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Synthesis and Biological Activity of Flurbiprofen Analogues as Selective Inhibitors of β-Amyloid$_{1-42}$ Secretion

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Flurbiprofen, a nonsteroidal antiinflammatory drug (NSAID), has been recently described to selectively inhibit β-amyloid$_{1-42}$ (Aβ42) secretion, the most toxic component of the senile plaques present in the brain of Alzheimer patients. The use of this NSAID in Alzheimer's disease (AD) is hampered by a significant gastrointestinal toxicity associated with cyclooxygenase (COX) inhibition. New flurbiprofen analogues were synthesized, with the aim of increasing Aβ42 inhibitory potency while removing anti-COX activity. In vitro ADME developability parameters were taken into account in order to identify optimized compounds at an early stage of the project. Appropriate substitution patterns at the alpha position of flurbiprofen allowed for the complete removal of anti-COX activity, while modifications at the terminal phenyl ring resulted in increased inhibitory potency on Aβ42 secretion. In rats, some of the compounds appeared to be well absorbed after oral administration and to penetrate into the central nervous system. Studies in a transgenic mice model of AD showed that selected compounds significantly decreased plasma Aβ42 concentrations. These new flurbiprofen analogues represent potential drug candidates to be developed for the treatment of AD.

Introduction

Alzheimer's disease (AD) is the most common form of dementia, affecting approximately 5% of the population over the age of 65. The basic pathological abnormalities in AD are amyloid plaques, neurofibrillary tangles and neuronal death. Their exact relationship is still unclear, but the major therapeutic efforts are presently directed in influencing amyloid metabolism.[1] Amyloid plaques consist of a proteinaceous core composed of 5−10 nm amyloid fibrils surrounded by dystrophic neurites, astrocytic processes and microglial cells. The β-amyloid peptide (Aβ) consists of 39−42 amino acids generated by the cleavage of the amyloid precursor protein (APP). The main form of Aβ contains 40 amino acids (Aβ40), but the carboxy-terminal-extended species made of 42 residues (Aβ42) is also produced. This longer form is more prone to aggregate into fibrils and Aβ42 makes up the major component of amyloid plaques. Plaques are in equilibrium with soluble monomers and oligomers and may not be the sole contributor to neuronal death, as there is evidence that soluble amyloid is also potentially neurotoxic.[2]

Epidemiological studies have documented a reduced prevalence of AD among patients using nonsteroidal antiinflammatory drugs (NSAIDs). The protective effects of NSAIDs appear proportional to the length of treatment and to depend on the specific chemical structure of the NSAIDs being used, with nonaspirin agents providing the lowest relative risk.[3] Despite these encouraging findings, all large, long-term, placebo-controlled clinical trials aimed at reducing inflammation in the brain of AD patients have produced negative results.[4] More recently, it has been shown that some NSAIDs decrease the production of Aβ42 in vitro and in vivo[5−7] and counteract the progression of Aβ42 pathology in transgenic mouse models of AD.[8−14] The proposed mechanism for this activity is an allosteric modulation of presenilin-1 (PS-1), the major component of the γ−secretase complex, the enzyme responsible for the formation of Aβ.[15−18] The inhibition of Aβ42 production is independent of the anti-cyclooxygenase activity[5,19] and is related to the chemical structure of the compounds, with some NSAIDs being active (ibuprofen, sulindac, flurbiprofen, indomethacin, diclofenac) and others not (naproxen, aspirin, celecoxib). This could explain the negative results of the large AD trials carried out so far, since they were performed with compounds (naproxen, hydroxychloroquine, dapsone, prednisone, rofecoxib and celecoxib) not able to decrease Aβ42 production.[20] Unfortunately, the generic use of NSAIDs in AD is hampered by a significant gastrointestinal toxicity associated with COX inhibition.

We tried to identify new NSAIDs analogues endowed with potent and selective Aβ42 lowering activity but devoid of COX inhibitory activity, thus suitable for chronic use in AD patients. Specifically, we concentrated our efforts on flurbiprofen and flurbiprofen derivatives, since this compound has been reported as the most

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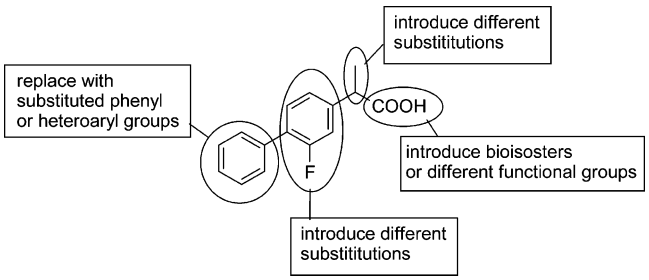
Scheme 1. Planned Chemical Variations on Flurbiprofen



active NSAID studied so far.[6] It is well-known that the *R*-enantiomer of flurbiprofen is much less active than the *S*-enantiomer on both COX-1 (IC_{50} of 44 versus 0.03 μM) and COX-2 (IC_{50} of 123 versus 0.91 μM) activity.[21] Indeed, the *R*-enantiomer (MPC-7869) is reported to be in Phase 3 clinical development for Alzheimer's disease in the USA,[20] with the rationale that the reduction of COX inhibitory activity should minimize the gastrointestinal toxicity potential. However, the IC_{50} of *R*-flurbiprofen on Aβ42 (280 μM) is still higher than that on both COX-1 (44 μM) and COX-2 (123 μM). Thus, if active concentrations of *R*-flurbiprofen on Aβ42 are reached in Alzheimer patients (i.e., around 280 μM) this will necessarily imply that a significant anti-COX activity is maintained. On the basis of these considerations, we developed a chemistry program with the following objectives: (a) to increase Aβ42 lowering properties, (b) to remove anti-COX activity from flurbiprofen.

Chemistry. Several possible chemical variations were considered and implemented on the basis of flurbiprofen structure (Scheme 1).

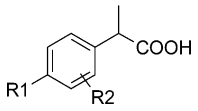
A first array of compounds was synthesized through a Suzuki coupling reaction between **4** and the suitable commercially available aryl or heteroaryl boronic acids. Intermediate **4** was prepared in four steps as outlined in Scheme 2; the anion of diethyl 2-methylmalonate was reacted with 2,4-difluoronitrobenzene to obtain **1**. After catalytic hydrogenation, the resulting amine **2** was converted into the corresponding diazonium salt that was reacted with potassium iodide to obtain **3**. Finally, hydrolysis and decarboxylation of **3** afforded **4** in 57% overall yield.

Suzuki couplings were performed on 1 mmol scale in a parallel way by employing a Myriad Personal Synthesizer. Intermediate **4** was reacted with the appropriate boronic acids in 1,2-dimethoxyethane at 80 °C in the presence of tetrakis(triphenylphosphine)palladium and aqueous sodium carbonate to obtain **5a**−**o** (Table 1).

The same reaction sequence was adopted to synthesize compounds **6a**−**c** that feature different substituents on the central aromatic ring.

After evaluation of the first array of biphenyl derivatives, a new synthetic pathway was developed in order to allow the synthesis of gem-disubstituted derivatives (Scheme 3). 4-Bromo-3-fluorotoluene was brominated with NBS to afford **7** that was reacted with potassium cyanide to obtain **8**. From this intermediate gem-disubstituted derivatives **9a**,**b** could be prepared by reaction with the appropriate methyl iodide or 1,2-dibromoethane. The cyano group was then hydrolyzed to afford **10a**,**b** that was submitted to a modified Suzuki coupling reaction to obtain the expected biaryl compounds **11a**−**h** (Table 5). The coupling was performed in ligand-free conditions[22] by reacting **10a**,**b** with the suitable boronic acids in aqueous sodium carbonate in the presence of TBAB and $Pd(OAc)_2$. Reactions were performed at 120 °C under microwave or conventional thermal heating in a sealed tube. These conditions gave a faster reaction time, improved yields and simplified the purification of crude materials.

Table 1. Variations of the Aromatic Rings of Flurbiprofen



compound	R_1	R_2	Aβ42 inhibition (@ 100 μM)	IC_{50} (μM)[a]
flurbiprofen	phenyl	3-F	30%	305
5a	3,4-dichlorophenyl	3-F	87%	75
5b	3,5-dichlorophenyl	3-F	75%	77
5c	2-chlorophenyl	3-F	13%	nd[a]
5d	3-chlorophenyl	3-F	58%	nd
5e	4-chlorophenyl	3-F	55%	nd
5f	4-cyclohexylphenyl	3-F	79%	21
5g	4-methylphenyl	3-F	44%	nd
5h	4-CF_3 phenyl	3-F	60%	129
5i	2-CF_3 phenyl	3-F	25%	nd
5j	3-benzothienyl	3-F	96%	83
5k	3-thienyl	3-F	40%	nd
5l	4-pyridyl	3-F	11%	nd
5m	4-methoxyphenyl	3-F	67%	67
5n	3,4 methylendioxyphenyl	3-F	42%	210
5o	4-hydroxyphenyl	3-F	27%	nd
13a	4-cyclohexyloxyphenyl	3-F	76%	41
16d	4-(4-hydroxyphenyl)phenyl	3-F	75%	35
6a	4-CF_3 phenyl	2-CF_3	57%	nd
6b	4-CF_3 phenyl	3-Cl	40%	nd
6c	4-CF_3 phenyl	H	2%	nd

[a] nd = not determined.

Derivatives **13a**−**d** were synthesized by alkylation or by Mitsunobu reaction on the corresponding ester of **5o** or **12** that were obtained through a traditional Suzuki coupling (Scheme 4).

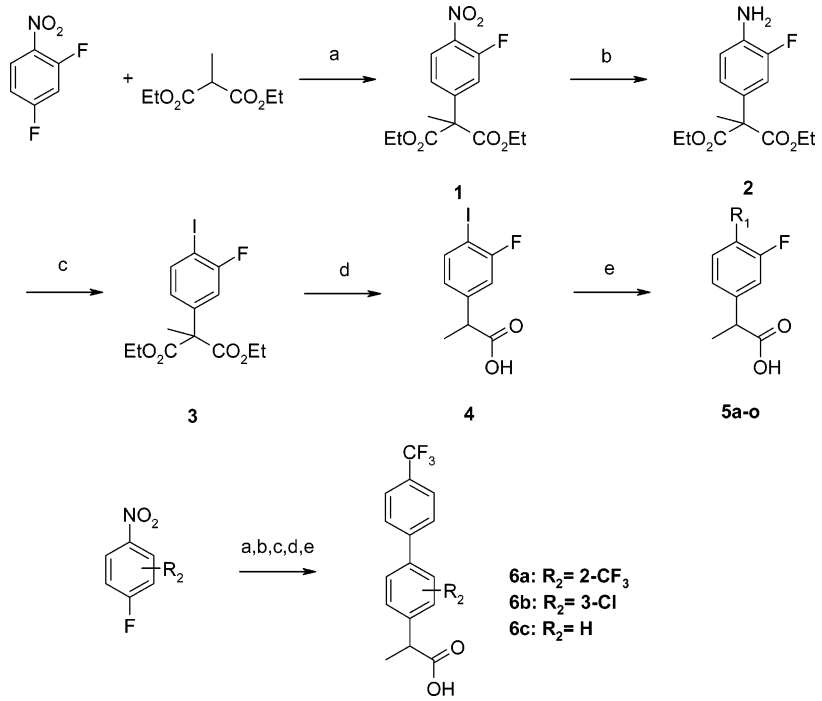
Terphenyl derivatives **16a**−**d** were prepared through a combination of the two Suzuki protocols previously employed (Scheme 5). Intermediate **10b** was converted into the corresponding iodo derivative **14** by a Ni-mediated halogen exchange[23] in order to allow a cleaner Suzuki coupling reaction with 4-bromobenzeneboronic and 4-bromo-3-fluorobenzeneboronic acids. Hence, **14** and **4** were submitted to a traditional Suzuki coupling leading to **15a**−**c** in about 60% yield, whereas the final coupling was performed in ligand free conditions as previously described, affording **16a**−**d**.

Compounds featuring bioisosteres of the carboxylic acid group (Table 3) were prepared according to Schemes 6 and 7. Hydroxamic acid **18** and acylsulfonamide **19** were prepared by reaction of the corresponding acyl chloride **17** with ammonia, hydroxylamine or methanesulfonamide, respectively (Scheme 6).

Tetrazole **21** and 2H-[1,2,4]-oxadiazol-5-one derivative **23** were obtained from nitrile **20** that in turn was synthesized from the corresponding acid **5h** in three steps. Compound **21** was prepared by reacting **20** with Bu_3SnN_3, whereas **23** was obtained by cyclization with CDI of N-hydroxyamidine **22**, obtained by reaction of **20** with hydroxylamine (Scheme 7).

