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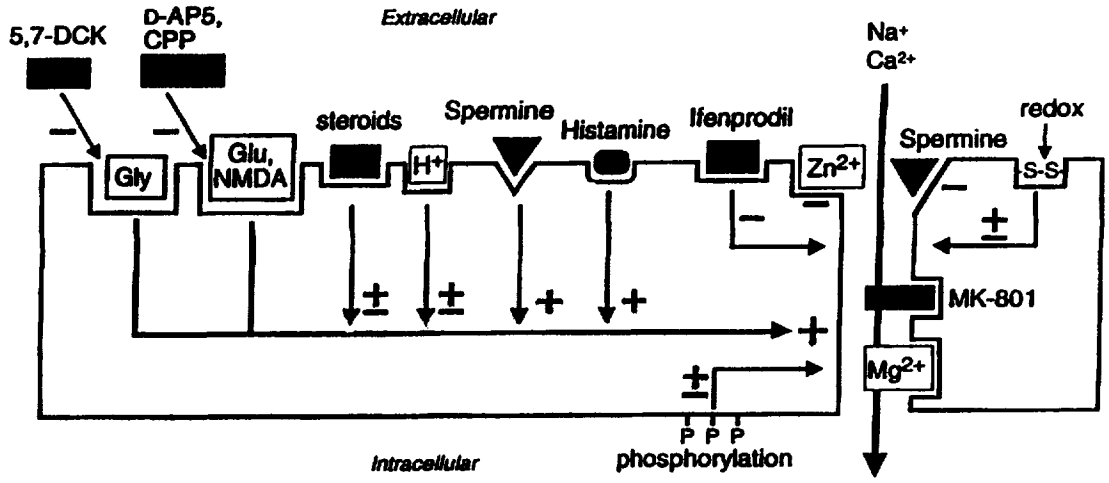

(57) Abstract

The present invention relates to functional NMDA antagonists and their ability to ameliorate aminoglycoside induced ototoxicity. These compositions are useful in the treatment or prevention of such hearing loss and/or loss of balance. A pharmaceutical composition comprising an aminoglycoside and at least one functional NMDA receptor antagonist administered simultaneously provides bacteriocidal activity and protection from aminoglycoside-induced ototoxicity.

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THE USE OF FUNCTIONAL N-METHYL-D-ASPARTATE ANTAGONISTS TO AMELIORATE OR PREVENT AMINOGLYCOSIDE-INDUCED OTOTOXICITY

SCOPE OF THE PRESENT INVENTION

The present invention relates to the use of a class of compounds to ameliorate and prevent aminoglycoside-induced ototoxicity. These compounds fall within the group of substances which reduce activity at N-methyl-D-aspartate (NMDA) receptors and are referred to as functional NMDA antagonists.

BACKGROUND

Aminoglycoside antibiotics are polycations consisting of amino sugars connected to an aminocyclitol nucleus, and are derived from the fungi *Streptomyces* and *Micromonospora* (1,2). Aminoglycoside antibiotics potently inhibit protein synthesis at the ribosomal level and compromise membrane integrity in a range of Gram-negative and some Gram-positive bacteria. These agents are clinically valued for their rapid onset of bacteriocidal activity, synergism with β-lactam antibiotics and low cost. They are commonly used to treat Gram-negative sepsis, endocarditis, nosocomial infections and tuberculosis.

While aminoglycosides are administered each year to approximately 3% of all clinical admissions in the United States (3), a major drawback to their use is serious ototoxicity (4). This ototoxicity results from the gradual destruction of sensory hair cells starting in the basal turns then ascending to the apex of the cochlea. The rapidity and degree of hearing loss in patients receiving aminoglycosides is dose-dependent and usually permanent. Moreover, measurable signs of hearing loss are found in approximately 20-33% of the patients receiving aminoglycoside antibiotics despite dosage limits and blood level monitoring (5).

Despite the widespread occurrence of

aminoglycoside-induced hearing impairments, the molecular mechanism(s) underlying this ototoxicity remain unknown. Aminoglycoside-induced ototoxicity has been ascribed to the inhibition of RNA synthesis (6), blockade of calcium-dependent potassium channels (7), and the inhibition of phosphoinositide metabolism (8) in cochlear hair cells. However, additional studies indicate that other cells in the cochlea may take up aminoglycosides without permanent toxic effects (7).

Recently, reports have been made of the presence of NMDA receptor mRNA in the synapses between cochlear hair cells and spiral ganglion neurons (9, 10), and of the enhancement of radioligand binding to the NMDA receptor by aminoglycosides in a polyamine-like fashion (11). It is also known that there are a number of isoforms of NMDA receptors, some of which are not responsive to polyamines. The reports on NMDA receptor mRNA did not discriminate among receptor isoforms, and, therefore, whether the isoforms in the tissues tested were sensitive or insensitive to polyamines. Moreover, since not all NMDA receptor isoforms exhibit potentiation by polyamines (22), it was not known on the basis of these reports whether any of the NMDA receptors present in cochlear hair cells would respond to polyamines or to compounds which mimic polyamines, such as aminoglycosides.

One object of the invention relates to the prevention and/or amelioration of aminoglycoside induced ototoxicity by use of NMDA antagonists.

Another object of the invention relates to the use of functional NMDA antagonists to provide substantial protection against aminoglycoside-induced ototoxicity in a patient.

A further object of the invention relates to the use of NMDA antagonists as a pharmaceutical composition in the prevention or amelioration of aminoglycoside induced ototoxicity.

Yet another object of the invention is to provide an improved method of antibiotic treatment to prevent aminoglycoside-induced ototoxicity comprising the step of administering a therapeutically effective amount of a NMDA antagonist in combination with an aminoglycoside antibiotic.

An additional object of the invention relates to a bacteriocidal antibiotic composition comprising a therapeutically effective amount of an aminoglycoside and functional NMDA antagonists to control ototoxicity.

SUMMARY OF THE INVENTION

The aminoglycoside antibiotics are bacteriocidal agents whose clinical utility is limited by their ototoxicity. This ototoxicity can result in significant hearing loss and/or a loss of balance. Recently, NMDA receptors were identified at the cochlear hair cell-spiral ganglion neuron synapse. Further, a recent *in vitro* study demonstrated the ability of aminoglycosides to modulate NMDA receptor function via the polyamine site. The present invention relates to the discovery that enhancement of glutamatergic neurotransmission at the hair cell synapse by aminoglycosides results in necrosis of either the hair cells and/or spiral ganglion neurons through excitotoxic mechanisms (12) which could be amenable to intervention with functional NMDA antagonists.

Accordingly, the present invention relates to the identification of functional NMDA antagonists, which are capable of ameliorating or preventing aminoglycoside-induced hearing loss or loss of balance. These antagonists act at a variety of sites on NMDA receptors including, but not limited to, the polyamine binding site, the ion channel, the glycine binding site and the glutamate binding site. The effect of these functional NMDA antagonists is to reduce the activity of NMDA receptors. Preferred compounds of the present invention

comprise quinoline and non-quinoline functional NMDA antagonists. The more preferred compounds are non-quinolines.

The compounds of the present invention are useful as a pharmaceutical composition either alone in the amelioration or prevention of aminoglycoside-induced ototoxicity, (as in the treatment of patients receiving aminoglycosides), or in combination with an aminoglycoside in order to prevent ototoxicity and simultaneously provide bacteriocidal activity. The present invention demonstrates that concurrent administration of functional NMDA antagonists and aminoglycosides limits cochlear damage, vestibular damage and hearing impairment produced by aminoglycoside antibiotics. Methods of using such pharmaceutical compounds in treating aminoglycoside-induced ototoxicity are also provided. These methods include an improved method of aminoglycoside antibiotic treatment which prevents aminoglycoside-induced ototoxicity.

In addition, the ability of aminoglycosides to modulate ligand binding to NMDA receptors also provides an effective screen for developing new aminoglycoside antibiotics with reduced ototoxic potential.

BRIEF DESCRIPTION OF THE DRAWINGS

Embodiments of the present invention are described by way of example with reference to the accompanying figures:

Figure 1. Schematic diagram of the NMDA receptor complex. The NMDA receptor complex is composed of several protein subunits clustered in such a way as to form a channel, which, when opened, is permeable to sodium and calcium ions. Excessive entry of calcium ions into a hair cell or neuron can result in the death of that cell. Surrounding and within the channel are numerous modulatory sites, including recognition sites for: glutamate;

glycine; multiple polyamine associated sites (one within the ion channel); and the dizocilpine (or MK-801) binding site within the ion channel.

Figure 2. NMDA antagonists limit neomycin-induced decrements in the Preyer reflex response to 5 (Panel A), 7 (Panel B), and 10 (Panel C) kHz tones. The Preyer reflex is a measure of hearing threshold well known in the art (17, 18). Two groups of guinea pigs received daily subcutaneous injections of either saline (●) or neomycin (O) for 14 days. Two other groups of guinea pigs received subcutaneous injections of neomycin together with either dizocilpine (1 mg/kg/d, ■) or ifenprodil (10 mg/kg/d, ▲) delivered by subcutaneous osmotic minipumps. The Preyer reflex was examined in these animals 2 weeks after completing the antibiotic regimen. At the maximum sound pressure level (120 dB) used in these studies, neomycin reduced the maximal response rates to <15% at all three frequencies, precluding an accurate estimate of the 50% response rate (ER_{50}). While dizocilpine and ifenprodil restored the maximal response rates in neomycin-treated animals to values indistinguishable from controls, modest but statistically significant increases in ER_{50} values were present at 7 and 10 kHz. In the absence of antibiotics, dizocilpine and ifenprodil treatment did not affect either the maximal response rates or ER_{50} values.

