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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 06-098637- -00004-0000

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Act. 538 PETICION Folios: 2

Señora Jefe de la
DIVISION DE NUEVAS CREACIONES
Superintendencia de Industria y Comercio
E. S. D.

Trámite : 011
Evento : 378
Actuación : 538

Referencia : Expediente No. 06.098.637
Asunto : Solicitud de Patente para **COMPOSICIONES Y METODOS PARA APLICACION TOPICA Y SUMINISTRO TRANSDERMICO DE TOXINAS BOTULINUM**
Solicitante : **REVANCE THERAPEUTICS, INC**
Clase : A61K 051/000
Publicada en : La Gaceta No. **583** del 18 de diciembre de 2007
Fecha de Solicitud : 29 de septiembre de 2006

1. Yo, Emilio FERRERO, identificado como aparece al pie de mi firma, actuando como apoderado en el asunto de la referencia, atentamente, solicito se efectúe el examen de patentabilidad de la solicitud en referencia presentada de acuerdo con el Capítulo I del PCT y según lo establecido en el artículo 44 de la Decisión 486 de la Comisión de la Comunidad Andina. Para tal efecto presento junto con este escrito el recibo por valor de \$ 420.000.00, que corresponde a la tasa fijada según lo dispuesto en la resolución No. 44372 de 26 de diciembre de 2007.

2. Ruego atentamente a la Señora Jefe se sirva ordenar que se anexe este recibo para que se efectúe dicho examen y se continúe con el trámite de la solicitud.

Señora Jefe,

EMILIO FERRERO
T.P.A. No. 31.929

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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

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***** CONSIGNACION *****

DEPOSITANTE	TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PGO	Vr. PAGO
CAVELIER ABOGADOS	CONSIGNACION	BANCO DE BOGOTA	062754387	2678328	09/06/2008	89,445,000.00

***** CONCEPTO *****

CANT. RENTISTICO	CONCEPTO	TOTAL CONCEPTO
50005-01-01 SOLICITUDES	85 EXAMEN DE PATENTABILIDAD DE INVEN	420,000.00
	TOTAL :	\$ 420,000.00

SON: CUATROCIENTOS VEINTE MIL PESOS

RESPONSABLE :

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DIVISIÓN DE NUEVAS CREACIONES

RADICACIÓN EXPEDIENTE No. 06-98637

EL EXTRACTO DE LA PRESENTE SOLICITUD DE **PATENTE DE INVENCION**

FUE PUBLICADO EN LA GACETA DE PROPIEDAD INDUSTRIAL No **583** DE

FECHA **18 DE DICIEMBRE DE 2007** EL TÉRMINO HÁBIL SEÑALADO POR LA

LEY EXPIRA EL **14 DE MARZO DE 2008**

NOTA: Dentro del plazo de los seis (6) meses siguientes a partir de la fecha de publicación en la gaceta de Propiedad Industrial, deberá solicitar y cancelar el examen de patentabilidad so pena de que la solicitud caiga en abandono, de acuerdo con lo establecido en el Artículo 44 de la Decisión 486/2000.

SE PRESENTARON OPOSICIONES POR PARTE DE TERCEROS.

SI _____

NO X



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196
106(19) **United States**(12) **Patent Application Publication**
Goodnough et al.(10) **Pub. No.: US 2003/0147921 A1**(43) **Pub. Date: Aug. 7, 2003**(54) **METHOD OF TARGETING
PHARMACEUTICALS TO MOTOR
NEURONS**(76) **Inventors: Michael C. Goodnough, Stoughton, WI
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MILWAUKEE, WI 53202-4497 (US)(21) **Appl. No.: 09/870,262**(22) **Filed: May 30, 2001****Related U.S. Application Data**(60) **Provisional application No. 60/208,516, filed on Jun.
1, 2000.****Publication Classification**(51) **Int. Cl.⁷ A61K 39/08**
(52) **U.S. Cl. 424/239.1; 514/12**(57) **ABSTRACT**

A method of targeting therapeutic molecules to motor neurons is disclosed. In one embodiment, this method comprises the steps of (a) synthesizing a prodrug comprising a therapeutic molecule covalently bound to a polymeric delivery vehicle, and (b) conjugating the prodrug to a botulinum neurotoxin heavy chain.

METHOD OF TARGETING PHARMACEUTICALS TO MOTOR NEURONS

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims priority to U.S. Serial No. 60/208,516, filed Jun. 1, 2000, which is incorporated by reference herein.

BACKGROUND OF THE INVENTION

[0002] Botulinum neurotoxin is an ongoing public health problem and is considered to be a potentially serious biological warfare agent. Recent worldwide events including the discovery of stockpiles of materials for manufacture of the toxin by extremist groups in Japan and Iraq have shown that the danger exists for biological terrorism. Intoxication from the toxin by consumption in food or water or from inhalation can lead to prolonged paralysis, incapacitation, and need for intensive supportive care. Depending on the route of administration, death can result from exposure to less than a microgram of toxin. There are no effective therapies once symptoms of botulism appear other than supportive treatments such as intubation and ventilation.

[0003] Within the last five years a number of potential therapies have been identified (e.g. aminoquinolines, metalloprotease inhibitors, aminopyridines. Adler, et al., 1996), and zinc chelators such as TPEN (Adler, et al., 1997). None of these are likely to work in vivo due to difficulty in penetration into the neuronal cytosol and high systemic toxicity. Although better metalloprotease inhibitors have recently been developed at the University of Wisconsin-Madison (Dr. Daniel Rich), these are likely to be limited by the same problems of membrane penetration and toxicity as the first generation of drugs. Anything that crosses lipid membranes easily will also be likely to cross the blood-brain barrier and have CNS toxicity. Any potential therapeutic compound that cannot be internalized into the nerve terminal cytosol will not be capable of antagonizing the active component of botulinum neurotoxin.

BRIEF SUMMARY OF THE INVENTION

[0004] In one embodiment, the present invention is a method of targeting therapeutic molecules to motor neurons. This method preferably comprises the step of (a) synthesizing a prodrug comprising a therapeutic molecule covalently bound to a polymeric delivery vehicle and (b) conjugating the prodrug to a botulinum neurotoxin heavy chain. In a preferred method of the present invention, the prodrug comprises at least two copies of the therapeutic molecule.

[0005] In another preferred method of the present invention, the botulinum neurotoxin heavy chain is type A botulinum neurotoxin and the therapeutic molecule is a metalloprotease inhibitor.

[0006] It is an object of the present invention to target therapeutic molecules to motor neurons.

[0007] Other objects, features and advantages of the present invention will become apparent after one has reviewed the specification, claims and drawings.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

[0008] FIG. 1. SDS-PAGE of purified type A neurotoxin (lane 1, unreduced; lane 2, reduced), light chain (lane 3), and heavy chain (lane 4).

[0009] FIG. 2. Covalent modification of free thiol groups in type A botulinum neurotoxin.

[0010] FIG. 3. Separation and purification of thiol modified type A botulinum heavy chain.

[0011] FIG. 4. Structure of amino-dextran.

[0012] FIG. 5. Structure of the heterobifunctional linker sulfo-succinimidyl-6-[alpha-methyl-alpha(2-pyridylthio)-toluamido]hexanoate (sulfo-LC-SMPT).

[0013] FIG. 6. sulfo-LC-SMPT activated dextran.

[0014] FIG. 7. Release of 2-thiopyridine leaving group from sulfo-LC-SMPT activated dextran.

[0015] FIG. 8. Inhibition of ACE by reduced prodrug.

[0016] FIG. 9. Structure of captopril.

[0017] FIG. 10. Captopril bound to functionalized amino-dextran.

[0018] FIG. 11. Oxidation of hydroxyl groups to carbonyl groups and subsequent reaction with PDPH.

[0019] FIG. 12. Overall reaction scheme for conjugation of prodrug to botulinum heavy chain.

[0020] FIG. 13. Cy 3.5 monofunctional dye molecule. Molecular weight 1102. Absorbance max=581 nm. Emission max=596 nm.

[0021] FIG. 14. Reaction of type A botulinum heavy chain with PDPH functionalized Cy 3.5 amino-dextran.

DETAILED DESCRIPTION OF THE INVENTION

[0022] Our solution to the above-stated problem is to use a modified, nontoxic form of the botulinum neurotoxin molecule to specifically target and deliver currently identified therapeutics as well as compounds developed in the future to the appropriate cholinergic motor neuron terminals. Once delivered to the presynaptic motor neuron membrane, the chimeric delivery vehicle preferably consisting of the heavy chain of the neurotoxin covalently linked with a polymeric molecule capable of delivering multiple copies of therapeutic compounds will bring the desired therapeutic across the membrane and into the neuron where the drug will be an effective therapeutic. In one embodiment, the drug may be an antagonist of botulinum neurotoxin. In another embodiment, the drug is an enzymatic inhibitor, including peptide inhibitors.

[0023] In one embodiment, the present invention is a method of targeting pharmaceuticals to motor neurons, comprising a first step of synthesizing prodrugs comprising at least one copy, and preferably multiple copies, of a therapeutic molecule (the Examples below describe the model metalloprotease inhibitor captopril as a therapeutic molecule) covalently bound to a polymeric delivery vehicle. The second step comprises conjugating the prodrug to the heavy chain of botulinum neurotoxin, preferably type A. This combination will yield a pharmaceutically active compound that specifically targets and internalizes desired substances into cholinergic neurons including active inhibitors of the active fragment of the neurotoxin itself.

[0024] We envision that the method of the present invention will be suitable for all heavy chains of botulinum

neurotoxin. The Examples below describe a preparation using type A heavy chain. Other heavy chains (i.e., all seven serotypes of heavy chains, A-G) can be prepared in a similar manner. One may obtain the heavy chain in a variety of manners. Preferably, one will obtain heavy chains as described below in the Examples. In another embodiment of the present invention, one will obtain the heavy chain via recombinant DNA methods.

[0025] Polymeric delivery vehicle is an inert carrier molecule ranging in size from 10-40 kD that can be chemically modified such that therapeutic molecules may be covalently linked to it.

[0026] The polymeric delivery vehicle preferably comprises amine reactive dextran, described below as having a molecular weight of approximately 10 kD and 40 kD. Other suitable polymers include polyethylene glycol and polyimines.

[0027] Dextran amines were preferably functionalized for conjugation with model inhibitors by addition of the water soluble heterobifunctional linker sulfo-succinimidyl-6-[alpha-methyl-alpha(2-pyridylthio)-toluamido]hexanoate (sulfo-LC-SMPT, FIG. 5). Other suitable linkers include heterobifunctional linkers containing amine and sulfhydryl reactive elements. Also useful are heterobifunctional linkers that are carboxy reactive as well as sulfhydryl reactive.

[0028] The conjugation of the prodrugs to the heavy chain of botulinum neurotoxin will typically take place as follows:

[0029] We envision two preferable approaches to the synthesis of prodrugs that contain high molar concentrations of model inhibitor and have a traceable label.

[0030] Approach #1. We currently use specific dextrans that are amine reactive and contain a fluorescent marker such as fluorescein, tetramethylrhodamine, or Cy3.5. The availability of fluorescent dextran conjugates with different sizes and charges will permit us to synthesize a wide variety of prodrugs. These labels are commercially available from Molecular Probes, Eugene, Oreg. and from Pharmacia Amersham, Piscataway, N.J.

[0031] The initial step in this approach will be to generate carbonyls on the dextran that are reactive to a hydrazide containing heterobifunctional linker. The preferred linker of choice is 3-(2-pyridylthio)propionyl hydrazide (PDPH). The generation of hydrazide reactive carbonyls on the dextran is accomplished by gentle oxidation using sodium periodate according to the method of Ranadive, et al. (G. Ranadive, et al., *Nucl. Med. Biol.* 20:719-726, 1993). Briefly, this reaction involves reacting the dextran with 5-10 mM sodium periodate in 100 mM sodium acetate, pH 5.5, for 20-30 minutes in the dark at room temperature. The oxidation reaction is quenched with addition of glycerol to 20 mM final concentration. The dextran is dialyzed against 100 mM sodium acetate, pH 5.5, to remove glycerol and periodate and linker is added to a final concentration of 5-10 mM. This reaction is allowed to proceed for 2 hours at room temperature at which time excess linker is removed from the dextran by applying the mixture to a desalting column such as the Pharmacia PD10.

[0032] The desalted, derivatized dextran is then reacted with captopril. The reaction can be monitored by appearance of the leaving group 2-thiopyridine having a characteristic

absorbance at 343 nm. After 2 hours incubation at room temperature, excess captopril is removed by desalting on PD10 columns. The amine groups on the dextran are then reacted with the linker sulfo-LC-SMPT. This generates free thiol groups that can then be conjugated directly to botulinum heavy chain via disulfide exchange.

