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Substituted thiazolamide coupled to a redox delivery system: a new γ-secretase inhibitor with enhanced pharmacokinetic profile

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Inhibition of γ-secretase, one of the enzymes responsible for the cleavage of the amyloid precursor protein (APP) to produce pathogenic Aβ peptides, is an attractive approach for the treatment of Alzheimer's disease. We have designed a new γ-secretase thiazolamide inhibitor bearing a dihydronicotinoyl moiety as Redox Delivery System which allows specific delivery of the drug to the brain. Through, on the one hand, Aβ peptide production measurements by specific *in vitro* assays (γ-secretase Cell Free assay and Cell Based assay on HEK 293 APP transfected cells) and, on the other hand, pharmacokinetic studies on animal models, the new inhibitor shows a good pharmacokinetic profile as well as a potent γ-secretase inhibitory activity *in vitro*. From the obtained results, it is expected that drug **2** will be mainly delivered to the CNS with low diffusion in the peripheral tissues. Consequently the side effects of this γ-secretase inhibitor on the immune cells could be reduced.

Introduction

Distribution of drugs to the Central Nervous System (CNS) is one of the major problems of current therapies of brain diseases. Difficulties in crossing the Blood Brain Barrier (BBB) often impair the efficacy of valuable drugs. In the past few years, most of the attempts to overcome this drawback have targeted the amelioration of the lipophilic properties through the preparation of prodrugs by formation of reversible linkages with suitable groups.[1,2] More recently, transporters that are involved in drug transfer across the BBB have been molecularly identified.[3]

Other strategies have been investigated, such as:

– liposomes targeted to the brain by exploiting receptor mediated transcytosis systems;[4]

– nanoparticules for drug delivery across the BBB;[5]

– implantation within the brain of either genetically engineered cells secreting a drug or a polymeric matrix or reservoir containing the drug;[6]

– neuroproteomic approaches and gene therapy for CNS disorders;[7]

– chimeric peptide technology, where a non-transportable drug is conjugated to a BBB transport vector.[8]

In addition to these promising strategies, Chemical Drug Delivery Systems (CDDS) still represent a systematic way of targeting active biological molecules to specific target sites or organs based on predictable enzymatic activation.[9] One of these systems called Redox Chemical Delivery Systems (RCDS) involves the linking of a drug to a lipophilic dihydropyridine carrier, creating a complex that after systemic administration readily transverses the BBB because of its lipophilicity. Once inside the brain parenchyma, the dihydropyridine moiety is enzymatically oxidized to the corresponding ionic pyridinium salt. The acquisition of charge has the dual effect of accelerating the rate of systemic elimination by the kidney and bile and trapping the drug pyridinium salt complex inside the brain. Subsequent cleavage of the drug from the pyridinium carrier leads to sustained drug delivery in the brain parenchyma.[10] This methodology increases intracranial concentrations of a variety of compounds, including neurotransmitters, antibiotics and antineoplastic agents.

One of the major fields of application of CDDS is the neurodegenerative diseases and particularly chemotherapy for Alzheimer's disease (AD). The available cholinergic therapies target essentially late aspects of AD, improving temporarily the performance of the undamaged neurones, but do not stop the progressive mental decline. In the past years, important progress has been made in the understanding of the pathogenic mechanism of AD. New therapeutic targets have become available and that should allow the underlying disease process to be tackled directly. In this respect, the "amyloid hypothesis" has become the dominant theory in this field. It is now believed that Aβ accumulation in plaques or as partial soluble filaments initiates a pathological cascade leading to tangle formation,[11] neuronal dysfunction and possibly inflammation and oxidative damage, with neurodegeneration and dementia as the final outcome.[12] Two enzymes known as β- and γ-secretases which cleave the Aβ precursor protein (APP) to yield Aβ peptide are now the potential therapeutic targets. Indeed, it is believed that lowering Aβ production will decrease the formation rate of senile plaques in Alzheimer's disease patients.[13] Today, one of the dominant strategies currently being pursued is the development of a drug that inhibits the activity of β- or γ-secretases and thereby reduces the production of Aβ peptide in the brain. Numerous very potent inhibitors of γ-secretase have been described so far. *N*-[*N*-(3,5-Difluorophenacetyl)-(*S*)-alanyl]-(*S*)-phenylglycine *tert*-butyl ester (DAPT) has been shown to dose-dependently reduce Aβ levels in the brains of two different strains of APP-transgenic mice after only a single dose injection.[14] A new series of highly potent benzodiazepine γ-secretase inhibitors active at the picomolar range have been also described.[15] Studies which came out from the use of benzodiazepine γ-secretase inhibitors demonstrate their ability to chronically reduce Aβ production. Nevertheless, these studies suggest also caution in the testing of γ-secretase inhibitors in humans and indicate specific side

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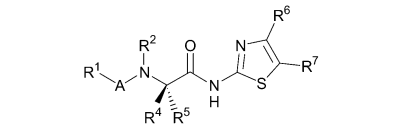
effects that should be monitored in any future clinical trials, mostly because Notch and the amyloid precursor protein are cleaved by similar γ-secretase.[16]

As it has been demonstrated that DAPT severely interferes with Notch signaling in zebrafish embryos,[17] it could be hypothesized that unwanted side-effects from the control of cellular differentiation could be observed during long-term treatment in humans with γ-secretase inhibitory drugs. Moreover, several potential liabilities seem to limit the potential promise of γ-secretase inhibitors mainly in the T cell development.[18] A possible way to overcome or to minimize these rather severe side-effects is to find drugs that are more rapidly and more effectively delivered to the CNS in order to reduce the concentration of the drug in the peripheral tissues by lowering the amount of drug to be administrated *in vivo*.

