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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: 523

Re: PI.-F. HOFFMANN-LA ROCHE AG y SIENA BIOTECH S.P.A.-Solicitud de Patente de Invención en Fase Nacional PCT para "FLUOROMETIL-5,6-DIHIDRO-4H-[1,3]OXAZINAS".-Clase C07D 401/12.-Expediente número 14-108.723.-

1. Me refiero al auto dictado por ese Despacho notificado por fijación en lista de 21 de abril de 2015.

2. Con el fin de responder las objeciones expuestas por el Señor Examinador en su Concepto Técnico correspondiente al Oficio No. **4109** en relación con la claridad del Capítulo Reivindicatorio y el Nivel Inventivo de la presente solicitud, me permito dirigir su atención a las siguientes modificaciones y argumentos técnicos por medio de los cuales no sólo se superan completamente dichas objeciones sino que además se establece de manera incontrovertible la patentabilidad de la solicitud en estudio.

3. CAPÍTULO REIVINDICATORIO.

3.1. **Modificaciones al Capítulo Reivindicatorio**

En primer lugar, me permito manifestar que es deseo del solicitante presentar un **capítulo reivindicatorio modificado**, que remplace al actualmente presentado, con el propósito de superar las objeciones presentadas como parte del concepto técnico realizado por ese Despacho.

En conexión con el mencionado **capítulo reivindicatorio modificado**, me permito resaltar que éste no constituye de ninguna manera una ampliación al alcance de la solicitud, tal como fue originalmente presentada, toda vez que define los novedosos compuestos derivados de fluorometil-5,6-dihidro-4H-[1,3]oxazinas con actividad inhibitoria de la BACE1 y sus respectivas composiciones farmacéuticas, los cuales son el objeto de la presente invención de acuerdo con las enseñanzas de la descripción. Dichas modificaciones se exponen a continuación:

- **Nueva Reivindicación 1:** tiene como base la reivindicación 1 actualmente presentada, la cual ha sido modificada en la definición de los sustituyentes de la fórmula general para restringir significativamente el alcance de la materia reclamada. Específicamente, se han definido los grupos funcionales particulares que caracterizan el sustituyente R^1 de la fórmula (I), con lo que se define de manera clara y concisa el objeto aquí reclamado. Dicha modificación tiene pleno sustento en las características técnicas de la reivindicación 14 actualmente presentada y en la página 18 de la memoria descriptiva.
- **Nuevas Reivindicaciones 2 a 9:** corresponden a las respectivas reivindicaciones 3 a 10 del capítulo actualmente presentado.
- **Nueva Reivindicación 10:** tiene como base la reivindicación 11 actualmente presentada, la cual ha sido modificada en la definición de los sustituyentes de la fórmula general para restringir significativamente el alcance de la materia reclamada. Específicamente, se han definido los grupos funcionales particulares que caracterizan el sustituyente R^1 de la fórmula (I), con lo que se define de manera clara y concisa el objeto aquí reclamado. Dicha modificación tiene pleno sustento en las características técnicas de la reivindicación 14 actualmente presentada y en la página 18 de la memoria descriptiva.
- **Nuevas Reivindicaciones 11 a 15:** corresponden a las respectivas reivindicaciones 15 a 19 del capítulo actualmente presentado.

De acuerdo a lo anterior, me permito señalar que la reivindicaciones **2, 12, 13 y 14** del Capítulo Reivindicatorio actualmente presentado fueron **eliminadas** del documento modificado. Asimismo, me permito aclarar que el orden de enumeración y dependencia de las nuevas reivindicaciones se ha reajustado de conformidad con las modificaciones previamente descritas.

De esta forma, **el capítulo reivindicatorio modificado queda constituido por 15 reivindicaciones**, las cuales se refieren de manera más clara y concisa a las características técnicas esenciales de los compuestos derivados de fluorometil-5,6-dihidro-4H-[1,3]oxazinas con actividad inhibitoria de la BACE1 que son objeto de la presente solicitud, y que cuentan con pleno sustento en las enseñanzas de la descripción.

3.2. Claridad del Capítulo Reivindicatorio

Con respecto a la claridad del capítulo reivindicatorio, el señor Examinador afirma lo siguiente:

- *"La reivindicación 1 carece de concisión y claridad (...) porque emplea definiciones tales como arilo, arilo sustituido, heteroarilo, heteroarilo sustituido, con las cuales se pueden presentar diferentes opciones de posibles compuestos pues el núcleo farmacofórico podría contener diversos anillos que no permiten conocer el objeto que se pretende proteger (...) Por tal motivo se obtienen un número indefinido de compuestos que no encuentran soporte en la descripción. Se sugiere a la solicitante restringir la solicitud a una razonable generalización del tipo de compuestos que han sido sintetizados o probados, es decir, que se encuentran debidamente sustentados en la descripción, como por ejemplo, se encuentran en las reivindicaciones 17 y 18". Además, el señor Examinador agrega que "la anterior objeción de claridad aplica para la reivindicación 11, que emplea tales definiciones ambiguas e imprecisas".*

Respecto al anterior señalamiento, me permito indicar que, tal y como se expuso en el numeral 3.1 del presente memorial, las reivindicaciones 1 y 11 (*nuevas reivindicaciones 1 y 10, respectivamente*) han sido modificadas de tal forma que el sustituyente R¹ ha sido restringido en su alcance, incorporando los específicos grupos funcionales que se encuentran en los compuestos de las reivindicaciones 17 y 18 (*nuevas reivindicaciones 13 y 14*), y que por lo tanto, están debidamente soportados por los Ejemplos de la memoria descriptiva. Así entonces, y de acuerdo con las sugerencias del señor Examinador, la nueva definición del sustituyente R¹ se lee de la siguiente manera:

"R¹ es 1-(difluormetil)-1H-pirazol-3-ilo, 2-(fluormetil)oxazol-4-ilo, 3,5-dicloro-piridin-2-ilo, 3-cloro-5-cianopiridin-2-ilo, 3-metilpirazin-2-ilo, 4-cloro-1-(difluormetil)-1H-pirazol-3-ilo, 5-(2,2,2-trifluoretoxi)-pirazin-2-ilo, 5-(2,2-difluoretoxi)pirazin-2-ilo, 5-(but-2-iniloxi)pirazin-2-ilo, 5-(difluormetoxi)-pirazin-2-ilo, 5-(difluormetil)pirazin-2-ilo, 5-(fluor-metoxi)pirazin-2-ilo, 5-(fluormetil)pirazin-2-ilo, 5-cloro-piridin-2-ilo, 5-ciano-3-metil-piridin-2-ilo, 5-ciano-piridin-2-ilo, 5-difluor-metoxi-pirazin-2-ilo, 5-difluor-metoxi-piridin-2-ilo, 5-difluormetil-piridin-2-ilo, 5-fluor-metoxi-piridin-2-ilo, 5-metoxi-pirazin-2-ilo, 5-metoxi-piridin-2-ilo, 5-metil-pirazin-2-ilo, 5-trifluoretoxi-piridin-2-ilo, 5-trifluor-metoxi-pirazin-2-ilo o 5-trifluor-metoxi-piridin-2-ilo;"

A la luz de lo anterior, es evidente que el nuevo capítulo reivindicatorio constituye la generalización del tipo de compuestos que han sido sintetizados y define de manera clara y concisa el sustituyente R¹ del compuesto de fórmulas I y Ia-1. Asimismo, dicha modificación limita la extensión del objeto de la invención, logrando la diferenciación de la materia reclamada con los compuestos del estado del arte previo.

Consecuentemente, me permito manifestar que dicho capítulo modificado supera completamente la objeción de claridad presentada por el Señor Examinador y, por ende, cumple a cabalidad con lo estipulado por el Artículo 30 de la Decisión Andina 486 de 2000.

4. NIVEL INVENTIVO.

Ahora bien, el Señor Examinador considera que la materia reclamada en la reivindicación 5 actualmente presentada carecería de altura inventiva con respecto a las enseñanzas del documento **WO 2011/069934 (D1)**.

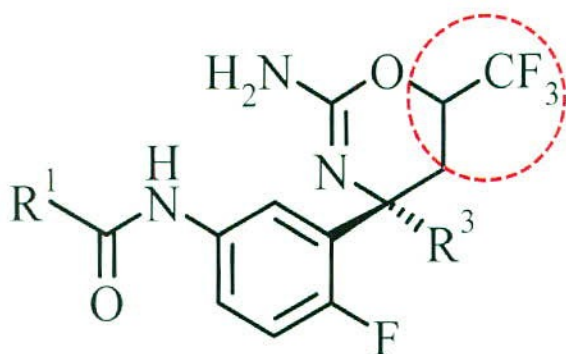
En conexión con lo anterior, el Señor Examinador asegura que la persona del oficio normalmente versada en la materia, basándose en los compuestos del documento D1 y en sus conocimientos básicos y simples en química heterocíclica, podría cambiar la posición de los átomos de halógeno en el anillo de oxazina, y así llegar al objeto de la reivindicación 1 de la solicitud.

