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(54) **Title**: USE OF SOX TRANSCRIPTION FACTOR ACTIVATORS FOR STIMULATING CHONDROGENESIS

(57) **Abstract**: The invention is directed to a method for stimulating chondrogenesis comprising administering to a subject in need thereof a pharmaceutical composition comprising a compound selected from the group consisting of phenazopyridine hydrochloride, cytochalasin B, methysticin, rhapontin, thiabendazole, indoprofen, oxybenzone, chrysin, pinocembrin, 7-hydroxyflavone, 6-methoxyluteolin, 6,7- dihydroxyflavone, tamarixetine, genistin, 5-methoxyflavone, pratol, 5,6- dehydrokawain, harringtonine, galangine, and kaempferol.

METHODS FOR STIMULATING CHONDROGENESIS

[001] This application claims the benefit of priority to U.S. Provisional Patent Application No. 61/101,946, filed October 1, 2008, the entirety of which is incorporated by reference.

[002] The multistep process of cartilage formation, or chondrogenesis, plays critical roles in skeletal development and maintenance. Chondrogenesis leads to the formation of permanent cartilaginous tissue, which provides major structural support for the respiratory and auditory tracts and the articular joints. In the latter, articular or hyaline cartilage covers the ends of bones to form the smooth articular surface of joints, protecting bones from load-bearing forces and allowing for joint motion. Chondrogenesis also leads to the formation of the cartilage growth plates. These growth plates, in turn, direct longitudinal growth of the skeleton and endochondral ossification, the process by which most of the bone in the skeleton is formed.

[003] Cartilage is a highly specialized tissue, consisting of chondrocytes embedded in a network of extracellular matrix components including collagens and proteoglycans. The chondrocytes maintain cartilage architecture by performing both formation and breakdown of critical extracellular matrix components. Steinert et al., *Arthritis Research & Therapy* 9(3):213 (2007).

[004] Chondrogenesis begins when mesenchymal cells committed to the chondrogenic lineage aggregate together to form cell clusters, or condensations, that express extracellular matrix. In the center of the condensations, prechondrocytes emerge and turn off expression of mesenchymal and condensation markers and increase the expression of Col2a1 and other early cartilage markers. Differentiation of these prechondrocytes is accompanied by increased expression of Col2a1 and

other extracellular matrix proteins, including but not limited to aggrecan and other collagens.

[005] In endochondral ossification, the chondrocytes within certain regions of the cartilage differentiate into hypertrophic chondrocytes. Once fully differentiated, hypertrophic cells become surrounded by a calcified extracellular matrix and die through apoptosis as the cartilage matrix is replaced by bone. Karsenty and Wagner, *Developmental Cell*, 2:389-406 (2002). Thus, the precursor cells responsible for cartilage formation also participate in bone formation. Although a few skeletal elements (including the lateral halves of the clavicles and some parts of the skull) form through an alternative osteogenic process known as intramembranous ossification, most bone in the vertebrate skeleton forms through this chondrocyte-mediated process of endochondral ossification.

[006] Nonhypertrophic chondrocytes include reserve and proliferating chondrocytes. They are considered to be the cartilage-forming cells because they express α1(II) collagen and aggrecan, the major components of cartilaginous extracellular matrix. The transcription factor *Sox9* is essential for differentiation of mesenchymal cells into chondrocytes at the mesenchymal condensation stage. *Sox5* and *Sox6* are coexpressed with *Sox9* in nonhypertrophic chondrocytes and are involved in the expression of various matrix genes. Increased expression and/or activity of *Sox5*, *Sox6*, and/or *Sox9* is associated with the maintenance of a chondrocytic phenotype, whereas these genes are down-regulated during chondrocyte hypertrophy. Consequently, increased *Sox5*, *Sox6*, and/or *Sox9* expression and/or activity is thought to be associated with inhibition of chondrocyte hypertrophy.

[007] Disruptions in the chondrogenesis process can lead to severe developmental consequences. In adults, impairment of chondrocyte function can cause cartilage degradation and osteoarthritis. Also, because cartilage exists as an avascular tissue, it has little or no capacity to repair itself when damaged by trauma or disease processes. In many cases, progenitor cells that could facilitate tissue repair do not migrate to the damaged site, resulting in permanent defects.

[008] Currently, the most common methods for cartilage repair involve surgical procedures such as autologous chondrocyte transplantation and delivery of matrices seeded with chondrogenic cells or chondrogenic factors, among other procedures. While several of these surgical methods have met with short-term success, long-term clinical results are difficult to achieve, and a critical need still exists for pharmacological agents that promote articular cartilage formation,healing, and maintenance. Known mediators of chondrogenesis include members of the transforming growth factor ß (TGF-ß) superfamily such as bone morphogenic proteins (BMPs) and fibroblast growth factors (FGFs). In addition, transcription factors have been considered for use in promoting chondrogenesis. However, these may not be ideal for therapeutic applications due to stability or delivery limitations.

[009] The invention is based in part on the discovery that compounds selected from phenazopyridine hydrochloride, cytochalasin B, methysticin, rhapontin, thiabendazole, indoprofen, oxybenzone, chrysin, pinocembrin, 7-hydroxyflavone, 6-methoxyluteolin, 6,7-dihydroxyflavone, tamarixetine, genistin, 5-methoxyflavone, pratol, 5,6-dehydrokawain, harringtonine, galangine, and kaempferol are effective to stimulate chondrogenesis. Accordingly, the invention provides methods for stimulating chondrogenesis in a subject, methods for treating a cartilage pathology or a chondrogenic disease, and methods for preventing or delaying onset of a cartilage

pathology by administering a chondrogenic composition formulated for pharmaceutical administration comprising an amount of one or more of these compounds effective to stimulate chondrogenesis in a subject.

[010] It will be apparent to the person of ordinary skill in the art that many of the chondrogenesis-stimulating compounds of the invention are nutraceuticals, i.e., they are found in, or can be derived from foodstuffs and can be used to make products such as fortified foods or dietary supplements. In particular, it will also be appreciated that many of the chondrogenesis-stimulating compounds are flavonoids, including chrysin, pinocembrin, 7-hydroxyflavone, 6-methoxyluteolin, tamarixetine, genistin, 5-methoxyflavone, pratol, galanganine, kaempferol, and 6,7-dihydroxyflavone.

[011] Exemplary embodiments of the invention are disclosed. Any embodiment described in connection with one aspect of the invention is intended to apply equally to other aspects of the invention. For example, each of the chondrogenic compositions disclosed may be used in each of the methods disclosed.

Brief Description of the Drawings

[012] Figure 1 shows expression of *Sox9* in mouse limb bud sections. E11.5 limb buds were serially sectioned and each region was transferred to individual microfuge tubes containing 700ml of RLT lysis buffer (Qiagen RNeasy kit). After homogenizing the sections in RLT lysis buffer by repeated pipetting, RNA was isolated according to the manufacturer's protocol.

[013] Figure 2 shows the effects of treating PML cultures with phenazopyridine hydrochloride, cytochalasin B, methysticin, thiabendazole, indoprofen, oxybenzone, chrysin, pinocembrin, 7-hydroxyflavone, 6-methoxyluteolin,

6,7-dihydroxyflavone, tamarixetine, genistin, 5-methoxyflavone, pratol, 5,6-dehydrokawain, harringtonine, galangine, and kaempferol. The data represent percent stimulation of pGL3(4X48) reporter activity compared to control cultures. Each compound was tested in quadruplicate in two separate experiments (4X48(1) and 4X48(2)).

[014] Figure 3 shows the effects of treating PLM cultures with various doses of rhapontin. The x-axis indicates rhapontin doses, and the y-axis represents percent stimulation of pGL3(4X48) reporter gene compared to control cultures.

[015] Figure 4 shows the activity of the pGL3(4X48) reporter in murine limb bud-derived cells in response to different treatment combinations. The x-axis indicates the different treatments and the y-axis represents percent stimulation of the pGL3(4X48) reporter construct relative to the control culture. PHCL = phenazopyridine hydrochloride, 4310 = pan RAR antagonist 4310, B4 = bone morphogenetic protein-4 (BMP-4). The reporter gene activities shown are from an experiment performed in triplicate.

[016] Figure 5 shows *Mmp13* mRNA levels in treated murine limb bud-derived cell cultures, as measured by a TaqMan® real-time PCR assay. *Mmp13* expression levels are expressed as a percent of control cells' expression level on day 5. 7 = phenazopyridine hydrochloride (PHCL), 4310 = pan RAR antagonist 4310, B4 = bone morphogenetic protein-4 (BMP-4). The expression levels shown are from an experiment performed in duplicate.

Definitions

[017] The terms "stimulating chondrogenesis" and "promoting chondrogenic activity" as used herein, include increasing differentiation of mesenchymal, chondroprogenitor, dedifferentiated chondrocytes or cells with chondrogenic

potential into chondrocytes, maintaining the nonhypertrophic chondrocyte phenotype by inhibiting their differentiation into hypertrophic chondrocytes, increasing cartilage formation, enhancing the production of cartilage matrix, and/or inducing cartilage repair. A "chondrogenesis stimulating agent" is a compound that stimulates one or more of these activities. A "chondrogenic composition" as used herein, refers to a composition comprising a compound selected from phenazopyridine hydrochloride, cytochalasin B, methysticin, rhapontin, thiabendazole, indoprofen, oxybenzone, chrysin, pinocembrin, 7-hydroxyflavone, 6-methoxyluteolin, 6,7-dihydroxyflavone, tamarixetine, genistin, 5-methoxyflavone, pratol, 5,6-dehydrokawain, harringtonine, galangine, and kaempferol together with a pharmaceutically acceptable carrier.

[018] A "chondrogenic protective agent" or "chondroprotective agent" is a compound that inhibits degeneration of cartilage. Examples of suitable chondroprotective agents include IL-1 receptor antagonists, TNF receptor antagonists, COX-2 inhibitors, viscosupplements (i.e. hyaluronic acid), and potentially glucosamine, or chondroitin sulphate. Additional chondrogenic protective agents are described in detail at columns 12 to 14 of United States Patent 7,067,144, incorporated by reference and in United States Patent 6,583,118, incorporated by reference in its entirety.

[019] A "cartilage defect" includes cartilage damage caused by injury, disease, or improper formation of cartilage during development. The cartilage defect may be caused by an injury to bone as well as cartilage, such as bone fractures or osteochondral defects. The cartilage defect may also result from surgical procedures.

[020] The terms "treatment," "therapeutic method," and their cognates include prophylactic/preventative measures. Thus, a subject in need of treatment

may include an individual already suffering from a particular cartilage defect, damage or disorder as well as a subject that may ultimately acquire the defect, damage or disorder. The term “treatment” and its cognates do not require full resolution/repair of the cartilage defect, damage, or disorder being treated, but include full, as well as partial resolution/repair, amelioration of associated symptoms, delayed onset, or attainment of a desired biological outcome, such as increased chondrogenesis. Typically, “treatment” will include healing or repair of cartilage damage or defect in a subject by regeneration of cartilage at the site of the injury or defect.

[021] The terms “therapeutically effective dose,” or “therapeutically effective amount,” refer to the amount of chondrogenic compound selected from phenazopyridine hydrochloride, cytochalasin B, methysticin, rhapontin, thiabendazole, indoprofen, oxybenzone, chrysin, pinocembrin, 7-hydroxyflavone, 6-methoxyluteolin, 6,7-dihydroxyflavone, tamarixetine, genistin, 5-methoxyflavone, pratol, 5,6-dehydrokawain, harringtonine, galangine, and kaempferol in a composition that results in prevention or delay of onset or amelioration of symptoms of cartilage damage or defects in a subject or an attainment of a desired biological outcome, such as increased chondrogenesis or prevention of hypertrophy. The effective amount can be determined by methods well-known in the art and will vary with the nature and severity of the condition treated.

