Scaffold of the Cyclooxygenase-2 (COX-2) Inhibitor Carprofen Provides Alzheimer γ-Secretase Modulators

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Abstract: N-Sulfonylated and N-alkylated carprofen derivatives were investigated for their inhibition and modulation of γ-secretase, which is associated with Alzheimer's disease. The introduction of a lipophilic substituent transformed the COX-2 inhibitor carprofen into a potent γ-secretase modulator. Several compounds (e.g., **9p**, **11f**) caused selective reduction of Aβ$_{42}$ and an increase of Aβ$_{38}$. The most active compounds displayed activities in the low micromolar range and no effect on the γ-secretase cleavage at the ε-site.

Despite tremendous progress in understanding Alzheimer's disease (AD), there remains the challenge to develop agents for its therapy. Approved drugs such as acetylcholinesterase inhibitors and memantine hydrochloride offer symptomatic treatment, but they do not address the basic pathology of the disease: deposition of amyloid plaques and development of neurofibrillary tangles. The metabolism of the β-amyloid precursor protein (APP), which is cleaved by the aspartic proteases β-secretase and γ-secretase, results in the generation and pathological deposition of the 40 and 42 amino acid long peptides Aβ$_{40}$ and Aβ$_{42}$ (Figure 1). These fragments are the major components of amyloid fibrils.[1,2]

Promising results for the treatment of AD were obtained with some cyclooxygenase-1 (COX-1) inhibitors,[3−7] both in vitro and in a prospective, population-based cohort study of 6989 patients.[8] This is not a class effect because nonsteroidal anti-inflammatory drugs (NSAIDs) (e.g., diclofenac (2-[2-(2,6-dichlorophenyl)aminophenyl]ethanoic acid) and naproxen ((+)-(*S*)-2-(6-methoxynaphthalen-2-yl)propanoic acid)) do not lower Aβ in vitro,[3,5] and neither naproxen nor rofecoxib (4-(4-methylsulfonylphenyl)-3-phenyl-5*H*-furan-2-one, a COX-2 inhibitor) slows cognitive decline in patients with mild-to-moderate AD.[9,10] The positive clinical results are still in need of a sound rationale and experimental validation.[11] The proof of concept is still missing. NSAIDs that modulate γ-secretase cleavage of APP affect the distance between APP and presenilin 1, the catalytic subunit of γ-secretase.[12] They seem to interfere with substrate recognition/cleavage and shift the precision of γ-secretase cleavage from the γ42 to the γ38 site (Figure 1) to generate more Aβ$_{38}$ and less Aβ$_{42}$.[3,12] Compounds with reverse shift were reported recently, and these enhanced Aβ$_{42}$ production.[13] Noncompetitive antagonism indicates an allosteric mechanism of action.[7] Flurbiprofen ((±)-2-(3-fluoro-4-phenylphenyl)-propanoic acid, **1**) (10 and 25 mg kg^{-1} d^{-1}) elicits nonselective reductions in Aβ$_{1-40}$ and Aβ$_{1-42}$ plasma levels but was found to be toxic. It produced small reductions in Aβ$_{1-40}$ in the cortex at 25 mg kg^{-1} d^{-1} but did not affect Aβ levels in the hippocampus or cerebrospinal fluid. Cyclopropylated flurbiprofen analogues without COX activity display improved potency on γ-secretase inhibition.[14] Contrary to previous reports, sulindac sulfide (2-((5*Z*)-1-(4-(methylthio)benzylidene)-5-fluoro-2-methyl-1*H*-inden-3-yl)acetic acid, **2**) and ibuprofen (2-(4-isobutylphenyl)propanoic acid, **4**) were found to be neither toxic nor efficacious at doses up to 50 mg kg^{-1} d^{-1}.[15] The kinetics of Aβ formation in the presence of the two NSAIDs and the displacement of an active site directed inhibitor support allosteric, noncompetitive modes of action of sulindac sulfide (**2**)[6] and *R*-flurbiprofen (**1**, Scheme 1) at low concentrations.[7] This resulted in selective inhibition of Aβ$_{42}$ production. However, both NSAIDs shift their modes of action from modulation to complete, nonselective inhibition of γ-secretase at high concentrations. Unfortunately, NSAID derivatives have escaped photoaffinity labeling techniques so far, except for biotinylated fenofibrate which labelled the C-terminal fragment of APP.[16]

