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The synthesis and structure–activity relationship of substituted *N*-phenyl anthranilic acid analogs as amyloid aggregation inhibitors

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ABSTRACT

It is believed that β-amyloid aggregation is an important event in the development of Alzheimer's disease. In the course of our studies to identify β-amyloid aggregation inhibitors, a series of *N*-phenyl anthranilic acid analogs were synthesized and studied for β-amyloid inhibition activity. The synthesis, structure–activity relationship, and in vivo activity of these analogs are discussed.

Genetic, pathological and biochemical evidence implicates aggregation of β-amyloid (Aβ) peptide in the pathogenesis of Alzheimer's disease.[1] Current marketed therapies for Alzheimer's disease are palliative and only modestly effective. A disease-modifying drug would be a major therapeutic breakthrough towards the treatment of this disease.

Our initial attempts at generating small molecule inhibitors for β-amyloid aggregation were based on Congo Red, a well-known stain for amyloid protein fibrils.[2] Figure 1 depicts the refinement of the structural class from a diazo-linked anthranilic acid derivative to an ethyl-linked moiety. The addition of a single methylene unit in the linker generated compounds with increased in vitro potency. Based on the promising in vitro and in vivo results[3] of initial leads PD 0118057 (**14**) and PD 0202091 (**57**), additional synthesis was pursued to generate a structure–activity relationship within this series of compounds.

In Scheme 1, olefin **3** was generated from heating a pyridine solution of *p*-nitrophenylacetic acid **1** and substituted aldehyde **2**. Hydrogenation of **3** gave amine **4** which was then coupled with

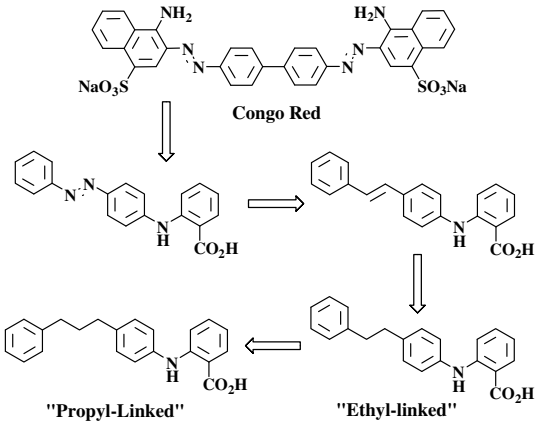

Figure 1. Evolution of *N*-phenyl anthranilic acid analogs as amyloid aggregation inhibitors.

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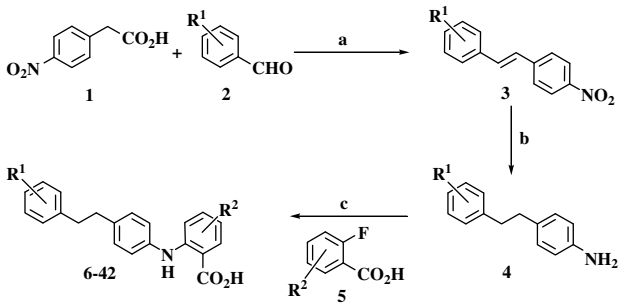
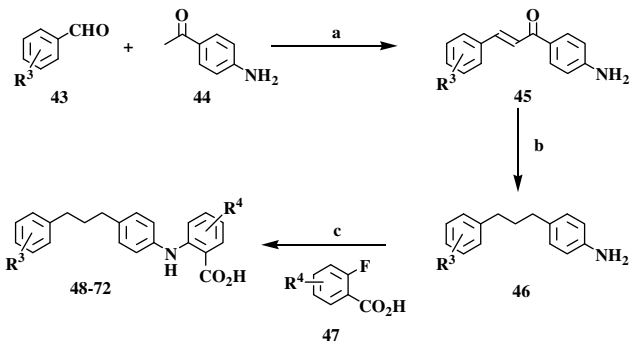
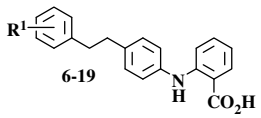


Scheme 1. Reagents: (a) Pyr; (b) RaNi, H_2, THF; (c) LHMDS, THF.



Scheme 2. Reagents: (a) NaOH, EtOH or H_2SO_4, AcOH; (b) Et_3SiH, TFA; (c) $LiNH_2$, THF.

Table 1
Modifications of ethyl-linked derivatives



Compound	R^1	BASSR IC_{50} (μM)	BASST IC_{50} (μM)
6	4-OMe	>100	>100
7	*N*-DHIQ[a]	>100	1
8	H	60	10
9	3,4-Me	>100	10
10	2-Cl	>100	5
11	3-Cl	>100	19
12	4-Cl	>100	3
13	3-F	>100	70
14	3,4-Cl	>100	2
15	2,4-Cl	>100	51
16	3,5-Cl	>100	15
17	3,4-F	>100	2
18	4-F, 3-CF_3	>100	2
19	3-Cl, 4-Me	88	1

[a] *N*-decahydroisoquinoline.