[022] A "subject" can be a mammal, e.g., a human, primate, ovine, bovine, porcine, equine, feline, canine, and a rodent (rat or mouse). The use of the word "a", "an" or “the” when used in conjunction with the term "comprising" in the claims and/or the specification may mean "one," but it is also consistent with the meaning of "one or more," "at least one," and "one or more than one."

Chondrogenic Compositions

[023] Chondrogenic compositions are formulated for pharmaceutical use in the methods of the invention and comprise at least one compound selected from phenazopyridine hydrochloride, cytochalasin B, methysticin, rhapontin, thiabendazole, indoprofen, oxybenzone, chrysin, pinocembrin, 7-hydroxyflavone, 6-methoxyluteolin, 6,7-dihydroxyflavone, tamarixetine, genistin, 5-methoxyflavone, pratol, 5,6-dehydrokawain, harringtonine, galangine, and kaempferol in an amount effective to stimulate chondrogenesis in a subject. In certain embodiments, the compound is selected from thiabendazole, indoprofen, oxybenzone, 5,6-dehydrokawain, harringtonine, phenazopyridine, cytochalasin B, rhapontin, and methysticin. In other embodiments, the compound is a flavonoid capable of increasing differentiation of mesenchymal cells into chondrocytes and/or increasing cartilage formation, enhancing the production of cartilage matrix, and/or inducing cartilage repair. In some embodiments, the flavonoid compound is selected from 6-methoxyluteolin, tamarixetin, genistin, pratol, and galangine In other embodiments, the flavonoid compound may be chosen from chrysin, pinocembrin, 7-hydroxyflavone, 5-methoxyflavone, and 6,7-dihydroxyflavone. Suitable excipients for inclusion in the chondrogenic compositions of the invention are well known in the art. In some embodiments, the chondrogenic compound is administered in an amount sufficient to increase mRNA, protein or activity levels of Sox 5, Sox 6, or Sox 9 (human GeneIDs 6660, 55553, and 6662, respectively) by at least 5, 10, 15, 20, 25, 30, 50, 70, or 90%; or 2, 4, 8, 10, 20, 40, or 80-fold. In certain embodiments, the chondrogenic compound is administered in an amount sufficient in increase expression of aggrecan (human, mouse, and rat GeneIDs 176, 11595, and 58968, respectively) or Col2a1 (human, mouse, and rat GeneIDs 1280, 12824, and 25412,

respectively) at the mRNA or protein level by at least 5, 10, 15, 20, 25, 30, 50, 70, or 90%; or 2, 4, 8, 10, 20, 40, or 80-fold. All information associated with all GeneIDs and reference protein and nucleic acid sequences referenced in this application, including references sequences and their associated annotations, is incorporated by reference.

[024] In some embodiments, the chondrogenic composition further comprises a at least one other therapeutic agent. In exemplary embodiments, the other therapeutic agent is a chondrogenesis promoter or chondrogenesis inducing agent such as, e.g., interleukin agonists, including IL-4, IL-10, IL-13 agonists, growth factors such as, e.g., TGF-ß1, TGF-ß2, and TGF-ß3; bone morphogenetic protein agonists such as, e.g., BMP-2, BMP-4, BMP-6, BMP-7, BMP-8, BMP-12, BMP-13; insulin like growth factors such as, e.g. IGF-1; growth and differentiation factors (GDFs); WNTs; Hedgehogs; MIA; fibroblast growth factors such as, e.g., bFGF; and retinoic acid receptor (RAR) antagonists. In other embodiments, the therapeutic agent is a chondrogenic protective agent such as, e.g., IL-1 receptor antagonists, TNF receptor antagonists, COX-2 inhibitors, MAP kinase inhibitors, nitric oxide synthase inhibitors, NFkB inhibitors, and flavonoid compounds such as those described in United States Patent 6,583,118. In an exemplary embodiment, the chondrogenic composition comprises a compound selected from phenazopyridine hydrochloride, cytochalasin B, methysticin, rhapontin, thiabendazole, indoprofen, oxybenzone, chrysin, pinocembrin, 7-hydroxyflavone, 6-methoxyluteolin, 6,7-dihydroxyflavone, tamarixetine, genistin, 5-methoxyflavone, pratol, 5,6-dehydrokawain, harringtonine, galangine, kaempferol, together with both another chondrogenesis inducing agent and a chondroprotective agent.

[025] In certain embodiments, the chondrogenic compositions of the invention may also exhibit chondroprotective activity. A composition can be evaluated for chondroprotective activity by a variety of means known in the art, including direct visualization of cells, histological grading of cartilage, or by molecular diagnostics, such as monitoring the expression level of one or more genes involved in cartilage metabolism. For example, the Mmp13 gene product (human, mouse, and rat GeneIDs: 4322, 17386, 171052, respectively) has been implicated in cartilage degeneration. Neuhold et al., *J. Clin. Invest.* 107(1): 35-44 (2001). Accordingly, in some embodiments, a compound's ability to reduce Mmp13 activity is indicative of chondroprotective activity. In some embodiments, a composition with chondroprotective activity reduces Mmp13 activity in a target cell by at least 10, 15, 20, 30, 50, or 90%; or 2, 4, 6, 8, 10, 20, 50, or 80-fold, relative to a control cell. In more particular embodiments, a composition with chondroprotective activity reduces Mmp13 mRNA or protein levels by at least 10, 15, 20, 30, 50, 90% or 2, 4, 6, 8, 10, 20, 50, 80-fold, relative to control cells.

Therapeutic Uses

[026] The chondrogenic compositions described above may be used in methods of inducing chondrogenesis, cartilage healing, and/or cartilage regeneration; methods of treating a cartilage pathology or chondrogenic disease; and methods of preventing or delaying the onset of a cartilage pathology or chondrogenic disease. The chondrogenic compositions may also be employed in methods of inducing de novo cartilaginous tissue formation.

[027] In certain embodiments, the methods of the invention include the repair of congenital cartilage defects, trauma-induced cartilage pathology, or cartilage defects of other origins. The methods of the invention may be used in connection

with surgical procedures for the attachment or repair of cartilage. In some embodiments, methods of the invention comprise the treatment and/or prophylaxis of articular cartilage tears, cartilage deformities, damage to hyaline cartilage, and other cartilage defects in humans and animals. Any of the chondrogenic compositions described above may be employed in methods to treat chondromalacia, chondrodysplasias, cartilage damage due to bone fracture, osteochondritis, congenital cartilage defects, osteochondritis dessecans, articular cartilage damage, herniated intervertebral disk, anotia, microtia cartilage defect, degenerative joint disease including arthritis (rheumatoid and osteoarthritis), and polychondritis.

[028] The chondrogenic compositions provide an environment to attract cartilage-forming cells, stimulate growth of cartilage-forming cells, induce differentiation of progenitors of cartilage-forming cells, or improve fixation of cartilage to bone or other tissues. Thus, the compositions may also be employed in tissue engineering of cartilage. The chondrogenic compositions may also be administered prophylactically to prevent damage to cartilaginous tissue.

[029] In certain embodiments, the methods of the invention comprise administration of a chondrogenic composition comprising a therapeutically effective amount of at least one compound selected from phenazopyridine hydrochloride, cytochalasin B, methysticin, rhapontin, thiabendazole, indoprofen, oxybenzone, chrysin, pinocembrin, 7-hydroxyflavone, 6-methoxyluteolin, 6,7-dihydroxyflavone, tamarixetine, genistin, 5-methoxyflavone, pratol, 5,6-dehydrokawain, harringtonine, galangine, and kaempferol in combination with cells that have chondrogenic potential, including stem cells, progenitor cells, mesenchymal stem cells, mesenchymal progenitor cells, chondroprogenitors, chondrocytes or dedifferentiated chondrocytes. In other embodiments, these compositions are administered in

combination with a chondroprotective or joint protective agent such as a viscosupplement (i.e. hyaluronic acid), glucosamine, or chondroitin sulphate. In still other embodiments, the methods of the invention comprise administration of a therapeutically effective amount of a chondrogenic composition of the invention in combination with another chondrogenesis inducing agent and/or with a chondroprotective agent. The individual components of the combination therapy may be administered simultaneously or sequentially.

[030] Administration of a chondrogenic composition to a subject according to the methods of the invention may be carried out using methods known to those of skill in the art. In certain embodiments, a chondrogenic composition is delivered systemically by injection, irrigation, ingestion, inhalation, or topical application. Alternatively, the chondrogenic composition is administered locally by injection (including intra-articular), topical application, or as a coating or component of a device or implant. The chondrogenic compositions may also be employed to treat cells *ex vivo* prior to implantation pursuant to the methods of the invention.

[031] Suitable doses of the chondrogenic compounds provided by the invention will be readily determined based on the desired outcome, using routine skill in the medical arts (see, for example *Physicians' Desk Reference*, 58th Edition, Thompson, P D R, Montvale, N.J. 2004). For example, effective doses identified through *in vitro* or *ex vivo* assays can be calibrated for use *in vivo*, while doses effective in *in vivo* animal models can be readily interconverted between species by means known in the art (see, for example, Freienreich et al., *Cancer Chemother. Rep.* 50(4):219-44 (1966)). In some embodiments, suitable doses of a chondrogenic compound are at least 0.01, 0.05, 0.1, 0.5, 1, 2, 5, 10, 15, 20, 30, 45, 50, 80, 100, 150, 200, 400, 600, 800, 1000, 2000, 4000, or 8000 μM. These doses may be used

ex vivo (e.g., for chondrocyte transplantation) or *in vivo* for direct administration. Doses may also be calculated based on subject's body weight, e.g., at least 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 20, 40, 80, 100, 200, 400, 800, 1000, 2000, 4000, 8000, 10000, 20000, 40000, or 80000 µg/kg. Dosing regimens may be carried out for a period of time sufficient to achieve the desired therapeutic outcome, *e.g.,* at least 2, 4, 6, 12, 24, 48, 72 hours, or 4, 5, 10, 15, 20, or 30 days.

Carriers and implants

[032] Carriers may aid in forming a chondrogenic composition that possesses appropriate handling characteristics for injectable application to the site of cartilage defect or damage. Adding the chondrogenic composition to a carrier or implant allows it to remain in the site of the disease or damage for a time sufficient to allow the composition to increase the regenerative chondrogenic activity of the infiltrating mammalian progenitor or other cells, and to form a space in which new tissue can grow and allow for ingrowth of cells. The carrier may also allow the chondrogenic composition to be released from the disease or damage site over a time interval appropriate for optimally increasing the rate of regenerative chondrogenic activity of the progenitor cells.

[033] In certain embodiments, the chondrogenic compositions of the invention may be administered using an appropriate implantable matrix, carrier, or device. For instance, the implant may provide a surface for cartilaginous tissue formation and/or other tissue formation (e.g., in mediation of bone growth or repair). Some embodiments include implantable mechanical physical devices, biodegradable carriers, biodegradable synthetic carriers, prostheses, demineralized allogenic bone and demineralized xenogenic bone. The implantable matrix, carrier, or device may provide slow release of the chondrogenic compound and/or the appropriate

environment for its presentation. Biodegradable materials, such as cellulose films, or surgical meshes, may also serve as matrices. Such materials could be sutured into an injury site, or wrapped around the cartilage. Some matrices include collagen-based materials, including sponges, such as Helistaf (Integra LifeSciences, Plainsboro, N.J.), or collagen in an injectable form.

