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NSAID-derived γ-secretase modulators. Part III: Membrane anchoring

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ABSTRACT

Selective lowering of $A\beta_{42}$ levels with small-molecule substrate targeting γ-secretase modulators (sGSMs), such as some non-steroidal anti-inflammatory drugs, is a promising therapeutic approach for Alzheimer's disease. Here we present N-substituted carbazole- and O-substituted fenofibrate-derived sGSMs and their activity data. Seven out of 19 screened compounds exhibited promising activity against $A\beta_{42}$ secretion at a low micromolar level. We presume that the sGSMs interact with lys624 at the membrane interface and that the lipophilic substituents anchor the compound orientation in the membrane.

γ-Secretase is one of two aspartyl proteases held responsible for the generation of the Alzheimer's disease causing pathology; amyloid β-peptide (Aβ) aggregation to amyloid plaques. The understanding of the precise mechanism of Aβ generation is crucial for the development of drugs targeting the disease. Small-molecule substrate targeting γ-secretase modulators (sGSMs) have shown promise in therapeutic treatment of Alzheimer's disease.[1] These sGSMs seem to interfere with substrate recognition/cleavage and shift the precision of γ-secretase cleavage from the beta-amyloid 42 to the beta-amyloid 38 site to generate more $A\beta_{38}$ and less $A\beta_{42}$. This activity is summarized as straight modulation of γ-secretase. The development of modulators will benefit from precise information on the binding site(s), but up to now this information is rather limited.

Selected NSAIDs, for example, ibuprofen, sulindac sulfide, and flurbiprofen were found to modulate the secretion of Aβ in vitro and in vivo.[2–4] Some cyclooxygenase-2 (COX-2) specific inhibitors, for example, celecoxib, and the peroxisome proliferator-activated receptor-γ (PPAR-γ) antagonist, fenofibrate (**3**, Fig. 1) were found to increase $A\beta_{42}$ levels selectively, resulting in inverse modulation of γ-secretase activity.[5,6]

We recently described N-substituted carprofen derivatives and carbozolyloxyactetic acids as γ-secretase modulators (Fig. 1).[7,8] The introduction of a lipophilic substituent, which may vary from arylsulfone to alkyl, turned 2-carbazolyloxyacetic acids into γ-secretase modulators (e.g., **4**: IC_{50} ($A\beta_{42}$) = 7.5 μM, **5**: IC_{50} ($A\beta_{42}$) = 2.9 μM). The most active compounds displayed activity in the low micromolar range and little or no effect on the γ-secretase cleavage at the ε-site. Furthermore, cell-based studies with $A\beta_{42}$-lowering compounds have revealed that sGSMs do not affect the cleavage of other γ-secretase substrates, such as Notch and others.[3,6,9–12]

Biotinylated, photoactivatable probes were developed to identify the target of flurbiprofen- and fenofibrate-derived sGSMs.[11] Surprisingly, these photoprobes did not label the core proteins of the γ-secretase complex, but instead labeled the β-amyloid precursor protein (APP), APP carboxy-terminal fragments (CTFs) and Aβ peptide in human neuroglioma H4-cells. sGSM interaction was localized to the residues 29–36 of Aβ, a region critical for

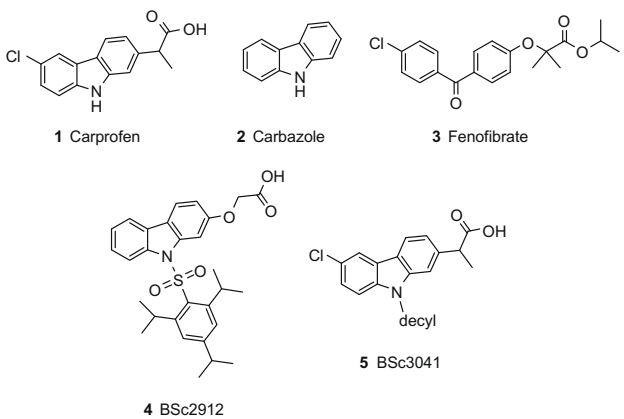

Figure 1. Carprofen (**1**), carbazole (**2**), fenofibrate (**3**) and the most active derivatives BSc2912 (**4**) and BSc3041 (**5**).

