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Design, synthesis and biological activity evaluation of novel 4-((1-cycloprop-yl-3-(tetrahydro-2*H*-pyran-4-yl)-1*H*-pyrazol-4-yl) oxy) pyridine-2-yl) amino derivatives as potent transforming growth factor-β (TGF-β) type I receptor inhibitors

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Abstract:

TGF-β type I receptor (also known as activin-like kinase 5 or ALK5) plays a critical role in the progression of fibrotic diseases and tumor invasiveness and metastasis, as well. The development of small inhibitors targeting ALK5 has been validated as a potential therapeutic strategy for fibrotic diseases and cancer. Here, we

developed various 4-((1-cyclopropyl-3-(tetrahydro-2*H*-pyran-4-yl)-1*H*-pyrazol-4-yl) oxy) pyridine-2-yl) amino derivatives as ALK5 inhibitors. The optimization led to identification of potent and selective ALK5 inhibitors **12r**. The compound **12r** exhibited strong inhibitory activity both *in vitro* and *in vivo*, and pharmacokinetics study showed an oral bioavailability of 57.6%. Thus, compound **12r** may provide as new therapeutic option as ALK5 TGF-βR1 inhibitor.

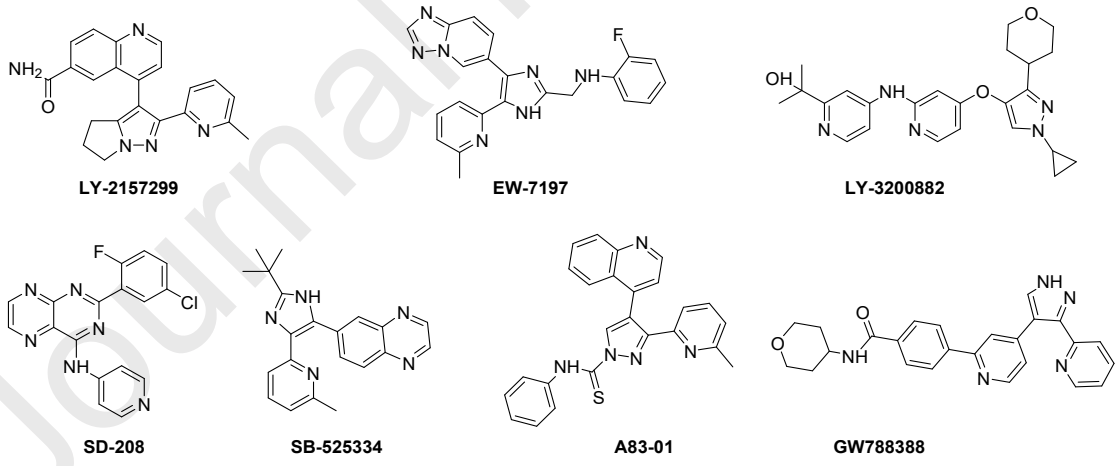
Keywords: TGF-β type I receptor; ALK5 inhibitors; inhibitory activity

TGF-β as a multifunctional growth factor, plays important role in regulating a wide range of cellular processes, including cell growth, differentiation, migration, adhesion, apoptosis, epithelial-mesenchymal transition (EMT), extracellular matrix (ECM) remodeling [1, 2]. TGF-β belongs to the TGF-β superfamily [3], and the TGF-β family members initiate intracellular downstream signaling by binding with cellular surface serine/threonine kinase receptors. It is categorized into three subfamilies: TGF-βRI, which is also known as activin receptor-like kinase 5 (ALK5), TGF-βRII and TGF-βRIII [4]. The TGF-βRIII is an auxiliary co-receptor and not directly involved in signal transduction. Its main function is to increase the binding of TGF-β and TGF-βR on the cell surface and deliver it to type I and type II receptors. The TGF-β/Smad signaling pathway is the main pathway for TGF-β to exert biological effects. The signal transduction of TGF-β pathway is rely on several signaling factors including microenvironment, microRNAs (miRNAs), TGF-β family ligands, TGF-β serine/threonine kinase receptors (ALK5 and TGFβRII), phosphorylation and dephosphorylation kinases, ubiquitin ligase and deubiquitinase (Smads) [6]. TGF-β signals through the binding of the TGF-β ligand to TGF-βRII receptor, which then phosphorylates the GS domain (a highly conserved glycine and serine residue domain) of ALK5. The activated ALK5 can trigger several signaling pathways by phosphorylating downstream signaling molecules such as the Smad2 and Smad3 [7], which form Smad2/3/4 heteromeric complex with another cytoplasmic transducer, Smad4. The complex is translocated to nucleus and binds to a region called Smad-binding element of TGF-β target genes through specific DNA-binding cofactors to