Sin embargo, sobre esta particular objeción **me permito señalar que las enseñanzas de dicho documento D1 de ninguna manera anticipan o sugieren de manera obvia los compuestos derivados de fluorometil-5,6-dihidro-4H-[1,3]oxazinas de fórmulas I y Ia-1, con todas y cada una de las definiciones específicas para sus sustituyentes y que a su vez conserven su específica actividad inhibitoria, tal y como se reclaman en el capítulo reivindicatorio modificado que se presenta junto con este memorial.**

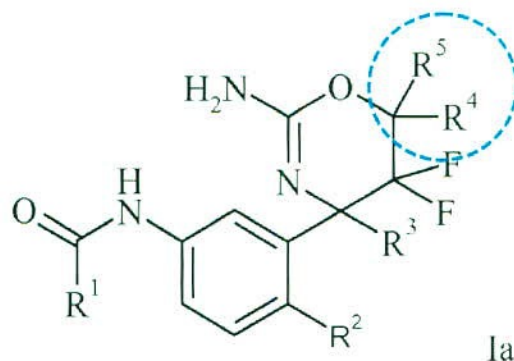
De conformidad con lo señalado por el señor Examinador, el documento D1 divulga compuestos derivados de 2-amino-5,5-difluoro-5,6-dihidro-4H-([1,3]-oxazin-4-il)-fenilamida con actividad inhibitoria contra la BACE1 y/o BACE2. Concretamente, el documento D1 presenta alrededor de 83 ejemplos de compuestos derivados tales oxazinas que tienen actividad inhibitoria contra Asp2, y por lo tanto, son útiles para el tratamiento terapéutico y/o profilático de enfermedades y desordenes caracterizados por niveles elevados de la proteína β amiloide.

Sin embargo, tras la atenta lectura y revisión de las enseñanzas de dicho documento, me permito resaltar que no existe razón alguna que motive a la persona versada en la materia a obtener los compuestos de la presente invención a partir de las enseñanzas de D1. A pesar de que dichos compuestos comprenden una porción de su estructura que es similar a la de los compuestos de la presente invención, **es evidente que existen varias diferencias estructurales significativas entre los compuestos derivados de la fórmulas I y Ia-1 de las nuevas reivindicaciones 1 y 10 de la presente invención y los compuestos del documento D1**, las cuales se exponen a continuación:

- i) En primer lugar, y de acuerdo al análisis del señor Examinador, los 49 compuestos derivados de fórmula (Ia-1) de la invención incluyen en la posición 6 del anillo de oxazina (*sustituyente R^4*) el grupo funcional trifluorometilo (*ver círculo rojo*) mientras que los compuestos de fórmula (Ia) de D1 incluyen en la misma posición (*Sustituyentes R^4 y R^5*) hidrógeno y/o un grupo metilo (*ver círculo azul*).

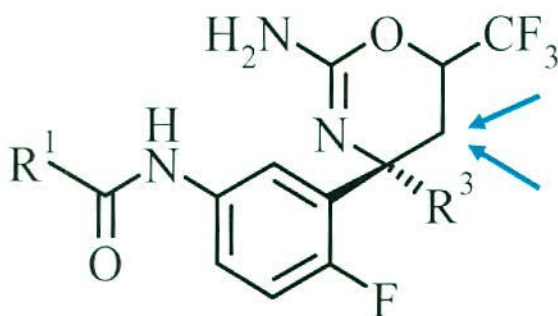


Fórmula Ia-1 de la presente Invención

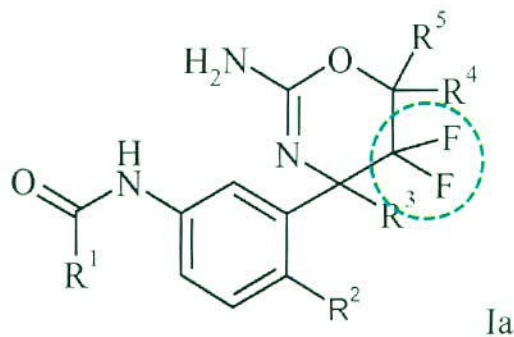


Fórmula Ia de D1

- ii) En segundo lugar, todos los 83 compuestos de D1 incluyen dos átomos de flúor en la posición 5 del anillo de la oxazina (*ver círculo verde*) mientras que ninguno de los 49 compuestos de la presente invención están sustituidos en dicha posición (*ver flecha azul*).

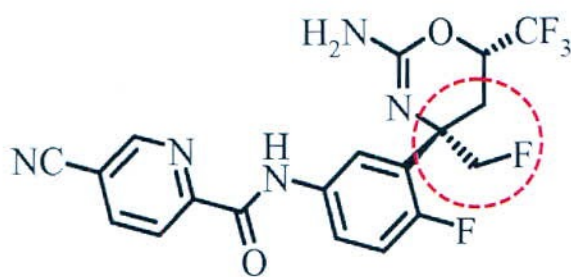


Fórmula Ia-1 de la presente Invención

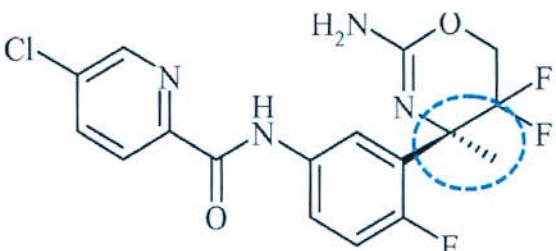


Fórmula Ia de D1

- iii) Sumado a las anteriores diferencias, se observa que, en los 83 ejemplos de compuestos de D1, el sustituyente R^3 de la posición 4 del anillo de oxazina corresponde a un grupo metilo (*ver círculo azul*). Por su parte, los compuestos de la presente invención para tal sustituyente R^3 en la misma posición, no sólo pueden incluir un grupo metilo sino también exhiben un fluorometilo o un difluorometilo (*ver círculo rojo*). Es importante resaltar que tales alquilos halogenados no se encuentran definidos en ninguna parte de D1 para tal sustituyente.



Compuesto 3 de la presente Invención



Compuesto 1 de D1

iv) Finalmente, es preciso mencionar que no todos los grupos funcionales definidos para el sustituyente R^1 de la fórmula de la presente invención (de acuerdo a la restricción al capítulo reivindicatorio en el numeral 3 del presente memorial) se encuentran divulgados en las enseñanzas del documento D1. En este sentido, la reivindicación 6 del documento D1 sólo revela 11 grupos funcionales de los 26 que se definen para R^1 en la nueva reivindicación 1 de la presente invención:

Grupos funcionales definidos para R^1 según la nueva reivindicación 1 de la presente invención

Grupos Funcionales para R^1 revelados en D1	Grupos Funcionales para R^1 <u>NO</u> revelados en D1
1-(difluormetil)-1H-pirazol-3-ilo, 2-(fluormetil)oxazol-4-ilo, 3,5-dicloro-piridin-2-ilo, 3-cloro-5-cianopiridin-2-ilo, 4-cloro-1-(difluormetil)-1H-pirazol-3-ilo, 5-cloro-piridin-2-ilo, 5-ciano-piridin-2-ilo, 5-difluor-metoxi-piridin-2-ilo, 5-fluor-metoxi-piridin-2-ilo, 5-metil-pirazin-2-ilo, 5-trifluoretoksi-piridin-2-ilo.	3-metilpirazin-2-ilo, 5-(2,2,2-trifluoretoksi)-pirazin-2-ilo, 5-(2,2-difluoretoksi)pirazin-2-ilo, 5-(but-2-iniloxi)pirazin-2-ilo, 5-(difluormetoxi)-pirazin-2-ilo, 5-(difluormetil)pirazin-2-ilo, 5-(fluor-metoxi)pirazin-2-ilo, 5-(fluormetil)pirazin-2-ilo, 5-ciano-3-metil-piridin-2-ilo, 5-difluor-metoxi-pirazin-2-ilo, 5-difluormetil-piridin-2-ilo, 5-metoxi-pirazin-2-ilo, 5-metoxi-piridin-2-ilo, 5-trifluor-metoxi-pirazin-2-ilo, 5-trifluor-metoxi-piridin-2-ilo.

De acuerdo a las anteriores observaciones, los compuestos derivados fluorometil-5,6-dihidro-4H-[1,3]oxazinas de la invención **no son sólo diferentes en cuanto a la posición de los átomos de flúor en el anillo de oxazina sino también a la naturaleza de los grupos funcionales de varios sustituyentes, en este caso la presencia del grupo funcional trifluorometilo en el sustituyente R⁴ (en comparación con el metilo de D1), la ausencia de dos átomos de flúor en la posición 5 del anillo de oxazina y la falta de sugerencia por la totalidad de los grupos funcionales para los sustituyentes R¹ y R³ en D1.**

Lo anterior implica, en primer lugar, que la persona del oficio medianamente versada en la materia tendría que realizar dos tipos diferentes de modificaciones a la molécula de oxazina de D1 para llegar a los compuestos que se reclaman en la presente solicitud: a) tendría que eliminar la sustitución de los átomos de flúor en la posición 5 del anillo de oxazina, y b) tendría que reemplazar en todos los compuestos el grupo trifluorometilo para la posición correspondiente al sustituyente R⁴.

Ahora, las anteriores modificaciones esenciales no son las únicas que la persona versada en la materia debería aplicar para poder obtener los compuestos de la invención. Al no revelar la totalidad de los grupos funcionales específicos para los sustituyentes R¹ y R³, la persona versada en la materia no podría derivar de manera obvia o evidente todos los compuestos que caen dentro de la definición de las fórmulas I y Ia-1 del capítulo reivindicatorio modificado.

