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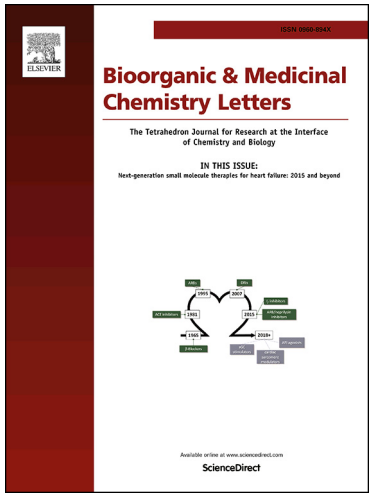

Discovery of amino-1,4-oxazines as potent BACE-1 inhibitors

Siem Jakob Veenstra, Heinrich Rueeger, Markus Voegtle, Rainer Lueoend, Philipp Holzer, Konstanze Hurth, Marina Tintelnot-Blomley, Mathias Frederiksen, Jean-Michel Rondeau, Laura Jacobson, Matthias Staufenbiel, Ulf Neumann, Rainer Machauer

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Graphical Abstract

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Discovery of amino-1,4-oxazines as potent BACE inhibitors

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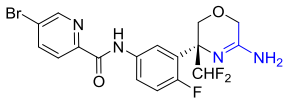
Siem Jakob Veenstra [a], Heinrich Rueeger [a], Markus Voegtle [a], Rainer Lueoend [a], Philipp Holzer [a], Konstanze Hurth [a], Marina Tintelnot-Blomley [a], Mathias Frederiksen [a], Jean-Michel Rondeau [b], Laura Jacobson [c], Matthias Staufenbiel [c], Ulf Neumann [c] and Rainer Machauer [a,*]



8a (rac.)

BACE-1 IC_{50}: 58 nM
cellular IC_{50}: 8 nM
-20% Aβ at 4 h & 30 mg/kg

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Discovery of amino-1,4-oxazines as potent BACE-1 inhibitors

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ABSTRACT

New amino-1,4-oxazine derived BACE-1 inhibitors were explored and various synthetic routes developed. The binding mode of the inhibitors was elucidated by co-crystallization of **4** with BACE-1 and X-ray analysis. Subsequent optimization led to inhibitors with low double digit nanomolar activity in a biochemical and single digit nanomolar potency in a cellular assays. To assess the inhibitors for their permeation properties and potential to cross the blood-brain-barrier a MDR1-MDCK cell model was successfully applied. Compound **8a** confirmed the *in vitro* results by dose-dependently reducing Aβ levels in mice in an acute treatment regimen.

BACE-1 (Beta-site Amyloid precursor protein Cleaving Enzyme) is a prime target for the treatment of Alzheimer's disease (AD) since it catalyzes the first step of the APP (amyloid precursor protein) processing.[1] The amyloid-β (Aβ) peptides resulting from the APP processing by BACE-1 and gamma-secretase are thought to trigger downstream events ultimately resulting in neurodegeneration and the development of AD following the widely accepted amyloid hypothesis.[2-4] However, Phase III trials with the BACE-1 inhibitor verubecestat have recently failed in AD patients at early and at mild-to-moderate disease states, which are known to have fully established amyloid-β pathology. This is widely interpreted as a strong hint that anti-amyloid-β treatment has to start early, possibly at pre-symptomatic stages of the disease. In line with this, clinical studies at pre-symptomatic AD stages have been initiated.[5]

From our work on macrocyclic peptidomimetic BACE-1 inhibitors carrying hydroxyethylene or ethanolamine transition state mimetics (TSM) [6, 7] and on cyclic sulfoxide / sulfone inhibitors [8, 9] we concluded that for drug-like, brain penetrant BACE-1 inhibitors it will be crucial to limit the number of H-bond donors and acceptors to an absolute minimum and, at the same time, to adjust the pKa of the moiety interacting with the catalytic aspartic acids of BACE-1 around the physiological pH, between 6 and 8.[6]

Recently a number of groups [10-18] have described BACE-1 inhibitors that employ a 2-aminoheterocycle/cyclic amidine, which engages in hydrogen bonding interactions with the catalytic aspartic acid residues of the protease. Those inhibitors often only need to fill the S1 and S3 sub-pockets of the protease (nomenclature according to Schechter and Berger[19], the parts of the inhibitor filling S1 and S3 are designated as P1 and P3, respectively), leading to potent inhibitors in the drug-like molecular weight range (<500 Da). In contrast, peptidomimetic inhibitors usually need to occupy multiple S and/or S' pockets to achieve nanomolar potency.

