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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Design, synthesis, and biological study of 6,7-dihydro-5*H*-pyrano[2,3-d]pyrimidine derivatives as novel hedgehog signaling pathway inhibitors

Minhang Xin · Liandi Zhang · Han Shen · Jun Wen · Chongxing Tu · Zhaoyu Liu · Lingfei Cheng · Xinge Zhao

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Abstract A novel series of hedgehog signaling pathway inhibitors were designed by replacing the pyrimidine nucleus of our earlier reported compounds with 6,7-dihydro-5*H*-pyrano[2,3-d]pyrimidine scaffold. Among this new class of hedgehog signaling pathway inhibitors, compounds **14** and **18** exhibited promising potency in vitro compared to GDC-0449. Compound **18** was advanced to profile its pharmacokinetic characteristics, and showed moderate pharmacokinetic properties in vivo, indicating that the 6,7-dihydro-5*H*-pyrano[2,3-d]pyrimidine skeleton is a promising scaffold for further exploration as hedgehog signaling pathway inhibitors.

Keywords Hedgehog signaling pathway · Inhibitors · 6,7-Dihydro-5*H*-pyrano[2,3-d]pyrimidine · Novel

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M. Xin (✉)
Department of Medicinal Chemistry, School of Pharmacy, Health Science Center, Xi'an Jiaotong University, No. 76, Yanta West Road, Xi'an 710061, People's Republic of China
e-mail: xmhcpu@163.com

M. Xin · L. Zhang · H. Shen · J. Wen · Z. Liu · L. Cheng · X. Zhao
Jiangsu Simcere Pharmaceutical Co. Ltd., Jiangsu Key Laboratory of Molecular Targeted Antitumor Drug Research, No. 699-18, Xuan Wu District, Nanjing 210042, People's Republic of China

H. Shen · J. Wen · C. Tu · L. Cheng · X. Zhao
Nanjing BioSciKin Co. Ltd., Small Molecule Drug Discovery Platform, No. 699-18, Xuan Wu District, Nanjing 210042, People's Republic of China

Introduction

The Hedgehog (Hh) signaling pathway plays very key roles in several normal biological processes including cell proliferation, survival signals control, embryological development, tissue patterning, and stem cells maintenance (Ng and Curran, 2011). However, when this signaling pathway becomes abnormally disrupted or hyperactivated, they express hallmarks of many cancer types. Mechanistic researches indicate that genetic mutations (mainly in receptor patched, GPCR-like receptor smoothened, or Suppressor of fused) or Hh ligand overexpression in this pathway are implicated in a variety of solid and hematologic tumors such as basal cell carcinoma (BCC), medulloblastoma, rhabdomyosarcoma, melanoma, glioblastoma, leukemia, lymphoma, hepatocellular, pancreatic, gastric, colorectal, esophageal, lung, ovarian, and prostate cancers (Onishi and Katano, 2011). Hence, inhibition of Hh signaling pathway is thought to have considerable therapeutic potential for addressing numerous unmet clinical needs.

To date, several small molecules which are capable of inhibiting the Hh signaling pathway have been advanced into clinical trials (Ruch and Kim, 2013). Among these, vismodegib was approved by FDA in 2012 for treatment of metastatic BCC (Dlugosz *et al.*, 2012). Moreover, it is still undergoing other clinical trials to profile its therapeutic potential against cancers, such as medulloblastoma, multiple myeloma, leukemia, chondrosarcoma, pancreatic, gastric, gastroesophageal, prostate, and small-cell lung cancers, presently. Likewise, other candidates including sonidegib (NVP-LDE225, Phase III), LY-2940680 (Phase II), BMS-833923 (XL-139, Phase II), NVP-LEQ506 (Phase I), PF-04449913(Phase I), and TAK-441 (Phase I), have recently been validated in clinical stages for combating some cancer types by a single or combinational

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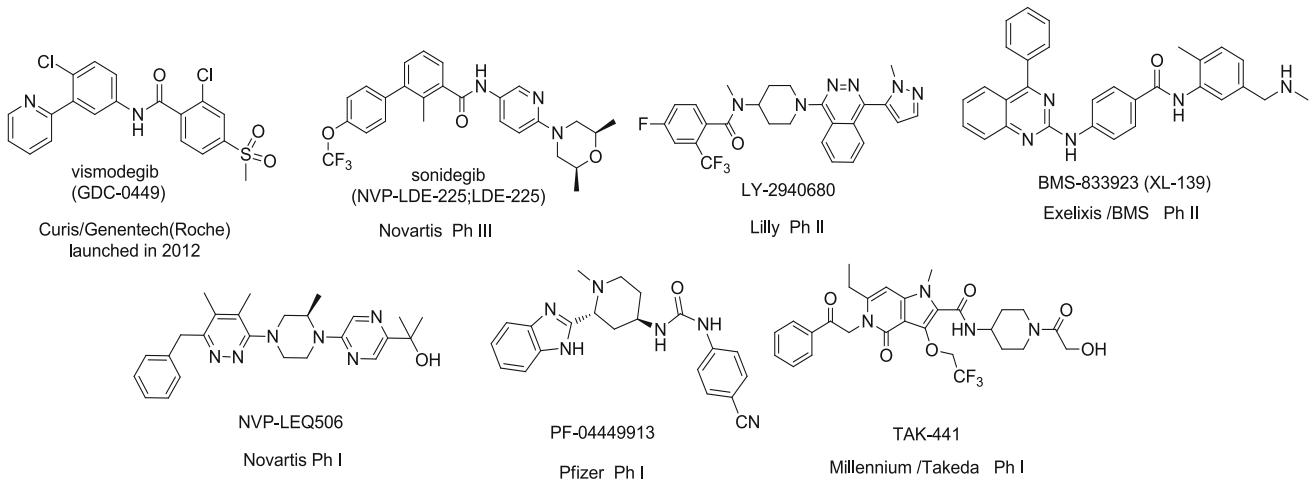


