



Industria y Comercio

SUPERINTENDENCIA

Delegatura de propiedad Industrial

División de Nuevas Creaciones

PCT
SOLICITUD

PATENTE DE INVENCION

PCT

06-98506

21. EXPEDIENTE No.

54. TÍTULO Uso de agonistas del receptor delta activado por el proliferador de peroxisoma para el tratamiento de esclerosis múltiple y otras enfermedades de desmielinización.

51. CLASIFICACIÓN INTERNACIONAL

C07D - 31

AGIX

31/197

71. SOLICITANTE

Aventis Pharmaceuticals Inc.

DOMICILIO

Bridgewater, New Jersey, Estado Unido

74. APODERADO

Luis Portino Leyva

22. BOGOTÁ, D. C.,

29 de Septiembre de 2006

FORMULARIO ÚNICO DE SOLICITUD DE PATENTE

PCT

ENTRADA EN FASE NACIONAL

DI-NEW-06

①

SOLICITUD DE :



Patente de Invención.



Patente de Modelo de Utilidad.



Capítulo I.



Capítulo II.

SOLICITANTE
(71)

Nombre: AVENTIS PHARMACEUTICALS INC.

Dirección: 300 Somerset Corporate Boulevard,
Bridgewater, NJ 08807-2854 Estados Unidos

Nacionalidad o Domicilio: Bridgewater, New Jersey,
Estados Unidos

Lugar de Constitución: Estados Unidos

Teléfono:

Fax:

IDENTIFICACION

C.C.



NIT



C.E.



Otro



Cual

Número

③
REPRESENTANTE
O APODERADO

Nombre: LUIS PATIÑO LEYVA

Dirección Cra. 11ª No. 94-76 - Of. 308

Teléfono: 6161948

Fax: 6163976

E-mail:

IDENTIFICACION

C.C.



NIT



C.E.



Otro



Cual

Número 2.930.616 TP 2698

④
INVENTOR (ES)
(72)

Nombre: CHANDROSS Karen

Dirección: 13 Staudt Court,

Nacionalidad o Domicilio: Somerset, NJ 08873, Estados Unidos

⑤ PCT (86)

Solicitud Internacional No. PCT/US2005/010371

Fecha Marzo 29 de 2005

WO 2005/097098 A2

Publicación Internacional No. WO 2005/097098 A3

Fecha Octubre 20 de 2005

⑥ Título (54) USO DE AGONISTAS DEL RECEPTOR DELTA ACTIVADO POR EL PROLIFERADOR DE PEROXISOMA PARA EL TRATAMIENTO DE ESCLEROSIS MÚLTIPLE Y OTRAS ENFERMEDADES DE DESMIELINIZACIÓN.

⑦ Clasificación Internacional (51) C07D 271/00, A61K 31/192, 31/426, A61P 25/02, 25/26

⑧ PRIORIDAD:

SI ☒ NO ☐

(33) País de Origen

Estados Unidos

(32) Fecha

Abril 1 de 2004

(31) Numero de Solicitud

60/558,533

⑨

Comprobante de pago: 06-74,961

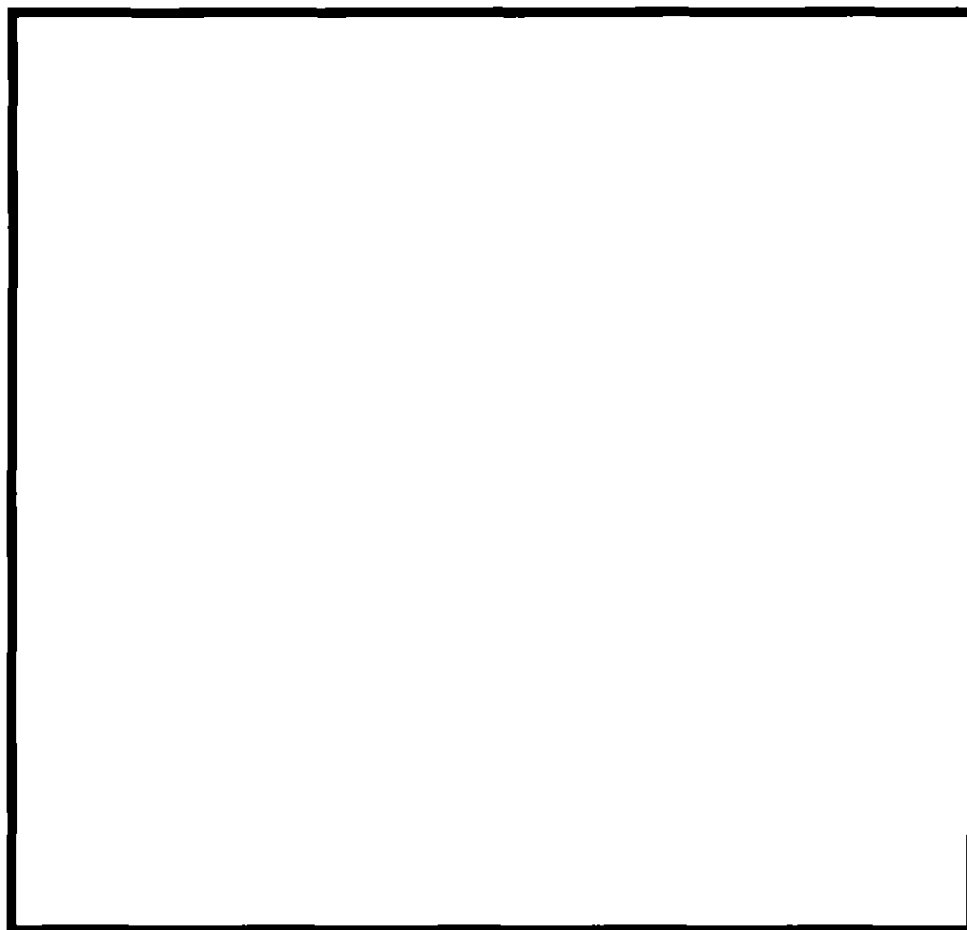
Fecha: Septiembre 27 de 2006

Instrucciones para el diligenciamiento del presente formulario, ver reverso de esta página.

ANEXOS

- ☒ Comprobante de pago de la tasa de presentación de la solicitud
- ☐ Documento que acredite 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 internacional efectuado por la Autoridad Internacional de Examen Preliminar (IPEA).
- ☐ Traducción del examen preliminar internacional 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.
- ☐ De ser el caso, modificaciones efectuadas a la solicitud internacional.
- ☒ Otros Reporte de Búsqueda Internacional.

(11) FIGURA CARACTERISTICA



(12) Solicito la concesión de la patente,

NOMBRE: LUIS PATIÑO LEYVA

FIRMA:

Luis Patiño

C.C. 2.930.616 Bogotá T.P. 2098

Instrucciones para el diligenciamiento del presente formulario , ver reverso de esta página.

Continuación nombre, dirección y domicilio de inventores:

<u>Nombre</u>	<u>Dirección</u>	<u>Domicilio</u>
MERRIL Jean	101 Alpine Lane,	Whippany, New Jersey 07981 Estados Unidos
MINNICH Anne	2 Knoll Court,	Flemington, NJ 08822 Estados Unidos
LEE Lan	Box 230 Pluckemin Park,	Pluckemin, NJ 07978 Estados Unidos
KHORKOVA Olga	570 Manchester Avenue,	North Haledon, New Jersey 07508 Estados Unidos
LIU Yun	42 McBride Way,	Bridgewater, NJ 08807 Estados Unidos

Otros anexos que se acompañan a la solicitud.

Formulario No. PCT/RO/101 correspondiente a la solicitud de patente internacional, con su traducción al castellano.

Formulario PCT/ISA/210 referente a Reporte de Búsqueda Internacional.

Formulario PCT/ISA/237 correspondiente a la opinión escrita de la Búsqueda Internacional autorizada.

Formulario PCT/IB/332 concerniente a la elección de oficinas notificación de su elección.

Formulario PCT/IB/308 correspondiente a la segunda y suplementaria noticia informando al solicitante de la comunicación de la solicitud internacional a las oficinas designadas.

Formulario PCT/IPA/408 relativa a la opinión escrita del Examen Preliminar Internacional autorizado.

Respuesta a la opinión escrita de la Autoridad de Búsqueda Internacional bajo el artículo 34 (2) PCT, junto con su traducción al español, la cual consiste en la modificación de las Reivindicaciones 1 a 7, para lo cual se acompaña nuevo juego de Reivindicaciones 1 a 5.

Respuesta a la opinión de la Autoridad Examinadora Internacional (Regla PCT 66, junto con su traducción al español, la cual consiste en la modificación de las Reivindicaciones 6 a 8, las cuales fueron reemplazadas por las Reivindicaciones 1 a 5.

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

NIT : 800176089-2

RECIBO OFICIAL DE CAJA : 06 - 74,961
FECHA : SEPTIEMBRE 27 DE 2006

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
Rad: 06-008506-00000-0000
Fec: 2006-09-29 16:21:51 Dep. 2080 NUECREACIONES
Tra. 11 PATENTEIYII
Eve: 379 FASENACIONALI
Act 411 PRESENTACION Folios: 110

***** CONSIGNACION *****

DEPOSITANTE	TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PGO	Vr. PAGO
HANUEL MIRANDA	CONSIGNACION	BANCO POPULAR	050-00110-6	53371884	27/09/2006	2,315,000.00

***** CONCEPTO *****

CANT. RENTISTICO	CONCEPTO	TOTAL CONCEPTO
1 50005-01-01 SOLICITUDES	1 TRAMITES DE SOL. DE PATENTE DE IN	463,000.00
	TOTAL :	\$ 463,000.00

CUATROCIENTOS SESENTA Y TRES MIL PESOS

Ref. J8954.

RESPONSABLE : 

RECIBO DE CAJA APLICADO AL EXPEDIENTE No. _____

75+ 5

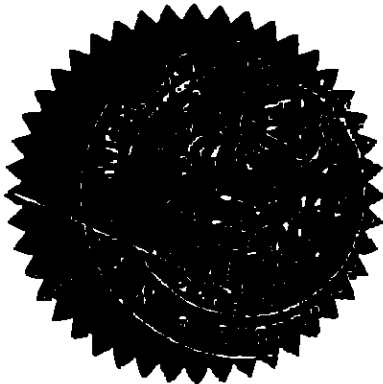
APOSTILLE

(CONVENTION DE LA HAYE DU 5 OCTOBRE 1961)

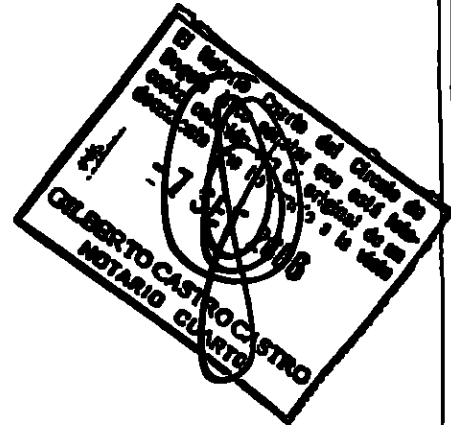
1. COUNTRY: UNITED STATES OF AMERICA
2. THIS PUBLIC DOCUMENT HAS BEEN SIGNED BY:
R. PETER WIDIN
3. ACTING IN THE CAPACITY OF:
COUNTY CLERK
4. BEARS THE SEAL/STAMP OF:
COUNTY OF SOMERSET

CERTIFIED

5. AT TRENTON, NEW JERSEY
6. THE 9TH DAY OF APRIL 2002
7. BY: John E McCormac, CPA, NEW JERSEY TREASURER
8. NO. A149012
9. SEAL/STAMP:
10. SIGNATURE



John E McCormac



G.HOLLMANN RESTREPO
ABOGADOS LTDAG. HOLLMANN RESTREPO
ABOGADOS LTDA

The undersigned, acting on behalf of and representing of AVENTIS PHARMACEUTICALS INC., domiciled in 300 Somerset Corporate Boulevard, Bridgewater, New Jersey, USA, hereby appoint(s) GUSTAVO HOLLMANN RESTREPO and/or LUIS PATIÑO LEYVA, domiciled in Bogotá D.C., Republic of Colombia, as our true and legal attorney with the necessary powers:

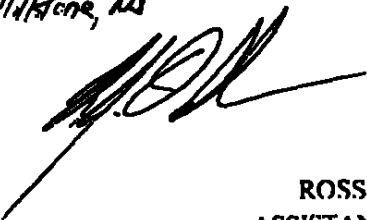
1) To apply to the administrative, judicial and police authorities of the Republic of Colombia, in any instance or appeal, for obtaining registration of patents, utility models, industrial designs and models, commercial engravings and names, trademarks, slogans, health licenses, copyrights and domain names as well as their assignments, extensions, renewals and changes of name; 2) To prepare, apply for, obtain, execute and register contracts, licenses, authorizations and registrations of any kind; 3) To intervene as plaintiff or defendant, in any instance or appeal, before any judge, court, tribunal, corporation, public officer or authority in any judicial, administrative or police action, such as: action dealing with commercial engravings and names; oppositions against trademark registrations; caducity and nullity actions; recovery actions, actions arising from obtainment and working of license agreements; actions dealing with unfair competition; criminal actions; actions claiming indemnity for damages, complaints or remarks on all sort of industrial property matters; the performance of precautionary measures, and in any other similar action, measure or appeal related to intellectual property matters.

To this effect, I (we) hereby authorize him to file applications, make descriptions, protests, amendments, declarations, oppositions, cancellations, appeals and claims; to pay taxes, quotas and liens as provided by Law; to waive or abandon granted rights or rights in process of being granted, to receive all kind of documents and securities; to issue the respective releases and, in general, to take, before said authorities, pertinent steps to the above mentioned purposes and take all measures they may deem advisable to protect my (our) interests, with all other necessary legal faculties, and I (we) hereby declare as good and valid whatever the attorneys should do to my (our) benefit.

This power comprises the authority to ratify or confirm on my (our) behalf any action, as well as the faculty of receiving, desisting, transacting, compromising, substituting and revoking substitutions, taking notifications, and appointing judicial or extra-judicial attorneys.

Signed in on this 1st day of March of 2002.

East Millstone, NJ



ROSS J. OEHLER
ASSISTANT SECRETARY

Yo (nosotros), obrando en nombre propio y representación de AVENTIS PHARMACEUTICALS INC., con domicilio en 300 Somerset Corporate Boulevard, Bridgewater, New Jersey, Estados Unidos de América, nombro(amos) a GUSTAVO HOLLMANN RESTREPO y/o LUIS PATIÑO LEYVA, con domicilio en Bogotá D.C., República de Colombia, Ora 11^a No 94-76, Oficina 308, apoderando verdadero y legal, con poder especial, amplio y suficiente:

1) Para recabar de las oficinas y autoridades colombianas administrativas, judiciales y de policía en cualquier instancia o recurso, la obtención de patentes de invención, modelos de utilidad, dibujos y modelos industriales, registro de marcas, registro de lemas comerciales, depósito de nombres y enseñanzas comerciales, registros sanitarios, derechos de autor y nombres de dominio así como sus traspasos, prórrogas, renovaciones y cambios de nombre; 2) Elaborar, tramitar, solicitar, obtener, realizar, ejecutar y registrar contratos, licencias, autorizaciones y registros de cualesquiera clases; 3) Intervenir como demandante o como demandado en cualquier instancia y recurso y ante cualquier juez, entidad, corporación, funcionario público o autoridad en acciones judiciales, administrativas y de policía, tales como acciones derivadas de nombre o enseña comerciales; acciones reivindicatorias; acciones derivadas de la obtención o explotación de licencias; acciones de competencia desleal, demandas penales y civiles; acciones de indemnización de perjuicios, reclamos, u observaciones a cualquier asunto sobre propiedad intelectual; al ejercicio de medidas cautelares y cualesquiera otras acciones, medidas o recursos similares relacionados con la propiedad intelectual.

A este efecto, lo facultamos para elevar solicitudes, formular descripciones, enmiendas, protestas, declaraciones, oposiciones, cancelaciones, apelaciones y reclamos; pagar impuestos, cuotas y gravámenes prescritos por la Ley; renunciar o desistir a derechos concedidos o en curso de concesión; recibir toda clase de documentos y valores dando al descargo respectivo, y en general, para dar ante dichas autoridades todos los pasos necesarios al efecto indicado y tomar todas las medidas que creyeren conducentes al resguardo de mis (nuestros) intereses, con todas las facultades necesarias, y por este documento desde ahora declaro(amos) válido y bueno todo cuanto los apoderados hicieren en mi (nuestro) beneficio.

Este poder comprende la facultad de ratificar, o confirmar en mi (nuestro) nombre lo actuado, así como la facultad de recibir, desistir, transigir, comprometer, sustituir y revocar sustituciones, recibir notificaciones y nombrar apoderados judiciales o extrajudiciales.

Firmado en el de de



NOTARIAL CERTIFICATE (Corporation)

UNITED STATES OF AMERICA)
STATE OF NEW JERSEY) ss.
COUNTY OF SOMERSET)

On this 1st day of March, 2002, personally appeared before me, Ross J. Oehler, Assistant Secretary, known or identified to me to be the individual described in and who executed the foregoing document, Power of Attorney, and acknowledged the same as a free act and deed for uses and purposes therein expressed by authority of the said Corporation incorporated under the laws of the State of Delaware, United States of America.

I further certify that said authority, dated November 29, 2000, is based on a Resolution of the Board of Directors of said Corporation, which I have seen, appointing the said individual Attorney-in-fact with full power to sign the foregoing document on behalf of the said Corporation and that the purposes for which said document is granted are within the scope of the objects or activities of said Corporation,

SEAL

Nancy J. Ingram
Notary Public

Nancy J. Ingram
Notary Public, State of New Jersey
My Commission Expires August 2, 2004

Notary Signature
Print or Type Name
of Notary, Expiration

STATE OF NEW JERSEY }
COUNTY OF SOMERSET. }

ss.:

I, PETER R. WIDIN, Clerk of the County of Somerset and also Deputy Clerk of the Superior Court in and for said County, the same being a Court of Record, Do Hereby Certify, that

Nancy J. Ingram
whose name is subscribed to the certificate of acknowledgement, proof or affidavit of the annexed instrument, was at the time of taking such acknowledgement, proof or affidavit, a NOTARY PUBLIC OF NEW JERSEY

in and for said State, duly appointed, qualified, commissioned and sworn and authorized by law to take affidavits, acknowledgements or proofs of deeds or conveyances for lands, tenements or hereditaments and other instruments in writing in said State of New Jersey. And I further Certify, that I am well acquainted with the handwriting of said official and verily believe the signature to the said certificate of acknowledgement, proof or affidavit is genuine.

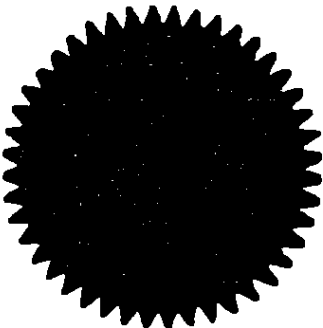
In Testimony Whereof, I have hereunto set my hand and affixed the seal of said County on

APR - 5 2002

R. PETER WIDIN

Clerk.

by *Robert J. Anderson* Special Deputy Clerk.



8

TRADUCCION OFICIAL DEL INGLES de las certificaciones y la
Apostilla adjuntas al poder que la compañía AVENTIS PHARMACEU-
TICALS INC domiciliada en Bridgewater, New Jersey, E.U.A.,
otorga a Gustavo Hollmann Restrepo y/o Luis Patiño Leyva.

CERTIFICADO NOTARIAL (Sociedad)

ESTADOS UNIDOS DE AMERICA

ESTADO DE NEW JERSEY

CONDADO DE SOMERSET

El día 1° de Marzo del año 2002 ante la suscrita Notaria com-
pareció personalmente Ross J. Oehler, Secretario Suplente,
quien yo sé o quien comprobó ante mí que es la persona mencio-
nada en el adjunto poder y quien lo ejecutó, y él reconoció
haber ejecutado dicho instrumento como un acto libre y volun-
tario destinado para los usos y propósitos allí expresados en
virtud a la autoridad que le concede la mencionada sociedad,
la cual está incorporada de conformidad con las leyes del Es-
tado de Delaware, Estados Unidos de América.

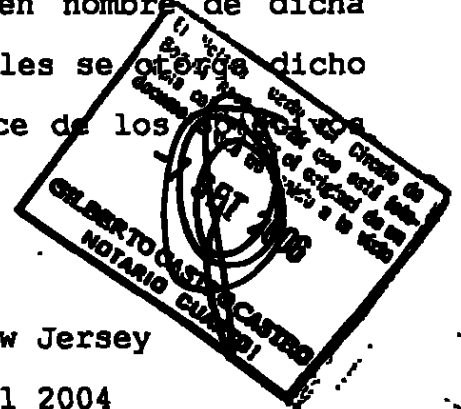
Certifico además que dicha autoridad, fechada el 29 de Noviem-
bre del 2000, se basa en una resolución de la Junta de Direc-
tores de la mencionada sociedad, documento que yo he tenido a
la vista, nombrando a la mencionada persona apoderado con todo
poder para firmar el instrumento adjunto en nombre de dicha
sociedad, y que los propósitos para los cuales se otorga dicho
instrumento se encuentran dentro del alcance de los
o actividades de la mencionada corporación.

Nancy J. Ingram

Notaria Pública del Estado de New Jersey

Mi comisión expira Agosto 2 del 2004

(Firma y Sello Oficial)



ESTADO DE NEW JERSEY, CONDADO DE SOMERSET

Yo, Peter S. Widin, Secretario del Condado de Somerset y Secretario Suplente de la Corte Superior de dicho Condado, la cual es una Corte de Registro, certifico que Nancy J. Ingram, cuyo nombre aparece suscrito al certificado de reconocimiento, prueba o declaración del instrumento adjunto, era en el momento de tomar tal reconocimiento, prueba o declaración Notaria Pública en y para el Estado de New Jersey, debidamente nombrada, calificada, comisionada y juramentada y autorizada por la ley para tomar declaraciones, reconocimientos o pruebas de escrituras o traspasos de tierras, tenencias o herencias y otros instrumentos escritos en el mencionado Estado. Y certifico además que conozco bien la firma de dicha funcionaria y estoy convencido de que la firma que aparece en el mencionado certificado de reconocimiento, prueba o declaración es genuina.

En testimonio de lo cual coloco mi firma y el sello del Condado el día de hoy 5 de Abril del 2002.

R. Peter Widin, Secretario

Deborah L. Anderson, Secretaria Especial Suplente

(Firma y Sello Oficial)

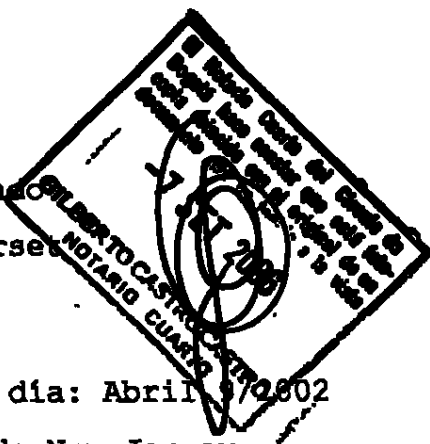
APOSTILLA

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

1. País: Estados Unidos de América
- Este documento público
2. Ha sido firmado por: R. Peter Widin
3. En su calidad de: Secretario del Condado
4. Y lleva el sello: del Condado de Somerset

Confirmado

5. En: Trenton, New Jersey
6. El día: Abril 5/2002
7. Por: John E. McCormac, CPA, Tesorero de New Jersey
8. Bajo el No.: A149012
9. Sello: Secretaría de Estado de New Jersey
10. Firma: (ilegible)



M. Méndez S.

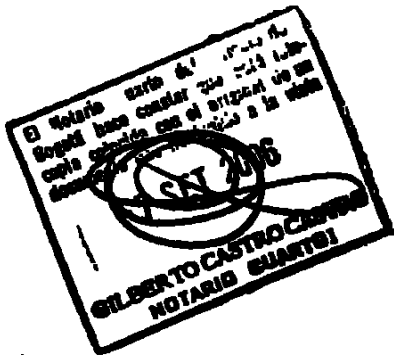
MINISTERIO DE RELACIONES
EXTERIORES

223893
JH. Mendez
CUYA FIRMA APARECE EN EL
DOCUMENTO DESEMPEÑA
LAS FUNCIONES INDICADAS

NO SE ASUME
RESPONSABILIDAD DEL TEXTO

2002 APR 22 P 12:19

JEFE DE DELEGACIONES
SANTAFE DE BOGOTA D.C.



(19) World Intellectual Property
Organization
International Bureau



(43) International Publication Date
20 October 2005 (20.10.2005)

PCT

(10) International Publication Number
WO 2005/097098 A2

(51) International Patent Classification⁷: A61K 31/192,
31/426, A61P 25/02, 25/28

(21) International Application Number:
PCT/US2005/010371

(22) International Filing Date: 29 March 2005 (29.03.2005)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/558,533 1 April 2004 (01.04.2004) US

(71) Applicant (for all designated States except US): AVEN-
TIS PHARMACEUTICALS INC. [US/US]; 300 Somer-
set Corporate Boulevard, Bridgewater, New Jersey 08807-
2854 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): CHANDROSS,
Karen [US/US]; 13 Standt Court, Somerset, NJ 08873
(US). MERRILL, Jean [US/US]; 101 Alpine Lane,
Whippany, New Jersey 07981 (US). MINNICH, Anne

[US/US]; 2 Knoll Court, Flemington, NJ 08822 (US).
LEE, Lan [US/US]; Box 230 Pluckemin Park, Pluckemin,
NJ 07978 (US). KHORKOVA, Olga [US/US]; 570 Man-
chester Avenue, North Haledon, New Jersey 07508 (US).
LIU, Yum [CN/US]; 42 McBride Way, Bridgewater, NJ
08807 (US).

(74) Agents: STRUPCZEWSKI, Joseph et al.; sanofi-aventis,
Route 202-206, P. O. Box 6800, Bridgewater, NJ 08807-
0800 (US).

(81) Designated States (unless otherwise indicated, for every
kind of national protection available): AE, AG, AL, AM,
AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN,
CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, 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, NA, NI, NO, NZ, OM, PG,
PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ,
TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA,
ZM, ZW.

(84) Designated States (unless otherwise indicated, for every
kind of regional protection available): ARIPO (BW, GH,
GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM,

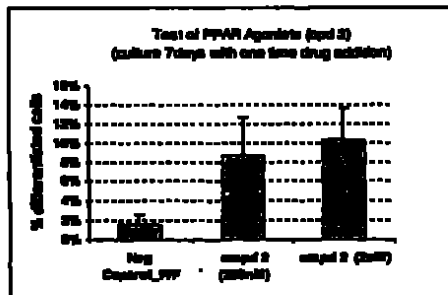
[Continued on next page]

(54) Title: USE OF PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR DELTA AGONISTS FOR THE TREATMENT
OF MS AND OTHER DEMYELINATING DISEASES

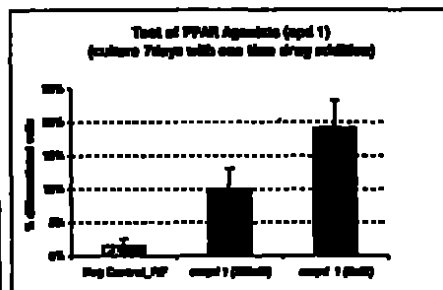
Myelin Basic Protein (MBP) Immunoreactivity is Enhanced in cultured
RAT Oligodendrocytes after Exposure to PPAR delta Agonists

Compound 1:
5-fold increase at 200 nM (7days)
10-fold increase at 2 μ M (7days)

Compound 2:
5-fold increase at 200 nM (7days)
6-fold increase at 2 μ M (7days)



*P+P = mitogens = 10 ng/ml PDGF-AA, 10 ng/ml bFGF



(57) Abstract: A method for treating demyelinating diseases in a patient in need thereof by treatment with an effective amount of a PPAR delta agonist is disclosed. Demyelinating diseases that may be effectively treated by this method include but are not limited to multiple sclerosis, Charcot-Marie-Tooth disease, Pelizaeus-Merzbacher disease, encephalomyelitis, neuromyelitis optica, adrenoleukodystrophy, Guillian-Barre syndrome and disorders in which myelin forming glial cells are damaged including spinal cord injuries, neuropathies and nerve injury.



ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),
European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI,
FR, GB, GR, HU, IE, IS, IT, LT, LU, MC, NL, PL, PT, RO,
SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN,
GQ, GW, ML, MR, NE, SN, TD, TG).

— of inventorship (Rule 4.17(iv)) for US only

Published:

— without international search report and to be republished
upon receipt of that report

Declarations under Rule 4.17:

— as to the applicant's entitlement to claim the priority of the
earlier application (Rule 4.17(III)) for all designations

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

5

10 **USE OF PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR DELTA
AGONISTS FOR THE TREATMENT OF MS AND OTHER DEMYELINATING
DISEASES**

FIELD OF THE INVENTION

15 This invention relates to the use of PPAR delta agonists for the treatment of multiple sclerosis (MS) and other demyelinating diseases. This invention also relates to the use of certain compounds that are selective PPAR delta agonists for the treatment of MS and other demyelinating diseases.

20 **BACKGROUND OF THE INVENTION**

The peroxisome proliferator-activated receptors (PPARs) comprise a subfamily of the nuclear receptor superfamily. Three closely related isoforms have been identified and cloned and are commonly known as PPAR alpha, PPAR gamma and PPAR delta. Each receptor subtype has a signature DNA binding domain (DBD) and a ligand-binding domain (LBD), both being necessary for ligand activated gene expression. PPARs bind as heterodimers with a retinoid X receptor. See J. Berger and D. E. Miller, *Ann. Rev. Med.*, 2002, 53, 409-435.

25 PPAR delta (also known as PPAR beta) is expressed in a broad range of mammalian tissue, but little information regarding its biological functions or the full array of genes regulated by the receptor have been elucidated. However, it has recently been found that agonists may be useful to treat conditions such as dyslipidemia and certain dermatological conditions, while antagonists may be useful to treat osteoporosis or colorectal cancer (D. Sternbach, in *Annual Reports in Medicinal Chemistry, Volume 38*, A. M. Doherty, ed., Elsevier Academic Press, 2003 pp. 71-80).

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PPAR delta appears to be significantly expressed in the CNS; however much of its function there still remains undiscovered. Of singular interest however, is the discovery that PPAR delta was expressed in rodent oligodendrocytes, the major lipid producing cells of the CNS (J. Granneman, et al., *J. Neurosci. Res.*, 1998, 51, 563-573). Moreover, it was also
5 found that a PPAR delta selective agonist was found to significantly increase oligodendroglial myelin gene expression and myelin sheath diameter in mouse cultures (I. Saluja et al., *Glia*, 2001, 33, 194-204).

Demyelinating conditions are manifested in loss of myelin—the multiple dense layers of lipids and protein which cover many nerve fibers. These layers are provided by
10 oligodendroglia in the central nervous system (CNS), and Schwann cells in the peripheral nervous system (PNS). In multiple sclerosis (MS), oligodendrocytes, the myelin forming cells in the CNS, are destroyed and axons are damaged, resulting in severely impaired neuronal activity and functional deficits, including plegia. In patients with demyelinating conditions, demyelination may be irreversible; it is usually accompanied or followed by axonal
15 degeneration, and often by cellular degeneration. Demyelination can occur as a result of neuronal damage or damage to the myelin itself—whether due to aberrant immune responses, local injury, ischemia, metabolic disorders, toxic agents, or viral infections (Prineas and McDonald, *Demyelinating Diseases. In Greenfield's Neuropathology*, 6.sup.th ed. (Edward Arnold: New York, 1997) 813-811, Beers and Berkow, eds., *The Merck Manual of Diagnosis and Therapy*, 17.sup.th ed. (Whitehouse Station, N.J.: Merck Research Laboratories, 1999)
20 1299, 1437, 1473-76, 1483). However, newly formed oligodendrocyte progenitor cells are present throughout areas of demyelination, suggesting the possibility of self-repair if these progenitor cells can be induced to undergo differentiation to mature oligodendrocytes.

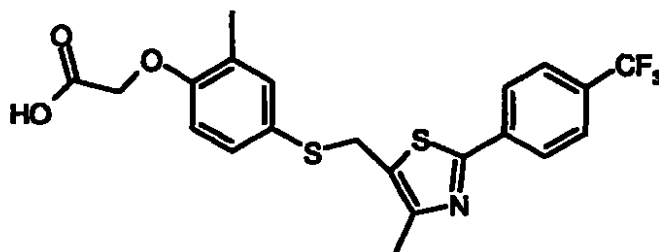
Central demyelination (demyelination of the CNS) occurs in several conditions, often
25 of uncertain etiology, that have come to be known as the primary demyelinating diseases. Of these, multiple sclerosis is the most prevalent. Other primary demyelinating diseases include adrenoleukodystrophy (ALD), adrenomyeloneuropathy, AIDS-vacuolar myelopathy, HTLV-associated myelopathy, Leber's hereditary optic atrophy, progressive multifocal leukoencephalopathy (PML), subacute sclerosing panencephalitis, and tropical spastic
30 paraparesis. In addition, there are acute conditions in which demyelination can occur in the CNS, e.g., acute disseminated encephalomyelitis (ADEM) and acute viral encephalitis. Furthermore, acute transverse myelitis, a syndrome in which an acute spinal cord transection

used for more severe progressive forms. However, they are not uniformly beneficial, and have significant toxic side effects. Several drugs (e.g., baclofen at 30-60 mg/day in divided doses) may reduce spasticity by inhibiting the spinal cord reflexes. Cautious and judicious use is required, though, because the drug-induced reduction in spasticity in MS patients often exacerbates weakness, thereby further incapacitating the patient.