2-fluoro benzoic acid **5** in the presence of a strong base to give ethyl-linked derivatives **6–42**.

Propyl-linked derivatives (Scheme 2) were prepared via an acid- or base-catalyzed Aldol reaction. Reaction of substituted aldehydes **43** and 4-aminoacetophenone **44** generated chalcone **45**. Reduction of chalcone **45** gave propyl-linked anilines **46** that were subsequently coupled with 2-fluoro benzoic acids **47** to yield the corresponding propyl-linked anthranilic acids **48–72**.

The in vitro data were obtained from two primary assays, BASSR and BASST, which were designed to determine the inhibition of amyloid fibril formation by the test compound. BASSR (Beta-Amyloid-Self-Seeding Radioassay) is a radiolabeled ^{125}I-Aβ(1-40) filtration assay. The BASST (Beta-Amyloid-Self-Seeding, Thioflavin T) is an amyloid-specific fluorescence assay. The test compound was incubated under the same conditions in both assays with Aβ(1–40)/buffer (30 μM unlabeled Aβ(1–40)/50 mM NaPi-150 mM NaCl, 1 M urea, 0.02% w/v NaN_3, pH 7.5). In the BASSR assay, a tracer amount of ^{125}I-labeled Aβ(1–40) was added to the assay. In the BASST assay, 5 μM ThioflavinT (ThT) was added after fibril formation.[4] BASST reports on the formation of the β-sheet structure of amyloid fibrils.[5] This measure can be complicated by false positives that result from the competition of test compounds with the extrinsic fluorescent ThT probe for binding to amyloid fibrils. Therefore, an additional assay, BASSR, was used to detect the inhibition of peptide aggregation. BASSR measures the formation of the radiolabeled fibrils that do not pass through a 0.2 μm filter. These two assays provided complementary information about the aggregation state and fibrillar amyloid conformation with compound present. Eight concentrations of each compound were used for IC_{50} determinations that were calculated by logit transformation of the titration curves. Variation of individual points was ±10%. The average IC_{50}s of two separate experiments are reported. At 30 μM of Aβ(1–40) peptide, an inhibitor with an IC_{50} of 1 μM targets a species that is less than 3% of the total peptide concentration. These assays were designed to detect inhibitors at an early stage in fibril formation with an amount of compound that would be less than the Aβ(1–40) peptide monomer concentration. An IC_{50} of 20 μM is approximately equivalent to a ratio of 0.7 moles compound/peptide monomer and thus, is borderline substoichiometric.[4,5] (The observed IC_{50} is a product of the true affinity of the compound, the stoichiometry of binding, and the concentration of the target aggregation intermediate.)

In vivo efficacy studies were performed in Tg2576 mice that over-express human $βAPP695_{Swe}$. These mice show an age-related increase in cerebral Aβ deposition, and therefore, are suitable models for testing β-amyloid aggregation inhibitors.[6] Bioavailable and brain-penetrant compounds were delivered orally by mixture in chow.

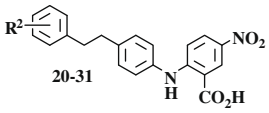
Our initial efforts in the ethyl-linked series produced a series of compounds (Table 1) that showed acceptable activity in the BASST assay, but were inactive in the BASSR assay.

By adding a nitro group to the anthranilic acid ring (Table 2), potency increased significantly in both assays. However, the nitro functionality is considered to be a risk for CNS compounds since it may decrease brain-penetration of the compound or have potential toxicity. Therefore, other substituents on the anthranilic acid ring were explored (Table 3).

When the carboxylic acid group was moved to the 4- and 5-positions on the aromatic ring (entries **32** and **34**, Table 3 vs entry **14**, Table 1), a 5-fold decrease in potency in BASST was observed. The methyl ester analog (entry **33**) of entry **32** exhibited no activity in either BASST or BASSR. Some of the most potent derivatives of **14** had a carboxylic acid group in the 6-position and an electron-deficient anthranilic acid ring (see Table 2 and entries **37, 39** and **41** in Table 3).