regulate the expression of TGFβ-downstream target genes, thereby regulating cell proliferation, differentiation, migration, apoptosis, and extracellular matrix production [8]. The overactivation of TGF-β/Smad signaling has been involved in various cancer such as breast cancer [9] and Hepatocellular Carcinoma (HCC) [10]. Therefore, the TGF-β signaling pathway seems to be a potential therapeutic target, especially the direct inhibition of ALK5 could be a good approach for the treatment of cancer.

Recently, a variety of small molecule ALK5 inhibitors has been developed such as **LY-2157299** [11], **EW-7197** [12], **LY-3200882** [13], **SD-208** [14], **SB-525334** [15], **A83-01** [16] and **GW788388** [17] (Figure 1) , which are able to specifically inhibit the TGF-β/Smad signaling pathway by occupying the bind site of TGF-βRI to intervene the binding of TGF-β and TGF-βRI. Among these inhibitors, **LY-2157299**, **EW-7197** and **LY-3200882** are currently under clinical evaluation (**Figure 1**). **LY-2157299** is a potent, selective TGF-βRI inhibitor, and granted the orphan drug qualification of HCC in 2013. **LY-3200882**, as a follow-up inhibitor of TGF-βRI, has improved inhibitory activity and selectivity than **LY-2157299**, and it is initiated Phase 1 clinical investigation.

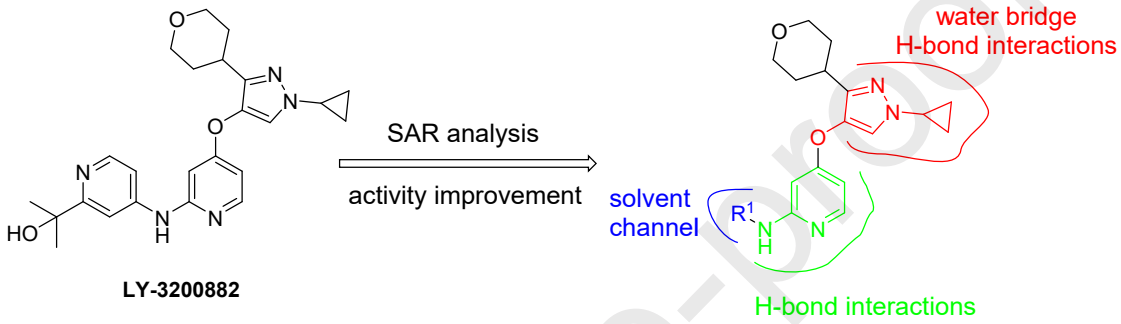
Figure 1 Example of ALK5 inhibitors under development