Fig. 1 Representative Hh signaling pathway inhibitors

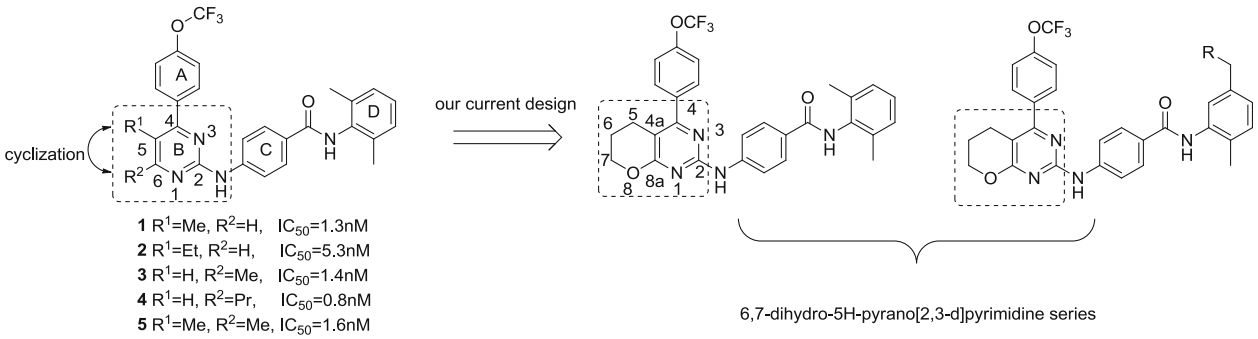


Fig. 2 Design of the 6,7-dihydro-5*H*-pyrano[2,3-d]pyrinidine derivatives

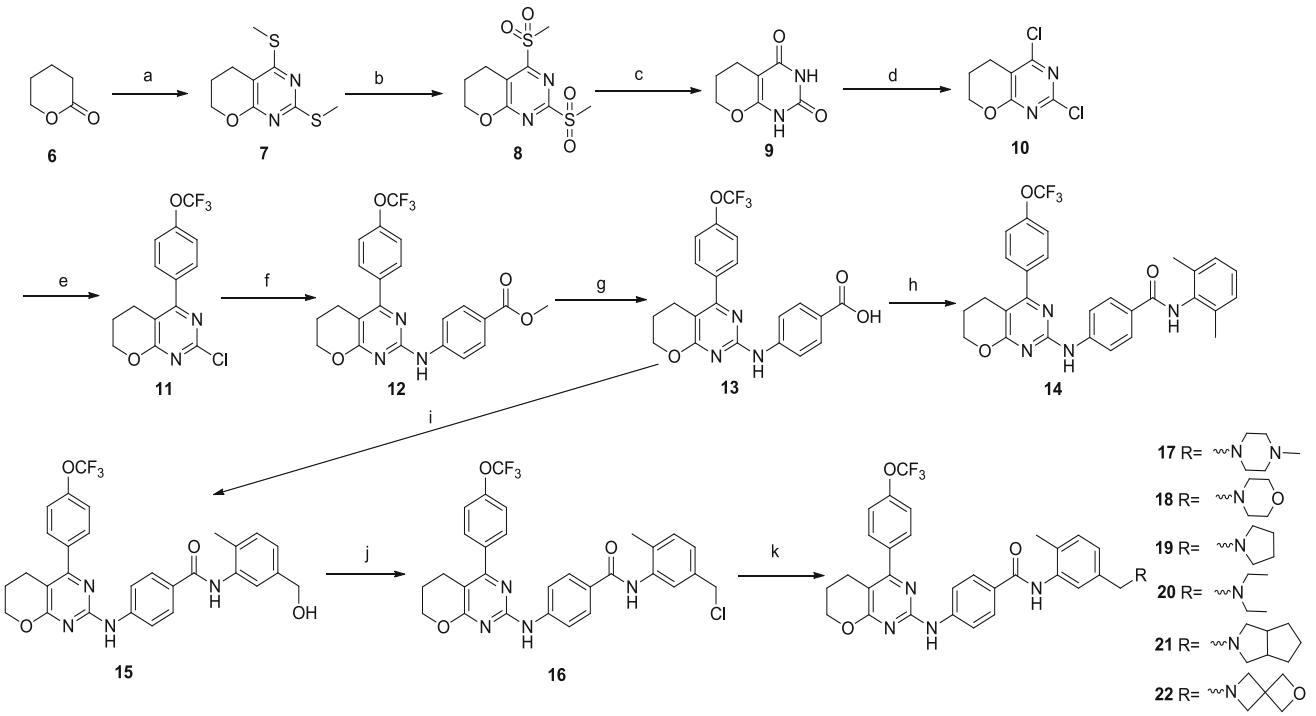
therapies (Fig. 1) (Hadden, 2013). Considering the promising therapeutic potential of Hh signaling pathway inhibitors in many solid and hematologic tumors, therefore, the novel Hh signaling pathway inhibitors are still needed in terms of exploring new anti-cancer therapeutic benefits for solving unmet clinical needs.

Results and discussion

Design of the 6,7-dihydro-5*H*-pyrano[2,3-d]pyrinidine derivatives

In previous studies, we detailed the discovery of a novel series of highly potent Hh signaling pathway inhibitors structurally featured by 4-(2-pyrimidinylamino)benzamides (Xin *et al.*, 2013; Xin *et al.*, 2014a). SAR studies of this series revealed that substituents outside the B-ring were limited to alter the substitutions at C5- and/or C6-position of the pyrimidine core, derivatizing some significantly potent analogs, such as 5-methyl (**1**), 5-ethyl (**2**), 6-methyl (**3**), 6-propyl (**4**), and 5,6-dimethyl (**5**) (Fig. 2). All of these derivatives bearing simple alkyl substituents at C5- and/or C6-position with suitable size showed similar potency, demonstrating that a sizable hydrophobic unit adjacent to the pyrimidine ring was allowed (Huang *et al.*, 2012). This result inspired us to attempt some scaffold modifications by conceptual cyclization of the 5-position and the 6-position of pyrimidine, thereby being capable of developing new scaffold as novel Hh signaling pathway inhibitors (Fig. 2).

