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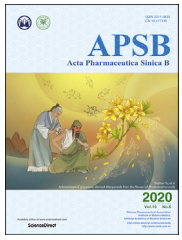

SHORT COMMUNICATION

Design and synthesis of selective sphingosine-1-phosphate receptor 1 agonists with increased phosphorylation rates

Qiong Xiao[a], Minwan Hu[b], Si Chen[a], Yifan Tang[c], Zeyu Shi[b], Jing Jin[d], Jinping Hu[b,*], Ping Xie[a,*], Dali Yin[a,*]

[a]*Department of Medicinal Chemistry, State Key Laboratory of Bioactive Substance and Function of Natural Medicines, Beijing Key Laboratory of Active Substances Discovery and Druggability Evaluation, Institute of Materia Medica, Peking Union Medical College, Chinese Academy of Medical Sciences, Beijing 100050, China*
[b]*Departments of Drug Metabolism, Beijing Key Laboratory of Non-Clinical Drug Metabolism and PK/PD Study, Institute of Materia Medica, Peking Union Medical College, Chinese Academy of Medical Sciences, Beijing 100050, China*
[c]*Beijing Union Pharmaceutical Factory, Beijing 102600, China*
[d]*Department of Pharmacology, State Key Laboratory of Bioactive Substance and Function of Natural Medicines, Institute of Materia Medica, Peking Union Medical College, Chinese Academy of Medical Sciences, Beijing 100050, China*

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$S1P_1$ agonist;
Prodrug;
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Abstract FTY720 and IMMH002, prodrugs for sphingosine-1-phosphate receptor 1 ($S1P_1$) agonists, show inadequate and inconsistent levels of phosphorylation in humans compared to that in rats. In this study, FTY720 or IMMH002 analogues (**21**−**24**) were designed and synthesized with modified head pieces to improve the biotransformation of the prodrugs to the active phosphorylated forms. Target compounds were synthesized *via* a convergent route using the key and optically pure building block **9**, which was first synthesized *via* asymmetrically catalyzed amination. The phosphorylation rates of these analogues in rat or human blood were compared. The new methyl-substituted analogue compound **21** showed higher phosphorylation rates in both rats and humans than the parent compound, whereas compound **23** showed improvements in rats, but not in humans. In pharmacokinetics studies of rats, compounds **21** and **23** both had higher levels of phosphorylation than FTY720 and IMMH002. Thus, our study not only yielded new compounds with therapeutic potential, but also showed species differences between rats

*Corresponding authors. Tel./fax: +86 10 63037952.
E-mail addresses: hujp@imm.ac.cn (Jinping Hu), xp@imm.ac.cn (Ping Xie), yindali@imm.ac.cn (Dali Yin).

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and humans in response to the structural modifications, which might be useful for predicting the biotransformation behavior and efficacy of this class of prodrugs in the clinic.

1. Introduction

FTY720 (fingolimod) belongs to a new class of orally active immune-modulating agents for multiple sclerosis[1] and is converted to the active form, its monophosphate ester (*S*)-FTY720-P, by sphingosine kinase 2 (SPHK2) *in vivo*[2]. The phosphorylated compound is a potent nonselective agonist for sphingosine-1-phosphate receptors 1 and 3−5 ($S1P_{1,3-5}$)[3]. It activates the $S1P_1$ receptor and sequesters lymphocytes in lymph nodes and secondary lymphoid organs, restricting autoimmune reactivity[4−6]. However, a number of adverse side effects, such as bradycardia, liver injury, and cardiovascular effects, are attributed to its broad agonist activities[7−9]. Therefore, potential drug candidates with high $S1P_1$ selectivity are required.

One major challenge in the prodrug strategy in S1P activation is the *in vivo* transformation of the prodrug to the active phosphate because this process requires either sphingosine kinase 1 (SPHK1) or sphingosine kinase 2 (SPHK2), whereas the phosphate can be hydrolyzed back to its original form by sphingosine-1-phosphate phosphatase[10,11]. The amount of phosphorylated prodrug reaches equilibrium under the simultaneous action of various enzymes. However, the different kinase activities in different species led to the phosphorylation of FTY720 in human blood being notably lower than in mouse blood[3]. This will undoubtedly increase the difficulty of activity evaluation in preclinical research, and the risk of the clinical curative effect and safety.

In our previous work, we had conducted a series of research to explore efficacious and safer $S1P_1$ agonists that led us to discovery one promising clinical candidate IMMH002[12−15]. This compound retained the polar head group of FTY720 and inserted aromatic substituent in order to increase structural rigidity, thus enhancing the selectivity to $S1P_1$ receptor, which is expected to have reduced clinical side effects compared with FTY720 (Fig. 1). In rats, IMMH002 showed superior safety and activity to FTY720 on a psoriasis model and a rheumatoid arthritis model, which progressed to phase I clinical trial.

During pharmacokinetic studies of IMMH002, we found that the phosphorylation level of IMMH002 was lower in human which may restrict its clinical efficacy. From the structure−activity relationship study on hundreds of compounds, we identified the polar group is critical for activity as the key region recognized and phosphorylated by SPHK2 while a rigid aromatic scaffold is important for both activity and selectivity. According to previous report[16], we speculate that the polar head is the key region affecting the phosphorylation rates. To design a better molecule with an improved phosphate profile in humans, we performed further structure modification focusing on the transformation of prodrugs to the corresponding phosphates. In order to keep the activity and selectivity of IMMH002 as much as possible, its rigid aromatic scaffold was retained. In this work, a series of chiral head-piece-modified analogues of FTY720 and IMMH002 were designed and synthesized. The target products had a chiral 2-methyl-2-aminoethanol or chiral 2-aminoethanol structure (Fig. 2).

Phosphorylation mainly occurs in the blood[17]. To simulate the human enzymatic environment and reflect the effects of cooperation among various enzymes in the *in vitro* experiments, the compounds were incubated with rat blood and human blood, and the phosphorylation rate was determined. In rat *in vivo* experiments, the concentrations of the prodrug and phosphorylated drug, as well as the reduction of the lymphocyte ratio, were detected. With comprehensive analysis of the correlation between *in vivo* and *in vitro* data, the phosphorylated drug conversion rate and activity can be predicted more accurately in humans.

2. Results and discussion

2.1. Retrosynthesis of desired compounds

We designed a convergent synthesis of target compounds (Scheme 1) using the key chiral phosphonium salt **9** which is obtained *via* asymmetrically catalyzed amination of α-methyl-α-protected hydroxymethyl aldehyde, a method developed by us. The method is economical, safe, and convenient.

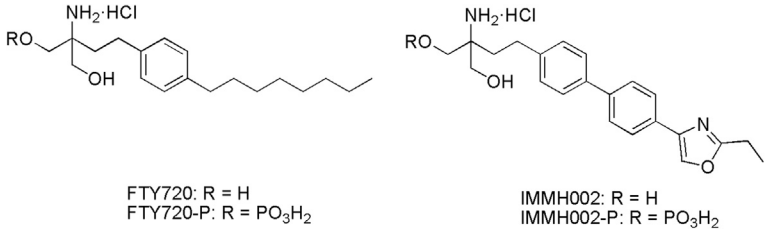


Figure 1 Structures of FTY720 and IMMH002.

