarbons, and/or other conventional solubilizing or dispersing agents.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects (a) approval by the agency of manufacture, use or sale for human administration, (b) directions for use, or both.
Effective Doses

[0119] Toxicity and therapeutic efficacy of compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD₅₀ (the dose lethal to 50% of the population) and the ED₅₀ (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD₅₀/ED₅₀. Compounds that exhibit large therapeutic indices are preferred. While compounds that exhibit toxic side effects can be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to unaffected cells and, thereby, reduce side effects.

[0120] The data obtained from cell culture assays and animal studies can be used in formulating a dose range for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED₅₀ with little or no toxicity. The dosage can vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose can be formulated in animal models to achieve a circulating plasma concentration range that includes the IC₅₀ (i.e., the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to optimize efficacious doses for administration to humans. Plasma levels can be measured by any technique known in the art, for example, by high performance liquid chromatography.

[0121] In addition, in vitro assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the disease or disorder, and should be decided according to the judgment of the practitioner and each subject's circumstances. Normal dose ranges used for particular therapeutic agents employed for specific diseases can be found in the Physicians' Desk Reference 54th Edition (2000).

EXAMPLES

[0122] The following examples are set forth to provide those of ordinary skill in the art with a description of how to make and use the methods and compositions of the invention, and are not intended to limit the scope thereof. Efforts have been made to insure accuracy of numbers used (e.g., amounts, temperature, etc.) but some experimental errors and deviations should be
accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is average molecular weight, temperature is in degrees Centigrade, and pressure is at or near atmospheric.

Example 1

Materials and methods

[0123] A 3mm in diameter defect was created in mouse calvaria into which was fit a collagen matrix. The matrix was soaked in either an adenosine $A_2A$ receptor agonist (CGS21680, 1mM) or an adenosine $A_1$ antagonist (DPCPX, 1mM). In addition, in some mice the defect was treated with dipyridamole, an agent that blocks adenosine uptake via the purine transporter ENT1, in order to raise endogenous adenosine levels at the site of the defect.

Results

[0124] As shown in Figure 1, stimulation of adenosine $A_2A$ receptors or blockade of $A_1$ receptors dramatically increases bone regeneration from approximately 30% to nearly 80%. Moreover, dipyridamole stimulates bone regeneration as well. These findings are surprising since previous work demonstrated that neither of these receptors regulates osteoblast formation, the cell which is primarily involved in bone formation. Although prior publications have taught dipyridamole as a stimulus for bone formation, they have not demonstrated any effect of dipyridamole on bone formation, and they have suggested alternative mechanisms of action of dipyridamole (as a pro-drug or as an inhibitor of phosphodiesterase). Thus, the effects of these agents are surprising.

Discussion

[0125] It was previously demonstrated that blockade of adenosine $A_1$ receptors or stimulation of adenosine $A_2A$ receptors diminishes osteoclast formation. It was also previously demonstrated that stimulation of $A_2A$ receptors inhibits wear particle-induced bone resorption in a murine model of prosthesis loosening. These data demonstrate that adenosine receptors play a role in promoting bone regeneration.

Example 2

Materials and Methods

[0126] A 3mm portion of the calvarium was trephined with a drill followed by implantation of a collagen matrix soaked in either a vehicle or a pharmacologic agent and/or receptor antagonists to promote bone regeneration. The matrix was reinjected daily with the agent for periods ranging
from 2-8 weeks prior to harvest of the mice and examination of the calvaria. We examined the
effect of varying doses of an adenosine AIAR antagonist, an A2AR agonist with and without
selective antagonists, an A2BAR agonist with and without a selective antagonist and an agent that
enhances extracellular adenosine concentrations by blocking cellular adenosine uptake
(dipyridamole) with and without a selective A2AR or A2BAR antagonist. Experiments were
performed with specific agonists and antagonists at varying doses, and specific receptor agonists
were tested on corresponding knockout mice to better confirm the role of the indicated receptor.