En su análisis, el señor Examinador resalta que "(...) *en la presente invención simplemente se proponen modificaciones como es cambiar la posición de átomos de halógeno en el anillo oxazina, pero que como se vio anteriormente, dicha modificación no demostró un efecto técnico inesperado o una ventaja técnica real y por tanto se consideran variaciones estructurales de rutina que no sustentan el requisito evaluado*".

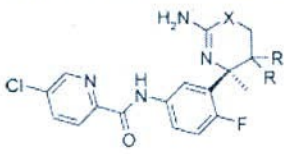
Frente lo anterior, muy respetuosamente me permito manifestar que el señor examinador hace un incorrecto análisis del nivel inventivo, toda vez que es claro que la persona versada en la materia no podría seleccionar dichas modificaciones al azar, teniendo en cuenta que D1 no cubre la totalidad de cambios y sugerencias necesarias para obtener los mismos compuestos de la invención. Es más, dicha persona versada en la materia tampoco podría derivar de manera evidente los compuestos de la invención dado que las enseñanzas de D1 no aseguran que las posibles modificaciones resulten en compuestos con la misma actividad inhibitoria satisfactoria de BACE1.

En realidad, no se habla de una simple modificación en la que los átomos de flúor de la posición 5 del anillo de oxazina –tal y como se ve en los compuestos de D1- pasan a otra posición en el anillo. Por el contrario, el cambio consiste en un nuevo grupo funcional (*un trifluorometilo*) que se agrega en otra posición del anillo (*Posición 6 en el*

sustituyente R^4). Así las cosas, la eliminación de los dos átomos de flúor y la posterior adición de un grupo funcional con propiedades fisicoquímicas muy diferentes en otra posición del anillo no se consideran una modificación de rutina. Más aún, **sin tener sugerencia alguna en el documento D1 sobre la existencia y/o interacción de tales grupos en los compuestos, la persona versada en la materia no encontraría razones suficientes para modificar la molécula de D1 para obtener compuestos que conserven o mejoren la actividad inhibitoria de BACE1.**

En este sentido, y como un complemento a los argumentos presentados anteriormente, me permito referirme al estudio realizado por Hilpert et al., que se adjunta al presente memorial, el cual demuestra claramente que (ver cuadro 1 de la página 3984) **las oxazinas difluoradas son las más adecuadas como inhibidores de BACE1**, esto en comparación con oxazinas sin fluoración y con tiazinas con y sin sustitución con flúor:

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.

Tabla 1. Documento Hilpert et al.

De acuerdo con 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 observa que las oxazinas fluoradas presentan mayor actividad como inhibidores de BACE1, al presentar menores valores de IC_{50} (μM) para la enzima BACE1 que aquellas sin fluoración. En este sentido, no se esperaría que la persona versada en la materia se viera motivada a eliminar dichos átomos de flúor del anillo principal con el fin de obtener compuestos con mejor actividad inhibitoria. En palabras más sencillas, ¿qué motivaría a la persona versada en la materia a utilizar oxazinas sin flúor directamente en el anillo de oxazina para inhibir BACE1, si la literatura sugiere que los mejores compuestos para tal inhibición son las oxazinas difluoradas?

Al leer los resultados de la Tabla 1 y la sección de "Resultados y Discusión" de dicho artículo, se puede concluir que el impacto de la eliminación de uno o dos átomos de flúor del anillo de oxazina sobre las actividades de hBACE1 y hBACE2, los ensayos celulares y la eficacia *in vivo* es muy complicado e involucra cambios extraordinarios.

Consecuentemente, **dicho artículo claramente evidencia que la eliminación de los átomos de flúor del anillo de oxazina no son cambios ni remplazos convencionales o de rutina para una persona versada en la materia.** Frente a tal afirmación, nuevamente me permito reiterar que no es un simple cambio de posición de los átomos de flúor. En realidad, la persona versada en la materia debería eliminarlos de la posición 5 del anillo de oxazina y luego incluir un grupo trifluorometilo en el sustituyente R⁴ de la fórmula, lo cual no es rutinario ni sugerido por D1.

Ahora, teniendo en cuenta que la literatura sugiere que anillos de oxazina difluorados exhibirían una menor inhibición de BACE1 y que los adición de un nuevo grupo funcional acarrea cambios relevantes en la actividad fisicoquímica del compuesto, es sorprendente e inesperado que los compuestos de la presente invención exhiban una actividad inhibitoria satisfactoria en comparación con los compuestos del estado del arte previo.

Consecuentemente, me permito afirmar que, contrario a lo que asegura el señor Examinador, los compuestos de la invención **sí** demuestran un efecto técnico sorprendente e inesperado dado que son oxazinas sin fluoración directa en el anillo central que cuentan con actividad inhibitoria de la BACE1. Esencialmente, la ventaja que presentan los compuestos de la presente invención consiste en que, esperando que no hubiese o no se conserve la actividad inhibitoria, las oxazinas reclamadas sin fluoración directa en el anillo central son capaces mostrar valores de inhibición entre los rangos de los compuestos del arte previo.

Frente a las anteriores consideraciones, me permito afirmar que **se descarta cualquier indicio de obviedad en la eliminación de los átomos de flúor de la posición 5 del anillo de oxazina y la posterior adición de un grupo trifluorometilo en el sustituyente R⁴ de D1 y se evidencia el nivel inventivo de los compuestos derivados de fluorometil-5,6-dihidro-4H-[1,3]oxazinas de la nueva reivindicación 1 de la presente solicitud.**

Ahora bien, la pregunta que en realidad que necesita ser resuelta, con el fin de decidir si la presente invención goza de carácter inventivo, es si la persona normalmente versada en la materia fue motivada por el documento D1 para modificar sus mismos compuestos, de manera que llegara a obtener los compuestos de la nueva reivindicación 1 con la selectiva y exclusiva actividad inhibitoria de BACE1.

Es más, **¿qué razón tendría la persona versada en la materia para que, a la luz del problema resuelto por la presente invención, decidiera modificar los compuestos de D1 de manera que las estructuras resultantes comprendan compuesto derivados de fluorometil-5,6-dihidro-4H- [1,3]oxazinas, y no otro tipo de modificación?**

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 particular modificación 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*". Conociendo de antemano la invención, el Señor Examinador está seleccionando partes específicas de las enseñanzas contenidas dentro del documento D1, las cuales modifica de forma que permiten construir la materia reclamada por la nueva reivindicación 1 de la presente solicitud. Dichas acciones o modificaciones de ningún modo 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 la eliminación de los átomos de flúor y la adición de un grupo trifluorometilo 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.

En vista de este señalamiento, y a la luz de lo discutido en el presente memorial, me permito resaltar que al no "*saltar a la vista de manera clara y directa*" a partir de las enseñanzas del documento D1 un compuesto derivado de fluorometil-5,6-dihidro-4H- [1,3]oxazinas, como el considerado en la presente invención, y al no tenerse "*certeza clara y manifiesta*" de que dicho compuesto conservaría el efecto inhibitorio de BACE1, es claro que el documento D1 no puede de ninguna manera afectar el Nivel Inventivo de la solicitud en estudio.

5. CONCLUSIÓN.

Para finalizar, me permito reiterar que el capítulo reivindicatorio modificado presentado junto con este memorial es totalmente claro y conciso al reclamar los novedosos compuestos derivados de fluorometil-5,6-dihidro-4H- [1,3]oxazinas que actúan como inhibidores de BACE1, y está plenamente soportado dentro del texto de la descripción, cumpliendo así con lo exigido por la mencionada Decisión Andina 486 de 2000.

Por otro lado, en virtud de los argumentos discutidos con respecto a los documentos citados, se estableció de manera incontrovertible que dichos compuestos de fórmulas I y Ia-1, los cuales son objeto de la solicitud en estudio, de ninguna manera resultarían evidentes para la persona versada en la materia a partir de las enseñanzas del documento D1, demostrando irrefutablemente la patentabilidad de la presente solicitud.

6. Anexo al presente memorial, me permito presentar:

- i) Copia de las páginas **84 a 91** que corresponden al **capítulo reivindicatorio modificado**, tal como se discutió en el presente memorial, constituido por **15** reivindicaciones; y
- ii) 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.

7. En vista de lo anterior, ruego a usted proceder a estudiar nuevamente la solicitud a la luz de las modificaciones y los argumentos presentados y conceder el privilegio de patente solicitado.