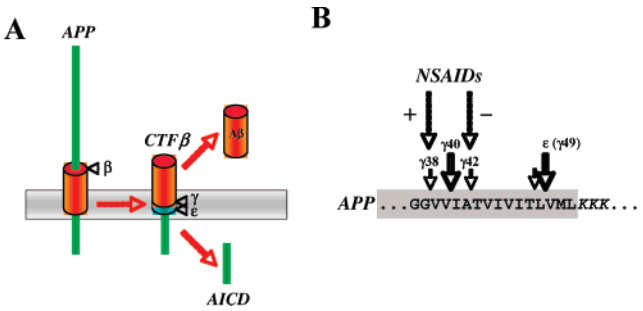
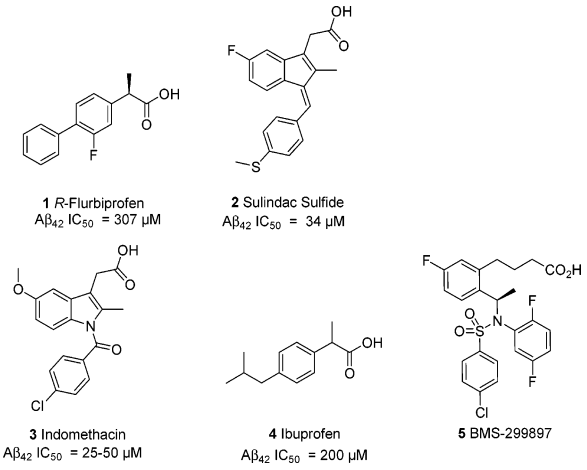


Figure 1. Schematic representation of APP processing and the modulation of γ-secretase cleavage by NSAIDs. (A) APP is first processed by β-secretase to generate the C-terminal fragment CTFβ, which is subsequently cleaved within its transmembrane domain at two principle sites, γ and ε, by γ-secretase to release Aβ and AICD. (B) Heterogeneous cleavage at the γ-site generates different Aβ species by cleavage at the γ38, γ40, and γ42 sites. A subset of NSAIDs increases the cleavage at the γ38 site while reducing cleavage at the γ42 site.

Scheme 1. Effect of NSAIDs (**1**−**4**) on Aβ Levels as Reported by Weggen[3] and Beher[7] and the Structure of BMS-299897 (**5**)



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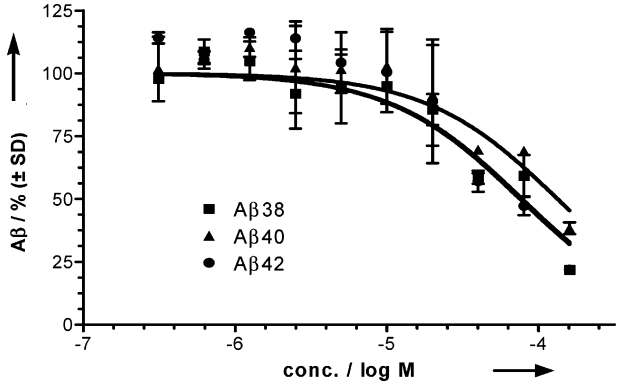
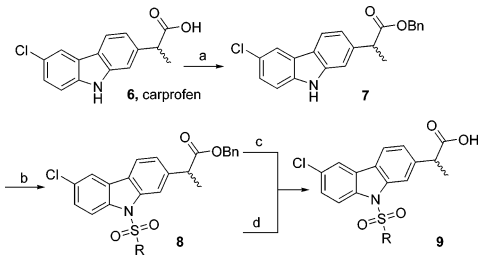


Figure 2. Dose response curve of carprofen (**6**).

