

LLOREDA CAMACHO & CO.**ABOGADOS**

Calle 72 No. 5-83 Piso 5, Bogotá D.C.

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

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Señor

SUPERINTENDENTE DE INDUSTRIA Y COMERCIO

DIVISIÓN DE NUEVAS CREACIONES

E. _____ S. _____ D. _____

Trámite: 11
Evento: 378
Actuación: 412

Re: PI.-F. HOFFMANN-LA ROCHE AG Y SIENA BIOTECH S.P.A.-Solicitud de Patente de Invención en Fase Nacional PCT para "COMPUESTOS DERIVADOS DE 2-AMINO-5,5-DIFLUOR-5,6-DIHIDRO-4H-OXAZINA COMO INHIBIDORES DE BACE1 Y/O BACE2".-Clase C07D 265/08.- Expediente número 12-113.850.-

Yo, **ALICIA LLOREDA RICAURTE**, mayor, vecina de esta ciudad, identificada con la cédula de ciudadanía número 39.690.713 de Usaquén, Representante Legal en Bogotá para todos los asuntos relacionados con la Propiedad Industrial de las sociedades **F. HOFFMANN-LA ROCHE AG**, organizada y existente de conformidad con las leyes de Suiza, domiciliada en Basilea, Suiza y **SIENA BIOTECH S.P.A.**, organizada y existente de conformidad con las leyes de Italia, domiciliada en Siena, Italia atentamente manifiesto a usted que interpongo **RECURSO DE REPOSICIÓN** contra la Resolución Número **76363**, dictada por el señor Superintendente de Industria y Comercio el 16 de Diciembre de 2014 y notificada por fijación en lista el 23 de Diciembre de 2014, en relación con la negación del privilegio de patente de invención para la solicitud en referencia, por una supuesta falta de **Nivel Inventivo** de dicha solicitud.

I. FUNDAMENTOS DE DERECHO

Me permito manifestar ante ese Despacho que el mencionado Recurso de Reposición encuentra fundamento en que el contenido de la Resolución Número **76363** evidencia que durante el examen técnico realizado a la solicitud denegada se realizó una evaluación incorrecta del objeto de la invención allí reclamada, sin considerarse su real alcance e importancia.

Es así que, a pesar de los argumentos proporcionados por el solicitante en el memorial de 15 de octubre de 2014 (en respuesta al Oficio No. **8504**), en la Resolución impugnada ese Despacho mantiene las objeciones con respecto al Nivel Inventivo de la solicitud frente al documento D1, afirmando categóricamente lo siguiente:

"En consecuencia, la persona del oficio normalmente versada en la materia, realizaría una selección del compuesto divulgado en el documento D1, sin generar ningún efecto técnico inesperado, para llegar así al objeto de la reivindicación 1 de la solicitud. Por lo cual se considera obvia".

Asimismo, ese Despacho presenta nuevas objeciones relacionadas con las enseñanzas del libro denominado "The Organic chemistry of Drug Design and Drug Action", el cual no fue mencionado en el concepto técnico previo, en este sentido el señor Examinador considera que:

"(...) el documento D1 divulga compuestos BACE-1 que incluyen en su estructura un anillo de oxazina enlazado con una fenilformamida, además, el documento "The Organic chemistry of Drug Desing and Drug Action(2)" revela conceptos sobre bioisoterismos y enseña que los anillos cíclicos que incluyen el átomo oxígeno y átomo azufre son isóteros clásicos entre sí".

En vista de lo anterior, a continuación me permito dar a conocer las modificaciones realizadas sobre el capítulo reivindicatorio y brindar argumentos técnicos que no sólo demostrarán a ese Despacho que la presente invención sí cumple con el requisito de Nivel Inventivo exigido por el Artículo 18 de la Decisión 486 de 2000, sino que además establecerán que el análisis realizado es incorrecto, toda vez que se interpreta de manera errónea tanto la invención como el arte previo; constituyéndose por lo tanto la negación de la solicitud en una clara violación al mencionado Artículo 18 de la Decisión Andina 486 de 2000.

1. MODIFICACIONES AL CAPÍTULO REIVINDICATORIO.

Inicialmente, me permito manifestar que el solicitante, al amparo de lo estipulado por el Artículo 34 de la Decisión Andina 486 de 2000, ha modificado la solicitud presentando un capítulo reivindicatorio modificado, que reemplaza al actualmente presentado.

En conexión con las señaladas modificaciones, me permito resaltar que estas no se constituyen de ninguna manera en una ampliación del alcance de la solicitud, tal como fue originalmente presentada, toda vez que tienen como fin **restringir considerablemente la solicitud a los compuestos de fórmula general I y Ia**, los cuales encuentran pleno sustento en la descripción de la solicitud y en el capítulo reivindicatorio actualmente presentado, tal como se expondrá a continuación:

- **Nueva Reivindicación 1.** Está basada en las reivindicaciones 1, 5, 8, 10 y 11 actualmente presentadas, en este sentido:

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- ✓ se ha restringido considerablemente el alcance del sustituyente R_1 , de manera que ahora éste únicamente se puede elegir entre piridinilo, pirazolilo, pirazinilo, pirimidinilo y piridinilo sustituido por 1-2 sustituyentes elegidos individualmente entre ciano, halógeno, y halógeno-alquilo C_{1-6} ;
 - ✓ se ha restringido considerablemente el alcance de los sustituyentes R_2 y R_3 , de manera que ahora R_2 es flúor y R_3 es metilo; y
 - ✓ se ha limitado n únicamente al número 1.
- **Nuevas Reivindicaciones 2 y 3.** Están basadas respectivamente en las reivindicaciones 3 y 6 actualmente presentadas, sin embargo ahora se ha restringido su alcance de acuerdo con las modificaciones realizadas a la reivindicación independiente 1.
 - **Nuevas Reivindicaciones 4 a 8.** Corresponden respectivamente a las reivindicaciones 7 y 12 a 15 actualmente presentadas, sin embargo su dependencia ha sido adaptada de acuerdo con la nueva numeración.
 - **Nuevas Reivindicaciones 9 a 12.** Están basadas respectivamente en las reivindicaciones 16 a 19 actualmente presentadas, sin embargo ahora se ha restringido su alcance de acuerdo con las modificaciones realizadas a la reivindicación independiente 1, además su dependencia ha sido adaptada según la nueva numeración.

Las reivindicaciones 2, 4, 5 y 8 a 11 actualmente presentadas han sido eliminadas del capítulo reivindicatorio.

Es así como, el capítulo reivindicatorio modificado está constituido por **12 reivindicaciones** las cuales enfocan la reclamación en las realizaciones preferidas de la invención.

En vista de estos hechos, y a la luz de la facultad que la Decisión Andina 486 otorga al solicitante mediante las disposiciones que establece el Artículo 34 de la misma:

"Artículo 34.- El solicitante de una patente podrá pedir que se modifique la solicitud en cualquier momento del trámite. La modificación no podrá implicar una ampliación de la protección que correspondería a la divulgación contenida en la solicitud inicial.

Del mismo modo, se podrá pedir la corrección de cualquier error material." (Énfasis añadido)

Resulta claro que la presentación del mencionado capítulo reivindicatorio modificado, está amparada bajo la legislación Andina, y por lo tanto muy comedidamente solicito a ese Despacho tenerlos en consideración en su análisis del presente Recurso.

2. CONSIDERACIONES TÉCNICAS FRENTE AL NIVEL INVENTIVO DE LA INVENCION DENEGADA.

Ahora bien, me permito señalar que el señor Examinador afirma que los compuestos reclamados en la presente solicitud carecerían de Nivel Inventivo con respecto a las enseñanzas del arte previo citado en la Resolución Número **76363**, esto es, el documento **EP1942105 (D1)**.

A este respecto, el Señor Examinador señala que la persona del oficio normalmente versada en la materia realizaría una selección de los compuestos divulgados en el documento D1, sin generar ningún efecto técnico inesperado, obteniendo así los compuestos reclamados en la reivindicación 1.

Frente a tal afirmación, muy respetuosamente me permito manifestar que las enseñanzas del mencionado documento D1, de ninguna manera pueden conducir a las estructuras de fórmula general I y Ia, mucho menos si se tiene en cuenta el capítulo reivindicatorio modificado que se presenta junto con este memorial, el cual ha sido considerablemente restringido.

Para soportar la anterior aseveración y con el ánimo de demostrar ante ese Despacho que el capítulo reivindicatorio modificado es inventivo frente a las enseñanzas del documento D1 atentamente me permito presentar las siguientes consideraciones técnicas:

2.1. La estructura de los compuestos reclamados, de ninguna manera puede ser derivada de las enseñanzas del documento D1.

En principio cabe señalar que el documento D1 reclama compuestos que comprenden una porción de su estructura que es similar a la de los compuestos de la presente invención, pero permite la sustitución de la misma de varias maneras, lo que conducen a un gran número de compuestos con un patrón de sustitución diferente.

En este sentido, es importante resaltar que el documento D1 da 1282 ejemplos que combinan los diferentes radicales de la molécula de varias maneras. Particularmente, la Tabla 170 en la página 260 de D1, muestra compuestos específicos con distintos valores de IC_{50} , y además en esa misma página del documento D1 se menciona que otros compuestos ejemplificados presentan valores de IC_{50} de menos de 100 μM . En este sentido, es claro que el experto en la materia se concentraría en aquellos ejemplos que presentan datos biológicos.

Se tiene entonces que el compuesto con mayor potencia que es divulgado en el documento D1 es el compuesto 1043, el cual presenta un valor de IC_{50} de 0.027 μM , seguido muy de cerca por los compuestos 1199 (IC_{50} : 0.032 μM), 1100 (IC_{50} : 0.037 μM) y 165 (IC_{50} : 0.0394 μM).

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Lo anterior significa que la persona del oficio medianamente versada en la materia tiene que realizar dos tipos diferentes de modificaciones a la molécula de tiazinilo amino-sustituido de D1, para llegar a los compuestos que se reclaman en la presente solicitud. En este sentido, el documento D1 no brinda ninguna sugerencia acerca de que estas modificaciones deban realizarse con el fin de llegar a obtener compuestos más adecuados.

Es claro que la fórmula general del documento D1 genera miles de compuestos como resultado de la sustitución de los diferentes radicales, razón por la cual, una persona del oficio normalmente versada en la materia, no encontraría motivación para derivar los compuestos de la invención a partir de D1, sino que necesitaría realizar pasos adicionales a nivel experimental para elegir los radicales y grupos específicos y combinarlos de manera tal, que lleve a la obtención de los compuestos de fórmula I y Ia de la presente invención.

Consecuentemente, la persona versada en la materia tampoco tendría ninguna motivación para elegir determinados compuestos de D1, modificar unos radicales específicos y su posición y obtener así los compuestos de la invención y además predecir que estos compuestos resultantes serían inhibidores de BACE 1 y/o BACE2.

En conexión con lo anterior, muy respetuosamente me permito señalar que la única motivación que tendría la persona versada en la materia para hacer esta selección y modificaciones particulares sería la revelación hecha por la presente invención, por lo que el análisis de nivel inventivo realizado por el Señor Examinador está enmarcado dentro de lo que en el ámbito de las patentes se conoce como "hindsight", o análisis "Ex post facto", toda vez que conociendo de antemano la invención el Señor Examinador está seleccionado partes específicas de las enseñanzas contenidas dentro de D1 y modificándolas de manera que permitan construir la materia reclamada por la presente solicitud, pasos que de ninguna manera podrían ser evidentes para la persona versada en la materia en el momento en el cual se desarrolló la invención.

En este sentido vale la pena resaltar que al tener conocimiento de la invención es de esperarse que dichas modificaciones particulares de las enseñanzas de D1 parezcan obvias, toda vez que ya se sabe de antemano que efectivamente estas modificaciones resuelven el problema planteado; sin embargo, esta información no era conocida por el inventor en el momento de desarrollar la invención, por lo que no podría tener certeza de la utilidad de realizar estos pasos.

Consecuentemente, **las enseñanzas del citado documento D1 no podrían llevar de ninguna manera a que la persona versada en la materia inequívocamente obtuviera las estructuras de los compuestos reivindicados en la presente invención.**

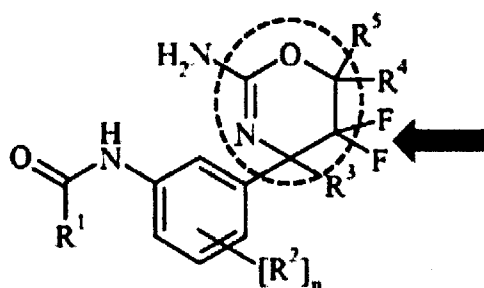
En este orden de ideas, cabe destacar que los 4 ejemplos de D1 mencionados anteriormente muestran un patrón de sustitución muy similar. Específicamente los 4 ejemplos **presentan como parte de su estructura un radical tiazinilo**. Igualmente, es claro que el documento **D1 enseña que los derivados de tiazinilo son el grupo preferido de la invención revelada en tal documento**. Incluso, el mismo título del documento D1 apoya la hipótesis de que un experto en la materia con interés en el desarrollo de inhibidores de BACE1 enfocaría su atención en los compuestos derivados de tiazinilo amino-sustituídos.

Cabe agregar que si bien, el documento D1 hace referencia a **3 compuestos que poseen un resto oxazinilo (compuestos 654, 783, 878) de un total de 1285**, es importante señalar que en tal documento no se encuentra ninguna información acerca de la actividad sobre BACE1 específicamente para esos tres compuestos. Por lo tanto, la persona experta en la materia simplemente deduciría que los compuestos de D1 que poseen un resto oxazinilo son menos adecuados que aquellos que presentan el grupo tiazinilo.

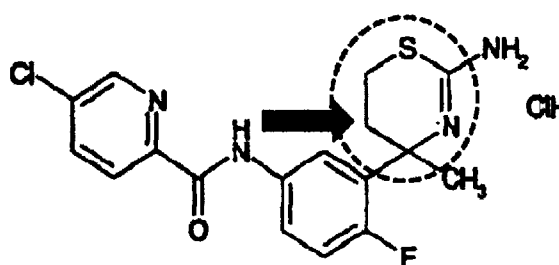
Asimismo, **ninguno de los 1285 compuestos ejemplificados en D1 presenta una disustitución con flúor sobre el núcleo central**, es más no existe ninguna enseñanza en D1 que hubiese motivado a la persona experta en la materia enfrentada con el problema técnico objetivo, a que modificara o adaptara D1 para llegar a la presente invención.

En síntesis, los compuestos reclamados en la presente solicitud difieren de las enseñanzas divulgadas en el documento D1 en dos aspectos importantes:

- (i) **los compuestos de la invención presentan un grupo oxazinilo** (ver círculo en rojo), **mientras que los compuestos divulgados en D1 presentan como parte fundamental de su molécula un grupo tiazinilo** (ver círculo en rojo), y
- (ii) **el anillo oxazinilo en los compuestos de la invención está disustituídos en la posición 5 por fluor** (5,5-difluor, ver flecha verde), mientras que los compuestos de D1 no presentan tal sustitución.



Compuestos de la presente
invención



Compuesto 1043 de D1

2.2. El intercambio del grupo tiazinilo por el grupo oxazinilo y la disustitución con flúor no pueden ser considerados como reemplazos convencionales para una persona experta en la materia.

Ahora bien, en este punto considero relevante referirme el argumento presentado por el señor Examinador, mediante el cual señala que:

"(...) además, el documento *"The Organic chemistry of Drug Design and Drug Action(2)"* revela conceptos sobre bioisoterismos y enseña que los anillos cíclicos **que incluyen el átomo oxígeno y átomo azufre son isómeros clásicos entre sí**" (Resaltado fuera de texto).

Frente a lo anterior, me permito indicar que si bien en Química Farmacéutica es conocido que existen una serie de estrategias generales de síntesis química que eventualmente pueden permitir la manipulación de la actividad farmacológica de un determinado compuesto activo o la obtención de nuevos compuestos derivados, el empleo de una técnica en particular es producto de un trabajo de laboratorio que significa un desarrollo específico para realizar dicha modificación, sin que ni siquiera ello implique o asegure la efectiva obtención del producto deseado. A este respecto, por ejemplo, considero que es bien sabido por la persona versada en la materia que un cambio en la estructura química de un compuesto puede o no resultar beneficiosa para la actividad biológica de dicho compuesto, y que incluso cualquier cambio en la estructura de una molécula puede dar origen a una estructura con propiedades totalmente distintas a las que presente la molécula sin modificar.

Adicionalmente y en relación específica con las enseñanzas del libro *Silverman R. B., "The organic Chemistry of Drugs Design and Drug Action", Elsevier, Northwestern University, Second Edition, Evanston, Illinois, 2004*, me permito aclarar ante ese Despacho que dicho documento menciona algunos ejemplos de bioisosterismo, pero ninguno de ellos llevaría a la persona medianamente versada en la materia técnica en cuestión a suponer de manera obvia que al sustituir un átomo de azufre del anillo de tiazina por un átomo de oxígeno dentro de la estructura química específica del compuesto 1241 del documento D1 (el cual es tomado por el señor Examinador para realizar su comparación) los compuestos resultantes mantendrían su actividad biológica como inhibidores de BACE1.

Igualmente, me permito señalar que desde el punto de vista estructural, de ninguna manera es predecible que un compuesto cuyo núcleo principal consiste en un oxazinilo y no en un tiazinilo, conserve su actividad como inhibidor de BACE 1 y/o BACE2, dado que en primer lugar **los reemplazos bioisostéricos son altamente impredecibles**, tal y como lo señala el documento *"Foye's Principles of Medicinal Chemistry"*¹:

¹ Foye's Principles of Medicinal Chemistry, Página 54, 1er párrafo, parte I, 7ma edición, 2013

*"Lo que puede ser un remplazo bioisostérico exitoso para una molécula dada interactuando con un receptor particular muy a menudo no tiene ningún efecto o elimina cualquier actividad biológica en otra. Así, el uso de remplazo bioisotérico (clásico o no clásico) en el diseño de medicamentos es altamente dependiente del sistema biológico que está siendo investigado. **No existen reglas invariables para determinar qué remplazo bioisostérico va a funcionar en una molécula dada.**"* (resaltado fuera de texto).