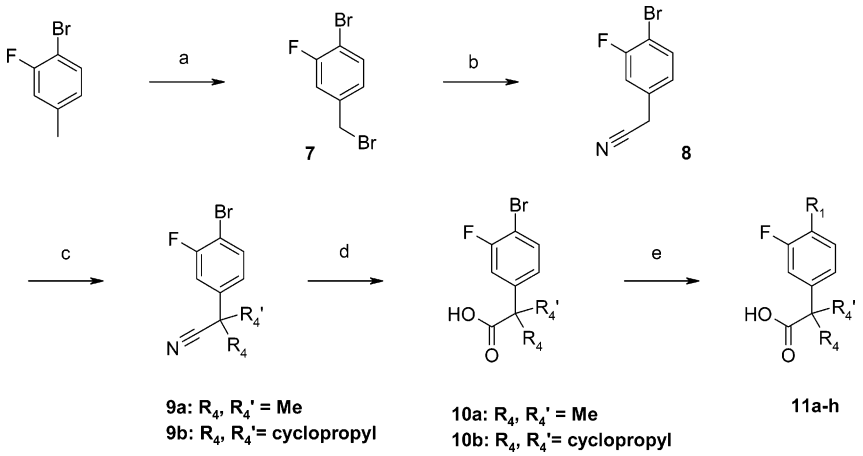
Similarly, compounds **24**, **25** and **26**, featuring a methyl ester, *tert*-butyl ester and a primary amide group

Scheme 2[a]



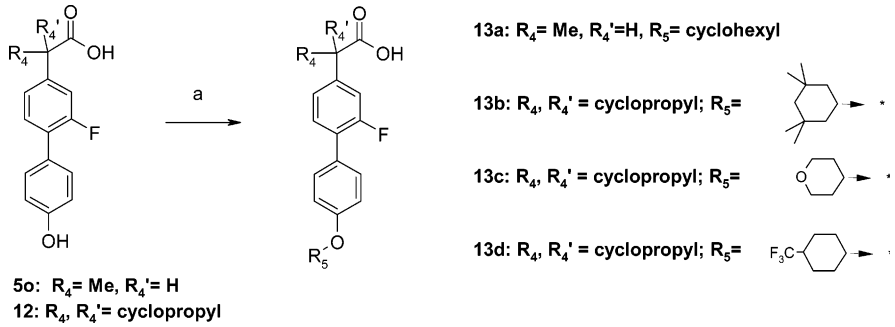
[a] Reagents and conditions: (a) NaH, DMSO; (b) H_2, Pd/C; (c) 1. $NaNO_2$, HCl, 0 °C; 2. KI; (d) NaOH, EtOH−H_2O, 100 °C, 57% (four steps); (e) $R_1B(OH)_2$, $Pd(PPh_3)_4$, Na_2CO_3, DME-H_2O, 80 °C.

Scheme 3[a]



[a] Reagents and conditions: (a) NBS, CCl_4; (b) KCN, EtOH; (c) RX or $Br(CH_2)_2Br$, NaOH, toluene-H_2O; (d) NaOH, EtOH−H_2O, 100 °C; (e) $R_1B(OH)_2$, $Pd(OAc)_2$, Na_2CO_3, TBAB, H_2O, 120 °C (MW or oil bath, sealed tube).

Scheme 4[a]

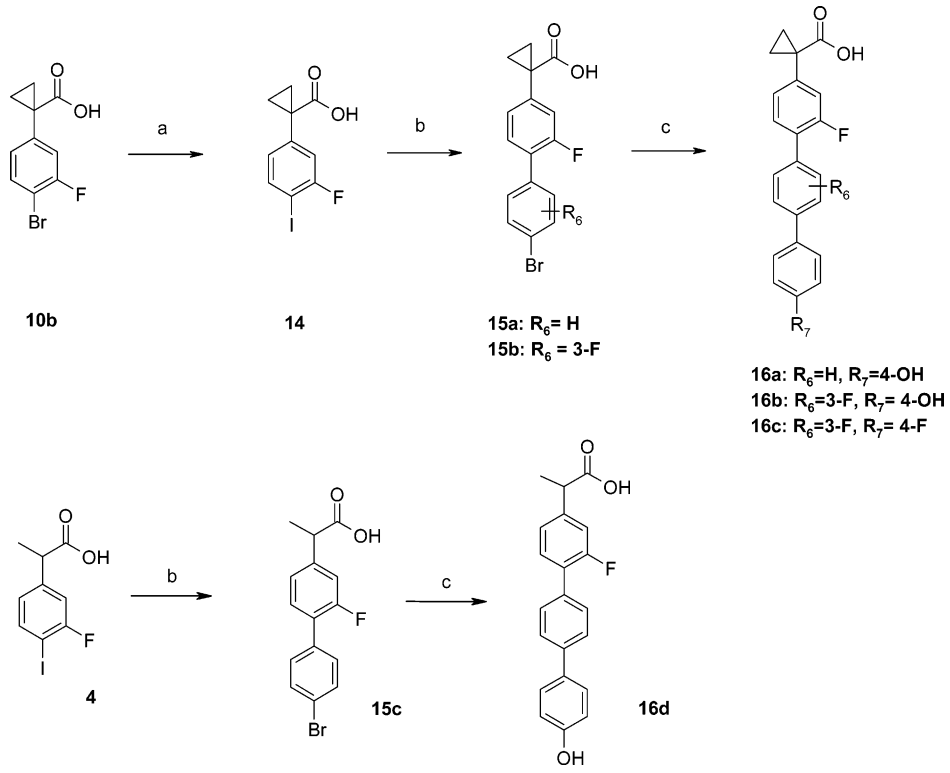


[a] Reagents and conditions: (a) 1. MeOH, H_2SO_4, reflux; 2. R_5OH, DEAD, PPh_3, THF, reflux; 3. KOH, THF/MeOH, reflux.

respectively, were obtained from the corresponding carboxylic acid **5b** by known procedures (Scheme 8).

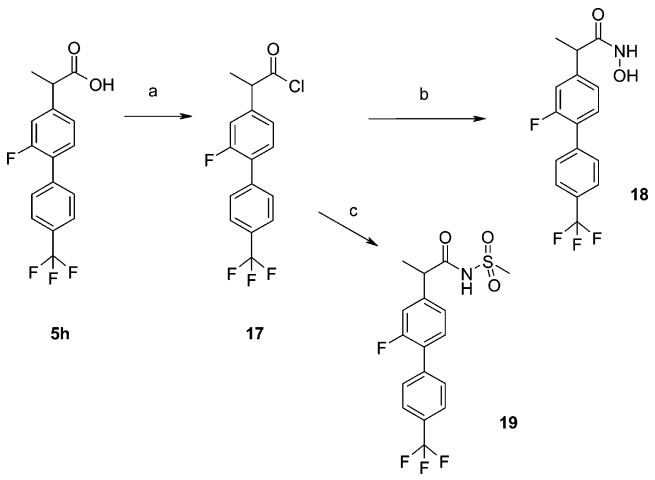
Biology. The effects of the compounds on Aβ42 and Aβ40 secretion were evaluated in human neuroglioma cells (H4-APP695NL) using a validated immunoassay (see Experimental Section). The effects of flurbiprofen and of a representative analogue (**11c**) on other Aβ species were studied using MALDI-TOF technique.

Scheme 5[a]



[a] Reagents and conditions: (a) KI, Ni, DMF, 150 °C; (b) 4-bromophenylboronic acid or 4-bromo-3-fluorophenylboronic acid, $Pd(PPh_3)_4$, Na_2CO_3, DME-H_2O, 80 °C; (c) 4-fluorophenylboronic acid or 4-hydroxyphenylboronic acid, $Pd(OAc)_2$, Na_2CO_3, TBAB, H_2O, 120 °C, MW or oil bath, sealed tube.

Scheme 6[a]



[a] Reagents and conditions: (a) $(COCl)_2$, DCM; (b) NH_2OH, Et_3N, DCM; (c) $MeSO_2NH_2$, Et_3N, DCM.

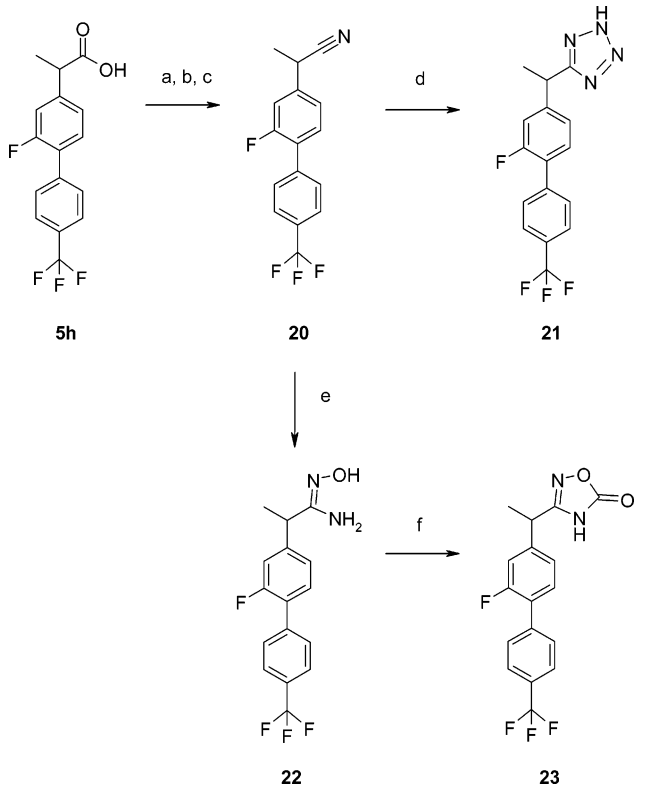
During the experiments on human neuroglioma cells, the effects of compounds on cell viability were simultaneously assessed using the MTT cytotoxicity assay: all compounds showed less than 15% of cytotoxicity at the concentrations used for the test. The pharmacological selectivity of selected compounds was evaluated on COX-1 and COX-2 enzyme activity assay. The effects of a few compounds on another substrate of γ-secretase, Notch-1, were assessed using a luciferase assay. In vitro ADME developability parameters of selected compounds were explored by evaluating membrane permeability in human colon adenocarcinoma (Caco-2) cell monolayers, interactions with major isoforms of human cytochrome P450, total body clearance and penetration in cerebrospinal fluid after continuous subcutaneous administration in rats.

Structure−Activity Relationships (SAR). Investigation of SAR for flurbiprofen analogues focused initially on substitution at the terminal phenyl ring of the molecule (Table 1). Derivatives bearing the 3-chloro (compound **5d**) or the 4-chloro substitution on the terminal phenyl ring (compound **5e**) showed an increased activity for the inhibition of Aβ42 production (about 50% at the concentration of 100 μM). On the contrary, derivative **5c** featuring the *o*-chloro substitution at the terminal phenyl ring was significantly less active (13% inhibition at the concentration of 100 μM). Combined 3,4-dichloro substitution (compound **5a**) showed a further improved activity, with an IC_{50} of 75 μM; compound **5b**, featuring the 3,5-dichloro substitution, showed a similar potency (IC_{50} of 77 μM). Comparison of compounds **5h** and **5i**, bearing a trifluoromethyl substituent at position para and ortho, respectively, seemed to confirm that para substitution is better than ortho substitution in inhibiting Aβ42 production.

Substitution with alkoxy derivatives (compounds **5m**, **5n**, **13a**) also led to an increase of Aβ42 inhibition, while the corresponding hydroxy derivative **5o** was less active; this effect was more prominent with bulky alkoxy groups (**13a**). Compound **13a** also showed to selectively inhibit Aβ42 formation, since Aβ40 inhibition (Table 1) was less significant (25% inhibition at the concentration of 100 μM).

Compounds bearing alkyl instead of alkoxy residues followed the same trend as the alkoxy derivatives, with bulky alkyl substituents (such as the cyclohexyl group,

Scheme 7[a]



[a] Reagents and conditions: (a) $(COCl)_2$, DCM; (b) NH_3, DCM; (c) $(CF_3CO)_2O$, Py, DCM; (d) Bu_3SnN_3, 100 °C; (e) NH_2OH, MeOH, 70 °C; (f) CDI, dioxane, 100 °C.

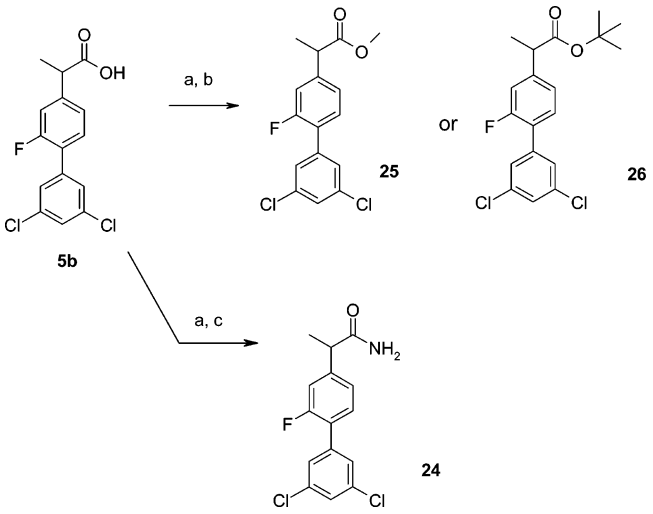
compound **5f**) displaying the best activity (IC_{50} = 21 μM). Compound **5f** showed also a moderate selectivity between Aβ42 and Aβ40 inhibition (IC_{50} = 21 μM for Aβ42 versus IC_{50} = 46.5 μM for Aβ40 inhibition). The terphenyl derivative **16d** similarly showed good activity for Aβ42 inhibition (IC_{50} = 35.3 μM).