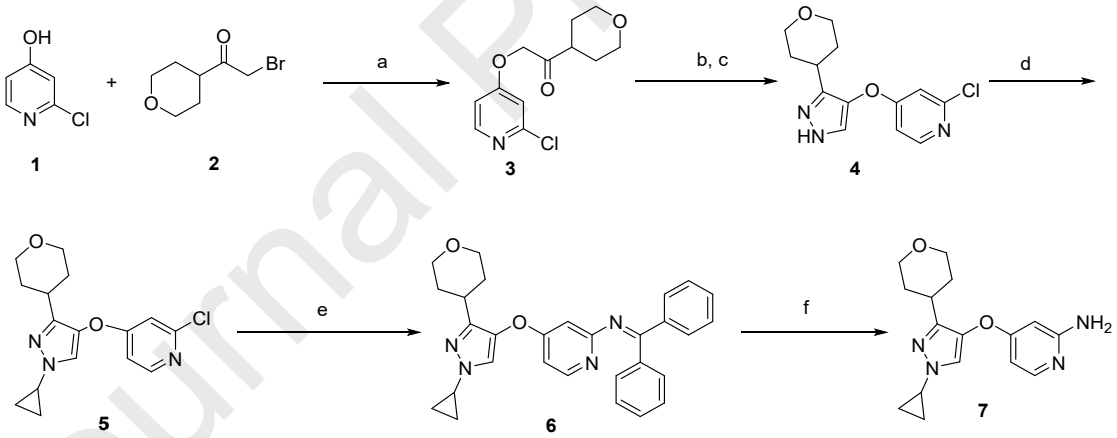


In order to rationalize the design idea (**Figure 2**), the binding mode of **LY-3200882** with TGF-βRI was studied. It was found that *N*-cyclopropyl-1H-pyrazole showed a strong hydrogen bond interaction through a water bridge, which was critical to maintain the binding efficacy. In addition, a hydrogen bond formed by hydroxyl moiety with Asp-290. The structural modification in this study was to replace the

hydroxyl substituted pyridine of LY-3200882 with various substituted aryl, aromatic and aliphatic heterocyclic derivatives, and the structure activity relationships of these compounds was discussed, as well. We evaluated the novel TGF-βRI inhibitors for their *in vitro* and *in vivo* activity, pharmacokinetic profile and preliminary toxicity. We provided the novel TGF-βRI inhibitor for further investigation of study and a promising clinical candidate.