Y en segundo lugar porque aun cuando documentos básicos en el campo técnico de la invención, señalen que existen reemplazos bioisostéricos que podrían permitir tener compuestos que mantuvieran la actividad biológica original, la persona versada en la materia reconocería que **existen muchas circunstancias en las cuales los reemplazos bioisostéricos resultan en una disminución de la actividad biológica del compuesto dependiendo de la manera en que este interactúe con la molécula biológica objetivo.**

En este sentido me permito resaltar que para la persona versada en la materia sería claro que la interacción de un medicamento con su molécula biológica objetivo está mediada por un gran número de factores, tal como por ejemplo se señala en el documento de consulta general **"Remington: The Science and practice of Pharmacy, 2000"**: *"Se debe reconocer que un número de factores pueden afectar la interacción de un medicamento con un receptor, incluyendo distancias interatómicas, forma, tamaño, configuración absoluta, rigidez, flexibilidad, y distribución de carga. Algunos o todos de estos factores juegan una parte en la consideración del diseño de un medicamento. Normalmente para empezar el proceso de diseño de medicamentos al nivel de receptores o enzimas, las variables tales como absorción, transporte metabolismo y excreción son descartados temporalmente con el fin de optimizar la afinidad y la potencia. Sin importar como el químico médico escoja modificar la estructura, el proceso de desarrollar un medicamento es muy complejo..."*²

De esta forma, la interacción de un medicamento con la molécula sobre la cual va a actuar dependerá directamente de diversos factores fisicoquímicos de la molécula, como su capacidad de formar puentes de hidrógeno, su polaridad, su conformación tridimensional, su estereoquímica, su acidez o basicidad, entre otros.

Por lo tanto todos estos factores deben ser tenidos en consideración cuando se realiza modificaciones dentro de una molécula buscando un bioisótero, tal como lo señala Thornber³: *"al hacer un reemplazo bioisostérico, los siguientes parámetros del grupo a ser cambiado pueden ser considerados: tamaño, forma (ángulos de enlace, hibridización), distribución electrónica (polarizabilidad, efectos inductivos, carga, dipolos), solubilidad lipídica, solubilidad acuosa, pK_a, reactividad química (incluyendo probabilidad de*

² Gennaro, A Editor "Remington: The Science and practice of Pharmacy" 20th edition, Philadelphia College of Pharmacy and Science, 2000. Pág. 464.

³ Thornber, C. W. "Isosterism and Molecular modification in drug design" Chem. Soc. Rev. 1979, 8, 563-580.

metabolismo), capacidad de uniones de hidrógeno.” **“Es poco probable que un reemplazo bioisostérico deje todos estos parámetros sin perturbar”**. (Resaltado fuera de texto).

Claramente, al depender directamente de la forma de interacción del medicamento con su molécula objetivo, un reemplazo bioisostérico que es útil para un sistema específico no necesariamente lo será para otro.

Ahora bien, para el caso que nos ocupa, me permito referirme nuevamente al artículo de **Hilpert et al⁴**, el cual fue allegado junto con mi memorial del 15 de octubre de 2014.

En este sentido, y contrario a lo que afirma el señor Examinador, es claro que tal documento es una prueba contundente de que **el intercambio del átomo de azufre del tiazinilo divulgado en D1 por un átomo de oxígeno como en los compuestos de la presente solicitud, de ninguna manera puede ser considerado como un reemplazo convencional para la persona experta en la materia**. Igualmente el intercambio de un átomo de hidrógeno de la posición 5 por flúor tampoco puede ser considerado como un reemplazo convencional.

Table 1. In Vitro and in Vivo Profiles of Oxazines 1a and 14a and Thiazines 112 and 113

Nc1nc2c(nc(=O)n2c1-c1ccc(F)cc1)C(R)(R)X

			IC ₅₀ (μM) ^a				shift BACE1 enzyme vs cell-based	brain Aβ40 reduction in mice at 30 mg/kg ^d (%)
compd	X	R	pK _s ^b	hBACE1 enzyme	hBACE2 enzyme	Aβ40 cell-based ^c		
1a	O	H	9.8	0.137	0.052	0.010	14	29
14a	O	F	6.3	0.026	0.015	0.017	2	69
112	S	H	9.0	0.016	0.009	0.001	16	92
113	S	F	6.1	0.014	0.010	0.004	3	0

^aIC₅₀ values are means of at least 2 independent experiments. ^bpK_s determined by capillary electrophoresis. ^cHEK293 cells transfected with wild-type human APP. ^dCompound given orally, n = 3–4, 4h post dose, mean reduction as compared to vehicle control.

Tabla 1, Documento Hilpert et al.

En este sentido, de la información consignada en la Tabla 1 de la página 3984 y tras la lectura atenta de la sección de resultados y discusión del artículo de Hilpert et al, se evidencia que las oxazinas di-fluoradas son las más adecuadas como inhibidores de BACE1, esto en comparación con oxazinas sin fluoración y con tiazinas con y sin sustitución con flúor, por lo que claramente se puede concluir que **el impacto de la disustitución con flúor sobre el grupo principal oxazinilo o tiazinilo y/o el reemplazo de un átomo de azufre por un átomo de oxígeno en el grupo principal, sobre las actividades BACE1 y las eficacias in vivo es complejo y conduce a resultados inesperados**.

⁴ Hilpert et al. β-Secretase (BACE1) Inhibitors with High in Vivo Efficacy Suitable for Clinical Evaluation in Alzheimer’s Disease. Journal of Medicinal Chemistry, 2013, 56, 3980–3995.

Por lo tanto, a la luz de las divulgaciones dadas en el artículo de Hilpert et al, es completamente evidente que **el reemplazo del grupo tiazinilo por el grupo oxazinilo y la disustitución con flúor NO son eventos convencionales en el campo técnico.**

En vista de lo anterior, me permito resaltar que claramente la persona versada en la materia reconocería que no sería predecible el efecto que tendría el remplazar el átomo de azufre por un átomo de oxígeno, aun cuando dicho remplazo se haga por un átomo considerado como bioisótero.

En conexión con los argumentos anteriores me permito agregar que, contrario a lo que afirma el señor Examinador cuando señala que " (...) *no se observa un efecto técnico sorprendente por parte de la solicitud (...) y es de resaltar que para que una selección se considere inventiva la totalidad de los compuestos derivados de la Markush deben tener efecto técnico inesperado y esto no ocurre en el presente caso.*", los compuestos de la invención sí poseen un efecto técnico sorprendente, teniendo en cuenta que:

- (i) el solicitante ha encontrado que a pesar de la presencia de un oxazinilo en lugar de un tiazinilo, los compuestos de la invención conservan una potente **actividad inhibitoria de BACE 1 y/o BACE 2**, que se evidencia por los valores de IC₅₀, los cuales para una gran mayoría de compuestos reclamados se encuentra por debajo de 0.02 μ m e incluso por debajo de 0.01 para BACE1 (Ver Págs. 61 a 76 de la presente solicitud de patente);
- (ii) los datos de actividad revelados por D1 de ninguna manera son comparables con los presentados para los compuestos de la invención, dado que la naturaleza de los ensayos realizados y las condiciones empleadas para realizar la determinación de tales datos son diferentes en ambos casos. Aún con esa salvedad, es claro que el dato de 0.027 μ m para el compuesto 1241 de D1 es mucho mayor que para una gran mayoría de compuestos ejemplificados y probados en la presente invención, tales como los compuestos de los ejemplos 11, 13, 15 y 21 los cuales presentan valores de IC₅₀ para la inhibición de BACE 1 de 0.018, 0.008, 0.009 y 0.007 μ m respectivamente, entre otros ejemplos.

En este sentido me permito resaltar que este comportamiento de ninguna manera sería evidente a partir del documento citado máxime cuando éste no sugiere cuál podría ser el efecto de tener compuestos cuyo núcleo principal sea un oxazinilo y no un tiazinilo, por lo tanto es evidente el Nivel Inventivo que conlleva la presente solicitud denegada; y

- (iii) los compuestos ensayados en la presente solicitud son compuestos representativos de la invención y permiten evidenciar el comportamiento y

actividad de los compuestos abarcados por el alcance de la reclamación. Por lo tanto, es completamente injusto soportar la negación del privilegio de patente con base en la premisa de que *"la totalidad de los compuestos derivados de la Markush deben tener efecto técnico inesperado"* cuando en realidad se ha demostrado que un grupo representativo de los compuestos de la invención demuestran una potente actividad como inhibidores de BACE1 y cuando es totalmente evidente que el sintetizar y probar todos y cada uno de los compuestos resultantes de la fórmula Markush resulta en una actividad poco práctica e innecesaria.

3. **REQUISITOS EXIGIDOS POR EL ARTÍCULO 18, LA EVALUACIÓN DEL NIVEL INVENTIVO DE UNA SOLICITUD DE PATENTE.**

Dentro del contexto de los argumentos discutidos anteriormente, me permito señalar que el Artículo 18 de la Decisión Andina 486 de 2000 exige de forma manifiesta que la invención no debe resultar obvia ni se debe poder derivar de forma evidente de lo existente en el arte previo, y por lo tanto es en este sentido que el examinador debe encaminar su análisis para sopesar si una solicitud es o no merecedora del beneficio de patente.

Así mismo, me permito llamar la atención a ese Despacho a que el Tribunal de Justicia de la Comunidad Andina se ha pronunciado con respecto a las definiciones de "obvio" y "evidente" en la evaluación del Nivel Inventivo de una solicitud de patente⁵:

"Resulta pertinente, a los efectos del artículo 4 de la Decisión 344, definir qué se entiende por "obvio" y por "evidente" a fin de determinar qué se debe entender por Nivel Inventivo.

Lo obvio es aquello que salta a la vista de manera clara y directa; lo "que se encuentra o pone delante de los ojos, muy claro o que no tiene dificultad". Mientras que lo evidente consiste en la "certeza clara y manifiesta de la que no se puede dudar; cierto, claro, patente y sin la menor duda". De forma que algo que resulte obvio no es necesariamente evidente; empero lo que es evidente, es también obvio.

En este orden de ideas, se puede concluir que una invención goza de Nivel Inventivo cuando a los ojos de un experto medio en el asunto de que se trate, se necesita algo más que la simple aplicación de los conocimientos técnicos en la materia para llegar a ella, es decir, que de conformidad con el estado de la técnica el invento no sea consecuencia clara y directa de dicho estado."

⁵ Tribunal de Justicia de la Comunidad Andina, PROCESO 192-IP-2005

Con respecto a este señalamiento me permito resaltar que resulta indiscutible que para la legislación Andina, para que exista afectación del requisito de Nivel Inventivo de una solicitud de patente, es necesario que la invención allí revelada sea derivable de forma clara y directa del arte previo, y que las enseñanzas de este arte previo sin lugar a dudas conduzcan a dicha invención.

Consecuentemente, para el caso que nos compete dado que a partir de las enseñanzas del documento D1 no "saltan a la vista de manera clara y directa" los compuestos que son objeto de la presente invención, y al no tenerse "certeza clara y manifiesta" a partir de las enseñanzas de dicho documento de que dichos compuestos serían potentes inhibidores de BACE 1 y/o BACE 2, es evidente que el arte previo no puede afectar el Nivel Inventivo de la solicitud en estudio.

II. CONCLUSIONES

Con base en los argumentos técnicos mostrados previamente, me permito manifestar ante ese Despacho que la materia revelada en la solicitud de patente en cuestión, presenta un inequívoco carácter inventivo frente a las enseñanzas del arte previo citado en la Resolución Número **76363** aquí impugnada, cumpliendo así a cabalidad las disposiciones establecidas en el Artículo 18 de la Decisión Andina 486 de 2000, y por lo tanto demostrando ser merecedora del privilegio de patente requerido.

Es así como, en virtud de los argumentos discutidos con respecto al documento D1, se estableció de manera incontrovertible que los compuestos que son objeto de la solicitud en estudio, de ninguna manera resultarían evidentes para la persona versada en la materia a partir de las enseñanzas de dicho documento, demostrando irrefutablemente la patentabilidad de la presente solicitud.

Como consecuencia de lo anterior, me permito resaltar que dado que la solicitud denegada sí se ajusta completamente a los requerimientos establecidos por la Decisión Andina 486, al negar el privilegio de patente ese Despacho claramente contraviene el Artículo 18 de dicha Decisión.

III. SOLICITUD

En vista de todo lo anterior, ruego a usted proceder a estudiar nuevamente la solicitud a la luz de las argumentaciones presentadas y revocar la Resolución Número **76363** impugnada para conceder el privilegio de patente solicitado.

LLOREDA CAMACHO & CO.

Calle 72 No. 5-83 Piso 5, Bogotá, D.C. Tel: 6069700 Fax 6069701

IV. PODER

En este punto me permito mencionar ante ese Despacho que el documento que acredita mi calidad de Representante Legal en Bogotá para asuntos relativos a la Propiedad Industrial de la sociedad peticionaria fue presentado junto con la Solicitud patente el 06 de julio de 2012.

V. ANEXOS

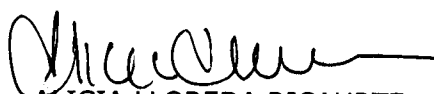
Anexo al presente recurso me permito presentar:

- (i) Recibo número 15-003842, expedido por la Superintendencia de Industria y Comercio, que acredita el pago de las modificaciones mencionadas;
- (ii) Copia de la páginas **143** a **148**, que corresponden al capítulo reivindicatorio modificado que consta de **12** reivindicaciones; y
- (iii) Artículo: Hilpert et al. β -Secretase (BACE1) Inhibitors with High in Vivo Efficacy Suitable for Clinical Evaluation in Alzheimer's Disease. Journal of Medicinal Chemistry, 2013, 56, 3980–3995.

VI. DOMICILIO

Mi dirección es Calle 72 No. 5-83, Piso 6º, Bogotá D.C.

Del Señor Superintendente,

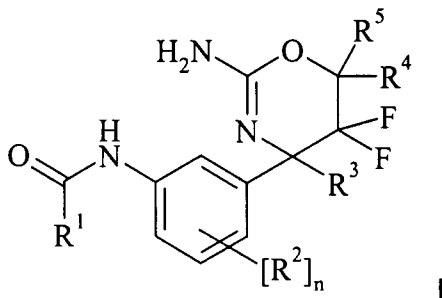


ALICIA LLOREDA RICAURTE

T.P. 53.215

REIVINDICACIONES

1. Un compuesto de la fórmula I,



5 en la que

R¹ se elige entre el grupo formado por

- i) piridinilo,
- ii) pirazolilo,
- iii) pirazinilo,
- 10 iv) pirimidinilo, y
- v) piridinilo sustituido por 1-2 sustituyentes elegidos individualmente entre ciano, halógeno, y halógeno-alquilo C₁-₆,

R² es flúor;

R³ es metilo;

15 R⁴ se elige entre el grupo formado por

- i) hidrógeno y
- ii) alquilo C₁-₆;

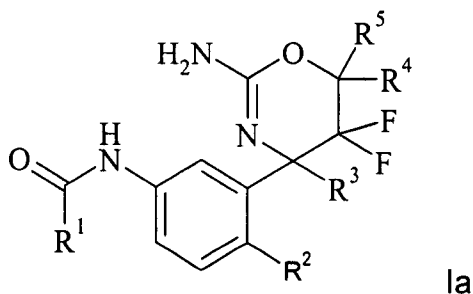
R⁵ se elige entre el grupo formado por

- i) hidrógeno y
- 20 ii) alquilo C₁-₆;

n es el número 1;

o sus sales farmacéuticamente aceptables.

2. Un compuesto de la fórmula Ia según la reivindicación 1,



en la que

R¹ se elige entre el grupo formado por

- i) piridinilo,
- ii) pirazolilo,
- 5 iii) pirazinilo,
- iv) pirimidinilo, y
- v) piridinilo sustituido por 1-2 sustituyentes elegidos individualmente entre ciano, halógeno, y halógeno-alquilo C₁₋₆,

R² es flúor;

10 R³ es metilo;

R⁴ se elige entre el grupo formado por

- i) hidrógeno y
- ii) alquilo C₁₋₆;

R⁵ se elige entre el grupo formado por

- 15 i) hidrógeno y
- ii) alquilo C₁₋₆;

o sus sales farmacéuticamente aceptables.

3. Un compuesto según una cualquiera de las reivindicaciones 1-2, en el que R¹ se elige entre el grupo formado por

- 20 3,5-dicloro-piridin-2-ilo, 3,5-difluor-piridin-2-ilo, 3-cloro-5-ciano-piridin-2-ilo, 3-cloro-5-fluor-piridin-2-ilo, 3-cloro-5-tri-fluormetil-piridin-2-ilo, 3-cloro-piridin-2-ilo, 3-fluor-piridin-2-ilo, 3-trifluormetil-piridin-2-ilo, 5-(trifluormetil)-piridin-2-ilo, 5-cloro-3-fluor-piridin-2-ilo, 5-cloro-3-metil-piridin-2-ilo, 5-cloro-piridin-2-ilo, 5-ciano-piridin-2-ilo, 5-fluor-piridin-2-ilo y piridin-2-ilo.

- 25 4. Un compuesto según una cualquiera de las reivindicaciones 1-3, en el que R¹ se elige entre el grupo formado por 5-cloro-piridin-2-ilo, 3-cloro-5-trifluormetil-piridin-2-ilo, 3-cloro-5-fluor-piridin-2-ilo, 3,5-dicloro-piridin-2-ilo, 5-ciano-piridin-2-ilo y 5-cloro-3-fluor-piridin-2-ilo.

- 5. Un compuesto según una cualquiera de las reivindicaciones de 1 a 4, en el que R⁴ es hidrógeno.
- 30

- 6. Un compuesto según una cualquiera de las reivindicaciones de 1 a 5, en el que R⁴ es metilo.