Replacement of the terminal phenyl ring with other heterocycles was also considered; while thienyl and pyridyl derivatives **5k** and **5l** were only moderately active, compound **5j** bearing the 3-benzothienyl group displayed good activity, with an IC_{50} of 83 μM.

The most interesting compounds were evaluated for their activity toward COX-1 enzyme, P450 interactions and permeability in the Caco2 cell layer model (Table 2). Benzothienyl derivative **5j** and compound **5a** bearing the 3,4-dichloro substitution on the terminal phenyl ring still showed a significant interaction with COX-1 (68% and 78% inhibition at the concentration of 100 μM, respectively), while introduction of bulkier substituents such as in compounds **5f**, **5h**, **13a**, **16d** caused a significant loss of COX-1 inhibitory activity. Significant interactions with one or more isoforms of P450 enzyme were observed for methoxy derivative **5m**, cyclohexyl derivative **5f**, dichloro derivative **5a**, cyclohexyloxy derivative **13a**, and terphenyl derivative **16d**; trifluoromethyl derivative **5h** showed no interactions at the concentration of 100 μM. Permeability in the Caco-2 assay proved to be medium to high for all the tested compounds.

The three most potent compounds (cyclohexyl derivative **5f**, cyclohexyloxy derivative **13a** and terphenyl derivative **16d**) were also tested in vivo in a steady-state model of metabolic clearance. All the derivatives showed

Scheme 8[a]



[a] Reagents and conditions: (a) $(COCl)_2$, (b) DCM, MeOH or tBuOH; (c) NH_3, DCM.

a high clearance; the concentration in the cerebrospinal fluid was about 1−2% of the concentration in plasma for **5f** and **16d** (similarly to what was observed with flurbiprofen) or below the detection limit for compound **13a**.

Substitution at the central aromatic ring was studied with derivatives **6a**, **6b**, **6c** (Table 1) bearing the 4-trifluoromethyl substitution on the terminal phenyl ring. Replacement of the fluorine atom with chlorine in position 3 (derivative **6b**) or with a trifluoromethyl group in position 2 (derivative **6a**) resulted in compounds with an inhibition of Aβ42 production comparable to **5h**. On the contrary, unsubstituted derivative **6c** was almost inactive at the concentration of 100 μM (Table 2).

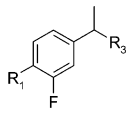
Carboxylic acid group bioisosteres were also considered, to verify if a better ADME developability profile could be obtained, especially for the penetration of compounds in the cerebrospinal fluid. Derivatives bearing the 4-trifluoromethyl substitution or the 3,5-dichlorophenyl substitution on the terminal phenyl ring were synthesized (Table 3). All carboxylic group bioisosteres (compounds **21**, **18**, **19**, **23**) displayed a lower activity in Aβ42 inhibition (Table 3) and significantly increased interactions with P450 enzymes (Table 4). The concentration in the cerebrospinal fluid was evaluated for compounds **19** and **23** and was not greater than 2%. Interestingly, two ester derivatives (methyl and *tert*-butyl, compounds **24** and **25**) were found to selectively raise Aβ42 (242% and 259% increase in Aβ42 compared to 45% and 20% inhibition of Aβ40 at 100 μM, respectively), supporting the allosteric nature of the γ-secretase modulation by NSAIDs.[18] Indeed, other authors have shown that simple chemical modifications of the carboxylic moiety of NSAIDs can generate selective Aβ42 raising agents.[24] A further modification of the carboxylic moiety with primary amide (compound **26**) led to the loss of selectivity in the Aβ42 inhibition (IC_{50}s of 64 and 12 μM on Aβ42 and Aβ40, respectively); the metabolic clearance was very high (193 mL/h), but penetration in the cerebrospinal fluid was improved (19% of the plasma levels). However, this last observa-

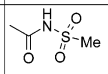
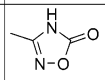
Table 2. Selectivity Assays and ADME Developability Parameters for Selected compounds

compound	Aβ40 inhibition	COX-1 inhibition	P450 interactions	Caco-2 absorption	clearance (mL/h)	CSF/plasma ratio
flurbiprofen	nd	IC_{50} = 170 nM	no interactions @ 100 μM	high	13.9	1.3%
5a	nd	78% @ 100 μM	inhibition >50% @ 100 μM for 2C19, 2C9*	high	nd[a]	nd
5b	IC_{50} = 94 μM	65% @ 100 μM	2C9[b]	high	nd	nd
5f	IC_{50} = 46 μM	18% @ 100 μM	inhibition >50% @ 100 μM for 1A2, 2C19, 2D6; 2C9[b]	medium	174	1.5%
5h	nd	40% @ 100 μM	no interactions @ 100 μM	high	nd	nd
5j	nd	68% @ 100 μM	2C9[b]	high	nd	nd
5m	nd	nd	IC_{50} = 87 μM (2C19)	high	nd	nd
13a	25% @ 100 μM	20% @ 100 μM	inhibition >50% @ 100 μM for 1A2, 3A4; 2C9[b]	high	148	below LLOQ[c]
16d	nd	17% @ 100 μM	IC_{50} = 58 μM (2C19), 74 μM (3A4), 4.7 μM (2C9)	high	85	1.1%

[a] nd = not determined. [b] Activation instead of inhibition was observed due to interference with the assay. [c] LLOQ = lower limit of quantitation.

Table 3. Carboxylic Group Replacement of Flurbiprofen Derivatives[a]



Compound	R_1	R_3	Aβ42 inhibition (@100μM)	IC_{50} (μM)[a]
5h	4-CF_3 phenyl	COOH	60%	129
21	4-CF_3 phenyl		43%	114
18	4-CF_3 phenyl	CONHOH	31%	nd
19	4-CF_3 phenyl		34%	nd
23	4-CF_3 phenyl		46%	nd
5b	3,5-dichloro phenyl	COOH	75%	77
24	3,5-dichloro phenyl	COOMe	increase + 242%	
25	3,5-dichloro phenyl	COO*t*Bu	increase + 259%	
26	3,5-dichloro phenyl	$CONH_2$	70%	64

[a] nd = not determined.

Table 4. Selectivity Assays and ADME Developability Parameters for Selected Compounds

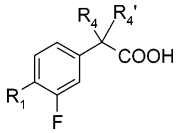
compound	Aβ40 inhibition	COX-1 inhibition	P450 interactions	Caco-2 absorption	clearance (mL/h)	CSF/plasma ratio
flurbiprofen	nd	IC_{50} = 170 nM	no interactions @ 100 μM	high	13.9	1.3%
5h	nd	40% @ 100 μM	no interactions @ 100 μM	high	nd[a]	nd
21	nd	9% @ 100 μM	inhibition >50% @ 100 μM for 1A2, 2C9, 2C19, 2D6, 3A4	nd	nd	nd
19	nd	nd	inhibition >50% @ 100 μM for 1A2, 2C9, 2C19, 2D6, 3A4	nd	3.1	1.7%
23	nd	nd	inhibition >50% @ 100 μM for 2C9, 2C19, 3A4	nd	5.3	2.1%
24	45% @ 100 μM	nd	nd	nd	nd	nd
25	20% @ 100 μM	nd	nd	nd	nd	nd
26	IC_{50}= 12 μM	nd	nd	nd	193	19%

[a] nd = not determined.

tion needs confirmation with further series of related compounds.

Substitution pattern at the alpha position of flurbiprofen was also considered (Table 5), in view of the reported different activity of the *R*- and *S*- enantiomers of flurbiprofen both on COX-1 activity and on Aβ42 inhibitory activity.[19] Introduction of the cyclopropyl substitution or the dimethyl substitution at the alpha position caused an almost complete loss of COX-1 inhibitory activity (Table 6), as for compounds **11a**, the alpha-cyclopropyl derivative of flurbiprofen, or for **11c** (the corresponding alpha-methyl derivative **5a** displayed 78% COX-1 inhibition at the concentration of 100μM). The cyclopropyl substitution also seemed to moderately increase the Aβ42 inhibitory activity (Table 5), as can be observed comparing the activity results for **11a** and flurbiprofen, **11c** and **5a**, **11b** and **5h**. On the contrary, cyclopropyl substitution did not significantly affect

Table 5. Combined Substitutions: Terminal Phenyl Ring and Alpha Position



compound	R_1	R_4, R_4'	Aβ42 inhibition (@ 100 μM)	IC_{50} (μM)
flurbiprofen	phenyl	H, Me	30%	305
11a	phenyl	cyclopropyl	40%	nd[a]
11b	4-CF_3 phenyl	cyclopropyl	55%	111
11c	3,4-dichlorophenyl	cyclopropyl	79%	41
11d	3-benzothienyl	cyclopropyl	49%	96.3
11e	4-(4,4-dimethylcyclohexyl)phenyl	cyclopropyl	75%	46
11f	4-tetrahydropyran-4-ylphenyl	cyclopropyl	52%	nd
11g	4-CF_3 phenyl	Me, Me	58%	70.6
11h	4-cyclohexylphenyl	Me, Me	77%	nd
11i	4-cyclohexylphenyl	cyclopropyl	60%	nd
16a	4-(4-hydroxyphenyl)phenyl	cyclopropyl	99%	31.6
16b	4-(4-hydroxyphenyl)-3-fluorophenyl	cyclopropyl	80%	33.6
16c	4-(4-fluorophenyl)-3-fluorophenyl	cyclopropyl	75%	57.9
13b	4-(3,3,5,5-tetramethylcyclohexyloxy)phenyl	cyclopropyl	97%	11
13c	4-(tetrahydropyran-4-yloxy)phenyl	cyclopropyl	28%	nd
13d	4-(4-trifluoromethylcyclohexyloxy)phenyl	cyclopropyl	70%	20

[a] nd = not determined.

Table 6. Selectivity Assays and ADME Developability Parameters for Selected Compounds

compound	Aβ40 inhibition	COX-1 inhibition	P450 interactions	Caco-2 absorption	clearance (mL/h)	CSF/ plasma ratio
flurbiprofen	nd	IC_{50} = 170 nM	no interactions @ 100 μM	high	13.9	1.3%
11a	−1% @ 100 μM	26% @ 100 μM	IC_{50} = 83 μM (2C9)	high	9.8	2.3%
11b	22% @ 100 μM	<1% @ 100 μM	IC_{50} = 71 μM (2C19)	high	0.52	1.8%
11c	14% @ 100 μM	<1% @ 100 μM	IC_{50} = 38 μM (2C9), 25 μM (2C19), 48 μM (3A4)	high	1.5	1.3%
11d	−5% @ 100 μM	<1% @ 100 μM	IC_{50} = 19 μM (2C9)	high	113.6	2.6%
11e	IC_{50} = 101 μM	17% @ 100 μM	IC_{50} = 17 μM (2C9), 13 μM (2C19), 5.3 μM (2D6), 84 μM (3A4)	low	145.8	below LLOQ[c]
11f	nd	nd	nd	nd	39	below LLOQ
11g	nd	nd	nd	nd	nd	nd
11h	47% @ 100 μM	12% @ 100 μM	IC_{50} = 59 μM (3A4), 28 μM (2C9)	nd	nd	nd
11i	nd	12% @ 100 μM	IC_{50} = 59 μM (3A4), 2C9[b]	nd	nd	nd
16a	IC_{50} = 409 μM	<1% @ 100 μM	IC_{50} = 89 μM (2C9), 35 μM (2C19), 21.5 μM (2D6)	high	nd	nd
16b	IC_{50} = 111 μM	<1% @ 100 μM	IC_{50} = 74 μM (2C19), 58 μM (3A4)	high	109	below LLOQ
16c	32% @ 100 μM	18% @ 100 μM	IC_{50}= 7 μM (2C9), 46 μM (2C19),) 38 μM (3A4)	low	8.1	1.8
13b	IC_{50} = 25.5 μM	<1% @ 100 μM	IC_{50}= 26 μM (1A2) 15 μM (2C9), 33 μM (2C19), 30 μM (2D6) 27 μM (3A4)	medium	134	below LLOQ
13c	nd	nd	nd	nd	70.5	1.1
13d	IC_{50} = 81 μM	<1% @ 100 μM	IC_{50} = 17.7 μM (2C9)	low	6.6	0.75

[a] nd = not determined. [b] Activation instead of inhibition was observed due to interference with the assay. [c] LLOQ = lower limit of quantitation.

either P450 profile and Caco2 permeability, or metabolic clearance and brain penetration.