Pyrano[2,3-d]pyrimidines and dihydro-5*H*-pyrano [2,3-d]pyrimidines ring systems are regarded as highly valuable heterocyclic scaffolds in drug discovery (EI-Agrody *et al.*, 2001). Numerous pyrano[2,3-d]pyrimidine derivatives have been abundantly described to have various interesting pharmocologic activities (EI-Agrody *et al.*, 2001; Huang *et al.*, 2012). Whereas 6,7-dihydro-5*H*-pyrano [2,3-d]pyrimidine nucleus is considered as a bioisoster of pyrano[2,3-d]pyrimidine. However, it was found in organic synthesis and medicinal chemistry that only few reports validated its pharmaceutical application (Herrera *et al.*, 2006). Considering that, in connection with our pursuit of developing novel Hh signaling pathway inhibitors containing new scaffold, we are interested in the development



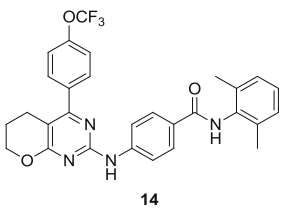
Scheme 1 Reagents and conditions: **a** CH_3SCN, Tf_2O, anhydrous CH_2Cl_2, −78–0 °C, 40 h, 23 %, **b** MCPBA, CH_2Cl_2, rt, 5 h, 98 %, **c** 10 % NaOH/MeOH, reflux, 2 h, 91 %, **d** $POCl_3$, DIPEA, reflux, 18 h, 52 %, **e** 4-Trifluoromethoxyphenylboronic acid, $Pd(PPh_3)_2Cl_2$, 2 M Na_2CO_3, dioxane, reflux, 2 h, 32 %, **f** methyl 4-aminobenzoate, $Pd(OAc)_2$, BINAP, Cs_2CO_3, dioxane, reflux, 13 h, 86 %, **g** 2 M NaOH, THF/MeOH, reflux, 5 h, 100 %, **h** 2,6-Dimethylaniline, HATU, TEA, DMF, rt for 16 h, 80 %, **i** 3-Amino-4-methylbenzyl alcohol, HATU, DMF, DIPEA, 80 °C, 17 h, 79 %, **j** $SOCl_2$, CH_2Cl_2, rt, 17 h, 93 %, **k** DMF, K_2CO_3, rt for 6 h, *N*-methylpiperazine for **17**, 71 %, morpholine for **18**, 80 %, pyrrolidine for **19**, 100 %, diethylamine for **20**, 94 %, octahydrocyclopenta[c]pyrrole for **21**, 81 %, 2-oxa-6-azaspiro[3.3]heptane for **22**, 90 %

of new scaffold containing this dihydro-5*H*-pyrano[2,3-d] pyrimidine heterocyclic system. Particularly, 6,7-dihydro-5*H*-pyrano[2,3-d]pyrimidine core could be considered as pyrimidine derivatizing nucleus by an ethoxy linker connected the 5-position of pyrimidine to the 6-position. Accordingly, herein we report the synthesis of this new 6,7-dihydro-5*H*-pyrano[2,3-d]pyrimidine series as well as the evaluation of their potencies toward the Hh signaling pathway (Fig. 2). Furthermore, the pharmacokinetic (PK) property of compound **18** is also profiled. To the best of our knowledge, this is the first report that 6,7-dihydro-5*H*-pyrano[2,3-d]pyrimidine is capable of a surrogate as pyrimidine derivatizing skeleton and its systemic synthesis in drug discovery.

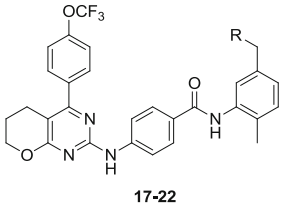
Synthesis of the 6,7-dihydro-5*H*-pyrano[2,3-d]pyrimidine derivatives (**14** and **17–22**)

The 6,7-dihydro-5*H*-pyrano[2,3-d]pyrimidine derivatives (**14** and **17–22**) were prepared as shown in Scheme 1. At first, we constructed the key intermediate 2,4-dichloro-6,7-dihydro-5*H*-pyrano[2,3-d]pyrimidine **10** following a four-step synthetic route. Starting from commercially available delta-valerolactone **6** and methyl thiocyanate (CH_3SCN), the bicyclic pyrano[2,3-d]pyrimidinyl **7** was constructed in the presence of trifluoromethanesulfonic anhydride (Tf_2O) with 23 % yield. The dimethylthio group of **7** was oxidized by 3-chloroperoxybenzoic acid (MCPBA) to provide bis-methylsulfonyl **8**. Nucleophilic displacement of both methylsulfonyl groups using reflux sodium hydroxide (NaOH) solution gave diketone **9**, followed by chlorination using phosphoryl chloride ($POCl_3$) to afford the key building block **10**. Then, the readily prepared **10** was reacted with p-trifluoromethoxylphenylboronic acid under palladium-catalyzed Suzuki–Miyaura coupling condition to yield 4-phenyl-substituted **11**, and then followed by Buchwald–Hartwig cross-coupling reaction of **11** with methyl 4-aminobenzoate under palladium acetate [$Pd(OAc)_2$] catalysis, to afford the intermediate **12** which was easily hydrolyzed to the free acid **13**. 6,7-Dihydro-5*H*-pyrano[2,3-d]pyrimidine derivative **14** was achieved by condensation of **13** with 2,6-dimethylaniline. Derivatives **17–22** were synthesized from carboxylic acid **13** following three steps, which were condensation with 3-amino-4-methylbenzyl alcohol to give **15**, chlorination of **15** with thionyl chloride ($SOCl_2$) to provide **16**, and nucleophilic

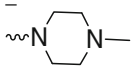
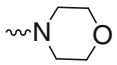
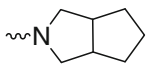
Table 1 Hh signaling pathway inhibitions of 6,7-dihydro-5*H*-pyrano[2,3-d]pyrimidine derivatives



14



17-22

Compounds	R	IC_{50} (nM)
14	–	5.2
17		16.7
18		6.9
19		16.1
20		15.4
21		17.6
22		15.7
1	–	1.3
GDC-0449	–	7.2

substitution of **16** with the corresponding amines to afford **17–22** (Scheme 1).

Hh signaling pathway inhibitions of 6,7-dihydro-5*H*-pyrano[2,3-d]pyrimidine derivatives

The Hh signaling pathway inhibitory activity of our series of 6,7-dihydro-5*H*-pyrano[2,3-d]pyrimidine derivatives (**14** and **17–22**) were assessed using a luciferase reporter in NIH3T3 cell carrying a stably transfected Gli reporter construct (Gli-luciferase reporter cell lines) (Ohashi *et al.*, 2012; Xin *et al.*, 2014b). GDC-0449 was used as positive control. The vitro IC_{50} for this seriesare illustrated in Table 1. It was found that all the analogs of 6,7-dihydro-5*H*-pyrano[2,3-d]pyrimidine derivatives showed good Hh signaling inhibitory activity with IC_{50} range from 5.2 to 17.6 nM. The B-ring of 6,7-dihydro-5*H*-pyrano[2,3-d]pyrimidine scaffold was proved to be an appropriate surrogate for the parent pyrimidine core; whereas the 6,7-dihydro-5*H*-pyrano[2,3-d]pyrimidine analog **14** displayed Hh inhibitory activity with an IC_{50} of 5.2 nM, despite showing weaker potency than the mother compound **1** (1.3 nM), showing slightly more potency than GDC-0449 (7.2 nM). Some aliphatic amine substitutions were incorporated on C-5 of D-ring, derivatizing compounds **17–22**, in order to improve the physicochemical properties

