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(74) Agents: **REPPER, George, R.** et al.; 1425 K Street, N.W.,
Suite 800, Washington, District of Columbia 20005 (US).

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(71) Applicants (*for all designated States except US*): **RE-GENERX BIOPHARMACEUTICALS, INC.** [US/US]; 3 Bethesda Metro Center, Suite 630, Bethesda, Maryland 20814 (US). **UNIVERSITAET ERLANGEN-NUERNBERG** [DE/DE]; Schlossplatz 4, D-91054 Erlangen (DE).

(72) Inventors; and

(75) Inventors/Applicants (*for US only*): **HANNAPPEL, Ewald** [DE/DE]; Schwalbenweg 1, 91080 Uttenreuth (DE). **HUFF, Thomas** [DE/DE]; Froebelstrasse 25, 91058 Erlangen (DE). **GOLDSTEIN, Allan, L.** [US/US]; 800 25th Street, N.W., Apt. 1005, Washington, District of Columbia 20037 (US). **CROCKFORD, David** [US/US]; 62 Kent Street, Newburyport, Massachusetts 01950 (US).

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(54) Title: CELL NUCLEUS-ENTERING COMPOSITIONS

(57) Abstract: A pharmaceutically acceptable composition and method for entering a cell nucleus utilizes a cell nucleus-entering polypeptide including at least one of amino acid sequence LKKTET, amino acid sequence LKKTNT or amino acid sequence KSKLKK, or a conservative variant thereof, linked to a physiologically active agent having at least one of therapeutic or diagnostic application in the cell nucleus.



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CELL NUCLEUS-ENTERING COMPOSITIONS

BACKGROUND OF THE INVENTION

CROSS-REFERENCE TO RELATED APPLICATIONS

[001] The present application claims benefit of U.S. Provisional Application Serial No. 60/684,993, filed May 27, 2005.

Field of the Invention

[002] The present invention relates to the field of compositions and methods for delivering physiologically active agents.

Description of the Background Art

[003] There is a need in the art for improved compositions and methods for delivering physiologically active agents.

SUMMARY OF THE INVENTION

[004] In accordance with the present invention, a pharmaceutically acceptable composition for entering a cell nucleus comprises a cell nucleus-entering polypeptide comprising at least one of amino acid sequence LKKTET, amino acid sequence LKKTNT or amino acid sequence KSKLKK, or a conservative variant thereof, linked to a physiologically active agent having at least one of therapeutic or diagnostic application in said cell nucleus.

DETAILED DESCRIPTION OF THE INVENTION

[005] The present invention provides compositions and methods utilizing actin-sequestering peptides such as thymosin β 4 (T β 4, TB4 or TBeta4) and other actin-sequestering peptides or peptide fragments containing amino acid sequence LKKTET, LKKTNT or KSKLKK, or conservative variants thereof. Included are N- or C-terminal variants such as KLKKTET and LKKTETO. These peptides and peptide fragments are useful for entering cell nuclei for treating and/or preventing various conditions, and affecting numerous

physiological functions. In some preferred embodiments, the cell-entering peptide is T β 4.

[006] The physiologically active agent can be linked to the cell-entering peptide at any suitable position. For example, the agent can be linked to the N-terminus of T β 4, or to an amino acid of the cell-entering peptide at another location, most preferably a glutamine residue. The agent can be a drug, chemotherapeutic agent, DNA sequence, RNA sequence, DNA- or RNA-activating or deactivating agent, diagnostic agent, or the like.

[007] In preferred embodiments, the cell-entering peptide penetrates the nuclear membrane so as to carry the linked agent into the nucleus. The nuclear membrane preferably is mammalian, more preferably human.

[008] The invention also is applicable to a method of administering an agent-carrying cell-entering peptide to a mammalian subject. The method may comprise contacting a nuclear membrane or tissue of a subject with a composition as defined herein, preferably a pharmaceutically acceptable composition. In preferred embodiments, the subject is mammalian, more preferably human.