[0033] Approach #2. The second approach to conjugating prodrug to heavy chain involves synthesis of prodrug using PDPH-activated amino dextran. Amine-reactive pharmaceutical compounds are added to PDPH-modified amino dextran and allowed to cross-link with the free amine groups present on the dextran. Following removal of excess pharmaceutical by desalting on PD10 columns or extensive dialysis in 25 mM phosphate buffered saline, pH 7.4, the prodrug is added to botulinum heavy chain and the free thiol group(s) on the heavy chain allowed to form disulfide bonds with the thiol reactive portion of the linker PDPH. The reaction is allowed to proceed overnight at 4° C. Excess prodrug is readily separated from the heavy chain-prodrug conjugates by addition of ammonium sulfate to 60% saturation. Under these conditions heavy chain-prodrug and unreacted heavy chain precipitate while the carbohydrate-based prodrug remains in solution.

[0034] A second approach to separation of unreacted dextran from botulinum heavy chain can be accomplished by immobilized-metal affinity chromatography (IMAC) according to the method of Schiavo, et al. (G. Schiavo, et al., *Infect. Immun.* 58:4136-4141, 1990). Briefly, chelating Sepharose (Pharmacia) charged with Zn is used to bind the heavy chain of botulinum toxin and heavy chain-polymer constructs. Unreacted dextran is washed from the column with 5-8 column volumes of running buffer and the heavy chain/polymer constructs eluted with 25 mM imidazole.

[0035] Preferred therapeutic agents include protease inhibitors. Specific inhibitors include aprotinin, chymostatin, amastatin, bestatin, leupeptin, antipain, trypsin inhibitors, chelating agents such as EDTA, TPEN, o-phenanthroline, and peptide inhibitors of proteases. Peptide inhibitors such as caspase inhibitors (apopain series) which are primarily four and five amino acid inhibitors of caspases are also preferred. Metalloprotease inhibitors such as phosphoramidon, pepstatin A, phebestin, and the TAPI series of matrix metalloprotease inhibitors are also preferred.

[0036] The prodrug-heavy chain may be targeted to specific muscle groups by directly injecting the affected muscles. Alternatively, one could deliver the prodrug-heavy chain combination orally.

EXAMPLES

[0037] Bacterial Strains and Culturing.

[0038] The Hall A strain of type A *C. botulinum* was used to produce crystalline type A complex. This strain was originally obtained from Dr. J. H. Mueller at Harvard University and was further screened for high toxin titers at Fort Detrick, Md. by Dr. E. J. Schantz and coworkers. This strain is routinely used for production of type A botulinum toxin due to high toxin titers and the rapid onset of cell lysis (usually within 48 hours).

[0039] Stock cultures of *C. botulinum* Hall A were grown statically in 15 ml Hungate tubes containing 10 ml of cooked meat medium +0.3% dextrose (CMM, [Difco Laboratories,

Detroit, Mich.)) under an anaerobic atmosphere (80% N₂, 10% CO₂, 10% H₂) at 37° C. for 24 hours and frozen at -20° C. until use. CMM cultures of the Hall A strain give toxin titers in excess of 10⁶ LD₅₀/ml in 48-72 hours.

[0040] For toxin production, cultures of Hall A were grown statically in 10 liter volumes of toxin production medium (TPM) consisting of 2.0% NZ TT (lot #9NC29) casein hydrolysate (Sheffield Laboratories, Norwich, N.Y.), 1.0% yeast extract (Difco), and 0.5% dextrose, pH 7.3-7.4, for 5-7 days at 37° C. Cultures of Hall A showed heavy growth in this medium during the first 24-48 hours followed by autolysis of the culture which was evident as a clearing and settling over the next 48-120 hours.

[0041] Toxin Purification.

[0042] The production and purification of type A neurotoxin for use as the starting reagent in subsequent chain separations and purifications was successful. As shown in FIG. 1, lanes 1 (unreduced) and 2 (reduced), purification of three batches of type A botulinum neurotoxin having an average specific toxicity of 1.5×10⁶ mouse intraperitoneal lethal doses per mg of protein was successful. Three separate batches of toxin were cultured and purified to homogeneity. Subsequent purification of the component light and heavy chains were performed with yields shown in Table 1.

TABLE 1

Yields of toxin at various stages of purification ¹ . (Roman numerals refer to individual purification steps described below.)			
	Batch 1	Batch 2	Batch 3
I. Crude culture ²	1.5 × 10 ⁶	2.0 × 10 ⁶	3.0 × 10 ⁶
II. Toxin complex ¹	200	225	275
III. Partially purified neurotoxin ¹	38	47	60
IV. Purified neurotoxin ¹	20	23	30
V. Light chain ¹	5	5.5	9
VI. Heavy chain ¹	6	7	11

¹mg of protein.

²LD₅₀/ml of crude culture.

[0043] We fortuitously determined that toxin production in crude culture could be increased by addition of specific mineral salts to production medium. The addition of magnesium (as magnesium chloride 100 mM final concentration) and calcium carbonate (400 mM final concentration) allowed us to double the amount of toxin produced in culture over the course of this research. This will be extremely important in subsequent scale-ups. As can be seen in Table 1, the amount of toxin complex obtained from Batch 3 vs. Batch 1 did not double as expected based on titers of crude cultures.

[0044] Toxin Purification Steps Noted in Table 1.

[0045] I. Cultures of *C. botulinum* were grown in 10 liter batches of 2% casein hydrolysate, 0.5% dextrose, 0.5% yeast extract, pH 7.4, for 4-5 days at 37° C. Cultures were harvested at this time by addition of 3N H₂SO₄ to a final pH of 3.4. Crude toxin precipitated and was collected by centrifugation.

[0046] II. Toxin complex was extracted from the acid precipitate by suspending in 200 mM sodium phosphate, pH 6.0. The first extraction was allowed to proceed for 2-3 hours at room temperature. Extracted

toxin was separated from cellular debris by centrifugation and the pellet reextracted in similar fashion overnight at 4° C. The extracts were pooled and precipitated with ammonium sulfate (39 g/100 ml). Precipitated extracts were collected by centrifugation and dissolved in 50 mM sodium citrate, pH 5.5. This material was loaded onto DEAE-Sephadex columns equilibrated with the same buffer. Toxin complex eluted essentially unretarded and was pooled and precipitated with ammonium sulfate (39 g/100 ml).

[0047] III. Neurotoxin was separated from toxin complex proteins by dialyzing precipitated complex against 3 changes of 20 mM sodium phosphate, pH 7.9. Dialyzed toxin complex was bound to DEAE-Sephadex equilibrated with the same buffer. Partially purified neurotoxin was eluted as the first peak with a 0-300 mM sodium chloride gradient.

[0048] IV. Purified neurotoxin was separated from minor amounts of contaminating complex proteins by dialyzing the neurotoxin peak eluted in step III against 20 mM sodium phosphate, pH 7.0, and applying the material to SP-Sephadex equilibrated with the same buffer. Under these conditions contaminating complex proteins did not bind to the matrix. Purified neurotoxin was eluted with a 200 mM sodium chloride step. Neurotoxin recovered from this step was subsequently modified with iodoacetamide thereby alkylating free thiol groups. The rationale for this is described in detail in the next section of this report.

[0049] V. Light chain was purified as a by-product of heavy chain purification. Purified neurotoxin was dialyzed against 20 mM sodium borate, 40 mM sodium phosphate, pH 8.4, and applied to QAE-Sephadex equilibrated with the same buffer. After binding neurotoxin to the column, one-half column volume of loading buffer plus 10 mM dithiothreitol (DTT) was applied to the column. A second one-half column volume of loading buffer plus 100 mM DTT and 2 M urea was applied and the column held over night at 4° C. to allow reduction of disulfide bonds. Light chain was eluted with loading buffer plus 10 mM DTT and 2 M urea. Heavy chain and contaminating unnicked neurotoxin were eluted with loading buffer containing 10 mM DTT, 2 M urea, and 500 mM sodium chloride.

[0050] VI. Heavy chain containing trace amounts of unnicked neurotoxin was dialyzed against 20 mM sodium phosphate, 5 mM DTT, pH 7.5. The heavy chain was then applied to a column of DEAE-Sephadex equilibrated with the same buffer. Contaminating unnicked neurotoxin was separated from heavy chain by use of a linear 0-600 mM sodium chloride gradient with unnicked neurotoxin eluting at around 100 mM sodium chloride and heavy chain eluting at about 400 mM sodium chloride.

[0051] Neurotoxin Modification.

[0052] Purified type A botulinum heavy chain has six cysteine residues. Of these six thiol groups, two are involved in an intrachain disulfide bond and are not available for reaction with other compounds as the reduced thiols are in such close proximity to each other that they quickly reform a disulfide linkage following removal of the reducing agent

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(FIG. 2). Treatment of intact neurotoxin with the thiol alkylating agent iodoacetamide yielded a molecule with 0-1.2 free thiols on average as assessed by titration with bis-dithionitrobenzoic acid (Ellman, 1959). This material retained greater than 80% of the starting toxicity as measured by mouse bioassay. Purification of heavy chain was subsequently performed according to the method of Sathyamoorthy and DasGupta (1985) as shown in FIG. 3.

[0053] Electrophoresis.

[0054] Protein samples were examined electrophoretically using the Pharmacia Phastsystem (Pharmacia LKB Inc., Piscataway, N.J.) according to the manufacturers instructions. Precast 12.5% acrylamide gels (Pharmacia) were stained with 0.1% coomassie brilliant blue R250 in 16.7% acetic acid, 41.7% methanol. Gels were destained in 7.5% acetic acid, 25% methanol. Samples for electrophoresis were solubilized in 50 mM Tris-HCl, 5 M urea, 5% SDS, 20% glycerol, pH 6.8. Some samples were reduced by addition of dithiothreitol to a final concentration of 0.5%. All samples for SDS-PAGE were boiled for >5 minutes prior to electrophoresis.

[0055] Toxin Assays.

[0056] Toxin titers were estimated in mice using the intravenous method of Boroff and Fleck (1966) and the intraperitoneal method of Schantz and Kautter (1978) in 18-22 g, female, ICR strain mice. Time-to-death values obtained from intravenous titration of type A toxin samples were converted to intraperitoneal LD_{50} /ml using a standard curve generated in our laboratory with type A complex. Botulinum toxin for titration was dissolved in 50 mM sodium phosphate, pH 6.8, and then further diluted as required in 30 mM sodium phosphate, 0.2% gelatin, pH 6.4.

[0057] Structure of Prodrugs.

[0058] Polymers selected for reaction with specific linkers included amine reactive dextrans (Molecular Probes, Eugene, Oreg.) having molecular weights of approximately 10 kD and 40 kD. These dextrans had on average 4.8 moles amine per mole of dextran (10 kD) and 6.7 moles amine per mole of dextran (40 kD). The general structures of these compounds are shown in FIG. 4.

[0059] Dextran amines were functionalized for conjugation with model inhibitors by addition of the water soluble heterobifunctional linker sulfo-succinimidyl-6-[α -methyl- α -(2-pyridylthio)-toluamido]hexanoate (sulfo-LC-SMPT, FIG. 5).

[0060] Linker was added to amine dextrans at 20:1 molar ratios (20 mole linker:1 mole amine). Linker addition reactions were performed in 100 mM sodium phosphate buffer, pH 9.2, and were allowed to continue for 2 hours at room temperature. This pH favored addition of linker to polymer by reaction of the succinimidyl ester on the linker with the primary amine of the polymer. Unreacted linker was removed by either size exclusion chromatography in the case of the 40 kD dextran on Pharmacia PD10 columns (Pharmacia Biotechnology, Piscataway, N.J.) equilibrated with 25 mM sodium phosphate buffered saline, pH 7.4, (PBS) or by extensive dialysis vs. PBS in Slide-A-Lyzer dialysis cassettes (3.5 kD cutoff, Pierce Biochemical Co., Rockford, Ill.) in the case of the 10 kD dextran. The resulting compounds are shown in FIG. 6.

[0061] Linker addition to dextran was confirmed by gentle reduction of functionalized polymer with dithiothreitol. This reaction liberated 2-thiopyridine from the linker which was detected by its characteristic absorbance at 343 nm. In one set of experiments, the final absorbance of 0.77 yielded a molar concentration of the leaving group of 96 μ M indicating that the amine groups on the dextran at a concentration of approximately 750 μ g/ml were over 75% modified by the linker. This is shown in FIG. 7. These reaction kinetics gave nearly identical results with both the 10 kD and the 40 kD amino-dextrans.

[0062] We have used the metalloprotease inhibitor captopril as a model pharmaceutical ligand. This ligand has been covalently coupled to linker modified amino dextran via the reactive 2-thiopyridine group. The structure of captopril is shown in FIG. 9.

[0063] The reaction kinetics (A_{343} increase) for the displacement of the 2-thiopyridine leaving group from functionalized amino-dextran by captopril were nearly identical to those generated by the reducing agent dithiothreitol indicating that captopril was a good nucleophile and was rapidly bound to the dextran polymer via disulfide bond exchange with the linker. These prodrugs are shown in FIG. 10.