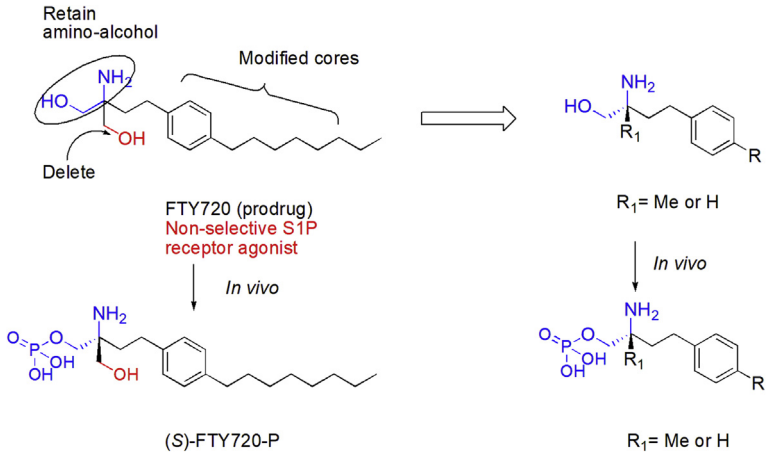


Figure 2 Design of $S1P_1$ modulators.

2.2. *Synthesis*

Initially, 3-(benzyloxy)-2-methylpropanal and dibenzyl azodicarboxylate were chosen as model substrate to determine to optimal reaction conditions. When L-proline (30%, mol/mol) was used as the catalyst[18], poor enantioselectivities (32% enantiomeric excess (ee)) were obtained. To improve enantioselectivity, a number of catalysts and solvents were screened and the result turned out that 3-(1-naphthyl)-L-alanine (**1**) as a catalyst (15%, mol/mol) in THF providing the aldehyde in 81% yield with 69% ee[19].

We investigated α-methyl-α-protected hydroxymethyl aldehydes with different protective groups and various azodicarboxylates. It is observed that 4-CF_3 benzyl-substituted aldehyde **2** and di-*p*-chlorobenzyl azodicarboxylate **3** provided desired product **4** in excellent yield (90%) and good enantioselectivity (up to 74% ee).

Upon recrystallization from 90% ethanol, aldehyde **4** was obtained in 97% ee (65% yield), and was subsequently converted to oxazolidinone **5** in 98% ee. The absolute configuration of **5** was determined to be (*R*) based on the circular dichroism spectrum. The benzyloxycarbonyl group was removed by hydrogenation using 10% Pd/C in methanol/acetic acid under ambient pressure. Cleavage of the hydrazine moiety by $NaNO_2$ gave alcohol **7**. **7** was treated with *p*-TsCl in pyridine, and the resulting tosylate was converted to iodide **8** with NaI in acetone under reflux. Reacting 8 with triphenylphosphine in DMF provided desired phosphonium salt **9** in moderate yield as a stable white solid[20] (Scheme 2). The synthesis of phosphonium salt **10** was performed by Sibi's method[21].

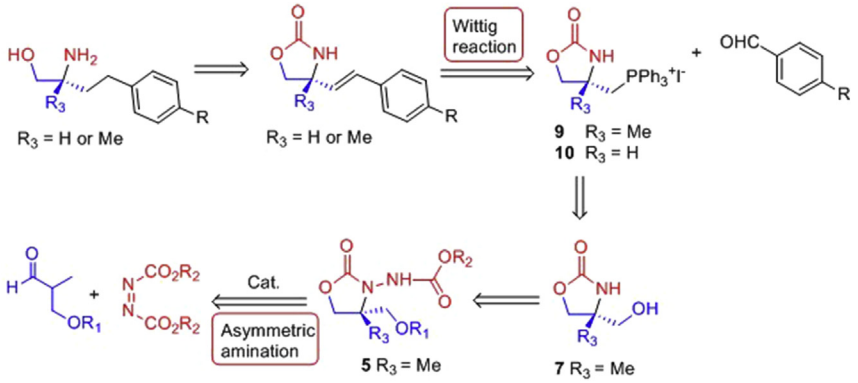
The Wittig reaction using phosphonium salt **9** or **10** with commercially available 4-octylbenzaldehyde or aldehyde **12** afforded corresponding olefin products **13**−**16** in good yields (Scheme 3). Aldehyde **12** was synthesized in good yields from 4-bromobenzaldehyde and dinary pinacol borate ester **11** *via* a Suzuki reaction with a Pd-dimer (dibromobis(tri-*tert*-butylphosphine)dipalladium) catalyst. Compounds **13**−**16** were reduced with 10% Pd-C in MeOH under a hydrogen atmosphere to obtain compounds **17**−**20** in good yields. Hydrolysis of the oxazolidinone moiety and acidification with 1 mol/L HCl in Et_2O gave chiral target products **21**−**24.**

2.3. *Phosphorylation level evaluation*

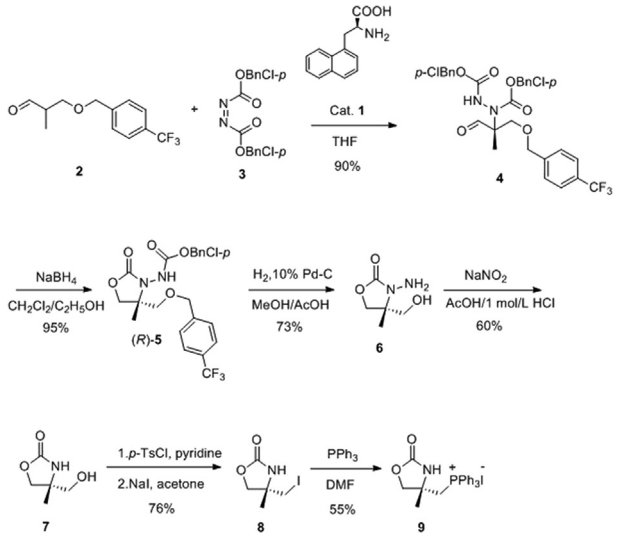
The phosphorylation levels of FTY720, IMMH002, and analogues **21**−**24** were evaluated *in vitro* and *in vivo*. Animal procedures were approved by the Institutional Animal Care and Use Committee at Institute of Materia Medica, CAMS & PUMC (Beijing, China). In whole blood, which has high SPHK2 activity[16], the phosphorylation rates of these compounds were significantly higher in rat blood than in human blood based on the Michaelis−Menten kinetics profiles. Based on the V_{max} and CL_{int} values, compound **21** showed improved phosphorylation rates in both rats and humans, whereas compound **23** showed improvements in rats, but not in humans (Table 1 and Fig. 3). These results exhibited different effects of the structural modifications on phosphorylation of the drugs by rat and human kinases. So, future studies are needed to identify the specific kinases involved in rats and humans and show why they are differentially affected by the structural changes, which may yield insights to how new modifications can be made to improve phosphorylation rates for IMMH002 derivatives and gain basic understanding of the structural basis for the species-selective effects of the chemical modifications.