In this paper, we report the design, synthesis and biological properties of new pseudopeptide thiazolamide derivatives with γ-secretase inhibitory properties. As shown in Fig. 1, a dihydropyridine RCDS was also introduced onto these new analogues in order to improve their BBB permeation. These derivatives, the syntheses of which have required original synthetic schemes, were assayed as γ-secretase inhibitors in cell free and cell based assays and their brain specific *in vivo* delivery was investigated.



general formula

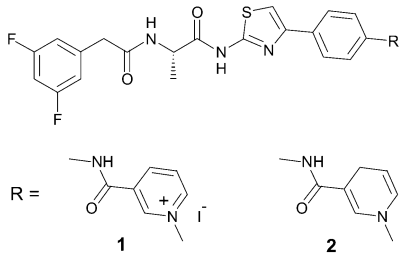


Fig. 1

Chemistry

The synthesis of the target molecule was performed as described in Scheme 1. The 2-thiazolamine, hydrobromide salt, **3** was the result of a modified conventional Hantzsch cyclocondensation achieved from 1-bromo-4′-nitroacetophenone and thiourea in refluxed ethanol.[19] The hydrobromide salt **3** was isolated quantitatively and in high purity by simple filtration and it was used for the next synthetic step without any further purification. This thiazolamine **3** was then acylated by the *N*-Boc protected amino acid (*S*)-alanine. Various methodologies were used to perform this acylation step. Unfortunately, conventional peptide coupling methods were almost completely ineffective. Indeed, the classical BOP reagent or the DCC–HOBt coupling system led to very poor yields in desired acylated derivative **4**, 19 and 5% yields respectively. No reaction was observed even after 24 hours by using uronium coupling reagent HBTU. These results highlight that *N*-acylation of such a weakly nucleophilic heterocyclic amine is not a straightforward reaction which can be achieved under any standard coupling conditions. The best results were obtained with the non-classical coupling system $POCl_3$ in pyridine.[20] This methodology led to a 77% yield in BocAla derivative **4**,[21] according to a known mechanism[20] involving the *in situ* formation of a mixed anhydride between the amino acid and $POCl_3$. Aminolysis of this anhydride by the thiazolamine species **3** led to the desired compound **4**. Use of pyridine as solvent for this reaction catalyses the nucleophilic attack. It also acts as a weak base which protects the *N*-Boc acid-labile function against acidolysis. During this kind of coupling reaction activated by $POCl_3$, we confirmed by chiral HPLC analysis that no racemization occurred.[21] The protecting group of the *N*-acylated thiazolamine **4** was released according to the conventional TFA deprotection methodology affording the corresponding TFA salt **5** in quantitative yield. Acylation of this deprotected amino acid **5** by 3,5-difluorophenylacetic acid by using BOP as coupling agent led to a 53% yield of acylated derivative **6**. The nitro function was then reduced in the presence of powdered iron in acidic conditions as the reducing system.[22] The nicotinoyl analogue **8** of this aniline was isolated after *N*-acylation of **7** by the hydrochloride salt of nicotinoyl chloride in pyridine. It was then methylated by using iodomethane into its iodide salt **1** in 39% yield for the last two steps. The methyl pyridinium moiety was reduced into its dihydropyridine form by using sodium dithionite in basic conditions, affording the Bodor-like target molecule **2**.[23]

Biology

In vitro γ-secretase cell free assay assay

The new compounds **1** and **2** were first tested for their ability to block specifically *in vitro* γ-secretase. This *in vitro* assay was performed using solubilized γ-secretase prepared essentially as described by Li *et al.*[24] This assay which uses solubilized γ-secretase and C100 flag as substrates reproduces both major cleavage events (giving rise to the $A\beta_{40}$ and $A\beta_{42}$ termini) that are ascribed to γ-secretase activity in cells. It should be recalled that to catalytically recover competent soluble γ-secretase the choice of detergent used for the membrane extraction is critical. CHAPSO (3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate) or CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) yield the active enzyme. This methodology is now widely used as standard *in vitro* γ-secretase assay.[25] Moreover, it is now well established that γ-secretase activity is catalyzed by a Presinilin-1 (PS-1) containing macromolecular complexes.

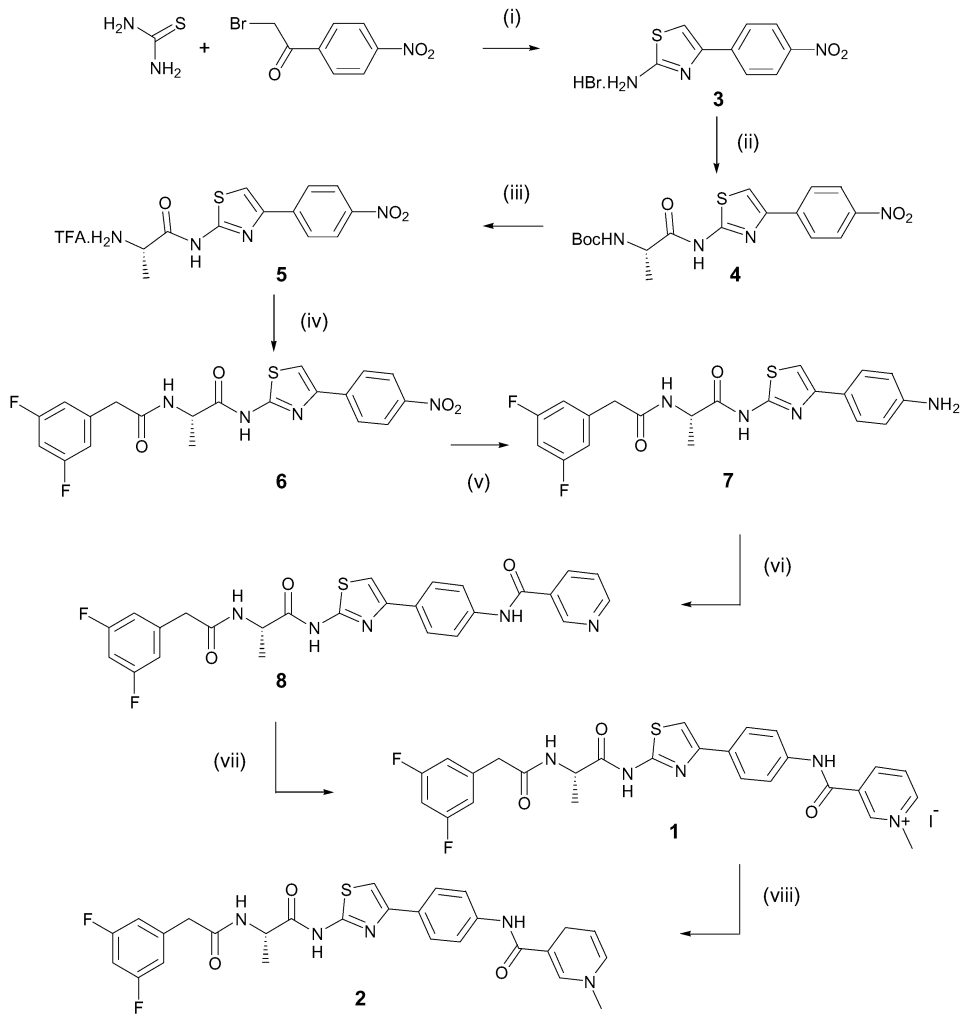
In this *in vitro* assay, the new tested analogues **1**, **2** and **7** exhibit very similar inhibition of Aβ-peptide production (EC_{50} values ranging from 0.1 to 1.0 μM).