7. Un compuesto según una cualquiera de las reivindicaciones de 1 a 6, en el que R⁵ es hidrógeno.

8. Un compuesto según una cualquiera de las reivindicaciones de 1 a 7, en el que R⁵ es metilo.

- 5 9. Un compuesto según una cualquiera de las reivindicaciones de 1 a 8, elegido entre el grupo formado por
- [3-((R)-2-amino-5,5-difluor-4-metil-5,6-dihidro-4H-[1,3]-oxazin-4-il)-4-fluor-fenil]-amida
del ácido 5-cloro-piridina-2-carboxílico,
- [3-((R)-2-amino-5,5-difluor-4-metil-5,6-dihidro-4H-[1,3]-oxazin-4-il)-4-fluor-fenil]-amida
10 del ácido 5-fluor-piridina-2-carboxílico,
- [3-((R)-2-amino-5,5-difluor-4-metil-5,6-dihidro-4H-[1,3]-oxazin-4-il)-4-fluor-fenil]-amida
del ácido 3-cloro-piridina-2-carboxílico,
- [3-((R)-2-amino-5,5-difluor-4-metil-5,6-dihidro-4H-[1,3]-oxazin-4-il)-4-fluor-fenil]-amida
del ácido 3,5-difluor-piridina-2-carboxílico,
- 15 [3-((R)-2-amino-5,5-difluor-4-metil-5,6-dihidro-4H-[1,3]-oxazin-4-il)-4-fluor-fenil]-amida
del ácido 3-cloro-5-trifluormetil-piridina-2-carboxílico,
- [3-((R)-2-amino-5,5-difluor-4-metil-5,6-dihidro-4H-[1,3]-oxazin-4-il)-4-fluor-fenil]-amida
del ácido 3-trifluormetil-piridina-2-carboxílico,
- [3-((R)-2-amino-5,5-difluor-4-metil-5,6-dihidro-4H-[1,3]-oxazin-4-il)-4-fluor-fenil]-amida
20 del ácido 3-cloro-5-fluor-piridina-2-carboxílico,
- [3-((R)-2-amino-5,5-difluor-4-metil-5,6-dihidro-4H-[1,3]-oxazin-4-il)-4-fluor-fenil]-amida
del ácido 3,5-dicloro-piridina-2-carboxílico,
- [3-((R)-2-amino-5,5-difluor-4-metil-5,6-dihidro-4H-[1,3]-oxazin-4-il)-4-fluor-fenil]-amida
del ácido piridina-2-carboxílico,
- 25 [3-((R)-2-amino-5,5-difluor-4-metil-5,6-dihidro-4H-[1,3]-oxazin-4-il)-4-fluor-fenil]-amida
del ácido 3-fluor-piridina-2-carboxílico,
- [3-((R)-2-amino-5,5-difluor-4-metil-5,6-dihidro-4H-[1,3]-oxazin-4-il)-4-fluor-fenil]-amida
del ácido 5-ciano-piridina-2-carboxílico,
- clorhidrato de la [3-((R)-2-amino-5,5-difluor-4-metil-5,6-dihidro-4H-[1,3]oxazin-4-il)-4-
30 fluor-fenil]-amida del ácido 5-ciano-piridina-2-carboxílico,
- [3-((R)-2-amino-5,5-difluor-4-metil-5,6-dihidro-4H-[1,3]-oxazin-4-il)-4-fluor-fenil]-amida
del ácido 5-ciano-piridina-2-carboxílico,

- [3-((R)-2-amino-5,5-difluor-4-metil-5,6-dihidro-4H-[1,3]-oxazin-4-il)-4-fluor-fenil]-amida del ácido 5-cloro-3-metil-piridina-2-carboxílico,
- [3-((R)-2-amino-5,5-difluor-4-metil-5,6-dihidro-4H-[1,3]-oxazin-4-il)-4-fluor-fenil]-amida del ácido 5-cloro-3-fluor-piridina-2-carboxílico,
- 5 [3-((R)-2-amino-5,5-difluor-4,6,6-trimetil-5,6-dihidro-4H-[1,3]oxazin-4-il)-4-fluor-fenil]-amida del ácido 5-cloro-piridina-2-carboxílico,
- [3-((R)-2-amino-5,5-difluor-4,6,6-trimetil-5,6-dihidro-4H-[1,3]oxazin-4-il)-4-fluor-fenil]-amida del ácido 3-cloro-5-trifluormetil-piridina-2-carboxílico,
- [3-((R)-2-amino-5,5-difluor-4,6,6-trimetil-5,6-dihidro-4H-[1,3]oxazin-4-il)-4-fluor-fenil]-
- 10 amida del ácido 5-ciano-piridina-2-carboxílico,
- clorhidrato de la (R)-N-(3-(2-amino-5,5-difluor-4,6,6-trimetil-5,6-dihidro-4H-1,3-oxazin-4-il)-4-fluorfenil)-3-cloro-5-cianopicolinamida,
- (R)-N-(3-(2-amino-5,5-difluor-4,6,6-trimetil-5,6-dihidro-4H-1,3-oxazin-4-il)-4-fluorfenil)-3-cloro-5-cianopicolinamida,
- 15 clorhidrato de la (R)-N-(3-(2-amino-5,5-difluor-4,6,6-tri-metil-5,6-dihidro-4H-1,3-oxazin-4-il)-4-fluorfenil)-3,5-dicloropicolinamida,
- (R)-N-(3-(2-amino-5,5-difluor-4,6,6-trimetil-5,6-dihidro-4H-1,3-oxazin-4-il)-4-fluorfenil)-3,5-dicloropicolinamida,
- (R)-N-(3-(2-amino-5,5-difluor-4,6,6-trimetil-5,6-dihidro-4H-1,3-oxazin-4-il)-4-fluorfenil)-
- 20 5-cloro-3-fluorpicolinamida,
- (R)-N-(3-(2-amino-5,5-difluor-4,6,6-trimetil-5,6-dihidro-4H-1,3-oxazin-4-il)-4-fluorfenil)-5-(trifluormetil)picolinamida, y
- [3-((R)-2-amino-5,5-difluor-4-metil-5,6-dihidro-4H-[1,3]-oxazin-4-il)-4-fluor-fenil]-amida del ácido 3-cloro-5-ciano-piridina-2-carboxílico,
- 25 o una sal farmacéuticamente aceptable de los mismos.

10. Un compuesto según una cualquiera de las reivindicaciones de 1 a 9, elegido entre el grupo formado por

- [3-((R)-2-amino-5,5-difluor-4-metil-5,6-dihidro-4H-[1,3]-oxazin-4-il)-4-fluor-fenil]-amida del ácido 5-cloro-piridina-2-carboxílico,
- 30 [3-((R)-2-amino-5,5-difluor-4-metil-5,6-dihidro-4H-[1,3]-oxazin-4-il)-4-fluor-fenil]-amida del ácido 5-fluor-piridina-2-carboxílico,

- [3-((R)-2-amino-5,5-difluor-4-metil-5,6-dihidro-4H-[1,3]-oxazin-4-il)-4-fluor-fenil]-amida del ácido 3-cloro-piridina-2-carboxílico,
- [3-((R)-2-amino-5,5-difluor-4-metil-5,6-dihidro-4H-[1,3]-oxazin-4-il)-4-fluor-fenil]-amida del ácido 3,5-difluor-piridina-2-carboxílico,
- 5 [3-((R)-2-amino-5,5-difluor-4-metil-5,6-dihidro-4H-[1,3]-oxazin-4-il)-4-fluor-fenil]-amida del ácido 3-cloro-5-tri-fluormetil-piridina-2-carboxílico,
- [3-((R)-2-amino-5,5-difluor-4-metil-5,6-dihidro-4H-[1,3]-oxazin-4-il)-4-fluor-fenil]-amida del ácido 3-trifluormetil-piridina-2-carboxílico,
- [3-((R)-2-amino-5,5-difluor-4-metil-5,6-dihidro-4H-[1,3]-oxazin-4-il)-4-fluor-fenil]-amida del ácido 3-cloro-5-fluor-piridina-2-carboxílico,
- 10 [3-((R)-2-amino-5,5-difluor-4-metil-5,6-dihidro-4H-[1,3]-oxazin-4-il)-4-fluor-fenil]-amida del ácido 3,5-dicloro-piridina-2-carboxílico,
- [3-((R)-2-amino-5,5-difluor-4-metil-5,6-dihidro-4H-[1,3]-oxazin-4-il)-4-fluor-fenil]-amida del ácido piridina-2-carboxílico,
- 15 [3-((R)-2-amino-5,5-difluor-4-metil-5,6-dihidro-4H-[1,3]-oxazin-4-il)-4-fluor-fenil]-amida del ácido 3-fluor-piridina-2-carboxílico,
- [3-((R)-2-amino-5,5-difluor-4-metil-5,6-dihidro-4H-[1,3]-oxazin-4-il)-4-fluor-fenil]-amida del ácido 5-ciano-piridina-2-carboxílico,
- [3-((R)-2-amino-5,5-difluor-4-metil-5,6-dihidro-4H-[1,3]-oxazin-4-il)-4-fluor-fenil]-amida del ácido 5-cloro-3-metil-piridina-2-carboxílico,
- 20 [3-((R)-2-amino-5,5-difluor-4-metil-5,6-dihidro-4H-[1,3]-oxazin-4-il)-4-fluor-fenil]-amida del ácido 5-cloro-3-fluor-piridina-2-carboxílico,
- [3-((R)-2-amino-5,5-difluor-4,6,6-trimetil-5,6-dihidro-4H-[1,3]oxazin-4-il)-4-fluor-fenil]-amida del ácido 5-cloro-piridina-2-carboxílico y
- 25 [3-((R)-2-amino-5,5-difluor-4,6,6-trimetil-5,6-dihidro-4H-[1,3]oxazin-4-il)-4-fluor-fenil]-amida del ácido 3-cloro-5-trifluormetil-piridina-2-carboxílico.

11. Un compuesto según una cualquiera de las reivindicaciones de 1 a 4, elegido entre el grupo formado por

- [3-((R)-2-amino-5,5-difluor-4-metil-5,6-dihidro-4H-[1,3]-oxazin-4-il)-4-fluor-fenil]-amida del ácido 5-cloro-piridina-2-carboxílico,
- 30 [3-((R)-2-amino-5,5-difluor-4-metil-5,6-dihidro-4H-[1,3]-oxazin-4-il)-4-fluor-fenil]-amida del ácido 3-cloro-5-tri-fluormetil-piridina-2-carboxílico,

- [3-((R)-2-amino-5,5-difluor-4-metil-5,6-dihidro-4H-[1,3]-oxazin-4-il)-4-fluor-fenil]-amida del ácido 3-cloro-5-fluor-piridina-2-carboxílico,
- [3-((R)-2-amino-5,5-difluor-4-metil-5,6-dihidro-4H-[1,3]-oxazin-4-il)-4-fluor-fenil]-amida del ácido 3,5-dicloro-piridina-2-carboxílico,
- 5 [3-((R)-2-amino-5,5-difluor-4-metil-5,6-dihidro-4H-[1,3]-oxazin-4-il)-4-fluor-fenil]-amida del ácido 5-ciano-piridina-2-carboxílico,
- [3-((R)-2-amino-5,5-difluor-4-metil-5,6-dihidro-4H-[1,3]-oxazin-4-il)-4-fluor-fenil]-amida del ácido 5-cloro-3-fluor-piridina-2-carboxílico,
- [3-((R)-2-amino-5,5-difluor-4,6,6-trimetil-5,6-dihidro-4H-[1,3]oxazin-4-il)-4-fluor-fenil]-
- 10 amida del ácido 5-cloro-piridina-2-carboxílico y
- [3-((R)-2-amino-5,5-difluor-4,6,6-trimetil-5,6-dihidro-4H-[1,3]oxazin-4-il)-4-fluor-fenil]-amida del ácido 3-cloro-5-trifluormetil-piridina-2-carboxílico.

12. Un compuesto según una cualquiera de las reivindicaciones de 1 a 11, el cual es clorhidrato de la [3-((R)-2-amino-5,5-difluor-4-metil-5,6-dihidro-4H-[1,3]oxazin-
- 15 4-il)-4-fluor-fenil]-amida del ácido 5-ciano-piridina-2-carboxílico.

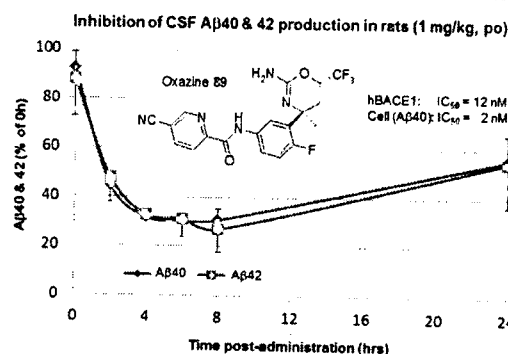
β -Secretase (BACE1) Inhibitors with High in Vivo Efficacy Suitable for Clinical Evaluation in Alzheimer's Disease

Hans Hilpert,^{*,†} Wolfgang Guba,[†] Thomas J. Woltering,[†] Wolfgang Wostl,[†] Emmanuel Pinard,[†] Harald Mauser,[†] Alexander V. Mayweg,[†] Mark Rogers-Evans,[†] Roland Humm,[†] Daniela Krummenacher,[†] Thorsten Muser,[†] Christian Schnider,[†] Helmut Jacobsen,[‡] Laurence Ozmen,[‡] Alessandra Bergadano,[‡] David W. Banner,[§] Remo Hochstrasser,[§] Andreas Kuglstatte,[§] Pascale David-Pierson,^{||} Holger Fischer,^{||} Alessandra Polara,[⊥] and Robert Narquizian[⊥]

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Supporting Information

ABSTRACT: An extensive fluorine scan of 1,3-oxazines revealed the power of fluorine(s) to lower the pK_a and thereby dramatically change the pharmacological profile of this class of BACE1 inhibitors. The CF_3 substituted oxazine 89, a potent and highly brain penetrant BACE1 inhibitor, was able to reduce significantly CSF A β 40 and 42 in rats at oral doses as low as 1 mg/kg. The effect was long lasting, showing a significant reduction of A β 40 and 42 even after 24 h. In contrast to 89, compound 1b lacking the CF_3 group was virtually inactive in vivo.

**INTRODUCTION**

Alzheimer's disease (AD) is a progressive neurodegenerative disease and the major cause of dementia in the elderly. The clinical symptoms of AD are progressive memory loss, learning impairment, and behavioral and psychiatric disturbances. The predominant pathological hallmarks of the disease are (1) amyloid plaques, i.e., extracellular deposits of polymerized amyloid- β peptides (A β), and (2) intracellular aggregates of mis-folded tau protein. Formation of these pathologies can precede the clinical disease symptoms by years. The final disease pathology is further characterized by pronounced neuroinflammation and widespread neuronal loss. As no causative treatment is currently available, there is an urgent need for new, efficacious therapies.¹ Amyloid aggregates are considered to be the earliest pathology and, according to the amyloid cascade hypothesis, the ultimate cause of the disease.^{2–5} Their main constituents, the amyloid- β peptides, are derived from the β -amyloid precursor protein (APP) via proteolytic processing by β -secretase (BACE1) and γ -secretase. Both APP and BACE1 are type-1 membrane proteins highly expressed in the brain. BACE1 is an aspartic protease of 501 amino acids length, organized into an extracellular domain with signal peptide, prodomain and catalytic domain, a trans-membrane domain and a short cytoplasmic stretch of 22 amino acids. Its principal cleavage site in the APP extracellular domain forms the N-terminus of A β (position D672 of the longest APP isotype); a secondary cleavage site is localized at

position E682. Processing of APP occurs predominantly in endosomal vesicles, in agreement with the "acidic pH" optimum of the enzyme.^{6–8} The unique and essential role of BACE1 for the generation of A β has been convincingly shown in mice where both alleles have been ablated by genetic means. These mice do not form A β , nor do they cleave APP at the known BACE1-positions.^{9,10} It has furthermore been demonstrated that upon crossing BACE1 knockout mice and APP-transgenic mice, the formation of brain amyloid is inhibited. Interestingly, ablation of only one BACE1 allele was enough to reduce amyloidosis significantly. These data suggest that pharmacological inhibition of BACE1 should also reduce amyloidosis and that partial inhibition should be sufficient to achieve therapeutic efficacy.^{11,12} Taken together, this makes BACE1 a prime target for anti-amyloid therapy. Partial inhibition of BACE1 may also be the preferred therapeutic target because a number of additional BACE1 substrates have been described. Of special interest is neuregulin 1 (NRG1) processing because close examination of BACE1 knockout mice has revealed that in these animals the myelination of peripheral and possibly central nerves is severely reduced, presumably due to the absence of NRG1 processing.^{13,14} Importantly, this adverse effect was not observed in animals that retained one functional allele.

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The aspartyl protease BACE2 is a close homologue of BACE1. BACE2, however, cleaves APP preferentially at a position downstream of the BACE1 cleavage sites at F691 of APP, which does not produce amyloidogenic A β peptides. BACE2 is primarily expressed in peripheral tissue, in particular in pancreatic islets.¹⁵ Its physiological function was poorly understood for some time,⁸ but recent evidence suggests that it may play a role in glucose homeostasis.¹⁵

Soon after the discovery of BACE1 in 1999,^{16–20} the enzyme became a prime target of both academic and industrial research. Initial inhibitor design focused on statine type structures engaging with the two aspartic acids in the catalytic site. While potent in vitro, the peptidic nature of these inhibitors rendered them poorly brain penetrant. To overcome this shortcoming, less peptidic hydroxyethylamine (HEA) isosters subsequently became the focus of research. Good brain penetration, however, remained the exception rather than the rule. This has been ascribed to the flexibility of HEAs and their high number of hydrogen bond donors and acceptors.²¹

Our research focused on cyclic, conformationally rigid structures that interact with the two aspartic acids of BACE1. An HTS campaign yielded a small fragment of a weakly active (IC₅₀ = 41.2 μ M) 2-amino-4*H*-5,6-dihydro-[1,3]thiazine derivative A (Figure 1) which could be cocrystallized with

been explored by various research groups^{23–28} as a headgroup interacting with the aspartic acids of BACE1.

Previously we described the identification and characterization of a number of cyclic amidines that were devoid of substantial in vivo activity due to low penetration into the CNS.²⁹ In this paper we disclose our efforts to convert our initial lead compound, the 2-amino-4*H*-5,6-dihydro-[1,3]-oxazine derivative 1a into fluorine containing oxazines B, a compound class characterized by high in vivo activity and good overall properties.

CHEMISTRY

The syntheses of oxazines 1a–b and difluoro-oxazines 14a–v (Scheme 1) started with elegant work developed by Ellman.³⁰ Condensation of the chiral auxiliary (*R*)-*tert*-butanesulfinamide and acetophenone 2 in the presence of Ti(OEt)₄ gave the sulfinyl imine 3. Transmetalation of the lithium enolate of ethyl acetate using TiCl(O*i*Pr)₃ generated the titanium enolate, which reacted with the sulfinyl imine 3 to furnish the ester 4 as a single diastereoisomer. Alternatively, the Reformatsky reaction of the sulfinyl imine 3 and ethyl bromodifluoroacetate³¹ furnished the α,α -difluoroester 5.

The ester group in 4 and 5 was then reduced to the alcohols 6 and 7 with LiBH₄, which was followed by cleavage of the chiral auxiliary under acidic conditions to give the amino alcohols 8 and 9.³² Cyclization of the amino alcohols 8 and 9 was readily accomplished with cyanogen bromide to give the oxazines 10 and 11, which were reduced to the corresponding anilines 12³³ and 13 by catalytic hydrogenation. Amide formation was accomplished without prior protection of the amidine moiety using 4-(4,6-dimethoxy[1,3,5]triazin-2-yl)-4-methylmorpholinium chloride hydrate,³⁴ furnishing the target compounds 1a–b and 14a–v (Table 2). The absolute configurations were assigned based on X-ray structures of 1b and 14d complexed to BACE1.