Figure 2 Design of ALK5 inhibitors

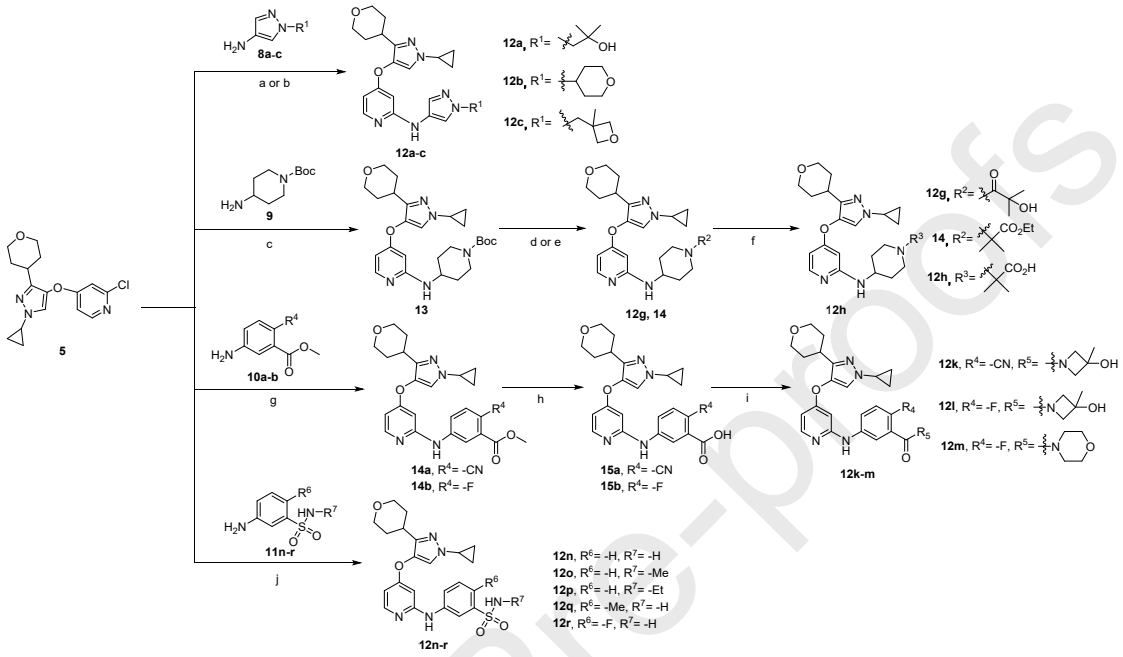




Scheme 1 Synthesis of intermediates **5** and **7.** Reagents and conditions: (a) K_2CO_3, DMF, 90 °C; (b) *N*, *N*-dimethylformamide dimethyl acetal, 100 °C; (c) AcOH, hydrazine hydrate, r.t.; (d) 2,2'-bipyridine, $Cu(OAc)_2$, Na_2CO_3, cyclopropylboronic acid, 1,2-dichloroethane, 80 °C; (e) diphenylmethanimine, $Pd(OAc)_2$, BINAP, Cs_2CO_3, toluene, 95 °C; (f) HCl/1,4-dioxane, r.t..

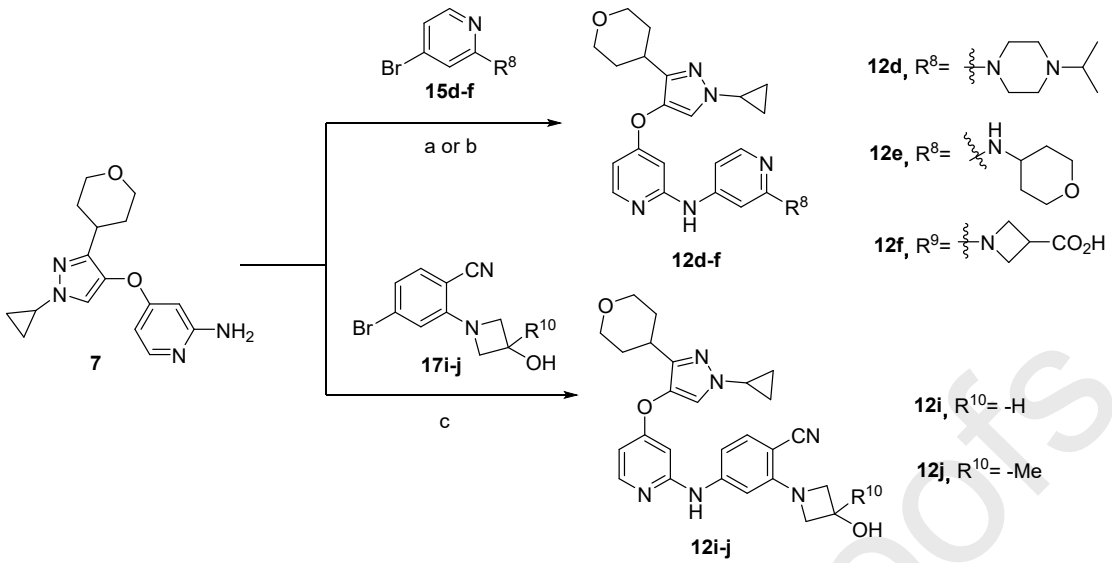
The intermediates **5** and **7** were synthesized as shown in **scheme 1**. The substitution reaction of pyridine **1** with brominated ketone **2** gave compound **3**, which

was cyclized to obtain pyrazole derivative **4**. Compound **4** was reacted with cyclopropylboronic acid catalyzed by $Cu(OAc)_2$ to afford intermediate **5**, which was further transformed to **7** via the palladium catalyzed coupling, followed by the acidic deprotection.