Similarly, current treatment for ALD, another devastating demyelinating disease, is relatively ineffective. Symptoms of ALD may include cortical blindness, corticospinal tract dysfunction, mental deterioration, and spasticity. Therapy to control the course of ALD may include bone marrow transplantation and dietary treatment (DiBiase et al., *Ann. Ist. Super. Sanita*, 35:185-92, 1999), but inexorable neurological deterioration invariably occurs, ultimately leading to death [Krivit et al., *Curr. Opin. Hematol.*, 6:377-82, 1999, (Beers and Berkow, eds., *The Merck Manual of Diagnosis and Therapy*, 17.sup.th ed. (Whitehouse Station, N.J.: Merck Research Laboratories, 1999) 1299, 1437, 1473-76, 1483). Some progress has been realized in the treatment of animals with EAE and EAN, by using glial cell transplants and growth factors, and by inhibiting adhesion molecules, autoantibodies, and cytokines (Njenga and Rodriguez, *Current Opinion in Neurology*, 9:159-64, 1996. However, none of these treatments has been shown to be beneficial in humans, and some require extensive neurosurgical intervention. Thus, it is clear from the foregoing that there exists a need for more effective, and less expensive and invasive, methods to treat the varied array of demyelinating conditions, without producing undesirable secondary effects.

The present invention entails the use of a small molecule-activated regenerative approach to significantly augment current neuromodulatory therapies for the treatment of demyelinating disorders.

Compounds that are known to be selective PPAR delta are known in the art, in particular, compound of formula (1) generally known as GW-501516 described in WO 01/00603.



(1)

of unknown cause affects both gray and white matter in one or more adjacent thoracic segments, can also result in demyelination.

MS is a chronic, devastating, neurological disease that affects mostly young adults.

The pathogenesis of MS is a complex process that leads to destruction of myelin and

5 oligodendroglia, as well as axonal damage, in the brain and spinal cord (Prineas and McDonald, *Demyelinating Diseases. In Greenfield's Neuropathology*, 6.sup.th ed. (Edward Arnold: New York, 1997) 813-811, Trapp et al., *N. Engl. J. Med.*, 338:278-85, 1998).

Histopathologically, MS is characterized by inflammation, plaques of demyelination infiltrating cells in the CNS tissue, loss of oligodendroglia, and focal axonal injury (Prineas

10 and McDonald, *Demyelinating Diseases. In Greenfield's Neuropathology*, 6.sup.th ed. (Edward Arnold: New York, 1997) 813-811).

The disease is thought to result from aberrant immune responses to myelin, and possibly non-myelin, self-antigens (Bar-Or et al., *J. Neuroimmunol.* 100:252-59, 1999, Hartung, H.-P., *Current Opinion in Neurology*, 8:191-99,

1995). Clinically, MS may follow a relapsing-remitting, or it may take a chronically

15 progressive course with increasing physical disability (Gold et al., *Mol. Med. Today*, 6:88-91, 2000). Typically, the symptoms of MS include lack of co-ordination, paresthesias, speech and visual disturbances, and weakness.

Current treatments for the various demyelinating conditions are often expensive, symptomatic, and only partially effective, and may cause undesirable secondary effects.

20 Corticosteroids (oral prednisone at 60-100 mg/day, tapered over 2-3 weeks, or intravenous methylprednisolone at 500-1000 mg/day, for 3-5 days) represent the main form of therapy for MS. While these may shorten the symptomatic period during attacks, they may not affect eventual long-term disability. Long-term corticosteroid treatment is rarely justified, and can cause numerous medical complications, including osteoporosis, ulcers, and diabetes (Beers and Berkow, eds., *The Merck Manual of Diagnosis and Therapy*, 17.sup.th ed. (Whitehouse

25 Station, N.J.: Merck Research Laboratories, 1999) 1299, 1437, 1473-76, 1483).

Immunomodulatory therapy with recombinant human interferon-.beta. (Betaseron and

Avonex) and with co-polymer (Copaxon) slightly reduces the frequency of relapses in MS,

30 and may help delay eventual disability (Beers and Berkow, eds., *The Merck Manual of Diagnosis and Therapy*, 17.sup.th ed. (Whitehouse Station, N.J.: Merck Research

Laboratories, 1999) 1299, 1437, 1473-76, 1483). Both forms of interferon-.beta. and co-

polymer are currently used as treatment modalities for MS, but all are exceedingly expensive.

Immunosuppressive drugs (azathioprine, cladribine, cyclophosphamide, and methotrexate) are

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mixing a solution of the compound according to the invention with a solution of a pharmaceutically acceptable acid such as hydrochloric acid, hydrobromic acid, sulfuric acid, methanesulfonic acid, 2-hydroxyethanesulfonic acid, p-toluenesulfonic acid, fumaric acid, maleic acid, hydroxymaleic acid, malic acid, ascorbic acid, succinic acid, glutaric acid, acetic acid, salicylic acid, cinnamic acid, 2-phenoxybenzoic acid, hydroxybenzoic acid, phenylacetic acid, benzoic acid, oxalic acid, citric acid, tartaric acid, glycolic acid, lactic acid, pyruvic acid, malonic acid, carbonic acid or phosphoric acid. The acid metal salts such as sodium monohydrogen orthophosphate and potassium hydrogen sulfate can also be formed. Also, the salts so formed may present either as mono- or di- acid salts and can exist either as hydrated or can be substantially anhydrous. Furthermore, where the compounds of the invention carry an acidic moiety, suitable pharmaceutically acceptable salts thereof may include alkali metal salts, e.g. sodium or potassium salts; alkaline earth metal salts, e.g. calcium or magnesium salts; and salts formed with suitable organic ligands, e.g. quaternary ammonium salts.

The term "therapeutically effective amount" as used herein means an amount of the compound, which is effective in treating the named disorder or condition.

As used herein, the expression "pharmaceutically acceptable carrier" means a non-toxic solvent, dispersant, excipient, adjuvant, or other material which is mixed with the compound of the present invention in order to permit the formation of a pharmaceutical composition, i.e., a dosage form capable of administration to the patient. One example of such a carrier is a pharmaceutically acceptable oil typically used for parenteral administration.

The invention also provides pharmaceutical compositions comprising one or more of the compounds according to this invention in association with a pharmaceutically acceptable carrier. Preferably these compositions are in unit dosage forms such as tablets, pills, capsules, powders, granules, sterile parenteral solutions or suspensions, metered aerosol or liquid sprays, drops, ampoules, auto-injector devices or suppositories; for oral, parenteral, intranasal, sublingual or rectal administration, or for administration by inhalation or insufflation.

Alternatively, the compositions may be presented in a form suitable for once-weekly or once-monthly administration; for example, an insoluble salt of the active compound, such as the decanoate salt, may be adapted to provide a depot preparation for intramuscular injection. An erodible polymer containing the active ingredient may be envisaged. For preparing solid compositions such as tablets, the principal active ingredient is mixed with a pharmaceutical carrier, e.g. conventional tableting ingredients such as corn starch, lactose, sucrose, sorbitol, talc, stearic acid, magnesium stearate, dicalcium phosphate or gums, and other pharmaceutical

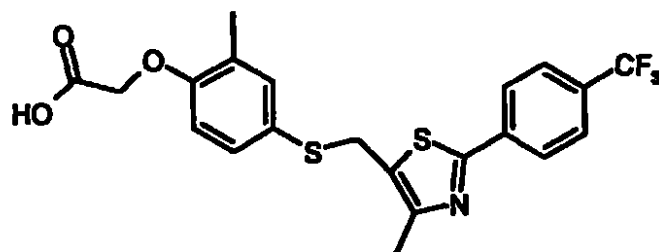
diluents, e.g. water, to form a solid preformulation composition containing a homogeneous mixture of a compound of the present invention, or a pharmaceutically acceptable salt thereof. When referring to these preformulation compositions as homogeneous, it is meant that the active ingredient is dispersed evenly throughout the composition so that the composition may be readily subdivided into equally effective unit dosage forms such as tablets, pills and capsules. This solid preformulation composition is then subdivided into unit dosage forms of the type described above containing from 0.1 to about 500 mg of the active ingredient of the present invention. Flavored unit dosage forms contain from 1 to 100 mg, for example 1, 2, 5, 10, 25, 50 or 100 mg, of the active ingredient. The tablets or pills of the novel composition can be coated or otherwise compounded to provide a dosage form affording the advantage of prolonged action. For example, the tablet or pill can comprise an inner dosage and an outer dosage component, the latter being in the form of an envelope over the former. The two components can be separated by an enteric layer which serves to resist disintegration in the stomach and permits the inner component to pass intact into the duodenum or to be delayed in release. A variety of materials can be used for such enteric layers or coatings, such materials including a number of polymeric acids and mixtures of polymeric acids with such materials as shellac, cetyl alcohol and cellulose acetate.

The liquid forms in which the novel compositions of the present invention may be incorporated for administration orally or by injection include aqueous solutions, suitably flavored syrups, aqueous or oil suspensions, and flavored emulsions with edible oils such as cottonseed oil, sesame oil, coconut oil or peanut oil, as well as elixirs and similar pharmaceutical vehicles. Suitable dispersing or suspending agents for aqueous suspensions include synthetic and natural gums such as tragacanth, acacia, alginate, dextran, sodium carboxymethylcellulose, methylcellulose, polyvinyl-pyrrolidone or gelatin.

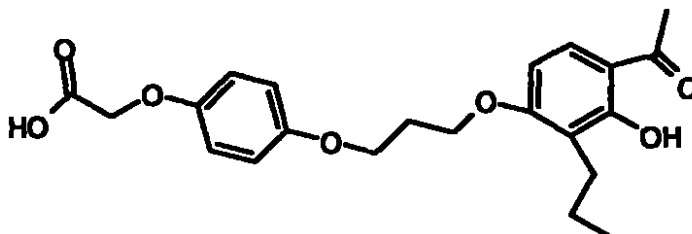
~~In the treatment of various disease states as described herein, a suitable dosage level is about 0.01 to 250 mg/kg per day, preferably about 0.05 to 100 mg/kg per day, and especially about 0.05 to 20 mg/kg per day. The compounds may be administered on a regimen of 1 to 4 times per day.~~

In one aspect of this invention there is disclosed a method for treating demyelinating diseases in a patient comprising administration of a therapeutically effective amount of a hPPAR delta agonist.

In a further aspect of this embodiment, the hPPAR delta agonist is a selective agonist.



(1)



(2)

In a further aspect of this embodiment is disclosed a pharmaceutical composition comprising an amount effective in treating multiple sclerosis.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Illustrates the enhancement of myelin basic protein (MBP) immunoreactivity in cultured rat oligodendrocytes by PPAR delta agonists.

Figure 2. This graph shows the enhancement of MBP mRNA in cultured rat oligodendrocytes by compound 1.

Figure 3. This graph shows the enhancement of MBP mRNA in cultured rat oligodendrocytes by compound 2.

Figure 4A. Illustrates the effect of compound 1 on transcriptional markers that confirm PPAR delta agonist pathway activation in cultured rat oligodendrocytes.

Figure 4B. Further illustrates the effect of compound 1 on transcriptional markers that confirm PPAR delta agonist pathway activation in cultured rat oligodendrocytes, showing that ADRP mRNA is upregulated in cultured rat oligodendrocytes.

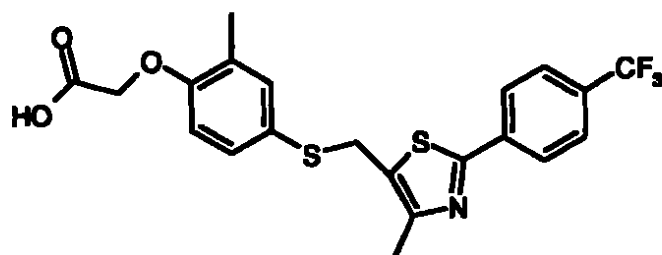
Figure 5. Shows the increase in the number of O4 immunopositive cells in mixed cultures of human oligodendrocytes effected by compound 1.

Figure 6. Shows the increase in the number of O4 immunopositive cells in mixed cultures of human oligodendrocytes effected by compound 2.

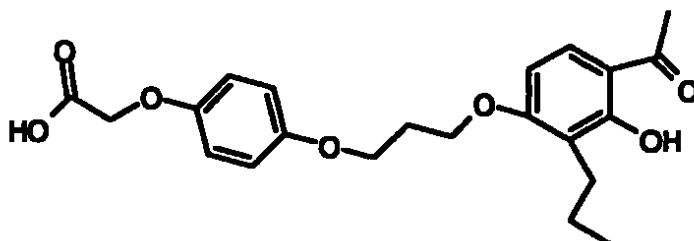
In another aspect of this embodiment is disclosed a method wherein the demylenating disease is selected from the group consisting of multiple sclerosis, Charcot-Marie-Tooth disease, Pelizaeus-Merzbacher disease, encephalomyelitis, neuromyelitis optica, adrenoleukodystrophy, Guillian-Barre syndrome and disorders in which myelin forming glial cells are damaged including spinal cord injuries, neuropathies and nerve injury.

In a further aspect of this embodiment is disclosed the method wherein the demylenating disease is multiple sclerosis.

In yet another aspect of this embodiment is disclosed the method wherein the agonist is selected from group consisting of compound of formula (1) and formula (2)



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In another embodiment disclosed in the present invention is a pharmaceutical composition comprising a compound selected from the group consisting of compound of formula (1) and formula (2) in an amount effective for treating multiple sclerosis, Charcot-Marie-Tooth disease, Pelizaeus-Merzbacher disease, encephalomyelitis, neuromyelitis optica, adrenoleukodystrophy, Guillian-Barre syndrome and disorders in which myelin forming glial cells are damaged including spinal cord injuries, neuropathies and nerve injury in combination with at least one pharmaceutically acceptable carrier

Compound Examples:

Compound of formula (1) (GW501516) can be prepared as published in WO 01/00603. Compound of formula (2) (L165,041) can be prepared as described in WO 97/28149.

Biological Examples:

The following test protocols are used to ascertain the biological properties of the compounds of this invention. The following examples are being presented to further illustrate the invention. However, they should not be construed as limiting the invention in any manner.

The PPAR delta agonists of the present invention are evaluated in *in vitro* and *in vivo* models for their ability to promote myelin expression and enhance regenerative processes.

The optimum nuclear receptor selectivity profile is determined by the GAL4/luciferase reporter assays. A rodent cellular assay shows the compound's ability to direct / accelerate differentiation of cultured oligodendrocyte progenitor cells to mature oligodendrocytes.

Specific biological assays suggesting efficacy for the treatment of MS are lysolecithin induced demyelination and experimental allergic encephalomyelitis performed in rodents.

Determination of EC50 values of PPAR agonists in the cellular PPAR delta assay**Principle**

The potency of substances, which bind to human PPAR delta and activate it in an agonistic manner, is analyzed using a stably transfected HEK cell line (HEK= human embryo kidney) which is referred to here as PPAR delta reporter cell line. The PPAR delta reporter cell line contains two genetic elements, a luciferase reporter element (pdeltaM-GAL4-Luc-Zeo) and a PPAR delta fusion protein (GR-GAL4-humanPPAR delta-LBD), which mediates expression of the luciferase reporter element depending on a PPAR delta ligand. The stably and constitutively expressed fusion protein GR-GAL4-humanPPAR delta-LBD binds in the cell nucleus of the PPAR delta reporter cell line via the GAL4 protein portion to the GAL4 DNA binding motifs 5'-upstream of the luciferase reporter element which is stably integrated in the genome of the cell line. There is only little expression of the luciferase reporter gene in the absence of a PPAR delta ligand if fatty acid-depleted fetal calf serum (cs-FCS) is used in the assay. PPAR delta ligands bind and activate the PPAR delta fusion protein and thereby

stimulate expression of the luciferase reporter gene. The luciferase, which is formed can be detected by means of chemiluminescence via an appropriate substrate.

Construction of the PPAR delta reporter cell line:

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The production of the stable PPAR delta reporter cell line is based on a stable HEK-cell clone which was stably transfected with a luciferase reporter element. This step was already described above in the section "construction of the PPAR alpha reporter cell line". In a second step, the PPAR delta fusion protein (GR-GAL4-humanPPAR delta-LBD) was stably introduced into this cell clone. For this purpose, the cDNA coding for the N-terminal 76 amino acids of the glucocorticoid receptor (Accession # P04150) was linked to the cDNA section coding for amino acids 1-147 of the yeast transcription factor GAL4 (Accession # P04386). The cDNA of the ligand-binding domain of the human PPAR delta receptor (amino acids S139-Y441; Accession # L07592) was cloned in at the 3'-end of this GR-GAL4 construct. The fusion construct prepared in this way (GR-GAL4-humanPPAR delta-LBD) was recloned into the plasmid pcDNA3 (Invitrogen) in order to enable constitutive expression by the cytomegalovirus promoter. This plasmid was linearized with a restriction endonuclease and stably transfected into the previously described cell clone containing the luciferase reporter element. The resulting PPAR delta reporter cell line which contains a luciferase reporter element and constitutively expresses the PPAR delta fusion protein (GR-GAL4-human PPAR delta-LBD) was isolated by selection with zeocin (0.5 mg/ml) and G418 (0.5 mg/ml).

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Assay procedure and evaluation:

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The activity of PPAR delta agonists is determined in a 3-day assay, which is described below:

Day 1

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The PPAR delta reporter cell line is cultivated to 80% confluence in DMEM (# 41965-039, Invitrogen) which is mixed with the following additions: 10% cs-FCS (fetal calf serum; #SH-30068.03, Hyclone), 0.5 mg/ml zeocin (#R250-01, Invitrogen), 0.5 mg/ml G418 (#10131-027, Invitrogen), 1% penicillin-streptomycin solution (#15140-122, Invitrogen) and 2 mM L-glutamine (#25030-024, Invitrogen). The cultivation takes place in standard cell culture

bottles (# 353112, Becton Dickinson) in a cell culture incubator at 37°C in the presence of 5% CO₂. The 80%-confluent cells are washed once with 15 ml of PBS (#14190-094, Invitrogen), treated with 3 ml of trypsin solution (#25300-054, Invitrogen) at 37°C for 2 min, taken up in 5 ml of the DMEM described and counted in a cell counter. After dilution to 500.000 cells/ml, 35,000 cells are seeded in each well of a 96 well microtiter plate with a clear plastic base (#3610, Corning Costar). The plates are incubated in the cell culture incubator at 37°C and 5% CO₂ for 24 h.

Day 2

PPAR delta agonists to be tested are dissolved in DMSO in a concentration of 10 mM. This stock solution is diluted in DMEM (#41965-039, Invitrogen) which is mixed with 5% cs-FCS (#SH-30068.03, Hyclone), 2 mM L-glutamine (#25030-024, Invitrogen) and the previously described antibiotics (zeocin, G418, penicillin and streptomycin).

Test substances are tested in 11 different concentrations in the range from 10 µM to 100 pM.

More potent compounds are tested in concentration ranges from 1 µM to 10 pM or between 100 nM and 1 pM.

The medium of the PPAR delta reporter cell line seeded on day 1 is completely removed by aspiration, and the test substances diluted in medium are immediately added to the cells. The dilution and addition of the substances is carried out by a robot (Beckman FX).

The final volume of the test substances diluted in medium is 100 µl per well of a 96 well microtiter plate. The DMSO concentration in the assay is less than 0.1 % v/v in order to avoid cytotoxic effects of the solvent.

Each plate was charged with a standard PPAR delta agonist, which was likewise diluted in 11 different concentrations, in order to demonstrate the functioning of the assay in each

individual plate. The assay plates are incubated in an incubator at 37°C and 5% CO₂ for 24 h.

Day 3

The PPAR delta reporter cells treated with the test substances are removed from the incubator, and the medium is aspirated off. The cells are lysed by pipetting 50 µl of Bright Glo reagent (from Promega) into each well of a 96 well microtiter plate. After incubation at room temperature in the dark for 10 minutes, the microtiter plates are measured in the luminometer (Trilux from Wallac). The measuring time for each well of a microtiter plate is 1 sec.

Evaluation:

The raw data from the luminometer are transferred into a Microsoft Excel file. Dose-effect plots and EC50 values of PPAR agonists are calculated using the XL.Fit program as specified by the manufacturer (IDBS).

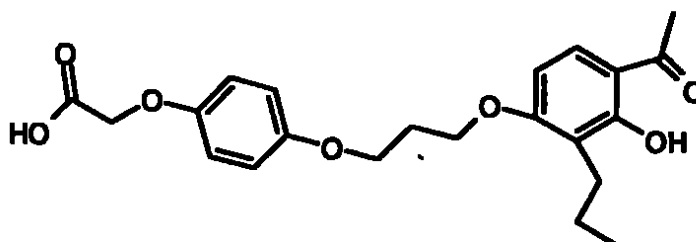
Determination of EC₅₀ values of PPAR agonists in the cellular PPAR alpha assay:**Principle**

The potency of substances which bind to human PPAR alpha and activate it in an agonistic manner is analyzed using a stably transfected HEK cell line (HEK= human embryo kidney) which is referred to here as PPAR alpha reporter cell line. It contains two genetic elements, a luciferase reporter element (pdeltaM-GAL4-Luc-Zeo) and a PPAR alpha fusion protein (GR-GAL4-humanPPAR alpha-LBD) which mediates expression of the luciferase reporter element depending on a PPAR alpha ligand. The stably and constitutively expressed fusion protein GR-GAL4-humanPPAR alpha-LBD binds in the cell nucleus of the PPAR alpha reporter cell line via the GAL4 protein portion to the GAL4 DNA binding motifs 5'-upstream of the luciferase reporter element which is stably integrated in the genome of the cell line. There is only weak expression of the luciferase reporter gene in the absence of a PPAR alpha ligand if fatty acid-depleted fetal calf serum (cs-FCS) is used in the assay. PPAR alpha ligands bind and activate the PPAR alpha fusion protein and thereby stimulate the expression of the luciferase reporter gene. The luciferase which is formed can be detected by means of chemiluminescence via an appropriate substrate.

Construction of the PPAR alpha reporter cell line:

The PPAR alpha reporter cell line was prepared in two stages. Firstly, the luciferase reporter element was constructed and stably transfected into HEK cells. For this purpose, five binding sites of the yeast transcription factor GAL4 (Accession # AF264724) were cloned in 5'-upstream of a 68 bp-long minimal MMTV promoter (Accession # V01175). The minimal MMTV promoter section contains a CCAAT box and a TATA element in order to enable efficient transcription by RNA polymerase II. The cloning and sequencing of the GAL4-

Compound of formula (2) also known as L165,041 and has been disclosed in European Patent Application 28063 and in W0 97/28149 wherein it was identified as a selective PPAR delta agonist.



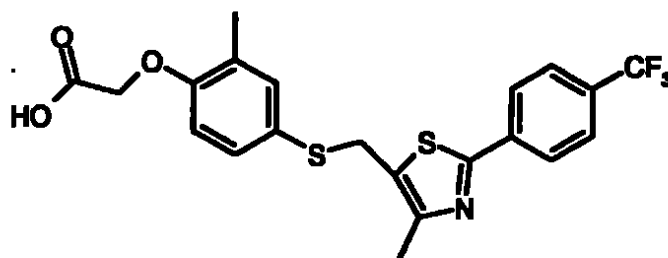
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Due to the potential ability of Peroxisome Proliferator Activated Receptor Delta (PPAR delta) agonists to accelerate the differentiation of acutely isolated oligodendrocyte progenitor cells from rodent cerebrum and to significantly increase both myelin sheath diameter and myelin gene expression, there exists the potential for PPAR delta agonists to activate the PPAR delta pathway in oligodendrocyte progenitor cells and enhance neuronal repair by restoring the myelin sheath to demyelinated axons in demyelinating disease, particularly MS.

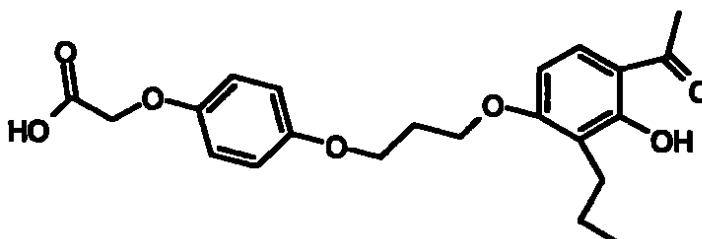
SUMMARY OF THE INVENTION

Thus in accordance with the practice of this invention there is provided a method of treating a variety of demyelinating disease conditions with PPAR delta agonists, and in particular multiple sclerosis. In general, the disease conditions that can be treated in accordance with the practice of this invention include but not limited to multiple sclerosis, Charcot-Marie-Tooth disease, Pelizaeus-Merzbacher disease, encephalomyelitis, neuromyelitis optica, adrenoleukodystrophy, Guillian-Barrte syndrome and disorders in which myelin forming glial cells are damaged including spinal cord injuries, neuropathies and nerve injury. The diseases as disclosed herein can be treated by administering to a patient in need of such treatment a therapeutically effective amount of a PPAR delta agonist.

The present invention is also directed to the use of compounds of formula (I) and formula (II) for the treatment of demyelinating diseases, and in particular multiple sclerosis.



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(2)

The present invention also comprises a method of treating multiple sclerosis in patients by administering a combination of a compound of formula (1) or formula (2) or pharmaceutically acceptable salt thereof, with another compound known to be effective for the treatment of multiple sclerosis in therapeutically effective amounts. Compounds that are currently used to treat the disease are the disease-modifying agents such as the interferons (interferon beta 1-a, beta 1-b and alpha 2), glatiramer acetate or corticosteroids such as methylprednisolone and prednisone. Also, chemotherapeutic agents such as methotrexate, azathioprine, cladribine cyclophosphamide and cyclosporine.

DETAILED DESCRIPTION OF THE INVENTION

As used herein, the expression "pharmaceutically acceptable carrier" means a non-toxic solvent, dispersant, excipient, adjuvant, or other material which is mixed with the compound of the present invention in order to permit the formation of a pharmaceutical composition, i.e., a dosage form capable of administration to the patient. One example of such a carrier is a pharmaceutically acceptable oil typically used for parenteral administration.

The term "pharmaceutically acceptable salts" as used herein means that the salts of the compounds of the present invention can be used in medicinal preparations. Other salts may, however, be useful in the preparation of the compounds according to the invention or of their pharmaceutically acceptable salts. Suitable pharmaceutically acceptable salts of the compounds of this invention include acid addition salts which may, for example, be formed by

MMTV construct took place in analogy to the description of Sambrook J. *et. al.* (Molecular cloning, Cold Spring Harbor Laboratory Press, 1989). Then the complete *Photinus pyralis* gene (Accession # M15077) was cloned in 3'-downstream of the GAL4-MMTV element. After sequencing, the luciferase reporter element consisting of five GAL4 binding sites, MMTV promoter and luciferase gene was recloned into a plasmid which confers zeocin resistance in order to obtain the plasmid pdeltaM-GAL4-Luc-Zeo. This vector was transfected into HEK cells in accordance with the statements in Ausubel, F.M. *et al.* (Current protocols in molecular biology, Vol. 1-3, John Wiley & Sons, Inc., 1995). Then zeocin-containing medium (0.5 mg/ml) was used to select a suitable stable cell clone which showed very low basal expression of the luciferase gene.

In a second step, the PPAR alpha fusion protein (GR-GAL4-humanPPAR alpha-LBD) was introduced into the stable cell clone described. For this purpose, initially the cDNA coding for the N-terminal 76 amino acids of the glucocorticoid receptor (Accession # P04150) was linked to the cDNA section coding for amino acids 1-147 of the yeast transcription factor GAL4 (Accession # P04386). The cDNA of the ligand-binding domain of the human PPAR alpha receptor (amino acids S167-Y468; Accession # S74349) was cloned in at the 3'-end of this GR-GAL4 construct. The fusion construct prepared in this way (GR-GAL4-humanPPAR alpha-LBD) was recloned into the plasmid pcDNA3 (Invitrogen) in order to enable constitutive expression therein by the cytomegalovirus promoter. This plasmid was linearized with a restriction endonuclease and stably transfected into the previously described cell clone containing the luciferase reporter element. The finished PPAR alpha reporter cell line which contains a luciferase reporter element and constitutively expresses the PPAR alpha fusion protein (GR-GAL4-human PPAR alpha-LBD) was isolated by selection with zeocin (0.5 mg/ml) and G418 (0.5 mg/ml).

Assay procedure:

The activity of PPAR alpha agonists is determined in a 3-day assay, which is described below:

Day 1

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G418 (#10131-027, Invitrogen), 1% penicillin-streptomycin solution (#15140-122, Invitrogen) and 2 mM L-glutamine (#25030-024, Invitrogen). The cultivation takes place in standard cell culture bottles (# 353112, Becton Dickinson) in a cell culture incubator at 37°C in the presence of 5% CO₂. The 80%-confluent cells are washed once with 15 ml of PBS (#14190-094, Invitrogen), treated with 3 ml of trypsin solution (#25300-054, Invitrogen) at 37°C for 2 min, taken up in 5 ml of the DMEM described and counted in a cell counter. After dilution to 500.000 cells/ml, 35,000 cells are seeded in each well of a 96 well microtiter plate with a clear plastic base (#3610, Corning Costar). The plates are incubated in the cell culture incubator at 37°C and 5% CO₂ for 24 h.

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Day 2

PPAR alpha agonists to be tested are dissolved in DMSO in a concentration of 10 mM. This stock solution is diluted in DMEM (#41965-039, Invitrogen) which is mixed with 5% cs-FCS (#SH-30068.03, Hyclone), 2 mM L-glutamine (#25030-024, Invitrogen) and the previously described antibiotics (zeocin, G418, penicillin and streptomycin). Test substances are tested in 11 different concentrations in the range from 10 µM to 100 pM. More potent compounds are tested in concentration ranges from 1 µM to 10 pM or between 100 nM and 1 pM.

15

The medium of the PPAR alpha reporter cell line seeded on day 1 is completely removed by aspiration, and the test substances diluted in medium are immediately added to the cells. The dilution and addition of the substances is carried out by a robot (Beckman FX). The final volume of the test substances diluted in medium is 100 µl per well of a 96 well microtiter plate. The DMSO concentration in the assay is less than 0.1 % v/v in order to avoid cytotoxic effects of the solvent.

20

Each plate was charged with a standard PPAR alpha agonist, which was likewise diluted in 11 different concentrations, in order to demonstrate the functioning of the assay in each individual plate. The assay plates are incubated in an incubator at 37°C and 5% CO₂ for 24 h.

25

Day 3

The PPAR alpha reporter cells treated with the test substances are removed from the incubator, and the medium is aspirated off. The cells are lysed by pipetting 50 µl of Bright Glo reagent (from Promega) into each well of a 96 well microtiter plate. After incubation at room temperature in the dark for 10 minutes, the microtiter plates are measured in the

30

luminometer (Trilux from Wallac). The measuring time for each well of a microtiter plate is 1 sec.

Evaluation:

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The raw data from the luminometer are transferred into a Microsoft Excel file. Dose-effect plots and EC50 values of PPAR agonists are calculated using the XL.Fit program as specified by the manufacturer (IDBS).

10

Determination of EC50 values of PPAR agonists in the cellular PPAR gamma assay

Cell based PPAR gamma Assay protocol

To perform cell based assays a luciferase assay is performed in 96 well plates as follows:

15

Day 1: Plating of cells:

- Wash cells grown to 80-90% confluency once in PBS
- Trypsinize for 2 min
- 20 • Add 15ml assay medium (DMEM, Invitrogen, Cat.No. 41965-039; 5% Charcoal/Dextran Treated FBS, Hyclone, Cat.No. SH30068; 0.5 mg/ml Zeocin, Invitrogen, Cat.No. 46-0072; 0.5mg/ml Geneticin, Invitrogen, Cat.No. 10131-027; 1% Penicillin/Streptomycin, Invitrogen, Cat.No. 15140-122; 2 mM L-Glutamine, Invitrogen, Cat.No. 25030-024; 7.5 µg/ml Blasticidin S HCl, Invitrogen, Cat.No. R210-01; 1 µg/ml Doxycycline, Clontech, Cat.No. 8634-1)
- 25 • Count cells
- Dilute cells in assay medium to 500.000 cells/ml
- Dispense 100 µl of cell suspension per well in clear bottom Corning plates (makes 50.000 cells/well)
- 30 • Incubate for 24 h at 37°C, 5% CO2

Day 2: Dosing with test compounds:

- Solve test compounds in DMSO to make a 10 mM stock solution
- Dilute compound to appropriate concentration in assay medium (DMEM, Invitrogen, Cat.No. 41965-039; 5% Charcoal/Dextran Treated FBS, Hyclone, Cat.No. SH30068; 0.5mg/ml Zeocin, Invitrogen, Cat.No. 46-0072; 0.5mg/ml Geneticin, Invitrogen, Cat.No. 10131-027; 1% Penicillin/Streptomycin, Invitrogen, Cat.No. 15140-122; 2mM L-Glutamin, Invitrogen, Cat.No. 25030-024; 7.5µg/ml Blasticidin S HCl, Invitrogen, Cat.No. R210-01; 1µg/ml Doxycycline, Clontech, Cat.No. 8634-1) (regular FCS is harboring\ free fatty acids interfering with the PPAR ligand binding domains).
- Aspirate medium (cells are quite sensitive at this step; make sure that cells are no longer than 1 min without being covered by medium)
- Transfer diluted compounds to 96 wells (100 µl medium including compound)
- Make controls with standard compound (e.g. Rosiglitazon) as well as a DMSO control (0,1 % DMSO)
- Incubate cells for 24 h at 37°C at 5% CO₂

Dilution steps and addition of diluted compounds is done using a Beckman Biomek 2000 or Beckman FX robot.

