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Research paper

Discovery of novel 4-(2-pyrimidinylamino)benzamide derivatives as highly potent and orally available hedgehog signaling pathway inhibitors

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ABSTRACT

A series of novel hedgehog signaling pathway inhibitors have been designed by structural modification based on the former reported scaffold of 4-(2-pyrimidinylamino)benzamide. The SAR for this series was described and many derivatives showed potent inhibitory activity. Among these compounds, compounds **12af** and **12bf** were identified to have high potency and optimal PK profiles. Although both of compounds **12af** and **12bf** did not show strong antitumor efficacy in LS-174T nude mice model, they were promising candidates as Hh signaling inhibitors due to great potency against Hh signaling pathway and outstanding PK properties, deserving further evaluation in other Hh signaling operative tumor models.

1. Introduction

Hedgehog (Hh) signaling pathway plays a critical role in regulation of the cell growth, embryonic morphogenesis, tissue patterning, and angiogenesis process [1]. Generally, Hh signaling is silence in the adult human; however, when tumor occurred, Hh signaling pathway became aberrantly activated. Strong evidences suggested that abnormal activation of Hh pathway has been linked to pathogenesis of a variety of human tumor types, such as basal cell carcinoma (BCC), medulloblastoma (MB), rhabdomyosarcoma (RMS), lymphoma, leukemia, lung, pancreatic, hepatocellular, gastric, esophageal, colorectal, ovarian, prostate, melanoma and glioblastoma [2]. Thus, Hh signaling pathway inhibitor is considered to have therapeutic potential for combating many human tumors.

Until now, there are many Hh signaling pathway inhibitors have been reported [3]. Two agents, vismodegib (GDC-0449, **1**) and sonidegib (LDE-225, **2**), have been approved by FDA for treatment of locally advanced BCC [4,5]. Besides, other Hh inhibitors, including taladegib (LY-2940680, **3**) [6], glasdegib (PF-04449913, **4**) [7] and BMS-833923 (XL-139, **5**) [8], are evaluated for their therapeutic use in clinical trials. Obviously, although Hh inhibitors have already been proved to be clinically significant effective in BCC treatment, they still remained to be undefined for their therapeutic potential against other solid and hematological tumors such as lymphoma, leukemia, hepatocellular and so on [9]. Therefore, there is still enthusiastic interest to develop new classes of Hh inhibitors for enlarging their medicinal use (see Fig. 1).

Recently, we have reported our medicinal chemistry effort on the discovery of a novel series of highly potent Hh inhibitors, which contained a central backbone of 4-(2-pyrimidinylamino)benzamide [10]. Subsequently, the systematic SAR of the A-ring, B-ring, C-ring and D-ring of *N*-(2-pyrimidinylamino) benzamide core has been established [11,12]. Furthermore, using scaffold hopping strategy, the structural modification on the B-ring has been explored, and several interesting novel scaffolds such as pyrrolo [2,1-*f*][1,2,4] triazine, thieno [2,3-*d*]pyrimidines, furo [3,2-*d*]pyrimidines, purines, and 6,7-dihydro-5*H*-pyrano [2,3-*d*]pyrinidine have first been developed [13–15]. Although these new scaffolds showed potent Hh signaling inhibitory activity in vitro, they displayed unsatisfactory physic-chemical properties and insufficient pharmacokinetic profiles, and that block their further investigation. Thus, our drug

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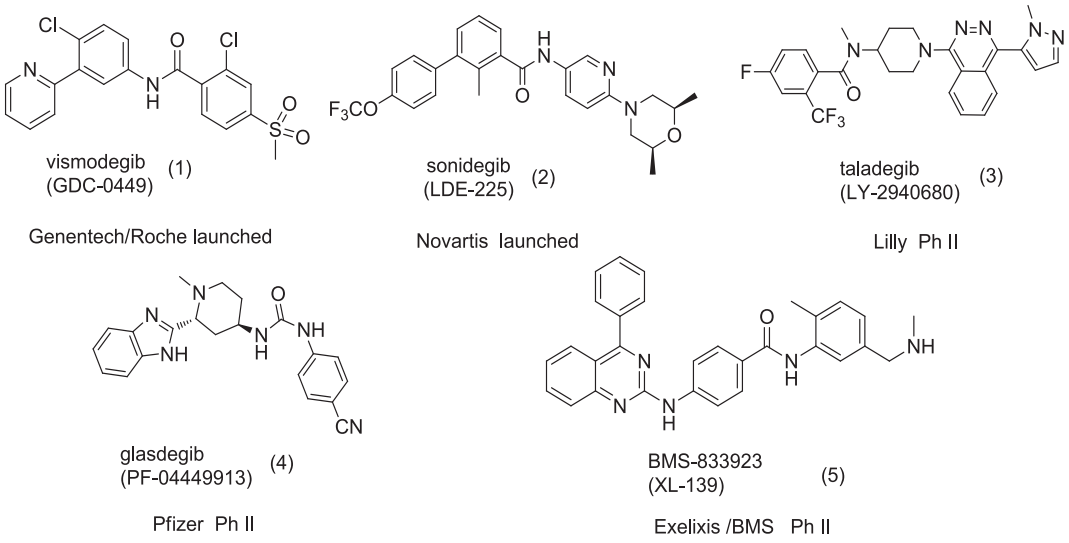


Fig. 1. Representative structures of clinical Hh signaling pathway inhibitors.

development attention was turned back towards the 4-(2-pyrimidinylamino)benzamide scaffold, which is proved to be a privileged skeleton and more druggable, and widely emerging in the drugs and drug candidates. Herein, in this report we reported our further structural derivatizations on the 4-(2-pyrimidinylamino)benzamide skeleton, and also reported the PK evaluations of these analogues in vivo. After the path to candidate nomination, compound **16b** was finally picked out as a drug candidate, which displayed highly potent inhibitory activity against Hh signaling in vitro, improving PK properties, and good anticancer activity in vivo (Fig. 2).

2. Chemistry

The novel 4-(2-pyrimidinylamino)benzamide derivatives were synthesized, as summarized in Table 1. The synthetic routes for all the target compounds were illustrated in Schemes 1–3.

As illustrated in Scheme 1, The commercially available 5-substituted 2,4-dichloropyrimidine (**6**) was treated with 4-trifluoromethoxyphenylboronic acid under Suzuki coupling condition of $Pd(PPh_3)_2Cl_2$ and 2 M aq Na_2CO_3 in reflux dioxane to give product **7**, which was subsequently treated with methyl 4-aminobenzoate under the Buchwald-Hartwig coupling condition of $Pd(OAc)_2$/BINAP/Cs_2CO_3 in reflux dioxane to yield benzoate **8**. Hydrolysis of **8** under basic condition of NaOH afforded free acid **9**. Condensation **9** with 3-amino-4-methylbenzyl alcohol gave benzamide **10**, which was followed by chlorination with thionyl chloride reagent to generate intermediate **11**. Then the target compounds **12a** and **12b** were conveniently synthesized by treating **11** with appropriate secondary amines using the nucleophilic substitution (Scheme 1).

The similar methods were used to prepare compounds **15** and **17**, which were depicted in Scheme 2. The picolinamides **15a-b** and nicotinamide **15c** were synthesized starting from the material **7**. After Buchwald-Hartwig coupling **7** with 5-aminopicolinic acid or 6-aminonicotinic acid, the picolicolinic acid intermediate **12a-b,** and the nicotinic acid intermediate **12c** were prepared, which were subsequently reacted with benzyl alcohol under the HATU/DIPEA catalytic condition to yield the intermediate **13**. The hydroxyl group of **13** was chlorinated, followed by substitution with morpholine to provide the desired product **15**. Likewise, the benzamide **17** was prepared starting from the intermediate **8b**. Using the similar procedures including acylation, chlorination, nucleophilic substitution, the desired products **17** were achieved (Scheme 2).

The synthetic approach to acquire the target product **28** was illustrated in Scheme 3. Claisen condensation of cyclopropylacetate with formate in the presence of LDA gave the β-keto ester **19**. Treating **19** with thiocarbamide followed by transformation of 2-thiocarbonyl group to 2-carbonyl group afforded pyrimidinedione **21**. Subsequent chlorination of **21** with $POCl_3$ followed by Suzuki coupling procedure provided the key intermediate **23**. Afterwards, following several procedures similar to that for preparation of **12a**, the target compound **28** was prepared (Scheme 3).

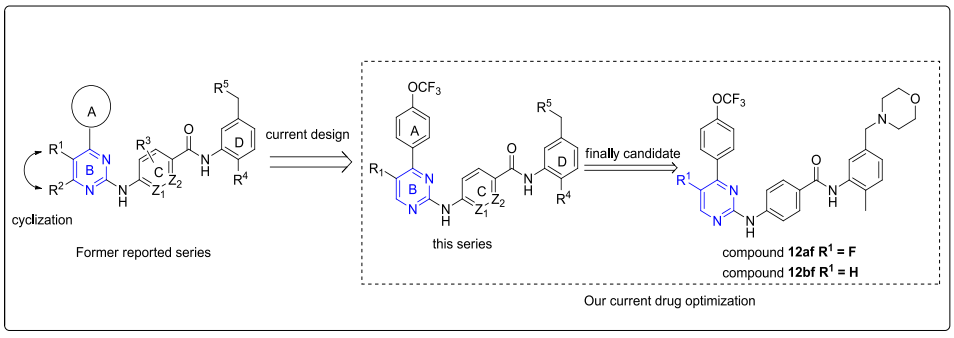
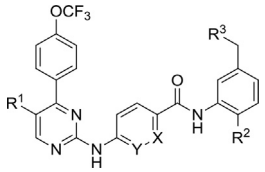


Fig. 2. Our new compounds design.

Table 1
Hh signaling pathway inhibition of 4-(2-pyrimidinylamino)benzamide derivatives.