Señor Superintendente,

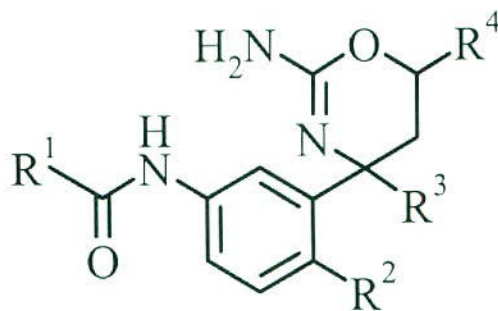


ALICIA LLOREDA RICAURTE

T.P. 53.215

REIVINDICACIONES

1. Un compuesto de la fórmula (I),



(I)

en la que:

R^1 es 1-(difluorometil)-1H-pirazol-3-ilo, 2-(fluorometil)oxazol-4-ilo, 3,5-dicloro-piridin-2-ilo, 3-cloro-5-cianopiridin-2-ilo, 3-metilpirazin-2-ilo, 4-cloro-1-(difluorometil)-1H-pirazol-3-ilo, 5-(2,2,2-trifluoroetoxi)-pirazin-2-ilo, 5-(2,2-difluoroetoxi)pirazin-2-ilo, 5-(but-2-iniloxi)pirazin-2-ilo, 5-(difluorometoxi)-pirazin-2-ilo, 5-(difluorometil)pirazin-2-ilo, 5-(fluoro-metoxi)pirazin-2-ilo, 5-(fluorometil)pirazin-2-ilo, 5-cloro-piridin-2-ilo, 5-ciano-3-metil-piridin-2-ilo, 5-ciano-piridin-2-ilo, 5-difluoro-metoxi-pirazin-2-ilo, 5-difluoro-metoxi-piridin-2-ilo, 5-difluorometil-piridin-2-ilo, 5-fluoro-metoxi-piridin-2-ilo, 5-metoxi-pirazin-2-ilo, 5-metoxi-piridin-2-ilo, 5-metil-pirazin-2-ilo, 5-trifluoroetoxi-piridin-2-ilo, 5-trifluoro-metoxi-pirazin-2-ilo o 5-trifluoro-metoxi-piridin-2-ilo;

R^2 se elige entre el grupo formado por:

- i) hidrógeno,
- ii) alquilo C_{1-6} y
- iii) halógeno;

R^3 se elige entre el grupo formado por:

- i) alquilo C_{1-6} y
- ii) halogeno-alquilo C_{1-6} ,

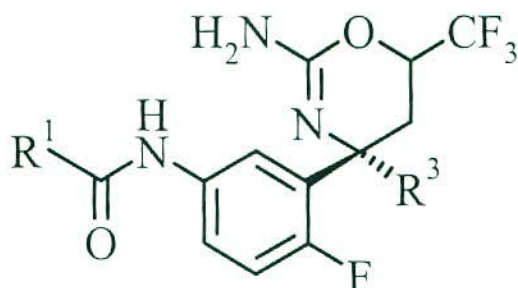
R^4 es halogeno-alquilo C_{1-6} ;

o las sales farmacéuticamente aceptables del mismo.

2. El compuesto según la reivindicación 1, en el que R^2 es halógeno.
3. El compuesto según cualquiera de las reivindicaciones 1 ó 2, en el que R^2 es F.
4. El compuesto según cualquiera de las reivindicaciones 1 a 3, en el que R^3 es fluoroo-alquilo C_{1-6} .
5. El compuesto según cualquiera de las reivindicaciones 1 a 4, en el que R^3 es fluorometilo.
6. El compuesto según cualquiera de las reivindicaciones 1 a 3, en el que R^3 es alquilo C_{1-6} .
7. El compuesto según cualquiera de las reivindicaciones 1 a 3 y 6, en el que R^3 es metilo.
8. El compuesto según cualquiera de las reivindicaciones 1 a 7, en el que R^4 es fluoroo-alquilo C_{1-6} .

9. El compuesto según cualquiera de las reivindicaciones 1 a 8, en el que R^4 es trifluorometilo.

10. El compuesto según una cualquiera de las reivindicaciones 1-10, que se ajusta a la fórmula Ia-1



Ia-1

en la que:

R^1 es 1-(difluorometil)-1H-pirazol-3-ilo, 2-(fluorometil)oxazol-4-ilo, 3,5-dicloro-piridin-2-ilo, 3-cloro-5-cianopiridin-2-ilo, 3-metilpirazin-2-ilo, 4-cloro-1-(difluorometil)-1H-pirazol-3-ilo, 5-(2,2,2-trifluoroetoxi)-pirazin-2-ilo, 5-(2,2-difluoroetoxi)pirazin-2-ilo, 5-(but-2-iniloxi)pirazin-2-ilo, 5-(difluorometoxi)-pirazin-2-ilo, 5-(difluorometil)pirazin-2-ilo, 5-(fluoro-metoxi)pirazin-2-ilo, 5-(fluorometil)pirazin-2-ilo, 5-cloro-piridin-2-ilo, 5-ciano-3-metil-piridin-2-ilo, 5-ciano-piridin-2-ilo, 5-difluoro-metoxi-pirazin-2-ilo, 5-difluoro-metoxi-piridin-2-ilo, 5-difluorometil-piridin-2-ilo, 5-fluoro-metoxi-piridin-2-ilo, 5-metoxi-pirazin-2-ilo, 5-metoxi-piridin-2-ilo, 5-metil-pirazin-2-ilo, 5-trifluoroetoxi-piridin-2-ilo, 5-trifluoro-metoxi-pirazin-2-ilo o 5-trifluoro-metoxi-piridin-2-ilo;

R^3 se elige entre el grupo formado por:

- i) alquilo C_{1-6} y
- ii) halogeno-alquilo C_{1-6} ,

o las sales farmacéuticamente aceptables del mismo.

11. El compuesto según cualquiera de las reivindicaciones 1 a 10, en el que R¹ es 5-ciano-piridin-2-ilo, 5-cloro-piridin-2-ilo, 5-(2,2-difluoroetoxi)pirazin-2-ilo, 5-(2,2,2-trifluoroetoxi)pirazin-2-ilo o 5-metoxipirazin-2-ilo.

12. El compuesto según cualquiera de las reivindicaciones 1 a 11, en el que R¹ es 5-ciano-piridin-2-ilo.

13. El compuesto según cualquiera de las reivindicaciones 1 a 12, elegido entre el grupo formado por:

N-(3-((4S,6S)-2-amino-4-metil-6-(trifluorometil)-5,6-dihidro-4H-1,3-oxazin-4-il)-4-fluorofenil)-5-cianopicolinamida,

N-(3-((4S,6S)-2-amino-4-metil-6-(trifluorometil)-5,6-dihidro-4H-1,3-oxazin-4-il)-4-fluorofenil)-5-cloropicolinamida,

N-(3-((4S,6S)-2-amino-4-(fluorometil)-6-(trifluorometil)-5,6-di-hidro-4H-1,3-oxazin-4-il)-4-fluorofenil)-5-cianopicolinamida,

N-(3-((4S,6S)-2-amino-4-(fluorometil)-6-(trifluorometil)-5,6-dihidro-4H-1,3-oxazin-4-il)-4-fluorofenil)-5-cloropicolinamida,

N-(3-((4S,6S)-2-amino-4-metil-6-(trifluoro-metil)-5,6-dihidro-4H-1,3-oxazin-4-il)-4-fluorofenil)-5-metoxipirazina-2-carboxamida,

N-(3-((4S,6S)-2-amino-4-metil-6-(trifluorometil)-5,6-dihidro-4H-1,3-oxazin-4-il)-4-fluoro-fenil)-5-(2,2-difluoroetoxi)pirazina-2-carboxamida,

N-(3-((4S,6S)-2-amino-4-metil-6-(trifluorometil)-5,6-dihidro-4H-1,3-oxazin-4-il)-4-fluorofenil)-5-(2,2,2-trifluoroetoxi)-pirazina-2-carboxamida,

N-(3-((4S,6R)-2-amino-4-metil-6-(tri-fluorometil)-5,6-dihidro-4H-1,3-oxazin-4-il)-4-fluorofenil)-5-cianopicolinamida,

N-(3-((4S,6R)-2-amino-4-metil-6-(trifluoro-metil)-5,6-dihidro-4H-1,3-oxazin-4-il)-4-fluorofenil)-5-cloro-picolinamida,

N-(3-((4S,6S)-2-amino-4-metil-6-(trifluoro-metil)-5,6-dihidro-4H-1,3-oxazin-4-il)-4-fluorofenil)-3,5-cloropicolinamida,

N-(3-((4S,6S)-2-amino-4-metil-6-(trifluoro-metil)-5,6-dihidro-4H-1,3-oxazin-4-il)-4-fluorofenil)-5-(fluorometoxi)picolinamida,

N-(3-((4S,6S)-2-amino-4-metil-6-(trifluorometil)-5,6-dihidro-4H-1,3-oxazin-4-il)-4-fluoro-fenil)-5-ciano-3-metilpicolinamida,

N-(3-((4S,6S)-2-amino-4-metil-6-(trifluorometil)-5,6-dihidro-4H-1,3-oxazin-4-il)-4-fluorofenil)-3-cloro-5-cianopicolinamida,

N-(3-((4S,6S)-2-amino-4-metil-6-(trifluorometil)-5,6-dihidro-4H-1,3-oxazin-4-il)-4-fluorofenil)-5-metoxipicolinamida,

N-(3-((4S,6S)-2-amino-4-metil-6-(trifluorometil)-5,6-dihidro-4H-1,3-oxazin-4-il)-4-fluorofenil)-5-(difluorometoxi)picolinamida,

N-(3-((4S,6S)-2-amino-4-metil-6-(trifluorometil)-5,6-dihidro-4H-1,3-oxazin-4-il)-4-fluorofenil)-5-(trifluorometoxi)-picolinamida,

