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2-(2-Phenylmorpholin-4-yl)pyrimidin-4(3*H*)-ones; A new class of potent, selective and orally active glycogen synthase kinase-3β inhibitors

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ABSTRACT

A series of 2-(2-phenylmorpholin-4-yl)pyrimidin-4(3*H*)-ones was synthesized and examined for their inhibitory activity against glycogen synthase kinase-3β (GSK-3β). We found **21**, **29** and **30** to possess potent in vitro GSK-3β inhibitory activity with good in vitro PK profiles. **21** demonstrated significant decrease of tau phosphorylation after oral administration in mice and excellent PK profiles.

Alzheimer's disease (AD) is a neurodegenerative disorder defined by the presence of extracellular plaques of β-amyloid (Aβ), and intracellular neurofibrillary tangles (NFTs). NFTs are composed of abnormally hyperphosphorylated microtubule-associated protein tau and their temporal and spatial distribution correlate well with severity of clinical symptoms, memory loss and cognitive impairment.[1]

Glycogen synthase kinase-3β (GSK-3β) is a serine/threonine kinase and is thought to be one of the key enzymes for aberrant tau phosphorylation.[2] Pathogenic activation of GSK-3β causes hyperphosphorylation of tau proteins, which induce their detachment from microtubules leading to microtubule de-polymerization and destabilization of neural cell processes. In parallel, hyperphosphorylated tau released from microtubules tends to misfold and aggregate into paired helical filaments (PHFs) inside the cells to form NFTs in the course of aging.[3] A conditional GSK-3β overexpressing transgenic mouse exhibits persistent tau hyperphosphorylation, pretangle-like somatodendritic localization of tau, neuronal death in hippocampus and cognitive deficits.[4] These studies suggest that GSK-3β is associated with AD progression and that GSK-3β inhibition is expected to be a promising therapeutic approach for AD.[5]

In the previous studies,[6] we obtained **1** as a lead compound which showed potent in vitro inhibitory activity against GSK-3β. However, **1** showed no significant decrease of tau phosphorylation after oral administration. We hypothesized that the N–H group, carbonyl group and linear connection of linker between the phenyl

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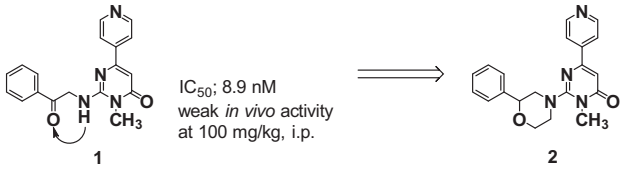


Figure 1. Design for in vivo active compound.

group and the pyrimidin-2-one moiety would prevent the increase of brain concentration by hydrogen bonding donation, moderate metabolic stability or its flexible conformation. In order to confer better in vivo inhibitory activity, we chose to transform the 2-oxo-ethylamine moiety into a morpholine group in the expectation that increases of metabolic stability and cell permeability would result in an increase of brain concentrations (cell permeability of **1** was better than that of propranolol in Caco-2 permeability and good brain concentration by intraperitoneal administration) (Fig. 1). A molecular docking study suggested that **2** would have protein-ligand interaction with GSK-3β as an possible inhibitor.[7] The scaffold possesses two hydrogen bonding interactions with GSK-3β, one between the nitrogen atom on the pyridine moiety and the H–N bond of Val135, and the other between the carbonyl oxygen atom of the pyrimidone moiety and the side chain of Lys85. The morpholine moiety fills the space between the N- and C-terminal domains, and the phenyl moiety makes a cation-π interaction with Arg141 in the hydrophobic site of GSK-3β. The docking study suggests only the (*S*)-isomer makes these interactions (Fig. 2).

2-(2-Phenylmorpholin-4-yl)-pyrimidin-4-ones **15**–**30** was synthesized by the condensation of the appropriate 2-phenylmorpholine and 2-chloropyrimidin-4-one.[8] β-Keto esters **4** were prepared from aryl carboxylic acid **3** with potassium monoethylmalonate, carbonyl diimidazole and magnesium chloride.[9] Condensation of the resulting keto esters **4** and *N*-methylthiourea afforded pyrimidine-4(3*H*)-one **5**, and successive treatment with phosphorous oxychloride and *N*,*N*-dimethylformamide yielded key intermediates **6** (Scheme 1).