In 2008, when we started our work on this class of inhibitors, a few early examples had emerged from fragment screening activities [20] like 6- and 5-membered acyl-guanidines **1** and **2** [21, 22] and amino-1,3-thiazines **3** [23].

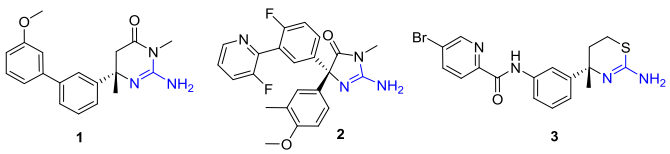


Figure 1. Examples of non-peptidic BACE-1 inhibitors.

Our own efforts led to the discovery of a series of amino-1,4-oxazine inhibitors exemplified by **4** (Figure 2). When we did a broad substructure search in the literature on this structural class we were surprised to see that the core structure **A** (Figure 2, with A = any atom, no ring annelated to the cyclic amidine) yielded only two 5-(dimethylamino)-3,6-dihydropyrazin-2(1H)-one examples published in 1995.[24] Similarly the corresponding

lactam precursor **B** (Figure 2) was barely known. Two literature references described them as intermediates for aldosterone synthase inhibitors.[25, 26]

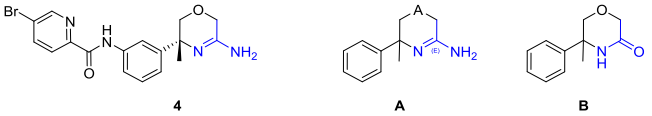


Figure 2. First example of new amino-1,4-oxazine inhibitors, corresponding Markush structure and potential precursor.

For the first examples of this new compound class we envisioned synthetic routes via suitable lactams **B**, whereas for later examples no lactam intermediates were isolated. Since all routes required at some point a chiral amino-alcohol intermediate **C**, the first challenge was always to establish high yielding, highly stereoselective approaches to those amino-alcohols. Classical amino-acid chemistry or more recent Ellman methodologies [27] proved to be very versatile. The amino-alcohols were then further transformed to intermediates which were cyclized under a broad variety of conditions, depending on the substitution pattern of the final amino-1,4-oxazine core **D** (Scheme 1).[28, 29]

fluorines next to the basic amidine functionality without significantly altering the core structure. Our first activities therefore focused on the fluorination pattern at the quaternary methyl group (table 1).

