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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIVISION DE NUEVAS CREACIONES
E. S. D.

Re: Expediente No. **05.022.398**
Solicitud de patente a nombre de **NOVARTIS AG.**

Yo, DILIA MARIA RODRIGUEZ D'ALEMAN, actuando como apoderada sustituta de acuerdo con la sustitución presentada el 17 de abril de 2009, identificada como aparece al pie de mi firma, domiciliada en la carrera 11 A No. 86-53, piso 6, obrando como apoderada de la sociedad **NOVARTIS AG.**, domiciliada en Basilea Suiza, solicitante de la patente de la referencia, en respuesta al oficio No. 452 notificado por fijación en lista el 15 de enero de 2010 relacionado con el concepto técnico de fondo No. 04234, me permito manifestar:

1. Con fundamento en la facultad que confiere el artículo 34 de la Decisión 486 al solicitante para modificar la solicitud de patente en cualquier momento del trámite, presento junto con este memorial nuevo juego de reivindicaciones que consta de 5 reivindicaciones y en el cual se realizaron las siguientes modificaciones.

1.1 Se cancelaron las antiguas reivindicaciones 1 y 6 y se renumeraron las antiguas cláusulas 2 a 5 y 7 como las nuevas reivindicaciones 1 a 5.

1.2 Se cancelaron de la antigua reivindicación 2, los compuestos (racemico)-3-(5-fenil-piridin-2-iloxi)-1-aza-biciclo[2.2.2]octano y (R)-3-(5-fenil-piridin-2-iloxi)-1-aza-biciclo[2.2.2]octano.

En cuanto a las modificaciones es deseo de la solicitante manifestar que las mismas no amplían el alcance de la presente invención y se realizan con el ánimo de cumplir con los requerimientos de novedad y altura inventiva, expuestos por el señor examinador en el concepto técnico No. 04234.

Teniendo en cuenta el concepto técnico emitido por el Señor Superintendente Delegado para la propiedad intelectual GianCarlo Marcenaro Jiménez a la doctora Ana Maria Castro Wey, que da alcance a las comunicaciones No. 09-001524 y 09-012566, no se presenta una tasa adicional, toda vez que el nuevo

capítulo reivindicatorio comprende menos de 15 reivindicaciones y la solicitud fue presentada con anterioridad al 1 de enero de 2009.

En ese orden de ideas, nos permitimos manifestar que a la luz de las modificaciones realizadas en el capítulo reivindicatorio, la invención cumple a cabalidad con los requisitos de patentabilidad pues los compuestos ahora incluidos en las reivindicaciones son novedosos y no habrían sido evidentes para una persona medianamente versada en la técnica a partir de lo divulgado en las anterioridades D1 y D5.

2. Claridad

En relación con la objeción planteada por el señor examinador en relación con el compuesto 3-(5-fenil-piridin-2-iloxi)-1-aza-biciclo[2.2.2]octano, en aras de no entrar en polémicas y de agilizar la concesión de la presente solicitud, el solicitante ha cancelado este término de la nueva reivindicación 1. Así las cosas, nos permitimos decir que quedan cabalmente atendidas y superadas las objeciones de claridad del concepto técnico No. 04234.

3. Novedad

El señor examinador objeta parcialmente la novedad de la solicitud en estudio en relación con el documento CA 2042860 (D5)

Con relación a la argumentación presentada en el concepto técnico No. 04234, resulta pertinente dirigir la atención del señor examinador al artículo 16 de la Decisión 486 donde se establece que: “Una invención se considera nueva cuando no está comprendida en el estado de la técnica.”

El estado de la técnica comprenderá todo lo que haya sido accesible al público por una descripción escrita u oral, utilización, comercialización o cualquier otro medio antes de la fecha de presentación de la solicitud de patente o, en su caso, de la prioridad reconocida....”(el resaltado es nuestro).

Si de acuerdo con el espíritu de la norma legal indicada, la invención no se considera como nueva si está “comprendida” en el estado de la técnica, dicha norma se está refiriendo, naturalmente, a la totalidad del invento y no a alguno o a algunos de los componentes que hacen parte de la nueva solicitud.

En efecto, para que la invención pierda su calidad de novedosa, es claro que ella, TODA ELLA, ha de estar contenida en el “conjunto de conocimientos existentes que son aplicables al proceso industrial” como define el estado de la técnica el Tribunal de justicia del Acuerdo de Cartagena (Proceso No. 6-IP-89, sentencia de 31 de octubre de 1989, publicada en Jurisprudencia del Tribunal de Justicia del Acuerdo de Cartagena, Tomo 11, BID-INTAL, Buenos Aires, 1994, página 60).

Esto se conoce como **NOVEDAD ABSOLUTA**, de conformidad con lo cual para desvirtuar el requisito de novedad de una invención será necesario que lo reivindicado por la invención bajo estudio corresponda de **forma total** con lo revelado en un documento o anterioridad señalada, conclusión a la que se llega después de un análisis exhaustivo mediante una comparación entre el capítulo reivindicatorio de la invención en estudio y la divulgación citada por ese despacho como anterioridad.

Ahora bien, si un determinado elemento, condición o circunstancia de la nueva creación ya hace parte del estado de la técnica no quiere ello decir, desde luego, que la invención deba ser rechazada por carecer del requisito de novedad. No sin razón ha dicho el mencionado Tribunal: *"hay que advertir que toda creación es en cierta forma, producto del estado de la técnica existente, en el sentido de que el inventor ha de tener en cuenta un conjunto de conocimientos técnicos que le permitan crear nuevos procedimientos aplicables a la industria"* (Ibidem, páginas 53 y 54) (El subrayado es nuestro).

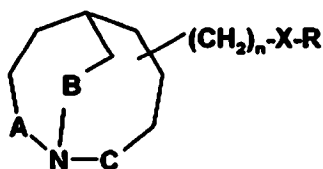
Por lo tanto y teniendo en cuenta lo anteriormente expuesto, como mínimo la novedad debe contemplar los siguientes aspectos:

-Que la invención en estudio esté contenida de forma **TOTAL** y **ABSOLUTA** en el documento que se menciona como anterioridad; y

-Que la invención en cuestión puede partir de conocimientos conocidos, caso en el cual habrá que hacer la comparación no de lo conocido sino de lo inédito y novedoso, teniendo que analizarse el **valor agregado** que aporta la invención con sus nuevas características al campo técnico al cual pertenece la solicitud en estudio.

Aplicando lo anterior al caso concreto, para que las razones que aduce ese despacho para desvirtuar la novedad fuesen ciertas, sería forzoso que se demostrara que la invención de mi mandante está comprendida en el estado de la técnica de forma **TOTAL**, lo cual no sucede como a continuación se demostrará.

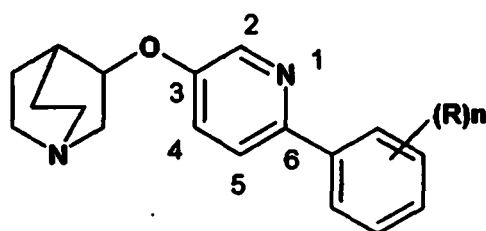
En efecto, la anterioridad D5 hace referencia de manera muy amplia y general a agonistas del receptor de acetilcolina muscarínico (mAChR agonistas) como se puede corroborar en la página 15, líneas 3 a 6, siendo dichos compuestos derivados de 1-azabicycloalcanos de la fórmula I:



4

En donde A, B ó C pueden ser CH_2 o un enlace sencillo; n puede ser 0, 1 ó 2 y X es oxígeno o azufre. Además, R es un alquilo, alquenilo, alquinilo, fenilo, bifenilo o anillo heterocíclico de 5 a 7 miembros (incluyendo un 3-piridil opcionalmente sustituido), todos ellos opcionalmente sustituidos. Aunque D5 ejemplifica 62 compuestos, incluyendo el compuesto 3-(piridin-iloxi)-1-azabicyclo[2.2.2]octano (ejemplo 1), D5 no ejemplifica un compuesto sencillo que sea 3-(6-fenil-piridin-3-il-oxi)-1-azabicyclo[2.2.2]octano.

En contraste, la presente invención se encuentra ahora restringida a un juego de once (11) quinuclidina-ésteres con características estructurales novedosas y actividad también novedosa. Los compuestos de la invención se encuentran unificados por la siguiente estructura



En donde n es 0 a 2 y R es seleccionado de halógeno, alquilo y haloalquilo. En este sentido, el señor examinador puede corroborar que la más notable característica estructural en el contexto de la presente invención es la presencia del novedoso anillo fenílico en la posición 6 de la parte del grupo pirid-3-il. Aún más, los compuestos de la invención son agonistas selectivos del receptor $\alpha_7\text{-nAChR}$, como así se expone en la página 15, líneas 7 y 8 de la memoria descriptiva.

Por consiguiente, teniendo en cuenta que ni las características estructurales antes mencionadas, es decir, el sustituyente fenílico en la posición 6 en el anillo piridínico, ni la actividad (agonismo receptor $\alpha_7\text{-nAChR}$) son revelados en la anterioridad D5, se concluye que los compuestos del nuevo juego de reivindicaciones son novedosos sobre las enseñanzas de esta anterioridad.

4. Nivel inventivo

El señor examinador manifiesta en los folios 186 y 187 de su concepto técnico que la invención en estudio es evidente para una persona medianamente versada en la materia en vista de la combinación de enseñanzas revelada por las anterioridades D1 y D5.

Sobre el particular, nos permitimos decir que como ya se expuso en el punto anterior de novedad, la anterioridad D5 describe agonistas mAChR que son útiles como medicamentos para el tratamiento de la hipofunción colinérgica, tal como el déficit cognoscitivo asociado con la enfermedad de Alzheimer como así se expone en la página 16, entre las líneas 13 a 18 de D5.

Por el contrario, la presente invención se encuentra relacionada con un conjunto novedoso de ésteres de quinuclidina que son agonistas selectivos de alfa7-nAChR, como se expone en la página 15, líneas 7-8 de la descripción, los cuales son útiles entre otras cosas, para el tratamiento del déficit cognoscitivo asociado con la enfermedad de Alzheimer como se establece en la página 16, línea 17 hasta la página 17 línea 10 de la descripción.

En ese orden de ideas, los datos biológicos que fueron presentados con nuestro memorial del 1 de diciembre de 2009, proporcionan evidencia en cuanto a que los compuestos de la invención muestran **selectividad** entre los receptores nicotínicos y muscarínicos AChRs, por ejemplo, el compuesto 63 tiene una afinidad por alfa7-nAChR de 81 nM, pero mayor de 10 µM para los receptores mAChRs. Esto demuestra que los compuestos de la invención en estudio tienen un **modo diferente de acción** en relación con los compuestos descritos en la anterioridad D5.

Sobre este particular, es importante señalar que todos los tipos de receptor mencionados son activados mediante el mismo neurotransmisor (acetilcolina) y son expresados en el sistema nervioso central. Por lo tanto, sus agonistas son considerados como fármacos colinérgicos para tratar entre otras cosas, el déficit cognoscitivo en la enfermedad de Alzheimer.

Es sabido que existen cinco receptores mAChR: M1 a M5. Se encuentra evidencia que el agonismo de M1 puede ser beneficioso para pacientes con déficit cognoscitivo asociado con la enfermedad de Alzheimer, como así se expone en MJ Sheardon, Expert Opinión on Therapeutic Patents (2002), 12(6), tabla 1, en la página 865 (véase anexo 1). No obstante el agonismo de M1 podría tener efectos laterales fuertes como (i) existe evidencia que sugiere que la activación de M1 en células post-ganglionares simpáticas pueden alterar las respuestas cardiovasculares, que podría conllevar a un incremento en la velocidad cardíaca (taquicardia) y en la presión arterial (SN Hardouin y colaboradores, Journal of Pharmacology and Experimental Therapeutics (2002), 301, página 136, líneas 22-26) (anexo 2) y (ii) existe evidencia que la activación de M1 puede afectar la regulación del ritmo circadiano lo cual puede tener un impacto sobre el funcionamiento del organismo de los pacientes durante los ciclos día-noche (MU Gillette y colaboradores, Life Sciences (2001), 68, figura 3, página 2470 y página 2470, línea 1 hasta la página 2471 línea 12. (Anexo 3).

Una dificultad significativa para la búsqueda de fármacos basados en agonistas de M1 es que al parecer es muy difícil encontrar agonistas que sean selectivos para M2 y M3. De hecho, como se evidencia por medio de la publicación Review de MJ Sheardown, en 2002 (anexo 1), documento que fue publicado once años después de la divulgación de D5), no se ha conocido ningún agonista relevante por lo menos 100 veces más selectivo en este campo, como así se puede apreciar en la página 866, columna izquierda, líneas 1 a 6 del

artículo del anexo 1. Incluso se ha sabido que con la activación en paralelo de los receptores M2/M3 se encuentran efectos secundarios severos relacionados como la fuerza y la velocidad de contracción reducidas del corazón (M2), una motilidad gastrointestinal aumentada y diarrea (M2/M3) y salivación (M3) como se puede apreciar en la página 865, columna izquierda entre las líneas 31-38 del artículo del anexo 1.

Comparado con el receptor M1, el receptor alfa7nAChR muestra muchas diferencias:

- (1) **Patrón de expresión:** aunque ambos receptores son expresados en el sistema nervioso central, el patrón de expresión es distinto incluso a nivel sub-celular. M1 es expresado principalmente de manera post-sináptica, en tanto que el receptor alfa7-nAChR se encuentra localizado de manera pre y postsináptica como también sobre la soma y las dendritas;
- (2) **Estructura molecular:** M1 es una proteína siete-trasmembranal unida a cascadas del mensajero secundario intracelular. Por su parte el receptor alfa7 nAChR es de canal de catión;
- (3) **Farmacología:** El sitio de unión del agonista (acetilcolina) de M1 es activado mediante muscarina, el sitio de unión del agonista del alfa7 nAChR es activado mediante nicotina. Aún más, el producto de degradación extracelular de acetilcolina,

Con base en la información anterior, es claro que existen diferencias significativas en el modo de acción de un agonista M1 y un agonista alfa7 nAChR.

Aunado a lo anterior, se cree que los agonistas alfa7nAChR tienen un perfil de efecto secundario más favorable y diferente, lo cual es particularmente importante para las medicaciones crónicas, tal como la terapia del déficit cognoscitivo en pacientes con la enfermedad de Alzheimer. Como evidencia de esto se ha probado en humanos el GTS-21, un agonista del nAChR en dosis de hasta 450 mg por día, mostrando mejoramientos en el comportamiento cognoscitivo pero no sin encontrarse ninguna seguridad clínica significativa como así se establece en el artículo de H. Kitagawa y colaboradores, *Neuropsychopharmacology* (2003), página 550, columna izquierda, líneas 1 a 3. (Anexo 4).

Adicionalmente, aunque se ha reconocido que los agonistas de M1 y alfa7nAChR son útiles para las mismas o comunes indicaciones, tal como hipofunciones del sistema colinérgico del tipo déficit de la cognición asociada con la enfermedad de Alzheimer, es un beneficio para los pacientes si los fármacos para la misma indicación existente tengan un modo diferente de acción dado que (i) esto abre una ruta terapéutica diferente ya que algunos pacientes pueden no responder a una terapia determinada y (ii) estos fármacos

pueden presentar perfiles de efectos laterales más favorables y diferentes puesto que incluso si la primera ruta funcionara, dicho paciente podría sufrir de, o no tolerar efectos secundarios asociados con la primera opción de tratamiento, siendo especialmente importante para los tratamientos crónicos.

Así las cosas, la propiedad de los compuestos de la presente invención de ser selectivos agonistas de $\alpha 7$ nAChR es una propiedad muy importante con una significativa relevancia clínica, toda vez que los compuestos pueden ser útiles para pacientes que no respondieran a la terapia de agonismo de M1, es decir, para una población distinta de pacientes que pudieran tener un perfil diferente de efectos laterales, lo cual puede ser beneficioso incluso para pacientes que responden al agonismo de M1 y que sufrirían efectos laterales asociados y que no los tolerarían, especialmente teniendo en cuenta el hecho de la selectividad potencial M1/M2/M3.

Por lo tanto, gracias a esta **propiedad inesperada** resulta claro entonces que los compuestos de la invención en estudio a la luz de las enseñanzas de D5, involucran un nivel inventivo y por lo tanto hace que sean merecedores de la protección a través de patente de invención.

4.1 Nivel inventivo sobre la combinación de las enseñanzas de D5 y D1 (WO2001/60821)

Como ya se señaló anteriormente, D5 describe agonistas mAChR y la anterioridad D1 reporta agonistas nAChR como se muestra en la página 31 entre las líneas 24 a 25 de esta anterioridad. Dado que ambas referencias describen agonistas que son efectivos para **dos receptores diferentes**, es decir, **diferentes farmacóforos**, la persona medianamente versada en la materia estaría bastante dudosa para aplicar las enseñanzas de un farmacóforo al otro. El argumento del señor examinador relacionado con que dicha persona habría combinado estas dos enseñanzas, es un argumento claramente basado en un enfoque de tipo hindsight, el cual se inicia del conocimiento completo de la invención en estudio y de ahí se parte para realizar la búsqueda del arte previo más cercano, sin tener en cuenta si la solución planteada en la solicitud, habría sido o no evidente para la persona versada en la materia **que no hubiera tenido en mente la solución ya propuesta por el solicitante**.

De hecho, esta clase de enfoque no es adecuado para la evaluación del nivel inventivo de la solicitud, pues al no hacer el esfuerzo intelectual de colocarse en el momento justo antes de la fecha de prioridad de la invención en estudio, se cae en el error de dar por obvia la solución propuesta y acabada de conocer en la solicitud, por el simple hecho que los documentos anteriores muestran agonistas similares a los de la solicitud pero con **actividades completamente diferentes** sobre receptores también completamente distintos.

Aún más, en gracia de discusión, incluso si la persona medianamente versada en la materia tratara de combinar las referencias D5 y D1, dichas enseñanzas en lugar de acercarlo a la presente invención, lo alejarían, toda vez que D1 muestra una clara preferencia por carboxamidas fenílicas, furílicas y tiofenílicas, como así se expone en la definición de Ar¹ en la página 4, entre las líneas 19 y 20 de D1 y no a un grupo parid-3-il como el de la presente solicitud. Por consiguiente, es claro que los compuestos de la presente solicitud a la luz de las enseñanzas de D1 y D5 también involucran altura inventiva. Adicionalmente, no resulta necesario ni procedente el evidenciar de manera comparativa con los compuestos de D5 que la solicitud en estudio presenta la actividad sobre el receptor alfa₇nAChR porque la actividad es totalmente distinta teniendo en cuenta el tipo de receptor y el farmacóforo en la estructura de los compuestos.

Habiendo atendido cabalmente las objeciones presentadas por ese despacho, atentamente solicitamos se conceda el privilegio de patente de invención a la solicitud en estudio o si el examinador considera que subsisten razones para objetar la presente invención rogamos expedir otro examen de fondo con los debidos motivos y razones que sustenten su objeción con el fin de que nuestro mandante tenga la posibilidad de exponer sus argumentos con base en la interpretación de los artículos 45 de la Decisión 486 -el cual otorga la facultad al examinador para emitir los exámenes de fondo-, 35 y 59 del Código Contencioso Administrativo que señala que: *"habiéndose dado oportunidad a los interesados para expresar sus opiniones, y con base en las pruebas e informes disponibles, se tomará la decisión que será motivada al menos en forma sumaria si afecta a particulares"* y *"(..) deberá proferirse la decisión definitiva. Esta se motivará en sus aspectos de hecho y de derecho, y en los de conveniencia si es el caso",* respectivamente (El subrayado es nuestro).

En consecuencia, solicitamos a ese despacho pronunciarse en relación con todos los argumentos presentados en este memorial dando estricta aplicación al principio del debido proceso consagrado en el artículo 29 de la Constitución Nacional

Señor Superintendente,



DILIA MARIA RODRIGUEZ D'ALEMAN
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ANEXO 1

Artículo de MJ Sheardon, Expert Opinión on Therapeutic Patents (2002), 12(6).

1. Introduction
2. Alzheimer's disease
3. Patents 1998 - 2001
4. Expert opinion



Muscarinic M₁ receptor agonists and M₂ receptor antagonists as therapeutic targets in Alzheimer's disease

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Alzheimer's disease is the most common form of senile dementia and is of increasing medical and economic importance. One of the earliest findings in the systematic study of Alzheimer's disease was the progressive loss of acetylcholinergic neurons. This led to the so-called cholinergic hypothesis of Alzheimer's disease and provoked a search for cholinergic replacement therapies. This has resulted in compounds capable of inhibiting the catabolism of acetylcholine (acetylcholinesterase inhibitors) being licensed for the treatment of Alzheimer's disease. However, the search for directly acting, selective, muscarinic M₁ receptor agonists and M₂ receptor antagonists, that are capable of increasing acetylcholine levels in the neocortex, has not yet proved successful. For the muscarinic agonists this failure has been largely due to the difficulty in finding genuinely selective compounds. As a result, there has been a reduction in the number published patents in this area. This review discusses the rationale, progress and the possible future for these two potential approaches to the therapy of Alzheimer's disease especially in light of some recent patents containing selective compounds.

Keywords: acetylcholine, Alzheimer's disease, M₁ receptor agonists, M₂ receptor antagonists, muscarinic receptors

Expert Opin. Ther. Patents (2002) 12(6):863-870

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease that is associated with a global impairment of higher mental function. These degenerative changes involve a progressive loss of cognitive abilities, including memory loss and disturbance of verbal and visuospatial ability. They are often accompanied by severe behavioural disturbances, such as psychosis, agitation, delusions, hallucinations and wandering. The disease course extends over a period of ~ 10 years and the deterioration mirrors a gradual expansion of degenerative changes in the brain.

One of the earliest observed pathological correlates of AD was the loss of acetylcholinergic neurons in the basal forebrain, particularly in the nucleus basalis of Meynert and the medial septum. This neuronal loss results in the impairment of acetylcholine (ACh) synthesis in the cerebral cortex and the hippocampus, where cholinergic transmission substantially contributes to normal cognitive function.

These findings resulted in the formulation of the cholinergic hypothesis of AD and in the search for ACh replacement therapies. The strategies used have included reducing ACh catabolism with acetylcholinesterase inhibitors, direct ACh replacement with muscarinic agonists and increasing ACh release with muscarinic antagonists. To date, the only approved therapeutic agents based on the cholinergic hypothesis are acetylcholinesterase inhibitors.

The number of people with AD will increase substantially in the first half of the 21st century as the proportion of the population over the age of 65 rises sharply.

Therefore, AD presents society with a major challenge and thus the requirement for effective pharmacotherapy of the disease represents a major unmet medical need. This review will assess the present status of the search for effective cholinergic agents and reassess their potential place in the armoury available to neurologists in the future.

2. Alzheimer's disease

AD is the leading cause of dementia in elderly people. At present it is estimated that it affects 15 million people worldwide and this number will increase sharply. The reason for this projected increase in the number of cases is that the prevalence of AD doubles every 5 years beyond the age of 65, and beyond the age of 85 47% of people are affected [1]. These figures, in a situation in which people from Western societies and parts of the so-called third world are living longer, and in which the baby boomers will be reaching 65 from 2011 onwards, add up to a potential AD crisis unless effective treatments are found. In 2001 the United States spent \$100 billion on treating AD and the estimated cost to US industry was \$33 billion, by mid-century these costs will escalate enormously.

AD is a progressive neurodegenerative disorder with a mean duration of 8.5 years between onset of clinical symptoms and death. Symptoms are typically characterised by gross and progressive impairments of cognitive function, with memory loss being the cardinal symptom. However, there is a gradual loss of all cognitive ability with verbal disturbances and loss of visuospatial ability being prominent relatively early in the disease. Ultimately, AD sufferers lose all ability for abstract thought and are not even able to recognise themselves in the mirror or walk through a door way. It is true to say that 'a poor memory is forgetting to make a telephone call but AD is not knowing what a telephone is for or in fact not recognising the telephone'. These cognitive deficits are often accompanied by severe behavioural changes, which can appear to be similar to those observed in psychosis, and include agitation, aggression, delusions, hallucinations, compulsiveness and wandering. These behavioural changes are very difficult to cope with and are often the reason for the institutionalisation of the patient [2]. This has been an opportunity for therapeutic intervention that has not, so far, been sufficiently investigated.

The diagnostic pathological features of AD, which were observed in Alzheimer's first patient in 1906, are the appearance of extracellular senile plaques that contain deposits of β -amyloid peptide, which is derived from amyloid precursor protein (APP), and intracellular neurofibrillary tangles (containing an abnormally phosphorylated form of a microtubule associated protein, tau). The most affected brain regions are those associated with higher cognitive functions, such as the hippocampus and the neocortex and in these regions one observes a significant loss of pyramidal neurons and their synapses [2].

2.1 The cholinergic hypothesis of Alzheimer's disease

In the late 1960s and early 1970s, several groups began to conduct systematic investigations of the brains of AD patients. The aim of these studies was to identify a clearly defined neurochemical abnormality that could provide the basis for the development of mechanistically based therapeutic agents. A breakthrough came in 1976, when it was reported that there were severe deficits in the enzyme responsible for the synthesis of Ach, choline acetyl transferase (ChAT), in the neocortex of AD patients [3-5]. Severe neuronal loss was also observed in neurotransmitter-specific subcortical nuclei that project to the neocortex and hippocampus, in particular the nucleus basalis of Meynert and the medial septum, both of which are cholinergic [6]. Further studies revealed a reduction in Ach uptake [7] and release [8] in brains from AD patients. Together, these studies revealed the presence of a substantial presynaptic cholinergic deficit in the brains of AD patients. These studies, together with the knowledge of the role of Ach in learning and memory [9], lead to the formulation of the cholinergic hypothesis of AD. This hypothesis proposed that the degeneration of cholinergic neurons in the basal forebrain and the associated loss of cholinergic transmission in the cerebral cortex and hippocampus contributed to the deterioration of cognitive function seen in AD patients.

Studies of biopsy tissue from AD patients taken 3.5 years after the onset of clinical symptoms has shown that this selective neurotransmitter deficit occurs early in the course of the disease [10]. It has also been demonstrated that reductions in ChAT activity and Ach synthesis are strongly correlated with the degree of cognitive deficit in patients with AD [10-13].

