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- (54) Title: METHODS AND COMPOSITIONS FOR TREATING OSTEOPOROSIS, MENOPAUSE AND OTHER AGE-RELATED DISEASES

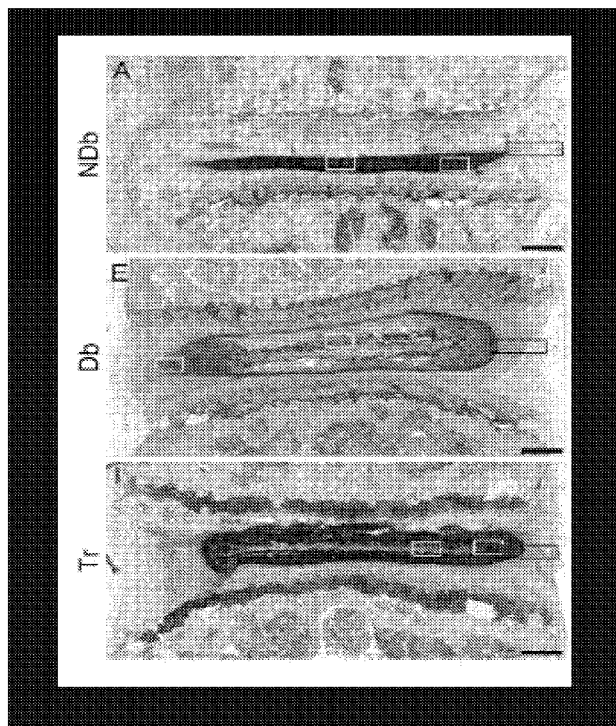


FIGURE 3

- (57) Abstract: Methods and compositions for treating diseases that arise from age-related loss of estrogen receptor function are disclosed. The compositions comprise at least one Advanced Glycation Endproduct (AGE) uptake inhibitor, such as sevelamer and at least one AGE formation inhibitor, such as pyridoxamine.



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**METHODS AND COMPOSITIONS FOR TREATING OSTEOPOROSIS,
MENOPAUSE AND OTHER AGE-RELATED DISEASES**

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority from US provisional application 61/824,753, filed May 17, 2013, the entire disclosure of which is incorporated herein by reference.

GOVERNMENT RIGHTS STATEMENT

[0002] This invention was made with U.S. Government support under DK-091231 and AG-017170 awarded by the National Institutes of Health. The U.S. Government has certain rights in the invention.

SEQUENCE LISTING

[0003] This application contains a Sequence Listing, created on May 15, 2014; the file, in ASCII format, is designated 3710005AWO_Sequence Listing_ST25.txt and is 1.86 kilobytes in size. The sequence listing file is hereby incorporated by reference in its entirety into the application.

BACKGROUND OF THE INVENTION

Technical Field

[0004] The present application relates generally to methods and compositions for treating diseases that arise from age-related loss of estrogen receptor function. The compositions comprise at least one Advanced Glycation Endproduct (AGE) uptake inhibitor and at least one AGE formation inhibitor.

Background Information

[0005] It has long been known that estrogen function in women decreases with age. While estrogen levels are decreased in aging, the levels remain sufficient to stimulate estrogen receptors, suggesting that the reason for the insensitivity to estrogen is due to estrogen receptor number or function. This lack of estrogen function may cause

effects including, for example, an increased risk of cardiovascular disease and kidney disease, premature or accelerated loss of mental functions, hot flashes, and skin changes.

[0006] At present, there is considerable discussion about the utility of estrogen replacement therapy (ERT) in post-menopausal women. Many feel that ERT is associated with unacceptable risks. Thus, many women in the peri- or post-menopausal period suffer from physiologic and pathophysiologic effects of estrogen "deprivation".

[0007] While estrogen function in women is commonly recognized as a target for therapeutic intervention, the problem of decreased estrogen function is not unique to females. ER α is uniquely important to women in the reproductive organs, but other sequelae of decreased ER α function, such as bone loss, disc degeneration, loss of cognitive or cerebral function and age-related skin changes, are shared by men and women.

[0008] Thus, a need exists for a safe therapy to increase the activity and/or expression of estrogen receptor α , rather than merely increasing the amount of estrogen.

SUMMARY OF THE INVENTION

[0009] The invention is directed to pharmaceutical compositions and methods for increasing the expression and/or action of estrogen receptor α .

[0010] The present invention provides, in a first aspect, a method of increasing estrogen receptor α expression and/or action in a patient comprising administering to a patient a therapeutically effective amount of at least one agent that decreases advanced glycation endproduct levels.

[0011] The present invention provides, in a second aspect, a method of treating a disease or condition chosen from osteoporosis, peri-menopause and menopause, degenerative disc disease, decreased cerebral function, and age-related skin changes. The method of treating involves administering a therapeutically effective amount of at least one AGE uptake inhibitor and at least one AGE formation inhibitor.

[0012] The present invention provides, in a third aspect, a pharmaceutical composition comprising a therapeutically effective amount of at least one AGE uptake inhibitor and at least AGE formation inhibitor.

[0013] The present invention provides, in a fourth aspect, a method of treating a disease or condition chosen from osteoporosis, peri-menopause and menopause, degenerative disc disease, decreased cerebral function, and age-related skin changes. The method of treating involves administering to a patient in need thereof a therapeutically effective amount of a composition comprising a therapeutically effective amount of at least one AGE uptake inhibitor and at least AGE formation inhibitor.

[0014] These, and other objects, features and advantages of this invention will become apparent from the following detailed description of the various aspects of the invention taken in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

[0015] FIG. 1 is a bar graph of discrimination ratio of mice fed low AGE diet and high AGE diet.

[0016] FIG. 2 is a line graph of discrimination ratio vs exposure session for mice fed low AGE diet and high AGE diet.

[0017] FIG. 3 is a comparison of three photomicrographs of mouse L4-L5 intervertebral discs.

[0018] FIG. 4 depicts cross sections of L4-L5 vertebrae showing (a) control, (b) low ER α and (c) low ER α treated with pyridoxamine and pentosan polysulfate.

DETAILED DESCRIPTION OF THE INVENTION

[0019] All publications, patents and other references cited herein are incorporated by reference in their entirety into the present disclosure.

[0020] It has been found that the number and function of estrogen receptors is decreased in aging, but that these receptors can be returned to functional number and function by reducing exposures to advanced glycation endproducts. It also has been found that estrogen receptors that had been suppressed as a consequence of aging can be normalized by reduction of oxidative stress and inflammation by inhibitors of AGE uptake or intracellular synthesis. This inhibition of AGE uptake may occur by reducing the AGE content in food and/or by utilizing a drug that binds AGEs in the gut and transports them out of the body in the stool.

[0021] In one aspect, the invention relates to a method for increasing/normalizing estrogen receptor α expression and/or action in a patient. This method includes administering to the patient a therapeutically effective amount of at least one agent that decreases advanced glycation endproduct levels. In some embodiments, increasing the estrogen receptor α expression and/or action is accomplished by administering a therapeutically effective amount of at least one AGE uptake inhibitor and at least one AGE formation inhibitor.

[0022] In another aspect, the invention relates to a method of treating a disease or condition by administering a therapeutically effective amount of at least one AGE uptake inhibitor and at least one AGE formation inhibitor.

[0023] In some embodiments, the disease or condition is osteoporosis. In osteoporosis, the bone mineral density is reduced, bone microarchitecture deteriorates, and the amount and variety of proteins in bone are altered. Osteoporosis is defined by the World Health Organization as a bone mineral density of 2.5 standard deviations or more below the mean peak bone mass (average of young, healthy adults) as measured by dual-energy X-ray absorptiometry. The disease may be classified as primary type 1, primary type 2, or secondary. The form of osteoporosis most common in women after menopause is referred to as primary type 1 or postmenopausal osteoporosis. Primary type 2 osteoporosis or senile osteoporosis occurs after age 75 and is seen in both females and males at a ratio of 2:1. Secondary osteoporosis may arise at any age and affect men and women equally. This form results from chronic predisposing medical problems or disease, or prolonged use of medications such as glucocorticoids. The osteoporosis treated by the methods and compositions of the present invention is most commonly primary osteoporosis.

