Industria y Comercio

SUPERINTENDENCIA

Delegatura de propiedad Industrial

División de Nuevas Creaciones

SOLICITUD

PATENTE DE INVENCIÓN

21. **EXPEDIENTE No.** 04-83750

54. **TÍTULO** Composiciones y Metodos para Modular Hemicanales de conexina

51. **CLASIFICACIÓN INTERNACIONAL** A61K38/08

71. **SOLICITANTE** Wyeth

DOMICILIO Madison, Estado de new Jersey Estados Unidos de América.

74. **APODERADO** Emilio Ferrero
código Nº 1092

22. **BOGOTÁ, D. C.,** 26/08/2004

(FORMA P-10)

Industria y Comercio
SUPERINTENDENCIA

PETITORIO

SUPERINDUSTRIA Y COMERCIO
Radicacion : 04063750 00000000 Folios: 345
Fecha (AMD): 2004-08-05 16:52:51
Tramite : 011 PCT-PATENT 379 FASE NACI 411 PRESENTAC
Dependencia: 2020 DIVISION DE NUEVAS CREACIONES

FORMULARIO UNICO DE SOLICITUD DE PATENTE
PCT
ENTRADA EN FASE NACIONAL

1 **SOLICITUD DE:**

☒ *Patente de Invención.* ☐ *Patente de Modelo de Utilidad.*

☐ Capítulo I. ☒ Capítulo II.

2 **SOLICITANTE (71)**	Nombre: **WYETH** sociedad constituida y existente bajo las leyes del estado de Delaware de Estados Unidos de América Dirección: Five Giralda Farms, Madison, New Jersey 07940, Estados Unidos de América Domicilio: Madison, New Jersey, Estados Unidos de América	**IDENTIFICACION** C.C. ☐ NIT ☐ C.E. ☐ Otro ☐ Cual_____ Número: _____
3 **REPRESENTANTE O APODERADO**	Nombre: EMILIO FERRERO Dirección: Carrera 4ª. No. 72-35 Teléfono: 347 36 11 Fax:: 217 92 11 E-mail: clientes@camyp.com. Domicilio: Bogotá, Colombia.	**IDENTIFICACION** C.C. ☒ NIT ☐ C.E. ☐ Otro ☐ Cual_____ Número: 438.019 T.P. 31.929

4 **INVENTOR (ES) (72)**

1. Jørgen Søberg PETERSEN
Hellebakken 1,
DK-3150 Hellebaek,
Dinamarca

2. Søren NEVE
Ellevaenget 4,
DK-2800 Lyngby,
Dinamarca

3. Morten Schak NIELSEN
Egebjergvej 51A,
DK-2750 Ballerup,
Dinamarca

4. Eddi MEIER
Bringebakken 88,
DK-3500 Vaerløse,
Dinamarca

5. Eva STEINESS
Sømarksvej 11,
DK-2900 Hellerup,
Dinamarca

6. Peter Holme JENSEN
Classensgade 11, 3. tv.,
DK-2100 Copenhagen Ø,
Dinamarca

7. Bjarne Due LARSEN
Arildsgaard 5, 1. th.,
DK-2700 Brønshøj,
Dinamarca

8. Lars-Bo Laurenborg HANSEN
Peberhaven 19, st. tv.,
DK-2730 Herlev,
Dinamarca

(5) **PCT (86)**

Solicitud Internacional No. PCT/DK03/00056 Fecha: 29 de enero de 2003

Publicación Interancional No. WO 03/063891 A1 Fecha: 07 de agosto de 2003

(6) **Título (54)**

COMPOSICIONES Y MÉTODOS PARA MODULAR HEMICANALES DE CONEXINA

(7) **Clasificación Internacional (51)** A61K 38/08

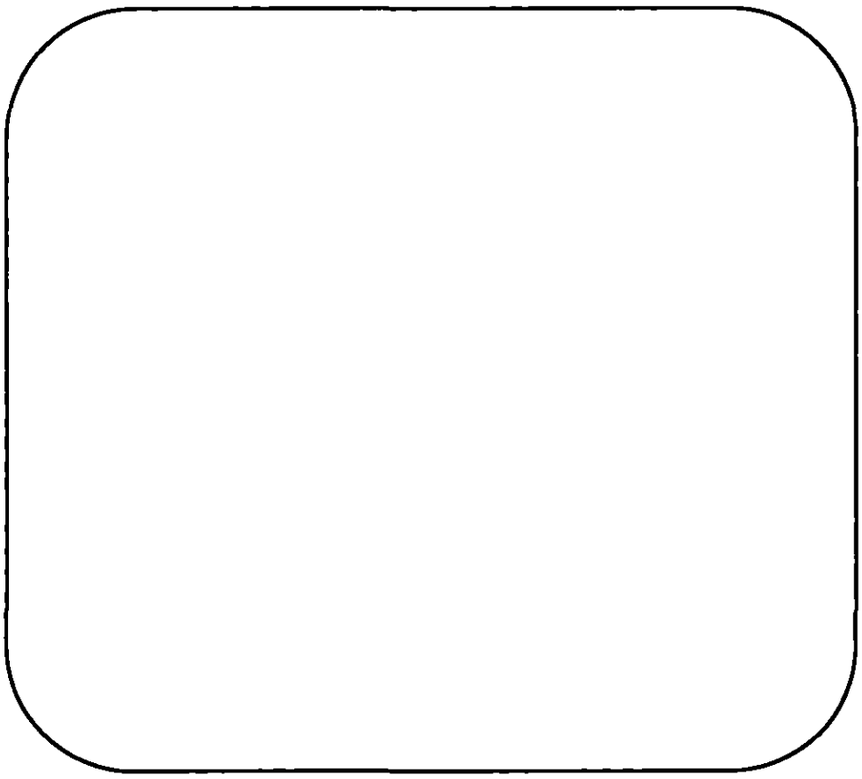
(8) **Prioridad**	(33) País de Origen	(32) Fecha	(31) Número de Solicitud
SI ☒ NO ☐	US	29 de enero de 2002	60/352,717

(9) **Comprobante de pago No.:** 04 – 63,348 **Fecha:** 10 de agosto de 2004

(10) **Anexos:**

- ☒ Comprobante de pago de la tasa de presentación de la solicitud por $ 422.000.oo.
- ☐ Comprobante de pago por reivindicación de prioridad.
- ☒ Documento que acredita la existencia y representación legal cuando el solicitante sea persona jurídica.
- ☒ Poderes, si fuere el caso.
- ☒ Documento de cesión del inventor al solicitante o a su causante.
- ☐ Declaraciones.
- ☒ Copia de la solicitud Internacional. (Resumen, descripción, reivindicaciones, dibujos)
- ☒ Traducción de la solicitud internacional al castellano. (Resumen, descripción, reivindicaciones, dibujos)
- ☐ Copia del examen preliminar efectuado por la IPEA.
- ☐ Traducción del examen preliminar efectuado por la IPEA.
- ☐ De ser el caso, copia del contrato de acceso.
- ☐ De ser el caso, documento que acredite la licencia o autorización de uso de conocimientos tradicionales de las comunidades indígenas.
- ☐ De ser el caso, certificado de depósito del material biológico.
- ☐ Arte final 12x12
- ☐ De ser el caso, información sobre otras solicitudes de patente o títulos obtenidos en el extranjero por el mismo titular o su causante, relacionadas parcial o totalmente con la invención de esta solicitud.
- ☐ Otros

11 **FIGURA CARACTERISTICA:**



12 **Solicito la concesión de la patente,** **NOMBRE:** EMILIO FERRERO

FIRMA :

C.C. 438.019 de Usaquén T.P. 31.929

DLM

4

13665
8070

APOSTILLE

(CONVENTION DE LA HAYE DU 5 OCTOBRE 1961)

1. COUNTRY: UNITED STATES OF AMERICA

2. THIS PUBLIC DOCUMENT HAS BEEN SIGNED BY:
MARILYN L MITCHELL

3. ACTING IN THE CAPACITY OF:
NOTARY PUBLIC OF NEW JERSEY

4. BEARS THE SEAL/STAMP OF:
MARILYN L MITCHELL, NOTARY

CERTIFIED

5. AT TRENTON, NEW JERSEY

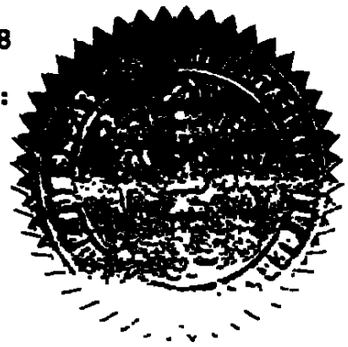
6. THE 26TH DAY OF JUNE 2003

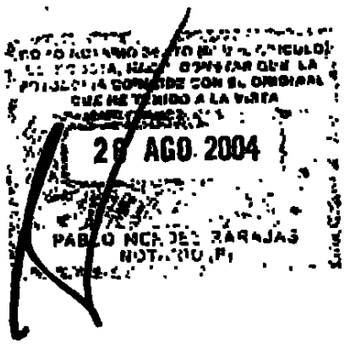
7. BY: John E McCormac, CPA, NEW JERSEY TREASURER

8. NO. A175618

9. SEAL/STAMP:

10. SIGNATURE





CAVELIER
ABOGADOS
EDIFICIO SIGLO
CARRERA 4a Nº 72-35
BOGOTA 8 - COLOMBIA

181

Representación y Poder con autenticación notarial y Apostille para patentes, modelos de utilidad, diseños industriales, marcas de fábrica, nombres comerciales, enseñas, nombres de dominio, lemas comerciales, licencias.

Representation and Power-of-Attorney with notarial authentication and Apostille for patents, utility models, industrial designs, trademarks, commercial names, trade emblems, domain names, slogans, licenses.

REPRESENTACION Y PODER

REPRESENTATION AND POWER-OF-ATTORNEY

Yo (nosotros), abajo firmado (s)/I (we), the undersigned

Wyeth

domiciliado (s) en/of Five Giralda Farms

Madison, NJ 07940, United States of America

por el presente nombramos a EMILIO FERRERO, tps. 31929; GONZALO PERDOMO, tps. 831; LUZ CLEMENCIA DE PAEZ, tps. 23555 y GERMAN CAVELIER, tps. 832, domiciliados en la Carrera 4a Nº 72-35, Bogotá, Colombia, en obedecimiento a lo dispuesto en el parágrafo 1o del Artículo 543 del Código de Comercio, el primero como principal y los demás como suplentes, en su orden, como mis (nuestros) representantes en todos los asuntos de propiedad industrial con facultad para recibir notificaciones y nombrar apoderados judiciales o extrajudiciales. El primer representante suplente reemplazará al principal en caso de ausencia o impedimento temporal o definitivo de aquél. La misma regla se aplicará cuando el segundo representante suplente haya de reemplazar al primero, o el tercero al segundo. En tales casos, el representante principal, o el primero, o segundo suplente, en su caso, o ambos, declararán su intención de no ejercer la representación mediante declaración autenticada por Notario Público en Colombia, o por Notario u otro Funcionario Público, o Cónsul de Colombia, en el extranjero; y si se tratare de impedimento, con el certificado respectivo. Si alguno de los nombrados faltare definitivamente, el siguiente tomará su lugar. Cuando cesare la ausencia o impedimento del representante principal, o de su primero o segundo suplentes, en su caso, podrán ejercer la representación, en su orden, uno u otros según el caso, asumiéndola por el solo hecho de ejercerla, cesando en ese momento el ejercicio de la representación por el primero, segundo o tercer suplente en su caso.

Además, confiero (confierimos) poder a los abogados arriba nombrados, para obtener la protección de mi (nuestra) propiedad industrial y contra la competencia desleal, a cuyo efecto autorizo (amos) a los dichos apoderados para que me (nos) representen ante cualesquiera entidades o personas, ejerzan o no jurisdicción, especialmente ante las del orden administrativo, comercio- administrativo y judicial, así como los tribunales de arbitramento, en todo lo concerniente a los siguientes asuntos: a) Obtención de patentes de invención, modelos de utilidad y diseños industriales: el registro de marcas de fábrica, colectivas, defensivas, de servicio, lemas comerciales, nombres comerciales, enseñas, nombres de dominio, variedades vegetales y denominaciones de origen; su renovación, traspaso, cambio de nombre o domicilio, cancelación voluntaria; y el registro de licencias de uso de esos derechos; b) Las acciones de competencia desleal, protección del consumidor, oposiciones u observaciones, cancelación administrativa y judicial, nulidad, medidas cautelares, concesión de licencias, la tasa, la protección de secretos industriales, y en general los juicios civiles, penales, administrativos o contencioso administrativos relacionados con

in due compliance with the terms of paragraph 1st. of article 543 of the Commercial Code, do hereby appoint EMILIO FERRERO, tps. 31929; GONZALO PERDOMO, tps. 831; LUZ CLEMENCIA DE PAEZ, tps. 23555 and GERMAN CAVELIER, tps. 832, domiciled at Carrera 4a Nº 72 35, Bogotá, Colombia, the first as principal and the others as alternates, in the order of their appointment, as our representatives for all industrial property matters with faculty to receive notifications and to appoint attorneys in and out of Court. The first alternate shall replace the principal in case of his absence or of temporal or definitive impediment. The same rule will apply when the second alternate representative is to replace the first one, or when the third is to replace the first or second alternates in their turn, or both of them, second. In such cases, the principal representative, or the first or second alternates in their turn, or both of them, shall state their intention not to act as representatives through a declaration given before a Notary Public in Colombia, or before a Notary or another Public Official or a Colombian Consul abroad; and in case of impediment, the appropriate certificate shall suffice. If any of the appointees were to be definitely absent, the following alternate shall take his place. When the absence or impediment of the principal representative, or of the first or second alternate in their turn ceases, the representation can be taken again in their sequence, by the principal, the first or second alternate depending on case, and the mere fact of its exercise will suffice. The exercise of the representation by the first, second or third alternates in their turn shall end at such time. In addition, we grant a Power of Attorney in favor of the above named attorneys at law so that they may obtain the protection of my (our) industrial property and against the unfair competition, for which purpose I (we) authorize the said attorneys to represent me (us) before any authorities or persons, with or without jurisdiction, specially those administrative, administrative commercious and judicial, as well as before Arbitration Court, in all matters concerning the following: a) Obtainment of patents, utility models and industrial designs; the registration of trademarks, collective, defensive and service marks, slogans, commercial names, trade emblems, domain names, vegetal varieties and denominations of origin; its renewal, assignment, change of name or domicile, voluntary cancellation; and the registration of licenses of use of the above rights; b) Actions of unfair competition, protection to the consumer, oppositions or observations, administrative or Court cancellation, nullity, infringement, granting of licenses, the action for writ mandamus, the protection of trade secrets, and in general the civil, penal, and administrative ~~and contentious~~ litigations and suits

COMO PRUEBA DE ESTO EN EL CIRCULO
Y EL PODERDANTE DEBE COTEJAR QUE LA
FOTOCOPIA CORRESPONDE CON EL ORIGINAL
QUE SE PRESENTA A LA VISTA

26 AGO 2004

PABLO MENDEZ BARAJAS
NOTARIO (E)

estos derechos; acciones y procesos que presentemos o se nos promuevan. Y para que en todos los asuntos arriba determinados, puedan ejercitar este mandato, transigir, conciliar, admitir los hechos del proceso, desistir, cancelar, recibir, renunciar, y ratificar los actos de agentes oficiosos.

instituted by me (us) or against me (us). And that in all the above matters they may substitute this Power of Attorney, compromise, conciliate, admit the facts of the case, desist, cancel, receive, resign, and ratify acts done by representatives without a power of attorney.

Wyeth

Dado y firmado hoy/Given and signed this 16th JUNE 2003

en/in Madison, New Jersey 07940; USA

por/by William H. Calnan

Assistant Secretary

Firma _____ Signature

COTEJO NOTARIAL HECHO EN ESTA CIUDAD DE BOGOTA, PARA CONSTAR QUE LA FOTOCOPIA CORRESPONDE CON EL ORIGINAL QUE HE TENIDO A LA VISTA

26 AGO 2004

PABLO MENDEZ E. [illegible]
NOTARIO (E)

7

CERTIFICADO NOTARIAL (INDIVIDUO)

a _____ días del mes de _____
200___ ante mí, Notario Público o Funcionario Autorizado

_____

apareció(ron) personalmente _____

_____

nien(es) doy fe de conocer y me consta que es(son) la(s) ona(s) firmante(s) del documento que precede, y quien(es) ne(n) capacidad legal para otorgarlo

NOTARIAL CERTIFICATE (INDIVIDUAL)

On this _____ day of _____
200___ before me, a Notary Public or Attesting Official of ___

_____

personally appeared _____

_____

to me known, and known to me to be the person(s) who executed that foregoing document, and who has(have) legal capacity to execute it.

_____ Notario Público o Funcionario Autorizado – Notary Public or Attesting Oficial _____

CERTIFICADO NOTARIAL (SOCIEDAD)

el 16 de Junio de 200 3
apareció(ron) ante mí William H. Calnan
ien(es) conozco personalmente y que firma(n) este cumento en representación de la Sociedad/compañía nominada Wyeth

_____

rifico:

Que he visto los siguientes documentos:
Certificado de Constitución replanteado
presentado el 22 de Abril 1988 ante el
Secretario del Estado de Delaware

Que dichos documentos prueban lo siguiente:

1. Que Wyeth

_____

una Sociedad/Compañía legalmente constituida y existente acuerdo con las leyes de Delaware

_____

2. Que el domicilio de dicha Sociedad/Compañía es Five Giralda Farms, Madison, New Jersey 07940; United States of America

_____

3. Que el objeto u objetos para los cuales se otorga el presente der están comprendidos dentro del objeto social de la ncionada Sociedad/Compañía.

4. Que el(los) Sr.(Sres.) William H. Calnan

_____

ha(n) sido designado(s) para otorgar el presente Poder.

Ciudad Morristown

Estado New Jersey

Hoy 16 de Junio de 200 3

NOTARIAL CERTIFICATE (CORPORATION)

On this 16th day of June 200 3
appeared before me William H. Calnan
who(m) I know personally and who(m) signed this document as representative(s) of the Corporation/Company named

_____

WYETH

I certify:

1. That I have seen the following documents:
Restated Certificate of Incorporation filed April 22, 1988 with Secretary of State of Delaware
2. That the above documents duly prove the following:

2.1. That Wyeth

_____

is a Corporation/Company legally constituted and in existence in accordance with the laws Delaware

_____

2.2. That the domicile of said Corporation/Company is Five Giralda Farms, Madison, New Jersey 07940; United States of America

2.3. That the purpose or purposes for which the present Power of Attorney is granted are comprised within the corporation purposes set forth in the charter of the mentioned Corporation/Company.

2.4. That Mr. (Messrs.) William H. Calnan

_____

is(are) authorized to grant the Power of Attorney.

City Morristown

State New Jersey

This 16th day of June 200 3

_____ Notario Público o Funcionario Autorizado – Notary Public or Attesting Oficial _____

Los documentos deben identificarse con fecha y origen

Documents must be identified with date and origin

CAVELIER ABOGADOS
PPODB01-181 (MINUTA N°12.1.1.2.6 USA)

MARILYN L. MITCHELL
NOTARY PUBLIC, STATE OF NEW JERSEY
My Commission Expires February 6, 2005

TRADUCCIÓN OFICIAL 101/2003 DE UN DOCUMENTO ESCRITO EN INGLÉS Y EN ESPAÑOL, REALIZADA POR EDUARDO CALADO NOGUERA, TRADUCTOR OFICIAL APROBADO POR RESOLUCIÓN 541 DEL 27 DE FEBRERO DE 1976 POR EL MINISTERIO DE JUSTICIA Y DEBIDAMENTE REGISTRADO ANTE EL MINISTERIO DE RELACIONES EXTERIORES DE LA REPÚBLICA DE COLOMBIA Y MEDIANTE EL CUAL **WYETH**, EMPRESA DOMICILIADA EN FIVE GIRALDA FARMS, MADISON, NJ 07940 ESTADOS UNIDOS DE AMÉRICA OTORGA PODER A EMILIO FERRERO, TPA 31929, GONZALO PERDOMO, TPA 831; LUZ CLEMENCIA DE PAEZ TPA 23555 Y A GERMÁN CAVELIER, TPA 832. EXPEDIDO Y FIRMADO EL 16 DE JUNIO DE 2003 EN MADISON, NEW JERSEY 07940, ESTADOS UNIDOS DE AMÉRICA. FIRMADO: WILLIAM H. CALNAN, SUBSECRETARIO.

CERTIFICADO NOTARIAL EN INGLES Y EN ESPAÑOL DE FECHA 16 DE JUNIO DE 2003 POR EL CUAL SE CERTIFICA LA COMPARECENCIA DE WILLIAM H. CALNAN PARA FIRMAR EL PODER ARRIBA CITADO A NOMBRE DE WYETH Y PARA CERTIFICAR LA EXISTENCIA LEGAL, EL DOMICILIO DE DICHA EMPRESA Y LA CAPACIDAD DE WILLIAM H. CALNAN PARA COMPROMETER A DICHA FIRMA. FIRMADO: MARILYN L. MITCHELL. SELLO: MARILYN L. MITCHELL, NOTARIO PÚBLICO, ESTADO DE NUEVA JERSEY. MI CARGO VENCE EL 6 DE FEBRERO DE 2005.

APOSTILLA:

(Convención de La Haya del 5 de octubre de 1961)

1. Estados Unidos de América

Este documento público

2. ha sido firmado por MARILYN L. MITCHELL

3. quien actúa en su calidad de NOTARIO PÚBLICO DE NUEVA JERSEY

4. lleva el sello de MARILYN L. MITCHELL, NOTARIO CERTIFICADO

5. en TRENTON, NUEVA JERSEY

6. el 26 DE JUNIO DE 2003

7. por JOHN E. MCCORMAC, CONTADOR PÚBLICO AUTORIZADO, TESORERO DE NUEVA JERSEY

8. Número A175618

9. Sello:

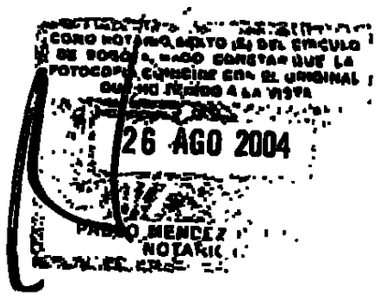
El sello del estado de Nueva Jersey

10. Firma: (ilegible)

COLO-13798-841-001-5

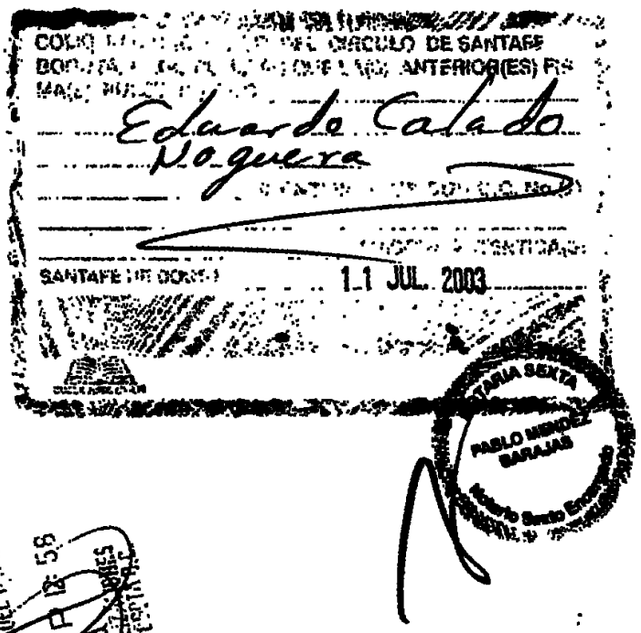
WPCL002G. ID. 4628

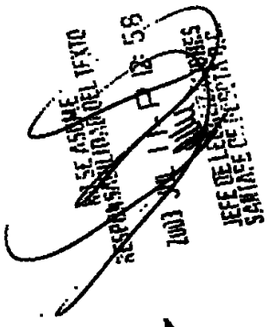
Bogotá, 12 de julio de 2003.



Eduardo Calad[illegible]

Eduardo [illegible]
"TRADUCTOR OFICIAL"
[illegible]
[illegible]


Eduardo Calado
Noguera
1 1 JUL. 2003
NOTARIA SEXTA
PABLO MENDEZ
BARAJAS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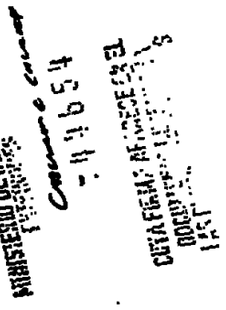
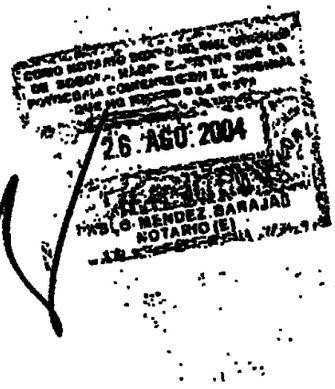

MINISTERIO DE RELACIONES


26 AGO. 2004
PABLO MENDEZ BARAJAS
NOTARIO (E)

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
7 August 2003 (07.08.2003)

PCT

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(25) Filing Language: English

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(30) Priority Data:
60/352,717 29 January 2002 (29.01.2002) US

(71) Applicant *(for all designated States except US)*: ZEALAND PHARMA A/S [DK/DK]; Smedeland 26B, DK-2600 Glostrup (DK).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: PETERSEN, Jørgen, Søberg [DK/DK]; Hellebakken 1, DK-3150 Hellebæk (DK). NEVE, Søren [DK/DK]; Ellevænget 4, DK-2800 Lyngby (DK). NIELSEN, Morten, Schak [DK/DK]; Egebjergvej 51A, DK-2750 Ballerup (DK). MEIER, Eddi [DK/DK]; Bringebakken 88, DK-3500 Værløse (DK). STEINESS, Eva [DK/DK]; Sømarksvej 11, DK-2900 Hellerup (DK). JENSEN, Peter, Holme [DK/DK]; Classensgade 11, 3.tv., DK-2100 Copenhagen ø (DK). LARSEN, Bjarne, Due [DK/DK]; Arildsgaard 5, 1. th., DK-2700 Brønshøj (DK). HANSEN, Lars, Bo, Laurenborg [DK/DK]; Peberhaven 19, st tv, DK-2730 Herlev (DK).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 03/063891 A1

(54) Title: COMPOSITIONS AND METHODS FOR MODULATING CONNEXIN HEMICHANNELS

(57) Abstract: Disclosed are compositions and methods for modulating hemichannel function in a cell, tissue or organ. The invention also relates to useful screens for detecting such compounds, particularly those capable of modulating connexin phosphorylation. Further provided are therapeutic methods for preventing or treating conditions impacted by undesired hemichannel function in a mammal such as heart arrhythmia.

ZEALAND
PHARMA

Zealand Pharma A/S
Smedeland 26 B
DK-2600 Glostrup
Denmark

Tel: +45 43 28 12 00
Fax: +45 43 28 12 12

E-mail: info@zp.dk
www.zp.dk

Telefax

To	Edwards & Angell, LLP
Attn.	Bob Buchanan
Fax No.	
Date	9 July 2004
From	Lone Skjøttgaard Due
No of pages	3 (incl. this page)
Subject	Change of applicant in PCT/DK03/00056
Text	Hi Bob:

Thank you for your email of 8 July 2004.

As requested I hereby send a copy of our letter to the Danish Patent Office of 21 June 2004 and our letter to WIPO of 8 July 2004.

Best regards
Lone

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ZEALAND
PHARMA

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Zealand Pharma A/S
Sydmarken 26 B
DK-2860 Gladstrup
Denmark

Tel: +45 43 28 12 00
Fax: +45 43 28 12 13

E-mail: info@zp.dk
www.zp.dk

Patent- og Varemærkestyrelsen
Helgeshøj Allé 81
2630 Taastrup

Att.: Marie Louise Rosendal

Date
21 June 2004

Page 1 of 1

International Patent Application No. PCT/DK03/00056
CHANGE OF APPLICANT
Our Ref: 018-2003 WO1

Dear Marie Louise Rosendal.

Please record the following change of the applicant for all designated states except US:

Wyeth
Five Giralda Farms
Madison, NJ 07940
U.S.A.

Yours sincerely,
ZEALAND PHARMA

Maj Hilligsøe

Cc.: Christine P. Bellon, Wyeth, Cambridge, MA, U.S.A.

ZEALAND
PHARMA

13

Zealand Pharma A/S
Smedeland 26 B
DK-2600 Glostrup
Denmark

Tel: +45 43 28 12 00
Fax +45 43 28 12 12

E-mail: info@zp.dk
www.zp.dk

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20
Switzerland

VIA FACSIMILE ONLY

Date
08 July 2004

International Patent Application No. PCT/DK03/00036
CHANGE OF APPLICANT
Our Ref: 016-2003 WO1

Dear Sirs,

We refer to our letter of 21 June 2004 to the Danish Patent Office about the change of applicant from Zealand Pharma A/S to

Wyeth
Five Giralda Farms
Madison, NJ 07940
U.S.A.

The new applicant has asked us to send a form PCT/IB/306 as soon as possible and before the application enters into the national phase on 29 July 2004.

We therefore kindly ask you to send the notification of the recording of the change at your earliest convenience.

Thank you.

Yours sincerely,
ZEALAND PHARMA

Lone Sigsgaard Due
Patent Assistant

COMPOSITIONS AND METHODS FOR MODULATING CONNEXIN HEMICHANNELS

CROSS-REFERENCE TO RELATED APPLICATION

The present application claims priority to U.S Provisional Application Serial No. 60/352,717 as filed on January 29, 2002, the disclosure of which is hereby incorporated by reference.

FIELD OF THE INVENTION

The present invention generally relates to compositions and methods for modulating connexin hemichannels. The invention also relates to useful screens for detecting such compounds.

BACKGROUND

There is recognition that gap junctions are important plasma membrane structures that help cells communicate with their environment. For example, most gap junctions are thought to assist passage of small molecules and ions between interconnected cells. Such movement is believed to exert profound effects on many aspects of cell physiology. Plasma membranes of adjacent cells are believed to include hemichannels, "connexons", formed by multimeric proteins called "connexins" that help form the gap junctions. In addition, hemichannels play an independent role in the exchange of small molecular weight compounds between the cell cytoplasm and the periplasmic or extracellular space. See generally Bennett, M. et al. (1991) *Neuron* 6: 305; Kumar, N. and Gilula, N.B. (1996) *Cell* 84: 381; and Quist, A.P et al (2000) *J. Cell Biol.* 148: 1063 and references cited therein.

In particular, there have been reports that many gap junctions are specialized regions of the cell membrane with clusters of densely packed channels. Such gap junction channels are thought to directly connect the cytoplasmic compartment of two neighbouring cells.

There is recognition that gap junction channels are composed of two hemichannels (connexons) provided by each of two neighbouring cells. Each connexon (hemichannel) has

been disclosed as consisting of six proteins called connexins. Each connexin is thought to share four transmembrane domains, two extracellular loops, and a cytoplasmic loop. The conduction of the electrical impulse is thought to take place through the gap junctions, thereby facilitating normal heart conduction and rhythm. See generally P. A. Guerrero, R. B. et al. *J Clin Invest* 1997, 99 1991; D. L. Lerner, K. A. et al., *Circulation* 1999, 99 1508; S. Kirchhoff, E. et al. *Curr Biol* 1998, 8 295.

Distribution of most heart connexins is thought to vary significantly throughout the organ. It has been disclosed that the Cx43 isoform is a major ventricular type while Cx40 is the most abundant isoform in the atrias and conductive system.

There are reports of strong links between connexin abnormalities and heart disease. See A. C. de Carvalho, et al., *J Cardiovasc Electrophysiol* 1994, 5 686; R. R. Kaprielian, et al., *Circulation* 1998, 97 651; N. S. Peters, et al., *Circulation* 1993, 88 864; and J. E. Saffitz, R. B. et al., *Cardiovasc Res* 1999, 42 309.

There is understanding that abnormal expression, distribution and regulation of gap junctions are involved in arrhythmias. The antiarrhythmic peptides disclosed by Larsen, B. et al. in PCT/DK01/00127 (WO01/62775) have been reported to increase gap junction intercellular communication (GJIC) in vertebrate tissue.

Particular mammalian gap junction proteins encoded by the connexin (Cx) gene family have been reported. See Bruzzone, R. et al. (1996) *Eur. J. Biochem.* 238: 1. The Cx family includes Cx26, 30, 31, 32, 37, 40, 43, 45, 46 and 50. Gap junction channels have also been found in invertebrates, where the channel forming proteins are called "innexins"

There is understanding that most connexins may be phosphorylated except for the Cx26 protein. The Cx43 protein is widely expressed in tissues. There are reports that phosphorylation of the Cx43 protein effects gap junction intracellular communication (GJIC). For example, there is acknowledgement that Cx43 turn over, trafficking, phosphorylation and gating are impacted by phosphorylation. See Darrow,B.J., et al. (1995). *Circ Res* 76: 381.

For example, Saffitz and co-workers have shown using conductance measurements that during ischemia there is an increase in connexin serine dephosphorylation within 15 minutes. See Figure 1 (showing a mammalian Cx43 transmembrane protein with several identified phosphorylation sites).

The phosphorylation and solubility of the connexins has attracted research interest. In particular, Cx43 was found to be phosphorylated in the myoepithelial cells of rat mammary glands. See Wang,Y., et al. (1995). *J Biol Chem* 270, 26581; and Yamanaka, I., et al. (1997). *Eur J Cell Biol* 72: 166.

As mentioned, gap junction channels are thought to be specialized pores that connect the cytoplasm of neighboring cells. Hemichannels communicate with the extracellular environment. There have been reports that metabolic inhibition of heart cells can activate an influx pathway that may be structured by connexin hemichannels. Metabolic inhibition is thought to open the hemichannel and enhance loss of potassium, and induce influx of protons, sodium and calcium, thereby damaging heart tissue. See Kondo et al *J.Mol.Cell.Cardiol.* 32:1859-72, 2000; Li et al *J. Mol. Cell. Cardiol.* 33: 2145-55, 2001).

Electrical uncoupling at gap junctions during acute myocardial ischemia is believed to contribute to conduction abnormalities and reentrant arrhythmias. Increased levels of intracellular Ca^{2+} and H^{+} and accumulation of amphipathic lipid metabolites during ischemia promote uncoupling. Other mechanisms may play a role. For instance, it has been reported that uncoupling induced by acute ischemia is associated with changes in phosphorylation of connexin43 (Cx43). Results have been reported that are consistent with rapid, reversible Cx43 dephosphorylation playing a role in myocardial uncoupling and arrhythmogenesis during acute ischemia. See Beardslee MA et al., *Circ Res.* 2000;87:656 and references cited therein.

The structure and function of hemichannels have attracted interest.

For example, atomic force microscopy (AFM), fluorescent dye uptake assay, and laser confocal immunofluorescence imaging, have suggested that hemichannels are involved in

extracellular calcium-dependent modulation of cell volume. As reported, cell volume changes were dependent on whether or not connexin43 was expressed. Changes were reported to be preventable by gap-junctional blockers (e.g., oleamide and beta-glycyrrhetinic acid) or were reversed by returning extracellular calcium to normal. It was suggested that nongap-junctional hemichannels regulate cell volume in response to the change in extracellular physiological calcium in an otherwise isosmotic situation. See Quist, A.P. et al, *supra*.

It has been proposed that open hemichannels, especially during ischemia or metabolic stress, may lead to cellular uptake of Ca^{2+}, protons and accumulation of amphipathic lipid metabolites in cells causing cellular swelling, cell damage or apoptosis. See Beardslee et al., *supra* and Quist et al. *supra*.

There have been reports Cx43 is phosphorylated a positions Tyr247 (Y247), Tyr265 (Y265) and perhaps other positions by the activated Src protein *in vitro*. Significantly, gap junction intercellular communication (GJIC) was reported to be resistant to disruption by phosphorylation mediated by v-Src. It was acknowledged that phosphorylation on Y247 and Y265 of Cx43 is important. See Lin R, et al. (2001) *J Cell Biol* 154(4):815. See also Figure 1.

See also Larsen, B.D et al. in a PCT application entitled *New Medical Uses of Intracellular Communication Facilitating compounds* as filed on 22 February 2002 as PCT/US02/05773 (WO 02/077017) in which a variety of GJIC modulating compounds have been disclosed.

It would be desirable to have compounds that modulate hemichannel function. Preferred compounds would assist opening or closing of the hemichannel. It would be especially desirable to have molecular screens to identify such compounds.

SUMMARY OF THE INVENTION

The invention generally features compounds and methods of using same to modulate hemichannel function. Particular compounds modulate hemichannel phosphorylation. Additional compounds of the invention modulate hemichannel function and in some instances also impact gap junction communication (GJIC) eg., by opening or closing gap junction channels. Useful screens for detecting and characterizing such compounds are also provided. The invention has many useful applications including providing therapies to treat or prevent various conditions modulated by unsuitable hemichannel function.

There is an urgent need to identify compounds that modulate (increase or decrease) hemichannel function. By "hemichannel function" is meant the opening or closing of connexin hemichannels to enhance or decrease passage of molecules or ions through the hemichannel. By the phrase "gap junction function" is meant the opening or closing of gap junctions to increase or reduce passage of molecules or ions through the gap junction. Preferred invention compounds modulate hemichannel phosphorylation and, typically also help open or close the hemichannel. Hemichannel function and gap junction function can be readily detected and optionally quantified by one or more of the standard assays disclosed herein.

More specific hemichannel modulating compounds modulate (increase or decrease) phosphorylation of a recognized connexin. Preferred sites of phosphorylation or dephosphorylation include one or more of a tyrosine, serine or threonine residue on the connexin. As will be discussed below, it has been found that modulation of phosphorylation on one or more of these residues impacts hemichannel function, particularly by opening and closing the hemichannel. Thus, and as described below, it is an object of the invention to provide screens adapted to monitor the phosphorylation state of the connexins as a means of detecting and optionally characterizing compounds with capacity to modulate hemichannel function.

For instance, certain compounds according to the invention are capable of phosphorylating at least one tyrosine residue of the connexin. In this embodiment, phosphorylation of the connexin will help close the hemichannel. Other suitable hemichannel modulating compounds decrease phosphorylation (dephosphorylate) at least one serine residue of

the connexin. In this example, dephosphorylation of the serine will help open the hemichannel. Still other compounds within the scope of the invention will enhance phosphorylation of at least one threonine residue of the connexin, typically to assist in the closure of the hemichannel. Additionally suitable invention compounds facilitate at least one of: an increase or decrease in serine phosphorylation, an increase or decrease in tyrosine phosphorylation, and an increase or decrease in threonine phosphorylation of the connexin. Preferably, one or more of the amino acid modifications will assist in a detectable opening or closing of the hemichannel.

Accordingly, and in one aspect, the invention provides a method to modulate hemichannel function preferably by closing the hemichannel. In one embodiment, the method involves closing the hemichannel in a cell, tissue or organ preferably exposed to stress including contacting the stressed cell, tissue or organ with a therapeutically effective amount of at least one of the compounds represented below as Formula I or II. Preferred contact according to this method embodiment is sufficient to close the hemichannel before, during or after exposure to the stress relative to a suitable control. Examples of such stress include one or more of metabolic inhibition, oxygen deprivation, lowering pH, or increasing extracellular potassium ion as described below.

In a more specific embodiment, the method further includes monitoring phosphorylation of a recognized connexin, preferably connexin 43 (Cx 43). Typically, the method will detect and report any increase or decrease in Cx43 phosphorylation on at least one of a tyrosine, serine, and threonine reside thereon, preferably an increase in phosphorylation in at least one of tyrosine and threonine. In this embodiment, the method also includes closing the hemichannel and, optionally, opening or closing gap junction channels relative to a suitable control.

The invention provides other methods to modulate hemichannel function. In one embodiment, the method involves monitoring phosphorylation of a recognized connexin, preferably Cx43, to detect any increase or decrease in Cx43 phosphorylation on at least one of a tyrosine, serine and threonine residue thereon, preferably a decrease in phosphorylation of one or more of those residues such as serine relative to a control. In this invention example, the method involves opening the hemichannel in a cell, tissue or organ exposed to stress including contacting

the stressed cell, tissue or organ with a therapeutically effective amount of at least one of the compounds represented below as Formula I or II. Preferred contact is sufficient to open the hemichannel and, optionally, open or close gap junction channels relative to a suitable control.

There is a need for screens to detect and characterize such hemichannel modulating compounds more specifically. Having such screens would be an important first step toward identifying and characterizing a range of new hemichannel modulating compounds e.g., by *in vitro* testing of a candidate compound relative to a control to detect capacity to help close hemichannels, for instance, by at least 5% more than a control compound. compounds identified by such a screen, including the compounds represented below as Formula I or II, can be used to in therapies that promote cell, tissue and organ homeostasis, for instance, by preventing, reducing or protecting against loss of cellular components to the extracellular environment.

Accordingly, the present invention also provides specific *in vitro* methods for screening candidate compounds that have capacity to modulate hemichannel function. Typically, the method involves contacting suitable cells, tissue or an organ with at least one compound represented below as Formula I or II. Preferably, the contact is under conditions conducive to modulating phosphorylation of a recognized connexin, preferably connexin 43 (Cx43) and detecting a change in Cx43 phosphorylation relative to a suitable control. Also preferably, the change in phosphorylation is taken to be indicative of a hemichannel modulating compound. Optionally, such compounds detected by the screening method may open or close gap junction channels according to assays disclosed herein.

In each of the foregoing methods, preferred phosphorylation changes according to the invention occur almost entirely at or near the intracellular C-terminus of the connexin. More preferred sites of phosphorylation and dephosphorylation of Cx43 are shown in Figure 1. By the phrase "C-terminus of connexin" is meant the region spanning about amino acid residues 240 to 281 as shown in Figure 1.

A particular *in vitro* screening assay of the invention for detecting connexin phosphorylation involves one or more steps designed to monitor hemichannel function, gap

junction function (or both). Such an assay generally includes at least one and preferably all of the following steps:

1) culturing a population of cells, a tissue or an organ such as those derived from the heart or muscle,

2) stressing the cells, tissue or the organ preferably by oxygen deprivation or metabolic inhibition,

3) adding a known or candidate hemichannel modulating compound such as those represented by Formula I or II below,

4) detecting a change in connexin phosphorylation (preferably Cx 43) relative to a suitable control; and

5) optionally measuring the change as being indicative of a compound that modulates hemichannel function and optionally gap junction function.

That assay can effectively measure capacity of the hemichannel modulating compound to increase or decrease phosphorylation of at least one of a serine, tyrosine and threonine residue of the preferred Cx43 protein. Reference herein to a "standard *in vitro* connexin phosphorylation assay" or related phrase refers to the above protocol of steps 1) through 5). The assay can be conducted with nearly any population of primary, secondary, or immortalized cells such as those derived from heart or muscle.

The foregoing standard *in vitro* assay is generally flexible. For instance, steps 1)-5) can be performed in nearly any order provided intended screening results are achieved. Thus in one embodiment of the assay, the candidate compound is added at step 1), step 2) (or both steps) instead of after step 2) exclusively.

The present invention provides other methods for screening candidate compounds for capacity to modulate hemichannel function. In one embodiment, the method includes contacting suitable cells, tissue or an organ with at least one compound selected from the group represented below as Formula I or II. Typically, any uptake of a detectable reporter by the cells, tissues or organ is monitored in the presence of the compound and relative to a suitable control. Preferred detectable reporters have a molecular size that is conducive to passage through an open

hemichannel. Thus, when the hemichannel is open in the assay, the detectable reporter enters the cell, tissue or organ. However when the hemichannel is closed, the detectable reporter is prevented from passing through the hemichannel. The contact is preferably under conditions conducive to detecting any change in the uptake of the detectable reporter with reference to a suitable control.

A hemichannel is "closed" in accord with the invention if at least one of the tyrosine residues in the C-terminal region of connexin, preferably Cx43 is phosphorylated, preferably at least the tyrosine at position 247 or position 265, more preferably both of same, as detected for instance in the standard in vitro connexin phosphorylation assay. By "closed" is also meant at least one of the threonine residues in the C-terminal region of the connexin is phosphorylated, which residues may be phosphorylated alone or in addition to tyrosine phosphorylation. A hemichannel is "open" if at least one of the serine residues in the C-terminal region of the connexin is dephosphorylated. Additionally preferred tyrosine, threonine and serine residues are shown in Figure 1 as kinase sites.

A more specific *in vitro* screening assay for detecting passage of the detectable reporter through the hemichannel involves one or more of the following steps:

1) culturing a population of cells, a tissue or an organ such as those derived from the heart or muscle,

2) stressing the cells, tissue or the organ preferably by oxygen deprivation or metabolic inhibition such as by adding a glucose derivative,

3) adding a known or candidate hemichannel modulating compound such as those represented below by Formula I or II,

4) adding a detectable reporter such as a fluorescent, chemiluminescent, or phosphorescent compound such as fluorescent dye such as calcein and related compounds,

5) detecting a change in uptake of the detectable reporter into the cells, tissue or organ relative to a suitable control; and

6) optionally measuring the change as being indicative of a compound that modulates hemichannel function.

That assay can effectively measure capacity of the candidate compound to open or close hemichannels in the cells, tissue or organ which change is readily detectable microscopically by visualizing the detectable reporter. Reference herein to a "standard *in vitro* uptake assay" or related phrase refers to the above protocol of steps 1) through 6). The assay can be conducted with nearly any population of primary, secondary, or immortalized cells such as those derived from heart or muscle. Other acceptable reports include suitable radioactive compounds also having a size permitting passage through the hemichannel. Particular compounds include those labeled with one or more of the following radionuclides : ^{3}H, ^{35}S, and ^{14}C.

Importantly, the standard *in vitro* uptake assay described generally above is not bound to any particular order of steps so long as intended assay results are achieved. Thus in one embodiment, at least one of the candidate compound and detectable reporter of steps 3) and 4), respectively, are added individually or together before step 2) in the method. In this example of the assay, the cells, tissue or organ is stressed in the presence of the compound and the detectable reporter. Alternatively, the detectable reporter can be "loaded" into the cells at step 1) to detect compounds with capacity to open hemichannels.

The invention provides further methods for screening one or more candidate compounds for capacity to modulate hemichannel function. In one embodiment, the method includes contacting cells, tissue or an organ with at least one compound selected from those represented by Formula I or II below. Typically, the cells, tissue or organ is loaded with a detectable reporter to measure volume. Preferred detectable reporters for use in the assay have a molecular size that is suited for passing through an open hemichannel. The contact is preferably under conditions conducive to detecting any change in the volume of the cells, tissue or organ as registered by the detectable reporter and by referring to a suitable control. Preferably, the cells, tissue or organ is stressed and the candidate compound closes the hemichannel so that cell volume is maintained or decrease more slowly when compared to a suitable control.

One particular *in vitro* screening assay for detecting cell volume changes involves one or more of the following steps:

1) culturing a population of cells, a tissue or an organ such as those derived from the heart or muscle,

2) loading the cells, tissue or organ with a detectable reporter such as a fluorescent, chemiluminescent, or phosphorescent compound such as dye, such as calcein or a related compound,

3) estimating the volume of the cells, tissue or organ by detecting and quantifying signal from the detectable reporter,

4) adding a known or candidate hemichannel modulating compound such as those represented by Formula I or II below,

5) stressing the cells, tissue or the organ preferably by oxygen deprivation or metabolic inhibition such as by adding a glucose derivative,

6) detecting a change in cell volume relative to a suitable control; and

7) optionally measuring the change as being indicative of a compound that modulates hemichannel function and optionally gap junction function.

The assay can effectively measure capacity of the candidate hemichannel modulating compound to open or close hemichannels in the cells, tissue or organ by observing cell volume changes microscopically. Reference herein to a "standard *in vitro* cell volume assay" or related phrase refers to the above protocol of steps 1) through 7). The assay can be conducted with nearly any population of primary or secondary cells derived from heart or muscle such as cardiomyocytes and related cells or tissue.

The standard *in vitro* cell volume assay is not bound to any particular order of steps so long as intended assay results are achieved. Thus in one embodiment, the candidate compound of step 4) is added after stressing the cells in step 3) to monitor ability of the compound to close hemichannels in already stressed cells as exemplified by a slower rate of cell volume decrease. Thus in one embodiment of the assay, a change in the rate of cell volume decrease or increase is monitored over a pre-determined time frame. However in another embodiment, the cell volume change can be monitored at a fixed time point e.g., at a time between about 1 to 120 minutes after stressing the cells, tissue or organ.

As discussed, the *in vitro* assays of the invention are flexible and can be adapted to suit an intended screening use. For instance, a particular candidate hemichannel modulating compound, such as those represented by Formula I or II below, can be employed as the sole active agent or in combination with other agents including other compounds to be tested. In most, but not all instances, the *in vitro* assays are performed with reference to a suitable control assay usually including the same or closely related test conditions as in the steps above, but without adding the compound to be tested to the culture medium. In such cases, a candidate hemichannel modulating compound can be identified by exhibiting at least 2% greater activity in the assay relative to the control, more preferably at least about 5% greater activity relative to the control assay, and still more preferably at least about 10% or greater activity, eg., about 20% to about 40% relative to the control assay.

As discussed, particular hemichannel modulating compounds will also impact gap junction channels. For instance, certain compounds may help close the hemichannels and assist in the opening of gap junction channels. However, other compounds may help open the hemichannels while assisting in the closure of gap junction channels. Still other compounds may open both hemichannels and gap junctions while others may close gap junctions and hemichannels.

Accordingly, the invention also provides a method of increasing gap junction intracellular communication (GJIC) in a cell, tissue or organ. In one embodiment, the method involves administering a therapeutically effective amount of at least one compound selected those represented by Formula I or II as described below. Preferably, the contact is sufficient to increase the GJIC in the cell, tissue or organ relative to a suitable control.

Also provided are combinations of *in vitro* assays in compounds are selected for capacity to modulate hemichannels and gap junctions. In one embodiment, at least one of the standard *in vitro* connexin phosphorylation assay, the standard *in vitro* uptake assay, and the standard *in vitro* cell volume assay, is combined with one or more of the GJIC assays disclosed by Larsen, B. et al. in a PCT application entitled *Novel Antiarrhythmic Peptides* as filed on 22 February 2001 as PCT/DK01/00127 (WO 01/62775) or as disclosed by Larsen, B. et al. in another PCT

application entitled *New Medical Uses of Intracellular Communication Facilitating compounds* as filed on 22 February 2002 as PCT/US02/05773 (WO 02/077017). Examples of such suitable GJIC assays include those measuring cell conductance by patch clamp, calcium wave measurements and dye transfer assays. The disclosures of the PCT/DK01/00127 (WO 01/62775) and PCT/US02/05773 (WO 02/077017) applications are hereby incorporated by reference.

By way of illustration and not limitation, the standard *in vitro* connexin phosphorylation assay described above is employed to select one or more hemichannel modulating compounds. One or more the compounds can subsequently be further screened in the cardiomyocyte patch clamp assay described in the PCT/US02/05773 (WO 02/077017) application. compounds providing suitable activity in both assays will have capacity to modulate hemichannels and gap junctions.

The invention further provides *in vivo* testing of the candidate hemichannel modulating compounds to help detect and optionally quantify therapeutic capacity to modulate a heart arrhythmia. As discussed, it is believed that most heart arrhythmias can be prevented, alleviated or treated by use of one or a combination of the compounds of this invention. A preferred *in vivo* testing model, referred to herein as a "standard *in vitro* mouse arrhythmia assay" or related phrase, has been disclosed in PCT/DK01/00127 (WO 01/62775) as well as in PCT/US02/05773 (WO 02/077017). Certain compounds according to the invention will desirably prolong the time until onset of induced atrial ventricular (AV) block by at least about 20% (score of at least 2) in the assay. Other compounds will exhibit a prolongation of at least about 60% or the time until onset of the induced AV block (score of at least 3) in the assay. In broad terms, the assay involves administering one or more compounds to a suitable mouse, injecting calcium chloride to induce arrhythmia, and detecting the time of onset of the arrhythmia (preferably 2nd degree AV-block) compared to a suitable control.

Significantly, use of multiple detection formats (ie., a combination of at least one of the standard *in vitro* assays and the *in vivo* arrhythmia assay as disclosed herein) can efficiently perform multiple analyses, thereby enhancing the accuracy and probability of identifying a hemichannel modulating compound (as represented by Formulae I and II, for instance) with good

therapeutic capacity. This feature of the invention is especially useful when large numbers of compounds are to be tested. For example, some or nearly all of the compounds according to Formula I or II can be tested. Alternatively, or in addition, suitable compounds could be made by standard synthetic methods including combinatorial-type chemical manipulations and then tested in accord with the invention.

In embodiments in which multiple detection formats are practiced, it is important to note that significant *in vitro* and *in vivo* activity as determined by the assays described herein is not a required feature of a hemichannel modulating compound. That is, certain compounds described herein will exhibit good activity in at least one of the standard *in vitro* assays described herein but will not exhibit significant activity in the standard *in vivo* arrhythmia assay. Alternatively, certain other compounds will exhibit significant activity in the *in vivo* arrhythmia assay but will not show good activity in one or more of the standard *in vitro* assays. However, a preferred hemichannel modulating compound will exhibit good activity in at least one of the *in vitro* and *in vivo* assays described in this application.

Compounds showing good activity in the standard in vivo arrhythmia assay will sometimes be called "antiarrhythmic compounds" or like phrase to denote capacity to prolong time to arrhythmia in the assay.

As will be discussed in more detail below, compounds of the invention can be used to prevent or treat a wide spectrum of medical conditions that are associated or suspected of being related to undesired or abnormal passage of molecules and/or ions through cell membranes. For instance, nearly all the medical indications disclosed in PCT/DK01/00127 (WO 01/62775) and PCT/US02/05773 (WO 02/077017) can be addressed in this way. Some of said medical indications are disclosed herein. The medical indications may be prevented, alleviated or treated by using one or a combination of compounds disclosed in the present application and particularly those showing good activity in at least one of the *in vitro* and *in vivo* assays provided herein.

Additional compounds suitable for testing and use with the present invention have been disclosed by Larsen, B.D et al. in PCT/US02/05773 (WO 02/077017).

In another aspect, the invention provides a method of preventing or treating tissue or organ stress in a mammal. In one embodiment, the method includes administering a therapeutically effective amount of at least one compound selected from the group of compounds represented by Formulae I and II above. Preferably, the contact is sufficient to prevent or treat the stress in tissue or organ.

The invention also provides for a method of treatment of burns comprising administering to a patient in need of such treatment a therapeutically effective amount of a compound that blocks connexin hemichannel opening.

Also provided is a method of treatment of thromboses. In one embodiment, the method includes administering to a patient in need of such treatment a therapeutically effective amount of a compound that blocks a connexin hemichannel from opening.

In another aspect, the invention also features a method of treatment of respiratory and metabolic acidosis. In one embodiment, the method includes administering to a patient in need of such treatment a therapeutically effective amount of a compound that blocks a connexin hemichannel from opening.

The invention also provides for a method of treatment of focal arrhythmia. In one example of the method, it includes administering to a patient in need of such treatment a therapeutically effective amount of a compound that blocks a connexin hemichannel from opening.

Further provided by the invention is a method of treating and preventing cell and tissue damage resulting from elevated levels of blood glucose. In one embodiment, the method includes administering to a patient in need of such treatment a therapeutically effective amount of a compound that blocks a connexin hemichannel from opening.

The invention also features a method of treatment of chronic atrial fibrillation. In one embodiment, the method includes administering to a patient in need of such treatment a therapeutically effective amount of a compound that blocks a connexin hemichannel from opening.

Also provided is a method of treatment of epilepsia. Typically, the method includes administering to a patient in need of such treatment a therapeutically effective amount of a compound that promotes a connexin hemichannel to open.

Further provided is a method of cytoprotecting tissue or an organ of a mammal in need of such treatment, the method comprising administering a therapuetically effective amount of at least one compound selected from the group consisting of the compounds represented by Formula I or II.

The invention also provides for a method of preventing or treating reperfusion injury in a mammal, the method comprising administering a therapuetically effective amount of at least one compound selected from the group consisting of the compounds represented by Formula I or II.

In each of the foregoing therapeutic methods, acompound according to the invention that features good anti-arrhythmic activity as determined by the standard *in vitro* mouse arrhythmia assay (ie., score of at least 2). Such compounds will sometimes be referred to herein as "antiarrhythmic" compounds or a related phrase.

Further uses and advantages of the present invention will be apparent from the following discussion and examples. Other aspects of the invention are also discussed below

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a drawing showing phosphorylation sites on a connexin (Cx43). As can be seen, the cytosolic domain of the Cx43 transmembrane protein has several potential serine and tyrosine phosphorylation sites.

Figure 2 is a graph showing metabolic stress induced by removal of glucose. As can be seen from the graph, administration of compound 1 reduces the stress as evidenced by a decrease in relative conduction delay.

Figures 3A-B are representations of immunoblots showing detection of tyrosine phosphorylation in connexin 43 (Cx43). Figure 3A shows results from compound 1 administration. Figure 3B shows results from administration of compound 1 and compound 2.

Figures 4A-B are representations of immunoblots showing detection of tyrosine phosphorylation (8A) and serine phosphorylation (8B) of Cx43.

Figure 5 is a graph showing effect of 10nM compound 1 on ischemia-induced uptake of calcein in cultured cardiomyocytes.

Figure 6A is a drawing showing a preferred ELISA assay format. Figure 6B is a graph showing ELISA results of phosphorylated Cx43 at Tyr-P in HeLa cells. Figure 6C is a graph illustrating ELISA results of phosphorylated Cx43 at Tyr-P in CHO cells.

Figure 7A-C are photomicrographs showing dye uptake in cultured cardiomyocytes. Fig. 7A: Cardiomyocytes under light microscopy; Fig. 7B: Fluorescence under control conditions (same as cells in Fig. 7A); Fig. 7C Fluorescence after 30 minutes of metabolic inhibition.

Figure 8 is a graph showing that compound 1 reduces stress-induced calcein uptake in a dose-dependent manner.

Figure 9A-B are graphs showing effect of compound 1 on stress-induced cell swelling. Fig. 9A Relative volume during metabolic inhibition in the presence or absence of compound 1 (0.1nM). Fig. 9B shows control data.

Figure 10 is a graph showing effect of compound 1 (0.1nM) on stress-induced cell swelling.

Figure 11 is a graph showing a reduction in heart weight to body weight ratio after administration of Compound 1 to rats subjected to myocardial infarction.

Figure 12 is a graph showing a reduction in infarct size in rats following administration of Compound 1.

Figure 13 is a graph showing improved cardiac function after administration of Compound 1 to rats subjected to myocardial infarction.

DETAILED DESCRIPTION OF THE INVENTION

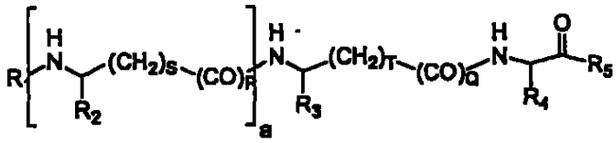
As discussed, the invention provides compounds and methods of using same to modulate hemichannel function. More particular compounds modulate hemichannel phosphorylation to assist opening or closing of hemichannel. Useful screens for detecting and characterizing such compounds are also provided which screens include *in vitro* assays, an *in vivo* assay, or a combination thereof. Further provided are therapeutic methods useful for the treatment or prevention of conditions impacted by unsuitable hemichannel function.

It is an object of the invention to demonstrate, for the first time, that cell, tissue and organ stress may be adversely impacted by the closing and opening of hemichannels. Without wishing to be bound to theory, it is believed that metabolic stress, such as oxygen deprivation during ischemia, glucose deprivation, uncoupling of the oxidative phosphorylation caused by HCN, or uncoupling of the citric acid cycle, may all contribute to uncoupling of the intercellular gap junctional communication (GJIC). It is further believed that this uncoupling is correlated with modulation of connexin phosphorylation, more particularly dephosphorylation of connexin-tyrosine residues and/or connexin-serine residues and/or threonine residues in the gap junction

channel. By way of illustration, it is believed that during atrial fibrillation, the atrial cells have increased metabolic demand due to the high frequency pacing. This is thought to lead to lactate acidosis, opening of hemichannels and uncoupling of gap junctions.

It is also an object of this invention to provide methods of modulating hemichannel function and, optionally, gap junction intercellular communication (GJIC) which methods include contacting cells, tissue or organs with a therapeutically effective amount of one or a combination of compounds that have such activity. Preferred compounds have been disclosed in the PCT/DK01/00127 (WO 01/62775) and PCT/US02/05773 (WO 02/077017) by B. Larsen et al.