Day 3: Cell Lysis and measurement of luciferase activity:

- Aspirate medium from cells
- Freeze plates at -20°C (optional)
- Thaw plates for 30 min (if necessary)
- Add 50 µl Bright-Glo-Luciferase Assay Reagent (Promega, Cat.No. E2650)
- Incubate for 10 min in the dark
- Measure luminescence 2 sec per well (Wallac Microbeta)

Data analysis:

Determination of EC₅₀ values is done with Microsoft Excel in combination with XLFit (develop by IDBS) using the fitting algorithm #205.

Determination of EC₅₀ values in the cellular human RXR receptor assay

Cell based RXR Assay protocol

To perform cell based assays a luciferase assay is performed in 96 well plates as follows:

5 Day 1: Plating of cells

- Wash cells grown to 80-90% confluency once in PBS
- Trypsinize for 2 min
- Add 15ml culture medium (DMEM, Invitrogen, Cat.No. 41965-039; 10%
10 Charcoal/Dextran Treated FBS, Hyclone, Cat.No. SH30068; 0.5mg/ml Zeocin, Invitrogen,
Cat.No. 46-0072; 0.5mg/ml Geneticin, Invitrogen, Cat.No. 10131-027; 1%
Penicillin/Streptomycin, Invitrogen, Cat.No. 15140-122; 2mM L-Glutamin, Invitrogen,
Cat.No. 25030-024)
- Count cells
- 15 • Dilute cells in culture medium to 175.000 cells/ml
- Dispense 200 µl of cell suspension per well in clear bottom Corning plates (makes 35.000
cells/well)
- Incubate for 24 h at 37°C, 5% CO₂

Day 2: Dosing with test compounds

20

- Solve test compounds in DMSO to make a 10 mM stock solution
- Dilute compound to appropriate concentration in assay medium (DMEM w/o phenol-red,
Invitrogen, Cat.No. 21063-029; 5% Charcoal/Dextran Treated FBS, Hyclone, Cat.No.
SH30068; 0.5mg/ml Zeocin, Invitrogen, Cat.No. 46-0072; 0.5mg/ml Geneticin,
25 Invitrogen, Cat.No. 10131-027; 1% Penicillin/Streptomycin, Invitrogen, Cat.No. 15140-
122; 2mM L-Glutamin, Invitrogen, Cat.No. 25030-024) (regular FCS is harboring free
fatty acids interfering with the PPAR ligand binding domains).
- Aspirate medium (cells are quite sensitive at this step; make sure that cells are no longer
than 1 min without being covered by medium)
- 30 • Transfer diluted compounds to 96 wells (100 µl medium including compound)
- Make controls with standard compound (e.g. RPR258134) as well as a DMSO control (0,1
% DMSO)
- Incubate cells for 24 h at 37°C at 5% CO₂

Dilution steps and addition of diluted compounds is done using a Beckman Biomek 2000 or Beckman FX robot.

Day 3: Cell Lysis and measurement of luciferase activity

5

- Aspirate medium from cells
- Freeze plates at -20°C (optional)
- Thaw plates for 30 min (if necessary)
- Add 50 μl Bright-Glo-Luciferase Assay Reagent (Promega, Cat.No. E2650)

10

- Incubate for 10 min in the dark
- Measure luminescence 2 sec per well (Wallac Microbeta)

Data analysis

15

Determination of EC50 values is done with Microsoft Excel in combination with XLFit (develop by IDBS) using the fitting algorithm #205.

Table 1 shows the results if the reporter assays. The results show that compounds 1 and 2 are selective PPAR delta activators with low PPAR alpha, gamma and RXR activity.

20

Table 1 Reporter Assays

Compound	Human PPAR delta EC ₅₀ (μM)	Mouse PPAR delta EC ₅₀ (μM)	Human PPAR alpha EC ₅₀ (μM)	Human PPAR gamma EC ₅₀ (μM)	Human PPAR RXR ¹ EC ₅₀ (μM)
1	< 0.00457 (29x)*	7.8 (4x)*, 6 (2x)*, 4(2x)*	1.3	3.97 (3.1x)*	No increase
2	0.039 (27x)*	1 (2x)*	1.1	1.1 (4.3x)*	No increase

*Value represents the fold increase over baseline luciferase activity. ¹Retinoid X receptor

5

RAT/MICE Oligodendrocyte culturesPreparation of cells:

10

1. Primary rat oligodendrocyte progenitor cells are obtained from the neocortex of newborn (postnatal days 2-3) rats or mice and are enriched, after removal of microglia, by mechanical separation from the astrocytic monolayer using a modification of the technique originally described by McCarthy and de Vellis (1980).

15

2. Remove the meninges from neonatal rat brain and mechanically dissociate tissue. Plate cells on T75 flasks and feed cells with DMEM/F12 + 10% FBS.

3. Collect oligodendrocytes growing on the astrocyte bed layer by shaking-off method fourteen days after the original prep date. Centrifuge the suspension and resuspend the cell pellet in serum free media (SFM; DMEM combined with 25 μg/ml transferring, 30 nM triiodothyronine, 20 nM hydrocortisone, 20 nM progesterone, 10 nM biotin, 1x trace elements, 30 nM selenium, 1 μg/ml putrescine, 0.1% BSA, 5 U/ml PenStrep, 10 μg/ml insulin) supplemented with the following growth factors: Platelet derived growth factor-AA (PDGF) and fibroblast growth factor-2 (FGF).

4. Plate the cells on PDL-coated dishes and incubate at 37°C with 6-7% CO₂.
5. Media components are replaced every 48 hr to keep the cells in a progenitor state.

Progenitor cell passaging to increase cell numbers for screening assays:

- 5 1. When the culture are confluent, rinse the culture with PBS, add trypsin and incubate
for ~2-3 min at 37°C.
2. Neutralize and centrifuge the cell suspension at 900g for 5 min.
3. Resuspend the cell pellet in SFM + PDGF/FGF.
4. Feed the cells with fresh growth factors every 48 hrs to keep enrich for rapidly
10 dividing progenitor cells.
5. Cells are passaged no more than 4-5 times prior to experimental assays.
6. All experiments involving oligodendrocyte progenitor cells were done using cells that
were continuously maintained under these conditions. Greater than 95% of all cells
were A2B5 immunopositive and expressed 2' 3' -cyclic nucleotide 3' -
15 phosphodiesterase II mRNA.
7. To generate mature oligodendrocytes, 24 h after plating progenitor cells were switched
to SFM supplemented with or without IGF-I and grown under these conditions for 7 d
prior to experimental assays.
8. Alternatively, the enriched rat Central Glia-4 (CG4) progenitor cell line may be used,
20 which is maintained in base media (DMEM, with 2 mM glutamine, 1mM sodium
pyruvate, biotin (40 nM), insulin (1 µM) and N1) supplemented with 30% conditioned
media from the B-104 neuroblastoma cell line. To induce differentiation, CG4 cells
are switched to base media with 1% fetal calf serum (removed after 2 days) and insulin
(500 nM). A2B5 and MBP immunoreactivity is used to confirm >95% enrichment in
25 immature and mature cultures, respectively.

Rat/Mouse Culture Compound Treatment:

1. Put 10,000 – 15,000 cells /well in 24-well PDL coated plates and culture the cells in presence of mitogen (10 ng/ml) overnight.
- 5 2. In the presence of mitogen:
 - a. Next day, remove the old medium and add compounds in fresh medium (with mitogen)
 - b. Compound dose response evaluations are performed at 6 different concentrations (10 μ M, 1 μ M, 100 nM, 10 nM, 1 nM, and 0.1 nM);
 - 10 c. Triplicates wells are run for each compound concentration.
3. In the absence of mitogen:
 - a. Next day, remove the old medium and add compounds in fresh medium (without mitogen)
 - b. Compound dose response evaluations are performed at 6 concentrations (10 μ M, 1 μ M, 100 nM, 10 nM, 1 nM, and 0.1 nM);
 - 15 c. Triplicates wells are run for each compound concentration.
4. Culture the treated cells for 7 d prior to using in experimental assays.

HUMAN Oligodendrocyte cultures**20 Preparation of cells:**

1. Human neurospheres collected from E19.5 – E22 human embryo cortex) are cultured for 2 weeks in progenitor media: DMEM/F12 containing 100 μ g/ml transferring, 30 nM triiodothyronine, 20 nM hydrocortisone, 20 nM progesterone, 10 nM biotin, 1x trace elements, 30 nM selenium, 60 μ M putrescine, 0.1% BSA, 5 U/ml PenStrep, 25 μ g/ml insulin) supplemented with PDGF and FGF.
- 25

2. Neurospheres are dissociated with 20 U/ml papain at 37°C for 30-50 min.
3. Cells are plated onto PDL coated dishes at density of 50,000-100,000 cell/well in progenitor media containing PDGF/FGF and incubated at 37°C with 5-6% CO₂.
4. Media and growth factors are replenished every 48 hr.

5

Human Culture Compound Treatment:

1. 24 to 48 hr after plating remove the old medium and add compounds in fresh medium (with mitogen)
2. Compound dose response evaluations are performed at 3-6 different concentrations (10
10 μ M, 1 μ M, 100 nM, 10 nM, 1 nM, and 0.1 nM)
3. Triplicates wells are run for each compound concentration.
5. Culture the treated cells for 7 d prior to using in experimental assays.

RAT/MOUSE/HUMAN Oligodendrocyte Specific Immunostaining:

- 15 Following compound exposure, oligodendrocyte-specific antibodies are used to assess ability of compound to accelerate/promote oligodendrocyte differentiation (for example, O4, O1, or myelin basic protein immunoreactivity is over time between compound treated and untreated cultures).

- 20 I. Cells are plated onto poly-D-lysine treated 4-well chamber slides at 5×10^3 to 20×10^3 cells/well and grown as described above. Sequential staining is performed on oligodendrocyte populations with increasing degrees of cellular differentiation, as determined by days *in vitro* without PDGF and FGF.
2. Live staining for 30 min at 37°C is used to detect oligodendrocyte stage specific cell surface marker expression (including A2B5, O4, and O1).
- 25 3. Subsequently, cells are fixed with 4% paraformaldehyde, 10 min, room temperature.

4. Fixed staining procedures are used to detect oligodendrocyte stage specific marker expression (including myelin basic protein, MBP).
5. Rinse with PBS.
6. Permeabilize with 0.1% Triton/0.01% NaAz diluted in 1X PBS for 10 min, room temperature.
7. Block with 5-10% goat serum in antibody dilution buffer (0.1% Triton-X 100 and 1% IgG-free bovine serum albumin; also used to dilute antibodies), 15 min, room temperature.
8. Add primary antibody diluted in antibody dilution buffer.
9. Incubate overnight, gently rocking, 4° C.
10. Next day, rinse with PBS 1X 5 min, followed by 3X 15 min each, room temperature.
11. Incubate with appropriate secondary antibodies, 45 min, room temperature.
12. Cell nuclei are stained with 4,6-diamidino-2-phenylindole (DAPI), 15min, room temperature.
13. Rinse several times with PBS and evaluate using fluorescent microscopy.
14. The following conditions are compared over time and at different compound doses:
PDGF/FGF alone, SFM alone, SFM-IGF1 alone, PDGF/FGF and compound, SFM and compound.

RAT/MOUSE/HUMAN Bromodeoxyuridine (BrdU) immunostaining:

To confirm that compounds do not promote cell proliferation.

1. Oligodendrocyte progenitor cells are labeled with 10 μ M BrdU for 20 hr and then fixed with either 70% ethanol or 4% paraformaldehyde.
2. The cells are incubated successively with biotinylated mouse anti-BrdU and Streptavidin-Peroxidase, with three intervening washes with PBS.

3. Colormetric visualization of the BrdU immunoreactivity is developed with DAB and total cell numbers are assessed using the counter-stain hematoxylin.
4. BrdU immunopositive cells are counted by two independent observers.

5 **RAT/MOUSE/HUMAN Culture Image analysis:** Fluorescent microscopy is used to quantitate the extent of oligodendrocyte differentiation after compound exposure. This assay demonstrates that selective agonists accelerate/promote oligodendrocytes differentiation.

1. **Manual Cell Counting:** Four fields are randomly selected for each experimental condition and 500-600 cells are counted in each field. The percentage of MBP (or O4) immunopositive cells (mature process bearing cells with or without myelin sheets) versus DAPI positive cells (total cell number) cells are compared in the control and drug-treated groups.
2. **Automated Cell Counting:** Fluorescent microscopy was used to quantitate the extent of oligodendrocyte differentiation after compound exposure. Six fields/well are randomly selected to assess the number of differentiating oligodendrocytes among the total population (~8 to 15x10³ cells are counted/well). Immunofluorescence images are obtained using a Zeiss AxioVision digital imaging system, with a Zeiss AxioCam HRc cooled CCD camera connected to the same microscope. All microscopic imaging parameters are set for acquiring images for the analysis of cellular immunofluorescence intensity. The percentage of MBP positive (differentiated) cells versus total cells (DAPI nuclear stained) is compared in the control versus drug-treated groups. Cellular autofluorescence was undetectable under the imaging conditions.
- a) 3. **Human oligodendrocyte differentiation assay:** manually count total number of O4 immunopositive cells/well (bipolar and multipolar)..

24

The results using rat oligodendrocyte cultures are shown in Figure 1 and the results using human oligodendrocyte mixed cultures are shown in Figures 5 and 6. As the results show, PPAR delta agonists enhance or accelerate rat and human oligodendrocyte differentiation, as measured by increased myelin basic protein expression compared to untreated controls. This novel finding would suggest that compound 1 and compound 2 and selective PPAR delta agonists in general would be enhance, accelerate, or stimulate oligodendrocyte differentiation and myelin formation *in vivo*, in the diseased or injured CNS, including MS and other demyelinating disorders.

10 **RAT/MOUSE/HUMAN Quantitative Polymerase Chain Reaction (PCR):** To evaluate compound induced PPAR delta pathway activation and the extent of oligodendrocyte maturation (changes in mRNA levels).

1. Total RNA is extracted from cultured oligodendrocytes using TriZol reagent.
2. Subsequently, mRNA is treated with RNase-free DNase, repurified, and then
15 converted to cDNA template using a RT reaction (Clontech Advantage RT for PCR Kit).
3. PPAR delta pathway member transcript expression is quantitated using Sybr Green PCR Master Mix.
4. The 18S ribosomal RNA primer/probe mix (186 bp product), suspended in Taqman
20 2X PCR Master Mix is used as an internal control.
5. Quantitative PCR is carried out using real-time Taqman™ technology (Gibson, et al., 1996) with a model 7700 Sequence Detector System (Applied Biosystems, Foster City, CA).
6. The results are analyzed using Sequence Detection Systems software version 1.91.

Results for these assays are shown in Figures 2, 3, 4A, and 4B. These results suggest that PPAR delta selective agonists bind the PPAR delta receptor and directly activate the PPAR delta pathway in oligodendrocytes and should act similarly *in vivo*.

RAT ELISA Assay: To evaluate compound induced PPAR delta pathway activation and the extent of oligodendrocyte maturation (changes in protein levels).

1. Plates are washed with PBS, and then keep on ice. Add 200 µl ice cold lysis buffer (Tris 50mM, pH7.4, MgCl₂ 2mM, EDTA 1mM, β-mercaptoethanol 5mM, Nonidet P-40 1%, Protease inhibitor cocktail (Roche): 1 tablet/50 ml) to each well.
2. Lyse cells by using pipette to up down and spin plates at 2000 rpm at 4°C for 5 min.
The supernatant is ready to use.
3. Pipet 50 µl of standard, controls and samples to the wells.
4. Add 50 µl of MBP Assay Buffer to each well.
5. Incubate the well, shaking at 500-700 rpm on orbital microplate shaker for 2 hr at room temperature.
6. Add 100ul of the MBP Antibody-Biotin Conjugate to each well.
7. Incubate the well, shaking at 500-700 rpm on orbital microplate shaker for 1 hr at room temperature.
8. Wash well 5 times with Wash Solution. Blot dry by inverting the plate on absorbent material.
9. Dilute the streptavidin-enzyme conjugate concentrate 1:50 with MBP Elisa Assay buffer. (must be diluted immediately prior to use in the assay).
10. Add 100 µl streptavidin-enzyme conjugate solutions to each well.
11. Incubate the well, shaking at 500-700 rpm on orbital microplate shaker for 30 min at room temperature.

12. Wash well 5 times with the Wash Solution. Blot dry by inverting the plate on absorbent material.
13. Add 100 µl of TMB Chromogen Solution to each well.
14. Incubate the well, shaking at 500-700 rpm on orbital microplate shaker for 10-20 min at room temperature. Avoid exposure to direct sunlight.
15. Add 100 µl of the Stopping Solution to each well.
16. Read the absorbance of the solution in the wells within 30 min, using a microplate reader set to 450 nM.

The above results taken in general and shown in Figures 1-6 illustrate that PPAR delta agonists promote oligodendrocyte differentiation even in the presence of mitogens, which normally keep cells mitotically active and inhibit cellular differentiation. Thus, it is expected that in the injured or diseased CNS selective PPAR delta agonists will cause dividing oligodendrocyte progenitor cells to express myelin proteins and ensheath demyelinated or hypomyelinated axons.

In Vivo Proof of Concept Models

Focal Lesions: (used to assess whether compounds protect myelin integrity or accelerate/enhance the rate of remyelination.)

1. Rats 7 weeks of age are given free access to food and water and acclimatized for a minimum of 4 days before use in experiments.
2. Prior to surgery each animal is weighed. The rat is then anaesthetized with ketamine (100 mg/ml) in combination with xylazine (20 mg/ml) in a ratio of 1.8 : 1. The rats are injected with 0.15ml/180g body weight i.p. of the anaesthetic solution prior to the surgical procedure. The animal is prepared for surgery using aseptic conditions in

accordance with the IACUC guidelines. All surgical instruments will be autoclaved. The hair is clipped between the ears and this region will then be scrubbed with Betadine, flushed with sterile saline and finally wiped with a pre-packaged sterile alcohol swab.

- 5 3. For the surgical procedure, the rat is placed on its ventral surface in a small animal stereotaxic instrument designed to hold the head steady. The incisor bar is always set at -3.9 mm, since this has been shown to achieve a flat-skull position for SD rats.
4. An incision is made in the previously shaven skin overlying the skull between the ears.
5. A small area of bone (0.75mm in diameter) is drilled at the following coordinates AP –
10 1.8, ML -3.1 from lambda.
6. The bone is removed and rats are injected with 2µl ethidium bromide, lysolecithin, or SIN-1 into the right caudal cerebellar peduncle, DV -7.1 mm, over a 2 min period by means of a Hamilton µl syringe and needle. Alternatively injections are made into the spinal cord, corpus callosum, or cortex.
- 15 7. The needle is left in position for the subsequent 2 min.
8. After withdrawal of the needle the incision is sutured.
9. Each rat receives an i.m. injection of 0.003mg buprenorphine into a hind leg.
10. The rat is placed in a warming cupboard until it regains consciousness. At which time it is returned to its home cage. Do not allow more than 2 rats per cage, as they will pull
20 each other's suture out.
11. Similar procedures are also done using mice.

Rat Experimental Allergic Encephalomyelitis (Rat EAE) Disease Model:

25 Experimental allergic encephalomyelitis (EAE) is a T-cell-mediated autoimmune disease of the nervous system that develops in susceptible animals following sensitization

with either whole spinal cord homogenate or a component (myelin basic protein). The EAE rodent model is an appropriate tool for studying the inflammation of the brain and spinal cord observed in MS patients. In rodents, injection of whole spinal cord or spinal cord components such as myelin basic protein induces an autoimmune response based on the activation of T-lymphocytes. Clinical disease typically becomes manifest around day 8-10 after inoculation, observed as a broad spectrum of behavioral anomalies ranging from mild gait disturbances and tail atony to complete paralysis and death. Weight loss typically occurs. In animals that survive, spontaneous recovery occurs, accompanied by variable recovery of most motor function. Depending on the species, allergen, and methodology used, animals tested by the EAE model may experience a single (acute EAE) or several (chronic relapsing EAE) attacks. Several treatment paradigms may be used: the drug or treatment of choice may be administered before immunization, during the nonsymptomatic period or during the clinical disease.

Animals:

Female Lewis rats, 160-220g (Charles River)

Antigen:

Whole Guinea Pig spinal cord (Harlan Biosciences).

Complete Freund's adjuvant H37 Ra [1mg/ml Mycobacterium Tuberculosis H37 Ra] (Difco).

Additional antigen:

Mycobacterium Tuberculosis (Difco).

Bordetella Pertussis [Heat Killed] (Difco).

Antigen preparation: (for approximately 720 animals):

1. Weigh 5 grams of frozen guinea pig spinal cord.
2. Add 5g spinal cord to 5ml 0.9% saline (1g/ml) in a round bottom centrifuge tube
3. Homogenize on ice with the Tissue-tech until the tissue is completely disrupted (approximately 5 minutes).
4. Add 10 ml Complete Freund's adjuvant H37 Ra supplemented with 200 mg Mycobacterium Tuberculosis (20 mg / ml Complete Freund's adjuvant H37 Ra).
5. Extract homogenate / adjuvant from tube by sucking it into a 10 ml syringe fitted with an 18 gauge emulsifying needle.

6. Emulsify between two 30 ml glass syringes until it becomes difficult to continue passing the material through the needle. (Approximately 5 minutes {there must be no separation between the oil phase and the aqueous phase}).
7. Use immediately or keep on ice until needed (not more than 30 min) (do not freeze).

5

Protocol

1. Female Lewis rats (Charles River) are given free access to food and water and should be acclimated a minimum of 3 days before use in experiments.
2. Rats weighing 160 and 220 grams are initially induced with 5% isoflurane (Aerrane, Fort Dodge), 30% O₂, 70% N₂O for 2-5 minutes.
3. The rat is then placed onto a circulating water heating blanket (Gaymar) (dorsal surface up) and into the nose cone for spontaneous respiration of anesthetic gases. The isoflurane is reduced to 2%.
4. Two subcutaneous injections (0.1 ml each) of either antigen or normal saline are made into ventral surface of the hind paws.
5. The animals are removed from the nose cone, weighed and numbered.
6. The rats are allowed to awake from anesthesia and are placed into individual cages.
7. The animals are observed daily for signs of EAE induction (see criteria below)

20 STAGE:0 NORMAL

STAGE 1 Abnormal gate and tail atony

STAGE 2 Mild but definite weakness of one or both hind legs

STAGE: 3 Severe weakness of one or both hind legs or mild ataxia

STAGE: 4 Severe paraparesis and minimal hind leg movement

25 STAGE: 5 No hind leg movement and paraplegia

STAGE: 6 Moribund state with no spontaneous movement and impaired respiration.

Increasing degree of front leg involvement and urinary and fecal incontinence may also occur

STAGE:7 DEATH

30 Treatment is begun on day 10 after immunization. Since the disease symptoms in this model typically appear 10-11 days after inoculation, this time point may be considered to represent the initial phase of an acute episode of MS. It is judged that this delay of the start of treatment mimics the clinical situation more closely than the traditionally used protocols

where drugs are administered at the time of, or even before, inoculation (Teitelbaum D. et al., Proc Natl Acad Sci USA 1999; 96: 3842-3847 and Brod S. A., et al., Ann Neurol 2000; 47: 127-131).

CLAIMS

What is claimed is:

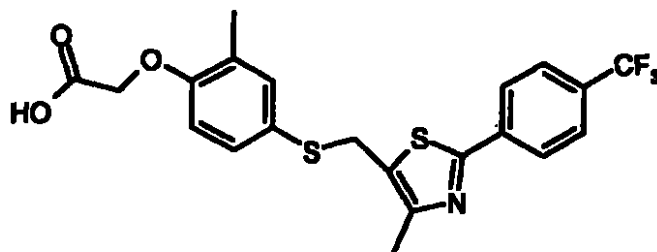
5 1. A method for the treatment of demyelinating diseases in a patient comprising the administration of a therapeutically effective amount of a hPPAR delta agonist.

2. The method according to claim 1 wherein the hPPAR delta agonist is a selective agonist.

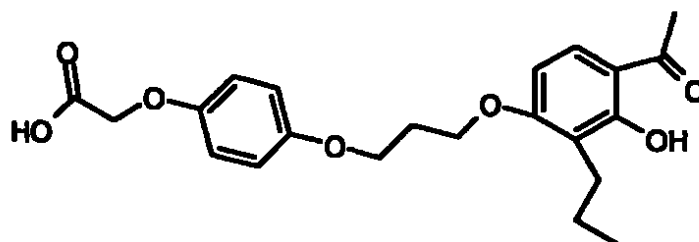
10 3. The method according to claim 1 wherein said demyelinating disease is selected from the group consisting of multiple sclerosis, Charcot-Marie-Tooth disease, Pelizaeus-Merzbacher disease, encephalomyelitis, neuromyelitis optica, adrenoleukodystrophy, Guillian-Barre syndrome and disorders in which myelin forming glial cells are damaged
15 including spinal cord injuries, neuropathies and nerve injury.

4. The method according to claim 3 wherein the demyelinating disease is multiple sclerosis.

20 5. The method according to claim 1 wherein the agonist is selected from the group consisting of compound of formula (1) and formula (2)

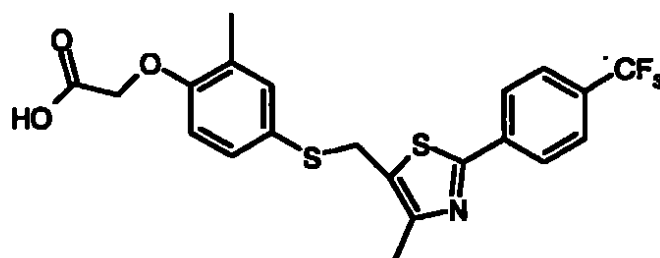


(1)

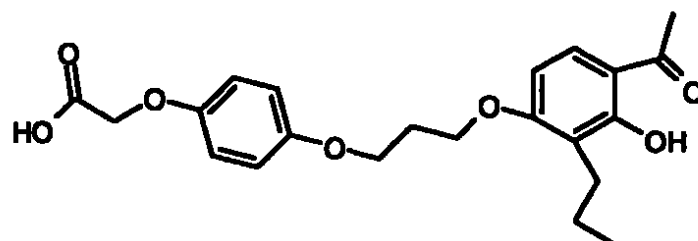


(2).

6. A pharmaceutical composition comprising a compound selected from the group consisting of compound of formula (1) and formula (2) in an amount effective for treating multiple sclerosis, Charcot-Marie-Tooth disease, Pelizaeus-Merzbacher disease, encephalomyelitis, neuromyelitis optica, adrenoleukodystrophy, Guillian-Barre syndrome and disorders in which myelin forming glial cells are damaged including spinal cord injuries, neuropathies and nerve injury in combination with at least on pharmaceutically acceptable carrier



(1)



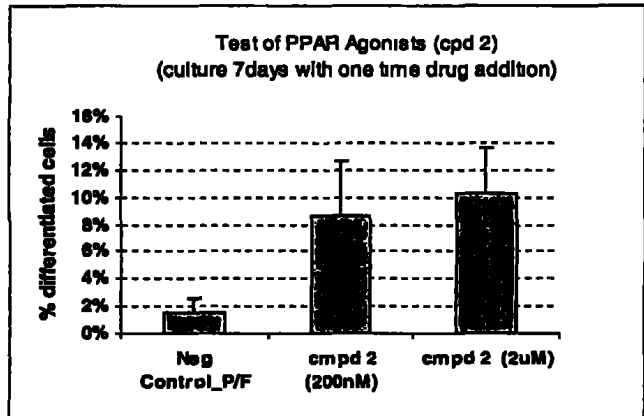
(2).

7. The pharmaceutical composition according to claim 6 comprising an amount effective for treating multiple sclerosis.

Figure 1 **Myelin Basic Protein (MBP) Immun reactivity Is Enhanced In cultured**
RAT Oligodendrocytes after Exposure to PPAR delta Agonists

Compound 1.
5-fold increase at 200 nM (7days)
10-fold increase at 2 μ M (7days)

Compound 2:
5-fold increase at 200 nM (7days)
6-fold increase at 2 μ M (7days)



*P+F = mitogens = 10 ng/ml PDGF-AA, 10 ng/ml bFGF

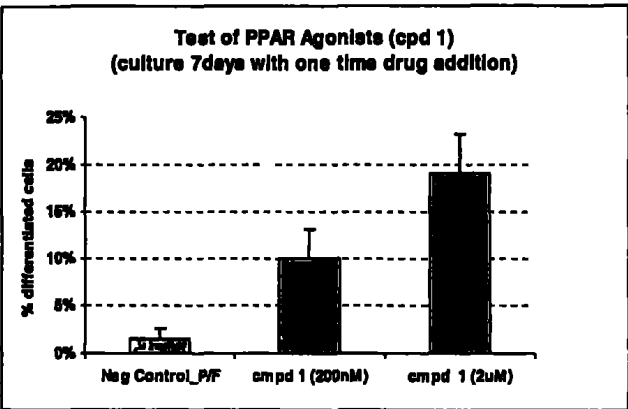
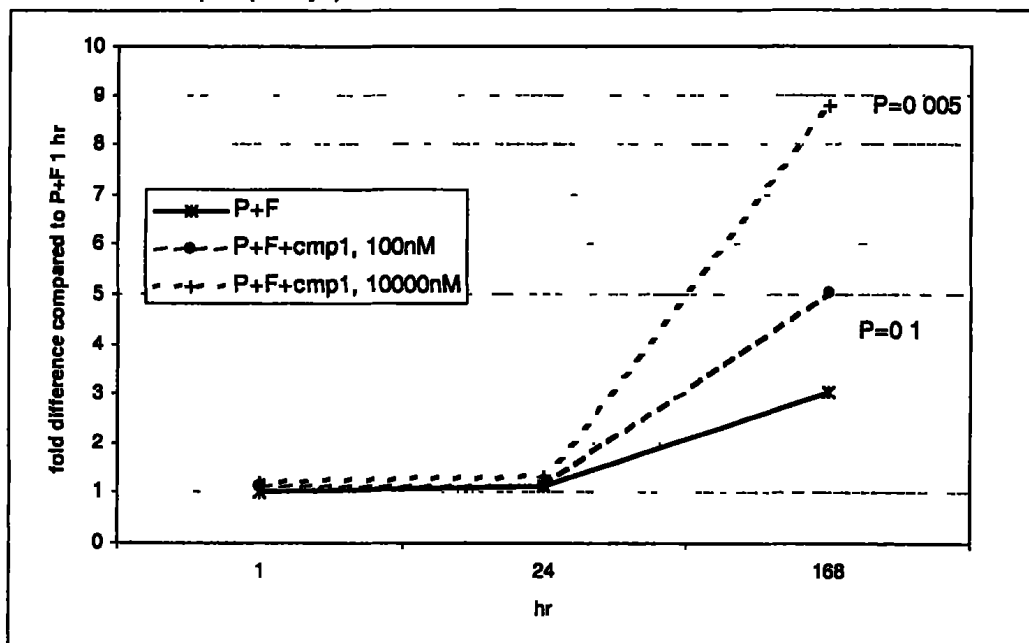


Figure 2 **Myelin Basic Protein (MBP) mRNA Is Enhanced in cultured**
RAT Oligodendrocytes after Exposure to PPAR delta Agonists

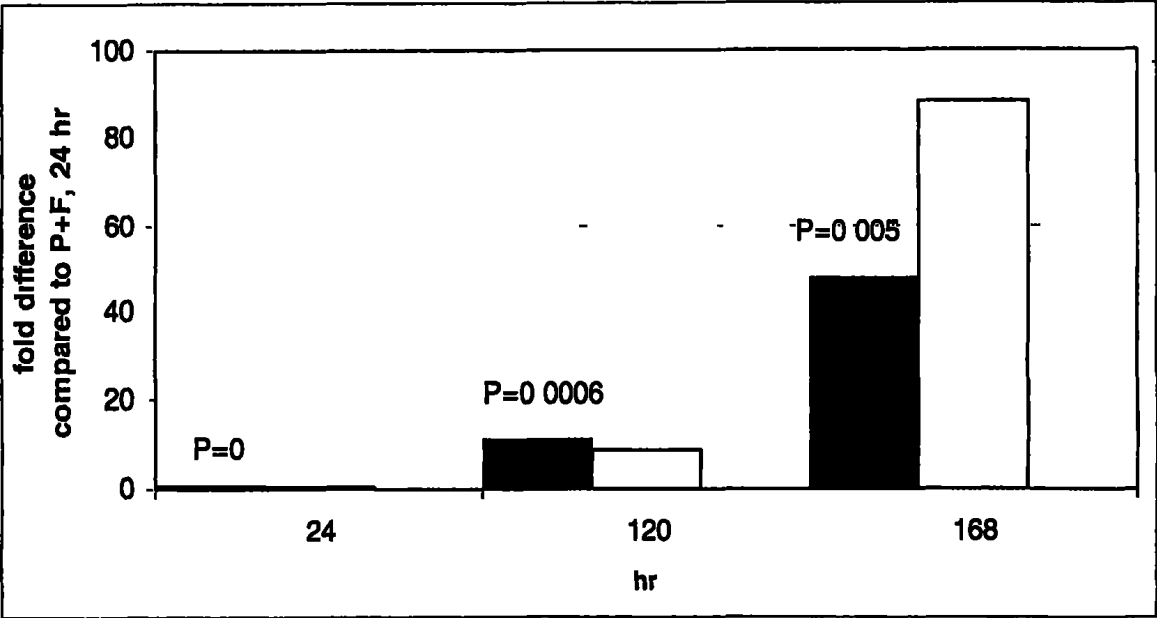
Compound 1.
2-fold increase at 100 nM (7 days)
3-fold increase at 10 μ M (7 days)



