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Novel inhibitors of the high-affinity L-proline transporter as potential therapeutic agents for the treatment of cognitive disorders

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ABSTRACT

The incidence of cognitive disorders such as Alzheimer's disease continues to increase unabated. While cures for such diseases have eluded investigators, progress is being made on alleviating certain symptoms of these diseases. Mouse knockouts of the proline transporter (PROT), a high affinity Na^+/Cl^--dependent transporter, indicated its potential as a novel therapeutic target for cognition improvement. Herein we report our investigation into a novel class of PROT inhibitors.

Cognitive disorders affect the ability to think, to process and store information, and to solve problems. Alzheimer's disease (AD), a progressive and fatal brain disease, is the most common form of dementia. It currently directly affects as many as 35 million people worldwide and is projected to double in incidence every 20 years, affecting 1 in 85 people globally by the year 2050.[1] While early symptoms are often mistaken for aging or stress, rapid advancement of the disease often leaves the patient completely dependent on caregivers.[2] Monetary costs of the disease have been estimated at $160 billion annually, but the incalculable cost of the burdens on main caregivers includes social, psychological, and physical aspects as well.

While AD currently has no known cure, efforts to alter the course of the disease and improve the quality of life for patients can have a dramatic affect not only for those with the disease, but those caring for them as well. Current treatments of the cognitive impairment associated with AD include acetylcholinesterase inhibitors[3] and an NMDA receptor antagonist. A known feature of AD is a reduction in the activity of the cholinergic neurons, which many researchers presume underlies much of the short-term memory loss associated with the disease. Acetylcholinesterase inhibitors ameliorate the effects of the loss of cholinergic neurons by reducing the degradation rate of acetylcholine and increasing its effective concentration in the brain. Memantine, a noncompetitive NMDA receptor antagonist approved for the treatment of moderate-to-severe AD, inhibits the overstimulation of NMDA receptors by glutamate, reducing the effects of this excitotoxicity pathway endemic to AD.

The amino acid L-proline has been reported to play a role in regulating synaptic transmission in the mammalian brain.[4] In general, neurotransmitter systems typically employ various mechanisms that inactivate signaling to regulate synaptic transmission and protect against excitotoxicity. Many of these rely on the action of a Na^+-dependent transporter to facilitate rapid re-uptake of neurotransmitter ligands.[5] The mammalian proline transporter (PROT) is a high affinity Na^+/Cl^--dependent transporter expressed in specific regions of the brain, including the cerebral cortex and hippocampus.[6,7] It displays sequence homology to other neurotransmitter transporters such as the glycine, dopamine, serotonin, and norepinephrine transporters.[7,9] PROT has been found to be expressed selectively within synaptic terminals of a subset of glutamatergic neurons and may play an important role in the regulation of excitatory neurotransmission. Knockout mice lacking functional PROT have demonstrated improved performance in learning and memory tests and do not differ from wild-type mice in locomotor

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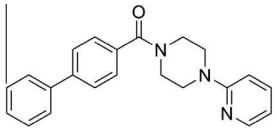


Figure 1. Initial HTS hit.

activity and motor coordination.[9] Three opioid peptide-related peptides were found to inhibit L-proline uptake by rat brain synaptosomes and were subsequently confirmed to inhibit proline uptake using recombinant PROT.[10,11] However, only a single small molecule inhibitor has been described for this transporter.[12]

Herein we report the discovery of a novel class of PROT inhibitors. These compounds are potent in vitro, metabolically stable, and exhibit good pharmacokinetics when dosed orally. The synthesis, representative structure–activity relationships (SAR), and in vivo activity are reported.

SAR and in vitro activity: In order to access in vitro potency of PROT inhibitors, cell-based assays were established. The inhibition levels were determined by measuring PROT mediated ^{3}H-proline uptake in the presence of increasing compound concentration. Nonspecific uptake was determined by measuring ^{3}H-proline uptake in the presence of 2 mM unlabeled proline.