Scheme 2 Preparation of **12a-c**, **12g-h** and **12k-r**. Reagents and conditions: (a) $Pd(OAc)_2$, XantPhos, Cs_2CO_3, 1,4-dioxane, 100 °C for **12a**; (b) $Pd_2(dba)_3$, XantPhos, NaOPh, 1, 4-dioxane, 100 °C for **12b** and **12c**; (c) $Pd_2(dba)_3$, XantPhos, t-BuONa, 1, 4-dioxane, 100 °C; (d) HCl, 1,4-dioxane, r.t. then HATU, DIPEA, DCM, r.t. for **12g**; (e) HCl, 1,4-dioxane, r.t. then K_2CO_3, MeCN, reflux for **14**; (f) NaOH, THF, H_2O, r.t.; (g) $Pd_2(dba)_3$, XantPhos, NaOPh, 1, 4-dioxane, 100 °C; (h) MeOH, H_2O, LiOH, r.t.; (i) HATU, DIPEA, DCM, r.t.; (j) $Pd_2(dba)_3$, XantPhos, NaOPh, 1, 4-dioxane, 100 °C.

Compound **5** was reacted with various amines via the palladium catalyzed coupling reaction to give corresponding target compounds **12a-c** and **12n-r**, as well as intermediates **13** and **14a-b**. The Boc-group in compound **13** was removed under acidic condition and then reacted with nucleophiles to obtain **12g** and **14**, and the **14** was further hydrolyzed at $LiOH/H_2O/MeOH$ system to **12h**. The cyanide and fluorine derivatives **14a** and **14b** were processed with hydrolysis and coupling with corresponding amines to obtain amide **12k-m**.



Scheme 3 Preparation of **12d-f** and **12i-j**. Reagents and conditions: (a) $Pd_2(dba)_3$, XantPhos, Cs_2CO_3, 1, 4-dioxane, 100 °C for **12d** and **12e**; (b) $Pd(OAc)_2$, Cs_2CO_3, XantPhos, 1, 4-dioxane, 100 °C for **12f**; (c) $Pd_2(dba)_3$, XantPhos, Cs_2CO_3, 1, 4-dioxane, 100 °C;

Compounds **15d-f** were coupled with compound **7** via Buchwald-Hartwig reaction to afford **12d-e** and **16**, and ester **16** was hydrolyzed under the basic condition to obtain acid **12f**. Compound **12i** and **12j** were synthesized from **7** with **17i-j** via Buchwald-Hartwig reaction, as well.

As shown in table 1, pyrazole derivative with propanol substitution **12a** gave the ALK5 inhibitory activity with IC_{50} = 77 nM, while compunds **12b** and **12c** substitution resulted in a great increase of inhibitory activity. The pyridine derivatives **12d** and **12e** displayed more potent ALK5 inhibitory activity which gave IC_{50} 13 and 17 nM, respectively. However, the carboxylic substitution (**Table 1**, **12f**) lead to significant decreased potency in the kinase assay. In addition, replacing the pyrazole and pyridine ring with piperidine (**Table 1**, **12g** and **12h**) resulted in the complete loss of the activity. Next, a series of phenyl substituted compounds were evaluated. Compound **12i** and **12j** were able to maintain the high efficacy with the value of IC_{50} 13 and 14 nM, while their amide analogues **12k-12m** displayed the reduction in potency. Phenylsulfonamide

derivatives exhibited highly inhibitory activity of ALK5. Phenyl sulfonamide **12n** gave the value of IC_{50} 9.1 nM, and the substitution on either nitrogen or phenyl ring possessed high potency as well (Table 1, **12o** to **12r**). The inhibitory activities of those compounds were further verified in the cell-based luciferase inhibition assay using NIH3T3 cell. In general, the ALK5 inhibitory activity of the compounds were well represented in cellular assay with only several exceptions. Compound **12d** possessed good inhibitory activity in both ALK5 enzyme and NIH3T3 cell-based assays. The methyl substituted phenlysulfonamides **12o** and **12q** exhibited 2-fold increase in inhibitory activity over **12n**, and fluorine-substituted compound **12r** showed the improved activity as well.

