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DESCRIPTION

FIELD OF INVENTION

[0001] The present invention relates to the treatment of sensorineural hearing loss.

BACKGROUND OF INVENTION

[0002] Approximately 360 million people worldwide suffer from disabling hearing loss. The percentage of adults with disabling hearing loss increases from about 2% of the population from 45 to 54 years old, to 8.5% ages 55 to 64, and about 25% of those aged 65 to 74 and 50% in subjects of more than 75 years old. Therefore, there is an important need of methods for treating or preventing hearing loss.

[0003] Sensorineural hearing loss is caused by damage to the sensory hair cells and neurons of the cochlea and is the most common type of permanent hearing loss. Among adults, the two main causes of sensorineural hearing loss are excessive noise exposure and aging, while other causes include illnesses (including high blood pressure and diabetes), ototoxic drugs, head trauma, tumors, blast exposure, autoimmune inner ear disease, idiopathic causes, viral and bacterial infections.

[0004] Patients suffering from hearing loss have difficulties communicating and frequently develop tinnitus, resulting in reduced quality of life due to feelings of loneliness, isolation, frustration, anxiety and depression. Hearing loss furthermore has a severe impact on language development in children, education and employment opportunities. A further complication of sensorineural hearing loss includes accelerated cognitive decline (Lin et al., 2013. JAMA Intern Med. 173(4):293-299).

[0005] Currently no approved pharmaceutical treatment exists and recent meta-analyses of the standard-of-care, off-label use of corticosteroid therapy have concluded that neither systemic nor intratympanic administration has any significant treatment effect (Crane et al., 2015. Laryngoscope. 125(1):209-17).

[0006] Inhibition of the protein phosphatase calcineurin (which mediates neurodegenerative signaling through inflammation, structural degeneration, oxidative stress and programmed cell death pathways after cellular stress and insult) by the immunosuppressive drugs cyclosporine A and tacrolimus has been demonstrated to reduce hearing loss in pre-clinical models of noise-induced hearing loss (Uemaetomari et al., 2005. Hearing Res. 209(1-2):86-90; Bas et al., 2009. Acta Otolaryngol. 129(4):385-9) and 2 cases of idiopathic sudden sensorineural hearing loss and autoimmune sudden sensorineural hearing loss (McClelland et al., 2005. Acta Otolaryngol. 125(12): 1356-60; Di Leo et al., 2011. Acta Otorhinolaryngol Ital. 31(6):399-401).

[0007] However, the use of the approved calcineurin inhibitors (CNIs) cyclosporin A and tacrolimus in transplant medicine is not only immunosuppressive, but also associated with severe adverse effects such as hyperlipidemia, diabetes mellitus, hypertension, nephrotoxicity and neurotoxicity as well as unpredictable individual pharmacokinetics and pharmacodynamics (Naesens & Sarwal, 2010. Transplantation. 89(11): 1308-9). While the combination of poor dose-level correlation, unpredictable level-effect association and unclear level-toxicity relation (Naesens et al., 2009. Clin J Am Soc Nephrol. 4(2):481-508) with severe adverse effects resulting in a narrow and individual therapeutic window (necessitating intensive monitoring) may be acceptable in life-threatening conditions such as post-transplant anti-rejection therapy, these characteristics do not render traditional CNIs acceptable drug candidates for the treatment of sensorineural hearing loss.

[0008] The inventors surprisingly showed that certain setron family members capable of inhibiting the protein phosphatase calcineurin effectively treated hearing loss in a pre-clinical animal model when administered after acoustic trauma. This treatment effect lead to significant better hearing outcomes than in placebo treated animals and even performed better than a traditional CNI, despite a much lower potency in terms of calcineurin inhibition. The treatment effect was not shared by setron compounds without calcineurin inhibition capacity.

[0009] The present invention is therefore directed to azasetron, or a pharmaceutically acceptable salt thereof, as a safe and effective therapeutic agent for use in the treatment of sensorineural hearing loss.

SUMMARY

[0010] The present invention thus relates to an inhibitor of calcineurin of the setron family for use in the treatment of sensorineural hearing loss in a subject in need thereof, wherein sensorineural hearing loss is idiopathic sensorineural hearing loss, noise-induced sensorineural hearing loss, age-related sensorineural hearing loss or ototoxic drug-induced sensorineural hearing loss, and wherein said inhibitor of calcineurin is azasetron or a pharmaceutically acceptable salt thereof.

[0011] In one embodiment, said sensorineural hearing loss is a decrease in hearing of at least 20 dB over at least three contiguous frequencies. In another embodiment, said sensorineural hearing loss is total deafness.

[0012] In one embodiment, said sensorineural hearing loss is unilateral or bilateral.

[0013] In one embodiment, said sensorineural hearing loss is sudden sensorineural hearing loss.

[0014] In one embodiment, said sensorineural hearing loss is noise-induced sensorineural

hearing loss.

[0015] In one embodiment, said sensorineural hearing loss is ototoxic drug-induced sensorineural hearing loss, with the ototoxic drug inducing sensorineural hearing loss being selected from the group consisting of aminoglycosides, chemotherapeutic agents, loop diuretics, antimetabolites and salicylates.

[0016] In one embodiment, said subject is affected with hearing loss for less than 7 days

[0017] In one embodiment, the inhibitor of calcineurin is to be systemically administered, topically administered or intra-tympanically injected.

[0018] In one embodiment, the inhibitor of calcineurin is to be administered 1 to 3 times a day.

[0019] In one embodiment, the inhibitor of calcineurin is to be administered for at least 30 days.

DEFINITIONS

[0020] In the present disclosure, the following terms have the following meanings:

- **"Hearing loss"** refers to a hearing loss of at least 10 dB, preferably of at least 20 dB, more preferably of at least 30 dB in three consecutive frequencies on a standard audiogram. Hearing loss may be unilateral (*i.e.*, it impacts only one ear of a subject) or bilateral (*i.e.*, it impacts both ears of a subject).
- **"Sensorineural hearing loss"** refers to a type of hearing loss caused by an abnormality and/or damage to the sensory hair cells and neurons of the cochlea, or of the auditory nerve or higher aspects of central auditory perception or processing. Sensory hair cells and neurons of the cochlea or the auditory nerve may be abnormal at birth or may be damaged during the life time (see below for a list of possible causes of sensorineural hearing loss).
- **"Treatment"** or **"treating"** refer to both therapeutic treatment and prophylactic or preventative measures; wherein the object is to prevent or slow down (lessen) hearing loss. Those in need of treatment include those already with hearing loss as well as those prone to have hearing loss or those in whom hearing loss is to be prevented. A subject is successfully "treated" for hearing loss if, after receiving a therapeutic amount of a compound according to the present invention, the subject shows observable and/or measurable improved hearing, and/or improvement in quality of life issues. The above parameters for assessing successful treatment and improvement in hearing loss are readily measurable by routine procedures familiar to a physician, such as, for example, by audiometry.
- **"Therapeutically effective amount"** means level or amount of a compound that is aimed at, without causing significant negative or adverse side effects to the target, (1)

delaying or preventing the onset of hearing loss; (2) slowing down or stopping the progression, aggravation, or deterioration of hearing loss; (3) bringing about ameliorations of the symptoms of hearing loss; (4) reducing the severity or incidence of hearing loss; or (5) curing hearing loss. A therapeutically effective amount may be administered prior to the onset of hearing loss, for a prophylactic or preventive action. Alternatively or additionally, the therapeutically effective amount may be administered after initiation of hearing loss, for a therapeutic action.

- By "**pharmaceutically acceptable**" is meant that the ingredients of a pharmaceutical composition are compatible with each other and not deleterious to the subject to which it is administered.
- A "**pharmaceutically acceptable excipient**" refers to an excipient that does not produce an adverse, allergic or other untoward reaction when administered to an animal, preferably a human. It includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents. For human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by regulatory offices, such as, for example, FDA Office or EMA.
- The term "**subject**" herein refers to a mammal, preferably a human. In one embodiment, a subject may be a "patient", *i.e.*, a warm-blooded animal, more preferably a human, who/which is awaiting the receipt of, or is receiving medical care or was/is/will be the object of a medical procedure, or is monitored for the development of a disease.
- "**Alkyl**" refers to any saturated linear or branched hydrocarbon chain, with 1 to 12 carbon atoms, preferably 1 to 8 carbon atoms, and more preferably methyl, ethyl, propyl, isopropyl, butyl, n-butyl, sec-butyl, isobutyl, tert-butyl, pentyl, hexyl, heptyl or octyl.
- "**Halogen**" refers to fluorine, chlorine, bromine or iodine.
- "**Alkoxy**" refers to any -O-alkyl group, wherein alkyl is as defined above. Suitable alkoxy groups include for example methoxy, ethoxy, *n*-propoxy, isopropoxy, n-butoxy, *f*-butoxy, sec-butoxy, n-pentoxo, pentyloxy, hexyloxy, heptyloxy or octyloxy.
- "**Amino**" refers to any compound derived from ammoniac NH₃ by substitution of one or more hydrogen atoms with an organic radical. Amino preferably refers to -NH₂, -NHR and -NRR', wherein R and R' are preferably alkyl groups. Therefore "amino" includes monoalkylamino and dialkylamino groups.
- "**Acylamino**" refers to the groups -NRC(O)alkyl, -NRC(O)cycloalkyl, -NRC(O)cycloalkenyl, -NRC(O)alkenyl, -NRC(O)alkynyl, -NRC(O)aryl, -NRC(O)heteroaryl and -NRC(O)heterocyclic, wherein R is hydrogen or alkyl. Preferably, "acylamino" refers to C₂₋₅ alkanoylamino such as, for example, acetylamino, propionylamino, butyrylamino or pivaloylamino.
- "**Alkylamino**" refers to any -N-alkyl group and includes groups wherein the amino moiety is substituted by mono- or di-alkyl, wherein alkyl is as defined above. Suitable alkylamino groups include methylamino, ethylamino, propylamino, isopropylamino, butylamino, hexylamino, octylamino, dimethylamino, diethylamino, dipropylamino, diisopropylamino, dibutylamino, dihexylamino or dioctylamino.
- "**Phenylalkyl**" refers to a phenyl group comprising an alkyl moiety, preferably said phenylalkyl is a "phenyl C₁₋₄ alkyl", *i.e.*, a group comprising an alkyl moiety bearing 1 to 4

carbon atoms. Suitable phenylalkyl compounds include benzyl, 2-phenylethyl, 1-phenylethyl, 3-phenylpropyl and 4-phenylbutyl. Substituent(s) of the phenyl nucleus may be selected from the group comprising 1 to 3 halogen atoms, alkoxy groups, alkyl groups, nitro groups, amino groups, trifluoromethyl groups, carboxy groups and alkoxy carbonyl groups.