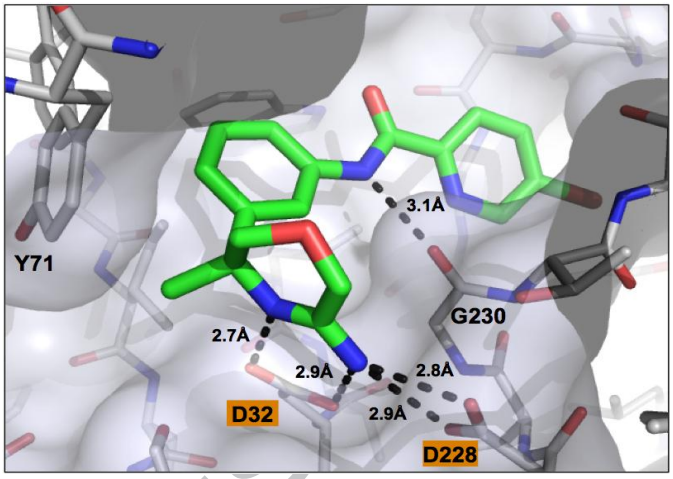
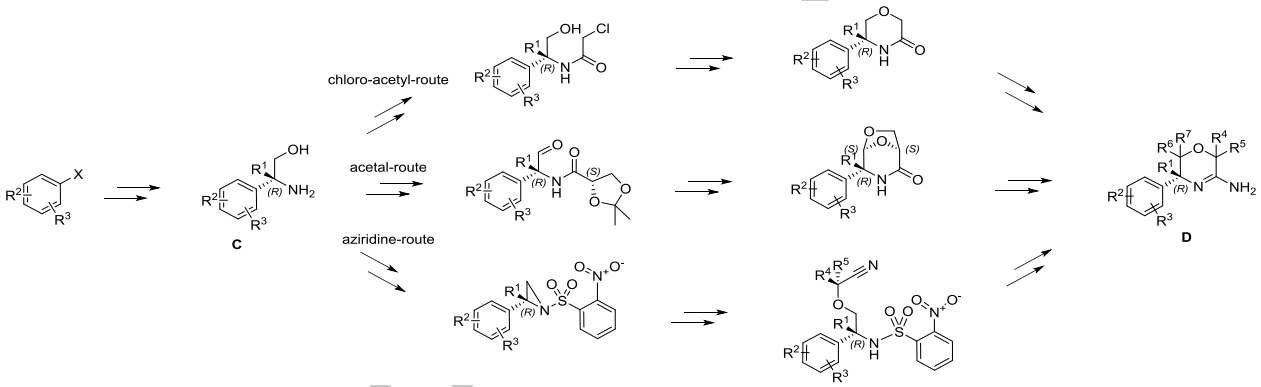


Figure 3. Crystal structure of BACE-1 in complex with **4**, showing key H-bonded interactions.



Scheme 1. General overview/key intermediates of selected approaches to amino-1,4-oxazine cores **D**.

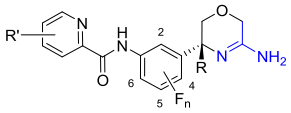
As shown in previous studies at Novartis, the pK_a of the moiety interacting with the catalytic aspartic acids of BACE-1 (Asp32 and Asp228) is of pivotal importance for cellular potency and brain penetration.[6] We also anticipated a relationship between pK_a and efflux.[8, 9, 30] Therefore it was important to have a flexible synthetic strategy allowing fine-tuning of the pK_a of the basic amidine unit.

The first compound prepared (**4**) was reasonably potent on BACE-1 with an IC_{50} of 70 nM; it had a pK_a of 9.5 and exhibited strong P-glycoprotein (P-gp)-mediated efflux, as measured in MDR1-transfected MDCK cells.[31] The binding mode of the compound to BACE-1 was elucidated by co-crystallization of racemic **4** with BACE-1 and X-ray analysis at 1.54 Å resolution (Figure 3).[32] The exocyclic nitrogen interacted with both catalytic aspartic acids and the endocyclic nitrogen interacted with Asp32. The P1/P3 amide NH formed a hydrogen bond to the carbonyl oxygen of Gly230. The oxazine ring oxygen and amide carbonyl were not involved in any direct H-bonded contacts. The quaternary methyl group was in close contact to Tyr71 (3.5Å) and Asp32 (3.5Å) while the P1 phenyl ring made hydrophobic interactions to Leu30, Tyr71, Phe108, Trp115 and Ile118.

To adjust the pK_a to the desired range we investigated the incremental effects of fluorine substituents in proximity of the amidine.[33] We looked for an opportunity to introduce one to three