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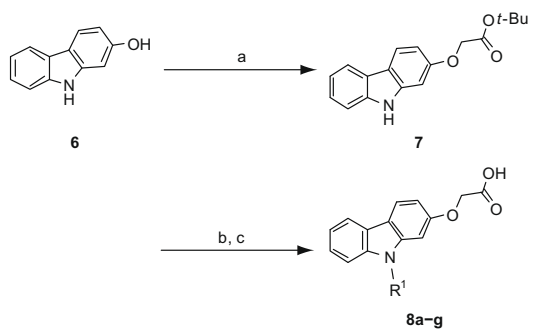
aggregation. This substrate targeting by sGSMs results in alteration of $A\beta_{42}$ production and inhibition of Aβ aggregation.[13] Substrate labeling has been competed by other sGSMs, and labeling of an APP γ-secretase substrate was more efficient than of a Notch substrate. The established binding site of flurbiprofen-derived sGSMs resides close to the membrane surface, and implies high lipophilicity in combination with an acidic functional group is necessary for binding. This combination is unusual for a therapeutic drug, but common to amphiphilic surfactants, thus creating an obstacle for drug development.

Fenofibrate (**3**) is used as lipid-regulating agent in humans. In H4-cells, **3** raises $A\beta_{42}$ by over 300% and decreases $A\beta_{38}$ by up to 60% in a dose-dependent fashion, while $A\beta_{40}$ levels are not altered.[5,14] At these concentrations, fenofibrate did not show toxic effects in the lactose dehydrogenase (LDH) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. Notably, the active metabolite of fenofibrate, the corresponding fenofibric acid, did not raise $A\beta_{42}$ at doses up to 250 μM.

The results with the N-substituted carprofen derivatives and carbazolyloxyacetic acids prompted us to investigate the detailed effects of the lipophilic substituents of N-substituted carbazolyloxyacetic acids. Therefore, lipophilic substituents like *trans*- and *cis*-oleic acid were introduced. *Cis*-configurated oleic acid is a component of biological lipid bilayers and should incorporate into the cellular membrane readily. Sterically more demanding substituents like aza-benzene, naphthalene, or biphenyl were linked to the carbazolic backbone testing space availability. These carbazolyloxyacetic acid derivatives have been synthesized according to Scheme 1 and are summarized in Table 1. One derivative (**8h**) features a compact photoreactive subunit aiming to minimize the tether used in previous crosslinking experiments. Such probes can be useful to identify binding sites on proteins either by mass spectrometry or via additional labeling.[13] The synthesis is outlined in Scheme 2 and described in the supplement.

In addition, we tried to convert the inverse γ-secretase modulating effects of fenofibrate into straight modulation. The corresponding O-alkylated oximes (Table 1) were readily accessible by straightforward synthesis (Scheme 3, **11a–g**).

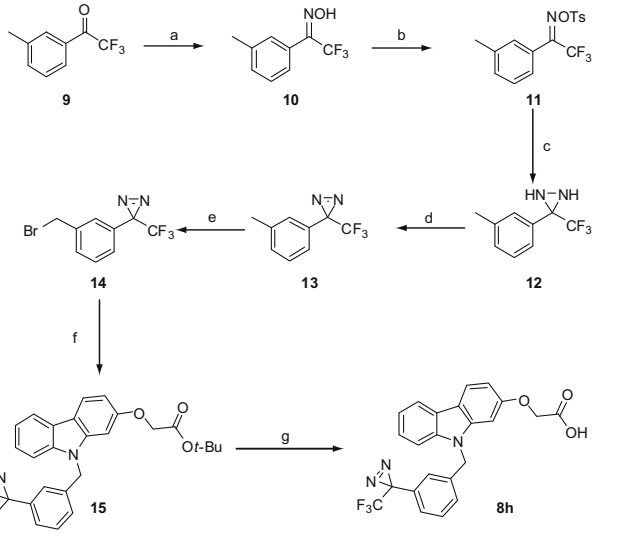
The ongoing discussion on the existence of two (or even more) binding sites in the γ-secretase complex advocates for an investigation into the potential distance between these sites. Two modulating monomer units linked by a variable spacer may form a more active dimer interacting with the two potential binding sites. The length of these spacers would thus provide information about the distance between both binding sites. Fenofibrate dimers with variable alkyl tethers offer access to such distance mapping. The conversion of the fenofibrate with 2,4-dinitrophenylhydrazine provided the hydrazone **19** (Scheme 3). The UV-activity and intrinsic color of **19** may be exploited in cellular mechanistic studies of γ-secretase modulation.



Scheme 1. Synthesis of carbazole-derived sGSMs. Reagents and conditions: (a) *t*-butyl 2-bromoacetate, K_2CO_3, acetone, reflux; (b) KO*t*-Bu, R^1X, THF, 0 °C to rt; (c) 20% TFA, CH_2Cl_2.