Figure 3. NMDA antagonists limit neomycin-induced reductions in distortion product otoacoustic emissions (DPOE). Each ear of each animal subject was tested; data points represent the mean DPOE amplitude determined from 12-20 ears. The DPOE from saline-treated animals (O, n= 12 ears) is presented for comparison (Panels A-D). Panels A and B: Chronic administration of neomycin (□, n= 16 ears) reduced DPOE amplitudes to values within ± 1 S.D. of the noise floor (dashed lines), indicating a complete loss of hearing over the range of 0.5-16 kHz. (The "noise floor" is a sound level below

that which can be detected by the measuring device at a particular frequency. Values at the noise floor for a frequency are recognized as indicating total hearing loss at that frequency.) Concurrent treatment with neomycin and dizocilpine (Panel A, ●, n = 20 ears) or ifenprodil (Panel B, ▲, n = 20 ears) maintained DPOE amplitudes above the noise floor, with a slight decrease in DPOE amplitude between 11500 and 16000 Hz. Panels C and D: Chronic administration of kanamycin (▵, n = 16 ears) also induced severe cochlear damage, as indicated by the reduction of DPOE amplitudes to within ± 1 S.D. of the noise floor. However, concurrent administration of kanamycin with dizocilpine (Panel C, ●, n = 20 ears) or ifenprodil (Panel D, ▲, n = 20 ears) maintained DPOE amplitudes above the noise floor, with moderate reductions in DPOE amplitude, particularly between 2500 and 6500 Hz. In >90% of the animals tested, the audiograms were qualitatively similar in both ears.

Figure 4. A summary of the changes in inner (A) and outer (B) hair cell populations of cochleae from individual guinea pigs treated with neomycin (■), neomycin + dizocilpine (●), or neomycin + ifenprodil (▲) in serial sections cut through the organ of Corti. Results are expressed as the percentage of cells missing compared to normal guinea pig cochleae. Note the total loss of both inner and outer hair cells between 13 to 18 mm from the apex of the cochlea in the neomycin treated guinea pig. Treatment of guinea pigs with either dizocilpine or ifenprodil resulted in complete protection against hair cell loss between 13 to 17 mm from the apex.

Figure 5. Effect of aminoglycosides on [^{3}H]dizocilpine binding to NMDA receptors: relationship to ototoxicity. A) Enhancement of [^{3}H]dizocilpine binding by representative aminoglycosides: streptomycin (O), amikacin (■), and spectinomycin (●). The biphasic modulation of [^{3}H]dizocilpine binding by streptomycin is

similar to that of the polyamine, spermine. B) The potencies of a series of ototoxic aminoglycosides in enhancing [^{3}H]dizocilpine binding is highly (Bonferroni corrected Pearson correlation coefficient = 0.928, $P<0.01$) correlated with their relative ototoxicities in humans (2). There was no significant correlation between the E_{max} and the relative aminoglycoside ototoxicity (Bonferroni corrected Pearson correlation coefficient = 0.046, $P=0.83$). Spectinomycin is not included in this correlation because it is not ototoxic. Streptomycin lies well outside this correlation. The anomalous behavior of streptomycin may be related to an open channel block (corresponding to the inhibition of [^{3}H]dizocilpine binding, panel A), which would limit its ototoxicity.

Figure 6. Chemical structures for some functional NMDA antagonists.

DETAILED DESCRIPTION OF THE INVENTION

The NMDA receptor is one of the major subtypes of fast excitatory neurotransmitter receptors in the mammalian central nervous system. These receptors are activated by the acidic amino acids glutamate and aspartate. The NMDA receptor/ion channel complex contains an integral ion channel that gates Na^+, K^+ and Ca^{2+} ions and is blocked in a voltage dependent manner by Mg^{2+}.

Excessive or abnormally prolonged stimulation of NMDA receptors leads to neuronal degeneration. Due to the structural arrangement of the NMDA receptor/channel, it is regulated at multiple sites. *See Figure 1.* First, activity can be blocked by competitive antagonists, that is, molecules that interact at the same site as glutamate or aspartate but which do not activate the receptor. Second, activity can be modulated by agents which block the ion channel, referred to as ion channel blockers. Third, a number of other regulatory sites have been identified from which neurotransmitter activity can be

modulated, including a receptor for glycine and two polyamine associated receptor sites, one of which lies within the ion channel. The present invention relates to the use of a specific group of compounds in the prevention and/or treatment of aminoglycoside-induced ototoxicity. These compounds are generally classified as functional NMDA antagonists since they reduce activity at NMDA receptors.

"Ototoxicity", as the term is used herein, includes cochleotoxicity which results in hearing loss, and vestibulotoxicity, which results in loss of balance. Although many of the examples are directed to cochlear toxicity, one skilled in the art will clearly recognize that the present invention encompasses treatment and prevention of aminoglycoside-induced ototoxicity in the form of vestibulotoxicity.

The functional NMDA antagonists of the present invention comprise compounds capable of acting on a number of different sites on the receptor (Figure 1). The antagonists of the present invention may act at a regulatory site known as the glycine binding site: examples include 7-chlorokynurenic acid, indole-2-carboxylic acid, L-687414, HA-966 and 1-aminocylopropane carboxylic acid (see Figure 5). Alternatively, the compounds may act at the glutamate binding site: examples include 2-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid ("CPP"). Further, the functional NMDA antagonists of the present invention may act at a polyamine associated binding site: examples include diethylenetriamine, ifenprodil, eliprodil, conantokin-G and arcaine. In addition, these compounds may act as ion channel blockers of the NMDA receptor: examples include 1-[1-(2-thienyl)cyclohexyl]-piperidine ("TCP"), (+)5-methyl-10,11-dihydro-5H-dibenzo[*a*,*d*]cyclohepten-5,10-imine maleate ("MK-801" or "Dizocilpine"), phencyclidine ("PCP"), ketamine and memantine. These compounds and several other

functional NMDA antagonists are illustrated in Figure 6.

Both quinoline and non-quinoline compounds can be used in the present method as long as they act as functional NMDA antagonists. Both *in vitro* and *in vivo* tests for determining whether a given compound is a functional NMDA antagonist are well known in the art. (*See, e.g.* references 12, 16, 19 and 20).

A particularly preferred group of functional NMDA antagonists comprises non-quinoline compounds, including but not limited to dizocilpine and ifenprodil. The comparable efficacies of these antagonists, which act at discrete sites on the NMDA receptor, indicates that aminoglycoside antibiotics damage vulnerable cochlear neurons through an excitotoxic process requiring NMDA receptor activation. Both their specificity and the modest dose range (approximately 1-20 mg/kg/day) required to limit aminoglycoside-induced hearing loss make these compounds ideal in its prevention and/or amelioration.

Four significant observations based on auditory, histological and pharmacological studies in guinea pigs provide concrete evidence for the use of functional NMDA antagonists in the amelioration of aminoglycoside-induced ototoxicity, and constitute the basis for a new screening assay to evaluate the ototoxic potential of new aminoglycosides. First, functional NMDA antagonists substantially ameliorate the aminoglycoside-induced loss of the Preyer reflex, a response indicative of the animals auditory threshold (Figure 2). Second, co-administration of functional NMDA antagonists substantially reduce the aminoglycoside-induced loss of the ability of guinea pigs to hear tones ranging from 500 to 16,500 cycles per second (Hz) (Figure 3). Third, administration of functional NMDA antagonists almost completely eliminates the aminoglycoside-induced destruction of inner and outer cochlear hair cells which underlies the loss of hearing sensitivity to 500-16,500 Hz tones (Figure 4). Fourth,

the potency of a series of aminoglycosides in enhancing the binding of [^{3}H]dizocilpine is significantly correlated with the ototoxic potential of these compounds (Figure 5). Moreover, the potency of aminoglycosides in enhancing [^{3}H]dizocilpine binding is within the range of ototoxic concentrations of these compounds in the cochlear perilymph (3). The ability of aminoglycosides to enhance the binding of ligands to the NMDA receptor complex at pathophysiologically relevant doses provides a solid mechanism to explain the ototoxic properties of these antibiotics. Also, this data provides a rational explanation for the use of functional NMDA antagonists in preventing and/or ameliorating the ototoxicity of aminoglycosides and constitutes an assay by which potential aminoglycoside antibiotics may be screened for their ototoxic liability (See examples 1-4).

Ototoxicity limits the clinical usefulness of aminoglycoside antibiotics. The present invention indicates that concurrent administration of functional NMDA antagonists in humans can attenuate aminoglycoside-induced ototoxic damage. Moreover, many functional NMDA antagonists are highly charged and have limited access to the central nervous system. Since access to the cochlea is not limited by the blood brain barrier, such agents may limit aminoglycoside-induced damage to cochlear neurons and be devoid of any behavioral side effects.