This evidence made it clear that neocortical cholinergic innervation is probably lost at an early stage of the disease and strongly suggested a causative link with the clinical symptoms of AD. Further studies have shown that whilst there is a reduction in the number of M₂ receptors, (which are mainly presynaptic) in the brains of AD patients, there is a relative sparing of the post synaptic M₁ receptors [14,15]. This sparing of postsynaptic M₁ receptors in the neocortex, despite the degeneration of the cholinergic innervation from the basal forebrain, indicated that muscarinic replacement therapy was feasible. Thus, the search for selective muscarinic agents for the treatment of AD was born.

2.2 Muscarinic receptors

Cholinergic muscarinic receptors are members of the superfamily of G-protein-coupled receptors, so-called GPCRs. In this family of receptors the amino acid chain spans the cell membrane seven times, hence the receptors are more accurately called seven transmembrane domain receptors, or 7 TMs, as there are members of this structural class that are not G protein-linked. Each of the hydrophobic transmembrane domains is formed into an α -helix and these helices are arranged around a central agonist/antagonist binding pocket, in which specific amino acid residues provide the sites for drug-receptor interactions.

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Table 1. M₁ selective agonists that have entered the clinic and reported efficacy data.

Compound	Company	Status	Efficacy (cognition or behaviour scores)	Comments
Xanomeline	Novo Nordisk/Lilly	P-II	Yes	Discontinued, high incidence of side effects/poor bioavailability
Milameline	Parke-Davis/Hoechst Marion Roussel	P-III	Yes	M ₂ side effects; discontinued
Sabcomeline	SmithKline Beecham	P-III	Yes	Discontinued, marginal efficacy
Cevimeline	Snow Brand/Teva/Forest	P-III	Yes	Significant M ₂ activity in rodents (salivation and lacrimation)
Tiltsclidine	Boehringer Ingelheim	P-III	Yes	High incidence of side effects; discontinued
Alvameline	Lundbeck	P-III	No	M ₁ partial agonist/M _{2/3} antagonist, discontinued

The muscarinic receptor genes encode for five receptor proteins, denoted as M₁, M₂, M₃, M₄ and M₅ subtypes [16]. The coupling of these receptors to their pharmacological responses is via so-called G proteins, that are located at the third intracellular loop. G proteins are composed of three separate peptide chains, each of different length and each existing in multiple forms. They are denoted α , β and γ . The α -subunit, which is the largest, can bind guanosine diphosphate (GDP) or guanosine triphosphate (GTP), hence the name G protein. Agonist activation of the receptor triggers a switch from GDP to GTP binding at the α -subunit of the protein. This in turn converts the protein to its active form, in which the α -subunit is cleaved from the β - and γ -subunits. This GTP bound activated α -subunit stimulates or inhibits a range of intracellular enzymes involved in the production of second messengers that are in turn linked to the cellular/tissue response, furthermore, different α -subunits are linked to different second messenger systems.

Muscarinic receptor subtypes are split into two families based on their α -subunit linkage. The M₁, M₃ and M₅ receptor subtypes are linked to the G_{q/11} α -subunit. This G protein subunit is linked to stimulation of phospholipase C (PLC), which hydrolyses phosphatidylinositol 4,5-bisphosphate (IP₂) and forms inositol 1,4,5-triphosphate (IP₃) and 1,2-diacylglycerol (DAG). The M₂ and M₄ subtypes, however, are linked through G_{vo} to adenylylcyclase, such that the activation of G_{vo} results in inhibition of adenylyl cyclase and a reduction in levels of cyclic adenosine 3', 5'-monophosphate (cAMP).

2.3 M₁ receptor agonists and Alzheimer's disease

M₁ receptors are most abundant in the neocortex and the hippocampus, where they are postsynaptically located and as stated above they are preserved in AD even after degeneration of the presynaptic terminals. In addition to their direct role in cognitive function, it has also been shown that M₁ receptor activation can increase cleavage of APP in the middle of its β -amyloid region. This cleavage (the α -secretase pathway) produces secreted nonamyloidogenic peptides, thus

preventing the formation of β -amyloid peptide [17]. There is also evidence that M₁ agonists can decrease the phosphorylation of tau and retard the formation of neurofibrillary tangles [18]. The potentially beneficial effects on β -amyloid have also been observed *in vivo*. Rabbits treated with the selective M₁ agonist AF102B (cevimeline) had reduced cerebrospinal fluid (CSF) levels of A β 1-40 and 1-42 compared to control animals [19]. Furthermore, promising clinical data has been reported from trials with two compounds. Tiltsclidine, a selective M₁ agonist, has been shown to stimulate the α -secretase pathway in model systems and to decrease the CSF levels of β -amyloid protein by 27%, compared to placebo, in a double-blind trial randomised to 24 AD patients [20]. Also, Ccevimeline reduced total β -amyloid protein levels (in the CSF) by 22% in 14 AD patients, in the same study the acetylcholinesterase inhibitor physostigmine was without effect on β -amyloid levels in AD patients [21]. Therefore, M₁ agonists, in addition to being useful for the treatment of the symptoms of AD, may be able to retard the progression of the disease process, hence the development of selective M₁ agonists for the treatment of AD has been a major goal for the pharmaceutical industry.

Several compounds have advanced to Phase II and Phase III clinical trials (Table 1) and most of these compounds have shown efficacy in improving both cognition and behaviour of Alzheimer's patients. The first relatively selective M₁ agonist to show efficacy in a Phase II trial was xanomeline from Eli Lilly and Novo Nordisk [22]. This compound produced a particularly profound effect on the behavioural problems in AD patients as well as the usual moderate improvement in cognition observed with cholinergics. One of the major problems with these compounds has been a modest degree of selectivity for the M₁ receptor. M₂ and M₃ receptor activation results in parasympathomimetic symptoms, such as reduced force and contraction rate of the heart (M₂), increased gastrointestinal (GI) motility and diarrhoea (M₂ and M₃) and salivation (M₃); many of the compounds developed thus far have not shown adequate selectivity against these receptors. This has resulted

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in either very modest efficacy at the doses used in the trials, or unacceptable levels of GI and cardiovascular side effects. To date, no M₁ agonists have shown an adequate level of clinical efficacy at doses that are free from, or produce acceptable levels of, cholinergic side effects. However, aside from selectivity, one reason for the modest degree of efficacy observed with some of the compounds may be that increased levels of β -amyloid protein may reduce the coupling of M₁ receptors to G proteins, leading to decreased signal transduction [23]. Furthermore, some of the side effects observed may be due to the activation of peripheral M₁ receptors on myenteric and submucous neurons in the gut [24]. If these peripheral receptors are identical to those in the CNS then the absence of GI side effects, even for highly selective M₁ agonists, will depend on the functional selectivity of the compounds and the degree of receptor reserve in the intestines compared to that in the CNS. However, the importance of these peripheral M₁ receptors will not be known until a truly selective agonist has been developed and the effects of M₂ and M₃ receptors on GI function can be ruled out. Another problem may have been that many of the M₁ receptor agonists developed to date have been derivatives of arecoline a naturally-occurring alkaloid, with no subtype selectivity and a very short biological half-life. This lack of chemical diversity has been a problem with companies constantly impinging on each others patents.

Using classical radioligand binding techniques, none of the compounds developed so far have shown any selectivity on the basis of receptor affinity, which is the classical measurement of pharmacological selectivity. However, when these compounds are studied *in vitro* in human recombinant receptor systems and isolated tissues or *in vivo*, so-called functional selectivity has been observed. Functional selectivity is primarily dependent on the level of receptor reserve in a particular tissue or cell line. Thus, a compound such as thiopilocarpine acts as a full agonist at M₁ receptors in the superior cervical ganglion, is a partial M₃ agonist in the guinea-pig ileum and a silent M₂ antagonist in the guinea-pig atria [25]. Therefore, screening for selective muscarinic agonists has revolved around the design of partial agonists and has been critically dependent on the effective receptor reserve associated with the assay used and the degree of agonism of the compounds at each of these receptors. With the use of human recombinant systems this makes the design of the cell line of vital importance, for example, a cell line with a high receptor density would predispose for detection of agonists with low intrinsic efficacy. Also, one has to be aware of the level of G protein expression in a cell line used for the expression of human receptors, as overexpression of G proteins results in the receptors becoming constitutively active and enhances agonist potency. Thus, it is evident that the screening and design of functionally selective M₁ agonists is problematic and in order to accurately determine the relative efficacy of a compound on all of the muscarinic subtypes, thorough pharmacological studies and analysis are required. The breakthrough required in the search for therapeutically useful M₁ agonists is for the

discovery of compounds with affinity based selectivity of at least 100-fold. Only when truly selective M₁ receptor agonists, that are free of debilitating muscarinic side effects, are available can their therapeutic potential be properly evaluated. This has not been possible with the crop of functionally selective agonists thus far discovered.

2.4 M₂ receptor antagonists and Alzheimer's disease

Evidence suggests that M₂ receptors are present on presynaptic terminals in the neocortex and the CA1 region of the hippocampus [26], where they act as autoreceptors. When synaptic Ach stimulates these receptors it inhibits further release of Ach. Studies have shown that blockade of M₂ receptors results in increased levels of Ach in the cortex [27] and the M₂ antagonist AF-DX-116 has been shown to improve cognitive performance in rats [28]. Recently, a number of compounds from Schering Plough, including SCH-217443, that are both potent and selective [29] have shown efficacy in increasing Ach release and improving cognitive function in rats. A potential problem with this approach is the fact that these receptors are located on presynaptic cholinergic fibres projecting to the neocortex and hippocampus from the basal forebrain and it is these fibres that degenerate in AD. Indeed, it has been shown that there is a progressive loss of M₂ receptors in AD [14,15] and this may limit the effectiveness of this approach in the clinic. Furthermore, there is no evidence to show that muscarinic antagonists have any direct activity that may correlate to inhibiting the progression of the disease, as is the case with M₁ agonists. The presence of M₂ receptors in the heart, where they mediate a reduction in both the rate and force of contraction, is also a cause for concern over the utility of M₂ receptor antagonists for the treatment of AD. One compound with M₂ receptor antagonist properties that has been in clinical trials is Alvimeline, from Lundbeck. This compound is a partial agonist at M₁ receptors and an antagonist at M_{2/3} receptors [30]; the compound has been withdrawn from development because of a lack of efficacy. Although Schering Corp. is reported to have an M₂ receptor antagonist in development, no official statement to that effect has been made. However, SCH-72788 is a selective M₂ receptor antagonist with an affinity of 0.5 nM at cloned M₂ receptors and of 42 nM at M₁ receptors. This compound has been shown to increase Ach release from the striatum of conscious rats and is active in a rodent model of cognition, the rat passive avoidance response paradigm [31]. SCH-217443 has very similar properties to SCH-72788 [29] and it is possible that a compound from this series has entered the early stages of development. Such a compound could be expected to have similar efficacy to the acetylcholinesterase inhibitors at present on the market.

3. Patents 1998 – 2001

3.1 M₁ receptor agonist patents

This area has become very quiet over the last few years, and relatively few patents have been published. One reason for the

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reduced level of interest in the development of M_1 agonists has been the number of high profile failures in the clinic, such as xanomeline, sabcomeline (SmithKline Beecham) and tilsacilidine (Boehringer Ingelheim). The mixture of poor efficacy and unacceptable side effect profiles observed has been, to a major degree, a result of the poor degree of selectivity seen with these compounds. An additional reason for the reduction in interest may well be the modest success of the acetylcholinesterase inhibitors that are being marketed as AD therapies. These compounds are the first examples of cholinergic replacement therapy available to neurologists. There are four such compounds on the market, tacrine (cognexTM, Warner-Lambert, now sold to First Horizon Pharmaceutical corporation), donepezil (ariceptTM, Eisai), comarketed with Pfizer), rivastigmine (exelonTM, Novartis) and galanthamine (reminyTM, Shire). Unfortunately, these compounds have not made a great contribution to the therapy of AD. The level of efficacy achieved has been modest and in a substantial number of patients the compounds show no efficacy at all.

In 1998, a patent was published by American Home Products [101] claiming a novel saturated and unsaturated pyridazino [4,5-b] indolizines. This claim increases the diversity of the chemistry in this area, which is dominated by arecoline derivatives. The biological selectivity data in the patent are based on radioligand binding studies, using displacement of pirenzepine in rat cerebral cortex membranes as the M_1 assay and displacement of quinuclidinyl benzylate (QNB) in rat cerebellum membranes as the M_2 assay. The specified compound, N-[4-(3-azabicyclo[3.2.2]non-3-yl)butyl-6,7,8,9-tetrahydro-4-methylpyridazino[4,5-b]indolizin-1-amine, produced K_i values for displacement of M_1 and M_2 binding of 1.34 and 13.35 nM, respectively, giving a selectivity ratio of only 10:1. This compares with values of 5 and 24 nM, respectively, for xanomeline in the same assays. Both of the radioligands used are muscarinic antagonists and the relevance of studying the binding of agonists to the antagonist-preferring conformation of the receptors is questionable. Furthermore, the degree of affinity based selectivity is low. The compound is a full agonist in a recombinant M_1 functional assay. From the data available, these compounds look similar to the functionally selective agonists that have so far failed in clinical trials and would not seem to represent the required breakthrough in selectivity.

The most active company in the search for selective muscarinic M_1 agonists has been Acadia, who published patents in 1999 [102] and 2001 [103] claiming M_1 -selective compounds. The assay technology used by Acadia is unique and proprietary, being based on a patent published in 1995 by Mark Brann [104]. This assay system is based on the ability of receptors to amplify NIH 3T3 cells. Receptors are transiently coexpressed in NIH 3T3 cells with β -galactosidase as a marker, and the receptors that can induce cell proliferation, select and amplify the cells that also express the marker. In this way, the ability of ligands to alter receptor activity is reported as a change in enzyme activity. Cells transfected with M_1 , M_3 and M_5 muscarinic receptors produce strong increases in enzyme

activity, although M_2 and M_4 receptors need to be coexpressed with a chimera between Gq and Gi (Gq-i5) in order to induce β -galactosidase. Brauner-Osborne and Brann [32] showed that the order of potency and intrinsic activity of a series of muscarinic ligands tested in this assay was in very good agreement with previously published results using cloned cell lines and isolated tissues. The advantage of this novel assay is that it can be performed in a 96-well format, using a colorimetric read-out.

These patents contain a wide range of heterocyclic compounds, and in both cases, particularly in the 2001 patent [103], which exemplifies 99 compounds, a lot of the chemistry lacks novelty. Only a small number of the claims are likely to be granted, as international search reports have produced a very large amount of prior art. However, in the 2001 patent [103] a number of compounds are claimed that show selectivity for M_1 and M_4 receptors, or both, with no effects on M_2 or M_3 receptors, which produce the majority of the dose limiting muscarinic side effects observed in the clinic with muscarinic agonists. Interestingly M_4 receptors are intact or actually increased in number in AD, even in the advanced stages [33]. They are located presynaptically in the hippocampus and it has been suggested that drugs targeting these receptor sites may augment cognitive function by increasing the release of excitatory amino acids. M_1 and M_4 receptors have also been shown, in knockout studies, to potentially mediate the antipsychotic effects of muscarinic agonists [34]. Thus, an $M_{1/4}$ -selective agonist could prove to have efficacy both on the cognitive and behavioural aspects of AD, similar to the very promising effects of xanomeline [22] which is also an efficacious M_4 agonist, but without the observed unacceptable levels of muscarinic side effects. The discovery of M_1 and M_4 -selective compounds, such as those exemplified by Acadia, may therefore represent a significant breakthrough in the search for a muscarinic agonist with utility in AD. Example number 19 [103] has 86% maximal efficacy and a pEC_{50} value of 7.3 at M_1 receptors, and 70% maximal efficacy and a pEC_{50} value of 6.5 at M_4 receptors, whilst having no effect on M_2 , M_3 or M_5 receptors. Such compounds may provide the most promising chance yet to investigate the efficacy of muscarinic agonists in AD.

3.2 M_2 receptor antagonist patents

As with M_1 receptor agonists, there has not been a great deal of activity in the search for M_2 receptor antagonists for the treatment of AD. The only compound with M_2 receptor antagonist activity to have entered clinical trials is alvamefine (Lundbeck), which also has partial agonist properties at M_1 receptors. However, in a double-blind, placebo-controlled trial, alvamefine was without effect on both the cognitive and behavioural symptoms of AD, and there was a trend for the patients on the highest drug dose to worsen [30].

In recent years, the majority of patents in this area have been produced by Schering Corp. (USA), who have published data on a series of selective M_2 receptor antagonists. The lead

compound in the literature was SCH-57790, (4-cyclohexyl-a[4-[4-methoxyphenyl]-(S)-sulphonyl]-phenyl]-1-piperazine-acetonitrile). In binding studies, using QNB as the radioligand, this compound has K_i values of 112, 2.8, 29, 14 and 309 nM against human M₁, M₂, M₃, M₄ and M₅ receptors respectively. Oral administration of this compound at 10 mg/kg produces an increased release of Ach in the CNS and improves cognitive function in young rats in the passive avoidance response model [29]. Schering Corp. has published a number of patents in recent years clearly aimed at developing such a compound for the treatment of AD.

In 1998, three patents were published by Schering Corp., one of which [105] claimed a series of novel di-*N*-substituted piperazine and 1,4-disubstituted piperidine compounds with muscarinic antagonist activity. These compounds were assayed using standard radioligand binding techniques in human recombinant receptors, using radiolabelled QNB as the ligand. The specified compound, 1- (4-cyclohexyl-2*R*-methylpiperazino)-5-(4-methoxyphenylsulfonyl)-1*H*-indane (one of seven specifically claimed), had K_i values of 1.23 nM for M₁, 0.11 nM for M₂ and 0.27 nM for M₄ receptors. The second patent [106] claimed a series of novel piperidine derivatives as selective M₂ antagonists; they were assayed as stated above and the specified compound (one of 23 specifically claimed), 4-(cyano[4-(3-methylenedioxypheylsulfonyl)phenyl]amino-1-cyclohexyl)piperidine, had K_i values for M₁, M₂ and M₄ receptors of 10, 0.44 and 1.6 nM, respectively. The final patent [107] claimed a series of novel 1,4-disubstituted piperidines with an ether linkage at the 4-position. Again, they were assayed using human recombinant receptors and QNB as the radioligand and the ranges of K_i values presented suggest selectivity for M₂ receptors. The patent also states that compounds were tested for M₂ antagonism *in vivo* in rats by measuring the Ach concentration in striatum microdialysate and increases in Ach levels were observed that were consistent with presynaptic M₂ receptor antagonism. No data were presented for specific compounds.

Schering Corp. has published two further muscarinic receptor patents. In 1999 they claimed a series of novel heteroaromatic derivatives of 1,4-substituted piperidines and 1,4-disubstituted piperazines as selective M₂ antagonists for the treatment of AD [108]. The selectivity assay was as for the patents listed above and no data were presented for specific compounds. However, a preferred compound had K_i values of 1.5 nM at M₂ receptors and 917 nM at M₁ receptors. In the second patent [109], a series of 1,4-disubstituted piperidines stated to be selective M₂ receptor antagonists, is claimed. The biological data relate to five compounds studied *in vivo* in a microdialysis assay. All the compounds produced an increase in Ach in the dialysate concentration consistent with presynaptic M₂ receptor antagonism.

Hoffman-La Roche published a patent in 1999 [110] claiming a series of 2-arylethyl-(piperidine-4-ylmethyl) amine derivatives as muscarinic antagonists, and included several disease states, including AD, in the claims. The biological

data presented were in anaesthetised dogs and rats, but no selectivity data were presented and it is doubtful if the prime claim of the patent was AD therapy.

Likewise, a series of patents from Alcon Labs were published in 1999 [111-114], claiming a range of heterocyclic compounds as muscarinic receptor antagonists. No biological data are presented in any of these patents and as the focus of Alcon is ocular therapy it is unlikely that AD therapy, although claimed, is the prime focus of this series of compounds.

An interesting patent was published in 2001 by Mediolum Farm [115] who claimed novel 5*H*-dibenzo[*b,f*]azepine derivatives as selective M₂ antagonists. The compounds were shown to display good selectivity for M₂ receptors with the specified compound, 5-[2-(4-[4-(dimethylamino)butyl]piperidine-1-yl)acetyl]-5*H*-dibenzo[*b,f*]azepine, having K_i values of 310, 2.6, 290 and 100 nM at human recombinant M₁, M₂, M₃ and M₄ receptors, respectively.

4. Expert opinion

The cholinergic hypothesis of AD was first proposed during the 1970s and it has dominated drug discovery efforts aimed at producing symptomatic improvements in AD patients. The only success has been the development of a number of acetylcholinesterase inhibitors that are now marketed for the treatment of AD. These compounds display a reasonable degree of efficacy and have proved to be useful clinical agents in many AD patients. However, they are ineffective in a significant number of patients and they have not proved a truly outstanding breakthrough in the therapy of AD.

One has to question whether a therapy that only treats the symptoms of AD, without inhibiting the progress of the disease, will ever make a truly important contribution to its treatment. The answer must be that drug discovery must move in the direction of inhibiting the progression of AD. Advances are being made in this direction and recent studies of both β and γ secretases have shown that inhibition of these enzymes can reduce A β deposition in β APP transgenic mice [35]. It has also been shown that A β can be removed by immunising against the protein and such treatment has reduced plaque formation, neuritic dystrophy and astrogliosis in PD-APP transgenic mice [36], although clinical trials using this approach have recently been halted by Elan because of the development of encephalitis in a number of patients. Therefore, it is unlikely that a purely symptomatic treatment, such as a selective muscarinic M₂ receptor antagonist, will make any significant contribution to AD therapy in the future. Moreover, as the mode of action is to increase the synaptic levels of Ach, the presynaptic terminals expressing the M₂ receptors are degenerating with time and the numbers of receptors are decreasing, it is unlikely that such compounds would provide any advantage over the acetylcholinesterase inhibitors already on the market. Furthermore, there is no evidence or real rationale to suggest that muscarinic M₂ receptor antagonists would have any beneficial effects on the behavioural problems associated with AD.

Selective muscarinic $M_{1/4}$ agonists may still have a role to play in AD therapy, if genuinely selective compounds can be discovered. There is good evidence in the scientific literature that M_1 agonists, in addition to improving cognitive function in AD patients, can inhibit the formation both of β -amyloid peptide and the phosphorylation of tau, and therefore potentially inhibit the formation of senile plaques and neurofibrillary tangles. In addition, compounds such as xanomeline have shown a remarkable degree of efficacy on the behavioural symptoms of AD, such as wandering. This evidence suggests that selective muscarinic agonists may be able to provide a global therapy for AD and both give symptomatic relief and inhibit

disease progression. The compounds developed by Snow Brand Milk Products, such as cevimeline, are claimed to be functionally selective M_1 agonists [23]; however recent publications have shown them to be efficacious M_3 receptor agonists and have proposed their use for treating salivary dysfunction [37]. The compounds discovered by Acadia, from the preliminary data available, seem to be genuinely selective and have no effect on M_2 and M_3 receptors, which are probably responsible for the majority of the side effects observed with the compounds developed to date. The further development of these and similarly selective compounds may see an awakening of interest in an area of drug discovery that has been in decline.

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ANEXO 2

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Altered Cardiovascular Responses in Mice Lacking the M₁ Muscarinic Acetylcholine Receptor

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ABSTRACT

Although the M₂ muscarinic acetylcholine receptor (mAChR) is the predominant functional mAChR subtype in the heart, some responses of the cardiovascular system to acetylcholine (ACh) may be mediated by other mAChR subtypes. The potential effect of M₁ mAChR on heart function was investigated using M₁ knockout (M₁-KO) mice. In vivo cardiodynamic analysis showed that basal values of heart rate (HR), developed left ventricular pressure (DLVP), left ventricular dP/dt_{max} (LV dP/dt_{max}), and mean blood pressure (MBP) were similar between wild-type (WT) and M₁-KO mice. Injection of the putative M₁-selective agonist 4-(*m*-chlorophenyl-carbamoyloxy)-2-butynyltrimethylammonium (McN-A-343) produced an increase in LV dP/dt_{max}, DLVP, HR, and MBP in WT mice but did not affect hemodynamic function in the M₁-KO mice. The stimulatory effect of McN-A-343 in WT mice was blocked by pretreatment

with propranolol, indicating that stimulation of the M₁ mAChRs on sympathetic postganglionic neurons evoked release of catecholamines. Intravenous injection of ACh in both WT and M₁-KO mice caused atrioventricular conduction block, without a significant change in the frequency of atrial depolarization, or atrial fibrillation. Immunoprecipitation and reverse transcriptase-polymerase chain reaction failed to detect the expression of M₁ mAChR in cardiac tissue from WT mice. The carbachol-induced increase of phospholipase C activity in cardiac tissues was not different between WT and M₁-KO mice. These results demonstrate that 1) activation of M₁ mAChR subtype on sympathetic postganglionic cells results in catecholamine-mediated cardiac stimulation, 2) M₁ mAChR is not expressed in mouse heart, and 3) administration of ACh to mice induces arrhythmia.

Stimulation of the parasympathetic postganglionic neurons causes the release of acetylcholine (ACh) that acts on muscarinic ACh receptors (mAChRs) on the myocardial cell membrane. There are five mammalian mAChR subtypes that preferentially couple to either the inhibition of adenylyl cyclase activity (M₂ and M₄ subtypes) or to the stimulation of phospholipase C (PLC) activity (M₁, M₃, and M₅ subtypes) (Bonner, 1989). The M₂ mAChR is the predominant muscarinic receptor subtype in mammalian heart (Brann et al., 1993; Caulfield, 1993). Activation of these M₂ mAChRs inhibits the activity of adenylyl cyclase, closes calcium channels, lowers the hyperpolarization-activated pacemaker current, and activates an inwardly rectifying potassium channel. These changes cause both negative chronotropic and inotropic effects on the heart (Caulfield, 1993).