On the basis of SAR results of the first set of compounds, a new set of derivatives was planned: the carboxylic group was maintained, as well as the 3-fluoro substitution at the central phenyl ring; the alpha-cyclopropyl moiety was introduced in order to gain COX-1 selectivity and a moderate activity enhancement, avoiding the presence of a troublesome stereogenic center. Functionalization of the terminal phenyl ring was the key feature to be introduced in order to improve the Aβ42 inhibitory activity; the substituents identified and outlined in Table 1 improved the activity but were not satisfactory for the interactions with P450 cytochrome, metabolic clearance and penetration in the cerebrospinal fluid.

A second array of compounds bearing combined substitution was then synthesized and tested (Table 5). Compound **11c**, bearing the 3,4-dichloro substitution, displayed a good Aβ42 activity; interestingly, no inhibition of the production of Aβ40 was observed (Table 6). This compound was selective toward COX-1 and showed a low metabolic clearance, but interactions with P450 cytochrome were significant for three isoforms, with an IC_{50} comparable to the Aβ42 inhibition activity. Compound **11b**, bearing the 4-trifluoromethyl substitution, showed good selectivity toward COX-1 and also a better developability profile (low clearance, only one significant P450 interaction), but it was less active in Aβ42 inhibition (IC_{50} = 111 μM, while inhibition of Aβ40 was only 22% at the concentration of 100 μM) if compared to **11c**. Benzothienyl derivative **11d** displayed no interactions with P450 cytochrome (except for 2C9 isoform, with IC_{50} = 19 μM), but only a moderate potency for Aβ42 inhibition and a very high clearance.

In view of these results, we reconsidered cycloalkyl derivatives trying to improve the activity and developability profile of compound **5f** (Table 1), which was one of the most active initially synthesized. Modifications of the terminal cyclohexyl moiety were introduced as in compounds **11e** and **11f**, but they did not result in significant improvement of the clearance profile or of

Table 7. Pharmacokinetic Parameters of Flurbiprofen and Novel Selected Compounds after Intravenous (iv) and Oral (po) Administration to Rats[a]

compound		dose (mg/kg)	$AUC_{0-\infty}$ (μg·h/mL)	C_{max} (μg/mL)	T_{max} (h)	$t_{1/2}$ (h)	CL (mL/h/kg)	F (%)
flurbiprofen	po	7.6	311	49.1	0.5	4.9		78.3
	iv	1.5	78	9.1	0.3	5.1	19.1	
11b	po	2.5	2613	54.9	4.0	32.8		90.9
	iv	0.5	575	14.4	0.08	36.2	0.9	
11c	po	2.5	435	16.2	4.0	20.7		50.2
	iv	0.5	173	6.1	0.08	29.4	2.9	
13d	po	0.5	6	0.3	8.0	11.8		24.2
	iv	0.2	11	0.6	0.25	13.2	18.8	

[a] C_{max} = maximum concentration obtained; $t_{1/2}$ = elimination phase half-life; CL = total body clearance; F = absolute oral bioavailability.

the P450 interactions. Similarly, all the terphenyl derivatives **16a**, **16b**, **16c** maintained good Aβ42 inhibition activity but displayed strong P450 interactions.

Optimization of alkoxy analogues of compound **13a** was then considered. While alkoxy moieties introduced in compounds **13b** and **13c** resulted in worse developability profiles or in loss of potency, derivative **13d**, featuring the trifluoromethyl cyclohexyloxy derivative, maintained a good Aβ42 inhibition activity, good selectivity toward COX-1, only one interaction with P450 cytochrome (2C9 isoform) and low metabolic clearance; permeability in the Caco-2 assay was low, as was the cerebrospinal fluid/plasma concentration ratio.

The three most interesting compounds, **13d**, **11b** and **11c**, were further profiled for their selectivity toward COX-2. At the concentration of 300 μM, both **11b** and **11c** were found to be completely inactive. At the same concentration **13d** retained a 52% inhibitory activity on COX-2 but at the concentration of 100 μM the compound did not inhibit the enzyme.

Compounds **11b** and **13d** were also tested in the CBF1-luciferase assay for their activity on Notch-1 cleavage. At the concentration of 100 μM, **11b** and **13d** did not significantly affect Notch-1 signaling (2.1 $\pm$ 15.1% and 9.3 $\pm$ 10.8% inhibition, respectively).

Since hepatotoxicity is an uncommon, but potentially lethal adverse reaction caused by NSAIDs,[25] we employed the MTT assay, that measures mitochondrial integrity and activity, to estimate hepatoxicity potential of compounds **11b, 11c** and **13d**. All compounds showed low hepatotoxicity (10% at the concentration of 100 μM for **11b**, IC_{50} = 284 μM for **11c**, and IC_{50} greater than 1000 μM for **13d**).

Pharmacokinetic profiles of compounds **11b**, **11c** and **13d** were then evaluated after single intravenous and oral administration in rats and compared to that of flurbiprofen. Plasma concentration profiles standardized for a 1 mg/kg dose versus time are shown in Figure 1 and main pharmacokinetic parameters are listed in Table 7. After oral dosing, flurbiprofen appeared to be quickly absorbed (T_{max} = 30 min) and cleared from plasma with a half-life of around 5 h. Absolute oral bioavailability was found to be 78.3% and total body clearance 19.1 mL/h/kg. After oral administration, compounds **11b**, **11c** and **13d** appeared to be absorbed slower than flurbiprofen (T_{max} = 4−8 h). Compounds were eliminated slowly with half-lives ranging from 12 to 33 h. Total body clearances were also lower than that of flurbiprofen (0.9, 2.9 and 18.8 mL/h/kg for **11b**, **11c** and **13d**, respectively). Absolute oral availability of compounds **11b**, **11c** and **13d** were 91%, 50% and 24% and were in line with the apparent permeability values derived by the Caco-2 model.

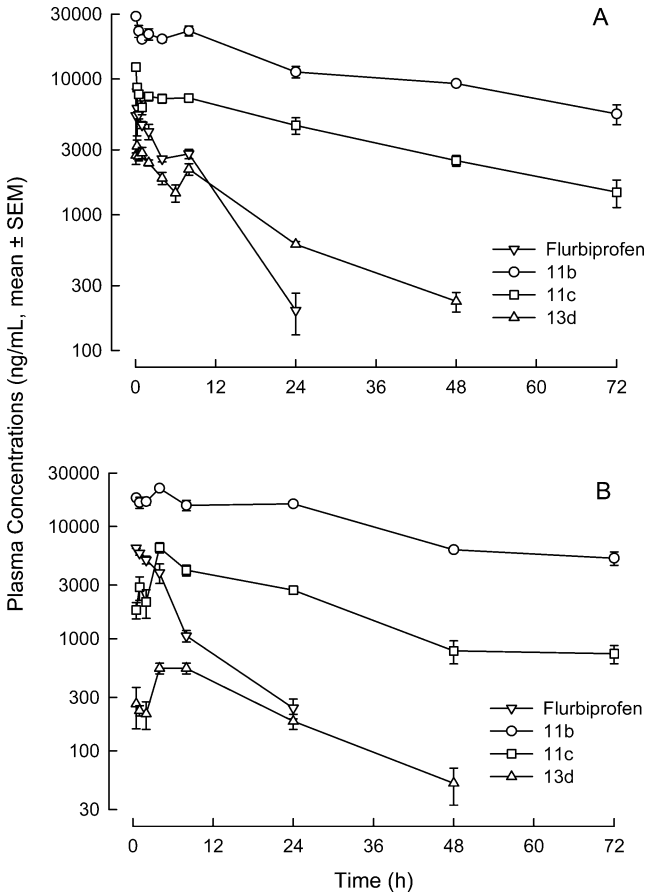


Figure 1. Plasma levels of flurbiprofen and selected analogues (**11b**, **11c** and **13d**) in the rat after intravenous (panel A) and oral (panel B) administration to rats. Plasma levels are standardized for a dose of 1 mg/kg.

Selectivity in the inhibition of Aβ42 species production versus other amyloid peptides was confirmed with compound **11c**, by MALDI-TOF analysis of cell supernatant during the Aβ42 inhibition assay. More than 20 different Aβ species in cell supernatant were detected (Figure 2). Compound **11c** determined a dose dependent decrease of Aβ42 and a simultaneous increase of the Aβ38 species.

The effects of a 7-day treatment (12.5 mg/kg twice-a-day) of *R*-flurbiprofen or compounds **13d**, **11c** and **26** on Aβ40 and Aβ42 plasma concentrations of transgenic mice are summarized in Figure 3. Compared to baseline, *R*-flurbiprofen did not decrease significantly either Aβ40 or Aβ42. Conversely, all the new compounds significantly ($p < 0.05$) reduced Aβ42 concentrations with mean differences compared to controls being −40% after **13d**, −29% after **11c** and −40% after **26**. The corresponding values for Aβ40 were −35%, −4% and −26%,

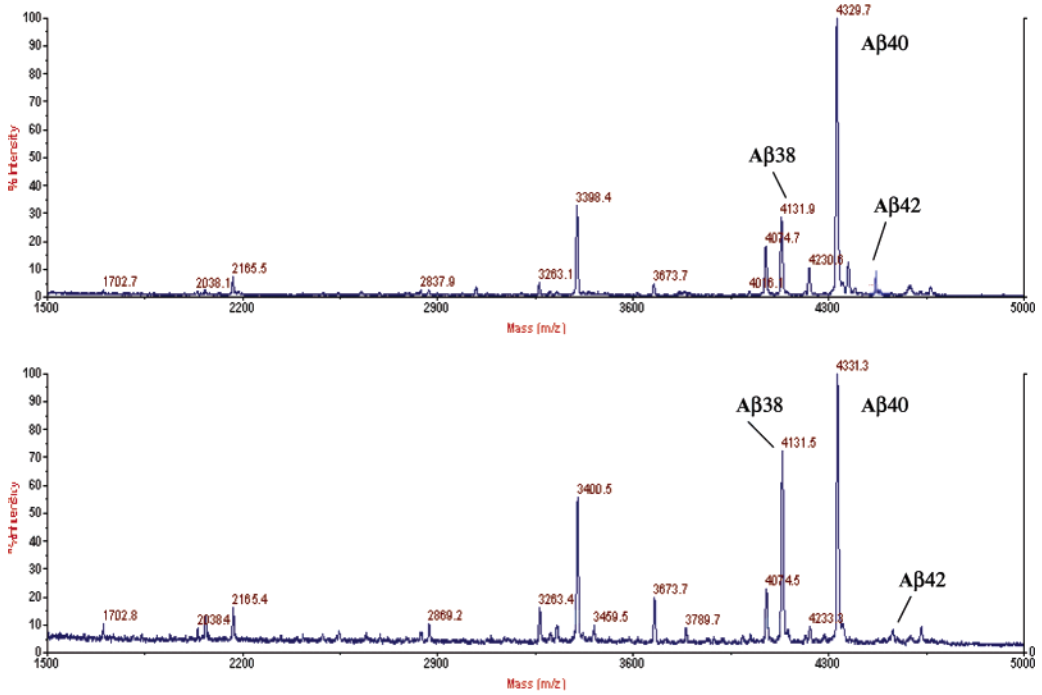


Figure 2. MALDI-TOF spectra of supernatants of immunoprecipitated H4-APP695NL cell cultures incubated for 42 h in absence (upper panel) and in the presence (lower panel) of **11c** (50 μM).

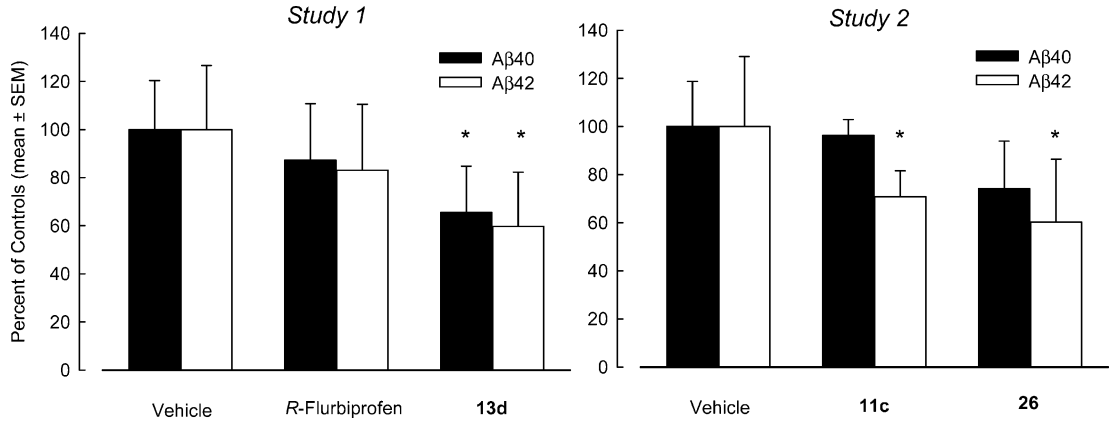


Figure 3. Effects of 7-day dosing (12.5 mg/kg twice-a-day) of *R*-flurbiprofen or compounds **13d**, **11c** or **26** on Aβ40 (black columns) and Aβ42 (white columns) concentrations in plasma of Tg2576 transgenic mice 3 h after the final dose. Columns represent mean (± SEM) Aβ concentrations expressed as percentage of the vehicle control. *$p < 0.05$ compared to baseline.

respectively. Brain concentrations of Aβ were not significantly affected by the short-term treatment of the compounds (data not shown). In Study 1, employing mice with 7−8 months of age (8 animals per treatment group), there were no deaths after vehicle, 2 deaths after *R*-flurbiprofen and 1 death after **13d**. At Day 8, animals treated with **13d** had a significant weight loss (−2.8 ± 0.4 g) compared to vehicle (−0.9 ± 0.7 g). In Study 2, employing mice of 3−4 months of age (12 animals per treatment group), there were four deaths after vehicle, three deaths after **11c** and three deaths after **26**. There were no significant differences between treatment groups on body weight gain.