To begin our investigation of inhibitors of PROT, we analyzed internal libraries of small molecules using high-throughput screening and a COS1 cell line expressing human PROT. A large number of compounds were found to have inhibitory activity; however, many of these compounds were ultimately judged to have poor ADME properties, and poor brain penetration in particular (Fig. 1). Compound **1** showed dose-dependent inhibition of PROT with an IC_{50} of 0.160 μM, however this compound showed poor mouse liver S9 stability.[13]

Replacement of the pyridine ring included substituted phenyl rings as well as other heteroaromatic rings (Fig. 2). The phenyl substituted compound **2** did improve the stability of the series (68% remaining after 30 min), but sacrificed significant potency. Similarly, substituted phenyl rings led to less active compounds, suggesting that substitution at the *para* position was not tolerated. Only two variations retained desirable levels of potency, 2-thiazolyl (**5**) and 2-pyrimidyl (**8**). While the thiazole substituted compound had similar potency to **1**, it also had poor metabolic stability. The modification of changing the pyridyl moiety to a less

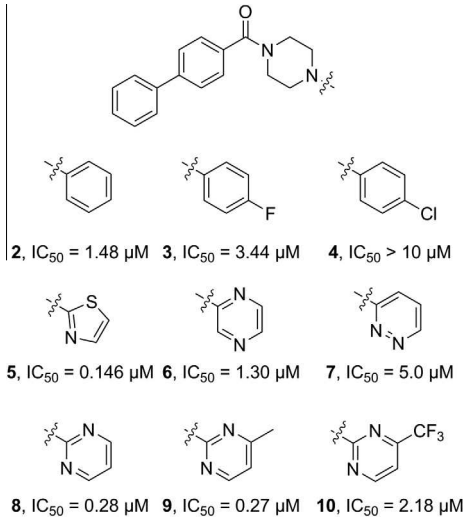


Figure 2. Replacement of the pyridyl moiety.

basic pyrimidine (**8**) sacrificed a small amount of potency for improved stability (S9 fraction, 100% remaining after 30 min.). Much of the subsequent SAR investigation retained the pyrimidine ring, and focused on changes to the central and western portions of the molecule.

Assessment of the favored orientation of the biphenyl moiety led to the synthesis of compounds **11** and **12** (Fig. 3). Increased radial deviation from the original 4-biphenyl substituted compound led to a decrease in potency. We therefore chose to continue investigation of this hit by retaining the *para* substitution pattern and focusing on changes to the distal phenyl ring.

Our general synthetic route for exploring the SAR of the biphenyl region is depicted in Scheme 1. Thermal reaction of 2-chloropyrimidine and piperidine gave amine **13**. Subsequent EDC coupling of **13** with 4-bromobenzoic acid afforded bromide **14**, our common intermediate for exploration of the terminal phenyl ring through Suzuki coupling reactions with a diverse set of boronic acids. The synthetic scheme was efficient, modular and straightforward, and allowed for investigation of each of the lead series' component pieces.

Investigation into the SAR of the biphenyl moiety began with the distal ring. We quickly found that the 2′ position did not tolerate groups larger than H or F (Table 1); for example, adding a methyl or trifluoromethyl group at the 2′ position reduced the potency by 2- and 4-fold, respectively, as compared to the unsubstituted compound. Therefore, our main focus was directed at the 3′ and 4′ positions. Substitution at the 4′ position was generally well tolerated, and improvements in potency were realized with small electron-withdrawing or neutral substituents such as methyl (**21**, PROT IC_{50} = 0.034 μM) and chloro (**23**, PROT IC_{50} = 0.052 μM). Electron-rich substituents such as methoxy led to a measurable decrease in potency (**26**, PROT IC_{50} = 0.23 μM), while some steric