The schedule by which functional NMDA antagonists can be administered to prevent or ameliorate aminoglycoside-induced ototoxicity in humans will vary somewhat according to the circumstances and is within the skill of the health care provider to determine. In many circumstances, it will be desirable to administer the functional NMDA antagonists concurrently with the aminoglycoside antibiotic. If the patient has already commenced aminoglycoside therapy, functional NMDA antagonists can be administered to ameliorate the

ototoxicity which might otherwise occur. If aminoglycoside administration is contemplated, treatment with one or more functional NMDA antagonists can be commenced before the administration of the aminoglycoside, and continued during administration of the aminoglycoside. While the practitioner may choose to cease administration of the functional NMDA antagonist or antagonists at or around the time of cessation of aminoglycoside administration, it will often be desirable to continue administration of the functional NMDA antagonist or antagonists for up to a month thereafter. This is so first, because the clearance of aminoglycosides from the body takes a substantial period of time and, second, because glutamate induced cell death continues for a time even after aminoglycoside administration ends. It is within the skill of the practitioner to determine how long to administer the functional NMDA antagonist or antagonists after cessation of aminoglycoside therapy based on the known pharmacokinetics of the particular aminoglycoside the practitioner has chosen to employ and the pharmacodynamics of the particular functional NMDA antagonists used. The functional NMDA antagonists could be used in conjunction with any aminoglycoside drug, or as a combination pharmaceutical preparation

It is contemplated that the ability to prevent or limit aminoglycoside-induced ototoxicity could result both in more frequent administration and in broader use of aminoglycosides. In the past, for example, aminoglycosides were used to irrigate the peritoneal cavity during abdominal surgery. The present invention may permit restoration of this practice, which was discontinued largely due to ototoxicity.

Efficacious amounts of a functional NMDA antagonist to be used in the method of the instant invention may be varied over a wide range. The typical range of the amount of a functional NMDA antagonist

compound is between about 0.5 mg/day and 800 mg/day.

Generally, the efficacious amount and concentration of the compound to be administered are those which result in the composition exhibiting a reduction or prevention of aminoglycoside-induced ototoxicity. Our results suggest that the amount of functional NMDA antagonists that provide substantial protection against hypoxic/ischemic insults (e.g., in models of rodent middle cerebral artery occlusion) also are sufficient to prevent aminoglycoside-induced ototoxicity. Physicians and other practitioners skilled in the art are familiar with standard techniques for determining appropriate dosages in individual patients, (*See, e.g.* reference 21). In addition, preferred amounts depend upon the rate of delivery of the active ingredient and the number of administrations which are used. Preferred amounts for any specific dose can be determined by normal pharmacological screening methods used in the art. The actual dosage amount administered can be determined by physical and physiological factors such as body weight, severity of condition, and idiopathy of the patient.

Carrier materials for the pharmaceutical preparations of the present invention are well known in the pharmaceutical formulations art and include those carriers referred to as diluents or vehicles.

The pharmaceutical compounds of the present invention can be administered orally, topically (for example, in the form of patches), or parenterally, in a vehicle comprising one or more pharmaceutically acceptable carriers, the proportion of which is determined by the solubility and chemical nature of the compounds, the chosen route of administration and standard biological practice. For oral administration, the compounds can be formulated in unit dosage forms such as capsules or tablets each containing a predetermined amount of active ingredient, ranging from about 0.5 mg to 800 mg, in a

pharmaceutically acceptable carrier.

The pharmaceutical composition of the present invention can take the form of a lyophilized powder of the active substance, to be dissolved immediately before use in a physiological solution for the purpose of injection. For parenteral administration, the compound is administered by either intravenous, subcutaneous or intramuscular injection, in compositions with pharmaceutically acceptable vehicles or carriers. For administration by injection, it is preferred to use the compound in solution in a sterile aqueous vehicle which may also contain other solutes such as buffers or preservatives as well as sufficient quantities of pharmaceutically acceptable salts or of glucose to make the solution isotonic. The pharmaceutical composition according to the invention can also take a form which is suitable for oral administration. For example, suitable forms are tablets, food gelatin capsules, dragées, powders and granules. The formulation of such oral forms is well-known to those skilled in the art. Any of the known formulations are useful in preparing the instant oral pharmaceutical compositions.

Suitable vehicles or carriers for the above noted formulations can be found in standard pharmaceutical texts, e.g. in "Remington's Pharmaceutical Sciences", 16th ed. Mack Publishing Company, Easton, Pa., 1980.

The present invention also relates to a kit for screening aminoglycosides for potential ototoxicity. One embodiment of such a kit comprises the following components: a source of NMDA receptor; a labeled ligand that binds to sites inside the NMDA receptor coupled ion channel, such as dizocilpine or phencyclidine; a non-specific binding molecule to estimate background binding levels; and a positive control. Many sources of NMDA receptor are known in the art. For example, rat brain provides a rich source of NMDA receptors. It may be

preferred to provide the NMDA receptor in a lyophilized form or in solution depending upon the format of the kit. Lyophilization may increase the shelf-life of the kit component. Labeled ligands may comprise any of the known and routinely used labels. For example, commonly used labels include radioisotopes, biotin, fluorescent and chemiluminescent compounds, and luciferase. Other labels known to the skilled artisan are also encompassed herein. Molecules to be used for determining non-specific binding comprise unlabelled compounds such as phencyclidine, dizocilpine, ketamine and memantine. For example, dizocilpine and phencyclidine are preferred molecules for determining the non-specific binding. Positive control molecules may include compounds that enhance the binding of labeled ligands and act at polyamine sites. Preferred examples are spermine and streptomycin.

All publications, patents and articles referred to herein are expressly incorporated herein *in toto* by reference thereto. The following examples are presented to illustrate the present invention but are in no way to be construed as limitations on the scope of the invention. It will be recognized by those skilled in the art that numerous changes and substitutions may be made without departing from the spirit and purview of the invention.

EXAMPLE 1

Studies of the Preyer Reflex were performed in albino male Hartley guinea pigs (250-200 g each, Charles River). The guinea pigs were maintained in AAALAC-accredited facilities which included a 12 hour day/night cycle (lights on at 0700) and free access to food and water. The animals were divided into 4 major groups. The normal control group of guinea pigs was administered a daily subcutaneous injection of physiological saline (1 ml) for 14 or 21 consecutive days. Aminoglycoside ototoxicity was induced by the administration of either

neomycin sulfate(13) (Sigma Chemicals, St. Louis MO, 150 mg/kg/D in 1 ml, subcutaneous (SC), for 14 days) or kanamycin sulfate (Sigma Chemicals, St. Louis MO, 250 mg/kg/D in ml, SC, for 21 days)(14). The third group consisted of guinea pigs receiving either dizocilpine maleate or ifenprodil tartrate (Research Biochemicals, Inc., Natick, MA) by subcutaneous osmotic minipumps (Alzet 200, Alza Scientific Instruments, Palo Alto, CA). Minipumps delivering 1 mg/kg of dizocilpine maleate per day in 40% DMSO/60% Milli-Q filtered water or 10 mg/kg ifenprodil tartrate per day in 40% DMSO/20% Emulphor/40% Milli-Q filtered water for 14 days were implanted subcutaneously under pentobarbital (25 mg/kg) and chloral hydrate (130 mg/kg) anesthesia. The fourth group consisted of guinea pigs receiving combinations of aminoglycosides and functional NMDA antagonists concurrently. At the end of 14 days, the depleted minipumps were surgically removed and a fresh pump implanted for another 14 day delivery schedule. Auditory testing of these guinea pigs began 4 weeks after initiating the drug or saline treatments.

Measurement of Hearing Threshold

The Preyer reflex consists of a flick or twitch of the pinnae (outer ear) in response to a tone of known frequency and intensity (17). Guinea pigs were evaluated beginning 4 weeks after the initiation of drug regimens. Animals were immobilized in mesh restraining cages and placed inside a sound attenuated chamber (18x12x12 in) facing a 3 in diameter speaker located 4 in from the subject. The speaker was connected to a signal generator with variable signal attenuator, a variable frequency narrow bandpass 10 dB/octave Butterworth filter, a pulse timing unit, and a 10 watt audio amplifier. The sound intensity of the speaker output at 4 in. was determined for each frequency using a calibrated microphone, and ranged from 50-120 dB SPL at 5 kHz to 50-105 dB SPL at 7

and 10 kHz, respectively. The stimulus tones consisted of a 300 msec long square-wave pulse (at 5,7, or 10 kHz) given once per second for five seconds. The number of ear flicks per stimulus train of 5 tones was recorded as the "percent response" (e.g. 3 ear flicks per train of 5 stimuli is a 60% response). The tone stimulus train was then repeated at a higher sound intensity until either the maximum response rate (100%) was achieved or the maximum sound output at that frequency attained. Sigmoidal curves were fitted to the data via non-linear regression techniques for determination of the ER_{50} and E_{max} values.

Dizocilpine and Ifenprodil Ameliorate Aminoglycoside-Induced Decrements in the Preyer Reflex.