By increasing the length of the alkyl linker by one methylene unit (i.e., propyl-linked, Table 4), a significant increase in potency was observed in the BASSR assay relative to the similarly substituted inactive ethyl-linked compounds (entries **50, 52, 54–56** in Table 4 vs **8–12** in Table 1). In addition to this observed increase in potency, another advantage of the propyl-linked analogs was the consistent activity in both BASSR and BASST. However, substitution on the anthranilic ring of the propyl-linked analogs with electron withdrawing groups (Table 5, entries **65, 66**) did not give

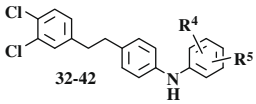
Table 2
4-Nitro substitution of ethyl-linked derivatives



Compound	R^2	BASSR IC_{50} (μM)	BASST IC_{50} (μM)
20	4-*N*(*n*Bu)$_2$	15	1
21	DHIQ[b]	5	1
22	H	70	1.5
23	3,4-Me	12	2
24	2-Cl	3	0.5
25	3-Cl	33	0.3
26	4-Cl	15	0.4
27	3,4-Cl	43	0.5
28	2,4-Cl	17	4
29	3,4-F	41	4
30	4-F, 3-CF_3	3	0.8
31	4-Cl, 3-CF_3	16	1

[b] *N*-decahydroisoquinoline.

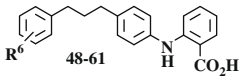
Table 3
Other modifications of ethyl-linked derivatives



Compound	R^4	R^5	BASSR IC_{50} (μM)	BASST IC_{50} (μM)
32	H	4-CO_2H	>100	10
33	H	4-CO_2Me	>100	>100
34	H	5-CO_2H	>100	10
35	4-CF_3	5-CO_2H	>100	35
36	4-F	5-CO_2H	>100	3
37	2-aza[c]	6-CO_2H	7	2
38	4-Me	6-CO_2H	>100	2
39	3-CF_3	6-CO_2H	27	4
40	2-CF_3	6-CO_2H	>100	30
41	5-CF_3	6-CO_2H	70	0.1
42	4-NEt_2	6-CO_2H	>100	53

[c] substituted pyridine ring.

Table 4
Propyl-linked derivatives

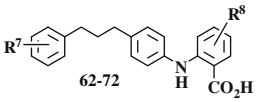


Compound	R^6	BASSR IC_{50} (μM)	BASST IC_{50} (μM)
48	4-NEt_2	3	1
49	4-OMe	30	17
50	H	14	5
51	4-Me	9	1
52	3,4-Me	6	3
53	3-Br	2	3
54	2-Cl	6	2
55	3-Cl	4	3
56	4-Cl	3	2
57	3,4-Cl	25	1
58	2,4-Cl	2	2
59	3,5-Cl	3	3
60	3,4-F	8	5
61	4-F, 3-CF_3	5	3

the same boost in potency as observed in the ethyl-linked series (Table 2).

The in vivo and pharmacokinetic data of **14** and **57**, pre-clinical chemical leads from the ethyl- and propyl-linked series, respectively, provided convincing evidence that this series of compounds could potentially generate clinical candidates for Alzheimer's disease therapy. Figures 2 and 3 illustrate the reduction of senile plaques (black spots in cortex) in treated Tg2576 mice with PD 0118057 (**14**) and PD 0202091 (**57**), respectively, compared to an untreated control mouse.

Table 5
Other modifications of propyl-linked derivative



Entry	R^7	R^8	BASSR IC_{50} (μM)	BASST IC_{50} (μM)
62	3,4-Cl	4-F	86	1
63	3,4-Cl	4-NO_2	>100	1
64	3,4-Cl	4-Me	>100	>100
65	3,4-Cl	2-aza	60	5
66	3,4-Me	4-NO_2	14	6
67	3,4-Me	4-F	>100	1
68	3,4-Me	2-aza	14	9
69	3,4-Me	3-F	12	5.4
70	3,4-Me	4-Me	16	3
71	4-OMe	3-F	14	9
72	4-OMe	4-F	14	10

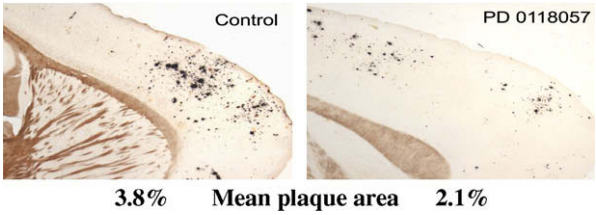


Figure 2. Plaque density of control versus PD 0118057-treated Tg2576 mice, 200 mg/kg/day.

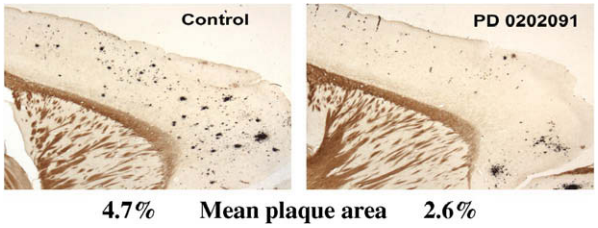


Figure 3. Plaque density of control versus PD 0202091-treated Tg2576 mice, 60 mg/kg/day.