The above kinetic results *in vitro* are consistent with those of the *in vivo* pharmacokinetic study on rats. The phosphorylation level at 6 h after dosing for FTY720 (80%), **21** (95%), **22** (69%), IMMH002 (69%), **23** (94%), and **24** (89%) indicated that methyl-substituted analogues **21** and **23** had a higher proportion of phosphorylation than FTY720 and IMMH002 (Fig. 4). These results are consistent with our observations in rat blood.

A fundamental consideration is that all of the tested compounds have different activities for the phosphorylation in rats and humans. The goal of this study is to develop new compounds that have higher phosphorylation rates in human blood than the parent compounds, particularly for the higher selective compound IMMH002. From the present results, even though compound **21** is li-



Scheme 1 Retrosynthesis of key intermediates of the S1P$_1$ modulators.



Scheme 2 Synthesis of chiral phosphonium salt **9**.

kely not a selective inhibitor, a >10-fold increase in bioactivation rate (as shown by intrinsic clearance) may prove beneficial in the balance between efficacy and adverse effects. Further studies are needed to provide biology data in human-based assays for efficacy and safety of compound **21**. Since compound **23** does not show improvement in phosphorylation rate in human blood, additional derivatives of IMMH002 would be studied in the future, given its selectivity.

2.4. *Biological evaluation*

The lymphopenia activities of head-piece-modified analogues **21**−**24** were evaluated *in vivo* in comparison with positive controls FTY720 and IMMH002. The number of lymphocytes in peripheral blood was monitored 24 h after oral administration of 3 mg/kg of each compound to Sprague−Dawley rats (Fig. 5). Compounds **21** and **23** showed similar lymphopenia activity compared with the positive controls, whereas compounds **22** and **24** had lower lymphopenia activity than the positive controls. The key structural differences among FTY720 or IMMH002 and their analogues **21** or **23** were the replacement of the second hydroxymethyl (pro-*R*) group on the quaternary carbon of the head moiety with a methyl group, suggesting that this structural change could keep the activity.

3. Conclusions

We developed an economical catalytic method for synthesizing the Wittig reagent involving a chiral 2-methyl-2-aminoethanol structure that could be applied to other syntheses when designed and synthesized head-piece-modified analogues (**21**−**24**). Compounds **21** and **23** that have the pro-*R* hydroxylmethyl replaced by a methyl group, facilitated higher phosphorylation rates in rats. Especially, compound **21** showed improvements of phosphorylation in both rat and human blood. Our study not only synthesized new compounds but also showed species differences in the response to the structural modifications in rats and human. These findings would provide a basis for predicting biotransformation behavior and efficacy of these compounds in clinic.

4. Experimental

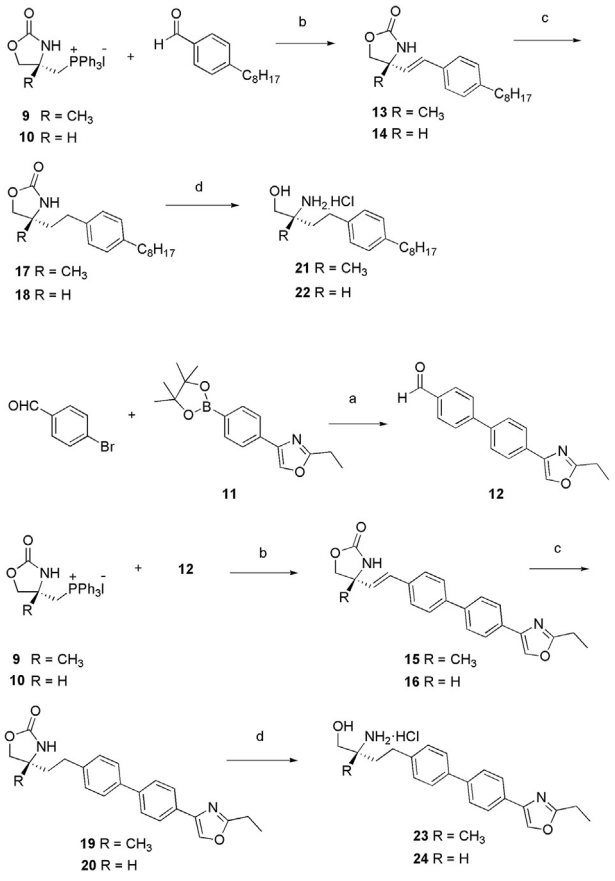
4.1. *General chemistry methods*

All melting points were measured with a micro melting point apparatus (MP-J3, Yanaco, Kyoto, Japan) and were uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on an NMR spectrometer (400 (100) or 600 (150) MHz; Mercury 400 or 600 (Varian, Palo Alto, CA, USA)). Electrospray ionization-mass spectrometry spectra were determined on a mass spectrometer (Exactive Plus, Thermo Fisher Scientific, MA, USA). Reactions were monitored by TLC (silica gel GF254, Qingdao, China). The ee values of the products were determined by high-performance liquid chromatography (1100 Series, Agilent, Palo Alto, CA, USA) using a Diacel Chiracel AD-H or AS-H column (250 mm × 4.6 mm). Rotational values were determined on a Rudolph Polarimeter (Rudolph, Wilmington, MA, USA) at λ = 589 nm (sodium D-line). Products were purified by column chromatography on silica gel (300−400 mesh, Qingdao, China) by using the solvent systems indicated. All other reagents were obtained from commercial suppliers and used without further purification.

4.2. *The synthesis of target compounds*

4.2.1. *3-(4-Chloro)benzyloxycarbonylamino-4-methyl-4-(4-trifluoro)benzyloxy-oxazolidin-2-one (**5**)*

Catalyst 1 (191 mg, 15% (mol/mol) in respect to the azodicarboxylate) was added to the suspension of 2-methyl-3-((4-



Scheme 3 Reagents and conditions: (a) Pd-dimer, K_2CO_3, PhMe/EtOH/H_2O (1:1:1 *v/v/v*); (b) aldehyde, *n*-BuLi, −78 °C, THF; (c) H_2, Pd-C, MeOH; (d) i) KOH, MeOH, H_2O; ii) HCl/Et_2O.

(trifluoromethyl)benzyl)oxy)propanal (2.2 g, 8.93 mmol) and di-*p*-chlorobenzyl azodicarboxylate (2.17 g, 5.93 mmol) in THF (50 mL). The mixture was stirred at RT under argon until the colour of the azodicarboxylate disappeared. The reaction was quenched by the addition of H_2O, then was extracted three times with Et_2O (50 mL × 3). The combined organic layers were dried over Na_2SO_4, filtered, and concentrated. The resulting crude was purified by flash chromatography on silica gel, eluting with light petroleum ether−ethyl acetate mixture (4:1, *v/v*) to afford 4 (3.27 g) as solid in 90% yield wtih 74% ee, m.p. 145−149 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.32 (s, 3H, CH_3), 3.71−3.87 (m, 2H, CH_2), 4.54 (s, 2 H, CH_2), 5.10−5.20 (m, 4H, 2 × CH_2), 6.71 (s, 1H, NH), 7.22−7.38 (m, 10H, H_{ar}), 7.62 (d, 2H, J = 4.0 Hz, H_{ar}), 9.62 (s, 1H, CHO). HR-MS *m/z* (ESI) 613.1150 $[M+H]^+$, $C_{28}H_{26}N_2O_6Cl_2F_3$ Calcd. for 613.1115. HPLC (Daicel Chirapak AD-H, hexane/isopropanol = 85:15, flow rate 1.0 mL/min, λ = 254 nm): t_R = 18.00 min (major), t_R = 20.29 min (minor).