In vitro cell based assay

The new compounds **1** and **2** were first tested for their ability to inhibit Aβ peptide production in a cell based assay according to a well known procedure using APP transfected HEK 293 cells.[26] This cell based assay confirms the efficiency of new analogues bearing a substituted 2-amino-thiazole moiety linked to the difluorophenylacetyl-alanyl moiety, to inhibit the production of total Aβ peptide ($A\beta_{40}$ + $A\beta_{42}$). The results are summarized in Table 1. As it can be seen, in our assay, the inhibitory potency for analogue **2** was found to be slightly better than for analogue **1**. The assay was validated by using DAPT as γ-secretase reference inhibitor. This result could be explained by the lower lipophilicity of the ionic derivative **1** compare to that of the neutral dihydropyridine **2**, as illustrated by their calculated log *P* values (Table 1).

BBB permeation evaluation

BBB permeation studies were performed on the dihydropyridine derivative **2** (reduced form), its oxidized form **1** and the unsubstituted aniline precursor **7**. These experiments were carried on anaesthetized healthy Sprague Dawley male rats, according to a well known methodology.[27] Solutions of each compound were freshly prepared in order to avoid any compound degradation reaction, mainly for **2** which is sensitive to oxidation and could regenerate its oxidized form **1**. This phenomenon could be a real



Scheme 1 *Conditions*: (i) EtOH, reflux, 5 min, quantitative; (ii) BocAla, $POCl_3$, pyridine, −15 °C to 80 °C, 16 h, 77%; (iii) TFA, CH_2Cl_2, RT, 16 h, quantitative; (iv) 3,5-difluorophenylacetic acid, BOP, DIEA, CH_2Cl_2, RT, 16 h, 53%; (v) Fe, NH_4Cl, H_2O–EtOH (v/v 6 : 10), reflux, 16 h, 90%; (vi) nicotinoyl chloride hydrochloride, pyridine, reflux, 18 h, 48%; (vii) CH_3I, DMF, 16 h, 81%; (viii) $Na_2S_2O_4$, H_2O–MeOH (v/v 1 : 1), RT, 4 h, 31%.

Table 1 Cell based γ-secretase inhibitory activities

Compound	Cell based assay[a] ED_{50}/μM	log *P*[b]
1	1.0 ± 0.5	2.93
2	0.2 ± 0.1	3.96
7	5.0 (30%)[c]	3.46

[a] Potency determination for the ability of compounds to reduce total Aβ production from HEK293 cells. [b] Log *P* determinations were performed using ACD (Advanced Chemistry Development, Inc.)/log *P* 1.0 base calculations. [c] 30% of inhibitory activity of **7** at 5 μM.

problem as our aim is to study the permeation of compound **2** through the BBB according to Bodor's concept. An unfortunate oxidation of the dihydropyridine moiety before permeation will lead to a lower diffusion and then will interfere with the demonstration of the efficiency of the concept.

These freshly prepared samples were injected into rats through the jugular vein at a dose of 20 mg Kg^{-1}. At various time intervals, a blood sample was withdrawn from the eyeball and after treatment was analysed by HPLC. At the end of this blood sampling, the animal was decapitated and the brain was submitted to treatment before HPLC analysis. HPLC profiles of each type of sample were compared to determine the relative permeation of analogue **2** (reduced form) compared to its oxidized form **1** and the aniline **7**.

As **2** is susceptible to oxidation, the total amount of drug **2** in the brain was determined as the amount of dihydropyridine **2** which has penetrated the brain in its intact form, its metabolites

Table 2 Concentration of the tested derivatives in the brain at suitable time intervals and comparison with the corresponding concentration in plasma

	2		**1**		**7**	
Time (hrs)	Brain[a,c]	Ratio[b]	Brain[a,d]	Ratio[b]	Brain[a]	Ratio[b]
0.25	n. q.	0	n. d.	0	n. d.	0
0.50	240	5.33	n. d.	0	85	1.60
1.00	320	12.31	**80**	4.44	230	8.21
2.00	**345**	38.33	60	12.00	**240**	24.00
4.00	255	n. d.	n. d.	n. d.	125	n. d.

[a] Concentration expressed in ng/g of brain.

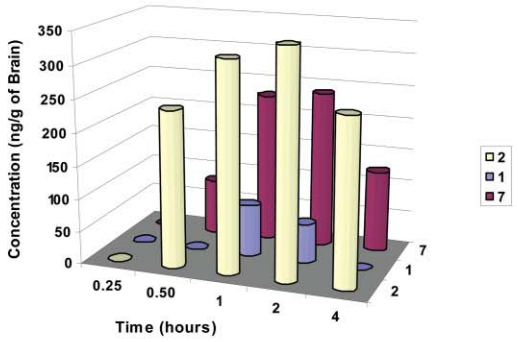
[b]
$$\text{Ratio} = \frac{\text{concentration of drug in brain (ng/g of brain)}}{\text{concentration of drug in blood (ng/g of blood)}} \times 10^3.$$

[c] Total concentration of **2** and its metabolites. [d] Total concentration of **1** and its metabolites. n. d.: not detected. n. q.: detected but not accurately quantified.