N-(3-((4S,6S)-2-amino-4-metil-6-(trifluoro-metil)-5,6-dihidro-4H-1,3-oxazin-4-il)-4-fluorofenil)-5-(di-fluorometil)picolinamida,

N-(3-((4S,6S)-2-amino-4-metil-6-(trifluorometil)-5,6-dihidro-4H-1,3-oxazin-4-il)-4-fluoro-fenil)-5-(2,2,2-trifluoroetoxi)picolinamida,

N-(3-((4S,6S)-2-amino-4-metil-6-(trifluorometil)-5,6-dihidro-4H-1,3-oxazin-4-il)-4-fluorofenil)-5-(fluorometil)pirazina-2-carboxamida,

N-(3-((4S,6S)-2-amino-4-metil-6-(trifluorometil)-5,6-dihidro-4H-1,3-oxazin-4-il)-4-fluorofenil)-5-(difluorometil)pirazina-2-carboxamida,

N-(3-((4S,6S)-2-amino-4-metil-6-(trifluorometil)-5,6-dihidro-4H-1,3-oxazin-4-il)-4-fluorofenil)-5-metil-pirazina-2-carboxamida,

N-(3-((4S,6S)-2-amino-4-metil-6-(tri-fluorometil)-5,6-dihidro-4H-1,3-oxazin-4-il)-4-fluorofenil)-5-(but-2-iniloxi)pirazina-2-carboxamida,

N-(3-((4S,6S)-2-amino-4-metil-6-(trifluorometil)-5,6-dihidro-4H-1,3-oxazin-4-il)-4-fluorofenil)-5-(fluorometoxi)pirazina-2-carboxamida,

N-(3-((4S,6S)-2-amino-4-metil-6-(trifluorometil)-5,6-dihidro-4H-1,3-oxazin-4-il)-4-fluorofenil)-5-(difluorometoxi)pirazina-2-carboxamida,

N-(3-((4S,6S)-2-amino-4-metil-6-(trifluorometil)-5,6-dihidro-4H-1,3-oxazin-4-il)-4-fluorofenil)-2-(fluorometil)-oxazol-4-carboxamida,

N-(3-((4S,6S)-2-amino-4-metil-6-(tri-fluorometil)-5,6-dihidro-4H-1,3-oxazin-4-il)-4-fluorofenil)-4-cloro-1-(difluorometil)-1H-pirazol-3-carboxamida,

N-(3-((4S,6S)-2-amino-4-(fluorometil)-6-(trifluorometil)-5,6-di-hidro-4H-1,3-oxazin-4-il)-4-fluorofenil)-5-ciano-3-metil-picolinamida,

N-(3-((4S,6S)-2-amino-4-(fluorometil)-6-(tri-fluorometil)-5,6-dihidro-4H-1,3-oxazin-4-il)-4-fluorofenil)-3-cloro-5-cianopicolinamida,

N-(3-((4S,6S)-2-amino-4-(fluoro-metil)-6-(trifluorometil)-5,6-dihidro-4H-1,3-oxazin-4-il)-4-fluorofenil)-3,5-dicloropicolinamida,

N-(3-((4S,6S)-2-amino-4-(fluorometil)-6-(trifluorometil)-5,6-dihidro-4H-1,3-oxazin-4-il)-4-fluorofenil)-5-metoxipicolinamida,

N-(3-((4S,6S)-2-amino-4-(fluorometil)-6-(trifluorometil)-5,6-dihidro-4H-1,3-oxazin-4-il)-4-fluorofenil)-5-(difluorometoxi)picolinamida,

N-(3-((4S,6S)-2-amino-4-(fluorometil)-6-(trifluorometil)-5,6-di-hidro-4H-1,3-oxazin-4-il)-4-fluorofenil)-5-(trifluorometoxi)-picolinamida,

N-(3-((4S,6S)-2-amino-4-(fluorometil)-6-(tri-fluorometil)-5,6-dihidro-4H-1,3-oxazin-4-il)-4-fluorofenil)-5-(difluorometil)picolinamida,

N-(3-((4S,6S)-2-amino-4-(fluoro-metil)-6-(trifluorometil)-5,6-dihidro-4H-1,3-oxazin-4-il)-4-fluorofenil)-5-(2,2,2-trifluoroetoxi)picolinamida,

N-(3-((4S,6S)-2-amino-4-(fluorometil)-6-(trifluorometil)-5,6-di-hidro-4H-1,3-oxazin-4-il)-4-fluorofenil)-5-(fluorometoxi)-picolinamida,

N-(3-((4S,6S)-2-amino-4-(fluorometil)-6-(tri-fluorometil)-5,6-dihidro-4H-1,3-oxazin-4-il)-4-fluorofenil)-5-metoxipirazina-2-carboxamida,

N-(3-((4S,6S)-2-amino-4-(fluorometil)-6-(trifluorometil)-5,6-dihidro-4H-1,3-oxazin-4-il)-4-fluorofenil)-5-(fluorometil)-pirazina-2-carboxamida,

N-(3-((4S,6S)-2-amino-4-(fluorometil)-6-(trifluorometil)-5,6-dihidro-4H-1,3-oxazin-4-il)-4-fluorofenil)-5-(but-2-iniloxi)-pirazina-2-carboxamida,
 N-(3-((4S,6S)-2-amino-4-(fluorometil)-6-(trifluorometil)-5,6-dihidro-4H-1,3-oxazin-4-il)-4-fluoro-fenil)-5-(difluorometoxi)pirazina-2-carboxamida,
 N-(3-((4S,6S)-2-amino-4-(fluorometil)-6-(trifluorometil)-5,6-di-hidro-4H-1,3-oxazin-4-il)-4-fluorofenil)-5-(2,2-difluoroetoxi)-pirazina-2-carboxamida,
 N-(3-((4S,6S)-2-amino-4-(fluorometil)-6-(trifluorometil)-5,6-dihidro-4H-1,3-oxazin-4-il)-4-fluoro-fenil)-5-(2,2,2-trifluoroetoxi)pirazina-2-carboxamida,
 N-(3-((4S,6S)-2-amino-4-(fluorometil)-6-(trifluorometil)-5,6-di-hidro-4H-1,3-oxazin-4-il)-4-fluorofenil)-5-metilpirazina-2-carboxamida,
 N-(3-((4S,6S)-2-amino-4-(fluorometil)-6-(tri-fluorometil)-5,6-dihidro-4H-1,3-oxazin-4-il)-4-fluorofenil)-5-(fluorometoxi)pirazina-2-carboxamida,
 N-(3-((4S,6S)-2-amino-4-(fluorometil)-6-(trifluorometil)-5,6-dihidro-4H-1,3-oxazin-4-il)-4-fluorofenil)-3-metilpirazina-2-carboxamida,
 N-(3-((4S,6S)-2-amino-4-(fluorometil)-6-(trifluorometil)-5,6-di-hidro-4H-1,3-oxazin-4-il)-4-fluorofenil)-5-(difluorometil)-pirazina-2-carboxamida,
 N-(3-((4S,6S)-2-amino-4-(fluorometil)-6-(trifluorometil)-5,6-dihidro-4H-1,3-oxazin-4-il)-4-fluoro-fenil)-4-cloro-1-(difluorometil)-1H-pirazol-3-carboxamida,
 N-(3-((4S,6S)-2-amino-4-(fluorometil)-6-(trifluorometil)-5,6-di-hidro-4H-1,3-oxazin-4-il)-4-fluorofenil)-2-(fluorometil)oxazol-4-carboxamida,
 N-(3-((4S,6R)-2-amino-4-(difluorometil)-6-(tri-fluorometil)-5,6-dihidro-4H-1,3-oxazin-4-il)-4-fluorofenil)-5-cianopicolinamida, y
 N-(3-((4S,6S)-2-amino-4-(difluorometil)-6-(trifluorometil)-5,6-dihidro-4H-1,3-oxazin-4-il)-4-fluoro-fenil)-5-cianopicolinamida;

o una sal farmacéuticamente aceptable de los mismos.

14. El compuesto según cualquiera de las reivindicaciones 1 a 13, elegido entre el grupo formado por:

N-(3-((4S,6S)-2-amino-4-metil-6-(trifluorometil)-5,6-dihidro-4H-1,3-oxazin-4-il)-4-fluorofenil)-5-cianopicolinamida,

N-(3-((4S,6S)-2-amino-4-metil-6-(trifluorometil)-5,6-dihidro-4H-1,3-oxazin-4-il)-4-fluorofenil)-5-cloropicolinamida,

N-(3-((4S,6S)-2-amino-4-(fluorometil)-6-(trifluorometil)-5,6-dihidro-4H-1,3-oxazin-4-il)-4-fluorofenil)-5-ciano-picolinamida,

N-(3-((4S,6S)-2-amino-4-(fluorometil)-6-(trifluorometil)-5,6-dihidro-4H-1,3-oxazin-4-il)-4-fluorofenil)-5-cloro-picolinamida,

N-(3-((4S,6S)-2-amino-4-metil-6-(trifluorometil)-5,6-dihidro-4H-1,3-oxazin-4-il)-4-fluorofenil)-5-metoxipirazina-2-carboxamida,

N-(3-((4S,6S)-2-amino-4-metil-6-(trifluorometil)-5,6-dihidro-4H-1,3-oxazin-4-il)-4-fluorofenil)-5-(2,2-difluoroetoxi)-pirazina-2-carboxamida, y

N-(3-((4S,6S)-2-amino-4-metil-6-(trifluorometil)-5,6-dihidro-4H-1,3-oxazin-4-il)-4-fluorofenil)-5-(2,2,2-trifluoroetoxi)-pirazina-2-carboxamida;

o una sal farmacéuticamente aceptable de los mismos.