*P+F = mitogens = 10 ng/ml PDGF-AA, 10 ng/ml bFGF

Figure 3 **Myelin Basic Protein (MBP) mRNA Is Enhanced in cultured**
RAT Oligodendrocytes after Exposure to PPAR delta Agonists

Compound 2
2-fold increase at 200 nM (7 days)



*P+F = mitogens = 10 ng/ml PDGF-AA, 10 ng/ml bFGF,
Black rectangles, P+F, empty rectangles, P+F+cmpd2, 200 nM

Figure 4A

Transcripti nal Markers Confirm PPAR delta Pathway Activation
In Cultured RAT Oligodendro ytes after Exposure to Agonists

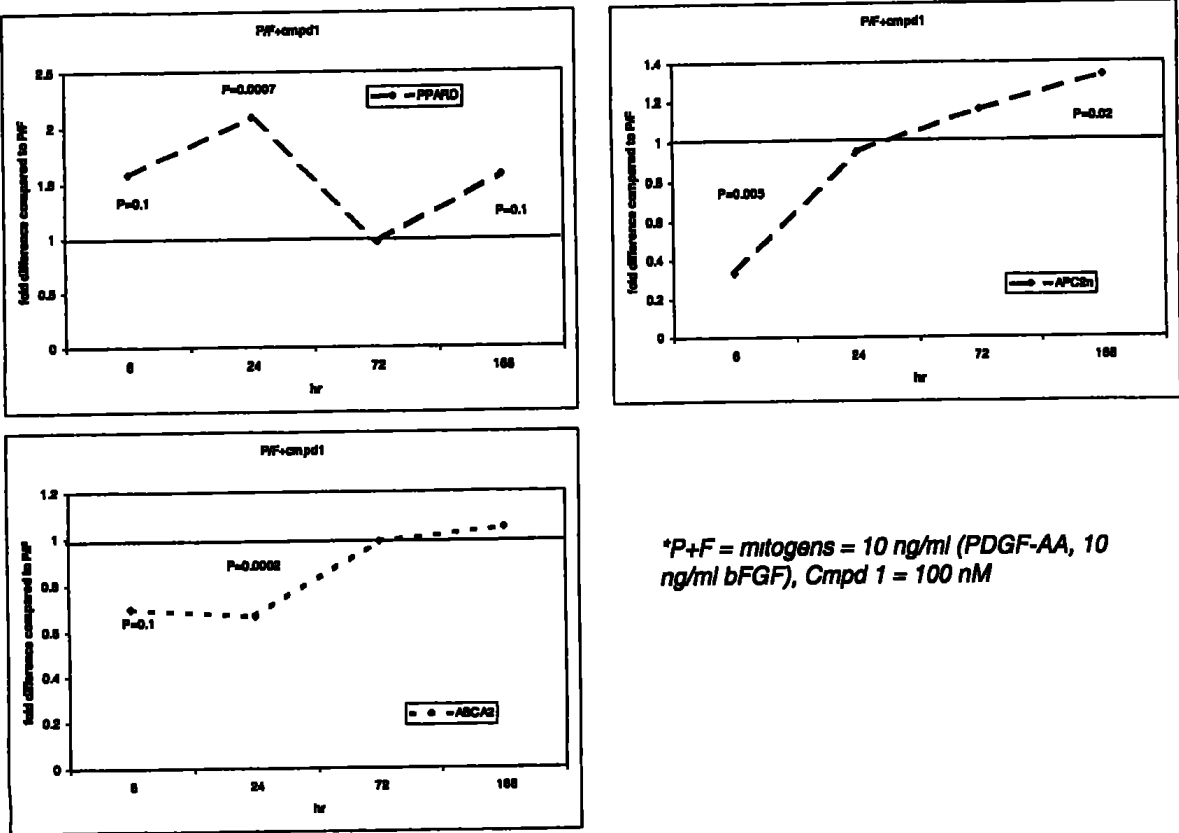
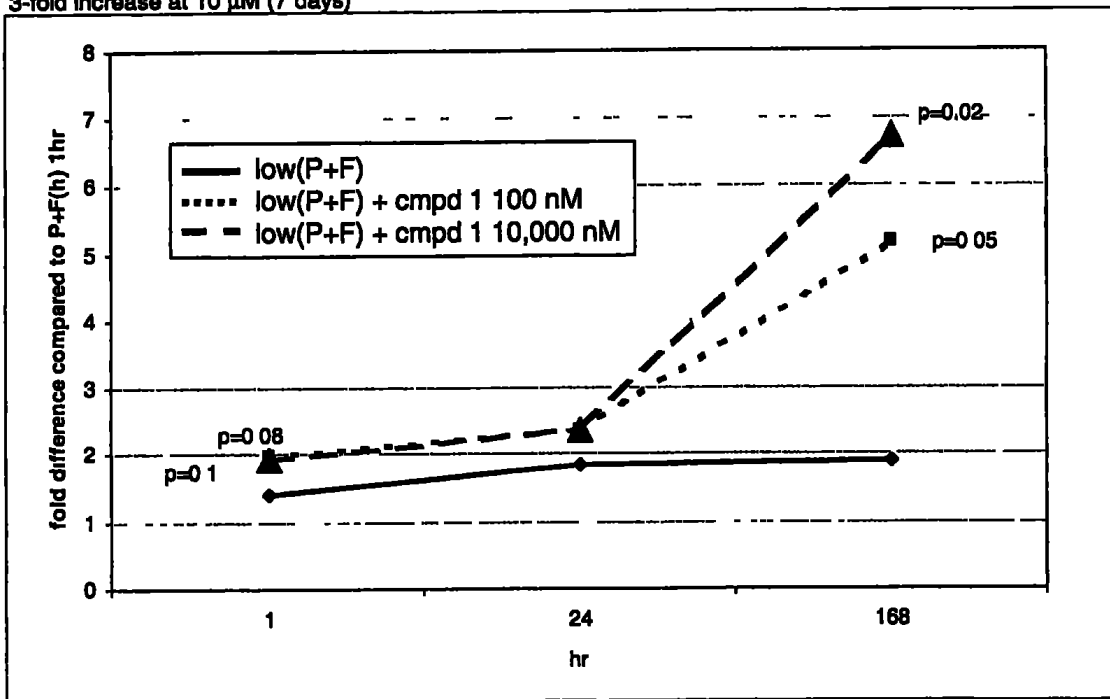


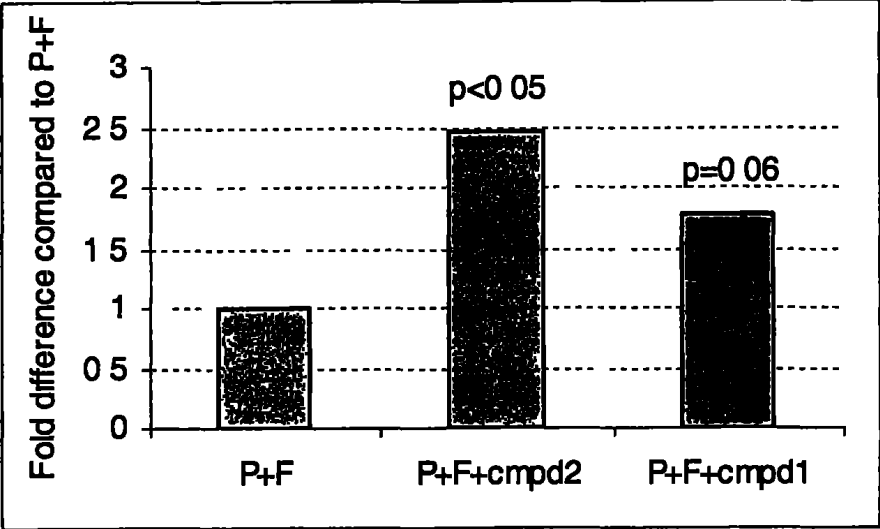
Figure 4B Transcriptional Markers Confirm PPAR delta Pathway Activation
ADRP (Adipose differentiation related protein) mRNA is upregulated in
Cultured RAT Oligodendrocytes after Exposure to Agonists

Compound 1
4-fold increase at 100 nM (7 days)
3-fold increase at 10 μ M (7 days)



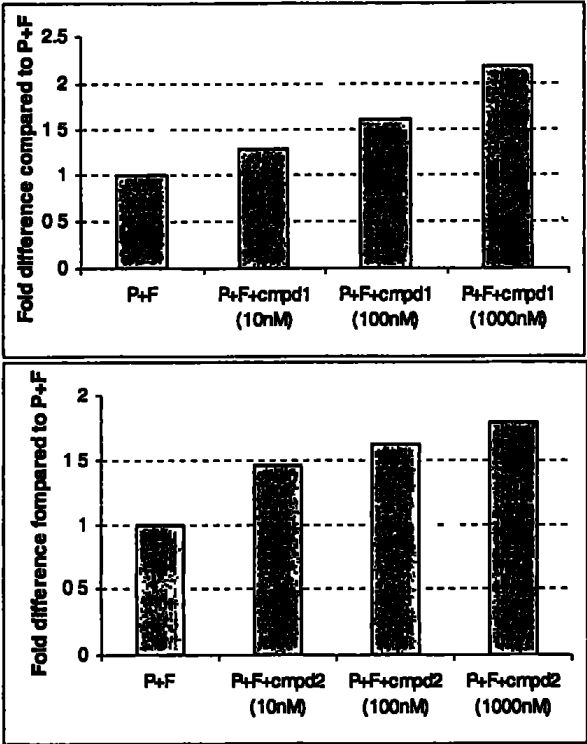
*P+F = mitogens = 1 ng/ml PDGF-AA, 1 ng/ml bFGF

Figure 5 PPAR delta Agonists Increase the Number of O4 Immunopositive Mature Cells (complex morphology) in Mixed Cultures of HUMAN Oligodendrocytes



Cultured for 8 days in the presence of P+F with or without compounds (200 nM) *P+F = mitogens = 10 ng/ml PDGF-AA, 10 ng/ml bFGF

Figure 6 PAR delta Agonists Increase the Number of O4 Immunopositive Mature Cells (complex morphology) in Mixed Cultures of HUMAN Oligodendrocytes



Cultured for 7 days with or without compounds (0 to 1000 nM)
*P+F = mitogens = 10 ng/ml PDGF-AA, 10 ng/ml bFGF

35

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- of inventorship (Rule 4.17(iv)) for US only

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For two-letter codes and other abbreviations, refer to the "Guid-
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(54) Title: USE OF PPAR DELTA AGONISTS FOR TREATING DEMYELINATING DISEASES

(57) Abstract: A method for treating demyelinating diseases in a patient in need thereof by treatment with an effective amount of a PPAR delta agonist is disclosed. Demyelinating diseases that may be effectively treated by this method include but are not limited to multiple sclerosis, Charcot-Marie-Tooth disease, Pelizaeus-Merzbacher disease, encephalomyelitis, neuromyelitis optica, adrenoleukodystrophy, Guillain-Barre syndrome and disorders in which myelin forming glial cells are damaged including spinal cord injuries, neuropathies and nerve injury.

WO 2005/097098 A3

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US2005/010371

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K31/192 A61K31/426 A61P25/02 A61P25/28

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B. FIELDS SEARCHED

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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, BIOSIS, EMBASE, BEILSTEIN Data, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 02/28434 A (GLAXO GROUP LIMITED; BUCHAN, KEVIN, WILLIAM) 11 April 2002 (2002-04-11) page 2, line 31 - page 3, line 3 page 7, lines 11,12; claims 1-8	1-7
X	WO 02/28433 A (GLAXO GROUP LIMITED; BUCHAN, KEVIN, WILLIAM) 11 April 2002 (2002-04-11) abstract page 3, lines 4-31 page 9, lines 1-26; example 2	1-7
-/--		

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

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US2005/010371

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
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X	WO 02/13812 A (PERSHADSINGH, HARRIHAR, A) 21 February 2002 (2002-02-21) abstract page 1, line 8 - page 2, line 5 page 7, line 18 - page 8, line 5 examples 9,10	1,3,4
X	WO 01/00603 A (GLAXO GROUP LIMITED; CHAO, ESTHER, YU-HSUAN; HAFFNER, CURT, DALE; LAMB) 4 January 2001 (2001-01-04) abstract; example 66	6,7
X	WO 97/28149 A (MERCK & CO., INC; LEIBOWITZ, MARK, D; BERGER, JOEL, P; MOLLER, DAVID,) 7 August 1997 (1997-08-07) abstract; example 7	6,7
X,P	WO 2004/093910 A (FUJISAWA PHARMACEUTICAL CO., LTD; KITA, YASUHIRO; YAMAZAKI, TAKAO; MUR) 4 November 2004 (2004-11-04) the whole document	1-7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2005/010371**Box II Observations where certain claims were found unsearchable (Continuation of Item 2 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. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Although claims 1-5 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
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 III Observations where unity of invention is lacking (Continuation of Item 3 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 SEARCH REPORT

Information on patent family members

International Application No

PCT/US2005/010371

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 0228434	A	11-04-2002	AU 9204601 A EP 1324774 A2 JP 2004510750 T US 2005131035 A1	15-04-2002 09-07-2003 08-04-2004 16-06-2005
WO 0228433	A	11-04-2002	AU 9204401 A EP 1324773 A2 JP 2004510749 T US 2004029938 A1	15-04-2002 09-07-2003 08-04-2004 12-02-2004
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WO 9728149	A	07-08-1997	AU 1856997 A	22-08-1997
WO 2004093910	A	04-11-2004	NONE	

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GR, HU, IE, IS, IT, LT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF,
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Publicada:

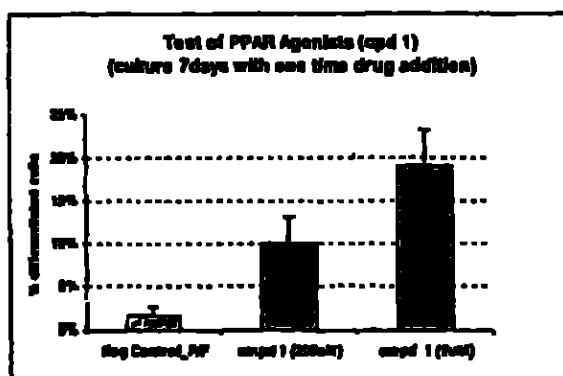
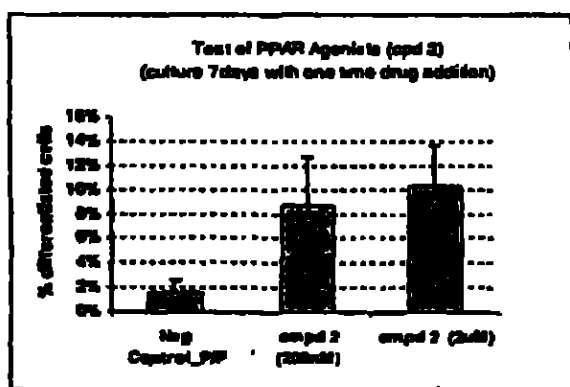
Con reporte de búsqueda internacional.

(54) Título: **USO DE AGONISTAS DEL RECEPTOR DELTA ACTIVADO POR EL PROLIFERADOR DE PEROXISOMA PARA EL TRATAMIENTO DE ESCLEROSIS MÚLTIPLE Y OTRAS ENFERMEDADES DE DESMIELINIZACIÓN.**

Myelin Basic Protein (MBP) Immunoreactivity is Enhanced in cultured RAT Oligodendrocytes after Exposure to PPAR delta Agonists

Compound 1:
5-fold increase at 200 nM (7days)
10-fold increase at 2 μ M (7days)

Compound 2:
5-fold increase at 200 nM (7days)
6-fold increase at 2 μ M (7days)



*P+F = mitogens = 10 ng/ml PDGF-AA, 10 ng/ml bFGF

(57) Resumen: Se describe un método para tratar enfermedades de desmielinización, en un paciente que necesita dicho tratamiento, con cantidad eficaz de un agonista del PPAR delta. Las enfermedades de desmielinización que se pueden tratar de forma eficaz con este método incluyen, aunque sin limitarse a ello, esclerosis múltiple, enfermedad de Charcot-Marie-Tooth, enfermedad de Pelizaeus-Merzbacher, encefalomiелitis, neuromielitis óptica, adrenoleucodistrofia, síndrome de Guillian-Barre y trastornos en los que están dañadas las células gliales formadoras de mielina, incluyendo lesiones de la médula espinal, neuropatías y lesiones nerviosas.

**USO DE AGONISTAS DEL RECEPTOR DELTA ACTIVADO POR EL
PROLIFERADOR DE PEROXISOMA PARA EL TRATAMIENTO DE
ESCLEROSIS MÚLTIPLE Y OTRAS ENFERMEDADES DE
DESMIELINIZACIÓN**

5 **Campo de la invención**

La presente invención se refiere al uso de agonistas de PPAR delta para el tratamiento de esclerosis múltiple (EM) y otras enfermedades de desmielinización. La presente invención también se refiere al uso de determinados
10 compuestos que son agonistas selectivos de PPAR delta para el tratamiento de EM y otras enfermedades de desmielinización.

ANTECEDENTES DE LA INVENCION

Los receptores activados por el proliferador de
15 peroxisoma (PPAR) comprenden una subfamilia de la superfamilia de los receptores nucleares. Se han identificado tres isoformas diferentes muy relacionadas que se han clonado y comúnmente se conocen como PPAR alfa, PPAR gamma y PPAR delta. Cada subtipo de receptor tiene un
20 dominio de unión de una secuencia de ADN (DBD) y un dominio de unión del ligando (LBD), ambos necesarios para la expresión génica activada por ligandos. Los PPAR se unen como heterodímeros con un receptor X retinoideo. Véanse J. Berger and D. E. Miller, *Ann. Rev. Med.*, 2002, 53, 409-435.

25 El PPAR delta (también conocido como PPAR beta) se expresa en una amplia gama de tejidos mamíferos, pero existe poca información sobre sus funciones biológicas o se ha puesto en claro muy poco sobre la gama completa de genes regulados por el receptor. No obstante, se ha descubierto
30 recientemente que los agonistas pueden ser útiles para tratar enfermedades como la dislipidemia y determinadas enfermedades dermatológicas, mientras que los antagonistas pueden ser útiles para tratar la osteoporosis o el cáncer

colorrectal (D. Sternbach, en *Annual Reports in Medicinal Chemistry*, Volumen 38, A. M. Doherty, ed., Elsevier Academic Press, 2003 pp. 71-80).

El PPARdelta parece expresarse significativamente en el SNC; sin embargo sigue sin descubrirse la mayor parte de su función en éste. Es de singular interés, no obstante, el descubrimiento de que el PPAR delta se expresaba en oligodendrocitos de roedores, las principales células productoras de lípidos del SNC (J. Granneman, et al., J. Neurosci. Res., 1998, 51, 563-573). Además, también se encontró que un agonista selectivo del PPAR delta aumentaba significativamente la expresión génica de mielina oligodendroglial y el diámetro de la vaina de mielina en cultivos de ratón (I. Saluja et al., *Glía*, 2001, 33, 194-204).

Los estados de desmielinización se ponen de manifiesto por la pérdida de mielina, las múltiples capas densas de lípidos y proteína que cubren muchas fibras nerviosas. Estas capas se proporcionan por la oligodendroglia en el sistema nervioso central (SNC), y las células Schwann en el sistema nervioso periférico (SNP). En la esclerosis múltiple (EM), los oligodendrocitos, las células formadoras de mielina en el SNC, son destruidas, y los axones son dañados, lo que provoca una actividad neuronal intensamente deteriorada y un déficit funcional, incluyendo plegia. En pacientes con estados de desmielinización, la desmielinización puede ser irreversible; normalmente va acompañada o seguida de degeneración axonal, y a menudo de degeneración celular. La desmielinización se puede producir como consecuencia de daño neuronal o daño a la propia mielina, sea debido a respuestas inmunitarias aberrantes, lesión local, isquemia, trastornos metabólicos, agentes tóxicos o infecciones víricas (Prineas and McDonald,

Demyelinating Diseases. In *Greenfield's Neuropathology*, 6.sup.th ed. (Edward Arnold: New York, 1997) 813-811, Beers and Berkow, eds., *The Merck Manual of Diagnosis and Therapy*, 17.sup.th ed. (Whitehouse Station, N.J.: Merck Research Laboratories, 1999) 1299, 1437, 1473-76, 1483). No obstante, las células progenitoras de oligodendrocitos están presentes en áreas de desmielinización, sugiriendo la posibilidad de una autorreparación, si se puede inducir a estas células a someterse a una diferenciación para madurar los oligodendrocitos.

La desmielinización central (desmielinización del SNC) se produce en diferentes estados, a menudo de etiología indeterminada, que se han conocido como las enfermedades de desmielinización primarias. De éstas, la esclerosis múltiple es la más prevalente. Otras enfermedades de desmielinización primarias incluyen adrenoleucodistrofia (ALD), adrenomieloneuropatía, mielopatía vacuolar asociada al sida, mielopatía asociada a HTLV, atrofia óptica hereditaria de Leber, leucoencefalopatía multifocal progresiva (LMP), panencefalitis esclerosante subaguda y paraparesia espástica tropical. Además, hay estados agudos en los que se puede producir la desmielinización en el SNC, p. ej., en la encefalomiелitis diseminada aguda (EMDA) y encefalitis vírica aguda. Además, la miелitis transversa aguda, que es un síndrome en el que una transección aguda de la médula espinal de causa desconocida afecta tanto a la materia gris como a la blanca en uno o más segmentos torácicos adyacentes, puede dar como resultado la desmielinización.

La EM es una enfermedad crónica, neurológica y devastadora que afecta en su mayoría a adultos jóvenes. La patogenia de la EM es un proceso complejo que conduce a la destrucción de la mielina y la oligodendroglia, como así

también a daño axonal en el cerebro y en la médula espinal (Prineas and McDonald, *Demyelinating Diseases*. In *Greenfield's Neuropathology*, 6.sup.th ed. (Edward Arnold: New York, 1997) 813-811, Trapp et al., *N. Engl. J. Med.*, 338:278-85, 1998). En términos histopatológicos, la EM se caracteriza por inflamación, placas desmielinización que infiltran las células del tejido del SNC, pérdida de oligodendroglia, y lesión axonal focal (Prineas and McDonald, *Demyelinating Diseases*. In *Greenfield's Neuropathology*, 6.sup.th ed. (Edward Arnold: New York, 1997) 813-811). Se cree que la enfermedad es consecuencia de respuestas inmunitarias aberrantes a la mielina, y posiblemente a los propios antígenos que no son mielina (Bar-Or et al., *J. Neuroimmunol.* 100:252-59, 1999, Hartung, H.-P., *Current Opinion in Neurology*, 8:191-99, 1995). Clínicamente, la EM puede seguir a una recidiva-recaída, o puede tomar un curso crónicamente progresivo con aumento de la discapacidad física (Gold et al., *Mol. Med. Today*, 6:88-91, 2000). Típicamente, los síntomas de la EM incluyen falta de coordinación, parestesias, trastornos del habla y la visión, y debilidad.

Los tratamientos actuales para los distintos estados de desmielinización por lo general son costosos, sintomáticos y solamente parcialmente eficaces, y pueden provocar efectos secundarios no deseados. Los corticoesteroides (prednisona oral en una dosis de 60-100 mg/día, disminuida progresivamente durante 2-3 semanas, o metilprednisolona intravenosa en una dosis de 500-1000 mg/día, durante 3-5 días) representan la forma principal de terapia para la EM. Si bien estas formas pueden acortar el período sintomático durante los ataques, no pueden modificar la discapacidad final en el largo plazo. El tratamiento con corticoesteroides a largo plazo muy pocas

veces se justifica y puede provocar diversas complicaciones
médicas que incluyen osteoporosis, úlceras y diabetes
(Beers and Berkow, eds., *The Merck Manual of Diagnosis and
Therapy*, 17.sup.th ed. (Whitehouse Station, N.J.: Merck
5 Research Laboratories, 1999) 1299, 1437, 1473-76, 1483).

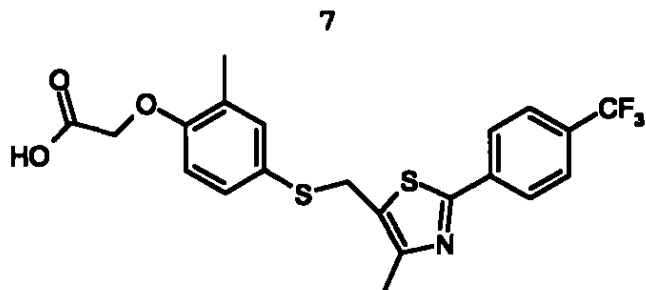
La terapia inmunomoduladora con interferón-.beta
humano recombinante. (Betaseron y Avonex) y con co-polímero
(Copaxon) reduce levemente la frecuencia de recidivas en la
EM y puede ayudar a demorar la discapacidad final (Beers
10 and Berkow, eds., *The Merck Manual of Diagnosis and
Therapy*, 17.sup.th ed. (Whitehouse Station, N.J.: Merck
Research Laboratories, 1999) 1299, 1437, 1473-76, 1483).
Ambas formas de interferón-.beta. y co-polímero se utilizan
actualmente como modalidades de tratamiento para la EM,
15 pero todas son excesivamente costosas. Los fármacos
inmunosupresores (azatioprina, cladribina, ciclofosfamida y
metotrexato) se utilizan para formas progresivas más
intensas. No obstante, no son uniformemente beneficiosos y
tienen efectos colaterales tóxicos importantes. Diversos
20 fármacos (p. ej., baclofen en una dosis de 30-60 mg/día en
administraciones separadas) pueden reducir la espasticidad,
inhibiendo los reflejos de la médula espinal. Se requiere
el uso cauteloso y juicioso, ya que la reducción de la
espasticidad inducida por el fármaco en pacientes con EM a
25 menudo exacerba la debilidad, incapacitando incluso más al
paciente.

De modo similar, el tratamiento actual para la ALD,
otra enfermedad de desmielinización devastadora, es
relativamente ineficaz. Los síntomas de la ALD pueden
30 incluir ceguera cortical, disfunción del tracto
corticoespinal, deterioro mental y espasticidad. La terapia
para controlar el curso de la ALD puede incluir el
trasplante de la médula ósea y el tratamiento con dieta

(DiBiase et al., *Ann. Ist. Super Sanita*, 35:185-92, 1999), pero invariablemente se produce un deterioro neurológico inexorable, que finalmente conduce a la muerte [Krivit et al., *Curr. Opin. Hematol.*, 6:377-82, 1999, (Beers and Berkow, eds., *The Merck Manual of Diagnosis and Therapy*, 17.sup.th ed. (Whitehouse Station, N.J.: Merck Research Laboratories, 1999) 1299, 1437, 1473-76, 1483). Se han producido ciertos avances en el tratamiento de animales con EAE y EAN, usando trasplantes de células gliales y factores de crecimiento, e inhibiendo las moléculas de adhesión, autoanticuerpos y citocinas (Njenga and Rodriguez, *Current Opinion in Neurology*, 9:159-64, 1996. No obstante, ninguno de estos tratamientos ha demostrado un beneficio para seres humanos, y algunos requieren una extensa intervención neuroquirúrgica. Por lo tanto, es claro que a partir de lo antedicho existe la necesidad de métodos más eficaces, más económicos y menos invasivos para tratar la amplia gama de estados de desmielinización, sin producir efectos secundarios no deseados.

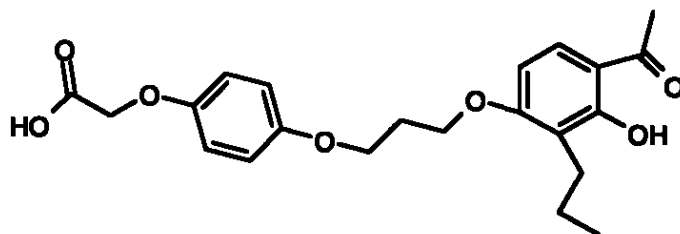
La presente invención abarca el uso de un planteamiento regenerativo activado por moléculas pequeñas para aumentar significativamente las terapias inmunomoduladoras actuales para el tratamiento de trastornos de desmielinización.

Los compuestos que se sabe son PPAR delta selectivos se conocen en la técnica, en particular, el compuesto de fórmula (1) en general conocido como GW 501516 descrito en el documento WO 01/00603.



(1)

El compuesto de fórmula (2), también conocido como L165,041, ha sido descrito en la solicitud de patente europea 28063 y en el documento W0 97/28149, donde fue
 5 identificado como un agonista selectivo de PPAR delta.



(2)

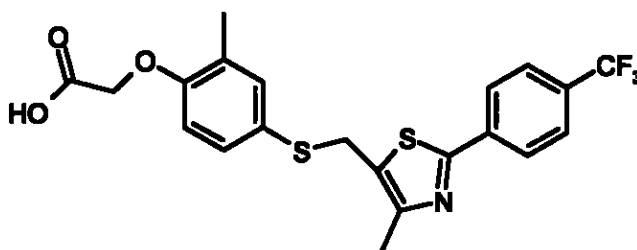
Debido a la capacidad potencial de los agonistas del
 10 Receptor Delta Activado por el Proliferador de Peroxisoma (PPAR delta) para acelerar la diferenciación de células progenitoras de oligodendrocitos agudamente aisladas del cerebro de roedores y para aumentar significativamente el diámetro de la vaina de mielina y la expresión génica de
 15 mielina, existe el potencial de que los agonistas de PPAR delta activen la vía del PPAR delta en células progenitoras de oligodendrocitos y aumenten la reparación neuronal, restaurando la vaina de mielina para los axones desmielinizados en la enfermedad de desmielinización,
 20 particularmente la EM.

SUMARIO DE LA INVENCION

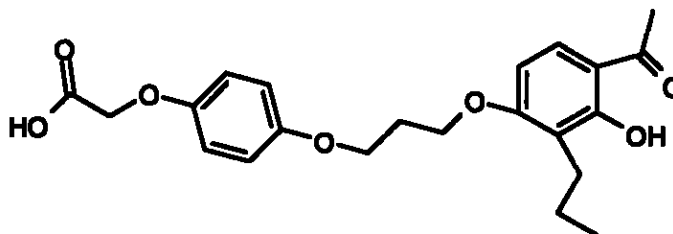
Por lo tanto, de acuerdo con la práctica de la presente invención, se provee un método para tratar estados de enfermedad desmielinizantes con agonistas del PPAR

delta, y en particular la esclerosis múltiple. En general, los estados de enfermedad que se pueden tratar de acuerdo con la práctica de la presente invención incluyen, aunque sin limitarse a ello, esclerosis múltiple, enfermedad de
5 Charcot-Marie-Tooth, enfermedad de Pelizaeus-Merzbacher, encefalomiелitis, neuromielitis óptica, adrenoleucodistrofia, síndrome de Guillian-Barre y trastornos en los que están dañadas las células gliales formadoras de mielina, incluyendo lesiones de la médula
10 espinal, neuropatías y lesiones nerviosas. Las enfermedades descritas en la presente memoria se pueden tratar administrando a un paciente que necesita dicho tratamiento, una cantidad terapéuticamente eficaz de un agonista de PPAR delta.

15 La presente invención también se refiere al uso de los compuestos de fórmula (I) y fórmula (II) para el tratamiento de enfermedades de desmielinización y, en particular, la esclerosis múltiple.



(1)



(2)

La presente invención también comprende un método para tratar la esclerosis múltiple en pacientes, administrando una combinación de un compuesto de fórmula (1) o fórmula (2) o su sal farmacéuticamente aceptable, con otro
5 compuesto conocido como eficaz para el tratamiento de la esclerosis múltiple en cantidades terapéuticamente eficaces. Los compuestos que actualmente se utilizan para tratar la enfermedad son agentes modificadores de la enfermedad tales como los interferones (interferón beta 1-
10 a, beta 1-b y alfa 2), acetato de glatirámero o corticoesteroides, tales como metilprednisolona y prednisona. Además, agentes quimioterapéuticos tales como metotrexato, azatioprina, cladribina ciclofosfamida y ciclosporina.

15 DESCRIPCIÓN DETALLADA DE LA INVENCION

Tal como se usa en esta memoria, la expresión "vehículo farmacéuticamente aceptable" significa un disolvente, dispersante, excipiente, adyuvante u otro material no tóxico, que se mezcla con el compuesto de la
20 presente invención, con el fin de permitir la formación de una composición farmacéutica, es decir, una forma de dosificación que se pueda administrar al paciente. Un ejemplo de dicho vehículo es un aceite farmacéuticamente aceptable que se utiliza de forma típica para la
25 administración parenteral.