Accumulating evidence indicates that other mAChR sub-

types also contribute to the regulation of heart rate and contractility. The novel muscarinic agonist McN-A-343 has pressor activity in mammals (Roszkowski, 1961). When injected in dogs and cats, McN-A-343 raised blood pressure, an effect blocked by pretreatment with the muscarinic antagonist atropine. The McN-A-343-mediated hypertension in vivo was also antagonized by adrenergic blockade, suggesting that McN-A-343 is a muscarinic agonist that acts as a sympathomimetic agent. Pretreatment of dogs with reserpine blocks the pressor effect of McN-A-343. In awake rats, McN-A-343 induced hypertension and tachycardia, which are antagonized by propranolol (Martin, 1996). Adrenal demedullation had no effect on the tachycardia, whereas treatment with guanethidine suppressed both tachycardia and hypertension (Martin, 1996). Taken together, these results demonstrate that the muscarinic agonist McN-A-343 evokes both tachycardia and hypertension by releasing catecholamines from the sympathetic nerve endings, with only a minor role for the adrenal glands.

This work was supported by National Institutes of Health Grants HL44948, HL58676, and NS26920. S.N.H. is a recipient of an American Heart Association Northwest Affiliate postdoctoral fellowship.

ABBREVIATIONS: ACh, acetylcholine; mAChR, muscarinic acetylcholine receptor; PLC, phospholipase C; McN-A-343, 4-(*m*-chlorophenyl-carbamoyloxy)-2-butynyltrimethylammonium; M₁-KO, M₁ mAChR knockout; MBP, mean blood pressure; LV dP/dt_{max}, maximum left ventricular dP/dt; DLVP, developed left ventricular pressure; HR, heart rate; bpm, beats per minute; RT-PCR, reverse transcriptase-polymerase chain reaction; QNB, quinuclidinyl benzilate; WT, wild-type; ANOVA, analysis of variance; HET, heterozygote.

The muscarinic subtype that mediates the cardiostimulatory effect of McN-A-343 has not been clearly identified. The increase in blood pressure and heart rate after McN-A-343 injection in adult rats was blocked by low concentrations of the M_1/M_4 muscarinic antagonist pirenzepine (Hammer and Giachetti, 1982; Wilffert et al., 1983), suggesting that either the M_1 or M_4 subtype mediated the cardiostimulatory effects of McN-A-343. In addition, functional studies with cloned mAChR indicate that McN-A-343 can activate multiple muscarinic receptor subtypes with greatest efficacy at the M_4 receptor (Caulfield and Birdsall, 1998). Furthermore, the M_1 , M_2 , and M_4 mAChR subtypes are all expressed in sympathetic ganglia (Caulfield, 1993).

Pharmacological studies have also shown that muscarinic agonists can elicit a stimulatory effect on the mammalian heart. ACh caused a positive inotropic effect in human heart (Du et al., 1995) and in sheep and in canine cardiac Purkinje fibers (Gilmour and Zipes, 1985; Iacono and Vassalle, 1989). In isolated mouse atria ACh induces a biphasic effect on both maximum upstroke velocity of the action potential and inotropic response (Islam et al., 1998; Nishimaru et al., 2000), whereas in isolated perfused rat heart, ACh caused a dose-dependent increase in perfusion pressure (Hoover and Neely, 1997). It has been suggested that the positive inotropic action of ACh is a protective mechanism to prevent excessive inhibition of cardiac function at high concentrations of ACh (Papano, 1991). However, the physiological effects of mAChR-mediated stimulation of the heart, particularly in vivo, are not well characterized and their mechanisms of action are poorly understood.

Other studies have demonstrated that other mAChR subtypes besides M_2 may be involved in cardiac function. For example, in canine atrial myocytes a novel K^+ current is regulated by an M_3 mAChR subtype (Shi et al., 1999a,b). The ACh-mediated increase in rate of contraction in neonatal ventricular myocytes was insensitive to the M_2 antagonist AFDX-116 but antagonized by the M_1/M_4 antagonist pirenzepine (Gallo et al., 1993). In guinea pig cardiomyocytes carbachol mediated a pirenzepine-sensitive increase in the L-type Ca^{2+} channel activity (Gallo et al., 1993). These studies suggest that the M_1 or M_4 mAChR may be involved in the regulation of the heart. More recently, RT-PCR analysis has shown the expression of the M_1 mAChR subtype in rat and guinea pig ventricular cells (Gallo et al., 1993; Sharma et al., 1996) and the M_3 and M_4 mAChR subtypes in dog atrial cells (Shi et al., 1999b).

Although there is data indicating that the M_1 receptor may regulate cardiovascular function by acting either in the heart and/or the sympathetic ganglia, there appear to be considerable differences due either to species differences or lack of adequate selectivity of pharmacological agents. We took advantage of the M_1 -KO mice previously generated in our laboratory (Hamilton et al., 1997) to determine the role of the M_1 mAChR in the regulation of cardiovascular function in mouse.

Materials and Methods

In Vivo Cardiodynamics. All procedures were approved by the University of Washington Institutional Animal Care and Use Committee. Mice (~6 months old) were anesthetized by intraperitoneal injection of tribromoethanol (Avertin, 0.4 mg/kg; Aldrich, Milwaukee, WI) and urethane- α -chloralose (750 mg/kg; 50 mg/kg). Mice

were ventilated with a volume displacement ventilator (Harvard 687; Harvard Apparatus, Holliston, MA) and end respiratory CO_2 was recorded (96282-ND-PR; Columbus Instruments, Columbus, OH) and maintained between 2.8 and 3.2%. Body temperature was maintained at 37°C by using a rectal probe and proportional temperature controller (Harvard 7129; Harvard Apparatus) with a heating blanket. Where indicated, mice were bilaterally vagotomized to avoid vagal reflexes. The jugular vein was catheterized for drug injections (25 μ l each).

A catheter tip pressure transducer (1.4F; Millar; Houston, TX) was inserted into the right carotid artery and advanced into the aorta (for determination of the aortic pressure) or the left ventricle (for the determination of developed left ventricular pressure). The left ventricular dp/dt_{max} was calculated online from the left ventricular pressure. The heart rate was calculated from the left ventricular pressure. The mean blood pressure (MBP) was calculated online from the aortic pressure. Each data point represents an average of five sequential measurements. Left ventricular dp/dt_{max} (LV dp/dt_{max}) is expressed in millimeters of mercury per second, developed left ventricular pressure (DLVP) and MBP are expressed in millimeters of mercury, and the heart rate is expressed in beats per minute (bpm). In some experiments, ECGs were recorded from electrodes placed under the skin. In these experiments the atrial rate was calculated from the P-P intervals.

RNA Extraction from Adult Heart. Total RNA from cortex, cerebellum, and whole heart from adult mice was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol with modification (Chomczynski and Sacchi, 1987).

RT-PCR. RT was performed at 42°C for 60 min by using 200 U of Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) in a volume of 50 μ l. The amplification reaction was carried out in the same tube in a final volume of 100 μ l with 1.25 U of a thermostable *Taq* DNA polymerase (M186A; Promega). The temperature steps were 1 min at 94°C, 1 min at 56°C, and 1 min at 73°C. Forty-five cycles were performed and one additional cycle was added with a final elongation time of 10 min at 73°C. Each cardiac preparation was tested for DNA contamination in a parallel reaction performed in the absence of reverse transcriptase. The primers used for PCR were the 5'-primer 5'-ggatccggatccaaagggtggc-3' and the 3'-primer 5'-gaattcgaattcttcttgggtggcctcttgacgtg-3'. The reverse transcription was performed with the same 3'-primer.

Southern Blot. The RT-PCR products were separated on a 1.0% agarose gel by electrophoresis and transferred to a nylon membrane. A ^{32}P -labeled 1.8-kilobase *KpnI-BamHI* genomic fragment (10⁶ cpm/mg) containing the entire coding M_1 receptor region (Shapiro et al., 1988) was used as a probe.

[³H]QNB Binding Assay and Immunoprecipitation. Membrane isolation and solubilization, [³H]QNB binding assays, and immunoprecipitation with specific anti- M_1 and anti- M_2 antibodies were performed as described in Hamilton et al. (1997).

Phosphatidylinositol Hydrolysis Assay. WT and M_1 -KO adult mice (~6 months old) were sacrificed by CO_2 intoxication. Hearts were quickly transferred to prewarmed Krebs' buffer containing 118 mM NaCl, 4.7 mM KCl, 3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4 , 0.5 mM EDTA, 25 mM $NaHCO_3$, and 10 mM glucose, pH 7.4 at 37°C. Atria and ventricles were cut into small pieces (1–3 mm) and incubated in 0.5 ml of oxygenated (95% O_2 , 5% CO_2) Krebs' buffer containing 4 μ Ci/ml of [³H]myo-inositol (specific activity 16 Ci/mmol; Amersham Biosciences, Piscataway, NJ) for 90 min at 37°C with gentle agitation. Tissues were then washed with phosphate saline solution-LiCl solution (118 mM NaCl, 4.7 mM KCl, 3 mM $CaCl_2$, 1.2 mM KH_2PO_4 , 10 mM glucose, 0.5 mM EDTA, 20 mM HEPES, and 50 mM LiCl) and incubated again for 30 min in this solution at 37°C. Carbachol (0.001–1 mM final concentration) was then added and the samples were incubated for an additional 30 min at 37°C.

The tissues were homogenized by hand with a glass-glass homogenizer and the [³H]inositol phosphates and total labeled inositol

phospholipids were extracted (Scherer and Nathanson, 1990; Mende et al., 1998). Samples of ventricular tissues were assayed in duplicate, whereas the amount of atrial tissue only allowed a single determination per sample of unstimulated and 1 mM carbachol-stimulated values. The normalized values (ratio of inositol phosphates to total inositol phospholipids) are expressed as percentage of stimulation over the control value. Statistics. Data are expressed as mean $\pm$ S.E.M. Two-tailed unpaired *t* tests with 95% confidence limits or ANOVA was performed as indicated in the figure legends. The results were considered statistically significant if *p* < 0.05.

Results

M₁ mAChR Have a Catecholamine-Dependent Stimulatory Effect on Heart in Vivo

Basal Function. Cardiodynamic analyses were carried out to determine whether the lack of M₁ mAChRs affected basal cardiac function. The basal functions of the heart (LV dP/dt_{max}, DLVP, HR) were recorded in vagotomized mice before administration of drugs (Table 1). There is no statistical difference in these variables between the WT and M₁-KO mice, indicating that the lack of M₁ mAChRs did not alter basal cardiac function.

Vagotomy. To ensure that there were no differences in basal cardiac function between WT and mutant mice that were obscured by vagotomy, the change of heart rate due to vagotomy was also determined. These recordings were performed in separate groups of mice without cardiac catheterization. The heart rate was determined from ECG before and after bilateral vagotomy. Although a moderate tachycardia was observed as expected in both groups after vagotomy, the change was not statistically different between the WT (+69 $\pm$ 19 bpm; *n* = 4) and M₁-KO mice (+50 $\pm$ 11 bpm; *n* = 5).

The mean blood pressure was also determined in mice after catheterization before and after bilateral vagotomy. Vagotomy induces a moderate increase in MBP in both groups (+7.5 $\pm$ 5.5 mm Hg in WT; +8.2 $\pm$ 3.3 mm Hg in M₁-KO mice). There is no statistical difference between WT and M₁-KO mice.

McN-A-343 Induces Stimulatory Effects on Mouse Heart Mediated by β -Adrenergic Receptors. Administration of the muscarinic agonist McN-A-343 (350 μ g/kg) into bilateral vagotomized WT mice increased DLVP, LV dP/dt_{max}, and heart rate (Fig. 1, A–C). This effect was totally inhibited by pretreatment with the β -adrenergic receptor antagonist propranolol (1 mg/kg).

Stimulatory Effect of McN-A-343 on Heart Is Mediated by M₁ mAChR. We took advantage of the M₁-KO mice

TABLE 1
Basal cardiac values
Basal hemodynamic variables were recorded after catheterization of the right jugular vein and insertion of a catheter tip manometer into the left ventricle via the right artery and after bilateral vagotomy but before administration of drugs. Results are expressed $\pm$ S.E.M. There was no statistical difference between WT and M₁-KO mice.

Genotype	LV dP/dt max	LVP	HR	<i>n</i>
	<i>m</i> Hg/s	<i>mm</i> Hg	<i>bpm</i>	
Wild-type	6.60 $\pm$ 0.6	87 $\pm$ 6	591 $\pm$ 22	9
M ₁ -KO	6.02 $\pm$ 0.7	93 $\pm$ 8	573 $\pm$ 35	8

n, number of mice.

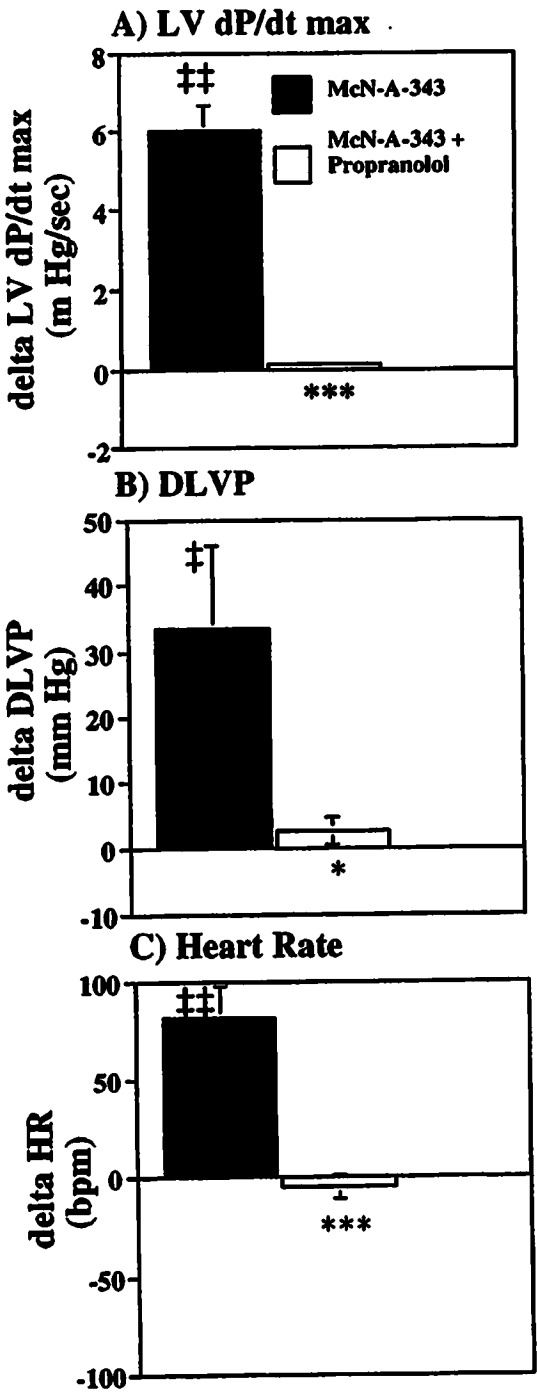


Fig. 1. Cardiac responses to McN-A-343 in bilateral vagotomized WT mice before and after β -adrenergic receptor blockade with propranolol. Results are presented as the change ($\pm$ S.E.M.) calculated from the baseline values of LV dP/dt_{max} (A), DLVP (B), and heart rate (C) preceding each drug injection (*n* = 6 WT mice). The muscarinic agonist McN-A-343 (350 μ g/kg i.v.) induces both positive inotropic and chronotropic effects in WT mice (■). In animals pretreated with 1 mg/kg i.v. propranolol, administration of 350 μ g/kg i.v. McN-A-343 no longer results in a significant change in hemodynamic variables. *, *p* < 0.05 McN-A-343 versus McN-A-343 + propranolol; ***, *p* < 0.001 McN-A-343 versus McN-A-343 + propranolol (*t* test). Administration of 1 mg/kg i.v. propranolol alone induced bradycardia. Effects of McN-A-343 in presence or in absence of propranolol were statistically different from those induced by propranolol alone. †, *p* < 0.05 McN-A-343 versus propranolol alone; ††, *p* < 0.01 McN-A-343 versus propranolol alone.

previously generated in this laboratory to determine whether the M_1 muscarinic receptor is responsible for the cardiac stimulatory effects of McN-A-343. All mice were vagotomized bilaterally before drug injection to avoid vagal reflexes. A recording from a WT mouse treated with the agonist McN-A-343 is shown in Fig. 2 and a comparison of the responses from WT and M_1 -KO mice is shown in Fig. 3, A–C. McN-A-343 caused a large increase in both DLVP and LV dP/dt_{max} as well as in the HR in the WT mice, with maximum increase observed at 350 $\mu\text{g/kg}$ (27.5 ± 8.6 mm Hg in DLVP, 5.1 ± 0.78 m Hg/s in LV dP/dt_{max} , 71 ± 20 bpm in HR). Administration of McN-A-343 did not have a significant effect on any of the cardiodynamic variables in the M_1 -KO mice.

McN-A-343 was also injected in a separate group of WT and M_1 -KO mice to record the mean blood pressure (Fig. 4). To ensure that vagotomy did not alter the responses due to drug administration, the effects of McN-A-343 administration were determined both before and after vagotomy. In the WT mice, McN-A-343 induced tachycardia and increased blood pressure before vagotomy (Fig. 4, A and C) (for 350 $\mu\text{g/kg}$ of McN-A-343, the increase was 30.0 ± 4.3 mm Hg in MBP and 68.3 ± 17.4 bpm in HR). No statistical difference was observed with results recorded after vagotomy (for 350 $\mu\text{g/kg}$ McN-A-343, $+18.5 \pm 6.7$ mm Hg in MBP, $+69.2 \pm 10.3$ bpm in HR) (Fig. 4, A and C). The M_1 -KO mice did not respond to injection of McN-A-343 (Fig. 4, B and D).

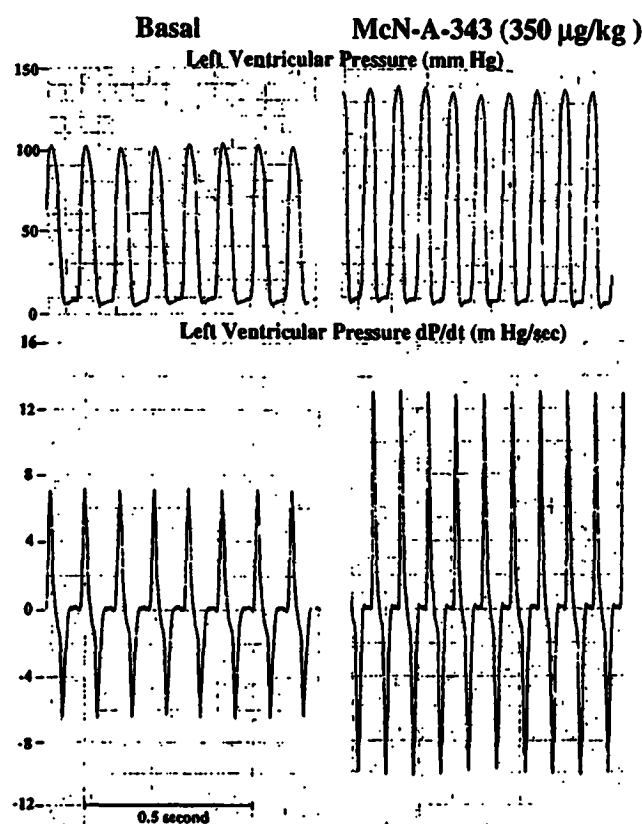


Fig. 2. Left ventricular pressure and LV dP/dt traces from bilateral vagotomized WT mouse before and after administration of McN-A-343. Administration of 350 $\mu\text{g/kg}$ i.v. McN-A-343 resulted in a positive inotropic response.

Nicotinic Responses Are Similar in Wild-Type and Knockout Mice. The agonist nicotine was injected at the end of each experiment as a positive internal control to test the effects of stimulation of nicotinic receptors in the sympathetic ganglia. As expected, nicotine induced a positive inotropic effect as well as tachycardia in both WT and M_1 -KO mice (Fig. 5). The responses were not statistically different between the two groups. The effects of nicotine in M_1 -KO mice demonstrate that the absence of a response to McN-A-343 was not due to an artifactual disruption of sympathetic effects on the heart.

ACh-Induced Responses Are Similar in WT and M_1 -KO Mice. To determine whether the lack of M_1 mAChR expression induced any change in M_2 mAChR responsiveness, ACh was injected in WT and M_1 -KO mice. Endogenously released ACh acts on M_2 mAChR in the heart to induce both negative chronotropic and inotropic responses (Fleming et al., 1992; Caulfield, 1993). We therefore expected that administration of ACh would result in sinoatrial node bradycardia, which could be recorded on an ECG trace as a longer P-P wave interval and a moderate decrease in maximum LV dP/dt_{max} (the negative inotropic effect).

Surprisingly, the atrial rate was not modified after ACh administration (see the P-wave intervals before and after ACh administration; Fig. 6). The main observed effects after administration of ACh, in mice, were 1) dropped QRS complexes, which is typical of atrioventricular conduction block; and 2) atrial flutter or atrial fibrillation alone or with atrioventricular block. The resultant change in DLVP is probably due to the change in ventricular rate rather than a direct inotropic effect. These effects were observed in bilaterally vagotomized propranolol-pretreated mice; similar effects were also observed in mice before bilateral vagotomy, without pretreatment with propranolol. These unexpected responses were observed in both WT (Fig. 6A) and M_1 -KO mice (Fig. 6B). (Among the 12 WT mice tested, 11 mice showed atrioventricular block and three mice exhibited atrial fibrillation or flutter after injection of ACh; of the seven M_1 -KO mice tested with ACh, six displayed atrioventricular conduction block and three mice had atrial fibrillation or flutter). The threshold of the responses to ACh (10–100 $\mu\text{g/kg}$) was similar in WT and M_1 -KO mice.

Mice Lacking M_1 mAChR Demonstrate No Change in M_2 mAChR Expression in Heart. M_1 mAChR Is Not Detectable in Wild-Type Mouse Heart

Immunoprecipitation. Because of the data indicating the presence of M_1 mAChR in the heart of rats and other mammals, we tested whether the M_1 receptor could be detected in WT mouse heart and whether the expression of mAChR was altered in M_1 -KO mice. The total number of mAChR in atrial and ventricular tissues was determined using the nonselective muscarinic antagonist [^3H]QNB. In both tissues, there was not a statistically significant difference in mAChRs number among the three genotypes: WT, heterozygote (HET), and M_1 -KO (Fig. 7).

M_1 and M_2 mAChRs were immunoprecipitated from atria and ventricles of WT, HET, and M_1 -KO mice (Fig. 7). Essentially all of the mAChR consisted of M_2 mAChRs (Fig. 7). The density of M_2 mAChRs is unchanged in the atria and ventricle of the M_1 mutant mice. M_1 mAChR expression in WT atria and ventricles is near or at background levels and is not

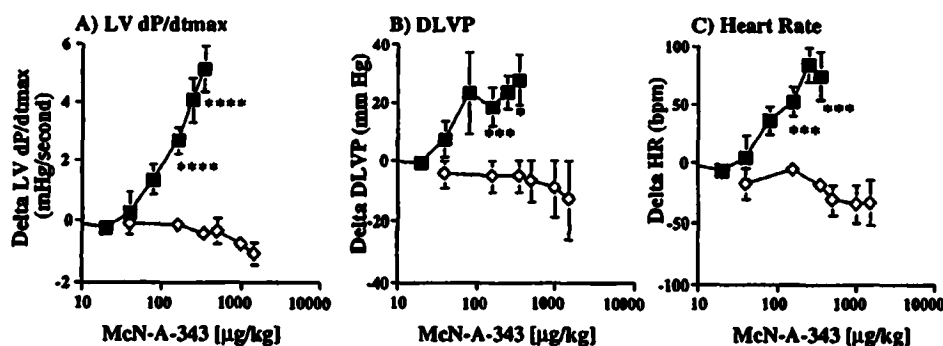


Fig. 3. A to C, cardiac responses to intravenous McN-A-343 in vagotomized WT and M_1 -KO mice. LV dP/dt_{max} (A), DLVP (B), and heart rate (C). WT (■) and M_1 -KO mice (◇) were treated with increasing concentrations of McN-A-343 (0 to 350 and 0 to 1500 µg/kg, respectively). The results are expressed as the change from the basal values ± S.E.M. The results are statistically different between WT ($n = 9$) and M_1 -KO mice ($n = 8$). ****, $p < 0.001$ WT versus M_1 -KO; ***, $p < 0.01$ WT versus M_1 -KO; *, $p < 0.05$ WT versus M_1 -KO (ANOVA). The graphs show an McN-A-343-mediated increase in both inotropic and chronotropic responses in WT mice. The data demonstrate that the M_1 mAChR-selective agonist McN-A-343 does not induce a positive regulation of the heart in M_1 -KO mice.

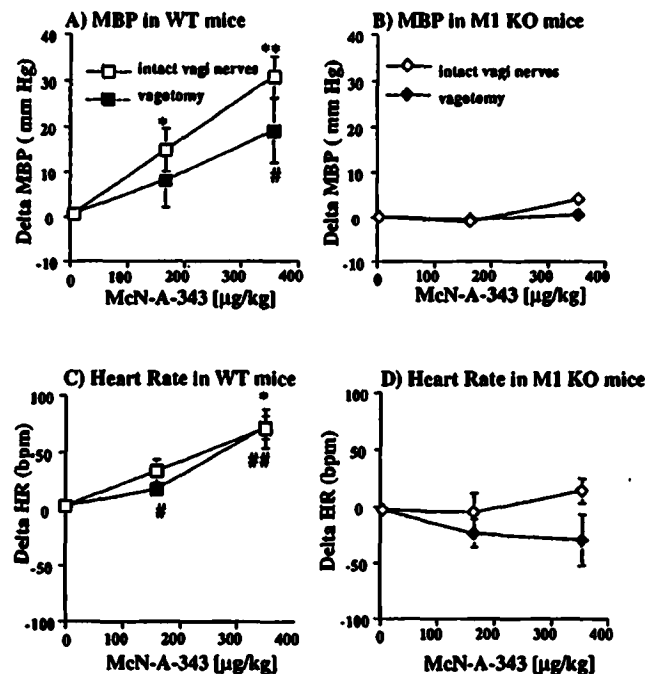


Fig. 4. Hemodynamic responses to intravenous McN-A-343 in WT and M_1 -KO mice before and after bilateral vagotomy. MBP in WT mice (A), MBP in M_1 -KO mice (B), HR in WT mice (C), and HR in M_1 -KO mice (D). WT (A and C) and M_1 -KO mice (B and D) were treated with McN-A-343 (160 and 350 µg/kg) before (□ for WT and ◇ for M_1 -KO) and after (■ for WT and ◆ for M_1 -KO) bilateral vagotomy. The results are expressed as the increase from the basal value (before or after vagotomy) ± S.E.M. The results are statistically different (t test) between WT ($n = 5$) and M_1 -KO ($n = 4$). **, $p < 0.01$ WT versus M_1 -KO before vagotomy; *, $p < 0.05$ WT versus M_1 -KO before vagotomy; ##, $p < 0.01$ WT versus M_1 -KO after vagotomy; #, $p < 0.05$ WT versus M_1 -KO after vagotomy. The graphs show an McN-A-343-caused increase in blood pressure in WT mice; the M_1 -KO mice are not responsive to the M_1 mAChR-selective agonist. The data also show that the McN-A-343-mediated effects are not the result of hypotension causing a baroreflex-induced tachycardia.

statistically different from the values obtained for HET or M_1 -KO.