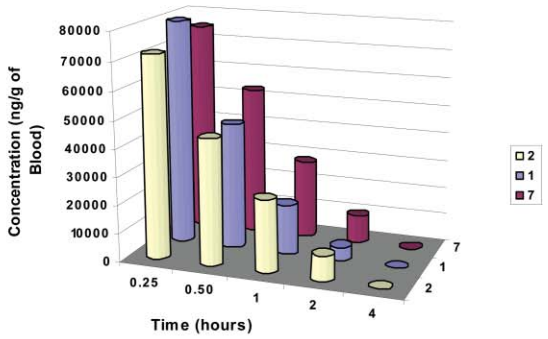
1 and the aniline **7** which comes from enzymatic hydrolysis of **2**. Similarly, the amount of compound **1** in the brain was estimated as the total amount of **1** which penetrated the brain in its intact form and its metabolite **7**. These results are summarized in Table 2 and Fig. 2a.

Rate of BBB permeation is a critical parameter. An optimal concentration of compound **7** in the brain was reached within 2 hours (240 ng/g of brain; Table 2). In contrast, for drug

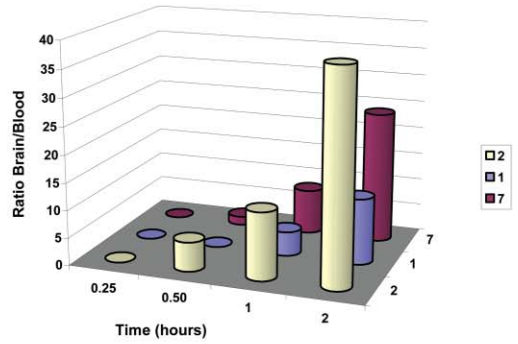
(a) Bioavailability in brain (ng/g of brain).



(b) Biovailability in blood (ng/g of blood).



(c) Ratio between brain and blood.



$$R = \frac{concentration\ of\ drug\ in\ brain\ (ng/g\ of\ brain)}{concentration\ of\ drug\ in\ blood\ (ng/g\ of\ blood)} \times 10^3$$

Fig. 2 Biological availability of the tested compounds in brain (a), in blood (b) and ratio between the concentrations in brain and blood (c).

2 and its metabolites, this optimal concentration is observed more rapidly, only after 30 minutes (240 ng/g of brain), while during this interval of time compound **1** and its metabolites were not detected. Indeed, it can be observed that about 1 hour after injection, compound **1** was almost undetectable in the brain except as its precursor **7** (80 ng/g of brain; Table 2). Compound **1** was unable to penetrate the BBB but it was hydrolysed into **7** which was found in a very low quantity in the CNS compartment.

In addition to the determination of drug levels in the brain, the concentration in the blood was also studied. As shown in Fig. 2b, the concentration level of each tested compound in the blood decreased gradually with time to completely disappear from the plasma after less than 4 hours.

Analysis of the ratio between the drug level in the brain and the drug level in the blood reinforces our findings about the better BBB permeation of **2** compared to **1** and **7**. Indeed, as shown in Table 2 and depicted in Fig. 2c, one can notice that this ratio is three times higher for the dihydropyridine **2** compared to that of the pyridinium derivative **1**. This ratio is even twice as high for aniline **7** than for **1**.

Discussion

We have synthesized a new γ-secretase inhibitor, the structure of which incorporates a thiazolamide moiety bearing a dihydronicotinoyl as well as a difluorophenyl-alanyl substituent. This inhibitor was identified as lowering Aβ production in intact cells (HEK 293) as well as in a cell free system. This result indicates that using the phenyl ring to introduce a substituent such as nicotinoyl moiety improved the inhibitory activity. We also demonstrate through *in vivo* experiments that the RCDS based on Bodor's concept inhibitor appears to be efficient, since the obtained results are consistent with those expected. Indeed, these studies revealed that the conjugation of the new thiazolamide to a BBB redox chemical drug delivery system allows the delivery of the inhibitor to the brain with improvement of the γ-secretase inhibitory activity.

As expected, the results confirmed that reduced compound **2** is metabolized into both its oxidized form **1** and its corresponding free anilino derivative **7**.

The remarkable pharmacokinetic parameter for compound **2** is its permeation rate in the brain. Indeed, from Table 2, it can be seen that after 30 minutes the concentration in the brain of compound **2** was around 240 ng/g of brain, which is 3 times higher than for its analogue **7** (85 ng/g of brain, after 30 minutes). Besides, the RCDS shows a more cumulative effect of compound **2** in the brain with time with maximum concentration after about 2 hours (345 ng/g of brain) as compared with compound **7** (240 ng/g of brain) with minimal systemic concentration of the system. This pharmacokinetic property is of interest since the use of this BBB-drug delivery system optimizes the therapeutic index of compound **2** by simultaneously increasing central nervous system drug uptake and decreasing drug uptake in peripheral tissues. As indicated in the introduction of the paper, the observed improved BBB permeation property of this new γ-secretase inhibitor **2** could reduce its diffusion into the peripheral tissues and consequently it could reduce its side-effects on the immune cells or on neural cell differentiation.[17]

In conclusion, we report here the synthesis of a new thiazolamide γ-secretase inhibitor, the design includes a dihydronicotinoyl moiety as Redox Delivery System, which allows efficient BBB permeation. Its favourable pharmacokinetic profile could be of interest *in vivo*. *In vivo* experiments in the Tg2576 mouse model of Alzheimer's disease are currently under way to validate the pharmacological potential of such compounds and to determine which of the drug or its metabolites is the most active γ-secretase inhibitor.