The profiling of the first examples showed that this series of 1,4-oxazines indeed is capable of inhibiting the BACE-1 enzyme in the low micromolar (**9**) to double-digit nanomolar (**8b**) activity range. The more active enantiomers have the (*R*) configuration. The introduction of one or two fluoro substituents at the quaternary methyl group gave potent inhibitors with incrementally lowered pK_a by approximately one unit for every fluorine introduced. Three fluoro substituents at the quaternary methyl (**7**) led to considerable loss of potency, the CF_3 group presumably being too bulky, in view of the close contacts to Tyr71 and Asp32 mentioned above. In cellular assays all compounds showed nanomolar activity for the inhibition of Aβ release. Introduction of fluorine substituents to the P1 phenyl ring led to measurable, albeit smaller, reduction of the pK_a. Introducing an additional fluorine in the 4-position of the P1 phenyl (**8a/b**), pointing toward Tyr71, reduced the pK_a from 7.1 (**6**) to 6.8 and the efflux ratio from 7 to 4-5, at the same time improving the potency by roughly a factor of 2 (Table 1). For these reasons, we decided to keep this fluorine in all later inhibitors. A second fluorine on the central aromatic core resulted in a more complex picture. The 4,6-difluoro substitution (**9**) led to considerable loss of potency. Presumably, a repulsive interaction between the 6-F substituent and the amide carbonyl affected the conformation of the latter, thereby weakening the H-bonded interaction to the main-chain carbonyl of Gly230 (Figure 3 and 4).

Table 1. Basic structure-activity / pK_a exploration of P1 phenyl and quaternary methyl of amino-1,4-oxazine



Compounds	R	R'	F_n	BACE-1 IC_{50} [nM]	cellular IC_{50} [nM]	measured pK_a	MDR1-MDCK cells flux [10^{-6}cm/s]	efflux ratio
4	CH_3	5-Br	-	70	17	9.5	19	30
5	CH_2F	5-Br	-	170	4	8.2	19	28
6	CHF_2	5-Br	-	38	15	7.1	15	7
7*	CF_3	5-Cl	-	352	324	6.2	33	1
8a*	CHF_2	5-Br	4-F	56	8	6.8	24	5
8b				14	3	6.8	16	4
9*	CHF_2	5-Br	4,6-di-F	1119	300	6.5	15	2
10*	CHF_2	5-Br	2,4-di-F	701	69	7.6	25	2
11	CHF_2	3-Me, 5-CN	4,5-di-F	20	9	6.6	29	2

* racemate [For synthetic reasons some of the compounds were prepared as racemates].

The 2,4-substitution (**10**) also led to a loss of potency, albeit to a lesser extent, presumably due to a direct repulsive interaction between the 2-F substituent and Gly230 of BACE-1 (Figure 4). This substitution pattern led to a highly congested structure with a pK_a of 7.6, approximately 1 unit higher than anticipated.

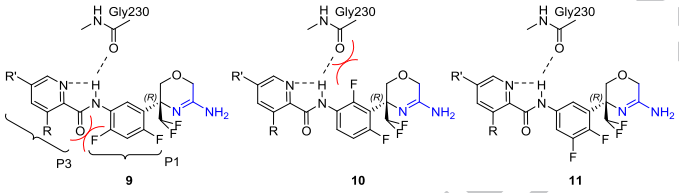


Figure 4. Fluorination patterns and amide conformations/interactions.

In contrast, the 4,5-difluoro derivative (**11**) was devoid of such intra- or inter-molecular repulsive interactions and was a potent BACE-1 inhibitor with its pK_a in the expected range (pK_a 6.6).

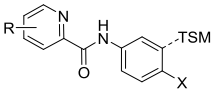
All compounds showed stronger potency in the cellular Aβ release assay in comparison to the biochemical enzyme inhibition assay. This ratio was not substantially influenced by the changes in pK_a. More striking was the effect of the pK_a on the efflux observed in the MDR1-MDCK cellular transport assay. All compounds showed good to very good flux, and a clear decrease in the P-gp-mediated efflux when going from the most basic amidines **4** and **5** to the less basic **6** and **7**. Based on our previous experience with other chemical series of BACE-1 inhibitors, the MDR1-MDCK efflux ratio is a good predictor of the *in vivo* brain permeation. Therefore, 1,4-oxazine inhibitors with MDR1-MDCK efflux ratios ≤ 5 appeared attractive to us.