[0064] We envision synthesizing prodrugs that contain 5-10 $\times$ more inhibitor (captopril) than the first generation prodrugs. These prodrugs will have a fluorescent label incorporated into them for subsequent in vitro and in vivo visualization and will be conjugated to botulinum heavy chain for targeting and internalization into motor neurons.

[0065] We will continue to use dextran based polymers for the synthesis of prodrugs because they are relatively non-toxic and are biologically inert due to the uncommon poly-(D-1,6-glucose) linkages. They also have low immunogenicity.

[0066] Prodrug Assay.

[0067] We have determined that prodrugs based on the metalloprotease inhibitor captopril described here inhibit angiotensin converting enzyme (ACE) only when they are released from the dextran carrier by reduction of the disulfide bond. This is advantageous because it sequesters potential therapeutic compounds until they are introduced into the reducing environment of the motor neuron. We used a modification of the assay described by Cushman and Cheung (1971) to measure inhibition of the enzyme by captopril. Cleavage of the substrate by ACE yields hippuryl which is soluble in ethyl acetate. Following ethyl acetate extraction of the reactions, the solvent is removed by evaporation and the residue dissolved in water. Hippuryl concentration is measured by absorption at 228 nm.

[0068] Inhibition of ACE was accomplished by addition of known quantities of free captopril. The IC_{50} of captopril for this particular reaction was calculated to be approximately 300 nM. When dextran-captopril conjugates were added to the reaction we did not obtain any inhibition until the disulfide bond between the dextran and captopril was reduced by addition of 2 mM mercaptoethanol. Results of the inhibition studies are shown in FIG. 8. Error bars represent extreme spread of samples tested in triplicate.

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201**[0069] Synthesis of Fluorescent Prodrugs.**

[0070] Cyanine dye reagents are useful as markers in biological systems due the fact that they are intensely fluorescent and highly water soluble. We have utilized the Cy 3.5 monofunctional dye (Amersham Pharmacia Biotech, Piscataway, N.J.) as a marker for determining uptake of dextran prodrugs into motor neurons. The Cy 3.5 dye is scarlet fluorescing and is readily detected using standard rhodamine filter sets. The dye is a monofunctional succinimidy ester that reacts readily with free amine groups. The structure of the dye is shown in FIG. 13.

[0071] Synthesis of dextran-based prodrugs (dextran vehicle plus dye) for conjugation to botulinum heavy chain involves two separate linker addition steps. The first step is oxidation of the aminodextran by addition of sodium periodate (10 mM final concentration) to solutions of 10 or 40 kD aminodextran. The reaction is carried out in 100 mM sodium acetate, pH 5.5, for 20 minutes at room temperature. The oxidation reaction is quenched by addition of glycerol to a final concentration of 50 mM. This results in the formation of hydrazide reactive aldehydes and ketones on the aminodextran. Excess periodate and glycerol are removed by extensive dialysis in 100 mM sodium acetate, pH 5.5. These carbonyl groups are reactive with the hydrazide portion of the linker PDPH (3-(2-pyridyldithio)propionyl hydrazide, Pierce Biochemical Co., Rockford, Ill.). PDPH is dissolved in DMSO and added to the dialyzed amino dextran at a final concentration of 10 mM. The reaction is allowed to proceed at room temperature for 2-4 hours.

[0072] After removal of unreacted linker by size exclusion chromatography or extensive dialysis, the amino dextran plus PDPH is reacted with the Cy 3.5 ester. This reaction adds the dye molecule to the free amine groups present on the dextran. Unreacted dye is removed by either size exclusion chromatography or dialysis and the prodrug conjugated to botulinum heavy chain via the pyridine-2-thione containing portion of the PDPH linker. This reaction scheme is shown in FIG. 14.

[0073] Reaction of 40 kD amino dextran prodrug with type A heavy chain results in molecules having molecular weights on SDS-PAGE ranging from 100 kD to approximately 160 kD. The molecules at 100 kD represent unreacted type A heavy chain while the 160 kD molecules represent heavy chain plus one or more dextran prodrugs with a combined molecular weight of approximately 60 kD. These species are all reducible to 100 kD starting heavy chain due to the disulfide bond involved in conjugation.

CONCLUSIONS

[0074] We have demonstrated that it is possible to chemically synthesize prodrugs consisting of the metalloprotease inhibitor captopril covalently bound to a relatively inert polymeric carrier. Carriers tested to date have included 10 and 40 kD amino dextrans. Additionally, we have successfully modified type A botulinum heavy chain thereby stabilizing the heavy chain relative to the unmodified native heavy chain and allowing us to target conjugation of prodrug to the sulfhydryl originally involved in the disulfide level connecting the heavy and light chains of the toxin.

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We claim:

1. A method of targeting therapeutic molecules to motor neurons, comprising the steps of:

- (a) synthesizing a prodrug comprising at least one therapeutic molecule covalently bound to a polymeric delivery vehicle, and
- (b) conjugating the prodrug to a botulinum neurotoxin heavy chain.
- 2. The method of claim 1 wherein the prodrug comprises at least two copies of a therapeutic molecule.
- 3. The method of claim 1 wherein the prodrug comprises at least four copies of a therapeutic molecule.
- 4. The method of claim 1 wherein the prodrug comprises at least two copies of different therapeutic molecules.
- 5. The method of claim 1 wherein the botulinum neurotoxin heavy chain is obtained via recombinant DNA methods.
- 6. The method of claim 1 wherein the botulinum neurotoxin heavy chain is obtained from native botulinum toxin.
- 7. The method of claim 1 wherein the polymeric delivery vehicle comprises amine-reactive carbohydrate units.
- 8. The method of claim 1 wherein the polymeric delivery vehicle comprises a polymer comprising reactive amines and reactive sulfhydryl groups before being bound to the therapeutic molecule via disulfide exchange.
- 9. The method of claim 1 wherein the therapeutic molecule is captopril.
- 10. The method of claim 1 wherein the heavy chain is type A botulinum neurotoxin.
- 11. The method of claim 1 wherein the therapeutic molecule is a metalloprotease inhibitor.
- 12. The method of claim 1 wherein the heavy chain has been covalently modified.

* * * * *

(12) **United States Patent**
Siller-Jackson et al.

(10) **Patent No.:** **US 6,544,548 B1**
(45) **Date of Patent:** ***Apr. 8, 2003**

(54) **KERATIN-BASED POWDERS AND HYDROGEL FOR PHARMACEUTICAL APPLICATIONS**

(75) **Inventors:** **Arlene J. Siller-Jackson**, Helotes, TX (US); **Mark E. Van Dyke**, Fair Oaks Ranch, TX (US); **Scott F. Timmons**, San Antonio, TX (US); **Cheryl R. Blanchard**, San Antonio, TX (US); **Robert A. Smith**, Jackson, MS (US)

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(*) **Notice:** Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

(21) **Appl. No.:** **09/638,643**

(22) **Filed:** **Aug. 14, 2000**

Related U.S. Application Data

(63) **Continuation-in-part of application No. 09/587,157**, filed on Jun. 5, 2000, which is a continuation-in-part of application No. 09/528,893, filed on Mar. 20, 2000, now Pat. No. 6,270,793, which is a continuation-in-part of application No. 09/512,918, filed on Feb. 25, 2000, now Pat. No. 6,274,155, which is a continuation-in-part of application No. 09/394,782, filed on Sep. 13, 1999, now Pat. No. 6,316,598.

(51) **Int. Cl.⁷** **A61F 13/00; A61K 7/06; A61K 9/00; A61K 6/00; A01N 25/34; A01N 37/18**

(52) **U.S. Cl.** **424/449; 424/400; 424/70.1; 424/70.11; 424/70.12; 424/70.21; 424/70.22; 424/70.24; 424/70.27; 424/401; 424/402; 424/443; 514/2; 514/21**

(58) **Field of Search** **424/70.1; 70.11; 424/70.12; 70.21; 70.22; 70.24; 70.27; 449; 443; 401**

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Primary Examiner—Thurman K. Page

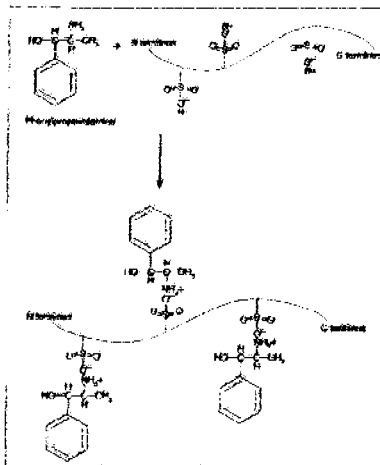
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(57) **ABSTRACT**

A hydratable, highly absorbent keratin solid fiber or powder capable of absorbing a large weight excess of water may be produced by partially oxidizing hair keratin disulfide bonds to sulfonic acid residues and reacting the sulfonic acid residues with a cation. The neutralized suspension can be filtered, washed, and dried, leaving keratin solid which can be shredded into fibers and further ground into powder. Addition of water to the solid produces a hydrogel. The powder or hydrogel may be useful as an absorbent material, as a therapeutic for skin, or as an excipient. The keratin materials can be incorporated into nonwoven films. The hydrogel can be used as a biocompatible viscoelastic filler for implant applications. Another use for the absorbent keratin and keratin hydrogel is as an excipient in pharmaceutical and cosmetic applications.

48 Claims, 12 Drawing Sheets



sion base, or water-soluble ointment bases as are well known in the pharmaceutical arts. Oleaginous bases that may be combined with the keratin compositions include ointments containing white wax and/or white petrolatum, ointments containing yellow wax and petrolatum, cetyl esters wax, oleic acids, and paraffins. Absorbent ointment bases or emulsifiable bases that may be used include those containing anhydrous lanolin, or combinations of cholesterol, stearyl alcohol, white wax and petrolatum, for example. Emulsion bases and components that may be used include ointments containing cetyl alcohol, and cold creams such as those containing cetyl esters wax, white wax, mineral oil, sodium borate and water, for example. Other ointments of the present invention may contain glyceryl monostearate, lanolin, stearic acid, or a combination of methylparaben, propylparaben, sodium lauryl sulfate, propylene glycol, stearyl alcohol and white petrolatum, for example, or an ointment containing cetyl esters wax, white wax, almond oil, sodium borate, stronger rose water, and rose oil, for example. Water soluble ointments and creams for use in the present invention may include glycol ethers and derivatives thereof, polyethylene glycols, polyoxyl 40 stearate, and/or polysorbates.

The preparations as described herein for topical applications may also include protectives and absorbents, demulcents such as benzoin, hydroxypropyl cellulose, hydroxypropyl methylcellulose, polyvinyl alcohol, propylene glycols, sodium alginates, and tragacanth. Emollients, astringents, or antiperspirants may also be included in the keratin containing formulations as described herein.

An aspect of the present disclosure is a method for augmenting soft tissue in a subject comprising injecting a keratin composition as described herein subdermally in an area in need of augmentation. A variety of such applications are available in light of the present disclosure and would include augmentation of soft tissue including, but not limited to bulking of a urinary sphincter in order to alleviate urinary incontinence, augmentation of vocal chords to restore elasticity, as well as improvement of the appearance of a subject by augmentation of breasts, lips, chin, gluteal area, or even to improve wrinkled or acne scarred skin, or skin scarred by other conditions, and including soft tissue voids or indentations. A keratin composition may be provided as a dry solid and hydrated after subdermal implantation, or a hydrogel or viscoelastic hydrogel may be prepared and implanted. In certain embodiments, a dry or hydrated keratin material may be contained in a biocompatible envelope, bag, or container for subdermal implantation, and hydrated after implantation by addition of water or absorption of body fluids, or a keratin material may be suspended in an injectable carrier and injected in the desired area of augmentation. In one embodiment, soft tissue augmentation is accomplished by injecting a preparation comprising a keratin hydrogel and a cell population.

Another aspect of the present invention is the use of keratin compositions for tissue engineering applications. One embodiment is an implantable preparation comprising a keratin hydrogel and a cell population. The cell population may include, but are not limited to, keratinocytes, fibroblasts, chondrocytes, hepatocytes, splenocytes, neurocytes, osteoblasts, or endothelial cells. The keratin hydrogel preparation may be prepared such that it is implantable by injection. Through processing and/or formulation parameters, the keratin hydrogel may be prepared such that it is resorbable and that such resorption is at a controlled rate. The keratin hydrogel may be prepared such that it is resorbed upon implantation after about 150 days, or

about 100 days, or about 90 days, or about 80 days, or about 70 days, or about 60 days, or about 50 days, or about 40 days, or about 30 days, or about 20 days, or about 10 days, or between about 10 and about 90 days, or between about 20 and about 90 days, or between about 50 and about 90 days, or greater than 90 days. The keratin hydrogel may also contain a therapeutic agent, which may be a water soluble peptide, which may be a mitogen.