Upon recrystallization from 90% ethanol, the aldehyde **4** (2.1 g) was obtained in 98% ee (65% yield). $NaBH_4$ (190 mg, 5.0 mmol) was added to the solution of **4** (900 mg, 1.65 mmol) in CH_2Cl_2/C_2H_5OH (4 mL). The reaction mixture was stirred for 1 h, and then it was quenched by adding 1 mol/L HCl until the mixture reached pH 7, and it was extracted with CH_2Cl_2. The combined organic solution was dried over Na_2SO_4, and the solvent was evaporated under reduced pressure. The resulting crude was purified by flash chromatography on silica gel eluted with light petroleum ether−ethyl acetate mixture (4:1, *v/v*) to afford **5** (1.53 g) was obtained in 95% yield with 98% ee. $[\alpha]_D^{20}$ −17.84 (*c* 0.7, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.34 (s, 3H, CH_3), 3.36 (d, 1H, J = 8.0 Hz, CH), 3.56 (d, 1H, J = 8.0 Hz, CH), 4.12 (d, 1H, J = 4.0 Hz, CH), 4.38 (d, 1H, J = 4.0 Hz, CH), 4.56−4.46 (m, 2H, CH_2), 5.18 (s, 2H, CH_2), 6.84 (bs, 1H, NH), 7.38−7.43 (m, 6H, H_{ar}), 7.63 (d, 2H, J = 4.0 Hz, H_{ar}); ^{13}C NMR (100 MHz, $CDCl_3$) δ 19.7, 61.2, 67.3, 71.4, 72.5, 125.4, 122.7, 125.5, 125.6, 127.5, 128.8, 129.6, 133.8, 134.4, 141.4, 156.1, 156.7. HR-MS *m/z* (ESI) 473.1086 $[M+H]^+$, $C_{21}H_{21}N_2O_5ClF_3$ Calcd. for 473.1084. HPLC (Daicel Chirapak AS-H, hexane/isopropanol = 70:30, flow rate 1.0 mL/min, λ = 254 nm): t_R = 34.57 min (major), t_R = 56.49 min (minor).

*4.2.2. (R)-3-Amino-4-(hydroxymethyl)-4-methyloxazolidin-2-one (**6**)*

To a solution of **5** (670 mg, 1.42 mmol) in 8 mL of methanol and 4 mL acetic acid, 360 mg of 10% palladium on charcoal was added. The mixture was hydrogenated at ambient pressure for 12 h and filtered. The filtrate was evaporated to dryness under reduced pressure. Column chromatography on silica gel (dichloromethane/methanol, 20:1−10:1) delivered 152 mg (1.03 mmol, 73%) of a colourless solid 6, m.p. 113−115 °C. $[\alpha]_D^{20}$ −3.86 (*c* 0.9, CH_3OH). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.20 (s, 3H, CH_3), 3.31 (dd, 1H, J = 12.0, 1 Hz, CH), 3.55 (bs, 3H, NH_2 and OH), 3.76−3.79 (m, 1H, CH), 3.96 (d, 1H, J = 8.0 Hz, CH), 4.40 (d, 1H, J = 8.0 Hz, CH). HR-MS *m/z* (ESI) 147.0764 $[M+H]^+$, $C_5H_{11}N_2O_3$ Calcd. for 147.0762.

*4.2.3. (R)-4-(Hydroxymethyl)-4-methyloxazolidin-2-one (**7**)*

146 mg (1 mmol) of $NaNO_2$ was added dropwise to a solution containing 45.0 mg (0.234 mmol) of **6** in 18 mL of acetic acid and 6 mL 1 mol/L HCl. The mixture was refluxed for 1 h. The solvent was evaporated to dryness under reduced pressure. Column chromatography on silica gel (dichloromethane/methanol, 20:1−10:1) delivered 79 mg (0.6 mmol, 60%) of a white solid **7**. $[\alpha]_D^{20}$ −8.8 (*c* 0.5, CH_3OH); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.34 (s, 3H, CH_3), 3.55 (dd, 1H, J = 12.0, 4.0 Hz, CH_2), 4.04 (d, 1H, J = 8.0 Hz, CH), 4.33 (d, 1H, J = 8.0 Hz, CH), 5.59 (bs, 1H, NH); ^{13}C NMR (150 MHz, $CDCl_3$) δ 22.6, 58.9, 67.5, 72.8, 159.4. HR-MS *m/z* (ESI) 132.0655 $[M+H]^+$, $C_5H_{10}NO_3$ Calcd. for 132.0654.

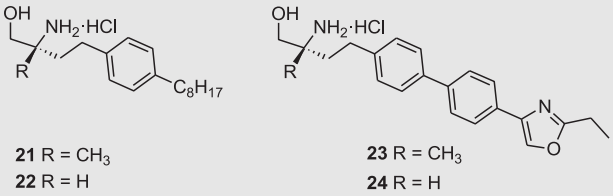
*4.2.4. General procedure for the synthesis of compound **12***

Catalyst Pd-dimor (1%, mol/mol in respect to 4-bromobenzaldehyde) was added to a suspension of 4-bromobenzaldehyde, K_2CO_3 and **11** in toluene/EtOH/H_2O = 1:1:1 (*v/v/v*). The mixture was refluxed for 4 h. Then the solvent was removed under vacuum. The crude material was extracted with Et_2O and washed with brine. The organic phase was dried (Na_2SO_4) and the solvent was evaporated under reduced pressure. The crude product was chromatographed (silica gel, light petroleum ether/ethyl acetate = 20:1) to afford the corresponding aldehyde **12** as a white solid, 81% yield, m.p. 125 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.40 (t, 3H, J = 8.0 Hz, CH_3), 2.88 (q, 2H, J = 8.0, 4.0 Hz, CH_2), 7.69 (d, 2H, J = 4.0 Hz, H_{ar}), 7.79 (d, 2H, J = 8.0 Hz, H_{ar}), 7.84 (d, 2H, J = 8.0 Hz, H_{ar}), 7.89 (s, 1H, H_{ar}), 7.96 (d, 2H, J = 8.0 Hz, H_{ar}), 10.06 (s, 1H, CHO). HR-MS *m/z* (ESI) $C_{18}H_{16}NO_2$ $[M+H]^+$ Calcd. for 278.1176, Found 278.1182.

*4.2.5. General procedure for the synthesis of compounds **13**−**16***

To a suspension of the phosphonium salt (2.0 equiv.) in THF, *n*-butyllithium (2.5 mol/L in hexane, 3.9 equiv.) was added at

Table 1 Michaelis–Menten kinetics for phosphorylation of FTY720, IMMH002, and compounds **21**–**24** in whole blood.