Table 1. Inhibitory activity of compounds **12** *in vitro*

Entry	Compound	IC_{50} (nM)[a]	
		ALK5	NIH3T3 cells Luciferase
1	**12a**	77(22)	215.2 (89.3)
2	**12b**	21(35)	128.3 (80.0)
3	**12c**	32(21)	338.0 (135.9)
4	**12d**	13(35)	54.9 (135.9)
5	**12e**	17(21)	133.4 (135.9)
6	**12f**	234(26)	N.D
7	**12g**	>10000	N.D
8	**12h**	>10000	N.D
9	**12i**	13(16)	N.D
10	**12j**	14(50)	85.9 (76.3)
11	**12k**	211(41)	N.D
12	**12l**	122(46)	N.D
13	**12m**	114(36)	N.D
14	**12n**	9.1(46)	167.7 (100.6)
15	**12o**	15(37)	92.7 (126.8)
16	**12p**	12(37)	N.D
17	**12q**	16(38)	69.5 (126.8)
18	**12r**	28(45)	126.4 (118.0)

[a] Data in parentheses were IC_{50} of the **LY-3200882** tested in parallel

N.D: Not detected

To investigate the pharmacokinetic profiles, the sulfonamide compounds **12n**, **12o**, **12q** and **12r** were administered to BALB/C mice (three male and three female mice for each compound) both oral gavage and intravenous injection in dosing of 10 mg/kg and 1 mg/kg, respectively. Compound **12n** was favorable with oral dosing and had a half-life of about 1.4 hours *in vivo*. The maximum concentration was 1508 ng/ml with 68.9% of bioavailability. The compound **12o** which showed good inhibitory activity *in vitro*, gave only 359 ng/ml of the C_{max} and short half-life ($T_{1/2}$ = 0.3 h). Although compound **12q** had similar value of C_{max} and bioavailability compared with **12n**, its relatively short $T_{1/2}$ was inappropriate for the further investigation of inhibitory activity *in vivo*. Compound **12r** had a superior PK profile, which exhibited over 3-fold improvement in both C_{max} and AUC with good bioavailability (F% = 74.3), and the half-life of the **12r** in plasma was 1.2 h.

Table 2. The results of PK for **12n**,**12o**, **12q** and **12r**.

		12n	**12o**	**12q**	**12r**
PO (10 mg/kg)	$T_{1/2}$ (h)	1.4	0.3	0.5	1.2
	C_{max} (ng/ml)	1508	359	1127	5134
	AUC (0-∞) (ng × h /ml)	2189	229	1244	7703
	F (%)	68.9	14.3	51.3	74.3
IV (1 mg/kg)	$T_{1/2}$ (h)	0.5	0.2	0.2	0.4
	C_0 (ng/ml)	549	562	797	3355
	AUC (0-∞) (ng × h /ml)	338	143	232	1061
	CL (L/h/kg)	3.1	7.1	4.4	1.0

Considering the good inhibitory activity in vitro and pharmacokinetic profile, compound **12r** was evaluated *in vivo* activity with BALB/C mice bearing xenograft tumors derived from H22 hepatocarcinoma cell. Compound **12r** was administrated at 30 mg/kg and 60 mg/kg twice daily by oral gavage, and the **LY-3200882** was tested at 60 mg/kg and 120 mg/kg twice daily as well. Treatment with both **12r** and **LY-3200882**

significantly reduced the tumor growth in comparison with the control group (**Figure 3**), and no significant loss of weight was observed (**Figure 4**). The compound **12r** with 30 mg/kg and 60 mg/kg cohorts gave the 30% and 27% T/C value, while **LY-3200882** gave 36% T/C value with dosing 120 mg/kg. The PK-PD analysis in treatment day 1 and day 19 were performed (**Table 3**). Dose-dependent exposal of the compound **12r** was observed, which gave 8907 h × ng/ml and 21045 h × ng/ml in the first treatment. However, after the 19 days of administration, the concentration of **12r** was decreased to 5933 and 8870 h × ng/ml.