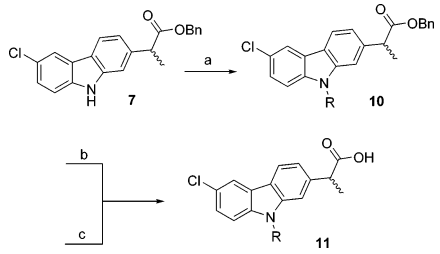
In summary, just a few NSAIDs (**1**−**4**, Scheme 1) were reported to modulate γ-secretase, and an even smaller number of NSAIDs display confirmed modulation at physiological concentrations. Despite these vague, sometimes contradictory results reported for NSAID activity, we and others[13,17] were encouraged to investigate NSAIDs as scaffolds for γ-secretase inhibitors. We commenced with the derivatization of the carboxylic acid common to the COX-1 inhibitors. Furthermore, we included COX-2 inhibitors that are different from the coxib class such as carprofen ((±)-2-(3-chloro-9*H*-carbazol-7-yl)-propanoic acid, **6**) and etodolac (2-(1,8-diethyl-1,3,4,9-tetrahydropyrano[3,4-*b*]indol-1-yl)acetic acid) because they are structurally closer to COX-1 than to COX-2 inhibitors. Although some of the esters and amides displayed moderate inhibition of the cleavage by γ-secretase (Aβ_{38}, Aβ_{40}, Aβ_{42}), this approach was soon abandoned. Few of these initial derivatives displayed increased, unselective inhibition of γ-secretase in comparison to their parent drugs. Most of the approximately 150 NSAID carboxylic acid derivatives (e.g., **7**) resulted in loss of activity. This indicated an important contribution of the carboxylic acid to target affinity. After a brief investigation of sulindac analogues we focused on carprofen, which is a COX-2 inhibitor approved for the use in dogs, cows, and horses. The selectivity of carprofen versus COX-2_{canine} and COX-1_{canine} is greater than 100:1 (COX-2_{canine} IC_{50}: *R*/*S*-carprofen 102 nM, *R*-carprofen 5.97 μM, *S*-carprofen 37 nM).[18] Original carprofen (as isolated from 500 mg tablets) was found to be a weak inhibitor of γ-secretase and reduced Aβ_{38}, Aβ_{40}, and Aβ_{42} at high concentration (Figure 2). This corresponds to the activity of α-methylated carprofen, which was reported to inhibit Aβ_{42} production by 40% at 100 μM.[17] This is in contrast to the inverse modulation of the COX-2 inhibitors that was reported recently.[13] The readily accessible sulfonamides were inspired by the γ-secretase inhibitor **5** (BMS-299897) and supported by a recent series from Merck Sharp & Dohme.[19,20] An analogue series of carprofen *N*-sulfonamides was prepared as outlined in Scheme 2. The acid functionality of carprofen was protected as a benzyl ester **7**. N-Sulfonylation of **7** was carried out using NaH and a sulfonyl chloride in THF. The benzyl group of **8** was removed by hydrogenation (**8a**−**m**,**p**−**r**) or base hydrolysis (**8n**−**o**,**s**) to give the acid **9**. We expected these compounds to be inhibitors of γ-secretase activity because of their resemblance to the γ-secretase inhibitor **5**. Serendipitously, they turned out to be modulators of γ-secretase activity, which control the cleavage pattern of γ-secretase. Such modulators may preserve the cleavage of substrates like Notch; thus, we adopted our initial objectives from inhibition to modulation. Notch−ligand interaction is a highly conserved mechanism that regulates specific cell fate during development.[21,22]

Scheme 2[a]



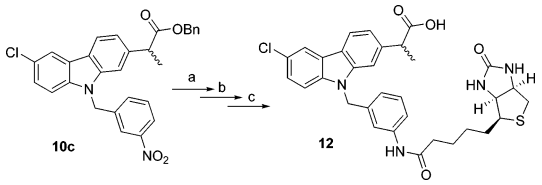
[a] Reagents: (a) BnBr, K_2CO_3, DMF, room temp, 1.5 h, 97%; (b) NaH, RSO_2Cl, THF, 0 °C to room temp, 2−8 h, 22−84%; (c) 10% Pd−C, MeOH/EtOAc (1:1), H_2, 3−18 h, 70−94%; (d) NaOH, THF/MeOH/H_2O (1:1:1), room temp, 3−12 h, 80−92%.