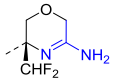
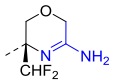
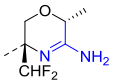
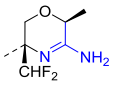
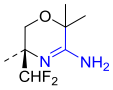
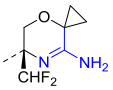
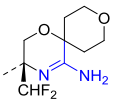
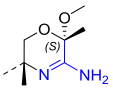
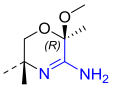
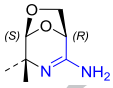
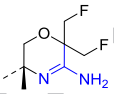
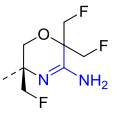
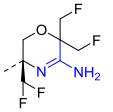
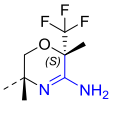
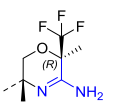
To double-check whether this correlation would also hold true in the case of this new series of BACE-1 inhibitors, compound **8a** was tested in the APP51/16 mouse model[34], using two oral doses and the 4 h time point. Indeed, brain/blood ratios of 1.3 and 1.4 and reductions of forebrain Aβ of 20% and 63% were observed at the lower (30 µmol/kg) and higher oral dose (100 µmol/kg), respectively. Since the *in vivo* potency of this early example was already in the range of the previously reported **NB-216**,[6] it strongly supported further exploration of this series.

We also wanted to investigate the effect of steric bulk on the efflux properties of this series of inhibitors. We hypothesized that steric shielding of the polar amidine functionality could reduce susceptibility to P-gp recognition and thereby lower the efflux. The effect of the introduction of various alkyl substituents next to the amidine moiety are shown in Table 2. A single methyl group slightly reduced (**12a**) or maintained (**12b**) potency, depending on the stereochemistry. Removal of the second stereo center by switching to the more bulky dimethyl substitution (**13**) led to weakly reduced potency, but overall the methyl substitutions had little influence on the pK_a or permeation properties. An attempt to slightly reduce the steric bulk by connecting the alkyl substituents led to the spiro-substituted cyclopropyl derivative **14**, which showed better potency but had surprisingly high efflux. This effect might be partially explained by the polar cyano-substituent on the P3 moiety which appears to trigger additional P-gp efflux in other compounds too (see also **8a/b** versus **8c**). When the ring size was increased to a 6-membered spiro cycle (**15**), the potency dropped only modestly versus the non-substituted **8b**. Despite the slight decrease in pK_a we observed an increase in the cellular efflux for the tetrahydro-pyran **15**, which might be due to improved recognition by the efflux pump through a polar interaction with the oxygen atom. We then prepared acetals to reduce the risk of increased interaction/recognition by P-gp, also hoping to achieve, at the same time, a lowering of the pK_a by the oxygen atom located in β-position to the amidine. Comparing the monocyclic acetals **16a** and **16b**, the (*3RS,6RS*)-stereoisomer **16b** is 10-fold more active than the *(3RS,6SR)*-stereoisomer **16a**. The bicyclic acetal **17** is less active (about 3-fold) than **16b**. The major draw-back of these surprisingly stable acetals is their higher pK_a of 7.6 (**16b**) and 8.2 (**17**), which translated to efflux ratios >10.

To further explore the correlation between brain permeation, the pK_a of the TSM and the P-gp-driven efflux we investigated whether steric shielding and the pK_a lowering effect of fluorine could be combined. The transfer of the fluorines from the quaternary methyl-group of **8a-c** to the 6,6-geminal di-methyl group gave compound **18**. To avoid the creation of a second stereo center one fluorine was introduced at each of the two methyl groups. By this, both fluorines are placed again β to the