- **"Phenoxyalkyl"** refers to a phenoxy group comprising an alkyl moiety, preferably said phenoxyalkyl is a "phenoxy C₁₋₄ alkyl", *i.e.*, a group comprising an alkyl moiety bearing 1 to 4 carbon atoms. Suitable phenylalkyl compounds include phoxymethyl, 2-phenoxyethyl, 3-phenoxypropyl and 4-phenoxybutyl. Substituent(s) of the phenyl nucleus may be selected from the group comprising 1 to 3 halogen atoms, alkoxy groups, alkyl groups, nitro groups, amino groups, trifluoromethyl groups, carboxy groups and alkoxy carbonyl groups.
- **"About"** preceding a figure means plus or less 10% of the value of said figure.

DETAILED DESCRIPTION

[0021] The invention is defined by the claims. Any subject-matter falling outside the scope of the claims is provided for information purposes only.

[0022] The inventors showed (see Examples), that some, but not all, compounds of the setron family act as calcineurin inhibitors. Moreover, these calcineurin inhibitors of the setron family were surprisingly proved to present a therapeutic potential for treating sensorineural hearing loss.

[0023] The present invention relates to azasetron or a pharmaceutically acceptable salt thereof, for use in treating sensorineural hearing loss in a subject, said sensorineural hearing loss being idiopathic sensorineural hearing loss, noise-induced sensorineural hearing loss, age-related sensorineural hearing loss or ototoxic drug-induced sensorineural hearing loss.

[0024] The setron family is a family of serotonin 5-HT₃ antagonists and includes the following compounds: azasetron, ondansetron, palonosetron, tropisetron, lerisetron, alosetron, granisetron, dolasetron, bemisetron, ramosetron, itasetron, zacopride, and cilansetron. Methods for determining if a compound of this family is a calcineurin inhibitor are known in the art. An example of such a method is shown in Example 1, and corresponds to the Test A described below.

[0025] In one aspect, the present disclosure relates to compounds of the setron family capable of inhibiting calcineurin activity in the conditions of Test A.

[0026] According to Test A, phosphatase calcineurin activity is assessed in cerebellar granule neurons (CGNs) using a colorimetric assay kit based on quantification of the green complex

formed between malachite green, molybdate and free phosphate released. CGNs are detached from plates, for example by scraping, rinsed in ice-cold tris buffer solution (TBS) and counted. About 10 million cells are lysed in 1 mL of the provided lysis buffer and centrifuged at 150,000×g at 4°C for 45 min, and the supernatant may be stored at -70°C until analysis. Prior to calcineurin activity assay, free phosphate and nucleotides are removed from cellular extract by gel filtration. To ensure the complete removal of phosphate, malachite green reagent may be added, which will turn the color green in presence of phosphate. A specific calcineurin substrate is added to phosphate-free cellular extracts and after appropriate incubation period, malachite green reagent is applied. The rapid green color formation from the reaction is measured, for example on a spectrophotometer. The developed color is proportional to the calcineurin phosphatase activity of samples. Absorbance values, read at 620 nm, may thus be transformed to percentage calcineurin activity relative to K25 control treatment (100%).

[0027] In one embodiment, the calcineurin inhibitor of the setron family of the invention is capable of inhibiting calcineurin activity by at least 20%, preferably at least 30, 40, 50, 60, 70, 80% or more when used at a dose of 10 nM, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900 or 1000 nM. In one embodiment, the calcineurin inhibitor of the setron family of the invention is capable of inhibiting calcineurin activity by at least 50%, preferably at least 60, 70, 80% or more when used at a dose of 1000 nM.

[0028] According to the present disclosure, the calcineurin inhibitor of the setron family is selected from the group comprising azasetron, ondansetron, tropisetron, ramosetron, salts, and analogs thereof.

[0029] Pharmaceutically acceptable salts of setron compounds that may be used in the present invention are pharmaceutically acceptable acid addition salts, such as, for example with inorganic acids, such as hydrochloric acid, sulfuric acid or a phosphoric acid, or with suitable organic carboxylic or sulfonic acids, for example aliphatic mono- or dicarboxylic acids, such as trifluoroacetic acid, acetic acid, propionic acid, glycolic acid, succinic acid, maleic acid, fumaric acid, hydroxymaleic acid, malic acid, tartaric acid, citric acid or oxalic acid, or amino acids such as arginine or lysine, aromatic carboxylic acids, such as benzoic acid, 2-phenoxybenzoic acid, 2-acetoxybenzoic acid, salicylic acid, 4-aminosalicylic acid, aromatic-aliphatic carboxylic acids, such as mandelic acid or cinnamic acid, heteroaromatic carboxylic acids, such as nicotinic acid or isonicotinic acid, aliphatic sulfonic acids, such as methane-, ethane- or 2-hydroxyethane-sulfonic, in particular methanesulfonic acid, or aromatic sulfonic acids, for example benzene-, p-toluene- or naphthalene-2-sulfonic acid.

[0030] According to the present disclosure, the calcineurin inhibitor of the setron family is azasetron, ondansetron, or a salt thereof, or an analog thereof.

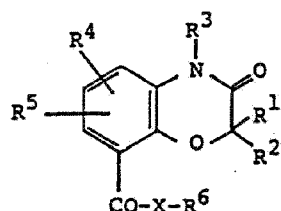
[0031] According to the invention, the calcineurin inhibitor of the setron family is azasetron or a salt thereof.

[0032] Azasetron is 6-chloro-3,4-dihydro-*N*-(8-methyl-8-azabicyclo-[3.2.1]-oct-3-yl)-2,4-

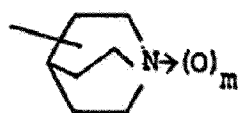
dimethyl-3-oxo-2*H*-1,4-benzoxazine-8-carboxamide, which may also be referred as *N*-(1-azabicyclo[2.2.2]octan-8-yl)-6-chloro-4-methyl-3-oxo-1,4-benzoxazine-8-carboxamide.

[0033] According to the present disclosure, an analog of azasetron is described in the US patent US4,892,872.

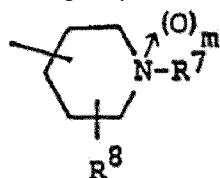
[0034] Analogs of azasetron described in US4,892,872 are benzoxazine compounds of the formula:



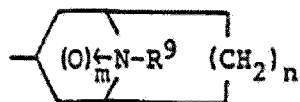
wherein R^1 and R^2 are the same or different, and each represents hydrogen or C_{1-8} alkyl; R^3 represents hydrogen, C_{1-8} alkyl, phenylalkyl or substituted phenylalkyl; R^4 and R^5 are the same or different, and each represents hydrogen, halogen, (C_1-C_8) alkyl, alkoxy, amino, acylamino, C_{2-5} alkylamino, hydroxy or nitro; X represents oxygen or NH; R^6 represents a group of the formula:



wherein m is 0 or 1,
or a group of the formula:



wherein R^7 represents C_{1-8} alkyl, phenyl C_{1-4} alkyl, phenoxyalkyl, substituted phenyl C_{1-4} alkyl or substituted phenoxyalkyl, R^8 represents hydrogen or C_{1-8} alkoxy and m is as defined above,
or a group of the formula:



wherein R^9 represents C_{1-8} alkyl, phenyl C_{1-4} alkyl or substituted phenyl C_{1-4} alkyl, n is 0 or 1, and m is as defined above,
or a pharmaceutically acceptable salt thereof.

[0035] Examples of analogs of azasetron include 6-chloro-3,4-dihydro-2-methyl-3-oxo-*N*-(3-quinuclidinyl)-2*H*-1,4-benzoxazine-8-carboxamide, 6-chloro-3,4-dihydro-2,4-dimethyl-3-oxo-*N*-(3-quinuclidinyl)-2*H*-1,4-benzoxazine-8-carboxamide, 6-chloro-2-ethyl-3,4-dihydro-4-methyl-3-oxo-*N*-(3-quinuclidinyl)-2*H*-1,4-benzoxazine-8-carboxamide, 6-chloro-3,4-dihydro-4-methyl-3-oxo-*N*-(3-quinuclidinyl)-2*H*-1,4-benzoxazine-8-carboxamide, 6-bromo-3,4-dihydro-2,4-dimethyl-3-

oxo-*N*-(3-quinuclidinyl)-2*H*-1,4-benzoxazine-8-carboxamide and 6-chloro-3,4-dihydro-2,2,4-trimethyl-3-oxo-*N*-(3-quinuclidinyl)-2*H*-1,4-benzoxazine-8-carboxamide, and pharmaceutically acceptable salts thereof.

[0036] As indicated hereinafter, according to the present invention, hearing loss is sensorineural hearing loss, said sensorineural hearing loss being idiopathic sensorineural hearing loss, noise-induced sensorineural hearing loss, age-related sensorineural hearing loss or ototoxic drug-induced sensorineural hearing loss.

[0037] In one embodiment, the present invention relates to the acute treatment of hearing loss. As used herein, the terms "acute treatment of hearing loss" refer to a treatment started as soon as possible after the onset of hearing loss, such as, at most 7 days after the onset of hearing loss. In one embodiment, the treatment starts at most 1 day, 2 days, 3 days, 4 days, 5 days, 6 days or at most 7 days after the onset of hearing loss.

[0038] In one embodiment, the term "onset of hearing loss" may refer to the exposure to the cause of hearing loss (such as, for example, exposure to loud noise for noise-induced hearing loss). In another embodiment, the term "onset of hearing loss" may refer to the diagnosis of hearing loss by a physician. In another embodiment, the term "onset of hearing loss" may refer to the perception by the subject of a loss, preferably of a significant loss, of hearing capacities, or to the perception by the subject of a loss of hearing capacities significantly impacting his/her quality of life.

[0039] Acute treatment of hearing loss is of particular interest because it allows preventing chronic hearing loss and tinnitus, as degenerative processes in the cochlea such as oxidative stress and apoptosis are not only initiated directly after an insult, but remain active for up to 4 weeks resulting in continued significant cell death for 2-3 weeks post-insult (Yang et al., 2004. *Hear Res.* 196(1-2):69-76; Yamashita et al., 2004. *Brain Res.* 1019(1-2):201-9). As significant degeneration and cell death is still ongoing after one week post-insult, treatment initiated at least up to this point has the potential to significantly improve long term outcomes.