Scheme 3[a]



[a] Reagents: (a) NaH, RX, THF, 0 °C to room temp, 2−8 h, 30−81%; (b) 10% Pd−C, MeOH/EtOAc (1:1), H_2, 3−18 h, 70−96%; (c) NaOH, THF/MeOH/H_2O (1:1:1), room temp, 3−12 h, 80−92%.

Scheme 4[a]



[a] Reagents: (a) $SnCl_2$, EtOH, reflux, 3 h, 90%; (b) EDCl, HOBT, biotin, Et_3N, DMF, room temp, 16 h, 65%; (c) 10% Pd−C, MeOH, H_2, room temp, 18 h, 90%.

N-Alkylated carprofen derivatives were prepared (Scheme 3) to evaluate the contribution of the sulfonamide moiety in **9r** and the most active derivative **9p**, where the sulfonamide is shielded by isopropyl substituents. The carprofen benzyl ester **7** was alkylated using NaH and RX in THF to yield the ester **10.** Subsequent benzyl deprotection by hydrogenation gave the desired N-alkylated carprofen **11**. The benzyl deprotection of **10c** was carried out by base hydrolysis.

The biotinylated carprofen derivative **12** was synthesized as depicted in Scheme 4 to identify the binding partner via immunoprecipitation.

To evaluate the compounds for their potency in modulating γ-secretase activity, we used the Aβ liquid-phase electrochemiluminescence assay to measure Aβ isoforms.[27] γ-Secretase cleavage activity at the ϵ-site was monitored by de novo production of AICD in vitro using the previously reported assay.[25]

Compounds **9a**−**s**, **11a**−**f**, and **12** turned out to be effective modulators of γ-secretase. They affected the cleavage at the γ38, γ40, and γ42 sites to a different extent and particularly suppressed the formation of Aβ_{42} while enhancing the formation of Aβ_{38} and thus showed the typical profile of effective NSAIDs (see Tables 1and 2, Figures 3 and 4). Compounds **9b**,**p** and **11a**,**e**,**f** were the most potent inhibitors of Aβ_{42}. Interestingly, the modulatory activity was preserved in the biotinylated **12**, which may therefore be used as an affinity reagent for

Table 1. Activity of Carprofen *N*-Sulfonamide Derivatives

			IC$_{50}$ (μM)		
entry	compd	R	Aβ_{38}[a]	Aβ_{40}	Aβ_{42}
1	**6**	H	78	133	76
2	**9a**	4-methylphenyl	43	>100	56
3	**9b**	3,5-bis(trifluoromethyl)phenyl	ND[b]	>30	11
4	**9c**	4,5-dibromothiophen-2-yl	ND[b]	ND	>40
5	**9d**	3,5-difluorophenyl	ND[b]	ND	>40
6	**9e**	4-biphenyl	ND[b]	ND	37
7	**9f**	2-fluorophenyl	ND[b]	ND	>40
8	**9g**	3-fluorophenyl	ND[b]	ND	>40
9	**9h**	4-fluorophenyl	ND[b]	ND	>40
10	**9i**	2-bromophenyl	ND[b]	ND	40
11	**9j**	4-bromophenyl	ND[b]	ND	>40
12	**9k**	phenyl	ND[b]	ND	>40
13	**9l**	4-chlorophenyl	ND[b]	ND	>40
14	**9m**	3-chlorophenyl	ND[b]	ND	>40
15	**9n**	3-nitrophenyl	ND[b]	ND	39
16	**9o**	4-nitrophenyl	ND[b]	ND	>40
17	**9p**	2,4,6-triisopropylphenyl	9.3	>20	8.5
18	**9q**	4-*n*-propylphenyl	ND[b]	>40	>40
19	**9r**	octyl	ND[b]	>40	25
20	**9s**	4-cyanophenyl	ND[b]	ND	>40

[a] EC$_{50}$ values are displayed for Aβ_{38} except **6**. The EC$_{50}$ is based on the maximum level with a slope approximating 0. [b] Maximum effect on Aβ_{38} not observed at 40 μM (except for **6**, 160 μM; **9a**, 200 μM; and **9b**, 200 μM).