[009] Thymosin β 4 initially was identified as a protein that is up-regulated during endothelial cell migration and differentiation *in vitro*. Thymosin β 4 is a 43 amino acid, 4.9 kDa ubiquitous polypeptide identified in a variety of tissues. Several roles have been ascribed to this protein including a role in a endothelial cell differentiation and migration, T cell differentiation, actin sequestration and vascularization.

[0010] Thymosin β 4 is a member of the β -thymosin family of highly conserved polar 5-kDa polypeptides found in various tissues and cell types. Originally purified from thymus and regarded as a thymic hormone, thymosin β 4 was then found to be involved in multiple biological processes. As the main G-actin sequestering peptide, it plays an important role in regulation of actin assembly during cell proliferation, migration, and differentiation. Numerous studies implicate thymosin β 4 in regulation of cancerogenesis, inflammation, angiogenesis, and wound healing. It was found that thymosin

$\beta 4$ expression regulated tumorigenicity and metastatic activity in malignant cell lines through actin-based cytoskeletal organization. Thymosin $\beta 4$ was found to be elevated in tube forming endothelial cells; it increases their attachment, spreading and migration thus promoting angiogenesis.

Thymosin $\beta 4$ was also found in ulcer extracts and wound fluids at high concentrations and was suggested to function as an antibacterial factor. The stimulating role of thymosin $\beta 4$ in wound healing was demonstrated in several studies with animal models. When added topically or administered intraperitoneally, thymosin $\beta 4$ enhanced dermal wound healing in a rat full thickness model. The ability to accelerate dermal wound healing has also been observed in db/db diabetic mice, steroid-immunosuppressed mice and in aged mice. Thymosin $\beta 4$ has also been shown to accelerate healing of the corneal epithelium after burn injuries and to down regulate a number of corneal cytokines and chemokines reducing the inflammatory response.

[0011] Activation of the coagulation cascade upon vascular injury results in generation of thrombin which converts fibrinogen into fibrin. Fibrin polymerizes spontaneously to form blood clots which seals damaged places thus preventing the loss of blood. Fibrin also serves as a provisional matrix on which various cell types adhere, migrate and proliferate replacing fibrin with normal tissues during subsequent wound healing processes.

[0012] Thymosin $\beta 4$ serves as a specific substrate for tissue transglutaminase and can be selectively cross-linked by it to collagen, actin, fibrinogen and fibrin, proteins which are also involved in the above mentioned processes. After activation of platelets with thrombin, thymosin $\beta 4$ is released and cross-linked to fibrin in a time- and calcium-dependent manner.

[0013] In preferred embodiments, the cell-entering polypeptide comprises amino acid sequence LKKTET, LKKTNT, KSKLKK, KLKKTET, LKKTETO, Thymosin $\beta 4$ (T $\beta 4$), an N-terminal variant of T $\beta 4$, a nucleus-entering C-terminal variant of T $\beta 4$, an N-terminal fragment of T $\beta 4$, an isoform of T $\beta 4$, a splice-variant of T $\beta 4$, oxidized T $\beta 4$, T $\beta 4$ sulfoxide, lymphoid T $\beta 4$, pegylated

T β 4 or any other actin sequestering or bundling proteins having actin binding domains, or peptide fragments comprising or consisting essentially of the amino acid sequence LKKTET, LKKTNT or KSKLKK, or conservative variants thereof. International Application Serial No. PCT/US99/17282, incorporated herein by reference, discloses isoforms of T β 4 which may be useful in accordance with the present invention as well as amino acid sequence LKKTET, and conservative variants thereof, which may be utilized with the present invention. International Application Serial No. PCT/GB99/00833 (WO 99/49883), incorporated herein by reference, discloses oxidized Thymosin β 4 which may be utilized in accordance with the present invention. Although the present invention is described primarily hereinafter with respect to T β 4 and T β 4 isoforms, it is to be understood that the following description is intended to be equally applicable to amino acid sequence LKKTET, LKKTNT, KSKLKK, KLKKTET, LKKTETO, peptides and fragments comprising or consisting essentially of LKKTET, LKKTNT, KSKLKK, KLKKTET, or LKKTETO, conservative variants thereof, as well as oxidized Thymosin β 4, T β 4 sulfoxide, lymphoid T β 4 and pegylated T β 4.