Table 2 PK profile of compound **18**

Compounds	C_{max} (ng/mL)	AUC (hr*ng/mL)	V_z (mL/kg)	Cl (mL/hr/kg)	$MRT_{(0-t)}$ (h)	$T_{1/2}$ (h)
GDC-0449	1,249.82	2,455.65	954.15	406.23	2.23	1.64
18	441.74	1,568.39	4,551.44	621.01	5.02	5.14

Compound **18** (as well as GDC-0449) was formulated using 5 % DMA + 5 % Tween-80, iv 1 mg/kg

based on the previous SAR study. It was found that only the morpholine **18** (6.9 nM) generated satisfactorily equipotent potency compared to GDC-0449; while other amine derivatives, such as *N*-methylpiperazine **17** (16.7 nM), pyrrolidine **19** (16.1 nM), diethylamine **20** (15.4 nM), and bicyclic amines **21** (17.6 nM) and **22** (15.7 nM), exhibited decreased activity compared to GDC-0449 (Table 1).

PK profile of compound **18**

Analog **18** was chosen as a representative of this new scaffold to profile the PK properties in vivo, and the result is illustrated in Table 2. After intravenous injection with 1 mg/kg in SD rats, positive control GDC-0449 displayed satisfactory PK profiles, with high exposure (AUC = 2,455.65 **h** × ng/mL) and lower clearance (Cl = 406.23 mL/hr/kg). Compared to GDC-0449, compound **18** exhibited moderate PK properties, with an acceptable exposure (AUC = 1,568.39 **h** × ng/mL) and relatively low clearance (Cl = 621.01 mL/hr/kg), and a large volume of distribution (V_z = 4,551.44 mL/kg). However, **18** showed lower peak plasma concentrations (C_{max} = 441.74 ng/mL), meaning that it should be administrated in a high dose for generating in vivo antitumor efficacy in Hh signaling dependent or operative solid tumor models. Thus, some more efforts should be made for optimizing the PK characteristics as well as in vitro biological activities for the next structural modification.

Conclusion

In this report, a novel series of 6,7-dihydro-5*H*-pyrano[2,3-d]pyrimidine derivatives as Hh signaling pathway inhibitors have been designed and synthesized, and their Hh signaling pathway inhibitory activities were evaluated by in vitro Gli-luciferase reporter assay. Among this new class of Hh signaling pathway inhibitors, compound **18** exhibited promising potency in vitro and moderate pharmacokinetic properties in vivo compared to GDC-0449. Based on this study, more extensive investigation is deserved to expand so as to understand the structure–activity relationship in depth and to further optimize the biological activities and PK properties.

Materials and methods

Chemistry

Melting points were measured with a Melt-Temp II apparatus and uncorrected. The Nuclear Magnetic Resonance of ^{1}H-NMR spectra (400 MHz) and ^{13}C-NMR (100 MHz) spectra were recorded on a Bruker BioSpin AG (Ultrashield Plus AV 400) spectrometer as deuterochloroform ($CDCl_3$) or dimethyl sulfoxide-d_6 (DMSO-d_6) solutions. The chemicals shift values were expressed in parts per million (ppm) relative to tetramethylsilane as an internal standard ($\delta = 0$) and signals were reported as s (singlet), d (doublet), d (double doublet), t (triplet), and m (multiplet). Mass spectra were recorded on an Agilent technologies 6120 quadrupole LC/MS spectrometer Electrospray Ionization (ESI) method. High-resolution mass spectra (HRMS) were measured on a Water Q-T of micro mass spectrometer. The purity of the compounds was verified by the HPLC study performed on an Agilent Eclipse C18 (4.6 × 150 mm, 5 μm, Agilent) column using a mixture of solvent methanol/water or CH_3CN/H_2O at the flow rate of 2 mL/min and peak detection at 254 nm under UV. The solvents (such as CH_2Cl_2, EtOAc, MeOH, EtOH, and others) were C.P. grade purchased from Nanjing Chemical Co., Ltd. and used without further purification. Column chromatography was carried out on silica gel (200–300 mesh, Qindao Ocean Chemical Company, China). Thin-layer chromatography (TLC) analyses were carried out on silica gel GF254 (Qindao Ocean Chemical Company, China). GDC-0449 was synthesized according to the literature (Robarge *et al.*, 2009). Concentration and evaporation of the solvent after reaction or extraction was carried out on rotary evaporator operated at reduced pressure.

*2,4-Dimethylthio-6,7-dihydro-5H-pyrano[2,3-d]pyrimidine (**7**) (Herrera et al., 2006)*

In a 250-mL reaction vial, a solution of Tf_2O (30 mL, 1.5 eq) in anhydrous CH_2Cl_2 (30 mL) was slowly added to a solution of delta-valerolactone (5 g, 1 eq) and CH_3SCN (13.5 mL, 4 eq) in anhydrous CH_2Cl_2 (50 mL) under −78 °C. The mixture was stirred for 2 h under −70 °C, then warmed to 0 °C, and stirred for 40 h at 0 °C. After the resulting mixture was adjusted to a pH 8–9 with saturated $NaHCO_3$ solution, the organic layer was separated and the aqueous layer was extracted by CH_2Cl_2. The organic layer was combined and dried by anhydrous Na_2SO_4, The solvent was removed in vacuo and the residue was crystallized from *n*-hexane to give the intermediate **7** (3.4 g, 23 %) as a white solid. $R_f = 0.64$ (PE : EtOAc = 5:1). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 4.32 (t, 2H, OCH_2), 2.57 (s, 3H, CH_3), 2.53 (s, 3H, CH_3), 2.52 (t, 2H, $ArCH_2$), 2.04 (m, 2H, CH_2) ppm; MS (ESI) m/z: $[M + H]^+ = 229.0$.

*2,4-Bis(methylsulfonyl)-6,7-dihydro-5H-pyrano[2,3-d]pyrimidine (**8**) (Herrera et al., 2006)*

In a 250-mL reaction vial, MCPBA (14 g, 6.85 eq) was added fractionally to a solution of **7** (2.66 g, 1 eq) in CH_2Cl_2 (70 mL). After 5 h of stirring at room temperature, the reaction was quenched by 5 % $Na_2S_2O_3$ solution (150 mL). The organic phase was separated and washed by saturated $NaHCO_3$ solution. After dried by anhydrous Na_2SO_4, the organic layer was concentrated in vacuo, and the residue was crystallized from CH_3OH, providing the intermediate **8** (2.54 g, 98 %) as a white solid. $R_f = 0.28$ (PE : EtOAc = 5:1). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 4.59 (t, 2H, OCH_2), 3.45 (s, 3H, CH_3), 3.34 (s, 3H, CH_3), 3.33 (t, 2H, $ArCH_2$), 2.16 (m, 2H, CH_2) ppm; MS (ESI) m/z: $[M + H]^+ = 293.1$.