Table 1
Compounds **8–19**

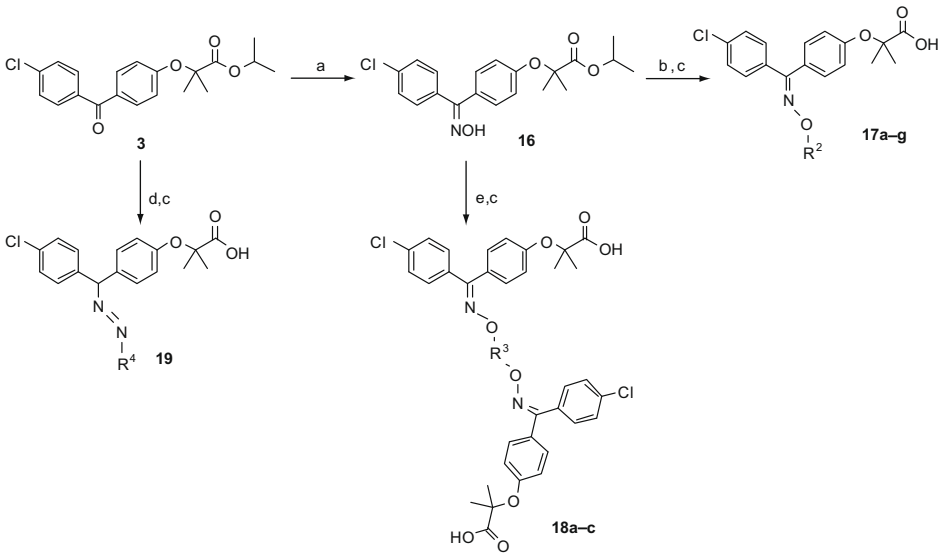
Entry	Compds	BSc number	R	Substituent
1	**8a**	3770	R^1	Octadecyl
2	**8b**	3853	R^1	*trans*-Oleic acid
3	**8c**	3854	R^1	Decacarbonyl
4	**8d**	3915	R^1	*cis*-Oleic acid
5	**8e**	3955	R^1	8-(*N*,*N*-Dimethyl)-4-sulfonylnaphthalene
6	**8f**	3956	R^1	4′-(*N*,*N*-Dimethyl)-1-sulfonylazabenzene
7	**8g**	3984	R^1	4′-Methylbiphenyl-3-carbonitrile
8	**8h**	3958	R^1	3-(3-Methylphenyl)-3-(trifluoromethyl)-3*H*-diazirine
9	**17a**	3934	R^2	Octyl
10	**17b**	3935	R^2	Nonyl
11	**17c**	3936	R^2	Decyl
12	**17d**	3937	R^2	Undecyl
13	**17e**	3938	R^2	Dodecyl
14	**17f**	3939	R^2	Tetradecyl
15	**17g**	3940	R^2	Hexadecyl
16	**18a**	3941	R^3	Octyl
17	**18b**	3942	R^3	Nonyl
18	**18c**	3943	R^3	Decyl
19	**19**	3989	R^4	2,4-Dinitrophenylhydrazyl



Scheme 2. Synthesis of a carbazole-derived sGSM **8h** equipped with a photoreactive unit. Reagents and conditions: (a) $NH_2OH{\cdot}HCl$, py, EtOH, reflux; (b) TsCl, DIEA, DMAP, CH_2Cl_2, 0 °C to rt; (c) NH_3, Et_2O, −78 °C to rt; (d) I_2, MeOH, Et3 N, pH >7; (e) NBS, AIBN, CCl_4, reflux; (f) **7**, KO*t*-Bu, THF, 0 °C to rt, 12 h; (g) TFA, CH_2Cl_2, 0 °C to rt, 2–8 h.

To evaluate the compounds **8a–h**, **17a–g**, **18a–c**, **19** for their potency to modulate Aβ secretion, we used the Aβ liquid phase electrochemiluminescence (LPECL) assay to measure Aβ isoforms.[7] Cell viability was measured by a colorimetric cell proliferation assay (CellTiter 96TM AQ assay, Promega) utilizing the bioreduction of MTS (Owen's reagent) to formazan. The results are summarized in Table 2 and the dose-dependent curves of the most active compounds are shown in Figure 2.