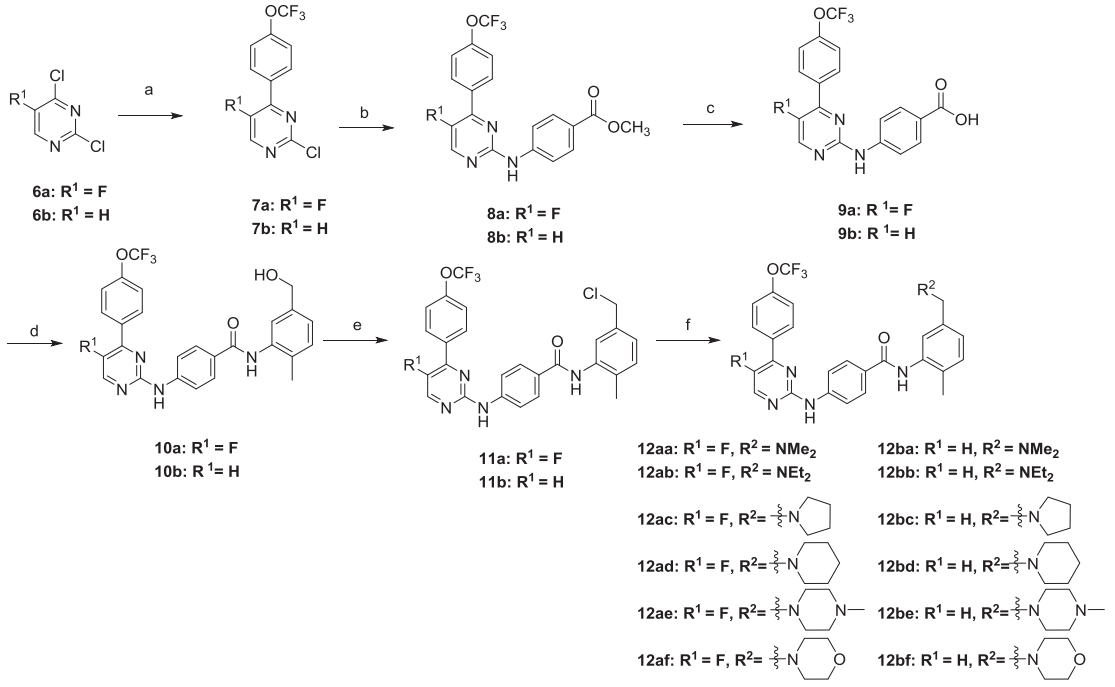


Compds	X	Y	R^1	R^2	R^3	Gli-luc reporter IC_{50} (nM)
12aa	CH	CH	F	Me	NMe_2	3.19
12ab	CH	CH	F	Me	NEt_2	2.56
12ac	CH	CH	F	Me		5.16
12ad	CH	CH	F	Me		3.12
12ae	CH	CH	F	Me		2.84
12af	CH	CH	F	Me		1.03
12ba	CH	CH	H	Me	NMe_2	3.04
12bb	CH	CH	H	Me	NEt_2	2.31
12bc	CH	CH	H	Me		1.83
12bd	CH	CH	H	Me		2.68
12be	CH	CH	H	Me		0.97
12bf	CH	CH	H	Me		0.89
15a	N	CH	F	Me		1.14
15b	N	CH	H	Me		0.69
15c	CH	N	F	Me		0.78
17	CH	CH	H	H		0.56
28	CH	CH	cyclopropyl	Me		11.95
GDC-0449	–	–	–	–	–	7.2
LDE-225	–	–	–	–	–	5.5

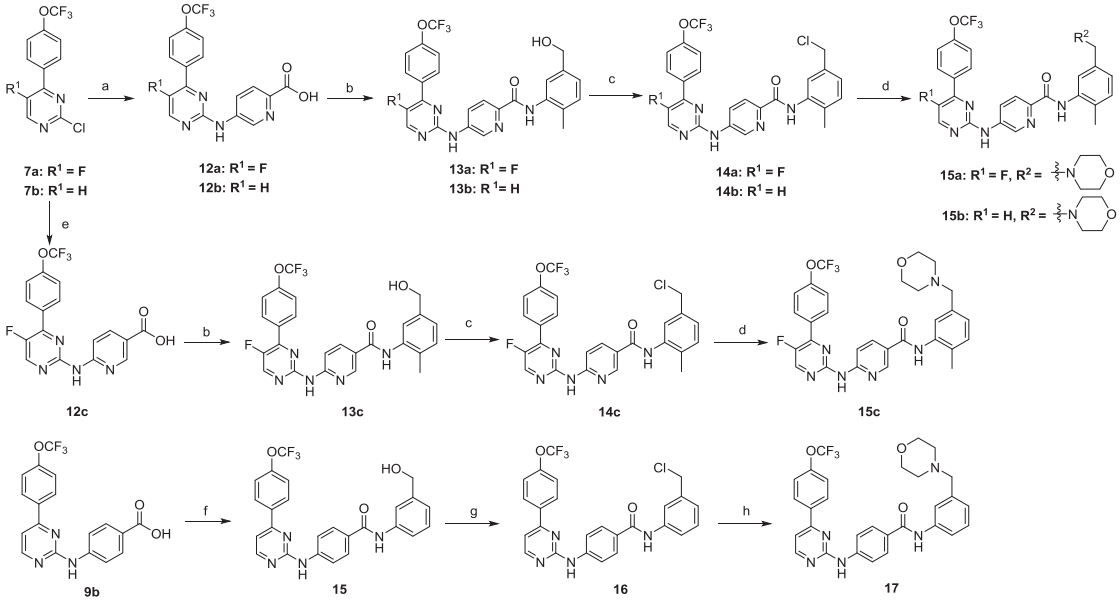
3. Result and discussion

3.1. Hh signaling inhibitory activities of 4-(2-pyrimidinylamino) benzamide derivatives

All the newly synthesized 4-(2-pyrimidinylamino)benzamide derivatives were screened for their inhibitory effect against Hh signaling pathway using a luciferase reporter in NIH3T3 cell carrying a stably transfected Gli-reporter construct (Gli-luciferase reporter cell lines), and the IC_{50} values were summarized in Table 1. On the basis of our previously established SAR, the preferred side chains were initially explored in this paper. It was found that all the newly synthetic compounds exhibited excellent Hh signaling pathway inhibitory activity with IC_{50} values varied from 0.56 nM to 11.95 nM. Except of compound **28**, the rest compounds also displayed more effective than the positive drugs GDC-0449 (IC_{50} = 7.2 nM) and LDE-225 (IC_{50} = 5.5 nM). These results indicated that the preferable substituents picked out from the previous SARs which were very useful information for designing and developing this series of novel 4-(2-pyrimidinylamino)benzamide derivatives. Nevertheless, it was found that substituent at R^3 positions could significantly affect the inhibitory activity. Exploration of substituents of R^3 on D-ring when the R^1 substituent was ensured as fluorine revealed that morpholine (**12af**, 1.03 nM) showed more effective inhibitory activity than dimethylamino (**12aa**, 3.19 nM), diethylamino (**12ab**, 2.56 nM), pyrrolidine (**12ac**, 5.16 nM), piperidine (**12ad**, 3.12 nM) and *N*-methyl piperazine (**12ae**, 2.84 nM). Meanwhile, when the R^1 substituent was fixed as hydrogen, the morpholine (**12bf**, 0.89 nM) also afforded the most potent activity, although (**12be**, 0.97 nM) showed nearly equivalent potency. After elucidating the SAR around P_3 and the morpholine group being picked out, we trend attention to other substituted position. Some representative variation examples of **15a**–**15c, 17** and **28** were attempted. Among these, the phenyl ring was changed to pyridine moiety lead to equivalent activity (**15a** vs **12af, 15b** vs **12bf, 15c** vs **12af**), while an attempt of removal the methyl group of D ring (**17**)



Scheme 1. Reagents and conditions: (a) 4-Trifluoromethoxyphenylboronic acid, $Pd(PPh_3)_2Cl_2$, 2 M aq Na_2CO_3, dioxane, reflux, 14 h, 64% for **7a**, 66% for **7b**; (b) methyl 4-aminobenzoate, $Pd(OAc)_2$, BINAP, Cs_2CO_3, dioxane, reflux, 16 h, 88% for **8a**, 88% for **8b**; (c) NaOH, MeOH/H_2O, reflux, 2 h, 97% for **9a**, 95% for **9b**; (d) 3-amino-4-methylbenzyl alcohol, HATU, DMF, DIPEA, 80 °C 15 h, 79% for **10a**, 73% for **10b**; (e) $SOCl_2$, CH_2Cl_2, rt, 5 h, 75% for **11a**, 91% for **11b**; (f) secondary amine, DMF, K_2CO_3, 80 °C, 5 h, 49–91%.

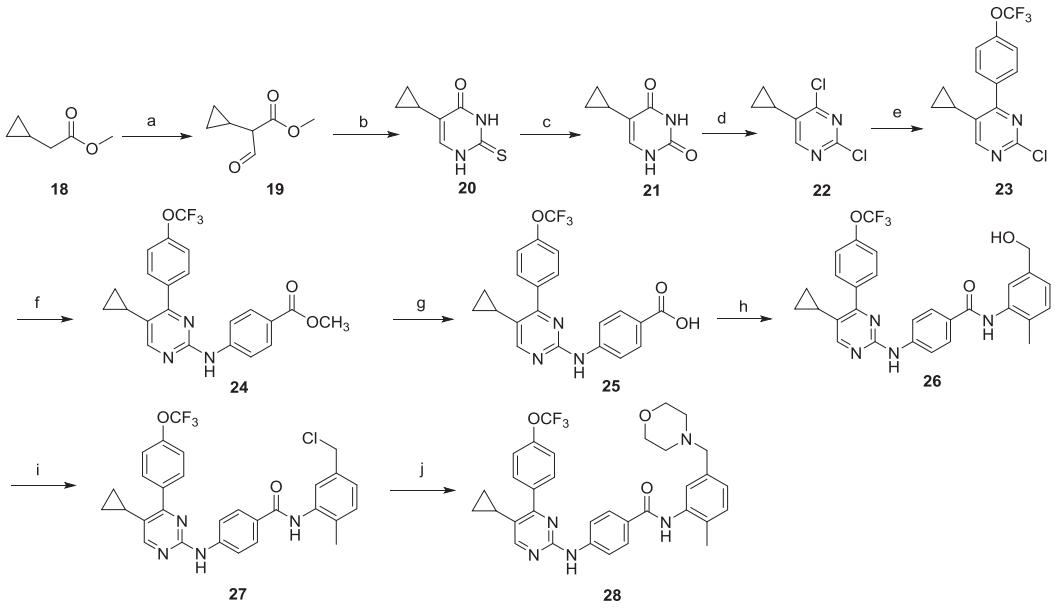


Scheme 2. Reagents and conditions: (a) 5-Aminopicolinic acid, $Pd(OAc)_2$, BINAP, Cs_2CO_3, dioxane, reflux, 16 h, 62% for **12a**, 87% for **12b**; (b) 3-amino-4-methylbenzyl alcohol, HATU, DMF, DIPEA, 80 °C 15 h, 43% for **13a**, 44% for **13b**, 35% for **13c**; (c) $SOCl_2$, CH_2Cl_2, rt, 5 h, 83% for **14a**, 100% for **14b**, 99% for **14c**; (d) morpholine, DMF, K_2CO_3, 80 °C, 5 h, 83% for **15a**, 40% for **15b**, 59% for **15c**; (e) 6-Aminonicotinic acid, $Pd(OAc)_2$, BINAP, Cs_2CO_3, dioxane, reflux, 16 h, 65%; (f) 3-aminobenzyl alcohol, HATU, DMF, DIPEA, 80 °C, 15 h, 97%; (g) $SOCl_2$, CH_2Cl_2, rt, 5 h, 70%; (h) morpholine, DMF, K_2CO_3, 80 °C, 5 h, 70%.

afforded more potent activity (IC_{50} = 0.56 nM). However, when structural modification was attempted by introducing a cyclopropyl group on B ring, the inhibitory activity showed reduced. Obviously, this series of compounds showed excellent potencies in the Gli-luciferase reporter assay, which were certainly significant Hh signaling pathway inhibitors. However, the precise target within Hh pathway for these compounds, such as SMO, Gli, or others, is still unknown. Nevertheless, considering the relevant similarity of this series of compounds with the reported structures such as BMS-833923, ALLO-2, it appears they probably target SMO, which should be identified in the further evaluation.