*6,7-Dihydro-5H-pyrano[2,3-d]pyrimidine-2,4-dione (**9**) (Herrera et al., 2006)*

In a 250-mL reaction vial, intermediate **8** (3.34 g, 1 eq) was suspended in a 10 % NaOH methanolic solution (115 mL), and the mixture was refluxed for 2 h. After cooled to room temperature, the reaction was poured into ice water, and adjusted to a pH 1–2 by 3 N HCl in an ice bath. The precipitated solid was collected by filtration and dried in vacuo to give **9** (1.5 g, 91 %) as a white solid. $R_f = 0.55$ (CH_2Cl_2 : CH_3OH = 20:1). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 11.19 (s, 1H, OH), 10.69 (s, 1H, OH), 4.24 (t, 2H, OCH_2), 2.17 (t, 2H, $ArCH_2$), 1.84 (m, 2H, CH_2) ppm; MS (ESI) m/z: $[M + H]^+ = 169.0$.

*2,4-Dichloro-6,7-dihydro-5H-pyrano[2,3-d]pyrimidine (**10**) (Bergeron et al., 2010)*

In a 25-mL reaction vial, DIPEA (8 mL) was slowly added to a mixture of **9** (1.0 g, 1 eq) and $POCl_3$ (8 mL); the reaction mixture was refluxed for 18 h. After cooled to room temperature, the reaction was poured slowly into ice and stirred vigorously for 30 min, followed by extraction with CH_2Cl_2. The organic layer was washed with water and saturated brine, and dried by anhydrous Na_2SO_4. The mother liquor was concentrated and the residue was purified by chromatography (PE : EtOAc = 5:1) to generate the intermediate **10** (636 mg, 52 %) as a white solid. $R_f = 0.25$ (PE : EtOAc = 5:1). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 4.43 (t, 2H, OCH_2), 2.78 (t, 2H, $ArCH_2$), 2.10 (m, 2H, CH_2) ppm; MS (ESI) m/z: $[M + H]^+ = 205.0$.

*2-Chloro-4-(4-(trifluoromethoxy)phenyl)-6,7-dihydro-5H-pyrano[2,3-d]pyrimidine (**11**)*

In a 25-mL reaction vial, a 2 M Na_2CO_3 solution (2 mL) was added to a suspension of $Pd(PPh_3)_2Cl_2$ (140 mg, 0.1 eq) and 4-trifluoromethoxylphenylboronic acid (416 mg, 1 eq) and intermediate **10** (412 mg, 1 eq) in dioxane (10 mL). The mixture was reflux for 2 h under N_2 atmosphere, then cooled to room temperature and filtrated. The filtrate was diluted with H_2O (100 mL) and extracted with EtOAc. After dried by anhydrous Na_2SO_4, the liquor was evaporated and purified by chromatography (PE : EtOAc = 5:1) to provide **11** (210 mg, 32 %) as a white solid. $R_f = 0.32$ (PE : EtOAc = 5:1). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 7.64 (d, 2H, $J = 8.4$ Hz, ArH), 7.31 (d, 2H, $J = 8.4$ Hz, ArH), 4.48 (t, 2H, OCH_2), 2.82 (t, 2H, $ArCH_2$), 2.01 (m, 2H, CH_2) ppm; MS (ESI) m/z: $[M + H]^+ = 331.0$.

*Methyl 4-((4-(4-(trifluoromethoxy)phenyl)-6,7-dihydro-5H-pyrano[2,3-d]pyrimidin-2-yl)amino)benzoate (**12**)*

In a 25-mL reaction vial, $Pd(OAc)_2$ (19 mg, 0.1 eq), BINAP (108 mg, 0.2 eq), and Cs_2CO_3 (850 mg, 3 eq) were added to a solution of **11** (301 mg, 1 eq) and methyl 4-aminobenzoate (131 mg, 0.95 eq) in dioxane (5 mL). The mixture was refluxed for 13 h under N_2 atmosphere. After cooled to room temperature and filtrated, the filtrate was evaporated and purified by chromatography (PE : EtOAc = 5:1) to provide the **12** (334 mg, 86 %) as a white solid. $R_f = 0.15$ (PE : EtOAc = 2:1). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 7.98 (d, 2H, $J = 8.4$ Hz, ArH), 7.70 (m, 4H, ArH), 7.32 (d, 2H, $J = 8.8$ Hz, ArH), 7.22 (s, 1H, NH), 4.46 (t, 2H, OCH_2), 3.86 (s, 3H, CH_3), 2.75 (t, 2H, $ArCH_2$), 1.99 (m, 2H, CH_2) ppm; MS (ESI) m/z: $[M + H]^+ = 446.0$.

*4-((4-(4-(Trifluoromethoxy)phenyl)-6,7-dihydro-5H-pyrano[2,3-d]pyrimidin-2-yl)amino)benzoic acid (**13**)*

In a 25-mL reaction vial, 2 M NaOH (1.5 mL) was added to a solution of **12** (334 mg, 1 eq) in THF (5 mL) and MeOH (5 mL). The reaction mixture was refluxed for 5 h, then cooled to room temperature, poured into ice water (30 mL), and stirred vigorously. After acidification with 1 N HCl, the precipitated solid was collected by filtration and washed with ice water, dried in vacuo to provide **13** (337 mg, 100 %) as a white solid. $R_f = 0.05$ (PE : EtOAc = 2:1). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 9.47 (s, 1H, NH), 7.81 (d, 2H, $J = 8.4$ Hz, ArH), 7.75 (d, 2H, $J = 8.0$ Hz, ArH), 7.64 (d, 2H, $J = 8.4$ Hz, ArH), 7.52 (d, 2H, $J = 8.4$ Hz, ArH), 4.37 (t, 2H, OCH_2), 2.66 (t, 2H, $ArCH_2$), 1.88 (m, 2H, CH_2) ppm; MS (ESI) m/z: $[M + H]^+ = 432.0$.