Introduction of a double bond by substitution with *cis*- and *trans*-configurated oleic acid did not show any differences in potency. Both isomers, *E* (**8b**) and *Z* (**8d**) inhibited $A\beta_{42}$ secretion with an IC_{50} of 13 μM. Remarkably, there is a twofold increase in potency compared to the saturated analogue **8a** (BSc3770). The introduction of an *E*- or *Z*-double bond seems to stabilize the membrane orientation of the lipophilic anchor resulting in increased potency. In addition linear lipophilic alkyl chains, even amides (**8c**) and sulfonamides are tolerated. We suggest that linear, lipophilic



Scheme 3. Synthesis of fenofibrate derived sGSMs. Reagents and conditions: (a) $NH_2OH{\cdot}HCl$, py, EtOH, 80 °C, 4 h; (b) NaH, R^2X, DMF, 0 °C to rt, 6–12 h; (c) 2 N NaOH, MeOH, rt, 4–12 h; (d) 2,4-dinitrophenylhydrazine, H_2SO_4, MeOH, rt, 2 h; (e) NaH, $X–R^3–X$ (0.5 equiv), DMF, 0 °C to rt, 6–12 h.

Table 2
Modulation and cell toxicity results for compounds **8–19**

Entry	Compds	BSc number	Cell toxicity (μM)	EC_{50} $Aβ_{38}$ (μM)	IC_{50} $Aβ_{40}$ (μM)	IC_{50} $Aβ_{42}$ (μM)
1	**8a**	3770	–	1.4	>80	26.0
2	**8b**	3853	n.t.[a]	9.3	>40	13.0
3	**8c**	3854	n.t.	12.6	>40	22.4
4	**8d**	3915	–	9.2	>40	13.1
5	**8e**	3955	80	>40	>40	>40
6	**8f**	3956	n.t.	7.6	>80	9.0
7	**8g**	3984	–	>80	>80	>80
8	**8h**	3958	–	>80	>80	>80
9	**17a**	3934	80	16.1	47.6	36.2
10	**17b**	3935	–	>40	>40	>40
11	**17c**	3936	–	>80	>80	36.1
12	**17d**	3937	–	>40	>40	>40
13	**17e**	3938	80	>40	>40	>40
14	**17f**	3939	–	>80	>80	>80
15	**17g**	3940	–	>80	>80	>80
16	**18a**	3941	–	47.6	37.3	23.7
17	**18b**	3942	80	>40	>40	>40
18	**18c**	3943	80	>40	>40	>32.6
19	**19**	3989	80	47.6	37.3	17.0

[a] n.t. = not tested.

substituents are useful anchors, given that *para*-substituted benzenes (**8f**) are modulators in contrast to *meta*-substituted benzenes (**8e, g, h**) showing no activity at all.

The activities of the fenofibrate derivatives have been determined at an initial concentration of 40 μM (see Supplementary data). As expected, fenofibrate (BSc3931) and the oxime **16** showed inverse modulation, the fenofibrate acid (BSc3932) displayed no activity at all (data not shown). The O-substituted compounds **17a** (BSc3934) and **17c** (BSc3936) displayed modulating effects. At a concentration of 40 μM the $Aβ_{42}$ level was reduced by 60%, while the $Aβ_{38}$ level was increased. The modulating effects of the dimers **18a**, **b** are not as strong, but still significantly stronger than those of flurbiprofen or indomethacin (see Supplementary data), which are benchmarks for GSM activity. The C12 dimer **18c** (BSc3943) does not show any effect on Aβ secretion.

Two out of eleven fenofibrate derivatives showed modulating effects at low concentrations with the most active being the O-alkylated C8 derivative **17a** (BSc3934). The modulating tendency is visible with all other alkylated derivatives **17b–g**, however, only at higher concentrations. Hydrazone **19** (BSc3989) inhibited total Aβ. The dimers **18a–c** showed modulatory effects at high concentrations. Some compounds showed cellular toxicity at a concentration of 80 μM in H4-cells. As fenofibrate is an approved drug, the in silico parameters are established and adequate. The introduction of the lipophilic substituent caused a dramatic increase of the *c*log *P* value (fenofibrate: tPSA: 52.6, *c*log *P*: 5.2; fenofibrate-C8-oxime: tPSA: 57.1, *c*log *P*: 10.2). We presume that the lipophilic anchor of the compounds is essential for orientation within the membrane. The maximum alkyl chain length should therefore not exceed the length of a natural phospholipid (see **8a–d**, Table 2). The results of the fenofibrate dimers indicate that the maximum chain length has been reached with C12. Furthermore, our results do not indicate an interaction with more than one binding site.

We compared the structures of highly active compounds (see supplement of Ref. 13) to rationalize the binding mode of straight sGSMs. Apparently, a carboxylic acid moiety is essential for potency.