3.2. Pharmacokinetic (PK) profiles of selective compounds

After carefully considering the screened results of the in vitro Hh signaling pathway inhibitory activities, several potent compounds



Scheme 3. Reagents and conditions: (a) $HCOOEt_2$,THF, LDA, −78 °C to rt, 2 h, 90%; (b) thiocarbamide, MeOH, reflux, overnight, 23%; (c) $ClCH_2COOH$, EtOH/H_2O, reflux, 7 h, 70%; (d) $POCl_3$, DMF, 100 °C, 2 h, 90%; (e) 4-trifluoromethoxyphenylboronic acid, $Pd(PPh_3)_2Cl_2$, TEA, DMF, H_2O, 80 °C, 6 h, 62%; (f) methyl 4-aminobenzoate, $Pd(OAc)_2$, BINAP, Cs_2CO_3, dioxane, reflux, overnight; 32%; (g) NaOH, MeOH/H_2O, reflux, overnight; 68%; (h) 3-amino-4-methylbenzyl alcohol, HATU, DMF, DIPEA, 85 °C overnight, 82%; (i) $SOCl_2$, CH_2Cl_2, rt, 5 h, 76%; (j) morpholine, DMF, K_2CO_3, 80 °C, 5 h, 72%.

Table 2
PK parameters of selective compounds in SD rats.

Compds	Dose (mg/kg)	C_{max} (ng/mL)	$AUC_{(0-t)}$ (hr × ng/mL)	Vz (mL/kg)	Cl (mL/h/kg)	$MRT_{(0-t)}$ (h)	$T_{1/2}$ (h)	F (%)
12af	1 (iv)[a]	1013.73	2031.06	4669.1	464.86	4.85	6.98	
	5 (po)[b]	494.48	4721.90	12033.24	927.77	7.42	9.08	37.2%
12bf	1 (iv)[a]	1197.64	2437.61	2865	401.49	4.22	4.96	
	5 (po)[b]	634.71	6222.13	7128.72	739.62	6.98	6.68	42.0%
15a	1 (iv)[c]	3139.28	1891.59	6281.04	502.06	4.23	8.85	
	5 (po)[b]	86.60	923.07	50455.46	4847.26	7.74	7.24	–
15b	1 (iv)	–	–	–	–	–	–	
	5 (po)[b]	137.20	660.16	59601.85	7406.95	4.83	5.56	
15c	1 (iv)[c]	769.64	624.66	4619.01	1581.91	1.98	2.02	
	5 (po)	–	–	–	–	–	–	
17	1 (iv)[c]	915.45	769.13	6334.30	1292.62	1.53	3.59	
	5 (po)	–	–	–	–	–	–	
GDC-0449	1 (iv)[a]	1249.82	2455.65	954.15	406.23	2.23	1.64	
	5 (po)[b]	3638.56	22403.25	1107.41	280.84	3.93	2.46	>100%
LDE-225	1 (iv)[a]	1929.11	2199.66	955.48	461.36	1.58	1.43	
	5 (po)[d]	1399.20	8825.89	3405.93	583.50	5.40	4.01	80.6%

[a] 5% DMA + 5% Tween-80.
[b] 0.5% HPMC + 0.3% Tween-80 pH = 2.8.
[c] 5% DMA + 5% Tween-80.
[d] PEG300/5% dextrose in water (75:25, v/v).

were selected to profile the PK properties in vivo. Table 2 illustrated the PK parameters for compounds **12af, 12bf, 15a, 15b, 15c**, **17**, as well as GDC-0449 and LDE-225. Despite their similar Hh inhibitory activities in vitro, these compounds exhibited significantly different PK properties. After intravenous (iv) injection with 1 mg/kg in SD rats, both compound **12af** containing a fluorine in B ring and compound **12bf** bearing no substituent at 5- position of B ring showed almost equivalently high plasma exposure (**12af**: AUC = 2031.06 h × ng/mL, and **12bf**: AUC = 2437.61 h × ng/mL), equivalently low clearance (**12af**: Cl = 464.86 mL/h/kg, and **12bf**: Cl = 401.49 mL/h/kg), in comparison to the positive drugs of GDC-0449 and LDE-225, while both compounds **15c** and **17** displayed disappointingly plasma exposure (**15c**: AUC = 624.66 h × ng/mL, and **17**: AUC = 769.13 h × ng/mL) and clearance (**15c**: Cl = 1581.91 mL/h/kg, and **17**: Cl = 1292.62 mL/h/kg), despite of their showing more effective Hh signaling inhibitory activity. Compound **15a** displayed the relatively satisfactory moderate area-under-curve (AUC = 1950.0 h × ng/mL) and clearance (Cl = 502.06 mL/h/kg), which suggested that incorporation of nitrogen atom into C ring was tolerable to maintain preferable PK properties. Therefore, the compounds **12af, 12bf, 15a** as well as **15b** were further investigated the PK properties by orally administration at 5 mg/kg dose in SD rats. Notably, **12af** and **12bf** showed optimal PK properties with long half-life (**12af**: 9.08 h and **12bf**: 6.68 h), high plasma exposure (**12af**: 4721.90 h × ng/mL and **12bf**: 6222.13 h × ng/mL), and good oral bioavailability (**12af**: 37.2% and **12bf**: 42.0%), although they were still not better than GDC-0449 and LDE-225. However unluckily, much lower exposure was observed for both **15a** and **15b** (**15a:** 923.07 h × ng/mL and **15b**: 660.16 h × ng/mL). Thus, it is no doubt that both of **12af** and **12bf**

Table 3
Inhibitory effects of selective compounds on given solid tumor cell proliferation.

Compounds	LS174T (μM)	PC3 (μM)	BxPC3 (μM)
12af	9.1	11.55	14.47
12bf	12.17	14.85	35.41
GDC-0449	45.81	66.78	47.95

possess enough ideal PK properties for further evaluation, and obviously, the PK profiles of compound **12bf** was superior to **12af** (Table 2).

3.3. Cellular anti-proliferative evaluation of compounds *12af* and *12bf*

Since the therapeutic efficacy to the solid tumors is an important clinical concern for Hh signaling pathway inhibitors, new prepared compounds with the capacity to address anti-proliferative effects against solid tumor cell lines are expected. In this regard, the compounds **12af** and **12bf** with high Hh signaling inhibitory activities and optimal PK properties were further evaluated for their anti-proliferative effects in three tumor cell lines including LS174T, PC3 and BxPC3. The anti-proliferative potency for the compounds was measured by ATP-dependent bioluminescence assay [16], and the results were shown in Table 3. It was found both of two compounds as well as GDC-0449 exhibited high potencies in the three cancer cell lines. Compound **12af** showed **3**–**6** fold more potent anti-proliferative activity than GDC-0449 in all of the three cell lines of LS174T, PC3 and BxPC3, with IC_{50} values of 9.1 μM, 11.55 μM and 14.47 μM, respectively. Compound **12bf** displayed similar inhibitory potency against the proliferations of LS174T, PC3 and BxPC3 cell lines, with IC_{50} values of 12.17 μM, 14.85 μM and 35.41 μM respectively, which are also higher efficacy than GDC-0449. This suggested Hh signaling pathway is significantly correlated to the growth of several specific solid cancers and Hh inhibitors has the cell-based potential to combat solid cancers (Table 3).

3.4. In vivo efficacy evaluation of compounds *12af* and *12bf*

To evaluate the in vivo efficacy of the best compounds against solid tumors, anti-tumor activity was initiatively investigated in the LS-174T nude mice xenograft model. For this model, tumor growth is specifically related to Hh signaling pathway, which is reported elsewhere, and also identified in our cell-based anti-proliferative assay. Compounds **12af** and **12bf** with both in-vitro potent inhibitory activities and optimal in-vivo PK profiles were evaluated, and the results were summarized in Table 4. Mice bearing LS-174T cells were orally administered with **12af** (18.75 mg/kg, 37.5 mg/kg and 75 mg/kg) twice daily, **12bf** (75 mg/kg) twice daily, as well as **12bf** (37.5 mg/kg, 75 mg/kg and 150 mg/kg) once daily for 10 days at indicated doses, and **GDC-0449** (75 mg/kg, twice daily) were also tested as comparison. The body weight and the volume of the tumor were measured twice per week. After continuous administration for 10 days, it was found that **12af** and **12bf** as well as **GDC-0449** at given doses showed good tumor volume growth inhibition (23.25%–42.93%), and 75 mg/kg twice daily regimen of **16b** provided the best tumor volume growth inhibition (42.93%). However, by the tumor weight growth inhibitory measure, **12af** and **12bf** as well as **GDC-0449** did not give strong tumor weight regression (only <25%) and there lacked of definite dose-efficacy dependent correlation. Nevertheless, it is noted that these drugs were well tolerated and no any significant loss of mice body weight after treatment (data not shown). On the whole, from these results, it appears that Hh signaling inhibitors as single-agent therapeutic regimen may be still inadequate for treatment of solid tumors such as colorectal cancer, in which abnormal Hh signaling activation was not the primary driver for tumor growth and development. Nevertheless, compounds **12af** and **12bf** were still considered to be the promising leading candidates as novel Hh signaling inhibitors, which exhibited superior Hh signaling inhibitory activity, optimal PK properties in oral administration, compared to GDC-0449 and LDE-225. Therefore, compounds **12af** and **12bf** deserved to be further evaluated in other Hh signaling operative tumor models (Table 4).

Table 4
Antitumor efficacy of selective compounds on the LS-174T nude mice xenograft model.[a]

Compounds	Dose	No. of death	IR (%, TW)	IR (%, TV)
GDC-0449	75 mg/kg, bid	0	11.8	34.49
12af	18.75 mg/kg, bid	0	18.4	33.99
12af	37.5 mg/kg, bid	0	17.3	34.92
12af	75 mg/kg, bid	0	14.4	33.12
12bf	75 mg/kg, bid	0	23.3	42.93
12bf	37.5 mg/kg, qd	0	10.2	32.99
12bf	75 mg/kg, qd	0	9.9	23.25
12bf	150 mg/kg, qd	0	21.1	38.90

[a] Selective compounds as well as GDC-0449 were orally administrated once (or twice) daily to the mice bearing LS-174T cells for 10 days at indicated doses. The body weight and the volume of the tumors were measured twice per week (n = 6 in treated group, n = 9 in vehicle group). GDC-0449 was used as a reference compound. The therapeutic effects of selective compounds at day 10 were determined by IR (%, TW) and IR (%, TV) values. IR (%, TW) = ($Control_{TW}$ − $Treated_{TW}$)/$Control_{TW}$; IR (%, TV) = ($Control_{TV}$ − $Treated_{TV}$)/$Control_{TV}$, TW: Tumor weight, TV: Tumor volume.