Another embodiment is a cell scaffold comprising a nonwoven film which comprises a hydratable keratin which contains sulfonate groups. The nonwoven film may comprise a synthetic polymer which may be resorbable. The hydratable keratin may also be made resorbable, thereby rendering the entire nonwoven film resorbable. The nonwoven film may also be a laminate composition. The hydratable keratin may contain a therapeutic agent which may be a water soluble peptide, which may be a mitogen.

Another embodiment of the present invention is a method of implanting a preparation comprising a population of cells and a keratin hydrogel into an animal. The cell populations may include, but are not limited to, keratinocytes, fibroblasts, chondrocytes, hepatocytes, splenocytes, neurocytes, osteoblasts, or endothelial cells. The hydrogel may contain a therapeutic agent which may be a water soluble peptide, which may be a mitogen. A further embodiment is a method of implanting a cell scaffold comprising a nonwoven film that comprises a hydratable keratin which contains sulfonate groups. Cells may be grown on the nonwoven cell scaffold in vitro. Thereby, the nonwoven cell scaffold may be seeded with cells prior to implantation. Such cells include, but are not limited to, keratinocytes, fibroblasts, chondrocytes, hepatocytes, splenocytes, neurocytes, osteoblasts, or endothelial cells. The nonwoven cell scaffold may be used to repair damaged hard and soft tissues. Hard tissues include, but are not limited to, bone and cartilage. Soft tissues include, but are not limited to, skin, mucosa and muscle. Mucosal tissue includes, but is not limited to, gingival tissue, which may be damaged bone or damaged cartilage. Another embodiment comprises implanting a keratin hydrogel cell scaffold. A therapeutic agent may be included in the keratin component of all the cell scaffolds of the present invention. Such a therapeutic agent may leach out of the keratin and may be a water soluble peptide, which may be mitogenic.

On one aspect of the present invention, the keratin composition is a preparation comprising a cross-linked insoluble oxidized keratin excipient. The preparation may be in the form of a powder, tablet, film, capsule, lotion, cream, gel, solution, suspension, emulsion or aerosol. The preparation may be a cosmetic preparation or may be a pharmaceutical preparation. The preparation may further comprise one or more additives such as diluents, fillers, lubricants, stabilizers, binders and gels.

It is an aspect of the present invention that a keratin composition as described herein, and in particular keratin obtained from human hair is also useful as an excipient for the delivery of an active agent. An embodiment of the invention may be described, therefore, as a composition comprising a keratin having oxidized cysteine groups and an active agent or as a cross-linked insoluble oxidized keratin excipient with an active agent. In certain embodiments the active agent is physically or sterically entrapped within the keratin excipient and released over time by diffusion, or as a keratin excipient is degraded. Further, in some embodiments the active agent may be associated with the keratin excipient. The association between the active agent and the keratin excipient may be by non-covalent attraction or

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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 06-098637- -00004-0000

Fecha: 2008-06-17 16:29:58 Dep. 2020 NULCREACION
Tra. 11 PATENTE/II Eve: 378 FASENACIONAL
Act. 538 PLICION Folios: 2

Señora Jefe de la
DIVISION DE NUEVAS CREACIONES
Superintendencia de Industria y Comercio
E. S. D.

Trámite : 011
Evento : 378
Actuación : 538

Referencia : Expediente No. 06.098.637
Asunto : Solicitud de Patente para **COMPOSICIONES Y METODOS
PARA APLICACION TOPICA Y SUMINISTRO
TRANSDERMICO DE TOXINAS BOTULINUM**
Solicitante : **REVANCE THERAPEUTICS, INC**
Clase : A61K 051/000
Publicada en : La Gaceta No. **583** del 18 de diciembre de 2007
Fecha de Solicitud : 29 de septiembre de 2006

1. Yo, Emilio FERRERO, identificado como aparece al pie de mi firma, actuando como apoderado en el asunto de la referencia, atentamente, solicito se efectúe el examen de patentabilidad de la solicitud en referencia presentada de acuerdo con el Capítulo I del PCT y según lo establecido en el artículo 44 de la Decisión 486 de la Comisión de la Comunidad Andina. Para tal efecto presento junto con este escrito el recibo por valor de \$ 420.000.00, que corresponde a la tasa fijada según lo dispuesto en la resolución No. 44372 de 26 de diciembre de 2007.

2. Ruego atentamente a la Señora Jefe se sirva ordenar que se anexe este recibo para que se efectúe dicho examen y se continúe con el trámite de la solicitud.

Señora Jefe,

EMILIO FERRERO
T.P.A. No. 31.929

COLO 15550-841-003-4
COLO-15861-845-003-1
SDC\MGMID-128536\WPC15861
No. M-2008-128536\SDC

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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

NIT : 800176089-2

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



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RECIBO OFICIAL DE CAJA : 08 - 46,079
FECHA : JUNIO 9 DE 2008

***** CONSIGNACION *****

DEPOSITANTE	TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PGO	Vr. PAGO
CAVELIER ABOGADOS	CONSIGNACION	BANCO DE BOGOTA	062754387	2678328	09/06/2008	89,445,000.00

***** CONCEPTO *****

CANT. RENTISTICO	CONCEPTO	TOTAL CONCEPTO
50005-01-01 SOLICITUDES	85 EXAMEN DE PATENTABILIDAD DE INVEN	420,000.00
	TOTAL :	\$ 420,000.00

SON: CUATROCIENTOS VEINTE MIL PESOS

RESPONSABLE :

RECIBO DE CAJA APLICADO AL EXPEDIENTE No. 0010 15861-895.003-1

DIVISIÓN DE NUEVAS CREACIONES

RADICACIÓN EXPEDIENTE No. 06-98637

EL EXTRACTO DE LA PRESENTE SOLICITUD DE **PATENTE DE INVENCION**

FUE PUBLICADO EN LA GACETA DE PROPIEDAD INDUSTRIAL No **583** DE

FECHA **18 DE DICIEMBRE DE 2007** EL TÉRMINO HÁBIL SEÑALADO POR LA

LEY EXPIRA EL **14 DE MARZO DE 2008**

NOTA: Dentro del plazo de los seis (6) meses siguientes a partir de la fecha de publicación en la gaceta de Propiedad Industrial, deberá solicitar y cancelar el examen de patentabilidad so pena de que la solicitud caiga en abandono, de acuerdo con lo establecido en el Artículo 44 de la Decisión 486/2000.

SE PRESENTARON OPOSICIONES POR PARTE DE TERCEROS.

SI _____

NO X



US 20030147921A1

196
1a6(19) **United States**(12) **Patent Application Publication** (10) **Pub. No.: US 2003/0147921 A1**
Goodnough et al. (43) **Pub. Date: Aug. 7, 2003**(54) **METHOD OF TARGETING
PHARMACEUTICALS TO MOTOR
NEURONS**(76) **Inventors: Michael C. Goodnough, Stoughton, WI
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MILWAUKEE, WI 53202-4497 (US)(21) **Appl. No.: 09/870,262**(22) **Filed: May 30, 2001****Related U.S. Application Data**(60) **Provisional application No. 60/208,516, filed on Jun.
1, 2000.****Publication Classification**(51) **Int. Cl.⁷ A61K 39/08**
(52) **U.S. Cl. 424/239.1; 514/12**(57) **ABSTRACT**

A method of targeting therapeutic molecules to motor neurons is disclosed. In one embodiment, this method comprises the steps of (a) synthesizing a prodrug comprising a therapeutic molecule covalently bound to a polymeric delivery vehicle, and (b) conjugating the prodrug to a botulinum neurotoxin heavy chain.

METHOD OF TARGETING PHARMACEUTICALS TO MOTOR NEURONS

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims priority to U.S. Serial No. 60/208,516, filed Jun. 1, 2000, which is incorporated by reference herein.

BACKGROUND OF THE INVENTION

[0002] Botulinum neurotoxin is an ongoing public health problem and is considered to be a potentially serious biological warfare agent. Recent worldwide events including the discovery of stockpiles of materials for manufacture of the toxin by extremist groups in Japan and Iraq have shown that the danger exists for biological terrorism. Intoxication from the toxin by consumption in food or water or from inhalation can lead to prolonged paralysis, incapacitation, and need for intensive supportive care. Depending on the route of administration, death can result from exposure to less than a microgram of toxin. There are no effective therapies once symptoms of botulism appear other than supportive treatments such as intubation and ventilation.

[0003] Within the last five years a number of potential therapies have been identified (e.g. aminoquinolines, metalloprotease inhibitors, aminopyridines. Adler, et al., 1996), and zinc chelators such as TPEN (Adler, et al., 1997). None of these are likely to work in vivo due to difficulty in penetration into the neuronal cytosol and high systemic toxicity. Although better metalloprotease inhibitors have recently been developed at the University of Wisconsin-Madison (Dr. Daniel Rich), these are likely to be limited by the same problems of membrane penetration and toxicity as the first generation of drugs. Anything that crosses lipid membranes easily will also be likely to cross the blood-brain barrier and have CNS toxicity. Any potential therapeutic compound that cannot be internalized into the nerve terminal cytosol will not be capable of antagonizing the active component of botulinum neurotoxin.

BRIEF SUMMARY OF THE INVENTION

[0004] In one embodiment, the present invention is a method of targeting therapeutic molecules to motor neurons. This method preferably comprises the step of (a) synthesizing a prodrug comprising a therapeutic molecule covalently bound to a polymeric delivery vehicle and (b) conjugating the prodrug to a botulinum neurotoxin heavy chain. In a preferred method of the present invention, the prodrug comprises at least two copies of the therapeutic molecule.

[0005] In another preferred method of the present invention, the botulinum neurotoxin heavy chain is type A botulinum neurotoxin and the therapeutic molecule is a metalloprotease inhibitor.

[0006] It is an object of the present invention to target therapeutic molecules to motor neurons.

[0007] Other objects, features and advantages of the present invention will become apparent after one has reviewed the specification, claims and drawings.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

[0008] FIG. 1. SDS-PAGE of purified type A neurotoxin (lane 1, unreduced; lane 2, reduced), light chain (lane 3), and heavy chain (lane 4).

[0009] FIG. 2. Covalent modification of free thiol groups in type A botulinum neurotoxin.

[0010] FIG. 3. Separation and purification of thiol modified type A botulinum heavy chain.

[0011] FIG. 4. Structure of amino-dextran.

[0012] FIG. 5. Structure of the heterobifunctional linker sulfo-succinimidyl-6-[alpha-methyl-alpha(2-pyridylthio)-toluamido]hexanoate (sulfo-LC-SMPT).

[0013] FIG. 6. sulfo-LC-SMPT activated dextran.

[0014] FIG. 7. Release of 2-thiopyridine leaving group from sulfo-LC-SMPT activated dextran.

[0015] FIG. 8. Inhibition of ACE by reduced prodrug.

[0016] FIG. 9. Structure of captopril.

[0017] FIG. 10. Captopril bound to functionalized amino-dextran.

[0018] FIG. 11. Oxidation of hydroxyl groups to carbonyl groups and subsequent reaction with PDPH.

[0019] FIG. 12. Overall reaction scheme for conjugation of prodrug to botulinum heavy chain.

[0020] FIG. 13. Cy 3.5 monofunctional dye molecule. Molecular weight 1102. Absorbance max=581 nm. Emission max=596 nm.

[0021] FIG. 14. Reaction of type A botulinum heavy chain with PDPH functionalized Cy 3.5 amino-dextran.

DETAILED DESCRIPTION OF THE INVENTION

[0022] Our solution to the above-stated problem is to use a modified, nontoxic form of the botulinum neurotoxin molecule to specifically target and deliver currently identified therapeutics as well as compounds developed in the future to the appropriate cholinergic motor neuron terminals. Once delivered to the presynaptic motor neuron membrane, the chimeric delivery vehicle preferably consisting of the heavy chain of the neurotoxin covalently linked with a polymeric molecule capable of delivering multiple copies of therapeutic compounds will bring the desired therapeutic across the membrane and into the neuron where the drug will be an effective therapeutic. In one embodiment, the drug may be an antagonist of botulinum neurotoxin. In another embodiment, the drug is an enzymatic inhibitor, including peptide inhibitors.

[0023] In one embodiment, the present invention is a method of targeting pharmaceuticals to motor neurons, comprising a first step of synthesizing prodrugs comprising at least one copy, and preferably multiple copies, of a therapeutic molecule (the Examples below describe the model metalloprotease inhibitor captopril as a therapeutic molecule) covalently bound to a polymeric delivery vehicle. The second step comprises conjugating the prodrug to the heavy chain of botulinum neurotoxin, preferably type A. This combination will yield a pharmaceutically active compound that specifically targets and internalizes desired substances into cholinergic neurons including active inhibitors of the active fragment of the neurotoxin itself.

[0024] We envision that the method of the present invention will be suitable for all heavy chains of botulinum

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neurotoxin. The Examples below describe a preparation using type A heavy chain. Other heavy chains (i.e., all seven serotypes of heavy chains, A-G) can be prepared in a similar manner. One may obtain the heavy chain in a variety of manners. Preferably, one will obtain heavy chains as described below in the Examples. In another embodiment of the present invention, one will obtain the heavy chain via recombinant DNA methods.