21 R = CH_3
22 R = H

23 R = CH_3
24 R = H

Compound	R	Rat			Human		
		V_{max} (pmol/min)	K_m (μmol/L)	CL_{int} (μL/min)	V_{max} (pmol/min)	K_m (μmol/L)	CL_{int} (μL/min)
FTY720	–	63.4 ± 10.2	68.2 ± 11.2	0.930	0.33 ± 0.05	25.7 ± 4.2	0.013
21	CH_3	221 ± 36.8	143 ± 25.4	1.545	3.36 ± 0.62	11.4 ± 1.9	0.295
22	H	28.4 ± 4.5	9.1 ± 1.8	3.121	1.24 ± 0.31	58.2 ± 10.6	0.021
IMMH002	–	20.1 ± 3.6	65.1 ± 11.5	0.309	1.11 ± 0.28	81.3 ± 14.8	0.014
23	CH_3	65.2 ± 8.2	129 ± 15.6	0.505	0.87 ± 0.09	65.5 ± 8.3	0.012
24	H	1.9 ± 0.3	128 ± 23.4	0.015	0.24 ± 0.05	40.2 ± 7.3	0.006

–Not applicable.

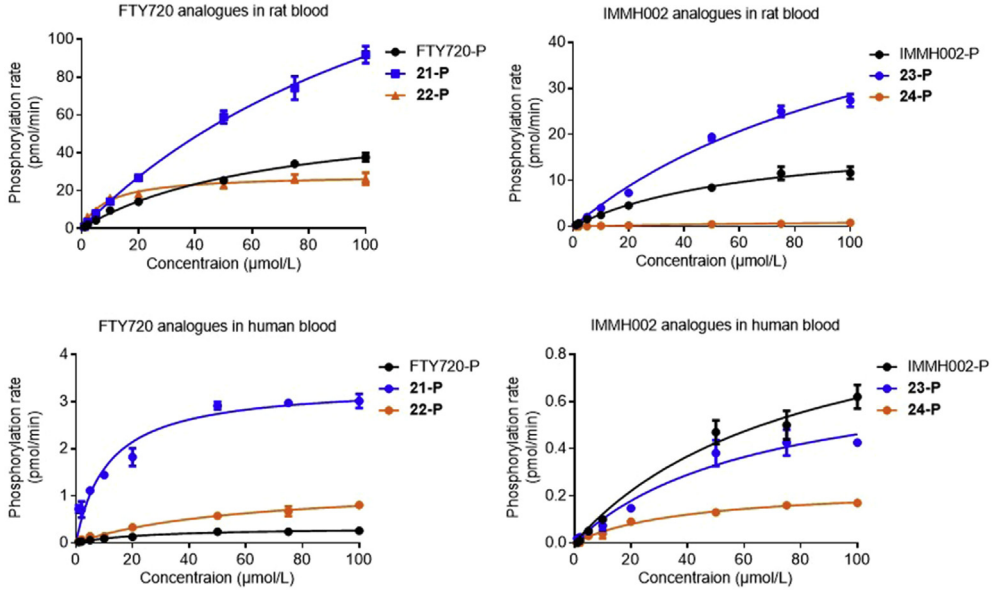


Figure 3 Substrate–velocity curves for phosphorylation of FTY720, IMMH002, and compounds **21**–**24** in whole blood.

−78 °C and then the solution was stirred for 30 min at the same temperature. After the addition of benzaldehyde (1.0 equiv.) at −78 °C, the reaction mixture was warmed to ambient temperature and stirred for 3 h. After quenching with saturated aq. NH_4Cl, the resulting biphasic mixture was extracted with AcOEt. The combined organic layer was washed with water and brine, dried over Na_2SO_4, filtered, and evaporated. Purification by silica gel column chromatography (hexane/AcOEt = 4:1 to 1:1) provided target products.

*4.2.5.1. (R,E)-4-Methyl-4-(4-octylstyryl)oxazolidin-2-one (**13**).* Oil, Yield 69%. $[\alpha]_D^{20}$ −44.8 (*c* 0.3, CH_3OH). ^1H NMR (400 MHz, $CDCl_3$) δ 0.86 (t, 3H, *J* = 8.0 Hz, CH_3), 1.24–1.28 (m, 10H, 5 × CH_2), 1.54–1.60 (m, 5H, CH_2 and CH_3), 2.56–2.59 (m, 2H, *J* = 8.0 Hz, CH_2), 4.17 (d, 1H, *J* = 8.0 Hz, CH), 4.23 (d, 1H, *J* = 8.0 Hz, CH), 5.07 (bs, 1H, NH), 6.17 (d, 1H, *J* = 16.0 Hz, CH), 6.57 (d, 1H, *J* = 16.0 Hz, CH), 7.13 (d, 2H, *J* = 8.0 Hz, H_{ar}), 7.27 (d, 2H, *J* = 8.0 Hz, H_{ar}). HR-MS *m/z* (ESI) 316.2261 $[M+H]^+$, $C_{20}H_{30}NO_2$ Calcd. for 316.2271.

*4.2.5.2. (R,E)-4-(4-Octylstyryl)oxazolidin-2-one (**14**).* Oil, Yield 73%. $[\alpha]_D^{20}$ −4.7 (*c* 0.3, CH_3OH). ^1H NMR (400 MHz, $CDCl_3$) δ 0.88 (t, 3H, *J* = 8.0 Hz, CH_3), 1.26–1.30 (m, 10H, 5 × CH_2), 1.58–1.61 (m, 2H, CH_2), 2.59 (t, 2H, *J* = 8.0 Hz, CH_2), 4.12–4.16 (m, 1H, CH), 4.54–4.62 (m, 2H, CH_2), 5.31 (bs, 1H, NH), 6.05–6.11 (m, 1H, CH), 6.58 (d, 1H, *J* = 16.0 Hz, CH),

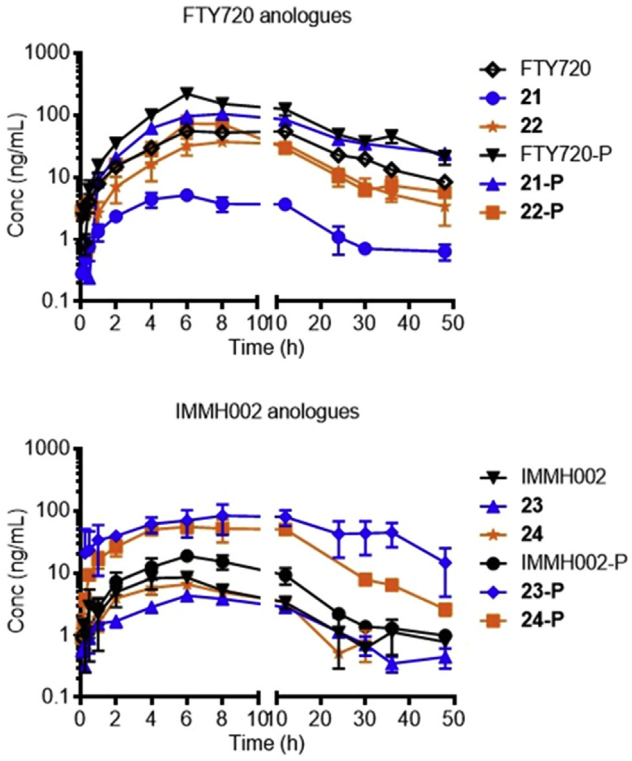


Figure 4 Blood concentration−time curves of parent compounds and phosphorylated compounds for FTY720, IMMH002, and compounds **21**−**24** in Sprague−Dawley rats.