More preferred compounds suitable for use with the present invention include those represented by the following Formula I:



(I)

wherein

R1 represents H or acetyl (Ac)

R2 represents a sidechain of one of the amino acids G, Y, D-Y, F and D-F,

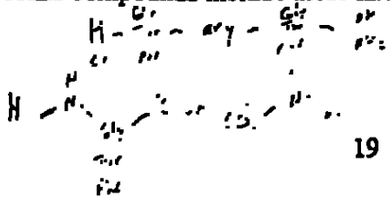
R3 represents any amino acid sidechain R4 represents a sidechain of one of the amino acids G, Y, D-Y, F and D-F,

R5 represents OH or NH2

and a, S, T, P and Q are integers are integers and independently = 0 or 1;

and salts thereof.

More specific compounds include those having the following Formula II:



R1-X1-X2-X3-R2

II

wherein,

X1 is 0, Ala, Gly, ß-Ala, Tyr, D-Tyr, Asp,

X2 is 0; Ala-Gly-T4c-Pro; Ala-Sar-Hyp-Pro;; Ala-Asn; D-Asn-D-Ala; D-Asn;; Gly; Ala; D-Ala; ß-Ala;; Asn; or;

X3 is Tyr; D-Tyr; Gly, , or Phe; and

R1 is H or Ac, with the proviso that X1 and X2 are not both 0; and salts thereof.

R2 is OH or NH2

More specific compounds represented by Formula I or II above include the following:

G-(DBF)-Y-NH2, , H-GA-Sar-Hyp-PY-NH2, H-GAG-T4c-PY-NH2,

Gly-Ala-Asn-Tyr , D-Tyr-D-Asn-D-Ala-Gly, D-Tyr-D-Asn-Gly, ,

, Gly-Gly-Tyr, Gly-Ala-Tyr, D-Tyr-D-Ala-Gly,

Gly-D-Asn-Tyr, Gly-βAla-Tyr, βAla-βAla-Tyr,

, Gly-βAla-Phe, Gly-Asn-Phe, , Asn-Tyr, Ac-Gly-Tyr,

Ac-Ala-Tyr, (reducedGly)-Gly-Tyr (H_2N-CH_2-CH_2-NH-CH_2-C(O)-Tyr),

gly-dab-gly-hyp-pro-tyr

gly-dapa-gly-hyp-pro-tyr

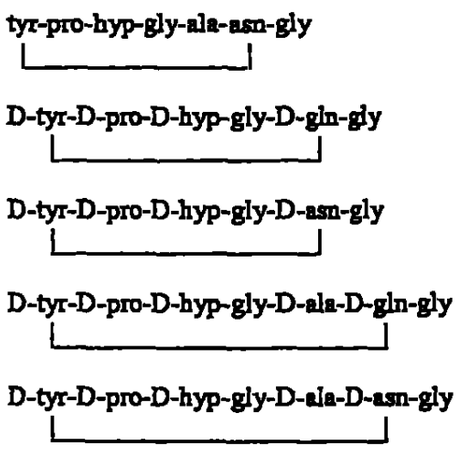
tyr-pro-hyp-gly-gln-gly

tyr-pro-hyp-gly-asn-gly

gly-dab-ala-gly-hyp-pro-tyr

gly-dapa-ala-gly-hyp-pro-tyr

tyr-pro-hyp-gly-ala-gln-gly



; and salts thereof.

Additionally preferred candidate compounds of the invention feature an oral bioavailability of more than about 5% as determined by an acceptable oral bioavailability assay. Generally preferred assays involve intraduodenal administration of a candidate components to a suitable test subject. Availability of the compounds in a biological sample, preferably a blood sample, is then detected and preferably quantified.

Further preferred candidate compounds generally satisfy Lipinski's rule of 5. As applied, it defines bioavailability. According to the Rule, less than satisfactory adsorption is more likely when one or more of the following features characterizes a particular candidate compound: 1) More than 5 H-bond donors (expressed as the sum OH's and NH's), 2) Molecular weight over 500, 3) Log P is over 5, 4) More than 10 H-bond acceptors (expressed as sum of N's and O's), and 5). Two parameters out of range are to be avoided for many invention embodiments.

Still further preferred compounds in accord with the invention are relatively stable in blood plasma. An acceptable assay involves contacting a desired candidate compound with plasma (rodent, rabbit or primate serum, for instance), incubating the compound with the plasma, and then detecting and preferably quantifying stability of that compound over time. Preferred plasma sources are rats, dogs, cats, mice, pigs, cows, horses, and humans. A preferred assay, sometimes referred to herein as "standard plasma stability assay" has been disclosed in the PCT application

PCT/US02/05773 (WO 02/077017). Typical compounds giving good activity in the assay feature C-terminal amidation or esterification, use of D-amino acids and derivatives of natural amino acids, N-terminal modifications, and the cyclic structures. One or a combination of such modifications can enhance stability while retaining substantial biological activity.

Especially preferred hemichannel modulating compounds for use with the present invention include: Ac-D-Tyr-D-Pro-D-4Hyp-Gly-D-Ala-Gly-NH2 (compound 1); Ac-Gly-Asn-Tyr-NH2 (compound 2); and salts thereof.

Preferred hemichannel modulating compounds exhibit significant activity in one or a combination of the standard *in vitro* and *in vivo* assays disclosed herein. Such compounds also have capacity to close or open hemichannels and, optionally, to modulate GJIC. Preferably, a suitable compound enhances or reduces phosphorylation of connexin 43 (Cx43) as determined by the standard *in vitro* connexin phosphorylation assay. A more specific assay monitors phosphorylation and/or dephosphorylation of one or more of the tyrosine, serine and threonine amino acid residues shown in Figure 1. A more particular assay format follows.

1) culturing a population of about 10^5 cells in medium such as confluent or semi-confluent rat cardiomyocytes or H9c2 cells,
2) stressing the cells by washing same and subsequently culturing them in glucose poor medium for about an hour or less,
3) adding compound 1 to the medium to a concentration of about 0.1 to about 200 nM for about 10 minutes to eight hours,
4) lysing the cells and then detecting a change in Cx 43 tyrosine, serine, and/or threonine phosphorylation relative to a suitable control; and
5) measuring the change in phosphorylation as being indicative of a compound that modulates hemichannel function.

In one embodiment of the method, the detecting step 4) further comprises testing the candidate compound in an immunological or cell sorting-based assay. In cases in which an immunological assay is employed that assay will typically include contacting cells with the

candidate compound under conditions sufficient to increase or decrease phosphorylation of the connexin. Preferably, the method further includes producing a lysate of the cells; and detecting increased or decreased connexin phosphorylation in the cell lysate. That increase or decrease is taken to be further indicative of the hemichannel modulating compound.

Preferred immunological assays for use with the method have been described and include immunoprecipitation assays, antibody capture assays, two-antibody sandwich assays, antigen capture assays, radioimmunoassays (RIAs) and the like. See generally E. Harlow and D. Lane in Antibodies: *A Laboratory Manual*, Cold Spring Harbor Laboratory (1988); Ausubel et al. (1989) in *Current Protocols in Molecular Biology*, J. Wiley & Sons, New York for a discussion relating to many standard immunological methods, the disclosures of which are incorporated herein by reference.

A preferred immunological assay is an ELISA assay. Thus in one embodiment, the forgoing method further includes at least one and preferably all of the following steps:

a) coating a solid support with a first antibody that specifically binds the connexin, preferably connexin 43 (Cx43),

b) contacting the cell lysate to the solid support under conditions conducive to forming a binding complex between the first antibody and the connexin,

c) contacting the first antibody:connexin binding complex with a second antibody, the contacting being under conditions sufficient to form a specific binding complex between the second antibody and any phosphorylated connexin in the first antibody:connexin binding complex,

d) contacting the first antibody:connexin:second antibody binding complex with a detectably-labeled third antibody that binds the second antibody; and

e) detecting presence of the third detectably-labeled antibody on the solid support as being further indicative of the compound.

In one embodiment of the method, the third antibody of the method is detectably-labeled with at least one of biotin, FITC, TRITC, radioactive iodine or peroxidase. In another embodiment, the second antibody is an anti-phosphotyrosine antibody. In still another embodiment, the second antibody is an anti-phosphoserine antibody. In some instances, detection of the detectably-labeled third antibody is performed by radio- or gama scintillation counting.

In another embodiment, the immunological method employed as the detecting step is a radioimmunoassay (RIA). Preferably, such an RIA includes at least one and preferably all of the following steps:

a) detectably-labeling the cells before producing the cell lysate, wherein the labeling is under conditions conducive to labeling phosphorylated connexin, preferably connexin 43 (Cx43),

b) contacting the cell lysate with an antibody that forms a specific binding complex with the connexin,

c) separating the binding complex from the detectably-labeled cell lysate; and

d) detecting the labelled and phosphorylated connexin as being further indicative of the gap junction modulating compound.

In one embodiment of the method, the antibody is an anti-connexin antibody. In another embodiment, the detection step further comprises performing a Western immunoblot assay. The RIA method is compatible with a wide range of detectable labels however for many applications administration of radioactive inorganic phosphorous will be preferred.

Antibodies for use with the foregoing invention method including anti-phosphotyrosine, anti-phosphoserine, and anti-Cx43 antibodies are commercially available from a variety of sources such as the American Type Culture Collection (ATCC); Sigma Chemical Co., St. Louis, MO; Zymed Lab, Inc., of California; and Amersham Biosciences, U.K.

Preferred hemichannel modulating compounds identified by the foregoing standard *in vitro* test show good capacity to modulate phosphorylation of Cx43, particularly at one or more of the tyrosine, serine and threonine residues shown in Figure 1. More preferred of such compounds exhibit at least a 5% change in such phosphorylation (measured as an increase of decrease in the presence of one or more of phosphotyrosine, phosphoserine, and/or phosphothreonine on Cx43), preferably at least about 10%, more preferably at least about 50% change relative to stressed cells in which the compound is present at a concentration of between about 0.1nM to about 200nM.

See Examples 2-5 below for a particular example of the standard *in vitro* connexin phophorylation assay.

As discussed, the invention also features a standard *in vitro* uptake assay that can be used to detect and optionally characterize hemichannel modulating compounds. The assay can be used alone or in combination with the standard *in vitro* connexin phosphorylation assay. A particular embodiment of the standard *in vitro* uptake assay is described as follows.

1) culturing a population of about 10^5 confluent rat ventricular myocytes in medium,

2) stressing the cells by replacing the medium with glucose poor medium for less than about 2 hours, preferably about 30 minutes,

3) adding compound 1 to the medium to a concentration of about 0.01pM to 100nM,

4) adding calcein dye to the medium to a concentration of about 10 to about 500 micromolar for less than about 2 hours and preferably about 30 minutes,

5) detecting a change in uptake of the calcein into the cells; and

6) measuring the change as being indicative of a compound that modulates hemichannel function.

The step of detecting the change in calcein uptake can be performed by one or a combination of standard methods including fluorescent light microscopy and/or related cell sorting methods. See Example 6 for a particular example of the standard *in vitro* uptake assay.

Preferred compounds detected by the forgoing standard *in vitro* uptake assay will exhibit at least about a 5% decrease in dye uptake (close cell hemichannels) relative to stressed cells, preferably at least about a 20% decrease and more preferably at least about a 50% decrease in the presence of between about 0.01pM to 100nM of the compound to be tested.

As also discussed, the invention provides the *in vitro* cell volume assay which assay can be alone or in combination with one or both of the standard *in vitro* connexin phosphorylation and the standard *in vitro* uptake assay. A particular embodiment of the standard *in vitro* cell volume assay is as follows.

1) culturing a population of about 10^5 confluent rat ventricular myocytes in medium,
2) loading the cells with between from about 0.5 to about 100 micromolar calcein-AM, preferably about 5 micromolar, for less than about 2 hours and preferably about 15 minutes
3) estimating the volume of the cells by detecting and preferably quantifying signal from the calcein-AM,
4) adding compound 1 to the medium to a concentration of about 0.01nM to about 100nM,
5) stressing the cells by culturing in glucose poor medium,
6) detecting a change in cell volume relative to a suitable control; and
7) measuring the change as being indicative of a compound that modulates hemichannel function.

The step of detecting the change in cell volume can be performed by one or a combination of standard methods including laser confocal microscopy and/or related cell sorting methods. Example 7 provides a particular example of the standard *in vitro* cell volume assay.

Preferred compounds detected by the forgoing standard *in vitro* cell volume assay will exhibit at least about a 5% decrease in cell volume (close hemichannels) relative to stressed cells, preferably at least about a 20% decrease and more preferably at least about a 50% decrease in the presence of between about 0.01nM to 100nM of the compound to be tested. Compounds selected in accord with this assay will inhibit flow of osmolytes through hemichannels and help maintain normal cell volume during conditions that produces cellular swelling. Importantly, cell swelling is associated with impaired perfusion in organs surrounded by a fibrous capsule (e.g., heart, kidney, skeletal muscle) or bone (brain, spinal cord) and therefore compounds with hemichannel blocking properties may be useful in the treatment of diseases associated with cellular swelling.

Suitable control experiments are generally tailored for use in a particular assay format. For example, most control experiments involve subjecting a test sample (e.g, a population of cultured rat cardiomyocytes) to non-stressful conditions such as incubation in medium. Typically, water, buffer, phosphate-buffered saline or the like is added to the assay instead of the compound(s) to be tested in parallel or separately. A desired assay is then conducted in accord with the present methods. Specific examples of suitable controls are provided in the Examples section.

Practice of the invention is compatible with a wide spectrum of conventional detection assays. Such assays can be performed manually, semi-manually, or in an automated format as needed. For applications in which rapid or large scale screening strategies are needed, the invention is additionally compatible with standard "high-throughput" and/or "ultra-high throughput" screening methodologies. Examples of such methods include immunological and cell sorting type assays such as those described herein.

As discussed, the present invention is compatible with a wide spectrum of testing strategies. Thus in some embodiments, further testing of candidate compounds will be performed *in vivo* to test for and preferably confirm hemichannel modifying activity. For instance, one or a combination of the standard *in vitro* methods described herein can be used to further test compounds for hemichannel modulating activity in the standard *in vivo* arrhythmia model. The standard *in vivo* mouse arrhythmia assay, described below as Reference Example 1, has been disclosed in the PCT/DK01/00127 (WO 01/62775) and PCT/US02/05773 (WO 02/077017) by B. Larsen et al.

Candidate compounds identified by one or more of the invention methods have a variety of important applications including use a probes for identifying gap junctions and particularly hemichannels in a range of cells, tissues and organs. Also, such compounds can be used as probes for detecting gap junctions and hemichannels in various developmental and disease states, and as probes for detecting those structures with distinct carbohydrate decoration and/or phosphorylation patterns. Such compounds find further use as solid support components for purifying gap junction components according to standard chromatographic procedures.

Compounds selected by the *in vitro* and/or *in vivo* tests described herein can be used as medicaments for preventing or treating conditions impacted by increased opening of hemichannels (increased cell membrane permeability to the extracellular compartments) eg., wounds such as burns, thromboses, respiratory and metabolic acidosis, tissue swelling eg., due to bacterial or environmental toxins, and focal arrhythmias. Other medicaments of the invention can be employed to protect cells from disruptive volume changes. Such changes can be facilitated by one or a combination of factors including excessive heat, ischemia, toxins, inflammation, oedema, disturbance of electrolytes, increased levels of glucose, and diabetic late complications. Treatment of chronic atrial fibrillation associated with increased serine-phosphorylation is also contemplated. Additional compounds can be used as medicaments to prevent, treat, alleviate, or reduce the severity of the following conditions. See also the PCT/DK01/00127 (WO 01/62775) and PCT/US02/05773 (WO 02/077017) application.

The therapeutic methods of the invention generally comprise administration of a therapeutically effective amount of one or a combination of the hemichannel modulating compounds disclosed herein to a subject in need of such treatment, such as a mammal, and particularly a primate such as a human. Treatment methods of the invention also comprise administration of an effective amount of a compound of Formula I or II as defined above to a subject, particularly a mammal such as a human in need of such treatment for an indication disclosed herein.

Typical subjects include mammals suffering from one or a combination of disorders as provided herein including the conditions described below in Sections A-R.

A. Nervous tissue

It is well known that microglia are the main immune effector of the central nervous system (CNS), and that they are activated in response to a wide range of injuries that trigger brain inflammatory responses, including head injury and ischemia, neurodegenerative diseases, autoimmune diseases, infectious diseases, prion diseases, and brain tumors. Activated microglia migrate to injured CNS areas, where they proliferate and gradually remove cell debris. Eugenin et al showed that microglia can communicate with each other through gap junctions that are induced by inflammatory cytokines (Eugenín, E A, et al. *Proc. Natl. Acad. Sci. USA*, Vol. 98, 4190-4195, 2001). This was demonstrated in the following experiments. At brain stab wounds, microglia progressively accumulated over several days and formed aggregates that frequently showed Cx43 immunoreactivity at interfaces between cells. In primary culture, microglia showed low levels of Cx43 determined by Western blotting, diffuse intracellular Cx43 immunoreactivity, and a low incidence of dye coupling. Treatment with the immunostimulant bacterial lipopolysaccharide (LPS) or the cytokines interferon-gamma (INF-gamma) or tumor necrosis factor-alpha (TNF-alpha) one at a time did not increase the incidence of dye coupling. However, microglia treated with INF-gamma plus LPS showed a dramatic increase in dye coupling that was prevented by coapplication of an anti-TNF-alpha antibody, suggesting the release and autocrine action of TNF-alpha . Treatment with INF-gamma plus TNF-alpha also greatly increased the incidence of dye coupling and the Cx43 levels with translocation of Cx43 to cell-cell contacts. The cytokine-induced dye coupling was reversibly inhibited by 18-

glycyrrhetinic acid, a gap junction blocker. Cultured mouse microglia also expressed Cx43 and developed dye coupling upon treatment with cytokines, but microglia from homozygous Cx43-deficient mice did not develop significant dye coupling after treatment with either INF-gamma plus LPS or INF-gamma plus TNF- alpha.

Forced expression of gap junction proteins, connexins, enables gap junction-deficient cell lines to propagate intercellular calcium waves. Cotrina et al. demonstrated that ATP secretion from poorly coupled cell lines, C6 glioma, HeLa, and U373 glioblastoma, is potentiated 5- to 15-fold by connexin expression. These observations indicate that cell-to-cell signaling associated with connexin expression results from enhanced ATP release mediated through connexin hemichannels (Cotrina ML et al. *Proc Natl Acad Sci U S A* 1998 Dec 22;95(26):15735-40; Cotrina et al.: *J Neurosci* 2000 Apr 15;20(8):2835-44). Moreover, during conditions with metabolic stress (e.g., ischemia), hemichannel-mediated ATP release from astrocytes may affect neighboring neurons and elicit elevations in intracellular calcium with in turn may turn in apoptosis and neuronal death .

Due, for instance, to the phosphorylation of Cx43 tyrosine by compound 1 and compound 2, administration of these compounds will assist in hemichannel closing and facilitate the intercellular communication of microglia and thereby augment or speed up the "healing" processes in the aforementioned diseases (brain inflammatory responses, including head injury and ischemia, neurodegenerative diseases, autoimmune diseases, infectious diseases, prion diseases, and brain tumors). Furthermore, it is expected that closing of hemichannels will prevent ATP release and thus reduce spreading of the primary injury.

B. Lung tissue: alveolar cells

Alveolar intercellular communication via gap junctions between alveolar cells is important for the propagation of ion transport, mechanochemical signal transduction, regulation of cell growth and secretion of surfactant factor (Ashino Y, et al. (*Am J Physiol Lung Mol Physiol* 2000; 279: L5-L13)). *In vivo* repair after acute and chronic inflammatory damage of the alveolar region of the lung involves formation of fibronectin as part of the extracellular matrix (Charash WE, et al. (*Am Rev Respir Dis* 1993; 148: 467-476) and Torikata C, et al. (*Lab Invest*

1985; 52: 399-408)). Alveolar epithelial cell culture studies have demonstrated an increased number of gap junctions in parallel to an increase of extracellular fibronectin concentration (Alford AI, Rannels DE . (*Am J Physiol Lung Cell Mol Physiol* 2001;280:L680-L688)). *In vivo* animal studies have found a decreased number of gap junctions after nitrogen dioxide induced severe pulmonary inflammation both in the alveolar tissue, the walls of the terminal bronchioles, alveolar ducts and peribronchiolar alveoli. These findings were dose dependent. However, if pretreated with taurin this loss of gap junctions was prevented in parallel with less pronounced inflammatory reactions. Similar findings were seen after irradiation of rat lung and after treatment with the chemotherapeutic compound, bleomycin.

Thus, maintaining the gap junctional communication in lung tissue is important for preventing lung fibrosis and decreased amount of connexin is seen as a reaction to inflammatory processes, to various toxic stimuli, such as gas inhalation, airborne destructive substance and irradiation. Pretreatment with a compound of the invention that phosphorylates Cx43 tyrosine residues, and facilitates hemichannel closing and/or gap junction opening or gap junctional communication will be indicated prior to therapeutic irradiation where the lungs are exposed, e.g. in lung cancer, treatment of breast cancer, thyroid and esophageal cancers.

Treatment with a compound that facilitates or mediates hemichannel closing and/or gap junction opening will prevent further deterioration of lung function in emphysema, asbestosis, silicosis, lung fibrosis, pneumonitis, drug induced lung fibrosis and in patients exposed to pulmonary toxic gasses such as nitrogen dioxide. Treatment will preferably be added on to conventional treatment of these conditions. The compound may be administered orally, parenterally, nasally, or via pulmonary inhalation.

C. Smooth muscle

1. Vascular system

Intercellular communication through gap junction channels plays a fundamental role in regulating and modulating vascular myocyte tone throughout the vascular tree (Christ GJ, et al. *Circ Res.* 1996; 79: 631 – 646)). Another important role of gap junction communication is the

spread of hyperpolarization among smooth muscle cells involved in vascular relaxation response (Benny JL, et al. *Physiol Heart Circ Physiol* 1994; 266: H1465-72)).

The specialized functions of the endothelium require gap junction intercellular communication between endothelial cells within the monolayer and between endothelium and other cells present in the vessel wall.. The communication between these different cell types via gap junctions in coronary capillaries as well as in all other vessels has been documented in several studies. Evidence of involvement in adaptive arteriogenesis has also been demonstrated (Cai W-J, et al. *J Mol Cell Cardiol* 2001; 33: 957-67), Wang H-Z, et al. *Am J Physiol Cell Physiol.* 2001; 281: C75-88), Schuster A, et al. *Am J Physiol Heart Circ Physiol.* 2001; 280: H1088-96)).

In different vascular patophysiological situations where the endothelial monolayer is disrupted as in diet induced hypercholestrolemic lesions the gap junction communication is decreased in the vascular smooth muscles (Polacek D, et al. *J Vasc Res* 1997; 34: 19-30). Injury at the endothelial cellular layer is seen during venous stasis and when thrombophlebitis is developed. Kwak BR, et al. *Molec Biol Cell* 2001; 12: 831-845 has clearly demonstrated that gap junction communication serves to coordinate cell migration during endothelial repair and also are important for capillary sprouting during angiogenesis.

Treatment with compounds of the invention that facilitate hemichannel closing and/or gap junction communication will improve the impaired inter cellular communication in the affected vascular areas, and will be particularly useful during organ ischemia, e.g. claudicatio intermittens and myocardial infarction..

However after baloon catheter injury in rat carotid the vascular healing process is characterised by increased gap junction communication .(Yeh HI, et al. *Arterioscle Thromb Vasc Biol* 1997;17:3174-84). A suitable invention compound will be administered before the balloon intervention and is preferably an add-on therapy to conventional medical treatment of this condition. Administration of the compound will preferably be parenterally. Effect can be tested in tissue sampled before and at different time after the balloon catheter injury. Faster healing of

the endothelial surface will be seen using conventional microscopy. Also improvement of gap junction communication will be found. See also *Arterioscle Thromb Vasc Biol* 1997;17:3174-84).

2. Erectile Dysfunction

In Corpus cavernosum a syncytial cellular network is established via gap junctions and is critical to erectile function and ensures that the corporal and arterial smooth muscle cells of the penis respond in a uniform and coordinated manner. (Christ GJ. (*Int J Impot Res.* 2000; 12 suppl. 4: S15-25), Melman A, Christ JC. (*Urolog Clin North America.* 2001; 28: 217-31)). Disturbed erectile function is seen in diabetes, arteriosclerosis, different neurological diseases and many chronic diseases. From studies in diabetes an inverse correlation between neural innervation and intercellular coupling point towards the potential functional plasticity of the corporal environment although not establishing the functional intercellular communication via gap junction.

Treatment with a compound that facilitates hemichannel closing and/or gap junction opening will improve the communication via the gap junction and thereby normalize the complex coordination between the smooth muscle cells in corpus cavernosum and the vessels.

In vivo pharmacological testing of erectile function of the compounds can be tested 10 weeks after streptozotocin (35 mg/kg i.p.) induced diabetes in rats (8 weeks old) as described by Rehman J,et al. (*Am J Physiol* 1997; 272: H1960-71). Penile reflexes and the intracavernous pressure are measured during local and systemic administration of different doses of the different hemichannel modulating compounds with measures and techniques described by the same research group. An increase in penile reflexes and in the intracavernous pressure of 25% or above can be seen.

Treatment of erectile dysfunction can be administered either locally in the penil corpus, as subcutanous injection or orally. Treatment will be either monotherapy or add-on to conventional treatment of this condition.

3. Incontinence

Smooth muscles in the urine bladder are characterized by phasic contractions and show spontaneous phasic contractions. However the bladder is in the healthy condition able to contain several hundred milliliters of urine without showing an increased intravesical pressure. In contrast to the normal bladder unstable bladders develop spontaneous increases in intravesical pressure related to urge incontinence (Turner WH, Brading AF. (*Pharmacol Therap.* 1997; 75: 77-110). Compared to gastrointestinal smooth muscle, bladder smooth muscles does not spontaneously generate co-ordinated contractions (Stevens RJ, et al. (*Am J Physiol.* 199; 2777: C448-60), Hashitani H, et al. (*J Physiol.* 2001; 530: 273-86)). Both electrical and morphological communications via gap junctions between smooth muscle cells in the bladder has recently been demonstrated (Hashitani H, et al. (*J Physiol.* 2001; 530: 273-86), Wang H-Z, et al. (*Urology.* 2001; Suppl 6A: 111)). The importance of these gap junctions was demonstrated by specific inhibition of the communication. Waves of spontaneous excitation in bladder smooth muscle propagate through gap junctions.

The uncontrolled urged incontinence will therefore be regulated via treatment with a hemichannel closing compound or a gap junction opener. Administration will be parenterally, orally or into the urinary bladder. Administration will preferably be as an add-on to treatment with drugs intended to normalize muscle contraction in the urine bladder.

Myoepithelial cells as presented in submandibular glandular ducts, in urether, in gall ducts, pancreatic ducts, tear duct are connected with gap junctions and intercellular communication is essential for the synchronization of contractile function of the myoepithelial cells (Taugner R, Schiller A. (*Cell Tissue Res.* 1980; 206: 65-72). Disturbed contractility in these ducts can be normalized by treatment with a hemichannel closing compound or a gap junction opener administered either parenterally or orally.

D. Healing

Prophylactic effect of treatment with a hemichannel closing agent or gap junction opener, such as compound 1 and compound 2, can be tested in an experimental set up as described by Yeh HI, et al. (*Arterioscle Thromb Vasc Biol* 1997;17:3174-84). compound 1 or

compound 2 can be administered before the balloon intervention using dosages in the range of 10-11 to 10-8 depending upon the compound's biological kinetics, e.g. as determined in the calciumchloride induced arrhythmia model described above. Tissue can be sampled before and at different time after the balloon catheter injury. Faster healing of the endothelial surface will be seen using conventional microscopy. Administration of the compound will be, e.g. parenterally.

Healing progresses in a series of overlapping phases beginning with haemostasis (coagulation). The second phase of the healing process is a cascade of inflammatory responses where microphages accumulates at the wound side and formulation of granulation tissue starts involving fibroblast and lymphocytes among other component. Epithelial cells will then start to migrate from the border of the wound to cover the area. Capillary spouting from the normal tissue into the wound is also involved in order to ensure supply of nutrients, oxygen and the different cells. All the cells and the capillary endothelium cells have an lively intercellular communication via gap junctions (Abdullah KM, et al. (*Endocrine.* 1999; 10: 35-41). Areas with low oxygen supply and/or high concentration of free radicals often seen in wounds with necrotic tissue, in diabetes, in arteriosclerosis, in surgery wounds, oedema, infection, burn wounds and in venous insufficiency will lower the gap junction communication (Nagy JI, et al. *Cell Growth Diff.* 1996; 7: 745-51)).

Treatment with a hemichannel closing compound or a gap junction opener will ensure maximal gap junction communication between the different cells considered to play an important role in the complicated repair process and thereby improve the wound repair. The compound will be administered topically, systemically or orally.

E. Diabetic retinopathy

Diabetic retinopathy can be diagnosed very early after onset of the disease by identifying alterations in the rate of blood flow (Bursell S-V, et al. (*Curr Eye Res.* 1992; 11: 287-95), breakdown in the blood-retinal barrier (Cunha-Vaz JG, et al. (*Br J Ophthalmol.* 1975; 59: 649-56), Do Carmo A, et al. (*Exp Eye Res.* 1998; 67: 569-75)) and/or loss of autoregulation (Kohner EM, Patel V, Rassam SMB.(*Diabetes* 1995; 44: 603-607)). By using both tracer transport and double cell patch clamp techniques Oku H, et al. (*Invest Ophthalmol Vis Sci.* 2001;42: 1915-

1920) have demonstrated an extensive cell-to-cell coupling. A closure of gap junction pathways disrupts the multicellular organization of retinal microvessels and contribute to diabetic retinal vascular dysfunction. Zhou ZY, et al. (*Neuroscience*. 2001; 102: 959-67) further demonstrated that reactive oxygen are involved in retinal gap junctional uncoupling and a recoupling when gluthation is supplied.

Hemichannel closers' effect on diabetic retinopathy can be studied *in vitro* using the streptozotocin induced diabetic rat model as described above. Freshly isolated retinal microvessels (Sakagami K, et al. *J Physiol (Lond)*. 1999; 521: 637-50) will be transferred to coverslip as described by Oku H, et al. (*Invest Ophthalmol Vis Sci*. 2001;42: 1915-1920). In this preperation the intercellular communication between the cells in the vascular wall will be measured either with dye or with tracer. Different concentrations in the range of 10-10 – 10-7 M of the compounds of the invention, eg., gap junction openers compound 1 or compound 2 can be tested and a significant increase in intercellular communication compared to baseline will be seen in the diabetic retina. Similar improvement will be seen when compared to controls (healthy animals). Treatment will be systemic, locally or orally. Therapy is preferably an add-on to conventional antidiabetic treatment.

Not only diabetic retinopathy but also other vascular abnormalities in the retina as for instance arteriosclerosis will benefit from increased closing of hemichannels or an improved gap junction communication by treatment with a compound that assist Cx tyrosine phosphorylation. compound will be administered parenterally.

F. Cardiac Disorders

1. Atrial-ventricular (AV) blockade

Intercellular communication in the cardiac av node is maintained via gap junctions. Decreased function lead to decreased conduction and may lead to total a-v blockade.

AV blockade is seen in acute myocardial infarction, in ischaemic heart disease, digitalis intoxication, calcium channel blocker intoxication and a hemichannel closing compound will

improve the av conduction. Administration of hemichannel closing compound shall be either parenterally or orally.

2. Atrial fibrillation

Nao et al. 2001 have found decreased serine phosphorylation of connexin 40 in myocardial cells of patients suffering from chronic atrial fibrillation (AF). Decreased serine phosphorylation of connexin is known to be connected with uncoupling of cells which may lead to AF. If this condition of chronic AF is also characterized in decreased tyrosine phosphorylation of connexins, then treatment with a compound such as Compound 1 or 2 herein may increase tyrosine phosphorylation, close hemichannels, open gap junction channels and remove the causes of AF.

3. Ischemia/reperfusion injury

During regional ischemia, hearts are exposed to metabolic stress that causes cell swelling which in turn increases tissue pressure and reduces perfusion of the ischemic border zone tissue. It is believed that the reduced perfusion in the ischemic border zone contributes to the gradual spreading of the infarct, which is associated with further impairment of cardiac function. As shown in Examples 7 and 8, Compound 1 prevents cell swelling during ischemia, reduces infarct size and prevents impairment of cardiac function after mypocardial infarction.

Moreover, coronary perfusion is restablished in patients with myocardial infarction by either administering a thrombolytic agent or by percutaneous transluminal coronary angioplasty (PTCA). Although restoration of blood flow is a prerequisite for myocardial salvage, reperfusion itself may lead to additional tissue injury beyond that generated by ischemia alone – this is known as "reperfusion injury". Mechanisms proposed to contribute to reperfusion injury are many, including oxygen free radical overload, neutrophil-mediated myocardial injury, intracellular calcium overload, and changes in osmotic environment (Wang et al.,*Cardiovasc Res.* 2002 Jul;55(1):25-37. Review).

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There is recognition that the osmolarity of bodily fluids is strictly controlled so that most cells do not experience changes in osmotic pressure under normal conditions, but osmotic changes can occur in pathological states such as ischemia, septic shock, and diabetic coma. The primary effect of a change in osmolarity is to acutely alter cell volume. If the osmolarity around a cell is decreased, the cell swells, and if increased, it shrinks. In order to tolerate changes in osmolarity, cells have evolved volume regulatory mechanisms activated by osmotic challenge to normalise cell volume and maintain normal function. In the heart, osmotic stress is encountered during a period of myocardial ischemia when metabolites such as lactate accumulate intracellularly and to a certain degree extracellularly, and cause cell swelling. This swelling may be exacerbated further on reperfusion when the hyperosmotic extracellular milieu is replaced by normosmotic blood and it may even cause rupture of the cardiac cell membranes (Wright AR, Rees SA.: *Pharmacol Ther.* 1998 Oct;80(1):89-121. Review). Thus, it is believed that compounds selected in accord with the present invention will prevent myocardial damage during reperfusion, eg., by a mechanism similar to the effect described in Example 7 (Figure 9A).

In addition, it has been reported that during the commonly used PTCA procedure, the atheroschlerotic plaque is ruptured which causes microembolization in the microcirculation distal to the site of the PTCA procedure, which is associated with accelerated progression of heart failure and increased mortality Henriques JP et al:. *Eur Heart J* 2002 Jul;23(14):1112-7). As shown in Examples 7 and 8, Compound 1 prevents cell swelling during ischemia, reduces infarct size and prevents impairment of cardiac function after mypocardial infarction and these properties of compounds will reduce injury during reperfusion.

Accordingly, the invention provides a method of cytoprotecting tissue or an organ of a mammal in need of such treatment. In one embodiment, the method includes administering a therapuetically effective amount of at least one compound selected from the group consisting of the compounds represented by Formula I or II. By the phrase "cytoprotecting" is meant reducing, preventing or alleviating symptoms associated with unwanted cell swelling. Particular tissues and organs that will benefit from the method include those confined or otherwise impacted by a fiborous capsule such as heart or

kidney. Also included are tissues associated with bone such as brain, spinal cord and bone marrow.

In one embodiment, the method further includes exposing the tissue or organ of the mammal to ischemic conditions such as those described herein. An example is heart infarction (heart attack) in which the ischemia is associated with harmful myocardial cell swelling. In one embodiment, such swelling can be reduced or avoided by administering at least one of Ac-D-Tyr-D-Pro-D-4Hyp-Gly-D-Ala-Gly-NH2 (Compound 1) and Ac-Gly-Asn-Tyr-NH2 (Compound 2) to the mammal.

As discussed a method of preventing or treating reperfusion injury in a mammal is also provided by the invention. In one embodiment, the method includes administering a therapuetically effective amount of at least one compound selected from the group consisting of the compounds represented by Formula I or II. In a more specific embodiment, the method further includes the heart of the mammal to infarct conditions. According to the method, it is desirable to establish coronary perfusion, typically as quickly as possible. In some embodiments, the method will further include administering a thrombolytic agent (eg., tissue plasminogen activator ("TPA")) or providing coronary angioplasty to facilitate coronary perfusion into the infarcted heart. In one embodiment, the compound is at least one of Ac-D-Tyr-D-Pro-D-4Hyp-Gly-D-Ala-Gly-NH2 (Compound 1) and Ac-Gly-Asn-Tyr-NH2 (Compound 2).

G. Immunology: cell maturation

Cell-to-cell interactions are crucial for lymphocyte maturation and activation. A wide rage of membrane molecules ensure intercellular adhesion and enabling cell-cell signalling during cell migration and activation in the immune system. Circulating human T, B and NK lymphocytes express Cx43 and active gap junctions between the cells have been demonstrated using dye methods as described previously. It has also been demonstrated that decrease in intercellular gap junctional coupling markedly decrease the secretion of IgM, IgG and IgA indicating that intercellular signaling across gap junctions is an important component of the

mechanisms underlying metabolic cooperation in the immune system (Oviedo-Orta E, et al. (*Immunology*. 2000; 99: 578-90), Oviedo-Orta E, et al. (*FASEB*. 2001; 15:768-774)).

In subchronic or chronic inflammation a local increase in synthesis of immunglobulins is desirable independent of aethiology. During inflammation the tissue is often different from the normal healthy tissue and low oxygen tension produces uncoupling of the intercellular gap junctional communication. The importance of low oxygen for GJIC uncoupling has been demonstrated in several different cell systems suggesting that oxygen tension is a universal regulator of GJIC. Said uncoupling is a result of altered tyrosine and/or serine and/or threonine phosphorylation of connexins in gap junction channels, and administration of a compound of the invention such as compound 1 or 2 herein will counteract this effect, close hemichannels and restore GJIC.

H. Improving GJIC

There are basically two ways of maintaining GJIC, either by keeping gap junction channels open or by facilitation of docking of hemichannels. In the treatment or prevention of disease states characterized in presence of ischemic conditions, both ways are preferred or recommended.

In primary cultures of neonatal rat ventricular cardiomyocytes, deprivation of oxygen and glucose leads to a decrease in the noradrenalin-induced stimulation of phosphoinositol (PI) turnover to app. 50% of the level at normal atmospheric and nutritional conditions. The gap junction modifier compound 1 has been shown to normalise this impaired noradrenalin-induced stimulation of PI turnover during oxygen and glucose deprivation by raising PI turnover to app. 90% of the normal level. Moreover is has been shown that compound 1 does not alter the noradrenalin-induced level of PI turnover during normal atmospheric and nutritional conditions (Meier, E and Beck, M M: 2001 International Gap Junction Conference, Aug 4-9, 2001, Hawaii, USA, abstract no. 132). Likewise, in cultured human osteoblast cultures and in osteoblastic rat osteosarcoma cell lines hypoxia decreased intracellular calcium wave propagation as measured as dye transfer after Lucifer Yellow injections. This decrease could be completely reversed by

treatment with compound 1 (Teilmann, S C, et al.: 2001 International Gap Junction Conference, Aug 4-9, 2001, Hawaii, USA, abstract no. 176).

Due to cellular uncoupling during inflammation, for instance, a compound that closes hemichannels and opens gap junctions will improve synthesis of immunglobulins during inflammation.

I. Peripheral neuropathy and neuropathic pain

Peripheral neuropathy and pain as seen in diabetes, during dialysis, liver cirrhosis and many other conditions are reported to involve both somatic and autonomic nerves. The exact mechanisms of the peripheral nerve injury in the various conditions are under investigation but nerve terminal destruction, decreased conductance, demyelination and increased inflammatory response have been described. Common for the various conditions in experimental set up are that increased free radicals, increased nitric oxide, oxygen stress and lack of free radical scavengers are seen and reduction of gap junction communication is recorded (Pitre DA, et al. (*Neurosci Lett.* 2001; 303: 67-71), Bolanos JP, Medina JM. (*J Neurochem.* 1996; 66: 2019-9), Low PA, Nickander, KK. (*Diabetes.* 1991; 40: 873-7), Levy D, et al. (*Neurosci Lett.* 1999; 260: 207-9), Bruzzone R, Ressot C. *J Eur Neurosci.* 1997; 9: 1-6)). Thus, a compound of the invention that assists hemichannel closing by phosphorylating hemichannel connexins will be beneficial in the treatment of peripheral neuropathy. Administration will be parenterally.

J. Hearing deficit

Noise induced hearing loss, presbycusis known to be associated with production of free radicals are related to inhibition of gap junction coupling between both Hensen cells and Deiters cells from Corti's organ in the cochlea (Todt I, et al. (*J Membrane Biol.* 2001;181: 107-114), Blasits S, et al. (*Phlugers Arch.* 2000; 440: 710-12) Lagostena L, et al. (*J Physiol.* 2001; 531: 693-707)). The gap junction communication between these supporting cochlear cells provides the important homeostasis for the sensory cells and thereby a normal neuronal activity of outer hair cells (Johnstone BM, et al. (*J Physiol* 1989; 408: 77-92)). This communication is disrupted during oxidative stress (Todt I, et al. (*J Membrane Biol.* 2001; 181: 107-114). Acquired or age dependent hearing loss will be prevented when treated with a compound which can increase

phosphorylation of tyrosine residues in connexin hemichannels (close the hemichannels) and maintain gap junction communication in the supportive cells. A suitable compound of the invention can be administered parenterally.

Melanocytes in the vestibular organ dark cell area are communicating heavily via gap junction and may play a role in transporting material between the endolymph and perilymph and also be of importance in maintaining the homeostasis of the microenvironment in the inner ear (Masuda M, et al. (*Anat Rec.* 2001; 262; 137-146)). Endolymphatic hydrops is related to various clinical conditions characterized by dizziness and reduced hearing. A decreased capacity of gap junction communication may be of importance in regulating transmembrane transport of several substances originally secreted or excreted via specific types of transporters.

K. Age dependent anemia and bone marrow transplantation

Existence of functional gap junctions between haematopoietic progenitor cells and stromal cells of the haematopoietic microenvironment was many years controversial but studies have now proofed the existence of human gap junction communication (Rosendaal M, et al. *Tissue Cell.* 1991; 23: 457-470), Dürig J, et al. (*Brit J Haematol.* 2000; 111: 416-25)). It has also been demonstrated that the communication is bi-directional favoring the hypothesis that stromal cells control the proliferative behaviour of the haematopoietic progenitor cells, but also their functional status can be regulated by immature haematopoietic cells (Gupta P, et al. (*Blood.* 1998; 91: 3724-3733)).

With age the functionality of the haematopoietic tissue is decreased and anemia is often seen in elderly people. Reduced capacity of haematopoietic tissue is also seen in haematological malignancies and after treatment with chemotherapeutics. Bone marrow transplantation from donor is used to prevent pancytopenia.

The effect of a compound of the invention that assists in hemichannel closing and/or facilitates gap junction communication will be studied in pretreated rats exposed to high dose cyclophosphamide. In these animals the bone marrow has stopped producing mature haematopoietic cells. Number of reticulocytes at different time intervals after cyclophosphamide

will be significantly higher in the animals pretreated with the connexin tyrosine phosphorylating compound 1 using doses of about 100 μL of 10^{-10} M to about 10^{-8} M compound 1 compared to non-pretreated animals. Administration of suitable compounds of the invention will be parenterally.

L. Pituitary and hypothalamic hypofunction

Hormones from the anterior pituitary gland show circadian variation in secretion within minutes, hours, days and seasons. The part of the nervous system responsible for most circadian rhythm is localized to a pair of structures in the hypothalamus known as the suprachiasmatic nucleus. In this center this biological clock is intrinsic in the individual cells. However coordinated electrical activity is mediated to neighboring cells via gap junction communication. (Colwell CS. *(J Neurobiol.* 2000; 43: 379-88)). Because also the anterior pituitary lacks direct innervations, gap junction-mediated cell-to-cell communication within the gland must be indispensable for the adequate cell-to-cell coordination and synchronization required to ensure appropriate and timed hormone secretion.(Vitale ML, et al. (*Biol Reporo.* 2001; 64: 625-633)). Guerineau NC, et al. (*J Biol Chem.* 1998; 273: 10389-95) concluded that spontaneously active endocrine cells are either single units or arranged in synchronized gap junction-coupled assemblies scattered throughout the anterior pituitary gland. Synchrony between spontaneously excitable cells may help shape the patterns of basal secretion. From the anterior pituitary gland, growth hormone, prolactin, adrenocortical hormone, thyroid hormone, and gonadotropin hormones are synthesized under control from hypothalamus stimulating hormones. One of the mechanism in dysrhythm of the complicated hypothalamic-pituitary-endocrine glands within one of the axis is therefore also related to reduced communication via gap junctions. The diseases are diabetes insipidus, hypogonadotrope hypogonadism, myxoedema, adrenocorticoid hypofunction, and dwarfism. Treatment with a suitable compound of the invention, preferably a gap junction opener, can improve the symptoms.

Also the neurons in the suprachiasmatic nucleus of the hypothalamus are dependent on optimal gap junction communication. In the axis mentioned above gap junction opener with mode of action in this region will also benefit patients with disturbed circadian rhythm (Shinohara K, et al. (*Neusosci Lett.* 2000; 286: 107-10).

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M. Renovascular hypertension and nephrotoxicity

Kidney and endothelial specific gap junctions are widely distributed in the kidney found in glomeruli, tubulus and vasculature including intraglomerular capillaries and juxaglomerular arterioles (Haefliger J-A, et al. (*Kidney Int.* 2001; 60: 190-201)). In that study the authors demonstrated the presence of gap junctions connecting renin-secreting cells of the afferent arteriole. The role of gap junction might contribute to the detection and propagation of blood borne signals, such as those elicited by increased blood pressure. Within the kidney, such signals need to be converted into autocrine, paracrine and endocrine stimuli by the endothelial cells of the afferent arteriole and the transmitted to the renin-secreting cells. Gap junction communication plays thus an important role in forming the interconnected juxtaglomerular apparatus. The rapid open to close transitions of gap junctions channels further imply a readily response to local vascular changes ensuring the continuous feedback required to match glomerular and tubular function as well as renin secretion to physiological demands. Diseases characterized by impaired renal gap junction communication will benefit from treatment with compound of the invention, preferably a specific gap junction opener, either administered orally or parenterally.

Heavy metals are nephrotoxic and causes renal injury. It has been demonstrated that the toxic metals cadmium (Fukumoto M, et al. (*Life Sciences.* 2001;69:247-54)) as well as mercury (Yoshida M, et al. (*Arch Toxicol.* 1998; 72: 192-96)) in primary cell cultures from rat proximal tubulus uncouple gap junctions and both groups suggest that renal dysfunction is related to the reduced intercellular communication. Treatment of heavy metal poisoning with a connexin tyrosine phosphorylating compound will reduce the tissue damage and prevent the progressive tissue devastation.

An *in vitro* test can be performed in cell cultures from tubulus cells and the compounds (compound 1 or compound 2 in a concentration of about $10^{-10} - 10^{-7}$ M) prevention of gap junction uncoupling when exposed to heavy metals will be investigated. Gap junction communication will be tested with Lucifer dye method as described previously.

After systemic administration of heavy metal to experimental animals (rats) renal function will be measured using ^{3}H-insulin as a clearance marker for glomerular filtration rate, ^{14}C-labelled tetraethylammonium as a clearance marker for renal plasma flow and lithium as a marker for proximal tubular function (Petersen JS, et al. *J. Pharmacol. Esp. Ther.* 1991, 258:1-7) before and after different time of chronic treatment with heavy metals. Chronic treatment with a specific compound of the invention, such as compound 1, will be initiated when renal function is compromised and an significant improvement of renal function parameters (glomerular filtration rate and blood pressure) will be seen following the treatment. Administration of a suitable invention compound will be parenterally.

Non-infectious inflammation as well as infections with different microbes induces significant non specific chronic changes in renal function also characterized by reduced glomerular filtration rate, decreased excretion of electrolytes and water and changes in blood pressure. Some of these symptoms will as well be treated with a specific connexin tyrosine phosphorylating compound and the symptoms will decline.

N. Developing and remodelling of teeth

Murakami S and Muramatsu T (Anat Embryol. 2001;203: 367-374) confirmed previous studies that gap junction communication exists between odontoblasts and that cellular activity is coordinated via these intercellular bridges (Iguchi Y, et al. *(Arch Oral Biol.* 1984; 29: 489-497)) but in their recent study they also demonstrated that these gap junction communications are present during the early development of teeth (pre-odontoblast) as well as in the odontoblasts in young and old odontoblast. Also the pulp cells subjacent to odotoblasts have gap junctions. These findings indicate that intercellular gap junction communication is important both during development of the teeth and during lifetime when teeth are remodeled or worned.

Treatment with a connexin phosphorylating compound of the invention, such as compound 2 which can be tested *in vitro* for effect on odontoblast intercellular communication in an assay which is essentially comparable to the osteoblast assays described herein, will normalize disturbed development of teeth. Treatment will also facilitate remodeling of teeth and make the teeth more resistant to caries.

O. Stem cells

Lumelsky et al (2001) have generated cells expressing insulin and other pancreatic endocrine hormones from mouse embryonic stem cells. See Nadya Lumelsky et al. in *Differentiation of Embryonic Stem Cells to Insulin-Secreting Structures Similar to Pancreatic Islets*. *Science*, 292, 1389-1394, 2001). The cells self-assemble to form three-dimensional clusters similar in topology to normal pancreatic islets where pancreatic cell types are in close association with neurons. Glucose triggers insulin release from these cell clusters by mechanisms similar to those employed *in vivo*. When injected into diabetic mice, the insulin-producing cells undergo rapid vascularization and maintain a clustered, islet-like organization.

In the clinical context, this embryonic stem cell-based system will allow simultaneous generation and assembly of insulin-secreting and other islet cell types known to play important role in regulation of insulin secretion into functional structural units. These units can provide material to optimize insulin production and analyze the fine control of glucose homeostasis. embryonic stem cells are ideal for these studies because genetic tools can be used to define the molecular basis of islet development and function. Potential for cell-based therapies is clearly an attractive goal for applications involving human and nonhuman embryonic stem and embryonic germ cells. Adult tissue may also be a useful source of functional pancreatic cells. The differentiation system described here may provide a source of functional pancreatic islets for treatment of diabetes. This is the first report showing that the several cell types of endocrine pancreas can be generated from embryonic stem cells *in vitro*. Although pancreatic islets obtained from cadavers can function in the liver after grafting, issues of tissue rejection and availability remain to be resolved. It is clear that engineering of embryonic stem cells to produce an abundant source of immunocompatible tissue for transplantation holds a growing promise for surmounting this and other problems associated with diabetes.

Myocardial infarction leads to loss of tissue and impairment of cardiac performance. The remaining myocytes are unable to reconstitute the necrotic tissue, and the post-infarcted heart deteriorates with time. Injury to a target organ is sensed by distant stem cells, which migrate to

the site of damage and undergo alternate stem cell differentiation; these events promote structural and functional repair. This high degree of stem cell plasticity prompted Orlic et al (*Nature* 410, 701 - 705 (2001)) to test whether dead myocardium could be restored by transplanting bone marrow cells in infarcted mice. They sorted lineage-negative (Lin-) bone marrow cells from transgenic mice expressing enhanced green fluorescent protein by fluorescence-activated cell sorting on the basis of c-kit expression. Shortly after coronary ligation, Lin- c-kitPOS cells were injected in the contracting wall bordering the infarct. They found that newly formed myocardium occupied 68% of the infarcted portion of the ventricle 9 days after transplanting the bone marrow cells. The developing tissue comprised proliferating myocytes and vascular structures. Their studies indicate that locally delivered bone marrow cells can generate de novo myocardium, ameliorating the outcome of coronary artery disease.

To characterize further the properties of these myocytes, they determined the expression of connexin 43. This protein is responsible for intercellular connections and electrical coupling through the generation of plasma-membrane channels between myocytes; connexin 43 was apparent in the cell cytoplasm and at the surface of closely aligned differentiating cells. These results were consistent with the expected functional competence of the heart muscle phenotype.

Since functional cells are generated from embryonic stem cells, and since connexins are indeed expressed in these cells in infarcted heart tissue, it is believed that this will be the case for other cells differentiated from embryonic stem cells. Since connexins play a dominating role in the function of these tissues (including pancreatic beta cells and heart muscle cells). compounds of the invention such as compound 1 and compound 2 that increase connexin tyrosine phosphorylation and hemichannel closing (resulting also in increased gap junction communication) will enhance the proliferation of embryonic stem cells into functional cells in organs wherein stem cells have been implanted.

Thus it is an object of the invention to provide connexin tyrosine phosphorylating compounds such as compound 1 and compound 2 to stimulate the transition of stem cells to functional cells in transplanted organs like pancreas for treatment of diabetes mellitus, heart for treatment of heart infarction, and basal ganglia of the brain for treatment of Parkinsons disease.

In general, it is believed that these compounds will accelerate diffentiation of stem cells, which may accelerate healing processes in all organs such as heart, brain, skin, bone, pancreas, liver, and other internal organs. Experiments can be performed using the general experimental design with myocardial infarction as described above by Orlic et al, *supra*, with administration of compound 1 and compound 2, for instance, repeatedly during the proliferation process. These experiments are believed to show an increase in connexin 43 expression by compound 1 and compound 2 or a faster regenerative process.

P. Cancer

1. Tumor progression

During tumorigenesis, the interruption of the physiological interaction of normal cells with their neighboring cells, and loss of features of differentiation are a common denominator in tumor progression. Alteration in gap junction communication is believed to be among the earliest changes during cell tumorgenesis (Wolburg H, Rohlmann A. *Int Rev Cytol.* 1995; 157: 315-73), Klaunig JE, Ruch RJ. 1990; 135-46)).

Kyung-Sun Kang, et al. (*Cancer Letters* 166 (2001) 147-153) have shown that pre- and co-incubation with GeO2 in TPA treated rat liver epithelial cells abolished down-regulation of GJIC by TPA suggesting that a substance that recovers the inhibition of GJIC may be used in the prevention or inhibition of tumor promotion. Suzuki J, Na H-K, et al. (*Nutrition and Cancer*, vol 36 No. 1 p. 122-8) have shown that the food additive lambda-carrageenan inhibits GJIC in rat liver epithelial cells similar to that of the well-documented tumor promotor phorbol ester (TPA), and therefore could play a role in carcinogenesis as a tumor promoting agent. Thus, the compounds of the present invention may be used in the prevention or treatment of cancer caused by tumor promoting agents, such as TPA and lambda-carrageenan.

2.Drug sensitivity resistance

Increased gap junction communication improves the microenvironment in tumors. See Chen et al.: Chem Biol Interact 1998 Apr 24;111-112:263-75

3. Metastasis

Loss of intercellular gap junction communication is associated with high metastatic potential in all cancers with metastatic potentials. (Saunders MM, et al. *Cancer Res.* 2001; 61: 1765-1767), Nicolson GI, et al. *Proc. Natl Acad Sci* USA. 1988; 85: 473-6)). Prevention of metastasis is established by treatment with a connexin tyrosine phosphorylating compound which will assist in preserving the gap junction communication in tumors. Treatment is an add on to conventional chemotherapy.

It is believed that administration of one or a combination of the invention compounds in a therapeutically effective amount (eg., compound 1 or compound 2) will be able to prevent or treat cancer.

Q. Miscellaneous Cells

Gap junctions also play an important role in intercellular communication, proliferation and differentiation in gastric mucosal cell. Gap junction opener will stimulate regenerative processes after induced injury (Endo K, et al. (*J Gastroenterol Hepatol.* 1995;10: 589-94)).

The cytoarchitecture of meniscal cells partly depends on gap junction communication. The fibrocartilage part of the meniscal as well as the fibrocartilage structure of tendons depends on intercellular communication. During injuries gap junction openers will improve the speed of repair.

R. Epilepsy

It may be desirable to treat disease states such as epilepsia characterized in abnormally high GJIC with a hemichannel opener, preferably as an adjuvant therapy in order to allow uptake of drugs or small regulatory molecules that serve to uncouple gap junction channels.

It is an object of the present invention to provide methods to treat or prevent one or more of the medical indications or conditions described herein. Typically, but not exclusively, such methods will include administration of at least one of the foregoing compounds, preferably one of same, in an amount sufficient to treat, prevent, or reduce the severity of the indication or

condition. Preferred compounds have been selected by one or a combination of the *in vitro* and *in vivo* assays described herein. Particular administration strategies will be apparent of those of skill in this field and will vary depending eg., on the sex, weight, general health and specific indication or condition to be treated or prevented. As discussed, the compounds disclosed herein can be employed as the sole active agent in invention methods. Alternatively, they can be used in "add-on" therapies such as those in which use of the compounds in conjunction with a recognized treatment method is indicated. Preferred indications or conditions to be treated or prevented in accord with the invention are generally associated with impaired cellular communication or impaired gap junction function. More specific indications and conditions relating to the invention have been discussed above.

Treatment methods in accord with the invention can employ one or more of the compounds disclosed herein as the sole active agent. Preferably, one of the compounds will be employed. If desired, such compounds can be used prophylactically ie., to prevent or reduce the severity of a particular indication or condition. Alternatively, the compounds can be used in conjunction with a recognized therapeutic approach. As an illustration in embodiments in which irradiation is treated, it is generally preferred that the treatment method be "add on" ie., in conjunction with a recognized therapy for treating the condition. Such "add on" treatment methods of the invention can be conducted at the same time or at a different time then the recognized therapy as needed. Established therapeutic approaches for a variety of diseases and medical conditions have been described. See generally, *Harrison's Principles of Internal Medicine* (1991) 12 ed., McGraw-Hill, Inc. and *The Pharmacological Basis of Therapeutics* (1996) Goodman, Louis S. 9th ed Pergammon Press, for example; the disclosures of which are incorporated herein by reference.

The concentration of one or more treatment compounds in a therapeutic composition will vary depending upon a number of factors, including the dosage of the hemichannel modulating compound to be administered, the chemical characteristics (e.g., hydrophobicity) of the composition employed, and the intended mode and route of administration. In general terms, one or more than one of the hemichannel modulating compounds may be provided in an aqueous

physiological buffer solution containing about 0.1 to 10% w/v of a compound for parenteral administration.

It will be appreciated that the actual preferred amounts of active compounds used in a given therapy will vary according to e.g. the specific compound being utilized, the particular composition formulated, the mode of administration and characteristics of the subject, e.g. the species, sex, weight, general health and age of the subject. Optimal administration rates for a given protocol of administration can be readily ascertained by those skilled in the art using conventional dosage determination tests conducted with regard to the foregoing guidelines. Suitable dose ranges may include from about 1 μg/kg to about 100 mg/kg of body weight per day ("therapeutically effective amount").

Therapeutic compounds of the invention are suitably administered in a protonated and water-soluble form, e.g., as a pharmaceutically acceptable salt, typically an acid addition salt such as an inorganic acid addition salt, e.g., a hydrochloride, sulfate, or phosphate salt, or as an organic acid addition salt such as an acetate, maleate, fumarate, tartrate, or citrate salt. Pharmaceutically acceptable salts of therapeutic compounds of the invention also can include metal salts, particularly alkali metal salts such as a sodium salt or potassium salt; alkaline earth metal salts such as a magnesium or calcium salt; ammonium salts such an ammonium or tetramethyl ammonium salt; or an amino acid addition salts such as a lysine, glycine, or phenylalanine salt. Additionally suitable salts are provided below.

More particular hemichannel modulating compounds of the invention are used in the form of a pharmaceutically acceptable salt, an alkyl ester, an amide, an alkylamide, a dialkylamide or a hydrazide formed with the C-terminal carboxylic acid function of a linear compound or a free carboxylic acid function, if present, of a cyclic compound. Amides and lower alkyl amides of linear compounds are among the preferred compounds of the invention. Salts include pharmaceutically acceptable salts, such as acid addition salts and basic salts. Examples of acid addition salts have already been described and include hydrochloride salts, sodium salts, calcium salts, potassium salts, etc. Examples of basic salts are salts where the cation is selected from alkali metals, such as sodium and potassium, alkaline earth metals, such as calcium, and

ammonium ions $^{+}N(R^3)_3(R^4)$, where R^3 and R^4 independently designate optionally substituted C_{1-6}-alkyl, optionally substituted C_{2-6}-alkenyl, optionally substituted aryl, or optionally substituted heteroaryl. Other examples of pharmaceutically acceptable salts are; e.g., those described in *Remington's Pharmaceutical Sciences* 17. Ed. Alfonso R. Gennaro (Ed.), Mark Publishing Company, Easton, PA, U.S.A., 1985 and more recent editions, and in *Encyclopedia of Pharmaceutical Technology*.