RT-PCR Experiments. RT-PCR was also used in an effort to detect low levels of M_1 mAChR mRNA in mouse heart. A representative result is shown in Fig. 8. As expected, a strong signal was present in the cortex where M_1 mAChR

constitutes 50% of the total mAChR (Hamilton et al., 1997). No signal was detected in any tissue from the M_1 -KO mouse, or in the cardiac tissues in the WT mouse.

Using immunoprecipitation experiments, Hamilton et al. (1997) were unable to detect M_1 mAChR in mouse cerebellum. The ability of the more sensitive RT-PCR technique to detect M_1 mAChR mRNA in cerebellum but not heart indicates that the M_1 mAChR is most likely not expressed in either mouse atria and ventricles.

Carbachol-Stimulated PLC Activity Is the Same in WT and M_1 -KO Mice

Two mechanisms have been advanced to account for the stimulatory effects of muscarinic agonists on the heart: one involves an increase in intracellular Na^+ concentration (by raising membrane permeability to Na^+); the second mechanism involves stimulation of PLC, via Gq protein, which induces Ca^{2+} release from the endoplasmic reticulum via the inositol triphosphate released after PLC activation (Nathanson, 1987). M_1 mAChRs have been reported to mediate activation of PLC activity in guinea pig cardiomyocytes in culture (Gallo et al., 1993). We measured mAChR agonist-stimulated PLC activity in atrial and ventricular tissues from adult WT and M_1 -KO mice (Fig. 9).

The cholinergic agonist carbachol (1 mM) induced an increase in PLC activity in both WT and M_1 -KO cardiac tissues. In ventricle, increases of 128 ± 36 and $94 \pm 27\%$ over basal were observed in WT and M_1 -KO, respectively (Fig. 9A). Incubation of atria with carbachol resulted in increases of 53 ± 12 and $43 \pm 11\%$ from WT and M_1 -KO mice, respectively (Fig. 9B). If there were M_1 mAChRs in cardiac tissue, a greater PLC activity would be expected in WT than M_1 -KO tissues; however, no significant difference in mAChR-mediated stimulation of PLC activity in hearts from WT and M_1 -KO mice was observed.

Discussion

The main goal of this study was to determine the effects of elimination of the M_1 mAChRs on the function of the heart in vivo. Although the M_2 receptor is the main muscarinic subtype present in the heart and is responsible for the inhibitory effects of ACh, there have been conflicting data on the poten-

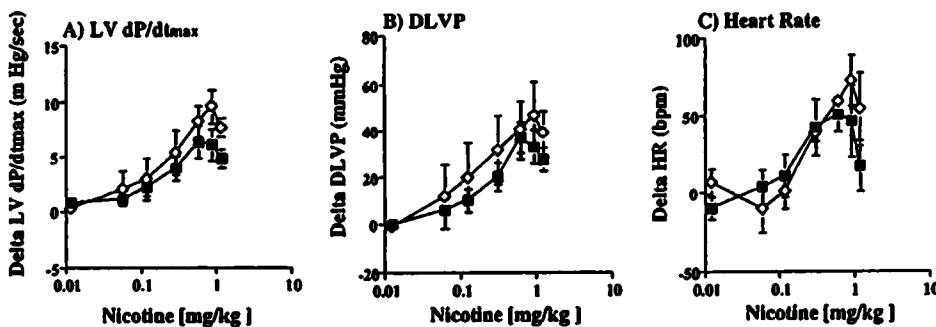


Fig. 5. Cardiac responses to intravenous nicotine in vagotomized WT and M₁-KO mice. LV dP/dt_{max} (A), DLVP (B), and heart rate (C). Both WT (n = 8; ■) and M₁-KO mice (n = 7; ◇) were treated with increasing doses of nicotine (0.01–1 mg/kg). The results are expressed as the difference from basal values ± S.E.M. WT and M₁-KO mice do not differ in their responses to nicotine (p > 0.05; ANOVA). This indicates that the lack of a response to the M₁ receptor agonist McN-A-343 in WT mice was not due to an unresponsive preparation.

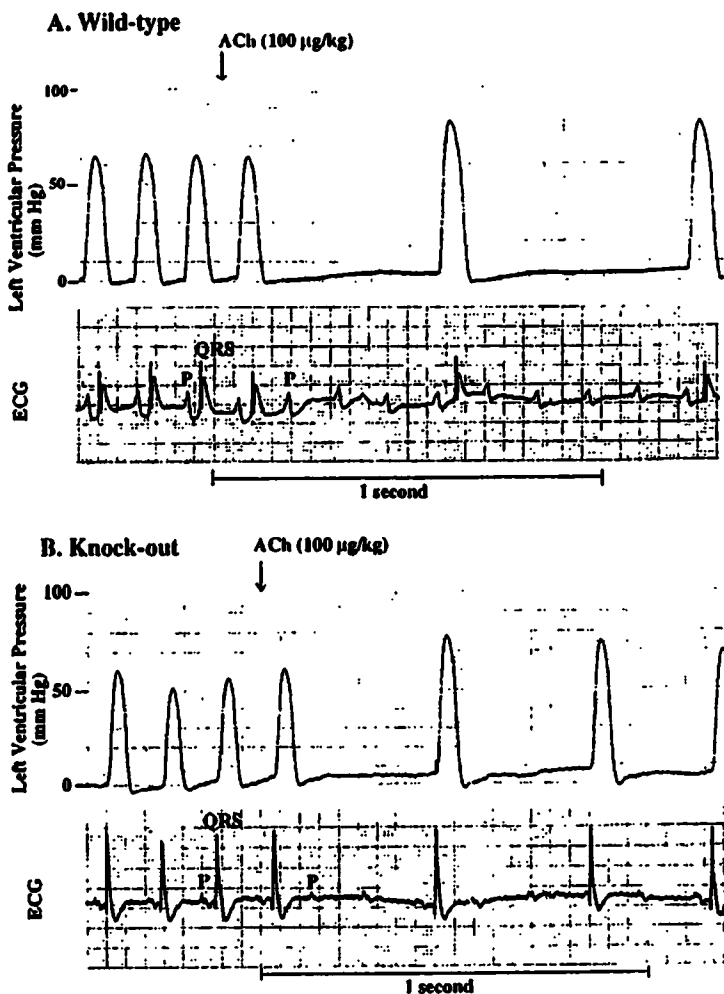


Fig. 6. Effect of ACh on atrioventricular conduction in WT (A) and M₁-KO (B) mice. Mice were pretreated with 1 mg/kg propranolol before injections of ACh to avoid sympathetic reflexes. Before injection of ACh, P waves are observable before the QRS complex. Immediately after injection of 100 µg/kg i.v. ACh there are persistent P waves with irregular QRS complexes, indicating atrioventricular heart block. The atrial rate did not change after the injection of ACh, indicating the absence of a bradycardic effect on the sinoatrial node. The initial threshold effect of intravenous ACh is atrioventricular block without slowing of the atrial rate. Similar results were observed in mice that did not receive a propranolol pretreatment.

tial role of other subtypes in mediating effects of ACh on the heart.

We first compared the basal hemodynamic variables to determine whether the lack of M₁ mAChR had any influence on cardiac function at rest. Both developed left ventricular pressure and LV dP/dt_{max} as well as HR are similar between WT and M₁-KO mice, showing that the loss of M₁ mAChR has no effect on basal cardiac function. We then determined the effects of administration of the muscarinic agonist McN-A-343 in WT and M₁-KO mice. In WT mice, the agonist McN-A-343 was able to induce tachycardia, positive inotropism, and an increase in blood pressure. The increase in HR and

MBP due to McN-A-343 administration observed in our study is in agreement with previous results reported on rat, dog, and cat (Roszkowski, 1961; Hammer and Giachetti, 1982; Wilffert et al., 1983).

None of the mice treated with McN-A-343 either before or after bilateral vagotomy showed a transient decrease in mean blood pressure before the McN-A-343-mediated increase in blood pressure. This indicates that McN-A-343 did not cause peripheral vasodilation and that the positive inotropic and chronotropic cardiac effects were not a baroreceptor reflex response to hypotension. On the other hand, the effect of McN-A-343 on the force of contraction in WT mice

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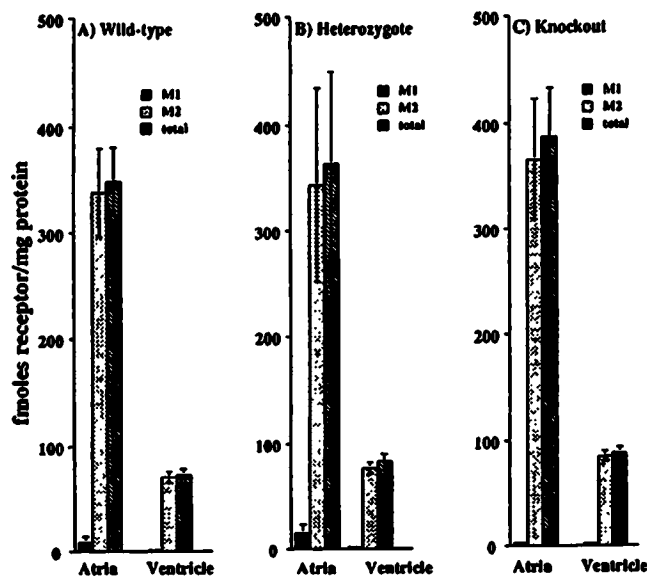


Fig. 7. Expression of muscarinic receptors subtypes in mouse heart. Total mAChR numbers were determined by [³H]QNB binding assay on tissue homogenates from WT (A), HET (B), and M₁-KO (C) mouse hearts. In all cases values are the mean ± S.E.M. of four individual pairs of ventricles and three pools (5–6 animals/pool) of atrial tissues. [³H]QNB-labeled receptors from WT, HET, and M₁-KO mouse heart were solubilized and immunoprecipitated with subtype-specific antisera. Values were compared by ANOVA. The total mAChR number is identical in all genotypes ($p > 0.05$). M₂ mAChR number is also not different between the genotypes ($p > 0.05$). The amount of receptor precipitated by the anti-M₁ mAChR antibody is near background levels and was not different between the three groups: $p > 0.2$ in atria (WT versus HET and WT versus M₁-KO); $p > 0.5$ in ventricle (WT versus HET and WT versus M₁-KO).

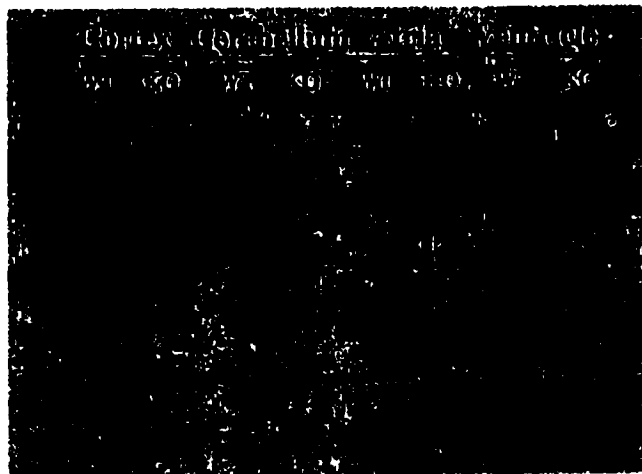


Fig. 8. RT-PCR and Southern blot analyses. Total RNA from WT and KO cortex (0.5 µg), and cerebellum, atria, and ventricles (1.5 µg each) were tested for M₁ mAChR mRNA expression. Each RNA sample was reverse transcribed in the presence (+) or absence (–) of reverse transcriptase (to test for genomic DNA contamination) before amplification by primers designed in the third cytoplasmic loop. The PCR products were analyzed on a 1.0% agarose gel, transferred to nylon membrane, and hybridized with the 1.8-kilobase fragment encoding the entire coding region of the M₁ mAChR. The M₁ mAChR mRNA is expressed in both cortex and cerebellum but is not detectable in mouse heart.

was blocked by the β -adrenergic receptor antagonist propranolol. These observations together indicate that McN-A-343 probably acts at the postganglionic sympathetic nerves,

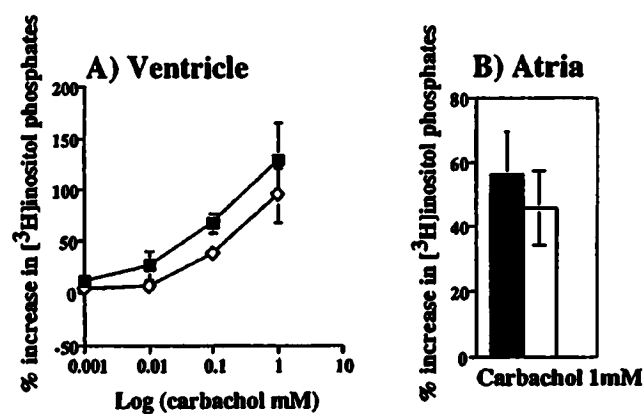


Fig. 9. mAChR-mediated PLC activation in WT and M₁-KO heart. The stimulation of PLC activity of ventricles (A) and atria (B) in response to muscarinic agonists was determined as described under *Materials and Methods*. The graphs show the percentage of increase in [³H]inositol phosphates formed after incubation with carbachol. The values are not statistically different ($p > 0.05$) between WT (filled) and M₁-KO mice (open) both in ventricle (A) ($n = 9$ for WT and $n = 8$ for M₁-KO mice; ANOVA) and in atria (B) ($n = 7$ in each group; t test). Results represent the average values ± S.E.M.

which is consistent with previous work reported in other species (Roszkowski, 1961; Martin, 1996).

In pithed normotensive rats, the dose-dependent increase in blood pressure after the injection of McN-A-343 was strongly reduced in the presence of the M₁/M₄ antagonist pirenzepine (Hammer and Giachetti, 1982; Wilffert et al., 1983). It has also been previously shown that M₁-mediated suppression of the M-current potassium channel is completely absent in sympathetic ganglia from M₁-KO mice (Hamilton et al., 1997). However M₁, M₂, and M₄ mAChR are all expressed in the sympathetic ganglia (Caulfield, 1993). In the present study WT mice displayed a dose-dependent increase in both heart rate and LV dP/dt_{max} when injected with McN-A-343, but the M₁-KO mice did not. This clearly demonstrates that McN-A-343-induced responses are mediated by M₁ mAChR.

Although the apparent decreases in hemodynamic values observed at higher doses of McN-A-343 (500–1500 µg/kg) in M₁-KO mice might be due to a nonselective stimulation of cardiac M₂ mAChR, these decreases were not statistically different from the basal values.

Activation of nicotinic receptors in sympathetic ganglia induces catecholamine release from the endings of postganglionic cells, which results in an increase in both force and rate of contraction by stimulating the cardiac β -adrenergic receptors. Administration of nicotine produced a similar response in both WT and M₁-KO mice, demonstrating that the lack of response to McN-A-343 in the KO mice was not due to a generalized defect in sympathetic transmission to the heart or an artifact of the surgical procedures.

Pharmacological studies have suggested a functional role for the M₁ mAChR in adult guinea pig (Gallo et al., 1993) and rat cardiomyocytes (Sharma et al., 1996). In rat cardiomyocytes, the stimulatory effects of mAChR agonists were significantly inhibited by antisense oligonucleotides directed against the M₁ mAChR subtype (Colecraft et al., 1998). In addition, RT-PCR analyses detected the expression of M₁ mAChR in adult rat and guinea pig ventricular myocytes in culture (Gallo et al., 1993; Sharma et al., 1996).

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We performed biochemical and functional studies on mouse cardiac tissue to determine whether M_1 mAChRs were also expressed in the mouse heart. Immunoprecipitation analyses showed that the M_1 -KO mice had unaltered levels of expression of M_2 receptors in the heart. Our results also show no statistically significant expression of the M_1 mAChR expression in WT mouse heart. We also used RT-PCR to detect M_1 mAChR mRNA in total RNA from WT heart. Despite multiple experimental conditions to increase sensitivity (two sets of primers to get a double amplification, radioactive PCR, variations in RT-PCR conditions, several RNA concentrations), we were not able to detect any signal in cardiac tissue from WT mice. Because we were able to detect M_1 mAChR mRNA in the cerebellum from WT mice, where the expression of M_1 mAChR represents less than 5% of total mAChR in cerebellum (Caulfield, 1993), the procedure was sensitive enough to detect very low levels of M_1 mAChR mRNA.

We also attempted to measure functional responsiveness of M_1 mAChRs in cardiac tissue. The mouse M_1 mAChR in transfected cells is preferentially coupled to the activation of PLC (Shapiro et al., 1988). Furthermore, M_1 mAChR is the main mAChR subtype coupled to activation of the PLC pathway in mouse cerebral cortical neurons (Hamilton and Nathanson, 2001). We measured the muscarinic agonist-mediated stimulation of PLC activity in heart from WT and M_1 -KO mice. If M_1 mAChRs are present in cardiac WT tissue, PLC activity would be expected to be higher in WT tissue than in M_1 -KO tissue. However, there was no significant difference in carbachol-mediated stimulation of PLC between WT mice compared with M_1 -KO.

These biochemical results indicate that, in contrast to rat and guinea pig hearts, M_1 mAChRs are not expressed in the mouse heart. This is consistent with our physiological data showing that the effects of McN-A-343 are the consequence of activation of M_1 mAChR in the sympathetic ganglia (present study) and with the fact that McN-A-343 did not have any effect on the isolated mouse atria (Nishimaru et al., 2000). Our results also are in agreement with the report of Roszkowski (1961) who observed, in dog, an McN-A-343-induced rise in blood pressure in vivo but did not report any effect on isolated heart from dog, rabbit, and cat, suggesting the absence of M_1 mAChR in this tissue (Roszkowski, 1961; Shi et al., 1999b).

To determine whether the lack of M_1 mAChR in the KO mice affected the responsiveness of M_2 mAChR, ACh was injected in both WT and M_1 -KO mice and the responsive effects were recorded by ECG. Both WT and M_1 -KO mice responded to ACh with a similar threshold (data not shown), demonstrating that the lack of M_1 mAChR did not result in any change in M_2 mAChR-mediated response. However, the prominent effect of ACh on cardiovascular function in many mammals is to slow the rate of the sinus node depolarization. Unexpectedly the main and immediate effects of intravenous ACh on the mouse heart were the induction of atrioventricular conduction block, atrial flutter, or atrial fibrillation. Over a wide range of doses (0.01 pg/kg-200 μ g/kg) sinus bradycardia was only barely and inconsistently detected. Similar Gi-coupled receptor-mediated arrhythmias have been also described in transgenic and knockout mice (Redfern et al., 2000; Kovoov et al., 2001). Because we never detected sympathetic reflex-mediated stimulation of the

heart in mice treated with ACh in the absence of propranolol (data not shown), it is unlikely that the absence of ACh-induced bradycardia is the result of a sympathetic reflex that might have buffered the bradycardia. The absence of ACh-induced bradycardia could result from the high level of acetylcholinesterase in the sinus node (Loffelholz and Lindmar, 1994). This would prevent ACh from acting at the sinus node level, but would allow an action on the atrioventricular node where the concentration of acetylcholinesterase is lower (Loffelholz and Lindmar, 1994). This hypothesis is supported by the results of Walker et al. (1999) and Feniuk and Large (1975), who were able to detect bradycardia after injection of mice with the agonist methacholine (which is partially resistant to hydrolysis) or after injection of ACh into mice pretreated with the cholinesterase inhibitor physostigmine.

Because pharmacologically administered ACh did not appear to affect the sinoatrial node, the role of vagal tone in regulating murine heart rate was examined. WT and M_1 -KO mice both exhibit tachycardia in response to bilateral vagotomy, indicating that the vagus did mediate a tonic inhibition of the sinoatrial node in resting mice.

In summary, we have shown that 1) McN-A-343 induces both tachycardia and a positive inotropic effect, as well as an increase in blood pressure, in vivo in mice, due to M_1 mAChR activation of postganglionic sympathetic neurons innervating the heart; 2) M_1 mAChRs are not detected in the WT mouse heart tissue; 3) the lack of M_1 mAChR does not modify M_2 mAChR cardiac responsiveness in vivo; and 4) pharmacological administration of ACh in mouse rarely results in sinus bradycardia but usually results in atrioventricular block, atrial fibrillation, or atrial flutter as the initial threshold effect.

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ANEXO 3

Artículo de M.U. Gillette y colaboradores, Life Sciences (2001), 68.

Role of the M₁ receptor in regulating circadian rhythms

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Abstract

Cholinergic stimuli are potent regulators of the circadian clock in the hypothalamic suprachiasmatic nucleus (SCN). Using a brain slice model, we have found that the SCN clock is subject to muscarinic regulation, a sensitivity expressed only during the night of the clock's 24-h cycle. Pharmacological and signal transduction characteristics are compatible with a response mediated by an M₁-like receptor. Molecular manipulation of muscarinic receptors will provide important insights as to the receptor subtype(s) regulating circadian rhythms. © 2001 Elsevier Science Inc. All rights reserved.

Keywords: Acetylcholine; cGMP/PKG signaling; Circadian rhythms; Muscarinic receptors; Suprachiasmatic nucleus

Fundamental behaviors, such as locomotory activity vs. sleep, exhibit alternating patterns of expression with characteristic relationships to the day-night cycle. Patterning is controlled by the master circadian clock that resides within the suprachiasmatic nucleus (SCN) at the base of the hypothalamus. The SCN circadian clock is an endogenous, dynamic set of cellular state progressions that generates a ~24-h timebase. Efferent signals, in turn, organize physiological, hormonal and behavioral functions into near 24-h cycles, termed *circadian rhythms*. In addition to this timekeeping role, the SCN integrates afferent signals relaying changes in external and internal temporal state. The clock restricts the timing of its sensitivity to these signals so that they communicate temporal desynchronization, readjusting the timekeeping mechanism. This gatekeeping property and the capacity for clock resetting are fundamental to appropriately orchestrating organismic functions over the day-night cycle [1].

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Acetylcholine (ACh) has long been implicated in nocturnal adjustment of circadian rhythms [2,3], although the behavioral context in which it functions is not yet established. The SCN is not intrinsically cholinergic [4], but receives direct cholinergic projections from the basal forebrain and brain stem tegmentum [5]. These brain regions regulate changes of sleep and arousal states, which occur in prominent day-night patterns that are timed by the SCN. Their cholinergic innervations of the SCN are potential feedback circuits [6].

Clock properties are preserved for at least three days in a hypothalamic brain slice containing the SCN. The neuronal ensemble within the SCN generates stable 24-h rhythms of spontaneous firing rate that peak near midday *in vitro* [7], as they do *in vivo* [8]; this peak provides an unambiguous marker of clock phase. Additionally, the SCN clock *in vitro* continues to gate its sensitivity to resetting stimuli [9,10]. Of note is the finding that clock sensitivity in the brain slice to glutamate, the neurochemical messenger transmitting environmental light signals from the eye to the SCN, matches in detail the timing and pattern of sensitivity of behavioral rhythms in animals to phase resetting by nocturnal light [11,12]. Thus, the suprachiasmatic brain slice offers an experimentally accessible preparation for investigating mechanisms that mediate cholinergic regulation of the SCN clock.

We have evaluated the direct action of the cholinergic agonist, carbachol, on the rhythm of SCN neuronal activity using extracellular electrophysiological recording techniques. We found that the SCN clock is sensitive to cholinergic stimulation such that the phase of the clock, as measured by timing of the peak in the spontaneous activity rhythm, is advanced (Fig. 1a, b). Phase advance means that clock processes jump ahead, as the timekeeping mechanism is immediately reset to a new state from which time is reckoned from that point on. Although the SCN brain slice is maintained under constant conditions *in vitro*, cholin-

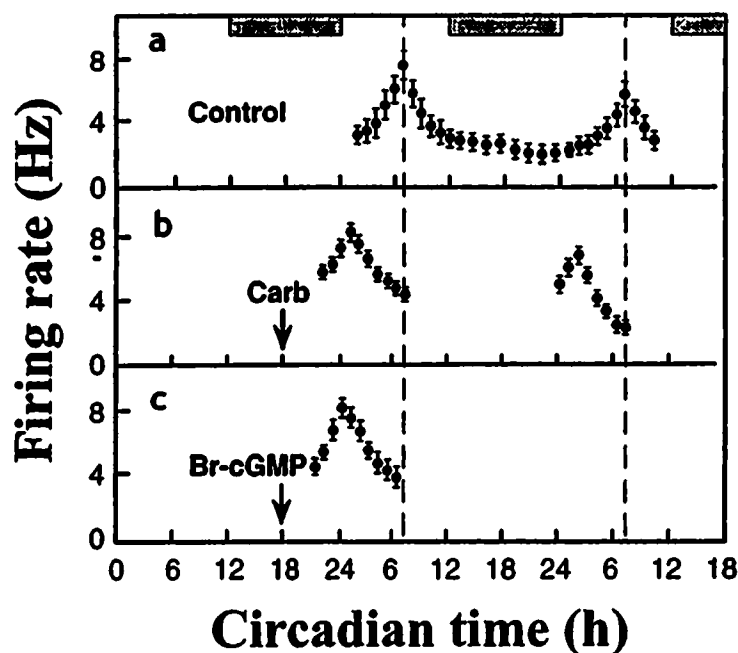


Fig. 1. The circadian rhythm of spontaneous neuronal activity (a) in the rat SCN brain slice is advanced by carbachol (Carb) (b) and 8-Br-cGMP (c) applied mid-subjective night (CT 18).