[0014] The cell-entering peptide with linked agent may be administered in any suitable effective amount. For example, the cell-entering peptide with linked agent may be administered in dosages within the range of about 0.1-50 micrograms, more preferably in amounts within the range of about 1-30 micrograms.

[0015] A composition in accordance with the present invention can be administered once, daily, every other day, every other week, every other month, etc., with a single application or multiple applications per day of administration, such as applications 2, 3, 4 or more times per day of administration.

[0016] T β 4 isoforms have been identified and have about 70%, or about 75%, or about 80% or more homology to the known amino acid sequence of T β 4. Such isoforms include, for example, T β 4^{ala}, T β 9, T β 10, T β 11, T β 12, T β 13, T β 14 and T β 15. Similar to T β 4, the T β 10 and T β 15 isoforms, as well as the T β 4

splice-variants, have been shown to sequester actin. T β 4, T β 10 and T β 15, as well as other isoforms share an amino acid sequence, LKKTET, that appears to be involved in mediating actin sequestration or binding. Although not wishing to be bound to any particular theory, the activity of T β 4 isoforms may be due, in part, to the ability to regulate the polymerization of actin. β -thymosins appear to depolymerize F-actin by sequestering free G-actin. T β 4's ability to modulate actin polymerization may therefore be due to all, or in part, its ability to bind to or sequester actin via the LKKTET sequence. Thus, as with T β 4, other proteins which bind or sequester actin, or modulate actin polymerization, including T β 4 isoforms having the amino acid sequence LKKTET, are likely to be effective, alone or in a combination with T β 4, as set forth herein, as are cell-entering peptides comprising sequence cell-entering.

[0017] Thus, it is specifically contemplated that known T β 4 isoforms, such as T β 4^{ala}, T β 9, T β 10, T β 11, T β 12, T β 13, T β 14 and T β 15, as well as T β 4 isoforms and T β 4 splice-variants not yet identified, will be useful in the methods of the invention. As such T β 4 isoforms are useful in the methods of the invention, including the methods practiced in a subject. The invention therefore further provides pharmaceutical compositions comprising agent-carrying T β 4, as well as T β 4 isoforms T β 4^{ala}, T β 9, T β 10, T β 11, T β 12, T β 13, T β 14 and T β 15, and a pharmaceutically acceptable carrier.

[0018] In addition, other proteins having actin sequestering or binding capability, or that can mobilize actin or modulate actin polymerization, as demonstrated in an appropriate sequestering, binding, mobilization or polymerization assay, or identified by the presence of an amino acid sequence that mediates actin binding, such as LKKTET, LKKTNT or KSKLKK, for example, can similarly be employed in the methods of the invention. Such proteins include gelsolin, vitamin D binding protein (DBP), profilin, cofilin, adsevertin, propomyosin, fincilin, depactin, DnaseI, villin, fragmin, severin, capping protein, β -actinin and acumentin, for example. As such methods include those practiced in a subject, the invention further provides pharmaceutical compositions comprising gelsolin, vitamin D binding protein

(DBP), profilin, cofilin, depactin, DnaseI, villin, fragmin, severin, capping protein, β -actinin and acumentin as set forth herein. Thus, the invention includes compositions and methods utilizing a polypeptide comprising the amino acid sequence LKKTET, LKKTNT or KSKLKK, (which may be within its primary amino acid sequence) and conservative variants thereof.