[0024] In other embodiments, the disease or condition is peri-menopause or menopause. Menopause typically occurs at around age 50, but can occur at much earlier or later ages. It can give rise to symptoms such as edema, hot flashes (or flashes), attacks of sweating, muscle and possibly joint pain, sleep disturbances, dysphoria, nervousness, mood swings, headache, palpitations (enhanced frequency of heart rate), dry mucous membranes, pain during intercourse and urinary disturbances. Hot flashes or flushing are characterized by a sudden onset of warmth in the face and neck, often progressing to the chest. Episodes generally last several minutes and are evidenced by a visible flushing of the skin. Often such episodes are accompanied by sweating, dizziness, nausea, palpitations and diaphoresis. Such symptoms can disrupt sleep and interfere with quality of life. Other symptoms of peri-menopause may include breast tenderness, and fatigue. Perimenopause literally means the time “around” menopause. The term is used by medical providers to describe the beginning of the decline in the estrogen/ER system leading to menopause. Perimenopause begins with the first signs or symptoms of menopause. For some women, this is as early as their thirties. By their mid-forties, most women notice at least occasional signs. Officially, perimenopause ends with the diagnosis of menopause, which is twelve consecutive months without a period.

[0025] Although the cause of hot flashes is not completely understood, they are thought to be a disorder of thermoregulation within the hypothalamus that is a consequence of declining estrogen levels. The administration of female sex hormones, such as estrogen, is effective in palliating these symptoms, but hormone therapy is fraught with undesirable side effects. Four out of five women have disturbing menopause disorders for at least one year and 25% of women have menopause disorders for more than 5 years. Half of all women have severe disorders..

[0026] In some embodiments, the disease or condition is degenerative disc disease. Fibrocartilage replaces the gelatinous mucoid material of the nucleus pulposus as the disc changes with age. There may be splits in the annulus fibrosis, permitting herniation of elements of nucleus pulposus. There may also be shrinkage of the nucleus pulposus that produces prolapse or folding of the annulus with secondary osteophyte formation at the margins of the adjacent vertebral body. The pathologic

findings in DDD include protrusion, spondylolysis, and/or subluxation of vertebrae (spondylolisthesis) and spinal stenosis

[0027] In other embodiments, the disease or condition is decreased cerebral function. Decreased cerebral function can include, for instance, reduced memory or motor function loss, including a loss of balance. Memory loss and impaired learning ability are features of a range of clinical conditions. For instance, loss of memory is the most common symptom of dementia states including Alzheimer's disease and senile dementia of the Alzheimer type (the two different terms here distinguish between young and old age onset cases). To the extent that it can be treated, Alzheimer's disease typically is treated by acetylcholine esterase inhibitors such as tacrine hydrochloride or donepezil. Early symptoms of AD include memory lapses and mild but progressive deterioration of specific cognitive functions, such as language (aphasia), motor skills (apraxia) and perception (agnosia). The earliest manifestation of AD is often memory impairment, which is required for a diagnosis of dementia in both the NINCDS/ADRDA criteria.

[0028] In some embodiments, the disease or condition is an age-related skin change. Age-related skin changes can be manifested in subcutaneous fat reduction and increased elastotic substance in the upper dermis, destruction of its fibrillar structure, augmented intercellular substance and the presence of moderate inflammatory infiltrate. The thickness of the epidermal layers and the number of cellular living layers is decreased in aging skin. The amount of keratohyaline granules is enlarged, the dermoepidermal junction is flattened and the presence of elastotic material in the dermis is pronounced with age. The fibrous trabeculae are thickened in older skin and the atrophy of the hypodermis is observed. Chronological ageing alters the fibroblasts' metabolism by reducing their life span, capacity to divide and produce collagen. During ageing, the enlargement of collagen fibrils diminishes the skin elasticity. As a practical consequence, wound healing speed is decreased.

[0029] In another aspect, the invention relates to a method of treating a disease or condition chosen from osteoporosis, peri-menopause and menopause, degenerative disc disease, decreased cerebral function, and age-related skin changes (all as described *supra*) by administering to a patient in need thereof a therapeutically effective amount of a composition described herein.

[0030] In some embodiments, disclosed is a pharmaceutical composition which includes a therapeutically effective amount of at least one AGE uptake inhibitor and at least one AGE formation inhibitor. In some embodiments, the pharmaceutical composition includes a therapeutically effective combination of at least one AGE uptake inhibitor and at least one vitamin B₆ vitamer. In other embodiments, the pharmaceutical composition includes a therapeutically effective combination of sevelamer and pyridoxamine. In some embodiments, the AGE uptake inhibitor is sevelamer carbonate and the AGE formation inhibitor is pyridoxamine. In some embodiments, the pharmaceutical composition also includes a pharmaceutically acceptable excipient, diluent, or carrier. In particular embodiments, the pharmaceutically acceptable excipient, diluent, or carrier is an excipient, diluent, or carrier for oral administration, and the medicament is an oral dosage form, particularly a unit dosage form.

[0031] In some embodiments, the AGE uptake inhibitor is an allylamine/epichlorohydrin copolymer. Examples of these include sevelamer carbonate, sevelamer hydrochloride and colesevelam. In other embodiments, the AGE uptake inhibitor is an ethyleneglycol dimethacrylate/ N-methylpyridylethylene hydrochloride copolymer. In some embodiments, the AGE uptake inhibitor is a 2-hydroxyethyl methacrylate-glycidyl methacrylate copolymer. In other embodiments, the AGE uptake inhibitor is a glycosideethyl methacrylate-glycidyl methacrylate copolymer. In some embodiments, the AGE uptake inhibitor is an ethylene glycol dimethacrylate cross-linking 3-amino-2-hydroxypropylmethacrylate hydrochloride copolymer. In other embodiments, the AGE uptake inhibitor is an ethylene glycol dimethacrylate cross-linking triethylenetetramine-substituted glycidyl methacrylate copolymer. In some embodiments, the AGE uptake inhibitor is a flavanoid. In other embodiments, the AGE uptake inhibitor is pentosan polysulfate.

[0032] In some embodiments, the AGE formation inhibitor is aminoguanidine. In some embodiments, the AGE formation inhibitor is ALT-462. In other embodiments, the AGE formation inhibitor is ALT-468. In other embodiments, the AGE formation inhibitor is tenilsitam. In some embodiments, the AGE formation inhibitor is OPB-9195. In some embodiments, the AGE formation inhibitor is benfotiamine. In other embodiments, the AGE formation inhibitor is TM-2002. In some embodiments, the

AGE formation inhibitor is thiamine pyrophosphate. In other embodiments, the AGE formation inhibitor is diaminophenazine. In some embodiments, the AGE formation inhibitor is lipoic acid. In some embodiments, the AGE formation inhibitor is penicillamine. In other embodiments, the AGE formation inhibitor is carnosine. In some embodiments, the AGE formation inhibitor is edaravone. In other embodiments, the AGE formation inhibitor is LR-90. In some embodiments, the AGE formation inhibitor is LR-9. In some embodiments, the AGE formation inhibitor is LR-20. In other embodiments, the AGE formation inhibitor is LR-59. In some embodiments, the AGE formation inhibitor is LR-74. In some embodiments, the AGE formation inhibitor is ALT-711. In other embodiments, the AGE formation inhibitor is losartan. In some embodiments, the AGE formation inhibitor is olmesartan. In other embodiments, the AGE formation inhibitor is R-147176. In some embodiments, the AGE formation inhibitor is triethylenetetramine. In other embodiments, the AGE formation inhibitor is ethylene diamine tetraacetic acid. In some embodiments, the AGE formation inhibitor is hydralazine. In some embodiments, the AGE formation inhibitor is a vitamin B₆ vitamer. In some embodiments, the vitamin B₆ vitamer is pyridoxamine. The structures of these and other AGE formation inhibitors are set forth in Nagai et al., *Diabetes* 61, 549-559 (2012), the disclosure of which is incorporated herein by reference.

[0033] In some embodiments, the invention relates to a pharmaceutical combination in which the AGE uptake inhibitor is selected from allylamine/epichlorohydrin copolymers, ethyleneglycol dimethacrylate/ N-methylpyridylethylene hydrochloride copolymers, 2-hydroxyethyl methacrylate-glycidyl methacrylate copolymers, glycosideethyl methacrylate-glycidyl methacrylate copolymers, ethylene glycol dimethacrylate cross-linking 3-amino-2-hydroxypropylmethacrylate hydrochloride copolymers, ethylene glycol dimethacrylate cross-linking triethylenetetramine-substituted glycidyl methacrylate copolymers, flavanoids, and pentosan polysulfate; and the AGE formation inhibitor is selected from aminoguanidine, ALT-462, ALT-468, a vitamin B₆ vitamer, tenilsitam, OPB-9195, benfotiamine, thiamine pyrophosphate, diaminophenazine, lipoic acid, penicillamine, carnosine, TM-2002, edaravone, LR-90, LR-9, LR-20, LR-59, LR-74, ALT-711, losartan, olmesartan, R-147176, triethylenetetramine, ethylene diamine tetraacetic acid and hydralazine. Any

combination of at least one AGE uptake inhibitor selected from the list above and at least one AGE formation inhibitor selected from the list above may be utilized.