Guinea pigs respond to intense 5-10 kHz tones with a pinna flick referred to as the Preyer Reflex (13). The presence of a Preyer reflex is dependent upon the sound pressure level (SPL) and the audibility magnitude to the animal. In control animals, 87±1.1, 72±0.9 and 75±0.9 dB are required to effect a 50% response rate (ER_{50}) to trains of 5, 7 and 10 kHz tones, respectively (Tables 1, 1a, Figure 2), while a 100% response rate is obtained with 5-10 kHz tones of ~100 dB SPL (Figure 2). Following chronic administration of either neomycin or kanamycin, the sound pressures required to elicit a 50% response rate to 5 and 7 kHz tones exceeded levels which could be measured in our apparatus (>120 dB SPL), with a response rate of < 20% obtained at 120 dB SPL. Antibiotic treated animals were completely unresponsive to 10 kHz tones at maximum sound pressure (120 dB SPL) (Fig 1, Table 1). Concurrent administration of either dizocilpine (1 mg/kg/day) or ifenprodil (10 mg/kg/day) significantly attenuated both the neomycin and kanamycin-induced loss of the Preyer reflex (Tables 1,1a, Figure 2). The ER_{50} values in response to 5 kHz tones were not significantly different (99 to 109% of control) from saline-treated

animals when these antibiotics were administered together with either NMDA antagonist (Tables 1, 1a, Figure 2). Small (14-35%) increases in ER_{50} values in response to 7 and 10 kHz tones were observed in animals treated with combinations of these antibiotics and NMDA antagonists. Despite these modest increases in ER_{50} values, concurrent administration of NMDA antagonists restored the maximum response rates in antibiotic-treated animals to values approaching 100% (Tables 1, 1a, Figure 2).

Table 1 and Table 1a. Functional NMDA Antagonists Ameliorate Aminoglycoside-Induced Ototoxicity: the Preyer Reflex.

TABLE 1.	Sound Pressure ED_{50} (dB)		
Treatment	5 kHz	7 kHz	10 kHz
Saline	87±1.1	72±0.9	75±0.9
Neomycin	>120**	>120**	>120**
Kanamycin	>120**	>120**	>120**
Dizocilpine	83±1.3	75±1.1	75±1.0
Ifenprodil	84±1.3	73±1.0	74±1.7
Dizocilpine + Neomycin	95±3.0	86±2.9**	86±2.9**
Dizocilpine + Kanamycin	95±4.1	97±4.8**	97±7.3**
Ifenprodil + Neomycin	86±1.4	85±2.8*	85±4.5
Ifenprodil + Kanamycin	88±1.4	82±1.1*	80±2.4

TABLE 1a	Maximal Response Rate (%)		
Treatment	5 kHz	7 kHz	10 kHz
Saline	100±0	100±0	100±0
Neomycin	13±3.9**	14±9.8**	0±0**
Kanamycin	13±8.4**	3.3±3.3**	6.7±4.2**
Dizocilpine	100±0	100±0	100±0
Ifenprodil	100±0	100±0	100±0
Dizocilpine + Neomycin	87±9.1	96±6.7	88±8.4
Dizocilpine + Kanamycin	77±17	83±17	67±21*
Ifenprodil + Neomycin	100±0	98±2.2	90±10
Ifenprodil + Kanamycin	100±0	100±0	100±0

The Preyer reflex was measured in male Hartley guinea pigs beginning 4 weeks after initiating ototoxic regimens of neomycin and kanamycin as described above. The ED_{50} is that sound pressure level eliciting a 50% response (pinnae flick) rate. Values represent the mean ± SEM of observations from 4 - 25 animals. *, **: Significantly different from the saline group, $P<0.05$, 0.01, respectively, ANOVA followed by Tukey's post-hoc multiple comparison test. All other groups were significantly different from either the neomycin or kanamycin only groups, $P<0.01$.

EXAMPLE 2

Distortion Product Otoacoustic Emissions (DPOE)

Male, albino Hartley guinea pigs (250-300 g, Charles Rivers, Raleigh, NC) were maintained under similar conditions, and received the same drug treatments as described in Example 1.

Measurement of distortion product otoacoustic emissions (DPOE) is one of several non-invasive means of evaluating outer hair cell and general cochlear integrity along the organ of Corti (23). The DPOE is most useful in determining alterations in cochlear function following aminoglycoside administration in animals (24, 25) and humans (26). Simultaneous presentation of two intense, primary tones (f1 and f2) to normal cochleae yields many fainter distortion tones. When an f1/f2 ratio of 1.2:1 is presented, the most powerful tone appears at a predictable frequency (2f1-f2), and is referred to as a 2f1-f2 DPOE. Thus, pairing a 65 dB SPL, 10 kHz tone (f1) with a 50 dB SPL, 12 kHz tone (f2) results in a cochlear emission of a third, 5-10 dB SPL, 8 kHz tone. The ability of a microphone to detect this third, faint tone provides a non-invasive acoustic means of assessing cochlear function (23). By varying f1 and f2, outer hair cell and general cochlear integrity can be evaluated along

the organ of Corti.

Following measurement of the Preyer Reflex, guinea pigs were anesthetized (ketamine 40 mg/kg and xylazine 4 mg/kg) and a CUBDISP™ system (consisting of microphones and high fidelity earphones (Etymotic Research, Oak Grove Village, IL) placed in each ear. Pairs of continuous tones (65 and 50 dB SPL for f1 and f2, respectively) ranging from 1-16 kHz were presented in descending sequences. The 2f1-f2 DPOE together with associated background noise were recorded by the CUBDISP™ system and plotted as a function of stimulus frequency.

The DPOE recorded from saline-treated guinea pigs (n=12) were characteristic of intact cochleae (24) (Figure 3). The DPOE of animals treated with either neomycin (n=16, Figure 3A,B) or kanamycin (n=16, Figure 3C,D) were indistinguishable from the noise floor, indicative of a severe loss of outer hair cells. Concurrent administration of NMDA antagonists increased DPOE amplitudes well above the noise floor (Figure 3) to values approaching those obtained in control subjects. Some residual hearing impairment was noted in guinea pigs treated with either neomycin + dizocilpine (Figure 3A) or neomycin + ifenprodil (Figure 3B) manifested as a significant ($P<0.01$, two-tailed paired t-test) mean decrease of 9.9 and 4.8 dB, respectively, in DPOE amplitudes at 11.5 and 16 kHz. Between these frequencies, a decrement in DPOE amplitudes of 17 and 4.2 dB was also noted in guinea pigs treated with kanamycin and either dizocilpine or ifenprodil, respectively ($P<0.01$, two-tailed paired t-test). A region of impaired responsiveness to f2 stimuli of 4 to 8.5 kHz averaging 17-18 dB (Fig 2C,D) was also observed in guinea pigs receiving kanamycin and either dizocilpine or ifenprodil.

Further assessment of the DPOE data indicated

that 80% (64/80) of the cochleae were within control values in animals receiving concurrent treatment with either dizocilpine or ifenprodil and aminoglycoside (Table 2). While 100% (28/28) of the DPOE recorded from animals receiving either neomycin or kanamycin were below the noise floor (indicative of total hearing loss), a similar DPOE was obtained from only one cochlea (1.25%) from guinea pigs treated concurrently with an NMDA antagonist and an aminoglycoside. There was no significant difference in the efficacy of dizocilpine and ifenprodil in this measure, nor was there a significant difference in the incidence (5-30%) of DPOE judged "partially impaired" between control animals and guinea pigs receiving NMDA antagonist +aminoglycosides (Table 2).

Table 2. NMDA Receptor antagonists attenuate aminoglycoside-induced hearing loss: Effects on DPOE amplitude.

Treatment	Number of Ears Tested	Normal Hearing	Partial Impairment	Complete Loss
Saline	12	11	1	0
Neomycin	16	0	0	16**
Kanamycin	12	0	0	12**
Dizocilpine	8	8	0	0
Ifenprodil	8	8	0	0
Dizocilpine + Neomycin	20	13	6[aa]	1
Dizocilpine + Kanamycin	20	17	3	0
Ifenprodil + Neomycin	20	15	5[aa]	0
Ifenprodil + Kanamycin	20	19	1	0

Table 2. Distortion product otoacoustic emissions (DPOE) were measured in male Hartley guinea pigs beginning 5 weeks after initiating ototoxic regimens of neomycin or kanamycin. A complete loss of hearing was defined as a DPOE amplitude within the noise floor. Partial hearing impairment was defined as a DPOE that was significantly lower than normal, but above the noise floor. **: Significantly different from saline values, $P<0.01$, χ^2 test. [aa]: Significantly different from corresponding antibiotic only group, $P<0.01$, χ^2 test.

EXAMPLE 3

Male, albino Hartley guinea pigs (250-300 g, Charles River, Raleigh, NC) were maintained under similar conditions, and received the same drug treatments as described in Example 1.

After DPOE measurements, some guinea pigs were euthanized with an overdose of chloral hydrate anesthesia. Guinea pigs treated with saline, neomycin, neomycin + dizocilpine, and neomycin + ifenprodil (2 animals/group) were then transcardially perfused with Tyrode's solution followed by mixed aldehydes (2% paraformaldehyde, 2.5% glutaraldehyde) in cacodylate buffer, pH 7.2. The cochleas were removed, fixed overnight in the mixed aldehyde solution, washed and decalcified in 8% EDTA for two weeks. They were then dehydrated and embedded in glycol methacrylate. Using a microtome, serial 4 μm sections were cut in the plane of the modiolus; every 8th section was mounted on a glass slide and stained with toluidine blue prior to coverslipping.

All sections through the organ of Corti were traced using a camera lucida. The numbers of inner and outer hair cells were counted and cytocochleograms constructed showing the number of hair cells along each millimeter of the basilar membrane. Analyses were performed by comparing the number of inner and outer hair cells/mm in each experimental group with the number of inner and outer hair cells/mm in saline control guinea pigs, and expressing the result as percentage of cells missing.