[0019] As used herein, the term "conservative variant" or grammatical variations thereof denotes the replacement of an amino acid residue by another, biologically similar residue. Examples of conservative variations include the replacement of a hydrophobic residue such as isoleucine, valine, leucine or methionine for another, the replacement of a polar residue for another, such as the substitution of arginine for lysine, glutamic for aspartic acids, or glutamine for asparagine, and the like.

[0020] The actual dosage, formulation or composition utilized may depend on many factors, including the size and health of a subject. However, persons of ordinary skill in the art can use teachings describing the methods and techniques for determining clinical dosages as disclosed in PCT/US99/17282, *supra*, and the references cited therein, to determine the appropriate dosage to use.

[0021] Suitable formulations may include the agent-carrying LKKTET, LKKTNT or KSKLKK peptide in a carrier at a concentration within the range of about 0.0001 - 10% by weight, more preferably within the range of about 0.01 - 0.1% by weight, most preferably about 0.05% by weight. Any suitable pharmaceutically acceptable carrier may be utilized, such as water for injection.

[0022] The invention also relates to methods for delivering a physiologically active agent to a cell nucleus comprising administering to the cell nucleus a pharmaceutically acceptable composition as described herein. The method may involve administering the composition to a cell containing a nucleus, to a nucleus within a cell, to a mammalian subject, or by contacting tissue of a subject with the inventive composition.

Example 1

[0023] Physiologically active agents having therapeutic and/or diagnostic application in a cell nucleus are linked to T β 4 as follows. The agents are selected from drugs, chemotherapeutic agents, DNA sequences, RNA sequences, DNA- or RNA-activity or deactivity agents and diagnostic agents.

[0024] Agent-linked thymosin β 4 is prepared by incubation of 240 μ g thymosin β 4 (200 μ M) with 120 μ g agent (1 mM) and 0.2 U guinea pig transglutaminase at room temperature in 240 μ l buffer consisting of 10 mM Tris-HCl, pH 7.4, 15 mM CaCl₂, 3 mM DTT. After 1 and 2 hours, 5 μ l of the reaction mixture is subjected to HPLC analysis. The reaction is stopped after 4 hours by addition of 5 μ l trifluoroacetic acid (TFA). Then the reaction mixture is subjected to preparative HPLC. Separated peptides are concentrated in vacuo and then characterised by amino acid analysis and mass spectrometry.

[0025] Proteolytic fragments of agent-linked thymosin β 4 are prepared by the following procedure: 50 μ g of peptide is incubated with 20 μ U AsnC-endoprotease in 100 μ l reaction buffer (50 mM sodium acetate, pH 5.0, 0.2 mM DTT, 0.2 mM EDTA) for 16 hours at room temperature. Then the reaction is stopped by adding 5 μ l 10% TFA and products are separated by preparative HPLC. Prior to analysis the samples are concentrated in vacuo.

Microinjection experiments

[0026] Microinjection is performed with an ECET cell injection system (Eppendorf, Hamburg, Germany) consisting of the micromanipulator 5170 and the microinjector 5242 adapted to an Axiovert 100 inverted microscope (Zeiss, Göttingen, Germany). Microinjections are visually controlled by a CCD camera on a TV monitor (SSM 121CE, Sony, Tokyo, Japan). Agent-linked thymosin β 4 and crosslinked ADP-ribosylated actin:thymosin β 4 complex are injected into the cytoplasm at a concentration of 32 μ M and 8.27 μ M respectively, in 135 mM KCl, 5 mM Na₂HPO₄, pH 7.2. The injection pressure is between 65 and 80 hPa (1 hPa=0.1 kPa) and the injection time between 0.5

and 0.7 seconds.

[0027] Agent-linked thymosin β 4 is microinjected into the cytoplasm of cells. Directly after microinjection the linked peptide is evenly distributed throughout the cytoplasm. After incubation for 1 hour a pronounced localization the cell nucleus is detected.

[0028] The N-terminal portion of thymosin β 4 contains a sequence stretch enriched in lysine residues ($^{14}\text{KSKLKK}^{19}$) which may be a functional nuclear localisation signal. An N-terminal fragment (thymosin β $^{27-43}_4$) containing the $^{14}\text{KSKLKK}^{19}$ sequence exhibits a pronounced nuclear localisation.