The commercially unavailable acids 23–26 (Scheme 2), required to introduce R¹ residues in difluoro-oxazine 14 (Table 2), were made available from pyridine and pyrazine derivatives

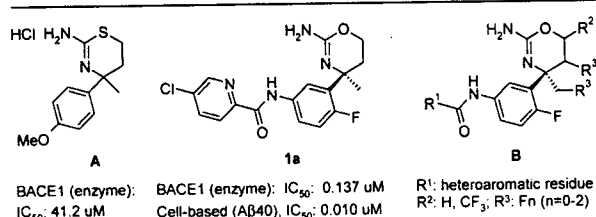
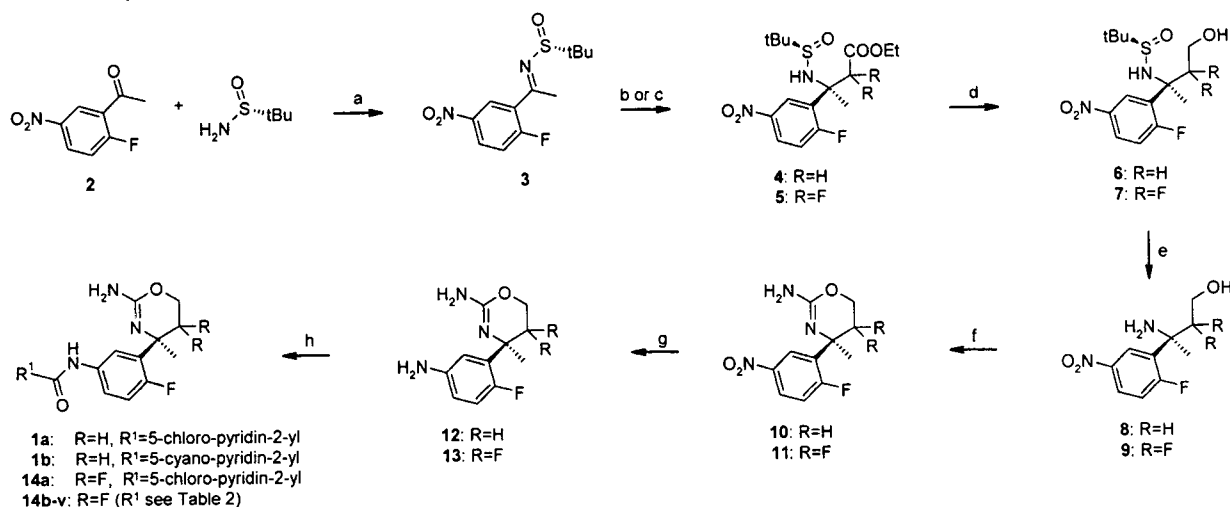


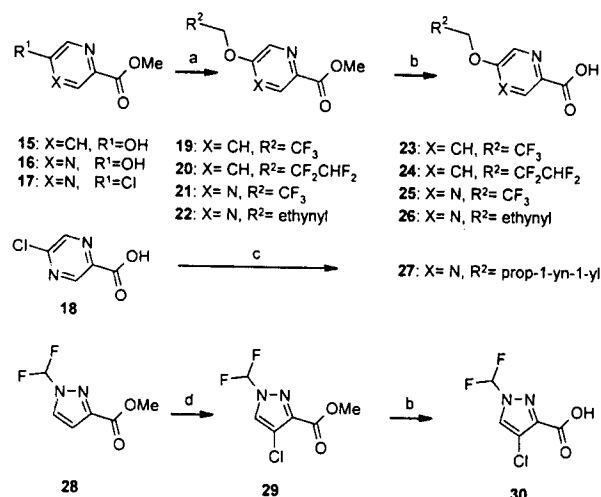
Figure 1. Lead compound 1a and targeted fluoro-oxazines B.

BACE1, offering unique opportunities for optimization. This heterocycle was first synthesized at Roche more than 30 years ago in a project looking for novel analgesics²² and has later

Scheme 1. Synthesis of Oxazines 1a–b and Difluoro-oxazines 14a–v^a



^aReagents and conditions: (a) Ti(OEt)₄, THF, 50–70 °C. (b) 3→4: MeCOOEt, LDA, TiCl(O*i*Pr)₃, THF, –78 °C. (c) 3→5: BrCF₂COOEt, Zn, CuCl, THF, 35 °C. (d) LiBH₄, THF, 0 °C. (e) HCl/1,4-dioxane/MeOH, 22 °C. (f) BrCN, EtOH, 85 °C. (g) H₂, Pd/C, EtOH, 22 °C. (h) 4-(4,6-Dimethoxy[1,3,5]triazin-2-yl)-4-methylmorpholinium chloride hydrate (DMTMM), MeOH, 22 °C.

Scheme 2. Synthesis of Commercially Unavailable Building Blocks 23–27 and 30^a

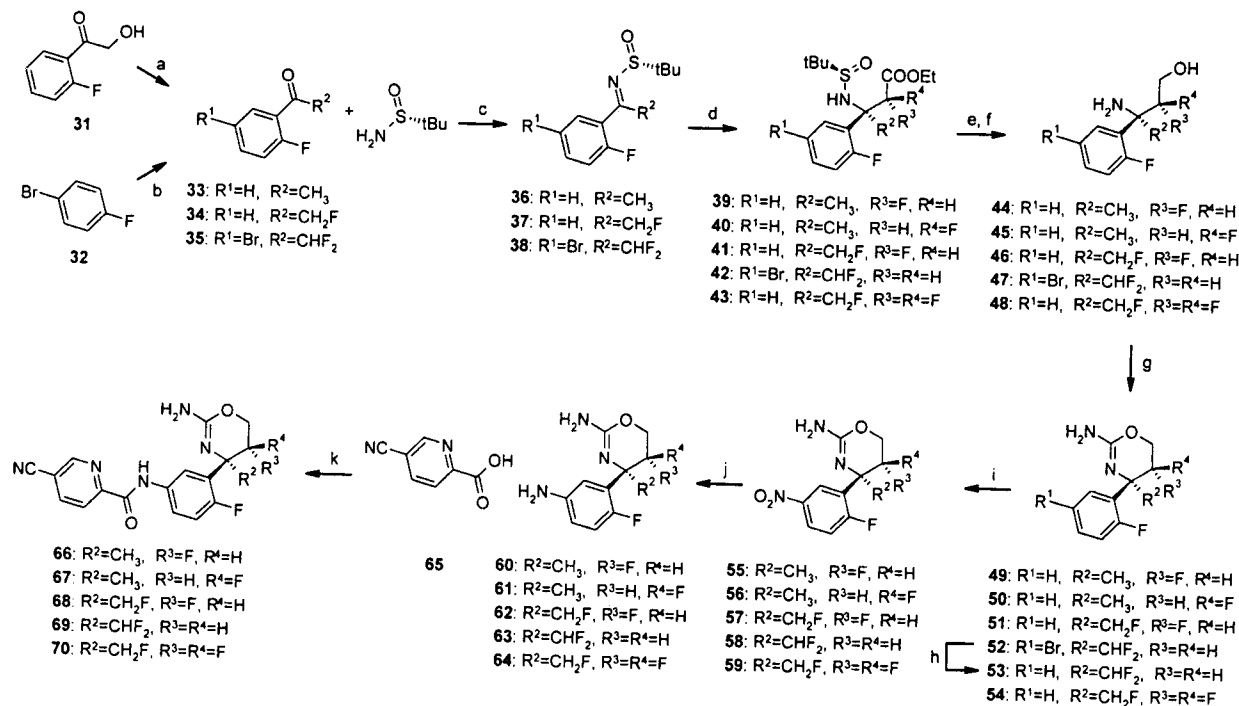
^aReagents and conditions: (a) 15→19: CF₃CH₂OTf, NaH, DMF, 22 °C. 15→20: CHF₂CH₂CH₂OTf, K₂CO₃, acetone. 17→21: CF₃CH₂OH, Cs₂CO₃, DMF. 17→22: prop-2-yn-1-ol, *tert*-BuOK, 1,4-dioxane, 22 °C. (b) LiOH or NaOH, THF/H₂O, 22 °C to reflux. (c) But-2-yn-1-ol, *tert*-BuOK, DMF, 65 °C. (d) *N*-chlorosuccinimide, DMF, 50 °C.

15 and 17 via the alkylated esters 19–22. Attempted alkylation of the hydroxy-pyrazine ester 16 with 3-bromopropyne did not lead to the expected *O*-alkylated product 22 but furnished the *N*-alkylated product (5-oxo-4-prop-2-ynyl-4,5-dihydro-pyrazine-2-carboxylic acid methyl ester, structure not shown).

Compound 27 was prepared directly from the acid 18 without proceeding via the corresponding ester. Finally, chlorination of the pyrazole ester 28 with *N*-chlorosuccinimide followed by saponification delivered the chloro-pyrazole derivative 30.

To access fluoro-oxazines 66–70 (Scheme 3) having fluorines attached at positions R²–R⁴, the synthetic strategy was modified. Occasionally the alcohol group in 6–9 (Scheme 1) can undergo an intramolecular aromatic substitution of the fluorine, which is activated by the nitro group, to give the corresponding nitro-chromane derivatives (structures not shown). We therefore introduced the nitro group at a later stage of the synthesis. The commercially unavailable ketones 34 and 35 (Scheme 3) were made available by fluorination of the alcohol 31 or by acylation of 32 using ethyl difluoroacetate. Sulfinyl imines 36–38, prepared as described in Scheme 1, were then subjected to the Reformatsky reaction to afford the esters 39–43. The reaction of 36 with BrCHFCOOEt yielded a separable 4:1-mixture of the epimers 39 and 40. Conversion of 37 with BrCHFCOOEt gave a separable 8:1-mixture of 41 as the major isomer and the corresponding epimer (R³ = H, R⁴ = F). Oxazines 49–51 and 53–54, prepared in analogy to Scheme 1, were then nitrated with fuming nitric acid, followed by reduction of the nitro group and amide formation in the usual manner, to give the targeted fluorinated oxazines 66–70. The relative and absolute configurations were assigned based on X-ray structures of 66–70 complexed to BACE1.

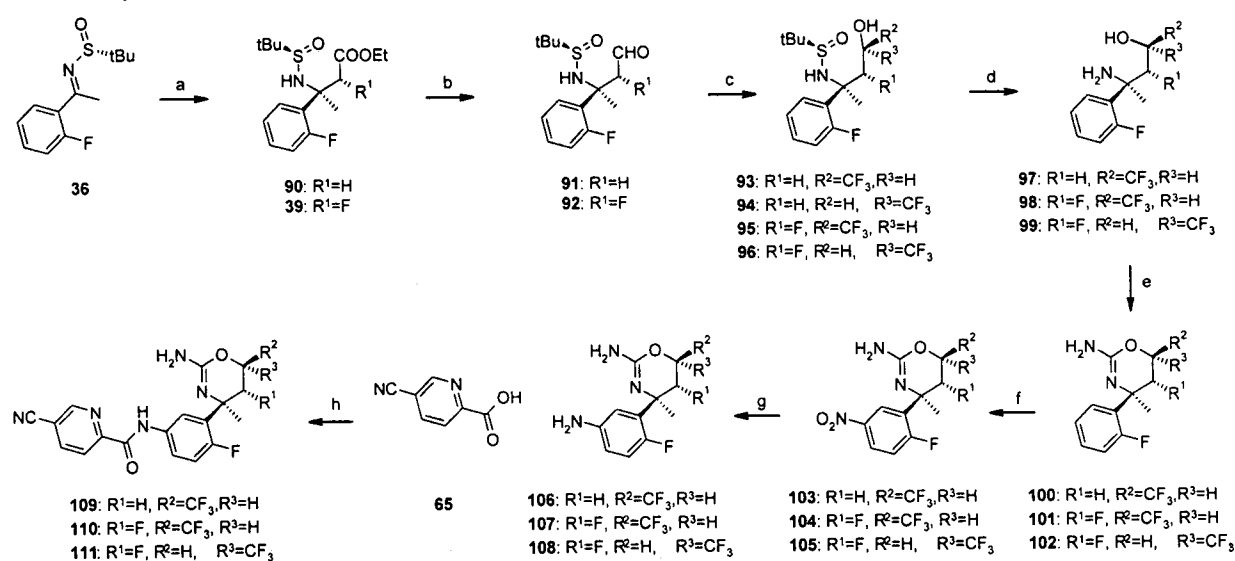
The synthesis of trifluoromethyl substituted oxazines 88 and 89 (Scheme 4) required a different strategy. Ready access to CF₃ substituted 3-amino-propan-1-ol derivatives 80 and 81 is achieved using a dipolar cycloaddition reaction of a nitrile oxide

Scheme 3. Synthesis of Fluoro-oxazines 66–70^a

^aReagents and conditions: (a) 31→34: nonafluoro-*n*-butansulfonyl fluoride, triethylamine trihydrofluoride, CH₂Cl₂, 0 to 22 °C. (b) 32→35: LDA, F₂CHCOOEt, THF, −70 to −10 °C. (c) Ti(OEt)₄, THF, 70 °C. (d) 39, 40, and 41: BrCHFCOOEt, 42; BrCH₂COOEt, 43; BrCF₂COOEt, Zn, CuCl, THF. (e) LiBH₄, EtOH, 22 °C. (f) aq HCl, THF, 22 °C. (g) BrCN, *i*-PrOH, or EtOH, 80 °C. (h) H₂, Pd/C, NaOAc, MeOH, 22 °C. (i) HNO₃, H₂SO₄, TFA, 0 °C. (j) H₂, Pd/C, Et₃N, EtOH, 22 °C. (k) DMTMM, MeOH, 0–22 °C.

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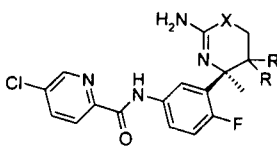
Scheme 5. Synthesis of Fluoro-oxazines 109–111^a



and an olefin³⁵ as the key step. Utilizing carefully optimized conditions, the nitrile oxide was slowly generated in situ from 2-chloro-2-hydroxy-imino ester **71** in a heterogeneous mixture of NaHCO₃ in ethyl acetate in the presence of 3,3,3-trifluoropropene (**72**) to give the 4,5-dihydro-isoxazole ester **73** in quantitative yield. Reduction of the ester **73** followed by fluorination of the alcohol **74** with Deoxo-Fluor furnished the desired fluoro compound **75**. 3-Methyl-4,5-dihydro-isoxazole derivative **76** was directly accessible by reacting nitroethane and phenyl isocyanate to generate the nitrile oxide in situ in the presence of **72**. Arylation of the imines **75** and **76** with 1-bromo-2-fluoro-benzene (**77**) and *n*-BuLi occurred from the sterically less hindered side to give the racemic isoxazolidines **78** and **79**, which were separated by chiral HPLC, affording the

The last set of CF₃-substituted oxazines 109–111 (Scheme 5), not accessible by methods hitherto described, again required a different synthetic strategy. Esters 90 and 39 were reduced with DIBALH at –76 °C to give the aldehydes 91 and 92, which were reacted with trimethyl(trifluoromethyl)silane (Ruppert–Prakash reagent), yielding the alcohols 93–96. The reaction of 91 afforded a separable 1:2-mixture of epimers 93 and 94, and

Table 1. In Vitro and in Vivo Profiles of Oxazines 1a and 14a and Thiazines 112 and 113



compd	X	R	pK_a^b	IC_{50} (μM) ^a			shift BACE1 enzyme vs cell-based	brain A β 40 reduction in mice at 30 mg/kg ^d (%)
				hBACE1 enzyme	hBACE2 enzyme	A β 40 cell-based ^c		
1a	O	H	9.8	0.137	0.052	0.010	14	29
14a	O	F	6.3	0.026	0.015	0.017	2	69
112	S	H	9.0	0.016	0.009	0.001	16	92
113	S	F	6.1	0.014	0.010	0.004	3	0

^a IC_{50} values are means of at least 2 independent experiments. ^b pK_a determined by capillary electrophoresis. ^cHEK293 cells transfected with wild-type human APP. ^dCompound given orally, $n = 3-4$, 4h post dose, mean reduction as compared to vehicle control.

aldehyde 92 gave a separable 2:1-mixture of epimers 95 and 96 with inverse selectivity. Conversion of alcohols 93, 95, and 96 to the target compounds 109–111 followed well-established procedures as described in previous Schemes. The relative and absolute configurations were assigned based on X-ray structures of 109–111 complexed to BACE1.

RESULTS AND DISCUSSION

At the outset of the BACE project, we envisaged both BACE1 and BACE2 as viable targets for AD and type 2 diabetes, respectively. The recently described oxazine 1a^{29,36,37} (for other 1,3-oxazine BACE1 inhibitors see^{26,27,38}) looked particularly suited for the diabetes target, which is primarily expressed in peripheral tissue. Compound 1a showed a high enzymatic inhibitory activity at BACE2 (Table 1, $IC_{50} = 0.052 \mu M$) and was hardly brain penetrant as evident from the low brain/plasma ratio in mice (0.13 at 30 mg/kg), resulting in a marginal activity of 29% reduction of A β 40 at 30 mg/kg in our wild-type mice model (vide infra) we use for the AD indication. An undesired potential liability of 1a, however, was the high basicity ($pK_a = 9.8$), giving rise to potential phospholipidosis³⁹ and polypharmacology.^{40,41} To overcome these liabilities, we introduced fluorines into the headgroup⁴² as depicted in difluoro-oxazine 14a.⁴³ Indeed, this modification reduced the pK_a by 3.5 log units compared to 1a and improved the enzymatic potency at BACE1 and BACE2 5- and 3-fold, respectively. Unexpectedly, however, 14a readily penetrated the brain (brain/plasma ratio in mice = 2.3 at 30 mg/kg) and therefore showed good in vivo activity in mice with a 69% reduction of A β 40 at 30 mg/kg. Obviously, this serendipitously found effect of fluorinated oxazines now rendered this compound class much more appropriate for the AD indication than type 2 diabetes.

For comparison, the thiazine 112²³ and its fluorine congener 113,³² both described by Shionogi, were also prepared. Interestingly, thiazine 112 was very potent in vivo (92% A β 40 reduction at 30 mg/kg), while the oxazine 1a was not. Most surprisingly, the fluorine congener 113, expected to be even more potent than 112, was inactive in vivo. Another remarkable difference was the impact of basicity on the shift between enzymatic and cellular BACE1 potency. Compounds 1a and 112 with high basicity ($pK_a \geq 9$) showed a significantly higher cellular activity (14- and 16-fold, respectively), whereas compounds 14a and 113 with low basicity (<6.5) did not (2

and 3-fold, respectively). The oxazine 14a served as a basis for further optimization of the in vivo activity.

SAR of R¹ Substituents. Next we explored various R¹ aromatic residues all having a nitrogen atom in *ortho* position to the amide group (Table 2) to direct the aromatic residue into the S3 pocket (vide infra).