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ergic sensitivity is expressed only during the subjective night (Fig. 1b, 2), that portion of the 24-h cycle that matches the dark (night) period in the animal colony.

The pattern of sensitivity of the SCN to carbachol provided clues as to the signaling pathway by which the cholinergic signal is transmitted to the clock. Carbachol induces phase advance across the night with peak sensitivity at circadian time (CT) 18. (CT is a designation for the temporal state of the clock under constant conditions that starts at CT 0 = “lights on” in the donor animal’s colony and continues for 24-h.) At CT 18, carbachol administration advances SCN clock state by ~6 h. The amplitude of this maximal shift and the timing of SCN sensitivity to carbachol are completely overlapping with clock sensitivity to membrane-permeable analogs of the intracellular messenger, cGMP (Fig. 1c, 2) [13,14].

When the predictions of these correlative data were probed experimentally, we found that carbachol directly up-regulated the guanylyl cyclase/cGMP/protein kinase G (PKG) pathway in the SCN of rat. At CT 18, carbachol induced a rapid, transient rise in cGMP in SCN tissue at 3 min, as well as enhanced phosphotransferase activity of PKG toward a preferred substrate; the former effect was block by the anti-muscarinic, atropine [14]. Furthermore, LY83583, a guanylyl cyclase inhibitor, blocked the carbachol-induced clock resetting, but not the shift induced by the analog of its product, cGMP, a downstream effector. On the other hand, the selective PKG inhibitor, KT5823, blocked the effects of carbachol and Br-cGMP, both of which act upstream of PKG. This evidence that the cholinergic signal may be mediated by cGMP implicates the odd-numbered muscarinic receptors (M_1 , M_3 , M_5) in SCN clock regulation. However, it does not identify a single receptor, nor does it exclude participation in the response by even-numbered receptor subtypes [15].

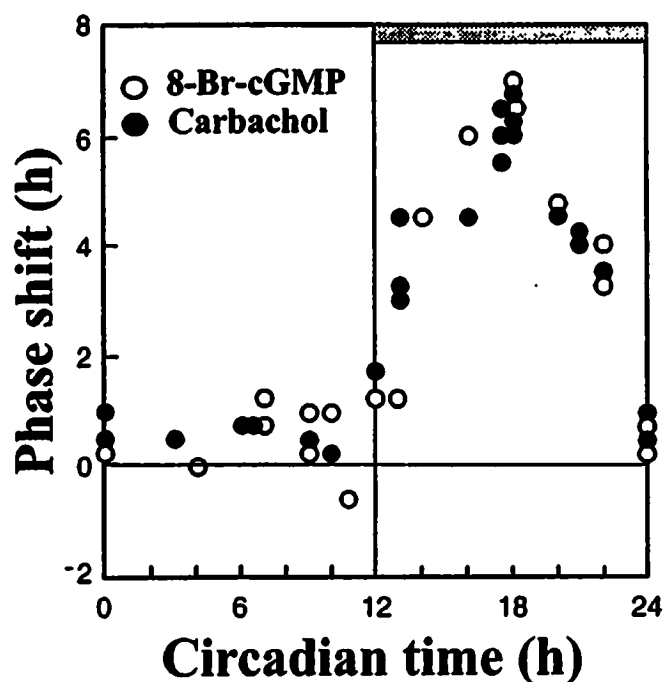


Fig. 2. Carbachol and 8-Br-cGMP induce phase advance of the SCN circadian clock during subjective night, with overlapping periods of sensitivity and amplitudes of response. Each circle is the result of a single experiment evaluating time-of-peak activity, as in Fig. 1b, c.

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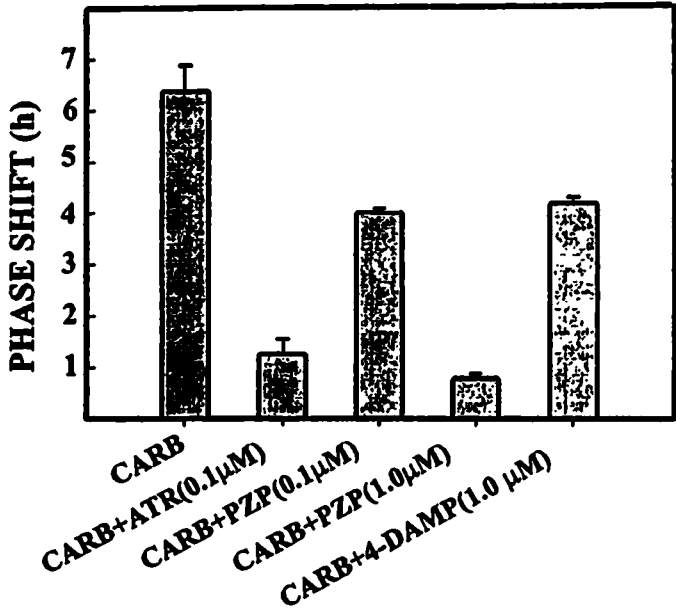


Fig. 3. Relative potency of muscarinic antagonists. atropine (ATR), pirenzepine (PZP) and 4-DAMP, in blocking the effect of carbachol at CT 18 suggests an M₁-like receptor mediated action.

Pharmacological analyses of the cholinergic effects on the SCN clock at CT 18 further characterized receptors mediating clock resetting. Carbachol is a nonselective cholinergic agonist that activates both muscarinic and nicotinic receptors, and both receptors are expressed in SCN [16]. Furthermore, nicotinic effects on the rat SCN have been reported [17,18]. When

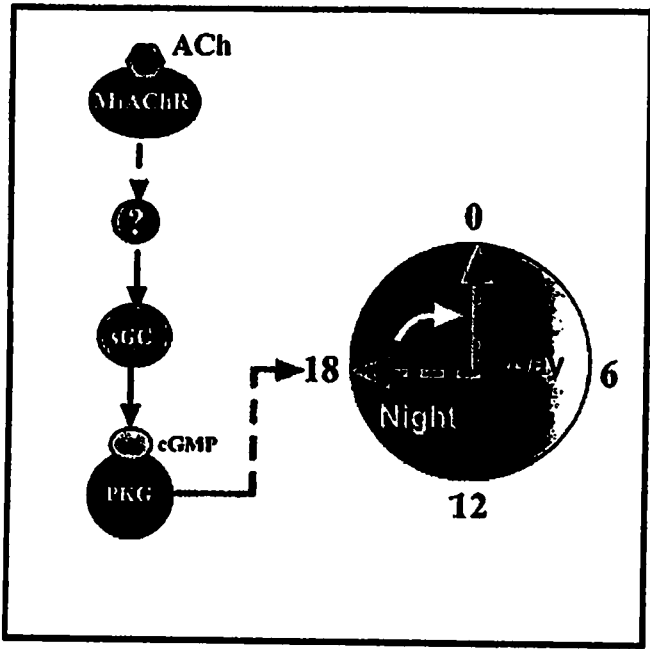


Fig. 4. Model of putative signaling elements that transduce the nocturnal cholinergic signal into phase advance of the SCN circadian clock.

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the dose-response relationship among various cholinergics applied by microdrop over a range of concentrations was analyzed for efficacy on phase-resetting, the following relative potency was observed: ACh > McN-A-343 > carbachol = muscarine >>> nicotine [19]. Nicotine was three orders of magnitude less effective than carbachol or muscarine. Nicotinic antagonists, dihydro- β -erythroidine (DH β E) and *d*-tubocurarine, each were ineffective in blocking the carbachol-induced phase advance. However, the effect of carbachol was blocked differentially by muscarinic antagonists, with a relative potency as follows: atropine > pirenzepine > 4,2-(4,4'-diacetoxydiphenylmethyl)pyridine (4-DAMP) (Fig. 3). Relative efficacy of McN-A-343 and of pirenzepine, putatively selective for the M₁ muscarinic subtype, concurred with pharmacological sensitivities in other reports [20,21]. These pharmacological data suggest that the cholinergic receptor mediating SCN clock resetting expresses M₁-like characteristics. A model linking putative elements in the cholinergic pathway signaling phase advance to the SCN clock appears in Fig. 4.

The specificity of these pharmacologic agents for the M₁ receptor subtype has been questioned [15]. However, the development of transgenic mice in which the gene for the M₁ receptor has been lost by homologous recombination (M₁-knock out mice, M₁KO) [22] offers the opportunity to genetically test M₁ receptor contribution to clock regulation. The M₁KO mice develop normally and are generally healthy. Up-regulation of M₂, M₃, or M₄ receptors was not detected in the hippocampus [22]. Deficits of function reported include loss of the muscarinic receptor-dependent M-current K⁺ channel activity in sympathetic ganglion neurons, reduced susceptibility to pilocarpine-induced cortical seizure [22], and disabled N- and L-type Ca²⁺-mediated neurotransmitter release in superior cervical ganglion [23]. Preliminary studies [24] suggest that the M₁KO mouse as well as transgenic mice with other muscarinic receptor deficits offer important models for critically evaluating cholinergic effects on the circadian clock.

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ANEXO 4

Artículo de H. Kitagawa y colaboradores, Neuropsychopharmacology (2003).

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Safety, Pharmacokinetics, and Effects on Cognitive Function of Multiple Doses of GTS-21 in Healthy, Male Volunteers

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This study was designed to determine the safety, tolerability, pharmacokinetics and effects on cognitive function of GTS-21 in healthy, male volunteers. A total of 18 subjects were randomized to GTS-21 (25, 75 and 150 mg) or placebo administered three times daily (first 4 days, once on Day 5) for three, 5-day sessions. GTS-21 was well tolerated up to doses of 450 mg/day, with no clinically significant safety findings. C_{max} and the area under the plasma concentration of GTS-21 and the metabolite 4-OH-GTS-21 increased in a dose-related fashion; although considerable intersubject variability occurred, it decreased with continued dosing. GTS-21 showed statistically significant enhancement of three measures of cognitive function (attention, working memory, episodic secondary memory) compared to placebo. A relationship between exposure to GTS-21 and the magnitude of the cognitive response was apparent, with maximal effect approached for doses between 75 and 150 mg three times a day. These data indicate that GTS-21 may represent a novel treatment for dementia. *Neuropsychopharmacology* (2003) 28, 542–551. doi:10.1038/sj.npp.1300028

Keywords: Alzheimer's disease; pharmacokinetics; cognition disorder

INTRODUCTION

Alzheimer's disease (AD) is a progressive, degenerative, disabling disease and is the leading cause of dementia in the elderly (Mayeux and Schofield, 1994). Approximately 4 million individuals in the United States, and about 25% of Americans who are 85 years or older, are afflicted with AD or a related dementia (Strock, 1994). The cholinergic hypothesis of the impairment of memory and cognition observed in AD patients postulates that the impairment is due to a reduction in cholinergic function (Bartus *et al*, 1982). Evidence for this hypothesis includes findings that

the degree of cognitive impairment in patients with AD is positively correlated with decreases in markers of cholinergic function measured in biopsied or post-mortem brains (Francis *et al*, 1985; Perry *et al*, 1978). In addition, the number of nicotinic acetylcholine receptors (nAChR) is reduced in the brains of AD patients (Schroder *et al*, 1991; Whitehouse *et al*, 1986). Consequently, it may be possible to improve the impairment in memory and cognitive function of AD patients and affect disease progression with nicotinic cholinergic agonists.

GTS-21 is a drug with a novel pharmacological profile, which has been shown to be an agonist at α -7 nAChRs. The chemical name of GTS-21 is (E)-3-(2,4-dimethoxybenzylidene)-3,4,5,6-tetrahydro-2,3'-bipyridine dihydrochloride; the structures of GTS-21 and the pharmacologically active metabolite, 4-OH-GTS-21, are shown in Figure 1. GTS-21 is active in many animal models that are widely used to measure effects on memory and learning. GTS-21 was also effective in promoting cell survival in several *in vitro* and *in vivo* studies. Based on its preclinical profile, GTS-21 is being developed for the treatment of both the cognitive dysfunction and neurodegeneration seen in patients with AD. A total of 87 healthy, human subjects have been enrolled in four Phase I studies to evaluate GTS-21. In the first three studies, GTS-21 was found to be well tolerated at single doses up to 250 mg. This article summarizes the results of the fourth study — a randomized, placebo-controlled, multiple dose study of the safety, pharmacokinetics and cognitive effects of GTS-21 in healthy volunteers.

The following parties contributed sufficiently to the research for, and writing of, this manuscript so as to take public responsibility for it, and affirm that this represents valid work whose results would be possible to reproduce with the same methods and conditions. Any financial or other affiliations with the sponsor are noted as follows: H Kitagawa, was employed by the Taiho Pharmaceutical Co. Ltd, during the time that the research discussed in this manuscript was conducted. KA Wesnes and WG Kramer were paid consultants for the Taiho Pharmaceutical Co. Ltd during the time that this research was conducted. DE Clody and AL Burnett worked under contract on behalf of the Taiho Pharmaceutical Co. Ltd at the time of this research.

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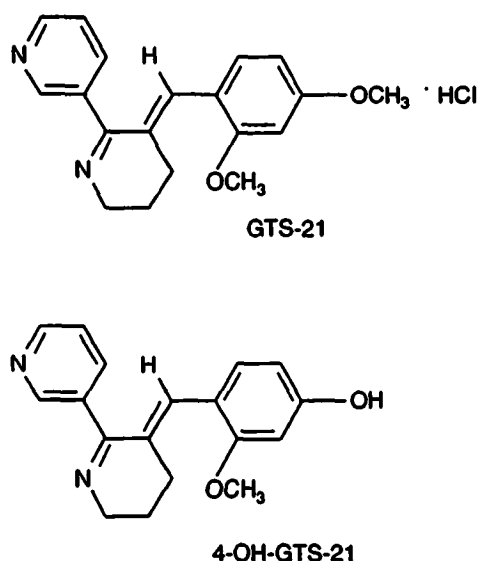


Figure 1 Chemical structures of GTS-21 and 4-OH-GTS-21.

SUBJECTS AND METHODS

Subjects

The study population comprised healthy, male volunteers aged 18–45 years; female volunteers were not included because the reproductive toxicity studies of GTS-21 had not been completed at the time of study initiation. Subjects were to be in good health as determined by a physical examination including vital signs, weight, clinical laboratory tests and electrocardiograms. Major exclusion criteria included the following: history of alcohol or drug dependency; positive result on urine drug screen; excessive caffeine use; and history of neurologic disorder, dementia, mental status change, severe head injury or serious psychiatric condition. The urine drug screen included the following: amphetamines, barbiturates, benzodiazepines, cocaine, morphine/opiate, methadone and tetrahydrocannabinol (THC). The study did not exclude subjects with a history of smoking, but smoking was not allowed during the study.

Study Protocol

This clinical trial was conducted at DDS Medicines Research Ltd in the UK in compliance with the Declaration of Helsinki and with regulatory requirements pertaining to informed consent and institutional review boards. The Ethics Review Board of the Tayside Committee on Medical Research, UK, approved the protocol prior to study initiation.

This was a single-center, randomized, placebo-controlled, double-blind, multiple dose study in a group of healthy, male volunteers. At baseline, the initial 16 eligible subjects who signed an informed consent were randomly assigned to either Group A (GTS-21, 12 subjects) or Group B (placebo, four subjects).

The treatment phase included three, 5-day treatment periods separated by 10-day wash-out periods. During each

of the three treatment sessions, subjects were restricted to the clinical site from the evening before the first dose until the morning after the last dose. Subject eligibility was verified by entry criteria and the urine drug screen at the beginning of each treatment period. The GTS-21 regimen was 25 mg (treatment period 1), 75 mg (treatment period 2) and 150 mg (treatment period 3). Doses were administered three times a day (t.i.d.) (08 00, 14 00, and 20 00 h) on Days 1–4 and once (08 00 h) on Day 5 of each treatment session. GTS-21 was supplied as coated tablets containing 25 mg of the active ingredient GTS-21; placebo tablets were identical in appearance. All doses were administered immediately after a meal. All subjects were to return to the clinical site 5–7 days after their last dose for a follow-up visit to assess safety parameters.

Plasma was collected predose; 15, 30 and 45 min; and 1, 1.25, 1.5, 2, 3, 4 and 6 h postdose on Day 1 and Day 5 (08 00 dose). Additional samples were collected predose on Day 3 (08 00 dose) and Day 4 (14 00 dose), and at 8 and 12 h postdose on Day 5. Urine was collected predose and 0–2, 2–4, and 4–6 h postdose on Day 1 (08 00 dose). Urine was also collected 0–2, 2–4, 4–6, 6–8 and 8–12 h postdose on Day 5. High-performance liquid chromatographic methods adapted from those described by Azuma were used to determine plasma (Azuma *et al*, 1996) and urine (internal report, Taiho Pharmaceutical Co.) concentrations of GTS-21 and 4-OH-GTS-21. Following solid-phase extraction, samples were chromatographed using a reverse-phase column with detection by visible absorbance at 405 nm. The method was linear for plasma concentrations of GTS-21 and 4-OH-GTS-21 from 1 to 200 ng/ml; the coefficient of variation for the parent was 7.3% at 5 ng/ml and 5.3% at 150 ng/ml; and corresponding values for the metabolite were 14.7 and 9.5%. For urine, the method was linear from 4 to 1000 ng/mL for both compounds, with coefficients of variation of 3.6 and 4.7% at GTS-21 concentrations of 10 and 750 ng/ml, respectively, and 7.3 and 8.9% at those concentrations of the metabolite. Urine samples were subjected to enzymatic hydrolysis using β -glucuronidase followed by reanalysis for GTS-21 and 4-OH-GTS-21 to determine the extent of formation of glucuronide conjugates.

All subjects were evaluated for performance on a computerized test battery to measure the effects of treatment on cognitive functioning. Using computerized tests developed by Cognitive Drug Research Ltd (CDR) (Reading, UK), changes in attention, memory and reaction time from predose measurements were assessed during each session (Wesnes *et al*, 1988). Cognitive tests included the following: attention-related tests (simple reaction time, choice reaction time, digit vigilance), numeric and spatial working memory tests, secondary episodic recognition memory tests (word and picture recognition, immediate and delayed word recall) and visual tracking. In addition, an overnight face recognition test was administered and Bond-Lader visual analog scales were used to assess subjective alertness, subjective contentment and subjective calmness (Bond and Lader, 1974). Two CDR training sessions were held prior to the first dosing period. Cognitive tests were performed on Day 1 (1 h predose) as well as on Days 2 and 4 of each treatment period (1 h predose and 1, 2, and 4 h after the 08 00-h dose).

STATISTICAL/PHARMACOKINETIC ANALYSIS

Safety

Descriptive statistical methods were used to summarize demographics, adverse events, ECG results and clinical laboratory tests.

Pharmacokinetics

Descriptive statistical methods were used to summarize plasma concentrations and amounts excreted in the urine of GTS-21 and 4-OH-GTS-21 and the resultant pharmacokinetic parameters. For GTS-21 and 4-OH-GTS-21, only those plasma or urine concentrations that were equal to or greater than the validated limit of the assay (1 ng/ml for either compound in plasma and 10 ng/ml in urine) were used in the pharmacokinetic analyses. Actual blood sampling and urine collection times were used for all pharmacokinetic calculations. For graphical presentation of mean data, nominal times were used in the calculation of mean plasma concentrations and amounts excreted in the urine.

Pharmacokinetic parameters for GTS-21 and 4-OH-GTS-21 were calculated using noncompartmental methods. C_{max} and T_{max} were taken directly from the data. The terminal elimination rate constant, λ_z , was calculated as the negative of the slope of the terminal log-linear portion of the plasma concentration time curve. The specific range of times was determined by graphical inspection of a semilogarithmic plot of concentration vs time. Elimination half-life ($t_{1/2}$) was calculated from λ_z according to $t_{1/2} = 0.693/\lambda_z$. The area under the plasma concentration (AUC)-time curves to the last time point (t_f) with a concentration above the validated limit of quantitation (C_{tl}) (AUC_{tl}) (Day 1) and during the 6-h dosing interval (Day 5) (AUC_r) were calculated using the linear trapezoidal method. AUC_{tl} on Day 1 was extrapolated to infinity (AUC_{∞}) using C_{tl} and λ_z , as shown below:

$$AUC_{\infty} = AUC_{tl} + \frac{C_{tl}}{\lambda_z}$$

On Day 5, if t_f occurred before the end of the dosing interval and a value was available for λ_z , AUC was extrapolated to the end of the dosing interval according to the following equation:

$$AUC_r = \frac{C_{tl}}{\lambda_z} (e^{-\lambda_z \cdot t_f} - e^{-\lambda_z \cdot 6})$$

The fraction of the dose excreted in the urine through 24 h (Day 1) or 6 h (Day 5) (fe), calculated from the sum of the amounts in each collection interval, was expressed as a percent of the dose; amounts of the 4-OH metabolite were corrected for the difference in molecular weight.

Cognitive Function

Both a primary analysis and a secondary (by dose period) analysis were performed on the cognitive assessment data. For the primary analysis, a two-way analysis of variance (ANOVA) was conducted. Five terms were fitted to the model: dosing condition, subjects nested within dosing condition, repeated testing, an interaction term between dosing condition and repeated testing, and an error term for this interaction. The two relevant statistical contrasts were

(1) a main effect of dosing condition, and (2) an interaction between dosing condition and repeated testing, reflecting an effect that depended on the time of assessment.

The secondary analysis was also performed to evaluate the treatment groups by dosing period. The difference from baseline data was averaged over each dosing period to derive a single score per measure for each subject. The data were subjected to a two-way ANOVA. Five terms were fitted to the model; these terms were the same as for the primary analysis except that the interaction term was between dosing condition and dosing periods. The two relevant statistical contrasts were (1) a main effect of dosing condition, and (2) an interaction between dosing condition and dosing period.

Pharmacokinetic/Pharmacodynamic Modeling

Potential relationships between the cognitive responses to GTS-21 and its pharmacokinetics were examined for immediate word recall accuracy, delayed word recall accuracy and digit vigilance. For each subject for each response, the area under the effect curve (AUEC) on Day 4 of dosing was calculated using the linear trapezoidal method, where effect was defined as the change from baseline. The difference in mean AUEC between GTS-21 and placebo at each dose was then plotted against the mean AUC_r on Day 5. Due to the short $t_{1/2}$ of GTS-21, concentrations on Day 5 were comparable to those on Day 1, and thus the AUC_r on Day 5 was considered to be representative of that on Day 4 when the cognitive responses were measured.

RESULTS

A total of 18 subjects, including two replacement subjects, were enrolled and received study drug (14 GTS-21, four placebo). Initially, 16 subjects were enrolled in the study (12 GTS-21, four placebo). Three subjects in the GTS-21 group discontinued early. One subject was discontinued in Session 3 at the sponsor's request because of elevated liver enzymes; one subject was chewing tablets instead of swallowing them and was discontinued in Session 1; and one subject left the study in Session 2 because he found employment. Two of these subjects were replaced; both were in the active treatment group. The demographics of the study population were similar between treatment groups. The mean age, height and weight was 33.2 years, 179.9 cm and 79.9 kg in the GTS-21 group, respectively, and 31.0 years, 175.0 cm and 72.2 kg in the placebo group, respectively. All subjects were Caucasians. Treatment compliance was controlled by the site, as the study drug was administered by the investigator or designee. Subject compliance with the study procedures was also assessed by the site staff.

Safety

GTS-21 was found to be well tolerated in healthy male volunteers up to a daily dose of 450 mg (150 mg t.i.d.). There were no clinically significant differences between treatment groups with respect to incidence of treatment-emergent adverse events. Table 1 shows the incidence of adverse events for all subjects. Although not considered to be

Table 1 Incidence of Treatment-Emergent Adverse Events

Body system/AE	GTS-21				Placebo
	25 mg t.i.d. (N = 12)	75 mg t.i.d. (N = 12)	150 mg t.i.d. (N = 12)	Total (N = 14)	(N = 4)
<i>Skin and appendages</i>					
Dermatitis	0	0	1 (8.3%)	1 (7.1%)	0
Eczema	1 (8.3%)	1 (8.3%)	0	2 (14.3%)	0
Rash	0	0	0	0	1 (25.0%)
<i>Musculoskeletal</i>					
Arthralgia	1 (8.3%)	0	0	1 (7.1%)	0
<i>Central and peripheral nervous system</i>					
Dizziness	1 (8.3%)	1 (8.3%)	1 (8.3%)	1 (7.1%)	1 (25.0%)
Headache	1 (8.3%)	3 (25.0%)	3 (25.0%)	6 (42.9%)	2 (50.0%)
Paresthesia	0	0	0	0	1 (25.0%)
<i>Psychiatric</i>					
Agitation	0	0	1 (8.3%)	1 (7.1%)	0
Euphoria	0	0	0	0	1 (25.0%)
Insomnia	0	1 (8.3%)	0	1 (7.1%)	0
Somnolence	0	1 (8.3%)	0	1 (7.1%)	1 (25.0%)
<i>Gastrointestinal</i>					
Abdominal pain	1 (8.3%)	0	0	1 (7.1%)	0
Appetite increased	0	1 (8.3%)	0	1 (7.1%)	0
Constipation	1 (8.3%)	0	0	1 (7.1%)	0
Toothache	0	0	1 (8.3%)	1 (7.1%)	0
<i>Liver and Biliary</i>					
SGOT increased	0	1 (8.3%)	3 (25.0%)	3 (21.4%)	1 (25.0%)
SGPT increased	0	2 (16.7%)	3 (25.0%)	3 (21.4%)	1 (25.0%)
GGT increased	0	0	1 (8.3%)	1 (7.1%)	1 (25.0%)
<i>Cardiovascular</i>					
Hypotension postural	0	1 (8.3%)	0	1 (7.1%)	0
<i>Respiratory</i>					
Coughing	1 (8.3%)	0	1 (8.3%)	2 (14.3%)	1 (25.0%)
Pharyngitis	2 (16.7%)	0	1 (8.3%)	2 (14.3%)	2 (50.0%)
Rhinitis	0	2 (16.7%)	3 (25.0%)	5 (35.7%)	4 (100.0%)
<i>Platelet, bleeding, clotting</i>					
Purpura	1 (8.3%)	1 (8.3%)	0	2 (14.3%)	0
<i>Body as a whole</i>					
Back pain	2 (14.3%)	0	0	2 (14.3%)	1 (25.0%)
Fatigue	0	1 (8.3%)	0	1 (7.1%)	0
Edema peripheral	0	0	0	0	1 (25.0%)
Pain	0	0	0	0	0
<i>Application site</i>					
Injection site pain	0	0	1 (8.3%)	1 (7.1%)	0
<i>Secondary terms</i>					
Inflicted injury	0	0	1 (8.3%)	1 (7.1%)	0
Abrasion not specified	0	1 (8.3%)	1 (8.3%)	2 (14.3%)	0

Note: Multiple occurrences of the same adverse event in the same subject were counted once per dose group.
This table presents adverse event data for all patients who received study drug (GTS-21 or placebo).

clinically significant, adverse events relating to hepatic function in four subjects were notable and are discussed below. There were no clinically significant, treatment-emergent changes reported with respect to ECG findings or physical examinations. No clinically significant, treatment-emergent trends were noted with respect to vital signs, although one subject was reported to have asympto-

matic postural hypotension at the 75 mg t.i.d. dose level. This subject did not experience dizziness, nor did the adverse event meet the following criteria for orthostatic hypotension: decrease of 20 mmHg in systolic blood pressure accompanied by an increase of 20 bpm in pulse.