Experimental

Chemistry

Unless otherwise noted, starting materials and reagents were obtained from commercial suppliers and were used without purification. All the protected amino acids and peptide coupling reagents were purchased from Bachem, Acros, Aldrich and Neosystem. Tetrahydrofuran (THF) was distilled over sodium benzophenone ketyl immediately prior to use. Methylene dichloride (CH_2Cl_2) was distilled over P_2O_5 just prior to use. Pyridine and dimethylformamide (DMF) were of anhydrous quality from commercial suppliers (Aldrich, Acros, Carlo Erba Reagents). Nuclear magnetic resonance spectra were recorded at 250 MHz for ^1H and 62.9 MHz for ^{13}C on a Brüker AC-250 spectrometer. Chemical shifts are expressed as δ units (part per million) downfield from TMS (tetramethylsilane). Electro-spray mass spectral analysis and LC-MS analysis were obtained on a Waters Micromass ZMD spectrometer for the ES-MS analysis by direct injection of the sample solubilized in acetonitrile. The LC-MS analysis was carried out by using a Waters model 2690 pump and a Waters C18 Symmetry column with a two-mobile phase system (0.1% formic acid in water and 0.1% formic acid in

acetonitrile). Microanalyses were carried out by Service Central d'Analyses du CNRS (Venaison, France) and were within 0.4% of the theoretical values. Analytical thin layer chromatographies (TLC) and preparative thin layers chromatographies (PLC) were performed using silica gel plates 0.2 mm thick and 1 mm thick respectively ($60F_{254}$ Merck). Preparative flash column chromatographies were carried out on silica gel (230–240 mesh, G60 Merck). The melting points were not determined because of the amorphous character of our peptides and derivatives.

2-Amino-4-(4-nitrophenyl)thiazole, hydrobromide salt, 3[19]. 1-Bromo-4′-nitroacetophenone (1.50 g, 1.0 equiv., 6.15 mmol) was dissolved in refluxed ethanol (25 mL). Thiourea (0.47 g, 1.0 equiv., 6.15 mmol) was then added in one lot. The refluxed reaction mixture was stirred and, after a few minutes, a yellow solid appeared. The solid was filtered, washed with diethyl ether and dried under reduced pressure to give the title compound **3** as a yellow solid (1.88 g, quantitative). (Found: C, 35.89; H, 2.83; N, 13.60. $C_9H_7N_3O_2S.HBr$ requires C, 35.78; H, 2.67; N, 13.91%). R_f 0.31 (CH_2Cl_2–MeOH 9 : 1). ^{1}H NMR (DMSO-d_6, 250 MHz) δ 7.47 (s, 1 H, =C*H*–S), 8.04 (d, 2 H, H_{m-NO2}, ^3J = 9.0 Hz), 8.15 (s, 2 H, NH_2), 8.24 (d, 2 H, H_{o-NO2}, ^3J = 9.0 Hz). ^{13}C NMR (DMSO-d_6, 62.9 MHz) δ 107.3, 124.1 (2 C), 126.7 (2 C), 136.0, 139.6, 164.9, 169.9. MS-ES m/z 222 $(M + H)^+$.

2-[*N*-(*N*-*tert*-Butoxycarbonyl)-(*S*)-alanyl]amino-4-(4-nitrophenyl)-thiazole, 4[21]. *N*-Boc-Ala (0.88 g, 1.0 equiv., 4.64 mmol) and the previous hydrobromide salt **3** (1.40 g, 1.0 equiv., 4.64 mmol) were dissolved in anhydrous pyridine (70 mL). The solution was cooled to −15 °C and phosphorus oxychloride (0.47 mL, 1.1 equiv., 5.10 mmol) was added dropwise under vigorous stirring. The reaction mixture was stirred at −15 °C for 30 min. The solution was allowed to warm to room temperature and then stirred overnight at 80 °C. The reaction was quenched by addition of crushed ice/water (50 mL). The desired compound was extracted into EtOAc (3 × 50 mL). The combined organic layers were dried over anhydrous $MgSO_4$, filtered and concentrated under reduced pressure. The crude material was purified by flash chromatography to lead to the title compound **4** as a yellow solid (1.40 g, yield: 77%). (Found: C, 51.93; H, 5.32; N, 14.48. $C_{17}H_{20}N_4O_5S$ requires C, 52.03; H, 5.14; N, 14.28%). R_f 0.70 (EtOAc–hexane 1 : 1). ^{1}H NMR (CD_3OD, 250 MHz) δ 1.44 (d, 3 H, CH_3 β Ala, ^3J = 7.3 Hz), 1.49 (s, 9 H, CH_3 Boc), 4.33 (m, 1 H, C*H* α Ala), 5.95–6.03 (m, 1 H, N*H* Ala), 7.74 (s, 1 H, = C*H*–S), 8.16 (d, 2 H, H_{m-NO2}, ^3J = 9.3 Hz), 8.29 (d, 2 H, H_{o-NO2}, ^3J = 9.3 Hz), 10.82 (s, 1 H, N*H* thiazole). ^{13}C NMR ($CDCl_3$, 62.9 MHz) δ 17.4, 28.3 (3 C), 50.8, 81.5, 111.4, 124.1 (2 C), 126.6 (2 C), 140.2, 147.1, 147.7, 157.7, 158.3, 171.0. MS-ES m/z 393 $(M + H)^+$.

2-(*N*-(*S*)-Alanyl)amino-4-(4-nitrophenyl)-thiazole, trifluoroacetic acid salt, 5. The previous *N*-Boc protected derivative **4** (1.0 equiv., 1.82 g, 4.64 mmol) was dissolved in CH_2Cl_2 (32 mL). Trifluoroacetic acid (10.0 equiv., 3.53 mL, 46.40 mmol) was then added and the reaction mixture was stirred overnight at room temperature. The solvent and excess TFA were removed under reduced pressure and the resulting solid was triturated in Et_2O. The desired trifluoroacetic acid salt **5** was isolated quantitatively after filtration as a yellow solid (1.88 g). ^{1}H NMR ($CDCl_3$, 250 MHz) δ1.67 (d, 3 H, CH_3 β Ala, ^3J = 7.2 Hz), 4.24 (m, 1 H, C*H* α Ala), 6.04 (d, 1 H, N*H* Ala, ^3J = 8.4 Hz), 7.80 (s, 1 H, =C*H*–S), 8.17 (d, 2 H, H_{m-NO2}, ^3J = 9.2 Hz), 8.30 (d, 2 H, H_{o-NO2}, ^3J = 9.2 Hz) 10.92 (s, 1 H, N*H* thiazole). ^{13}C NMR ($CDCl_3$, 62.9 MHz) δ 17.3, 66.9, 113.3, 125.0 (2 C), 127.7 (2 C), 141.6, 148.5, 159.1, 169.5, 183.1. MS-ES m/z 293 $(M + H)^+$.