Table 2. In vitro data of transition-state-mimic (TSM) exploration



Compounds	TSM	X	R	BACE-1 IC_{50} [nM]	cellular IC_{50} [nM]	measured pK_a	MDR1-MDCK cells flux [10^{-6}cm/s]	efflux ratio
8b		F	5-Br	14	3	6.8	16	4
8c		F	3-Me, 5-CN-	27	7	6.7	17	11
12a* [rac. trans]		F	5-Br	92	11	7.3	13	3
12b* [rac. cis]		F	5-Br	38	5	7.1	31	2
13		F	5-Br	87	26	7.0	16	5
14		F	3-Me, 5-CN	25	4	7.4	15	15
15		F	3-Me, 5-CN	28	11	6.5	14	20
16a*		F	3-Me, 5-CN	207	27	7.8	17	12
16b*		F	3-Me, 5-CN	16	4	7.6	14	21
17		F	3-Me, 5-CN	53	25	8.2	7	18
18		F	3-Me, 5-CN	24	3	7.3	15	15
19		F	3-Me, 5-CN	12	13	6.4	24	4
20		F	3-Me, 5-CN	76	663	n.d.	34	3
21a		H	5-Br	1077	1161	7.3	5	2
21b		H	5-Br	20	32	7.3	10	2

* racemate; n.d. = not determined.

basic amidine functionality. Compound **18** nicely maintained the enzymatic and cellular potency but the pK_a increased by half a unit compared to **8a-c**. In line with the higher pK_a, the efflux in the cellular MDR1-MDCK assay increased. To counter-balance this effect, and in order to avoid a second stereo center, we introduced an additional fluorine back into the quaternary methyl group resulting in a significantly lower pK_a of 6.4 for compound **19**. As a result, the efflux ratio was reduced to the desired level. When a second fluorine was added to the quaternary methyl group a significant drop in activity in the enzymatic and cellular BACE assays was observed (**20**). While there was no compound available for pK_a measurements, the previously observed incremental pK_a reduction (Table 1) predicts a pK_a of **20** significantly below 6.0. Possibly, this amidine was not protonated enough for the interaction with the catalytic aspartates of the enzyme. In contrast to the disappointing potency, the permeation properties showed some improvement. Overall, the introduction of three fluorines seemed optimal, we therefore decided to explore another arrangement, concentrating all three fluorines in one methyl of the geminal di-methyl group. Since this design generates a new stereocenter both epimers were explored. The (*6S*)-epimer **21a** showed a significant drop in enzymatic and cellular BACE-1 inhibition compared to **13**, with IC_{50}s greater than 1 μM in both assays. Its pK_a of 7.3 was closer to that of **13** rather than **19,** and minimal efflux was observed in the MDR1-MDCK assay. The higher pK_a is noteworthy, considering the missing fluorine in P1 (expected effect: 0.3 units). The same pK_a and similarly low efflux was also observed for the (*6R*)-epimer **21b**. The enzymatic and cellular activities of **21b** were in the same range as the other most potent examples of the amino-1,4-oxazine BACE-1 inhibitors described herein. Trifluoromethyl analogues of **21a/b** lacking the second geminal methyl group were also prepared but were stereochemically unstable and were therefore not further pursued.

Overall the amino-1,4-oxazines proved to be a highly interesting chemotype for BACE-1 inhibition. This scaffold lends itself to fine-tuning of the pK_a, enabling the optimization of brain permeation, an essential pre-requisite for a potential CNS drug that needs to be applied over a long treatment period. The presented SAR suggests that the desired pK_a window for highly brain penetrant inhibitors is even more narrow between 6.5 and 7.5. Due to their good biochemical and cellular potency, good permeation in the MDR1-MDCK assay and little P-gp efflux examples **8b**, **12b**, **19** and **21b** are particularly attractive chemical starting points for further optimization. The optimization of the P1 and P3 moieties of this inhibitor series and other aspects like selectivity over other aspartyl proteases, metabolic stability, etc. will be subject of future communications.

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Supplementary Material

Supplementary data (supplementary material with experimental details for the preparation of compounds **14**, **15**, **16a**, **16b**, **17** and **20** as well as for the in vitro and in vivo assays and X-ray analysis data is available) associated with this article can be found, in the online version, at http://xxxxx

Highlights:

The structure activity relationship of new amino-1,4-oxazine derived BACE-1 inhibitors is elucidated
In vivo activity in a mouse model is demonstrated already with an early example
The pKa range of 6.5 to 7.5 is identified as optimal fo this class of compounds
Several starting for further optimization are presented