[0034] Combination therapy can be achieved by administering two or more agents, each of which is formulated and administered separately, or by administering two or more agents in a single formulation. Other combinations are also encompassed by combination therapy. For example, two agents can be formulated together and administered in conjunction with a separate formulation containing a third agent. While the two or more agents in the combination therapy can be administered simultaneously, they need not be. For example, administration of a first agent (or combination of agents) can precede administration of a second agent (or combination of agents) by minutes, hours, days, or weeks. Thus, the two or more agents can be administered within minutes of each other or within 1, 2, 3, 6, 9, 12, 15, 18, or 24 hours of each other. In some cases even longer intervals are possible. While in many cases it is desirable that the two or more agents used in a combination therapy be present in within the patient's body at the same time, this need not be so. Combination therapy can also include two or more administrations of one or more of the agents used in the combination. For example, if agent X and agent Y are used in a combination, one could administer them sequentially in any combination one or more times, e.g., in the order X-Y-X, X-X-Y, Y-X-Y, Y-Y-X, X-X-Y-Y, etc.

[0035] As used herein, "treatment" or "treating," or "palliating" or "ameliorating" are used interchangeably herein. These terms refers to an approach for obtaining beneficial or desired results including but not limited to therapeutic benefit and/or a prophylactic benefit. By therapeutic benefit is meant eradication or amelioration of the underlying disorder being treated. Also, a therapeutic benefit is achieved with the eradication or amelioration of one or more of the physiological symptoms associated with the underlying disorder such that an improvement is observed in the patient, notwithstanding that the patient may still be afflicted with the underlying disorder. For example, "treating menopause" means alleviating at least one symptom associated with menopause; it is not required or that the patient no longer exhibit any symptoms of menopause or that estrogen levels or responses thereto be brought to a pre-menopausal state. The compositions may be administered to a patient at risk of developing a particular disease, or to a patient reporting one or more of the

physiological systems of a disease, even though a diagnosis of this disease may not have been made.

[0036] As used herein, the terms “comprising” and “including” or grammatical variants thereof are to be taken as specifying the stated features, integers, steps or components but do not preclude the addition of one or more additional features, integers, steps, components or groups thereof. This term encompasses the terms “consisting of” and “consisting essentially of”. The phrase “consisting essentially of” or grammatical variants thereof when used herein are to be taken as specifying the stated features, integers, steps or components but do not preclude the addition of one or more additional features, integers, steps, components or groups thereof but only if the additional features, integers, steps, components or groups thereof do not materially alter the basic and novel characteristics of the claimed composition or method. As used herein, the singular forms “a”, “an” and “the” are intended to include the plural forms as well, unless the context clearly indicates otherwise. It will be further understood that the terms “comprise” (and any form of comprise, such as “comprises” and “comprising”), “have” (and any form of have, such as “has” and “having”), “include” (and any form of include, such as “includes” and “including”), and “contain” (and any form contain, such as “contains” and “containing”) are open-ended linking verbs. As a result, a method that “comprises”, “has”, “includes” or “contains” one or more steps or elements possesses those one or more steps or elements, but is not limited to possessing only those one or more steps or elements.

[0037] The formulations include those suitable for oral, parenteral (including subcutaneous, intradermal, intramuscular, intravenous and intraarticular), rectal and topical (including dermal, buccal, sublingual and intraocular) administration. The most suitable route may depend upon the condition and disorder of the recipient. It will, of course also depend on whether an AGE-uptake inhibitor of the non-absorbable type is present. The formulations may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. Formulations of the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets or tablets each containing a predetermined amount of the active ingredient; as a powder or granules; as a solution or a suspension in an aqueous liquid or a non-aqueous liquid; or as an oil-in-water

liquid emulsion or a water-in-oil liquid emulsion. The active ingredient may also be presented as a bolus, electuary or paste. Preferred unit dosage formulations are oral unit dosage forms containing an effective dose, or an appropriate fraction thereof, of the active ingredient.

[0038] It should be understood that in addition to the ingredients particularly mentioned above, the formulations of this invention may include other agents conventional in the art. For example those suitable for oral administration may include other therapeutic ingredients, anti-caking agents, preservatives, sweetening agents, colorants, flavors, desiccants, plasticizers, dyes, disintegrants, lubricants and the like.

[0039] EXAMPLES

Example 1: Methods For Estrogen Receptor/ROS/AGEs in Bone and Brain:

[0040] Animal studies in Brain:

[0041] *Reagents:* Anti-SIRT1, anti-IRS1, anti-IRS2, and anti-phospho-IRS1 (Ser307) were from Millipore (Temecula, CA). Anti-NAMPT was from BETHYL (Montgomery, TX). Anti-AGER1, anti-insulin receptor antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Human SIRT1 cDNA was a gift of Dr. J. C. He (Mount Sinai School of Medicine, NY, NY), human RAGE cDNA was from Dharmacon RNAi Technologies, anti-RAGE antibody (Affinity BioReagents), anti-SIRT1 and anti-ADAM10 antibodies, anti-NeuN (Millipore), anti-AGER1 and anti-PPAR γ antibodies (Santa Cruz Biotechnology), anti-NAMP antibody (BETHYL), anti-APP (6E10) and anti-sAPPbeta antibodies (COVANCE). Plasma 8-isoprostanes (Cayman, Ann Arbor, MI), VCAM-1 (R&D Systems, Inc) and adiponectin (Invitrogen, Camarillo, CA) were assessed by commercial kits as per manufacturer's procedures.

[0042] *Animals and treatments:* C57BL/6 mice (National Institutes on Aging, CR colony), which were bred for over ten generations on defined diets, termed MG+ and MG-, were used in these studies. Offspring (F5-F9, n=18-20/group, 1:1 M/F, age 18-19 mos), after weaning, were assigned to 2 pair-fed groups, receiving either standard diet (NIH-31 open formula) prepared without heat exposure (Low-AGE or MG-) or

the same with synthetic MG-BSA added (1 mg/g food, MG+), and air-dried, at RT. These diets were identical in nutritional and caloric content but MG⁺ diet contained ~x2-fold more MG-H1 and anti-carboxy-methyl lysine (CML) than MG⁻ diet and equivalent to the regular heat-treated chow (REG). Age-matched mice (n=8, 4:4 M/F), fed NIH-31 open formula (REG) were also used as controls. An intraperitoneal glucose tolerance test (IGTT) was performed at 6 month intervals after age 12 months. All mice were maintained in a pathogen-free environment, (72°F, 50% humidity, and 12:12-hour light: dark cycles) at the Center for Laboratory Animal Science, Mount Sinai School of Medicine. All experimental procedures complied with the guide for the Care and Use of Laboratory Animals (Department of Health, Education, And Welfare, NIH 78-23,1996).

[0043] *AGE determination:* AGEs in sera (mouse and human), mouse brain tissue and cultured neuronal cells were determined by two competitive ELISAs, based on non-cross-reactive MAbs for MG-derivatives, i.e. MG-H1 (3D11 MAb), and for anti-carboxy-methyl-lysine (CML) (4G9 MAb). Test sensitivity for CML and MG is 0.1 U/ml and 0.004 nmol/ml, respectively; the intra-assay variation is $\pm 2.6\%$ (for CML) and $\pm 2.8\%$ (for MG) and the inter-assay variation is $\pm 4.1\%$ (CML) and $\pm 5.2\%$ (MG). Brain AGE-lipids were measured by direct ELISA using the above antibodies immediately after total brain tissue lipid extraction (2:1 chloroform-methanol, v/v, 20mM BHT [Butylated hydroxytoluene, Sigma]).