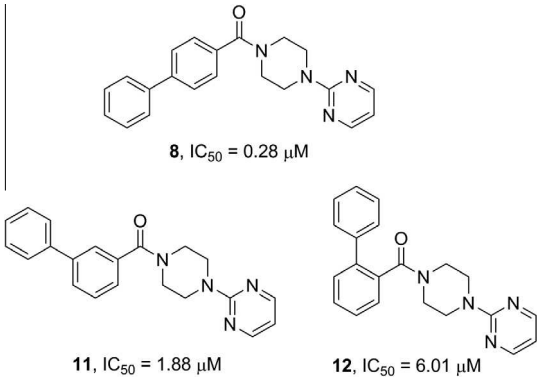
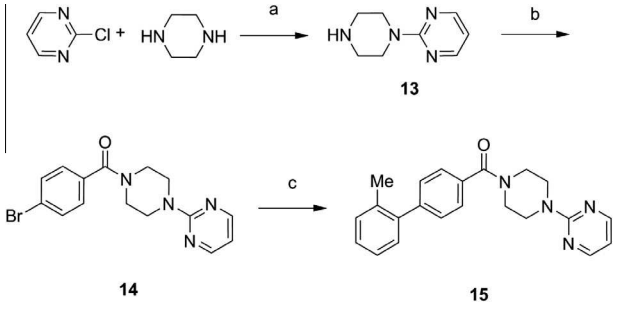
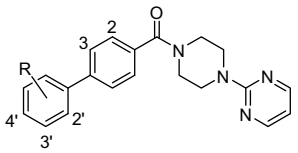


Figure 3. Optimal phenyl orientation.



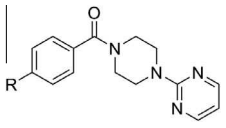
Scheme 1. Reagents and conditions: (a) triethylamine, EtOH, 100 °C; (b) 4-bromobenzoic acid, EDC, HOBt, DIPEA, DMF; (c) 2-methylphenylboronic acid, $Pd(dppf)Cl_2$, K_3PO_4, DME, H_2O, 80 °C.

Table 1
Distal phenyl ring SAR



Compd	R	PROT IC_{50} (μM)
8	H	0.28
15	2′-Me	0.653
16	2′-CF_3	1.26
17	3′-Cl	0.036
18	3′-CF_3	0.071
19	3′-OCF_3	0.073
20	3′-CN	0.19
21	4′-Methyl	0.034
22	4′-Ethyl	0.077
23	4′-Cl	0.052
24	4′-CF_3	0.076
25	4′-CN	0.19
26	4′-MeO	0.23
27	4′-F	0.26

Table 2
Biphenyl replacements



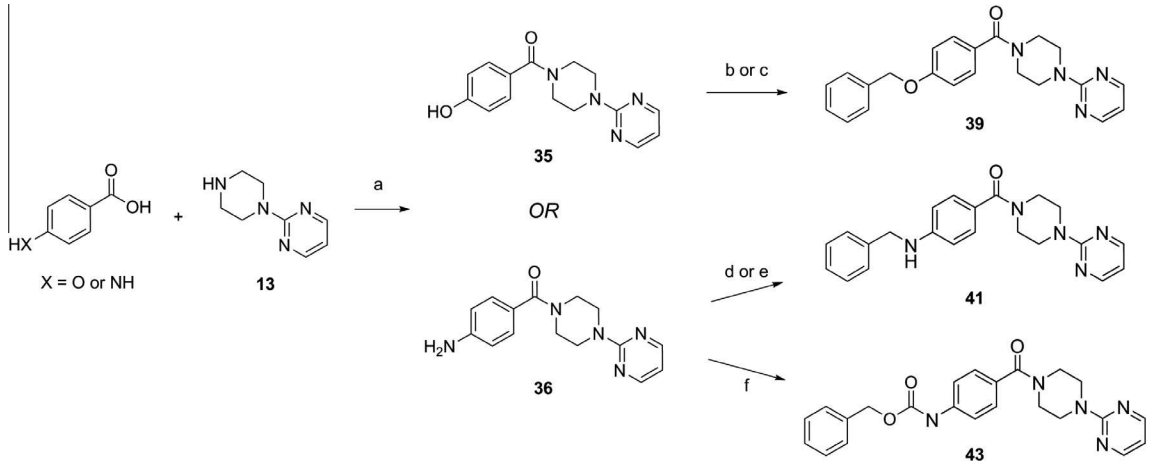
Compd	R	PROT IC_{50} (μM)
8	Phenyl	0.28
28	Cyclohexyl	7.01
29	2-Pyridyl	7.08
30	1-Pyrolo	2.23
31	1-Imidazolo	>10
32	5-Chloro-2-thiophenyl	0.15
33	4-Methyl-2-thiophenyl	0.034
34	5-Methyl-2-thiophenyl	0.061