Histological examination of cochleae following treatment with neomycin (Figure 4) revealed a complete (100%) loss of inner and outer hair cells in half-turns I and II (basal 7 mm of the cochlea), with severe (30-80%) hair cell loss in half-turns III-IV (7-11 mm from the apex), and moderate (0-20%) hair cell loss in half-turns V-VIII (1-7 mm from the apex, Figure 4). In contrast,

only moderate (17-20%) to severe (27-40%) hair cell loss in basal half-turn I was present in guinea pigs receiving neomycin and either dizocilpine (Fig. 3B, 4) or ifenprodil (Figure 4). In the more apical turns (1-17 mm from the base) of the cochlea, hair cell loss was minor (0-7%) following treatment with either NMDA antagonist, with a considerable reduction in damage to other structural elements of the cochlea compared to neomycin-treated animals (Figure 4).

EXAMPLE 4

Male, Sprague-Dawley rats (200-300 g, Taconic Farms, Germantown, NY) provided the brain tissue used for the radioligand binding assays. These animals were maintained under similar conditions as described in Example 1 for guinea pigs, but in separate facilities.

Radioligand Binding Assays

Rat cerebral cortex was homogenized in preparation buffers (15,16) with a Polytron (Brinkmann Instruments, Westbury, New York) and extensively "washed" (resuspended and recentrifuged) to remove endogenous materials (e.g. glycine, glutamate, polyamines) that affect radioligand binding to NMDA receptors. Extensively washed membranes were suspended in 5-10 volumes (original wet weight) of 5 mM HEPES/4.5 mM Tris (HTS) buffer (pH 7.4) at stored at -70°.

[^{3}H]Dizocilpine binding: Forebrain homogenates were thawed, diluted to a concentration of 1:50 with HTS, and washed twice. After the final centrifugation step, the pellet was resuspended in 25 vol of HTS. Assays were performed in duplicate using 1.5 ml plastic microtiter plates and consisted of 50 μl of aminoglycoside or spermine (1-3000 μM), 300 μl of tissue suspension, 50 μl [^{3}H]dizocilpine (final concentration, 3.5 nM), and HTS buffer to a volume of 500 μl. Nonspecific binding was

determined using dizocilpine (10 μM). Incubations were conducted at room temperature and terminated after 2 hours by rapid filtration over Schleicher and Schuell #32 filters (Keene, NH) pretreated with 0.03% polyethylenimine. Filters were rapidly washed with 2 x 2.5 ml of ice cold HTS.

[^{3}H]Ifenprodil binding: Extensively washed cortical homogenates were thawed, diluted to a concentration of 1:50 with HTS, and washed an additional two times. After the final centrifugation step, the pellet was resuspended in 5 volumes of HTS. Assays were performed in (e.g. 12 x 75 mm) glass tubes. Duplicate tubes contained: 50 μl of aminoglycoside (0.1 - 1000 μM), 50 μl membrane suspension, 50 μl (+)-pentazocine (10 μM, to block σ sites), 50 μl of [^{3}H]ifenprodil (5 nM) and HTS buffer to a final volume of 500 μl. Non-specific binding was determined using 10 μM ifenprodil. The assay was incubated at 4°C for 2 hours and terminated by rapid filtration over S+S #32 filters pretreated with 0.03% polyethylenimine. Filters were washed with 2 x 2.5 ml of ice-cold HTS. All binding data were analyzed by non-linear regression.

Aminoglycoside Modulation of Radioligand Binding to the NMDA Receptor

All seven aminoglycosides examined enhanced [^{3}H]dizocilpine binding to NMDA receptors prepared from rat forebrain (Table 3; Figure 5). Spectinomycin, which exhibits little or no ototoxicity (27, 28) was >20-fold less potent (EC_{50}=220±42 μM) than the ototoxic aminoglycosides. The potencies of these aminoglycosides to enhance [^{3}H]dizocilpine binding was highly correlated with their relative ototoxicities in humans (2) (Bonferroni-corrected Pearson correlation coefficient: 0.928, $P < 0.01$, Figure 5B), with streptomycin a prominent outlier. Endogenous polyamines such as spermine initially

enhance, and at higher concentrations inhibit [^{3}H]dizocilpine binding (29, 30). Among the ototoxic antibiotics examined, only neomycin, gentamicin and streptomycin exhibited this biphasic effect on [^{3}H]dizocilpine binding (Figure 5A, Table 3), and the potencies (IC_{50}) of these antibiotics to produce the secondary inhibition was negatively correlated with their relative ototoxicities in humans (2) (Bonferroni-corrected Pearson correlation coefficient = -0.838, $P < 0.01$).

Aminoglycoside antibiotics also inhibit [^{3}H]ifenprodil binding to polyamine-associated sites (15,31) on NMDA receptors with potencies similar to those required to enhance [^{3}H]dizocilpine binding (Table 3a). However, the IC_{50} values for aminoglycosides in this measure were not significantly correlated with their relative ototoxicities in humans (Bonferroni-corrected Pearson correlation coefficient: 0.36, $P = 0.09$).

Table 3. Aminoglycoside Modulation of [^{3}H]Dizocilpine Binding to NMDA receptors.

Compound	EC_{50}, [^{3}H]Dizocilpine, (μM)	E_{max} [^{3}H]Dizocilpine, (% increase)	IC_{50} [^{3}H]Dizocilpine, (μM)
Streptomycin	4.9±0.1	92±2.8	570±120
Neomycin	6.4±0.3	110±14	5700±1200
Amikacin	8.1±0.3	110±3.0	
Kanamycin	8.3±0.2	140±8.2	
Tobramycin	9.5±0.4	120±0.7	
Gentamicin	10±0.2	110±7.5	1300±78
Spectinomycin	220±42	100±9.7	
Spermine	5.3±0.7	110±13	580±190

Table 3a Aminoglycoside Modulation of [^{3}H]Ifenprodil Binding to NMDA Receptors.

Compound	IC_{50} [^{3}H]Ifenprodil, (μM)
Streptomycin	6.2±0.7
Neomycin	4.8±0.3
Amikacin	7.4±0.4
Kanamycin	5.6±0.3
Tobramycin	7.5±0.7
Gentamicin	5.7±0.3
Spectinomycin	230±25
Spermine	6.9*

EC_{50} values are the aminoglycoside concentrations that increase [^{3}H]dizocilpine (3.5 nM) binding to rat forebrain homogenates by 50% of maximum. E_{max} is the maximal enhancement of [^{3}H]dizocilpine binding expressed as the % increase above basal (non-stimulated) values. The E_{max} values were not significantly correlated with relative ototoxicities (Bonferroni-corrected Pearson correlation coefficient: 0.046, P=0.83). IC_{50} values are presented for those aminoglycosides exhibiting a biphasic effect on [^{3}H]dizocilpine binding. There was no apparent relationship between the IC_{50} values for inhibition of [^{3}H]ifenprodil binding and the relative ototoxicities of these aminoglycosides. Parameter values were obtained using nonlinear regression fitting of data to sigmoidal curves with the IC_{50} values for [^{3}H]dizocilpine binding determined by constraining the fit to control levels. Each value represents the mean ± SEM of 4 observations.

EXAMPLE 5

A variety of functional NMDA antagonists are tested in humans being treated with the aminoglycosides neomycin, gentamycin and kanamycin.

Patients are treated with 1-3 mg/kg gentamycin 3 times daily for the duration of an infection; 500-2000 mg neomycin 4 times daily for a period of 4 days; or 5-7.5 mg/kg amikacin every 8-12 hr. for the duration of the infection. The functional NMDA antagonists used in combination with these aminoglycosides include at least one of ifenprodil, memantine or dizocilpine.

EXAMPLE 6

The functional NMDA antagonists, dizocilpine and ifenprodil are administered to patients at dosages of 0.1 mg/kg/day and 2 mg/kg/day respectively prior to beginning aminoglycoside treatment. The bacterial infection is cured and the subject suffers no ototoxic damage.

The person of skill in the art will recognize that the invention herein can be modified in ways still within the scope of the invention. All references cited herein and in the section following are hereby incorporated by reference.

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CLAIMS:

1. A method of preventing or ameliorating aminoglycoside-induced ototoxicity comprising administering to a subject in need of preventing or ameliorating aminoglycoside-induced ototoxicity a therapeutically effective amount of a functional NMDA antagonist.

2. The method of claim 1 wherein the functional NMDA antagonist is administered preceding administration of an aminoglycoside antibiotic.

3. The method of claim 1 wherein the functional NMDA antagonist is administered concurrently with an aminoglycoside antibiotic.

4. The method of claim 1 wherein administration of the functional NMDA is continued for up to a month after the cessation of aminoglycoside antibiotic therapy.

5. The method of claim 1 wherein the functional NMDA antagonist is a quinoline compound.

6. The method of claim 1 wherein the functional NMDA antagonist is a non-quinoline compound.

7. The method of claim 6, wherein said NMDA receptor antagonist is administered over a period of time corresponding to the two half lives of said aminoglycoside antibiotic.

8. The method of claim 6, wherein the aminoglycoside antibiotic is selected from the group consisting of:

- gentamycin;

- streptomycin;
- kanamycin; and
- neomycin.