[034] One family of carriers that may be used in the methods of the invention comprises collagenous materials, and can be in a form suitable for injection, such as a gel. Such gels may be crosslinked or non-crosslinked. Other forms of collagen, such as dispersions or fibrillar collagen, may also be useful in the methods of the present invention. Another family of carriers includes cellulosic materials such as alkylcellulose, including hydroxyalkylcellulose, methylcellulose, ethylcellulose, hydroxyethylcellulose, hydroxypropylcellulose, hydroxypropylmethylcellulose, and carboxymethylcellulose (CMC).

[035] In some embodiments using cellulosic carriers and collagen gels, the carrier may be in the form of a hydrated cellulosic viscous gel. Viscosity may be increased through mechanical means, such as high agitation for a suitable period of time, followed by autoclaving, or chemically. The active agent and cellulosic carrier may be formulated in a solution of a suitable buffer.

[036] Another class of materials for injectable carriers is resorbable hydroxyapatites, as well as minerals, ceramics and phosphates. Resorbable hydroxyapatites, for example, can be formulated at various porosities with varying resorption rates; their handling characteristics vary from hard implantable types, to gel-like consistency, to those that are injectable but harden at body temperature. Suitable hydroxyapatite and ceramic carriers are described, for example in W096/36562; and United States Patents 5,543,019; 5,306,305; 5,258,044;

5,496,399; 5,455,231; 5,336,264; 5,178,845; 5,053,212; 5,047,031; 5,129,905; 5,034,059; 4,880,610; 5,290,763; and 5,563,124; all of which are incorporated by reference for their disclosure of suitable carriers.

[037] In other embodiments, the carrier is an injectable polymer, which may be viscous and which may optionally include a sequestering agent. Suitable polymers and sequestering agents include those described in United States Patent 5,171,579, incorporated by reference. Other polymers include pluronics, which are liquid (and hence syringeable) at 4°C and gel at body temperature. The pluronic Poloxamer 407, MW 12,500, is excreted unchanged in the urine after systemic absorption and has reportedly been shown to be non-toxic in animals. In certain embodiments, the polymer may be a polylactide and/or polyethylene glycol, including poly(lactide)/poly(ethylene glycol) gel. Polylactides may be dissolved in polyethylene glycols, such as low molecular weight (2000) PLA dissolved in PEG to produce a syringeable solution that precipitates PLA upon injection into an aqueous environment, resulting in a relatively firm gel.

[038] In some embodiments, the chondrogenic composition may include a sequestering agent such as hyaluronic acid, sodium alginate, poly(ethylene glycol), polyoxyethylene oxide, carboxyvinyl polymer or poly(vinyl alcohol), or a cellulosic material, such as hydroxycellulose or carboxymethylcellulose. These sequestering agents may themselves be useful as carriers for injection. In addition, combinations of sequestering agents may be used.

[039] The choice of a carrier material will be based on biocompatibility, biodegradability, mechanical properties, cosmetic appearance and interface properties. The particular application of the chondrogenic compositions will define the appropriate formulation. Potential matrices for the compositions may be

biodegradable and chemically defined. Other matrices may be comprised of pure proteins or extracellular matrix components. Additional potential matrices are non-biodegradable and chemically defined.

Chondrocyte Transplantation

[040] In certain embodiments, chondrocytes or chondrocyte precursor cells may be implanted along with any of the chondrogenic compositions described above. Some embodiments include culturing the cells with a composition comprising a chondrogenic compound *ex vivo,* prior to implantation at a defect site. In some embodiments, the chondrocytes or precursor cells may be harvested from the subject in need of treatment (e.g., autologous chondrocyte transplantation). The chondrocytes can be implanted along with a matrix, carrier or device. An autologous bone graft may also be implanted with the chondrocytes for certain subjects in need of bone repair.

[041] In other embodiments, genetically engineered cells may be administered along with any of the chondrogenic compositions described herein, and optionally in combination with an appropriate matrix or carrier that can provide a surface for cartilage and/or other connective tissue growth. The cells may be engineered to express proteins, growth factors, extracellular matrix materials or other chondrogenesis stimulating agents. In some embodiments, various collagenous and non-collagenous proteins are expected to be upregulated and secreted from the engineered cells. This phenomenon accelerates tissue regeneration by enhancing extracellular matrix deposition, and can enhance the engraftment and attachment of transplanted cells into the defect site. A carrier or implantable matrix may be used to provide slow release of the chondrogenic composition and/or differentiated cells.

[042] Other embodiments of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein. It is intended that any of the chondrogenic compounds of the invention may be used in any of the formulations described above to treat any of the conditions described herein. It is further intended that the embodiments provided in this specification are exemplary only, with a true scope and spirit of the invention being indicated by the following claims.

[043] The following examples illustrate various embodiments of the invention and are not intended to limit the scope of the invention.

Example 1: Establishment and Staining of Primary Limb Mesenchymal Cultures

[044] Primary limb mesenchymal (PLM) cultures were established from CD-1 murine embryonic limbs (E11.5) as previously described (Hoffman et al., *J. Cell Biol.*, 174:101-13; Weston et al., *J. Cell* Biol., 158:39-51 (2002)). Limb mesenchyme was dissociated by dispase treatment and a single cell suspension was obtained by filtration through a 40mM cell strainer (BD Biosciences). PLM cells were pelleted by centrifugation at 200 x g and resuspended to produce a stock cell suspension at a concentration of $2.0x10^7$ cells/ml. Cells were used for transfection (Example 2) or for establishment of cultures for Alcian blue staining.

[045] For the latter, 10 µL of PLM cells were spotted into the well of a 24-well plate, and allowed to adhere for 1h. Culture medium consisting of 60% Ham's F12 nutrient mix/40% Dulbecco's modified Eagle's medium (DMEM) and supplemented with 10% FBS (Qualified, Invitrogen) was added to each well, and test compounds were added at approximately 15 micromolar concentrations to each well 24 hrs following culture establishment; this time was considered T=1. Cultures were

maintained for a period of up to 4 days, and culture media was replaced on alternate days to minimize handling.

[046] For Alcian blue staining, culture medium was aspirated and cells were washed once with PBS. Cultures were fixed in 95% ethanol at -20°C overnight. Fixative was removed by aspiration and cells were sequentially washed once with PBS, followed by 0.2M HCl. Cells were stained overnight with a 1% Alcian blue solution prepared in 0.2M HCl.

Example 2: Primary Cell Transfections and Expression

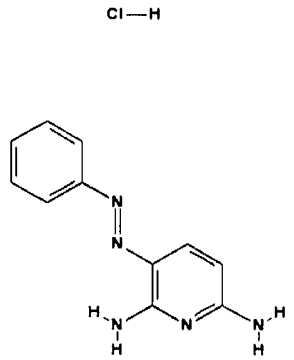
[047] Stock plasmid DNAs were standardized to a concentration of 1mg/ml. For transfections, a ratio of 20:1 of reporter gene pGL3(4X48) to control reporter gene (phRL-SV40) was used and combined with Effectene transfection reagent according to the manufacturers instructions (Qiagen); trehalose to a final concentration of 0.4 M was added to the EC buffer. Luciferase reporter genes consist of a firefly reporter gene, pGL3(4X48), and a *Renilla* (*Renilla reniformis*) luciferase reporter phRL-SV40 to normalize for transfection efficiency. The pGL3(4X48) reporter contains four repeats of a Sox5/6/9 binding site. Reporter plasmids containing Sox5/6/9 binding sites (pGL3[4X48]) were previously described (Weston et al., *J. Cell Biol.*, 158:39-51 (2002)).

[048] Test compounds were added to the culture medium of transfected cells 24 hours following transfection at approximately 15 micromolar concentrations. After 24 hours, cells were washed with PBS and lysed in 40ml of passive lysis buffer (Promega) per well. Plates were assayed for luciferase activity according to the manufacturer's instructions (Promega).

[049] To follow the expression of transcripts for *Sox9* or other chondrogenic markers, quantitative real-time PCR was performed using the 7500 Fast Sequence

Detection System (Applied Biosystems). The primer/probe set used for detection of *Sox9* was as described in Weston et al., *J. Cell Biol.*, 158:39-51 (2002). For detection of all other transcripts, TaqMan Gene Expression Assays (Applied Biosystems) were used. Total RNA was isolated from primary cultures, and an aliquot was reverse transcribed to cDNA using a High Capacity cDNA Archive kit (Applied Biosystems). Quantification was performed using ~10 ng of total RNA and the expression of all genes relative to endogenous *rRNA* was determined using TaqMan Ribosomal Control Reagents (Applied Biosystems).

Example 3: Chondrogenic Activity of Phenazopyridine Hydrochloride

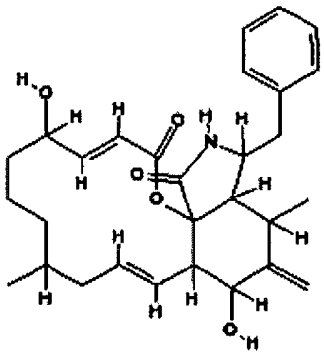


[050] Phenazopyridine hydrochloride, also known as 3-phenyldiazenylpyridine-2,6-diamine hydrochloride, is most commonly known as an anesthetic compound. It is commercially available and may also be prepared according to procedures set forth in the literature. See, e.g., U.S. Patent 1,680,109. Phenazopyridine hydrochloride has been reported to have bacteriostatic properties. The toxicity profile for phenazopyridine hydrochloride has been described by by Becker and Swift, *Toxicol. Appl. Pharmacol.* 1:42 (1959). Shang et al., *Anal. Bioanal. Chem.* 382:216 (2005) reports on pharmacokinetics and LC-MS determination in plasma. A review of clinical experience with phenazopyridine

hydrochloride is provided by Zelenitsky and Zhanel, *Ann. Pharmacother.* 30:866-868 (1996).

[051] Using procedures based on those described in Example 2, phenazopyridine hydrochloride was found to significantly increase the activity of the chondrogenic responsive reporter gene pGL3(4X48), indicating that it is a chondrogenesis stimulating agent (Figure 2). These PLM transfection assays, as well as Alcian blue staining experiments, indicate that low micromolar concentrations of phenazopyridine hydrochloride stimulate chondrogenic activity. Treatment of PLM cultures with phenazopyridine hydrochloride for two days also increased aggrecan expression by more than two fold in proximal mesenchymal cells and over four fold in distal mesenchymal cells, as determined by RT-qPCR.

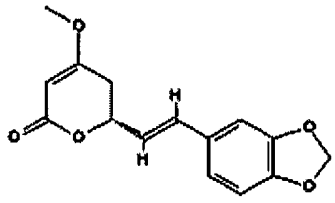
Example 4: Chondrogenic Activity of Cytochalasin B



[052] Cytochalasin B is a cell permeable fungal toxin isolated from *Helminthosporium determatioideum*. It is one of the most important and biologically studied cytochalasins. It is commercially available from a number of sources. Cytochalasin B may also be isolated from *Phoma exigua* var. *heteromorpha* using the process described by Bottalico et al., *Appl. Biochem. Biotech.* 48(1):33-36 (1994).

[053] Using procedures based on those described in Example 2, cytochalasin B was found to significantly increase the activity of the chondrogenic responsive reporter gene pGL3(4X48), indicating that it is a chondrogenesis stimulating agent (Figure 2). PLM transfection assays and Alcian blue staining experiments both indicate that low micromolar concentrations of cytochalasin B stimulate chondrogenic activity. In addition, treatment of PLM cultures with cytochalasin B for two days increased aggrecan expression by over 9 fold in proximal mesenchymal cells, and over 4 fold in distal mesenchymal cells, as determined by RT-qPCR.