[0040] In one embodiment, the present invention relates to the chronic treatment of hearing loss, in particular for the treatment of age-induced hearing loss or for the prevention of worsening of age-induced hearing loss. In one embodiment, the term "chronic treatment" means a treatment for an indefinite period of time, such as, for example, for 1 year, or for 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20 years or more.

[0041] In one embodiment, the term "hearing loss" refers to decrease in hearing of at least 10 dB, or 20, 30, 40, 50, 60, 70, 80, 90 dB or more over at least three contiguous frequencies. In one embodiment, "hearing loss" refers to total deafness.

[0042] In one embodiment, hearing loss is bilateral hearing loss, which means that decrease in hearing is observed in both ears of the subject. In another embodiment, hearing loss is unilateral, *i.e.* only observed in one ear, whereas the hearing capacity of the other stands

normal.

[0043] Normal hearing range usually is from 20 Hz to 20000 Hz. In one embodiment, hearing loss may affect all sound frequencies. In another embodiment, hearing loss only affects some sound frequencies, such as, for example, hearing loss in the low frequency (such as, for example, up to 500, 1000 or 2000 Hz, whereas hearing at higher frequencies is normal) or hearing loss in the high frequency (such as, for example, hearing loss beginning at 4000, 6000, 8000 or 10000 Hz).

[0044] Methods for measuring hearing loss are well-known by the skilled artisan. Examples of such methods include tuning fork test, bone conduction test, pure tone audiogram, ABR (auditory brainstem responses) measurement, DPOAE (distortion product otoacoustic emissions) measurement, TEOAE (transiently evoked otoacoustic emissions) measurement, speech in noise test, and word comprehension test.

[0045] Hearing loss include sensorineural hearing loss, conductive hearing loss and mixed hearing loss (wherein mixed hearing loss is a combination of both sensorineural and conductive hearing loss).

[0046] Conductive hearing loss results from abnormalities of the external ear, tympanic membrane, middle ear space or ossicles. It may result from cerumen impaction, middle ear fluid, otitis media, foreign bodies, perforated tympanic membrane, canal edema from otitis externa, otosclerosis, trauma or cholesteatoma (all these conditions may be diagnosed by otoscopy).

[0047] Sensorineural hearing loss and conductive hearing loss may be distinguished according to tests well known of the skilled artisan, including Weber and Rinne tests, otoscopy and audiometry.

[0048] According to the present invention, hearing loss is sensorineural hearing loss, said sensorineural hearing loss being idiopathic sensorineural hearing loss, noise-induced sensorineural hearing loss, age-related sensorineural hearing loss or ototoxic drug-induced sensorineural hearing loss.

[0049] According to the present invention, hearing loss is not conductive hearing loss, or does not include conductive hearing loss.

[0050] In one aspect of the disclosure, hearing loss is sudden hearing loss (SHL). Sudden hearing loss may be defined by the following audiometric criterion: decrease in hearing of at least 10 dB, 20 dB, 30 dB or more over at least three contiguous frequencies, evolving within 3 days or less. Because premorbid audiometry is generally unavailable, for unilateral hearing loss, hearing loss may be defined as related to the opposition ear's thresholds. In a particular embodiment, sensorineural hearing loss is sudden sensorineural hearing loss (SSNHL).

[0051] Sudden hearing loss, preferably SSNHL, may result from vestibular schwannoma (acoustic neuroma), stroke, malignancy, vascular ischemia of the inner ear, perilymph fistula or autoimmune causes (including IgE or IgG allergy) or other causes (such as, for example, causes listed below). However, a cause of SSNHL is identified in only 10 to 15% of patients at the time of presentation.

[0052] In one embodiment, SSNHL is idiopathic, which means that no cause was identified despite adequate investigation.

[0053] Examples of causes of sensorineural hearing loss include excessive noise exposure (such as, for example, exposure to a noise of more than 70 dB, 80 dB, 90 dB, 100 dB, 110 dB, 120 dB, 130 dB or more), aging, inner ear involvement by infectious agents (such as, for example, viral and bacterial infections), autoimmunity (such as, for example, autoimmune inner ear disease) or vasculopathy, illnesses (including high blood pressure and diabetes), ototoxic drugs, head trauma, tumors or blast exposure.

[0054] In one embodiment, sensorineural hearing loss is congenital, *i.e.*, sensory hair cells and neurons of the cochlea or the auditory nerve were abnormal at birth. Examples of causes of congenital hearing loss, preferably congenital sensorineural hearing loss include a lack of development (aplasia) of the cochlea, chromosomal syndromes, congenital cholesteatoma, delayed familial progressive, congenital rubella syndrome, and human Cytomegalovirus (HCMV) transmission to a developing fetus during pregnancy.

[0055] In another embodiment, sensorineural hearing loss is acquired, *i.e.*, sensory hair cells and neurons of the cochlea or the auditory nerve were normal at birth, but were subsequently damaged.

[0056] Examples of causes of acquired hearing loss, preferably acquired sensorineural hearing loss include inflammatory causes, ototoxic drugs, physical trauma (such as, for example, a head injury, a fracture of the temporal bone affecting the cochlea and middle ear, or a shearing injury affecting cranial nerve VIII), noise exposure, presbycusis, tumors or diseases.

[0057] Examples of inflammatory causes of sensorineural hearing loss include autoinflammatory diseases (such as, for example, Muckle-Wells Syndrome), suppurative labyrinthitis, meningitis, mumps or measles.

[0058] Examples of viral causes of sensorineural hearing loss include syphilis, mumps or measles.

[0059] Examples of ototoxic drugs that may cause sensorineural hearing loss include aminoglycosides (such as, for example, tobramycin), chemotherapeutic agents (such as, for example, platinum drugs, including cisplatin, carboplatin, oxaliplatin, satraplatin, picoplatin, tetraplatin, transplatin, nedaplatin, ormaplatin, PtCl₂[R,RDACH], pyriplatin, ZD0473, BBR3464

and Pt-1C3), loop diuretics (such as, for example, furosemide), antimetabolites (such as, for example, methotrexate) and salicylates (such as, for example, aspirin).

[0060] Presbycusis refers to hearing loss that typically, but not only, occurs in the high frequency range (4000 Hz to 8000 Hz), mainly in the elderly. It is considered by some to be a degenerative process, although there has never been a proven link to aging.

[0061] Examples of tumors that may induce sensorineural hearing loss include cerebellopontine angle tumor, such as, for example, acoustic neuroma and meningioma.

[0062] An example of disease leading to sensorineural hearing loss is Meniere's disease. It typically, but not only, causes sensorineural hearing loss in the low frequency range (125 Hz to 1000 Hz). According to the present invention, sensorineural hearing loss does not result from or is not associated with Meniere's disease.

[0063] Other examples of causes of sensorineural hearing loss include autoimmune diseases (such as, for example, granulomatosis with polyangiitis), adenoids that do not disappear by adolescence resulting in obstruction of the Eustachian tube, causing conductive hearing impairment and nasal infections that can spread to the middle ear, AIDS, ARC, HIV (and subsequent opportunistic infections), chlamydia, fetal alcohol syndrome, premature birth, otosclerosis, posterior circulation infarct and Charcot-Marie-Tooth disease.

[0064] In one embodiment, sensorineural hearing loss is inherited, for example, it results from a genetic predisposition of the subject. More than 40 genes have been identified to cause deafness, including DFNB1 (also known as Connexin 26 or GJB) and MT-TL1. Examples of syndromes resulting in hearing loss include Stickler syndrome, Waardenburg syndrome, Pendred syndrome, large vestibular aqueduct syndrome and Usher syndrome.

[0065] In one embodiment, sensorineural hearing loss is idiopathic, *i.e.*, without identified cause.

[0066] In one embodiment, sensorineural hearing loss is noise-induced sensorineural hearing loss. Hearing loss may for example be induced by exposure to loud noises (such as, for example, of more than 90 dB, 100 dB, 110 dB, 120 dB, 130 dB or more), or by prolonged exposure to less loud noises. Indeed, the louder the noise, the shorter the safe amount of exposure. For example, the safe daily exposure amount at 85 dB is of 8 hours, while it is of only 2 hours at 91dB.

[0067] In one embodiment, sensorineural hearing loss is age-induced sensorineural hearing loss. Therefore, in one embodiment, the subject to be treated according to the present invention is at least 30, 40, 50, 60, 70, 80 years old or more.

[0068] In one embodiment, the subject receiving the setron compound of the invention experiences hearing loss for at least 24 hours, *i.e.*, according to this embodiment, subjects with

only temporary hearing loss are excluded from the scope of the present invention.

[0069] Therefore, in one embodiment, the subject starts receiving the treatment of the invention between 24 hours and 7 days after the onset of hearing loss. In one embodiment, the subject starts receiving the treatment of the invention between 24 hours and 2, 3, 4, 5 or 6 days after the onset of hearing loss. According to this embodiment, the term "acute treatment of hearing loss", may thus refer to a treatment beginning between 24 hours and 2, 3, 4, 5, 6 or 7 days after hearing loss onset.

[0070] In one embodiment, the subject is at risk of hearing loss. Examples of risk of hearing loss include genetic predisposition to hearing loss, familial history of hearing loss, history of previous episodes of hearing loss, noise exposure, exposure to ototoxic agent (including ototoxic drugs), and aging.

[0071] In one embodiment, the compound of the invention may be administered prior to the onset of hearing loss, such as, for example, prior to the exposure to a noise or agent that may cause hearing loss. As used herein, when the compound of the invention is used for treating or preventing noise-induced hearing loss, "prior to" may refer to an administration a few hours or minutes before exposition to noise. In another embodiment, the compound of the invention may also be administered during or after the exposure to a noise or agent that may cause hearing loss, such as, for example, a few minutes or hours after.

[0072] In another embodiment, the compound of the invention may be administered to diminish ongoing age-related hearing loss. As used herein, when the compound of the invention is used for treating or preventing age-related hearing loss, "prior to" refers to administration after the risk of, or beginning of, age related hearing loss has been determined, but prior to its continued worsening.

[0073] In one embodiment, the subject is a child or an adolescent. In another embodiment, the subject is an adult, *i.e.*, a subject of at least 18 or 20 years old or more. In another embodiment, the subject is at least 30, 40, 50, 60, 70, 80 years old or more.

[0074] In one embodiment, the subject is a male. In another embodiment, the subject is a female.