Adverse events relating to abnormal hepatic function occurred in three (21.4%) subjects in the GTS-21 group and

Table 2 Summary of Adverse Events Related to Liver Function

Subject	Treatment	Session/Day	Value (U/L)	Out of range	AE	Relationship
1204	GTS-21 150mg t.i.d.	S3/Day 6	SGOT 39	1.1 × ULN	SGOT incr	Probably
			SGPT 68	1.7 × ULN	SGPT incr	Probably
1205	GTS-21 75mg t.i.d.	S2/Day 6	SGPT 50	1.3 × ULN	SGPT incr	Possibly
	GTS-21 150mg t.i.d.	S3/Day 3	SGOT 107	3.0 × ULN	SGOT incr	Possibly
			SGPT 114	2.9 × ULN	SGPT incr	Possibly
			GGT 89	1.9 × ULN	GGT incr	Possibly
		S3/Day 4	SGOT 122	3.4 × ULN		
			SGOT 97	2.7 × ULN		
			SGPT 158	4.1 × ULN		
			SGPT 165	4.2 × ULN		
			GGT 125	2.7 × ULN		
			GGT 137	3.0 × ULN		
		S3/Day 5	SGOT 72	2.0 × ULN		
			SGPT 149	3.8 × ULN		
			GGT 140	3.0 × ULN		
		S3/Day 6	SGOT 3.8	1.1 × ULN		
			SGPT 101	2.6 × ULN		
			GGT 112	2.4 × ULN		
1213	GTS-21 75mg t.i.d.	S2/Day 1	SGOT 47	1.3 × ULN	SGOT incr	Remotely
			SGPT 70	1.8 × ULN	SGPT incr	Remotely
	GTS-21 150mg t.i.d.	S3/Day 6	SGOT 40	1.1 × ULN	SGOT incr	Possibly
			SGPT 59	1.5 × ULN	SGPT incr	Possibly
1209	Placebo	S1/Day 6	SGPT 62	1.6 × ULN	SGPT incr	Possibly
		Washout	GGT 51	1.1 × ULN	GGT incr	Possibly
	Placebo	S2/Day 6	SGOT 98	2.7 × ULN	SGOT incr	Possibly
	Placebo	S3/Day 6	SGPT 52	1.3 × ULN	SGPT incr	Probably

Note: All liver enzyme values returned to clinically acceptable limits by the 7-day follow-up visit.
Normal ranges were as follows: SGOT 10–36 U/L, SGPT 8–39 U/L, GGT 2–46 U/L

one (25.0%) subject in the placebo group (Table 2). Of these four subjects, one GTS-21 subject and one placebo subject showed liver enzyme values > 2 times the upper limit of the normal range (ULN). The maximum SGOT, SGPT and GGT values for the GTS-21 subject were 3.4 × ULN, 4.2 × ULN and 3.0 × ULN, respectively. This subject was withdrawn from the study at the sponsor's request. The maximum SGOT, SGPT and GGT values for the placebo subject were 2.7 × ULN, 1.3 × ULN and 1.1 × ULN, respectively. Only one of these four subjects was discontinued early, as stated above; the other three subjects with liver enzyme elevations all completed the study. None of the subjects who experienced liver enzyme elevations showed evidence of hepatic dysfunction and all liver enzyme values had returned to clinically acceptable limits by the 7-day follow-up visit.

Pharmacokinetics

The mean plasma GTS-21 and 4-OH-GTS-21 concentration-time profiles on Days 1 and 5 after administration of GTS-21 daily for 5 days under fed conditions are shown in Figure 2. The mean ± SD and range of the pharmacokinetic parameters derived from the individual subject data are listed in Table 3 for GTS-21 and Table 4 for the metabolite.

Plasma GTS-21 concentrations reached their peak at 1–1.4 h after the first dose and 1–1.2 h after 5 days of dosing, consistent with values for *T*_{max} observed in previous studies (internal reports, Taiho Pharmaceutical Co.) (Table 3).

The pharmacokinetics of the metabolite, 4-OH-GTS-21, paralleled those of the parent. Concentrations reached their peak at 0.9–1.5 h after the first dose and 1.1–1.3 h after 5 days of dosing, again consistent with values for *T*_{max}

observed in previous studies (internal reports, Taiho Pharmaceutical Co.) (Table 4).

Urinary excretion of unchanged GTS-21 and 4-OH-GTS-21 accounted for less than 0.5% of the dose (Tables 3 and 4). Following enzymatic hydrolysis, there was evidence for small amounts of a conjugated form of GTS-21 (Table 5). Conjugated 4-OH-GTS-21, however, accounted for 6–7% of the dose on Day 1 and 10–12% on Day 5.

Cognitive Function

The data in this study indicate a clear effect of GTS-21 over placebo on a range of cognitive functions. Table 6 summarizes the results of the ANOVA analysis of the cognitive data. For the primary analysis, statistically significant effects of GTS-21 were observed for at least one measure of all three attention-related tasks (simple reaction time, choice reaction time and digit vigilance), two secondary episodic recognition memory tasks (word recognition and picture recognition), and for the numeric working memory task. Additionally, both the visual tracking tasks and the immediate word recall task exhibited statistically significant improvements in performance following dosing with GTS-21. Tasks for which there were no effects on performance as indicated by the primary analysis were the spatial working memory task, the delayed word recall task, overnight face recognition task, and the Bond-Lader self-ratings of mood and alertness. The secondary analysis confirmed the main effects of GTS-21 seen in the primary analysis (Figures 3–5) and, in addition, showed significant improvement for immediate and delayed word recall (Figures 6 and 7) as well as one measure of spatial working memory.

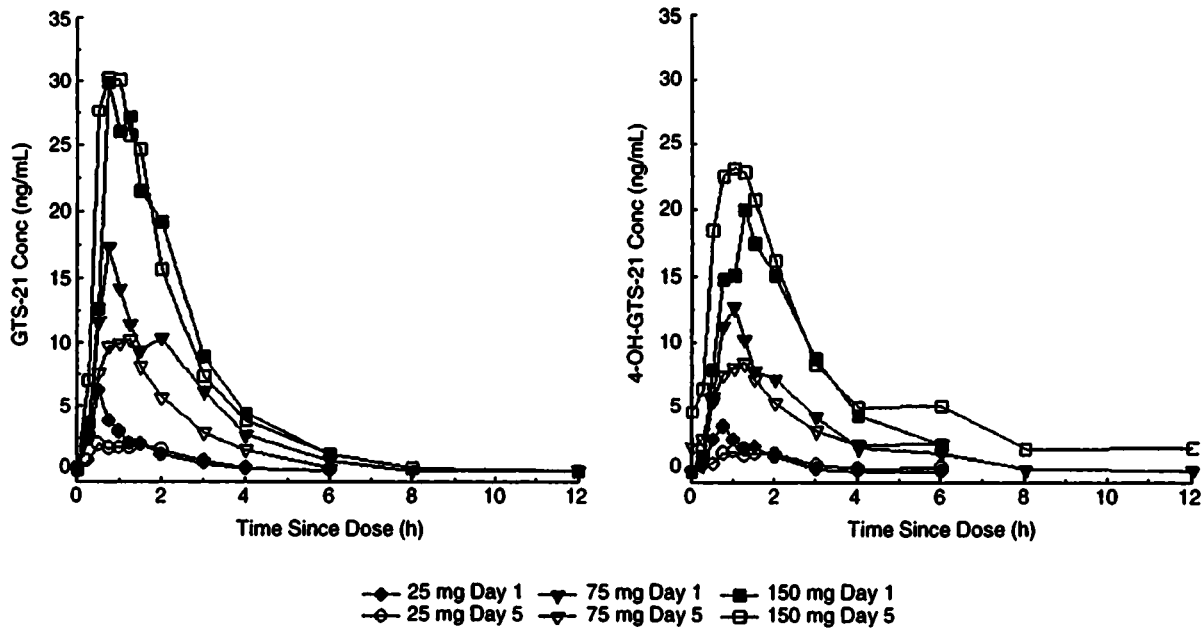


Figure 2 Mean plasma concentrations of GTS-21 (left panel) and 4-OH-GTS-21 (right panel) after administration of 25, 75, and 150 mg t.i.d. for 4 days and q.d. for 1 day.

Table 3 Mean $\pm$ SD (Range) Pharmacokinetic Parameters for GTS-21 after Oral Administration of 25, 75, and 150 mg of GTS-21 Daily for 5 Days

Parameter	25 mg	75 mg	150 mg
Day 1			
C_{max} (ng/ml)	8.58 $\pm$ 11.2 (1.71 $\rightarrow$ 36.3)	20.0 $\pm$ 20.5 (3.49 $\rightarrow$ 68.5)	40.1 $\pm$ 48.1 (8.57 $\rightarrow$ 147)
T_{max} (h)	1.00 $\pm$ 0.78 (0.25 $\rightarrow$ 3.0)	1.17 $\pm$ 0.69 (0.5 $\rightarrow$ 3.0)	1.42 $\pm$ 0.71 (0.27 $\rightarrow$ 3.0)
ACU_4 (hng/ml)	8.11 $\pm$ 13.2 (0.99 $\rightarrow$ 46.1)	37.9 $\pm$ 52.9 (6.14 $\rightarrow$ 195)	66.0 $\pm$ 65.3 (15.5 $\rightarrow$ 236)
AUC_{∞} (hng/ml)	13.0 $\pm$ 16.1 (2.09 $\rightarrow$ 47.9)	44.4 $\pm$ 59.6 (7.07 $\rightarrow$ 203)	77.4 $\pm$ 69.0 (18.2 $\rightarrow$ 241)
$t_{1/2}$ (h)	0.76 $\pm$ 0.38 (0.35 $\rightarrow$ 1.47)	0.93 $\pm$ 0.32 (0.53 $\rightarrow$ 1.55)	0.93 $\pm$ 0.19 (0.52 $\rightarrow$ 1.16)
fe (% dose)	0.012 $\pm$ 0.008 (0.006 $\rightarrow$ 0.027)	0.012 $\pm$ 0.005 (0.005 $\rightarrow$ 0.023)	0.017 $\pm$ 0.007 (0.007 $\rightarrow$ 0.034)
Day 5			
C_{max} (ng/ml)	3.49 $\pm$ 0.99 (1.76 $\rightarrow$ 4.85)	12.8 $\pm$ 8.23 (5.18 $\rightarrow$ 33.0)	47.8 $\pm$ 28.4 (21.1 $\rightarrow$ 117)
T_{max} (h)	1.19 $\pm$ 0.60 (0.5 $\rightarrow$ 2.0)	1.09 $\pm$ 0.28 (0.75 $\rightarrow$ 1.5)	1.02 $\pm$ 0.38 (0.5 $\rightarrow$ 1.5)
ACU_4 or AUC_4 (hng/ml)	4.57 $\pm$ 2.62 (2.70 $\rightarrow$ 12.3)	32.7 $\pm$ 16.4 (21.1 $\rightarrow$ 44.4)	85.1 $\pm$ 32.9 (46.7 $\rightarrow$ 138)
fe (% dose)	0.009 $\pm$ 0.008 (0.0 $\rightarrow$ 0.022)	0.017 $\pm$ 0.005 (0.008 $\rightarrow$ 0.025)	0.021 $\pm$ 0.009 (0.0 $\rightarrow$ 0.036)

Note: C_{max} — maximum plasma concentration, T_{max} — time to C_{max} , AUC — area under the curve, $t_{1/2}$ — elimination half-life, fe — percent of dose excreted in the urine.

Pharmacokinetic/Pharmacodynamic Relationship

The relationship between the cognitive responses to GTS-21 (as measured by immediate word recall accuracy, delayed word recall accuracy and digit vigilance task) and drug exposure is illustrated in Figure 8. For the three tests

Table 4 Mean $\pm$ SD (Range) Pharmacokinetic Parameters for 4-OH-GTS-21 after Oral Administration of 25, 75, and 150 mg of GTS-21 Daily for 5 Days

Parameter	25 mg	75 mg	150 mg
Day 1			
C_{max} (ng/ml)	5.75 $\pm$ 4.63 (1.49 $\rightarrow$ 16.3)	14.2 $\pm$ 14.8 (2.52 $\rightarrow$ 54.4)	27.4 $\pm$ 25.8 (7.22 $\rightarrow$ 86.3)
T_{max} (h)	0.86 $\pm$ 0.33 (0.5 $\rightarrow$ 1.5)	1.25 $\pm$ 0.65 (0.75 $\rightarrow$ 3.0)	1.48 $\pm$ 0.64 (0.5 $\rightarrow$ 3.0)
ACU_4 (hng/ml)	5.59 $\pm$ 5.75 (1.38 $\rightarrow$ 19.7)	27.3 $\pm$ 25.1 (2.54 $\rightarrow$ 82.8)	50.0 $\pm$ 35.3 (15.8 $\rightarrow$ 124)
AUC_{∞} (hng/ml)	9.39 $\pm$ 6.81 (3.45 $\rightarrow$ 22.8)	32.4 $\pm$ 29.2 (4.19 $\rightarrow$ 85.6)	55.7 $\pm$ 38.0 (20.9 $\rightarrow$ 126)
$t_{1/2}$ (h)	0.81 $\pm$ 0.30 (0.59 $\rightarrow$ 1.25)	1.18 $\pm$ 0.41 (0.73 $\rightarrow$ 1.68)	1.15 $\pm$ 0.37 (0.74 $\rightarrow$ 1.86)
fe (% dose)	0.031 $\pm$ 0.022 (0.005 $\rightarrow$ 0.075)	0.030 $\pm$ 0.018 (0.009 $\rightarrow$ 0.061)	0.047 $\pm$ 0.037 (0.016 $\rightarrow$ 0.15)
Day 5			
C_{max} (ng/ml)	2.71 $\pm$ 1.12 (1.73 $\rightarrow$ 4.61)	9.87 $\pm$ 5.19 (3.81 $\rightarrow$ 19.3)	32.9 $\pm$ 18.3 (12.8 $\rightarrow$ 80.8)
T_{max} (h)	1.30 $\pm$ 0.58 (0.75 $\rightarrow$ 2.0)	1.23 $\pm$ 0.51 (0.5 $\rightarrow$ 2.0)	1.11 $\pm$ 0.48 (0.5 $\rightarrow$ 2)
AUC_4 (hng/ml)	7.49 $\pm$ 4.73 (0.77 $\rightarrow$ 11.9)	26.2 $\pm$ 5.72 (18.2 $\rightarrow$ 37.7)	71.3 $\pm$ 24.9 (37.2 $\rightarrow$ 136)
fe (% dose)	0.062 $\pm$ 0.038 (0.008 $\rightarrow$ 0.14)	0.074 $\pm$ 0.042 (0.035 $\rightarrow$ 0.18)	0.11 $\pm$ 0.085 (0.013 $\rightarrow$ 0.30)

examined, there appeared to be a relationship between exposure to GTS-21 and the magnitude of the response.

DISCUSSION

This study was conducted to evaluate the safety, pharmacokinetics and cognitive effects of GTS-21 after multiple doses of 25, 75 and 150 mg per day for 5 days in healthy male volunteers.

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Table 5 Mean (± SD) Urinary Excretion^a of GTS-21, 4-OH-GTS-21, and Conjugates after Administration of 25, 75, and 150 mg of GTS-21 tid × 5

Day	Dose	GTS-21		4-OH-GTS-21	
		Free	Conjugate	Free	Conjugate
1	25	0.011 ± 0.008	0.047 ± 0.030	0.029 ± 0.024	6.97 ± 2.77
	75	0.012 ± 0.005	0.058 ± 0.037	0.031 ± 0.018	7.29 ± 3.20
	150	0.017 ± 0.007	0.053 ± 0.032	0.049 ± 0.039	5.78 ± 1.62
5	25	0.010 ± 0.008	0.114 ± 0.056	0.042 ± 0.026	10.2 ± 3.03
	75	0.016 ± 0.005	0.128 ± 0.056	0.054 ± 0.027	12.1 ± 3.81
	150	0.019 ± 0.009	0.132 ± 0.062	0.083 ± 0.066	11.0 ± 4.31

^aBased on urine collected from 0 to 6 h on each study day.

Table 6 Summary of Cognitive Function Testing (P-Values from ANOVA Analysis)

	Primary analysis		Secondary analysis (by dosing period)		
	Main effect of GTS-21 (N = 16)	Interaction with repeated testing (N = 16)	Treatment period 1 (N = 16)	Treatment period 2 (N = 16)	Treatment period 3 (N = 16)
<i>Attention-related tasks</i>					
Simple reaction time	0.0237	0.0443	0.0059	0.0042	0.0019
Choice reaction time	NS	0.0276	0.0003	NS	0.0703
<i>Digit vigilance</i>					
Correct detections	0.0130	0.0026	0.0419	0.0052	0.0001
Speed of detections	NS	0.002	0.0087	NS	NS
<i>Working memory tasks</i>					
<i>Numeric working memory</i>					
Sensitivity	NS	0.0001	NS	NS	0.0023
Speed	NS	NS	0.0446	0.0524	0.0992
<i>Spatial working memory</i>					
Sensitivity	NS	NS	0.0420	NS	NS
Speed	NS	NS	0.0053	0.0491	0.0529
<i>Secondary episodic recognition memory tasks</i>					
<i>Word recognition</i>					
Sensitivity	0.0293	0.0048	0.0004	0.0007	0.0245
Speed	NS	NS	0.0969	0.0557	NS
<i>Picture recognition</i>					
Sensitivity	NS	0.0358	NS	0.0515	0.0155
Speed	NS	NS	NS	0.0325	NS
Immediate word recall	0.1039	NS	0.0020	0.0002	0.0001
Delayed word recall	NS	NS	0.0005	0.0001	0.0005
<i>Other tasks (coordination)</i>					
Visual tracking	NS	0.0293	NS	NS	0.0428

Note: Trends toward statistical significance are shown in italics.
NS — Not statistically significant.

GTS-21 was well tolerated up to a daily dose of 450 mg (150 mg t.i.d.). There were no clinically significant, treatment-emergent changes reported with respect to ECG findings, physical examinations and vital signs; one subject experienced asymptomatic postural hypotension at the 75 mg t.i.d. dose level. There were no clinically significant differences between treatment groups with respect to the incidence of adverse events. However, due to the elevated liver enzymes in four subjects, the potential for hepatotoxicity was examined carefully. The literature suggests that laboratory results in healthy subjects should be interpreted cautiously in Phase I studies, as LFT elevations may be a result of nondrug factors such as diet or environment, and do occur in placebo

subjects as well as those treated with study drug (Purkins *et al*, 1997, Rosenzweig *et al*, 1999, Merz *et al*, 1997). In this study, none of the adverse events relating to hepatic function were determined to be clinically significant, and none of the subjects exhibited signs of hepatic dysfunction. This finding was consistent with preclinical data. The no observable effect level of GTS-21 in a 4-week rat study was determined to be 150 mg/kg/day; significant increases in liver weight and hepatocellular hypertrophy were only seen at 450 mg/kg/day. Nevertheless, future studies of GTS-21 will track LFT abnormalities carefully.

The pharmacokinetics of GTS-21 and 4-OH-GTS-21 appeared to be linear over the range of doses studied, as

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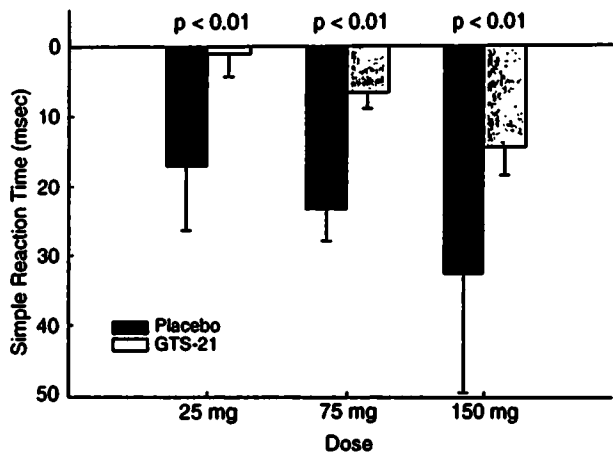


Figure 3 Effect of GTS-21 on simple reaction time as a function of dose and treatment group.

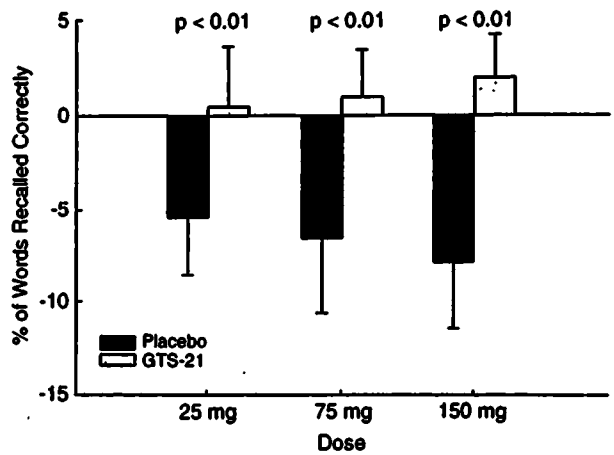


Figure 6 Effect of GTS-21 on immediate word recall accuracy as a function of dose and treatment group.

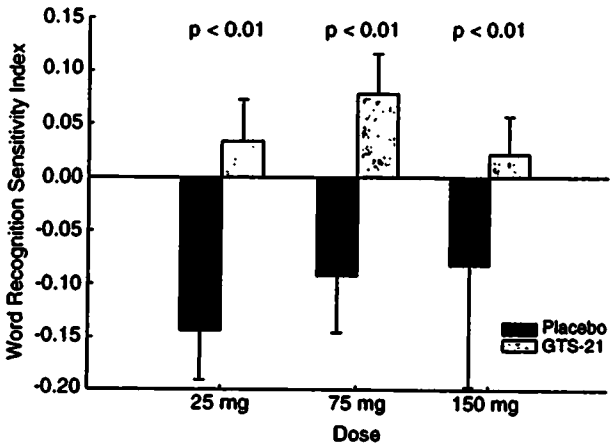


Figure 4 Effect of GTS-21 on word recognition sensitivity as a function of dose and treatment group.

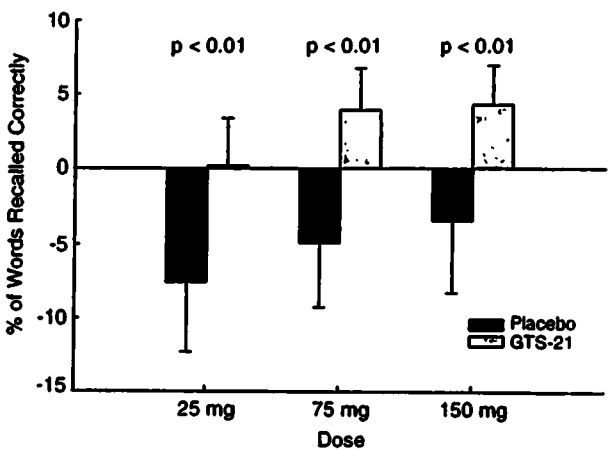


Figure 7 Effect of GTS-21 on delayed word recall accuracy as a function of dose and treatment group.

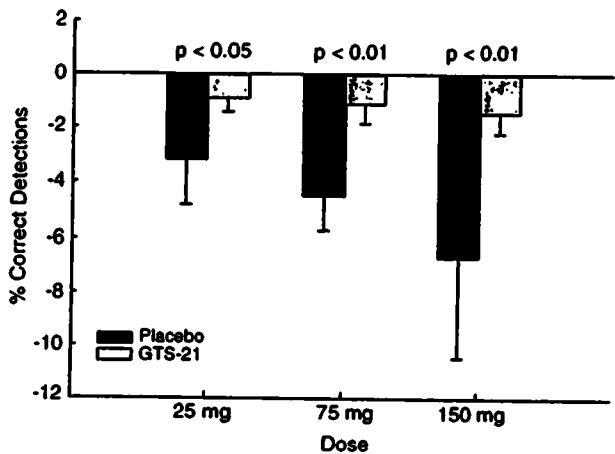


Figure 5 Effect of GTS-21 on digit vigilance as a function of dose and treatment group.