tolerance was noted for the 4-ethyl (**22**, PROT IC_{50} = 0.077 μM) and 4-trifluoromethyl analogs (**24**, PROT IC_{50} = 0.076 μM). Substitution at the 3′ position of the phenyl ring led to slight improvements in potency relative to the 4′ position. In particular, the 3-chloro substituted compound **17** proved to be the most potent compound (PROT IC_{50} = 0.036 μM) and had good metabolic stability (S9 fraction, 90% remaining at 30 min) and good selectivity over the dopamine (DAT) and glycine (GlyT) transporters (DAT IC_{50} = 3.4 μM and GlyT IC_{50} > 10 μM, respectively). These compounds had, in general, poor solubility and high plasma protein binding (>98%).[14] To improve these characteristics, changes to the biphenyl ring system were pursued.

Replacing the distal ring with saturated or heteroaromatic rings generally led to substantial decreases in potency (Table 2). The cyclohexyl analog and basic nitrogen-containing heterocycles were found to be micromolar inhibitors of PROT. The exception to these results came from substituted thiophene derivatives. Improved potencies relative to the initial HTS compounds were observed when thiophene rings with small substituents replaced the distal phenyl ring (**32**, **33**, and **34**). The potencies of these compounds were similar to the previously synthesized phenyl analogs, but the compounds did not afford any significant improvement in solubility or in the protein binding of the series.

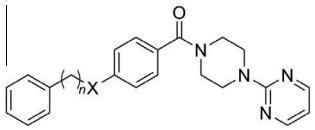
Attempts to investigate the effects of the linker groups between the two phenyl rings to possibly address the low solubility led to the synthesis of a number of linker-spaced biphenyl analogs. As shown in Scheme 2, the synthesis of these analogs began with the acylation of piperidine **13** with either 4-amino- or 4-hydroxybenzoic acid by treatment with HOBt and EDC affording intermediates **35** and **36**, respectively, in good yields. Subsequent functionalization of the phenol **35** by either alkylation under Finklestein-type reaction conditions or by Mitsunobu reaction with appropriate alcohols afforded ethers of the general form **39**. Monoalkylated derivatives of the aniline intermediate were prepared by either treatment of **36** with alkyl bromides and potassium carbonate at 80 °C in DMF or by reductive amination with aldehydes employing sodium cyanoborohydride as the reducing reagent. In the case of the reductive aminations, acetic acid and molecular sieves were necessary to achieve good yields. Alternatively, the anilines could be derivatized as carbamates by treatment with carbonyl diimidazole (CDI) and then with an alcohol to afford the desired compounds.

The synthesized compounds were tested for inhibition of PROT, and Table 3 summarizes the results from these efforts. Insertion of amide or carbamate linkers between the two phenyl rings rendered the compounds inactive (**42** and **43**). More subtle changes such as insertion of a carbonyl (**37**), oxygen (**38**) or amine (**41**) afforded compounds that retained potency. However, while some of these compounds did show some improved protein binding



Scheme 2. Reagents and conditions: (a) EDC, HOBt, DMF, rt; (b) BnBr, NaI, K_2CO_3, acetone, 50 °C; (c) BnOH, DEAD, PPh_3, THF, 0 °C to rt; (d) BnBr, K_2CO_3, DMF, 80 °C; (e) PhCHO, $NaCNBH_3$, HOAc, 4 Å molecular sieves, EtOH; (f) carbonyldiimidazole, BnOH, THF, rt.