2-(*S*)-Phenylmorpholine **14** was prepared from the corresponding 2-chloroacetophenone **7** in six steps. An asymmetric catalytic reduction of **7** with BH_3·THF and (*S*)-CBS afforded (*S*)-alcohol **8** with high enantioselectivity,[10] and successive treatment of **8** with potassium hydroxide yielded epoxide **9**. After reaction of **9** and benzylamine, the resulting amino alcohol **10** was acylated with chloroacetyl chloride followed by cyclization with potassium hydroxide to afford morpholin-2-one **12**. Reduction of **12** with in situ generated borane by chlorotrimethylsilane and lithium

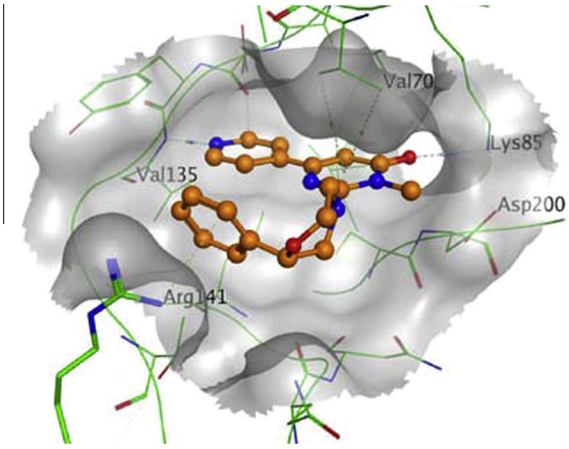
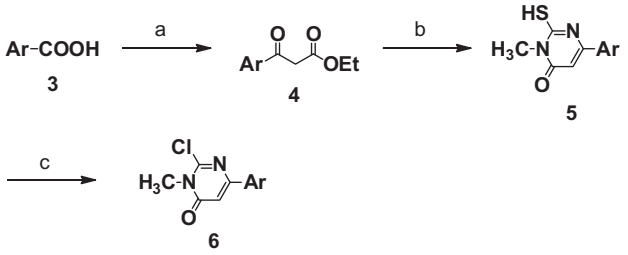


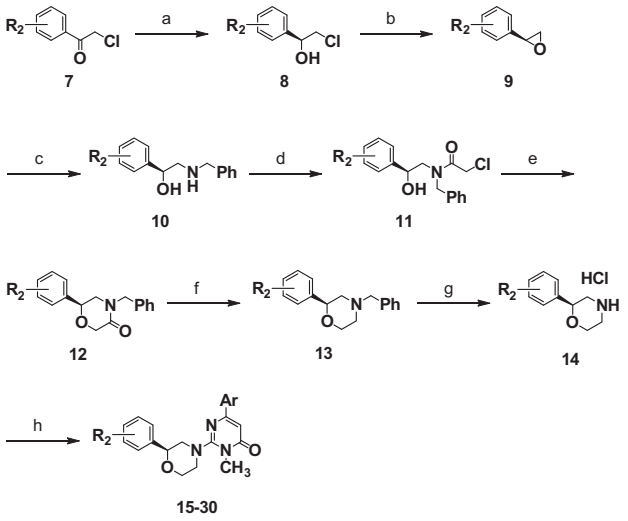
Figure 2. Predicted binding mode of **2** with GSK-3β by docking calculation.



Scheme 1. Reagent and conditions: (a) potassium monoethyl malonate, carbonyl diimidazole, $MgCl_2$, THF, 50 °C; (b) *N*-methylthiourea, DBU, EtOH, 80 °C; (c) $POCl_3$, DMF, dichloroethane, 60 °C.

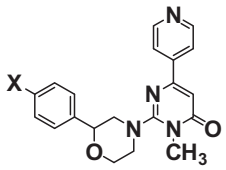
borohydride[11] yielded 2-phenylmorpholine **13**, and removal of the benzyl group by using 1-chloroethyl chloroformate[12] yielded 2-(*S*)-phenylmorpholine hydrochloride **14**. Desired compounds **15**–**31** were obtained by treatment of **6** with appropriate morpholines **14** in the presence of triethylamine at room temperature (Scheme 2). Phenylmorpholines containing a cyano group was prepared by substitution of the corresponding N-protected bromomorpholine by zinc cyanide catalyzed by tetrakis(triphenylphosphine)palladium or tris(dibenzylideneacetone)dipalladium(0) - 1,1′-bis(diphenylphosphino)ferrocene in *N*,*N*-dimethylformamide. 2-(*S*)-(2,6-Disubstituted-phenyl)morpholines were prepared by separation of the racemate by chiral HPLC because of poor enantioselectivity in an asymmetric reduction of 2,6-disubstituted acetophenones. (*R*)-**15** was synthesized using (*R*)-CBS instead of (*S*)-CBS.