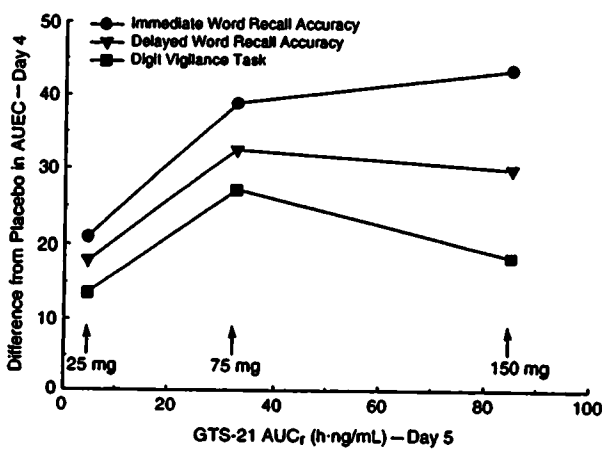


Figure 8 Relationship between the effects of GTS-21 on immediate and delayed word recall accuracy and digit vigilance (AUEC) and drug exposure (AUC).

evidenced by increases in C_{max} , AUC_{∞} and/or AUC_t that were proportional to dose, within the limits of the observed variability. Elimination half-life was also indepen-

dent of dose, providing further evidence of linearity. As demonstrated in a previous study (internal report, Taiho Pharmaceutical Co.), the wide range in pharmacoki-

netic parameters of GTS-21 and 4-OH-GTS-21 is due to between-subject variability, with essentially no intrasubject contribution. Consistent with these observations, there was considerable intersubject variability in C_{max} and AUC_{∞} of GTS-21 (Table 3). However, the ranges at steady state (Day 5) were 2.1 to 6.4-fold, considerably less than the 13 to 29-fold observed after the first dose, indicating that the intersubject variability in pharmacokinetics may decrease with continued dosing. The ranges in C_{max} and AUC at steady state (Day 5) for the metabolite were 2.1 to 15-fold, somewhat less than the 6 to 22-fold observed after the first dose. This is consistent with the parent and provides further evidence that the intersubject variability in pharmacokinetics may decrease with multiple dosing. Consistent with a $t_{1/2}$ of approximately 1 h and a dosing interval of at least 6 h, there was essentially no accumulation of either the parent or metabolite after 5 days of dosing (Figure 2).

The CDR computerized assessment system has been designed to identify changes in the quality of several major aspects of human cognitive function, including attention and memory. It is currently the most widely used system of its type in worldwide clinical research. The core tests on the system were initially developed to identify the cognitive effects of drugs acting on brain cholinergic systems, primarily nicotine and scopolamine (Wesnes and Warburton, 1984). The various tests in the system are highly sensitive to a variety of compounds that can enhance cognitive function, particularly those that influence the cholinergic system (van Harten *et al*, 1996; Parrott *et al*, 1996).

The data in this study strongly supported the hypothesis that GTS-21 has a favorable effect on cognitive function and are very encouraging for the future development of GTS-21 as a cognition enhancer. Firstly, the effects are consistent with the pharmacological actions of the compound; for example, nicotine has long been known to enhance vigilance (Wesnes and Warburton, 1984) and has also been shown to produce similar effects on the vigilance task used in this study (Parrott *et al*, 1996). Secondly, the effects detected also go beyond the simple effects of nicotine and physostigmine. For example, there is no evidence that nicotine has favorable effects on episodic memory, in contrast to the clear effects of GTS-21 on this function. In addition, although nicotine has been shown to affect attention, and physostigmine affects both attention and episodic memory, the results indicate that GTS-21 shows positive effects on both these aspects of cognitive function as well as working memory.

As illustrated in Figure 8, there appears to be a relationship between the exposure to GTS-21, as measured by AUC_{∞} , and its cognitive effects, based on the difference from placebo in AUEC. Response increased between 25 and 75 mg, and appeared to plateau between 75 and 150 mg. The 24-h exposure on Day 4 is essentially the same as that on Day 1, for which minimal or no cognitive effects were seen. The effect may thus be a function of both the magnitude and duration of exposure. A relationship identical to that shown in Figure 8 was also observed for the active metabolite and the sum of parent and metabolite (not shown), and the observed effect may be related to both compounds.

In conclusion, GTS-21 was well tolerated up to doses of 450 mg/day (150 mg t.i.d.) and shows improvements in cognitive behavior in normal subjects. The pharmacokinetics appear to be linear over the range of dose studies and the variability, primarily intersubject, decrease with continued dosing. The data provide important evidence that GTS-21 has the potential to improve human cognitive function. The range of the effects is important, clear benefits being seen to three major cognitive functions (attention, working memory, episodic secondary memory). In addition, there appears to be a relationship between the duration and magnitude of exposure to GTS-21 and/or 4-OH-GTS-21 and the magnitude of effect, with doses between 75 and 150 mg t.i.d. approaching maximal effect. GTS-21 may represent a novel treatment for dementia, and should be investigated further for its potential therapeutic effects in a variety of conditions in which cognitive function is compromised, not least of all Alzheimer's disease.

ACKNOWLEDGEMENT

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REIVINDICACIONES

5 1. Un derivado de aza-bicicloalquilo seleccionado del grupo que consiste de:

(R)-3-(6-p-tolil-piridin-3-iloxi)-1-aza-biciclo[2.2.2]octano,

(R)-3-(6-fenil-piridin-3-iloxi)-1-aza-biciclo[2.2.2]octano,

10 (R)-3-(6-(3-trifluorometil-fenil)-piridin-3-iloxi)-1-aza-biciclo[2.2.2]octano,

(R)-3-(6-(2-cloro-fenil)-piridin-3-iloxi)-1-aza-biciclo[2.2.2]octano,

(R)-3-(6-o-tolil-piridin-3-iloxi)-1-aza-biciclo[2.2.2]octano,

15 (R)-3-(6-m-tolil-piridin-3-iloxi)-1-aza-biciclo[2.2.2]octano,

(R)-3-(6-(2,3-dimetil-fenil)-piridin-3-iloxi)-1-aza-biciclo[2.2.2]octano,

(R)-3-(6-(4-etil-fenil)-piridin-3-iloxi)-1-aza-biciclo[2.2.2]octano,

20 (R)-3-(6-(3,4-dimetil-fenil)-piridin-3-iloxi)-1-aza-biciclo[2.2.2]octano,

(R)-3-(6-(2-cloro-4-metil-fenil)-piridin-3-iloxi)-1-aza-biciclo[2.2.2]octano, y

(R)-3-(6-(2-cloro-5-metil-fenil)-piridin-3-iloxi)-1-aza-biciclo[2.2.2]octano;

25 en forma de base libre o de sal de adición de ácido.

2. Un derivado de aza-bicicloalquilo de acuerdo con la reivindicación 1, caracterizado porque dicho derivado es

30 (R)-3-(6-p-tolil-piridin-3-iloxi)-1-aza-biciclo[2.2.2]octano, en forma de base libre o de sal de adición de ácido.

3. Un derivado de aza-bicicloalquilo de acuerdo con la reivindicación 1, caracterizado porque dicho derivado es

35 (R)-3-(6-fenil-piridin-3-iloxi)-1-aza-biciclo[2.2.2]octano, en forma de base libre o de sal de adición de ácido.

4. Un derivado de aza-bicicloalquilo de acuerdo con la reivindicación 1, caracterizado porque dicho derivado es

40 (R)-3-(6-(3,4-dimetil-fenil)-piridin-3-iloxi)-1-aza-

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biciclo[2.2.2]octano, en forma de base libre o de sal de adición de ácido.

5. Una composición farmacéutica caracterizada porque comprende un derivado de aza-bicicloalquilo de acuerdo con cualquiera de las reivindicaciones 1 a 4 en forma de base libre o en forma de la sal de adición de ácido farmacéuticamente aceptable en asociación con un portador o diluyente farmacéutico.

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Consommation
et Corporations Canada

Consumer and
Corporate Affairs Canada (21) (A1)

2,042,860

Bureau des brevets

Patent Office

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Ottawa, Canada
K1A 0C9

(43)

1991/11/20

DS
238

(51) INTL.CL.⁵ C07D-453/02; C07D-471/08; A61K-031/44; A61K-031/505

(19) (CA) **APPLICATION FOR CANADIAN PATENT** (12)

(54) **Bicyclic 1-Aza-Cycloalkanes**

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Kufner-Muhl, Ulrike - Germany (Federal Republic of) ;
Stransky, Werner - Germany (Federal Republic of) ;
Weber, Karl-Heinz - Germany (Federal Republic of) ;
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(73) **Boehringer Ingelheim International G.m.b.H. - Germany**
(Federal Republic of) ;

(30) (DE) P 40 16 212.5 1990/05/19
(DE) P 40 18 886.8 1990/06/13

(57) **24 Claims**

5,026,670

Notice: The specification contained herein as filed

Canada

CCA 3254 (10-89) 41

enantiomer.

(+)-3-quinuclidinylmethanol

5 The accumulated mother liquors obtained during the
production of (-)-3-quinuclidinylmethanol are evaporated
down in vacuo. The residue is taken up in a little
water, made alkaline with potash and extracted with
chloroform. The resulting 19.5 g of optically enriched
10 3-quinuclidinylmethyl acetate are converted into the
diastereomeric salt with 16.0 g of D-(+)-tartaric acid
in 200 ml of 95% ethanol. After 5 recrystallisations
from 95% ethanol, 12.7 g of salt are obtained, with a
rotary value $[\alpha]_D^{20} = 16.8$ ($c = 2$, H_2O), from which 4.7 g
15 of (+)-3-quinuclidinylmethanol are liberated as a light
coloured oil with $[\alpha]_D^{20} = +66.5$ ($c = 1$, in 1N HCl),
analogously to the (-)-enantiomer, using 75 ml of 2N
sodium hydroxide solution.

20 After derivatisation with phenylisocyanate, the
enantiomeric purity was determined by HPLC on a
chiracel-OD column with 97.4% (+) - : 2.6% (-)-
enantiomer.

25 3a: (+)-3-(Propargyloxymethyl)-1-azabicyclo[2,2,2]-
octane

30 5.6 g of (+)-3-quinuclidinylmethanolhydrochloride,
1.32 g of sodium borohydride and 100 ml of
tetrahydrofuran are stirred overnight. The solvent is
removed from the filtrate, the residue is taken up in
ethyl acetate and the resulting solution is washed with
saturated saline solution. After drying and removal of
the solvent, 3.4 g of (+)-3-quinuclidinylmethanol-boran
complex remain in the form of a light coloured oil.

35 3.4 g of the boran complex are stirred in 100 ml of
tetrahydrofuran for 30 minutes with 2.63 g of 60% sodium
hydride at ambient temperature, then reacted with 4.89 g
of 80% propargyl bromide and stirred for a further 6

claims 10 to 14.

5 17. Pharmaceutical compositions comprising as active ingredient at least one compound of formula I or Id, as claimed in claim 1 or claim 7, or a physiologically acceptable acid addition salt thereof in association with one or more pharmaceutically acceptable carriers, diluents or excipients.

10 18. Compositions as claimed in claim 17 substantially as herein defined and with reference to the Examples.

15 19. Compounds of general formula I or Id as claimed in claim 1 or claim 7 for use in therapy.

20 20. The use of a compound of general formula I or Id as claimed in claim 1 or claim 7; or the use of a compound of formula Ib or Ic as defined in claim 7 and all racemic forms, diastereomers and mixtures thereof and physiologically acceptable acid addition salts thereof and additionally the quaternary salts thereof; for the preparation of a medicament for use in the treatment of diseases or conditions caused by the reduced function of the cholinergic system.

25 21. The use as claimed in claim 20 wherein the medicament is used in the treatment of Alzheimers disease, senile dementia, cognitive disorders or glaucoma and in the improvement of memory performance.

30 22. A method of treatment of diseases or conditions in a subject which arise from the reduced function of the cholinergic system which comprises administering to said subject an effective amount of a compound of formula I or Id, as claimed in claim 1 or claim 7, or an effective amount of a compound of formula Ib or Ic as defined in claim 7 and all racemic forms, diastereomers and

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

2020
Bogotá, D.C.,

Abogado
DILIA MARIA RODRÍGUEZ D'ALEMAN
Apoderado

ASUNTO	Radicación	05-022398
	Trámite	002
	Evento	001
	Actuación	430
	Oficio	Nº 10469
	Folios	08

Patente de Invención ☒ Patente de Modelo de Utilidad ☐

Vía Nacional ☐

Vía PCT (fase nacional) ☐ Cap. I ☐ Cap. II ☐

No. Sol. Internacional PCT/EP2003/009772
Fecha de Presentación Internal. 03 de Septiembre de 2003

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

1. Documentos estudiados

Descripción: Folios: 5 a 52 como se radicó inicialmente

Reivindicaciones: Folios 53 a 59 como se radicó inicialmente
Folios 90 a 95 modificadas
Folios 127 a 138 modificadas
Folios 164 a 166 modificadas
Folios 236 a 237 modificadas

Prioridad: Folios: 1
04 de Septiembre de 2002, UK, 0220581.3

Respuesta del solicitante: Folios 119 a 126, Folios 191 a 235

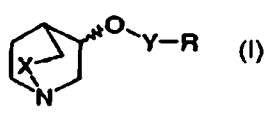
2. Análisis de la Prioridad

La fecha de prioridad del 04 de Septiembre de 2002 y su correspondiente documento, son aceptados para el estudio de patentabilidad.

3. Objeto de la invención

Título de la solicitud: **"AZA-BICICLO-ALQUIL-ÉTERES Y SU USO COMO AGONISTAS DE ALFA7-NACHR"** Folio 1.

El objeto de la presente solicitud de patente de invención consiste en derivados de aza-bicicloalquilo de fórmula I:



El problema planteado en esta solicitud es obtener compuestos útiles como agonistas de los receptores de acetilcolina nicotínicos $\alpha 7$ (nAChRs).

Para solucionar este problema técnico la solicitud en estudio proporciona un compuesto de fórmula I, sus composiciones farmacéuticas y su proceso de preparación.

- **Clasificación Internacional de Patentes (IPC):** A61K31/501; A61K31/506

4. **Determinación del Estado de la Técnica**

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

- Fecha de presentación de la solicitud prioritaria: 04 de Septiembre de 2002

Considerando que la fecha de presentación de la primera solicitud de patente de la cual se reivindica prioridad es: **04/09/2002** se encontraron los siguientes documentos del estado de la técnica relacionados, que pueden afectar la patentabilidad del objeto de la presente solicitud:

Nº	Documento	Título	Fecha de publicación
D5	CA 2042860	Bicyclic 1-Aza-cycloalkanes	20/11/1991

5. **Análisis de patentabilidad (artículo 14 D486)**

Nivel inventivo (artículo 18 D486)

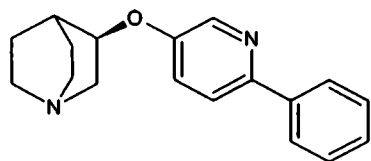
La reivindicación 1 consiste en:

Un derivado de aza-bicicloalquilo seleccionado del grupo:

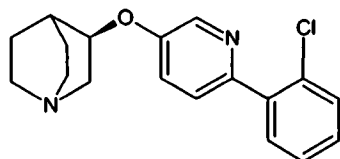
- (R)-3-(6-p-tolil-piridin-3-iloxi)-1-aza-biciclo[2.2.2]octano,
- (R)-3-(6-fenil-piridin-3-iloxi)-1-aza-biciclo[2.2.2]octano,
- (R)-3-(6-(3-trifluorometil-fenil)-piridin-3-iloxi)-1-aza-biciclo[2.2.2]octano,
- (R)-3-(6-(2-cloro-fenil)-piridin-3-iloxi)-1-aza-biciclo[2.2.2]octano,
- (R)-3-(6-o-tolil-piridin-3-iloxi)-1-aza-biciclo[2.2.2]octano,
- (R)-3-(6-m-tolil-piridin-3-iloxi)-1-aza-biciclo[2.2.2]octano,
- (R)-3-(6-(2,3-dimetil-fenil)-piridin-3-iloxi)-1-aza-biciclo[2.2.2]octano,
- (R)-3-(6-(4-etil-fenil)-piridin-3-iloxi)-1-aza-biciclo[2.2.2]octano,
- (R)-3-(6-(3,4dimetil-fenil)-piridin-3-iloxi)-1-aza-biciclo[2.2.2]octano,
- (R)-3-(6-(2-cloro-4-metil-fenil)-piridin-3-iloxi)-1-aza-biciclo[2.2.2]octano,
- (R)-3-(6-(2-cloro-5-metil-fenil)-piridin-3-iloxi)-1-aza-biciclo[2.2.2]octano,

En forma de base libre o de sal de adición del ácido

La característica **esencial** de los compuestos de la reivindicación 1 objeto de invención, es un anillo 1-azabicyclo-[2.2.2]-octano, con un grupo oxo sustituido con un anillo de piridina, sustituido éste a su vez con un anillo fenilo, con la siguiente estructura por ejemplo para el compuesto 2 o 4:

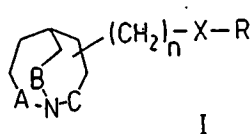


(3R)-3-[(6-phenylpyridin-3-yl)oxy]-1-azabicyclo[2.2.2]octane

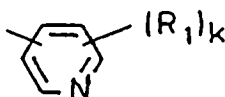


(3R)-3-[[6-(2-chlorophenyl)pyridin-3-yl]oxy]-1-azabicyclo[2.2.2]octane

El estado de la técnica más cercano es **D5**, pues da a conocer compuestos con fórmula I, los cuales son derivados aza-biciclo-alquil-éteres, útiles para el tratamiento de la enfermedad de Alzheimer, demencia senil, desórdenes cognitivos o glaucoma y el mejoramiento del desempeño de la memoria. (Ver reivindicación 21)



D5 enseña las sustituciones para A, B, C independientemente cuando cada uno representan CH₂ o un enlace sencillo (Ver página 38, líneas 31-32) (Ver página 24, un ejemplo con 1-azabicyclo-[2.2.2]-octano, línea 25), n representa cero (Ver página 38, línea 34), X representa oxígeno (Ver página 38, línea 34), R representa un grupo heterocíclico de 5, 6 o 7 miembros sustituido o no (Ver página 38, líneas 21-22) y donde la sustitución puede ser un anillo de piridina (Ver página 40, línea 19, 2ª columna) y R¹ es un grupo fenilo sustituido o no y k es 1, 2 o 3 y cuando k es mayor de 1 el sustituyente puede ser idéntico o diferente. (Ver página 41, líneas 28).



Y cuando se refiere al término fenilo sustituido, el cual puede estar con grupos mono, di o tri sustituidos, por un átomo de halógeno y una cadena alquílica C₁₋₅ ramificada o no. Y además, cuando entre los ejemplos de las sustituciones en el anillo fenilo se incluyen, entre otros: 2-clorofenil, 2-metilfenil, 4metil-fenil, 3-trifluorometilfenil; las cuales se habían divulgado previamente en **D5**.

Los compuestos objeto de la presente solicitud se diferencian de los compuestos de **D5**, en que los compuestos no se encuentran explícitamente, reportados en dicha anterioridad.

El hecho de incluir estos compuestos específicos en las estructuras en los compuestos de **D5**, causa que los compuestos tengan actividad sobre los receptores de acetilcolina nicotínicos α7 de forma selectiva.

El Problema Técnico Objetivo era obtener compuestos "agonistas de los receptores de acetilcolina nicotínicos α7(nAChRs), útiles en el tratamiento y prevención de desórdenes psíquicos tales como esquizofrenia, manías, depresión y ansiedad, Alzheimer, entre otros"

D5 ya resolvía el problema técnico de obtener compuestos, azabicyclo [2.2.2]-octano, útiles en el tratamiento de Alzheimer, entre otros.

La reivindicación es obvia, porque el Experto en la Materia enfrentado al problema de

“obtener compuestos aza biciclo alquil éteres”, realizaría “todos los reemplazos sugeridos y revelados” en la estructura Markush de **D5**, de compuestos útiles para la enfermedad de Alzheimer, logrando de esta forma los compuestos objeto de esta solicitud. Por tanto, la reivindicación 1 no tiene Nivel Inventivo.

Las reivindicaciones dependientes 2-5 no mencionan alguna característica que haga inventiva a los compuestos de la reivindicación 1, por lo cual se considera que no tienen nivel inventivo.

7. Conclusión:

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

N°	Documento N°	Reivindicaciones afectadas	Requisito que afecta
D5	CA 2042860	1-5	Nivel Inventivo

6. Respuesta a los argumentos del solicitante:

El solicitante en el Folio 191 se refiere a las modificaciones realizadas a las reivindicaciones y anexa un nuevo capítulo reivindicatorio, el cual es aceptado para el estudio de patentabilidad.

El solicitante en el Folio 192, se refiere a la novedad de la solicitud frente al documento CA 2042860 (**D5**):

De acuerdo con las aclaraciones del solicitante, se acepta la novedad de los compuestos objeto de invención, más no su nivel inventivo.

El solicitante se refiere en el Folio 194: **D5** no ejemplifica un compuesto sencillo que sea 3-(6-fenil-piridin-3-iloxi)-1-aza-biciclo[2.2.2]octano:

Esta Oficina encuentra que aunque el compuesto no se encuentra de forma explícita, el compuesto se encuentra sugerido en **D5** en todas sus sustituciones lo cual motivaría al experto en la materia a realizar los reemplazos, y obtener los compuestos de la solicitud presentada, como se mencionó anteriormente en el análisis de nivel inventivo, derivándose los compuestos de forma obvia de **D5**

El solicitante se refiere en el Folio 194 en adelante al nivel inventivo y en el Folio 195, afirma: “los compuestos de la invención en estudio tienen un modo diferente de acción en relación con los compuestos descritos en la anterioridad **D5**”

Al respecto, el solicitante no ha evidenciado dichas diferencias en comparación con los compuestos del arte más cercano. Tanto de los compuestos de la solicitud presentada como los de **D5**, son útiles para la enfermedad de Alzheimer, es decir presentan el mismo efecto terapéutico y que el hecho que el mecanismo de acción sea diferente, no evidencia un efecto inesperado de los compuestos frente a **D5**.

El solicitante en el Folio 195 y adelante, se refiere a los receptores muscarínicos M1 a M5 y anexa resultados sobre la farmacología y diseño de nuevos fármacos selectivos y reitera “es claro que existen diferencias significativas en el modo de acción de un agonista M1 y un agonista alfa7nAChR”.

Es claro que el solicitante realiza los estudios de mecanismos de acción, y que los pudo haber obtenido con un resultado diferente, sin embargo los compuestos ya se encontraban sugeridos en **D5** en todos sus elementos esenciales y que el experto en

la materia motivado a realizar dichas sustituciones, los obtendría de forma obvia con la misma utilidad en la enfermedad de Alzheimer, es decir con el mismo efecto. El solicitante lo único que realiza son ensayos experimentales *a posteriori* sobre dichos compuestos para dilucidar el mecanismo de acción, sin evidenciarse habilidad inventiva.

El solicitante se refiere en el Folio 197: "gracias a esta propiedad inesperada resulta claro entonces que los compuestos de la invención en estudio a la luz de las enseñanzas de D5, involucran un nivel inventivo"

Se reitera al solicitante que D5 ya sugería los compuestos de la solicitud presentada y que no se ha evidenciado que para el diseño de dicha estructura se haya empleado habilidad inventiva, pues se derivan de forma obvia del estado de la técnica y que el resultado en terapéutica es el mismo, pues tanto los compuestos de la solicitud presentada como los de la solicitud presentada sirven para la enfermedad de Alzheimer por lo tanto el efecto era esperado.

El solicitante en el Folio 197 se refiere a la combinación de los documentos D5 y D1:

De acuerdo con la modificación realizada al capítulo reivindicatorio D1 se encuentra alejado de los compuestos de la nueva reivindicación 1, por lo tanto fue retirado del presente concepto técnico.

El solicitante en el Folio 198, se refiere "no resulta necesario ni procedente evidenciar de manera comparativa con los compuestos de D5 que la solicitud en estudio presenta la actividad sobre el receptor alfa7nAChR teniendo en cuenta el tipo de receptor y el farmacóforo en la estructura de los compuestos

Se reitera al solicitante que los compuestos de la solicitud presentada como los de D5, presentan el mismo efecto esperado en la utilidad en la enfermedad de Alzheimer y que todos los elementos esenciales de la estructura química ya se encontraban sugeridos en D5, derivándose de forma obvia y sin evidenciarse habilidad inventiva. La solicitud carece por lo tanto de nivel inventivo.

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

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


ALIX CARMENZA CESPEDES DE VERGEL

Directora de Nuevas Creaciones (C)

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

27 ABO. 2010

CONCEPTO TÉCNICO No. 02829

EXAMINADOR TÉCNICO
Código No. 202070

Clarke, Modet & C^o

COLOMBIA

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 05-022398- -00017-0000

Fecha 2010-11-08 14.57 23 Dep. 2020 DIR.NUEVASCR
Tra. 11 PATENTEIYII Eve. 379 FASENACIONALII
Act 444 RESPUESTAREQUE Folios 7

P-1892

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

DIVISION DE NUEVAS CREACIONES

E.

S.

D.

Re: Expediente No. 05.022.398

Solicitud de patente a nombre de **NOVARTIS AG.**

Yo, DILIA MARIA RODRIGUEZ D'ALEMAN, actuando como apoderada sustituta de acuerdo con la sustitución presentada el 17 de abril de 2009, identificada como aparece al pie de mi firma, domiciliada en la carrera 11 A No. 86-53, piso 6, obrando como apoderada de la sociedad **NOVARTIS AG.**, domiciliada en Basilea Suiza, solicitante de la patente de la referencia, en respuesta al oficio No. 10469 notificado por fijación en lista el 20 de agosto de 2010 relacionado con el concepto técnico de fondo No. 02829, me permito manifestar:

1. Con fundamento en la facultad que confiere el artículo 34 de la Decisión 486 al solicitante para modificar la solicitud de patente en cualquier momento del trámite, presento junto con este memorial nuevo juego de reivindicaciones que consta de 5 reivindicaciones y en el cual se realizaron las siguientes modificaciones.

1.1 Se cancelaron los compuestos (R)-3-(6-(3-trifluoroetil-fenil)-piridin-3-iloxi)-1-aza-biciclo[2.2.2]octano y (R)-3-(6-(4-etil-fenil)-piridin-3-iloxi)-1-aza-biciclo[2.2.2]octano de la antigua reivindicación 1.

En cuanto a las modificaciones es deseo de la solicitante manifestar que las mismas no amplían el alcance de la presente invención y se realizan con el ánimo de cumplir con los requerimientos de novedad y altura inventiva, expuestos por el señor examinador en el concepto técnico No. 02829.

Teniendo en cuenta el concepto técnico emitido por el Señor Superintendente Delegado para la propiedad intelectual GianCarlo Marcenaro Jiménez a la doctora Ana Maria Castro Wey, que da alcance a las comunicaciones No. 09-001524 y 09-012566, no se presenta una tasa adicional, toda vez que el nuevo capítulo reivindicatorio comprende menos de 15 reivindicaciones y la solicitud fue presentada con anterioridad al 1 de enero de 2009.