Out of a number of 6-membered heterocycles explored, pyridines, pyrimidines, and pyrazines showed promising in vitro activities. Pyridines 14a–k having one substituent in *para* position to the amide showed enzymatic inhibition at IC_{50} s values below $0.3 \mu M$, most of them with good cellular activity. The nitrile 14d displayed already an improved in vivo activity compared to the chloride 14a with A β 40 reductions of 78 and 28%, respectively, at a dose of 10 mg/kg. In addition, favorable mouse PK data for 14d (Cl = 22 mL/min/kg, $T_{1/2} = 1.8$ h, Vss = 2.9 L/kg, $F = 52\%$) encouraged us to explore further substitution patterns. The *o*- and *p*-disubstituted pyridines 14l and 14m were virtually equipotent to the monosubstituted chloride 14a both in the enzymatic as well as the cell-based assay, but 14m showed a higher in vivo activity compared to 14a (77% vs 28% reduction of A β 40 at 10 mg/kg). The pyrimidine 14n was 3-fold less active in terms of enzymatic potency compared to the pyridine 14a. Pyrazines 14o–14r showed good enzymatic inhibition activity ($IC_{50} = 0.027-0.006 \mu M$), and examples 14o and 14p were clearly more potent in the enzyme/cell-based assays compared to their corresponding pyridine counterparts 14g (2/3-fold) and 14j (4/3-fold), respectively. Interestingly, the propynyloxy-pyrazine 14q was very potent in vivo (93% reduction of A β 40 at 10 mg/kg). Out of a number of 5-membered aromatic heterocycles explored, pyrazoles looked particularly promising. A first example, the methyl pyrazole 14s, was only moderately active in the enzyme/cell-based assays ($IC_{50} = 0.446/0.110 \mu M$) and showed very low metabolic stability in mouse microsomes (Cl = 200 μL /min/mg protein). Potential labile positions, i.e., the methyl group and position 4 of the pyrazole, were therefore blocked by fluorines and a chlorine substituent, respectively. As expected, metabolic stability in mouse microsomes improved considerably in 14t–v (Cl = 98, 59, and 9 μL /min/mg protein, respectively) and higher enzymatic potencies were also observed ($IC_{50} = 0.151, 0.036, \text{ and } 0.019 \mu M$, respectively). Disappointingly, however, the in vivo activity of 14v remained rather modest, with a 57% reduction of A β 40 at 30 mg/kg. The shift between enzyme and cell-based activity was in the majority of examples rather small (6-fold and less, with the exception of a 13-fold shift of the alkyne derivative 14q) and in general in

Table 2. SAR of R1 Substituents and in Vivo Properties of Difluoro-oxazines 14a–v

14a	14b	14c	14d	14e	14f	14g	
14h	14i	14j	14k	14l	14m	14n	
14o	14p	14q	14r	14s	14t	14u	
14v							
compd	IC ₅₀ (μM) ^a		selectivity BACE 2/1	IC ₅₀ (μM) ^a		shift BACE1 enzyme vs cell- based	brain Aβ40 reduction in mice at 30/10 mg/kg ^c (%)
	hBACE1 enzyme	hBACE2 enzyme		Aβ40 cell-based ^b			
14a	0.026	0.015	0.6	0.017	2	69/28	
14b	0.294	0.036	0.1	0.230	1		
14c	0.066	0.022	0.3	0.013	5		
14d	0.023	0.024	1	0.021	1	84/78	
14e	0.087	0.030	0.3	0.087	1		
14f	0.032	0.047	1	0.024	1	27/–	
14g	0.051	0.045	1	0.061	1	64/–	
14h	0.057	0.245	4	0.054	1	64/–	
14i	0.184	0.904	5	0.480	–3		
14j	0.108	7.490	69	0.290	–3		
14k	0.042	9.440	225	0.100	–2		
14l	0.017	0.010	0.6	0.016	1		
14m	0.026	0.012	0.5	0.013	2	91/77	
14n	0.067	0.023	0.3	0.023	3		
14o	0.027	0.119	4	0.022	1	95/66	
14p	0.025	0.943	38	0.110	–4		
14q	0.013	0.175	13	0.001	13	98/93	
14r	0.006	0.101	17	0.001	6	94/28	
14s	0.446	0.106	0.2	0.110	4		
14t	0.151	0.031	0.2	0.131	1		
14u	0.036	0.018	0.5	0.017	2		
14v	0.019	0.011	0.6	0.013	1	57/–	

^aIC₅₀ values are means of at least two independent experiments. ^bHEK293 cells transfected with wild-type human APP. ^cCompound given orally, *n* = 3–4, 4 h post dose, mean reduction as compared to vehicle control.

favor of higher cellular activity. The (fluoro)alkoxy derivatives 14i–14k and 14p, however, were found to have “negative” shifts (2–4-fold) in favor of higher enzymatic potencies, clearly demonstrating that not only the p*K*_a but also the nature of R¹ substituents had an impact on the shift.

X-ray Structures and BACE2 Selectivity. The catalytic aspartate residues D93 and D289 form the central recognition motif in the BACE1 binding site (Figure 2). The active site is partially covered by a flexible hairpin loop commonly referred to as the “flap” (Y129–E138).⁴⁴ In apo structures of BACE1 or in complexes with small molecule inhibitors containing an amidine binding motif, the flap backbone is approximately 12 Å away from the side-chains of D93 and D289 (the “open” position). In complexes with peptidomimetic transition state analogues, the flap moves approximately 4 Å closer to the

binding site (the “closed” position).⁴⁴ There is an additional flexible region, the “10s loop”⁴⁴ (G69–G74), forming one side of the S3 pocket. In BACE1, the 10s loop is either in an “up” or “down” conformation,⁴⁵ whereas in BACE2 the 10s loop has been observed in a down conformation by Ostermann et al.⁴⁶ and by ourselves in the complex with 14i (Figure 3). The S3 pocket is much larger in the up conformation, so this difference can be exploited to modulate BACE1/2 selectivity as well as selectivity against other single chain aspartyl proteases.⁴⁴ The binding pocket in S1 and the region at the catalytic aspartates are almost identical between BACE1 and BACE2. Conformational variation around the active site of BACE1 has recently been discussed in detail by Xu et al.;⁴⁷ we will analyze BACE2 flexibility in detail separately.⁴⁸

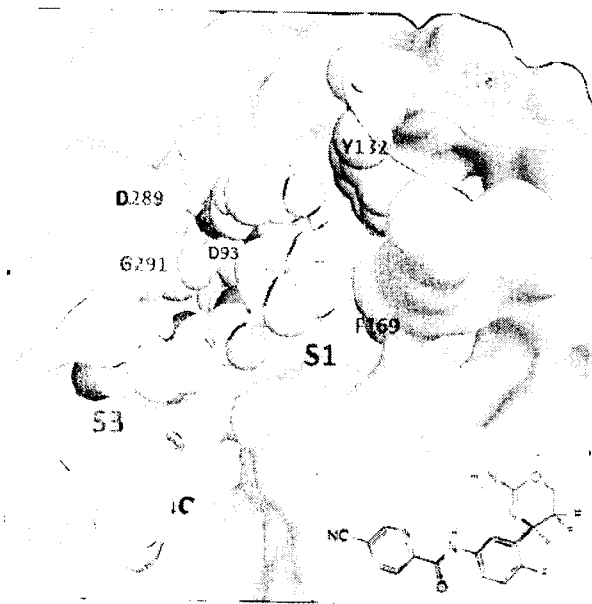


Figure 2. 14d bound to the BACE1 binding site. The amidine recognition motif is tightly bound to the catalytic aspartates D289 and D93 (partially hidden) in the bottom of the pocket. The fluorine atoms in the headgroup and the *o*-F substituent of the phenyl ring (marked in green) form a network of vdW interactions to Y132 and to F169. An additional H-bond is established between the NH of the amide bond of the ligand and the backbone carbonyl oxygen of G291. The *para* nitrile substituent displaces bound water molecules from the S3 pocket.



Figure 3. 14i bound to BACE1 (yellow, ball and stick representation) and BACE2 (magenta, stick representation). The molecular surface has been generated for BACE1, and the 10s loops of BACE1 and BACE2 are depicted separately. The ethoxy side chain in 14i prefers a planar orientation around the C–O bond. When bound to BACE2, the corresponding torsion angle deviates significantly from planarity because the S3 pocket in BACE2 is tighter than in BACE1 due to the “down” conformation of the 10s loop in BACE2.

In Figure 2, the topology of the BACE1 active site bound to 14d is highlighted. Tight hydrogen bonds are formed between

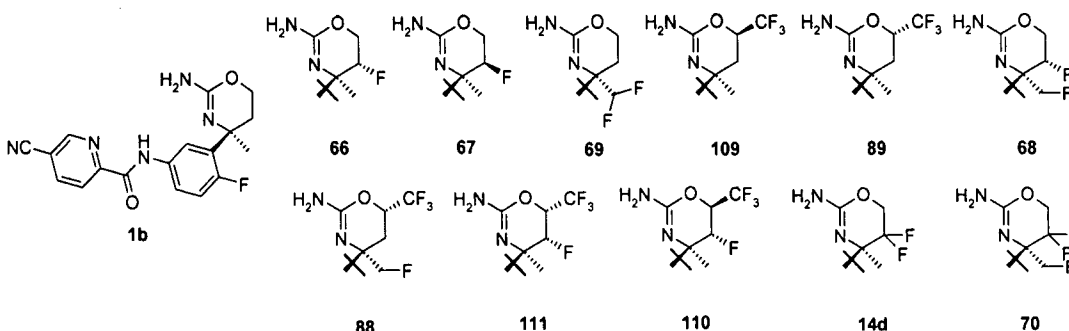
the protonated amidine motif in the oxazine headgroup and the catalytic aspartates. The fluorine atoms in position 5 and the *o*-F substituent of the phenyl ring (S1 pocket) are engaged in a network of van der Waals interactions to Y132 located in the flap and to F169 at the rim of the S1 pocket. A hydrogen bond is formed between the NH of the amide bond in 14d and the backbone carbonyl oxygen of G291. The *para* nitrile substituent projects into the S3 pocket and displaces bound water molecules seen in the apo structure, thereby enhancing binding affinity. The proper orientation in the S3 pocket is supported by a stabilization of the conformation of the pyridine ring via a favorable electrostatic interaction between the pyridyl N and the NH of the amide bond in 14d.

The role of the 10s loop in modulating BACE1/2 selectivity can be seen in the ratios of inhibition constants of the compounds 14g–k (Table 2). As is evident in Figure 3, the S3 pocket in BACE2 is smaller than in BACE1 because the 10s loop in BACE2 is observed here in a down conformation. The IC₅₀ ratio between BACE2 and BACE1 increases with the growing chain length of the S3 alkyl substituent. Longer chain lengths can only be accommodated in the BACE2 S3 pocket by adopting an unfavorable ligand conformation as depicted in Figure 3. The ethoxy side chain in 14i prefers a planar orientation around the C–O bond. When bound to BACE2, the corresponding torsion angle deviates significantly from planarity, and this conformational strain translates into a reduced inhibition of BACE2. A similar rationale applies for the increase in selectivity with an increasing number of F substituents. To avoid repulsive contacts between F-substituents and carbonyl oxygen atoms of the protein, the tighter S3 pocket in BACE2 requires more strained conformations of the S3 substituent than the more spacious pocket in BACE1.

Fluorine Scan of Oxazines. From various R1 aromatic residues explored, we selected the nitrile 14d for further optimization based on its balanced profile, i.e., good in vitro activities, no shift between enzymatic and cell-based assay, and a favorable in vivo activity. The crucial role of the pK_a on the pharmacological profile as found in the difluoro-oxazine 14a prompted us to investigate extensively the impact of fluorines on various parameters. Oxazines with fluorines appended at different positions in the headgroup (Table 3) were prepared spanning a pK_a range of almost five log units. The head groups in Table 3 are ordered according to decreasing pK_a values. Below we discuss the impact of fluorines on the pK_a, the lipophilicity, the enzymatic potency, the shift of enzyme vs cell-based activity, P-gp efflux, and hERG inhibition, most of these parameters eventually affecting in vivo activity.

The configuration of a fluorine atom attached directly to the oxazine ring has a strong impact on the pK_a. Thus, the “down” configured fluorine in 66 showed a pK_a decrement of 1.6 (Δ pK_a of 1b and 66), whereas the “up” configured fluorine in 67 induced a larger pK_a decrement of 2.3. Similar effects were also observed in fluoro-piperidines.^{49,50} The difluoro oxazine 14d showed exactly the sum of both contributions, i.e., a pK_a decrement of 3.9. The two fluorines in compound 69,^{37,51} also located at the β -position to the ring nitrogen, induced a lower pK_a decrement of 2.4 compared to those of difluoro oxazine 14d. It appears that fluorines with limited conformational flexibility as present in 14d induced a larger pK_a decrement compared to those in 69 having more flexibility. The CF₃ group in compounds 109 and 89 still showed a remarkably large pK_a decrement of 2.4 and 2.7, respectively, despite the longer distances through which the fluorines exert

Table 3. Head Group Fluorine Scan and in Vitro/in Vivo Properties of Fluorinated Oxazines



compd	pK _a ^b	ΔpK _a to 1b	log D ^c	log P	Δlog D/log P	hBACE1 enzyme	Aβ40 cell-based ^d	shift BACE1 enzyme vs cell-based	P-gp ER ^e	hERG inhibition at 10 μM (%)	brain Aβ40 reduction in mice at 10/3/1 mg/kg ^f (%)
1b	9.7	0	0.0	2.3	2.3	0.051	0.014	4	14.8	88	13/–/–
66	8.1	–1.6	1.1	1.9	0.8	0.028	0.001	28	4.9	85	89/43/–
67	7.4	–2.3	1.3	1.6	0.3	0.435	0.027	16	2.1	91	2/–/–
69	7.3	–2.4	1.7	1.9	0.2	0.054	0.009	6	1.7	85	68/34/–
109	7.3	–2.4	2.8	3.1	0.3	0.776	0.230	3			
89	7.0	–2.7	2.8	2.9	0.1	0.012	0.002	6	1.9	75	95/88/78
68	6.7	–3.0	1.6	1.7	0.1	0.077	0.025	3	2.6	65	55/26/–
88	6.3	–3.4	3.0	3.0	0	0.019	0.013	1	1.6	39	88/64/11
111	5.9	–3.8	3.1	3.1	0	0.013	0.010	1	1.5	48	93/71/41
110	5.8	–3.9	3.1	3.1	0	0.040	0.150	–4	1.5		
14d	5.8	–3.9	2.5	2.5	0	0.023	0.021	1	1.5	43	78/39/4
70	5.1	–4.6	2.3	2.3	0	0.049	0.400	–8	1.6	14	

^aIC₅₀ values are means of at least two independent experiments. ^bpK_a determined by capillary electrophoresis. ^clog D determined in 1-octanol/phosphate buffer, pH = 7.4. ^dHEK293 cells transfected with wild-type human APP. ^eER = efflux ratio in LLC-PK1 cells stably expressing human MDR1. ^fCompound given orally, n = 3–4, 4 h post dose, mean reduction as compared to vehicle control.

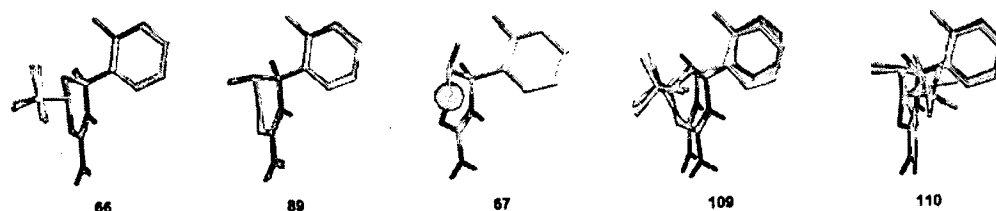


Figure 4. X-ray structures (yellow) of fluorinated head groups with the S1 phenyl ring were energy minimized in the unbound state by a semiempirical geometry optimization with the PM3 Hamiltonian (magenta). The coordinates of 66 and 89 remain almost unchanged as compared to the bound conformation. Therefore, the oxazine rings are without conformational strain. The geometry optimization of unbound 109 and 110 resulted in different ring puckers, indicating that those ligands are not in their lowest energy conformations. For 67, the position of the marked C-atom cannot be clearly assigned because of weak electron density. This indicates the presence of multiple ring conformations.

their impact on the nitrogens. This observation can be rationalized based on the equidistance of the fluorines to both nitrogens in the δ-position having an impact not just on one but on both nitrogens of the amidine moiety. Fluorines located at more than one position (as in 68, 88, 111, 110, and 70⁵¹) showed a more complex picture, with pK_a decrements not being additive.

The lipophilicity of compounds having 1–3 fluorines attached to the headgroup is reduced or similar (log P = 1.6–2.5 in 66–70 and 14d) compared to the nonfluorinated oxazine headgroup 1b (log P = 2.3). Compounds substituted by a CF₃ group showed increased lipophilicity (log P = 2.9–3.1 in 88, 89, 109–111) compared to 1b. As expected, the Δlog P/D approaches a value of 0 at a pK_a of 6.3.