[0127] Staining of decalcified bone sections for osteoclasts (TRAP staining) and osteoblasts
(alkaline phosphatase staining) was performed. Immunostaining for other markers of osteoclast
(cathepsin K) lineage and other molecules of interest (e.g. semaphorin 4D, plexin-B1, nuclear b-
catenin) were performed. As our initial experiments tested only a single time point (8 weeks), at
which point bone regeneration was complete, we harvested calvaria at biweekly intervals to
better understand the timing and occurrence of those events involved in promoting bone growth.

[0128] Some of the calvaria were decalcified, and histochemical and immunohistochemical
staining and microscopic analyses were performed. Other calvaria were cleaned of adherent
tissue and subject to microCT analysis for bone defect size and percent regeneration measures.
These experiments were performed with 10 mice in each group for histologic and
immunohistologic experiments, to allow 90% power to detect as little as a 25% difference with a
standard deviation of 15% using a 2-sided analysis and a 95% confidence interval, differences
much smaller than those observed in initial experiments although earlier timepoints and dose-
response analyses yielded smaller differences. Because calvaria that have been subject to
microCT cannot be utilized for histology, and decalcified calvaria are not useful for microCT
analysis, each treatment at all timepoints required 20 animals.

[0129] Finally, we determined the effect of the selective A2AR deletions on bone formation at the
site of wear particle-induced osteolysis using Xenolight Redject Bone Probe 680 with scanning
and quantitation of fluorescence in the IVIS apparatus. We have been able to use this
fluorophore, which binds to hydroxyapatite, to determine dynamic bone formation over time in
mice without having to sacrifice the mice at any of the timepoints. This technique permits
accurate assessment of bone formation while reducing the number of animals required to
determine bone formation over time. All conditions were tested in groups of five animals and
repeated at least once.
Results

[0130] Figure 2 demonstrates that the adenosine A<sub>i</sub> receptor antagonist DPCPX, the A<sub>2A</sub> agonist CGS21680 and the A<sub>2B</sub> agonist BAY606583 all stimulate bone regeneration. Similarly the adenosine uptake inhibitor dipyridamole, which increases local adenosine concentration, promotes bone regeneration as well. In contrast, the adenosine A<sub>3</sub> receptor agonist has no effect on bone regeneration. Thus blockade of adenosine A<sub>i</sub> receptors and stimulation of both adenosine A<sub>2A</sub> and A<sub>2B</sub> receptors promotes bone regeneration. As shown in Figure 2B, the effects are first detectable after as little as 2-4 weeks for the A<sub>2A</sub> agonist, the A<sub>i</sub> antagonist and dipyridamole. Figure 3 demonstrates that the effects of all four of these agents, the adenosine A<sub>i</sub> receptor antagonist DPCPX, the A<sub>2A</sub> agonist CGS21680, the A<sub>2B</sub> agonist, and the adenosine A<sub>3</sub> receptor agonist are dose-dependent and appear to achieve maximal effect at 1µM. Figure 4 demonstrates that the adenosine A<sub>2A</sub> receptor antagonist ZM241385 blocks the effect of both the A<sub>2A</sub> agonist CGS21680 and dipyridamole indicating that the A<sub>2A</sub> receptor plays a role in promoting bone regeneration. Moreover, the A<sub>2B</sub> receptor antagonist MRS1764 blocks the effect of dipyridamole as well indicating that the A<sub>2B</sub> receptor is also involved in promoting bone regeneration by extracellular adenosine.