2. Nivel inventivo

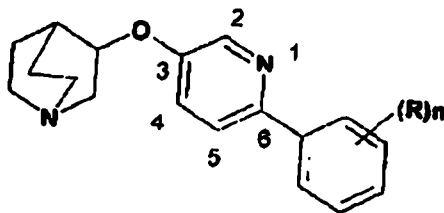
El señor examinador indica en su concepto técnico que “D5 ya sugería los compuestos de la solicitud presentada y que no se ha evidenciado que para el diseño de dicha estructura se haya empleado habilidad inventiva, pues se derivan de forma obvia del estado de la técnica y que el resultado en terapéutica es el mismo, pues tanto los compuestos de la solicitud presentada como los de la solicitud presentada sirven para la enfermedad de Alzheimer por lo tanto el efecto era esperado.”

Sobre particular, respetuosamente nos permitimos decir de nuevo que la anterioridad D5 hace referencia a agonistas del receptor mACh, siendo útiles como medicamentos para el tratamiento de la hipofunción colinérgica, tal como déficit cognoscitivos asociados con la enfermedad de Alzheimer como se puede apreciar en la página 16 de D5, específicamente entre las líneas 13 a 18 a continuación:

“ In the light of the pharmacological findings the compounds of general formulae I, Id, Ib and Ic as defined above are therefore useful for treatment of, for example, the following diseases: Alzheimer's disease, senile dementia and cognitive disorders; the compounds may also be used to improve memory performance. ”

En contraste, la presente invención se encuentra dirigida a un juego de nueve (9) quinuclidina-éteres que son selectivos como agonistas del receptor $\alpha 7$ -nACh como se puede apreciar en la página 15 de la memoria descriptiva de la solicitud en estudio, principalmente entre las líneas 7 y 8 cuando se afirma que: “En particular, los agentes de la invención son agonistas del receptor de acetilcolina nicotínico $\alpha 7$ (nAChR).”, en donde los compuestos de la invención, son útiles entre otras cosas, para el tratamiento de déficit cognoscitivo asociado con la enfermedad de Alzheimer como así se expone desde la página 16 línea 17 hasta la página 17 línea 10 de la memoria descriptiva de la solicitud en estudio.

En ese orden de ideas, también se hace necesario hacer énfasis sobre el hecho que los compuestos e la invención se encuentran unificados por la siguiente estructura:



en donde n es un entero de 0 a 2 y R es seleccionado de cloro y metilo.

El señor examinador ha manifestado en el folio 244 antepenúltimo párrafo que: *“Al respecto, el solicitante no ha evidenciado dichas diferencias en comparación con los compuestos del arte más cercano. Tanto de los compuestos de la solicitud presentada como los de D5, son útiles para la enfermedad de Alzheimer, es decir presentan el mismo efecto terapéutico y que el hecho que el mecanismo de acción sea diferente, no evidencia un efecto inesperado de los compuestos frente a D5.*

Al respecto, es deseo del solicitante poner en consideración del señor examinador más datos experimentales que demuestra que el compuesto estructuralmente más cercano reportado en D5 y los compuestos de la presente invención poseen **potencias gramaticalmente diferentes en cuanto a la actividad relacionada con el receptor alfa7-nACh**, lo cual es el modo de acción descrito para los compuestos de la invención. En efecto, en los datos del Anexo 1, se muestra que la potencia en cuanto al receptor alfa7-nACh del ejemplo 1 de la anterioridad D5 se compara con la potencia de todos los 9 compuestos reclamados en la presente invención. En tanto que el compuesto de la técnica anterior tiene una potencia de 1950nM, los compuestos de la invención reportan potencias de 39 a 269 nM, es decir, son de **siete (7) veces a cincuenta (50) veces más potentes.**

En consecuencia, la potencia alfa7-nACh de los compuestos de la invención es una propiedad inesperada y debido a esta propiedad inesperada, los compuestos de la invención son, a la luz de las enseñanzas de D5, inventivos y merecedores de obtención de protección bajo la figura de patente de invención.

Habiendo atendido cabalmente las objeciones presentadas por ese despacho, atentamente solicitamos se conceda el privilegio de patente de invención a la solicitud en estudio o si el examinador considera que subsisten razones para objetar la presente invención rogamos expedir otro examen de fondo con los debidos motivos y razones que sustenten su objeción con el fin de que nuestro mandante tenga la posibilidad de exponer sus argumentos con base en la interpretación de los artículos 45 de la Decisión 486 -el cual otorga la facultad al examinador para emitir los exámenes de fondo-, 35 y 59 del Código Contencioso Administrativo que señala que: *“habiéndose dado oportunidad a los interesados para expresar sus opiniones, y con base en las pruebas e informes disponibles, se tomará la decisión que será motivada al menos en forma sumaria si afecta a particulares” y “(..) deberá proferirse la decisión definitiva. Esta se motivará en sus aspectos de hecho y de derecho, y en los de conveniencia si es el caso”, respectivamente (El subrayado es nuestro).*

En consecuencia, solicitamos a ese despacho pronunciarse en relación con todos los argumentos presentados en este memorial dando estricta aplicación al principio del debido proceso consagrado en el artículo 29 de la Constitución Nacional

Señor Superintendente,



DILIA MARÍA RODRÍGUEZ D' ALEMAN
C.C. 41.654.882 de Bogotá
T.P. 20824

ANEXO 1

**Datos Biológicos que sustentan la Solicitud de Patente Colombiana
No. 05.022.398**

Se evaluó la potencia de los compuestos de la invención (Compuesto N. 58, 61, 63, 65 a 67, 67, 70 y 74) y el compuesto de la técnica anterior D5: 3-(piridin-3-iloxi)-1-aza-biciclo[2.2.2]octano (ejemplo 1 de CA2042860, página 26) en la activación del receptor acetilcolina nicotínico alfa 7 (alfa 7-nAChR) en un ensayo funcional como se describió en nuestra respuesta del 1 de Diciembre de 2009.

Los resultados se condensan en la siguiente tabla:

Compuesto No.	nAChRα7 (EC ₅₀ nM)	nAChRα7 Eficacia ¹
Tecnica anterior. Ejemplo 1 de Ca2042860 D5)	<u>1950</u>	<u>89</u>
58	39	73
61	93	79
63	81	81
65	170	94
66	50	83
67	269	48
69	52	45
70	158	72
74	123	103

¹. Eficacia comparada a Epibatidina (la cual es un agonista total de nAChRα7, como se indica en D. Feuerbach y colaboradores, 2005).

REIVINDICACIONES

5 1. Un derivado de aza-bicicloalquilo seleccionado del grupo que consiste de:

(R)-3-(6-p-tolil-piridin-3-iloxi)-1-aza-biciclo[2.2.2]octano,
(R)-3-(6-fenil-piridin-3-iloxi)-1-aza-biciclo[2.2.2]octano,
10 (R)-3-(6-(2-cloro-fenil)-piridin-3-iloxi)-1-aza-biciclo[2.2.2]octano,
(R)-3-(6-o-tolil-piridin-3-iloxi)-1-aza-biciclo[2.2.2]octano,
(R)-3-(6-m-tolil-piridin-3-iloxi)-1-aza-biciclo[2.2.2]octano,
(R)-3-(6-(2,3-dimetil-fenil)-piridin-3-iloxi)-1-aza-
15 biciclo[2.2.2]octano,
(R)-3-(6-(3,4-dimetil-fenil)-piridin-3-iloxi)-1-aza-biciclo[2.2.2]octano,
(R)-3-(6-(2-cloro-4-metil-fenil)-piridin-3-iloxi)-1-aza-biciclo[2.2.2]octano, y
20 (R)-3-(6-(2-cloro-5-metil-fenil)-piridín-3-iloxi)-1-aza-biciclo[2.2.2]octano;
en forma de base libre o de sal de adición de ácido.

25 2. Un derivado de aza-bicicloalquilo de acuerdo con la reivindicación 1, caracterizado porque dicho derivado es (R)-3-(6-p-tolil-piridin-3-iloxi)-1-aza-biciclo[2.2.2]octano, en forma de base libre o de sal de adición de ácido.

30 3. Un derivado de aza-bicicloalquilo de acuerdo con la reivindicación 1, caracterizado porque dicho derivado es (R)-3-(6-fenil-piridin-3-iloxi)-1-aza-biciclo[2.2.2]octano, en forma de base libre o de sal de adición de ácido.

35 4. Un derivado de aza-bicicloalquilo de acuerdo con la reivindicación 1, caracterizado porque dicho derivado es (R)-3-(6-(3,4-dimetil-fenil)-piridin-3-iloxi)-1-aza-biciclo[2.2.2]octano, en forma de base libre o de sal de adición de ácido.

- 252
5. Una composición farmacéutica caracterizada porque comprende un derivado de aza-bicicloalquilo de acuerdo con cualquiera de las reivindicaciones 1 a 4 en forma de base libre o en forma de la sal de adición de ácido farmacéuticamente aceptable en asociación con un portador o diluyente farmacéutico.
- 5
- 7

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

Radicación No. 05-022398

Clasificación Internacional (IPC): A61K31/501; A61K31/506

Concepto Técnico No: 04351

Patente de Invención ☐ Patente de Modelo de Utilidad ☐

Vía Nacional ☐

Vía PCT (fase nacional) ☐ Cap. I ☐ Cap. II ☐

No. Sol. Internacional PCT/EP2003/009772

Fecha de Presentación Internal. 03 de Septiembre de 2003

Como resultado del examen de fondo, realizado con fundamento en el artículo 48 de la Decisión 486 de la Comisión de la Comunidad Andina, se le comunica:

1. Documentos estudiados

Descripción: Folios: 5 a 52 como se radicó inicialmente

Reivindicaciones: Folios 53 a 59 como se radicó inicialmente
Folios 90 a 95 modificadas
Folios 127 a 138 modificadas
Folios 164 a 166 modificadas
Folios 236 a 237 modificadas
Folios 251 a 252 modificadas

Prioridad: Folios: 1
04 de Septiembre de 2002, UK, 0220581.3

Respuesta del solicitante: Folios 119 a 126, Folios 191 a 235, Folios 246 a 249

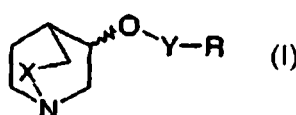
2. Análisis de la Prioridad

La fecha de prioridad del 04 de Septiembre de 2002 y su correspondiente documento, son aceptados para el estudio de patentabilidad.

3. Objeto de la invención

Título de la solicitud: **"AZA-BICICLO-ALQUIL-ÉTERES Y SU USO COMO AGONISTAS DE ALFA7-NACHR"** Folio 1.

El objeto de la presente solicitud de patente de invención consiste en derivados de aza-bicicloalquilo de fórmula I:



El problema planteado en esta solicitud es obtener compuestos útiles como

11. Análisis de patentabilidad (artículo 14 D486)

Novedad (artículo 16 D486)

Los compuestos presentados en la solicitud se consideran novedosos de acuerdo con las enseñanzas del estado de la técnica, puesto que no se encontraban divulgados compuestos azabicyclo[2.2.2]octano sustituidos con un anillo piridin-3-iloxi sustituido.

Nivel Inventivo (Art18 D486)

No se encontró dentro del estado de la técnica, tal como lo estipulado en el numeral 10, documento alguno que afecte el nivel inventivo.

La solicitud presentada tiene nivel inventivo pues resuelve el problema de obtener compuestos agonistas de los receptores de acetilcolina nicotínicos $\alpha 7$ (nAChRs), mediante un anillo azabicyclo[2.2.2]octano sustituidos con un anillo piridin-3-iloxi sustituido, en la estructura química de los compuestos más cercanos del estado de la técnica; esta modificación no es obvia y el Experto en la Materia, no solucionaría fácilmente el problema por lo cual tiene nivel inventivo, de acuerdo con el artículo 18 de la Decisión 486.

6. Conclusión:

En consecuencia y por todo lo expuesto anteriormente, se recomienda **Conceder** la reivindicación: 1 a 5 (folios: 251 a 252).

Bogotá, D.C.,

Se sugiere al solicitante modificar el título de la invención a: "Compuestos piridin-3-iloxi sustituidos-azabicyclo[2.2.2]octanos"

CONCEPTO TÉCNICO No. 04351	EXAMINADOR TÉCNICO Código No. 202070
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/102



AVISO DE NOTIFICACION
CORREO CERTIFICADO



Radicación : 5-22398- -19-0 Fecha: 2010-12-27 10:22:03
Trámite : 11 PATENTEIYII
Evento : 379 FASENACIONALII
Actuación : 432 COMUNICACTOADM
Destinatario : DILIA MARIA RODRIGUEZ D' ALEMAN Folios 1

Señor (a) (es)
DILIA MARIA RODRIGUEZ D' ALEMAN
CARRERA 11 NO. 86-53 PISO 6
BOGOTA D.C.

Referencia RESOLUCION : 72873
Fecha : 24/12/2010
Expediente : 5-22398
Trámite : 11 PCT-PATENTE DE INVENCION CAPITULOS I Y II
Evento : 379 FASE NACIONAL INCLUIDO CAPITULO II
Actuación : 432 COMUNICACION ACTO ADMINISTRATIVO

Comedidamente le solicito presentarse a este Despacho ubicado en la Carrera 13 No. 27 - 00 Piso Tercero (3), de la ciudad de Bogotá D.C., dentro del término de cinco (5) días hábiles contados a partir del envío de la presente citación, en horario de 8:00 a.m. a 4:30 p.m. jornada continua, con el fin de notificarle personalmente el contenido de la resolución de la referencia proferida dentro del expediente tramitado con el número de radicación citado en el asunto.

De no hacerse presente en el término indicado anteriormente, la providencia en mención se notificará por edicto, el cual se fijará por el término de diez (10) días en el Centro de Documentación e Información.

Para su comodidad el texto completo de la resolución se encontrará disponible en la página web de la Entidad desde el día hábil siguiente al de su notificación, la cual podrá ser consultada desde un computador con acceso a Internet, en la página www.sic.gov.co, con el número de resolución a través de la opción trámites y servicios - de carácter general transversal - consulta actos administrativos y providencias.

En caso de que se haya notificado, haga caso omiso de este aviso.

Secretaría General
SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

Al contestar favor indique el número de radicación consignado en el sticker

Sede Centro: Carrera 13 No 27-00 pisos 3, 5, 7 y 10 PBX: 5870000
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Web: www.sic.gov.co e-mail: info@sic.gov.co
Bogotá D C Colombia

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Industria y Comercio
SUPERINTENDENCIA

REPUBLICA DE COLOMBIA
SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

RESOLUCION No. 8773

Por la cual se otorga una patente de invención

CERTIFICADO No. 877

Rad. PCT/ 05-22398

EL SUPERINTENDENTE DE INDUSTRIA Y COMERCIO
en ejercicio de sus facultades legales, en especial de las conferidas en el numeral 38 del artículo 3° del Decreto 1687 de 2010, y

CONSIDERANDO:

PRIMERO: Que mediante escrito radicado en esta Superintendencia el día 10 de marzo de 2005 con el N° 05022398-00000000, la doctora Dilia Maria Rodríguez D'Aleman, en su calidad de apoderada de Novartis AG, presentó la solicitud de patente de invención titulada "AZA-BICICLO-ALQUIL-ÉTERES Y SU USO COMO AGONISTAS DE ALFA7-NACHR", correspondiente a la solicitud internacional PCT/EP 2003/009772 de fecha 03 de septiembre de 2003, para que se iniciara la fase nacional en virtud del Tratado de Cooperación en Materia de Patentes (PCT).

SEGUNDO: Que el extracto de esta solicitud fue publicado en la Gaceta de la Propiedad Industrial N° 573 del 28 de febrero de 2007, sin que se hubieran presentado oposiciones por parte de terceros.

TERCERO: Que realizado el examen de fondo mediante Oficio N° 10469 y notificado por fijación en lista el 20 de agosto de 2010 se requirió al solicitante en los términos del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, para que presentara respuesta a las observaciones de carácter técnico visibles a folios 238 a 245 del cuaderno administrativo, relacionadas con la patentabilidad o cumplimiento de los requisitos establecidos por esta Decisión, para la concesión de la patente.

CUARTO: Que el solicitante mediante escrito radicado bajo el N° 05-022398-00017-0000 del 08 de noviembre de 2010, respondió oportunamente el requerimiento formulado, y presentó nuevas reivindicaciones 1 a 5 (Fls. 251 y 252) que reemplazan las originalmente presentadas, atendiendo de esta manera las observaciones efectuadas en el examen de fondo. Se aceptó el nuevo capítulo reivindicatorio presentado, como quiera que se ajusta a las prescripciones contenidas en el artículo 34 de la Decisión 486.

QUINTO: Que en virtud de lo dispuesto en el artículo 14 de la Decisión 486 expedida por la Comisión de la Comunidad Andina "*[l]os países miembros otorgarán patentes para las invenciones, sean de producto o de procedimiento, en todos los campos de la tecnología, siempre que sean nuevas, tengan nivel inventivo y sean susceptibles de aplicación*"

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Industria y Comercio
SUPERINTENDENCIA

REPUBLICA DE COLOMBIA
SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

RESOLUCION No. 2173
Por la cual se otorga una patente de invención

CERTIFICADO No. 971

Rad. PCT/ 05-22398

industrial."

SEXTO: Que en el presente caso las reivindicaciones 1 a 5 contenidas en los folios 251 y 252 cumplen los requisitos indicados en el considerando anterior y, en consecuencia, este Despacho encuentra procedente conceder para las mismas la patente solicitada.

SEPTIMO: Que de acuerdo con el objeto concedido se modifica el titulo de la invención, el cual quedará de la siguiente manera: "COMPUESTOS PIRIDIN-3ILOXI SUSTITUIDOS-AZABICICLO [2.2.2]OCTANOS".

RESUELVE:

ARTICULO PRIMERO: Otorgar patente de invención a la solicitud que entró en fase nacional en virtud del Tratado de Cooperación en Materia de Patentes (PCT), para la creación titulada:

" COMPUESTOS PIRIDIN-3ILOXI SUSTITUIDOS-AZABICICLO [2.2.2]OCTANOS "

Clasificación IPC: A 61 K 31/501, 31/506.

Reivindicaciones: 1 a 5 **Folios:** 251 y 252.

Titular: NOVARTIS AG.

Domicilio: Basilea, Suiza.

Inventores: Dominik Feuerbach, Konstanze Hurth y Timothy John Ritchie.

Prioridad N°: 0220581.3 **Fecha:** 04 de septiembre de 2002 **Pais:** GB.

Solicitud Internacional N°: PCT/EP 2003/009772 **Fecha:** 03 de Sep. de 2003.

Vigente desde: 03 de septiembre de 2003 **Hasta:** 03 de septiembre de 2023.

ARTICULO SEGUNDO: El titular tendrá los derechos y las obligaciones establecidos en la Decisión 486 de la Comisión de la Comunidad Andina y en las demás disposiciones legales vigentes sobre propiedad industrial, precisando que para mantener vigente la patente se

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Industria y Comercio
SUPERINTENDENCIA

REPUBLICA DE COLOMBIA
SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

RESOLUCION No. 13,
Por la cual se otorga una patente de invención

CERTIFICADO No: 31 ;

Rad. PCT/ 05-22398

deberá cancelar la tasa anual de mantenimiento, conforme lo dispone el artículo 80 de la referida norma comunitaria.

ARTICULO TERCERO: Entregar al titular copia auténtica de esta resolución y el certificado de patente, previas las anotaciones en los libros de registro.

ARTICULO CUARTO: Notificar personalmente el contenido de la presente resolución a la doctora Dilia María Rodríguez D'Aleman, en su calidad de apoderada de Novartis AG, o a quien haga sus veces, entregándole copia de la misma y advirtiéndole que contra ella procede el recurso de reposición, ante el Superintendente de Industria y Comercio, el cual podrá ser interpuesto en el momento de la notificación o dentro de los cinco 5 días hábiles siguientes a ella.

COPIA AUTÉNTICA DEL VALOR DE
7105
DECRETOS ADMINISTRATIVOS

NOTIFÍQUESE Y CUMPLASE
Dada en Bogotá, D.C., a los

EL SUPERINTENDENTE DE INDUSTRIA Y COMERCIO,


JOSÉ MIGUEL DE LA CALLE RESTREPO

Doctora
DILIA MARÍA RODRÍGUEZ D'ALEMAN
Carrera 11 N° 86 – 53, Piso 6
Bogotá D C

202021

Superintendencia de Industria y Comercio

Otorga la Patente de Invención a:

Novartis Ag.

Basilea, Suiza

A la Creación denominada:

"Compuesto piridin-3iloxi sustituidos-azabicyclo [2.2.2] octanos"

Inventor(es):

Dominik Feuerbach

Konstanze Hourth

Timothy John Ritchie

Certificado No. 877

Solicitud Internacional No.:

PCT/EP2003/009772

Clasificación Internacional:

A61K 31/501

de Patentes

A61K 31/506

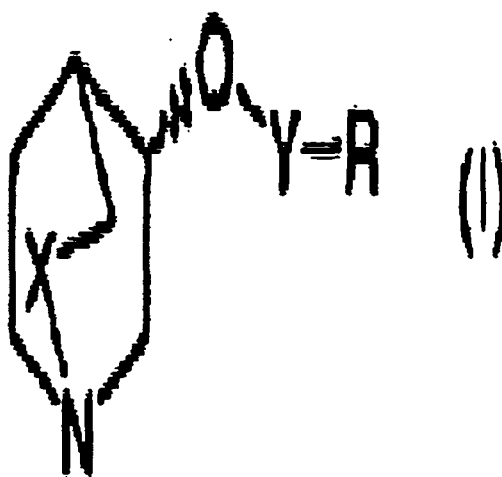
5 Reivindicaciones

Vigente Desde: 03/09/2003

Hasta: 03/09/2023

Prioridad No.:

GB 0220581.3 04/09/2002



Que la solicitud de Patente de Invención No. 5 22398 cumple con los requisitos previstos en las disposiciones legales vigentes, Resolución No. 72873 de 24/12/2010, en testimonio de ello se estampa la firma del funcionario competente y el sello de esta Entidad.

Superintendente de Industria y Comercio

Clarke, N

COL

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 05-022398-00020-0000

Fecha: 2011-02-22 15:29:01 Dep. 2020 DIR.NUEVASCR
Tra. 11 PATENTEIYII Eve: 379 FASENACIONALII
Act. 329 CTOINFORMACION Folios: 1

Señores

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIVISIÓN DE NUEVAS CREACIONES

EXPEDIENTE: 05.022.398

SOLICITUD : ENTREGA DE TITULO

ASUNTO : P-1892

Estimados señores,

Yo, DILIA RODRÍGUEZ D' ALEMAN, identificada como aparece al pie de mi firma, domiciliada en Bogotá, en la Carrera 11 No. 86-53 Piso 6, obrando como apoderada de este asunto, mediante resolución No. 72873 de 24 de diciembre de 2010, se concedió el registro de la patente en referencia a favor de mi mandante. Ya me fue entregada copia íntegra, auténtica y gratuita de dicha resolución.

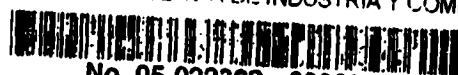
Solicito que se proceda a numerar el certificado de la patente y se ordene la entrega del título correspondiente.

Cordialmente,

DILIA MARÍA RODRÍGUEZ D' ALEMAN
C.C. 41.654.882 de Bogotá.
T.P. 20824 CSJ

Pedro Castillo Pineda & Asociados
ABOGADOS

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 05-022398-00023-0003

Fecha: 2013-09-27 17:01:15 Dep: 2020 DIR NUEVASCR
Tra 11 PATENTEIYII Eve 323 ANUALIDADES
Act 411 PRESENTACION Folios: 2

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
División de Nuevas Creaciones
E. S. D.

Ref: PAGO DE ANUALIDAD-EXPEDIENTE No.05-022398
Solicitud de Patente de Invención a nombre de **NOVARTIS AG.**
Certificado 877

Por Resolución No.72873 del 24 de Diciembre de 2010 ese Despacho concedió el privilegio de Patente de Invención para **COMPUESTO PIRIDIN-3ILOXI SUSTITUIDOS-AZABICICLO [2.2.2] OCTANOS**, certificado 877 a nombre de **NOVARTIS AG.**

Para cumplir con lo dispuesto por el artículo 80 de la Decisión 486 de la Comisión de la Comunidad Andina, adjunto el recibo N° por concepto de la tasa anual para el mantenimiento de la patente, correspondiente al periodo comprendido entre el 03 de Septiembre de 2013 y el 03 de Septiembre de 2014.

Atentamente,

JERONIMO CASTILLO BONILLA
C.C. 79.462.057 Bogotá
T.P. 104167 C.S. Judicatura

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

NIT : 800.176.089-2

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252



RECIBO DE CAJA

No. 13 - 0096708

Bogotá D.C., Septiembre 25 de 2013 - 16:24:47

RECIBIDO DE : CASTILLO PINEDA SAS

NI 860.046.502

*** Soporte del Pago ***

TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PAGO	VR PAGO
CONSIGNACION	BANCO DE BOGOTA	062754387	175318835	25/09/2013	39.900.000.00

*** Conceptos Pagados ***

CANT.	RENTISTICO	CONCEPTO	Vr. UNIDITARIO	Vr. CONCEPTO
1 50005-01-09	OTROS SERVICIOS DE PROPIEDAD INDUSTRIAL	1612 TASA ANUAL A#QS 9 a 12 CUATRENIO PARA MANTENIMINETO DE	480.000.00	480.000.00
				\$480.000.00

SON: **CUATROCIENTOS OCHENTA MIL PESOS MONEDA CORRIENTE***

Responsable: _____

Recibo de Caja Aplicado al Expediente No. _____

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 05-022398-00023-0003

Fecha 2013-09-27 17:01:15 Dep. 2020 DIR.NUEVASCR
Tra 11 PATENTE IYII Eve 323 ANUALIDADES
Act 411 PRESENTACION Folios: 2

Sede Centro: Carrera 13 No. 27 - 00 Pisos 3,4,5 y 10 Bogotá, D.C.- Colombia

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, Septiembre 25 de 2013 - 16:24:53

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