In the therapeutic methods of the invention, a treatment compound can be administered to a subject in any of several ways. For example, a hemichannel modulating compound selected in one or a combination of the *in vitro* and/or *in vivo* assays provided can be administered as a prophylactic to prevent the onset of or reduce the severity of a targeted condition. Alternatively, the compound can be administered during the course of a targeted condition.

A treatment compound can be administered to a subject, either alone or in combination with one or more therapeutic agents, as a pharmaceutical composition in mixture with conventional excipient, i.e. pharmaceutically acceptable organic or inorganic carrier substances suitable for parenteral, enteral or intranasal application which do not deleteriously react with the active compounds and are not deleterious to the recipient thereof. Suitable pharmaceutically acceptable carriers include but are not limited to water, salt solutions, alcohol, vegetable oils, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, perfume oil, fatty acid monoglycerides and diglycerides, petroethral fatty acid esters, hydroxymethyl-cellulose, polyvinylpyrrolidone, etc. The pharmaceutical preparations can be sterilized and if desired mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, colorings, flavorings and/or aromatic substances and the like which do not deleteriously react with the active compounds.

Such compositions may be prepared for use in parenteral administration, particularly in the form of liquid solutions or suspensions; for oral administration, particularly in the form of tablets or capsules; intranasally, particularly in the form of powders, nasal drops, or aerosols; vaginally; topically e.g. in the form of a cream; rectally e.g. as a suppository; etc.

The pharmaceutical agents may be conveniently administered in unit dosage form and may be prepared by any of the methods well known in the pharmaceutical arts, e.g., as described in *Remington's Pharmaceutical Sciences* (Mack Pub. Co., Easton, Pa., 1980). Formulations for parenteral administration may contain as common excipients such as sterile water or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, hydrogenated naphthalenes and the like. In particular, biocompatible, biodegradable lactide polymer, lactide/glycolide copolymer, or polyoxyethylene-polyoxypropylene copolymers may be useful excipients to control the release of certain hemichannel modulating compounds of the invention.

Other potentially useful parenteral delivery systems include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for inhalation administration contain as excipients, for example, lactose, or may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate, or oily solutions for administration in the form of nasal drops, or as a gel to be applied intranasally. Formulations for parenteral administration may also include glycocholate for buccal administration, methoxysalicylate for rectal administration, or citric acid for vaginal administration. Other delivery systems will administer the therapeutic agent(s) directly at a surgical site, e.g. after balloon angioplasty a hemichannel modulating compound may be administered by use of stents.

Abbreviations and Formulae:

Throughout the description and claims the three letter code for natural amino acids is used as well as generally accepted three letter codes for other α-amino acids, such as Sarcosin (Sar), α-Amino-iso-butanoic acid (Aib), Naphthylalanine (Nal) including 1-naphthylalanine (1Nal) and 2-naphthylalanine (2Nal), Phenylglycine Phg, 2,4-Diaminobutanoic acid (Dab), 2,3-Diaminopropanoic acid (Dapa), and Hydroxyproline (Hyp). Where nothing is specified Hyp represents 4-hydroxyproline. The natural or essential amino acids are the amino acid constituents of proteins. The aromatic amino acids are Phe, Tyr, Trp, 1Nal, 2Nal and His. Where the L or D form has not been specified it is to be understood that the amino acid in question has the natural L form, cf. *Pure & Appl. Chem.* Vol. 56(5) pp595-624 (1984). Where nothing is specified it is to

be understood that the C-terminal amino acid of a compound of the invention exists as the free carboxylic acid, this may also be specified as "-OH". The C-terminal amino acid of a compound of the invention may be shown to have the terminal function "-OH/NH_2" which means that there are two preferred forms of the compound: the free carboxylic acid and the amidated derivative.

The following specific definitions apply unless otherwise specified: ASAL refers to 4-azidosalicyloyl radical; AB refers to 4-azidobenzoyl radical; Fmoc refers to 9-fluorenylmethyloxycarbonyl radical; Ac refers to acetyl radical. Throughout the present disclosure unless otherwise specified the following definitions apply; Acm refers to acetamidomethyl radical; T4c refers to L-thiazolidin-4-carboxylic acid radical; Pc refers to L-pipecolic acid radical; DNP refers to dinitrophenyl; ; DBF is defined as 2-aminoethyl-6-dibenzofuranpropionic acid;; Acm refers to acetamidomethyl; and Sar refers to sarcosinyl radical; DNP functions is a hapten for antibody recognition, and compounds of the invention that contain a DNP moiety may be preferably used as research tools.

See also PCT/DK01/00127 (WO 01/62775) and PCT/US02/05773 (WO 02/077017) for additional information.

All references disclosed herein are incorporated by reference. The following Examples are illustrative of the invention.

Reference Example 1: Standard *in vivo* Mouse Arrythmia Assay

It is possible to test for antiarrythmic effects by performing a calcium-induced arhythmia model in mice.

Briefly, the antiarrhythmic effects of compounds can be tested in an *in vivo* model of calcium-induced arrythmias according to the model of J. J. Lynch, R. G. et al. *J Cardiovasc. Pharmacol.* 1981, 3 49-60. Mice (25-30 g) were anaesthetised with a neurolept anaesthetic combination (Hypnorm® (fentanyl citrate 0.315 mg/ml and fuanisone 10 mg/ml) +

midazolam (5 mg/ml)). Commercial solutions of hypnorm and midazolam were diluted 1:1 in distilled water and one part diluted Hypnorm® is mixed with one part diluted midazolam.

The anaesthesia was induced by s.c. administration in a dose of 0.05– 0.075 µl/10 gram mouse. An *i.v.* cannula was inserted into the tail vein. The lead II ECG signal was recorded continuously by positioning of a stainless steel ECG electrodes on the right forelimb and on the left hind limb. The ground electrode was placed on the right hind limb. The signal was amplified (x 5.000–10.000) and filtered (0.1-150 Hz) via a Hugo Sachs Electronic model 689 ECG module. The analogue signal was digitised via a 12 bit data acquisition board (Data Translation model DT321) and sampled at 1000 Hz using the Notocord HEM 3.1 software for Windows NT. After a 10-min equilibration period, the test sample of drug was injected into the tail vein. Mice pre-treated with vehicle were tested as a measure of the control level in untreated animals. The injection volume was 100 µl in all experiments. Infusion of $CaCl_2$ (30 mg/ml, 0.1 ml/min ≈ 100 mg/kg/min (calciumchlorid-2-hydrat, Riedel-de Haën, Germany)) was started 3 min after *i.v.* administration of drug or vehicle. The time lag to onset of 2nd degree AV-block was determined as the time from the start of $CaCl_2$ infusion until the first arrhythmic event occured. An event of 2nd degree AV-block was defined as intermittent failure of the AV conduction characterised by a P-wave without the concomitant QRS complex.

Responses were expressed relative to the time until 2nd degree AV-block occurred in vehicle treated mice. Maximal effect of each of the tested substances has been summarized.

More particular methods for detecting candidate compounds with good gap junction modifying activity further include selecting candidate compounds that prolong the time until onset of the induced atrial ventricular (AV) block by at least about 20% (score at least 2) in the standard *in vivo* mouse arrythmia assay. More preferred compounds exhibit a prolongation of at least about 60% of the time until onset of the induced AV block (score at least 3) in the assay.

The following examples provide a functional assay that shows the ability of compound 1 to both open gap junction channels and close hemichannels in rat neonate cardiomyocytes. Thus,

it is possible to obtain differentiated regulation of GJIC by tyrosine phosphorylation of both hemichannels and gap junction channels.

Example 1- Antiarrhythmic compounds

compounds used in the invention include Ac-D-Tyr-D-Pro-D-4Hyp-Gly-D-Ala-Gly-NH2 (compound 1) and Ac-Gly-Asn-Tyr-NH2 (compound 2). The compounds are synthesised according to standard solid phase synthesis as described in WO01/62775. A further example of compounds useful in the invention is trans-resveratrol and CAPE (caffeic acid phenethyl ester). Methods for making the compounds have been described. See e.g, the PCT/DK01/00127 (WO 01/62775) and PCT/US02/05773 (WO 02/077017) applications.

Example 2- Analysis of Metabolic Stress Induced by Glucose Removal

Cardiac arrhythmias can be caused by disturbances of both formation and conduction of the action potential. The propagation of an action potential from cell to cell is mediated by the intercellular gap junctions. These gap junctions consist of channels, which are built by specialised proteins called connexins. There are several lines of evidence showing that a reduced expression and/or a disturbed distribution of the connexins, can be deleterious for normal impulse propagation and thereby arrhythmogenic. One example is the alterations seen during and after ischemia. During acute ischemia uncoupling of the cells are mainly believed to be caused by intracellular acidification, increased intracellular Ca2+, reduced intracellular ATP and by elevated concentrations of long-chain acylcarnitines and fatty acids [1, 2]. After the acute phase of ischemia, remodelling occurs where the architecture of the infarcted area and its border zone is altered. This remodelling has been associated with areas of connexin disarray and the development of potential reentrant circuits, which are believed to act as arrhythmogenic substrates [3]. In vivo and in vitro studies have shown that a group of antiarrhythmic peptides can delay and inhibit the onset of arrhythmia, and subsequent studies have shown that these peptides increase the electrical coupling between cells [2].

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A Methods

1. Cell culture: In short the hearts from 20 neonate rats (age 1-2 days) are excised aseptically. The ventricles are isolated and cut into 4-6 pieces, which are digested in several steps in a Hanks buffered saline with trypsin and DNAse. The cells are then centrifuged and resuspended in MEM with 5 % FCS. To eliminate non-muscle cells the suspension is preplated in petri dishes for 30 minutes at 37°C. The cells in suspension are then seeded onto collagen coated coverslips. Seeding density will be adjusted to give confluent cultures.

2. Electrophysiology: The cover slips with confluent cardiomyocytes are mounted in an open chamber on the stage of an inverted microscope and superfused with Dulbeccos phosphate buffered saline (PBS) by gravity driven flow at 1 ml/min, 37°C. The solution (PBS) contain (in mM): Na+ 152, K+ 4.2, Cl- 141.5, PO43- 9.5, Ca2+ 0.9, Mg2+ 0.5, pH 7.4. Patch clamp pipettes are pulled from 1.5 mm glass capillaries (GC150F-15, Harvard Apparatus) on a Sutter Flaming-Brown P-87 microelectrode puller and fire polished to a resistance of 4-6 MW. Pipettes are filled with an intracellular like solution containing in mM: K+ 150, Na+ 15, Cl- 5, Gluconate- 150.2, EGTA 5, HEPES 5, Ca2+ 2 mM, Mg2+ 1.6, pH 7.2.

The patch clamp set-up consists of a synchronised discontinuous amplifier (SEC-05LX, NPI electronics) and data is digitised using an INT-10 interface (NPI electronics) and a PC1200 data acquisition board (National Instruments). Both current and voltage signals are low pass filtered at 1 kHz using the internal filters of the amplifiers and digitised at 10 kHz.

A cell is approached with an electrode using a PatchMan 5173 micromanipulator (Eppendorf). When contact with the cell is obtained (seen as a sudden increase in input resistance), suction is applied until the Giga seal configuration is established. Then a brief pulse of suction is applied to break the membrane under the pipette. The amplifier is then put in the zero-current clamp mode to monitor the membrane potential.

Some distance (»1 cm) away a bipolar platinum electrode is used to pace the culture. The delay between the stimulus artifact and the appearance of an action potential in the patched cell is then a measure of the conduction velocity.

The cells were perfused with control solution with 0.1 g/l BSA until a stable baseline in the stimulus-activation interval (SAI) was established. Then the perfusate was changed to solution without glucose for 15 minutes and subsequently challenged with compound 1 (10^{-8} M) for 20 minutes. In parallel, control experiments were performed which were conducted on a similar timescale without adding compound 1. Date are expressed as percent change relative to baseline prior to removal of glucose from the media.

B. Results

As illustrated in Figure 2, metabolic stress induced by removal of glucose produced a slight increase in the stimulus-activation interval suggesting that removal of glucose delayed the conduction velocity. In contrast, superfusion with a 10 nM concentration of compound 1 decreased the stimulus-activation interval, i.e. compound 1 increased conduction velocity in cultured primary cardiomyocytes. These data indicate that compound 1 increases cell-to-cell coupling in cardiomyocytes.

References:

[1] Carmeliet, E. Cardiac ionic currents and acute ischemia: from channels to arrhythmias. *Physiol Rev.*, 1999, 79: 917-1017.

[2] Dhein, S. *Cardiac gap junctions. Physiology, regulation, pathophysiology and pharmacology.* Karger, Basel, 1998.

[3] Peters, N. S. and Wit, A. L. Myocardial architecture and ventricular arrhythmogenesis. *Circulation*, 1998, 97: 1746-1754.

Example 3- Immunoprecipitation And Analysis Of Phosphorylated Connexins

Compounds used in this example were made using standard solid phase Fmoc chemistry. See the prior examples. Identification was performed by mass spectrometry, and the purity, determined by RP-HPLC. *In situ* binding and animal studies have shown that the following peptide (compound 1) binds to the receptor in the nano-molar range. Initial studies with compound 1 in 1nM, 10nM, 50nM and 100nM will determine whether studies with the following peptides compound 3 (H-GAG-4-Hyp-PY-NH2) and compound 4 (H-GNY-NH2) are conducted.

These studies will also determine whether studies involving specific kinase inhibitors are conducted.

H9c2 cells were seeded in 24-multi well dishes in a density of 7,900 cells/cm^2 (~ 15,000 cells/well) and grown for 3 days in MEM suplemented with 10% foetal calf serum (FCS) and 1000 units penicillin/1000 µg streptomycin (pen/strep) in an atmosphere of 5% CO_2 and 100% humidity at 37° C.

The labelled cells were incubated in Triton X-100 lysis buffer (~5 *10^7 cells/ml) for 1 hr at 4°C. The lysate is centrifuged 30 min at 20,000 * g for 30 min. The supernatant was precleared by adding 10 ul Sepharose per 200 µl supernatant. The precleared supernatant were then added 0.5-1µg phospho-tyrosine or phospho-serine antibodies (Sigma) and incubated for 1.5 hr at 4°C in an orbital shaker. The antibody-complex was then captured by incubation with 50 µl of a 1:1 slurry of protein G Sepharose for 1.5 hr. Finally the complex were extensively washed with 0.1% (w/v) Triton X-100, 50 mM Tris, pH 7.4, 300 mM NaCl and 5 mM EDTA.

The complexes were loaded onto a 12% SDS gel and blotted onto a PVDF membrane. The membrane is blocked with 0.1% Tween 20, 50 mM Tris, pH 7.4, 300 mM NaCl, 5% dry milk (TBST) for minimum 2h. The membrane was subsequently incubated with connexin 43 specific antibodies (Zymed) for 1.5 h at RT and developed with ECL+ (Amersham).

Figures 3A-B show immunoblots in which the anti-phosphotyrosine antibody was used. Cx43 fragment phosphorylation is depicted as a function of compound 1 (3A-B) and compound 2 (3B) concentration.

Figures 4A-B show immunoblots in which the anti-phosphoserine antibody (4A) or the anti-phosphotyrosine (4B) antibody was used.

Example 4: Effect of compound 1 Peptide On Hemichannel Activity in Confluent Cardiac Myocytes

1. *Cell culture:* Briefly, the hearts from 20 neonate rats (age 1-2 days) were excised aseptically. The ventricles were isolated and cut into 4-6 pieces, which are digested in several steps in a Hanks buffered saline with trypsin and DNAse. The cells were then centrifuged and resuspended in MEM with 5 % FCS. To eliminate non-muscle cells the suspension was preplated in petri dishes for 30 minutes at 37°C. The cells in suspesion were then seeded onto collagen coated coverslips.

2. *Dye-uptake:* The cover slips with cardiomyocytes were incubated for 30 minutes with control solution (PBS) containing in mM: Na^+ 152, K^+ 4.2, Cl^- 141.5, PO_4^{3-} 9.5, Ca^{2+} 0.9, Mg^{2+} 0.5, glucose 6, pH 7.4. or an ischmia mimicking (injury) solution (as control only K^+ 8.2, no glucose, 10 mM deoxy-glucose, pH 6.5), both solutions containing 200 µM calcein. Then the cells were washed for 10 minutes with control solution.

The cover slips were mounted in an open chamber on the stage of an inverted microscope. Ten random fields were chosen and from each field three cells were chosen under conventional light microscopy. Then the cells were excited by 480 nm light and the fluorescence emmision measured at 510 nm, where the emission intensity is a measure of dye-uptake.

The effect of the compound 1 on dye-uptake was investigated under two conditions:

Experiment 1: In this series of experiments the cells were be incubated with control solution with calcein for 30 minutes and washed for 10 minutes without dye. In paired experiments the same experiment was performed where the peptide is added during the 30 minutes incubation.

Experiment 2: As experiment 1 only performed with injury solution instead of control solution.

3. Results Experiments using uptake of calcein in cultured cardiomyocytes from neonate rats show that the dye uptake is 38 % higher than in control cells, when the cells are exposed to metabolic stress (in the form of low pH, increased extracellular K^+ and deoxyglucose 5-10 mM).

This effect could be significantly reduced to 5 % when the cells were co-incubated with compound 1 (10 nM, $P<0.017$ in paired t-test versus cells exposed to deoxyglucose alone, $n=6$). These results show that the increased membrane permeability through connexin hemi-channels seen after metabolic stress can be inhibited by compound 1. It is likely that this effect will be beneficial for cellular function and survival. Furthermore, phosphorylation experiments on cultured H9c2 cardiomyocytes have shown that compound 1 mediates tyrosine phosphorylation of connexin 43. Moreover, Cardiomyocytes exposed for 4 h with 0,1, 10, 50 and 100 nM compound 1 an increase connexin 43 tyrosine phosphorylation. Tyrosine phosphorylation has been reported to mediate a disruption of gap junction intercellular communication and could easily explain the decrease in membrane permeability seen in the calcein experiments. In some experiments, the compound 1 was found to decrease Ser-phosphorylation.

Figure 5 shows effects of 10nM compound 1 on ischemia-induced uptake of the calcein in the cultured cardiomyocytes.

Example 5- ELISA Assay Of Site-specific Connexin Phosphorylation

An ELISA sandwich assay has been developed which enables measurements of site-specific phosphorylation of connexins in tissue samples as well as in cell cultures in a multi-well format (24 – 96 wells). Wells are coated with antibody (capture antibody) against the connexin type in question. Cell or tissue extracts are then reacted with capture antibody-coated plates at 4° C o/n. The captured connexins that are phosphorylated are detected with an antibody directed against specific phosphorylation sites and conjugated with either biotin, FITC, TRITC or peroxidase.

The amounts of antibody bound to the specific phosphorylation sites may alternatively be measured with a radioactive labelled or an enzyme conjugated antibody against the species IgG of the detection antibody.

The principle has been demonstrated using mouse antiCx43 as capture antibody, rabbit anti-Tyr-P (tyrosine phosphorylation site) as detection antibody and anti-rabbit IgG either labelled with ^{125}I or HRP (horse radish peroxidase) to measure the amounts of bound anti-Tyr-P.

The principle may also be demonstrated using rabbit antiCx43 (5 μg/ml; cat. no. 710700, Zymed Lab. Inc., California, USA) as capture antibody, a monoclonal mouse anti-phosphotyrosine (50 μg/ml; cat. no. P-3300, clone pt66, Sigma, MO, USA) as detection-antibody and anti-mouse whole antibody either labelled with ^{125}I (sheep anti-mouse Ig cat. no. IM 131, Amersham Biosciences, Wales, UK) or peroxidase (donkey anti-mouse Ig; cat. no. 715-035-151) to measure the amounts of bound anti-phosphotyrosine.

Figure 6A shows in schematic form a preferred ELISA detection format. Figures 6B and 6C show results of this example.

The present example and discussion provides evidence that metabolic stress opens connexin hemi-channels in cardiac cells, and that this opening can be circumvented by incubation with compound 1. Moreover, Examples 2-5 show that compound 1 closes hemichannels during metabolic stress. In agreement with the inhibitory effect of compound 1 on calcein uptake by hemichannels, i.e. a closing of hemichannels, the Examples also show that compound 1 assists in modulating the tyrosine phosphorylation state of connexin 43. Compound 1 is known to open gap junctions, cf. WO01/62775 and it is known that the inhibition of dephosphorylation of connexins prevents closing of gap junction channels. Thus, it is believed that compounds provided herein which can be shown to have beneficial gap junction opening or modulating effect of compound 1, i.e. all antiarrhythmic peptides disclosed in WO01/62775 and known peptides such as AAP, AAP10, HP5 and the like are useful in the present invention as modulators of hemichannel opening or closing. It is also believed that compound 1 maintains or increases the tyrosine phosphorylation of Cx43, thereby modulating hemichannel function.

Example 6- Dye uptake in cardiomyocytes

1. Methods Ventricular myocytes were isolated from neonate rats by trypsin digestion and plated onto collagen-coated cover slips. After four days the myocytes formed confluent and synchronously beating sheets of cells. To measure dye uptake, cells were incubated in buffer containing 200 μM calcein for 30 minutes at room temperature. Then coverslips were thoroughly washed with control buffer and mounted in an open bath chamber and superfused with control buffer (RT). Cells were excited with 480 nm light by means of a Xenon lamp and a

monochromator. Images of the fluorescence emitted at 510 nm were collected by a cooled CCD camera (Sensicam). Excitation control and image acquisition was controlled using Imaging workbench (Axon).

Under light microscopy three regions were placed over cardiomyocytes in an image. Then average fluorescence intensity was measured, and the procedure was repeated until 30 regions in 10 images had been measured. The average of these measurements were used as value for the given experimental condition, that is n=1. In each series one control incubation was performed and all experimental values are given as relative to this value.

Solutions: Control buffer (in mM): NaCl 136, KCl 4, $MgCl_2$ 0.8, $CaCl_2$ 1.8 HEPES 5, MES 5, Glucose 6, pH 7.3 Stress buffer (in mM): NaCl 136, KCl 8, $MgCl_2$ 0.8, $CaCl_2$ 1.8 HEPES 5, MES 5, Deoxy-glucose 10, pH 6.2

2. Results Incubation of cells with calcein under control condition gave some fluorescence staining, which was mostly of a localized particulate nature (see Figure 7A-C, panel B). On top of the cultured myocytes, curled up cells that immediately stained with trypan blue, were often found. These cells stained heavily with calcein and care was always taken not to place the regions of measurements over these cells. When cells were exposed to metabolic inhibition the pattern of staining changed, and diffuse staining throughout the cytosol was observed (figure 7C). The average intensity during stress was 36.2 ± 4.18 % higher compared to control (n=5, $P<0.001$ in paired t-test).

This confirms findings in cardiac and other cell types, that metabolic inhibition activates the uptake of fluorescent dyes, probably via connexin hemi-channels. We hypothesized that one of the mechanisms by which the AAP family of peptides could exert its positive effects on cardiac tissue, could be to interrupt activation of hemi-channels, thereby improving cellular homeostasis. To investigate this, cells were exposed to stress in the presence of ZP123 in concentrations between 0.1 pM and 10 nM. The results are presented in Figure 8, where each data point represent 5 experiments. As can be seen ZP123 dose-dependently reduced the uptake of calcein. Using a sigmoidal function ED_{50} was estimated to 3.3 ± 5.3 pM with a Hill coefficient

of 2.5 ± 1.0. Average intensity during maximal inhibition by compound 1 was 6.9 ± 1.7% above control.

Example 7- Volume measurements in cardiomyocytes:

1. Methods: Ventricular myocytes were isolated from neonate rats by trypsin digestion and plated onto collagen-coated cover slips. After four days the myocytes formed confluent and synchronously beating sheets of cells. To measure cell volume cells were loaded with calcein-AM (5 μM in control buffer) for 15 minutes at 37 °C and the cover slips mounted in an open bath chamber. The following experiments were performed at room temperature. Using a Leica laser confocal microscope, an optical cross-section was performed every 20 seconds throughout the duration of the experiments.

The optical cross-sections were analyzed using Metamorph (Universal Imaging). The area of the cell was determined as the area of pixels with fluorescence intensities higher than a threshold value chosen to minimize background induced fluctuations. Data are presented as relative volumes by dividing each value by the average area under control conditions (the last measurement before metabolic stress).

Solutions: Control buffer (in mM): NaCl 136, KCl 4, $MgCl_2$ 0.8, $CaCl_2$ 1.8 HEPES 5, MES 5, Glucose 6, pH 7.3 Stress buffer (in mM): NaCl 136, KCl 8, $MgCl_2$ 0.8, $CaCl_2$ 1.8 HEPES 5, MES 5, Deoxy-glucose 10, pH 6.2

2. Results Under control conditions cells on average tended to slightly decrease their volume with time (Figure 9B shows average data from 5 experiments). When cells were challenged with metabolic inhibition, the fall in volume was reversed to a net-increase (filled squares in Figure 9A). On average the relative volume was 1.06 ± 0.02 (last five minutes of stress, n=5), whereas the volume of control cells in this period was 0.94 ± 0.04.

Dye uptake measurements in myocytes have shown that compound 1 inhibits open connexin hemi-channels. It is believed that the hemi-channels might contribute to the observed swelling. To test this compound 1 (0.1 nM) was included in the stress medium and volume

monitored. Data from six experiments are shown in Figure 9A (open squares) and as can be seen the stress induced swelling was prevented. Average relative volume during stress in the presence of compound 1 was 0.93 ± 0.05.

When comparing data from control, stress and stress plus compound 1 using a one-way ANOVA, significant differences were detected (see Figure 10). Post.hoc. testing (LSD) showed that stress was significantly different from control ($P<0.05$) and stress plus compound 1 ($P<0.05$), whereas control was not different from stress plus compound 1 ($P>0.76$).

Example 8 – Reduction of infarct size after myocardial infarction in rats.

1. Methods:

Male Lewis rats (300-350 g; M&B, Ll. Skendsved, Denmark) were anaesthetized with a neurolept anaesthesic combination (Hypnorm® (fentanyl citrate 0.315 mg/ml and fluanisone 10 mg/ml) + midazolam (5 mg/ml)). Commercial solution of midazolam was diluted 1:2 in distilled water. Three parts of the diluted midazolam is mixed with one part hypnorm®. Anesthesia was induced by s.c. administration of 0.2 ml of this solution per 100 gram rat. When surgical anesthesia was established, an endotracheal cannula is inserted and the animal is artificially ventilated using a Harvard rodent ventilator adjusted to maintain arterial pH at 7.3–7.5 during surgery.

Compound 1 was delivered by an osmotic minipump (Alzet model 2ML4) that was inserted into the intraperitoneal (i.p.) cavity immediately prior to induction of the myocardial infarction. The pump was filled with vehicle (isotonic saline) or Compound 1 and primed at 37°C for 24 hours prior to the operation. In order to ensure that the therapeutic plasma concentration was obtained already at the time of infarction, a loading dose of Compound 1 was given s.c. prior to ligation of the LAD. With the s.c. loading dose, a steady-state plasma level was reached already after 3 min.

After administration of the s.c. loading dose of Compound 1 and after i.p. insertion of the osmotic minipump, a left thoracotomy was performed, and the left anterior decending artery was

ligated using a 6-0 silk suture. The thorax is closed in layers, and a negative pressure was induced in the pleural cavity to unfold the lungs. Sham-operated animals were subjected to the same procedure without ligation of the LAD. Postoperatively, the animal were allowed to recover in a heated cabinet at 30°C with 50% oxygen until the next morning. To prevent dehydration during the recovery period, the rat was given 5 ml 5% glucose s.c. immediately after completion of surgery and another 5 ml 5% glucose at 4 p.m. To relieve postoperative pain, the rat was treated with buprenorphine (20 μg/100 g s.c. b.i.d.) and meloxicam (0.1 mg/100 g s.c. once daily) for three days after the myocardial infarction. In this study the infusion dose given to rats with myocardial infarction (MI) was adjusted to produce plasma concentrations at 0 [vehicle=isotonic saline], 1.7±0.4 nM [dose 1], 5.7±0.4 [dose 2], or 93.7±12.3 nM [dose 3]. Plasma concentrations of Compound 1 were measured by mass spectrometry following solid phase extraction. Doses used are shown below in Table I:

TABLE I

Group	Plasma concentration after 3 weeks nM	S.c. loading dose of Compound 1 (nmol/kg)	I.p. infusion dose of Compound 1 (pmol/kg/min)
Sham-MI	0	0	0
MI	0	0	0
MI	1.7±0.4	0.25	11
MI	5.7±0.4	2.5	110
MI	93.7±12.3	25	1100

Three weeks after ligation of LAD, the rats were anesthetized with an i.p, injection of pentobarbital (50 mg/kg). The animals were placed on a heating blanket in order to maintain body temperature at 37°C. Tracheotomy was performed and the rat was ventilated with oxygen using a Harvard rodent ventilator. The ventilator was adjusted to maintain arterial pH at 7.35-7.45. PE50 catheters were placed in the femoral vein and artery for i.v. administration and arterial pressure measurements, respectively. After insertion of the femoral i.v. catheter, the animal was paralysed by i.v. administration of pancuronium, 1 mg/kg. Anaesthesia and respiratory paralysis is maintained by continuous i.v. infusion of pentobarbital (2.5 mg/kg/h) and

pancuronium (1 mg/kg/h) delivered in isotonic saline (50 µl/min). A tygon catheter was inserted into the left ventricle via the right carotid artery for determination of left ventricular end-diastolic pressure. After recording of left ventricular end-diastolic pressure (LVEDP), The hearts are removed from the bodies by cutting the veins and arteries a few mm from the hearts. The hearts were carefully rinsed for blood with isotonic saline, weighed and placed in 4% neutral buffered formalin.

After fixation the large vessels were cut close to the heart. Thereafter the hearts were cut into parallel slices with a thickness of 1.5 mm using an aggregate consisting of parallel razor blades with a distance of 1.5 mm. The slices are perpendicular to the long axis of the heart.

The slices were placed in two histological capsules so that the first slice is placed in one of the capsules by random number and the second in the other, the third in the first capsule and so on. After embedding, the slices were cut into 4 µm thick sections that are stained by Hematoxylin-eosin and by Masson-trichrome.

Using a microscope with a stage that can be moved in predetermined steps, both Masson-trichrome stained slides were examined and the number of points hitting fibrous tissue (blue stained in Masson-trichrome) and normal tissue are counted. The microscope was connected to a video camera and the image is displayed on a screen. The morphometry system used was Cast2 from Olympus, Denmark. Infarct size was calculated as % fibrous tissue in the heart.

2. Results:

As illustrated in Fig. 11, the heart weight-body weight ratio was reduced by the two highest doses of Compound 1 suggesting that the compound reduces the hypertrophic consequences (i.e., remodelling) of myocardial infarction.

As shown in Fig. 12, treatment with Compound 1 for three weeks reduced infarct size by 20-50% relative to infarct size in rats subjected to a myocardial infarction, but treated with vehicle. Tyhis indicates that Compound 1 has cytoprotective actions during myocardial ischemia. In line with what has been described herein, these data indicate that Compound 1 reduces the

size of the myocardial infarct by a mechanism that may involve prevention of cell swelling. Thus, prevention of cell swelling may prevent compression of surrounding healthy tissue, and therefore prevent compression of the microcirculation in surrounding healthy tissue. Therefore, in this application we claim that this principle may prevent the consequence of any ischemic lesion in any organ, preferably but not limited to organs with a confined fibrous capsule (e.g., heart, kidney) or organs surrounded by bone (e.g., brain, spinal cord, bone marrow).

As shown in Fig. 13, rats subjected to myocardial infarction but treated with either dose of Compound 1 for three weeks, had better an improved cardiac function with less congestion in the left ventricle as demonstrated by a reduced left ventricular end-diastolic pressure. This data indicate that Compound 1, prevents the impairment of cardiac function following a myocardial infarction.

All references disclosed herein are incorporated by reference. The invention has been described with reference to preferred embodiments thereof. However, it will be appreciated that those skilled in the art, upon consideration of this disclosure, may make modifications and improvements within the spirit and scope of the invention.

What is claimed is:

1. A method of closing a hemichannel in a cell, tissue or organ exposed to stress, the method comprising contacting the stressed cell, tissue or organ with a therapeutically effective amount of at least one compound selected from the group consisting of compounds represented as Formula I or II , wherein the contact is sufficient to close the hemichannel in the stressed cell, tissue or organ.

2. The method of claim 1, wherein the method further comprises phosphorylating a tyrosine residue of connexin 43 (Cx 43) and closing the hemichannel.

3. A method for opening a hemichannel in a cell, tissue or organ, the method comprising contacting the cell, tissue or organ with a therapeutically effective amount of at least one compound selected from the group consisting of compounds represented as Formula I or II , wherein the contact is sufficient to open the hemichannel in the cell, tissue or organ.

4. The method of claim 3, wherein the method further comprises dephosphorylating a serine residue of connexin 43 (Cx 43) and opening the hemichannel.

5. A method of preventing or treating tissue or organ stress in a mammal, the method comprising administering a therapeutically effective amount of at least one compound selected from the group consisting of compounds represented as Formula I or II as described above, wherein the contact is sufficient to prevent or treat the stress in tissue or organ.

6. The method of claim 5, wherein the method further comprises phosphorylating a tyrosine residue of connexin 43 (Cx 43) and closing the hemichannel.

7. A method of increasing gap junction intracellular communication (GJIC) in a cell, tissue or organ, the method comprising administering a therapeutically effective amount of at least one

compound selected from the group consisting of compounds represented as Formula I or II, wherein the contact is sufficient to increase the GJIC in the cell, tissue or organ.

8. A method of treatment of burns comprising administering to a patient in need of such treatment a therapeutically effective amount of a compound according to Formula I or II that blocks connexin hemichannel opening.

9. A method of treatment of thromboses comprising administering to a patient in need of such treatment a therapeutically effective amount of a compound according to Formula I or II that blocks connexin hemichannel opening.

10. A method of treatment of respiratory and metabolic acidosis comprising administering to a patient in need of such treatment a therapeutically effective amount of a compound according to Formula I or II that blocks connexin hemichannel opening.

11. A method of treatment of focal arrhythmia comprising administering to a patient in need of such treatment a therapeutically effective amount of a compound according to Formula I or II that blocks connexin hemichannel opening.

12. A method of treating and preventing cell and tissue damage resulting from elevated levels of blood glucose comprising administering to a patient in need of such treatment a therapeutically effective amount of a compound according to Formula I or II that blocks connexin hemichannel opening.

13. A method of treatment of chronic atrial fibrillation comprising administering to a patient in need of such treatment a therapeutically effective amount of a compound according to Formula I or II that blocks connexin hemichannelopening.

14. A method of treatment of epilepsia comprising administering to a patient in need of such treatment a therapeutically effective amount of a compound according to Formula I or II that promotes connexin hemichannel opening.

15. A method for screening candidate compounds that modulate hemichannel function, the method comprising contacting cells with at least one compound selected from the group consisting of antiarrhythmic peptides and compounds represented as Formula I or II, the contacting being under conditions conducive to modulating phosphorylation of connexin 43 (Cx43); and detecting a change in Cx43 phosphorylation, wherein the change in phosphorylation is taken to be indicative of a hemichannel modulating compound.

16. The method of claim 15, wherein the testing step further comprises testing the candidate compound in an immunological or cell sorting-based assay.

17. The method of claim 16, wherein the immunological assay comprises contacting cells with the candidate compound under conditions sufficient to increase or decrease phosphorylation of the Cx43, producing a lysate of the cells; and detecting increased or decreased Cx43 phosphorylation in the cell lysate as being indicative of the hemichannel modulating compound.

18. The method of claims 16-17, wherein the immunological assay is an ELISA assay.

19. The method of claim 18, wherein the ELISA method further comprises:

a) coating a solid support with a first antibody that specifically binds the connexin,

b) contacting the cell lysate to the solid support under conditions conducive to forming a binding complex between the first antibody and the connexin,

c) contacting the first antibody:connexin binding complex with a second antibody, the contacting being under conditions sufficient to form a specific binding complex between the second antibody and any phosphorylated connexin in the first antibody:connexin binding complex,

d) contacting the first antibody:connexin:second antibody binding complex with a detectably-labeled third antibody that binds the second antibody; and

e) detecting presence of the third detectably-labeled antibody on the solid support as being further indicative of the compound.

20. The method of claim 19, wherein the third antibody is detectably-labeled with at least one of biotin, FITC, TRITC, radioactive iodine or peroxidase.

21. The method of claims 19-20, wherein the second antibody is an anti-phosphotyrosine antibody.

22. The method of claims 19-21, wherein the second antibody is an anti-phosphoserine antibody.

23. The method of claims 19-22, wherein the detectably-labeled third antibody is detected by radioscintillation counting.

24. The method of claim 16, wherein the immunological method is a radioimmunoassay (RIA)

25. The method of claim 24, wherein the immunological method is an RIA assay and the method further comprises:

a) detectably-labeling the cells before producing the cell lysate, wherein the labeling is under conditions conducive to labeling phosphorylated connexin,

b) contacting the cell lystate with an antibody that forms a specific binding complex with the connexin,

c) separating the binding complex from the detectably-labeled cell lysate; and

d) detecting the labelled and phosphorylated connexin as being further indicative of the gap junction modulating compound.

26. The method of claim 25, wherein the antibody is an anti-connexin antibody.

27. The method of claims 25-26, wherein the detection step further comprises performing a Western immunoblot assay.

28. The method of claims 25-27, wherein the detectable label is radioactive phosphorous.

29. The method of claims 15-29, wherein the method is performed in a high throughput or ultra high throughput screening format.

30. The method of claims 1-30, wherein the compound facilitates phosphorylation of Cx43 intracellular C-terminal region.

31. The method of claims 1-30, wherein the compound facilitates dephosphorylation of Cx43 C-terminal region.

32. The method of claim 31, wherein the phosphorylation site is a tyrosine residue.

33. The method of claim 32, wherein the dephosphorylation site is a serine residue.

34. A method of screening candidate compounds that modulate hemichannel function, the method comprising:

1) culturing a population of cells, a tissue or an organ such as those derived from the heart or muscle,

2) stressing the cells, tissue or the organ preferably by oxygen deprivation or metabolic inhibition such as by adding a glucose derivative,

3) adding a known or candidate hemichannel modulating compound represented by Formula I or II,

4) adding a detectable reporter such as a fluorescent, chemiluminescent, or phosphorescent compound such as fluorescent dye such as calcein and related compounds,

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5) detecting a change in uptake of the detectable reporter into the cells tissue or organ relative to a suitable control and

6) optionally measuring the change as being indicative of a compound that modulates hemichannel function

35 A method of screening candidate compounds that modulate hemichannel function, the method comprising:

1) culturing a population of cells a tissue or an organ such as those derived from the heart or muscle,

2) loading the cells, tissue or organ with a detectable reporter such as a fluorescent, chemiluminescent, or phosphorescent compound such as dye, such as calcein or a related compound,

3) estimating the volume of the cells tissue or organ by detecting and quantifying signal from the detectable reporter

4) adding a known or candidate hemichannel modulating compound represented by Formula I or II

5) stressing the cells tissue or the organ preferably by oxygen deprivation or metabolic inhibition such as by adding a glucose derivative,

6) detecting a change in cell volume relative to a suitable control and

7) optionally measuring the change as being indicative of a compound that modulates hemichannel function and optionally gap junction function

36 A method of cytoprotecting tissue or an organ of a mammal in need of such treatment, the method comprising administering a therapeutically effective amount of at least one compound selected from the group consisting of the compounds represented by Formula I or II.

37 The method of claim 37 wherein the method further comprises exposing the tissue or organ of the mammal to ischemic conditions

8 P

38 The method of claim 38 wherein the organ to be cytoprotected is associated with a fibrous capsule or bone.

39 The method of claim 39 wherein the organ is heart, kidney brain, spinal cord or bone marrow

40 The method of claim 40 wherein the heart has been subjected to an infarction and the ischemia is associated with myocardial cell swelling

41 The method of claim 41 wherein the compound is Ac-D-Tyr D Pro-D-4Hyp-Gly-D-Ala-Gly-NH2 (Compound 1) or (Ac-Gly Asn-Tyr NH2) Compound 2

42 A method of preventing or treating reperfusion injury in a mammal the method comprising administering a therapuetically effective amount of at least one compound selected from the group consisting of the compounds represented by Formula I or II

43 The method of claim 43 wherein the method further comprises exposing the heart of the mammal to infarct conditions and establishing coronary perfusion

44 The method of claim 44 wherein the method further comprises administering a thrombolytic agent or providing coronary angioplasty to facilitate coronary perfusion into the infarcted heart.

45 The method of claim 45 wherein the compound is Ac-D-Tyr D Pro-D-4Hyp-Gly-D Ala-Gly NH2 (Compound 1) or (Ac-Gly Asn Tyr NH2) Compound 2

89

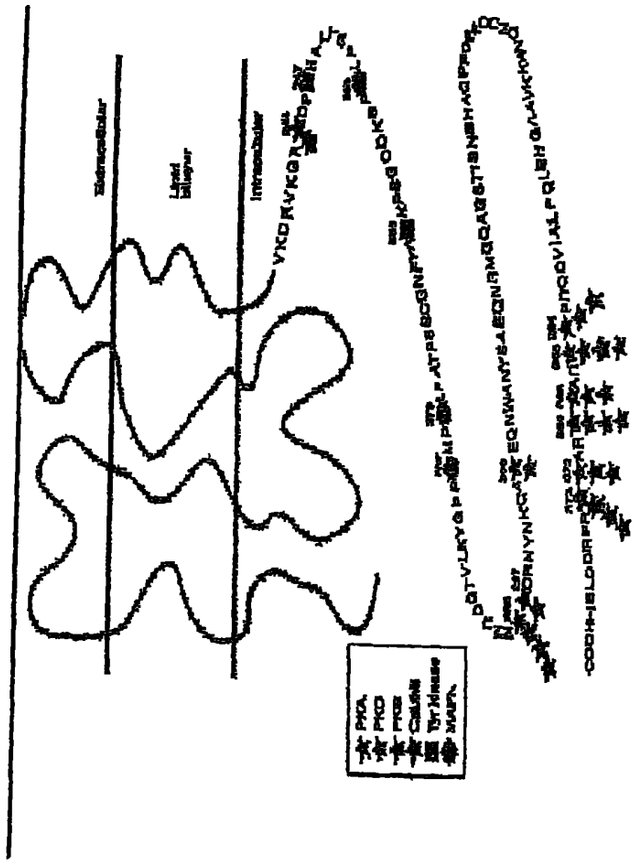

Extracellular
Lipid bilayer
Intracellular
PKA
PKD
PKB
Calcium
Tyr kinase
MAPK

Figure 1

90

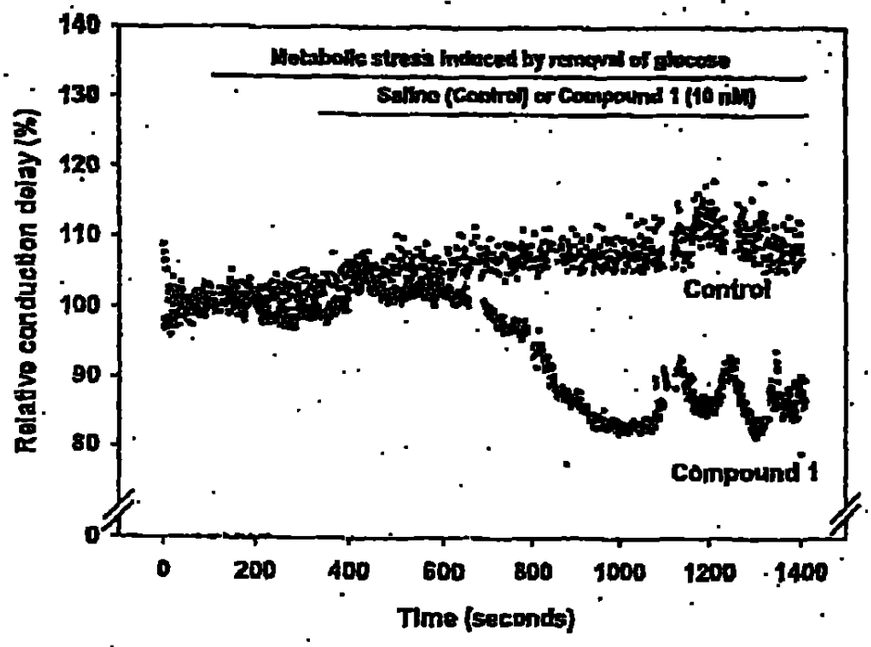

Metabolic stress induced by removal of glucose
Saline (Control) or Compound 1 (10 nM)
Relative conduction delay (%)
140
130
120
110
100
90
80
0
Control
Compound 1
0
200
400
600
800
1000
1200
1400
Time (seconds)

Figure 2

Figure 3A

Figure 3B

92

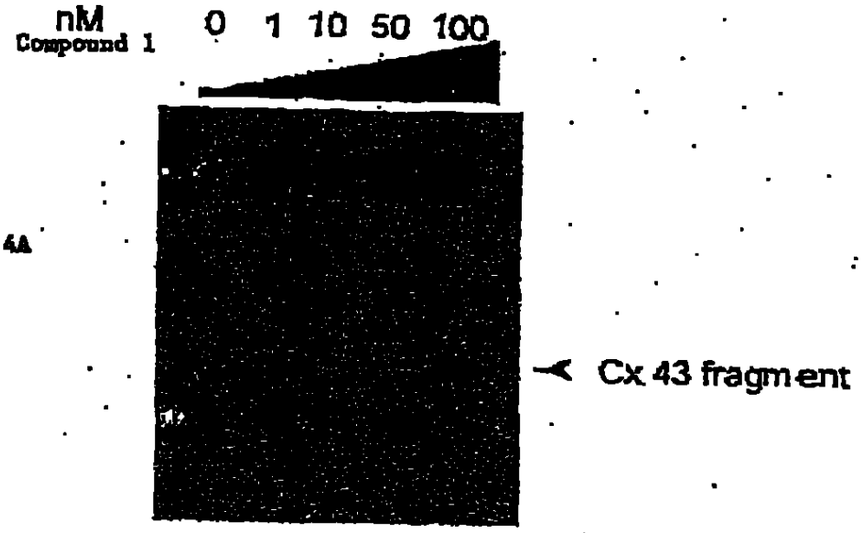


Figure 4A

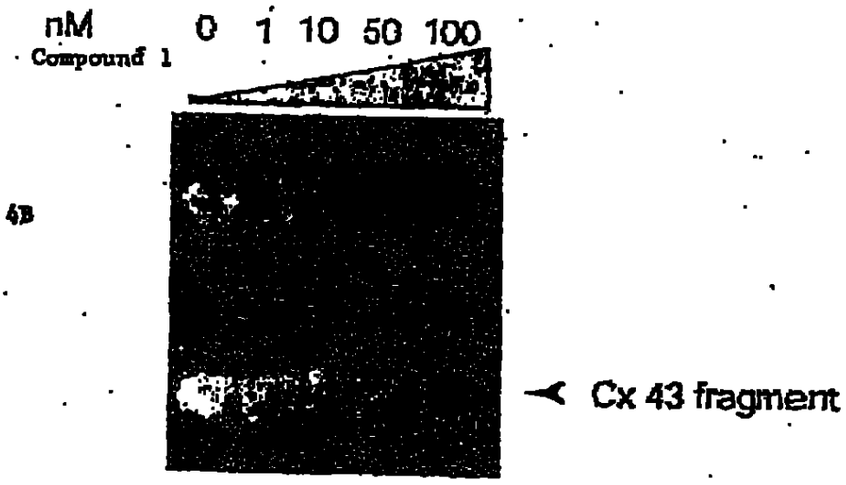


Figure 4B

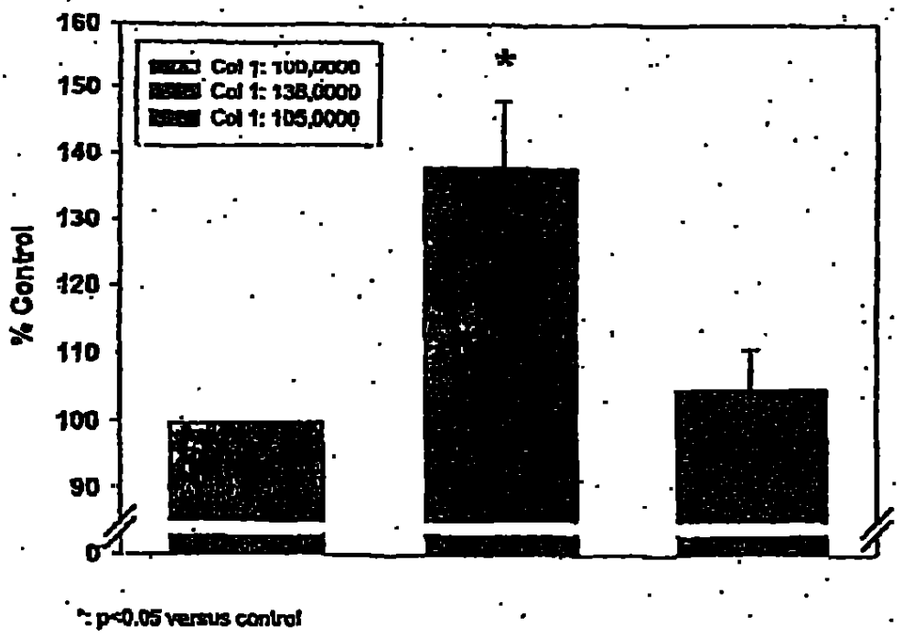

160
150
140
130
120
110
100
90
0
% Control
Col 1: 100,0000
Col 1: 138,0000
Col 1: 105,0000
*
*: p<0.05 versus control

Figure 5

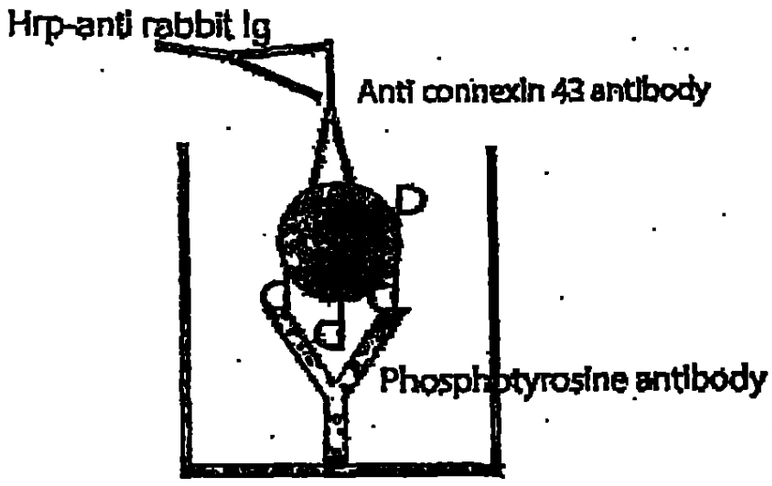

Hrp-anti rabbit Ig
Anti connexin 43 antibody
P
Phosphotyrosine antibody

Figure 6A

95

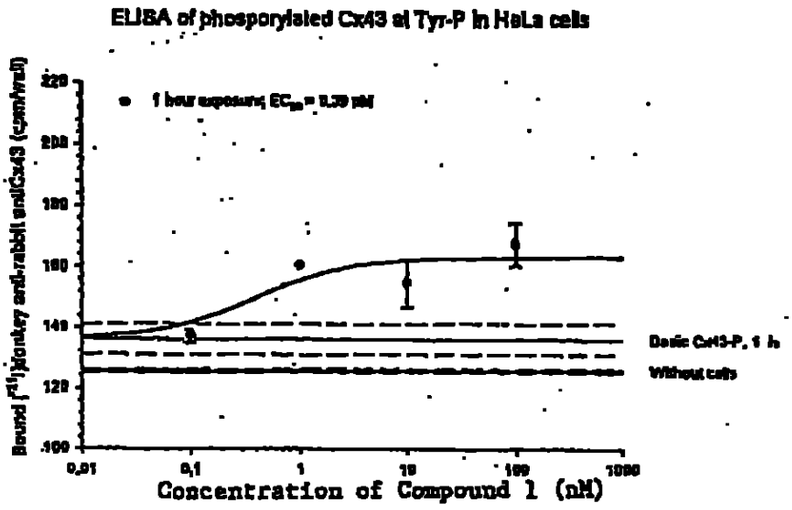


Figure 6B

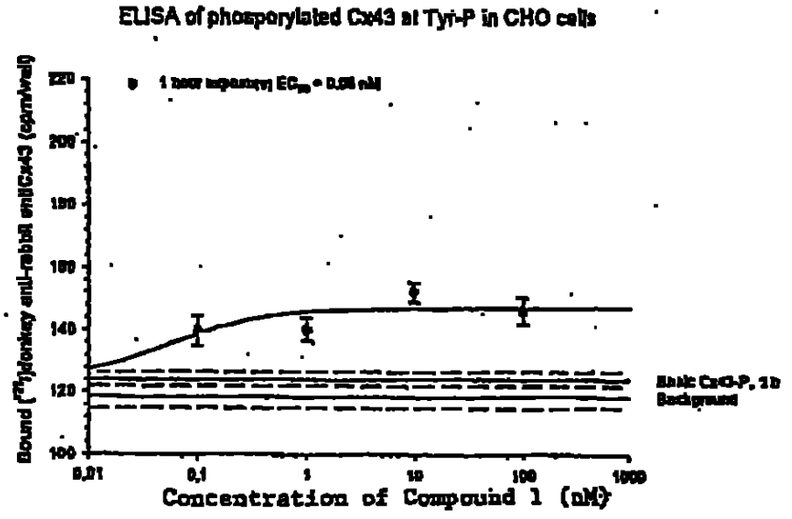


Figure 6C

96

Fig. 7A

Fig. 7B

Fig. 7C

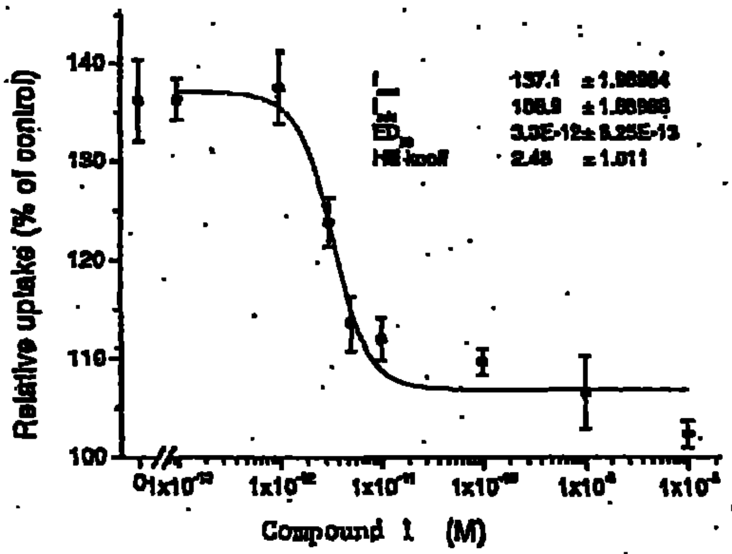


Fig. 8

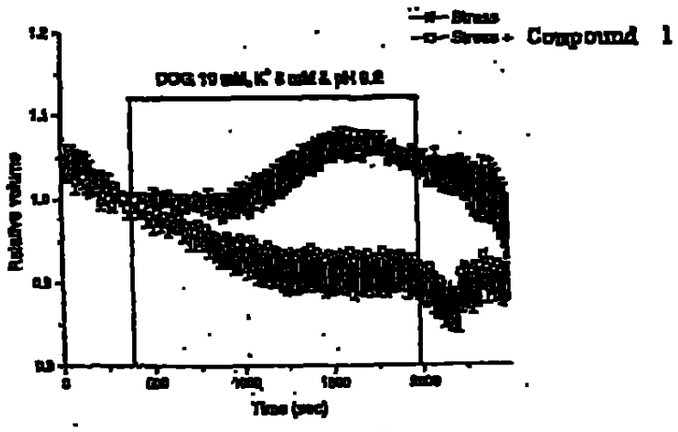

Stress
Stress + Compound 1
Relative volume
Time (sec)

Fig. 9A

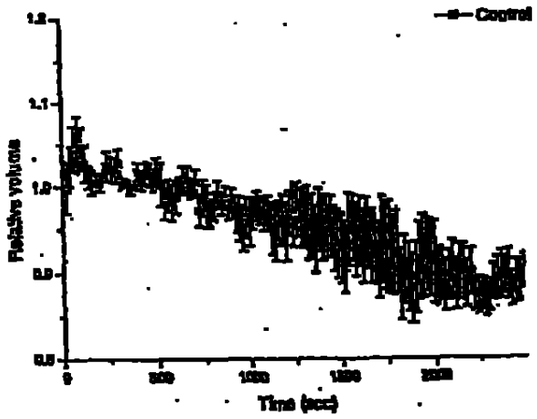

Control
Relative volume
Time (sec)

Fig. 9B

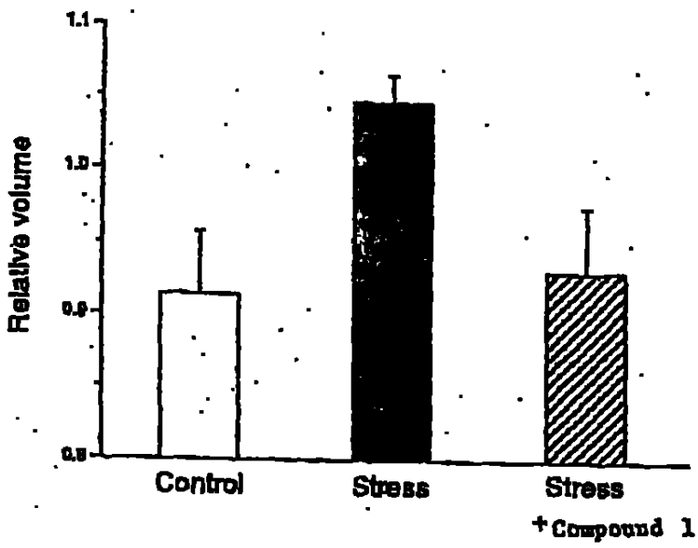

Relative volume
1.1
1.0
0.9
0.8
Control
Stress
Stress
+Compound 1

Fig. 10

100

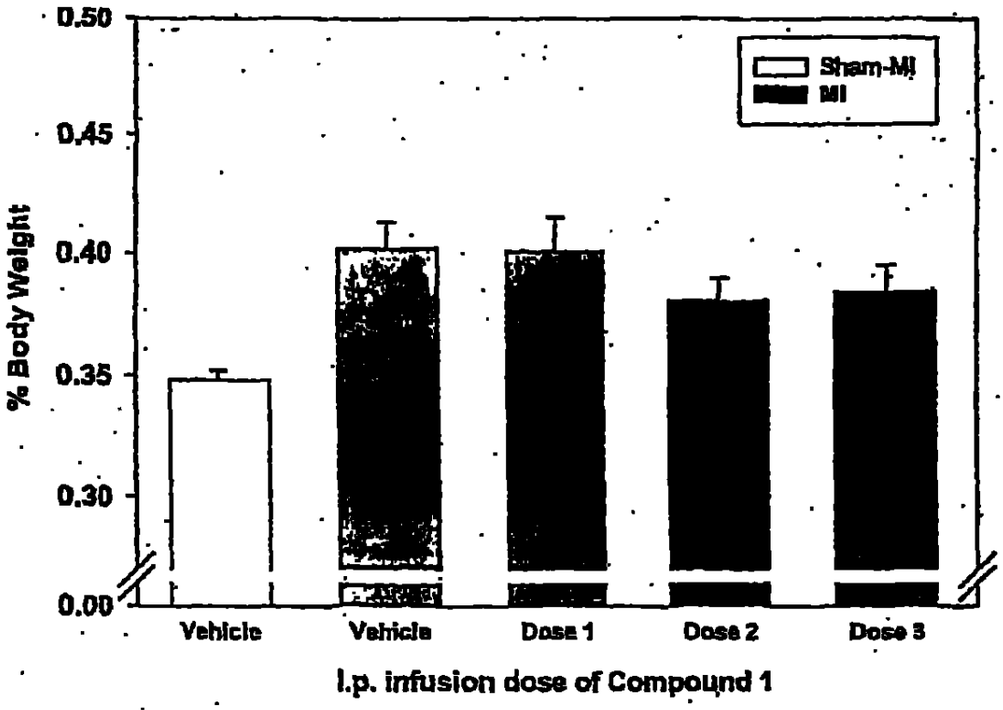
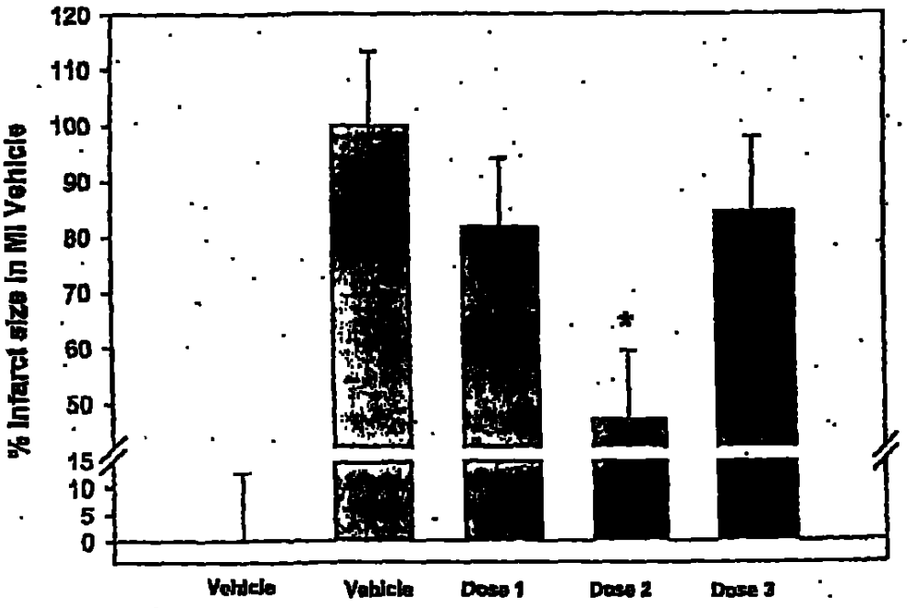

0.50
0.45
0.40
0.35
0.30
0.00
% Body Weight
Sham-MI
MI
Vehicle
Vehicle
Dose 1
Dose 2
Dose 3
I.p. infusion dose of Compound 1

Fig. 11



*: p<0.05 *versus* MI Vehicle

Fig. 12

102

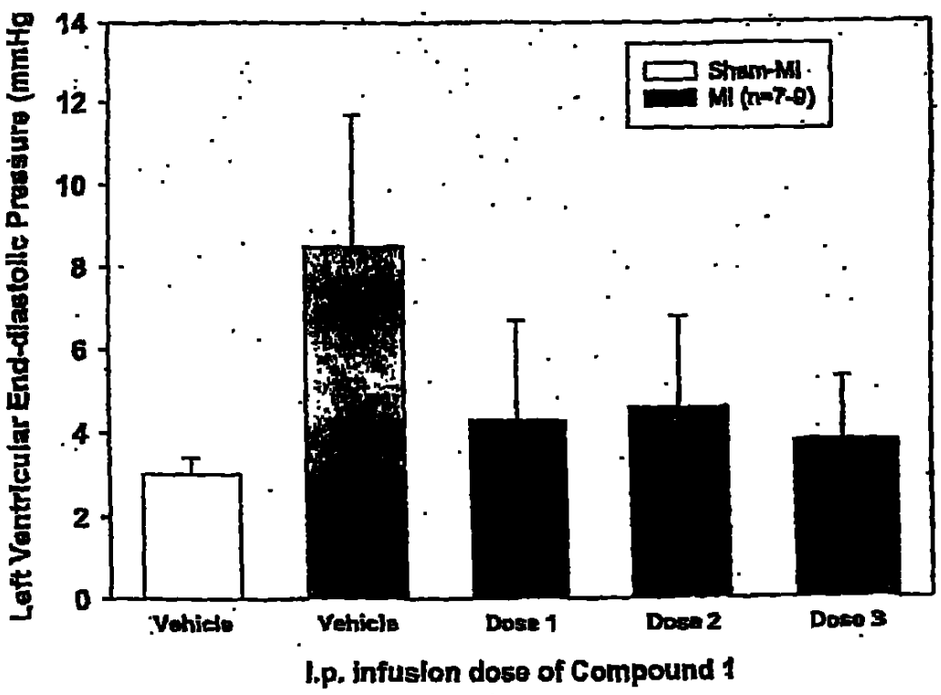

Left Ventricular End-diastolic Pressure (mmHg
14
12
10
8
6
4
2
0
Sham-MI
MI (n=7-9)
Vehicle
Vehicle
Dose 1
Dose 2
Dose 3
I.p. infusion dose of Compound 1

Fig. 13

International Application No
:T/DK 03/00056

103

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K38/08 A61P9/06

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

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 practical, search terms used)
EPO-Internal, CHEM ABS Data, WPI Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 02 077017 A (HOLSTEIN-RATHLOU NIELS-HENRIK ;KJOLBYE ANNE LOUISE (DK); LARSEN BJ) 3 October 2002 (2002-10-03) the whole document	1-46
X	WO 01 62775 A (HOLSTEIN RATHLOU NIELS HENRIK ;JOERGENSEN NIKLAS RYE (DK); LARSEN) 30 August 2001 (2001-08-30) the whole document	1-46

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

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 May 2003	26. 05. 2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	CAROLINA LAGERLÖF /ELY

Form PCT/ISA/210 (second sheet) (July 1998)

International Application No. PCT/DK 03/00056

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.1

Claims Nos.: 1-14,37-46

Claims 1-14, 37-46 relate to methods of treatment of the human or animal body by surgery or by therapy/diagnostic methods practised on the human or animal body/Rule 39.1.(iv). Nevertheless, a search has been executed for these claims. The search has been based on the alleged effects of the compounds/compositions.