*N-(2,6-Dimethylphenyl)-4-((4-(4-(trifluoromethoxy)phenyl)-6,7-dihydro-5H-pyrano[2,3-d]pyrimidin-2-yl)amino)benzamide (**14**)*

In a 25-mL reaction vial, HATU (114 mg, 1.25 eq) and TEA (56 mg, 2 eq) were added to a solution of **13** (51 mg, 1 eq) and 2,6-dimethylaniline (15 mg, 1 eq) in DMF (10 mL). The mixture was stirred at room temperature for 16 h under N_2 atmosphere and then poured into ice water (30 mL), following extraction with EtOAc. The liquor was concentrated in vacuo and the residue was purified by chromatography (PE : EtOAc = 2:1) to acquire the product **14** (42 mg, 80 %) as a white solid, mp 246–248 °C. $R_f = 0.18$ (PE : EtOAc = 2:1). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 9.84 (s, 1H, CONH), 9.55 (s, 1H, NH), 7.93 (m, 4H, ArH), 7.84 (d, 2H, $J = 8.4$ Hz, ArH), 7.54 (d, 2H, $J = 8.0$ Hz, ArH), 7.12 (s, 3H, ArH), 4.41 (t, 2H, OCH_2), 2.69 (t, 2H, $ArCH_2$), 2.18 (s, 6H, 2 × CH_3), 1.91 (m, 2H, CH_2) ppm; ^{13}C-NMR (100 MHz, DMSO-d_6) δ 167.67, 164.56, 164.20, 157.66, 148.74, 143.83, 136.97, 135.70, 135.60, 130.81, 129.09, 128.21, 127.61, 127.52, 126.46, 126.23, 120.62, 118.24, 117.37, 104.20, 67.50, 22.29, 22.05, 18.06; MS (ESI) m/z: $[M + H]^+ = 535.3$; HRMS(ESI) m/z calcd for $C_{29}H_{25}N_4O_3F_3Na$ $[M + Na]^+$ 557.1784, found 557.1776.

*N-(5-(Hydroxymethyl)-2-methylphenyl)-4-((4-(4-(trifluoromethoxy)phenyl)-6,7-dihydro-5H -pyrano[2,3-d]pyrimidin-2-yl)amino)benzamide (**15**)*

In a 25-mL reaction vial, HATU (356 mg, 1.2 eq) and DIPEA (201 mg, 2 eq) were added to a solution of **13** (337 mg, 1 eq) and 3-amino-4-methylbenzyl alcohol (107 mg, 1 eq) in DMF (5 mL). The reaction mixture was stirred at 80 °C for 17 h under N_2 atmosphere. The resulting mixture was cooled to room temperature, poured into ice water (50 mL), and extracted by EtOAc. After dried over anhydrous Na_2SO_4, the organic layer was concentrated and purified by chromatography (CH_2Cl_2 : CH_3OH = 50:1) to provide **15** (340 mg, 79 %) as a white solid. $R_f = 0.27$ (CH_2Cl_2 : CH_3OH = 50:1). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 9.85 (s, 1H, CONH), 9.63 (s, 1H, NH), 7.88 (s, 4H, ArH), 7.83 (d, 2H, $J = 8.4$ Hz, ArH), 7.51 (d, 2H, $J = 8.4$ Hz, ArH), 7.29 (s, 1H, ArH), 7.21 (d, 1H, $J = 8.0$ Hz, ArH), 7.10 (d, 1H, $J = 7.6$ Hz, ArH), 5.17 (t, 1H, OH), 4.46 (d, 2H, $J = 5.6$ Hz, $\underline{CH_2}OH$), 4.40 (t, 2H, OCH_2), 2.68 (t, 2H, $ArCH_2$), 2.20 (s, 3H, CH_3), 1.90 (m, 2H, CH_2) ppm; MS (ESI) m/z: $[M + H]^+ = 551.0$.

*N-(5-(Chloromethyl)-2-methylphenyl)-4-((4-(4-(trifluoromethoxy)phenyl)-6,7-dihydro-5H -pyrano[2,3-d]pyrimidin-2-yl)amino)benzamide (**16**)*

In a 25-mL reaction vial, $SOCl_2$ (730 mg, 10 eq) was added to a solution of **15** (340 mg, 1 eq) in CH_2Cl_2

(10 mL). After stirred at room temperature for 17 h, the reaction mixture was washed with saturated $NaHCO_3$ solution and water, and dried over anhydrous Na_2SO_4. The solvent was removed in vacuo and the residue was purified by chromatography (CH_2Cl_2 : CH_3OH = 100:1) to give the chloride **16** (329 mg, 93 %) as a white solid. R_f = 0.52 (CH_2Cl_2 : CH_3OH = 50:1). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 9.87 (s, 1H, CONH), 9.69 (s, 1H, NH), 7.91 (s, 4H, ArH), 7.83 (d, 2H, J = 8.4 Hz, ArH), 7.53 (d, 2H, J = 8.0 Hz, ArH), 7.44 (s, 1H, ArH), 7.28 (d, 1H, J = 8.0 Hz, ArH), 7.23 (d, 1H, J = 8.0 Hz, ArH), 4.76 (s, 2H, $\underline{CH_2}Cl$), 4.40 (t, 2H, OCH_2), 2.68 (t, 2H, $ArCH_2$), 2.23 (s, 3H, CH_3), 1.91 (m, 2H, CH_2) ppm; MS (ESI) m/z: $[M + H]^+$ = 569.0.

*N-(2-Methyl-5-((4-methylpiperazin-1-yl)methyl)phenyl)-4-((4-(4-(trifluoromethoxy)phenyl)-6,7- dihydro-5H-pyrano[2,3-d]pyrimidin-2-yl)amino)benzamide (**17**)*