[0075] In one embodiment, the subject is not affected and/or diagnosed with a vestibular disease, *i.e.*, labyrinthine, vestibular nerves or vestibuli nuclei diseases responsible of vestibular dysfunction. Examples of vestibular diseases include vertigo (such as, for example, benign paroxysmal vertigo and familial episodic vertigo), vestibular neuritis, Meniere's disease (such as, for example, chronic Meniere's disease), endolymphatic hydrops, perilymphatic fistula, vestibular disorders resulting from head trauma, labyrinthine haemorrhage, chronic or acute labyrinthine (such as, for example, of viral, immune or bacterial origin), serous labyrinthine, barotraumatism of the vestibule, autoimmune inner ear disease, vestibular migraine, migraine associated with vestibular syndromes, vestibular syndromes resulting from

chirurgical treatment of the middle ear, middle ear tumor, endolymphatic sac tumor, pontocerebellar angle tumor, cerebellopontine or temporal bone tumors involving the vestibular nerves and/or the labyrinth, brainstem tumors or lesions associated with vestibular disorders (such as, for example, multiple sclerosis, stroke or angeitis), inner ear channelopathies, vestibular schwannomas, and presbyvestibulia. In one embodiment, the subject is not affected with acute vestibular dysfunction (such as, for example, barotraumatism, Meniere's disease or migraine associated vertigo). In another embodiment, the subject is not affected with chronic vestibular dysfunction (such as, for example, sequellae from acute vestibular dysfunction or presbyvestibulia).

[0076] In another embodiment, the subject does not present any vestibular damage or vestibular lesion. Examples of lesional vestibular disorder include vestibular neuritis, viral neuronitis, labyrinthitis, viral endolymphatic labyrinthitis, drug-induced ototoxicity, Meniere's disease, endolymphatic hydrops, head trauma with lesional vestibular deficits, labyrinthine haemorrhage, chronic or acute labyrinthine infection, serous labyrinthine, barotraumatism, autoimmune inner ear disease, presbyvestibulia and toxic vestibular impairments.

[0077] In another embodiment, the subject is not affected by tinnitus.

[0078] Also disclosed herein is a composition comprising or consisting of a calcineurin inhibitor of the setron family as described hereinabove.

[0079] Also disclosed herein is a pharmaceutical composition comprising or consisting of or consisting essentially of a calcineurin inhibitor of the setron family as described hereinabove in combination with at least one pharmaceutically acceptable excipient.

[0080] Also disclosed herein is a medicament comprising or consisting of or consisting essentially of a calcineurin inhibitor of the setron family as described hereinabove.

[0081] Also disclosed herein is a nutraceutical composition comprising or consisting of or consisting essentially of an inhibitor of calcineurin of the setron family, as described hereinabove. In particular, disclosed herein is a nutraceutical composition for treating age-related hearing loss in a subject in need thereof.

[0082] As used herein, the term "consisting essentially of", with reference to a pharmaceutical composition or medicament or nutraceutical composition of the disclosure, means that the at least one inhibitor of calcineurin of the setron family is the only one therapeutic agent or agent with a biologic activity within said pharmaceutical composition or medicament or nutraceutical composition.

[0083] In the composition, pharmaceutical composition, medicament or nutraceutical composition disclosed herein, the setron compound, alone or in combination with another active principle, can be administered in a unit administration form, as a mixture with conventional pharmaceutical supports, to a subject. Suitable unit administration forms

comprise oral-route forms such as tablets, gel capsules, powders, granules and oral suspensions or solutions, sublingual and buccal administration forms, aerosols, implants, subcutaneous, transdermal, topical, intraperitoneal, intramuscular, intravenous, subdermal, transdermal, intrathecal, intra-tympanic and intranasal administration forms and rectal administration forms.

[0084] Preferably, the composition, pharmaceutical composition, medicament or nutraceutical composition contains vehicles which are pharmaceutically acceptable for a formulation capable of being injected, preferably systemically or intra-tympanically injected. These may be in particular isotonic, sterile, saline solutions (monosodium or disodium phosphate, sodium, potassium, calcium or magnesium chloride or mixtures of such salts), or dry, especially freeze-dried compositions which upon addition, depending on the case, of sterilized water or physiological saline, permit the constitution of injectable solutions. In one aspect of the disclosure, the composition, pharmaceutical composition, medicament or nutraceutical composition is in the form of a gel.

[0085] In one aspect of the disclosure, the composition, pharmaceutical composition, medicament or nutraceutical composition comprises sustained-release matrices, such as biodegradable polymers.

[0086] Examples of pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions; gel formulation; formulations including sesame oil, peanut oil or aqueous propylene glycol; and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases, the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi.

[0087] Solutions comprising a setron compound of the invention as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

[0088] The setron compound of the invention can be formulated into a composition in a neutral or salt form. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, and mandelic. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, and procaine.

[0089] The carrier can also be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol), suitable

mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, and thimerosal. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

[0090] Sterile injectable solutions may be prepared by incorporating the setron compound in the required amount in the appropriate solvent with one or several of the other ingredients enumerated in the present disclosure, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

[0091] Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms, such as the type of injectable solutions described above, but drug release capsules can also be employed.

[0092] For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage could be dissolved in 1 mL of isotonic NaCl solution and either added to 1000 mL of hypodermoclysis fluid or injected at the proposed site of infusion. Some variation in dosage will necessarily occur depending on the condition of the subject being treated.

[0093] The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. The setron compound of the invention may be formulated within a therapeutic mixture to comprise 0.01 to 1000 mg, preferably from 1 to 400 mg, more preferably from 5 to 100 mg per dose or so. Multiple doses can also be administered.

[0094] In addition to the compounds of the invention formulated for parenteral administration, such as intravenous or intramuscular injection, other pharmaceutically acceptable forms include, e.g., tablets or other solids for oral administration; liposomal formulations; time release capsules; and any other form currently used.

[0095] In one embodiment, a therapeutically effective amount of said calcineurin inhibitor of the setron family of the invention is to be administered to the subject.

[0096] In one embodiment, the therapeutically effective amount ranges from 1 to 10000 mg/mL of the setron compound of the invention, preferably 50 to 5000 mg/mL, more preferably from 200 to 2000 mg/mL of the setron compound of the invention. In one embodiment, the therapeutically effective amount ranges from 1 to 10000 mg/mL of the setron compound of the invention, preferably 2 to 2000 mg/mL, more preferably from 5 to 500 mg/mL of the setron compound of the invention.

[0097] In one embodiment, the therapeutically effective amount ranges from 1 to 10000 mg/g of the setron compound of the invention, preferably 50 to 5000 mg/g, more preferably from 200 to 2000 mg/g of the setron compound of the invention. In another embodiment, the therapeutically effective amount ranges from about 1 to 10000 mg/g of the setron compound of the invention, preferably 2 to 2000 mg/g, more preferably from 5 to 500 mg/g of the setron compound of the invention.

[0098] It will be understood that the total daily usage of the setron compound of the invention, composition, pharmaceutical composition, medicament or nutraceutical composition of the present disclosure will be decided by the attending physician within the scope of sound medical judgment. The specific therapeutically effective dose level for any particular patient will depend upon a variety of factors including the hearing loss being treated and the severity of the hearing loss; activity of the specific setron compound employed; the specific composition employed, the age, body weight, general health, sex and diet of the subject; the time of administration, route of administration, and rate of excretion of the specific setron compound employed; the duration of the treatment; drugs used in combination or coincidental with the specific polypeptide employed; and like factors well known in the medical arts. For example, it is well within the skill of the art to start doses of the compound at levels lower than those required to achieve the desired therapeutic effect and to gradually increase the dosage until the desired effect is achieved.

[0099] However, the daily dosage of the setron compound of the invention may be varied over a wide range from 0.01 to 500 mg per adult per day. In another embodiment, the daily dosage of the setron compound of the invention may be varied over a wide range from 1 to 10000 mg per adult per day, preferably from 2 to 2000 mg per adult per day, more preferably from 5 to 500 mg per adult per day. Preferably, the compositions contain 0.01, 0.05, 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0, 20.0, 25.0, 50.0, 100, 150, 200, 250, 300, 350, 400, 450, 500, 1000 or 2000 mg of the active ingredient for the symptomatic adjustment of the dosage to the patient to be treated. A medication typically contains from 0.01 mg to 500 mg of the active ingredient, preferably from 1 mg to 100 mg of the active ingredient. In one embodiment, the composition of the disclosure contains from 1 mg to 10000 mg of the active ingredient, preferably from 2 mg to 2000 mg, more preferably from 5 mg to 500 mg of the active ingredient. The composition of the disclosure may comprise the setron compound in a range of 1 mg to 100 mg, preferably from 5 mg to 20 mg, more preferably of 10 mg. The composition of the

disclosure may also comprise the setron compound in a range of 1 mg to 10000 mg, preferably from 2 mg to 2000 mg, more preferably from 5 mg to 500 mg. An effective amount of the drug is ordinarily supplied at a dosage level from 0.01 mg/kg to 2 mg/kg of body weight per day, especially from 0.05 mg/kg to 0.4 mg/kg of body weight per day, more preferably of 0.1-0.3 mg/kg of body weight per day. In one embodiment, an effective amount of the setron compound is supplied at a dosage level from 0.01 mg/kg to 100 mg/kg of body weight per day, especially from 0.02 mg/kg to 20 mg/kg of body weight per day, more preferably of 0.05 mg/kg to 5 mg/kg of body weight per day.

[0100] In one embodiment, the compound of the invention is to be topically administered, preferably is to be topically applied on the outer ear or on the ear canal. Examples of formulations adapted to topical administration include ear drops, solutions or topical gels.

[0101] In one embodiment, the compound of the invention is to be intra-tympanically injected. Examples of formulations adapted to intra-tympanic injection include solutions, such as, for example, sterile aqueous solutions, gels, dispersions, emulsions, suspensions, solid forms suitable for using to prepare solutions or suspensions upon the addition of a liquid prior to use, such as, for example, powder, and liposomal forms.

[0102] In a preferred embodiment, the setron compound of the invention is to be systemically administered, such as, for example, orally administered, intranasally administered or injected (including, for example, intraperitoneal, intravenously, subcutaneously or intramuscularly injected).

[0103] In one embodiment, the setron compound of the invention, the composition, the pharmaceutical composition, the medicament or the nutraceutical composition of the disclosure is to be orally administered. Examples of forms adapted for oral administration include tablets, orodispersing/orally disintegrating tablets, effervescent tablets, powders, granules, pills (including sugarcoated pills), dragees, capsules (including soft gelatin capsules), syrups, liquids, gels or other drinkable solutions, suspensions, slurries, and liposomal forms.