Figure 3 Tumor volume change of the mice in **LY-3200882** and **12r** treatment of H22 s.c xenografts

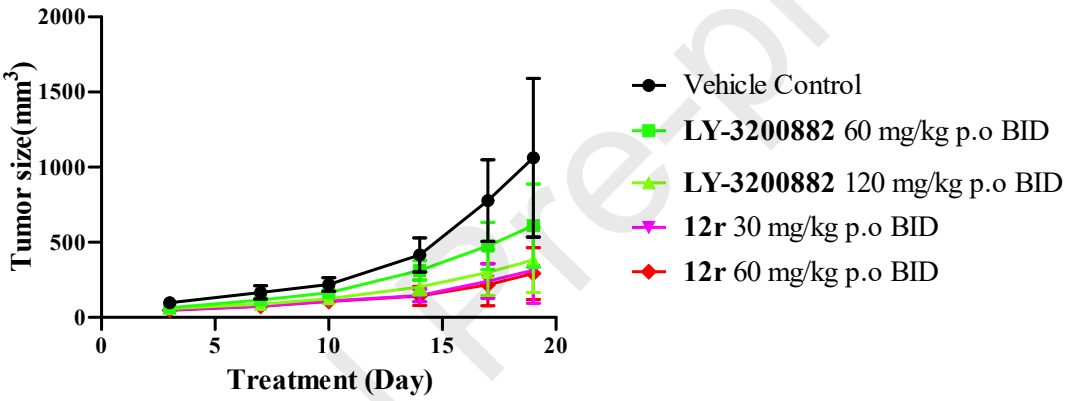


Figure 4 Mice body weight change in **LY-3200882** and **12r** treatment of H22 s.c xenografts