4. Conclusion

A series of novel 4-(2-pyrimidinylamino)benzamide derivatives have been designed by structural modification based on the former reported hedgehog signaling pathway inhibitors. The expanded comprehensive SAR was described and many derivatives were found to show potent Hh signaling inhibitory activity. Among these compounds, compounds **12af** and **12bf** were selected to profile their PK properties, measure their anti-proliferative activity against LS-174T cells in vitro, and further evaluate their antitumor efficacy in vivo. Despite of both of compounds **12af** and **12bf** showing weak antitumor efficacy in LS-174T nude mice model, they have showed highly potency against Hh signaling pathway and optimal PK properties, deserving to further evaluation in Hh signaling operative tumor models.

5. Experimental

5.1. General methods

^{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. Mass spectra (MS) were recorded on an Agilent technologies 6120 quadrupole LC/MS spectrometer, and High-resolution mass spectra (HRMS) were obtained on a Water Q-Tof micro mass spectrometer. The purity of the compounds was verified by the HPLC study using a mixture of solvent methanol/water or acetonitrile/water at the flow rate of 2 mL/min and peak detection at 254 nm under UV. Column chromatography was carried out on silica gel (200–300 mesh). All the reactions were monitored using thin layer chromatography (TLC) on silica gel plates. All the reagents were purchased from commercial sources, and used without further purification unless especially stated.

*5.1.1. 2-Chloro-5-fluoro-4-(4-(trifluoromethoxy)phenyl)pyrimidine (**7a**)*

It was prepared according to the method we recently described [10], slightly yellow solid (0.92 g, 64%), MS (ESI) *m/z*: $[M+H]^+ = 293.0$. ^1H NMR (400 M, $CDCl_3$) δ 8.55 (d, 1H, $J = 2.8$ Hz, ArH), 8.23 (d, 2H, $J = 8.8$ Hz, ArH), 7.38 (d, 2H, $J = 8.8$ Hz, ArH) ppm.

*5.1.2. Methyl 4-((5-fluoro-4-(4-(trifluoromethoxy)phenyl) pyrimidin-2-yl)amino) benzoate (**8a**)*

It was prepared according to the method we recently reported [10], as white product (0.28 g, 88%), MS (ESI) *m/z*: $[M+H]^+ = 408.1$. ^1H NMR (400 M, DMSO-d_6) δ 10.31 (s, 1H, NH), 8.76 (d, 1H, $J = 2.8$ Hz, ArH), 8.21 (d, 2H, $J = 8.4$ Hz, ArH), 7.93 (s, 4H, ArH), 7.63 (d, 2H, $J = 8.4$ Hz, ArH), 3.82 (s, 3H, OCH_3) ppm.

*5.1.3. 4-((5-Fluoro-4-(4-(trifluoromethoxy)phenyl)pyrimidin-2-yl) amino)benzoic acid (**9a**)*

It was synthesized as we recently described [10], as white product (0.24 g, 94%), MS (ESI) *m/z*: $[M+H]^+ = 394.3$. ^1H NMR (400 M, DMSO-d_6) δ 12.57 (brs, 1H, COOH), 10.26 (s, 1H, NH), 8.76 (d, 1H, $J = 2.8$ Hz, ArH), 8.21 (d, 2H, $J = 8.4$ Hz, ArH), 7.93 (s, 4H, ArH), 7.63 (d, 2H, $J = 8.4$ Hz, ArH) ppm.

*5.1.4. 4-((5-Fluoro-4-(4-trifluoromethoxyphenyl)pyrimidin-2-yl) amino)-N-(5-(hydroxymethyl)-2-methylphenyl)benzamide (**10a**)*

To a solution of **9a** (75 mg, 0.15 mmol) and 3-amino-4-methylbenzyl alcohol (36 mg, 0.26 mmol) in DMF (10 mL), HATU (108 mg, 0.28 mmol) and DIPEA (126 mg, 0.98 mmol) were added. The mixture was then stirred at 80 °C for 15 h under N_2 atmosphere. After cooling to room temperature, the reaction mixture was poured into ice water (50 mL) followed by extraction with EtOAc. The organic layer was dried over anhydrous Na_2SO_4 and concentrated. The residue was purified by chromatography (DCM/MeOH, 80:1) to give the compound **10a** (77 mg, 79%) as a slight yellow solid, MS (ESI) *m/z*: $[M+H]^+ = 513.2$. ^1H NMR (400 M, DMSO-d_6) δ 10.20 (s, 1H, CONH), 9.70 (s, 1H, NH), 8.76 (d, 1H, $J = 2.8$ Hz, ArH), 8.22 (d, 2H, $J = 8.4$ Hz, ArH), 7.99 (d, 2H, $J = 8.8$ Hz, ArH), 7.93 (d, 2H, $J = 8.8$ Hz, ArH), 7.63 (d, 2H, $J = 8.4$ Hz, ArH), 7.30 (s, 1H, ArH), 7.22 (d, 1H, $J = 7.6$ Hz, ArH), 7.11 (d, 1H, $J = 7.6$ Hz, ArH), 5.18 (s, 1H, OH), 4.49 (d, 2H, $J = 5.6$ Hz, $ArCH_2O$), 2.21 (s, 3H, $ArCH_3$) ppm.

*5.1.5. N-(5-(Chloromethyl)-2-methylphenyl)-4-((5-fluoro-4-(4-trifluoromethoxyphenyl)pyrimidin-2-yl)amino)benzamide (**11a**)*

$SOCl_2$ (0.5 mL) was added to a solution of **10a** (554 mg, 1.08 mmol) in DCM (10 mL). The mixture was stirred at room temperature for 5 h. The reaction mixture was washed with water and the organic layer dried over anhydrous Na_2SO_4 and then concentrated to give the intermediate **11a** (430 mg, 75%) as a yellow solid, MS (ESI) *m/z*: $[M+H]^+ = 531.2$. ^1H NMR (400 M, DMSO-d_6) δ 10.21 (s, 1H, CONH), 9.75 (s, 1H, NH), 8.77 (d, 1H, $J = 3.2$ Hz, ArH), 8.22 (d, 2H, $J = 8.8$ Hz, ArH), 7.99 (d, 2H, $J = 8.8$ Hz, ArH), 7.93 (d, 2H, $J = 8.8$ Hz, ArH), 7.63 (d, 2H, $J = 8.4$ Hz, ArH), 7.44 (s, 1H, ArH), 7.27 (d, 1H, $J = 7.6$ Hz, ArH), 7.24 (d, 1H, $J = 7.6$ Hz, ArH), 4.76 (s, 2H, $ArCH_2Cl$), 2.24 (s, 3H, $ArCH_3$) ppm.

*5.1.6. N-(5-((Dimethylamino)methyl)-2-methylphenyl)-4-((5-fluoro-4-(4-trifluoromethoxyphenyl)pyrimidin-2-yl)amino) benzamide (**12aa**)*

Dimethylamine hydrochloride (58 mg, 0.72 mmol) and K_2CO_3 (124 mg, 0.9 mmol) were added to a solution of **11a** (190 mg, 0.36 mmol) in DMF (10 mL). The mixture was stirred at 80 °C for 5 h. The reaction mixture was cooled to room temperature and poured into ice water (50 mL) and then extracted with EtOAc. The organic layer was dried over anhydrous Na_2SO_4 and concentrated. The residue was purified by chromatography (DCM/MeOH, 50:1) to afford compound **12aa** (68 mg, 35%) as a slight yellow solid, MS (ESI) *m/z*: $[M+H]^+ = 540.2$. ^1H NMR (400 M, DMSO-d_6) δ 10.19 (s, 1H, CONH), 9.68 (s, 1H, NH), 8.75 (d, 1H, $J = 2.8$ Hz, ArH), 8.22 (d, 2H, $J = 8.8$ Hz, ArH), 7.99 (d, 2H, $J = 8.4$ Hz, ArH), 7.93 (d, 2H, $J = 8.4$ Hz, ArH), 7.63 (d, 2H, $J = 8.8$ Hz, ArH), 7.29 (s, 1H, ArH), 7.22 (d, 1H, $J = 8.0$ Hz, ArH), 7.08 (d, 1H, $J = 7.6$ Hz, ArH), 3.38 (s, 2H, $ArCH_2N$), 2.22 (s, 3H, $ArCH_3$), 2.17 (s, 6H, $N(CH_3)_2$) ppm. ^{13}C NMR (100 M, DMSO-d_6) δ 164.72, 156.12, 156.10, 151.56, 150.01, 149.73, 149.64, 149.04, 148.07, 147.81, 143.44, 137.64, 136.43, 132.15, 132.11, 130.96, 130.90, 129.96, 128.47, 126.98, 126.81, 126.12, 121.28, 121.13, 118.72, 117.39, 62.85, 44.81, 17.65. HPLC: 96.21%.

*5.1.7. N-(5-((Diethylamino)methyl)-2-methylphenyl)-4-((5-fluoro-4-(4-trifluoromethoxyphenyl)pyrimidin-2-yl)amino)benzamide (**12ab**)*

Similar procedure of **12aa** was performed to give compound **12ab** (41 mg, 76%) as a yellow solid, MS (ESI) *m/z*: $[M+H]^+ = 568.3$. ^1H NMR (400 M, DMSO-d_6) δ 10.19 (s, 1H, CONH), 9.69 (s, 1H, NH), 8.75 (d, 1H, $J = 3.2$ Hz, ArH), 8.22 (d, 2H, $J = 8.4$ Hz, ArH), 7.99 (d, 2H, $J = 8.4$ Hz, ArH), 7.93 (d, 2H, $J = 8.4$ Hz, ArH), 7.63 (d, 2H, $J = 8.4$ Hz, ArH), 7.29 (s, 1H, ArH), 7.20 (d, 1H, $J = 7.6$ Hz, ArH), 7.10 (d, 1H, $J = 7.6$ Hz, ArH), 3.50 (s, 2H, $ArCH_2N$), 2.51 (m, 4H, $N(CH_2)_2$), 2.21 (s, 3H, $ArCH_3$), 1.05–0.90 (m, 6H, $(CH_3)_2$) ppm. ^{13}C NMR (100 M, DMSO-d_6) δ 164.70, 156.12, 156.10, 151.56, 150.01, 149.71, 149.62, 149.03, 148.07, 147.81, 143.43, 137.64, 136.40, 132.15, 132.09, 131.94, 130.96, 130.89, 129.87, 128.48, 126.97, 126.58, 125.94, 121.28, 121.12, 118.72, 117.39, 56.44, 46.03, 22.05, 11.60. HPLC: 97.81%.