The enzymatic potency was strongly affected by fluorines introduced into the oxazine headgroup. A down configured CF₃

group in 89 improved the binding affinity considerably (IC₅₀ = 0.012 μM) compared to unsubstituted 1b (IC₅₀ = 0.051 μM). According to the X-ray structure, the CF₃ group does not interact with BACE1 but points toward the solvent. A possible rationale for this unexpected increase in binding affinity might be the pK_a of 89, which is 2.7 log units lower than for 1b. Therefore, 1b should be more strongly solvated than 89 due to its higher basicity. The lower binding affinity of 1b compared to 89 could be rationalized by the higher desolvation enthalpy upon binding to the active site. The configurations of the fluorine in 66 and the CF₃ group in 89 strongly affected the binding affinity, which increased 16-fold and 65-fold compared to their epimers 67 and 109, respectively. This effect can be rationalized by conformational strain of the head groups. The head groups of the X-ray structures were submitted to a semiempirical geometry optimization⁵² (PM3 Hamiltonian)

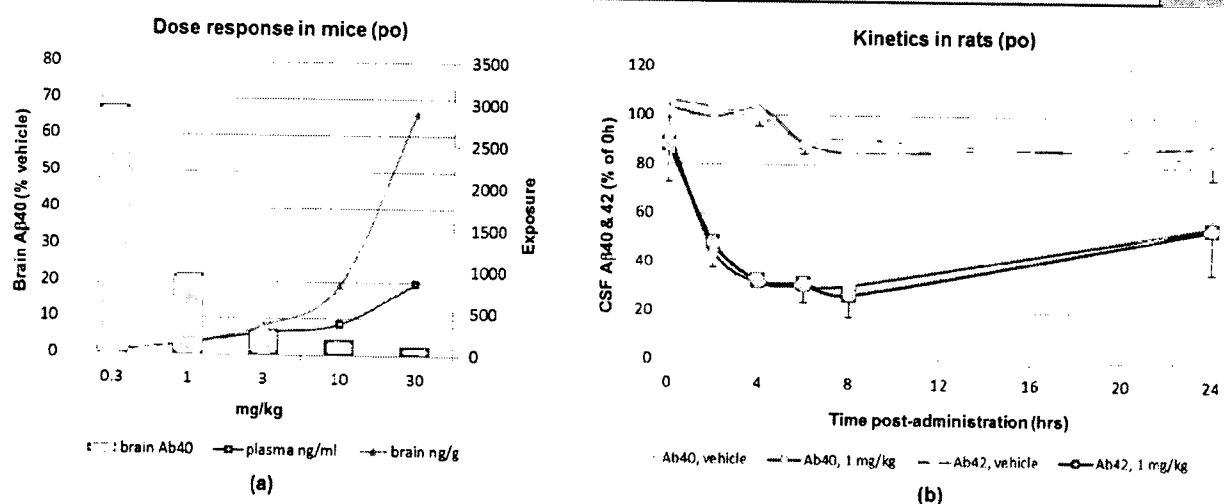


Figure 5. (a) Dose dependent inhibition of brain Aβ40 production in mice by compound 89 determined 4 h oral post dose, $n = 3-4$, mean reduction as compared to vehicle control. (b) Time dependent inhibition of CSF Aβ40/42 production in rats after an oral dose of 1 mg/kg of compound 89, $n = 2-3$. CSF was sampled at -1 and 0 h to define an average Aβ40/42 level for each individual rat; all post-treatment Aβ-levels were expressed as percentage of this pretreatment average.

outside the binding pocket (unbound geometry). As shown in Figure 4, the coordinates of bound as well as unbound 66 and 89 remain almost unchanged, therefore, the oxazine rings are without conformational strain. The geometry optimization of 109 resulted in a different ring pucker, indicating that the bound ligand is not in its lowest energy conformation. This conformational strain results in a reduced binding affinity. The geometry optimization of 110 also yields a strained conformation in the bound state. However, the effect on binding affinity is much lower than expected. A reason for this result might be the involvement of the F-substituent in the network of van der Waals interactions involving Y132, which could overcompensate the conformational energy penalty. For 67, the position of the methylene carbon next to the oxazine oxygen atom cannot be clearly assigned because of weak electron density. This is probably due to the presence of multiple ring conformations, indicating that 67 is not well bound in the active site.

The shift of cellular vs enzymatic activity depends on the pK_a : basic compounds with a $pK_a \geq 7$ show a trend for higher activity in the cell-based assay, whereas oxazines with a $pK_a < 7$ are more balanced or can even revert to have a higher potency in the enzymatic assay.⁵³ BACE1 inhibitors with enhanced basicity have been associated with increased cellular potency by favoring the acidic intracellular compartment where BACE1 is located.⁵⁴

P-gp mediated efflux is clearly correlated with the pK_a .⁵⁵ Lowering of the pK_a from 9.7 to 8.1 to 7.4 in compounds 1b, 66, and 67 resulted in a continuous reduction of the efflux ratio from 15 to 5 to 2, respectively, which remained low at a $pK_a < 7.4$. There was in general no species differences between human and mouse P-gp efflux, e.g., for compound 89 (efflux ratio = 1.9/1.9, respectively) and compound 14d (efflux ratio = 1.5/2.3, respectively).

hERG inhibition is strongly affected by the pK_a .⁵⁶ Reduction of the pK_a from 9.7 to 5.1 correlated well with hERG inhibition, which declined from 88% to 14% at 10 μ M.

The low in vivo activity of compound 1b (13% reduction of Aβ40 at 10 mg/kg) is now better understood because of the high P-gp efflux ratio of 15. Attachment of one fluorine in the

relaxed down configuration in compound 66 boosted the cellular activity 14-fold and reduced P-gp efflux 3-fold as compared to 1b, resulting in good in vivo activity (43% reduction of Aβ40 at a lower dose of 3 mg/kg). The cellular activity of compounds 88 and 111 ($IC_{50} = 0.013$ and 0.010 μ M) was lower compared to 66 ($IC_{50} = 0.001$ μ M) but the 3-fold lower P-gp efflux of 1.6/1.5 may account for the higher in vivo activity (64 and 71% reduction of Aβ40 at 3 mg/kg) compared to 66. Most gratifyingly, the CF₃ substituted oxazine 89 with combined favorable properties (cellular $IC_{50} = 0.002$ μ M, P-gp efflux of 1.9) was most potent showing a reduction of Aβ40 of 78% at a dose as low as 1 mg/kg.

In Vivo Pharmacokinetics (PK) and Pharmacodynamics (PD) of Oxazine 89. The oxazine 89 was selected for further investigation of pharmacokinetic and dynamic parameters. Aβ40 lowering was determined after oral administration in wild-type mice (C57Bl/6J mice, $n = 3-4$) 4 h post dosing. Wild-type mice were used as they have a normal physiological level of APP expression and are thus expected to provide a better physiological model for Aβ-peptide production than APP-transgenic organisms. Compound 89 showed a dose-dependent inhibition of Aβ40 production in the brain of mice (Figure 5a) with a brain/plasma ratio of 1–1.5 between 0.3 and 3 mg/kg, gradually increasing to 2.3 and 3.4 at the higher doses of 10 and 30 mg/kg. In a rat PK experiment (Table 4), oxazine 89 showed a low to moderate systemic plasma clearance (16.8 mL/min/kg), a high volume of distribution (11.5 L/kg), and a long terminal half-life time (9–11 h). This, together with a good oral bioavailability (68%) and a moderately high fraction

Table 4. Pharmacokinetic Properties of Oxazine 89 in Rats^a

dose (mg/kg)	AUC _{inf} (ng·h/mL)	Cl (mL/min/kg)	Vd _{ss} (L/kg)	t _{1/2} (h)	F (%)	Fu ^c (%)
1.2 mg iv ^b	1195	16.8	11.5	8.9		
5.2 mg po ^b	3520			10.9	68	20.5

^aAll values given are means of two experiments. ^bpo: microsuspension of 89 in gelatin/aqueous NaCl. iv: solution of 89 in aqueous NaCl/N-methyl-2-pyrrolidone. ^cFu = fraction unbound.

unbound (20.5% in plasma), would predict a favorable PD effect in rats.

Wild-type Wistar rats were used with a cannula inserted into the cisterna magna, and the kinetics of changes in the CSF A β 40/42 in an acute treatment study was followed.⁵⁷ An oral dose as low as 1 mg/kg (Figure 5b) was sufficient to induce a long lasting effect, with CSF levels remaining most inhibited 4 h post dosing and still showing a significant reduction of A β 40/42 after 24 h as a result of favorable PK properties.

The changes in plasma A β 40/42 (Figure 5b) were analyzed in relation to the measured plasma exposure in rats (Table 4). For the modeling of the plasma exposure data, a one-compartment model with first-order drug absorption and elimination was considered. For the modeling of A β 40/42 changes in plasma, an indirect response model was considered with the zero-order production rate being modulated by the pharmacological effects of the oxazine 89. The proposed model fitted the data well, and the in vivo potency was estimated to be 0.020 μ M free, which compares favorably to the in vitro measurement of IC₅₀ = 0.012 μ M in the hBACE1 enzyme assay. Thus, it can be concluded that the free plasma concentration of 89 equals the free brain concentration, which can be considered as the pharmacological relevant concentration.

In terms of its in vitro toxicological profile, compound 89 did not inhibit CYP₄₅₀ 3A4, 2D6, and 2C9 (IC₅₀ > 25 μ M) nor was there a time-dependent inhibition of CYP₄₅₀ 3A4. Furthermore, 89 was very selective against other aspartyl proteases such as human cathepsin D/E and the peptidases renin and pepsin (IC₅₀ > 200 μ M).

SUMMARY AND CONCLUSION

Starting from a very basic, in vivo marginally active 1,3-oxazine 1a, the introduction of two fluorine atoms into the headgroup reduced the pK_a considerably and led unexpectedly to good in vivo activity, as found in compound 14a. Encouraged by this finding, we performed an extensive fluorine scan to study the impact of fluorine(s) on the pK_a and a number of other parameters. Lowering the pK_a had a profound effect on enzymatic and cellular activity, hERG activity, P-gp efflux, and eventually on in vivo activity. The knowledge acquired by the fluorine headgroup scan delivered crucial information on how to improve in vivo efficacy and reduce in vitro toxicological liabilities. The CF₃-substituted oxazine 89 was found to be an orally active BACE1 inhibitor with an excellent PD profile in mice and a long lasting A β 40/42 reduction in the CSF of rats at a dose as low as 1 mg/kg. A compound from this chemical class is currently undergoing clinical evaluation for the treatment of Alzheimer's Disease.

EXPERIMENTAL SECTION

General. All solvents and reagents were obtained from commercial sources and were used as received. All reactions were followed by TLC (TLC plates F254, Merck) or LCMS (liquid chromatography–mass spectrometry) analysis. Silica gel chromatography was either performed using cartridges packed with silica gel (ISOLUTE Columns, TELOS Flash Columns) or silica-NH₂ gel (TELOS Flash NH₂ Columns) on ISCO Combi Flash Companion or on glass columns on silica gel 60 (32–60 mesh, 60 Å). Proton NMR spectra were obtained on Bruker Avance 300, 400, or 600 MHz spectrometer with chemical shifts (δ in ppm) reported relative to tetramethylsilane or the residual solvent peak as the internal reference (i.e., CDCl₃ = 7.26 ppm, DMSO-*d*₆ = 2.50 ppm) as the internal reference. ¹H resonances are reported to the nearest 0.01 ppm. NMR abbreviations are as follows: s,

singlet; d, doublet; t, triplet; q, quadruplet; quint, quintuplet; sext, sextuplet; m, multiplet; br, broadened. Coupling constants (*J*) are reported to the nearest 0.1 Hz. Purity was analyzed by reverse phase HPLC and for specific compounds by elemental analysis. HPLC was performed on Finnigan LTQ (Thermo Fisher Scientific) and Agilent RRLC 1200 equipment. Column: Agilent XDB C15, 30 mm $\times$ 4.6 mm, 3.5 μ m. Analytical conditions: gradient used, 5–95% acetonitrile in water containing 0.1% trifluoroacetic acid in 3 min. Flow: 4.5 mL/min. UV-detector: DAD 190–400 nm. Sample solvent: in water/acetonitrile (8/2). The UV detection was an averaged signal from wavelengths of 190–400 nm. Elemental analyses were performed by Solvias AG (Mattenstrasse, Postfach, CH-4002 Basel, Switzerland). Mass spectra were recorded on an SSQ 7000 (Finnigan-MAT) spectrometer for electron impact ionization. The purities of final test compounds as measured by HPLC were found to be above 95%. Melting points were determined on a Büchi melting point B-540: heating rate 1 °C/min starting 15 °C below melting point. LC-HRMS spectra were recorded with an Agilent LC-system consisting of an Agilent 1290 high pressure system, a CTC PAL auto sampler, and an Agilent 6520 QTOF. The separation was achieved on a Zorbax Eclipse Plus C18 1.7 μ m 2.1 mm $\times$ 30 mm column at 55 °C; eluent A = 0.01% formic acid in water; B = 0.01% formic acid in acetonitrile:2-propanol (8:2); flow, 1 mL/min. Gradient: 0 min 5% of B, 0.3 min 5% of B, 4.5 min 99% of B, 5 min 99% of B. The injection volume was 2 μ L. All assay compounds had a purity of >95%. LC-MS (ESI, positive or negative ion) data were recorded on Waters UPLC-MS Systems equipped with Waters Acquity, a CTC PAL auto sampler, and a Waters SQD single quadrupole mass spectrometer using ES ionization modes (positive and/or negative). The separation was achieved on a Zorbax Eclipse Plus C18 1.7 μ m 2.1 mm $\times$ 30 mm column at 50 °C; A = 0.01% formic acid in water, B = acetonitrile at flow 1; gradient: 0 min 3%B, 0.2 min 3%B, 2 min 97%B, 1.7 min 97%B, 2.0 min 97%B. The injection volume was 2 μ L. MS (ESI, positive or negative ion): FIA (flow injection analysis)-MS were recorded on a Applied Biosystem API150 mass spectrometer. Sample introduction was made with a CTC PAL auto sampler and a Shimadzu LC-10ADVP pump. The samples were directly flushed to the ESI source of the mass spectrometer with a flow 50 μ L/min of a mixture of acetonitrile and 10 mM ammonium acetate (1:1) without a column. The injection volume was 2 μ L.

(*R*)-2-Methyl-propane-2-sulfinic Acid [1-(2-Fluoro-5-nitro-phenyl)-(E)-ethylidene]-amide (3).³² To a solution of the (*R*)-(+)-*tert*-butylsulfonamide (10.88 g, 89.8 mmol) in THF (400 mL) was added subsequently 1-(2-fluoro-5-nitro-phenyl)-ethanone (2) (16.45 g, 89.8 mmol) and titanium(IV)ethoxide (40.70 g, 178.4 mmol), and the solution was stirred at reflux temperature for 5 h. The mixture was cooled to 22 °C and treated with brine (400 mL), and the suspension was stirred for 10 min and filtered over Dicalite. The layers were separated, the aqueous layer was extracted with EtOAc, and the combined organic layers were washed with water, dried, and evaporated. The residue was purified by flash column chromatography on silica gel using a mixture of cyclohexane/EtOAc (2:1) to give the title compound 3 (21.56 g, 84%) as a pale-yellow solid: mp 83–85 °C. ¹H NMR (600 MHz, CDCl₃) δ ppm 8.57 (dd, *J* = 6.3, 2.9 Hz, 1 H), 8.33 (ddd, *J* = 9.0, 3.2, 3.1 Hz, 1 H), 7.30 (t, *J* = 9.5 Hz, 1 H), 2.82 (d, *J* = 3.5 Hz, 3 H), 1.34 (s, 9 H). MS (ISP): *m/z* = 287.0 [*M* + *H*]⁺. Anal. (C₁₂H₁₃FN₂O₃S) Calcd: C 50.34%, H 5.28%, N 9.78%, S 11.20%. Found: C 50.49%, H 5.02%, N 9.94%, S 11.31%.

(*R*)-2,2-Difluoro-3-(2-fluoro-5-nitro-phenyl)-3-((*R*)-2-methyl-propane-2-sulfinylamino)-butyric Acid Ethyl Ester (5).³² In a dry apparatus, a suspension of freshly activated zinc powder (37.5 g, 574 mmol) and CuCl (5.99 g, 60.5 mmol) in dry THF (100 mL) was heated under an inert atmosphere to reflux for 30 min. The suspension was cooled to 22 °C and treated with a solution of ethyl 2-bromo-2,2-difluoroacetate (30.7 g, 151 mmol) in dry THF (50 mL) over 1 h, and stirring was continued for 30 min. The black mixture was treated with a solution of (*R*)-2-methyl-propane-2-sulfinic acid [1-(2-fluoro-5-nitro-phenyl)-(E)-ethylidene]-amide (3) (17.5 g, 60.5 mmol) in THF (50 mL), and stirring was continued at 22 °C for 2 h. The suspension was filtered through Dicalite and washed with *t*-butyl methyl ether. The

combined filtrates were subsequently washed with aqueous citric acid (5%) and aqueous saturated NaHCO_3 solution, the organic layer was dried over Na_2SO_4 and filtered, and the filtrate was evaporated. The residue was purified by flash column chromatography on silica gel using a mixture of *n*-heptane/EtOAc (2:1) to give the title compound **5** (17.24 g, 69%) as a yellow oil. ^1H NMR (300 MHz, CDCl_3) δ ppm 8.51 (dd, $J = 6.6, 2.7$ Hz, 1 H), 8.29 (ddd, $J = 9.1, 3.8, 3.0$ Hz, 1 H), 7.26 (dd, $J = 11.3, 9.1$ Hz, 1 H), 4.72 (s, 1 H), 4.34 (qd, $J = 7.2, 1.5$ Hz, 2 H), 2.09–2.17 (m, 3 H), 1.32 (t, $J = 7.3$ Hz, 3 H), 1.32 (s, 9 H). MS (ISP): $m/z = 411.2$ [$M + H$] $^+$. Anal. ($\text{C}_{16}\text{H}_{21}\text{F}_3\text{N}_2\text{O}_5\text{S}$) Calcd: C 46.82%, H 5.16%, N 6.83%, S 7.81%. Found: C 46.73%, H 4.89%, N 6.81%, S 7.66%. The configuration of the newly introduced stereogenic center was confirmed by X-ray crystallographic analysis of the target compound **14d** complexed with BACE1 (3ZMG).

(R)-2-Methyl-propane-2-sulfinic Acid [(R)-2,2-Difluoro-1-(2-fluoro-5-nitro-phenyl)-3-hydroxy-1-methyl-propyl]-amide (7).³² To a solution of (R)-2,2-difluoro-3-(2-fluoro-5-nitro-phenyl)-3-((R)-2-methyl-propane-2-sulfinylamino)-butyric acid ethyl ester (**5**) (3.78 g, 9.2 mmol) in dry THF (24 mL) was added at 0 °C lithium borohydride (9.0 mmol), and stirring was continued at 0 °C for 1.5 h. The reaction mixture was quenched by addition of acetic acid (1 mL) and water, the mixture was extracted with EtOAc, and the organic layers were washed with brine, dried over Na_2SO_4 , and evaporated. The residue was purified by flash column chromatography on silica gel using a mixture of *n*-heptane/EtOAc (3:2) to give the title compound **7** (2.90 g, 86%) as a pale-yellow solid. ^1H NMR (600 MHz, CDCl_3) δ ppm 8.46 (dd, $J = 6.8, 2.8$ Hz, 1 H), 8.25 (ddd, $J = 9.0, 3.7, 2.9$ Hz, 1 H), 7.25 (dd, $J = 11.7, 9.0$ Hz, 1 H), 5.36 (br s, 1 H), 4.51–5.03 (br s, 1 H), 4.21 (ddd, $J = 25.0, 13.4, 2.7$ Hz, 1 H), 3.84 (td, $J = 12.8, 7.9$ Hz, 1 H), 2.19 (s, 3 H), 1.31 (s, 9 H). MS (ISP): $m/z = 369.0$ [$M + H$] $^+$. A small sample was recrystallized from EtOAc/*n*-heptane: mp 154–155 °C. Anal. ($\text{C}_{14}\text{H}_{19}\text{F}_3\text{N}_2\text{O}_4\text{S}$) Calcd: C 45.65%, H 5.20%, N 7.60%, S 8.70%. Found: C 45.60%, H 5.07%, N 7.79%, S 8.78%.