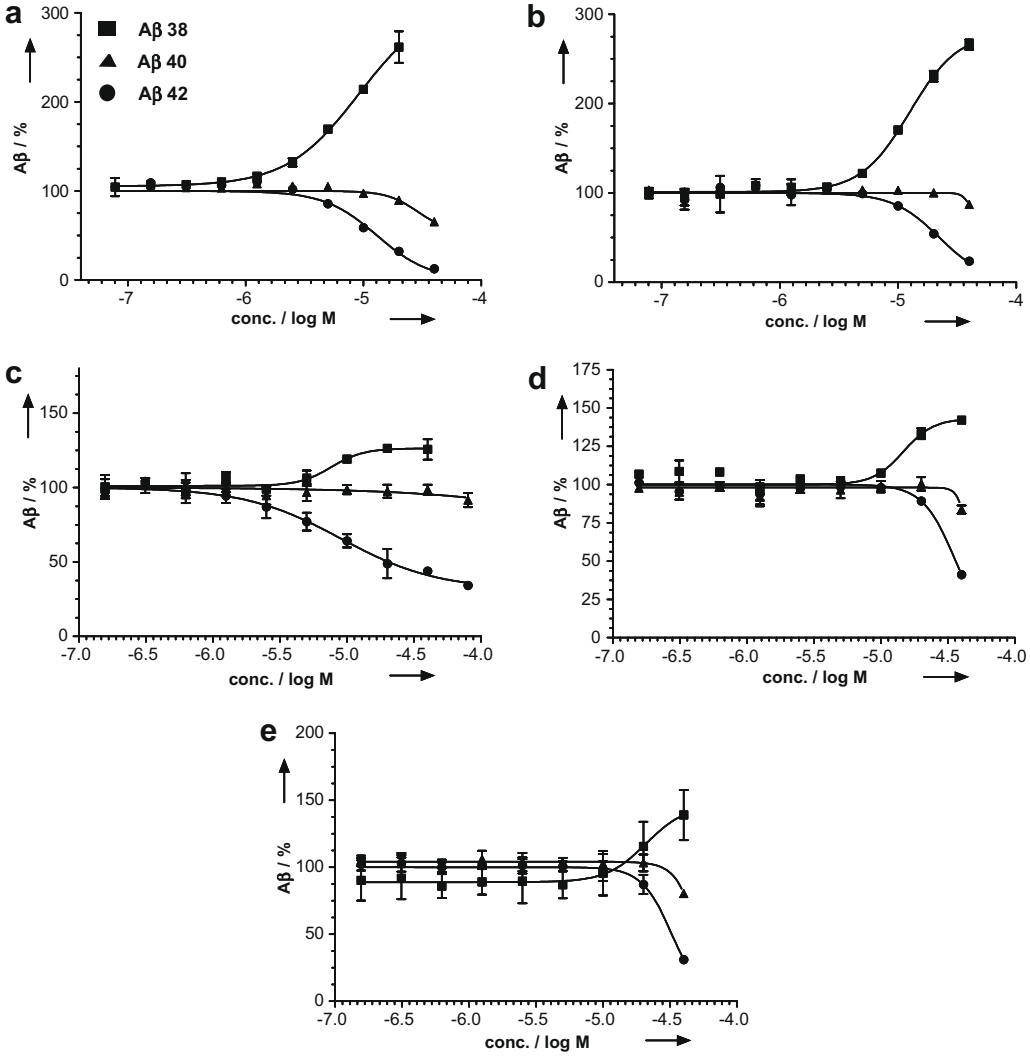


Figure 2. Dose–response curves for the most active N-substituted carbazolyloxyacetic acid and fenofibrate derivatives; Aβ (% of control). (a) Compound **8d**. (b) Compound **8c**. (c) Compound **8f**. (d) Compound **17a** (e) Compound **18c**.

We assume that this functionality interacts with a lysine (maybe lys624) of the substrate APP, which is located next to the membrane interface. Thereby, the lipophilic substituents of the sGSMs serve as membrane anchors. Binding to the substrate may avoid substrate dimerization involving the GAIIG motif (see Supplementary data), thereby driving the cleavage shift away from $A\beta_{42}$.

In summary, the introduction of a lipophilic substituent turned fenofibrate- and carbazole-derived carboxylic acids into sGSMs, which display selective reduction of $A\beta_{42}$ and an increase of the less aggregatory $A\beta_{38}$ fragment. The most active compounds display activity on APP overexpressing cell lines in the low micromolar range. Some of these sGSMs do not target the γ-secretase complex directly, but rather bind to its substrate APP, thereby changing the cleavage pattern. The dimerization of APP may be influenced by this sGSM binding. The lipophilic substituents anchor the compound in the membrane.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.10.035.

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