In a 10-mL reaction vial, *N*-methylpiperazine (44 mg, 5 eq) and K_2CO_3 (24 mg, 3 eq) were added to a solution of **16** (50 mg, 1 eq) in DMF (2 mL). The mixture was stirred at room temperature for 6 h and poured into ice water (20 mL), extracted by EtOAc. After dried over anhydrous Na_2SO_4, the organic layer was concentrated and purified by chromatography (CH_2Cl_2 : CH_3OH = 20:1) to give the product **17** (40 mg, 71 %) as a white solid, mp 184–186 °C. R_f = 0.15 (CH_2Cl_2 : CH_3OH = 20:1). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 9.88 (s, 1H, CONH), 9.67 (s, 1H, NH), 7.92 (s, 4H, ArH), 7.84 (d, 2H, J = 8.4 Hz, ArH), 7.55 (d, 2H, J = 8.0 Hz, ArH), 7.34 (s, 1H, ArH), 7.25 (d, 1H, J = 7.6 Hz, ArH), 7.12 (d, 1H, J = 6.4 Hz, ArH), 4.41 (t, 2H, OCH_2), 3.55 (s, 2H, $PhCH_2N$), 2.69 (t, 2H, $ArCH_2$), 2.68 (m, 8H, $N(CH_2CH_2)_2N$), 2.23 (s, 3H, CH_3), 1.91 (m, 2H, CH_2) ppm; ^{13}C-NMR (100 MHz, DMSO-d_6) δ 167.65, 164.78, 164.20, 157.63, 148.74, 143.94, 136.95, 136.60, 130.82, 130.11, 128.37, 126.24, 120.65, 117.27, 104.24, 67.52, 60.58, 52.75, 49.63, 45.38, 21.54, 17.64; MS (ESI) m/z: $[M + H]^+$ = 633.3; HRMS(ESI) m/z calcd for $C_{34}H_{36}N_6O_3F_3$ $[M + H]^+$ 633.2809, found 633.2801.

*N-(2-Methyl-5-(morpholinomethyl)phenyl)-4-((4-(4-(trifluoromethoxy)phenyl)-6,7-dihydro-5H-pyrano[2,3-d]pyrimidin-2-yl)amino)benzamide (**18**)*

Similar procedure of **17** was employed to provide compound **18** (42 mg, 90 %) as a white solid. mp 237–239 °C. R_f = 0.18 (CH_2Cl_2 : CH_3OH = 20:1). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.95 (s, 1H, CONH), 7.86 (d, 2H, J = 8.4 Hz, ArH), 7.79 (d, 2H, J = 8.4 Hz, ArH), 7.68 (d, 2H, J = 8.0 Hz, ArH), 7.62 (s, 1H, NH), 7.44 (s, 1H, ArH), 7.35 (d, 2H, J = 8.0 Hz, ArH), 7.18 (d, 1H, J = 7.6 Hz, ArH), 7.09 (d, 1H, J = 8.0 Hz, ArH), 4.46 (t, 2H, OCH_2), 3.71 (m, 4H, $(CH_2)_2O$), 3.51 (s, 2H, $PhCH_2N$), 2.74 (t, 2H, $ArCH_2$), 2.47 (s, 4H, $N(CH_2)_2$), 2.31 (s, 3H, $PhCH_3$), 2.00 (m, 2H, CH_2) ppm; ^{13}C-NMR (100 MHz, $CDCl_3$) δ 168.10, 165.38, 165.16, 157.69, 149.93, 143.26, 136.36, 135.95, 130.38, 128.21, 127.78, 125.90, 123.76, 120.73, 118.12, 104.19, 68.05, 66.94, 63.08, 53.52, 29.70, 17.57; MS (ESI) m/z: $[M + H]^+$ = 620.3; HRMS(ESI) m/z calcd for $C_{33}H_{33}N_5O_4F_3$ $[M + H]^+$ 620.2440, found 620.2434.

*N-(2-Methyl-5-(pyrrolidin-1-ylmethyl)phenyl)-4-((4-(4-(trifluoromethoxy)phenyl)-6,7-dihydro-5H-pyrano[2,3-d]pyrimidin-2-yl)amino)benzamide (**19**)*

Similar procedure of **17** was employed, and the compound **19** (56 mg, 100 %) was obtained as a white solid. mp 116–118 °C. R_f = 0.16 (CH_2Cl_2 : CH_3OH = 20:1). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 9.86 (s, 1H, CONH), 9.64 (s, 1H, NH), 7.91 (s, 4H, ArH), 7.84 (d, 2H, J = 8.4 Hz, ArH), 7.54 (d, 2H, J = 8.0 Hz, ArH), 7.30 (s, 1H, ArH), 7.21 (d, 1H, J = 8.0 Hz, ArH), 7.10 (d, 1H, J = 7.6 Hz, ArH), 4.41 (t, 2H, OCH_2), 3.58 (s, 2H, $PhCH_2N$), 2.69 (t, 2H, $ArCH_2$), 2.47 (s, 4H, $N(CH_2)_2$), 2.21 (s, 3H, CH_3), 1.90 (m, 2H, CH_2), 1.70 (s, 4H, CH_2CH_2) ppm; ^{13}C-NMR (100 MHz, DMSO-d_6) δ 167.65, 164.75, 164.19, 157.64, 148.75, 143.89, 136.95, 136.43, 131.99, 130.82, 129.95, 128.34, 126.56, 126.30, 120.65, 117.27, 104.21, 59.01, 53.39, 23.05, 22.06, 21.54, 17.63; MS (ESI) m/z: $[M + H]^+$ = 604.3; HRMS(ESI) m/z calcd for $C_{33}H_{33}N_5O_3F_3$ $[M + H]^+$ 604.2543, found 604.2536.

*N-(5-((Diethylamino)methyl)-2-methylphenyl)-4-((4-(4-(trifluoromethoxy)phenyl)-6,7-dihydro- 5H-pyrano[2,3-d]pyrimidin-2-yl)amino)benzamide (**20**)*

Similar procedure of **17** was employed, and the title compound **20** (50 mg, 94 %) was obtained as a white solid. mp 168–170 °C. R_f = 0.23 (CH_2Cl_2 : CH_3OH = 20:1). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 9.87 (s, 1H, CONH), 9.65 (s, 1H, NH), 7.91 (s, 4H, ArH), 7.84 (d, 2H, J = 8.8 Hz, ArH), 7.54 (d, 2H, J = 8.4 Hz, ArH), 7.31 (s, 1H, ArH), 7.21 (d, 1H, J = 7.6 Hz, ArH), 7.11 (d, 1H, J = 7.6 Hz, ArH), 4.41 (t, 2H, OCH_2), 3.54 (s, 2H, $PhCH_2N$), 2.69 (t, 2H, $ArCH_2$), 2.51 (m, 4H, $N(CH_2)_2$), 2.21 (s, 3H, $PhCH_3$), 1.91 (m, 2H, CH_2), 1.00 (m, 6H, 2 × CH_3) ppm; ^{13}C-NMR (100 MHz, DMSO-d_6) δ 168.15, 165.24, 164.69, 158.14, 149.25, 144.39, 137.44, 136.94, 131.31, 130.41, 128.84, 127.21, 126.80, 121.13, 117.77, 104.70, 68.00, 56.82, 46.49, 29.48, 22.79, 22.04, 18.13, 11.89; MS (ESI) m/z: $[M + H]^+$ = 606.3; HRMS(ESI) m/z calcd for $C_{33}H_{35}N_5O_3F_3$ $[M + H]^+$ 606.2701, found 606.2692.