El término "sales farmacéuticamente aceptables", tal como se usa en la presente memoria, significa que las sales de los compuestos de la presente invención pueden usarse en preparaciones medicinales. Sin embargo, pueden resultar
30 útiles otras sales en la preparación de los compuestos acordes con la invención, o de sus sales farmacéuticamente aceptables. Las sales farmacéuticamente aceptables de los compuestos de esta invención incluyen sales de adición de

ácido que se pueden formar, por ejemplo, mezclando una disolución del compuesto de acuerdo con la invención, con una disolución de un ácido farmacéuticamente aceptable tal como ácido clorhídrico, ácido bromhídrico, ácido sulfúrico, ácido metansulfónico, ácido 2-hidroxietanosulfónico, ácido p-toluensulfónico, ácido fumárico, ácido maleico, ácido hidroximaleico, ácido málico, ácido ascórbico, ácido succínico, ácido glutárico, ácido acético, ácido salicílico, ácido cinámico, ácido 2-fenoxiacético, ácido hidroxibenzoico, ácido fenilacético, ácido benzoico, ácido oxálico, ácido cítrico, ácido tartárico, ácido glicólico, ácido láctico, ácido pirúvico, ácido malónico, ácido carbónico o ácido fosfórico. También pueden formarse sales de metales ácidos, como monohidrógeno-ortofosfato de sodio e hidrógeno-sulfato de potasio. Además, las sales así formadas pueden estar presentes como sales de mono o di-ácido y pueden existir en forma hidratada o pueden ser sustancialmente anhidras. Además, cuando los compuestos de la invención tienen un resto ácido, sus sales farmacéuticamente aceptables adecuadas pueden incluir sales de metales alcalinos, por ejemplo sales de sodio o de potasio; sales de metal alcalinotérreo, por ejemplo, sales de calcio o magnesio; y sales formadas con ligandos orgánicos adecuados, por ejemplo, sales de amonio cuaternarias.

La expresión "cantidad terapéuticamente eficaz", tal como se usa en la presente memoria, significa una cantidad del compuesto, que es eficaz para tratar la enfermedad o el trastorno mencionado.

Tal como se emplea en esta memoria, la expresión "vehículo farmacéuticamente aceptable" significa un disolvente, dispersante, excipiente, adyuvante u otro material no tóxico, que se mezcla con el compuesto de la

presente invención, con el fin de permitir la formación de una composición farmacéutica, es decir, una forma de dosificación que se pueda administrar al paciente. Un ejemplo de dicho vehículo es un aceite farmacéuticamente
5 aceptable que se utiliza de forma típica para la administración parenteral.

La invención también proporciona composiciones farmacéuticas que comprenden uno o más de los compuestos de acuerdo con esta invención, junto con un vehículo
10 farmacéuticamente aceptable. Preferiblemente, estas composiciones están en formas de dosificaciones unitarias tales como comprimidos, píldoras, cápsulas, polvos, gránulos, disoluciones o suspensiones parenterales estériles, aerosoles o pulverizadores líquidos dosificados,
15 gotas, ampollas, dispositivos de autoinyección o supositorios; para la administración oral, parenteral, intranasal, sublingual o rectal, o para la administración por inhalación o insuflación. Alternativamente, las composiciones pueden presentarse en una forma adecuada para
20 la administración una vez semanal o una vez mensual; por ejemplo, una sal insoluble del compuesto activo, como la sal decanoato, puede adaptarse para proporcionar una preparación de depósito para la inyección intramuscular. Puede contemplarse un polímero erosionable que contiene el
25 ingrediente activo. Para preparar composiciones sólidas como comprimidos, el ingrediente activo principal se mezcla con un vehículo farmacéutico, p.ej. ingredientes convencionales para formar comprimidos, tal como almidón de maíz, lactosa, sacarosa, sorbitol, talco, ácido esteárico,
30 estearato de magnesio, fosfato de dicalcio o gomas, y otros diluyentes farmacéuticos, p.ej. agua, para formar una composición de preformulación sólida que contiene una mezcla homogénea de un compuesto de la presente invención,

o su sal farmacéuticamente aceptable. Cuando se indica que estas composiciones de preformulación son homogéneas, significa que el ingrediente activo está disperso de modo uniforme a través de la composición, de forma que la

5 composición puede subdividirse con facilidad en formas de dosificación unitarias igualmente eficaces, como comprimidos, píldoras y cápsulas. Esta composición de preformulación sólida se subdivide entonces en formas de dosificación unitarias del tipo descrito anteriormente, que

10 contienen desde 0,1 a aproximadamente 500 mg del ingrediente activo de la presente invención. Las formas de dosificación unitaria aromatizadas contienen desde 1 a 100 mg, por ejemplo 1, 2, 5, 10, 25, 50 ó 100 mg, del ingrediente activo. Los comprimidos o píldoras de la nueva

15 composición pueden revestirse o componerse de otro modo para proporcionar una forma de dosificación que permita la ventaja de una acción prolongada. Por ejemplo, el comprimido o píldora puede comprender un componente de dosificación interno y uno de dosificación externo, estando

20 éste último en forma de una envuelta sobre el primero. Los dos componentes pueden estar separados por una capa entérica, que actúa para resistir la disgregación en el estómago, y permite que el componente interno pase intacto hacia el duodeno, o sea liberado con retraso. Puede

25 utilizarse una diversidad de materiales para estas capas o revestimientos entéricos, incluyendo dichos materiales una serie de ácidos poliméricos y mezclas de ácidos poliméricos con materiales tales como goma de laca, alcohol cetílico y acetato de celulosa.

30 Las formas líquidas en las que las nuevas composiciones de esta memoria invención pueden incorporarse para su administración por vía oral o mediante inyección incluyen disoluciones acuosas, jarabes aromatizados de

forma apropiada, suspensiones acuosas u oleosas, y emulsiones aromatizadas con aceites comestibles tales como aceite de semilla de algodón, aceite de sésamo, aceite de coco o aceite de cacahuete, así como elixires y vehículos farmacéuticos similares. Los agentes dispersantes o suspensores adecuados para las suspensiones acuosas incluyen gomas sintéticas y naturales como tragacanto, goma arábiga, alginato, dextrano, carboximetilcelulosa de sodio, metilcelulosa, polivinilpirrolidona o gelatina.

10 En el tratamiento de los diversos estados patológicos como se describe en la presente memoria, un nivel de dosificación adecuado es desde aproximadamente 0,01 a 250 mg/kg diarios, preferiblemente desde aproximadamente 0,05 a 100 mg/kg diarios, y en especial desde aproximadamente 0,05
15 a 20 mg/kg diarios. Los compuestos pueden administrarse en un régimen de 1 a 4 veces diarias.

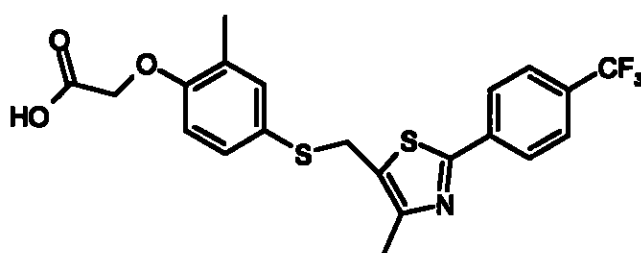
En un aspecto de la presente invención, se describe un método para tratar enfermedades de desmielinización en un paciente, que comprende la administración de una cantidad
20 terapéuticamente eficaz de un agonista de hPPAR delta.

En otro aspecto de la presente invención, el agonista de hPPAR delta es un agonista selectivo.

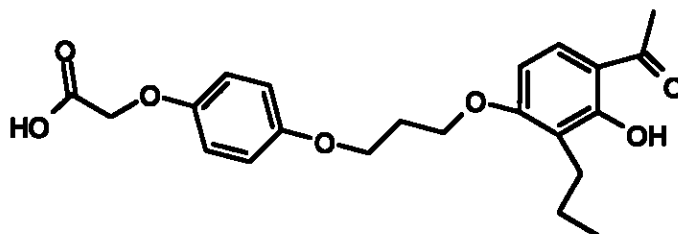
En otro aspecto de esta realización se describe un método en el que la enfermedad de desmielinización se
25 selecciona del grupo que consiste en esclerosis múltiple, enfermedad de Charcot-Marie-Tooth, enfermedad de Pelizaeus-Merzbacher, encefalomiелitis, neuromielitis óptica, adrenoleucodistrofia, síndrome de Guillian-Barre y trastornos en los que están dañadas las células gliales
30 formadoras de mielina, incluyendo lesiones de la médula espinal, neuropatías y lesiones nerviosas.

En otro aspecto de esta realización se describe un método en el que la enfermedad de desmielinización es la esclerosis múltiple.

Incluso en otro aspecto de esta realización se describe el método en el que el agonista se selecciona del grupo que consiste en el compuesto de fórmula (1) y fórmula (2)



(1)

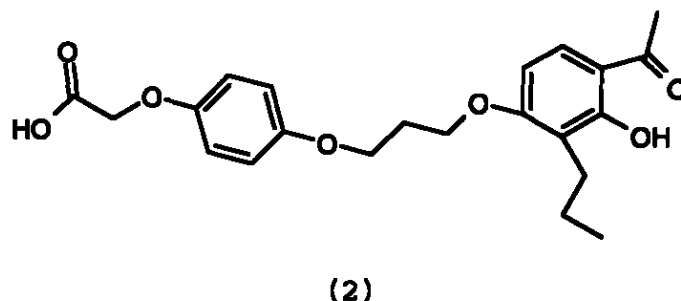
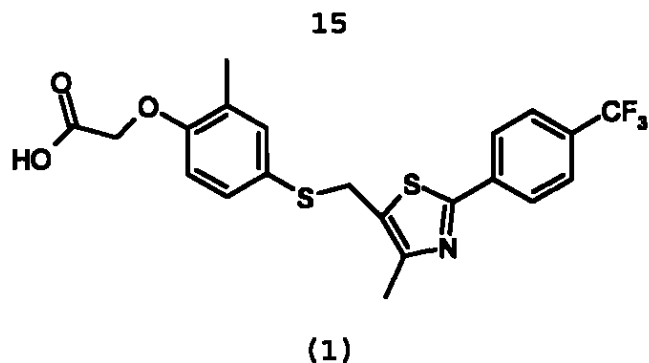


(2)

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Otra realización descrita en la presente invención consiste en una composición farmacéutica que comprende un compuesto seleccionado del grupo que consiste en el compuesto de fórmula (1) y fórmula (2) en una cantidad eficaz para tratar la esclerosis múltiple, enfermedad de Charcot-Marie-Tooth, enfermedad de Pelizaeus-Merzbacher, encefalomiелitis, neuromielitis óptica, adrenoleucodistrofia, síndrome de Guillian-Barre y trastornos en los que están dañadas las células gliales formadoras de mielina, incluyendo lesiones de la médula espinal, neuropatías y lesiones nerviosas, en combinación con por lo menos un vehículo farmacéuticamente aceptable

20



5 En otro aspecto de esta realización se describe una composición farmacéutica que comprende una cantidad eficaz para tratar la esclerosis múltiple.

BREVE DESCRIPCIÓN DE LOS DIBUJOS

10 Figura 1. Ilustra el aumento de inmunorreactividad de la proteína básica de mielina (MBP) en cultivos de oligodendrocitos de rata por los agonistas del PPAR delta.

 Figura 2. Este gráfico muestra el aumento de ARNm de MBP en cultivos de oligodendrocitos de rata por el compuesto 1.

15 Figura 3. Este gráfico muestra el aumento de ARNm de MBP en cultivos de oligodendrocitos de rata por el compuesto 2.

20 Figura 4A. Ilustra el efecto del compuesto 1 en marcadores transcripcionales que confirman la activación de la vía agonista de PPAR delta en cultivos de oligodendrocitos de rata.

 Figura 4B. Ilustra también el efecto del compuesto 1 en marcadores transcripcionales que confirman la activación de la vía agonista de PPAR delta en cultivos de

oligodendrocitos de rata, demostrando que el ARNm de ADRP aumenta en forma ascendente en cultivos de oligodendrocitos de rata.

Figura 5. Muestra el incremento en la cantidad de células inmunopositivas O4 en cultivos mixtos de oligodendrocitos humanos efectuado por el compuesto 1.

Figura 6. Muestra el incremento en la cantidad de células inmunopositivas O4 en cultivos mixtos de oligodendrocitos humanos efectuado por el compuesto 2.

Ejemplos de compuestos:

El compuesto de fórmula (1) (GW501516) se puede preparar tal como se publica en el documento WO 01/00603. El compuesto de fórmula (2) (L165,041) se puede preparar tal como se describe en el documento WO 97/28149.

Ejemplos biológicos:

Los siguientes protocolos de ensayo se usan para evaluar las propiedades biológicas de los compuestos de esta invención. Los siguientes ejemplos se presentan para ilustrar adicionalmente la invención. Sin embargo, no deben considerarse limitantes de la invención de ninguna manera.

Se evalúa la capacidad de los agonistas del PPAR delta de la presente invención en modelos *in vitro* e *in vivo* para promover la expresión de mielina y aumentar los procesos regenerativos.

El perfil de selectividad del receptor nuclear óptimo se determina mediante ensayos del indicador de luciferasa/GAL4. Un ensayo de células de roedores muestra la capacidad del compuesto para dirigir/acelerar la diferenciación de células progenitoras de oligodendrocitos cultivadas para madurar los oligodendrocitos.

Los ensayos biológicos específicos que sugieren eficacia en el tratamiento de la EM son desmielinización

inducida por lisolecitina y encefalomiелitis alérgica experimental en roedores.

Determinación de valores de CE50 de los agonistas de PPAR en el ensayo de PPAR delta celular

5 Principio

La potencia de las sustancias, que se unen al PPAR delta humano y lo activan en un modo agonista, se analiza usando una línea celular HEK transfectada estable (HEK-rifión embrionario humano) que aquí se denomina línea celular de indicador de PPAR delta. Contiene dos elementos genéticos, un elemento indicador de luciferasa (pdeltaM-GAL4-Luc-Zeo) y una proteína de fusión de PPAR delta (GR-GAL4-PPAR delta humano-LBD), que media en la expresión del elemento indicador de luciferasa que depende de un ligando del PPAR delta. La proteína de fusión GR-GAL4-PPAR delta humano-LBD expresada constitutiva y establemente, se une en el núcleo celular de la línea celular de indicador de PPAR delta por la parte de la proteína GAL4 a los patrones de unión del ADN de GAL4, 5' corriente arriba del elemento indicador de luciferasa que es integrado establemente en el genoma de la línea celular. En ausencia de un ligando de PPAR delta sólo hay una expresión débil del gen indicador de luciferasa, si en el ensayo se usa suero de ternero fetal con reducción de ácidos grasos (cs-FCS). Los ligandos del PPAR delta se unen y activan la proteína de fusión de PPAR delta, y así estimulan la expresión del gen indicador de luciferasa. La luciferasa que se forma se puede detectar por quimiluminiscencia mediante un sustrato adecuado.

20 Construcción de la línea celular de indicador de PPAR delta:

La producción de la línea celular de indicador de PPAR delta estable se basa en un clon de células HEK estables que se transfectó establemente con un elemento indicador de

luciferasa. Esta etapa ya fue descrita en la sección "construcción de la línea celular de indicador de PPAR alfa". En una segunda etapa, la proteína de fusión de PPAR delta (GR-GAL4-PPAR delta humano-LBD) se introdujo establemente en este clon celular. Para este propósito, el ADNc que codifica los 76 aminoácidos N-terminales del receptor de glucocorticoides (n° de acceso # P04150) se unió a la sección de ADNc que codifica los aminoácidos 1-147 del factor de transcripción de levadura GAL4 (n° de acceso # P04386). El ADNc del dominio de unión del ligando del receptor PPAR delta humano (aminoácidos S139-Y441 n° de acceso # L07592) se clonó en el extremo 3' de esta construcción GR-GAL4. La construcción de fusión preparada de esta forma (GR-GAL4-PPAR delta humano-LBD) se volvió a clonar en el plásmido pcDNA3 (Invitrogen) con el fin de permitir la expresión constitutiva en él mediante el promotor de citomegalovirus. Este plásmido se linealizó con una endonucleasa de restricción y se transfectó establemente en el clon celular previamente descrito que contenía el elemento indicador de luciferasa. La línea celular del receptor PPAR delta acabada que contiene un elemento indicador de luciferasa y expresa constitutivamente la proteína de fusión de PPAR delta (GR-GAL4-PPAR delta-humano-LBD) se aisló por selección con zeocina (0,5 mg/ml) y G418 (0,5 mg/ml).

Procedimiento de ensayo y evaluación:

La actividad de los agonistas de PPAR delta se determina en un ensayo de 3 días, que se describe a continuación:

30 Día 1

La línea celular de indicador de PPAR delta se cultiva hasta 80% de confluencia en DMEM (#° 41965-039, Invitrogen) que se mezcla con las siguientes adiciones: cs-FCS al 10%

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(suero de ternero fetal; #SH-30068.03, Hyclone), 0,5 mg/ml zeocina (#R250-01, Invitrogen), 0,5 mg/ml G418 (#10131-027, Invitrogen), disolución al 1% penicilina-estreptomicina (#15140-122, Invitrogen) y L-glutamina 2 mM (#25030-024, Invitrogen). El cultivo se lleva a cabo en botellas de cultivo celular patrón (# 353112, Becton Dickinson) en un incubador de cultivo celular a 37°C en presencia de CO₂ al 5%. Las células 80% confluentes se lavan una vez con 15 ml de PBS (#14190-094, Invitrogen), se tratan con 3 ml de disolución de tripsina (#25300-054, Invitrogen) a 37°C durante 2 min, se absorben en 5 ml del DMEM descrito y se cuentan en un contador de células. Después de diluir a 500.000 células/ ml, se siembran 35.000 células en cada pocillo de una placa de microvaloración de 96 pocillos con una base de plástico transparente (n° 3610, Corning Costar). Las placas se incuban en el incubador de cultivo celular a 37°C y con CO₂ al 5% durante 24 h.

Día 2

Los agonistas de PPAR delta que se van a ensayar se disuelven en DMSO con una concentración 10 mM. Esta disolución madre se diluye en DMEM (#41965-039, Invitrogen) que se mezcla con 5% cs-FCS (#SH-30068.03, Hyclone), L-glutamina 2 mM (#25030-024, Invitrogen) y los antibióticos previamente descritos (zeocina, G418, penicilina y estreptomicina).

Las sustancias de ensayo se ensayan con 11 concentraciones diferentes en el intervalo de 10 µM a 100 pM. Los compuestos más potentes se ensayan con concentraciones en el intervalo de 1 µM a 10 pM o entre 100 nM y 1 pM.

El medio de la línea de células con el indicador de PPAR delta sembrada el día 1 se elimina completamente por aspiración, y a las células se les añaden inmediatamente

las sustancias de ensayo diluidas en medio. La dilución y adición de las sustancias se lleva a cabo mediante un robot (Beckman FX). El volumen final de las sustancias de ensayo diluidas en medio es 100 μ l por pocillo de una placa de microvaloración de 96 pocillos. La concentración de DMSO en el ensayo es menor que 0,1% en vol/vol con el fin de evitar efectos citotóxicos del disolvente.

Cada placa se cargó con un agonista de PPAR delta patrón, que se diluyó igualmente en 11 concentraciones diferentes, con el fin de demostrar el funcionamiento del ensayo en cada placa individual. Las placas de ensayo se incuban en un incubador a 37°C y CO₂ al 5% durante 24 h.

Día 3

Las células con el indicador de PPAR delta tratadas con las sustancias de ensayo se sacan del incubador y se aspira el medio. Las células se lisan pipeteando 50 μ l de reactivo Bright Glo (de Promega) en cada pocillo de una placa de microvaloración de 96 pocillos. Después de incubar a temperatura ambiente en la oscuridad durante 10 minutos, las placas de microvaloración se miden en el luminómetro (Trilux de Wallac). El tiempo de medición para cada pocillo de la placa de microvaloración es 1 segundo.

Evaluación:

Los datos sin procesar del luminómetro se transfieren a un fichero de Microsoft Excel. Se calculan las gráficas del efecto de la dosis y los valores CE50 de los agonistas del PPAR, usando el programa XL.Fit como especifica el fabricante (IDBS).

Determinación de valores de CE₅₀ de los agonistas de PPAR en el ensayo de PPAR alfa celular

Principio

La potencia de las sustancias, que se unen al PPAR alfa humano y lo activan en un modo agonista, se analiza

usando una línea celular HEK transfectada estable (HEK=riñón embrionario humano) que aquí se denomina línea celular de indicador de PPAR alfa. Contiene dos elementos genéticos, un elemento indicador de luciferasa (pdeltaM-GAL4-Luc-Zeo) y una proteína de fusión de PPAR alfa (GR-GAL4-PPAR alfa humano-LBD), que media en la expresión del elemento indicador de luciferasa que depende de un ligando del PPAR alfa. La proteína de fusión GR-GAL4-PPAR alfa humano-LBD expresada constitutiva y establemente, se une en el núcleo celular de la línea celular de indicador de PPAR alfa por la parte de la proteína GAL4 a los patrones de unión del ADN de GAL4, 5' corriente arriba del elemento indicador de luciferasa que es integrado establemente en el genoma de la línea celular. En ausencia de un ligando de PPAR alfa sólo hay una expresión débil del gen indicador de luciferasa, si en el ensayo se usa suero de ternero fetal con reducción de ácidos grasos (cs-FCS). Los ligandos del PPAR alfa se unen y activan la proteína de fusión de PPAR delta, y así estimulan la expresión del gen indicador de luciferasa. La luciferasa que se forma se puede detectar por quimiluminiscencia mediante un sustrato adecuado.

Construcción de la línea celular de indicador de PPAR alfa:

La línea celular de indicador de PPAR alfa se preparó en dos etapas. Primero, se construyó el elemento indicador de luciferasa y se transfectó establemente en células HEK. Para este propósito, se clonaron cinco sitios de unión del factor de transcripción de levadura GAL4 (n° de acceso # AF264724) 5' corriente arriba de un promotor mínimo de MMTV de 68 pb de longitud (n° de acceso # V01175). La sección promotora mínima de MMTV contiene una caja CCAAT y un elemento TATA con el fin de permitir una transcripción eficaz por la RNA-polimerasa II. La clonación y

secuenciación de la construcción de GAL4-MMTV se produce de forma análoga a la descrita por Sambrook J. et. al. (Molecular cloning, Cold Spring Harbor Laboratory Press, 1989). Después se clonó el gen de *Photinus pyralis* (n° de acceso # M15077) 3' corriente abajo del elemento GAL4-MMTV. Después de la secuenciación, el elemento indicador de luciferasa que consistía en cinco sitios de unión de GAL4, promotor de MMTV y gen de luciferasa, se volvió a clonar en un plásmido que confiere resistencia a la zeocina con el fin de obtener el plásmido pdeltaM-GAL4-Luc-Zeo. Este vector se transfectó en células HEK de acuerdo con lo expuesto por Ausubel, F.M. et al. (Current protocols in molecular biology, Vol. 1-3, John Wiley & Sons, Inc., 1995). Después se utilizó medio que contenía zeocina (0,5 mg/ml) para seleccionar un clon de célula estable adecuado que presentó muy poca expresión basal del gen de luciferasa.

En una segunda etapa, la proteína de fusión de PPAR alfa (GR-GAL4-PPAR alfa humano-LBD) se introdujo establemente en el clon descrito. Para este propósito, inicialmente el ADNc que codifica los 76 aminoácidos N-terminales del receptor de glucocorticoides (n° de acceso # P04150) se unió a la sección de ADNc que codifica los aminoácidos 1-147 del factor de transcripción de levadura GAL4 (n° de acceso # P04386). El ADNc del dominio de unión del ligando del receptor PPAR alfa humano (aminoácidos S167-Y468; n° de acceso # S74349) se clonó en el extremo 3' de esta construcción GR-GAL4. La construcción de fusión preparada de esta forma (GR-GAL4-PPAR alfa humano-LBD) se volvió a clonar en el plásmido pcDNA3 (Invitrogen) con el fin de permitir la expresión constitutiva en él mediante el promotor de citomegalovirus. Este plásmido se linealizó con una endonucleasa de restricción y se transfectó

establemente en el clon celular previamente descrito que contenía el elemento indicador de luciferasa. La línea celular del receptor PPAR alfa acabada que contiene un elemento indicador de luciferasa y expresa
5 constitutivamente la proteína de fusión de PPAR alfa (GR-GAL4-PPAR alfa-humano-LBD) se aisló por selección con zeocina (0,5 mg/ml) y G418 (0,5 mg/ml).

Procedimiento de ensayo:

La actividad de los agonistas de PPAR alfa se
10 determina en un ensayo de 3 días, que se describe a continuación:

Día 1

La línea celular de indicador de PPAR alfa se cultiva hasta 80% de confluencia en DMEM (# 41965-039, Invitrogen)
15 que se mezcla con las siguientes adiciones: cs-FCS al 10% (suero de ternero fetal; #SH-30068.03, Hyclone), 0,5 mg/ml zeocina (#R250-01, Invitrogen), 0,5 mg/ml G418 (#10131-027, Invitrogen), disolución al 1% penicilina-estreptomina (#15140-122, Invitrogen) y L-glutamina 2 mM (#25030-024,
20 Invitrogen). El cultivo se lleva a cabo en frascos de cultivo celular patrón (# 353112, Becton Dickinson) en un incubador de cultivo celular a 37°C en presencia de CO₂ al 5%. Las células 80% confluentes se lavan una vez con 15 ml de PBS (#14190-094, Invitrogen), se tratan con 3 ml de
25 disolución de tripsina (#25300-054, Invitrogen) a 37°C durante 2 min, se absorben en 5 ml del DMEM descrito y se cuentan en un contador de células. Después de diluir a 500.000 células/ ml, se siembran 35.000 células en cada pocillo de una placa de microvaloración de 96 pocillos con
30 una base de plástico transparente (n° 3610, Corning Costar). Las placas se incuban en el incubador de cultivo celular a 37°C y con CO₂ al 5% durante 24 h.

Día 2

Los agonistas de PPAR alfa que se van a ensayar se disuelven en DMSO con una concentración 10 mM. Esta disolución madre se diluye en DMEM (#41965-039, Invitrogen) que se mezcla con 5% cs-FCS (#SH-30068.03, Hyclone), L-glutamina 2 mM (#25030-024, Invitrogen) y los antibióticos previamente descritos (zeocina, G418, penicilina y estreptomicina).

Las sustancias de ensayo se ensayan con 11 concentraciones diferentes en el intervalo de 10 μ M a 100 pM. Los compuestos más potentes se ensayan con concentraciones en el intervalo de 1 μ M a 10 pM o entre 100 nM y 1 pM.

El medio de la línea de células con el indicador de PPAR alfa sembrada el día 1 se elimina completamente por aspiración, y a las células se les añaden inmediatamente las sustancias de ensayo diluidas en medio. La dilución y adición de las sustancias se lleva a cabo mediante un robot (Beckman FX). El volumen final de las sustancias de ensayo diluidas en medio es 100 μ l por pocillo de una placa de microvaloración de 96 pocillos. La concentración de DMSO en el ensayo es menor que 0,1% en vol/vol con el fin de evitar efectos citotóxicos del disolvente.

Cada placa se cargó con un agonista de PPAR alfa patrón, que se diluyó igualmente en 11 concentraciones diferentes, con el fin de demostrar el funcionamiento del ensayo en cada placa individual. Las placas de ensayo se incuban en un incubador a 37°C y CO₂ al 5% durante 24 h.

Día 3

Las células con el indicador de PPAR alfa tratadas con las sustancias de ensayo se sacan del incubador y se aspira el medio. Las células se lisan pipeteando 50 μ l de reactivo Bright Glo (de Promega) en cada pocillo de una placa de

microvaloración de 96 pocillos. Después de incubar a temperatura ambiente en la oscuridad durante 10 minutos, las placas de microvaloración se miden en el luminómetro (Trilux de Wallac). El tiempo de medición para cada pocillo de la placa de microvaloración es 1 segundo.

Evaluación:

Los datos sin procesar del luminómetro se transfieren a un fichero de Microsoft Excel. Se calculan las gráficas del efecto de la dosis y los valores CE50 de los agonistas del PPAR, usando el programa XL.Fit como especifica el fabricante (IDBS).

Determinación de valores de CE50 de los agonistas de PPAR en el ensayo de PPAR gamma celular

Protocolo de ensayo de PPAR gamma celular

Para realizar los ensayos celulares se lleva a cabo un ensayo de luciferasa en placas de 96 pocillos de la siguiente manera:

Día 1: Preparación de las células en las placas:

- Las células lavadas se desarrollan hasta 80-90% de confluencia una vez en PBS
- Se las sumerge en tripsina durante 2 min
- Se añaden 15 ml de medio de ensayo (DMEM, Invitrogen, Cat.No. 41965-039; 5% FBS tratado con Dextrano/Carbón, Hyclone, Cat.No. SH30068; 0,5 mg/ml Zeocina, Invitrogen, Cat.No. 46-0072; 0,5mg/ml Geneticina, Invitrogen, Cat.No. 10131-027; 1% Penicilina/Estreptomicina, Invitrogen, Cat.No. 15140-122; L-Glutamina 2 mM, Invitrogen, Cat.No. 25030-024; 7,5 µg/ml Blastocidin S HCl, Invitrogen, Cat.No. R210-01; 1 µg/ml Doxiciclina, Clontech, Cat.No. 8634-1)
- Se cuentan las células
- Se diluyen las células en medio de ensayo hasta 500.000 células/ml

- Se dispensan 100 μ l de suspensión celular por pocillo en placas Corning de fondo claro (se producen 50.000 células/pocillo)

- Se incuba durante 24 h a 37°C, 5% CO₂

5 Día 2: Administración de los compuestos de ensayo:

- Se disuelven los compuestos de ensayo en DMSO para formar una disolución madre 10 mM

- Se diluye el compuesto hasta una concentración adecuada en el medio de ensayo (DMEM, Invitrogen, Cat.No. 41965-039; 5% FBS tratado con Dextrano/Carbón, Hyclone, Cat.No. SH30068; 0,5 mg/ml Zeocina, Invitrogen, Cat.No. 46-0072; 0,5mg/ml Geneticina, Invitrogen, Cat.No. 10131-027; 1% Penicilina/Estreptomicina, Invitrogen, Cat.No. 15140-122; L-Glutamina 2mM, Invitrogen, Cat.No. 25030-024; 7,5 μ g/ml Blasticidin S HCl, Invitrogen, Cat.No. R210-01; 1 μ g/ml Doxiciclina, Clontech, Cat.No. 8634-1) (El FCS regular hospeda ácidos grasos libres que interfieren con los dominios de unión del ligando del PPAR).

- Se aspira el medio (las células son bastante sensibles en esta etapa; es necesario asegurarse que las células no pasen más de 1 min sin estar cubiertas por el medio)

- Se transfieren los compuestos a 96 pocillos (100 μ l de medio que incluye el compuesto)

- Se efectúan los controles con el compuesto patrón (p. ej., Rosiglitazon) como también con un control DMSO (0,1% DMSO)

- Las células se incuban durante 24 h a 37°C a CO₂ al 5%.

Las etapas de dilución y adición de los compuestos diluidos se realizan usando un robot Beckman Biomek 2000 o Beckman FX robot.

Día 3' Lisis celular y medición de la actividad de luciferasa:

- Se aspira el medio de las células
- Se congelan las placas a -20°C (opcional)
- 5 • Se descongelan las placas durante 30 min (si es necesario)
- Se añaden 50 µl de Reactivo de Ensayo Bright-Glo-Luciferasa (Promega, Cat.No. E2650)
- Se incuba durante 10 min en la oscuridad
- 10 • Se mide la luminiscencia 2 seg por pocillo (Wallac Microbeta)

Análisis de datos:

La determinación de los valores de CE50 se realiza con Microsoft Exel combinado con XLFit (desarrollado por IDBS),
15 usando el algoritmo de ajuste #205.

Determinación de los valores de CE50 en el ensayo del receptor RXR humano celular

Protocolo de ensayo de RXR celular

Para realizar los ensayos celulares se lleva a cabo un
20 ensayo de luciferasa en placas de 96 pocillos de la siguiente manera:

Día 1: Preparación de las células en las placas

- Las células lavadas se desarrollan hasta 80-90% de confluencia una vez en PBS
- 25 • Se las sumerge en tripsina durante 2 min
- Se añaden 15 ml de medio de cultivo (DMEM, Invitrogen, Cat.No. 41965-039; 10% FBS tratado con Dextrano/Carbón, Hyclone, Cat.No. SH30068; 0,5 mg/ml Zeocina, Invitrogen, Cat.No. 46-0072; 0,5mg/ml Geneticina, Invitrogen, Cat.No. 10131-027; 1% Penicilina/Estreptomicina, Invitrogen, Cat.No. 15140-122; L-Glutamina 2mM, Invitrogen, Cat.No. 25030-024)
- 30

- Se cuentan las células
- Se diluyen las células en medio de ensayo hasta 175.000 células/ml
- Se dispensan 200 μ l de suspensión celular por pocillo
5 en placas Corning de fondo claro (se producen 35.000 células/pocillo)
- Se incuba durante 24 h a 37°C, 5% CO₂

Día 2: Administración de los compuestos de ensayo

- Se disuelven los compuestos de ensayo en DMSO para
10 formar una disolución madre 10 mM
- Se diluye el compuesto hasta una concentración adecuada en el medio de ensayo (DMEM sin rojo fenol, Invitrogen, Cat.No. 21063-029; 5% FBS tratado con Dextrano/Carbón, Hyclone, Cat.No. SH30068; 0,5 mg/ml
15 Zeocina, Invitrogen, Cat.No. 46-0072; 0,5mg/ml Geneticina, Invitrogen, Cat.No. 10131-027; 1% Penicilina/Estreptomina, Invitrogen, Cat.No. 15140-122; L-Glutamina 2mM, Invitrogen, Cat.No. 25030-024) (El FCS regular hospeda ácidos grasos libres que interfieren con
20 los dominios de unión del ligando del PPAR).
- Se aspira el medio (las células son bastante sensibles en esta etapa; es necesario asegurarse que las células no pasen más de 1 min sin estar cubiertas por el medio)
- Se transfieren los compuestos a 96 pocillos (100 μ l de
25 medio que incluye el compuesto)
- Se efectúan los controles con el compuesto patrón (p. ej., RPR258134) como también con un control DMSO (0,1% DMSO)
- Las células se incuban durante 24 h a 37°C, CO₂ al 5%.
- 30 Las etapas de dilución y adición de los compuestos diluidos se realizan usando un robot Beckman Biomek 2000 o Beckman FX robot.