Table 3
Linker investigation



Compd	X	*n*	PROT IC_{50} (μM)
8	—	0	0.28
37	C=O	0	2.70
38	O	0	1.95
39	O	1	0.27
40	O	2	1.02
41	NH	1	0.87
42	C(O)NH	1	>10
43	OC(O)NH	1	>10

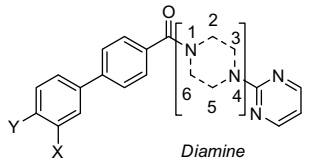
(**41**, 95% bound), most did not improve either the solubility or PPB, and metabolic stability was also problematic.[15] Unable to improve the ADME properties of the series relative to compound **37**, we chose to investigate the eastern portion of the compounds holding the biphenyl portion of the series constant.

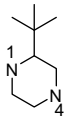
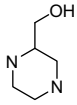
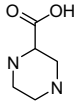
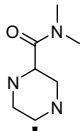
Having established the SAR of the biphenyl moiety, we turned our attention to the piperazine ring. Substitution on the piperazine was generally well tolerated (Table 4). Substitution at the 2 and 6 positions by small alkyl groups was found to improve the potency of the compounds. Gem dimethylation (**45**), 2,6-dimethyl substitution (**46**), and the beta epimer of the mono-methylated compounds (**51**) had the most advantageous effect on the potency of the series. Substitution at the 3 position was disfavored (**47**), as were larger alkyl groups such as *tert*-butyl (**44**). Polar groups such as alcohols (**48**) and carboxylic acids (**49**) were also generally tolerated, though some potency was lost in certain cases (**50**).

As shown in Figure 4, fragmentation of the piperazine ring to the open chain compound **54** led to a dramatic reduction of potency. Homologation to larger ring systems such as the 7-membered homopiperazine (**53**) improved the potency by twofold. Replacement of the piperazine ring with analogous cyclic diamine moieties yielded positive results. The fused bicyclic [3.3.0] ring system (**56**) retained the potency and demonstrated improved PK properties, specifically increased brain levels after oral dosing. Constraining the cyclic diamine portion with bridging groups led to compounds with improved PK and superior potency. While the bicyclo-[2.2.1] compound (**55**), which forces the six-member ring into a boat conformation, was significantly less active at inhibiting PROT (IC_{50} = 1.34 μM), the compounds that exhibit a chair conformation, bicyclo[3.2.1]octane compounds **57** and **58**, exhibit even greater potency (PROT IC_{50} = 0.027 and 0.018 μM, respectively). Although these compounds continued to exhibit higher than desired protein binding, the pharmacokinetic profiles of **57** and **58** led us to investigate them further.

Compounds **57** and **58** were determined to be quite selective within a class of associated small molecule transporters. The dopamine transporter (DAT) was not inhibited by **57** up to a maximum tested concentration of 10 μM, and it was 37-fold selective against the glycine transporter. Compound **58** was slightly less selective for DAT (IC_{50} = 6.1 μM), but more selective for GlyT (IC_{50} > 10 μM). The metabolic stability of both compounds was acceptable, with 75% and 70% remaining when exposed to the mouse S9 fraction, respectively. Compound **58** was found to inhibit HERG at 59% at 10 μM concentration, and dosing of mice (IV, 1 mpk) resulted in lethargic animals and, as a result, in vivo investigation of this compound was not progressed. Following a single oral administration of Compound **57** to C57BL/6 albino mice at 100 mpk, the compound was rapidly absorbed, well tolerated and exhibited good exposure with a T_{max} of 0.38 h and a C_{max} of 4.5 μg/mL and a

Table 4
Piperazine modifications



Compd	Diamine	X	Y	PROT IC_{50} (μM)
44		Cl	H	0.70
45		Cl	H	0.053
46		Cl	H	0.024
47		H	H	0.477
48		Cl	H	0.076
49		Cl	H	0.128
50		Cl	H	0.392
51		H	Cl	0.021
52		H	Cl	0.057