[0104] In one embodiment, the setron compound of the invention, the composition, the pharmaceutical composition, the medicament or the nutraceutical composition of the disclosure is to be injected. Therefore, according to this embodiment, the setron compound of the invention, the composition, the pharmaceutical composition, the medicament or the nutraceutical composition of the disclosure is in a form adapted for injection, such as, for example, for intramuscular, subcutaneous, intradermal, transdermal or intravenous injection or infusion. Examples of forms adapted for injection include solutions, such as, for example, sterile aqueous solutions, gels, dispersions, emulsions, suspensions, solid forms suitable for using to prepare solutions or suspensions upon the addition of a liquid prior to use, such as, for example, powder, and liposomal forms.

[0105] In one aspect of the disclosure, the nutraceutical composition disclosed herein is to be orally administered to the subject. Examples of forms adapted for oral administration include

tablets, orodispersing/orally disintegrating tablets, effervescent tablets, powders, granules, pills (including sugarcoated pills), dragees, capsules (including soft gelatin capsules), syrups, liquids, gels or other drinkable solutions, suspensions, slurries, and liposomal forms. In one aspect of the disclosure, the nutraceutical composition disclosed herein is in the form of a food additive, drink additive, dietary supplement or nutritional product.

[0106] In one embodiment, the setron compound of the invention is for administration three times a day, twice a day, once a day, once every 2 days, once every 3 days, twice a week, once a week or less, preferably, the setron compound of the invention is for administration one to three times a day.

[0107] In one embodiment, the setron compound is for administration for a period of time of about 1 week, or of 2, 3, 4, 5, 6, 7, 8 weeks or more, or of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 months or more. In one embodiment, the setron compound is for administration until normal hearing, or until the subject recovered 50%, 60, 70, 80, 90% or more of his hearing capacity. Recovering of the hearing capacity may be measured by methods well known by the skilled artisan, including tuning fork test, bone conduction test, pure tone audiogram, ABR (auditory brainstem responses) measurement, DPOAE (distortion product otoacoustic emissions) measurement, TEOAE (transiently evoked otoacoustic emissions) measurement, speech in noise test, and word comprehension test.

[0108] In one embodiment, the setron compound of the invention is the only one therapeutic compound to be administered to the subject for treating hearing loss.

[0109] In another embodiment, the setron compound of the invention may be administered in combination with another therapeutic compound for treating hearing loss, including sodium thiosulfate, vasodilators, magnesium, acetyl-L-carnitine (ALCAR), n-AcetylCysteine (NAC), ebselen, D-methionine, anti-oxidants and ROS scavengers, ginkgo biloba, glutamate receptor antagonists, JNK kinase inhibitors, alpha lipoic acid, prostaglandin agonists, steroids, vitamin E, A and/or C. In one embodiment, the setron compound of the invention and the additional therapeutic compound are to be administered simultaneously or sequentially.

[0110] Also disclosed herein is a calcineurin inhibitor of the setron family for use in improving hearing in a subject in need thereof. Preferably, a therapeutically effective amount of the calcineurin inhibitor of the setron family is to be administered to the subject.

[0111] According to this disclosure, the subject is affected by, preferably is diagnosed with, hearing loss, preferably sensorineural hearing loss, more preferably noise-induced sensorineural hearing loss or age-related sensorineural hearing loss.

[0112] According to this disclosure, the improvement of hearing capacities may correspond to a decreased ABR threshold and/or to an increased DPOAE amplitude.

[0113] Also disclosed herein is a calcineurin inhibitor of the setron family for use in reducing

loss of outer hair cells in the cochlea in a subject in need thereof. Preferably, a therapeutically effective amount of the calcineurin inhibitor of the setron family is to be administered to the subject.

[0114] According to this disclosure, the loss of outer hair cells may be induced by an acoustic trauma, such as, for example, loud noise exposure, aging, illnesses (including high blood pressure and diabetes), ototoxic drugs, head trauma, tumors, blast exposure, autoimmune inner ear disease, idiopathic causes, viral or bacterial infections.

BRIEF DESCRIPTION OF THE DRAWINGS

[0115]

Figure 1 is a set of histograms showing the effect of setron compound application on calcineurin phosphatase activity after 4 DIV. (A) Ondansetron, (B) Granisetron, (C) Tropisetron, (D) Ramosetron and (E) Azasetron. * means $p \leq 0.05$.

Figure 2 is a set of histograms showing the auditory brainstem response (ABR) thresholds recorded for the 6 treatment groups at baseline (A), 24 hours after acoustic trauma induction (B) and 14 days after acoustic trauma (C).

Figure 3 is a set of histograms showing the distortion product otoacoustic emissions (DPOAEs) amplitudes determined for the 6 treatment groups at baseline (A), 24 hours after acoustic trauma induction (B) and 14 days after acoustic trauma (C).

Figure 4 is a histogram showing the ABR threshold shift from baseline to D14 for all treatment groups.

Figure 5 is a histogram showing the ABR threshold recovery from 24 hours after acoustic trauma to D14 for all treatment groups.

Figure 6 is a histogram showing DPOAE amplitude loss from baseline to D14 for all treatment groups.

Figure 7 is a histogram showing DPOAE amplitude recovery from 24 hours after acoustic trauma to D14 for all treatment groups.

Figure 8 is a graph showing mean outer hair cell loss as a function normalized distance from apex for groups treated with placebo, granisetron, tacrolimus or azasetron in cochleae fixed at D14 after acoustic trauma.

EXAMPLES

[0116] The present invention is further illustrated by the following examples.

Example 1: *In vitro* inhibition of calcineurin activity by setron compounds

Cell culture

[0117] Cerebellar granule cells were dissociated from the cerebellum of 7-day-old Wistar rat pups as described (Kramer et al., 2003. Mol Cell Neurosci. 23(2):325-30). Briefly, the cerebella were removed, rinsed in HBSS-BSA, minced, digested with 0.025% trypsin and incubated at 37°C for 15 minutes. To stop the digestion, DMEM containing 10% fetal calf serum was added; then a single cell suspension was obtained through pipetting up and down the sedimented tissue. Following centrifugation, cells were counted using trypan blue exclusion test in a Burkner chamber. This method is based on the ability of viable cells to exclude trypan blue due to their intact cell membranes, leaving them unstained while nonviable cells take up the dye. Cells at $3.2 \times 10^5/\text{cm}^2$ were seeded in DMEM Hepes modification supplemented with 10% fetal calf serum, 100 µg/mL pyruvate, and 100 µg/mL gentamicin on Polystyrene 12-well tissue culture plates (CELLSTAR) coated with poly-L-lysine. After 24 hours, 10 µM cytosine arabinofuranoside was added to inhibit the growth of non-neuronal cells. All pharmacological interventions begun at this time in the culture medium containing 25 mM KCl (K25), which caused depolarization of granule cells and consequently activated gene expression machinery. Moreover, the viability of granule neurons was higher in this milieu and therefore provides the possibility to maintain cells for several days. Indeed, 25 mM KCl is required in the medium for CGN cultures to maintain adequate calcineurin activity and cell maturation and depolarization (Kramer et al., 2003. Mol Cell Neurosci. 23(2):325-30; Vallano et al., 2006. Neuropharmacology. 50(6):651-60).

[0118] Cultures received simple medium (K25 control) or medium containing a setron compound in HCl salt form (Tocris Bioscience, Bristol, UK) at concentrations of 10 nM, 100 nM or 1000 nM for 4 days *in vitro* (DIV).

Calcineurin activity assay

[0119] Phosphatase calcineurin activity was assessed in CGNs using a colorimetric assay kit based on quantification of the green complex formed between malachite green, molybdate and free phosphate released. CGNs were detached from plates by scraping, rinsed in ice-cold tris buffer solution (TBS) and counted. About 10 million cells are lysed in 1 mL of the provided lysis buffer and centrifuged at 150,000×g at 4°C for 45 minutes, and the supernatant was stored at -70°C until analysis. Prior to calcineurin activity assay, free phosphate and nucleotides were removed from cellular extract by gel filtration. To ensure the complete removal of phosphate,

malachite green reagent was added, which will turn the color green in presence of phosphate. A specific calcineurin substrate was added to phosphate-free cellular extracts and after appropriate incubation period, malachite green reagent was applied. The rapid green color formation from the reaction was measured on a spectrophotometer. The developed color is proportional to the calcineurin phosphatase activity of samples. Absorbance values, read at 620 nm, were transformed to percentage calcineurin activity relative to K25 control treatment (100%). For every single treatment, five independent cultures are assessed and each sample run in triplicate.

Statistics

[0120] Statistical analysis was performed using One-way ANOVA followed by Tukey post-hoc test for multiple comparisons. Results are given as mean $\pm$ SD and a p value less than or equal to 0.05 considered significant.

Results

[0121] As shown in **Figure 1**, ondansetron HCl, tropisetron HCl, ramosetron HCl and azasetron HCl all significantly inhibited calcineurin phosphatase activity, while granisetron HCl had no effect. For ondansetron HCl and tropisetron HCl, the calcineurin inhibition was significant from 10-1000 nM, for ramosetron HCl at 100 and 1000 nM while the calcineurin inhibitory effect of azasetron HCl only reached significance at 1000 nM due to increased variability of both control and test conditions for this particular assay run. Calcineurin inhibition increased to different degree with increasing concentrations of tropisetron HCl, ramosetron HCl and azasetron HCl while it conversely decreased with increasing concentration of ondansetron HCl. This demonstrates that calcineurin inhibition by setron family compounds is compound specific and not a class effect.

Example 2: *In vivo* treatment of acoustic trauma induced hearing loss by setron compounds

Animals

[0122] All experiments were performed using 7 week old male Wistar rats (CERJ, Le Genest, France) in accordance with the French Ministry of Agriculture regulations and European Community Council Directive no. 86/609/EEC, OJL 358. The rats were fed a standard diet ad libitum and maintained on a 12-hour light-dark cycle.

Audiometry

[0123] Auditory Brainstem Responses (ABR) and Distortion Product Otoacoustic Emissions (DPOAE) were recorded using a RZ6 Auditory Workstation (Tucker-Davis Technologies, Alachua, FL, USA) with animals deeply anesthetized using 90 mg/kg ketamine and 10 mg/kg xylazine and placed on a 35°C recirculating heating pad inside a sound attenuating cubicle (Med Associates Inc., St. Albans, VT, USA) throughout the experiment.

[0124] For ABR recordings, three stainless steel needle electrodes were placed subdermally over the vertex, the right mastoid and right hind leg of each animal. Tone-pips (5 msec duration presented at a rate of 21/s) at 8, 16 and 24 kHz were delivered to the right ear using a calibrated MF-1 speaker in closed-field configuration (Tucker-Davis Technologies, Alachua, FL, USA) at attenuating intensity until no reproducible response could be recorded. Close to the ABR threshold, the responses to 1000 acoustic stimuli in 5 dB steps were averaged. Responses were low-pass filtered at 3 kHz.