*5.1.8. 4-((5-Fluoro-4-(4-trifluoromethoxyphenyl)pyrimidin-2-yl) amino)-N-(2-methyl-5-((1-pyrrolidinyl)methyl)phenyl)benzamide (**12ac**)*

Similar procedure of **12aa** was performed to give compound **12ac** (41 mg, 76%) as a yellow solid, MS (ESI) *m/z*: $[M+H]^+ = 566.3$. ^1H NMR (400 M, DMSO-d_6) δ 10.20 (s, 1H, CONH), 9.70 (s, 1H, NH), 8.76 (d, 1H, $J = 3.2$ Hz, ArH), 8.23 (d, 2H, $J = 8.4$ Hz, ArH), 8.00 (d, 2H, $J = 8.4$ Hz, ArH), 7.93 (d, 2H, $J = 8.8$ Hz, ArH), 7.64 (d, 2H, $J = 8.4$ 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), 3.56 (s, 2H, $ArCH_2N$), 2.55–2.35 (m, 4H, pyrrolidine-H), 2.22 (s, 3H, $ArCH_3$), 1.78–1.65 (m, 4H, pyrrolidine-H) ppm. ^{13}C NMR (100 M, DMSO-d_6) δ 164.74, 156.12, 156.09, 151.56, 150.01, 149.73, 149.63, 149.03, 148.06, 147.80, 143.44, 137.09, 136.39, 132.14, 132.09, 131.94, 130.96, 130.90, 129.94, 128.48, 126.97, 126.52, 125.85, 121.27, 121.13, 118.72, 117.39, 59.10, 53.41, 28.94, 17.64. HPLC: 98.16%.

*5.1.9. 4-((5-Fluoro-4-(4-trifluoromethoxyphenyl)pyrimidin-2-yl) amino)-N-(2-methyl-5-((1-piperidyl)methyl)phenyl)benzamide (**12ad**)*

Similar procedure of **12aa** was performed to give compound **12ad** (43 mg, 79%) as a yellow solid, MS (ESI) *m/z*: $[M+H]^+ = 579.3$. ^1H NMR (400 M, DMSO-d_6) δ 10.19 (s, 1H, CONH), 9.69 (s, 1H, NH), 8.76 (d, 1H, $J = 3.2$ Hz, ArH), 8.22 (d, 2H, $J = 8.4$ Hz, ArH), 7.98 (d, 2H, $J = 8.4$ Hz, ArH), 7.92 (d, 2H, $J = 8.4$ Hz, ArH), 7.63 (d, 2H, $J = 8.4$ Hz, ArH), 7.26 (s, 1H, ArH), 7.21 (d, 1H, $J = 7.2$ Hz, ArH), 7.08 (d, 1H, $J = 7.2$ Hz, ArH), 3.39 (s, 2H, $ArCH_2N$), 2.40–2.25 (m, 4H, piperidine-H), 2.21 (s, 3H, $ArCH_3$), 1.54–1.42 (m, 4H, piperidine-H), 1.40–1.35 (m, 2H, piperidine-H) ppm. ^{13}C NMR (100 M, DMSO-d_6) δ 164.73, 156.12, 156.09, 151.56, 150.01, 149.72, 149.63, 149.03, 148.06, 147.80, 143.44, 136.38, 132.14, 132.08, 130.96, 130.90, 129.91, 128.48, 126.88, 126.29, 121.27, 121.13, 118.71, 117.38, 62.37, 53.78, 25.45, 23.94, 17.65. HPLC: 93.34%.

*5.1.10. 4-((5-Fluoro-4-(4-trifluoromethoxyphenyl)pyrimidin-2-yl) amino)-N-(2-methyl-5-((4-methyl-1-piperazinyl)methyl)phenyl) benzamide (**12ae**)*

Similar procedure of **12aa** was performed to give compound **12ae** (67 mg, 74%) as a yellow solid, MS (ESI) *m/z*: $[M+H]^+ = 595.3$. ^{1}H NMR (400 M, DMSO-d_6) δ 10.20 (s, 1H, CONH), 9.70 (s, 1H, NH), 8.76 (d, 1H, $J = 3.2$ Hz, ArH), 8.22 (d, 2H, $J = 8.8$ Hz, ArH), 7.99 (d, 2H, $J = 8.8$ Hz, ArH), 7.93 (d, 2H, $J = 8.8$ Hz, ArH), 7.63 (d, 2H, $J = 8.4$ Hz, ArH), 7.27 (s, 1H, ArH), 7.22 (d, 1H, $J = 7.6$ Hz, ArH), 7.08 (d, 1H, $J = 7.6$ Hz, ArH), 3.43 (s, 2H, $ArCH_2N$), 2.48–2.30 (m, 8H, piperazine-H), 2.21 (s, 3H, $ArCH_3$), 2.20 (s, 3H, NCH_3) ppm. ^{13}C NMR (100 M, DMSO-d_6) δ 164.72, 156.12, 151.56, 150.00, 149.72, 149.63, 149.04, 148.08, 147.83, 143.45, 136.44, 135.87, 132.17, 130.97, 130.91, 129.98, 128.50, 126.92, 126.88, 126.28, 121.27, 121.15, 118.72, 117.37, 61.51, 54.46, 52.13, 45.34, 17.66. HPLC: 98.21%.

*5.1.11. 4-((5-Fluoro-4-(4-trifluoromethoxyphenyl)pyrimidin-2-yl) amino)-N-(2-methyl-5-((4-morpholinyl)methyl)phenyl)benzamide (**12af**)*

Similar procedure of **12aa** was performed to give compound **12af** (50 mg, 91%) as a yellow solid, MS (ESI) *m/z*: $[M+H]^+ = 582.2$. ^{1}H NMR (400 M, DMSO-d_6) δ 10.21 (s, 1H, CONH), 9.72 (s, 1H, NH), 8.76 (d, 1H, $J = 3.2$ Hz, ArH), 8.22 (d, 2H, $J = 8.8$ Hz, ArH), 7.99 (d, 2H, $J = 8.4$ Hz, ArH), 7.93 (d, 2H, $J = 8.4$ Hz, ArH), 7.63 (d, 2H, $J = 8.4$ Hz, ArH), 7.29 (s, 1H, ArH), 7.23 (d, 1H, $J = 7.6$ Hz, ArH), 7.11 (d, 1H, $J = 7.6$ Hz, ArH), 3.65–3.50 (m, 4H, morpholine-H), 3.44 (s, 2H, $ArCH_2N$), 2.44–2.32 (m, 4H, morpholine-H), 2.22 (s, 3H, $ArCH_3$) ppm. ^{13}C NMR (100 M, DMSO-d_6) δ 164.75, 156.10, 151.55, 150.00, 149.72, 149.63, 149.03, 148.06, 147.81, 143.45, 136.44, 135.48, 132.25, 132.14, 132.08, 130.96, 130.90, 130.01, 128.50, 126.98, 126.90, 126.39, 121.27, 121.14, 118.71, 117.37, 66.14, 62.00, 53.08, 17.65. HPLC: 98.11%.

*5.1.12. 2-Chloro-4-(4-trifluoromethoxyphenyl)pyrimidine (**7b**)*

It was prepared according to the method we recently described [10], as a white solid (0.3 g, 66%), MS (ESI) *m/z*: $[M+H]^+ = 275.0$. ^{1}H NMR (400 M, $CDCl_3$) δ 8.68 (d, 1H, $J = 5.2$ Hz, ArH), 8.16 (d, 2H, $J = 8.4$ Hz, ArH), 7.64 (d, 1H, $J = 5.2$ Hz, ArH), 7.37 (d, 2H, $J = 8.8$ Hz, ArH) ppm.

*5.1.13. Methyl 4-((4-(4-trifluoromethoxyphenyl)pyrimidin-2-yl) amino)benzoate (**8b**)*

It was prepared according to the method we recently reported [10], as a white solid (0.69 g, 88%), MS (ESI) *m/z*: $[M+H]^+ = 390.1$.

*5.1.14. 4-((4-(4-Trifluoromethoxyphenyl)pyrimidin-2-yl)amino) benzoic acid (**9b**)*

It was synthesized as we recently described [10], as a white product (0.64 g, 95%), MS (ESI) *m/z*: $[M+H]^+ = 376.1$. ^{1}H NMR (400 M, DMSO-d_6) δ 12.51 (s, 1H, COOH), 10.13 (s, 1H, NH), 8.66 (d, 1H, $J = 5.2$ Hz, ArH), 8.34 (d, 2H, $J = 8.4$ Hz, ArH), 7.98–7.87 (m, 4H, ArH), 7.64–7.54 (m, 3H, ArH) ppm.

*5.1.15. N-(5-(Hydroxymethyl)-2-methylphenyl)-4-((4-(4-trifluoromethoxyphenyl)pyrimidin-2-yl)amino)benzamide (**10b**)*

Similar procedure of **10a** was performed to give compound **10b** (611 mg, 73%) as a white solid, MS (ESI) *m/z*: $[M+H]^+ = 495.2$. ^{1}H NMR (400 M, DMSO-d_6) δ 10.11 (s, 1H, CONH), 9.70 (s, 1H, NH), 8.67 (d, 1H, $J = 5.2$ Hz, ArH), 8.35 (d, 2H, $J = 8.4$ Hz, ArH), 7.98 (s, 4H, ArH), 7.59 (m, 3H, ArH), 7.30 (s, 1H, ArH), 7.22 (d, 1H, $J = 7.6$ Hz, ArH), 7.11 (d, 1H, $J = 7.6$ Hz, ArH), 5.18 (s, 1H, OH), 4.49 (d, 2H, $J = 5.2$ Hz, $ArCH_2O$), 2.22 (s, 3H, $ArCH_3$) ppm.

*5.1.16. N-(5-(Chloromethyl)-2-methylphenyl)-4-((4-(4-trifluoromethoxyphenyl)pyrimidin-2-yl)amino)benzamide (**11b**)*

Similar procedure of **11a** was performed to give compound **11b** (580 mg, 91%) as a yellow solid, MS (ESI) *m/z*: $[M+H]^+ = 513.2$. ^{1}H NMR (400 M, DMSO-d_6) δ 10.12 (s, 1H, CONH), 9.76 (s, 1H, NH), 8.67 (d, 1H, $J = 5.2$ Hz, ArH), 8.35 (d, 2H, $J = 8.4$ Hz, ArH), 7.99 (s, 4H, ArH), 7.59 (m, 3H, ArH), 7.45 (s, 1H, ArH), 7.30 (d, 1H, $J = 7.6$ Hz, ArH), 7.24 (d, 1H, $J = 8.0$ Hz, ArH), 4.77 (s, 2H, $ArCH_2Cl$), 2.25 (s, 3H, $ArCH_3$) ppm.