Example 5: Chondrogenic Activity of Methysticin

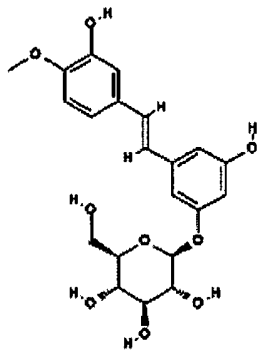


[054] Methysticin, also known as 6-[2-(1,3-benzodioxol-5-yl)ethenyl]-4-methoxy-5,6-dihydropyran-2-one, is a white, silky, crystalline substance extracted from the thick rootstock of a species of pepper (*Piper methysticum*) of the South Sea Islands. Synthesis of the racemate was described by Klohs et al., *J. Org. Chem.* 24, 1829 (1959). Structural characterizations, including absolute configuration (Snatzke et al., *Tetrahedron Lett.* 9:1797 (1968)) and HPLC determination (Smith et al., *J. Chromatogr.* 283, 303 (1984)) have also been described. Biological activities of methysticin include anticonvulsant effects (Kretzschmar, *Arch. Int. Pharmacodyn. Ther.* 177, 261 (1969)) and neuroprotective activity (Backhauss et al., *Eur. J.*

Pharmacol. 215, 265 (1992)). Methysticin is commercially available from a number of sources.

[055] Using procedures based on those described in Example 2, methysticin was found to significantly increase the activity of the chondrogenic responsive reporter gene pGL3(4X48), indicating that it is a chondrogenesis stimulating agent (Figure 2). The PLM transfection assays and Alcian blue staining experiments demonstrate that low micromolar concentrations of methysticin stimulate chondrogenic activity.

Example 6: Chondrogenic Activity of Rhapontin

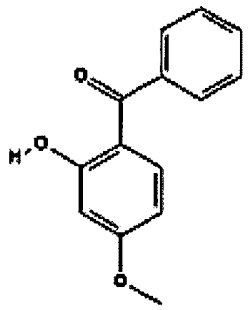


[056] Rhapontin, also known as 2-[3-hydroxy-5-[2-(3-hydroxy-4-methoxyphenyl) ethenyl]phenoxy]-6-(hydroxymethyl) oxane-3,4,5-triol, is isolated from rhubarb (Rheum rhaponticu) (Hesse, *J. Prakt. Chem.* 77, 321 (1908); Schürhoff et al., *Arch. Pharm.* 275, 281 (1937)). Rhapontin has been shown to inhibit growth and induce apoptosis in KATO III stomach cancer cells. Habasami et al., *Oncol. Rep.* 18, 347 (2007). Rhapontin is also commercially available from numerous sources.

[057] Using procedures based on those described in Example 2, rhapontin was found to significantly increase the activity of the chondrogenic responsive

reporter gene pGL3(4X48), indicating that it is a chondrogenesis stimulating agent (Figure 2). As shown in Figure 3, a dose response study of rhapontin indicated that it was most effective at stimulating 4X48 activity at concentrations between 100nM and 1000nM. These assays and Alcian blue staining experiments show that, ~100 nanomolar to low micromolar concentrations of rhapontin stimulate chondrogenic activity.

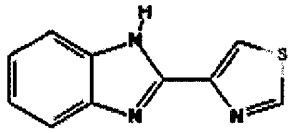
Example 7: Chondrogenic Activity of Oxybenzone



[058] Oxybenzone, also known as 2-hydroxy-4-methoxyphenyl)-phenylmethanone, is a derivative of benzophenone. It forms colorless crystals that are readily soluble in most organic solvents. Methods of preparing this compound are disclosed in United States Patents 2,773,903; 2,861,104; 2,861,105; and 3,073,866. Oxybenzone is commercially available.

[059] Using procedures based on those described in Example 2, oxybenzone was found to significantly increase the activity of the chondrogenic responsive reporter gene pGL3(4X48), indicating that it is a chondrogenesis stimulating agent (Figure 2). These assays and Alcian blue staining experiments indicate that low micromolar concentrations of oxybenzone stimulate chondrogenic activity.

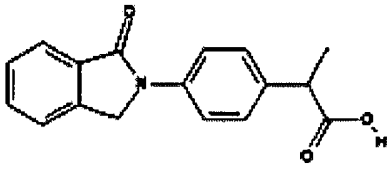
Example 8: Chondrogenic Activity of Thiabendazole



[060] Thiabendazole, also know as 2-(1,3-thiazol-4-yl)benzimidazole, exists as a white to off-white powder, and has low solubility in water but can readily dissolve in dilute acid and alkali solutions. The compound is used to control parasitic worm infections in humans and other animals, and as an agricultural fungicide. Gordon, *Nature* 191, 1409 (1961); Dalvie et al., *Drug Metab. Dispos.* 34, 709 (2006). Thiabendazole is commercially available from multiple sources.

[061] Using procedures based on those described in Example 2, thiabendazole was found to significantly increase the activity of the chondrogenic responsive reporter gene pGL3(4X48), indicating that it is a chondrogenesis stimulating agent (Figure 2) and that low micromolar concentrations of thiabendazole stimulate chondrogenic activity. At the single dosage that was tested, this compound did not significantly increase Alcian blue staining relative to controls, but it is expected that experiments with varying conditions (e.g., dosage, duration) will further support the chondrogenic activity indicated in the reporter assay.

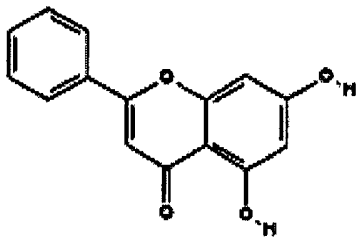
Example 9: Chondrogenic Activity of Indoprofen



[062] Indoprofen, also known as 4-(1,3-dihydro-1-oxo-2H-isoindol-2-yl)-alpha-methyl-benzeneacetic acid, is a non-steroidal anti-inflammatory drug that may also upregulate the survival motor neuron (SMN) protein. Lunn et al., *Chem. Biol.* 11, 1489 (2004). Methods of preparing indoprofen are disclosed in DE 2258088. Indoprofen is commercially available.

[063] Using procedures based on those described in Example 2, indoprofen was found to significantly increase the activity of the chondrogenic responsive reporter gene pGL3(4X48), indicating that it is a chondrogenesis stimulating agent (Figure 2). The PLM transfection assays indicate that low micromolar concentrations of indoprofen stimulate chondrogenic activity. At the single dosage that was tested, this compound did not significantly increase Alcian blue staining relative to controls, but it is expected that experiments with varying conditions (e.g., dosage, duration) will further support the chondrogenic activity indicated in the reporter assay.

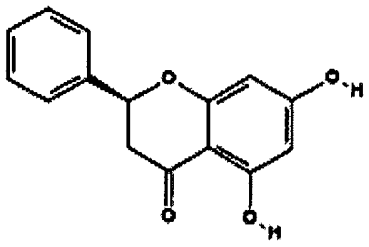
Example 10: Chondrogenic Activity of Chrysin



[064] Chrysin, also known as 5,7-dihydroxy-2-phenylchromen-4-one, is a flavonoid compound extracted from multiple plant sources and tree bark. Medina et al., *Biochem. Pharmacol.* 40, 2227 (1990); Liu et al., *J. Nat. Prod.* 55, 357 (1992). The synthesis of chrysin was disclosed in Hutchins et al., *J. Chem. Soc.* 1, 91 (1939). Chrysin has been used as an herbal supplement by bodybuilders, and may have anti-inflammatory effects (Woo et al., *FEBS Lett.* 579, 705 (2005)). The compound is commercially available from multiple sources.

[065] It has been reported in US 6,583,118 that chrysin may reduce loss of glycosaminoglycan content in chondrocyte matrix in the presence of a proteoglycan depleting agent, thus identifying chrysin as a potential chondroprotective agent. Using procedures based on those described in Example 2, chrysin has now been found to significantly increase the activity of the chondrogenic responsive reporter gene pGL3(4X48), indicating that it is also a chondrogenesis stimulating agent (Figure 2) that it is effective at low micromolar concentrations. At the single dosage that was tested, this compound did not significantly increase Alcian blue staining relative to controls, but it is expected that experiments with varying conditions (e.g., dosage, duration) will further support the chondrogenic activity indicated in the reporter assay.

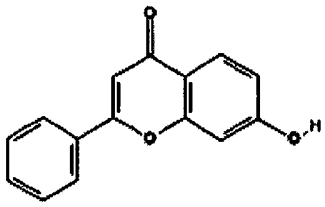
Example 11: Chondrogenic Activity of Pinocembrin



[066] Pinocembrin, also known as 5,7-dihydroxyflavone, is a flavonoid compound that comes from European eucalyptus honeys. Martos et al., *J. Agric. Food Chem.* 48, 4744 (2000). It was isolated from propolis and identified as a bacteriostatic substance. Villaneuva et al., *Ann. Inst. Pasteur* 118, 84 (1970). Pinocembrin is also available from multiple commercial sources.

[067] It has been reported in US 6,583,118 that pinocembrin, like chrysin, may reduce loss of glycosaminoglycan content in chondrocyte matrix in the presence of a proteoglycan depleting agent and that consequently, pinocembrin may function as a chondroprotective agent. Using procedures based on those described in Example 2, pinocembrin has now been found to significantly increase the activity of the chondrogenic responsive reporter gene pGL3(4X48), indicating that it is also a chondrogenesis stimulating agent (Figure 2) that it is effective at low micromolar concentrations. At the single dosage that was tested, this compound did not significantly increase Alcian blue staining relative to controls, but it is expected that experiments with varying conditions (e.g., dosage, duration) will further support the chondrogenic activity indicated in the reporter assay.

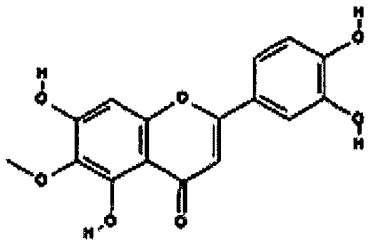
Example 12: Chondrogenic Activity of 7-hydroxyflavone



[068] 7-hydroxyflavone, also known as 7-hydroxy-2-phenylchromen-4-one, is present in many edible vegetables. It is a well characterized flavonoid compound, shown to act as an antimetastatic agent (Yang et al., *Arch Oral Biol* 53(3):287-94.(2008)), an aromatase inhibitor (Ta and Walle, *Steroid Biochem Mol Biol* 107(1-2):127-9.(2007)), and a potent competitive inhibitor of xanthine oxidase, i.e, it is an antioxident (Mei-Ling et al., *Free Radical Biology and Medicine* 20(1):35-43 (1996)). 7-hydroxyflavone is commercially available.

[069] While it has not been specifically reported in US 6,583,118 that 7-hydroxy flavone will reduce loss of glycosaminoglycan content in chondrocyte matrix in the presence of a proteoglycan depleting agent, it is possible that this flavonoid may function as a chondroprotective agent. However, it has now been confirmed, using procedures based on those described in Example 2, that 7-hydroxyflavone significantly increases the activity of the chondrogenic responsive reporter gene pGL3(4X48), indicating that it is also a chondrogenesis stimulating agent (Figure 2) that it is effective at low micromolar concentrations. At the single dosage that was tested, this compound did not significantly increase Alcian blue staining relative to controls, but it is expected that experiments with varying conditions (e.g., dosage, duration) will further support the chondrogenic activity indicated in the reporter assay.