[0125] DPOAEs were recorded and using ER10B+ Low Noise DPOAE microphone (Etymotic Research, Inc., Elk Grove Village, IL, USA) with acoustic stimuli delivered by two calibrated MF-1 speakers in closed-field configuration (Tucker-Davis Technologies, Alachua, FL, USA). DPOAEs were recorded at fixed stimulus levels ($L_1=L_2=70$ dB SPL), with an f_2/f_1 ratio of 1.2. Responses were recorded at 4, 8, 16, 24 and 32 kHz.

Acoustic trauma

[0126] Animals were exposed to 121 dB octave band noise (8-16 kHz) for 2 hours in groups of 4 rats, placed in individual compartments of a custom-built circular cage placed on a 30 cm diameter platform rotating at 3 turns/minute (Aquila Innovation, Valbonne, France). The calibrated octave band noise generated by the RZ6 SigGen software was further amplified by a Crown D-75 amplifier in bridge mode (Crown Audio, Elkhart, IN, USA) and delivered by four Beyma CP16 compression tweeters (Acustica Beyma S.L., Moncada, Valencia, Spain) positioned 39 cm above the rotating platform, each 10 cm from the platform center.

Drug treatment

[0127] Animals were treated by intraperitoneal injection daily for 14 days beginning immediately after acoustic trauma induction and subsequently repeated every 24 hours. The setron compounds granisetron HCl, ondansetron HCl, tropisetron HCl and azasetron HCl (Tocris Bioscience, Bristol, UK) were delivered dissolved in saline solution (1.6 mL/kg) while Tacrolimus (Selleck Chemicals, Houston, TX, USA) was dissolved in solution composed of 10% ethanol-100%/10% Kolliphor EL/1% Tween 80/79% saline (1.0 mL/kg). Placebo treated animals received injections of 1.6 mL/kg saline following the same treatment schedule.

Histology

[0128] After audiometry on day 14, animals were deeply anaesthetized with an intraperitoneal injection of pentobarbital (100 mg/kg). The cochleae were removed, fixed with 4% paraformaldehyde, pH 7.4, decalcified with 10% EDTA, pH 7.4 and then stained with myosin VIIa 1/1000. Inner and outer hair cells (IHCs and OHCs) were observed with the aid of a Zeiss Axiomager Z1/Apotome (Zeiss, France) and counted in 0.20 mm long segments of sensory epithelium from the apex to the base to obtain a cochleogram. The mean loss of OHCs per group was subsequently calculated as a function of normalized distance from the apex in bins of 2.5% of the total length, using the total length of each histological specimen. Cell counts could reliably be obtained for up to 90% of the total distance from the apex (corresponding to the tonotopic region up to from 0 kHz to -50 kHz in rat, Viberg & Canlon, 2004. *Hear Res.* 197(1-2): 1-10), while the last 10% was too variable due to damage incurred during dissection.

Statistics

[0129] Statistical analysis was performed using two-way ANOVA (Audiometry data: frequency and treatment group. Cochleograms: normalized distance from apex and treatment group) followed by Holm-Sidak post-hoc test for multiple comparisons. Results are given as mean $\pm$ SEM and *p* value less than or equal to 0.05 considered significant.

Results

[0130] The capacity of three calcineurin inhibiting setron compounds (azasetron HCl, 4.22 mg/kg, ondansetron HCl, 4 mg/kg and tropisetron HCl, 3.5 mg/kg) and one setron compound with no calcineurin inhibition effect (granisetron HCl, 3.8 mg/kg) to reduce hearing loss after acoustic trauma at equimolar doses were compared to the effect of a reference calcineurin inhibitor (tacrolimus, 1.5 mg/kg) under randomized and placebo controlled conditions. Baseline line audiometry (ABR and DPOAE measures) was performed for each animal 3 days before acoustic trauma was induced. Subsequently, audiometry measures were repeated 24 hours and 14 days after acoustic trauma.

[0131] **Figures 2 & 3** illustrate the audiometry measures (ABR thresholds and DPOAE amplitudes) determined for all treatment groups at baseline, 24 hours and 14 days after acoustic trauma. For ABR thresholds exceeding 90 dB, a maximal value of 95 dB was attributed. Following baseline audiometry, a strong positive ABR threshold shift (up to -70 dB) was seen for all groups accompanied by a severe DPOAE amplitude loss across all frequencies, signifying strongly reduced auditory signal transmission and reduction of outer hair cell amplificatory function. At 14 days after acoustic trauma, auditory functions are

differentially improved among treatment groups and across frequencies: ABR thresholds improved by ~7-33 dB and DPOAE amplitudes by ~0-17 dB. Compared to placebo treated animals, the group treated with azasetron HCl tendentially showed better hearing outcomes across all frequencies and the tacrolimus and ondansetron HCl treated group showed improvement at some frequencies, while the granisetron HCl or tropisetron HCl treated groups tendentially had both worse ABR thresholds and DPOAE amplitudes than the placebo group. Note that out of 11 tacrolimus treated animals, 5 died between treatment initiation and end of the study (only 6 animals completed the study) while only 2 animals out of 30 were lost for the 5 other groups combined (due to anesthesia complications). This further underscores the potential for adverse effects following repeated treatment with classic calcineurin inhibitors.

[0132] To quantify and compare the difference in treatment effects between groups while taking into account individual baseline and trauma variability, the hearing loss (ABR threshold shift & DPOAE amplitude loss) at D14 relative to baseline, as well as the functional recovery of hearing from 24 hours to D14 (ABR threshold shift & DPOAE amplitude loss) were determined for all treatment groups.

[0133] The azasetron HCl treated group had both significantly lower ABR threshold shifts at day 14 (**Figure 4**, $p = 0.003$) and better ABR threshold recovery from 24 hours to day 14 (**Figure 5**, $p < 0.001$) compared to placebo, while the ondansetron HCl treated group had a lower ABR threshold shift at day 14 (**Figure 4**) compared to placebo (not reaching significance). The granisetron HCl and tropisetron HCl treated groups showed strong trends towards higher ABR threshold shifts (**Figure 4**) and decreased threshold recovery. Treatment with the specific calcineurin inhibitor tacrolimus had no major effect on ABR threshold measures.

[0134] Compared to the placebo treated group, azasetron HCl treatment resulted in significantly reduced loss of DPOAE amplitudes ($p < 0.001$) from baseline to day 14 (**Figure 6**) as well as significantly improved recovery of DPOAE amplitudes ($p < 0.001$) from 24 hours post trauma to day 14 (**Figure 7**), suggesting reduced loss of outer hair cells in the cochlea. While there was trend towards reduced loss of DPOAE amplitude and improved recovery in the ondansetron HCl and tacrolimus treated groups at some frequencies, this did not reach statistical significance. Neither did the trend towards increased DPOAE amplitude loss and reduced recovery after granisetron HCl or tropisetron HCl treatment.

[0135] To further support the conclusions from the functional audiometry data, the loss of outer hair cells in four treatment group was determined by constructing cytochleograms from histological preparations of cochleae fixed after audiometry on day 14.

[0136] Consistent with the significantly reduced loss of DPOAE amplitudes after azasetron HCl treatment, this group also displayed significantly lower mean loss (**Figure 8**) of outer hair cells (OHC, the source of the DPOAE signals) compared to placebo ($p < 0.05$). Neither the granisetron HCl nor the tacrolimus treated groups were significantly different from placebo.

[0137] Altogether, the data demonstrate a significant and strong treatment effect of the calcineurin inhibiting setron family member azasetron, and similar trends for ondansetron, on functional and histological measures of noise-induced hearing loss.

[0138] Indeed, compared to placebo treatment, ABR thresholds shifts induced by azasetron treatment were reduced by ~35-60% (for ondansetron treatment, ~10-30%), DPOAE amplitudes loss by ~60% (for ondansetron ~10-30%) and mean outer hair cell loss by ~50-75%.

[0139] Conversely, granisetron (a setron compound with no calcineurin inhibiting effect) and tropisetron had a negative impacts on ABR threshold shifts (~17-40% higher threshold shift at day 14 than placebo) a trend towards worse DPOAE amplitudes and OHC loss. Treatment with the specific calcineurin inhibitor tacrolimus showed trends towards improving DPOAE amplitude loss at some frequencies, but had no effect on ABR threshold shifts in this realistic hearing loss (initial severe threshold shifts of up to 70 dB) and treatment (treatments only initiated after acoustic overexposure) paradigm. This in contrast to previously published results with specific calcineurin inhibitors using either a milder hearing loss paradigm (~10 dB initial ABR threshold shifts), treatment initiation prior to acoustic trauma or both (Uemaetomari et al., 2005. Hearing Res. 209(1-2):86-90; Bas et al., 2009. Acta Otolaryngol. 129(4):385-9).

[0140] In conclusion, these data demonstrate significant protective effects of treatment with the calcineurin inhibiting setron family member azasetron with similar trends for ondansetron in model of severe sensorineural hearing loss (even outperforming the specific calcineurin inhibitor tacrolimus), while the setron family member granisetron lacking the capacity to inhibit calcineurin had no or even deleterious effects on hearing outcomes. These data supports the use of calcineurin inhibiting setron family members, in particular azasetron and ondansetron, in the treatment of sensorineural hearing loss. The significant ABR threshold improvements determined for the azasetron treated group corresponds to "marked improvement to complete recovery" compared to only "slight improvement" for the placebo group, according to typical clinical audiological criteria (Furuhashi et al., 2002. Clin Otolaryngol Allied Sci. 27(6):458-63). Similarly, recent clinical research data has demonstrated that the early preservation or improvement of DPOAE responses (a significant effect of azasetron treatment) is highly predictive for significant long-term improvement of hearing capability in patients suffering idiopathic sudden sensorineural hearing loss (Shupak et al., 2014. Otol Neurotol. 35(10): 1691-7).