As further noted in Figure 4, **57** showed a significant reduction in plaque load over a 7-month and then a 3-month treatment period at a lower dose versus **14**. A 3-fold lower dose of **57** versus **14** gave the same reduction in plaque load in the Tg2576 mouse.[7]

Additional evidence of these types of inhibitors preventing the aggregation of the Aβ protein was provided by an atomic force microscopy (AFM) study.[8] Results indicated that smaller, fragmented Aβ species were present after in vitro treatment of the Aβ protein with, for example, **14** (Fig. 5).

This AFM result correlated with the smaller size Aβ peptide species observed by analytical ultracentrifugation (fibrils > 200S, fibrils treated with **14** = 15S).[9] These Aβ species, although multimeric, seem to be non-toxic based on results from cell culture studies and the lack of cell loss in the mice. In contrast to amyloid fibrils, the fragmented Aβ species were protease-sensitive and thus could be cleared from the brain.[9] The size of these accumulated Aβ

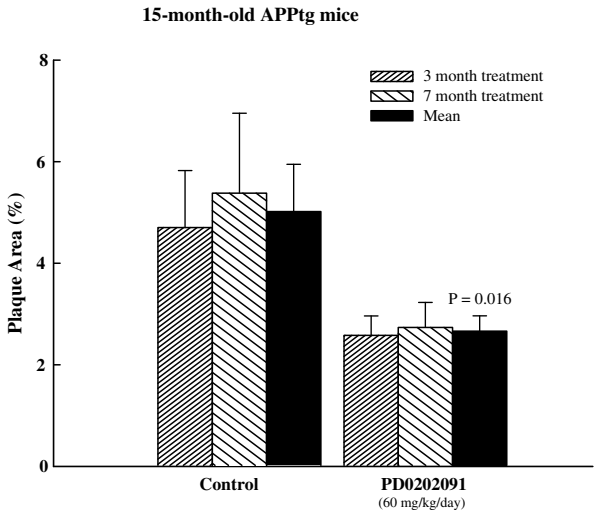


Figure 4. 3- and 7-month treatment of Tg2576 mice with PD 0202091.

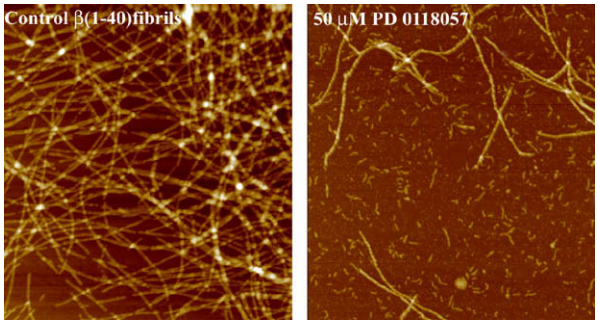


Figure 5. AFM results with PD 0118057 (14).

forms may indicate the inhibition site in the process of amyloid fibril formation, perhaps at an early, non-toxic pre-protofibril stage. Further study of the interruption of amyloid formation would be needed to establish the relationship of fibril deposition per se to the progression of Alzheimer's disease. Compounds such as **14** and **57** with brain penetration and significant in vivo activity could be useful in probing the role of fibril formation in disease progression.

Following the discovery of **14** and **57**, many amyloid aggregation inhibitors with low micromolar inhibition and in vivo efficacy were synthesized. However, **14** and **57** possessed the most acceptable drug-like properties, such as brain penetration, bioavailability, no toxicity and in vivo efficacy. Thus, the in vitro and in vivo effects of **14** and **57** indicate that in vitro inhibition of amyloid aggregation translated to efficacy in the Tg2576 mouse model as measured by the reduction in senile plaque load. These results suggest that agents limiting the self-polymerization of Aβ in brain and maintaining the Aβ in a metabolizable form can impede senile plaque formation, and hence, could lead to a disease-modifying drug for the treatment of Alzheimer's disease.

References and notes

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6. Callahan, M. J.; Lipinski, W. J.; Bian, F.; Durham, R. A.; Pack, A.; Walker, L. C. *Am. J. Pathol.* **2001**, *158*, 1173.
7. Plaque reduction for **14** and **57** seems to be at maximum effect expected in this model: **14** (200 mg/kg/day, PO: 7 months (n = 15, 44% reduction); **57** (60 mg/kg/day, PO: 7 months (n = 14, 47% reduction) and 3 months (n = 16, 45% reduction).
8. Thank you to P. Lansbury/J. Harper for AFM studies and J. Kelly/H. Lashuel for the analytical ultracentrifugation studies.
9. Unpublished results.