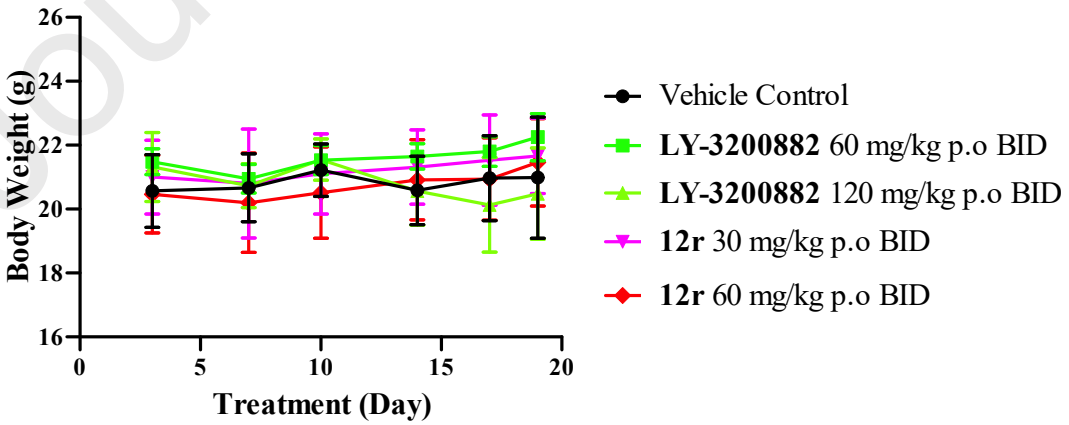


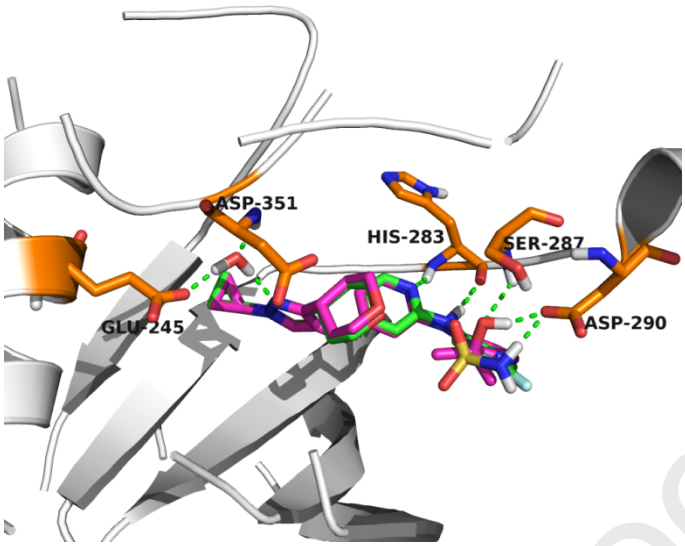
Table 3 PK-PD analysis of the **LY-3200882** and **12r**

Compound	Dosing (mg/kg)	AUC（h × ng/ml）		T/C (%)
		DAY1	DAY19	
LY-3200882	60	9949	1658	57
	120	20232	4864	36
12r	30	8907	5933	30
	60	21045	8870	27

Compound **12r** showed no inhibitory activity to hERG channel (IC_{50} > 30μM) and the mini-Ames experiment indicated that no potential mutagenicity was observed as well. In order to confirm the selectivity of the **12r** to ALK5 over other kinases, a broad spectrum of kinases was tested against compound **12r**. The compound was treated in single dose duplicate model at a concentration 10 μM. Among the 374 tested kinases, ALK1, 2 and 4 which were the isoforms of ALK5 remained 2-29% enzyme activity after treatment with **12r**, none of the other kinases showed obvious inhibition with less than 30% enzyme activity remaining (see Supplementary Material for details).

In order to rationalize the observed activities of these compounds, binding mode of compound **12r** and **LY-3200882** in the binding pocket of TGF-β RI (PDB code: 2WOU) were studied (**Figure 5**). As displayed by the docking model, the pyrazole of **12r** formed strong hydrogen bond with Glu-245 and Asp-351 through a water bridge, which was an essential interaction to maintain inhibitory activity. The replacement of the tertiary alcohol in **LY-3200882** with sulfonamide in **12r** could keep the key hydrogen bonding interaction through bind with Asp-290.

Figure 5 Docking mode of **LY-3200882** (pink) and compound **12r** (green).



We have synthesized a series of 4-((1-cyclopropyl-3-(tetrahydro-2H-pyran-4-yl)-1H-pyrazol-4-yl) oxy) pyridine-2-yl) amino derivatives, which were evaluated as TGF-βR1 inhibitors. The structure-activity relationship in this series of compounds were discussed. Optimization of the structure led to the eventual identification of a potent and selective TGF-β R1 inhibitor **12r**. The introduction of phenlysulfonamides not only improved the inhibitory activity but also increased the oral bioavailability. Compound **12r** exhibited good enzyme inhibitory activity (IC_{50} = 28 nM), and luciferase inhibition assay of **12r** in NIH3T3 cells demonstrated that **12r** had high inhibition activity which IC_{50} was 126.4 nM, as well. Pharmacokinetics study with **12r** gave an oral bioavailability of 74.3% with high AUC of 7703 h × ng/mL and C_{max} of 5134 ng/mL. The *in vivo* study of **12r** with H22 xenograft model showed 27% of T/C in dosing of 60mg/kg B.I.D. Based on these results, compound **12r** was currently undergoing with toxicology study as a promising candidate for clinical investigation.

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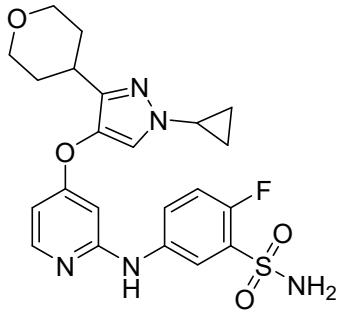
Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:



12r

ALK5 IC_{50}=28 nM
T/C=30%(30 mg/kg), 27%(60 mg/kg)
F=74.3%
hERG IC_{50} > 30 uM