Día 3: Lisis celular y medición de la actividad de luciferasa

- Se aspira el medio de las células
- Se congelan las placas a -20°C (opcional)
- 5 • Se descongelan las placas durante 30 min (si es necesario)
- Se añaden 50 µl de Reactivo de Ensayo Bright-Glo-Luciferasa (Promega, Cat.No. E2650)
- Se incuba durante 10 min en la oscuridad
- 10 • Se mide la luminiscencia 2 seg por pocillo (Wallac Microbeta)

Análisis de datos

La determinación de los valores de CE50 se realiza con Microsoft Exel combinado con XLFit (desarrollado por IDBS),
15 usando el algoritmo de ajuste #205.

La Tabla 1 muestra los resultados de los ensayos del indicador. Los resultados demuestran que los compuestos 1 y 2 son activadores selectivos de PPAR delta con baja actividad de PPAR alfa, gamma y RXR.

20 Tabla 1
 Ensayos de indicador

Compuesto	PPAR delta humano CE ₅₀ (μM)	PPAR delta de ratón CE ₅₀ (μM)	PPAR alfa humano CE ₅₀ (μM)	PPAR gamma humano CE ₅₀ (μM)	PPAR RXR ¹ humano CE ₅₀ (μM)
1	< 0,00457 (29x)*	7,8 (4x)*, 6 (2x)*, 4 (2x)*	1,3	3,97 (3,1x)*	Ningún incremento
2	0,039 (27x)*	1 (2x)*	1,1	1,1 (4,3x)*	Ningún incremento

*El valor representa el incremento en veces en comparación con la actividad de luciferasa basal. ¹Receptor X retinoideo

5 Cultivos de oligodendrocitos de RATAS/RATONES

Preparación de las células:

1. Se obtienen oligodendrocitos primarios de rata, a través de la neocorteza de ratas o ratones recién nacidos (2-3 días después del nacimiento) y se enriquecen, después
10 de la extirpación de las células microgliales, por separación mecánica de la monocapa astrocítica usando una modificación de la técnica originalmente descrita por McCarthy y de Vellis (1980).
2. Se extirpan las meninges del cerebro de rata neonatal
15 y se disocia el tejido mecánicamente. Se disponen las células en placas con matraces T75 y se alimentan las células con DMEM/F12 + 10% FBS.
3. Se recogen los oligodendrocitos que se desarrollan en la capa del lecho de astrocitos por el método de agitación
20 durante catorce días después de la fecha de prep original. Se centrifuga la suspensión y se resuspende el sedimento celular en medio libre de suero (SFM; DMEM combinado con 25 μg/ml transferencia, triyodotironina 30 nM, hidrocortisona

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20 nM, progesterona 20 nM, biotina 10 nM, 1x oligoelementos, selenio 30 nM, 1 µg/ml putrescina, 0,1% BSA, 5 U/ml PenStrep, 10 µg/ml insulina) enriquecido con los siguientes factores de crecimiento: Factor de
5 crecimiento: Factor de crecimiento AA derivado de plaquetas (PDGF) y factor de crecimiento de fibroblastos 2 (FGF).

4. Se colocan las células en placas recubiertas con PDL y se incuban a 37°C con CO₂ al 6-7%.

10 5. Los componentes del medio se reemplazan cada 48 h para mantener las células en un estado progenitor..

Se realiza el pasaje de células progenitoras para aumentar la cantidad de células para los ensayos:

15 1. Cuando el cultivo es confluyente, se enjuagua el cultivo con PBS, se añade tripsina y se incuba durante -2-3 min a 37°C.

2. Se neutraliza y centrifuga la suspensión celular a 900g durante 5 min.

3. Se resuspende el sedimento celular en SFM + PDGF/FGF.

20 4. Se alimentan las células con factores de crecimiento frescos cada 48 horas para mantener enriquecido a fin de dividir rápidamente las células progenitoras.

5. El pasaje de las células se realiza no más de 4-5 veces antes de los ensayos experimentales.

25 6. Todos los experimentos que incluyen células progenitoras de oligodendrocitos se llevaron a cabo usando células que se mantuvieron continuamente bajo estas condiciones. Más de 95% de todas las células fueron A2B5 inmunopositivas y expresaron 2' 3'-nucleótido cíclico 3' - fosfodiesterasa II mRNA.

30 7. Para generar oligodendrocitos maduros, 24 h después de disponerse en placas, las células progenitoras se pasaron a SFM enriquecido con o sin IGF-I y se desarrollaron bajo

estas condiciones durante 7 días antes de los ensayos experimentales.

8. Alternativamente, se puede utilizar la línea celular progenitora Central Glia-4 (CG4) enriquecida de rata, que se mantiene en un medio base (DMEM, con glutamina 2 mM, piruvato sódico 1mM, biotina (40 nM), insulina (1 μ M) y N1) enriquecido con 30% medio condicionado a partir de la línea celular de neuroblastoma B-104. Para inducir la diferenciación, las células CG4 se pasan al medio base con 1% de suero de ternero fetal (que se elimina después de 2 días) e insulina (500 nM). Se utiliza la inmunorreactividad de A2B5 y MBP para confirmar un enriquecimiento >95% en cultivos inmaduros y maduros, respectivamente.

Tratamiento con el compuesto de cultivo de Rata/Ratón:

1. Se disponen 10.000 - 15.000 células /pocillo en placas de 24 pocillos recubiertas con PDL y se cultivan las células en presencia de mitógeno (10 ng/ml) durante toda la noche.
2. En presencia de mitógeno:
 - a. Al día siguiente se elimina el medio usado y se añaden compuestos en medio nuevo (con mitógeno)
 - b. Las evaluaciones de la dosis y el efecto del compuesto se llevaron a cabo con 6 concentraciones (10 μ M, 1 μ M, 100 nM, 10 nM, 1 nM y 0,1 nM);
 - c. Se preparan pocillos por triplicado para cada concentración del compuesto.
3. En ausencia de mitógeno:
 - a. Al día siguiente se elimina el medio usado y se añaden compuestos en medio nuevo (sin mitógeno)
 - b. Las evaluaciones de la dosis y el efecto del compuesto se llevaron a cabo con 6 concentraciones (10 μ M, 1 μ M, 100 nM, 10 nM, 1 nM y 0,1 nM);

c. Se preparan pocillos por triplicado para cada concentración del compuesto.

4. Se cultivan las células tratadas durante 7 días antes de usarse en los ensayos experimentales.

5 Cultivos de oligodendrocitos HUMANOS

Preparación de las células:

1. Se cultivan neuroesferas humanas de corteza de embrión humano E19.5 - E22) durante 2 semanas en medio progenitor: DMEM/F12 que contiene 100 µg/ml transferencia, triyodotironina 30 nM, hidrocortisona 20 nM, progesterona 20 nM, biotina 10 nM, 1x oligoelementos, selenio 30 nM, putrescina 60 uM, 0,1% BSA, 5 U/ml PenStrep, 25 µg/ml insulina) enriquecido con PDGF y FGF.

15 2. Las neuroesferas se disocian con 20 U/ml papaína a 37°C durante 30-50 min.

3. Las células se disponen en placas recubiertas con PDL a una densidad de 50.000-100.000 célula/pocillo en medio progenitor que contiene PDGF/FGF y se incuban a 37°C con CO2 al 5-6%.

20 4. El medio y los factores de crecimiento se vuelven a llenar cada 48 h.

Tratamiento del compuesto de cultivo humano:

1. 24 a 48 h después de disponer en placas, se elimina el medio usado y se añaden compuestos en medio nuevo (con mitógeno)

2. Las evaluaciones de la dosis y el efecto del compuesto se llevaron a cabo con 3 -6 concentraciones diferentes (10 µM, 1 µM, 100 nM, 10 nM, 1 nM y 0,1 nM);

30 3. Se preparan pocillos por triplicado para cada concentración del compuesto.

5. Se cultivan las células tratadas durante 7 días antes de usarse en los ensayos experimentales.

Inmunotinción específica de oligodendrocitos de
RATA/RATON/SERES HUMANOS:

Después de la exposición del compuesto, se utilizan anticuerpos específicos de oligodendrocitos para evaluar la capacidad del compuesto para acelerar/promover la diferenciación de oligodendrocitos (por ejemplo, la inmunorreactividad de O4, O1 o proteína básica de mielina en tiempo entre el compuesto tratado y cultivos no tratados).

1. Las células se disponen en placas de 4 pocillos tratadas con poli-D-lisina a 5×10^3 hasta 20×10^3 células/pocillo y se inoculan tal como se describió anteriormente. Se realiza la tinción secuencial en las poblaciones de oligodendrocitos con grados en aumento de diferenciación celular, según lo determinado por los días *in vitro* sin PDGF y FGF.
2. Se utiliza tinción *in vivo* durante 30 min a 37°C para detectar la expresión del marcador de superficie celular específica de la etapa de oligodendrocitos (incluyendo A2B5, O4 y O1).
3. Posteriormente, las células se fijan con 4% paraformaldehído, 10 min a temperatura ambiente.
4. Se utilizan procedimientos de tinción fija para detectar la expresión del marcador específica de la etapa de oligodendrocitos (incluyendo proteína básica de mielina, MBP).
5. Se enjuaga con PBS.
6. Se permeabiliza con 0,1% Triton/0,01% NaAz diluido en 1X PBS durante 10 min a temperatura ambiente.
7. Se bloquea con 5-10% suero de cabra en tampón de dilución de anticuerpos (0,1% Triton-X 100 y 1% albúmina de suero bovino libre de IgG; también se utiliza para diluir anticuerpos), 15 min, temperatura ambiente.

8. Se añade anticuerpo primario diluido en tampón de dilución de anticuerpos.

9. Se incuba durante toda la noche, se agita moderadamente a 4° C.

5 10. Al día siguiente se enjuaga con PBS 1X 5 min y luego con 3X 15 min cada uno a temperatura ambiente.

11. Se incuba con anticuerpos secundarios adecuados durante 45 min a temperatura ambiente.

10 12. Los núcleos celulares se tiñen con 4,6-diamidino-2-fenilindol (DAPI), 15 min a temperatura ambiente.

13. Se enjuaga varias veces con PBS y se evalúa usando microscopía fluorescente.

14. Las siguientes condiciones se comparan en tiempo y en distintas dosis del compuesto: PDGF/FGF solo, SFM solo,
15 SFM-IGF1 solo, PDGF/FGF y compuesto, SFM y compuesto.

Inmunotinción con bromo desoxiuridina (BrdU) en RATA/RATÓN/SER HUMANO:

Para confirmar que los compuestos no promueven la proliferación celular.

20 1. Se marcan células progenitoras de oligodendrocitos con 10 µM BrdU durante 20 h y luego se fijan bien con etanol al 70% o con paraformaldehído al 4%.

2. Las células se incuban sucesivamente con BrdU anti-ratón biotinilado y Estreptavidina-Peroxidasa,
25 interrumpiendo con tres lavados de PBS.

3. La visualización colorimétrica de la inmunorreactividad de BrdU se desarrolla con DAB y se evalúan las cantidades de células totales usando hematoxilina contra-tinción.

30 4. Las células con BrdU inmunopositivas son contadas por dos observadores independientes.

Análisis de las imágenes de los cultivos de RATA/RATÓN/SERES HUMANOS: Se emplea microscopía

fluorescente para cuantificar el grado de diferenciación de oligodendrocitos después de la exposición al compuesto. Este ensayo demuestra que los agonistas selectivos aceleran/promueven la diferenciación de oligodendrocitos.

5 1. Recuento manual de células: Se seleccionan aleatoriamente cuatro campos para cada condición experimental y se cuentan 500-600 células en cada campo. El porcentaje de células inmunopositivas MBP (u O4) (células con un proceso de madurez con o sin láminas de mielina)
10 frente a células positivas con DAPI (cantidad total de células) se compara entre el control y los grupos tratados con el fármaco.

2. Recuento automático de células: Se emplea microscopía fluorescente para cuantificar el grado de diferenciación de
15 oligodendrocitos después de la exposición al compuesto. Se seleccionaron aleatoriamente seis campos/pocillo para evaluar la cantidad de oligodendrocitos de diferenciación entre la población total (se cuentan ~8 a 15x10³ células/pocillo). Las imágenes de inmunofluorescencia se
20 obtuvieron usando un sistema de imágenes digitales Zeiss AxioVision, con una cámara de CCD Zeiss AxioCam HRc enfriada conectada al mismo microscopio. Se ajustaron todos los parámetros de imágenes microscópicas para adquirir imágenes para el análisis de intensidad de la
25 inmunofluorescencia celular. Se comparó el porcentaje de células MBP positivas (diferenciadas) frente a las células totales (tinción nuclear DAPI) entre el control y los grupos tratados con el fármaco. La autofluorescencia celular no fue detectable bajo las condiciones de las
30 imágenes.

a) 3. Ensayo de diferenciación de oligodendrocitos humanos: cantidad total con recuento manual de células inmunopositivas O4/pocillo (bipolar y multipolar).

Los resultados del uso de cultivos de oligodendrocitos de rata se muestran en la figura 1 y los resultados del uso de cultivos mixtos de oligodendrocitos humanos se muestran en las Figuras 5 y 6. Tal como lo indican los resultados, los agonistas del PPAR delta aumentan o aceleran la diferenciación de oligodendrocitos de ratas y seres humanos, según lo medido por el aumento de la expresión de proteína básica de mielina en comparación con los controles no tratados. Este nuevo hallazgo sugeriría que el compuesto 1 y el compuesto 2 y los agonistas selectivos de PPAR delta en general aumentarían, acelerarían o estimularían la diferenciación de oligodendrocitos y la formación de mielina *in vivo*, en el SNC enfermo o dañado, incluyendo la EM y otras enfermedades de desmielinización.

Reacción en cadena por la polimerasa (PCR) cuantitativa en RATA/RATÓN/SER HUMANO: Para evaluar la activación de la vía de PPAR delta inducida por el compuesto y el grado de maduración de los oligodendrocitos (cambios en los niveles de ARNm).

1. Se extrae ARN total de oligodendrocitos cultivados usando el reactivo TriZol.
2. Posteriormente, se trata el ARNm con DNasa libre de RNasa, se repurifica y luego se convierte al molde de ADNc usando una reacción RT (Clontech Advantage RT para Kit de PCR).
3. La expresión de transcripción de miembros de la vía de PPAR delta se cuantifica usando Sybr Green PCR Master Mix.
4. Se utiliza la mezcla de cebador de 18S ribosómico/sonda (producto 186 bp), suspendida en Taqman 2X PCR Master Mix como control interno.
5. Se lleva a cabo la PCR cuantitativa usando tecnología en tiempo real Taqman® (Gibson, et al., 1996) con un

Sistema Detector de Secuencias modelo 7700 (Applied Biosystems, Foster City, CA).

6. Los resultados se analizan usando los Sistemas de Detección de Secuencias, versión de software 1.91.

5 Los resultados para estos ensayos se muestran en las Figuras 2, 3, 4A y 4B. Estos resultados sugieren que los agonistas selectivos del PPAR delta se unen al receptor PPAR delta y activan directamente la vía de PPAR delta en oligodendrocitos, y deberían actuar de modo similar *in*
10 *vivo*.

Ensayo ELISA en RATAS: Para evaluar la activación de la vía de PPAR delta inducida por el compuesto y el grado de maduración de los oligodendrocitos (cambios en los niveles de proteínas).

15 1. Se lavan las placas con PBS y luego se mantienen en hielo. Se añaden 200 μ l de tampón de lisis usado (Tris 50mM, pH7,4, MgCl₂ 2mM, EDTA 1mM, β -mercaptoetanol 5mM, Nonidet P-40 1%, cóctel inhibidor de proteasa (Roche): 1 comprimido /50 ml) a cada pocillo.

20 2. Se lisan las células usando una pipeta y se centrifugan las placas a 2000 rpm a 4°C durante 5 min. El sobrenadante está listo para usar.

3. Se introducen con pipeta 50 μ l de controles y muestras patrón a los pocillos.

25 4. Se añaden 50 μ l de Tampón de Ensayo MBP a cada pocillo.

5. Se incuba el pocillo, agitando a 500-700 rpm en un agitador de microplacas orbital durante 2 h a temperatura ambiente.

30 6. Se añaden 100 μ l de Conjugado de Biotina-Anticuerpo MBP a cada pocillo.

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7. Se incuba el pocillo, agitando a 500-700 rpm en un agitador de microplacas orbital durante 1h a temperatura ambiente.
8. Se lava 5 veces con Disolución de Lavado. Se seca invirtiendo la placa en material absorbente.
9. Se diluye el conjugado de estreptavidina-enzima a una concentración de 1:50 con tampón de ensayo Elisa MBP. (se debe diluir inmediatamente antes de usarse para el ensayo.
10. Se añaden 100 μ l de disoluciones con conjugado de estreptavidina-enzima a cada pocillo.
11. Se incuba el pocillo, agitando a 500-700 rpm en un agitador de microplacas orbital durante 30 h a temperatura ambiente.
12. Se lava 5 veces con Disolución de Lavado. Se seca invirtiendo la placa en material absorbente.
13. Se añaden 100 μ l de Disolución TMB Chromogen a cada pocillo.
14. Se incuba el pocillo, agitando a 500-700 rpm en un agitador de microplacas orbital durante 10 - 20 min a temperatura ambiente. Se evita la exposición a la luz solar directa.
15. Se añaden 100 μ l de Disolución Stopping a cada pocillo.
16. Se lee la absorbancia de la disolución en los pocillos dentro de los 30 min, usando una lectora de microplacas ajustada a 450 nM.

Los resultados anteriormente expuestos tomados en general y exhibidos en las Figuras 1-6 ilustran que los agonistas de PPAR delta promueven la diferenciación de oligodendrocitos incluso en presencia de mitógenos, que normalmente mantienen las células mitóticamente activas e inhiben la diferenciación celular. Por lo tanto, se espera que en el SNC dañado o enfermo, los agonistas selectivos

del PPAR delta provoquen que las células progenitoras de oligodendrocitos expresen proteínas de mielina y envainen axones desmielinizados o hipomielinizados.

Prueba in vivo de los modelos conceptuales

- 5 Lesiones focales: (se utilizan para evaluar si los compuestos protegen la integridad de la mielina o aceleran/aumentan el índice de remielinización).
1. A ratas de 7 semanas de vida se les provee acceso ilimitado a agua y alimento, y se las aclimata durante un
10 mínimo de 4 días antes de ser usadas en los experimentos.
2. Antes de la cirugía se pesa a cada animal. La rata luego se anestesia con cetamina (100 mg/ml) combinada con xilazina (20 mg/ml) en una relación de 1,8: 1. A las ratas se les inyecta 0,15 ml/180 g peso corporal i.p. de la
15 disolución anestésica antes del procedimiento quirúrgico. Se prepara al animal para la cirugía usando condiciones asépticas de acuerdo con las pautas IACUC. Todos los instrumentos quirúrgicos se esterilizan en autoclave. El pelo se recoge con una grapa entre de las orejas y esta
20 región se frota con Betadina, se lava con disolución salina estéril y finalmente se frota con un hisopo estéril embebido en alcohol.
3. Para el procedimiento quirúrgico, la rata se dispone en su superficie ventral en un instrumento estereotáxico
25 para animales diseñado para sostener firme la cabeza. La barra de incisión está siempre regulada a -3,9 mm, ya que se ha demostrado que así se logra una posición plana del cráneo en ratas SD.
4. Se realiza una incisión en la piel previamente
30 rasurada que cubre el cráneo entre las orejas.
5. Se perfora una pequeña área de hueso (0,75 mm de diámetro) en las coordenadas AP -1,8, ML -3,1 de lambda.

6. Se extrae el hueso, y a las ratas se les inyectan 2 μ l de bromuro de etidio, lisolecitina o SIN-1 en el pedúnculo cerebeloso caudal derecho, DV -7,1 mm, durante un período de 2 min mediante jeringa y aguja μ l Hamilton.
- 5 Alternativamente, las inyecciones se aplican en la médula espinal, el cuerpo calloso o la corteza.
7. Se deja allí la aguja durante los 2 min. subsiguientes.
8. Después de retirar la aguja, se sutura la incisión.
- 10 9. Cada rata recibe una inyección i.m. de 0,003 mg de buprenorfina en una de las patas traseras.
10. Se dispone a la rata en un armario de calentamiento hasta que vuelve en sí. En ese momento, se la transfiere nuevamente a su jaula. No se permiten más de 2 ratas por
- 15 jaula, ya que se quitarán las suturas entre sí.
11. Se realizan procedimientos similares usando ratones.

Modelo de encefalomiелitis alérgica experimental (EAE) en la rata:

- a encefalomiелitis alérgica experimental (EAE) es una
- 20 enfermedad autoinmunitaria mediada por las células T del sistema nervioso central que se desarrolla en animales susceptibles después de la sensibilización, sea con homogeneizados de la médula espinal total o solo un componente de ésta (proteína básica de mielina). El modelo
- 25 de roedor de EAE es una herramienta apropiada para estudiar la inflamación del cerebro y la médula espinal, que se observa en pacientes con EM. En roedores, la inyección de la médula espinal total o algunos componentes de la médula espinal, tales como la proteína básica de mielina, induce
- 30 una respuesta autoinmunitaria que se basa en la activación de los linfocitos T. La enfermedad clínica típicamente se manifiesta alrededor del día 8-10 después de la inoculación, y se observa como un amplio espectro de

anomalías del comportamiento que van desde una alteración leve en la marcha o atonía del rabo hasta parálisis completa y muerte. Típicamente, se produce una pérdida de peso. En los animales que sobreviven se produce una
 5 recuperación espontánea, acompañada de la recuperación variable de la mayor parte de la función motora. Dependiendo de la especie, el alérgeno y la metodología que se utilice, los animales ensayados por el modelo EAE podrán experimentar un solo ataque (EAE aguda) o diversos ataques
 10 (EAE crónica con recidiva). Se pueden utilizar diversos paradigmas de tratamiento: el fármaco o el tratamiento de elección se puede administrar antes de la inmunización, durante el período asintomático o durante la enfermedad clínica.

15 Animales:

Ratas Lewis hembra, 160-220g (Charles River)

Antígeno:

Médula espinal de cobaya entera (Harlan Biosciences).

Adyuvante de Freund completo H37 Ra [1mg/ml Mycobacterium
 20 Tuberculosis H37 Ra] (Difco).

Antígeno adicional:

Mycobacterium Tuberculosis (Difco).

Bordetella Pertussis [exterminado por calor] (Difco).

Preparación de antígenos: (para aproximadamente 720
 25 animales):

1. Se pesan 5 gramos de médula espinal congelada de cobaya.

2. Se añaden 5g de médula espinal a 5 ml de disolución salina al 0,9% (1g/ml) en un tubo centrífugo de fondo
 30 redondo

3. Se homogeneiza en hielo con Tissue-tech hasta que el tejido se rompe por completo (aproximadamente 5 minutos).

4. Se añaden 10 ml de adyuvante de Freund completo H37 Ra enriquecido con 200 mg de Mycobacterium Tuberculosis (20 mg / ml adyuvante de Freund completo H37 Ra).
5. Se extrae el homogeneizado / adyuvante del tubo aspirándolo con una jeringa de 10 ml equipada con una aguja emulsionante de 18 gauge.
6. Se emulsiona entre dos jeringas de vidrio de 30 ml hasta que se torna difícil continuar pasando el material a través de la aguja. (Aproximadamente 5 minutos {no debe haber separación entre la fase oleosa y la fase acuosa}).
7. Se usa de inmediato o se conserva en hielo hasta que se usa (no más de 30 min) (no congelar).

Protocolo

1. A ratas Lewis hembra (Charles River) se les provee acceso ilimitado a alimento y agua, y se las aclimata durante un mínimo de 3 días antes de usarse en los experimentos.
2. A las ratas que pesan 160 y 220 gramos se las induce inicialmente con isoflurano al 5% (Aerrane, Fort Dodge), O₂ al 30%, N₂O al 70% durante 2-5 minutos.
3. La rata se dispone luego en una manta de calentamiento con agua circulante (Gaymar) (superficie dorsal hacia arriba) y se le coloca un cono nasal para respiración espontánea de gases anestésicos. El isoflurano se reduce al 2%.
4. Se aplican dos inyecciones subcutáneas (0,1 ml cada una) bien de antígeno o de disolución salina normal en la superficie ventral de las garras traseras.
5. Se les retira el cono nasal, se pesan y se numeran.
6. Se permite que las ratas despierten de la anestesia y se disponen en jaulas individuales.

7. Los animales son observados diariamente para detectar signos de inducción de EAE (véanse criterios a continuación)

ETAPA: 0 NORMAL

5 ETAPA: 1 Marcha anormal y atonía del rabo

ETAPA: 2 Debilidad leve pero concreta de una o ambas patas traseras

ETAPA: 3 Debilidad intensa de una o ambas patas traseras o ataxia leve

10 ETAPA: 4 Paraparesia intensa y movimientos mínimos de las patas traseras

ETAPA: 5 Ningún movimiento de las patas traseras y paraplegia

15 ETAPA: 6 Estado moribundo sin movimiento espontáneo y dificultad respiratoria. También puede haber un grado en aumento de la implicación de la pata delantera e incontinencia urinaria y fecal

ETAPA: 7 MUERTE

20 El tratamiento comienza en el día 10 después de la inmunización. Ya que los síntomas de enfermedad en este modelo típicamente aparecen en los días 10-11 después de la inoculación, se puede considerar que este punto de tiempo representa la fase inicial de un episodio agudo de EM. Se juzga que esta demora en el inicio del tratamiento imita la
25 situación clínica más precisamente que los protocolos tradicionalmente utilizados en los que los fármacos se administran al momento de la inoculación o incluso antes (Teitelbaum D. et al., Proc Natl Acad Sci USA 1999;96: 3842-3847 y Brod S. A., et al., Ann Neurol 2000; 47: 127-
30 131).

REIVINDICACIONES

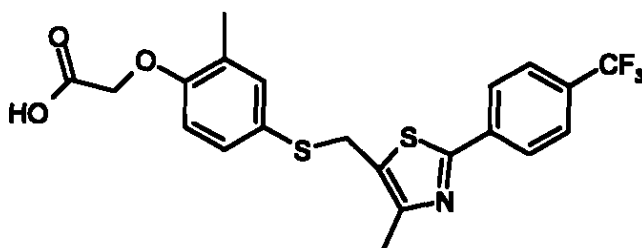
1. Un método para tratar enfermedades de desmielinización en un paciente, que comprende la administración de una cantidad terapéuticamente eficaz de un agonista de hPPAR delta.

2. El método según la reivindicación 1, en el que el agonista de hPPAR delta es un agonista selectivo.

3. El método según la reivindicación 1, en el que la enfermedad de desmielinización se selecciona del grupo que consiste en esclerosis múltiple, enfermedad de Charcot-Marie-Tooth, enfermedad de Pelizaeus-Merzbacher, encefalomielitis, neuromielitis óptica, adrenoleucodistrofia, síndrome de Guillian-Barre y trastornos en los que están dañadas las células gliales formadoras de mielina, incluyendo lesiones de la médula espinal, neuropatías y lesiones nerviosas.

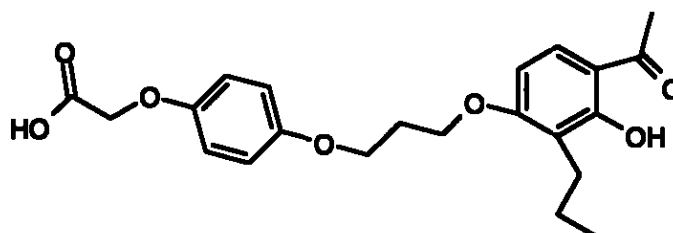
4. El método según la reivindicación 3, en el que la enfermedad de desmielinización es la esclerosis múltiple.

5. El método según la reivindicación 1, en el que el agonista se selecciona del grupo que consiste en el compuesto de fórmula (1) y fórmula (2)



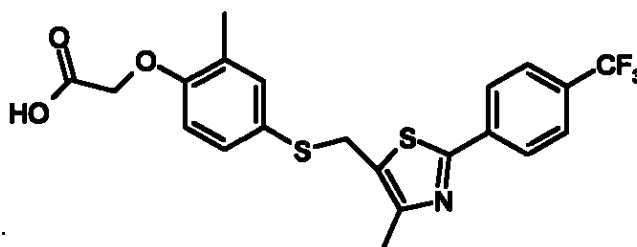
(1)

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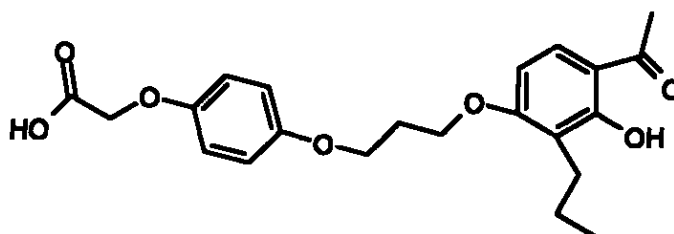


(2)

6. Una composición farmacéutica que comprende un compuesto seleccionado del grupo que consiste en el compuesto de fórmula (1) y fórmula (2) en una cantidad eficaz para tratar la esclerosis múltiple, enfermedad de Charcot-Marie-Tooth, enfermedad de Pelizaeus-Merzbacher, encefalomiелitis, neuromielitis óptica, adrenoleucodistrofia, síndrome de Guillian-Barre y trastornos en los que están dañadas las células gliales formadoras de mielina, incluyendo lesiones de la médula espinal, neuropatías y lesiones nerviosas, en combinación con por lo menos un vehículo farmacéuticamente aceptable



(1)



(2).

7. La composición farmacéutica según la reivindicación 6, que comprende una cantidad eficaz para tratar la esclerosis múltiple.

RESUMEN

Se describe un método para tratar enfermedades de desmielinización, en un paciente que necesita dicho tratamiento, con una cantidad eficaz de un agonista del PPAR delta. Las enfermedades de desmielinización que se pueden tratar de forma eficaz con este método incluyen, aunque sin limitarse a ello, esclerosis múltiple, enfermedad de Charcot-Marie-Tooth, enfermedad de Pelizaeus-Merzbacher, encefalomyelitis, neuromielitis óptica, adrenoleucodistrofia, síndrome de Guillian-Barre y trastornos en los que están dañadas las células gliales formadoras de mielina, incluyendo lesiones de la médula espinal, neuropatías y lesiones nerviosas.