*5.1.17. N-(5-((Dimethylamino)methyl)-2-methylphenyl)-4-((4-(4-trifluoromethoxyphenyl)pyrimidin-2-yl)amino)benzamide (**12ba**)*

Similar procedure of **12aa** was performed to give compound **12ba** (25 mg, 49%) as a white solid, MS (ESI) *m/z*: $[M+H]^+ = 522.2$. ^{1}H NMR (400 M, DMSO-d_6) δ 10.10 (s, 1H, CONH), 9.69 (s, 1H, NH), 8.67 (d, 1H, $J = 5.2$ Hz, ArH), 8.35 (d, 2H, $J = 8.4$ Hz, ArH), 7.99 (s, 4H, ArH), 7.59 (m, 3H, ArH), 7.30 (s, 1H, ArH), 7.22 (d, 1H, $J = 7.6$ Hz, ArH), 7.09 (d, 1H, $J = 7.6$ Hz, ArH), 3.40 (s, 2H, $ArCH_2N$), 2.23 (s, 3H, $ArCH_3$), 2.18 (s, 6H, $N(CH_3)_2$) ppm. ^{13}C NMR (100 M, DMSO-d_6) δ 164.77, 162.33, 159.88, 159.43, 150.20, 143.53, 136.45, 135.67, 132.14, 129.98, 129.11, 128.44, 126.92, 126.83, 126.14, 121.24, 118.74, 117.79, 108.81, 62.82, 44.79, 17.66. HPLC: 94.74%.

*5.1.18. N-(5-(Diethylamino)methyl)-2-methylphenyl)-4-((4-(4-trifluoromethoxyphenyl)pyrimidin-2-yl)amino)benzamide (**12bb**)*

Similar procedure of **12aa** was performed to give compound **12bb** (41 mg, 76%) as a white solid, MS (ESI) *m/z*: $[M+H]^+ = 550.3$. ^{1}H NMR (400 M, DMSO-d_6) δ 10.10 (s, 1H, CONH), 9.70 (s, 1H, NH), 8.67 (d, 1H, $J = 4.8$ Hz, ArH), 8.35 (d, 2H, $J = 8.8$ Hz, ArH), 7.99 (s, 4H, ArH), 7.59 (m, 3H, ArH), 7.30 (s, 1H, ArH), 7.21 (d, 1H, $J = 8.0$ Hz, ArH), 7.10 (d, 1H, $J = 7.6$ Hz, ArH), 3.51 (s, 2H, $ArCH_2N$), 2.51–2.40 (m, 4H, $N(CH_2)_2$), 2.22 (s, 3H, $ArCH_3$), 1.05–0.94 (m, 6H, $(CH_3)_2$) ppm. ^{13}C NMR (100 M, DMSO-d_6) δ 164.75, 162.32, 159.88, 159.42, 150.20, 143.52, 136.42, 135.67, 131.86, 129.89, 129.11, 128.45, 126.92, 126.60, 125.97, 121.29, 121.24, 118.74, 117.78, 108.79, 56.43, 46.03, 17.66, 11.58. HPLC: 96.85%.

*5.1.19. N-(2-Methyl-5-((1-pyrrolidinyl)methyl)phenyl)-4-((4-(4-trifluoromethoxyphenyl)pyrimidin-2-yl)amino)benzamide (**12bc**)*

Similar procedure of **12aa** was performed to give compound **12bc** (47 mg, 88%) as a white solid, MS (ESI) *m/z*: $[M+H]^+ = 548.2$. ^{1}H NMR (400 M, DMSO-d_6) δ 10.10 (s, 1H, CONH), 9.71 (s, 1H, NH), 8.67 (d, 1H, $J = 5.2$ Hz, ArH), 8.35 (d, 2H, $J = 8.4$ Hz, ArH), 8.00 (s, 4H, ArH), 7.59 (m, 3H, ArH), 7.30 (s, 1H, ArH), 7.21 (d, 1H, $J = 7.6$ Hz, ArH), 7.10 (d, 1H, $J = 7.6$ Hz, ArH), 3.56 (s, 2H, $ArCH_2N$), 2.38–2.29 (m, 4H, piperidine-H), 2.22 (s, 3H, $ArCH_3$), 1.52–1.44 (m, 4H, piperidine-H), 1.42–1.35 (m, 2H, piperidine-H) ppm. ^{13}C NMR (100 M, DMSO-d_6) δ 164.80, 162.33, 159.87, 159.42, 150.20, 143.52, 137.16, 136.40, 135.65, 131.92, 129.94, 128.45, 126.91, 126.51, 125.84, 121.29, 121.23, 118.74, 117.79, 108.79, 59.12, 53.41, 23.08, 17.64. HPLC: 94.74%.

*5.1.20. N-(2-Methyl-5-((1-piperidyl)methyl)phenyl)-4-((4-(4-trifluoromethoxyphenyl)pyrimidin-2-yl)amino)benzamide (**12bd**)*

Similar procedure of **12aa** was performed to give compound **12bd** (51 mg, 93%) as a white solid, MS (ESI) *m/z*: $[M+H]^+ = 562.3$. ^{1}H NMR (400 M, DMSO-d_6) δ 10.10 (s, 1H, CONH), 9.71 (s, 1H, NH), 8.67 (d, 1H, $J = 4.8$ Hz, ArH), 8.35 (d, 2H, $J = 8.4$ Hz, ArH), 7.99 (s, 4H, ArH), 7.58 (m, 3H, ArH), 7.27 (s, 1H, ArH), 7.21 (d, 1H, $J = 7.6$ Hz, ArH), 7.08 (d, 1H, $J = 8.0$ Hz, ArH), 3.40 (s, 2H, $ArCH_2N$), 2.38–2.28 (m, 4H, piperidine-H), 2.22 (s, 3H, $ArCH_3$), 1.54–1.45 (m, 4H, piperidine-H), 1.43–1.30 (m, 2H, piperidine-H) ppm. ^{13}C NMR (100 M, DMSO-d_6) δ 164.77, 162.33, 159.88, 159.41, 150.20, 143.53, 136.40, 132.04, 129.90, 129.10, 128.45, 126.90, 126.86, 126.25, 121.29, 121.23, 118.74, 117.79, 108.79, 62.41, 53.80, 25.49, 23.96, 17.66. HPLC: 97.72%.

*5.1.21. N-(2-Methyl-5-((4-methyl-1-piperazinyl)methyl)phenyl)-4-((4-(4-trifluoromethoxyphenyl)pyrimidin-2-yl)amino)benzamide (**12be**)*

Similar procedure of **12aa** was performed to give compound **12bf** (75 mg, 83%) as a white solid, MS (ESI) *m/z*: $[M+H]^+ = 577.3$. ^{1}H NMR (400 M, DMSO-d_6) δ 10.11 (s, 1H, CONH), 9.72 (s, 1H, NH), 8.67 (d, 1H, $J = 5.2$ Hz, ArH), 8.35 (d, 2H, $J = 8.8$ Hz, ArH), 7.99 (s, 4H, ArH), 7.59 (m, 3H, ArH), 7.28 (s, 1H, ArH), 7.22 (d, 1H, $J = 7.6$ Hz, ArH), 7.08 (d, 1H, $J = 7.6$ Hz, ArH), 2.48–2.37 (m, 8H, piperazine-H), 2.22 (s, 3H, $ArCH_3$), 2.22 (d, 6H, $ArCH_3$+NCH_3) ppm. ^{13}C NMR (100 M, DMSO-d_6) δ 164.78, 162.32, 159.87, 159.43, 150.18, 143.53, 136.46, 135.89, 135.66, 132.17, 129.98, 129.11, 128.46, 126.88, 126.27, 121.25, 118.74, 117.77, 108.80, 61.53, 54.48, 52.16, 51.99, 45.55, 45.37, 17.67. HPLC: 98.89%.

*5.1.22. N-(2-Methyl-5-((4-morpholinyl)methyl)phenyl)-4-((4-(4-trifluoromethoxyphenyl)pyrimidin-2-yl)amino)benzamide (**12bf**)*

Similar procedure of **12aa** was performed to give compound **12bf** (30 mg, 91%) as a white solid, MS (ESI) *m/z*: $[M+H]^+ = 564.2$. ^{1}H NMR (400 M, DMSO-d_6) δ 10.11 (s, 1H, CONH), 9.71 (s, 1H, NH), 8.67 (d, 1H, $J = 5.2$ Hz, ArH), 8.35 (d, 2H, $J = 8.8$ Hz, ArH), 7.99 (s, 4H, ArH), 7.59 (m, 3H, ArH), 7.30 (s, 1H, ArH), 7.23 (d, 1H, $J = 7.6$ Hz, ArH), 7.10 (d, 1H, $J = 7.6$ Hz, ArH), 3.62–3.53 (m, 4H, morpholine-H), 3.44 (s, 2H, $ArCH_2N$), 2.40–2.32 (m, 4H, morpholine-H), 2.22 (s, 3H, $ArCH_3$). ^{13}C NMR (100 M, DMSO-d_6) δ 164.77, 162.33, 159.88, 159.43, 150.20, 143.54, 136.49, 135.67, 135.53, 132.22, 130.00, 129.11, 128.46, 126.95, 126.89, 126.35, 121.24, 117.78, 108.81, 66.17, 62.03, 53.11, 17.67. HPLC: 99.17%.

*5.1.23. 5-((5-Fluoro-4-(4-trifluoromethoxyphenyl)pyrimidin-2-yl)amino)picolinic acid (**12a**)*

Similar Buchwald coupling procedure of **8a** was performed to give compound **12a** (248 mg, 62%) as a yellow solid, MS (ESI) *m/z*: $[M+H]^+ = 395.1$.

*5.1.24. N-(5-(Hydroxymethyl)-2-methylphenyl)-5-((5-fluoro-4-(4-trifluoromethoxyphenyl)pyrimidin-2-yl)amino)picolinamide (**13a**)*

Similar procedure of **10a** was performed to give compound **13a** (138 mg, 43%) as a yellow solid, MS (ESI) *m/z*: $[M+H]^+ = 514.2$. ^{1}H NMR (400 M, DMSO-d_6) δ 10.52 (s, 1H, CONH), 10.08 (s, 1H, NH), 9.03 (d, 1H, $J = 4.0$ Hz, ArH), 8.80 (d, 1H, $J = 4.0$ Hz, ArH), 8.53 (dd, 1H, $J_1 = 8.0$ Hz, $J_2 = 4.0$ Hz, ArH), 8.22 (d, 2H, $J = 8.0$ Hz, ArH), 8.16 (d, 1H, $J = 8.0$ Hz, ArH), 7.86 (s, 1H, ArH), 7.64 (d, 2H, $J = 8.0$ Hz, ArH), 7.23 (d, 1H, $J = 8.0$ Hz, ArH), 7.07 (d, 1H, $J = 8.0$ Hz, ArH), 5.25 (t, 1H, OH), 5.25 (d, 2H, OCH_2Ar), 2.30 (s, 3H, $ArCH_3$) ppm.