[0044] *Cell culture and treatment:* Embryonic E14 primary brain neuronal cell cultures derived from MG⁺, MG⁻ and REG mice were prepared as described in the literature with minor modification. Briefly, brain tissue sections in cold DMEM medium (FBS, 10% and antibiotics, 1%) were mechanically dissociated by using a fire-polished Pasteur pipette, or a syringe and needle as needed (at RT). The dissociated tissues were filtered through a mesh (BD Falcon, 40uM) and seeded on 6-well plates pre-coated with poly-L-lysine (0.1mg/ml; Sigma). After 30min the medium was replaced with neurobasal medium (Invitrogen), 2% (v/v) B-27, 0.5% glutamine (2mM), and 0.2% gentamicin (0.1mg/ml), refreshed after 24h. The cultures were maintained at 37°C in 5% CO₂ for 3-4 days. In separate studies, neuronal cells from REG mice were stimulated with different doses of MG-BSA or BSA for 72 h. Total RNA was extracted by Trizol (Molecular Probes, Inc). The extracted RNA had

an A260/280 ratio between 1.8 and 2.0. Total RNA was reverse-transcribed using Superscript III RT (Invitrogen). For the clinical study, human mononuclear cells (MNC) were separated from fasting, EDTA anti-coagulated blood by Ficoll-Hypaque Plus gradient (American Biosciences, Uppsala, Sweden) and used to isolate mRNA as above.

[0045] *Western blotting:* Mouse whole brain or cultured E14 neuronal cells were homogenized in radio-immune precipitation assay buffer (RIPA, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS, 50mM Tris, pH 8.0) including Complete Protease Inhibitor mixture (Roche). Lysates were then separated by SDS-PAGE and subjected to Western blot analysis with the indicated antibodies.

[0046] *RNA isolation and real-time quantitative PCR (RT-PCR):* Total RNA from mouse brain or human mononuclear cells was extracted using Trizol (Sigma). For real-time quantitative PCR, first-strand cDNA was synthesized from total RNA and PCR was performed in the presence of CYBR green (Bio-Rad). The murine ADAM10 specific primers were: forward: TGGAACACGAGAAGCTGTGA (SEQ ID NO: 1); reverse: GGGAAACGGAAAGGATTTGT (SEQ ID NO: 2). Primer sequences for human AGER1, RAGE, and SIRT1 were: for AGER1, forward: 5'-CTGGGGCTCTTCATCTTCAG-3' (SEQ ID NO: 3), reverse : 5'-GTTGCATCTCCACAGAGGT-3' (SEQ ID NO: 4), for RAGE, forward : 5'-AGGAGCGTGCAGAACTGAAT-3'(SEQ ID NO: 5), reverse : 5'-TTGGCAAGGTGGGGTTATAC-3'(SEQ ID NO: 6), and for SIRT1, forward :5'-CGGAAACAATACCTCCACCT-3'(SEQ ID NO: 7), reverse : 5'-CACCCCAGCTCCAGTTAGAA (SEQ ID NO: 8). β -actin and GAPDH housekeeping genes were used for internal normalization. The transcript copy number of target genes was determined based on their Ct values.

[0047] *A β assay:* A β 1-42 (A β) levels in mouse brain were measured by ELISA for A β 42 (Biosource, Camarillo CA), according to the manufacturer's directions. Briefly, brain tissue extracts were loaded onto plates pre-coated with a monoclonal antibody specific for the NH₂-terminus of A β . After co-incubating with a monoclonal antibody specific for the COOH-terminus of A β , antibody binding was detected by a

horseradish peroxidase-conjugated secondary antibody and the color produced was assessed in proportion to the concentration of A β present.

[0048] *H₂O₂ assay*: Intracellular reactive oxygen species (iROS) in primary E14 brain neuronal cells, freshly isolated from each group of mice (n=6/group) or after incubation of REG E14 cells with MG-BSA or BSA (30ug/ml and 60ug/ml for 48 hrs), were measured by incubation with 2',7'-dichlorofluorescein diacetate (DCFDA) (Molecular Probes, Eugene, OR) (5 uM for 45 min), DCF was tested (exc 485-nm and em 530-nm).

[0049] *Immunohistochemistry*: Anesthetized mice were transcardially perfused with 4% paraformaldehyde (PF). The brain was removed and fixed overnight in 4% PF followed by paraffin embedding. Then 20- μ m-thick coronal sections were de-paraffinized, rehydrated, and microwave-irradiated for antigen retrieval. Hippocampal area was identified by staining with a neuron-specific nuclear protein marker (NeuN, Millipore) at 1:500). Sections were incubated with anti-glial fibrillary acidic protein (GFAP) at 1:500, and anti-AGE (4G9) at 1:50 as primary antibodies overnight at 4°C. Alexa Fluor-488 and Alexa Fluor 594 at 1:500 (Invitrogen) were used as secondary antibodies. All images were captured by a fluorescent microscope (Zeiss Axioplan 2 Imaging system) at the MSSM-Microscopy Shared Resource Facility.

[0050] *Rotarod Test*: Motor coordination, balance and motor learning were tested on the accelerating-rotarod device with automatic timers and falling sensors (Series 8, IITC Life Science, Inc., Woodland Hills, CA). The device speed accelerated from 4 rpm to 40 rpm over 2 min. The latency to fall from the rotating rod was scored automatically. Mice were given 3 trials/day with 15 min inter-trial intervals for 4 days with 1 day rest between 3rd and 4th day. The latency of falling from the rod was scored as an index of their motor co-ordination as was the improvement in the latency between each session, indicating motor learning.

[0051] *Object recognition and placement memory*: For recognition memory the mice were allowed to explore two different objects for 5 min up to 4 days (training phase) followed by a test phase on the 5th day with the replacement of one familiar object with a novel object. The exploration of the object was video-recorded and the time taken to explore each object was noted. The data was analyzed by measuring the

time spent by the animal with the familiar vs. the novel object, a *difference score* (novel object interaction – familiar object interaction) and *discrimination ratio* (novel object interaction/total interaction with both objects). Object recognition memory was reflected in *longer* interaction with the novel rather than the familiar object, with a positive *difference score* and *discrimination ratio* above 0.5. A similar protocol was followed for object placement memory only differing in the placement of a familiar object in a different location on the test day. Results are shown in Figures 1 and 2. Mice exposed to higher levels of AGEs showed statistically significant impairment of object recognition. Mice exposed to higher levels of AGEs showed statistically significant impairment of learning and memory.

[0052] *Statistical analysis:* Animal data were expressed as mean $\pm$ SEM, using Student's t-test to determine the significance between groups. One-way ANOVA with Bonferroni correction analysis was performed for comparisons among the three groups. A probability value of $p < 0.05$ was considered significant. The behavioral data were analyzed using repeated-measure analysis (ANOVA) with multiple comparison tests performed with Bonferroni's adjustment. Significance was set at $P < 0.05$.

[0053] **Bone** (vertebral bodies and intervertebral discs)

[0054] *Study design:* Eight to twelve week old female ROP Os/+mice were maintained for 6.5 months. Mice were randomized in experimental and control mice (21 mice, $n=6-8$ per group). Body weight was determined weekly; and in experimental mice (Db), diabetes was induced over a 2 week period using low dose streptozotocin injections ($50 \mu\text{g/g}$ body wt of STZ). STZ injections induced hyperglycemia, which was controlled to $<250 \text{ mg/dl}$ with lente-insulin as needed. Non-diabetic control mice (NDb) obtained vehicle solution (citrate buffer) correspondingly. Glycemic levels were monitored, and mice that maintained blood glucose levels ($> 200 \text{ mg/dl}$) for 1 month were then randomized to two groups: Diabetic (Db) mice which were maintained without insulin treatment for another 5 months, and treated mice that underwent the same diabetes induction protocol but were given 5 months of treatments including: Pyridoxamine (PYR), and Pentosan Polysulphate (PPS). All diabetic mice received enalapril.