Table 2. Activity of N-Alkylated Carprofen Derivatives.

			activity (μM)		
entry	compd	R	EC$_{50}$ Aβ_{38}	IC$_{50}$ Aβ_{40}	IC$_{50}$ Aβ_{42}
1	**12**		ND[a]	>150	88
2	**11a**	3-(trifluoromethoxy)benzyl	ND[a]	>40	7.5
3	**11b**	3-methoxybenzyl	ND[a]	>40	39
4	**11c**	3-nitrobenzyl	ND[a]	>40	22
5	**11d**	octyl	ND[a]	>40	6.9
6	**11e**	nonyl	8.1	>40	3.0
7	**11f**	decyl	5.8	>40	2.9

[a] Maximum effect on Aβ_{38} not observed at 40 μM (except for **12**, 150 μM).

γ-secretase. We observed from another series that N-alkylation of carbazole with shorter *n*-alkyl chains such as *n*-butyl and *n*-hexyl diminishes the γ-secretase modulatory activity (unpublished results). The observed differences for octyl, nonyl, or decyl substitution are small and so is the difference for alkylsulfone amides of equivalent length (see **11e** versus **9r**). The sulfone amides display an increased topological polar surface area (tPSA) from 49 to approximately 93 Å^2, which makes blood−brain barrier penetration less likely.[23] The calculated increase in tPSA is only partially compensated by a small reduction of the clogP (− 0.5) in comparison to the analogue alkyl derivatives. This favors the alkyl derivatives over the sulfone amides for further investigation in animal models. The N-benzylated or N-arylsulfonated derivatives benefit from fluorinated substituents (**9b**, **11a**) in the meta position. Ortho fluorination as in (**9f**) did not alter the activity in comparison to the parent sulfonamide (**9k**). The replacement of the methoxy group in **11b** by a trifluoromethoxy resulted in a 5-fold improvement of the IC$_{50}$ (Aβ_{42}). This fluorine-derived enhancement may be due to lipophilicity or polar interactions. We speculate that the lipophilic substituent anchors the N-substituted carprofen in the required orientation within the membrane; thus, the maximum tolerated length should be similar to those of natural phospholipids. The effect of the most potent compounds on γ-secretase cleavage at the ϵ-site was assessed by an in vitro assay that monitors the de novo generation of the APP intracellular domain (AICD).[24,25] The generation of AICD was

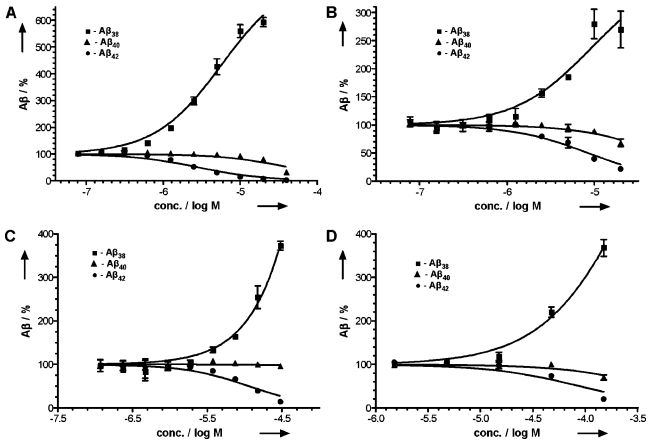


Figure 3. Dose response curves for the most active carprofen derivatives (Aβ % of control): (A) **11f**; (B) **9p**; (C) **9b**; (D) **12**; (■) Aβ_{38}; (▲) Aβ_{40}; (●) Aβ_{42}).