Preliminary results of cyclization of the linker are shown in Table 1.[13] Racemic phenylmorpholine **2** showed a severalfold decrease in inhibitory activity with maintained in vitro metabolic stability and improved CYP450 inhibition profiles. We thought that the deletion of the carbonyl group would afford moderate microsomal clearance by increasing the electron density of the phenyl moiety, and a fluorine atom was introduced at the 4-position. Racemic **15** showed increased inhibitory activity and metabolic stability with maintained CYP450 inhibition. (*S*)-Enantiomer of **15** had threefold more potent inhibitory activity against GSK-3β than its (*R*)-enantiomer, which was consistent with the prediction of the docking study for **2**. From these results we thought that the transformation including deletion of a hydrogen bonding donor as



Scheme 2. Reagent and conditions: (a) (*S*)-CBS (20 mol %), BH_3·THF, −30 °C; (b) KOH, H_2O, Et_2O, r.t.; (c) benzylamine, 80 °C; (d) chloroacetyl chloride, NaOH, H_2O, CH_2Cl_2, r.t.; (e) KOH, 2-PrOH, r.t.; (f) $LiBH_4$, TMSCl, THF, r.t.; (g) 1-chloroethyl chloroformate, dichloroethane, r.t., then MeOH, reflux; (h) **6**, Et_3N, THF, r.t..

Table 1
Preliminary result of the effects of the linker transformation

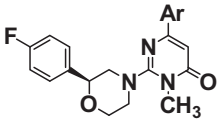


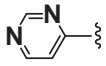
2 (X=H)
15 (X=F)

Compd	GSK-3β IC_{50}[a] (nM)	CL[b] (ml/min/mg)	CYP450 inhibition[c] (IC_{50}; μM)		
			1A2	2D6	3A4
1	8.9	0.172	5.70	>50.0	>50.0
(*RS*)-**2**	51	0.155	23.1	47.4	>50.0
(*RS*)-**15**	27	0.104	23.9	35.8	>50.0
(*S*)-**15**	22	n.t.	17.1	44.2	>50.0
(*R*)-**15**	77	n.t.	n.t.	n.t.	n.t.

[a] See Ref. 13 for inhibition assay conditions for human GSK-3β cell free enzyme.
[b] Rat microsome.
[c] Recombinant human CYP450 isoform inhibition against 1A2, 2D6 and 3A4.

Table 2
GSK-3β inhibition and recombinant human CYP450 inhibition



Compd	Ar		GSK-3β IC_{50}[a] (nM)	CYP450 inhibition[b] (IC_{50}; μM)		
		R^1		1A2	2D6	3A4
(*S*)-**15**		H	22	17.1	44.2	>50.0
16		3-F	7.4	5.5	22.1	>50.0
17		3-Cl	430	n.t.	n.t.	n.t.
18		3-CH_3	2466	n.t.	n.t.	n.t.
19		3-OCH_3	791	n.t.	n.t.	n.t.
20		2-Cl	903	n.t.	n.t.	n.t.
21			12	29.4	39.7	>50.0

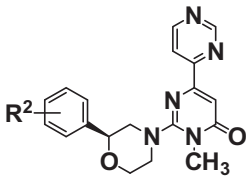
n.t.: not tested.
[a] See Ref. 13 for inhibition assay conditions for human GSK-3β cell free enzyme.
[b] Recombinant human CYP450 isoform inhibition against 1A2, 2D6 and 3A4.

well as a metabolically labile group and conformational restriction would afford desired compounds by further modifications. Further optimization of **15** was conducted using (*S*)-enantiomer.

Effects of the nitrogen atom of the pyridine moiety was summarized in Table 2. Reduced GSK-3β inhibitory activity of pyridin-2-yl and 3-yl derivatives (data not shown) suggested that the nitrogen atom at the 4-position of pyridine was essential, and introduction of a fluorine atom at the 3-position of pyridine (**16**) showed an increase in inhibitory activity together with increased CYP 1A2 inhibition. Other substituents at the 3-position, such as chlorine (**17**), methyl (**18**), methoxy (**19**) decreased the activity independently of their electronic features. 2-Chloropyridine derivative **20** showed a further decrease in in vitro activity compared to that of 3-chloro isomer **17**. These facts suggested that the steric bulkiness of the substituent on the pyridine moiety perturbed the inhibitory activity. Transformation of the pyridine moiety into pyrimidine increased the inhibitory activity against GSK-3β with improved in vitro pharmacokinetic profiles (**21**). Based on this compound, we further examined the effect of substituents on the phenyl group of the 2-phenylmorpholine moiety (Table 3). **22**, having an electron-withdrawing cyano group at the 4-position maintained in vitro activity with potent CYP 2D6 inhibition. Introduction of a methoxy group increased the inhibitory activity. 4-Methoxy derivative **23** and 3-methoxy derivative **24** showed a moderate increase of inhibitory activity, and more than 10-fold increase of activity was observed in 2-methoxy derivative **26**. Further introduction of a methoxy group at the ortho position gave 2,6-dimethoxy analogue **28**, which was the most potent compound, showing a nearly 50-fold increase of inhibitory activity. Effects of the introduction of substituents at the 2-position were also observed in the case of the fluorine atom containing compound, regardless of its electronic profile, which showed a several fold increase of GSK-3β inhibitory activity (**21** vs **25**). Although no effect was observed against impaired microsomal human intrinsic clearance of methoxy analogues by introducing an electron-withdrawing fluorine atom at the 4-position (**27**), further introduction of an electron-withdrawing substituent to the 2-fluorophenyl moiety resulted in **29** and **30** with increased in vitro activity and desirable in vitro pharmacokinetic profiles.