[0055] *Spine harvest and μ Ct* Following sacrifice, lumbar spines were dissected and fixed in z-fix. Before μ Ct, spines were washed in PBS. Trabecular bone of vertebrae and end plate as well as cortical bone analyses were performed using a Pre-Clinical Specimen Micro-computed Tomography system eXplore Locus SP. (GE Healthcare, London, Ontario, Canada). Lumbar vertebrae (L5) were used to measure bone structure as described below. Three-dimensional images of the entire lumbar spine were obtained with a short scan at an 8.7 micron voxel size (acquisition parameters: 400 views at 0.5 degree increment (9 pictures/view) 80kVp, 80uA, 3 second exposure). Cross-sections were analyzed for the amount of tissue (trabecular (Tb.) bone: Tissue Mineral Density, TMD) Tb. number (Tb.N.) Tb. Spaces (Tb.Sp.) and Bone volume fraction (BVF); cortical bone: marrow, cortical and total area Bone mineral density (BMD) and bone mineral content (BMC); total bone volume). Figure 4 depicts cross sections of L4-L5 vertebrae showing (a) control, (b) low ER α and (c) low ER α treated with pyridoxamine and pentosan polysulfate. Reversion to a state similar to control can be seen in the treated mice. Intervertebral disc and lumbar vertebrae (L4-L5) height measurements were calculated by specifying contour coordinates in Microview ABA 2.2 (GE healthcare) and calculating the difference between the coordinates via MATLAB 2010 (Mathworks). Contours were selected by manually approximating the contour outline with the polygon advanced ROI tool, then the "Shrink Wrap" tool was used to generate a finer outline of the contour with a resolution of 10 nodes/10 pixels. Vertebrae heights were automatically calculated by subtracting the y coordinates of nodes which are aligned on the x coordinate. Disc height index (DHI) and disc wedge index (DWI) were calculated based on sagittal IVD and vertebrae height measurements. The DHI reflects the disc height relative to the vertebrae ($DHI = 2 \times IVD \text{ height} / (L4 + L5)$), while the DWI correlates to the IVD shape. ($DWI = \text{disc height anterior} / \text{disc height posterior}$). A DWI value above 1 indicates that the anterior side of the disc is thicker than the posterior side, corresponding to a relative loss of the posterior disc height (i.e., hyperlordosis). Figure 3 shows three sections of vertebral discs. The section labeled Tr (treated) illustrates the effect of pentosan polysulfate on preventing disc degeneration.

[0056] *Histomorphometric grading scheme:* Sections of plastic embedded IVDs were used to score the degree of degeneration based on histological appearances of the NP. Safranin-O/fast green sections were graded within 8 parameters for signs of degeneration, based on a modified scoring system from Gries *et al.* and Sive *et al* 2002. Scores within 3 categories were added together for a final score out of 8. The final scores were classified in 3 groups: no to minimal degeneration (score 0-2), moderate degeneration (score 3-5), severe degeneration (score 6-7). Three specimens per group were analyzed for the scoring system.

[0057] *Histology and immunohistochemistry:* IVD-vertebrae segments of L3-4 and L4-5 were decalcified and embedded either in plastic (n=2-3 per group) or paraffin (n=3-4 per group); Sections of plastic and Paraffin sections were used for Histology (Safranin-O/fast green). Immunohistochemistry for CML, MG, TNF α , ADAMTS-5 and MMP13 was performed on plastic embedded IVD-vertebrae sections.

[0058] *GAG content quantification:* GAG intensity was measured on histology slides of plastic and paraffin embedded IVD slides. Regions of interest were defined manually (ImageJ; <http://rsbweb.nih.gov/ij/>) to include centrally located areas within the morphologically distinct AF and endplate regions. The staining intensity was normalized to the background on the same slide and GAG quantification was performed using a custom written Matlab code. Slides from Tr and Db mice were normalized to control mice and only sections of the same staining procedure were compared.

[0059] *Statistical analyses:* For statistical analyses unpaired t-tests with Bonferoni correction were used (GraphPad Prism5). Error Bars were displayed as $\pm$ SD. For all statistical analyzes a *p*-value < 0.025 was considered significant.

[0060] An experiment was undertaken in human subjects. Human subjects with HbA1c >6.5 and eGFR 25-80ml/min/1.73m² and albumin excretion >300mg/day were randomized in an intention-to-treat trial to SevCarb (4800mg/day) or Ca₂CO₃ (1950mg/day) for 6 months. Analysis at 3 months showed a robust increase in levels of ER α (p=0.003) and two markers of anti-oxidant and anti-AGE defenses; Nrf2 (p=0.009) and AGER1 (p=0.028) in both males and females.

Sevelamer Carbonate (n = 56)**Calcium Carbonate (n = 50)**

PBMC	Women (Age: 62.5±9.3)	Men (Age: 67.5±9.9)	Women (Age: 64.9±8.8)	Men (Age 60.1±10.7	p value*	p value†
ERα	1.89±4.16	0.41±0.99	-0.05±0.11	-0.52±0.99	0.038	0.002
Nrf2	0.45±1.08	0.13±0.36	-0.03±0.33	-0.13±0.49	0.061	0.043
AGER1	0.20±0.35	0.03±0.18	-0.11±0.55	0.07±0.23	0.040	0.603

SevCarb treatment restored ERα gene function in both women and men. This was coupled with reduced OS/infl, improved anti-oxidant defenses.

[0061] While several aspects of the present invention have been described and depicted herein, alternative aspects may be effected by those skilled in the art to accomplish the same objectives. Accordingly, it is intended by the appended claims to cover all such alternative aspects as fall within the true spirit and scope of the invention.

CLAIMS

1. A pharmaceutical composition comprising a therapeutically effective amount of at least one AGE uptake inhibitor and at least one AGE formation inhibitor.
2. A pharmaceutical composition according to claim 1, wherein said AGE uptake inhibitor is selected from allylamine/epichlorohydrin copolymers, ethyleneglycol dimethacrylate/ N-methylpyridylethylene hydrochloride copolymers, 2-hydroxyethyl methacrylate-glycidyl methacrylate copolymers, glycosideethyl methacrylate-glycidyl methacrylate copolymers, ethylene glycol dimethacrylate cross-linking 3-amino-2-hydroxypropylmethacrylate hydrochloride copolymers, ethylene glycol dimethacrylate cross-linking triethylenetetramine-substituted glycidyl methacrylate copolymers, flavanoids, and pentosan polysulfate; and wherein said AGE formation inhibitor is selected from aminoguanidine, ALT-462, ALT-468, a vitamin B₆ vitamer, tenilsitam, OPB-9195, benfotiamine, thiamine pyrophosphate, diaminophenazine, lipoic acid, penicillamine, carnosine, TM-2002, edaravone, LR-90, LR-9, LR-20, LR-59, LR-74, ALT-711, losartan, olmesartan, R-147176, triethylenetetramine, ethylene diamine tetraacetic acid and hydralazine.
3. A pharmaceutical composition according to claim 1, wherein said AGE formation inhibitor is a vitamin B₆ vitamer.
4. A pharmaceutical composition according to claim 1, comprising a therapeutically effective combination of sevelamer and pyridoxamine.
5. A pharmaceutical composition according to any one of claims 1-4, further comprising a pharmaceutically acceptable excipient, diluent, or carrier.
6. A pharmaceutical composition according to claim 5, wherein said pharmaceutically acceptable excipient, diluent, or carrier is a pharmaceutically acceptable excipient, diluent, or carrier for oral administration.
7. Use of a pharmaceutical composition according to any of claims 1 to 6 for treating a disease or condition chosen from:
 - (a) osteoporosis,
 - (b) peri-menopause,

- (c) degenerative disc disease,
- (d) decreased cerebral function, and
- (e) age-related skin changes,

8. Use of an agent that decreases advanced glycation endproduct levels to increase estrogen receptor α expression and/or action.

9. Use, according to claim 8, of at least one AGE uptake inhibitor and at least one AGE formation inhibitor to increase estrogen receptor α expression.

10. Use of at least one AGE uptake inhibitor and at least one AGE formation inhibitor to treat a disease or condition chosen from:

- (a) osteoporosis,
- (b) peri-menopause or menopause,
- (c) degenerative disc disease,
- (d) decreased cerebral function, and
- (e) age-related skin changes.

11. Use according to claim 10, wherein said AGE uptake inhibitor is chosen from allylamine/epichlorohydrin copolymers, ethyleneglycol dimethacrylate/ N-methylpyridylethylene hydrochloride copolymers, 2-hydroxyethyl methacrylate-glycidyl methacrylate copolymers, glycosideethyl methacrylate-glycidyl methacrylate copolymers, ethylene glycol dimethacrylate cross-linking 3-amino-2-hydroxypropylmethacrylate hydrochloride copolymers, ethylene glycol dimethacrylate cross-linking triethylenetetramine-substituted glycidyl methacrylate copolymers, flavanoids, and pentosan polysulfate.