[0025] Polymeric delivery vehicle is an inert carrier molecule ranging in size from 10-40 kD that can be chemically modified such that therapeutic molecules may be covalently linked to it.

[0026] The polymeric delivery vehicle preferably comprises amine reactive dextran, described below as having a molecular weight of approximately 10 kD and 40 kD. Other suitable polymers include polyethylene glycol and polyimines.

[0027] Dextran amines were preferably functionalized for conjugation with model inhibitors by addition of the water soluble heterobifunctional linker sulfo-succinimidyl-6-[α -methyl- α -(2-pyridylthio)-toluamido]hexanoate (sulfo-LC-SMPT, FIG. 5). Other suitable linkers include heterobifunctional linkers containing amine and sulfhydryl reactive elements. Also useful are heterobifunctional linkers that are carboxy reactive as well as sulfhydryl reactive.

[0028] The conjugation of the prodrugs to the heavy chain of botulinum neurotoxin will typically take place as follows:

[0029] We envision two preferable approaches to the synthesis of prodrugs that contain high molar concentrations of model inhibitor and have a traceable label.

[0030] Approach #1. We currently use specific dextrans that are amine reactive and contain a fluorescent marker such as fluorescein, tetramethylrhodamine, or Cy3.5. The availability of fluorescent dextran conjugates with different sizes and charges will permit us to synthesize a wide variety of prodrugs. These labels are commercially available from Molecular Probes, Eugene, Oreg. and from Pharmacia Amersham, Piscataway, N.J.

[0031] The initial step in this approach will be to generate carbonyls on the dextran that are reactive to a hydrazide containing heterobifunctional linker. The preferred linker of choice is 3-(2-pyridylthio)propionyl hydrazide (PDPH). The generation of hydrazide reactive carbonyls on the dextran is accomplished by gentle oxidation using sodium periodate according to the method of Ranadive, et al. (G. Ranadive, et al., *Nucl. Med. Biol.* 20:719-726, 1993). Briefly, this reaction involves reacting the dextran with 5-10 mM sodium periodate in 100 mM sodium acetate, pH 5.5, for 20-30 minutes in the dark at room temperature. The oxidation reaction is quenched with addition of glycerol to 20 mM final concentration. The dextran is dialyzed against 100 mM sodium acetate, pH 5.5, to remove glycerol and periodate and linker is added to a final concentration of 5-10 mM. This reaction is allowed to proceed for 2 hours at room temperature at which time excess linker is removed from the dextran by applying the mixture to a desalting column such as the Pharmacia PD10.

[0032] The desalted, derivatized dextran is then reacted with captopril. The reaction can be monitored by appearance of the leaving group 2-thiopyridine having a characteristic

absorbance at 343 nm. After 2 hours incubation at room temperature, excess captopril is removed by desalting on PD10 columns. The amine groups on the dextran are then reacted with the linker sulfo-LC-SMPT. This generates free thiol groups that can then be conjugated directly to botulinum heavy chain via disulfide exchange.

[0033] Approach #2. The second approach to conjugating prodrug to heavy chain involves synthesis of prodrug using PDPH-activated amino dextran. Amine-reactive pharmaceutical compounds are added to PDPH-modified amino dextran and allowed to cross-link with the free amine groups present on the dextran. Following removal of excess pharmaceutical by desalting on PD10 columns or extensive dialysis in 25 mM phosphate buffered saline, pH 7.4, the prodrug is added to botulinum heavy chain and the free thiol group(s) on the heavy chain allowed to form disulfide bonds with the thiol reactive portion of the linker PDPH. The reaction is allowed to proceed overnight at 4° C. Excess prodrug is readily separated from the heavy chain-prodrug conjugates by addition of ammonium sulfate to 60% saturation. Under these conditions heavy chain-prodrug and unreacted heavy chain precipitate while the carbohydrate-based prodrug remains in solution.

[0034] A second approach to separation of unreacted dextran from botulinum heavy chain can be accomplished by immobilized-metal affinity chromatography (IMAC) according to the method of Schiavo, et al. (G. Schiavo, et al., *Infect. Immun.* 58:4136-4141, 1990). Briefly, chelating Sepharose (Pharmacia) charged with Zn is used to bind the heavy chain of botulinum toxin and heavy chain/polymer constructs. Unreacted dextran is washed from the column with 5-8 column volumes of running buffer and the heavy chain/polymer constructs eluted with 25 mM imidazole.

[0035] Preferred therapeutic agents include protease inhibitors. Specific inhibitors include aprotinin, chymostatin, amastatin, bestatin, leupeptin, antipain, trypsin inhibitors, chelating agents such as EDTA, TPEN, o-phenanthroline, and peptide inhibitors of proteases. Peptide inhibitors such as caspase inhibitors (apopain series) which are primarily four and five amino acid inhibitors of caspases are also preferred. Metalloprotease inhibitors such as phosphoramidon, pepstatin A, phebestin, and the TAPI series of matrix metalloprotease inhibitors are also preferred.

[0036] The prodrug-heavy chain may be targeted to specific muscle groups by directly injecting the affected muscles. Alternatively, one could deliver the prodrug-heavy chain combination orally.

EXAMPLES

[0037] Bacterial Strains and Culturing.

[0038] The Hall A strain of type A *C. botulinum* was used to produce crystalline type A complex. This strain was originally obtained from Dr. J. H. Mueller at Harvard University and was further screened for high toxin titers at Fort Detrick, Md. by Dr. E. J. Schantz and coworkers. This strain is routinely used for production of type A botulinum toxin due to high toxin titers and the rapid onset of cell lysis (usually within 48 hours).

[0039] Stock cultures of *C. botulinum* Hall A were grown statically in 15 ml Hungate tubes containing 10 ml of cooked meat medium +0.3% dextrose (CMM, [Difco Laboratories,

Detroit, Mich.)) under an anaerobic atmosphere (80% N₂, 10% CO₂, 10% H₂) at 37° C. for 24 hours and frozen at -20° C. until use. CMM cultures of the Hall A strain give toxin titers in excess of 10⁶ I.D.₅₀/ml in 48-72 hours.

[0040] For toxin production, cultures of Hall A were grown statically in 10 liter volumes of toxin production medium (TPM) consisting of 2.0% NZ TT (lot #9NC29) casein hydrolysate (Sheffield Laboratories, Norwich, N.Y.), 1.0% yeast extract (Difco), and 0.5% dextrose, pH 7.3-7.4, for 5-7 days at 37° C. Cultures of Hall A showed heavy growth in this medium during the first 24-48 hours followed by autolysis of the culture which was evident as a clearing and settling over the next 48-120 hours.

[0041] Toxin Purification.

[0042] The production and purification of type A neurotoxin for use as the starting reagent in subsequent chain separations and purifications was successful. As shown in FIG. 1, lanes 1 (unreduced) and 2 (reduced), purification of three batches of type A botulinum neurotoxin having an average specific toxicity of 1.5×10⁸ mouse intraperitoneal lethal doses per mg of protein was successful. Three separate batches of toxin were cultured and purified to homogeneity. Subsequent purification of the component light and heavy chains were performed with yields shown in Table 1.

TABLE 1

Yields of toxin at various stages of purification ¹ . (Roman numerals refer to individual purification steps described below.)				
	Batch 1	Batch 2	Batch 3	
I. Crude culture ²	1.5 × 10 ⁶	2.0 × 10 ⁶	3.0 × 10 ⁶	
II. Toxin complex ¹	200	225	275	
III. Partially purified neurotoxin ¹	38	47	60	
IV. Purified neurotoxin ¹	20	23	30	
V. Light chain ¹	5	5.5	9	
VI. Heavy chain ¹	6	7	11	

¹mg of protein.

²1.D50/ml of crude culture.

[0043] We fortuitously determined that toxin production in crude culture could be increased by addition of specific mineral salts to production medium. The addition of magnesium (as magnesium chloride 100 mM final concentration) and calcium carbonate (400 mM final concentration) allowed us to double the amount of toxin produced in culture over the course of this research. This will be extremely important in subsequent scale-ups. As can be seen in Table 1, the amount of toxin complex obtained from Batch 3 vs. Batch 1 did not double as expected based on titers of crude cultures.

[0044] Toxin Purification Steps Noted in Table 1.

[0045] I. Cultures of *C. botulinum* were grown in 10 liter batches of 2% casein hydrolysate, 0.5% dextrose, 0.5% yeast extract, pH 7.4, for 4-5 days at 37° C. Cultures were harvested at this time by addition of 3N H₂SO₄ to a final pH of 3.4. Crude toxin precipitated and was collected by centrifugation.

[0046] II. Toxin complex was extracted from the acid precipitate by suspending in 200 mM sodium phosphate, pH 6.0. The first extraction was allowed to proceed for 2-3 hours at room temperature. Extracted

toxin was separated from cellular debris by centrifugation and the pellet reextracted in similar fashion overnight at 4° C. The extracts were pooled and precipitated with ammonium sulfate (39 g/100 ml). Precipitated extracts were collected by centrifugation and dissolved in 50 mM sodium citrate, pH 5.5. This material was loaded onto DEAE-Sephadex columns equilibrated with the same buffer. Toxin complex eluted essentially unretarded and was pooled and precipitated with ammonium sulfate (39 g/100 ml).

[0047] III. Neurotoxin was separated from toxin complex proteins by dialyzing precipitated complex against 3 changes of 20 mM sodium phosphate, pH 7.9. Dialyzed toxin complex was bound to DEAE-Sephadex equilibrated with the same buffer. Partially purified neurotoxin was eluted as the first peak with a 0-300 mM sodium chloride gradient.

[0048] IV. Purified neurotoxin was separated from minor amounts of contaminating complex proteins by dialyzing the neurotoxin peak eluted in step III against 20 mM sodium phosphate, pH 7.0, and applying the material to SP-Sephadex equilibrated with the same buffer. Under these conditions contaminating complex proteins did not bind to the matrix. Purified neurotoxin was eluted with a 200 mM sodium chloride step. Neurotoxin recovered from this step was subsequently modified with iodoacetamide thereby alkylating free thiol groups. The rationale for this is described in detail in the next section of this report.

[0049] V. Light chain was purified as a by-product of heavy chain purification. Purified neurotoxin was dialyzed against 20 mM sodium borate, 40 mM sodium phosphate, pH 8.4, and applied to QAE-Sephadex equilibrated with the same buffer. After binding neurotoxin to the column, one-half column volume of loading buffer plus 10 mM dithiothreitol (DTT) was applied to the column. A second one-half column volume of loading buffer plus 100 mM DTT and 2 M urea was applied and the column held over night at 4° C. to allow reduction of disulfide bonds. Light chain was eluted with loading buffer plus 10 mM DTT and 2 M urea. Heavy chain and contaminating unnicked neurotoxin were eluted with loading buffer containing 10 mM DTT, 2 M urea, and 500 mM sodium chloride.

[0050] VI. Heavy chain containing trace amounts of unnicked neurotoxin was dialyzed against 20 mM sodium phosphate, 5 mM DTT, pH 7.5. The heavy chain was then applied to a column of DEAE-Sephadex equilibrated with the same buffer. Contaminating unnicked neurotoxin was separated from heavy chain by use of a linear 0-600 mM sodium chloride gradient with unnicked neurotoxin eluting at around 100 mM sodium chloride and heavy chain eluting at about 400 mM sodium chloride.

[0051] Neurotoxin Modification.

[0052] Purified type A botulinum heavy chain has six cysteine residues. Of these six thiol groups, two are involved in an intrachain disulfide bond and are not available for reaction with other compounds as the reduced thiols are in such close proximity to each other that they quickly reform a disulfide linkage following removal of the reducing agent

200
L60

(FIG. 2). Treatment of intact neurotoxin with the thiol alkylating agent iodoacetamide yielded a molecule with 0-1.2 free thiols on average as assessed by titration with bis-dithionitrobenzoic acid (Ellman, 1959). This material retained greater than 80% of the starting toxicity as measured by mouse bioassay. Purification of heavy chain was subsequently performed according to the method of Sathyamoorthy and DasGupta (1985) as shown in FIG. 3.

[0053] Electrophoresis.

[0054] Protein samples were examined electrophoretically using the Pharmacia Phastsystem (Pharmacia LKB Inc., Piscataway, N.J.) according to the manufacturers instructions. Precast 12.5% acrylamide gels (Pharmacia) were stained with 0.1% coomassie brilliant blue R250 in 16.7% acetic acid, 41.7% methanol. Gels were destained in 7.5% acetic acid, 25% methanol. Samples for electrophoresis were solubilized in 50 mM Tris-HCl, 5 M urea, 5% SDS, 20% glycerol, pH 6.8. Some samples were reduced by addition of dithiothreitol to a final concentration of 0.5%. All samples for SDS-PAGE were boiled for >5 minutes prior to electrophoresis.