[0131] Figure 5 provides further evidence for the involvement of the A<sub>i</sub> and A<sub>2B</sub> receptors. Deletion of the A<sub>i</sub> receptor (AiKO), like blockade of that receptor, increases bone regeneration. Moreover, deletion of the A<sub>2B</sub> receptor (A<sub>2BKO</sub>) reverses the effect of the A<sub>2A</sub> agonist CGS21680 and dipyridamole as well as the A<sub>i</sub> receptor antagonist DPCPX further supporting the role of the A<sub>2B</sub> receptor. These results further support the effect of A<sub>i</sub> and A<sub>2B</sub> receptors in bone regeneration and do not rule out a role for the A<sub>2A</sub> receptor as well. Figure 6 directly demonstrates the effect of A<sub>i</sub> receptor blockade and A<sub>2A</sub> receptor stimulation on bone formation using a fluorescent indicator of bone formation. Figure 7 provides further evidence for the effect of the A<sub>i</sub> antagonist DPCPX, the A<sub>2A</sub> receptor agonist CGS21680 and dipyridamole on bone formation in which all three of these agents increase alkaline phosphatase positive osteoblasts and reduce TRAP stained osteoclasts in the regenerating bone. Figure 8 demonstrates a reduction of both RANK+ osteoblasts, RANKL+ osteoclasts and an increase in osteoprotegerin+ cells in the regenerating bone of mice treated with the A<sub>i</sub> antagonist DPCPX, the A<sub>2A</sub> receptor agonist CGS21680 and the adenosine uptake inhibitor dipyridamole. Figure 9 demonstrates histologically the bone formation that occurs associated with treatment with the A<sub>i</sub> antagonist DPCPX, the A<sub>2A</sub> agonist CGS21680 and the adenosine uptake inhibitor dipyridamole.

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CLAIMS:

1. A method for stimulating or promoting bone regeneration comprising
administering to a subject a therapeutically effective amount of an adenosine receptor agonist, or
an analog or derivative thereof.

2. The method of claim 1 wherein the adenosine receptor is selected from the group
consisting of A₁, A₂A, A₂B and A₃.

3. The method of claim 2 wherein the adenosine receptor is an A₂A receptor.

4. The method of claim 1 wherein and the agonist is an adenosine receptor A₂A agonist.

5. The method of claim 4 wherein and the adenosine receptor A₂A agonist is a selective
adenosine receptor agonist.

6. The method of claim 4 wherein the adenosine A₂A receptor agonist is selected from the
group consisting of CGS 21680, IB-MECA and R-PIA.

7. The method of claim 4 wherein the adenosine A₂A receptor agonist is administered in
combination with one or more of other compounds or agents for inhibiting bone resorption or
osteoclast differentiation or for stimulating osteoblast differentiation or activation.

8. A method for stimulating or promoting bone regeneration comprising administering to a
subject a therapeutically effective amount of an adenosine receptor antagonist, or an analog, or
derivative thereof.

9. The method of claim 8 wherein the adenosine receptor is selected from the group
consisting of A₁, A₂A, A₂B and A₃.

10. The method of claim 8 wherein the adenosine receptor is an Aᵢ receptor.

11. The method of claim 8 wherein and the antagonist is an adenosine receptor Aᵢ antagonist.
12. The method of claim 11 wherein and the adenosine receptor A \textit{i} antagonist is a selective adenosine receptor antagonist.

13. The method of claim 11 wherein the adenosine A \textit{i} receptor antagonist is administered in combination with one or more of other compounds or agents for inhibiting bone resorption or osteoclast differentiation or for stimulating osteoblast differentiation or activation.

14. A method for stimulating or promoting bone regeneration comprising administering to a subject a therapeutically effective amount of adenosine or a therapeutically effective amount of an agent that upregulates, increases the amount of or increases the biological activity of adenosine or an analog or derivative thereof.

15. The method of claim 14 wherein the agent is dipyridamole.

16. The method of claim 14 wherein the agent is administered in combination with one or more of other agents for inhibiting bone resorption or osteoclast differentiation or for stimulating osteoblast differentiation or activation.

17. A method for stimulating differentiation or activation of osteoblasts comprising administering to a subject a therapeutically effective amount of an adenosine receptor agonist, or an analog, or derivative thereof.

18. The method of claim 17 wherein the adenosine receptor is selected from the group consisting of A \textit{1}, A \textit{2A}, A \textit{2B} and A \textit{3}.

19. The method of claim 18 wherein the adenosine receptor is an A \textit{2A} receptor.

20. The method of claim 17 wherein and the agonist is an adenosine receptor A \textit{2A} agonist.

21. The method of claim 20 wherein and the adenosine receptor A \textit{2A} agonist is a selective adenosine receptor agonist.