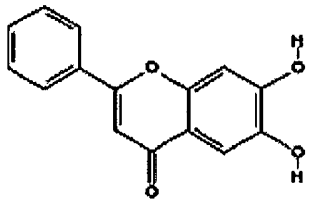
Example 13: Chondrogenic Activity of 6-Methoxyluteolin



[070] 6-Methoxyluteolin, also known as nepetin, 3',4',5,7-tetrahydroxy-6-methoxyflavone, and 2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-6-methoxychromen-4-one, has been isolated from Eupatorium arnottianum Griseb and Eupatorium ballotaefolium. 6-Methoxyluteolin has been shown to inhibit tumor cells. Militao et al., *Pharmazie* 59(12):965-6 (2004). It has also been shown to possess anti-inflammatory properties. Clavin et al., *Ethnopharmacol* 112(3):585-9 (2007). 6-Methoxyluteolin is commercially available.

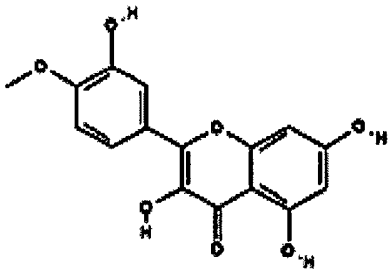
[071] Using procedures based on those described in Example 2, 6-methoxyluteolin was found to significantly increase the activity of the chondrogenic responsive reporter gene pGL3(4X48), indicating that it is a chondrogenesis stimulating agent (Figure 2). The PLM transfection assays show that low micromolar concentrations of 6-methoxyluteolin stimulate chondrogenic activity. At the single dosage that was tested, this compound did not significantly increase Alcian blue staining relative to controls, but it is expected that experiments with varying conditions (e.g., dosage, duration) will further support the chondrogenic activity indicated in the reporter assay.

Example 14: Chondrogenic Activity of 6,7-dihydroxyflavone



[072] 6,7-Dihydroxyflavone, also known as 6,7-dihydroxy-2-phenylchromen-4-one, is commercially available. It is reported to have a weak antibacterial effect on methicillin-resistant Staphylococcus aureus by itself, but has been shown to dramatically intensify the susceptibility of methicillin-resistant or -sensitive Staphylococcus aureus to beta-lactams. Sato et al., *Antimicorb Agents Chemother* 48(4):1357-60 (2004). Using procedures based on those described in Example 2, 6,7-dihydroxyflavone was found to significantly increase the activity of the chondrogenic responsive reporter gene pGL3(4X48), indicating that it is a chondrogenesis stimulating agent (Figure 2). PLM transfection assays and Alcian blue staining assays indicate that low micromolar concentrations of 6,7-dihydroxyflavone stimulate chondrogenic activity.

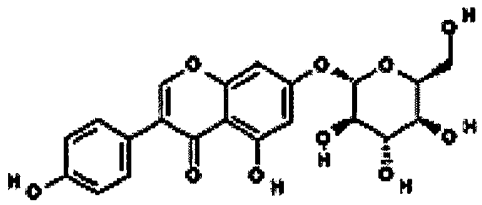
Example 15: Chondrogenic Activity of Tamarixetine



[073] Tamarixetine, also known as 3,5,7-trihydroxy-2-(3-hydroxy-4-methoxyphenyl)chromen-4-one, is commercially available. Tamarixetine may be isolated from plants or synthesized as described by, e.g., Peng et al. *Carbohydr Res*

340(10):1682-8 (2005). It is believed to possess antioxidant properties. Using procedures based on those described in Example 2, tamarixetine has now been found to significantly increase the activity of the chondrogenic responsive reporter gene pGL3(4X48), indicating that it is capable of stimulating chondrogenesis at low micromolar concentrations. At the single dosage that was tested, this compound did not significantly increase Alcian blue staining relative to controls, but it is expected that experiments with varying conditions (e.g., dosage, duration) will further support the chondrogenic activity indicated in the reporter assay.

Example 16: Chondrogenic Activity of Genistin

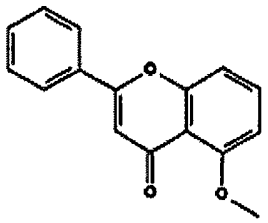


[074] Genistin, also known as 5-hydroxy-3-(4-hydroxyphenyl)-7-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxychromen-4-one, is a flavonoid derivative. Genistin has been shown to promote the proliferation of primary mouse bone MSC and osteoblasts. Li et al., *Acta Pharmacol Sin* 26(9):1081-6 (2005). Genistin is also believed to have anticancer properties. Choi et al. *Life Sci* 80(15):1403-8 (2007).

[075] Using procedures based on those described in Example 2, genistin was found to significantly increase the activity of the chondrogenic responsive reporter gene pGL3(4X48), indicating that it is a chondrogenesis stimulating agent (Figure 2). The PLM transfection assays demonstrate that low micromolar concentrations of genistin stimulate chondrogenic activity. At the single dosage that was tested, this compound did not significantly increase Alcian blue staining relative

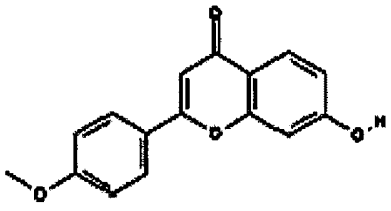
to controls, but it is expected that experiments with varying conditions (e.g., dosage, duration) will further support the chondrogenic activity indicated in the reporter assay.

Example 17: Chondrogenic activity of 5-methoxyflavone



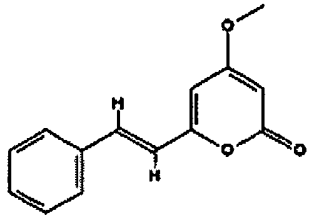
[076] 5-Methoxyflavone, also known as 5-methoxy-2-phenylchromen-4-one, is widely available from commercial sources. Large-scale synthesis of 5-methoxyflavone has been described by Ares et al., *J. Org. Chem.* 58(27):7903-05 (1993). Using procedures based on those described in Example 2, 5-methoxyflavone was found to significantly increase the activity of the chondrogenic responsive reporter gene pGL3(4X48), indicating that it is capable of stimulating chondrogenesis at low micromolar concentrations (Figure 2). At the single dosage that was tested, this compound did not significantly increase Alcian blue staining relative to controls, but it is expected that experiments with varying conditions (e.g., dosage, duration) will further support the chondrogenic activity indicated in the reporter assay.

Example 18: Chondrogenic Activity of Pratol



[077] Pratol, also known as 7-hydroxy-4-methoxyflavone and 7-hydroxy-2-(4-methoxyphenyl)chromen-4-one, is commercially available through a variety of sources. It has been described in WO/2001/003681 as useful to treat viral and parasitic infections. Using procedures based on those described in Example 2, pratol was found to significantly increase the activity of the chondrogenic responsive reporter gene pGL3(4X48), indicating that it is a chondrogenesis stimulating agent (Figure 2). The PLM transfection assays show that low micromolar concentrations of pratol stimulate chondrogenic activity. At the single dosage that was tested, this compound did not significantly increase Alcian blue staining relative to controls, but it is expected that experiments with varying conditions (e.g., dosage, duration) will further support the chondrogenic activity indicated in the reporter assay.

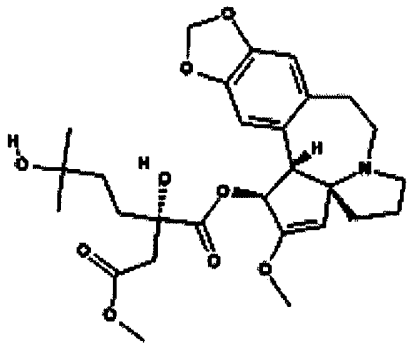
Example 19: Chondrogenic Activity of 5,6-Dehydrokawain



[078] 5,6-Dehydrokawain, also known as 4-methoxy-6-[(E)-2-phenylethenyl] pyran-2-one, is commercially available. It may be isolated from Alpinia speciosa rhizoma. Teng et al., *Chin J Physiol* 33(1):41-8 (1990). It is believed to possess antiplatelet properties. Following procedures outlined in Example 2, 5,6-dehydrokawain was found to significantly increase the activity of the chondrogenic responsive reporter gene pGL3(4X48), indicating that it is capable of stimulating chondrogenesis at low micromolar concentrations. At the single dosage that was tested, this compound did not significantly increase Alcian blue staining relative to controls, but it is expected that experiments with varying conditions (e.g., dosage,

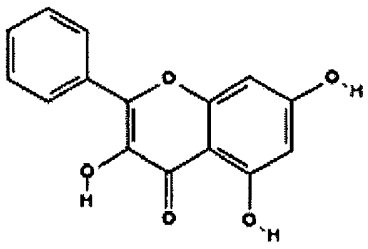
duration) will further support the chondrogenic activity indicated in the reporter assay.

Example 20: Chondrogenic Activity of Harringtonine



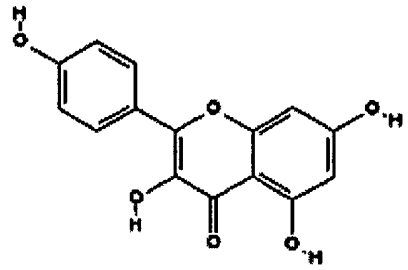
[079] Harringtonine, also know as 4-methyl-, 2-hydroxy-2-(3-hydroxy-3-methylbutyl) butanedioate-cephalotaxine is a commercially available natural product that has been shown to possess anti-cancer properties. It has now been shown, using the procedures outlined in Example 2, that harringtonine significantly increases the activity of the chondrogenic responsive reporter gene pGL3(4X48), indicating that it is a chondrogenesis stimulating agent (Figure 2). The PLM transfection assays show that low micromolar concentrations of harringtonine stimulate chondrogenic activity. At the single dosage that was tested, this compound did not significantly increase Alcian blue staining relative to controls, but it is expected that experiments with varying conditions (e.g., dosage, duration) will further support the chondrogenic activity indicated in the reporter assay.

Example 21: Chondrogenic Activity of Galangine



[080] Galangine, also known as 3,5,7-trihydroxyflavone and 3,5,7-trihydroxy-2-phenylchromen-4-one, is a mutagen believed to posses both anti-cancer and antibacterial properties. Using procedures based on those described in Example 2, galangine was found to significantly increase the activity of the chondrogenic responsive reporter gene pGL3(4X48), indicating that it is capable of stimulating chondrogenesis at low micromolar concentrations (Figure 2). At the single dosage that was tested, this compound did not significantly increase Alcian blue staining relative to controls, but it is expected that experiments with varying conditions (e.g., dosage, duration) will further support the chondrogenic activity indicated in the reporter assay.

Example 22: Chondrogenic Activity of Kaempferol



[081] Kaempferol, also known as 3,5,7-trihydroxy-2-(4-hydroxyphenyl)chromen-4-one, is commercially available and may be isolated from a variety of plants. Kaempferol is believed to possess a number of biological activities, including antioxidant, antimicrobial, anti-inflammatory, and anti-cancer activities. Using procedures based on those described in Example 2, kaempferol was found to significantly increase the activity of the chondrogenic responsive reporter gene pGL3(4X48), indicating that it is a chondrogenesis stimulating agent (Figure 2). These PLM transfection assays, as well as Alcian blue staining experiments, indicate that low micromolar concentrations of kaempferol stimulate chondrogenic activity.