[0055] Toxin Assays.

[0056] Toxin titers were estimated in mice using the intravenous method of Boroff and Fleck (1966) and the intraperitoneal method of Schantz and Kautter (1978) in 18-22 g, female, ICR strain mice. Time-to-death values obtained from intravenous titration of type A toxin samples were converted to intraperitoneal LD₅₀/ml using a standard curve generated in our laboratory with type A complex. Botulinum toxin for titration was dissolved in 50 mM sodium phosphate, pH 6.8, and then further diluted as required in 30 mM sodium phosphate, 0.2% gelatin, pH 6.4.

[0057] Structure of Prodrugs.

[0058] Polymers selected for reaction with specific linkers included amine reactive dextrans (Molecular Probes, Eugene, Oreg.) having molecular weights of approximately 10 kD and 40 kD. These dextrans had on average 4.8 moles amine per mole of dextran (10 kD) and 6.7 moles amine per mole of dextran (40 kD). The general structures of these compounds are shown in FIG. 4.

[0059] Dextran amines were functionalized for conjugation with model inhibitors by addition of the water soluble heterobifunctional linker sulfosuccinimidyl-6-[alpha-methyl-alpha-(2-pyridylthio)-toluamido]hexanoate (sulfo-I.C-SMPT, FIG. 5).

[0060] Linker was added to amine dextrans at 20:1 molar ratios (20 mole linker:1 mole amine). Linker addition reactions were performed in 100 mM sodium phosphate buffer, pH 9.2, and were allowed to continue for 2 hours at room temperature. This pH favored addition of linker to polymer by reaction of the succinimidyl ester on the linker with the primary amine of the polymer. Unreacted linker was removed by either size exclusion chromatography in the case of the 40 kD dextran on Pharmacia PD10 columns (Pharmacia Biotechnology, Piscataway, N.J.) equilibrated with 25 mM sodium phosphate buffered saline, pH 7.4, (PBS) or by extensive dialysis vs. PBS in Slide-A-Lyzer dialysis cassettes (3.5 kD cutoff, Pierce Biochemical Co., Rockford, Ill.) in the case of the 10 kD dextran. The resulting compounds are shown in FIG. 6.

[0061] Linker addition to dextran was confirmed by gentle reduction of functionalized polymer with dithiothreitol. This reaction liberated 2-thiopyridine from the linker which was detected by its characteristic absorbance at 343 nm. In one set of experiments, the final absorbance of 0.77 yielded a molar concentration of the leaving group of 96 μ M indicating that the amine groups on the dextran at a concentration of approximately 750 μ g/ml were over 75% modified by the linker. This is shown in FIG. 7. These reaction kinetics gave nearly identical results with both the 10 kD and the 40 kD amino-dextrans.

[0062] We have used the metalloprotease inhibitor captopril as a model pharmaceutical ligand. This ligand has been covalently coupled to linker modified amino dextran via the reactive 2-thiopyridine group. The structure of captopril is shown in FIG. 9.

[0063] The reaction kinetics (A_{343} increase) for the displacement of the 2-thiopyridine leaving group from functionalized amino-dextran by captopril were nearly identical to those generated by the reducing agent dithiothreitol indicating that captopril was a good nucleophile and was rapidly bound to the dextran polymer via disulfide bond exchange with the linker. These prodrugs are shown in FIG. 10.

[0064] We envision synthesizing prodrugs that contain 5-10 $\times$ more inhibitor (captopril) than the first generation prodrugs. These prodrugs will have a fluorescent label incorporated into them for subsequent *in vitro* and *in vivo* visualization and will be conjugated to botulinum heavy chain for targeting and internalization into motor neurons.

[0065] We will continue to use dextran based polymers for the synthesis of prodrugs because they are relatively non-toxic and are biologically inert due to the uncommon poly-(α -D-1,6-glucose) linkages. They also have low immunogenicity.

[0066] Prodrug Assay.

[0067] We have determined that prodrugs based on the metalloprotease inhibitor captopril described here inhibit angiotensin converting enzyme (ACE) only when they are released from the dextran carrier by reduction of the disulfide bond. This is advantageous because it sequesters potential therapeutic compounds until they are introduced into the reducing environment of the motor neuron. We used a modification of the assay described by Cushman and Cheung (1971) to measure inhibition of the enzyme by captopril. Cleavage of the substrate by ACE yields hippuryl which is soluble in ethyl acetate. Following ethyl acetate extraction of the reactions, the solvent is removed by evaporation and the residue dissolved in water. Hippuryl concentration is measured by absorption at 228 nm.

[0068] Inhibition of ACE was accomplished by addition of known quantities of free captopril. The IC₅₀ of captopril for this particular reaction was calculated to be approximately 300 nM. When dextran-captopril conjugates were added to the reaction we did not obtain any inhibition until the disulfide bond between the dextran and captopril was reduced by addition of 2 mM mercaptoethanol. Results of the inhibition studies are shown in FIG. 8. Error bars represent extreme spread of samples tested in triplicate.

[0069] Synthesis of Fluorescent Prodrugs.

[0070] Cyanine dye reagents are useful as markers in biological systems due the fact that they are intensely fluorescent and highly water soluble. We have utilized the Cy 3.5 monofunctional dye (Amersham Pharmacia Biotech, Piscataway, N.J.) as a marker for determining uptake of dextran prodrugs into motor neurons. The Cy 3.5 dye is scarlet fluorescing and is readily detected using standard rhodamine filter sets. The dye is a monofunctional succinimidyl ester that reacts readily with free amine groups. The structure of the dye is shown in FIG. 13.

[0071] Synthesis of dextran-based prodrugs (dextran vehicle plus dye) for conjugation to botulinum heavy chain involves two separate linker addition steps. The first step is oxidation of the aminodextran by addition of sodium periodate (10 mM final concentration) to solutions of 10 or 40 kD aminodextran. The reaction is carried out in 100 mM sodium acetate, pH 5.5, for 20 minutes at room temperature. The oxidation reaction is quenched by addition of glycerol to a final concentration of 50 mM. This results in the formation of hydrazide reactive aldehydes and ketones on the aminodextran. Excess periodate and glycerol are removed by extensive dialysis in 100 mM sodium acetate, pH 5.5. These carbonyl groups are reactive with the hydrazide portion of the linker PDPH (3-(2-pyridyldithio)propionyl hydrazide, Pierce Biochemical Co., Rockford, Ill.). PDPH is dissolved in DMSO and added to the dialyzed amino dextran at a final concentration of 10 mM. The reaction is allowed to proceed at room temperature for 2-4 hours.

[0072] After removal of unreacted linker by size exclusion chromatography or extensive dialysis, the amino dextran plus PDPH is reacted with the Cy 3.5 ester. This reaction adds the dye molecule to the free amine groups present on the dextran. Unreacted dye is removed by either size exclusion chromatography or dialysis and the prodrug conjugated to botulinum heavy chain via the pyridine-2-thione containing portion of the PDPH linker. This reaction scheme is shown in FIG. 14.

[0073] Reaction of 40 kD amino dextran prodrug with type A heavy chain results in molecules having molecular weights on SDS-PAGE ranging from 100 kD to approximately 160 kD. The molecules at 100 kD represent unreacted type A heavy chain while the 160 kD molecules represent heavy chain plus one or more dextran prodrugs with a combined molecular weight of approximately 60 kD. These species are all reducible to 100 kD starting heavy chain due to the disulfide bond involved in conjugation.

CONCLUSIONS

[0074] We have demonstrated that it is possible to chemically synthesize prodrugs consisting of the metalloprotease inhibitor captopril covalently bound to a relatively inert polymeric carrier. Carriers tested to date have included 10 and 40 kD amino dextrans. Additionally, we have successfully modified type A botulinum heavy chain thereby stabilizing the heavy chain relative to the unmodified native heavy chain and allowing us to target conjugation of prodrug to the sulfhydryl originally involved in the disulfide level connecting the heavy and light chains of the toxin.

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We claim:

1. A method of targeting therapeutic molecules to motor neurons, comprising the steps of:

- (a) synthesizing a prodrug comprising at least one therapeutic molecule covalently bound to a polymeric delivery vehicle, and
- (b) conjugating the prodrug to a botulinum neurotoxin heavy chain.
- 2. The method of claim 1 wherein the prodrug comprises at least two copies of a therapeutic molecule.
- 3. The method of claim 1 wherein the prodrug comprises at least four copies of a therapeutic molecule.
- 4. The method of claim 1 wherein the prodrug comprises at least two copies of different therapeutic molecules.
- 5. The method of claim 1 wherein the botulinum neurotoxin heavy chain is obtained via recombinant DNA methods.
- 6. The method of claim 1 wherein the botulinum neurotoxin heavy chain is obtained from native botulinum toxin.
- 7. The method of claim 1 wherein the polymeric delivery vehicle comprises amine-reactive carbohydrate units.
- 8. The method of claim 1 wherein the polymeric delivery vehicle comprises a polymer comprising reactive amines and reactive sulfhydryl groups before being bound to the therapeutic molecule via disulfide exchange.
- 9. The method of claim 1 wherein the therapeutic molecule is captopril.
- 10. The method of claim 1 wherein the heavy chain is type A botulinum neurotoxin.
- 11. The method of claim 1 wherein the therapeutic molecule is a metalloprotease inhibitor.
- 12. The method of claim 1 wherein the heavy chain has been covalently modified.

* * * * *

(12) **United States Patent**
Siller-Jackson et al.

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(45) **Date of Patent:** ***Apr. 8, 2003**

(54) **KERATIN-BASED POWDERS AND HYDROGEL FOR PHARMACEUTICAL APPLICATIONS**

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(*) **Notice:** **Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.**

This patent is subject to a terminal disclaimer.

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(51) **Int. Cl.⁷** **A61F 13/00; A61K 7/06; A61K 9/00; A61K 6/00; A01N 25/34; A01N 37/18**

(52) **U.S. Cl.** **424/449; 424/400; 424/70.1; 424/70.11; 424/70.12; 424/70.21; 424/70.22; 424/70.24; 424/70.27; 424/401; 424/402; 424/443; 514/2; 514/21**

(58) **Field of Search** **424/70.1, 70.11, 424/70.12, 70.21, 70.22, 70.24, 70.27, 449, 443, 401**

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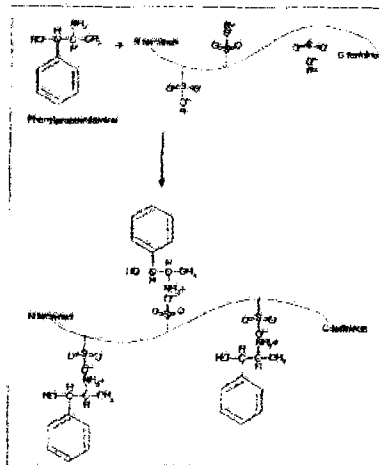
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(57) **ABSTRACT**

A hydratable, highly absorbent keratin solid fiber or powder capable of absorbing a large weight excess of water may be produced by partially oxidizing hair keratin disulfide bonds to sulfonic acid residues and reacting the sulfonic acid residues with a cation. The neutralized suspension can be filtered, washed, and dried, leaving keratin solid which can be shredded into fibers and further ground into powder. Addition of water to the solid produces a hydrogel. The powder or hydrogel may be useful as an absorbent material, as a therapeutic for skin, or as an excipient. The keratin materials can be incorporated into nonwoven films. The hydrogel can be used as a biocompatible viscoelastic filler for implant applications. Another use for the absorbent keratin and keratin hydrogel is as an excipient in pharmaceutical and cosmetic applications.

48 Claims, 12 Drawing Sheets



sion base, or water-soluble ointment bases as are well known in the pharmaceutical arts. Oleaginous bases that may be combined with the keratin compositions include ointments containing white wax and/or white petrolatum, ointments containing yellow wax and petrolatum, cetyl esters wax, oleic acids, and paraffins. Absorbent ointment bases or emulsifiable bases that may be used include those containing anhydrous lanolin, or combinations of cholesterol, stearyl alcohol, white wax and petrolatum, for example. Emulsion bases and components that may be used include ointments containing cetyl alcohol, and cold creams such as those containing cetyl esters wax, white wax, mineral oil, sodium borate and water, for example. Other ointments of the present invention may contain glyceryl monostearate, lanolin, stearic acid, or a combination of methylparaben, propylparaben, sodium lauryl sulfate, propylene glycol, stearyl alcohol and white petrolatum, for example, or an ointment containing cetyl esters wax, white wax, almond oil, sodium borate, stronger rose water, and rose oil, for example. Water soluble ointments and creams for use in the present invention may include glycol ethers and derivatives thereof, polyethylene glycols, polyoxyl 40 stearate, and/or polysorbates.

The preparations as described herein for topical applications may also include protectives and absorbents, demulcents such as benzoin, hydroxypropyl cellulose, hydroxypropyl methylcellulose, polyvinyl alcohol, propylene glycols, sodium alginates, and tragacanth. Emollients, astringents, or antiperspirants may also be included in the keratin containing formulations as described herein.