22. The method of claim 21 wherein the adenosine A \textit{2A} receptor agonist is selected from the group consisting of CGS 21680, IB-MECA and R-PIA.
23. The method of claim 20 wherein the adenosine $A_2A$ receptor agonist is administered in combination with one or more of other compounds or agents for inhibiting bone resorption or osteoclast differentiation or for stimulating osteoblast differentiation or activation.

24. A method for stimulating differentiation or activation of osteoblasts comprising administering to a subject a therapeutically effective amount of an adenosine receptor antagonist, or an analog, or derivative thereof.

25. The method of claim 24 wherein the adenosine receptor is selected from the group consisting of $A_1$, $A_2A$, $A_2B$ and $A_3$.

26. The method of claim 24 wherein the adenosine receptor is an $A_i$ receptor.

27. The method of claim 24 wherein the antagonist is an adenosine receptor $A_i$ antagonist.

28. The method of claim 27 wherein the adenosine receptor $A_i$ antagonist is a selective adenosine receptor antagonist.

29. The method of claim 27 wherein the adenosine $A_i$ receptor antagonist is administered in combination with one or more of other compounds or agents for inhibiting bone resorption or osteoclast differentiation or for stimulating osteoblast differentiation or activation.

30. A method for stimulating differentiation or activation of osteoblasts comprising administering to a subject a therapeutically effective amount of adenosine or a therapeutically effective amount of an agent that upregulates, increases the amount of or increases the biological activity of adenosine or an analog or derivative thereof.

31. The method of claim 30 wherein the agent is dipyridamole.

32. The method of claim 30 wherein the agent is administered in combination with one or more of other compounds or agents for inhibiting bone resorption or osteoclast differentiation or for stimulating osteoblast differentiation or activation.
A method for treating, ameliorating or preventing a bone disease or a condition in a subject having such disease or condition or at risk for developing such disease or condition, comprising administering to the subject a therapeutically effective amount of an adenosine receptor agonist, or an analog or derivative thereof, by administering to a subject a therapeutically effective amount of an adenosine receptor antagonist, or an analog or derivative thereof, or by administering to a subject a therapeutically effective amount of adenosine or a therapeutically effective amount of an agent that upregulates or increases the amount of or the biological activity of adenosine.

34. The method of claim 33 wherein the bone disease or condition is selected from the group consisting of osteoporosis, juvenile osteoporosis, onset of menopause, osteoporotic fractures, giant cell tumors of bone, renal osteodystrophy, osteogenesis imperfecta, hypercalcemia, hyperparathyroidism, osteomalacia, osteohalisteresis, osteolytic bone disease, osteonecrosis, Paget's disease, rheumatoid arthritis, inflammatory arthritis, osteomyelitis, corticosteroid treatment, metastatic bone diseases, malignancy-induced osteoporosis and bone lysis, childhood idiopathic bone loss, periodontal bone loss, age-related loss of bone mass, osteotomy, bone loss associated with prosthetic ingrowth, osteopenia, bone fractures, bone defects, plastic surgery, and implantations.

35. A pharmaceutical composition comprising an adenosine receptor agonist or antagonist.

36. The pharmaceutical composition of claim 35 wherein the agonist is an adenosine A<sub>2A</sub> receptor agonist.

37. The pharmaceutical composition of claim 35 wherein the antagonist is an adenosine A<sub>1</sub> receptor antagonist.

38. The pharmaceutical composition of claim 35 further comprising adenosine or a therapeutically effective amount of an agent that upregulates, increases the amount of or increases the biological activity of adenosine or an analog or derivative thereof.
FIG. 3B

Original defect | Control | CGS21680
---|---|---
|  |  | $10^{-9} M$
|  |  | $10^{-8} M$
|  |  | $10^{-7} M$
|  |  | $10^{-6} M$

![Images of bone formation with control and different concentrations of CGS21680.]