15. La composición farmacéutica que contiene un compuesto de la fórmula I según cualquiera de las reivindicaciones 1 a 14 y un vehículo farmacéuticamente aceptable y/o una sustancia auxiliar farmacéuticamente aceptable.

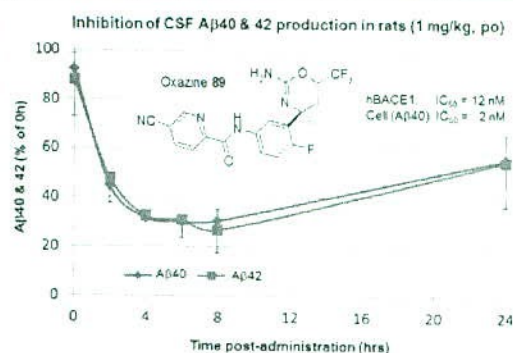
β -Secretase (BACE1) Inhibitors with High in Vivo Efficacy Suitable for Clinical Evaluation in Alzheimer's Disease

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 Kuglstatter,[§] Pascale David-Pierson,^{||} Holger Fischer,^{||} Alessandra Polara,[⊥] and Robert Narquizian[⊥]

[†]Discovery Chemistry, [‡]DTA Neuroscience, [§]Discovery Technologies, ^{||}Drug Metabolism and Pharmacokinetics, [⊥]Roche Partnering, F. Hoffmann-La Roche Ltd, pRED, Pharma Research & Early Development, Grenzacherstrasse 124, Basel CH-4070, Switzerland

S 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 (IC_{50} = 41.2 μ M) 2-amino-4*H*-5,6-dihydro-[1,3]thiazine derivative **A** (Figure 1) which could be cocrystallized with

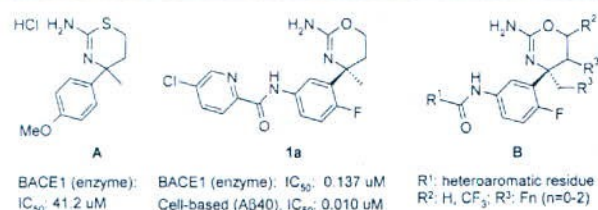


Figure 1. Lead compound **1a** and targeted fluoro-oxazines **B**.

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

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.

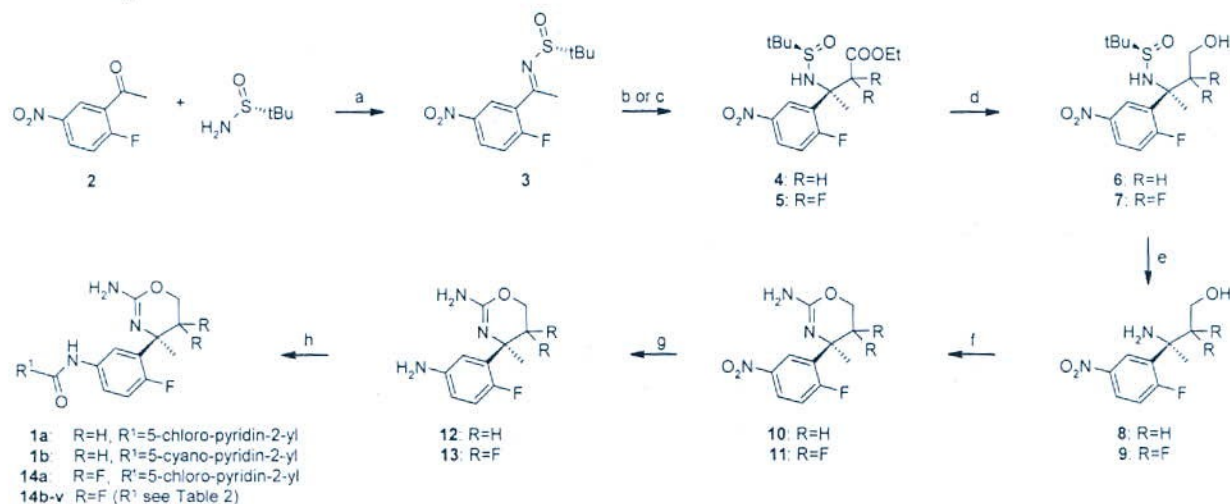
CHEMISTRY

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**.

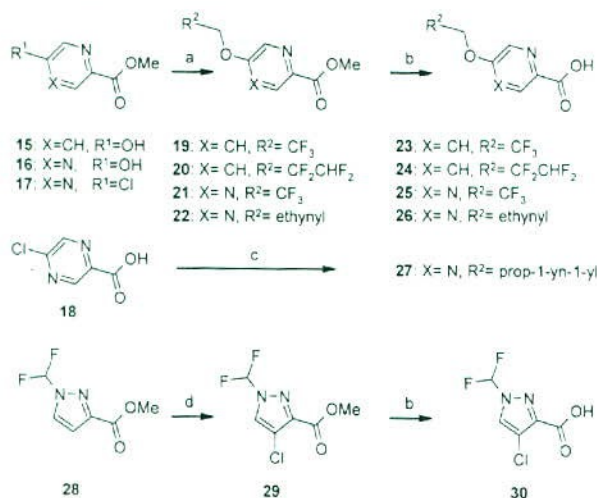
The ester group in **4** and **5** was then reduced to the alcohols **6** and **7** with LiBH₄, which was followed by cleavage of the chiral auxiliary under acidic conditions to give the amino alcohols **8** and **9**.³² Cyclization of the amino alcohols **8** and **9** was readily accomplished with cyanogen bromide to give the oxazines **10** and **11**, which were reduced to the corresponding anilines **12**³³ 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 R^1 residues in difluoro-oxazine **14** (Table 2), were made available from pyridine and pyrazine derivatives

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

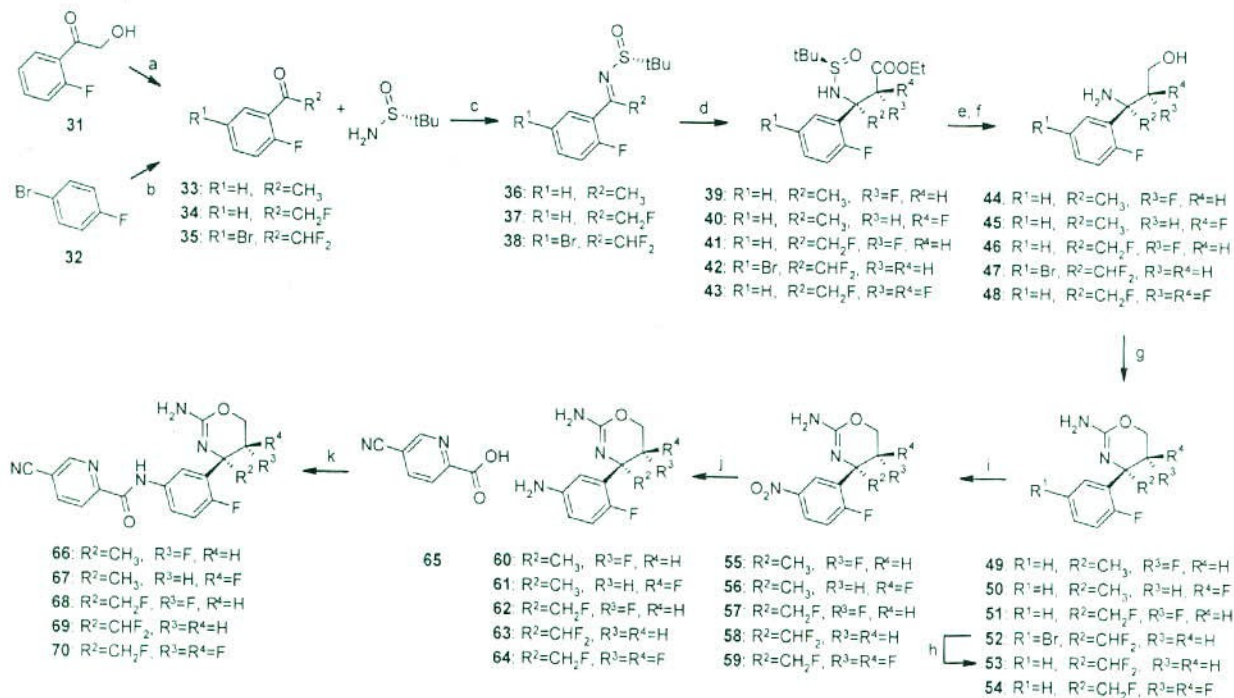
^aReagents and conditions: (a) 15→19: CF₃CH₂OTf, NaH, DMF, 22 °C. 15→20: CHF₂CH₂OTf, K₂CO₃, acetone. 17→21: CF₃CH₂OH, Cs₂CO₃, DMF. 17→22: prop-2-yn-1-ol, *tert*-BuOK, 1,4-dioxane, 22 °C. (b) LiOH or NaOH, THF/H₂O, 22 °C to reflux. (c) But-2-yn-1-ol, *tert*-BuOK, DMF, 65 °C. (d) *N*-chlorosuccinimide, 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).