9. The method of claim 6, wherein the non-quinoline NMDA receptor antagonist is selected from the group consisting of:

- dizocilpine; and
- ifenprodil.

10. A bacteriocidal antibiotic composition comprising:

(a) a therapeutically effective amount of an aminoglycoside; and

(b) a functional NMDA antagonist provided in a dosage to control ototoxicity.

11. The antibiotic of claim 10, wherein said aminoglycoside is selected from the group consisting of:

- gentamycin;
- streptomycin;
- kanamycin; and
- neomycin.

12. The antibiotic composition according to claim 10 wherein said functional NMDA antagonist is capable of acting at an NMDA receptor glycine binding site.

13. The antibiotic composition according to claim 10 wherein said functional NMDA antagonist is capable of acting at an NMDA receptor glutamate binding site.

14. The antibiotic composition according to claim 10 wherein said functional NMDA antagonist is

capable of acting at an NMDA receptor polyamine/spermine binding site.

15. The antibiotic composition according to claim 10 wherein said functional NMDA antagonist is an NMDA receptor ion channel blocker.

16. The antibiotic composition according to claim 10 wherein said functional NMDA antagonist is selected from the group consisting of:

- 7-chlorokynurenic acid;
- indole-2-carboxylic acid;
- L-687414;
- HA-966; and
- 1-aminocyclopropane carboxylic acid.

17. The antibiotic composition according to claim 13 wherein said functional competitive NMDA antagonist is selected from the group consisting of:

- 2-(2-carboxypiperazin-4-yl) propyl-1-phosphonic acid;
- CGP 39653;
- CGP 40116;
- LY 274614; and
- LY 235959.

18. The antibiotic composition according to claim 14 wherein said functional polyamine site NMDA antagonist is selected from the group consisting of:

- diethylenetriamine;
- ifenprodil;
- eliprodil;
- conantokin-G; and
- arcaine.

19. The antibiotic composition according to claim 15 wherein said functional NMDA antagonist is selected from the group consisting of:

- 1-[1-(2-thienyl)cyclohexyl]-piperidine;
- dizocilpine;
- ketamine; and
- memantine.

20. The composition of claim 10, wherein said composition further comprises: a pharmaceutically acceptable carrier.

21. The composition of claims 10, wherein said composition is a solution for parenteral administration.

22. The composition of claims 1, wherein said composition is administered orally.

FIGURE 1

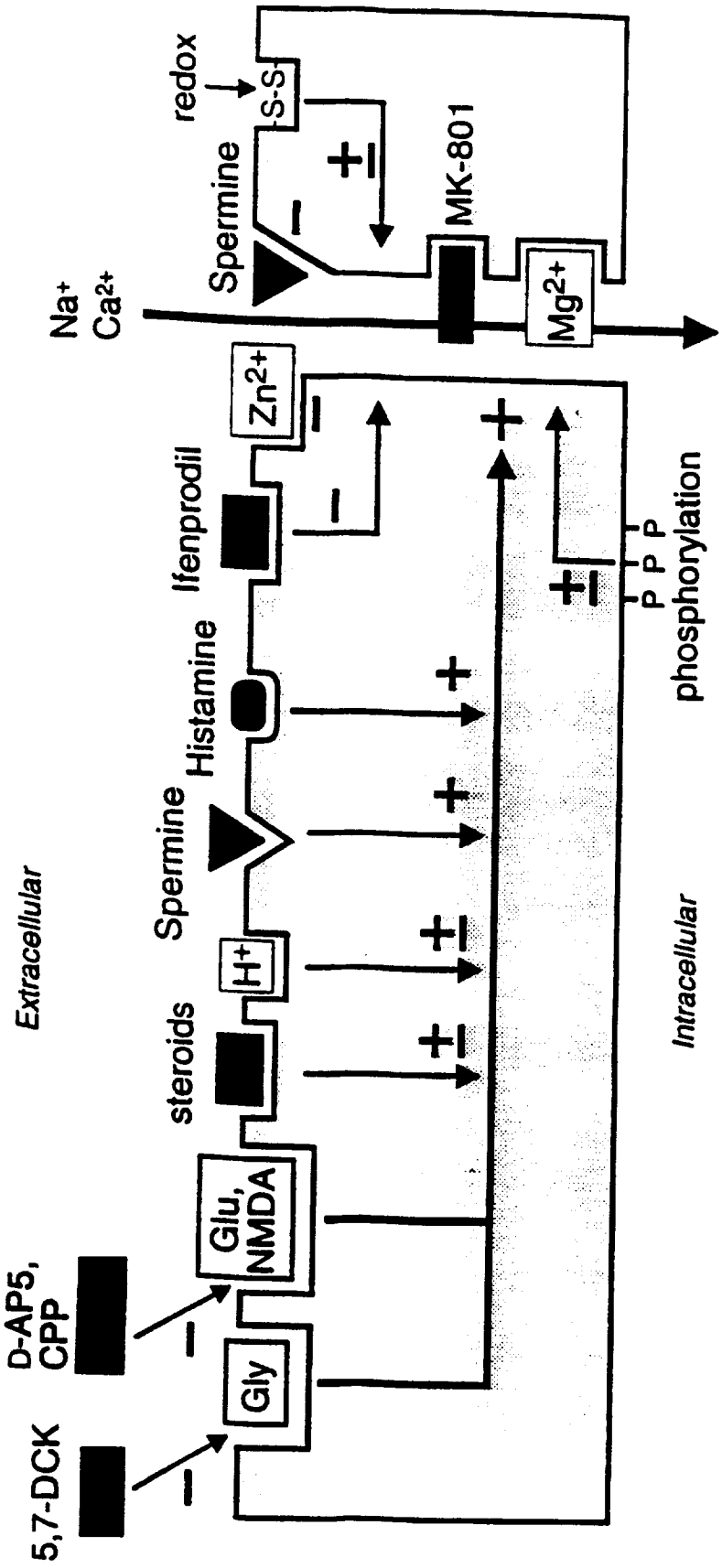

redox
S-S
Spermine
MK-801
Na+
Ca2+
Mg2+
Zn2+
Ifenprodil
Histamine
Spermine
H+
steroids
Glu, NMDA
Gly
D-AP5, CPP
5,7-DCK
Extracellular
Intracellular
P P P
phosphorylation

FIGURE 2

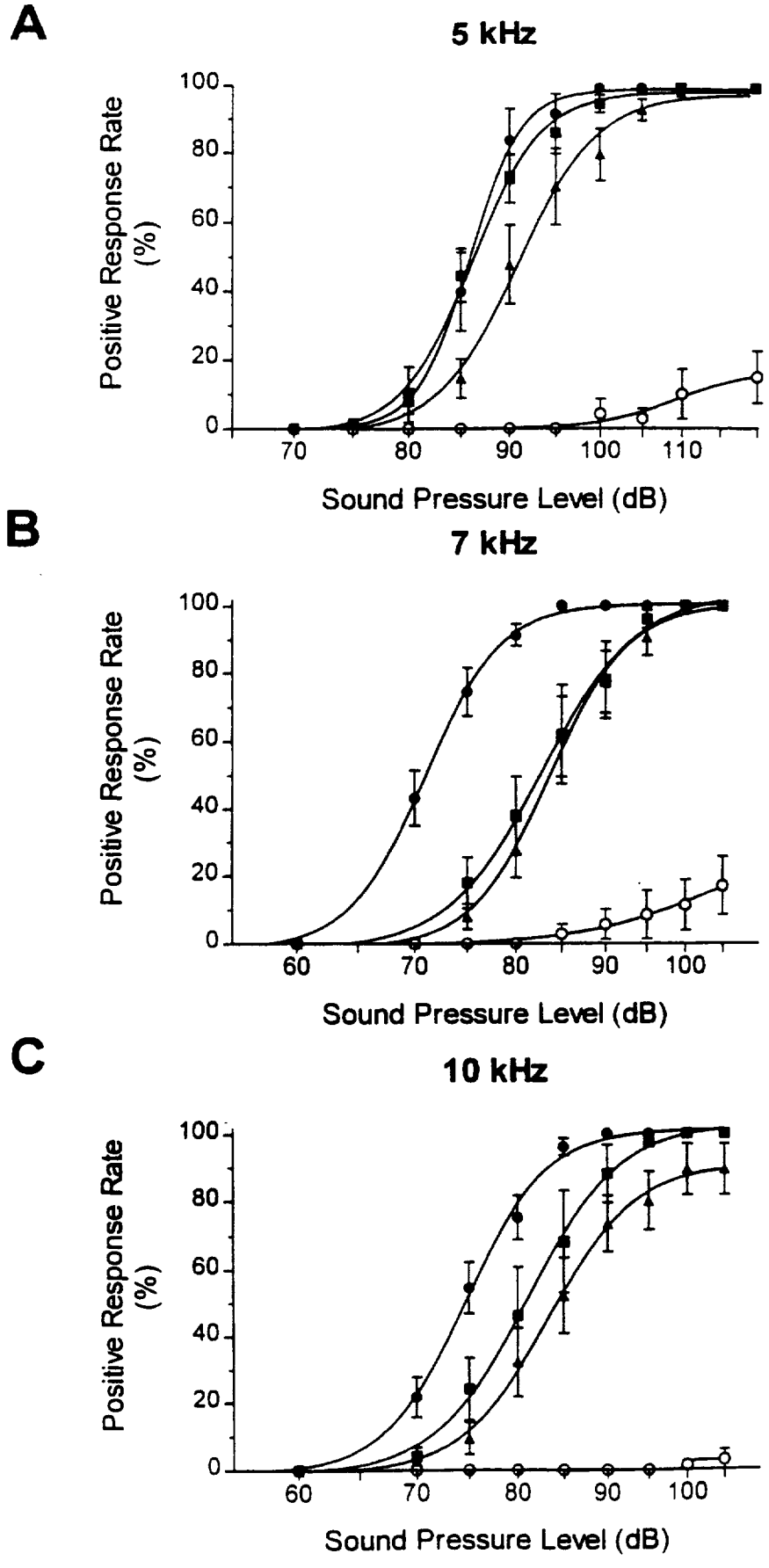

A
5 kHz
Positive Response Rate (%)
100
80
60
40
20
0
70
80
90
100
110
Sound Pressure Level (dB)
B
7 kHz
Positive Response Rate (%)
100
80
60
40
20
0
60
70
80
90
100
Sound Pressure Level (dB)
C
10 kHz
Positive Response Rate (%)
100
80
60
40
20
0
60
70
80
90
100
Sound Pressure Level (dB)