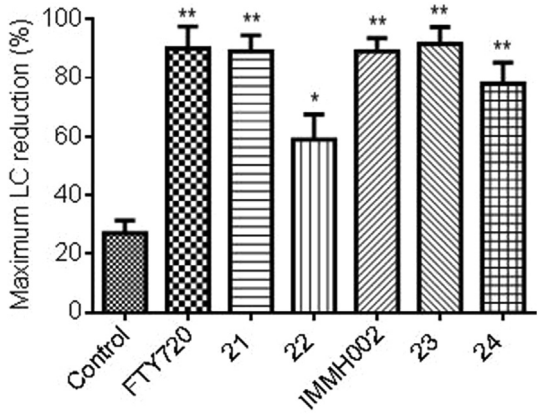


Figure 5 Maximum lymphocyte (LC) reduction of compounds FTY720, IMMH002, and **21**−**24** in Sprague−Dawley rats. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$.

7.15 (d, 2H, J = 8.0 Hz, H_{ar}), 7.29 (d, 2H, J = 8.0 Hz, H_{ar}). HR-MS *m/z* (ESI) 302.2112 $[M+H]^+$, $C_{19}H_{28}NO_2$ Calcd. for 302.2115.

*4.2.5.3. (R,E)-4-(2-(4′-(2-Ethyloxazol-4-yl)-[1,1′-biphenyl]-4-yl)vinyl)-4-methyloxazolidin-2-one (**15**).* White solid, Yield 72%, m.p. 220 °C. $[\alpha]_D^{20}$ −41.1 (*c* 0.3, CH_3OH). ^1H NMR (400 MHz, $CDCl_3$) δ 1.40 (t, 3H, J = 8.0 Hz, CH_3), 1.60 (s, 3H, CH_3), 2.86−2.92 (m, 2H, CH_2), 4.21 (d, 1H, J = 8.0 Hz, CH), 4.28 (d, 1H, J = 8.0 Hz, CH), 5.24 (bs, 1H, NH), 6.28 (d, 1H, J = 16.0 Hz, CH), 6.65 (d, 1H, J = 16.0 Hz, CH), 7.45 (d, 2H, J = 8.0 Hz, H_{ar}), 7.59−7.64 (m, 2H, J = 8.0 Hz, H_{ar}), 7.79−7.81 (m, 2H, H_{ar}), 7.87 (s, 1H, H_{ar}); ^{13}C NMR (100 MHz, $CDCl_3$) δ 13.7, 20.7, 25.5, 30.2, 58.5, 125.9, 127.1, 127.2, 127.2, 129.8, 130.5, 131.1, 133.2, 134.7, 139.7, 140.1, 140.6, 158.7, 165.5. HR-MS *m/z* (ESI) 375.1701 $[M+H]^+$, $C_{23}H_{23}N_2O_3$ Calcd. for 375.1703.

*4.2.5.4. (R,E)-4-(2-(4′-(2-Ethyloxazol-4-yl)-[1,1′-biphenyl]-4-yl)vinyl)oxazolidin-2-one (**16**).* White solid, Yield 71%, m.p. 230 °C. $[\alpha]_D^{20}$ 23.0 (*c* 0.1, CH_3OH). ^1H NMR (400 MHz, $CDCl_3$) δ 1.39 (t, 3H, J = 8.0 Hz, CH_3), 2.87−2.92 (m, 2H, CH_2), 4.16−4.19 (m, 1H, CH), 4.57−4.64 (m, 2H, CH_2), 5.06 (bs, 1H, NH), 6.15−6.21 (m, 1H, CH), 6.64 (d, 1 H, J = 16.0 Hz, CH), 7.44 (d, 2H, J = 4.0 Hz, H_{ar}), 7.59−7.64 (m, 4H, H_{ar}), 7.80 (d, 2H, J = 4.0 Hz, H_{ar}), 7.86 (s, 1H, H_{ar}); ^{13}C NMR (100 MHz, $CDCl_3$) δ 11.3, 21.8, 55.2, 70.2, 125.9, 126.3, 127.2, 127.2, 127.3, 130.4, 133.2, 133.6, 134.3, 139.7, 140.0, 140.8, 158.9, 166.4. HR-MS *m/z* (ESI) 361.1541 $[M+H]^+$, $C_{22}H_{21}N_2O_3$ Calcd. for 361.1547.

*4.2.6. General procedure for the synthesis of compounds **17**−**20*** To a solution of **13**−**16** (1.0 equiv.) in methanol was added 10% Pd/C (1.0 equiv.), and then the suspension was stirred for 2 h under a hydrogen atmosphere at ambient temperature. The reaction mixture was filtered and evaporated *in vacuo*, providing the products **17**−**20**.

*4.2.6.1. (R)-4-Methyl-4-(4-octylphenethyl)oxazolidin-2-one (**17**).* Oil, Yield 94%. $[\alpha]_D^{20}$ 5.60 (*c* 0.8, $CHCl_3$). ^1H NMR (400 MHz, $CDCl_3$) δ 0.84−0.88 (m, 3H, CH_3), 1.24−1.28 (m, 10H, 5 × CH_2), 1.39 (s, 3H, CH_3), 1.57 (m, 2H, CH_2), 1.88−1.91 (m, 2H, CH_2), 2.52−2.57 (m, 2H, CH_2), 2.60−2.64 (m, 2H, CH_2), 4.04 (d, 1H, J = 12.0 Hz, H_{ar}), 4.16 (d, 1H, J = 8.0 Hz, H_{ar}), 5.61 (bs, 1H, NH), 7.05−7.10 (m, 4H, H_{ar}). HR-MS *m/z* (ESI) 318.2424 $[M+H]^+$ $C_{20}H_{32}NO_2$, Calcd. for 318.2428.

*4.2.6.2. (R)-4-(4-Octylphenethyl)oxazolidin-2-one (**18**).* Oil, Yield 91%. $[\alpha]_D^{20}$ 8.8 (*c* 0.2, CH_3OH). ^1H NMR (400 MHz, $CDCl_3$) δ 0.84−0.88 (m, 3H, CH_3), 1.24−1.28 (m, 10H, 5 × CH_2), 1.56−1.57 (m, 2H, CH_2), 1.86−1.92 (m, 2H, CH_2), 2.53−2.57 (m, 2H, CH_2), 2.61−2.65 (m, 2H, CH_2), 3.81−3.88 (m, 1H, CH), 3.97−4.01 (m, 1H, CH), 4.42−4.47 (m, 1H, CH), 5.42 (bs, 1H, NH), 7.05 (d, 2H, H_{ar}), 7.05 (d, 2H, H_{ar}), 7.10 (d, 2H, H_{ar}). HR-MS *m/z* (ESI) 304.2255 $[M+H]^+$, $C_{19}H_{30}NO_2$ Calcd. for 304.2271.