PATENT COOPERATION TREATY

MODTAGET
16 OKT. 2003

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

ZEALAND PHARMA A/S
Smedeland 26B
DK-2600 Glostrup
DANEMARK

PCT

WRITTEN OPINION
(PCT Rule 66)

Date of mailing *(day/month/year)*	13.10.2003
Applicant's or agent's file reference 016-2003 WO1,MHI,LSD	REPLY DUE within 3 month(s) from the above date of mailing

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/DK03/00056	29.01.2003	29.01.2002

International Patent Classification (IPC) or both national classification and IPC
A61K38/08

Applicant
ZEALAND PHARMA A/S et al.

1. This written opinion is the first drawn up by this International Preliminary Examining Authority.

2. This opinion contains indications relating to the following items:

I	☒	Basis of the opinion
II	☐	Priority
III	☒	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
IV	☐	Lack of unity of invention
V	☒	Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
VI	☐	Certain documents cited
VII	☐	Certain defects in the international application
VIII	☐	Certain observations on the international application

3. The applicant is hereby invited to reply to this opinion.

When? See the time limit indicated above. The applicant may, before the expiration of that time limit, request this Authority to grant an extension, see Rule 66.2(d).

How? By submitting a written reply, accompanied, where appropriate, by amendments, according to Rule 66.3. For the form and the language of the amendments, see Rules 66.8 and 66.9.

Also: For an additional opportunity to submit amendments, see Rule 66.4.
For the examiner's obligation to consider amendments and/or arguments, see Rule 66.4 bis.
For an informal communication with the examiner, see Rule 66.6.

If no reply is filed, the international preliminary examination report will be established on the basis of this opinion.

4. The final date by which the international preliminary examination report must be established according to Rule 69.2 is: 29.05.2004

Name and mailing address of the international preliminary examining authority:

European Patent Office
D-80298 Munich
Tel. +49 89 2399 - 0 Tx: 523656 epmu d
Fax: +49 89 2399 - 4465

Authorized Officer
Deck, A

Formalities officer (incl. extension of time limits)
Ladurner, Y
Telephone No. +49 89 2399-7913

WRITTEN OPINION International application No. PCT/DK03/00056

I. Basis of the opinion

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this opinion as "originally filed")*

Description, Pages

1-68 as originally filed

Claims, Numbers

1-45 as originally filed

Drawings, Sheets

1/14-14/14 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
   ☐ the language of publication of the international application (under Rule 48.3(b)).
   ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   ☐ contained in the international application in written form.
   ☐ filed together with the international application in computer readable form.
   ☐ furnished subsequently to this Authority in written form.
   ☐ furnished subsequently to this Authority in computer readable form.
   ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

   ☐ the description, pages:
   ☐ the claims, Nos.:
   ☐ the drawings, sheets:

5. ☐ This opinion has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)).

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this opinion.)

6. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been and will not be examined in respect of:

   ☐ the entire international application,

   ☒ claims Nos. 1-14, 30-33, 36-45

   because:

   ☒ the said international application, or the said claims Nos. 1-14, 30-33, 36-45 relate to the following subject matter which does not require an international preliminary examination (specify):

   see separate sheet

   ☐ the description, claims or drawings *(indicate particular elements below)* or said claims Nos. are so unclear that no meaningful opinion could be formed *(specify)*:

   ☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

   ☐ no international search report has been established for the said claims Nos.

2. A written opinion cannot be drawn due to the failure of the nucleotide and/or amino acid sequence listing to comply with the Standard provided for in Annex C of the Administrative Instructions:

   ☐ the written form has not been furnished or does not comply with the Standard.

   ☐ the computer readable form has not been furnished or does not comply with the Standard.

V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	1-14, 30-33, 36-45 (NO)
Inventive step (IS)	Claims	1-45 (NO)
Industrial applicability (IA)	Claims	see separate sheet

2. Citations and explanations

   see separate sheet

Concerning section III

Claims 1-14, 30-33, 36-45 relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT).

Concerning section V

1. The following document is referred to in this communication; the numbering will be adhered to in the rest of the procedure:

   D1: WO 01 62775 A (HOLSTEIN RATHLOU NIELS HENRIK ;JOERGENSEN NIKLAS RYE (DK); LARSEN) 30 August 2001 (2001-08-30)

2. The document D1 from the applicant discloses the use of the antiarrhythmic peptides of the present application for the treatment of the same disorders, see claims 112-161.
   Hence the subject-matter of claims 1-14, 30-33, 36-45 is not novel.

3. The screening methods claimed in claims 15-29, 34, 35 are well-known methods in the art. Applying these methods to the peptides of D1 does not need particular inventive skills, hence the subject-matter of claims 15-29, 34 and 35 is not seen as being inventive.

4. For the assessment of the present claims 1-14, 30-33, 36-45 on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

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ZEALAND
PHARMA

European Patent Office
Erhardtstrasse 27
D-80331 München
Germany

Zealand Pharma A/S
Smedeland 26 B
DK-2600 Glostrup
Denmark

Tel: +45 43 28 12 00
Fax: +45 43 28 12 12

E-mail: info@zp.dk
www.zp.dk

International Patent Application No. PCT/DK03/00056
DEMAND
Our Ref: 016-2003 WO1, MHI/LSD

Date
20 August 2003

Dear Sirs,

Enclosed please find a Demand for preliminary examination in respect of the above PCT application. Please perform a detailed preliminary examination in accordance with Notice of the President of the European Patent Office dated 2 November 2001.

The handling fee and examination fee as specified in the fee calculation sheet will be debited from our account No. 28030041.

Yours sincerely
ZEALAND PHARMA

Maj Hilligsøe
GA 47210

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The demand must be filed directly with the competent International Preliminary Examining Authority or, if two or more Authorities are competent, with the one chosen by the applicant. The full name or two-letter code of that Authority may be indicated by the applicant on the line below:

IPEA/ EP

PCT

CHAPTER II

DEMAND

under Article 31 of the Patent Cooperation Treaty:
The undersigned requests that the international application specified below be the subject of international preliminary examination according to the Patent Cooperation Treaty and hereby elects all eligible States (except where otherwise indicated).

For International Preliminary Examining Authority use only	
Identification of IPEA	Date of receipt of DEMAND

Box No. I IDENTIFICATION OF THE INTERNATIONAL APPLICATION		Applicant's or agent's file reference
		016-2003 WO1, MHI/LSD
International application No.	International filing date *(day/month/year)*	(Earliest) Priority date *(day/month/year)*
PCT/DK03/00056	29 January 2003 (29/01/2003)	29 January 2002 (29/01/2002)
Title of invention		
COMPOSITIONS AND METHODS FOR MODULATING CONNEXIN HEMICHANNELS		

Box No. II APPLICANT(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*	Telephone No.
ZEALAND PHARMA A/S Smedeland 26B DK-2600 Glostrup Denmark	+45 43 28 12 00
	Facsimile No. +45 43 28 12 12
	Teleprinter No.
	Applicant's registration No. with the Office

State *(that is, country)* of nationality:	State *(that is, country)* of residence:
DK	DK

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*
PETERSEN, Jørgen Søberg
Hellebakken 1
DK-3150 Hellebæk
Denmark

State *(that is, country)* of nationality:	State *(that is, country)* of residence:
DK	DK

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*
NEVE, Søren
Ellevænget 4
DK-2800 Lyngby
Denmark

State *(that is, country)* of nationality:	State *(that is, country)* of residence:
DK	DK

[X] Further applicants are indicated on a continuation sheet.

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Sheet No. 2.

International application No.
PCT/DK03/00056

Continuation of Box No. II APPLICANT(S)

If none of the following sub-boxes is used, this sheet should not be included in the demand.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*
NIELSEN, Morten Schak
Egebjergvej 51A
DK-2750 Ballerup
Denmark

State *(that is, country)* of nationality:	State *(that is, country)* of residence:
DK	DK

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*
MEIER, Eddi
Bringebakken 88
DK-3500 Værløse
Denmark

State *(that is, country)* of nationality:	State *(that is, country)* of residence:
DK	DK

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*
STEINESS, Eva
Sømarksvej 11
DK-2900 Hellerup
Denmark

State *(that is, country)* of nationality:	State *(that is, country)* of residence:
DK	DK

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*
JENSEN, Peter Holme
Ahlefeldtsgade 18A, 1. th.
DK-1359 Copenhagen K
Denmark

State *(that is, country)* of nationality:	State *(that is, country)* of residence:
DK	DK

☒ Further applicants are indicated on another continuation sheet.

Sheet No. 3.

International application No.
PCT/DK03/00056

Continuation of Box No. II APPLICANT(S)

If none of the following sub-boxes is used, this sheet should not be included in the demand.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

LARSEN, Bjarne Due
Arildsgaard 5, 1. th.
DK-2700 Brønshøj
Denmark

State *(that is, country)* of nationality:	State *(that is, country)* of residence:
DK	DK

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

HANSEN, Lars Bo Laurenborg
Peberhaven 19, st. tv.
DK-2730 Herlev
Denmark

State *(that is, country)* of nationality:	State *(that is, country)* of residence:
DK	DK

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

State *(that is, country)* of nationality:	State *(that is, country)* of residence:

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

State *(that is, country)* of nationality:	State *(that is, country)* of residence:

☐ Further applicants are indicated on another continuation sheet.

Sheet No. 4

International application No.
PCT/DK03/00056

Box No. III AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The following person is ☐ agent ☒ common representative

and ☒ has been appointed earlier and represents the applicant(s) also for international preliminary examination.

☐ is hereby appointed and any earlier appointment of (an) agent(s)/common representative is hereby revoked.

☐ is hereby appointed, specifically for the procedure before the International Preliminary Examining Authority, in addition to the agent(s)/common representative appointed earlier.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

ZEALAND PHARMA A/S
Smedeland 26B
DK-2600 Glostrup
Denmark

Telephone No.
+45 43 28 12 00

Facsimile No.
+45 43 28 12 12

Teleprinter No.

Agent's registration No. with the Office

☐ Address for correspondence: Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Box No. IV BASIS FOR INTERNATIONAL PRELIMINARY EXAMINATION

Statement concerning amendments:*

1. The applicant wishes the international preliminary examination to start on the basis of:

☒ the international application as originally filed

- the description ☐ as originally filed
  ☐ as amended under Article 34
- the claims ☐ as originally filed
  ☐ as amended under Article 19 (together with any accompanying statement)
  ☐ as amended under Article 34
- the drawings ☐ as originally filed
  ☐ as amended under Article 34

2. ☐ The applicant wishes any amendment to the claims under Article 19 to be considered as reversed.

3. ☐ The applicant wishes the start of the international preliminary examination to be postponed until the expiration of 20 months from the priority date unless the International Preliminary Examining Authority receives a copy of any amendments made under Article 19 or a notice from the applicant that he does not wish to make such amendments (Rule 69.1(d)). *(This check-box may be marked only where the time limit under Article 19 has not yet expired.)*

* Where no check-box is marked, international preliminary examination will start on the basis of the international application as originally filed or, where a copy of amendments to the claims under Article 19 and/or amendments of the international application under Article 34 are received by the International Preliminary Examining Authority before it has begun to draw up a written opinion or the international preliminary examination report, as so amended.

Language for the purposes of international preliminary examination: English

☒ which is the language in which the international application was filed.

☐ which is the language of a translation furnished for the purposes of international search.

☐ which is the language of publication of the international application.

☐ which is the language of the translation (to be) furnished for the purposes of international preliminary examination.

Box No. V ELECTION OF STATES

The applicant hereby elects all eligible States *(that is, all States which have been designated and which are bound by Chapter II of the PCT)*

excluding the following States which the applicant wishes not to elect:

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Sheet No. .5.

International application No.
PCT/DK03/00056

Box No. VI CHECK LIST

The demand is accompanied by the following elements, in the language referred to in Box No. IV, for the purposes of international preliminary examination:			For International Preliminary Examining Authority use only: received	not received
1. translation of international application	:	sheets	☐	☐
2. amendments under Article 34	:	sheets	☐	☐
3. copy (or, where required, translation) of amendments under Article 19	:	sheets	☐	☐
4. copy (or, where required, translation) of statement under Article 19	:	sheets	☐	☐
5. letter	:	sheets	☐	☐
6. other *(specify)*	:	sheets	☐	☐

The demand is also accompanied by the item(s) marked below:

1. ☒ fee calculation sheet
2. ☐ original separate power of attorney
3. ☐ original general power of attorney
4. ☐ copy of general power of attorney; reference number, if any:
5. ☐ statement explaining lack of signature
6. ☐ sequence listings in computer readable form
7. ☐ tables in computer readable form related to sequence listings
8. ☒ other *(specify)*: Letter and Form 1037

Box No. VII SIGNATURE OF APPLICANT, AGENT OR COMMON REPRESENTATIVE

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the demand).

Maj Hilligsøe, Representative of Applicant
GA 47210

For International Preliminary Examining Authority use only

1. Date of actual receipt of DEMAND:
2. Adjusted date of receipt of demand due to CORRECTIONS under Rule 60.1(b):
3. ☐ The date of receipt of the demand is AFTER the expiration of 19 months from the priority date and item 4 or 5, below, does not apply. ☐ The applicant has been informed accordingly.
4. ☐ The date of receipt of the demand is WITHIN the period of 19 months from the priority date as extended by virtue of Rule 80.5.
5. ☐ Although the date of receipt of the demand is after the expiration of 19 months from the priority date, the delay in arrival is EXCUSED pursuant to Rule 82.

For International Bureau use only

Demand received from IPEA on:

CHAPTER II

PCT

FEE CALCULATION SHEET

Annex to the Demand

International application No. PCT/DK03/00056	For International Preliminary Examining Authority use only
Applicant's or agent's file reference 016-2003 WO1, MHI/LSD	Date stamp of the IPEA
Applicant ZEALAND PHARMA A/S	

CALCULATION OF PRESCRIBED FEES

1. Preliminary examination fee	EUR 1,530	P	
2. Handling fee *(Applicants from certain States are entitled to a reduction of 75% of the handling fee. Where the applicant is (or all applicants are) so entitled, the amount to be entered at H is 25% of the handling fee.)*	EUR 159	H	
3. Total of prescribed fees Add the amounts entered at P and H and enter total in the TOTAL box	EUR 1,689	TOTAL	

MODE OF PAYMENT

[X] authorization to charge deposit account with the IPEA (see below)	[ ] cash
[ ] cheque	[ ] revenue stamps
[ ] postal money order	[ ] coupons
[ ] bank draft	[ ] other *(specify)*:

AUTHORIZATION TO CHARGE (OR CREDIT) DEPOSIT ACCOUNT

(This mode of payment may not be available at all IPEAs)

IPEA/ EP

[X] Authorization to charge the total fees indicated above.

[X] *(This check-box may be marked only if the conditions for deposit accounts of the IPEA so permit)* Authorization to charge any deficiency or credit any overpayment in the total fees indicated above.

Deposit Account No.: 28030041

Date: 20 August 2003

Name: Maj Hilligsøe

Signature:

See Notes to the fee calculation sheet

1/6

PCT REQUEST

016-2003 WO

Original (for SUBMISSION) - printed on 29.01.2003 09:05:17 PM

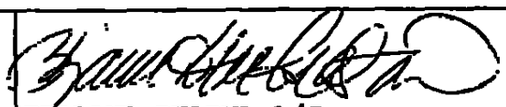
0	For receiving Office use only	
0-1	International Application No.	
0-2	International Filing Date	
0-3	Name of receiving Office and "PCT International Application"	

0-4	Form - PCT/RO/101 PCT Request	
0-4-1	Prepared using	PCT-EASY Version 2.92 (updated 01.01.2003)
0-5	Petition The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty	
0-6	Receiving Office (specified by the applicant)	Danish Patent and Trademark Office (RO/DK)
0-7	Applicant's or agent's file reference	016-2003 WO
I	Title of invention	COMPOSITIONS AND METHODS FOR MODULATING CONNEXIN HEMICHANNELS
II	Applicant	
II-1	This person is:	applicant only
II-2	Applicant for	all designated States except US
II-4	Name	ZEALAND PHARMA A/S
II-5	Address:	Smedeland 26B DK-2600 Glostrup Denmark
II-6	State of nationality	DK
II-7	State of residence	DK
II-8	Telephone No.	+45 43 28 12 00
II-9	Facsimile No.	+45 43 28 12 12
II-10	e-mail	lsd@zp.dk
II-11	Applicant's registration No. with the Office	111111
III-1	Applicant and/or inventor	
III-1-1	This person is:	applicant and inventor
III-1-2	Applicant for	US only
III-1-4	Name (LAST, First)	PETERSEN, Jørgen, Søberg /
III-1-5	Address:	Hellebakken 1 DK-3150 Hellebæk Denmark
III-1-6	State of nationality	DK
III-1-7	State of residence	DK

III-2	Applicant and/or Inventor	
III-2-1	This person is:	applicant and inventor
III-2-2	Applicant for	US only
III-2-4	Name (LAST, First)	NEVE, Søren
III-2-5	Address:	Ellevænget 4 DK-2800 Lyngby Denmark
III-2-6	State of nationality	DK
III-2-7	State of residence	DK
III-3	Applicant and/or Inventor	
III-3-1	This person is:	applicant and inventor
III-3-2	Applicant for	US only
III-3-4	Name (LAST, First)	NIELSEN, Morten, Schak
III-3-5	Address:	Egebjergvej 51A DK-2750 Ballerup Denmark
III-3-6	State of nationality	DK
III-3-7	State of residence	DK
III-4	Applicant and/or Inventor	
III-4-1	This person is:	applicant and inventor
III-4-2	Applicant for	US only
III-4-4	Name (LAST, First)	MEIER, Eddi
III-4-5	Address:	Bringebakken 88 DK-3500 Værløse Denmark
III-4-6	State of nationality	DK
III-4-7	State of residence	DK
III-5	Applicant and/or Inventor	
III-5-1	This person is:	applicant and inventor
III-5-2	Applicant for	US only
III-5-4	Name (LAST, First)	STEINESS, Eva
III-5-5	Address:	Sømarksvej 11 DK-2900 Hellerup Denmark
III-5-6	State of nationality	DK
III-5-7	State of residence	DK
III-6	Applicant and/or Inventor	
III-6-1	This person is:	applicant and inventor
III-6-2	Applicant for	US only
III-6-4	Name (LAST, First)	JENSEN, Peter, Holme
III-6-5	Address:	Ahlefeldtsgade 18A, 1.th DK-1359 Copenhagen K Denmark
III-6-6	State of nationality	DK
III-6-7	State of residence	DK

III-7	Applicant and/or inventor	
III-7-1	This person is:	applicant and inventor
III-7-2	Applicant for	US only
III-7-4	Name (LAST, First)	LARSEN, Bjarne, Due
III-7-5	Address:	Arildsgaard 5, 1. th. DK-2700 Brønshøj Denmark
III-7-6	State of nationality	DK
III-7-7	State of residence	DK
III-8	Applicant and/or inventor	
III-8-1	This person is:	applicant and inventor
III-8-2	Applicant for	US only
III-8-4	Name (LAST, First)	HANSEN, Lars, Bo, Laurenborg
III-8-5	Address:	Peberhaven 19, st tv DK-2730 Herlev Denmark
III-8-6	State of nationality	DK
III-8-7	State of residence	DK
V	Designation of States	
V-1	Regional Patent (other kinds of protection or treatment, if any, are specified between parentheses after the designation(s) concerned)	AP: GH GM KE LS MW MZ SD SL SZ TZ UG ZM ZW and any other State which is a Contracting State of the Harare Protocol and of the PCT EA: AM AZ BY KG KZ MD RU TJ TM and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT EP: AT BE BG CH&LI CY CZ DE DK EE ES FI FR GB GR HU IE IT LU MC NL PT SE SI SK TR and any other State which is a Contracting State of the European Patent Convention and of the PCT OA: BF BJ CF CG CI CM GA GN GQ GW ML MR NE SN TD TG and any other State which is a member State of OAPI and a Contracting State of the PCT
V-2	National Patent (other kinds of protection or treatment, if any, are specified between parentheses after the designation(s) concerned)	AE AG AL AM AT AU AZ BA BB BG BR BY BZ CA CH&LI CN CO CR CU CZ DE DK DM DZ EC EE ES FI GB GD GE GH GM HR HU ID IL IN IS JP KE KG KP KR KZ LC LK LR LS LT LU LV MA MD MG MK MN MW MX MZ NO NZ OM PH PL PT RO RU SC SD SE SG SK SL TJ TM TN TR TT TZ UA UG US UZ VC VN YU ZA ZM ZW

V-5	Precautionary Designation Statement In addition to the designations made under items V-1, V-2 and V-3, the applicant also makes under Rule 4.9(b) all designations which would be permitted under the PCT except any designation(s) of the State(s) indicated under item V-6 below. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit.		
V-6	Exclusion(s) from precautionary designations	NONE	
VI-1	Priority claim of earlier national application		
VI-1-1	Filing date	29 January 2002 (29.01.2002)	
VI-1-2	Number	60/352,717	
VI-1-3	Country	US	
VII-1	International Searching Authority Chosen	European Patent Office (EPO) (ISA/EP)	
VIII	Declarations	Number of declarations	
VIII-1	Declaration as to the identity of the inventor	-	
VIII-2	Declaration as to the applicant's entitlement, as at the international filing date, to apply for and be granted a patent	-	
VIII-3	Declaration as to the applicant's entitlement, as at the international filing date, to claim the priority of the earlier application	-	
VIII-4	Declaration of inventorship (only for the purposes of the designation of the United States of America)	-	
VIII-5	Declaration as to non-prejudicial disclosures or exceptions to lack of novelty	-	
IX	Check list	number of sheets	electronic file(s) attached
IX-1	Request (including declaration sheets)	5	-
IX-2	Description	68	-
IX-3	Claims	7	-
IX-4	Abstract	1	EZABST00.TXT
IX-5	Drawings	14	-
IX-7	TOTAL	95	
	Accompanying Items	paper document(s) attached	electronic file(s) attached
IX-8	Fee calculation sheet	✓	-
IX-17	PCT-EASY diskette	-	Diskette
IX-19	Figure of the drawings which should accompany the abstract		
IX-20	Language of filing of the international application	English	

X-1	Signature of applicant, agent or common representative	
X-1-1	Name	ZEALAND PHARMA A/S
X-1-2	Name of signatory	Bjarne Due Larsen
X-1-3	Capacity	Representative of Applicants

FOR RECEIVING OFFICE USE ONLY

10-1	Date of actual receipt of the purported international application	
10-2	Drawings:	
10-2-1	Received	
10-2-2	Not received	
10-3	Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application	
10-4	Date of timely receipt of the required corrections under PCT Article 11(2)	
10-5	International Searching Authority	ISA/EP
10-6	Transmittal of search copy delayed until search fee is paid	

FOR INTERNATIONAL BUREAU USE ONLY

11-1	Date of receipt of the record copy by the International Bureau	

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PCT (ANNEX - FEE CALCULATION SHEET)

016-2003 WO

Original (for SUBMISSION) - printed on 29.01.2003 09:05:17 PM

(This sheet is not part of and does not count as a sheet of the international application)

0	For receiving Office use only				
0-1	International Application No.				
0-2	Date stamp of the receiving Office				
0-4	Form - PCT/RO/101 (Annex) PCT Fee Calculation Sheet				
0-4-1	Prepared using	PCT-EASY Version 2.92 (updated 01.01.2003)			
0-9	Applicant's or agent's file reference	016-2003 WO			
2	Applicant	ZEALAND PHARMA A/S, et al.			
12	Calculation of prescribed fees		fee amount/multiplier	Total amounts (DKK)	
12-1	Transmittal fee	T	⇨	1,500	
12-2-1	Search fee	S	⇨	7,030	
12-2-2	International search to be carried out by		EP		
12-3	International fee Basic fee (first 30 sheets)	b1	3,300		
12-4	Remaining sheets		65		
12-5	Additional amount	(X)	80		
12-6	Total additional amount	b2	5,200		
12-7	b1 + b2 =	B	8,500		
12-8	Designation fees Number of designations contained in international application		94		
12-9	Number of designation fees payable (maximum 5)		5		
12-10	Amount of designation fee	(X)	710		
12-11	Total designation fees	D	3,550		
12-12	PCT-EASY fee reduction	R	-1,020		
12-13	Total international fee (B+D-R)	I	⇨	11,030	
12-17	TOTAL FEES PAYABLE (T+S+I+P)		⇨	19,560	
12-19	Mode of payment		cheque		

VALIDATION LOG AND REMARKS

13-2-7	Validation messages Contents	Green? Figure of the drawings which should accompany the abstract not specified. Please verify.
		Green? Priority 1. The priority document is not enclosed. (The applicant must furnish it within 16 months from the earliest priority date claimed)

PCT

016-2003 WO

Original (for SUBMISSION) - printed on 29.01.2003 09:05:17 PM

PCT-EASY INFORMATION SHEET

(For applicant use only, DO NOT submit this sheet with the international application)

VALIDATION LOG

	Contents
Green?	Figure of the drawings which should accompany the abstract not specified. Please verify.
Green?	Priority 1. The priority document is not enclosed. (The applicant must furnish it within 16 months from the earliest priority date claimed)

Before submitting the International Application, please carefully verify that:

-the information contained on printed Request form is correct;
-Box X of the Request form has been signed;
-all elements of the international application as indicated in Boxes VIII and IX of the Request form have been attached; and,
-the diskette containing the PCT-EASY zip file of the International Application has been enclosed and has been clearly labeled "PCT-EASY", with the applicant's or agent's file reference, and the first applicant's name.

ATTENTION

DO NOT modify any indications on the Request form printout. The electronic version of the PCT-EASY application has been locked. If an error or an omission is discovered at this time, you must reopen the stored form for submission, perform necessary amendments and immediately resubmit the form. Finally, a NEW submission diskette must be created manually by resending the corrected stored form to the diskette. The previously created printout and submission diskette must be destroyed in order to prevent the possibility of erroneously sending it to the RO.

Disclosed are compositions and methods for modulating hemichannel function in a cell, tissue or organ. The invention also relates to useful screens for detecting such compounds, particularly those capable of modulating connexin phosphorylation. Further provided are therapeutic methods for preventing or treating conditions impacted by undesired hemichannel function in a mammal such as heart arrhythmia.

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MODTAGET
30 MAJ 2003

PATENT COOPERATION TREAT

From the INTERNATIONAL SEARCHING AUTHORITY

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

To:
ZEALAND PHARMA
Smedeland 26 B
DK-2600 Glostrup
DENMARK

→ HU, BG, SK, CZ

Applicant's or agent's file reference 016-2003 WO	Date of mailing (day/month/year) 26/05/2003 FOR FURTHER ACTION See paragraphs 1 and 4 below
International application No. PCT/DK 03/00056	International filing date (day/month/year) 29/01/2003
Applicant ZEALAND PHARMA A/S	

1. [X] The applicant is hereby notified that the International Search Report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:
The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the International Search Report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Facsimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. [ ] The applicant is hereby notified that no International Search Report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. [ ] With regard to the protest against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

[ ] the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

[ ] no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. Further action(s): The applicant is reminded of the following:

Shortly after 18 months from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90*bis*.1 and 90*bis*.3, respectively, before the completion of the technical preparations for international publication.

Within 19 months from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within 20 months from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the International Searching Authority	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Sylvia Hermier

Form PCT/ISA/220 (July 1998)

NOTES TO FORM PCT/ISA/220

These Notes are intended to give the basic instructions concerning the filing of amendments under article 19. The Notes are based on the requirements of the Patent Cooperation Treaty, the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the PCT Applicant's Guide, a publication of WIPO.

In these Notes, "Article", "Rule", and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions, respectively.

INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purposes of provisional protection or has another reason for amending the claims before international publication. Furthermore, it should be emphasized that provisional protection is available in some States only.

What parts of the international application may be amended?

Under Article 19, only the claims may be amended.

During the international phase, the claims may also be amended (or further amended) under Article 34 before the International Preliminary Examining Authority. The description and drawings may only be amended under Article 34 before the International Examining Authority.

Upon entry into the national phase, all parts of the international application may be amended under Article 28 or, where applicable, Article 41.

When?

Within 2 months from the date of transmittal of the international search report or 16 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

Where not to file the amendments?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been/is filed, see below.

How?

Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Administrative Instructions, Section 205(b)).

The amendments must be made in the language in which the international application is to be published.

What documents must/may accompany the amendments?

Letter (Section 205(b)):

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confused with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

The letter must be in English or French, at the choice of the applicant. However, if the language of the international application is English, the letter must be in English; if the language of the international application is French, the letter must be in French.

NOTES TO FORM PCT/ISA/220 (continue..,

The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped),whether

(i) the claim is unchanged;

(ii) the claim is cancelled;

(iii) the claim is new;

(iv) the claim replaces one or more claims as filed;

(v) the claim is the result of the division of a claim as filed.

The following examples illustrate the manner in which amendments must be explained in the accompanying letter:

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers; claims 30, 33 and 36 unchanged; new claims 49 to 51 added."

2. [Where originally there were 15 claims and after amendment of all claims there are 11]:
"Claims 1 to 15 replaced by amended claims 1 to 11."

3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."

4. [Where various kinds of amendments are made]:
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

"Statement under article 19(1)" (Rule 46.4)

The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

It must be in the language in which the international application is to be published.

It must be brief, not exceeding 500 words if in English or if translated into English.

It should not be confused with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)."

It may not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

Consequence if a demand for international preliminary examination has already been filed

If, at the time of filing any amendments and any accompanying statement, under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the time of filing the amendments (and any statement) with the International Bureau, also file with the International Preliminary Examining Authority a copy of such amendments (and of any statement) and, where required, a translation of such amendments for the procedure before that Authority (see Rules 55.3(a) and 62.2, first sentence). For further information, see the Notes to the demand form (PCT/IPEA/401).

Consequence with regard to translation of the international application for entry into the national phase

The applicant's attention is drawn to the fact that, upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Offices, instead of, or in addition to, the translation of the claims as filed.

For further details on the requirements of each designated/elected Office, see Volume II of the PCT Applicant's Guide.

PATENT COOPERATION TREATY

PCT

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INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
016-2003 WO		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/DK 03/00056	29/01/2003	29/01/2002
Applicant		
ZEALAND PHARMA A/S		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 5 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the language, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [X] Certain claims were found unsearchable (See Box I).

3. [ ] Unity of invention is lacking (see Box II).

4. With regard to the title,

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the abstract,

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the drawings to be published with the abstract is Figure No. _____

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [X] None of the figures.

INTERNATIONAL SEARCH REPORT

ernational application No.
PCT/DK 03/00056

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 1-14,37-46
because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

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

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

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

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

International Application No. PCT/DK 03/00056

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.1

Claims Nos.: 1-14,37-46

Claims 1-14, 37-46 relate to methods of treatment of the human or animal body by surgery or by therapy/diagnostic methods practised on the human or animal body/Rule 39.1.(iv). Nevertheless, a search has been executed for these claims. The search has been based on the alleged effects of the compounds/compositions.

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

PCT

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To:

ZEALAND PHARMA A/S
Smedeland 26B
DK-2600 Glostrup
DANEMARK

NOTIFICATION OF RECEIPT OF DEMAND BY COMPETENT INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

(PCT Rules 59.3(e) and 61.1(b), first sentence and Administrative Instructions, Section 601(a))

Date of mailing *(day/month/year)*	05-09-2003
Applicant's or agent's file reference: 016-2003 WO1,MHI/LSD	IMPORTANT NOTIFICATION

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/DK 03/00056	29/01/2003	29/01/2002

Applicant: ZEALAND PHARMA A/S et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority considers the following date as the date of receipt of the demand for international preliminary examination of the international application:

25/08/2003

2. This date of receipt is:

- [X] the actual date of receipt of the demand by this Authority (Rule 61.1(b)).
- [ ] the actual date of receipt of the demand on behalf of this Authority (Rule 59.3(e)).
- [ ] the date on which this Authority has, in response to the invitation to correct defects in the demand (Form PCT/IPEA/404), received the required corrections.

3. [ ] ATTENTION: That date of receipt is AFTER the expiration of 19 months from the priority date. Consequently, the election(s) made in the demand does (do) not have the effect of postponing the entry into the national phase until 30 months from the priority date (or later in some Offices) (Article 39(1)). Therefore, the acts for entry into the national phase must be performed within 20 months from the priority date (or later in some Offices) (Article 22). For details, see the *PCT Applicant's Guide*, Volume II.

[ ] *(If applicable)* This notification confirms the information given by telephone, facsimile transmission or in person on:

4. Only where paragraph 3 applies, a copy of this notification has been sent to the International Bureau.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. (+49-89) 2399-0, Tx: 523656 epmu d Fax: (+49-89) 2399-4465	FERRO VASCONCELOS M Tel. ext: 7905

Form PCT/IPEA/402 (July 1998) P20452 (02/09/2003)

MODTAGET
08 SEP. 2003

PATENT COOPERATION TREAT

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

PCT

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To:

ZEALAND PHARMA A/S
Smedeland 26B
DK-2600 Glostrup
DANEMARK

COMMUNICATION IN CASES FOR WHICH NO OTHER FORM IS APPLICABLE

	Date of mailing (day/month/year) 05. 09. 03
Applicant's or agent's file reference 016-2003 WO1,MHI/LSD	REPLY DUE See paragraph 1 below
International application No. PCT/DK 03/00056	International filing date (day/month/year) 29/01/2003
Applicant ZEALAND PHARMA A/S et al.	

1. ☒ REPLY DUE within 1 month/days from the above date of mailing
   ☐ NO REPLY DUE

2. COMMUNICATION:

Dear Madam or Sir,

The address of "Jensen, Peter Holme" indicated on the demand differs from the one indicated on WIPO data. (Please see attachment - IB306)

You are invited to correct the defect within the time limit indicated above.
Yours very truly

Name and mailing address of the IPEA/ European Patent Office D-80298 Munich Tel. (+49-89) 2399-0, Tx: 523656 epmu d Fax: (+49-89) 2399-4465	Authorized officer Monica Ferro ext. 7905

Form PCT/IPEA/424 (January 1994) (02/09/2003)

PATENT COOPERATION TREATY

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From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

ZEALAND PHARMA A/S
Smedeland 26B
DK-2600 Glostrup
Denmark

Date of mailing (day/month/year)
23 May 2003 (23.05.03)

Applicant's or agent's file reference
016-2003 WO

IMPORTANT NOTIFICATION

International application No.
PCT/DK03/00056

International filing date (day/month/year)
29 January 2003 (29.01.03)

1. The following indications appeared on record concerning:

[X] the applicant [X] the inventor [ ] the agent [ ] the common representative

Name and Address
JENSEN, Peter, Holme
Ahlefeldtsgade 18A, 1.th
DK-1359 Copenhagen K
Denmark

State of Nationality: DK
State of Residence: DK
Telephone No.:
Facsimile No.:
Teleprinter No.:

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[ ] the person [ ] the name [X] the address [ ] the nationality [ ] the residence

EPO - DG1
16. 05. 2003
104

Name and Address
JENSEN, Peter, Holme
Classensgade 11, 3.tv.
DK-2100 Copenhagen ø
Denmark

State of Nationality: DK
State of Residence: DK
Telephone No.:
Facsimile No.:
Teleprinter No.:

3. Further observations, if necessary:

4. A copy of this notification has been sent to:

[X] the receiving Office
[X] the International Searching Authority
[ ] the International Preliminary Examining Authority
[X] the designated Offices concerned
[ ] the elected Offices concerned
[ ] other:

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Facsimile No. (41-22) 338-7690

Authorized officer
Marek LASKOWSKI
Telephone No. (41-22) 338 8162

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Europäisches Patentamt | European Patent Office | Office européen des brevets

D-80298 München
(+49-89) 2399-0
Tx 523 656 epmu d
Fax (+49-89) 2399-4465

P.B. 5818 Patentlaan 2
NL-2280 HV Rijswijk
(+31-70) 340 2576
Tx 31 651 epo nl
Fax (+31-70) 340 3075

D-10958 Berlin
(+49-30) 25901-0
Fax (+49-30) 25901-840

Zealand Pharma A/S
Smedeland 26B
DK- DK-2600 Glostrup
Denmark

EPO - Munich
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25 AUG 2003

MODTAGET
28 AUG. 2003

Acknowledgement of receipt at the European Patent Office of subsequently filed items relating to patent applications / patents.

Date and place of receipt are shown by the perforation appearing on this receipt (M + date = Munich as place of receipt; date alone = The Hague as place of receipt; date + B = Berlin as place of receipt)

Items filed

No.	Application (and Directorate) No. / Patent No.	Your reference	Nature and date of items (optional)	
1	PCT/DK03/00056	016-2003 WO1	Demand Letter	20-08-2003

PATENT COOPERATION TREAT

PCT/DK03/00056

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Corrected V rsi n / Versi n Corrigée

From the INTERNATIONAL BUREAU

PCT

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

To:

ZEALAND PHARMA A/S
Smedeland 26B
DK-2600 Glostrup
DANEMARK

MODTAGET
08 OKT. 2003

Date of mailing (day/month/year)	
07 August 2003 (07.08.03)	
Applicant's or agent's file reference	IMPORTANT NOTICE
016-2003 WO	

International application No.	International filing date (day/month/year)	Priority date (day/month/year)
PCT/DK03/00056	29 January 2003 (29.01.03)	29 January 2002 (29.01.02)

Applicant: ZEALAND PHARMA A/S

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this notice:

   AU, AZ, BY, CH, CO, DE, DZ, GH, HU, KG, KP, KR, MD, MK, MZ, RU, TM, US

   In accordance with Rule 47.1(c), third sentence, those Offices will accept the present notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:

   AE, AG, AL, AM, AP, AT, BA, BB, BG, BR, BZ, CA, CN, CR, CU, CZ, DK, DM, EA, EC, EE, EP, ES, FI, GB, GD, GE, GM, HR, ID, IL, IN, IS, JP, KE, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MG, MN, MW, MX, NO, NZ, OA, OM, PH, PL, PT, RO, SC, SD, SE, SG, SK, SL, TJ, TN, TR, TT, TZ, UA, UG, UZ, VC, VN, YU, ZA, ZM, ZW

   The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49.1(a-*bis*)).

3. Enclosed with this notice is a copy of the international application as published by the International Bureau on 07 August 2003 (07.08.03) under No. WO 03/063891

4. **TIME LIMITS for filing a demand for international preliminary examination and for entry into the national phase**

   The applicable time limit for entering the national phase will, subject to what is said in the following paragraph, be 30 MONTHS from the priority date, not only in respect of any elected Office if a demand for international preliminary examination is filed before the expiration of 19 months from the priority date, but also in respect of any designated Office, in the absence of filing of such demand, where Article 22(1) as modified with effect from 1 April 2002 applies in respect of that designated Office. For further details, see *PCT Gazette* No. 44/2001 of 1 November 2001, pages 19926, 19932 and 19934, as well as the *PCT Newsletter*, October and November 2001 and February 2002 issues.

   In practice, time limits other than the 30-month time limit will continue to apply, for various periods of time, in respect of certain designated or elected Offices. For regular updates on the applicable time limits (20, 21, 30 or 31 months, or other time limit), Office by Office, refer to the *PCT Gazette*, the *PCT Newsletter* and the *PCT Applicant's Guide*, Volume II, National Chapters, all available from WIPO's Internet site, at http://www.wipo.int/pct/en/index.html.

   For filing a demand for international preliminary examination, see the *PCT Applicant's Guide*, Volume I/A, Chapter IX. Only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination (at present, all PCT Contracting States are bound by Chapter II).

   It is the applicant's sole responsibility to monitor all these time limits.

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Authorized officer: Judith Zahra

Facsimile No. (41-22) 740.14.35

Telephone No. (41-22) 338.91.11

Moscow
Dr. Mikhail Gorodissky (1927-2002)
Dip. Eng. Valery Medvedev, PA, EPA, TMA, DA
Dr. Econ. Anatoly Pavlovsky, PA, EPA
Dip. Eng. Sergey Dudushkin, PA, EPA, TMA, DA
Dip. Econ. Anatoly Shelkhov, TMA
Dip. Eng. Natalia Lebedeva, PA, EPA
Dip. Eng. Elena Tomskaya, PA, EPA
Dip. Law. Vladimir Biriulin, L, PA
Dip. Eng. Yury Kuznetsov, PA, EPA, TMA, L
Dip. Ling. Galina Egorova, PA
Dip. Ling. Ludmila Kirtushina, PA, EPA, TMA, DA
Dip. Eng. Alexander Vasiliev, DA, PA, TMA
Dip. Chem. Elena Nazina, PA, EPA
Dip. Ling. Irina Korzun, TMA
Dip. Eng. Sergey Dorofeev, PA, EPA, TMA
Dip. Eng. Evgeny Ermilenov, PA, EPA
Dip. Eng. Vladimir Meschersky, PA
Dip. Eng. Alexander Mitta, PA

Dip. Eng. Valery Kalinovsky, TMA, PA*
Dip. Law. Igor Reikovsky, L, PA, TMA*

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Dip. Eng. Natalia Potenina, PA
Dip. Eng. Elena Chuporina, DA
Dip. Law. Maria Nosova, L

N. Novgorod
Dip. Eng. Irina Shishko
Dip. Chem. Ludmila Pozina

Krasnodar
Dip. Eng. Tatiana Titova
Dip. Law. Vadim Bloshentsev, L

Samara
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Dip. Law. Nadezhda Zagumennikova, L

Ekaterinburg
Dip. Eng. Sergey Egorov
Dip. Eng. Nina Andreeva, PA
Dip. Eng. Ekaterina Glebova

Kiev (Ukraine)
Dip. Eng. Nina Moshinskaya, L, PA, TMA, DA
Dip. Ling. Sergey Novikov, L, PA, TMA, DA
Dip. Ling. Yuliya Grabovska, L, TMA, DA, PA
Dip. Eng. Natalia Breus, L, PA, DA, TMA

PA - Patent Attorney
EPA - Eurasian Patent Attorney
TMA - Trademark Attorney
DA - Design Attorney
L - Lawyer
** - Consultant*

ZEALAND PHARMA A/S

MODTAGET 29 SEP. 2003

Date 26 August 2003
Our reference 2421-2608P55

Dear Sirs

Re: PCT/DK03/00056

The above captioned application has been published in the PCT Gazette. If you plan to obtain patent protection in Russia and/or the ex-USSR republics through national patent(s) or Eurasian Patent, we would be very glad if we could assist you in this matter.

We are the premier Patent Attorneys firm in Russia with more than 40 years of experience in IP matters. Our rating as the No 1 firm in patents and trademarks in Russia has been confirmed by international surveys conducted by the "Managing Intellectual Property" magazine in 1998, 1999, 2000, 2001 and 2002.

To get more information about us, please visit our web site: **www.gorodissky.com.**

We can effectively assist you in all the ex-USSR republics through our reliable network of associated patent attorneys in these republics, including our office in Ukraine.

Upon request you can get a more comprehensive disclosure of our firm's activities, as well as other documents, including the schedule of fees, Power of Attorney form etc.

Yours sincerely,

Val Medvedev

Valery Medvedev
Managing Partner

Address:
Law firm "Gorodissky & Partners" Ltd.
B. Spasskaya str., 25, strenie 3

Telephone:
+7 (095) 937 6116 / 6109

Fax:
+7 (095) 937 6104 / 6123

Internet:
E-mail: pat@gorodissky.ru
http://www.gorodissky.com

PATENT COOPERATION TREATY

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From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

MODTAGET
20 JUNI 2003

ZEALAND PHARMA A/S
Smedeland 26B
DK-2600 Glostrup
Denmark

Date of mailing (day/month/year)	
23 May 2003 (23.05.03)	
Applicant's or agent's file reference	**IMPORTANT NOTIFICATION**
016-2003 WO	
International application No.	**International filing date (day/month/year)**
PCT/DK03/00056	29 January 2003 (29.01.03)

1. The following indications appeared on record concerning:

[X] the applicant [X] the inventor [ ] the agent [ ] the common representative

Name and Address	State of Nationality	State of Residence
JENSEN, Peter, Holme Ahlefeldtsgade 18A, 1.th DK-1359 Copenhagen K Denmark	DK	DK
	Telephone No.	
	Facsimile No.	
	Teleprinter No.	

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[ ] the person [ ] the name [X] the address [ ] the nationality [ ] the residence

Name and Address	State of Nationality	State of Residence
JENSEN, Peter, Holme Classensgade 11, 3.tv. DK-2100 Copenhagen ø Denmark	DK	DK
	Telephone No.	
	Facsimile No.	
	Teleprinter No.	

3. Further observations, if necessary:

4. A copy of this notification has been sent to:

[X] the receiving Office
[X] the International Searching Authority
[ ] the International Preliminary Examining Authority
[X] the designated Offices concerned
[ ] the elected Offices concerned
[ ] other:

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland	Authorized officer Marek LASKOWSKI
Facsimile No. (41-22) 740-1435	Telephone No. (41-22) 338 8152

PATENT COOPERATION TREAT

From the INTERNATIONAL BUREAU

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PCT

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

To:

MODTAGET

18 AUG. 2003

ZEALAND PHARMA A/S
Smedeland 26B
DK-2600 Glostrup
DANEMARK

Date of mailing *(day/month/year)*	
07 August 2003 (07.08.03)	
Applicant's or agent's file reference	IMPORTANT NOTICE
016-2003 WO	

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/DK03/00056	29 January 2003 (29.01.03)	29 January 2002 (29.01.02)

Applicant

ZEALAND PHARMA A/S

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this notice:

   None

   In accordance with Rule 47.1(c), third sentence, those Offices will accept the present notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:

   None

   The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49.1(a-*bis*)).

3. Enclosed with this notice is a copy of the international application as published by the International Bureau on 07 August 2003 (07.08.03) under No. 03/063891

4. TIME LIMITS for filing a demand for international preliminary examination and for entry into the national phase

   The applicable time limit for entering the national phase will, subject to what is said in the following paragraph, be 30 MONTHS from the priority date, not only in respect of any elected Office if a demand for international preliminary examination is filed before the expiration of 19 months from the priority date, but also in respect of any designated Office, in the absence of filing of such demand, where Article 22(1) as modified with effect from 1 April 2002 applies in respect of that designated Office. For further details, see *PCT Gazette* No. 44/2001 of 1 November 2001, pages 19926, 19932 and 19934, as well as the *PCT Newsletter*, October and November 2001 and February 2002 issues.

   In practice, time limits other than the 30-month time limit will continue to apply, for various periods of time, in respect of certain designated or elected Offices. For regular updates on the applicable time limits (20, 21, 30 or 31 months, or other time limit), Office by Office, refer to the *PCT Gazette*, the *PCT Newsletter* and the *PCT Applicant's Guide*, Volume II, National Chapters, all available from WIPO's Internet site, at http://www.wipo.int/pct/en/index.html.

   For filing a demand for international preliminary examination, see the *PCT Applicant's Guide*, Volume I/A, Chapter IX. Only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination (at present, all PCT Contracting States are bound by Chapter II).

   It is the applicant's sole responsibility to monitor all these time limits.

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.(41-22) 740.14.35

Authorized officer

Judith Zahra

Telephone No.(41-22) 338.91.11

PATENT COOPERATION TREATY

MODTAGET
22 SEP. 2003

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From the INTERNATIONAL BUREAU

PCT

INFORMATION CONCERNING ELECTED OFFICES NOTIFIED OF THEIR ELECTION

(PCT Rule 61.3)

To:

ZEALAND PHARMA A/S
Smedeland 26B
DK-2600 Glostrup
Denmark

Date of mailing (day/month/year)	
10 September 2003 (10.09.03)	
Applicant's or agent's file reference	**IMPORTANT INFORMATION**
016-2003 WO	

International application No.	International filing date (day/month/year)	Priority date (day/month/year)
PCT/DK03/00056	29 January 2003 (29.01.03)	29 January 2002 (29.01.02)

Applicant
ZEALAND PHARMA A/S et al

1. The applicant is hereby informed that the International Bureau has, according to Article 31(7), notified each of the following Offices of its election:

   EP :AT,BE,BG,CH,CY,CZ,DE,DK,EE,ES,FI,FR,GB,GR,HU,IE,IT,LU,MC,NL,PT,SE,SI,
   SK,TR
   National :BG,CA,CN,DE,GB,IL,JP,KP,KR,MN,NO,PL,RO,RU,SK,US

2. The following Offices have waived the requirement for the notification of their election; the notification will be sent to them by the International Bureau only upon their request:

   AP :GH,GM,KE,LS,MW,MZ,SD,SL,SZ,TZ,UG,ZM,ZW
   EA :AM,AZ,BY,KG,KZ,MD,RU,TJ,TM
   OA :BF,BJ,CF,CG,CI,CM,GA,GN,GQ,GW,ML,MR,NE,SN,TD,TG
   National :AE,AG,AL,AM,AT,AU,AZ,BA,BB,BR,BY,BZ,CH,CO,CR,CU,CZ,DK,DM,DZ,EC,
   EE,ES,FI,GD,GE,GH,GM,HR,HU,ID,IN,IS,KE,KG,KZ,LC,LK,LR,LS,LT,LU,LV,MA,MD,
   MG,MK,MW,MX,MZ,NZ,OM,PH,PT,SC,SD,SE,SG,SL,TJ,TM,TN,TR,TT,TZ,UA,UG,UZ,VC,
   VN,YU,ZA,ZM,ZW

3. The applicant is reminded that he must enter the "national phase" before the expiration of 30 months from the priority date before each of the Offices listed above. This must be done by paying the national fee(s) and furnishing , if prescribed, a translation of the international application (Article 39(1)(a)), as well as, where applicable, by furnishing a translation of any annexes of the international preliminary examination report (Article 39(3)(b) and Rule 74.1).

   Some offices have fixed time limits expiring later than the above-mentioned time limit. For detailed information about the applicable time limits and the acts to be performed upon entry into the national phase before a particular Office, see Volume II of the PCT Applicant's Guide.

   The entry into the European regional phase is postponed until 31 months from the priority date for all States designated for the purposes of obtaining a European patent.

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740-1435

Authorized officer:
Junko TAKEUCHI (Fax: 338 71 30)

Telephone No. (41-22) 338 8448

PATENT COOPERATION TREA

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From the INTERNATIONAL BUREAU

PCT

COMMUNICATION IN CASES FOR WHICH NO OTHER FORM IS APPLICABLE

To:
ZEALAND PHARMA A/S
Smedeland 26B
DK-2600 Glostrup
DANEMARK

MODTAGET
21 JULI 2003

Date of mailing (*day/month/year*) 10 July 2003 (10.07.03)	
Applicant's or agent's file reference 016-2003 WO	REPLY DUE see paragraph 1 below
International application No. PCT/DK03/00056	International filing date (*day/month/year*) 29 January 2003 (29.01.03)
Applicant ZEALAND PHARMA A/S	

1. ☐ REPLY DUE within _____ months/days from the above date of mailing

☐ NO REPLY DUE, however, see below

☒ IMPORTANT COMMUNICATION

☐ INFORMATION ONLY

2. COMMUNICATION:

The attention of the applicant is drawn to the fact that according to Rule 91.1(e)(ii) "*no rectification shall be made except with the express authorization of the International Searching Authority if the error is in any part of the international application other than the request or in any paper submitted to that Authority*", which is the case.

Consequently, pursuant to Rule 91.1(e)(ii), the International Bureau has forwarded the request of modification to ISA/EP, in its capacity as International Searching Authority, for authorization.

cc : ISA/EP

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland	Authorized officer SUAREZ Javier
Facsimile No. (41-22) 740-1435	Telephone No. (41-22) 338.99.99

Form PCT/IB/345 (July 1992)

ZEALAND
PHARMA

Zealand Pharma A/S
Smedeland 26 B
DK-2600 Glostrup
Denmark

Tel: +45 43 28 12 00
Fax: +45 43 28 12 12

E-mail: info@zp.dk
www.zp.dk

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20
Switzerland

International Patent Application No. PCT/DK03/00056
Amendment of the description under Article 34
Our Ref: 016-2003 WO1

Date
24 June 2003

Dear Sirs,

Enclosed please find an amended page 17 in the description of the above international patent application. In the first line of the brief description of the drawings we have added the details of the article referred to.

The amendment does not go beyond the disclosure in the international application as filed.

Yours sincerely,

Maj Hilligsøe
Patent Manager

Enclosure: New page 17

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a drawing showing phosphorylation sites on a connexin (Cx43) (Hossain M Z et al. (*Science's stke* 2000; pp 1-5)). As can be seen, the cytosolic domain of the Cx43 transmembrane protein has several potential serine and tyrosine phosphorylation sites.

Figure 2 is a graph showing metabolic stress induced by removal of glucose. As can be seen from the graph, administration of compound 1 reduces the stress as evidenced by a decrease in relative conduction delay.

Figures 3A-B are representations of immunoblots showing detection of tyrosine phosphorylation in connexin 43 (Cx43). Figure 3A shows results from compound 1 administration. Figure 3B shows results from administration of compound 1 and compound 2.

Figures 4A-B are representations of immunoblots showing detection of tyrosine phosphorylation (8A) and serine phosphorylation (8B) of Cx43.

Figure 5 is a graph showing effect of 10nM compound 1 on ischemia-induced uptake of calcein in cultured cardiomyocytes.

Figure 6A is a drawing showing a preferred ELISA assay format. Figure 6B is a graph showing ELISA results of phosphorylated Cx43 at Tyr-P in HeLa cells. Figure 6C is a graph illustrating ELISA results of phosphorylated Cx43 at Tyr-P in CHO cells.

Figure 7A-C are photomicrographs showing dye uptake in cultured cardiomyocytes. Fig. 7A: Cardiomyocytes under light microscopy; Fig. 7B: Fluorescence under control conditions (same as cells in Fig. 7A); Fig. 7C Fluorescence after 30 minutes of metabolic inhibition.

Figure 8 is a graph showing that compound 1 reduces stress-induced calcein uptake in a dose-dependent manner.

PATENT COOPERATION TREATY

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From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF RECEIPT OF RECORD COPY

MODTAG

17 MRS 2003 (PCT Rule 24.2(a))

To:

ZEALAND PHARMA A/S
Smedeland 26B
DK-2600 Glostrup
Denmark

Date of mailing (day/month/year)	IMPORTANT NOTIFICATION
06 March 2003 (06.03.03)	
Applicant's or agent's file reference	**International application No.**
016-2003 WO	PCT/DK03/00056

The applicant is hereby notified that the International Bureau has received the record copy of the international application as detailed below.

Name(s) of the applicant(s) and State(s) for which they are applicants:

ZEALAND PHARMA A/S (for all designated States except US)
PETERSEN, Jørgen, Søberg et al (for US)

International filing date	:	29 January 2003 (29.01.03)
Priority date(s) claimed	:	29 January 2002 (29.01.02)
Date of receipt of the record copy by the International Bureau	:	19 February 2003 (19.02.03)
List of designated Offices	:	

AP :GH,GM,KE,LS,MW,MZ,SD,SL,SZ,TZ,UG,ZM,ZW
EA :AM,AZ,BY,KG,KZ,MD,RU,TJ,TM
EP :AT,BE,BG,CH,CY,CZ,DE,DK,EE,ES,FI,FR,GB,GR,HU,IE,IT,LU,MC,NL,PT,SE,SI,SK,TR
OA :BF,BJ,CF,CG,CI,CM,GA,GN,GQ,GW,ML,MR,NE,SN,TD,TG
National :AE,AG,AL,AM,AT,AU,AZ,BA,BB,BG,BR,BY,BZ,CA,CH,CN,CO,CR,CU,CZ,DE,DK,DM,DZ,EC,EE,ES,FI,GB,GD,GE,GH,GM,HR,HU,ID,IL,IN,IS,JP,KE,KG,KP,KR,KZ,LC,LK,LR,LS,LT,LU,LV,MA,MD,MG,MK,MN,MW,MX,MZ,NO,NZ,OM,PH,PL,PT,RO,RU,SC,SD,SE,SG,SK,SL,TJ,TM,TN,TR,TT,TZ,UA,UG,US,UZ,VC,VN,YU,ZA,ZM,ZW

ATTENTION

The applicant should carefully check the data appearing in this Notification. In case of any discrepancy between these data and the indications in the International application, the applicant should immediately inform the International Bureau.