Discussion

The flurbiprofen analogues we synthesized appear more potent than flurbiprofen and almost completely inactive on COX-activity, especially regarding COX-1 activity, the most dangerous one for the gastrointestinal toxicity. The improvement in potency was reached mainly with appropriate substitutions on the distal phenyl ring of flurbiprofen, while selectivity toward COX-1 and COX-2 was achieved by alpha-cyclopropyl substitution.

Flurbiprofen is exclusively metabolized by CYP2C9 isoenzyme.[26] We found that both racemic and *R*-flurbiprofen interact bimodally with CYP2C9 with activation at lower concentrations and inhibition at higher concentrations. Several flurbiprofen derivatives synthesized during this project showed the same behavior. While flurbiprofen does not interact with the other cytochrome isoenzymes, many analogues showed marked inhibitory activity, especially on CYP2C19. Structure−activity relationships of these interactions were quite complex, with aromatic substitution on the distal phenyl ring and replacement of the carboxylic moiety with the tetrazole group producing the major interferences with CYP isoenzymes.

It has been shown that different chemical classes of prototypical γ-secretase inhibitors do not discriminate between APP and Notch-1 cleavage sites.[27] In vitro studies showed that application of a peptidomimetic γ-secretase inhibitor to fetal thymus organ cultures results in inhibition of T cell development in a manner consistent with loss or reduction of Notch-1 function.[28] More recently, an in vivo study in TgCRND8 APP transgenic mice showed that LY-411,575, a nonpeptidic γ-secretase inhibitor, produces marked effects on lymphocyte development and on the intestine tissue morphology.[29] In addition, it has been shown that a 5-day treatment with γ-secretase inhibitors with dibenzazepine and benzodiazepine structure causes dose-dependent intestinal goblet cell metaplasia in rats.[30] In cell-based assays, sulindac sulfide has been shown not altering either ϵ or S3 cleavage sites.[5] Additional in vitro studies demonstrated that sulindac sulfide and ibuprofen preserve release of APP, Notch-1 and ErbB-4 intracellular domains.[6] Although these observations were confirmed by other groups, at high concentrations of NSAIDs an inhibition of the S3 cleavage site of Notch-1 is observed, reminiscent of classical γ-secretase inhibition.[16] Thus, it appears that the separation of the inhibitory effects on the various cleavage sites is concentration dependent leaving a "window of modulation". Our preliminary results indicated that compounds **11b** and **11c** evaluated at concentrations (100 μM) near or above the IC_{50} on Aβ42 secretion do not significantly inhibit Notch-1 cleavage. In vivo studies are required to confirm the lack of biological effects of these compounds on Notch-1 processing.

The two main metabolites of flurbiprofen are the 4′-hydroxy and 3′,4′-dihydroxy derivatives.[31] Indeed, flurbiprofen analogues with para and meta positions of the distal phenyl ring protected from metabolic oxidation showed a much lower total body clearance than that of flurbiprofen. Successful protection of 3′ and 4′ positions was reached with halogens or trifluoromethyl moiety (**11b** and **11c**). Interestingly, these compounds showed higher inhibitory potency on Aβ42 secretion than flurbiprofen. Total body clearances calculated with a single blood sample taken at steady-state after continuous subcutaneous infusion with osmotic mini-pumps were found in good agreement with values calculated for selected compounds (flurbiprofen, **11b**, **11c**, **13d**) in traditional experiments with single intravenous doses and drug concentrations measured at multiple time points. After intravenous or oral administration to rats, **11b** and **11c** were eliminated slowly with plasma half-life of 20−36 h. The low total body clearance of **11b** and **11c** is probably due to their limited oxidative metabolism, the 3′ and 4′ positions being protected from hydroxylation.

Penetration in cerebrospinal fluid of flurbiprofen was low with concentrations representing only 1.5% of the corresponding plasma levels. This is probably linked to the formation of a covalent adduct with proteins via reactive acyl glucuronide metabolites that prevent from crossing of the blood−brain barrier.[32] Indeed the in vivo plasma unbound percent fraction of flurbiprofen in rats is 1.4%,[33] a value in very good agreement with the percent penetration in cerebrospinal fluid we found in our studies. Penetration in cerebrospinal fluid for the new compounds was in the range of 1−2% of the corresponding plasma levels. Interestingly, the primary amide **26** appeared to penetrate more efficiently the blood brain barrier with cerebrospinal fluid levels reaching concentrations equal to 19% of those in plasma. This suggests that this modification of the carboxylic moiety could be a good strategy to improve CNS penetration of these compounds. Further studies are needed to address this important issue.

Studies in transgenic mice expressing the Swedish mutated form of APP showed that a 7-day oral treatment with compounds **13d**, **11c**, and **26** decreased significantly Aβ42 plasma concentrations and to a lesser extent Aβ40. Compound **11c** appeared to be the most selective inhibitor on the two Aβ peptides (−30% on Aβ42 and −4% on Aβ40, compared to controls) and this agreed with its in vitro activity profile (IC_{50} = 41 μM on Aβ42 and 14% inhibition of Aβ40 at 100 μM). Short-term treatment with the new compounds, as well as *R*-flurbiprofen, was not able to significantly modify Aβ levels in the brain. A lack of central effects was also recently observed with a 3-day treatment of racemic flurbiprofen in Tg2576 transgenic mice.[34] This could be due to insufficient brain concentrations reached by these compounds not compatible with their micromolar potency. On the other hand, long-term treatments (3−6 months) with ibuprofen,[8,9,11] indomethacin[12] or flurbiprofen derivatives[10,14] have demonstrated a significant reduction in brain Aβ pathology in transgenic mice. Different reasons may explain the different efficacy of selected NSAIDs on central Aβ after short-term and long-term treatment. First, it could be that NSAIDs accumulate within the neuronal membrane where γ−secretase is localized. Alternatively, it is possible that NSAIDs do not act centrally but instead reduce peripheral Aβ42 levels, which results in enhanced efflux of Aβ42 from the brain.[20] Compared to vehicle, the new compounds appeared to be relatively well tolerated, although no formal biochemistry or histopathological examinations were done. Mortality rate was similar in vehicle-treated animals and in animals treated with the new compounds. However, at Day 8 animals treated with **13d** had a significant body weight loss of about 2 g compared to controls. Conversely, mean body weight of animals treated with **11c** and **26** were similar to that of vehicle-treated animals along the entire study period. In a 2-week toxicity study in mice, no behavioral, laboratory or histopathological toxic effects were observed with **11b** at doses of 30 or 100 mg/kg/day.

Conclusions

Novel flurbiprofen analogues have been identified with potent and selective inhibitory activity on Aβ42.[35] The new Aβ42 lowering agents appear to be almost devoid of anti-COX-1 and COX-2 activity. Compared to flurbiprofen, the new compounds show improved potency on Aβ42 (up to 27 times) and improved total body clearance (up to 29 times). In rats, compounds **13d**, **11b**, **11c** showed good oral bioavailability and long elimination half-life. Short-term studies in a transgenic mice showed that compounds **13d**, **11c** and **26** decreased significantly plasma Aβ42 concentrations. These new flurbiprofen analogues are worthy of being tested after long-term administration in animal models of AD to

verify their ability to ameliorate brain Aβ pathology and could become viable clinical candidates once the ongoing preclinical development activities are successfully completed.

Experimental Section

Chemistry. ^{1}H NMR spectra were recorded on a Bruker ARX 300 (300 MHz); chemical shifts are reported downfield in parts per million (ppm) relative to TMS utilizing the solvent peaks as the reference. EI Mass spectra analysis was recorded on a Thermo Finnigan TSQ700 spectrometer; ESI-LCMS analysis was performed on a Phenomex Luna C-18, 3 μm, 4.6 $\times$ 50 mm column, with an AQA Thermo Finnigan single quadrupole instrument or with a Waters Micromass ZQ2000 instrument; high performance liquid chromatography (HPLC) analysis was performed on a Shimadzu SCL-10A equipped with an SIL-10AD injector and an SPD-M10A detector normally operating in a 200−360 nm range, with a Waters Symmetry C-18, 3.5 μm, 4.6 $\times$ 75 mm column, using a 10 min gradient of 0−100% solvent B, where solvent A is 90:10:0.05 CH_3CN-H_2O-TFA and solvent B is 90:10:0.05 H_2O-CH_3CN-TFA; preparative HPLC purifications were performed on a Waters SymmetryPrep C-18, using a 20 min gradient of 0−100% solvent B, where solvent A is 99.9:0.1 H_2O-TFA and solvent B is 99.9:0.1 CH_3CN-TFA; reactions were monitored by TLC using 0.25 mm Merck silica gel plates (60 F_{254}); column chromatography was performed on Merck silica gel 60 (particle size 0.063−0.2 mm); flash chromatography was conducted using a Biotage-Quad3 apparatus and prepacked silica gel columns (KP−SIL, particle size 32−60 μm). Solid liquid extraction (SLE) cartridges were purchased from Varian ("Chem Elut", 3 mL, unbuffered, part no. 12198003). Anhydrous solvents were purchased from Aldrich and used as received. "Brine" refers to a saturated aqueous solution of NaCl. Unless otherwise specified, solutions of common inorganic salts used in workups are aqueous solutions. Common reagents and solvents are abbreviated as follows: CDI, carbonyldiimidazole; DCM, dichloromethane; DEAD, diethyl azodicarboxylate; DME, 1,2-dimethoxyethane; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; NBS, *N*-bromosuccinimmide; TBAB, tetrabutylammonium bromide; TFA, trifluoroacetic acid; THF, tetrahydrofuran.

2-(3-Fluoro-4-nitrophenyl)-2-methylmalonic Acid Diethyl Ester (1). Anhydrous DMSO (100 mL) was added over 30 min to a 60% dispersion of sodium hydride in mineral oil (6.12 g, 153 mmol), then the mixture was cooled to 15 °C and a solution of diethyl-2-methylmalonate (24.0 g, 138 mmol) in anhydrous DMSO (100 mL) was added dropwise over 30 min. A solution of 2,4-difluoronitrobenzene (20.3 g 128 mmol) in anhydrous DMSO (200 mL) was added dropwise over 60 min to the resulting mixture. After 30 min, the reaction mixture was poured into crushed ice (5 kg) and extracted with ethyl acetate (150 mL $\times$ 3). The organic solution was washed with brine, dried over Na_2SO_4 and concentrated to afford a crude oil (40.0 g). Chromatography on silica gel (hexane, then hexane/ethyl acetate 9:1) afforded **1** as yellow oil (35.0 g, 87%). ^{1}H NMR ($CDCl_3$): δ 8.04(dd, 1H); 7.39(dd, 1H); 7.33(ddd, 1H); 4.26(q, 2H); 4.25(q, 2H); 1.88(s, 3H); 1.27(t, 6H). MS (EI; TSQ 700; source 180 °C; 70 V; 200 μA): 313 (M^+); 268; 241; 213; 195; 184; 166. Anal. ($C_{14}H_{16}FNO_6$) C, H, N, F.

2-(4-Amino-3-fluorophenyl)-2-methylmalonic Acid Diethyl Ester (2). To a solution of **1** (35 g, 112 mmol) in ethanol (500 mL), 10% Pd on charcoal (2.0 g) was added. The mixture was hydrogenated for 1 h under atmospheric pressure, then filtered through a Celite pad and concentrated to afford **2** as a colorless oil (30.2 g, 95%). ^{1}H NMR ($CDCl_3$): δ 7.06(dd, 1H); 6.95(ddd, 1H); 6.72(dd, 1H); 4.22(q, 2H); 4.21(q, 2H); 1.80(s, 3H); 1.25(t, 6H). Anal. ($C_{14}H_{18}FNO_4$) C, H, N, F.