Claims

1. A pharmaceutically acceptable composition for entering a cell nucleus, comprising a cell nucleus-entering polypeptide comprising at least one of amino acid sequence LKKTET, amino acid sequence LKKTNT or amino acid sequence KSKLKK, or a conservative variant thereof, linked to a physiologically active agent having at least one of therapeutic or diagnostic application in said cell nucleus.
2. The composition of claim 1, wherein said physiologically active agent comprises a drug, chemotherapeutic agent or nucleic acid sequence.
3. The composition of claim 1 wherein said cell nucleus-entering polypeptide comprises amino acid sequence LKKTET or amino acid sequence KSKLKK.
4. The composition of claim 1 wherein said cell nucleus-entering polypeptide comprises thymosin beta 4.
5. The composition of claim 1 wherein said polypeptide comprises an N-terminal fragment of thymosin beta 4.
6. The composition of claim 1 wherein said cell-entering polypeptide linked to said agent is present in a pharmaceutically acceptable carrier.
7. The composition of claim 6 wherein said cell nucleus-entering polypeptide linked to said agent is present in said carrier in concentration within a range of about 0.0001-10% by weight of said carrier.
8. A method of delivering a physiologically active agent to a cell nucleus comprising administering to said cell nucleus the composition of claim 1.
9. The method of claim 8 comprising administering said composition to a mammalian subject.

10. The method of claim 9 comprising contacting a tissue of said subject with said composition.
11. The method of claim 8 wherein said physiologically active agent comprises a drug, chemotherapeutic agent or nucleic acid sequence.
12. The method of claim 8 wherein said cell nucleus-entering polypeptide comprises amino acid sequence LKKTET or amino acid sequence KSKLKK.
13. The method of claim 8 wherein said cell nucleus-entering polypeptide comprises thymosin beta 4.
14. The method of claim 8 wherein said polypeptide comprises an N-terminal fragment of thymosin beta 4.
15. The method of claim 8 wherein said cell nucleus-entering polypeptide linked to said agent is present in a pharmaceutically acceptable carrier.
16. The method of claim 15 wherein said cell nucleus-entering polypeptide linked to said agent is present in said carrier in a concentration within a range of about 0.0001 – 10% by weight of said carrier.
17. The composition of claim 4 wherein said physiologically active agent is linked to an N-terminal portion of said thymosin beta 4.
18. The composition of claim 5 wherein said physiologically active agent is linked to an N-terminal portion of said N-terminal fragment.
19. The method of claim 13 wherein said physiologically active agent is linked to an N-terminal portion of said thymosin beta 4.
20. The method of claim 14 wherein said physiologically active agent is linked to an N-terminal portion of said N-terminal fragment.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US05/35716

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) : A61K 38/00

US CL : 530/324

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/324

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

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

Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	WO 2004/0099768 A1 (REGENEREX BIOPHARMACEUTICALS, INC.) 18 November 2004 (18.11.2004), entire document, claims, especially claims 1, 5, 19-20.	1-7 ----- 8-20
X --- Y	WO 2004/091550 A2 (REGENEREX BIOPHARMACEUTICALS, INC.) 28 October 2004 (28.10.2004), entire document, claims, especially claims 1, 5-6, 8, 12, 17.	1-7 ----- 8-20

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

☐ See patent family annex.

* Special categories of cited documents:	
"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E" earlier application or patent published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US
Commissioner of Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

Facsimile No. (571) 273-3201

Authorized officer

Maury Audet

Telephone No. 571-272-0960

INTERNATIONAL SEARCH REPORT

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Continuation of B. FIELDS SEARCHED Item 3:

STIC-Search of sequences LKKTET, LKKTNT, and KSKLKK (hand-entry into databse, no Sequenc List). East (Internal USPTO).