In addition, the applicant's attention is drawn to the information contained in the Annex, relating to:

- [X] time limits for entry into the national phase - see updated Important Information (as of April 2002)
- [ ] confirmation of precautionary designations (if applicable)
- [X] requirements regarding priority documents (if applicable)

A copy of this Notification is being sent to the receiving Office and to the International Searching Authority.

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland	Authorized officer: Leslie BARRIOS
Facsimile No. (41-22) 338.89.70	Telephone No. (41-22) 338 9715

ANNEX TO FORM PCT/IB/301

International application No.
PCT/DK03/00056

INFORMATION ON TIME LIMITS FOR ENTERING THE NATIONAL PHASE

The applicant is reminded that the "national phase" must be entered before each of the designated Offices indicated on the cover sheet of this Notification by paying national fees and furnishing translations, as prescribed by Articles 22 and 39 and the applicable national laws. In addition, the applicant may also have to comply with other special requirements applicable in certain Offices. It is the applicant's responsibility to ensure the necessary steps to enter the national phase are taken in a timely fashion. Most Offices do not issue reminders to applicants in connection with the entry into the national phase.

The applicable time limit for entering the national phase will, subject to what is said in the following paragraph, be **30 MONTHS** from the priority date, not only in respect of any elected Office where a demand for international preliminary examination is filed before the expiration of 19 months from the priority date (see Article 39(1)), but also in respect of any designated Office, in the absence of filing of such demand, where Article22(1) as modified with effect from 1 April 2002 applies in respect of that designated Office. For further details, see PCT Gazette No. 44/2001 of 1 November 2001, pages 19828, 19832 and 19834, as well as the PCT Newsletter, October and November 2001 and February 2002 issues. .

In practice, time limits other than the 30-month time limit will continue to apply, for various periods of time, in respect of certain designated or elected Offices. For regular updates on the applicable time limits (20, 21, 30 or 31 months, or other time limit), Office by Office, refer to the PCT Gazette("Section IV" part published on a weekly basis), to the PCT Newsletter (on a monthly basis) and to the relevant National Chapters in Volume II of the PCT Applicant's Guide (the paper version of which is updated usually twice a year and the Internet version of which is updated usually on a weekly basis). Finally, a cumulative table of all applicable time limits for entering the national phase is available from WIPO's Internet site, via links from various pages the site including those of the Gazette, Newsletter and Guide, at http://www.wipo.int/pct/en/index.html.

Information about the requirements for filing a demand for international preliminary examination is set out in the PCT Applicant's Guide, Volume I/A, Chapter IX. Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination (at present, all PCT Contracting States are bound by Chapter II).

CONFIRMATION OF PRECAUTIONARY DESIGNATIONS

This notification lists only specific designations made under Rule 4.9(a) in the request. It is important to check that these designations are correct. Errors in designations can be corrected where precautionary designations have been made under Rule 4.9(b). The applicant is hereby reminded that any precautionary designations may be confirmed according to Rule 4.9(c) before the expiration of 15 months from the priority date (this time limit may not be extended). If it is not confirmed, it will automatically be regarded as withdrawn by the applicant. There will be no reminder and no invitation. Confirmation of a designation consists of the filing of a notice specifying the designated State concerned (with indication of the kind of protection or treatment desired) and the payment of the designation and confirmation fees. The Notice of confirmation and payment must reach the receiving Office within the 15-month time limit.

REQUIREMENTS REGARDING PRIORITY DOCUMENTS

For applicants who have not yet complied with the requirements regarding priority documents, the following is recalled.

Where the priority of an earlier national, regional or international application is claimed, the applicant must submit a copy of the said earlier application, certified by the authority with which it was filed ("the priority document") to the receiving Office (which will transmit it to the International Bureau) or directly to the International Bureau, before the expiration of 16 months from the priority date, provided that any such priority document may still be submitted to the International Bureau before that date of international publication of the international application, in which case that document will be considered to have been received by the International Bureau on the last day of the 16-month time limit (Rule 17.1(a)).

Where the priority document is issued by the receiving Office, the applicant may, instead of submitting the priority document, request the receiving Office to prepare and transmit the priority document to the International Bureau. Such request must be made before the expiration of the 16-month time limit and may be subjected by the receiving Office to the payment of a fee (Rule 17.1(b)).

If the priority document concerned is not submitted to the International Bureau or if the request to the receiving Office to prepare and transmit the priority document has not been made (and the corresponding fee, if any, paid) within the applicable time limit indicated under the preceding paragraphs, any designated State may disregard the priority claim, provided that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within the time limit which is reasonable under the circumstances.

Where several priorities are claimed, the priority date to be considered for the purposes of computing the 16-month time limit is the filing date of the earliest application whose priority is claimed.

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION CONCERNING SUBMISSION OR TRANSMITTAL OF PRIORITY DOCUMENT

(PCT Administrative Instructions, Section 411)

To:

ZEALAND PHARMA A/S
Smedeland 26B
DK-2600 Glostrup
Denmark

Date of mailing (day/month/year) 08 April 2003 (08.04.03)	
Applicant's or agent's file reference 016-2003 WO	**IMPORTANT NOTIFICATION**
International application No. PCT/DK03/00056	International filing date (day/month/year) 29 January 2003 (29.01.03)
International publication date (day/month/year) Not yet published	Priority date (day/month/year) 29 January 2002 (29.01.02)
Applicant ZEALAND PHARMA A/S et al	

1. The applicant is hereby notified of the date of receipt (except where the letters "NR" appear in the right-hand column) by the International Bureau of the priority document(s) relating to the earlier application(s) indicated below. Unless otherwise indicated by an asterisk appearing next to a date of receipt, or by the letters "NR", in the right-hand column, the priority document concerned was submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b).

2. This updates and replaces any previously issued notification concerning submission or transmittal of priority documents.

3. An asterisk(*) appearing next to a date of receipt, in the right-hand column, denotes a priority document submitted or transmitted to the International Bureau but not in compliance with Rule 17.1(a) or (b). In such a case, the attention of the applicant is directed to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

4. The letters "NR" appearing in the right-hand column denote a priority document which was not received by the International Bureau or which the applicant did not request the receiving Office to prepare and transmit to the International Bureau, as provided by Rule 17.1(a) or (b), respectively. In such a case, the attention of the applicant is directed to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

Priority date	Priority application No.	Country or regional Office or PCT receiving Office	Date of receipt of priority document
29 Janu 2002 (29.01.02)	60/352,717	US	10 Marc 2003 (10.03.03)

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland	Authorized officer Allie Patricia
Facsimile No. (41-22) 338.88.70	Telephone No. (41-22) 338 9304

MODTAGE
05 MRS. 2003

PATENT COOPERATION TREAT.

From the INTERNATIONAL SEARCHING AUTHORITY

PCT

NOTIFICATION OF RECEIPT OF SEARCH COPY

(PCT Rule 25.1)

To:
ZEALAND PHARMA
Smedeland 26 B
DK-2600 Glostrup
DENMARK

Date of mailing *(day/month/year)*	04/03/2003
Applicant's or agent's file reference: 016-2003 WO	IMPORTANT NOTIFICATION

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/DK 03/ 00056	29/01/2003	29/01/2002

Applicant

ZEALAND PHARMA A/S

1. **Where the International Searching Authority and the Receiving Office are not the same office:**

   The applicant is hereby notified that the search copy of the international application was received by this International Searching Authority on the date indicated below.

   Where the International Searching Authority and the Receiving Office are the same office:

   The applicant is hereby notified that the search copy of the international application was received on the date indicated below.

   17/02/2003 *(date of receipt).*

2. ☐ The search copy was accompanied by a nucleotide and/or amino acid sequence listing in computer readable form.

3. **Time limit for establishment of International Search Report**

   The applicant is informed that the time limit for establishing the International Search Report is 3 months from the date of receipt indicated above or 9 months from the priority date, whichever time limit expires later

4. A copy of this notification has been sent to the International Bureau and, where the first sentence of paragraph 1 applies, to the Receiving Office.

Name and mailing address of the International Searching Authority	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	ISA/EP

Form PCT/ISA/202 (July 1998)

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MODTAGET
05 MRS. 200

PATENT COOPERATION TREAT.

From the INTERNATIONAL SEARCHING AUTHORITY

PCT

To:
ZEALAND PHARMA
Smedeland 26 B
DK-2600 Glostrup
DENMARK

COMMUNICATION IN CASES FOR WHICH
NO OTHER FORM IS APPLICABLE

Applicant's or agent's file reference 016-2003 WO	Date of mailing (day/month/year) 04/03/2003
International application No. PCT/DK 03/ 00056	REPLY DUE See paragraph 1 below
Applicant ZEALAND PHARMA A/S	International filing date (day/month/year) 29/01/2003

1. [ ] REPLY DUE within _____ days from the above date of mailing

   [X] NO REPLY DUE

2. COMMUNICATION:

The applicant is informed that establishment of the international search report (ISR) for non first-filings may be delayed due to a current search backlog.

Although the time limit for entering the national phase before designated offices under Article 22(1) PCT and elected offices under Article 39(1) PCT has, with effect from 1 April 2002 (see PCT Gazette 44/2001 Section IV) been set at 30 months from the priority date (before the EPO the time limit is 31 months from the priority date - see Rule 107 EPC as amended with effect from 2 January 2002 - OJ EPO 8-9/2001, 373) not all PCT contracting states have yet made the necessary changes to their national laws and will for the time being continue to require entry to the national phase at 20/21 months from the priority date if a demand has not been filed before the end of 19 months from the priority date - see PCT Gazette/PCT Newsletter available on the WIPO internet site at http://www.wipo.int/pct/en/index.html for an up to date list of the applicable time limits.

In these circumstances, the EPO acting as IPEA will accept, without any late payment fee under Rule 58*bis* PCT, the handling fee and the preliminary examination fee due in respect of the demand relating to the present application, even if they are not paid within the time limit prescribed in Rules 57.3 and 58.1(b) PCT, provided that they are paid within one month from the date of transmittal of the ISR; i.e., the EPO will only send an invitation pursuant to Rule 58*bis*.1(a) PCT after expiry of this one-month period. In all cases where the EPO has sent an invitation to pay and the applicant has not paid in full the amount due, the demand shall be considered as if it had not been submitted (Rule 58*bis*.1(b)-(d) PCT). A loss of rights may well be the consequence in designated states where the time limit for entry into the national phase under Article 22 PCT has already expired (see also Article 37(4) PCT).

Note that if the competent IPEA chosen by the applicant is not the EPO and if the fees mentioned above are not paid within the time limit prescribed in Rules 57.3 and 58.1(b) PCT, the competent IPEA is entitled to apply Rule 58*bis*.1(a) PCT immediately thereafter.

If your application is affected, we apologise for any inconvenience caused.

Finally, applicants are reminded that as of 3 January 2002 a rationalised PCT II procedure may apply, see OJ EPO 11/2001, 539 and that the EPO as ISA will not carry out international search on an application which relates to no more than a method of doing business, see OJ EPO 10/2001,482. Applicants should also bear in mind the restriction of the EPO's competence as ISA and IPEA in certain technical fields in respect of certain international applications, see OJ EPO 1/2002, 52 and PCT Newsletter 1/2002 for further details.

Name and mailing address of the International Searching Authority European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer ISA/EP

Form PCT/ISA/224 (January 1994)

MOTTAGET
05 MAR 2003

PATENT COOPERATION TREATY

From the RECEIVING OFFICE

To:

ZEALAND PHARMA A/S
Smedeland 26 B
DK-2600 Glostrup.

PCT

NOTIFICATION OF DATE OF RECEIPT OF PRIORITY DOCUMENT OR OF PRIORITY APPLICATION NUMBER

(PCT Administrative Instructions, Sections 323(a), (b) and (c))

Applicant's or agent's file reference	Date of mailing (day/month/year)
016-2003 WO	04 March 2003
International application No.	**International filing date (day/month/year)**
PCT/DK 03/00056	29 January 2003
Applicant	
ZEALAND PHARMA A/S et.al.	

1. ☒ This receiving Office hereby gives notice of the receipt of the priority document(s) identified below on:

   22 February 2003

2. ☐ This receiving Office hereby gives notice of the receipt of a request (made under Rule 17.1(b)) to prepare and transmit to the International Bureau the priority document(s) identified below on:

   _____

Identification of the priority document(s):

Priority date	Priority application No.	Country or regional Office or PCT receiving Office
29 January 2002	60/352,717	US

Name and mailing address of the receiving Office	Authorised officer
PATENT- OG VAREMÆRKESTYRELSEN Helgesvej Alle 81, DK-2630 Taastrup	Marie Louise Rosendal Head Clerk
Facsimile No.	Telephone No.

Form PCT/RO/135 (July 1998)

PCT REQUEST **KOPI** til orientering. 2/5

Original (for SUBMISSION) - printed on 29.01.2003 06:05:17 PM

016-2003 WO

MODTA
05 MRS.

III-2	Applicant and/or inventor	
III-2-1	This person is:	applicant and inventor
III-2-2	Applicant for	US only
III-2-4	Name (LAST, First)	NEVE, Søren
III-2-5	Address:	Ellevænget 4 DK-2800 Lyngby Denmark
III-2-6	State of nationality	DK
III-2-7	State of residence	DK
III-3	Applicant and/or inventor	
III-3-1	This person is:	applicant and inventor
III-3-2	Applicant for	US only
III-3-4	Name (LAST, First)	NIELSEN, Morten, Schak
III-3-5	Address:	Egebjergvej 51A DK-2750 Ballerup Denmark
III-3-6	State of nationality	DK
III-3-7	State of residence	DK
III-4	Applicant and/or inventor	
III-4-1	This person is:	applicant and inventor
III-4-2	Applicant for	US only
III-4-4	Name (LAST, First)	MEIER, Eddi
III-4-5	Address:	Bringebakken 88 DK-3500 Værløse Denmark
III-4-6	State of nationality	DK
III-4-7	State of residence	DK
III-5	Applicant and/or inventor	
III-5-1	This person is:	applicant and inventor
III-5-2	Applicant for	US only
III-5-4	Name (LAST, First)	STEINESS, Eva
III-5-5	Address:	Sømarksvej 11 DK-2900 Hellerup Denmark
III-5-6	State of nationality	DK
III-5-7	State of residence	DK
III-6	Applicant and/or inventor	
III-6-1	This person is:	applicant and inventor
III-6-2	Applicant for	US only
III-6-4	Name (LAST, First)	JENSEN, Peter, Holme
III-6-5	Address:	~~Ahlefeldtsgade 18A, 1.th~~ Classensgade 11, 3.tv. ~~DK-1359~~ DK-2100 Copenhagen K Denmark
III-6-6	State of nationality	DK
III-6-7	State of residence	DK

▲ Corrected by RO/DK

MODTAGET
14 FEB. 2003

PATENT COOPERATION TREATY

From the RECEIVING OFFICE

PCT

INVITATION TO CORRECT DEFECTS IN THE INTERNATIONAL APPLICATION

(PCT Articles 3(4)(i) and 14(1) and Rule 26)

To:

ZEALAND PHARMA A/S
Semdeland 26 B
DK-2600 Glostrup.

Date of mailing *(day/month/year)* 13 February 2003	
Applicant's or agent's file reference 016-2003 WO	REPLY DUE within 1 months/days from the above date of mailing
International application No. PCT/DK 03/00056	International filing date *(day/month/year)* 29 January 2003
Applicant ZEALAND PHARMA A/S et.al.	

1. ☒ The applicant is hereby invited, within the time limit indicated above, to correct, in the international application as filed, the defects specified on the attached:
   - ☒ Annex A
   - ☒ Annex B1 *(text matter of the international application as filed)*
   - ☒ Annex C1 *(drawings of the international application as filed)*

2. ☐ The applicant is hereby invited, within the time limit indicated above, to correct, in the translation of the international application furnished under Rule 12.3 or 12.4, the defects specified on the attached:
   - ☐ Annex A
   - ☐ Annex B2 *(text matter of the translation of the international application)*
   - ☐ Annex C2 *(drawings of the translation of the international application)*

Additional observations (if necessary):

HOW TO CORRECT THE DEFECTS?

Corrections must be submitted by filing a replacement sheet embodying the correction and a letter accompanying the replacement sheet, which shall draw attention to the difference between the replaced sheet and the replacement sheet. A correction may be stated in a letter only if it is of such a nature that it can be transferred from the letter to the record copy without adversely affecting the clarity and direct reproducibility of the sheet onto which the correction is to be transferred (Rule 26.4).

ATTENTION

Failure to correct the defects will result in the international application being considered withdrawn by this receiving Office (see Rule 26.5 for further details).

A copy of this invitation and any attachments has been sent to the International Bureau
☒ and the International Searching Authority

Name and mailing address of the receiving Office PATENT- OG VAREMÆRKESTYRELSEN Helgeshøj Allé 81, DK-2630 Taastrup	Authorized officer Marie Louise Rosendal Head Clerk
Facsimile No.	Telephone No.

Form PCT/RO/106 (January 2003)

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ANNEX A TO FORM PCT/RO/106

International application No.
PCT/DK 03/00056

The receiving office has found the following defects in the international application:

1. As to signature* of the international application (Rules 4.15 and 90.4), the request:

   a. ☐ is not signed.

   b. ☐ is not signed by all the applicants.

   c. ☐ is not accompanied by the statement referred to in the check list in Box No. VIII of the request explaining the lack of the signature of an applicant for the designation of the United States of America.

   d. ☒ is signed by what appears to be an agent/common representative but

      ☒ the international application is not accompanied by a power of attorney appointing him.

      ☐ the power of attorney accompanying the international application was not signed by all the applicants.

   e. ☐ other *(specify)*:

* All applicants must sign, including inventors if they are also applicants (e.g. where the United States of America is designated).

2. As to indications concerning the applicant, the request (Rules 4.4 and 4.5):

   a. ☐ does not properly indicate the applicant's name *(specify)*:

   b. ☐ does not indicate the applicant's address.

   c. ☐ does not properly indicate the applicant's address *(specify)*:

   d. ☐ does not indicate the applicant's nationality.

   e. ☐ does not indicate the applicant's residence.

   f. ☐ other *(specify)*:

3. As to the language of some parts of the international application (Rule 12.1):

   a. ☐ the request is not one in (one of) the admitted language(s) which is (are): _____

   b. ☐ the text matter of the drawings is not in (one of) the admitted language(s) which is (are): _____

   c. ☐ the abstract is not in (one of) the admitted language(s) which is (are): _____

4. The title of the invention:

   a. ☐ is not indicated in Box No. I of the request (Rule 4.1(a)).

   b. ☐ is not indicated at the top of the first sheet of the description (Rule 5.1(a)).

   c. ☐ as appearing in Box No. I of the request is not identical with the title heading the description (Rule 5.1(a)).

5. As to the abstract (Rule 8):

   ☐ the international application does not contain an abstract

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ANNEX B1 TO FORM PCT/RO/106

International application No. PCT/NL03/00056

This receiving Office has found that, with regard to the presentation of the text matter of the international application as filed, the physical requirements are not complied with to the extent that compliance therewith is necessary for:

1. ☐ Reasonably uniform international publication (Rule 11 and 26.3(a)(i)) *(defects to be specified):*

		Request	Description	Claims	Abstract
a. ☐	The sheets do not admit of direct reproduction.	☐	☐	☐	☐
b. ☐	The element does not commence on a new sheet	☐	☐	☐	☐
c. ☐	Sheets are not free from creases, cracks, folds.	☐	☐	☐	☐
d. ☐	Sheets are not used in the upright position.	☐	☐	☐	☐
e. ☐	One side of the sheets is not left unused.	☐	☐	☐	☐
f. ☐	The paper of the sheets is not flexible/strong/white/smooth/non-shiny/durable.	☐	☐	☐	☐
g. ☐	The sheets are not connected as prescribed (Rule 11.4(b)).	☐	☐	☐	☐
h. ☐	Sheets are not A4 size (29,7cm × 21cm).	☐	☐	☐	☐
i. ☐	The minimum margins on the sheets are not as prescribed (top: 2cm; left side 2.5cm; right side: 2cm; bottom: 2cm).		☐	☐	☐
j. ☐	The file reference number indicated on the sheets does not appear in the left-hand corner of the sheets, within 1.5 cm of the top of the sheets.		☐	☐	☐
k. ☐	The file reference number exceeds the maximum of 12 characters.	☐	☐	☐	☐
l. ☒	The ~~sheets of the description, claims and abstract~~ are not numbered in consecutive Arabic numerals.		☐	☒	☐
m. ☐	The sheet numbers are not centred at the top or bottom of the sheets.	☐	☐	☐	☐
n. ☐	The sheet numbers are in the margin (see i. above for the size of the margins)		☐	☐	☐
o. ☐	The text matter is not typed or printed.	☐	☐	☐	☐
p. ☐	The typing on the sheets is not 1½-spaced.		☐	☐	☐
q. ☐	The characters in the text matter on the sheets are less than 0.21 cm high in capital letters.	☐	☐	☐	☐
r. ☐	The text matter on the sheets is not in dark, indelible colour.	☐	☐	☐	☐
s. ☐	The element contains drawings.	☐	☐	☐	☐
t. ☐	The sheets contain alterations/overwritings/interlineations/too many erasures.	☐	☐	☐	☐
u. ☐	The sheets contain photocopy marks.	☐	☐	☐	☐

2. ☐ satisfactory reproduction (Rule 11 and 26.3(b)(i)).

Further observations (if necessary):

Claim no 28 is missing - please renumber

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ANNEX C1 TO FORM PCT/RO/106

International application No.
PCT/DK 03/00056

This receiving Office has found that, with regard to the presentation of the drawing of the international application as filed, the physical requirements are not complied with to the extent that compliance therewith is necessary for:

1. ☐ reasonably uniform international publication (Rule 11 and 26.3(a)(i)) *(defects to be specified)*:

Sheets containing drawings:

a. ☐ the sheets do not admit of direct reproduction.
b. ☐ the sheets are not free from creases, cracks, folds.
c. ☐ one side of the sheets is not left unused.
d. ☐ the paper of the sheets is not flexible/strong/white/smooth/non-shiny/durable.
e. ☐ the drawings do not commence on a new sheet.
f. ☐ the sheets are not connected as prescribed (Rule 11.4(b)).
g. ☐ the sheets are not A4 size (29.7cm × 21cm).
h. ☐ the minimum margins on the sheets are not as prescribed (top: 2.5cm; left side 2.5cm; right side: 1.5cm; bottom: 1cm).
i. ☐ the file reference number indicated on the sheets does not appear in the left-hand corner of the sheets, within 1.5 cm of the top of the sheets
j. ☐ the file reference number exceeds the maximum of 12 characters.
k. ☐ the sheets are not free from frames around usable or used surfaces.
l. ☒ the sheets are not numbered in consecutive Arabic numerals (e.g. 1/3, 2/3, 3/3).
m. ☐ the sheet numbers are not centred at the top or bottom of the sheets.
n. ☐ the sheet numbers are in the margin (see h. above for the size of the margins).
o. ☐ the sheets contain alterations/overwritings/interlineations/too many erasures.
p. ☒ the sheets contain photocopy marks. and text from the faxmachine

II. **Drawings (Rule 11.13):**

a. ☐ do not admit of direct reproduction.
b. ☐ contain unnecessary text matter.
c. ☐ contain words so placed as to prevent translation without interference with lines thereof.
d. ☐ are not executed in durable black colour; the lines are not uniformly thick and well defined.
e. ☐ contain cross sections not properly hatched.
f. ☐ would not be properly distinguishable in reduced reproduction.
g. ☐ contain scales not represented graphically.
h. ☐ contain numbers, letters and reference lines lacking simplicity and clarity.
i. ☐ contain lines drafted without the aid of drafting instruments.
j. ☐ contain disproportionate elements of a figure not necessary for clarity.
k. ☐ contain numbers and letters of height less than 0.32 cm.
l. ☐ contain letters not conforming to the Latin, and where customary, Greek alphabets.
m. ☐ contain figures on two or more sheets which form a single complete figure but which are not able to be assembled without concealing parts thereof.
n. ☐ contain figures, which are not properly arranged and clearly separated.
o. ☐ contain different figures not numbered in consecutive Arabic numerals.
p. ☐ contain different figures not numbered independent of the numbering of the sheets.
q. ☐ are not restricted to reference signs mentioned in the description.
r. ☐ do not contain reference signs that are mentioned in the description.
s. ☐ contain the same feature denoted by different reference signs.

2. ☐ satisfactory reproduction (Rule 11 and 26.3(b)(i)).

Further observations (if necessary):

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ZEALAND
PHARMA

Marie Louise Rosendal
Patent- og Varemærkestyrelsen
Helgeshøj Allé 81
2630 Taastrup

Zealand Pharma A/S
Smedeland 26 B
DK-2600 Glostrup
Denmark

Tel: +45 43 28 12 00
Fax: +45 43 28 12 12

E-mail: info@zp.dk
www.zp.dk

Date
21 February 2003

International Patent Application No. PCT/DK03/00056
ur ref: 016-2003 US1

We refer to your Invitation to Correct Defects of 13 February 2003.

In reply to the Invitation we enclose

- a Power of Attorney from each applicant/inventor to Zealand Pharma A/S
- a renumbered set of claims in which claim no. 28 is not missing
- a numbered set of drawings without photocopy marks and text from facsimile transmission

Furthermore, we enclose a certified copy of 60/352,717 from which priority is claimed.

Please acknowledge safe receipt of this letter and its enclosures by returning the enclosed copy of this letter.

Yours sincerely,

Lone S. Due

Lone Skjøttgaard Due
Patent Assistant

Enclosures: 8 Power of Attorney forms, set of claims, set of drawings, certified copy of 60/352,717, copy of this letter

PCT

154

POWER OF ATTORNEY

(for an international application filed under the Patent Cooperation Treaty)

(PCT Rule 90.4)

The undersigned applicant(s) *(Names should be indicated as they appear in the request)*:

PETERSEN, Jørgen Søberg
Hellebakken 1
DK-3150 Hellebæk
Denmark

hereby appoints (appoint) the following person as: ☐ agent ☒ common representative

Name and address
(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)

ZEALAND PHARMA A/S
Smedeland 28B
DK-2600 Glostrup
Denmark

to represent the undersigned before

☒ all the competent International Authorities
☐ the International Searching Authority only
☐ the International Preliminary Examining Authority only

in connection with the international application identified below:

Title of the invention: COMPOSITIONS AND METHODS FOR MODULATING CONNEXIN HEMICHANNELS.

Applicant's or agent's file reference: 016-2003 WO1

International application number (if already available): PCT/DK03/00056

filed with the following Office Danish Patent and Trademark Office as receiving Office and to make or receive payments on behalf of the undersigned.

Signature of the applicant(s) *(where there are several applicants, each of them must sign; next to each signature, indicate the name of the person signing and the capacity in which the person signs, if such capacity is not obvious from reading the request or this power)*:

Jørgen Søberg Petersen
Inventor and Applicant for the US only

Date: Feb. 14, 2003 Jørgen S. Petersen

Form PCT/Model of power of attorney (for a given international application) (July 1992)

PCT

POWER OF ATTORNEY

(for an international application filed under the Patent Cooperation Treaty)

(PCT Rule 90.4)

The undersigned applicant(s) *(Names should be indicated as they appear in the request)*:

NEVE, Søren
Ellevænget 4
DK-2800 Lyngby
Denmark

hereby appoints (appoint) the following person as: ☐ agent ☒ common representative

Name and address
(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)

ZEALAND PHARMA A/S
Smedeland 26B
DK-2600 Glostrup
Denmark

to represent the undersigned before

☒ all the competent International Authorities
☐ the International Searching Authority only
☐ the International Preliminary Examining Authority only

in connection with the international application identified below:

Title of the invention: COMPOSITIONS AND METHODS FOR MODULATING CONNEXIN HEMICHANNELS

Applicant's or agent's file reference: 016-2003 WO1

International application number (if already available): PCT/DK03/00058

filed with the following Office Danish Patent and Trademark Office as receiving Office and to make or receive payments on behalf of the undersigned.

Signature of the applicant(s) *(where there are several applicants, each of them must sign; must be each signature, indicate the name of the person signing and the capacity in which the person signs, if such capacity is not obvious from reading the request or this power)*:

X

Søren Neve
Inventor and Applicant for the US only

Date: 15-2-2003

Form PCT/Model of power of attorney (for a given international application) (July 1992)

PCT

1/6

POWER OF ATTORNEY

(for an international application filed under the Patent Cooperation Treaty)

(PCT Rule 90.4)

The undersigned applicant(s) *(Names should be indicated as they appear in the request)*:

NIELSEN, Morten Schak
Egebjergvej 51A
DK-2750 Ballerup
Denmark

hereby appoints (appoint) the following person as: ☐ agent ☒ common representative

Name and address
(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)

ZEALAND PHARMA A/S
Smedeland 26B
DK-2600 Glostrup
Denmark

to represent the undersigned before

☒ all the competent International Authorities
☐ the International Searching Authority only
☐ the International Preliminary Examining Authority only

in connection with the international application identified below:

Title of the invention: COMPOSITIONS AND METHODS FOR MODULATING CONNEXIN HEMICHANNELS

Applicant's or agent's file reference: 016-2003 WO1

International application number (if already available): PCT/DK03/00058

filed with the following Office Danish Patent and Trademark Office as receiving Office and to make or receive payments on behalf of the undersigned.

Signature of the applicant(s) *(where there are several applicants, each of them must sign; next to each signature, indicate the name of the person signing and the capacity in which the person signs, if such capacity is not obvious from reading the request or this power)*:

X

Morten Schak Nielsen
Inventor and Applicant for the US only

Date: Mort Schak Niel

Form PCT/Model of power of attorney (for a given international application) (July 1992)

PCT

POWER OF ATTORNEY

(for an international application filed under the Patent Cooperation Treaty)

(PCT Rule 90.4)

The undersigned applicant(s) *(Names should be indicated as they appear in the request)*:

MEIER, Eddi
Bringebakken 88
DK-3500 Værløse
Denmark

hereby appoints (appoint) the following person as: ☐ agent ☒ common representative

Name and address
(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)

ZEALAND PHARMA A/S
Smedeland 26B
DK-2600 Glostrup
Denmark

to represent the undersigned before

☒ all the competent International Authorities
☐ the International Searching Authority only
☐ the International Preliminary Examining Authority only

in connection with the international application identified below:

Title of the invention: COMPOSITIONS AND METHODS FOR MODULATING CONNEXIN HEMICHANNELS

Applicant's or agent's file reference: 016-2003 WO1

International application number (if already available): PCT/DK03/00058

filed with the following Office Danish Patent and Trademark Office as receiving Office and to make or receive payments on behalf of the undersigned.

Signature of the applicant(s) *(where there are several applicants, each of them must sign; next to each signature, indicate the name of the person signing and the capacity in which the person signs, if such capacity is not obvious from reading the request or this power)*:

Eddi Meier
Inventor and Applicant for the US only

Date: 19-02-2003

Form PCT/Model of power of attorney (for a given international application) (July 1992)

PCT

POWER OF ATTORNEY

(for an international application filed under the Patent Cooperation Treaty)

(PCT Rule 90.4)

The undersigned applicant(s) *(Names should be indicated as they appear in the request)*:

STEINESS, Eva
Sømarksvej 11
DK-2900 Hellerup
Denmark

hereby appoints (appoint) the following person as: ☐ agent ☒ common representative

Name and address
(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)

ZEALAND PHARMA A/S
Smedeland 26B
DK-2600 Glostrup
Denmark

to represent the undersigned before

☒ all the competent International Authorities
☐ the International Searching Authority only
☐ the International Preliminary Examining Authority only

in connection with the international application identified below:

Title of the invention: COMPOSITIONS AND METHODS FOR MODULATING CONNEXIN HEMICHANNELS

Applicant's or agent's file reference: 016-2003 WO1

International application number (if already available): PCT/DK03/00056

filed with the following Office Danish Patent and Trademark Office as receiving Office and to make or receive payments on behalf of the undersigned.

Signature of the applicant(s) *(where there are several applicants, each of them must sign; next to each signature, indicate the name of the person signing and the capacity in which the person signs, if such capacity is not obvious from reading the request or this power)*:

Eva Steiness
Inventor and Applicant for the US only

Date: Feb. 14, 2003

Form PCT/Model of power of attorney (for a given international application) (July 1992)

PCT

POWER OF ATTORNEY

(for an international application filed under the Patent Cooperation Treaty)

(PCT Rule 90.4)

159

The undersigned applicant(s) *(Names should be indicated as they appear in the request)*:

JENSEN, Peter Holme
Classensgade 11, 3. tv.
DK-2100 Copenhagen Ø
Denmark

hereby appoints (appoint) the following person as: ☐ agent ☒ common representative

Name and address
(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)

ZEALAND PHARMA A/S
Smedeland 26B
DK-2600 Glostrup
Denmark

to represent the undersigned before

☒ all the competent International Authorities
☐ the International Searching Authority only
☐ the International Preliminary Examining Authority only

in connection with the international application identified below:

Title of the invention: COMPOSITIONS AND METHODS FOR MODULATING CONNEXIN HEMICHANNELS

Applicant's or agent's file reference: 016-2003 WO1

International application number (if already available): PCT/DK03/00058

filed with the following Office Danish Patent and Trademark Office as receiving Office and to make or receive payments on behalf of the undersigned.

Signature of the applicant(s) *(where there are several applicants, each of them must sign; next to each signature, indicate the name of the person signing and the capacity in which the person signs, if such capacity is not obvious from reading the request or this power)*:

X
Peter Holme Jensen
Inventor and Applicant for the US only

Date: X 18-02-03 Peter Holme

Form PCT/Model of power of attorney (for a given international application) (July 1992)

PCT

160

POWER OF ATTORNEY

(for an international application filed under the Patent Cooperation Treaty)

(PCT Rule 90.4)

The undersigned applicant(s) *(Names should be indicated as they appear in the request)*:

HANSEN, Lars Bo Laurenborg
Peberhaven 19, st. tv.
DK-2730 Herlev
Denmark

hereby appoints (appoint) the following person as: ☐ agent ☒ common representative

Name and address
(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)

ZEALAND PHARMA A/S
Smedeland 26B
DK-2600 Glostrup
Denmark

to represent the undersigned before

☒ all the competent International Authorities
☐ the International Searching Authority only
☐ the International Preliminary Examining Authority only

in connection with the international application identified below:

Title of the invention: COMPOSITIONS AND METHODS FOR MODULATING CONNEXIN HEMICHANNELS

Applicant's or agent's file reference: 016-2003 WO1

International application number (if already available): PCT/DK03/00056

filed with the following Office Danish Patent and Trademark Office as receiving Office and to make or receive payments on behalf of the undersigned.

Signature of the applicant(s) *(where there are several applicants, each of them must sign; next to each signature, indicate the name of the person signing and the capacity in which the person signs, if such capacity is not obvious from reading the request or this power)*:

Lars Bo Laurenborg Hansen
Inventor and Applicant for the US only

Date: 7/2 2003

Form PCT/Model of power of attorney (for a given international application) (July 1992)

What is claimed is:

1. A method of closing a hemichannel in a cell, tissue or organ exposed to stress, the method comprising contacting the stressed cell, tissue or organ with a therapeutically effective amount of at least one compound selected from the group consisting of compounds represented as Formula I or II , wherein the contact is sufficient to close the hemichannel in the stressed cell, tissue or organ.

2. The method of claim 1, wherein the method further comprises phosphorylating a tyrosine residue of connexin 43 (Cx 43) and closing the hemichannel.

3. A method for opening a hemichannel in a cell, tissue or organ, the method comprising contacting the cell, tissue or organ with a therapeutically effective amount of at least one compound selected from the group consisting of compounds represented as Formula I or II , wherein the contact is sufficient to open the hemichannel in the cell, tissue or organ.

4. The method of claim 3, wherein the method further comprises dephosphorylating a serine residue of connexin 43 (Cx 43) and opening the hemichannel.

5. A method of preventing or treating tissue or organ stress in a mammal, the method comprising administering a therapeutically effective amount of at least one compound selected from the group consisting of compounds represented as Formula I or II as described above, wherein the contact is sufficient to prevent or treat the stress in tissue or organ.

6. The method of claim 5, wherein the method further comprises phosphorylating a tyrosine residue of connexin 43 (Cx 43) and closing the hemichannel.

7. A method of increasing gap junction intracellular communication (GJIC) in a cell, tissue or organ, the method comprising administering a therapeutically effective amount of at least one

compound selected from the group consisting of compounds represented as Formula I or II, wherein the contact is sufficient to increase the GJIC in the cell, tissue or organ.

8. A method of treatment of burns comprising administering to a patient in need of such treatment a therapeutically effective amount of a compound according to Formula I or II that blocks connexin hemichannel opening.

9. A method of treatment of thromboses comprising administering to a patient in need of such treatment a therapeutically effective amount of a compound according to Formula I or II that blocks connexin hemichannel opening.

10. A method of treatment of respiratory and metabolic acidosis comprising administering to a patient in need of such treatment a therapeutically effective amount of a compound according to Formula I or II that blocks connexin hemichannel opening.

11. A method of treatment of focal arrhythmia comprising administering to a patient in need of such treatment a therapeutically effective amount of a compound according to Formula I or II that blocks connexin hemichannel opening.

12. A method of treating and preventing cell and tissue damage resulting from elevated levels of blood glucose comprising administering to a patient in need of such treatment a therapeutically effective amount of a compound according to Formula I or II that blocks connexin hemichannel opening.

13. A method of treatment of chronic atrial fibrillation comprising administering to a patient in need of such treatment a therapeutically effective amount of a compound according to Formula I or II that blocks connexin hemichannelopening.

14. A method of treatment of epilepsia comprising administering to a patient in need of such treatment a therapeutically effective amount of a compound according to Formula I or II that promotes connexin hemichannel opening.

15. A method for screening candidate compounds that modulate hemichannel function, the method comprising contacting cells with at least one compound selected from the group consisting of antiarrhythmic peptides and compounds represented as Formula I or II, the contacting being under conditions conducive to modulating phosphorylation of connexin 43 (Cx43); and detecting a change in Cx43 phosphorylation, wherein the change in phosphorylation is taken to be indicative of a hemichannel modulating compound.

16. The method of claim 15, wherein the testing step further comprises testing the candidate compound in an immunological or cell sorting-based assay.

17. The method of claim 16, wherein the immunological assay comprises contacting cells with the candidate compound under conditions sufficient to increase or decrease phosphorylation of the Cx43, producing a lysate of the cells; and detecting increased or decreased Cx43 phosphorylation in the cell lysate as being indicative of the hemichannel modulating compound.

18. The method of claims 16-17, wherein the immunological assay is an ELISA assay.

19. The method of claim 18, wherein the ELISA method further comprises:

a) coating a solid support with a first antibody that specifically binds the connexin,

b) contacting the cell lysate to the solid support under conditions conducive to forming a binding complex between the first antibody and the connexin,

c) contacting the first antibody:connexin binding complex with a second antibody, the contacting being under conditions sufficient to form a specific binding complex between the second antibody and any phosphorylated connexin in the first antibody:connexin binding complex,

d) contacting the first antibody:connexin:second antibody binding complex with a detectably-labeled third antibody that binds the second antibody; and

e) detecting presence of the third detectably-labeled antibody on the solid support as being further indicative of the compound.

20. The method of claim 19, wherein the third antibody is detectably-labeled with at least one of biotin, FITC, TRITC, radioactive iodine or peroxidase.

21. The method of claims 19-20, wherein the second antibody is an anti-phosphotyrosine antibody.

22. The method of claims 19-21, wherein the second antibody is an anti-phosphoserine antibody.

23. The method of claims 19-22, wherein the detectably-labeled third antibody is detected by radioscintillation counting.

24. The method of claim 16, wherein the immunological method is a radioimmunoassay (RIA)

25. The method of claim 24, wherein the immunological method is an RIA assay and the method further comprises:

a) detectably-labeling the cells before producing the cell lysate, wherein the labeling is under conditions conducive to labeling phosphorylated connexin,

b) contacting the cell lystate with an antibody that forms a specific binding complex with the connexin,

c) separating the binding complex from the detectably-labeled cell lysate; and

d) detecting the labelled and phosphorylated connexin as being further indicative of the gap junction modulating compound.

26. The method of claim 25, wherein the antibody is an anti-connexin antibody.

27. The method of claims 25-26, wherein the detection step further comprises performing a Western immunoblot assay.

28. The method of claims 25-27, wherein the detectable label is radioactive phosphorous.

29. The method of claims 15-29, wherein the method is performed in a high throughput or ultra high throughput screening format.

30. The method of claims 1-30, wherein the compound facilitates phosphorylation of Cx43 intracellular C-terminal region.

31. The method of claims 1-30, wherein the compound facilitates dephosphorylation of Cx43 C-terminal region.

32. The method of claim 31, wherein the phosphorylation site is a tyrosine residue.

33. The method of claim 32, wherein the dephosphorylation site is a serine residue.

34. A method of screening candidate compounds that modulate hemichannel function, the method comprising:

1) culturing a population of cells, a tissue or an organ such as those derived from the heart or muscle,

2) stressing the cells, tissue or the organ preferably by oxygen deprivation or metabolic inhibition such as by adding a glucose derivative,

3) adding a known or candidate hemichannel modulating compound represented by Formula I or II,

4) adding a detectable reporter such as a fluorescent, chemiluminescent, or phosphorescent compound such as fluorescent dye such as calcein and related compounds,

5) detecting a change in uptake of the detectable reporter into the cells, tissue or organ relative to a suitable control; and

6) optionally measuring the change as being indicative of a compound that modulates hemichannel function.

35. A method of screening candidate compounds that modulate hemichannel function, the method comprising:

1) culturing a population of cells, a tissue or an organ such as those derived from the heart or muscle,

2) loading the cells, tissue or organ with a detectable reporter such as a fluorescent, chemiluminescent, or phosphorescent compound such as dye, such as calcein or a related compound,

3) estimating the volume of the cells, tissue or organ by detecting and quantifying signal from the detectable reporter,

4) adding a known or candidate hemichannel modulating compound represented by Formula I or II ,

5) stressing the cells, tissue or the organ preferably by oxygen deprivation or metabolic inhibition such as by adding a glucose derivative,

6) detecting a change in cell volume relative to a suitable control; and

7) optionally measuring the change as being indicative of a compound that modulates hemichannel function and optionally gap junction function.

36. A method of cytoprotecting tissue or an organ of a mammal in need of such treatment, the method comprising administering a therapuetically effective amount of at least one compound selected from the group consisting of the compounds represented by Formula I or II.

37. The method of claim 37, wherein the method further comprises exposing the tissue or organ of the mammal to ischemic conditions.

38. The method of claim 38, wherein the organ to be cytoprotected is associated with a fibrous capsule or bone.

39. The method of claim 39, wherein the organ is heart, kidney, brain, spinal cord or bone marrow.

40. The method of claim 40, wherein the heart has been subjected to an infarction and the ischemia is associated with myocardial cell swelling.

41. The method of claim 41, wherein the compound is Ac-D-Tyr-D-Pro-D-4Hyp-Gly-D-Ala-Gly-NH2 (Compound 1) or (Ac-Gly-Asn-Tyr-NH2) Compound 2.

42. A method of preventing or treating reperfusion injury in a mammal, the method comprising administering a therapuetically effective amount of at least one compound selected from the group consisting of the compounds represented by Formula I or II.

43. The method of claim 43, wherein the method further comprises exposing the heart of the mammal to infarct conditions and establishing coronary perfusion.

44. The method of claim 44, wherein the method further comprises administering a thrombolytic agent or providing coronary angioplasty to facilitate coronary perfusion into the infarcted heart.

45. The method of claim 45, wherein the compound is Ac-D-Tyr-D-Pro-D-4Hyp-Gly-D-Ala-Gly-NH2 (Compound 1) or (Ac-Gly-Asn-Tyr-NH2) Compound 2.

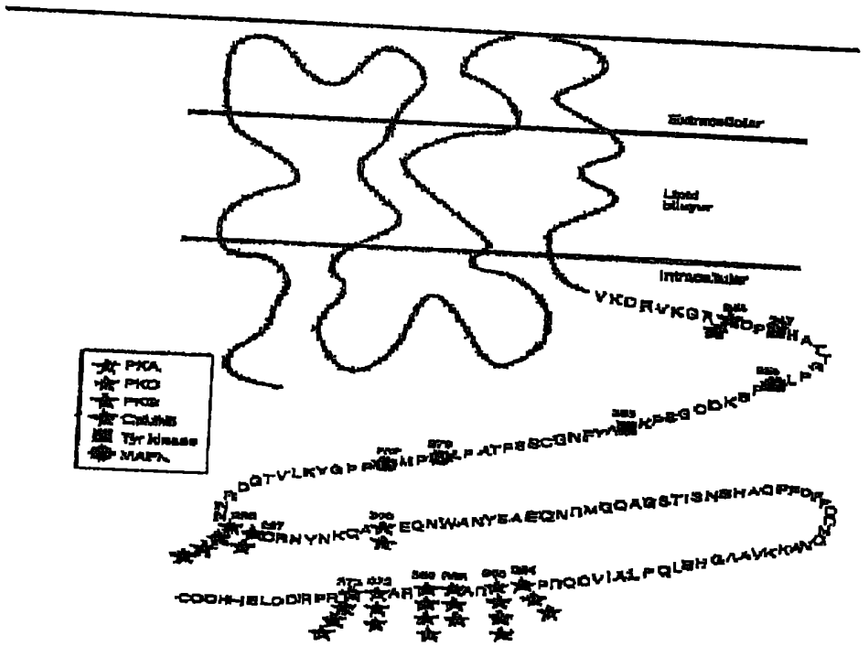
Extracellular
Lipid bilayer
Intracellular
PKA
PKC
PKG
Cdk/MB
Tyr kinase
MAPK

Figure 1

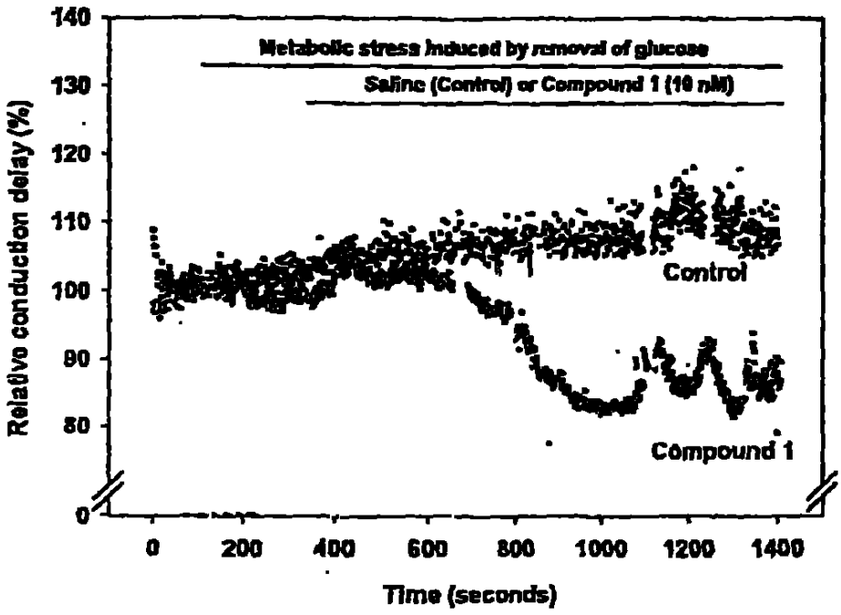
Metabolic stress induced by removal of glucose
Saline (Control) or Compound 1 (10 nM)
Control
Compound 1
Relative conduction delay (%)
Time (seconds)
140
130
120
110
100
90
80
0
0
200
400
600
800
1000
1200
1400

Figure 2

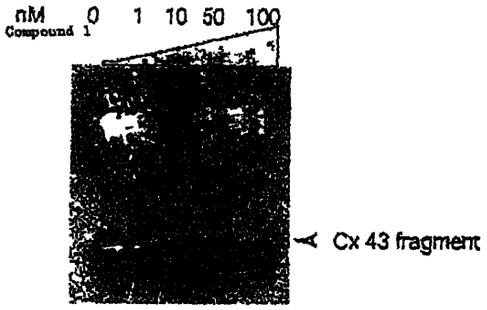

Figure 3A

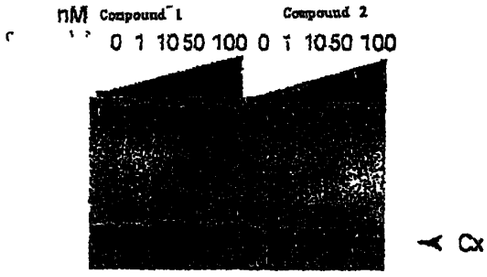

Figure 3B

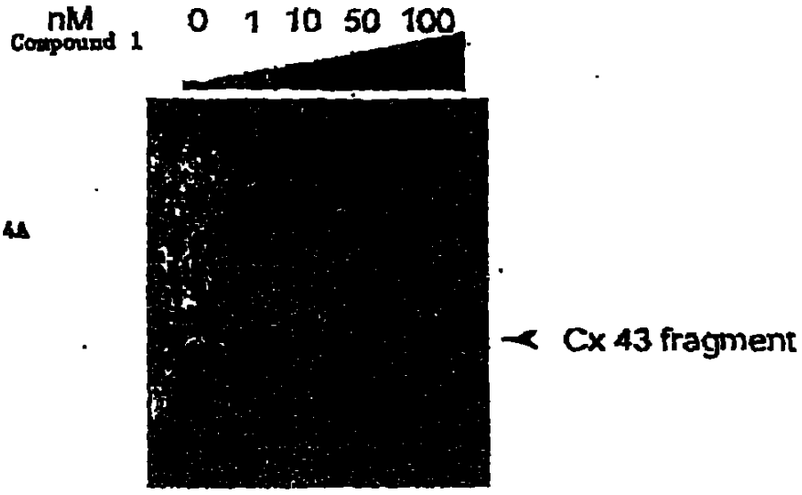

Figure 4A

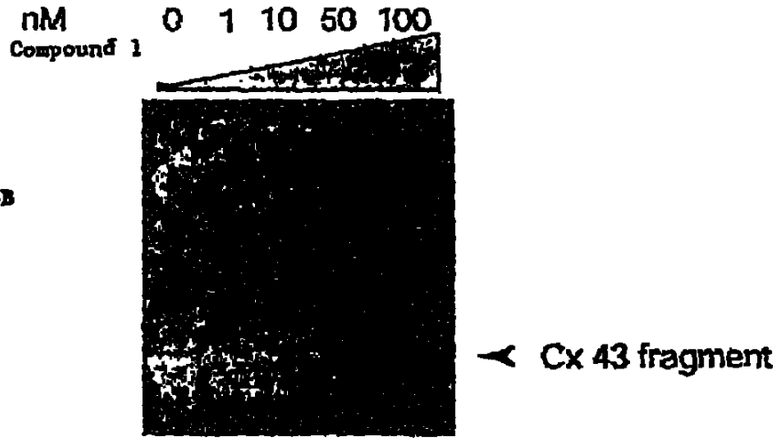

Figure 4B

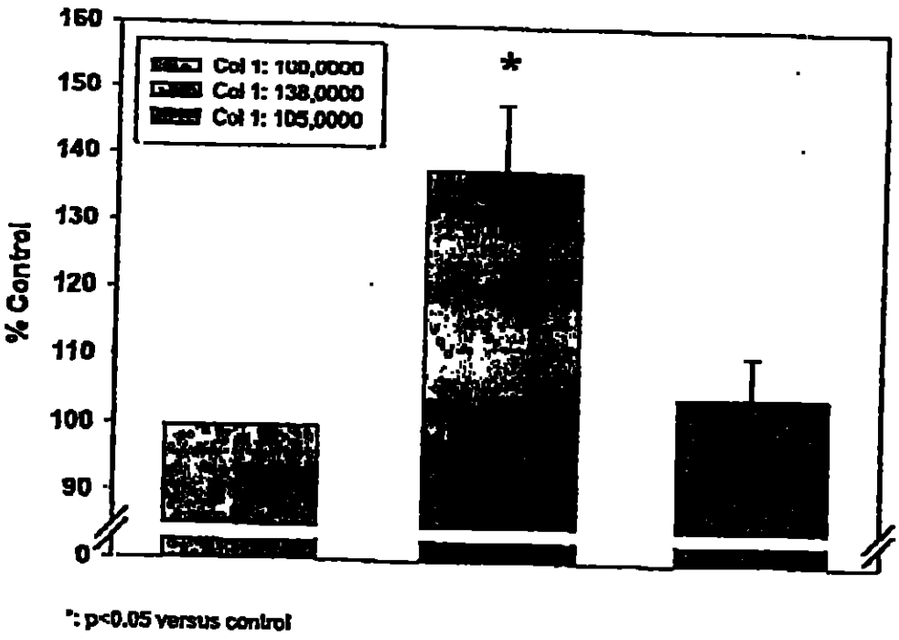
160
150
140
130
120
110
100
90
0
% Control
Col 1: 100,0000
Col 1: 138,0000
Col 1: 105,0000
*
*: p<0.05 versus control

Figure 5

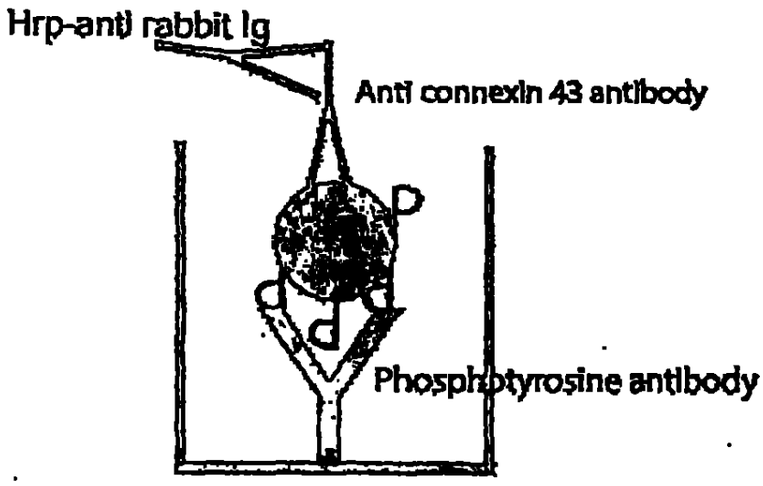
Hrp-anti rabbit Ig
Anti connexin 43 antibody
P
P
P
Phosphotyrosine antibody

Figure 6A

ELISA of phosporylated Cx43 at Tyr-P in HeLa cells

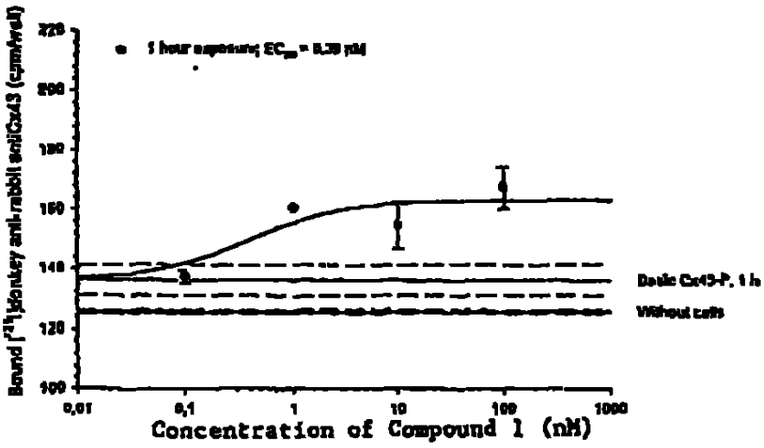

Figure 6B

ELISA of phosporylated Cx43 at Tyr-P in CHO cells

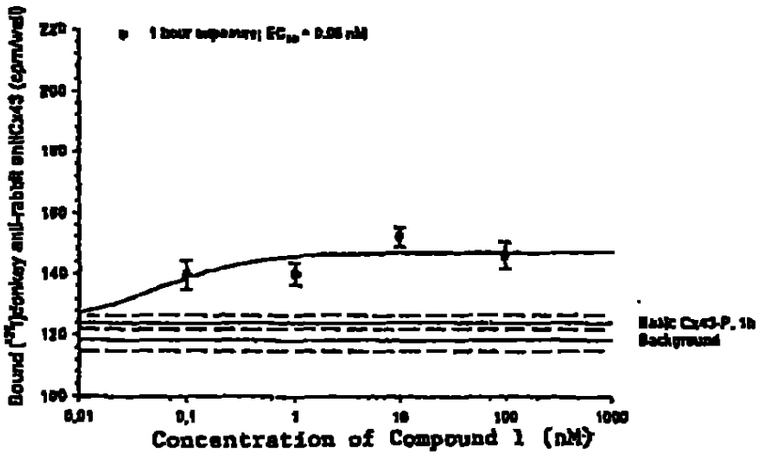

Figure 6C

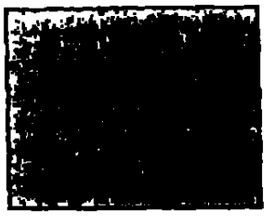

Fig. 7A

Fig. 7B

Fig. 7C

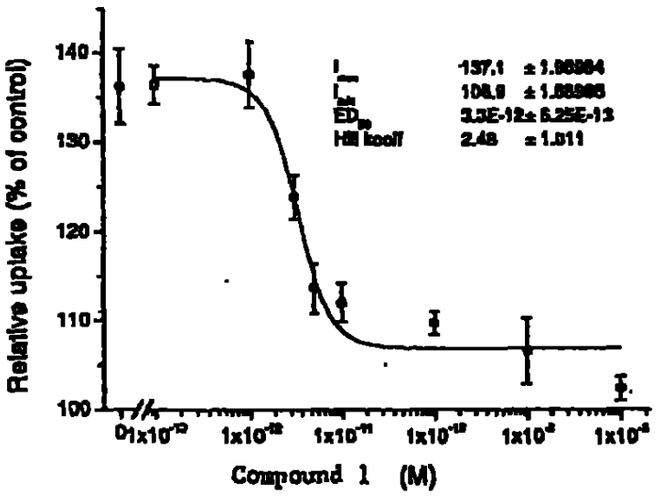
Relative uptake (% of control)
140
130
120
110
100
0
1x10⁻¹²
1x10⁻¹⁰
1x10⁻⁹
1x10⁻⁸
1x10⁻⁷
1x10⁻⁶
Compound 1 (M)
137.1 ± 1.88884
108.9 ± 1.88986
3.3E-12± 5.25E-13
2.48 ± 1.011

Fig. 8

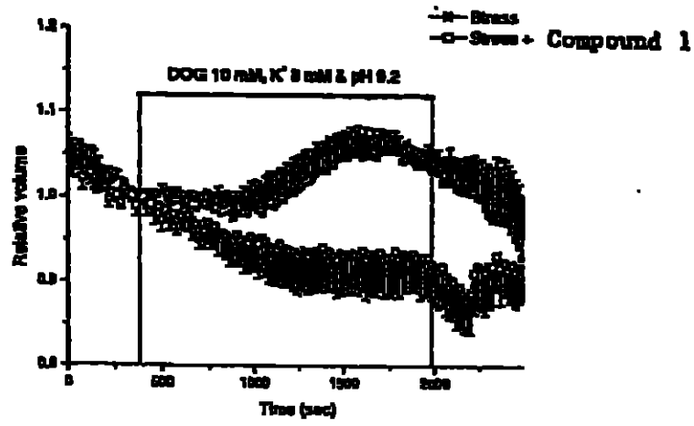
Stress
Stress + Compound 1
DOG 10 mM, K+ 8 mM & pH 6.2
Relative volume
Time (sec)

Fig. 9A

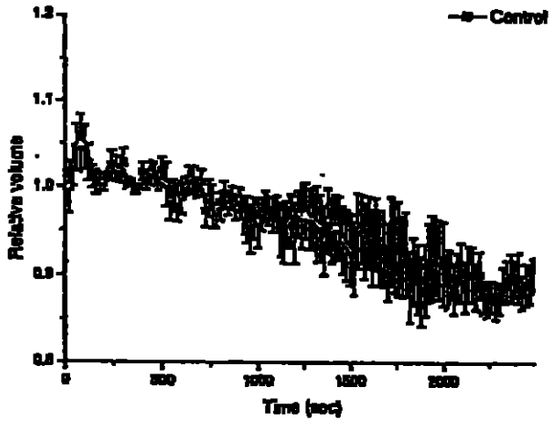
Control
Relative volume
Time (sec)

Fig. 9B

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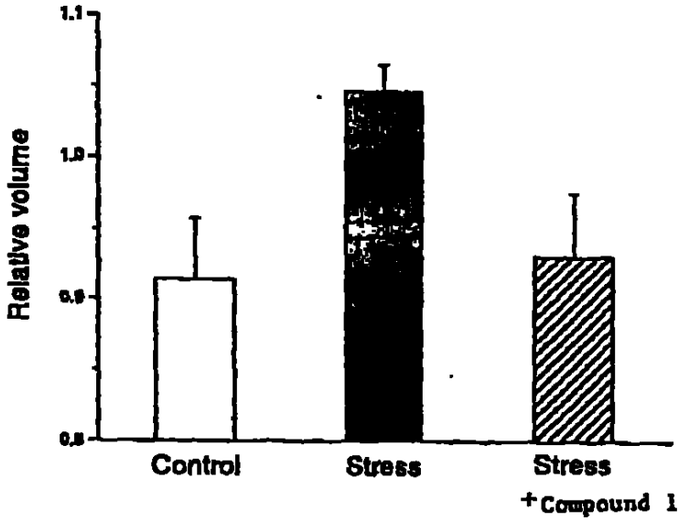

Relative volume
1.1
1.0
0.9
0.8
Control
Stress
Stress
+Compound 1

Fig. 10

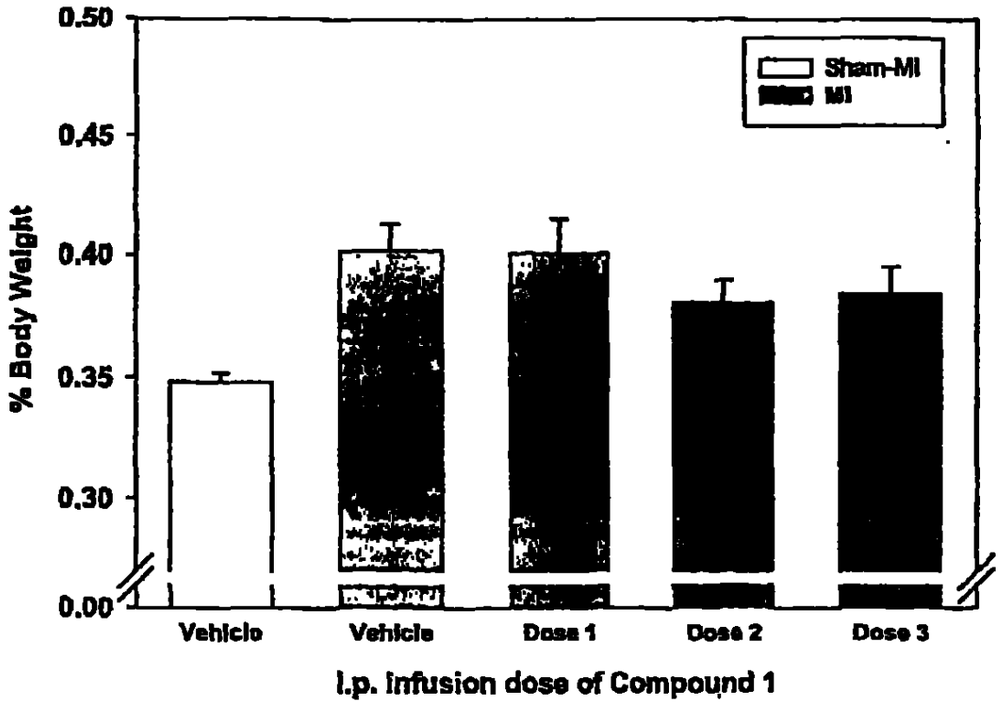
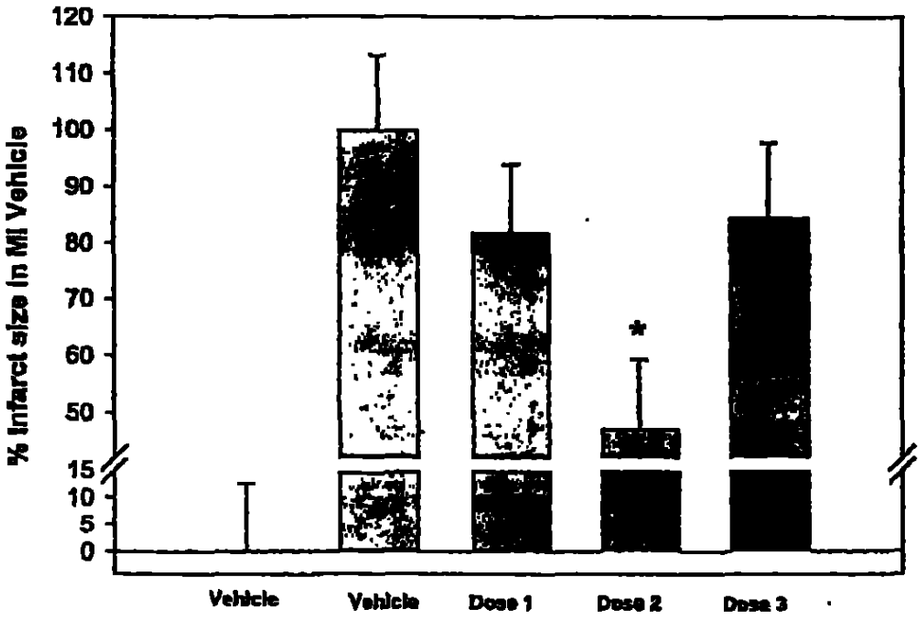

0.50
0.45
0.40
0.35
0.30
0.00
% Body Weight
Sham-MI
MI
Vehicle
Vehicle
Dose 1
Dose 2
Dose 3
I.p. infusion dose of Compound 1

Fig. 11



*: p<0.05 *versus* MI Vehicle

Fig. 12

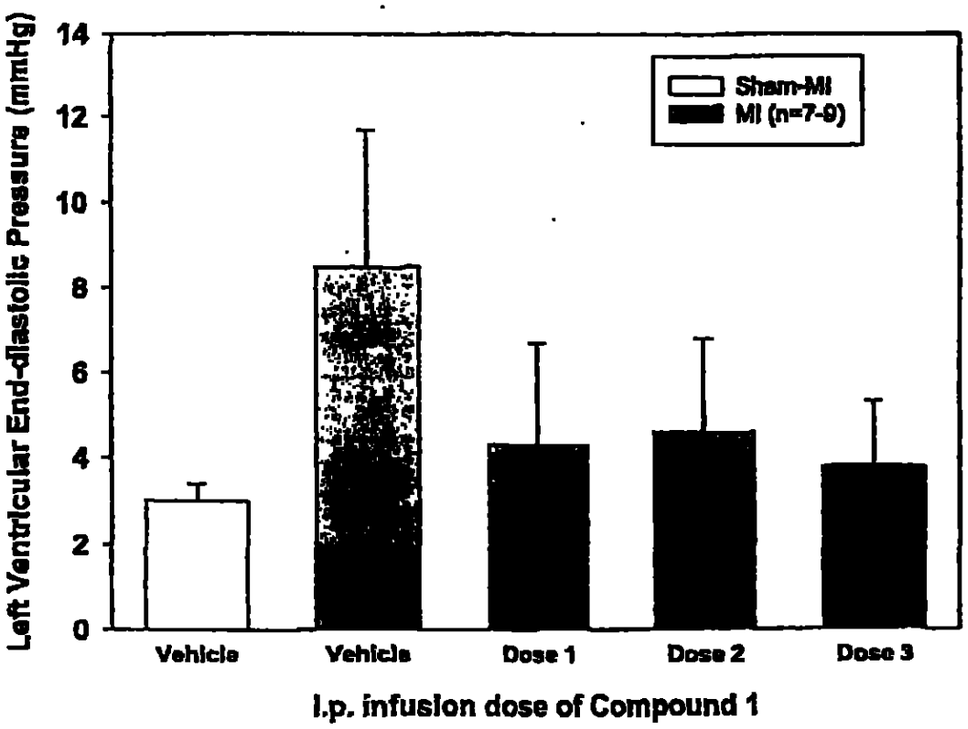

Left Ventricular End-diastolic Pressure (mmHg)
14
12
10
8
6
4
2
0
Sham-MI
MI (n=7-9)
Vehicle
Vehicle
Dose 1
Dose 2
Dose 3
I.p. infusion dose of Compound 1

Fig. 13

COMPOSICIONES Y MÉTODOS PARA MODULAR HEMICANALES DE CONEXINA

REFERENCIA CRUZADA A SOLICITUDES RELACIONADAS

La presente solicitud reivindica prioridad de la solicitud provisional estaunidense serie No. 60/352,717 presentada en enero 29, 2002, cuya descripción se incorpora aquí como referencia.