2-(3-Fluoro-4-iodophenyl)-2-methylmalonic Acid Diethyl Ester (3). To a solution of **2** (30 g, 106 mmol) in 6 N HCl (180 mL) cooled to 0 °C, a solution of $NaNO_2$ (7.3 g, 106 mmol) in water (40 mL) was added dropwise. The resulting solution was added dropwise to a solution of KI (73 g, 440 mmol) in water (75 mL), keeping the temperature at 0 °C. The reaction mixture was allowed to warm to room temperature and stirred for 3 h, then extracted with ethyl acetate. The combined layers were washed in sequence with 10% $Na_2S_2O_3$ and brine, then dried over Na_2SO_4 and concentrated under vacuum to afford **3** as a brown oil (37 g, 88%), which was used without further purification in the next step. ^{1}H NMR ($CDCl_3$): δ 7.70(dd. 1H); 7.14(dd, 1H); 6.94(dd, 1H); 4.23(q, 2H); 4.22(q, 2H); 1.83(s, 3H); 1.26(t, 6H). MS (EI; TSQ 700; source 180 °C; 70 V; 200 μA): 394 (M^+); 321; 293; 276; 247; 166. Anal. ($C_{14}H_{16}FIO_4$) C, H, F, I.

2-(3-Fluoro-4-iodophenyl)propionic Acid (4). To a solution of **3** (37 g, 94 mmol) in ethanol (130 mL), 2 N NaOH (130 mL) was added. The mixture was heated to 100 °C for 8 h, then concentrated under vacuum. The residue was dissolved in water (300 mL) and washed with ethyl ether (2 $\times$ 100 mL), then the aqueous layer was acidified (pH 2) with HCl and extracted with ethyl acetate (3 $\times$ 150 mL). The organic extracts were washed with brine, dried over Na_2SO_4 and concentrated to afford a brown oil (27 g). Chromatography on silica gel (methylene chloride) afforded a yellow solid that crystallized from methanol/water 1:3 yielding **4** as a white solid (16 g, 58%). ^{1}H NMR ($CDCl_3$): δ 7.69(dd, 1H); 7.05(dd, 1H); 6.87(dd, 1H); 3.71(q, 1H); 1.50(d, 3H). MS (EI; TSQ 700; source 180 °C; 70 V; 200 μA): 294 (M^+); 249. Anal. ($C_9H_8FIO_2$) C, H, F, I.

1-Bromo-4-bromomethyl-2-fluorobenzene (7). To a solution of 1-bromo-2-fluoro-4-methylbenzene (10.0 g, 53 mmol) in carbon tetrachloride (100 mL) NBS (14.0 g, 80 mmol) was added. The mixture was heated to 80 °C, then dibenzoyl peroxide (0.1 g, 0.4 mmol) was added. The mixture was stirred at this temperature for 1 h then cooled to room temperature and washed in sequence with water and brine, dried over Na_2SO_4 and concentrated to afford an oil (16 g) that was used without further purification in the next step.

(4-Bromo-3-fluorophenyl)acetonitrile (8). A solution of crude **7** (16 g) and NaCN (2 g, 0.04 mol) in ethanol (100 mL) was heated to 80 °C for 2 h, then cooled to room temperature and concentrated under vacuum. The residue was suspended in water and extracted with ethyl acetate. The organic solution was washed with brine, dried over Na_2SO_4 and concentrated to afford a brown oil. Chromatography over silica gel (hexane/ethyl ether 7:3) yielded **8** as a light brown solid (5 g, 44% over two steps). ^{1}H NMR ($CDCl_3$): δ 7.57(dd, 1H); 7.13(dd, 1H); 7.02(dd, 1H); 3.72(s, 2H). MS (EI; TSQ 700; source 180 °C; 70 V; 200 μA): 213 (M^+); 134; 114; 107; 87. Anal. (C_8H_5BrFN) C, H, N, Br, F.

1-(4-Bromo-3-fluorophenyl)cyclopropanecarbonitrile (9b). To a solution of **8** (5 g, 23 mmol) and 1,2-dibromoethane (3 mL, 35 mmol) in toluene (20 mL), 50% NaOH (20 mL) and TBAB (1.6 g, 5 mmol) were added. The mixture was vigorously stirred at room temperature for 4 h, then diluted with water and extracted with ethyl acetate. The organic solution was washed with 1 N HCl, then with brine, dried over Na_2SO_4 and concentrated to afford a brown solid (5.7 g). Chromatography on silica gel (hexane/ethyl ether 1:1) afforded **9b** as yellow solid (5.1 g, 91%). ^{1}H NMR ($CDCl_3$): δ 7.53(dd, 1H); 7.03(dd, 1H); 6.98(dd, 1H); 1.78(m, 2H); 1.40(m 2H). Anal. ($C_{10}H_7BrFN$) C, H, N, Br, F. Compound **9a** was obtained following the same procedure and employing MeI instead of 1,2-dibromoethane.

1-(4-Bromo-3-fluorophenyl)cyclopropanecarboxylic Acid (10b). To a solution of **9b** (5.1 g, 21 mmol) in methanol (10 mL) 35% NaOH (40 mL) was added and the mixture was heated to 100 °C for 8 h. After cooling to room temperature, the mixture was acidified (pH 2) with 2 N HCl, and the precipitate formed was filtered, washed with water and redissolved in 5% $NaHCO_3$. Insoluble materials were filtered off and the solution acidified with 2 N HCl. The precipitate was filtered, washed with water and dried under reduced pressure to afford **10b** as a white solid (4.2 g, 76%). ^{1}H NMR (DMSO-d_6): δ 7.60(dd, 1H); 7.34(dd, 1H); 7.13(dd, 1H); 1.45-(m, 2H); 1.18(m, 2H). Anal. ($C_{10}H_8BrFO_2$) C, H, Br, F. Compound **10a** was obtained starting from **9a** and following the same procedure.

General Procedure for Preparing Biaryl Compounds 5a−o. Reactions were performed in 2 mL vessels under argon atmosphere employing a Myriad Personal Synthesizer. 2 M Na_2CO_3 (0.75 mL) and 3 mL of a solution prepared dissolving **4** (4.7 g, 16 mmol) and $Pd(PPh_3)_4$ (0.92 g, 0.8 mmol) in DME (70 mL) were added in sequence to 1 mmol of the suitable arylboronic acid. Reaction vessels were maintained at 80 °C for 15−24 h under stirring. Workup was performed by adding 2 N HCl (1.5 mL) and ethyl acetate (3 mL) and removing the aqueous phase with SLE cartridges. The organic phase was concentrated under vacuum and crude materials purified by flash chromatography (DCM/MeOH 97:3) or preparative HPLC. Products were obtained on average 50% isolated yield and purity greater than 95% (HPLC−UV 215 nm).

Compound 5a. MS (EI; TSQ 700; source 180 °C; 70 V; 200 μA): 312 (M^+.); 267 ($M^+ - CO_2$).

Compound 5b. ^{1}H NMR (DMSO): δ (DMSO): 12.45(s br, 1H, COO*H*); 7.65(dd, $J = 1.9$, 1.9 Hz, 1H, 4′); 7.60(dd, $J = 1.9$, 1.9 Hz, 2H, 2′ and 6′); 7,57(dd, $J = 8.5$, 8.5 Hz, 1H, 6); 7,27-(dd, $J = 12.3$, 1.6 Hz, 1H, 3); 7,25(dd, $J = 8.5$, 1.6 Hz, 1H, 5);3.80(q, $J = 7.2$ Hz, 1H, CH3−C*H*); 1.41(d, $J = 7.2$ Hz, 3H, C*H3*−CH). MS (EI; TSQ 700; source 180 °C; 70 V; 200 μA): 312 (M^+); 267 ($M^+ - CO_2$). Anal. ($C_{15}H_{11}Cl_2FO_2$) C, H, Cl, F.

General Procedure for Preparing Biaryl Compounds 11a−h. A suspension of **10a** or **10b** (0.39 mmol), the appropriate arylboronic acid (0.47 mmol), TBAB (125 mg, 0.39 mmol) and $Pd(OAc)_2$ (10 mg) in 2 M Na_2CO_3 (1 mL) was heated to 120 °C in a sealed tube for 1 h. 2 N HCl and ethyl acetate were added and the mixture was filtered through a Celite pad. The organic layer was separated, washed with brine, dried over Na_2SO_4 and concentrated under vacuum. Crude materials were purified by flash chromatography on silica gel (hexane/ethyl acetate 8:2). Final products were obtained in average 70% isolated yield and purity greater than 95% (HPLC−UV 215 nm).

Compound 11a. ^{1}H NMR (DMSO-d_6): δ 12.41(s br, 1H, COO*H*); 7.56−7.35(m, 6H, CH−Ar); 7.27(m, 1H, C*H*-Ar); 7.24-(s, 1H, C*H*-Ar); 1.48(m, 2H, *CH*$_2$-cyclopr.); 1.22(m, 2H, C*H*$_2$-cyclopr.). MS (EI; TSQ 700; source 180 °C; 70 V; 200 μA): 256 (M^+); 210; 196. Anal. ($C_{16}H_{13}FO_2$) C, H, F.

General Procedure for Preparing Alkoxy Derivatives 13a−d. 5o or **12** were submitted to esterification by refluxing overnight a solution of the compound (3.8 mmol) and 98% sulfuric acid (1 mL) in methanol (50 mL). After extractions with ethyl acetate, washings with brine and drying over Na_2SO_4, the methyl ester was obtained as a white solid and employed without further purification.

The methyl ester (3.6 mmol), triphenylphosphine (1.15 g, 4.3 mmol) and 3.6 mmol of the suitable alcohol were dissolved in anhydrous THF (10 mL) under inert atmosphere. To this mixture, a solution of DEAD (0.8 mL, 4.3 mmol) in THF (5 mL) was added dropwise. The reaction mixture was heated to 65 °C overnight, then diluted with ethyl acetate, washed in sequence with 1 N HCl, 1N NaOH and brine, then dried over Na_2SO_4 and concentrated to dryness. The residue was chromatographed on silica gel (hexane/ethyl acetate 95:5 to 90:10) and then submitted to hydrolysis as follows: 1.6 mmol were dissolved in a mixture of THF and MeOH 3:1 (15 mL), then 2 N KOH (5 mL) was added and the solution was refluxed overnight. Extractions with ethyl acetate, washings with brine and drying over Na_2SO_4 yielded a crude material that was purified by crystallization from ethanol to afford the expected compounds in 25% average yield (three steps).

Compound 13a. ^{1}H NMR (DMSO-d_6): δ 11.80(s br, 1H, COO*H*); 7.47−7.35(m, 3H, C*H*-Ar); 7.20−7.13(m, 2H, C*H*-Ar); 7.01(d, 2H, C*H*-Ar); 4.38(m, 1H, C*H*-cyclohex.); 3.63(q, 1H, C*H*-benzylic); 1.95(m, 2H, C*H*$_2$-cyclohex.); 1.73(m, 2H, C*H*$_2$-cyclohex.); 1.58−1.22(m, 6H, C*H*$_2$-cyclohex.); 1.35(d, 3H, C*H*$_3$). MS (EI; TSQ 700; source 180 °C; 70 V; 200 μA): 342 (M^+); 260; 215. Anal. ($C_{21}H_{23}FO_3$) C, H, F.

1-(3-Fluoro-4-iodophenyl)cyclopropanecarboxylic Acid (14). A suspension of **10b** (1.0 g, 3.9 mmol), KI (6.5 g, 39 mmol), Ni (2.5 g, 40 mmol) and I_2 (50 mg, 0.2 mmol) in DMF (10 mL) was heated to 140 °C for 2 h, then cooled and diluted with 1 N HCl (100 mL) while decanting metallic Ni. The precipitate was filtered, washed with water and redissolved in ethyl acetate, then dried over Na_2SO_4, and concentrated to yield a crude solid (0.84 g) that was employed without further purification.

General Procedure for Preparing Compounds 15a−c. To a solution of **14** or **4** (2 mmol), the suitable 4-bromoarylboronic acid (2.3 mmol) and $Pd(PPh_3)_4$ (0.1 mmol) in DME (20 mL) in 2 M Na_2CO_3 (5 mL) were added and the mixture was heated to 80 °C for 2 h. After extractions with ethyl acetate, washings with brine and drying over Na_2SO_4, the residue was chromatographed on silica gel (hexane/ethyl acetate 7:3) to afford the expected products in 60% average yield.

General Procedure for Preparing Compounds 16a−d. A suspension of the suitable acid **15a−c** (1.0 mmol), the suitable arylboronic acid (1.5 mmol), TBAB (1.5 mmol) and $Pd(OAc)_2$ (0.05 mmol) in 2 M K_2CO_3 was heated to 130 °C in a sealed Pyrex tube for 1 h. 2 N HCl and ethyl acetate were added and the mixture was filtered through a Celite pad. The organic layer was separated, washed with brine, dried over Na_2SO_4 and concentrated under vacuum. Crude materials were purified by preparative HPLC. Final products were obtained in 30% average yield and purity greater than 95% (HPLC−UV 215 nm).

Compound 16a. ^{1}H NMR (DMSO-d_6): δ 12.48(s br, 1H, COO*H*); 9.55(s, 1H, O*H*); 7.69(d, 2H, C*H*-Ar); 7.61−7.45(m, 5H, C*H*-Ar); 7.28(m, 1H, C*H*-Ar); 7.25(s, 1H, C*H*-Ar); 6.87(d, 2H, C*H*-Ar); 1.48(m, 2H, *CH*$_2$-cyclopr.); 1.23(m, 2H, C*H*$_2$-cyclopr.). MS (EI; TSQ 700; source 180 °C; 70 V; 200 μA): 348 (M^+); 302. Anal. ($C_{22}H_{17}FO_3$) C, H, F.