REFERENCES CITED IN THE DESCRIPTION

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Patentkrav

1. Hæmmer af calcineurin fra setronfamilien til anvendelse i behandling af sensorineuralt høretab hos en patient med behov derfor, hvor sensorineuralt høretab er idiopatisk
5 sensorineuralt høretab, støjinduceret sensorineuralt høretab, aldersrelateret sensorineuralt høretab eller ototoksisk lægemiddelinduceret sensorineuralt høretab, og hvor hæmmeren af calcineurin er azasetron eller et farmaceutisk acceptabelt salt deraf.
2. Hæmmer af calcineurin til anvendelse ifølge krav 1, hvor det sensorineurale høretab er
10 en hørenedsættelse på mindst 20 dB over mindst tre tilstødende frekvenser.
3. Hæmmer af calcineurin til anvendelse ifølge krav 1 eller 2, hvor det sensorineurale høretab er total døvhed.
- 15 4. Hæmmer af calcineurin til anvendelse ifølge et hvilket som helst af kravene 1 til 3, hvor det sensorineurale høretab er unilateralt eller bilateral.
5. Hæmmer af calcineurin til anvendelse ifølge et hvilket som helst af kravene 1 til 4, hvor det sensorineurale høretab er pludseligt sensorineuralt høretab.
20
6. Hæmmer af calcineurin til anvendelse ifølge et hvilket som helst af kravene 1 til 5, hvor det sensorineurale høretab er støjinduceret sensorineuralt høretab.
7. Hæmmer af calcineurin til anvendelse ifølge et hvilket som helst af kravene 1 til 4, hvor
25 det sensorineurale høretab er ototoksisk lægemiddelinduceret sensorineuralt høretab, og hvor det ototoksiske lægemiddel, der inducerer sensorineuralt høretab, er valgt fra gruppen bestående af aminoglycosider, kemoterapeutisk midler, sløjfediuretika, antimetabolitter og salicylater.
- 30 8. Hæmmer af calcineurin til anvendelse ifølge et hvilket som helst af kravene 1 til 7, hvor patienten har lidt af sensorineuralt høretab i mindre end 7 dage.

9. Hæmmer af calcineurin til anvendelse ifølge et hvilket som helst af kravene 1 til 8, hvor hæmmeren af calcineurin skal indgives systemisk, anvendes topisk eller injiceres intratympanisk.
- 5 10. Hæmmer af calcineurin til anvendelse ifølge et hvilket som helst af kravene 1 til 9, hvor hæmmeren af calcineurin skal indgives 1 til 3 gange dagligt.
11. Hæmmer af calcineurin til anvendelse ifølge et hvilket som helst af kravene 1 til 10, hvor hæmmeren af calcineurin skal indgives i mindst 30 dage.