CAMPO DE LA INVENCIÓN

La presente invención se relaciona de manera general con composiciones y métodos para modular hemicanales de conexina. La invención también se relaciona con tamices útiles para detectar tales compuestos,

ANTECEDENTES

Existe reconocimiento de que las uniones puentes son estructuras de membrana de plasma importantes que ayudan a las células a comunicarse con su ambiente. Por ejemplo, la mayoría de las uniones puente se cree que ayuda al paso de moléculas pequeñas iones entre células

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interconectadas. Tal movimiento se cree que ejerce profundos efectos en muchos aspectos de la fisiología celular. Las membranas de plasma de las células adyacentes se cree que incluyen hemicanales, "conexiones", formadas por proteínas multiméricas denominada "conexinas" que ayudan a formar las uniones puente. Además, los hemicanales juegan un papel independiente en el intercambio de compuestos de peso molecular pequeño entre el citoplasma de la célula y el espacio periplásmico o extra celular. Ver de manera general Bennett, M. et al. (1991) *Neuron* 6:305; Kumar, and Gilula, N.B. (1996) *Cell* 84: 381; y Quist, A.P et al (2000) J. Cell Biol. 148: 1063 y las referencias citadas aquí.

En particular, existen reportes de que muchas uniones puente son regiones especializadas de la membrana celular con agrupados de canales densamente empacados. Tales canales de uniones puente se creen que conectan directamente el compartimiento citoplasmático de dos células vecinas.

Se reconoce que los canales de las uniones puente estás compuesta de dos hemicanales (conexiones) provistas por

cada una de las dos células vecinas. Cada conexión (hemicanal) ha sido descrito como consistente de seis proteínas denominadas conexinas. Cada conexina se cree que comparte cuatro dominios de transmembrana, dos ciclos extracelulares, y un ciclo citoplasmático. La conducción del impulso eléctrico se cree que tiene lugar a través de las uniones puente, facilitando de esta manera la conducción y el ritmo normal del corazón. Ver de manera general P. A. Guerrero, R. B. et al. *J Clin Invest* 1997, 99 1991; D.L. Lerner, K. A. et al., *Circulation* 1999, 99 1508; S. Kirchhoff, E. et al. *Curr Biol* 1998, 8 295.

La distribución de la mayoría de las conexinas del corazón se cree que varía significativamente a través del órgano. Sea descrito que la isoforma Cx43 es el mayor tipo ventricular aunque la Cx40 es la isoforma más abundante en las arterias y el sistema conductivo.

Existen reportes de fuertes nexos entre anormalidades de conexina y enfermedades del corazón. Ver a A.C. de Carvalho, et al., *J Cardiovas Electrophysiol* 1994, 5 686; R.R. Kaprielian, et al., Circulation 1998, 97 651; N.S. Peters, et al., *Circulation* 1993, 88 864; y J.E. Saffitz, R.B: et al., Cardiovasc Res 1999, 42 309.

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Existe entendimiento de que la expresión anormal, distribución y regulación de las uniones puente están involucras en arritmias. Los péptidos arrítmicos descritos por Larsen, B. et al., en la PCT/DK01/00127 (WO01/62775) se han reportado por incrementar la comunicación intracelular de la unión puente (GJIC) en tejido de vertebrados.

Se han reportado proteínas particulares de unión puente de mamíferos codificadas por la familia de gen conexina (Cx). Ver Bruzzone, R. et al. (1996) *Eur. J. Biochem* 238: 1. La familia Cx incluye Cx 26, 30. 31, 32, 37, 40. 43, 45, 46 y 50. Los canales e la unión puente también se han encontrado en invertebrados, donde las proteínas formadoras de canal son denominadas "inexinas".

Existe entendimiento de que la mayoría de las conexinas pueden ser fosforiladas excepto para la proteína Cx26. La proteína Cx43 es ampliamente expresada en tejidos. Existen reportes de que la fosforilación de la proteína Cx43 efectúa la comunicación intracelular de la unión puente (GJIC). Por ejemplo, se reconoce que la transferencia, tráfico, fosforilación y recte de la Cx43

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es impactada por la fosforilación. Ver Darrow, B.J., et al. (1995). *Circ Res* 76: 381.

Por ejemplo, Saffitz y los coautores han demostrado utilizando mediciones de conductancia que durante la isquemia existe un incremento en la desfosforilación de la cerina conexina en quince minutos. Ver figura 1 (que muestra una proteína de transmembrana Cx43 de mamífero con varios sitios de fosforilación identificados) La fosforilación y la solubilidad de las conexinas ha atraído el interés de los investigadores. En particular, se encontró que Cx43 era fosforilado en las células mió epiteliales de las glándulas mamarias de rata. Ver Wang, Y., et al. (1995). J Biol Chem 270. 26581; y Yamanaka, I., et al. (1997). Eur J Cell Biol 72: 166.

Como se mencionó, los canales de la unión puente se cree que son poros especializados que conectan el citoplasma de las células encinas. Los hemicanales se comunican con el ambiente extracelular. Han existido reportes de que la inhibición metabólica de las células del corazón puede activar una senda de influjo que se puede estructurar mediante hemicanales de conexina. La inhibición metabólica se cree que abre el hemicanal y mejora la

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perdida de potasio, e induce el influjo de protones, sodio y calcio, dañando de esta manera el tejido cardiaco. Ver Kondo et al J Mol. Cell. Cardiol. 32: 1859-72, 2000; Li et al J. Mol. Cell. Cardiol. 33: 2145-55, 2001).

El desacoplamiento eléctrico en las unión puentees durante la isquemia aguda del miocardio se cree que contribuye a la conducción de anormalidades y arritmias reentrantes. Los niveles crecientes de Ca^{2+} y H^{+} intracelular y la acumulación de metabolitos e lípido anfipático durante la isquemia promueve el desacoplamiento. Otros mecanismos pueden jugar un papel. Por ejemplo, se ha reportado que el desacoplamiento inducido por isquemia aguda se asocia con cambios en la fosforilación e la conexina 43 (Cx43). Se han reportado resultados que son consistentes con la desfosforilación rápida, reversible de la Cx43 que juega un papel en el desacoplamiento del miocardio y la arritmogénesis durante isquemia aguda. Ve Beardslee MA et al., Cir Res. 2000; 87: 656 y las referencias citadas aquí.

La estructura y la función de los hemicanales han atraído el interés.

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Por ejemplo, la microscopia de fuerza atómica (AFM), el ensayo de toma de tinte fluorescente, y las imágenes inmunofluorescentes con focales láser han sugerido que los hemicanales están involucrados en al modulación dependiente de calcio extracelular del volumen de la célula. Como se reportó los cambios del volumen de la célula eran dependientes sea fuera o no que se expresará la conexina 43. Fueron reportados cambios que eran evitables por los bloqueadores de la unión puente (por ejemplo oleamida y ácido beta-gliciretinico) o era reversado al regresar el calcio extracelular a normal. Se sugirió que los hemicanales sin unión puente regulan el volumen de la célula en respuesta al cambio en al calcio de la fisiología extracelular en una situación de otra manera isosmótica. Ver Quist, A. P. et al, supra.

Se ha propuesto que los hemicanales abiertos, especialmente durante la isquemia o la tensión metabólica, puedan conducir a la toma celular de protón Ca^{2+}, y la acumulación de metabolitos lípidos anfipáticos en células que originan el hinchamiento celular, en daño de célula o la apoptosis. Ver Beardlee et al., supra y Quist et al. supra.

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Han existido reportes del que Cx43 es fosforilado en las posiciones Tyr247 (Y247), Tyr265 (Y265) y quizás otras posiciones mediante la proteína Src activada in Vitro. Significativamente, la comunicación intracelular de unión puente (GJIC) se reportó por ser resistente a la disrupción mediante la fosforilación mediada por v-Src. Se reconoció que la fosforilación en el Y247 y Y265 de Cx43 es importante. Ver Lin R, et al. (2001) J Cell Biol 154 (4): 815. Ver también figura 1.

Ver también Larsen, B.D et al. en la solicitud PCT titulada New Medical Uses o Intracellular Communication Facilitating compounds presentada el 22 de febrero e 2002 como PCT/US02/05773 (WO 02/77017) en la cual una variedad de compuestos modulantes GJIC e han descrito.

Sería deseable tener compuestos que modules la función de hemicanal. Los compuestos preferidos ayudarían a abrir o cerrar el hemicanal. Sería especialmente deseable tener tamices moleculares para identificar tales compuestos.

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RESUMEN DE LA INVENCIÓN

La invención se caracteriza de manera general por compuestos y métodos para utilizar los mismos para modular la función hemicanal. Los compuestos particulares modulan la fosforilación del hemicanal.

Compuestos adicionales de la invención modulan la función hemicanal y en algunos casos también impactan la comunicación del unión puente (GJIC) por ejemplo, al operar o cerrar los canales de la unión puente. También se suministran tamices útiles para detectar y caracterizar tales compuestos. La invención tiene muchas aplicaciones útiles incluyendo el suministro de terapias para tratar o evitar varias condiciones modulada por la función inadecuada del hemicanal.

Existe urgente necesidad de identificar compuesto que modulen (incrementen o diminuyan) la función del hemicanal. Por "función del hemicanal" se significa la abertura o cierre de los hemicanales de conexina para mejorar o disminuir el paso de moléculas o iones a través del hemicanal. Por la frase "función de unión de puente" se significa la apertura o cierre de la unión puente para

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incrementar o reducir el paso de moléculas o iones a través de la unión puente. Los compuestos de la invención preferidos modulan la fosforilación del hemicanal y, típicamente también ayudad a la abertura o cierre del hemicanal. La función del hemicanal y la función de la unión puente pueden ser fácilmente dirigida y opcionalmente cuantificada por uno o más de los ensayos estándares descritos aquí.

La mayoría de los compuestos de modulación de hemicanal específicos modula (incrementan o disminuyen) la fosforilación o una conexina reconocida. Los sitios preferidos de fosforilación o desfosforilación incluyen uno o más de un residuo de tirosina, serina o treonina sobre la conexina. Como se discutirá adelante se ha encontrado que la modulación de la fosforilación en uno o más de estos residuos impacta la función hemicanal, particularmente al abrir y cerrar el hemicanal. Así, como se describe adelante es un objeto de la invención suministrar tamices adaptados para monitorear el estado de fosforilación de las conexinas como un medio para detectar y opcionalmente caracterizar compuestos con capacidad para modular la función hemicanal.

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Por ejemplo, ciertos compuestos de acuerdo con la invención son capaces de fosforilar al menos un residuo de tirosina de la conexina. En esta modalidad, la fosforilación de la conexina ayudara a cerrar el hemicanal. Otros compuestos para modular el hemicanal adecuados disminuyen la fosforilación (desfosforilar) al menos una serie de residuos de la conexina. Es este ejemplo, la desfosforilación de la serina ayudara a abrir el hemicanal. Aun otros compuestos dentro el alcance de la invención mejoraran la fosforilación de al meno un residuo de treonina de la conexina, típicamente para ayudar en el cierre del hemicanal. Adicionalmente, los compuestos adecuados de la invención facilitan al menos uno de un incremento o disminución en la fosforilación de la serina, un incremento o disminución en la fosforilación de tirosina, y un incremento o disminución en la fosforilación de treonina de la conexina. Preferiblemente, uno o más de las modificaciones de aminoácido ayudaran en una abertura o cierre detectable del hemicanal.

De acuerdo con esto, en un aspecto, la invención suministra un método para modular la función hemicanal preferiblemente al cerrar el hemicanal. En una modalidad,

el método involucra cerrar el hemicanal en una célula, tejido u órgano preferiblemente expuesto a tensión incluyendo poner en contacto la célula, tejido u órgano tensionado con una cantidad terapéuticamente efectiva de al menos uno de los compuestos representados adelante como formula I o II. El contacto preferido de acuerdo con esta modalidad de método es suficiente para cerrar el hemicanal antes, durante o después de la exposición a la tensión con relación al control adecuado. Ejemplos de tales tensones incluyen uno o más de la inhibición metabólica, privación de oxígeno, disminución de pH, o incremento del ión potasio extracelular como se describe adelante.

En una modalidad más especifica, el método incluye además monitorear la fosforilación de una conexina reconocida, preferiblemente la conexina 43 (Cx43). Típicamente, el método detectará y reportará cualquier incremento o disminución en la fosforilación del Cx43 sobre al menos uno de un residuo de tirosina, serina y treonina, preferiblemente un incremento en la fosforilación de al menos una de tirosina y treonina. En esta modalidad, el método también incluye cerrar el hemicanal y,

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opcionalmente, abrir y cerrar los canales de unión puente relativos a un control adecuado.

La invención suministra otros métodos para modular la función hemicanal. En una modalidad, el método involucra monitorear la fosforilación de una conexina reconocida, preferiblemente Cx43, para detectar cualquier incremento o disminución en la fosforilación del Cx43 en al menos uno de un residuo de tirosina, serina y treonina, preferiblemente una disminución en la fosforilación de uno o más de aquellos residuos tales como serina con relación a un control. En este ejemplo de la invención, el método involucra abrir el hemicanal en una célula tejido u órgano expuesto a tensión incluyendo conectar la célula, tejido u órgano tensionado con una cantidad terapéuticamente efectiva de al menos uno de los compuestos representados adelante como formula I o II. El contacto preferido suficiente para abrir el hemicanal y, opcionalmente abrir y cerrar los canales e unión puente con relación a un control adecuado.

Subsiste la necesidad de tamices para detectar y caracterizar tales compuestos que modulan el hemicanal más específicamente. Teniendo tales tamices sería un

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primer paso importante hacia la identificación y caracterización un rango de nuevos compuestos que modulan el hemicanal por ejemplo. Mediante ensayo in Vitro de un compuesto candidato con relación a un control para detectar la capacidad para ayudar a cerrar hemicanales, por ejemplo, por al menos 5% mas que un compuesto e control. Los compuestos identificados mediante tal tamiz, incluyendo los compuestos representados adelante como formula I o II, se pueden utilizar en terapias que promueven la homeostasis celular de tejido y órgano, por ejemplo, al evitar reducir o proteger contra la perdida de los componentes celulares al ambiente extracelular.

De acuerdo con esto la presente invención también suministra métodos específicos in Vitro para tamizar compuestos candidatos que tengan la capacidad de modular la función hemicanal. Típicamente, el método involucra pone en contacto célula, tejido y o un órgano adecuado con al menos un compuesto representado adelante como formula I o II. Preferiblemente, el contacto bajo condiciones que conducen a modular la fosforilación de una conexina reconocida preferiblemente la conexina 43 (Cx43) y detectar un cambio en la fosforilación del Cx43 con relación a un control adecuado. También

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preferiblemente, el cambio en la fosforilación es tomado por ser indicativo de u compuesto modulador de hemicanal. Opcionalmente, tales compuestos detectados mediante el método de tamizado pueden abrir o cerrar canales de unión puente de acuerdo con ensayos descritos aquí.

En cada uno de los métodos anteriores, los cambios de fosforilación preferidos de acuerdo con la invención ocurren casi completamente o cerca al Terminal C intracelular de la conexina. Los sitos mas preferidos de fosforilación y defosforilación de Cx43 son mostrados en la figura 1. Mediante la frase "Terminal C de conexina" se significa la región amarrada alrededor de los residuos aminoácidos 240 a 281 como se muestra en la figura 1.

Un ensayo de tamizado particular in Vitro de la invención para detectar fosforilación de conexina involucra una o más etapas diseñadas para monitorear la función hemicanal, la función de unión puente (o ambas). Tal ensayo generalmente incluye al menos uno y preferiblemente todas las siguientes etapas:

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1) Cultivar una población de células, un tejido o un órgano tal como aquellos derivados del corazón o músculo,
2) Tensionar las células, tejido o el órgano preferiblemente mediante la privación e oxígeno o inhibición metabólica,
3) Agregar un compuesto que modula hemicanal conocido o candidato tal como aquellos representados por la formula I o II dado adelante,
4) Detectar un cambio en la fosforilación de la conexina (preferiblemente Cx43) con relación a un control adecuado; y
5) Medir opcionalmente el cambio como siendo indicativo de un compuesto que modula la función hemicanal y opcionalmente la función de unión puente.

Esos ensayos pueden medir efectivamente la capacidad del hemicanal que modula el compuesto para incrementar o medir la fosforilación de al menos un residuo de serina, tirosina y treonina de la proteína preferida Cx43. La referencia aquí a un "ensayo estándar de fosforilación de conexina in Vitro" o una frase relacionada se refiere al protocolo anterior de las etapas 1) hasta 5). El ensayo puede ser conducido con aproximadamente cualquier

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población de células primarias, secundarias o inmortalizadas tales como aquellas derivas del corazón o músculo.

El ensayo estándar in Vitro anterior es generalmente flexible. Por ejemplo, las etapas 1)-5) pueden ser desarrolladas en aproximadamente cualquier orden siempre y cuando se logre los resultados del tamizado pretendido. Así en una modalidad el ensayo, el compuesto candidato se agrega en la etapa 1) etapa 2) (o ambas etapas) en lugar de después de la etapa 2) exclusivamente.

La presente invención suministra otros métodos para tamizar compuestos candidatos por la capacidad para modular la función hemicanal. En una modalidad, el método incluye poner en contacto células, tejido o un órgano adecuado con al menos un compuesto seleccionado del grupo representado adelante como formula I o II. Típicamente, cualquier toma de un reportero detectable por las células, tejidos u órganos es monitoreado en la presencia del compuesto y con relación a un control adecuado. Los reporteros detectables preferidos tienen un tamaño molecular que conduce a un paso a través de un hemicanal abierto. Así, cuando el hemicanal es abierto en el ensayo

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el reportero detectable ingresa a la célula, tejido u órgano. Si embargo cuando el hemicanal es cerrado, el reportero detectable evita pasar a través el hemicanal. Así, cuando el hemicanal es abierto en el ensayo, el reportero detectable entra en la célula, tejido u órgano. Sin embargo, cuando el hemicanal se cierra, el reportero detectable evita pasar al través del hemicanal. El contacto es preferiblemente bajo condiciones que conducen a detectar cualquier cambio en la tomo del reportero detectable con referencia a un control adecuado.

Un hemicanal esta "cerrado" de acuerdo con la invención si al menos uno de los residuos de tirosina en la región C-Terminal de la conexina, preferiblemente Cx43 es fosforilado, preferiblemente al menos la tirosina en la posición 247 o la posición 265, más preferiblemente ambos, detectados por ejemplo en el ensayo estándar de fosforilación de conexina in Vitro. Por "cerrado" se quiere significar al menos que un de los residuos de treonina en la región C-terminal de la conexina es fosforilado, cuyos residuos pueden ser fosforilados solos o en adición a la fosforilación de tirosina. Un hemicanal es "abierto" si al menos uno de los residuos e serina en la región C-terminal e la conexina es desfosforilado.

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Adicionalmente los residuos de tirosina, trionina y serina preferidos son mostrados en la figura 1 como sitios de kinasa.

Un ensayo de tamizado más específico in Vitro para detectar el paso del reportero detectable a través del hemicanal involucra uno o más de las siguientes etapas:

1) Cultivar una población de células, un tejido o un órgano tal como aquellos derivados del corazón o músculo,
2) Tensionar las células, tejido o el órgano preferiblemente mediante privación de oxigeno o inhibición metabólica tal como al agregar un agregado de glucosa,
3) Agregar un compuesto modulador de hemicanal conocido o candidato tal como aquellos representados adelante mediante la formula I o II,
4) Agregar un reportero detectable tal como un compuesto fluorescente, quimioluminiscente, o fosforescente tal como un tinte fluorescente tal como calceina y compuestos relacionados,

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5) Detectar un cambio en la toma del reportero detectable en las células, tejido u órgano con relación a un control adecuado; y
6) Medir opcionalmente el cambio como indicativo del compuesto que modula la función hemicanal.

Ese ensayo puede medir efectivamente la capacidad del compuesto candidato a abrir o cerrar los hemicanales en las células, tejidos u órganos que cuyo cambio es fácilmente detectable microscópicamente mediante la visualización del reportero detectable. Se le referencia aquí a "ensayo de toma estándar in Vitro" o frase relacionadas se refieren al protocolo anterior de etapas 1) hasta 6). El ensayo puede ser conducido con aproximadamente cualquier población de células primarias, secundarias, o inmortalizadas tales como aquellas derivadas del corazón o músculo. Otros reporteros aceptables incluyen compuestos radiactivos adecuados que tiene también un tamaño que permite el paso a través del hemicanal. Los compuestos particulares incluyen aquellos etiquetados con uno o más de los siguientes radio núcleos: ^{3}H, ^{35}S, y ^{14}C.

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De manera importante, el estándar del ensayo de toma in Vitro descrito de manera general anteriormente no esta unido a ningún orden particular de etapas en tanto no se logre los resultados pretendidos del ensayo. Así, en una modalidad, al menos uno de los compuestos candidatos y el reportero detectable de las etapas 3) 4) respectivamente, son agregados individualmente o juntos antes de la etapa 2) en el método. En este ejemplo del ensayo, las células, tejido u órgano es tensionado en la presencia del compuesto y el reportero detectable. De manera alternativa, el reportero detectable puede ser "cargado" en las células en la etapa 1) para detectar el compuesto con capacidad de abrir hemicanales.

La invención suministra métodos adicionales para tamizar uno o más compuestos candidatos por la capacidad para modular la función hemicanal. En una modalidad, el método incluye poner en contacto poner en contacto células, tejido u órgano con al menos un compuesto relacionado con aquellos representados median te la formula I o II. Típicamente, las células, tejido u órgano es cargado con un reportero detectable para medir el volumen. Los reporteros detectables preferidos para uso en el ensayo tiene un tamaño molecular que es adecuado para pasar a

través de un hemicanal abierto. El contacto es preferiblemente bajo condiciones que conducen a detectar cualquier cambio en el volumen de las células, tejido u órgano como registrado por el reportero detectable y al referirse a un control adecuado. Preferiblemente, las células, tejido u órgano es tensionado y el compuesto candidato cierra el hemicanal de tal forma que el volumen celular es mantenido o disminuye más lentamente cuando se compara con un control adecuado.

Un ensayo particular de tamizado in Vitro para detectar los cambios en volumen celular involucran una o más de las siguientes etapas:

1) Cultivar una población de células, un tejido o un órgano tal como aquellos derivados del corazón o músculo,
2) Cargar las células, tejido o el órgano con un reportero detectable tal como un compuesto fluorescente, quimioluminiscente, o fosforescente tal como un tinte, tal como calceina o un compuestos relacionado,

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3) Estimular el volumen de las células, tejido u órgano al detectar una señal cuantificante del reportero detectable,
4) Agregar un compuesto que modula hemicanal conocido o candidato tal como aquellos representados mediante la formula I o II adelante,
5) Tensionar las células, tejido o el órgano preferiblemente mediante privación de oxígeno o inhibición metabólica tal como al agregar un agregado de glucosa
6) Detectar un cambio en el volumen celular con relación a un control adecuado; y
7) Medir opcionalmente el cambio como para ser indicativo de un compuesto que modula la función hemicanal y opcionalmente la función de unión puente.

El ensayo puede medir efectivamente la capacidad del compuesto que modula hemicanal candidato para abrir o cerrar los hemicanales en las células, tejido u órgano al observar los cambios de volumen celular microscópicamente. La referencia aquí "ensayo estándar de volumen celular in vitro" o frase relacionada se refiere al protocolo anterior de las etapas 1) hasta 7). El

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ensayo puede ser conducido con aproximadamente cualquier población de células primarias, secundarias, o inmortalizadas tales como aquellas derivadas del corazón o músculo tal como cardiomiocitos y células o tejidos relacionados.

El ensayo estándar de volumen celular in Vitro no esta unido a un orden particular de etapas en tanto que se logren los resultados pretendidos del ensayo. Así en una modalidad, el compuesto candidato de la etapa 4) se agrega después de estresar las células en la etapa 3) para monitorear la capacidad del compuesto para cerrar los hemicanales en las células ya tensionadas como se ejemplifico mediante un índice más lento de disminución de volumen celular. Así en una modalidad del ensayo se monitorea un cambio en el índice de disminución o incremento del volumen celular durante un marco de tiempo predeterminado. Sin embargo en otra modalidad, el cambio de volumen celular se puede monitorear en un punto de tiempo fijo, por ejemplo, en un tiempo entre aproximadamente 1 a 120 minutos después de tensionar las células, tejido u órgano.

Como se discutió, los ensayos in Vitro de la invención son flexibles y pueden ser adaptados para adecuarse el uso de tamizado pretendido. Por ejemplo, un compuesto modulador de hemicanal candidato particular, tal como aquellos representaos mediante las formulas I o II adelante, se pueden emplear como el único agente activo o en combinación con otros agentes que incluyen otros compuestos a ser ensayados. En la mayoría, pero no es todos los casos, los ensayos in Vitro son desarrollados con referencia aun ensayo de control adecuado que usualmente incluye las mismas condiciones o condiciones de ensayo cercanamente relacionada como en las etapas anteriores, pero sin agregar el compuesto a ser ensayado al medio de cultivo. En tales casos el compuesto modulador de hemicanal candidato puede ser identificado al exhibir al menos 2% de mayor actividad en el ensayo con relación al control, más particularmente al menos más aproximadamente 5% de mayor actividad con relación al ensayo de control, y aun más preferiblemente al menos aproximadamente 10% o mayor actividad, por ejemplo, aproximadamente 20% a aproximadamente 405 con relación al ensayo de control.

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Como se discutió, los compuestos moduladores de hemicanal particulares también impactaran los canales de unión puente. Por ejemplo, ciertos compuestos pueden ayudar a cerrar los hemicanales y ayudar en la abertura de los canales de unión puente. Sin embargo, otros compuestos pueden ayudar a abrir los hemicanales aunque ayudando en el cierre de los canales de unión puente. Aun otros compuestos pueden abrir tanto los hemicanales como la unión puente mientras otros pueden cerrar las uniones puente y los hemicanales.

De acuerdo con esto, la invención también suministra un método para incrementar la comunicación intracelular de unión puente (GJIC) en una célula, tejido u órgano. En una modalidad, el método involucra administrar una cantidad terapéuticamente efectiva de al menos un compuesto seleccionado de aquellos representados mediante la formula I o II como se describe adelante. Preferiblemente el contacto es suficiente para incrementar GJIC en la célula, tejido u órgano con relación a un control adecuado.

Se suministran también combinaciones de ensayos in Vitro en compuestos que son seleccionados por la capacidad para

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modular hemicanales y uniones puentes. En una modalidad, al menos uno de los ensayos de fosforilación estándar de conexina in Vitro, el ensayo estándar de tomo in Vitro y el ensayo estándar de volumen celular in Vitro, se combinan con uno o más de los ensayos GJIC descritos por Larsen, B. et al. en la solicitud PCT titulada Novel Antiarrhythmic Peptides presentada el 22 de febrero de 2001 como PCT/DK01/00127 (WO 01/62775) como se describió por Larsen, B. et al. en otra solicitud PCT titulada New Medical Uses of Intracellular Communication Facilitating compounds presentada el 22 de febrero de 2002 como PCT/US02/05773 (WO 02/077017). Los ejemplos de cada uno de los ensayos adecuados GJIC incluyen aquellas mediciones de conductancia celular mediante mediciones de onda de calcio con abrazadera parche y ensayos de transferencia de tinte. Las descripciones de las solicitudes PCT/DK01/00127 (WO 01/62775) y PCT/US02/05773 (WO 02/077017) son incorporadas aquí como referencia.

Por vía de ilustración y no de limitación, el ensayo de fosforilación estándar de conexina in Vitro descrito anteriormente se emplea para seleccionar 1 o más de compuestos moduladores de hemicanal. Uno o mas de los compuestos pueden subsecuentemente ser tamizados

adicionalmente en ensayo de abrazadera parece de cardiomiocito descrito en la solicitud PCT/US02/05773 (WO 02/077017). Los compuestos que suministra actividad adecuada en ambos ensayos tendrán capacidad para modulas los hemicanales y las uniones puente.

La invención suministra adicionalmente ensayos in vivo de los compuestos moduladores de hemicanal candidatos para ayudar a detectar y opcionalmente cuantificar la capacidad terapéutica para modular una arritmia cardiaca. Como se discutió, se cree que la mayoría de las arritmias cardíacas se pueden evitar, aliviar o tratar mediante el uso de una o un combinación de los compuestos de esta invención. Un modelo de ensayo in Vitro preferido denominado aquí como un "ensayo estándar de arritmia in Vitro en ratón" o frase relacionada a sido descrita en la PCT/DK01/00127 (wo 01/62775) así como también PCT/US02/05773 (WO 02/077017). Ciertos compuestos de acuerdo con la invención prolongaran deseablemente el tiempo la irrupción del bloque ventricular atrial inducido (AV) mediante al menos aproximadamente 20% (calificación de al menos 2) en el ensayo. Otros compuestos exhibirán una prolongación de al menos 60% o el tiempo hasta la irrupción de le bloque AV inducido

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(calificación de la menos 3) en el ensayo. En términos amplios, el ensayo involucra administrar uno o más compuestos aun ratón adecuado inyectando cloruro de calcio para inducir arritmia y detectar el tiempo de la irrupción de la arritmia preferiblemente bloque AV de segundo grado comparado con un control adecuado.

Significativamente, el uso de formato de detección múltiples es decir, una combinación de al menos uno de los ensayos estándares in Vitro y el ensayo de arritmia in vivo como se describió aquí pueden desarrollar eficientemente ensayos múltiples, mejorando de esta manera la precisión y probabilidad de identificar un compuesto modulador de hemicanal (como se representó mediante las formula I y II, por ejemplo) con buena capacidad terapéutica. Esta característica de la invención es especialmente útil cuando grandes números de compuestos van a ser ensayaos. Por ejemplo, algunos o casi todos los compuestos de acuerdo con las formulas I o II pueden ser ensayados. Alternativamente, o adicionalmente los compuestos adecuados pueden ser hechos mediante métodos sintéticos estándar que incluyen manipulación química de tipo combinatorio y luego ensayados de acuerdo con la invención. En las modalidades

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en la cual son practicados los formatos de detección múltiple, es importante notar que la actividad in vitro e in vivo significativa como se determinó mediante los ensayos descritos aquí no es una característica requerida de un compuesto modulador de hemicanal. Esto es, ciertos compuestos descritos aquí exhibirán buena actividad en al menos de los ensayos estándar in Vitro descritos aquí pero no exhibirán actividad significativa en el ensayo estándar de arritmia in vivo. Alternativamente, ciertos otros compuestos exhibirán actividad significativa en el ensayo de arritmia in vivo pero no mostraran buena actividad en uno o mas de los ensayos in Vitro estándar. Sin embargo, un compuesto modulador de hemicanal exhibirá buena actividad en al menos uno de los ensayos in Vitro e in vivo descritos en esta solicitud.

Los compuestos que muestran buena actividad en el ensayo estándar de arritmia in vivo algunas veces serán llamados "compuestos anti arrítmicos" o frases similares para denotar la capacidad para prolongar el tiempo de arritmia en el ensayo.

Como se discutirá con más detalle adelante, los compuestos de la invención pueden ser utilizados para

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evitar o tratar un amplio espectro de condiciones médicas que están asociadas o se sospecha que esta relacionada al paso indeseado o anormal de moléculas y/o iones a través de membranas celulares. Por ejemplo, casi todas las indicaciones médicas descritas en la PCT/DK01/00127 (WO 01/62775) y PCT/US02/05773 (WO 02/077017) pueden estar dirigidas de esta manera. Algunas de dichas indicaciones médicas son descritas aquí. Las indicaciones médicas pueden ser evitadas, aliviadas o tratadas al utilizar una o una combinación de los compuestos descritos en la presente solicitud y particularmente aquellos que muestran actividad en al menos uno de los ensayos in Vitro o in vivo suministrados aquí.

Los compuestos adicionales adecuados para ensayar y utilizar con la presente invención se han descrito por Larsen, B. D. et al. en PCT/US02/05773 (WO 02/077017).

En otro aspecto, la invención suministra un método para evitar o tratar tensión en tejido u órgano en un mamífero. En una modalidad el método incluye administrar una cantidad terapéuticamente efectiva de al menos un compuesto seleccionado del grupo de compuestos representados por las formulas I y II anteriores.

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Preferiblemente el contacto es suficiente par evitar o tratar la tensión en el tejido u órgano.

La invención también suministra un método para tratar quemaduras que comprende administrar a un paciente necesitado de tal tratamiento una cantidad terapéuticamente que bloquea la abertura del hemicanal de conexina.

También se suministra un método de tratamiento de trombosis. En una modalidad el método incluye administrar a un paciente necesitado de tal tratamiento una cantidad terapéuticamente efectiva de un compuesto que bloquea la abertura de un canal de conexina.

En otro aspecto, la invención también caracteriza un método de tratamiento de acidosis respiratoria y metabólica. En una modalidad, el método incluye administrar a un paciente necesitado de tal tratamiento una cantidad terapéuticamente efectiva de un compuesto que bloquea la abertura de un canal de un hemicanal de conexina.

La invención también suministra un método de tratamiento de arritmia focal. En un ejemplo del método este incluye administrar aun paciente necesitado de tal tratamiento una cantidad tal tratamiento una cantidad terapéuticamente efectiva de un compuesto que bloquee la abertura de un hemicanal de conexina.

La invención suministra además un método para tratar y evitar el daño celular y de tejido que resulta de niveles elevados de glucosa en la sangre. En una modalidad, el método incluye administrar a un paciente necesitado de tal tratamiento una cantidad terapéuticamente efectiva de un compuesto que bloque la abertura de un hemicanal de conexina.

La invención también caracteriza un método de tratamiento de fibrilación atrila crónica. En una modalidad el método incluye administrar aun paciente necesitado de tal tratamiento una cantidad terapéuticamente efectiva de un compuesto que bloquee la abertura de un canal de conexina.

También se suministra un método de tratamiento de epilepsia. Típicamente, el método incluye administrar a

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un paciente necesitado tal tratamiento una cantidad terapéuticamente efectiva de un compuesto que promueva la abertura de un hemicanal de conexina.

Se suministra adicionalmente un método de citoproteger tejido o un órgano de un mamífero necesitado de tal tratamiento, el método comprende administrar una cantidad terapéuticamente efectiva de al menos un compuesto seleccionado del grupo que consiste de los compuesto representados mediante la formula I o II.

La invención también suministra u método para evitar o tratar daño por repercusión en un mamífero, el método comprende administrar una cantidad terapéuticamente efectiva de un compuesto de al menos un compuesto seleccionado del grupo que consiste de los compuesto representados mediante la formula I o II.

En cada uno de los métodos terapéuticos anteriores, un compuesto de acuerdo con la invención que caracteriza una buena actividad antiarrítmica como se determino mediante el ensayo estándar de arritmia en ratón in vitro (es decir calificación de al menos 2). Tales compuestos serán algunas veces denominadas aquí como compuestos "anti-

arrítmicos" a una frase relacionada .Usos y ventajas adicionales de la presente invención serán evidentes de la siguiente discusión y ejemplos. Otros aspectos de la invención también se discutirán adelante.

BREVE DESCRIPCIÓN DE LOS DIBUJOS

La figura 1 es un dibujo que muestra los sitios de fosforilación sobre una conexina (Cx43). Como se puede ver, el dominio citosólico de la proteína de trasmembrana Cx43 tiene varios sitios de fosforilación de serina y tirosina potenciales.

La figura 2 es una grafica que muestra tensión metabólica inducida por la remoción de glucosa. Como se puede ver de la gráfica, la administración de incompuesto uno reduce la tensión como se evidencio mediante la disminución en el retraso de conducción relativo

Las figuras 3A-B son las representaciones de inmunoabsorciones que muestran detección de reportero detectable de tirosina en conexina 43 (Cx43). La figura 3A muestra los resultados de la administración del

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compuesto 1. La figura 3B muestran los resultados de la administración del compuesto 1 y compuesto 2.

Las figuras 4A -B son representaciones de inmunoabsorciones que muestran la detección de reportero detectable de tirosina (8A) y fosforilación de serina (8b) de Cx43.

La figura 5 es una gráfica que muestra el efecto 10 nM del compuesto 1 sobre toma de calceina inducida por isquemia en cardiomocitos cultivados.

La figura 6 A es un dibujo que muestra un formato de ensayo Elisa preferido. La figura 6B es una gráfica que muestra los resultados Elisa de Cx43 fosforilados e Tyr-P en célula HeLa. La figura 6c es una gráfica que ilustra los resultados Elisa de Cx43 fosforilado en Tyr-P en células hemicanal.

La figura 7A-c Son foto micrografías que muestran toma de tinte en cardiomiocitos cultivados. Figura 7A: cardiomiocitos bajo la microscopia de luz; figura 7B: fluorescencia bajo condiciones de control (lo mismo que

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las células e la figura 7A); figura 7C fluorescencia después de 30 minutos de inhibición metabólica.

La figura 8 es una gráfica que muestra que el compuesto 1 reduce la toma de calceina inducida por estrés de una manera dependiente de dosis.

La figura 9 A-B son gráficas que muestran el efecto de un compuesto uno sobre el hinchamiento inducido por estrés de las células. La figura 9A relativa al volumen mediante la inhibición metabólica en la presencia o ausencia del compuesto (0,1 nM) la figura 9 muestra datos de control.

La figura 10 es una gráfica que muestra el efecto del compuesto 1 (0,1 nM) obre el hinchamiento celular inducido por estrés.

La figura 11 es una gráfica que muestra una reducción en el índice del peso del corazón al peso del cuerpo después de la administración del compuesto uno a rata sometidas a infarto del miocardio.

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La figura 12 es una gráfica que muestra una reducción en el tamaño del infarto en ratas luego de la administración del compuesto.

La figura 13 es una grafica que muestra la función cardiaca mejorada después de la administración del compuesto uno a ratas sometidas al infarto del miocardio.

DESCRIPCIÓN DETALLADA DE LA INVENCIÓN

Como se discutió, la invención suministra compuestos y métodos para utilizar las mismas para modular la función del hemicardio: Para modular la función de hemicanal los compuestos más particulares modulan la fosforilación el hemicanal para ayudar a la abertura y cierre del hemicanal. Los tamices útiles para detectar y utilizar tales compuestos también se suministràn, cuyos tamices incluyen ensayos in Vitro o ensayos in vivo o una combinación de estos. Se suministran adicionalmente métodos terapéuticos útiles para el tratamiento o prevención de condiciones impactadas por función hemicanal inadecuada.

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Es un objeto de la invención demostrar, por primera vez, que la tensión de la célula, tejido y órgano puede se adversamente impactada por el cierre y abertura de hemicanales. Sin desear estar ligados a la teoría se cree que la tensión metabólica tal como la privación de oxígeno durante la isquemia, privación e glucosa, desacoplamiento de la fosforilación oxidativa causada por el HCN, o desacoplamiento del ciclo de ácido cítrico pueden contribuir todos al desacoplamiento de la comunicación de la unión puente intercelular (GIC). Se cree además que este desacoplamiento esta correlacionado con la modulación de la fosforilación de conexina, más particularmente la desfosforilación de residuos de conexina-tirosina y/o residuos de conexina-serina y/o residuos de treonina en el canal de unión puente. Por vía de ilustración, se cree que durante la fibrilación atrial, las células atriales tienen demanda metabólica incrementada debido al paso altamente frecuente. Se cree que esto conduce a acidosis de lactato, abertura de los hemicanales y desacoplamiento de las uniones puente.

También es objeto de esta invención suministrar métodos para modular las funciones hemicanal y, opcionalmente, la comunicación intercelular de unión puente (GJIC) cuyos métodos incluyen contactar células, tejidos u órganos con una cantidad

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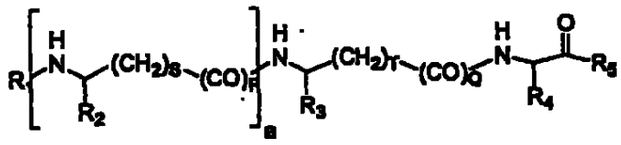
terapéuticamente efectiva de una o una combinación de compuestos que tienen tal actividad. Los compuestos preferidos han sido descritos en la PCT/DK01/00127 (WO 01/62775) y PCT/US02/05773 (WO 02/077017) por B. Larsen et al.

Los compuestos más preferidos adecuados para uso de la presente invención incluyen aquellos representados por la siguiente formula I:



(I)

en donde

R1 representa H o acetil (Ac)

R2 representa una cadena lateral de uno de los aminoácidos G, Y, D-Y, F y D-F,

R3 represente cualquier cadena lateral de aminoácido R4 representa una cadena lateral de uno de los aminoácidos G, Y, D-Y, F y D-F,

R5 representa OH o NH_2

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Y a, S, P y Q son enteros e independientemente igual a 0 o 1:

Y sales de estos.

Los compuestos más específicos incluyen aquellos que tiene la siguiente formula II:

R1-X1-X2-X3-R2

II

En donde,

X1 es 0. Ala, Gly, β-Ala, Tyr, D-Tyr, Asp,

X2 es 0. Ala-Gly-$T4_c$-Pro; Ala-Sar-Hyp-Pro; Ala-Asn; D-Asn-D-Ala; D-Asn; Gly; Ala;

D-Ala; β-Ala; Asn; o;

X3 es Tyr; D-Tyr; Gly, o Phe: y

R1 es H o Ac, con la condición de que X1 y X2 no son ambos 0; y sales de estos

R2, es OH o NH_2

Los compuestos más específicos representados por la formula I o II anterior incluye los siguientes:

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G-(DBF)-Y-NH2, , H-GA-Sar-Hyp-PY-NH2, H-GAG-T4c-PY-NH2,
Gly-Ala-Asn-Tyr , D-Tyr-D-Asn-D-Ala-Gly, D-Tyr-D-Asn-Gly, ,
, Gly-Gly-Tyr, Gly-Ala-Tyr, D-Tyr-D-Ala-Gly,
Gly-D-Asn-Tyr, Gly-βAla-Tyr, βAla-βAla-Tyr,
, Gly-βAla-Phe, Gly-Asn-Phe, , Asn-Tyr, Ac-Gly-Tyr,
Ac-Ala-Tyr, (reducedGly)-Gly-Tyr (H_2N-CH_2-CH_2-NH-CH_2-C(O)-Tyr),

gly-dab-gly-hyp-pro-tyr

gly-dapa-gly-hyp-pro-tyr

tyr-pro-hyp-gly-gln-gly

tyr-pro-hyp-gly-asn-gly

gly-dab-ala-gly-hyp-pro-tyr

gly-dapa-ala-gly-hyp-pro-tyr

tyr-pro-hyp-gly-ala-gln-gly

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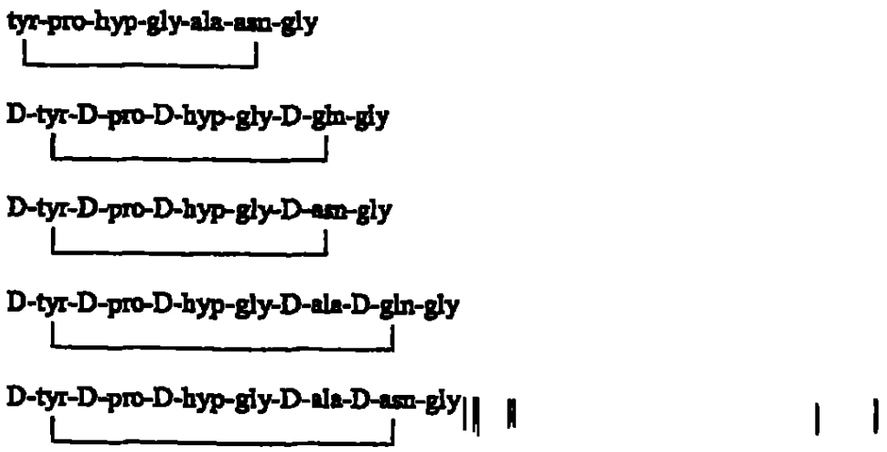

; y sales de estos

Los compuestos candidatos adicionalmente preferidos de la invención se caracterizan por una biodisponibilidad oral de más de aproximadamente el 5% como se determinó por un ensayo de biodisponibilidad oral aceptable. Los ensayos generalmente preferidos involucran administración intraduodenal de componentes candidatos a un sujeto con ensayo adecuado. La biodisponibilidad de los compuestos en una muestra biológica, preferiblemente en una muestra de sangre, es entonces detectada y preferiblemente cuantificada.

Los compuestos candidatos adicionales preferidos generalmente satisfacen la regla Lipinski de 5. Se

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aplica, esta define la biodisponibilidad. De acuerdo con la regla, una absorción menos que satisfactoria es más probable cuando uno o más de las siguientes características caracteriza un compuesto candidato particular:

1) Mas de 5 donadores de enlaces H (expresados como la suma de OHs y NHs), 2) peso molecular sobre 500. 3)Log P esta sobre 5, 4) A de 10 aceptadores de enlaces de uniones H (expresadas como la suma de Ns y Os), y 5)Dos parámetros por fuera del rango deben ser evitados para muchas modalidades de la invención.

Aún compuestos preferidos adicionales de acuerdo con la invención son relativamente estables en el plasma de la sangre. Un ensayo aceptable involucra poner en contacto un compuesto candidato deseado con plasma (suero de roedor, conejo o primate, por ejemplo), incubando el compuesto con el plasma, y luego detectando y preferiblemente cuantificando a la estabilidad de ese componente durante el tiempo. Las fuentes de plasma preferido son las ratas, perros, gatos, ratones, cerdos, vacas, caballos, y humanos. Un ensayo preferido, algunas veces denominado aquí como "ensayo de estabilidad

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estándar de plasma" se ha descrito en la solicitud PCT/US 02/05773 (WO 02/077017). Los compuestos típicos que dan buena actividad en la amidación o esterificación C-terminal característica del ensayo, utiliza D-aminoácidos y derivados de aminoácidos naturales, modificaciones N-termina, y estructuras cíclicas. Una o una combinación de tales modificaciones puede mejorara la estabilidad aunque reteniendo actividad biológica substancial.

Los compuestos moduladote de hemicanal especialmente preferidos para uso con la presente invención incluyen: Ac-D-Tyr-D-Pro-D-4Hyp-Gly-D-Ala-Gly-NH_2 (compuesto 1); Ac-Gly-Asn-Tyr-NH_2 (compuesto 2); y sales de esta.

Los compuestos moduladores de hemicanal preferidos exhiben una actividad significativa en uno o en una combinación de ensayos estándar .in nitro e in vivo descritos aquí. Tales compuestos también tiene capacidad para cerrar y abrir hemicanales y, opcionalmente, para modular GTC. Preferiblemente, un compuesto adecuado mejora o reduce la fosforilación de conexina c43 (Cx43) como se determinó mediante el ensayo de fosforilación de conexina estándar in Vitro. Un ensayo más específico monitorea la fosforilación y/o la desfosforilación de uno

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o más de los residuos de aminoácido tirosina. Serina y treonina mostrado en al figura 1. Sigue un formato de ensayo más particular.

1) Cultivar una población de aproximadamente 10^5 células, en medio tal como cardiomiocitos de rata confluente o semi confluentes o células H9c2,
1) Tensionar las células, a lavar las mismas y subsecuentemente cultivarlas en medio pobre de glucosa durante aproximadamente una hora o menos,
1) Agregar el compuesto 1 al medio a una concentración de aproximadamente 0,1 a aproximadamente 200 nM durante aproximadamente 10 minutos a 8 horas,
1) Lisar las células y luego detectar un cambio en la fosforilación de tirosina, serina, y/o treonina de Cx43 con relación a un control adecuado,
1) Medir el cambio en fosforilación por ser indicativo de un compuesto que modula la función de hemicanal.

En una modalidad del método, la etapa e detectar 4) comprende además ensayar el compuesto candidato en un ensayo basado en una clase inmunológica o de célula. En casos en los cuales se emplea un ensayo inmunológico ese ensayo incluirá típicamente poner en contacto células con

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el compuesto candidato bajo condiciones suficientes para incrementar o disminuir la fosforilación de la conexina. Preferiblemente, el método incluye además producir un lisado de las células; y detectar el incremento o disminución de la fosforilación de conexina en el lisado de célula. Ese incremento o disminución es tomado por ser indicativo adicional del compuesto modulador de hemicanal.

Los ensayos inmunológicos preferidos para uso con el método se han descrito e incluyen ensayos de inmunoprecipitación, ensayos e captura de anticuerpos, ensayo "sándwich" de dos anticuerpos, ensayos de captura de antígeno, radio inmuno ensayos (RIAs) y similares. Ver de manera general E. Harlow y D. Lane en Antibodies: A Laboratory Manual, Cold Spring Harbor Laoratory (1988); Ausubel et al. (1989) in Current Protocols in Molécula Biology, J. Wiley & Sons, New York para una discusión con relación a muchos métodos inmunológicos estándar, cuyas descripciones son incorporadas aquí como referencia.

Un ensayo inmunológico preferido es un ensayo ELISA. Así en una modalidad, el método anterior incluye además al menos y preferiblemente todas las siguientes etapas:

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a) Recubrir un soporte sólido con un primer anticuerpo que se une específicamente a la conexina, preferiblemente la conexina 43 (Cx43),

b) Poner en contacto el lisado de célula con el soporte sólido bajo condiciones que conducen a formar un complejo de unión entre un primer anticuerpo y la conexina,

c) Poner en contacto el primer complejo de unión de anticuerpo: conexina con el segundo anticuerpo, el contacto siendo bajo condiciones suficientes para formar un complejo de unión especifica entre el segundo anticuerpo y cualquier conexina fosforilada en el primer complejo de unión de anticuerpo: conexina,

d) Poner en contacto el complejo de unión primer anticuerpo: conexina: segundo anticuerpo con un tercer anticuerpo detectablemente marcadó que se une al segundo anticuerpo; y

e) Detectar la presencia del tercer anticuerpo detectablemente marcado sobre el soporte sólido por ser indicativo adicional del compuesto.

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En una modalidad del método, el tercer anticuerpo del método es detectablemente marcado con al menos uno de biotina, FITC, TRITC, yodo radiactivo o peroxidasa. En otra modalidad, el segundo anticuerpo es un anticuerpo anti-fosfotirosina. En aun otra modalidad, el segundo anticuerpo es una anticuerpo anti-fosfoserina. En algunos casos, la detección del tercer anticuerpo detectablemente marcado se desarrolla mediante conteo de radio o gama centelleo.

En otra modalidad, el método inmunológico empleado para detectar la etapa es un radioinmunoensayo (RIA). Preferiblemente, tal RIA incluye al menos y preferiblemente todas las siguientes etapas:

a) Marcar detectablemente las células antes de producir el lisado de célula, en donde el marcado es bajo condiciones que conducen a marcar conexina fosforilada, preferiblemente conexina43 (Cx43),
b) Poner en contacto el lisado de célula con un anticuerpo que forma un complejo de unión específica con la conexina,

c) Separar el complejo de unión del lisado de célula detectablemente marcado, y

d) Detectar el marcado y la conexina fosforilada por ser indicadoras adicionales del compuesto de modulación de unión puente.

En una modalidad del método, el anticuerpo es un anticuerpo anti-conexina. En otra modalidad, la etapa de detección comprende además desarrollar un ensayo de inmunoabsorción Western. El método RIA es compatible con un amplio rango de etiquetas de marcadores detectables sin embargo para muchas aplicaciones la administración de fósforo inorgánico radiactivo será preferido.

Los anticuerpos para uso con el método de invención anterior incluye anticuerpos anti-fosfotirosina, anti-fosfoserina, y anti-Cx43 están comercialmente disponibles de una variedad e fuentes tal como la American Type Culture Collection (ATCC); Sigma Chemical Co., St Louis, MO; Zymed Lab, Inc., de California y Amersham Biosciences, U.K.