Example 23: Combinations of Phenazopyridine Hydrochloride and Pro-chondrogenic Compounds Increase Pro-chondrogenic Activity

[082] High-density murine limb bud-derived cultures were established as previously described (Hoffman 2006). Media was replaced every two days. Cells were transfected on day 0 with pGL3(4X48) (reporter) and phRL-SV40 (to normalize transfection efficiency) plasmids using Effectene transfection reagent (Qiagen). Twenty-four hours after transfection, cells were treated with the following final concentrations of compounds, either individually or in combination: BMP4 (20 ng/ml), phenazopyridine hydrochloride (PHCL) (10 μM), and the pan-RAR antagonist 4310 (100 nM). Lysates were collected at 24h and 72h after treatment. Luciferase activity was measured using the Dual-Luciferase® kit according to the manufacturer's instructions (Promega). Reporter activity was normalized to untreated controls (-) on the same day. Results of an experiment performed in triplicate are shown in Figure 4.

[083] PHCL, 4310, and BMP4 all increased reporter gene activity individually. Reporter gene activity was further increased by combinations of PHCL with 4310 or BMP4. These results demonstrate that PHCL, either alone or in combination with BMP4 or 4310, significantly increases pGL3(4X48) reporter activity, indicating its effectiveness as a pro-chondrogenic compound.

Example 24: Phenazopyridine Hydrochloride and Pan-RAR Antagonist 4310 Reduce Mmp13 Expression in Limb Mesenchymal Cultures

[084] MMP13 is a major contributor to cartilage degradation in osteoarthritis, and inhibiting its activity is of therapeutic interest. RAR antagonists promote chondrocyte differentiation and inhibit expression of a hypertrophic phenotype. BMPs stimulate both chondrogenesis and chondrocyte hypertrophy. Accordingly, in

this study, 4310 and BMP4 were used as negative and positive controls for chondrocyte hypertropertrophy-inducing activity, respectively.

[085] Murine limb bud-derived high-density cultures were established as previously described (Hoffman 2006). Media was replaced every three days. After 24 hours of culture, compounds were added at the following final concentrations: BMP4 (20 ng/ml), phenazopyridine hydrochloride (PHCL, #7) (10 μM), and 4310 (100 nM). After 72 hours of culture, ascorbic acid and beta-glycerol phosphate were added to the culture at final concentrations of 126 μM and 1 mM, respectively. RNA was isolated from cultures at five and ten days. Gene expression was assessed using real-time PCR with TaqMan® probe and primer sets (Applied Biosystems,Taqman Gene Expression Assay Mm00439491_m1). All gene expression levels were normalized to the 5-day control (set at 100%). Results from an experiment performed in duplicate are shown in Figure 5.

[086] Control cells exhibited an approximately three-fold increase in *Mmp13* expression between 5 and 10 days. Consistent with previous reports, BMP4 increased the expression of *Mmp13*. Phenazopyridine hydrochloride and 4310 both markedly decreased the expression of *Mmp13*, indicating that they have chondroprotective activity. Thus, these compounds are attractive alternatives to decrease MMP13 activity, without the side-effects associated with MMP inhibitors.

WHAT IS CLAIMED IS:

1. A method for stimulating chondrogenesis comprising administering to a subject in need thereof a pharmaceutical composition comprising an amount of at least one compound selected from phenazopyridine hydrochloride, cytochalasin B, methysticin, rhapontin, tiabendazole, indoprofen, oxybenzone, chrysin, pinocembrin, 7-hydroxyflavone, 6-methoxyluteolin, 6,7-dihydroxyflavone, tamarixetine, genistin, 5-methoxyflavone, pratol, 5,6-dehydrokawain, harringtonine, galangine, and kaempferol effective to stimulate chondrogenesis.

2. A method for preventing chondrocyte hypertrophy or maturation comprising administering to a subject in need thereof a composition comprising a therapeutically effective amount of a compound selected from phenazopyridine hydrochloride, cytochalasin B, methysticin, rhapontin, thiabendazole, indoprofen, oxybenzone, chrysin, pinocembrin, 7-hydroxyflavone, 6-methoxyluteolin, 6,7-dihydroxyflavone, tamarixetine, genistin, 5-methoxyflavone, pratol, 5,6-dehydrokawain, harringtonine, galangine, and kaempferol.

3. A method for treating a chondrogenic disease comprising administering to a subject in need thereof a pharmaceutical composition comprising a therapeutically effective amount of a compound selected from phenazopyridine hydrochloride, cytochalasin B, methysticin, rhapontin, thiabendazole, indoprofen, oxybenzone, chrysin, pinocembrin, 7-hydroxyflavone, 6-methoxyluteolin, 6,7-dihydroxyflavone, tamarixetine, genistin, 5-methoxyflavone, pratol, 5,6-dehydrokawain, harringtonine, galangine, and kaempferol.

4. The method of claim 3, wherein the chondrogenic disease is selected from the group consisting of chondromalacia, chondrodysplasias, osteochondritis, congenital cartilage disease, osteochondritis dessecans, degenerative or fibrotic joint disease, rheumatoid arthritis, osteoarthritis, and polychondritis.

5. A method for treating or repairing a cartilage defect comprising administering to a subject in need thereof a composition comprising a therapeutically effective amount of a compound selected from phenazopyridine hydrochloride,

cytochalasin B, methysticin, rhapontin, thiabendazole, indoprofen, oxybenzone, chrysin, pinocembrin, 7-hydroxyflavone, 6-methoxyluteolin, 6,7-dihydroxyflavone, tamarixetine, genistin, 5-methoxyflavone, pratol, 5,6-dehydrokawain, harringtonine, galangine, and kaempferol.

6. The method of claim 5, wherein the cartilage defect is selected from a group consisting of articular cartilage tears, congenital cartilage defects, and cartilage damage induced by bone fractures.

7. A method of treating, ameliorating or repairing a skeletal defect, a large segmental skeletal gap or a non-union fracture arising from trauma or surgery comprising administering to a subject in need thereof a composition comprising a therapeutically effective amount of a compound selected from phenazopyridine hydrochloride, cytochalasin B, methysticin, rhapontin, thiabendazole, indoprofen, oxybenzone, chrysin, pinocembrin, 7-hydroxyflavone, 6-methoxyluteolin, 6,7-dihydroxyflavone, tamarixetine, genistin, 5-methoxyflavone, pratol, 5,6-dehydrokawain, harringtonine, galangine, and kaempferol.

8. The method of claim 7, wherein the composition is provided at the site of surgery, and wherein the composition mediates the formation of new bone tissue.

9. The method of claim 7, wherein the composition is delivered at the site of the segmental skeletal gap or non-union fracture, and wherein the composition mediates the formation of new bone tissue.

10. A method for the *ex vivo* engineering of chondrocytes comprising:

(a) culturing a population of precursor cells of chondrocyte lineage with a composition comprising a compound selected from phenazopyridine hydrochloride, cytochalasin B, methysticin, rhapontin, thiabendazole, indoprofen, oxybenzone, chrysin, pinocembrin, 7-hydroxyflavone, 6-methoxyluteolin, 6,7-dihydroxyflavone, tamarixetine, genistin, 5-methoxyflavone, pratol, 5,6-dehydrokawain, harringtonine, galangine, and kaempferol for a time sufficient to stimulate chondrogenesis; and

(b) implanting the cells from (a) into a desired site in a subject.

11. The method of claim 10, wherein the cells from step (a) are applied to an implantable device selected from the group consisting of a mechanical physical device, biodegradable carrier; biodegradable synthetic carrier, prostheses, demineralized allogenic bone and demineralized xenogenic bone, and implanted into the desired site.

12. The method of any one of claims 1 to 11, wherein the subject is a mammal.

13. The method of any one of claims 1 to 7, wherein the composition is administered systemically.

14. The method of any one of claims 1 to 7, wherein the composition is administered locally.

15. The method of any one of claims 1 to 9, wherein the composition is administered by injection.

16. The method of any one of claims 1 to 9, wherein the composition is administered as a coating on a device or implant.

17. The method of any one of claims 1 to 9, wherein said composition is used in conjunction with an implantable device selected from the group consisting of a mechanical physical device, biodegradable carrier, biodegradable synthetic carrier, prostheses, demineralized allogenic bone, and demineralized xenogenic bone.

18. The method of any one of claims 1 to 17, wherein the composition further comprises at least one other therapeutic agent.

19. The method of claim 18, wherein at least one other therapeutic agent is a chondrogenesis inducing agent.

20. The method of claim 19, wherein the chondrogenesis inducing agent is selected from BMPs, GDFs, FGFs, WNTs, Hedgehog, MIA or RAR antagonists.

21. The method of claim 18, wherein at least one other therapeutic agent is a chondroprotective agent.

22. The method of claim 21, wherein the chondroprotective agent is selected from IL-1 receptor antagonists, TNF receptor antagonists, COX-2 inhibitors, MAP kinase inhibitors, nitric oxide synthase inhibitors, and NFkB inhibitors.

23. The method of any one of claims 1 to 16, wherein the composition is administered simultaneously or sequentially with a chondrogenesis inducing agent and/or a chondroprotective agent.

24. The method according to any one of claims 1 to 23, wherein the compound is selected from thiabendazole, indoprofen, oxybenzone, 5,6-dehydrokawain, harringtonine, phenazopyridine, cytochalasin B, rhapontin, and methysticin.

25. A method for stimulating chondrogenesis comprising administering to a subject in need thereof a pharmaceutical composition comprising an amount of at least one flavonoid effective to stimulate chondrogenesis.

26. The method of claim 25, wherein the flavonoid is chrysin, pinocembrin, 7-hydroxyflavone, 6-methoxyluteolin, tamarixetine, genistin, 5-methoxyflavone, pratol, galanganine, kaempferol, and 6,7-dihydroxyflavone.

27. Use of at least one compound selected from phenazopyridine hydrochloride, cytochalasin B, methysticin, rhapontin, tiabendazole, indoprofen, oxybenzone, chrysin, pinocembrin, 7-hydroxyflavone, 6-methoxyluteolin, 6,7-dihydroxyflavone, tamarixetine, genistin, 5-methoxyflavone, pratol, 5,6-dehydrokawain, harringtonine, galangine, and kaempferol in the preparation of a medicament for stimulating chondrogenesis in a subject in need thereof.

28. At least one compound selected from phenazopyridine hydrochloride, cytochalasin B, methysticin, rhapontin, tiabendazole, indoprofen, oxybenzone, chrysin, pinocembrin, 7-hydroxyflavone, 6-methoxyluteolin, 6,7-dihydroxyflavone, tamarixetine, genistin, 5-methoxyflavone, pratol, 5,6-dehydrokawain, harringtonine, galangine, and kaempferol for stimulating chondrogenesis in a subject in need thereof.

29. Use of at least one compound selected from phenazopyridine hydrochloride, cytochalasin B, methysticin, rhapontin, tiabendazole, indoprofen, oxybenzone, chrysin, pinocembrin, 7-hydroxyflavone, 6-methoxyluteolin, 6,7-dihydroxyflavone, tamarixetine, genistin, 5-methoxyflavone, pratol, 5,6-dehydrokawain, harringtonine, galangine, and kaempferol in the preparation of a medicament for preventing chondrocyte hypertrophy or maturation in a subject in need thereof.

30. At least one compound selected from phenazopyridine hydrochloride, cytochalasin B, methysticin, rhapontin, tiabendazole, indoprofen, oxybenzone, chrysin, pinocembrin, 7-hydroxyflavone, 6-methoxyluteolin, 6,7-dihydroxyflavone, tamarixetine, genistin, 5-methoxyflavone, pratol, 5,6-dehydrokawain, harringtonine, galangine, and kaempferol for preventing chondrocyte hypertrophy or maturation in a subject in need thereof.