12. Use according to claim 10, wherein said AGE formation inhibitor is chosen from aminoguanidine, ALT-462, ALT-468, a vitamin B₆ vitamer, tenilsitam, OPB-9195, benfotiamine, thiamine pyrophosphate, diaminophenazine, lipoic acid, penicillamine, carnosine, TM-2002, edaravone, LR-90, LR-9, LR-20, LR-59, LR-74, ALT-711, losartan, olmesartan, R-147176, triethylenetetramine, ethylene diamine tetraacetic acid and hydralazine.

13. Use according to claim 11, wherein said AGE uptake inhibitor is selected from sevelamer carbonate, sevelamer hydrochloride, and colesevelam.
14. Use according to claim 13, wherein said AGE uptake inhibitor is sevelamer carbonate.
15. Use according to claim 12, wherein said AGE formation inhibitor is a vitamin B₆ vitamer.
16. Use according to claim 15, wherein said AGE uptake inhibitor is sevelamer carbonate and said vitamin B₆ vitamer is pyridoxamine.
17. A method of increasing estrogen receptor α expression and/or action in a patient comprising administering to said patient a therapeutically effective amount of at least one agent that decreases advanced glycation endproduct levels.
18. A method according to claim 17, wherein said increasing estrogen receptor α expression is accomplished by administering a therapeutically effective amount of at least one AGE uptake inhibitor and at least one AGE formation inhibitor.
19. A method of treating a disease or condition chosen from:
 - (a) osteoporosis,
 - (b) peri-menopause or menopause,
 - (c) degenerative disc disease,
 - (d) decreased cerebral function, and
 - (e) age-related skin changes,comprising administering a therapeutically effective amount of at least one AGE uptake inhibitor and at least one AGE formation inhibitor.
20. A method according to claim 19, wherein said AGE uptake inhibitor is chosen from allylamine/epichlorohydrin copolymers, ethyleneglycol dimethacrylate/ N-methylpyridylethylene hydrochloride copolymers, 2-hydroxyethyl methacrylate-glycidyl methacrylate copolymers, glycosideethyl methacrylate-glycidyl methacrylate copolymers, ethylene glycol dimethacrylate cross-linking 3-amino-2-hydroxypropylmethacrylate hydrochloride copolymers, ethylene glycol

dimethacrylate cross-linking triethylenetetramine-substituted glycidyl methacrylate copolymers, flavanoids, and pentosan polysulfate.

21. A method according to claim 19, wherein said AGE formation inhibitor is chosen from aminoguanidine, ALT-462, ALT-468, a vitamin B₆ vitamer, tenilsitam, OPB-9195, benfotiamine, thiamine pyrophosphate, diaminophenazine, lipoic acid, penicillamine, carnosine, TM-2002, edaravone, LR-90, LR-9, LR-20, LR-59, LR-74, ALT-711, losartan, olmesartan, R-147176, triethylenetetramine, ethylene diamine tetraacetic acid and hydralazine.

22. A method according to claim 20, wherein said at least one AGE uptake inhibitor is selected from sevelamer carbonate, sevelamer hydrochloride, and colesevelam.

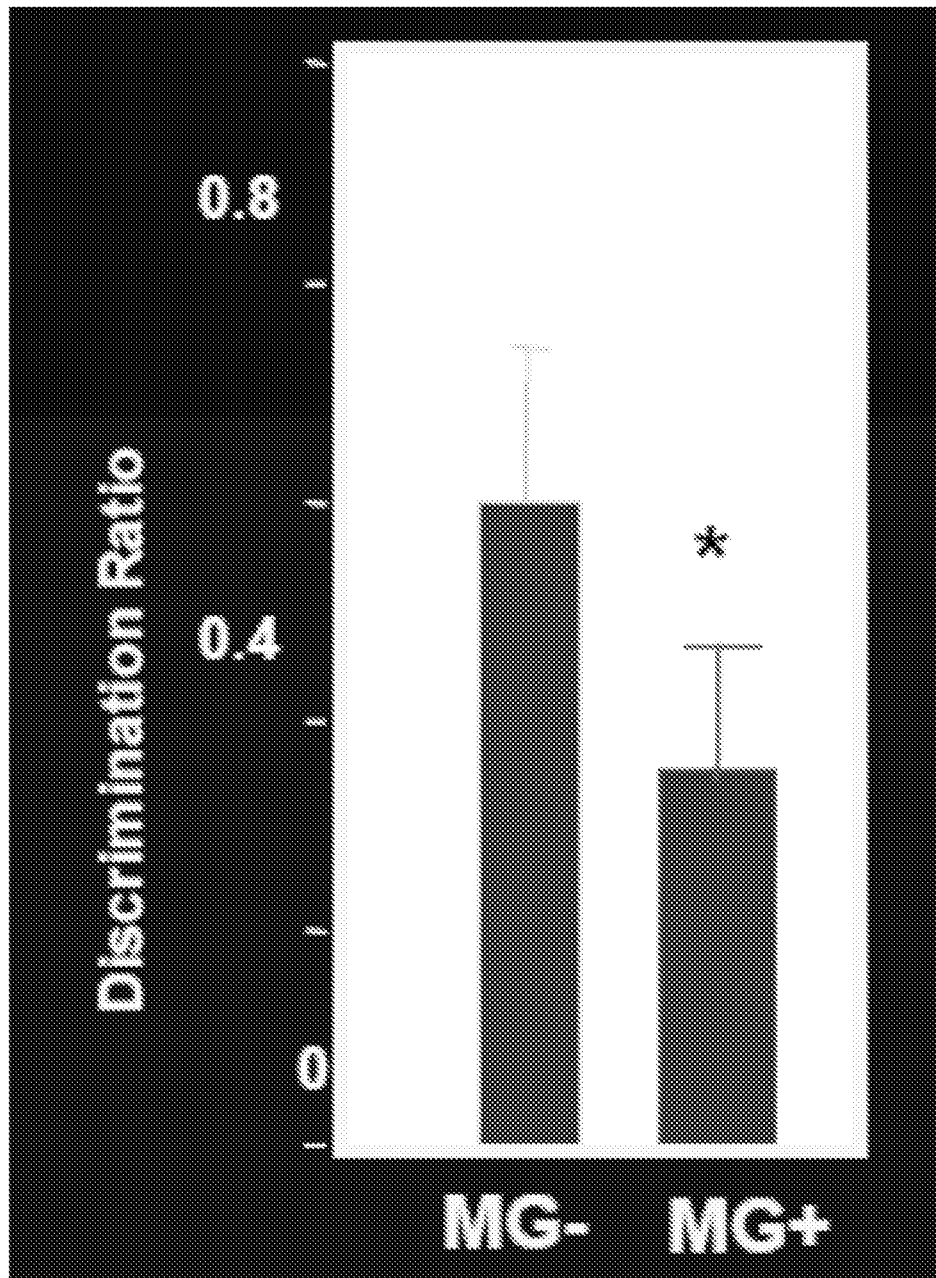
23. A method according to claim 20, wherein said at least one AGE uptake inhibitor is sevelamer carbonate.

24. A method according to claim 21, wherein said AGE formation inhibitor is a vitamin B₆ vitamer.

25. A method according to claim 18, wherein said AGE uptake inhibitor is sevelamer carbonate and said vitamin B₆ vitamer is pyridoxamine.