*N-(5-((Hexahydrocyclopenta[c]pyrrol-2(1H)-yl)methyl)-2-methylphenyl)-4-((4-(4-(trifluoro methoxy)phenyl)-6,7-dihydro-5H-pyrano[2,3-d]pyrimidin-2-yl)amino)benzamide (**21**)*

Similar to the preparation of **17**, the title compound **21** (46 mg, 81 %) was obtained as a white solid. mp 194–196 °C. $R_f = 0.18$ (CH_2Cl_2 : CH_3OH = 20:1). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 9.87 (s, 1H, CONH), 9.65 (s, 1H, NH), 7.92 (s, 4H, ArH), 7.84 (d, 2H, J = 8.4 Hz, ArH), 7.54 (d, 2H, J = 8.0 Hz, ArH), 7.31 (s, 1H, ArH), 7.22 (d, 1H, J = 7.6 Hz, ArH), 7.11 (d, 1H, J = 7.2 Hz, ArH), 4.41 (t, 2H, OCH_2), 3.57 (s, 2H, $PhCH_2N$), 2.69 (t, 2H, $ArCH_2$), 2.67 (m, 2H, $N(CH_2)_2$), 2.22 (s, 3H, CH_3), 2.21 (m, 2H, $N(CH_2)_2$), 1.90 (m, 2H, CH_2), 1.61 (m, 2H, 2 × CH), 1.26-1.60 (m, 6H, octahydrocyclopentane-3 × CH_2) ppm; ^{13}C-NMR (100 MHz, DMSO-d_6) δ, 167.65, 164.77, 164.18, 157.64, 148.74, 143.92, 136.94, 136.46, 130.81, 130.02, 128.35, 126.65, 126.28, 125.93, 120.63, 118.78, 117.28, 104.20, 67.50, 60.57, 58.40, 41.67, 32.39, 22.30, 22.06, 17.63; MS (ESI) m/z: $[M + H]^+$ = 644.3; HRMS(ESI) m/z calcd for $C_{36}H_{37}N_5O_3F_3$ $[M + H]^+$ 644.2857, found 644.2849.

*N-(5-(2-Oxa-6-azaspiro[3.3]heptan-6-ylmethyl)-2-methylphenyl)-4-((4-(4-(trifluoromethoxy)phenyl)-6,7-dihydro-5H-pyrano[2,3-d]pyrimidin-2-yl)amino)benzamide (**22**)*

Similar to the preparation of **17**, the title compound **22** (30 mg, 90 %) was acquired as a white solid. mp 175–177 °C. $R_f = 0.18$ (CH_2Cl_2 : CH_3OH = 20:1). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 9.86 (s, 1H, CONH), 9.62 (s, 1H, NH), 7.90 (s, 4H, ArH), 7.83 (d, 2H, J = 8.4 Hz, ArH), 7.54 (d, 2H, J = 8.4 Hz, ArH), 7.22 (s, 1H, ArH), 7.19 (d, 1H, J = 7.6 Hz, ArH), 7.03 (d, 1H, J = 7.6 Hz, ArH), 4.60 (s, 4H, $N(CH_2)_2$), 4.40 (t, 2H, OCH_2), 3.49 (s, 2H, $PhCH_2N$), 3.32 (s, 4H, $(CH_2)_2O$), 2.68 (t, 2H, $ArCH_2$), 2.19 (s, 3H, $PhCH_3$), 1.89 (m, 2H, CH_2) ppm; ^{13}C-NMR (100 MHz, DMSO-d_6) δ 167.65, 164.72, 164.18, 157.64, 148.75, 143.91, 136.95, 136.51, 132.11, 130.82, 130.04, 128.35, 117.27, 126.32, 126.29, 125.63, 120.65, 118.78, 104.22, 79.91, 67.51, 62.73, 61.72, 28.98, 22.30, 22.06, 17.63; MS (ESI) m/z: $[M + H]^+$ = 632.3; HRMS(ESI) m/z calcd for $C_{34}H_{33}N_5O_4F_3$ $[M + H]^+$ 632.2493, found 632.2485.

In vitro Gli-luciferase reporter assay

The Hh signaling inhibition of the 6,7-dihydro-5*H*-pyrano[2,3-d]pyrimidine derivatives were assessed through a luciferase reporter assay in NIH3T3 cells carrying a stably transfected Gli reporter construct (Gli-luc reporter cell line) (Ohashi *et al.*, 2012; Xin *et al.*, 2013). NIH3T3/Gli-luc cells were treated with DMEM and 10 % FBS and 1 μg/mL Puromycin. The cells were seeded onto 96-well plates at 2 × 10^4cells/well and cultured in the condition of 5 % CO_2 and 37 °C overnight. After incubation, all the prepared compounds (including GDC-0449, as an internal standard control) diluted in a serial 8 × solution (0.05–300 nM) containing 0.5 % FBS and 0.7 μg/mL Sonic Hh agonist were added to each well (n = 4 wells per concentration). The cells were incubated for an additional 48 h. To determine the assay window, cells were incubated in media containing 0.1 % DMSO with or without Sonic Hh (0 % or 100 % inhibition control), respectively. Cells were then harvested and lysed in reporter lysis buffer, and luciferase activities were measured using a Dual-Luciferase® Reporter Assay System (Promega E1910). The activity of the Gli reporter was defined as the ratio of Firefly/Renilla luciferase activities.

In vivo pharmacokinetic Study in SD rats

Six healthy male SD rats (weight ranging from 180 to 240 g, three for GDC-0449 and three for compound **18**) were administered with GDC-0449 or compound **18** at a dose of 1 mg/kg body weight for iv administration. The dosing volume was 5 mL/kg. After administration, blood samples collected at each time point (2 min, 5 min, 15 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h, 10 h, and 24 h) for PK analyses (n = 3) were centrifuged at 4,000 rpm for 5 min at 4 °C. Plasma samples were analyzed after protein precipitation with CH_3CN acidified with 1 % HCOOH. LC/MS/MS (Agilent 6418B) analysis of compound **18** was performed under optimized conditions to obtain the best sensitivity and selectivity of the analyte in the selected reaction monitoring mode containing an internal standard. Plasma concentration–time data were measured by a non-compartmental approach using the software WinNonlin Enterprise, version 5.2 (Pharsight Co.,Mountain View, CA, USA) (Xin *et al.*, 2014b).

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