*4.2.6.3. (R)-4-(2-(4′-(2-Ethyloxazol-4-yl)-[1,1′-biphenyl]-4-yl)ethyl)-4-methyloxazolidin-2-one (**19**).* White solid, Yield 92%, m.p. 210 °C. $[\alpha]_D^{20}$ 6.7 (*c* 0.1, $CHCl_3$). ^1H NMR (400 MHz, $CDCl_3$) δ 1.38 (t, 3H, J = 8.0 Hz, CH_3), 1.42 (s, 3H, CH_3), 1.94−1.95 (m, 2H, CH_2), 2.69−2.72 (m, 2H, CH_2), 2.82−2.88 (m, 2H, CH_2), 4.08 (d, 1H, J = 8.0 Hz, CH), 4.20 (d, 1H, J = 8.0 Hz, CH), 5.32 (bs, 1H, NH), 7.23 (d, 1H, J = 8.0 Hz, CH), 7.53−7.55 (m, 2H, H_{ar}), 7.58−7.60 (m, 2H, H_{ar}), 7.75−7.77 (m, 2H, H_{ar}), 7.83 (s, 1H, H_{ar}). HR-MS *m/z* (ESI) 377.1864 $[M+H]^+$, $C_{23}H_{25}N_2O_3$ Calcd. for 377.1860.

*4.2.6.4. (R)-4-(2-(4′-(2-Ethyloxazol-4-yl)-[1,1′-biphenyl]-4-yl)ethyl)oxazolidin-2-one (**20**).* White solid, yield 90%, m.p. 250 °C. $[\alpha]_D^{20}$ 23.0 (*c* 0.1, CH_3OH). ^1H NMR (400 MHz, $CDCl_3$) δ 1.40 (t, 3H, J = 8.0 Hz, CH_3), 1.94−1.98 (m, 2H, CH_2), 2.71−2.75 (m, 2H, CH_2), 2.85−2.92 (m, 2H, CH_2), 3.90−3.94 (m,

1H, CH), 4.05−4.08 (m, 2H, CH_2), 4.48−4.52 (m, 2H, CH_2), 5.15 (bs, 1H, NH), 7.25 (d, 2H, J = 8.0 Hz, H_{ar}), 7.57−7.78 (m, 4H, H_{ar}), 7.80 (d, 2H, J = 8.0 Hz, H_{ar}), 7.86 (s, 1H, H_{ar}). HR-MS *m/z* (ESI) 363.1706 $[M+H]^+$, $C_{22}H_{23}N_2O_3$ Calcd. for 363.1703.

*4.2.7. General procedure for the synthesis of compounds **21**−**24***

Compounds **17**−**20** were diluted with methanol/H_2O = 10:1 (*v/v*), then potassium hydroxide (10 equiv.) was added, and the reaction was refluxed for 18 h. After cooling to room temperature, water was added to the reaction mixture and extracted with CH_2Cl_2. The combined organic layer was washed with water and brine, dried over Na_2SO_4, filtered, and evaporated. The crude product was chromatographed (silica gel, dichloromethane/methanol = 10:1), and then acidification with 1 mol/L HCl in Et_2O (2 mL) to afford the desired products **21**−**24**.

*4.2.7.1. (R)-2-Amino-2-methyl-4-(4-octylphenyl)butan-1-ol hydrochloride (**21**).* Oil, Yield 84%. $[\alpha]_D^{20}$ −1.53 (*c* 0.5, CH_3OH). ^1H NMR (600 MHz, $CDCl_3$) δ 0.84−0.88 (m, 3H, CH_3), 1.21−1.26 (m, 10H, 5 × CH_2), 1.28 (s, 3H, CH_3), 1.55−1.57 (m, 2H, CH_2), 1.80−1.86 (m, 1H, CH), 1.89−1.94 (m, 1H, CH), 2.52−2.55 (m, 2H, CH_2), 2.58−2.65 (m, 2H, CH_2), 3.50 (d, 1H, J = 12.0 Hz, CH), 3.60 (d, 2 H, J = 12.0 Hz, CH), 7.06−7.11 (m, 4H, H_{ar}); ^{13}C NMR (150 MHz, $CDCl_3$) δ 13.0, 18.8, 22.3, 28.5, 28.8, 28.9, 29.1, 29.3, 31.3, 31.6, 35.0, 37.4, 57.3, 64.8, 127.7, 128.2, 137.9, 140.7. HR-MS *m/z* (ESI) 292.2631 $[M+H]^+$, $C_{19}H_{34}NO$ Calcd. for 292.2635. HPLC purity >95%, retention time = 4.37 min.

*4.2.7.2. (R)-2-Amino-4-(4-octylphenyl)butan-1-ol hydrochloride (**22**).* Oil, Yield 87%. $[\alpha]_D^{20}$ −4.28 (*c* 0.7, CH_3OH). ^1H NMR (600 MHz, $CDCl_3$) δ 0.86 (t, J = 12.0 Hz, CH_3), 1.25−1.30 (m, 10H, 5 × CH_2), 1.53−1.58 (m, 2H, CH_2), 1.82−1.88 (m, 1H, CH), 1.89−1.96 (m, 1H, CH), 2.53−2.55 (m, 2H, CH_2), 2.62−2.71 (m, 2H, CH_2), 3.14−3.18 (m, 1H, CH), 3.55−3.59 (m, 1H, CH), 3.75−3.77 (m, 1H, CH), 7.08−7.12 (m, 4H, H_{ar}); ^{13}C NMR (150 MHz, $CDCl_3$) δ 14.5, 23.8, 30.4, 30.5, 30.6, 32.8, 33.1, 36.5, 54.2, 62.0, 129.3, 129.8, 139.0, 142.2. HR-MS *m/z* (ESI) 278.2476 $[M+H]^+$, $C_{18}H_{32}NO$ Calcd. for 278.2478. HPLC purity >96%, retention time = 4.05 min.

*4.2.7.3. (R)-2-Amino-4-(4′-(2-ethyloxazol-4-yl)-[1,1′-biphenyl]-4-yl)-2-methylbutan-1-ol hydrochloride (**23**).* White solid, Yield 82%, m.p. 210 °C. $[\alpha]_D^{20}$ −2.24 (*c* 0.7, CH_3OH). ^1H NMR (600 MHz, $CDCl_3$) δ 1.34−1.37 (m, 6H, 2 × CH_3), 1.89−1.91 (m, 1H, CH), 1.97−1.99 (m, 1H, CH), 2.70−2.71 (m, 2H, CH_2), 2.83−2.86 (m, 2H, CH_2), 3.53 (d, 1H, J = 8.0 Hz, CH), 3.63 (d, 1H, J = 8.0 Hz, CH), 7.31 (d, 2H, J = 4.0 Hz, H_{ar}), 7.58 (d, 2H, J = 4.0 Hz, H_{ar}), 7.63 (d, 2H, J = 4.0 Hz, H_{ar}), 7.77 (d, 2H, J = 4.0 Hz, H_{ar}), 8.16 (s, 1H, H_{ar}); ^{13}C NMR (150 MHz, $CDCl_3$) δ 11.7, 20.3, 22.5, 30.8, 38.8, 58.8, 66.3, 127.0, 128.1, 128.2, 129.9, 131.2, 135.5, 140.0, 141.3, 141.7, 141.8, 168.3. HR-MS *m/z* (ESI) 351.2056 $[M+H]^+$, $C_{22}H_{27}N_2O_2$ Calcd. for 351.2067. HPLC purity >94%, retention time = 3.27 min.