31. Use of at least one compound selected from phenazopyridine hydrochloride, cytochalasin B, methysticin, rhapontin, tiabendazole, indoprofen, oxybenzone, chrysin, pinocembrin, 7-hydroxyflavone, 6-methoxyluteolin, 6,7-dihydroxyflavone, tamarixetine, genistin, 5-methoxyflavone, pratol, 5,6-dehydrokawain, harringtonine, galangine, and kaempferol in the preparation of a medicament for treating a chondrogenic disease in a subject in need thereof.

32. At least one compound selected from phenazopyridine hydrochloride, cytochalasin B, methysticin, rhapontin, tiabendazole, indoprofen, oxybenzone, chrysin, pinocembrin, 7-hydroxyflavone, 6-methoxyluteolin, 6,7-dihydroxyflavone, tamarixetine, genistin, 5-methoxyflavone, pratol, 5,6-dehydrokawain, harringtonine, galangine, and kaempferol for treating a chondrogenic disease in a subject in need thereof.

33. Use of at least one compound selected from phenazopyridine hydrochloride, cytochalasin B, methysticin, rhapontin, tiabendazole, indoprofen, oxybenzone, chrysin, pinocembrin, 7-hydroxyflavone, 6-methoxyluteolin, 6,7-dihydroxyflavone, tamarixetine, genistin, 5-methoxyflavone, pratol, 5,6-

dehydrokawain, harringtonine, galangine, and kaempferol in the preparation of a medicament for treating or repairing a cartilage defect in a subject in need thereof.

34. At least one compound selected from phenazopyridine hydrochloride, cytochalasin B, methysticin, rhapontin, tiabendazole, indoprofen, oxybenzone, chrysin, pinocembrin, 7-hydroxyflavone, 6-methoxyluteolin, 6,7-dihydroxyflavone, tamarixetine, genistin, 5-methoxyflavone, pratol, 5,6-dehydrokawain, harringtonine, galangine, and kaempferol for treating or repairing a cartilage defect in a subject in need thereof.

35. Use of at least one compound selected from phenazopyridine hydrochloride, cytochalasin B, methysticin, rhapontin, tiabendazole, indoprofen, oxybenzone, chrysin, pinocembrin, 7-hydroxyflavone, 6-methoxyluteolin, 6,7-dihydroxyflavone, tamarixetine, genistin, 5-methoxyflavone, pratol, 5,6-dehydrokawain, harringtonine, galangine, and kaempferol in the preparation of a medicament for treating, ameliorating or repairing a skeletal defect, a large segmental skeletal gap or a non-union fracture arising from trauma or surgery in a subject in need thereof.

36. At least one compound selected from phenazopyridine hydrochloride, cytochalasin B, methysticin, rhapontin, tiabendazole, indoprofen, oxybenzone, chrysin, pinocembrin, 7-hydroxyflavone, 6-methoxyluteolin, 6,7-dihydroxyflavone, tamarixetine, genistin, 5-methoxyflavone, pratol, 5,6-dehydrokawain, harringtonine, galangine, and kaempferol for treating, ameliorating or repairing a skeletal defect, a large segmental skeletal gap or a non-union fracture arising from trauma or surgery in a subject in need thereof.

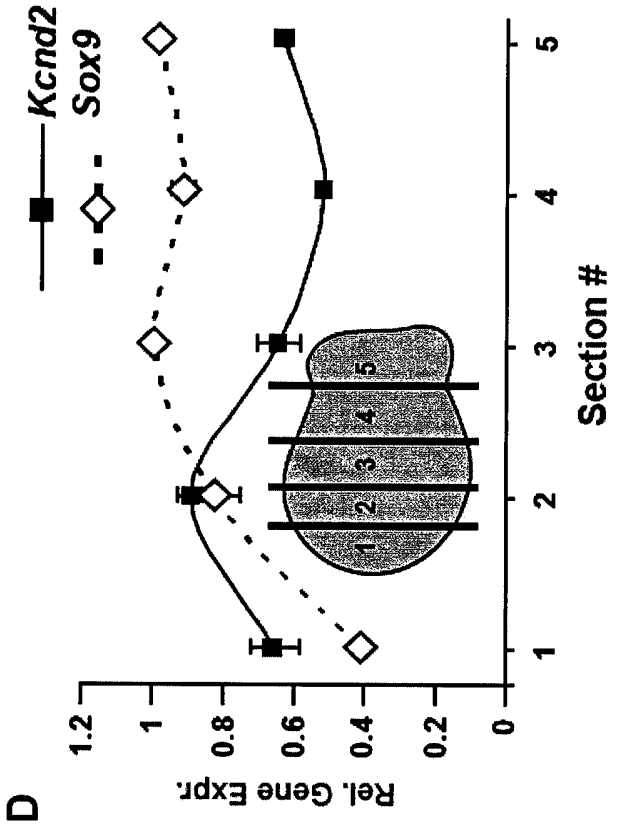
37. The use of any one of claims 29, 31, 33, or 35, or the compound of any one of claims 30, 32, 34, or 36, wherein the compound is selected from thiabendazole, indoprofen, oxybenzone, 5,6-dehydrokawain, harringtonine, phenazopyridine, cytochalasin B, rhapontin, and methysticin.

38. Use of a flavonoid in the preparation of a medicament for stimulating chondrogenesis in a subject in need thereof.

39. A flavonoid for stimulating chondrogenesis in a subject in need thereof.

40. The use of claim 38 or the flavenoid of claim 39, wherein the flavonoid is chrysin, pinocembrin, 7-hydroxyflavone, 6-methoxyluteolin, tamarixetine, genistin, 5-methoxyflavone, pratol, galanganine, kaempferol, and 6,7-dihydroxyflavone.

FIG. 1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Sox9
Rel. Gene Expr.
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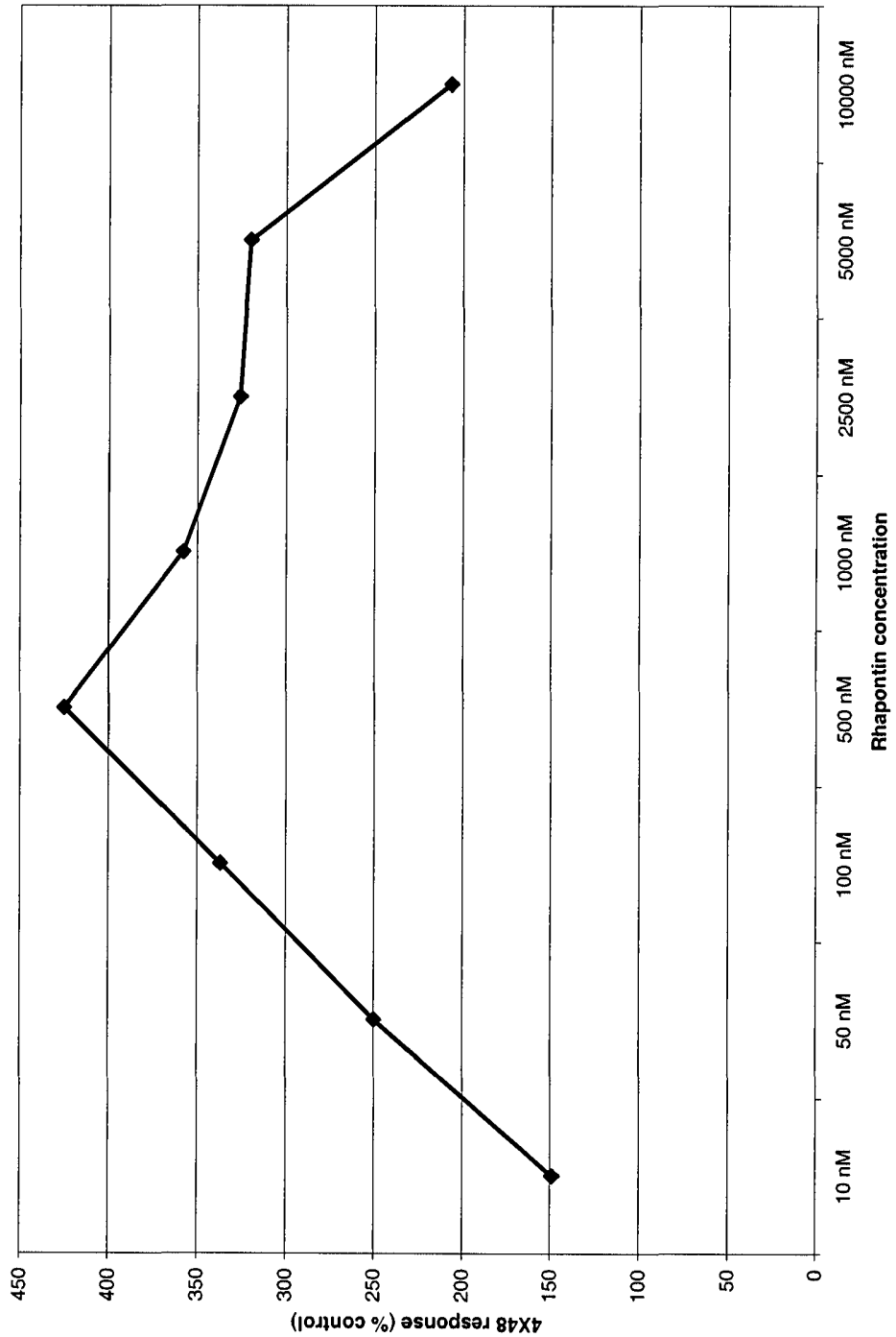
0.4
0.6
0.8
1
1.2
1
2
3
4
5
Section #
1
2
3
4
5

FIG. 2

Compound Name	4X48 (1)	4X48(2)
Thiabendazole	253	350
Indoprofen	167	194
Oxybenzone	224	421
Chrysin	251	485
Pinocembrin	192	712
7-Hydroxyflavone	181	588
6-Methoxyluteolin	155	221
Tamarixetine	205	603
Genistin	211	471
5-Methoxyflavone	155	485
Pratol	129	400
5,6-Dehydrokawain	275	588
Harringtonine	173	606
Galangine	293	763
Kaempferol	273	305
Phenazopyridine HCL	209	559
Cytochalasin B	2149	2204
6,7-dihydroxyflavone	411	537
Methysticin	368	376

FIG. 3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450
400
350
300
250
200
150
100
50
0
10 nM
50 nM
100 nM
500 nM
1000 nM
2500 nM
5000 nM
10000 nM
Rhapontin concentration