An aspect of the present disclosure is a method for augmenting soft tissue in a subject comprising injecting a keratin composition as described herein subdermally in an area in need of augmentation. A variety of such applications are available in light of the present disclosure and would include augmentation of soft tissue including, but not limited to bulking of a urinary sphincter in order to alleviate urinary incontinence, augmentation of vocal chords to restore elasticity, as well as improvement of the appearance of a subject by augmentation of breasts, lips, chin, gluteal area, or even to improve wrinkled or acne scarred skin, or skin scarred by other conditions, and including soft tissue voids or indentations. A keratin composition may be provided as a dry solid and hydrated after subdermal implantation, or a hydrogel or viscoelastic hydrogel may be prepared and implanted. In certain embodiments, a dry or hydrated keratin material may be contained in a biocompatible envelope, bag, or container for subdermal implantation, and hydrated after implantation by addition of water or absorption of body fluids, or a keratin material may be suspended in an injectable carrier and injected in the desired area of augmentation. In one embodiment, soft tissue augmentation is accomplished by injecting a preparation comprising a keratin hydrogel and a cell population.

Another aspect of the present invention is the use of keratin compositions for tissue engineering applications. One embodiment is an implantable preparation comprising a keratin hydrogel and a cell population. The cell population may include, but are not limited to, keratinocytes, fibroblasts, chondrocytes, hepatocytes, splenocytes, neurocytes, osteoblasts, or endothelial cells. The keratin hydrogel preparation may be prepared such that it is implantable by injection. Through processing and/or formulation parameters, the keratin hydrogel may be prepared such that it is resorbable and that such resorption is at a controlled rate. The keratin hydrogel may be prepared such that it is resorbed upon implantation after about 150 days, or

about 100 days, or about 90 days, or about 80 days, or about 70 days, or about 60 days, or about 50 days, or about 40 days, or about 30 days, or about 20 days, or about 10 days, or between about 10 and about 90 days, or between about 20 and about 90 days, or between about 50 and about 90 days, or greater than 90 days. The keratin hydrogel may also contain a therapeutic agent, which may be a water soluble peptide, which may be a mitogen.

Another embodiment is a cell scaffold comprising a nonwoven film which comprises a hydratable keratin which contains sulfonate groups. The nonwoven film may comprise a synthetic polymer which may be resorbable. The hydratable keratin may also be made resorbable, thereby rendering the entire nonwoven film resorbable. The nonwoven film may also be a laminate composition. The hydratable keratin may contain a therapeutic agent which may be a water soluble peptide, which may be a mitogen.

Another embodiment of the present invention is a method of implanting a preparation comprising a population of cells and a keratin hydrogel into an animal. The cell populations may include, but are not limited to, keratinocytes, fibroblasts, chondrocytes, hepatocytes, splenocytes, neurocytes, osteoblasts, or endothelial cells. The hydrogel may contain a therapeutic agent which may be a water soluble peptide, which may be a mitogen. A further embodiment is a method of implanting a cell scaffold comprising a nonwoven film that comprises a hydratable keratin which contains sulfonate groups. Cells may be grown on the nonwoven cell scaffold in vitro. Thereby, the nonwoven cell scaffold may be seeded with cells prior to implantation. Such cells include, but are not limited to, keratinocytes, fibroblasts, chondrocytes, hepatocytes, splenocytes, neurocytes, osteoblasts, or endothelial cells. The nonwoven cell scaffold may be used to repair damaged hard and soft tissues. Hard tissues include, but are not limited to, bone and cartilage. Soft tissues include, but are not limited to, skin, mucosa and muscle. Mucosal tissue includes, but is not limited to, gingival tissue, which may be damaged bone or damaged cartilage. Another embodiment comprises implanting a keratin hydrogel cell scaffold. A therapeutic agent may be included in the keratin component of all the cell scaffolds of the present invention. Such a therapeutic agent may leach out of the keratin and may be a water soluble peptide, which may be mitogenic.

On one aspect of the present invention, the keratin composition is a preparation comprising a cross-linked insoluble oxidized keratin excipient. The preparation may be in the form of a powder, tablet, film, capsule, lotion, cream, gel, solution, suspension, emulsion or aerosol. The preparation may be a cosmetic preparation or may be a pharmaceutical preparation. The preparation may further comprise one or more additives such as diluents, fillers, lubricants, stabilizers, binders and gellants.

It is an aspect of the present invention that a keratin composition as described herein, and in particular keratin obtained from human hair is also useful as an excipient for the delivery of an active agent. An embodiment of the invention may be described, therefore, as a composition comprising a keratin having oxidized cysteine groups and an active agent or as a cross-linked insoluble oxidized keratin excipient with an active agent. In certain embodiments the active agent is physically or sterically entrapped within the keratin excipient and released over time by diffusion, or as a keratin excipient is degraded. Further, in some embodiments the active agent may be associated with the keratin excipient. The association between the active agent and the keratin excipient may be by non-covalent attraction or

association, through electrostatic, hydrophilic or ionic interaction, for example, or it may be covalently attached to a keratin excipient by covalent bonding to an oxidized keratin as described herein. In one embodiment, the active agent is in a cationic form that ionically binds to the sulfonate groups of the ionized keratin. In another embodiment the active agent is associated with the keratin excipient by Van der Waal's forces. Association of the active agent with a keratin excipient allows for the sustained and/or controlled release of active agents. In some embodiments, the controlled release of the active agent is provided by the hydrolysis of the keratin excipient. Such a formulation may include a hydratable keratin solid excipient, or a keratin hydrogel depending on the particular application. In some embodiments the active agent is a pharmaceutical agent while in other embodiments the active agent is a cosmetic agent.

In the practice of the invention, a dry hydratable keratin as described herein may be mixed with a powdered pharmaceutical agent and water added to hydrate the mixture, or alternatively such a solid mixture may be formulated as a compressed tablet to be orally administered or for extemporaneous preparations for injection, or as a molded tablet, or it may be enclosed in a capsule for oral administration or subdermal implantation, for example. In certain embodiments a solution containing a water soluble drug or pharmaceutical agent may be added to a hydratable keratin so that the agent is carried into a hydrogel along with the water. A prepared hydrogel, or dry formulation may also be enclosed in a digestible or biodegradable capsule, such as a hard gelatin capsule for oral administration. In certain embodiments, the described pharmaceutical preparations may be formulated for injection, either intravenous, subcutaneous, or intramuscular, for example, or for inhalant, for eye, ear, or nose drops, or for administration as a suppository.

It is understood that the pills formulated for oral administration, including a hydratable keratin solid, or even pills, capsules or tablets containing a keratin hydrogel may contain ingredients to serve as coatings, additional fillers, binders and for color coding purposes. These ingredients are in common use in present pharmaceutical formulations and may include, but are not limited to, gelatin, lactose, corn starch, calcium phosphate, povidone, magnesium stearate, stearic acid, colloidal silicon dioxide, hydroxypropyl methylcellulose, polyethylene glycol and one or more of the following dyes: FD&C Blue No. 1 Lake, FD&C Blue No. 2 Aluminum Lake, D&C Green No. 3, D&C Yellow No. 10, FD&C Yellow No. 6 or FD&C Red No. 3. Of course these are only exemplary fillers and dyes, those of skill in the art will recognize that other inactive ingredients may be used in the preparation of the formulations of the present invention.

Keratin excipient preparations as described herein may be prepared for oral administration, and would also include injectable solutions or suspensions for intramuscular or subcutaneous implantation including long acting injections, suppositories, topical ointments, transdermal applications such as skin patches, and preparations delivered by inhalation. Other ingredients may include 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 may contain a preservative to prevent the growth of microorganisms. Keratin excipient preparations may also include other compounds such as diluents, fillers, lubricants, stabilizers, binders and gelants. Diluents and fillers are added to increase bulk formation, and lubricants to

reduce friction during the tableting or other formulation process. Binders are used in tableting and provide the cohesiveness necessary for bonding together the ingredients under compression. They also increase the strength of the compressed tablet and decrease its friability, leading to an improvement in the both appearance and mechanical characteristics.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersion, sterile powders and hydrogels for the extemporaneous preparation of sterile injectable solutions, dispersions or gels. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride.

Suitable pharmaceutical agents for use with the excipients described herein would include any pharmaceutical agent that can form an association with the keratin formulations through non-covalent, covalent, or steric interaction. These agents would include protein therapeutic agents, such as growth factors. In regard to oral administration, such agents may include compounds such as acetaminophen, tetracyclines, penicillins, vitamins, antacids, non-steroidal antiinflammatory agents, anesthetics, breath fresheners, and minerals, for example.

In those embodiments in which transdermal administration is desired, the disclosed compositions may be formulated to be administered by use of a skin patch, or transdermal delivery system. Transdermal administration may be accomplished by any of a number of systems known in the art. Examples of systems that may be adapted for use with the compositions described herein include those systems of transdermal administration described in U.S. Pat. No. 4,816,252; U.S. Pat. No. 5,122,382; U.S. Pat. No. 5,198,223; U.S. Pat. No. 5,023,084; U.S. Pat. No. 4,906,169; U.S. Pat. No. 5,145,682; U.S. Pat. No. 4,624,665; U.S. Pat. No. 4,687,481; U.S. Pat. No. 4,834,978; and U.S. Pat. No. 4,810,499 (all incorporated herein by reference).

These methods typically include an adhesive matrix or drug reservoir system and may include a skin permeation enhancement agent such as ethanol, polyethylene glycol 200 dilaurate, isopropyl myristate, glycerol trioleate, linolenic acid saturated ethanol, glycerol monooleate, glycerol monolaurate, n-decyl alcohol, capric acid, and certain saturated and unsaturated fatty acids, and their esters, alcohols, monoglycerides, acetate, diethanolamides and N,N-dimethylamides (See for examples, U.S. Pat. No. 4,906,169).

The release rate of an active agent from a keratin excipient preparation, when that active agent is not associated with the keratin excipient, is determined by the rate at which water is absorbed and the keratin solid disintegrates. The water absorption rate of the solid keratin can be controlled by the number of sulfonic acid residues generated in the oxidation procedure. By exposing the keratin source material to extremes of oxidant concentration, temperature, and time, extremes of absorption rate can be obtained. For example, at low oxidant concentration, colder temperatures and short time periods, relatively few disulfide residues will be converted to sulfonic acid residues. Such a keratin solid, further

processed as described herein will absorb relatively little water and disintegrate relatively slowly. Conversely, a keratin solid prepared at high oxidant concentration, at boiling temperature for a long time period, further processed as described herein, will absorb relatively large amounts of water and disintegrate relatively quickly. Disintegration rates between these extremes can be obtained by processing the keratin source material using intermediate conditions. When the active agent is associated, such as ionically, with the keratin excipient, the release rate is determined by both the rate at which water is absorbed and the keratin solid disintegrates and the rate of disassociation of the active agent from the keratin excipient. For some materials which are so tightly bound that release by disassociation alone is ineffectual, degradation of the keratin solid must occur before the drug molecule can become dissolved in the surrounding media. The release rate under these conditions can be controlled by the degradation rate of the keratin solid. In general, oxidation and formulation conditions will effect the hydrolytic stability of the hydrogel containing the drug compound. Peracetic acid oxidized keratin provides a more hydrolytically stable gel than does hydrogen peroxide oxidized keratin, for example. Parameters such as oxidant, oxidation time and solids content of the hydrogel have been shown to be important parameters in controlling in-vitro stability at body temperature.

In some embodiments of the invention, a composition for the delivery of pharmaceutical agents is in the form of a nonwoven film comprising a synthetic polymer and a keratin material. The synthetic polymer may be, but is not limited to, α -olefins, acrylates, urethanes, acetates, nylons, esters, and copolymers thereof. In some embodiments the nonwoven composition is a laminate, which may be a tri-laminate comprising two outer layers of synthetic polymer and a middle layer of keratin material. The keratin material in the middle layer may be partially exposed by openings in the two outer nonwoven synthetic polymer layers. In some embodiments of the invention the synthetic polymer layers are nonwoven synthetic polymer webs. The nonwoven film pharmaceutical delivery composition may be used externally or internally.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments herein.

FIG. 1. Release of albumin from keratin tablets.

FIG. 2. Schematic of proposed mechanism for amine drug binding to oxidized keratin.

FIG. 3. Hydrogen peroxide oxidized hair pH 7 titration curve.

FIG. 4. Release of norephedrine from keratin tablets in simulated gastric fluid.

FIG. 5. Release of acetaminophen from keratin tablets in simulated gastric fluid.

FIG. 6. Loading of acetaminophen into keratin tablets. Loading based on amount used in ion exchange process.

FIG. 7. Stability of keratin hydrogel prepared by peracetic acid oxidation and at a solid to liquid weight ratio of 1:8. Columns of <1 day did not form a gel.