*5.1.25. N-(5-(Chloromethyl)-2-methylphenyl)-5-((5-fluoro-4-(4-trifluoromethoxyphenyl)pyrimidin-2-yl)amino)picolinamide (**14a**)*

Similar procedure of **11a** was performed to give compound **14a** (119 mg, 83%) as a yellow solid, MS (ESI) *m/z*: $[M+H]^+ = 532.2$. ^{1}H NMR (400 M, $CDCl_3$) δ 9.96 (s, 1H, CONH), 8.81 (d, 1H, ArH), 8.47 (d, 1H, $J = 4.0$ Hz, ArH), 8.42–8.36 (m, 2H, ArH), 8.31 (d, 1H, $J = 8.0$ Hz, ArH), 8.21 (d, 2H, $J = 8.0$ Hz, ArH), 7.45 (s, 1H, NH), 7.41 (d, 2H, $J = 8.0$ Hz, ArH), 7.24 (d, 1H, $J = 8.0$ Hz, ArH), 7.14 (d, 1H, $J = 4.0$ Hz, ArH), 4.61 (s, 2H, $ClCH_2Ar$), 2.43 (s, 3H, $ArCH_3$) ppm.

*5.1.26. 5-((5-Fluoro-4-(4-trifluoromethoxyphenyl)pyrimidin-2-yl)amino)-N-(2-methyl-5-((1-morpholinyl)methyl)phenyl)benzamide (**15a**)*

Similar procedure of **12aa** was performed to give compound **15a** (108 mg, 83%) as a yellow solid, MS (ESI) *m/z*: $[M+H]^+ = 583.3$. ^{1}H NMR (400 M, $CDCl_3$) δ 9.91 (s, 1H, CONH), 8.82 (d, 1H, $J = 2.4$ Hz, ArH), 8.47 (d, 1H, $J = 3.2$ Hz, ArH), 8.37 (m, 1H, ArH), 8.31 (d, 1H, $J = 8.4$ Hz, ArH), 8.24 (s, 1H, NH), 8.20 (d, 2H, $J = 8.4$ Hz, ArH), 7.48 (s, 1H, ArH), 7.41 (d, 2H, $J = 8.4$ Hz, Ar), 7.19 (d, 1H, $J = 7.6$ Hz, ArH), 7.08 (d, 1H, $J = 7.6$ Hz, ArH), 3.96–3.90 (m, 4H, morpholine-H), 3.52 (s, 2H, $PhCH_2N$), 3.03 (d, 2H, NCH_2), 2.50–2.42 (s, 4H, morpholine-H), 2.41 (s, 3H, $ArCH_3$) ppm. ^{13}C NMR (100 M, DMSO-d_6) δ 164.76, 164.23, 157.79, 152.48, 150.06, 149.96, 149.33, 148.11, 147.85, 142.07, 139.99, 138.53, 135.96, 135.69, 131.87, 131.82, 131.02, 130.96, 130.02, 128.30, 125.24, 124.88, 123.04, 122.62, 121.26, 121.12, 118.70, 66.11, 62.21, 53.06, 17.17. HPLC: 99.55%.

*5.1.27. 5-((4-(4-Trifluoromethoxyphenyl)pyrimidin-2-yl)amino) picolinic acid (**12b**)*

Similar Buchwald coupling procedure of **8a** was performed to give compound **12b** (674 mg, 87%) as a yellow solid, MS (ESI) *m/z*: $[M+H]^+ = 377.1$.

*5.1.28. N-(5-(Hydroxymethyl)-2-methylphenyl)-5-((4-(4-trifluoromethoxyphenyl)pyrimidin-2-yl)amino)picolinamide (**13b**)*

Similar procedure of **10a** was performed to give compound **13b** (197 mg, 44%) as a yellow solid, MS (ESI) *m/z*: $[M+H]^+ = 496.2$.

*5.1.29. N-(5-(Chloromethyl)-2-methylphenyl)-5-((4-(4-trifluoromethoxyphenyl)pyrimidin-2-yl)amino)picolinamide (**14b**)*

Similar procedure of **11a** was performed to give compound **14b** (197 mg, 100%) as a yellow solid, MS (ESI) *m/z*: $[M+H]^+ = 514.2$.

*5.1.30. N-(2-Methyl-5-((1-morpholinyl)methyl)phenyl)-5-((4-(4-trifluoromethoxyphenyl)pyrimidin-2-yl)amino)picolinamide (**15b**)*

Similar procedure of **12aa** was performed to give compound **15b** (90 mg, 40%) as a yellow solid, MS (ESI) *m/z*: $[M+H]^+ = 565.3$. ^{1}H NMR (400 M, DMSO-d_6) δ 10.44 (s, 1H, CONH), 10.09 (s, 1H, NH), 9.07 (d, 1H, $J = 2.2$ Hz, ArH), 8.71 (d, 1H, $J = 5.2$ Hz, ArH), 8.60 (dd, 1H, $J_1 = 8.6$ Hz, $J_2 = 2.3$ Hz, ArH), 8.35 (d, 2H, $J = 8.8$ Hz, ArH), 8.17 (d, 1H, $J = 8.6$ Hz, ArH), 7.90 (s, 1H, ArH), 7.60 (m, 3H, ArH), 7.23 (d, 1H, $J = 7.6$ Hz, ArH), 7.05 (d, 1H, $J = 7.4$ Hz, ArH), 3.59 (s, 4H, morpholine-H), 3.45 (s, 2H, $PhCH_2N$), 2.38 (s, 4H, morpholine-H), 2.31 (s, 3H, $ArCH_3$) ppm. HPLC: 97.2%.

*5.1.31. 6-((5-Fluoro-4-(4-trifluoromethoxyphenyl)pyrimidin-2-yl)amino)nicotinic acid (**12c**)*

Similar Buchwald coupling procedure of **8a** was performed to give compound **12c** (158 mg, 65%) as a yellow solid, MS (ESI) *m/z*: $[M+H]^+ = 395.1$.

*5.1.32. 6-((5-Fluoro-4-(4-trifluoromethoxyphenyl)pyrimidin-2-yl)amino)-N-(5-(hydroxymethyl)-2-methylphenyl)nicotinamide (**13c**)*

Similar procedure of **10a** was performed to give compound **13c** (96 mg, 35%) as a yellow solid, MS (ESI) *m/z*: $[M+H]^+ = 514.2$.

*5.1.33. N-(5-(Chloromethyl)-2-methylphenyl)-6-((5-fluoro-4-(4-trifluoromethoxyphenyl)pyrimidin-2-yl)amino)nicotinamide (**14c**)*

Similar procedure of **11a** was performed to give compound **14c** (88 mg, 99%) as a yellow solid, MS (ESI) *m/z*: $[M+H]^+ = 531.2$.

*5.1.34. 6-((5-Fluoro-4-(4-trifluoromethoxyphenyl)pyrimidin-2-yl)amino)-N-(2-methyl-5-((1-morpholinyl)methyl)phenyl) nicotinamide (**15c**)*

Similar procedure of **12aa** was performed to give compound **15c** (66 mg, 59%) as a yellow solid, MS (ESI) *m/z*: $[M+H]^+ = 583.2$. ^{1}H NMR (400 M, DMSO-d_6) δ 10.56 (s, 1H, CONH), 9.95 (s, 1H, NH), 8.90 (s, 1H, ArH), 8.80 (d, 1H, $J = 3.2$ Hz, ArH), 8.35 (s, 2H, ArH), 8.24 (d, 2H, $J = 8.4$ Hz, ArH), 7.63 (d, 2H, $J = 8.4$ Hz, ArH), 7.28 (s, 1H, ArH), 7.25 (d, 1H, $J = 7.7$ Hz, ArH), 7.13 (d, 1H, $J = 8.1$ Hz, ArH), 3.57 (s, 4H, morpholine-H), 3.38 (s, 2H, NCH_2Ph), 2.35 (s, 4H, morpholine-H), 2.22 (s, 3H, $ArCH_3$) ppm. ^{13}C NMR (100 M, DMSO-d_6) δ 163.48, 155.16, 155.07, 152.10, 150.08, 148.17, 147.97, 137.50, 136.05 135.62, 132.29, 131.96, 131.09, 131.02, 130.08, 126.95, 126.61, 123.40, 121.19,

110.72, 66.16, 61.98, 53.10, 17.67. HPLC: 98.2%.

*5.1.35. N-(3-(Hydroxymethyl)phenyl)-4-((4-(4-trifluoromethoxyphenyl)pyrimidin-2-yl)amino)benzamide (**15**)*

Similar procedure of **10a** was performed to give compound **15** (145 mg, 97%) as a white solid, MS (ESI) *m/z*: $[M+H]^+ = 481.2$.

*5.1.36. N-(3-(Chloromethyl)phenyl)-4-((4-(4-trifluoromethoxyphenyl)pyrimidin-2-yl)amino)benzamide (**16**)*

Similar procedure of **11a** was performed to give compound **16** (105 mg, 70%) as a white solid, MS (ESI) *m/z*: $[M+H]^+ = 499.2$.

*5.1.37. N-(3-((1-Morpholinyl)methyl)phenyl)-4-((4-(4-trifluoromethoxyphenyl)pyrimidin-2-yl)amino)benzamide (**17**)*

Similar procedure of **12aa** was performed to give compound **17** (59 mg, 70%) as a white solid, MS (ESI) *m/z*: $[M+H]^+ = 550.3$. ^{1}H NMR (400 M, DMSO-d_6) δ 10.14 (s, 1H, CONH), 10.10 (s, 1H, NH), 8.68 (d, 1H, J = 8.0 Hz, ArH), 8.35 (d, 2H, J = 8.0 Hz, ArH), 8.00 (s, 4H, ArH), 7.74 (m, 2H, ArH), 7.57 (m, 3H, ArH), 7.30 (m, 1H, ArH), 7.04 (s, 1H, ArH), 3.60 (s, 4H, morpholine-H), 3.46 (s, 2H, $PhCH_2N$), 2.38 (s, 4H, morpholine-H) ppm. ^{13}C NMR (100 M, DMSO-d_6) δ 164.96, 162.31, 159.85, 159.44, 150.17, 143.61, 139.39, 138.20, 135.66, 129.10, 128.54, 128.30, 127.14, 123.96, 121.25, 120.70, 118.98, 118.73, 117.69, 108.83, 66.16, 62.62, 53.18. HPLC: 96.2%.

*5.1.38. Methyl 2-cyclopropyl-3-oxopropanoate (**19**)*

It was synthesized as we recently described [10], as an oil (600 mg, 90%), MS (ESI) *m/z*: $[M+H]^+ = 143.1$.

*5.1.39. 5-Cyclopropyl-2-thioxo-2,3-dihydropyrimidin-4(1H)-one (**20**)*

It was synthesized as we recently described [10], as a white solid (187 mg, 23%), MS (ESI) *m/z*: $[M+H]^+ = 169.0$. ^{1}H NMR (400 M, DMSO-d_6) δ 12.38 (s, 1H, NH), 12.18 (s, 1H, NH), 7.02 (s, 1H, ArH), 1.65–1.56 (m, 1H, CH), 0.78–0.72 (m, 2H, CH_2), 0.61 (d, 2H, J = 4.4 Hz, CH_2) ppm.