DRAWINGS

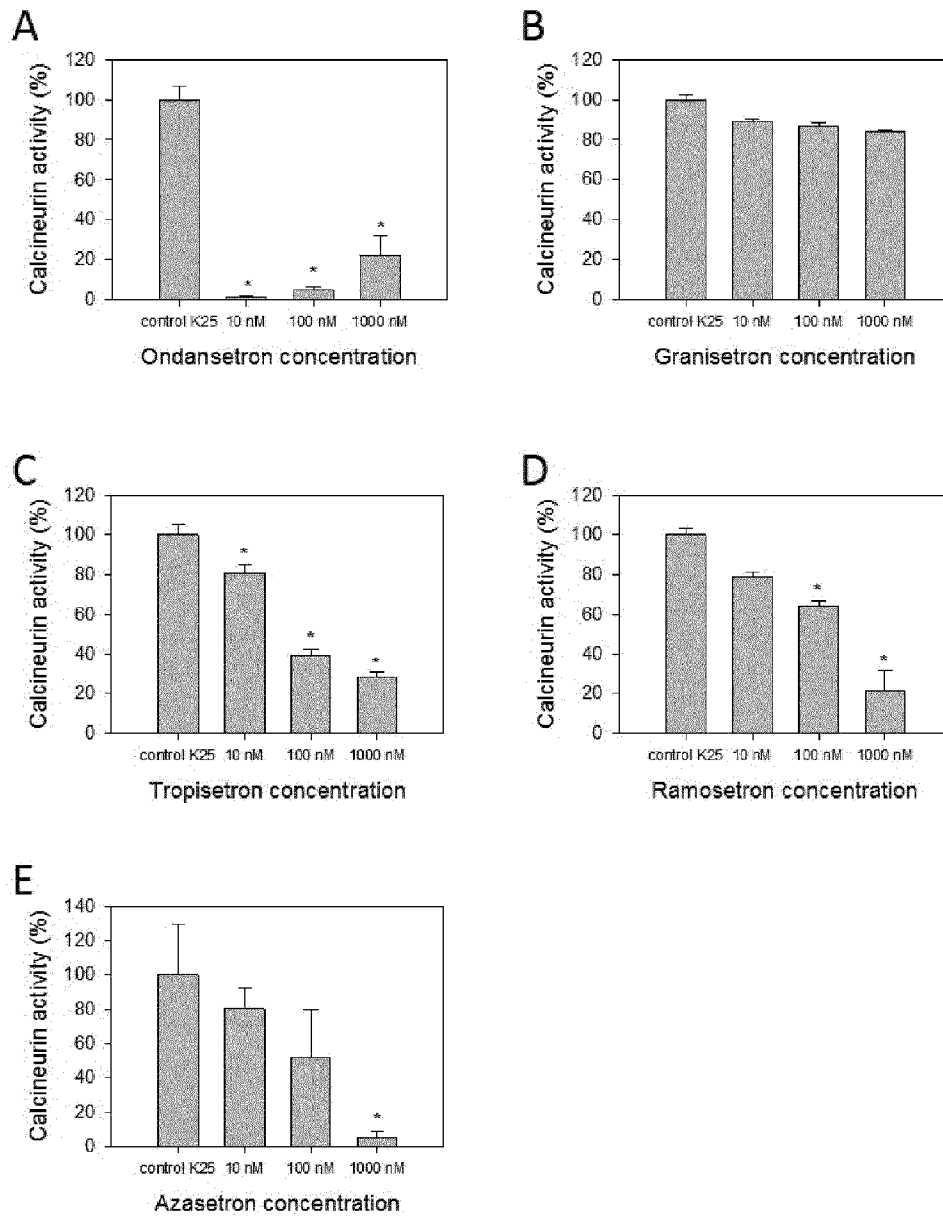


FIG. 1

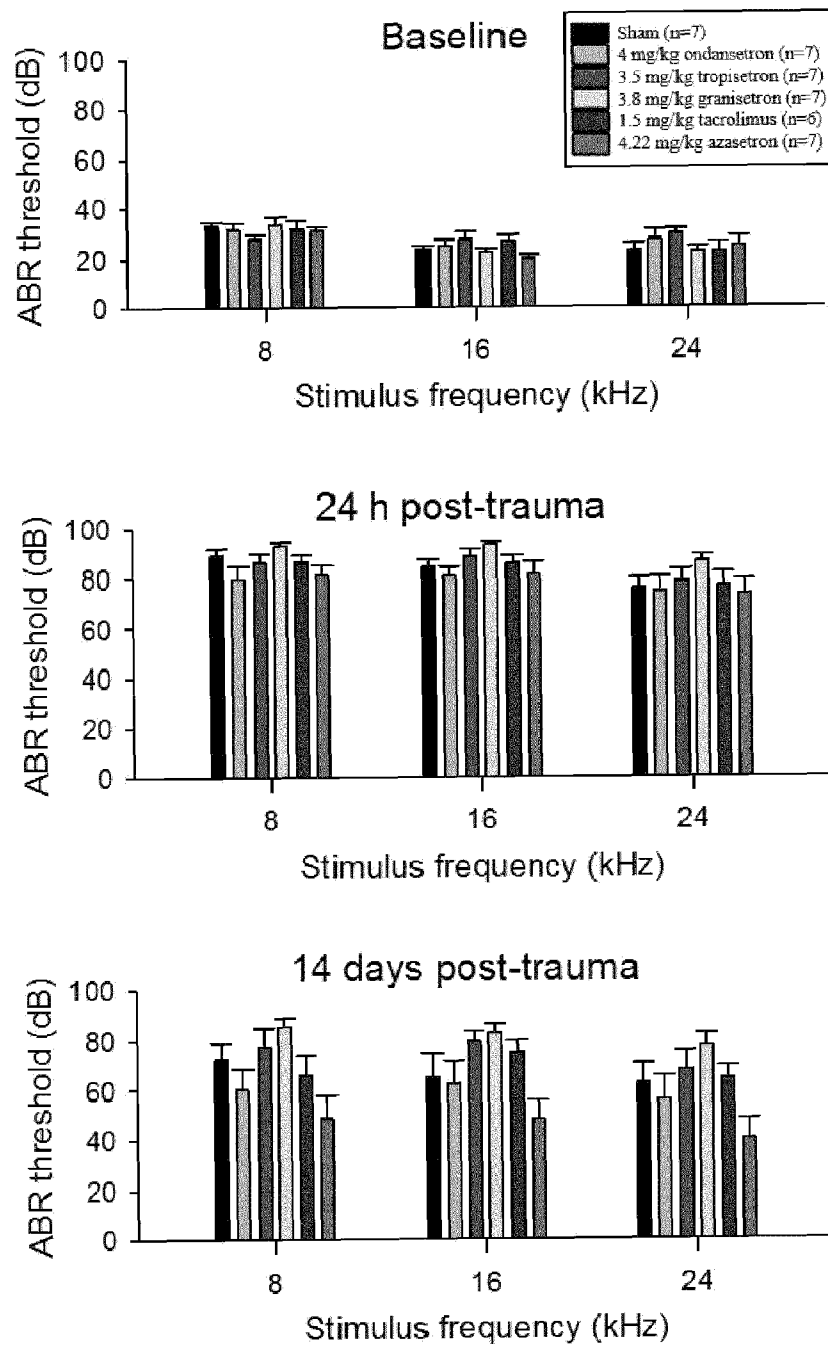


FIG. 2

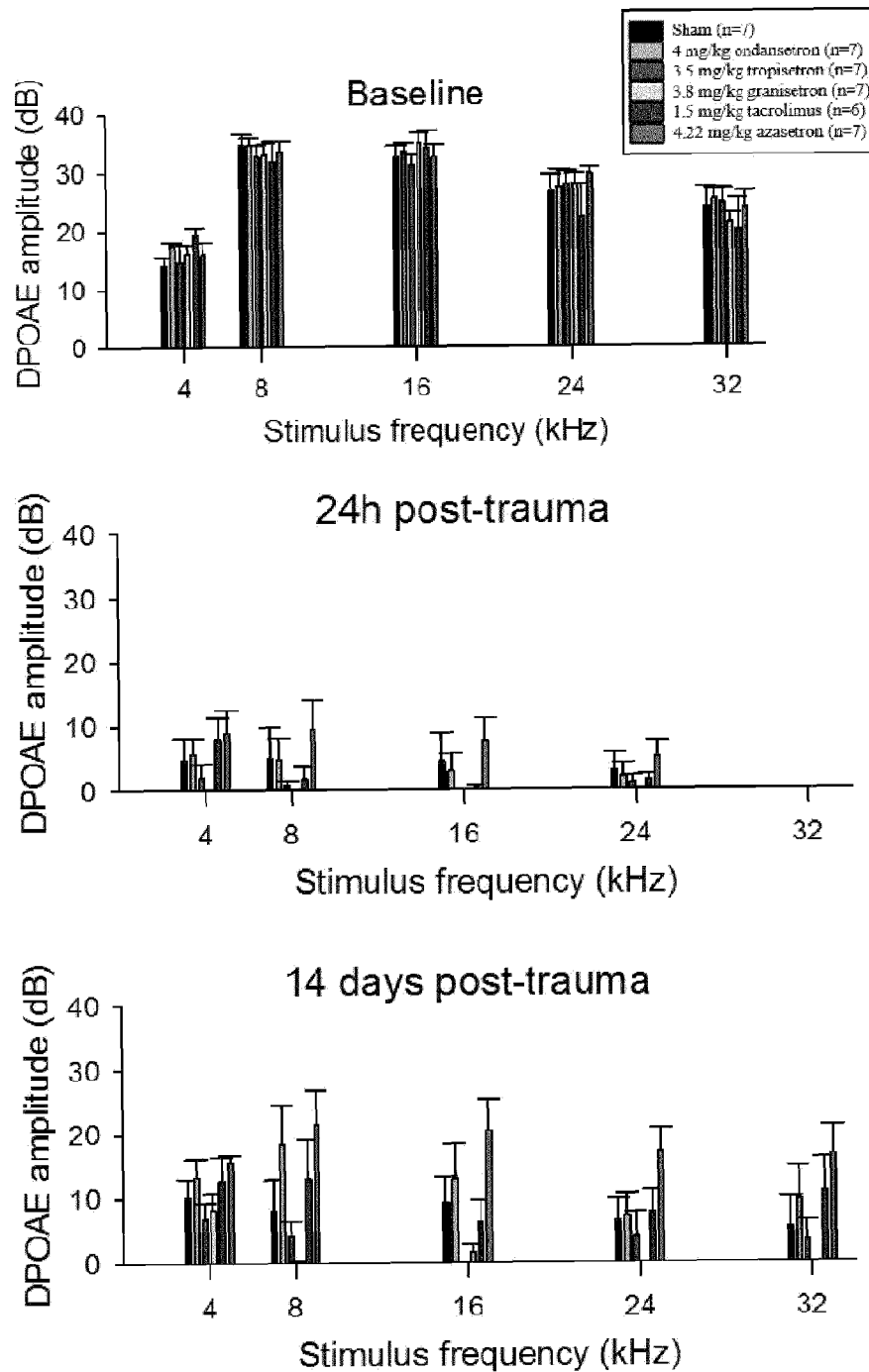


FIG. 3

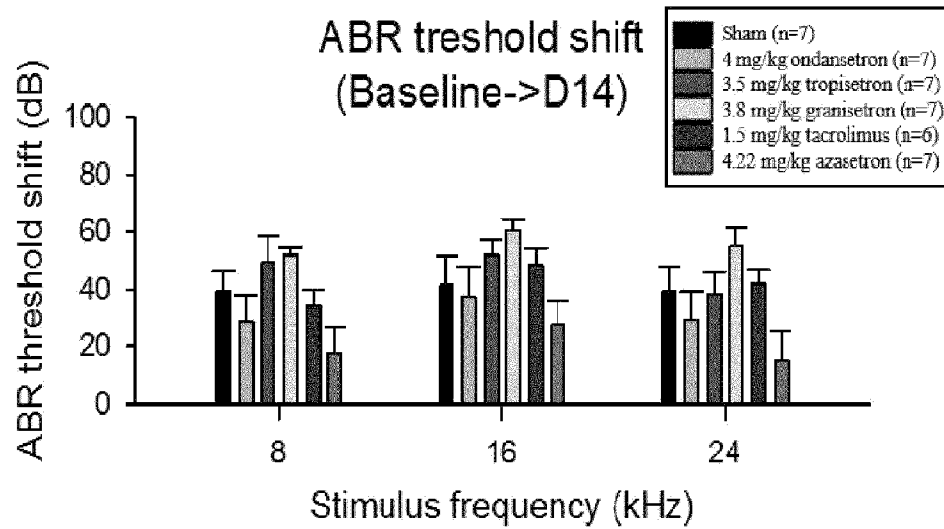


FIG. 4

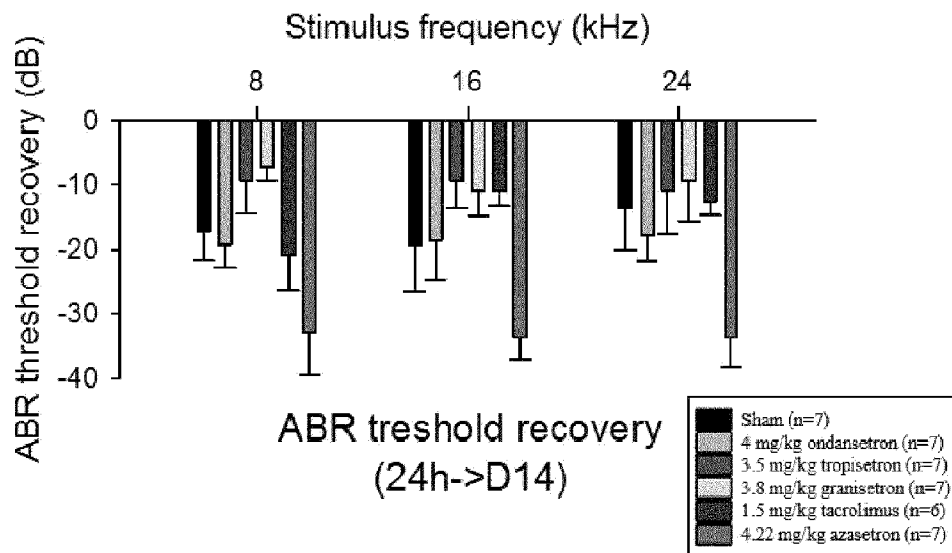


FIG. 5

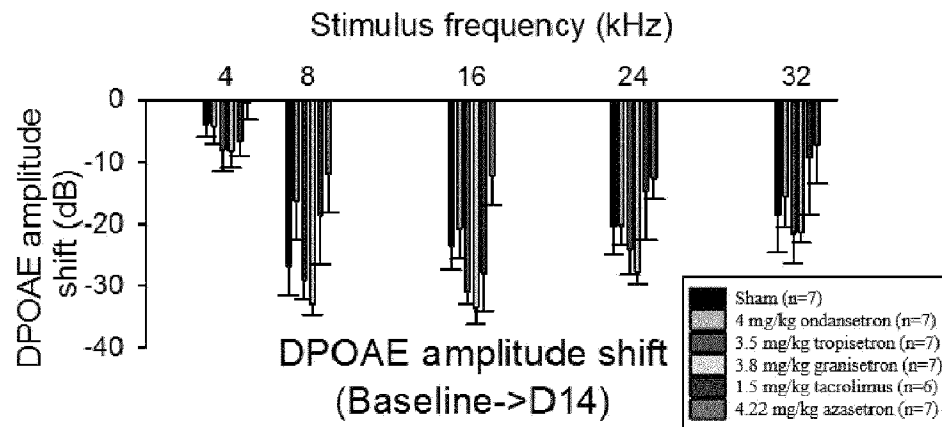


FIG. 6

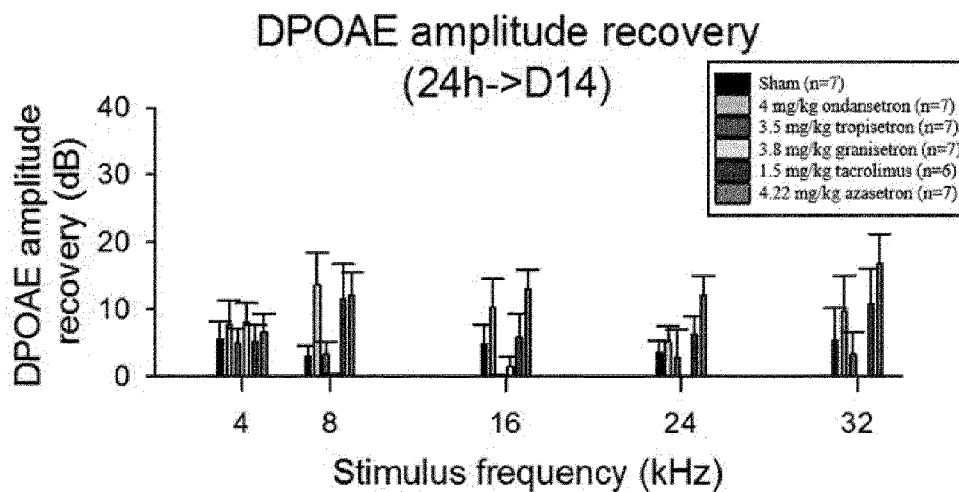


FIG. 7

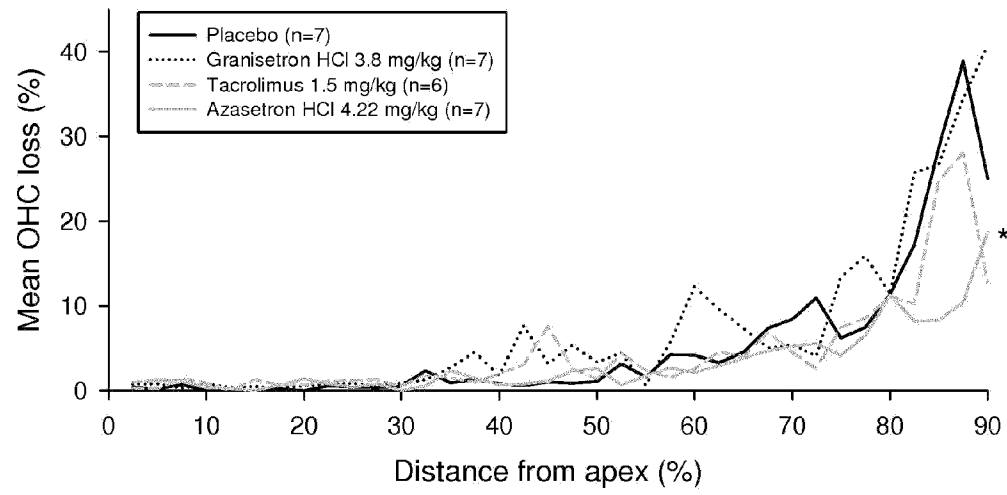


FIG. 8