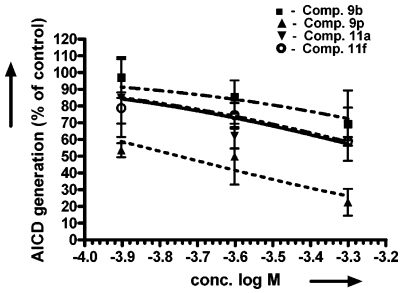


Figure 4. Dose−response curves for carprofen derivatives (**9b**, **9p**, **11a** and **11f**) on in vitro AICD generation. Results are the average of three independent experiments. Error bars indicate the standard error of the mean.

affected by the compounds to various extent (Figure 4). However, consistent with previous results,[3,7] generally much higher compound concentrations than those determined to be modulatory were required to inhibit the ϵ-cleavage. One of the most active carprofen derivatives **11f** was selected for evaluation in COX-1 and COX-2 assays to rule out COX-1 or COX-2 mediated effects at the necessary concentrations for γ-secretase modulation. The assays were performed at CEREP (www.cerep.com) using indomethacin as a standard for COX-1 and NS398 (*N*-(2-(cyclohexyloxy)-4-nitrophenyl)methanesulfonamide) for COX-2. **11f** displayed no activity on COX-1 and only marginal activity on COX-2 at 10 μM. No toxicity was observed at 40 μM in H4 cells except for **9p** and **12**, which significantly decreased viability at 40 μM, respectively (data not shown).

In conclusion, the introduction of a single lipophilic substituent, which may vary from arylsulfone to alkyl substituents, turns the COX-2 inhibitor carprofen into a γ-secretase modulator and improved potency 10-fold or more. Thus, several compounds (e.g., **9p**, **11f**) caused the selective reduction of Aβ_{42} and an increase of the less aggregatory Aβ_{38}. The most active compounds are more potent than the best reported NSAIDs, and they are devoid of COX-1 and COX-2 activity at the critical concentration; thus, they do not interfere with the delicate COX-1/COX-2 balance. Some of the sulfonamides are comparable in potency to the best N-alkylated analogues (**11b**), but the 50% increase of the tPSA (**9p**: 93.4 Å^2) makes a penetration of the blood−brain barrier less likely. Therefore, the N-alkylated derivatives are favored over the sulfonamides for further investigations. The properties of these N-alkylated lead candidates are close to the range of approved drugs or preclinical candidates except for their clogP.[26] The carboxylic acid group

may interfere with uptake, but the tPSA of **11b** is just 62.9 Å^2. This compares favorably to lumiracoxib ({2-[(2-chloro-6-fluorophenyl)amino]-5-methylphenyl}acetic acid, tPSA = 58 Å^2). The more polar **11c** has similar properties (tPSA = 96.1, clogP = 5.83) as the carboxylic acid **5** (clogP = 5.92, tPSA = 93.4 Å^2).[19] The lipophilic substituents cause amphiphilic properties of the carboxylic acids, which may interact with membranes. However, the placement of a polar end group, as in **12**, weakened but did not reverse the modulatory effect. Again, we favor the N-alkylated derivatives for the investigation of potential membrane interactions, as they allow the incorporation of phospholipids analogues and membrane disrupting fragments.

If affected at all, the ϵ-cleavage of γ-secretase was inhibited at much higher compound concentrations than those determined to be modulatory at the γ-site (Figure 4). The compounds are therefore expected to have little or no impact on γ-secretase-mediated signaling via the AICD or via intracellular domains of other γ-secretase substrates. However, the interaction site of these compounds has not been identified yet. The improvement of potency and the investigation of in vivo activity are subject to further investigations.

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Supporting Information Available: Synthetic procedure and spectral data for the tested compounds and experimental procedure for assay. This material is available free of charge via the Internet at http://pubs.acs.org.

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