*5.1.40. 5-Cyclopropylpyrimidine-2,4(1H,3H)-dione (**21**)*

It was synthesized as we recently described [10], as a white solid (55 mg, 70%), MS (ESI) *m/z*: $[M+H]^+ = 153.1$. ^{1}H NMR (400 M, DMSO-d_6) δ 10.99 (s, 1H, NH), 10.62 (s, 1H, NH), 6.99 (d, 1H, ArH), 1.65–1.56 (m, 1H, CH), 0.78–0.72 (m, 2H, CH_2), 0.65–0.58 (m, 2H, CH_2) ppm.

*5.1.41. 2,4-Dichloro-5-cyclopropylpyrimidine (**22**)*

It was synthesized as we recently described [10], as an oil (70 mg, 90%), MS (ESI) *m/z*: $[M+H]^+ = 189.0$. ^{1}H NMR (400 M, DMSO-d_6) δ 8.49 (s, 1H, ArH), 2.03–1.95 (m, 1H, CH), 1.11–1.07 (m, 2H, CH_2), 0.95–0.89 (m, 2H, CH_2) ppm.

*5.1.42. 2-Chloro-5-cyclopropyl-4-(4-trifluoromethoxyphenyl) pyrimidine (**23**)*

Similar procedure of **7a** was performed to give compound **23** (74 mg, 62%) as a white solid, MS (ESI) *m/z*: $[M+H]^+ = 315.0$. ^{1}H NMR (400 M, DMSO-d_6) δ 8.54 (s, 1H, ArH), 7.95 (d, 2H, J = 8.4 Hz, ArH), 7.56 (d, 2H, J = 8.4 Hz, ArH), 1.98–1.90 (m, 1H, CH), 1.04–1.00 (m, 2H, CH_2), 0.97–0.91 (m, 2H, CH_2) ppm.

*5.1.43. Methyl 4-((5-cyclopropyl-4-(4-trifluoromethoxyphenyl) pyrimidin-2-yl)amino) benzoate (**24**)*

Similar procedure of **8a** was performed to give compound **24** (160 mg, 32%) as a white solid, MS (ESI) *m/z*: $[M+H]^+ = 430.1$. ^{1}H NMR (400 M, DMSO-d_6) δ 10.13 (s, 1H, NH), 8.39 (s, 1H, ArH), 7.99 (d, 2H, J = 8.0 Hz, ArH), 7.94 (m, 4H, ArH), 7.55 (d, 2H, J = 8.4 Hz, ArH), 3.81 (s, 3H, OCH_3), 1.98–1.90 (m, 1H, ArCH), 0.95–0.89 (m, 2H, CH_2), 0.74–0.69 (m, 2H, CH_2) ppm.

*5.1.44. 4-((5-Cyclopropyl-4-(4-trifluoromethoxyphenyl) pyrimidin-2-yl)amino)benzoic acid (**25**)*

Similar procedure of **9a** was performed to give compound **25** (350 mg, 68%) as a white solid, MS (ESI) *m/z*: $[M+H]^+ = 416.1$.

*5.1.45. 4-((5-Cyclopropyl-4-(4-trifluoromethoxyphenyl) pyrimidin-2-yl)amino)-N-(5-(hydroxymethyl)-2-methylphenyl) benzamide (**26**)*

Similar procedure of **10a** was performed to give compound **26** (160 mg, 82%) as a white solid, MS (ESI) *m/z*: $[M+H]^+ = 535.2$. ^{1}H NMR (400 M, DMSO-d_6) δ 10.02 (s, 1H, CONH), 9.66 (s, 1H, NH), 8.40 (s, 1H, ArH), 8.01 (d, 2H, J = 8.4 Hz, ArH), 7.94 (s, 4H, ArH), 7.57 (d, 2H, J = 8.0 Hz, ArH), 7.30 (s, 1H, ArH), 7.22 (d, 1H, J = 7.6 Hz, ArH), 7.11 (d, 1H, J = 7.6 Hz, ArH), 5.18 (t, 1H, OH), 4.49 (d, 2H, J = 4.8 Hz, $ArCH_2O$), 2.21 (s, 3H, $ArCH_3$) 1.98–1.90 (m, 1H, ArCH), 0.95–0.89 (m, 2H, CH_2), 0.74–0.69 (m, 2H, CH_2) ppm.

*5.1.46. N-(5-(Chloromethyl)-2-methylphenyl)-4-((5-cyclopropyl-4-(4-trifluoromethoxyphenyl)pyrimidin-2-yl)amino)benzamide (**27**)*

Similar procedure of **11a** was performed to give compound **27** (86 mg, 76%) as a yellow solid, MS (ESI) *m/z*: $[M+H]^+ = 553.2$. ^{1}H NMR (400 M, DMSO-d_6) δ 10.04 (s, 1H, CONH), 9.73 (s, 1H, NH), 8.40 (s, 1H, ArH), 8.01 (d, 2H, J = 8.4 Hz, ArH), 7.94 (s, 4H, ArH), 7.57 (d, 2H, J = 8.4 Hz, ArH), 7.44 (s, 1H, ArH), 7.30 (d, 1H, J = 8.0 Hz, ArH), 7.24 (d, 1H, J = 8.0 Hz, ArH), 4.77 (s, 2H, $ArCH_2Cl$), 2.24 (s, 3H, $ArCH_3$) 1.93–1.89 (m, 1H, ArCH), 0.95–0.87 (m, 2H, CH_2), 0.75–0.70 (m, 2H, CH_2) ppm.

*5.1.47. 4-((5-Cyclopropyl-4-(4-trifluoromethoxyphenyl) pyrimidin-2-yl)amino)-N-(2-methyl-5-((1-morpholinyl)methyl) phenyl)benzamide (**28**)*

Similar procedure of **12aa** was performed to give compound **28** (35 mg, 72%) as a white solid, MS (ESI) *m/z*: $[M+H]^+ = 604.3$. ^{1}H NMR (400 M, DMSO-d_6) δ 10.01 (s, 1H, CONH), 9.66 (s, 1H, NH), 8.40 (s, 1H, ArH), 8.00 (d, 2H, J = 8.4 Hz, ArH), 7.93 (s, 4H, ArH), 7.57 (d, 2H, J = 8.4 Hz, ArH), 7.29 (s, 1H, ArH), 7.23 (d, 1H, J = 7.6 Hz, ArH), 7.10 (d, 1H, J = 7.2 Hz, ArH), 3.60–3.52 (m, 4H, morpholine-H), 3.37 (s, 2H, $ArCH_2N$), 2.40–2.35 (m, 4H, morpholine-H), 2.24 (s, 3H, $ArCH_3$), 1.93–1.88 (m, 1H, ArCH), 0.94–0.89 (m, 2H, CH_2), 0.73–0.69 (m, 2H, CH_2) ppm. ^{13}C NMR (100 M, DMSO-d_6) δ 164.76, 164.23, 157.79, 157.48, 148.83, 146.26, 143.78, 137.12, 136.47, 135.47, 132.18, 131.09, 129.97, 129.54, 128.39, 126.93, 126.44, 126.32, 124.10, 123.89, 121.34, 120.51, 118.79, 117.31, 116.90, 116.24, 114.54, 66.15, 62.78, 62.02, 53.20, 53.09, 17.63, 10.76, 7.84. HPLC: 96.79%.

5.2. In vitro Gli-luciferase reporter assay to evaluate the Hh signaling pathway inhibitory activity

The target compounds were assessed for their Hh signaling pathway inhibitory activity, using a luciferase reporter assay in NIH3T3 cells carrying a stably transfected Gli-reporter construct (Gli-luc reporter cell line). NIH3T3/Gli-luc cells were treated with DMEM and 10% FBS and1 μg/mL Puromycin. The cells were seeded onto 96-well plates at 2×10^4 cells/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.

5.3. Pharmacokinetic studies of the selected compounds in SD rats

The selected compounds were administered to 3 male SD rats (weight ranging from 180 g to 240 g) at doses of 1 mg/kg for iv administration or doses of 5 mg/kg for po administration [17]. The dosing volume was 5 mL/kg. After administration, blood samples were collected at the point including 2 min, 5 min, 15 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h, 10 h, and 24 h for analyses, the collected blood samples were centrifuged at 4000 rpm for 5 min at 4 °C, and then analyzed after protein precipitation. LC/MS/MS analysis of compounds were performed under optimized conditions to obtain the best sensitivity and selectivity of the analyte in selected reaction monitoring mode (SRM) containing an internal standard. Plasma concentration-time data were measured by a noncompartmental approach using the software WinNonlin Enterprise, version 5.2 (Pharsight Co., Mountain View, CA).

5.4. Cell proliferation assay of the selected compounds

The in vitro anti-proliferative potency of the selected compounds was measured by the ATP-dependent bioluminescence assay. Measurements were performed according to manufacturer's instructions of CellTiter-Glo assay. Briefly, LS-174T, PC3 or BxPC3 cells were seeded in 96-well microplates at the appropriate density for each cell line and cultured in DMEM 10% FBS, and incubated at 37 °C for 24 h. When the cells were settling well to the bottom of assay plates, and compounds were prepared in DMSO and further diluted in medium to the desired concentration. Cells were treated with a variety of concentrations (starting for 100 μM) of test compounds simultaneously and incubated at 37 °C CO_2 for 72 h. After removal of the plates, the equal volume of CellTiter-Glo reagent was added directly to the wells, then plates were incubated at room temperature for 30 min and the relative luminescence were measured using CellTiter-Glo® Luminescent Cell Viability Assay (Promega G7570), and the IC_{50} values were calculated by concentration-response curve fitting using a SoftMax pro-based four-parameter method.

5.5. In vivo anti-tumor effects in LS-174T nude mice xenograft model

Mice (BALB/C, male, 18–20 g) were obtained from Shanghai SLAC laboratory Animal Co., Ltd (Shanghai, China) and maintained in a specific-pathogen free (SPF) conditions. The animals were fed standard rodent chow and water ad libitum. The LS-174T cells at a density of 2.5×10^7 cells/mL were injected subcutaneously into the right flank of mice and allowed tumor xenografts to reach 100 mm [3]. All the tumor-bearing nude mice were randomly assigned into 9 groups with 6 mice in each group. The control group was given the same volume of solvent and the treatment groups were given with the compounds as indicated doses orally for once or twice daily for 10 days, respectively. Tumor volumes and body weights were measured twice per week. Tumor volume was calculated as follows: V = length (mm) × width^2 (mm^2)/2. Results are expressed as the mean ± standard error. The therapeutic effect of the compounds on indicated days was presented as (IR%, TW) and (IR%, TV).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2016.01.018.

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