***N*-[*N*-(3,5-Difluorophenylacetyl)-(*S*)-alanyl]-*N*-[4-(4-nitrophenyl)]-thiazol-2-ylamine, 6.** 3,5-Difluorophenylacetic acid (232 mg, 1.0 equiv., 1.35 mmol) was dissolved in freshly distilled CH_2Cl_2 (30 mL) with 1.0 equiv. (652 mg, 1.35 mmol) of BOP reagent. The reaction mixture was cooled to 0 °C then 1.0 equiv. of DIEA (240 µL, 1.35 mmol) was added dropwise. The reaction mixture was stirred for 1 h at room temperature then once again cooled to 0 °C. A CH_2Cl_2 solution (10 mL) of 0.9 equiv. (500 mg, 1.23 mmol) of the previous trifluoroacetic acid salt **5** and 3.0 equiv. (720 µL, 4.05 mmol) of DIEA was added dropwise. The solution was allowed to warm and stirred overnight at room temperature. The solvent was removed under reduced pressure and the residue was dissolved in EtOAc (100 mL). The organic layer was washed successively by using H_2O (50 mL), 5% aqueous citric acid (2 × 50 mL), brine (50 mL), 5% aqueous $NaHCO_3$ (2 × 50 mL) and brine (50 mL), was dried over anhydrous $MgSO_4$, filtered and concentrated under reduced pressure. The crude residue was purified by flash chromatography (EtOAc–hexane 1 : 1) to give the title compound **6** (290 mg, yield: 53%) as a yellow solid. (Found: C, 53.68; H, 3.52; N, 12.82. $C_{20}H_{16}F_2N_4O_4S$ requires C, 53.81; H, 3.61; N, 12.55%). R_f 0.36 (EtOAc–hexane 1 : 1). ^{1}H NMR ($CDCl_3$, 250 MHz) δ 1.48 (d, 3 H, CH_3 β Ala, ^3J = 7.0 Hz), 3.64 (s, 2 H, -CH_2), 4.76 (m, 1 H, C*H* α Ala), 5.96 (d, 1 H, N*H* Ala, ^3J = 8.8 Hz), 6.82 (m, 3 H, Ar *H*), 7.36 (s, 1 H, =C*H*–S), 7.99 (d, 2 H, H_{o-NO2}, ^3J = 9.0 Hz), 8.27 (d, 2 H, H_{m-NO2}, ^3J = 9.0 Hz), 10.02 (s, 1 H, N*H* thiazole). ^{13}C NMR (DMSO-d_6, 62.9 MHz) δ 17.6, 40.9, 48.5, 101.8 (t, 1 C, ^2J = 25.3 Hz), 112.2 (d, 2 C, ^2J = 24.2 Hz), 112.5, 124.1 (2 C), 126.4 (2 C), 140.4 (t, 1 C, ^3J = 10.0 Hz), 146.4, 146.7, 158.2, 162.1 (dd, 2 C, ^1J = 245.4 Hz, ^3J = 13.4 Hz), 169.1, 171.8, 181.2. MS-ES m/z 447 $(M + H)^+$.

***N*-[*N*-(3,5-Difluorophenylacetyl)-(*S*)-alanyl]-*N*-[4-(4-aminophenyl)]-thiazol-2-ylamine, 7.** The previous nitro derivative **6** (280 mg, 1.0 equiv., 0.62 mmol) was dissolved in a H_2O–EtOH solution (6 : 10 v/v; 15 mL). Iron (173 mg, 5.0 equiv., 3.10 mmol) and 2.0 equiv. of NH_4Cl (66 mg, 2.0 equiv., 1.24 mmol) were then added and the reaction mixture was refluxed overnight. The solution was cooled to room temperature, filtered and concentrated under reduced pressure. The crude material was purified by flash chromatography (EtOAc–hexane 1 : 1) to provide the desired compound **7** as a yellow solid (230 mg, yield: 90%). (Found: C, 57.82; H, 4.61; N, 13.25. $C_{20}H_{18}F_2N_4O_2S$ requires C, 57.68; H, 4.36; N, 13.45%). R_f 0.10 (EtOAc–hexane 1 : 1). ^{1}H NMR ($CDCl_3$, 250 MHz) δ 1.45 (d, 3 H, CH_3 β Ala, ^3J = 7.0 Hz), 3.55 (s, 2 H, -CH_2), 4.80 (m, 1 H, C*H* α Ala), 6.40 (d, 1 H, N*H* Ala, ^3J = 8.4 Hz), 6.70 (d, 2 H, H_{o-NH2}, ^3J = 8.7 Hz), 6.80 (m, 3 H, Ar *H*), 7.95 (s, 1 H, =C*H*–S), 7.62 (d, 2 H, H_{m-NH2}, ^3J = 8.9 Hz), 10.04 (s, 1 H, N*H* thiazole), 10.96–11.07 (m, 2 H, -NH_2). ^{13}C NMR (CD_3OD, 62.9 MHz) δ 17.8, 42.6, 50.7, 103.1 (t, 1 C, ^2J = 25.5 Hz), 105.5, 113.3 (d, 2 C, ^2J = 24.8 Hz), 116.3 (2 C), 126.2, 128.1 (2 C), 140.9 (t, 1 C, ^3J = 9.7 Hz), 148.9, 151.9, 158.9, 164.4 (dd, 2 C, ^1J = 246.8 Hz, ^3J = 13.1 Hz), 172.7, 172.8. MS-ES m/z 417 $(M + H)^+$.