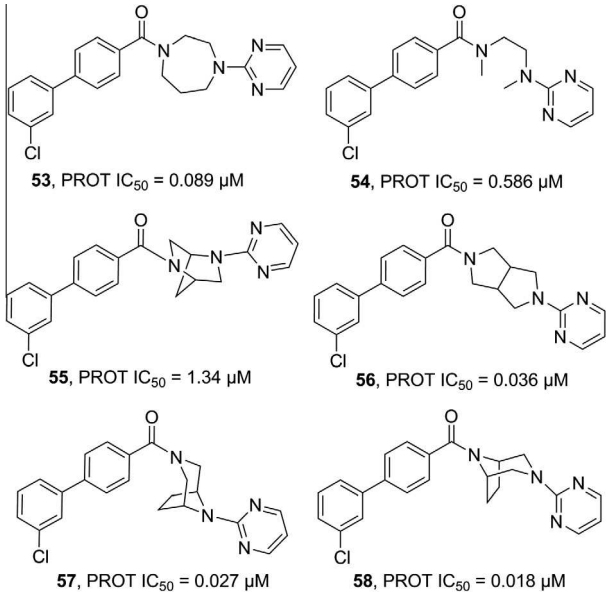


Figure 4. Piperazine modifications.

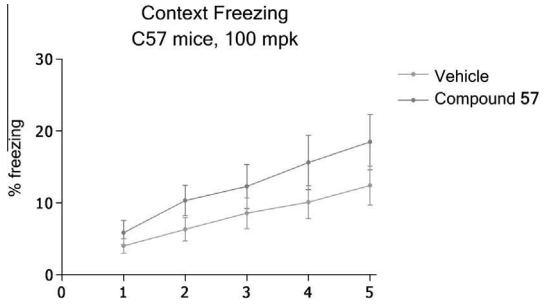


Figure 5. Trace conditioning results of compound **57**.

$AUC_{0-\infty}$ of 24 μg h/mL. Analysis of total brain concentration at 6 h after dosing revealed a level of brain concentration of 2.26 μg/mL, resulting in a calculated brain-to-plasma ratio of 1.7.

After demonstrating no effects distinguishable from vehicle in an Open Field experiment,[16] this compound was selected for testing using a trace conditioning model to evaluate for potential cognitive improvement.[17] In the experiment, subjects are presented with a neutral conditioned stimulus (CS) that is paired, after an interval of time, with an unconditioned stimulus (US) that is averse in nature. This is an associative learning task that by the nature of the delay, or trace interval, will engage the hippocampus and prefrontal cortex. Mice learn to associate the two stimuli and will exhibit behavioral responses such as freezing when the CS is presented alone. Mice will also associate the context, or environment, in which the training of the CS–US occurs, and will exhibit similar behavior even in the absence of the conditioned stimulus. Compound **57** was dosed in a cohort of C57 mice ($n = 13$) at 100 mpk PO along with a vehicle control group.[18] As shown in Figure 5, the results of the context freezing portion of the experiment indicated a possible improvement in response to conditioned stimulus (CS) in the drug treated subjects as compared to the vehicle control group, however the data was not statistically significant ($P = 0.16$).

In summary, we have reported a novel series of highly potent inhibitors for the mammalian proline transporter (PROT). Investigation of the modular SAR of this series has yielded compounds that have demonstrated stability in vivo, and have achieved good plasma and brain levels in oral dosing pharmacokinetic experiments. While the pharmacological effects of these compounds have not been conclusively demonstrated, continued SAR investigation of this series and the identification of compounds which demonstrate reproducible effects in in vivo models will be subsequently reported.

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13. After 30 min of incubation with the S9 fraction of mouse liver homogenate 53% of the parent compound remained.
14. Compound **17**, for instance, was determined to be 99.2% protein bound in an equilibrium dialysis assay. Compound **23**: 97.8%.
15. After 30 min of incubation with the S9 fraction of mouse liver homogenate, 45% of compound **41** and 63% of compound **39** remained.
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