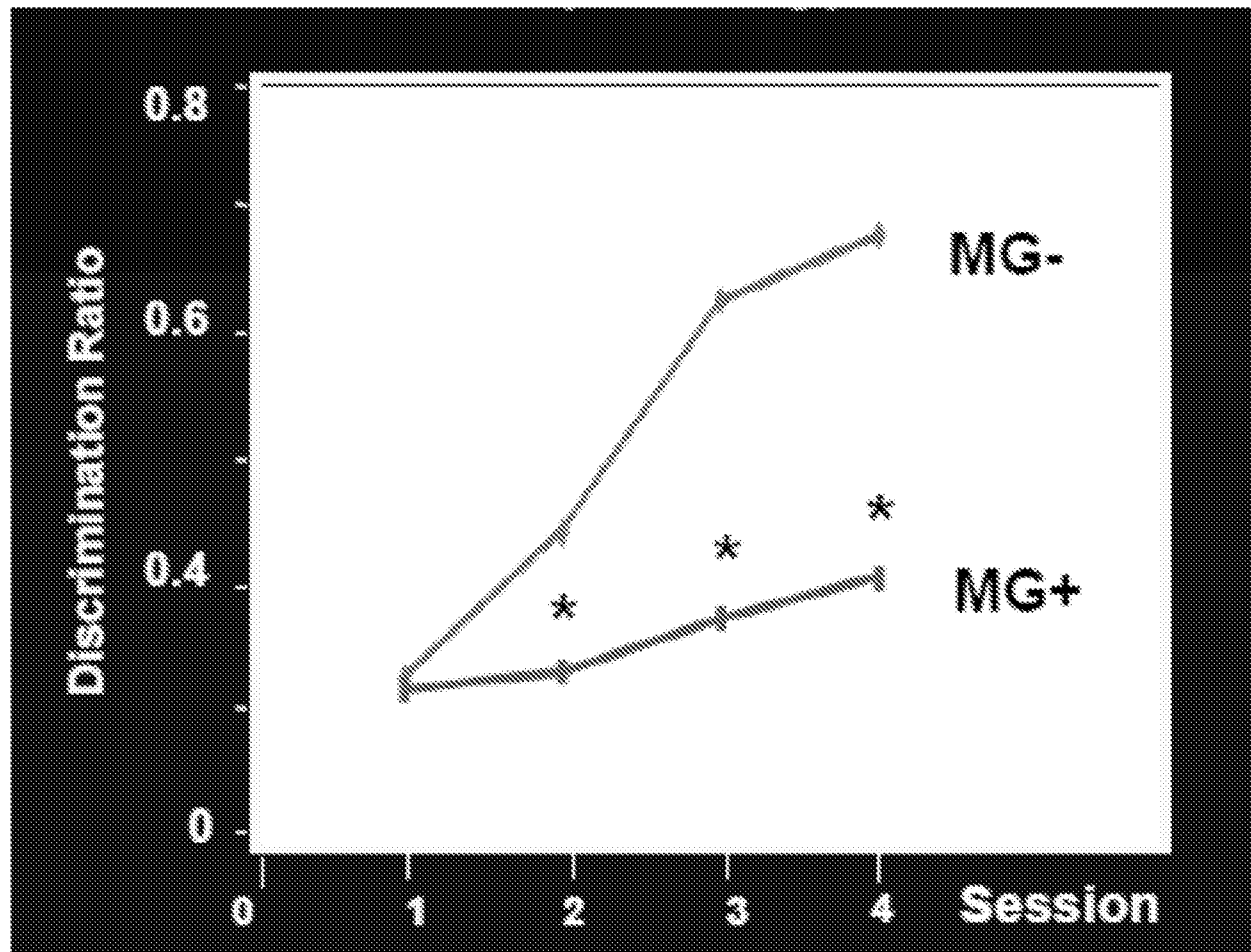
1/6

Object Recognition

**FIGURE 1**

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Memory / Learning
(Latency)