2-(2-Fluoro-4′-trifluoromethylbiphenyl-4-yl)propionyl Chloride (17). To a solution of **5h** (1.5 g, 4.8 mmol) in DCM (50 mL), oxalyl chloride (1.7 mL, 19.2 mmol) was added dropwise and the solution was stirred overnight at room temperature. Concentration yielded **17** as a yellow oil (1.6 g) that was employed without further purification.

2-(2-Fluoro-4′-trifluoromethylbiphenyl-4-yl)-N-hydroxypropionamide (18). Triethylamine (0.60 mL, 4.9 mmol) was added dropwise to a suspension of hydroxylamine hydrochloride (240 mg, 3.4 mmol) in DCM (5 mL). The mixture was stirred for 30 min at room temperature, then the insoluble salt was filtered off. To the clear solution, cooled to 0 °C, a solution of **17** (200 mg, 0.60 mmol) in DCM (1 mL) was added dropwise. After 1 h the mixture was extracted with ethyl acetate, washed with brine and dried over Na_2SO_4.The crude material was chromatographed on silica gel (DCM/MeOH 95:5) to afford **18** as a white solid (60 mg, 30%). ^{1}H NMR (DMSO-d_6): δ 10.68-(s, 1H); 8.83(s, 1H); 7.83(d, 2H); 7.76(d, 2H); 7.54(dd, 1H); 7.32−7.23(m, 2H); 3.53(q, 1H); 1.39(d, 3H). MS (EI; TSQ 700; source 180 °C; 70 V; 200 μA): 327(M^+); 267. Anal. ($C_{16}H_{13}F_4NO_2$) C, H, N, F.

***N*-[2-(2-Fluoro-4′-trifluoromethylbiphenyl-4-yl)propionyl]methanesulfonamide (19).** To a solution of methanesulfonamide (120 mg, 1.26 mmol) and Et_3N (0.2 mL, 1.64 mmol) in DCM (5 mL), a solution of **17** (200 mg, 0.60 mmol) in DCM (1 mL) was added dropwise. After stirring for 1 h, the mixture was extracted with ethyl acetate, washed with brine and dried over Na_2SO_4. The crude material crystallized from MeOH to afford **19** (85 mg, 36%) as a white solid. ^{1}H NMR (DMSO-d_6): δ 11.93(s br, 1H); 7.84(d, 2H); 7.78(d, 2H); 7.59-(dd, 1H); 7.32−7.24(m, 2H); 3.84(q, 1H); 3.24(s, 3H); 1.42(d, 3H). MS (EI; TSQ 700; source 180 °C; 70 V; 200 μA): 389-(M^+); 294; 267. Anal. ($C_{17}H_{15}F_4NO_3S$) C, H, N, F, S.

2-(2-Fluoro-4′-trifluoromethylbiphenyl-4-yl)propionitrile (20). A solution of **17** (1.3 g, 3.9 mmol) in DCM (10 mL) was added dropwise to a saturated solution of gaseous ammonia in DCM (100 mL). The mixture was stirred for 30 min at room temperature, then concentrated, the residue washed with water (3 × 20 mL) and desiccated to afford the corresponding primary amide as white solid. The raw product was dissolved in dioxane in 1,4-dioxane (15 mL) and pyridine (5 mL, 62 mmol) was added; the resulting solution was cooled to 0 °C and trifluoroacetic anhydride (5 mL, 35 mmol) was added

dropwise. The reaction mixture was allowed to warm to room temperature and stirred for 4 h. The mixture was then poured into crushed ice (150 g) and extracted with ethyl acetate. The organic solution was washed in sequence with 1 N HCl, 10% $NaHCO_3$ and brine, then dried over Na_2SO_4 and concentrated to afford **20** as a yellow solid (0.9 g, 95%).

5-[1-(2-Fluoro-4′-trifluoromethylbiphenyl-4-yl)ethyl]-2H-tetrazole (21). A suspension of **20** (0.5 g, 1.7 mmol) in azido tributyltin (3.4 g, 10 mmol; caution: hazardous material) was heated to 100 °C for 4 h, then cooled to room temperature and quenched with a 1:1 mixture of MeOH and 37% HCl (10 mL). The mixture was stirred for 2 h at room temperature, then diluted with water and extracted with ethyl acetate. The organic solution was washed with brine, dried over Na_2SO_4 and concentrated to afford a residue that was washed with ether and desiccated to afford **21** as a white solid (0.49 g, 86%). ^{1}H NMR ($CDCl_3$): δ 15.46(s br, 1H); 7.67(d, 2H); 7.60(d, 2H); 7.37(dd, 1H); 7.20−7.12(m, 2H); 4.52(q, 1H); 1.80(d, 3H). MS (EI; TSQ 700; source 180 °C; 70 V; 200 μA): 336(M^+); 267. Anal. ($C_{16}H_{12}F_4N_4$) C, H, N, F.

2-(2-Fluoro-4′-trifluoromethylbiphenyl-4-yl)-N-hydroxypropionamidine (22). A suspension of **20** (250 mg, 0.85 mmol), hydroxylamine hydrochloride (65 mg, 0.94 mmol) and $NaHCO_3$ (80 mg, 0.95 mmol) in MeOH (2 mL) was refluxed for 4 h, then concentrated and the residue was extracted with ethyl acetate, washed with brine and dried over Na_2SO_4 to afford a crude material (205 mg, 74%) that was employed without further purification.

3-[1-(2-Fluoro-4′-trifluoromethylbiphenyl-4-yl)-ethyl]-4H-[1,2,4]oxadiazol-5-one (23). A solution of **22** (200 mg, 0.61 mmol) and CDI (150 mg, 0.93 mmol) in 1,4-dioxane (5 mL) was heated to 100 °C for 30 min, then cooled to room temperature, diluted with water and extracted with ethyl acetate. The organic solution was washed with brine, dried over Na_2SO_4 and concentrated to afford a crude material that was washed with ether and desiccated to obtain **23** as a white solid (125 mg, 58%). ^{1}H NMR (DMSO-d_6): δ 12.33(s br, 1H); 7.85(d, 2H); 7.78(d, 2H); 7.61(dd, 1H); 7.37(dd, 1H); 7.30(dd, 1H); 4.23(q, 1H); 1.57(d, 3H). MS (EI; TSQ 700; source 180 °C; 70 V; 200 μA): 352 (M^+); 307; 293. Anal. ($C_{17}H_{12}F_4N_2O_2$) C, H, N, F.

2-(2-Fluoro-3′,5′-dichloromethylbiphenyl-4-yl)-propionic Acid Methyl Ester (24). Thionyl chloride (130 mg, 1.09 mmol) was added dropwise to a solution, cooled to 0 °C, of **5b** (110 mg, 0.35 mmol) in MeOH (3 mL). The solution was stirred overnight at room temperature, then was concentrated under vacuum. The crude material was diluted in diethyl ether, washed with $NaHCO_3$ 10% and dried over Na_2SO_4. The concentration of the organic solution afforded a colorless oil (72 mg; 63%). ^{1}H NMR (200 MHz, $CDCl_3$, T = 303 K): 7.45−7.39 (m, 2H, Ar-*H*); 7.38−7.30 (m, 2H, Ar-*H*); 7.20−7.15 (m, 1H, Ar-*H*); 7.15−7.08 (d,d, J = 6.52 Hz, J = 1.57 Hz, 1H, Ar-*H*); 3.85−3.66 (q+s, J = 7.30 Hz, 4H, O−C*H*$_3$, C*H*-CH$_3$); 1.60−1.48 (d, J = 7.30 Hz, 3H, C*H*$_3$). MS (EI; TSQ 700; source 180 °C; 70 V; 200 μA): 349 (M^+Na +).

2-(2-Fluoro-3′,5′-dichloromethylbiphenyl-4-yl)-propionic Acid *tert*-butyl ester (25). In a solution, cooled to 0 °C, of **5b** (110 mg, 0.35 mmol) in dichloromethane 5 drops of H_2SO_4 96% were added and isobutylene was bubbled for 5 min. The solution was stirred at room temperature in a sealed Pyrex tube overnight. After concentration the crude material was diluted in diethyl ether, washed with water and dried over Na_2SO_4. The concentration of the organic solution afforded a colorless oil (62 mg; 48%). ^{1}H NMR (200 MHz, $CDCl_3$, T = 303 K): 7.47−7.40 (m, 2H, Ar-*H*); 7.39−7.31 (m, 2H, Ar-*H*); 7.20−7.14 (m, 1H, Ar-*H*); 7.14−7.07 (d,d, J = 13.30 Hz, J = 4.85 Hz, 1H, Ar-*H*); 3.72−3.57 (q, J = 7.26 Hz, 1H C*H*-CH$_3$); 1.53−1.37 (d+s, J = 7.26 Hz 12H, 4C*H*$_3$). MS (EI; TSQ 700; source 180 °C; 70 V; 200 μA): 391 (M^+Na +)

2-(2-Fluoro-3′,5′-dichloromethylbiphenyl-4-yl)-propionamide (26). The methyl ester **24** (200 mg, 0.61 mmol) was stirred in a solution of 8 M NH_3 in MeOH (5 mL) for 2 days. After concentration the crude material was chromatographed on silica gel (*n*-hexane/ethyl acetate 50:50) to afford **26** as a white solid (54 mg, 28%). ^{1}H NMR (200 MHz, DMSO-d_6 T = 303 K): 7.73−7.50 (m, 4H, Ar-*H*); 7.50−7.38 (br, 1H, N*H*); 7.33−7.18 (M, 2H, Ar-*H*); 6.98−6.81 (br, 1H, N*H*); 3.76−3.54 (q, J = 7.05 Hz, 1H, C*H*); 1.42−1.20 (d, J = 7.05 Hz, 3H, C*H*$_3$). MS (EI; TSQ 700; source 180 °C; 70 V; 200 μA): 334 (M^+Na +)

Biology. Aβ42 and Aβ40 Assay. Aβ42 and Aβ40 were measured in the culture medium of H4-APP695NL cells, a human neuroglioma cell line carrying the double Swedish mutation of the APP695 isoform (K595N/M596L). Cells were seeded onto 24-well plates (2×10^5 cell/well) and allowed to grow to confluence for 24 h, in 5% CO_2/95% air in a humidified atmosphere. Increasing concentrations of the compounds were added to the cells overnight in a final volume of 0.5 mL. DAPT (*N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *tert*-butyl ester) and flurbiprofen were used as positive controls at 1 μM and 100 μM final concentrations, respectively. DMSO (1%) was used as negative control. At the end of the incubation, 100 μL of supernatants were removed and treated with a biotinylated mouse monoclonal antibody (4G8, Signet Laboratories Inc., Dedham, MA), specifically recognizing the 17−24 amino acid region of Aβ and two rabbit polyclonal antibodies (C-term 42 and C-term 40, Biosource International Inc., Camarillo, CA), specifically recognizing the C-terminus of Aβ42 and Aβ40, respectively. Antigen−antibody complexes were recognized by TAG-donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Soham, UK). Streptavidin-coated magnetic beads captured the complexes and the signals were read by an electrochemiluminescence instrument (Origen M8 Analyzer, BioVeris Corporation, Gaithersburg, MD).

Cytotoxicity in Human Neuroglioma Cells. The cytotoxicity potential of test compounds was assessed in the same cells of the Aβ assay (H4-APP695NL) with the MTT assay. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) is a soluble pale yellow salt that is reduced by mitochondrial succinate dehydrogenase to form an insoluble dark blue formazan product to which the cell membrane is impermeable. The ability of cells to reduce MTT provides an indication of mitochondria integrity and activity and it may be interpreted as a measure of viability and/or cell number. After medium removal for Aβ42 and Aβ40 determination, cells were incubated for 3 h with 500 μL culture medium containing 0.5 mg/mL MTT, at 37 °C, 5% CO_2 and saturated humidity. After removal of the medium, 500 μL of 100% DMSO were added to each well. The amount of formed formazan was determined reading the samples at 570 nm (background 630 nm) using a microplater reader (model 450, Biorad, Hercules, CA).