FIG. 8. Stability of keratin hydrogel prepared by peracetic acid oxidation and at a solid to liquid weight ratio of 1:6.

Columns of <1 day did not form a gel. Columns with data labels indicate gel stable at last observation point.

FIG. 9. Stability of keratin hydrogel prepared by peracetic acid oxidation and at a solid to liquid weight ratio of 1:4. Columns of <1 day did not form a gel. Columns with data labels indicate gel stable at last observation point.

FIG. 10. Stability of keratin hydrogel prepared by hydrogen peroxide oxidation and at a solid to liquid weight ratio of 1:8. Columns of <1 day did not form a gel.

FIG. 11. Stability of keratin hydrogel prepared by hydrogen peroxide oxidation and at a solid to liquid weight ratio of 1:6. Columns of <1 day did not form a gel. Columns with data labels indicate gel stable at last observation point.

FIG. 12. Stability of keratin hydrogel prepared by hydrogen peroxide oxidation and at a solid to liquid weight ratio of 1:4. Columns of <1 day did not form a gel. Columns with data labels indicate gel stable at last observation point.

DETAILED DESCRIPTION OF THE INVENTION

The present invention includes a hydratable solid derived from keratin that is highly absorbent and can form a hydrogel or viscoelastic hydrogel upon the application of water. The keratin solid can include protein having an ionizable pendant group such as sulfonic acid that can be derived from an oxidized protein disulfide linkage. A preferred source of protein is keratin, and particularly preferred is keratin obtained from hair, including human hair. While hair is a preferred source of keratinous material, other keratinous materials are also believed suitable for use in the present invention. Examples of other sources include animal hair, skin, hooves, feathers, beaks, feet and horns. The patient or a human donor are some preferred sources of hair, as hair from these sources is most likely to result in a non-immunogenic product, although animal hair may be acceptable for many individuals for many applications. In one method according to the present invention, hair is provided, preferably clean and unbleached. In another method, the hair is washed with Versa-Clean™ (Fisher Scientific, Pittsburgh, Pa.), rinsed with deionized water, and allowed to dry.

A. Preparation of Oxidized Keratin

The hair can be oxidized in peracetic acid or another suitable reagent such as H_2O_2 . One method utilizes between about 1% to 32% peracetic acid, at a temperature between about 0 degrees C. and 100 degrees C. for between 0.5 and 24 hours. In one method, about 1 weight/volume percent peracetic acid is used. One method treats 30 grams of hair with 500 ml. of 4% peracetic acid at 4 degrees C. for 24 hours. Another method treats the hair at room temperature for 24 hours. Yet another method treats the hair at about 90 degrees C. for about 10 hours. In a preferred method, the hair is treated by heating the hair in the oxidizing agent for between about 1 and 4 hours at a temperature between about 20 and 100 degrees C. In a more preferred method, the hair is treated by heating the hair in the oxidizing agent for between about 1 and 2 hours at a temperature between about 80 and 100 degrees C. In a most preferred method, the hair is treated by heating the hair in about 2 weight/volume percent oxidizing agent for about 2 hours at a temperature of about 100 degrees C. The oxidation is believed to cleave a significant portion of keratin disulfide bonds forming cysteic acid residues having sulfonic acid groups. The sulfonic acid groups are believed to be hydrophilic in nature and will ionically bond to cations later in the process, forming a salt of the keratin and cation. The partial oxidation is also

**SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIRECCIÓN DE NUEVAS CREACIONES**

2020
Bogotá, D.C.,

Abogado
EMILIO FERRERO
Apoderado

ASUNTO	Radicación	06-98637
	Trámite	002
	Evento	001
	Actuación	430
	Oficio	
	Folios	002

Como resultado del examen de búsqueda de anterioridades y con fundamento en el artículo 46 de la Decisión 486 de la Comisión de la Comunidad Andina, se le comunica:

1. SOLICITUD DE SUMINISTRO DE INFORMACIÓN

El artículo 46 de la Decisión 486 de la Comisión de la Comunidad Andina establece que: "La Oficina Nacional competente podrá requerir el informe de expertos o de organismos científicos o tecnológicos que se consideren idóneos, para que emitan opinión sobre la patentabilidad de la invención. Asimismo, cuando lo estime conveniente, podrá requerir informes de otras oficinas de propiedad industrial. De ser necesario, a efectos del examen de patentabilidad y a requerimiento de la oficina nacional competente, el solicitante proporcionará, en un plazo que no excederán de 3 meses, uno o más de los siguientes documentos relativos a una o más de las solicitudes extranjeras referidas total o parcialmente a la misma invención que se examina: ... b) copia de los resultados de exámenes de novedad o de patentabilidad efectuados respecto a esa solicitud extranjera; ... Si el solicitante no presentara los documentos requeridos dentro del plazo señalado en el presente artículo la oficina nacional competente denegará la patente".

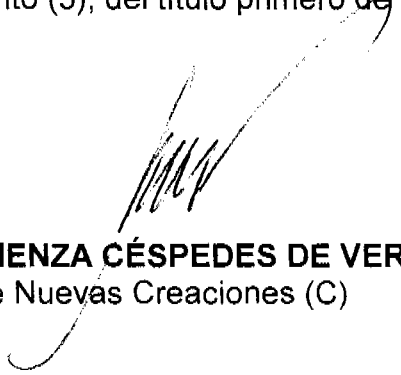
Como consecuencia de la búsqueda de anterioridades de materia relacionada con la de la presente invención, se determinó que existen en el estado de la técnica los siguientes documentos, que eventualmente afectarían los requisitos de patentabilidad de esta solicitud:

SHALABY , SW. Polymers for augmenting R Botulinum vaccine efficacy, crisp data base National Institutes of Health, 2002. Todo el documento.
CROSLAND, R. Detection of sparse Botulinum toxin A binding sites using fluorescent latex microspheres, June 1999, vol 22, no. 2, pages 113-115.
SCHANTZ et al., properties and use of botulinum toxin and other microbial neurotoxins in medicine, microbiological reviews, march 1992, p. 80-99. Todo
CONSOLE et al, antennapedia and HIV transactivator of transcription (TAT) protein transduction domains promote endocytosis of cell surface glycosaminoglycans, the journal of biological chemistry, 2003, vol. 276, No. 37, p. 35109 – 35224. todo

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Siendo que las publicaciones referenciadas hacen parte del resultado del examen de búsqueda realizado esta solicitud colombiana, y no siendo viable la consecución de dichos artículos por este despacho, se requiere que el solicitante, en virtud de lo establecido en el artículo 46 de la Decisión 486 de la Comisión de la Comunidad Andina, proporcione las publicaciones completas, con lo que podrá realizarse un análisis absoluto de la patentabilidad de la invención correspondiente a la presente solicitud.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto (5), del título primero de la circular única N° 10 de 2001.



ALIX CARMENZA CÉSPEDES DE VERGEL
Directora de Nuevas Creaciones (C)

El anterior requerimiento fue notificado por fijación en lista, de fecha 04 JUN 2010

CONCEPTO TÉCNICO Nº: 1125	EXAMINADOR TÉCNICO Código Nº: 202087	EXAMINADOR JURÍDICO Código Nº:
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**SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIRECCION DE NUEVAS CREACIONES**

2020
Bogotá, D.C.,

Abogado
EMILIO FERRERO
Apoderado

ASUNTO	Radicación	06-98637
	Trámite	011
	Evento	379
	Actuación	430
	Oficio	
	Folios	211

Patente de Invención	☒	Patente de Modelo de Utilidad	☐
Vía Nacional	☐		
Vía PCT (fase nacional)	☒	Cap. I	☒
		Cap. II	☐
No. Sol. Internacional	PCT/US2005/007524		
Fecha de Presentación Internal.	2005/03/03		

Como resultado del examen de fondo, realizado con fundamento en el artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le comunica:

1. Documentos estudiados

Descripción: Folios: 66 a 120

Reivindicaciones: Folios 121 a 142 como se radicó inicialmente

Prioridad: Fecha: 2004/03/03, país: US, número: 60/550.015

2. Objeto de la invención

- Título de la solicitud: Composiciones y métodos para aplicación tópica y suministro transdermico de toxinas botulinum. Número de folio: 1

El problema planteado en esta solicitud es la necesidad de la aplicación tópica de la toxina botulinica que es más segura y deseable debido a la naturaleza dolorosa de la aplicación, a las largas superficies de tratamiento que se deben cubrir, a la habilidad de formular una toxina pura con una mas alta especificidad de actividad, a las menores dosis que son necesarias para producir el efecto deseado, etc, por lo tanto es necesario un medio para la entrega y administración transdermica (tipo parche) de la toxina sin requerir inyección.

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Para solucionar este problema técnico la solicitud en estudio proporciona una composición para aplicación tópica de toxina botulínica.

Clasificación Internacional de Patentes (IPC.): A61K 51/00

3. Unidad de invención (artículo 25 D 486)

Reivindicaciones 41- 50: Se refiere a un kit para administración de una toxina botulinum a un sujeto. Los componentes del kit (toxina, portador, aplicador habitual, frasco, dispositivo, composición, empaque, instrucciones) pertenecen a diferentes campos técnicos de manera que no dan cabida al principio de unidad de invención.

4. Excepción a la patentabilidad (artículo 20 D486 literal a)-d)

Reivindicaciones 51- 150: Se refieren a un método para administrar una toxina botulinum a un sujeto por lo tanto están exceptuadas de patentabilidad según la legislación vigente.

5. Determinación del Estado de la Técnica

La fecha que se ha tenido en cuenta para determinar el Estado de la Técnica es:

Fecha de presentación de la solicitud prioritaria 2004/03/03

Teniendo en cuenta la fecha indicada se realizó la búsqueda en el estado de la técnica de documentos relacionados con la solicitud encontrándose los siguientes documentos más relevantes:

Nº	Documento	Título	Fecha de publicación*
D ₁	US 2003/0147921	METHOD OF TARGETING PHARMACEUTICALS TO MOTOR NEURONS	2003/08/7
D ₂	US 6544548	KERATIN- BASED POWDERS AND HYDROGEL FOR PHARMACEUTICAL APPLICATIONS	2003/04/8

6. Análisis de patentabilidad (artículo 14 D486)

Nivel inventivo (artículo 18 D486)

La presente Solicitud de Patente reivindica:

Reivindicación 1: Composición que comprende toxina botulinum en asociación no covalente a un portador que comprende una estructura polimérica que tiene grupos ramificados adheridos cargados positivamente.

Dicha composición está afectada por los siguientes documentos del Estado de la Técnica:

D1 enseña la aplicación médica de la toxina botulínica y composiciones que comprenden la toxina botulínica en asociación a un portador polimérico.

D2 enseña que la asociación de un agente activo con un excipiente puede llevarse a cabo mediante atracción no covalente (folio 203 ln 65-67) o asociación a través de interacción electrostática, hidrofilita o iónica, en donde el agente activo puede ser una forma catiónica que se une iónicamente al excipiente. La práctica de la invención puede administrarse mediante implantación subcutánea (folio 204) la cual incluye inyecciones de larga duración, supositorios, ungüentos tópicos o aplicaciones transdérmicas como parches cutáneos (folio 204 ln 58).

La única **diferencia con D₁** es que esta solicitud **es que la unión entre la toxina y el portador polimérico es no covalente**.

El efecto técnico logrado por esta diferencia es una forma de aplicación tópica para la toxina botulínica.

El Problema Técnico Objetivo se puede definir en como lograr **una forma de aplicación vía tópica** para la toxina botulínica **alternativa a lo previamente conocido**.

Por tanto, la solicitud, tal como se reivindica, es obvia, porque el experto medio en la materia enfrentado al problema de necesitar una vía tópica para la administración de la toxina botulínica y conociendo el estado de la técnica fabricaría las composiciones reveladas en D1 en donde la toxina botulínica se asocia a un portador polimérico pero utilizando las enseñanzas de D2 en donde se asocia un agente activo a un excipiente de forma no covalente que puede ser administrable en forma de parches transdermicos, y así de esta forma lograría lo reivindicado.

En conclusión, no se considera que la presente solicitud merezca reconocimiento de nivel inventivo.

6. Conclusión:

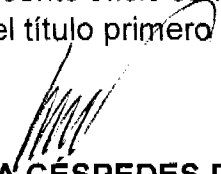
Los requisitos de patentabilidad de la presente solicitud se encuentran afectados así:

Nº	Documento Nº	Reivindicaciones afectadas	Requisito que afecta
D ₁	US 2003/0147921	1-40	NIVEL INVENTIVO
D ₂	US 6544548	1-40	NIVEL INVENTIVO

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En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de 60 días contados a partir de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única N° 10 de 2001.


ALIX CARMENZA CÉSPEDES DE VERGEL
Directora de Nuevas Creaciones (C)

El anterior requerimiento fue notificado por fijación en lista, de fecha
14 de mayo de 2006

CONCEPTO TÉCNICO No. 1125	EXAMINADOR TÉCNICO Código No. 202087
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