Los compuestos modulantes de hemicanal preferidos identificados mediante el estándar anterior en ensayo in

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Vitro muestran buena capacidad para modular fosforilación de Cx43, particularmente en uno o más de los residuos tirosina, serina y treonina, mostrados en al figura 1. El más preferido de tales compuestos exhibe al menos un cambio de 5% en tal fosforilación (medida como un incremento de la disminución en la presencia de uno o más de fosfotirosina, fosfoserina y/o fosfotreonina e Cx43), preferiblemente un cambio de al menos 10% mas preferiblemente al menos aproximadamente 50% con relación a las células tensionadas en las cuales el compuesto esta presente en una concentración entre aproximadamente 0,1 nM a aproximadamente 200 nM.

Ver ejemplo 2-5 adelante para un ejemplo particular el ensayo de fosforilación de conexina estándar in Vitro.

Como se discutió, la invención se caracteriza por un ensayo de toma in Vitro estándar que se puede utilizar para detectar y opcionalmente caracterizar compuestos moduladores de hemicanal. El ensayo puede ser utilizado solo o combinación con el ensayo estándar de fosforilación de conexina in vitro. Una modalidad particular del ensayo de toma de estándar in Vitro es descrito como sigue,

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1) Cultivar una población de aproximadamente 10^5 miocitos ventriculares de rata confluentes en medio,

2) Estresar las células, al remplazar el medio con medio pobre en glucosa durante menos de aproximadamente 2horas, preferiblemente aproximadamente 30 minutos,
3) Agregar el compuesto 1 al medio a una concentración de aproximadamente 0,1 pM a aproximadamente 100 nM,
4) Agregar tinte de calceina al medio a una concentración de aproximadamente 10 a aproximadamente 500 micromolar durante menos de aproximadamente 2 horas y preferiblemente aproximadamente 30 minutos,
5) Detectar un cambio en la toma de la calceina en las células.
6) Medir el cambio en por ser indicativo de un compuesto que modula la función de hemicanal.

La etapa de detectar el cambio en la toma de calceina puede desarrollarse mediante una combinación de métodos estándar que incluye microscopia de luz fluorescente y/o métodos de la clase de célula relacionada. Ver ejemplo 6

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para un ejemplo particular del ensayo de toma estándar in Vitro.

Los compuestos preferidos detectados mediante el ensayo de tomo estándar in Vitro anterior exhibirá al menos aproximadamente un 5% de disminución en la toma de tinte (hemicanales de célula cerrada) con relación a las células tensionadas, preferiblemente al menos aproximadamente un 20% de disminución y más preferiblemente al menos aproximadamente un 50% de disminución en la presencia de entre aproximadamente 0,01 pM a 100 nM del compuesto ha ser ensayado.

Como también se discutió la invención suministra el ensayo de volumen de célula in Vitro cuyo ensayo puede ser solo o en combinación con uno o ambos de los ensayos de fosforilación estándar de conexina in Vitro y ensayo de toma estándar in Vitro. Una modalidad particular del ensayo de volumen de célula estándar in Vitro es como sigue.

1) Cultivar una población de aproximadamente 10^5 miocitos ventriculares de rata confluentes en medio,

2) Cargar las células con entre desde aproximadamente 0,5 a aproximadamente 100 micromolar de calceina-AM, preferiblemente aproximadamente 5 micromolar durante menos de aproximadamente 2 horas y preferiblemente aproximadamente 15 minutos,
3) Estimar el volumen en células al detectar y preferiblemente cuantificar la señal proveniente de la calceina-AM,
4) Agregar el compuesto 1 al medio a una concentración de aproximadamente 0,01 nM a aproximadamente 100 nM,
5) Tensionar las células al cultivarlas en un medio pobre de glucosa,
6) Detectar un cambio en el volumen celular con relación a u control adecuado,
7) Medir el cambio por ser indicativo de un compuesto que modula la función de hemicanal.

La etapa de detectar el cambio en el volumen celular se puede desarrollar mediante uno o una combinación de métodos estándar que incluyen microscopia con focal láser y/o métodos de la clase de célula relacionada. El ejemplo 7 suministra un ejemplo particular del ensayo estándar de volumen celular in Vitro exhibirá al menos aproximadamente una disminución del 5% en el volumen

celular (hemicanales cerrados) con relación a las células tensionadas, preferiblemente al menos aproximadamente un 20% de disminución y mas preferiblemente al menos aproximadamente un 50% de disminución en la presencia de entre aproximadamente 0,01nM a 100nM del compuesto a ser ensayado. Los compuestos seleccionados de acuerdo con este ensayo exhibirán flujo de osmolitos a través de los hemicanales y ayudaran a mantener el volumen celular normal durante las condiciones que produce el hinchamiento celular. De manera importante el hinchamiento celular esta asociado con perfusión dañada en órganos secundados por una cápsula fibrosa (por ejemplo, corazón, riñón, músculo del esqueleto) o hueso (cerebro, espina dorsal) y por lo tanto compuestos con propiedades de bloqueo de hemicanal pueden ser utilizado en el tratamiento de enfermedades asociadas con el hinchamiento celular.

Los experimentos de control adecuados son generalmente ajustados para uso en un formato de ensayo particular. Por ejemplo, la mayoría de los experimentos e control involucran someter una muestra de ensayo (por ejemplo una población de cardiomiocitos de rata) cultivados a condiciones de no tensión tal como incubación en medio.

Típicamente, el agua, amortiguador, la solución salina amortiguada con fosfato o similar se agregan al ensayo en lugar de el o los compuestos a ser ensayados en paralelo o por separado. Un ensayo deseado es entonces conocido de acuerdo con los métodos presentes. Los ejemplos específicos de los controles adecuados son suministrados en la sección de ejemplos.

La práctica de la invención es compatible con un amplio espectro de ensayos de detección convencionales. Tales ensayos pueden ser desarrollados manualmente, semi-manualmente, o en un formato automatizado según sea necesario. Para las aplicaciones en las cuales se requieren estrategias de tamizado rápido o a gran escala, la invención es adicionalmente compatible con las metodologías de tamizado estándar de "alta capacidad" y/o "ultra alta capacidad". Ejemplos de tales métodos incluyen ensayos del tipo de láser inmunológica o de célula tales como los descritos aquí.

Como se discutió, la presente invención es compatible con un amplio espectro de estrategias de ensayo. Así en algunas modalidades, los ensayos adicionales de compuestos candidato se desarrollaran in vivo para

ensayar y confirmar preferiblemente la actividad modificadora de hemicanal. Por ejemplo, una o una combinación de los métodos estándar in Vitro descritos aquí se puede utilizar para ensayar adicionalmente los compuestos para la actividad moduladora de hemicanal en el modelo de arritmia estándar in vivo. El ensayo de arritmia de ratón estándar in vivo, descrito adelante como ejemplo de referencia 1 se ha descrito en la PCT/DK 01/00127 (WO 01/62775) y PCT/US 02/05773 (WO 02/077017) por B. Larsen et al.

Los compuestos candidatos identificados por uno o más de los métodos de la invención tiene una variedad de aplicaciones importantes que incluyen el uso de sondas para identificar uniones puente y particularmente hemicanales en un rango de células, tejidos y órganos. También, tales compuestos se pueden utilizar como sondas para detectar las uniones puente y los hemicanales en varios desarrollos y estados de enfermedad y como sondas para detectar aquellas estructuras con diferente decoración de carbohidrato y/o patrones de fosforilación. Tales compuestos encuentran uso adicional como componentes de soporte sólido para purificación de

componentes de unión puente de acuerdo con procedimientos cromatográficos estándar.

Los compuestos seleccionados mediante los ensayos in Vitro y/o in vivo descritos aquí se pueden utiliza r como medicamentos para evitar o tratar condiciones impactadas mediante la abertura incrementada de hemicanales (permeabilidad de membrana de célula incrementada a los compartimientos extracelulares), por ejemplo, heridas tales como quemaduras, trombosis, acidosis respiratoria y metabólica, hinchamiento de tejido por ejemplo debido a toxinas bacteriales y ambientales y arritmias focales. Otros métodos de la invención se pueden emplear para proteger las células de cambios de volumen destructores. Tales cambios pueden ser facilitados por una o una combinación de factores que incluyen tales Cambios se pueden facilitar por uno o una combinación de factores que incluyen calor excesivo, isquemia, toxina, inflamación, edema, alteración de electrolitos, niveles incrementados de glucosa, y complicaciones diabéticas tardías. El tratamiento de fibrilación atril crónica asociado con fosforilación de serina implementada también se contempla. Los compuestos adicionales se pueden utilizar como medicamentos, para prevenir, tratar,

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aliviar, o reducir la severidad de las siguientes condiciones. Ver también la solicitud PT/DK01/00127 (WO 01/62775) y PCT/US02/05773 (WO 02/077017).

Los métodos terapéuticos de la invención comprenden generalmente la administración de una cantidad terapéuticamente efectiva de uno o una combinación de compuestos modulares de mi canal descritos aquí a un sujeto necesitado de tal tratamiento, tal como un mamífero, y particularmente un primate tal como un humano. Los métodos de tratamiento de la invención también comprenden la administración de una cantidad efectiva de un compuesto de formula I, o II como se definió anteriormente a un sujeto, particularmente un mamífero tal como un humano necesitado de tal tratamiento para una indicación descrita aquí.

Los sujetos típicos incluyen mamíferos que sufren de una combinación de desordenes que se suministran aquí incluyendo las condiciones descritas adelante en las secciones A-R.

A. Tejido nervioso

Es bien conocido que la microglia es el principal efectuador inmune del sistema nervioso central (CNS), y que ellos son activados en respuesta a un amplio rango de daños que disparan las respuestas inflamatorias del cerebro, incluyendo daño de la cabeza e isquemia, enfermedades neurodegenerativas, enfermedades autoinmunes, enfermedades infecciosas, enfermedades de priones tumores cerebrales. La microglia activada migra a las áreas CNS dañadas, donde ellas proliferan, y remuden gradualmente los desechos celulares. Eugenin et al mostró que la microglia puede comunicarse una con la otra a través de las uniones puente que son inducidas por citoquinas inflamatorias (Eugenin, *E A, et al. Proc. Natl. Acad. Sci. USA*, VOL.98, 4190-4195, 2001). Esto se demostró en los siguientes experimentos. En heridas de arma blanca en el cerebro, microglia progresivamente acumulada durante varios días y agregados formados que frecuentemente muestran inmunoreactividad Cx43 e interfaces entre células. En cultivos primarios la microglia mostró bajos niveles de Cx43 determinada mediante bandas de proteína, inmunoreactividad intracelular difusa de Cx43 y baja incidencia de acoplamiento de tinte. El tratamiento con lipopolisacárido bacterial inmunoestimulante (LPS) o las

citoquinas interferón-gama (INF-gama) o el factor alfa de de necrosis tumoral (TNF-alfa) uno a la vez no incrementa la incidencia del acoplamiento de tinte. Sin embargo, la microbia tratada con INF-gama más LPS mostró un dramático incremento en el acoplamiento de tinte que fue evitado por la coaplicación de un anticuerpo anti-TNF-alfa sugiriendo la liberación y acción autocrina de TNF-alfa. El tratamiento con TNF-gama más TNF-alfa también incrementó grandemente la incidencia del acoplamiento de tinte y los niveles de Cx43 con translocación de Cx43 a los contactos célula-célula.

El acoplamiento de tinte inducido por citoquina fue inhibido reversiblemente mediante ácido 18-glicirretínico, un bloqueador de la unión puente. Microglia cultivada de ratón también expreso Cx43 y desarrollo acoplamiento de tinte luego de tratamiento con citoquinas, pero la microbia de ratones o homocigotos deficientes en Cx43 no desarrollo acoplamiento significativo de tinte después del tratamiento con INF-gama más LPS o INF-gama más TNF-alfa.

La expresión forzada de las proteínas de unión puente, conexinas, posibilita que las líneas de célula deficientes en unión puente propaguen las ondas de calcio

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intercelular. Cotrina et al. Demostró que la secreción de ATP proveniente de lineas celulares pobremente acopladas, glioma C6, HeLa, y gliobastoma U373 es potenciada 5 a 15 veces mediante la expresión de conexina. Estas observaciones indican que la señalización célula a célula asociada con expresiones de conexina da como resultado una liberación mejorada de ATP mediada a través de hemicanales de conexina (Cotrina ML et al. Proc. Natl Acad Sci U S A 1998 Dec 22, 95 (26):15735-40; Cotrina et al.: J Neurosci 2000 Apr 15;, 20(8):2835-44). Más aun, durante las condiciones con intensión metabólica (por ejemplo isquemia). La liberación de ATP mediada por hemicanal proveniente de astrositos puede afectar las neuronas vecinas y elicitar elevaciones en calcio intracelular lo que a su vez puede tornarse en apoptósis y muerte neuronal.

Debido, por ejemplo, a la fosforilación de la tirosina Cx43 mediante el compuesto I y el compuesto II, la administración de estos compuestos ayudara en el cerramiento del hemicanal y facilitará la comunicación intercelular de microglia y aumentará de esta manera o acelerará el proceso de "curación" en las enfermedades anteriormente mencionadas (respuestas inflamatorias del

cerebro, incluyendo daño en la cabeza e isquemia, enfermedades neurodegenerativas, enfermedades autoinmunes, enfermedades infecciosas, enfermedades de prion, y tumores cerebrales). Adicionalmente, se espera que en cierre de hemicanales evitará la liberación de ATP y así reducirá
el esparcimiento de daño primario.

B. **Tejido del pulmón: Células alveolares**

La comunicación intercelular alveolar por vía de las uniones puente entre células alveolares es importante para la propagación del transporte de ión, la transducción de señal mecanoquímica, la regulación del crecimiento celular y la secreción del factor de tensoactivo (Ashino Y, et al. (*Am J Physiol Lung Mol Phisiol* 2000; 279:L5-L13)). La reparación *in vivo* después de un daño inflamatorio agudo o crónico de la región alveolar del pulmón involucra la formación de fibronectinas como parte de la matriz extracelular. (Charash EW, et al. (*Am Rev Respir Dis* 1993; 148: 467-476) y Toricata C, et al. (*Lab Invest* 1985; 52:399-408)). Los estudios de cultivos de célula epitelial alveolar han demostrado un número creciente de uniones fuente en

paralelo a un incremento de la concentración de fibronectina extracelular (Alford AI, Rannels DE. (*Am J Physiol Lung Cell Mol Physiol* 2001; 280:L680-L688)). Estudios de animales in vivo han encontrado un número decreciente de uniones puente después de inflamación pulmonar severa inducida por dióxido de nitrógeno tanto en el tejido alveolar, las paredes de los bronquiolos terminales, los ductos alveolares y los alvéolos peribronquiolares. Estos hallazgos fueron dependientes de dosis. Sin embargo, si se pretrata con taurina esta perdida de uniones puente se evita en paralelo con menos reacciones inflamatorias pronunciadas. Hallazgos similares fueron vistos después de la irradiación del pulmón de rata y después del tratamiento con el compuesto quimioterapeútico, bleomicina.

Así, el mantenimiento de la comunicación de la unión puente en el tejido del pulmón es importante para evitar la fibrosis del pulmón y la cantidad disminuida de conexina es vista como una reacción a los procesos inflamatorios, a varios estímulos tóxicos. Tales como inhalación de gas, sustancias destructivas aéreas e irradiación. El pretratamiento con un compuesto de la invención que fosforila los residuos de tirosina Cx43,

facilita el cerramiento de hemicanal y/o la abertura de la unión puente o la comunicación de la unión puente se indicará antes de la irradiación terapéutica donde los pulmones son expuestos, por ejemplo, en cáncer de pulmón, tratamiento de cáncer de seno, cánceres de tiroides y del esófago.

El tratamiento con un compuesto que facilita o media el cerramiento hemicanal y/o la abertura de unión puente evitará el deterioro adicional de la función pulmonar en el enfisema, asbestosis, silicosis, fibrosis pulmonar, neumonitis, fibrosis de pulmón inducida por droga y en pacientes expuestos a gases tóxicos pulmonares tales como dióxido de nitrógeno. El tratamiento preferiblemente se agregará al tratamiento convencional de estas condiciones. El compuesto puede ser administrado oralmente, parenteralmente, nasalmente o por vía de inhalación pulmonar.

C. Músculo suave.

1. Sistema vascular.

La comunicación intercelular a través de canales de unión puente juega un papel fundamental en la regulación y modulación del tono de miocito vascular a través del árbol vascular (Christ GJ, et al. *Circ Res.* 1996; 79: 631-646)). Otro papel importante de la comunicación de la unión puente es el esparcimiento de la hiperpolarización entre células de músculo liso involucradas en la respuesta a la relajación vascular (Benny JL, et al. *Physiol Herat Circ Physiol* 1994; 266: H1465-72)).

Las funciones especializadas del endotelio requieren comunicación intercelular de la unión puente entre las células endoteliales dentro de la monocapa y entre el endotelio y otras células presentes en la pared vascular. La comunicación entre estos diferentes tipos de célula por vía de las uniones puente en los capilares coronarios así como también en otros vasos se han documentado en varios estudios. Evidencia de la involucración en arteriogénesis adaptativa también ha sido demostrada (Cai W-J, et al. *J Mol Cell Cardiol* 2001; 33:957-67), Wang H-Z, et al. *Am J Physiol Cell Physiol.* 2001 ;281: C75-88), Schuster A, et al. *Am J Physiol Heart Circ Physiol.* 2001; 280: H1088-96)).

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En diferentes situaciones patofisiológicas vasculares donde la monocapa endotelial es rota como en las lesiones hipercolestrolémicas inducidas por dieta la comunicación de la unión puente se disminuye en los músculos lisos vasculares (Polacek D, et al, *J Vasc Res* 1997; 34: 19-30) El daño en la capa celular endotelial es visto durante la estasis venosa y cuando la tromboflebitis es desarrollada. Kwak BR, et al. *Molec Biol Cell* 2001; 12:831-845 ha demostrado claramente que la comunicación de la unión puente sirve para coordinar la migración celular durante el reparamiento endotelial y también es importante para el brote capilar durante la angiogénesis.

El tratamiento con los compuestos de la invención que facilitan el cerramiento del hemicanal y/o la comunicación de la unió puente mejorará la comunicación intercelular dañada en las áreas vasculares afectadas, y será particularmente útil durante la isquemia de órgano, por ejemplo claudicatio intermitent e infarto del miocardio.

Sin embargo después del daño de catéter de globo en carótidas de ratas el proceso de curación vascular es caracterizado por la comunicación de unión puente

incrementada. (Yeh HI, et al. *Arterioscle Thromb Vasc Biol* 1997; 17:3174-84). Un compuesto adecuado de la invención será administrado antes de la intervención del globo y es preferiblemente una terapia agregada al tratamiento médico convencional de esta condición. La administración del compuesto será preferiblemente parenteral. El efecto puede ser ensayado en muestras de tejido antes y en diferentes momentos después del daño de catéter de globo. El más rápido curaminto de la superficie endotelial será vista utilizando microscopía convencional. También se encontrará lamedora de la comunicación de la unión puente. Ver también *Arterioscle Thromb Vasc Biol* 1997;17:3174-84).

2. Disfunsión eréctil

En el cuerpo cavernoso una red celular sincital se establece por vía de la unión puente y es crítica para la función eréctil y asegura que las células del músculo liso corporal y arterial del pene respondan de una manera uniforme y coordinada. (Christ GJ. (*Int J Impot Res.* 2000; 12 suppl. 4:S15-25), Merman A, Christ JC. (*Urolog Clin North America*. 2001; 28:217-31)). La función eréctil dañada se ve en diabetes, arteriosclerosis, diferentes

enfermedades neurológicas y muchas enfermedades crónicas. De los estudios en diabetes una correlación inversa entre la inervación neural y el punto de acoplamiento intercelular hacia la plasticidad funcional potencial del ambiente corporal aunque no estableciendo la comunicación intercelular funcional por vía de la unión puente.

El tratamiento con un compuesto que facilita el cerramiento del hemicanal y/o la abertura de la unión puente mejorará la comunicación por vía de la unión puente y normalizará de esta manera la coordinación compleja entre las células de músculo liso en el cuerpo cavernoso y los vasos.

Los ensayos farmacológicos in vivo de la de la función eréctil del compuesto puede ser ensayada 10 semanas después de diabetes inducida por estreptozotocina (35 mg/kg i.p) en ratas (de 8 semanas de edad) como se describió por Rheman J, et al. (*Am J Physiol* 1997;272:H1960-71). Los reflejos del pene y la presión intracavernosa son medidos durante la administración local y sistémica de diferentes dosis de los compuestos diferentes modulantes de hemicanal con mediciones y técnicas descritas por el mismo grupo de investigación.

Un incremento en los reflejos del pene y en la presión intracavernosa del 25% o superior puede ser vista. El tratamiento de la disfunción eréctil se puede administrar bien sea localmente en el cuerpo del pene, como una inyección subcutánea u oral. El tratamiento puede ser de monoterapia o agregado al tratamiento convencional de esta condición.

3. **Incontinencia.**

Los músculos lisos en la vejiga de orina son caracterizados por contracciones fásicas y muestran contracciones fásicas espontáneas. Sin embargo la vejiga es en condición saludable capaz de contener varios cientos de mililitros de orina sin mostrar una presión intravesical incrementada. Como contraste a la vejiga normal las vejigas inestables desarrollan espontáneos en la presión intravesical relacionada con el estimulo de incontinencia (Turner WH, Brading AF. *(Pharmacol Therap* 1997; 75:77-110). Comparado con el músculo liso gastrointestinal los músculos lisos de la vejiga no generan espontáneamente contracciones coordinadas (Stevens RJ. Et al. (*Am J Physicol,* 199; 2777:C448-60), Hashitani H, et al. (*J Physiol.* 2001; 530:273-86)). Ambas

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comunicaciones eléctricas y morfológicas por vía de las uniones Puente entre las células del músculo liso en la vejiga han sido recientemente demostradas (Hashitania H., et al. (J Physiol. 2001; 530:273-86), Wang H-Z, et al. (*Urology* 2001; Suppl 6A: 111)). La importancia de estas señales Puente se demostró mediante la inhibición específica de la comunicación. Las ondas de la exitación espontánea en el músculo liso o de la vejiga se propagan a través de las uniones puente.

La incontinencia estimulada no controlada por lo tanto será regulada por vía del tratamiento con un compuesto de cierre de hemicanal o un abridor de unión puente. La administración será parenteralmente, oralmente o dentro de la vejiga urinaria. La administración será preferiblemente como un agregado a un tratamiento con drogas destinadas a normalizar la contracción muscular en la vejiga de orina.

Las células mioepiteliales son presentadas en ductos glandulares submandibulares, en la uretra, en los ductos de bilis, los ductos pancreáticos, los ductos de lágrimas están conectados con las uniones puente y la comunicación intercelular es esencial para la sincronización de la

función contráctil de las células mioepiteliales (Taugner R, Schiller A.(*Cell Tissue Res.* 1980;206: 65:72). La contractilidad afectada en estos ductos se puede normalizar mediante tratamientos con incompuesto de cierre de hemicanal o un abridor de unión puente administrado parenteral u oralmente.

D. **Curación**

El efecto profiláctico del tratamiento con un agente de cierre de hemicanal o un abridor de unión puente, tal como el compuesto 1 y el compuesto 2, se puede ensayar en una configuración experimental como se describió por Yeh HI, et al.(*Arterioscle Thromb Vasc Biol* 1997; 17:3174-84). El compuesto 1 o el compuesto 2 se pueden administrar antes de la intervención del globo utilizando dosis en el rango de 10-11 a 10-8 dependiendo de las cinéticas biológicas del compuesto, por ejemplo como se determinó en el modelo de arritmia inducido por cloruro de calcio descrito anteriormente. El tejido puede ser muestreado antes y en diferentes momentos después del daño con catéter de globo. La curación más rápida de la superficie endotelial será vista utilizando microscopia

convencional. La administración del compuesto será, por ejemplo, parenteralmenet.

Los progresos de la curación en una serie de fases traslapantes iniciando con hemostasis (coagulación). La segunda fase del proceso de curación es una cascada de respuestas inflamatorias donde los micrófonos se acumulan en el lado de la herida y la formulación de tejidos en granulación inicia involucrando fibrolastos linfocitos entre otros componentes. Las células epiteliales iniciaran entonces a migrar desde la frontera de la herida para cubrir el área. Los conductos capilares provenientes de tejido normal en la herida también se involucran con el fin de asegurar el suministro de nutrientes, oxígeno y las diferentes células. Todas las células y las células endoteriales capilares tienen unas uniones fuente por vía de comunicación intracelular viva (Abdullah KM, et al. (Endocrine. 1999; 10:35-41). Las áreas con suplemento de bajo oxígeno y/o alta concentración de radicales libres a menudo se ven en las heridas con tejido necrótico, en diabetes, en arteriosclerosis, en heridas de cirugía, edema, infección, heridas de quemada y en insuficiencia venosa

disminuirán la comunicación de unión puente (Nagy JI, et al. *Cell Growth Diff.* 1996; 7:745-51)).

El tratamiento con un compuesto de cierre de hemicanal o un abridor de unión puente asegurará la comunicación máxima de la unión punte entre las diferentes células que se considera que juegan un papel importante en el proceso de reparación complicado y mejora de esta manera la reparación de la herida. El compuesto será administrado tópicamente, sistemáticamente u oralmente.

E. **Retinopatía diabética**

La retinopatía diabética puede ser diagnosticada muy temprano después del ataque de la enfermedad al identificar alteraciones en el índice de la ruptura de flujo sanguíneo (Bursell S-V, et al. (*Curry Eye Res.* 1992; 11:287-95), en la barrera sangre-retina (Cunha-Vaz JG. Et al. (Br JG. Et al. (Br J Ophthalmol. 1975; 59: 649-56), Do Carmo A, et al. (Exp Eye Res. 1998;67: 569-75)) y/o perdida de autorregulación (Kohner EM, Patel V, Rassam SMB. (*Diabetes* 1995; 44:603-607)). Al utilizar tanto transporte de trazador como técnicas de abrazadera de parche de doble célula Oku H, et al. (*Invest*

Ophthalmol Vis Sci.2001; 42: 1915-1920) ha demostrado un acoplamiento extensivo célula a célula. El cierre de las sendas de la función puente rompe la organización multicelular de los microvasos de la retina y contribuye a la disfusión vascular diabética de la retina. Zhou ZY, et al. (Neuroscience. 2001:102:959-67) demostró adicionalmente que el oxígeno reactivo está involucrado en el desacoplamiento de la unión puente de la retina y un reacoplamiento cuando se suministra la glutación.

El efecto de los cerradores de hemicanal sobre la retinopatía diabética puede ser estudiada in Vitro utilizando el modelo de rata diabética inducida por estreptozotocina como se describió anteriormente. Los microvasos de retina recientemente aislados (Sakagami K, et al. J *Physiol (Lond)*. 1999; 521: 637-50) se transformará vidrios de cubierta como se describió por Oku H, et al. (*Invest Ophthalmo Vis Sci*. 2001; 42:1915-1929). En esta preparación la comunicación intercelular entre las células en la pared vascular se medirán bien sea con tinte o con trazador. Las diferentes concentraciones en el rango de 10-10-10-7 M de los compuestos de la invención, por ejemplo, el compuesto 1 de abridores de unión puente o el compuesto 2 se pueden ensayar y se verá un incremento significativo en la

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comunicación intercelular comparado con la línea base en la retina diabética. Una mejora similar se verá cuando se comparan con los controles animales saludables). El tratamiento será sistémico, local u oral. La terapia es preferiblemente una agregada al tratamiento antidiabético convencional.

No solo la retinopatía diabética sino también otras anormalidades basculares en la retina como por ejemplo la arteriosclerosis se beneficiarán del cierre incrementado de los hemicanales o una comunicación de unión puente mejorada mediante el tratamiento con un compuesto que ayuda a la fosforilación de tirosina Cx. El compuesto será administrado parenteralmenet.

F. **Desordenes cardiacos.**

1. **Bloque Atrial-ventricular (AB)**

La comunicación intercelular en el nodo av cardiaco se mantiene por vía de las uniones puente. La función diminuida lleva a una conducción disminuida y puede llevar a un bloqueo total a-v.

El bloqueo AV se ve en el infarto agudo del miocardio, en enfermedades isquémicas cardiacas, intoxicación con digitales, Intoxicación del bloqueador de canal de calcio y un compuesto de cierre de hemicanal mejorará la conducción av. La administración del compuesto de cierre del hemicanal deberá ser parenteral u oral.

2. **Fibrilación atrial**

Nao et al. 2001 ha encontrado fosforilación de serina disminuida de conexina 40 en las células del miocardio de pacientes que sufren de fibrilación atrial crónica (AF). La fosforilación de serina disminuida de conexina es conocida por estar conectada con el desacoplamiento de células que pueden conducir a AF. Sí esta condición de AF crónica también se caracteriza por la fosforilación de tirosina disminuida de conexinas, entonces el tratamiento con un compuesto tal como el compuesto 1 o el 2 de aquí puede incrementar la fosforilación de tiroxina, cerrar los hemicanales, abrir los canales de unión puente y remover las causas de AF.

3. **Daño de isquemia/reperfusión**

Durante la isquemia regional, los corazones son expuestos a tensión metabólica que origina el inchamiento de las células que a su vez incrementa la perfusión del tejido en la zona limite isquémica. Se cree que la perfusión reducida en la zona de limite esquémico contribuye al gradual esparcimiento del infarto, que esta asociado con el daño adicional de la función cardiaca. Como se muestra en los ejemplos 7 y 8, el compuesto 1 evita el hinchamiento de la célula durante la isquemia, reduce el tamaño del infarto y evita el daño de la función cardiaca después del infarto del miocardio.

Más aún, la perfusión coronaria se restablece en pacientes con infarto del miocardio al administrar un agente trombolítico o mediante angioplastia coronaria transluminal percutánea (PTCA). Aunque el restablecimiento de flujo sanguíneo es un prerrequisito el salvamente del miocardio, la reperfusión misma puede conducir al daño adicional del tejido más allá de aquel generado por la isquemia sola-esta es conocida como "daño por repercusión". Los mecanismos propuestos para contribuir al daño por reperfusión son mismos, incluyendo la sobrecarga de radicales libres de oxígeno, daño del miocardio mediado por neutrófilo, sobrecarga de calcio

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intracelular, y cambios en el ambiente osmótico (Wang et al., Cardiovasc Res. 2002 Jul; 55(1):25-37. Revisión).

Existe reconocimiento de que la osmolaridad de los fluidos corporales es estrictamente controlada de tal forma que la mayoría de las células no experimenten cambios en la presión osmótica bajo condiciones normales, sino que pueden ocurrir cambios osmóticos en los estados patológicos tales como la isquemia, el choque séptico, y el coma diabético. El efecto primario de un cambio es la os molaridad es alterar agudamente el volumen celular. Si la os molaridad al rededor de una célula se disminuye, las células se inchan, y si se disminuyen, este encoge. Con el fin de tolerar los cambios en la osmolaridad, las células han evolucionado mecanismos regulatorios de volumen activados mediante el reto osmótico al normalizar el volumen celular y mantener la función normal. En el corazón, la tensión osmótica de encuentro durante un periodo de isquemia del miocardio cuando los metabolitos tales como lactato se acumulan intracelularmente y en cierto grado extracelularmente, y originan el inchamiento de la célula. Este inchamiento puede ser exacerbado adicionalmente en la reperfusión cuando el ambiente extracelular hiperosmótico es reemplazado por sangre

normosmótica y esta puede causar a un la ruptura de las membranas de la célula cardiaca (Wriggth AR, Rees SA: Pharmacol Ther, 1998 Oct; 80(1): 89-121. Review). Así, se cree que los compuestos seleccionados de acuerdo con la presente invención evitarán el daño del miocardio durante la reperfusión, por ejemplo, mediante un mecanismo similar para el efecto descrito en el ejemplo 7 (figura 9A).

Además, se ha reportado que durante el procedimiento PTCA comúnmente utilizado, la placa ateroesclerótica es rota lo cual origina la microembolización en la microcirculización distante del sitio del procedimiento PTCA, lo cual está asociado con la progresión acelerada de la falla cardiaca y la mortalidad incrementada Henriques JP et al:. *Eur Herat J* 2002 Jul; 23 (14): 1112-7). Como se mostró en los ejemplos 7 y 8, el compuesto 1 evita el hinchamiento celular durante la isquemia, reduce e tamaño del infarto y evita el daño de a función cardiaca después del infarto del miocardio y estas propiedades de compuesto reducirán el daño durante la reperfusión.

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De acuerdo con esto, la invención suministra un método de citoprotección de tejido o un órgano de un mamífero necesitado de tal tratamiento. En una modalidad, el método incluye administrar una cantidad terapéuticamente efectiva de al menos n compuesto seleccionado del grupo que consiste de los compuestos representados por la formula I o II. Por la frase "citoprotector" que significa reducir, evitar o aliviar síntomas asociados con hinchamiento celular no deseado. Los tejidos y órganos particulares que se beneficiarán del método incluyen aquellos confinados o impactados de otra manera con una cápsula fibrosa tal como corazón o riñón. También están incluidos los tejidos asociados con el hueso tal como cerebro, espina dorsal y médula ósea.

En una modalidad, el método incluye adicionalmente exponer el tejido u órgano del mamífero condiciones isquémicas tal como aquellas descritas aquí. Un ejemplo es el infarto del corazón (ataque cardiaco) en el cual la isquemia está asociada con el hinchamiento celular dañino del miocardio. En una modalidad, tal inchamiento se puede reducir o evitar administrar al menos uno de Ac-D-Tyr-D-Pro-D-4Hyp-Gly-D-Ala-Gly-NH2 (compuesto 1) y Ac-Gly-Asn-Tyr-NH2 (compuesto 2) al mamífero.

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Como se discutió un método para evitar o tratar el daño por reperfusión en un mamífero también se suministra por mediante la invención. En una modalidad, el método incluye administrar una cantidad terapéuticamente efectiva de al menos un compuesto seleccionado del grupo que consiste de los compuestos representados por la formula I o II. En una modalidad más especifica, el método incluye además el corazón del mamífero a condiciones de infarto. De acuerdo con el método, es deseable establecer la perfusión coronaria, típicamente tan rápidamente como sea posible. En algunas modalidades, el método adicionalmente incluirá administrar un agente trombolítico (por ejemplo activador de plasminógeno de tejido ("TPA")) O suministrar angioplastia coronaria para facilitarla perfusión coronaria en el corazón infartado. En una modalidad, el compuesto es al menos uno de Ac-D-Tyr-D-Pro-D-4Hyp-Gly-D-Ala-Gly-NH2 (compuesto 1) Y Ac-Gly-Asn-Tyr-NH2 (compuesto 2).

G. **Inmunología: Maduración celular**

Las interacciones célula a célula son cruciales para la maduración y activación del, linfocito. Un amplio rango

de moléculas de membrana asegura la adhesión intercelular y posibilita el señalamiento célula-célula durante la migración de la célula y la activación del sistema inmune. Los linfocitos humanos T, B y NK circulantes expresan Cx43 y las uniones fuente activas entre las células han sido demostradas utilizando métodos de tinte como se describió previamente. También se ha demostrado que disminuir el acoplamiento de la unión de puente intercelular disminuye marcadamente la secreción de IgM, IgG e IgA indicando que la señalización intercelular a través de las uniones puente es un componente importante del mecanismo subyacente de cooperación metabólica en el sistema inmune (Oviedo-Orta E, et al. (Inmunology, 2000; 99:578-90), Oviedo -Orta E et al. (*FASEB*. 2001; 15:768-774)).

En inflamación subcrónica o crónica es deseable un incremento local en las síntesis de inmunoglobulinas independientes de la etiología. Durante la inflamación el tejido es a menudo diferente del tejido normal saludable y una tensión baja de oxígeno produce el desacoplamiento de la comunicación de la unión puente intercelular. La importancia del oxígeno bajo para el desacoplamiento GJIC se ha demostrado en varios diferentes sistemas de célula

sugiriendo que la tensión de oxígeno es un regulador universal del GJIC. Dicho desacoplamiento es un resultado de la fosforilación alterada de tirosina y/o serina y/o treonina de las conexinas en los canales de unión puente, y la administración de un compuesto de la invención tal como el compuesto 1 o 2 contra restará este efecto, cerrar los hemicanales y restablecerá GJIC.

H. **Mejorar GJIC**

Existen básicamente dos maneras de mantener el GJIC, o al mantener los canales de la unión puente abiertos o al facilitar el corte de los hemicanales. En el tratamiento o prevención de los estados de enfermedad caracterizados por la presencia de condiciones isquémicas, ambas vías son preferidas o recomendadas.

En cultivos primarios de cardiomiocitos ventriculares en rata neonatal, la privación de oxígeno y glucosa conduce a una disminución en la simulación inducida por noradrenalina de fosfoinositol (PI) cambiado a aplicación 50% del nivel en condiciones atmosféricas y nutricionales normales. El compuesto 1 modificador de la unión puente se ha mostrado que normaliza esta estimulación dañada

inducida por noradrenalina de vuelco de PI durante la privación de oxígeno y glucosa al elevar el vuelco de PI a una aplicación de 90% del nivel normal. Más aún se ha mostrado que el compuesto 1 no altera el nivel inducido por noradrenalina de vuelco de PI durante las condiciones atmosféricas y nutricionales normales 8Meier, E y Beck, M M: 2001 International Gap Junction Conference, Aug 4-9, 2001, Hawai, USA, abstract no. 132). De manera similar, en cultivos de osteoblasto humano cultivados y en hipoxia de líneas celulares de osteosarcoma en rata osteoblástica disminuye la propagación de la onda de calcio intracelular medida como transferencia de tinte después de inyecciones de amarillo lucifer. Esta disminución podría ser completamente reversada mediante tratamiento con el compuesto 1 (Teilman, S C, et al.: 2001 International Gap Junction Conference, Aug 4-9 2201, Hawai, USA, abstract no. 176).

Debido al desacoplamiento celular durante la inflamación, por ejemplo, un compuesto que cierra hemicanales y abre las uniones puente mejorará la síntesis de las inmunoglobulinas durante la inflamación.

1. **Neuropatía Periférica y dolor neuropático**

La neuropatía periférica y el dolor como se ven en la diabetes, durante la diálisis, cirrosis del hígado y muchas otras condiciones son reportadas por involucrar tanto nervios somáticos co o autonómicos. Los mecanismos exactos del daño del nervio periférico en varias condiciones están bajo investigación pero la destrucción Terminal del nervio, disminución de la conductancia, desmielinización y repuesta inflamatoria creciente han sido descritas. Común a varias condiciones en una configuración experimental son que son vistos radicales libres incrementados, óxido nítrico incrementado, tensión y falta de oxígeno y de los absorbedores de radical libre y se registra la reducción de la comunicación de la unión puente (Pitre DA, et al. (Neurosci Lett. 2001; 303: 67-71), Bolanos JP, Medina JM. (J Neurochem. 1996; 66:2019-9), Low PA, Nickander, KK. (diabetes 1991; 40:873-7), Levy D, et al. Neurosci Lett. 1999; 260: 207-9), Bruzzone R, Ressot C. J. Eur Neurosci. 1997;9:1-6). Así, un compuesto de la invención que ayuda al cierre de hemicanal mediante al fosforilar las conexinas de hemicanal será benéfico en el tratamiento de neuropatía periférica. La administración será parenteral.

J. **Déficit de escucha.**

La pérdida de escucha inducido por ruido, presbicusia conocida por estar asociada con la producción de radicales libres está relacionada con la inhibición del acoplamiento de la unión puente entre ambas células Hensen y células Deiters del órgano e Corti en la coclea (Todt I, et al. (J *Membrana Biol.* 2001; 181:107-114), Blasits S, et al. (*Phlugers Arch.* 2000; 440:710-12)Lagostena L. et al. (J Physiol, 2001;531:693-707)) . La comunicación de unión puente entre estas células cocleares de soporte suministra la homeostasis importante para las células sensoriales y de esta manera una actividad neuronal normal de las células de cabello exterior (Johnstone BM, et al. (J Physiol 1989; 408:77-92)). Esta comunicación es dañada durante la tensión oxidativa (Todt I, et al. (J *Membrana Biol.* 2001;181:107-114). La perdida de escucha dependiente de la edad o adquirida se evitará cuando se trata con un compuesto que puede incrementar la fosforilación de los residuos de tirosina en los hemicanales de conexina (cerrar los hemicanales) y mantener la comunicación de la unión puente en las células de soporte. Un compuesto adecuado de la invención se puede administrar parenteralmenet.

Los melanocitos en el área celular oscura del órgano vestibular están fuertemente comunicados por vía de la unión puente y puede jugar un papel en el transporte de material entre la endolinfa y la perilinfa y también ser de importancia para mantener la homeostasis del microambiente en el oído interior (Masuda M, et al. (*Anat Rec.* 2001;262;137-146)). La hidropesía endolinfática está relacionada con varias condiciones clínicas caracterizadas por vértigo y escucha reducida. Una capacidad disminuida de la comunicación de la unión puente puede ser de importancia para regular el transporte de transmembrana de varias sustancias originalmente secretadas o excretadas por vía de tipos específicos de transportadores.

K. **Anemia dependiente de la edad y transplante de médula ósea**

La Existencia de las uniones puente funcionales entre células progenitoras hematopoyéticas y células estromales del microambiente hematopoyético fueron controvertidas durante muchos años pero los estudios han probado ahora la existencia de comunicación de la unión puente humana

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(Rosendaal M, et al. *Tissue Cell.* 1991; 23:457-470), Dúrig J, Et al. (*Brit J Haematol.* 200; 11:416-25)). También se ha demostrado que la comunicación es bidireccional favoreciendo la hipótesis de que las células estromales controlan el comportamiento proliferativo de las células progenitoras hematopoyéticas, pero también su estatus funcional se puede regular por células hematopoyéticas inmaduras (Guptat P, et al. (*Blood.* 1998; 91: 3724-3733)).

Con la edad la funcionalidad del tejido hematopoyético se disminuye y la anemia es a menudo vista en personas de edad. La capacidad reducida del tejido hematopoyético también se ve en malignidades hematológicas y después del tratamiento con quimioterapéuticos. El trasplante de médula ósea proveniente de donadores se utiliza para evitar la pancitopenia.

El efecto de un compuesto de la invención que ayuda en el cierre de hemicanal y/o facilita la comunicación de la unión puente será estudiado en ratas pretratadas expuestas a altas dosis de ciclofosfamida. En estos animales la médula ósea ha detenido la producción de células hematopoyéticas maduras. El número de

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reticulocitos a diferentes intervalos de tiempo después de la ciclofosfamida se incrementará significativamente en los animales pretratados con el compuesto 1 fosforilante de tirosina conexina utilizando dosis de aproximadamente 100µL de 10^{-10}M a aproximadamente 10^{-8}M del compuesto 1 comparado con animales no pretratados. La administración de compuestos adecuados de la invención será parenteralmenet.

L. Hipofunción de la pituitaria e hipotalámica

Las hormonas de la glándula pituitaria anterior muestran variación circadiana en la secreción en minutos, horas, días y estaciones. La parte del sistema nervioso responsable por la mayoría de ritmo circadiano está localizada a un par de estructuras del hipotálamo conocido como núcleo supraquiasmático. En el centro este reloj biológico es intrínseco en las células individuales. Sin embargo la actividad eléctrica coordinada es mediada a las células vecinas por vía de la comunicación de la unión puente. (Cowell CS. (J *Neurobiol*. 2000;43: 379-88)). Por que también a la pituitaria anterior le falta inervaciones directas, la comunicación célula a célula mediada por la unión puente

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d entro de la glándula debe ser indispensable para la adecuada coordinación célula a célula y la sincronización requerida para asegurar la secreción hormonal apropiada y a tiempo. (Vitale ML, et al. (*Biol Reporo*. 2001;64:625-633)). Guerineau NC, et al. (*J Biol Chem*. 1998; 273: 10389-95) concluyó que activar espontáneamente las células endocrinas son unidades simples o dispuestas en ensambles acoplados a la unión puente esparcidos a través de la glándula pituitaria anterior. La sincronia entre las células espontáneamente excitables puede ayudar a la forma de los patrones de la secreción basal. Desde la glándula pituitaria anterior, la hormona del crecimiento, prolactina, hormona adrenocortical, la hormona tiroides, y las hormonas de gonadotropina son sintetizadas bajo control de las hormonas estimulantes del hipotálamo. Uno de los mecanismos en la disrritmia de las glándulas hipotalámicas-pituitaria-endocrina complicadas dentro de uno de los ejes estás por lo tanto también relacionada con las uniones puente por vía de la comunicación reducida. Las enfermedades son diabetes insipidus, hipogonadotropo hipogonadismo, mixoedema, hipofunción adrenocortical, y enanismo. El tratamiento con incompuesto adecuado de la invención, preferiblemente un abridor de unión puente, puede mejorar los síntomas.

También las neuronas en el núcleo supraquiasmático del hipotálamo son dependientes de la comunicación de la unión puente óptima. En el eje mencionad anteriormente el abridor de la unión puente con modo de acción en esta región también beneficiará los pacientes con ritmo circadiano perturbado (Shinohara K, et al. (*Neusosci Lett.* 2000; 286:107-10)

M. **Hipertensión renovascular y nefrotoxicidad**

Las uniones puentes específicas del riñón y endotelial son ampliamente distribuidas en el riñón encontrado en los glomérulos, túmulos y vasculatura incluyendo los capilares intraglomedulares y las arteriolas juxaglomedulares (Haefliger J-A, et al. (*Kidney Int.* 2001;60:190-201)). En ese estudio los autores demostraron la presencia de uniones puente que conectan las células secretadas de renina de la arteriola aferente. El papel de la unión puente podría contribuir a la detección y propagación de las señales que se originan en la sangre, tales como aquellas elicitadas por la presión sanguínea incrementada. Dentro del riñón, tales señales necesitan ser convertidas en estímulos autocrinos, paracrinos y

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endocrinos mediante las células endoteliales de la arteriola aferente y la transmitida a las células secretoras de renina. La comunicación de unión puente juega así un papel importante en la formación del aparato juxtaglomerular interconectado. Las rápidas transiciones de abertura a cierre de las uniones de los canales de las uniones puente implican adicionalmente una repuesta pronta a los cambios vasculares asegurando la retroalimentación continua requerida para casar la función glomerular y tubular así como también la secreción de renina a las demandas fisiológicas. Las enfermedades caracterizadas por una comunicación de unión puente renal dañada se beneficiarán del tratamiento con el compuesto de la invención, preferiblemente un abridor de unión puente específico, administrado oralmente o parenteralmente.

Los métales pesados son nefrotóxicos y causan daño renal. Se ha demostrado que los métales tóxicos de cadmio (FukumotoM, et al. (*Life Sciences*. 2001; 69:247-54)) así como también el mercurio (Yoshida M, et al. (*Arch Toxicol*. 1998; 72:192-96) en cultivos de célula primaria provenientes de uniones puente desacopladas de túbulo próximo de rata y ambos grupos sugieren que la disfunción

renal está relacionada con la comunicación intracelular reducida. El tratamiento del envenenamiento con metal pesado con un compuesto de fosforilación de tirosina conexina se reducirá el daño del tejido y evitará la devastación de tejido progresiva.

Como un ensayo in Vitro se puede desarrollar en cultivos de células provenientes de células túmulos y los compuestos (compuesto 1 compuesto 2 en concentraciones de aproximadamente 10^{-10}-10^{-7} M) prevención del desacoplamiento de la unión puente cuando se dispone a metales pesados será investigada. La comunicación de la unión puente será ensayada con el método de tinte Lucifer como se describió previamente.

Después de la administración sistémica de metales pesados a animales experimentales (ratas) la función renal será medida utilizando ^{3}H-insulina como un marcador de claridad para el índice de filtración glomerular, tetraetilamonio marcado con ^{14}C como un marcador de claridad para flujo de de plasma renal y litio como un marcador para la función tubular próxima (Petersen JS, et al. J. Pharmacol. Esp. Ther. 1991, 258:1-7) antes y después de diferente tiempo de tratamiento crónico con

metales pesados. El tratamiento crónico con un compuesto específico de la invención, tal como el compuesto 1, será iniciado cuando la función renal se comprometa y será vista una mejora significativa de los parámetros de función renal luego del tratamiento. La administración de un compuesto adecuado a la invención será parenteralmente.

La inflamación no infecciosa así como también las infecciones con diferentes microbios induce cambios crónicos no especificos significativos en la función renal también caracterizada por el índice de filtración glomerular reducido, la descresión disminuida de los electrolitros y el agua y los cambios en la presión sanguínea. Algunos de estos síntomas serán también tratados con un compuesto de fosforilación de tirosina conexina específico y los síntomas declinaran.

N. Desarrollo y remodelación de los dientes

Murakami S y Muramatsu T (Anat embryol. 2001;203:367-374) confirman estudios previos de que existe la comunicación de la unión puente entre odontoblastos y la actividad celular se coordina por vía de estos puentes

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intercelulares (Iguchi Y, et al. (Arch Oral Biol. 1984; 29: 489-497)) peo en su reciente estudio ellos también demostraron que estas comunicaciones de unión puente están presentes durante el desarrollo temprano de los dientes (pre-odontoblasto) así como también en odontoblastos jóvenes y viejos. También las célula e pulpa subyacentes los odontoblastos tienen uniones puente. Estos hallazgos indican que la comunicación de la unión puente intercelular es importante tanto durante el desarrollo de los dientes como durante la vida cuando los dientes son remodelados o sacados. El tratamiento con un compuesto fosforilante de conexina de la invención tal como el compuesto 2 que puede ser ensayado un Vitro para efectuarse sobre comunicación intercelular de odontoblasto en un ensayo que es esencialmente comparable al ensayo de osteoblasto descrito aquí, normalizara el desarrollo dañado de los dientes. El tratamiento también facilitará la remodelación de los diente y hará los dientes más resistentes a la caries.

0. **Célula madre.**

Lumelsky et al (2001) han generado células que expresan insulina y otras hormonas endocrinas pancreáticas

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provenientes de células madre embriónicas de ratón. Ver Nadya Lumelsky et al. En *Differentiation of Embryonic Stem Cells to Insulin-Secreting Structures similar to pancreatic Islets, Science,* 292, 1398-1394, 2001). Las células autoensambladas para formar grupos tridimensionales similares en topología Islets pancreáticos normales donde los tipos de célula pancreática están en asocio cercano con las neuronas. La glucosa dispara la nivelación de insulina proveniente de estos grupos de células mediante mecanismos similares a aquellos empleados in vivo. Cuando se inyecta en ratones diabéticos, lasa células productoras de insulina sufren rápida vascularización y mantienen una organización similar a islets agrupada.

En el contexto clínico, este sistema basado en célula madre embriónica permitirá a la generación simultánea y el ensamblaje de secreción de insulina y otros tipos de célula islets conocidos por jugar un papel importante en la regulación de la secreción de insulina en las unidades estructurales funcionales. Estas unidades pueden suministrar material para optimizar la producción de insulina y analizar el control fino de la homeostasis de glucosa. Las células madre embriónicas son ideales para

estos estudios por que las herramientas genéticas se pueden utilizar para definir la base molecular del desarrollo y función del islet. El potencial de las terapias basadas en células es claramente una meta atractiva para la aplicación que involucre células madre embriónicas y germinales embriónicas no humanas. El tejido adulto también puede ser una fuente útil de células pancreáticas funcionales. El sistema de diferenciación descrito aquí puede suministrar una fuente de islets pancreáticos funcionales para el tratamiento de diabetes. Este es el primer reporte que muestra que varios tipos de células de páncreas endocrino pueden ser generados de células madre embriónicas *in vitro*.

Aunque los islets pancreáticos obtenidos de cadáveres pueden funcionar en el hígado después de injerto, los temas de rechazo de tejido y disponibilidad permanecen por resolverse. Es claro que la ingeniería de las células madre embriónicas para producir una fuente abundante de tejido inmunocompatible para el trasplante mantiene una promesa creciente para sobremontar esto y otros problemas asociados con la diabetes.

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El infarto de miocardio conduce a la perdida de tejido y al daño del desempeño cardiaco. Los miocitos restantes son incapaces de reconstruir el tejido necrótico, y el corazón post infartado se deteriora con el tiempo. El daño al órgano objetivo es sensibilizado por las células madre distantes, que migran al sitio del daño y sufren diferenciación de célula madre alternativa; estos eventos promueven en la reparación estructural y funcional. Este alto grado de plasticidad de célula madre urge el ensayo Orlic et al (Nature 410. 701-705 (2001)) sea que la muerte del miocardio se pueda restablecer mediante trasplante de células de médula ósea en el ratón infartado. Ellos clasificaron las células de médula ósea negativas lineales ((Lin-) de ratones transgénicos que expresan proteína fluorescente verde mejorada mediante células activadas fluorescentes clasificadas sobre la base de la expresión del kit-c. Poco después de la ligación coronaria, las células Lin-c-kitPOS fueron inyectadas en la pared de contracción que bordea el infarto. Ellos encontraron que el miocardio recientemente formado ocupaba el 68% de la porción infartada en el ventrículo 9 días después del trasplante de las células de médula ósea. El tejido desarrollado comprendía miocitos proliferantes y estructuras vasculares. Sus

estudios indicaron que las células de médula ósea localmente suministradas pueden generar miocardio de novo, mejorando el resultado de la enfermedad de arteria coronaria. Para caracterizar adicionalmente las propiedades de estos miocitos, ellos determinaron la expresión de la conexión a 43. Esta proteína es responsable de las conexiones intercelulares y el acoplamiento eléctrico a través de la generación de los canales de plasma-membrana entre los miocitos; la conexión a 43, era evidente en el citoplasma de la célula y en la superficie de las células diferenciadoras cercanamente alineadas. Estos resultados fueron consistentes con la competencia funcional esperada del fenotipo del músculo cardiaco. En razón a que las células funcionales son generadas de célula madre embriónica, y en razón a que las conexinas son de hecho expresadas en estas células en tejido cardiaco infartado, se cree que este será el caso de otras células que se diferencien de las células madre embriónicas. En razón a que las conexinas juegan un papel dominante en la función de los tejidos (incluyendo células beta pancreáticas y células del músculo cardiaco). Los compuestos de la invención tal como el compuesto 1 y el compuesto 2 que incrementan la fosforilación de tirosina conexina y el cierre de

hemicanal (dando como resultado una comunicación de unión puente incrementada) mejorará la proliferación de células madre embriónicas en células funcionales en órganos en donde se han implantado células madre.

De esta manera, es un objeto de la invención suministrar compuestos fosforilantes de tirosina conexina tal como el compuesto 1 y el compuesto 2 para estimular la transición de las células madre a células funcionales en órganos trasplantados como el páncreas para el tratamiento de diabetes melitus, corazón para tratamiento de infarto cardiaco, y ganglios basales en el cerebro para el tratamiento de la enfermedad de Parkinson.

En general, se cree que estos compuestos acelerarán la diferenciación de las células madre, que pueden acelerar los procesos de curación en todos los órganos tal como el corazón, cerebro, piel, hueso, páncreas, hígado y otros órganos internos. Los experimentos pueden ser desarrollados utilizando el diseño experimental general y con infarto del miocardio como se describió anteriormente por Orlic et al, *supra*, con administración del compuesto 1 y el compuesto 2, por ejemplo, repetidamente durante el proceso de proliferación. Estos experimentos se cree que

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muestran un incremento en la expresión de conexina 43 mediante el compuesto 1 y el compuesto 2 o un proceso regenerativo más rápido.

P. **Cáncer**

1. Progresión del tumor

Durante la tumorigénesis, la interrupción de la interacción fisiológica de las células normales con las células vecina, y la perdida de características de diferenciación son un denominador común en la provisión de tumor. La alteración en la comunicación de la unión puente se cree que está entre los cambios más templados durante la tumorgénesis celular (*WolburgH, Rohlmann A. Int Rev Cytol.* 1995; 157:315-73), Klaunig JE, Ruch RJ. 1990; 135-46)).

Kyung-Sun Kang, et al. (*Cancer Letters* 166 (2001) 147-153) ha mostrado que la pre y co-incubación con GeO2 en células epiteliales de hígado de rata tratada con TPA abolen la infra regulación del GJIC mediante el TPA que sugiere que una sustancia que recupera la inhibición del GJIC puede ser utilizada en la prevención o inhibición de

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la promoción del tumor. Suzuki J, Na, H-K al. (*Nutrition and Cancer*, vol 36 No.1 p.122-8) han mostrado que los inhibidores de carragenina Lambda aditivo de comida GJIC en células epiteliales de hígado de rata similares a aquellas del forbol éster promotor de tumor bien documentado (TPA), y por lo tanto puede jugar un papel en la carcinogénesis como un agente promotor del tumor. Así, los compuestos de la presente invención pueden ser utilizados en la prevención o tratamiento de cáncer causado por los agentes promotores de tumor, tal como el TPA y la carragenina lambda.

2. Resistencia a la sensibilidad de la droga.

La comunicación de la unión puente incrementada mejora el microambiente en los tumores. Ver Chen et al.: Chem Biol Interact 1998 Abril 24; 111-112:263-75.

3. Metástasis

La perdida de la comunicación de la unión puente intercelular está asociada con alto potencial metastático en todos los canceres con potenciales metastáticos (Saunders MM, et al. *Cáncer Res.* 2001; 61; 1765-1767), Nicolson GI, et al. Proc. Natl. Acad Sci USA. 1988;

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85:473-6)). La prevención de la metástasis se establece mediante el tratamiento con un compuesto fosforilante de tirosina conexina que ayudará en la prevención de la comunicación de la unión puenteen los tumores. El tratamiento es una ayuda la quimioterapia convencional.

Se cree que la administración de una o una combinación de los compuestos de la invención en una cantidad terapéuticamente efectiva (por ejemplo compuesto 1 o compuesto 2) será capaz de evitar o tratar el cáncer.

Q. **Células misceláneas.**

Las uniones puente también juegan un papel importante en la comunicación intercelular (proliferación y diferenciación en la célula de mucosa gástrica. El abridor de unión puente estimulará los procesos regenerativos después del daño inducido. (Endo K, et al. (*J. Gastoenterol Hepatol.* 1995;10:589-94)).

La citoarquitectura de las células meniscales depende parcialmente de la comunicación de la unión puente. La parte del fibrocartílago del menisco así como también de la estructura del fibrocartílago de los tendones

dependerá de la comunicación intercelular. Durante los daños los abridores de la unión puente mejorarán la velocidad de reparación.

R. **Epilepsia**

Puede ser deseable tratar estados de enfermedades tales como epilepsia caracterizada por GJIC anormalmente alto con un abridor de hemicanal, preferiblemente como una terapia activante con el fin de permitir la toma de drogas o de pequeñas moléculas regulatorias que sirven para desacoplar los canales de unión puente.

Es un objeto de la presente invención suministrar métodos para tratar o evitar una o más de las indicaciones médicas o condiciones descritas aquí. Típicamente, pero no exclusivamente, tales métodos incluirán la administración de al menos uno de los compuestos anteriores, preferiblemente uno de los mismos, en una cantidad suficiente para tratar, evitar, o reducir la severidad de la indicación o condición. Los compuestos preferidos han sido seleccionados por uno o una combinación de los ensayos in Vitro e in vivo descritos aquí. Las estrategias de administración particular serán

evidentes para aquellos expertos en el campo y variedad dependiendo por ejemplo del sexo, peso, salud general e indicación o condición especifica a ser tratada o evitada. Como se discutió, los compuestos descritos aquí se pueden emplear como el agente activo solo en los métodos de la invención. Alternativamente, ellos pueden ser utilizados en terapias "agregadas" tal como aquellas en las cuales el uso de los compuestos en conjunto con un método de tratamiento reconocido se indica. Las indicaciones o condiciones preferidas a ser tratadas o evitadas de acuerdo con la invención están generalmente asociadas con comunicación celular dañada o funciones de unión puente dañadas. Indicaciones más especificas o condiciones que se relacionan con la invención se han discutido anteriormente.

Los métodos de tratamiento de acuerdo con la invención pueden emplear uno o más de los compuestos descritos aquí como un agente activo solo. Preferiblemente, uno de los compuestos será empleado. Si se desea, tales compuestos pueden ser utilizados profilácticamente es decir, para evitar o reducir la severidad de una condición indicación o condición particular. Alternativamente, los compuestos se pueden utilizar en conjunto con una aproximación

terapéutica reconocida. Como una ilustración de las modalidades en la cual se trata con irradiación, es generalmente preferido que el método de tratamiento sea "agregado" es decir en conjunto con una terapia reconocida para el tratamiento de la condición. Tales métodos de tratamiento "agregados" de la invención se pueden conducir al mismo tiempo o en diferente tiempo que la terapia reconocida según sea necesaria. Las aproximaciones terapéuticas establecidas para una variedad de enfermedades y condiciones médicas han sido descritas. Ver de manera general, Harrison Principles of Internal Medicine (1991) 12 ed., McGraw-hill, Inc. and the Pharmacological Basis of Therapeutics (1966) Goodman, Louis S. 9th edición Pergammon Press, por ejemplo, cuya descripción se incorpora aquí como referencia.