From these studies, **21**, **29** and **30** proved to have preferable in vitro GSK-3β inhibitory activity and in vitro PK profiles. Among them, **21** was selected for further examinations of pharmacological and pharmacokinetic profiles.

Table 3
GSK-3β inhibition and recombinant human CYP450 inhibition



Compd	R^2	GSK-3β; IC_{50}[a](nM)	$CL_{int,\ human}$[b] (mL/min/mg)	CYP inhibition[c] (IC_{50}; μM)		
				1A2	2D6	3A4
21	4-F	12	0.06	29.4	39.7	>50.0
22	4-CN	13	0.03	>50.0	4.5	n.t.
23	4-OCH_3	2.9	0.11	>50.0	6.9	n.t.
24	3-OCH_3	4.6	0.13	>50.0	26.6	>50.0
25	2-F	3.2	0.10	23.3	32.4	n.t.
26	2-OCH_3	0.87	0.19	15.7	16.0	n.t.
27	2-OCH_3, 4-F	0.47	0.18	16.5	18.0	n.t.
28	2,6-di-OCH_3	0.23	0.20	>50.0	>50.0	n.t.
29	2-F, 4-CN	6.5	0.03	>50.0	26.7	n.t.
30	2-F, 6-Cl	0.64	0.07	19.5	32.3	n.t.

n.t.: not tested.
[a] Conditions for human GSK-3β cell free enzyme inhibition assay, see Ref. 13.
[b] Hunan liver microsome assay.
[c] Recombinant human CYP450 isoform inhibition for 1A2, 2D6 and 3A4.

Table 4
Pharmacokinetic properties of **21**

CL_{tot}[a] (L/h/kg)	$T_{1/2}$[b] (h)	V_{dss}[a] (L/kg)	C_{max}[b] (ng/L)	$AUC_{0-\infty}$[b] (ng·h/ml)	$Kp_{0-\infty}$[c]	F (%)[d]	Caco-2 ($\times 10^{-7}$ cm/s)	P_{gp} (MDR_1/LLC)
2.9	1.7	2.4	221	1305	3.1	37	583	1.0

[a] SD rats were given intravenously at 1.0 mg/kg (n = 5, 6 W, female, 0.5% HPMC suspension).
[b] SD rats were given orally at 10 mg/kg (n = 5, 6W, female, 0.5% HPMC suspension).
[c] K_p was measured at 1.0 mg/kg oral administration in ICR mice (n = 4, 7 W, male, 0.5% Tween suspension).
[d] Bioavailability in rats.