MALDI-TOF Analysis of Aβ Species. H4-APP695NL cells were treated with flurbiprofen (250 μM) or DAPT (1 μM) or **11c** (50 and 100 μM) or DMSO (1%) and then incubated in 5% CO_2 at 37 °C for different periods (18 and 42 h). The conditioned media were harvested and centrifuged (10 000 rpm for 5 min). The supernatants (0.5 mL) of centrifuged conditioned media were collected and treated for 18 h at 4 °C with 4 μg of monoclonal antibody selective for the 17−24 region of Aβ (4G8, Signet Laboratories Inc., Dedham, MA). Precipitation of the Aβ-4G8 complexes was obtained using 30 μL of protein G/A-agarose beads (SantaCruz Biotechnology Inc., Santa Cruz, CA) left for 3 h at 4 °C. Protein complexes were separated by centrifugation at 10 000 rpm for 2 min. The agarose beads were washed twice with ice-cold immunoprecipitation buffer (0.1% *n*-octyl glucoside, 140 mM NaCl, 0.025% sodium azide and 10 nM Tris-HCl, pH = 8) and once with 10 mM Tris-HCl, pH = 8, containing 0.025% sodium azide. Proteins were then extracted with 3 μL of saturated solution of α-cyano-4-hydroxycinnamic acid in TFA/H_2O/CH_3CN (1:20:20). One μL of the resulting solution was deposited on a stainless steel sample holder and allowed to dry before analysis. MALDI-TOF measurements were performed using a Voyager-DE PRO instrument (Applied Biosystem, Foster City, CA). Linear, positive operating mode was adopted. Ions, formed by a pulsed UV laser beam (λ = 337 nm), were accelerated to 25 keV. Instrumental conditions were: grid voltage = 93%, extraction

delay time = 300 ns and grid wire = 0.3%. External mass calibration was performed by using the Calibration Mixture 2 of Sequazime Peptide Mass Standard Kit (Applied Biosystem, Foster City, CA), based on the average values of [M + H]+ of angiotensin I, ACTH (clip 1−17), ACTH (clip 18−39), ACTH (clip 7−38) and bovine insulin at *m/z* 1297.51, 2094,-46, 2466.72, 3366.19 and 5734.59, respectively.

COX-1 Assay. Freshly taken rat blood (0.5 mL) was preincubated for 1 h at 37 °C with 0.5 μL of vehicle (DMSO, 0.3% final concentration) or test compounds (100 μM or at different concentrations). Blood was then stimulated with the Ca^{2+} ionophore A23187 (calcimycin; 1 μL, 50 μM) for 30 min. Afterward, samples were centrifuged at 12 000 × g for 3 min at 4 °C and the plasma obtained was immediately frozen at −80 °C, until analysis. Concentrations of [3H]thromboxane B2 were determined by radioimmunoassay, using a commercial kit (NEK007, Perkin-Elmer, Milan, Italy).

COX-2 Assay. Freshly taken human blood (0.5 mL) was preincubated at 37 °C in the presence of aspirin (0.5 μL, 10 μg/mL, final concentration in the sample) to inactivate COX-1. Thirty minutes later, blood was treated with lipopolysaccharide from Escherichia Coli (1 μL, 100 μg/mL, final concentration in the sample) plus 0.5 μL of vehicle (DMSO, 0.4% final concentration) or test compounds (100 μM). Afterward, incubation was continued for 18 h at 37 °C and was terminated by centrifugation at 12 000 × g for 3 min at 4 °C. Plasma was separated and immediately frozen at −80 °C, until analysis. Concentrations of [^{125}I]prostaglandin E2 were determined by radioimmunoassay, using a commercial kit (NEK020, Perkin-Elmer, Milan, Italy).

Membrane Permeability. Intestinal drug absorption of test compounds was estimated in vitro using human colon adenocarcinoma (Caco-2) cell monolayers (Areta International, Gerenzano, Italy) as previously described.[36] Cells were suspended in DMEMB and seeded (2×10^6 cells in 0.3 mL) in 24-well plates (Transwell, Costar, Cambridge, MA) using polycarbonate microporous cell culture inserts. Cells were grown for 21 days. The integrity of cell monolayers was checked by measuring transepithelial electrical resistance (1000 Ω) and by the transport of the paracellular leakage marker sodium fluorescein. One hundred μL of test compound solution (50 μM in 1% DMSO) was applied to the apical side. After 2 h incubation at 37 °C the apical and basolateral side solutions were removed and analyzed by liquid chromatography and tandem mass spectrometry with selected reaction monitoring. The apparent permeability (P_{app}) was calculated as $P_{app} = C_b V_b/(C_0 \cdot At)$, in which C_b = basolateral test compound concentration at time t (μM), V_b = basolateral volume (cm^3), C_0 = apical test compound concentration at $t = 0$ (μM), A = filter surface area (cm^2), t = time (s). The rank order of apparent permeability of the test compounds was compared with that of known reference compounds tested in the same experiment as internal standards including flurbiprofen, propranolol and cimetidine. P_{app} values below the limit of 10 nm/s were classified as "low", "medium" when $10 = P_{app} = 50$ nm/s and "high" when $P_{app} > 50$ nm/s.

Interaction with Cytochrome P450 Isoenzymes. The assay was performed in a 96-well microtiter plates as previously described.[37] Microsomes from baculovirus-insect cells (Supersomes, Gentest Corporation, Woburn, MA) expressing human cytochrome P450 isoforms (CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4) were utilized. Test compounds or standard inhibitors (dissolved in DMSO or acetonitrile) were serially diluted in a solution containing 16.3 μM $NADP^+$, 0.83 mM glucose-6-phosphate, 0.83 mM $MgCl_2$, 0.4 U/mL of glucose-6-phosphate dehydrogenase and 0.1 mg/mL microsomal protein prepared from wild-type baculovirus-infected insect cells. Appropriate control wells without inhibitor and without microsomes were also added. The plates were then pre-warmed at 37 °C for 10 min, and the reaction was started by adding pre-warmed enzyme/substrate mix (cytochrome P450 isoforms with their specific substrates in a 0.35 M potassium phosphate buffer at pH 7.4). Specific substrates were 3-cyano-7-ethoxycoumarin (CEC) for CYP1A2 and CYP2C19, 7-methoxy-4-trifluoromethylcoumarin (MFC) or dibenzylfluorescein (DBF) for CYP2C9, 3-[2(*N*,*N*-diethyl-*N*-methylamino)ethyl]-7-methoxy-4-methylcoumarin (AMMC) for CYP2D6 and 7-benzyloxy-4-(trifluoromethyl)-coumarin (BFC) for CYP3A4. Reaction was terminated at various times, depending on the assays, by addition of a 4:1 acetonitrile: 0.5 M Tris base solution. Known inhibitors for each isoenzyme (furafylline for CYP1A2, sulfaphenazole for CYP2C9, tranylcypromine for CYP2C19, quinidine for CYP2D6 and ketoconazole for CYP3A4) were tested in all assays as positive controls. Plates were read on a fluorometer (Fluoroskan Ascent, Thermo Electron Corporation, Helsinki, Finland) at the appropriate emission/excitation wavelengths. Median inhibitory concentration (IC_{50}) was determined by nonlinear regression (GraFit software, Erithacus Software, Horley, UK).

CBF1−Luciferase Notch Assay. Human embryonic kidney 293 (HEK293) cells were seeded in 96-well plates and grown at 37 °C in the presence of 5% CO_2 to subconfluency with Dulbecco modified Eagle's minimum essential medium (DMEM) medium supplemented with 10% fetal bovine serum, 100 units/mL penicillin and 100 μg/mL streptomycin. Cells were transiently cotransfected with a truncated form of Notch-1 (Notch-1 Δ extracellular domain, N1Δ*E*C) and a C-promoter binding factor 1 (CBF1)-firefly luciferase reporter construct using a cationic lipid transfection agent (Lipofectamine 2000, Invitrogen, Carlsbad, CA). N1ΔEC contains the cytoplasmic portion and the membrane-spanning region of Notch-1 and leads to rapid accumulation of the C terminus of Notch1 in the nucleus and to transcriptional activation of the CBF1 reporter construct. Eight hours after the cotransfection, vehicle or test compounds were added to the cells for an additional period of 36 h. The assay was performed in triplicate using a commercial kit (LucLite, Perkin-Elmer, Milan, Italy) and a luminometer (LKB 1251, LKB−Wallac, Turku, Finland). Data were normalized to total protein content and expressed as counts per second/μg protein.

Cytotoxicity in Human Hepatocytes. The cytotoxicity in human hepatocytes was assessed by the MTT assay. Cryopreserved human hepatocytes (25 000−30 000 cells/well) were seeded into 96-well microplates and treated overnight with the test compounds or 1% DMSO (vehicle). Cell medium was then removed and the cells were incubated for 2 h in the presence of 100 μL of MTT (0.5 mg/mL), at 37 °C, 5%CO_2 and saturated humidity. After removal of the medium, 100 μL of 100% DMSO were added to each well. The amount of formed formazan was determined reading the samples at 570 nm (background 630 nm) using a microplater reader (model 450, Biorad, Hercules, CA).

Pharmacokinetic Studies. Metabolic stability and central nervous system penetration of the new compounds were determined in rats after continuous subcutaneous infusion by osmotic mini-pumps (model 2001, Alzet Osmotic Pumps, Cupertino, CA) using the cassette dosing technology. This approach consists of the simultaneous administration of low doses of two to six different compounds to a single animal and estimating total body clearance by measuring the compound concentration in a single plasma sample collected at steady-state. Brain penetration was estimated by measuring compounds in the cerebrospinal fluid (CSF) and calculating the CSF to plasma ratio (as reported in Tables 1a, 2a, 3a). Doses from 2 to 65 μg/h were infused for 4−7 days to rats. One mL of plasma and 100−150 μL of CSF were collected at the end of infusion. Three to four animals were used for each dose session. Samples were prepared by adding 300 μL of acetonitrile and 40 μL of phosphoric acid 40% to 100 μL of plasma or CSF and placing the mixture in a vortex for 20 s. Then, samples were centrifuged at 14 000 rpm for 15 min and the supernatants (30 μL) were injected into the HPLC system. Equipment systems with fluorescence (Waters 470, Waters, Guyancourt, France) or mass spectrometry (API 2000, Applied Biosystems, Foster City, CA) detectors were used. The chromatographic conditions were adapted to each mixture of compounds to obtain good peak separation and detection sensitivity. A mixture of ammonium formate (20 mM) buffer−

acetonitrile−methanol was used as mobile phase for the fluorescence detector, while a mixture of ammonium formate (20 mM) buffer−acetonitrile was used for the mass spectrometry detector API 2000. Pharmacokinetic profile after single intravenous and oral administration in rats was determined for flurbiprofen, **11b**, **11c** and **13d**. Plasma samples (100 μL) were drawn in groups of 4 animals at 0.083, 0.25, 0.5, 1, 2, 4, 8, 24 h after intravenous administration and at 0.5, 1, 2, 4, 8, 24, 48 and 72 h after oral administration of the compounds. Intravenous doses were 0.2 mg/kg for **13d**, 0.5 mg/kg for **11b** and **11c** and 1.5 mg/kg for flurbiprofen. Oral doses were 0.5 mg/kg for **13d**, 2.5 mg/kg for **11b** and **11c** and 7.6 mg/kg for flurbiprofen. To permit a proper comparison between different compounds, plasma levels were standardized for a 1 mg/kg dose.

Studies in Transgenic Mice. The effects of selected compounds of Aβ secretion in vivo were studied in transgenic mice (Tg2576) overexpressing the human APP gene with the Swedish double mutation (K670N/M671L) under the transcriptional control of the hamster prion promoter.[38] Animals (B6SJLF17J strain) were bought from Taconic (Germantown, NY). All experiments were performed in compliance with the guidelines of the European Union for the use of laboratory animals. In Study 1, groups of 8 animals of 7−8 months of age were used. In Study 2, groups of 12 animals of 3−4 months of age were employed. One day before starting treatments, animals were anesthetized with ether and blood samples (150 μL) were collected via retro-bulbar puncture in EDTA-coated tubes for measuring baseline Aβ40 and Aβ42 concentrations. Vehicle (7.5 mL/kg of Kool-Aid) or test compounds (12.5 mg/kg in Kool-Aid) were administered, by oral gavage, twice-a-day for 7 days. The dose used (12.5 mg/kg twice-a-day) was selected based on preliminary tolerability experiments with *R*-flurbiprofen. On Day 8, animals were given a dose of 25 mg/kg of the test drugs and sacrificed 3 h later. Blood samples were again collected in EDTA-coated tubes and plasma separated by centrifugation (800 × g for 20 min) and stored at −80 °C until assay. The brains were quickly removed on an ice-cold plate and fronto-parietal cortex and hippocampus were dissected, immediately frozen on dry ice and stored at −80 °C. Total Aβ was extracted from brain tissues as described by Lanz and colleagues.[34] In brief, cortices and hippocampi were homogenized in 10 vol/weight of 5 M guanidium HCl in 50 mM Tris HCl, pH 8.0, agitated by rotation for 3 h at 4 °C, diluted 1:10 with phosphate-buffered saline. These diluted homogenates were spun at 35 000 × g (25 min at 4 °C). Aβ40 and Aβ42 concentrations in supernatants were assayed by ELISA using commercial kits (Genetics Company, Zurich, Switzerland). All ELISA's detections were conducted in duplicate.

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Supporting Information Available: Spectroscopic data and elemental analyses for compounds **5c**−**o**, **6a**−**c**, **11b**−**i**, **13b**−**d**, and **16b**−**d**. This material is available free of charge via the Internet at http://pubs.acs.org.

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