La concentración de 1 o más compuestos de tratamiento en la composición terapéutica variará dependiendo de un número de factor, incluyendo la dosis del compuesto modulador de hemicanal a ser administrado las características químicas (por ejemplo (hidrofobicidad) de la composición empleada, y el modo y ruta de administración pretendido. En términos finales, uno o más de uno de los compuestos moduladores de hemicanal puede

ser suministrado en una solución amortiguadora fisiológica acuosa que contiene cerca de 0,1 a 10 % p/v de un compuesto para administración parenteral.

Podrá apreciarse que las cantidades reales preferidas de los compuestos activos usadas en una terapia dada variarán de acuerdo a por ejemplo el compuesto específico que está siendo utilizado, la composición particular formulada, el modo de administración y las características del sujeto, por ejemplo la especie, sexo, peso, salud general y edad del sujeto. Las ratas de administración óptimas para un protocolo dado de administración pueden ser determinadas fácilmente por aquellas versados en la técnica usando ensayos de determinación de dosis convencional conducidos con respecto a las líneas de guía anteriores. Los rangos de dosis adecuados pueden incluir entre cerca de 1 µg/kg a cerca de 100 mg/kg del peso del cuerpo por día ("cantidad terapéuticamente efectiva").

Los compuestos terapéuticos de la invención son adecuadamente administrados en una forma protonada y soluble en agua, por ejemplo, como una sal

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farmacéuticamente aceptable, típicamente una sal de adición de ácido tal como una sal de adición de ácido inorgánico, por ejemplo, hidrocloruro, sulfato, o sal de fosfato, o como una sal de adición de ácido orgánico tal como un acetato, maleato, fumarato, tartrato, o sal citrato. Las sales farmacéuticamente aceptables de los compuestos terapéuticos de la invención también pueden incluir sales metálicas, particularmente sales de metal álcali tales como sal de sodio o sal de potasio; las sales de metales de tierras alcalinas tales como sales de magnesio o calcio; sales de amonio tales como una sal de tetrametil amonio o de amonio; y sales de adición de aminoácidos tales como una sal de lisina, glicina, o fenilalanina. Adicionalmente las sales adecuadas están suministradas más adelante.

Compuestos moduladores de hemicanal más particulares de la invención son usados en la forma de sales farmacéuticamente aceptables, un alquil éster, una amida, una alquilamida, una dialquilamida o una hidrazida formada con una función de ácido carboxílico en el terminal C de un compuesto lineal o de una función ácido carboxílica libre, si está presente, de un compuesto cíclico. Las amidas y las alquilamidas inferiores de

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compuestos lineales están entre los compuestos preferidos de la invención. Las sales incluyen sales farmacéuticamente aceptables, tales como sales de adición de ácido y sales básicas. Ejemplos de sales de adición de ácido ya han sido descritos e incluyen las sales de hidrocloruro, sales de sodio, sales de calcio, sales de potasio, etc. Ejemplos de sales básicas son las sales en donde en catión es seleccionado de metales álcali, tales como sodio y potasio, metales de tierras alcalinas, tales como calcio, e iones amonio $^{+}N(R^3)_3(R^4)$, donde R^3 y R^4 independientemente designan alquilo-C_{1-6} opcionalmente sustituido, alquenilo-C_{2-6} opcionalmente sustituido, arilo opcionalmente sustituido, o heteroarilo opcionalmente sustituido. Otros ejemplos de sales farmacéuticamente aceptables son; por ejemplo, aquellas descritas en *Remington's Pharmaceutical Sciences* 17. Ed. Alfonso R Gennaro (Ed.), Mark Publishing Company, Easton, PA, U.S.A., 1985 y ediciones más recientes, y en la *Encyclopedia of Pharmaceutical Technology*.

En los métodos terapéuticos de la invención, un compuesto de tratamiento puede ser administrado a un sujeto en cualquiera de las diversas maneras. Por ejemplo, un compuesto modulador de hemicanal seleccionado en una

combinación de los ensayos *in vitro* y/o *in vivo* suministrados pueden administrados como un profiláctico para impedir el establecimiento de o reducir las severidad de una condición objetivada. Alternativamente, el compuesto puede ser administrado durante el curso de una condición objetivada.

Un compuesto de tratamiento puede ser administrado a un sujeto, sea solo o en combinación con uno o más agentes terapéuticos, como una composición farmacéutica en mezcla con excipientes convencionales, es decir sustancias portadoras orgánicas o inorgánicas farmacéuticamente aceptables adecuadas para aplicación parenteral, enteral o intranasal que no reaccionen de manera adversa con los compuestos activos y que no causen daño al receptor de los mismos. Los portadores o vehículos farmacéutivamente aceptables adecuados incluyen pero no se limitan a agua, soluciones salinas, alcohol, aceites vegetales, polietilen glicoles, gelatina, lactosa, amilosa, estearato de magnesio, talco, ácido silícico, parafina viscosa, aceite de perfume, monoglicéridos de ácidos grasos y diglicéridos de ácidos grasos, ésteres de ácido graso petroetral, hidroximetil-celulosa, polivinilpirrolidona, etc. Las preparaciones

farmacéuticas pueden ser esterilizadas y se desea mezcladas con agentes auxiliares, por ejemplo, lubricantes, preservativos, estabilizadores, agentes humectantes, emulsificadores, sales para influenciar la presión osmótica, amortiguadores, colorantes, saborizantes, y/o sustancias aromáticas y similares que no reaccionen adversamente con los compuestos activos.

Tales composiciones pueden ser preparadas para uso en administración parenteral, particularmente en la forma de soluciones o suspensiones líquidas; para administración oral, particularmente en la forma de tabletas o cápsulas; intranasalmente, particularmente en la forma de polvos, gotas nasales, o aerosoles; vaginalmente; tópicamente por ejemplo, en la forma de una crema; rectalmente por ejemplo, como un supositorio; etc.

Los agentes farmacéuticos pueden ser administrados convenientemente en formas de dosificación unitarias y pueden ser preparados por cualesquiera de los métodos bien conocidos en las técnicas farmacéuticas, por ejemplo, como se describe en la *Remington's Pharmaceutical Sciences* (Mack Pub. Co., Easton, Pa., 1980). Las formulaciones para administración parenteral

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pueden contener como excipientes comunes tales como agua estéril o salina, polialquilen glicoles tales como polietilen glicol, aceites de origen vegetal, naftalenos hidrogenados y similares. En particular, polímero láctido biodegradable, biocompatible, copolímero de láctido/glicolida, o copolímeros de polioxietileno-poliixipropileno pueden ser excipientes útiles para controlar la liberación de ciertos compuestos moduladores del hemicanal de la invención.

Otros sistemas de suministra parenteral potencialmente útiles incluyen las partículas de copolímero de etileno-acetato de vinilo, bombas osmóticas, sistemas de infusión implantable, y liposomas. Las formulaciones para administración por inhalación contienen como excipientes, por ejemplo, lactosa, o pueden ser soluciones acuosas que contienen, por ejemplo, polioxietileno-9-lauril éter, glicolato y deoxicolato, o soluciones aceitosas para administración en la forma de gotas nasales, o como un gel que es aplicado intranasalmente. Las formulaciones para administración parenteral también pueden incluir glicocolato para adminiostración bucal, metoxisalicilato para administración rectal, ácido cítrico para administración vaginal. Otros sistemas de suministra

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administrarán los agentes terapéuticos directamente en un sitio quirúrgico, por ejemplo, después de una angioplastia de balón un compuesto modulador de hemicanal puede ser administrado por el uso de stents.

Abreviaciones y Fórmulas:

Por toda la descripción y reivindicaciones los tres códigos de letra para los aminoácidos naturales son usados lo mismo que los tres códigos de letras generalmente aceptados para otros α-aminoácidos, tales como Sarcosina (Sar), ácido α-Amino-iso-butanoico (Aib), Naftilalanina (Nal) incluyendo 1-naftilalanina (1Nal) y 2-naftilalanina (2Nal), Fenilglicina Phg, ácido 2,4-Diaminobutanoico (Dab), ácido 2,3-Diaminopropanoico (Dapa), y Hidroxiprolina (Hyp). En donde no se especifica nada Hyp representa 4-hidroxiprolina. Los aminoácidos naturales o esenciales son los aminoácidos constituyentes de las proteínas. Los aminoácidos aromáticos son Phe, Tyr, Trp, 1Nal, 2Nal, y His. En donde la forma L o D no ha sido especificada debe entenderse que el aminoácido en cuestión tiene la forma natural L, es decir, *Pure & Appl. Chem.* Vol. 56(5) pp. 595-624 (1984). En donde no se

especifica nada debe entenderse que el aminoácido del terminal C de un compuesto de la invención existe como el ácido carboxílico libre, esto puede también ser especificado como "-OH". El aminoácido de terminal C de un compuesto de la invención puede ser mostrado como que tiene la función de terminal "-OH/NH_2" lo que significa que hay dos formas preferidas del compuesto: el ácido carboxílico libre y el derivado amidado.

Las siguientes definiciones específicas aplican a menos que se especifique otra cosa: ASAL se refiere a radical 4-azidosaliciloilo; AB se refiere a radical 4-azidobenzoilo; Fmoc se refiere al radical 9-fluorenilmetiloxicarbonil; Ac se refiere al radical acetilo. Por toda la divulgación presente a menos que se especifique otra cosa las siguientes definiciones aplican; Acm se refiere al radical acetamidometilo; T4c se refiere al radical ácido L-tiazolidin-4-carboxílico; Pc se refiere al radical ácido L-pipecólico; DNP se refiere a dinitrofenilo; DBF se define como ácido 2-aminoetil-6-dibenzofuranpropiónico; Acm se refiere a acetamidometilo; y Sar se refiere al radical sarcosinilo; las funciones DNP son un hapteno para el reconocimiento del anticuerpo, y compuestos de la invención que

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contienen partes de DNP pueden ser usadas preferiblemente como herramientas de investigación.

Ver también PCT/DK01/00127 (WO 01/62775) y PCT/US02/05773 (WO 02/077017) para información adicional.

Todas las referencias descritas aquí se incorporan como referencia. Los siguientes ejemplos son ilustrativos de la invención.

Ejemplo de Referencia 1: Ensayo de Arritmia Estándar en Ratón *in vivo*

Es posible ensayar los efectos antiarrítmicos llevando a cabo un modelo de arritmia inducida por calcio en ratones.

Brevemente, los efectos antiarrítmicos de los compuestos pueden ser ensayados en un modelo *in vivo* de arritmias inducidas por calcio de acuerdo con el modelo de J.J. Lynch, R.G. et al. *J Cardiovasc.Pharmacol.* 1981, 3 49-60. Los ratones (25-30 g) fueron anestesiados con una combinación de anestésico neuroléptico (Hypnorm® (citrato de fentanilo 0,315 mg/ml y fuanisona 10 mg/ml) +

midazolam (5 mg/ml)). Las soluciones comerciales de hypnorm y midazolam fueron diluidas 1:1 en agua destilada y una parte de Hypnorm® diluida es mezclada con una parte de midazolam diluida.

La anestesia fue inducida por administración s.c. en una dosis de 0,05-0,075 µl/10 gramos de ratón. Una cánula *i.v.* fue insertada en la vena de la cola. La señal guiadora II ECG fue registrada continuamente por medio de posicionar los electrodos ECG de acero inoxidable sobre el miembro delantero derecho y sobre el miembro trasero izquierdo. El electrodo de tierra fue colocado sobre el miembro trasero derecho. La señal fue amplificada (x 5.000-10,000) y filtrada (0,1-150 Hz) por medio del módulo Hugo Sachs Electronic model 689 ECG. La señal análoga fue digitalizada por medio de una tarjeta de adquisición de datos de 12 bit (Data Traslation model DT321) y muestreada a 1000 Hz usando el software Notocord HEM 3.1 para Windows NT. Después de un periodo de equilibración de 10-min, la muestra de ensayo de la droga fue inyectada en la vena de la cola. Los ratones pretratados con el vehículo fueron ensayados como una medida del nivel de control en animales no tratados. El

volumen de inyección fue de 100 µl en todos los experimentos. La infusión de $CaCl_2$ (30 mg/ml, 0,1 ml/min ≈ 100 mg/kg/min (calciumchlorid-2-hydrat, Riedel-de Haën, Alemania)) fue iniciado 3 minutos después de la administración *i.v.* de la droga o vehículo. El tiempo de retardo para el establecimiento del segundo grado de bloque AV fue determinado como el tiempo desde el inicio de la infusión de $CaCl_2$ hasta que el primer evento arrítmico ocurrió. Un evento del segundo grado de bloque AV fue definido como falla intermitente de la conducción AV caracterizada por una onda P sin el complejo QRS concomitante.

Las respuestas fueron expresadas con relación al tiempo hasta que el segundo grado de bloque AV ocurrió en los ratones tratados con el vehículo. El efecto máximo de cada una de las sustancias ensayadas se ha resumido.

Métodos más particulares para detectar compuestos candidatos con buena unión de espacio modificando la actividad adicionalmente incluyen compuestos candidatos de selección que prolongan el tiempo hasta el establecimiento del bloque ventricular atrial inducido

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(AV) por lo menos cerca del 20% (marcador de por lo menos 2) en el ensayo de arritmia de ratón estándar *in vivo*. Los compuestos más preferidos exhiben una prolongación de por lo menos cerca del 60% del tiempo hasta el establecimiento del bloque AV inducido (marcador por lo menos 3) en el ensayo.

Los siguientes ejemplos suministran un ensayo funcional que muestra la habilidad del compuesto 1 tanto para los canales de unión de espacio abierto y hemicanales cerrados en cardiomiocitos de ratones neonatos. Así, es posible obtener la regulación diferenciada de GJIC por la fosforilación de tirosina tanto de los hemicanales como los canales de unión de espacio.

Ejemplo 1 – Compuestos antiarrítmicos

Los compuestos usados en la invención incluyen Ac-D-Tyr-D-Pro-D-4Hyp-Gly-D-Ala-Gly-NH2 (compuesto 1) y Ac-Gly-Asn-Tyr-NH2 (compuesto). Los compuestos son sintetizados de acuerdo con síntesis de fase sólida estándar como se describió en la WO 01/62775. Un ejemplo adicional de compuestos útiles en la invención es el trans-resveratrol y CAPE (éster de fenetil de ácido cafeico). Los métodos

para hacer los compuestos han sido descritos. Ver por ejemplo, las solicitudes PCT/DK01/00127 (WO 01/62775) y PCT/US02/05773 (WO 02/077017).

Ejemplo 2 – Análisis del Esfuerzo Metabólico Inducido por Remoción de Glucosa

Las arritmias cardíacas pueden ser causadas por disturbios tanto de formación y conducción de la acción potencial. La propagación de una acción potencial de célula a célula es mediada por las uniones de espacio intercelular. Estas uniones de espacio consisten de canales, que son construidos por proteínas especializadas llamadas conexinas. Hay diversas líneas de evidencia que muestran que una expresión reducida y/o una distribución alterada de las conexinas pueden ser dañosas para la propagación del impulso normal y por lo tanto arritmogénicas. Un ejemplo son las alteraciones vistas durante y después de la isquemia. Durante isquemia aguda el no acoplamiento de las células se cree principalmente que es causado por la acidificación intracelular, la Ca2+ intracelular incrementada, ATP intracelular reducida y por concentraciones elevadas de acilcarnitinas de cadena larga y ácidos grasos [1,2]. Después de la fase aguda de

isquemia, el remodelado ocurre en donde la arquitectura del área infartada y su zona borde es alterada. Este remodelamiento ha sido asociado con áreas de desarreglo de conexina y el desarrollo de circuitos reentrantes potenciales, que se cree que actúan como sustratos arritmogénicos [3]. Estudio in vivo e in Vitro han mostrado que un grupo de péptidos antiarrítmicos pueden retrasar e inhibir el establecimiento de la arritmia, y estudios subsecuentes han mostrado que estos péptidos incrementan el acoplamiento eléctrico entre las células [2].

A. Métodos

1. Cultivo de células: En corto los corazones de 20 ratas neonatas (edad de 1-2 días) son cortados asépticamente. Los ventrículos son aislados y cortados en 4-6 piezas que son digeridos en diversas etapas en una salina amortiguada Hanks con tripsina y DNAse. Las células son entonces centrifugadas y resuspendidas en MEM con 5% de FCS. Para eliminar las células no musculosas la suspensión es preplaneada en platos petri durante 30 minutos a 37° C. Las células en suspensión son entonces sembradas en cubiertas deslizantes recubiertas con

colágeno. La densidad del sembrado será ajustada para dar cultivos confluentes.

2. Electrofisiología: La cubierta deslizante con cardiomiocitos confluentes es montada en una cámara abierta en una etapa de un microscopio invertido y superfusionada con salina amortiguada con fosfato Dulbeccos (PBS) por gravedad causada por el flujo a 1 ml/min, 37° C. La solució (PBS) contiene (en mM): Na+ 152, K+ 4,2, Cl- 141,5, PO43- 9,5, Ca2+ 0,9, Mg2+ 0,5, pH 7,4. Pipetas abrazadas con parches son haladas de capilarios de vidrio de 1,5 mm (GC150F-15, Harvard Apparatus) en un halador de micro electrodo Sutter Flaming-Brown P-87 y polichadas por fuego a un resistencia de 4-6 MW. Las pipetas son llenadas con una solución similar intracelular que contiene en mM: K+ 150. Na+ 15, Cl- 5, Gluconato- 150,2, EGTA 5, HEPES 5, Ca2+ 2 mM, Mg2+ 1,6, pH 7,2.

El conjunto de abrazadera de parche consiste de un amplificador discontinuo sincronizado (SEC-05LX, NPI electronics) y los datos son digitalizados usando una interfaz INT-10 (NPI electronics) y una tarjeta de adquisición de datos PC1200 (Nacional Instruments). Ambas

señales de corriente y voltaje son filtradas en pasa bajos de 1 kHz usando filtros internos de los amplificadores y digitalizando en 10 kHZ.

Una célula es acercada con un electrodo usando el micromanipulador PatchMan 5173 (Eppendorf). Cuando se hace contacto con la célula (visto como un incremento súbito en la resistencia de entrada), se aplica la succión hasta que la configuración Giga sello es establecida. Entonces un breve pulso de succión es aplicado para romper la membrana debajo de la pipeta. El amplificador es entonces colocado en el modo de amarre de corriente cero para monitorear el potencial de la membrana.

Alguna distancia (>>1 cm) lejana de un electrodo de platino bipolar es usada para apaciguar el cultivo. El retraso entre el artefacto de estímulo y la aparición de una acción potencial en la célula marcada o parchada es entonces una medida de la velocidad de conducción.

Las células fueron regadas con una solución de control con 0,1 g/l BSA hasta que una línea de base estable en el intervalo de estímulo-activación (SAI) fue establecido.

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Entonces el rociado fue cambiado a solución sin glucosa durante 15 minutos y subsecuentemente retado con el compuesto 1 (10^{-8} M) durante 20 minutos. En paralelo, los experimentos de control fueron llevados a cabo en donde fueron conducidos sobre una escala de tiempo similar sin agregar el compuesto 1. La fechas son expresadas como porcentaje de cambio en relación a la línea base anterior a la remoción de la glucosa del medio.

B. Resultados

Como se ilustró en la Figura 2, el estrés metabólico inducido por la remoción de glucosa produjo un incremento ligero en el intervalo de estímulo-activación sugiriendo que la remoción de glucosa retardó la velocidad de conducción. En contraste, el superrociazo con una concentración de 10 nM del compuesto 1 disminuyó el intervalo de estímulo-activación, es decir, el compuesto 1 incrementó la velocidad de conducción en los cardiomiocitos primarios cultivados. Estos datos indican que el compuesto 1 incrementa el acoplamiento de célula a célula en los cardiomiocitos.

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Ejemplo 3 - Inmunoprecipitación y Análisis de Conexinas Fosforiladas

Los compuestos usados en este ejemplo fueron hechos usando la química Fmoc de fase sólida estándar. Ver los ejemplos anteriores. La identificación fue llevada a cabo por espectrometría de masa, y la pureza determinada por RP-HPLC. La aglomeración *in situ* y los estudios animales han mostrado que el siguiente péptido (compuesto 1) se une al receptor en el rango nano-molar. Los estudios iniciales con el compuesto 1 en 1 nM, 10 nM, 50 nM y 100

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nM determinarán si los estudios con los siguientes compuestos de péptidos 3 (H-GAG-4-Hyp-PY-NH2) y el compuesto 4(H-GNY-NH2) son conducidos. Estos estudios también determinarán si los estudios involucran inhibidores específicos de quinasa son conducidos.

Las células H9c2 fueron sembradas en platos de 24 multi pozos en una densidad de 7.900 células/cm^2 (~15.000 células/pozo) y crecidas durante 3 días en MEM suplementado con 10% de suero de ternero fetal (FCS) y 1000 unidades de penicilina/1000 µg estreptomicina (pen/strip) en una atmósfera de CO_2 al 5% y una humedad del 100% a 37° C.

Las células marcadas fueron incubadas en un amortiguador Triton X-100 de lisis (~$5*10^7$ células/ml) durante 1 hora a 4° C. El lisato es centrifugado 30 minutos a 20,000*g durante 30 min. El sobrenadante fue preclarificado agregando 10 µl de Sefarosa por 200 µl de sobrenadante. El sobrenadante preclarificado fue entonces agregado con 0,5-1 µg de fosfo-tirosina o fosfo-serina como anticuerpos (Sigma) e incubados durante 1,5 hr a 4° C en un agitador orbital. El complejo de anticuerpos fue

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entonces capturado por incubación con 50 µl de una suspensión 1:1 de proteína G Sefarosa durante 1,5 hr. Finalmente el complejo fue lavado extensivamente con 0,1% (p/v) Triton X-100. 50 mM de Tris, pH 7,4, 300 mM de NaCl y 5 mM de EDTA.

Los complejos fueron cargados en un gel SDS al 12% y secados en una membrana PVDF. La membrana es bloqueada con 0,1% de Tween 20. 50 mM de Tris, pH 7,4, 300 mM de NaCl, leche seca al 5% (TDST) durante mínimo 2 h. La membrana fue incubada subsecuentemente con anticuerpos de conexina específicos 43 (Zymed) durante 1,5 h a RT y desarrollados con ECL+ (Amersham).

Las Figuras 3A-B muestran inmunomanchas en las cuales el anticuerpo anti-fosfotirosina fue usado. El fragmento Cx43 de fosforilación es ilustrado como una función del compuesto 1 (3A-B) y el compuesto 2 (3B) en concentración.

Las Figuras 4A-B muestran inmunomanchados en los cuales el anticuerpo anti-fosfoserina (4A) o el anticuerpo anti-fosfotirosina (4B) fue usado.

Ejemplo 4: Efecto del Péptido del compuesto 1 en la Actividad Hemicanal en Miocitos Cardíacos Confluentes

1. Cultivo celular: Brevemente, los corazones de 20 ratas neonatas (edad 1-2 días) fueron cortadas asépticamente. Los ventrículos fueron aislados y cortados en 4-6 piezas, que fueron digeridas en diversas etapas en una salina amortiguada Hanks con tripsina y DNAse. Las células fueron entonces centrifugadas y resuspendidas en MEM con 5% de FCS. Para eliminar las células no musculosas la suspensión fue preplaneada en platos petri durante 30 minutos a 37° C. Las células en suspensión fueron entonces sembradas cubiertas deslizantes recubiertas con colágeno.

2. Toma coloreada: Las cubiertas deslizantes con cardiomiocitos fueron incubadas durante 30 minutos con una solución de control (PBS) que contiene en mM: Na^+ 152, K^+ 4,2, Cl^- 141,5, PO_4^{3-} 9,5, Ca^{2+} 0,9, Mg^{2+} 0,5, glucosa 6, pH 7,4 o una solución de mimetización de isquemia (daño) (como control solamente K^+ 8,2, ninguna glucosa, 10 mM de deoxi-

glucosa, pH 6,5), ambas soluciones contienen 200 µM de calceína. Entonces las células fueron lavadas durante 10 minutos con la solución de control.

Las cubiertas deslizantes fueron montadas en una cámara abierta sobre la etapa de un microscopio invertido. Diez campos aleatorios fueron escogidos y de cada campo tres células fueron escogidas bajo la microscopía de luz convencional. Entonces las células fueron excitadas por luz de 480 nm y la emisión de fluorescencia fue medida en 510 nm, en donde la intensidad de emisión es una medida de la toma de coloreado.

El efecto del compuesto 1 sobre la toma de coloreado fue investigado bajo dos condiciones:

Experimento 1: En esta serie de experimentos las células fueron incubadas con la solución de control con calceína durante 30 minutos y lavada por 10 minutos sin la tinta. En experimentos apareados el mismo experimento fue llevado a cabo en donde el péptido es agregado durante los 30 minutos de incubación.

Experimento 2: Como el experimento 1 solamente se llevo a cabo con la solución de daño en lugar de la solución de control.

3. Resultados Los experimentos que usaron la toma de calceína en cardiomiocitos cultivados de ratas neonatas mostró que la toma de coloreado o de tinta es 38% mayor que en las células de control, cuando las células son expuestas a esfuerzo metabólico (en la forma de bajo pH, el K^+ extracelular se incrementó y la deoxiglucosa 5-10 mM). Este efecto podría ser reducido significativamente a 5% cuando las células fueron co-incubadas con el compuesto 1 (10 nM, $P<0,017$ en el ensayo-t apareado contra las células expuestas a la deoxiglucosa solamente, $n=6$). Estos resultados muestran que la permeabilidad de membrana incrementada por los hemicanales de conexina visto después del esfuerzo metabólico pueden ser inhibidos por el compuesto 1. También es probable que este efecto será benéfico para la función celular y la supervivencia. Mas aún, los experimentos de fosforilación sobre cardiomiocitos H9c2 cultivados han mostrado que el compuesto 1 media la fosforilación de tirosina de la conexina

43. Más aún, los Cardiomiocitos expuestos durante 4 h con 0,1, 10. 50 y 100 nM del compuesto 1 un incremento de la fosforilación de tirosina de la conexina 43. La fosforilación de tirosina ha sido reportada para mediar una ruptura de la unión de espacio de comunicación intercelular y podría explicar fácilmente la disminución en la permeabilidad de la membrana visto en los experimentos con calceína. En algunos experimentos, el compuesto 1 se encontró que diminuía la fosforilación del Ser.

La Figura 5 muestra los efectos de 10 nM del compuesto 1 en la toma de la calceína inducida por isquemia en los cardiomiocitos cultivados.

Ejemplo 5 - Ensayo ELISA de la Fosforilación de la Conexina en Sitio Específico

Un ensayo de emparedado ELISA ha sido desarrollado el cual permite medir la fosforilación en sitio específico de las conexinas en muestras de tejido lo mismo que en cultivos de células en un formato multi-pozo (24-96 pozos). Los pozos son recubiertos con anticuerpo (captura

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de anticuerpo) contra el tipo de conexina en cuestión. Las células o los extractos de tejido se hacen reaccionar entonces con las placas recubiertas con el anticuerpo capturado a 4° C o/n. Las conexinas capturadas que son fosforiladas son detectadas con un anticuerpo dirigido contra sitios de fosforilación específicos y conjugado sea con biotina, FITC, TRITC o peroxidasa.

Las cantidades de anticuerpo ligadas a los sitios de fosforilación específicos pueden alternativamente ser medidos con una radioactiva marcada o una enzima conjugada con anticuerpo contra las especies IgG del anticuerpo de detección.

El principio ha sido demostrado usando antiCx43 de ratón como anticuerpo capturado, anti-Tyr-P de conejo (sitio de fosforilación de la tirosina) como anticuerpo de detección y anti-IgG de conejo sea marcado con ^{125}I o HRP (peroxidasa de rábano) para medir las cantidades de enlaces anti-Tyr-P. El principio también puede ser demostrado usando antiCx43 del conejo (5 µg/ml; cat. No. 710700. Zymed Lab. Inc., California, USA) como anticuerpo de captura, una anti-fosfotirosina monoclonal de ratón (50 µg/ml; cat. no. P-3300. clon pt66, Sigma, MO, USA)

como anticuerpo de detección y anticuerpo completo anti-ratón sea marcado con ^{125}I (antiratón de oveja Ig cat. no. IM 131, Amersham Biosciences, Wales, UK) o peroxidasa (anti-Ig de ratón de burro; cat. no. 715-035-151) para medir las cantidades de anti-fosfotirosina ligada.

La Figura 6A muestra en forma esquemática un formato de detección ELISA preferido. Las Figuras 6B y 6C muestran los resultados de este ejemplo.

El presente ejemplo y la discusión suministran evidencia de que el estrés metabólico abre los hemicanales de conexina en células cardíacas, y que esta abertura puede ser proporcionada por la incubación con el compuesto 1. Mas aún, los Ejemplos 2-5 muestran que el compuesto 1 cierra los hemicanales durante el estrés metabólico. De acuerdo con el efecto inhibitorio del compuesto 1 sobre la toma de calceína por los hemicanales, es decir un cerrado de los hemicanales, los ejemplos también muestran que el compuesto 1 ayuda a modular el estado de fosforilación de tirosina de la conexina 43. El compuesto 1 es conocido como que abre las uniones de espacios, CF. WO01/62775 y es conocido que la inhibición de la desfosforilación de las conexinas impide que se cierren

los canales de unión de espacios. Así, se cree que los compuestos suministrados aquí los cuales pueden ser mostrados como que tienen efectos de abertura o modulación de la unión del espacio benéficos del compuesto 1, es decir todos los péptidos antiarrítmicos descritos en WO01/62775 y péptidos conocidos tales como AAP, AAP10. HP5 y similares son útiles enla presente invención como moduladores de la abertura y cierre de los hemicanales. También se cree que el compuesto 1 mantiene o incrementa la fosforilación de tirosina del Cx43, modulando así la función del hemicanal.

Ejemplo 6 - Toma de coloreado en cardiomiocitos

1. Métodos Los miocitos ventriculares fueron aislados con ratas neonatas por digestión de tripsina y puestas en placas sobre cubiertas deslizantes recubiertas con colágeno. Después de cuatro días los miocitos formaron láminas de células confluentes y golpeándose sincrónicamente. Para medir la toma de coloreado, las células fueron incubadas en un amortiguador que contiene 200 µM de calceína durante 30 minutos a temperatura ambiente. Entonces las cubiertas deslizantes fueron lavadas muy bien con el

amortiguador de control y montadas en una cámara de baño abierta y superfundidas con el amortiguador de control (RT). Las células fueron excitadas con luz a 480 nm por medio de una lámpara Xenon y un monocromador. Las imágenes de la fluorescencia emitida a 510 nm fueron recolectadas por una cámara CCD enfriada (Sensicam). El control de la excitación y la adquisición de la imagen fueron controlados usando un sitio de trabajo de creación de Imágenes (Axon).

Bajo la microscopia de luz tres regiones fueron colocadas sobre los cardiomiocitos en una imagen. Entonces la intensidad de fluorescencia promedio fue medida y el procedimiento fue repetido hasta que 30 regiones en 10 imágenes habían sido medidas. El promedio de estas medidas fue usado como valor para la condición experimental dada, esto es n=1. En cada serie una incubación de control fue llevada a cabo y todos los valores experimentales son dados como relativos a este valor.

Soluciones: Amortiguador de control (en mM): NaCl 136, KCl 4, $MgCl_2$ 0,8, $CaCl_2$ 1,8 HEPES 5, MES 5, Glucosa 6, pH

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7,3 Amortiguador de esfuerzo (en mM): NaCl 136, KCl 8, $MgCl_2$ 0,8, $CaCl_2$ 1,8 HEPES 5, MES 5, Deoxi-glucosa 10. pH 6,2.

2. Resultados La incubación de las células con calceína bajo la condición de control dio algún manchado de fluorescencia, el cual fue principalmente de una naturaleza particulada localizada (ver Figura 7A-C, panel B). En la parte superior de los miocitos cultivados, el curvado o enroscado de las células que se mancharon inmediatamente con tripan azul, fueron frecuentemente encontradas. Estas células manchadas grandemente con calceina y con cuidado fueron siempre tomadas para colocar las regiones de las medidas sobre estas células. Cuando las células fueron expuestas a la inhibición metabólica el patrón de manchado cambio, y el manchado difuso por el citosol fue observado (figura 7 C). La intensidad promedio durante el esfuerzo fue de 36.2 ± 4.18 % mayor comparada con el control (n=5, P< 0,001 en el ensayo t pareado).

Esto confirma los hallazgos en tipo de células cardiacas y otros tipos de células, que la inhibición metabólica

activa la toma de manchados fluorescentes, probablemente vía los hemi-canales de conexina. Hemos supuesto que uno de los mecanismos por medio de los cuales la familia AAP de péptidos podría ejercer sus efectos positivos sobre el tejido cardiaco, podría ser interrumpir la activación de los hemi-canales, y mejorando así la homeostasis celular. Para investigar esto, las células fueron expuestas a esfuerzo en la presencia de ZP123 en concentraciones entre 0,1 pM y 10nM. Los resultados son presentados en la figura 8, en donde cada punto de datos representa cinco experimentos. Como puede verse la dosis dependientemente de ZP123 redujo la toma de calceina. Usando una función sigmoidal ED_{50} fue estimado en 3.3 ± 5.3 pM con un coeficiente Hill de 2.5 ± 1.0. la intensidad promedio durante la inhibición máxima por el compuesto 1 fue de 6.9 ± 1.7 % por encima del control.

Ejemplo 7- Medidas de volumen en cardiomiocitos:

Métodos: los miocitos ventriculares fueron aislados de ratas neonatas por digestión de tripsina y puestas en placas sobre cubiertas deslizantes recubiertas con colágeno. Después de cuatro días los miocitos formaron laminas de células confluentes y golpeadas

sincrónicamente. Para medir el volumen de células las células fueron cargadas con calceina AM (5 µm en amortiguador de control) durante 15 minutos a 37 °C y las cubiertas deslizantes fueron montadas en una cámara de baño abierta. Los experimentos siguientes fueron llevados a cabo a temperatura ambiente. Usando un microscopio con focal láser Leica, una sección transversal óptica fue llevada a cabo cada 20 segundos por la duración de los experimentos.

Las secciones transversales ópticas fueron analizadas usando Metamorf (creación de Imágenes Universales). El área de las células fue determinada como el área de pixeles con intensidades de fluorescencia mayores que el valor umbral escogido para minimizar las fluctuaciones inducidas en el fondo. Los datos son presentados como volúmenes relativos por medios de dividir cada valor por el área promedio bajo las condiciones de control (la ultima medida antes del esfuerzo metabólico).

Soluciones: amortiguador de control (en mM): NaCl 136, KCl 4, $MgCl_2$ 0,8, $CaCl_2$ 1.8 HEPES 5, MES 5, Glucosa 6, pH 7.3 amortiguador de esfuerzo (en mM): NaCl 136, KCl 8,

$MgCl_2$ 0,8, $CaCl_2$ 1.8 HEPES 5, MES 5, deoxi-glocosa 10. pH 6.2

Resultados bajo condiciones de control las células en promedio tienden a disminuir ligeramente su volumen con el tiempo (Figura 9B muestra datos promedio de 5 experimentos). Cuando las células fueron retadas con la inhibición metabólica, la caída en volumen fue invertida a un incremento neto (cuadrados llenos en la figura 9A). En promedio el volumen relativo fue de 1.06 ± 0,02 (últimos cincos minutos de esfuerzo, n=5), en donde el volumen de las células de control en este periodo fue de 0,94 ± 0,04.

Las medidas de toma de tinturado en miocitos han mostrado que el compuesto 1 inhibe los hemi-canales de conexina abierto. Se cree que los hemi-canales pueden contribuir al hinchado observado. Para ensayar este compuesto 1 (0,1 nM) fue incluido en el medio de esfuerzo y el volumen fue monitoreado. Los datos de seis experimentos son mostrados en la figura 9A. (cuadrados abiertos) y como puede verse el estrés que indujo el hinchamiento fue impedido. El volumen relativo promedio durante el esfuerzo en la presencia del compuesto 1 fue de 0,93 ± 0,05.

Cuando se comparar los datos de control, esfuerzo y esfuerzo mayor del compuesto 1 usando ANOVA de una vía, las diferencias significativas fueron detectadas (ver figura 10). En ensayos Post.hoc (LSD) mostraron que el esfuerzo fue significativamente diferente del control ($P<0,05$) y el esfuerzo plus del compuesto 1 ($P<0,05$), mientras que el control no fue diferente del esfuerzo plus del compuesto 1 ($P>0,76$).

Ejemplo 8- Reducción del tamaño infartado después de infartación miocardial en ratas.

1. Métodos:

Las ratas macho Lewis (300-350 g; M&B, L1. Skendsved, Dinamarca) fueron anestesiadas con una combinación de anestésico neurolepto (Hypnorm® (citrato de fentanil 0,315mg/ml y fluanisona 10 mg/ml) + midazolam (5 mg/ml)). La solución comercial de midazolam fue diluida 1:2 en agua destilada. Tres partes del midazolam diluido es mezclado con una parte de hypnorm®. La anestesia fue inducida por administración s.c. de 0,2 ml de esta solución por

100 gramos de rata. Cuando la anestesia quirúrgica fue establecida, una canula endotraqueal es insertada y el animal es artificialmente ventilado usando un ventilador para rodentes Harvard ajustado para mantener el pH arterial en 7,3-7,5 durante la cirugía.

El compuesto 1 fue suministrado por una minibomba osmótica (Alzet modelo 2ML4) que fue insertada dentro de la cavidad intraperitoneal (i.p.) inmediatamente antes de la inducción del infarto miocardial. La bomba fue llenada con vehículo (salina isotónica) o el compuesto 1 y se va a 37 °C durante 24 horas antes de la operación. Para poder asegurar que la concentración de plasma terapéutico fue obtenida ya en el momento del infarto, una dosis de carga del compuesto 1 fue dada s.c. antes del ligado del LAD. Con la dosis de carga s.c. un nivel de plasma estable fue alcanzado ya después de 3 minutos.

Después de la administración de la dosis de carga s.c. en el compuesto 1 y después de la inserción i.p. de la minibomba osmótica, una toracotomia izquierda fue llevada a cabo, y la arteria descendente anterior izquierda fue ligada usando una sutura de 6-0 hilos. El tórax es

cerrado con capas y una presión negativa fue inducida en la cavidad pleural para desdoblar los pulmones. Los animales Sham-operados fueron sometidos al mismo procedimiento sin el ligado del LAD. Postoperativamente, al animal se le permitió recuperarse en una cabina calentada a 30 °C con 50% de oxígeno hasta la siguiente mañana. Para impedir la deshidratación durante el periodo de recuperación, a las ratas se les dio 5 ml de 5% de glucosa s.c. inmediatamente después de completar la cirugía y otros 5 ml de glucosa al 5% a las 4 p.m. Para aliviar el dolor postoperativo, las ratas fueron tratadas con buprenorfina (20 µg/100 g s.c. b.i.d) y meloxicam (0,1 mg/100 g s.c. una vez diariamente) durante tres idas despues del infarto miocardial. En este estudio la dosis de infusión dada a las ratas con infarto miocardial (MI) fue ajustada para producir concentraciones de plasma en 0 [vehículo = salina isotónica], 1,7 ± 0,4 nM [dosis 1] 5.7 ± 0,4 [dosis 2], o 93,7 ± 12,3 nM [dosis 3]. Las concentraciones de plasma del compuesto 1 fueron medidas por espectrometría de masa seguida por extracción de fases sólida. Las dosis usadas son mostradas mas abajo en la Tabla I:

TABLA I

Grupo	Concentración de plasma después de 3 semanas nM	Dosis de carga s.c. del compuesto 1 (nmol/kg)	Dosis de infusión i.p del compuesto 1 (pmol/kg/min)
Sham-MI	0	0	0
MI	0	0	0
MI	1,7±0,4	0,25	11
MI	5,7±0,4	2,5	110
MI	93,7±12,3	25	1100

Tres semanas después del ligado de LAD, las ratas fueron anestesiadas con una inyección i.p., de pentobarbital (50 mg/kg). Los animales fueron colocados sobre un tapete de calentamiento para poder mantener la temperatura del cuerpo en 37 °C. La traceotomio fue llevada a cabo y las ratas fueron ventiladas con oxígeno üsando el ventilador de rodentes Harvard. El ventilador fue ajustado para mantener el pH arterial en 7,35-7,45. Catéteres PE50 fueron colocados en la vena femoral y la arteria para administración i.v. y las medidas de presión arterial, respectivamente. Después de la inserción del catéter por i.v. en la femoral, el animal fue paralizado por administración i.v. de pancuronio, 1 mg/kg. La anestesia

y la parálisis respiratoria es mantenida por infusión i.v. continuo de pentobarbital (2.5 mg/kg/h) y pancuronio (1 mg/kg/h) suministrado en salina isotónica (50 µl/min). Un catéter tigon fue insertado en el ventrículo izquierdo por vía de la arteria carótida derecha para la determinación de la presión diastolica final ventricular izquierda. Después de registrar la presión diastolica final ventricular izquierda (LVEDP), los corazones son removidos de los cuerpos por medio de cortar las venas y arterias unos pocos mm del corazón. Los corazones fueron enjuagados cuidadosamente de la sangre con salina isotónica, pesados y colocados en formalina al 4% amortiguada neutral.

Después de fijación las venas grandes fueron cortadas cerca al corazón. De allí en adelante los corazones fueron cortados en tajadas paralelas con un espesor de 1,5 mm usando un agregado que consiste de cuchillas de afeitar paralelas con una distancia de 1,5 mm. Las tajadas son perpendiculares al eje longitudinal del corazón.

Las tajadas fueron colocadas en dos cápsulas histológicas de modo que la primera taja es colocada en una de las

cápsulas por numero aleatorio y la segunda en la otra, la tercera en la primera cápsula y así en adelantes. Después de enbeber, las tajadas fueron cortadas en secciones de 4µ de gruesas que son manchadas por Hematoxilin-eosina y por Masson-tricromo.

Usando un microscopio con una etapa que puede ser movida en etapas predeterminadas, tanto las tajadas manchadas con Masson-tricromo fueron examinadas y el numero de puntos de golpeado del tejido fibroso (manchado azul en Masson-tricromo) y el tejido normal fueron contados. El microscopio fue conectado a una video cámara y la imagen es visualizada en una pantalla. El sistema de morfometría usado fue Cast2 de Olympus, Dinamarca. El tamaño del infarto fue calculado como % de tejido fibroso en el corazón.

2. Resultados:

Como se ilustró en la figura 11, el cuerpo de peso del corazón y proporción de peso fue reducido en dos dosis muy altas del compuesto 1 sugiriendo que el compuesto redujo las consecuencias hipertroficas (es decir, remodelado) del infarto miocardial.

Como se muestra en la figura 12, el tratamiento con el compuesto 1 durante tres semanas redujo el tamaño del infarto en 20-50% en relación con el tamaño infartado en las ratas sometidas a un infarto miocardial, pero tratadas con el vehículo. Esto indica que el compuesto 1 tiene acciones citoprotectoras durante la isquemia miocardial. En línea con lo que ha sido descrito aquí, estos datos indican que el compuesto 1 reduce el tamaño del infarto miocardial por un mecanismo que puede involucrar el impedimento del hinchamiento celular. Así, la prevención del hinchamiento celular puede impedir o prevenir la compresión del tejido saludable vecino, y por lo tanto impedir la compresión de la micro circulación en la vecindad del tejido saludable. Por lo tanto, en esta solicitud reclamamos que este principio puede impedir la consecuencia de cualquier lección isquemica en cualquier órgano, preferiblemente pero no limitados a órganos con una cápsula fibrosa confinada (por ejemplo, corazón, riñones) u órganos rodeados por hueso (por ejemplo, cerebro, medula espinal, tejido del hueso).

Como se muestra en la figura 13, las ratas sometidas al infarto miocardial pero tratadas sea con dosis del compuesto 1 durante tres semanas, tuvieron una función cardiaca mejorada con menos congestión en el ventrículo izquierdo como se demostró por una presión diastolica final ventricular izquierda reducida. Este dato indica que el compuesto 1, impide la producción de la función cardiaca luego de un infarto miocardial.

Todas las referencias descritas aquí son incorporadas como referencia. La invención ha sido descrita con referencia a modalidades preferidas de la misma. Sin embargo, podrá apreciarse que aquellos versados en la técnica, luego de la consideración de la descripción, pueden hacer modificaciones y mejoras dentro del espíritu y alcance de la invención.

Lo que se reivindica es:

1. Un método para cerrar un hemicanal en una célula, tejido u órgano expuesto a esfuerzo, el método comprende poner en contacto la célula estresada, el tejido o el órgano estresados con una cantidad terapéuticamente efectiva de por lo menos un compuesto seleccionado del grupo que consiste de compuestos representados como formulas I o II, en donde el contacto es suficiente para cerrar los hemicanales en las células, tejido u órganos estresados.

2. El método de la reivindicación 1, en donde el método comprende adicionalmente fosforilar un residuo de tirosina de la conexina 43 (Cx 43) y cerrar el hemicanal.

3. Un método para abrir un hemicanal en una célula, tejido u órgano, el método comprende poner en contacto la célula, tejido u órgano con una cantidad terapéuticamente efectiva de por lo menos un compuesto seleccionado del grupo que consiste de compuestos representados

como formula I o II, en donde el contacto es suficiente para abrir el hemicanal en la célula, tejido u órgano.

4. El método de la reivindicación 3, en donde el método comprende adicionalmente defosforilar un residuo de serina de la conexina 43 (Cx 43) y abrir el hemicanal.

5. Un método para impedir o tratar tejido u órgano estresado en un mamífero, el método comprende administrar una cantidad terapéuticamente efectiva de por lo menos un compuesto seleccionado del grupo que consiste de compuestos representados como formula I o II según se describió anteriormente, en donde el contacto es suficiente para impedir o tratar el estrés en el tejido u órgano.

6. El método de la reivindicación 5, en donde el método comprende adicionalmente fosforilar un residuo de tirosina de la conexina 43 (Cx 43) y cerrar el hemicanal.

7. Un método para incrementar la comunicación intracelular de la unión de espacios (GJIC) en una célula, tejido u órgano, el método comprende administrar una cantidad terapéuticamente efectiva de por lo menos un compuesto seleccionado del grupo que consiste de compuestos representados como formula I o II, en donde el contacto es suficiente para incrementar el GJIC en la célula, tejido u órgano.

8. Un método para tratar quemaduras que comprende administrar a un paciente en necesidad de tal tratamiento una cantidad efectiva terapéuticamente de un compüesto de acuerdo con la formula I o II que bloquea la abertura del hemicanal de la conexina.

9. Un método de tratar trombosis que comprende administrar a un paciente en necesidad de tal tratamiento una cantidad terapéuticamente efectiva de un compuesto de acuerdo con formula I o II que bloquea la abertura del hemicanal de conexina.

10. Un método de tratar la acidosis respiratoria y metabólica que comprende administrar a un paciente en necesidad de tal tratamiento una cantidad terapéuticamente efectiva de un compuesto con la formula I o II que bloque la abertura del hemicanal de conexina.

11. Un método de tratar arritmia focal que comprende administrar a un paciente en necesidad de tal tratamiento una cantidad terapéuticamente efectiva de un compuesto de acuerdo con formula I o II que bloquea la abertura del hemicanal de conexina.

12. Un método de tratar e impedir daño celular y en tejidos un resultante de niveles elevados de glucosa en la sangre que comprende administrar a un paciente en necesidad de tal tratamiento una cantidad terapéuticamente efectiva de un compuesto de acuerdo con la formula I o II que bloquea la abertura del hemicanal de conexina.

13. Un método para tratar la fibrilación atrial crónica que comprende administrar a un paciente en necesidad de tal tratamiento una cantidad terapéuticamente efectiva de un compuesto de acuerdo con la formula I o II que bloquea la abertura del hemicanal de conexina.

14. Un método de tratar epilepsia que comprende administrar a un paciente en necesidad de tal tratamiento una cantidad terapéuticamente efectiva de un compuesto de acuerdo con la formula I o II que promueve la abertura del hemicanal de conexina.

15. Un método para tamizar compuestos candidatos que modulan la función del hemicanal, el método comprende poner en contacto células con por lo menos un compuesto seleccionado del grupo que consiste de péptidos antiarrítmicos y compuestos representados como formula I o II, el contacto se hace bajo condiciones conducentes a modular la fosforilación de la conexina 43 (Cx43); y detectar un cambio en la fosforilación de la Cx43, en donde el cambio en

la fosforilación es tomado como indicación de un compuesto modulador del hemicanal.

16. El método de la reivindicación 15, en donde la etapa de ensayo comprende adicionalmente ensayar el compuesto candidato en un ensayo con base en escogencia inmunología o celular.

17. El método de la reivindicación 16, en donde el ensayo immunológico comprende poner en contacto células con el compuesto candidato bajo condiciones suficientes para incrementar o disminuir la fosforilación del Cx43, producir un lisato de las células; y detectar fosforilación de las Cx43 incrementada o disminuida en el lisato de células como indicativo del compuesto modulador del hemicanal.

18. El método de las reivindicaciones 16-17, en donde el ensayo immunológico es un ensayo ELISA.

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19. El método de la reivindicación 18, en donde el método ELISA comprende adicionalmente:

a) recubrir un soporte sólido con un primer anticuerpo que liga específicamente la conexina,

b) poner en contacto el lisato de células con el soporte sólido bajo condiciones conducentes para formar un complejo de ligado entre el primer anticuerpo y la conexina,

c) Poner en contacto el primer complejo ligado de anticuerpo: conexina con un segundo anticuerpo, el contacto se hace bajo condiciones suficientes para formar un complejo ligado especifico entre el segundo anticuerpo y cualquier conexina fosforilada en el primer complejo de ligado anticuerpo: conexina,

d) El complejo del primer anticuerpo: conexina: segundo anticuerpo con un tercer anticuerpo marcado detectablemente que liga el segundo anticuerpo; y

e) Detectar la presencia del tercer anticuerpo marcado detectablemente sobre el soporte sólido como indicativo adicional del compuesto.

20. El método de la reivindicación 19, en donde el tercer anticuerpo es marcado detectablemente con por lo menos una entre biotina FITC, TRITC, yodina radioactiva, o peroxidasa.

21. El método de las reivindicaciones 19-20. en donde el segundo anticuerpo es un anticuerpo anti-fosfotirosina.

22. El método de las reivindicaciones 19-21, en donde el segundo anticuerpo es un anticuerpo anti-fosfoserina.

23. El método de las reivindicaciones 19-22, en donde el tercer anticuerpo marcado detectablemente es detectado por cuenta de radiocintilación.

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24. El método de la reivindicación 16, en donde el método immunológico es un radioimmunoensayo (RIA).

25. El método de la reivindicación 24, en donde el método immunológico es un ensayo RIA y el método comprende adicionalmente:

a) marcar detectablemente las células antes de producir el lisato de células, en donde la marcación se hace bajo condiciones conducentes a marcar conexina fosforilada,

b) Poner en contacto el lisato de células con un anticuerpo que forma un complejo de ligado especifico con la conexina,

c) Separar el complejo ligado del lisado de células marcado detectablemente; y

d) Detectar la conexina marcada y fosforilada como indicativo adicional del compuesto modulador de la unión de espacios.

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26. El método de la reivindicación 25, en donde el anticuerpo es un anticuerpo anti-conexina.

27. El método de las reivindicaciones 25-26, en donde la etapa de detección comprende adicionalmente llevar a cabo un ensayo de immunoblot Western.

28. El método de las reivindicaciones 25-27, en donde el marcado detectable es fosforoso radioactivo.

29. El método de las reivindicaciones 15-29, en donde el método es llevado a cabo en un formato de tamizado de alta salida o ultra alta salida.

30. El método de las reivindicaciones 1-30. en donde el compuesto facilita la fosforilación del Cx43 en la región de terminal C intracelular.

31. El método de las reivindicaciones 1-30. en donde el compuesto facilita la desfosforilación del Cx43 en la región de terminal C

32. El método de la reivindicación 31, en donde el sitio de fosforilación es un residuo de tirosina.

33. El método de la reivindicación 32, en donde el sitio de fosforilación es un residuo de serina.

34. Un método para tamizar o escoger compuestos candidatos que modulan la función del hemicanal, el método comprende:

1) Cultivar una población de células, un tejido o un órgano tal como aquellos del corazón o de los músculos,

2) estresar las células, el tejido u órganos preferiblemente por eliminación de oxígeno o inhibición metabólica tal como por medio de agregar un derivado de glucosa,

3) Agregar un compuesto modulador del hemicanal conocido o candidato representado por las formula I o II.

4) Agregar un reportero detectable tal como un compuesto fluorescente, quimioluminicente, o fosforescente tal como una tinta fluorescente como la calceina y compuestos relacionados,

5) Detectar un cambio en la toma del reportero detectable en las células, tejidos u órgano con relación a un control adecuado;

6) Medir opcionalmente el cambio como indicativo de un compuesto que modula la función del hemicanal.

35. Un método para tamizar o escoger compuestos candidatos que modulan la función hemicanal, el método comprende:

1) Cultivar una población de células, un tejido o un órgano como aquellos del corazón o de los músculos,

2) Cargar las células, el tejido u órganos con un reportero detectable tal como un compuesto fluorescente, quimioluminicente, o

fosforescente tal como una tinta, como la calceina o compuestos relacionados,

3) estimar el volumen de las células, tejidos u órgano por medio de detectar y cuantificar la señal del reportero detectable,

4) Agregar un compuesto modulador conocido o candidato del hemicanal representado por las formula I o II.

5) Someter a esfuerzo las células, tejido u órgano preferiblemente por privarlo de oxígeno o inhibición metabólica de modo tal como por medio de agregar un derivado de glucosa,

6) Detectar un cambio en el volumen de la célula en relación a un control adecuado;

7) Medir opcionalmente el cambio como indicativo de un compuesto que modula la función del hemicanal y opcionalmente la función de la unión de espacio.

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36. Un método de citoproteger tejido o un órgano de un mamífero en necesidad de tal tratamiento, el método comprende administrar una cantidad terapéuticamente efectiva de por lo menos un compuesto seleccionado del grupo que consiste de los compuestos representados por formula I o II.

37. El método de la reivindicación 36, en donde el método comprende adicionalmente exponer el tejido u órgano del mamífero a las condiciones isquemicas.

38. El método de la reivindicación 37, en donde el órgano al ser citoprotegido esta asociado con una cápsula fibrosa o hueso.

39. El método de la reivindicación 38, en donde el órgano es el corazón, riñón, cerebro, medula espinal o medula ósea.

40. El método de la reivindicación 39, en donde el corazón ha sido sometido a un infarto y la

isquemia esta asociada con hinchamiento de las células miocardiales.

41. El método de la reivindicación 40. en donde el compuesto es Ac-D-Tyr-D-Pro-D-4Hyp-Gly-D-Ala-Gly-NH2 (compuesto 1) o (Ac-Gly-Asn-Tyr-NH2) compuesto 2.

42. Un método para impedir o tratar el daño por repercusión en un mamífero, el método comprende administrar una cantidad terapéuticamente efectiva de por lo menos un compuesto seleccionado del grupo que consiste de los compuestos representados por formula I o II.

43. El método de la reivindicación 42, en donde el método comprende adicionalmente exponer el corazón del mamífero a condiciones de infarto y establecer la perfusión coronaria.

44. El método de la reivindicación 43, en donde el método comprende adicionalmente administrar un agente trombolitico o suministrando

angioplastia coronaria para facilitar la perfusión coronaria en el corazón infartado.

45. El método de la reivindicación 44, en donde el compuesto es Ac-D-Tyr-D-Pro-D-4Hyp-Gly-D-Ala-Gly-NH2 (compuesto 1) o (Ac-Gly-Asn-Tyr-NH2) compuesto 2.

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SUPERINDUSTRIA Y COMERCIO
Radicacion : 04003750 00000000 Folios: 345
NIT : 800176089-2 Fecha (AMD): 2004-08-26 16:52:51
Tramite : 011 PCT-PATENT 379 FASE NACI 411 PRESENTAC
Dependencia: 2020 DIVISION DE NUEVAS CREACIONES

RECIBO OFICIAL DE CAJA : 04 - 63,348
FECHA : AGOSTO 10 DE 2004

C O N S I G N A C I O N

DEPOSITANTE	TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PAG	Vr. PAGO
CAVELIER ABOGADOS	CONSIGNACION	BANCO POPULAR	050-00110-6	23490088	10/08/2004	43,469,600.00

C O N C E P T O

CANT.	RENTISTICO	CONCEPTO	TOTAL CONCEPTO
1	50005-01-01 SOLICITUDES	1 TRAMITES DE SOL. DE PATENTE DE IN	422,000.00
		TOTAL : $	422,000.00

SON: CUATROCIENTOS VEINTIDOS MIL PESOS

RESPONSABLE : _____

RECIBO DE CAJA APLICADO AL EXPEDIENTE No._____

Cdo 13665-841-001-6

345

SUPERINTENDENCIA Y COMERCIO
Radicacion : 04083750 00000000 Folios 345
Fecha (AMD) 2004-08-05 16 52 51
Tramite 811 PCT-PATENT 379 FASE NACI 411 PRESENTAC
Dependencia: 2020 DIVISION DE NUEVAS CREACIONES

REQUISITOS NECESARIOS PARA PRESENTACION Y AGILIZACION DEL TRAMITE

	USUARIO	RADICADOR
PATENTE DE INVENCIÓN ✓ **MODELO DE UTILIDAD**	( )	(✓)
Indicación de que se solicita la concesión de una patente	( )	(✓)
Datos de identificación del solicitante o de la persona que se presenta la solicitud.	( )	(✓)
Descripción de la invención.	( )	( )
Dibujos de ser estos pertinentes.	( )	(✓)
Comprobante de Pago de las tasas establecidas		
COMPLETA (✓) **INCOMPLETA**	( )	( )
DISEÑO INDUSTRIAL ( )		
Indicación de que se solicita el registro de Diseño Industrial	( )	( )
Datos de identificación del solicitante ò de la persona que presenta la solicitud	( )	( )
Representación gráfica o fotográfica del Diseño Industrial o muestra del Material que incorpora el diseño	( )	( )
Comprobante de pago de las tasas establecidas	( )	( )
COMPLETA ( ) **INCOMPLETA**	( )	( )
ESQUEMA DE TRAZADO ( )		
Indicación de que solicita el registro de un Esquema de trazado	( )	( )
Datos de identificación del solicitante o de la persona que presenta La solicitud	( )	( )
Representación gráfica del esquema de trazado	( )	( )
Comprobante de pago de las tasas establecidas	( )	( )
COMPLETA ( ) **INCOMPLETA**	( )	( )

Firma Responsable

Fecha

347

Industria y Comercio
SUPERINTENDENCIA

2020
Bogotá D C

06475

Doctor(a)
EMILIO FERRERO
Apoderado
WYETH

Oficio N°

ASUNTO:

Radicación N°	04-83750
Solicitud internacional	PCT/DK03/00056
Fecha inicio fase nacional	29/08/2004
Trámite	011
Evento	379
Actuación	416
Folios	001

Como resultado del examen de forma, realizado con fundamento en el artículo 38 de la Decisión 486 de la Comisión de la Comunidad Andina, artículo 27 del Tratado de Cooperación en Materia de Patentes (PCT), regla 51 bis del Reglamento y Circular Única de la Superintendencia de Industria y Comercio, se le comunica que:

- La solicitud no contiene la copia y traducción del documento en que consta la cesión del derecho de patente del inventor al solicitante o a su causante ó en su defecto la declaración bajo la regla PCT 4.17 junto con su traducción. (Art. 26-k) Decisión 486).

- La solicitud no contiene la traducción de las reivindicaciones, tal como fueron modificadas. Folios 161-181. (Numeral 5.2.5 capítulo quinto del título décimo de la Circular Unica de la SIC)

En cumplimiento de lo dispuesto por el artículo 39 de la Decisión 486, se le requiere para que dentro del término de dos meses siguientes a la fecha de notificación del presente oficio, complete los requisitos anteriormente enunciados. En caso de no dar cumplimiento al presente requerimiento, la solicitud se considerará abandonada y perderá su prelación.

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 CESPEDES DE VERGEL
Jefe de la División de Nuevas Creaciones.

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

202044

Sede Centro: Carrera 13 No. 27-00 Pisos 2, 5, 7 y 10
Conmutador: 3 820840 Fax: 3 505220
Sede CAN: Tr. 40A No. 38-50 3153265 al 69
Fax: 3 505220. Línea 9800-910 165
Web: www.sic.gov.co e-mail: info@sic.gov.co
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