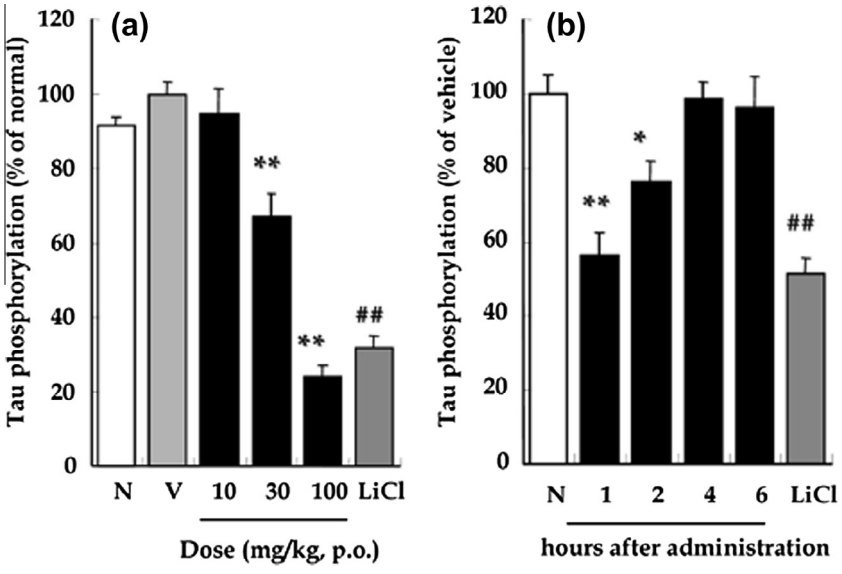


Figure 3. (a) Dose–response of **21** for decrease of phosphorylated tau protein after 2 h. N: male normal mice, V: vehicle-treated mice, LiCl: LiCl (20 mmol/kg, i.p. 8 h). $^{**}p$ <0.01 versus vehicle, Dunnett's test $^{\#\#}p$ <0.01 versus vehicle, t-test, n = 6. (b) Time course for **21** (30 mg/kg, p.o.)-evoked decrease phosphorylated tau protein after p.o. administration in normal mice. N: male normal mice. LiCl: LiCl (20 mmol/kg, i.p. 8 h) $^{*}p$ <0.05, $^{**}p$ <0.01 versus normal, Dunnett's test $^{\#\#}p$ <0.01 versus normal, t-test, n = 6.

Rats and mice PK experiments of **21** were performed and pharmacokinetic parameters are summarized in Table 4. Caco-2 permeability was high and no effects were observed on P-glycoprotein (*P*-gp) efflux. The systemic clearance of **21** after iv dosing averaged 2.9 L/h/kg with volume of distribution of 2.4 L/kg and the AUC after p.o. dosing of 1305 ng h/mL in rats. **21** demonstrated moderate oral bioavailability (37%) with reasonable half-lives in rats, and excellent blood-brain-barrier penetration (brain/plasma 3.1) in mice for CNS drugs.

A kinase selectivity study revealed that **21** had more than 1000-fold selectivity over 50 kinases except CK_1 (IC_{50} = 1.5 μM). Results of the inhibitory effects on in vivo Ser396 tau phosphorylation in mice[14] are shown in Figure 3. A dose response study showed **21** significantly decreased tau phosphorylation two hours after administration at a dose of 30 mg/kg or higher doses and a time course study showed **21** effectively inhibited in vivo tau phosphorylation for two hours after oral administration at a dose of 30 mg/kg.

In conclusion, transformation of the 2-oxoethylamine moiety into a morpholine moiety provided potent inhibitors of GSK-3β with good in vitro pharmacokinetic profile such as **21**, **29** and **30**. Among them, **21** was orally active, inhibited in vivo tau phosphorylation in mice and showed moderate bioavailability in rats with high kinase selectivity. A detailed profile of **21** will be published elsewhere.

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13. Human GSK-3β cell free enzyme inhibition assay; 7.5 μM of prephosphorylated GS1 peptide and 10 μM [γ-^{32}P]ATP were incubated in 50 mM *N*-(2-hydroxyethyl)piperazine-*N*'-(2-ethanesulfonic acid) (HEPES)-sodium hydroxide (pH 7.2), 1 mM dithiothreitol (DTT), 1 mM magnesium chloride, 0.02% Tween-20 buffer for 1 h at room temperature in the presence of human recombinant GSK-3β. The reaction was stopped with 0.1 volume of 21% perchloric acid. An aliquot of the reaction mixture was then transferred onto Whatman P81 cation exchange filters and the filters were washed 3 times with 75 mM phosphoric acid solution, once in water and once in acetone. Incorporated ^{32}P radioactivity was determined by liquid scintillation spectrometry. The prephosphorylated GS1 peptide had the following sequence; NH_2-YRRAAVPPSPSLSRHSSPHQS(P)EDEE-COOH. IC_{50} values are the mean of at least two experiments.
14. ICR mice (male, 5–6 w) were orally treated with **21** (10–100 mg/kg, 0.5% Tween80 suspension). Each group was then sacrificed at a given time, from 1 up to 6 h after drug administration. Prefrontal cortexes were sonicated with homogenization buffer (62.5 mM Tris–HCl pH 6.8, 2.3% sodium dodecyl sulfate (SDS), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethylene glycol tetraacetic acid (EGTA), 1 mM DTT, Protease inhibitor cocktail and Phosphatase inhibitor cocktail), boiled for 5 min and centrifuged at 15000*g* for 15 min at 4 °C. Protein content was measured and samples were anti-pS396-tau antibody and bands were quantified by using a fluorescent scanner and ImageQuant software. Data were analyzed mixed with Laemmli buffer, then applied on Western blot. Phosphorylated tau protein was detected by Dunnett's test and *t*-test.