(R)-3-Amino-2,2-difluoro-3-(2-fluoro-5-nitro-phenyl)-butan-1-ol (9).³² A solution of (R)-2-methyl-propane-2-sulfinic acid [(R)-2,2-difluoro-1-(2-fluoro-5-nitro-phenyl)-3-hydroxy-1-methyl-propyl]-amide (**7**) (3.79 g, 10.3 mmol) in MeOH (50 mL) was treated with a solution of HCl in 1,4-dioxane (4 M, 13 mL), and stirring was continued at 22 °C for 1 h. The mixture was evaporated, the residue was partitioned between EtOAc and aqueous Na_2CO_3 solution (2 M), and the organic layers were washed with brine, dried over Na_2SO_4 , filtered, and evaporated to give the crude title compound **9** (2.50 g, 92%) as a pale-yellow solid, which was used without further purification. ^1H NMR (600 MHz, CDCl_3) δ ppm 8.49 (dd, $J = 7.0, 2.8$ Hz, 1 H), 8.25 (ddd, $J = 9.0, 3.8, 2.9$ Hz, 1 H), 7.25 (dd, $J = 11.5, 9.0$ Hz, 1 H), 4.02 (ddd, $J = 19.7, 12.7, 8.6$ Hz, 1 H), 3.86 (dd, $J = 23.5, 11.8$ Hz, 1 H), 2.01–3.63 (3 H), 1.85 (d, $J = 0.9$ Hz, 3 H). MS (ISP): $m/z = 265.1$ [$M + H$] $^+$. A small sample was triturated with 2-propanol: mp 110–111 °C. Anal. ($\text{C}_{10}\text{H}_{11}\text{F}_3\text{N}_2\text{O}_3$) Calcd: C 45.46%, H 4.20%, N 10.60%. Found: C 45.67%, H 4.20%, N 11.03%.

(R)-5,5-Difluoro-4-(2-fluoro-5-nitro-phenyl)-4-methyl-5,6-dihydro-4H-[1,3]oxazin-2-ylamine (11).³³ To a solution of (R)-3-amino-2,2-difluoro-3-(2-fluoro-5-nitro-phenyl)-butan-1-ol (**9**) (1.5 g, 5.7 mmol) in EtOH (30 mL) at 22 °C was added under argon cyanogen bromide (0.91 g, 8.6 mmol), and the light-yellow solution was stirred in a sealed tube at 85 °C for 24 h. The mixture was cooled to 22 °C, evaporated, and the residue partitioned between EtOAc and saturated aqueous Na_2CO_3 solution. The organic layers were dried over Na_2SO_4 , filtered, and evaporated, and the residue was purified by flash column chromatography on silica gel using a mixture of *n*-heptane/EtOAc (5:2) to afford the title compound **11** (1.30 g, 79%) as a pale-yellow solid. ^1H NMR (600 MHz, CDCl_3) δ ppm 8.59 (dd, $J = 6.6, 2.9$ Hz, 1 H), 8.21 (ddd, $J = 9.0, 3.8, 3.0$ Hz, 1 H), 7.20 (dd, $J = 10.6, 8.9$ Hz, 1 H), 4.25–4.45 (2 H), 4.27 (ddd, $J = 23.2, 11.8, 3.8$ Hz, 1 H), 4.06 (ddd, $J = 11.7, 10.4, 8.2$ Hz, 1 H), 1.79 (t, $J = 2.3$ Hz, 3 H). MS (ISP): $m/z = 290.2$ [$M + H$] $^+$. A small sample was recrystallized from EtOAc/*n*-heptane: mp 134–135 °C. Anal. ($\text{C}_{11}\text{H}_{10}\text{F}_3\text{N}_3\text{O}_3$) Calcd: C 45.68%, H 3.49%, N 14.53%. Found: C 45.63%, H 3.41%, N 14.65%.

(R)-4-(5-Amino-2-fluoro-phenyl)-5,5-difluoro-4-methyl-5,6-dihydro-4H-[1,3]oxazin-2-ylamine (13).⁴³ To a solution of (R)-5,5-difluoro-4-(2-fluoro-5-nitro-phenyl)-4-methyl-5,6-dihydro-4H-[1,3]oxazin-2-ylamine **11** (1.29 g, 4.47 mmol) in EtOH (35 mL) was added at 22 °C under an inert atmosphere palladium on carbon (10% Pd, 238 mg), and the mixture was stirred under a hydrogen atmosphere (balloon) at 22 °C for 1 h. The catalyst was filtered off and washed twice with EtOH, and the solvent was removed under reduced pressure to give the crude title compound **15** (1.14 g, 98%) as a colorless foam which was used without further purification. ^1H NMR (600 MHz, CDCl_3) δ ppm 6.85 (dd, $J = 11.7, 8.6$ Hz, 1 H), 6.79 (dd, $J = 6.5, 3.0$ Hz, 1 H), 6.58 (ddd, $J = 8.6, 3.2, 3.1$ Hz, 1 H), 4.10–4.90 (br s, 2 H), 4.10 (ddd, $J = 13.2, 11.5, 7.5$ Hz, 1 H), 3.99 (ddd, $J = 18.2, 11.6, 5.9$ Hz, 1 H), 3.55 (br s, 2 H), 1.75 (t, $J = 2.3$ Hz, 3 H). MS (ISP): $m/z = 260.1$ [$M + H$] $^+$. A small sample was purified by flash column chromatography on silica gel using a mixture of dichloromethane/methanol (19:1) containing 1% of ammonium hydroxide, followed by crystallization from *tert*-butylmethyl ether: mp 122–123 °C. Anal. ($\text{C}_{11}\text{H}_{12}\text{F}_3\text{N}_3\text{O}$) Calcd: C 50.97%, H 4.67%, N 16.21%. Found: C 51.03%, H 4.52%, N 16.34%.

General Procedure for the Synthesis of the Amides 14a and 14d. To a solution of the carboxylic acid (0.23 mmol) in MeOH (5 mL) at 0 °C was added 4-(4,6-dimethoxy[1.3.5]triazin-2-yl)-4-methylmorpholinium chloride hydrate (DMTMM) (80 mg, 0.27 mmol). The colorless solution was stirred at 0 °C for 30 min, then a solution of (R)-4-(5-amino-2-fluoro-phenyl)-5,5-difluoro-4-methyl-5,6-dihydro-4H-[1,3]oxazin-2-ylamine (**13**) (54 mg, 0.21 mmol) in MeOH (5 mL) was added dropwise at 0 °C and the mixture was stirred at 22 °C for 20 h. The mixture was partitioned between aqueous Na_2CO_3 solution (1 M) and dichloromethane, and the organic layers were washed with brine, dried over Na_2SO_4 , and evaporated. The residue was purified by flash column chromatography on silica gel using a mixture of methanol/methylene chloride (0–10%) to give the pure amides **14a** and **14d**.

5-Chloro-pyridine-2-carboxylic Acid [3-((R)-2-Amino-5,5-difluoro-4-methyl-5,6-dihydro-4H-[1,3]oxazin-4-yl)-4-fluoro-phenyl]-amide (14a).⁴³ The condensation of **13** (110 mg, 0.42 mmol) and 5-chloro-pyridine-2-carboxylic acid (67 mg, 0.42 mmol) yielded the title compound **14a** (122 mg, 72%) as a colorless solid. ^1H NMR (300 MHz, $\text{DMSO}-d_6$) δ ppm 10.70 (s, 1 H), 8.79 (d, $J = 2.0$ Hz, 1 H), 8.13–8.22 (m, 2 H), 7.95 (dd, $J = 7.1, 2.6$ Hz, 1 H), 7.83–7.91 (m, 1 H), 7.15 (dd, $J = 11.8, 8.8$ Hz, 1 H), 4.29 (ddd, $J = 17.8, 12.0, 5.8$ Hz, 1 H), 4.02 (ddd, $J = 17.8, 11.8, 5.8$ Hz, 1 H), 3.30 (s, 2 H), 1.64 (s, 3 H). LC-HRMS: m/z 399.0839 [($M + H$)] $^+$ calcd for $\text{C}_{17}\text{H}_{15}\text{ClF}_3\text{N}_4\text{O}_2$, 399.0830]. MS (ISP): $m/z = 399.2$ [$M + H$] $^+$.

5-Cyano-pyridine-2-carboxylic Acid [3-((R)-2-Amino-5,5-difluoro-4-methyl-5,6-dihydro-4H-[1,3]oxazin-4-yl)-4-fluoro-phenyl]-amide (14d).⁴³ The condensation of **13** (80 mg, 0.31 mmol) and 5-cyano-pyridine-2-carboxylic acid (50 mg, 0.34 mmol) yielded the title compound **14d** (78 mg, 65%) as a colorless solid. ^1H NMR (600 MHz, $\text{DMSO}-d_6$) δ ppm 10.86 (s, 1 H), 9.20 (dd, $J = 2.1, 0.9$ Hz, 1 H), 8.58 (dd, $J = 8.2, 2.1$ Hz, 1 H), 8.28 (dd, $J = 8.2, 0.8$ Hz, 1 H), 7.98 (dd, $J = 7.2, 2.8$ Hz, 1 H), 7.88 (ddd, $J = 8.8, 3.8, 2.9$ Hz, 1 H), 7.17 (dd, $J = 11.8, 8.8$ Hz, 1 H), 5.91 (br s, 2 H), 4.30 (ddd, $J = 18.5, 11.9, 5.4$ Hz, 1 H), 4.03 (ddd, $J = 17.4, 11.8, 5.7$ Hz, 1 H), 1.65 (s, 3 H). LC-HRMS: m/z 390.1183 [($M + H$)] $^+$ calcd for $\text{C}_{18}\text{H}_{15}\text{F}_3\text{N}_5\text{O}_2$, 390.1172]. MS (ISP): $m/z = 390.2$ [$M + H$] $^+$. X-ray PDB accession code: 3ZMG.

3-Methyl-5-(trifluoromethyl)-4,5-dihydroisoxazole (76). To diethyl ether (640 mL) was added subsequently at –78 °C 3,3,3-trifluoroprop-1-ene (65.3 g, 680 mmol), nitroethane (30.0 g, 400 mmol), Et_3N (405 mg, 4 mmol), and phenyl isocyanate (97.2 g, 816 mmol), the solution was warmed to 22 °C, and stirring was continued for 74 h. The suspension was filtered and the filtrate distilled from bulb to bulb at 50–60 °C/1.0 mbar to give a 9:1 mixture of the title compound **76** and its corresponding position isomer (37.3 g, 61%) as a pale-yellow liquid. ^1H NMR (600 MHz, CDCl_3) δ ppm 4.80–4.87 (m, 1 H), 3.24 (ddq, $J = 17.8, 11.5, 1.0, 1.0, 1.0$ Hz, 1 H), 3.08 (ddq, $J = 17.8, 5.8, 0.9, 0.9, 0.9$ Hz, 1 H), 2.04 (t, $J = 1.1$ Hz, 3 H). ^1H NMR signals of position isomer 3-methyl-4-(trifluoromethyl)-4,5-dihydrois-

soxazole: 4.44–4.53 (m, 2 H), 3.83–3.91 (m, 1 H), 2.12 (t, $J = 0.9$ Hz, 3 H). MS: $m/z = 153$ [M] $^+$. Anal. ($C_5H_6F_3NO$) Calcd: C 39.22%, H 3.95%, N 9.15%. Found: C 39.27%, H 3.60%, N 9.34%.

(3S,5S)-3-(2-Fluoro-phenyl)-3-methyl-5-trifluoromethyl-isoxazolidine (79). To a stirred solution of 1-bromo-2-fluoro-benzene (77) (11.2 g, 64 mmol) in THF (50 mL) and toluene (140 mL) was added at -78 °C n -BuLi (1.6 M in hexane, 38 mL, 61 mmol), keeping the temperature below -70 °C, and stirring was continued at -78 °C for 1 h. To a solution of 3-methyl-5-(trifluoromethyl)-4,5-dihydroisoxazole (76) (4.68 g, 30.6 mmol) in toluene (330 mL) was added at -78 °C $BF_3 \cdot Et_2O$ (8.68 g, 61.1 mmol), which was followed by the addition of the phenyl lithium reagent prepared above using an insulated cannula over 10 min keeping the temperature below -70 °C. The mixture was stirred at -78 °C for 1 h, quenched with saturated aqueous NH_4Cl , and extracted with EtOAc. The organic layers were washed with brine, dried, and evaporated, and the residue was purified by flash column chromatography on silica gel using a mixture of n -heptane/EtOAc (50:1) to give the racemic title compound 79 (4.275 g, 56%) as a pale-yellow liquid. The racemate was resolved on a chiral HPLC column (Chiralpack AD) using n -heptane/EtOH (95:5) to give the desired (3S,5S)-3-(2-fluoro-phenyl)-3-methyl-5-trifluoromethyl-isoxazolidine (79) (1.719 g, 23%) as the slower eluting enantiomer with positive optical rotation and an $ee > 99.5\%$. ^1H NMR (300 MHz, $CDCl_3$) δ ppm 7.78 (td, $J = 8.0, 1.3$ Hz, 1 H), 7.22–7.31 (m, 1 H), 7.14 (td, $J = 7.7, 1.2$ Hz, 1 H), 7.05 (ddd, $J = 12.0, 8.1, 1.1$ Hz, 1 H), 5.67 (s, 1 H), 4.27 (sxt, $J = 7.4$ Hz, 1 H), 3.10 (ddd, $J = 13.4, 8.2, 1.0$ Hz, 1 H), 2.37 (dd, $J = 13.3, 7.5$ Hz, 1 H), 1.62 (s, 3 H). MS: $m/z = 250.4$ [$M + H$] $^+$. The relative and absolute configuration of the newly introduced stereogenic centers were confirmed by X-ray crystallographic analysis of the target compound 89 complexed with BACE1 (4J1F). (3R,5R)-3-(2-Fluoro-phenyl)-3-methyl-5-trifluoromethyl-isoxazolidine (1.738 g, 23%) was obtained as the faster eluting enantiomer with negative optical rotation and an $ee > 99.5\%$.

(2S,4S)-4-Amino-1,1,1-trifluoro-4-(2-fluorophenyl)pentan-2-ol (81). To a solution of (3S,5S)-3-(2-fluoro-phenyl)-3-methyl-5-trifluoromethyl-isoxazolidine (79) (1.50 g, 6.02 mmol) in EtOH (35 mL) was added 22 °C ammonium formate (3.04 g, 48.2 mmol) and Pd/C (10%, 320 mg), and stirring of the mixture was continued at 22 °C for 4 h. The suspension was filtered, the filtrate was evaporated, and the residue was partitioned between EtOAc and saturated aqueous $NaHCO_3$ solution. The organic layer was dried and evaporated to give the crude title compound (1.50 g, 99%) as a colorless solid which was used without further purification. ^1H NMR (300 MHz, $CDCl_3$) δ ppm 7.45 (td, $J = 8.1, 1.8$ Hz, 1 H), 7.28–7.37 (m, 1 H), 7.19 (td, $J = 7.5, 1.2$ Hz, 1 H), 7.09 (ddd, $J = 12.6, 8.1, 1.3$ Hz, 1 H), 3.61 (dq, $J = 11.4, 6.7, 6.7, 2.0$ Hz, 1 H), 2.50 (dt, $J = 14.3, 1.6$ Hz, 1 H), 1.75–2.75 (br, 2 H), 1.82 (dd, $J = 14.3, 11.5$ Hz, 1 H), 1.69 (d, $J = 1.2$ Hz, 3 H) (OH not detectable). MS: $m/z = 252.2$ [$M + H$] $^+$. A small sample was purified by flash column chromatography on silica gel using a mixture of n -heptane/EtOAc: mp 113–115 °C. Anal. ($C_{11}H_{13}F_4NO$) Calcd: C 52.59%, H 5.22%, N 5.58%. Found: C 52.49%, H 5.24%, N 5.73%.

(4S,6S)-4-(2-Fluorophenyl)-4-methyl-6-(trifluoromethyl)-5,6-dihydro-4H-1,3-oxazin-2-amine (83). To a solution of (2S,4S)-4-amino-1,1,1-trifluoro-4-(2-fluorophenyl)pentan-2-ol (81) (1.834 g, 7.30 mmol) in EtOH (38 mL) was added a solution of Br-CN (5 M in CH_3CN , 2.2 mL, 11.0 mmol), and the mixture was stirred in a sealed tube at 85 °C for 15 h. The mixture was evaporated and the residue partitioned between EtOAc and saturated aqueous Na_2CO_3 solution, and the organic layer was dried and evaporated, and the residue was purified by chromatography (silica- NH_2) using a mixture of n -heptane/EtOAc (5:1 to 0:1) to afford the title compound 83 (817 mg, 41%) as a colorless oil. ^1H NMR (600 MHz, $CDCl_3$) δ ppm 7.38 (td, $J = 8.1, 1.9$ Hz, 1 H), 7.24–7.29 (m, 1 H), 7.13 (td, $J = 7.6, 1.3$ Hz, 1 H), 7.04 (ddd, $J = 12.4, 8.1, 1.3$ Hz, 1 H), 4.35 (br s, 2 H), 3.97 (dq, $J = 12.4, 6.0, 6.0, 6.0, 2.8$ Hz, 1 H), 2.77 (dd, $J = 13.6, 2.8$ Hz, 1 H), 1.86 (dd, $J = 13.6, 12.6$ Hz, 1 H), 1.63 (d, $J = 1.3$ Hz, 3 H). MS: $m/z = 277.1$ [$M + H$] $^+$. A small sample was subjected again to flash column chromatography on silica- NH_2 gel using a mixture of n -

heptane/EtOAc: Anal. ($C_{12}H_{12}F_4N_2O$) Calcd C 52.18%, H 4.38%, N 10.14%. Found: C 52.04%, H 4.14%, N 10.05%.