***N*-[*N*-(3,5-Difluorophenylacetyl)-(*S*)-alanyl]-*N*-{4-[4-(*N*-pyridyl-3-carbonyl)-aminophenyl]}-thiazol-2-ylamine, 8.** The anilino derivative **7** (100 mg, 1.0 equiv., 0.24 mmol) and the hydrochloride salt of nicotinoylchloride (42 mg, 1.0 equiv., 0.24 mmol) were dissolved in anhydrous pyridine (3 mL). The reaction mixture was refluxed for 18 h. The solvent was removed under reduced pressure. The solid residue was triturated, filtered and dried by using successively EtOAc (20 mL), H_2O (10 mL) and Et_2O (20 mL). The resulting solid was dried under reduced pressure. The desired compound **8** was obtained as a white solid (50 mg, yield: 48%). (Found: C, 59.98; H, 4.34; N, 13.18. $C_{26}H_{21}F_2N_5O_3S$ requires C, 59.88; H, 4.06; N, 13.43%). R_f 0.57 (EtOAc–hexane 1 : 2). ^{1}H NMR (DMSO-d_6, 250 MHz) δ 1.58 (d, 3 H, CH_3 β Ala, ^3J = 7.1 Hz), 3.80 (s, 2 H, -CH_2), 4.75 (m, 1 H, C*H* α Ala), 7.30 (m, 3 H, diF Ar *H*), 7.81 (t, 2 H, =C*H*–S + $H_{5-pyridine}$, ^3J = 7.4 Hz), 8.11 (d, 4 H, Ar *H*, ^3J = 6.4 Hz), 8.53 (dt, 1 H, $H_{6-pyridine}$, ^3J = 8.0 Hz, ^4J = 1.9 Hz), 8.85 (d, 1 H, -N*H* Ala, ^3J = 6.6 Hz), 9.00 (dd, 1 H, $H_{4-pyridine}$, ^3J = 4.9 Hz, ^4J = 1.6 Hz), 9.34 (d, 1 H, $H_{2-pyridine}$, ^3J = 2.9 Hz), 10.78 (s, 1 H, N*H* thiazole), 12.64 (s, 1 H, N*H*). ^{13}C NMR (DMSO-d_6, 62.9 MHz) δ 17.5, 41.0, 48.5, 101.8 (t, 1 C, ^2J = 25.5 Hz), 107.2,

112.2 (d, 2 C, ^2J = 24.4 Hz), 120.3 (2 C), 123.4 (2 C), 126.0 (2 C), 130.0, 130.5, 137.0, 140.5 (t, 1 C, ^3J = 9.8 Hz), 148.6, 148.7, 152.1, 157.7, 162.0 (dd, 2 C, ^1J = 245.6 Hz, ^3J = 13.6 Hz), 169.2, 171.6, 181.1. MS-ES *m/z* 522 $(M + H)^+$.

***N*-[*N*-(3,5-Difluorophenylacetyl)-(*S*)-alanyl]-*N*-(4-{4-[*N*-(*N*-methyl)pyridinium-yl-3-carbonyl]-aminophenyl})-thiazol-2-yl-amine, iodide salt, 1.** The previous pyridino derivative **8** (1.00 g, 1.0 equiv., 1.92 mmol) was dissolved in anhydrous DMF (50 mL). Iodomethane (2.3 mL, 20.0 equiv., 38.40 mmol) was then added. The reaction mixture was stirred overnight at room temperature. The solvent and excess iodomethane were removed under reduced pressure. The solid residue was dissolved in MeOH (10 mL) and the pyridinium salt was precipitated by addition of Et_2O. The resulting yellow solid was filtered to afford the desired compound **1** (1.03 g, yield: 81%). (Found: C, 48.62; H, 3.83; N, 10.35. $C_{27}H_{24}F_2N_5O_3S.I$ requires C, 48.88; H, 3.65; N, 10.56%). ^{1}H NMR (DMSO-d_6, 250 MHz) δ 1.35 (d, 3 H, C*H*$_3$ β Ala, ^3J = 7.2 Hz), 3.56 (s, 2 H, -C*H*$_2$), 4.41 (m, 4 H, C*H* α Ala + N–C*H*$_3$), 7.06 (m, 3 H, diF Ar *H*), 7.61 (s, 1 H, =C*H*–S), 7.84–7.91 (m, 4 H, Ar *H*), 8.31 (dt, 1 H, $H_{5-pyridine}$, ^3J = 6.1 Hz, ^4J = 1.9 Hz), 8.62 (d, 1 *H*, -N*H* Ala, ^3J = 6.5 Hz), 9.04 (d, 1 H, $H_{6-pyridine}$, ^3J = 8.0 Hz), 9.15 (d, 1 H, $H_{4-pyridine}$, ^3J = 6.0 Hz), 9.52 (s, 1 H, $H_{2-pyridine}$), 10.91 (s, 1 H, N*H* thiazole), 12.39 (s, 1 H, N*H*). ^{13}C NMR (DMSO-d_6, 62.9 MHz) δ 17.5, 40.9, 48.2, 48.5, 101.8 (t, 1 C, ^2J = 25.7 Hz), 107.7, 112.4 (d, 2 C, ^2J = 24.4 Hz), 120.4 (2 C), 126.2 (2 C), 127.3 (2 C), 130.7, 140.4 (t, 2 C, ^3J = 10.0 Hz), 137.6, 143.3, 145.7, 147.3, 160.3, 162.1 (dd, 2 C, ^1J = 231.8 Hz, ^3J = 13.3 Hz), 169.1, 171.6, 181.2. MS-ES *m/z* 268.5 $(M + H)^+$.