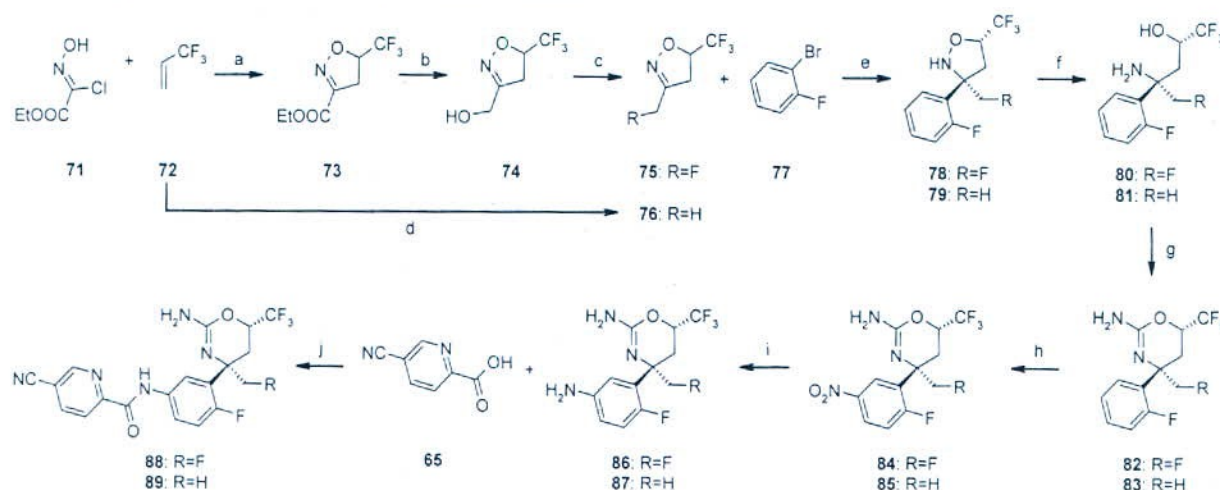
Compound 27 was prepared directly from the acid 18 without proceeding via the corresponding ester. Finally, chlorination of the pyrazole ester 28 with *N*-chlorosuccinimide followed by saponification delivered the chloro-pyrazole derivative 30.

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.

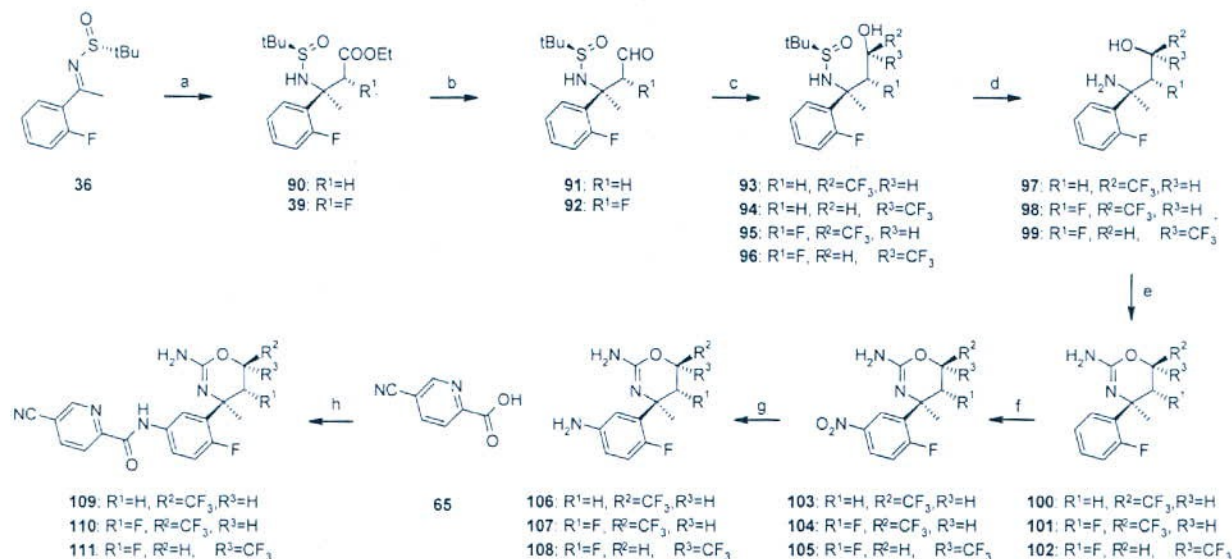
The synthesis of trifluoromethyl substituted oxazines 88 and 89 (Scheme 4) required a different strategy. Ready access to CF₃ substituted 3-amino-propan-1-ol derivatives 80 and 81 is achieved using a dipolar cycloaddition reaction of a nitrile oxide

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, 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.

Scheme 4. Synthesis of Trifluoromethyl-oxazines 88 and 89^a

^aReagents and conditions: (a) NaHCO₃, EtOAc, -65 to 22 °C. (b) NaBH₄, EtOH, 0 °C. (c) Deoxo-Fluor, CH₂Cl₂, -78 to 22 °C. (d) 72→76: nitroethane, phenylisocyanate, Et₃N (cat.), Et₂O, -78 to 22 °C. (e) THF, *n*-BuLi, BF₃·Et₂O, -78 to 22 °C. (f) HCOONH₄, Pd/C, EtOH, 22 °C. (g) BrCN, EtOH, 85 °C. (h) H₂SO₄, HNO₃, 0 °C. (i) H₂, Pd/C, EtOH, Et₃N, 22 °C. (j) DMTMM, MeOH, 0 to 22 °C.

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

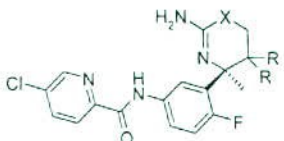
^aReagents and conditions: (a) 36→39: BrCHFCOOEt, Zn, CuCl, THF. 36→90: BrCH₂COOEt, Zn, CuCl, THF. (b) DIBAH, toluene, -76 °C. (c) Trimethyl(trifluoromethyl)silane, THF, TBAF, 0 °C. (d) aq HCl, THF, 22 °C. (e) BrCN, EtOH, 85 °C. (f) H₂SO₄, HNO₃, 0 °C. (g) H₂, Pd/C, EtOH, Et₃N, 22 °C. (h) DMTMM, MeOH, 0–22 °C.

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

chiral compounds 78 and 79. Transfer hydrogenation employing ammonium formate and Pd/C led to the amino alcohols 80 and 81, which were then converted to the CF₃-substituted oxazines 88 and 89 following the procedures employed in Scheme 3. The relative and absolute configurations were assigned based on X-ray structures of 88 and 89 complexed to BACE1.

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 DIBAH 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 **112**²³ 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} values below 0.3 μM , 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, $V_{ss} = 2.9$ 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 ($IC_{50} = 0.151, 0.036$, 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

compd	IC ₅₀ (μM) ^a		selectivity BACE 2/1	IC ₅₀ (μM) ^a Aβ40 cell-based ^b	shift BACE1 enzyme vs cell- based	brain Aβ40 reduction in mice at 30/10 mg/kg ^c (%)
	hBACE1 enzyme	hBACE2 enzyme				
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

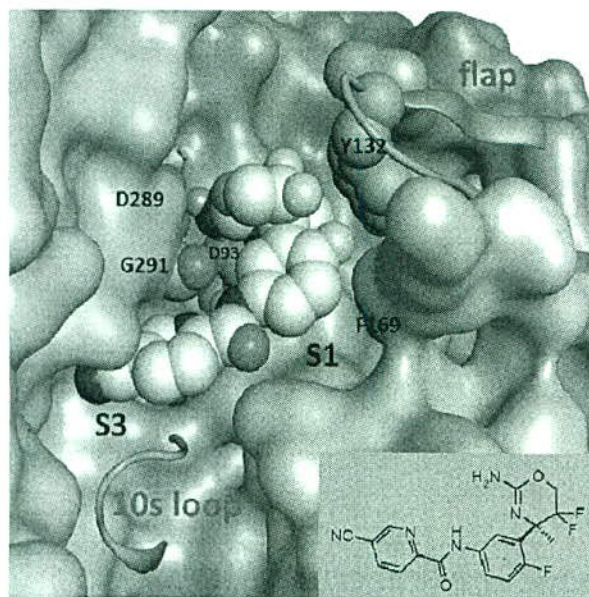


Figure 2. 14d bound to the BACE1 binding site. The amidine recognition motif is tightly bound to the catalytic aspartates D289 and D93 (partially hidden) in the bottom of the pocket. The fluorine atoms in the headgroup and the *o*-F substituent of the phenyl ring (marked in green) form a network of vdW 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.