(4S,6S)-4-(2-Fluoro-5-nitrophenyl)-4-methyl-6-(trifluoromethyl)-5,6-dihydro-4H-1,3-oxazin-2-amine (85). To concentrated sulfuric acid (11 mL) was added portion wise (4S,6S)-4-(2-fluorophenyl)-4-methyl-6-(trifluoromethyl)-5,6-dihydro-4H-1,3-oxazin-2-amine (83) (760 mg, 2.75 mmol) at 22 °C, the solution obtained was cooled to 0 °C and treated with red fuming HNO_3 (0.17 mL, 3.85 mmol) over 20 min, and stirring was continued at 0 °C for 30 min. The reaction mixture was slowly added to crushed ice/water (150 mL), the pH was adjusted to 10 using NaOH, the aqueous layer was extracted with EtOAc, the organic layer was dried and evaporated, and the residue was chromatographed on silica gel using a mixture of n -heptane/EtOAc (3:1) to afford the title compound 85 (800 mg, 91%) as a pale-yellow oil. ^1H NMR (300 MHz, $CDCl_3$) δ ppm 8.37 (dd, $J = 7.0, 2.9$ Hz, 1 H), 8.19 (ddd, $J = 8.9, 4.0, 3.0$ Hz, 1 H), 7.21 (dd, $J = 10.7, 8.9$ Hz, 1 H), 4.31 (br s, 2 H), 3.87–4.01 (m, 1 H), 2.78 (dd, $J = 13.8, 2.7$ Hz, 1 H), 1.95 (dd, $J = 13.8, 12.8$ Hz, 1 H), 1.64 (d, $J = 1.2$ Hz, 3 H). MS: $m/z = 322.5$ [$M + H$] $^+$.

(4S,6S)-4-(5-Amino-2-fluorophenyl)-4-methyl-6-(trifluoromethyl)-5,6-dihydro-4H-1,3-oxazin-2-amine (87). A suspension of (4S,6S)-4-(2-fluoro-5-nitrophenyl)-4-methyl-6-(trifluoromethyl)-5,6-dihydro-4H-1,3-oxazin-2-amine (85) (765 mg, 2.38 mmol) in EtOH (45 mL) and Et_3N (0.33 mL, 2.38 mmol) was treated with Pd/C (10%, 127 mg), and the mixture was hydrogenated at atmospheric pressure and 22 °C for 3 h. The mixture was filtered and the filtrate evaporated to give the crude title compound 87 (680 mg, 98%) as a colorless foam which was used without further purification. ^1H NMR (300 MHz, $CDCl_3$) δ ppm 6.83 (dd, $J = 11.7, 8.5$ Hz, 1 H), 6.67 (dd, $J = 6.9, 2.8$ Hz, 1 H), 6.52 (ddd, $J = 8.5, 3.0, 3.0$ Hz, 1 H), 4.04 (dq, $J = 12.3, 6.0, 6.0, 6.0, 2.8$ Hz, 1 H), 3.90–4.60 (br s, 2 H), 3.57 (br s, 2 H), 2.76 (dd, $J = 13.5, 2.8$ Hz, 1 H), 1.81 (dd, $J = 13.4, 12.6$ Hz, 1 H), 1.60 (d, $J = 1.2$ Hz, 3 H). MS: $m/z = 292.5$ [$M + H$] $^+$. Anal. ($C_{12}H_{13}F_4N_3O$) Calcd: C 49.49%, H 4.50%, N 14.43%. Found: C 49.25%, H 4.51%, N 14.15%.

N-(3-((4S,6S)-2-Amino-4-methyl-6-(trifluoromethyl)-5,6-dihydro-4H-1,3-oxazin-4-yl)-4-fluorophenyl)-5-cyanopicolinamide (89). The coupling of (4S,6S)-4-(5-amino-2-fluorophenyl)-4-methyl-6-(trifluoromethyl)-5,6-dihydro-4H-1,3-oxazin-2-amine (87) and 5-cyano-pyridine-2-carboxylic acid (65), carried out according to the general procedure for 14a and 14d, yielded a crude material which was purified by chromatography (silica- NH_2) using a mixture of n -heptane/EtOAc (1:1) to give the title compound 89 (69% yield) as a colorless solid. ^1H NMR (300 MHz, $CDCl_3$) δ ppm 9.84 (s, 1 H), 8.89 (dd, $J = 2.0, 0.8$ Hz, 1 H), 8.43 (dd, $J = 8.1, 0.8$ Hz, 1 H), 8.20 (dd, $J = 8.3, 2.0$ Hz, 1 H), 7.99 (ddd, $J = 8.7, 4.1, 2.9$ Hz, 1 H), 7.48 (dd, $J = 7.1, 2.8$ Hz, 1 H), 7.11 (dd, $J = 11.4, 8.8$ Hz, 1 H), 4.24 (br s, 2 H), 4.03 (dq, $J = 12.2, 6.0, 6.0, 6.0, 2.6$ Hz, 1 H), 2.81 (dd, $J = 13.7, 2.6$ Hz, 1 H), 1.89 (dd, $J = 13.6, 12.8$ Hz, 1 H), 1.64 (d, $J = 1.0$ Hz, 3 H). LC-HRMS m/z 422.1243 [($M + H$)] $^+$ calcd for $C_{19}H_{16}F_4N_5O_2$, 422.1235]. MS: $m/z = 422.5$ [$M + H$] $^+$. X-ray PDB accession code: 4J1F.

BACE1 and BACE2 Enzyme Assay. Enzyme inhibition assays were performed in 384-well microtiter plates (black with clear bottom, nonbinding surface from Corning, cat. no. 3655) in a final volume of 51 μ L. Test compounds dissolved in DMSO at a concentration of 10 mM were serially diluted in DMSO (15 concentrations, 1/3 dilution steps, final concentration range: 200–0.0004 μ M). Then 1 μ L of diluted compounds were mixed with 40 μ L of enzyme on an H&P Teleshaker for 4 min. After addition of the MR121-labeled substrate, the plates were again strongly shaken for 2 min. The enzymatic reaction was followed by reading the fluorescence emission on a plate: vision reader (Perkin-Elmer) (excitation wavelength, 630 nm; emission, 695 nm) for at least 30 min in a kinetic measurement detecting an increase of MR121 fluorescence following the cutting of the quenched substrate. The slope in the linear range of the kinetic was calculated and the IC_{50} of the test compounds were determined using a four parameter Hill equation for curve fitting on the calculated % inhibition values referenced to wells without enzyme and wells with enzyme but no test compound, respectively. BACE1 enzyme used in

the assay was prepared and purified as described.⁵⁸ The preparation of BACE2 was carried out following exactly the known protocol.⁴⁶ BACE1/2 assay: The final enzyme concentration in the assay was 30 and 125 nM, respectively. The assay buffer was 100 mM sodium acetate, 20 mM EDTA, 0.05% BSA, pH 4.0. The substrate peptide was of sequence WSEVNLDAEFRG-MR121 and used at a final concentration of 300 nM.

Cathepsin D Enzyme Assay. Cathepsin D from human liver was purchased from Calbiochem (cat. no. 219401) and used at a final concentration of 200 nM. The assay buffer was 100 mM sodium acetate at pH 5.5 with 0.05% BSA. The labeled substrate peptide was of sequence WTSVLMAAPC-MR121 (measurement time >60 min).

Cathepsin E Enzyme Assay. Recombinant human cathepsin E was purchased from R&D Systems (cat. no. 1294-AS) and used at a final concentration of 0.01 nM. The assay buffer was 100 mM sodium acetate at pH 4.5 with 0.05% BSA. The labeled substrate was of sequence MR121-CKLVFFAEDW.

Renin Enzyme Assay. Renin was purchased from Anaspec (cat. no. 72041) and used at a final concentration of 5 nM. The assay buffer was 50 mM MES at pH 6.2 with 0.5% BSA and 2 mM EDTA. The substrate peptide was of sequence WPHPPFHLVIHTC-MR121.

Pepsin Enzyme Assay. Pepsin from porcine stomach mucosa was purchased from Calbiochem (cat. no. 516360) and used at a final concentration of 8 nM. The assay buffer was 100 mM sodium acetate at pH 4.0 with 0.05% BSA, and the substrate was the same as for cathepsin D. All substrate peptides were prepared and purified by Biosyntan, Berlin, Germany, and purchased as lyophilized powders. After reconstitution in DMSO small aliquots were kept at -20°C .

Cell-Based Assay. HEK293 cells transfected with wild-type human APP cDNA were seeded in 96-well Microtiter plates in cell culture medium (Iscove's, plus 10% (v/v) fetal bovine serum, penicillin/streptomycin) to about 80% confluency, and the compounds were added at a $3\times$ concentration in 1/3 volume of culture medium (final DMSO concentration was kept at 1% v/v). After 18–20 h incubation at 37°C and 5% CO_2 in a humidified incubator, the culture supernatants were harvested for the determination of A β 40 concentrations by AlphaLISA technique using PerkinElmer Human Amyloid β 1–40 kit (A β 1–40, high specificity, cat. no. AL275 C/F). In a PerkinElmer OptiPlate –384 (cat. no. 6007290), 6 μL of culture supernatants were added to 2 μL of AlphaLISA Anti hu A β acceptor beads (50 $\mu\text{g}/\text{mL}$), 5 nM Biotinylated Antibody Anti A β 1–40, 1 $\times$ AlphaLISA HiBlock buffer solution and incubated for 1 h at room temperature. Then 16 μL of a Streptavidin Donor beads (250 ng/mL in 1 $\times$ AlphaLISA solution) were added, and the plate was incubated for 30 min at room temperature. Light emission at 615 nm was then recorded on an EnVision-2104 multilabel reader. Levels of A β 40 in the culture supernatants were calculated as percentage of maximum signal (cells treated with 1% DMSO without inhibitor). The IC_{50} values were calculated using the Excel XLfit software.

P-gp Assay. Bidirectional transport was examined in LLC-PK1 cells (Lewis-lung cancer porcine kidney 1 I, ATCC CL-101) stably expressing human MDR1. The cell lines were obtained from Dr. Alfred Schinkel, The Netherlands Cancer Institute (Amsterdam, The Netherlands) and used under a license agreement. Both cell lines were cultured on semipermeable insert plates (Costar, surface area 0.33 cm^2 , pore size 3.0 μm), and transport experiments were performed on day 4 after seeding using medium without phenol red. Bidirectional transport was assessed in triplicate inserts with or without inhibitor (elacridar, 1 μM), and the cell monolayer integrity was assessed via the permeability of lucifer yellow (10 μM). Before each assay, the culture medium in the receiver compartment was replaced with fresh medium. Bidirectional transport was then initiated by the addition of medium consisting of test compound (1 μM) and lucifer yellow to the donor compartments. The plates were incubated for 3.5 h at 37°C , 5% CO_2 , and air-saturated humidity. At the end of incubation, samples were taken from the donor and the receiver compartments, and the levels of each compound were determined either by LC-MS/MS or scintillation counting. Lucifer yellow in the receiver samples was quantified using a fluorescence-based plate reader set at excitation and emission wavelengths of 430 and 535 nm,

respectively. Experiments showing lucifer yellow permeation superior to 1%/h were rejected. The apparent permeability coefficient (P_{app}) was calculated using the following equation: $P_{\text{app}} = 1/(A \cdot C_0) \cdot (dQ/dt)$, where dQ/dt is the amount of compound transported over time, A is the surface area of the transwell, and C_0 is the initial concentration of the test compound. Mean P_{app} value and standard deviation were calculated from triplicate inserts. Active efflux by MDR1 was assessed by determining the vectorial transport or efflux ratio (ER), calculated from the basolateral-to-apical P_{app} divided by the apical-to-basolateral P_{app} . Digoxin was included in each experiment as a positive control.

HERG Inhibition. HERG inhibition was tested as described.⁵⁹

Protein Crystallography. X-ray crystal structures of BACE1 and BACE2 were determined as described by Kuglstatter et al.⁶⁰ and Ruf et al.⁴⁸

Pharmacokinetic Experiments in Mice and Rats. Animals and Housing Conditions. Animals were maintained in a 12/12 h light/dark cycle, with lights starting at 6 a.m., and experiments were conducted during the light phase. Animal housing and experimental procedures were in line with ethical and legal guidelines and were authorized by local veterinary authorities.

Experiment. Compound 89 was administered to female C57Bl/6NJ mice. At indicated time points plasma and brain samples were quenched with organic solvent. (1 vol of sample + 3 vol of acetonitrile/methanol, 1:1 (v/v) containing an internal standard). After centrifugation for min at 4000g, the supernatant was diluted with one volume of aqueous 10 mM ammonium acetate at pH 3. Samples were then directly injected onto an LC-MS/MS system to quantify compound concentrations against a calibration curve using analyte specific transitions. Pharmacokinetic studies in rats were conducted accordingly using the following formulations: PO microsuspension in gelatin/NaCl (7.5%/0.62% in water) at 1.25 mg 89/mL; IV 0.5 mg 89/mL 0.9% NaCl in water/NMP (30/70).

Inhibition of A β 40 in Brain of Wild-Type Mice. Animals and Housing Conditions. Animals were maintained in a 12/12 h light/dark cycle, with lights starting at 6 a.m., and experiments were conducted during the light phase. Animal housing and experimental procedures were in line with ethical and legal guidelines and were authorized by local veterinary authorities.

Experiment. Female C57Bl/6J mice were treated with different doses of the compounds, 3–4 animals per treatment group. The test compound was dissolved in 5% EtOH, 10% Solutol, and was applied per os at 10 mL/kg. After 4 h, the animals were sacrificed and brain and plasma were collected. The brain was cut into halves and immediately frozen on dry ice. Brain was used for measurement of A β 40 and plasma was used for determination of compound exposure. The method for A β 40 determination in brain lysates followed the known procedure.⁶¹ Brain tissue was homogenized in 2% DEA buffer in a Roche MagnaLyser (20", 4000 rpm) and subsequently centrifuged for 1 h at 10000g. DEA was reduced to 0.2% in 50 mM NaCl and one-half of the DEA lysate was passed over an Oasis Solid phase extraction plate (Waters; cat. no. 186000679), which had been activated with MeOH and equilibrated in dH_2O (1 mL each). After washes in 10% and 30% MeOH (1 mL each), the A β -peptides were eluted in 0.8 mL of 2% NH_4OH in 90% MeOH. The eluate was dried over a N_2 flow, and the dried sample was reconstituted in 30 μL of AlphaLISA assay buffer. A β 40 was determined by an AlphaLISA assay (Perkin-Elmer). In a white 96-well, half area microplate (Perkin-Elmer cat. no. 6005561), 20 μL of the reconstituted sample were mixed with 5 μL of biotinylated BAP-24 (specific for C-terminus of A β 40⁶²), stock = 4.4 mg/mL, f.c.s.5 $\mu\text{g}/\text{mL}$), and 5 μL 252Q6 acceptor beads (252Q6 antibody, Invitrogen AMB0062) had been previously conjugated with AlphaLISA Acceptor beads (Perkin-Elmer cat. no. 6772002); final dilution 1:500). The mix was incubated for 1 h at RT in the dark. Then 20 μL of Streptavidin-coated Donor Beads (Perkin-Elmer cat. no. 6760002, final dilution 1:125) were added and this final mix was incubated in the dark for another 30 min at RT before RFU was measured in an AlphaScreen Reader (Perkin-Elmer Envision 2104).

Inhibition of CSF A β 40 in Wild-Type Rats. Animals and Housing Conditions. Animals were maintained in a 12:12 h

light:dark cycle, with lights starting at 6 a.m., and experiments were conducted during the light phase. Animal housing and experimental procedures were in line with ethical and legal guidelines, and were authorized by local veterinary authorities.

Experiment. In this *in vivo* model, we followed the kinetics of changes in rat CSF A β 40 in an acute treatment study. For this purpose, male rats (Wistar) were prepared with a cannula inserted into the Cisterna magna.⁵⁷ Approximately 1 week after surgery, 2–3 animals were treated with one dose of the test compound by gavage (compound prepared in 10% solutol, 5% ethanol, and 85% water, 4 mL/kg). CSF was drawn at 2 and 1 h before compound application and then at different time points postapplication, the volume of CSF withdrawn at each time-point was about 10 μ L. For the determination of A β -peptide in the rat CSF a commercial ELISA kit specific for A β 40 was used according to the protocol of the manufacturer (WAKO, cat. no. 294–64701, human/rat β -amyloid 40). Because of interanimal differences in baseline A β 40 level in CSF the pretreatment samples were used to define the 100% A β 40 level for each individual animal and the subsequent post-treatment values were expressed as percentage of this control value.

□ ASSOCIATED CONTENT

Supporting Information

Preparation details and analytical data for compounds 1a–b, 14b–c, 14e–v, 66–70, 88, 109–113. Log *D*, p*K*_a determinations, stability in mouse microsomes, inhibition of CYP₄₅₀ and time dependent inhibition of CYP₄₅₀ 3A4. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Accession Codes

Crystal structure coordinates and structure factors have been deposited to the PDB with the accession numbers 4JOP (1b), 3ZMG (14d), 4JOT (14i), 4JOV (66), 4JOY (67), 4JOZ (68), 4J17 (69), 4J1C (70), 4J1E (88), 4J1F (89), 4J1H (109), 4J1I (110), and 4J1K (111). The structure of BACE2 in complex with 14i has been deposited with the accession number 3ZLQ.

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Notes

The authors declare no competing financial interest.

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□ ABBREVIATIONS USED

HERG, human ether-à-go-go related gene; DCM, dichloromethane; Deoxo-Fluor, bis(2-methoxyethyl)aminosulfur trifluoride; DIBAH, diisobutylaluminumhydride; DMF, *N,N*-dimethylformamide; DMSO, dimethyl sulfoxide; DMTMM, 4-(4,6-dimethoxy[1.3.5]triazin-2-yl)-4-methylmorpholinium chloride hydrate; EtOAc, ethyl acetate; EtOH, ethanol; MeOH, methanol; TBME, *tert*-butylmethylether; TEA, triethylamine; TFA, trifluoro-acetic acid; T3P, (2,4,6-tripropyl-1,3,5,2,4,6-trioxatriphosphorinane-2,4,6-trioxide; TBAF, tetrabutylammonium fluoride; THF, tetrahydrofuran

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RECIBO DE CA	999999999	131491	10/12/2014		128.000.00
RECIBO DE CA	999999999	131492	10/12/2014		128.000.00
RECIBO DE CA	999999999	131493	10/12/2014		128.000.00
RECIBO DE CA	999999999	131778	11/12/2014		2.000.00
RECIBO DE CA	999999999	133412	16/12/2014		128.000.00
RECIBO DE CA	999999999	133413	16/12/2014		128.000.00
RECIBO DE CA	999999999	133417	16/12/2014		106.000.00
RECIBO DE CA	999999999	133420	16/12/2014		106.000.00

*** Conceptos Pagados ***

CANT. RENTISTICO

CONCEPTO

Vr.UNDITARIO

Vr.CONCEPTO

1 50005-01-03 CAMBIO DE MODALIDAD Y MODIFICACIONES EN CREACION CORRECCION A SOLICITUD EN TRAMITE / MODIFICA

132.000.00

132.000.00

\$132.000.00

SON: **CIENTO TREINTA Y DOS MIL PESOS MONEDA CORRIENTE**

Responsable: _____

Recibo de Caja Aplicado al Expediente No. _____

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 12-113850-00007-0000

Fecha: 2015-01-23 16:38:38 Dep. 2020 DIR.NUEVASCR
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Enero 15 de 2015 - 08:50:44