FIGURE 3

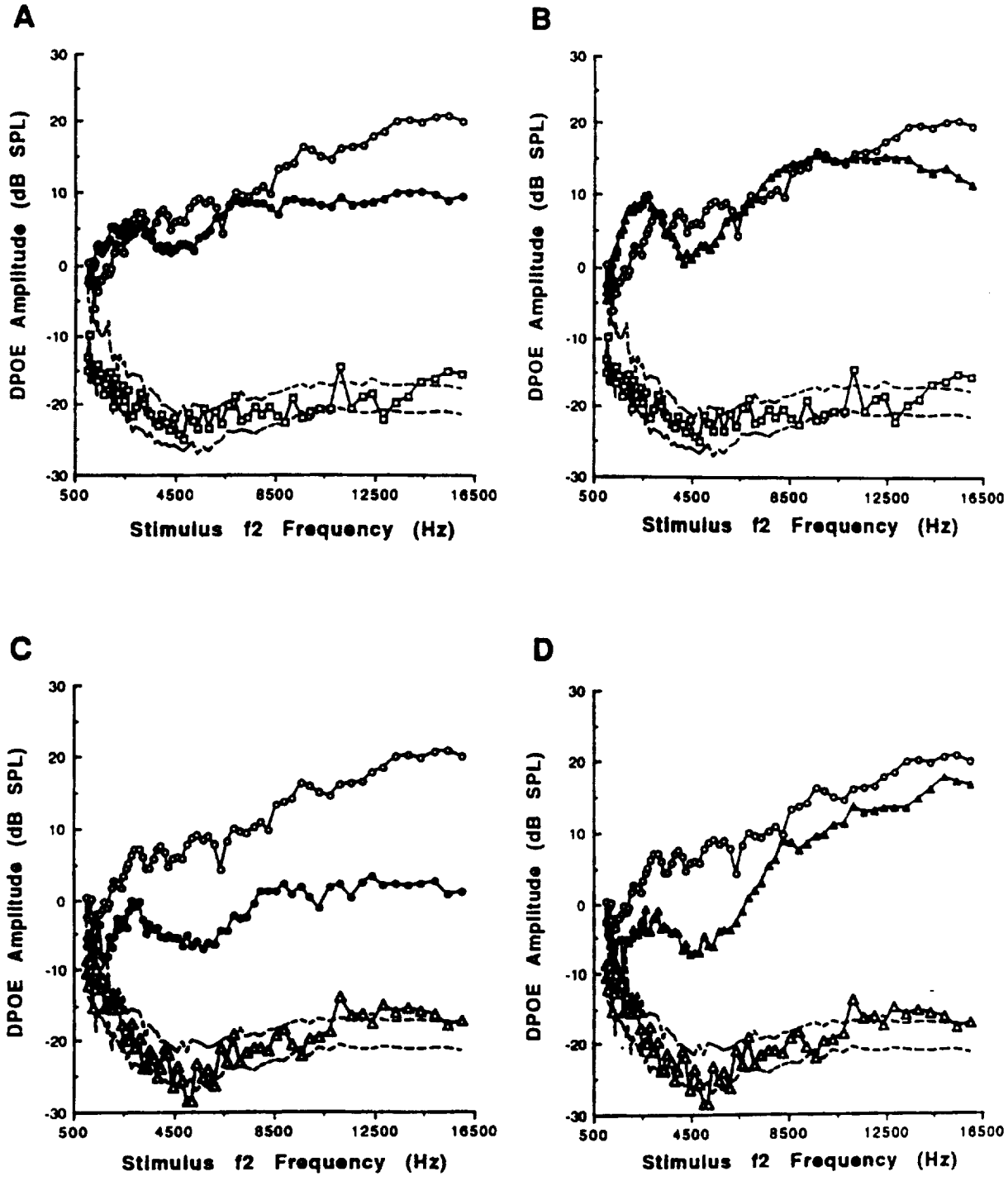
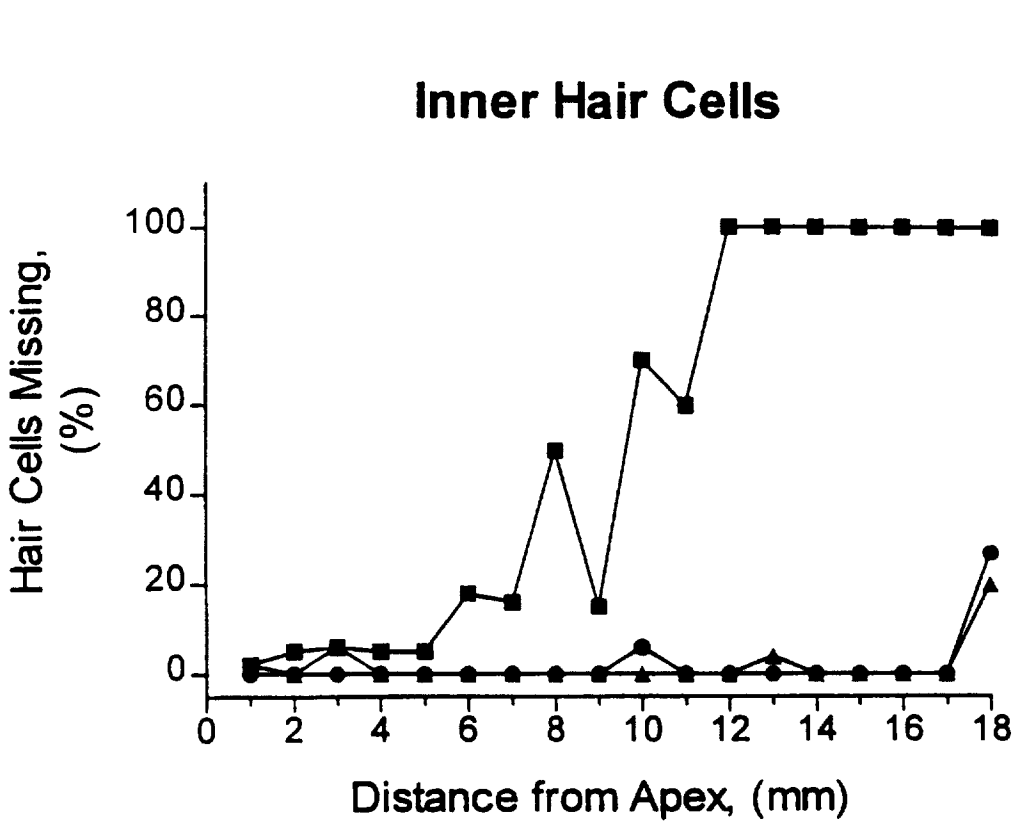

A
B
C
D
DPOE Amplitude (dB SPL)
30
20
10
0
-10
-20
-30
500
4500
8500
12500
16500
Stimulus f2 Frequency (Hz)

FIGURE 4

A


Inner Hair Cells
Hair Cells Missing, (%)
100
80
60
40
20
0
0
2
4
6
8
10
12
14
16
18
Distance from Apex, (mm)