% bone formation

- Control
- $10^{-9} M$
- $10^{-8} M$
- $10^{-7} M$
- $10^{-6} M$

Log [CGS21680]

---

Bone volume

- Control
- $10^{-9} M$
- $10^{-8} M$
- $10^{-7} M$
- $10^{-6} M$

Log [CGS21680]
FIG. 5C

% bone formation

- Control
- A1KO
- A2AKO
- A2BKO

***
*

FIG. 6A

Control
DPCPX
CGS21680
Dipyridamole

FIG. 6B

Total flux in %

- Control
- DPCPX
- CGS21680
- Dipyridamole

Weeks since trephination

SUBSTITUTE SHEET (RULE 26)
FIG. 7A

Alkaline Phosphatase

TRAP staining

Original defect

Control

DPCPX

CGS21680

Dipyridamole
FIG. 7B

- Control
- DPCPX
- CGS21680
- Dipyridamole

ALKP positive cells/hpf

Weeks since trephination

FIG. 7C

- Control
- DPCPX
- CGS21680
- Dipyridamole

TRAP positive cells/hpf

Weeks since trephination
FIG. 8A

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<thead>
<tr>
<th>Control</th>
<th>DPCPX</th>
<th>CGS21680</th>
<th>Dipyridamole</th>
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<tr>
<td>RANK</td>
<td>RANKL</td>
<td>Osteoprotegerin</td>
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[Images of tissue sections labeled Control, DPCPX, CGS21680, and Dipyridamole, showing RANK, RANKL, and Osteoprotegerin expressions]
FIG. 9

Original defect

Control

DPCPX

CGS

IB-MECA

Dipyridamole
INTERNATIONAL SEARCH REPORT
International application No. PCT/US2013/027097

A. CLASSIFICATION OF SUBJECT MATTER

A61K 31/70(2006.01)i, A61K 31/7076(2006.01)i, A61K 31/52(2006.01)i, A61P 19/00(2006.01)i

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)

A61K 31/70; A61K 31/7076

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

Korean utility models and applications for utility models

Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords: adenosine receptor, antagonist, agonist, bone, osteoporosis

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>
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<tbody>
<tr>
<td>X</td>
<td>US 2009-0123510 A1 (CRONSTEIN et al.) 14 May 2009 See abstract; and claims 28 and 29.</td>
<td>35, 36, 38</td>
</tr>
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<td>A</td>
<td>US 2007-0191279 A1 (CRONSTEIN et al.) 16 August 2007 See abstract and claims 44-47.</td>
<td>35, 37</td>
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<td>A</td>
<td>WO 2009-086077 A2 (ENDACEA, INC.) 09 July 2009 See abstract and page 1, lines 5-30.</td>
<td>35-38</td>
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</table>

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

* Special categories of cited documents:
  "A" document defining the general state of the art which is not considered to be of particular relevance
  "E" earlier application or patent but published on or after the international filing date
  "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)
  "O" document referring to an oral disclosure, use, exhibition or other means
  "P" document published prior to the international filing date but later than the priority date claimed
  "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
  "X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
  "Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
  "K" document member of the same patent family

Date of the actual completion of the international search 19 June 2013 (19.06.2013)

Date of mailing of the international search report 19 June 2013 (19.06.2013)

Name and mailing address of the ISA/KR
Korean Intellectual Property Office 189 Cheongsa-ro, Seo-gu, Daejeon Metropolitan City, 302-701 Republic of Korea
Facsimile No. 82-42-472-7140

Authorized officer CHOL Sung Hee
Telephone No. 82-42-481-8740

Form PCT/ISA/210 (second sheet) (July 2009)
INTERNATIONAL SEARCH REPORT

Box No. II  Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 1-34
   because they relate to subject matter not required to be searched by this Authority, namely:
   Claims 1-34 pertain to methods for treatment of the human body by therapy, and thus relate to a subject matter which this International Searching Authority is not required, under Article 17(2)(a)(i) of the PCT and Rule 39.1(iv) of the Regulations under the PCT, to search.

2. ☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III  Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☑ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☑ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☒ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☒ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☒ No protest accompanied the payment of additional search fees.
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