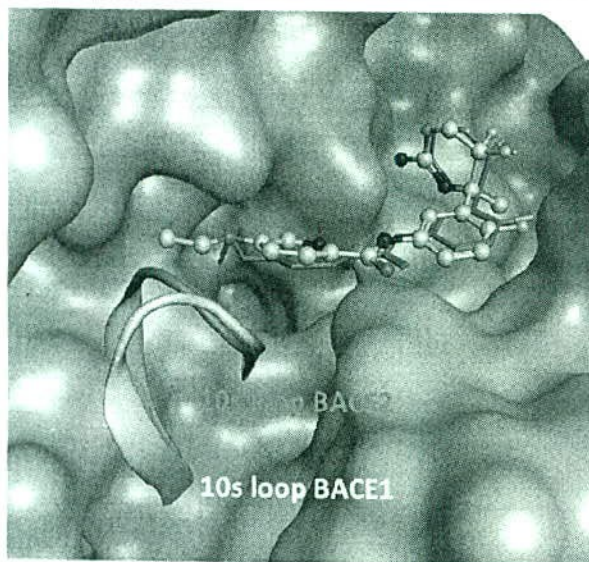


Figure 3. 14i bound to BACE1 (yellow, ball and stick representation) and BACE2 (magenta, stick representation). The molecular surface has been generated for BACE1, and the 10s loops of BACE1 and BACE2 are depicted separately. The ethoxy side chain in 14i prefers a planar orientation around the C–O bond. 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

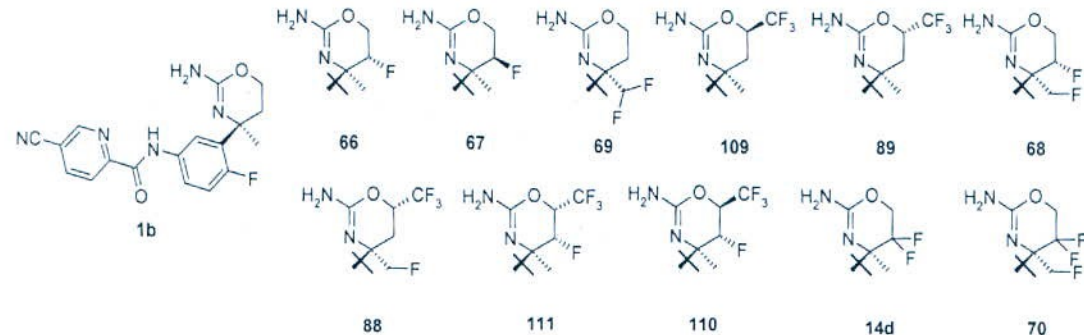
the protonated amidine motif in the oxazine headgroup and the catalytic aspartates. The fluorine atoms in position 5 and the *o*-F substituent of the phenyl ring (S1 pocket) are engaged in a network of van der Waals interactions to Y132 located in the flap and to F169 at the rim of the S1 pocket. A hydrogen 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,^{47,51} 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

											
IC ₅₀ (μM) ^a											
compd	pK _a ^b	ΔpK _a to 1b	log D ^c	log P	Δlog D/log P	hBACE1 enzyme	A/40 cell-based ^d	shift BACE1 enzyme vs cell-based	P-gp ER ^e	hERG inhibition at 10 μM (%)	brain A/40 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.

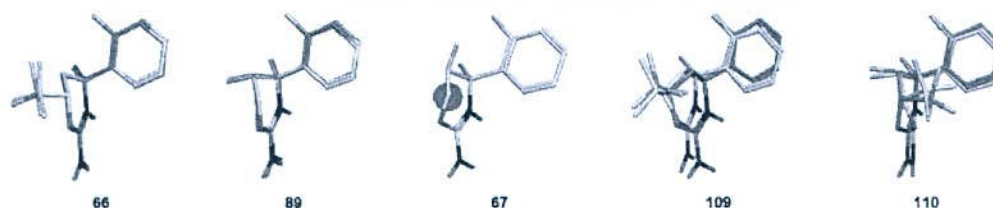


Figure 4. X-ray structures (yellow) of fluorinated head groups with the S1 phenyl ring were energy minimized in the unbound state by a semiempirical geometry optimization with the PM3 Hamiltonian (magenta). The coordinates of 66 and 89 remain almost unchanged as compared to the bound conformation. Therefore, 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)

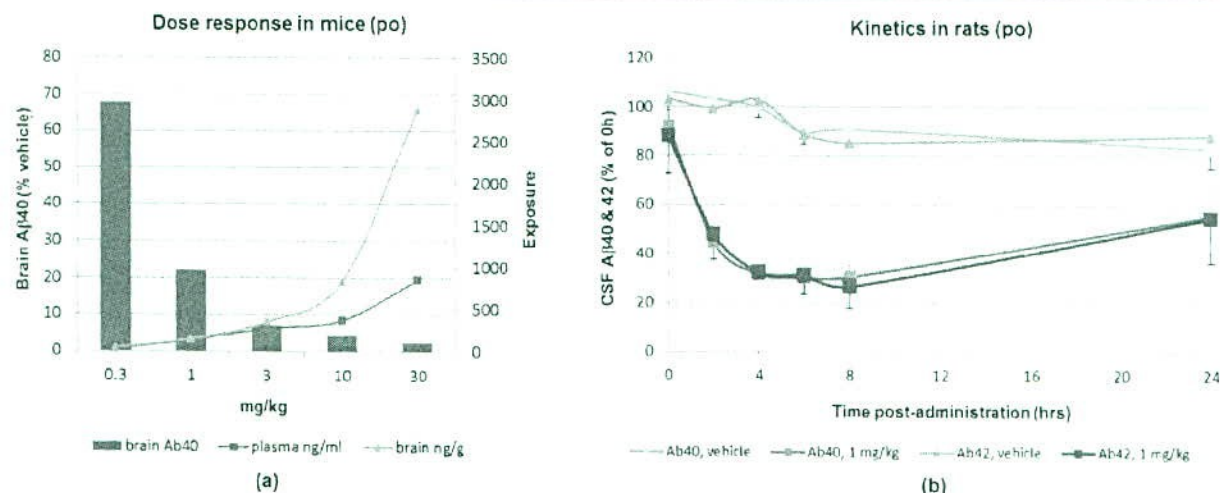


Figure 5. (a) Dose dependent inhibition of brain Aβ40 production in mice by compound 89 determined 4 h oral post dose, $n = 3-4$, mean reduction as compared to vehicle control. (b) Time dependent inhibition of CSF Aβ40/42 production in rats after an oral dose of 1 mg/kg of compound 89, $n = 2-3$. CSF 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 μM .

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 $0.010 \mu M$) was lower compared to 66 ($IC_{50} = 0.001 \mu M$) 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 $IC_{50} = 0.002 \mu M$, 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 (CS7Bl/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: microcapsule 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 an 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-sulfonic Acid [1-(2-Fluoro-5-nitro-phenyl)-(E)-ethylidene]-amide (3).³² To a solution of the (R)-(+)-tert-butylsulfinamide (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. (C₁₂H₁₅FN₂O₃S) 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-sulfonic 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. ($\text{C}_{16}\text{H}_{21}\text{F}_3\text{N}_3\text{O}_5\text{S}$) 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. ($\text{C}_{14}\text{H}_{19}\text{F}_3\text{N}_3\text{O}_5\text{S}$) 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. ($\text{C}_{10}\text{H}_{11}\text{F}_3\text{N}_3\text{O}_3$) 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 **13** (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 $\text{C}_{15}\text{H}_{13}\text{F}_3\text{N}_5\text{O}_2$, 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 (Chiralpak 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, $J = 12.4, 6.0, 6.0, 2.8$ 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, $J = 12.3, 6.0, 6.0, 2.8$ 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, $J = 12.2, 6.0, 6.0, 2.6$ 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 WSEVNLDAEFR-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 WIPHPHLVHTC-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× 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% CO₂ 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 μ L of culture supernatants were added to 2 μ L of AlphaLISA Anti hu A β acceptor beads (50 μ g/mL), 5 nM Biotinylated Antibody Anti A β 1–40, 1× AlphaLISA HiBlock buffer solution and incubated for 1 h at room temperature. Then 16 μ L of a Streptavidin Donor beads (250 ng/mL in 1× 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 IC₅₀ values were calculated using the Excel XLfit software.

P-gp Assay. Bidirectional transport was examined in LLC-PK1 cells (Lewis-lung cancer porcine kidney 11, 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 cm², pore size 3.0 μ m), 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 μ M), and the cell monolayer integrity was assessed via the permeability of lucifer yellow (10 μ M). 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 μ M) and lucifer yellow to the donor compartments. The plates were incubated for 3.5 h at 37 °C, 5% CO₂, 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: $P_{app} = 1/(A \cdot C_0) \cdot (dQ/dt)$, 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 MagnaLysor (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 dH₂O (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% NH₄OH in 90% MeOH. The eluate was dried over a N₂ flow, and the dried sample was reconstituted in 30 μ L 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 μ L of the reconstituted sample were mixed with 5 μ L of biotinylated BAP-24 (specific for C-terminus of A β 40⁶²), stock = 4.4 mg/mL, f.c.5.5 μ g/mL), and 5 μ L 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 μ L 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.³⁷ 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 4J0P (1b), 3ZMG (14d), 4J0T (14i), 4J0V (66), 4J0Y (67), 4J0Z (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

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■ ABBREVIATIONS USED

HERG, human ether-à-go-go related gene; DCM, dichloromethane; Deoxo-Fluor, bis(2-methoxyethyl)aminosulfur tri-fluoride; 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*-butylmethyl ether; TEA, triethylamine; TFA, trifluoroacetic 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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