***N*-[*N*-(3,5-Difluorophenylacetyl)-(*S*)-alanyl]-*N*-(4-{4-[*N*-(*N*-methyl)dihydropyridinyl-3-carbonyl]-aminophenyl})-thiazol-2-yl-amine, 2.** The iodide salt **1** (200 mg, 1.0 equiv., 0.30 mmol) was dissolved in a H_2O–MeOH solution (1 : 1 v/v; 20 mL). The resulting solution was degassed under a nitrogen flush at 0 °C for 20 min. Na_2CO_3 (191 mg, 6.0 equiv., 1.8 mmol) and sodium dithionite ($Na_2S_2O_4$, 208 mg, 4.0 equiv., 1.20 mmol) were then added. The reaction mixture was stirred at room temperature for 4 h. The aqueous layer was extracted with CH_2Cl_2 (3 × 5 mL). The combined organic layers were dried over anhydrous $MgSO_4$, filtered and concentrated under reduced pressure. The desired compound **2** was obtained as a yellow solid (50 mg, yield: 31%). (Found: C, 60.61; H, 4.97; N, 13.31. $C_{27}H_{25}F_2N_5O_3S$ requires C, 60.32; H, 4.69; N, 13.03%). ^{1}H NMR ($CDCl_3$, 250 MHz) δ 1.41 (d, 3 H, C*H*$_3$ β Ala, ^3J = 6.8 Hz), 2.92 (s, 3 H, N–C*H*$_3$), 3.26 (s, 2 H, $H_{4-pyridine}$), 3.51 (s, 2 H, -C*H*$_2$), 4.57–4.87 (m, 2 H, C*H* α Ala + $H_{5-pyridine}$), 5.70 (dd, 1 H, $H_{6-pyridine}$, ^3J = 8.1 Hz, ^4J = 1.3 Hz), 5.97 (d, 1 H, N*H* Ala, ^3J = 8.6 Hz), 6.67–6.78 (m, 3 H, diF Ar *H*), 7.01 (m, 1 H, $H_{2-pyridine}$), 7.12 (s, 1 H, =C*H*–S), 7.55–7.64 (m, 4 H, Ar *H*), 11.03 (s, 1 H, N*H* thiazole), 12.27 (s, 1 H, N*H*). ^{13}C NMR ($CDCl_3$, 62.9 MHz) δ 17.2, 28.3, 38.6, 39.4, 50.6, 52.1, 102.7 (t, 1 C, ^2J = 24.5 Hz), 103.4, 110.2, 112.4 (d, 2 C, ^2J = 26.3 Hz), 120.9 (2 C), 126.3 (2 C), 127.2 (2 C), 132.1, 138.2, 139.1, 150.8, 163.8 (d, 2 C, ^1J = 237.0 Hz, ^3J = 13.5 Hz), 164.2, 170.9, 172.7, 182.6. MS-ES *m/z* 538 $(M + H)^+$.

Biology

***In vitro* γ-secretase cell free assay.** The ability to block specifically *in vitro* γ-secretase was performed by using solubilized γ-secretase prepared as described by Li *et al.*[24] A DNA fragment encoding amino acids 596–695 of the 695-aa isoform of APP (APP695) and the Flag sequence (DYKDDDDK) at the C terminus was generated by PCR amplification with suitably designed oligonucleotides and the APP695 cDNA. The Met that serves as the translation start site is residue 596 of APP695 (the P1 residue with respect to the β-secretase cleavage site). C100Flag (1.7 μM) was incubated with cell membranes (0.5 mg ml^{-1}) in the presence of CHAPSO. The reactions were stopped by adding RIPA.

***In vitro* cell based assay.** The ability to inhibit Aβ peptide production in a cell based assay was measured according to a well known procedure using APP transfected HEK 293 cells.[26] HEK 293 E cells were maintained in DMEM supplemented with 10% fetal bovine serum, 25 μg mL^{-1} penicillin, 25 μg mL^{-1} streptomycin, and 250 μg mL^{-1} G 418. Cells were transfected following specifications provided by the manufacturer (Gibco-BRL). One day after transfection, the conditioned medium was replaced with selection medium (media as above containing in addition 250 μg mL^{-1} hygromycin B). For immunoblotting analysis, the 24 h serum-free conditioned medium was harvested and cellular debris was removed by centrifugation for 10 min. The monolayers were washed with PBS, and cell lysates were prepared by the addition of SDS–sample buffer containing 50 mM dithiothreitol. For Aβ determination by ELISA, confluent cultures were incubated in serum-free medium containing 0.2% BSA for 16 h. To test the effects of compounds on Aβ formation, cells were plated at confluency (1 × 10^6 cells cm^{-2}) in 96-well tissue culture dishes in serum-free medium containing 0.2% BSA in the presence of the indicated concentration of either γ-secretase reference inhibitor DAPT. After 16 h, the conditioned medium was harvested and analyzed using the HA 11 Aβ-specific ELISA.

BBB permeation evaluation[27]. Five groups, each of four Sprague Dawley male rats of average weight of 55–65 g, were anaesthetized with urethane. A freshly prepared solution (50 mmol) of each compound in DMSO (diluted to 20% with water) was injected through the jugular vein at a dose of 20 mg kg^{-1}.

Blood sample analysis. At time intervals (0.25, 0.50, 1.00, 2.00 and 4.00 hours) blood samples were withdrawn from the eyeball, immediately added to previously weighed centrifuge tubes containing acetonitrile (4 mL) and weighed to determine the amount of blood added. The blood samples were centrifuged at about 4000 rpm for 10 minutes and the supernatant analyzed by HPLC.

Brain sample analysis. The animals were decapitated and the brains were taken, weighed and immediately homogenized with 1 mL saline and diluted with 4 mL of 5% DMSO in acetonitrile, homogenized again and centrifuged at 4000 rpm for 10 minutes. The supernatants were analyzed by HPLC.

HPLC analysis method. HPLC method with UV detection.

Equipment: Knauer HPLC with Marathon Plus Autosampler.

Column: HPLC column C18, 4.6 mm × 25 cm, 5 μm.

Detector: UV detector at wavelength about 280 nm.

Injection volume: 50 μL.

Temperature: room temperature.

Flow rate: 1 ml min^{-1}.

Software: Eurochrom 2000.

Mobile phase: Filtered and degassed mixture of 0.2% hexane–sulfonic acid (prepared by pH adjustment of 0.2% solution of hexane–sulfonic acid sodium salt with 20% trifluoroacetic acid to pH about 2.7) : acetonitrile (30 : 70).

Retention time: **1a** (about 6.3 minutes), **6** (about 11.2 minute) and **1b** (about 15 minutes).

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