**FIGURE 2**

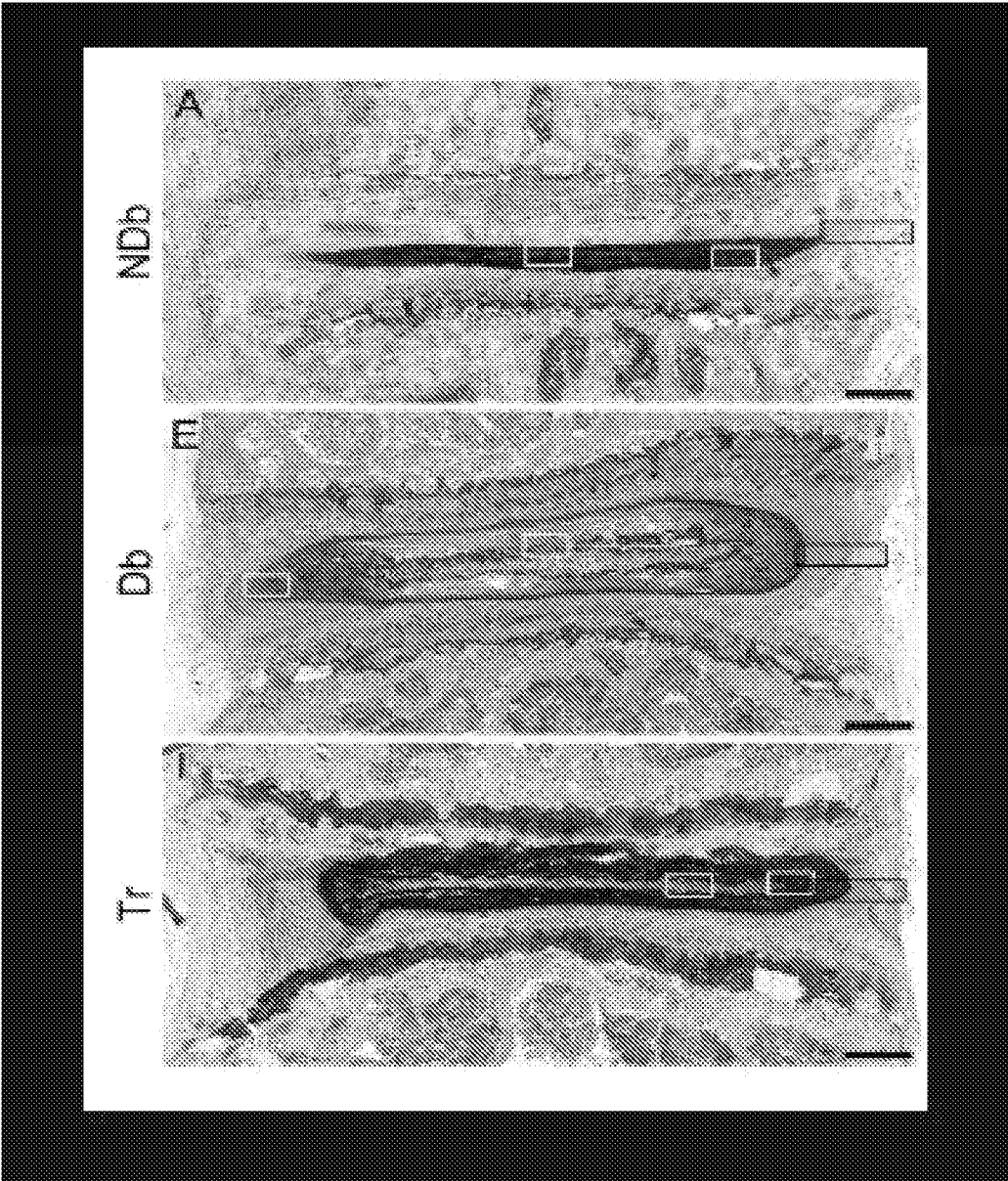


FIGURE 3

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Control

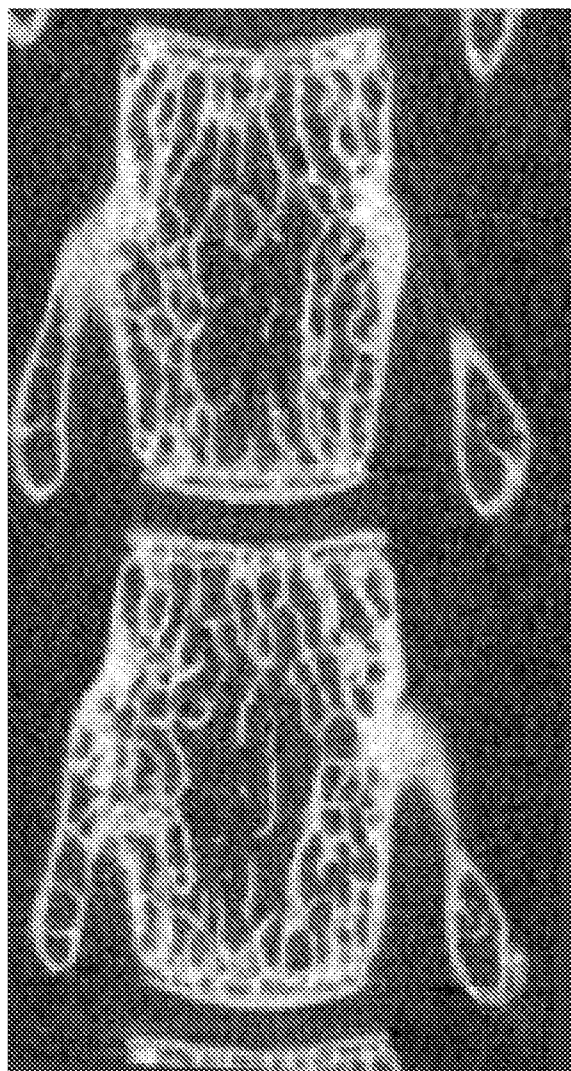


FIGURE 4A

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Low ER α

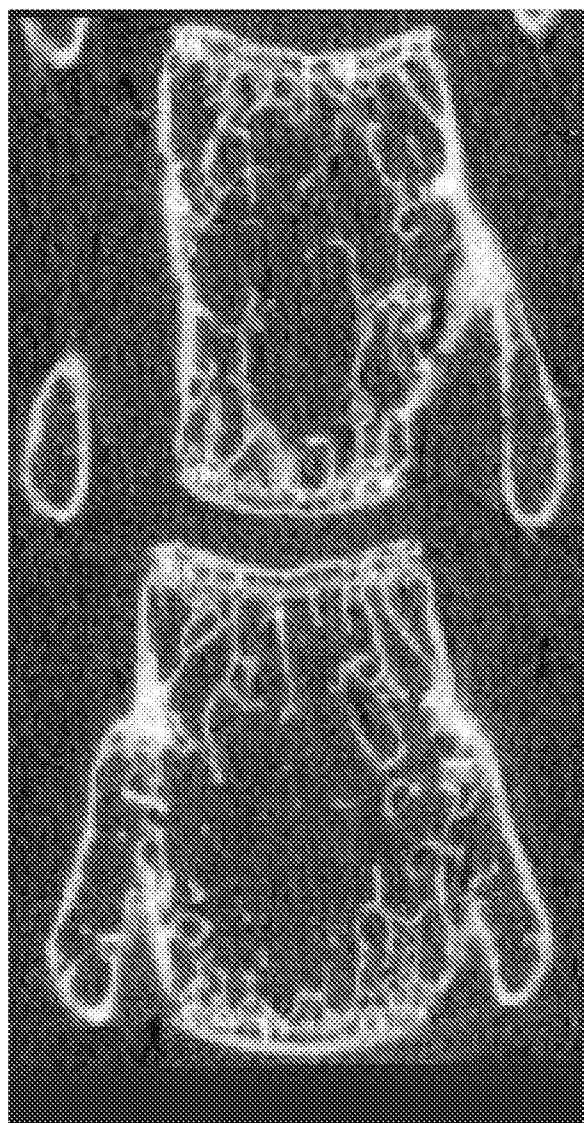


FIGURE 4B

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Treated with PY and PPS

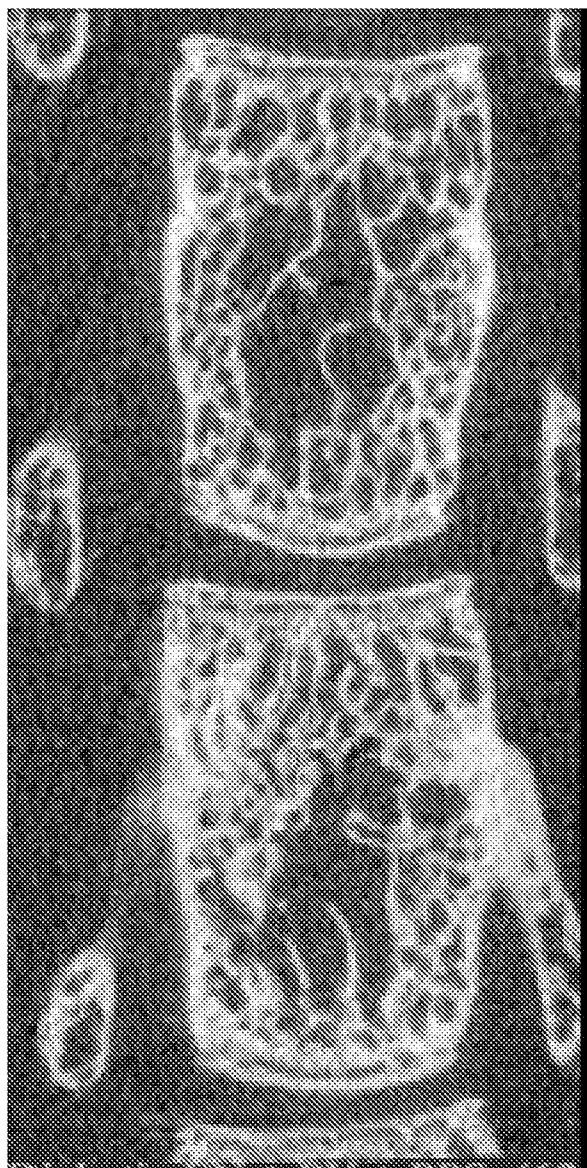


FIGURE 4C

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 14/38427

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 38/22, A61K 38/00 (2014.01)

CPC - A61K 38/00, A61K 38/09

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 (8): A61K 38/22, A61K 38/00 (2014.01)

CPC: A61K 38/00, A61K 38/09

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

IPC(8): A61K 31/714, A61P 5/30 (2014.01)

CPC: A61K 31/714, A61K 47/48215

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

Google patents, Google scholar, Google web, PatBase, Proquest Dialog

osteoporosis/peri-menopause/menopause/degenerative disc disease/decreased cerebral function/age-related skin changes; age related; oestrogen/estrogen; function/activity/action/expression; AGE; uptake/movement/transport; formation/synthesis/biosynthesis; inhibitor

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	VLAASARA et al. Effects of sevelamer on HbA1c, inflammation, and Advanced glycation end products in diabetic kidney disease Clin J Am Soc Nephrol June 2012. Vol. 7. pp 934-942 especially, page 935; Col. 1, para 2; page 939, Col. 2, para 1; page 939, Col. 2, para 2; page 940, Col. 1, para 1	1-6, 10-16, 19-24
Y	US 2003/0013746 A1 (HUDSON et al.) 16 January 2003 (16.01.2003) para [0035]; para [0020]; claim 10; para [0336]	1-6, 10-16, 19-24
Y	US 2008/0057025 A1 (BURKE) 6 March 2008 (06.03.2008) para [0047]; Abstract; para [0053]	10-16, 19-24
Y	WO 2008/090201 A1 (GORNE) 31 July 2008 (31.07.2008) Citations are from the machine translated version; page 3, para 2	10-16, 19-24
A	GARCIA DE ARRIBA et al. Advanced glycation endproducts induce changes in glucose consumption, lactate production, and ATP Levels in SH-SY5Y neuroblastoma cells by a redox-sensitive mechanism. Journal of Cerebral Blood Flow & Metabolism. 2003. Vol. 23. pp1307-1313 especially, page 1309, Col. 1, para 2; page 1309, Col. 2, para 2	8-9, 17-18, 25

☒ Further documents are listed in the continuation of Box C.

* 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

1 September 2014 (01.09.2014)

Date of mailing of the international search report

07 OCT 2014

Name and mailing address of the ISA/US

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Facsimile No. 571-273-3201

Authorized officer:

Lee W. Young

PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 14/38427

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MUKHERJEE et al. Differential effect of estrogen receptor alpha and beta agonists on the receptor for advanced glycation end product expression in human microvascular endothelial cells. <i>Biochimica et Biophysica Acta</i> . 2005. Vol.1745. pp 300-309 especially, page 307, Col.1, para 2; page 307, Col. 2, para 1	8-9, 17-18, 25
A	MUKHOPADHYAY et al. Bridging advanced glycation end product, receptor for advanced glycation end product and nitric oxide with hormonal replacement/estrogen therapy in healthy versus diabetic postmenopausal women: A perspective. <i>Biophysica Acta</i> . 2005. Vol.1745. pp 145-155 especially, page 148, Col. 2, para 2	8-9, 17-18, 25
A	TANAKA et al. The receptor for Advanced glycation end products is induced by the glycation products themselves and Tumor necrosis factor- α through Nuclear factor- κ B, and by 17 beta-estradiol through Sp-1 in human vascular endothelial cells. <i>The Journal of Biological Chemistry</i> . 18 August 2000. Vol. 275. pp 25781-25790. especially, page 25788, Col. 2, para 3; page 25789, Col. 2, para 1	8-9, 17-18, 25
A	XU et al. Calycosin protects HUVECs from advanced glycation end products-induced macrophage infiltration. <i>Journal of Ethnopharmacology</i> . 2011. Vol. 137. pp 359- 370. especially, page 359, Col. 2, para 3; page 360, Col. 1, para 1; page 365, Col. 1, para 1	8-9, 17-18, 25

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 14/38427

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

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

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

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

3. ☒ Claims Nos.: 7
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying 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 claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.