B

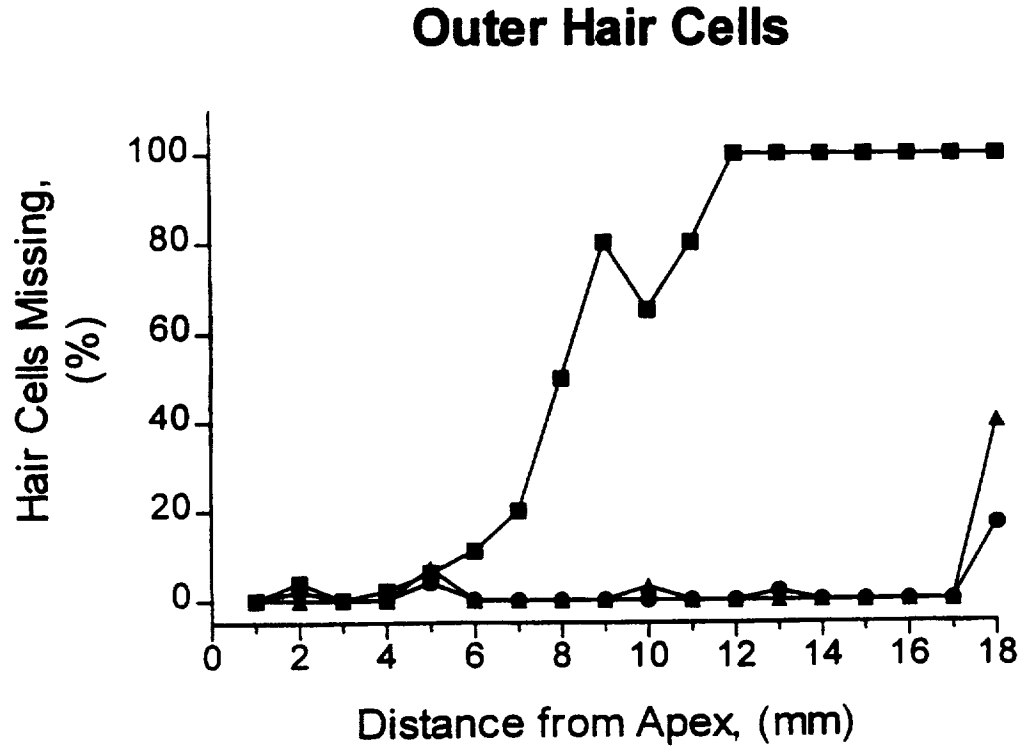

Outer Hair Cells
Hair Cells Missing, (%)
100
80
60
40
20
0
0
2
4
6
8
10
12
14
16
18
Distance from Apex, (mm)

FIGURE 5

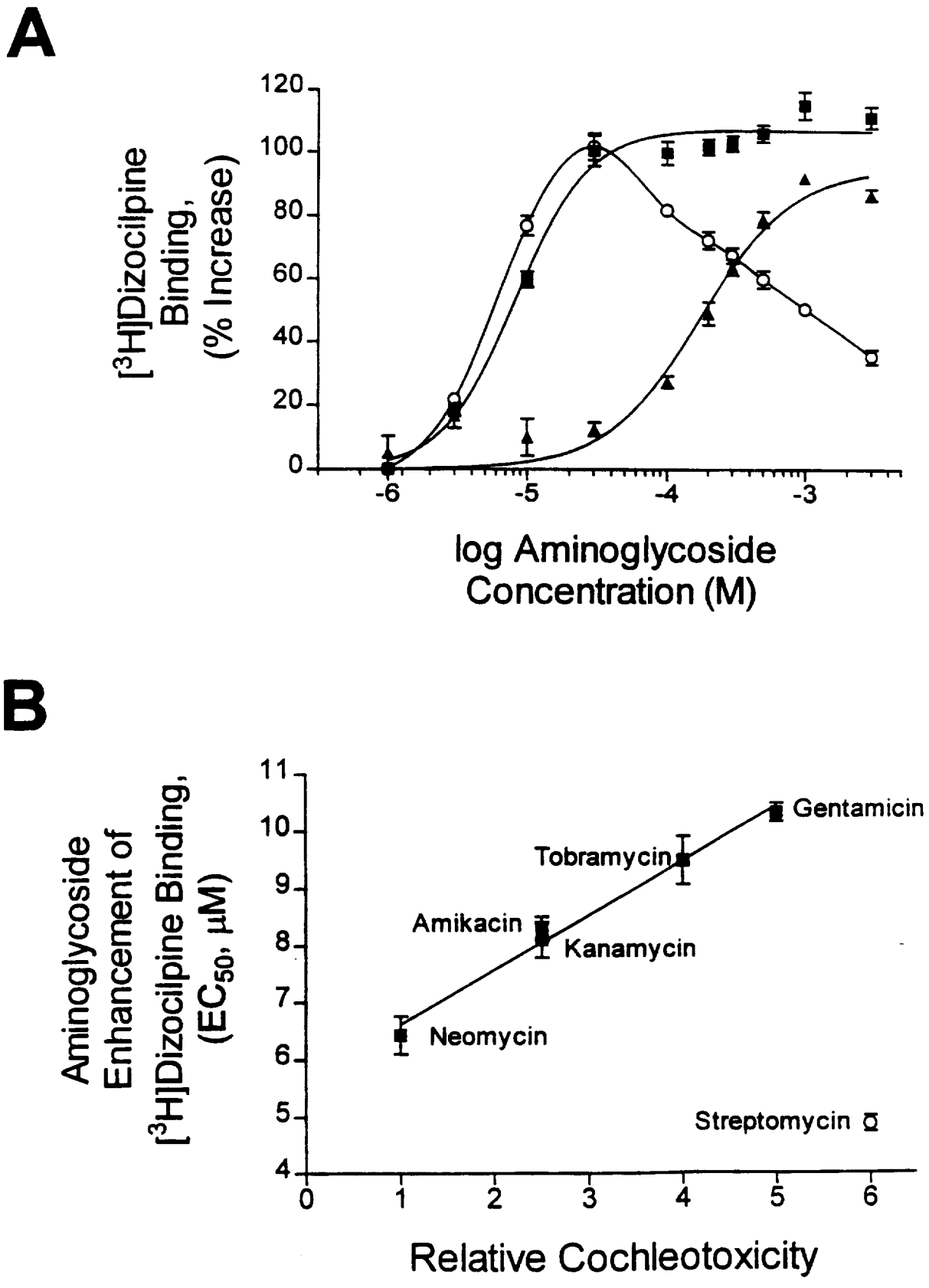
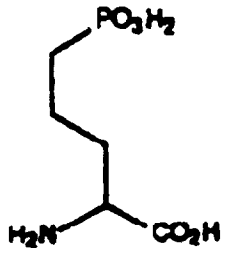
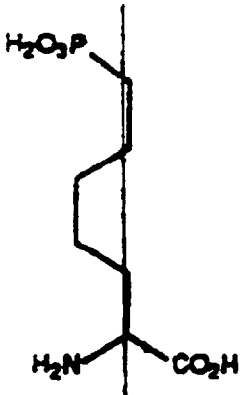

A
[3H]Dizocilpine Binding, (% Increase)
120
100
80
60
40
20
0
-6
-5
-4
-3
log Aminoglycoside Concentration (M)
B
Aminoglycoside Enhancement of [3H]Dizocilpine Binding, (EC50, μM)
11
10
9
8
7
6
5
4
0
1
2
3
4
5
6
Gentamicin
Tobramycin
Amikacin
Kanamycin
Neomycin
Streptomycin
Relative Cochleotoxicity

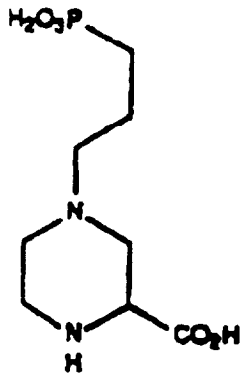
FIGURE 6



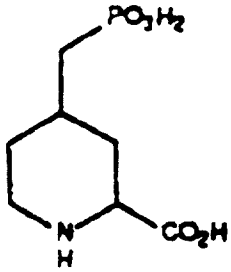
APS



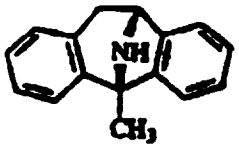
APT



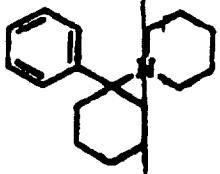
CPP



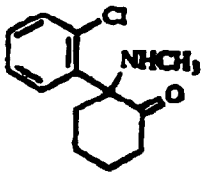
CGS 19755



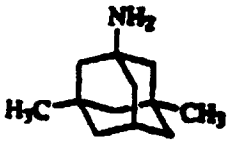
MK-801 (Dizocilpine)



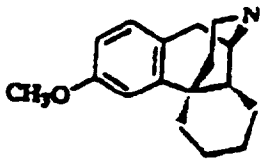
Phencyclidine (PCP)



Ketamine

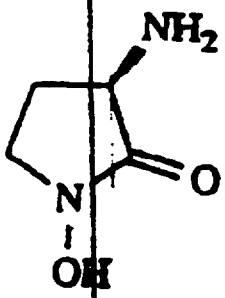


Memantine

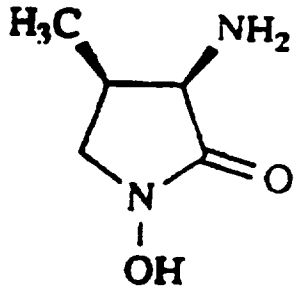


Dextromethorphan

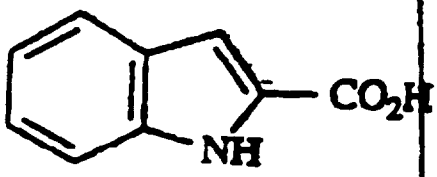
ACPC



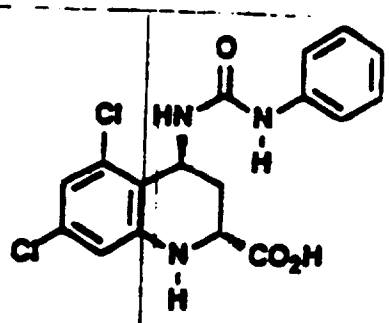
HA-966



L-687,414



Indole-2-carboxylic acid



(±)-*trans*-2-carboxy-5,7-dichlorotetrahydroquinoline-4-phenylurea (L689560; 0.0078 μM)