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PCT REQUEST

Original (for SUBMISSION)

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-SAFE [EASY mode] Version 3.50 (Build 0002.169)
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)	United States Patent and Trademark Office (USPTO) (RO/US)
0-7	Applicant's or agent's file reference	US20020240WO
I	Title of Invention	USE OF PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR DELTA AGONISTS FOR THE TREATMENT OF MS AND OTHER DEMYELINATING DISEASES
II	Applicant	
II-1	This person is	applicant only
II-2	Applicant for	all designated States except US
II-4	Name	AVENTIS PHARMACEUTICALS INC.
II-5	Address	300 Somerset Corporate Boulevard Bridgewater, New Jersey 08807-2854 United States of America
II-6	State of nationality	US
II-7	State of residence	US
II-8	Telephone No.	(908) 231-3800
II-9	Facsimile No.	(908) 231-2626

US20020240WO

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PCT REQUEST

Original (for SUBMISSION)

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)	CHANDROSS, Karen
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III-1-6	State of nationality	US
III-1-7	State of residence	US
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)	MERRILL, Jean
III-2-5	Address	101 Alpine Lane Whippany, New Jersey 07981 United States of America
III-2-6	State of nationality	US
III-2-7	State of residence	US
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)	MINNICH, Anne
III-3-5	Address	2 Knoll Court Flemington, NJ 08822 United States of America
III-3-6	State of nationality	US
III-3-7	State of residence	US
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)	LEE, Lan
III-4-5	Address	Box 230 Pluckemin Park Pluckemin, NJ 07978 United States of America
III-4-6	State of nationality	US
III-4-7	State of residence	US

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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)	KHORKOVA, Olga
III-5-5	Address	570 Manchester Avenue North Haledon, New Jersey 07508 United States of America
III-5-6	State of nationality	US
III-5-7	State of residence	US
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)	LIU, Yun
III-6-5	Address	42 McBride Way Bridgewater, NJ 08807 United States of America
III-6-6	State of nationality	CN
III-6-7	State of residence	US
IV-1	Agent or common representative; or address for correspondence The person identified below is hereby/ has been appointed to act on behalf of the applicant(s) before the competent International Authorities as:	agent
IV-1-1	Name (LAST, First)	STRUPCZEWSKI, Joseph
IV-1-2	Address	sanofi-aventis Route 202-206 P. O. Box 6800 Bridgewater, NJ 08807-0800 United States of America
IV-1-3	Telephone No.	(908) 231-2387
IV-1-4	Facsimile No.	(908) 231-2626
IV-1-5	e-mail	joseph.strupczewski@sanofi-aventis.com
IV-1-6	Agent's registration No.	50903
IV-2	Additional agent(s)	additional agent(s) with same address as first named agent
IV-2-1	Name(s)	HARNEY, Charlotte, L. (35060); BENDER, Kelly (52610); BOLCSAK, James, W. (39912); COPPOLA, William, C. (41686); DAHLING, Gerald, V. (29632); DARKES, Paul, R. (33862); DOLAN, Peter, L. (46307); GERMAN, Joel, B. (48676); GUPTA, Balaram (40009); KIRK, Joseph, P.,

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		Jr. (36844); KNIGHT, Julie, Anne (48867); KRUPEN, Karen, I. (34647); KURYS, Barbara, E. (34650); LIN, Jiang (51065); MOON, Carolyn, D. (33022); NELLIGAN, Mark, C. (36389); NEWMAN, Irving (22638); OEHLER, Ross, J. (33270); ORT, Ronald, G. (36969); PARKER, Raymond, S., III (34893); ROSSI, Joseph (47038)
V	DESIGNATIONS	
V-1	The filing of this request constitutes under Rule 4.8(a), the designation of all Contracting States bound by the PCT on the international filing date, for the grant of every kind of protection available and, where applicable, for the grant of both regional and national patents.	
VI-1	Priority claim of earlier national application	
VI-1-1	Filing date	01 April 2004 (01.04.2004)
VI-1-2	Number	60/558,533
VI-1-3	Country	US
VI-2	Priority document request The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) identified above as item(s):	VI-1
VI-1	International Searching Authority Chosen	European Patent Office (EPO) (ISA/EP)
VII	Declarations	Number of declarations
VII-1	Declaration as to the identity of the inventor	-
VII-2	Declaration as to the applicant's entitlement, as at the international filing date, to apply for and be granted a patent	-
VII-3	Declaration as to the applicant's entitlement, as at the international filing date, to claim the priority of the earlier application	1
VII-4	Declaration of inventorship (only for the purposes of the designation of the United States of America)	1
VII-5	Declaration as to non-prejudicial disclosures or exceptions to lack of novelty	-

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VII-3-1	Declaration: Entitlement to claim priority Declaration as to the applicant's entitlement, as at the international filing date, to claim the priority of the earlier application specified below, where the applicant is not the applicant who filed the earlier application or where the applicant's name has changed since the filing of the earlier application (Rules 4.17(II) and 51bis.1(a)(II)) Name	in relation to this international application AVENTIS PHARMACEUTICALS INC. is entitled to claim priority of earlier application No. 60/558,533 by virtue of the following:
VIII-3-1(i) v)		an assignment from CHANDROSS, Karen to AVENTIS PHARMACEUTICALS INC., dated 13 May 2004 (13.05.2004)
VIII-3-1(i) v)		an assignment from MERRILL, Jean to AVENTIS PHARMACEUTICALS INC., dated 16 May 2004 (16.05.2004)
VIII-3-1(i) v)		an assignment from MINNICH, Anne to AVENTIS PHARMACEUTICALS INC., dated 14 May 2004 (14.05.2004)
VIII-3-1(i) v)		an assignment from LEE, Lan to AVENTIS PHARMACEUTICALS INC., dated 14 May 2004 (14.05.2004)
VIII-3-1(i) v)		an assignment from KHORKOVA, Olga to AVENTIS PHARMACEUTICALS INC., dated 14 May 2004 (14.05.2004)
VIII-3-1(i) v)		an assignment from LIU, Yun to AVENTIS PHARMACEUTICALS INC., dated 13 May 2004 (13.05.2004)
VIII-3-1(i) x)	This declaration is made for the purposes of:	all designations

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VII-4-1	Declaration: Inventorship (only for the purposes of the designation of the United States of America) Declaration of Inventorship (Rules 4.17(iv) and 51bis.1(a)(iv)) for the purposes of the designation of the United States of America:	I hereby declare that I believe I am the original, first and sole (if only one inventor is listed below) or joint (if more than one inventor is listed below) inventor of the subject matter which is claimed and for which a patent is sought. This declaration is directed to the international application of which it forms a part (if filing declaration with application). I hereby declare that my residence, mailing address, and citizenship are as stated next to my name. I hereby state that I have reviewed and understand the contents of the above-identified international application, including the claims of said application. I have identified in the request of said application, in compliance with PCT Rule 4.10, any claim to foreign priority, and I have identified below, under the heading "Prior Applications", by application number, country or Member of the World Trade Organization, day, month, and year of filing, any application for a patent or inventor's certificate filed in a country other than the United States of America, including any PCT international application designating at least one country other than the United States of America, having a filing date before that of the application on which foreign priority is claimed.
VIII-4-1-1	Prior applications:	

		I hereby acknowledge the duty to disclose information that is known by me to be material to patentability as defined by 37 C.F.R. § 1.56, including for continuation-in-part applications, material information which became available between the filing date of the prior application and the PCT international filing date of the continuation-in-part application. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.
VIII-4-1-1-1	Name (LAST, First)	CHANDROSS, Karen
VIII-4-1-1-2	Residence: (city and either US State, if applicable, or country)	Somerset,
VIII-4-1-1-3	Mailing address:	13 Staudt Court Somerset, NJ 08873 United States of America
VIII-4-1-1-4	Citizenship:	US
VIII-4-1-1-5	Inventor's Signature: (If not contained in the request, or if declaration is corrected or added under Rule 28ter after the filing of the international application. The signature must be that of the inventor, not that of the agent)	
VIII-4-1-1-6	Date: (of signature which is not contained in the request, or of the declaration that is corrected or added under Rule 28ter after the filing of the international application)	

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PCT REQUEST

Original (for SUBMISSION)

VIII-4-1-2-1	Name (LAST, First)	MERRILL, Jean
VIII-4-1-2-2	Residence: (city and either US State, if applicable, or country)	Whippany, New Jersey
VIII-4-1-2-3	Mailing address:	101 Alpine Lane Whippany, NJ 07981 United States of America
VIII-4-1-2-4	Citizenship:	US
VIII-4-1-2-5	Inventor's Signature: (if not contained in the request, or if declaration is corrected or added under Rule 28ter after the filing of the international application. The signature must be that of the inventor, not that of the agent)	
VIII-4-1-2-6	Date (of signature which is not contained in the request, or of the declaration that is corrected or added under Rule 28ter after the filing of the international application)	
VIII-4-1-3-1	Name (LAST, First)	MINNICH, Anne
VIII-4-1-3-2	Residence: (city and either US State, if applicable, or country)	Flemington,
VIII-4-1-3-3	Mailing address:	2 Knoll Court Flemington, NJ 08822 United States of America
VIII-4-1-3-4	Citizenship:	US
VIII-4-1-3-5	Inventor's Signature: (if not contained in the request, or if declaration is corrected or added under Rule 28ter after the filing of the international application. The signature must be that of the inventor, not that of the agent)	
VIII-4-1-3-6	Date (of signature which is not contained in the request, or of the declaration that is corrected or added under Rule 28ter after the filing of the international application)	

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Original (for SUBMISSION)

VIII-4-1-4-1	Name (LAST, First)	LEE, Lan
VIII-4-1-4-2	Residence: (city and either US State, if applicable, or country)	Pluckemin,
VIII-4-1-4-3	Mailing address:	Box 230 Pluckemin Park Pluckemin, NJ 07978 United States of America
VIII-4-1-4-4	Citizenship:	US
VIII-4-1-4-5	Inventor's Signature: (If not contained in the request, or if declaration is corrected or added under Rule 26ter after the filing of the International application. The signature must be that of the inventor, not that of the agent)	
VIII-4-1-4-6	Date (of signature which is not contained in the request, or of the declaration that is corrected or added under Rule 26ter after the filing of the International application)	
VIII-4-1-5-1	Name (LAST, First)	KHORKOVA, Olga
VIII-4-1-5-2	Residence: (city and either US State, if applicable, or country)	North Haledon, New Jersey
VIII-4-1-5-3	Mailing address:	570 Manchester Avenue North Haledon, NJ 07508 United States of America
VIII-4-1-5-4	Citizenship:	US
VIII-4-1-5-5	Inventor's Signature: (If not contained in the request, or if declaration is corrected or added under Rule 26ter after the filing of the International application. The signature must be that of the inventor, not that of the agent)	
VIII-4-1-5-6	Date (of signature which is not contained in the request, or of the declaration that is corrected or added under Rule 26ter after the filing of the International application)	

PCT REQUEST

Original (for SUBMISSION)

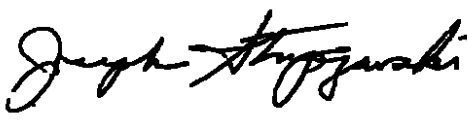
VIII-4-1- 8-1	Name (LAST, First)	LIU, Yun
VIII-4-1- 8-2	Residence: (city and either US State, if applicable, or country)	Bridgewater,
VIII-4-1- 8-3	Mailing address:	42 McBride Way Bridgewater, NJ 08807 United States of America
VIII-4-1- 8-4	Citizenship:	CN
VIII-4-1- 8-5	Inventor's Signature: (If not contained in the request, or if declaration is corrected or added under Rule 28ter after the filing of the international application. The signature must be that of the inventor, not that of the agent)	
VIII-4-1- 8-6	Date (of signature which is not contained in the request, or of the declaration that is corrected or added under Rule 28ter after the filing of the international application)	

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IX	Check list	number of sheets	electronic file(s) attached
IX-1	Request (including declaration sheets)	11	✓
IX-2	Description	33	-
IX-3	Claims	2	-
IX-4	Abstract	1	✓
IX-5	Drawings	7	-
IX-7	TOTAL	54	
	Accompanying items	paper document(s) attached	electronic file(s) attached
IX-8	Fee calculation sheet	✓	-
IX-9	Original separate power of attorney	✓	-
IX-17	PCT-SAFE physical media	-	✓
IX-18	Figure of the drawings which should accompany the abstract	1	
IX-20	Language of filing of the international application	English	
X-1	Signature of applicant, agent or common representative		
X-1-1	Name (LAST, First)	STRUPCZEWSKI, Joseph	
X-1-2	Name of signatory		
X-1-3	Capacity		

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

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference US20020240WO	FOR FURTHER ACTION see Form PCT/ISA/220 as well as, where applicable, Item 5 below.	
International application No. PCT/US2005/010371	International filing date (day/month/year) 29/03/2005	(Earliest) Priority Date (day/month/year) 01/04/2004
Applicant AVENTIS PHARMACEUTICALS INC.		

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 4 sheets.



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, see Box No. I.

2. ☐ Certain claims were found unsearchable (See Box II).

3. ☐ Unity of invention is lacking (see Box III).

4. With regard to the title,



the text is approved as submitted by the applicant.



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

USE OF PPAR DELTA AGONISTS FOR TREATING DEMYELINATING DISEASES

5. With regard to the abstract,



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 No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. With regard to the drawings,

- a. the figure of the drawings to be published with the abstract is Figure No. _____



as suggested by the applicant.



as selected by this Authority, because the applicant failed to suggest a figure.



as selected by this Authority, because this figure better characterizes the invention.

- b. ☒ none of the figures is to be published with the abstract.

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K31/192 A61K31/426 A61P25/02 A61P25/28

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 A61P

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, BIOSIS, EMBASE, BEILSTEIN Data, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
✓ X	WO 02/28434 A (GLAXO GROUP LIMITED; BUCHAN, KEVIN, WILLIAM) 11 April 2002 (2002-04-11) page 2, line 31 - page 3, line 3 page 7, lines 11,12; claims 1-8	1-7
✓ X	WO 02/28433 A (GLAXO GROUP LIMITED; BUCHAN, KEVIN, WILLIAM) 11 April 2002 (2002-04-11) abstract page 3, lines 4-31 page 9, lines 1-26; example 2	1-7
	-/-	



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.
- "Z" document member of the same patent family

Date of the actual completion of the international search

20 October 2005

Date of mailing of the international search report

28/10/2005

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentstein 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax (+31-70) 340-3016

Authorized officer

A. Jakobs

INTERNATIONAL SEARCH REPORT

Inter. Patent Application No.
PC., JS2005/010371

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 02/13812 A (PERSHADSINGH, HARRIHAR, A) 21 February 2002 (2002-02-21) abstract page 1, line 8 - page 2, line 5 page 7, line 18 - page 8, line 5 examples 9,10	1,3,4
✓ X	WO 01/00603 A (GLAXO GROUP LIMITED; CHAO, ESTHER, YU-HSUAN; HAFFNER, CURT, DALE; LAMB) 4 January 2001 (2001-01-04) abstract; example 66	6,7
✓ X	WO 97/28149 A (MERCK & CO., INC; LEIBOWITZ, MARK, D; BERGER, JOEL, P; MOLLER, DAVID,) 7 August 1997 (1997-08-07) abstract; example 7	6,7
✓ X,P	WO 2004/093910 A (FUJISAWA PHARMACEUTICAL CO., LTD; KITA, YASUHIRO; YAMAZAKI, TAKAO; MUR) 4 November 2004 (2004-11-04) the whole document	1-7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2005/010371

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Box II Observations where certain claims were found unsearchable (Continuation of item 2 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. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 1-5 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
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 III Observations where unity of invention is lacking (Continuation of item 3 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 SEARCH REPORT

Information on patent family members

International Application No

PC./US2005/010371

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Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 0228434	A	11-04-2002	AU 9204601 A EP 1324774 A2 JP 2004510750 T US 2005131035 A1	15-04-2002 09-07-2003 08-04-2004 16-06-2005
WO 0228433	A	11-04-2002	AU 9204401 A EP 1324773 A2 JP 2004510749 T US 2004029938 A1	15-04-2002 09-07-2003 08-04-2004 12-02-2004
WO 0213812	A	21-02-2002	AU 8827101 A	25-02-2002
WO 0100603	A	04-01-2001	AT 297384 T AU 765347 B2 AU 5817100 A BR 0011891 A CA 2377126 A1 CN 1358179 A CZ 20014664 A3 DE 60020701 D1 EP 1189895 A1 HU 0201858 A2 JP 3490704 B2 JP 2003503399 T JP 2003313141 A MX PA01013244 A NO 20016078 A NZ 515676 A PL 353135 A1 TR 200103612 T2 US 6710063 B1 ZA 200109804 A	15-06-2005 18-09-2003 31-01-2001 05-03-2002 04-01-2001 10-07-2002 13-03-2002 14-07-2005 27-03-2002 28-09-2002 26-01-2004 28-01-2003 06-11-2003 02-07-2002 13-12-2001 28-05-2004 20-10-2003 21-05-2002 23-03-2004 28-02-2003
WO 9728149	A	07-08-1997	AU 1856997 A	22-08-1997
WO 2004093910	A	04-11-2004	NONE	

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PATENT COOPERATION TREATY

WO 2005/097098
PCT/US2005/010371

AUG 09 2006

From the INTERNATIONAL BUREAU

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PATENT DEPT. PCT

SECOND AND SUPPLEMENTARY NOTICE
INFORMING THE APPLICANT OF THE
COMMUNICATION OF THE INTERNATIONAL
APPLICATION (TO DESIGNATED OFFICES
WHICH APPLY THE 30 MONTH TIME
LIMIT UNDER ARTICLE 22(1))

(PCT Rule 47.1(c))

To:

~~STRUPCZEWSKI, Joseph~~
sanofi-aventis
Route 202-208
P. O. Box 6800
Bridgewater, NJ 08807-0800
ETATS-UNIS D'AMERIQUE

Craig Bell

COMPUTER ENTERED

es 8/10/06

I.A.

IMPORTANT NOTICE

Date of mailing (day/month/year)

03 August 2006 (03.08.2006)

Applicant's or agent's file reference

US20020240WO

International application No.

PCT/US2005/010371

International filing date (day/month/year)

29 March 2005 (29.03.2005)

Priority date (day/month/year)

01 April 2004 (01.04.2004)

Applicant

AVENTIS PHARMACEUTICALS INC. et al

1. **ATTENTION:** For any designated Office(s), for which the time limit under Article 22(1), as in force from 1 April 2002 (30 months from the priority date), does not apply, please see Form PCT/IB/308 (First Notice) issued previously.

2. Notice is hereby given that the following designated Office(s), for which the time limit under Article 22(1), as in force from 1 April 2002, does apply, has/have requested that the communication of the international application, as provided for in Article 20, be effected under Rule 93bis.1. The International Bureau has effected that communication on the date indicated below:
20 October 2005 (20.10.2005)

AU, AZ, BY, CN, CO, DZ, EP, HU, KG, KP, KR, MD, MK, MZ, NA, PG, RU, SY, TM, US

In accordance with Rule 47.1(c-bis)(i), 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).

3. The following designated Offices, for which the time limit under Article 22(1), as in force from 1 April 2002, does apply, have not requested, as at the time of mailing of the present notice, that the communication of the international application be effected under Rule 93bis.1:

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

In accordance with Rule 47.1(c-bis)(ii), those Offices accept the present notice as conclusive evidence that the Contracting State for which that Office acts as a designated Office does not require the furnishing, under Article 22, by the applicant of a copy of the international application.

4. **TIME LIMITS** for entry into the national phase

For the designated or elected Office(s) listed above, 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.

In practice, time limits other than the 30-month time limit will continue to apply, for various periods of time, in respect of certain of the designated or elected Office(s) listed above. For regular updates on the applicable time limits (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>.

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 22 338 82 70

Authorized officer

Ellen Moyse

e-mail: pt05@wipo.int

J. Newman
COMPUTER ENTERED
Jes 2.16.06 **PCT**

From the INTERNATIONAL BUREAU

**INFORMATION CONCERNING ELECTED
OFFICES NOTIFIED OF THEIR ELECTION**

(PCT Article 31(7) and Rule 61.3)

To:

STRUPCZEWSKI, Joseph
sanofi-aventis
Route 202-208
P. O. Box 6800
Bridgewater, NJ 08807-0800
ETATS-UNIS D'AMERIQUE

Date of mailing (day/month/year) 02 February 2006 (02.02.2006)		IMPORTANT INFORMATION
Applicant's or agent's file reference US2002024WO		
International application No. PCT/US2005/010371	International filing date (day/month/year) 29 March 2005 (29.03.2005)	Priority date (day/month/year) 01 April 2004 (01.04.2004)
Applicant AVENTIS PHARMACEUTICALS INC. et al		

- 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, IS, IT, LT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR
National: BG, CA, CN, CZ, DE, JP, KP, KR, MN, NO, PL, RO, RU, SK, SM, US
- 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: BW, GH, GM, KE, LS, MW, MZ, NA, 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, BW, BY, BZ, CH, CO, CR, CU, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, KE, KG, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MW, MX, MZ, NA, NI, NZ, OM, PG, PH, PT, SC, SD, SE, SG, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VC, VN, YU, ZA, ZM, ZW
- Since the election(s) was (were) made after the expiration of 19 months from the priority date, the applicant is reminded that he must, subject to the following paragraph, enter the national phase within 20 months from the priority date (or later in some Offices) before some of the designated Offices in respect of which Article 22(1), as modified with effect of 1 April 2002, does not apply, by paying the national fee(s) and furnishing, if prescribed, a translation of the international application.

However, in respect of most other designated Offices, the time limit of 30 months (or later) may nevertheless apply. See the Annex to Form PCT/IB/301 and, for details about the applicable time limits, Office by Office, see the *PCT Applicant's Guide*, Volume II, National Chapters, the *PCT Newsletter* and the WIPO Internet site, updated regularly.

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland	Authorized officer Ellen Moyse
Facsimile No. +41 22 740 14 35	Facsimile No. +41 22 338 89 75

From the
INTERNATIONAL SEARCHING AUTHORITY

To:

see form PCT/ISA/220

PCT

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY
(PCT Rule 43bis.1)

Date of mailing
(day/month/year) see form PCT/ISA/210 (second sheet)

Applicant's or agent's file reference
see form PCT/ISA/220

FOR FURTHER ACTION
See paragraph 2 below

International application No.
PCT/US2005/010371

International filing date (day/month/year)
29.03.2005

Priority date (day/month/year)
01.04.2004

International Patent Classification (IPC) or both national classification and IPC
A61K31/192, A61K31/426, A61P25/02, A61P25/28

Applicant
AVENTIS PHARMACEUTICALS INC.

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

- ☒ Box No. I Basis of the opinion
☒ Box No. II Priority
☒ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
☐ Box No. IV Lack of unity of invention
☒ Box No. V Reasoned statement under Rule 43bis.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
☒ Box No. VI Certain documents cited
☐ Box No. VII Certain defects in the international application
☐ Box No. VIII Certain observations on the international application

2. FURTHER ACTION

If a demand for international preliminary examination is made, this opinion will usually be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA"). However, this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 68.1bis(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of three months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

3. For further details, see notes to Form PCT/ISA/220.

ACTION DUE

DUE DATE
ENTRY

ATTY

Authorized Officer

Name and mailing address of the ISA:



European Patent Office - P.B. 5818 Patentplan 2
NL-2280 HV Rijswijk - Pays Bas
Tel. +31 70 340 - 2040 Tx: 31 851 epo nl
Fax: +31 70 340 - 3018

A. Jakobs

Telephone No. +31 70 340-2817



Prior Art Entry - 10/1/06
Enter in US Case if/when created in M3 office

**WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING
AUTHORITY (SEPARATE SHEET)**

International application No.

PCT/US2005/010371

Re Item III.

Claims 1-5 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).

Present claims 1-4 relate to a method defined by reference to the following parameters: a hPPAR delta agonist, a selective hPPAR delta agonist, disorders in which myelin forming glial cells are damaged .

The use of these parameters in the present context is considered to lead to a lack of clarity within the meaning of Article 6 PCT. It is impossible to compare the parameters the applicant has chosen to employ with what is set out in the prior art. The lack of clarity is such as to render a meaningful complete search impossible. Consequently, the search has been restricted to the use of the compounds specifically mentioned in claim 5 in relation to the disorders specifically mentioned in claim 3 and 4.

No Written Opinion will be formulated with respect to subject matter which is not covered by the search report.

Re Item V.

- 1 Reference is made to the following documents:
D1 : WO 02/28434 A (GLAXO GROUP LIMITED; BUCHAN, KEVIN, WILLIAM) 11 April 2002 (2002-04-11)
D2 : WO 02/28433 A (GLAXO GROUP LIMITED; BUCHAN, KEVIN, WILLIAM) 11 April 2002 (2002-04-11)
D3 : WO 02/13812 A (PERSHADSINGH, HARRIHAR, A) 21 February 2002 (2002-02-21)

2 CLAIMS 1-7

**WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING
AUTHORITY (SEPARATE SHEET)**

International application No.

PCT/US2005/010371

- 2.1** The present application does not meet the criteria of Article 33(1) PCT, because the subject-matter of claim 1-7 is not new in the sense of Article 33(2) PCT.
Document D1 discloses (see the passages cited in the search report) the use of the PPAR delta agonist {2-methyl-4- [4-methyl-2- (4- trifluoromethyl phenyl) thiazol-5-ylmethylthio] phenoxy}-acetic acid for treating multiple sclerosis.
- 2.2** The present application does not meet the criteria of Article 33(1) PCT, because the subject-matter of claim 1-7 is not new in the sense of Article 33(2) PCT.
Document D2 discloses (see the passages cited in the search report) the use of the PPAR delta agonist {2-methyl-4- [4-methyl-2- (4- trifluoromethyl phenyl) thiazol-5-ylmethylthio] phenoxy}-acetic acid and pharmaceutically acceptable salts to treat multiple sclerosis.
- 2.3** The present application does not meet the criteria of Article 33(1) PCT, because the subject-matter of claim 1,3,4 is not new in the sense of Article 33(2) PCT.
Document D3 discloses (see the passages cited in the search report) the use of PPAR delta ligands to treat demyelinating diseases.
- 3 CLAIMS 1-7**
Claims 1-7 do not contain any features which, in combination with the features of any claim to which they refer, meet the requirements of the PCT in respect of novelty and/or inventive step (Article 33(2) and (3) PCT).

**WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY**

International application No.
PCT/US2005/010371

Box No. I Basis of the opinion

1. With regard to the language, this opinion has been established on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.
 - ☐ This opinion has been established on the basis of a translation from the original language into the following language , which is the language of a translation furnished for the purposes of international search (under Rules 12.3 and 23.1(b)).
2. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, this opinion has been established on the basis of:
 - a. type of material:
 - ☐ a sequence listing
 - ☐ table(s) related to the sequence listing
 - b. format of material:
 - ☐ in written format
 - ☐ in computer readable form
 - c. time of filing/furnishing:
 - ☐ contained in the international application as filed.
 - ☐ filed together with the international application in computer readable form.
 - ☐ furnished subsequently to this Authority for the purposes of search.
3. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
4. Additional comments:

Box No. II Priority

1. ☒ The validity of the priority claim has not been considered because the International Searching Authority does not have in its possession a copy of the earlier application whose priority has been claimed or, where required, a translation of that earlier application. This opinion has nevertheless been established on the assumption that the relevant date (Rules 43bis.1 and 64.1) is the claimed priority date.
2. ☐ This opinion has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid (Rules 43bis.1 and 64.1). Thus for the purposes of this opinion, the international filing date indicated above is considered to be the relevant date.
3. Additional observations, if necessary:

**WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY**

International application No.
PCT/US2005/010371

Box No. III Non-establishment of opinion with regard to novelty, inventive step and Industrial applicability

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 examined in respect of:

- ☐ the entire international application,
- ☒ claims Nos. 1-5 (partially)

because:

- ☒ the said international application, or the said claims Nos. 1-5 with respect to industrial applicability 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 whole application or for said claims Nos. 1-4 (partially)
- ☐ the nucleotide and/or amino acid sequence listing does not comply with the standard provided for in Annex C of the Administrative Instructions in that:
 - the written form
 - ☐ has not been furnished
 - ☐ does not comply with the standard
 - the computer readable form
 - ☐ has not been furnished
 - ☐ does not comply with the standard
- ☐ the tables related to the nucleotide and/or amino acid sequence listing, if in computer readable form only, do not comply with the technical requirements provided for in Annex C-bis of the Administrative Instructions.
- ☐ See separate sheet for further details

**WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY**

International application No.
PCT/US2005/010371

Box No. V Reasoned statement under Rule 43bis.1(a)(I) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes: Claims	
	No: Claims	1-7
Inventive step (IS)	Yes: Claims	
	No: Claims	1-7
Industrial applicability (IA)	Yes: Claims	see separate sheet
	No: Claims	

2. Citations and explanations

see separate sheet

Box No. VI Certain documents cited

1. Certain published documents (Rules 43bis.1 and 70.10)

and / or

2. Non-written disclosures (Rules 43bis.1 and 70.9)

see form 210

PATENT COOPERATION TREATY

89 ✓
RECEIVED

JUN 12 2006

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

PCT PATENT DEPT.

To:

STRUPCZEWSKI, Joseph
Aventis Pharmaceuticals Inc.
Route 202-206
P. O. Box 6800
Bridgewater, NJ 08807-0800
ETATS-UNIS D'AMERIQUE

WRITTEN OPINION OF THE
INTERNATIONAL PRELIMINARY
EXAMINING AUTHORITY
(PCT Rule 66)

Date of mailing
(day/month/year)

08.08.2006

Applicant's or agent's file reference
UAS V2002/0240

REPLY DUE

within 2 month(s)
from the above date of mailing

International application No.
PCT/US2005/010371

International filing date (day/month/year)
29.03.2005

ACTION DUE

Priority date (day/month/year)
01.04.2004

Response Reply - 7/6/06

International Patent Classification (IPC) or both national classification and IPC
INV. A61K31/192 A61K31/426 A61P25/02 A61P25/28

DUE DATE

Reply Due 8/8/06

Applicant
AVENTIS PHARMACEUTICALS INC. et al

DKTD BY

ATTY

J. D. 6/12/06

I. Newman

- ☒ The written opinion established by the International Searching Authority.
☒ is ☐ is not
considered to be a written opinion of the International Preliminary Examining Authority
- This second report contains indications relating to the following items:
 - ☒ Box No. I Basis of the opinion
 - ☐ Box No. II Priority
 - ☒ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
 - ☐ Box No. IV Lack of unity of invention
 - ☒ Box No. V Reasoned statement under Rule 66.2(a)(II) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
 - ☐ Box No. VI Certain documents cited
 - ☐ Box No. VII Certain defects in the international application
 - ☐ Box No. VIII Certain observations on the international application
- 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(a).
 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 the examiner's obligation to consider amendments and/or arguments, see Rule 66.4bis. For an informal communication with the examiner, see Rule 66.6. For an additional opportunity to submit amendments, see Rule 66.4.
 If no reply is filed, the international preliminary examination report will be established on the basis of this opinion.
- The final date by which the international preliminary report on patentability (Chapter II of the PCT) must be established according to Rule 69.2 is: 01.08.2006

Name and mailing address of the international preliminary examining authority:

European Patent Office - P.B. 5818 Patentplan 2
NL-2280 HV Rijswijk - Pays Bas
Tel. +31 70 340 - 2040 Tlx 31 651 epo nl
Fax: +31 70 340 - 3018

Authorized Officer

A. Jakobs

Telephone No. +31 70 340-2617



**WRITTEN OPINION OF THE INTERNATIONAL
PRELIMINARY EXAMINING AUTHORITY**

International application No.
PCT/US2005/010371

Box No. I Basis of the opinion

1. With regard to the language, this opinion is based on the international application in the language in which it was filed, unless otherwise indicated under this item.
 - ☐ This opinion is based on translations from the original language into the following language, which is the language of a translation furnished for the purposes of:
 - ☐ international search (under Rules 12.3 and 23.1(b))
 - ☐ publication of the international application (under Rule 12.4)
 - ☐ international preliminary examination (under Rules 55.2 and/or 55.3)
2. With regard to the elements of the international application, this opinion is based on *(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

1-33 as originally filed

Claims, Numbers

1-5 received on 02.01.2006 with letter of 02.01.2006

Drawings, Sheets

1/7-7/7 as originally filed

- ☐ a sequence listing and/or any related table(s) - see Supplemental Box Relating to Sequence Listing.

3. ☒ The amendments have resulted in the cancellation of:
 - ☐ the description, pages
 - ☒ the claims, Nos. 6,7
 - ☐ the drawings, sheets/figs
 - ☐ the sequence listing *(specify)*:
 - ☐ any table(s) related to sequence listing *(specify)*:
4. ☐ 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, as indicated in the Supplemental Box (Rule 70.2(c)).
 - ☐ the description, pages
 - ☐ the claims, Nos.
 - ☐ the drawings, sheets/figs
 - ☐ the sequence listing *(specify)*:
 - ☐ any table(s) related to sequence listing *(specify)*:

**WRITTEN OPINION OF THE INTERNATIONAL
PRELIMINARY EXAMINING AUTHORITY**

International application No.
PCT/US2005/010371

Box No. 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 examined in respect of:

- ☐ the entire international application,
- ☒ claims Nos. 1-3 (partially)

because:

- ☒ the said international application, or the said claims Nos. 1-3 with respect to industrial applicability 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 opinion has been established for the said claims Nos.
- ☐ the nucleotide and/or amino acid sequence listing does not comply with the standard provided for in Annex C of the Administrative Instructions in that:
 - the written form ☐ has not been furnished
 - ☐ does not comply with the standard
 - the computer readable form ☐ has not been furnished
 - ☐ does not comply with the standard
- ☐ the tables related to the nucleotide and/or amino acid sequence listing, if in computer readable form only, do not comply with the technical requirements provided for in Annex C-bis of the Administrative Instructions.
- ☐ See supplemental sheet for further details

**WRITTEN OPINION OF THE INTERNATIONAL
PRELIMINARY EXAMINING AUTHORITY**

International application No.
PCT/US2005/010371

Box No. 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)	Yes: Claims	
	No: Claims	1-5
Inventive step (IS)	Yes: Claims	
	No: Claims	1-5
Industrial applicability (IA)	Yes: Claims	see separate sheet
	No: Claims	

2. Citations and explanations:

see separate sheet

**WRITTEN OPINION OF THE INTERNATIONAL
PRELIMINARY EXAMINING AUTHORITY
(SEPARATE SHEET)**

International application No.

PCT/US2005/010371

Re Item III.

Claims 1-3 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).

No Written Opinion will be formulated with respect to subject matter which is not covered by the search report.

Re Item V.

1 Reference is made to the following documents:

D1 : WO 02/28434 A (GLAXO GROUP LIMITED; BUCHAN, KEVIN, WILLIAM) 11 April 2002 (2002-04-11)

D2 : WO 02/28433 A (GLAXO GROUP LIMITED; BUCHAN, KEVIN, WILLIAM) 11 April 2002 (2002-04-11)

D3 : WO 02/13812 A (PERSHADSINGH, HARRIHAR, A) 21 February 2002 (2002-02-21)

2 CLAIMS 1-5

2.1 The present application does not meet the criteria of Article 33(1) PCT, because the subject-matter of claim 1-5 is not new in the sense of Article 33(2) PCT.

Document D1 discloses (see the passages cited in the search report) the use of the PPAR delta agonist {2-methyl-4- [4-methyl-2- (4- trifluoromethyl phenyl) thiazol-5-ylmethylthio] phenoxy}-acetic acid for treating multiple sclerosis and pharmaceutical compositions per se comprising {2-methyl-4- [4-methyl-2- (4- trifluoromethyl phenyl) thiazol-5-ylmethylthio] phenoxy}-acetic acid.

2.2 The present application does not meet the criteria of Article 33(1) PCT, because the subject-matter of claim 1-5 is not new in the sense of Article 33(2) PCT.

**WRITTEN OPINION OF THE INTERNATIONAL
PRELIMINARY EXAMINING AUTHORITY
(SEPARATE SHEET)**

International application No.

PCT/US2005/010371

Document D2 discloses (see the passages cited in the search report) the use of the PPAR delta agonist {2-methyl-4- [4-methyl-2- (4- trifluoromethyl phenyl) thiazol-5-ylmethylthio] phenoxy}-acetic acid and pharmaceutically acceptable salts to treat multiple sclerosis and pharmaceutical compositions per se comprising {2-methyl-4- [4-methyl-2- (4- trifluoromethyl phenyl) thiazol-5-ylmethylthio] phenoxy}-acetic acid.