FIG. 4

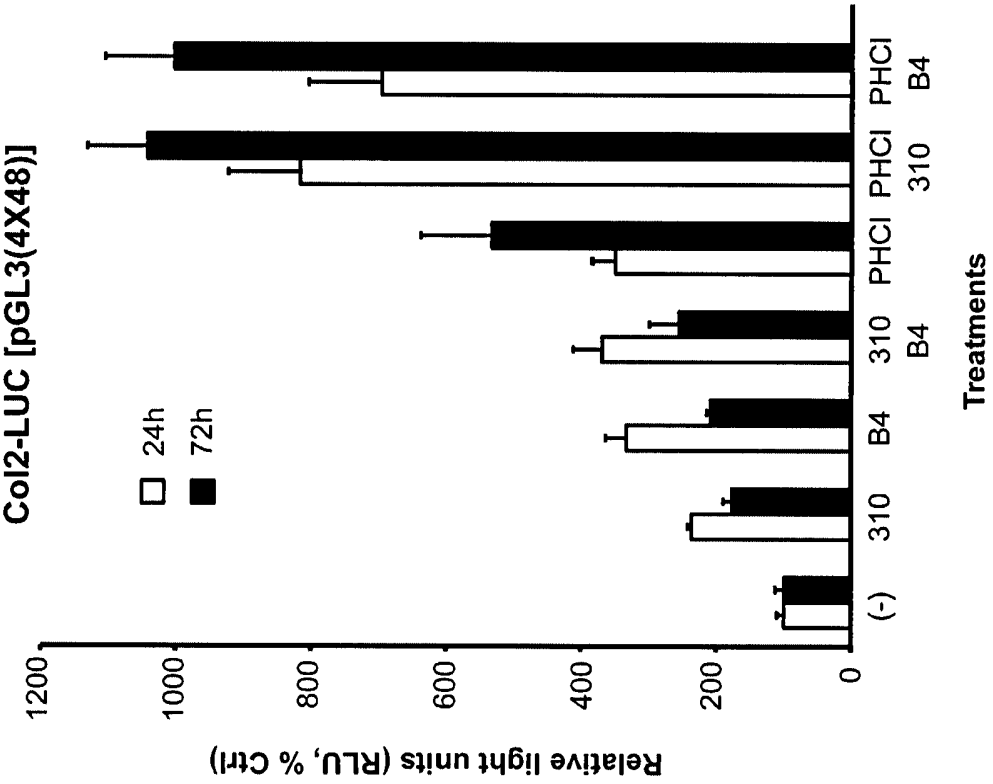
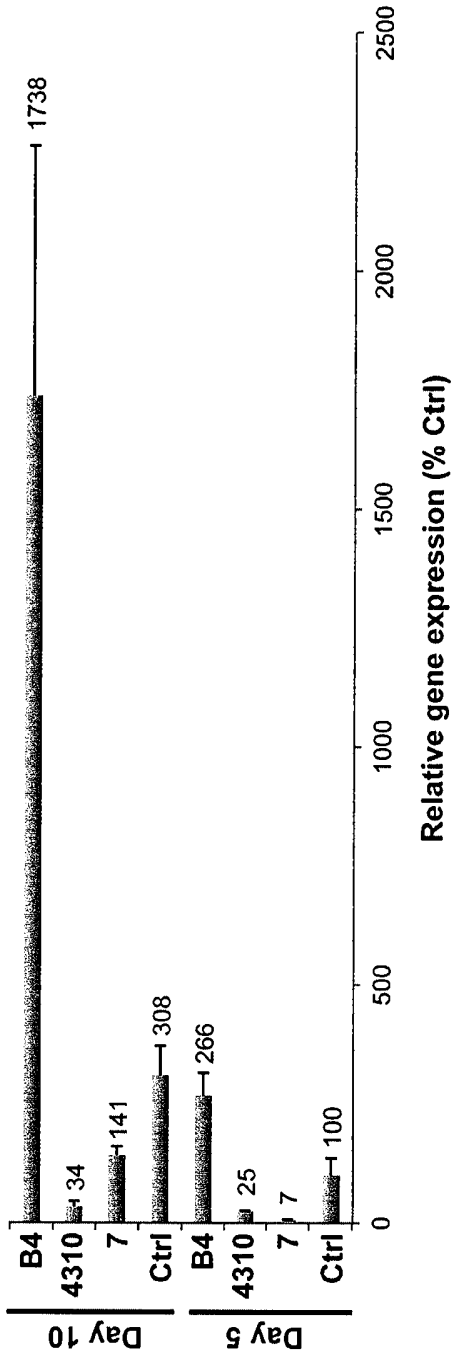

Col2-LUC [pGL3(4X48)]
24h
72h
Relative light units (RLU, % Ctrl)
0
200
400
600
800
1000
1200
(-)
310
B4
310
B4
PHCl
310
B4
PHCl
PHCl
Treatments

FIG. 5


Mmp13
Day 10
Day 5
B4
4310
7
Ctrl
1738
34
141
308
266
25
7
100
0
500
1000
1500
2000
2500
Relative gene expression (% Ctrl)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2009/007131

A. CLASSIFICATION OF SUBJECT MATTER
IPC (2010.01): ***A61K 31/352*** , ***A61K 31/12*** , ***A61K 31/366*** , ***A61K 31/4035*** , ***A61K 31/427*** , ***A61K 31/55*** , ***A61K 31/655*** , ***A61K 31/7034*** , ***A61P 19/00*** , ***C12N 5/077*** , *A61K 35/32* , *A61L 27/38* , *A61L 27/54*
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)
IPC (2010.01): **A61K 31/*** , **A61P 19/*** , **C12N 5/077**

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
IPC (2010.01): *C07D 213/**

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)
Canadian Patent Database, EPOQUE (EPODOC, TXTEN, WPI, MEDLINE), Scopus
Keywords: phenazopyridine, cartilage, bone, chondrogenesis, chondrogenic, osteogenesis, osteogenic, sox

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 1680109 A (OSTROMILENSKY) 7 August 1928 (07-08-1928) See page 1, lines 17-40. --	28, 30, 32, 34, and 36
A	WO 96/17057 A1 (KOOPMAN, *et al.*) 6 June 1996 (06-06-1996) See page 11, line 1 to page 12, line 10; page 14, lines 14-19; and claims 7-10. --	1-24 and 27-37
A	CA 2357549 A1 (UNDERHILL, *et al.*) 21 March 2002 (21-03-2002) See page 7, lines 4-13; page 13, lines 17-29; page 21, lines 3-5; and claims 1-6. --	1-24 and 27-37
A	WESTON, *et al.*, JOURNAL OF CELL BIOLOGY, Vol. 158, No. 1, 8 July 2002 (08-07-2002), pages 39-51. See abstract; and the section entitled "Introduction" on pages 39-40. ----	1-24 and 27-37

[ ] Further documents are listed in the continuation of Box C.

[X] 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 another 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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 February 2010 (22-02-2010)	26 February 2010 (26-02-2010)

Name and mailing address of the ISA/CA	Authorized officer
Canadian Intellectual Property Office Place du Portage I, C114 - 1st Floor, Box PCT 50 Victoria Street Gatineau, Quebec K1A 0C9 Facsimile No.: 001-819-953-2476	Philip O. Brown, Ph.D. (819) 994-1622

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2009/007131

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of the 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. [X] Claim Nos. : Claims 1-24

because they relate to subject matter not required to be searched by this Authority, namely :

Said claims are directed to methods for treatment of the human or animal body by surgery or therapy which, under Article 34(4)(a)(i) and Rule 67.1(iv), the International Search Authority is not required to search. However, this Authority has carried out a search based on the alleged effect or purpose/use of the products appearing in said claims.

2. [X] Claim Nos. : Claims 1-3, 5, 7, 19-23, and 27-36

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 :

The above claims do not clearly and concisely define the subject matter for which protection is sought, and thus fail to comply with Article 6 of the PCT. By the same token, the subject matter encompassed by said claims has not been clearly and completely disclosed in the description, such that the application does not comply with Article 5 of the PCT. **See Extra Sheets for further details.**

3. [ ] Claim 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 :

See Extra Sheets for further details.

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 additional fees, this Authority did not invite payment of additional fees.

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 claim Nos. :

4. [X] 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 claim Nos. :

Claims 1-24 (in part) and 27-37 (in part)

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.

Continuation of Box II:

Claims 1-3, 5, 7, and 27-36 do not clearly define the matter for which protection is sought, and therefore fail to comply with Article 6 of the PCT. In the above claims, the medicinal use is functionally defined in terms of various mechanisms of action (such as "stimulating chondrogenesis" , " preventing chondrocyte hypertrophy or maturation", "chondrogenic disease", "cartilage defect", and "skeletal defect"). Such definitions fail to clearly and concisely state the specific pathological disorder or biological effect on an organism that is to be achieved. As a result, it is unclear how the claimed compositions and methods can be practically applied to treat pathological disorders or achieve organism-wide biological effects encompassed by these functional definitions. As a result, these claims have been searched on the basis of the applications appearing in the disclosure and other claims, namely the treatment of selected skeletal and cartilaginous disorders mentioned in claims 4 and 6.

Claims 19-23 do not clearly define the matter for which protection is sought, and therefore fail to comply with Article 6 of the PCT. In each of the above claims, components of the compositions are functionally defined in terms of a desired mechanism of action (i.e., a "chondrogenesis inducing agent", a "chondroprotective agent", antagonists of specific enzymes, and inhibitors of specific enzymes). Such expressions fail to clearly and explicitly define what compounds actually fall within the scope of these claims. Furthermore, the disclosure relating to such genera is so incomplete with regards to the meaning of Article 5 such that said claims appear to lack support with regards to the meaning of Article 6. This renders a search across the entire breadth of these claims impossible. These claims have instead been searched on the basis of the use of phenazopyridine hydrochloride with other drugs suitable for the treatment of cartilaginous or skeletal disorders, regardless of mechanism of action.

Continuation of Box III:

This international application does not comply with Rules 13.1 and 13.2 of the PCT, as it is directed to a plurality of alleged inventions that do not share a special technical feature. The International Searching Authority has identified the following alleged inventions:

Group A – Claims 1-24 (in part) and 27-37 (in part) are directed to use of phenazopyridine hydrochloride for the treatment of cartilaginous and skeletal disorders linked to decreased chondrogenesis, methods of use of said compound, and compositions comprising said compound;

Group B – Claims 1-24 (in part) and 27-37 (in part) are directed to use of cytochalasin B for the treatment of cartilaginous and skeletal disorders linked to decreased chondrogenesis, methods of use of said compound, and compositions comprising said compound;

Group C – Claims 1-24 (in part) and 27-37 (in part) are directed to use of methysticin for the treatment of cartilaginous and skeletal disorders linked to decreased chondrogenesis, methods of use of said compound, and compositions comprising said compound;

Group D – Claims 1-24 (in part) and 27-37 (in part) are directed to use of rhapontin for the treatment of cartilaginous and skeletal disorders linked to decreased chondrogenesis, methods of use of said compound, and compositions comprising said compound;

Group E – Claims 1-24 (in part) and 27-37 (in part) are directed to use of oxybenzone for the treatment of cartilaginous and skeletal disorders linked to decreased chondrogenesis, methods of use of said compound, and compositions comprising said compound;

Group F – Claims 1-24 (in part) and 27-37 (in part) are directed to use of thiabendazole for the treatment of cartilaginous and skeletal disorders linked to decreased chondrogenesis, methods of use of said compound, and compositions comprising said compound;

Group G – Claims 1-24 (in part) and 27-37 (in part) are directed to use of indoprofen for the treatment of cartilaginous and skeletal disorders linked to decreased chondrogenesis, methods of use of said compound, and compositions comprising said compound;

Group H – Claims 1-24 (in part), 25, 26, 27-37 (in part) and 38-40 are directed to use of flavonoids for the treatment of cartilaginous and skeletal disorders linked to decreased chondrogenesis, methods of use of said compound, and compositions comprising said compound; and

Group I – Claims 1-24 (in part) and 27-37 (in part) are directed to use of harringtonine for the treatment of cartilaginous and skeletal disorders linked to decreased chondrogenesis, methods of use of said compound, and compositions comprising said compound;

As is evident from review of examples 3-22 on pages 19-35, the above groups each consist of diverse families compounds with no structural feature in common to serve as a linking technical feature. Furthermore, it is noted on page 18, paragraph 047 that use of compounds to modulate of *Sox*5/6/9 is known in the art to affect chondrogenesis. As a result, the mechanism of action of the above families also cannot act as a linking technical feature that differentiates the alleged inventions from the prior art. As no other technical features appear to be shared by the above groups, this results in a lack of unity.

As no additional fees were timely paid by applicant, this International Search Report is limited to the claims of **Group A**.

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