*4.2.7.4. (R)-2-Amino-4-(4′-(2-ethyloxazol-4-yl)-[1,1′-biphenyl]-4-yl)butan-1-ol hydrochloride (**24**).* White solid, Yield 85%, m.p. 200 °C. $[\alpha]_D^{20}$ −6.04 (*c* 0.5, CH_3OH). ^1H NMR (600 MHz, $CDCl_3$) δ 1.37 (t, 3H, J = 12.0 Hz, CH_3), 1.92−2.00 (m, 2H, CH_2), 2.76−2.77 (m, 2H, CH_2), 2.86−2.90 (m, 2H, CH_2), 3.14−3.18 (m, 1H, CH), 3.59−3.62 (m, 1H, CH), 3.78−3.81 (m, 1H, CH), 7.32 (d, 2H, J = 6.0 Hz, H_{ar}), 7.59 (d, 2H, J = 12.0 Hz, H_{ar}), 7.64 (d, 2H, J = 12.0 Hz, H_{ar}), 7.64 (d, 2H, J = 12.0 Hz, H_{ar}), 7.77 (d, 2H, H_{ar}), 8.22 (s, 1H, H_{ar}); ^{13}C NMR (150 MHz, $CDCl_3$) δ 11.4, 22.5, 25.5, 32.2, 32.3, 54.2, 62.0, 127.1, 128.2, 128.3, 130.0, 135.8, 140.0, 140.7, 141.3, 142.0, 168.7. HR-MS *m/z* (ESI) 337.1905 $[M+H]^+$, $C_{21}H_{25}N_2O_2$ Calcd. for 337.1911. HPLC purity >95%, retention time = 3.15 min.

4.3. Biological evaluation

4.3.1. In vitro assays of compound phosphorylation

To assay the phosphorylation of compounds in whole blood, rat or human blood was collected in heparin-coated tubes. Different concentrations of the compound (1, 2, 5, 10, 20, 50, 75, and 100 μmol/L) were added directly to rat or human blood (200 μL), and then incubated at 37 °C for 60 min. Reactions were stopped with the addition of ice-cold acetonitrile (600 μL). All samples were vortex-mixed and centrifuged at 14,000 rpm (Thermo Fisher, MA, USA) for 5 min. The supernatant was then subjected to liquid chromatography-tandem mass spectrometry (LC−MS/MS). The kinetic parameters (K_m and V_{max}) for phosphate formation in rat and human blood were calculated using a nonlinear curve fitting program based on the Michaelis−Menten equation (GraphPad Prism 5.0, GraphPad Software Inc., San Diego, CA, USA).

4.3.2. In vivo pharmacokinetic assay

Male Sprague−Dawley rats (three per group, purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd., China) were fasted for 12 h before receiving the agonists and fed 4 h after administration. The animal experiments were performed in accordance with the guidelines of China for animal care, which conforms to the internationally accepted principles in the care and use of experimental animals. The dosing solutions used for the animal studies were prepared by dissolving the required amounts of agonist in 0.5% hydroxypropyl methylcellulose. After oral administration of the agonist at a dose of 3 mg/kg to the rats, blood samples (∼0.2 mL) were collected in heparinized 1.5 mL polythene tubes by orbital bleeding *via* capillary tubes at 0.08, 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24, 36, and 48 h postdose. Each blood sample (100 μL) was spiked with acetonitrile (300 μL), and then vortexed and centrifuged at 14,000 rpm (Thermo Fisher, MA, USA) for 5 min to precipitate protein. The supernatant (10 μL) was injected into the LC−MS/MS system for analysis.

4.3.3. LC−MS/MS analysis

The LC−MS/MS system consisted of a Surveyor auto-sampler, a Surveyor LC pump, a TSQ Quantum Access™ triple quadrupole mass spectrometer with an electrospray ionization (ESI) source and Xcalibur 2.0 software for data acquisition and analysis spectrometer (Thermo Finnigan, Santa Clara County, CA, USA). The analytes were chromatographed by injection of a 10 μL sample into a ZorbaxSB-C18 column (3.5 μm, 2.1 mm × 100 mm). The mobile phase consisted of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in methanol).The analytes were eluted, at a flow rate of 0.2 mL/min, with the following gradient elution: from 0 to 1.5 min, 5% B; linear increase to 95% B in 0.2 min; 95% B during 3 min, decrease to 5% B in 0.2 min; and stabilization at initial conditions for 3 min. The mass spectrometer was set for multiple-reaction monitoring and was operated in a positive-ion mode with ESI source. The spray voltage was set at 3700 V, and the sheath gas and auxiliary gas at 30 and 15

psi. The capillary temperature was set at 350 °C. The transition ion pairs were at *m/z* 308.3/255.3 for FTY720, *m/z* 388.3/255.3 for FTY720-P, *m/z* 292.2/257.2 for **21**, *m/z* 372.2/257.2 for **21-P**, *m/z* 278.2/243.2 for **22**, *m/z* 358.2/243.2 for **22-P**, *m/z* 367.3/350.3 for IMMH002, *m/z* 447.2/320.2 for IMMH002-P, *m/z* 351.3/334.3 for **23**, *m/z* 431.3/316.3 for **23-P**, *m/z* 337.3/320.3 for **24**, *m/z* 417.3/302.2 for **24-P**.

4.3.4. In vivo peripheral lymphocyte reduction test

Male Sprague−Dawley rats (three per group) were administered 3 mg/kg test agonist, positive control, or vehicle (0.5% hydroxypropyl methylcellulose) orally. Blood (20 μL) was collected from the tail vein. The peripheral blood lymphocytes were counted with a hematology analyzer (MEK-7222 K, Nihon Kohden, Tokyo, Japan) at 0, 2, 4, 8, 12, and 24 h postdose.

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Author contributions

Qiong Xiao and Jinping Hu conceived and designed the study. Qiong Xiao, Jinping Hu, Minwan Hu, Si Chen, Yifan Tang, Zeyu Shi, Jing Jin performed the experiments. Qiong Xiao and Jinping Hu analyzed all the data and written the manuscript. Dali Yin and Ping Xie revised the manuscript. Qiong Xiao obtained the funding and supervised the whole study. All the authors approved the final version of the manuscript.

Conflicts of interest

The authors have no conflicts of interest to declare.

Appendix A. Supporting information

Supporting data to this article can be found online at https://doi.org/10.1016/j.apsb.2019.11.005.

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