- 2.3 The attention of the applicant is drawn to the fact that the disclosure of a document has to be considered as a whole and not only on the basis of the examples thereof. It is the overall disclosure which the skilled person can unambiguously take from a document which is decisive for establishing whether such disclosure is novelty destroying or not. This means that, when examining novelty, different passages of one document (here D1 or D2) may be combined provided that there are no reasons which would prevent a skilled person from such a combination. In general the technical teaching of examples may be combined with that disclosed elsewhere in the same document, e.g. in the description of a patent document, provided that the example concerned is indeed representative for the general technical teaching disclosed in the respective document.

3 CLAIMS 1-5

Claims 1-5 do not contain any features which, in combination with the features of any claim to which they refer, meet the requirements of the PCT in respect of novelty and/or inventive step (Article 33(2) and (3) PCT).

- 4 For the assessment of the present claims 1-5 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.

**WRITTEN OPINION OF THE INTERNATIONAL
PRELIMINARY EXAMINING AUTHORITY
(SEPARATE SHEET)**

International application No.

PCT/US2005/010371

PCT

In the
European Patent Office

Applicants File Reference:
USA V2002/0240 WO PCT

International Filing Date:
29/March/2005 (29.03.2005)

International Application No.:
PCT/US2005/010371

Priority Date:
01/April/2004 (01.04.2004)

Title of Invention:
**USE OF PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR DELTA
AGONISTS FOR THE TREATMENT OF MS AND OTHER DEMYELINATING
DISEASES**

Applicant(s):
AVENTIS PHARMACEUTICALS INC., et al

**REPLY TO THE WRITTEN OPINION BY THE INTERNATIONAL SEARCHING
AUTHORITY UNDER PCT ARTICLE 34(2)(b)**

European Patent Office
Erhardstr. 27
D-80298 Munich, Germany

This is a reply to the Written Opinion of the International Searching Authority of the European Patent Office on October 28, 2005 in the above-captioned application.

AMENDMENTS IN THE CLAIMS

Applicants have amended the claims. Claims 1 to 7 have been canceled and replaced by amended claims 1 to 5. No new matter has been added by these amendments.

RESPONSE

According to the Written Opinion of the ISA, claims 1-7 lack novelty and inventive step.

Claims 1 to 7 are replaced by amended claims 1 to 5. Applicants comment below on effect of the amended claims.

Amended claims 1 to 5 are directed to a second medical use of two known compounds for the treatment of demyelinating diseases and in particular multiple sclerosis. The references D1 and D2 disclose that compound of formula (I) may be useful for treating an extremely broad category of inflammatory diseases, which under said broad definition of inflammatory diseases includes multiple sclerosis (MS). The documents however offer no specific data to support the use in treating MS and in particular demyelinating diseases. As such, Applicants disclosure should be considered as a selection invention over the prior art. Applicants' provide data in the instant application that readily supports the secondary medical use of the compounds for the treatment of demyelinating diseases and MS.

If in the opinion of the Examiner a favorable IPER cannot be issued on the basis of the above claims and arguments, Applicants expressly request issuance of an additional written opinion under Rule 66.4 and/or institution of a telephone interview under Rule 66.6. It is submitted that such additional communication, if needed, will facilitate the issuance of a favorable IPER, and provide efficiencies in subsequent regional and national phase examination.

Respectfully submitted,

By 
Joseph Strupczewski, Reg. No. 50,903
Attorney/Agent for Applicant

Aventis Pharmaceuticals Inc.
Patent Department
Route #202-206 / P.O. Box 6800
Bridgewater, NJ 08807-0800
Telephone (908) 231-2387
Telefax (908) 231-2626

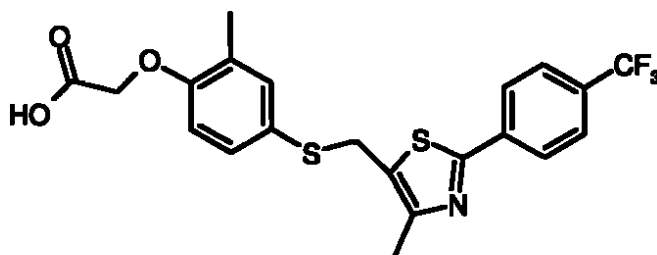
Aventis Docket No. USA V2002/0240 WO PCT
Dated: January 2, 2006

Attachments: Substitute sheets: 34 to 35.

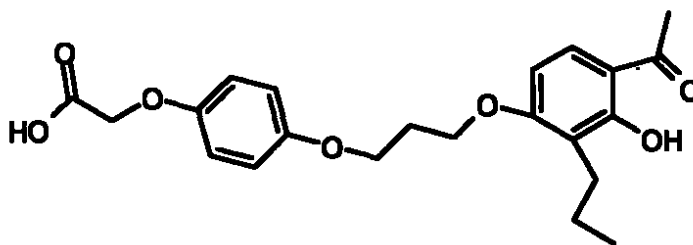
CLAIMS

What is claimed is:

- 5 1. A method for the treatment of demyelinating diseases in a patient comprising the administration of a therapeutically effective amount of a compound selected from the group consisting of compound of formula (1) and formula (2)



(1)

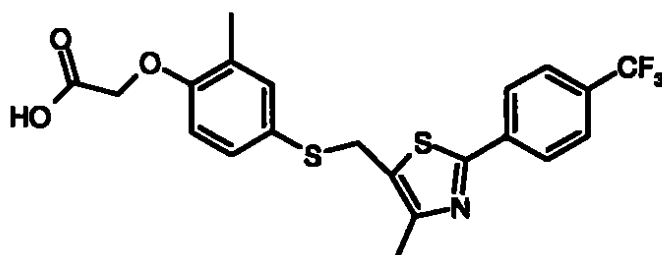


(2).

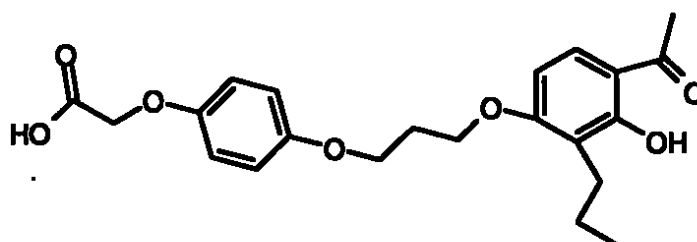
- 15 2. The method according to claim 1 wherein said demyelinating disease is selected from the group consisting of multiple sclerosis, Charcot-Marie-Tooth disease, Pelizaeus-Merzbacher disease, encephalomyelitis, neuromyelitis optica, adrenoleukodystrophy, Guillian-Barre syndrome and disorders in which myelin forming glial cells are damaged including spinal cord injuries, neuropathies and nerve injury.

- 20 3. The method according to claim 2 wherein the demyelinating disease is multiple sclerosis.

4. A pharmaceutical composition comprising a compound selected from the group consisting of compound of formula (1) and formula (2) in an amount effective for treating multiple sclerosis, Charcot-Marie-Tooth disease, Pelizaeus-Merzbacher disease, encephalomyelitis, neuromyelitis optica, adrenoleukodystrophy, Guillian-Barre syndrome and disorders in which myelin forming glial cells are damaged including spinal cord injuries, neuropathies and nerve injury in combination with at least one pharmaceutically acceptable carrier



(1)



(2).

5. The pharmaceutical composition according to claim 4 comprising an amount effective for treating multiple sclerosis.

PCT	
En la Oficina de Patentes Europea	
Referencia del solicitante USAV2002/0240 WO PCT	Fecha de presentación 29 de marzo 2005 (29.03.2005)
Solicitud internacional PCT/US2005/010371	fecha de la prioridad 01 de abril de 2004 (01.04.2004)
Titulo de la Invención: Uso de agonistas del receptor delta activado por proliferador de peroxisoma para el tratamiento de esclerosis múltiple (MS) y otras enfermedades de desmielinización	
Solicitante: Aventis Pharmaceuticals Inc., et al.	

respuesta a la opinión escrita de la Autoridad de Búsqueda internacional bajo el artículo 34 (2)(b)PCT

Oficina Europea de Patentes
Erhardstr 27
D- 80298 Munich, Alemania

Esta es una respuesta a la opinión escrita de la autoridad de búsqueda Internacional de la oficina Europea de patentes de octubre 28, 2005 en el caso arriba señalado

MODIFICACIONES A LAS REIVINDICACIONES

El Solicitante ha modificado las reivindicaciones. Reivindicaciones 1 a 7 han sido canceladas y reemplazadas por las reivindicaciones 1 a 5. No se ha introducido nueva materia en estas modificaciones.

RESPUESTA

De acuerdo con la opinión escrita de la ISA, las reivindicaciones 1-7 adolecen de novedad y de nivel inventivo. Las reivindicaciones 1-7 son reemplazadas por las reivindicaciones 1 a 5. El solicitante comenta a continuación el efecto de las reivindicaciones modificadas.

Las nuevas reivindicaciones 1 a 5 están dirigidas a un segundo uso medico de dos compuestos conocidos en el estado del arte para el tratamiento de las enfermedades de desmielinización y en particular a la esclerosis múltiple. Las referencias hechas a D1 y D2 revelan un compuesto de la formula (I) que puede ser útil para el tratamiento de un estado extremo de enfermedades inflamatorias, que bajo tal definición de enfermedades inflamatorias incluye la esclerosis múltiple (MS). Los documentos, sin embargo, no ofrecen una información específica que soporte el uso el tratamiento de MS y en particular de enfermedades de desmielinización. Por lo anterior, el solicitante provee de información en la presente solicitud que realmente soporta los uso secundarios de los compuestos para el tratamiento de enfermedades de desmielinización y MS.

Si en la opinión del examinador, un IPER no puede ser favorable sobre la base de las reivindicaciones y argumentos señalados, el solicitante, pide una opinión escrita adicional bajo la regla 66.4 y/o una conferencia telefónica bajo la regla 66.6. Se entiende que esa comunicación adicional permitirá obtener un IPER favorable y provea de eficiencia a la solicitud en las fases nacionales y regionales.

Respetuosamente presentado,

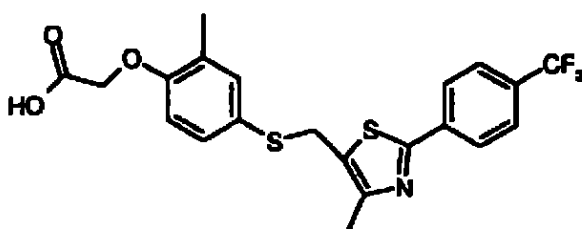
Por: _____
Joseph Strupczewski, Reg No. 50.903
Representante/agente del solicitante

Aventis Pharmaceuticals Inc.
(miembro del grupo sanofi aventis)
Operaciones Patentes Estados Unidos
Route No. 2002 - 206/P.O BOX 6800
Bridgewater, NJ 08807-0800
Telefono: (908)231-3679
Telefax (908) 231-2626
Aventis Docket No. USAV2004/0240 WO PCT
Fecha Enero 2, 2006
Anexos: Hojas sustituidas 34 a 35

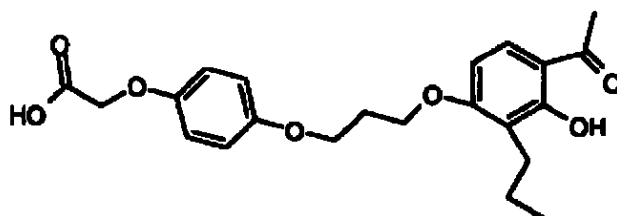
REIVINDICACIONES

Lo que se reivindica es:

1. Un método para el tratamiento de enfermedades de desmielinización en pacientes que comprende la administración de una cantidad terapéuticamente eficaz de un compuesto seleccionado de un grupo consistente en la formula (1) y la Formula (2)

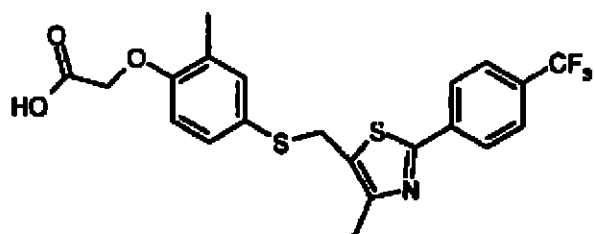


(1)

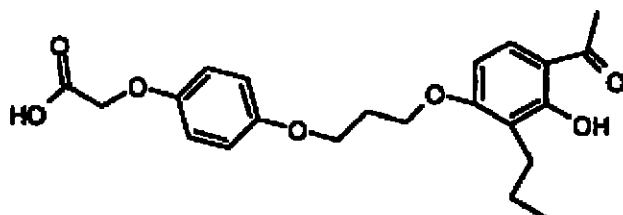


(2).

2. Un método según la reivindicación No. 1 en el que la enfermedad de desmielinización se selecciona de un grupo consistente en esclerosis múltiple, enfermedad de Charcot-Marie Tooth, enfermedad de Pelizaeus-Merzbacher, encefalomielitis, neuromielitis óptica, adrenoleucodistrofia, síndrome de Guillian-Barre y trastornos en los que están dañadas las células gliales formadoras de mielina, incluyendo lesiones de la médula espinal, neuropatías y lesiones nerviosas
3. Un método según la reivindicación No. 2 en el que la enfermedad de desmielinización es la esclerosis múltiple.
4. Una composición farmacéutica que comprende un compuesto seleccionado del grupo que consiste en el compuesto de la formula (I) y de la formula (2) en una cantidad eficaz para tratar la esclerosis múltiple, enfermedad de Charcot-Marie Tooth, enfermedad de Pelizaeus-Merzbacher, encefalomielitis, neuromielitis óptica, adrenoleudodistrofia, síndrome de Guillén-Barre y trastornos en los que están dañadas las células gliales formadoras de mielina, incluyendo lesiones de la médula espinal, neuropatías y lesiones nerviosas, en combinación con por lo menos un vehículo farmacéuticamente aceptable.



(1)



(2).

5. La composición farmacéutica, según la reivindicación No. 4 que comprende una cantidad eficaz para tratar la esclerosis múltiple.

PCT

In the
European Patent Office

Applicants File Reference:
USA V2002/0240 WO PCT

International Filing Date:
29 March 2005 (29/03/2005)

International Application No.:
PCT/US2005/010371

Priority Date:
01 April 2004 (01/04/2004)

Title of Invention:
USE OF PPAR DELTA AGONISTS FOR TREATING DEMYELINATING DISEASES

Applicant(s):
AVENTIS PHARMACEUTICALS INC. et al.

**REPLY TO THE WRITTEN OPINION OF THE INTERNATIONAL PRELIMINARY
EXAMINING AUTHORITY (PCT Rule 66)**

European Patent Office
Erhardstr. 27
D-80298 Munich, Germany

This is a reply to the Written Opinion of the International Preliminary Examining Authority on June 8, 2006 in the above-captioned application.

AMENDMENTS IN THE CLAIMS

Applicants have amended the claims. Claims 6 to 8 have been added and amended claims 1 to 5 have been retained. No new matter has been added by these amendments.

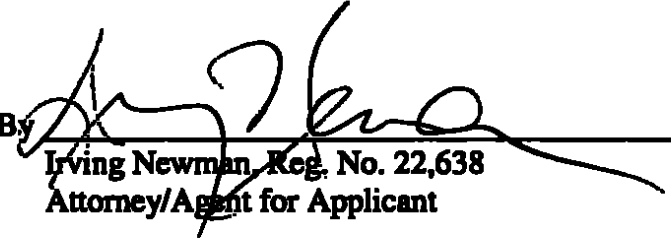
RESPONSE

According to the Written Opinion of the IPEA, claims 1-5 lack novelty and inventive step and fail to meet the criteria of industrial applicability in, e.g. IEPO.

Claims 6 to 8 are added to conform to the industrial applicability requirement discussed in item 4. the penultimate page of the written opinion. Applicants comment below on effect of the amended claims.

Amended claims 1 to 8 are directed to a second medical use of two known compounds for the treatment of demyelinating diseases and in particular multiple sclerosis. The references D1 and D2 disclose that compound of formula (I) may be useful for treating an extremely broad category of inflammatory diseases, which under said broad definition of inflammatory diseases includes multiple sclerosis (MS). The documents however offer no specific data to support the use in treating MS and in particular demyelinating diseases. As such, Applicants disclosure should be considered as a selection invention over the prior art. Applicants' provide data in the instant application that readily supports the secondary medical use of the compounds for the treatment of demyelinating diseases and MS. Claims 1-5 conform to the requirements of the USPTO whereas claims 6-9 conform to the requirements of e.g., the EPO.

Respectfully submitted,

By 
Irving Newman, Reg. No. 22,638
Attorney/Agent for Applicant

Aventis Pharmaceuticals Inc.
Patent Department
Route #202-206 / P.O. Box 6800
Bridgewater, NJ 08807-0800
Telephone (908) 231-2785
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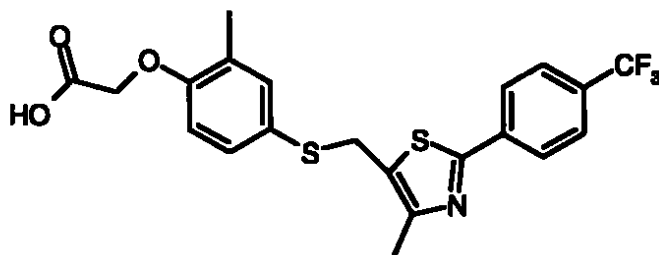
Aventis Docket No. USA V2002/0240 WO PCT
Dated: August 8, 2006

Attachments: Substitute sheets: 34 to 36.

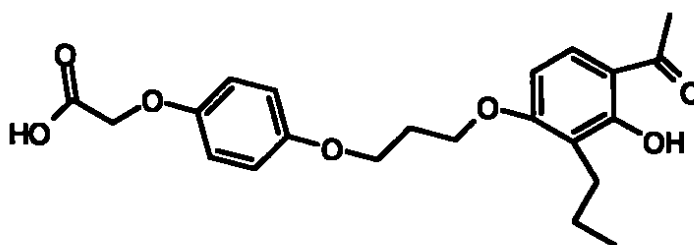
CLAIMS

What is claimed is:

- 5 1. A method for the treatment of demyelinating diseases in a patient comprising the administration of a therapeutically effective amount of a compound selected from the group consisting of compound of formula (1) and formula (2)



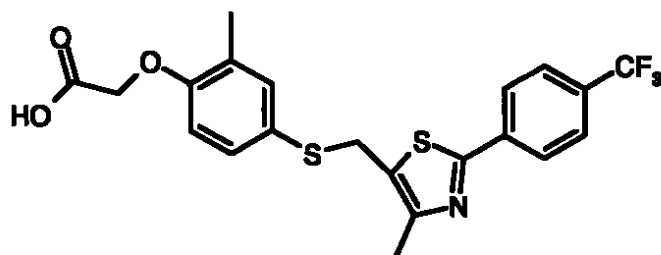
(1)



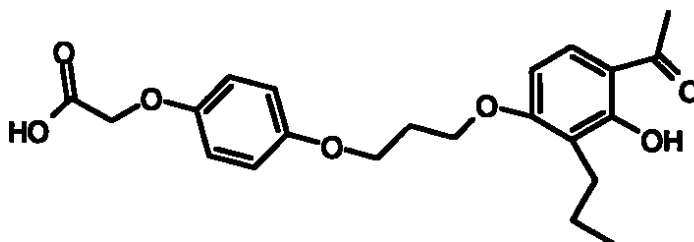
(2).

- 15 2. The method according to claim 1 wherein said demyelinating disease is selected from the group consisting of multiple sclerosis, Charcot-Marie-Tooth disease, Pelizaeus-Merzbacher disease, encephalomyelitis, neuromyelitis optica, adrenoleukodystrophy, Guillian-Barre syndrome and disorders in which myelin forming glial cells are damaged including spinal cord injuries, neuropathies and nerve injury.
- 20 3. The method according to claim 2 wherein the demyelinating disease is multiple sclerosis.
- 25

4. A pharmaceutical composition comprising a compound selected from the group consisting of compound of formula (1) and formula (2) in an amount effective for treating multiple sclerosis, Charcot-Marie-Tooth disease, Pelizaeus-Merzbacher disease, encephalomyelitis, neuromyelitis optica, adrenoleukodystrophy, Guillian-Barre syndrome and disorders in which myelin forming glial cells are damaged including spinal cord injuries, neuropathies and nerve injury in combination with at least on pharmaceutically acceptable carrier



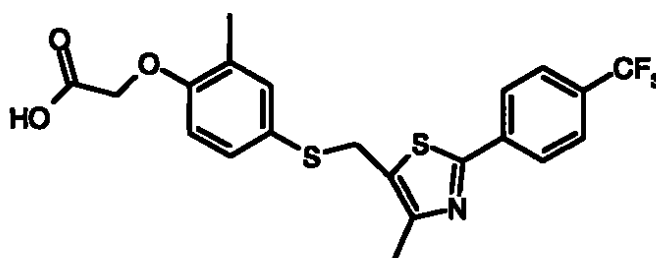
(1)



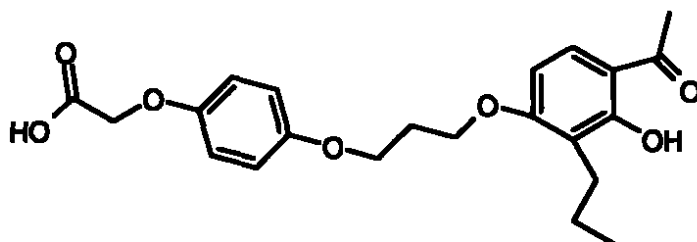
(2).

5. The pharmaceutical composition according to claim 4 comprising an amount effective for treating multiple sclerosis.

6. The use of a compound selected from the group consisting of compound of formula (1) and formula (2)



(1)

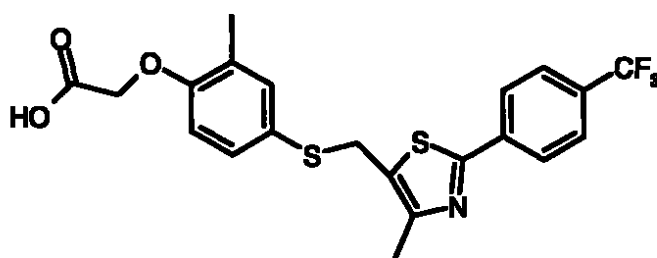


(2)

to treat a demyelinating disease.

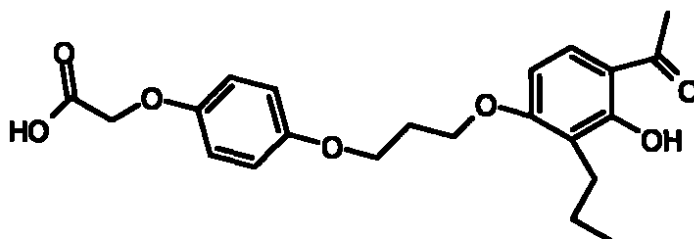
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7. The use of a compound selected from the group consisting of compound of formula (1) and formula (2)



(1)

10



(2)

to prepare a pharmaceutical composition for the treatment of a demyelinating disease.

15

8. The use of claim 6 or 7 wherein said demyelinating disease is selected from multiple sclerosis, Charcot-Marie-Tooth disease, Pelizaeus-Merzbacher disease, encephalomyelitis, neuromyelitis optica, adrenoleukodystrophy, Guillian-Barre syndrome and disorders in which myelin forming glial cells are damaged including spinal cord injuries, neuropathies and nerve injury

20

PCT

**En la
Oficina de Patentes Europea**

Referencia del solicitante
USAV2002/0240 WO PCT

Fecha de presentación
29 de marzo 2005 (29.03.2005)

Solicitud internacional
PCT/US2005/010371

fecha de la prioridad
01 de abril de 2004 (01.04.2004)

Título de la Invención:

Uso de agonistas del receptor delta activado por proliferador de peroxisoma para el tratamiento de esclerosis múltiple (MS) y otras enfermedades de desmielinización

Solicitante:

Aventis Pharmaceuticals Inc., et al.

**respuesta a la opinión de la Autoridad Examinadora internacional
(Regla PCT 66)**

Oficina Europea de Patentes
Erhardstr 27
D- 80298 Munich, Alemania

Esta es una respuesta a la opinión de la autoridad de examinadora Internacional de la oficina Europea de patentes de junio 8, 2005 en el caso arriba señalado

MODIFICACIONES A LAS REIVINDICACIONES

El Solicitante ha modificado las reivindicaciones. Reivindicaciones 6 a 8 han sido adicionadas y modificadas y las reivindicaciones 1 a 5 han sido mantenidas. No se ha introducido nueva materia en estas modificaciones.

RESPUESTA

De acuerdo con la opinión escrita de la ISA, las reivindicaciones 1-7 adolecen de novedad y de nivel inventivo.

Las reivindicaciones 1-7 son reemplazadas por las reivindicaciones 1 a 5. El solicitante comenta a continuación el efecto de las reivindicaciones modificadas.

Las nuevas reivindicaciones 1 a 5 están dirigidas a un segundo uso medico de dos compuestos conocidos en el estado del arte para el tratamiento de las enfermedades de desmielinización y en particular a la esclerosis múltiple. Las referencias hechas a D1 y D2 revelan un compuesto de la formula (I) que puede ser útil para el tratamiento de un estado extremo de enfermedades inflamatorias, que bajo tal definición de enfermedades inflamatorias incluye la esclerosis múltiple (MS). Los documentos, sin embargo, no ofrecen una información específica que soporte el uso el tratamiento de MS y en particular de enfermedades de desmielinización. Por lo anterior, el solicitante provee de información en la presente solicitud que realmente soporta los uso secundarios de los compuestos para el tratamiento de enfermedades de desmielinización y MS. Las reivindicaciones 1 a 5 cumplen con los requisitos de la USPTO y en las reivindicaciones 6 a 9 cumplen con los requisitos de la EPO

Respetuosamente presentado,

Por: _____
Irving Newman

Reg. No. 22.638
solicitante

Representante/agente del

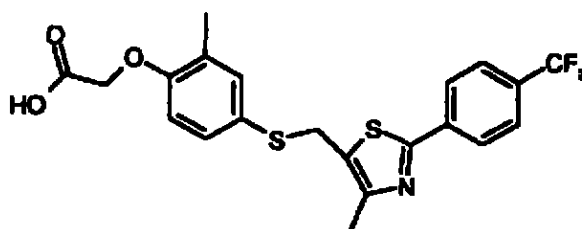
Aventis Pharmaceuticals Inc.
(miembro del grupo sanofi aventis)
Operaciones Patentes Estados Unidos
Route No. 2002 - 206/P.O BOX 6800
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Telefono: (908)231-3679
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Aventis Docket No. USAV2004/0240 WO PCT
Fecha Enero 2, 2006
Anexos: Hojas sustituidas 34 a 36

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109

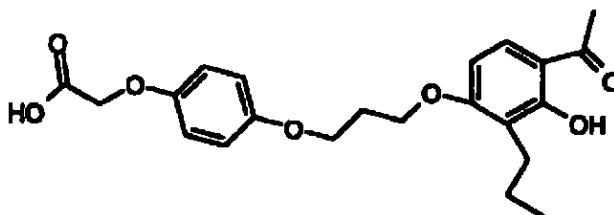
REIVINDICACIONES

Lo que se reivindica es:

1. Un método para el tratamiento de enfermedades de desmielinización en pacientes que comprende la administración de una cantidad terapéuticamente eficaz de un compuesto seleccionado de un grupo consistente en la formula (1) y la Formula (2)

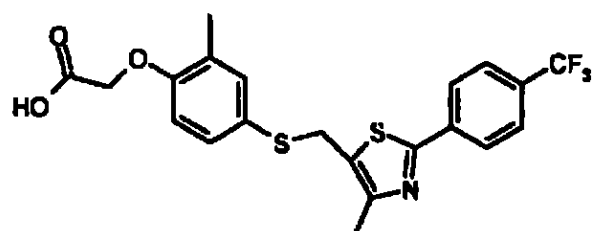


(1)

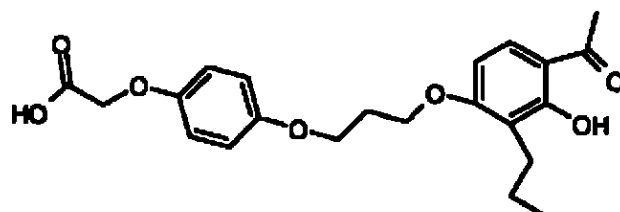


(2).

2. Un método según la reivindicación No. 1 en el que la enfermedad de desmielinización se selecciona de un grupo consistente en esclerosis múltiple, enfermedad de Charcot-Marie Tooth, enfermedad de Pelizaeus-Merzbacher, encefalomielitis, neuromielitis óptica, adrenoleucodistrofia, síndrome de Guillian-Barre y trastornos en los que están dañadas las células gliales formadoras de mielina, incluyendo lesiones de la médula espinal, neuropatías y lesiones nerviosas
3. Un método según la reivindicación No. 2 en el que la enfermedad de desmielinización es la esclerosis múltiple.
4. Una composición farmacéutica que comprende un compuesto seleccionado del grupo que consiste en el compuesto de la formula (1) y de la formula (2) en una cantidad eficaz para tratar la esclerosis múltiple, enfermedad de Charcot-Marie Tooth, enfermedad de Pelizaeus-Merzbacher, encefalomielitis, neuromielitis óptica, adrenoleudodistrofia, síndrome de Guillén-Barre y trastornos en los que están dañadas las células gliales formadoras de mielina, incluyendo lesiones de la médula espinal, neuropatías y lesiones nerviosas, en combinación con por lo menos un vehículo farmacéuticamente aceptable.

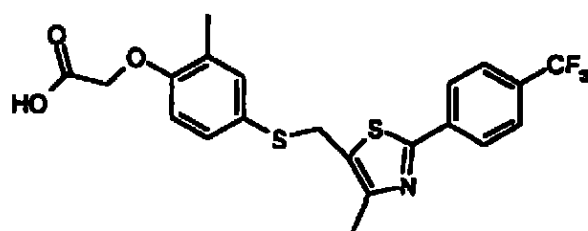


(1)

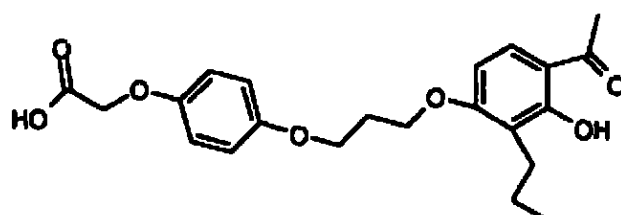


(2).

5. La composición farmacéutica, según la reivindicación No. 4 que comprende una cantidad eficaz para tratar la esclerosis múltiple.
6. El uso del compuesto seleccionado del grupo consistente en el compuesto de la formula (1) y de la formula (2)



(1)

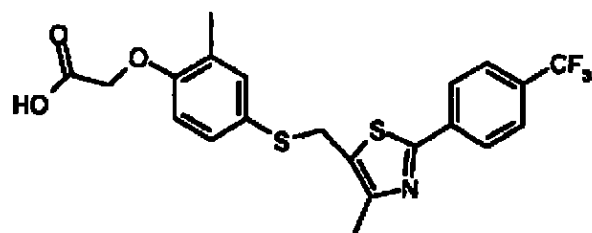


(2)

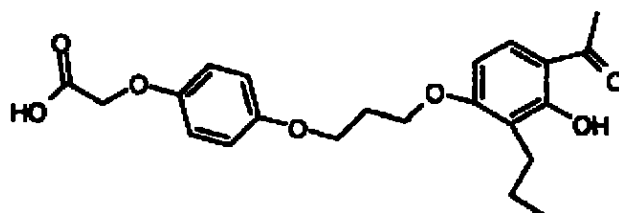
Para trata las enfermedades de desmielinización

7. El uso de un compuesto seleccionado del grupo consistente en el compuesto de la formula (1) y la formula (2)

140
111



(1)



(2)

Para preparar una composición farmacéutica para el tratamiento de las enfermedades de desmielinización

8. El uso de las reivindicaciones 6 y 7 en el que dicha enfermedad de desmielinización es seleccionada de la esclerosis múltiple, Charcot-Marie Tooth, enfermedad de Pelizaeus-Merzbacher, encefalomielitis, neurtomielitis óptica, adrenoleucodistrofia, síndrome de Guillian-Barre y trastornos en los que están dañadas las células gliales formadoras de mielina, incluyendo lesiones de la médula espinal, neuropatías y lesiones nerviosas



Industria y Comercio

SUPERINTENDENCIA

112 HT

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
Rad: 06-008506-00000-0000
Fec: 2006-09-29 16:21:51 Dep. 2080 NUECREACIONES
Tm: 11 PATENTEYII
Eve: 378 FASENACIONALII
Adi 411 PRESENTACION FOME 110

Delegatura de Propiedad Industrial División de Nuevas Creaciones

REQUISITOS NECESARIOS PARA PRESENTACIÓN Y AGILIZACIÓN DEL TRAMITE

PATENTE DE INVENCION ()	MODELO DE UTILIDAD ()	USUARIO ()	RADICADOR ()
-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 o de la persona que se presenta la solicitud.	()	()	
-Representación gráfica y 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 se solicita el registro de Diseño Industrial.	()	()	
-Datos de identificación del solicitante o de la persona que se presenta la solicitud.	()	()	
-Representación gráfica del Esquema Trazado.	()	()	
-Comprobante de Pago de las tasas establecidas.	()	()	
COMPLETA ()	INCOMPLETA ()	()	()

Firma Responsable:

Fdo B

Fecha:

29. Sep. 6